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Bovine mastitis and ecology of *Streptococcus uberis*

A thesis submitted in partial fulfilment
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by

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Abstract

Bovine mastitis caused by *Streptococcus uberis* is a common problem in pasture-based dairying systems. This study examines both the ecology of *S. uberis* and infection of the bovine mammary gland on a New Zealand dairy farm. Initially, the REP-PCR strain typing method was developed and the potential of MALDI-TOF mass spectrometry evaluated as a strain typing method. While strain-specific mass spectra were obtained with MALDI-TOF mass spectrometry, the irreproducibility of spectra was its major downfall. With further work, this rapid method could be very useful for strain typing *S. uberis* on a large scale.

Using optimised REP-PCR and anchored typing methods, multiple *S. uberis* strains were isolated and strain typed from the dairy environment, including farm races and paddocks, faeces, teat skin, the cow body and from intramammary infections. High strain diversity was observed in all sampled locations; however, some strains were found at more than one site, suggesting transmission may occur between the environment and cows.

The most likely means of *S. uberis* distribution throughout the dairy farm was via excretion with faeces and, although not all cow faeces contained this pathogen, the gastrointestinal tract of some cows appeared to be colonised by specific strains, resulting in persistent shedding of this bacteria in the faeces.

Infection of the mammary gland is likely to occur through contamination of the teat skin with highly diverse environmental strains of *S. uberis*. However, only one or two strains are generally found in milk from mastitis cases, suggesting that, although infection may arise from a random or opportunistic event, a strain selection process may take place. Intramammary challenge with multiple strains of *S. uberis* revealed that

selection of a single 'infective' strain can occur within the mammary gland. The predominance of one strain over others may be related to production of virulence factors allowing enhanced ability to establish in the gland and evade the immune response, or due to direct competition between strains through the production of antimicrobial factors such as bacteriocins. In addition to strain-specific factors, the individual cow and quarter response may play an important role in the development of infection and selection of the infective strain.

Using results from this study, a model of *S. uberis* strain transmission has been proposed, which includes potential mechanisms of infection and persistence of *S. uberis* within the mammary gland.

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Abbreviations

Standard International Unit abbreviations were used throughout this thesis.

ACN	Acetonitrile
AP-PCR	Arbitrarily primed – polymerase chain reaction
a.u.	Arbitrary units
AU	Absorbance units
BHI	Brain heart infusion
bp	Base pairs
CCD	Charge-coupled device
cfu	Colony forming units
cfu/ml	Colony forming units per ml
CHCA	α-cyano-4-hydroxycinnamic acid
CI	Confidence interval
CM	Clinical mastitis
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
EBA	Esculin blood agar
EC	Electrical conductivity
ECMPs	Extracellular matrix proteins
EDTA	Ethylenediamine tetra acetic acid
ERIC	Enterobacterial repetitive intergenic consensus
GI	Gastrointestinal
HA	Hyaluronic acid
Kb	Kilobases
LB	Left back quarter
LF	Left front quarter
Log	Logarithm
MALDI-TOF	Matrix assisted laser desorption ionisation - time of flight
MLST	Multi-locus sequence typing
MM-A	PCR master-mix A
MM-B	PCR master-mix B
MS	Mass spectrometry
MtuA	Metal transporter uberis A
MQ-H_2O	Deionised double distilled water
m/z	Mass/charge
NI	Not infected
OD	Optical density
Q	Quarter
PauA	Plasminogen activator uberis A
PCR	Polymerase chain reaction
PEG	Polyethylene glycol

PFGE	Pulsed field gel electrophoresis
P-typing	Producer-typing
RAPD	Randomly amplified polymorphic DNA
RB	Right back quarter
rDNA	Ribosomal DNA
REF	Restriction endonuclease fingerprinting
REP elements	Repetitive extragenic palindromic elements
REP-PCR	Repetitive element PCR
RF	Right front quarter
RNase	Ribonuclease
rRNA	Ribosomal ribonucleic acid
SA	Sinapinic acid
SB	Sodium hydroxide, boric acid buffer
SCC	Somatic cell count
SCM	Subclinical mastitis
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SK	Streptokinase
S/N	Signal to noise
ST	Strain type
SUAM	*S. uberis* adhesion molecule
TBE	Tris, boric acid, EDTA buffer
TE	Tris, EDTA buffer
TFA	Trifluoroacetic acid
Tris	Tris-hydroxymethyl-methylamine
UHT	Ultra-heat treated
UPGMA	Unweighted Pair group Method using Arithmetic averages
UV	Ultraviolet
w/v	Weight to volume

General introduction

Inflammation of the bovine mammary gland, commonly known as mastitis, has been researched for many years. Mastitis reduces milk quality and quantity (Deluyker *et al.*, 1993) and can lead to loss of the cow when severe or ongoing infection occurs. The treatment regimes for mastitis are also costly and the overall result is a reduction in farm productivity and profitability. Besides the trauma of mastitis, the disease poses a considerable problem economically. The cost of mastitis to the average supplier using representative management techniques is approximately $11,500 for the herd, which equates to a loss of some $180 million annually for the New Zealand dairy industry (National Mastitis Advisory Committee, 2006). Therefore, the prevention and control of mastitis is important to every dairy farmer and to the New Zealand economy.

Streptococcus uberis, an opportunistic environmental pathogen, is a common cause of bovine mastitis in pasture-based dairying systems. The ecology and infection mechanism of *S. uberis* is poorly understood with the majority of isolated strains being distinct from each other and no particular niches identified as the primary source of mammary gland infection. This pathogen can be isolated from many sites on the cow's body and the dairy environment, and contagious cow-to-cow transmission may also occur.

The main aims of this study were to elucidate the major niches of *S. uberis* on a New Zealand dairy farm and any possible transmission between these sites and intramammary infections. The interaction between multiple strains during development of infection by this pathogen was investigated in an experimental infection study involving multiple *S. uberis* strain types.

The first chapter of this thesis reviews the current knowledge of bovine mastitis and the mechanism of infection by *S. uberis*, including virulence

factors known to be produced by this pathogen to overcome host defence mechanisms. While this is not an exhaustive review of these factors, it focuses on those considered to be important to the initial infection event and development of persistent or recurrent infections of the mammary gland.

In the second chapter, the use of REP-PCR and MALDI-TOF mass spectrometry are evaluated with regard to their ability to discriminate between strains of *S. uberis* while maintaining good reproducibility and efficiency for use as strain typing methods. The third chapter describes the use of an optimized REP-PCR method to study *S. uberis* ecology. Isolates collected from the environment, faeces, cow body, teat skin and intramammary infections were strain typed and compared to identify potential transmission of strains between these sites. Additionally, a more intensive study of strains found on the body of cows with clinical and subclinical mastitis was carried out.

Chapter 4 describes an intramammary challenge study performed using five strains of *S. uberis* infused individually or as a mixture into the mammary gland. This experiment was conducted to observe if selection of a predominant strain(s) occurs during the infection process. The production of bacteriocins by these five strains was also investigated as a potential means of direct competition between strains during infection of the mammary gland.

In the final chapter the findings of this study are discussed and conclusions drawn about *S. uberis* ecology on the New Zealand dairy farm and potential mechanisms of infection and selection of the infective strain during mastitis.

Chapter 1:

Bovine mastitis and *Streptococcus uberis*

1.1 The bovine mammary gland

The mammary gland is typically a sterile environment; therefore, entry of any foreign body usually results in a localised immune response leading to inflammation of the infected portion of the gland. This inflammatory response is commonly known as mastitis, and on most occasions arises as a result of infection by bacteria.

The bovine udder is divided into halves by the central supporting ligament and each half into quarters by a fine membrane (Bray & Shearer, 1993)(Figure 1.1). Each quarter contains a complex network of ducts, blood vessels and secretory tissue and is effectively closed off from all others, thus an infection in one quarter cannot be transferred internally to any of the other three quarters. The only route of transmission of bacteria infecting one quarter is through exiting the infected quarter and subsequent infection of another teat canal on an adjacent quarter.

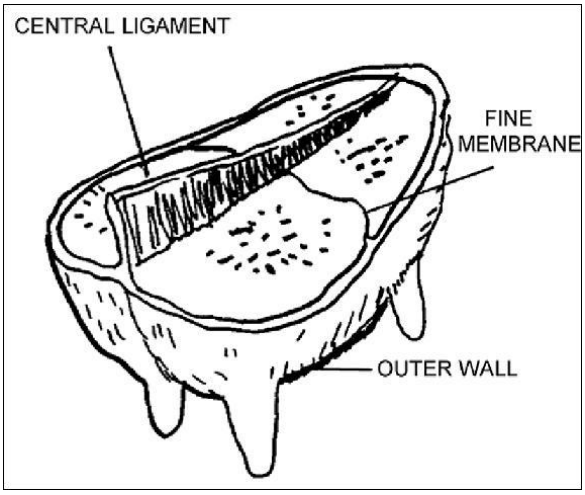


Figure 1.1. Four quarters of the bovine udder.
Diagram adapted from Bray and Shearer (1993).

Although it is assumed that the four quarters of an udder are independent, Adkinson *et al.* (1993) noted that quarters within a cow tended to be more alike than expected with regard to their natural susceptibility to mastitis

and Merle *et al.* (2007) found that infection of one udder quarter can influence the immune response of neighbouring quarters.

1.2 Subclinical and clinical mastitis

Bacterial invasion of the mammary gland can result in a clinical form of mastitis, characterised by the presence of visible signs of inflammation, or a subclinical form of the disease, with no outwardly visible signs of infection. Due to the lack of outward signs, the main indication of subclinical mastitis is an increase in the level of somatic cells in milk from the infected gland (Leigh, 1999). Somatic cells are a reflection of the inflammatory response to an intramammary infection and consist of lymphocytes, macrophages, neutrophils and some epithelial cells (Harmon, 1994). Milk produced from an uninfected gland normally contains somatic cells at levels less than 200,000 cells/ml of milk (Harmon, 1994). However, with a subclinically infected gland the cell count is usually in excess of 200,000 cells/ml. Although subclinically infected mammary glands can undergo spontaneous bacteriological cure (McDougall *et al.*, 2004; Zadoks *et al.*, 2003), bacteria can become established and persist as a chronic infection for many days or weeks, existing as the subclinical form of the disease with periodic, more acute, clinical episodes occurring from time to time.

When an infection is classed as clinical mastitis, visible abnormalities such as clots are apparent in the milk in addition to pain and swelling of the infected gland. In severe cases, there may be systemic signs such as elevated body temperature and loss of appetite; in some cases the infection may lead to death of the cow. The somatic cell count associated with a clinical mastitis infection is normally in excess of 2,000,000 cells/ml of milk (Leigh, 1999).

With both clinical and subclinical mastitis, inflammation of the infected gland results in decreased milk production and a reduction in milk quality.

Also, when an intramammary infection occurs during the non-lactating period, a reduction in the milk yield is observed in the subsequent lactation (Smith *et al.*, 1968).

1.3 Mastitis-causing bacteria

Bacteria that commonly cause mastitis are generally classified as either 'contagious' or 'environmental' pathogens, depending on the likely source of the pathogen and mode of transmission. The main contagious pathogens are *Staphylococcus aureus* and *Streptococcus agalactiae*. These pathogens have adapted to survive within the mammary gland and are spread from cow-to-cow at or around the time of milking (Bradley, 2002; Radostits *et al.*, 1994). Environmental pathogens are best described as opportunistic invaders. They are found within the cows' environment and are not thought to be adapted for survival within the mammary gland (Bradley, 2002). The main characteristic setting environmental and contagious pathogens apart is that non-contagious transmission can occur at other times and through exposure to other sources than the milking process (Zadoks *et al.*, 2001a).

Historically, mastitis was mainly caused by infection with contagious pathogens. However, during the 1960s, the implementation of antibiotic dry cow therapy, post-milking teat disinfection, and routine maintenance of milking machines, reduced the incidence of mastitis caused by these bacteria (Neave *et al.*, 1969). Although these control programs were efficient in reducing the problem of contagious pathogens, they were not as effective against environmental pathogens (Schukken *et al.*, 1990). It is hypothesised that the niche vacated by the contagious pathogens actually became occupied by the environmental mastitis pathogens (Jayarao *et al.*, 1999).

The main group of environmental pathogens known to cause mastitis are *Escherichia coli*, *Klebsiella pneumoniae, Streptococcus dysgalactiae* and *Streptococcus uberis* (Paulin-Curlee *et al.*, 2007; Todhunter *et al.*, 1995). Of

these environmental pathogens, *S. uberis* has been reported to be the most prevalent in pasture-based systems (Hillerton *et al.*, 1995).

1.4 *Streptococcus uberis*

A Gram-positive, catalase-negative cocci, the key biochemical diagnostic characteristics of *S. uberis* are the hydrolysis of hippurate and esculin (Watts, 1988) and acid production from inulin (McDonald & McDonald, 1976; Seeley, 1951; Watts, 1988). The *Streptococcus* genus is divided into groups, of which *S. uberis* is included in either the pyogenic group (Hardie & Whiley, 1997; Kawamura *et al.*, 1995) or the parapyogenic group (Bridge & Sneath, 1983), along with another environmental pathogen, *S. dysgalactiae*. Garvie and Bramley (1979) determined that the *S. uberis* species could actually be divided into two distinct groups, type I and type II; then, just over a decade later, the *S. uberis* type II group was reclassified as *S. parauberis* (Williams & Collins, 1990). Although the two species are genotypically different, phenotypically both species appear very similar making differentiation of *S. uberis* from *S. parauberis* difficult by biochemical and physiological tests (Jayarao *et al.*, 1991b). However, the two species are now easily differentiated based on their 16S rRNA (Forsman *et al.*, 1997; Williams & Collins, 1990).

Streptococcus uberis is one of the most significant causes of bovine mastitis in countries like New Zealand and Australia where the industry is pasture-based (Douglas *et al.*, 2000; McDougall *et al.*, 2004; Pankey *et al.*, 1996), with 63.2% of clinical mastitis cases being attributed to *S. uberis* in one New Zealand study (McDougall *et al.*, 2004). Another potential problem with *S. uberis* mastitis is that the infection often remains subclinical during long periods of time and, in the absence of treatment, this causes serious losses in milk production (Khan *et al.*, 2003). Not only is *S. uberis* found within the dairy environment (Bramley, 1982; Pullinger *et al.*, 2006; Zadoks *et al.*, 2005b), but it has also been isolated from the body of cows including the

skin, genital tract, rumen, tonsils, lips, udder, teat skin and faeces (Buddle *et al.*, 1988; Cullen & Little, 1969; Kruze & Bramley, 1982; Razavi-Rohani & Bramley, 1981; Zadoks *et al.*, 2005b). Therefore, minimising exposure of the mammary gland to this pathogen can be difficult.

Generally, *S. uberis* isolates from one infected quarter tend to be the same strain, indicating that infection with multiple strains of *S. uberis* within the same quarter is not common (Oliver *et al.*, 1998b; Phuektes *et al.*, 2001; Wieliczko *et al.*, 2002). However, when several herds were tested, no common strains were found (Phuektes *et al.*, 2001; Wieliczko *et al.*, 2002), indicating that the environment is the most likely source of infection by *S. uberis* rather than contagious transmission. Interestingly, Phuektes *et al.* (2001) found the same strain predominating over two lactations in one of the herds studied. Whether this strain was widespread in the environment and stable over time resulting in reinfection, or persistent in the chronically infected quarter between lactations, are questions that remain unanswered. This predominant strain of *S. uberis* appeared to be more virulent than other strains, with infection more likely to result in clinical mastitis. It was also found to be more resistant to antibiotics than any of the other *S. uberis* strains investigated (Phuektes *et al.*, 2001).

1.4.1 Incidence of *S. uberis* mastitis

Subclinical mastitis caused by *S. uberis* frequently occurs before parturition and during drying-off, whereas clinical mastitis caused by *S. uberis* is observed more frequently in the first five weeks of lactation (Jayarao *et al.*, 1999; Khan *et al.*, 2003). Buddle *et al.* (1988) found that the greatest numbers of *S. uberis* isolations from a herd in New Zealand were made during winter when rainfall was highest, average daily temperature was lowest and the cows had dried off. Other research however, has found that there is an increased incidence of mastitis during early or peak lactation, which is suggested to be a result of negative energy balance resulting in reduced

competence of the immune system (Zadoks *et al.*, 2001b). High-yielding cows are especially susceptible to mastitis, possibly because they are immunocompromised due to the physiological stress of lactogenesis (Pyorala, 2002). Also, milking machine factors such as vacuum pressure have been associated with the incidence rate of clinical mastitis caused by *S. uberis* (Barkema *et al.*, 1999), which is thought to be due to teat end damage thereby affecting the first line of defence for the mammary gland.

Some cows or quarters may be more susceptible to intramammary infection than others (Adkinson *et al.*, 1993; Batra *et al.*, 1977; Zadoks *et al.*, 2001a). The cow characteristics that influence susceptibility to mastitis include parity, stage of lactation and genetic make-up (Zadoks *et al.*, 2001b). Also, the incidence of clinical mastitis caused by any pathogen appears to be different for the four different quarters of the udder, with the rear two quarters more likely to be infected than the front two quarters (Adkinson *et al.*, 1993; Batra *et al.*, 1977; Zadoks *et al.*, 2001a). The effect of age of the cow on incidence of *S. uberis* mastitis has also been investigated and was found to have no influence over the number of *S. uberis* isolations (Buddle *et al.*, 1988).

In terms of reinfection, the incidence rate of mastitis in quarters that had recovered from *S. uberis* infection was 7.5-fold higher than the incidence rates in quarters that had never experienced *S. uberis* infection (Zadoks *et al.*, 2001b), indicating that recovery from infection does not confer immunity to reinfection with the same pathogen. In contrast, Phuektes *et al.* (2001) found that the prevalence of *S. uberis* infections decreased significantly from one lactation to the next in all herds that were studied.

1.4.2 Persistent intramammary infections

Persistent infections of the mammary gland can also occur (McDougall *et al.*, 2004; Milne *et al.*, 2005; Pullinger *et al.*, 2007; Wang *et al.*, 1999; Zadoks *et*

al., 2003), with infection continuing for up to 300 days in some cases (Pullinger *et al.*, 2007). The duration of infection appears mainly to be influenced by host factors such as age and lactation stage (Pullinger *et al.*, 2007), as cows were observed to have a longer duration of infection than heifers (McDougall *et al.*, 2004). When the strain types of both persistent and rapidly cleared infections were identified it was found that the same strain could cause both types of infection, indicating the lack of influence that the strain type has on the duration of infection (Pullinger *et al.*, 2007). While it is possible that persistent infections actually represent multiple acquisitions from the environment, this interpretation seems unlikely in view of the diversity of isolates found within herds; also, the majority of infections were found to be due to the same strain over weeks or months (Pullinger *et al.*, 2007; Wang *et al.*, 1999; Zadoks *et al.*, 2003). However, some chronic infection cases have been associated with different strains over time, suggesting that mammary quarters can clear one infection and become re-infected with a different strain within the same lactation (McDougall *et al.*, 2004). Therefore, persistent infections of *S. uberis* can occur; however, the duration of infection appears to be determined more by unique cow factors such as the immune response rather than pathogenicity of the strain that is infecting the mammary gland. Persistent infections with the same strain can fail to respond to conventional antibiotic treatment, while those infections caused by different strains over time can be more responsive (Milne *et al.*, 2005), indicating that there may be differences in the mammary gland when strains are persistent over long periods of time.

1.5 Udder defence mechanisms

The teat canal and associated keratin is the first line of defence against invading pathogens (Grindal *et al.*, 1991; Hogan *et al.*, 1988; Lacy-Hulbert & Hillerton, 1995). Keratin in the teat canal physically plugs the opening (Seykora & McDaniel, 1985) and also has antibacterial properties due to the

presence of lipids that are capable of inhibiting the growth of environmental pathogens such as *S. uberis* (Hogan *et al.*, 1988).

Capuco *et al.* (1992) found that partial removal of keratin from the teat canal rendered the mammary gland more susceptible to infection by *Streptococcus agalactiae*. In support of this, Lacy-Hulbert and Hillerton (1995) found the incidence of *S. uberis* infection was significantly higher among quarters with less keratin. Environmental pathogens, such as *S. uberis*, are less able than contagious bacteria to adapt and colonize the teat canal due to growth inhibition by fatty acids present in the keratin (Hogan *et al.*, 1988) and differences exist between *S. agalactiae* and *S. uberis* in mechanisms of transfer through the canal, with *S. uberis* passing through the teat canal in a shorter length of time than *S. agalactiae* (Lacy-Hulbert & Hillerton, 1995).

Passage of *S. uberis* through the teat canal is influenced more by the length of the canal than by the diameter (Lacy-Hulbert & Hillerton, 1995), but there is lack of agreement on relationships between other teat traits such as teat shape, milk flow rate and overall resistance to mastitis (Seykora & McDaniel, 1985). More work is required to examine interactions between *S. uberis* and keratin and transmission of this environmental pathogen through the teat canal.

1.5.1 Host immune response

After entry through the teat canal into the mammary gland, the first line of defence that is encountered is the non-specific innate immune defence system, including lysozyme, a bactericidal protein that lyses the cell wall of Gram-positive bacteria, and lactoferrin, an iron-binding glycoprotein that prevents supply of iron for bacterial growth. However, these compounds are thought to have only a minor role in preventing mastitis (Fang *et al.*, 1998a; Reiter, 1985; Smith & Oliver, 1981).

The main cellular defence mechanism includes macrophages, lymphocytes and in particular, neutrophils. After invasion of the mammary gland, bacteria spread rapidly within the udder and have been found intracellularly within macrophages (Thomas *et al.*, 1994) and neutrophils (Pedersen *et al.*, 2003), although the impact of neutrophils in the control of invading *S. uberis* has been questioned (Leigh *et al.*, 1999; Thomas *et al.*, 1994).

Antimicrobial peptides produced by the bovine mammary gland epithelium also have an important defensive role. One group of these peptides, known to have an antibacterial effect against both Gram positive and Gram negative bacteria, are known as the β-defensins (Ryan *et al.*, 1998). The level of β-defensin 1 has been shown to be constant; however, production of β-defensin 2 is stimulated by the presence of bacteria (Tunzi *et al.*, 2000).

These varied immune responses play an important role in defence of the mammary gland against invading bacterial species including *S. uberis*. McDougall *et al.* (2004) found that the majority of mammary quarters subclinically infected with *S. uberis* actually underwent spontaneous bacteriological cure. However, despite this host immune response, *S. uberis* can establish infection in the bovine mammary gland, which may in part be due to an array of virulence factors produced by this pathogen.

1.6 *Streptococcus uberis* virulence factors

Virulence factors can facilitate the establishment of bacteria in the host through mechanisms such as enhanced nutrient uptake and growth, adherence of the bacteria to cells in the mammary gland, invasion of the host tissue and providing protection against the host immune defences.

Several supposed virulence factors have been identified in *S. uberis*, with most of them having similarity to those found in other streptococci including the human pathogen, *Streptococcus pyogenes*.

1.6.1 Hyaluronic acid capsule

A particularly well-studied virulence factor is the hyaluronic acid (HA) capsule. These polysaccharide capsules, which are also present in the Group A streptococcus *S. pyogenes* (Wilson, 1959), are composed of repeating units of N-acetylglucosamine and glucuronic acid. Polysaccharide capsules have been shown to facilitate survival of many pathogenic bacteria by interfering with antibodies, complement, and phagocyte-mediated host defence mechanisms (Boulnois & Roberts, 1990).

The enzymes required to synthesise the HA capsule are hyaluronan synthase (encoded by the gene *hasA*) (Dougherty & van de Rijn, 1994), UDP-glucose dehydrogenase (*hasB*) (Dougherty & van de Rijn, 1993) and UDP-glucose pyrophosphorylase (*hasC*) (Crater *et al.*, 1995). In *S. pyogenes* the *hasC* gene is dispensable for hyaluronic acid biosynthesis as there is a homologue elsewhere in the genome (Ashbaugh *et al.*, 1998; DeAngelis & Weigel, 1994). In contrast, *S. uberis* has only one gene encoding UDP-glucose pyrophosphorylase; therefore the *hasC* gene, in addition to *hasA*, is necessary for synthesis of the HA capsule (Ward *et al.*, 2001).

The hyaluronic acid of the capsule is identical to the polysaccharide produced in higher animals and therefore is thought to be relatively nonimmunogenic (Dougherty & van de Rijn, 1994). Studies conducted both *in vitro* and *in vivo* have confirmed that the HA capsules of the human pathogen *S. pyogenes* allow these micro-organisms to resist phagocytosis by neutrophils upon invasion of the host (Gryllos *et al.*, 2001; Moses *et al.*, 1997; Wessels *et al.*, 1994).

The protection provided by the capsule for *S. uberis* invasion of the bovine mammary gland has been researched both *in vitro* and *in vivo* with conflicting results (Hill, 1988; Leigh & Field, 1991; Ward *et al.*, 2001). Hill (1988) investigated the *in vivo* pathogenicity of two strains of *S. uberis*, one that was encapsulated and the other that had no capsule. The encapsulated strain showed increased infectivity and virulence with experimental challenge of the lactating mammary gland compared with the acapsular strain. Likewise, Ward *et al.* (2001) studied the resistance of capsular and acapsular strains to phagocytosis by bovine neutrophils *in vitro* and found that the capsular strains were more resistant to killing by neutrophils than the acapsular strains. In contrast to these studies, Leigh and Field (1991) found that, although the resistance to phagocytic killing by neutrophils was enhanced by growth of *S. uberis* in media containing casein, this effect was not due to the HA capsule. Field *et al.* (2003) were able to reproduce the *in vitro* results of Ward *et al.* (2001), however, when they experimentally challenged the lactating bovine mammary gland with either an encapsulated, or genetically altered acapsular mutant, both strains caused similar levels of infection. Further investigation revealed that rather than the HA capsule, there was another extracellular factor produced by *S. uberis* that appeared to reduce the action of neutrophils (Field *et al.*, 2003).

To date, research appears to show that the HA capsule may only provide minimal protection against bovine neutrophils in the invasion of the bovine mammary gland *in vivo*. However, the HasABC genotype occurs at a higher frequency in isolates associated with disease, suggesting that the capsule is required for some aspects of pathogenesis (Field *et al.*, 2003). In addition, the *hasA* gene was found in the majority of clinical mastitis isolates, while *hasA*-negative isolates were more commonly associated with subclinical mastitis; therefore, carriage of the *hasA* gene could either have a direct influence on virulence or else it may correlate with the presence of other genes involved in virulence (Pullinger *et al.*, 2006).

1.6.2 Acquisition of essential amino acids

During infection of the mammary gland, *S. uberis* is found predominantly in the luminal areas of secretory alveoli and ductular tissue of the mammary gland (Thomas *et al.*, 1994); therefore, this pathogen must be able to grow in residual and newly synthesised milk. This environment is likely to be deficient in free and peptide-associated amino acids (Aston, 1975), and *S. uberis* is unable to specifically hydrolyse casein to release these essential amino acids (Kitt & Leigh, 1997; Leigh, 1993). However, this pathogen secretes an enzyme, plasminogen activator uberis A (PauA), which assists in this process.

PauA is a 30 kDa extracellular protein produced by *S. uberis* (Leigh, 1994) that specifically converts plasminogen (which occurs naturally in bovine milk) to the active serine protease, plasmin (Leigh, 1993; Leigh, 1994). Plasmin then hydrolyses casein, releasing the essential amino acids that can be taken up by *S. uberis* through the oligopeptide ABC transporter encoded with the Opp operon, another important factor allowing growth in milk (Smith *et al.*, 2002; Taylor *et al.*, 2003). *Streptococcus uberis* has also been shown to bind the active plasmin, allowing the pathogen to be in close proximity to the liberated amino acids (Leigh & Lincoln, 1997). Fang *et al.* (1998a) found that removal of casein from skim milk samples reduced bacterial growth, consistent with the idea that casein is used as a nutritional source for *S. uberis* through expression of the plasminogen activator.

Studies of 112 isolates of *S. uberis* from bovine milk showed that approximately 80% carried the *pauA* allele (Ward & Leigh, 2002), indicating that, although not essential, this gene may be involved in infection of the mammary gland. However, Ward *et al.* (2003) found that intramammary challenge with PauA-negative mutant strains of *S. uberis* showed little difference in the levels of inflammation, bacterial numbers in the milk or any discernable difference in the clinical signs associated with infection

compared with wild-type strains. This suggests that PauA may not play a major role in the ability of *S. uberis* to either grow in milk or infect the bovine mammary gland. At present, no firm conclusions can be made about the role of PauA as a virulence factor of *S. uberis*.

In addition to amino acid uptake, acquisition of metal ions such as manganese also appears to be important. The metal transporter uberis A (mtuA) gene product, which is involved in uptake of Mn^{2+}, was found to be essential for growth of *S. uberis* in milk both *in vitro* and in experimental infection of the bovine mammary gland (Smith *et al.*, 2003).

1.6.3 Adherence and internalisation into epithelial cells

Invading bacteria need to adhere within the mammary gland to resist the flushing effect of milk secretions (Oliver *et al.*, 1998a), and adherence of *S. uberis* to both ductular and secretory epithelia was observed in experimentally infected mammary glands (Thomas *et al.*, 1994). A number of factors have been suggested to aid adherence of *S. uberis* to mammary epithelial cells.

1.6.3.1 Lactoferrin binding protein

Lactoferrin is an iron-binding glycoprotein that is present as a non-specific antibacterial system in bovine milk (Smith & Oliver, 1981). The antibacterial action of lactoferrin is believed to be through iron deprivation, thus reducing bacterial growth (Arnold *et al.*, 1977). However, *S. uberis* has a minimal iron requirement and is therefore resistant to the antibacterial effects of lactoferrin (Smith & Oliver, 1981). Lactoferrin may actually be used as a bridging molecule for adherence of *S. uberis* to mammary epithelial cells (Fang *et al.*, 2000). Fang and Oliver (1999) found that all strains of *S. uberis* studied could bind lactoferrin, which in turn could bind to bovine mammary epithelial cells (Rejman *et al.*, 1994). Fang *et al.* (2000) found that *S. uberis* adherence to the bovine mammary epithelial cell line,

MAC-T, was enhanced in the presence of either purified lactoferrin or milk, indicating the involvement of lactoferrin in the adherence of *S. uberis* to epithelial cells.

Moshynskyy *et al.* (2003) also found that *S. uberis* expressed a specific lactoferrin-binding protein. However, in contrast to the reports by Fang *et al*. (2000), this group demonstrated that lactoferrin binding was not necessary to observe adherence of *S. uberis* to MAC-T cells. They suggest that other proteins must be expressed by *S. uberis* to mediate adherence to these host cells. More recently, a novel protein with affinity for lactoferrin was discovered and named *S. uberis* adhesion molecule (SUAM) (Almeida *et al.*, 2006). This protein appears to play an important role in adherence of *S. uberis* to mammary epithelial cells and also as a trigger for uptake of *S. uberis* by host cells.

1.6.3.2 Other binding proteins

The expression of surface-associated proteins of *S. uberis* is upregulated by the presence of extracellular matrix proteins (ECMPs) from the mammary gland, particularly collagen (Almeida *et al.*, 1999a; Almeida & Oliver, 2001), and by bovine mammary epithelial cells (Fang *et al.*, 1998b; Gilbert *et al.*, 1997). Pre-incubation with collagen has also been found to enhance the subsequent adherence and internalisation of *S. uberis* into mammary epithelial cells (Almeida *et al.*, 1999b; Almeida & Oliver, 2001), indicating that changes in surface-associated protein expression must occur upon exposure to collagen. *Streptococcus uberis* has been shown to invade epithelial cells and could be found in this intracellular location for up to 24 hours (Matthews *et al.*, 1994a). This invasion caused little damage to the host cells (Tamilselvam *et al.*, 2006) and is thought to provide protection for the micro-organism from the immune defence mechanisms in the mammary gland (Matthews *et al.*, 1994a).

Almeida *et al.* (1999a) noted that *S. uberis* exploited host proteoglycans to adhere and internalise bovine mammary cells. This attachment to epithelial cell surfaces using host factors as bridging molecules is a common mechanism among bacterial pathogens (Duensing *et al.*, 1999). It is thought to be a possible strategy to increase the efficiency of adherence in the very early stages of infection, before upregulation of binding protein expression occurs. It must be noted however, that experiments on adherence and invasion of bovine mammary epithelial cells by *S. uberis* have all been conducted *in vitro*. Whether these results are also true *in vivo* is yet to be determined.

The hyaluronic acid capsule has been investigated for its involvement in adherence of Group A streptococci to human pharyngeal epithelial cells. This adherence is believed to occur through the binding of hyaluronic acid to CD44, a hyaluronic acid-binding protein expressed on the surface of the epithelial cells in the human pharynx (Cywes & Wessels, 2001; Schrager *et al.*, 1998). Bovine mammary epithelial cells have also been shown to express CD44 (Bosworth *et al.*, 1991), suggesting the possibility that *S. uberis* may also adhere to the mammary epithelial cells by a similar mechanism. However, this has yet to be shown.

Overall, despite a lack of understanding of the processes involved, adherence and internalisation of *S. uberis* into mammary epithelial cells is thought to be an important virulence mechanism allowing protection from the flushing effects of milk and also providing protection from the host immune response.

1.7 *Streptococcus uberis* mastitis

In conclusion, *S. uberis* is one of the most prevalent environmental pathogens causing mastitis in pasture-based dairy cows. This bacterial species can be found throughout the dairy environment and on the cow

body; therefore, minimising exposure to this pathogen could be difficult. Both clinical and subclinical mastitis can occur after infection with *S. uberis*, with clinical cases more common during early lactation and subclinical infections commonly occurring during the dry period. Many potential virulence factors of *S. uberis* have been investigated by different groups with contrasting results. Hence, there are few consistent theories as to the methods of *S. uberis* establishment in the mammary gland and evasion of host immune defence systems. Questions remain as to whether there are particular reservoirs of *S. uberis* that have enhanced virulence and ability to cause mastitis compared with others.

The main objective of this study is to investigate the diversity of strains in the dairy environment, on the cow body and teats and compare with those causing mastitis. Reservoirs of particularly virulent strains can be identified by comparing similar strain types in each of these locations and the most likely means of strain transmission proposed. The second objective is to determine the process by which the infective strain is selected during the initial infection of the mammary gland. A suitable strain typing method is required to identify and differentiate between strains of *S. uberis*, therefore the initial aim of the study was to optimize and evaluate both a PCR-based method and mass spectrometry as strain typing methods for *S. uberis*.

Chapter 2:

Development of strain typing methods

Summary

A suitable method of strain typing is required to study *S. uberis* ecology. After evaluating commonly used bacterial strain typing techniques, a PCR-based method that utilised repetitive extragenic palindromic sequences within bacterial DNA (REP-PCR) was chosen and optimized. A variation of the REP-PCR method was also developed and called anchored typing. Both REP-PCR and anchored typing had a high discriminatory power but the combination of these two methods yielded the highest strain differentiation. A set of isolates was also strain typed by Multi-Locus Sequence Typing (MLST) and the results compared to those obtained by REP-PCR. Although REP-PCR had a high discriminatory power, the MLST method yielded even higher strain discrimination that could only be matched by combining both REP-PCR and anchored typing. The reproducibility of REP-PCR band patterns was also examined and the main concern identified as the reliance on agarose gels, a previously reported problem of PCR-based methods. In addition to PCR-based methods, MALDI-TOF mass spectrometry was investigated as a potential strain typing technique for *S. uberis*. Although strain-specific mass spectra were obtained with this method, the reproducibility of these mass spectra was a problem and further development of the method is required.

2.1 Introduction

2.1.1 Bacterial strain typing

Bacterial typing at the subspecies level is an essential tool for investigating the epidemiology of pathogenic bacteria. A strain is an isolate or group of isolates that can be distinguished from other isolates of the same genus and species by phenotypic characteristics or genotypic characteristics or both (Tenover *et al.*, 1995). A variety of methods have been used in an attempt to type strains of *S. uberis*. Prior to DNA-based methods, techniques such as phage typing (Hill & Brady, 1989), serotyping (Jayarao *et al.*, 1991a), antimicrobial susceptibility testing (Phuektes *et al.*, 2001) and bacteriocin-like inhibitor typing schemes (Buddle *et al.*, 1988; Jayarao *et al.*, 1991b) were used. These methods, although capable of broadly classifying strains of *S. uberis*, were found to be unsuitable for the discriminatory analysis of closely related strains (Oliver *et al.*, 1998b; Phuektes *et al.*, 2001). The use of DNA macrorestriction analysis methods such as restriction endonuclease fingerprinting (REF) (Hill & Leigh, 1989; Jayarao *et al.*, 1991b) and pulsed-field gel electrophoresis (PFGE) (Baseggio *et al.*, 1997; Douglas *et al.*, 2000; Khan *et al.*, 2003; Phuektes *et al.*, 2001) have shown more potential for this type of analysis. PCR-based strain typing techniques have also been utilised for *S. uberis* including the use of short random sequence primers and low stringency PCR for arbitrarily primed PCR (AP-PCR) and a randomly amplified polymorphic DNA (RAPD) assay (Gillespie *et al.*, 1998; Oliver *et al.*, 1998b; Zadoks *et al.*, 2003). PCR reactions based on repetitive elements within the prokaryotic genome (REP-PCR) (Wieliczko *et al.*, 2002) have also been performed. More recently, Multi-Locus Sequence Typing (MLST) (Coffey *et al.*, 2006; Pullinger *et al.*, 2006; Zadoks *et al.*, 2005a) and Multiple-Locus Variable-Number Tandem-Repeats Analysis (MLVA) (Gilbert *et al.*, 2006) have been employed to study *S. uberis* epidemiology.

A suitable strain typing method must be appropriate for the biological investigation to be carried out. To study *S. uberis* ecology, comparative typing is required, which assesses the relatedness of a set of isolates without reference to a database or library of previously strain typed isolates (van Belkum *et al.*, 2007). The strain typing method also requires typability, reproducibility and strain discrimination (Hunter & Gaston, 1988; van Belkum *et al.*, 2007). Typability refers to the ability to obtain unique typing markers for distinct bacterial strains, while discriminatory power refers to the ability of the method to distinguish between unrelated strains. Reproducibility is the ability to obtain similar results when the same isolate is repeatedly typed. The chosen *S. uberis* strain typing method needs to achieve all three of these important factors while remaining uncomplicated, rapid and economical to implement. To determine the best method for strain typing *S. uberis*, some of the available methods were reviewed (Table 2.1).

Method	Description	Analysis	Advantages	Disadvantages	References
REF	Digestion of chromosomal DNA with restriction enzyme.	Electrophoresis of fragments (2-24 kb) and compare band patterns	Simple to perform and discriminatory	Insufficient separation of large fragments on agarose gels	Hill and Leigh (1989) Jayarao *et al.* (1991b)
PFGE		Electrophoresis of fragments with an electrical field periodically changing in orientation	Accurately resolves large DNA fragments (>600 kb)	Time-consuming and expensive, requires specialised equipment	Baseggio *et al.* (1997) Douglas *et al.* (2000) Phuektes *et al.* (2001) McDougall *et al.* (2004)
AP-PCR/ RAPD	Short random sequence primers and low stringency PCR	Electrophoresis of PCR products and compare band patterns	Rapid, easy to perform. Easily accessible equipment.	Difficult to compare complex band patterns between gels. Poor reproducibility due to arbitrary priming of reactions.	Zadoks *et al.* (2003) Gillespie *et al.* (1998)
REP-PCR	Primers designed for repetitive elements within the genome. Stringent PCR conditions	Electrophoresis of PCR products and compare band patterns	More reproducible than AP-PCR/RAPD. Rapid and easy to perform.	May still be arbitrary priming. Reproducibility issues when comparing band patterns between agarose gels	Wieliczko *et al.* (2002)
MLST	PCR and sequencing of 6-8 housekeeping genes	DNA sequences compared between isolates	Does not require agarose gels, suitable for inter-laboratory comparisons. Highly discriminatory.	Method is expensive. May not capture content of virulence-associated genes associated with particular phenotypes.	Zadoks *et al.* (2005) Coffey *et al.* (2006) Pullinger *et al.* (2006)
MLVA	Evaluates tandem-repeat polymorphisms using PCR	Electrophoresis of PCR products and band size compared between isolates.	Discriminatory, fast and easy to perform. No reproducibility issues.	None noted to date	Gilbert *et al.* (2006)

Table 2.1. Comparison of some genotypic techniques previously used for strain typing *S. uberis*.

2.1.2 DNA-based methods

Both REF and PFGE have been used to strain type *S. uberis* and involve the digestion of isolated chromosomal DNA with a restriction enzyme and agarose gel electrophoresis of the strain-specific fragments (Baseggio *et al.*, 1997; Hill & Leigh, 1989; Jayarao *et al.*, 1991b). While REF utilises a conventional unidirectional electrical field, separating fragments between two and 24 kb in size, PFGE has the advantage of improved separation of large fragments (>600 kb) through the use of an electrical field that periodically changes in orientation. While REF was found to be fairly reproducible, it was not as discriminatory as PFGE and comparison of individual bands between strains was difficult due to insufficient separation on agarose gels (Hill & Leigh, 1989).

PFGE has been regarded as the 'gold-standard' in strain typing for certain bacterial species such as methicillin resistant *Staphylococcus aureus* (Stranden *et al.*, 2003) and Salmonella species (Fakhr *et al.*, 2005) due to the method having a high discriminatory power. However, the main disadvantage of the method is the time and specialised equipment required to obtain strain-specific band patterns. These constraints limit the number of isolates that can be strain typed (Baseggio *et al.*, 1997; McDougall *et al.*, 2004) and therefore is not suitable for large-scale *S. uberis* strain typing studies.

2.1.3 PCR-based methods

2.1.3.1 Arbitrarily-primed PCR

PCR-based methods have become popular due to the ease and speed that strain-specific band patterns can be obtained. Short random sequence primers can be used with low stringency PCR for AP-PCR and RAPD assays (Welsh & McClelland, 1990; Williams *et al.*, 1990). The primers anneal randomly, some within a few hundred base pairs of each other and on opposite strands, therefore sequences between these positions will be PCR amplifiable. The number and location of the random primer sites

varies for different strains of a bacterial species giving different band patterns when PCR products are electrophoresed on agarose gels (Caetano-Anolles, 1996).

Both Zadoks *et al.* (2003) and Gillespie *et al.* (1998) found that RAPD gave discrete DNA fingerprint patterns for *S. uberis* isolates. However, they found the agarose gels were often difficult to standardize and compare thereby making it difficult to interpret complex band patterns (Zadoks *et al.*, 2003). With each agarose gel that is prepared, inconsistency can occur due to variations in preparation such as the temperature of molten agarose when poured into the gel-casting apparatus and the room temperature in which the gel sets. Also, during electrophoresis, discrepancy may occur between agarose gels due to slight variations in buffer components and temperature. Artifactual variation of band patterns can also occur due to the arbitrary priming of the reactions. Differences in primer to template concentration ratio, annealing temperatures and magnesium ion concentrations in the PCR mixture (Ellsworth *et al.*, 1993; Kar *et al.*, 2006) can affect the resulting band pattern, therefore a high level of standardization and internal controls are necessary if the RAPD method is to provide reproducible results (Ellsworth *et al.*, 1993).

2.1.3.2 REP-PCR

REP-PCR relies on hybridization of primers to repetitive elements interspersed throughout prokaryotic genomes and amplification of differently sized DNA fragments between these elements (Versalovic *et al.*, 1991; Versalovic *et al.*, 1994). Unrelated organisms can be distinguished because individual bacterial strains vary with respect to the distances between the repetitive sequences (Versalovic *et al.*, 1994). As the primer sequences are homologous to defined sequences, stringent amplification conditions can be used, thus removing the arbitrary nature and potential variation inherent in the RAPD technique (Johnson & O'Bryan, 2000). The

repetitive sequences commonly used for bacterial strain typing are repetitive extragenic palindromic (REP) elements (Stern *et al.*, 1984), Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences (Hulton *et al.*, 1991) and BOX elements (Martin *et al.*, 1992).

Hermans *et al.* (1995) compared PFGE and REP-PCR (with a primer directed toward ERIC elements) for strain typing clinical isolates of *Streptococcus pneumoniae*. Although the discriminatory powers of the various techniques were found to differ, the resulting genetic clustering of the pneumococcal strains was comparable for each of the methods. Overall, REP-PCR typing was found to be both an easy and rapid method, especially in comparison to PFGE, and it was concluded that this technique was potentially very useful for strain typing (Hermans *et al.*, 1995).

2.1.3.3 REP, ERIC and BOX sequences

Between 0.5% and 1.0% of the total genome is devoted to REP elements that consist of a highly conserved 33 base pair inverted repeat sequence (Stern *et al.*, 1984). They have been utilised as efficient primer binding sites in PCR to produce fingerprints of genomes of both Gram-negative and Gram-positive microorganisms (Alam *et al.*, 1999; Del Vecchio *et al.*, 1995; Kang & Dunne, 2003; Versalovic *et al.*, 1991; Versalovic *et al.*, 1992) including *S. uberis* (Wieliczko *et al.*, 2002).

ERIC sequences are present in multiple copies throughout the genomes of enterobacteria (Hulton *et al.*, 1991). They are longer in length than REP elements at 126 base pairs (bp) and have no similarity to the REP sequence. ERIC sequences have been used for Gram-negative bacterial strain typing (Rasschaert *et al.*, 2005), and despite a lack of evidence for ERIC elements in Gram-positive bacteria, some research groups have utilised these elements for strain typing *Bacillus subtilis* (Versalovic *et al.*, 1992), viridans group streptococci (Alam *et al.*, 1999), and *S. pneumoniae* (Hermans *et al.*, 1995). It

has been suggested however, that ERIC-PCR performed on non-enterobacterial targets may actually be acting as a highly reproducible variant of the arbitrarily-primed RAPD method (Alam *et al.*, 1999; Gillings & Holley, 1997; Sander *et al.*, 1998).

BOX sequences were the first group of highly conserved repetitive DNA elements found in Gram-positive bacteria (Martin *et al.*, 1992). Located within intergenic regions of the *Streptococcus pneumoniae* genome, this element differs from REP and ERIC sequences in that it contains three highly conserved subunits, boxA (59 bp), boxB (45 bp) and boxC (50bp). BOX sequences have been used as a target for strain typing of *S. pneumoniae* (van Belkum *et al.*, 1996), yielding excellent discrimination between strains. However, DNA amplification could not be achieved across all streptococcal species, implying that the BOX element may be species-specific (Hermans *et al.*, 1995; van Belkum *et al.*, 1996) or that only some subunits rather than the entire BOX sequence may be conserved (Koeuth *et al.*, 1995).

2.1.4 MLST

Unlike the previously discussed methods, MLST does not rely on the use of agarose gels; therefore reproducibility problems due to gel-to-gel variation are eliminated. MLST involves PCR amplification and sequencing of a 450-500 bp internal fragment of six to eight housekeeping genes from each bacterial isolate (Enright & Spratt, 1999). The evolution of housekeeping genes is constrained by their requirement to encode functional products; therefore, the sequence of these genes can provide useful information about the genetic relationships between strains. Sequences are compared between bacterial isolates and differences of ≥ 1 nucleotide are assigned as different alleles. The alleles at the six to eight loci provide a profile which defines the sequence type (ST) of each isolate (Enright & Spratt, 1999).

The first MLST scheme was designed for *S. uberis* by Zadoks *et al.* (2005), and included sequencing of potential virulence genes along with housekeeping genes. Coffey *et al.* (2006) also developed a MLST scheme but noted that inclusion of virulence genes rendered it unsuitable for the analysis of population structure and evolutionary relationships; therefore a more conventional scheme was used that included only housekeeping genes. Both schemes resulted in high strain discrimination, exceeding that of RAPD typing, and showed excellent agreement with the epidemiological origin of isolates. Additionally, the value of MLST in terms of inter-laboratory comparisons and the global study of *S. uberis* epidemiology was highlighted (Pullinger *et al.*, 2006).

It has been noted however, that strains of *Streptococcus pneumoniae* and *Streptococcus agalactiae* assigned the same strain type by MLST, actually differed in gene content (Pettigrew *et al.*, 2006; Silva *et al.*, 2006; Tettelin *et al.*, 2005). Also, isolates with the same MLST type behaved differently in an infection model, indicating that genetic differences, undetected by MLST, may actually affect the phenotype. This is due to MLST focusing on the core genome rather than accessory genes that result in 'specialist' phenotypes (Turner & Feil, 2007). Therefore MLST may be more suited as a strain typing method for understanding global epidemiology rather than short-term epidemiological studies, where a strain typing method that characterizes rapidly accumulated genetic variation would be more useful (Enright & Spratt, 1999). Another disadvantage of the MLST method is the cost involved due to the sequencing of multiple PCR products for each isolate to be typed (Gilbert *et al.*, 2006); thus this method does not lend itself to large high-throughput studies of *S. uberis* epidemiology.

2.1.5 MLVA

The most recently developed strain typing method, Multiple-Locus Variable-Number Tandem-Repeats Analysis (MLVA) evaluates tandem

repeat polymorphisms within different bacterial strains. The number of repeat units at the same locus often varies from strain to strain and can be detected by PCR with flanking primers (Sabat *et al.*, 2003). This method has been used to type *Staphylococcus aureus* (Sabat *et al.*, 2003), *Escherichia coli* 0157:H7 (Lindstedt *et al.*, 2004) and most recently *S. uberis* (Gilbert *et al.*, 2006), with discriminatory power equally as suitable as PFGE or MLST (Malachowa *et al.*, 2005). The MLVA method was easy and rapid to perform and would have potential for use in short-term epidemiological studies.

2.1.6 Selection of a *S. uberis* strain typing method

It has become evident that high genetic diversity may exist between bacterial strains of the same species with Tettelin *et al.* (2005) finding only approximately 80% of any single genome was shared between six strains of *Streptococcus agalactiae*. The rest of the genome consisted of partially shared and strain-specific genes. This research group also found that commonly used strain classification techniques did not reflect the real genetic diversity described by the whole genome analysis, particularly when using methods that failed to take into account the variable genome in which many of the virulence-related genes might reside. As an environmental pathogen, it is also likely that *S. uberis* has a high genetic diversity between strains; therefore any strain typing method chosen may only reveal a small fraction of the genetic variety present. The only definitive method for discriminating between strains would be to sequence the entire genomes of the isolates in question; however, as this is not practical in large epidemiological studies, a more simple strain typing method needs to be chosen.

To study *S. uberis* ecology, the strain typing method is required to assess relatedness of isolates without reference to a definitive library of isolates (comparative typing). At the time this research was initiated the PCR-based

methods appeared most promising with the capability of discriminating between strains in a rapid and inexpensive manner. Due to observations of lower artifactual variation and improved reproducibility with REP-PCR rather than AP-PCR or RAPD, it was decided that REP-PCR would be optimized for use in this study.

2.1.7 MALDI-TOF mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) involves co-crystallisation of a sample with a UV-absorbing organic acid matrix (Karas *et al.*, 1987; Karas & Hillenkamp, 1988; Tanaka *et al.*, 1988). The analyte-matrix crystals are irradiated with a laser, volatizing and ionizing the analyte which is then accelerated through an electric field and its time-of-flight in a vacuum tube recorded. The mass of the analyte is proportional to the square of the flight time. Desorption of analytes of different masses results in a profile of peaks called a mass peak spectrum. MALDI-TOF MS can be used for the analysis of proteins, peptides, oligosaccharides and DNA (Hurst *et al.*, 1996; Papac *et al.*, 1996; Tanaka *et al.*, 1988).

2.1.7.1 MALDI-TOF MS for bacterial identification

MALDI-TOF mass spectrometry has been used for bacterial identification to both the species (Conway *et al.*, 2001; Holland *et al.*, 1996; Krishnamurthy & Ross, 1996; Mandrell *et al.*, 2005; Smole *et al.*, 2002; Wang *et al.*, 1998) and strain level (Bernardo *et al.*, 2002; Kumar *et al.*, 2004; Majcherczyk *et al.*, 2006; Rupf *et al.*, 2005; Satten *et al.*, 2004) and is based on the generation of mass spectra through the desorption of proteins and peptides specific to each bacterial species or strain. Many researchers have investigated the use of both intact cells and cellular lysates (Bernardo *et al.*, 2002; Chong *et al.*, 1997) to generate reproducible mass spectra. Both methods appear to generate a unique bacterial 'fingerprint' (Conway *et al.*, 2001; Gantt *et al.*, 1999; Holland *et al.*, 1996; Ruelle *et al.*, 2004); however, the intact cell method is

advantageous due to sample preparation being more rapid. In the intact cell method, the resulting mass spectrum is likely to be due to either peptides, proteins or other macromolecules, including complex carbohydrates (Bernardo *et al.*, 2002).

Although it is possible to desorb molecules weighing up to 500 kDa by MALDI-TOF MS (Gantt *et al.*, 1999), the majority of spectra are in the mass/charge (m/z) range of 2000-20,000, corresponding to protein masses of 2 – 20 kDa (Bernardo *et al.*, 2002; Demirev *et al.*, 1999; Gantt *et al.*, 1999; Ruelle *et al.*, 2004; Vaidyanathan *et al.*, 2002). Conway *et al.* (2001) attempted to detect larger molecules up to 100 kDa by varying instrument settings and preparation of bacteria, but were unsuccessful in obtaining any mass peaks greater than 25 kDa. In contrast, mass spectra were obtained in two mass ranges (2000 – 20,000 and 20,000 – 200,000 m/z) by Vaidyanathan *et al.* (2002) by modifying the sample preparation method. A database search revealed that the molecular mass distribution of known prokaryotic proteins has a peak centred around 12 kDa (Demirev *et al.*, 1999); therefore it may be expected that unique combinations of protein masses between 2 and 20 kDa may serve to identify bacteria.

2.1.7.2 Analysis of MALDI-TOF mass spectra

Three general approaches for the use of MALDI-TOF mass spectra have been developed for bacterial characterization. The first of these methods utilises specific biomarker proteins that are found to be indicative of a particular bacterial species (Holland *et al.*, 1999; Ruelle *et al.*, 2004). Although this method is not suitable for strain typing, it has potential for use in identifying bacterial species. Another approach, developed by Demirev *et al.* (1999), utilises a database of known proteins to identify organisms. For this, mass peaks in a spectrum of an unknown organism are compared against a database containing the sequence-derived molecular masses of all proteins present in known organisms. This method also

works well for bacterial species identification but is limited to the proteins that have been entered in the database; some bacterial species may be under-represented in the database, therefore are unlikely to be identified by this method (Rupf *et al.*, 2005).

The third approach for analysis relies on comparison of the mass spectra that are generated for each isolate to identify similar mass peaks. Those mass spectra that are highly similar are likely to have originated from the same strain (Holland *et al.*, 1996; Krishnamurthy & Ross, 1996). Of the three approaches to MALDI-TOF MS analysis, this final method appears to be the most useful for strain typing. A study of *E. coli* isolates using this method showed strain-specific differences in the mass spectra (Wang *et al.*, 1998). However, this method also requires the highest level of spectral reproducibility (Demirev *et al.*, 1999; Williams *et al.*, 2003); thus Wang *et al.* (1998), in their study of *E. coli* strains, found it difficult to verify the strain-based differences because reproducibility was compromised by slight variations in extraction, sample preparation and salt content.

2.1.7.3 Reproducibility of MALDI-TOF mass spectra

The reproducibility of MALDI-TOF mass spectra has been an issue in some studies (Conway *et al.*, 2001; Wang *et al.*, 1998), while in other studies good reproducibility was observed (Kumar *et al.*, 2004; Majcherczyk *et al.*, 2006; Mandrell *et al.*, 2005). The mass spectra from whole organisms can be quite complex and have poor reproducibility between spectra when compared to other mass spectrometry techniques (Satten *et al.*, 2004). Also, the sample preparation strategy has been found to be crucial to improve reproducibility (Marvin-Guy *et al.*, 2004). Even inconsistencies in the number of bacterial cells applied to the target plate can have an effect on the mass spectra and result in poor reproducibility (Williams *et al.*, 2003). Despite this, many researchers have noted good reproducibility of mass spectra. Majcherczyk *et al.* (2006) obtained highly reproducible spectra

when 12 replicates of *Staphylococcus aureus* isolates were strain typed and Kumar *et al.* (2004) found that 90% of the peaks in the mass spectra were reproduced in all replicates.

As well as experimental reproducibility, the 'biological' reproducibility also needs to be high since MALDI-TOF MS is sensitive enough to detect variations in proteins associated with growth phase and the culture media (Conway *et al.*, 2001; Holland *et al.*, 1999). These spectral variations are comparable to the variability observed with different strains, indicating the importance of using a standardised method of bacterial growth and preparation for strain typing to ensure high reproducibility (Majcherczyk *et al.*, 2006). In contrast, Mandrell *et al.* (2005) and Bernardo *et al.* (2002) noticed little difference in mass spectra produced from Campylobacter species and *Staphylococcus aureus* with different growth media and observed good repeatability between experiments. However, bacteria were only identified to the species level in both of these studies, therefore the spectra may have appeared less reproducible when comparing at a level required for strain differentiation.

Investigations have also been made into the inter-laboratory reproducibility of mass spectra using the same bacterial isolates. This type of comparison was difficult as the sample preparation techniques had to be exactly replicated (Williams *et al.*, 2003) and slight differences in instrument sensitivities and settings could lead to variations in mass spectra (Wang *et al.*, 1998). Walker *et al.* (2002) found lower reproducibility of mass spectra when comparing between two different machines in separate labs than when the same machine was used. With the same instrument, greater than 75% of peaks remained constant between experiments; however, between instruments the number of consistent peaks was reduced to approximately 60%. Therefore, at this stage a method involving MALDI-TOF MS is unlikely to be useful for a large-scale, inter-laboratory study of bacterial

strain typing. However, with care and standardisation, reproducibility of mass spectra can be achieved and strain typing of bacteria is possible within a laboratory.

2.1.7.4 Bacteria strain typed by intact cell MALDI-TOF MS

As yet, no reports have been found on the use of intact cell MALDI-TOF MS for strain typing of *S. uberis*; however, this technique has been used to differentiate strains of other streptococcal species such as *Streptococcus pneumoniae* (Satten *et al.*, 2004), beta haemolytic streptococci (Kumar *et al.*, 2004) and mutans streptococci (Rupf *et al.*, 2005).

2.1.7.5 Sample preparation methods

Many different sample preparation and MALDI-TOF MS methods have been used to obtain high quality mass spectra for bacterial identification. In general, a good mass spectrum contains a large number of peaks with good signal intensity, mass resolution and a high signal-to-noise ratio. To be useful for bacterial strain typing, these mass peaks also need to be strain-specific and reproducible (Ruelle *et al.*, 2004). Important variables in the sample preparation method include choice of matrix, solvents used for sample crystallisation and sample/matrix application methods to the MALDI target plate.

(i) Choice of matrix

The distribution of the analytes in the matrix crystals and the nature of the crystals are known to influence MALDI-TOF MS signals (Cohen & Chait, 1996; Ruelle *et al.*, 2004; Williams *et al.*, 2003). Many research groups have found Sinapinic acid to be superior to CHCA (α-cyano-4-hydroxycinnamic acid) due to better mass accuracy, less shot-to-shot variability and improved reproducibility of mass peaks between 5 and 10 kDa (Bernardo *et al.*, 2002; Conway *et al.*, 2001; Demirev *et al.*, 1999; Vaidyanathan *et al.*, 2002; Williams *et al.*, 2003). However, other researchers have chosen CHCA as the matrix

(Holland *et al.*, 1996; Kumar *et al.*, 2004; Ruelle *et al.*, 2004; Rupf *et al.*, 2005), indicating that the choice of matrix is user-specific and may depend on the bacterial species in question and other sample preparation and application methods.

(ii) Extraction solvents

Various experiments have revealed that the choice of extraction solvents has an effect on the resulting mass spectra (Ruelle *et al.*, 2004; Wang *et al.*, 1998) including differences in the number and intensity of protein mass peaks observed. Williams *et al.* (2003) found the use of acetonitrile as the solvent consistently provided better mass spectra between 3 and 14 kDa than did acetone, yielding a higher number and intensity of peaks. These researchers also found that inclusion of trifluoroacetic acid (TFA) resulted in a greater number of intense peaks compared to when ferulic acid was used.

(iii) Sample and matrix application methods

The method of applying sample and matrix to the target plate affects the mass spectra produced (Cohen & Chait, 1996; Dai *et al.*, 1999; Onnerfjord *et al.*, 1999; Ruelle *et al.*, 2004); therefore it is beneficial to optimize the sample and matrix application for each bacterial species. The ideal sample spot has a homogeneous layer of thin matrix and sample crystals (Landry *et al.*, 2000) and to achieve this, many different methods have been attempted. The most common technique is direct application of a sample-matrix mixture, also known as the "dried droplet" method. However, one disadvantage of this method is that the sample spots can crystallise inhomogeneously and suitable crystals or "sweet spots" within the dried spot have to be carefully chosen to obtain mass peaks of suitable intensity and reproducibility (Miliotis *et al.*, 2002; Onnerfjord *et al.*, 1999; Vaidyanathan *et al.*, 2002). Other sample/matrix deposition techniques include two-layer methods that basically consist of either a bacterial layer then a matrix layer (or vice versa) (Dai *et al.*, 1999), and three-layer methods, which are similar to two layer

except they may also include an application of ethanol to the dried spot (Ruelle *et al.*, 2004).

2.1.7.6 MALDI-TOF MS as a strain typing method

MALDI-TOF MS of intact cells has been used successfully in many bacterial identification and strain typing studies, including those for streptococcal species. Bacterial growth conditions, sample preparation and application to the target plate need to be determined and standardized for each bacterial species to be studied, so that optimal and reproducible mass spectra are produced. Overall, MALDI-TOF MS has been found to be a rapid and practical method for strain typing as bacteria can be taken from overnight colonies and processed within an hour to obtain mass spectra (Conway *et al.*, 2001; Holland *et al.*, 1996; Walker *et al.*, 2002). Therefore MALDI-TOF MS has potential for the strain typing of *S. uberis*.

2.1.8 Aims

The aim was to optimize and refine the REP-PCR method for strain typing *S. uberis*. The isolates of *S. uberis* to be typed and compared were from one farm, therefore a method suitable for comparative typing was required.

It has been established that an acceptable comparative strain typing method, while remaining a rapid, simple and low cost method, also needs to provide:

(i) Strain-specific band patterns

(ii) High discriminatory power

(iii) Good reproducibility to allow day-to-day comparisons.

The use of MALDI-TOF mass spectrometry was also evaluated as a comparative strain typing method for *S. uberis*.

2.2 Materials and Methods

2.2.1 General materials

Common laboratory chemicals were sourced from BDH laboratory supplies (Poole, Dorset, UK), Sigma-Aldrich Ltd. (Auckland, NZ), Ajax chemicals (NSW, Australia) and USB (Cleveland, OH, USA). Double distilled water, deionised using a Barnstead E-Pure water system (>18 mΩ; MQ-H_2O), was used to prepare all buffers and solutions except for electrophoresis buffers where double distilled (ddH_2O) water was used.

2.2.2 *Streptococcus uberis* isolates

Isolates of *S. uberis* were obtained from the milk of infected mammary quarters, vat milk, teat skin, keratin, the cow body, faeces, farm races, paddock soil and grass. Isolates were collected from Dexcel Lye farm (Appendix 1) over a two year time period using procedures described in Appendix 2. Known strains of *S. uberis* were also utilised including 0140J and EF20 (J. Leigh, previously of Institute of Animal Health, Compton, Berkshire, UK), and SR115 (S. McDougall, Animal Health Centre, Morrinsville, NZ).

2.2.3 DNA isolation

Single colonies of each *S. uberis* isolate were cultured on brain-heart infusion (BHI) agar (Difco; Becton Dickinson, Sparks, MD, USA) for 18-24 h at 37°C. A loopful of this culture was resuspended in 50 µl of lysozyme (10 mg/ml; Sigma-Aldrich Ltd., Auckland, NZ) in TE buffer (10 mM Tris-hydroxymethyl-methylamine (Tris); 1 mM Ethylenediaminetetraacetic acid (EDTA); pH 8.0) and incubated at 37°C for 30 min in a thermomixer (Eppendorf AG, Hamburg, Germany) at 750 rpm. Following this, 350 µl of lysis solution (100 mM Tris, pH 9.0; 50 mM EDTA; 100 mM NaCl; 1.0% sodium dodecyl sulfate (SDS)) was added and samples incubated at 65°C for 20 min to complete cell lysis. To the lysate, 350 µl of 5 M LiCl and 750 µl

chloroform were added and the biphasic solution rotary mixed for 30 min. Centrifugation at 16,000 x g for 10 min facilitated separation of the phases and the aqueous phase was collected and nucleic acids precipitated with an equal volume of isopropanol. The precipitated nucleic acids were pelleted by centrifugation at 16,000 x g for 20 min, washed with 1.0 ml of 70% ethanol and the final pellet resuspended in 50 µl of TE buffer. To assist the resuspension of the pellet, samples were incubated in a thermomixer for 1-3 hours at 37°C and 750 rpm.

2.2.4 DNA sample concentration and dilution

Two different methods were used to determine the concentration of DNA in each of the samples:

2.2.4.1 Gel-based method

Initially, a gel-based method was used whereby 5 µl of DNA sample was electrophoresed in a 1.0 % (w/v) agarose gel in TBE buffer as described in section 2.2.9. A 1 kb ladder (0.5 µg/lane; New England Biolabs, Ipswich, MA) was also included and the concentration of DNA in the sample estimated by comparing the relative intensity of the sample band with the standard DNA bands in the ladder. This comparison was facilitated by utilising the pixel intensity value obtained in the Scion Image program (Scion Corporation, MD, USA).

2.2.4.2 Nanodrop method

The concentration of nucleic acids in each sample was calculated by measuring absorbance at a wavelength of 260 nm using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The DNA samples had not been treated with an RNase previous to this so the absorbance measured actually reflected the total nucleic acids present, rather than DNA concentration.

All DNA samples were diluted in TE buffer as required and stored at 4°C until subsequent PCR analysis.

2.2.5 PCR master-mix preparation

Two PCR master-mixes with final concentrations of 5x (master-mix A) or 1x (master-mix B) were prepared and stored ready for addition to PCR reactions.

2.2.5.1 Master-mix A

Master-mix A (MM-A) was prepared to a 5x concentration by mixing 2.5 ml of 10x HotMaster™ *Taq* buffer containing 25 mM Mg^{2+}, pH 8.5 (Eppendorf AG, Hamburg, Germany), 10 µl of each of the deoxyribonucleotide triphosphates (dNTPs); dATP, dCTP, dGTP, dTTP (100mM; Invitrogen, Carlsbad, California), and 2.46 ml of sterile filtered (0.2 µm) MQ-H_2O. The final concentration of components in MM-A was 5x HotMaster™ buffer, 12.5 mM Mg^{2+}, and 200 µM of each dNTP. The prepared master-mix was divided into aliquots and stored at -20°C. MM-A (5 µl) was added to 25 µl PCR reactions to achieve a final concentration of 1x buffer, 2.5 mM Mg2+ and 40 µM dNTPs.

2.2.5.2 Master-mix B

Master-mix B (MM-B) was prepared to a 1x concentration by mixing 1.0 ml of 10x HotMaster™ *Taq* buffer containing 25 mM Mg^{2+}, 20 µl of each dNTP, and 8.92 ml of sterile filtered (0.2 µm) MQ-H_2O. To ensure all contaminating DNA was removed from the master-mix, 50 Units of DNase (Roche Diagnostics NZ, Ltd., Auckland, NZ) was added to the 10 ml mixture and incubated at 37°C for 30 minutes. DNase was inactivated by incubation at 80°C for 30 minutes and the master-mix divided into aliquots and stored at -20°C. The final concentration of components in MM-B was 1x HotMaster™ buffer, 2.5 mM Mg^{2+} and 200 µM of each dNTP. No further

dilution was required before use in PCR reactions; *Taq* polymerase, primers and DNA could be added directly to the thawed solution.

2.2.6 Oligonucleotide primers

Primer sequences (Table 2.2) for amplification of the 16S-23S rRNA intergenic spacer region (confirmation of *S. uberis*) were obtained from Forsman *et al.* (1997). A total of 13 previously published primers were tested for REP-PCR (Versalovic *et al.*, 1991; Versalovic *et al.*, 1994; Versalovic *et al.*, 1995; Wieliczko *et al.*, 2002). For anchored typing, primers were designed to amplify the streptokinase (SK) and metal transporter uberis A (mtuA) genes using Genamics Expression 1.1 (Genamics, Hamilton, NZ) based on the *S. uberis* gene sequence data for SK (skc gene; GenBank accession AJ006413) and mtuA (GenBank accession AJ539135). Designed primer sequences were entered into NetPrimer (www.premierbiosoft.com/netprimer) to check melting temperatures and possible secondary structures.

All primers were synthesised by Sigma-Aldrich Ltd. (Auckland, NZ) and reconstituted in TE buffer to 200 μM according to the concentration given on the information sheet received with the primer. Reconstituted primers were stored at -20°C until required and were used at a final concentration of 0.4 μM in 25 μl PCR reactions.

Method	Primers	Primer sequence (5′ – 3′)	Ref
***S. uberis* confirmation**	STRU-UbI	TAAGGAACACGTTGGTTAAG	1
	STRU-UbII	TCCAGTCCTTAGACCTTCT	
REP-PCR typing	REP-2	RCGYCTTATCMGGCCTAC	2
	REP1R-DT	IIINCGNCGNCATCNGGC	3
	REP2-I	ICGICTTATCIGGCCTAC	
	REP1R-I	IIIICGICGICATCIGGC	
	REP2-DT	NCGNCTTATCNGGCCTAC	4
	DRREP1	GCGGACTGGGACAGCTCG	
	DRREP1R	CGAGCTGTCCCAGTCCGC	
	ERIC2	AAGTAAGTGACTGGGGTGAGCG	
	ERIC1R	ATGTAAGCTCCTGGGGATTCAC	
	BOXB1	TTCGTCAGTTCTATCTACAACC	
	BOXC1	TGCGGCTAGCTTCCTAGTTTGC	
	BOXA1R	CTACGGCAAGGCGACGCTGACG	
	MBOREP1	CCGCCGTTGCCGCCGTTGCCGCCG	5
Anchored typing	MtuA-F	CCTATGGCGTTCCTTCAGCA	
	MtuA-R	CTGTCGCCGTTTTTTCCTTTC	
	SK-F	TGCAACGCTACCCTGACTATATG	
	SK-R	TTGAGTTTCACCGAGTTCTTTTCC	

Table 2.2. Oligonucleotide primers for *S. uberis* confirmation, REP-PCR and anchored typing. (1) Forsman *et al.* (1997); (2) Wieliczko *et al.* (2002); (3) Versalovic *et al.* (1991); (4) Versalovic *et al.* (1994); Versalovic *et al.* (1995). I = Inosine; M = A or C; N = A, G, C, or T; R = A or G; Y = C or T.

2.2.7 *Taq* DNA polymerase and thermal cycler

Two different brands of *Taq* DNA polymerase were used throughout this study: Thermo-Start® DNA polymerase (ABgene, Epsom, Surrey, UK) and HotMaster™ *Taq* DNA polymerase (Eppendorf, Hamburg, Germany). A PTC-100 Peltier thermal cycler (MJ Research Inc., Waltham, MA, USA) was used for PCR amplification unless otherwise indicated.

2.2.8 Confirmation of *S. uberis* DNA

To confirm bacterial isolates as *S. uberis*, the 16S-23S rRNA intergenic spacer region was amplified as described in Forsman *et al.* (1997). Prepared master-mix B (section 2.2.5.2) was used in 25 µl reactions with 0.4 µM of each primer (STRU-UbI, -UbII; Table 2.2), 0.625 U of HotMaster™ *Taq* DNA polymerase (Eppendorf AG, Hamburg, Germany) and 150-300 ng of DNA. Thermal cycling conditions involved an initial denaturation step of 94°C for 2 min followed by 40 cycles of 94°C for 20 sec, annealing at 55°C for 20 sec and extension at 68°C for 40 sec. Negative PCR controls were always included to check for contaminating DNA by preparing an identical PCR reaction without any DNA template. PCR products (10 µl) were electrophoresed in a 1.5% agarose gel along with a 100 bp ladder (Invitrogen, Carlsbad, California) as described in section 2.2.9.

2.2.9 Agarose gel electrophoresis

Agarose gels were prepared in two different types of buffer: TBE (90 mM Tris; 88 mM Boric acid; 2 mM EDTA; pH 8.0) or SB (0.45 M boric acid; ~0.11 M NaOH; pH 8.5). SB buffer was used as an alternative to TBE as SB showed superior resolution of DNA under high-voltage gel running conditions, with a faster run time (Brody & Kern, 2004). The general electrophoresis method was as follows: PCR products (normally 10 µl unless indicated) were mixed with 1 µl of loading dye (0.1 M Tris, pH 8.0; 0.1 M EDTA, pH 8.0; 1% SDS, 50% glycerol, 1% bromophenol blue) and electrophoresed in 1.5 – 1.8% (w/v) agarose gels containing 0.5 µg/ml ethidium bromide at 16 V/cm (SB) or 11 V/cm (TBE) in Owl electrophoresis tanks (Owl separation systems Inc, Portsmouth, NH, USA). A 100-bp DNA ladder (0.5 µg/lane; Invitrogen, Carlsbad, California or New England Biolabs, Ipswich, MA, USA) was included at least once on every gel. Bands were visualized by ultraviolet (UV) transillumination (312 nm), photographed with a high performance CCD camera (Cohu Inc., Electronics

division, San Diego, CA, USA) and saved as a bitmap of digitized densitometric values.

2.2.10 REP-PCR strain typing

Variations of PCR reaction components and primers were tested and are described in the results. In general, 25 µl PCR reactions were performed with either master-mix A or B, 25 different primer (Table 2.2) combinations, Thermo-start® or HotMaster™ *Taq* DNA polymerase and various DNA concentrations. Negative PCR controls were always included. Two different thermal cycling programs were used with initial denaturation/activation times adjusted depending on the DNA polymerase used. The activation times for Thermo-start® DNA polymerase are shown here as this was the main enzyme used.

2.2.10.1 REP-PCR Program 1

Six cycles of a denaturation/activation step of 94°C for 2 min, annealing (45°C, 20 s) and extension (68°C, 2 min). These initial activation cycles were followed by 40 amplification cycles of denaturation (94°C, 20 s); annealing (45°C, 20 s) and extension (68°C, 2 min). The temperature ramp rate before and after annealing remained at the default setting of the machine (~1.2°C/s).

2.2.10.2 REP-PCR Program 2

Initial activation/denaturation at 94°C for 7 min, 30 s followed by 40 cycles of denaturation (94°C, 20 s); annealing (45°C, 30 s) and extension (68°C, 2 min) and a final extension step of 72°C for 5 min. The temperature ramp rate before and after the annealing step was set to 0.5°C/s. PCR products (10 µl) were electrophoresed in 1.5% agarose gels prepared in SB or TBE buffer as described in section 2.2.9.

2.2.11 Anchored typing

As for REP-PCR, different primer combinations were tested in this study. In general, PCR reactions were prepared with master-mix B, 0.4 µM of each primer to be tested (as described in results), 1 U of *Taq* DNA polymerase (1 U/µl; Roche Diagnostics NZ, Ltd., Auckland, NZ), and approximately 200 ng DNA to a total volume of 25 µl. Amplification reactions were performed under the following conditions: An initial denaturation step of 94°C for 2 min followed by 40 cycles of 94°C for 20 s, 45°C for 30 s, and 68°C for 2 min. Ramp rate was the default setting for the machine. PCR products (10µl) were electrophoresed in 1.8% agarose gels prepared in SB buffer as described in section 2.2.9.

2.2.12 Analysis of band patterns

Agarose gels were photographed as described in section 2.2.9 and the bitmap of densitometric values for each gel transferred and stored as an image file of tagged image file format. REP-PCR and anchored typing band patterns were analyzed with GelComparII software (version 4.0; Applied Maths BVBA, Belgium) using the 100-bp ladder as the reference standard for normalization of each gel. The standard instructions within the GelComparII manual were used for preparation and normalization of all gels and the following settings were used: Spot removal set at 1 pts and background subtraction and least square filtering were applied based on the scores given with the spectral analysis of curves. Spot removal and background subtraction allowed gel scans with irregular background and spots or artifacts to be cleaned up to a certain extent, whereas the least square filtering applied to the smoothing of profiles and removal of any remaining background noise. Unless indicated otherwise, similarity between band patterns was determined using Pearson product-moment correlation (Pearson correlation) and a dendrogram created with the Unweighted Pair group Method using Arithmetic averages (UPGMA). Optimization values between 1% and 5% were used as indicated, which

allowed a shift between any two patterns to give the best possible matching of profiles.

Composite data sets were also generated that contained REP-PCR and anchored typing band patterns for each isolate. These composite data sets were compared using the 'Average of Experiments' option in GelComparII with equal weighting given to both REP-PCR and anchored typing band patterns. Comparison using the composite data set firstly involved calculation of individual similarity matrices using REP-PCR and anchored typing band patterns and then from these matrices, a combined matrix was determined by averaging the values.

2.2.13 Reproducibility of REP-PCR band patterns

Similarity between REP-PCR band patterns was calculated using Pearson correlation with an optimization of 1.5 – 2.0% and dendrograms created based on UPGMA. The similarity values for each pair of band pattern comparisons were imported into Microsoft Excel (2003) for further analysis and any significant difference in similarity values was calculated using a single factor analysis of variance (ANOVA).

2.2.14 MLST

DNA samples were sent to the Institute for Animal Health, Compton, Berkshire, UK for MLST using the scheme developed by Coffey *et al.* (2006). The actual methods used are described at the *S. uberis* MLST website (http://pubmlst.org/suberis/info/protocol.shtml) that is sited at the University of Oxford. Fragments of the following seven house-keeping genes were PCR amplified and sequenced: Glucose kinase (gki), transketolase (recP), D-ala-D-ala ligase (ddl), thymidine kinase (tdk), carabamate kinase (arcC), triosephosphate isomerase (tpi), and acetyl CoA acetyl-transferase (yqiL). ST numbers were assigned based on the allelic profiles.

