Psychrophilic and Psychrotrophic Clostridium *spp. Associated with Meat Spoilage*

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Dorota Maria Broda

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Summary

Twenty two isolates of psychrophilic and psychrotrophic *Clostridium* spp. associated with 'blown pack' spoilage of vacuum-packed meats were studied with respect to their identification, differentiation and taxonomy, as well as spoilage aetiology. The aetiological study was conducted to establish whether a causal relationship exists between psychrophilic and psychrotrophic clostridia and the production of abundant gas in vacuum-packed chilled meats. The attempt was then made to identify these clostridia using conventional microbiological methods. Genotypic approaches to differentiation of psychrophilic and psychrotrophic clostridia at inter- and intra-species level were evaluated and applied to tracing the 'blown pack' spoilage causing clostridia back to their contamination source in the abattoir. Where phenotypic and genotypic characteristics indicated that some isolates of psychrophilic and psychrotrophic clostridia differed from those previously described, taxonomic studies were conducted to determine whether these isolates could be described as new clostridial species. Four clostridial isolates were found to cause premature vacuum pack distension during chilled storage. Conventional phenotype based methods were found to be ineffective for identification of psychrophilic and psychrotrophic clostridia. Although RFLP analysis of the amplified 16S rDNA gene allowed differentiation of psychrophilic and psychrotrophic clostridia at the genotypic species level and below, comparison of PCR-RFLP patterns and 16S rDNA sequences of meat isolates with patterns and sequences of reference strains did not effect ready identification of some of these microorganisms. Consequently, the use of these methods for identification of unknown clostridial isolates needs to be approached carefully. Differentiation of some meat-derived isolates of psychrophilic and psychrotrophic clostridia at intra-species or inter-strain level was achieved with methods based on polymorphism of genomic DNAs and/or the 16S-23S rDNA internal transcribed spacers from these organisms. With the 16S-23S rDNA internal transcribed spacer polymorphism analysis, inter-species differentiation of psychrophilic and psychrotrophic clostridia may be achieved in instances where little inter-strain variation is expected e.g. in typing of isolates from the same meat plant or spoilage incident. Hides and faeces of slaughter animals were identified as an abundant

abattoir source of psychrophilic and psychrotrophic clostridia representing many species. Seven isolates of these microorganisms, representing four distinct taxonomic groups, were found to differ in their phenotypic, genotypic and phylogenetic characteristics from known clostridial species. With a polyphasic approach, three new clostridial species, *C. frigidicarnis*, *C. gasigenes* and *C. algidixylanolyticum* have been named and characterised. It is hoped that this study has added to our understanding of the aetiology and causative agents of 'blown pack' spoilage and, thus, has advanced the process of developing measures to control this type of spoilage of vacuum packed chilled meats.

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General Introduction

Microflora of dressed carcasses

At the time of slaughter, internal muscle tissues from healthy, hygienically killed animals are traditionally thought to be either sterile or to contain very few bacteria (Gill, 1979; Mackey and Derrick, 1979). In contrast, the intestinal and respiratory tracts, and external surfaces, such as the skin, hide and fleece carry large numbers of microorganisms. During slaughter and dressing some of these microorganisms are invariably transferred onto dressed carcass and, thus, become its initial microflora. Both the qualitative and quantitative composition of this microflora can vary (Blickstad *et al.*, 1981; Dainty *et al.*, 1983; Jackson *et al.*, 1992). However, approximately 90 % or more of the initial bacterial load of the dressed carcass is thought to be aerobic and mesophilic (West *et al.*, 1972; Newton *et al.*, 1978).

Only bacteria that are present on meat will be able to participate in its spoilage. Therefore, the shelf life of fresh meat would appear to primarily depend on the numbers and types of microorganisms constituting its initial flora and those introduced subsequently during boning and packaging. However, subject to human intervention, the meat microflora is exposed to the directed selection of the bacterial species that develop into a spoilage microflora. Subsequently, the shelf life of fresh meat will depend directly on the composition of spoilage microflora and indirectly on choice of processing and/or extrinsic environmental conditions that were applied to the meat during its storage. These conditions, chilling and anaerobic storage effectively prolong the shelf life while retaining most of the original characteristics of fresh meat.

Selective effect of chilled aerobic storage on development of spoilage microflora

From the initial microflora present on dressed carcasses, chilled storage selects by cold enrichment psychrophilic and psychrotrophic bacterial populations while suppressing mesophilic microorganisms. Up to 10 % (during winter in temperate regions), and often only 1 % (during summer in temperate regions) of the initial microflora can

colonise meat stored at temperatures between 0°C and 5°C (West *et al.*, 1972; Newton *et al.*, 1978). Most bacteria detected by culturing on chilled meat stored in air are Gram negative rods, the majority of which belong to the genera *Pseudomonas*, *Acinetobacter* (including some species previously assigned to the genus *Moraxella*) and *Psychrobacter* (previously *Moraxella*-like) (Gardner *et al.*, 1967; Patterson, 1970; Dainty *et al.*, 1983; Dainty and Mackey, 1992). *Pseudomonas* spp. usually dominate the bacterial populations, and microorganisms belonging to this genus account for more than 50 % and sometimes up to 90 % of the total flora (Ayres, 1955). With other genera, *Acinetobacter* spp. can form from 1 to 10 % of the total flora and *Psychrobacter* spp. can form from 1 to 50 % (Erobo *et al.*, 1985). *Brochothrix thermosphacta,* Enterobacteriaceae (usually represented by *Serratia liquefaciens*, *Enterobacter agglomerans* and *Hafnia alvei*), lactic acid bacteria, *Shewanella putrefaciens, Staphylococcus* spp. and *Micrococcus* spp. can also be found on chilled aerobically stored meat (Nortjé *et al.*, 1990; Dainty and Mackey, 1992; Prieto *et al.*, 1993; Desmarchelier *et al.*, 1999). Among other factors, intrinsic properties of the meat and hygiene of the slaughter process influence the proportion that these microorganisms represent in the total spoilage microflora.

Under aerobic storage conditions, chilled fresh meats have a relatively short shelf life. This shelf life may vary from days to 2 to 3 weeks (Gill and Molin, 1991; Blickstadt and Molin, 1983) largely depending on initial numbers of *Pseudomonaceae* that are recognised to have the high growth rate and spoilage potential (Gill and Molin, 1991). At 0°C, meat with a normal ultimate pH between 5.4 and 5.8 typically spoils after about 3 weeks and this type of spoilage is manifested as a sweet or sometimes putrid odour, slime formation and greening.

Selective effect of chilled anaerobic storage on development of spoilage microflora

At low temperatures and under anaerobic atmospheres colonisation of meat by obligately aerobic and/or mesophilic microorganisms is suppressed. When the residual oxygen is consumed in a vacuum pack by muscle-tissue respiration and the gaseous

atmosphere is enriched with carbon dioxide (Roberts and Mead, 1986), growth of strict aerobes is inhibited and colonisation of meat by obligate anaerobes, facultative anaerobes and microaerophilic organisms is initiated.

On chilled anaerobically stored meat the growth of *Pseudomonas*, *Acinetobacter* and *Psychrobacter* spp. that prevail in the flora of chilled meat stored in air is usually inhibited. In contrast, the dominant microorganisms found on anaerobically stored chilled meats are lactic acid bacteria (Roth and Clark, 1972; Beebe *et al.*, 1976; Seideman *et al.*, 1976; Patterson and Gibbs, 1978; Dainty *et al.*, 1979; Erichsen and Molin, 1981) belonging to genera *Carnobacterium*, *Lactobacillus, Leuconostoc* and *Lactococcus* (Shaw and Harding, 1984; Collins *et al.*, 1987; Schillinger and Lücke, 1987; Borch and Molin, 1988). In addition, in processed vacuum-packed meat products, genera *Pediococcus*, and recently characterised *Weissella* and *Oenococcus* are commonly encountered (Collins *et al.*, 1993; Dicks *et al.*, 1995). Enterobacteriaceae, *Brochothrix thermosphacta*, *Shewanella putrefaciens*, *Pseudomonas* spp., *Staphylococcus* spp. and *Micrococcus* spp. may also grow (Dainty *et al.*, 1983; Gill and Penney, 1988; Venugopal *et al.*, 1994). The growth of these microorganisms on chilled anaerobically stored meats may be enhanced by high initial contamination levels (Gill and Penney, 1988), high ultimate pH of meat (Campbell *et al.*, 1979), a higher gas permeability of the packaging film (Newton and Rigg, 1979) or elevated (abusive) storage temperatures (Beebe *et al.*, 1976).

Under anaerobic storage conditions shelf life of chilled fresh meats can be extended from the 2 to 3 weeks that is achievable with storage in air, to 10 to 14 weeks (Egan, 1983; Gill, 1991). Commercial experience indicates that at storage temperatures between –1.5°C and 0°C (Moorhead and Bell, 1999) and a vacuum packaging film permeability of less than 40 ml of oxygen m^{-2} 24 h^{-1} atm^{-1} at 25°C and 100 % r.h. meat with a normal ultimate pH between 5.4 and 5.8 typically spoils after approximately 12 weeks. This shelf life varies only slightly with different meat species (pork<lamb<beef) or with different bacterial species constituting the spoilage microflora.

Sensory evaluation of vacuum-packed meats at the end of their shelf life usually identifies inoffensive 'sour', 'cheesy' or 'acid' odours that dissipate quickly after opening of the packs (Gill, 1986). The packaging typically remains tight around the meat and no gas or only small gas bubbles are present inside the pack. The meat has the purple colour typical of anoxic meat, has a firm texture and only a relatively small volume of accumulated drip within the vacuum pack.

Characteristics of 'blown pack' spoilage

'Blown pack' spoilage of chilled anaerobically stored meat is manifested by gross pack distension. This type of spoilage can usually be attributed to temperature abuse (Hanna *et al.*, 1979; De Lacy and Cook, unpublished data). Since 1989, however, a number of 'blown pack' spoilage incidents where temperature abuse has not occurred were reported in New Zealand and overseas (Dainty *et al.*, 1989; Kalchayanand *et al.*, 1989; Broda *et al.*, 1996b; Kalinowski and Tompkin, 1999). Products affected included vacuum-packed fresh beef, lamb and venison all of normal ultimate pH, as well as vacuum-packed roasted beef, dog rolls packed in gas-impermeable plastic casings and processed poultry product. Products spoiled at storage temperatures between –1.5 and 2°C with pack distension becoming evident within 4 to 6 weeks of storage.

Spoilage of New Zealand product was characterised by copious gas production causing gross pack distension (Fig. A). The meat was dark in colour, often with a green or reddish-green discolouration. Large amounts of drip accumulated in all blown vacuum packs. Meat odours shortly after pack opening were decidedly offensive ranging from 'sulphurous' to 'strong dairy' or 'cheesy' (Broda *et al.*, 1996b).

Analysis of the meat from blown packs using conventional microbiological methods, both overseas and in New Zealand, did not show significant departures from the expected norm (Dainty *et al.*, 1989; Kalchayanand *et al.*, 1989; Broda *et al.*, 1996b). The spoilage microflora found was typical of that present on anaerobically stored chilled meat and was dominated by lactic acid bacteria. Members of Enterobacteriaceae and other spoilage microorganisms were either not detected or present in low numbers.

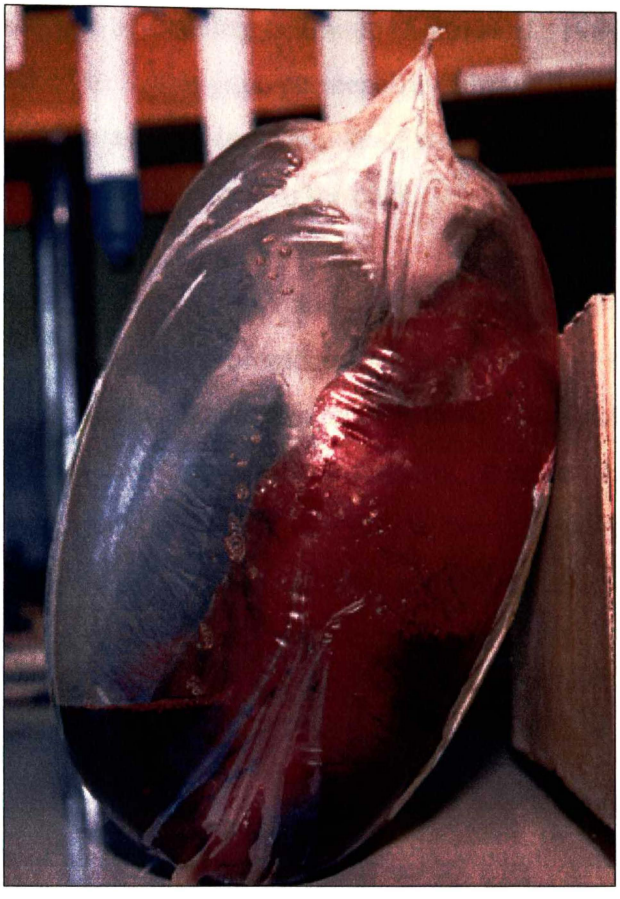

Figure A. Commercial sample of vacuum-packed venison showing gross pack distension and excessive drip.

Chemical analysis of headspace volatiles that accumulated in the packs showed presence of large amounts of carbon dioxide and hydrogen, together with significant amounts of butanol, butyric and acetic acids and a range of butyl esters (Dainty *et al.*, 1989; Broda *et al.*, 1996b). The presence of these volatile compounds indicated that the most likely cause of the 'blown pack' spoilage was the metabolic activity of microorganisms.

Probable cause of 'blown pack' spoilage: traditional spoilage microflora?

Lactic acid bacteria. Spoilage of chilled anaerobically stored meats due to metabolic activity of lactic acid bacteria occurs because of the slow accumulation of volatile fatty acids produced from valine and leucine (Nakae and Elliot, 1965). Some species, e.g. *Lactobacillus sake*, may produce hydrogen sulphide and dimethyl sulphide, but only

when the glucose and oxygen availability is limited (Egan *et al.*, 1989). At 0°C, meat of normal ultimate pH packed in low oxygen permeability film will spoil as a result of the growth of lactic acid bacteria after 10 to 12 weeks of storage and, at this time, no gas or only small gas bubbles are usually present in the pack.

Recently, homofermentative lactobacilli and *Leuconostoc* spp. were surprisingly associated with 'blown pack' spoilage of vacuum-packed processed meat products (von Holy *et al.*, 1991; Mäkelä *et al.*, 1992; Dykes *et al.*, 1994; Yang and Ray, 1994). This spoilage was characterised by gross pack distension, souring and excessive drip formation. Spoilage occurred in product carrying a large initial load of lactic acid bacteria (e.g. re-contaminated with starter cultures) and stored under conditions of mild temperature abuse (von Holy *et al.*, 1991; Yang and Ray, 1994; Dykes, G., personal communication).

Enterobacteriaceae. Many strains belonging to this group are able to produce large quantities of gas, including hydrogen sulphide (McMeekin and Patterson, 1975; Patterson and Gibbs, 1977, 1978; Dainty *et al.*, 1989). However, at temperatures approaching 0°C the growth rate of lactic acid bacteria is approximately seven times higher than that of psychrotrophic Enterobacteriaceae and the later may even be excluded from the spoilage flora of anaerobically stored meats. The growth of Enterobacteriaceae on anaerobically stored chilled meats is favoured under conditions of increased storage temperature, pH and film permeability (Enfors *et al.*, 1979; Grau 1981; Blickstad and Molin, 1983).

Product failure due to pack distension and gas production that was attributed to the activity of *Hafnia alvei* and heterofermentative lactobacilli has been reported (Hanna *et al.*, 1979). This spoilage occurred with vacuum-packed beef stored at an abusive temperature. It has been shown recently that *Hafnia alvei* dominate among Enterobacteriaceae on vacuum-packed chilled meat stored at 4°C rather than at –1.5°C (Borch *et al.*, 1996).

Brochothrix thermosphacta. The major end product of the anaerobic metabolism of this microorganism is lactic acid and small amounts of volatile fatty acids (Davidson *et al.*, 1968). *Brochothrix thermosphacta* will not grow anaerobically on meat of pH 5.8 or lower (Campbell *et al.*, 1979, Grau, 1980).

Shewanella putrefaciens. While this microorganism is able to produce large volumes of hydrogen sulphide under anaerobic conditions (Freeman *et al.*, 1976; Herbert and Shewan, 1976), no strains are able to grow at chiller temperatures on meat of pH less than 6.0 (Gill and Newton, 1979; Seelye and Yearbury, 1979).

Pseudomonas *spp.* A number of *Pseudomonas* species can produce volatiles, including hydrogen sulphide and other sulphur containing compounds (Nicol *et al.*, 1970; Kadota and Ishida, 1972; Dainty *et al.*, 1984). Although during anaerobic storage the growth of obligately aerobic bacteria is normally inhibited, *Pseudomonas* strains may be found in significant numbers on chilled vacuum-packed meats (Christopher *et al.*, 1979). The reason for that is thought to be their ability to grow at low concentrations of residual oxygen (Shaw and Nicol, 1969; Newton *et al.*, 1977) and short generation times at chiller temperatures (Gill and Newton, 1977; Newton and Gill, 1978). Growth of *Pseudomonas* species is, however, considerably suppressed or inhibited on chilled meat stored in packs with film permeability lower than 190 ml of oxygen per m^{-2} 24 h^{-1} atm^{-1} at 2°C and 100 % r.h. (Newton and Rigg, 1979).

Staphylococcus *and* Micrococcus *spp.* Little is known about the spoilage ability of *Staphylococcus* and *Micrococcus* species. The presence of these microorganisms on chilled vacuum-packed beef, however, has not resulted in premature meat spoilage due to gas production and pack distension (Venugopal *et al.*, 1993). It is believed that on raw meat *Staphylococcus* and *Micrococcus* species compete poorly with other spoilage microorganisms.

Probable cause of 'blown pack' spoilage: unknown *Clostridium* spp.?

In the absence of temperature abuse it was unlikely that the species constituting typical microfloras of chilled anaerobically stored meat could cause 'blown pack' spoilage of meat of normal pH packed in low permeability packaging film. Consequently, it was concluded that 'blown pack' spoilage is a previously unknown type of spoilage where no causative microorganisms could be isolated using routine media and techniques for isolation and enumeration of common spoilage bacteria from chilled meats.

In both New Zealand and overseas incidents of 'blown pack' spoilage, microscopic examination of drip from blown packs revealed the presence of large Gram positive sporeforming rods (Dainty *et al.*, 1989; Kalchayanand *et al.*, 1989; Broda *et al.*, 1996b). The presence of carbon dioxide and hydrogen in the headspace was consistent with fermentative metabolism of the butyric type (Jones and Woods, 1986, 1989) and typical of clostridia (Dainty, 1996). Similarly, butanol and butyric acid were likely to be products of clostridial metabolism. When eventually isolated in pure culture, two clostridial species, *C. laramiense* (Kalchayanand *et al.*, 1989; Trüper and De' Clari, 1997) and *C. estertheticum* (Collins *et al.*, 1992) reproduced the characteristics of the original 'blown pack' spoilage under experimental conditions and were, consequently, confirmed as the microorganisms responsible for this type of spoilage.

During early 1990s, 22 clostridial isolates were obtained in New Zealand from blown vacuum-packed beef, lamb and venison, and from dog rolls packed in gas-impermeable plastic casings. The majority of these isolates appeared to belong to a phenotypically heterogenous group of dominantly saccharolytic, butanol-producing organisms (Broda *et al.*, 1996a,b,c). All 22 isolates were able to grow at 4°C and, therefore, could be considered as being either psychrophilic or psychrotrophic.

Psychrophilic and psychrotrophic microorganisms on chilled meats

Presently, the most widely accepted definition of physiological groups among low-temperature-growing bacteria distinguishes psychrophiles and psychrotrophs (psychrotolerants) (Morita, 1975; Olson and Nottingham, 1980; Russell and Hamamoto,

1998). The term 'psychrophile' is applied to organisms with an optimum growth temperature of about 15°C or lower, a maximum temperature of about 20°C or lower and a minimum temperature of 0°C or lower. The term 'psychrotroph' is, most often, applied to organisms with an optimum growth temperature between 25°C and 30°C a maximum temperature between 30°C and 35°C and a minimum temperature of 5°C or lower (Olson and Nottingham, 1980). In practice, the distinction between psychrophiles and psychrotrophs is often not clear-cut and minimum, optimum and maximum temperatures for growth of microorganisms belonging to different physiological groups often merge (Kraft, 1992). Therefore, in this study the bacterial isolates that are able to grow below 5°C but do not meet the optimum and maximum growth temperatures for psychrophiles will be regarded as psychrotrophic (Eddy, 1960; Morita, 1975).

Psychrophiles are considered to be thermolabile and, consequently, it is thought that they occur only in permanently cold habitats (Gounot, 1991). In contrast, psychrotrophic microorganisms appear to tolerate temperature variation and, therefore, are commonly present in seasonally cold environments. Psychrotrophs are also thought to adapt more easily than psychrophiles to changing nutritional conditions. With vacuum-packed meat, the lowest storage temperature at which the original characteristics of fresh meat are still retained is –1.5°C (Gill and Molin, 1991). However, during pre- and post-storage processing and handling meat invariably is subjected to temperature variation. Consequently, the majority of microorganisms that grow on chilled meat have been found to be psychrotrophic and not psychrophilic (Grau, 1986). Similarly, except for chill-stored seafood, true psychrophiles have been rarely encountered in other types of foods (Baross and Morita, 1978).

It is estimated that up to 10 % of the initial microflora of dressed carcasses is able to grow at refrigeration temperatures (Grau, 1986) and can, therefore, be considered psychrophilic or psychrotrophic. Almost all genera of bacteria that may cause either aerobic or anaerobic spoilage include low-temperature-growing species (Olson and Nottingham, 1980), some being able to grow down to –3°C (Barnes, 1976). The ratio of psychrophiles/psychrotrophs to mesophiles on beef carcasses has shown a negative

correlation with ambient temperature (Empey and Scott, 1939). Consequently, this ratio has been found to be lower for carcasses that have been processed during summer in temperate regions (Newton *et al.*, 1978) and for those processed in warm climates (Empey and Scott, 1939).

Psychrophilic and psychrotrophic clostridia in food spoilage: historical background

Clostridia are widely distributed in the environment and, since a great proportion of the earth's surface is permanently or temporarily cold, growth of *Clostridium* spp. at low temperatures would be expected to be a common occurrence. However, of the over 120 known clostridial species, only eight are currently described as being psychrophilic or psychrotrophic (Cato *et al.*, 1986; Collins *et al.*, 1992; Kalchayanand *et al.*, 1993; Lawson *et al.*, 1994; Kotsyurbenko *et al.*, 1995; Mountfort *et al.*, 1997). Early studies frequently failed to isolate low-temperature-growing clostridia (Roberts and Hobbs, 1968). However, psychrophilic and psychrotrophic clostridia have later been isolated from environmental sources, such as soil, water, mud and sewage (Starka and Stokes, 1960; Sinclair and Stokes, 1964; Beerens *et al.*, 1965) and marine sediments (Liston *et al.*, 1969; Finne and Matches, 1974) in cold, as well as temperate, climates.

Clostridia have been previously isolated from fresh (Ingram and Dainty, 1971; Smart *et al.*, 1979), chilled (Ayres, 1960) and some cured meats (Roberts and Smart, 1976). Historically, these microorganisms have been associated with 'bone taint' spoilage, a condition where offensive odours are present in deep muscle tissues, frequently near the bone. 'Bone taint' of beef carcasses that were cooled too slowly and stifle joint taint (Mundt and Kitchen, 1951) have been thought to occur when meat was temperature abused. 'Bone taint' spoilage of brine-cured hams (Sturges and Drake, 1927; Jensen and Hess, 1941) has been attributed to the growth of psychrotrophic *C. putrefaciens* introduced into deep tissues during unhygienic processing. This spoilage condition has affected product stored at temperatures between 1°C and 2°C for less than 4 weeks (McBryde, 1911, cited in Ross, 1965). Spoilage of canned meat by clostridial gas and butyric acid production during storage at temperatures above 15°C has also been

reported (Gardner, 1983; Eyles and Adams, 1986). With the exception of 'bone taint' spoilage of brine-cured ham, it has been believed that clostridial spoilage of meat is caused by the growth of mesophilic clostridia unable to proliferate at refrigeration temperatures. Consequently, the significance of clostridia in the spoilage of chilled meats has been thought to be limited (Roberts and Mead, 1986). However, this traditional perception underwent significant change during late 1980s and 1990s when instances of 'blown pack' spoilage of vacuum-packed meat (Dainty *et al.*, 1989; Kalchayanand *et al.*, 1989; Broda *et al.*, 1996b; Kalinowski and Tompkin, 1999) and spoilage of cooked vacuum-packed pork due to production of offensive odours have been reported (Lawson *et al.*, 1994). These instances of meat spoilage have occurred during storage at or below 4°C and have been caused by psychrophilic and/or psychrotrophic clostridia.

In dairy products, mesophilic *Clostridium* spp. may produce gas, resulting in late blowing of hard cheeses during maturation at temperatures above 5°C (Cousin, 1982; Walker, 1988; Dasgupta and Hull, 1989). However, milk spoilage due to growth of psychrophilic clostridia has also been reported (Bhadsavle *et al.*, 1972). With other foods, psychrophilic and psychrotrophic clostridia have been associated with soft rot spoilage of potatoes (Brocklehurst and Lund, 1982) and a psychrotrophic *Clostridium* sp. resembling *C. arcticum* has been implicated in the spoilage of canned crabmeat stored at temperatures below 2°C (Segner, 1992).

Of the pathogenic clostridia, psychrotrophic non-proteolytic *C. botulinum* types B, E and F sometimes occur on chilled vacuum-packed meats (Dodds, 1993a) but more frequently in fish and fish products (Dodds, 1993a; Hyytiä *et al.*, 1998; Korkeala *et al.*, 1998). These bacteria can grow and produce toxin at temperatures as low as 3.3°C (Schmidt *et al.*, 1961; Michener and Elliot, 1964; Eklund *et al.*, 1967), or below (Graham *et al.*, 1997; Moorhead and Bell, 1999). Growth of *C. botulinum* in foods, including vacuum-packed meat stored at 0°C or below, is usually not accompanied by noticeable spoilage symptoms (Siddiqui *et al.*, 1979; Moorhead and Bell, 1999).

Ultimate goal of meat producer is 'blown pack' spoilage control

To a meat producer, an economic loss may be an indirect consequence of the activity of two main groups of microorganisms, one that causes disease in man, the other which causes product spoilage. Recently, the attention of meat producers has focused on the first of these two groups, because incidents of food poisoning due to ingestion of contaminated meat may result in loss of market. The awareness of meat producers to market vulnerability resulting from 'meat scares' (e.g. BSE in beef, *Campylobacter* in chicken) is particularly strong in New Zealand and other countries that depend economically on trade of primary agricultural products.

In addition to microbial safety of meat, the interest of a meat producer lies in extension of the shelf life of the product because it enables a producer of fresh meat to trade in distant markets. During the 1970s and 1980s, significant progress in the shelf life extension of fresh meats was achieved by developing measures to minimise the initial contamination levels of certain meat cuts (e.g. inverted dressing system), to remove bacteria from carcasses (e.g. decontaminating sprays) or to inhibit growth of bacteria on meat (e.g. vacuum or modified atmosphere packaging)(Gould *et al.*, 1983; Mossel, 1989; Mossel and Struijk, 1992). However, instances of premature meat spoilage, such as 'blown pack' spoilage, still occur resulting in product and, when recurrent, market loss.

Once the clinical signs of infection are observed in humans, animals or plants, considerable diagnostic effort is directed towards establishing the cause of a disease. Traditionally, this is initiated by detection and, whenever possible physical isolation of the probable causative agent. Subsequently, a causal relationship between microbe and disease is assessed to confirm that Koch's postulates are fulfilled. These postulates aim to test that the presence of a specific microorganism causes specific disease symptoms. Koch's postulates require that (1) the organism is found among isolates from the disease, (2) it is isolated in pure culture, (3) under experimental conditions this pure culture reproduces the disease symptoms identical to those observed originally and (4) the microorganism is recovered again from this artificially reproduced disease (Wilson

and Miles, 1955). Alternatively, the traditional serological diagnostic approach requires demonstration of the presence of a high level of antibody specific to a human or animal pathogen.

Subsequent to fulfilment of Koch's postulates, that is confirmation of an aetiological agent, come epidemiological investigations of the disease. It is assumed that outbreak-related microorganisms are derived from the same parent source as descendants of a single cell and, therefore, can be treated as identical or near identical clones (Swaminathan and Matar, 1993). To establish the source(s) of the aetiological agent that causes a specific disease, isolates present in the probable source and host are identified at the species level and then, within the bacterial species, differentiated and grouped (typed) according to intra-species discriminatory characteristics. If source and host strains are indistinguishable from each other, the outbreak source is considered to have been established in a definitive manner and, ultimately, procedures to prevent the transmission of pathogen from the source onto a host can be initiated. Consequently, detection, isolation, identification, differentiation and typing of pathogenic microorganisms are considered the 'gold standard' for development of measures for the control of a particular disease.

While cause and control of pathogenic organisms from meat are usually extensively studied, until recently, bacteria associated with meat spoilage have attracted considerably less attention. Many species, genera and families of meat spoilage bacteria have been described only recently (Shaw and Harding, 1985, 1989; Juni and Heym, 1986; Molin *et al.*, 1986; Collins *et al.*, 1987, 1993; Rossau *et al.*, 1991; Dicks *et al.*, 1995). Little is known about the source and distribution of specific species of meat spoilage bacteria in the farm environment and abattoir. Although methods have been developed to enable differentiation and rapid detection of many spoilage microorganisms (Brooks *et al.*, 1992; Dykes and von Holy, 1993; Grant *et al.*, 1993; Nissen *et al.*, 1994; Dykes *et al.*, 1995; Björkroth and Korkeala, 1996; Björkroth *et al.*, 1996; van der Vossen and Hofstra, 1996; Sanz *et al.* 1998), studies applying these methods to trace abattoir source(s) of meat contamination (e.g. Björkroth and Korkeala,

1997) are still rarely reported. Similarly, currently available information on cause and control of 'blown pack' spoilage appears to be fragmented. Although the relationship between presence of *C. estertheticum* or *C. laramiense* and 'blown pack' spoilage has been established (Dainty *et al.*, 1989; Kalchayanand *et al.*, 1989), this relationship has been only partially confirmed with the clostridial isolates obtained in New Zealand (Broda *et al.*, 1996a,b). While the overseas studies led to the isolation of single strains of psychrophilic or psychrotrophic *Clostridium*, a group of strains, most likely belonging to many clostridial species, was obtained from New Zealand incidents of 'blown pack' spoilage (Broda *et al.*, 1996b). Today, it is not known whether there are one or more clostridial species capable of causing 'blown pack' spoilage. No diagnostic tools are available that enable ready identification of psychrophilic and psychrotrophic clostridia associated with 'blown pack' spoilage of chilled meats. Little or no information is available on the source of carcass contamination with these clostridia in the abattoir and no methods are available to enable tracing the 'blown pack' spoilage causing clostridial species back to their abattoir source.

When an incident of meat spoilage occurs, the meat producer is interested in immediate remedial action. Consequently, 'the cure before the cause' approach is often adapted in these spoilage instances. However, the development of effective measures to control specific spoilage incidents is rarely possible without a thorough knowledge of the causative agent(s). A multi-disciplinary approach that expands our knowledge on diagnosis, taxonomy and epidemiology of spoilage-causing microorganism(s) and that applies this knowledge at the meat processing level is, therefore, required if effective long-term spoilage control is to be achieved. 'Knowing the cause before developing the cure' is the rationale of this approach (Fig. B).

It was hoped that this study would help to widen our understanding of 'blown pack' spoilage aetiology and that this knowledge would, ultimately, be used to reduce product loss through the control of 'blown pack' spoilage of vacuum-packed meats.

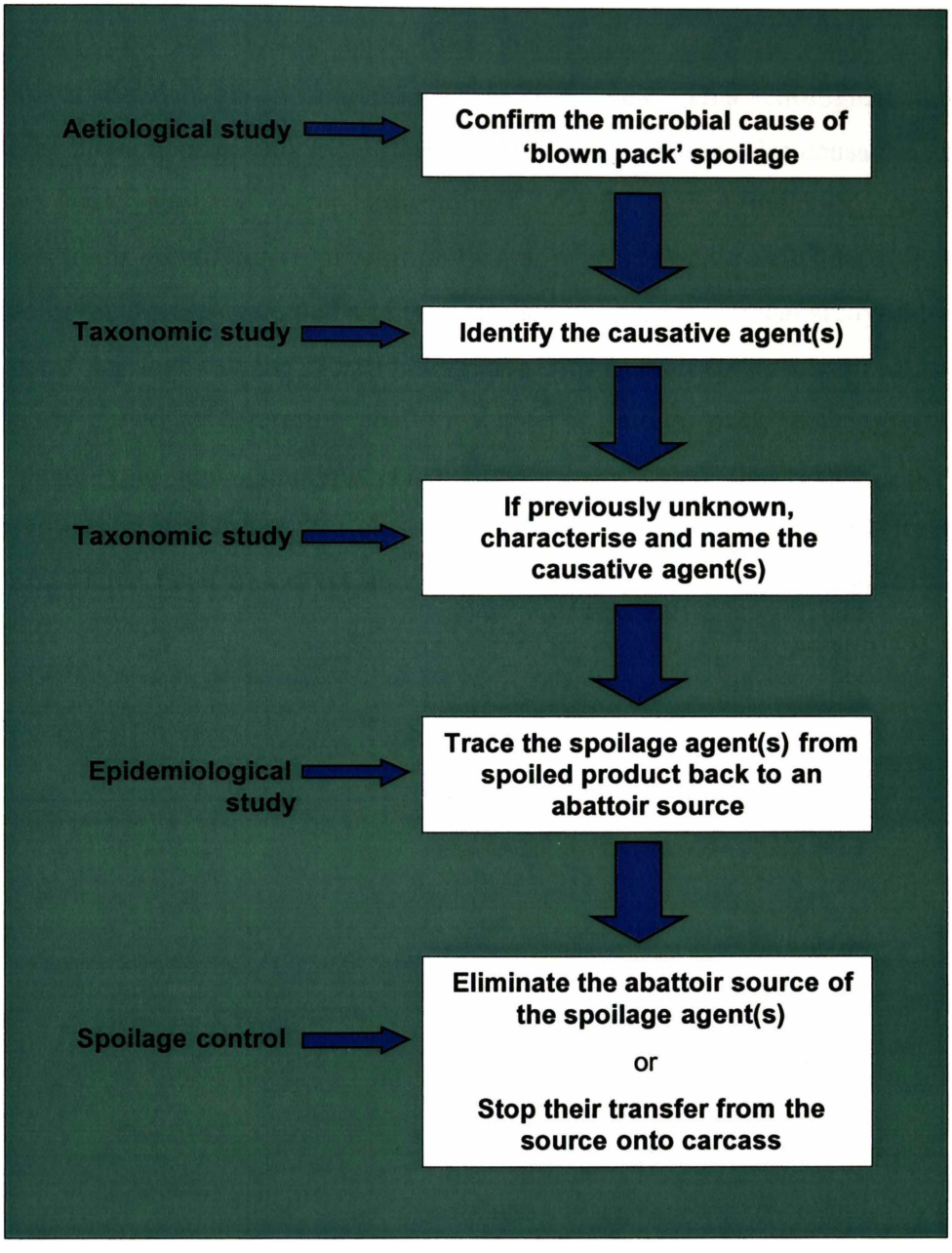

Figure B. 'The cause before the cure' – a pragmatic approach to 'blown pack' spoilage.

Objectives

The objective of this study was to establish a causal relationship between psychrophilic and psychrotrophic clostridia isolated from 'blown pack' spoilage incidents and the production of abundant gas in vacuum-packed chilled meat. This aetiological study was followed by a taxonomic study that aimed to identify and characterise causative agent(s) of 'blown pack' spoilage using conventional microbiological methods. Genotypic approaches to differentiation of psychrophilic and psychrotrophic clostridia at inter- and intra-species level were evaluated and then applied in epidemiological study to trace the 'blown pack' spoilage causing clostridial species back to their abattoir source. This part of the study aimed to determine abattoir source of 'blown pack' causing clostridia. Where phenotypic and genotypic characteristics indicated that some isolates of psychrophilic and psychrotrophic clostridia differed from those described previously, new species have been described and characterised by means of detailed taxonomic studies.

Chapter 1

Conventional Identification of Psychrophilic and Psychrotrophic Clostridia

Introduction

Identification: a useful application of taxonomy

In the past, numerous attempts have been made to arrange (classify) microorganisms into groups (taxa) according to taxonomic rules (Cowan, 1965; Sneath and Sokal, 1973). Traditionally, classifications generated groups by comparing observable properties of microorganisms and, thus, reflected their present morphological or biochemical similarities. If equal weighting has been assigned to each microbial trait during this comparison, the classification was considered to be phenetic (Cowan, 1978; Goodfellow and O'Donnell, 1993).

The major aim of early classifications was to enable ready recognition of unknown microorganisms. Classifications for diagnosis aimed to achieve a single purpose objective and, consequently, often gave unequal weight to disease related properties of microorganisms (e.g. toxigenicity or antibiotic resistance). The resulting classifications of microorganisms have often contained a combination of phenetic and non-phenetic groups. Despite being artificial, classifications for diagnosis satisfied the practical requirements of clinical, veterinary and food microbiologists and the majority of these classifications are still being used today.

The alternative, more academic aim of classification was to arrange microorganisms into clades according to their natural relationships in time and this type of classification

has been termed cladistic (Cain and Harrison, 1960; Swofford *et al.*, 1996). Historically, cladistic relationships were derived from various types of phenetic, phenotype based, relationships by applying theoretical models of evolution. Unequal weight was attached to those phenotypic properties that were thought to reflect ancestral relationships of microorganisms and, consequently, cladistic classifications were considered to be special purpose classifications.

Because higher organisms have complex morphologies, phenotypic properties enabled their discrimination and cladistic classification. For these organisms, groupings obtained with phenetic and cladistic classifications were usually congruent and fossil records and/or sediment procedures evidenced linkage between phenotype and ancestry. Phenotypic properties of microorganisms were, however, too simple and too variable for them to be used in cladistic reconstructions. Additionally, the anticipated evolutionary changes could not be supported by historical evidence due to limited availability of fossil records. Consequently, in the past, phenotype based cladistic classifications of microorganisms were unsuccessful (Stanier *et al.*, 1963).

More recently, molecular sequence data were being included to describe phenetic relationships of microorganisms. Similarly, these data were being used to construct cladistic (phylogenetic) classifications and, for the first time, more objective, rather than intuitive, classification of microorganisms above the genus level has been achieved. Increasingly, however, phylogenetic reconstructions produce groupings that are distinctly different from those obtained in phenetic classifications. These groupings are often confusing to clinical, veterinary or food microbiologists and, currently, their practical value in recognition of microorganisms is limited. Consequently, practical and academic classifications still appear to diverge, with microbial classification more than ever becoming a series of classifications, each with different purpose (Cowan, 1971), rather than forming a universal general purpose classification system that could be used by various interest groups.