2.2.15 BOX and ERIC sequences in *S. uberis* genome

Consensus BOX (Martin *et al.*, 1992) and ERIC (Hulton *et al.*, 1991) nucleotide sequences along with primer sequences directed towards these elements were used to search the genome sequence database of *S. uberis* strain 0140J produced by the *S. uberis* sequencing group at the Sanger Institute (http://www.sanger.ac.uk/Projects/s_uberis). To further explore the *S. uberis* genome the nucleotide sequence data was obtained (ftp://ftp.sanger.ac.uk/pub/pathogens/su) and viewed using the Microsoft Windows version of Artemis, release 8 (Rutherford *et al.*, 2000).

2.2.16 Discriminatory power of typing methods

To investigate the discriminatory power of REP-PCR, anchored typing and both methods combined, a numerical index of discriminatory ability (Simpson's index of diversity) was calculated using the following equation obtained from Hunter and Gaston (1988):

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j (n_j - 1)$$

Where N is the total number of isolates in the sample set, S is the total number of strain types found, and n_j is the number of isolates belonging to the jth type. As the calculated diversity indices are only estimates of the unknown true value for the population from which the sample was taken, confidence intervals were calculated using the following equation obtained from Gaia *et al.* (2005):

$$\sigma^2 = \frac{4}{N}\left\{\sum_{i=1}^{n}\left(\frac{x_i}{N}\right)^3 - \left[\sum_{i=1}^{n}\left(\frac{x_i}{N}\right)^2\right]^2\right\}$$

Where σ is the standard deviation, N is the total number of isolates, and x_i is the number of isolates in the ith category. Approximate 95% confidence intervals were then calculated by using the following formula:

$$95\%\,\mathrm{CI} = D - 1.96\sqrt{\sigma^2}, D + 1.96\sqrt{\sigma^2}$$

2.2.17 MALDI-TOF MS of intact bacterial cells

2.2.17.1 Preparation of *S. uberis* samples

Throughout these experiments, >18 mΩ deionised H_2O was used for preparation of reagents unless otherwise indicated. Isolates of *S. uberis* were cultured in either brain heart infusion (BHI) broth (Difco) or on BHI agar for 24 hours at 37°C. A 1.5 ml aliquot of each broth culture was centrifuged at 16,000 x g for 5 min and the cell pellet washed once with H_2O before resuspending in 1 ml of H_2O. When grown on agar, the culture was removed from the plate using a sterile wire loop and resuspended in 1.0 ml H_2O before washing as described above for the broth culture. The optical density at 610 nm (OD_{610}) of each culture was measured and adjusted to approximately 1.8 absorbance units (AU) with water. From this suspension, 0.8 ml was removed into a fresh tube and centrifuged at 16,000 x g for 5 min to pellet the cells. The bacterial pellet was washed once again with water and then all remaining water removed from the cell pellet. The bacterial pellet was resuspended in 150 µl of freshly prepared 50% acetonitrile (ACN) with 0.1% trifluoroacetic acid (TFA) and mixed well. These prepared samples were then stored at 4°C until ready to crystallise on the MALDI target plate.

2.2.17.2 Matrix preparation

Two matrix solutions were prepared by dissolving 3, 5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid (SA); Sigma-Aldrich, Auckland, NZ) in either 50% ACN/0.1% TFA (SA-ACN/TFA) or in acetone (SA-acetone) to obtain a saturated solution. To assist this process, solutions were sonicated for 10 minutes and then centrifuged 16,000 x g for 5 min to pellet any excess matrix.

2.2.17.3 Sample crystallisation on MALDI target plate

Unless indicated otherwise, the homogeneous, transparent phase of the freshly prepared SA-ACN/TFA matrix solution was mixed in a 1:1 ratio with the prepared bacterial samples. This mixture was applied to the target

plate using a two-layer method. For this method, 1 µl of the transparent phase of the SA-acetone matrix solution was applied to the target plate as the first layer and dried. This was followed by 1 µl of the 1:1 bacteria and SA-ACN/TFA mixture which was deposited over the top and air dried. At least two spots were prepared for each sample.

Protein calibration standards (Protein standard I; Bruker Daltonik GmbH, Liepzig, Germany) were prepared in 0.1% TFA and mixed with the SA-ACN/TFA matrix solution in a 1:1 ratio before applying to the target plate using the same application method as for the samples.

2.2.17.4 Mass spectrometry and analysis

MALDI-TOF mass spectrometry was performed on an AutoFlex II TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Liepzig, Germany) using linear mode with a mass/charge (m/z) range between 2000 and 14,000. The instrument was first calibrated using a standard mixture of proteins (Protein standard I, Bruker) before collecting positive ion spectra for each of the samples. The samples were desorbed with a 337 nm Nitrogen laser with power settings between 28-35% and the finalised spectra were a sum of up to 210 laser shots over 3 – 6 different positions on the sample surface. At all times a signal intensity of at least 50 counts/shot was maintained by adjusting the laser power. The spectra were analysed using FlexAnalysis software (Bruker Daltonik GmbH) and a modification of the centroid peak detection method to find peaks within 2000 – 20,000 m/z with a signal-to-noise ratio greater than eight after background subtraction. The resulting peak masses were recorded in a mass list. Unless indicated, all spectra were generated the same day that the samples were prepared and applied to the plate.

2.3 Results

2.3.1 Confirmation of *S. uberis* DNA

DNA from all isolates was confirmed as that of *S. uberis* by the presence of a 330 bp band (Figure 2.1) according to Forsman *et al.* (1997). Once confirmed, DNA samples were then used in further strain typing studies.

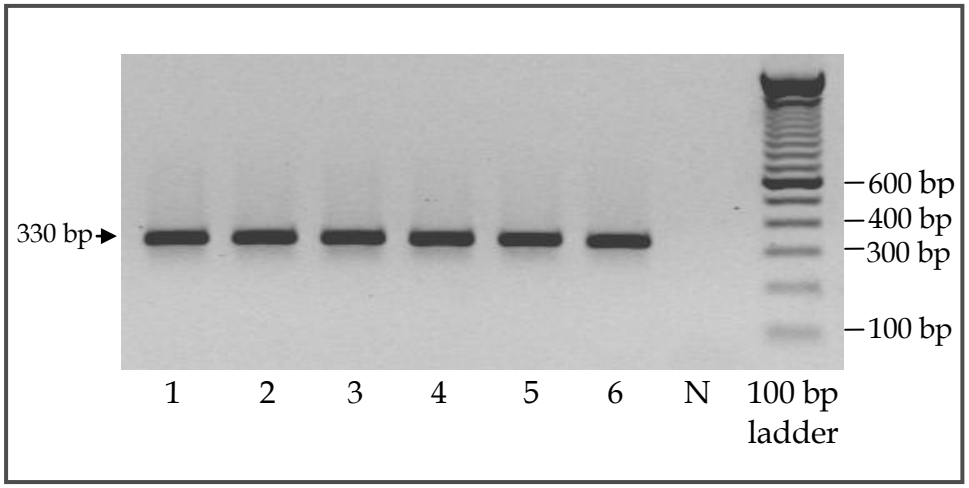


Figure 2.1. **Amplification of 16S-23S rRNA intergenic spacer region of *S. uberis*.** DNA was amplified from six potential *S. uberis* isolates (1-6). The 100 bp ladder and the negative PCR control (N) are indicated.

2.3.2 Optimizing the REP-PCR strain typing method

To optimize the REP-PCR strain typing method and obtain reproducible band patterns that differentiated strains of *S. uberis*, it was necessary to experiment with various features of the REP-PCR reactions and cycling conditions. The various parameters and factors that were investigated included: Combinations of primers directed towards REP, BOX or ERIC repetitive elements, primer annealing temperature, DNA template concentration, thermal cycling conditions, brand of *Taq* DNA polymerase used, and the concentration of deoxyribonucleotide triphosphates (dNTPs) in the PCR reaction mixture.

2.3.2.1 REP, BOX and ERIC primer combinations

Twenty-five different combinations of primers directed towards REP, BOX and ERIC elements were investigated with each combination being tested on ten or more *S. uberis* isolates obtained from mastitic milk, teat skin and farm race material. All primers were used in 25 µl PCR reactions at a final concentration of 0.4 µM along with MM-A, 0.625 U of Thermo-Start® DNA polymerase and 150-250 ng DNA. REP-PCR program 1 was used for amplification and PCR products electrophoresed in 1.5% agarose gels prepared in TBE buffer. The number of bands obtained with each of these combinations varied with a maximum of 6 bands observed (Table 2.3). No bands were produced when any primer was combined with BOXB1, MBOREP1 or REP1R-I. The best primer combinations were ERIC2-ERIC1R (2-4 bands), BOXC1-BOXA1R (3-6 bands), REP2-REP1R-DT (1-4 bands), and BOXA1R-ERIC1R (1-6 bands) as these gave the most unique band patterns and allowed adequate differentiation of the tested isolates (Figure 2.2). Of the primer combinations that appeared to be useful for strain typing of *S. uberis*, BOXC1-BOXA1R (Figure 2.2-B) and BOXA1R-ERIC1R (Figure 2.2-D) appeared the most promising as each of the isolates had a varied number of bands in different positions that would easily allow strain differentiation.

PRIMER 1													
REP2	REP1R-DT	REP2-DT	ERIC2	BOXB1	BOXC1	BOXA1R	ERIC1R	DRREP1	DRREP1R	MBOREP1	REP2-I	REP1R-I	
	1-4												REP2
		0-1				**0-2**	**1-2**						REP1R-DT
						1	**0-1**		**0-1**			**0**	REP2-DT
				1-2		**0-1**	**2-4**				**0-2**		ERIC2
						0	**0**						BOXB1
						3-6	**0-1**						BOXC1
							1-6	**0-1**				**0**	BOXA1R
											0		ERIC1R
									0			**0**	DRREP1
										0		**0**	DRREP1R
													MBOREP1
												0	REP2-I

PRIMER 2

Table 2.3. Number of bands obtained with different primer combinations. Each primer combination gave either no bands (0) or up to 6 bands. Grey boxes indicate primer combinations that gave the most discriminatory band patterns.

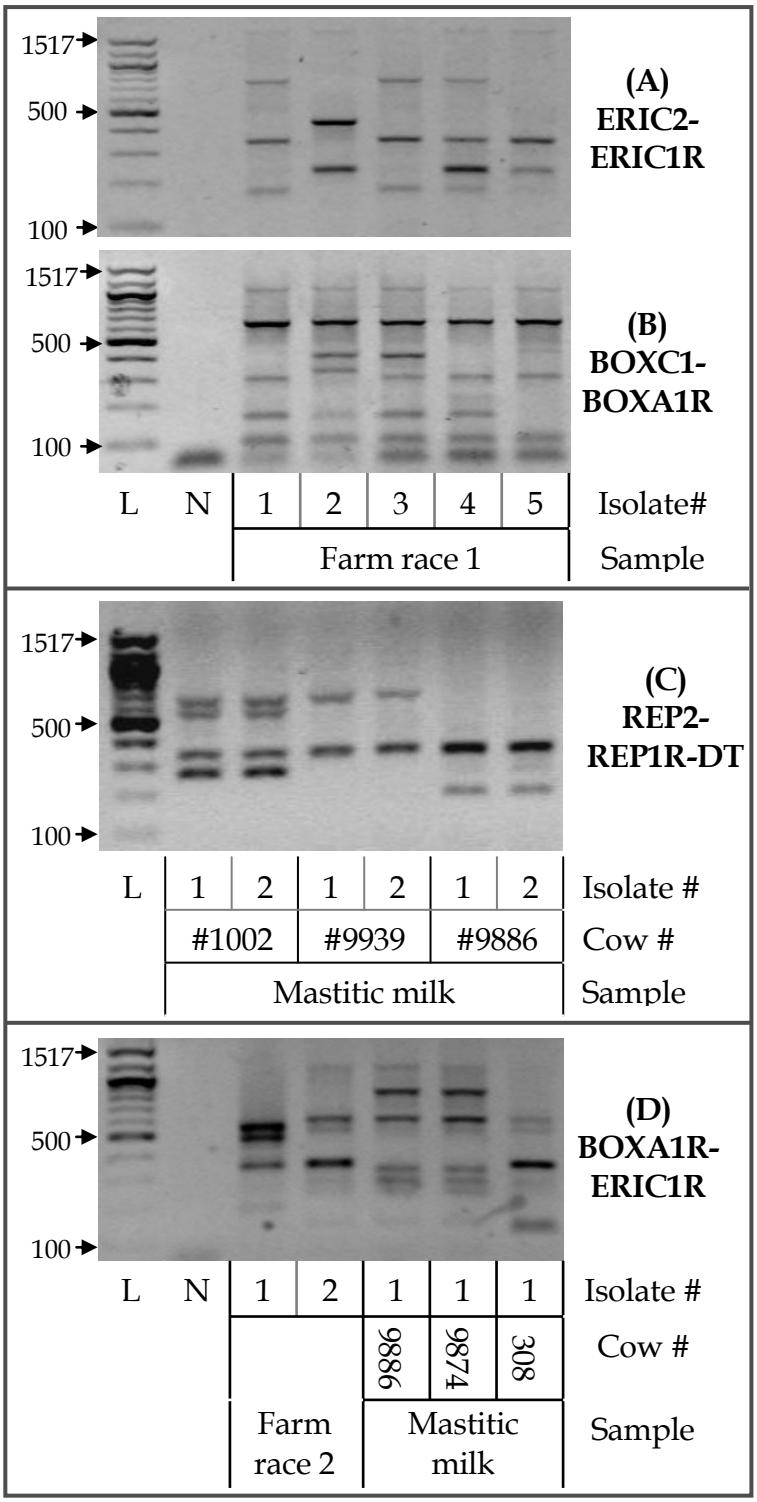


Figure 2.2. Band patterns produced with optimal primer combinations. (A) ERIC2-ERIC1R, (B) BOXC1-BOXA1R, (C) REP2-REP1R-DT and (D) BOXA1R-ERIC1R. The same DNA samples were used for both (A) and (B). Different DNA samples were used in (C) and (D) except for mastitic milk isolate #1 from cow #9886. L = 100 bp DNA ladder (NEB) with DNA fragment sizes (in bp) indicated. N = Negative PCR control.

The ERIC2, ERIC1R and BOXA1R primers were also tested singularly on three *S. uberis* isolates, one from mastitic milk and two from the teat skin of the same cow (#1033). PCR reactions consisted of MM-A with 0.4 μM of one or two primers, 1.25 U of Thermo-Start® DNA polymerase and 200 ng DNA. REP-PCR program 2 was used and PCR products electrophoresed in 1.5% agarose gels prepared in SB buffer. Resulting band patterns were compared with those obtained when these primers were used in combination (Figure 2.3). A maximum of three bands (1 – 2 major bands) were obtained when only one primer was used compared to the more complex band pattern obtained when primers were combined. Consequently, a combination of primers may be more suitable for the differentiation of *S. uberis* strain types. In this case, all three of the isolates were from mastitic milk and teat skin of the same quarter, therefore were likely to be the same strain type, as confirmed by the highly similar band patterns obtained with both BOXA1R-ERIC1R and ERIC2-ERIC1R primer combinations.

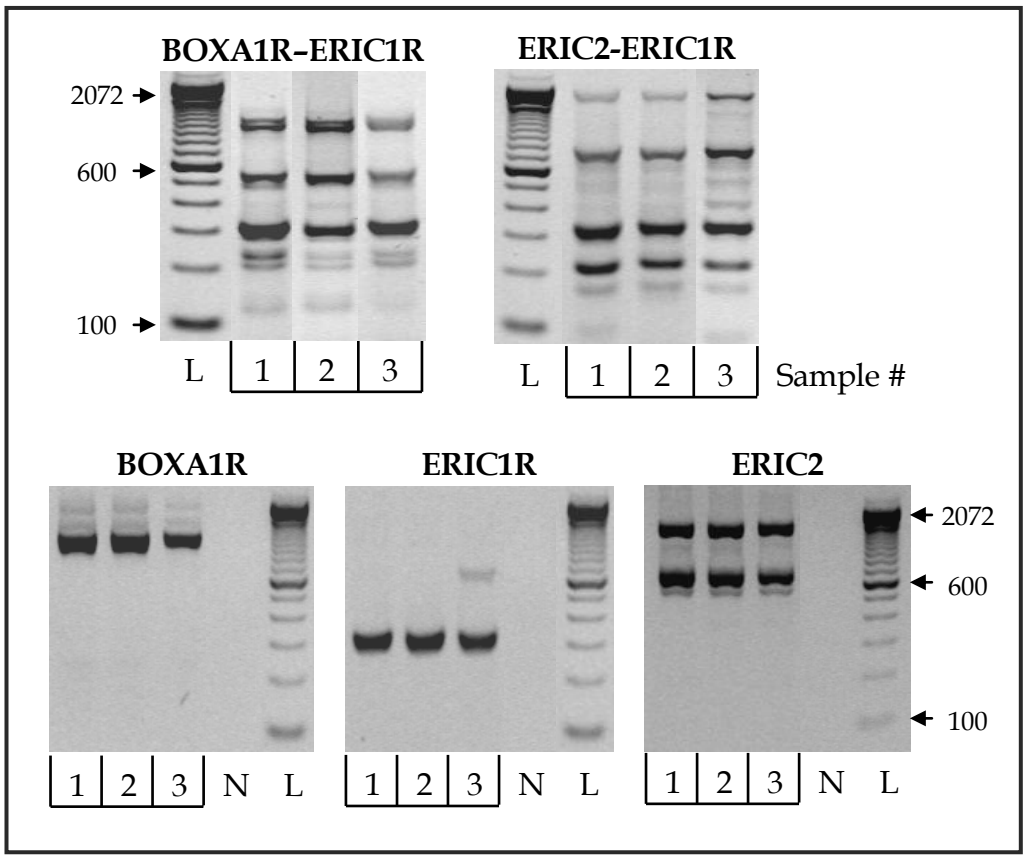


Figure 2.3. Band patterns obtained using one or two primers. Three *S. uberis* DNA samples were amplified with primer combinations BOXA1R-ERIC1R or ERIC2-ERIC1R, or with BOXA1R, ERIC1R or ERIC2 primers individually. Sample 1 = mastitic milk isolate; 2 and 3 = teat skin isolates. Some of the gel photos displayed are a composite of photos taken separately. The 100 bp ladder (L; Invitrogen) was included on every gel, and several fragment sizes (bp) are indicated. The negative PCR control (N) is shown for PCR reactions with individual primers.

2.3.2.2 Searching for BOX and ERIC elements in the genome

As amplification products were generated using primers directed toward BOX and ERIC elements, these DNA sequences may be present within the *S. uberis* genome. To investigate this further, the sequenced *S. uberis* genome (strain 0140J) was searched to identify regions where the BOXA1R, BOXB1, BOXC1, ERIC2 and ERIC1R primers may be binding (Table 2.4; Appendix 3).

		Best match	
Primer	**# Matches to genome**	**Homology**	**Region of primer**
BOXB1	1	50%	5′
BOXC1	4	50%	3′
BOXA1R	4	68%	5′
ERIC2	4	82%	3′
ERIC1R	5	82%	3′

Table 2.4. Homology observed between primers and the *S. uberis* genome. Sequence alignments can be found in Appendix 3.

One match was found in the *S. uberis* genome when searching with the BOXB1 primer sequence and only 11 of the 22 primer nucleotides were identical to the genomic DNA sequence, giving an overall homology of 50%. This region of homology was at the 5′ end of the primer which, under stringent conditions, would not lead to priming of the amplification reaction (as priming occurs from the 3′ end of the primer), which may explain the lack of PCR products when this primer was used. BOXC1 and BOXA1R were homologous to four different regions of the *S. uberis* genome respectively, but again similarities of only 50-55% and 50-68% were obtained for each primer. However, while 50% homology was observed close to the 3′ end of the BOXC1 primer, homologies between BOXA1R and the genome were all closer to the 5′ end of the primer, indicating that non-specific binding may have occurred to result in amplification with these primers.

Searching the *S. uberis* genome with the ERIC2 primer sequence resulted in 4 matches, again three of the matches were only short stretches but one match spanned the entire length of the primer, with a few mismatches, giving a homology 82%. The similarity of the primer to the 0140J sequence indicates that this primer may specifically bind to this region of DNA, even

under stringent conditions, resulting in amplification. Likewise, the ERIC1R primer had five matches with the genome, with homologies ranging from 50% to 82%. Three of these matches were actually between the 3' end of the primer and the genome sequence, again suggesting that priming could occur from ERIC1R when hybridized to the 0140J genome under stringent conditions. Overall, the only homologous regions found at the 3' end of the primers were for BOXC1, ERIC2 and ERIC1R. Amplification with the other primers was most likely due to non-specific hybridisation of the primers under non-stringent PCR conditions.

The regions of the genome where the primers may possibly be binding were further explored by viewing *S. uberis* sequence data with Artemis. The nucleotide sequences where BOX primers may anneal were found but no surrounding sequences corresponded with any other part of the BOX elements (data not shown). Likewise for the ERIC primers, the regions of the genome showing highest homology to the ERIC primers did not contain any evidence of the ERIC consensus sequence or the core inverted repeat that is found in all ERIC sequences (Hulton *et al.*, 1991). If this core inverted repeat was used in a BLAST search of the *S. uberis* genome, no significant matches were found (data not shown). Therefore, it can be concluded that although the BOX and ERIC primers may bind to *S. uberis* DNA, it is unlikely that they are binding to BOX and ERIC repetitive elements previously characterised in *S. pneumoniae* and Enterobacteria respectively. Instead, they may actually be arbitrarily binding to short homologous regions within the *S. uberis* genome.

2.3.2.3 Primer annealing temperature

The annealing temperature used during PCR amplification determines the specificity of primer hybridization. Based on previous work (Wieliczko *et al.*, 2002) an annealing temperature of 45°C was initially used for this REP-PCR strain typing method. However, a low annealing temperature may result in the production of non-specific bands that occur randomly and result in irreproducible band patterns. To investigate the effect of various annealing temperatures on the BOXA1R-ERIC1R band pattern, a single DNA sample was amplified in identical PCR reactions at 11 different annealing temperatures ranging from 40–60°C. Each PCR reaction contained MM-A, 0.4 μM BOXA1R and ERIC1R primers, 1.25 U Thermo-Start DNA polymerase and 200 ng DNA. PCR was performed using the gradient function of a PTC-200 thermal cycler (MJ Research, Inc., Waltham, MA, USA) and REP-PCR program 2. Products were electrophoresed in a 1.5% agarose gel prepared in SB buffer (Figure 2.4).

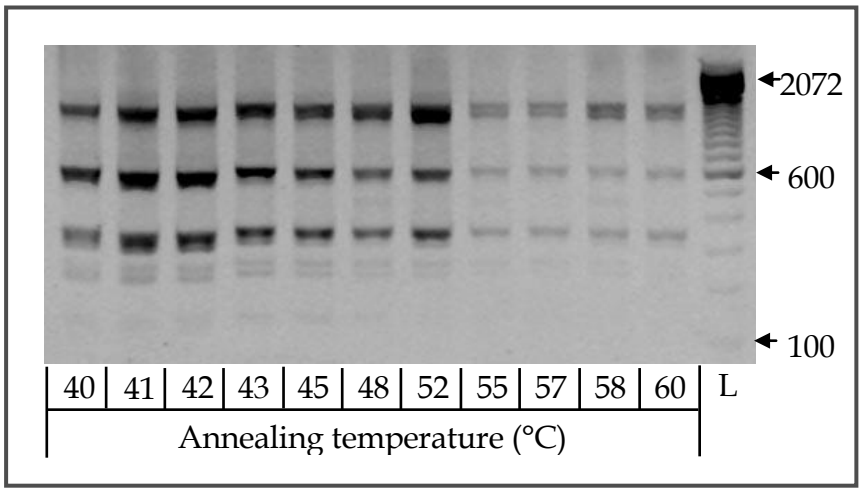


Figure 2.4. REP-PCR band patterns obtained with annealing temperatures of 40 – 60°C. L = 100 bp ladder.

Elevation of the annealing temperature above 52°C resulted in a decreased band intensity, but had little affect on the number of bands observed. Similar band patterns at both 45°C and 60°C indicates that these bands may be the result of specific hybridization of the primers to sequences within the DNA template.

The annealing temperature may also affect the reproducibility of the band patterns with a low temperature giving irreproducible bands between PCR runs. Three *S. uberis* isolates obtained from the farm race, faeces and vat milk were used to test the reproducibility of band patterns when different annealing temperatures were used. Three identical PCR reactions were prepared with BOXA1R and ERIC1R primers and amplified with REP-PCR program 2 and annealing temperatures of 45, 50 and 55°C on two separate days with a PTC-200 thermal cycler. Both sets of PCR products were electrophoresed on the same 1.5% agarose gel prepared in SB buffer and the band patterns compared (Figure 2.5). Negative PCR controls were included for each PCR reaction and no bands were apparent other than primer dimers below 100 bp (data not shown).

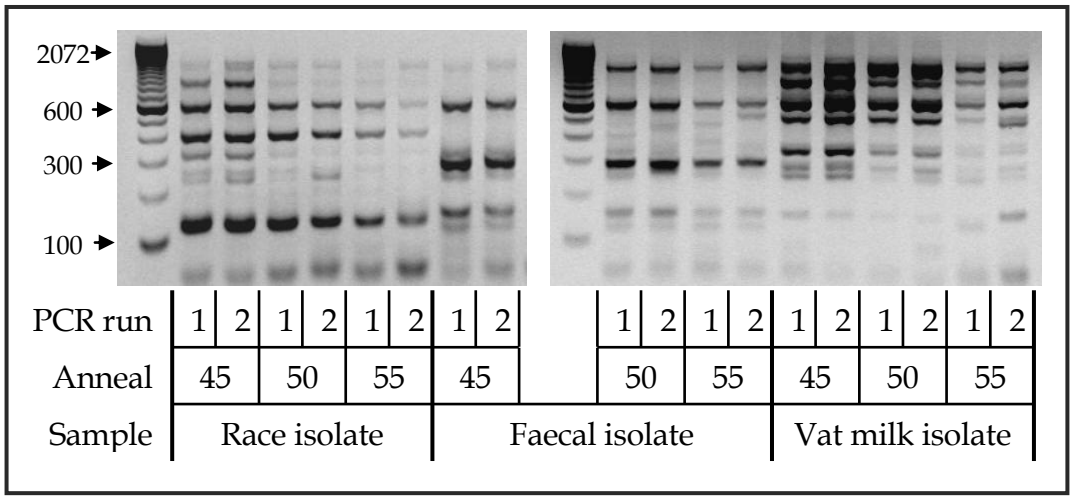


Figure 2.5. Band pattern reproducibility at three annealing temperatures. Three *S. uberis* isolates were amplified with annealing temperatures of 45, 50 and 55°C on two separate days (PCR run 1 and 2). A 100 bp ladder was included in the left lane of each gel.

Again the band intensity, but not the number of observed bands, decreases with elevation of annealing temperature. Very little difference was apparent between the high intensity bands obtained on different days, indicating that annealing temperature had little affect on the reproducibility of band patterns. However, a slight variation in the position of faint bands was observed between days: for example, the faecal isolate had different

faint bands between PCR runs at an annealing temperature of 55°C. This suggests that the major bands produced with BOXA1R-ERIC1R primers may be due to specific hybridization of the primers, while the faint, less reproducible bands are due to non-specific hybridization and could vary between PCR runs.

2.3.2.4 DNA template concentration

The effect of DNA concentration on the REP-PCR band pattern was investigated using DNA isolated from four *S. uberis* isolates (two isolates from a teat skin sample and two from a faecal sample). DNA samples (5 µl) were electrophoresed in a 1.0% gel in TBE buffer (Figure 2.6, A) and the DNA concentrations estimated to be approximately 80 ng/µl in each of the two teat isolates and 800 ng/µl in the two faecal DNA samples. A total of 200 ng and 2 µg of each teat DNA and faecal DNA samples respectively were amplified in 25 µl PCR reactions with MM-A, 0.4 µM REP2 and REP1R-DT primers and 1.25 U Thermo-Start DNA polymerase using REP-PCR program 2. PCR products were electrophoresed in a 1.5% gel in TBE buffer (Figure 2.6, B). While band patterns were obtained for the two teat isolates, no bands were obtained using the DNA from the two faecal isolates, suggesting that the higher DNA concentration in the faecal DNA samples inhibited the PCR reaction. Alternatively, substances inhibitory to PCR may have been present in these samples.

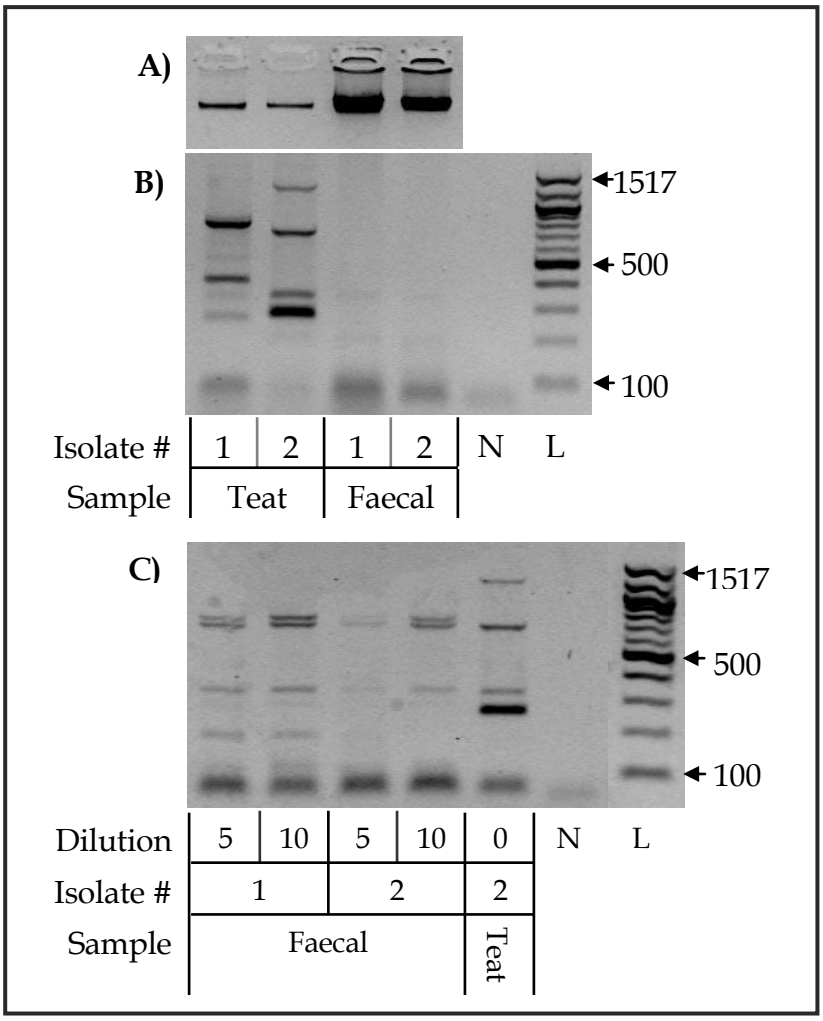


Figure 2.6. Effect of DNA concentration on REP-PCR band patterns. DNA samples from teat and faecal isolates were electrophoresed (A) and amplified with REP2-REP1R-DT primers using undiluted DNA (B) or 5- and 10-fold dilutions of the DNA (C). N = negative PCR control. L = 100 bp ladder (NEB).

The faecal isolates were diluted 5- and 10-fold in TE buffer, amplified again and the band patterns compared to one of the undiluted teat skin isolates (Figure 2.6, C). Unlike the undiluted DNA, both the 5- and 10-fold dilutions gave band patterns with electrophoresis of PCR products with higher intensity bands observed with the 10-fold dilution than with the 5-fold dilution. Therefore, approximately 200 ng of DNA appears to be optimal in these REP-PCR reactions.

2.3.2.5 Thermal cycling conditions

The effect of thermal cycling conditions on REP-PCR band patterns was investigated by testing two different PCR programs; REP-PCR program 1 (section 2.2.10.1) and REP-PCR program 2 (section 2.2.10.2) with four *S. uberis* isolates from the faeces, teat skin and farm race. Duplicate PCR reactions were prepared containing MM-A, 0.4 µM ERIC2 and ERIC1R primers, 1.25 U Thermo-Start DNA polymerase and 200 ng DNA and PCR products were electrophoresed in 1.5% agarose gels in TBE buffer (Figure 2.7). Up to five bands were produced with program 2 compared to only two bands with program 1. A noticeable feature of band patterns produced with program 1 was the lack of bands above 500 bp whereas with program 2, bands were observed up to 1000 bp.

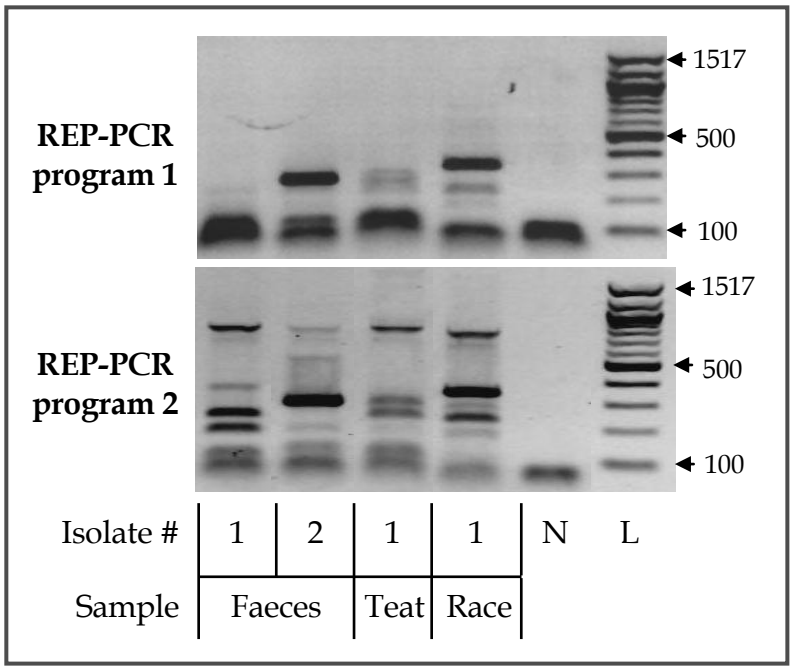


Figure 2.7. Band patterns produced with different PCR cycling conditions. Duplicate PCR reactions were amplified with either REP-PCR program 1 or program 2. L = 100 bp DNA ladder (NEB). N = Negative control for PCR reactions.

The main difference between the two programs was a slow temperature ramping (0.5°C/s) both before and after the annealing step in program 2 compared to the default settings of ~1.2°C/s in program 1. Also, the time allowed for primer annealing was extended slightly in program 2. These

small changes appear to increase the number of bands observed when these *S. uberis* isolates were amplified, allowing the production of more discriminatory band patterns. Therefore, REP-PCR program 2 was chosen for strain typing *S. uberis*.

2.3.2.6 Brand of *Taq* DNA polymerase

Two brands of *Taq* DNA polymerase, HotMaster™ Taq DNA polymerase (Eppendorf AG, Hamburg, Germany) and Thermo-Start® DNA polymerase (Abgene, Epsom, Surrey, UK), were tested with two different primer sets, ERIC2-ERIC1R and BOXA1R-ERIC1R to investigate any effect on REP-PCR band patterns. DNA samples were obtained from four faecal isolates and 200 ng used in 25 µl PCR reactions with MM-A, 0.4 µM primers, and 1.25 U of either brand of DNA polymerase. REP-PCR program 2 was used with the 94°C activation time adjusted to 2 min when HotMaster *Taq* polymerase was used and PCR products electrophoresed in 1.5% agarose gels in TBE buffer. The band patterns obtained with either HotMaster or Thermo-Start DNA polymerase were very similar for each primer combination tested (Figure 2.8). The main difference observed was variation in the presence and position of faint bands within the pattern. For example isolate #1 amplified with BOXA1R-ERIC1R primers had more faint bands with Thermo-Start DNA polymerase than with the HotMaster brand. The intensity of the bands was also slightly different for each DNA polymerase used.

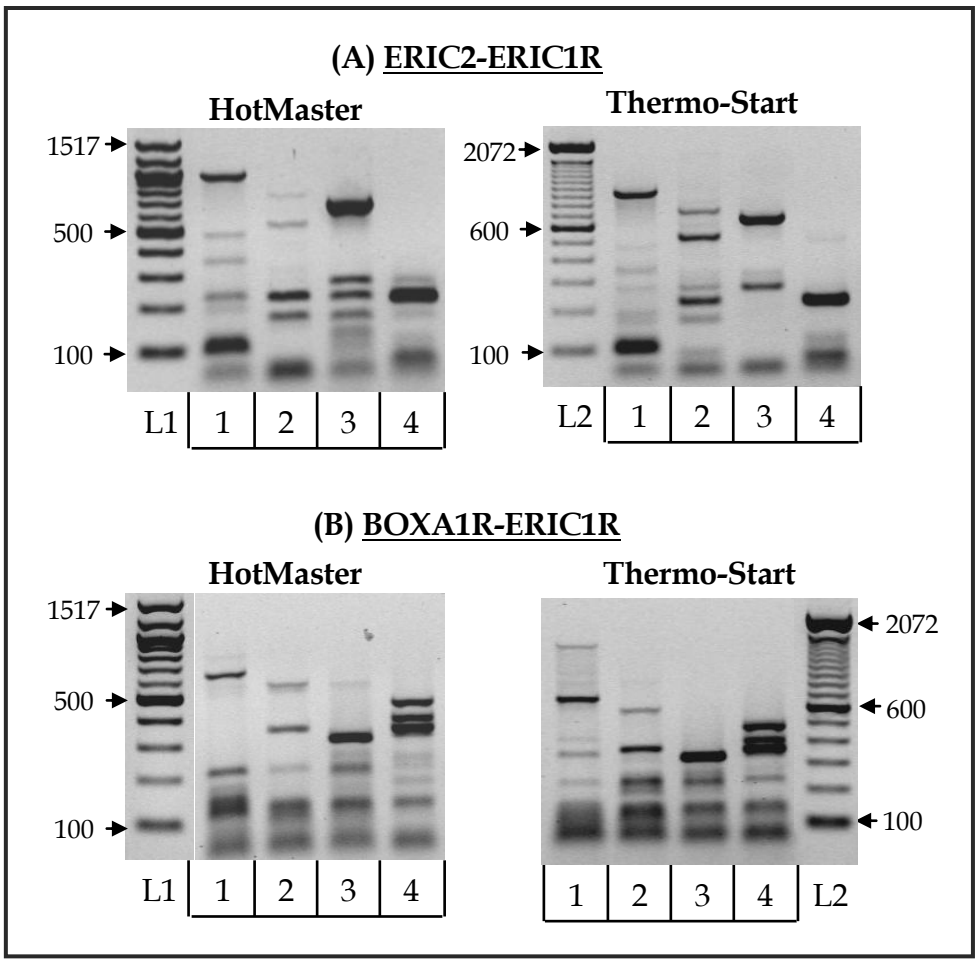


Figure 2.8. Effect of two brands of DNA polymerase on REP-PCR band patterns. PCR reactions were set up using four faecal isolates (1-4) with ERIC2-ERIC1R (A) or BOXA1R-ERIC1R (B) primers and either HotMaster or Thermo-Start DNA polymerase. The 100 bp ladders were from either NEB (L1) or Invitrogen (L2) with DNA fragment sizes (in bp) labelled.

2.3.2.7 Concentration of dNTPs

Differences in deoxyribonucleotide triphosphate (dNTP) concentration were also investigated for any effect on REP-PCR band patterns. Two different PCR master-mixes, MM-A and MM-B (section 2.2.5) were used for the amplification of two *S. uberis* DNA samples (200 ng) with 0.4 µM ERIC2 and ERIC1R primers and 1.25 U HotMaster *Taq* DNA polymerase. The final concentration of dNTPs in each reaction was 40 µM MM-A or 200 µM for MM-B. REP-PCR program 2 was used and products electrophoresed in 1.5% agarose gels in TBE buffer. Negative PCR controls were included for each master mix and no bands were apparent (data not shown).

Band intensity was much higher with 200 µM dNTPs than with 40 µM, and also less bands were observed with sample #2 when only 40 µM dNTPs were used (Figure 2.9). Therefore, to accurately compare band patterns for strain typing, the dNTP concentration needed to be constant between PCR reactions. Overall, the clarity of the band patterns was higher when 40 µM dNTPs were used, especially when two bands ran close together on the gel. Therefore, despite one of the samples showing fewer bands with 40 µM, MM-A was used in all subsequent strain typing analyses.

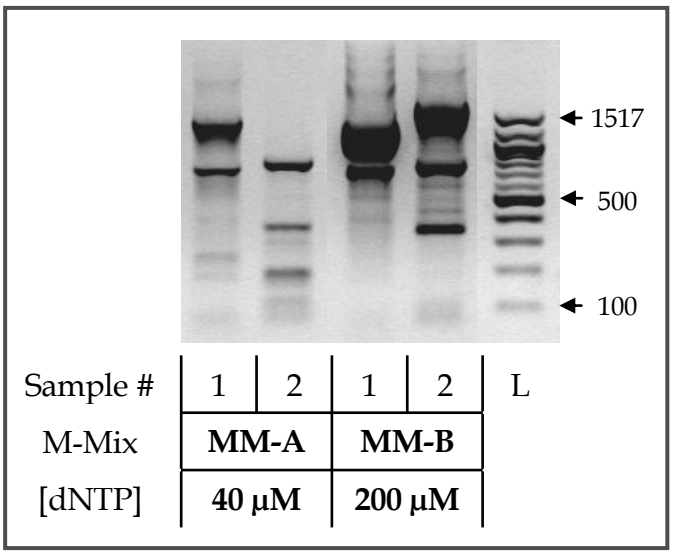


Figure 2.9. The effect of dNTP concentration on REP-PCR band patterns. PCR reactions were prepared with either PCR master-mix A (MM-A) or master-mix B (MM-B) for two DNA samples. L = 100 bp ladder.

2.3.2.8 Finalised REP-PCR method for strain typing

Analysis of different primer combinations, variations in PCR cycling conditions, annealing temperature, DNA template concentration, brand of DNA polymerase and dNTP concentration, allowed the optimal REP-PCR method for strain typing *S. uberis* to be determined as follows:

(i) REP-PCR reactions

Each 25 µl PCR reaction should contain 1x HotMaster buffer, 2.5 mM Mg^{2+}, 40 µM dNTPs, 0.4 µM primers (in one of these combinations: ERIC2-ERIC1R, BOXC1-BOXA1R, REP2-REP1R-DT, or BOXA1R-ERIC1R), 1.25 U of Thermo-Start DNA polymerase and 200 ng of DNA.

(ii) PCR cycling conditions

Optimal results were obtained using REP-PCR program 2 (section 2.2.10.2), therefore this cycling program was used in all subsequent *S. uberis* strain typing studies. The annealing temperature was set to 45°C in all cases.

2.3.3 Alternative PCR-based method – anchored typing

The combination of REP, BOX or ERIC primers with a primer specific for a known *S. uberis* gene was investigated as an alternative strain typing method. This method variation was named "anchored typing" due to the specific hybridization of one of the primers to a known gene (the anchor) while the other primer binds to multiple sites. The specific primers (or anchored primers) chosen to test this method were directed toward two genes encoding potential *S. uberis* virulence factors, the streptokinase (SK) gene (Johnsen *et al.*, 1999) and a gene designated metal transporter uberis A (mtuA)(Smith *et al.*, 2003).

Three isolates of *S. uberis* from faeces, milk and hocks that gave BOXA1R-ERIC1R band patterns (produced using finalised REP-PCR method, section 2.3.1.8) less than 62% similar when analysed using GelCompar II (Figure 2.10) were used to investigate anchored typing.

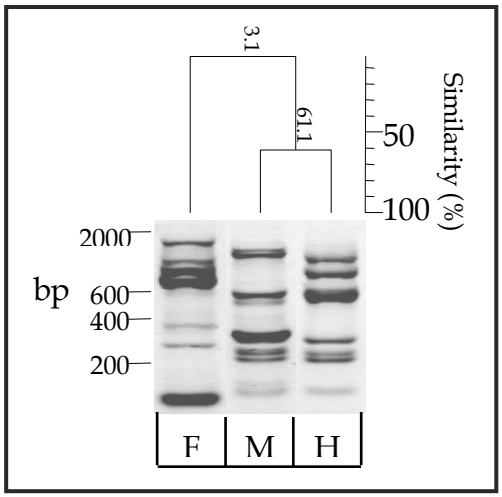


Figure 2.10. REP-PCR band patterns of three isolates used to test anchored typing. Band patterns for faecal (F), milk (M) and hock (H) isolates were compared with Pearson correlation (optimization 2.0%) and a UPGMA dendrogram generated. Relative positions of a 100 bp ladder are displayed to the left of the gel photos. Numbers above each branch indicate the average similarity for each branch or cluster.

The three DNA samples were amplified with either the MtuA-F or MtuA-R anchored primers in combination with ERIC1R, BOXA1R, or BOXA1R-ERIC1R together (Figure 2.11). All primer combinations resulted in the production of 1 – 3 very intense bands and multiple faint bands for each of the isolates. Despite the milk and hock isolates having different REP-PCR patterns, some of the anchored typing primer combinations gave band patterns that were almost identical for these two isolates. The faecal isolate gave a unique band pattern with all primer combinations tested, indicating that this method may have potential if more discriminatory primer combinations could be found.

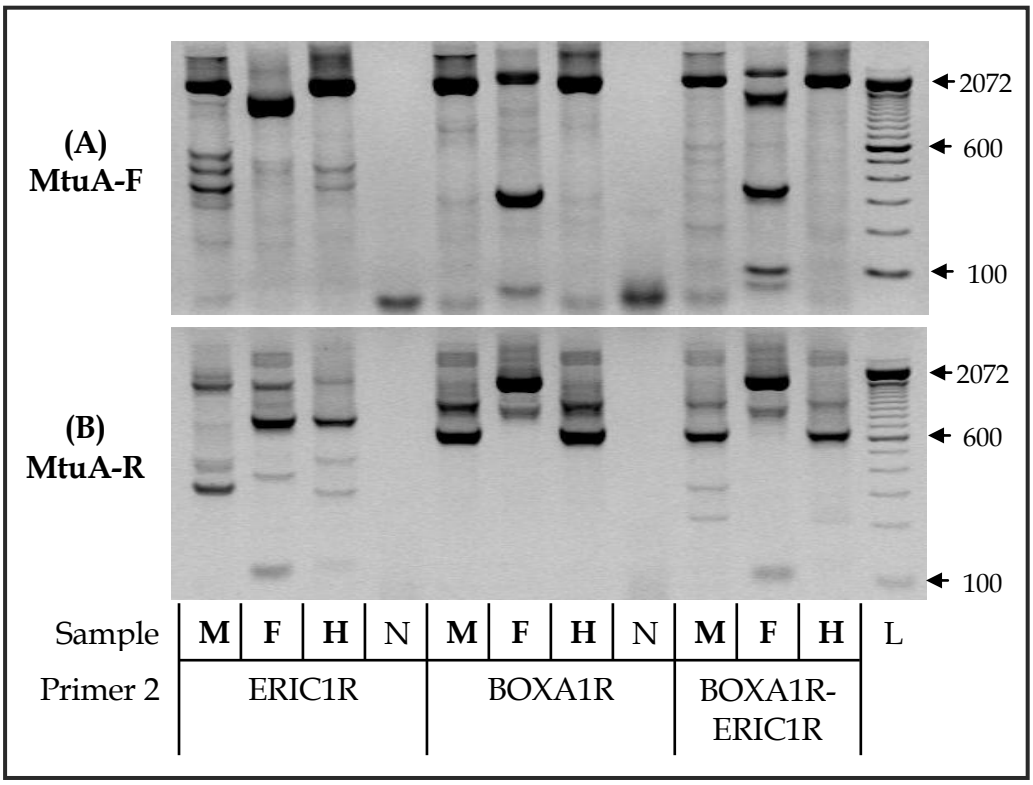


Figure 2.11. Anchored typing with MtuA-F or -R and BOXA1R or ERIC1R primers. DNA from milk (M), faecal (F), or hock (H) isolates was amplified with: MtuA-F (A) or MtuA-R (B) primer combined with ERIC1R, BOXA1R or BOXA1R-ERIC1R together. N = negative PCR control; L = 100 bp ladder.

To screen more primer combinations, two well-documented strains of *S. uberis* were used, 0140J and EF20. DNA from these strains was amplified with either the streptokinase forward (SK-F), reverse (SK-R) or the MtuA-R primer in combination with ERIC1R, BOXA1R, REP2, REP1R-DT, ERIC2 or BOXC1 primers (Figure 2.12). The band patterns obtained with the MtuA-R primer were more satisfactory, with a higher number of intense bands, than those obtained with either the forward or reverse streptokinase primers.

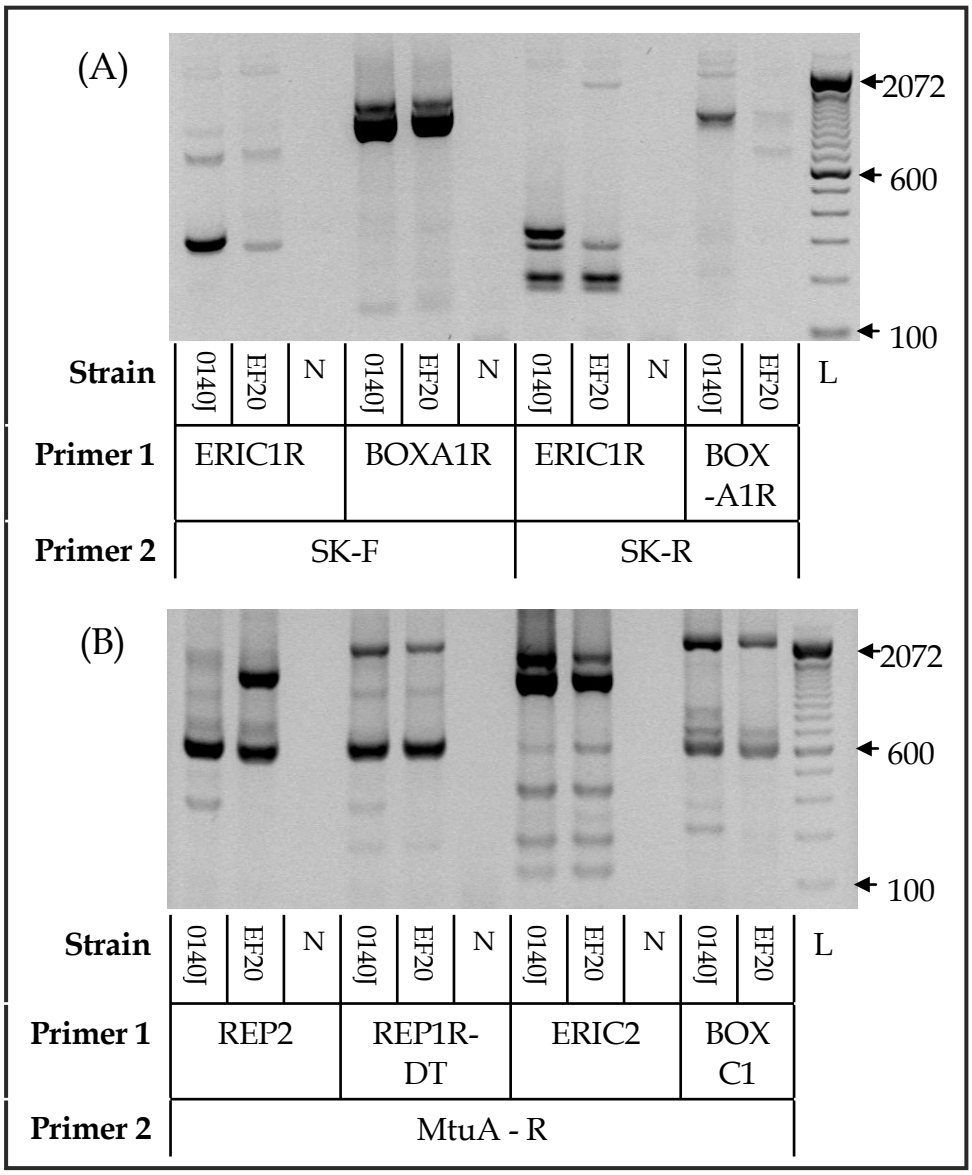


Figure 2.12. Anchored typing primer combinations. Strains 0140J and EF20 were amplified using streptokinase forward (SK-F) or reverse (SK-R) primers with both ERIC1R and BOXA1R (A) and MtuA-R primer with REP2, REP1R-DT, ERIC2 or BOXC1 (B). A negative PCR control (N) and a 100 bp ladder (L) were also included.

Although 0140J and EF20 are two different strains (Hill, 1988), the majority of the primer combinations yielded very similar band patterns for each, indicating the lack of discriminatory power of some combinations. Of the MtuA-R primer combinations, only REP2 and MtuA-R yielded a band pattern that was clearly unique to each strain.

2.3.4 Discriminatory power of typing methods

The ability of a typing method to distinguish between unrelated strains is known as discriminatory power. The REP-PCR method and some anchored typing primer combinations appeared to give varied and unique band patterns for the different isolates tested, indicating that these methods have discriminatory power. To investigate this further and obtain a numerical index of discrimination ability, the Simpson's index of diversity was calculated (Hunter & Gaston, 1988). This index is based on the probability that two unrelated strains sampled from a set of isolates will be placed into different strain type groups.

A set of 124 *S. uberis* isolates obtained from the milk of infected quarters, teat skin, and the mouths, tails and feet of different cows were strain typed using both the optimized REP-PCR method and anchored typing with primers MtuA-R and REP2. The band patterns were compared using Pearson correlation (optimization 2% for both REP-PCR and anchored typing) and those isolates with patterns ≥90% similar were classed as the same strain type. In addition, a composite data set was generated that contained both REP-PCR and anchored typing band patterns for each isolate and this was compared using the average of experiments option in GelComparII.

Of the 124 isolates, 72 strain types were defined by REP-PCR typing and 48 by anchored typing. However, when the two methods were combined as a composite data set, 80 different strain types were observed. The calculated discrimination indices for each of the methods were 0.985, 0.967, and 0.989 for REP-PCR, anchored typing and the composite data set respectively. Therefore, if two strains were sampled randomly from the population, then on 98.5% (REP-PCR), 96.7% (anchored typing) or 98.9% (composite) of occasions they would fall into different strain type groups. Both the upper and lower 95% confidence intervals of these indices were all above 0.95.

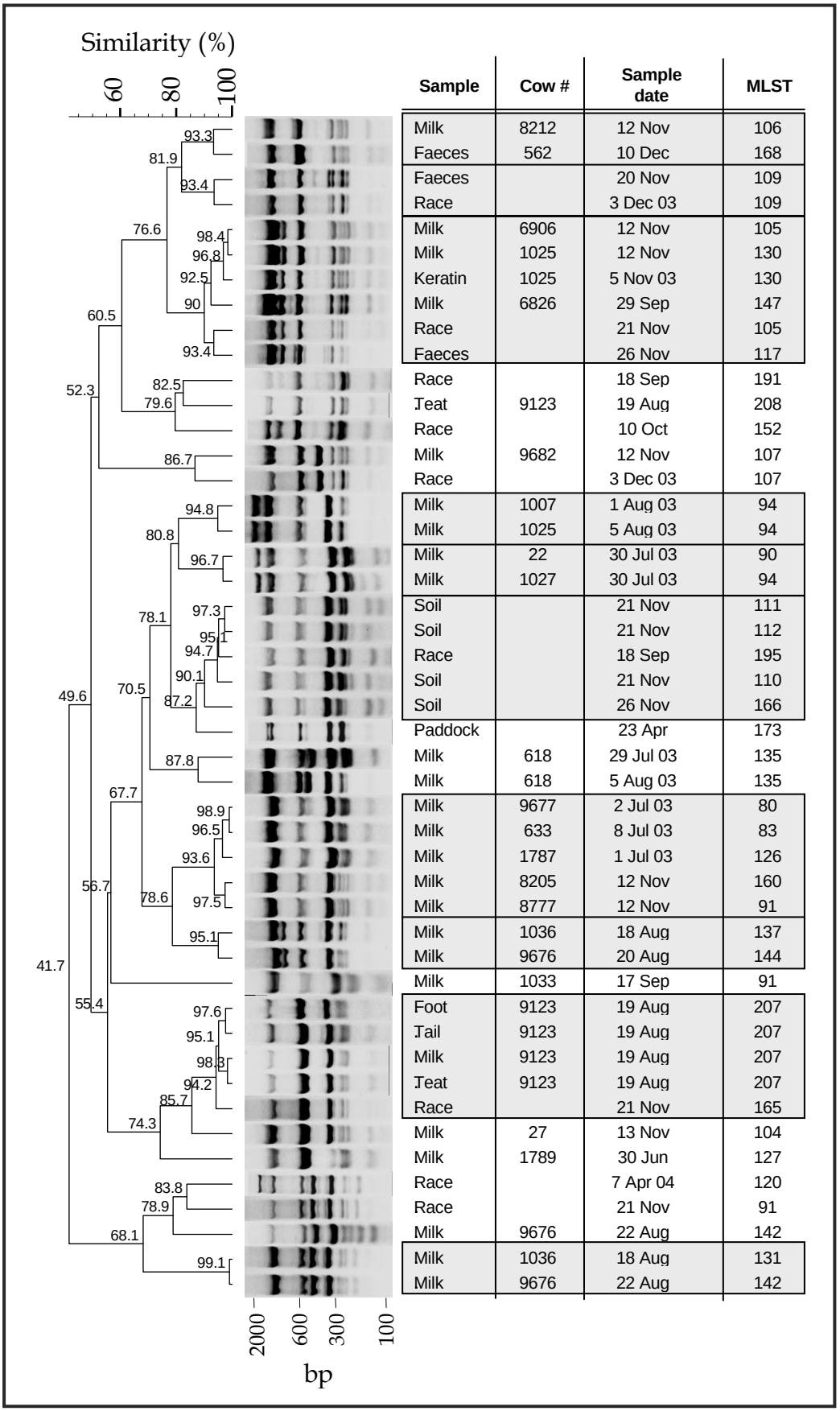
Each of these methods yielded a discrimination index greater than 0.9 which, according to Hunter and Gaston (1988), is desirable if typing results are to be interpreted confidently. Of the methods, the composite data set containing both REP-PCR and anchored typing band patterns yielded the highest discriminatory power.

2.3.5 REP-PCR, anchored typing and MLST

2.3.5.1 Comparison of REP-PCR and MLST strain typing results

A set of 47 *S. uberis* isolates from various sources that had been strain typed by REP-PCR (using the finalised method, section 2.3.1.8) were also typed using the Multi-Locus Sequence Typing (MLST) scheme (Coffey *et al.*, 2006). REP-PCR band patterns obtained with BOXA1R-ERIC1R primers were compared using Pearson correlation (optimization 2.0%) and a UPGMA dendrogram generated (Figure 2.13). Isolates with band patterns ≥90% similar were classed as the same strain type to give a total of 24 identified strains, of which, ten strain types were represented by more than one isolate. The REP-PCR strain type groups and MLST strain type (ST) did not always correlate with only two of the ten assigned REP-PCR groups containing isolates with the same ST, indicating potential differences in discriminatory power of the two methods.

Figure 2.13 (next page). REP-PCR strain typing of *S. uberis* isolates.
Band patterns were compared using Pearson correlation with 2.0% optimization. Strain type groups were assigned when band patterns were ≥90% similar as indicated by grey boxes.

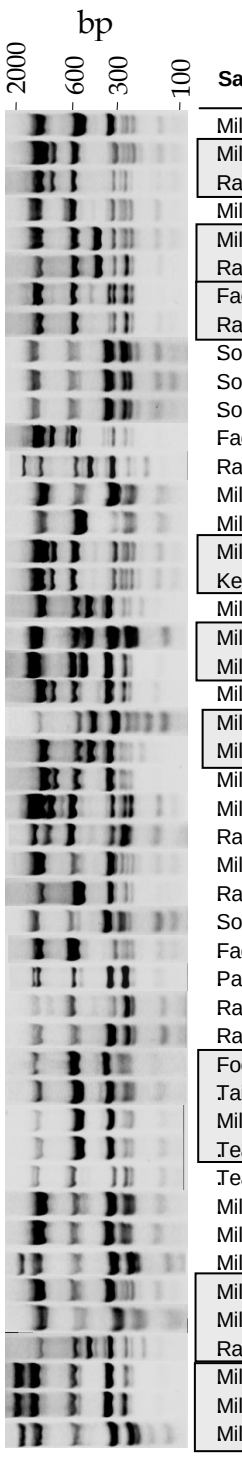


Sample	Cow #	Sample date	MLST
Milk	8212	12 Nov	106
Faeces	562	10 Dec	168
Faeces		20 Nov	109
Race		3 Dec 03	109
Milk	6906	12 Nov	105
Milk	1025	12 Nov	130
Keratin	1025	5 Nov 03	130
Milk	6826	29 Sep	147
Race		21 Nov	105
Faeces		26 Nov	117
Race		18 Sep	191
Teat	9123	19 Aug	208
Race		10 Oct	152
Milk	9682	12 Nov	107
Race		3 Dec 03	107
Milk	1007	1 Aug 03	94
Milk	1025	5 Aug 03	94
Milk	22	30 Jul 03	90
Milk	1027	30 Jul 03	94
Soil		21 Nov	111
Soil		21 Nov	112
Race		18 Sep	195
Soil		21 Nov	110
Soil		26 Nov	166
Paddock		23 Apr	173
Milk	618	29 Jul 03	135
Milk	618	5 Aug 03	135
Milk	9677	2 Jul 03	80
Milk	633	8 Jul 03	83
Milk	1787	1 Jul 03	126
Milk	8205	12 Nov	160
Milk	8777	12 Nov	91
Milk	1036	18 Aug	137
Milk	9676	20 Aug	144
Milk	1033	17 Sep	91
Foot	9123	19 Aug	207
Tail	9123	19 Aug	207
Milk	9123	19 Aug	207
Teat	9123	19 Aug	207
Race		21 Nov	165
Milk	27	13 Nov	104
Milk	1789	30 Jun	127
Race		7 Apr 04	120
Race		21 Nov	91
Milk	9676	22 Aug	142
Milk	1036	18 Aug	131
Milk	9676	22 Aug	142

Figure 2.13. REP-PCR strain typing of *S. uberis* isolates. Figure details on preceding page.

When isolates were re-ordered according to ST number rather than REP-PCR band pattern similarity (Figure 2.14), only four ST groups contained isolates that had been grouped together based on the REP-PCR band pattern, ST105, 109, 130 and 207. Band patterns were visually compared for isolates within each ST group and in some cases they were actually very similar. For example, two isolates with ST 135 had the same bands present, however the position and intensity of some bands was slightly different resulting in a calculated similarity of only 87.8% when band patterns were compared using Pearson correlation. Therefore, as this value was just below the cut-off level of 90% for inclusion in a REP-PCR strain group they were deemed to be different strains by this method.

In contrast, the ST 91 group contained two isolates with very similar band patterns when compared manually (but less than 90% similar with Pearson correlation and UPGMA), while another isolate, obtained from a farm race, had a clearly distinct band pattern. In this case, strain typing using the REP-PCR method may have resulted in higher strain differentiation than obtained with the MLST method.



Sample	Cow #	Sample date	MLST
Milk	27	13 Nov 03	104
Milk	6906	12 Nov 03	105
Race		21 Nov 03	105
Milk	8212	12 Nov 03	106
Milk	9682	12 Nov 03	107
Race		3 Dec 03	107
Faeces		20 Nov 03	109
Race		3 Dec 03	109
Soil		21 Nov 03	110
Soil		21 Nov 03	111
Soil		21 Nov 03	112
Faeces		26 Nov 03	117
Race		7 Apr 04	120
Milk	1787	1 Jul 03	126
Milk	1789	30 Jun 03	127
Milk	1025	12 Nov 03	130
Keratin	1025	5 Nov 03	130
Milk	1036	18 Aug 03	131
Milk	618	29 Jul 03	135
Milk	618	5 Aug 03	135
Milk	1036	18 Aug 03	137
Milk	9676	22 Aug 03	142
Milk	9676	22 Aug 03	142
Milk	9676	20 Aug 03	144
Milk	6826	29 Sep 03	147
Race		10 Oct 03	152
Milk	8205	12 Nov 03	160
Race		21 Nov 03	165
Soil		26 Nov 03	166
Faeces	562	10 Dec 03	168
Paddock		23 Apr 04	173
Race		18 Sep 03	191
Race		18 Sep 03	195
Foot	9123	19 Aug 04	207
Tail	9123	19 Aug 04	207
Milk	9123	19 Aug 04	207
Teat end	9123	19 Aug 04	207
Teat end	9123	19 Aug 04	208
Milk	9677	2 Jul 03	80
Milk	633	8 Jul 03	83
Milk	22	30 Jul 03	90
Milk	8777	12 Nov 03	91
Milk	1033	17 Sep 04	91
Race		21 Nov 03	91
Milk	1007	1 Aug 03	94
Milk	1025	5 Aug 03	94
Milk	1027	30 Jul 03	94

Figure 2.14. Isolates of *S. uberis* grouped according to MLST. Grey boxes indicate where the same ST is shared by more than one isolate.

To determine the discriminatory power of REP-PCR and MLST with this set of isolates, Simpson's index of diversity was again calculated and values of 0.953 and 0.983 obtained for REP-PCR and MLST respectively. The upper

95% confidence intervals were above 0.95 for both typing methods and the lower confidence intervals were 0.93 for REP-PCR and 0.97 for MLST. Therefore, the true index of diversity for each method was above 0.9, meaning each method should have suitable discriminatory power for strain typing. The discriminatory power of the MLST method was slightly higher than that for REP-PCR.

2.3.5.2 MLST and REP-PCR with anchored typing confirmation

The combination of REP-PCR and anchored typing band patterns as a composite data set previously revealed a higher discriminatory power than for either method alone. Therefore, both PCR typing methods were combined again in an attempt to improve correlation with MLST results. Initially, five isolates of *S. uberis* (three milk isolates, one from a farm race and one from paddock soil) of different MLST strain types, were strain typed by REP-PCR, band patterns compared with Pearson correlation (optimization 0%) and a UPGMA dendrogram generated (Figure 2.15)

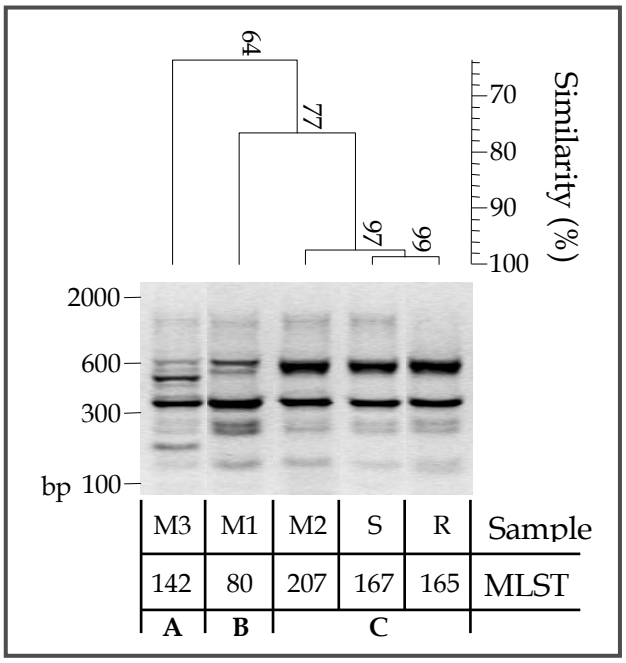


Figure 2.15. REP-PCR band patterns for *S. uberis* isolates of different ST. Band patterns for milk isolates (M1, M2, M3), a race isolate (R) and a soil isolate (S) were compared. Letters A-C indicate isolate groups with band patterns ≥90% similar.

Although all five isolates had a different strain type by MLST, REP-PCR only differentiated three strain types with M2, S and R isolates having band patterns >97% similar.

Anchored typing alone was also unable to differentiate between all five isolates with only three different strain types revealed with comparison of band patterns (Pearson correlation, optimization 1%; Figure 2.16). With this method only the soil isolate (S) was classed as a unique strain.

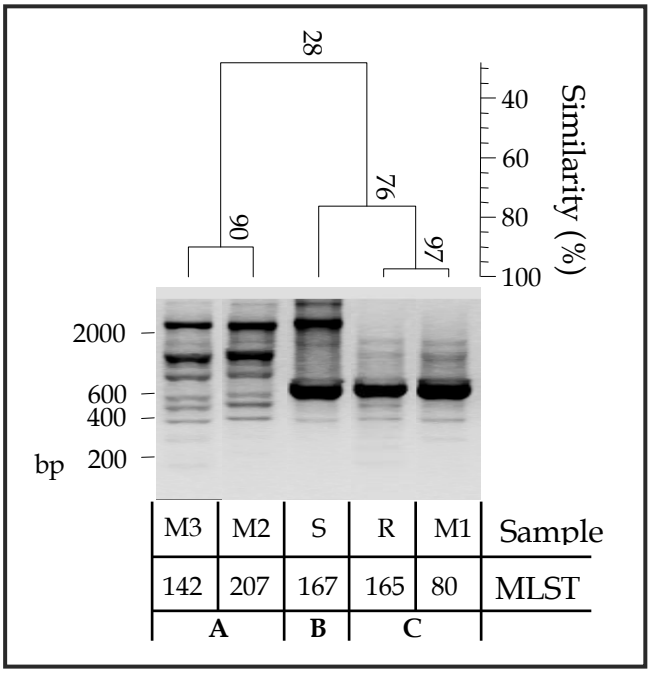


Figure 2.16. Anchored typing band patterns for *S. uberis* isolates. Band patterns for milk isolates (M1, M2, and M3), a race isolate (R) and a soil isolate (S) were compared. Letters A-C indicate clusters of isolates with band patterns ≥90% similar.

Combination of both REP-PCR and anchored typing band patterns (using the average of experiments option in GelComparII) correctly differentiated the five strains (Figure 2.17).

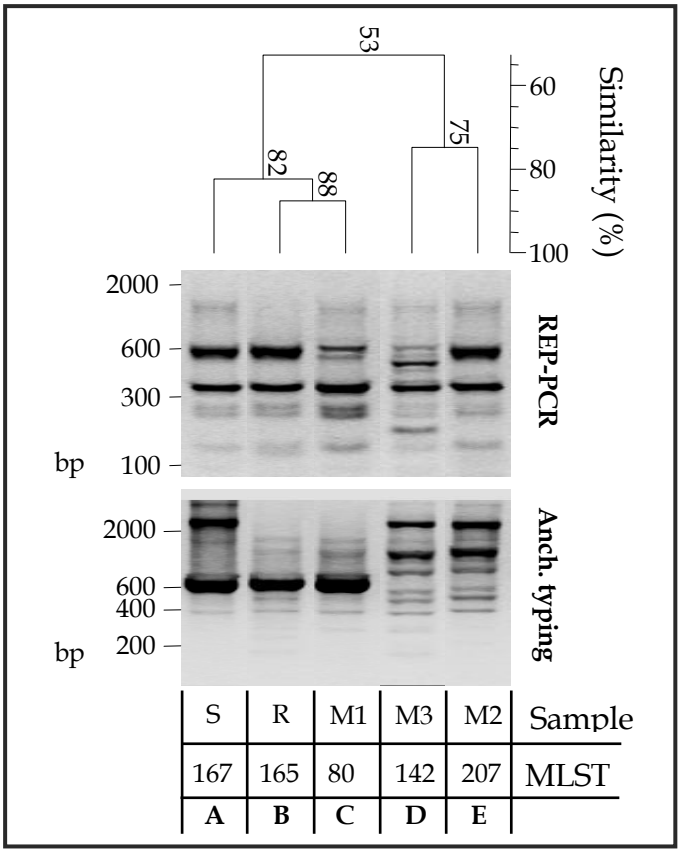


Figure 2.17. Comparison using both REP-PCR and anchored typing band patterns. A composite data set of REP-PCR and anchored typing band patterns for the five isolates was analysed using the average of experiments option in GelComparII. Letters A-E indicate each defined strain type.

Overall, the use of each individual method did not provide sufficient discriminatory power to differentiate between the isolates. Although the index of discriminatory power calculated for both REP-PCR and anchored typing was sufficient, combining the band patterns obtained with both methods vastly improved correlation with MLST results.

2.3.6 Reproducibility of REP-PCR band patterns

Band pattern reproducibility is important to allow accurate comparison of samples strain typed at different times. Potential reproducibility problems were investigated in the optimised REP-PCR typing system as summarised in Table 2.5. Reproducibility experiments were repeated up to three times in some cases and a representative example is shown for each experiment.

Potential reproducibility problem	Method of investigation
DNA extract	Compare band patterns using different extracts of the same strain of *S. uberis*
Intra-PCR run	Duplicate PCR reactions with the same DNA sample in the same PCR run
Inter-PCR run	Duplicate PCR reactions with the same DNA sample run at different times and on different thermal cyclers
Inter-gel run	The same PCR products run on two different agarose gels
Intra-gel run	The same PCR reactions run on the same agarose gel but at different positions

Table 2.5. Potential reproducibility problems to be investigated.

2.3.6.1 REP-PCR band patterns of selected *S. uberis* strains

Three different strains of *S. uberis* were used: SR115, 0140J, and an isolate from mastitic milk. Despite the different origins of these three strains, very similar band patterns were obtained using BOXA1R-ERIC1R primers (Figure 2.18). Only SR115 gave a distinct band pattern due to the lack of an intense band at ~300 bp that was present in the other two strains. It was unlikely that the milk isolate and 0140J were the same strain type, therefore the similarity between band patterns again indicated the lack of discriminatory power in the REP-PCR method.

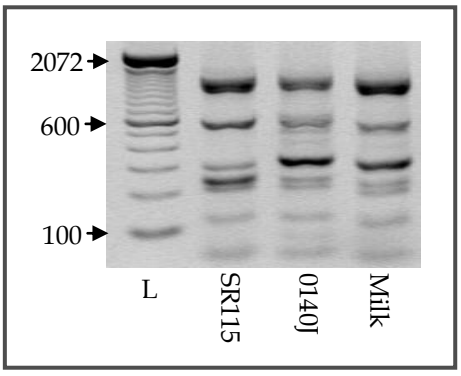


Figure 2.18. REP-PCR band patterns for three *S. uberis* strains.
L = 100 bp ladder.

2.3.6.2 DNA extract reproducibility

DNA was extracted from BHI agar cultures of the three *S. uberis* strains on two separate occasions. The DNA samples were amplified on the same day, electrophoresed in the same agarose gel and band patterns compared with Pearson correlation and optimization of 1.5% (Figure 2.19). Despite isolating DNA on different days, the REP-PCR band patterns were greater than 94% similar between the two DNA extracts for all three strains.

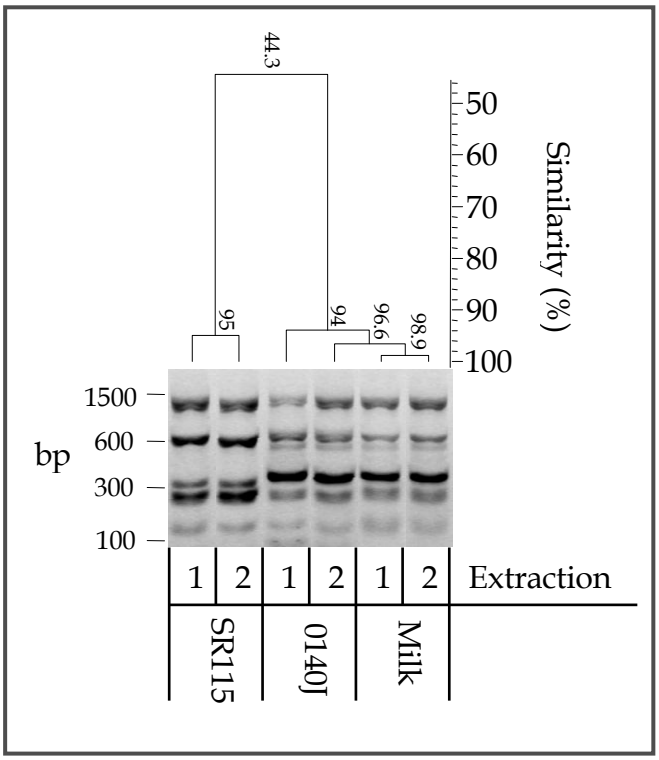


Figure 2.19. REP-PCR band pattern reproducibility between two DNA extracts (1, 2) of the same isolate.

2.3.6.3 Intra-PCR run variability – duplicate PCR reactions

Two identical PCR reactions were prepared for each sample, amplified at the same time and electrophoresed in the same agarose gel. Band patterns were compared using Pearson correlation with 2.0% optimization (Figure 2.20). Band pattern reproducibility was high between duplicate reactions with a minimum similarity of 96% observed between duplicates for each strain.

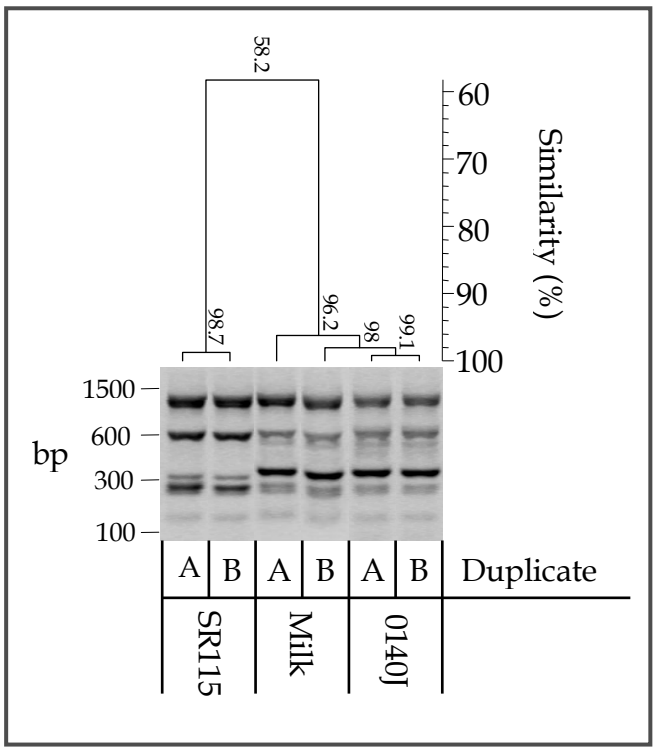


Figure 2.20. Band pattern reproducibility with duplicate PCR reactions (A and B).

2.3.6.4 Variability between PCR runs and thermal cyclers

Two sets of PCR reactions were prepared with the same DNA samples, one set was amplified using the PTC-100 thermal cycler (MJ Research) while the second set was prepared the next day and amplified using the PTC-200 thermal cycler. PCR products were run on the same agarose gel and band patterns compared using Pearson correlation and an optimization of 2% (Figure 2.21).

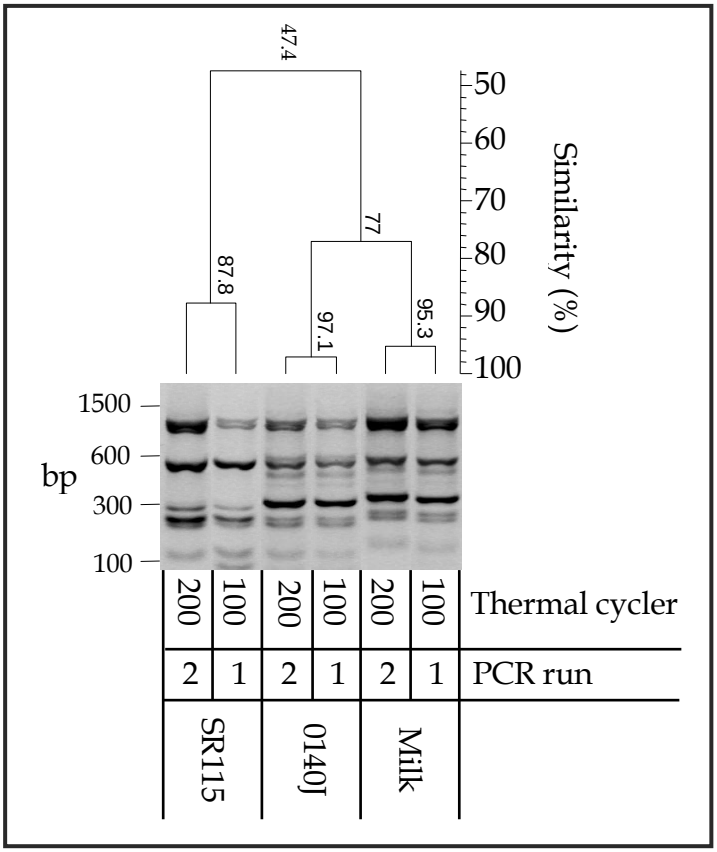


Figure 2.21. Band reproducibility between days and thermal cyclers.

Band patterns were very similar between different models of thermal cycler and PCR runs on two different days, although SR115 band patterns were only 87.8% similar. This lower similarity may be due to reduced band intensity when the PTC-100 cycler was used. Pearson correlation considers band intensity along with band presence when comparing patterns, therefore those that have identical numbers and band position may actually be given a lower similarity value due to the different band intensity.

2.3.6.5 Band pattern reproducibility between gels (inter-gel)

Reproducibility of band patterns between agarose gels was investigated by loading REP-PCR products (10 µl) on two separate agarose gels, electrophoresing under the same conditions and comparing band patterns (Pearson correlation; optimization 2%; Figure 2.22).

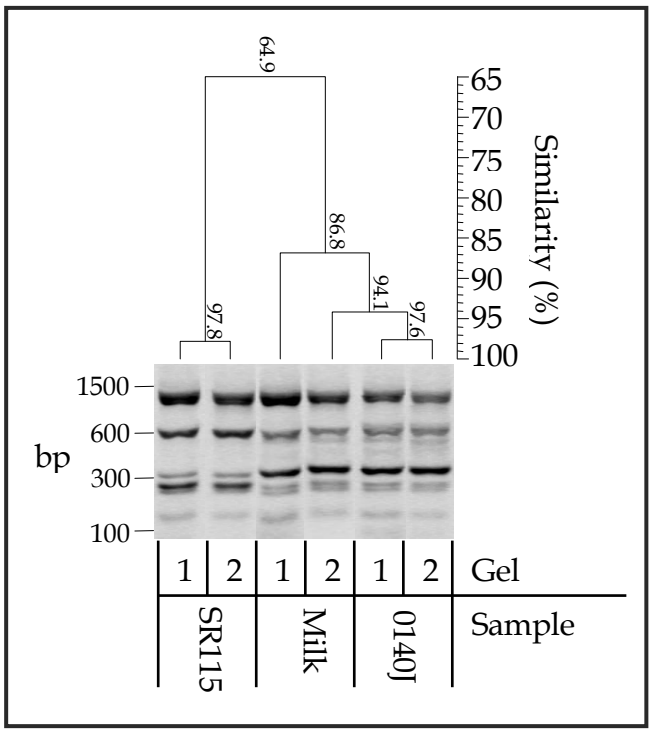


Figure 2.22. Band pattern reproducibility between agarose gels.

Band patterns for SR115 and 0140J were very similar between the two agarose gels (97.8% and 97.6% respectively); however, the milk band patterns only had a similarity of 86.8% between the two gels. This may have been due to imperfections in gel 1 that resulted in a slight curvature of the bands. Therefore, gel-to-gel variation may be an issue if slight variation in the agarose gels or electrophoresis conditions occurs.

2.3.6.6 Band pattern reproducibility within gels (intra-gel)

The electrophoresis apparatus used in this study allowed PCR products to be loaded in wells on both the top half and halfway through the gel. Any variations in band pattern that may occur due to the electrophoresis of PCR products on the top or bottom half of the gels was investigated by loading the same PCR products on both the top and bottom half of the gel and comparing the resulting band patterns (Pearson correlation, optimization 2%; Figure 2.23).

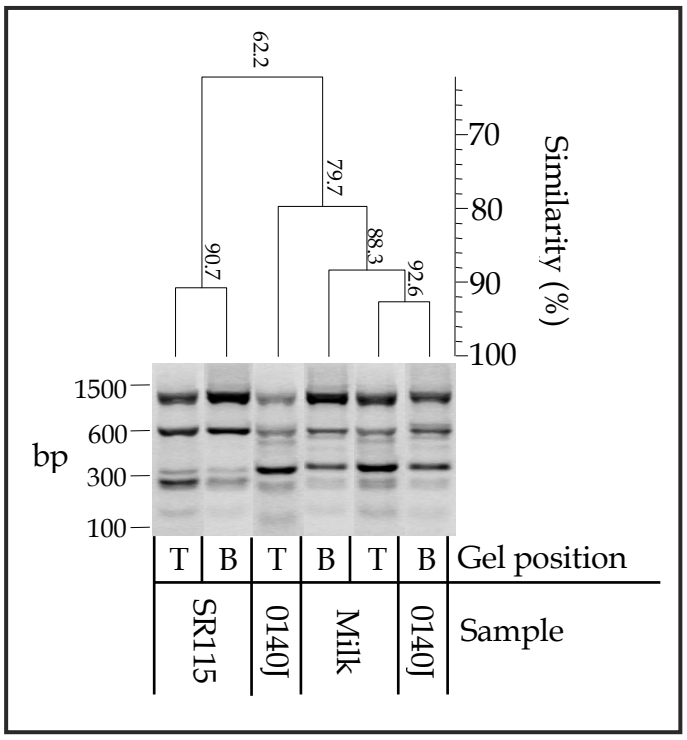


Figure 2.23. Band pattern variability due to gel loading position.
Top (T) and bottom (B) half of the gel.

Similarity between band patterns was lower than observed in the other reproducibility experiments. The SR115 band patterns were the most reproducible of the three strains with a similarity of 90.7%. The 0140J and milk isolate band patterns clustered together with a minimum similarity of 79.7%. These similarity values indicate that the position that the sample is loaded on the gel is very important for ensuring reproducibility of band patterns.

2.3.6.7 REP-PCR band pattern reproducibility

The similarity between band patterns for each strain and reproducibility experiment was tabulated for easier comparison (Table 2.6). These values were obtained by comparing band patterns for each strain independently from the other two strains (using Pearson correlation, optimization 2.0%). Calculating the similarity values independently was a more accurate reflection of band pattern reproducibility as the presence of other strains' band patterns appeared to affect the similarity values obtained. Therefore

the independently calculated similarity values in Table 2.6 differ slightly from those observed in Figures 2.19 – 2.23. In some cases the reproducibility experiment was repeated up to three times, therefore similarity values for each of these experiments are given.

		Similarity between band patterns (%)		
Reproducibility Experiment	**Expt.#**	**SR115**	**0140J**	**Milk**
DNA extract	1	94.9	96.3	97.5
Intra-PCR	1	98.7	96.1	97.3
	2	87.6	99.1	97.5
Inter-PCR	1	87.8	94.9	94.8
	2	97.1	97.6	95.5
Inter-Gel	1	97.8	97.6	86.9
Intra-Gel	1	90.4	85.7	86.6
	2	88.7	87.5	80.5
	3	82.3	85.9	73.7

Table 2.6. Similarity values between band patterns for each strain. Results of replicate experiments (1-3) are shown. Grey boxes indicate where less than 90% similarity was obtained between band patterns.

Similarity values from Table 2.6 were averaged across the repeated experiments and the three different strains for each reproducibility experiment (Figure 2.24).

The same sample, run on the top and bottom half of the gel (intra-gel) resulted in a significantly lower similarity between band patterns ($p < 0.05$) than for any other reproducibility experiment that was performed. Also, while comparison of band patterns between agarose gels (inter-gel) results, on average, in greater than 90% similarity, on some occasions a similarity below 90% can occur.

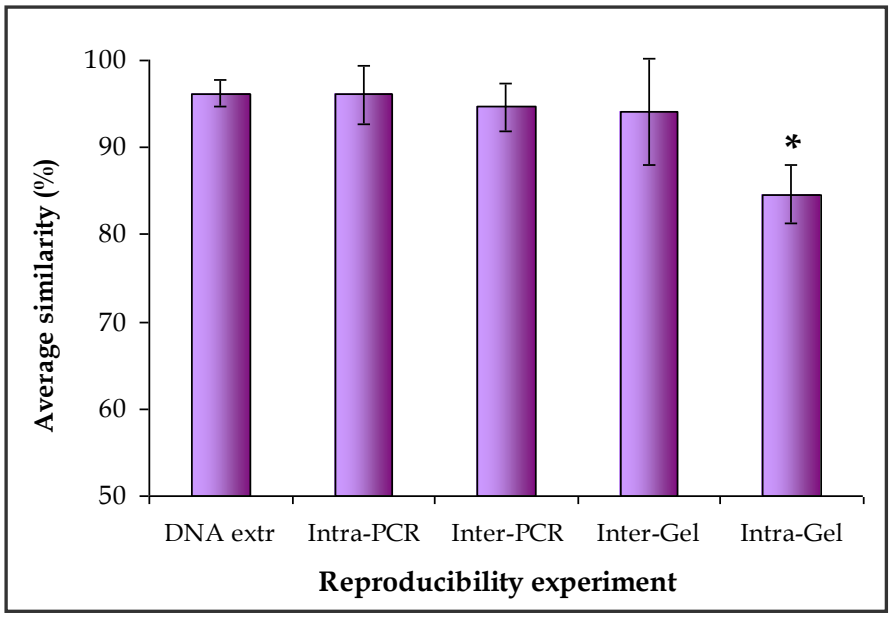


Figure 2.24. Average similarity between band patterns for each reproducibility experiment. DNA extr. (n=3), Intra-PCR (n=6), Inter-PCR (n=6), Intra-gel (n=9), Inter-gel (n=3). Error bars indicate 95% confidence intervals, *$p < 0.05$.

Overall, a similarity of at least 90% can be expected between band patterns generated from the same strain, unless the samples are run on the top and bottom half of the gel before comparison. Therefore, any unknown isolates with band patterns greater than 90% similar are most likely the same strain.

2.3.7 MALDI-TOF MS for strain typing

2.3.7.1 Obtaining strain-specific mass spectra

To investigate MALDI-TOF MS as a strain typing method, the first step was to determine if strain-specific mass spectra could be obtained. For this, two known *S. uberis* strains, 0140J and EF20 were prepared, mixed with sinapinic acid matrix and applied to the MALDI-TOF target plate using the two-layer method. Mass spectra between 2000 – 14000 mass/charge (m/z) were obtained by firing 210 laser shots over at least 3 different positions of the sample spot. Laser power of 30-35% was used to maintain signal intensity (in arbitrary units) of at least 50 counts/shot (Figure 2.25).

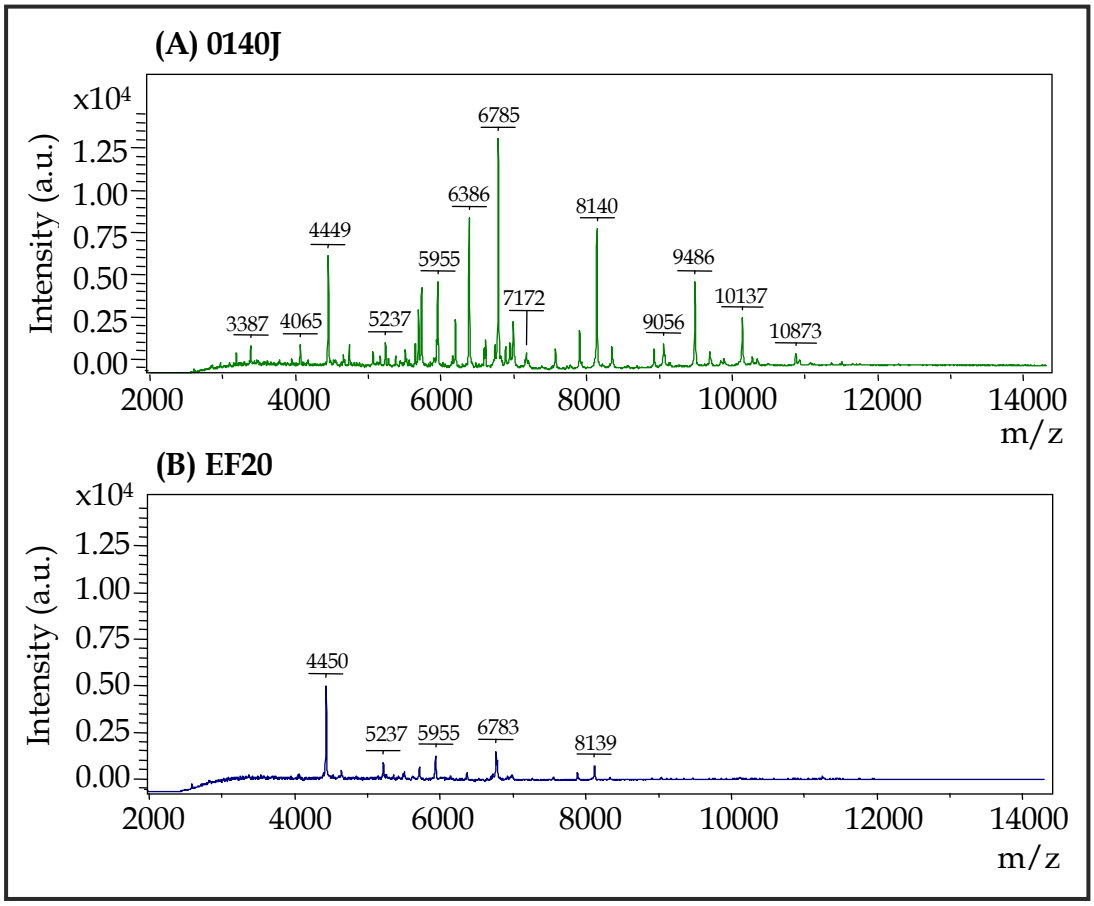


Figure 2.25. Example of mass spectra for *S. uberis* strains 0140J and EF20. Mass peaks obtained for 0140J (A) and EF20 (B) between 2000 – 14000 m/z. Height of the peak indicates signal intensity in arbitrary units (a.u.) and some of the more prominent peaks are labelled with the actual m/z value.

The most obvious difference between the two mass spectra was the higher number of mass peaks for 0140J compared to EF20. The most intense peaks in the EF20 spectrum were approximately the same mass as those obtained for 0140J. The intensity of peaks was noted as being of little use in previous studies, therefore this information was not included in the analysis. Instead, the signal-to-noise (S/N) ratio (the height of the mass peak above its baseline relative to the standard deviation of the noise) was used for defining significant peaks. A high S/N threshold reduces the number of significant peaks, while if the selected S/N threshold value is low, noise may be regarded as peaks. The recommended S/N range in the FlexAnalysis software was from three to ten and after analysis of 0140J and EF20 mass spectra and background subtraction, peaks with a S/N ratio

greater than eight were considered significant and included in the mass peak list (Table 2.7).

Peaks within ±10 m/z in both 0140J and EF20 mass spectra were considered to be the same as some mass inaccuracy was to be expected due to the limited resolution of the instrument in this mass range, especially when an external calibrant was used (Holland *et al.*, 1999; Walker *et al.*, 2002). The main difference between 0140J and EF20 was the additional 26 peaks present in the 0140J spectrum. Therefore strain typing by MALDI-TOF MS may be possible due to the production of strain-specific mass spectra. Also, the presence of conserved peaks between the two strains may indicate that species-specific peaks exist.

	O140J		EF20	
Peak #	**m/z**	**S/N**	**m/z**	**S/N**
1	3188	9		
2	3387	14		
3	4066	15		
4	**4450**	**80**	**4450**	**87**
5			4658	8
6	4740	15		
7	5066	9		
8	**5237**	**16**	**5237**	**15**
9	5507	11		
10	5645	16		
11	5690	40		
12	**5732**	**56**	**5732**	**11**
13	**5955**	**61**	**5956**	**21**
14	6197	33		
15	6386	107		
16	6593	13		
17	6613	19		
18	6742	15		
19	**6786**	**165**	**6784**	**25**
20			6801	18
21	6888	13		
22	6946	17		
23	6992	32		
24	7173	9		
25	7571	12		
26	7905	25		
27	**8140**	**99**	**8140**	**12**
28	8347	13		
29	8926	12		
30	9056	15		
31	9487	60		
32	9693	10		
33	10138	34		
34	10873	9		

Table 2.7. Mass peak lists for 0140J and EF20 strains. Shared peaks within ±10 m/z in 0140J and EF20 mass spectra are shown in bold. S/N indicates the signal-to-noise ratio for that peak.

2.3.7.2 Sample application methods

To optimize the MALDI-TOF MS method of strain typing, a comparison of different sample application methods was conducted using both 0140J and EF20. Two saturated matrix solutions were prepared using sinapinic acid in either 50% ACN/0.1% TFA (SA-ACN/TFA) or acetone (SA-acetone) and bacteria prepared (section 2.2.17.1) and resuspended in 50% ACN/0.1%TFA. Bacteria and matrix were applied to the target plate using

seven different methods: dried droplet (A), bottom layer (B), four variations of the two layer method (C1 – C4) and a three-layer method (D).

(A) Dried droplet

The bacterial preparation was mixed with an equal volume of SA-ACN/TFA matrix and 1 µl of the mixture deposited on the MALDI target plate.

(B) Bottom layer

Bacterial preparation (1 µl) was applied to the target plate and dried. SA-ACN/TFA (1 µl) was then applied over the top and dried.

(C) Two-layer

Either 1 µl of SA-acetone matrix (C1 and C3) or 1 µl of SA-ACN/TFA (C2 and C4) were applied to the target plate and dried. Over the top of the dried spot, 1 µl of bacterial preparation (C1 and C2) or 1 µl of bacterial preparation mixed 1:1 with SA-ACN/TFA matrix (C3 and C4) was applied.

(D) Three-layer

Bacterial preparation (1 µl) was deposited on the target plate and 1 µl of 100% ethanol applied to the dried spot. Finally 1 µl of SA-ACN/TFA matrix was deposited over the top and dried.

A sample spot with homogeneous crystals is superior to one where patches of crystals are evident. With the dried droplet method, crystals tended to clump together, therefore the quality of the mass spectrum was determined by the ability to find adequate crystals. When the bottom layer (B), two-layer (C1, C3) and the three-layer methods (D) were used, the crystals were homogeneous across the surface of the sample spot and good mass spectra were obtained without having to search for a sufficient aggregation of crystals. Good mass spectra were defined as those containing well defined peaks above baseline (a minimum of 50 counts/shot) and peaks not flattened due to detector saturation. The crystals tended to be found in clumps, similar to those observed with the dried droplet method, when the

variants of the two-layer method (C2 and C4) were used, and the resulting mass spectra were not high quality. With the C4 application method it was almost impossible to obtain satisfactory mass spectra for both 0140J and EF20, therefore this method was not analysed further.

In general, the collection of mass spectra for the EF20 strain was more difficult than for 0140J. With most of the different application techniques high noise was observed between 3000 – 4000 m/z for EF20 and the C1 and C4 two-layer application methods yielded no significant mass peaks for this strain and could not be analysed further. The layering methods, particularly the two-layer method, C3, gave the best results for both 0140J and EF20 strains.

Mass spectra obtained using each of these application methods were analysed and peaks with a S/N ratio greater than eight included in mass peak lists. While 12 different peaks were observed between 4000 – 9000 m/z for EF20 (Table 2.8), 0140J mass spectra showed 51 different peaks between 3000 – 12000 m/z across the six application methods (Table 2.9). Of these, only two EF20 peaks and eight 0140J mass peaks were present with every application method.

	Application method				
	A	**B**	**C2**	**C3**	**D**
Peak #	m/z	m/z	m/z	m/z	m/z
1	**4456**	**4449**	**4455**	**4450**	**4454**
2		4484			
3		4656		4658	4659
4			5243	5237	5240
5					5512
6		5731		5732	
7	5741				5736
8	**5957**	**5953**	**5962**	**5956**	**5960**
9		6784		6784	
10	6799	6798	6792	6801	
11			6809		6806
12	8140	8139		8140	

Table 2.8. Strain EF20 mass peaks with different application methods. Peaks conserved across all methods shown in bold.

	Application method					
	A	B	C1	C2	C3	D
Peak #	m/z	m/z	m/z	m/z	m/z	m/z
1	3191				3188	
2	3390	3387			3387	3385
3	3950					
4	4068	4064		4070	4066	
5	**4453**	**4449**	**4448**	**4451**	**4450**	**4446**
6	4660					
7	4746	4740		4745	4740	4738
8	5069			5071	5066	
9	5240		5234		5237	
10	5510		5505		5507	
11	5650		5642		5645	
12	**5693**	**5688**	**5687**	**5695**	**5690**	**5688**
13	**5736**	**5730**	**5729**	**5736**	**5732**	**5730**
14	**5958**	**5954**	**5952**	**5959**	**5955**	**5953**
15	**6200**	**6196**	**6193**	**6203**	**6197**	**6194**
16	**6390**	**6384**	**6383**	**6393**	**6386**	**6384**
17	6596		6590		6593	
18			6609			6609
19	6614			6619	6613	
20		6718				6715
21		6740	6739		6742	
22	**6789**	**6784**	**6782**	**6791**	**6786**	**6783**
23		6819				
24		6885	6884		6888	6885
25	6891			6895		
26		6943	6946		6946	6942
27	6949			6954		
28	**6995**	**6992**	**6990**	**6994**	**6992**	**6990**
29			7168	7164	7173	
30				7181		
31			7567		7571	
32	7908	7905	7901		7905	7904
33				7914		
34				7943		
35	8143	8139	8136		8140	8138
36				8149		
37	8349		8343		8347	
38						8565
39			8921		8926	
40				8938		
41	9059	9054			9056	9053
42				9066		
43	9073					
44	9489	9487	9483		9487	9486
45				9499		
46	9695	9694			9693	9693
47	10141	10138	10133		10138	10136
48				10149		
49				10287		
50		10872			10873	
51				11523		

Table 2.9. Strain 0140J mass peaks with different application methods. Conserved peaks are shown in bold.

The C2 application method gave mass peaks that were just over 10 m/z higher than with the other application methods for both 0140J and EF20 strains, therefore these could not be classed as the same peak and resulted in less conserved peaks across the mass spectra. This suggests that either some protein modification or alkali cation adducts may be occurring with the C2 application method, as has been observed previously (Demirev *et al.*, 1999).

The peak lists were analysed further with the average intensity and signal-to-noise ratio of the peaks compared for each strain across the application methods (Table 2.10).

Strain	Method	Number of peaks	Lowest peak m/z	Highest peak m/z	Average peak intensity	Average peak S/N
0140J	A	30	3191	10141	1352	**39**
	B	23	3387	10872	1320	37
	C1	24	4448	10133	2332	36
	C2	25	4070	11523	1516	28
	C3	**32**	**3188**	**10873**	**2732**	31
	D	20	3385	10136	1861	34
EF20	A	5	4456	8140	714	19
	B	**8**	**4449**	**8139**	806	**27**
	C1	-	-	-	-	-
	C2	5	4455	6809	328	20
	C3	**8**	**4450**	**8140**	**1417**	25
	D	7	4454	6806	1150	31

Table 2.10. Comparison of peak information for 0140J and EF20 mass spectra with application methods A – D. Optimal results are indicated by grey boxes.

The C3 application method (with SA-acetone as the first layer and bacteria mixed with SA-ACN/TFA as the second layer) yielded the highest number of peaks, the largest mass range (lowest to highest m/z) and the best average peak intensity. While the highest average S/N was obtained with different application methods for each of the strains, the C3 method also gave a good average S/N ratio. Also, with this application method, the dried sample crystals were homogeneous and a mass spectrum for 0140J was easily obtained. Adequate mass spectra were also obtained from EF20,

which was the problematic strain in regard to obtaining good mass spectra overall.

Attempts to collect mass spectra in a higher mass range were also carried out for each of these sample application methods and for each of the strains. Three mass ranges were investigated including 12,000 – 20,000 m/z, 20,000 – 50,000 m/z and greater than 50,000 m/z. In all cases, no significant mass peaks were observed above the background noise in any of these mass ranges or with any of the different sample application methods (data not shown).

2.3.7.3 Analysis of 12 isolates of unknown strain type

A blind test was set-up with 12 cultures of *S. uberis* grown and prepared by the Mastitis Research Group (Dexcel, Ltd) and actual strain identities were not revealed until after MALDI-TOF MS had been performed and strain types assigned using this method.

Bacterial preparations were applied to the target plate using the two-layer application method (C3) with SA-acetone as the first layer matrix. Mass peaks were obtained between 2000 and 12000 m/z (some examples are shown in Figure 2.26) and, upon initial observation, it was apparent that some isolates had highly similar spectra compared to others. For example isolate #1 and #2 had very similar spectra, while isolate #6 was obviously different with a very intense peak between 2000 – 4000 m/z.

Peak lists were generated and aligned for each of the 12 isolates (Table 2.11). A total of 57 different mass peaks were observed across the 12 unknown isolates. Of these, nine peaks were present in the mass spectrum of all 12 unknown isolates with average peak masses of 4449, 5731, 5955, 6196, 6386, 7905, 8139, 9487, and 10138 m/z. These conserved peaks may be species-specific and could potentially be used to identify *S. uberis* to the species

level; however, not all of these peaks were present in the EF20 mass spectrum observed earlier. Upon initial examination of the mass peaks lists it was apparent that unknown isolates #1 and 2 were either very similar or the same strain type, as 25 of the total 28 peaks were the same mass. In contrast, isolate #6 had quite a different list of peaks with 22 or 18 different mass peaks when compared to isolate #1 or 2 respectively, indicating that isolate #6 may be a different strain type than either isolate #1 or 2.

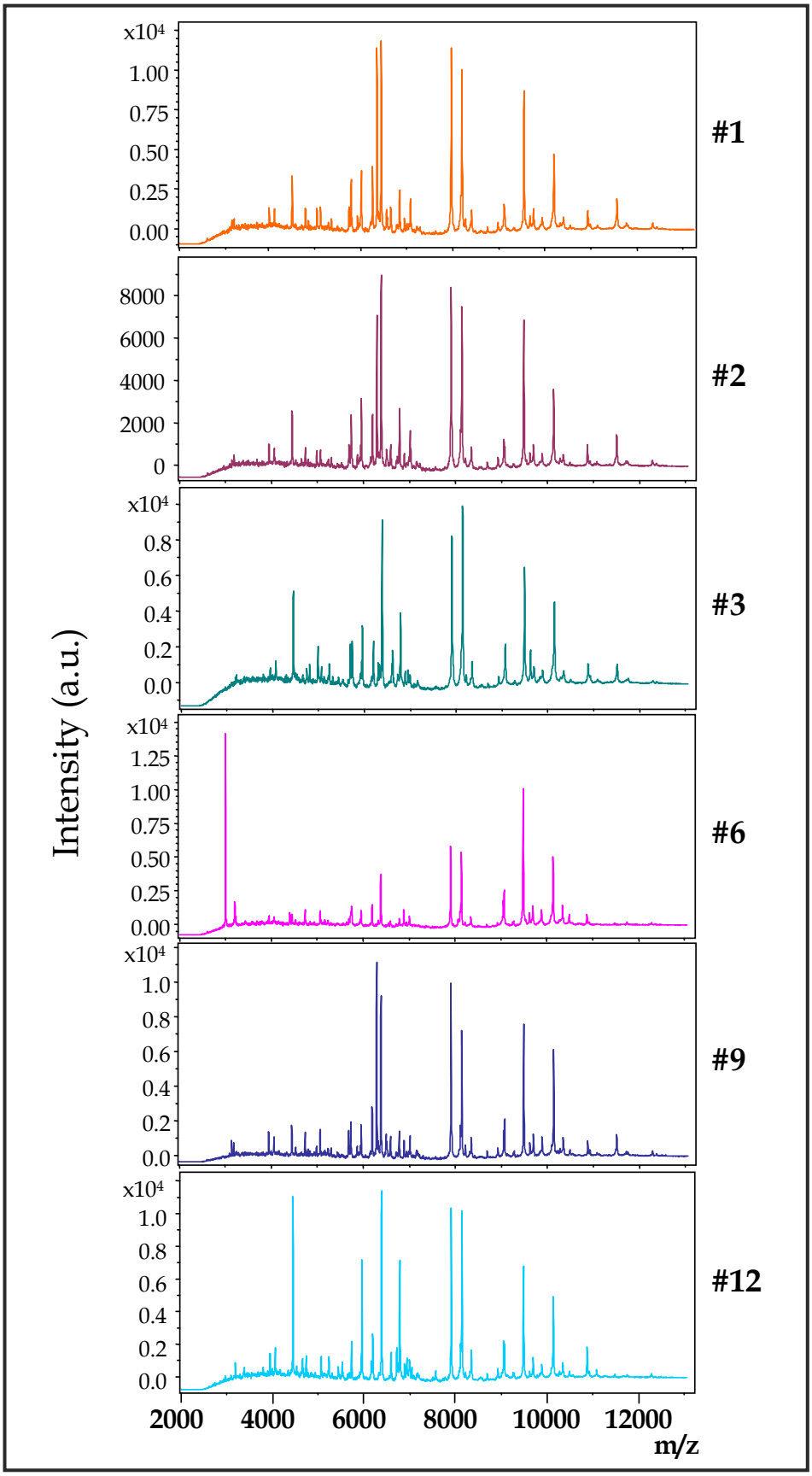


Figure 2.26. Example of mass spectra obtained for six of the unknown isolates. Mass spectra were collected for each isolate between 2000 and 12,000 m/z. Isolates #1, 2, 3, 6, 9, and 12 are shown.

	UNKNOWNS											
PEAK #	1 m/z	2 m/z	3 m/z	4 m/z	5 m/z	6 m/z	7 m/z	8 m/z	9 m/z	10 m/z	11 m/z	12 m/z
1						3001						
2					3142				3141	3139		
3											3186	
4						3208						
5						3225						
6	3949	3948		3950	3949		3946	3949	3947	3945	3945	3947
7	4066	4066	4068	4067	4065		4063	4065	4065	4064	4063	4064
8						4401						
9	**4450**	**4449**	**4452**	**4450**	**4450**	**4451**	**4447**	**4448**	**4448**	**4447**	**4447**	**4448**
10								4655				4655
11	4741	4740			4740	4740	4738	4740	4738	4738	4737	4739
12			4805									
13	4990	4989	4991	4990			4987			4986		
14	5068	5066			5066	5067	5065		5065	5064	5064	5064
15								5077				
16			5236	5235								5233
17												5526
18	5685	5686			5684		5684		5684			
19			5693	5692								
20	**5732**	**5731**	**5735**	**5733**	**5731**	**5732**	**5730**	**5732**	**5730**	**5729**	**5729**	**5730**
21						5750						
22											5937	
23	**5956**	**5955**	**5958**	**5957**	**5954**	**5957**	**5954**	**5955**	**5954**	**5953**	**5954**	**5954**
24								5994				
25												6160
26	**6197**	**6196**	**6199**	**6198**	**6196**	**6197**	**6195**	**6198**	**6195**	**6194**	**6194**	**6195**
27	6295	6295	6298		6294		6294		6293	6292		
28	6327		6332		6326		6326	6329	6326		6326	
29	**6386**	**6386**	**6390**	**6388**	**6386**	**6387**	**6385**	**6387**	**6385**	**6384**	**6383**	**6385**
30	6502	6502			6501		6501		6500	6500		
31					6518		6518		6517	6516		
32	6593	6593	6597		6593		6592		6592	6592	6590	6591
33			6615	6613								
34												6717
35	6785	6784	6788	6786	6784		6783	6785	6783	6782	6782	6782
36					6887	6888		6888	6887		6886	
37												6941
38												6989
39											7003	
40	7020	7019			7018				7018	7017		
41	**7905**	**7905**	**7909**	**7907**	**7904**	**7906**	**7904**	**7906**	**7904**	**7903**	**7902**	**7904**
42					8112				8111	8111	8110	8112
43	**8140**	**8140**	**8143**	**8142**	**8138**	**8140**	**8139**	**8140**	**8138**	**8138**	**8137**	**8138**
44					8215					8213	8214	
45	8346	8347	8351	8349	8345		8346	8347	8346	8344	8344	8345
46							8926					
47	9055	9055			9054	9056	9054	9057	9054	9054	9053	9054
48		9071	9075	9073	9071	9073	9072		9072	9071	9070	9071
49	**9486**	**9486**	**9491**	**9489**	**9485**	**9487**	**9487**	**9489**	**9486**	**9486**	**9484**	**9487**
50	9617		9623	9620		9618		9619				
51	9692	9694			9691	9694	9694		9693	9692	9691	9693
52					9883	9885	9885		9885	9884	9882	
53	**10137**	**10136**	**10142**	**10140**	**10136**	**10137**	**10138**	**10140**	**10138**	**10136**	**10135**	**10138**
54					10342	10344	10345		10343	10343	10342	
55						10492						
56	10873	10872	10879	10874	10872	10874	10875		10873	10873	10872	10873
57	11507	11507	11514		11505		11510		11509	11507		

Table 2.11. Mass peak lists for 12 isolates of unknown strain type.
Conserved peaks across all 12 isolates are indicated in bold.

The similarity of mass peak lists was calculated by dividing the number of shared peaks by the total number of possible peaks for each pair-wise combination of isolates. This calculation was performed for all 12 isolates and the result expressed as the percentage similarity of each two isolates' mass lists; for example a comparison of isolate #1 and #2 mass lists revealed 25 identical peaks out of 28 possible peaks, resulting in a similarity of 89% between these two isolates (Table 2.12).

Unknown isolate #	1	2	3	4	5	6	7	8	9	10	11
2	89%										
3	65%	61%									
4	60%	59%	76%								
5	71%	74%	50%	43%							
6	41%	42%	34%	36%	45%						
7	78%	81%	59%	52%	80%	45%					
8	55%	47%	48%	52%	46%	39%	46%				
9	74%	76%	54%	44%	97%	46%	82%	47%			
10	71%	78%	49%	46%	88%	44%	79%	41%	85%		
11	56%	57%	43%	44%	72%	50%	64%	52%	69%	67%	
12	53%	59%	44%	50%	53%	39%	53%	48%	54%	55%	58%

Unknown isolate #

Table 2.12. Similarity of mass peak lists for the 12 unknown isolates. Isolates with mass peak lists ≥70% similar are indicated by pink, blue or grey boxes as defined in the text.

The highest similarity (97%) was observed between isolate #5 and #9. When an arbitrary threshold of 70% similarity was used for defining strains, groups of isolates were observed; for example isolates #1, 2, 5, 7, 9, and 10 (pink boxes, Table 2.12) shared greater than 70% similarity between their mass lists compared to a range of 41% to 69% similarity (average 55%) with any of the other six unknown isolates. The only anomalous result was for isolate #11 which had a mass list similar to that of isolate #5 (72%; grey box, Table 2.12) and yet it had only an average similarity of 63% with the other five isolates in the same proposed group as isolate #5. Isolates #3 and #4 had mass lists that were 76% similar (blue box, Table 2.12), while being only, on average, 50% similar to the other ten isolates. Unknown isolates

#6, 8, and 12 did not have greater than 70% similarity with any other isolates' mass list, indicating that they may be unique strains within this set.

Using this calculation method, the following groups of isolates were obtained: Isolates #1, 2, 5, 7, 9, and 10 grouped as strain type A, while isolates #3 and 4 were grouped as strain type B. Isolate #11, although very similar to isolate #5, was different from all other isolates in strain group A, therefore #11 was assigned as a unique strain type along with isolates #6, 8, and 12.

The lists were also analysed using STATISTICA software (version 7.1; StatSoft, Inc. Tulsa, OK, USA) to obtain a dendrogram using the unweighted pair-group average (UPGMA) linkage rule (Figure 2.27). Because 1-Pearson r correlation was used the unknowns were clustered based on difference rather than similarity, with a linkage distance of 0% indicating that the mass peak lists were identical.

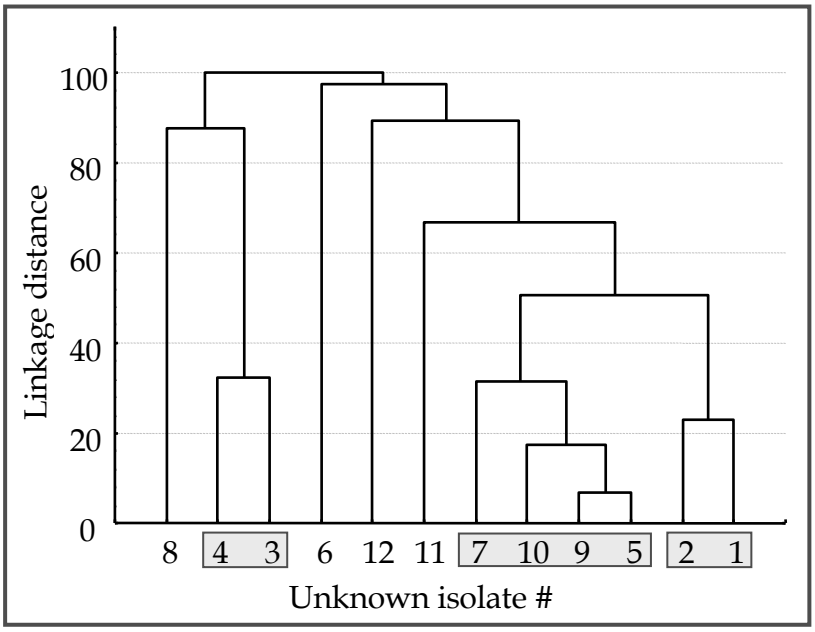


Figure 2.27. Comparison of mass peak lists of 12 unknown isolates.
Grey boxes indicate clusters of unknowns that are potentially based on strain type.

Three main clusters of closely related mass peak lists were obvious; however, the level of linkage distance to set as the threshold for defining strain type groups was difficult to determine. If a linkage distance of 35% was arbitrarily set as the cut-off level (based on visual assessment of the clusters), seven different strain types were revealed. The first cluster contained isolates #3 and 4 (strain type B), the second cluster (strain type A) contained isolates #5, 7, 9, and 10 and the only other cluster defined by a threshold setting of 35% contained isolates #1 and 2 (strain type C). The other isolates #6, 8, 11, and 12 had mass lists that were different by more than 35% therefore could not be clustered with any other isolates.

The strain type groups obtained using this method were very similar to those observed with the pair-wise similarity comparison method; however, there was further differentiation of isolates #1, 2, 5, 7, 9, and 10 by this dendrogram method into two different strain types.

2.3.7.4 Comparison with REP-PCR typing results

The 12 isolates used in this investigation were also strain typed by REP-PCR using the finalised method (section 2.3.1.8) with BOXA1R-ERIC1R primers. REP-PCR band patterns were compared with Pearson correlation (optimization 2%) and a UPGMA dendrogram generated (Figure 2.28). The final groupings generally correlated with the clustering of isolates observed after typing by MALDI-TOF MS. With a cut-off level of 90% similarity for defining strains by REP-PCR, two groups of isolates were created with only isolate #6 having a unique band pattern in this set of 12 isolates. If the similarity cut-off level was increased to 95%, there was increased discrimination and five strain types were distinguished. The isolate groups were similar to the clusters observed after MALDI-TOF MS results were analysed using the dendrogram method (Table 2.13). The main exceptions were that REP-PCR did not differentiate between isolate #11 and 12 and the REP-PCR band pattern for isolate #8 was over 95% similar to that of isolates #3 and 4. Also, while showing less differentiation with some isolates, REP-

PCR further differentiated isolates #1 and 2 into different strain type groups from isolates #5, 7, 9, and 10 when the 95% similarity cut-off was used.

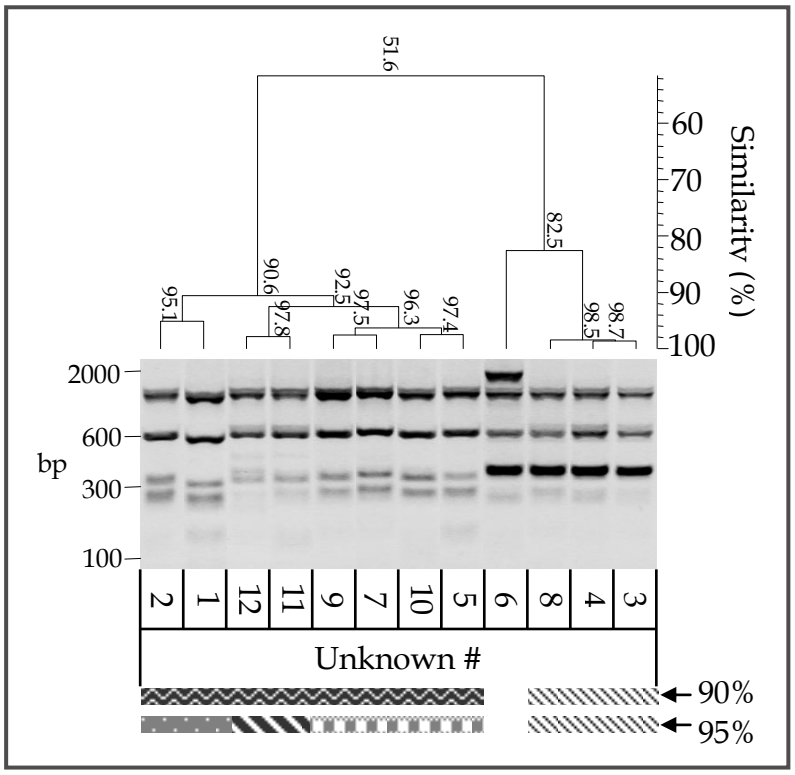


Figure 2.28. Comparison of REP-PCR band patterns for 12 unknown isolates. Band patterns were compared using Pearson correlation (optimization 2.0%) and UPGMA. Patterned bars indicate strain type groups when either a 90% or 95% similarity cut-off level is used.

		MALDI-TOF MS		REP-PCR	
		Pair-wise comparison	**Dendrogram**	**90% similarity**	**95% similarity**
Assigned group	A	1,2,5,7,9,10	5,7,9,10	1,2, 5,7,9,10,11,12	5,7,9,10
	B	3,4	3,4	3,4,8	3,4,8
	C		1,2		1,2
	D	11*	11		11,12
	E	12	12		
	F	6	6	6	6
	G	8	8		

Table 2.13. Assigned strain types with MALDI-TOF MS or REP-PCR typing. MALDI-TOF MS mass lists were analysed by pair-wise comparison or dendrogram. For REP-PCR either a 90% or 95% similarity between band patterns was used to define strain types. (* This isolate showed >70% similarity with one isolate in group A but not the other isolates in this group).

Overall, the 12 isolates were differentiated into seven strain types by MALDI-TOF MS, while a maximum of five strain types were distinguished by REP-PCR, despite applying a more discriminatory threshold of 95%.

After differentiating the isolates into groups by both MALDI-TOF MS and REP-PCR, the actual identities of these isolates were revealed. Isolates #1, 2, 5, 7, 9, and 10 were actually individual cultures of the same strain, SR115. Isolates #3 and #4 were strain 0140J, while isolates #6, 8, 11, and 12 had been obtained from different cases of clinical mastitis and were highly likely to be different strain types. MALDI-TOF MS gave superior differentiation of isolates into the correct strain groups (Table 2.14). In particular, pair-wise comparison of MALDI-TOF mass lists by direct calculation of percentage similar peaks gave the highest correlation with actual strain identities. The only irregular result with MALDI-TOF MS was for unknown isolate #11

which had high similarity with isolate #5 but no other members of the group assigned as strain type A. Isolate #11 was actually revealed to be a unique strain in this set of isolates.

Actual strain type	Unknown isolate #	MALDI-TOF MS Assigned strain type (Pair-wise analysis)	REP-PCR Assigned strain type (95% similarity)
0140J	**3**	B	B
	4	B	B
SR115	**1**	A	**C** *
	2	A	**C** *
	5	A	A
	7	A	A
	9	A	A
	10	A	A
Clinical strain 1	**6**	F	F
Clinical strain 2	**8**	G	**B** *
Clinical strain 3	**11**	D	**D** *
Clinical strain 4	**12**	E	**D** *

Table 2.14. Identity of 12 unknown isolates and assigned strain types based on MALDI-TOF MS and REP-PCR. * Incorrectly assigned strain types.

2.3.7.5 Reproducibility of MALDI-TOF MS method

To be suitable for strain typing the MALDI-TOF MS method needed to generate reproducible mass spectra. While the variable peaks in mass spectra allowed strain differentiation, they may also be a source of irreproducibility, making strain typing difficult. To investigate reproducibility, mass peak lists for the six isolates corresponding to the SR115 strain (from the experiment in section 2.3.6.3) were compared (Table 2.15). Each of these mass lists was generated from six different cultures of SR115 that were prepared and applied to the target plate as six different spots with mass spectra collected on the same day as application.

	SR115 strain					
	1	2	3	4	5	6
Peak #	m/z	m/z	m/z	m/z	m/z	m/z
1			3142		3141	3139
2	**3949**	**3948**	**3949**	**3946**	**3947**	**3945**
3	**4066**	**4066**	**4065**	**4063**	**4065**	**4064**
4	**4450**	**4449**	**4450**	**4447**	**4448**	**4447**
5	**4741**	**4740**	**4740**	**4738**	**4738**	**4738**
6	4990	4989		4987		4986
7	**5068**	**5066**	**5066**	**5065**	**5065**	**5064**
8	5685	5686	5684	5684	5684	
9	**5732**	**5731**	**5731**	**5730**	**5730**	**5729**
10	**5956**	**5955**	**5954**	**5954**	**5954**	**5953**
11	**6197**	**6196**	**6196**	**6195**	**6195**	**6194**
12	**6295**	**6295**	**6294**	**6294**	**6293**	**6292**
13	6327		6326	6326	6326	
14	**6386**	**6386**	**6386**	**6385**	**6385**	**6384**
15	**6502**	**6502**	**6501**	**6501**	**6500**	**6500**
16			6518	6518	6517	6516
17	**6593**	**6593**	**6593**	**6592**	**6592**	**6592**
18	**6785**	**6784**	**6784**	**6783**	**6783**	**6782**
19			6887		6887	
20	7020	7019	7018		7018	7017
21	**7905**	**7905**	**7904**	**7904**	**7904**	**7903**
22			8112		8111	8111
23	**8140**	**8140**	**8138**	**8139**	**8138**	**8138**
24			8215			8213
25	**8346**	**8347**	**8345**	**8346**	**8346**	**8344**
26				8926		
27	**9055**	**9055**	**9054**	**9054**	**9054**	**9054**
28		9071	9071	9072	9072	9071
29	**9486**	**9486**	**9485**	**9487**	**9486**	**9486**
30	9617					
31	**9692**	**9694**	**9691**	**9694**	**9693**	**9692**
32			9883	9885	9885	9884
33	**10137**	**10136**	**10136**	**10138**	**10138**	**10136**
34			10342	10345	10343	10343
35	**10873**	**10872**	**10872**	**10875**	**10873**	**10873**
36	**11507**	**11507**	**11505**	**11510**	**11509**	**11507**

Table 2.15. Mass peak lists for six replicate spectra of the SR115 strain. Six preparations of SR115 were deposited on the MALDI target plate and mass spectra generated from each. Conserved peaks are indicated in bold. Peaks within grey lines indicate those that may be species-specific biomarkers for *S. uberis*.

Of a possible 36 mass peaks only 22 (61%) were conserved across all six replicate SR115 mass spectra, indicating that reproducibility within one experiment is not high. The mass peaks that were thought to be species-specific based on the previous experiment are indicated and, if these are disregarded, 13 reproducible mass peaks remain, which may be sufficient for further discrimination of these isolates as SR115 strain types.

Based on the observed reproducibility between the SR115 mass lists, any two mass lists with greater than 61% similarity were likely to be the same strain type. Therefore, where similarity was less than 61% then the two isolates were likely to be different strain types. If this rule had been applied to the experiment with 12 unknown isolates however (section 2.3.6.3), incorrect strain types would have been assigned.

To further investigate the reproducibility of the MALDI-TOF MS method, *S. uberis* strain 0140J was cultured in either broth or on agar and prepared for MALDI-TOF MS on four different days. On days 1, 3 and 4 mass spectra were collected on the same day as the bacterial preparation; for day 2, mass spectra were collected either on the same day as the bacterial preparation (i) or the next day (ii) using the same preparation that had been stored at 4°C. For day 4 the bacterial preparation was deposited at two positions of the MALDI target plate to give two duplicate spots, A and B. All bacteria were prepared in the same way and applied to the target plate using the same two-layer method. Mass lists were generated containing peaks with a S/N ratio greater than eight (Table 2.16).

	Bacterial preparation					
	Agar culture			Broth culture		
	Day 1	Day 2		Day 3	Day 4	
		(i)	(ii)		Spot A	Spot B
Peak #	m/z	m/z	m/z	m/z	m/z	m/z
1	3188					
2	3387					
3				3951		3950
4	**4066**	**4067**	**4067**	**4065**	**4068**	**4067**
5	**4450**	**4451**	**4450**	**4449**	**4452**	**4450**
6	4740		4741			
7					4805	
8				4990	4991	4990
9	5066			5067		
10	5237	5237	5237		5236	5235
11	5507					
12	5645		5645			
13	5690	5691	5690		5693	5692
14	**5732**	**5734**	**5732**	**5735**	**5735**	**5733**
15			5940			
16	**5955**	**5955**	**5956**	**5956**	**5958**	**5957**
17	**6197**	**6197**	**6197**	**6199**	**6199**	**6198**
18					6298	
19					6332	
20	**6386**	**6385**	**6387**	**6387**	**6390**	**6388**
21	6593	6592	6593	6597	6597	
22	**6613**	**6611**	**6612**	**6612**	**6615**	**6613**
23	6742	6740				
24	**6786**	**6785**	**6786**	**6787**	**6788**	**6786**
25	6888		6888			
26	6946	6944	6948			
27	6992	6991	6993			
28			7157			
29	7173					
30	7571					
31	**7905**	**7907**	**7905**	**7909**	**7909**	**7907**
32		7932	7934			
33	**8140**	**8139**	**8140**	**8141**	**8143**	**8142**
34	**8347**	**8346**	**8346**	**8349**	**8351**	**8349**
35	8926			8930		
36	9056	9061	9055			
37				9072	9075	9073
38	**9487**	**9484**	**9486**	**9492**	**9491**	**9489**
39				9624	9623	9620
40	9693		9693			
41		9881		9884		
42	**10138**	**10139**	**10137**	**10140**	**10142**	**10140**
43				10348		
44	10873	10870		10876	10879	10874
45		10894				
46			11508		11514	

Table 2.16. Mass peak lists for 0140J grown on BHI agar or in broth and prepared on different days. Conserved peaks are shown in bold and peaks thought to be species-specific are in grey.

A total of 46 possible peaks were observed across the six mass spectra for 0140J. Of these, only 13 peaks (28%) were present in all six mass lists. Most of these conserved peaks were thought to correspond to the species-specific mass peaks observed in previous experiments with other strains of *S. uberis*. Visual comparison of the six mass lists revealed that most variability occurred due to the use of either agar or broth culture before collection of mass spectra. The percentage similarity of 0140J mass lists across the different experiments was calculated by taking the total number of conserved peaks and dividing by the total number of possible peaks between each pair-wise combination of spectra (Table 2.17).

Preparation								
	Agar	**2(i)**	60%					
		2(ii)	64%	65%				
	Broth	**3**	45%	52%	39%			
		4A	45%	55%	50%	64%		
		4B	44%	57%	47%	72%	76%	
			1	**2(i)**	**2(ii)**	**3**	**4A**	
			Agar			Broth		
			Bacterial preparation					

Table 2.17. Similarity of 0140J mass lists across different bacterial growth media and preparations. 1 and 2(i): spectra collected same day as prep; 2(ii): spectra collected next day using 2(i) prep; 3 and 4: spectra collected same day as prep; 4A and 4B: duplicate spots of day 4 prep.

Mass peak lists showed higher similarity when the same growth media was used for bacterial culture (green and yellow boxes, Table 2.17) with similarities above 60%. However when comparing mass lists between broth and agar cultures the similarities ranged between 39% and 57% similarity, indicating the influence that growth media has on the resulting mass spectrum.

Mass lists from experiments where only agar culture was used had 19 conserved peaks out of a possible 38 mass peaks, or 50% similarity between the experiments. For the broth culture experiments the mass lists were 59% similar with 17 peaks conserved out of a total of 29 possible peaks. Overall,

it appeared that reproducibility was improved when 0140J was cultured in broth.

The highest similarity between mass spectra was observed between duplicate spots (4A and 4B) of 0140J, however the same bacterial preparation applied to the target plate on separate days (2(i) and 2(ii)) showed inconsistencies between mass spectra, indicating that inherent variability may exist between mass spectra collected at different times.

Several mass peaks appeared unique to 0140J (average of 4990, 6613, 9073, 9622 m/z) or to SR115 (average of 3947, 4739, 5065, 6294, 6501, 6592, 9054, 9693, 11508 m/z) when the reproducible mass peaks from experiments where only broth culture was used were compared (Table 2.18). Therefore, despite variation in the presence of mass peaks, there were reproducible peaks that appeared strain-specific.

	Average m/z	
Peak #	**SR115**	**0140J**
1	**3947**	
2	4065	4067
3	4449	4450
4	**4739**	
5		**4990**
6	**5065**	
7	5731	5734
8	5954	5957
9	6195	6199
10	**6294**	
11	6385	6388
12	**6501**	
13	**6592**	
14		**6613**
15	6783	6787
16	7904	7908
17	8139	8142
18	8346	8349
19	**9054**	
20		**9073**
21	9486	9491
22		**9622**
23	**9693**	
24	10137	10141
25	10873	10876
26	**11508**	

Table 2.18. Potential strain-specific mass peaks in SR115 and 0140J. Average m/z of reproducible mass peaks across six (SR115) or three (0140J) replicate mass spectra. Strain-specific mass peaks are indicated in bold.

Overall, strain-specific mass spectra were obtained using this MALDI-TOF MS method, however it was difficult to obtain reproducible spectra with greater than 76% similarity. Because of this low reproducibility it may be difficult to establish which variations were strain-specific rather than experimental. An additional problem was the level of similarity at which to set the threshold for differentiating isolates into strain types. If these problems were addressed then MALDI-TOF MS could be a useful method for strain typing *S. uberis*.

2.4 Discussion

2.4.1 Selection and optimization of the REP-PCR method

The study of *S. uberis* ecology firstly requires the development of a suitable strain typing method that is both discriminatory and reproducible. Many methods have been used for strain typing *S. uberis* including pulsed-field gel electrophoresis (PFGE), arbitrarily primed PCR, REP-PCR and most recently, MLST and MLVA. As well as discriminatory power and reproducibility, other important factors in a strain typing method are ease of use and time required to obtain results. The PCR method based on repetitive elements within bacterial DNA appeared to be the most appropriate method as it has been used to differentiate strain types in numerous bacterial species (Alam *et al.*, 1999; Versalovic *et al.*, 1991; Versalovic *et al.*, 1992; Wieliczko *et al.*, 2002) and is rapid and easy to perform. This method would also be suitable for comparative strain typing, which would be carried out in this study. Although there are some questions concerning reproducibility (Johnson & O'Bryan, 2000; van Belkum *et al.*, 1996), for the most part REP-PCR is as reproducible as any other method relying on gel-based band pattern comparison, i.e. PFGE and RAPD.

2.4.1.1 REP-PCR primer selection

The REP-PCR method was optimized to obtain sufficient discrimination and reproducible band patterns. Primers directed toward repetitive elements were tested and the combinations ERIC2-ERIC1R, BOXC1-BOXA1R, REP2-REP1R-DT, and BOXA1R-ERIC1R were the most suitable. Of these, only REP2-REP1R-DT had been published as a successful combination of primers for typing *S. uberis* (Wieliczko *et al.*, 2002).

2.4.1.2 BOX and ERIC elements in *S. uberis*

The other primer combinations that worked well were directed toward BOX and ERIC repetitive elements that have not yet been identified in *S. uberis*. BOX elements are typically found in *S. pneumoniae* (Hermans *et al.*, 1995; Martin *et al.*, 1992) and in some strains of *Streptococcus sanguis* (van Belkum *et al.*, 1996) but not in many other streptococcal species (Hermans *et al.*, 1995; Koeuth *et al.*, 1995). The BOX element in *S. pneumoniae* consists of three subunits, boxA, boxB and boxC, which the primers BOXA1R, BOXB1 and BOXC1 are directed towards respectively. While PCR products were obtained with primer combinations including BOXA1R and BOXC1, any combination including the BOXB1 primer did not yield any PCR products, which may indicate that the boxB subunit is not present in *S. uberis*. When the sequenced genome of strain 0140J was searched with the BOX primers and consensus BOX sequences, none of the three BOX subunits were identified. Although, the BOXA1R and BOXC1 primers generally showed some homology to the genome, the BOXB1 primer only had one low homology match, which may explain the lack of PCR products when this primer was used.

ERIC sequences were initially characterised in Enterobacteria (Hulton *et al.*, 1991) and although they have not been characterised in many Gram-positive bacterial species, primers directed toward these elements have been used for strain typing of viridans group streptococci (Alam *et al.*, 1999) and *S. pneumoniae* (Hermans *et al.*, 1995). However, these primers were possibly binding to DNA in an arbitrary manner more similar to that of RAPD than REP-PCR (Alam *et al.*, 1999; Gillings & Holley, 1997; Sander *et al.*, 1998). In the present study, PCR products were observed with primers directed toward ERIC elements, yet few regions of the *S. uberis* genome showed homology to ERIC primers, and no ERIC elements were identified.

Overall, only BOXC1, ERIC2 and ERIC1R primers showed homology between the 3′ end of the primers and the 0140J DNA sequence. Therefore, these are the only primers that may initiate amplification under stringent conditions. The other primers with homology at the centre or 5′ end of the primer are more likely to require non-stringent PCR conditions for annealing to the DNA template and priming of the amplification reaction.

It must be noted however, that these results were from searching the genome of only one *S. uberis* strain, it is possible that other strains may actually contain these repetitive elements.

2.4.1.3 Arbitrary versus specifically-primed PCR

The lack of evidence for BOX and ERIC elements within the *S. uberis* genome suggested that the primers were binding arbitrarily to the DNA. The 45°C annealing temperature used in the REP-PCR method was much lower than the melting temperatures (T_m) of the BOXC1, BOXA1R, ERIC2 and ERIC1R primers (68°C, 76°C, 66°C and 65°C respectively). This annealing temperature (45°C) was used previously with REP primers (Versalovic *et al.*, 1992; Wieliczko *et al.*, 2002) with successful strain typing results, which is why it was chosen for use in the present study. Experimenting with the annealing temperatures revealed that all of the PCR products obtained at the low annealing temperature of 40°C were still present at a more stringent temperature of 60°C, indicating that priming may not be completely arbitrary. However, even the highest temperature used (60°C) was still more than 5°C below the T_m of these primers therefore it would be interesting to observe band patterns as the annealing temperature was increased further and approached the high T_m of the primers. Gillings and Holley (1997) also investigated a range of annealing temperatures with ERIC primers and found that the band patterns remained constant up to 56°C but from 57°C to 68°C the bands progressively failed to amplify. In contrast, PCR of *Salmonella enterica* with

ERIC2 and BOXA1R primers generated robust fingerprints at annealing temperatures as high as 72°C (Johnson & Clabots, 2000). Whether the same would be true in *S. uberis* with these primers is unfortunately not known.
Band patterns produced by arbitrarily-primed PCR are highly irreproducible (Ellsworth *et al.*, 1993; Kar *et al.*, 2006), therefore if the REP-PCR band patterns were due to non-specific binding of primers, a variation in band patterns would have been expected between PCR reactions performed on different days. However, despite observing a decrease in the intensity of bands above 600 bp at annealing temperatures of 50°C and 55°C compared to 45°C, the major bands remained almost identical between the two days, suggesting they were produced by specific rather than arbitrary priming. Some of the faint bands were variable between the two PCR runs, even at 50°C and 55°C annealing temperature, indicating that these bands may be the due to non-specific priming or possibly single-stranded PCR products.

Although it has not been determined if these REP-PCR reactions were the result of specific priming, strain-specific band patterns were obtained and the major bands in these patterns were reproducible over time, even with an annealing temperature of 45°C. The inclusion of any faint bands in the analysis of patterns is not recommended as these bands may occur randomly and will not be a good indicator of strain type.

2.4.1.4 Optimization of REP-PCR reactions

In addition to primer combinations and annealing temperature, variations in PCR cycling conditions, DNA template concentration, brand of DNA polymerase and dNTP concentration were investigated. In some cases method variations did little to affect the band pattern obtained, for example the use of two brands of *Taq* DNA polymerase. This was in contrast to the results published by van Belkum *et al.* (1996) where different brands of *Taq*

DNA polymerase lead to an alteration of the specificity of BOX primers in their PCR typing assay.

Other variables in the PCR reaction, such as DNA concentration, appeared to have a much larger effect on the REP-PCR band pattern produced. A high DNA concentration appears to be inhibitory to the PCR reactions with an optimal concentration found to be approximately 200 ng of DNA per 25 µl reaction. Alternatively, the observed results may also have been due to inhibitory substances present in the DNA sample. Dilution of the sample reduced these effects and allowed PCR to occur. Due to the sensitivity of the PCR reactions to DNA concentration and possible inhibitory substances, it was important to ensure that all DNA samples were quantified and diluted accordingly before PCR. Ellsworth *et al.* (1993) noted that arbitrarily-primed methods were very sensitive to variations in PCR reactions including template concentration, therefore the observed sensitivity of these REP-PCR reactions to DNA concentration again suggests that these PCR reactions may be more similar to the arbitrarily-primed method than the specific priming associated with the original REP-PCR method.

The concentration of dNTPs and thermal cycling conditions also affected the REP-PCR band pattern. Reducing the dNTP concentration resulted in a marked decrease in band intensity as well as the disappearance of bands of around 1000 bp in some isolates. The thermal cycling conditions also played a large role in the production of band patterns with a slower ramp rate (0.5°C/sec) before and after a slightly extended annealing step resulting in an improved band pattern and PCR products greater than 600 bp in size.

Once optimized, PCR reaction conditions were maintained to avoid any artificial changes in band pattern due to set-up rather than strain type. The

sensitivity of these reactions means that inter-laboratory comparison of REP-PCR results would be difficult unless methods could be identically repeated in a different laboratory, as has also been found previously (van Belkum *et al.*, 1996).

2.4.1.5 Anchored typing and discriminatory power

An alternative PCR-based method, anchored typing, was developed that utilised a gene-specific primer in combination with a primer directed towards a repetitive element. A primer directed toward the MtuA gene of *S. uberis*, in combination with the REP2 primer, yielded the most useful band patterns. The calculated discriminatory power of REP-PCR and anchored typing was above the recommended threshold for the confident interpretation of typing results (Hunter & Gaston, 1988) and, when both methods were combined, the discriminatory power was higher than when the two methods were used individually. Similar results have been observed previously where combined band patterns from two primers delivered superior discriminating power than when either of the individual primers were used (Johnson & Clabots, 2000; Johnson & O'Bryan, 2000). Therefore, the anchored typing method appears to be useful for improving the discriminatory power of REP-PCR.

2.4.1.6 Comparison of REP-PCR and MLST

MLST has an advantage in that results are not reliant on agarose gels and band patterns, improving both intra- and inter-laboratory comparisons of strain typing results. However, a disadvantage of the MLST method that is becoming apparent is that the strain type results do not always reflect differences in gene content between strains (Silva *et al.*, 2006). This appears particularly important in pathogenic bacteria where two isolates, indistinguishable by MLST, actually have different virulence-related genes (Pettigrew *et al.*, 2006). Therefore, as MLST only focuses on the

housekeeping genes of the core-genome, the discriminatory power may not be as high as initially proposed.

The opportunity to type a set of *S. uberis* isolates by both MLST and REP-PCR allowed a comparison of these two typing methods. Poor correlation was observed between the two sets of results despite the high discriminatory power of each method. One explanation for the lack of correlation was that the MLST method focused on variations in the more stable core-genome, while the REP-PCR method detected variations throughout the genome (Enright & Spratt, 1999). Overall, MLST differentiated more strain types than the REP-PCR method; however, the use of anchored typing in addition to REP-PCR improved correlation with MLST, indicating that the combination of REP-PCR and anchored typing gave an enhanced discriminatory power more similar to that of MLST.

It has been noted that when a set of isolates are strain typed using different methods, the results do not always correlate (Goldberg *et al.*, 2006; Hermans *et al.*, 1995; Malachowa *et al.*, 2005) with some methods resulting in discrimination of more strain types than others. Goldberg *et al.* (2006) found that the maximum correspondence achieved between their REP-PCR method and MLST results was 84.5% with *E. coli* isolates. Also, MLST had a higher discriminatory power than REP-PCR and isolates with visually indistinguishable REP-PCR band patterns were differentiated by MLST, similar to what was observed in the present study.

Although MLST is becoming the method of choice for many researchers (Coffey *et al.*, 2006; Pullinger *et al.*, 2006; Zadoks *et al.*, 2005a), another disadvantage of the method is that multiple sequencing reactions are required for each isolate, which can be both time-consuming and expensive, prohibiting large-scale studies of epidemiology (Goldberg *et al.*, 2006). REP-PCR in comparison, is easy to perform, relatively inexpensive and, when

combined with anchored typing, has a discriminatory power very similar to that of MLST. The main disadvantage of REP-PCR is the possibility of band pattern irreproducibility.

2.4.1.7 Reproducibility of REP-PCR band patterns

Of the PCR-based methods, REP-PCR has superior reproducibility compared to any arbitrarily-primed PCR method (Gillings & Holley, 1997), although it has also been known to result in irreproducible band patterns (Johnson & O'Bryan, 2000; Johnson *et al.*, 2004; Malathum *et al.*, 1998; van Belkum *et al.*, 1996). Investigations into band pattern reproducibility in the present study revealed that consistently prepared PCR reactions resulted in highly similar band patterns. These results are similar to those obtained by Rasschaert *et al.* (2005) where the reproducibility of DNA samples prepared at different times was higher than the reproducibility observed between PCR runs. The reproducibility between PCR runs was also investigated in the present study and, in contrast to that found by Rasschaert *et al.* (2005); the band patterns from two PCR runs were almost identical, even when two different PCR machines were used.

The main reproducibility problem encountered with the REP-PCR method was when PCR products from the same isolate were run at different positions on the same agarose gel, in particular when they were run on the top and bottom halves of the gel. This variation in band patterns may have been due to the migration of ethidium bromide incorporated into the agarose gel during preparation. As electrophoresis progresses ethidium bromide migrates toward the cathode, therefore the DNA on the bottom half of the gel will have progressively less ethidium bromide bound to it as the DNA advances through the gel. As a result, the bands may have decreased intensity and increased migration through the bottom half of the gel compared to those on the top half of the gel. The band patterns from top and bottom halves of the gel will appear slightly different and the

normalization of gels in GelCompar II software does not entirely correct for this. Ideally, ethidium bromide should have been omitted from the gels until after electrophoresis had been performed, as migration of DNA on agarose gels is also influenced by the concentration of PCR products loaded on the gel when ethidium bromide is present.

Methods that involve DNA separation and band patterns have been noted as lacking reproducibility between agarose gels (Johnson & O'Bryan, 2000) and Johnson *et al.* (2004) noted that REP-PCR band patterns of the same isolate had an average similarity of only 88% when compared between gels. Therefore, the higher the number of agarose gels included in the analysis the larger the discrepancy between band patterns may be as each gel brings a slight variation to the comparison. Attempts to improve the reliability of gel-based band pattern comparisons have been made in the past with the use of fluorescently labelled REP-PCR bands and internal ROX-labelled molecular weight markers to improve accuracy of band pattern comparisons (Johnson *et al.*, 2004). The use of these standards and controls allows accurate alignment of gels before the band patterns are compared. Using this method, REP-PCR band patterns had an average similarity of 92% between gels compared to the 88% similarity observed when a standard REP-PCR was performed. Ideally, a strain typing method that does not rely on gel-based comparisons would be superior. Healy *et al.* (2005) accomplished this by replacing agarose gels with a microfluidics chip that size fractionates REP-PCR fragments. However, in terms of ease of use and expense, agarose gels are superior for separating REP-PCR fragments compared to any of these methods and were used in the present study. To minimise the reproducibility issues, gel preparation and running conditions were kept as similar as possible.

2.4.1.8 Analysis of band patterns

Comparison software such as GelComparII can be used to calculate the percentage similarity between band patterns for each isolate and a dendrogram created that shows clusters of isolates with the most similar band patterns. Various settings and coefficients can be chosen for these calculations which can lead to quite different results. In this study the Pearson product-moment correlation coefficient was chosen over any band-based comparison algorithms as it was thought to be a less subjective method. Band-based comparisons rely on the operator to determine which bands are to be included in the analysis, whereas Pearson correlation is a curve-based coefficient that uses the entire densitometric track of the band pattern and the intensity of each band as variables. Previous research has found that the use of Pearson correlation is superior to band-matching algorithms for assigning known isolates into correct source groups (Hassan *et al.*, 2005; Johnson *et al.*, 2004). In addition, a study by Goldberg *et al.* (2006) found high correspondence between REP-PCR and MLST results when the densitometric profiles (i.e. Pearson correlation) were compared rather than when the presence or absence of individual bands was scored by band-matching algorithms. Also, with Pearson correlation, more emphasis is placed on the most intense bands in the pattern which were observed to be more reproducible than faint bands. Therefore, the use of a curve-based correlation such as Pearson correlation was preferable for comparing REP-PCR band patterns in the present study.

2.4.1.9 Differentiation of strain type groups based on similarity

After comparison of band patterns using Pearson correlation, a dendrogram was generated using UPGMA and clusters of isolates with similar band patterns were revealed. Application of a similarity cut-off level allowed discrimination of isolates into different strain types. Theoretically, isolates are designated genetically indistinguishable if their band patterns have the same numbers of bands and the corresponding bands are the same

apparent size (Tenover *et al.*, 1995); therefore having 100% similarity. However, in the reproducibility study, lower similarity values were obtained between band patterns for identical isolates, indicating that 100% similarity is difficult to achieve. Rasschaert *et al.* (2005) based their delineation levels for strain discrimination on the minimum similarity coefficient between the isolates in the reproducibility studies. Therefore, the cut-off levels were between 92.5% and 95% similarity depending on the primer used. If this approach was taken for the REP-PCR method developed here then a similarity cut-off level of 90% would be most appropriate. Therefore, isolates with band patterns less than 90% similar are very likely to be a different strain type. A similarity cut-off level of 90% also correlates with what other researchers have used previously for defining strain type groups (Johnson *et al.*, 2004; van Belkum *et al.*, 1996).

2.4.2 Optimal REP-PCR strain typing method

In conclusion, the REP-PCR method appears to be a suitable method for the comparative strain typing of *S. uberis*. REP-PCR reactions require standardized concentrations of dNTPs and DNA template and a slower ramping rate both before and after an annealing step of 30 seconds. The discriminatory power of REP-PCR is enhanced if a second confirmatory method such as anchored typing is also performed. Although reproducibility of the REP-PCR band patterns could be an issue, particularly when comparing within gels, attempts could be made to minimise these problems. The use of band comparison software also helps to improve reproducibility between gels and Pearson correlation minimises any subjectivity in the analysis. Finally a band pattern similarity cut-off level of 90% appeared most suitable for defining strain types based on the results of the reproducibility experiments and previous studies.

2.4.3 MALDI-TOF mass spectrometry for strain typing

As an alternative to PCR-based methods, MALDI-TOF mass spectrometry of intact bacterial cells was investigated as a rapid strain typing method.

2.4.3.1 Collection of mass spectra

MALDI-TOF mass spectrometry has been used for bacterial identification to both the species and strain level (Majcherczyk *et al.*, 2006; Mandrell *et al.*, 2005), yet to our knowledge there has not been any research into the ability of MALDI-TOF MS to discriminate between strains of *S. uberis*. The method used in the present study generated different mass spectra for two known *S. uberis* strains, 0140J and EF20.

There has previously been debate over the origin of proteins that contribute to the intact cell MALDI-TOF mass spectrum (Krishnamurthy & Ross, 1996; Ruelle *et al.*, 2004; Vaidyanathan *et al.*, 2002); however, it is likely that the mass peaks are originating from both cell wall-associated and intracellular proteins due to some cell lysis during sample preparation. More mass peaks were obtained for 0140J than EF20, suggesting that EF20 may either have less cell-surface associated proteins or may be less likely than 0140J to lyse during sample preparation, therefore less intracellular proteins were exposed for desorption during the MALDI process.

The number of acquired mass peaks varied considerably between *S. uberis* isolates with a maximum of 33 peaks observed in one isolate. These results were similar to those observed with other bacterial species; *E. coli* and other Enterobacteriaceae yielded complex spectra of approximately 50 mass peaks (Conway *et al.*, 2001; Smole *et al.*, 2002), while fewer peaks were found in Campylobacter species (Mandrell *et al.*, 2005) and *Staphylococcus capitis* (Conway *et al.*, 2001) which averaged only 20 mass peaks in the resulting spectra.

Peaks were observed between 2000 and 12000 m/z, which also correlates well with previous observations. It has been noted that this mass range and the number of peaks should be sufficient for differentiation of strain types (Demirev *et al.*, 1999).

As found in other studies (Holland *et al.*, 1996; Mandrell *et al.*, 2005; Walker *et al.*, 2002), the peak intensity was of little use for strain identification due to variation in intensities, even between replicates of the same isolate. Therefore, emphasis was placed on choosing mass peaks that were significantly above the baseline noise. Marvin-Guy *et al.* (2004) only considered mass peaks that had a signal-to-noise ratio greater than 15, while in the present study a signal-to-noise ratio greater than eight was required for mass peaks to be considered significant based on the recommendations given in the Bruker AutoFlex software and through observation of the most significant peaks in the mass spectra.

Once the lists of significant mass peaks had been generated, they were aligned to allow comparison of the presence or absence of peaks for different isolates. Due to limited resolution of the MALDI-TOF instrument in this mass range and the use of external rather than internal calibration, some mass inaccuracy can occur. Therefore, peak masses within 0.1% - 0.2% are likely to correspond to the same protein (Bernardo *et al.*, 2002; Holland *et al.*, 1996; Satten *et al.*, 2004; Walker *et al.*, 2002; Wang *et al.*, 1998) and mass peaks that varied by 10 m/z between different spectra in the present study were deemed to be the same. Although the use of an external calibrant may lead to some mass inaccuracy, it has been preferred over the use of an internal calibrant which can lead to suppression of the sample mass peak intensities (Gantt *et al.*, 1999; Satten *et al.*, 2004).

2.4.3.2 Sample and matrix application methods

The application of sample and matrix to the MALDI-TOF target plate can influence the appearance of the mass spectrum (Cohen & Chait, 1996; Ruelle *et al.*, 2004), and the results of this study were no exception. The dried droplet method is one of the more commonly used application methods (Arnold & Reilly, 1998; Gantt *et al.*, 1999; Krishnamurthy & Ross, 1996; Satten *et al.*, 2004), and in these experiments this method resulted in good mass spectra for 0140J; however, it was more difficult to achieve this with the EF20 strain. Both this and previous studies have noted that the dried droplet method leads to inhomogeneous crystals, making it difficult to obtain high quality mass spectra (Miliotis *et al.*, 2002; Onnerfjord *et al.*, 1999; Vaidyanathan *et al.*, 2002).