The concept of bacterial species

The basic taxonomic group in bacterial classification is the species and, consequently, most identification systems use the species as the basic unit. In contrast to higher organisms, bacterial species could not be precisely defined in biological terms (e.g on the basis of reproductive separation) and, historically, the definition of species has been the source of much debate (van Niel, 1955; Cowan, 1968). Generally, a bacterial species has been regarded as a group of strains that are similar to each other in a high proportion of characteristics; this group, however, can be distinguished from other groups of strains (Staley and Krieg, 1986). Alternatively, bacteria were considered too pleomorphic for a bacterial species to be anything more than an artificial concept (Stanier and van Niel, 1962; Cowan, 1968).

Generally, it has been thought that there is sufficient stability in clonal populations of bacteria for them to be recognised as ecological entities. However, in the past an arbitrary judgement of a microbiologist has sometimes been required to recognise a group of bacterial strains as a separate species (Staley and Krieg, 1986). More recently (as discussed in more detail in Chapter 4) genotypic data are being used in an attempt to introduce more precision into the definition of a bacterial species (Wayne *et al.*, 1987; Vandamme *et al.*, 1996).

As posited by Staley and Krieg (1986) "...bacterial classifications are devised for microbiologists, not for the entities being classified". Phenotype based microbial classifications, while artificial in character and often encompassing arbitrarily assigned taxa/species, have, in most instances, performed satisfactorily in identification of microorganisms associated with disease and/or spoilage. For practical reasons, in most laboratories, classification for diagnosis and routine identification of microorganisms remains phenotype based. Alternative identification approaches are usually restricted to the instances where their superiority over conventional methods, e.g. with respect to specificity or speed, can clearly be demonstrated.

Principles of conventional identification of microorganisms

Traditionally, identification procedures are applied to microorganisms after their isolation in pure culture. Conventional identification involves comparison of phenotypic characteristics of these unknown microorganisms with those of known microorganisms, using procedures that are conducted uniformly for both unknown isolates and reference microorganisms, to determine whether the unknown organism belongs to previously described taxa (Cato and Stackebrandt, 1989). For disease/spoilage diagnosis, the identification of bacterial isolates to the species level is usually sufficient.

Generally, taxonomical studies determine phenotypic criteria for inclusion in appropriate taxa. These criteria should be based on properties of bacterial strains within the taxa that are consistent, stable, common to all strains and independent of growth conditions (Krieg, 1986; Cato and Stackebrandt, 1989). The choice of the identifying properties depends on the adequate characterisation of strains within a taxon. Ready recognition is assured when a low number of properties of an unknown microorganism can provide high discrimination from known microbial groups.

Conventional identification of clostridia at genus level

Historically, the genus *Clostridium* was established to include obligately anaerobic spore-forming Gram positive rods that do not carry out a dissimilatory sulphate reduction (Cato *et al.*, 1986). Conventional identification of members of this genus was, therefore, essentially based on phenotypic, mainly morphological criteria. Today, however, many clostridial species do not conform to the traditional criteria in one or more characteristics. Aerotolerant or microaerophilic species (e.g. *C. histolyticum* or *C. carnis*), species that stain Gram negative (e.g. *C. aerotolerans* or *C. lentocellum*), species forming cocci rather than rods (e.g. *C. coccoides*) and species for which spore formation is rarely or never demonstrated (e.g. *C. perfringens*, *C. proteoclasticum* or *C. acetireducens*) are all considered to be members of the genus *Clostridium*. There are no strict biochemical or physiological criteria for inclusion in the genus *Clostridium*.

The members of this genus are or are not saccharolytic and/or proteolytic and can be psychrophilic, psychrotrophic, mesophilic or thermophilic. Similar diversity of clostridia is observed with respect to other morphological, biochemical and physiological properties.

When encountered in food-borne disease and/or food spoilage, phenotype of obligately anaerobic, Gram positive, catalase and oxidase negative, metronidazole sensitive, large rods carrying terminal or subterminal spores usually indicates involvement of clostridia.

Conventional identification of clostridia at species level

Because the genus *Clostridium* has been defined in wide phenotypic terms, this genus now contains over 120 morphologically and metabolically heterogenous species. Consequently, it is rarely possible to limit criteria for identification of clostridial species within this genus to only a few phenotypic characters and acquisition of a wide range of morphological and biochemical data is often required. This applies especially to identification of clostridia other than those causing human and animal diseases, because selective isolation media, specific detection procedures or serological tests are usually unavailable.

Conventional identification systems. Phenotypic characteristics that are used for the conventional identification of clostridia usually include cellular and colony morphology and a large selection of biochemical and physiological criteria. These criteria, however, are not applied uniformly to all clostridial species. Identification of pathogenic clostridia is based primarily on the production of toxins or serological tests, while morphological and biochemical criteria are considered of secondary value. The identification of non-pathogenic clostridia is based entirely on morphological, biochemical and physiological criteria. The two widely recognised identification systems for clostridia are based on dichotomous keys (Holdeman *et al.*, 1977; Cato *et al.*, 1986). These identification schemes consider criteria related to the more stable properties of clostridia, particularly metabolic activity. Thus, with minor exceptions, the keys are based on such biochemical reactions as gelatine hydrolysis, egg yolk

reaction, nitrate reduction, indole production, urease activity and carbohydrate fermentation (Holdeman *et al.*, 1977; Cato *et al.*, 1986). Since the late 1960s, the composition of end products of carbohydrate or amino acid metabolism, as analysed by gas chromatography, have routinely been included in identification of clostridia (Moore *et al.*, 1966; Moss and Lewis, 1967).

Miniaturised identification systems. The majority of miniaturised systems were developed in the 1970s (Phillips *et al.*, 1985). These systems usually carry out the conventional biochemical tests in sets of mini-tubes or mini-cupules. Tests for a wide range of substrates are conducted simultaneously and the microorganism's biochemical profile is read against those in a database. Systems like API (Starr *et al.*, 1973; Moore *et al.*, 1975; Nord *et al.*, 1975), Minitek (Hanson *et al.*, 1979) or Anaerobe-Tek (Buesching *et al.*, 1983) use conventional biochemical tests. More recently, with systems like Rapid ID32 A (BioMerieux) or BBL Crystal Anaerobe (Cavallaro *et al.*, 1997), combinations of enzymatic activities towards specific substrates and conventional biochemical tests are used for identification. Presumpto Plates (Whaley *et al.*, 1995) utilise conventional media in multiple plates. The majority of miniaturised systems usually target a range of anaerobic microorganisms of clinical importance, including a selection of clostridia. In contrast to conventional methods that often require prolonged incubation of test media, the miniaturised identification systems aim to provide identification of isolates within 4 hours.

Limitations of conventional identification of clostridia

Conventional identification of pathogenic clostridia are often based on toxin production, but transfer of neurotoxigenicity between different clostridial species has been reported (Eklund and Poysky, 1974; Eklund *et al.*, 1974; Eklund *et al.*, 1988; Zhou *et al.*, 1993). Consequently, toxin production does not appear to be a criterion suitable for species identification. With clostridial species that are represented by many strains, large variability of biochemical properties is frequently observed between different strains of the same species (Cato *et al.*, 1986; Hobbs, 1986).

Non-pathogenic clostridia are often represented by single strains whose phenotype may not adequately represent all phenotypes of the species.

Clostridia are fastidious microorganisms and the media commonly used for biochemical tests often do not support the growth of a wide range of clostridia. Consequently, a negative reaction may mean there was no growth in the medium. With some biochemical tests, difficulties are commonly experienced in obtaining reproducible results and in interpretation of positive or negative reactions, for example with carbohydrate fermentation (Hobbs, 1986). Many biochemical tests yield weak reactions and even with the GC analysis of fermentation products, considered to give the most stable data, variations in the concentrations of individual products between replicate cultures of the same strain can occur (Hobbs, 1986).

Miniaturised systems are quite reliable with many anaerobes, but often fail to identify clostridial species (Hanson *et al.*, 1979; Cavallaro *et al.*, 1997; Brett, 1998). While the conventional systems for identification of clostridia can be applied universally to both pathogenic and non-pathogenic clostridia, miniaturised systems target mesophilic clostridia of medical importance, and so their databases are usually limited to selection of these microorganisms (Lombard *et al.*, 1982; Buesching *et al.*, 1983). As with conventional systems, biochemical reactions obtained with miniaturised systems for non-pathogenic clostridia and for wild strains of pathogenic clostridia frequently give weak reactions that are difficult to interpret (Brett, 1998). These weak reactions may confound the final species identification, e.g. when profiles of the two most closely related species differ only by their reaction with one substrate (Brett, 1998).

It appears that the present identification schemes are unsatisfactory for recognition of many pathogenic and non-pathogenic clostridia. The limitations of conventional identification of clostridia are mainly due to the phenotypic diversity of bacteria in the genus *Clostridium* and the lack of stable identification criteria.

Conventional identification of psychrophilic and psychrotrophic clostridia

The majority of non-pathogenic clostridia are routinely identified using conventional phenotype based tests and dichotomous keys. With food spoilage clostridia, few exceptions from the conventional identification of clostridia have been reported (Magot *et al.*, 1983; Klijn *et al.*, 1994, 1995). For these clostridia, including meat spoilage associated species, alternative identification methods have either not been developed or are not available to industry testing laboratories.

Because the presently available identification schemes were developed mainly for pathogenic mesophilic clostridia, they may have limited applications for identification of psychrophilic and psychrotrophic clostridia associated with meat spoilage. Since 1986, five new psychrophilic and psychrotrophic species have been added to genus *Clostridium* (Collins *et al.*, 1992; Kalchayanand *et al.*, 1993; Lawson *et al.*, 1994; Kotsyurbenko *et al.*, 1995; Mountfort *et al.*, 1997). These species have not been included in the presently available dichotomous keys (Holdeman *et al.*, 1977; Cato *et al.*, 1986). With the majority of low-temperature-growing clostridial species, only single strains are described, and variation in biochemical reactions between these and unknown strains may preclude identification of the latter. Because some psychrophilic and psychrotrophic clostridia require long incubation at low temperatures, prolonged exposure of biochemical tests to atmospheres containing 5 % carbon dioxide may cause acidification of the growth medium. Consequently, biochemical reactions, including carbohydrate fermentation tests, may be difficult to interpret. Miniaturised systems using incubation temperatures favourable for growth of mesophiles and some psychrotrophs but not for psychrophilic clostridia, and with their associated databases limited to clostridia of medical importance (Lombard *et al.*, 1982; Buesching *et al.*, 1983), are also not likely to be suitable for identification of psychrophilic and psychrotrophic species.

It was thought appropriate, despite the limitations just discussed, to identify meat strains of psychrophilic and psychrotrophic clostridia using methods available to industry laboratories that might attempt to investigate the causative agent(s) of 'blown pack' spoilage incidents.

Objective

The aim of this study was to confirm a causal relationship between clostridia isolated from meat spoilage incidents and the production of abundant gas in vacuum-packed chilled meat and to identify meat strains of psychrophilic and psychrotrophic clostridia associated with meat spoilage. To fulfil Koch's 3rd postulate and to establish clostridial aetiology of 'blown pack' spoilage, attempts were made to reproduce the original spoilage characteristics in artificially inoculated meat under laboratory conditions. Subsequently, the species identification of 22 clostridial isolates associated with meat spoilage was attempted using conventional phenotype based methods.

Methods and Materials

General procedures

Unless stated otherwise, stringent anaerobic procedures (Holdeman *et al.*, 1977) were used for culturing all *Clostridium* strains and for physiological and substrate utilisation tests. All media used were pre-reduced inside an anaerobic chamber (Forma Scientific).

Bacteria

A total of 22 meat derived isolates of psychrophilic and psychrotrophic clostridia were obtained from spoiled and unspoiled vacuum-packed raw beef, lamb and venison, and from spoiled cooked dog rolls (Table 1.1). These isolates were termed meat strains in this study.

Table 1.1. Origins and cultivation conditions of meat strains of psychrophilic and psychrotrophic *Clostridium* spp. associated with spoilage of vacuum-packed meats and dog rolls packed in gas-impermeable plastic casings.

Strain designation	Source*	Cultivation temperature and time†	Ability to grow anaerobically at 37°C ‡
DB2	Chill stored lamb	30°C /48 h	+
SPL17	Fresh lamb	30°C /48 h	+
SPL242	Temperature abused beef	30°C /48 h	+
KDL358	Chill stored dog rolls	30°C /48 h	+
M14	Chill stored venison	30°C /48 h	+
M33	Chill stored venison	30°C /48 h	+
PP1	Chill stored lamb	30°C /48 h	+
SPL4	Chill stored lamb	30°C /48 h	+
SPL9	Chill stored lamb	30°C /48 h	+
SPL58	Temperature abused lamb	30°C /48 h	+
SPL111	Temperature abused beef	30°C /48 h	+
SPL141	Temperature abused beef	30°C /48 h	+
SPL148	Temperature abused beef	30°C /48 h	+
SPL281	Temperature abused beef	30°C /48 h	+
SPL290	Temperature abused beef	30°C /48 h	+
DB1A	Chill stored lamb	20°C /72 h	–
R26	Chill stored lamb	20°C /72 h	–
SPL73	Temperature abused lamb	25°C /72 h	–
SPL77A	Temperature abused beef	30°C /48 h	+
SPL77B	Temperature abused beef	30°C /48 h	+
K21	Chill stored venison	15°C /96 h	–
K24	Chill stored venison	15°C/96 h	–

*, with the exception of dog rolls that were packed in gas impermeable plastic casings, all meat was vacuum-packed. Chill stored meat was stored at temperatures not exceeding 2°C. Fresh meat was obtained immediately after processing and packaging, transported chilled and sampled on receipt in the laboratory. Temperature abused meat was stored at elevated temperatures not exceeding 15°C. †, temperature and time of incubation required under anaerobic conditions to obtain exponential growth on fresh Columbia Blood Agar with 5 % sheep blood or in pre-reduced Peptone Yeast Extract Glucose Starch broth (culture conditions within the optimal growth range). ‡, ability to grow at 37°C (a major property of mesophilic clostridia) was tested on fresh Columbia Blood Agar with 5 % sheep blood. +, strain able to grow; –, strain unable to grow.

The meat strains were isolated at 15°C using methods described previously (Broda *et al.*, 1996b). Briefly, clostridia were isolated from heat or ethanol treated meat or drip samples by plating onto either Shahidi Ferguson Perfringens Agar (SFP, Oxoid CM587) or Tryptone Sulfite Cycloserine Agar (TSC, Oxoid CM587), each agar supplemented with 10 % v/v egg-yolk solution (Oxoid SR047C) and 10 % w/v glucose. In addition, clostridia were isolated from untreated samples that were plated onto a Reinforced Clostridial Agar (RCM, Oxoid CM149) containing 5 % v/v sheep blood, 0.5 % w/v glucose and 1.5 % w/v agar. Immediately before use the surface of the latter agar was treated with sterile bovine catalase (2000 U per plate). The isolated microorganisms were confirmed as mainly Gram positive, obligately anaerobic, sporeforming, catalase and oxidase negative, and metronidazole sensitive rods. Regardless of their optimum growth temperature, all strains were able to grow at temperatures below 4°C, but only some were able to grow at 37°C (Table 1.1). After their initial isolation, meat strains were maintained as freeze-dried cultures. Before characterisation, strains were revived from freeze-dried material in Peptone Yeast Extract Glucose Starch (PYGS) broth (Lund *et al.*, 1990), subcultured onto Columbia Blood Agar (CBA; Oxoid CM331) containing 5 % v/v sheep blood (Life Technologies) and incubated for 48 to 96 h at temperatures within their optimum growth range (Table 1.1). Strains were checked for purity before use.

Reference strain of *C. estertheticum* DSM 8809^T (T = type strain) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. This strain was revived in sterile anaerobic milk and was subcultured onto Columbia Blood Agar (CBA, Oxoid) containing 5 % v/v sheep blood. The strain was grown at 10°C for 7 days.

Morphological characteristics

Colony, cell and spore morphology. For the description of colony morphology, meat strains were grown on the surface of CBA with 5 % v/v sheep blood at temperatures within their optimum growth range for 48 to 96 h. The morphology of well-isolated colonies was described and documented using conventional photography.

For description of vegetative cell and spore morphology, meat strains were grown in PYGS broth at temperatures within their optimum growth range for 18 to 72 h and 5 to 60 days, respectively. A light microscope (Leitz Orthoplan) in phase contrast mode was used to examine and photograph vegetative cells and spores of each bacterium.

Gram reaction and Gram type. Cells of meat strains exponentially growing in PYGS broth were Gram stained using the method of Johnson *et al.* (1995). A KOH test (Powers, 1995) was also performed to determine the Gram type of cells grown on the surface of CBA with 5 % v/v sheep blood at temperatures within their optimum growth range for 48 to 96 h.

Biochemical characteristics

All biochemical tests were conducted in duplicate. Unless otherwise stated, consensus duplicate results were required for the final results to be recorded as positive or negative. When a consensus of duplicate results was not reached, the test was repeated and the consensus of two out of three results was recorded as positive or negative.

Substrate utilisation. Substrate utilisation was tested according to Holdeman *et al.* (1977) at temperatures within each strain's optimum growth range and using an initial pH of 7.0 in PY broth containing 0.1 % w/v yeast extract. The following initial concentrations of substrates were used: adonitol, 0.5 % w/v; arabinose, 0.5 % w/v; cellobiose, 1.0 % w/v; dextran, 1.0 % w/v; fructose, 1.0 % w/v; galactose, 1.0 % w/v; glucose, 1.0 % w/v; inositol, 1.0 % w/v; inulin, 1.0 % w/v; lactose, 1.0 % w/v; maltose, 1.0 % w/v; mannitol, 1.0 % w/v; rhamnose, 1.0 % w/v; raffinose, 1.0 % w/v; salicin, 1.0 % w/v; sorbitol, 1.0 % w/v; sorbose, 1.0 % w/v; sucrose, 1.0 % w/v; trehalose, 0.5 % w/v; and xylose 1.0 % w/v. Hungate tubes containing the medium with substrate, and duplicate controls with no substrate, were inoculated with 2 % v/v of exponentially growing PYGS culture incubated at the appropriate temperature.

Growth was determined by monitoring an increase in the optical density at 550 nm, as measured with a spectrophotometer (Novaspec II, Pharmacia), against three uninoculated controls. Tubes showing an increase in optical density of more than 0.1 OD unit, relative to the controls, were recorded as being positive with respect to substrate utilisation. Tubes showing a pH drop of more than 1.0 pH unit, compared with controls, were recorded as being positive with respect to substrate fermentation.

Gelatine hydrolysis, lecithinase and lipase activity, milk reaction, meat and casein digestion, indole production, nitrate reduction, urea hydrolysis, ammonia and hydrogen sulphide production, esculin and starch hydrolysis, and growth in the presence of bile, and Tween 80 were tested following methods of Holdeman *et al.* (1977).

Fermentation products. Fermentation products were determined for all meat strains grown in PYGS broth at temperatures within their optimum growth range until stationary growth phase was reached. Ether extracts of volatile fatty acids and alcohols, and methyl esters of non-volatile fatty acids were prepared according to Holdeman *et al.* (1977). Extracts were analysed on a Hewlett Packard 5890/II gas chromatograph (Hewlett Packard) equipped with a 30 m x 0.53 mm, 1.0 μm FFAP capillary column (J&W Scientific), a flame ionisation detector (FID) and a Hewlett Packard model 7673 automatic sampler. The gas chromatographic parameters were as follows: column head pressure, 47.4 kPa; split vent flow of helium carrier gas, 50 ml min^{-1}; column flow measured at 60°C, 9.9 ml min^{-1}; the resultant split ratio, 5:1; the split/splitless injector and FID detector temperature, 260°C. Run conditions were: initial temperature 60°C for 2 minutes, raised to 180°C at 5°C min^{-1}, final temperature of 240°C held for 2 minutes after raising at a rate of 20°C min^{-1}. Peaks were automatically integrated (Chemstation v.4) and fatty acids were identified and quantified against retention times and peak areas of known standards. Results of duplicate experiments were recorded as a concentration range for a product. Production of H_2 and CO_2 was determined on a Varian gas chromatograph (Varian) equipped with a Porapak Q 80/100-mesh column (Supelco) and a thermal conductivity detector. This test was qualitative only.

Selected enzymatic reactions. With the meat strains, except strains DB1A, R26, SPL73, K21 and K24, the Rapid ID 32 A system (BioMerieux, MedBio Enterprises) was used to study an additional 29 enzymatic reactions: urea hydrolysis, arginine dihydrolase activity, fermentation of 11 sugars, glutamic acid decarboxylase activity, nitrate reduction, indole production, alkaline phosphatase activity and arylamidase activity towards 12 amino acids. Test strips were inoculated according to the manufacturer's instructions and read after aerobic incubation at 37°C for 4 h.

Identification of psychrophilic and psychrotrophic clostridia

Morphological and biochemical characteristics of meat strains were compared with those of known psychrophilic and psychrotrophic clostridia to determine whether the meat strains were isolates of any currently described low-temperature-growing clostridial species (Cato *et al.*, 1986; Collins *et al.*, 1992; Kalchayanand *et al.*, 1993; Lawson *et al.*, 1994; Kotsyurbenko *et al.*, 1995; Mountfort *et al.*, 1997). Subsequently, the dichotomous keys of Bergey (Cato *et al.*, 1986) and Holdeman *et al.* (1977) were used in an attempt to identify the meat strains. This approach assumed that the meat strains were included in the known clostridial species forming the identification databases. With these identification schemes, selected morphological, biochemical and physiological characteristics were used, as required in a key. The enzyme activity profiles of meat strains obtained with Rapid ID32 A system were compared with profiles of anaerobic microorganisms available in the manufacturer's database.

Ability to cause vacuum pack 'blowing'

Ability to cause 'blown pack' spoilage in a laboratory meat model system was demonstrated for meat strains and reference strain *C. estertheticum* DSM 8809^T. Each strain was grown in 10 ml of PYGS broth until a heavy suspension (approximately 10^8 ml^{-1}) was obtained. Cells of each strain were harvested by centrifugation at 6,000 *g* for 15 minutes and washed five times with ice-cold saline (0.85 % w/v NaCl). These washed suspensions were used as inocula.

Chilled boneless lamb chumps were obtained from a local meat plant one day after boning and placed individually into barrier bags (Cryovac BB4L) pending inoculation. Duplicate packs were prepared for each strain tested. The packs were inoculated with 0.5 ml of a cell suspension of each strain to obtain a concentration of approximately 10^3 cfu cm^{-2}. Inoculated packs and two uninoculated controls were immediately vacuum-packed using a controlled atmosphere packaging machine (Securepack 10) and were stored at 2°C for up to 84 days (the maximum shelf life expected for vacuum-packed chilled lamb). Packs were examined regularly for the presence of gas bubbles in the meat drip followed by loss of vacuum and substantial pack distension.

Results

Morphological and biochemical characteristics

Colony, cell and spore morphology. Colonies of all meat strains grown on the surface of CBA agar had circular shape and varied in size (Table 1.2).

Table 1.2. Colony and vegetative cell measurements of meat strains of psychrophilic and psychrotrophic clostridia associated with meat spoilage.

Strain designation	Colony diameter	Vegetative cell length/width (in µm)
DB2, SPL17, SPL242, KDL358, M14, M33, PP1	2.0–3.9	3.0–6.0/1.2–1.4
SPL4, SPL9, SPL58, SPL111, SPL141, SPL281, SPL290	1.0–1.7	2.4–4.0/0.9–1.1
SPL148	1.6–2.0	2.4–4.0/0.9–1.1
DB1A, R26	0.7–3.0	2.0–7.5/0.4–0.9
SPL73	0.8–2.5	1.8–2.8/0.5–0.8
SPL77A, SPL77B	2.2–7.2	4.5–9.4/1.3–1.6
K21, K24	0.7–2.6	6.3–12.2/0.9–1.3

Examples of appearance of colonies representing different colony morphology types that occurred among meat strains of psychrophilic and psychrotrophic clostridia are shown in Fig. 1.1.

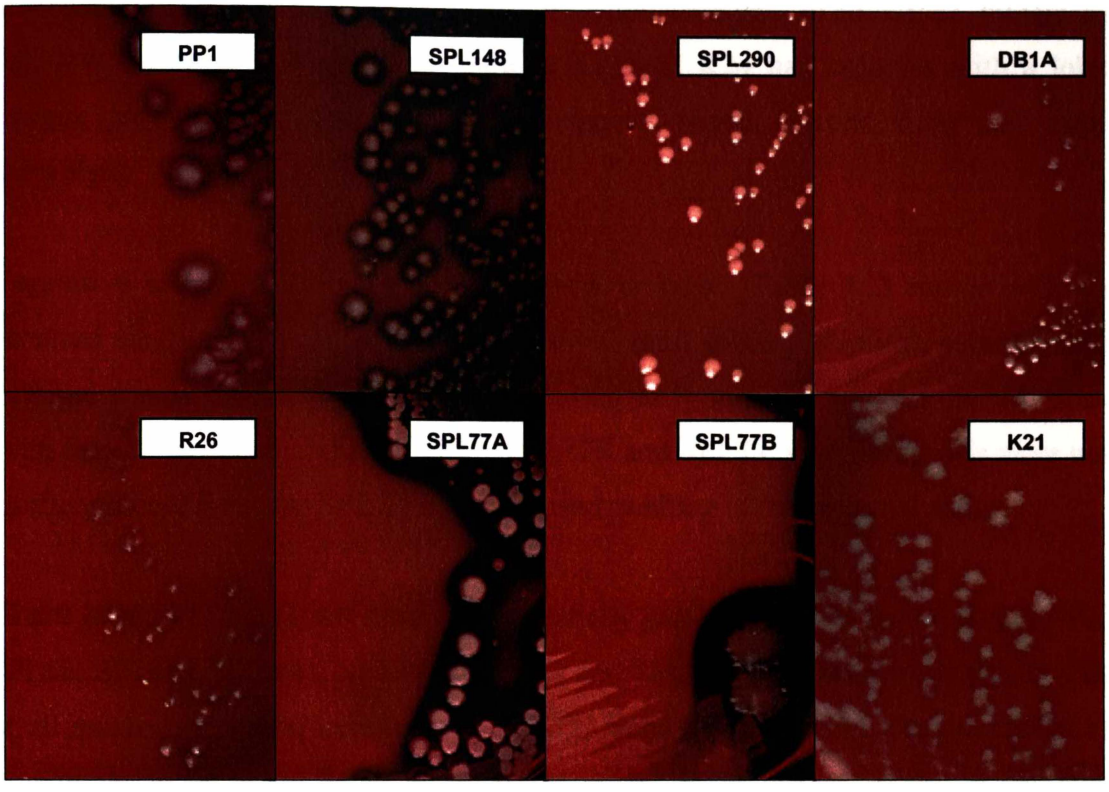

Figure 1.1 Different colony morphotypes (actual size) encountered among strains of psychrophilic and psychrotrophic clostridia grown anaerobically on fresh Columbia Blood Agar with 5 % sheep blood. Strains PP1, SPL77A and SPL77B were grown at 30°C for 48 h; strains SPL148 and SPL290 were grown at 30°C for 72 h; strains DB1A and R26 were grown at 20°C for 96 h; and strains K21 was grown at 15°C for 7 d.

With strains DB2, SPL17, SPL242, KDL358, M14, M33 and PP1, surface colonies had irregular margins, were grey to colourless, semi-opaque with translucent edges, flat, low convex, shiny and haemolytic. With strains SPL4, SPL9, SPL58, SPL111, SPL141, SPL281 and SPL290, colonies had an entire margin and were cream-grey, opaque, raised, convex, shiny, smooth and non-haemolytic. Strain SPL148 had characteristics similar to the latter group, with the exception that the margin was irregular and colonies were haemolytic. Meat strains DB1A, R26 and SPL73 had colonies with an entire margin, grey-white, semi-translucent, raised, convex, shiny and weakly haemolytic to haemolytic. Colonies of strain SPL77A had an entire to irregular margin and were grey-cream, opaque, raised, low convex, shiny and haemolytic.

Colonies of strain SPL77B had a lobate margin and were grey to colourless, translucent, flat, low convex and haemolytic. Strains K21 and K24 had colonies with a lobate margin, grey-cream or dirty yellow, semi-opaque, raised, convex, shiny and weakly haemolytic.

Vegetative cells of all strains were medium to large (Table 1.2) motile rods that occurred singly or in pairs (Fig. 1.2). With all meat strains, elliptical, subterminal to terminal spores (Fig. 1.2) were observed during early to late stationary growth phase. With the exception of strains SPL77A, SPL77B and SPL73, sporulated cells were non-motile. Except for strain SPL73, spores caused swelling of the maternal cells.

Gram reaction and Gram type. Exponentially growing cells of strains SPL4, SPL9, SPL58, SPL111, SPL141, SPL148, SPL281 and SPL290 stained Gram positive. Cells of all remaining strains, except strain SPL73, were Gram variable. Regardless of the growth phase, strain SPL73 stained Gram negative. With all meat strains except strain SPL73, the KOH reaction was negative (i.e. characteristic of Gram positive cells). With strain SPL73, the KOH reaction was positive.

Substrate utilisation. The substrate utilisation by meat strains of psychrophilic and psychrotrophic clostridia is summarised in Tables 1.3 and 1.4. No meat strain was positive for indole production, nitrate reduction or growth in bile. Tween 80 did not stimulate the growth of any strain.

Fermentation products. All strains produced acetate, butyrate and ethanol as their major fermentation products. Detailed composition of fermentation products formed by each strain is given in Table 1.3.

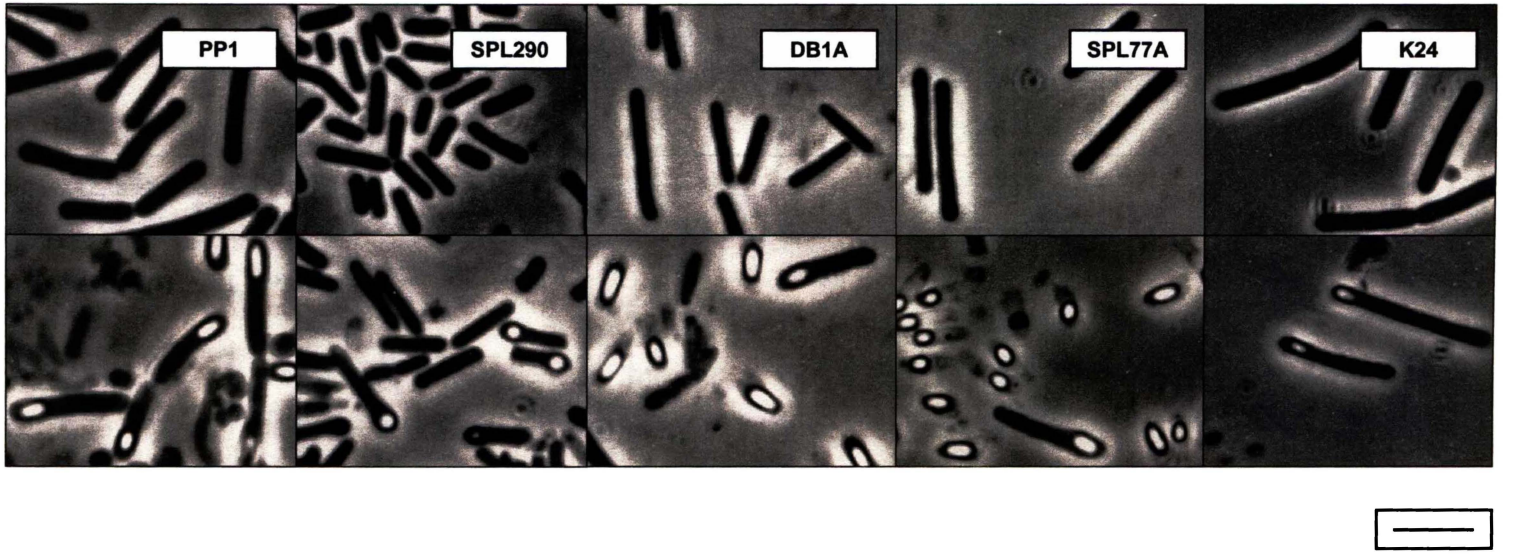

Figure 1.2. Typical morphology of vegetative (upper panel) and sporulating (lower panel) cells of meat strains of psychrophilic and psychrotrophic clostridia. Upper and lower panels represent the same strain. With strain K24, the bar equals 3.5 μm; with the remaining strains, the bar equals 4 μm.

Table 1.3. Some biochemical characteristics of meat strains of psychrophilic and psychrotrophic clostridia associated with meat spoilage.

Strain designation	Haemolysis	Gelatine hydrolysis	Casein digestion	Milk reaction	Meat digestion	Lipase activity	Lecithinase activity	Starch hydrolysis	Esculin hydrolysis	Ammonia production	Fermentation products
DB2, SPL17, SPL242, KDL358, M14, M33, PP1	+	+	–	C	–	+	+	+	–	–	B, A, L, o, 2
SPL4, SPL9	–	–	–	C	–	–	–	+	+	+	A, B, L, iV, ib, 2
SPL58, SPL111, SPL141, SPL281, SPL290	–	–	–	–	–	–	–	–	–	+	A, B, L, iV, ib, 2
SPL148	+	–	–	–	–	–	–	–	–	+	A, B, L, iV, ib, 2
DB1A	w+	+	–	C	–	–	–	+	+	–	A, B, L, 2, 4
R26	+	+	–	C	–	–	–	+	+	–	A, B, L, 2, 4
SPL73	+	–	–	–	–	–	–	+	–	–	A, F, B, l, 2, 4
SPL77A	+	+	+	C, D	+	–	+	–	–	+	A, B, ib, iv, l, o, 2, 4
SPL77B	+	+	w+	C, wD	w+	–	+	–	–	+	A, B, ib, iv, l, o, 2, 4
K21	+	+	–	C	–	–	+	–	–	+	A, B, ic, l, p, 2
K24	+	+	–	–	–	–	+	–	–	+	A, B, ic, l, p, 2

Indole production, nitrate reduction and growth in bile were negative for all meat strains. +, positive; –, negative; w, weak reaction; C, curd; D, digestion; A, acetate; B, butyrate; L or l, lactate; ib, isobutyrate; ic, isocaproate, iV or iv, isovalerate; f, formate; o, oxalacetate; py, pyruyvate; 2, ethanol; 4, butanol. Upper case letters indicate major and lower case letters indicate minor fermentation product. Fermentation products are listed in order of quantities of compounds produced by meat strains.

Table 1.4. Fermentation of selected carbohydrates and alcohols by meat strains of psychrophilic and psychrotrophic clostridia associated with meat spoilage.

Strain designation	Adonitol	Arabinose	Cellobiose	Galactose	Inositol	Inulin	Lactose	Maltose	Mannose	Rhamnose	Raffinose	Salicin	Sorbitol	Sucrose	Trehalose	Xylose
DB2, SPL17, SPL242, KDL358, M14, M33, PP1	+	–	–	–	–	–	–	+	+	–	–	–	+	+	+	–
SPL4, SPL9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
SPL58, SPL111, SPL141, SPL281, SPL290	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–
SPL148	–	–	–	–	+	–	–	–	+	–	–	–	–	–	–	+
DB1A	–	–	+	–	+	–	–	+	+	–	–	+	–	–	+	–
R26	–	–	+	w	–	–	–	+	+	–	–	+	–	+	+	–
SPL73	–	+	+	+	–	+	+	+	+	+	+	+	–	+	–	+
SPL77A	–	–	–	–	–	–	–	+	+	–	–	–	+	–	+	–
SPL77B	–	–	–	–	–	–	–	+	+	–	–	–	+	–	+	–
K21	–	–	–	–	–	–	–	+	–	+	–	–	–	–	+	–
K24	–	–	w	–	–	–	–	+	w	+	–	–	–	–	+	–

Strains were grown at temperatures within their optimum growth range (see Table 1.1). Glucose and fructose were fermented by all meat strains, and no strains fermented dextran, mannitol and sorbose. +, positive; –, negative; w, weak reaction (pH from 5.5 to 5.9).

Selected enzymatic reactions. With the Rapid ID 32 A, α-glucosidase activity was present in strains DB2, SPL17, SPL242, KDL358, PP1, M14 and M33. With strains SPL4, SPL9, SPL58, SPL111, SPL141, SPL148, SPL281 and SPL290, positive reactions were observed for pyroglutamic acid arylamidase and proline and arginine arylamidases. With strain SPL77A positive reactions were observed for β-N-acetyl-glucosaminidase and proline arylamidase and weak reactions were observed for arginine arylamidase and pyroglutamic acid arylamidase. Strain SPL77B was positive or negative for the same reactions as strain SPL77A, except β-N-acetyl-glucosaminidase activity was not present in strain SPL77B.

Identification of psychrophilic and psychrotrophic clostridia

With the identification of meat strains, the same results were obtained with the dichotomous keys of both Cato *et al.* (1986) and Holdeman *et al.* (1977). Consequently, only identification results obtained with the latter are reported.

Using the key of Holdeman *et al.* (1977), strains DB2, SPL17, SPL242, KDL358, PP1, M14 and M33 were identified as *C. lituseburense.* The meat strains would have shown similarity to non-proteolytic *C. botulinum* types B, E or F (Cato *et al.*, 1986), if it had not been for the positive lecithinase reaction and adonitol fermentation results obtained for these strains. With the Rapid ID32 A system, a 'presumptive identification' result was obtained with the meat strains being identified as *C. botulinum* (98.1 % ID, 0.75 T; where % ID is an estimate of how closely the profile matches profiles of other taxa in the database and T is an estimate of how closely the profile matches the typical set of reactions of the identified taxon).

Strains SPL4, SPL9, SPL58, SPL111, SPL141, SPL148, SPL281 and SPL290 differed in their ability to hydrolyse esculin. Consequently, strains SPL4 and SPL9 that were able to hydrolyse this compound would have been, according to the key, identified as *C. fallax*, and the remaining strains would have been identified as *C. tyrobutyricum.* All these meat strains showed characteristics similar to another psychrotroph *C. algidicarnis* (Lawson *et al.*, 1994). Identification with Rapid ID32 A system was

returned with a 'doubtful profiles' note. The meat strains were identified as either *C. sporogenes* (44.7 % ID, 0.49 T) or *C. histolyticum* (39.5 % ID, 0.42 T).

Strain DB1A would have been identified as *C. oceanicum*, strain R26 as *C. perfringens* and strain SPL73 as *C. thermosaccharolyticum*. Strains SPL77A and SPL77B were identified as *C. novyi* type B and strains K21 and K24 as either *C. novyi* type A or *C. sporogenes*. None of these seven strains appear to share major morphological, biochemical and physiological characteristics with known psychrophilic and psychrotrophic clostridia. In addition, 'low discrimination' was achieved when strains SPL77A and SPL77B were tested with Rapid ID32 A system. With this system, these strains were identified as *C. sporogenes* (66.4 % ID, 0.92 T).

Ability to cause vacuum pack 'blowing'

In packs inoculated with vegetative cells of the meat strains DB1A and R26, small gas bubbles first appeared in the meat drip after 14 days storage at 2°C. In comparison, gas production in packs inoculated with vegetative cells of the meat strains K21 and K24 was first evident at 24 and 27 days of storage, respectively. Gas production in packs inoculated with vegetative cells of the reference strain of *C. estertheticum* DSM 8809^T was first evident at 11 days of storage. While packs inoculated with strains K21, K24 and *C. estertheticum* reached the tightly distended state after 35 days of storage, a smaller volume of gas was produced in packs inoculated with strains DB1A and R26. These packs did not reach the tightly distended state within 84 days storage. No gas was present in uninoculated control packs stored at 2°C for 84 days.

No gas production or pack distension was observed in packs inoculated with vegetative cells of any of the remaining meat strains.

Discussion

Phenotypically, psychrophilic and psychrotrophic clostridia characterised in this study appeared to belong to a very diverse group. The majority of clostridia in this group included dominantly Gram positive organisms with heterogeneous colony morphology. However, meat strain SPL73, as determined with the KOH reaction, seemed to be a Gram negative bacterium. Possession of a Gram negative type cell wall may appear to be an unusual property of clostridia in the traditional sense (Prazmowski 1880, as cited in Cato *et al.*, 1986). However, many organisms that give Gram negative reaction, e.g. *C. aerotolerans* and *C. xylanolyticum* (van Gylswyk and van der Toorn, 1987; Rogers and Baecker, 1991), are included in the genus *Clostridium*. Recent reports indicate that a conventional Gram stain may not always reflect a true cell wall type and that *C. xylanolyticum* may in fact possess a Gram positive cell wall (Rogers and Messner, 1992).

Most of the psychrophilic and psychrotrophic meat clostridia investigated in this study were metabolically active, saccharolytic or saccharolytic and proteolytic, organisms showing typical butyric type metabolism (Jones and Woods, 1986). These microorganisms varied in their substrate utilisation and fermentation product patterns. Isolates DB2, SPL17, SPL242, KDL358, PP1, M14 and M33, however, were indistinguishable in their biochemical reactions and consistently grouped together regardless of phenotypic test used. It is likely that these strains belong to the same clostridial species and possibly represent the same clostridial strain. According to the key, these isolates would have been identified as *C. lituseburense*. However, this reference strain is mesophilic, digests milk and produces ammonia, while the meat strains are psychrotrophic, produce curd in milk and do not produce ammonia. In addition, *C. lituseburense* produces a wider variety of fermentation products than the meat strains, which fermented a wider range of sugars than *C. lituseburense* (Holdeman *et al.*, 1977). With the key of Holdeman *et al.* (1977), strains DB2, SPL17, SPL242, KDL358, PP1, M14 and M33 would have been identified as one of the non-proteolytic *C. botulinum* types B, E and F, if not for the positive lecithinase reaction and adonitol

fermentation obtained for the meat strains. In a related study, these strains with the exception of isolate KDL358 were tested for production of botulinal neurotoxin. Culture supernatants from the tested strains have been subsequently found to be non-toxic to mice and botulinal neurotoxin genes could not be detected in these meat strains (Broda *et al.*, 1998a; Moorhead and Bell, 1999). Although the results of phenotypic characterisation and identification with the Rapid ID32 A system indicated similarity of the meat strains to non-proteolytic *C. botulinum* types B, E and F, the identity of these strains remains to be confirmed. This is especially important from a public health perspective, considering the recent findings of Brett (1998) which demonstrated that with the Rapid ID32 A system some clostridial strains might be mis-identified.

With the dichotomous keys, strains SPL4, SPL9, SPL58, SPL111, SPL141, SPL148, SPL281 and SPL290 would have been identified as *C. fallax* or *C. tyrobutyricum*. But both *C. fallax* and *C. tyrobutyricum* are mesophilic, haemolytic and do not produce isovaleric acid, while these meat strains are psychrotrophic, mainly non-haemolytic and isovaleric acid is a major product of fermentation in PYGS broth. In addition, *C. tyrobutyricum* does not produce ethanol, a major fermentation product of the meat strains. Strains SPL4, SPL9, SPL58, SPL111, SPL141, SPL148, SPL281 and SPL290 showed characteristics similar to another psychrotrophic bacterium, *C. algidicarnis* (Lawson *et al.*, 1994). However, none of the meat strains showed the weak casein digestion characteristic of *C. algidicarnis*. Some of these meat strains fermented different sugars to those fermented by *C. algidicarnis*, e.g. strains SPL4 and SPL9 did not ferment mannose, while *C. algidicarnis* slowly fermented this carbohydrate and strain SPL148 fermented inositol, while *C. algidicarnis* did not. Strain SPL148 was also haemolytic, while the remaining meat strains in this group and *C. algidicarnis* were not. Similarly, some of meat strains hydrolysed esculin and starch and produced curd in milk, none of these characteristics are typical of the *C. algidicarnis* reference strain. It is not known whether the phenotypic variability of individual meat strains within this group of isolates observed in this study indicated intra- or inter-species heterogeneity. Further work is needed to test if strains SPL4, SPL9, SPL58, SPL111, SPL141, SPL148, SPL281 and SPL290 belong to the species *C. algidicarnis*.

With both the keys of Cato *et al.* (1986) and Holdeman *et al.* (1977), strains SPL77A and SPL77B were identified as *C. novyi* type B. The majority of *C. novyi* type B are, however, able to produce indole, do not ferment sorbitol, are mesophilic and in PYGS broth have propionic acid as their major fermentation product. In contrast, strains SPL77A and SPL77B did not produce indole, but did ferment sorbitol, were psychrotrophic, and acetic, butyric, isovaleric, isobutyric acids and ethanol and butanol were their major PYGS broth fermentation products. According to their major morphological, biochemical and physiological characteristics these two meat strains (as discussed in detail in Chapter 4) could not be classified as strains of any currently described species of low-temperature-growing or mesophilic clostridia.

With the dichotomous keys, strain DB1A would have been identified as *C. oceanicum*, strain R26 as *C. perfringens* and strains K21 and K24 as either *C. novyi* type A or *C. sporogenes*. All these reference strains are, however, mesophilic and strains DB1A, R26, K21 and K24 were not able to grow at 30°C or 37°C. Because the ability to grow at 37°C is incorporated into the key for differentiation of clostridial species not liquefying gelatine, psychrotrophic strain SPL73 which was unable to grow at temperatures above 32°C, would have been identified as *C. thermosaccharolyticum*, a thermophilic *Clostridium* sp. that grows optimally at temperatures between 55 and 62°C. Strains DB1A, R26, K21, K24 and SPL73 did not appear to share major morphological, biochemical and physiological characteristics with any known psychrophilic and psychrotrophic clostridia (as discussed in detail in Chapter 4). Consequently, it was not possible to identify these meat strains as any of the currently described clostridial species.

The Rapid ID32 A system proved unsatisfactory with the majority of psychrotrophic meat strains. This system utilises incubation temperatures suitable for mesophiles and some psychrotrophic but not for psychrophilic clostridia. Consequently, it was used with the 17 psychrotrophic meat strains able to grow at 37°C. However, the unsatisfactory identification that was achieved with 10 of these strains is most likely due

to the database containing few if any *Clostridium* spp. not associated with human disease (Lombard *et al.*, 1982; Buesching *et al.*, 1983; Williams *et al.*, 1992).

Only four of the 22 meat strains of psychrophilic and psychrotrophic clostridia produced gas in the artificially inoculated packs stored at 2°C. The variability in reproducing the original spoilage characteristics was previously reported with some strains of these clostridia (Broda *et al.*, 1996b). This finding indicated that not all low-temperature-growing clostridia associated with meat spoilage cause 'blown pack' spoilage. Indeed, meat strains examined in the present study are likely to belong to numerous species, some that cause 'blown pack' spoilage and some that are benign. Therefore, the detection of psychrophilic and/or psychrotrophic *Clostridium* spp. in vacuum-packed meat may no longer be sufficient to account for premature meat spoilage at refrigeration temperatures.

Conclusions

When recognised dichotomous identification keys were used, none of the 22 meat strains of psychrophilic and psychrotrophic clostridia could be recognised as previously described mesophilic clostridial species. When the conventional phenotypic characteristics of meat strains were compared with those of known psychrophilic and psychrotrophic clostridia, some cross-similarities between meat and known strains were evident. None of the meat strains, however, showed characteristics indistinguishable from those of any previously described species of psychrophilic and psychrotrophic clostridia. Similarly, none of the psychrotrophic meat strains investigated in this study could be confidently identified using the Rapid ID32 A system. The large variability of conventional phenotypic characters that was observed with the majority of meat strains precluded their differentiation into consistent phenotypic groups. Consequently, it is concluded that alternative methods are needed to achieve reliable identification and differentiation of psychrophilic and psychrotrophic meat borne clostridia.

Chapter 2

Genotypic Differentiation of Psychrophilic and Psychrotrophic Clostridia

Introduction

Molecular techniques promise objective identification and differentiation of bacteria

Conventional methods may confound identification of some bacterial groups (e.g. psychrophilic and psychrotrophic *Clostridium* spp., as demonstrated in Chapter 1) and in such instances alternative identification and differentiation methods are generally sought. The majority of alternative, molecular methods were introduced in the 1970s and 1980s. The majority of molecular methods aimed to analyse the composition of molecules that are most directly expressed by the bacterial cell, in anticipation that these molecular methods would not be, or would be only minimally, influenced by the growth conditions of bacteria. However, variety of both directly and indirectly expressed cell constituents, such as cellular fatty acids, proteins, quinones, peptidoglycans and nucleic acids have been used in the identification and differentiation of bacteria and analyses based on these molecules have been found applicable to the majority of bacterial species (Schleifer and Stackebrandt, 1983). The most commonly used molecular methods include cellular fatty acid analysis, whole cell proteins analysis, multilocus enzyme electrophoresis and nucleic acid based analysis (Swaminathan and Matar, 1993).

Cellular fatty acid (CFA) analysis. Over 300 fatty acids and related compounds that constitute part of cellular lipids can be found in bacterial cells. The types and relevant proportions of cellular fatty acids are considered characteristic of specific bacterial

groups. To obtain CFA profiles, the lipid components of bacterial cells are saponified and the fatty acids are methylated to convert them into volatile derivatives. The methylated fatty acids are then extracted into an organic solvent and separated by gas chromatography.

Standardised procedures for cellular fatty acid analysis in combination with a computerised database, such as MIDI Microbial Identification System, have been used for the identification of various bacterial species (Tunér *et al.*, 1992; Kellogg *et al.*, 1996), including clostridia (Allen *et al.*, 1995; Ghanem *et al.*, 1991). The discriminatory power of cellular fatty acid analysis is usually adequate for inter-species differentiation (Welch, 1991; Swaminathan and Matar, 1993). However, CFA profiles can be affected by the growth conditions (Swaminathan and Matar, 1993; Wauthoz *et al.*, 1995; Vandamme, 1998), such as growth medium or incubation temperature. Consequently, to effect reproducible and consistent identification of bacterial isolates standardised culture conditions must be applied to both unknown isolates and previously described bacterial species. It is also possible for different species belonging the same genus to have similar fatty acid compositions (Vandamme, 1998) and an additional phenotypic data may be needed to allocate an unknown isolate to some species.

Electrophoretic analysis of proteins. Numerous protein molecules can be synthesised in a bacterial cell (Jackman, 1985) and under the same growth conditions a bacterial strain is thought to produce the same set of proteins (Kersters and De Ley, 1980). With SDS-PAGE, bacterial cells are usually mechanically disrupted and denatured in presence of SDS. Soluble whole cell proteins are then separated using the discontinuous buffer system of Laemmli (1970) in polyacrylamide gel, usually under constant voltage. To detect the protein banding pattern, gels are stained with Coomassie brilliant blue solution, de-stained and dried, and the banding patterns are analysed.

Whole cell protein profiles as determined by PAGE have frequently been used for identification and differentiation of bacteria (Kersters and De Ley, 1975; Moore *et al.*,

1980) and these profiles are similar or almost identical for closely related bacterial strains. PAGE has been applied to study a wide selection of clostridial species and was found to correlate well with DNA-DNA homology data (Cato *et al.*, 1982). Consequently, whole cell protein analysis is particularly useful for inter-species differentiation of bacteria but may lack discriminatory power for differentiation at intra-species level. Whole cell protein profiles can be affected by the growth medium and strain cultivation conditions (Kersters and DeLey, 1980). To effect reproducible and consistent identification and differentiation of bacterial isolates, whole cell protein profiles to be compared between laboratories should be generated for isolates grown in the same growth medium, under similar conditions and to the same growth phase (Kersters and De Ley, 1980).

Multilocus enzyme electrophoresis (MEE). MEE proposes to identify or differentiate bacterial strains according to the differences in the relative electrophoretic mobilities of constitutive cellular enzymes. It is thought that, within one species, the presence of mobility variants of an enzyme reflects changes in the DNA that lead to a change in charge of the polypeptide. It has been thought that these variants represent different alleles at the corresponding gene locus (Selander *et al.*, 1986). Because MEE aims to determine the genetic diversity of a structural gene locus it has been thought that, in contrast to cellular fatty acids analysis and proteins analysis, results obtained with this method can be directly related to genotype (Swaminathan and Matar, 1993).

With some bacterial strains, e.g. those of *Listeria monocytogenes*, multilocus enzyme electrophoresis has been found useful in inter-strain differentiation (Baloga and Harlander, 1991). However, this method may not discriminate between other strains, e.g. those of *Escherichia coli* O157:H7 (Whittam *et al.*, 1988). With multilocus enzyme electrophoresis, analysis of a large number of isolates is labour intensive.

Nucleic acid based analysis. Cellular fatty acids, whole cell proteins and multilocus enzyme electrophoresis analyses, while valuable for identification and differentiation, discriminate between bacteria on the basis of the composition of indirectly expressed

cell constituents, which are in fact phenotypic characteristics. The phenotype is determined by both genetic and environmental information and, consequently, unless strict precautions are taken, quantitative and qualitative results obtained with any phenotypic method are subject to variation, especially when data are compared between different laboratories. This variation often confounds bacterial identification. In contrast, nucleic acid based methods use the most directly expressed molecules and, therefore, their composition is independent of cell cultivation conditions or nucleic acid preparation methods.

Most nucleic acid based methods for identification and differentiation of bacteria use DNA rather than RNA, because it is the most directly expressed molecule. Similarly to DNA, the sequence of ribosomal RNA is not affected by the environment. However, DNA is chemically more stable than RNA. Many bacteria are RNase rich and isolation of intact RNA may be difficult. DNA can be extracted relatively easily from the majority of bacterial cultures and DNA based identification and differentiation methods are, thus, universally applicable. Since DNA encodes the basis of all genetic diversity that exists between bacterial strains and species, DNA based methods have the potential for discrimination at the inter- and intra-species level. Consequently, such methods are able to distinguish between both closely and remotely related strains.

Numerous methods are currently available for DNA based identification and differentiation of bacteria. The most frequently used are DNA sequencing, DNA probing, genomic DNA based methods, PCR product based methods and plasmid profiling.

DNA sequencing based identification and differentiation of bacteria

Genomic DNA sequencing. The most accurate way to determine precisely whether two bacterial strains are different is to directly compare their genomic DNA sequences. For many years, however, DNA sequencing methods were laborious and time consuming.

High throughput genomic sequencing and analysis has improved significantly with the introduction of the polymerase chain reaction (PCR) technique (Mullis and Faloona, 1987) and automated DNA sequencing.

PCR consists of consecutive cycles of *in vitro* DNA synthesis. The specificity of amplification is obtained by using single stranded oligonucleotides (primers) complementary to the regions flanking the two ends of the DNA fragment that is to be amplified. At the appropriate temperature these primers anneal to their complementary sequences on a single stranded template and initiate DNA synthesis. In the presence of DNA polymerase, deoxynucleotides (dNTPs) and magnesium cations, starting from the annealed primers, a strand complementary to that of the template is synthesised and, consequently, the amount of target DNA is doubled. With PCR, more than 10^6 copies of a specific DNA fragment can be obtained from template DNA in 20 to 30 repeated cycles of denaturation, primer annealing and strand extension by a thermostable *Thermus aquaticus (Taq)* or other thermophilic DNA polymerase (Saiki *et al.*, 1988).

Most frequently employed DNA sequencing strategies utilise the dideoxynucleotide (ddNTP) chain termination method of Sanger *et al.* (1977). This sequencing method involves enzymatic synthesis of the DNA and is carried out in the presence of a preferably single stranded DNA template, single sequencing primer, the mixture of all four dNTPs, one dideoxy analog of dNTP, and DNA polymerase. Either primer or dNTPs are radioactively labelled. Synthesis of a strand complementary to the template DNA is initiated by annealing, and continued by extension, of a sequencing primer. The dideoxy sugar lacks the 3' hydroxyl group and when it is incorporated into the synthesised DNA strand, extension of the strand cannot occur and is randomly terminated. During this incomplete extension process, DNA fragments of variable length, each ending with a dideoxynucleotide, are created and when the ratio of the dNTPs to ddNTP is appropriately balanced, these fragments represent termination at every nucleotide relative to the ddNTP used.

To determine the sequence, four DNA sequencing reactions, each with different ddNTP, must be performed. The denatured DNA fragments obtained in four reactions are separated by electrophoresis in four lanes of a polyacrylamide gel. The sequence complementary to that of the DNA template strand can be read from the positions of radioactive bands present in all four lanes, as visualised on an autoradiograph of the gel.

An automated DNA sequencing procedure uses the similar principle as conventional sequencing. However, in this procedure one of four different fluorescent dyes is used to identify one of A, G, C and T extension reaction products. Consequently, four differently labelled products can be identified in a single DNA sequencing reaction and the electrophoresis can be conducted in one gel lane instead of four. Fluorescent dye labels can be incorporated into DNA extension products using either dye 5'-labelled primers or dye 3'-labelled terminators. With dye primer sequencing chemistry, extension products are identified using primers labelled with four different fluorescent dyes. The products from these four reactions are then combined and loaded into a single gel lane. With dye terminator labelling, each of the four dideoxy terminators is labelled with a different fluorescent dye and four sequencing reactions are performed simultaneously in one tube. Each of the A, G, C or T dyes emits light at a different wavelength when excited with laser light, and this signal is detected with a scanner as the marked fragments are electrophoresed through the gel.

Once determined, DNA sequences are routinely deposited in nucleotide databases such as the GenBank database (Benson *et al.*, 1998), the European Molecular Biology Laboratories (EMBL) database (Stoesser *et al.*, 1999) and the DNA Data Bank of Japan (DDBJ). An additional database, the Ribosomal Database Project (RDP), contains 16S rRNA gene sequences that have been aligned to fit a standard model of rRNA structure, so sequence heterogeneities or errors are easily identifiable (Maidak *et al.*, 1999). A variety of search tools, such as BLAST (Altschul *et al.*, 1997) or FASTA, are available via the Internet for accessing the sequencing data and for comparison of the sequences of bacterial strains. However, while the combination of PCR amplification and automated DNA sequencing has enabled sequencing of complete microbial genomes

(e.g. Fleischmann *et al.*, 1995; Blattner *et al.*, 1997; Cole *et al.*, 1998), to date (June 1999), finished sequences of about 14 bacterial genomes have been published. Direct comparison of genomic DNA sequences is still too expensive and too complex to be routinely applied to identification and differentiation of bacteria. Consequently, until replaced by discrimination based on genome sequencing, alternative methods that are either based on DNA sequence comparison of specific genes or derive a snapshot of genetic diversity rather than its direct, quantitative measurements are likely to be used for identification and differentiation of microorganisms.

16S rRNA gene sequencing. Direct sequence comparison of 16S ribosomal RNA (as derived from the sequence of rDNA) is considered a preferred method for identification and species differentiation of microorganisms (Amann *et al.*, 1994).

Ribosomes, primarily responsible in the cell for the translation of proteins, consist of one small and one large subunit. In prokaryotes, the small subunit contains a 16S rRNA molecule and approximately 20 proteins, while a large subunit contains a 23S rRNA molecule, a 5S rRNA molecule and approximately 30 proteins. In prokaryotes, the rRNA (*rrn*) genes are arranged continuously, have common promoters and can all be controlled together; these genes, therefore, show operon organisation. Within a single operon, *rrn* genes are typically organised 5'-16S, 23S and 5S-3', the individual genes being often separated by internal spacers that may contain tRNA encoding gene(s). rRNA operons cover only about 0.1 % of genomic DNA. While most bacterial genes are present in only one copy, *rrn* operons can be present in 2 to 11 copies per bacterial cell.

In clostridia, rRNA appears to be encoded by several *rrn* operons (Canard and Cole, 1989; Gurtler *et al.*, 1991;Campbell *et al.*, 1993b; Rainey *et al.*, 1996) but, for the majority of clostridial species, the exact number of operons per species is unknown (Gürtler and Stanisich, 1996). In *Clostridium perfringens*, all of the ten *rrn* operons are located in a region that represents about one third of the genome and these operons account for about 1.5 % of the organism coding capacity (Canard and Cole, 1989).

With this microorganism, all copies of the rRNA operons show canonical organisation and all but one appear to be transcribed from dual promoters (Garnier *et al.*, 1991). Some of the internal transcribed spacers that separate individual genes in clostridial *rrn* operons have been shown to contain sequences encoding tRNA (Barry *et al.* 1991; Garnier *et al.*, 1991), while others did not contain these sequences (Campbell *et al.*, 1993b). It is generally believed that only minor sequence heterogeneities exist between rRNA genes located on different *rrn* operons. However, it has been shown recently that multiple copies of 16S rRNA genes of *C. paradoxum* that were located on different operons differed significantly, with some copies containing intervening sequences of more than 100 bp (Rainey *et al.*, 1996).

In the cell, rRNA is involved in protein translation and in assembly of the ribosome. The structural constraints of rRNA were thought to impose considerable conservation of rRNA genes and to ensure low level of mutability of these genes during evolution. Consequently, the genes coding for rRNA, such as 16S rRNA gene, were thought to have mutated slowly and at a constant rate throughout evolution and, therefore, their sequences were thought to relate to the genealogy of an organism (Woese *et al.*, 1975; Woese and Fox, 1977). The sequencing of the 16S rRNA genes has allowed reconstruction of the phylogeny of life (Fox *et al.*, 1980; as discussed in more detail in Chapter 4).

16S rRNA genes are composed of conserved, slow evolving and variable, relatively fast evolving regions (Neefs *et al.*, 1991; Woese *et al.*, 1990), and the latter is believed to contain species specific information (Gendel, 1996). Universal PCR primers complementary to DNA sequences within regions conserved in almost all known bacteria are commonly used to amplify 16S rDNA gene. To identify an unknown microorganism, the 16S rDNA gene is PCR amplified, purified and sequenced. The 16S rDNA gene sequence can then be used to search the GenBank, EMBL, DDBJ or RDP database libraries to establish whether the unknown sequence resembles those of known bacterial species and to identify taxonomically related species.

Direct sequence comparison appears to be a universal, practical and an increasingly inexpensive method for identifying unknown isolates. For the majority of bacterial species, it is thought that, in contrast to other nucleic acid based methods, 16S rRNA gene sequence analysis will reveal the identity, as well as the taxonomic relatives, of unknown isolates. Because a large number of microbial 16S rRNA gene sequences are contained in and can readily be accessed from, the databases (e.g. more than 9700 prokaryotic 16S rRNA gene sequences are included in the Ribosomal Database Project database), this method does not require acquisition and testing the reference cultures of bacteria. However, 16S rRNA genes sequences may significantly vary between different operons of the same strain or different strains of the same species (Clayton *et al.*, 1995; Cilia *et al.*, 1996; Rainey *et al.*, 1996). Because bacterial species in the databases are usually represented by 16S rRNA gene sequences of single strains and/or single operons, comparison of 16S rRNA genes may fail to identify bacterial isolates whose 16S rRNA gene sequences represent unknown strains or unknown operons of known species. Similarly, sequences in the databases often contain sequencing or other errors (Gendel, 1997). Such errors may impair successful identification of unknown isolates. It is also possible for different species to have nearly identical 16S rRNA sequences (Fox *et al.*, 1992; Stackebrandt and Goebel, 1994) and, consequently, the results of sequence comparisons need to be interpreted carefully.

DNA probes for specific detection/identification of bacteria

By increasing the pH or the temperature, the double helix DNA structure can be denatured and separated into two single strands. Under appropriate conditions, single stranded nucleic acid molecules that consist of complementary sequences can reassociate and form the double stranded duplex. The formation of this DNA/DNA duplex can be visualised with appropriate reporter molecules carried on one of the strands. This principle has been used to develop methods for specific detection/identification of bacteria with DNA probes (Tenover and Unger, 1993).

Probes are DNA or RNA fragments that have been labelled with reporter molecules, such as enzymes (e.g. alkaline phosphatase), antigenic substrates, chemiluminescent

molecules (e.g. acridinium esters), affinity labels (e.g. biotin, digoxigenin) or radioisotopes. Radioactive and fluorescent probes allow direct, while enzyme and other reporter molecules allow indirect, detection of hybrids. Although probes can be 20 to thousands of bases long, oligonucleotide probes including less than 50 bp hybridise more rapidly to target molecules and, consequently, large probes are frequently fragmented to increase affinity. Under stringent conditions, i.e. those which do not allow formation of mismatched base pairs in probe-nucleic acid hybrids, probes can detect the change of a single nucleotide within a given nucleic acid sequence.

Hybridisation can be conducted in liquid or solid format. In liquid format, both the target nucleic acid and the probe are free to interact in a solution. With this format, hybrids are most often detected with nuclease protection methods or the hybridisation protection assay. In solid format, target nucleic acid is transferred onto a solid support (membrane) and then hybridised with the probe. Alternatively, the probe may be immobilised prior to hybridisation. The most common solid format hybridisations include the Southern blot (Southern, 1975), dot blot (Barbour and Tice, 1997) or sandwich hybridisation assays.

Depending on the information and the level of discrimination that is required, probes may target specific or non-specific DNA sequences. While it is possible to achieve both inter- and intra- species discrimination of bacterial isolates using broad spectrum DNA probes (as detailed later in the paragraph on combination of RFLP analyses and hybridisation techniques), DNA probing is most commonly used for specific detection/identification of bacteria. The use of probes for specific detection depends on the identification of a DNA sequence fragment that can serve as a surrogate for the identification of the organism as a whole. With specific detection, DNA probes that target variable regions of 16S rRNA gene; antigen coding genes; virulence coding genes, such as verotoxin gene (Willshaw *et al.*, 1985), staphylococcal enterotoxin B gene (Notermans *et al.*, 1988), haemolysin gene (Datta *et al.*, 1988) or botulinal neurotoxin gene (e.g. Szabo *et al.*, 1992, 1993; Campbell *et al.*, 1993a); the *hdc*A gene; genes encoding selected metabolic activities, such as thermonuclease (Liebl *et al.*,

1987) or cystein protease (Fernández *et al.*, 1994); or an outer membrane protein are usually used.

With DNA probing, the detection may be performed using pure or crude genomic DNA and, consequently, this method is suitable for indication of the presence of microorganisms that are difficult to grow. Although specific, DNA probing often lacks sensitivity and is only able to detect microorganisms when their numbers exceed 10^5 to 10^6 ml^{-1} (Wilson *et al.*, 1990; Hill and Keasler, 1991; Mabilat *et al.*, 1996). Probing with specific DNA probes cannot be used for quantitative assessments or to study genetic diversity of microorganisms, because the results of this analysis indicate only the presence or absence of the target gene.

Genomic DNA based methods for differentiation and identification of bacteria

Restriction fragment length polymorphism (RFLP) analysis. With this analysis, total genomic DNA is extracted and digested with infrequently cutting restriction endonuclease. The resulting DNA fragments are then separated by agarose gel electrophoresis and visualised with ethidium bromide. Bacterial strains are compared on the basis of the number and the size of fragments produced by DNA digestion. Qualitative and quantitative differences in restriction profiles of the two bacterial strains are called restriction fragment length polymorphisms (RFLP) and can result from sequence rearrangements, insertion or deletion of DNA, or base substitution within the endonuclease cleavage sites (Tenover *et al.*, 1995).

RFLP analysis of genomic DNA is universally applicable, relatively easy to perform and sensitive because the entire genome is evaluated. It has a high discriminatory power because it is possible to distinguish between strains that differ by only one nucleotide residue in their DNA sequences, provided that this variable nucleotide is within an appropriate restriction site. However, pure and intact DNA is required for RFLP analysis of genomic DNA. Presence of impurities may inhibit restriction endonucleases and result in lack of or incomplete digestion.

Digestion of partially degraded DNA usually results in a smear of small fragments that cannot be separated on the gel. Therefore, RFLP analysis of genomic DNA analysis may not be suitable for some Gram positive bacteria for which intact DNA may be difficult to obtain due to the presence of active DNases (Blaschek and Klacik, 1984; Swiatek *et al.*, 1987). The additional disadvantage of RFLP analysis of genomic DNA is that the genomic restriction fragments are usually very numerous and closely spaced. Consequently, the interpretation of RFLPs between a large number of bacterial isolates may be difficult. However, alternative techniques, such as DNA probing or pulsed field gel electrophoresis (PFGE), attempt to reduce the complexity of restriction patterns obtained with RFLP analyses of genomic DNA.

RFLP analysis in combination with DNA probing. With this method, genomic DNA is cleaved by restriction endonuclease, the restriction fragments are separated by agarose gel electrophoresis and are then transferred to solid, usually nitrocellulose or nylon, membranes using Southern blotting (Southern, 1975). The single stranded DNA fragments are then allowed to hybridise to single stranded, labelled DNA probe. After hybridisation, only those genomic DNA restriction fragments that hybridise with the probe are revealed in the restriction profile. For identification and/or differentiation of bacteria, probes may be derived from randomly cloned genomic DNA fragments, genes coding for virulence, insertion sequences or repetitive fragments, bacteriophage DNA or *E. coli* ribosomal RNA. Of those, however, probes based on conserved ribosomal genes can be used as broad spectrum probes useful in identification and differentiation of wide range of bacterial species (Stull *et al.*, 1988). Grouping of bacteria on the basis of restriction profiles obtained after hybridisation with these probes is called ribotyping.

Ribotyping involves isolation and restriction of genomic DNA, separation of the restriction fragments and their transfer to a membrane. Hybridisation is usually performed with a probe containing the genes encoding *E. coli* 16S and 23S rRNA (Grimont and Grimont, 1986). After probing, only those fragments of genomic DNA containing all or part of these ribosomal genes will be visualised, creating a restriction pattern containing approximately 1 to 15 bands.

Ribotyping has been useful for inter- and intra-species discrimination of diverse bacteria (Stull *et al.*, 1988; Grimont and Grimont, 1991; Bingen *et al.*, 1994). For a bacterial strain, the restriction pattern (ribotype) generated with ribotyping appears to be stable after *in vitro* and *in vivo* passage (LiPuma *et al.*, 1991) and this method may thus be used in long term epidemiological studies. The discriminatory power of this method depends on the number of copies and diversity of a *rrn* operon present in a particular bacterial species. Generally, the higher the number of these operons, the more discriminatory the ribotyping will be for a bacterium. For some bacteria, e.g. *Salmonella* serotype *typhimurium*, ribotyping provides sufficient level of strain discrimination for this method to be used in epidemiological studies (Barrett, 1997). With other bacteria, e.g. *Mycobacterium* spp., ribotyping does not enable intra-species discrimination due to the low number of *rrn* loci present in different strains.

The main advantages of ribotyping include very good pattern reproducibility, universal applicability and ease of interpretation. A single probe can be used to differentiate almost all bacteria at both inter- and intra-species level and because most bacteria contain multiple copies of the ribosomal operon, moderate numbers of fragments are obtained after probing. However, because the ribotyping procedure includes multiple steps (DNA isolation, restriction, electrophoresis and Southern blotting), ribotyping is considered more time and labour intensive than DNA sequencing and also derives less information on the genes of interest. Since *rrn* operons represent a minor proportion of genomic DNA, in comparison to RFLP of genomic DNA, ribotyping has a lower discriminatory power. This method may not be suitable for inter-strain differentiation of bacteria that contain only 1 or 2 *rrn* loci and for screening a large number of bacterial isolates.

Pulsed field gel electrophoresis (PFGE). With pulsed field gel electrophoresis, genomic DNA is extracted and then digested with an infrequently cutting restriction endonuclease (Finney, 1993; Maslow *et al.*, 1993). As a result of using an infrequently cutting enzyme, 30 to 500 bp DNA fragments are generated and, with conventional electrophoresis, these migrate in the electric field at the similar rate (i.e. they cannot be

separated). However, such DNA fragments can be separated by cyclically alternating the direction of the electric field during electrophoresis in starch gel (Schwartz and Cantor, 1984). Before the molecules can begin to migrate, they must align themselves in the electric field according to the molecule charge and this process takes longer for large than for small molecules. At the end of electrophoresis, total genomic DNA can be resolved into a moderate number of restriction fragments of different electrophoretic mobility.

PFGE allows the generation of easy to interpret genomic restriction patterns without probe hybridisation. It is universally applicable, reproducible and discriminatory, and can be used for both identification and inter- and intra-species differentiation of bacteria (Arbeit, 1995). For some bacteria, e.g. *Listeria monocytogenes*, PFGE provides a higher level of discrimination than ribotyping or probe based methods (Brosch *et al.*, 1991). With PFGE, however, intact, pure high molecular weight DNA is required. To avoid shearing, the DNA isolation procedure is conducted *in situ* with bacteria embedded in agarose plugs. Currently used PFGE procedures are, therefore, considered long, tedious and expensive. PFGE may not be suitable for identification and differentiation of bacteria that carry active DNases and for the discrimination of large number of bacterial isolates.

PCR product based methods for differentiation and identification of bacteria

PCR that targets specific genes. With this method, identification or differentiation is based on the presence or absence of a specific gene and not on genetic heterogeneity of the whole bacterial genome. Typically, DNA is isolated and used in PCR amplification with specific primers encoding a target gene. The PCR product is visualised on an agarose gel. The presence of the target gene in a bacterial genome is detected by obtaining a PCR product of the expected size, but gene identity should be additionally confirmed for this product with the RFLP procedure, probing or sequencing.

Genes encoding virulence, metabolic properties or phylogenetic information are commonly targeted in this procedure. Among medically important genes, the verotoxin gene (Witham *et al.*, 1996), the gene encoding heat-labile enterotoxin (Meyer *et al.*, 1991), the haemolysin gene (Cooray *et al.*, 1994) or the botulinal neurotoxin gene (e.g. Szabo *et al.*, 1992, 1993; Campbell *et al.*, 1993a) have been used for specific detection/identification of bacteria. Among genes encoding metabolic properties, those encoding specific enzymes, like β-galactosidase (Bej *et al.*, 1990) or β-glucuronidase (Bej *et al.*, 1991) have been used for bacterial discrimination. PCR based assays that detect genes encoding virulence or metabolic properties indicate only the genetic potential to express virulence or produce enzyme, rather than expression of the encoded phenotypic feature. Phylogenetically informative 16S rRNA genes occur in virtually all organisms and, therefore, their presence or absence in microorganisms is not a differentiating property. 16S rRNA genes can, however, be amplified from any bacterium using universal bacterial primers (Lane, 1991; Marchesi *et al.*, 1998), so 16S rRNA gene amplification is universally applicable. Subsequently, the PCR-amplified gene may be cleaved with a frequently cutting restriction endonuclease to generate patterns discriminating at species level (Gurtler *et al.*, 1991; Heyndrickx *et al.*, 1996; Pukall *et al.*, 1998).

PCR that targets specific genes is highly reproducible and easy to perform but usually has low discriminatory power. However, as demonstrated with 16S rRNA genes, the level of discrimination can be improved by incorporation of a confirmatory procedure, such as RFLP (e.g. Nachamkin *et al.*, 1993), probing or sequencing. Comparison of restriction fragments of PCR amplified 16S rRNA genes is useful for identification of bacteria at species level. For effective identification, restriction patterns of reference strains and unknown isolates are best compared when obtained on the same gel or under the same conditions of electrophoresis.

16S-23S rRNA gene internal transcribed spacer polymorphism (ITSP) analysis. This analysis is a modification of the previous method but instead of a specific gene, the PCR reaction targets an internal transcribed 16S-23S rRNA gene spacer.

It is believed that the spacer region, in contrast to conserved 'house keeping' genes, is a fast evolving part of a bacterial genome and its sequence should vary more between microorganisms than sequences of 16S or 23S ribosomal RNA genes (Barry *et al.*, 1991; Gürtler and Stanisich, 1996). Consequently, higher discrimination should be obtained with ITSP analysis than with any analysis based on conserved 16S or 23S rRNA genes.

With ITSP analysis, DNA is isolated and used in a standard PCR amplification with conserved bacterial primers targeting the 3' end of the 16S rRNA gene and the 5' end of the 23S rRNA gene. PCR product is visualised on an agarose gel and the bacteria are differentiated on the basis of comparison of the resulting patterns.

Both sequence and length heterogeneity in 16S-23S rDNA spacer may exist between different bacterial species (Barry *et al.*, 1991; Scheinert *et al.*, 1996) and between different strains of the same species (Jensen *et al.*, 1993). Consequently, both inter- and intra-species discrimination of bacteria can be achieved with ITSP analysis (Leblond-Bourget *et al.*, 1996; Graham *et al.*, 1997; Hain *et al.*, 1997). On the other hand, most bacteria contain multiple copies of the *rrn* operon. While with some bacterial species/strains little sequence and length polymorphism occurs between different ribosomal operons of a single strain (Barry *et al.*, 1991), with others, the spacer has shown inter-operon variability (Campbell *et al.*, 1993b; Nagpal *et al.*, 1998). When spacer length polymorphism occurs within a single bacterial strain, the multiple bands after amplification represent the spacer regions of different lengths present in different ribosomal operons.

The ITSP analysis is reproducible and easy to perform and to interpret. Because conserved primers can be used for many species, it is also universally applicable. However, the level of discrimination that this analysis provides may be species/strain dependent and needs to be interpreted carefully.

Repetitive sequence amplification. Repetitive extragenic palindromic (REP) or enterobacterial repetitive intergenic consensus (ERIC) sequences are typically noncoding regions that may contribute to the tertiary structure of the folded genome. These sequences consist of exact copies of DNA fragments that repeat throughout the genome. Repetitive DNA elements are separated by heterogenous fragments of DNA whose length varies between all the repeat sequences on the genome. Consensus primers based on REP or ERIC sequences are used in the PCR to amplify DNA sequences located between repetitive elements (Versalovic *et al.*, 1991; Versalovic *et al.*, 1994). Consequently, amplification yields DNA fragments of various sizes, resulting in patterns that differ between the individual bacterial strains or species.

REP and ERIC sequences have been initially identified for Enterobacteriaceae and related Gram negative bacteria, and, consequently, REP or ERIC based methods are most frequently used in differentiation of these organisms (e.g. Burr *et al.*, 1998). REP and ERIC sequence based differentiation gives reproducible, although only moderately discriminatory results. A highly conserved repetitive BOX sequences have been identified for some Gram positive microorganisms (Martin *et al.*, 1992).

Arbitrarily primed PCR (or randomly amplified polymorphic DNA). In contrast to previous PCR product based methods for identification and differentiation of bacteria, which required prior knowledge about the DNA sequence, arbitrarily primed PCR targets an unknown sequence. With this method, genomic DNA is used in the PCR amplification, together with a single, typically 10-mer primer whose homology to this DNA is unknown (Welsh and McClelland, 1990; Williams *et al.*, 1990). The amplification is conducted at low stringency (low annealing temperature and high $MgCl_2$) that allows the primer to anneal exactly, as well as imperfectly, to template hybridisation sites matching the primer's complement sequence. The primer usually anneals to several sites on the DNA strands, and when two annealing sites occur within around 200 to 2000 bp of each other and in the correct orientation (5' to 3') on opposite DNA strands, the fragment sequence between the sites can be amplified.