In contrast to the dried droplet method, the bottom layer and two-layer methods resulted in homogenous crystals across the surface of the dried spot, correlating well with results observed previously (Miliotis *et al.*, 2002; Onnerfjord *et al.*, 1999; Vaidyanathan *et al.*, 2002). The theory behind the two-layer method involves an initial layer of matrix that gives smaller, densely packed crystals that are homogeneous across the dried spot. These matrix crystals then 'seed' the crystallisation of the second layer that contains sample (in this case bacterial cells) and matrix, thereby enhancing crystal formation and sensitivity (Dai *et al.*, 1999; Onnerfjord *et al.*, 1999). The use of SA-acetone in the first layer, followed by bacteria and matrix in ACN/TFA as the second layer (C3 method) gave optimal results for both 0140J and EF20 strains. This was most likely because the acetone in the first matrix layer evaporated almost instantly, enhancing the formation of matrix seed crystals and improving the signal-to-noise ratio of resulting mass peaks (Miliotis *et al.*, 2002).

For EF20, no mass spectrum was obtained when the bacterial preparation was applied as the second layer without additional matrix (C1 method).

Dai *et al.* (1999) noted that the addition of matrix to the second-layer was critical for analysing proteins in a large mass range, therefore, with the more difficult EF20 strain, the lack of matrix in this second layer may have reduced sensitivity so no mass spectra were obtained.

With all sample application methods tested, attempts to find mass peaks above 12,000 m/z were unsuccessful. Other studies have also failed to find mass peaks in a higher mass range (Conway *et al.*, 2001) and the majority found the best mass range was between 2000 and 20,000 m/z. In contrast, Vaidyanathan *et al.* (2002) obtained mass peaks in two mass ranges 2000 – 20,000 and 20,000 – 200,000 m/z using similar sample application methods as were used here; however, they also noted that the peaks in the higher mass range had poor resolution and mass accuracy. The strain typing of beta haemolytic streptococci using MALDI-TOF MS revealed peaks in a mass range of 200 to 2000 m/z (Kumar *et al.*, 2004); however, this may not have been the best mass range to use as the majority of peaks associated with growth medium and matrix occur between 500 and 1000 m/z (Walker *et al.*, 2002). In the present study any peaks below 2000 m/z were not included in the analyses for this very reason.

Overall, most application methods resulted in average to high quality mass spectra in terms of number of mass peaks, the range of peak masses and peak intensity. However, the two-layer method with matrix in acetone as the first layer showed consistently good mass spectra and was the method of choice for further use in this study.

2.4.3.3 Strain typing of 12 unknown isolates

MALDI-TOF mass spectrometry of 12 isolates in a blind test revealed that strain-specific mass spectra could be generated by this method. A total of 57 different mass peaks were observed across the 12 isolates. Of these, nine peaks were conserved in all 12 isolates, indicating that they may be specific

to *S. uberis* and could potentially be used for bacterial identification to the species level. However, no other bacterial species were subjected to MALDI-TOF MS analysis in this study so the presence of these same mass peaks in other species is not known. Previous studies have used mass peaks that are unique to a species as biomarkers in bacterial identification (Holland *et al.*, 1999; Ruelle *et al.*, 2004); therefore, it is likely that there will also be species-specific peaks in *S. uberis*.

The other, less-conserved mass peaks found across the 12 isolates were used to differentiate the isolates according to strain type. Analysis was carried out by direct calculation of the percentage similarity for each pair-wise combination of the 12 isolates' mass lists or through the use of statistical analysis software. The difficulty in the analysis of these methods was deciding where to set the cut-off level for classing two isolates as the same strain type. Despite this, isolates were grouped into strain types that correlated very well with the actual strain identities of the 12 isolates, particularly when percentage similarity of pair-wise combinations was used as the analysis method. MALDI-TOF MS also appeared more discriminatory than REP-PCR, differentiating the isolates into a higher number of strain types, similar to what was found by Siegrist *et al.* (2007) using strains of *E. coli*. However, the disadvantage of the MALDI-TOF MS method was a lower repeatability level compared to REP-PCR.

2.4.3.4 Reproducibility of mass spectra

In previous studies, contrasting degrees of reproducibility were observed for MALDI-TOF mass spectra (Conway *et al.*, 2001; Kumar *et al.*, 2004; Mandrell *et al.*, 2005; Siegrist *et al.*, 2007). Kumar *et al.* (2004) noted that 90% of mass peaks were reproducible using their method with various streptococcal species and Walker *et al.* (2002) found that spectra generated from the same strain on the same instrument had 75% similar peaks, while between instruments this was reduced to 60%. In the present study, when

comparing mass spectra collected on the same day from six individual preparations of the same strain, only 61% of mass peaks were conserved.

Variability in mass spectra can occur due to slight changes in bacterial preparation (Marvin-Guy *et al.*, 2004; Wang *et al.*, 1998), salt content of the sample (Ruelle *et al.*, 2004) and even the number of bacterial cells applied to the target plate (Williams *et al.*, 2003). While the method in the present study was kept as standardized as possible, slight variations could occur, especially in the final number of bacterial cells applied to the plate. Efforts were made to adjust samples to an identical optical density before mass spectrometry; however, it was difficult to get this exact. In addition, the bacterial growth time was not standardized, which may have been necessary to prevent variability. Some studies have noted the importance of bacterial growth phase on reproducibility (Conway *et al.*, 2001; Holland *et al.*, 1999), while others have found no influence on the reproducibility of mass spectra (Bernardo *et al.*, 2002; Mandrell *et al.*, 2005). In this particular case, growth time may have affected mass spectra, therefore, this may need to be optimized in future.

The highest variability between mass spectra was observed when comparing the same strain cultured either in broth or on agar, similar to the study by Conway *et al.* (2001) where growth phase and choice of media significantly influenced MALDI-TOF mass spectra generated from *E. coli*. These researchers found that the variability of mass peaks between experiments was comparable to variability observed among the diverse *E. coli* strains that were examined and concluded that strain specific identification was not yet possible due to the inconsistency of the method.

Walker *et al.* (2002) noted that mass spectra produced from *S. aureus* grown on Columbia agar produced fewer peaks and had more reproducible profiles than when grown in broth. However, in the present study, the

mass spectra of broth-cultured strains were more reproducible than when the same strains were cultured on agar. Despite the variation observed between replicate mass spectra of the same strain of *S. uberis* in this study, there were still many reproducible peaks. Although many of these peaks may have been species-specific, there were also reproducible peaks that appeared to be strain-specific and would allow differentiation of isolates according to strain type.

2.4.3.5 MALDI-TOF MS as a strain typing method for *S. uberis*

In summary, mass spectra were obtained from isolates of *S. uberis* using an optimized intact cell MALDI-TOF mass spectrometry method. A two-layer application of sample and matrix lead to the generation of complex and strain-specific mass spectra that allowed the differentiation of *S. uberis* isolates into correct strain types. Some of these mass peaks were conserved across almost all of the different strains studied indicating that these may be species-specific peaks, which were of little use in discriminating between strain types. To our knowledge this was the first study to show that *S. uberis* can be strain typed using intact cell MALDI-TOF MS; however, further work is required before this method could be utilised in any large-scale strain typing studies. The main problem was the lack of reproducibility between mass spectra for the same strain, however further alterations and standardization of the bacterial growth and preparation method may improve the reproducibility.

Chapter 3:

Streptococcus uberis ecology

Summary

Streptococcus uberis is characterised as an environmental pathogen and as such has been found throughout the dairy environment. This study was no exception with many different strains identified from farm races and paddocks, faeces, teat skin, cow body and mammary gland infections. Strain typing of isolates from these sites showed that some strains could be found both in the environment and on the teat skin as well as infecting the mammary gland, consistent with environmental transmission of this pathogen. However, there was also some evidence of contagious transmission with the same strain found on the teats and in the mammary gland of multiple cows in the same herd. Persistent or recurrent mastitis was often found to be caused by the same strain of *S. uberis*, indicating that a mechanism must exist for these strains to either persist in the mammary gland or possibly colonise the teat skin to allow recurrent infection of the same quarter. The most likely method for spread of *S. uberis* within the dairy environment is through excretion of this species in the faeces. Although *S. uberis* could not be found in the faeces of all cows studied, some appeared to be persistent shedders and the same strain could be found across multiple sample dates indicating possible colonization of the gastrointestinal tract of these animals. Despite finding *S. uberis* in the faeces, there was very little indication that these strains were directly linked to mastitis in these cows. Overall, the results of this study have shown that some strains of *S. uberis* can be found at multiple sites on the cow and in the environment, allowing possible mechanisms of infection to be proposed, and to facilitate and direct further studies into *S. uberis* mastitis.

3.1 Introduction

As an environmental pathogen, *S. uberis* is ubiquitous in the dairy cow environment. Cows are continuously exposed to this pathogen during lactation and the non-lactating period (Smith *et al.*, 1985) and it is assumed that teats become contaminated or colonized through contact with *S. uberis* in the dairy environment which may then lead to intramammary infections (Pullinger *et al.*, 2006). The primary source of exposure is unknown but could originate from several sources, including soil and faeces in the environment of the cow, teat skin flora or existing intramammary infections (Pankey, 1989). Also, it has not been established whether the strains causing mammary gland infections actually reflect a random selection of all environmental *S. uberis* strains or a limited subset of strains with enhanced pathogenicity (Zadoks *et al.*, 2005b). A precise niche for *S. uberis* has not been established (Baseggio *et al.*, 1997) and it is thought unlikely that a common environmental source is present on different farms (Phuektes *et al.*, 2001). However, it is accepted that infection of dairy cows with *S. uberis* is largely due to opportunistic infection with a great variety of strains present on the cows and in their immediate environment (Douglas *et al.*, 2000; McDougall *et al.*, 2004; Phuektes *et al.*, 2001; Zadoks *et al.*, 2003). It is also likely that mastitis management schemes, nutrient management and farm ecological factors may influence the ecology of *S. uberis*, however these were not investigated in the present study.

3.1.1 Location of *S. uberis*

3.1.1.1 *S. uberis* on dairy cows

Many regions of the dairy cow body have yielded *S. uberis* upon sampling, including the skin surface, genital tract, intestinal tract, tonsils, udder and teats (Buddle *et al.*, 1988; Cullen & Little, 1969; Razavi-Rohani & Bramley, 1981). Also, it has been found that *S. uberis* could be isolated more frequently from the body of cows in herds that experience high rates of intramammary infection (Kruze & Bramley, 1982).

Cullen and Little (1969) also found evidence of *S. uberis* in the rumen of cattle, possibly in large quantities, and also on the lips. The lips are thought to be an important means of spreading infection from one cow to another as cows frequently lick each other and their surroundings. Also, the bacteria present on the lips could possibly pass through the gut to be excreted with the faeces (Cullen & Little, 1969).

In an early study, it was thought that the skin of teats may be a comparatively unfavourable site for *S. uberis*, and the abdominal wall and lips may be more important reservoirs of infection (Cullen, 1966). However, later studies have found that the skin and teat canal of the bovine teat may be colonized by a greater variety of streptococcal species than suspected previously (Watts, 1988) and *S. uberis* has also been found on the udder skin (Buddle *et al.*, 1988; Sharma & Packer, 1970). In addition, *S. uberis* has been isolated simultaneously from the teat surface and milk from the same quarter (Buddle *et al.*, 1988), indicating that strains of *S. uberis* that are present on the teats may be able to invade the mammary gland and cause infection. It is now assumed that teats become contaminated by *S. uberis* from the dairy environment and the degree of contamination, or colonisation, of the teat end subsequently predisposes the cow to infection. There does appear to be a seasonal variation in the presence of *S. uberis* on the teat skin, with isolates obtained only intermittently during the lactation period and more frequent isolations observed during the dry period (Lacy-Hulbert *et al.*, 2006). These differences in isolation of *S. uberis* may actually reflect the use of teat spraying during lactation, resulting in minimal presence of *S. uberis* on the teats compared to the dry period, when no teat spraying is performed.

There also appears to be a link between mastitis and cleanliness of the cow and environment, with cleaner dairy environments and cows resulting in

herds that produced milk with a lower somatic cell count compared to herds that were classed as dirty (Barkema *et al.*, 1998). Also, the prevalence of isolation of environmental mastitis pathogens was significantly associated with udder hygiene, indicating an important link between contamination from environmental sources and mastitis, particularly subclinical mastitis (Schreiner & Ruegg, 2003).

3.1.1.2 *S. uberis* in the dairy environment

The high heterogeneity of *S. uberis* has been suggested to be consistent with an environmental reservoir of infections (Wang *et al.*, 1999); therefore many different sites within the dairy farm, including soil, water, grass, hay and farm races, have been sampled for the presence of this pathogen (Bramley, 1982; Pullinger *et al.*, 2006; Zadoks *et al.*, 2005b). While *S. uberis* was found at all of these sites, it is thought that moisture, mud and faeces are the primary sources of exposure for environmental pathogens (Schreiner & Ruegg, 2003). This widespread occurrence of *S. uberis* in the environment may indicate that soil is a natural niche for *S. uberis* and bacterial multiplication can occur in the environment (Zadoks *et al.*, 2005b). However, there does appear to be seasonal variation in the presence of *S. uberis* in the dairy environment, with higher numbers of this species observed when air and soil temperatures are low and soil moisture is high, corresponding to winter conditions (Lopez-Benavides *et al.*, 2005). Interestingly, on this particular farm, these higher levels of *S. uberis* in the environment correspond with an increase in the incidence of clinical mastitis cases caused by this pathogen.

Although, detection of a pathogen in an environmental site does not necessarily imply that this site acts as a source of infection (Zadoks *et al.*, 2005b), areas where cows gather or pass frequently appear particularly likely to pose a high risk of *S. uberis* exposure (Cullen & Little, 1969; Zadoks *et al.*, 2005b). In support of this, several of the most common strain types

isolated from the New Zealand dairy farm were also isolated from the cow body and milk from infected quarters, consistent with intramammary infections resulting from contact with the contaminated environment (Pullinger *et al.*, 2006). Also, the multitude of strains of *S. uberis* strengthens the classification of *S. uberis* as an environmental pathogen with multiple habitats (Douglas *et al.*, 2000).

The presence of cows appears to be an important factor in the detection of *S. uberis* in environmental samples. Soil samples collected from close to a stream where cows tended to congregate yielded *S. uberis* (Cullen & Little, 1969) and isolation of *S. uberis* from paddock material was only possible one day after cows had grazed the paddocks (Lacy-Hulbert *et al.*, 2006). Also, areas that tested positive for the presence of *S. uberis* throughout one year included 90% of samples from outdoor lying and gathering areas, 42% of water samples and 67% of samples from grazing areas (Zadoks *et al.*, 2005b). In contrast, *S. uberis* could not be isolated from a nearby field where a non-infected herd grazed (Cullen & Little, 1969) and none of the soil samples from non-dairy environments yielded *S. uberis* (Zadoks *et al.*, 2005b). Therefore the presence of cattle must be important for the introduction and/or maintenance of *S. uberis* in the dairy environment.

3.1.1.3 *S. uberis* in faeces

One of the most likely mechanisms of spreading *S. uberis* within the dairy environment is through contamination by cows, in particular by excretion of *S. uberis* in the faeces. As *S. uberis* has been identified in the rumen of cows (Cullen & Little, 1969), it is also likely to be present in their faeces and, indeed, cows have been found to excrete *S. uberis* in their faeces (Bramley, 1982; Cullen & Little, 1969; Kruze & Bramley, 1982). Zadoks *et al.* (2005) found that one in four cows tested positive for *S. uberis* in their faeces; however there appeared to be a seasonal variation in the presence of this bacterium in faeces. It has also been suggested that the numbers of *S. uberis*

excreted in faeces could be influenced by the diet although this was not tested at the time (Kruze & Bramley, 1982).

Kruze and Bramley (1982) observed persistent faecal shedding within a dairy herd. However, a later study (Zadoks *et al.*, 2005b) found that only one cow of 22 sampled repeatedly tested positive for *S. uberis* over time, indicating a lack of persistent shedding in the faeces of these cows. A closer examination of the *S. uberis* strain types present in cow faeces did not support the concept of persistent shedding. In the same study, 59% of cow faecal samples contained multiple strains of *S. uberis* and when *S. uberis* was isolated from the faeces of the same cow on multiple occasions, the strain types differed (Zadoks *et al.*, 2005b).

In summary, the presence of *S. uberis* in the rumen and faeces indicates that the gastrointestinal tract may become colonised by *S. uberis*, even if only for a short time. It also appears likely that the presence of *S. uberis* in the dairy environment is linked to excretion of this pathogen in cow faeces and it has been suggested that once a herd is infected with *S. uberis* it may remain so and cases of *S. uberis* mastitis will continue to occur (Cullen & Little, 1969).

3.1.2 Environmental versus contagious transmission

The environment is thought to be the most likely source of infection by *S. uberis* (Douglas *et al.*, 2000; Zadoks *et al.*, 2003). Many new infections with *S. uberis* occur in dry cows and in heifers before calving, and these infections cannot be attributed to transmission via the milking machine. However, evidence of contagious transmission of *S. uberis* has been reported.

Some studies have isolated indistinguishable strains of *S. uberis* from intramammary infections occurring in different cows within the same herd, indicating that direct transmission from cow-to-cow may have occurred (Baseggio *et al.*, 1997; Khan *et al.*, 2003). However, the possibility that all

cows had been infected from the same environmental source can not be ruled out. In some cases, multiple quarters of individual cows were also infected with identical strains of *S. uberis*, suggesting that either the onset of infection was simultaneous in the multiple quarters or that direct transmission from infected to uninfected quarters had occurred (Phuektes *et al.*, 2001; Zadoks *et al.*, 2003). Adkinson *et al.* (1993) found that the incidence of infections in multiple quarters within a cow actually appeared to be related to the physical distance between each of the teat ends. The distance between teats of diagonal quarters is usually greatest, followed by teats of the front quarters, teats of ipsilateral quarters, and teats of the rear quarters. They observed that frequencies of simultaneously infected pairs of quarters followed this same order (Adkinson *et al.*, 1993).

The direct transmission of pathogens between quarters and cows is presumed to be occurring during the milking process (Adkinson *et al.*, 1993; Zadoks *et al.*, 2001a) and the milking machine may play an important role in determining which quarters are more likely to be infected (Adkinson *et al.*, 1993). In support of this hypothesis, *S. uberis* was isolated from teat cup liners after the milking of infected quarters and also after subsequent milking of uninfected quarters (Zadoks *et al.*, 2001a); indicating a possible mode of contagious transmission. In contrast to this, other studies have noted that the majority of cases where multiple quarters of individual cows were infected involved different strain types (Pullinger *et al.*, 2007). Also, McDougall *et al.* (2004) noted that only 14% of cows with multiple quarters infected simultaneously with *S. uberis* had the same strain type in two or more of the quarters. It has also been found that, provided the teats are effectively disinfected immediately after milking, the spread of some mastitis pathogens via teat-cups does not greatly affect the incidence of new infections (Neave *et al.*, 1966).

Although no common infection-causing strain has been found (Phuektes *et al.*, 2001; Wieliczko *et al.*, 2002), there has been evidence of one strain predominating within a herd (Gillespie *et al.*, 1998; Phuektes *et al.*, 2001; Zadoks *et al.*, 2003) and each farm commonly has a unique set of *S. uberis* strains (Coffey *et al.*, 2006; McDougall *et al.*, 2004; Zadoks *et al.*, 2003). The predominance of one strain may indicate that this strain is either widespread in the environment and stable over time resulting in reinfection, or it could be persisting in chronically infected quarters between lactations (Phuektes *et al.*, 2001). This predominant strain appeared to be more virulent than other strains, with infection more likely to result in clinical mastitis, and it was also found to be more resistant to antibiotics than any of the other *S. uberis* strains investigated (Phuektes *et al.*, 2001). In the study by Zadoks *et al.* (2003) however, where a predominant strain was also observed using RAPD typing, it was suggested that this result could be attributed to the inability of the typing system to discriminate between closely-related strains rather than a true predominant strain, indicating the importance of the strain typing method chosen. In contrast to both of these studies, McDougall *et al.* (2004) found that neither subclinical nor clinical mastitis could be associated with any particular strain type, so no predominant strain was evident.

The high genetic diversity of *S. uberis* strains observed across multiple studies also supports an environmental contamination rather than contagious transmission as the major route of infection. Khan *et al.* (2003) analysed 69 arbitrarily selected strains from mastitis cases using pulsed-field gel electrophoresis (PFGE); of these, 55 displayed different DNA patterns. Observation of a large variation in strain types has been repeated using PFGE (McDougall *et al.*, 2004) and MLST (Coffey *et al.*, 2006; Pullinger *et al.*, 2006) systems to strain type isolates of *S. uberis* from infected mammary glands. Also, 86% of samples collected from around a dairy farm harboured multiple *S. uberis* strain types (Zadoks *et al.*, 2005b).

Zadoks *et al.* (2003) were able to divide *S. uberis* isolates into two subpopulations. The first subpopulation contained the majority of strains, which were those isolated from both environmental sources and intramammary infections. These infections occurred at any time during the lactating and non-lactating period and were generally of short duration. The second subpopulation of strains was those that predominantly caused subclinical or chronic infections and were probably spread from cow-to-cow during the milking process. They concluded that, although *S. uberis* is mainly an environmental pathogen, it can infect the mammary gland through contagious cow-to-cow transmission.

3.1.3 Overview of *S. uberis* ecology

In summary, the majority of mastitis cases are caused by a diverse range of *S. uberis* strains, indicative of environmental transmission as the primary mechanism of infection. However, there is evidence of contagious cow-to-cow transmission between infected and uninfected quarters. This highly diverse environmental pathogen can be found on the cow's body and it appears that strains present on the teat skin can infect the mammary gland. The most likely source of *S. uberis* in the environment is through excretion in the faeces. However, although many potential environmental reservoirs have been identified, their association with the occurrence of mastitis in a herd is still unclear.

3.1.4 Aims

i) Investigate the similarity of *S. uberis* strain types between infected mammary glands and the environment, faeces, teat skin, and cow body.

Hypothesis 1: Strains that cause mastitis will also be found in the dairy environment.

Hypothesis 2: Particular environmental reservoirs (e.g. farm races) will be more likely to contain strains that also cause mastitis.

ii) Evaluate the *S. uberis* strain diversity in samples from each of these sites.

Hypothesis: There is high strain diversity in the environment.

iii) Determine the presence of three genes required for the production of the hyaluronic acid capsule in *S. uberis* isolates from the environment, faeces, teat skin, cow body and infected mammary glands.

3.2 Materials and methods

3.2.1 Isolation of *S. uberis*

Streptococcus uberis isolates were obtained from several different studies conducted on the Dexcel Lye farm (Appendix 1) between June 2003 and August 2005. During these various studies samples were acquired from the milk of infected mammary quarters, teat skin, teat canal, faeces, various anatomical locations on the cows' body, and the dairy environment, including farm races, paddock soil and grass. The sampling schedules for each study are listed below. Sample processing and bacterial isolation procedures are described in Appendix 2. DNA was isolated and confirmed as *S. uberis* as described in Chapter 2. In total, 713 isolates were strain typed by REP-PCR, including 143 milk, 199 teat skin, 56 teat canal, 102 faecal, 90 cow body, and 123 environmental isolates.

3.2.2 Sampling schedules

3.2.2.1 Clinical and subclinical mastitis cases

Clinical mastitis (CM) was diagnosed when cows presented at milking time with visible clinical symptoms (i.e. clots, discoloured milk) whereas sub-clinical mastitis (SCM) was diagnosed when cows did not have abnormal physical changes in milk appearance yet were found to be positive for *S. uberis* in the bacteriological foremilk samples and had elevated foremilk somatic cell counts (SCC). Single colonies of *S. uberis* were isolated as described in Appendix 2.2. Isolates from random CM and SCM cases were chosen for strain typing during the period of June 2003 to August 2005.

3.2.2.2 Teat skin sampling

The teat skin of 33 cows was swabbed, *S. uberis* obtained and isolates strain typed on either one or two different dates between 15 September 2003 and 25 August 2005 (Table 3.1). The majority of swabs were collected during the dry period (21 May 04 and 1 June 04) from quarters deemed uninfected at

dry-off. Other samples were collected from quarters that were either subclinically or clinically infected with *S. uberis*. In some cases the skin of the teat barrel and teat end (teat orifice) of both infected and uninfected quarters were individually sampled, while for other samples both front, both back or all four teats were sampled with the same swab. Teat swabs were aseptically collected before sampling milk from that cow and *S. uberis* isolated from all samples as described in Appendix 2.3.1.

3.2.2.3 Faecal sampling

On a monthly basis, between July 2003 and November 2004, faecal samples were obtained from a herd of approximately 100 cows by inserting a sterile cotton-tipped swab, moistened in 0.1% peptone diluent, into the rectum of cows. Samples were processed and *S. uberis* isolated as described in Appendix 2.5. For cows where *S. uberis* was isolated, additional swabs were collected weekly for a period of up to 2 weeks after the monthly screening sample. Not all samples that contained *S. uberis* isolates were strain typed however.

	Cow #																															
	51	7111	7160	561	9149	9146	1027	1034	1036	1038	1048	1003	1023	545	546	6277	6278	8112	8111	9101	9102	1033	2031	9123	8138	1034	8212	1058	680	9139	3213	2659	3105
15 Sep 03	S	S																														
29 Sep 03			N																													
10 Dec 03				N																												
12 Mar 04					N	N																										
21 May 04						N	N	N	N	N	N	N	N	N	N	N	N	N														
1 Jun 04												N	N	N	N	N	N	N	N	N	N											
12 Aug 04																						C										
17 Aug 04																							C									
19 Aug 04																								C								
6 Sep 04																									S							
17 Sep 04																						C				C						
24 Oct 04																											C					
16 Nov 04																										S		S	S	S		
18 Aug 05																															C	
22 Aug 05																																C
25 Aug 05																																C

Table 3.1. Samples collected from the teat skin of cows between September 2003 and August 2005.
N – No infection in any quarter, S – subclinical mastitis in one quarter, C – clinical mastitis in one quarter.

3.2.2.4 Environmental sampling

Samples were collected from farm races, paddock soil and paddock grass using the following sampling regimes.

(i) Farm races

On a fortnightly basis between November 2003 and November 2004, surface material was acquired from three sampling points positioned along four high-traffic races and three low-traffic races. Race material samples were processed and *S. uberis* isolated as described in Appendix 2.6.1. Of the isolates obtained, only 111 were strain typed.

(ii) Paddocks

Three paddocks were monitored for *S. uberis* contamination at three stages of lactation: mid lactation (November 2003), late lactation (April 2004) and during the dry period (August 2004). Each paddock was sampled at nine locations either before or after grazing. On each sampling occasion, a sample of grass, surface soil and sub soil (obtained at 5 cm depth) were collected and processed as described in Appendix 2.6. Twelve of the *S. uberis* isolates obtained were strain typed by REP-PCR.

3.2.3 Sampling of the body of cows with mastitis

Nine cows (during seven confirmed clinical and four subclinical intramammary infections) were subjected to an extensive sampling procedure to determine if the infective strain could also be isolated from other locations on the body of the cow. One of the nine cows had two cases of clinical mastitis (CM) while another cow had clinical and then subclinical mastitis (SCM) two months later. CM was indicated by visible symptoms (e.g. clots, discoloured milk) and *S. uberis* in foremilk samples, while SCM was characterized by *S. uberis* in the mid-lactation bacteriological foremilk sample, elevated foremilk somatic cell count (SCC), with the absence of any visible symptoms.

The sampling procedure involved swabbing 15 areas of the body including the teat ends (teat orifice) and teat barrels of the infected and uninfected quarters, udder skin, inside surface of the leg hock nearest to the infected quarter, coronet band of both back hooves, lips, nostrils, tail swish, and rectum. Foremilk samples were collected aseptically after collecting the teat swab samples and before treating the clinically infected quarters with antibiotics. The sampling procedures are described in Appendix 2.4 (body swab samples), Appendix 2.3.1 (teat skin swabs), Appendix 2.5 (faecal/rectum samples) and Appendix 2.2 (milk samples). Where possible five isolates from each sample were strain typed by REP-PCR. If less than five isolates were obtained from any sample then all isolates were typed.

3.2.4. Sampling of the teat end and teat canal of infected mammary quarters

The milk, teat end and teat canals of two random quarters from 22 cows (44 quarters total) were sampled to establish the presence of *S. uberis* at these locations. The teat ends were initially sampled by swabbing with a sterile cotton swab before thorough cleaning of the teats with 70% ethanol-soaked cotton wool. Next, the teat canals were reemed using a sterile 1.0 mm diameter interdental brush (DentalPro; Jacks Co. Ltd) and the teats cleaned again before aseptically collecting milk from these quarters. The milk, teat end and teat canal swabs were processed and *S. uberis* isolated as described in Appendices 2.2 and 2.3 respectively.

Two sampled quarters were clinically infected with *S. uberis* and two quarters had *S. uberis* present in the milk without any clinical symptoms (subclinical infections). Three isolates from the milk and 16 – 20 isolates from the teat end and teat canal from both of these clinically infected quarters and one of the subclinically infected quarters were strain typed by REP-PCR.

3.2.5 Strain typing of *S. uberis* isolates

Isolates were strain typed using the REP-PCR and anchored typing methods developed in Chapter 2; the optimized PCR reactions used are given below. A total of 127 isolates from infected quarters (56 isolates), teat skin (8), faeces (19) and the environment (44 isolates) were also sent to the Institute for Animal Health, Compton, UK for typing by MLST using the methods described in Chapter 2.2.14.

3.2.5.1 REP-PCR

Each 25 µl PCR reaction was prepared with master-mix A (Chapter 2.2.5.1), which consisted of 1x HotMaster buffer, 2.5 mM Mg^{2+}, 40 µM dNTPs, 0.4 µM BOXA1R and ERIC1R primers (Chapter 2.2.6), and 1.25 U of Thermo-Start® DNA polymerase (ABgene, Epsom, Surrey, UK). To each PCR reaction, 2.5 µl of DNA was added to give a final concentration of 200 ng. REP-PCR program 2 was used for thermal cycling, which included an initial activation/denaturation step of 94°C for 7 min, 30 s followed by 40 cycles of denaturation (94°C, 20 s); annealing (45°C, 30 s) and extension (68°C, 2 min) and a final extension step of 72°C for 5 min. The temperature ramp rate before and after the annealing step was set to 0.5°C/s. The same thermal cycler, model PTC-100 (MJ Research Inc., Waltham, MA, USA), was used for all PCR reactions and PCR products (10 µl) were electrophoresed in 1.5% agarose gels prepared in SB buffer as described in Chapter 2.2.9.

3.2.5.2 Anchored typing

A random selection of isolates were also typed using the Anchored typing method developed in Chapter 2. PCR reactions (25 µl) were prepared with master-mix B (Chapter 2.2.5.2) to give 1x HotMaster buffer, 2.5 mM Mg^{2+}, 200 µM dNTPs, 0.4 µM of MtuA-R and REP2 primers (Chapter 2.2.6), 1 U of *Taq* DNA polymerase (1 U/µl; Roche Diagnostics NZ, Ltd., Auckland, NZ), and approximately 200 ng DNA. All amplification reactions were performed on the same PTC-100 thermal cycler (MJ Research Inc., Waltham,

MA, USA) with the following conditions: An initial denaturation step of 94°C for 2 min followed by 40 cycles of 94°C for 20 s, 45°C for 30 s, and 68°C for 2 min. Ramp rate was set to the default setting for the machine. PCR products (10µl) were electrophoresed in 1.8% agarose gels prepared in SB buffer as described in Chapter 2.2.9.

3.2.6 Selection of isolates representing each strain type

Reproducibility experiments in Chapter 2 gave an average band pattern similarity of >90% when the same isolate was repeatedly typed; consequently, isolates with band patterns less than 90% similar were likely to be different strain types. Therefore, correlation between isolates or strain types was defined as REP-PCR band patterns with ≥90% similarity after comparison with Pearson correlation and UPGMA using the settings in GelCompar II as described in Chapter 2.2.12.

Initially, band patterns of isolates obtained from one sample were compared and different strain types identified. Then, one isolate representing each strain type was chosen as the 'representative isolate' for that sample. Representative isolates were compared between collected samples and the presence of the same strain type across the samples identified. From this comparison, the total number of unique strain types could be determined. For example, out of 80 representative isolates from different samples, there may only be 70 unique strain types due to the same strain type being found in multiple samples.

3.2.7 Estimation of strain diversity

To obtain an estimate of strain diversity within each set of isolates (e.g. all faecal isolates, or all race isolates) that were typed, representative isolates from each sample were assigned strain type numbers. These numbers were entered into the EstimateS 7.5 freeware software application for Windows (Robert K. Colwell, Department of Ecology and Evolutionary Biology,

University of Connecticut, USA; available at http://viceroy.eeb.uconn.edu/EstimateS) as format 3 with species, sample and abundance triplets. A rarefaction curve was generated based on 50 randomizations with replacement (as detailed in the EstimateS 7.5 User's Guide available from the internet site listed above) and results (including values for Sobs (Mao Tau)) were exported into Microsoft Excel (2003) for analysis.

3.2.8 Has A, B, C genotyping PCR reactions

3.2.8.1 Oligonucleotide primers

Oligonucleotide primers for genes *hasA*, *hasB* and *hasC* (Table 3.2) were designed based on the *S. uberis* gene sequence data for *hasA* and *hasB* (GenBank accession AJ242946) and *hasC* (GenBank accession AJ400707). Primers were synthesised by Sigma-Aldrich, Ltd. (Auckland, NZ) and reconstituted in TE buffer to 200 µM according to the information sheet received with the primers.

Primers	Primer sequence (5′ – 3′)
hasA-F	CATAGGGGATGACCGTTGTT
hasA-R	GCAACACTTGGTGTGGCTAA
hasB-F	AACCCTTCTTTTGGCTATGGA
hasB-R	TCTTCATTCATCATTGGCTCA
hasC-F	CGCTCCATTGAAGACCATTT
hasC-R	CATCGTAGCGTTTGCCAGTA

Table 3.2. HasA, B and C gene forward (F) and reverse (R) primer sequences.

3.2.8.2 PCR reactions

Each 25 µl PCR reaction was prepared using master-mix B (Chapter 2.2.5.2), 0.4 µM of each primer, 0.625 units of HotMaster™ *Taq* DNA polymerase (Eppendorf AG, Hamburg, Germany) and 2.5 µl of prepared DNA to obtain

between 200 and 500 ng/µl total. Amplification conditions involved an initial activation/denaturation step of 94°C for 2 minutes followed by 40 cycles of 94°C for 20 s, annealing at 55°C for 20 s and extension at 68°C for 1 min. PCR products (10 µl) were electrophoresed in a 1.5% agarose gel prepared in SB buffer as described in Chapter 2.2.9. A negative PCR control (PCR reaction mix without DNA template) was always included, along with a positive control that contained DNA from strains known to contain all three genes.

3.3 Results

3.3.1 Analysis of strains from intramammary infections

Isolates of *S. uberis* were obtained from the milk of 37 cows that had either clinical or subclinical infections. Some of these cows had recurrent clinical infections, while others had cases of both subclinical and clinical mastitis. Also, in some cows, multiple quarters were simultaneously infected; therefore milk from all quarters was sampled, *S. uberis* isolated and strain typed. Between one and five colonies from each milk sample were strain typed using REP-PCR, and in some cases the results were confirmed by anchored typing.

3.3.1.1 Number of strains found in each mastitis case

Of all the cases of mastitis that were sampled, multiple *S. uberis* isolates were collected from 13 cases of clinical mastitis (CM) and 4 cases of subclinical mastitis (SCM). After REP-PCR and anchored typing, two infected quarters had two different strains of *S. uberis* present in the milk. One quarter (#1058) was subclinically infected while the other (#3105) had a clinical infection (Figure 3.1). All other clinical and subclinical infections had only one strain present in the milk of the infected quarter; some examples are given in Figure 3.1.

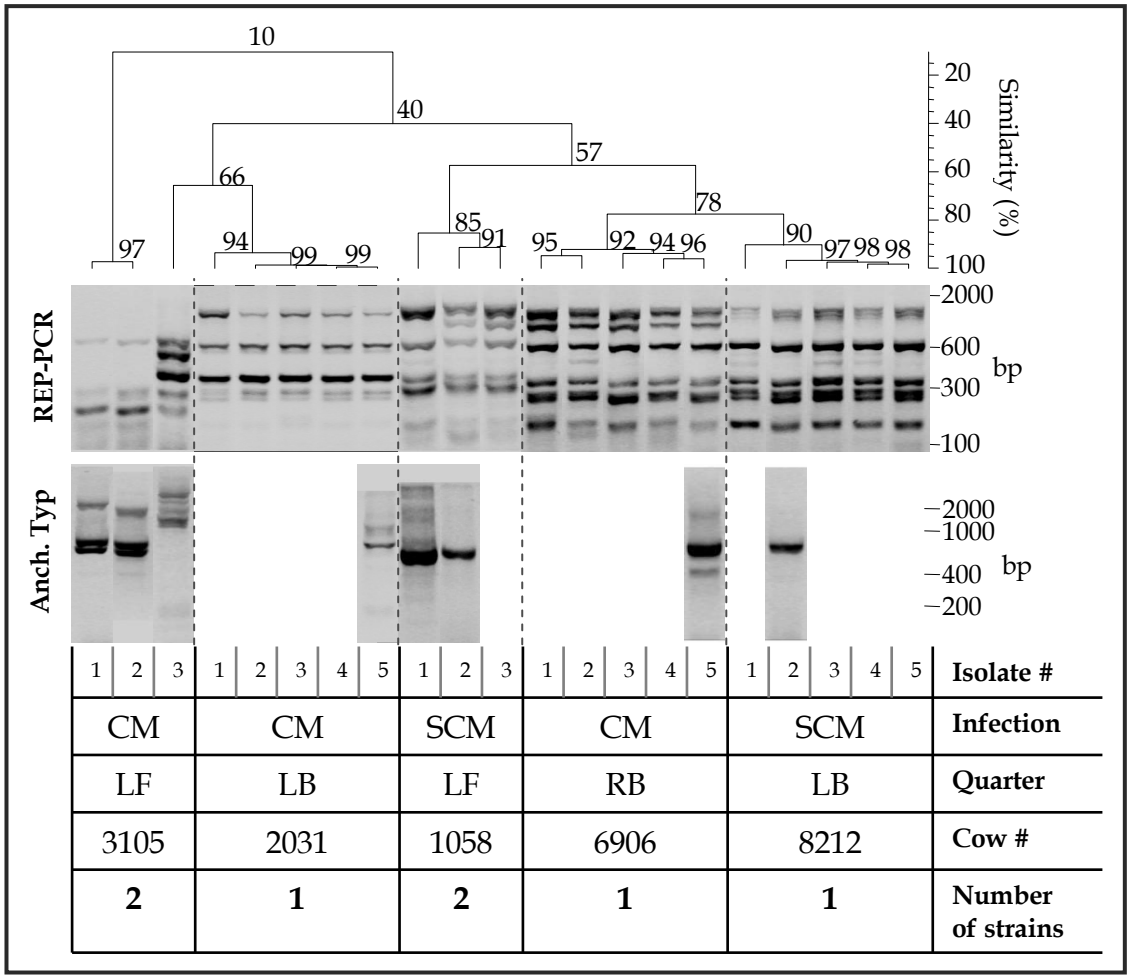


Figure 3.1. Strain typing of multiple isolates from clinical and subclinical infections. Three to five isolates of *S. uberis* from the milk of quarters with clinical mastitis (CM) or subclinical mastitis (SCM) were typed by REP-PCR and anchored typing. Band patterns were compared with Pearson correlation (optimization 1.0% for REP-PCR; 2.0% for anchored typing) and a UPGMA dendrogram created. LF – Left front; LB – Left back; RB – Right back.

3.3.1.2 Infection of multiple quarters with *S. uberis*

Seventeen of the 37 cows had *S. uberis* present in the milk of more than one quarter on the same sampling day. Generally only two quarters were infected simultaneously, however three cows (#1024, #27, and #6826) had three infected quarters and one cow (#8777) had all four quarters infected at the same time. When isolates from each of the infected quarters were strain typed, nine of the 17 cows had the same strain present in each of the infected quarters (Table 3.3), while the other eight cows had different strains in each quarter (Table 3.4).

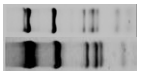
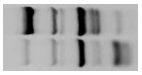
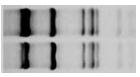
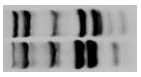
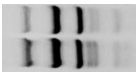
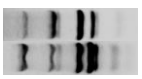
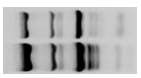
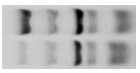
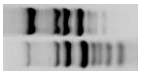
	Cow #	Date	Quarter	Infection	MLST	REP-PCR pattern
Same strain between quarters	26	27Apr04	RF	SCM		
			LB	SCM		
	51	27Aug03	RF	SCM		
			RB	SCM		
	532	27Apr04	RF	SCM		
			LB	SCM		
	1027	30Jul03	LB	SCM	94	
			RB	CM	94	
	6826	30Aug03	LF	SCM		
			RF	SCM		
	8124	9Sep03	LF	SCM		
			LB	CM		
	8777	4Sep03	LB	SCM		
			RB	SCM		
	9673	13Aug03	RF	CM	91	
			LB	CM	139	
	9676	22Aug03	RF	SCM	142	
			RB	SCM	142	

Table 3.3. Same strain in two infected quarters of each cow. REP-PCR band patterns run from left to right, 2000 to 100 bp. MLST was performed on isolates in some cases and the strain type (ST) listed

Two of the cows with three infected quarters (#27 and #1024) had a different strain in each quarter, while cow #6826 had the same strain in two quarters and a different strain in the third quarter. Cow #8777, with four infected quarters, had the same strain in two quarters (LB and RB), which was different to the strains found in the other two quarters (LF and RF). For cow #9673 (Table 3.3), the REP-PCR band patterns of isolates from each quarter looked very similar so were deemed to be the same strain; however, the MLST result was different for each isolate, suggesting that they may be different strain types.

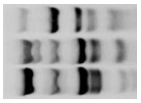
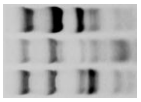
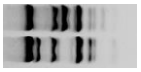
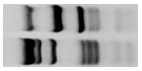
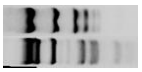
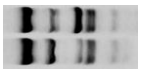
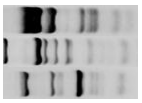
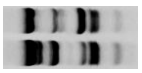
	Cow #	Date	Quarter	Infection	MLST	REP-PCR pattern
Different strains between quarters	27	13Aug03	RF	SCM	104	
			LB	SCM	137	
			RB	SCM	141	
	1024	7Aug03	RF	SCM	91	
			LB	SCM	138	
			RB	SCM	140	
	1036	18Aug03	LB	SCM	131	
			RB	CM	137	
	6826	30Aug03	RF	SCM		
			LB	SCM		
	6904	27Aug03	LF	SCM		
			RB	SCM		
	8126	9Jul03	RF	SCM		
			RB	SCM		
	8777	4Sep03	LF	SCM		
			RF	SCM		
			RB	SCM		
	9677	2Jul03	RF	CM	80	
			RB	SCM	81	

Table 3.4. Different strains between infected quarters of the same cow. REP-PCR band patterns run from left to right, 2000 to 100 bp. MLST was performed on isolates in some cases and the ST is listed.

While some cows had different strains in each quarter, some of these strains appeared to be the same as those in other cows. For example isolates from cow #1036 RB and from cow #27 LB, which were collected around the same time, had very similar REP-PCR patterns and strain types by MLST (ST 137), suggesting that the same strain may be present in two different cows. This correlation of strain types between cows will be investigated in more detail later in this chapter.

3.3.1.3 Recurrent and persistent infections over time

Four cows had two cases of clinical mastitis with 3 to 21 weeks between infection events. Likewise, six cows were found to have more than one case of subclinical mastitis, although the time between detection of these infections ranged from just under two weeks (therefore likely to be the same infection event) up to 37 weeks between detection of infection events (therefore could be recurrent rather than one continuous infection).

Of the four cows with two cases of clinical mastitis, the same strain was isolated from both infection events for cows #461, #1024, #9677 (Table 3.5), while two different strains were isolated from the two cases for the other cow (#1033; Table 3.6). For those cows with subclinical mastitis, five of the cows had the same strain present each time the infection was detected (Table 3.6), and only one cow had a different strain present each time it was sampled (Table 3.7). Unfortunately it is unknown if the collection of *S. uberis* isolates from subclinical mastitis cases over time was due to a continuous infection of the mammary quarter or was actually reinfection at a later date after the previous infection had been cleared, as samples were not collected between the two dates shown. For the five cows where the same strain was found over time, particularly for cow #1043 that was sampled twice 13 days apart, it was perhaps more likely that these quarters were continuously infected by the same strain rather than cure followed by re-infection. However, where the strain type changed between sampling it was more likely that the initial infection was cleared and then the same quarter re-infected with a different strain of *S. uberis*.

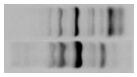
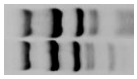
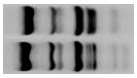
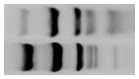
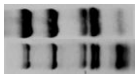
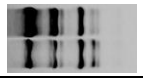
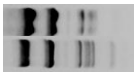
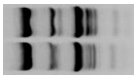
	Cow #	Date	Quarter	Infection	MLST	REP-PCR pattern
Recurrent infection with same strain	461	29Sep03	RB	CM	89	
		23Feb04	RB	CM		
	1024	12Aug03	RF	CM	91	
		4Sep03	RF	CM		
	9677	2Jul03	RF	CM	80	
		5Sep03	RF	CM		
	27	13Aug03	RF	SCM	104	
		13Nov03	RF	SCM	104	
	1002	26Jul03	RB	SCM	134	
		2Apr04	RB	SCM		
	1043*	10Jul03	RB	SCM		
		23Jul03	RB	SCM		
	8212	27Aug03	LB	SCM		
		12Nov03	LB	SCM	106	
	8777	4Sep03	RB	SCM		
		12Nov03	RB	SCM	91	

Table 3.5. Recurrent infections of the same quarter with the same strain of *S. uberis*. REP-PCR band patterns run from left to right, 2000 to 100 bp. *Less than one month between samples therefore likely to be the same infection event.

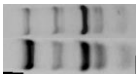
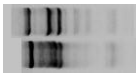
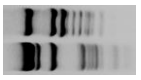
	Cow #	Date	Quarter	Infection	MLST	REP-PCR pattern	Anch. typing
Different strains	1033	12Aug04	LF	CM			
		17Sep04	LF	CM	91		
	6906	22Aug03	RB	SCM	143		
		12Nov03	RB	SCM	105		

Table 3.6. Recurrent infections of the same quarter by different strains of *S. uberis*. REP-PCR and anchored (Anch.) typing band patterns run from left to right, 2000 to 100 bp.

3.3.1.4 Progression from subclinical to clinical mastitis

Fourteen cows with subclinical mastitis developed clinical mastitis at a later date. Of these, 11 cows had the same strain present with both infection events (Table 3.7), while four cows had different strains present (Table 3.8). Also, one cow (#1161) that had clinical mastitis was later found to have subclinical mastitis in the same quarter with the same strain found each time (Table 3.7).

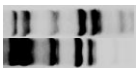
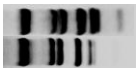
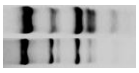
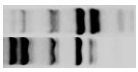
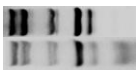
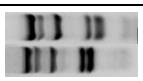
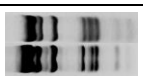
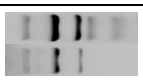
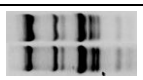
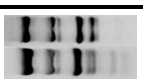
	Cow #	Date	Quarter	Infection	MLST	REP-PCR pattern
Same strain between SCM and CM	22	30Jul03	LB	SCM	90	
		4Aug03	LB	CM	90	
	618	29Jul03	LB	SCM	135	
		5Aug03	LB	CM	135	
	633	8Jul03	RF	SCM	83	
		14Jul03	RF	CM		
	1007	26Jul03	LB	SCM	94	
		1Aug03	LB	CM	94	
	1024	7Aug03	RF	SCM	91	
		12Aug03	RF	CM	91	
	1025	5Aug03	LB	SCM	94	
		10Aug03	LB	CM	94	
	1034	17Sep04	RB	CM		
		16Nov04	RB	SCM		
	6826	30Aug03	LB	SCM		
		29Sep03	LB	CM	147	
	8217	8Jul03	LB	SCM	85	
		11Jul03	LB	CM		
	8777	4Sep03	RB	SCM		
		19Mar04	RB	CM		
	1161	19Jul03	LB	SCM		
		2Sep03	LB	CM		

Table 3.7. Same strain found between subclinical and clinical mastitis. REP-PCR band patterns run from left to right, 2000 to 100 bp. MLST was performed in some cases and the ST is listed.

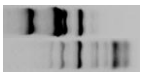
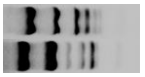
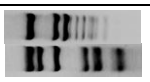
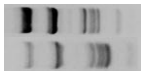
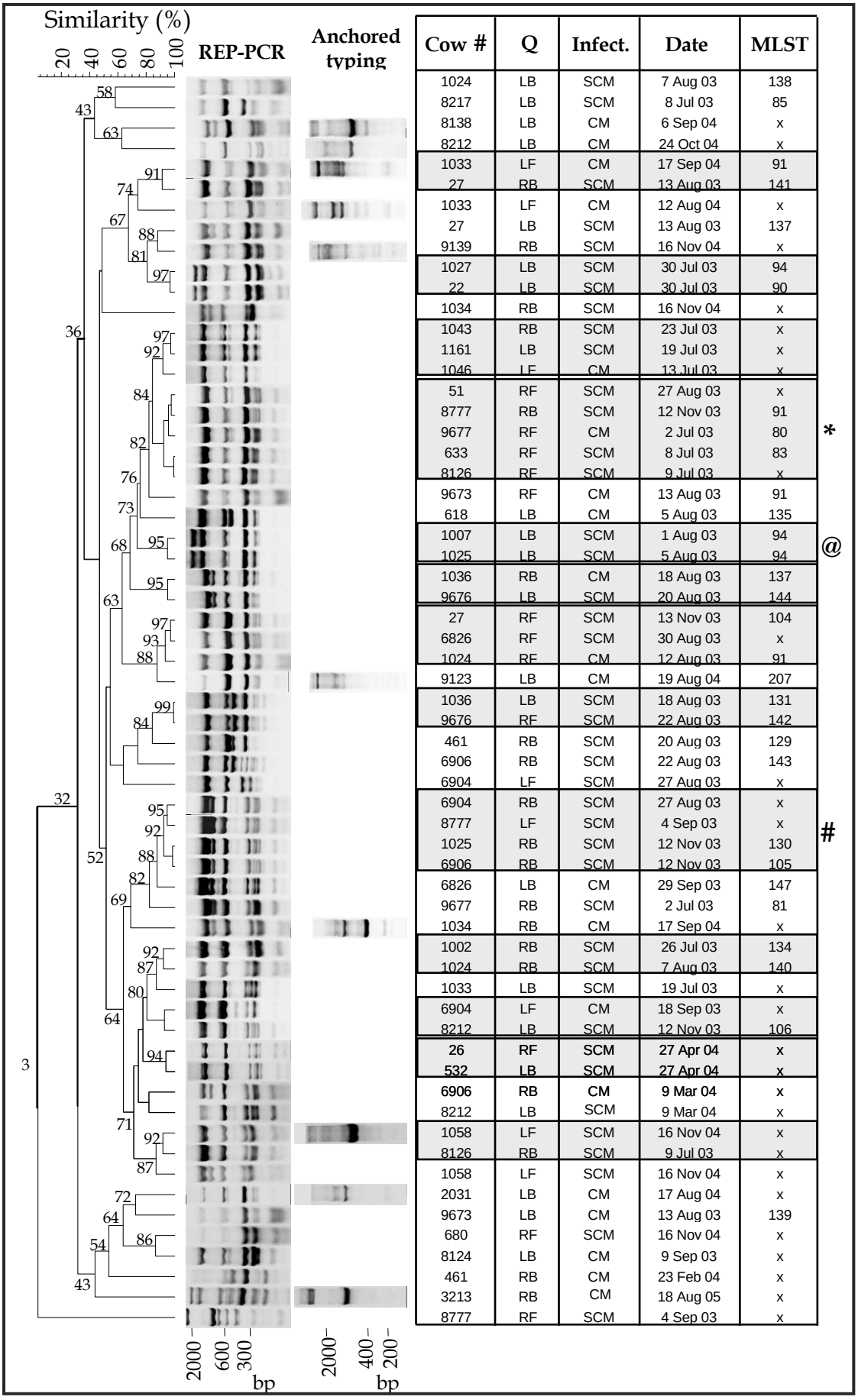
	Cow #	Date	Quarter	Infection	MLST	REP-PCR pattern
Different strain SCM to CM	461	20Aug03	RB	SCM	129	
		29Sep03	RB	CM	89	
	6904	27Aug03	LF	SCM		
		18Sep03	LF	CM		
	6906	22Aug03	RB	SCM	143	
		9Mar04	RB	CM		
	8212	12Nov03	LB	SCM	106	
		24Oct04	LB	CM		

Table 3.8. Different strain found between subclinical and clinical mastitis. REP-PCR band patterns run from left to right, 2000 to 100 bp.

3.3.1.5 Correlation of infective strain types between cows

REP-PCR and anchored typing band patterns for representative isolates of each identified milk strain were compared across cows (Figure 3.2). Correlation of strain types between cows was defined as REP-PCR band patterns with ≥90% similarity after comparison by Pearson correlation and UPGMA. Thirteen different strains were associated with infections in more than one cow while all other strain types (28) appeared to be unique to a single cow. The most common strain type (*) was prevalent between 2 July and 12 November 2003, during which time four cows had subclinical mastitis with this strain and one cow had clinical mastitis. Another commonly observed strain type (#) caused subclinical mastitis in four different cows over a period of 11 weeks.

Figure 3.2 (next page). Comparison of infective strains across cows.
REP-PCR and anchored typing band patterns were compared for each strain isolated from infected (infect.) quarters (Q) using the average of experiments option in GelComparII and a UPGMA dendrogram created. Grey boxes indicate isolates with ≥90% band pattern similarity. Notations (*, # and @) are explained in the text. Some isolates were not typed by MLST (x).



Cow #	Q	Infect.	Date	MLST	
1024	LB	SCM	7 Aug 03	138	
8217	LB	SCM	8 Jul 03	85	
8138	LB	CM	6 Sep 04	x	
8212	LB	CM	24 Oct 04	x	
1033	LF	CM	17 Sep 04	91	
27	RB	SCM	13 Aug 03	141	
1033	LF	CM	12 Aug 04	x	
27	LB	SCM	13 Aug 03	137	
9139	RB	SCM	16 Nov 04	x	
1027	LB	SCM	30 Jul 03	94	
22	LB	SCM	30 Jul 03	90	
1034	RB	SCM	16 Nov 04	x	
1043	RB	SCM	23 Jul 03	x	
1161	LB	SCM	19 Jul 03	x	
1046	LF	CM	13 Jul 03	x	
51	RF	SCM	27 Aug 03	x	
8777	RB	SCM	12 Nov 03	91	
9677	RF	CM	2 Jul 03	80	*
633	RF	SCM	8 Jul 03	83	
8126	RF	SCM	9 Jul 03	x	
9673	RF	CM	13 Aug 03	91	
618	LB	CM	5 Aug 03	135	
1007	LB	SCM	1 Aug 03	94	
1025	LB	SCM	5 Aug 03	94	@
1036	RB	CM	18 Aug 03	137	
9676	LB	SCM	20 Aug 03	144	
27	RF	SCM	13 Nov 03	104	
6826	RF	SCM	30 Aug 03	x	
1024	RF	CM	12 Aug 03	91	
9123	LB	CM	19 Aug 04	207	
1036	LB	SCM	18 Aug 03	131	
9676	RF	SCM	22 Aug 03	142	
461	RB	SCM	20 Aug 03	129	
6906	RB	SCM	22 Aug 03	143	
6904	LF	SCM	27 Aug 03	x	
6904	RB	SCM	27 Aug 03	x	
8777	LF	SCM	4 Sep 03	x	#
1025	RB	SCM	12 Nov 03	130	
6906	RB	SCM	12 Nov 03	105	
6826	LB	CM	29 Sep 03	147	
9677	RB	SCM	2 Jul 03	81	
1034	RB	CM	17 Sep 04	x	
1002	RB	SCM	26 Jul 03	134	
1024	RB	SCM	7 Aug 03	140	
1033	LB	SCM	19 Jul 03	x	
6904	LF	CM	18 Sep 03	x	
8212	LB	SCM	12 Nov 03	106	
26	RF	SCM	27 Apr 04	x	
532	LB	SCM	27 Apr 04	x	
6906	RB	CM	9 Mar 04	x	
8212	LB	SCM	9 Mar 04	x	
1058	LF	SCM	16 Nov 04	x	
8126	RB	SCM	9 Jul 03	x	
1058	LF	SCM	16 Nov 04	x	
2031	LB	CM	17 Aug 04	x	
9673	LB	CM	13 Aug 03	139	
680	RF	SCM	16 Nov 04	x	
8124	LB	CM	9 Sep 03	x	
461	RB	CM	23 Feb 04	x	
3213	RB	CM	18 Aug 05	x	
8777	RF	SCM	4 Sep 03	x	

Figure 3.2. Comparison of infective strains across cows.

Unlike the tables shown earlier in this Chapter, strain types assigned by REP-PCR and MLST rarely corresponded when REP-PCR band patterns were compared using Pearson correlation. Only one of the 13 strain types isolated from more than one cow as assessed by REP-PCR actually had the same strain type by MLST (ST 94; @). REP-PCR band patterns for isolates with the same MLST appeared very similar, indicating that they may have been incorrectly grouped when Pearson correlation was used to compare band patterns.

3.3.1.6 Incidence of infection in each quarter

Of the 60 infection events that were observed, 41 of these occurred in the rear quarters of cows while only 19 were in the front two quarters, indicating that the rear quarters were more likely to become infected than the front two quarters. Infections in the left or right side of the udder were similar with 31 infections in the two right quarters of the udder and 29 infected quarters on the left side of the udder.

3.3.2 Isolation of *S. uberis* from cow's teats

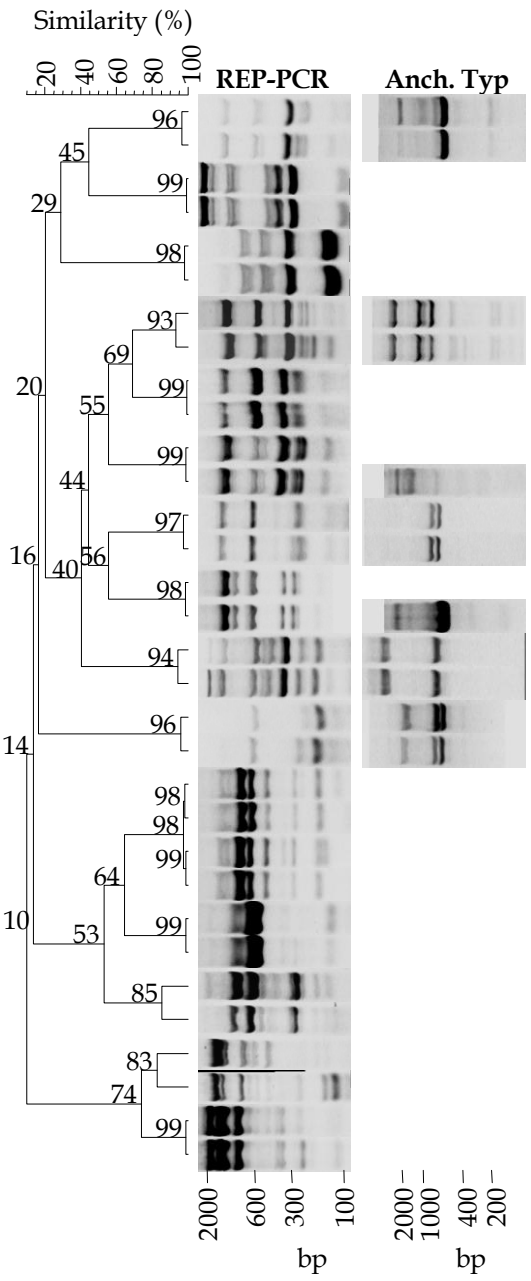
Streptococcus uberis was isolated from the teat skin of both infected and uninfected quarters. Between two and 20 isolates of *S. uberis* were strain typed from a total of 42 different swab samples of cow teat skin. These included samples collected during May and June from dry cows that had no infection present in any quarter at dry off.

3.3.2.1 Single strain of *S. uberis* on teat skin

In 16 teat swab samples, only one strain type was identified (Figure 3.3), but for eight of these samples only two isolates were typed, therefore more strain types may have been present but were not identified. Cow #9123 and #9139 had both an infected and uninfected quarter sampled and, while only one strain was found on each, this was different between the two teats of the same cow, indicating that one strain type was not prevalent across all

teats of the same cow. Interestingly, the observed strain types appear to be unique for each teat, except for cows #1038 and #1027, which appear to have the same strain present on their teats on the same date.

Only one strain of *S. uberis* was found on the teat skin of the six clinically or subclinically infected quarters; therefore it was possible that the strain on the teats was the same as that in the milk from the infected gland (milk or infective strain). To assess this, teat skin and milk isolates from infected quarters were compared by REP-PCR and anchored typing (Table 3.9).



Cow details				Date	Actual No. typed*
Cow #	Q	Swab	Infect.		
9123	RB	End	NI	19Aug04	4
6277	All	Teat	NI	21May04	2
6278	All	Teat	NI	21May04	2
1033	LF	Teat	CM	12Aug04	10
9123	LB	Barrel	CM	19Aug04	5
9139	RB	End	SCM	16Nov04	3
2659	RF	End	SCM	22Aug05	18
9139	LB	Barrel	NI	16Nov04	3
3213	RB	End	CM	18Aug05	18
3105	LF	End	CM	25Aug05	18
1038	B	Teat	NI	21May04	2
1027	F	Teat	NI	21May04	2
1034	B	Teat	NI	21May04	2
8112	All	Teat	NI	21May04	2
1023	B	Teat	NI	21May04	2
1048	B	Teat	NI	21May04	2

Figure 3.3. Comparison of teat samples where only one strain was detected. Band patterns of two representative isolates from a maximum of 18 (*) from each sample were compared using the average of experiments option in GelComparII (optimization 2% REP-PCR; 0.85% anchored typing). Either the entire teat was swabbed (teat) or the teat barrel (barrel) or teat end (end; around teat orifice) was swabbed independently. Isolates were obtained from swabs of individual quarters (Q), or combined samples from both front teats (F), both back teats (B), or all four teats (All). Strains found on the teat skin of more than one cow are indicated by grey boxes. NI – not infected.

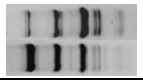
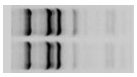
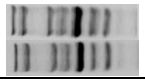
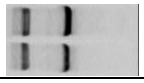
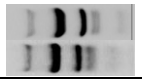
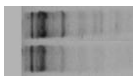
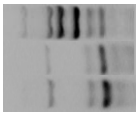
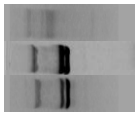
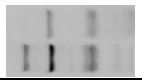
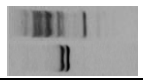
	Cow #	Q	Infection	Sample	REP-PCR pattern	Anchored typing
A	1033	LF	CM	**Milk**		
				Teat barrel		
	3213	RB	CM	**Milk**		
				Teat end		
	9123	LB	CM	**Milk**		
				Teat barrel		
	9139	RB	SCM	**Milk**		
				Teat end		
B	3105	LF	CM	**Milk 1**		
				Milk 2		
				Teat end		
C	2659	RF	SCM	**Milk**		
				Teat end		

Table 3.9. Comparison of teat skin and milk strains from infected quarters. (A) Same strain in milk and on teat skin, (B) Two strains in milk and one of those on teat, (C) Different strain in milk and on teat skin.

The infective milk strain was isolated from the teat skin of five of the six infected quarters. Only cow #2659 had a different strain on the teat from that in the milk. Cow #3105 had two strains in milk; but only one was detected on the teat end of this same quarter.

3.3.2.2 Multiple strains of *S. uberis* on teat skin

A further 26 teat swab samples yielded multiple strain types and the proportion of strains identified to isolates typed was 0.8 (Table 3.10). The number of strain types detected on teat skin, although not significant, differed between uninfected and infected quarters. Of the five clinical quarters, four had only one strain present on the teat skin. In contrast, only two of the four subclinically infected quarters and 10 of 33 uninfected quarters had only one strain present on the teat skin.

Cow	Q	Sample	Infection	Date	No. of isolates*	No. of strains#
545	All	Teat	NI	21 May 04	**2**	**2**
545	All	Teat	NI	1 Jun 04	**2**	**2**
546	All	Teat	NI	21 May 04	**2**	**2**
546	All	Teat	NI	1 Jun 04	**2**	**2**
1034	LF	Teat end	NI	16 Nov 04	**2**	**2**
1034	RF	Teat end	NI	16 Nov 04	**2**	**2**
1036	F	Teat	NI	21 May 04	**4**	**3**
1048	F	Teat	NI	21 May 04	**5**	**2**
1058	LB	Teat end	NI	16 Nov 04	**3**	**3**
1058	LF	Teat barrel	SCM	16 Nov 04	**3**	**3**
1058	RF	Teat barrel	NI	16 Nov 04	**3**	**3**
6278	All	Teat	NI	1 Jun 04	**3**	**2**
8111	All	Teat	NI	1 Jun 04	**2**	**2**
8112	All	Teat	NI	1 Jun 04	**4**	**3**
8138	LB	Teat barrel	CM	6 Sep 04	**3**	**2**
8138	LF	Teat end	NI	6 Sep 04	**3**	**3**
9101	All	Teat	NI	1 Jun 04	**2**	**2**
9102	All	Teat	NI	1 Jun 04	**3**	**2**
9123	RF	Teat barrel	NI	19 Aug 04	**5**	**4**
9123	RF	Teat end	NI	19 Aug 04	**5**	**4**
9139	RF	Teat barrel	NI	16 Nov 04	**3**	**2**
9139	RF	Teat end	NI	16 Nov 04	**3**	**2**
9139	RB	Teat barrel	SCM	16 Nov 04	**3**	**2**
9146	All	Teat	NI	21 May 04	**5**	**5**
9146	All	Teat	NI	12 Mar 04	**5**	**5**
9149	All	Teat	NI	12 Mar 04	**6**	**2**
				TOTAL	**85**	**68**
Proportion of strains identified/isolates typed:					**0.8**	

Table 3.10. Multiple strains on teat skin. Multiple isolates (*) were typed from swabs of individual teats, or combined samples from both front teats (F), both back teats (B), or all four teats (All). The number of strain types was identified (#) and the proportion of strains identified per isolate calculated.

The multiple strain types from the teat skin of infected quarters were compared to the infective milk strain from that quarter and all were found to be different (Table 3.11). Cow #1058 actually had two different strains present in the milk from the infected quarter, but neither of these two strains were found on the teat skin.

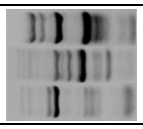
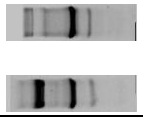
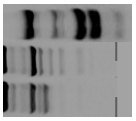
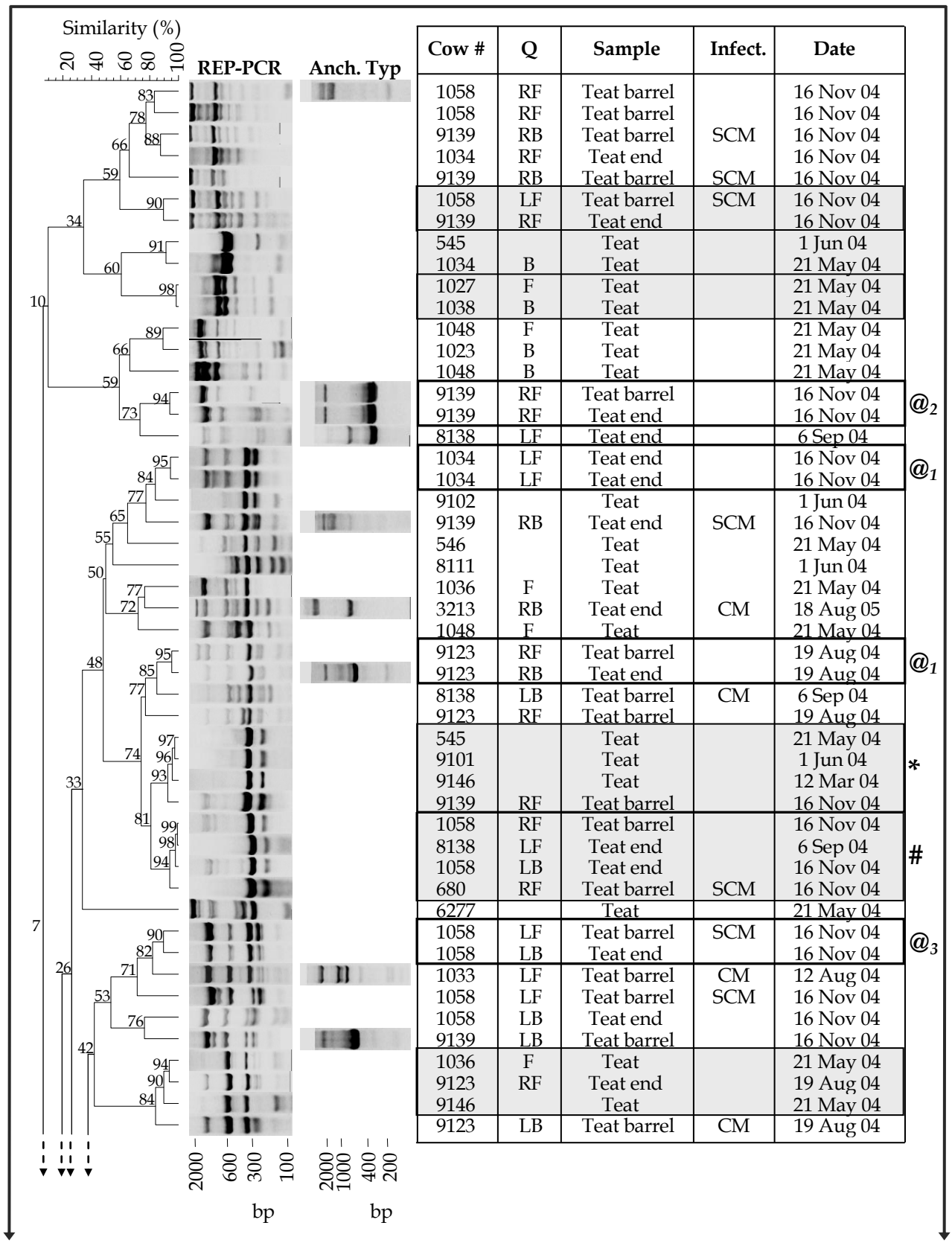
Cow #	Q	Infection	Sample	REP-PCR pattern	Anchored typing
1058	LF	SCM	**Milk 1**		
			Milk 2		
			Teat barrel 1		
			Teat barrel 2		
			Teat barrel 3		
8138	LB	CM	**Milk**		
			Teat barrel 1		
			Teat barrel 2		
9139	RB	SCM	**Milk**		
			Teat barrel 1		
			Teat barrel 2		

Table 3.11. Comparison of multiple teat skin strains with milk strains from infected quarters.

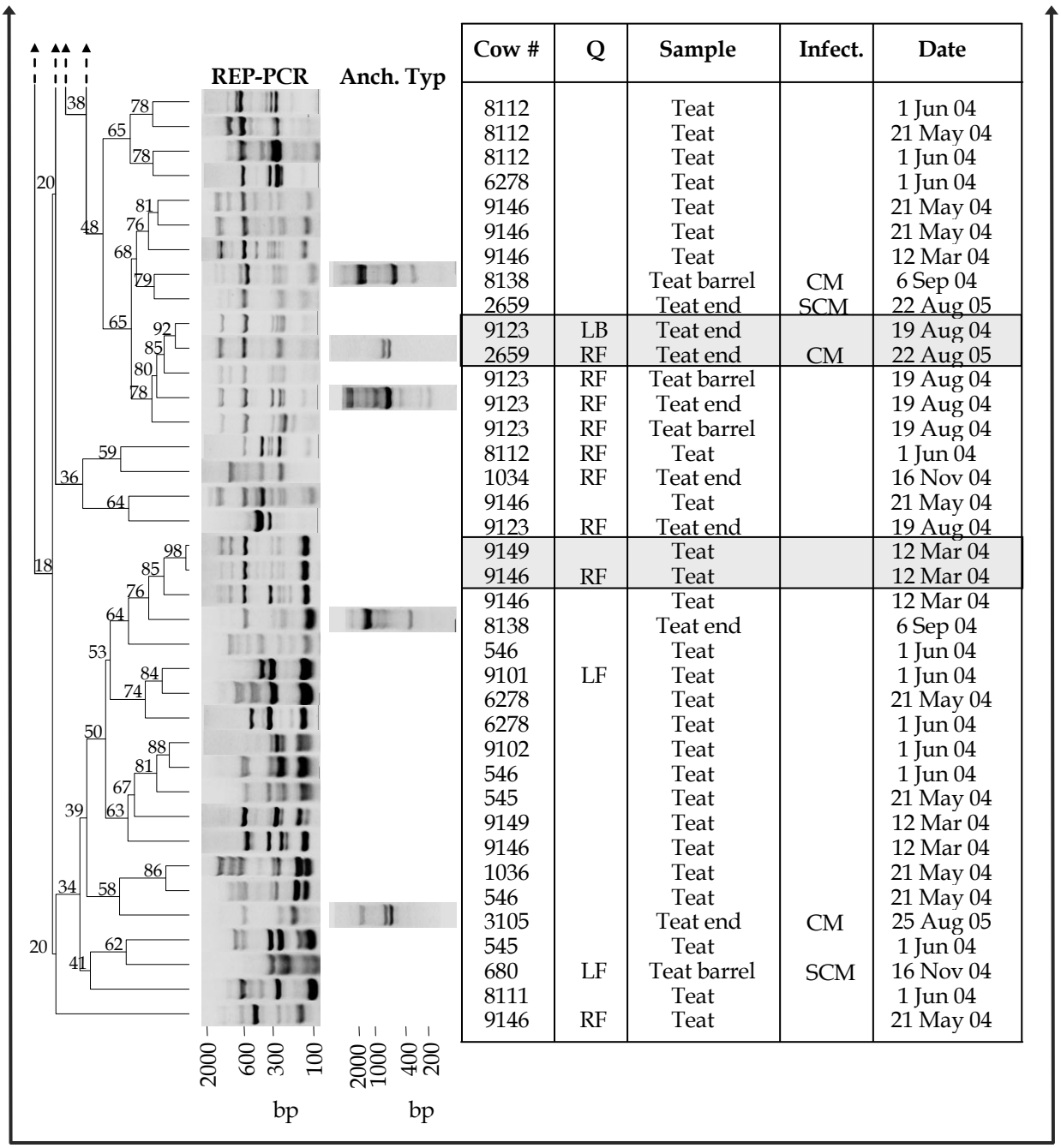
3.3.2.3 Correlation of teat strains between cows

Of the 87 different strain types obtained from teat skin swabs, eight were found on at least two different cows' teats as assessed by comparing REP-PCR and anchored typing band patterns (Figure 3.4, grey boxes). The most frequently isolated strain type (*) was found on the teat skin of four different cows (#545, #9101, #9146, and #9139) between 12 March 2004 and 16 November 2004. This group of band patterns appeared very similar to the next group in the dendrogram (#), with markedly different band patterns from all others; however, the presence of faint bands between 1000 – 2000 bp resulted in a similarity of only 81% between the two groups.