The arbitrarily primed PCR usually generates strain specific patterns consisting of several bands that vary in intensity. This analysis is highly discriminatory, rapid and easy to perform, and does not require prior knowledge of the DNA sequence. The polymorphisms observed with arbitrarily primed PCR may be due to genetic variability of DNA, but they may also include amplification artifacts that result from variation in amplification reagents, conditions or techniques (Ellsworth *et al.*, 1993; Jutras *et al.*, 1995; McEwan *et al.*, 1998). The reproducibility of this technique is poor and it is not suitable for long term epidemiological studies that involve comparison of bacterial isolates from different sources (Tyler *et al.*, 1997). The interpretation of similarities between bacterial isolates, that produce banding patterns with bands of the same size, but different intensity, may also be difficult.

Plasmid profiling as a method for differentiation of bacteria

Plasmids are autonomous, self-replicating extrachromosomal DNA elements that are carried by many bacteria. With plasmid profiling, plasmid DNA is extracted and separated in agarose gel electrophoresis. In the obtained profile, bands of a different size are assumed to represent different plasmids, and bacteria are differentiated according to their plasmid number and sizes (Swaminathan and Matar, 1993).

Plasmid profiling is rapid and easy to perform. However, some bacterial strains do not carry plasmids and are, therefore, untypable by this method (Eisgruber *et al.*, 1995). Plasmids are commonly transferred between microorganisms in the environment and under laboratory conditions, so the presence of a plasmid in the bacterial genome cannot be considered a stable property suitable for identification of bacteria. Because different bacterial species/strains can carry plasmids of the same size, plasmid profiling usually provides low discrimination if plasmid size is used as the differential criterion. Plasmids of identical size may differ in their nucleotide sequence and, consequently, treatment with a restriction nuclease may be necessary to determine degree of heterogeneity of plasmids from different bacteria.

DNA based methods for identification and differentiation of clostridia

A number of studies have included, among other bacteria, selection of clostridial species. Identifications of eight species of medically important clostridia have been conducted with RFLP of PCR amplified 16S rDNA genes (Gurtler *et al.*, 1991). The internal transcribed spacer length polymorphism analysis has also been used for differentiation of selected clostridial species (Barry *et al.*, 1991; Scheinert *et al.*, 1996). This analysis was thought to achieve a moderate, strain specific level of discrimination (Scheinert *et al.*, 1996). However, profile polymorphism detected with the ITSP analysis may occur due to inter-operon, as well as inter-strain variability in the spacer region (Campbell *et al.*, 1993b; Nagpal *et al.*, 1998).

With *C. difficile*, PCR assays have been developed that detect either species specific sequences of the 16S rRNA gene (Gumerlock *et al.*, 1991) or the toxin A and/or B genes (Gumerlock *et al.*, 1993; Kato *et al.*, 1998). The clinical epidemiological studies using *C. difficile* required a high level of discrimination to differentiate outbreak related from unrelated strains. The methods that were used to differentiate strains of *C. difficile* included plasmid profiling (Clabots *et al.*, 1988a,b), restriction endonuclease analysis (Devlin *et al.*, 1987; Kuijper *et al.*, 1987; Clabots *et al.*, 1993), ribotyping (Bowman *et al.*, 1991), pulsed field gel electrophoresis (Kato *et al.*, 1994), arbitrarily primed PCR (Killgore and Kato, 1994) and the 16S-23S rDNA internal transcribed spacer analysis (Gürtler, 1993; Cartwright *et al.*, 1995; Stubbs *et al.*, 1999). A number of studies compared various DNA based approaches for strain discrimination of *C. difficile* (Kristjánsson *et al.*, 1994, Martirosian *et al.*, 1995; Collier *et al.*, 1996; Samore *et al.*, 1996, 1997; Rafferty *et al.*, 1998). Generally, the results obtained with RFLP of genomic DNA or PFGE analysis compared well with those obtained with the ITSP analysis, with the spacer analysis being applicable to the majority of *C. difficile* isolates, rapid, reproducible and the least laborious of the three methods. Types obtained with the ITSP analysis were stable after *in vivo* and *in vitro* transfers (Gürtler, 1993). With *C. difficile*, ribotyping was found less discriminatory than other methods (Kristjánsson *et al.*, 1994), while arbitrarily primed PCR was the least reproducible (Samore *et al.*, 1996).

Specific detection of *C. botulinum* has been frequently based on detection of BoNT genes (Szabo *et al.*, 1992, 1993, 1994; Campbell *et al.*, 1993a; Ferreira *et al.*, 1994; Franciosa *et al.*, 1994; Fach *et al.* 1995; Sciacchitano and Hirschfield, 1996). For specific detection, these genes are PCR amplified with specific or degenerate primers and their identity is confirmed by probe hybridisation. This method has a low discriminatory power and detects both proteolytic and non-proteolytic *C. botulinum* species, as well as non-botulinal species that carry genes encoding the same type of toxin (Campbell *et al.*, 1993a). Similarly, the unexpressed BoNT genes may also be detected (Franciosa *et al.*, 1994). On the other hand, non-toxigenic *C. botulinum* strains that do not harbour BoNT gene are also known (Yamakawa *et al.*, 1997) and these may be detected with a probe based on the 16S-23S rDNA spacer (Campbell *et al.*, 1993b). Specific detection of *C. botulinum* was also attempted with PCR that targets 16S rRNA gene sequences, and with a capture probe immobilised to a microtiter plate (Galindo *et al.*, 1993). To date, DNA based methods have rarely been used for epidemiological studies of *C. botulinum*. It was postulated that this might be due to difficulties with obtaining intact DNA from clostridial strains rich in DNases (Blaschek and Klacik, 1984; Swiatek *et al.*, 1987; Kristjánsson *et al.*, 1994). However, pulsed field gel electrophoresis has been used for genotypic analysis of *C. botulinum* type A (Lin and Johnson, 1995) and with modified procedure for *in situ* DNA isolation, for non-proteolytic *C. botulinum* types B, E and F (Hielm *et al.*, 1998a,b). Recently, the use of arbitrarily primed PCR and repetitive sequence amplification for differentiation of *C. botulinum* has also been described (Hyytiä *et al.*, 1999).

PCR amplification strategies, including multiple PCR and nested PCR, have been developed for specific detection of *C. perfringens* enterotoxin gene (*cpe*) (Baez and Juneja, 1995a; Yoo *et al.*, 1997; Miserez *et al.*, 1998; Miwa *et al.*, 1998). This gene, however, may be transferred by a phage or carried on the chromosome or on plasmids (Cornillot *et al.*, 1995; Collie and McClane, 1998) and, consequently, absence of *cpe* gene amplification does not exclude the possibility that non-toxigenic *C. perfringens* isolates are present in the sample.

Non-toxigenic *C. perfringens* isolates can be detected by amplification of a species specific 16S rRNA gene fragments (Rönner and Stackebrandt, 1994; Wang *et al.*, 1994). Specific probes for detection of toxigenic *C. perfringens* have also been developed (van Damme-Jongsten *et al.*, 1990; Schlapp *et al.*, 1995), and, more recently, a non-isotopic probe targeting the *cpe* gene has been used in dot blot hybridisation assays (Baez and Juneja, 1995b; Rodríguez-Romo *et al.*, 1998). DNA based methods for differentiation of *C. perfringens* include plasmid profiling (Mahony *et al.*, 1987; Eisgruber *et al.*, 1995), ribotyping (Forsblom *et al.*, 1995; Schalch *et al.*, 1997) and pulsed field gel electrophoresis (Collie and McClane, 1998; Ridell *et al.*, 1998). All these methods achieve inter-strain and/or intra-species discrimination.

With food spoilage clostridia, 16S rRNA gene based PCR assays and species specific probes have been used for specific detection of vegetative cells and spores of *C. tyrobutyricum*, which causes late blowing of cheese (Herman *et al.*, 1995; Klijn *et al.*, 1995). A method combining of specific amplification of the 16S rRNA gene fragment and RFLP analysis is being prepared for detection of *C. estertheticum* in meat and in environmental samples (J.E.L. Corry, personal communication).

DNA based methods for identification and differentiation of psychrophilic and psychrotrophic clostridia

Molecular techniques should be first choice methods for the identification and detection of organisms that are difficult to identify or difficult to cultivate. Because such difficulties have been previously encountered with psychrophilic and psychrotrophic clostridia associated with meat spoilage (Dainty *et al.*, 1989; Kalchayanand *et al.*, 1989; Broda *et al.*, 1996b, 1998b,c), these techniques appear to be ideally suited for identification and differentiation of these microorganisms, as well as for their specific detection.

Generally, the choice of the genotypic method depends on the level of discrimination and the epidemiological information that are sought. To date, no single method exists

that would assure identification/differentiation of every microorganism and that would satisfy every epidemiological requirement. The suitability of a DNA based method for identification and differentiation of psychrophilic and psychrotrophic clostridia would need to be determined empirically with respect to their genomic diversity, the applicability of the protocol and epidemiological relevance of results. For a comprehensive assessment, a number of methods, each fulfilling different purpose, was used in this study.

Methods for species identification and differentiation. Today, sequencing of the 16S rRNA gene followed by a database search would appear to be the preferred method for identification of microorganisms and this method was used in this study for speciation of psychrophilic and psychrotrophic clostridia. Where full 16S rRNA gene sequence determination is impractical (e.g. due to a large number of isolates), RFLP analysis of PCR amplified 16S rRNA genes was used to provide inter-species discrimination. 16S rRNA gene based analyses are highly reproducible and would suit long term trace back studies. However, because of the low discriminatory power of this analysis, epidemiological relevance of the information provided for use in trace back studies of psychrophilic and psychrotrophic clostridia remains to be determined.

Because 16S rRNA gene analysis may fail to differentiate between some closely related species (Fox *et al.*, 1992; Stackebrandt and Goebel, 1994), phenotypic characters determined in Chapter 1 were used to further evaluate groupings obtained with the 16S rRNA gene based techniques. Additionally, the 16S-23S rDNA ITSP analysis was used in an attempt to achieve inter-species discrimination of these microorganisms.

Methods for strain differentiation. Tracing of psychrophilic and psychrotrophic clostridia back to their source(s) in the abattoir was likely to require methods that were more discriminating than those used for their inter-species differentiation. It was not, however, known what level of strain discrimination would be required in studies tracing clostridia from 'blown packs' back to their abattoir sources.

DNA based methods are very sensitive and often are able to detect single genetic event differences, such as nucleotide insertion, deletion or a point mutation resulting in acquisition or loss of a restriction site within a genome. While strains showing identical genomic profiles can be considered epidemiologically related, strains that display differences between their profiles may or may not be the same origin clones. During chilled storage pack distension generally occurs 4 to 8 weeks after slaughter and packaging, and a retrospective study was conducted to trace 'blown pack' causing clostridia from spoiled product back to their abattoir source. The possibility existed that, even if the microorganisms in a spoiled pack and those obtained at the time of slaughter in anticipation of spoilage originally produced indistinguishable profiles, differences in these profiles may be observed at the time trace back is conducted due to genetic drift that occurs naturally over time. Similarly, the extent of genetic diversity of psychrophilic and psychrotrophic clostridia is also unknown. The effects that genetic drift over a period of time and natural genetic diversity among psychrophilic and psychrotrophic clostridia might have on epidemiological conclusions concerning relatedness of strains remain to be assessed. In this study, use of more then one typing method would help to determine the influence of genetic drift on differences observed between strains.

With long term trace back studies, the use of a highly reproducible method is desirable. Highly discriminatory methods, such as restriction fragment length polymorphism of genomic DNA or pulsed field gel electrophoresis may be used with a limited number of isolates. Both these methods have similar discriminatory power and similar reproducibility, and both require intact DNA. However, RFLP of genomic DNA is considered less laborious than PFGE (Swaminathan and Matar, 1993) and, consequently, this method was given priority in this study. With a large number of isolates, PCR based differentiation methods appear rapid and reproducible. The 16S-23S rRNA gene internal transcribed spacer polymorphism analysis was used in this study to evaluate whether, with this method, intra-species discrimination of psychrophilic and psychrotrophic clostridia may be obtained.

Methods for identification/specific detection. With microorganisms that are difficult to cultivate, DNA based methods enable identification/detection of specific organisms without recourse to their physical isolation. Of these methods, PCR has demonstrated wide potential for specific recognition of bacteria directly in foods (Scheu *et al.*, 1998). With PCR, problems experienced with releasing DNA from spores, the need to concentrate bacteria or DNA polymerase inhibition by the food matrix and other substances, leading to false-negative results (Rossen *et al.*, 1992; Katcher and Schwartz, 1994; Wiedbrauk *et al.*, 1995), can usually be solved.

Little is known about the genomes of psychrophilic and psychrotrophic clostridia with respect to genes that may be used as a target for specific amplification and that are carried universally by all these clostridia. It is possible, however, that species specific primers could be derived from sequences of 16S rRNA gene or the 16S-23S rDNA internal transcribed spacer of psychrophilic and psychrotrophic clostridia.

PCR can detect the presence of target DNA in the sample, rather than the presence of viable and/or culturable microorganisms. In foods, DNA that remains intact may be detected with PCR after extended periods (Allmann *et al.*, 1995). From the spoilage control perspective, the significance of the presence of non-viable psychrophilic and psychrotrophic clostridia whose DNA might have been detected in meat or meat plant environment needs to be interpreted carefully. Methods that use sample enrichment, immunomagnetic separation or the detection of intact RNA, may need to be incorporated to confirm an active metabolism and the viability of these clostridia.

DNA based methods that enable identification and specific detection of psychrophilic and psychrotrophic clostridia directly in foods and the environment will be developed in the future. However, because these methods would not lead to the isolation of the organisms, their applicability in studies tracing clones of psychrophilic and psychrotrophic clostridia to their abattoir sources may be limited.

Objective

The aim of the study reported in this chapter was to evaluate the genotypic approaches that could be applied in epidemiological and/or taxonomical studies to effect identification and trace back of psychrophilic and psychrotrophic clostridia associated with meat spoilage. 16S rRNA gene sequencing and the RFLP analysis were used in an attempt to identify and differentiate these clostridia at an inter-species level. The RFLP of genomic DNA and the 16S-23S rDNA ITSP analysis were used to differentiate between clostridial strains at intra-species level.

Methods and Materials

Bacteria

Stringent anaerobic procedures and pre-reduced media (Holdeman *et al.*, 1977) were used for culturing all *Clostridium* strains.

Reference strains of *C. estertheticum* DSM 8809^T (T = type strain), *C. fimetarium* DSM 9179^T, *C. putrefaciens* DSM 1291^T and *C. vincentii* DSM 10228^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. A reference strain of *C. algidicarnis* NCFB 2931^T was obtained from the National Collection of Food Bacteria, Reading, UK. Reference strains of psychrotrophic non-proteolytic *C. botulinum* type B (17B) and *C. botulinum* type E (Beluga) were obtained from Dr E. A. Szabo, CSIRO, Sydney, Australia. With the exception of *C. estertheticum*, reference strains were revived from freeze-dried material in Peptone Yeast Extract Glucose Starch (PYGS) broth (Lund *et al.*, 1990) and subcultured onto Columbia Blood Agar (CBA, Oxoid) containing 5 % v/v sheep blood. *C. estertheticum* was revived in sterile anaerobic milk. With the exception of non-proteolytic *C. botulinum* cultures, reference strains were grown at their optimum growth temperatures, as recommended by the source culture collections. The two non-proteolytic *C. botulinum* strains were grown at 30°C.

Twenty-two meat strains of psychrophilic and psychrotrophic clostridia were isolated as detailed in Chapter 1. The meat strains, maintained as freeze-dried cultures, were revived in PYGS broth, plated onto CBA supplemented with 5 % v/v sheep blood and incubated for 48 to 96 h at temperatures within their optimum growth range (Table 1.1).

Genomic DNA isolation

To isolate DNA, cells from the reference and meat strains growing exponentially at their optimum temperatures were harvested by centrifugation at 12,000 *g* for 15 min. Cells were washed twice with saline-EDTA (0.15 M NaCl, 1 mM EDTA, pH 8.0). DNA was isolated using the spooling method of Marmur (1961) modified as described by Johnson (1994). Briefly, cells were suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA) and lysed with lysozyme at a final concentration (FC) of 50 mg ml^{-1}, followed by SDS (FC 2.5 % w/v) and Proteinase K (FC 50 μg ml^{-1}) lysis. The lysate was repeatedly deproteinised with phenol, phenol-chloroform (1:1) and chloroform, and DNA was precipitated with absolute ethanol and spooled onto a glass rod. The DNA was washed, dried and dissolved in TE buffer. RNase A treatment (FC 50 μg ml^{-1}) was used to remove RNA from the solution, followed by repeated deproteinisation. The DNA solution was dialysed overnight against ice-cold TE and then the DNA concentration was measured by a fluorimetric dye-binding method (Hoechst 33258) using a fluorimeter (Hoeffer DynaQuant 200, Biolab Scientific). The procedure recommended by the fluorimeter's manufacturer was followed. Calf thymus and *Clostridium perfringens* DNAs (Sigma) were used as a standard. After quantification, the purified DNA was diluted in TE to contain 0.5 mg ml^{-1} and stored at –20°C.

Restriction fragment length polymorphism (RFLP) analysis of genomic DNA (DNA-RFLP)

The 22 meat strains were initially screened using RFLP analysis of genomic DNA to investigate inter-strain polymorphism of the isolates and to limit 16S rDNA gene sequencing to genetically heterogenous isolates. DNA from reference strains were not subjected to genomic DNA-RFLP analysis.

With many bacterial species, RFLP analysis of genomic DNA is considered highly discriminatory and detects inter-strain differences (Clabots *et al.*, 1993; Swaminathan and Matar, 1993). Consequently, it was unlikely that genomic DNA restriction patterns of reference strains would have a high degree of homology with those of meat strains thereby precluding ready identification of the latter.

The DNA of the meat strains was digested with five infrequently cutting restriction endonucleases: *Sma*I, *Hin*dIII, *Hin*fI, *Eco*RI and *Kpn*I (Boehringer Mannheim GmbH). Restriction digests containing 1 μg of genomic DNA, 2 μl of the appropriate buffer and 5 U of enzyme in a total volume of 20 μl were prepared and incubated according to the manufacturer's recommendations. Digestion products were separated by gel electrophoresis in 0.7 % w/v agarose (Seakem) gels at 1.7 V cm^{-1} for 12 h with a 1 kb DNA Ladder (Gibco BRL, Life Technologies) used as a size marker. Restriction patterns resulting from enzyme digestion were visualised with ethidium bromide by UV transillumination. Efficiency of digestion was checked with λ DNA (Gibco BRL, Life Technologies), that was digested and restriction patterns were visualised as described for meat and reference strains.

Scanned images of the gels containing DNA-RFLP fragments were analysed with a Molecular Imager and Molecular Analyst software (Bio-Rad). Isolates were grouped on the basis of restriction pattern similarity, as assessed by comparison of sizes of individual fragments larger than 4 kbp. Isolates whose DNA, digested with a single endonuclease, yielded indistinguishable restriction patterns were considered members of the same DNA-RFLP group. Eleven DNA-RFLP groups, differentiated by different restriction patterns, were identified and numbered from 1 to 11.

Restriction fragment length polymorphism (RFLP) analysis of 16S rDNA gene (PCR-RFLP)

For amplification of the 16S rDNA gene, genomic DNA was used as the PCR template. PCR was performed with universal bacterial primers complementary to conserved regions of the 5'and 3'ends of the 16S rRNA gene.

The primers' sequences were pA (forward) 5'-AGA GTT TGA TCC TGG CTC AG-3' and pH* (reverse) 5'-AAG GAG GTG ATC CAG CCG CA-3' (Hutson *et al.*, 1993a).

The PCR mix was prepared according to the manufacturer's (Boehringer Mannheim GmbH) recommendation and contained: PCR buffer 10x (10 µl), 0.2 mmol of each deoxynucleoside triphosphate, 0.5 µmol of each primer, 2.5 units of *Taq* polymerase and 10 µl of template DNA (100 ng) in a total volume of 100 µl. Amplifications were performed in a heated lid thermal cycler (Techne Genius). After initial denaturation for 3 min at 93°C, target DNA was amplified in 30 cycles. Each cycle consisted of denaturation for 1 min at 92°C, annealing for 1 min at 5°C and extension for 2 min at 72°C. The final extension was for 3 min at 72°C. The PCR reaction tubes, each containing an amplified 16S rDNA fragment, were held at 4°C until further analysis. The concentration of amplified PCR product was measured by a fluorimetric dye-binding procedure described for the genomic DNA quantification. After quantification, the unpurified PCR products were stored at –20°C.

Amplified 16S rDNA genes of meat and reference strains were digested with eight frequently cutting restriction endonucleases: *Alu*I, *Hae*III, *Taq*I, *Cfo*I, *Rsa*I, *Hin*cII, *Msp*I and *Nde*II (Boehringer Mannheim GmbH). Restriction digests containing 10 µl of the PCR product (approximately 2 µg DNA), 2 µl of the appropriate buffer and 10 U of enzyme in a total volume of 20 µl were prepared and incubated according to the manufacturer's recommendations. Digestion products were separated by gel electrophoresis in 2.0 % w/v agarose (Seakem) gels at 2.5 V cm^{-1} for 1.5 h. The DNA molecular weight marker VI (Boehringer Mannheim GmbH) or 1 kb DNA Ladder (Gibco BRL, Life Technologies) was used as a size marker. Restriction patterns were visualised with ethidium bromide by UV transillumination. Efficiency of digestion was checked with the 16S rDNA gene from *Escherichia coli* using *E. coli* strain B DNA (Sigma) as a PCR template. Digestion of the gene and visualisation of restriction patterns were conducted as described for the meat and reference strains.

Scanned images of the gels containing PCR-RFLP fragments were analysed with a Molecular Imager and Molecular Analyst software (Bio-Rad). Isolates were grouped on the basis of restriction pattern similarity, as assessed by comparison of sizes of individual fragments larger than 154 bp. Isolates whose 16S rDNA genes, digested with a single endonuclease, yielded indistinguishable restriction patterns were considered members of the same PCR-RFLP group. With each endonuclease, PCR-RFLP groups represented by different restriction patterns were assigned different lower case letters. For each strain, the 16S rDNA genotype was assigned according to the PCR-RFLP grouping obtained with four endonucleases. The 16S rDNA genotypes were assigned upper case letters.

Sequencing of 16S rDNA gene

Species identity of reference strains of *C. algidicarnis* NCFB 2931^T, *C. fimetarium* DSM 9179^T and *C. putrefaciens* DSM 1291^T; of 14 meat strains representing each of 11 different DNA-RFLP groups; and of three isolates untypable with DNA-RFLP analysis, was confirmed with 16S rDNA gene sequencing. With reference strains, sequencing with both forward and reverse primers was conducted. With meat strains, sequencing with reverse primers was completed only for isolates that had different forward sequences ($\geq$ 1 nucleotide mismatch over the total determined sequence).

In addition to primers pA and pH*, the following sequencing primers complementary to internal regions of the 16S rRNA gene were used (Hutson *et al.*, 1993a): Sef 1 (forward) 5'-CGT GCC AGC AGC CGC GGT AAT-3', Sef 2 (forward) 5'-GGA GCA TGT GGT TTA ATT CG-3', Ser 1 (reverse) 5'-CAC GAC ACG AGC TGA CGA CAA C-3', Ser 2 (reverse) 5'-CTA CGC ATT TCA CCG CTA CAC-3' and Ser 3 (reverse) 5'-GCC GTG TCT CAG TCC CAA TGT-3'. Approximate hybridisation sites for sequencing primers are shown in Fig. 2.1.

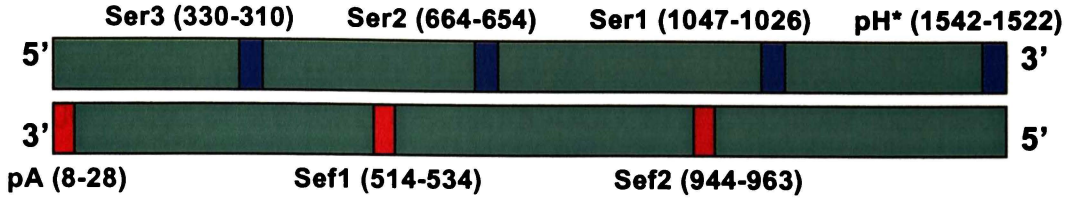

Figure 2.1. Diagram showing approximate hybridisation sites for forward (red) and reverse (blue) sequencing primers. Approximate primer positions refer to respective *E. coli* numbering.

The PCR-amplified 16S rDNA genes were purified with the Wizard purification kit (Promega, Dade Diagnostics) according to the manufacturer's instructions. The DNA sequencing was performed by the Waikato DNA Sequencing Facility, University of Waikato, Hamilton, New Zealand. 16S rDNA fragments were sequenced directly using dideoxy terminator cycle sequencing chemistry in an ABI 377 automated sequencer (Perkin Elmer, Applied Biosystems). The typical sequencing reaction contained: 8 μl of Terminator Ready Reaction Mix with Ampli*Taq* DNA polymerase (Perkin Elmer), 3.2 pmol of sequencing primer and 40 ng of template PCR product in a total volume of 20 μl. Cycle sequencing has been conducted in 25 cycles. Each cycle consisted of denaturation for 10 s at 96°C, annealing for 5 s at 50°C and extension for 4 min at 60°C with a rapid thermal ramp (1°C s^{-1}) to reach each temperature. The sequencing reaction tubes were held at 4°C until purification, separation of extension products in a polyacrylamide gel and sequence scanning were conducted.

The sequences were aligned using Sequence Navigator (Perkin Elmer), and were corrected in agreement with the electropherograms and the reading of complementing and overlapping fragments. The complete sequences were compared with the sequences in the GenBank (Benson *et al.*, 1998), the EMBL Nucleotide Sequence Database and the DNA Data Bank of Japan libraries using the gapped version of the BLAST program (Altschul *et al.*, 1997). Similarly, the sequences were compared with sequences aligned by the Ribosomal Database Project (RDP) (Maidak *et al.*, 1999).

Internal transcribed spacer polymorphism (ITSP) analysis

16S-23S rDNA internal transcribed spacers (ITS) from reference and meat strains were PCR amplified with universal bacterial primers ISRA (forward) 5'-AAG TCG TAA CAA GGT ARC-3' and ISRC (reverse) 5'-GGG TTB CCC CAT TCR G-3' (Lane, 1991). These primers are complementary to conserved regions of the 3' end of 16S rRNA and 5' end of 23S rRNA genes directly flanking the spacer. The PCR mix was prepared and amplifications were performed as with the PCR-RFLP analysis. The PCR reaction tubes, each containing an amplified ITS fragment(s), were held at 4°C until further analysis.

A 5 µl aliquot of the amplified PCR product was subjected to electrophoresis in a 1.5 % w/v agarose gel at 3.5 V cm^{-1} for 1.5 h. The DNA molecular weight marker VI (Boehringer Mannheim GmbH) was used as a size marker. Banding patterns were visualised with ethidium bromide by UV transillumination.

Scanned images of the gels containing ITS fragments were analysed with Molecular Imager and Molecular Analyst software (Bio-Rad). The sizes of the fragments produced in the amplification were estimated from the positions of these fragments relative to the position of the molecular weight markers located in the two outer lanes of the gel. Isolates for which PCR amplification produced fragment(s) of the same size and that were also indistinguishable with respect to their overall banding patterns, were considered the members of the same ITS group and were assigned Roman numerals.

Results

Restriction fragment length polymorphism (RFLP) analysis of genomic DNA (DNA-RFLP)

High molecular weight DNA was isolated for all but two meat strains (Fig. 2.2). Despite repeated DNA isolation partially degraded DNA have been obtained for strains K21 and K24.

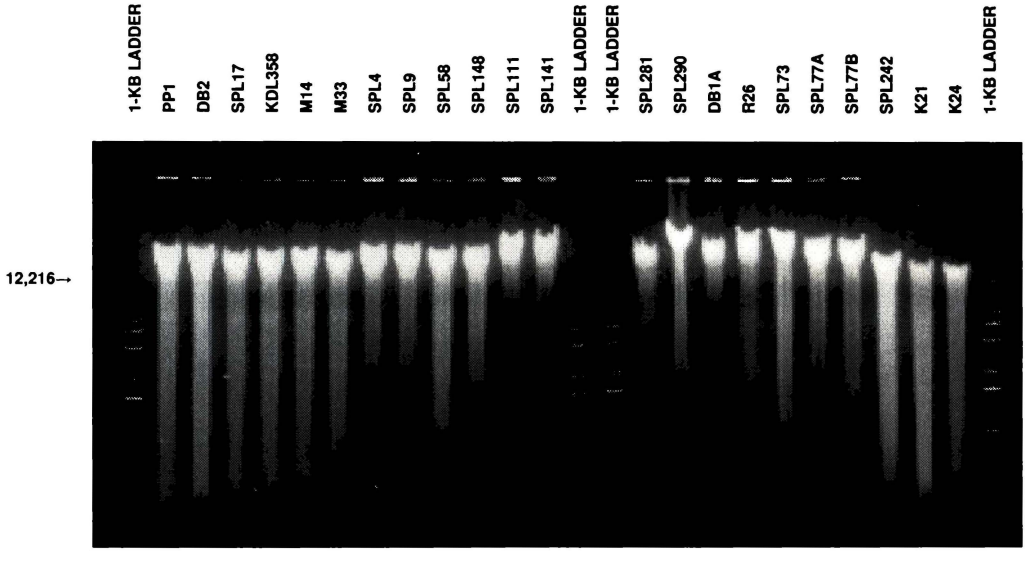

Figure 2.2 Quality of DNA isolated from meat strains of psychrophilic and psychrotrophic clostridia. DNA was isolated using the spooling method of Marmur (1961) modified as described by Johnson (1994). For each strain, approximately 500 ng of DNA was loaded onto a lane of 1 % w/v agarose gel and electrophoresed at approximately 4 V cm^{-1} for 1 h. DNA was visualised with ethidium bromide by UV transillumination. 1-kb DNA Ladder (Gibco BRL, Life Technologies) was used as a size marker.

On the basis of restriction patterns similarity of *Hin*dIII-digested DNA, 19 of the 22 meat strains were assigned into 11 DNA-RFLP groups (Fig. 2.3a), each showing a distinct restriction pattern.

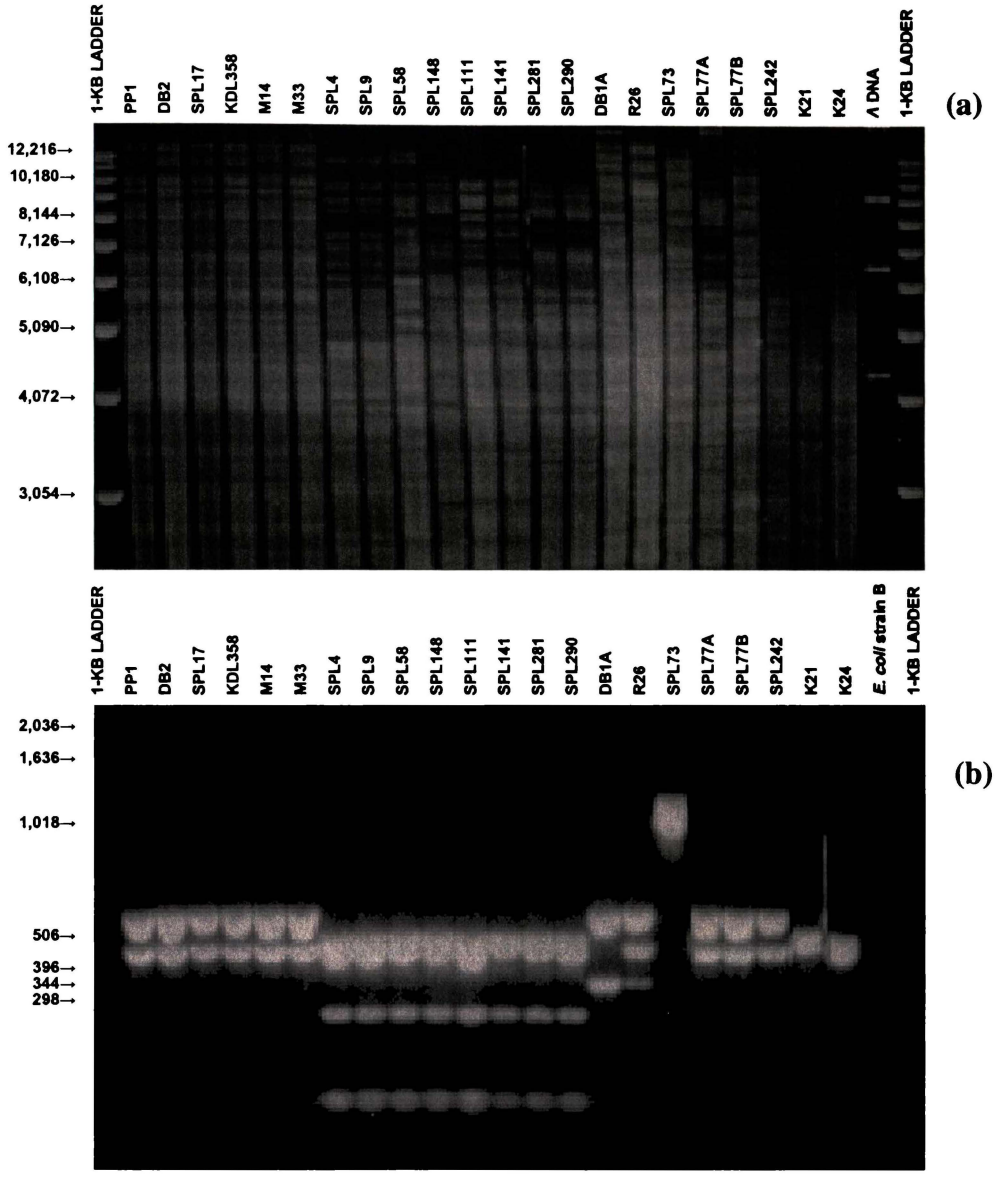

Figure 2.3. Restriction patterns of meat strains of psychrophilic and psychrotrophic clostridia obtained in RFLP analysis of genomic DNA with *Hin*dIII endonuclease (a) and in RFLP analysis of PCR-amplified 16S rDNA genes digested with *Hae*III (b). Digestion products were separated by gel electrophoresis in 0.7 % w/v agarose at 1.7 V cm^{-1} for 12 h (a) or in 2.0 % w/v agarose at 2.5 V cm^{-1} for 1.5 h (b). Restriction patterns were visualised with ethidium bromide by UV transillumination. 1-kb DNA Ladder (Gibco BRL, Life Technologies) was used as a size marker. Λ DNA (Gibco BRL, Life Technologies) and *E. coli* strain B DNA (Sigma) were used to check efficiency of digestion.

DNA-RFLP group 1 included six isolates that had restriction patterns indistinguishable from each other. DNA-RFLP groups 2, 5 and 6 each included two isolates that had indistinguishable restriction patterns. DNA-RFLP groups 3, 4, 7, 8, 9, 10 and 11 were represented by individual isolates whose restriction patterns differed from those of other isolates and each other. The remaining three isolates produced patterns consisting unresolved or badly resolved DNA fragments. With these isolates, the similar results have been obtained with DNAs that were re-isolated on two more occasions. These isolates were considered untypable with the DNA-RFLP differentiation method. Identical groupings of the meat strains were obtained with four other endonucleases, *Sma*I, *Hin*fI, *Eco*RI and *Kpn*I.

Restriction fragment length polymorphism (RFLP) analysis of 16S rDNA gene (PCR-RFLP)

With both reference strains and meat strains, the combination of four endonucleases (*Alu*I, *Hae*III, *Taq*I and *Cfo*I) had the same discriminatory power as the combination of all eight enzymes. Consequently, only PCR-RFLP results obtained with these four endonucleases are reported in this study. Endonucleases *Alu*I and *Hae*III produced the highest number of differentiating bands in restriction patterns for the majority of the reference and meat strains. During digestion of PCR-amplified 16S rDNA genes with *Alu*I, *Hae*III and *Cfo*I, distinct restriction patterns were obtained for five of the seven reference strains used in this study. With *Taq*I, distinct patterns were obtained for four reference strains. Digestion with *Hae*III differentiated eight PCR-RFLP groups among the 22 meat strains (Fig. 2.3b). With *Alu*I, six distinct PCR-RFLP restriction patterns were present among the meat strains, and with either *Cfo*I or *Taq*I, five distinct patterns were present. The grouping of reference and meat strains obtained with each of the four enzymes and the assigned PCR-RFLP genotypes are listed in Table 2.1.

Table 2.1. Grouping of psychrophilic and psychrotrophic clostridia obtained with RFLP analysis of genomic DNA (DNA-RFLP) and with RFLP analysis of the amplified 16S rDNA genes (PCR-RFLP).

Strain designation	DNA-RFLP group	PCR-RFLP group obtained during digestion of 16S rDNA genes with				PCR-RFLP genotype
		*Alu*I	*Hae*III	*Taq*I	*Cfo*I	
C. botulinum type B (17B), *C. botulinum* type E (Beluga)	Not available	a	a	a	a	A
PP1, DB2, SPL17, KDL358, M14, M33	1	a	a	a	a	A
SPL242	Untypable	a	a	a	a	A
C. algidicarnis NCFB 2931^T	Not available	b	b	b	b1	B1
C. putrefaciens DSM 1291^T	Not available	b	b	b	b2	B2
SPL4, SPL9	2	b	b	b	b1	B1
SPL58	3	b	b	b	b1	B1
SPL148	4	b	b	b	b1	B1
SPL111, SPL141	5	b	b	b	b1	B1
SPL281, SPL290	6	b	b	b	b1	B1
DB1A	7	c	c1	c	a	C1
R26	8	c	c2	c	a	C2
SPL73	9	d	d	d	d	D
SPL77A	10	e	e	e	e	E
SPL77B	11	e	e	e	e	E
C. estertheticum DSM 8809^T	Not available	f	f1	f1	f	F1
K21	Untypable	f	f2	f2	f	F2
K24	Untypable	f	f1	f2	f	F3
C. vincentii DSM 10228^T	Not available	g	g	b	b2	G
C. fimetarium DSM 9179^T	Not available	h	h	h	h	H

Irrespective of type of endonuclease used in digestion, the patterns of *C. fimetarium* and *C. estertheticum* were readily differentiated from each other and from the remaining reference strains (Fig 2.4 and data not shown). With either *Alu*I or *Hae*III, the restriction pattern of *C. vincentii* was also easily distinguished from those of the other reference strains. On digestion with *Alu*I, *Hae*III and *Taq*I, restriction patterns of *C. algidicarnis* and *C. putrefaciens* were indistinguishable. These two species were differentiated, however, on subsequent digestion with *Cfo*I (Fig. 2.4). The PCR-RFLP patterns of non-proteolytic *C. botulinum* type B (17B) and type E (Beluga) obtained by digestion with *Alu*I, *Hae*III, *Taq*I and *Cfo*I were indistinguishable.