Cow #	Q	Sample	Infect.	Date	
1058	RF	Teat barrel		16 Nov 04	
1058	RF	Teat barrel		16 Nov 04	
9139	RB	Teat barrel	SCM	16 Nov 04	
1034	RF	Teat end		16 Nov 04	
9139	RB	Teat barrel	SCM	16 Nov 04	
1058	LF	Teat barrel	SCM	16 Nov 04	
9139	RF	Teat end		16 Nov 04	
545		Teat		1 Jun 04	
1034	B	Teat		21 May 04	
1027	F	Teat		21 May 04	
1038	B	Teat		21 May 04	
1048	F	Teat		21 May 04	
1023	B	Teat		21 May 04	
1048	B	Teat		21 May 04	
9139	RF	Teat barrel		16 Nov 04	
9139	RF	Teat end		16 Nov 04	@2
8138	LF	Teat end		6 Sep 04	
1034	LF	Teat end		16 Nov 04	
1034	LF	Teat end		16 Nov 04	@1
9102		Teat		1 Jun 04	
9139	RB	Teat end	SCM	16 Nov 04	
546		Teat		21 May 04	
8111		Teat		1 Jun 04	
1036	F	Teat		21 May 04	
3213	RB	Teat end	CM	18 Aug 05	
1048	F	Teat		21 May 04	
9123	RF	Teat barrel		19 Aug 04	
9123	RB	Teat end		19 Aug 04	@1
8138	LB	Teat barrel	CM	6 Sep 04	
9123	RF	Teat barrel		19 Aug 04	
545		Teat		21 May 04	
9101		Teat		1 Jun 04	*
9146		Teat		12 Mar 04	
9139	RF	Teat barrel		16 Nov 04	
1058	RF	Teat barrel		16 Nov 04	
8138	LF	Teat end		6 Sep 04	#
1058	LB	Teat end		16 Nov 04	
680	RF	Teat barrel	SCM	16 Nov 04	
6277		Teat		21 May 04	
1058	LF	Teat barrel	SCM	16 Nov 04	
1058	LB	Teat end		16 Nov 04	@3
1033	LF	Teat barrel	CM	12 Aug 04	
1058	LF	Teat barrel	SCM	16 Nov 04	
1058	LB	Teat end		16 Nov 04	
9139	LB	Teat barrel		16 Nov 04	
1036	F	Teat		21 May 04	
9123	RF	Teat end		19 Aug 04	
9146		Teat		21 May 04	
9123	LB	Teat barrel	CM	19 Aug 04	

Figure 3.4. Comparison of all teat skin strains.
(Continued on next page)



Cow #	Q	Sample	Infect.	Date
8112		Teat		1 Jun 04
8112		Teat		21 May 04
8112		Teat		1 Jun 04
6278		Teat		1 Jun 04
9146		Teat		21 May 04
9146		Teat		21 May 04
9146		Teat		12 Mar 04
8138		Teat barrel	CM	6 Sep 04
2659		Teat end	SCM	22 Aug 05
9123	LB	Teat end		19 Aug 04
2659	RF	Teat end	CM	22 Aug 05
9123	RF	Teat barrel		19 Aug 04
9123	RF	Teat end		19 Aug 04
9123	RF	Teat barrel		19 Aug 04
8112	RF	Teat		1 Jun 04
1034	RF	Teat end		16 Nov 04
9146		Teat		21 May 04
9123	RF	Teat end		19 Aug 04
9149		Teat		12 Mar 04
9146	RF	Teat		12 Mar 04
9146		Teat		12 Mar 04
8138		Teat end		6 Sep 04
546		Teat		1 Jun 04
9101	LF	Teat		1 Jun 04
6278		Teat		21 May 04
6278		Teat		1 Jun 04
9102		Teat		1 Jun 04
546		Teat		1 Jun 04
545		Teat		21 May 04
9149		Teat		12 Mar 04
9146		Teat		12 Mar 04
1036		Teat		21 May 04
546		Teat		21 May 04
3105		Teat end	CM	25 Aug 05
545		Teat		1 Jun 04
680	LF	Teat barrel	SCM	16 Nov 04
8111		Teat		1 Jun 04
9146	RF	Teat		21 May 04

Figure 3.4. Comparison of all teat skin strains. (Continued from previous page)**.** REP-PCR and anchored typing band patterns for isolates representing each teat strain type were compared using the average of experiments option in GelComparII. Unlabelled infection status (Infect.) indicates an uninfected quarter at time of sampling. Teat skin of individual quarters (Q) was swabbed or a single swab was used to sample the two front (F) or two back quarters (B), or all four quarters together (no label). Grey boxes and notations (@, *, #) are described in the text.

Some strains were found across two teat samples from the same cow ($@_{1\text{-}3}$). For cow #1034 and #9123 ($@_1$), the band patterns within each group were >90% similar despite being different upon manual comparison. Because of

the high similarity between three bands in the patterns, the similarity score was higher than would be expected given that one isolate contained an extra band in both cases. Therefore, these two strain types were not the same, but as they originated from the same teat end for cow #1034, and from two different teats of the same cow for #9123, they may be closely-related strains. The same strain was found on both the teat barrel and the teat end of the same quarter in cow #9139 ($@_2$), which was not unexpected and suggests that the entire teat may be colonised or contaminated by this strain. Cow #1058 had the same strain on two different teats ($@_3$), indicating either transfer of the strain between teats or that both teats came in contact with this strain from some other source at the same time.

3.3.2.4 General correlation of teat skin and infective strains

Previously in this section it was shown that the majority of cows with only one strain on the teat skin also had this same strain infecting that quarter. In contrast, those cows with multiple strains on the teat did not have the same strains in the milk. To further investigate any potential correlation between teat skin and infective strains, REP-PCR and anchored typing band patterns of 87 unique teat strains and 61 unique milk strains were compared. A 65% similarity cut-off level was arbitrarily chosen to simplify the dendrogram and the general clusters are shown (Figure 3.5). These general clusters consisted mainly of either teat or milk strains. For example, a large cluster (*) with at least 54% similarity between band patterns, contained 40 strains from milk and only 12 strains from teat skin. Similarly, at the bottom end of the dendrogram, five major clusters contained teat strains with only one milk strain present in one of the clusters. This indicated that the general band pattern obtained may be quite different for teat skin and milk isolates and strains from each location may not be closely-related.

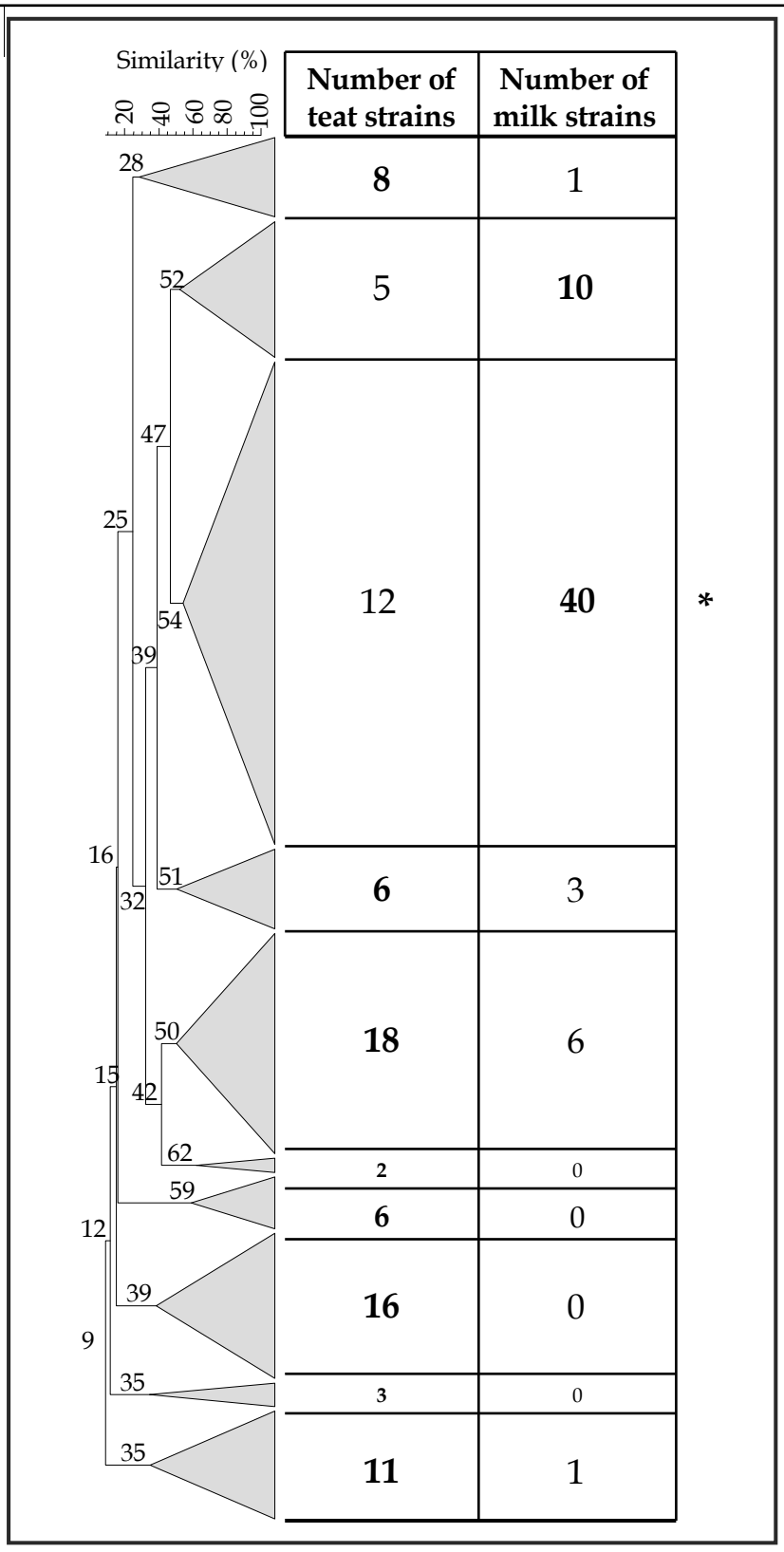
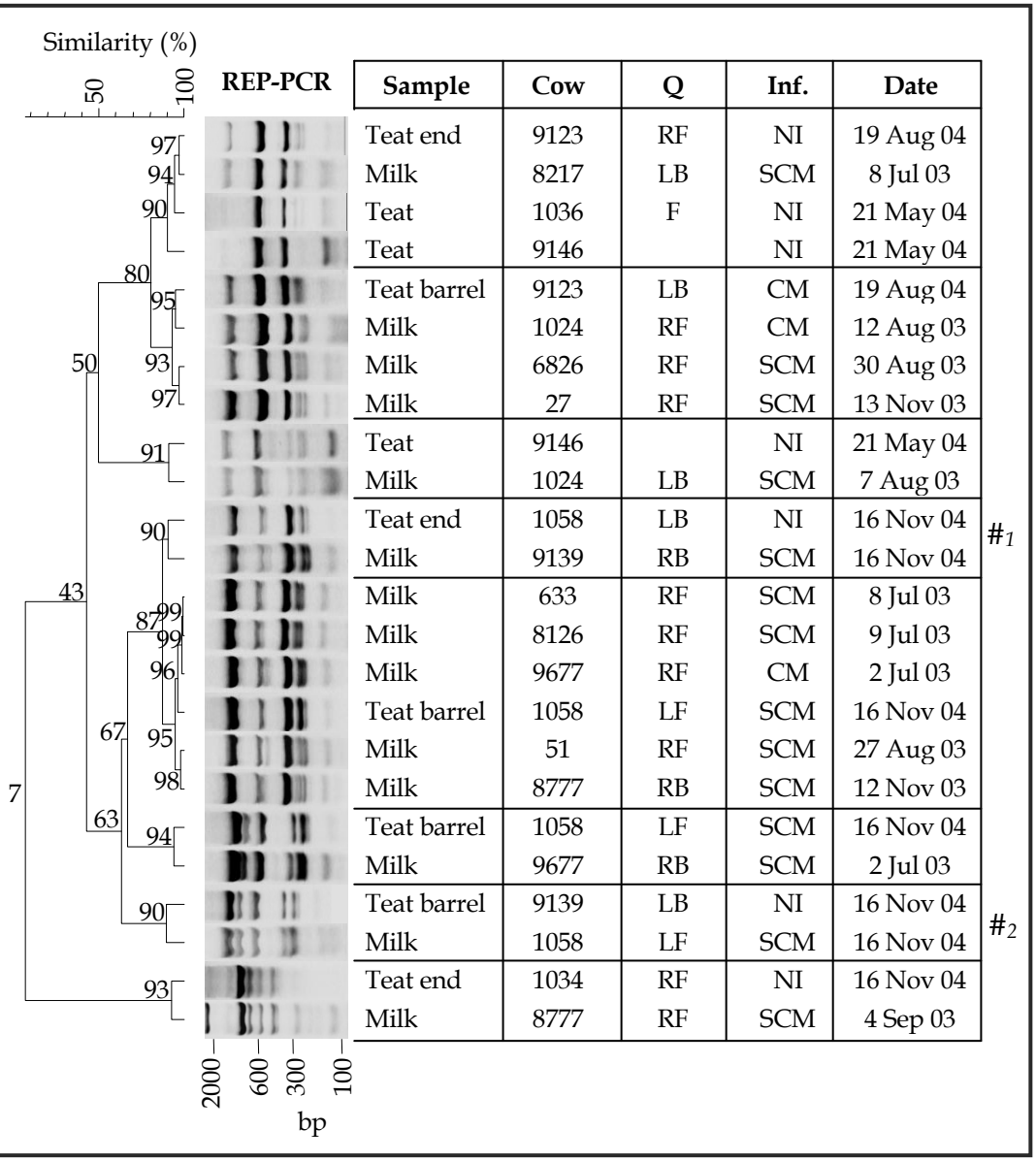


Figure 3.5. Major clusters of milk and teat strains. REP-PCR and anchored typing band patterns for isolates representing the milk and teat strain types were compared using Pearson correlation (optimization 2%) and a UPGMA dendrogram created. Major clusters with more than 65% similarity were collapsed for simplicity and the number of teat and milk strains in each of the clusters noted.

Eight clusters from this simplified dendrogram are shown in detail in Figure 3.6 as they contain both teat and milk strains with ≥90% similarity between band patterns. In this figure only the correlation between teat and milk strains from different cows is shown.

The majority of teat skin and milk strains with ≥90% similar band patterns appeared unrelated in regard to sample collection date. The first two clusters of the dendrogram contained milk strains identified approximately one year earlier than when the same strain was found on the teats. The only strain types that were found on the teat skin and the milk from another cow on the same date were those from 16 November 2004 (#$_{1, 2;}$ Figure 3.6). In the first cluster (#$_1$), cow #1058 had a strain on the teat end with a highly similar band pattern to the infective milk strain of cow #9139, which had subclinical mastitis. The other cluster (#$_2$) contained a strain from the teat barrel of cow #9139, which was similar to the strain detected in the milk of cow #1058, also with subclinical mastitis. Therefore, the two cows, #1058 and #9139, had subclinical mastitis in one quarter that was caused by different strains, but each of these respective strains could be found on the other cow's uninfected teats.



Sample	Cow	Q	Inf.	Date	
Teat end	9123	RF	NI	19 Aug 04	
Milk	8217	LB	SCM	8 Jul 03	
Teat	1036	F	NI	21 May 04	
Teat	9146		NI	21 May 04	
Teat barrel	9123	LB	CM	19 Aug 04	
Milk	1024	RF	CM	12 Aug 03	
Milk	6826	RF	SCM	30 Aug 03	
Milk	27	RF	SCM	13 Nov 03	
Teat	9146		NI	21 May 04	
Milk	1024	LB	SCM	7 Aug 03	
Teat end	1058	LB	NI	16 Nov 04	#1
Milk	9139	RB	SCM	16 Nov 04	
Milk	633	RF	SCM	8 Jul 03	
Milk	8126	RF	SCM	9 Jul 03	
Milk	9677	RF	CM	2 Jul 03	
Teat barrel	1058	LF	SCM	16 Nov 04	
Milk	51	RF	SCM	27 Aug 03	
Milk	8777	RB	SCM	12 Nov 03	
Teat barrel	1058	LF	SCM	16 Nov 04	
Milk	9677	RB	SCM	2 Jul 03	
Teat barrel	9139	LB	NI	16 Nov 04	#2
Milk	1058	LF	SCM	16 Nov 04	
Teat end	1034	RF	NI	16 Nov 04	
Milk	8777	RF	SCM	4 Sep 03	

Figure 3.6. Similarity between teat skin and milk strains from different cows. REP-PCR and anchored typing band patterns for representative teat skin and milk isolates were clustered using Pearson correlation (optimization 2%) and a UPGMA dendrogram generated. Only those isolates with ≥90% similarity between teat and milk band patterns from different cows are shown. No entry in the quarter (Q) column indicates where a single swab was used to sample all four teats. Notations (#*1, 2*) are explained in the text.

Overall, of the 87 teat strains and 61 milk strains that were compared, only eight strains were found on the teat skin and in the milk of different cows, suggesting that strains on the teat skin and those causing infections may be distinct. Also, only five of the nine cows with mastitis had the same strain both in the milk and on the teat skin of the same quarter. The infective

strain from the milk was usually found exclusively on the teat skin and, when multiple strains were present on the teat, the infective strain was not detected. Another three cows also had mastitis but the milk and teat samples were collected months apart and there was no evidence that the strains causing the infection were the same as those found on the teat skin (data not shown).

The low sampling level, particularly of infected cows around the same time period may be part of the reason why few correlations were observed between strains found on the teats and those causing mastitis in these cows. Therefore, firm conclusions cannot be drawn regarding this lack of correlation between teat and milk strains.

3.3.2.5 Isolation of *S. uberis* from the teat canal

Bacteria were isolated from the teat canal of 43 mammary quarters, with *S. uberis* isolated from nine quarters in numbers ranging from 20-9600 cfu/swab (Table 3.12). Four quarters had intramammary infections with *S. uberis*; two were clinical mastitis due to obvious clinical signs such as clotted milk, while the other two quarters had no clinical signs therefore were classed as subclinical. Pure cultures of *S. uberis* were obtained from the teat canal of one clinical and one subclinical quarter, indicating possible colonisation rather than contamination of the teat canal by this pathogen. The teat canal of the other clinical quarter contained *S. uberis* along with other bacterial species, and *S. uberis* could not be detected in the teat canal of the second subclinical quarter.

Six uninfected quarters had *S. uberis* in the teat canal, although other bacterial species were also detected. In cow #3215, *S. uberis* was not detected in the teat canal of the subclinically infected quarter, yet the teat canal of the diagonally opposite, uninfected quarter contained this pathogen. Another cow (#3105) had *S. uberis* in the teat canal of both the

clinically infected and an uninfected quarter. Although the number of cfu/swab was high in the teat canal of these particular uninfected quarters, other bacterial species were also found.

Thirty of the sampled quarters also had *S. uberis* on the teat end in numbers ranging from 10-5200 cfu/swab. When these samples were plated on EBA, it was apparent that other bacterial species were also present, even those with mammary glands infected with *S. uberis*.

			Numbers of *S. uberis* (cfu/swab)		**Bacterial species in teat canal**
Cow #	**Quarter**	**Mastitis**	**Teat end**	**Teat canal**	
1692	LB	NI	160	30	SU + others
2201	RB	NI	70	20	SU + others
2659	**RF**	**SCM**	**1080**	**380**	**SU only**
3105	**LF**	**CM**	**5200**	**9600**	**SU only**
	RB	NI	1400	6880	SU + others
3211	LB	NI	160	440	SU + others
3213	**RB**	**CM**	**1840**	**1200**	**SU + others**
3215	RF	NI	520	1720	SU + others
	LB	**SCM**	**180**	**0**	**Other species**
3217	LF	NI	0	40	SU + others

Table 3.12. Number of *S. uberis* (SU) cfu isolated on selective media from teat end and canal swab samples. NI – quarter not infected.

Between 16-20 isolates from the teat end and teat canal and three isolates from the milk of two clinically infected quarters, and one subclinically infected quarter were strain typed (Figure 3.7; Table 3.13). Only one infective strain was identified in the milk of cow #2659 and #3213, while two strains were identified in the milk of cow #3105.

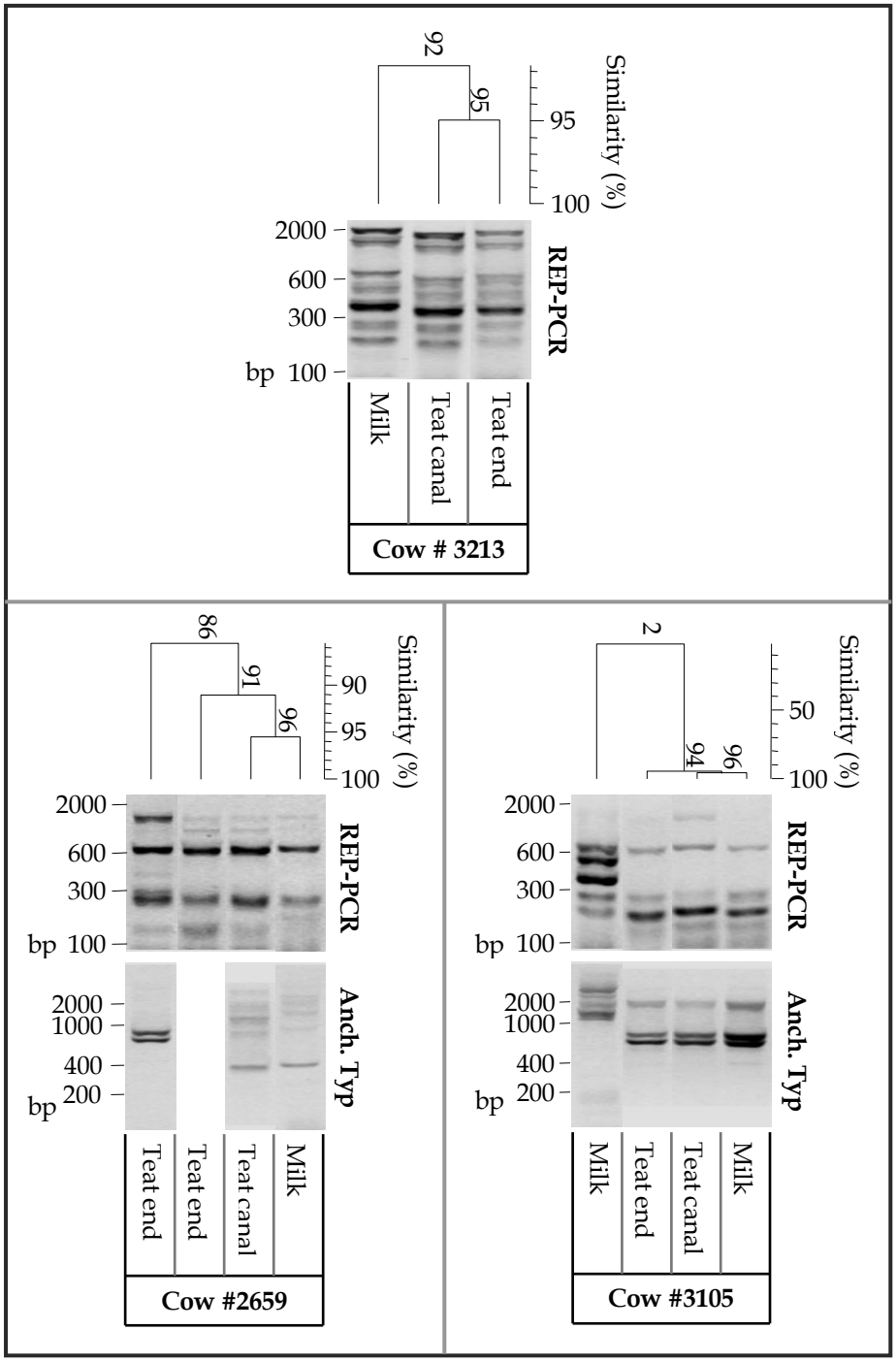


Figure 3.7. Band patterns of strains in milk, the teat canal and on the teat end of infected quarters.

			Strain types found		
Cow #	**Quarter**	**Mastitis**	**Milk**	**Teat canal**	**Teat end**
3213	RB	CM	A^{3}	A^{20}	A^{20}
2659	RF	SCM	B^{3}	B^{20}	B^{1} , C^{19}
3105	LF	CM	D^{2} , E^{1}	D^{16}	D^{18}

Table 3.13. Strains in milk, the teat canal and on the teat end of infected quarters. Each unique strain type was assigned a letter (A – E). The number of isolates of each strain type is shown in superscript.

For cow #3213, the infective milk strain was found exclusively in the teat canal and on the teat end. In contrast, the subclinically infected quarter of cow #2659 had the infective strain in the teat canal but two different strains were detected on the teat end, with only one of the 20 typed isolates the same strain as that in the milk and teat canal. In cow #3105, only one infective strain from the milk was also found in the teat canal and on the teat end of this quarter, with the other infective strain not detected at either location. Unfortunately, isolates of *S. uberis* from the teat canal and teat end of the uninfected quarter in this cow (#3105 RB) were not strain typed, therefore it is unknown if the strain causing clinical mastitis was the same as that in the teat canal and on the teat end of the uninfected quarter.

3.3.3 Isolation of *S. uberis* from faeces

Streptococcus uberis was isolated from the rectum of 5.6% (range 1.2% to 9.7%) of 100 cows sampled monthly between July 2003 and November 2004. However, only a selection of these positive samples were strain typed as follows: Isolates of *S. uberis* were strain typed from the faeces of 13 cows on either one or multiple occasions over a period of nine months between December 2003 and September 2004. In total 102 *S. uberis* isolates were typed by REP-PCR with 53 unique strains identified. Between one and six isolates were typed from faecal samples collected between 5 December 2003

and 17 September 2004 from eight cows and the identified strain types compared (Table 3.14).

Streptococcus uberis was isolated from the faeces of one cow (#9149) on all sampling occasions, from six of the seven samples for cow #144 and five out of seven samples from cow #561. These three cows appeared to be more persistent shedders of *S. uberis* than the other six cows whose faecal samples only contained *S. uberis* sporadically. Alternatively, the negative faecal samples may actually have contained *S. uberis*; however this species may not have been detected using the culture-based isolation method.

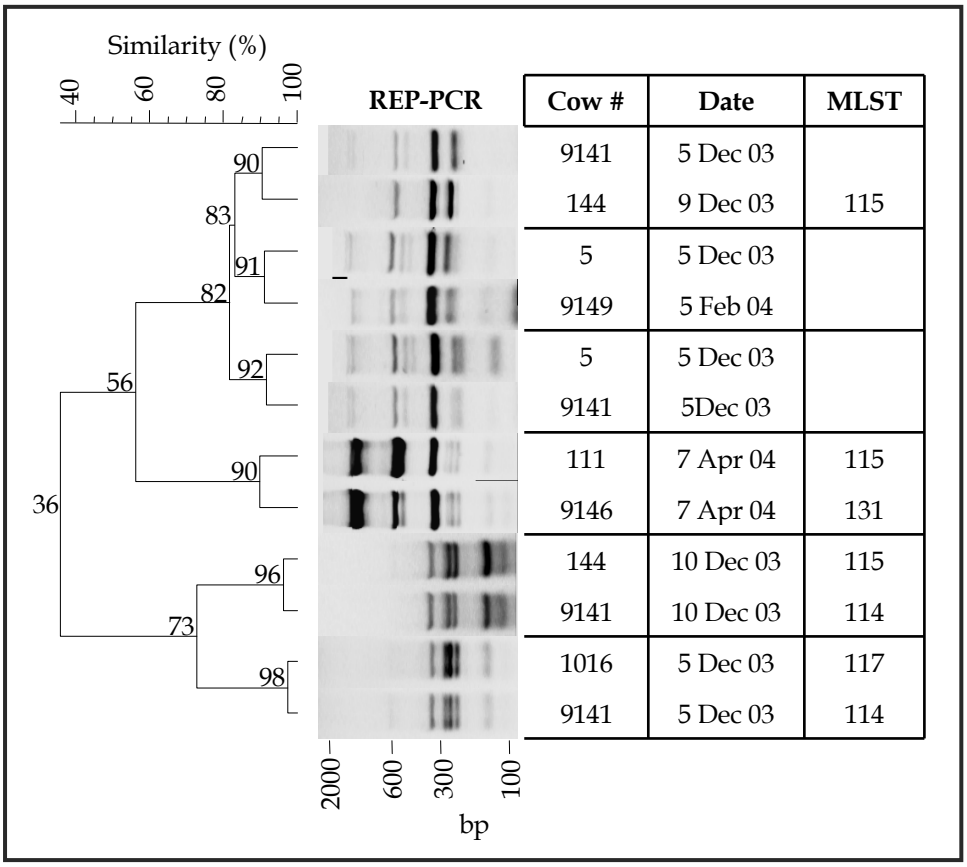
When the strain types of the *S. uberis* isolates in the faeces were determined it appeared that the persistent shedders (cow #9149, #144 and #561) were more likely to contain the same strain of *S. uberis* in the faeces over two to three sampling dates compared to the other cows that were studied, although this was not statistically significant However, the same strain could also be found on two occasions in cow #9141 which was not classed as a persistent shedder. These results suggest that the gastrointestinal (GI) tract of the cow may become colonised by particular strains of *S. uberis*. However, where the same strain was found on two different sampling dates, months apart, a sample from an intervening month sometimes yielded no *S. uberis*. In these particular cases it was possible that either the initially identified strain disappeared and then later re-colonised the GI tract of this same animal, or alternatively, the strain was maintained within the GI tract throughout these intervening months but was not shed in the faeces at high enough levels to be detected by the culture-based isolation system used.

	Cow #							
Date	**#1033**	**#1034**	**#144**	**#5**	**#561**	**#9141**	**#9146**	**#9149**
5 Dec 03	**A^{1}**	*0*	**A^{4}, B^{1}, C^{1}**	**A^{3}, B^{1}, C^{2}**	**A^{4}, B^{1}, C^{1}**	**A^{5}, B^{1}**	*0*	**A^{1}, B^{1}**
9 Dec 03	*NS*	*NS*	**C^{1}**	*0*	**D^{1}, E^{1}**	*0*	*0*	**C^{1}**
10 Dec 03	*NS*	*NS*	**D^{1}**	*0*	**D^{1}, F^{1}**	**B^{1}**	*0*	**D^{1}, E^{1}**
29 Jan 04	*0*	*0*	*NS*	*NS*	*NS*	*0*	*0*	**+**
5 Feb 04	*0*	*NS*	**E^{5}**	*0*	**+**	*0*	*NS*	**F^{3}, G^{2}**
18 Feb 04	*NS*	*NS*	*0*	*0*	*0*	*0*	*NS*	**F^{5}**
4 Mar 04	*0*	*0*	**+**	*0*	**D^{4}, G^{1}**	**C^{5}**	**A^{5}**	**H^{5}**
7 Apr 04	*0*	*0*	**+**	*0*	*0*	*0*	**B^{1}**	**H^{1}**
21 May 04	*0*	*0*	*NS*	*NS*	*NS*	**D^{5}**	*0*	*NS*
12 Aug 04	**B^{3}, C^{1}, D^{1}**	*0*	*NS*	*NS*	*NS*	*0*	*0*	*NS*
17 Sep 04	**E^{1}, F^{1}, G^{1}**	**A^{1}, B^{1}**	*NS*	*NS*	*NS*	*0*	*0*	*NS*

Table 3.14. Strain types found over time in faeces of eight cows. Strain types are indicated by A – G within each cow and the number of isolates of each strain type is in superscript. On some dates faeces were not sampled (*NS*) or they were sampled but negative for *S. uberis* (*0*) or positive for *S. uberis* but isolates not typed (+). Use of the same letters between cows is not an indication that the same strains were found between cows.

3.3.3.1 Correlation of faecal strains between cows

In addition to persistent shedding of the same strain of *S. uberis* over time, the same strain types also appeared in the faeces of different cows (Figure 3.8).



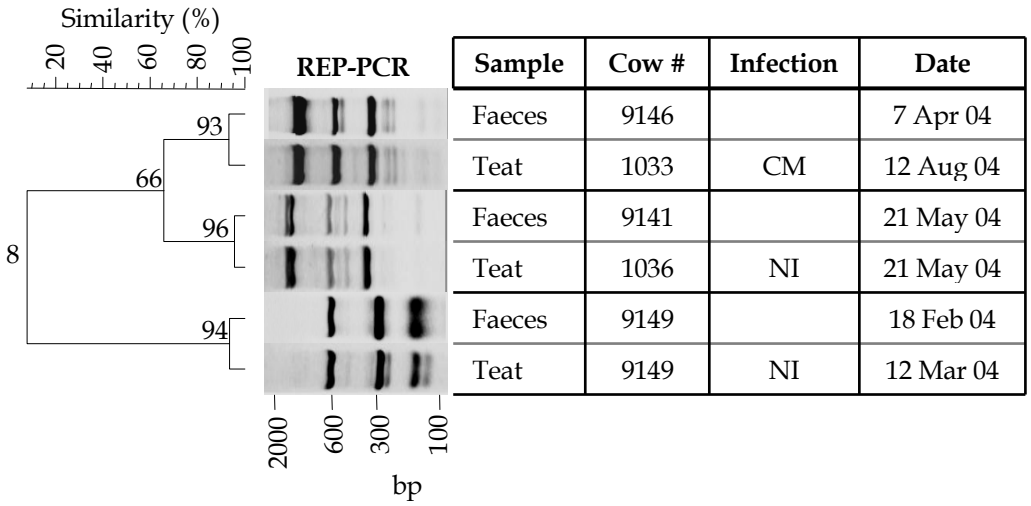
Cow #	Date	MLST
9141	5 Dec 03	
144	9 Dec 03	115
5	5 Dec 03	
9149	5 Feb 04	
5	5 Dec 03	
9141	5Dec 03	
111	7 Apr 04	115
9146	7 Apr 04	131
144	10 Dec 03	115
9141	10 Dec 03	114
1016	5 Dec 03	117
9141	5 Dec 03	114

Figure 3.8. Comparison of faecal strains between cows. REP-PCR band patterns of representative strain types from each faecal sample were compared with Pearson correlation (optimization 1%).

Six different strains were found in the faeces of two cows on the same sampling day as assessed by REP-PCR. Although the MLST strain types did not correspond to the REP-PCR results, MLST also demonstrated that some strains were present in the faeces of more than one cow. MLST 115 was found in cow #144 and #111, which were sampled four months apart; however, the corresponding REP-PCR band patterns were visibly different.

3.3.3.2 Comparison of faecal and teat strains

When representative REP-PCR band patterns of 53 faecal strains and 74 teat skin strains were compared with Pearson correlation (optimization 2%) only three faecal strains had band patterns >90% similar to teat strains (Figure 3.9). One strain was identified in the faeces of cow #9149 on the 18 February 2004 and then again on an uninfected teat of this same cow almost one month later, suggesting that the teat of this cow may have been contaminated with faecal material. Other correlations were observed between faecal and teat skin strains from different cows, suggesting that a few faecal strains may have been spread throughout the environment, resulting in contamination of cows' teats.



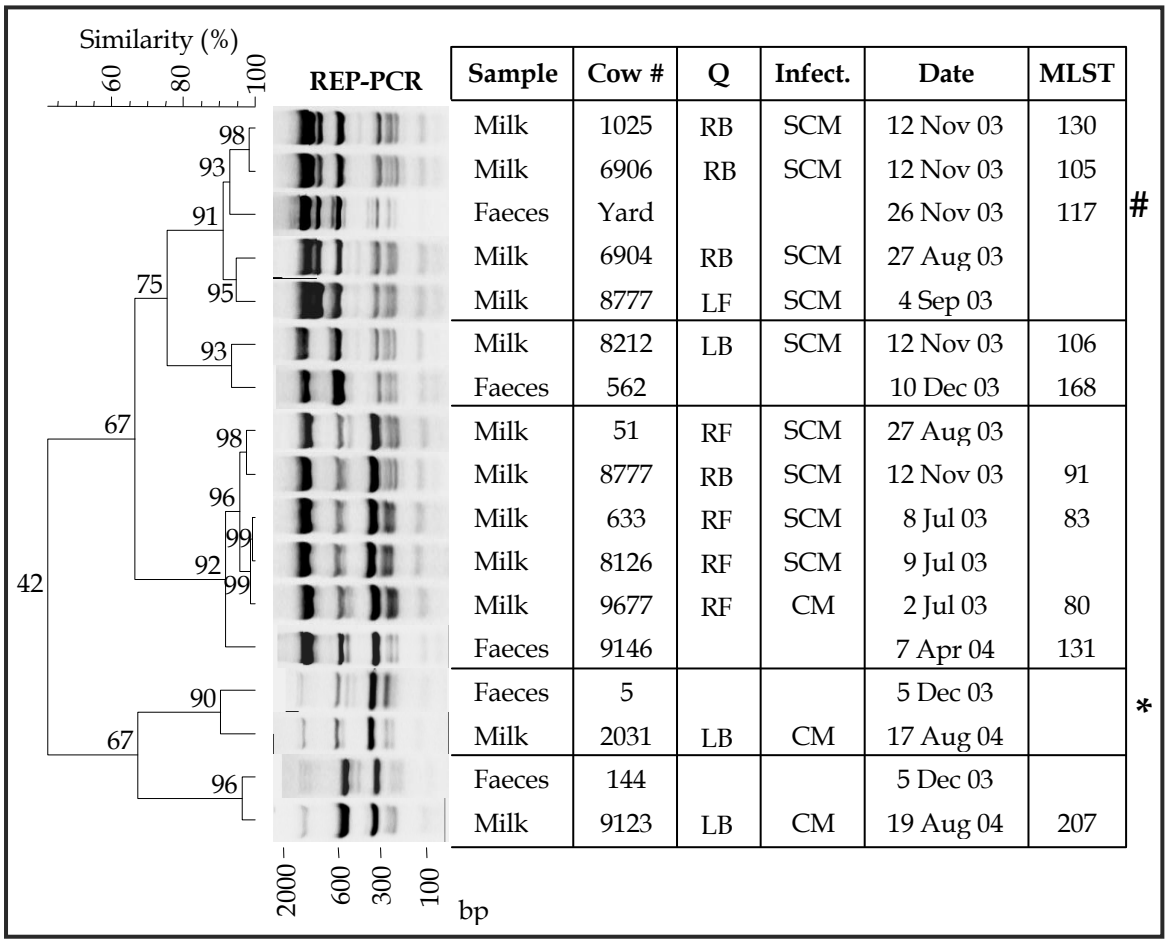
Sample	Cow #	Infection	Date
Faeces	9146		7 Apr 04
Teat	1033	CM	12 Aug 04
Faeces	9141		21 May 04
Teat	1036	NI	21 May 04
Faeces	9149		18 Feb 04
Teat	9149	NI	12 Mar 04

Figure 3.9. Correlation of strains between faeces and teat skin. REP-PCR band patterns for faecal and teat skin strains were compared using Pearson correlation (optimization 2%) and a UPGMA dendrogram generated. NI – not infected.

3.3.3.3 Comparison of faecal and infective milk strains

The strains found in faeces were also compared with strains isolated from clinical or subclinical cases of mastitis within the same herd. If faeces were a major source of *S. uberis* on the farm, then it would be expected that some faecal strains would also be found causing mastitis. However, based on the REP-PCR band patterns (Figure 3.10), only five strains were found in both

faeces and milk from infected quarters and almost all of these isolations occurred at different time points, indicating that the infection may not be directly related to the strain being shed in the faeces. For example, a strain isolated from the faeces of cow #5 on 5 December 2003 (*) was then identified again in the milk from the infected quarter of cow #2031 over eight months later on 17 August 2004.



Sample	Cow #	Q	Infect.	Date	MLST	
Milk	1025	RB	SCM	12 Nov 03	130	
Milk	6906	RB	SCM	12 Nov 03	105	
Faeces	Yard			26 Nov 03	117	#
Milk	6904	RB	SCM	27 Aug 03		
Milk	8777	LF	SCM	4 Sep 03		
Milk	8212	LB	SCM	12 Nov 03	106	
Faeces	562			10 Dec 03	168	
Milk	51	RF	SCM	27 Aug 03		
Milk	8777	RB	SCM	12 Nov 03	91	
Milk	633	RF	SCM	8 Jul 03	83	
Milk	8126	RF	SCM	9 Jul 03		
Milk	9677	RF	CM	2 Jul 03	80	
Faeces	9146			7 Apr 04	131	
Faeces	5			5 Dec 03		*
Milk	2031	LB	CM	17 Aug 04		
Faeces	144			5 Dec 03		
Milk	9123	LB	CM	19 Aug 04	207	

Figure 3.10. Correlation of faecal and infective strains isolated from milk. REP-PCR band patterns for faecal and milk strains were compared using Pearson correlation (optimization 2%) and a UPGMA dendrogram generated. Notations (*, #) are explained in the text.

A faecal sample collected from the cow yard yielded an isolate with a REP-PCR band pattern highly similar to one isolated from subclinical infections in four different cows within a similar time period (#), suggesting that this strain may be cycling between the mammary gland, faeces and the yard. However, it was not possible to determine whether faeces were the primary

source as it was also possible that the yard became contaminated by milk dripping from infected quarters.

Overall, the lack of correlation between strains found in faeces, on the teat skin and in milk from infected quarters suggests that it is unlikely that the faeces may be harbouring and spreading particularly virulent strains of *S. uberis* on this dairy farm. However, samples were not collected at the same time, making it difficult to draw firm conclusions. It has also been noted that REP-PCR was not capable of fully differentiating strains, so the correlations described so far may not reflect a true picture. Different results may have been obtained if the faeces, teat skin and intramammary isolates had been collected from the same cows repeatedly through time. The correlation of infection-causing strains and strains of the teat skin and faeces are more thoroughly investigated for individual cows in section 3.3.4.

3.3.4 Identification of *S. uberis* strains on the cow's body

To investigate if the infective strain could be isolated from other parts of the cow body, nine cows diagnosed with clinical or subclinical mastitis due to *S. uberis* were subjected to an extensive sampling procedure of various regions of the body. Clinical mastitis was indicated by visible signs of infection (e.g. clots, discoloured milk) and *S. uberis* in foremilk samples, while subclinical mastitis was diagnosed when foremilk somatic cell count was elevated and *S. uberis* was detected in the mid-lactation foremilk sample with the absence of any visible signs of infection. Milk, teat and body samples were collected before the onset of antibiotic treatment for clinical mastitis cases.

Two cows were sampled during two separate infections of the same quarter, giving a total of seven cases of clinical mastitis and four cases of subclinical mastitis. The milk from all four quarters of each cow was sampled together with swabs of the teat end and teat barrel of infected and

uninfected quarters. Other sites included: udder skin, the hock of the hind leg closest to the infected quarter, coronet band of both hind feet, lips, nostrils, tail swish, and rectum. Where possible five single *S. uberis* colonies from each sample were strain typed by REP-PCR and anchored typing and the band patterns compared between samples and with those isolated from milk of the infected quarter. Where less than five isolates were available, all were typed.

3.3.4.1 Isolation of *S. uberis*

Infections were located in only one quarter of each cow, with no *S. uberis* detected in the milk from the other quarters of each cow (grouped together as uninfected quarters; Table 3.15). Isolates of *S. uberis* were more likely to be obtained from the teat barrel than the teat end of the infected quarter with six of the 11 mastitis cases (55%) having *S. uberis* on the barrel of the infected teat, compared with four of the 11 cases (36%) having this species on the teat end. *S. uberis* could also be found on the teat end and teat barrel of the uninfected quarters, but this only occurred in less than half of the cows studied.

Of the 44 teat skin samples collected from any of the teats from the 11 mastitis cases, 18 (41%) were positive for *S. uberis*, suggesting that the teat skin does not always harbour *S. uberis*. It must be noted however, that the swabbing and recovery procedure was limited in its ability to detect *S. uberis*, with a minimum detection level of 10 cfu/ml of swab rinsing. Therefore, *S. uberis* may have been present but not detected on these quarters.

				S. uberis isolates					
				Infected quarter			Uninfected quarters		
				Milk	Teat		Milk	Teat	
Cow #	Infection	Quarter	Date		End	Barrel		End	Barrel
1033	CM	LF	12-Aug	+	+	+	-	-	-
2031	CM	LB	17-Aug	+	-	-	-	-	-
9123	CM	LB	19-Aug	+	-	+	-	+	+
8138	CM	LB	6-Sep	+	+	+	-	+	-
1033	CM	LF	17-Sep	+	-	-	-	-	-
1034	CM	RB	17-Sep	+	-	-	-	-	-
8212	CM	LF	24-Oct	+	-	-	-	-	-
680	SCM	RF	16-Nov	+	-	+	-	-	-
1034	SCM	RB	16-Nov	+	-	-	-	+	-
1058	SCM	LF	16-Nov	+	+	+	-	+	+
9139	SCM	RB	16-Nov	+	+	+	-	+	+
	Percentage of positive cases:			**100%**	36%	**55%**	0%	45%	27%

Table 3.15. Isolation of *S. uberis* from milk and the teat skin of infected and uninfected quarters. LF – left front, LB – left back, RF – right front, RB – right back.

Only three of the seven clinical cases had *S. uberis* on the teat skin of infected or uninfected quarters, while all four subclinical cases had *S. uberis* on the teat skin of at least one quarter, suggesting that *S. uberis* could be more prevalent on the teats of subclinical rather than clinical quarters. As the subclinically infected quarters may have remained undetected for multiple days, it was perhaps more likely that the teat skin became more contaminated than with clinical mastitis which was diagnosed and treated promptly.

For swabs collected across other regions of the body, the number of samples positive for *S. uberis* was low with only 29 of the 77 samples (38%) yielding *S. uberis*. The presence of *S. uberis* in the body samples was more similar between clinical or subclinical mastitis cases (37% and 39% respectively) compared to the teat skin swab samples.

Streptococcus uberis was most commonly isolated from the hind feet (91%) followed by lips (64%), and tail (45%), (Table 3.16). The udder skin was the only site where *S. uberis* was not found in any of the 11 cases of mastitis.

Although only three of the seven clinical mastitis cases had *S. uberis* present on the lips, all four subclinical mastitis cases had *S. uberis* located at this site.

				S. uberis isolates						
Cow #	**Infection**	**Quarter**	**Date**	**Udder**	**Hock**	**Feet***	**Lips**	**Nostrils**	**Tail**	**Rectum**
1033	CM	LF	12-Aug	-	+	+	-	-	+	+
2031	CM	LB	17-Aug	-	-	+	-	+	-	-
9123	CM	LB	19-Aug	-	-	+	+	+	+	-
8138	CM	LB	6-Sep	-	-	+	-	-	-	-
1033	CM	LF	17-Sep	-	-	+	-	-	-	+
1034	CM	RB	17-Sep	-	-	-	+	-	-	+
8212	CM	LF	24-Oct	-	-	+	+	-	+	-
680	SCM	RF	16-Nov	-	-	+	+	-	-	-
1034	SCM	RB	16-Nov	-	-	+	+	-	-	-
1058	SCM	LF	16-Nov	-	+	+	+	-	+	-
9139	SCM	RB	16-Nov	-	-	+	+	-	+	-
	Percentage of positive cases:			0%	18%	**91%**	**64%**	18%	**45%**	27%

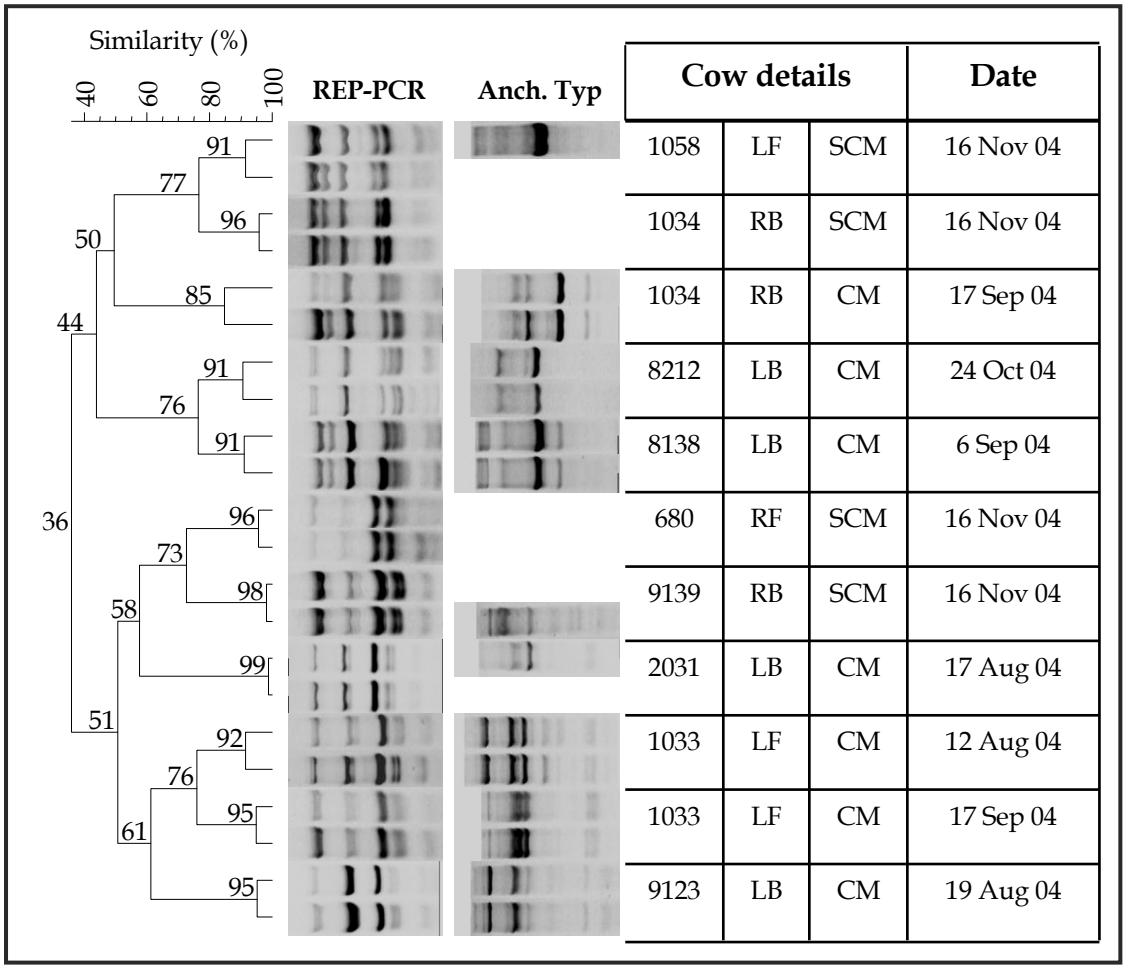
Table 3.16. Isolation of *S. uberis* from the body of cows with mastitis.
* Both hind feet were sampled separately and the results pooled.

Only two of the nine cows, #1033 and #1034 had *S. uberis* isolated from the rectum, and both of these cows had more than one infection within a short period of time. However, cow #1033 had *S. uberis* present in the rectum on both sampling dates, but cow #1034 showed evidence of *S. uberis* only on the first sampling date (September), indicating that shedding of *S. uberis* in the faeces may have been sporadic for this cow.

For cow #1033, isolation of *S. uberis* from regions of the body differed between the two clinical events, with more prevalent isolations with the first case of clinical mastitis. However, isolates of *S. uberis* from the feet and rectum were common to both events. For cow #1034, *S. uberis* was isolated on both occasions from the lips, with the presence of *S. uberis* at other sampled body sites varying between the two infections events.

3.3.4.2 Strain typing of isolates from the cows' body

Between one and five *S. uberis* colonies from each sample were strain typed by REP-PCR and the results confirmed by anchored typing. Usually only one strain was found in milk from the infected quarter, although two strains were identified in milk from a cow with subclinical mastitis (#1058). The strains were unique to each mastitis case, except for cow #1034, which had clinical mastitis and subclinical mastitis two months later, caused by the same strain (Figure 3.11). Some problems occurred with incorrect clustering of milk isolates based on their band patterns. The isolates from cow #1034 on the two infection dates were not tightly clustered on the dendrogram; however, visual inspection suggested the REP-PCR band patterns were highly similar and likely to be from the same or very closely-related strain types. Also, the two isolates from cow #1058 had a similarity of 91% on this dendrogram, despite having an obviously different band pattern. This high similarity, despite the extra band present, is most likely due to the other bands of the pattern being in almost identical positions with similar intensity. The two milk isolates from cow #1034 on 17 Sep 04 also showed a similarity of only 85% between band patterns, yet the REP-PCR and anchored typing band patterns appeared highly similar on visual inspection. These results indicate that in the majority of cases, comparing band patterns using Pearson correlation with optimization of 2% resulted in correct clustering; however, for these few cows the similarity values were erroneous compared to visual comparison of the band patterns.



Cow details			Date
1058	LF	SCM	16 Nov 04
1034	RB	SCM	16 Nov 04
1034	RB	CM	17 Sep 04
8212	LB	CM	24 Oct 04
8138	LB	CM	6 Sep 04
680	RF	SCM	16 Nov 04
9139	RB	SCM	16 Nov 04
2031	LB	CM	17 Aug 04
1033	LF	CM	12 Aug 04
1033	LF	CM	17 Sep 04
9123	LB	CM	19 Aug 04

Figure 3.11. Representative milk isolates from each cow.
REP-PCR and anchored typing band patterns for two milk isolates from each of the nine cows were compared using Pearson correlation (optimization 2%) and a UPGMA dendrogram generated.

3.3.4.3 Location of infective strains on the teats

The strain detected in the milk sample was termed the infective strain, and the presence of this strain type was investigated in the samples obtained from the teats of that same cow. Generally, the infective strain was most likely to be found on the teat end of the infected quarter (Table 3.17) with three of the four samples that were positive for *S. uberis* containing this infective strain. Overall, however, only three of the 11 teat end samples yielded the infective strain.

	Infection details		Milk strain	*S. uberis* strains isolated			
				Infected teat		Uninfected teats	
	Cow #	Date		End	Barrel	End	Barrel
Clinical mastitis	1033	12 Aug	**A^{5}**	**A^{5}**	**A^{5}**	-	-
	2031	17 Aug	**A^{5}**	-	-	-	-
	9123	19 Aug	**A^{5}**	-	**A^{5}**	**A^{2}** B^{4}, **C^{1}**, G^{1}, H^{1}	**A^{2}** D^{1}, F^{1}, I^{1}
	8138	6 Sep	**A^{5}**	**A^{3}**	C^{2}, G^{1}	**B^{1}**, D^{1}, E^{1}	-
	1033	17 Sep	**A^{3}**	-	-	-	-
	1034	17 Sep	**A^{3}**	-	-	-	-
	8212	24 Oct	**A^{5}**	-	-	-	-
	Percentage with milk strain:			29%	29%	14%	14%
Subclinical mastitis	680	16 Nov	**A^{3}**	-	B^{2}, C^{1}	-	-
	1034	16 Nov	**A^{3}**	-	-	**B^{1}**, C^{1}, D^{1}, E^{1}	-
	1058	16 Nov	**A^{2}, B^{1}**	M^{1}	**C^{1}**, **G^{1}**, I^{1}	**B^{1}** **F^{1}**, H^{1}	**F^{1}**, N^{1}, O^{1}
	9139	16 Nov	**A^{3}**	**A^{3}**	F^{2}, I^{1}	**B^{1}**, C^{2}	**B^{1}**, **D^{2}**, E^{3}
	Percentage with milk strain:			25%	0%	25%	0%

Table 3.17. Presence of the infective strain on the teat skin of infected and uninfected quarters. Each identified strain type was assigned a letter and the total number of typed isolates of that strain is shown in superscript. Those strain types shown in red were non-infective strains found at more than one site on the same cow (also see body samples Table 3.18). The use of the same letters between cows does not indicate correlation of strain types between cows.

While the teat end of the infected quarter tended to yield only the infective strain, the teat barrel and teat ends of the uninfected quarters frequently yielded other strain types, particularly when the cow had subclinical mastitis. There appeared to be a difference in the presence of the infective strain on teat skin of cows with clinical or subclinical mastitis. Of the eight samples collected from clinical mastitis cases that were positive for *S. uberis*,

the infective strain was found in six of these samples (75%). However, for subclinical quarters, only two of the 10 teat skin samples (20%) that yielded *S. uberis* also had the infective strain present. Therefore, although not statistically significant, the infective strain was more likely to be found on the teat skin with clinical rather than subclinical mastitis.

3.3.4.4 Location of the infective strain on other areas of the body

The infective strain was found on other areas of the cow body in five of the 11 mastitis cases (Table 3.18). The infective strain was most prevalent on cow #9123, where it was found in three teat skin samples as well as the feet, lips, nostrils and tail. However, of the 29 body samples that yielded *S. uberis*, only nine actually contained the infective strain. There was also a difference in the presence of the infective strain on the body between cows with clinical and subclinical mastitis. With clinical mastitis, eight of the 18 samples positive for *S. uberis* also contained the infective strain (44%), while only one of the 11 *S. uberis*-positive samples (9%) from subclinical cases contained the infective strain. Therefore, the infective strain was more likely to be found on the body with clinical rather than subclinical mastitis. Indeed, for subclinical mastitis cases the infective strain was only found on the lips of one cow in addition to teat skin, compared to eight other body sites for the clinical mastitis cases.

	Infection details		*S. uberis* strains isolated						
	Cow	**Date**	**Milk strain**	Hock	Feet	Lips	Nostrils	Tail	Rectum
Clinical mastitis	1033	12 Aug	**A^{5}**	B^{4}, C^{1}	**A^{1}**	-	-	D^{2}, E^{2}, F^{1}	G^{2}, H^{1}, I^{1}, J^{1}
	2031	17 Aug	**A^{5}**	-	**A^{1}**, D^{1}	-	**A^{1}**, B^{1}, C^{1}	-	-
	9123	19 Aug	**A^{5}**	-	**A^{3}**, C^{1}	**A^{4}**, E^{1}	**A^{5}**	**A^{2}**	-
	8138	6 Sep	**A^{5}**	-	**A^{2}**, B^{2}, F^{1}	-	-	-	-
	1033	17 Sep	**A^{3}**	-	B^{1}, C^{1}	-	-	-	D^{1}, E^{1}, F^{1}
	1034	17 Sep	**A^{3}**	-	-	B^{3}	-	-	C^{1}, D^{1}
	8212	24 Oct	**A^{5}**	-	C^{2}, D^{2}	B^{5}	-	E^{1}, F^{1}, G^{1}, H^{1}, I^{1}	-
	Percentage with milk strain:			0%	57%	14%	29%	14%	0%
Subclinical mastitis	680	16 Nov	**A^{3}**	-	D^{2}, E^{3}, F^{1}	**A^{1}**	-	-	-
	1034	16 Nov	**A^{3}**	-	F^{1}, G^{1}, H^{1}, I^{1}, J^{1}, K^{1}	B^{1}	-	-	-
	1058	16 Nov	**A^{2}, B^{1}**	F^{2}	E^{2}, J^{1}	C^{1}, D^{2}	-	G^{1}, K^{1}, L^{1}	-
	9139	16 Nov	**A^{3}**	-	D^{2}, G^{1}	H^{3}	-	G^{2}, J^{1}	-
	Percentage with milk strain:			0%	0%	25%	0%	0%	0%

Table 3.18. Location of the infective strain on other areas of the cow body. Each identified strain was assigned a letter and the total number of typed isolates of that strain is shown in superscript. Those strain types shown in red are non-infective strains found at more than one site on the same cow (also see teat skin samples Table 3.17).

Although isolates of *S. uberis* were most prevalent on the hind feet, the infective strain was only found on the feet of four cows, all with clinical mastitis. *Streptococcus uberis* was not commonly isolated from the nostrils, however for the two cows where *S. uberis* was found, the infective strain was present in both samples. The only sampled areas of the body where the infective strain was not detected in any of the cows were the leg hock and the rectum. Only cow #1033 (17 Sep), #1034 (17 Sep and 16 Nov), and

#8212 did not have the infective strain present on either the teat skin or body sites sampled.

In addition to the infective strain, other strains were found at multiple sites on the cow body. For cow #9123, 8138, 1034 (16 Nov), 1058 and 9139, the same strain of *S. uberis* was found on two or three different areas of the body. Usually, a particular strain was observed on the teat skin and one other region of the body such as the feet, lips, tail or hock.

Overall, 110 samples were collected from the teat skin and body of cows with mastitis, yet *S. uberis* was only found in 47 samples, indicating that this species was not highly prevalent across the teats and bodies of infected cows. The strain causing the infection was only present in 17 of these samples, indicating that it was not widespread across the body. Differences in prevalence of *S. uberis* and the infective strain were also observed between clinical and subclinical mastitis cases (Table 3.19).

		Clinical mastitis			**Subclinical mastitis**			**All cases**
		Teat skin	Body	**Total**	Teat skin	Body	**Total**	
No. of samples	Total samples	28	42	70	16	24	40	110
	SU-positive	8	18	26	10	11	21	47
	Milk strain	6	8	14	2	1	3	17
Percentage of samples	**SU-positive**	44%	43%	37%	63%	46%	53%	**43%**
	Milk strain	21%	19%	20%	12.5%	4%	7.5%	**15%**
	SU-positive and milk strain	75%	44%	54%	20%	9%	14%	**36%**

Table 3.19. Number and percentage of samples positive for *S. uberis* and/or the infective strain. The percentage of samples with *S. uberis* (SU-positive), with the infective strain from milk (milk strain) and with both *S. uberis* and the infective strain (SU-positive and milk strain) was calculated for the teat skin and body of clinical and subclinical mastitis cases.

A total of 70 samples were collected from the teat skin and body of clinical cases and only 26 (37%) of these had *S. uberis* present. Of these positive samples, 14 (54%) also contained the infective strain from that cow. Forty

samples were collected from subclinical mastitis cases and 21 (53%) were positive for *S. uberis*, but the infective strain was only detected in three of the positive samples (14%). Therefore, although *S. uberis* was more prevalent on cows with subclinical mastitis, the infective strain was more likely to be found on the teat skin and body with clinical mastitis. The question that remains unanswered is whether the infective strain was present on cows before clinical mastitis developed or through post-infection contamination of the teat skin and body with the infective strain from milk.

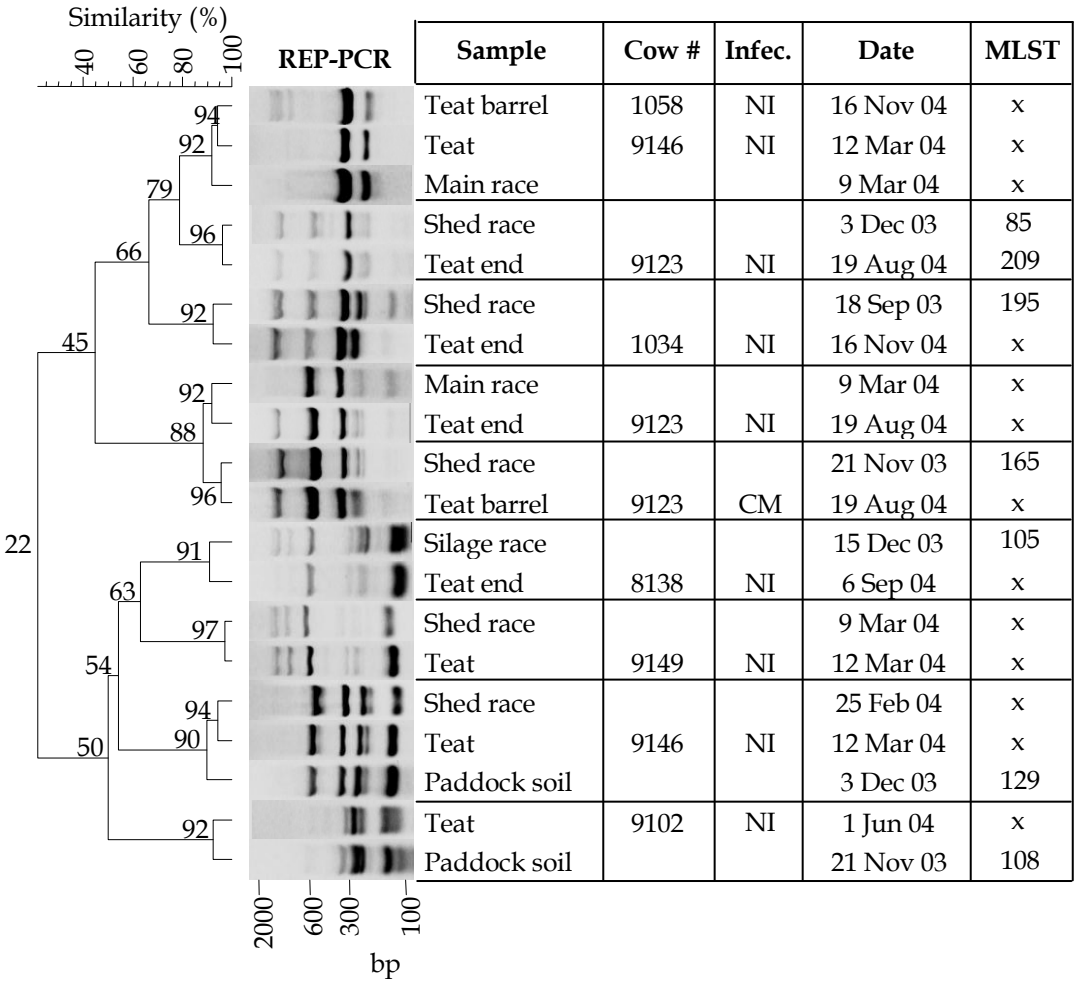
3.3.5 Isolation of *S. uberis* from the dairy environment

The majority of strain typed environmental isolates originated from farm races (110 isolates), while only 11 isolates from paddock soil and two isolates from paddock grass were typed. Six farm races were sampled on the Dexcel Lye farm including two races with high cow traffic (main race and shed race) and four with lower cow traffic (B race, east race, west race and silage race). Samples were collected from three permanent sampling points on a fortnightly basis over the course of one year. Isolates of *S. uberis* were strain typed from one sample from the east race and silage race, two samples from the B race, four samples from the west race, seven samples from the main race and 18 samples from the shed race.

Of the 123 environmental isolates, 81 different strain types were observed and band patterns representing each unique environmental strain type were compared to those found on the teat skin, in milk from infected quarters and in faeces.

3.3.5.1 Correlation of environmental and teat skin strains

The 81 environmental and 74 teat skin strains were compared, and nine of these had REP-PCR band patterns ≥90% similar (Figure 3.12), suggesting very little correlation between environmental and teat skin strains overall. However, in March 2004 the same strain was found on the main race and the teat skin of two different cows (#1058 and 9146; *) and on 9 March 2004 a strain from the shed race was also identified on the teat skin of cow #9149 three days later (#), suggesting that strains may be transferred from race material to the teat skin. All other observed correlations between the environment and teat skin strains were from different sample dates over a long time period. Therefore, the overall lack of correlation may reflect insufficient sampling around the same time points rather than low transmission of strains between the environment and teat skin. The use of teat spraying on these cows may also have had an affect on the correlation of strains between the environment and on the teat skin.



Sample	Cow #	Infec.	Date	MLST	
Teat barrel	1058	NI	16 Nov 04	x	
Teat	9146	NI	12 Mar 04	x	*
Main race			9 Mar 04	x	
Shed race			3 Dec 03	85	
Teat end	9123	NI	19 Aug 04	209	
Shed race			18 Sep 03	195	
Teat end	1034	NI	16 Nov 04	x	
Main race			9 Mar 04	x	
Teat end	9123	NI	19 Aug 04	x	
Shed race			21 Nov 03	165	
Teat barrel	9123	CM	19 Aug 04	x	
Silage race			15 Dec 03	105	
Teat end	8138	NI	6 Sep 04	x	
Shed race			9 Mar 04	x	#
Teat	9149	NI	12 Mar 04	x	
Shed race			25 Feb 04	x	
Teat	9146	NI	12 Mar 04	x	
Paddock soil			3 Dec 03	129	
Teat	9102	NI	1 Jun 04	x	
Paddock soil			21 Nov 03	108	

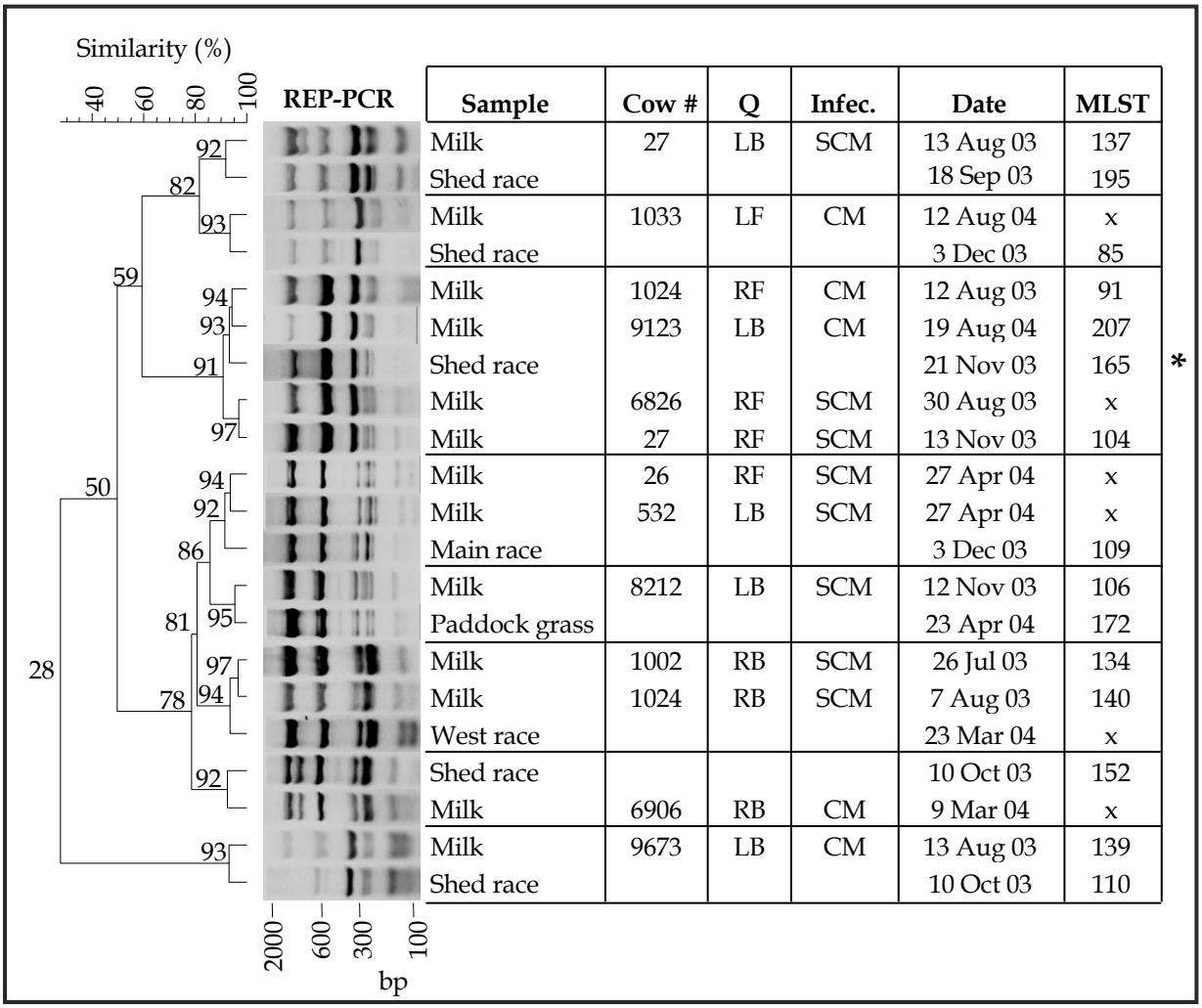
Figure 3.12. Same strain in the environment and on cow teats. REP-PCR band patterns for each unique environmental and teat skin strains were compared using Pearson correlation (optimization 2%) and a UPGMA dendrogram generated. Only those strains with band patterns ≥90% similar between isolates from each source are shown. An 'x' indicates where MLST was not performed. Notations (* and #) are explained in the text.

3.3.5.2 Correlation of environmental and cow body strains

Comparison of 81 environmental strains with 36 unique strains identified on the cow body revealed five strain types with band patterns greater than 90% similar between the two sources (data not shown). However, farm race and paddock isolates were not collected during the same months that cow bodies were sampled, thus, it was difficult to determine if strains may have been directly transferred from the environment to the cow body.

3.3.5.3 Correlation of environmental and infective milk strains

Very few correlations were observed between REP-PCR band patterns of environmental strains and those isolated from the milk of infected quarters collected around the same time (Figure 3.13). The largest group, with band patterns ≥90% similar (*), contained four milk strains and one strain from the shed race, but these were obtained over the course of one year from 12 August 2003 to 19 August 2004. Therefore, it was difficult to determine any direct link between the presence of the strain in the environment and the infection of cows with this same strain.



Sample	Cow #	Q	Infec.	Date	MLST	
Milk	27	LB	SCM	13 Aug 03	137	
Shed race				18 Sep 03	195	
Milk	1033	LF	CM	12 Aug 04	x	
Shed race				3 Dec 03	85	
Milk	1024	RF	CM	12 Aug 03	91	
Milk	9123	LB	CM	19 Aug 04	207	
Shed race				21 Nov 03	165	*
Milk	6826	RF	SCM	30 Aug 03	x	
Milk	27	RF	SCM	13 Nov 03	104	
Milk	26	RF	SCM	27 Apr 04	x	
Milk	532	LB	SCM	27 Apr 04	x	
Main race				3 Dec 03	109	
Milk	8212	LB	SCM	12 Nov 03	106	
Paddock grass				23 Apr 04	172	
Milk	1002	RB	SCM	26 Jul 03	134	
Milk	1024	RB	SCM	7 Aug 03	140	
West race				23 Mar 04	x	
Shed race				10 Oct 03	152	
Milk	6906	RB	CM	9 Mar 04	x	
Milk	9673	LB	CM	13 Aug 03	139	
Shed race				10 Oct 03	110	

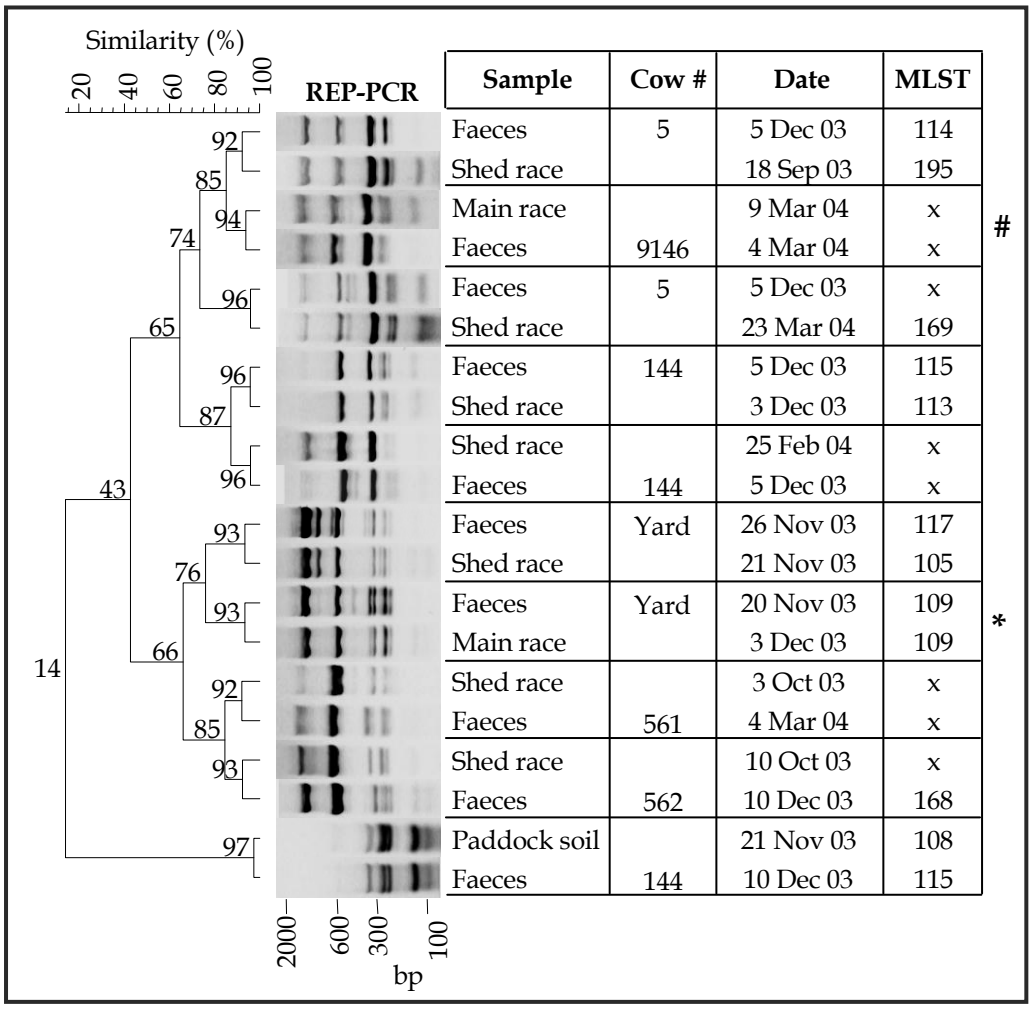
Figure 3.13. Comparison of strains from the environment and mastitis. REP-PCR band patterns from representative environmental and milk strains from infected quarters were compared using Pearson correlation (optimization 2%) and a UPGMA dendrogram generated. Only those strains with band patterns ≥90% similar between an isolate from each source are shown. Notations (*) are explained in the text.

A total of 81 strains were found in the environment and 41 strains isolated from infections, but of these only eight strains had a highly similar band pattern from the different sources. Once again however, this low correlation of strains could be due to lack of sampling rather than the absence of any direct link between environmental and infective strains.

3.3.5.4 Correlation of environmental and faecal strains

Shedding of *S. uberis* in the faeces is a potential source of dairy environment contamination; therefore, any correlation between strains found in the environment and in the faeces was investigated by comparing REP-PCR band patterns of strains from each of these sources. Ten strains found in the environment had band patterns ≥90% similar to strains identified in the faeces (Figure 3.14).

Some of the more interesting correlations observed were those found close together in time. For example, the same strain was found in both the faeces of cow #9146 on 4 March 2004, and five days later in a sample from the main race (#), perhaps indicating that the race may have been contaminated by faeces from this cow. Also, the same strain was identified in a sample of faeces from the cow yard and the main race 13 days later (*), suggesting that the dairy environment may have been contaminated by *S. uberis* from the faeces of a cow not sampled in this study.



Sample	Cow #	Date	MLST	
Faeces	5	5 Dec 03	114	
Shed race		18 Sep 03	195	
Main race		9 Mar 04	x	#
Faeces	9146	4 Mar 04	x	
Faeces	5	5 Dec 03	x	
Shed race		23 Mar 04	169	
Faeces	144	5 Dec 03	115	
Shed race		3 Dec 03	113	
Shed race		25 Feb 04	x	
Faeces	144	5 Dec 03	x	
Faeces	Yard	26 Nov 03	117	
Shed race		21 Nov 03	105	
Faeces	Yard	20 Nov 03	109	*
Main race		3 Dec 03	109	
Shed race		3 Oct 03	x	
Faeces	561	4 Mar 04	x	
Shed race		10 Oct 03	x	
Faeces	562	10 Dec 03	168	
Paddock soil		21 Nov 03	108	
Faeces	144	10 Dec 03	115	

Figure 3.14. Correlation of strains between the environment and faeces. REP-PCR band patterns from representative environmental and faecal strains were compared using Pearson correlation (optimization 2%) and a UPGMA dendrogram generated. Only those strains with band patterns ≥90% similar from each isolate set are shown in this figure. Notations (* and #) are explained in the text.