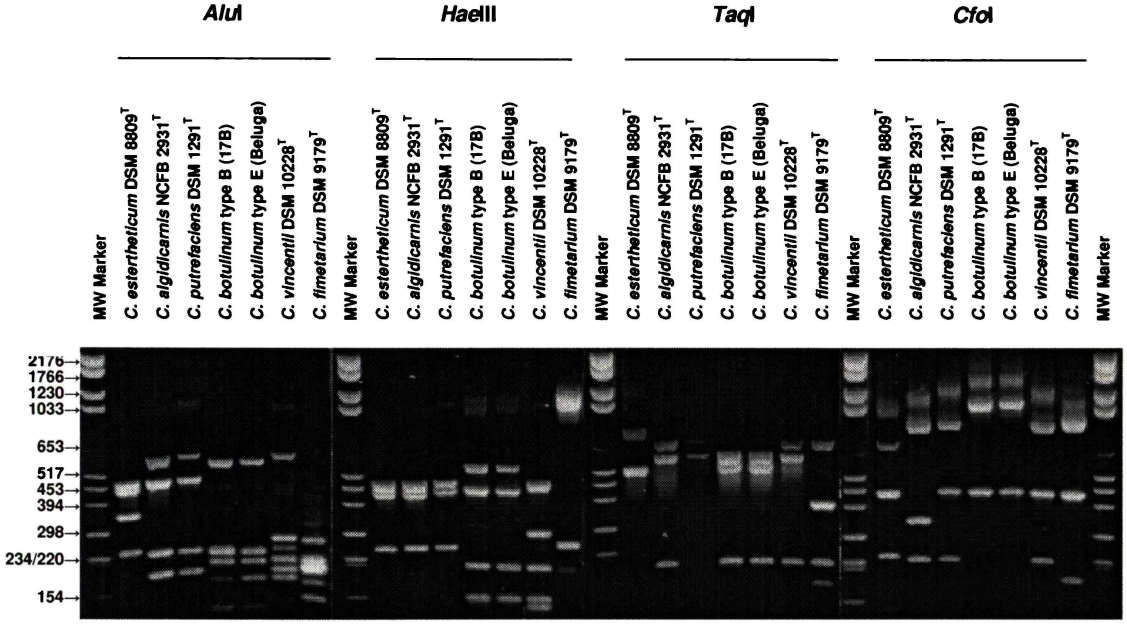

Figure 2.4. Restriction patterns of reference strains of psychrophilic and psychrotrophic clostridia obtained in RFLP analysis of PCR-amplified 16S rDNA genes digested with four restriction enzymes. Digestion products were separated by gel electrophoresis in 2.0 % w/v agarose at 2.5 V cm^{-1} for 1.5 h. Restriction patterns were visualised with ethidium bromide by UV transillumination. The DNA molecular weight marker VI (Boehringer Mannheim GmbH) was used as a size marker.

Six meat strains, PP1, DB2, SPL17, KDL358, M14 and M33, had PCR-RFLP restriction patterns that were indistinguishable from each other and from the PCR-RFLP pattern of reference strains of non-proteolytic *C. botulinum* type B (17B) and E (Beluga). These strains were assigned to PCR-RFLP genotype A.

With each of four endonucleases, this genotype consistently included isolates previously classified to DNA-RFLP group 1.

When the PCR products delineating the 16S rDNA genes of meat strains SPL4, SPL9, SPL58, SPL111, SPL141, SPL148, SPL281 and SPL290 were digested with *Alu*I, *Hae*III and *Taq*I, their PCR-RFLP restriction patterns were indistinguishable from each other and from the PCR-RFLP patterns of the reference strain of *C. algidicarnis* NCFB 2931^T and of *C. putrefaciens* DSM 1291^T. With *Cfo*I, restriction patterns of these isolates were indistinguishable from the pattern of reference strain of *C. algidicarnis.* These isolates were assigned to PCR-RFLP genotype B1. Isolates included in this genotype belonged to DNA-RFLP groups 2, 3, 4, 5 and 6.

Two meat strains, DB1A and R26, which were assigned to DNA-RFLP groups 7 and 8, had identical patterns when 16S rDNA genes of these isolates were digested with *Alu*I (Fig. 2.5), *Taq*I or *Cfo*I endonucleases. With *Hae*III, however, the restriction pattern polymorphism of these two meat strains was demonstrated by the presence of an additional band at approximately 450 bp in the pattern of strain R26. Restriction patterns of isolates DB1A and R26 with all enzymes were dissimilar to those of the reference strains of psychrophilic and psychrotrophic clostridia obtained with the same enzyme. Isolates DB1A and R26 were assigned to the PCR-RFLP genotypes C1 and C2.

With all eight endonucleases used singly, the restriction patterns of meat strain SPL73 did not match the patterns of any the reference strains. This isolate was assigned to PCR-RFLP genotype D.

With all eight endonucleases used singly, meat strains SPL77A and SPL77B had restriction patterns indistinguishable from each other. These patterns, however, differed from the restriction patterns of the reference strains obtained with the same endonucleases. These meat strains, included previously in DNA-RFLP groups 10 and 11, were assigned to PCR-RFLP genotype E.

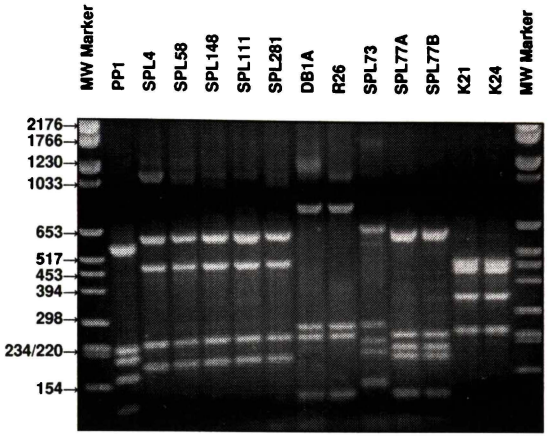

Figure 2.5. Restriction patterns of meat strains of psychrophilic and psychrotrophic clostridia representing 11 different DNA-RFLP groups and strains K21 and K24 that were untypable with DNA-RFLP analysis. Patterns were obtained in RFLP analysis of PCR-amplified 16S rDNA genes digested with *Alu*I. The PCR-RFLP restriction pattern (not shown) of meat strain SPL242 that was untypable in DNA-RFLP analysis was indistinguishable from the pattern of strains in PCR-RFLP group a, here represented by meat strain PP1. Digestion products were separated by gel electrophoresis in 2.0 % w/v agarose at 2.5 V cm^{-1} for 1.5 h. Restriction patterns were visualised with ethidium bromide by UV transillumination. The DNA molecular weight marker VI (Boehringer Mannheim GmbH) was used as a size marker.

The three isolates that were untypable with the DNA-RFLP analysis produced clearly resolved PCR-RFLP restriction patterns. With four endonucleases, strain SPL242 had restriction patterns indistinguishable from those of non-proteolytic *C. botulinum* strains 17B and Beluga, and from those of meat strains in PCR-RFLP genotype A. Consequently, this strain was assigned to genotype A. The remaining meat strains, K21 and K24, had PCR-RFLP restriction patterns indistinguishable from each other when *Alu*I (Fig. 2.5), *Taq*I and *Cfo*I digestions were compared. Pattern polymorphism between these two isolates was observed, however, when *Hae*III was used. Isolate K21 showed some *Alu*I and *Cfo*I digest pattern cross-similarity, and isolate K24 showed some *Alu*I, *Hae*III and *Cfo*I digest pattern cross-similarity, with the reference strain of *C. estertheticum*.

However, the *Taq*I restriction patterns of strains K21, K24, and *C. estertheticum* differed in the positions of two major bands. These two isolates were, therefore, assigned to the PCR-RFLP genotypes F2 and F3.

Sequencing of 16S rDNA gene

PCR amplified rDNAs were sequenced (Fig. 2.6) and very little background noise was observed in all resulting sequences (Fig. 2.7). On average, over 500 bp of the sequence was read from a single sequencing run.

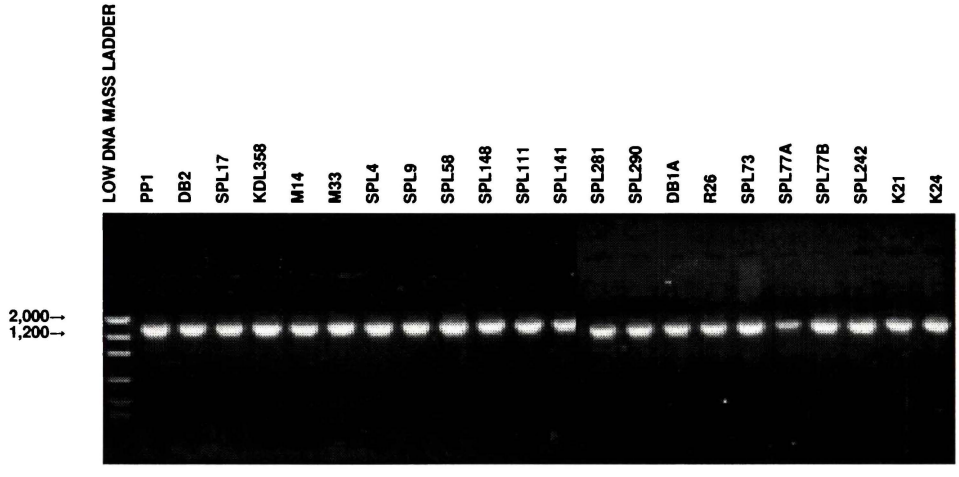

Figure 2.6. Quality of sequencing-ready PCR-amplified 16S rDNA genes from meat strains of psychrophilic and psychrotrophic clostridia. PCR products were purified with Wizard purification system. For each strain, approximately 200 ng of PCR product has been loaded onto a lane of 1.5 % w/v agarose gel and electrophoresed at approximately 4 V cm^{-1} for 1 h. DNA has been visualised with ethidium bromide by UV transillumination. Low DNA Mass Ladder (Gibco BRL, Life Technologies) was used as a size marker.

The near-complete 16S rDNA gene sequences of *C. putrefaciens* DSM 1291^T, *C. algidicarnis* NCFB 2931^T and *C. fimetarium* DSM 9179^T, consisting of 1487, 1481 and 1497 bp respectively, were produced in the present study. These sequences have been deposited in the GenBank database. Their accession numbers are AF127024, AF127023 and AF126687 respectively. When the sequences of *C. algidicarnis* NCFB 2931^T and *C. putrefaciens* DSM 1291^T were aligned, they exhibited 99.5 % similarity.

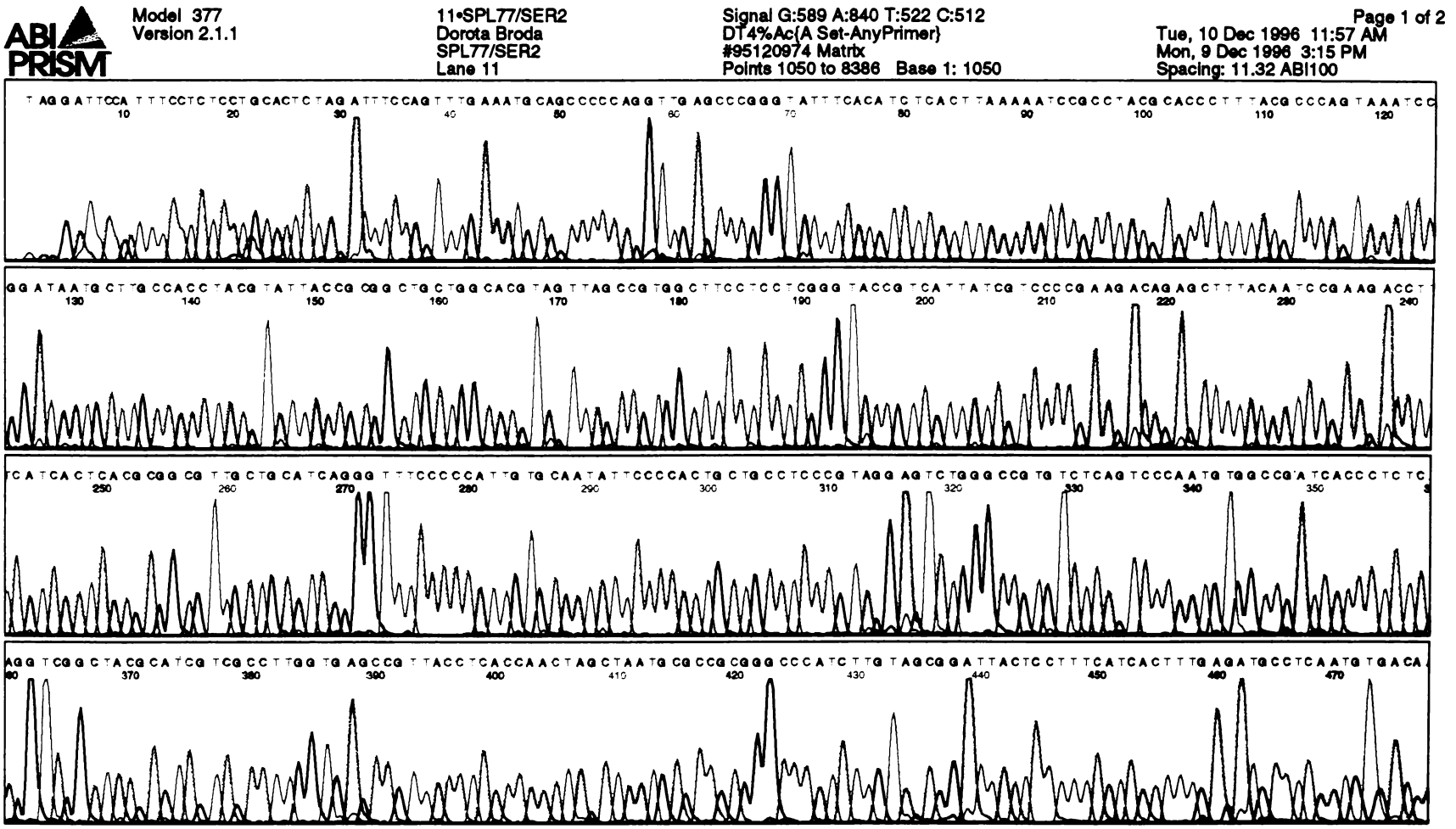

Figure 2.7. Example of quality of the 16S rDNA gene sequence obtained for meat strains of psychrophilic and psychrotrophic clostridia.

The forward and reverse sequencing of the 16S rDNA gene was performed for the meat strain PP1, representing PCR-RFLP genotype A. Database searches showed that this sequence had 99.7 % similarity to the sequences of non-proteolytic *C. botulinum* types E (accessions L37592 and X68170), F (accession X68171) and B (accession X68173) deposited in the GenBank and EMBL databases.

The forward sequences of the 16S rDNA genes from meat strains representing DNA-RFLP groups 2, 3, 4, 5 and 6 (Table 2.1), all belonging to PCR-RFLP genotype B1, were identical. Forward and reverse sequencing of the 16S rDNA gene from meat strain SPL290, representing PCR-RFLP genotype B1, was completed. Aligned forward sequences of individual isolates and the full sequence of strain SPL290 were identical to the sequence of *C. algidicarnis* NCFB 2931^T obtained in this study. However, when GenBank, EMBL and DDBJ databases searches were conducted, using the *C. algidicarnis* sequence obtained in this study, no similarity between this sequence and the sequence of *C. algidicarnis* deposited in the EMBL database library under accession X77676 (Lawson *et al.*, 1994) was indicated. The sequence of *C. algidicarnis* in the database library did not appear in the list of 70 sequences that most closely matched our sequence. The 16S rDNA gene sequences most similar to the *C. algidicarnis* sequence obtained in this study was that of *C. corinoforum* (accession X76742) at a similarity level of less than 94 %.

The original *C. algidicarnis* sequence (X77676) was retrieved from the EMBL database library and aligned with the *C. algidicarnis* sequence obtained in this study (AF127023). With both sequences, 1481 nucleotides were used in alignment. For the first 746 and the last 723 positions, the aligned sequences of *C. algidicarnis* AF127023 and *C. algidicarnis* X77676 were nearly identical (99.9 % and 99.4 % identity, respectively). However, the similarity of the two full sequences was only 98.9 %, and 11 out of 16 mismatching nucleotides were located within a continuous stretch of 14 nucleotides (positions 753 to 766 of *C. algidicarnis* X77676 sequence). When aligned against the RDP database sequences, the *C. algidicarnis* 16S rDNA sequence obtained

in this study was most similar to the sequence for *C. algidicarnis* NCFB 2931^T (Lawson *et al.*, 1994).

For the meat strains DB1A and R26 (PCR-RFLP genotypes C1 and C2), and SPL77A and SPL77B (PCR-RFLP genotype E), both the forward and reverse 16S rDNA gene sequences were determined. The sequences of the 16S rDNA gene of strains DB1A and R26 differed by three nucleotides within 1483 bp used in the comparison. The forward and reverse sequences of 16S rDNA genes of meat strains SPL77A and SPL77B were identical. The 16S rDNA gene sequences of all these meat strains failed to match any of the GenBank, EMBL, DDBJ and RDP database sequences at a similarity level above 97.0 %, regardless of whether database searches were conducted with full or partial (500 bp fragments) sequences.

The forward and reverse 16S rDNA gene sequences of meat strain SPL73 were also determined. Database searches showed that 16S rDNA gene sequence of this meat strain was most similar to the sequences of *C. aerotolerans* (accession X76163) and *C. xylanolyticum* (accessions X71855 and X76739) at a sequence similarity level of 97.7 % and 97.6 %, respectively.

For the meat strains K21 and K24 (PCR-RFLP genotypes F2 and F3), both forward and reverse 16S rDNA gene sequences were determined. The sequences of strains K21 and K24 differed by five nucleotides within 1507 bp used in the comparison. Database searches revealed that these sequences were most similar to the sequence of *C. estertheticum* (accession X68181) at a sequence similarity level of 97.9 %.

Internal transcribed spacer polymorphism (ITSP) analysis

PCR-amplification of the internal transcribed spacer regions from the reference strains of psychrophilic and psychrotrophic clostridia generated banding patterns unique for each microorganism (Fig. 2.8).

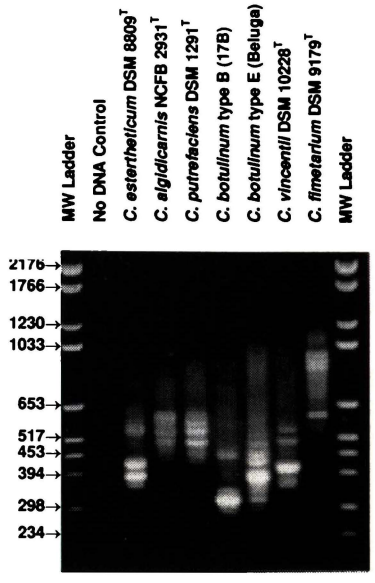

Figure 2.8. The banding patterns of reference strains of psychrophilic and psychrotrophic clostridia obtained by PCR amplification of the 16S-23S rDNA internal transcribed spacer. PCR products have been electrophoresed in a 1.5 % w/v agarose gel at 3.5 V cm^{-1} for 1.5 h and banding patterns were visualised with ethidium bromide by UV transillumination. The DNA molecular weight marker VI (Boehringer Mannheim GmbH) was used as a size marker.

These patterns consisted of one or more major bands of ITS products, presumably of the size(s) of spacers occurring in different *rrn* operons within the genome of the microorganism, and a number of minor bands. All reference strains were differentiated by the size of the PCR products and overall banding pattern. With *C. algidicarnis* and *C. putrefaciens*, bands representing their ITS products occupied a similar size range. However, these two reference strains differed in the size and intensity of individual bands. Reference strains of non-proteolytic *C. botulinum* type B (17B) and E (Beluga) had major ITS products that differed significantly in size by approximately 70 bp.

ITS products amplified from the DNAs of the meat strains are shown in Fig. 2.9. On the basis of ITS product size, as well as overall banding patterns, all the 22 meat strains were differentiated into ten ITS groups (Table 2.2).

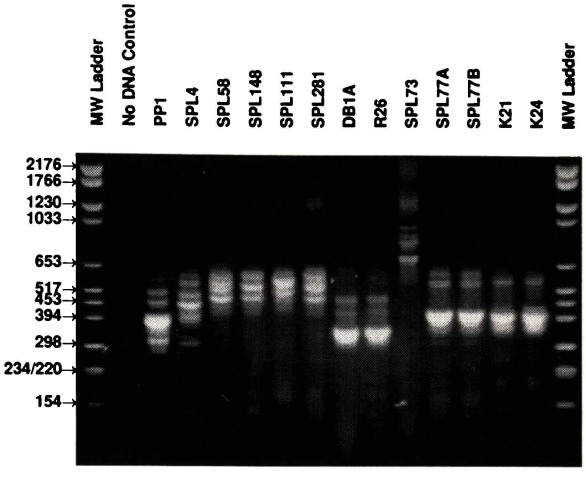

Figure 2.9. The banding patterns of meat strains of psychrophilic and psychrotrophic clostridia obtained by PCR amplification of 16S-23S rDNA internal transcribed spacer. PCR products have been electrophoresed in a 1.5 % w/v agarose gel at 3.5 V cm^{-1} for 1.5 h and banding patterns were visualised with ethidium bromide by UV transillumination. The DNA molecular weight marker VI (Boehringer Mannheim GmbH) was used as a size marker.

PCR amplification of ITS from meat strains PP1, DB2, SPL17, SPL242, KDL 358, M14 and M33 produced banding patterns indistinguishable from each other (results not shown). These meat strains were assigned to ITS group I. The size of the primary PCR product for these isolates was similar to that of the spacer from the reference strain *C. vincentii* DSM 10228^T. Of the other meat strains, the size of the main PCR product from ITS group I strains was similar only to those of strains SPL77A and SPL77B. The overall banding patterns for meat strains in ITS group I differed, however, from the patterns of the reference and other meat strains in positions and intensities of minor products.

When the ITS regions of meat strains SPL4, SPL9, SPL 58, SPL111, SPL141, SPL148, SPL 281 and SPL290 were amplified, their banding patterns consisted of multiple bands of similar intensity in the size range from 595 to 454 bp. The ITS banding patterns for isolates SPL4 and SPL9; SPL 111 and SPL141; and SPL281 and SPL290, were, within each pair of isolates, indistinguishable from each other and were assigned to ITS groups

II, V and VI, respectively. The remaining strains SPL58 and SPL148 were assigned to ITS groups III and IV, respectively. Between ITS groups, the banding patterns of meat strains were easily distinguished from each other. With respect to ITS product size(s), none of the meat strains belonging to groups II, III, IV, V and VI had a banding pattern identical to the patterns of *C. algidicarnis*, *C. putrefaciens* or the other reference strains (Table 2.2).

Table 2.2. Grouping of psychrophilic and psychrotrophic clostridia obtained with ITSP analysis, compared with those obtained with RFLP analysis of the amplified 16S rDNA genes (PCR-RFLP).

Strain designation	ITS product size (in bp)	ITS group*	PCR-RFLP genotype
C. botulinum type B (17B)	318	-	A
C. botulinum type E (Beluga)	389	-	A
PP1, DB2, SPL17, KDL358, M14, M33, SPL242	410	I	A
C. algidicarnis NCFB 2931^T	569, 541, 502	-	B1
C. putrefaciens DSM 1291^T	541, 520	-	B2
SPL4, SPL9	502, 454	II	B1
SPL58	595, 556, 520, 470	III	B1
SPL148	595, 541, 486	IV	B1
SPL111, SPL141	575, 520	V	B1
SPL281, SPL290	595, 556, 486	VI	B1
DB1A	346	VII	C1
R26	346	VII	C2
SPL73	835, 729	VIII	D
SPL77A, SPL77B	410	IX	E
C. estertheticum DSM 8809^T	431, 379	-	F1
K21	396	X	F2
K24	396	X	F3
C. vincentii DSM 10228^T	410	-	G
C. fimetarium DSM 9179^T	993, 944, 875, 614	-	H

*, ITS grouping assigned according to the overall banding pattern. DSM, the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. NCFB, the National Collection of Food Bacteria, Reading, UK. CSIRO, Division of Food Science and Technology, Sydney, Australia. -, reference strains were not assigned to ITS group.

The meat strains DB1A and R26 had ITS banding patterns indistinguishable from each other and were assigned to ITS group VII. Strain SPL73 was assigned to ITS group VIII. Banding patterns of group VII and VIII isolates differed from each other and from patterns of any reference and other meat strains in the sizes of ITS products.

Strain SPL77A had a banding pattern indistinguishable from the pattern of strain SPL77B. These two strains were assigned to ITS group IX. The sizes of the main ITS products from strains SPL77A and SPL77B, from meat strains grouped in ITS group I and from *C. vincentii* DSM 10228^T were similar. However, the overall banding patterns of strains SPL77A and SPL77B differed from patterns of isolates in ITS group I and *C. vincentii* in the positions and intensities of minor products. The banding patterns of these two strains differed from patterns of other reference and meat strains.

Strain K21 had a pattern indistinguishable from the pattern of strain K24 and these two meat strains were assigned to ITS group X. With ITS group X, banding patterns of strains differed from those of other meat strains in the sizes of ITS products. The size of the major PCR product for the two ITS group X isolates was similar to that of the spacer from only one reference strain, non-proteolytic *C. botulinum* type E (Beluga). The ITS banding patterns of meat strains K21 and K24 and this reference strain differed, however, in the positions and intensities of minor products.

Discussion

DNA- and PCR-RFLP analysis, and 16S rDNA gene sequencing for identification and differentiation of psychrophilic and psychrotrophic clostridia

In this study six out of seven reference strains of psychrophilic and psychrotrophic clostridia were differentiated by a comparison of *Alu*I or *Hae*III, and *Cfo*I, restriction patterns of PCR products encompassing 16S rDNA genes. The two reference strains that could not be differentiated by digestion with four restriction endonucleases were

both non-proteolytic *C. botulinum* strains. These two strains produce different toxin types but belong to the same genotypic species (Collins and East, 1998). PCR-RFLP patterns of reference strains of *C. algidicarnis* NCFB 2931^T and *C. putrefaciens* DSM 1291^T differed only in their *Cfo*I restriction patterns. These results were consistent with results of 16S rDNA gene sequencing that demonstrated close sequence similarity (99.5 %) between these two reference strains. With *C. algidicarnis* and *C. putrefaciens,* it is not known whether the minor variation in PCR-RFLP genotypes of these closely related strains reflects, in the absence of DNA-DNA homology or chemotaxonomic characterisation data, intra- or inter-species 16S rDNA gene sequence heterogeneity (Fox *et al.*, 1992; Stackebrandt and Goebel, 1994). Consequently, while reference strains that represented six distinct phenotypic species were differentiated with PCR-RFLP analysis in this study, further work is required to establish whether, for some strains, this differentiation was obtained at the genotypic species level or below.

Based on the results of RFLP analysis of 16S rDNA genes, the 22 meat strains of psychrophilic and psychrotrophic clostridia were differentiated into eight PCR-RFLP genotypes. The results of PCR-RFLP differentiation were consistent with the results of 16S rDNA gene sequencing, as each genotype was represented by a distinct sequence. With the majority of the meat strains, these genotypes are likely to represent different genotypic species. However, genotypes of meat strains DB1A and R26 differed only in their *Hae*III restriction patterns. As demonstrated with 16S rDNA gene sequencing, this difference corresponded to only three out of 1483 nucleotides, with the overall similarity between sequences of the two meat strains reaching 99.8 %. Similarly, genotypes of meat strains K21 and K24 differed only in their *Hae*III restriction patterns, and 16S rDNA gene sequence similarity between these isolates was 99.7 %. These results, along with results of phenotypic and chemotaxonomic characterisation (as detailed in Chapters 1 and 3), indicate that, with meat strains DB1A and R26, and with strains K21 and K24, *Hae*III restriction sites variation is probably due to intra-specific nucleotide diversity. Consequently, in this study, a restriction with *Alu*I was sufficient to differentiate six genotypic species among the 22 meat strains of psychrophilic and psychrotrophic clostridia (Fig. 2.5).

Restriction patterns of seven meat strains matched the patterns of reference strains of non-proteolytic *C. botulinum* types B (17B) and E (Beluga). The similarity of 16S rDNA genes of these isolates to gene of *C. botulinum* was confirmed with sequencing. The majority of these meat strains, however, did not produce botulinal neurotoxin (BoNT) (Moorhead and Bell, 1999) and did not carry BoNT genes (Broda *et al.*, 1998a). Similarly, these isolates differed from non-proteolytic *C. botulinum* types B (17B) and E (Beluga) in some phenotypic and chemotaxonomic characteristics, e.g. substrate utilisation and cellular fatty acid composition (as detailed in Chapters 1 and 3). Current species delineation of *C. botulinum* is based on phenotypic, rather than genotypic traits (Campbell *et al.*, 1993b; Collins and East, 1998). Therefore, while on the basis of 16S rDNA similarity the seven meat strains may be identified as non-proteolytic *C. botulinum*, further work is required to determine if these microorganisms belong to the phenetic species *C. botulinum*.

Eight meat strains had PCR-RFLP genotypes indistinguishable from the genotype of the reference strain *C. algidicarnis* NCFB 2931^T. Similarly, 16S rDNA gene sequences of these isolates and the reference strain were identical. However, the eight meat strains could not be identified as *C. algidicarnis* when full sequences of these strains were used to search GenBank, EMBL and DDBJ databases. Failure to indicate *C. algidicarnis* as one of the close relatives of the meat strains during 16S rDNA gene sequence searches was most likely a result of sequence uncertainty between nucleotide positions 753 to 766, for the *C. algidicarnis* NCFB 2931^T sequence deposited previously (Lawson *et al.*, 1994). The variability of the sequence in this stretch of 14 nucleotides may be due to inter-operon variability within the *C. algidicarnis* NCFB 2931^T strain, or to PCR, sequencing or data transmission error (Clayton *et al.*, 1995; Gendel, 1996). These results demonstrate that some sequence regions may confound search algorithms (Altschul *et al.*, 1994), e.g. those used in the original or gapped BLAST program, resulting in missing close similarities between sequences and, ultimately, in failure to identify a microorganism.

The restriction patterns of four meat strains (DB1A, R26, SPL77A and SPL77B), representing three PCR-RFLP genotypes C1, C2 and E, did not match the patterns of reference strains of any psychrophilic or psychrotrophic clostridia that are currently available from culture collections. The absence of bacterial 16S rDNA gene sequences of high similarity (>97.0 %) to the sequences of the four meat strains was confirmed when full and partial sequences of these strains were used to search the GenBank, EMBL, DDBJ and RDP database libraries. These results, together with results of phenotypic characterisation and phylogenetic analyses (as detailed in Chapters 1 and 4), indicate that the four meat strains may represent two new clostridial species. Although meat strain SPL73 (genotype D) showed 16S rDNA gene sequence similarity to *C. aerotolerans* and *C. xylanolyticum*, it differed from these microorganisms in key phenotypic and chemotaxonomic characteristics (as detailed in Chapters 1 and 4). Similarly, while meat strains K21 and K24 (genotypes F2 and F3) showed 16S rDNA gene sequence similarity to *C. estertheticum*, they differed from this bacterium in phenotypic and chemotaxonomic characteristics (as detailed in Chapters 1 and 4). Strains SPL73, K21 and K24 may also represent previously undescribed clostridial species. Species descriptions of isolates DB1A and R26, SPL77A and SPL77B, SPL73, K21 and K24 are reported in Chapter 4.

16S rRNA gene sequencing followed by database searching is frequently used as a preliminary tool for bacterial identification and for taxonomic studies (Amann *et al.*, 1994). With this method, however, inter-operon or inter-strain 16S rRNA gene sequence variation within a species (Clayton *et al.*, 1995; Cilia *et al.*, 1996) may preclude identification of microorganisms that are represented in the database by only single operons and/or a single strain sequences. Misidentification may also result from PCR, sequencing or data transfer errors (Doolittle, 1994; Domenighini *et al.*, 1995; Gendel, 1996). With psychrophilic and psychrotrophic clostridia, this identification method may be unsatisfactory for species that are described but not sequenced (e.g. *C. arcticum* and *C. laramiense*) and with species that contain sequence heterogeneities that may confound some alignment algorithms (e.g. *C. algidicarnis* in the present study). The present study has also questioned the reliability of both 16S rDNA gene

sequencing and RFLP analysis of this gene for identification of previously undescribed species of psychrophilic and psychrotrophic clostridia and species described on the basis of one or more phenotypic traits. As reported previously (Fox *et al.*, 1992; Stackebrandt and Goebel, 1994), 16S rDNA based analyses may not resolve the detailed taxonomic position of closely related microorganisms (e.g. *C. algidicarnis* and *C. putrefaciens* in this study). The results of the present study (Broda *et al.*, *in press* a) show that while RFLP analysis of amplified 16S rDNA gene allows differentiation of psychrophilic and psychrotrophic clostridia at the genotypic species level and below, the results of identification of these clostridia using the 16S rDNA based method need to be approached carefully. Further 16S rDNA gene sequencing would provide a better database for future studies.

In the present study, PCR-RFLP analysis supported by sequencing found a high diversity of 16S rDNA gene sequences of psychrophilic and psychrotrophic clostridia that permitted differentiation of unknown meat strains at genotypic species level and below. PCR-RFLP analysis of 16S rDNA genes could, therefore, be applied in trace back investigations of 'blown pack' spoilage incidents, to obtain indicative speciation of the psychrophilic and psychrotrophic clostridial isolates involved. More highly discriminatory typing methods, such as DNA-RFLP, could then be applied to individual genotypic species/PCR-RFLP groups of these microorganisms, to differentiate clostridial isolates apparently causing 'blown pack' spoilage from those not causing this condition.

ITSP analysis for identification and differentiation of psychrophilic and psychrotrophic clostridia

The groupings of psychrophilic and psychrotrophic meat clostridia obtained with ITSP analysis differed from groupings obtained with RFLP analysis of both genomic DNA and 16S rDNA genes. With RFLP of genomic DNA, the 22 meat strains of psychrophilic and psychrotrophic clostridia were differentiated into 13 groups. With RFLP of 16S rDNA genes, these isolates were differentiated into eight genotypes. In this study, ITSP analysis differentiated these 22 meat strains into 10 groups.

With ITSP analysis, a lower discrimination of psychrophilic and psychrotrophic clostridial isolates was obtained than with RFLP analysis of genomic DNA and a higher discrimination was obtained than with RFLP analysis of 16S rDNA genes.

With ITSP analysis, all reference strains of psychrophilic and psychrotrophic clostridia were differentiated by the size of their main PCR products. In contrast to RFLP analysis of 16S rDNA genes, in the present study reference strains of non-proteolytic *C. botulinum* type B (17B) and E (Beluga) were differentiated on the basis of polymorphism of major ITS products representing 16S-23S rDNA spacer sizes of these reference strains. Variation in spacer length was observed previously between strains of non-proteolytic *C. botulinum* types B or E, and F (Campbell *et al.*, 1993b). While 16S-23S rDNA internal spacers from strains Eklund 17B (type B) and Hazen 36208 (type E) have been very similar in both sequence and length, with strain Eklund 202F (type F) a 71-nucleotide insertion has been found in the 16S-23S rDNA spacer (Campbell *et al.*, 1993b). In the present study similar spacer length polymorphism was observed between strains of non-proteolytic *C. botulinum* 17B (type B) and Beluga (type E), confirming inter-strain variation within this species. It is not known if the difference in spacer lengths observed in the present study is associated with the presence of an insertion or the presence of a region encoding for a tRNA molecule.

With ITSP analysis, most meat strains were differentiated into the same groups as with RFLP analysis of 16S rDNA genes. However, meat strains SPL4, SPL9, SPL 58, SPL111, SPL141, SPL148, SPL 281 and SPL290 that were previously classified into the same PCR-RFLP group (genotype B1) and were identified as members of the species *C. algidicarnis*, produced five different ITS banding patterns (Fig 2.4). Similar ITS polymorphism was not observed with meat strains within the remaining PCR-RFLP groups. Inter-strain ITS length polymorphism is known to occur with some clostridial species (Campbell *et al.*, 1993b; Gürtler, 1993), but not with others (Jensen *et al.*, 1993; Scheinert *et al.*, 1996). It appears that the inter-strain ITS polymorphism observed in this study with *C. algidicarnis*, but not with other meat strains, may also be species specific.

Internal transcribed spacer length polymorphism (ITSP) analysis has been used previously for differentiation and identification of many bacteria (Barry *et al.*, 1991; Jensen *et al.*, 1993; Scheinert *et al.*, 1996), and the size of this spacer has generally been thought to be species specific (Jensen *et al.*, 1993). In the present study ITS products of similar length were generated for meat strains belonging to ITS groups I and IX, and for reference strain of *C. vincentii* DSM 10228^T. According to phenotypic, genotypic and phylogenetic characteristics, the meat strains in ITS groups I and IX are unlikely to be closely related and probably belong to different clostridial species. Similarly, the PCR-RFLP analysis of these meat strains and *C. vincentii* followed by 16S rDNA gene sequencing indicated gross heterogeneities between the 16S rDNA sequences of meat strains representing ITS groups I and IX and the reference strain. A major ITS product of similar length was generated for meat strains belonging to ITS group X (K21 and K24) and for the reference strain *C. botulinum* type E (Beluga). However, PCR-RFLP analysis, 16S rDNA gene sequencing and phenotypic characteristics of strains K21 and K24 preclude the possibility that these meat strains and the reference strain of *C. botulinum* type E (Beluga) belong to the same species. In contrast to previous reports (Gürtler and Stanisich, 1996), the results of the present study indicate that differentiation based solely on spacer length polymorphism may miss gross heterogeneities between some clostridial isolates. Despite the ITS size similarity, the spacer sequences from strains representing different bacterial species may exhibit a high degree of heterogeneity that may only be demonstrated with further sequencing of the spacer region. However, the sequence of PCR derived ITS product may contain combinations of species, strain and operon specific information (Jensen *et al.*, 1993; Nagpal *et al.*, 1998) and differentiation of some isolates with ITSP analysis may not be feasible. Consequently, use of PCR-amplified spacer region sequencing as a means for clostridial species differentiation would depend on inter-strain and inter-operon variability that exists between strains of individual species.

Reference and meat strains confirmed with RFLP analysis and sequencing of 16S rDNA gene as the same species, *C. algidicarnis,* differed from each other in their banding patterns and/or the length of their ITS products. The spacer length polymorphism is

likely to be strain specific. However, it is also possible for the ITS pattern to contain artifactual bands that are present due to strain specific heteroduplex and single-stranded DNA structure formation (Jensen and Straus, 1993). With heterogenous species, ITS polymorphism analysis is likely to deliver strain-specific, rather than species-specific, differentiation of psychrophilic and psychrotrophic clostridia. Consequently, 16S-23S rDNA spacer length polymorphism analysis may complement RFLP analysis of 16S rDNA genes, providing rapid and more discriminatory differentiation of psychrophilic and psychrotrophic clostridia. However, the level of discrimination that ITSP analysis provides needs to be assessed individually with each clostridial species.