A total of 81 environmental and 53 faecal strains were identified, but only 10 strain types were found in both sources. If faecal shedding of *S. uberis* was responsible for the spread of strains then it would be expected that these faecal strains would be found more frequently throughout the dairy environment, particularly on farm races with high cow traffic. However, the level of environmental sampling would have had an effect on the observed correlation of faecal strains and, since it was not possible to strain type all isolates found in each sample, the isolates that were typed reflect only a small collection of a much larger community.

3.3.6 Common REP-PCR band patterns

REP-PCR bands were commonly observed at 600 bp, 300-400 bp and slightly less often between 1000 and 1500 bp. The presence of these common bands was analysed for all identified milk, teat skin, faecal and environmental strains (Table 3.20).

		Three common bands	
	Total strains	All present	None present
Milk	41	34 (83%)	1 (2%)
Teat	74	25 (34%)	10 (14%)
Faeces	53	19 (36%)	0 (0%)
Environment	81	55 (68%)	0 (0%)

Table 3.20. Number of strains from different sources with or without three common REP-PCR bands.

Strains from infected quarters (milk) were most likely to contain all three bands, while only 34% and 36% of teat and faecal strains respectively had all three bands. The teat skin strains gave quite varied band patterns compared to milk strains and 10 identified strain types did not contain any common bands.

Generally, the 300-400 bp band was the most regularly observed for strains from all sources with over 80% of REP-PCR patterns containing this band (Figure 3.15). Similarly, the 600 bp band was commonly found in faecal, environmental and milk strains with over 80% of the different strains having this band. However, only 62% of the strains from the teat skin had a band at this position. A clear difference was observed in the presence of the 1500 bp band between the different sets of strains. While 85% of milk strains had this band, only 43% of strains from the teat skin and faeces contained this band upon REP-PCR typing. The environmental strains gave

a more similar result to the milk strains with 72% of the different environmental strain types containing a band at 1500 bp.

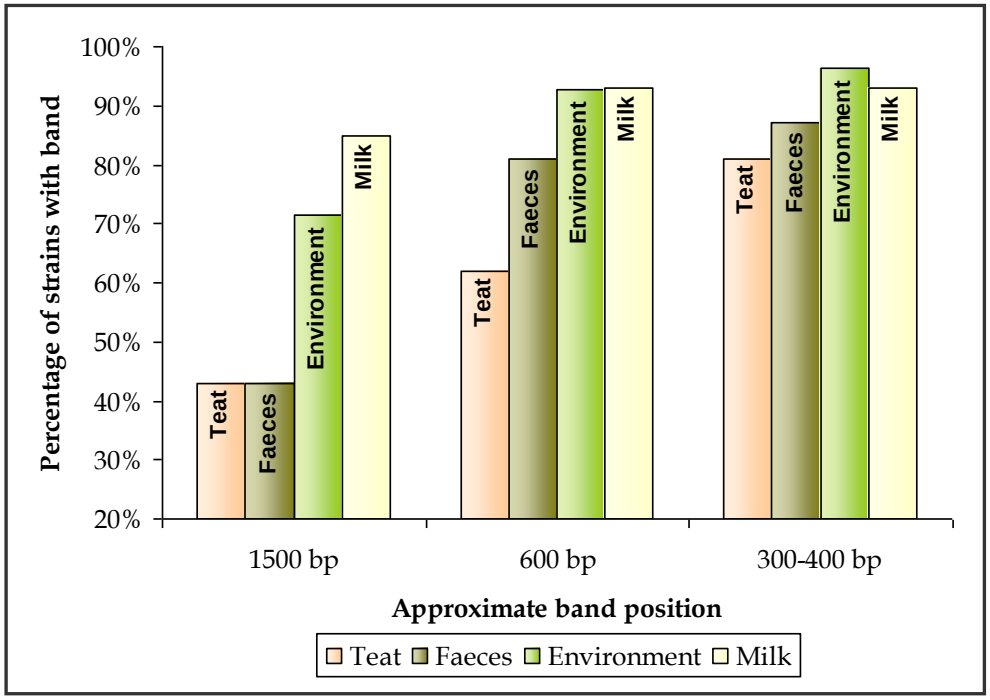


Figure 3.15. Percentage of strains from each source with three common bands. Bands at approximately 1500 bp, 600 bp and 300-400 bp were assessed for each strain from the teat skin, faeces, environment, and milk. This is shown as a percentage of the total number of strains identified from each source.

If the overall similarity between strains from the different sources was determined by the presence of these three common bands, then it would appear that the teat and faecal strains were quite different populations to those isolated from the milk and environment. Based on the band patterns, isolates from the environment may actually be more closely-related to those strains causing mastitis than strains found on the teat skin or in faeces.

3.3.7 Overall correlation of strain types between sources

The teat skin strains were most likely to be found causing mastitis, with 14 different strains identified both on the teat skin and in milk from infected quarters (Table 3.21). This was because the infective strain was often found on the teat skin of infected quarters. Only three faecal strains were also found on the teat skin, suggesting that faeces may not be a common source

of teat skin contamination. Eight and nine correlations were observed between strains found in the environment and from intramammary infection and on teat skin respectively, indicating that the environment may be an important reservoir of *S. uberis*, which is sustained by spreading of *S. uberis* via the faeces.

	Teat (74)	**Faeces** (53)	**Environment** (81)
Milk (41)	14	5	8
Teat (74)	-	3	9
Faeces (53)	-	-	10

Table 3.21. Number of strain types found in two locations. The numbers in brackets indicate how many unique strains were found in samples from each source.

3.3.8 Comparison of samples from similar time periods

As mentioned throughout this chapter, lack of sampling around similar time points lead to difficulty in determining transmission of strains between different locations and intramammary infections. With this in mind, isolates collected between 1 July 2003 and 31 July 2004 were compared according to date of isolation. Ten time periods, three months in length were set and all isolates' band patterns for each three month period were compared using Pearson correlation and UPGMA. Those isolates after 31 July 2004 were omitted from the comparison as they had been collected during the study of cow body sites and the teat end and teat canal study and were thoroughly investigated in earlier sections.

Any clusters on the dendrogram that contained isolates from different sources (e.g. Milk and faeces), with band patterns ≥90% similar were noted (Table 3.22). As was expected, more clusters were observed for the time period where a higher number of isolates were collected and compared.

A	2003						2004							
	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Total
Milk	82			8			31				0			121
Teat	5			1			6				56			68
Faeces	0			43			44				5			92
Race	6			58			46				0			110
Soil	0			10			1				0			11
Grass	0			0			2				0			2
TOTAL	**93**			**120**			**130**				**61**			
REP-PCR clusters*	**1**			**6**			**7**				**0**			

B	2003						2004						
	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul
Milk		49			8				21				
Teat		5			1				41				
Faeces		0			47				28				
Race		49			15				29				
Soil		0			10				1				
Grass		0			0				2				
TOTAL		**103**			**81**				**122**				
REP-PCR clusters*		**0**			**6**				**8**				

C	2003						2004						
	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul
Milk			25			10			9				
Teat			5			1			56				
Faeces			4			60			8				
Race			53			28			2				
Soil			9			1			1				
Grass			0			0			2				
TOTAL			**96**			**100**			**78**				
REP-PCR clusters*			**4**			**4**			**1**				

Table 3.22. Number of isolates obtained from different sources over ten different, three month time periods.
The number of REP-PCR clusters that consisted of isolates from different sources with band patterns ≥90% similar are indicated (*). Samples were only collected on one day in January 2004 (29th), therefore these were included in the Feb – Apr time period.

When the isolates within each of the ≥90% similarity clusters were analysed, it was apparent that many of the clusters consisted of faecal and race isolates (Table 3.23). While this may indicate a direct route of transmission between the faeces and farm races, it must be noted that faecal and race isolates were present in higher numbers in the comparison (92 and 110 respectively; Table 3.22,A), compared with other isolates, except for those from milk.

		Time period					
		Jul - Sep 2003	Sep - Nov 2003	Oct - Dec 2003	Dec - Feb 2004	Jan - Apr 2004	Mar - May 2004
Isolates in cluster	Milk (SCM) & Race	1	1	1			
	Milk (CM) & Race					2	
	Faeces & Race		1	3	2	3	
	Teat & Race		1			2	2
	Teat & Faeces			1			1
	Soil & Faeces			1			
	Milk (SCM), Faeces & Race			1			
	Milk (CM), Soil & Race		1				

Table 3.23 Number of REP-PCR clusters containing isolates from different sources across different time periods.

Another frequently observed cluster contained teat and race isolates, indicating possible routes of transmission from faeces to the race and from the race to the teat skin.

Despite a high number of milk isolates being compared in total, very few of these correlated with isolates of *S. uberis* from other sources. However, this may be due to the majority of milk isolates being collected during 1 July – 30 September 2003 when the only others collected during this time were six isolates from the race and five from the teat skin (Table 3.22,A).

Overall, it appears that the higher the number of isolates collected, the more likely that clustering of band patterns will be observed. The most frequently observed clusters consisted of faecal and race isolates which may indicate a potential mechanism of *S. uberis* transmission from the cow to the environment. Also, the number of clusters containing teat skin isolates and race isolates may indicate that *S. uberis* may also be transferred from the race to the teat skin.

3.3.9 Correspondence of REP-PCR and MLST results

Where isolates were typed by MLST and REP-PCR, it became apparent that the results of the two typing methods rarely corresponded, although better correlations were observed between MLST and REP-PCR for milk isolates than for isolates from any other source. Isolates from milk, teats, faeces and the environment were also compared based on the MLST results and any isolates from different sources with the same ST are shown in Table 3.24. A total of 56 milk isolates, 44 environmental, 19 faecal and 8 teat isolates were MLST typed and of these, 77 different strain types were determined. Where the same strain was identified from different sources, the largest group (ST 91) contained seven isolates obtained from milk of quarters with clinical and subclinical mastitis, together with two race isolates. Therefore, based on MLST typing, *S. uberis* strains that caused mastitis were also found on farm races (ST 91, 105, 107, 145), on the teat skin of uninfected quarters of other cows (ST 105, 129), and in the faeces of other cows on the farm (ST 131). The same strain of *S. uberis* could also be identified as causing both clinical and subclinical mastitis in different cows over time (ST 91, 94, 145) and found in three different locations within the dairy environment (ST 109).

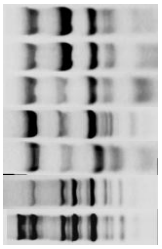
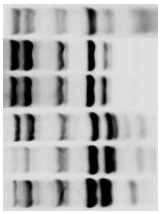
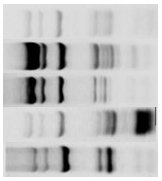
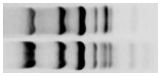
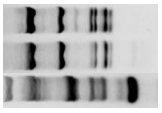
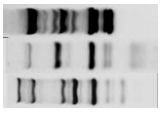
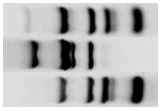
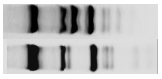
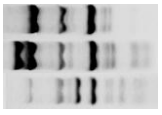
MLST	Sample	Cow #	Q	Infect.	Date	REP-PCR
91	Milk Milk Milk Milk Milk Shed race West race	1024 1024 9673 8777 1033	RF RF RF RB LF	SCM CM CM SCM CM	7 Aug 03 12 Aug 03 13 Aug 03 12 Nov 03 17 Sep 04 21 Nov 03 7 Apr 04	
94	Milk Milk Milk Milk Milk Milk	1025 1007 1025 1027 1007 1027	LB LB LB LB LB RB	CM CM SCM SCM SCM CM	10 Aug 03 1 Aug 03 5 Aug 03 30 Jul 03 26 Jul 03 30 Jul 03	
105	Teat barrel Milk Shed race Silage race West race	9123 6906	RF RB	NI SCM	19 Aug 04 12 Nov 03 21 Nov 03 15 Dec 03 3 Dec 03	
107	B race Milk	9682	RF	SCM	3 Dec 03 12 Nov 03	
109	Faeces Main race Paddock soil	Yard			20 Nov 03 3 Dec 03 21 Nov 03	
120	Faeces Main race West race	Yard			20 Nov 03 15 Dec 03 7 Apr 04	
129	Teat Milk Paddock soil	51 461	RB RB	SCM SCM	15 Sep 03 20 Aug 03 3 Dec 03	
131	Milk Faeces	1036 9146	LB	SCM	18 Aug 03 7 Apr 04	
145	Milk Milk Shed race	8705 1026	LF LF	SCM CM	16 Aug 03 3 Nov 03 15 Dec 03	

Table 3.24. Comparison of isolates with the same strain type by MLST.

Several MLST strain types contained isolates with highly similar REP-PCR band patterns (for example ST 94, 105, 107), while other groups (ST 91, 109, 120, 131, 145) contained isolates with visibly different band patterns. In these cases it was possible that the REP-PCR method was showing a higher

discriminatory power than that obtained by MLST by differentiating these isolates as unique strain types.

To investigate this further, the Simpson's index of diversity was calculated as described in Chapter 2.2.16. A set of 85 isolates were assigned strain types based on MLST or REP-PCR band and of these, MLST yielded 59 strain types, while REP-PCR yielded 62 strain types. The diversity index calculations revealed that if two strains were sampled randomly from the population, then on 99.1% (REP-PCR) or 98.8% (MLST) of occasions they would fall into different strain type groups. The lower 95% confidence intervals were above 98% for both REP-PCR and MLST. Therefore, for this set of isolates, REP-PCR had a slightly higher discriminatory power than MLST, although overall the results were very similar, indicating high strain differentiation with both methods. In conclusion, each method was capable of discriminating between strains; however, the strain type groups formed with each method tended not to contain the same isolates.

3.3.10 Comparison of strain diversity

Variations were observed in the number of unique strains identified across different sources (Table 3.25).

Isolate set	Total no. isolates typed	No. of isolates typed per sample	Total no. unique strains	% unique strains per total typed
Milk	143	1-5	41	29%
Teat skin	199	1-20	74	37%
Faeces	102	1-6	53	52%
Environment	123	1-12	81	66%

Table 3.25. Unique strains as a percentage of total isolates typed.

Generally, only one strain type was observed within milk samples, which may account for the relatively low proportion of unique strains (29% of total typed). In contrast, the faecal and environmental samples were less likely to contain only one strain type with 52% and 66% of typed isolates corresponding to unique strains respectively. This suggested that strain diversity may differ between infective (milk), teat skin, faecal and environmental strains.

3.3.10.1 Rarefaction curves to estimate strain diversity

Application of rarefaction techniques to estimate microbial diversity is reviewed fully by Hughes and Hellman (2005). For this study, rarefaction curves represent the theoretical average number of strains observed when isolates were drawn repeatedly, with replacement, from the entire sample set. REP-PCR band patterns of isolates were compared to obtain representative isolates from each sample and strain type numbers assigned as described in section 3.2.3 – 3.2.4. Assigned strain types and frequency of occurrence were entered into the EstimateS 7.5 program for each set of isolates from milk, teat skin, faeces and the environment. The default setting of 50 randomizations were calculated using the 'with replacement' option to produce the total number of strains observed at each level of sampling (number of isolates; Figure 3.16).

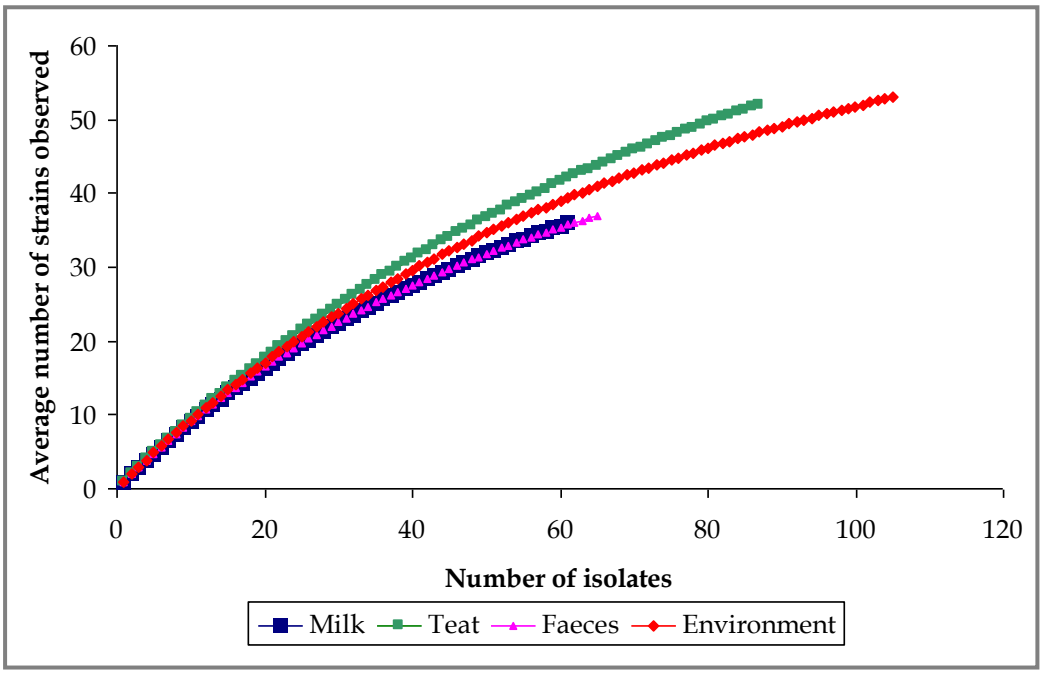


Figure 3.16. Estimation of strain diversity in milk, teat, faecal and environmental isolates. Diversity statistics (Sobs Mao Tau) were plotted to show the average number of strains as the number of isolates in the set increases.

The number of theoretical strains obtained was very similar in each of the sets when only a few isolates were typed. However, after typing 40 isolates, the number of strains began to vary between sources. After typing 60 isolates, more strains would theoretically be found in samples from the teat skin and the environment, compared to milk from infected quarters or faeces.

As this was just an estimation of strain diversity, 95% confidence intervals (CIs) were also calculated in EstimateS and applied to the graphs as error bars to give a measure of variance around the average rarefaction curve. The error bars for all sets of isolates from different sources were found to overlap (Figure 3.17), indicating that the estimated strain diversity was not significantly different for each set of isolates, even at the highest level of sampling obtained.

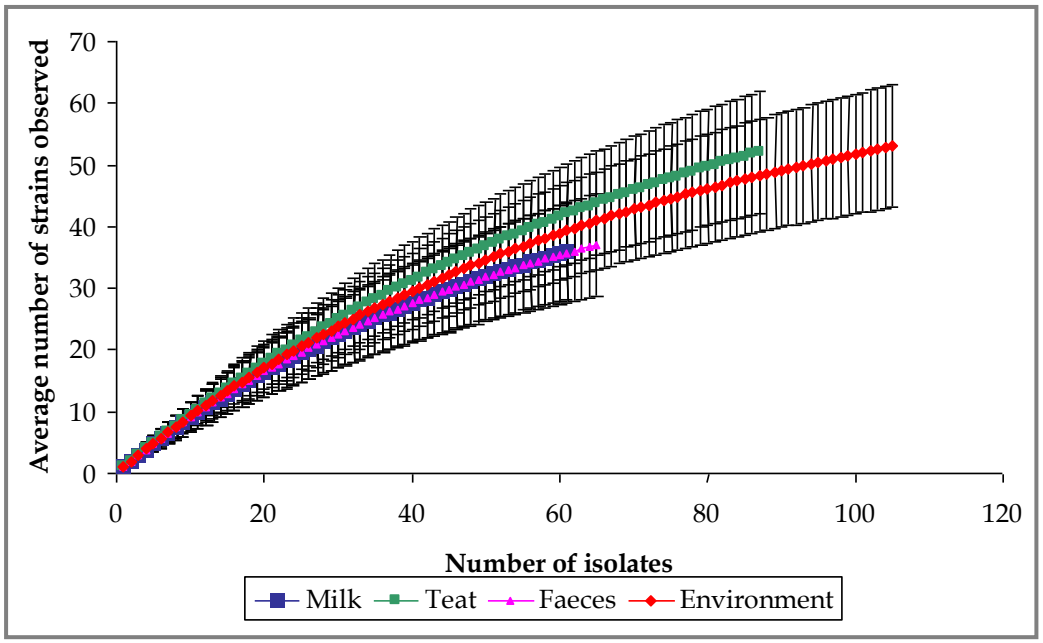


Figure 3.17. Estimation of strain diversity including 95% CI error bars. The same graph as in Figure 3.16 was reproduced with the inclusion of error bars representing 95% confidence intervals for each set of isolates.

The comparison of rarefaction curves between sample sets only indicates whether the diversity of the samples in the sets was significantly different. Had more isolates from each source been typed, then it may have been possible to observe a significant difference in the strain diversity of *S. uberis*. The calculated rarefaction curves were still increasing at the highest level of sampling, but if they had become asymptotic, it could perhaps be said with more certainty that a measure of the true diversity of the community had been obtained.

3.3.11 Capsule genes in *S. uberis* isolates

Three genes (*hasA*, *hasB* and *hasC*) are required for the formation of a hyaluronic acid capsule in *S. uberis*, a potential virulence factor. Therefore, the presence of these three genes in *S. uberis* isolates from different sources was also investigated. Based on the nucleotide sequence for the *hasA*, *hasB* and *hasC* genes, the designed primers resulted in a product of 190 bp for *hasA*, 320 bp for *hasB* and 592 bp for *hasC* (Figure 3.18).

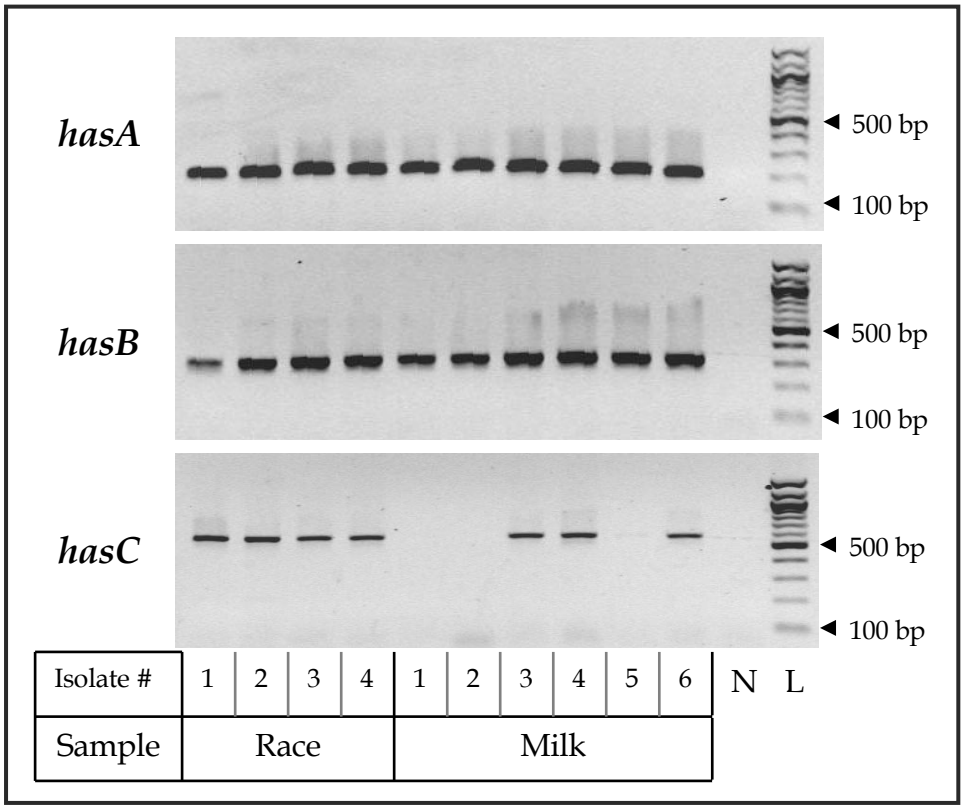


Figure 3.18. Amplification of DNA from race and milk isolates with primers specific to the *hasA*, *hasB* or *hasC* capsule genes. A negative control (N) was included for each primer set and a 100 bp ladder (L; NEB) included on each gel.

PCR for all three genes was performed on a total of 58 *S. uberis* isolates from milk of infected quarters (mixture of clinical and subclinical infections), teat and udder skin, mouth, tail, feet, faeces and the dairy farm environment. Of these, 85% had the three *has* genes, with the cow body and environment the only sources where all tested isolates contained all three genes (Table 3.26).

	Number of isolates	**Number positive for *hasABC***	**Percentage positive**
Milk	20	17	85%
Teat/udder	7	4	57%
Cow body	3	3	100%
Faeces	12	9	75%
Environment	16	16	100%
All isolates	58	49	**85%**

Table 3.26. Isolates positive for all three capsule genes.

Additional isolates were also randomly tested for either of the *hasA*, *hasB* or *hasC* genes. Amplification of the *hasA* gene was performed on 79 isolates in total and all were found to be positive (Table 3.27). A few isolates were found to lack the *hasB* gene with 73 of 79 isolates (92%) that were amplified, containing this gene. Of the 69 isolates subjected to PCR for the *hasC* gene, 58 isolates (84%) were found to be positive. Therefore, of the three genes, *hasC* was most likely to be absent.

Gene	**Number of isolates**	**Number positive for gene**	**Percentage positive**
hasA	79	79	**100%**
hasB	79	73	**92%**
hasC	69	58	**84%**

Table 3.27. Percentage of all isolates positive for each capsule gene.

3.4 Discussion

The main objective of this study was to compare strains of *S. uberis* within the dairy environment, faeces, teat skin, cow body and those from intramammary infections. However, due to samples being obtained from various studies over a period of two years, there was a lack of samples around similar time points, making it difficult to draw conclusions about the presence of the same strain in more than one location. Also, it has recently been noted that *S. uberis* appears to have a substantial recombination rate (Tomita *et al.*, 2008). In the present study, samples were often collected months apart, therefore it was possible that an identified strain would not be detected in subsequent samples due to recombination events occurring between sampling dates. Despite these setbacks, this study confirmed the findings of many others in that *S. uberis* could be isolated from different locations throughout the dairy farm environment and on the cow itself. Isolates of *S. uberis* were found in material from farm races and paddocks in addition to the teat skin and teat canal of infected and uninfected mammary quarters, faeces, and other areas of the cow body including lips, nostrils, tail swish, hocks and hind feet.

It must also be noted that the validity of conclusions that were drawn depend on the strain typing method that was used. While attempts were made to standardise the REP-PCR typing method, some problems were observed both with the method and analysis; therefore it was possible that not all strain type assignations were correct. However, it is thought that this would not greatly affect the general conclusions reached. Also, the MLST typing of a set of 127 isolates support the findings that the same strain of *S. uberis* can be found within the mammary gland, causing mastitis, and on teat skin, farm races, paddock soil and in faeces.

3.4.1 Suitability of REP-PCR as the strain typing method

3.4.1.1 REP-PCR typing and analysis

The REP-PCR strain typing method allowed comparison of strains obtained from different sources; however, the use of REP-PCR alone may not have been sufficiently discriminatory, particularly when large sets of band patterns were compared. Many observed correlations between strains from various sources may have been due to a lack of differentiation between strains. However, as very few correlations were actually observed then it is unlikely that additional strain typing would have resulted in different conclusions overall.

Problems were sometimes identified with the use of Pearson correlation for analysis of REP-PCR band patterns. As this type of comparison is based on the densitometric curves of each band pattern, the intensity of each band is important. If the majority of bands were in the same position and of almost identical intensity, any slight differences such as the presence of an additional, less intense, band may still result in a very high percentage similarity (sometimes >90%). Unfortunately this problem could not be avoided with the parameters in GelComparII that were deemed most appropriate for analysis of REP-PCR band patterns. In future, new settings and parameters may need to be investigated to improve any incorrect strain type assignments. Also, analysis programs other than GelComparII could be tested as different programs can give slightly different results when the same band patterns are analysed (Cardinali *et al.*, 2002; Rementeria *et al.*, 2001). However, any computer program may not provide indisputably correct analysis and any conclusions drawn from computerized analyses should always be accepted with caution (Rementeria *et al.*, 2001). As found in Chapter 2 and by Duck *et al.* (2003), software-based algorithms were unable to match band patterns of identical isolates at 100%, therefore these algorithms cannot entirely replace visual interpretation of banding patterns.

Despite these problems, REP-PCR was rapid and easy to perform and the main advantage was the ability to strain type multiple isolates from each sample, unlike previous studies where only one *S. uberis* colony from each sample was typed due to the time-consuming nature of the typing methods (Douglas *et al.*, 2000; McDougall *et al.*, 2004). In future, REP-PCR could be refined further, improving the discriminatory power and eliminating the need for secondary anchored typing.

3.4.1.2 Analysis of REP-PCR band patterns

Johnson *et al.* (2004) noted that results were improved by removing isolates with duplicate band patterns from the analysis; identical band patterns most likely represented clonal isolates, which artificially biased the subsequent analyses. In the present study, removal of duplicate band patterns from each sample also improved analysis of strains across different samples. With removal of duplicates, the resulting clusters determined with Pearson correlation more closely reflected clusters determined by visual interpretation. Therefore, all sets of isolates from milk, teat skin, faeces and the environment were analysed in exactly the same way with duplicate band patterns removed as the analysis progressed, resulting in a final set of unique strain types from each source.

3.4.1.3 Correlation between REP-PCR and MLST results

As found in Chapter 2, REP-PCR and MLST strain type assignments rarely corresponded, although results were more similar when REP-PCR patterns for milk isolates were manually compared rather than when Pearson correlation was used. While the reliance on software analysis may have caused this disparity, it may also have been due to differences in the discriminatory power of the two methods. In Chapter 2 it was noted that the use of anchored typing improved the discriminatory power of REP-PCR. However, this secondary typing method was only performed on 14% of all isolates collected, and improved correlations may have been observed

if all isolates had been subjected to this technique. While on some occasions MLST appeared more discriminatory, at other times REP-PCR gave different band patterns between isolates despite an identical MLST strain type. It has been reported that two different typing methods rarely give similar results when the same isolates are typed (Goldberg *et al.*, 2006; Hermans *et al.*, 1995; Malachowa *et al.*, 2005), and this also appears to be the case in the present study. As MLST method focuses on variations in the more stable core-genome, while the REP-PCR method detects variations throughout the genome (Enright & Spratt, 1999), it would not be expected that results from each method would be completely congruent.

3.4.2 *Streptococcus uberis* in the dairy environment and faeces

The dairy environment is considered to be the main source of contamination of the cow's body and teats by *S. uberis* and this pathogen has been found to be widespread throughout the dairy environment (Cullen & Little, 1969; Douglas *et al.*, 2000; Zadoks *et al.*, 2005b). However, detection of a pathogen in an environmental site does not necessarily imply that this site acts as a source of infection (Zadoks *et al.*, 2005b). It is assumed that teats become colonized by *S. uberis* through contact with the contaminated dairy environment which then leads to intramammary infections (Pullinger *et al.*, 2006), and locations where cows gather are particularly likely to pose a high risk of *S. uberis* exposure (Cullen & Little, 1969; Zadoks *et al.*, 2005b).

In the present study, many different strains of *S. uberis* were found on farm races. However, only a few strains from this location were also found on the teat skin or causing mastitis, as observed previously (Zadoks *et al.*, 2005b). With such strain diversity within the environment, more sampling may have been required to find the same strain between these locations. Despite these limitations, the same strain was isolated from a farm race and teat skin of two cows a few days later, indicating that the teat skin may have

been contaminated with race material containing *S. uberis*. Also, when isolate band patterns' were compared within three month time periods, several clusters were observed to contain isolates from farm races and teat skin, indicating that transmission between these two sites may occur. The same strain was mainly isolated from farm races and intramammary infections at different times, so any direct link between strains in the environment and mastitis is not clear.

The presence of cows appears to be required for contamination of the dairy environment as *S. uberis* could not be found in non-dairy environments (Cullen & Little, 1969; Zadoks *et al.*, 2005b), and could not be isolated from fields before grazing (Lacy-Hulbert *et al.*, 2006), or where non-infected herds grazed (Cullen & Little, 1969). Given the high diversity of *S. uberis* in the dairy environment, the most likely source of contamination was through excretion of *S. uberis* in faeces.

3.4.2.1 *Streptococcus uberis* in faeces

Cows have previously been found to contain *S. uberis* in the rumen and to excrete this species in their faeces (Bramley, 1982; Cullen & Little, 1969; Kruze & Bramley, 1982; Zadoks *et al.*, 2005b), which may provide a mechanism for spread of *S. uberis* around the dairy environment. Animal nutrition has been associated with the incidence rate of clinical mastitis caused by *S. uberis* (Barkema *et al.*, 1999) and, although this result could be due to other physiological factors in the cow, it is also possible that nutrition may affect the level of colonisation of the gastrointestinal (GI) tract by *S. uberis* (Cullen, 1966; Kruze & Bramley, 1982), and subsequently change the level of exposure to these pathogens through faecal contamination.

In the present study, some cows were found to have multiple strains of *S. uberis* in the faeces, similar to observations made by Zadoks *et al.* (2005). This may explain the high diversity of strains observed in the dairy

environment. Also, the persistent shedding of *S. uberis* in some cows suggests that the GI tract had become colonised by this species. Although the same strains of *S. uberis* were not present in every sample from each cow, persistent shedders were more likely to have the same strain in their faeces over time compared to those that excreted *S. uberis* more sporadically. Some strains were found infrequently in the faeces of a cow, which may reflect repeated colonisation and clearance of *S. uberis* from the GI tract. However, this was unlikely with such diversity in the environment. Instead, the GI tract of particular cows may have been continuously colonised by certain strains of *S. uberis*, but the level excreted in the faeces varied over time and on some occasions could be below detectable levels (<10 cfu/rectal swab). These results again support those of Zadoks *et al.* (2005), where faecal samples that were positive for *S. uberis* changed over time; however this was attributed to seasonal variation rather than any host or strain effect.

The same strain of *S. uberis* was sometimes found in the faeces of different cows, indicating that either the strain had been transferred through cow-to-cow contact or that it was prevalent in the environment and had been ingested by more than one cow. This may indicate the potentially cyclic nature of strain transmission between the environment, through the GI tract of cows, and excretion in the faeces on to pasture which is then ingested by a different cow in the herd, continuing the cycle.

3.4.2.2 Distribution of *S. uberis* via the faeces

To identify if *S. uberis* was being spread around the dairy farm by excretion in the faeces, the identified faecal strains were compared to those found in the environment. Although the overall correlation between strains was not high, a strain found in the faeces of one cow was then identified again five days later on the main race. Also, another strain found in faeces from the cow yard was identified on the main race 13 days later. Therefore, the same

strains can be found both in the faeces and environment, indicating that *S. uberis* may be spread throughout the farm via the faeces. When isolates from three month time periods were compared, more clusters were observed containing faecal and race isolates than any other combination of isolates. This may indicate contamination of races via the faeces, or these results may have been due to faecal and race isolates being present in higher numbers than other isolates in the comparison. Overall, had more isolates from these collected samples been strain typed more correlations may have been observed.

Zadoks *et al.* (2005) noted that persistent faecal shedders were not involved in the spread of *S. uberis* around the dairy farm. Instead, it was suggested that the widespread occurrence of *S. uberis* in the environment was due to bacterial multiplication within the farm race material and paddock soil, which are natural niches for *S. uberis*. Another possibility may involve leaking of milk from the udders of clinically and subclinically infected cows, resulting in direct contamination of the environment with these infective strains.

3.4.2.3 Faecal strains and mastitis

The presence of *S. uberis* in faeces may be directly linked to mastitis (Hogan *et al.*, 1989; Ward *et al.*, 2002). However, in this study only five faecal strains were also found causing intramammary infections and in all cases the strain was first detected in the milk before being isolated from faeces at a later date. Also, strains isolated from faeces of cows with mastitis were not the same as the milk strain from the infected quarter. When isolates were compared within three month time periods, only one cluster was observed to contain both a faecal isolate and a milk isolate from an infected quarter. Similarly, Lopez-Benavides *et al.* (2006) observed that cows shedding *S. uberis* in faeces did not have a higher risk of developing subclinical or clinical mastitis during their lifetime and Cullen (1966) noted that faecal

contamination was unlikely to play an important role in mastitis due to the low incidence of positive rectal swabs.

Faeces were unlikely to be the main source of *S. uberis* contamination of the teat skin as the same strains were rarely found in both locations. However, one individual strain was found both in faeces and on the teat skin of the same cow one month later, indicating that contamination of the teat skin with the faeces may be possible.

The overall lack of correlation between strains found in faeces and those on the teat skin or from intramammary infections may indicate that either the presence of *S. uberis* in the faeces does not directly relate to infection of the mammary glands of the cows in this herd, or that insufficient samples were strain typed from the teat skin and faeces of the same cows immediately prior to development of a new intramammary infection.

3.4.3 *Streptococcus uberis* on the body of cows with mastitis

Many areas of the cow body have yielded *S. uberis* upon sampling, including the skin surface, genital tract, intestinal tract, lips and tonsils (Cullen & Little, 1969; Razavi-Rohani & Bramley, 1981). In the present study, *S. uberis* was found on many different parts of the body, but it was not ubiquitous, with only 43% of all body and teat samples containing this pathogen. This perhaps reflected contamination of the cow body rather than true colonisation of these sites. Of the sampled sites, *S. uberis* was most commonly found on the hind feet of cows with clinical mastitis and the lips of cows with subclinical mastitis. Despite previous studies that found *S. uberis* on the udder skin (Buddle *et al.*, 1988; Cullen, 1966; Sharma & Packer, 1970), this was the only sampled site where *S. uberis* was not detected in the nine cows studied.

Several strains were found at multiple sites on one cow, particularly when the cow had subclinical rather than clinical mastitis. As teat and body swab

samples were collected before antibiotic treatment of clinically infected quarters, these observed differences can not be attributed to use of antibiotics in clinical cases compared to the absence of treatment for subclinical cases. The majority of similar strains were found on the teat skin and one or two other areas of the cow's body, unlike the results of Buddle *et al.* (1988), where no clear associations were found between the strains of *S. uberis* on the teat skin compared to other skin sites. The lips have been suggested to play a role in spreading of *S. uberis* from one part of the body to another (Cullen, 1966), which may explain why the same strain was sometimes found on the lips and other areas of the body in the present study.

With further examination, a higher proportion of *S. uberis*-positive samples from the body of cows with clinical mastitis also contained the infective strain (44%) compared to cows with subclinical mastitis (9%). With clinical mastitis, the infective strain was not found on the hocks or in the rectum in any of the cases and was present more often on the feet than any other site, which may reflect human involvement rather than any natural contamination or colonisation. During detection and confirmation of mastitis, milk was stripped from the infected quarter onto the ground, possibly splashing the hind feet and contaminating them with the infective strain. This technique was not conducted on cows with subclinical mastitis and thus the infective strain was not found on the feet of these cows. The only site that yielded the infective strain with subclinical mastitis was the lips. The strains causing mastitis in these nine cows were not isolated from the rectum, indicating again that the faeces were unlikely to be the source of the infective strains.

The infective strain was prevalent across the body of one cow with clinical mastitis and was found in three teat skin samples and on the feet, lips, nostrils and tail. However, three other cows in the study did not show any

evidence of the infective strain on the teats or body. Kruze and Bramley (1982) noted that *S. uberis* could be isolated more frequently from the body of cows in herds that experience high rates of intramammary infection; however, as the bodies of uninfected cows were not sampled in the present study, this comparison was unable to be made. Also, the two cows with two infection events within a short period of time were not particularly contaminated with the infective or other strains of *S. uberis* compared to other cows in this study, indicating that a particularly high load of *S. uberis* on the teats and body was not necessary for an infection to develop.

Cows with clinical mastitis appeared to have the infective strain at more locations on the body than did subclinically infected cows. However, it was impossible to determine if the cow body became contaminated with this strain before or after mastitis developed.

Overall, 110 samples from the teat skin and body were analysed from nine cows with mastitis and only 47 were positive for *S. uberis*, indicating that this species was not ubiquitous across the body sites sampled, although lack of recovery and culture of live bacteria from samples may have affected this result. The infective strain was only found in 17 of the samples positive for *S. uberis*, therefore this was also not widespread across the body.

Isolates of *S. uberis* were obtained more frequently from the skin of cows during the winter than in the spring or autumn (Buddle *et al.*, 1988; Sharma & Packer, 1970), indicating that the weather or other physiological factors relating to the season may influence the colonisation or contamination of the cow body by *S. uberis*. In the present study, cows were sampled from August through to November, corresponding with late winter through to spring. It would be interesting to sample cows during the summer months to see the effect of increased sunshine hours on the level of *S. uberis* contamination of the cow body and also to repeatedly sample cows to

determine if particular areas of the body are transiently or persistently contaminated by *S. uberis*.

3.4.4 The teat skin and teat canal

Initially, bacteria need to adhere to teat skin and gain access through the teat canal to establish infection within the mammary gland. Sharma and Packer (1970) noted that *S. uberis* was most commonly found on the teat skin and udder surface and, in some studies, the incidence of mammary gland infection was correlated with the number of pathogens present on the teat end (Neave *et al.*, 1966; Pankey, 1989). However, others have found the teat skin to be a comparatively unfavourable site for *S. uberis* with other skin sites being of more importance as reservoirs of infection (Cullen, 1966).

In the present study, *S. uberis* was found on the teat skin of both infected and uninfected quarters, although less than half of all teat skin samples collected from 11 mastitis cases yielded *S. uberis*, indicating that not all teats were colonised or contaminated by this species, supporting the hypothesis of Cullen (1966).

Teat skin of the majority of infected quarters was exclusively contaminated/colonised by the infective strain from that quarter. In contrast, teat skin of uninfected quarters usually contained multiple strains either with or without the infective strain from the neighbouring quarter. This suggests that high numbers of the infective strain may out-compete any other strains on the teat skin of infected quarters. Alternatively, this may indicate complete colonisation of the teat skin by this one strain, with subsequent infection of that mammary quarter. At this stage it is unknown whether the infective strain was prevalent on the teat skin before or after mastitis developed.

Almost all teat canals that were sampled had various bacterial species present, but only a minority contained *S. uberis*. *Streptococcus uberis* was found exclusively in the teat canals of two clinical and one subclinically infected quarter, and only one strain was detected, the same as that infecting the mammary gland. This suggested colonisation of the teat canal before the infection ascended into the mammary gland, despite King (1981) noting that *S. uberis* does not colonize the teat canal, perhaps due to the growth-inhibiting effects of keratin fatty acids (Hogan *et al.*, 1988). Rather than being colonised, it was also possible that the teat canal became contaminated post-infection. However, the teat canals of two uninfected quarters also contained *S. uberis* along with other bacterial species, which indicates that *S. uberis* can be found within the teat canal without an infection in the mammary gland itself. Also, one of the clinically infected quarters had two strains present in the milk, but only one of these was found in the teat canal. Thus, if the presence of the infective strain in the teat canal was through post-infection contamination then it would be unlikely that uninfected quarters would contain *S. uberis* at this location and this second milk strain would also have been found within the teat canal.

Further investigations are required before establishing whether colonisation of the teat canal leads to development of a mammary gland infection, or whether post-infection contamination occurs. In particular, *S. uberis* isolates from the teat canal of uninfected quarters need to be strain typed to establish if single or multiple strains of *S. uberis* colonise the teat canals of uninfected quarters.

In summary, the teat skin of both infected and uninfected quarters was found to harbour *S. uberis*, although clinically infected quarters were more likely to have the infective strain exclusively on the teats, while uninfected quarters often harboured multiple strain types. Teat canals of infected quarters exclusively contained the infective strain from that quarter.

However, at this stage it is unknown if the infective strain was present on the teat skin or the teat canal before mastitis developed, or whether it was found after milk from the infected gland contaminated the teat canal and teat skin.

3.4.4.1 Transmission of *S. uberis* strains between teats

The majority of teat skin samples contained multiple strains of *S. uberis*, similar to the observations of Buddle *et al.* (1988). The same strains were often found on at least two cows' teats and, at most, the same strain was detected on the teats of four different cows over the course of eight months, indicating that transmission of strains between teats may occur. Zadoks *et al.* (2003) found that two types of strain transmission were possible; the first involved transfer of strains from the contaminated dairy environment to the mammary gland and the second involved cow-to-cow transmission of strains, perhaps during the milking process. Either of these two mechanisms may have occurred for these cows.

One strain, that caused subclinical mastitis in one cow, was also found on the teat skin of a second cow in the same herd. This second cow also had subclinical mastitis and the strain from this infection was found on the teat skin of the first cow. This connection between these two cows suggests that the infective strain was transferred from the milk of one cow to the other cows' teats, perhaps through the milking process or other direct contact between the cows.

Despite these few correlations, most strains found in milk and on the teat skin of different cows showed little correspondence in regard to sample date, suggesting that strains from each of these locations may not be closely-related.

3.4.5 Transmission of *S. uberis* strains between sites

As noted by Pullinger *et al.* (2006), strains that cause mastitis can be found in the dairy environment and on the teat skin, consistent with infections resulting from environmental reservoirs. However, direct transmission between these sites is difficult to trace in the present study as samples were not collected around the same time points. Also, it was difficult to differentiate whether strains on the teat skin or cow body were present before or after the mammary gland became infected. A more focused set of experiments may be required to determine how strain transmission occurs and the main reservoirs of strains involved in mammary gland infection.

3.4.6 *Streptococcus uberis* strains causing mastitis

In many studies only one isolate from each infected quarter was strain typed (Douglas *et al.*, 2000; McDougall *et al.*, 2004; Pullinger *et al.*, 2007; Zadoks *et al.*, 2005b); therefore, it was impossible to determine the number of different strains infecting the mammary gland at any one time. Other studies have shown, that when multiple (2 – 8) colonies were typed from a single quarter, usually only one strain was detected (Oliver *et al.*, 1998b; Phuektes *et al.*, 2001), with two strain types identified occasionally (Wieliczko *et al.*, 2002; Zadoks *et al.*, 2003). Similarly, in the present study, the majority of clinical and subclinical mastitis cases were caused by only one strain. However, one clinical and one subclinical infection had two different strain types present within the milk.

3.4.6.1 Multiple infected quarters

Adkinson *et al.* (1993) noted that simultaneous infection of multiple quarters within a cow occurred at a higher rate than expected based on the independence of quarters. This was thought to be due to within cow transmission of pathogens. In the present study, multiple quarters of the same cow were often simultaneously infected and, in approximately half of the cases, the same strain of *S. uberis* was detected in each quarter. Others

have also found that exposure to quarters infected with *S. uberis* was associated with an increased rate of infection in neighbouring uninfected quarters (Zadoks *et al.*, 2001b), and that multiple quarters were often found to be infected by the same strain type (Douglas *et al.*, 2000; Khan *et al.*, 2003; Zadoks *et al.*, 2003). In contrast, other studies have shown that a different strain was generally found between multiple infected quarters (McDougall *et al.*, 2004; Pullinger *et al.*, 2007). The infection of multiple quarters of the same cow with one strain of *S. uberis* suggests that either the onset of infection was simultaneous due to direct contact with the same environmental source, or that direct transmission from infected to uninfected quarters occurred. As most of these cases were actually subclinical mastitis it was not known when the infection began in each quarter, therefore it was difficult to differentiate between the different modes of infection. However, those cases where a different strain type was found in each quarter were more indicative of individual infection of each quarter through contact with a different source.

3.4.6.2 Incidence of infection in each udder quarter

When all infections were considered, the two rear quarters of the udder were more likely to be infected than the front two quarters, which has also been observed previously (Adkinson *et al.*, 1993; Batra *et al.*, 1977; Lancelot *et al.*, 1997; Zadoks *et al.*, 2001b). This suggests that the two rear quarters may have increased exposure to potential pathogens compared to the front two quarters. Lancelot *et al.* (1997) found that the only significant factor associated with distribution of mastitis between quarters was parity, with clinical mastitis more frequent in rear quarters in primiparous than multiparous cows. In contrast, Adkinson *et al.* (1993) found a higher proportion of clinical cases in the rear quarters with increasing parity. Therefore the influence of parity and other factors on the incidence of infection in front and rear quarters has yet to be described.

It has also been noted that the quarters on the right side of the udder had a higher rate of infection than the left quarters with mastitis pathogens in general (Barkema *et al.*, 1997; Zadoks *et al.*, 2001b), which may be associated with lying behaviour of cows (Ewbank, 1966). However, as in the present study, Adkinson *et al.* (1993) found little difference between quarters.

3.4.6.3 Recurrent or persistent infections of the same quarter

Some quarters were found to be subclinically or clinically infected on more than one occasion when sampled over a period of up to 37 weeks. Some of the time periods between infection events were quite short, possibly indicating continuous rather than recurrent infection, particularly when the quarter was infected at a subclinical level.

As clinical mastitis was usually detected and treated quickly, recurrent cases were more likely to be separate infection events rather than one continuous infection. The majority of cows with recurrent clinical mastitis infections (with at least one month between the infection events) had the same strain present on each date. Given the observed strain diversity of *S. uberis*, it was unlikely that the same quarter became infected on both occasions by the same strain; therefore, this strain may not have been completely cleared from the mammary gland after the first infection event. These results are in contrast to those of Pullinger *et al.* (2007), where infections first detected on different dates were usually associated with different MLST strain types.

The majority of cows with subclinical mastitis on two different dates up to 12 months apart were also infected with the same strain of *S. uberis*. Therefore, it was possible that each cow was persistently infected with the same strain at a subclinical level over this time period. Again however, the possibility of reinfection with the same strain could not be ruled out as samples were not collected between the two dates. These results are similar

to those obtained using other strain typing methods where the majority of long duration infections were actually continuous infection by the same strain rather than re-infection with a different strain, even between lactations (Oliver *et al.*, 1998b; Phuektes *et al.*, 2001; Pullinger *et al.*, 2007; Wieliczko *et al.*, 2002; Zadoks *et al.*, 2003). In contrast to these studies, McDougall *et al.* (2004) found that only 55% of mammary glands had the same strain isolated on more than one occasion, though this number did vary between herds.

For some of the cows infected at a subclinical level, clinical mastitis developed between 3 to 6 days later, or in some cases 1 to 11 months after the subclinical infection was detected. Of these, the same strain was isolated from both the subclinical and clinical infections in almost 75% of the cases. The most likely scenario was that the same strain persistently infected the mammary gland, resulting in subclinical infection, before an unknown event triggered a clinical episode. Again it was unlikely that the infection was cleared and that the same quarter became re-infected by the exact same strain at a later date.

As the same strain types were often isolated from both subclinical and clinical mastitis, it was most likely that the host response, rather than the virulence of particular strains, determined the severity of mastitis that developed in the mammary gland. While these results support those of other studies (Coffey *et al.*, 2006; McDougall *et al.*, 2004; Pullinger *et al.*, 2007), other researchers have observed differences between *S. uberis* isolates obtained from cows with clinical and subclinical mastitis (Jayarao *et al.*, 1993; Phuektes *et al.*, 2001), indicating that in some herds there were groups of *S. uberis* strains that were more likely to cause either clinical or subclinical mastitis.

3.4.6.4 Possible mechanisms of mammary gland re-infection

Although the location of *S. uberis* in quarters with recurrent clinical mastitis was not investigated, it was possible that *S. uberis* avoided elimination by persisting intracellularly within the mammary gland. *S. uberis* has been shown to adhere and internalize into mammary epithelial cells (Almeida *et al.*, 1996; Matthews *et al.*, 1994a; Thomas *et al.*, 1994), where they can survive for extended lengths of time (Tamilselvam *et al.*, 2006). Additionally, Denis *et al.* (2006) observed that *S. uberis* was able to replicate inside milk macrophages, which had low bactericidal activity against this pathogen *in vitro*. This access to the intracellular environment may provide protection from the immune system and allow viable microorganisms to be maintained that serve as a reservoir for persistent infections (Denis *et al.*, 2006; Tamilselvam *et al.*, 2006). In the present study, it was unknown if the persistent subclinical or recurrent clinical mastitis cases were due to adherence and internalization into the mammary epithelial cells or interaction with macrophages, but these mechanisms provide a plausible explanation for persistent or recurrent infections by the same strain.

An alternative explanation may be that the teat skin of infected cows becomes colonised with the infective strain after the first infection event, which provides a source of pathogens to reinfect the gland at a later date. This theory is supported by the observation that the infective strain was found exclusively on teat skin of the majority (80%) of clinically infected quarters sampled. However, subclinically infected quarters were not as likely to have the infective strain exclusively on the teat skin; therefore persistence within the mammary gland itself is a more likely scenario for subclinical infections that occur over long time periods.

Further sampling and strain typing of *S. uberis* isolates from the teat skin following an initial infection event may address whether colonisation of the teat skin is involved in recurrent or persistent mammary gland infection.

These experiments would determine if the infective strain remains exclusively on the teat skin of previously infected quarters over time, or whether other strain types would contaminate or colonise the area, making a secondary infection with the initial infective strain less likely.

3.4.6.5 Contagious transmission of infective strains

Some mastitis-causing strains were similar between different cows with thirteen different strains causing mastitis in more than one cow. Similar results were observed previously (Jayarao *et al.*, 1993; Khan *et al.*, 2003; Zadoks *et al.*, 2001a), indicating that contagious transmission may occur between cows in a herd. This transmission was most likely happening during the milking process as Zadoks et al (2001a) noted that *S. uberis* was detected in teat cup liners after milking of up to two uninfected cows following the milking of an infected cow. In another study, *Serratia marcescens* was infused into one teat cup of the first cow milked and was subsequently detected on the teat skin of four other cows milked with the same teat cup (Phillips, 1982). Alternatively, transmission of infective strains may occur less directly through contamination of the dairy, milker's hands or other areas of the environment through milk leaking (Zadoks *et al.*, 2001a). Flies have also been suggested as a mechanism of transmission (Zadoks *et al.*, 2001a), and they have been shown to transfer another mastitis pathogen, *S. aureus*, between cows (Owens *et al.*, 1998). However, evidence of spread of environmental pathogens in this manner has not been reported to date.

Contagious transmission, by whatever means of contact, is probably only likely for those infection events that were observed within a short period of time. Those mastitis cases that were detected months apart were more likely due to infection of quarters from the same environmental source, contaminated with this particular strain.

In the present study, no single strain appeared to predominate within the herd at any one time, as also observed by McDougall *et al.* (2004). The most frequent occurrence was a strain that appeared in the milk of five different cows with clinical and subclinical mastitis over a period of four months. Based on the diversity of strains found, especially those causing mastitis, it can be concluded that the environment was the main source of strains that infected the mammary gland. However, some contagious transmission of strains between cows may also have occurred along with transfer of strains between the teat skin of different cows. This strain transfer may have happened at any time through cow-to-cow contact, but was most likely occurring during the milking process. Therefore cow and milking hygiene are of high importance in minimizing both contagious transmission and the environmental load of *S. uberis*.

3.4.7 Common REP-PCR band patterns

Differences were observed in the presence of three common REP-PCR bands for strains from milk, teat skin, faeces and the environment. While all three bands were present in 83% of milk strains and 68% of environmental strains, only 34% of teat skin strains and 36% of faecal strains contained these bands. These differences in common REP-PCR bands may explain why little correlation was observed between strains from different sources. The percentage of strains with the three common REP-PCR bands may give an indication of which sets of strains were more closely-related. If so, the mastitis-causing strains on this farm were more closely-related to strains from the environment, particularly farm races, than those strains found either on teat skin or in faeces.

3.4.8 *Streptococcus uberis* strain diversity

Zadoks *et al.* (2005) suggested that high strain diversity may be due to bacterial multiplication in the environment, which leads to a high degree of variation in *S. uberis* strain types over time. Lateral transfer of genomic

DNA between strains may also account for high strain diversity, which could occur by transduction since *S. uberis* is not naturally competent but does carry phages (Hill & Brady, 1989). Coffey *et al.* (2006) suggested that substantial recombination had occurred in the evolutionary history of *S. uberis* and lateral acquisition of genes between and among streptococci pathogenic to humans has also been shown (Davies *et al.*, 2005); therefore, transfer of DNA between strains of *S. uberis* may be possible. Tettelin *et al.* (2005) noted a high genetic diversity between bacterial strains of *Streptococcus agalactiae*, where genome sequencing of six strains revealed that only approximately 80% of a single genome was shared between strains and the rest of the genome consisted of partially shared and strain-specific genes. Based on these results, it is highly likely that the *S. uberis* species also consists of many genetically diverse strains.

Slight differences in strain diversity were observed for isolates obtained from different sources. While 143 milk isolates were typed and only 41 different strains observed, 123 environmental isolates yielded 81 different strains. Therefore, there appears to be fewer unique strains causing infections compared to those found in the environment. Despite this, the use of rarefaction curves revealed that strain diversity between each source was actually very similar at the level of sampling obtained. However, the number of samples and isolates strain typed were not sufficient to capture a true estimate of the actual strain diversity. Based on the results of Dopfer *et al.* (2005), where approximately 20 isolates of *S. uberis* need to be typed to identify almost all strain types within a sample, the lower number of isolates typed in the present study would have revealed only a very small fraction of all possible strain types present in each sample.

Davies *et al.* (2005) found that the plasticity of the group G streptococcus genome, with its apparent ability to continually acquire genes from other related pathogenic species, blurred the epidemiological conclusions based

on molecular markers. Therefore similar studies of *S. uberis* may also encounter such problems due to apparent high strain diversity.

3.4.9 HasABC genotyping

Field *et al.* (2003) found that isolates without a hyaluronic acid capsule lacked the *hasA* gene, whereas *hasC* was ubiquitous. In the present study, *hasA* was found in all isolates tested, *hasB* in 92% of isolates and *hasC* in 84% of isolates. All three genes (*hasA, hasB* and *hasC*) are essential for the production of the hyaluronic acid capsule (Ward *et al.*, 2001); therefore it is likely that the isolates lacking *hasB* or *hasC* were acapsular, despite having the *hasA* gene.

There is some controversy over the involvement of the hyaluronic acid capsule in virulence of *S. uberis* (Field *et al.*, 2003; Hill, 1988; Ward *et al.*, 2001). Previous studies have shown that the hyaluronic acid capsule may not actually be required for virulence (Field *et al.*, 2003), and acapsulate bacteria lacking the *hasA* gene were isolated from milk of infected quarters (Coffey *et al.*, 2006; Pullinger *et al.*, 2006). Results of the present study support this with only 85% of strains isolated from the milk of clinically and subclinically infected quarters containing all three *has* genes; therefore, 15% were likely to be acapsular. However, in contrast to previous studies, the isolates would have been acapsular due to a lack of *hasB* or *hasC* genes rather than *hasA*. The 16 isolates sourced from the dairy environment had all three *has* genes present, therefore were capable of producing a capsule. These results differ from those of Field *et al.* (2003) where the *hasABC* genotype actually occurred at a higher frequency in isolates associated with disease rather than those from environmental sources.

Overall, despite only examining a small set of isolates (10% of all collected isolates) for *hasABC* genes, it was clear that any acapsular phenotype observed in these strains was more likely due to absence of *hasB* or *hasC*

genes, rather than *hasA*. Therefore, the analysis of all three genes is required to determine the ability of a strain to generate the hyaluronic acid capsule, unlike previous studies where detection of the *hasA* gene alone was used to designate potential capsular phenotype (Coffey *et al.*, 2006; Pullinger *et al.*, 2006). Also, these results support the conclusions of previous studies, that the hyaluronic acid capsule is not always necessary for the establishment of infection, as strains lacking the *hasABC* genes were also isolated from mastitis cases.

3.4.10 Conclusions

Due to lack of sampling around similar time points and a high recombination rate, problems with the reproducibility of REP-PCR strain typing, and the analysis of band patterns, it was difficult to draw any firm conclusions from this study; however, some generalisations could be made: Strains of *S. uberis* were identified as highly diverse and were found throughout the dairy environment and on the teat skin and other regions of the cow body. Some strain types were found in more than one location and it is impossible to rule out transmission of strains between the faeces, environment, teat skin and mastitis. Although no reservoirs of particularly virulent strains were apparent, the farm races were thought to be the most likely source of teat skin contamination resulting in mastitis. Although no obvious links were observed between faecal and infection-causing strains, the faeces were the most likely means of spreading *S. uberis* throughout the dairy environment. Also, if more samples had been collected then more clustering may have been observed.

The infective strain was often found on the teat skin of the infected quarter; however, there was very little correlation between teat skin strains and those causing mastitis in different cows. Although some transfer of strains may occur between the teats of different cows, the majority of teat strains appeared to be unique to each of the cows sampled. Presence of the

infective strain on the teat skin and in the teat canal was difficult to explain based on the results and could have been due to pre- or post-infection contamination or colonisation of the teat skin and teat canal.

Persistent and recurrent infection of the mammary gland by the same strain of *S. uberis* was also common, however further investigation is required to determine whether the strains persisted within the mammary gland over time or the mammary gland was re-infected at a later date with the same strain, perhaps due to colonisation of the teat skin of this quarter.

While poor correlation was observed between MLST and REP-PCR, the MLST results also showed that the same strain of *S. uberis* could be found in the dairy environment, on the teat skin and causing mastitis. Another strain type was observed both in the faeces of one cow, on the farm race and in paddock soil. Therefore, despite only a limited number of isolates being typed with this method, the results support those of REP-PCR in that strains of *S. uberis* could potentially be transferred from the environment on to cows and/or spread through the environment through the movement of contaminated cows.

3.4.11 Future work

The results of the present study have raised further questions regarding the mechanism of mammary gland infection and *S. uberis* ecology on the dairy farm. Overall, more strain typing is required to fully predict strain diversity and identify definite correlations between strains found on the cow and in the environment. Also, further investigation is required into possible enrichment and isolation methods to increase isolation of *S. uberis* from environmental and cow skin samples as, in the present study, it was possible that samples apparently negative for *S. uberis* using selective media isolation may actually have had *S. uberis* present.

A more focused approach may be required to trace the infective strains over longer periods of time. A study, whereby the same cows are followed over the course of a year (or lactation), with frequent and repeated sampling of teat skin, faeces and other areas of the body, would be most useful, especially if some of these cows develop mastitis (clinical or subclinical) during this time. Over the same time period, the farm races and paddocks, particularly after grazing, could also be monitored at regular intervals. In this way a better overall picture of strain transfer between each of these sites could be established. This study would also allow a comparison of *S. uberis* strains on the body of cows without mastitis, something that was not investigated in the present study, and also whether there is a seasonal variation in *S. uberis* numbers on the cow body. The introduction of a unique and recognisable 'tracer' strain into a small contained environment (small number of cows maintained in a small area) may also allow investigation into the transmission of strains between the environment and cows.

Colonisation of the gastrointestinal tract by *S. uberis* could be studied by strain typing isolates from the lips, rumen and rectum of cows shedding *S. uberis* in their faeces. This study could be run in conjunction with

environmental sampling, particularly of paddock grass, to determine if some strains of *S. uberis* are cycled from the environment, through the GI tract to the faeces.

Further study of the teat canals of uninfected and infected quarters is also required, as detailed strain typing was only performed on isolates from three infected quarters. Isolates from teat canals of uninfected quarters need to be strain typed to establish if multiple strains are present when the quarter is uninfected, compared to the findings obtained in infected quarters. Also, sampling of teat canals of uninfected quarters adjacent to those with mastitis would establish if these quarters were also contaminated or colonised by the same strain as that causing the infection. This study would provide further evidence as to whether multiple or single strains may be gaining entry into the mammary gland, leading to mastitis.

The examination of *S. uberis* on the teat skin of infected quarters also needs to be extended since the infective strain was often found exclusively on clinically infected quarters. Sampling the teat skin of the infected quarter every day of the infection and after the infection is cleared would determine if the infective strain remained on the teat skin over time, possibly resulting in re-infection of the mammary gland, or the time required for other strain types to re-appear on the teat skin.

The main disadvantage of these studies however, is the high number of samples generated and strain typing required. While the REP-PCR method of strain typing is rapid and inexpensive compared to other typing methods, this method needs to be further refined to be acceptable as a high-throughput analysis technique. In particular, it would be preferable if the secondary confirmation method, anchored typing, was not required as this addition to the method can be time-consuming.

In conclusion, further sampling and strain typing would aid in confirming the conclusions already drawn and shed more light on the transmission of strains from the environment, between cows and the mechanism of infection resulting in mastitis.

Chapter 4:

Mammary gland infection with multiple strains of *S. uberis*

Summary

Many different strains of *S. uberis* can be found throughout the dairy environment and on the cow itself; however, only one strain or at most two are usually found in the milk when clinical or subclinical mastitis is detected. This suggests that, at some stage of the infection process, a selection of this 'infective' strain must be occurring. Based on the strain diversity observed in the dairy environment and on the teat end it is unlikely that only one strain actually gains entry through the teat canal and into the mammary gland at any one time; therefore, this selection of the infective strain may be occurring within the mammary gland itself. To investigate this, five different strains of *S. uberis* were infused together into ten mammary quarters and the milk from these quarters monitored for each strain type until clinical mastitis developed. While all five strains were capable of causing mastitis when individually infused, when infused together into the mammary gland, only one strain could be detected in the majority of quarters by the end of the trial. The same strain did not always predominate however, indicating the unique effect of each cow on development of infection and selection of the predominant strain. The predominance of particular strains was thought to be due to an enhanced ability to evade the host immune response and, potentially, due to bacteriocin production, which inhibits the growth of other strains. However, *in vitro* studies showed no single strain predominating, indicating that the natural infection event resulted in a higher selection pressure than could be achieved during culture *in vitro*. In conclusion, this study showed that when multiple strains gain entry to the mammary gland, usually only one strain will predominate, which may explain why only one infective strain is usually identified when mastitis is detected.

4.1 Infection of the mammary gland

Despite many years of investigation into bovine mastitis, the mechanism of mammary gland infection by *S. uberis* remains poorly understood. The main theory that has persisted over time links *S. uberis* present on the teat skin with intramammary infections (Pullinger *et al.*, 2006; Sharma & Packer, 1970). In support of this, studies have shown that the incidence of mammary gland infection correlates with the number of pathogens present on the teat end (Neave *et al.*, 1966; Pankey, 1989). Also, *S. uberis* has been isolated simultaneously from the teat surface and the milk from the same quarter (Buddle *et al.*, 1988), indicating that strains present on the teats may be able to invade the mammary gland and cause infection.

The first barrier to infection is the teat canal and associated keratin (Lacy-Hulbert & Hillerton, 1995); then, once inside the mammary gland, survival depends on the ability of the pathogen to grow in milk, adhere to mammary epithelia and avoid the flushing effect of milk secretions, and resist the host immune response (Capuco *et al.*, 1992). Despite these host defence mechanisms, *S. uberis* is still capable of establishing infection in the bovine mammary gland, which is in part due to the extensive array of virulence factors produced by this invading pathogen (Almeida *et al.*, 1999a; Fang *et al.*, 2000; Leigh & Field, 1991; Leigh, 1994; Taylor *et al.*, 2003; Ward *et al.*, 2001).

Development of infection within the mammary gland can be monitored by the somatic cell count (SCC) and the electrical conductivity (EC) of milk. SCC provides an indication of the inflammatory response to an infection within the mammary gland (Schukken *et al.*, 2003) and is valuable for monitoring the progress of infection (Harmon, 1994). An elevated SCC is indicative of an increase in lymphocytes, macrophages and neutrophils in the milk. In addition to an increase in somatic cells, the onset of an infection

may result in damage to the mammary epithelium, leading to increased levels of sodium and chloride ions in the milk and an increase in milk electrical conductivity (Wheelock *et al.*, 1966). Therefore, elevated SCC, EC in addition to bacteria in the milk are clear indicators of mammary gland infection.

4.1.1 Selection of the infective strain

The environment is thought to be the most likely source of teat end contamination and infection by *S. uberis* (Douglas *et al.*, 2000; Zadoks *et al.*, 2003). However, despite the high diversity of strains found in the environment, *S. uberis* isolates from one infected quarter are generally all found to be the same strain type (Oliver *et al.*, 1998b; Phuektes *et al.*, 2001; Wieliczko *et al.*, 2002), although two strains can be found occasionally (Zadoks *et al.*, 2003). As it is unlikely that only one strain type would gain access to the mammary gland at any particular time, a selection of one predominant strain must be occurring during the infection process. This strain selection process may be occurring on the teat skin or in the teat canal, thereby only one strain gains entry to the mammary gland. Alternatively, multiple strains may gain entry and then selection occurs within the mammary gland itself to result in one or sometimes two infective strains.

4.1.1.1 Selection of the infective strain on teat skin

In Chapter 3 it was noted that the majority of all the infected quarters studied had the infective strain present almost exclusively on the teat skin, while the teats of uninfected quarters generally had multiple strain types present. This suggested that one strain may have been able to out-compete other strains residing on the teat skin, but it was unknown whether the prevalence of this infective strain on the teat skin occurred before the onset of infection or was due to high levels of teat skin contamination by milk from the infected mammary gland.

It was also found that the same strain could be isolated both from the milk and the teat canals of clinically and subclinically infected cows. However, it is unknown if these strains within the teat canal indicated an ascending infection of a single strain or contamination of the teat canal after infection of the mammary gland. It has previously been noted that *S. uberis* does not appear to colonize or multiply within the teat canal (King, 1981); therefore in the present study it may have been more likely that the teat canals were actually contaminated with the infective strain present in the milk.

Although very little research has been carried out between the interactions of different strains of *S. uberis* on the teat skin, other studies have investigated interactions between different bacterial species present at this location. De Vleigher *et al.* (2004) found that natural bacterial flora on the teat skin influenced the somatic cell count in that animal and heifer quarters' colonised with *Staphylococcus chromogenes* (commonly found on the teat ends and in the teat canal of mammary quarters (Matthews *et al.*, 1992)) were less likely to have an elevated somatic cell count during the first five days of lactation. These *S. chromogenes* isolates showed inhibitory activity against all *Staphylococcus aureus*, *Streptococcus dysgalactiae* and *S. uberis* isolates that were tested *in vitro* (De Vliegher *et al.*, 2004). Additionally, Green *et al.* (2005) found that the risk of isolating *S. uberis* from the mammary gland was lower when *Corynebacterium* species were also isolated during the dry period, indicating that these minor pathogens may influence infection of the mammary gland by *S. uberis*. In contrast, an earlier study noted that, of the commonly studied mastitis pathogens, *S. uberis* was the least inhibited by normal flora isolates from the teat skin (Woodward *et al.*, 1987). However, it is thought that maintaining or inducing a protective flora on the teat skin may be a successful mastitis prevention strategy (De Vliegher *et al.*, 2004; Woodward *et al.*, 1987; Woodward *et al.*, 1988). If bacterial interactions on the teat skin and within the mammary gland are occurring at the species level, it is also possible that

there may be interaction and competition between different strains of *S. uberis*.

4.1.1.2 Selection of the infective strain in the mammary gland

Selection of the infective strain may be occurring within the mammary gland itself. Multiple strains of *S. uberis* present on the teat skin may gain access through the teat canal, then, once inside the mammary gland, a selection process may result in one strain prevailing. This dominant strain would be that which is detected in the milk when mastitis is confirmed. The predominance of one particular strain within the mammary gland may be due to a superior array of virulence factors that enhance the ability of one strain to grow under conditions found within the gland and allow evasion of the host immune response. Alternatively, production of antibacterial factors by some strains may inhibit the growth of other strains of *S. uberis*.

4.1.1.3 Virulence factors of *S. uberis*

Different virulence factors produced by *S. uberis* are thought to facilitate establishment of bacteria in the host through mechanisms such as enhanced nutrient up-take and growth, adherence of the bacteria to cells in the mammary gland, invasion of the mammary tissue and providing protection against the immune response. These factors are reviewed more fully in Chapter 1.

As the mammary gland environment is deficient of free and peptide-associated amino acids (Aston, 1975), *S. uberis* requires specific enzymes such as plasminogen activator uberis A (PauA) and Opp proteins (Smith *et al.*, 2003; Taylor *et al.*, 2003) to release and take up essential amino acids from casein (Leigh, 1993; Leigh, 1994; Leigh & Lincoln, 1997). However, not all strains of *S. uberis* that cause intramammary infections contain the PauA gene, indicating that this enzyme is not essential for growth in milk (Ward & Leigh, 2002).

After invasion of the mammary gland, adherence of *S. uberis* to secretory and ductular epithelia has been observed (Thomas *et al.*, 1994). Adherence of bacteria to the host tissue is an important virulence mechanism. In the case of the mammary gland, adherence needs to be strong enough to resist the flushing effect of the milk secretions and also specific enough that the bacteria have a source of nutrients (Oliver *et al.*, 1998a). Many *in vitro* studies of adherence and internalisation of *S. uberis* into mammary epithelial cells have been conducted (Almeida *et al.*, 1999a; Almeida *et al.*, 1999b; Almeida & Oliver, 2001; Fang *et al.*, 2000), and several *S. uberis* proteins have been identified that mediate this, including lactoferrin binding protein (Almeida *et al.*, 2006; Fang & Oliver, 1999; Moshynskyy *et al.*, 2003) and proteins that bind to host proteoglycans (Almeida *et al.*, 1999a). Once internalised into mammary epithelial cells, *S. uberis* may have evolved mechanisms to survive intracellularly and maintain low levels of viable bacterial cells that are protected from host defence mechanisms and may serve as a reservoir for persistent infections (Matthews *et al.*, 1994a; Tamilselvam *et al.*, 2006).

Another potential virulence factor that may aid resistance to phagocytosis is the hyaluronic acid capsule. Although conflicting results have been obtained regarding protection provided by this polysaccharide capsule, capsular strains of *S. uberis* were more resistant to killing by neutrophils (Ward *et al.*, 2001) and showed increased infectivity with experimental challenge of the mammary gland (Hill, 1988). There may also be other, as yet unknown factors, that also contribute to resistance to phagocytosis by neutrophils (Field *et al.*, 2003).

Thomas *et al.* (1994) noted that macrophages were present in high numbers within the mammary gland after experimental infection with *S. uberis*; however, Denis *et al.* (2006) observed that macrophages had low bactericidal activity against this pathogen *in vitro* and *S. uberis* could

actually replicate inside these macrophages. Therefore internalisation into mammary epithelial cells and/or macrophages may provide protection and allow viable microorganisms to be maintained (Denis *et al.*, 2006; Tamilselvam *et al.*, 2006).

In summary, many virulence factors of *S. uberis* have been investigated for their importance in the infection of the mammary gland, with contrasting results obtained by different groups. Also, many more virulence factors are likely to exist that have not yet been discovered. Thus, there are few consistent theories as to the methods of *S. uberis* establishment in the mammary gland and evasion of host immune defence systems.

4.1.1.4 Bacteriocins

Direct competition between different strains of *S. uberis* may also aid predominance of some strains over others. This competition could be facilitated through the production of bacteriocins; small (<10 kDa) antimicrobial peptides produced by many lactic acid bacteria, including *S. uberis,* which typically kill other bacteria of the same or closely-related species (Tagg *et al.*, 1976).

The two most recently discovered bacteriocins in *S. uberis* are nisin U (Wirawan *et al.*, 2006) and uberolysin (Wirawan *et al.*, 2007). Nisin U was characterized as a novel variant of nisin, which belongs to the lantibiotic class of bacteriocins (Guder *et al.*, 2000). The lantibiotics are ribosomally synthesised, heat stable peptides characterized by their content of post-translationally modified amino acids, including lanthionine and/or β-methyl-lanthionine (McAuliffe *et al.*, 2001). Lantibiotic loci typically comprise a structural gene that encodes the actual bacteriocin peptide and other essential genes that encode proteins responsible for post-translational modification of the prepeptide, proteolytic processing, transport, producer self-protection, and regulation of biosynthesis (Guder *et al.*, 2000; McAuliffe

et al., 2001). The elimination of any of these genes may result in the lack of functional nisin U production or producer immunity.

Uberolysin, a bacteriolytic compound, has also been isolated from some strains of *S. uberis* and characterized as a novel member of the circular bacteriocins (Wirawan *et al.*, 2007). Despite bearing no primary sequence similarity to any of the currently known circular bacteriocins, it does share the defining characteristic of head-to-tail linkage of its N- and C-terminal amino acids. As with the lantibiotic class of bacteriocins, many other genes are also required for uberolysin production in addition to the structural gene itself. One of these genes produces a designated immunity peptide, although the mode of action that confers immunity has not yet been elucidated (Wirawan *et al.*, 2007).

4.1.2 Infection of the mammary gland by *S. uberis*

Very little information is available on the dynamics of growth, establishment and infection during the development of mastitis. However, it is clear that while there is high strain diversity within the environment and often on the teat skin of cows', only one or occasionally two strains are actually recovered from the mammary quarter during a case of clinical or subclinical mastitis. Therefore, at some stage of the infection process a selection of the infective strain must take place. It is possible that this selection could be occurring on the teat skin, as studies have shown that bacterial interactions at the species levels affect colonization of the teat skin. However, this selection process may also take place within the mammary gland, with multiple strains gaining entry and one strain predominating to become the infective strain. This selection process may be facilitated by differences in the expression of virulence factors between strains that allow enhanced growth in milk, adherence to mammary epithelia and, importantly, evasion of the host immune response. Additionally, some

strains may produce bacteriocins that inhibit the growth of or kill other strains that may be present.

4.1.3 Aims

To our knowledge, there have not been any studies published on the infusion of more than one strain of *S. uberis* into the mammary gland. Therefore, the overall aim of this study was to determine the outcome of challenging the mammary gland with a mixture of multiple strains of *S. uberis*.

Specific aims included:

i) Determine the ability of five different strains of *S. uberis*, isolated from clinical mastitis and the dairy environment, to infect the mammary gland when infused individually into mammary quarters.
Hypothesis: Each strain will cause clinical mastitis in the infused quarter

ii) Determine the ability of the five strains to infect the mammary gland when infused as a mixture into mammary quarters.
Hypothesis: One strain will predominate over all others

iii) Investigate potential competition between the five strains while culturing *in vitro* in sterile skimmed milk.

iv) Examine the production of two bacteriocins, uberolysin and nisin U, by these five strains.

4.2 Materials and Methods

4.2.1 Selection of five *S. uberis* strains

Five *S. uberis* strains were selected from a collection of 253 isolates from Lye farm that had previously been typed by MLST (Table 4.1). The set of five strains included three from different clinical infections and two isolated from the dairy farm environment (farm race and paddock soil); all with a different MLST type but from the same clonal complex. The five strains were labelled A-E.

Assigned code	Isolate source	Cow #	Mastitis	Isolation date	MLST
A	Milk	9677	CM	2 Jul 03	80
B	Race	-	-	21 Nov 03	165
C	Milk	9123	CM	19Aug 04	207
D	Soil	-	-	26 Nov 03	167
E	Milk	9676	CM	22 Aug 03	142

Table 4.1. Five *S. uberis* strains selected for experiments.
CM – clinical mastitis.

REP-PCR and anchored typing (as described in Chapter 3.2.2) were performed on strains A-E to ensure that they could be differentiated by their band patterns once mixed together. Additionally, the presence of three genes (*hasA*, *hasB* and *hasC*) required for the production of a hyaluronic acid capsule was also assessed for all five strains, using the methods given in Chapter 3.2.5.

4.2.1.1 Ability of the five strains to grow in milk

To test the ability of the five chosen strains to grow in milk, individual vials of 5 ml sterile ultra-heat treated (UHT) skimmed milk were inoculated with 250 µl of an 18 h Brain Heart Infusion (BHI) broth culture of each strain. All milk cultures were incubated at 37°C with shaking (180 rpm) for 20 h at

which time a sample was taken, serial tenfold dilutions made to 10^{-5} in sterile UHT skimmed milk and 100 µl spread plated on Esculin Blood Agar (EBA; Fort Richard Laboratories, Ltd., Otahuhu, NZ).

4.2.2 Five strain intramammary challenge trial

4.2.2.1 Trial outline

Ten Friesian-Jersey-cross lactating cows of mixed age were randomly selected. Of these, only one cow had a recorded case of mastitis prior to the trial (cow #1041, CM, July 2005), but it is unknown if this was due to *S. uberis* or some other pathogen. In general cows were treated as for the rest of the herd except that they were separated from the rest of the herd 24 h before commencing infusions, and kept in this mob for the duration of the trial. General farm management practices are described in Appendix 1.

Milk samples were collected at two milkings before infusion (M-7, M-1), directly before infusion (M0) and at morning and afternoon milkings for three days post-infusion (M1-5) (Table 4.2). The foremilk electrical conductivity (EC) and somatic cell count (SCC), presence of bacteria and evidence of clinical mastitis were assessed throughout the trial. Antibiotic treatment commenced at the fifth milking (M5) when the majority of infused quarters showed signs of clinical infection. No milk samples were collected during antibiotic treatment until follow up bacteriology was performed 36 days after infusion.

Ethical approval for this experiment was obtained from the Ruakura Animal Ethics Committee (RAEC # 10837). The infection trial was conducted in early April, which corresponded to late lactation in these dairy cows.

			Milk tests			Clinical signs assessed	Antibiotic treatment
	Trial day	**Milking #**	**Bact.**	**EC**	**SCC**		
Pre-trial	-3	**M -7**	Y		Y		
	0	**M -1**	Y	Y	Y	Y	
Trial		**M 0 ***	Y	Y	Y	Y	
	+1	**M 1**	Y	Y	Y	Y	
		M 2	Y	Y	Y	Y	
	+2	**M 3**	Y	Y	Y	Y	
		M 4	Y	Y	Y	Y	
	+3	**M 5**	Y	Y	Y	Y	Y
Post-trial		**M 6**				Y	Y
	+4	**M 7**				Y	
		M 8				Y	Y
	+6	**M 12**					Y
	+8	**M 16**					Y
	+36	**M 72**	Y				

Table 4.2. Trial outline.
Milk samples collected before, during and after the trial were tested for bacteria (Bact.), somatic cells (SCC), electrical conductivity (EC) and signs of clinical mastitis as indicated (Y). Infusion of quarters was carried out at milking 0 (*).

4.2.2.2 Preparation of cultures for infusion of mammary glands

Five BHI broths (10 ml) were inoculated with a single colony of strains A-E from BHI agar, and incubated for 20 h at 37°C before serial tenfold dilutions were prepared to 10^{-7} in sterile UHT skimmed milk (stock dilutions). The 10^{-3} to 10^{-7} dilutions were spread plated (100 µl) on EBA plates in duplicate and incubated at 37°C for 48 hours. Broths and dilutions were stored at 4°C during this time. The colony forming units per ml (cfu/ml) of each stock dilution was calculated and used to prepare inocula.

The aim was to obtain approximately 1250-1500 cfu/ml for each individual strain inoculum and 250-300 cfu/ml of each of the five strains in the mixed culture (SU5) inoculum. A high inoculum was chosen as cows were infused during late lactation and were less susceptible to developing an infection. Each individual strain inoculum was prepared to a total volume of at least

10 ml by diluting in sterile UHT skimmed milk and the SU5 mixture was prepared by mixing 2.5 ml of each individual strain inoculum. A 1 ml volume of each prepared inoculum was aseptically aspirated into a 1 ml sterile disposable syringe and capped with an 18G blunt-ended teat infusion cannula (J-12; Jorgensen Laboratories Inc., Loveland, Colorado, USA). Four syringes were prepared for each individual strain and 10 syringes of SU5 inoculum. Extra syringes were prepared for each inocula and used to confirm bacterial numbers by plating on EBA upon return to the laboratory. All syringes were incubated at 37°C for 1 h to allow acclimatisation directly prior to infusion into mammary quarters.

4.2.2.3 Infusion technique

Each mammary quarter was challenged by infusion through the teat canal, immediately following the afternoon milking. After cluster removal, the teat end was scrubbed with cotton wool soaked in 70% ethanol and dried. The tip of the cannula was gently inserted into the teat orifice and the entire 1 ml inoculum expelled into the teat canal. The teat was then massaged to ensure dispersal of the inoculum.

The mixed strain inoculum (SU5) was infused into 10 quarters total, either one front or one back quarter of each cow (Table 4.3). Two different single strain inocula were administered to either both front or both back quarters of each cow, so in total, each strain was administered to four quarters across four cows. The fourth quarter of each cow, including the blind quarter in cow #2208, was not infused.

Cow #	Inoculum delivered to each mammary quarter	
	LF	**RF**
	LB	**RB**
145	A	B
	-	SU5
857	C	A
	-	SU5
939	-	SU5
	B	D
1041	B	C
	SU5	-
1647	-	SU5
	C	E
2058	-	SU5
	D	C
2202	E	B
	-	SU5
2208	(BQ)	SU5
	A	D
3218	-	SU5
	E	A
3219	D	E
	-	SU5

Table 4.3. Infusion schedule for strains A-E and the five strain mixture (SU5). Control quarters (-) of each cow were not infused. BQ – blind quarter.

4.2.2.4 Milk sampling and analysis

Milk samples were collected both morning and afternoon for five milkings after infusion until quarters showed significant clinical signs and required antibiotic treatment. Foremilk was stripped to check electrical conductivity (EC) using a hand-held digital mastitis detector (Technipharm, Rotorua, New Zealand), and to perform clinical assessment of the milk and udder (section 4.2.2.5). Teats were then scrubbed with cotton wool soaked in 70% ethanol and samples of milk collected for bacteriological analysis, somatic cell counting and strain typing. Cows were then milked as normal. Milk

samples for viable cell plate count and strain typing were stored at -20°C before analysis.

Bacteriological analysis was conducted by plating 10 µl of each sample on one quarter of an EBA plate with a sterile loop. Together with EC measurement, these tests allowed a rapid assessment of the health of each quarter and to follow any infections as they progressed. Somatic cell count was determined using a fluorometric cell counting technique (Fossomatic, Foss Electric, Hillerød, Denmark; operated by LIC, Hamilton, New Zealand).

(i) Bacterial plate count

Milk samples for bacterial plate count and strain typing were thawed and tenfold serial dilutions performed to 10^{-6} in 0.1% peptone (Fort Richard Laboratories, Ltd., Otahuhu, NZ). Each dilution (100 µl) was spread plated on EBA, incubated at 37°C for 48 h and the number of colony forming units enumerated. For milk samples from SU5-infused quarters the number of golden yellow-coloured (corresponding to strain C) and white colonies (strain A, B, D, and E) on the plate were counted individually along with the total number of colony forming units. The total number of cfu/ml of milk was calculated and these results $\log_{10}$ transformed before further analysis.

(ii) Colony selection, DNA isolation and strain typing

Between two and five colonies were selected for strain typing from quarters infused with individual strains A-E. For quarters infused with SU5, 20-30 colonies were chosen to identify all strain types within the sample, based on the recommendations of Dopfer *et al.* (2005). Strain C was easily identified due to a different colony morphology (golden yellow colour). Therefore, only 5-10 yellow colonies were chosen from each sample to confirm as

strain C and 15-20 white colonies to identify as strain A, B, D or E by REP-PCR and anchored typing.

DNA was isolated from all selected colonies using the alkaline polyethylene glycol (PEG)-based method of Chomczynski and Rymaszewski (2006). Briefly, a single bacterial colony was mixed with 25 µl of an alkaline PEG solution which consisted of 60% PEG 200 (Sigma-Aldrich NZ, Ltd., Auckland, NZ) and 20 mM KOH and had a pH of 13.4. This mixture was incubated at 95°C for 10 minutes to lyse the cells and then mixed thoroughly by flicking the tube. The crude DNA preparations were diluted with TE buffer to obtain approximately similar levels of nucleic acid concentration as measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Strain typing was performed using both REP-PCR and anchored typing reactions as described in Chapter 3.2.2.

(iii) Calculations and statistical analysis

At each milking, treatment comparisons were carried out in GenStat (9th edition, VSN International Ltd, Hemel Hempstead, UK) for SCC and EC using linear mixed models that took account of the experimental design, animal and quarter effects and the repeated measures nature of the data. Specific comparisons of interest were evaluated by using a nested treatment model. For SCC, examination of residuals showed that $\log_{10}$ transformed counts could be safely compared using a normal distribution assumption.

Trends in bacterial numbers over milkings 1-5 were calculated for each cow for strains A-E and SU5 separately. T-tests were performed on slopes to determine if the trend significantly departed from zero.

Band patterns from REP-PCR and anchored typing reactions were identified as those of strains A-E and the percentage of each strain type present calculated.

To analyse the predominance of one strain; firstly, confidence intervals were calculated for the proportion of cows having a mixture of strains (Clopper & Pearson, 1934; Owen, 1962). Secondly, a Chi-square test was performed to test if the dominant strain occurred by chance. This was carried out using maximum likelihood by fitting a generalized linear model with a log link and a Poisson distribution using GenStat (10.1 edition, VSN International Ltd, Hemel Hempstead, UK).

4.2.2.5 Diagnosis of infection and antibiotic therapy

Intramammary infections were diagnosed when the SCC of quarter foremilk samples was >2.0x10^5 cells/ml and bacteria were detected in the milk and/or clinical signs were apparent. Diagnosis of clinical infections was primarily by cow-side assessment of the milk appearance and status of the udder and assisted by measurements of electrical conductivity. Clinical signs included: heat, pain and/or swelling of the udder, clots and/or discolouration of the milk. The degree of milk clotting was classified on a 4 point scale:

C1 = 1-3 small clots or flakes in the sample.

C2 = 1 large clot or more than 3 clots, affecting up to 5% of the sample.

C3 = 5-30% of secretion contains clots.

C4 = Secretion is mostly clots.

Antibiotic treatment began when signs of clinical mastitis were reasonably well established, but no more than 48 h after appearance of the first clinical signs. All glands were treated by intramammary infusion with Orbenin LA (Pfizer Animal Health, Auckland, New Zealand). In total, five tubes of

antibiotic were used per quarter, with the first two tubes given at the fifth and sixth milking after infusion and subsequent tubes administered after 24 h then at 48 h intervals. All five *S. uberis* strains had shown sensitivity to this antibiotic *in vitro* prior to the trial. Six cows showed systemic signs of inflammation (i.e. elevated temperature, loss of appetite, high pain) and were administered a non steroidal anti-inflammatory drug (Metacam; Boehringer-Ingelheim GmbH, Ingelheim, Germany).

4.2.3 Growth of the five strain mixture *in vitro*

4.2.3.1 Experiment 1

Individual 24 h BHI broth cultures of the five strains A-E were used to inoculate 20 ml of UHT skimmed milk to obtain approximately 300 cfu/ml of each strain. Three replicates were prepared and, after mixing, a 0.5 ml sample was immediately taken from each replicate mixture (time 0) before incubating at 37°C, 100 rpm. After 74 h of incubation, samples were collected again and stored at -20°C before analysis.

4.2.3.2 Experiment 2

Similar to experiment 1 except that the initial BHI broth cultures were incubated for 5 h and only 10 ml of UHT skimmed milk was inoculated to give 300 cfu/ml of each of the five strains. Two replicates were prepared and 0.5 ml of each initial mixture removed (time 0). As for experiment 1, the mixtures were incubated at 37°C and 100 rpm before further sample collection after 74 h.

4.2.3.3 Colony selection and strain typing

For bacterial plate counts, 100 µl of undiluted time 0 samples and a 10^{-6} dilution of 74 h samples (in 0.1% peptone) were spread plated on EBA. Plates were incubated for 48 h before colony forming units were counted and 16-25 colonies selected for strain typing. Strain typing by REP-PCR and anchored typing was performed as described in Chapter 3.2.2.

4.2.4 Analysis of bacteriocin production

4.2.4.1 Deferred antagonism

Cultures of strains A-E were prepared by streaking a single colony of strains A-E on EBA and incubating at 37°C for 48 h. A sterile cotton-tipped swab was charged with growth and used to inoculate a single diametric streak (~1 cm wide) across the surface of five EBA plates (1 strain/plate – test strains). Also, using a sterile loop, individual BHI broths were inoculated with a single colony from each of the five strains before incubating, along with the prepared plates, at 37°C for 18 h.

All visible growth of test strains on the EBA plates was removed by scraping with the edge of a glass microscope slide and wiping over the area with a cotton-tipped swab. The surface of each plate was sterilised by inverting over a circle of chloroform-soaked dish cloth for approximately 30 min. Then, after air drying plates for at least 30 min, each of the five 18 h BHI broth cultures were cross-streaked across the surface of the sterilised blood agar plate at right angles to the original line of the test strain growth using a cotton-tipped swab (indicator strains; Figure 4.1). After incubation at 37°C for 18-24 h, each plate was scored for absence of indicator strain growth across the area originally occupied by the test strain.

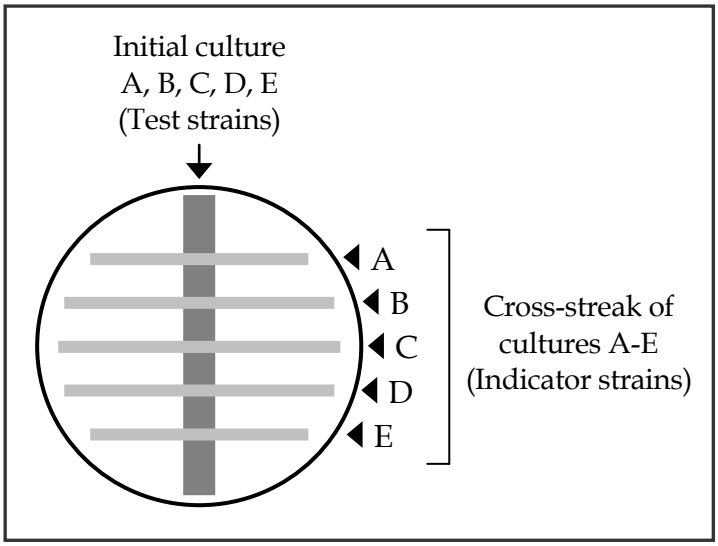


Figure 4.1. Illustration of deferred antagonism plate after cross-streaking. Initial cultures of strains A, B, C, D, or E (test strains) were grown in one line on each plate before removing and sterilising the plate surface. The five strains A-E (indicator strains) were cross-streaked across the line of initial test strain growth.

Absence of growth provided evidence that the test strain was producing an inhibitory substance. The production of an inhibitory substance by the test strain was scored by measuring the clearance where each indicator strain was unable to grow:

0 = No inhibition, indicator strains grew across test strain line
2 = Inhibition, ~2 cm clear across the test strain line
3 = Inhibition, ~3 cm clear across the test strain line
R = Resistant colonies – some individual colonies observed but the majority of growth inhibited across the test strain line.

4.2.4.2 Identification of bacteriocins in the five strains A-E

The five strains A-E were also sent to the Department of Microbiology and Immunology, University of Otago, for identification of bacteriocins by producer typing (P-typing) as described in Tagg and Bannister (1979). The P-typing scheme uses nine standard indicator species and points are assigned when the indicator is inhibited (using the deferred antagonism method described above) and a triplet code assigned (Table 4.4). Media both with and without blood was used for the analyses. When all

indicators are inhibited, a P-type of 777 is given, whereas no inhibition results in a 000 P-type.

Group	Code	Indicator species and strain	Points
1	I1	*Micrococcus luteus,* T-18	4
	I2	*Streptococcus pyogenes,* FF22	2
	I3	*Streptococcus anginosus,* T-29	1
2	I4	*S. uberis,* ATCC 27958	4
	I5	*S. pyogenes,* 71-679	2
	I6	*Lactococcus lactis,* T-21	1
3	I7	*S. pyogenes,* 71-698	4
	I8	*S. pyogenes,* W-1	2
	I9	*Streptococcus equisimilis,* T-148	1

Table 4.4. Nine standard indicators used for P-typing.
Points are assigned when the indicator species is inhibited by the test strain and summed for each of the groups to obtain a triplet code.

PCR was also performed by this same group to determine the presence of structural genes for two known *S. uberis* bacteriocins, uberolysin and nisin U. The oligonucleotide primers are listed in Table 4.5. PCR reactions were performed as described in Wirawan *et al.* (2007) for the uberolysin gene and Wirawan *et al.* (2006) for the nisin U structural gene.

Bacteriocin	Primer	Primer sequence (5′-3′)	Product size
Uberolysin	ublyF	GGGATTTAACATGGACATTTTATTAG	~300 bp
	ublyR	AGCTCCTTTATCAAATTATTAGTCG	
Nisin U	nsuA-F	GGATCTCATCAAAATCTCAAAGG	~130 bp
	nsuA-R	CACAGGTTGCAGTTTTTAGTGGAC	

Table 4.5. Primers for amplification of uberolysin and nisin U structural genes.

4.2.4.3 Sequencing of the nisin U gene locus

To fully characterise the presence and position of genes required for full nisin U production and immunity, the entire 15,653 bp locus consisting of 11 genes was sequenced and compared to the published *S. uberis* nisin U gene locus (GenBank accession DQ146939).

(i) PCR

The 11 different oligonucleotide primers (Table 4.6) directed toward overlapping regions of the nisin U locus were obtained from the University of Otago and reconstituted in DNA-free MQ H_2O. They were used in the following combinations:

a) nsu1 + nsu2
b) nsu3 + nsu2
c) nsu4 + nsu5
d) nsu6 + nsu7
e) nsu8 + nsu9
f) nsu10 + nsu11

Primer	Sequence (5′-3′)
nsu1	CAAGTAAATGGAACTGTACAAGATA
nsu2	TCAGTCCCCTCAGGCGTTTTG
nsu3	AATCGGATGCATTATTTCGTTCAA
nsu4	GTCTTTTCTCCGGTATGATTAC
nsu5	CAACAAATACTACTAGTAATGAGC
nsu6	GAAATCGAAAGATAGGGGGATTAGAAAG
nsu7	CTTTCTCGGATAAATCAACAATG
nsu8	CTTCAAATTAAATTTCTTAACCGC
nsu9	TTGCTAATAACTTTATGGGTCT
nsu10	GGATCTCATCAAAATCTCAAAGG
nsu11	CTACAAGGTGTGAATCAAAAATAC

Table 4.6. Primers for sequencing of the nisin U locus

For 50 µl PCR reactions, master-mix B was used (Chapter 2.2.5.2) together with each primer at a concentration of 0.2 µM and 1.25 U HotMaster *Taq* DNA polymerase (Eppendorf AG, Hamburg, Germany). To each PCR reaction, approximately 300 ng of DNA was added. Positive and negative controls were always included with the positive control DNA (obtained from University of Otago) extracted from a strain known to produce nisin U. After an initial activation step of 94°C for 2 minutes, amplification conditions involved a touchdown PCR of 30 cycles including five cycles of 94°C for 20 s, 65°C for 20 s and 68°C for 2 min. The next set of 15 cycles had the same denaturation and extension settings; however, the annealing temperature decreased by 0.6°C/cycle. The final set of cycling parameters involved ten cycles of denaturation (same as previous) and annealing at 55°C for 20 s followed by extension (same as previous).

(ii) Purification of PCR products

Success of the PCR was confirmed by electrophoresis of 5µl PCR product in a 1% agarose gel. Once confirmed, the remaining PCR products were precipitated using the polyethylene glycol method of Schmitz and Riesner (2006). As the PCR products were all >900 bp the method used was as follows: To the 45 µl remaining PCR products, 1.26 µl 500 mM EDTA, 10.8 µl 50% PEG_{6000}, and 6.3 µl 5M NaCl were added. The mixture was then incubated for 10 min at room temperature before centrifuging 16,000 x g, 10 min and the resulting precipitate washed in 125 µl of 70% ethanol. After removing all traces of ethanol, the pellet was resuspended in 25 µl 5 mM Tris-HCl and the nucleic acid concentration measured on a Nanodrop ND-1000 spectrophotometer.

(iii) Sequencing of PCR products and analysis

Sequencing of purified PCR products was conducted by the University of Waikato DNA sequencing facility with reactions performed using both the forward and reverse primers for each PCR product. Resulting sequences

were compared to the published *S. uberis* nisin U gene locus (GenBank accession DQ146939) using ClustalW multiple sequence alignment (www.ebi.ac.uk/clustalW).

4.3 Results

4.3.1 Characterization of selected *S. uberis* strains

Five strains of *S. uberis* were selected for use in the intramammary challenge experiments and *in vitro* investigations, including three strains from different clinical mastitis cases, one strain from a farm race and another isolated from the paddock soil of the same farm.

4.3.1.1 REP-PCR and anchored typing

Both REP-PCR and anchored typing reactions were required to discriminate between the five different strain types. With REP-PCR, very similar band patterns were observed for strains B, C and D; while similar patterns were obtained for strains A and B with anchored typing (Figure 4.2). However, the combination of both typing methods allowed clear differentiation between all five strains. Discrimination between strains was important to allow identification of strain types once the mixture of five strains was infused into the mammary gland.

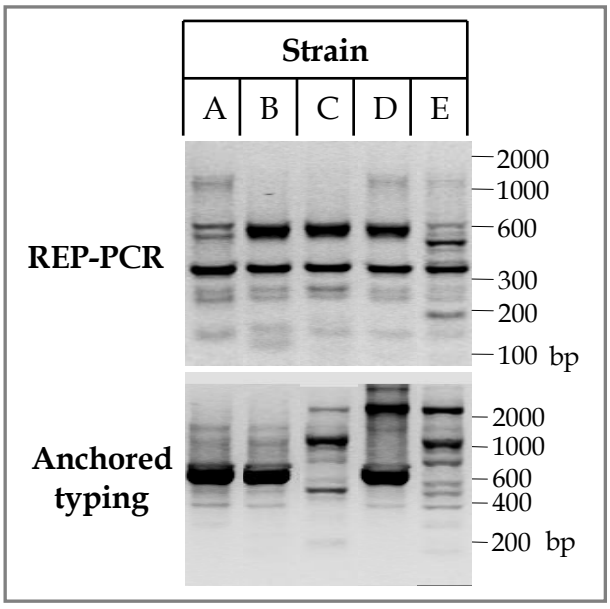


Figure 4.2. REP-PCR and anchored typing band patterns for strains A-E. REP-PCR and anchored typing reactions were performed on DNA extracted from strains A-E. PCR products (10 µl) were electrophoresed in 1.5% agarose gels and band patterns aligned using GelComparII. A 100 bp DNA ladder (Invitrogen) was run on each gel and the relative positions of bands indicated to the right of the gel photos.

4.3.1.2 HasABC genotype

All five strains gave a positive result when DNA was amplified with primers directed toward the *hasA*, *hasB* and *hasC* genes (Figure 4.3), indicating that strains A-E contained the genes required for production of a hyaluronic acid capsule, however it is unknown if these genes were expressed in these strains to result in a capsular phenotype.

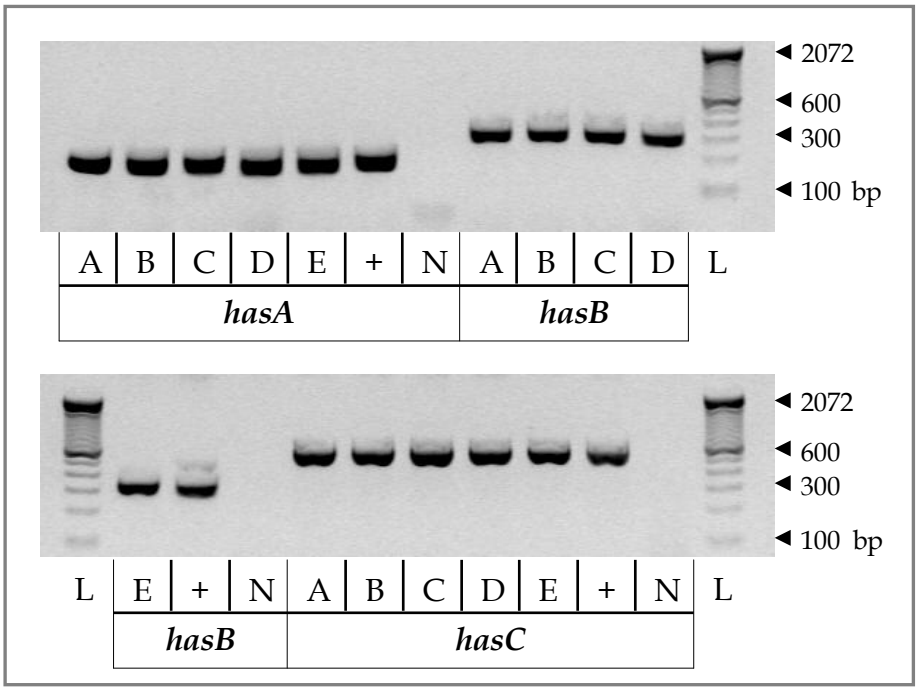


Figure 4.3. Presence of *hasABC* genes in the five strains.
DNA from strains A-E and a positive control, *S. uberis* strain 0140J (+), was amplified with primers directed toward the *hasA*, *hasB*, or *hasC* genes. Negative controls (no DNA added to PCR reaction; N) were included with each set of primers. A 100 bp ladder (L; Invitrogen) was included on each gel and the size of some bands noted.

4.3.1.3 Colony morphology on esculin blood agar

When cultured on EBA, differences were observed between the five strains in terms of colony morphology (Figure 4.4). Strains A, B and D were fairly similar, generating white, circular colonies that were smooth and umbonate when viewed from the side. Strain C was distinct, producing a larger sized, circular colony, which was flat, shiny and golden in colour. These colonies also tended to merge when in close proximity. Strain E differed by

producing colonies that were large and circular, similar to strain C, but were grey in colour and typically had a white ring within the grey colony (Figure 4.4). The observed golden colour of strain E is an artifact of the photograph with colonies being grey in colour in reality.

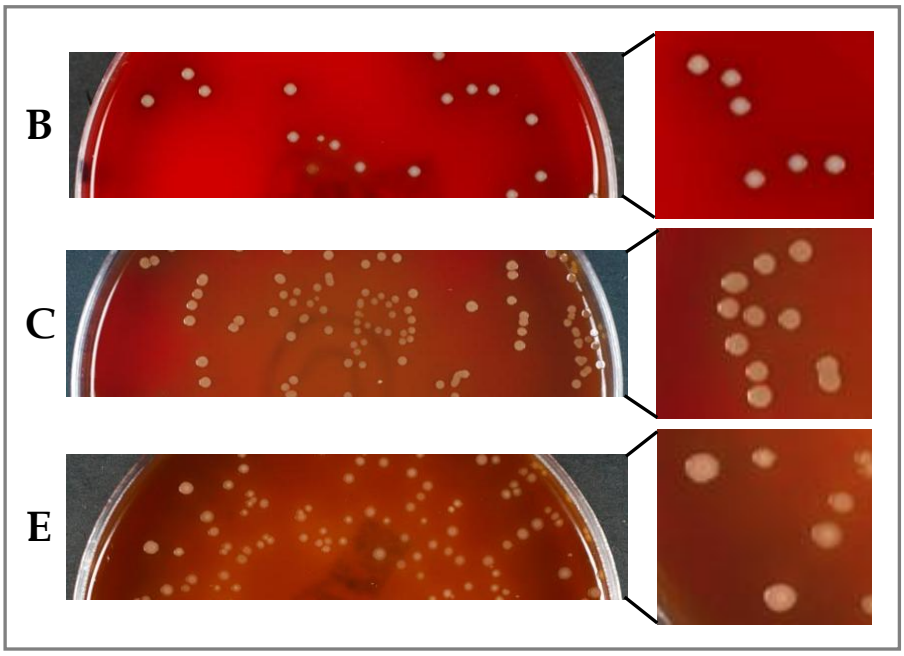


Figure 4.4. Colony morphology of strains B, C and E after 48 h growth on EBA. A few colonies from each plate have been enlarged to show specific morphology details characteristic of each strain.

Differences were also observed in the ability to resuspend each strain in TE buffer. Typically, strains C and E were the most difficult to emulsify, as colonies were viscous and mucoid and difficult to remove from the loop. In contrast, strain D was easily removed from the loop and a uniform suspension was obtained in the solution. Strains A and B were also viscous but easier to emulsify than strains C or E.

These differences in colony morphology and ability to emulsify suggested that variation in capsule expression may exist between strains A-E when cultured on blood agar. The mucoid nature of strain C and E colonies was characteristic of a capsular phenotype, while strains A, B and D, despite

containing the required genes, may have been acapsular under these growth conditions.

4.3.1.4 Ability of strains A-E to grow in milk

When inoculated into sterile UHT milk, all five strains were capable of growth, with each strain reaching at least 10^8 cfu/ml after 20 h incubation at 37°C (Table 4.7). Therefore, each strain was considered suitable for use in the experimental infection study.

Strain	Bacterial numbers (cfu/ml)
A	3.9×10^9
B	1.1×10^9
C	5.1×10^9
D	4.9×10^9
E	7.0×10^8

Table 4.7. Bacterial numbers after 20 h culture in UHT milk.
Bacterial numbers were counted as colony forming units per ml of milk (cfu/ml).

4.3.2 Preparation for the five strain cow infection trial

4.3.2.1 Bacterial numbers in inocula

Individual strains (A-E) were cultured and diluted to obtain approximately 1250-1500 cfu/ml for each individual strain inoculum and 250-300 cfu/ml of each of the five strains in the mixed culture (SU5) inoculum. The actual number of bacteria in each inoculum was assessed directly after infusion of mammary glands by plating extra prepared syringes. Despite attempting to generate similar numbers in each inoculum, bacterial numbers varied slightly between individual strains and SU5 (Table 4.8).

Strain	Average numbers in inocula (cfu/ml)	±SEM
A	2778	268
B	2267	571
C	1840	161
D	2018	41
E	3285	288
SU5	2666	671

Table 4.8. Average cfu/ml in each inoculum, A-E (n=3), SU5 (n=2).

The slight differences between preparations may have been due to incubation of syringes for 1 h at 37°C before infusion, resulting in selected growth of some strains. Alternatively, there may have been slight errors in the performed dilutions and plate count.

4.3.2.2 Pre-trial cow assessment

Prior to infusion, most quarters were found to be free from bacteria and had foremilk SCC of less than $2.0x10^5$ cells/ml. Those quarters where bacteria were found in the milk or with a SCC greater than $2.0x10^5$ cells/ml are noted in Table 4.9.

		Day -3, milking -7		Day 0, milking -1	
Cow #	Quarter	Bacteria	SCC (x1000)	Bacteria	SCC (x1000)
1647	LF	**CNS**	<100	0	155
	RF	*CS*	117	**CNS**	162
2058	RF	0	1919	0	1918
939	LB	0	781	0	298

Table 4.9. Pre-trial assessment of udder health.
Bacteriology and somatic cell counts (SCC) were performed on foremilk collected 3 days before (Day -3, milking -7) and one milking before infusion (Day 0, milking -1). One milk sample was contaminated (*CS*) and could not be interpreted while others had coagulase-negative staphylococci (CNS) present.

Some cows showed evidence of coagulase negative staphylococci (CNS) in the milk; however the SCC remained below $2.0x10^5$ cells/ml. In contrast, bacteria were not detected in milk from a quarter with the highest SCC (cow #2058 RF). Although some quarters had bacteria present and/or a high SCC, none showed visible signs of a clinical infection.

Five quarters had an SCC greater than $2.0x10^5$ cells/ml immediately prior to infusion, although bacteria were not detected in the milk (Table 4.10). Two quarters were infused with the five strain mixture, SU5, while another two quarters were infused with individual strains B or C. Cow #2058 again had the highest SCC of all quarters.

Cow #	Quarter	Bacteria	SCC (x1000)	Inoculum used
939	LB	0	316	B
1647	LF	0	242	-
	RF	0	225	SU5
2058	RF	0	1399	SU5
	RB	0	227	C

Table 4.10. Cow quarters with high SCC immediately prior to infusion. Bacteriology and SCC of foremilk collected on Day 0, milking 0 immediately prior to infusion with prepared inocula. Uninfused control quarter (-).

4.3.3 Evaluation of control quarters

Uninfused control quarters (n=9) were monitored for milk EC, SCC and presence of bacteria. At no stage of the trial were bacteria detected in these quarters. The EC of milk remained constant (Figure 4.5) with no significant difference between milkings M0 – M5 ($p > 0.05$). Similarly, the SCC also remained constant, except for the first milking after infusion (M1) where a slight decrease in average SCC was observed (Figure 4.5). This decrease in SCC was unexpected and it is unknown why this may have occurred. A maximum SCC of $8.4x10^5$ cells/ml and maximum EC of 7.0 were observed in the control quarters over the course of the trial.

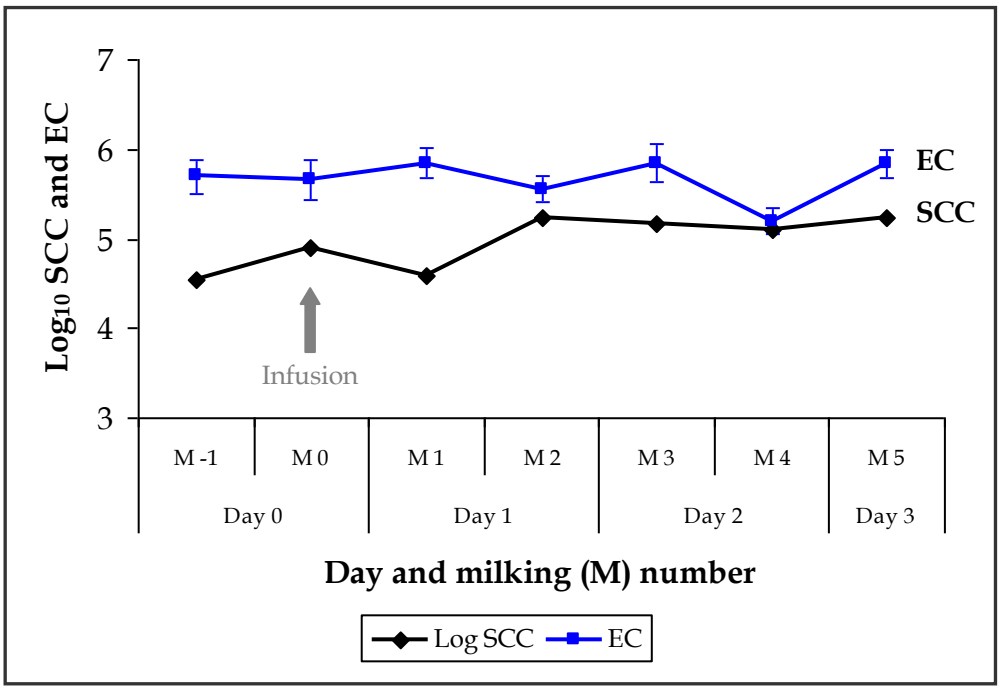


Figure 4.5. Average SCC and EC (±SEM) of control quarter foremilk (n=9).

4.3.4 Development of infection

All infused quarters showed one or more signs of infection, which included increased milk electrical conductivity, elevated SCC (>5.0x10^5 cells/ml), bacteria in the milk and/or clinical signs during the study.

4.3.4.1 Milk electrical conductivity

Foremilk electrical conductivity (EC) of all infused quarters was significantly higher (p < 0.01) than the EC of control quarters from the second milking after infusion (M2; Figure 4.6). There was no significant difference in EC between any of the quarters infused with A-E or SU5 (p > 0.05).

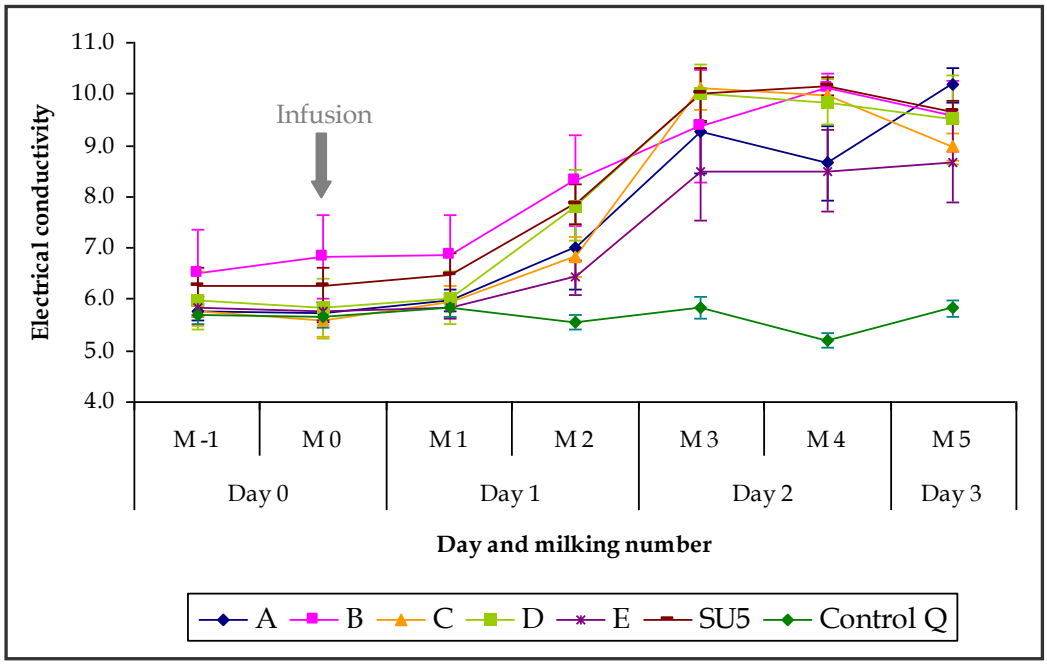


Figure 4.6. Average foremilk EC (±SEM) from quarters infused with strains A-E (n=4), SU5 (n=10) or control quarters (n=9).

Although not significant, the average EC for strain B-infused quarters appeared slightly higher at almost all milkings as one of the quarters (cow #939, LB) had a high EC before (9.0) and after infusion. Pre-trial assessment of cow #939 revealed a high SCC in the LB quarter before and at infusion, indicative of infection (Table 4.9, 4.10), however no bacteria were detected in the milk from this quarter before infusion.

4.3.4.2 SCC of milk from infused quarters

The SCC varied widely between quarters of different cows infused with the same strain and two examples are shown in Figure 4.7. For some cows, wide variations in SCC were also observed between infused quarters and an example (cow #1647) is shown in Figure 4.8. Other cows however, had similar levels of somatic cells in all infused quarters throughout the study (e.g. cow #3218; Figure 4.8). Therefore, the inflammatory response appeared unique to each mammary quarter rather than reflecting cow- or strain-specific differences.

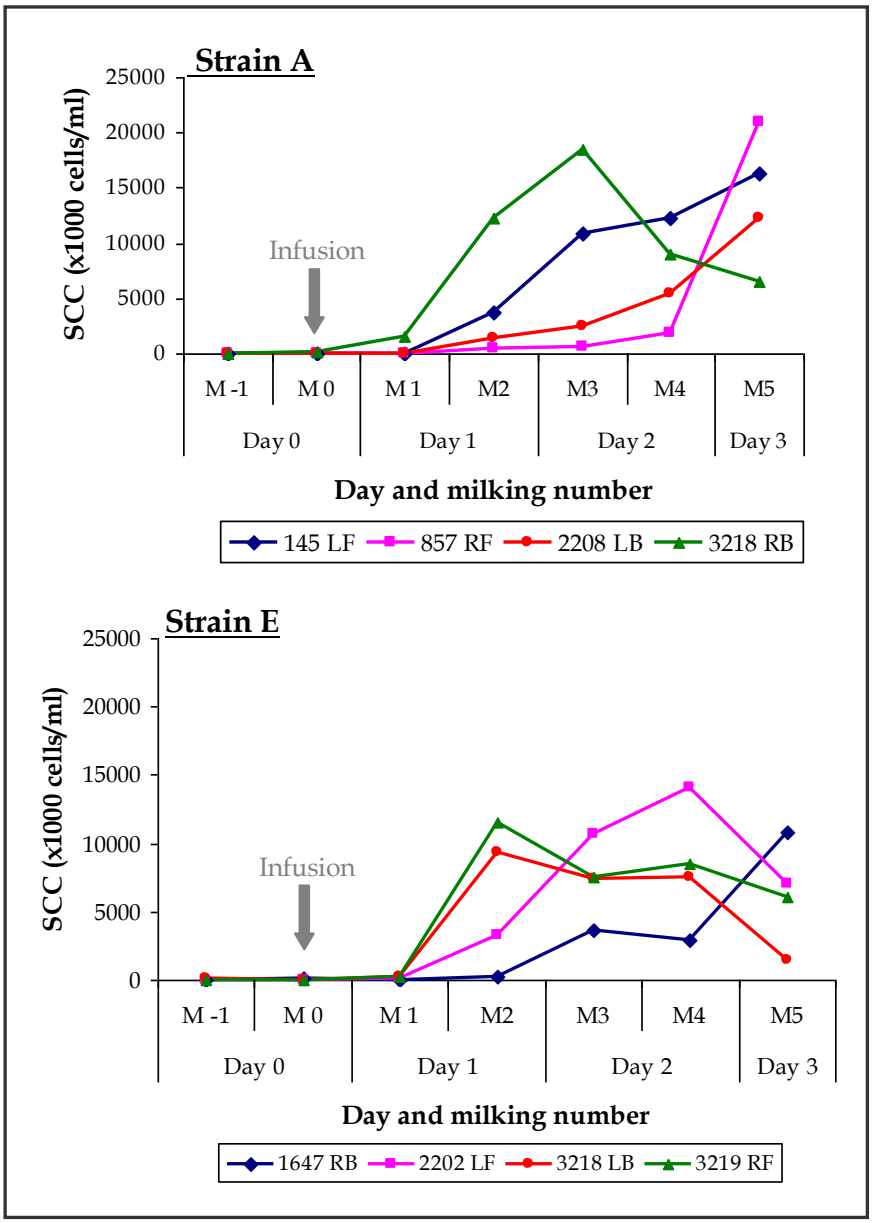


Figure 4.7. Milk SCC in quarters infused with strains A or E.

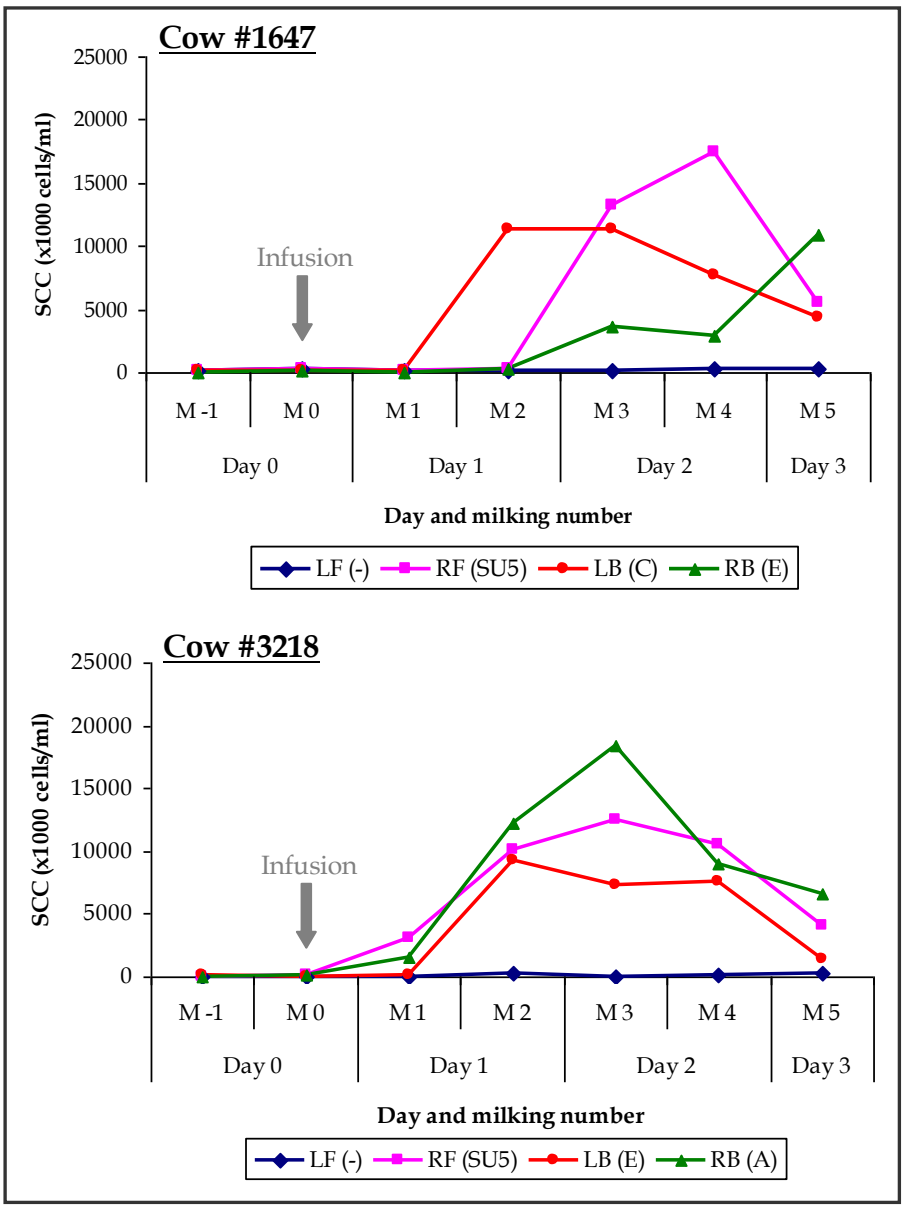


Figure 4.8. Milk SCC of four quarters from the same cow.
SCC in three infused quarters (SU5, A, C, E) and uninfused control (-) quarters of cow #1647 (i) and #3218 (ii).

Overall however, there was no significant difference in $\log_{10}$ SCC of all quarters infused individually with strains A-E or SU5 ($p > 0.05$) across all milkings of the trial. All infused quarters were significantly higher ($p < 0.01$) than control quarters from the second milking (M2) after infusion (Figure 4.9).

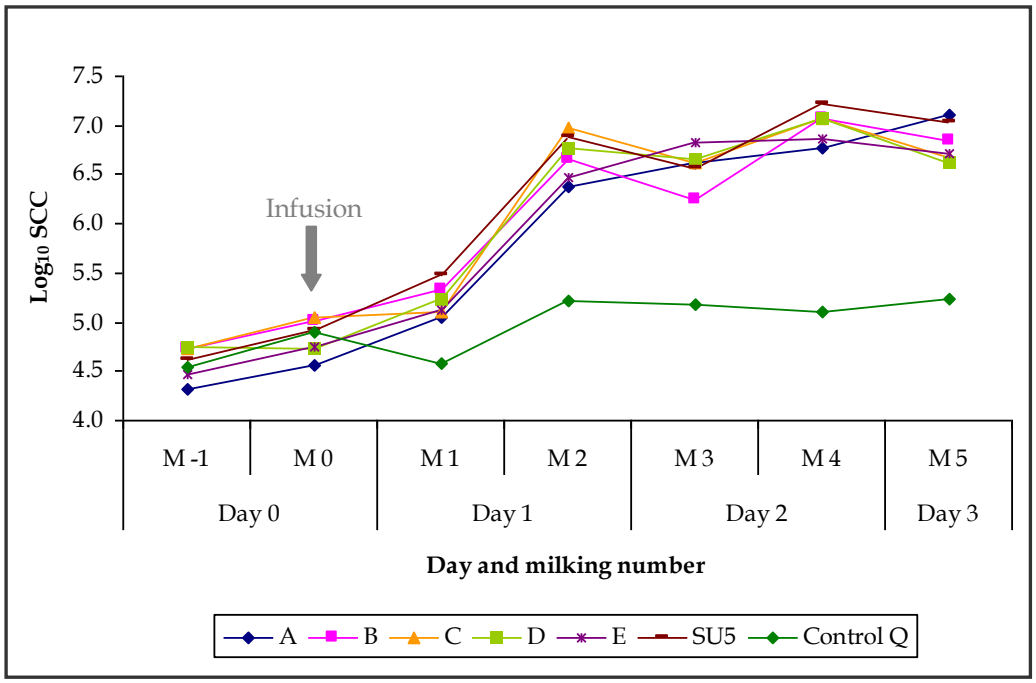


Figure 4.9. Average SCC in quarters infused with A-E (n=4), SU5 (n=10) or control quarters (n=9).

The average SCC for all infused quarters was greater than $4.5x10^6$ cells/ml by the second milking (M2), indicative of establishment of an inflammatory response to infection, compared to control quarters which had an average SCC of $3.7x10^5$ cells/ml. Similar levels of somatic cells were maintained in the infused quarters until the fifth milking (M5), at which time quarters showed signs of clinical infection and antibiotics were administered.

4.3.4.3 Total bacterial numbers in milk

At the first milking (M1), less than 24 hours after infusion, total bacterial numbers ranged from 300 cfu/ml (strain A) to $9.5x10^5$ cfu/ml (SU5) in milk, suggesting rapid establishment within all infused quarters (Figure 4.10). By the fifth milking (M5) a larger range in bacterial numbers was observed

with less than 10 cfu/ml detected in the milk of one quarter (SU5) up to $4.5x10^8$ cfu/ml in the milk of a strain A-infused quarter.

Generally, the number of bacteria isolated from the foremilk samples remained similar from the first milking to the fifth milking after infusion, with no significant difference observed between strains A-E and SU5 across these milkings ($p > 0.05$).

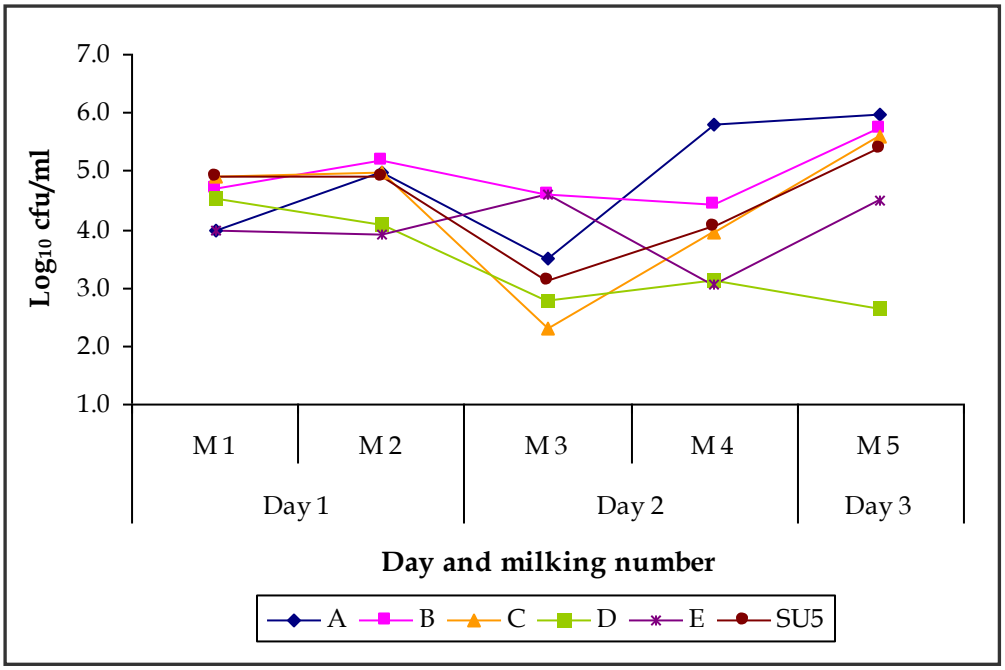


Figure 4.10. Average bacterial numbers in milk from quarters infused with strains A-E or SU5. M1, M2, M4: strains A-E (n=4); SU5 (n=10). M3: strains A, B, C, D (n=3); E (n=1); SU5 (n=8). M5: strains A, C, D, E (n=4); B (n=3); SU5 (n=10).

4.3.4.4 Signs of clinical infection

Although some quarters had clots in the foremilk (indicative of clinical infection) by the second milking (Table 4.11), the majority of infused quarters remained free of clots until the fifth, sixth and seventh milking after infusion. Antibiotic treatment commenced at the fifth milking. Quarters infused with strain B and SU5 tended to be the first to show clinical signs of infection. Two SU5-infused quarters were classified as C2 and C3 by the second milking, while one quarter (cow #939 LB) infused

with strain B, was classed as C2. Cow #939 LB had a high SCC ($3.2x10^5$ cells/ml) and a high EC (9.0) before infusion, which may be why clinical signs were observed sooner than for other strain B-infused quarters.

	Milking Number								
	0 Infusion	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
						Antibiotic treatment			
A	0	0	0	1	0	1	3	3	2
B	0	0	1	2	1	3	3	4	1
C	0	0	0	0	0	2	3	2	1
D	0	0	0	0	0	1	2	1	1
E	0	0	0	0	0	2	2	2	1
SU5	0	0	2	1	1	8	6	10	6
% of total infused quarters	0%	0%	10%	13%	7%	**57%**	**63%**	**73%**	**40%**

Table 4.11. Development of clinical signs in the infused quarters.
Number of quarters with clots in milk after infusion with each strain (A-E; n=4) or the five strain mixture (SU5; n=10). Infusion was at milking 0 and antibiotic treatment started at milking 5 until clinical signs abated.

4.3.4.5 Mammary gland infection with strains A – E and SU5

All five strains and the five strain mixture, SU5, were capable of establishing an intramammary infection with an elevated immune response and foremilk electrical conductivity from the second milking, 24 hours after infusion and throughout the trial period. The SCC varied between quarters, suggesting that each quarter had a unique response that was not strain- or cow-specific. Viable bacteria were detected at the first milking after infusion and overall this level was maintained until the onset of antibiotic treatment of the infused quarters. Although strains B and D were originally isolated from the dairy environment, they were equally as capable of infecting the mammary gland as strains A, C, and E, which were isolated from clinical mastitis cases. No difference was observed in the development of infection for the five strains infused individually or as a mixture.

4.3.5 Strain identification in A-E-infused quarters

To confirm that the infused strain established infection in the quarters receiving the single strain inocula, between two and five single colonies were strain typed from each milk sample from quarters infused with individual strains A-E. In all cases except one, the strain obtained was the same as the inoculum strain for that quarter. One milk sample, from cow #3218 LB at the fifth milking, had two different strain types present. The milk from this quarter had been directly plated (100 µl) without dilution and only five colony forming units were obtained. One of the five colonies was identified as strain E, the infused strain, while the other four colonies corresponded to strain C. The previous milk sample yielded only one colony after direct plating (100 µl) which, when strain typed, was identified as strain E. This unexpected result at the fifth milking may indicate contamination of the milk sample during collection, dilution or plating, or that this quarter became infected at a low level with strain C between the fourth and fifth milking.

No other 'contaminating' strains of unknown identity were detected in any of the milk samples; however, as only between two and five colonies were typed it was possible that other strains may have been present and not detected.

4.3.6 Strain identification in SU5-infused quarters

Infusion of the five strain mixture resulted in mastitis; however, it was unknown if some or all of the strains were responsible for the infection. Therefore the strain types of isolates from milk of SU5-infused quarters were identified for up to five milkings after infusion.

4.3.6.1 Colony selection and strain typing

The yellow colour of colonies corresponding to strain C (see section 4.3.1.3) was useful in identifying this strain within each milk sample (Figure 4.11).

After counting the total number of yellow and white colonies on the plate, between five and ten yellow colonies and 15-20 white colonies were chosen for strain typing, based on the recommendations of Dopfer *et al.* (2005). After strain typing of the selected colonies, the relative proportions of the five strains within each of the milk samples were calculated.

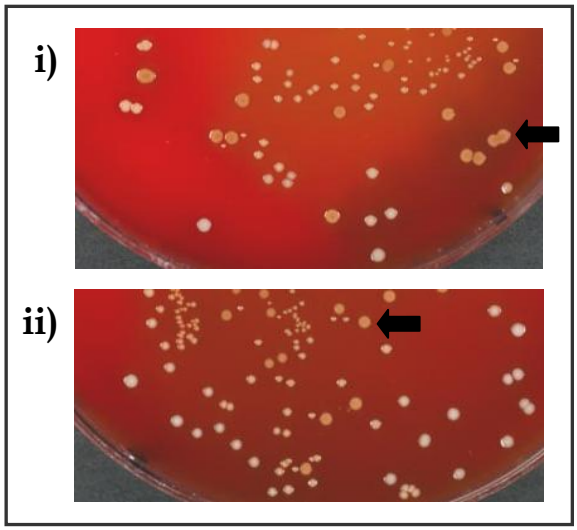


Figure 4.11. Colony morphologies after plating milk from two SU5-infused quarters on EBA. (i) Cow #1041 LB quarter; (ii) cow #3219 RB quarter (ii) from the second milking after infusion. Arrows indicate yellow-coloured colonies corresponding to strain C.

4.3.6.2 Proportion of each strain in SU5 inocula

In the initial SU5 inocula, strains A, D, and E were present in approximately equal proportions with each representing 22 – 25% of the total bacterial number, whilst strains B and C were present in lesser amounts, representing only 14% and 15% respectively of the total bacteria (Figure 4.12). Despite these differences in the starting amount of each strain, there appeared to be little effect on the subsequent detection of these strain types in the milk from the inoculated quarters.

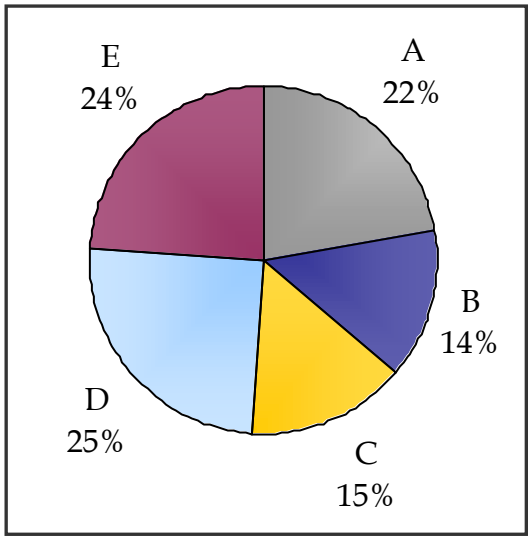


Figure 4.12. Proportion of each strain A–E in the SU5 inocula.

4.3.6.3 Relative levels of strains A-E in SU5-infused quarters

At the first milking after infusion, varied results were obtained for each of the quarters in regard to the proportion of each strain types detected (Figure 4.13). All five strains were only detected in one quarter (cow #2202 RB), whilst in other quarters the number of detected strain types ranged from two to four. Although 22% of the inoculum corresponded to strain A, this strain was only detected in four of the ten quarters, strains B and E were found in six of the ten quarters, strain D in eight and strain C, the most prevalent, was found in nine of the ten infused quarters.

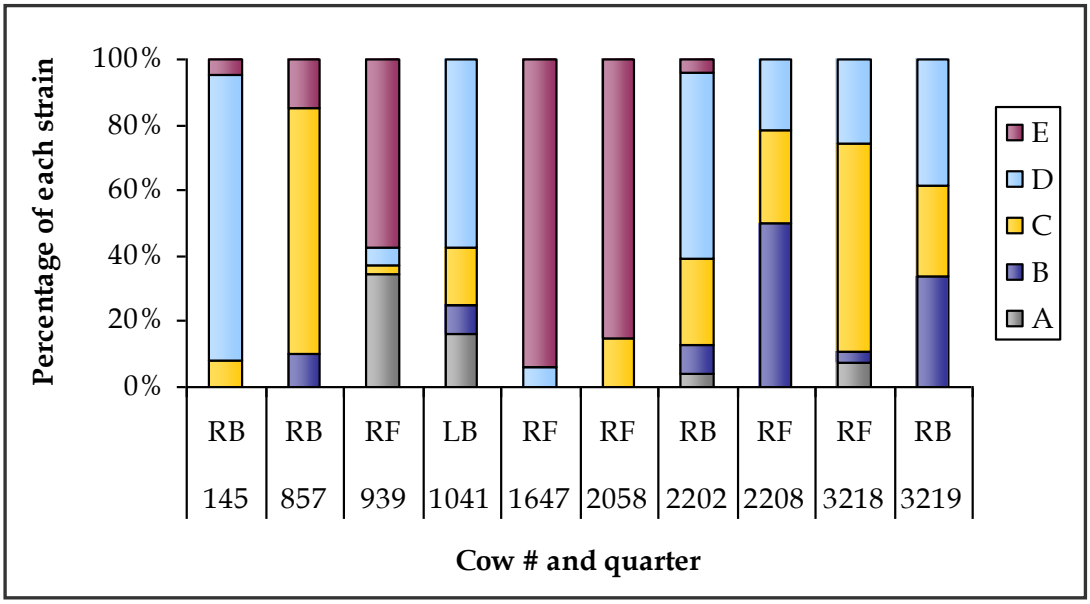


Figure 4.13. Relative proportion of each strain A-E at the first milking after infusion.

As the infection progressed, fewer strain types were detected in most quarters and by the fifth milking after infusion, when antibiotic treatment commenced, seven of the ten quarters had only one strain type detectable in the milk (Figure 4.14).

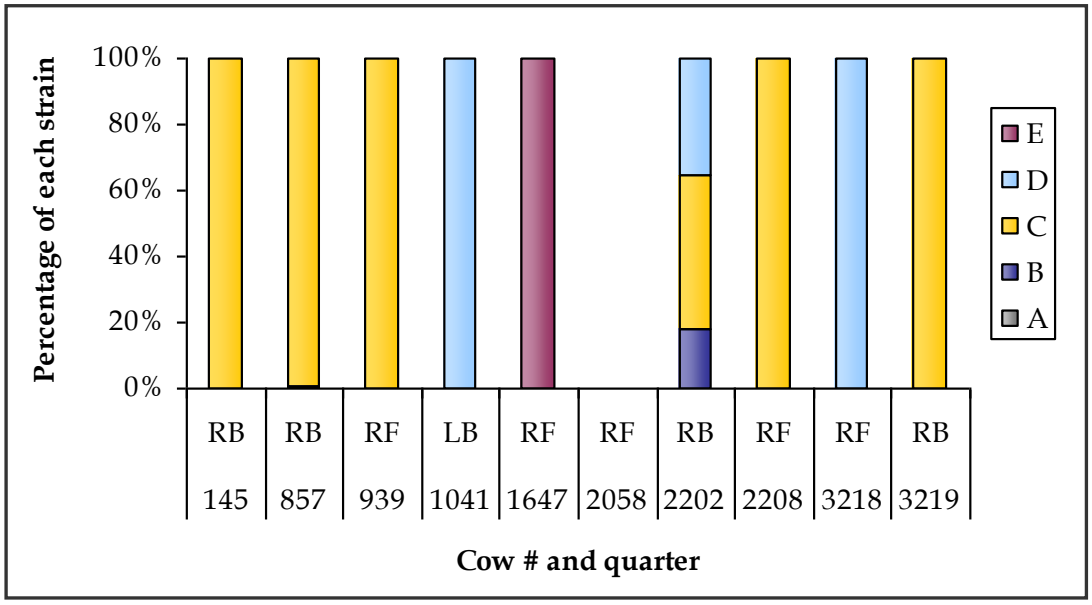


Figure 4.14. Relative proportion of each strain A-E at the fifth milking after infusion. Bacteria were not detected in the milk from cow #2058 RF using these methods.

In cow #857 two strains were detected but strain A was only present in very small numbers (1%). Only one of the ten quarters (#2202 RB) had three strain types present in the milk, whilst in cow #2058 bacteria were not isolated after plating at this milking, indicating that there must have been less than 10 cfu/ml milk. However, strain E alone was observed at the fourth milking after infusion.

At the fifth milking after infusion, strain C predominated in five of the ten quarters, while strains D and E predominated in two and one quarter respectively. The only quarter with a mixture of strains had similar proportions of strains B, C, and D present, whilst strain A was only detected in one quarter (#857 RB) at very low levels, suggesting that this strain was not as capable of infecting the mammary gland when the other four strains were present. Performing a Chi-square test showed that there were significant differences among the strains in the proportion of times that each strain was dominant ($P<0.026$), however this test was unable to determine where those significant differences occurred. Most likely this was the difference between strain C (predominated in 5/10 quarters) and strain A (0/10 quarters).

4.3.6.4 Change in strain types over time

i) Predominance of strain C

For the five quarters where strain C predominated, this predominance was observed either early in the trial, for two cows (Figure 4.15) or in the last two milk samples collected (three cows; Figure 4.16). Strain C was exclusively detected in cow #145 RB (Figure 4.15i) from the second milking onwards, despite the first milk sample consisting mainly of strain D with only small proportions of strains C and E present. Therefore, in the few hours between these two milkings there was major growth of strain C while strains D and E were reduced to undetectable levels. Of the SU5-infused

quarters, the total number of bacteria was consistently highest in cow #145 RB across the five milkings.

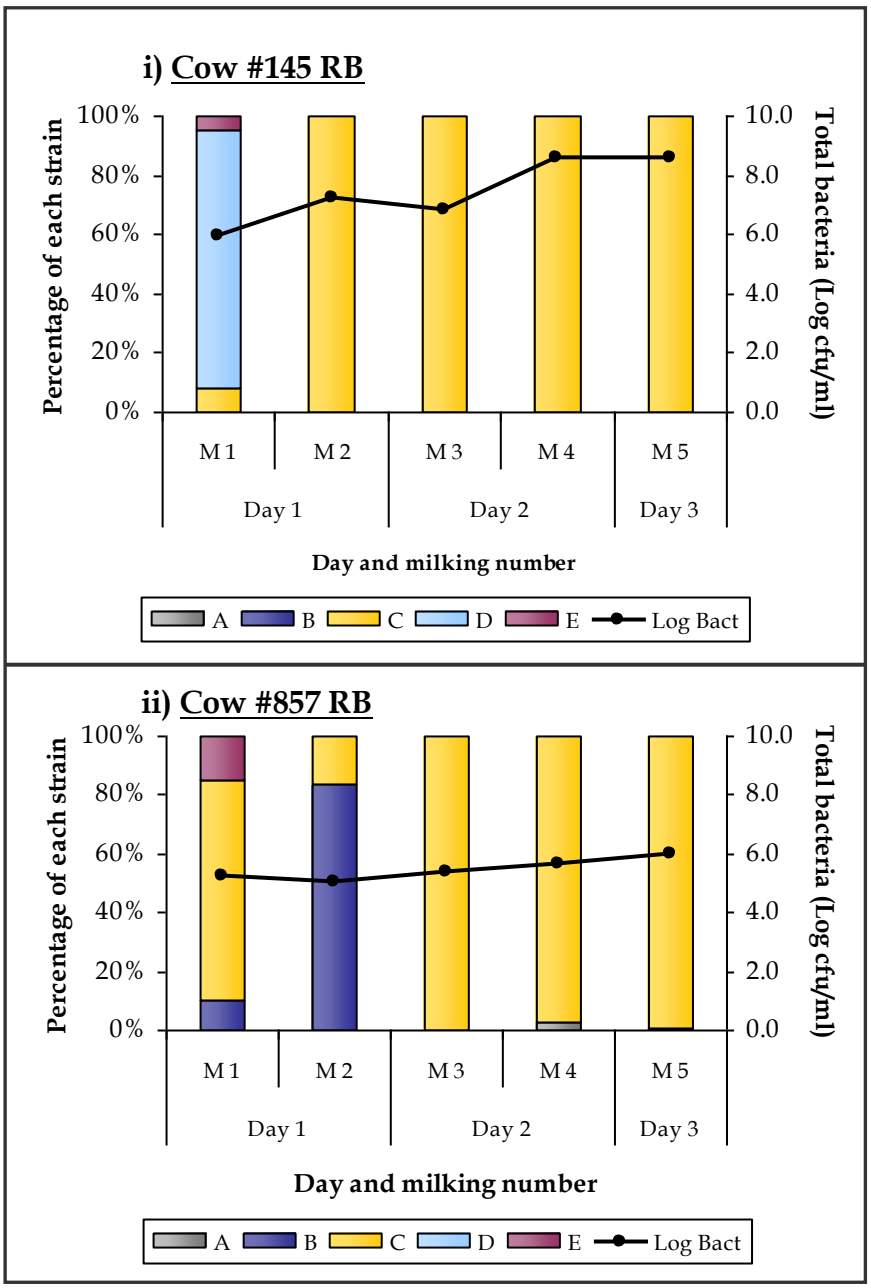


Figure 4.15. Strain C predominance early in the trial.
Percentage of each strain type and total number of bacteria (log_{10} cfu/ml) are shown for each milking after infusion for cow #145 RB (i) and cow #857 RB (ii).

Cow #857 RB (Figure 4.15ii) also showed a similar trend to #145 RB, although more time passed before strain C predominated. From the third milking after infusion (M3), only strain C was detected and this strain predominated for the rest of the trial. A small amount of strain A was

detected at M4, suggesting that this strain was still present in the mammary gland but at undetectable levels in the earlier milk samples.

Strain C predominated late in the trial in three other cows (Figure 4.16). Of these, cow #2208 RF and #3219 RB had a very similar trend in strains detected, with strain C predominating from the fourth milking. Predominance of strain C in the other quarter (cow #939 RF; Figure 4.16iii) was not apparent until the last milking (M5) of the trial as, previous to this, a mixture of four or five strains was found. Unfortunately, milk samples collected at M3 from cow #3219 RB and #939 RF were contaminated and total number of bacteria and strain types present could not be determined.

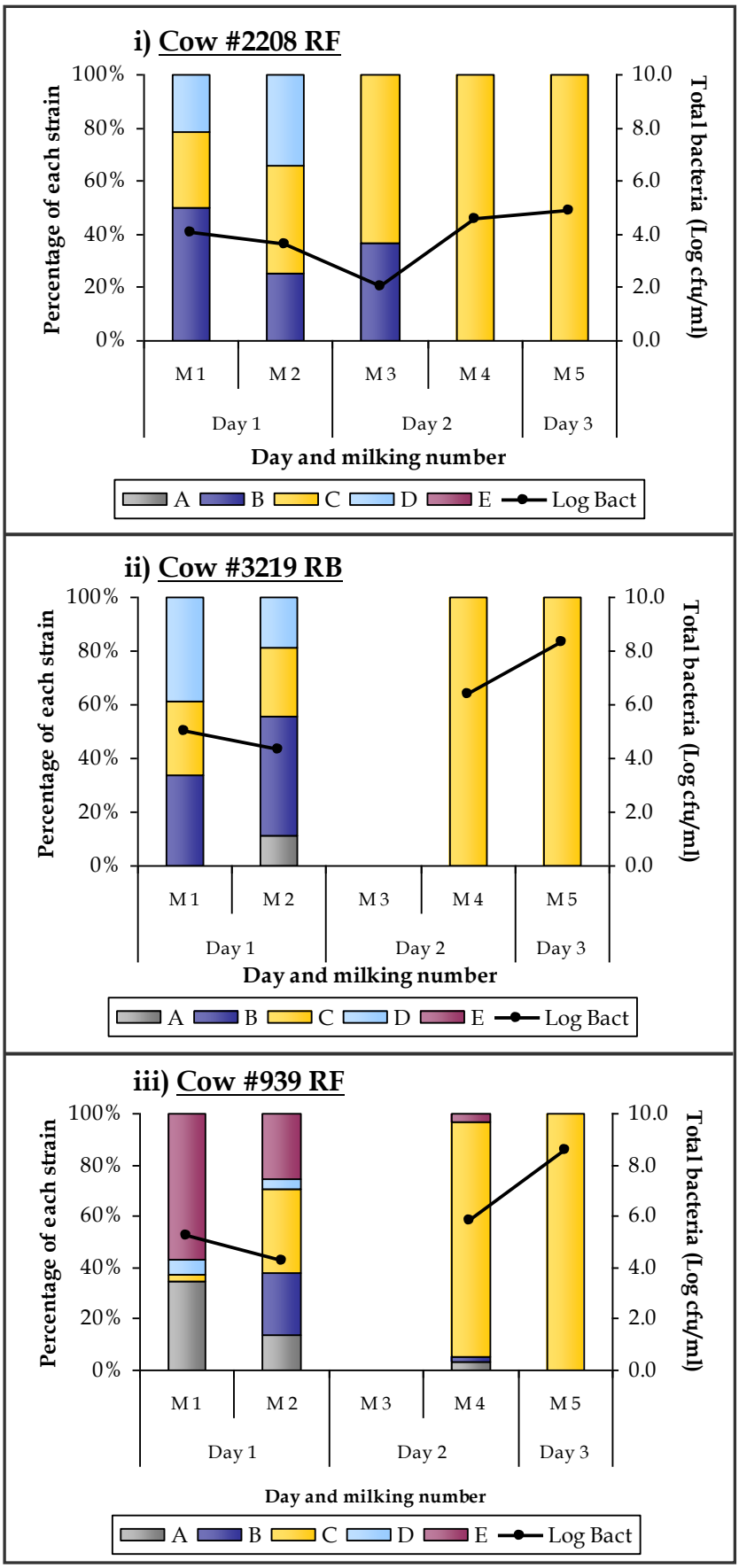


Figure 4.16. Predominance of strain C late in the trial. (i) Cow #2208 RF; (ii) Cow #3219 RB; (iii) Cow #939 RF. Data missing for cow #3219 and #939 at M3 due to contamination of milk samples.

ii) Predominance of strain E

Strain E predominated in two of the infused quarters (Figure 4.17). In cow #1647 RF (Figure 4.17i), 94% of total bacteria in the milk were strain E and 6% strain D at the first milking after infusion (M1). At the second milking, strain D was no longer detected but strain C was found, indicating that this strain must also have been present at M1, but below detectable levels.

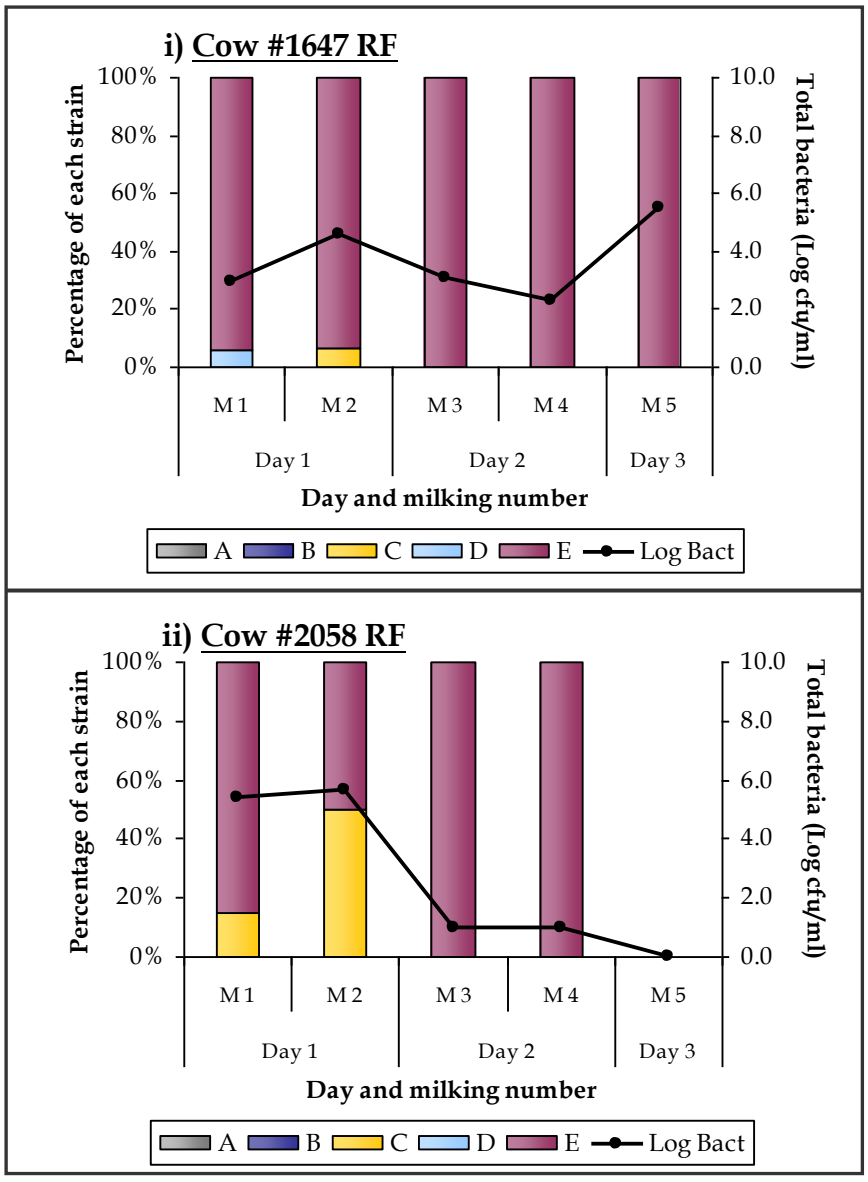


Figure 4.17. Strain E predominance during the trial.
(i) Cow #1647 RF; (ii) Cow #2058 RF.