Conclusions

Restriction fragment length polymorphism (RFLP) analysis of the amplified 16S rDNA gene allowed differentiation of psychrophilic and psychrotrophic clostridia at the genotypic species level and below. 16S-23S rDNA internal transcribed spacer length polymorphism analysis was more discriminatory than RFLP analysis of the 16S rDNA gene, but was less discriminatory than RFLP of genomic DNA. With some clostridial species, the spacer length polymorphism analysis may complement RFLP analysis of 16S rDNA genes, providing rapid and strain specific differentiation of psychrophilic and psychrotrophic clostridia. Utility of ITSP analysis for species differentiation of these clostridia, however, remains to be evaluated.

Although in this study PCR-RFLP restriction patterns of 15 of the 22 meat strains matched those of non-proteolytic *C. botulinum* or *C. algidicarnis* reference strains, comparison of PCR-RFLP patterns and 16S rDNA gene sequences of unknown clostridial isolates with patterns and sequences of reference strains may not effect ready identification of psychrophilic and psychrotrophic clostridia.

Seven meat strains had RFLP restriction patterns of 16S rDNA genes that differed from those of any reference strains. Further work is required to confirm whether these strains represent previously undescribed species.

Chapter 3

Source of Psychrophilic and Psychrotrophic Clostridia Causing 'Blown Pack' Spoilage

Introduction

Routes of contamination of dressed carcasses and muscle tissues

Historically, contamination of internal meat tissues of slaughter animals was believed to either not occur or to occur via two routes, internal and external.

A scenario of an internal or endogenous contamination route has assumed that bacteria can naturally reside in the deep tissues of healthy animals. These intrinsic bacteria (Ingram and Hauge, 1949, Ingram, 1952) were thought to enter the blood stream or lymphatic system of an animal from the gut (Ingram and Dainty, 1971). The access of bacteria via the gastrointestinal mucosa and their transfer in body fluids to the deep tissues was thought to be possible before, at and/or after slaughter (Reith, 1926; Jensen and Hess, 1941).

However, the numbers of bacteria found in the deep tissues were frequently very low. It has been suggested that these bacteria may have resulted from compromised asepticity of sampling procedures (Gill, 1979) and that the presence of intrinsic contaminants in the meat tissues may have been overstated (Gill, 1979; Roberts and Mead, 1986).

Currently, it is believed that at the time of slaughter the deep tissues of a healthy, hygienically slaughtered animal are in principle free from bacteria (Buckley *et al.*, 1976; Gill *et al.*, 1978; Mackey and Derrick, 1979).

Consequently, it is likely that the bacterial contamination present on dressed carcasses and meat cuts comprises extrinsic microorganisms that are transferred from exogenous sources via an external route. The contamination of meat is, therefore, thought to be limited to its surface.

Sources of contamination with extrinsic microorganisms

The essentially sterile internal tissues are sandwiched between the animal's heavily contaminated skin and surfaces of its gastrointestinal, respiratory and the lower part of the urogenital tracts. With current slaughtering technology, aseptic removal of meat tissues from between these potential contamination sources is not possible (Eustace, 1981; Dickson and Anderson, 1992).

Animals are frequently considered the main reservoir of mesophilic human pathogens (Oosterom, 1991; Weijtens *et al.*, 1993; Berends *et al.*, 1997), such as *Campylobacter*, *Salmonella* or *Escherichia coli* O157:H7 and carcass contamination with these microorganisms is thought to be effected by their transfer from the gastrointestinal tract (Hathaway, 1997). In addition, many spoilage microorganisms are carried in the animal's intestine. However, the viscera of a slaughtered animal normally remains intact during carcass dressing and, consequently, direct carcass contamination from the gastrointestinal tract rarely occurs at the meat plant (Narasimha and Ramesh, 1992; Bell and Hathaway, 1996). The floras of respiratory or urogenital tracts are not thought to contribute greatly to contamination of dressed carcasses.

Most of bacteria present on a carcass are usually transferred from the skin of the dressed animal or derive through cross-contamination from other animals dressed in its proximity (Nottingham, 1982; Narasimha and Ramesh, 1992; Bell and Hathaway, 1996; Bell, 1997). The microorganisms present on hide/fleece are mainly those residing in soil, water, vegetation and animal feed (Ayres, 1955) but usually also include faecal contaminants. Consequently, the exogenous environment of an animal is considered the primary reservoir for direct carcass contamination.

The majority of carcass contamination is likely to occur during dressing. Significant contamination has been found at carcass sites close to opening cuts and/or subject to hide contact during hide removal (Bell and Hathaway, 1996) and, generally, little increase in carcass contamination has been thought to occur after skin removal (Narasimha and Ramesh, 1992; Bell and Hathaway, 1996).

Although the primary source of bacterial contamination in the meat plant is the animal itself (Eustace, 1981), air, structural and work surfaces, workers' hands and clothes, knives, cutting boards and other equipment have been recognised as potential secondary sources of bacteria on dressed carcasses (West *et al.*, 1972; Nortjé *et al.*, 1990; Bell and Hathaway, 1996; Desmarchelier *et al.*, 1999). However, in the meat plant environment, air and non-contact surfaces, such as walls or floors, are not generally thought to contribute significantly to the microflora present on the dressed carcasses (Eustace, 1981; Widders *et al.*, 1995; Eisel *et al.*, 1997), especially when the initial level of carcass contamination is high. In contrast, contact surfaces, knives and cutting boards can be a major source of bacteria transferred onto the primal cuts (Widders *et al.*, 1995).

Sources of contamination with psychrotrophic microorganisms

Psychrotrophic bacteria are common in soil, water, animal feeds and vegetation (Stokes and Redmond, 1966; Druce and Thomas, 1970; Warskow and Juni, 1972; Jones, 1973; Gounot, 1991) but may also be present in animal faeces. Consequently, soil particles and faecal residues attached to the hide/fleece are likely to contain a significant proportion of psychrotrophic microorganisms. In the abattoir, the hides/fleeces of slaughter animals have been found to be the immediate source of carcass contamination with psychrotrophic microorganisms (Newton *et al.*, 1978; Patterson and Gibbs, 1978) and the primary reservoir of these microorganisms is thought to be the exogenous environment of the slaughter animal. Gram negative psychrotrophic bacteria, such as *Pseudomonas*, *Acinetobacter*, *Moraxella* and Enterobacteriaceae, are the most common psychrotrophs occurring on hides of slaughter animals and on dressed carcasses (Newton *et al.*, 1978).

Psychrotrophic microorganisms are known to be able to colonise surfaces within a meat plant (Newton *et al.*, 1978; Mäkelä and Korkeala, 1987; Nortjé *et al.*, 1990) and, consequently, such abattoir sites may act as secondary contamination sources with these bacteria. In contrast to other bacteria, the carcass contamination with psychrotrophs via an air route has been demonstrated (Mäkelä and Korkeala, 1987; Borch *et al.*, 1988; Gustavsson and Borch, 1993).

Clostridium spp. as intrinsic contaminants of muscle tissue

Reports exploring the internal route of meat tissue contamination have consistently demonstrated the presence of small numbers of clostridia in muscle and organs (liver, kidney, lymph nodes and spleen) of healthy animals (Jensen and Hess, 1941; Lepovetsky *et al.*, 1953; Smith and Jasmin, 1956; Canada and Strong, 1964; Zagaevskii, 1973; Eisgruber and Stolle, 1996). While the presence of organisms other than clostridia could be attributed to the use of inappropriate sampling procedures (Gill, 1979), the presence of clostridial species in the deep tissues was thought to warrant more detailed investigation. This was due largely to a fact that the falling redox potential in deep tissues after slaughter would appear to favour the growth of anaerobes. However, little growth of clostridia that occur naturally in deep tissues has been reported. It has been suggested that a high oxygen concentration in the tissue may, for the first few hours after slaughter, inhibit proliferation of *Clostridium* sp. in the musculature (Barnes and Ingram, 1956; Roberts and Mead, 1986). After this initial period, while falling tissue redox potential would favour the growth of clostridia, falling temperature and pH would inhibit their proliferation.

Traditionally, the occurrence of bone taint spoilage has been regarded as evidence for the presence of intrinsic clostridia (Ingram and Dainty, 1971; Roberts and Mead, 1986). The term 'bone taint' has been traditionally used to describe the presence of sour or putrid odours in deep musculature, bone marrow, hip or stifle joints, or shoulder region in both fresh and cured meats (Haines, 1941; Mundt and Kitchen, 1951; Ingram, 1952).

The occurrence of bone taint was believed to be associated with inadequate carcass cooling that, pending high pH and tissue temperature and low muscle tissue-oxygen concentration, may allow germination and proliferation of clostridia in deep tissues (Callow and Ingram, 1955; Ingram and Dainty, 1971).

However, attempts to induce bone taint by temperature abuse and to isolate clostridia from tainted tissues in high enough numbers for them to be recognised as the primary causative agents of spoilage have, however, been unsuccessful (Cosnett *et al.*, 1956; Shank *et al.*, 1962). Documented conditions of redox potential, pH and temperature appear unlikely to prevent the multiplication of clostridia, therefore, other factors such as the bactericidal activity of body fluids, may contribute to a slowing of bacterial growth for some hours after slaughter (Roberts and Mead, 1986). Alternatively, the growth of clostridia might have occurred but high numbers of these microorganisms might not have been detected with conventional microbiological culture techniques.

Route of contamination of muscle tissue with intrinsic clostridia

The confusion about the possibility of clostridia being intrinsic has been exacerbated by the lack of information on a possible mode of entry of these microorganisms into deep tissues. It was thought that clostridia might gain access into the tissues of live animal during ante-mortem infection, contamination at slaughter or post-mortem invasion from the intestine.

Ante-mortem, clostridia may enter the tissues from skin abrasions and/or from the respiratory, urogenital and gastrointestinal tracts. In live animals, however, a balance between invasion of tissues and removal of invading bacteria is usually maintained by the bactericidal activities of the immune system (Koneman, 1970; Corry, 1978). The animal's immune defence is normally active for some time after slaughter and, consequently, bacteria that may be introduced into the blood stream from contaminated slaughter instruments (Jensen and Hess, 1941; Mackey and Derrick, 1979) are also likely to be eliminated by the activity of animal's body fluids (Gill and Penney, 1979).

Similarly, there is no evidence that post-mortem translocation of bacteria from the gut occurs shortly after slaughter (Koneman, 1970; Gill and Penney, 1977).

Some clostridia are relatively resistant to the bactericidal activity of the animal's immune system (Labadie and Guinet, 1981) and ingested clostridial spores can persist in the visceral organs (Bagadi and Sewell, 1974). Similarly, while in a healthy animal small numbers of clostridia are likely to be readily eliminated (Gill *et al.*, 1981), in a stressed animal they may persist longer in the system (Frank *et al.*, 1961; Nowicki, 1976; Gill, 1979). The contamination of deep tissues with clostridia might, therefore, occur ante-mortem in immuno-compromised animals, such as those requiring emergency slaughter or animals that are stressed or sick. The internal mode of entry of these microorganisms may be either from the gastrointestinal tract or through body abrasions.

Route of contamination of muscle tissue with extrinsic clostridia

In the past, the vast majority of known clostridia were mesophilic, obligately anaerobic, often pathogenic microorganisms. Mesophilic clostridia, including those able to cause disease, are part of the normal flora of an animal's gastrointestinal tract (Cato *et al.*, 1986; Roberts and Mead, 1986). Consequently, the carriage of clostridia in the intestine has appeared to fit well with the scenario of an internal mode of entry into the tissues based on their translocation from the gut. However, the primary sources of many clostridial species are soil, water, sewage, animal feed (such as silage) or aerial plant surfaces (Roberts *et al.*, 1982; Lund, 1986; Dasgupta and Hull, 1989; Saito, 1990; Dodds, 1993b; Ercolani, 1997), as well as animal faeces (Fontaine *et al.*, 1942; Dasgupta and Hull, 1989). Clostridial spores are thus likely to be carried on hides/fleece of slaughter animals and contamination with extrinsic clostridia during dressing is, in principle, also possible. The exogenous sources of clostridial contamination of meat are, however, rarely reported (Dempster *et al.*, 1973; Roberts and Mead, 1986).

Source of carcass contamination with psychrophilic and psychrotrophic clostridia

Little is known about the possible source(s) of carcass contamination with psychrophilic and psychrotrophic clostridia. Psychrotrophic non-proteolytic *C. botulinum* types B, E, and F are commonly present in soil, natural or farm freshwater or marine sediments and animal faeces (Huss, 1980; Dodds, 1993b; Hielm *et al.*, 1998b,c). With these microorganisms, the external route of carcass contamination appears likely. Similarly, with 'blown pack' spoilage causing clostridia, the vacuum pack distension during chilled storage was reported to be associated with abattoir foci of contamination, such as the conveyers carrying unwrapped meat prior to vacuum packaging or boning room air vents (Kalchayanand *et al.*, 1991).

In contrast, an internal mode of access of *C. putrefaciens* into the deep tissues (Ingram, 1952) and porcine carriage of this psychrotrophic *Clostridium* sp. have been implicated in instances of bone taint in cured hams. More recently, psychrophilic and psychrotrophic clostridia have been associated with stifle joint taint in chilled vacuum-packed lamb (Broda *et al.*, 1996a) and with bone taint in temperature abused beef (De Lacy *et al.*, 1998). With stifle joint taint, it has been suggested that clostridia were present in the deep tissues at slaughter rather than introduced as a surface contaminant during dressing and packaging (Broda *et al.*, 1996a).

Psychrotrophic microorganisms, including clostridia, because of their ability to tolerate higher temperatures, have been isolated previously from both permanently cold environments like the deep sea Antarctic (Jordan and McNicol, 1979; Mountfort *et al.*, 1997) and from seasonally cold environments (Baross and Morita, 1978; Kotsyurbenko *et al.*, 1995). Although psychrophilic microorganisms, including clostridia, are frequently unable to proliferate at temperatures above 22°C clostridial spores survive exposure to sub-optimal environmental conditions, including exposure to oxygen and elevated temperatures. Therefore, spores entering the abattoir in soil particles attached to an animal's hide and/or in animal faeces, as well as those that reside within the abattoir environment can be considered potential source(s) for psychrophilic clostridial

contamination of carcasses. Similarly, in immuno-compromised animals, spores of psychrophilic clostridia may be able to enter the blood stream and deep muscle tissue from the gastrointestinal tract or through the skin abrasions. Although under a correct chilling regime the growth of mesophilic intrinsic clostridia will not occur (Barnes *et al.*, 1963), germination and proliferation of psychrophilic and psychrotrophic clostridia may occur because of their lower optimum growth temperatures. The synovial fluid of the leg joints, because its pH is generally higher and more stable during rigour onset than those of muscle tissue (Gracey, 1986), may provide conditions particularly favourable to the outgrowth of clostridial spores.

With psychrophilic and psychrotrophic clostridia, neither exogenous nor endogenous route of carcass and/or muscle tissue contamination can be excluded. This contamination may occur directly from animal carriers and/or from secondary sources harboured within the meat plant.

Objective

The aim of this study was to identify the abattoir source(s) and possible route(s) of deer carcass contamination with psychrophilic clostridia causing 'blown pack' spoilage of vacuum-packed chilled meats. A study tracing these microorganisms back to meat plant reservoir(s) was conducted using genotypic approaches described in Chapter 2. Faeces were examined to determine if the gastrointestinal tract could be the source of psychrophilic *Clostridium* spp. contamination of carcass meat; similarly, hide samples were examined to determine if hides, or soil or faecal matter attached to them, could be the source of contamination. Faeces/hides were examined to establish whether carcass contamination with 'blown pack' causing clostridia occurs via an extraneous route. Tonsils were examined to establish whether this contamination might occur via an internal route. The environmental samples have been screened to assess whether the source of 'blown pack' causing clostridia may be contained within the meat plant.

Methods and Materials

Bacteria

Stringent anaerobic procedures and pre-reduced media (Holdeman *et al.*, 1977) were used for culturing all *Clostridium* strains.

Reference strain of *C. estertheticum* DSM 8809^T (T = type strain) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. This strain was revived in sterile anaerobic milk and was subcultured onto Columbia Blood Agar (CBA, Oxoid) containing 5 % v/v sheep blood. The strain was grown at 10°C for 7 days.

Psychrophilic clostridia of meat origin, DB1A, R26, K21 and K24, were isolated using methods described previously (Broda *et al.*, 1996b). These strains were obtained between 1993 and 1997 from blown packs of chilled vacuum-packed lamb (strains DB1A and R26) and venison (strains K21 and K24). With meat strains, the ability to cause pack 'blowing' was checked under laboratory conditions (see Chapter 1). At 2°C all four strains produced the first signs of gas production in vacuum packs within 11 to 14 d storage. The meat strains, maintained as freeze-dried cultures, were revived in PYGS broth, plated onto CBA supplemented with 5 % v/v sheep blood and incubated at 20°C (strains DB1A and R26) or 15°C (strains K21 and K24) for 72 or 96 h, respectively.

A total of 359 strains of psychrotrophic and psychrophilic clostridia were isolated from hide swabs, faeces and tonsils of 100 slaughter animals, and from 33 environmental samples collected at various points on a venison processing chain. A multi-step isolation procedure was followed using media and techniques described previously (Broda *et al.*, 1998b,c). Briefly, vegetative cells and spores were recovered on Shahidi Ferguson Perfringens (SFP, Oxoid) agar with 5 % egg-yolk. Spores were recovered after ethanol treatment (sample mixed with equal volume of absolute ethanol, incubated for 60 min at 15°C) on Peptone Yeast Extract Glucose agar (Lund *et al.*, 1990) with 625

U ml^{-1} lysozyme and after heat treatment (80°C for 10 min) on Glucose Starch agar (Nakamura *et al.*, 1985). All inoculated plates were incubated at 15°C for 14 days.

Isolates representing every colony morphotype (one per type) on each primary isolation plate were streaked onto CBA agar and incubated anaerobically at 15°C for 7-10 days to confirm purity. Isolates that were obligately anaerobic, catalase negative, oxidase negative, Gram-positive (sometimes Gram-variable) large rods with terminal or subterminal spores, able to grow at 4°C were considered to be psychrophilic or psychrotrophic clostridia. Of those, isolates that were unable to grow at 25°C and above were considered to be psychrophilic (Morita, 1975). Following their original isolation, the isolates, referred to as industry strains, were stored anaerobically on CBA agar at 10°C for 3 months without further transfers. Before use in molecular typing, these strains were subcultured onto CBA plates and incubated at 15°C for 7-10 days to obtain individual colonies.

DNA isolation

To isolate DNA, cells from the exponentially growing reference, meat and industry strains of psychrophilic and psychrotrophic clostridia were harvested from the surface of CBA plates. Cells were suspended in 1 ml of sterile TE buffer and the optical density of the cell suspension was adjusted to approximately 1.0 OD (equivalent of 10^9 cells ml^{-1}). Genomic DNA was isolated using a HighPure DNA preparation kit (Boehringer Mannheim GmbH). The manufacturer's recommended protocol for isolation of nucleic acids from bacteria and yeast was followed, except that, to improve lysis, cells were resuspended in 200 µl of 50 mg ml^{-1} and incubated at 37°C for 60 min. From this step the recommended protocol, incorporating Proteinase K lysis, guanidine-HCl binding and subsequent isopropanol precipitation and ethanol washes, was followed. Eluted DNA was stored at –20°C pending PCR amplification.

Restriction fragment length polymorphism (RFLP) analysis of 16S rDNA gene (PCR-RFLP)

For amplification of 16S rDNA gene, genomic DNA was used as the PCR template. PCR was performed with universal bacterial primers complementary to conserved regions of the 5' and 3' ends of the 16S rRNA gene, as described in Chapter 2. The primers' sequences were: pA (forward) 5'-AGA GTT TGA TCC TGG CTC AG-3' and pH* (reverse) 5'-AAG GAG GTG ATC CAG CCG CA-3' (Hutson *et al.*, 1993a).

The PCR mix was prepared according to the manufacturer's (Boehringer Mannheim) recommendation and contained: PCR buffer 10x (10 µl), 0.2 mM of each deoxynucleoside triphosphate, 0.5 µM of each primer, 2.5 units of *Taq* polymerase and 10 µl of template DNA (100 ng) in a total volume of 100 µl. Amplifications were performed in a heated lid thermal cycler (Techne Genius, John Morris Scientific). After initial denaturation for 3 min at 93°C, target DNA was amplified in 30 cycles.

Each cycle consisted of denaturation for 1 min at 92°C, annealing for 1 min at 55°C and extension for 2 min at 72°C. The final extension was for 3 min at 72°C. The PCR reaction tubes, each containing an amplified 16S rDNA fragment were held at 4°C until further analysis.

PCR-amplified 16S rDNA genes of the reference, meat and industry strains were digested with *Alu*I endonuclease (Boehringer Mannheim GmbH). Restriction digests containing 10 µl of the PCR product, 2 µl of the appropriate buffer and 10 U of enzyme in a total volume of 20 µl were prepared and incubated according to the manufacturer's recommendations. Digestion products were separated by gel electrophoresis in 2.0 % w/v agarose (Seakem) gels at 90 V for 1.5 h. The DNA molecular weight marker VI (Boehringer Mannheim GmbH) was used as a size marker. Banding patterns were visualised with ethidium bromide by UV transillumination.

Isolates were grouped on the basis of their banding pattern similarity as assessed by comparison of resolved fragments larger than 154 bp. Isolates whose PCR-amplified

16S rDNA genes digested with *Alu*I yielded indistinguishable banding patterns were considered the members of the same PCR-RFLP type. PCR products from the isolates that had *Alu*I restriction patterns similar to patterns of known 'blowers' *C. estertheticum* DSM 8809^T, DB1A, R26, K21 and K24 were then digested with an additional 3 restriction endonucleases, *Hae*III, *Taq*I and *Cfo*I (Boehringer Mannheim GmbH). With these three enzymes, digestion and restriction fragment separation was conducted as with *Alu*I.

Internal transcribed spacer polymorphism (ITSP) analysis

16S-23S rDNA internal transcribed spacers (ITS) from the reference strain of *C. estertheticum* DSM 8809^T, meat and industry strains that had *Alu*I restriction patterns similar to the known 'blowers' were PCR amplified with universal bacterial primers ISRA (forward) 5'-AAG TCG TAA CAA GGT ARC-3' and ISRC (reverse) 5'-GGG TTB CCC CAT TCR G-3' (Lane, 1991). These primers are complementary to conserved regions of the 3' end of 16S rRNA and 5' end of 23S rRNA genes directly flanking the spacer. The PCR mix was prepared and ITS amplifications were performed as described for the RFLP analysis of 16S rDNA genes.

A 5 µl aliquot of the PCR reaction was electrophoresed on a 1.5 % w/v agarose gel at 90 V for 1.5 h. The DNA molecular weight marker VI (Boehringer Mannheim GmbH) was used as a size marker. Banding patterns were visualised with ethidium bromide by UV transillumination.

Confirmation of 'blowing' ability

Ability to cause 'blown pack' spoilage in a laboratory meat model was determined, as described in Chapter 1, for 6 isolates representing each of the two PCR-RFLP *Alu*I group that showed pattern similarity to known 'blowers'. In addition, 2 isolates representing each of the remaining PCR-RFLP *Alu*I groups were also tested. Reference strain *C. estertheticum* DSM 8809^T was used as a positive control. Each strain was grown in 10 ml of PYGS broth until a heavy suspension (approximately 10^8 ml^{-1}) was obtained.

Cells of each strain were harvested by centrifugation at 6,000 *g* for 15 min and washed five times with ice-cold saline (0.85 % w/v NaCl). These washed suspensions were used as inocula.

Chilled boneless lamb chumps were obtained one day after boning from a local meat plant and placed individually into barrier bags (Cryovac BB4L) pending inoculation. Duplicate packs were prepared for each strain tested. The packs were inoculated with 0.5 ml of a cell suspension of each strain to obtain a concentration of approximately 10^3 cfu cm^{-2}. Inoculated packs and two uninoculated controls were immediately vacuum-packed using a controlled atmosphere packaging machine (Securepack 10) and were stored at 2°C for up to 84 days (the maximum shelf life expected for vacuum-packed chilled lamb). Packs were examined regularly for the presence of gas bubbles in the meat drip followed by a loss of vacuum and subsequently by substantial pack distension.

Results

Restriction fragment length polymorphism analysis of 16S rDNA gene (PCR-RFLP)

On the basis of RFLP analysis of the 359 PCR-amplified 16S rDNAs, the majority of the industry strains were classified into 6 distinct groups. Each of these groups contained multiple isolates that had banding patterns similar to each other, but that were readily differentiated from other PCR-RFLP groups. Only two PCR-RFLP groups (named groups I and II) showed *Alu*I restriction pattern similarity with known 'blowers' DB1A, R26, K21, K24 and *C. estertheticum*. Isolates in both groups were unable to grow at or above 25°C. An additional 12 isolates had banding patterns that differed from those of the 6 major groups, the reference or meat strains. These isolates represented eight unique banding patterns and were for convenience designated as miscellaneous.

PCR-RFLP group I contained 11 isolates (3.1 % of the total number) that had *Alu*I banding patterns indistinguishable from those of meat strains DB1A and R26 (Fig. 3.1). Similarly, the subsequent digestion of 16S rDNAs of these industry isolates with *Taq*I and *Cfo*I yielded, with each endonuclease, restriction patterns indistinguishable from those of strains DB1A and R26. However, *Hae*III digestion of 16S rDNAs demonstrated restriction pattern polymorphism within PCR-RFLP group I. Within this group, 5 isolates had *Hae*III restriction patterns indistinguishable from the pattern of strain DB1A, whereas 6 other isolates had *Hae*III restriction patterns similar to the pattern of strain R26.

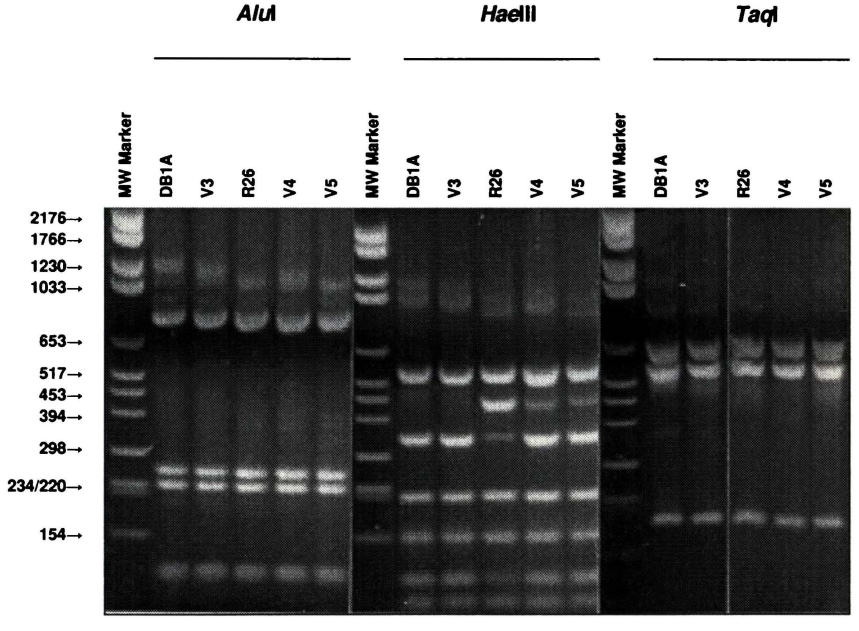

Figure 3.1. Restriction patterns of digested 16S rDNA genes of meat strains DB1A and R26, and three industry isolates representing PCR-RFLP group I. The remaining eight isolates in this group had restriction patterns indistinguishable from pattern of either industry isolate V3 or V4. Digestion products were separated by gel electrophoresis in 2.0 % w/v agarose at 2.5 V cm^{-1} for 1.5 h. Restriction patterns were visualised with ethidium bromide by UV transillumination. The DNA molecular weight marker VI (Boehringer Mannheim GmbH) was used as a size marker.

PCR-RFLP group II contained 17 isolates (4.7 % of the total number) that, with *Alu*I, *Hae*III, *Taq*I or *Cfo*I, had restriction patterns indistinguishable from each other. These isolates shared some *Alu*I and *Hae*III pattern similarity with the reference strain *C. estertheticum* DSM 8809^T and meat strains K21 and K24 (Fig. 3.2).

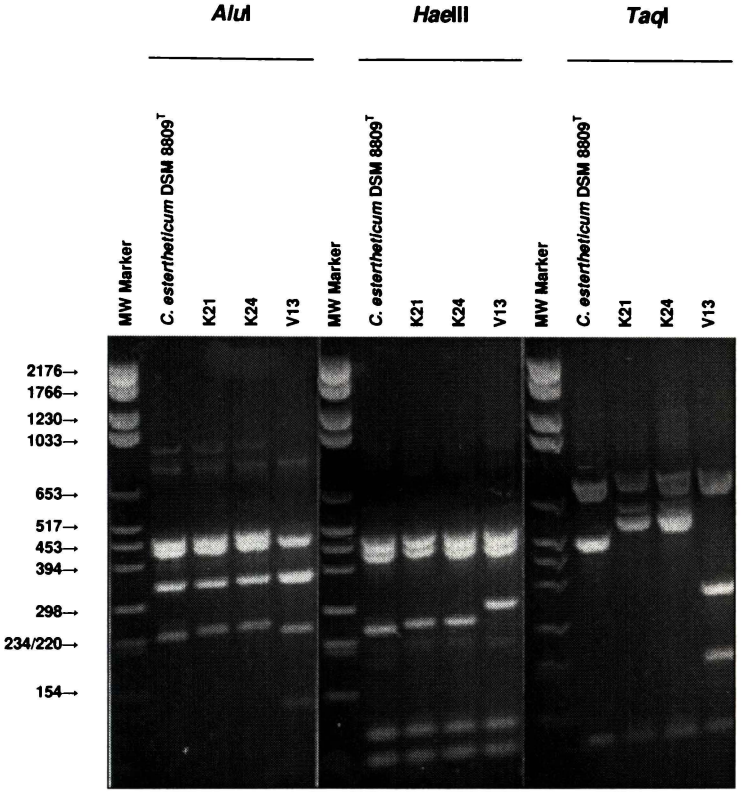

Figure 3.2. Restriction patterns of digested 16S rDNA genes of reference strain *C. estertheticum* DSM 8809^T, meat strains K21 and K24, and industry isolate V13 representing PCR-RFLP group II. The remaining sixteen isolates in this group had PCR-RFLP restriction patterns indistinguishable from pattern of isolate V13. Digestion products were separated by gel electrophoresis in 2.0 % w/v agarose at 2.5 V cm^{-1} for 1.5 h. Restriction patterns were visualised with ethidium bromide by UV transillumination. The DNA molecular weight marker VI (Boehringer Mannheim GmbH) was used as a size marker.

With *Alu*I, however, the restriction patterns of industry isolates lacked one band at approximately 450 bp while an additional band below 154 bp was present in restriction patterns of PCR-RFLP group II isolates, but not in patterns of the reference or meat strains.

With *Hae*III, a change in position of one major band was observed between patterns of the industry isolates and strains K21, K24 and *C. estertheticum.* On subsequent digestion with *Taq*I the restriction patterns of the industry isolates were readily differentiated from the reference strain *C. estertheticum* and meat strains K21 and K24. With the same enzyme, restriction patterns of *C. estertheticum*, and strains K21 and K24, were distinctly different from each other (Fig. 3.3).

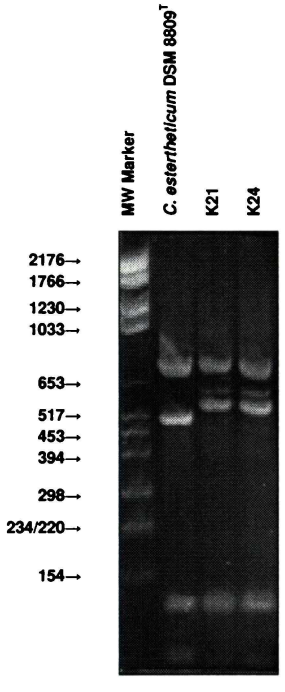

Figure 3.3. Restriction patterns of 16S rDNA genes of reference strain *C. estertheticum* DSM 8809^T, and meat strains K21 and K2 digested with *Taq*I restriction endonuclease. Digestion products were separated by gel electrophoresis in 2.0 % w/v agarose at 2.5 V cm^{-1} for 1.5 h. Restriction patterns were visualised with ethidium bromide by UV transillumination. The DNA molecular weight marker VI (Boehringer Mannheim GmbH) was used as a size marker.

Internal transcribed spacer polymorphism (ITSP) analysis

With PCR-RFLP group I, the ITS banding patterns represented by isolates V3, V4 and V5 on Fig. 3.4 consisted of one main band at approximately 346 bp (major ITS product) and multiple minor bands (minor ITS products). All PCR-RFLP group I isolates and meat strains DB1A and R26 had indistinguishable overall banding patterns and a similar sized main spacer PCR-amplification product visualised on the gel (Fig. 3.4).

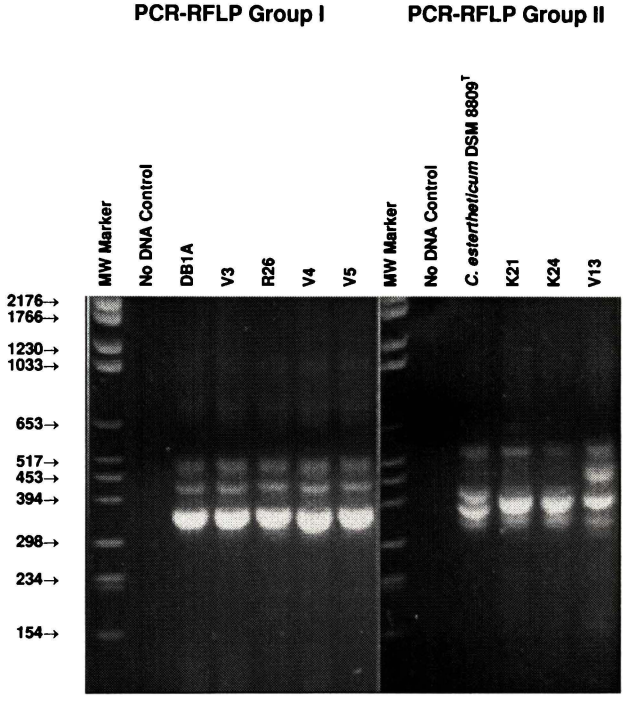

Figure 3.4. The banding patterns of reference, meat and industry strains of psychrophilic clostridia representing PCR-RFLP group I and PCR-RFLP group II obtained in PCR amplification of 16S-23S rDNA internal transcribed spacer. Industry isolates V3, V4 and V5 represent PCR-RFLP group I. The remaining eight isolates in this group had banding patterns indistinguishable from patterns of these isolates. Industry isolate V13 represents PCR-RFLP group II. The remaining sixteen isolates in this group had banding patterns indistinguishable from pattern of isolate V13. PCR products have been electrophoresed in a 1.5 % w/v agarose gel at 3.5 V cm^{-1} for 1.5 h and banding patterns were visualised with ethidium bromide by UV transillumination. The DNA molecular weight marker VI (Boehringer Mannheim GmbH) was used as a size marker.

With PCR-RFLP group II, the ITS banding patterns represented by isolate V13 on Fig. 3.4 consisted of two main bands at approximately 490 and 409 bp and multiple minor bands. Within PCR-RFLP group II, all the industry isolates had indistinguishable ITS banding patterns and major ITS products of similar size. The industry isolates differed, however, from *C. estertheticum* in overall ITS banding pattern and in the size of the two major ITS products (Fig. 3.4). These isolates also differed from meat strains K21 and K24 in overall ITS banding pattern. In contrast to the industry isolates whose patterns consisted of two main bands, banding patterns of strains K21 and K24 had only one main band.

Confirmation of 'blowing' ability

In packs inoculated with vegetative cells of PCR-RFLP group I isolates, small gas bubbles were first present in the meat drip after 14 days storage at 2°C. In comparison, gas production in packs inoculated with vegetative cells of the reference strain of *C. estertheticum* DSM 8809^T was first evident at 11 days of storage. While packs inoculated with *C. estertheticum* reached a grossly distended state after 35 days of storage, a smaller volume of gas was produced in packs inoculated with PCR-RFLP group I isolates. These packs did not reach a grossly distended state within 84 days storage. No gas was present in uninoculated control packs stored at 2°C for 84 days.

No gas production or pack distension was observed in packs inoculated with PCR-RFLP group II isolates or those from any other PCR-RFLP group.

Source of psychrophilic clostridia

With PCR-RFLP group I, all isolates were obtained from either hide swabs or faecal samples. A similar proportion of hide and faecal samples (six and five per cent of total number of samples, respectively) was positive for psychrophilic clostridia belonging to PCR-RFLP group I. None of the isolates belonging to PRC-RFLP group I was obtained from tonsil or environmental samples.

With PCR-RFLP group II, 15 out of 17 isolates were obtained from faecal samples. Of the two remaining isolates within this RFLP group, one was obtained from a hide swab and one from an environmental sample. The single environmental isolate was found in a swab sample taken from an inedible offal room. None of the isolates belonging to PCR-RFLP group II was obtained from tonsil samples.

Discussion

PCR-RFLP group I psychrophilic clostridia caused gas production and pack distension in vacuum-packed lamb stored at 2°C for 14 days. With both RFLP analysis of 16S rDNA genes and ITSP analysis, banding patterns of these clostridia were found to be indistinguishable from patterns of either meat strain DB1A or R26 that were previously confirmed as the causative organisms of 'blown pack' spoilage. In this study PCR-RFLP group I psychrophilic clostridia were obtained from either hides or faeces of slaughter animals. Since none of the isolates belonging to PCR-RFLP group I was obtained from environmental samples, it appears that, with these microorganisms, the primary sources of carcass contamination in the abattoir are the slaughter animals. With 'blown pack' spoilage causing psychrophilic clostridia, this finding may explain the variability in presence of pack distension that occurs between different vacuum packs within the same processing batch (unpublished data) or the same shipment (Dainty *et al.*, 1989). Similarly, none of the isolates belonging to PCR-RFLP group I was obtained from tonsils of slaughter animals. Consequently, carcass contamination with PCR-RFLP group I clostridia most likely occurs with spores that either survive passage through the digestive system of slaughter animals or those from environmental sources carried on the coats of slaughter stock. Dressing procedure hygiene, therefore, remains paramount for controlling the spread of PCR-RFLP group I psychrophilic clostridia in a meat plant.

Although in the abattoir the immediate source of carcass contamination with psychrotrophic microorganisms is usually the hides/fleeces of slaughter animals (Newton *et al.*, 1978), a high proportion of PCR-RFLP group I and the majority of PCR-RFLP group II psychrophilic clostridia were found in faecal samples. The presence of clostridia from the gastrointestinal tract on hides can be anticipated as a consequence of faecal contamination occurring during the transport and pre-slaughter holding of stock. Mesophilic clostridia, including those able to cause disease, are part of the normal flora of an animal's gastrointestinal tract (Cato *et al.*, 1986; Roberts and Mead, 1986). The results of this study have shown that, in addition to mesophilic clostridia, the spores of psychrophilic clostridia are commonly carried in animals' intestines. Since the PCR-RFLP group I and group II isolates are unable to grow at temperatures above 25°C, it is likely that, as with the majority of spoilage organisms in vacuum-packed meats, these microorganisms originate from the exogenous environment of an animal (Gill, 1979; Nottingham, 1982). It is likely that, as with many other clostridial species, the primary sources of these microorganisms in the farm environment are soil, animal feed or aerial plant surfaces (Lund, 1986; Ercolani, 1997).