The strain types found in cow #2058 RF (Figure 4.17ii) were similar to those of cow #1647 in that strain E was the major strain present at M1 and again by the third milking, only strain E was detected. This was the only quarter

where bacteria were reduced to undetectable levels (<10 cfu/ml) by the end of the trial (M5). This quarter had a particularly high SCC at the time of infusion ($1.4x10^6$ cells/ml), and also one of the highest observed SCC levels at M3 and M4. This high initial SCC may be the cause of the reduction in bacterial numbers to undetectable levels in this quarter by the fifth milking.

Interestingly, both of the quarters where strain E predominated had a high SCC at the time of infusion (although cow #1647 RF only had a SCC of $2.3x10^5$ cells/ml compared to $1.4x10^6$ for #2058 RF; Table 4.10) This may indicate that, of the five strains inoculated into these quarters, strain E was more resistant to a high initial immune response within the mammary gland.

iii) Predominance of strain D

Strain D predominated in two of the infused quarters (Figure 4.18). In cow #1041 LB (Figure 4.18i), four of the five strains were found at the first milking after infusion; however, by the third milking (M3) only strain B was detected. Strain D was found again at M4 and this re-emergence was followed by rapid growth so that by M5 strain B had disappeared and only strain D was detected.

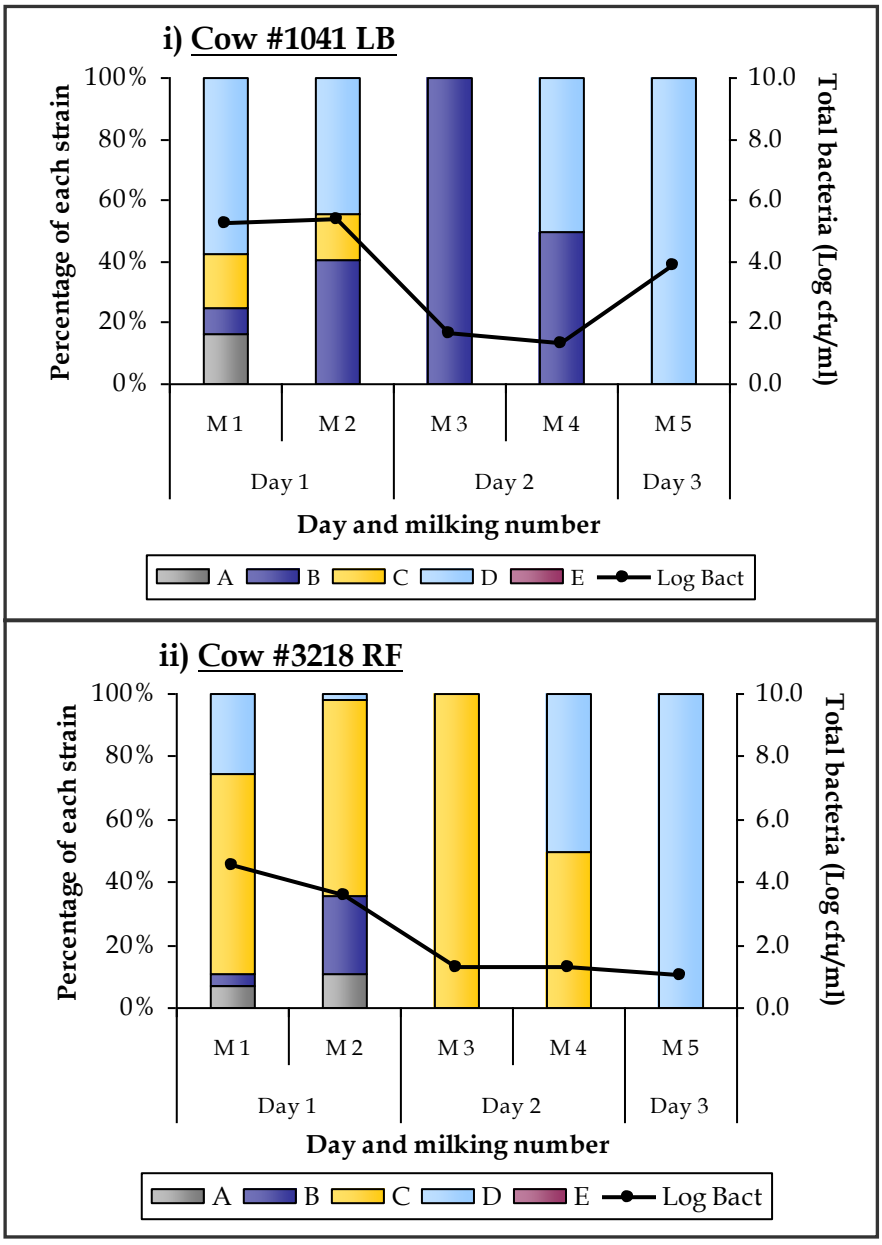


Figure 4.18. Predominance of strain D during the trial.
(i) Cow #1041 LB; (ii) Cow #3218 RF.

Similarly for cow #3218 RF (Figure 4.18ii), four strains were found in the milk at the start of the trial, but over time these were reduced until only strain D remained on the last day of the trial (M5).

iv) Absence of a predominant strain

Only one of the ten SU5-infused quarters lacked a predominant strain by the end of the trial (Figure 4.19). Interestingly, this was the only quarter where all five strains were detected at the first milking after infusion, although all five strains were also found in cow #939 RF at M2. By the second milking after infusion, strain E was no longer detected, then at M3 and M4 strains A and B were also undetectable. This elimination of strain types over time was similar to the other quarters, but instead of one strain becoming predominant, strain B recovered to give three different strain types at M5 (strains B, C and D) and an increase in total number of bacteria. If the trial had been extended for a longer period of time then perhaps, based on the results of the other nine quarters, this quarter may also have developed one predominant strain type.

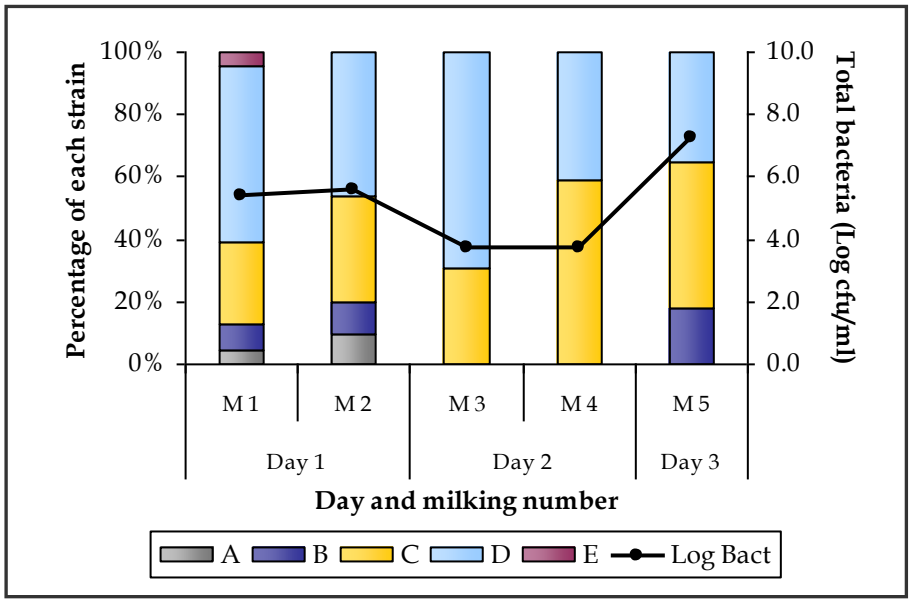


Figure 4.19. Multiple strains detected on the last day of the trial in cow #2202 RB.

4.3.7 Emergence of a predominant strain

Infusion with a mixture of five strains resulted in the predominance of one strain type over time in nine of the ten quarters. However, the predominating strain was not always the same and a summary of the

number of strain types detected in each quarter at each milking after infusion is shown in Table 4.12.

	Milking number					Strains present	
Cow #	**M 1**	**M 2**	**M 3**	**M 4**	**M 5**	**M 3**	**M 5**
145	3	1	1	1	1	**C**	**C**
857	3	2	1	2	2	**C**	**C/A**
939	4	5		4	1		**C**
1041	4	3	1	2	1	**B**	**D**
1647	2	2	1	1	1	**E**	**E**
2058	2	2	1	1	0	**E**	**-**
2202	5	4	2	2	3	**C, D**	**B, C, D**
2208	3	3	2	1	1	**B, C**	**C**
3218	4	4	1	2	1	**C**	**D**
3219	3	4		1	1		**C**

Table 4.12. Number of strain types detected at each milking and strains observed at the third and fifth milking. No results were available for cow #939 and 3219 at M3 due to contamination of milk samples.

The number of strain types decreased from the first to the third milking after infusion, at which time only one strain was detected in six of the eight quarters. Although the number of strain types increased again in some quarters by the fourth milking, only one strain was found in the majority (7) of quarters by the fifth milking.

4.3.8 Post-trial cow assessment

When bacteriology was conducted on the foremilk of all infused quarters 36 days after infusions took place, *S. uberis* was isolated from the milk of four quarters, despite completion of an extended course of intramammary antibiotics administered from the fifth milking onwards. Three of these quarters had been infused originally with strain E and the other quarter

with strain C. After strain typing a single colony from each quarter sample, the infused strains were found in each quarter, suggesting persistence of these strains within the mammary gland over time.

4.3.9 Growth of the five strain mixture *in vitro*

To investigate if strain predominance occurred in the absence of unique cow factors such as the immune response, mixtures of the five strains were prepared in UHT milk and the level of each strain type determined before and after incubation at 37°C for 74 hours. Total bacterial numbers ranged from $9.7x10^2$ to $2.0x10^3$ cfu/ml before incubation and all five strains were detected, although not in equal proportions (Figure 4.20). Experiment 2 had more similar proportions of each strain present prior to incubation than experiment 1, which may indicate better preparation and mixing of cultures in the second experiment.

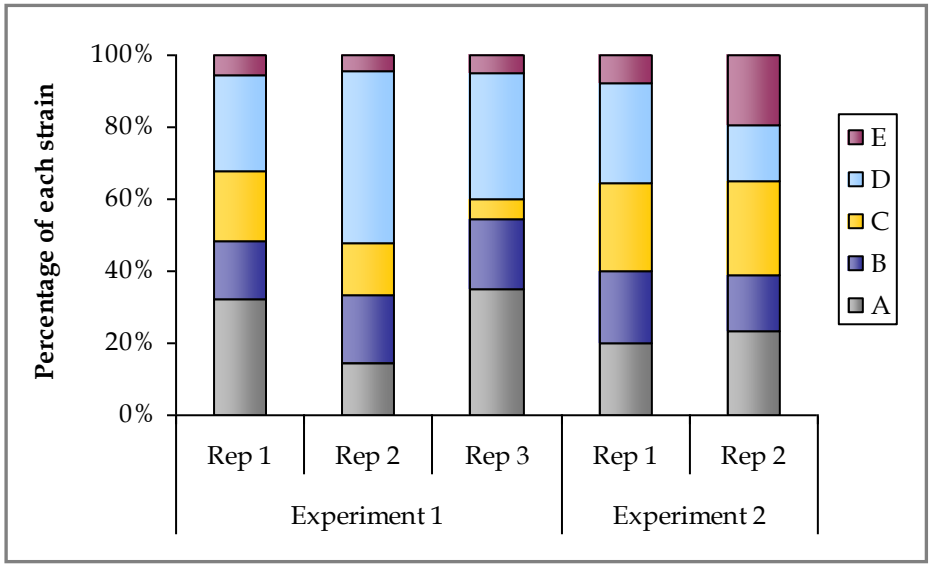


Figure 4.20. Relative proportions of strains A-E before incubation in replicates (Rep 1 – 3) from two experiments.

After 74 hours of incubation, total bacterial numbers increased in all replicates, ranging from $4.1x10^8$ through to $7.6x10^8$ cfu/ml and all five strains were still detected (Figure 4.21).

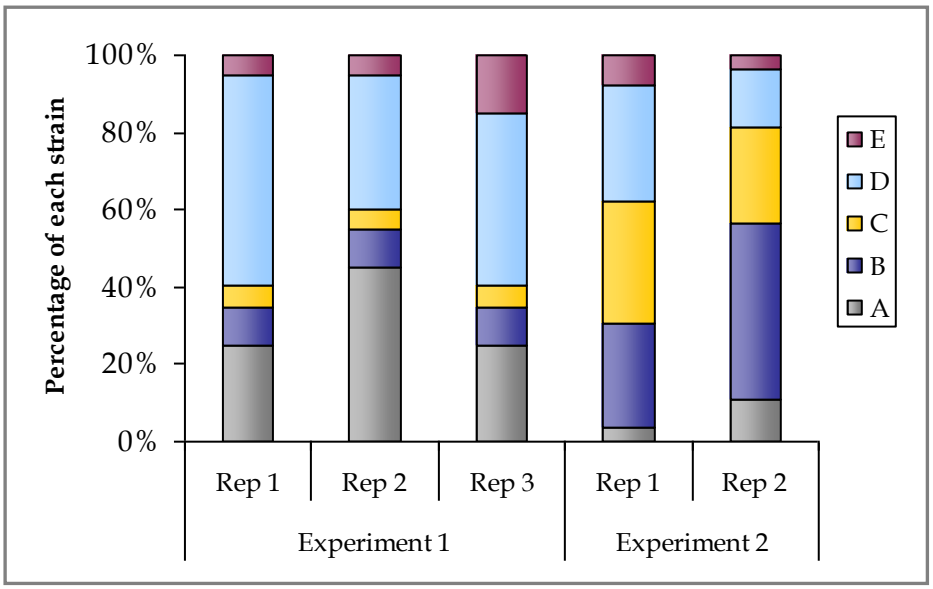


Figure 4.21. Relative proportions of strains A-E after 74 h incubation in replicates from two experiments.

Although total bacterial numbers increased during incubation, the relative proportion of each strain remained similar after the 74 h incubation period, when all replicates were averaged (Figure 4.22). Therefore, when cultured together in UHT milk, no strains predominated, in contrast to when the same five strains were infused together into the mammary gland.

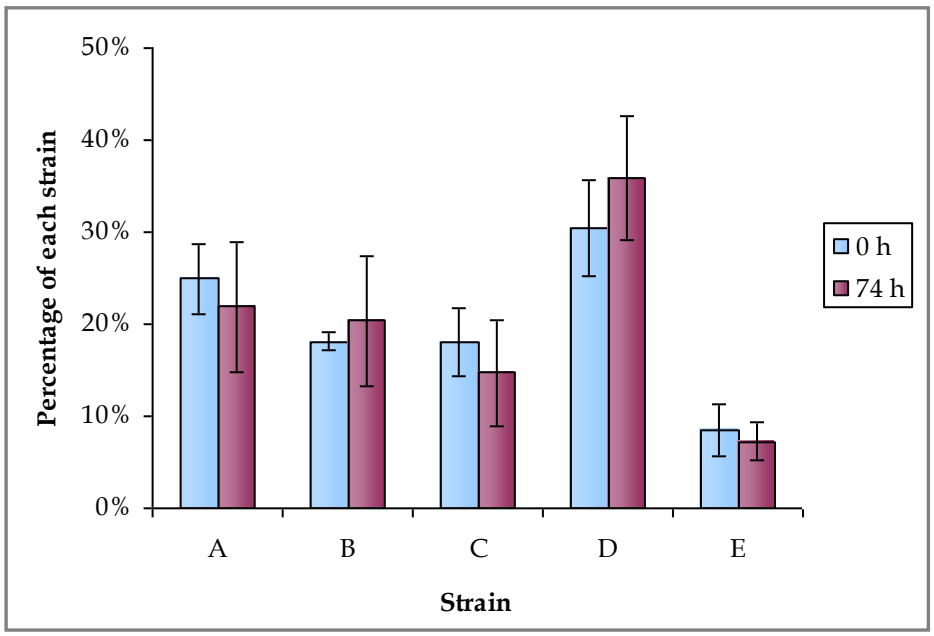


Figure 4.22. Relative proportion of strains A-E (±SEM) detected at 0 and after 74 hours incubation at 37°C in UHT milk (n=5).

4.3.10 Production of bacteriocins by strains A-E

The production of bacteriocins was investigated as a mechanism of direct competition between the five strains.

4.3.10.1 Deferred antagonism

Strain C completely inhibited the growth of all other strains on EBA with a large zone of clearance around the original line of test strain growth (Figure 4.23; Table 4.13). As expected, strain C showed self-immunity to the inhibitory substance being produced and could grow across the test strain line. Strain B also showed inhibitory activity towards strains A, D and E, although not to the extent of strain C, with only partial inhibition of D and E with some resistant colonies observed. Strain D did not inhibit any other strains; however, it partially inhibited itself with only a few resistant colonies observed, indicating that this strain did not have strong self-immunity towards the inhibitory substance being produced.

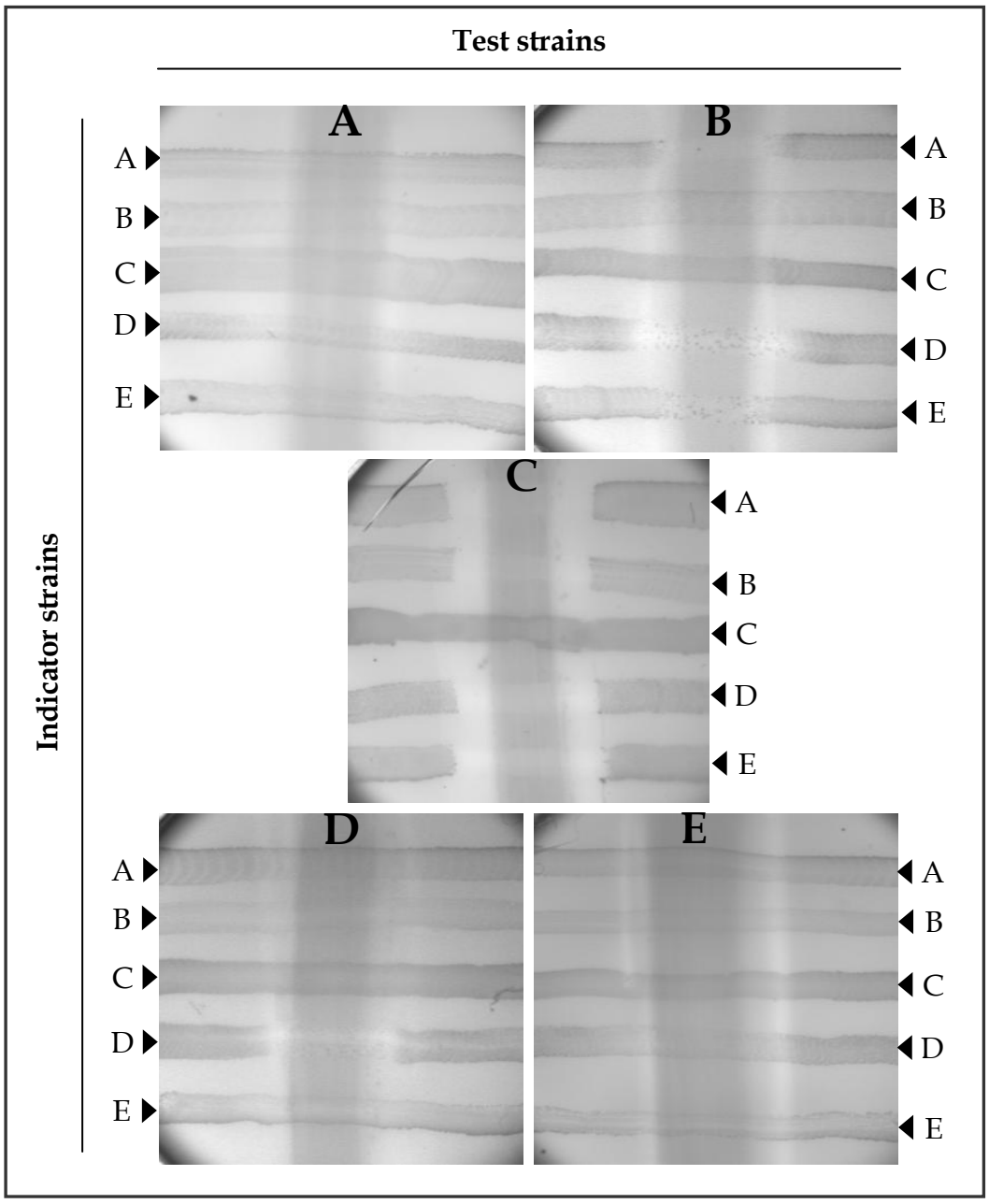


Figure 4.23. Deferred antagonism EBA plates for strains A-E.
Test strains A-E were grown in the centre of each plate and, after removal of each culture, the five indicator strains (A-E) were cross-streaked perpendicular to this line. Absence of growth across the central test strain line indicated sensitivity to an inhibitory substance produced by the test strain.

	Indicator strain				
Test strain	**A**	**B**	**C**	**D**	**E**
A	0	0	0	0	0
B	2	0	0	**R**	**R**
C	**3**	**3**	0	**3**	**3**
D	0	0	0	**R**	0
E	0	0	0	0	0

Table 4.13. Deferred antagonism results for strains A-E.
0 = No inhibition; 2 = Inhibition (~2 cm clearance); 3 = Inhibition (~3 cm clearance); R = Inhibition with resistant colonies

4.3.10.2 Identification of bacteriocins in strains A-E

Following analysis by PCR, all five strains were found to contain the structural gene for uberolysin. Of the five strains, only strain C contained the structural gene for nisin U, which may explain the ability of this strain to inhibit growth of all others in the deferred antagonism experiment. However, the presence of structural genes does not necessarily confirm the production of a bacteriocin since additional genes are required for inhibitor production, export and self-immunity.

To investigate production, producer (P)-typing was performed. Production of uberolysin typically gives a triplet code of 777 upon P-typing due to inhibition of all indicator strains; therefore, based on this, strains B, C, and D produced uberolysin when cultured on EBA, while strains A and E only weakly produced this bacteriocin (Table 4.14). The production of uberolysin by strains A and E was also dependent on blood in the media with each strain inhibiting fewer indicator strains on blood-free agar compared to EBA.

	Triplet codes	
Strain	**Esculin blood agar**	**Blood-free agar**
A	616	200
B	777	777
C	777	777
D	777	777
E	610	000

Table 4.14. P-typing results for strains A-E on esculin blood agar (EBA) or blood-free agar.

Production of nisin U typically inhibits all indicator species except I4 (a nisin U producer), resulting in a P-type of 737. However, if both uberolysin and nisin U were produced by the same test strain the uberolysin P-type of 777 would obscure that of nisin U. Therefore, to determine if strain C was capable of producing nisin U, the presence of genes required for production, processing and export of this bacteriocin were investigated by sequencing the entire nisin U gene locus. The sequences for strain C were almost identical to those given in the published nisin U gene locus (GenBank accession DQ146939) with all eleven genes present and in the same position within the locus (data not shown), indicating that strain C contained all the genes required for producing nisin U.

In summary, all five strains were capable of producing uberolysin, although some strains (B, C, and D) had enhanced production on media containing blood, suggesting that different regulatory mechanisms may be involved in bacteriocin production in these strains. Strain C also contained the necessary genes for production of nisin U and completely inhibited growth of all other strains with deferred antagonism. However, despite production on EBA, bacteriocins may not have been produced with culture in UHT milk as all five strains co-existed during 74 h of incubation. At this stage it

is unknown if bacteriocins were produced during the infection of the mammary gland and were responsible for the predominance of a single strain over time.

4.4 Discussion

4.4.1 Selection of an infective strain

While many strains of *S. uberis* are found throughout the dairy environment and on the teat skin, usually only one, or at most, two strains are found in the milk from mammary quarters infected with *S. uberis* (Oliver *et al.*, 1998b; Phuektes *et al.*, 2001; Wieliczko *et al.*, 2002; Zadoks *et al.*, 2003). From the multitude of strains at the teat end, it is unlikely that only one or two strains would gain access at any one time. It is more likely that a number of strains gain access to the teat canal or mammary gland and that a single strain is selected during the invasion process, to become the infective strain.

This selection process could be occurring on the teat skin, resulting in colonisation of the skin by one strain that is better adapted to survival at this site. Given an appropriate opportunity, this strain would then be in an ideal position to infect the mammary gland and cause mastitis. The results reported in Chapter 3 provided little evidence of teat ends becoming colonised by a single strain of *S. uberis* prior to infection of the mammary gland. Single strain types were generally only isolated from teat ends of infected quarters, and on most occasions this was the infective strain; whilst a variety of strains were isolated from the skin of uninfected quarters. These results suggest that colonisation of the teat skin with a single strain was more likely to arise from a post-infection contamination rather than a pre-infection event. However, more data is required to confirm this conclusion, in particular investigating the diversity of strains present on teats of uninfected quarters and on recently infected quarters.

Selection of the infective strain may be occurring within the teat canal, with different strains more suited to access through the teat canal compared to others. However, selection of the infective strain by this natural defence cannot be further explored until the mechanism by which this pathogen

generally gains access through the teat canal is better understood (Lacy-Hulbert & Hillerton, 1995).

Selection of the infective strain could be occurring within the mammary gland itself. In this scenario, multiple strain types on the teat end gain entry through the teat canal into the mammary gland. Then once inside, selection pressures arising from competition between strains, the immune response, changes in available nutrients and/or the flushing effects of milk may select for one or a few of the strains that are able to rapidly adapt and establish within the gland under these conditions. The main aim of this study was to determine if selection of a single infective strain can occur within the mammary gland.

4.4.2 Development of infection

Several factors may be involved in determining which strains survive and multiply over any other that are present. These include the ability of individual strains to actively grow in milk (Rambeaud *et al.*, 2003), adhere to mammary epithelia and resist the flushing effect of the milk (Almeida *et al.*, 2003; Frost *et al.*, 1977), evade the host immune response (Hill, 1988; Tamilselvam *et al.*, 2006) and finally, compete with other strains that may be present (Riley & Gordon, 1999).

All five strains selected for this study were capable of establishing an intramammary infection as defined by an increase in somatic cells (SCC), electrical conductivity (EC) and presence of bacteria within the milk across the course of the trial. While SCC and EC of all infused quarters significantly increased above control quarters from 24 h after infusion, the actual number of somatic cells in the milk varied widely, even when the same strain was present or between quarters of the same cow, indicating the unique immune response of each udder quarter. Zadoks *et al.* (2003) also found no significant associations between the quarter milk SCC and the

strain of *S. uberis* that was present. Thus the cow itself may influence the development of infection and perhaps the selection of a predominant infective strain. Indeed, Burvenich *et al.* (2003) noted that the severity of mastitis caused by *Escherichia coli* was determined by cow factors rather than pathogenicity of the microorganism.

Zadoks *et al.* (2003) considered a quarter to be infected when ≥1000 cfu/ml of *S. uberis* were cultured from a single sample or when ≥100 cfu/ml were cultured from three consecutive milk samples. Using this criteria, all quarters were infected at the second milking, within 24 hours of infusion, when ≥1000 cfu/ml of *S. uberis* were detected in all quarters, with corresponding increases in foremilk SCC and EC.

Although initially high, total bacterial numbers did not change significantly across the days of the trial. In contrast, Rambeaud *et al.* (2003) observed the level of the challenge strain to be very low in milk for the first 42 hours after experimental infusion followed by a 1500-fold increase in numbers between 48 and 120 h later, suggesting that an adaptation time or lag phase was required before *S. uberis* could actively grow within the mammary gland. In the present study, this lag phase was not apparent, suggesting that these five strains easily adapted for survival and growth in the mammary gland.

Bacterial numbers reached a maximum of 4.5×10^8 cfu/ml in one quarter during the course of infection, much higher than that observed in other studies. Fang *et al.* (1998) and Rambeaud *et al.* (2003) reported maximal levels of 2.6×10^5 and 2.0×10^5 cfu/ml respectively of the challenge strain, despite the initial inocula being higher than that used in the present study. It has been noted that the stage of lactation influences bacterial growth (Oliver, 1991; Rambeaud *et al.*, 2004); the study by Rambeaud *et al.* (2003) was conducted using cows in early lactation compared to the present study where cows were in late lactation. These cows would be producing much

less milk and this may have influenced the number of bacteria observed. Also both of these earlier studies were conducted using different strains of *S. uberis* which may not have been as well adapted for growth within the mammary gland as the strains used in the present study.

Although the total number of bacteria in milk ranged from $3.0x10^2$ to $9.5x10^5$ cfu/ml less than 24 hours after infusion, clinical signs were not apparent in the majority of quarters until the fifth milking (2.5 days after infusion). Similarly, Hill (1988) observed that cows inoculated with a single strain excreted large numbers of bacteria in their milk before the appearance of obvious signs of clinical mastitis and Fang *et al.* (1998) noted that clinical signs were not apparent until 48 to 72 hours after experimental challenge.

Hill (1988) found a significant difference in infectivity and virulence between different strains of *S. uberis*; however, the five strains used here were equally capable of infecting the mammary gland, despite two of the strains being isolated from environmental sites. The mixture of all five strains was equally as capable of establishing an infection as the individual strains with no significant difference in SCC and EC between SU5-infused and individual strain-infused quarters.

4.4.3 Strain predominance

A single strain predominated in nine of the ten quarters that were infused with the mixture of five strains, indicating that selection of a single strain can occur within the mammary gland when multiple strains gain entry. This may explain why, with such strain diversity in the environment and on the teat skin, only one strain or at most two strains are detected from clinical or subclinical mastitis cases (Wieliczko *et al.*, 2002; Zadoks *et al.*, 2003). The same strain did not always predominate however, with strain C prevailing in five of the ten quarters and strains D or E prevailing in two other quarters

respectively. Interestingly, strain A could not be detected in the milk of any SU5-infused quarters by the last day of the trial, yet when individually infused, the development of infection was comparable to all other strains in regard to SCC, EC and number of bacteria. This suggests that strain A was not able to establish infection in the mammary gland when other strains were present.

Strain C was the most prevalent predominant strain and it is possible that this was the most virulent strain. However, as it did not predominate in all quarters receiving the mixed inocula, other factors, relating to the cow itself or the unique quarter immune response, may have influenced selection of the final infective strain.

4.4.3.1 Adapting to growth within the mammary gland

It has been noted that some strains may require a longer period of adaptation to the mammary environment, after which they are able to colonize and infect the mammary gland (Rambeaud *et al.*, 2003). Although this may explain why some strains are more capable of establishing infection in the mammary gland than others, in the present study, all five strains were detected in similar numbers less than 24 hours after they were individually infused into the mammary gland. Therefore the ability to quickly adapt and grow may not have created the selection pressure for the predominance of particular strain types in this study.

4.4.3.2 Evasion of the host immune response

The host immune response aims to eliminate all invading pathogens; therefore the ability to evade phagocytosis would be advantageous for survival within the mammary gland. Strains of *S. uberis* which differ markedly in their ability to infect the mammary gland have also shown differences in their ability to resist phagocytosis and killing by bovine neutrophils *in vitro* (Grant & Finch, 1997). The five strains chosen in the

present study may also have different virulence factors that allow evasion of the host immune response. The increase in somatic cells observed in the milk 24 hours after infusion (M2) was followed by a decline in the number of strain types detected in SU5-infused quarters at the next milking (M3), suggesting that this initial influx may have reduced or eliminated strains that were unable to quickly adapt and evade the immune cells. However, despite a sustained immune response, some strains were able to return to detectable levels, indicating that they had only been reduced by the initial immune response, not eliminated. The differences observed between strains when individually infused, rather than in the presence of other strains, suggest that expression of virulence factors that enable infection may differ in a competitive situation.

Mechanisms for evading the host immune response have been investigated in *S. uberis*, although there is still controversy over the actual importance of some of these factors during a natural infection event. The first example is production of a hyaluronic acid capsule, which may increase the chances of surviving phagocytosis by the small number of neutrophils usually present in milk when the pathogen gains access to the gland (Hill, 1988). However, subsequent studies have questioned the relevance of the hyaluronic acid capsule and other extracellular factors may be more important for enabling resistance to phagocytosis (Field *et al.*, 2003; Leigh & Field, 1991).

All five strains contained the genes required for the production of the hyaluronic acid capsule; however, it is unknown whether this capsule was actually produced by each of these strains when they were infused either individually or together into the mammary gland. The colony phenotype of the five strains when grown on blood agar indicated that there may be differences in capsule production between strains. Strains C and E appeared mucoid on this media compared to strains A, B, and D, indicating that, under these conditions, less capsule may be produced by these three

strains. Although this does not reflect whether a capsule was produced during infection of the mammary gland, it indicates that there may be differences in the expression of capsule between the strains. Matthews *et al.* (1994b) demonstrated that medium supplemented with milk and lipid components actually enhanced production of *S. uberis* capsule, therefore it was possible that strains C and E had extra protection from phagocytosis by enhanced production of a hyaluronic acid capsule, and may help to explain why these two strains predominated in five and two quarters respectively by the end of the trial.

Strain E may be superior in resisting the initial onslaught of immune cells. The two SU5-infused quarters where strain E predominated had an elevated SCC (>2x10^5 cells/ml) prior to infusion. Strain E was also found to persist in three of the four individually infused quarters, with recovery observed 36 days after infusion, indicating that this strain was able to resist phagocytosis and other immune responses, as well as the presence of administered antibiotics. All strains had proved to be susceptible to the antibiotics used to treat infections when tested *in vitro* prior to the trial. This evasion of the immune response and later re-establishment of infection may have been facilitated through internalisation of the bacteria into mammary epithelial cells or macrophages during the initial infection, with subsequent re-emergence when the immune response subsided.

Adherence and internalization of *S. uberis* into mammary epithelial cells has been identified as a potential mechanism for evasion of the immune response by a number of authors (Almeida *et al.*, 1999a; Almeida & Oliver, 2001; Matthews *et al.*, 1994a; Oliver *et al.*, 1998a). Additionally, *S. uberis* has been found to survive and replicate within macrophages (Denis *et al.*, 2006), which would also provide another protective environment. As most of these studies have been conducted *in vitro* it is unknown if *S. uberis* is internalized into mammary epithelial cells or can survive within

macrophages during a natural infection event. Frost *et al.* (1977) noted that some but not all strains of *S. uberis* were capable of adherence, which may indicate strain-specific differences in adherence and internalisation into mammary epithelial cells. Therefore, strain E may have an enhanced ability to internalise within mammary epithelial cells compared to the other four strains, which may explain why it was more resistant to an elevated immune response and persisted in the mammary gland over time.

As all five strains were equally capable of infecting the mammary gland when infused individually, the ability to evade the immune response may not entirely account for the predominance of strains C and E in SU5-infused quarters. Also, strain D predominated in two of the quarters, yet this strain did not appear to have any particular characteristics that may explain this; the presence of a hyaluronic acid capsule was not obvious when cultured on blood agar and the two quarters where this strain predominated did not appear to have any difference in SCC or EC compared to other infused quarters. Therefore, strain D may have some other advantage that can not be explained by evasion of the immune system or ability to adhere or internalise into mammary epithelial cells.

4.4.3.3 Direct competition between bacteria

In addition to virulence factors, the production of antimicrobial peptides (bacteriocins) may have facilitated direct competition between the five strains (Riley & Gordon, 1999). All five strains produced uberolysin, a cyclic bacteriocin characterized by Wirawan *et al.* (2007), when grown on blood agar. However, strains A, D and E appeared somewhat susceptible to this substance when it was produced by strain B, indicating that immunity to uberolysin varied between strains. Strain C was particularly interesting as it produced nisin U in addition to uberolysin and strongly inhibited the growth of all four other strains on esculin blood agar. This additional bacteriocin, previously characterised in *S. uberis* by Wirawan *et*

al. (2006), may have contributed to the predominance of strain C in the SU5-infused quarters.

Unfortunately, however, it is unknown if the bacteriocins were produced during the infection situation. Culture media has been shown to affect bacteriocin production: Al-Qumber and Tagg (2006) found that strains of bacilli that were particularly inhibitory to most other strains had maximum activity when grown on solid media, while unshaken cultures in various liquid media failed to produce detectable inhibitory activity. Also, Yan *et al.* (2003) found that the production of antimicrobials by some strains of bacilli was dependent on biofilm-specific signalling associated with critical cell densities in cultures. Therefore, further experiments are required to determine if uberolysin and nisin U are produced by these strains during experimental infection of the mammary gland.

4.4.3.4 *In vitro* experiments

Culture of the five strains together in UHT milk *in vitro* did not reflect the results obtained after experimental infection of the mammary glands. While one strain tended to predominate *in vivo*, the same strains could co-exist and multiply in UHT milk over a 74 hour time period, suggesting that eliminating the influence of the immune response and unique cow factors also removes the selection pressure that leads one strain to predominate. Matthews *et al.* (1994b) noted that the growth media can affect the expression of virulence factors by bacteria; therefore, it is possible that the differences between UHT and fresh milk may have had an effect on the expression of virulence factors and bacteriocins that gave some of the strains a superior advantage during the infection trial.

4.4.4 Conclusions

In conclusion, all five strains and a mixture of these strains were capable of establishing infection within the mammary gland. The two strains originally isolated from the environment (strains B and D), were equally as capable as those from clinical mastitis cases (strains A, C and E) to infect the mammary gland. However, differences were observed between the strains when they were infused as a mixture into the gland. Strain C fared particularly well in this competitive situation and predominated in five of the ten quarters, while strain A was reduced to undetectable levels by the end of the trial in all SU5-infused quarters. Strains D and E predominated in two quarters respectively. The predominance of strain C may be attributed to an enhanced capsule production and/or production of nisin U which could inhibit the growth of all other strains. Likewise, predominance of strain E may be attributed to capsule production and an unknown mechanism for evasion of the host immune response and persistence within the mammary gland. The predominance of strain D was peculiar as no particular factors were apparent for this strain. The two quarters where it predominated may have aided in the exclusive survival of this strain. It must also be noted that these experiments were performed during late lactation in these cows; perhaps different results may have been observed had the trial been conducted during early lactation.

Although certain strains predominated during experimental infection, each strain was equally capable of surviving and multiplying when grown together in UHT milk, suggesting that no direct competition was occurring between the five strains *in vitro*. Further work is required to investigate the presence of virulence factors and bacteriocin production by these strains; however, this study has shown that a single strain can predominate when multiple strains gain entry to the mammary gland, which may explain why only one or at most two strains are detected in the milk with clinical or subclinical mastitis.

4.4.5 Future work

The experiments performed in the present study have shown that selection of a predominant strain can occur within the mammary gland. However, many questions have arisen from this work that need addressing. Further studies could involve repeating the infection trial using the same strains but with different cows, to determine if strain C again predominates in the majority of quarters. Also, repeating the same trial at different stages of lactation, for example early lactation, may give different results. A different set of five strains could also be used to determine if one will always be superior to the others or whether, in the choice of strains in the present study, a particularly virulent strain was found by chance. Isolates could be obtained from the teat skin and used to experimentally infect the gland either individually or in a mixture, which would more closely mimic the natural infection event. Also, including a strain within the set of five that did not contain any of the known bacteriocins may indicate whether these antimicrobial peptides are important in the selection of a predominant strain within the mammary gland.

The expression of potential virulence factors by these five strains also needs to be examined. In particular, the ability of strain E to evade the immune response and persist within the mammary gland for extended periods of time despite antibiotic treatment needs to be investigated. The MAC-T mammary epithelial cell line (Huynh *et al.*, 1991) could be utilised for adherence and internalisation assays to compare the ability of all five strains to adhere, internalise and persist within mammary epithelial cells.

The expression of known virulence genes thought to be required for growth in milk (e.g. PauA), adherence and internalisation into mammary epithelial cells, and evasion of the immune response could also be investigated for each of the five strains to determine if any of these genes, or the regulation of them, is important for establishing infection. In particular the production

of a hyaluronic acid capsule by each of these strains may shed light on whether this controversial virulence factor is indeed important during the establishment of infection in the mammary gland.

Additionally, the production of uberolysin and nisin U during experimental infection and *in vitro* culture needs to be explored further to determine whether these bacteriocins are involved in direct competition between strains of *S. uberis*. During an infection trial the milk from quarters infused with individual strains or a mixture of strains could be tested for the presence of these antimicrobial peptides. This would determine if bacteriocins are produced and if multiple strain types need to be present to elicit their production.

In regard to the *in vitro* experiments, it would perhaps be more useful to use sterile fresh milk after removal of somatic cells rather than UHT milk to better mimic the infection situation. As the strains may express different virulence factors and bacteriocins in UHT milk rather than fresh milk, this may explain why different results were obtained between the infection trial and the *in vitro* cultures of the five strains.

Such studies would help to confirm and elucidate the conclusions reached in the present study, and provide further insights into the mechanism of infection of the mammary gland by *S. uberis*.

Chapter 5:

Streptococcus uberis ecology and mastitis

5.1 *Streptococcus uberis* ecology

Although an environmental pathogen, *S. uberis* can also be isolated from cow faeces and body, including skin, genital tract, rumen, tonsils, lips, udder and teat skin (Buddle *et al.*, 1988; Cullen & Little, 1969; Kruze & Bramley, 1982; Razavi-Rohani & Bramley, 1981; Zadoks *et al.*, 2005b). Isolates from these different locations may be the same strain type (Chapter 3) (Pullinger *et al.*, 2006), which is consistent with the hypothesis that infections arise from contact of the teat end with pathogens located in environmental reservoirs.

Due to a lack of sampling around similar time points it was difficult to determine transfer of strains between each of these sites. Also, because of problems with the REP-PCR strain typing method and analysis of band patterns, it was difficult to draw firm conclusions about *S. uberis* ecology in this study. However, from the observations made in Chapter 3 it was possible to propose a model of the most likely route of *S. uberis* transmission between the dairy environment, teat skin, cow body and mammary gland (Figure 5.1).

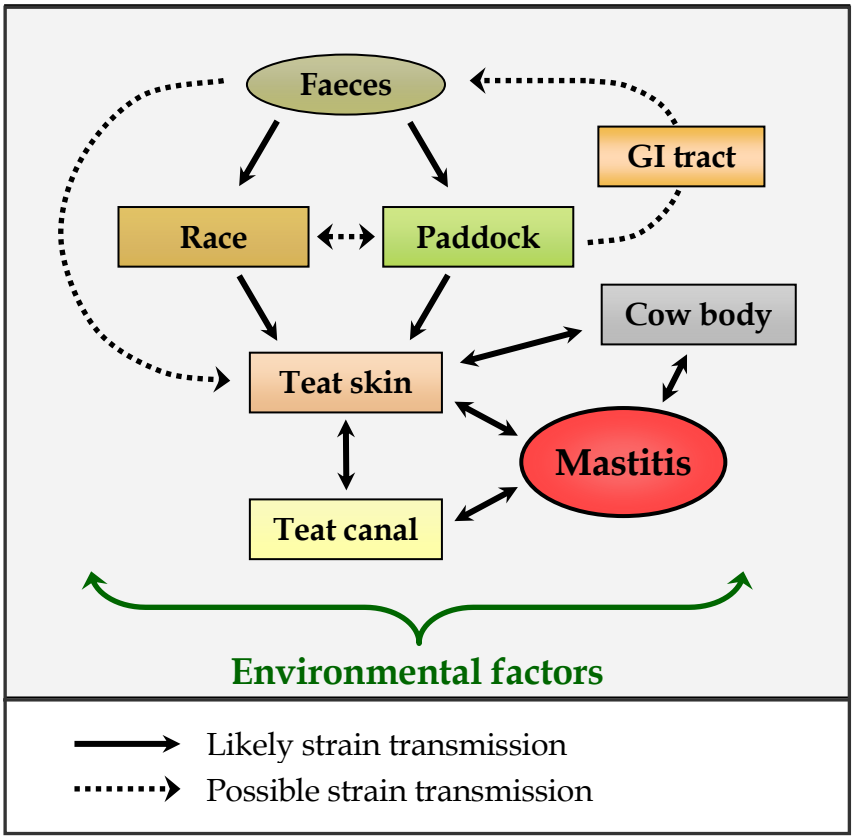


Figure 5.1. Potential transmission of *S. uberis* strains on the dairy farm.

Central to the model is the dairy environment, including farm races, paddock grass and soil. Of these, the high traffic farm races are likely to be a major reservoir of *S. uberis* strains, with high strain diversity observed at this site. Others have also noted that areas where cows gather or that experience high cow traffic are particularly likely to pose a high risk of *S. uberis* exposure (Cullen & Little, 1969; Zadoks *et al.*, 2005b). Lopez-Benavides *et al.* (2005) found that weather conditions affected the survival of *S. uberis* in this part of the dairy environment, with higher numbers observed on farm races when air and soil temperatures were low, soil moisture was high and solar radiation levels were low. These weather conditions also corresponded to the calving period where rates of clinical mastitis were high, suggesting a possible association between level of *S. uberis* in the environment and mastitis. However, since cows are not usually required to lie down or calve on farm races, the specific detail of the

mechanisms by which bacterial strains move from the farm race material onto and into teats remains unknown.

The most likely mechanism for creating high strain diversity in the environment was via excretion of *S. uberis* in faeces. In addition, strains could be spread from races into paddocks through the movement of race material on the cows' feet. In support of this, Lacy-Hulbert *et al.* (2006) found that *S. uberis* was only detected in paddocks after grazing, suggesting that cow presence was required for spreading of this pathogen within the environment. In contrast, Cullen (1966) noted that faecal contamination was unlikely to play a role in the spread of *S. uberis* due to the low incidence of positive rectal swabs.

Detection of the same strain repeatedly through time from faecal material of some animals in the herd suggests that the GI tract may be colonised by individual strains, as suggested previously (Kruze & Bramley, 1982). Therefore, *S. uberis* may be cycled from the environment through the GI tract and excreted in the faeces due to cows eating contaminated pasture or by licking of other contaminated areas, contributing to the strain diversity observed. There were few indications that the faeces may directly contaminate the teat skin with *S. uberis*, although faecal material is a common contaminant of teat skin. This link can only be recognized as a possible mode of strain transmission.

Despite an overall lack of correlation between teat skin and infective strains, the most likely connection between strains in the dairy environment and intramammary infections was via contamination of the teat skin. This contamination could be via direct contact between teats and contaminated grass and paddock material, or soiled legs and hooves, or via splashing or flicking of contaminated race material onto cows' teats when they are walked around the farm. Cow and udder hygiene has been linked to the

incidence of mastitis (Schreiner & Ruegg, 2003; Ward *et al.*, 2002) and the use of teat sanitation in the late dry period, prior to calving (Lopez-Benavides *et al.*, 2006a) or pre- and post-milking teat sanitation for lactating cows (Pankey *et al.*, 1987), tends to result in a lower incidence of mastitis and a lower somatic cell count in the herd (Barkema *et al.*, 1998).

Although intramammary infections may develop through the presence of *S. uberis* on the teat skin, transfer of strains between teat skin and the mammary gland is likely to be bidirectional as the teat skin may also become contaminated post-infection. *Streptococcus uberis* can be found within the teat canal of both infected and uninfected mammary glands, providing the link between *S. uberis* on the teat skin and infection of the mammary gland. However, further strain typing of isolates from the teat canals of uninfected quarters would shed light on the contamination or colonisation of this site and association with strains on the teat skin.

It was also likely that the cow body may become contaminated through contact with the environment; however, this was not studied in detail as cow body and environmental samples were collected at different times. Location of the infective strain on the cow's body may indicate that mastitis can develop via direct contact between the teats and a contaminated or colonised part of the cow's body. However, the study reported here could not rule out the possibility that body sites became contaminated with infected milk after the infection event, rather than prior to establishment of an intramammary infection.

Environmental factors such as farm management systems may also impact on the transmission of *S. uberis* from the dairy environment on to cows and between cows. Strategies such as teat spraying, pasture management and nutrition of animals, among others, may all influence the overall numbers of *S. uberis* within the dairy environment and on the cows, thereby effecting

transmission of this pathogen. In this study, only one farm was sampled, therefore the presence of *S. uberis* and transmission of this microorganism may vary on other farms with different management practices.

5.2 Selection of the 'infective' strain

Infection with multiple strains of *S. uberis* within the same quarter is not common (Oliver *et al.*, 1998a; Phuektes *et al.*, 2001; Wieliczko *et al.*, 2002), although does occur occasionally (Chapter 3.3.1.1). At some point in the initial infection process, selection of a dominant infective strain must be occurring. In Chapter 3 it was noted that the teat skin of infected quarters usually yielded the infective strain at the exclusion of all others, whilst the teat skin of uninfected quarters often yielded multiple strains. Therefore, it is possible that one strain, better suited for survival at this location, predominates on the teat skin and then gains entry to the mammary gland to cause mastitis. However, the presence of this strain on the teat skin may also have been due to post-infection contamination or colonisation of the teat skin.

Experimental infection of mammary glands with multiple strains of *S. uberis* (Chapter 4) revealed that a single strain predominates over time in the majority of cases, indicating that selection of the infective strain may occur within the mammary gland itself. This slightly artificial scenario created a situation whereby multiple strains on the teat skin simultaneously gained entry to the mammary gland. It was observed that, within a few days, some strains demonstrated an enhanced capability to grow and out-compete other strains in the milk, adhere within the mammary gland and evade the host immune response. This process may have been aided by the production of bacteriocins, which inhibited the growth of non-producing strains.

Wang *et al.* (1999) thought it unlikely that virulence would be associated with any specific strain type, while Hill (1988) noted that two different strains differed in their ability to infect the mammary gland. Each of the five strains that were studied (as reported in Chapter 4) was able to establish infection when individually infused into the mammary gland; however, when in a competitive situation, some strains appeared more capable than others at infecting the mammary gland. Three of the strains were originally isolated from clinical mastitis cases, yet while two of these strains (C and E) predominated in five and two of the ten infused quarters respectively, the third strain (A) could only be found in low numbers and was undetectable by the end of the study period. These differences in ability to infect the gland in competitive situations suggest that virulence factor expression differs between strains and may be affected by the presence of other strains within the mammary gland.

It is generally thought that infection with *S. uberis* is mainly due to opportunistic infection because of the great variety of strains present on the cows and in the immediate environment (Douglas *et al.*, 2000; Khan *et al.*, 2003). The successful experimental infection of mammary glands with strains originally isolated from the farm race and paddock soil (Chapter 4) supports this. Also, in a competitive situation the soil strain actually predominated over all other strains in two of the ten mammary glands infused. However, while the contamination of teat skin with *S. uberis* strains may be opportunistic, once inside the mammary gland selection of the infective strain appears to occur. Different strains predominated in different cows in the study, suggesting that the host response may play an important role in selection of the infective strain. Indeed, when the same five strains were cultured together in UHT milk, all strains could equally grow and no selection of a superior strain occurred.

5.3 Persistent *S. uberis* infections

The same strain of *S. uberis* could be isolated from recurrent clinical mastitis cases and persistent subclinical cases (Chapter 3), suggesting that *S. uberis* is capable of persisting within the mammary gland, avoiding the host immune response and antibiotic treatment (of clinical mastitis cases) for extended periods of time. This persistence may be facilitated by internalisation into mammary epithelial cells (Almeida *et al.*, 1996; Matthews *et al.*, 1994a; Tamilselvam *et al.*, 2006) or macrophages (Denis *et al.*, 2006).

Some strains may have an enhanced ability to adhere and internalize within epithelial cells and therefore persist within the mammary gland. Frost *et al.* (1977) noted that some, but not all, strains of *S. uberis* were capable of adherence and Tamilselvam *et al.* (2006) also found that strains of *S. uberis* known to induce persistent infections *in vivo* were able to persist intracellularly for extended periods of time during *in vitro* experiments. One of the strains (E) in the experimental infection study described in Chapter 4 was found within three of the four mammary glands 36 days after they were originally infused. Therefore, despite an elevated immune response during the study and subsequent antibiotic treatment, this strain appears to have persisted within the mammary gland, unlike the other four strains studied, which were eliminated by antibiotic treatment.

5.4 Proposed mechanism of infection and persistence within the mammary gland

Based on previous studies and the results obtained in this study, selection of the predominant 'infective' strain and a mechanism of persistence within the mammary gland can be proposed as follows:

1. *Streptococcus uberis* is spread around the dairy environment via the faeces and cow movement along races and in paddocks. This

pathogen is capable of replicating within the soil/race material resulting in a high diversity of strains.

2. Strains from the environment and, occasionally, faeces are transferred to the teat skin of cows through contact with contaminated race material or paddocks.

3. The teat skin becomes colonized by multiple strain types and two scenarios are hypothesised:

    a. Competition occurs between strains on the teat skin; one strain predominates and exclusively colonizes the teat skin. This then gains access through the teat canal to the mammary gland.

    b. Multiple strains gain access through the teat canal into the mammary gland.

4. During establishment of infection, two outcomes are possible, depending on the number of strains that gained entry to the mammary gland:

    a. Only one strain gains access through the teat canal, this multiplies and engenders a host response. Mastitis develops.

    b. Multiple strains gain entry to the mammary gland. Competition occurs resulting in one dominant strain that becomes the infective strain. (This is thought to be the more likely scenario based on strain diversity within the environment and the results of the intramammary challenge study).

5. During the infection of the mammary gland three scenarios are possible:

    a. All bacterial cells are detected and killed by the immune response and/or infused antibiotics. The infection is cleared and bacteria cannot be detected in the milk from this quarter.

    b. High numbers of bacteria present in the milk are able to contaminate or colonise the teat skin of that quarter and other areas of the cow body, providing a new niche of subsequent infectious pathogens.

    c. Some bacterial cells adhere to and invade epithelial cells and macrophages, thus evading the host response and any

administered antibiotic. They remain within the host cells until the immune response has subsided.

6. At a later date, another infection develops and the same strain is detected. This could be due to either:

   a. The strain contaminating/colonizing the teat after the first infection event re-gains entry into the mammary gland and provokes an immune response.

   b. Emergence of the bacterial cells from within the mammary gland epithelial cells and/or macrophages, which then multiply and the immune response is re-activated. (This is thought to be the most likely scenario for the majority of medium to long term persistent infections of around 1 – 4 months duration).

Further work is required before the actual mechanism of infection, selection of the infective strain and persistence within the mammary gland can be confirmed. This work would need to include strain typing of teat canal isolates from otherwise uninfected quarters, which may shed light on whether single or multiple strains of *S. uberis* can be found at this location. Typing of isolates from the teat skin during and after resolution of infection may reveal whether the infective strain remains exclusively on the teat skin for extended periods of time.

5.5 Conclusions

This study has revealed that the same strains can be found in the dairy environment, faeces, teat skin and intramammary infections. No obvious reservoirs of particularly virulent strains were apparent and this study fell short of identifying direct transfer between each of the studied sites, although it was possible to develop a proposed model of strain transmission. Further work is required to ascertain if this model is correct.

The results support previous studies in showing that mastitis caused by *S. uberis* is largely initiated through opportunistic infection due to contact with

the contaminated environment, although some contagious transmission of strains can occur. Despite this, some strains appear to be more virulent than others when present within the mammary gland along with other strains of *S. uberis*. This superior virulence cannot be attributed to strains from different niches however, as environmental isolates were equally capable of infecting the mammary gland as those known to cause mastitis, even under competitive situations. In addition to strain-specific factors, the individual cow response plays an important role in the development of infection and the selection of a predominant infective strain.

Previously, *Streptococcus uberis* was classified as an environmental pathogen; however, in this study this microorganism was also commonly found on the cow itself and appeared to persist within the mammary gland over months at a time. Therefore, as *S. uberis* appears to have multiple niches and can readily adapt to life both in the dairy environment and within the mammary gland, it can not strictly be classed as an environmental pathogen.

Appendix 1: Dairy farm and animals

The majority of *S. uberis* isolates were obtained from the Dexcel Lye farm, which is located near Hamilton, in the North Island of New Zealand. This farm milks, on average, 340 Friesian-Jersey cross cows off a 90 ha area. All cows are maintained on ryegrass/white clover pasture, with grass silage used to fill feed deficits. Farm races are 5 m wide and consist of a 400 mm layer of 'rotten rock' beneath a 50 mm layer of pit sand. They are used to move stock between paddocks and the farm dairy for milking twice a day. All cows calve in early spring, from July onwards, and are then dried off again in the autumn, in late April or early May. For lactating animals, bacteriology is carried out routinely on foremilk from individual quarters at four times during lactation: before the first milking after calving, on two occasions in mid and late lactation, and at dry-off. Additional samples were collected at detection of new cases of clinical mastitis, prior to treatment with antibiotics.

Appendix 2: Isolation of *S. uberis*

Isolates of *S. uberis* were obtained from various sources including milk from cows with clinical or subclinical mastitis, vat milk, teat skin and teat canal keratin, various anatomical locations on the cows' body and from the dairy environment including paddock material (soil and grass) and farm races. The Ruakura Animal Ethics Committee approved all animal manipulations (RAEC #4777) and samples were collected by trained field technicians (Dexcel, Ltd.).

A2.1 *Streptococcus uberis* selective media

All samples, other than milk samples, were plated onto a selective inulin based media for *S. uberis* developed by the University of Waikato and Dexcel and incubated at 37°C for 48 h – 72 h as described in Pullinger *et al.* (2006). Colonies were provisionally identified as *S. uberis* on the selective

media when they were esculin-positive (observed by using a UV transilluminator) and fermented inulin (producing khaki-coloured colonies). Isolates were further confirmed as *S. uberis* by subculture on Trypticase agar supplemented with mannitol and 4-methylumbelliferyl β-D-glucuronide with bromocresol purple as the pH indicator (Pullinger *et al.*, 2006) and by PCR amplification of the 16S-23S rRNA intergenic spacer region (Forsman *et al.*, 1997) as described in Chapter 2.2.8.

A2.2 Isolation of *S. uberis* from milk samples

Milk samples were aseptically collected from cows with clinical or sub-clinical mastitis. Clinical mastitis (CM) was diagnosed when cows presented at milking time with visible clinical symptoms (i.e. clots, discoloured milk) whereas sub-clinical mastitis (SCM) was diagnosed when cows did not have abnormal physical changes in milk appearance yet were found to be positive for *S. uberis* in the bacteriological foremilk samples and had foremilk somatic cell counts (SCC) greater than 200,000 cells/ml. All milk samples were collected using aseptic techniques and analysed according to guidelines provided by the National Mastitis Council (USA). Essentially this involved scrubbing of the teat ends with cotton wool swabs soaked in 70% ethanol before stripping the foremilk sample (20 – 30 ml) into a sterile container. A 10 µl volume of this sample was then plated directly onto one quarter of an Esculin Blood agar (EBA; Fort Richard Laboratories, Ltd. Otahuhu, NZ) plate using a sterile loop and incubated at 37°C for 48 hours (National Mastitis Council, 1999).

A2.2.1 Vat milk

Vat milk samples were aseptically collected in a sterile bottle, 100 µl spread plated on *S. uberis* selective media and incubated at 37°C for 72 h (A2.1).

A2.3 Isolation from teat skin and teat canal

A2.3.1 Teat skin

The skin of the teat barrel and teat end (teat orifice) of both infected and uninfected quarters were sampled by rigorous scrubbing of the teat using sterile cotton-tipped swabs moistened in 0.1% peptone diluent (Fort Richard Laboratories Ltd., Otahuhu, NZ). In addition to swabbing of individual teats, on some occasions one swab was used to sample either both front teats, both back teats, or all four teats. The swabs were placed into 1 ml 0.1 % peptone diluent, shaken vigorously, and 100 µl spread plated onto selective media (see A2.1). Where milk was also collected, teat skin was first swabbed then cleaned thoroughly before milk samples were aseptically collected as described in A2.2.

A2.3.2 Teat canal

Teats were thoroughly cleaned by scrubbing with 70% ethanol-soaked cotton wool and teat canals reemed using a sterile 1.0 mm diameter interdental brush (DentalPro; Jacks Co. Ltd., Osaka, Japan). Brushes were shaken in 0.1% peptone diluent to remove bacteria and 100 µl spread plated onto *S. uberis* selective media and EBA. Where necessary, a serial dilution of each sample was prepared in peptone diluent to obtain countable numbers of colony forming units (30-300 cfu). Plates were incubated at 37°C for 48-72 hours before counting *S. uberis* colonies on selective media (cfu/brush) and evaluation of other bacterial species present on EBA.

A2.4 Isolation from the cow body

Various regions of the cow body were sampled for the presence of *S. uberis*, including udder skin, hocks of the hind legs, coronet band of the hind feet, lips, nostrils, and tail swish. All animal manipulations were approved by the Ruakura Animal Ethics Committee (RAEC #4777, modification #95). Each site was sampled using a cotton-tipped swab which was then shaken in 1 ml 0.1% peptone diluent (foot swabs) or 1 ml sterile skimmed milk (all

other swabs) before spread plating 100 µl onto selective media (see A2.1). Significantly greater colony forming units were achieved when swabs were diluted in sterile skimmed milk immediately before plating.

A2.5 Isolation from faeces

Faecal isolates were obtained by swabbing the rectum of cows using sterile cotton-tipped swabs moistened in sterile peptone diluent. Rectal swabs were treated in the same way as teat skin swabs (A2.3.1) before plating on selective media.

Mixed faecal samples were collected from the yard or paddock by scooping approximately 100 g of faecal material into a sterile container and mixing. In the laboratory, 1 g of faeces was added to 9 ml of 0.1% peptone diluent, vortex mixed and 100 µl spread plated on selective media (A2.1).

A2.6 Isolation from environmental sites

A2.6.1 Farm races

Samples of race material were collected by scooping approximately 10 g of race surface material from an area of approximately 25 cm^2 into a sterile container. On return to the laboratory, 1 g of race material was added to 9 ml of 0.1% peptone diluent and shaken vigorously for 2 min before spread plating 100 µl onto selective media (A2.1) and incubating for 72 hours.

A2.6.2 Paddock soil

Approximately 5 g of paddock surface soil and soil 5 cm below the surface was collected into sterile plastic vials after clearing a 100 cm^2 area of grass. Each sample (1 g of wet weight soil) was processed as described for farm races (A2.6.1).

A2.6.3 Paddock grass

Leaves of grass were cut from a 200 cm^2 area with scissors cleaned with 70% ethanol, leaving a 5 cm grass residual. The cut grass was placed in a clean plastic bag and transferred to the laboratory. Approximately 1 g of the grass leaves were cut into smaller pieces (~1-2 cm in length) and mixed vigorously for 2 min in 9 ml of 0.1% peptone diluent before spread plating 100 µl on selective media (A2.1).

Appendix 3: BLAST search with BOX and ERIC primers

A3.1 BLAST results

The *S. uberis* 0140J genome was searched using BLAST at the Sanger Institute (http://www.sanger.ac.uk/Projects/s_uberis) and primer sequences BOXB1, BOXC1, BOXA1R, ERIC2 and ERIC1R as given in Chapter 2.2.6. BLAST results were as follows:

A3.1.1 BOXB1 primer

```
Database:  SU.dbs
           1 sequences; 1,852,352 total letters.
                                                                  High
Probability
Sequences producing High-scoring Segment Pairs:                   Score  P(N)
N

Streptococcus_uberis_strain_0140J                                   55  0.98
1

>Streptococcus_uberis_strain_0140J
[Full Sequence]

        Length = 1,852,352

Minus Strand HSPs:

 Score = 55 (14.3 bits), Expect = 4.0, P = 0.98
 Identities = 11/11 (100%), Positives = 11/11 (100%), Strand =
Minus / Plus
[HSP Sequence]

Query:     11 GAACTGACGAA 1
              ||||||||||
Sbjct: 527611 GAACTGACGAA 527621
```

A3.1.2 BOXC1 primer

```
                                                                  High
Probability
Sequences producing High-scoring Segment Pairs:                  Score  P(N)
N

Streptococcus_uberis_strain_0140J                                  60   0.98
1
>Streptococcus_uberis_strain_0140J
[Full Sequence]
        Length = 1,852,352

Plus Strand HSPs:
 Score = 60 (15.1 bits), Expect = 4.0, P = 0.98
 Identities = 12/12 (100%), Positives = 12/12 (100%), Strand =
Plus / Plus
[HSP Sequence]

Query:      6 CTAGCTTCCTAG 17
              ||||||||||||
Sbjct: 334147 CTAGCTTCCTAG 334158


Score = 55 (14.3 bits), Expect = 4.0, P = 0.98
 Identities = 11/11 (100%), Positives = 11/11 (100%), Strand =
Plus / Plus
[HSP Sequence]

Query:       3 CGGCTAGCTTC 13
              ||||||||||||
Sbjct: 1016438 CGGCTAGCTTC 1016448

 Score = 55 (14.3 bits), Expect = 4.0, P = 0.98
 Identities = 11/11 (100%), Positives = 11/11 (100%), Strand =
Plus / Plus
[HSP Sequence]

Query:     11 TTCCTAGTTTG 21
              ||||||||||||
Sbjct: 175998 TTCCTAGTTTG 176008

Minus Strand HSPs:

 Score = 55 (14.3 bits), Expect = 4.0, P = 0.98
 Identities = 11/11 (100%), Positives = 11/11 (100%), Strand =
Minus / Plus
[HSP Sequence]

Query:      21 CAAACTAGGAA 11
              ||||||||||||
Sbjct: 1512272 CAAACTAGGAA 1512282

 Score = 55 (14.3 bits), Expect = 4.0, P = 0.98
 Identities = 11/11 (100%), Positives = 11/11 (100%), Strand =
Minus / Plus
[HSP Sequence]

Query:     12 AAGCTAGCCGC 2
              ||||||||||||
Sbjct: 934444 AAGCTAGCCGC 934454
```

A3.1.3 BOXA1R primer

```
                                                                  High
Probability
Sequences producing High-scoring Segment Pairs:                 Score  P(N)
N

Streptococcus_uberis_strain_0140J                                 66   0.98
1

>Streptococcus_uberis_strain_0140J
[Full Sequence]

        Length = 1,852,352

Plus Strand HSPs:

 Score = 66 (16.0 bits), Expect = 4.0, P = 0.98
 Identities = 14/15 (93%), Positives = 14/15 (93%), Strand = Plus
/ Plus
[HSP Sequence]

Query:      3 ACGGCAAGGCGACGC 17
              ||||||||||| |||
Sbjct: 360819 ACGGCAAGGCGCCGC 360833

 Score = 61 (15.2 bits), Expect = 4.0, P = 0.98
 Identities = 15/17 (88%), Positives = 15/17 (88%), Strand = Plus
/ Plus
[HSP Sequence]

Query:      1 CTACGGCAAGGCGACGC 17
              |||||||||||  | |||
Sbjct: 1455337 CTACGGCAAGG-GTCGC 1455352

 Score = 55 (14.3 bits), Expect = 4.0, P = 0.98
 Identities = 11/11 (100%), Positives = 11/11 (100%), Strand =
Plus / Plus
[HSP Sequence]

Query:      2 TACGGCAAGGC 12
              ||||||||||
Sbjct: 408126 TACGGCAAGGC 408136

Minus Strand HSPs:

 Score = 55 (14.3 bits), Expect = 4.0, P = 0.98
 Identities = 11/11 (100%), Positives = 11/11 (100%), Strand =
Minus / Plus
[HSP Sequence]

Query:     16 CGTCGCCTTGC 6
              ||||||||||
Sbjct: 74594 CGTCGCCTTGC 74604
```

A3.1.4 ERIC2 primer

```
                                                                  High
Probability
Sequences producing High-scoring Segment Pairs:                  Score  P(N)
N

Streptococcus_uberis_strain_0140J                                  64   0.98
1

>Streptococcus_uberis_strain_0140J
[Full Sequence]

        Length = 1,852,352

Plus Strand HSPs:

 Score = 64 (15.7 bits), Expect = 4.0, P = 0.98
 Identities = 18/23 (78%), Positives = 18/23 (78%), Strand = Plus
/ Plus
[HSP Sequence]

Query:      1 AAGT-AAGTGACTGGGGTGAGCG 22
              |||  || ||||||||||| | |
Sbjct: 304316 AAGCGAATTGACTGGGGTGGGTG 304338

 Score = 61 (15.2 bits), Expect = 4.0, P = 0.98
 Identities = 13/14 (92%), Positives = 13/14 (92%), Strand = Plus
/ Plus
[HSP Sequence]

Query:       1 AAGTAAGTGACTGG 14
              |||||||||| ||
Sbjct: 1044999 AAGTAAGTGACAGG 1045012

 Score = 60 (15.1 bits), Expect = 4.0, P = 0.98
 Identities = 14/15 (93%), Positives = 14/15 (93%), Strand = Plus
/ Plus
[HSP Sequence]

Query:       5 AAGTGACTGGGGTGA 19
              |||||||||| |||
Sbjct: 1519019 AAGTGACTGGG-TGA 1519032

 Score = 57 (14.6 bits), Expect = 4.0, P = 0.98
 Identities = 13/15 (86%), Positives = 13/15 (86%), Strand = Plus
/ Plus
[HSP Sequence]

Query:       7 GTGACTGGGGTGAGC 21
              |||||||||| | |
Sbjct: 1777143 GTGACTGGGGTAAAC 1777157
```

A3.1.5 ERIC1R primer

```
                                                                  High
Probability
Sequences producing High-scoring Segment Pairs:                   Score   P(N)
N

Streptococcus_uberis_strain_0140J                                   60    0.98
1
```

Plus Strand HSPs:

```
 Score = 60 (15.1 bits), Expect = 4.0, P = 0.98
 Identities = 12/12 (100%), Positives = 12/12 (100%), Strand =
Plus / Plus
[HSP Sequence]

Query:       1 ATGTAAGCTCCT 12
               ||||||||||||
Sbjct: 1290340 ATGTAAGCTCCT 1290351

 Score = 55 (14.3 bits), Expect = 4.0, P = 0.98
 Identities = 11/11 (100%), Positives = 11/11 (100%), Strand =
Plus / Plus
[HSP Sequence]

Query:       1 ATGTAAGCTCC 11
               ||||||||||||
Sbjct: 958728 ATGTAAGCTCC 958738

 Score = 55 (14.3 bits), Expect = 4.0, P = 0.98
 Identities = 11/11 (100%), Positives = 11/11 (100%), Strand =
Plus / Plus
[HSP Sequence]

Query:      12 TGGGGATTCAC 22
               ||||||||||||
Sbjct: 855187 TGGGGATTCAC 855197
```

Minus Strand HSPs:

```
 Score = 58 (14.8 bits), Expect = 4.0, P = 0.98
 Identities = 18/23 (78%), Positives = 18/23 (78%), Strand = Minus
/ Plus
[HSP Sequence]

Query:      22 GTGAAT-CCCCAGGAGCT-TACA 2
               | || | |||||||||||| | ||
Sbjct: 452629 GGGATTGCCCCAGGAGCTGTCCA 452651

 Score = 56 (14.5 bits), Expect = 4.0, P = 0.98
 Identities = 12/13 (92%), Positives = 12/13 (92%), Strand = Minus
/ Plus
[HSP Sequence]

Query:       22 GTGAATCCCCAGG 10
               | ||||||||||||
Sbjct: 1345834 GAGAATCCCCAGG 1345846
```

A3.2 Overview of best matches between primers and 0140J genome

The homologous regions between the 0140J genome and each of the primers were summarised (Figure A.1) to identify which of the best matches would most likely result in amplification. Ideally, a best match would show high homology and occur at the 3′ end of the primer to result in amplification.

Figure A.1 (next page). Summary of BLAST search results with BOX and ERIC primers. Best homologous matches between each primer and the 0140J DNA sequence are shown with any mismatches indicated and the actual nucleotide present in 0140J given. Gaps required in the DNA sequence to improve homology are shown by a hyphen while gaps required in the primer sequences are not shown but indicated by an (*). (+/-) indicates the DNA strand to which the primer shows homology. Best matches that are most likely to result in amplification with each primer are marked by (►).

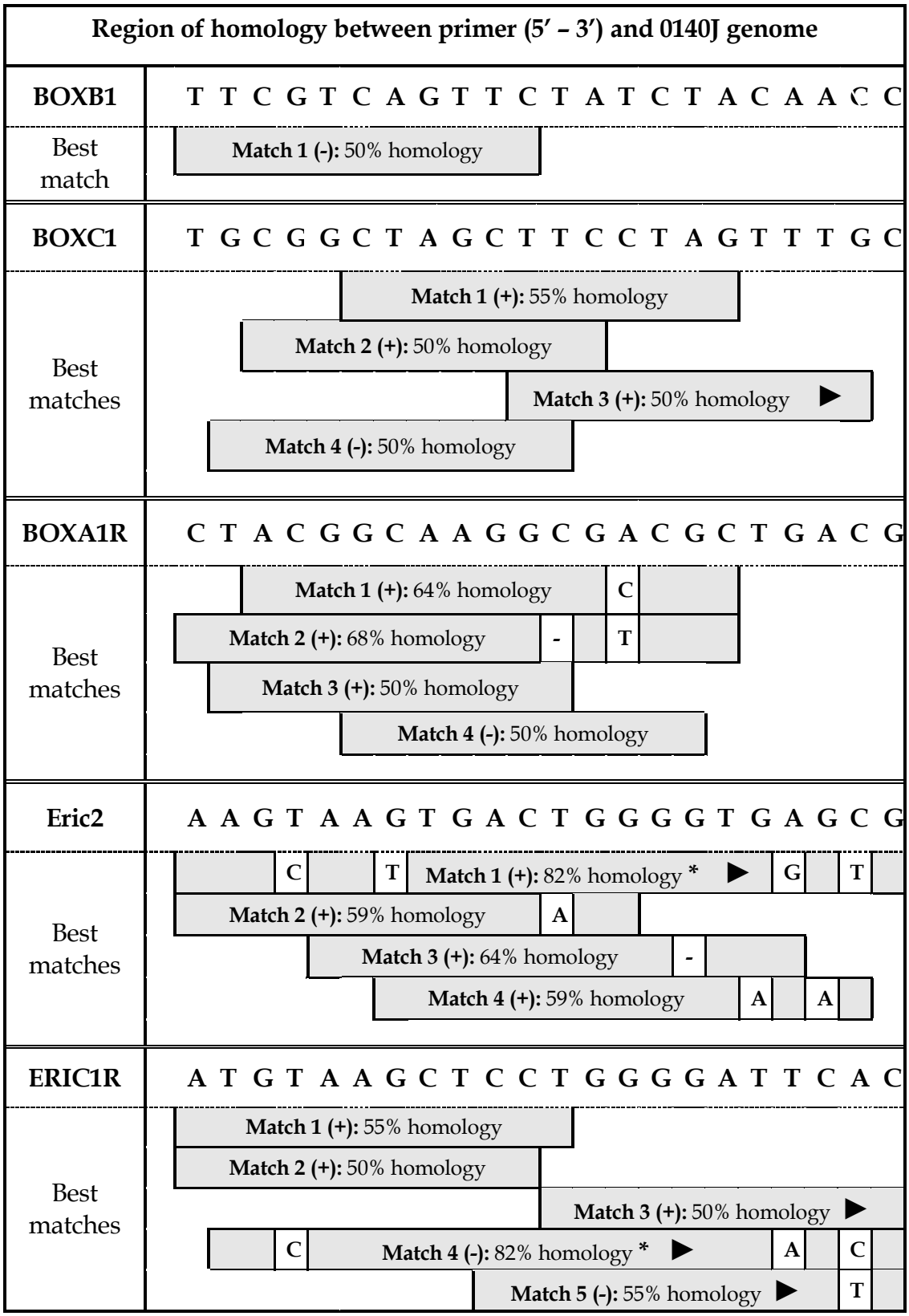


Figure A.1. Summary of BLAST search results with BOX and ERIC primers. Figure details on preceding page.

Appendix 4: Commercial suppliers

ABgene	Epsom, Surrey, UK
Ajax chemicals	NSW, Australia.
BDH laboratory supplies	Poole, Dorset, UK
Becton Dickinson and Co.	Sparks, MD, USA
Boehringer-Ingelheim GmbH	Ingelheim, Germany
Bruker Daltonik GmbH	Liepzig, Germany
Eppendorf AG	Hamburg, Germany
Fort Richard Laboratories, Ltd.	Otahuhu, NZ
Foss Electric	Hillerod, Denmark
Invitrogen	Carlsbad, California
Jacks Co. Ltd.	Osaka, Japan
Jorgensen Laboratories Inc.	Loveland, Colorado, USA
MJ Research Inc.	Waltham, MA, USA
Nanodrop Technologies	Wilmington, DE, USA
New England Biolabs (NEB)	Ipswich, MA, USA
Owl separation systems Inc.	Portsmouth, NH, USA
Pfizer Animal Health	Auckland, NZ
Roche Diagnostics NZ, Ltd.	Auckland, NZ
Sigma-Aldrich NZ, Ltd.	Auckland, NZ
Technipharm	Rotorua, New Zealand
USB Corporation	Cleveland, OH, USA

A4.1 Software

EstimateS, version 7.5	Robert K. Colwell. Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, CT 0689-3043, USA. http://viceroy.eeb.uconn.edu/EstimateS
FlexAnalysis	Bruker Daltonik, Liepzig, Germany
GelComparII, version 4.0	Applied Maths BVBA, Belgium
Genamics	Genamics, Hamilton, NZ
Genstat, 9th Edition	VSN International Ltd, Hemel Hempstead, UK
Scion Image	Scion Corporation, MD, USA
STATISTICA	StatSoft, Inc. Tulsa, OK, USA

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