With PCR-RFLP group I isolates, *Hae*III restriction pattern polymorphism of digested 16S rDNA genes was observed in this study. Despite this polymorphism very little 16S-23S rDNA spacer length or overall ITS banding pattern differences were evident between individual isolates in PCR-RFLP group I, indicating that with industry isolates obtained under the same set of conditions there is very little inter-strain variability in length of major and minor spacer amplification products. Similarly little variability in ITS region was demonstrated between PCR-RFLP group I strains and meat strains DB1A and R26 that were originally obtained from spoiled product. If this minor inter-strain spacer polymorphism is species specific remains, however, to be confirmed with a larger number of isolates from different sources. With both PCR-RFLP group I isolates and meat strains DB1A and R26, the ITS banding pattern included one major ITS product. In contrast to RFLP group I, the ITS banding patterns of RFLP group II isolates consisted of two major ITS products, indicating that inter-operon spacer length polymorphism exists within this group.

Despite some *Alu*I and *Hae*III restriction pattern cross similarity between PCR-RFLP group II isolates and known 'blowers' (*C. estertheticum*, strain K21 and strain K24) these isolates did not cause pack blowing. Furthermore, the *Taq*I patterns of PCR-RFLP group II isolates were readily differentiated from patterns of the reference and meat strains. Distinct differences between restriction patterns of *C. estertheticum* and meat strains K21 and K24, were also observed after digestion of 16S rDNA genes with *Taq*I. Subsequent sequencing of 16S rDNA genes from two PCR-RFLP group II isolates and phylogenetic analysis (data nor shown) showed that these industry isolates are closely related to *C. subterminale* (approximately 98% 16S rDNA sequence similarity) and, together with *C. estertheticum*, are members of the same phylogenetic clade. However, PCR-RFLP group II isolates differed from their nearest phylogenetic neighbours in key biochemical and physiological characteristics (data not shown). ITSP analysis demonstrated polymorphism in spacer lengths of the reference strain *C. estertheticum*, meat strains K21 and K24, and PCR-RFLP group II psychrophilic clostridia. The results of the present study indicate a considerable degree of 16S rDNA gene and internal transcribed spacer heterogeneity exists between *C. estertheticum*, meat strains K21 and K24, and the RFLP group II isolates. The detailed taxonomic status of these meat and RFLP group II strains remains uncertain.

The possibility exists that microorganisms causing 'blown pack' spoilage, *C. estertheticum*, *C. laramiense* or meat strains K21 and K24, were not present in the meat plant. It is more likely, however, that in this study the failure to isolate clostridia that cause gross pack distension of vacuum-packed chilled meats reflects commonly encountered difficulties with the conventional isolation of these microorganisms (Dainty *et al.*, 1989; Kalchayanand *et al.*, 1989; Broda *et al.*, 1996b). Isolation temperatures have been found to markedly influence the type of microorganisms recovered (unpublished data). The development of molecular microbiological methods may, therefore, offer an attractive alternative to conventional methods for specific detection of 'blown pack' causing clostridia in vacuum-packed meats and in the meat plant environment.

This study demonstrated that PCR-RFLP of 16S rDNA genes accompanied by ITSP analysis could successfully determine the probable source of carcass contamination with psychrophilic clostridia. However, within each 'blown pack' spoilage causing clostridial species, more discriminatory typing methods must be used to establish epidemiological relatedness of clostridial strains from spoiled packs to strains obtained *a priori*, in anticipation of spoilage, from slaughter animals. Only when such relatedness is established can the route of carcass contamination with psychrophilic clostridia be confirmed in a definitive manner.

Conclusion

Within the abattoir, the primary source of carcass contamination with psychrophilic clostridia causing 'blown pack' spoilage are the slaughter animals. The route of this contamination appears to be extrinsic.

Chapter 4

Delineation of Previously Undescribed Species of Psychrophilic and Psychrotrophic Clostridia

Introduction

Methods in classification and delineation of bacterial taxa

Taxonomic studies are most commonly performed to establish a basis for the recognition of microorganisms. However, the additional aim of taxonomy is to describe and name new taxa, to determine relationships between new and previously described taxa and to place a new taxon within an established hierarchy of all organisms.

Until the 1980s, the relationships between bacteria were generally determined using a low number of morphological, biochemical or physiological tests that usually described a small proportion of bacterial phenotype. To bring more objectivity and to minimise the effect that extrachromosomally encoded traits might have on the classification of bacteria, computer assisted or numerical taxonomy has been introduced in which a large range of characters were compared.

In the 1980s, in addition to conventional morphological, biochemical or physiological tests, a wide variety of methods have been applied to study relationships between bacteria. Generally, these methods have been based on chemotaxonomic (such as cellular fatty acids, polysaccharides of bacterial cell walls, quinones and soluble cellular proteins), genotypic (DNA base composition, DNA-rRNA homology, DNA-DNA homology, tRNA and rRNA sequence comparison) and phylogenetic (16S rRNA) markers.

DNA-DNA hybridisation has made it possible to include genomic data for describing phenetic relationships at species level. Similarly, the 16S rRNA gene sequencing has enabled the systematisation of phenetic relationships at the genus level and above. In addition, for the first time, cladistic relationships between bacteria could be described more objectively and phylogenetic classifications of bacteria, mainly on the basis of 16S rRNA gene sequence, have been constructed.

Phenotypic methods in phenetic classification of bacteria

Numerical taxonomic methods. With these methods, large sets of phenotypic characters for large number of strains can be compared (Sneath, 1957). Typically, 50 to 60 phenotypic characters of bacterial isolates, including the reference and type strains, are tested (Sokal, 1985; Sneath, 1986) and the result of each test is assigned a score. With each character being given equal weight, similarity and dissimilarity coefficients are computed using coded test scores and, for each pair of isolates, a similarity matrix is derived. According to this matrix, clusters are formed using an unweighted pair group method using arithmetic averages (UPGMA) or other average linkage clustering techniques (Sneath and Sokal, 1973). The results of this clustering and, consequently, phenetic relationships between bacterial isolates, are graphically displayed as dendrograms or phenograms.

Numerical taxonomy has been successful in defining homogeneous clusters of strains and in integrating a variety of data.

Chemotaxonomic methods. With these methods, microorganisms are grouped and/or identified according to the qualitative and quantitative composition of those chemical compounds of bacterial cells (Goodfellow and Minnikin, 1985) that are universally distributed among bacteria and easily analysed in large numbers of isolates (Komagata and Suzuki, 1987). Compounds that have been found useful in classification include lipids (fatty acids, mycolic acids, phospholipids, glycolipids or isoprenoid quinones), proteins (e.g. ribosomal or whole cell proteins), cell wall components (peptidoglycan, amino acid or sugar composition) and pyrolysis fragments of the whole bacteria.

Of these, analysis of cellular fatty acids, whole cell proteins and cell wall components are most commonly employed for taxon delineation.

While cellular fatty acid (CFA) analysis by gas chromatography is most commonly used for the identification of bacteria at the species level (Welch, 1991), some workers have also attempted to use this analysis to construct classification of bacteria. On the basis of cellular fatty acid composition bacteria have been divided into two major groups. Gram positive aerobic organisms, such as *Bacillus* or *Micrococcus*, contain mainly iso- and/or anteiso-branched fatty acids (Girard, 1971; Kaneda, 1977). Straight chain saturated and mono unsaturated fatty acids are found in other Gram positive or Gram negative bacteria, e.g. lactic acid bacteria (Veerkamp, 1971), clostridia (Johnston and Goldfine, 1983) or some Enterobacteriaceae (Bergen *et al.*, 1983). The qualitative and quantitative composition of cellular fatty acids has been shown to be culture dependent and, consequently, with CFA analysis, classifications of large number of taxa that vary in their culture condition requirements, as well as inter-laboratory comparisons, may be difficult. However, the CFA analysis is useful for taxonomic comparisons of a small number of taxa and, with delineation of new taxa, this analysis is commonly used to confirm dissimilarity of an unknown isolate and its taxonomic neighbours.

The analysis of whole cell soluble protein patterns has been applied in taxonomic studies of many bacterial species, including *Clostridium* (Cato *et al.*, 1982), *Corynebacterium* (Carlson and Vidaver, 1982), *Streptomyces* (Manchester *et al.*, 1990) and *Campylobacter* (Vandamme *et al.*, 1990). Separation of proteins on polyacrylamide gels results in protein banding patterns that are similar or almost identical for closely related bacterial strains (Kersters and De Ley, 1975). With many bacterial taxa, the classifications on the basis of whole cell protein profiles usually compare well with those based on results of DNA-DNA hybridisation (Kersters and De Ley, 1975; Cato *et al.*, 1982; Dicks *et al.*, 1990; Dijkshoorn *et al.*, 1990). With clostridia, strains with greater than 80 % DNA-DNA homology produced identical protein patterns and strains that differed grossly in their protein profiles displayed low DNA-DNA hybridisation values (Cato *et al.*, 1982).

The SDS-PAGE analysis depends on the growth medium and the culture conditions (Kersters and De Ley, 1980). Consequently, with this analysis, classifications of large number of taxa that vary in their culture condition requirements may be impossible. However, the analysis of whole cell proteins are useful for taxonomic comparisons of closely related taxa (Jackman, 1987; Vauterin *et al.*, 1993) and, similarly to CFA analysis, it is often used to preclude a close relationship of previously undescribed taxon and its taxonomic neighbours.

Peptidoglycan composition of the bacterial cell wall has been found useful for differentiation of Gram positive and Gram negative bacteria. Walls of Gram positive bacteria usually contain more than 30 % peptidoglycan and almost no lipids, and walls of Gram negative bacteria contain less than 10 % peptidoglycan and high levels of lipids. There are almost one hundred peptidoglycan types (Schleifer and Kandler, 1972; Schleifer and Stackebrandt, 1983) and their primary structure varies greatly in Gram positive bacteria. Peptidoglycan types are traditionally determined using thin layer chromatography, and either the chemical type or the amino acid composition of the peptidoglycan can be used as chemotaxonomic marker. Qualitative amino acid and amino sugar analyses of cell walls have been found useful in delineating taxa at the generic and at the species level, respectively (Schleifer and Kandler, 1972), and these analyses have been applied to classification of clostridia (Cummins, 1970; Cummins and Johnson, 1971). Differences between ultrastructure of cell walls of Gram positive and Gram negative bacteria can also be visually demonstrated with the transmission electron microscopy. The cell wall of Gram positive bacteria consists of one thick (30-100 nm) and usually homogeneous layer, whereas Gram negative bacteria have a thinner, multi-layered cell wall.

Genotypic methods in phenetic classification of bacteria

With numerical or chemotaxonomic methods, relationships between many bacteria could not be resolved because closely related microorganisms often have different phenotypes. Since the early 1960s, analysis of the genomic DNA of microorganisms has been used to study phenetic relationships between microorganisms and to classify

and identify bacteria. Genotypic features that have been and are still commonly used for this purpose are DNA base composition ratio, DNA homology and 16S rRNA gene similarity.

DNA base composition ratio. The ratio of guanine (G) plus cytosine (C) to adenine (A) plus thymine (T), that is the mol% G+C, has been traditionally determined using the thermal denaturation or the buoyant density in cesium chloride methods. With the thermal denaturation method, the DNA base ratio is derived from a linear plot of the melting temperature (T_m) versus mol% G+C (Marmur and Doty, 1962). The T_m is defined as the temperature at the midpoint of the thermal stability curve obtained by plotting stepwise increasing temperature applied to the solution of double stranded DNA versus increasing absorbance of this solution. With the buoyant density method, DNA is subjected to centrifugation in a cesium chloride density gradient. The mol% G+C value of the DNA is positively correlated with the density of the cesium chloride solution (Schildkraut *et al.*, 1962). More recently, the mol% G+C ratio is determined using high performance liquid chromatography (HPLC) (Mesbah *et al.*, 1989). With the HPLC method, DNA is denatured and digested enzymatically with P1 nuclease and alkaline phosphatase, and the resulting nucleosides are separated using a HPLC column. The DNA base composition ratio is then calculated against a known standard (Mesbah *et al.*, 1989).

The mol% G+C in bacteria varies between different taxa within the range of 23 to 78 (Holländer and Pohl, 1980). DNAs from unrelated microorganisms may have identical G+C content, therefore, the similarity of DNA base composition of microorganisms does not necessarily indicate their taxonomic relatedness. Consequently, the G+C ratio is best used as an excluding marker in the classification of microorganisms. Bacteria, whose DNAs differ by more than 5 % should not be classified to the same species and whose DNAs differ by more than 10 % should not be assigned to the same genus (Goodfellow and Minnikin, 1985).

DNA-DNA homology. Under defined conditions, single stranded DNA of one microorganism can hybridise with single stranded DNA from a different microorganism (Schildkraut *et al.*, 1961) if the two strands contain less than approximately 15 % base mispairing (Ullman and McCarthy, 1973). The extent of hybridisation of these two genomes is commonly expressed as percent homology and can be determined using liquid or solid format hybridisation methods, of which membrane competition, hydroxyapatite and spectrophotometric methods are considered the most reproducible (Grimont *et al.*, 1980).

In the membrane competition method, as with other binding methods (Gillespie and Gillespie, 1971), unlabelled denatured DNA from one strain is immobilised on a nitrocellulose membrane (Gillspie and Spiegelman, 1966). The membrane is then incubated with single stranded, fragmented and radioactively labelled homologous DNA, but increasing amounts of single stranded, fragmented and unlabelled DNA from a competitor strain are added to the homologous reassociation system. If the competitor DNA is homologous to the labelled and to the immobilised DNA, it will hybridise with both DNAs. Consequently, the percentage of labelled DNA that hybridises with the immobilised DNA will be lower than that without competition. If the competitor DNA is not related, it will not hybridise with the labelled and immobilised DNAs and both DNAs will fully hybridise. With the membrane competition method, the percent DNA-DNA homology is derived from the ratio of the heterologous competition to the homologous competition, as measured by the amount of radioactivity detected on the membrane after unbound radioactivity is eluted.

In the hydroxyapatite method, a low concentration of fragmented, labelled DNA from one strain is mixed with an excess of fragmented, unlabelled DNA from another strain (Lachance, 1980). This mixture is then denatured and incubated to allow hybridisation of fragments. If the labelled DNA is identical with the unlabelled DNA, it will reassociate with this DNA but if the two DNAs are unrelated most of the labelled DNA will remain single stranded, because the rate of DNA reassociation is a function of DNA concentration. To separate single and double stranded DNA in the solution

hydroxyapatite is used. Adsorption of double stranded DNA to hydroxyapatite allows single stranded DNA to be eluted (Brenner *et al.*, 1969). Hydroxyapatite can then be desorbed and the radioactivity that remains in the eluted double stranded DNA solution can be measured. The percent of DNA-DNA homology is estimated as the ratio of the radioactivity bound to the eluted DNA duplexes to the total radioactivity of labelled DNA.

The spectrophotometric method for determining the degree of DNA-DNA homology is based on the optical measurement of DNA reassociation from renaturation rates (De Ley *et al.*, 1970; Gillis *et al.*, 1970), as obtained under optimal hybridisation conditions (Huss *et al.*, 1983). To estimate homology of two organisms, the renaturation rates of equivalent concentrations from each of the organisms are measured separately and compared with that of an equal mixture of the two DNA preparations. If the DNAs from the two organisms are homologous, the renaturation rates in individual and mixed DNA solutions will be the same. If the two organisms are heterologous, the reassociation rate in the mixed solution will be one half of those in individual solutions. The percent of DNA-DNA homology is estimated as the ratio of the renaturation rate in the mixed to that in the individual DNA preparations.

The DNA-DNA homology values strongly depend on the sodium ion concentration and the hybridisation temperature. Consequently, hybridisation is usually carried out under standardised optimal conditions, that is, at a sodium ion concentration of about 0.4 M and hybridisation temperature of about 25°C below the midpoint of the thermal stability profile (analogous to T_m of native DNA) of the heterologous duplex (Marmur and Doty, 1961).

With DNA-DNA homology, the difference between the 'T_m's of a heterologous and homologous duplex is defined as the ΔT_m and is used to describe the degree of base pair mismatching in the heterologous duplex. The ΔT_m value for heterologous duplexes of DNAs from organisms that have more than 80 % DNA homology and for homologous duplexes is usually very similar and can range from 0 to 3°C. However, for

heterologous duplexes of DNAs from organisms that have approximately 60 to 70 % DNA homology the ΔT_m is substantially higher and can range from 6 to 9°C (Johnson, 1994). Consequently, DNA homology between 60 % and 70 % has been thought to signify a point of genetic divergence between microorganisms and, with majority of bacteria, strains that have DNA homology above 70 % (Johnson, 1973; Wayne *et al.*, 1987) are considered to be the same genomic species.

The DNA-DNA homology experiments have allowed determination of close relationships between bacteria, especially those at inter- and intra-species, but below inter-generic level. In addition, these experiments allowed introduction of a more unifying concept of bacterial species. Because with DNA-DNA hybridisation entire bacterial genomes are compared the bacterial relationships can be described more precisely than with techniques that compare individual genes or gene products (Stackebrandt and Woese, 1984). However, since the methods for DNA-DNA hybridisation are laborious and subject to inter- and intra-laboratory inconsistencies they have been rarely applied to large taxonomic studies. More often, these methods are used in delineation of lower taxa, especially new bacterial species.

DNA-rRNA homology. When RNA is mixed with single stranded DNA it can hybridise to a complementary DNA strand to form RNA/DNA hybrids. DNA-rRNA homology were initially determined by hybridisation method, in which labelled rRNA from one bacterial strain is incubated under optimal to stringent conditions with excess single stranded, membrane immobilised DNA from another strain. After the hybridisation, each membrane is washed, unpaired segments of rRNA are removed with RNases and the membrane bound radioactivity is measured. Because DNA-rRNA homology depends not only on the sequence similarity but also on the genome size, the number of RNA operons per genome and its state of replication, the relatedness of bacterial strains is indicated by the thermal stability of the DNA-rRNA duplexes. This stability is defined as the temperature at which half of the paired rRNA is eluted from membrane bound DNA (De Ley and De Smedt, 1975).

DNA-rRNA hybridisation has been used in the taxonomic studies of many bacteria including *Desulfovibrio* spp. (Pace and Campbell, 1971), *Agrobacterium* spp. (De Smedt and De Ley, 1977), streptococci, enterococci and lactococci (Schleifer and Kilpper-Bälz, 1987). Because the ribosomal RNA has evolved at a slower rate than genomic DNA, DNA-rRNA homology can be used to determine distant bacterial relationships, for example at the genus level and above. Therefore, rRNA-DNA hybridisation can be considered supplementary to DNA-DNA hybridisation which enables determination of similarities between closely related organisms.

Similarity analysis of 16S rRNA genes. Because DNA-DNA or DNA-rRNA hybridisations require pairwise comparison of all organisms under study (Johnson, 1985) these methods have rarely been applied to classification of large groups of microorganisms. More recently, DNA and/or RNA sequencing has been replacing hybridisation based methods to estimate homologies of bacterial genomes. It is still impossible to construct phenetic classifications of large bacterial groups on the basis of sequences of entire bacterial genomes and, consequently, hybridisation remains the only method available to study DNA-DNA homology. However, thermal stabilities of DNA-rRNA duplexes have been found to correlate well with rRNA sequence similarity values (Johnson and Harich, 1983) and, consequently, rRNA gene sequencing has replaced DNA-rRNA hybridisation.

Because the 16S rRNA gene sequences have been commonly used to study cladistic relationships of bacteria (Woese, 1987) this sequence data is often considered phylogenetic. In fact, sequence data belong to currently observable genotypic characteristics of microorganisms and, thus, are phenetic. Consequently, numerical taxonomic analyses can be applied to the aligned sequence data and by integration of genotypic and phenotypic clusters phenetic classification of microorganisms can be derived. Distance methods, such as UPGMA or neighbour joining (as described in paragraph on methods in phylogenetic reconstructions), are considered suitable for generating phenetic groupings on the basis of sequence data (Sneath, 1989).

16S rRNA gene sequence similarities can be used to suggest relatedness of an unknown bacterial species within higher taxa. Usually, the similarity of the unknown sequence to each of the previously known sequences can be determined by a database search. If the unknown sequence is much more similar to one of the known sequences than to other sequences in the database, the unknown and highly similar sequences can be considered related (Olsen *et al.*, 1986). However, 16S rRNA gene sequences of unrelated organisms can also be very similar (Fox *et al.*, 1992; Stackebrandt and Goebel, 1994). In addition, alignment tools used for searching databases can sometimes miss important sequence similarities (Doolittle, 1994; Gendel, 1997). Consequently, the percent 16S rRNA gene sequence similarity or dissimilarity value should not be used as a sole marker when describing the taxonomic position of an unknown microorganism but it preferably should be supported by additional phenotypic or genotypic markers.

Molecular chronometers in cladistic classification of microorganisms

On morphological or physiological levels, the prokaryotes do not have characteristics that allow their cladistic classification, especially above the genus level. This has been thought to change with the introduction of DNA based methods to study relationships of microorganisms.

Modern cladistics (phylogeny) assumes that all existing organisms have evolved from a common ancestor, the last relative of all existing organisms. Consequently, it supposes that there is an orthologous (i.e. similar in different species because it is derived from a common ancestral character) molecule that is shared by all these organisms and that reflects the genealogy of the whole organisms. For valid phylogenetic interpretation, transfer of this molecule between organisms can only be vertical (inherited). Modern cladistics, therefore, assumes stability of the genome and the monophyletic origins of present day taxa. Consequently, it also assumes that there is natural hierarchy of all organisms in which each taxa belongs to only one higher taxa.

Modern cladists postulate that evolution has been recorded within molecules at a genetic (DNA sequences) or epigenetic (sequences of homologous transcription or translation products, either RNAs or proteins) level. When the amino acid sequences of corresponding proteins from two organisms or their nucleotide sequences are very similar, the organisms are presumed to be phylogenetically related. With these molecules, phylogenetic methods construct clades and, then, build trees that graphically present cladistic relationships. These trees may or may not accurately depict the actual evolutionary relationships of the taxa. Whether phylogenetic methods deliver the 'true' phylogeny of examined taxa depends on many variables, one of which is selection of the phylogenetic marker molecule, or molecular chronometer (Zuckerkandl and Pauling, 1965).

A molecular chronometer is preferably a large molecule whose sequence changes as randomly as possible and which accumulates mutations at a constant rate that reflects the overall evolutionary rate in a line of descent (Woese, 1987). Conserved genes encoding cytochrome *c*, ferredoxin, DNA gyrase, RNA polymerase, β-subunit of the ATPase (*atp*D gene), ribosomal protein S12, elongation factor Ef-TU (the *tuf* gene) or ribosomal RNA genes have been found to contain characteristics of a molecular chronometer and are considered useful in determining phylogenetic relationships (Vandamme, 1998). With the eukaryotes, homologous proteins, such as cytochrome *c*, ferredoxin, globin, lysozyme and serum albumin, have been found suitable for phylogenetic analyses. With the prokaryotes, however, the identification of homologous proteins that occur universally in all groups of microorganisms is difficult. Similarly, of ribosomal genes, the 5S rRNA, 23S rRNA and tRNA genes have been found unsuitable for assessing phylogenetic relationships of microorganisms due to either their size, inappropriate rate of sequence change or structural constraints (Pace *et al.*, 1986).

16S rRNA gene as a phylogenetic marker

Many reasons have been described to demonstrate the suitability of the use of 16S rRNA molecule as a molecular chronometer (Woese, 1987). It has been suggested that

the 16S rRNA genes accumulate mutations at a constant, the slowest of the rRNAs, rate over time and are functionally and evolutionally homologous in all organisms. The 16S rRNA genes occur in all cellular forms of life and, consequently, allow estimation of a universal phylogeny of all organisms. Only a small part of the 16S rRNA gene has accumulated mutations at a fast rate and, in consequence, the 16S rRNA molecule has a highly conserved primary and secondary structure. The 16S rRNA gene is sufficiently long to give statistically significant sequence comparisons. In addition, it was initially thought that lateral gene transfer, i.e. the exchange of a chromosomal gene between different species (e.g. Kroll *et al.*, 1998), is only possible with small adaptive genes (Goodfellow *et al.*, 1997) and that transfer of 'house keeping' genes, such as 16S rRNA gene, do not occur between contemporary organisms (Olsen *et al.*, 1986). However, no evidence has been shown by early phylogeneticists to prove that lateral transfer of 16S rRNA gene could not occur.

The mutations in 16S rRNA gene sequence are mainly nucleotide replacements, rather than insertions or deletions. Different sequence sites in this molecule mutate at different rates. With some positions, the primary nucleotide sequence changes within lower taxa, while other positions may vary between higher taxa or be conserved among almost all organisms (Woese *et al.*, 1985). This characteristic of the 16S rRNA gene allows estimation of both close and distant cladistic relationships between microorganisms.

Initially, phylogenetic relationships of microorganisms were computed using numerical methods from pairwise comparisons of catalogues of oligonucleotide sequences (Fox *et al.*, 1977) or, later, from comparisons of complete 16S rRNA sequences determined by reverse transcriptase sequencing (Lane *et al.*, 1985). Direct sequencing of a PCR amplified 16S rRNA gene, however, has allowed fast generation of sequence data that have been then deposited in nucleic acid databases.

Implications, limitations and taxonomic usefulness of 16S rRNA gene based phylogenies

The availability of 16S rRNA gene sequences of a variety of organisms has allowed reconstruction of the universal tree of life (Fox *et al.*, 1980) and has been thought to significantly enhance the search for the universal ancestor, represented in phylogenetics as the consensus sequence of all existing organisms. Initially, comparisons of 16S rRNA genes suggested that life began with primitive bacteria. These bacteria diverged into two branches, one leading to the modern bacteria and the other producing the *Archaea* and later branching again to produce the *Eucarya*. Consequently, phylogenies derived from analyses of 16S rRNA genes has showed that, in contrast to the previous division of organisms into two main lines based on the presence or absence of a nucleus, there were three main lines of evolutionary descent (Woese *et al.*, 1990; Winker and Woese, 1991). The 16S rRNA based phylogenies have suggested that the eukaryotic line of descent is as ancient as the bacterial genotype. However, these phylogenies have indicated that the major organelles in eukaryotic organisms, the mitochondria and chloroplasts, are probably of bacterial origin (Woese and Fox, 1977; Fox *et al.*, 1980).

Generally, congruence between phylogenetic trees based on different phylogenetic markers is thought to provide strong evidence of an evolutionary relationship. The major pitfall of 16S rRNA gene derived phylogenies has, however, been a lack of a consensus with the phylogenies derived from some alternative molecules. Phylogenies constructed on the basis of 5S and 23S rRNA genes, RNA polymerases, membrane lipids and fatty acid synthetase, tRNA, flagella and β-subunit of the ATPase have appeared to support the three domain proposal suggested by 16S rRNA analyses (Woese *et al.*, 1990; Schleifer and Ludwig, 1994). However, phylogenies based on glutamine synthetases, glutamine dehydrogenases or homologues of the 70 kD heat shock proteins (Gogarten *et al.*, 1996) have supported a close association between the *Archaea* and the Gram positive bacteria, and therefore do not support the three domain proposal of Woese and Fox (1977). A similar lack of consistency has been observed in many phylogenies that are based on protein molecules (Woese, 1998).

The reason why different molecules give different phylogenies for the same organism was attributed to lateral transfer, duplication or back mutations of genes encoding these molecules. Highly homologous genes have been found in unrelated bacteria (Syvanen, 1994) and this phenomenon has been proposed to result from lateral gene transfer. Similarly, homologous genes have been observed in organisms belonging to different domains, e.g. *Bacteria* and *Archaea* (Nelson *et al.*, 1999). The presence of foreign functional genes in bacterial genomes, as well as the existence of an integron gene system with the specific function of facilitating gene exchange (Hall and Collins, 1995; Mazel *et al.*, 1998) have also been thought to support lateral gene transfer in bacteria. While lateral transfer of house-keeping genes or chromosome located key metabolic genes has not been well documented, the possibility of transfer of parts of 16S rRNA gene between genomic species in *Aeromonas* (Sneath, 1993) and, more recently, between *E.coli* and *Proteus vulgaris* (Asai *et al.*, 1999) has been demonstrated.

Some researchers point out that lateral gene transfer describes how different molecules may produce incongruent phylogenies and, therefore, indicate an outcome of, rather than a real evolutionary process. Consequently, they remain hopeful that lateral gene transfer did not affect 'house keeping' genes and that 16S rRNA gene derived phylogeny may still depict true ancestral relationships. It is not known at present how widespread lateral gene transfer has been in the past and what genes are more likely to have been laterally transferred during evolution. Consequently, it is possible that the genes that disagree with the 16S rRNA derived phylogeny (e.g. HSP70, glutamine synthetase) have been laterally transferred from *Bacteria* to *Archaea* (Kandler, 1998; Olendzenski and Gogarten, 1998). With this scenario, lateral gene transfer is thought to introduce a 'noise' into phylogenetic analyses but phylogeny based on the 16S rRNA gene, the bacterial origin of the last common ancestor and placement *Bacteria* and *Archaea* in two distinct domains are believed to remain essentially correct. Alternative theories, however, question the correctness of the tree of life based on ribosomal genes. Phylogenies based on the presence or absence of insertions/deletions in genes coding for highly conserved proteins, such as those involved in DNA synthesis indicate that the last common ancestor of all organisms resembled a Gram positive bacterium.

With this scenario, Gram positive bacteria and salt tolerant *Archaea* would form one, while Gram negative bacteria and methano- and acidophilic *Archaea* would form the other, of two distinct domains of the tree of life. An eukaryotic cell would then be formed by fusion of an archaeon with a Gram negative bacterium (Martin and Müller, 1998). Another, even more radical theory suggests that a primitive eukaryotic cell is the last common ancestor. This theory argues that placement of bacteria at the base of phylogenetic tree is an artifact of insufficient compensation for different rates of evolution of genes of different organisms. Consequently, organisms having a higher number of genes evolving at a faster rate would appear more ancient than those containing slowly evolving genes (Forterre, 1998, 1999).

It is now believed that the lateral transfers of bacterial genes have been commonly occurring not only in the distant evolutionary past (Cohan, 1994; Jaenecke *et al.*, 1996; Lawrence and Ochman, 1998) but, as shown with some bacterial and even slowly evolving archaeal genomes, is a major factor in the their present-day evolution (Lan and Reeves, 1996). It is probable that modern microbial genomes amalgamate genes from different organisms. Consequently, the phylogeny of 16S rRNA gene, as well as other genes, does not represent the phylogeny of the whole organism but instead describe relatedness of each single gene. The high prevalence of the lateral gene transfer among ancestral cells also suggests the absence of the one common ancestor of the main evolutionary lines (Kandler, 1998; Woese, 1998). It is, therefore, likely that the natural populations of bacteria cannot be organised into the inclusive hierarchy and, thus, universal, gene based, 'tree of life' does not exist. Currently, occurrence of lateral gene transfer in lower organisms contradicts all basic phylogenetic assumptions. To date, bacterial genome data point to complexity of, rather than allow firm conclusions about, the true origin and development of life.

Lateral transfer of bacterial genes hinders our ability to trace the ancestry of *Bacteria* in the way resembling that of higher organisms. The 16S rRNA gene has been found, however, to be extremely valuable in studies of natural, in contrast to culturable, populations of microorganisms (Amann *et al.*, 1991). Similarly, this gene has proved

very useful in determining the relatedness of bacterial taxa in a more precise manner (Woese *et al.*, 1975; Woese and Fox, 1977) and in deriving descriptions of taxonomic ranks. Today, the taxonomic utility of 16S rRNA gene sequence analysis resides in the description of the approximate taxonomic position of unknown isolates, indication of their taxonomic neighbourhood and their identification. Traditionally, however, determination of the approximate phylogenetic position is required when delineating a new taxon (Vandamme, 1996). This is effected by means of phylogenetic analysis of 16S rRNA gene sequences.

Methods for phylogenetic reconstruction

The general processes of phylogenetic reconstruction. Phylogenetic reconstruction generally includes three steps: alignment of sequences, building and selection of a best-fit tree, and estimating confidence of the best-fit tree (Olsen *et al.*, 1986).

During sequence alignment the nucleotide positions of a sequence are matched with the evolutionarily homologous positions in each of the other sequences. Consequently, the process of alignment selects sequence regions that are more similar among the analysed sequences than could be expected from random variation. Because the length of both terminal and internal sequence regions can vary, alignments commonly involve exclusion of terminal fragments and insertion of alignment gaps.

To build a phylogenetic tree, the aligned sequence data are fitted into a tree branching order. Tree building is most often carried out using distance, parsimony and maximum likelihood reconstructions (Olsen, 1987; Lake, 1987; Penny, 1991). All these reconstructions assume that the analysed sequence sites change independently, i.e. that change of a nucleotide in one site/lineage does not affect a change in any other site/lineage, respectively, and that each nucleotide position or site has the same probability of change (Lento *et al.*, 1995).

Once the tree with a particular branching order is ready, alternative branching orders are tested to see whether better branch arrangements exist. To find the best-fit tree, exact methods, such as an exhaustive search or branch and bound search, evaluate all, or the majority, of possible trees for set of sequences under study. However, these methods are slow and impractical to use with large number of taxa. Consequently, inexact methods, such as heuristic searches that evaluate a limited number of trees, are commonly applied with these analyses.

Confidence in phylogenetic trees is commonly estimated with a bootstrap analysis (Felsenstein, 1985). With this analysis, sequence sites are sampled at random from the original sequence to create many new sequences (pseudosamples), each of the same size as the original sequence. After each sampling, sites in the original sequence are replaced, so in each of the pseudosamples some of the original sites will be represented more than once and some will be omitted. Sets of the new re-sampled sequences representing all required taxa are then analysed to find the best-fit tree. A consensus tree is constructed from the optimal trees of each replicate set. The bootstrap value for a branch is the percentage of re-sampled trees that have this branch. Generally, bootstrap values over 80 % indicate the consistency of the phylogenetic signal in the character data under study (Zharkikh and Li, 1992). Confidence in phylogenetic trees may also be estimated comparing trees that are equally optimal or sub-optimal (Cracraft and Helm-Bychowski, 1991). With maximum parsimony, when branching orders of equally optimal trees are significantly different, a consensus tree can be constructed. The 50 % majority rule consensus tree is a tree in which branches are present if they are supported by at least half the trees considered.

Distance methods. With these methods, DNA sequences are converted into a matrix of evolutionary distances calculated from the number of nucleotide mismatches between each two aligned sequences (Olsen and Woese, 1993). A phylogenetic tree is then fitted to this matrix. The best tree branching order is considered the one that produces the lowest standard deviation between observed and expected pairwise evolutionary distances (West and Faith, 1990).

There are several distance methods of which UPGMA (unweighted pair group method using arithmetic averages) (Sneath and Sokal, 1973), Fitch-Margoliash (Fitch and Margoliash, 1967) and the neighbour joining (Saitou and Nei, 1987) are most often used. Uncorrected, these methods are often considered to be clustering, rather then phylogenetic, methods. Common distance methods assume that every site in the data changes at an equal rate but 75 % of the 16S rRNA gene is not subject to evolutionary changes. Consequently, for a valid phylogenetic conclusion, gamma shape parameters, transitions (purine to purine or pyrimidine to pyrimidine substitutions) to transversions (purine to pyrimidine or *vice versa* substitutions) ratio and base composition ratio should be examined in the data under study and, if appropriate, a correction factor should be selected to allow for the underlying model of evolution.

The distance methods are recommended for the reconstruction of phylogenies of very divergent lineages and/or lineages with varying evolution rate (Olsen, 1987). The neighbour joining method, in which an initial tree is sequentially modified to minimise the total branch length, is the most often used method for phylogenetic classifications of large numbers of bacterial taxa. Distance methods are often criticised because with conversion of sequences to distances a large proportion of the information contained in rRNA sequence is discarded. In addition, distance methods, such as the neighbour joining, are usually sensitive to a violation of the independence of characters (Tillier and Collins, 1995).

Parsimony reconstructions. Parsimony analysis examines the nucleotide substitutions at every site of the sequence and then attempts to construct the shortest phylogenetic tree, i.e. the tree having branching order that requires the least number of mutational changes to occur (Olsen and Woese, 1993). Usually, several optimality criteria have to be selected to build the tree and under a selected criterion several exact or inexact methods can be used to find the shortest tree.

Parsimony analysis is based on 'parsimony informative sites', and sites that do not change or where only one of examined taxa varies are ignored. Parsimony reconstructions are usually recommended when the rates of evolution in various lineages are similar. When some branches of the tree are much longer than other branches, the parsimony tree tends to group the long branches together (Nei, 1991; Olsen and Woese, 1993; Kuhner and Felsenstein, 1994) and, consequently, this tree may not show the 'true' phylogeny.

Maximum likelihood reconstructions. These reconstructions estimate the likelihood that the mutations in sequences under study result from a random process (Olsen and Woese, 1993). The maximum likelihood reconstruction analyses the sequences on a site-by-site basis and fits data to a model of sequence evolution. With a defined model of sequence evolution, parameters such as base ratio and transitions to transversions ratio should be selected prior to the analysis. Maximum likelihood then aims to find the most probable tree, i.e. the tree having branching order that most likely represents the number of mutational changes occurring at all sites of observed sequences (Felsenstein, 1981; Kishino and Hasegawa, 1989).

Maximum likelihood reconstructions allow selection of different evolution rates for different sites and, consequently, these analyses generally perform well when, within a branch, sequences contain unequal evolution rates at different sites (Kuhner and Felsenstein, 1994). These reconstructions also produce good results when rates of substitution vary in different lineages (Kuhner and Felsenstein, 1994). However, maximum likelihood reconstructions generally disregard sequence information from insertions and deletions (Penny *et al.*, 1991).

Selection of tree building methods. Theoretically, with repeated analysis of the same data, various tree building methods should produce a single tree representing the true phylogeny of the analysed taxa. In practice, however, these methods produce different branching orders. The results obtained with different tree building methods can be influenced by violation of the assumption of independence of mutations, number of

taxa, correctness of alignment, outgroup selection, site to site differences in mutation rate, variation in evolutionary rate among different lineages, degree of genetic divergence, branch lengths, transitions to transversions ratio, base composition ratio and/or other factors (Olsen and Woese, 1993; DeBry and Abele, 1995). Tree building methods also vary in their consistency, computational speed and discriminating ability (Nei, 1991). Because each method compromises one or more of these areas, selection of a method should be based on the desired application and on the characteristics of the data (Hillis, 1995). With a large number of taxa, the neighbour joining method is frequently selected to reconstruct the phylogeny of the 16S rRNA gene. This method, however, is sensitive to a violation of the assumption of independence of characters (Tillier and Collins, 1995). With the 16S rRNA gene, this assumption may be breached by complementary base pairing and tertiary interactions across helical regions located in the stem regions of the gene. Consequently, it is recommended that an alternative method be used to determine whether this violation has a significant effect on the reconstructions of phylogeny in a study. Parsimony using PAUP* (Swofford, 1996) or other parsimony analyses can be used to search for optimal trees with a large number of taxa and this analysis can be performed in a relatively short time. Agreement between trees estimated by different methods, e.g. neighbour joining and parsimony, generally confirms that the reconstruction has not been affected by errors due to incorrect assumptions (Kim, 1993).

With a small number of taxa and in the absence of large rate variations across sites/lineages or very long branches, maximum parsimony and maximum likelihood reconstructions are recommended for analysis of 16S rRNA gene sequences. These methods use more information contained in the data than distance methods and usually achieve accurate phylogeny. In addition, the maximum likelihood method is robust to violations of many of its assumptions and the appropriateness of the selected model of evolution can be tested using likelihood ratio tests.

Polyphasic criteria for delineation of new taxa

Regardless of whether gene based phylogeny is in principle able to reflect evolutionary relationships of bacteria or not, it is not likely that phylogeny will reflect the full diversity of bacterial relationships. Currently, these relationships are being described, and taxa are being delineated and classified, according the polyphasic concept (Colwell, 1970). With this concept, classification of bacterial taxa should suit general purpose application and should be derived by means of phenetic studies (Cowan, 1978). Therefore, with its original meaning, polyphasic classification has intended to delineate and group microorganisms by their overall similarity, with all examined characters being equally weighted (Cowan, 1978; Goodfellow and O'Donnell, 1993).

In 1987 the *ad hoc* committee on the reconciliation of approaches to bacterial systematics suggested that DNA-DNA homology values provide an objective measure of what is a bacterial species and that this measure should be used when describing a new bacterial taxa. The species was defined as the population of strains that share more than 70 % DNA-DNA homology with a ΔT_m of less than 5°C (Wayne *et al.*, 1987). Because the sequence of rRNA gene has become easier to determine than DNA-DNA homology values, and also because the level of DNA sequence similarity can be measured more precisely than these values, 16S rRNA analysis was later proposed to replace DNA-DNA homology studies for delineation of new species (Stackebrandt and Goebel, 1994). It has been established that strains sharing less than 97 % of their 16S rRNA sequences never show DNA-DNA homology above 70 % (Fox *et al.*, 1992; Stackebrandt and Goebel, 1994). However, strains sharing more than 97 % 16S rRNA gene sequence similarity may have highly homologous genomes or may have genomes related at less than 70 % DNA-DNA homology. Therefore, it has been suggested that the 16S rRNA similarity data can be used to distinguish unrelated taxa rather than to confirm the relatedness of closely related taxa. On the basis of 16S rRNA gene sequence data a new bacterial species can be proposed when its gene sequence displays less than 97 % similarity to this gene sequence of any other bacterium. With strains showing 16S rRNA gene sequence similarity above 97 %, DNA-DNA homology

studies are required to determine whether such strains belong to the same or to the different species.

Initially, genomic data have been recognised as central to classification and delineation of bacterial taxa and DNA-DNA hybridisation has been suggested as the standard for delineating species. Although the utility of rRNA/DNA sequencing has been found to lie mainly in delineating taxa at generic level and above, the use of 16S rRNA gene sequence data for delineating individual species has also been permitted. It was expected that, similarly to higher animals, groupings of bacterial taxa defined on the basis of genetic information would be supported by chemotaxonomic or phylogenetic groupings (Wayne *et al.*, 1987; Vandamme *et al.*, 1996). This principle, the traditional interpretation of polyphasic taxonomy, has been modified to include both phenetic (based on overall similarities, general purpose) and phylogenetic (based on ancestral relationships, special purpose) data. Consensus of groupings obtained with phenotypic, genotypic and phylogenetic data has been required for delineation of bacterial taxa (Vandamme *et al.*, 1996).

More recently, the emphasis of taxonomy has been to describe natural, rather than artificial, relationships among bacteria using phylogenetic analysis of the 16S rRNA gene. Consequently, the current strategy for taxa delineation has significantly departed from the phenetic principle originally included in the polyphasic taxonomy (Colwell, 1970), in that the unequal weighting was given to one special purpose character of a microorganism, its phylogenetic position. In this strategy, a cladistic principle is applied for delineation of higher taxa. Within these taxa, delineation of lower taxa is, theoretically, to be conducted according to phenetic principle (Vandamme *et al.*, 1996). In practice, however, phylogenetic analyses are now being seen as principal to all taxa delineation. Delineation of a new species is usually initiated with phylogenetic analysis that indicates the phylogenetic neighbourhood of an unknown microorganism. Phenotypic and genotypic characteristics are then used to distinguish the unknown taxon from neighbouring taxa. To describe a new taxon, a consensus of all available information on the organism is required. The considered importance of phylogenetic

information in classification of bacteria has prompted proposals for extensive taxonomic revisions and nomenclatural changes of many bacterial groups, genus *Clostridium* inclusive (Collins *et al.*, 1994).

Implications and limitations of modern polyphasic taxonomy

DNA-DNA homology studies and 16S rRNA gene sequence similarity analyses have formed quantifiable guidelines for designation of new bacterial species represented by a limited number of strains. However, much of the precision of the newly formed definitions of bacterial species has been lost when these guidelines have been applied to existing species (Ursing *et al.*, 1995). With DNA-DNA homology studies, several human pathogenic bacteria have been found genomically indistinguishable from similar strains that, due to their non-pathogenicity to humans, have been classified as separate species. With these microorganisms, the need to retain bacterial nomenclature that reflects a connection between a pathogen and a disease has been considered principal over DNA-DNA homology consistency in bacterial classification. Consequently, *Mycobacterium bovis* and *M. tuberculosis* or *Yersinia pestis* and *Y. pseudotuberculosis* are still considered different species. Similarly, the closely related genera *Citrobacter*, *Enterobacter*, *Klebsiella*, *Salmonella* and *Shigella* within the family Enterobacteriaceae (Brenner, 1984) remain classified as separate taxa despite genomic similarity of some bacterial groups, for example those belonging to *Escherichia* and *Shigella* (Brenner and Falkow, 1971). In contrast, strains of some *Acidovorax* species have been found to be related at a level of only approximately 40 % DNA-DNA homology (Willems *et al.*, 1990). With these strains, phenotypic homogeneity has been considered paramount in their recognition as separate species and not as separate genera or families.

Similarly, further studies of 16S rRNA gene sequence similarities have indicated significant levels of 16S rRNA sequence variability with some bacterial taxa, while other taxa have showed very little variability. Because the databases frequently contain only single 16S rRNA gene sequences, standing for single operons of individual bacterial species, it is not possible at the moment to assess whether this sequence variability reflects inter-operon, inter-strain or inter-species variation (Clayton *et al.*,

1995). Similarly, this variability may be due to inadequate taxon delimitation, sequencing error or other laboratory error. The level of 16S rRNA gene sequence similarity, while useful when describing a limited number of strains belonging to a previously undescribed bacterial species, may, thus, be less informative with respect to quantifiable definition of species.

Recently, it has also been found that species established on the basis of DNA-DNA hybridisation or 16S rRNA gene sequences often fail to correspond to ecologically distinct populations (Palys *et al.*, 1997). It has been proposed that, when supported by consistent phenotypic characteristics, DNA clusters defined on the basis of sequences of protein-coding genes should be recognised as a separate species (Palys *et al.*, 1997).

Regardless of whether it is defined in terms of DNA-DNA homology or 16S rRNA gene sequence similarity, with a polyphasic approach, bacterial species have been expected to show consensus of their morphological, biochemical, chemotaxonomic or genotypic characteristics (Wayne *et al.*, 1987; Vandamme *et al.*, 1996). However, by using a wide range of methods it is possible to characterise strains more precisely, it is increasingly difficult to produce consensus groupings of bacterial taxa using different methods (Stackebrandt and Rainey, 1997). Consequently, different relationships between strains within and between species are indicated with different methods. These difficulties have been amplified with inclusion of phylogenetic data for species description and with attaching large weighting to phylogenetic analyses (Vandamme *et al.*, 1996). Considering that phylogenetic analysis arranges taxa according to their historical relationships and, therefore, derives special purpose groupings, it is not surprising that consensus of phylogenetic clades and groupings based on biochemical or physiological characters often cannot be achieved.

It was thought that, with the increased use of genotypic, phylogenetic or chemotaxonomic methods, various taxonomic groups including bacterial species could be described more precisely (Goodfellow and O'Donnell, 1993; Vandamme *et al.*, 1996; Goodfellow *et al.*, 1997). It was also thought that bacterial species, as defined

with a polyphasic approach, would be a stable and a more objective biological entity than its early artificial conception (Stanier and van Niel, 1962). It appears, however, that bacterial species cannot be recognised as a group of strains that is separated in a definite manner from neighbouring taxa regardless of whether a phenotypic, genotypic or phylogenetic approach or their combination is used. Consequently, while it is possible to apply a polyphasic approach to the delineation of new taxa, the emphasis of the classification of existing bacterial groups should be directed to diagnostic utility that should lead to the ready recognition of distinct bacterial populations by special interest groups. Future classification of bacteria should, therefore, aim to deliver a useful, if not a 'natural' and consistent, system.

Classification and speciation of clostridia

Few, mainly morphological, criteria have been required to classify microorganisms to the genus *Clostridium* and, consequently, this genus has become a heterogenous assemblage that includes numerous morphologically, metabolically and physiologically diverse bacteria. Because of the large number of species that are included in the genus *Clostridium*, there have been a number of attempts to sub-group clostridia (Holdeman *et al.*, 1977; Cato *et al.*, 1986). Widely adapted classifications have usually grouped clostridia according to their metabolic characteristics, such as gelatine hydrolysis or glucose fermentation. The 83 clostridial species described in the Bergey's Manual of Systematic Bacteriology (Cato *et al.*, 1986) have been grossly classified into four groups: one including species that ferment glucose and hydrolyse gelatine, one with species that ferment glucose but cannot hydrolyse gelatine, one with species that cannot ferment glucose but can hydrolyse gelatine, and one with species that cannot ferment glucose or hydrolyse gelatine. The main purpose of this artificial classification has been to provide groupings that enable ready recognition and identification of clostridia (Cato *et al.*, 1986), especially those associated with disease. Since the genus *Clostridium* contains 35 species that are considered pathogenic, in addition to metabolism, characteristics related to pathogenicity have featured prominently in phenotype based classification.

The systematic sub-grouping of clostridia has also been proposed with genotypic approaches. Members of the genus *Clostridium* have been found to have G+C content of DNA between 21 to 55 mol% (Hippe *et al.*, 1992). This mol% G+C diversity has been thought to be too great for microorganisms included in a single genus (Goodfellow and Minnikin, 1985) and, consequently, it has been suggested that the genus *Clostridium* should be divided into two main genera, one including species with a mol% G+C from 21 to 34, the other including species with a mol% G+C from 40 to 55 (Cato *et al.*, 1986). Both DNA-DNA and rRNA-DNA homologies of clostridia have been extensively studied (Johnson, 1970, 1973; Johnson and Francis, 1975). According to rRNA-DNA homology values, three main groups have been distinguished among *Clostridium* spp. Group I contained 30 species that had low G+C content ranging from 21 to 28 mol%. This group included the type species *C. butyricum*. Group II also had low G+C content but the 11 species in this group had low rRNA similarity with species within Group I. The six species in the Group III showed low rRNA similarities with members of Group I and II. A number of clostridial species, including five species that had G+C content above 40 mol%, could not be assigned to the three DNA-rRNA homology groups. DNA-rRNA homology groups correlated with only some phenotypic properties (Johnson and Francis, 1975).

The 16S rRNA gene sequences have been determined for over 100 clostridial species and have been used to study phylogenetic relationships of clostridia and related microorganisms (Lawson *et al.*, 1993; Rainey and Stackebrandt, 1993; Collins *et al.*, 1994). Within the Gram positive bacteria phylum, *Clostridium* spp. have been located in the low G+C subdivision (subphylum) (Fox *et al.*, 1980; Woese, 1987). Members of this subphylum have the G+C content of DNA below 55 mol%. *Clostridium* spp. have been found to represent several ancient lineages within the low G+C subdivision and are considered descendants of a common Gram positive ancestor (Lawson *et al.*, 1993; Rainey and Stackebrandt, 1993; Stackebrandt and Rainey, 1997).

On the basis of phylogenetic data, it has been proposed that the genus *Clostridium* may be divided into 19 clusters (Collins *et al.*, 1994). Almost half of all currently described

clostridial species are members of a single clade (Cluster I). Phylogenetic analyses of clostridial and non-clostridial 16S rRNA gene sequences have demonstrated that many clostridia representing the remaining clusters are historically more closely related to bacterial species from non-clostridial genera than to members of their own genus. For example, Cluster XIV has been shown to include 11 clostridial species as well as members of the genera *Eubacterium*, *Ruminococcus*, *Roseburia*, *Butyrivibrio* and *Lachnospira*. The phylogenetic analyses have also placed some *Sarcina* spp. in the Cluster I (Willems and Collins, 1994). Extensive taxonomic reclassification of the genus *Clostridium* and formation of at least five new genera and 11 new combinations, including clostridial and non-clostridial species, has been proposed (Collins *et al.*, 1994).

Phylogenetically defined clusters within the genus *Clostridium* frequently contain a mixture of rods and cocci, Gram positive and Gram negative, anaerobic and aerobic, spore forming and non-spore forming phenotypes. It is thought that anaerobic, rod shaped and endospore forming phenotype is more ancient than aerobic metabolism, coccoid shape and lack of spore formation. Consequently, it has been suggested that loss of spore formation and changes in morphology that have occurred during the evolution of *Clostridium* species led to the formation of non-clostridial phenotypes. However, according to the 16S rRNA gene based phylogeny, a Gram negative type cell wall appears to be an ancestral feature of a pre-proteobacterial organism, with a reduction of peptidoglycan layers typical of the Gram-positive cell wall occurring later in evolution (Stackebrandt and Rainey, 1997).

Many of the phylogenetic groupings proposed within the genus *Clostridium* are not supported by the presence of consistent phenotypic or genotypic characteristics. The best correlation of genotypic and phylogenetic classifications appears to exist between DNA-rRNA homology Group I *sensu* Johnson and Francis (1975) and 16S rRNA based phylogenetic Cluster I *sensu* Collins *et al.* (1994) and it has been proposed that this group/cluster should retain the genus name *Clostridium*. Since correlation between the phenetic and phylogenetic groupings is best observed with bacterial taxa that evolved

more recently, it is perhaps not surprising that this correlation is not seen with ancient clostridial lineages.

A number of nomenclatural problems have arisen with the proposed phylogenetic classification of clostridia. Since *Sarcina* spp. have been placed in Cluster I and this genus name has priority over the name of genus *Clostridium*, unless Rule 56a of the Bacteriological Code (Lapage *et al.*, 1992) is used, *Clostridium* species should be renamed as *Sarcina* species. Similarly, if only Group I/Cluster I clostridia were to retain the genus name, clostridial species including some pathogenic clostridia, for example *C. difficile* and *C. sordelli*, that phylogenetically belong to other genera would lose their traditional generic epithet. Because of the considerable public impact of such nomenclatural changes the generic epithet of many clostridial species, especially human and animal pathogens, while inconsistent with phylogenetic classification, should be conserved (Lapage *et al.*, 1992).

Phylogenetic classification of clostridia would also produce nomenclatural dilemmas for strains of *C. botulinum* and related species. Since this species epithet has been assigned solely on the basis of a diagnostically important trait, botulinal neurotoxin (BoNT) production, a phenotype based classification has divided *C. botulinum* strains into types A, B, C, D, E, F and G. Highly related strains of *C. botulinum* type G have been reclassified as *C. argentinense* (Suen *et al.*, 1988) and *C. botulinum* strains appear today to represent three genomically and phylogenetically consistent groups (Collins and East, 1998). Nucleic acid hybridisation and 16S rRNA sequencing studies have revealed the high degree of relatedness between strains of proteolytic *C. botulinum* types A, B and F (Lee and Riemann,, 1970a,b; Nakamura *et al.*, 1977; Hutson *et al.*, 1993b). Similarly, strains of non-proteolytic *C. botulinum* types B, E and F have shown the high degree of DNA and 16S rRNA homogeneity (Lee and Riemann, 1970a,b; Nakamura *et al.*, 1977; Hutson *et al.*, 1993a). While some genetic diversity exists among strains of *C. botulinum* types C and D, these strains can still be readily distinguished from other phenotypic and phylogenetic groups of *C. botulinum*. It has been suggested that the traditional species epithet is retained for strains of proteolytic *C. botulinum* types A, B

and F, while the remaining two groups are designated as different species (Collins and East, 1998). However, assigning a different species epithet to non-proteolytic *C. botulinum* types B, E and F and *C. botulinum* types C and D would cause significant problems in communication in medical microbiology and diagnosis of botulism. From the perspective of the medical and diagnostic utility of the nomenclature, there is also a need to distinguish phenotypically, genomically and phylogenetically highly related non-toxigenic and toxigenic strains of non-proteolytic *C. botulinum* types B, E and F that are currently classified as the same species (Collins and East, 1998). A precedent for such a division has been established when the *C. sporogenes* species epithet was maintained for non-toxigenic strains genomically related to strains of proteolytic *C. botulinum* types A, B and F (Olsen *et al.*, 1995; Judicial Commision of the International Committee on Systematic Bacteriology, 1999). However, problems with phenotype based classification and nomenclature of *C. botulinum* also exist. These particularly apply to strains of *C. botulinum* that produce more than one neurotoxin type (Franciosa *et al.*, 1997), those that carry silent (unexpressed) BoNT genes (Franciosa *et al.*, 1994; Hutson *et al.*, 1996) or to BoNT producing strains of clostridial species other than *C. botulinum* (Meng *et al.*, 1997).

Currently available classifications of clostridia use a number of approaches, of which the most significant are those based on special purpose characters, such as pathogenicity or phylogenetic markers. At the moment, phenotypic, genotypic and phylogenetic groupings within the genus *Clostridium* appear to lack the consistency that is required with a polyphasic taxa delineation. Unless additional taxonomic markers to complement the present classifications are found, the lack of consistency of various taxonomic approaches may preclude construction of a general purpose polyphasic classification of *Clostridium* species. Because many clostridial species are considered pathogenic the medical and diagnostic utility of classification of these microorganisms is best served by a stable nomenclature that reflects the connection between a pathogen and a disease (e.g. Williams, 1983). The overriding factor in a future reclassification of, and taxa delineation within, the genus *Clostridium* should be the stability of the recognised nomenclature.

Delineation of new species of psychrophilic and psychrotrophic clostridia

Generally, a new taxon is described to provide evidence of its novelty and to enable its future recognition. To prove that an unknown microorganism represents a new taxon, the taxonomic relatives of the unknown isolate need to be identified and differences between the isolate and these relatives need to be demonstrated. Consequently, in the present study, delineation of new clostridial taxa was initiated with 16S rDNA gene sequence determination and similarity analysis. With this analysis, the degree of sequence similarity between the unknown microorganism and its nearest 16S rDNA gene relatives was determined and sequences were selected for phylogenetic analysis.

To satisfy the requirements of a polyphasic taxonomy, phylogenetic analyses were used to identify whether an unknown microorganism belongs to *Clostridium* subphylum and to select its nearest phylogenetic neighbours. To minimise the possibility of producing artifactual groupings two types of analysis were used. Parsimony has been selected for the analysis of 16S rDNA gene sequence data because this reconstruction utilises more information contained in the data than distance methods. Parsimony reconstruction with PAUP* was used because this program contains a heuristic algorithm suitable for searching for optimal trees with a large number of taxa. When possible, maximum likelihood reconstruction was used as a confirmatory method. Bootstrapping was used to assess consistency of the derived branching order.

It is thought that chemotaxonomic methods should contribute to the description of a species (Wayne *et al.*, 1987; Goodfellow and O'Donnell, 1993; Vandamme *et al.*, 1996; Goodfellow *et al.*, 1997). Cellular fatty acids analysis (Cato and Stackebrandt, 1989) and soluble cell protein profiling (Moore *et al.*, 1980; Cato *et al.*, 1982) have demonstrated their potential for differentiation of distinct genotypic species of clostridia. These analyses, therefore, were used to demonstrate differences between each unknown isolate and its nearest taxonomic neighbours.

Due to involvement of psychrophilic and psychrotrophic clostridia in meat spoilage and their presence in food intended for human consumption it was considered imperative to

study phenotypic characteristics of these clostridia, particularly those that may be relevant to pathogenicity and spoilage potential. Detailed characterisation may reveal information useful in spoilage control (e.g. physiological properties such as minimum growth temperature may help to identify appropriate chilling regime). In addition, since the recognition of clostridia from sources other than man is most frequently conducted using conventional keys that are mainly based on phenotypic properties, characterisation of these properties would help to establish species specific identification procedures that in the future would facilitate correct identification of these clostridia. Of the phenotypic properties that should be characterised for novel taxa (Trüper and Kramer, 1981), descriptions of morphological characteristics, such as colony and cell morphology, cell ultrastructure and staining behaviour, physiological properties, such as temperature and pH optima and range for growth, and biochemical properties were included in the present study.

Objective

The aim of the study described in this chapter was to determine whether seven strains of psychrophilic and psychrotrophic clostridia that differed from previously described clostridial species represented new species. An extensive range of phenotypic and genotypic properties of previously undescribed meat derived clostridial isolates was compared with those of previously described clostridial species. Phenetic and cladistic relationships between these meat isolates and their taxonomic neighbours were also determined.

Methods and Materials

Stringent anaerobic techniques (Holdeman *et al.*, 1977) were used for media preparation and culturing of all *Clostridium* strains. The handling of microorganisms and inoculation of test media were conducted inside an anaerobic chamber (Forma Scientific).

Bacteria

Strains SPL77A^T (T = type strain) and SPL77B were initially isolated from the same 'blown' temperature-abused vacuum pack of raw beef; strains DB1A^T, R26, K21 and K24 were isolated from 'blown' vacuum-packed lamb, which spoiled within less than 8 weeks chilled storage, in the absence of temperature abuse; and strain SPL73^T had been isolated from a 'blown', temperature-abused vacuum pack of raw lamb (Table 1.1). An additional 34 SPL77A^T-like and SPL77B-like isolates were subsequently obtained from faeces, hides and tonsils of bovine slaughter stock (Boerema, Broda and Bell, unpublished results). Preliminary morphological and biochemical characteristics were determined for these additional isolates and they were found to be identical to those of either SPL77A^T or SPL77B. Similarly, additional isolates similar to strains DB1A^T or R26 were obtained on different occasions from cervine and bovine slaughter stock. In a preliminary study, phenotypic properties and total soluble cell protein profiles of these additional isolates were compared with those of the original strains. All isolates resembled either strain DB1A^T or R26. Consequently, only characteristics of the original meat-derived strains SPL77A^T, SPL77B, DB1A^T and R26, as well as strains K21, K24 and SPL73^T represented by single isolates are described in this study.

After their initial isolation, meat strains were maintained as freeze-dried cultures. Before characterisation, strains were revived from freeze-dried material in Peptone Yeast Extract Glucose Starch (PYGS) broth (Lund *et al.*, 1990), subcultured onto Columbia Blood Agar (CBA; Oxoid CM331) containing 5 % v/v sheep blood (Life Technologies) and incubated at temperatures within their optimum growth range for 48 to 96 h (Table 1.1). Strains were checked for purity before use.

Designations of reference strains and their sources are listed in Table 4.1. With the exception of *C. estertheticum,* reference strains were revived from freeze-dried material in PYGS broth and plated onto CBA supplemented with 5 % v/v sheep blood. *C. estertheticum* was revived in sterile anaerobic milk. *C. vincentii* and *C. estertheticum* were grown at 10°C and both psychrotrophic *C. botulinum* strains and *C. algidicarnis* were grown at 30°C. Remaining reference strains were grown at 37°C for 48 h.

Table 4.1. Designations and source of reference strains of *Clostridium* spp. that are taxonomically related to the seven meat strains of psychrophilic and psychrotrophic clostridia characterised in this study.

Strain	Designation	Source
C. aerotolerans	DSM 5434^T	DSMZ
C. algidicarnis	NCFB 2931^T	NCFB
C. botulinum type B	17B (= ATCC 25765)	CSIRO
C. botulinum type E	Beluga	CSIRO
C. cadaveris	DSM 1284^T	DSMZ
C. carnis	DSM 1293^T (= ATCC 25777^T)	DSMZ
C. celerecrescens	DSM 5628^T	DSMZ
C. chauvoei	NZRM 99^T (= ATCC 10092^T)	NZCC
C. estertheticum	DSM 8809^T (= NCIMB 12511^T)	DSMZ
C. fallax	DSM 2631^T	DSMZ
C. intestinale	DSM 6191^T	DSMZ
C. septicum	NZRM 18^T (= ATCC 12464^T)	NZCC
C. sphenoides	DSM 632^T	DSMZ
C. subterminale	DSM 6970^T	DSMZ
C. vincentii	DSM 10228^T	DSMZ
C. xylanolyticum	DSM 6555^T	DSMZ

ATCC, the American Type Culture Collection, Rockville, MD, USA. DSMZ, the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. NCFB, the National Collection of Food Bacteria, Reading, UK. NCIMB, the National Collection of Industrial and Marine Bacteria, Aberdeen, UK. NZCC, the New Zealand Culture Collection, Communicable Disease Centre, Porirua, New Zealand. CSIRO, Division of Food Science and Technology, Sydney, Australia.

All reference strains were checked for purity by plating onto CBA supplemented with 5 % v/v sheep blood.

Phenotypic characterisation

Colony, and cell and spore morphology. Morphology and size of well-isolated colonies, and vegetative cell and spore morphology have been studied using methods described in Chapter 1.

The presence of flagella. For this determination, exponentially growing cultures of meat strains were washed with water, allowed to settle onto Formvar-coated copper grids and negatively stained with 1 % to 2 % w/v uranyl acetate (Beveridge *et al.*, 1994). Stained grids were dried and examined with a transmission electron microscope (Philips EM 400).

Vegetative cell and spore ultrastructure. The ultrastructure of vegetative cells and spores was determined for the isolates grown in PYGS broth at temperatures with an optimum growth range for 18 to 36 h and 5 to 30 days, respectively. The bacteria were fixed overnight at 4°C in a solution containing 2.5 % v/v glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.2). The fixed cells were washed in 100 mM sodium cacodylate buffer and post-fixed with 1 % w/v aqueous osmium tetroxide. The cells were then *en bloc* stained with 4 % w/v aqueous uranyl acetate, dehydrated and embedded in Spurr embedding medium. Ultra-thin sections were then prepared and stained with a 4 % w/v uranyl acetate and lead citrate solution (Beveridge *et al.*, 1994). The grids containing sections were examined using the transmission electron microscope.

Gram reaction, Gram type and cell wall type. Gram reaction has been performed as described in Chapter 1. A KOH test (Powers, 1995) was also conducted to determine the Gram type of cells grown on the surface of CBA with 5 % v/v sheep blood. Cell wall type was determined using transmission electron microscopy, as described in the previous section.

Physiological tests. For physiological tests, growth was determined by monitoring an increase in the optical density at 550 nm, as measured with a spectrophotometer (Novaspec II, Pharmacia), against three uninoculated controls.

The potential for growth at 25 pH values ranging from 4.23 to 9.82 was determined at 30°C for strains SPL77A^T and SPL77B, at 20°C for strains DB1A^T and R26, at 26°C for strain SPL73^T and at 15°C strains K21 and K24. The growth was determined in

Peptone Yeast Extract (PY) broth (Holdeman *et al.*, 1977) with glucose concentration adjusted to 0.5 % w/v. Broths were inoculated with 2 % v/v of culture growing exponentially at pH 7.0. The pH of the broth was kept constant (±0.1 unit) by adjustment with potassium phosphate buffer. The broth pH was measured with a pH meter (Schott CG 837, Labsupply Pierce) equipped with a combination electrode. Cultures were incubated for up to 10 days to determine whether growth occurred.

Growth rates were measured for 42 temperatures ranging from -1.5°C to 47°C. The temperature range for growth was determined at pH 7.0 in PY broth with 0.5 % w/v glucose. For each meat strain, Hungate tubes containing medium were inoculated with 2 % v/v of exponentially growing culture and incubated in a temperature gradient incubator of local manufacture. Throughout the study, temperatures were monitored with copper constatan thermocouples and a Comark 1624 electronic thermometer. Tubes were incubated for up to 21 days to determine whether growth occurred.

Biochemical characteristics. Types of biochemical tests and their detailed procedures were conducted as described in Chapter 1.

The ability of meat strains to degrade xylan was determined on PY agar containing 3 % w/v oat spelts xylan (Sigma) after incubation at their optimum growth temperatures (Table 1.1). To determine xylanase activity of strain SPL73^T that was positive in this initial test, the strain was grown at 26°C for 72 h in PY broth supplemented with 0.5 % w/v oat spelts xylan. A cell-free culture supernatant was prepared by centrifuging the culture at 12,000 *g* for 15 min and subsequent sonication of the supernatant followed by another centrifuging cycle. Enzyme activity was measured at both pH 5.6 and pH 7.0 by determining reducing sugar (xylose) release. Enzyme activity at each pH was tested in the reaction mixtures containing culture supernatant, oat spelts and potassium phosphate buffer. Final concentrations of xylan and potassium phosphate in each mixture was 1 % w/v and 80 mM, respectively. These reaction mixtures were incubated at 26°C for 1 h. The reducing sugar formed was measured by the dinitrosalicilic acid method (Miller, 1959).

One unit of xylanase activity was defined as the activity that released 1 μmol of xylose in 1 min.

Toxicity test. To test for toxicity, procedures described in the FDA Bacteriological Analytical Manual (Solomon *et al.*, 1995) were followed. National Animal Ethics Committee approval was granted before testing began. Meat strains SPL77A^T, DB1A^T, K21 and SPL73^T were grown for 5 to 21 days at their optimum growth temperatures in cysteine and resazurin deficient PYGS broth. An undiluted, trypsin-treated and heat-treated culture supernatant was prepared and injected intraperitoneally into mice. For each sample a separate pair of mice was injected. Mice were observed periodically over 72 h.

Composition of headspace volatiles. Headspace volatiles of packs inoculated with strains DB1A^T and R26 were analysed using gas chromatography and mass spectrometry. Chilled whole lamb legs, obtained from a local meat plant, were placed individually into barrier bags (Cryovac BB4L) pending inoculation. Duplicate packs were prepared for each strain tested. Each inoculum was prepared and packs were inoculated as described in the previous section. In addition, two uninoculated control packs were also prepared. Inoculated packs and uninoculated controls were immediately vacuum-packed using a controlled atmosphere packaging machine (Securepack 10) and were stored at 2°C for 84 days.

At the end of the storage trial, headspace volatiles produced in each inoculated vacuum pack and in the uninoculated control packs (released by introducing ultra pure oxygen-free nitrogen into the packs) were collected onto pre-conditioned Tenax TA (Alltech Associates) traps and analysed, as described previously (Broda *et al.*, 1996b). Briefly, traps carrying volatiles were thermally desorbed, volatile compounds were cryofocused at the head of the FFAP column and then separated on a Fisons 8000 series gas chromatograph fitted with a Fisons MD800 mass spectral detector (Fisons Instruments). Unknown compounds were identified by comparison of their mass spectra with those of known compounds in the National Institute of Standards and Technology library

(Gaithersburg, MD, USA) and quantified against retention times and peak areas of authentic compounds.

The oxygen content of the headspace in each pack was measured with an oxygenmeter (Gaspace Systech Instruments). The carbon dioxide and hydrogen contents were determined on a Varian gas chromatograph using a Porapak Q 80/100-mesh column (Supelco) and a thermal conductivity detector.

Genotypic characterisation

DNA isolation. DNA was isolated as described in Chapter 2.

Determination of DNA base composition. Mol% G+C was determined in DNA from meat strains and in DNA from *Escherichia coli* strain B (51.0 mol% G+C; Sigma) by the method of Mesbah *et al.* (1989). DNA was denatured and digested enzymatically with P1 nuclease (FC 36 ng μl^{-1}) and alkaline phosphatase (FC 0.01 U μl^{-1}). The resulting nucleosides were separated using a reverse-phase HPLC equipped with a 1.5 m x 4.6 mm, 3 µm Econosphere C18 column (Alltech Associates), and fractions were quantified. Mol% G+C was calculated using the mol fractions of deoxyguanosine and thymidine against the *Escherichia coli* standard.

16S rRNA sequence determination and similarity analysis. The 16S rRNA gene sequences of seven meat strains were determined by direct sequencing of the purified PCR-amplified 16S rDNA fragments following the protocol described in Chapter 2. The sequences were aligned using Sequence Navigator (Perkin Elmer) and corrected manually in agreement with the electropherograms and the reading of complementing and overlapping fragments.

To find the nearest relative of the meat strains, the sequences of these strains were compared with sequences of microorganisms in the EMBL and GenBank databases. Sequence analysis was performed on a Silicon Graphics Indigo 2 using the GCG package (Devereux *et al.*, 1984). Sequences of meat strains and the sequences of their

closest relations belonging to the *Clostridium* subphylum were aligned using the program PILEUP (Feng and Doolittle, 1987) with a low gap weighting and low gap extension weightings. When necessary, the alignment was corrected manually to accommodate for the secondary structure of RNA.

Phylogenetic characterisation

To derive the phylogenetic position of strains SPL77A^T and SPL77B, approximately 1350 sites of the aligned sequences were used. A phylogenetic tree was constructed using pairwise analysis and the neighbour joining method (Saitou and Nei, 1987). The stability of relationships was assessed using programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the PHYLIP package (Felsenstein, 1989). At least 1,000 bootstrap trees were generated for each data set.

With the remaining meat strains, phylogenetic analysis was performed with both maximum parsimony (MP) and maximum likelihood (ML) using the program PAUP* (Swofford, 1996). Maximum likelihood based analyses were performed using the HKY85 (Hasegawa *et al.*, 1985) substitution model. The consistency of the obtained groupings was estimated by MP bootstrap analysis of 2000 replicates.

To derive the phylogenetic position of strains DB1A^T and R26, 1408 sites of the aligned sequences were used, and gaps were treated as missing data. A single most parsimonious tree was generated using a heuristic search of 1000 replicates with the random addition of sequences. With maximum likelihood, rates were assumed to be equal and only 10 heuristic replicates were performed because of time limitations with this number of taxa.

With strain SPL73^T, 1395 sites, and with strains K21 and K24, 1344 sites, of the aligned sequences were used for the phylogenetic reconstruction, with gaps treated as missing data. To derive the phylogenetic position of these three strains, maximum parsimony analyses were performed using a branch and bound search, while with maximum likelihood, rates were assumed to follow a gamma distribution and the gamma shape

parameter, and transition to transversion ratios were estimated from the most parsimonious tree. ML bootstrap analysis was performed using the same model with 100 heuristic replicates.

Relatedness of meat strains to their taxonomic relatives

For sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of total soluble cell proteins and for cellular fatty acids (CFA) analysis, meat strains and the reference strains of related clostridia were grown in PYGS broth at their optimum growth temperatures to reach an exponential growth phase. Cells were harvested by centrifugation at 6,000 *g* for 15 min, washed and suspended in sterile ultra-pure water to obtain an optical density of the bacterial suspension of 1.5 at 550 nm.

Soluble cell protein profiles. For SDS-PAGE, a 0.3 to 0.5 ml volume of the bacterial suspension was sonicated on ice with an ultrasound probe (Kontes CV 18, Biolab Scientific) at a maximum output of 90 W. Three 1 min bursts, with 5 min periods of cooling on ice between each burst, were used to disrupt the cells. Sonicated samples were mixed in a proportion 1:1 with an SDS sample buffer containing Tris-HCl, SDS 20 % w/v, 2-mercaptoethanol, bromophenol blue and water. Low- and broad-range protein standards (Bio-Rad) were mixed with the sample SDS buffer in the proportion of 1:20. Samples and standards were denatured by boiling for 5 min. Portions (30 µl) of these denatured samples and 10 µl of the standards were loaded in the wells of a gradient Tris-HCl polyacrylamide gel (Bio-Rad) and subjected to electrophoresis. The 4-15 % gradient gels were used with strains SPL77A^T, SPL77B, DB1A^T and R26, and the 8-16 % gradient gels were used with strains SPL73^T, K21 and K24. SDS-PAGE was performed using the discontinuous buffer system of Laemmli (1970) in a Mini Protean II vertical gel apparatus (Bio-Rad). Electrophoresis was carried out at constant voltage of 150 V for 1 hr at 15°C. The gel was stained with Coomassie blue R-250 (BDH), 0.125 % w/v, dissolved in aqueous methanol, 50 % v/v and aqueous acetic acid, 10 % v/v. The gel was de-stained in a solution containing aqueous methanol, 50 % v/v and aqueous acetic acid, 10 % v/v.

Cellular fatty acids analysis. For CFA analysis, fatty acid methyl esters (FAMEs) were extracted as described by Kuykendall *et al.* (1988). Briefly, 40 mg of wet weight cells of each strain were saponified by heating at 100°C for 30 min with 1 ml of NaOH, 15 % w/v, in aqueous methanol, 50 % v/v. Samples were cooled at ambient temperature, then 1.5 ml of HCl, 25 % v/v, in aqueous methanol, 50 % v/v, was added and the mixture was heated at 100°C for 15 min. FAMEs were extracted with a mixture of hexane and methyl-tert-butyl ether (1:1 v/v).

The C10 to C20 fatty acids were identified and quantified using a Hewlett Packard 6890 gas chromatograph (Hewlett Packard) equipped with a 60 m x 0.25 mm, 0.25 μm BPX 70 column (GBC Scientific), a flame ionisation detector and a Hewlett Packard model 7673 automatic sampler. The gas chromatographic parameters were as follows: average linear velocity, 22 cm sec^{-1}; column head pressure, 210 kPa; split vent flow of helium carrier gas, 44 ml min^{-1}; constant column flow, 5.9 ml min^{-1}; the resultant split ratio, 50:1; the split/splitless injector and FID detector temperature, 250°C. Run conditions were: initial temperature 100°C for 4 min, raised to 130°C at 10°C min^{-1}, then raised to 150°C at 1°C min^{-1}, raised to 200°C at 2.5°C min^{-1}, final temperature of 240°C held for 2 min after raising at a rate of 25°C min^{-1}. Peaks were automatically integrated (Chemstation v.4) and FAMEs were identified and quantified against retention times and peak areas of known standards. Peaks that did not have retention times identical to those of the standards were reported as unknown compounds at their respective equivalent chain length (Christie, 1989).

Results

Where possible, new species are represented by two strains. Where characters are the same for both these strains results obtained for type strain will be used as an example.

Characterisation of meat strains SPL77A^T and SPL77B

Phenotypic characterisation. The colonies of strain SPL77A^T grown on the surface of CBA agar containing 5 % v/v sheep blood were circular to irregular with undulate to lobate margin, creamy grey, raised, low convex, shiny and opaque. The colonies measured from 2.2 to 3.2 mm in diameter. The colonies of strain SPL77B were irregular with an erose margin, grey, flat to raised, shiny, semi-opaque to translucent and measured from 3.3 to 7.2 mm. Both strains produced large zones of β-haemolysis. With both these strains, cells in exponential growth phase were round-ended straight to slightly bent rods 4.5 to 9.4 μm long and 1.3 to 1.6 μm wide (Fig. 4.1a). These rods occurred singly or in pairs.

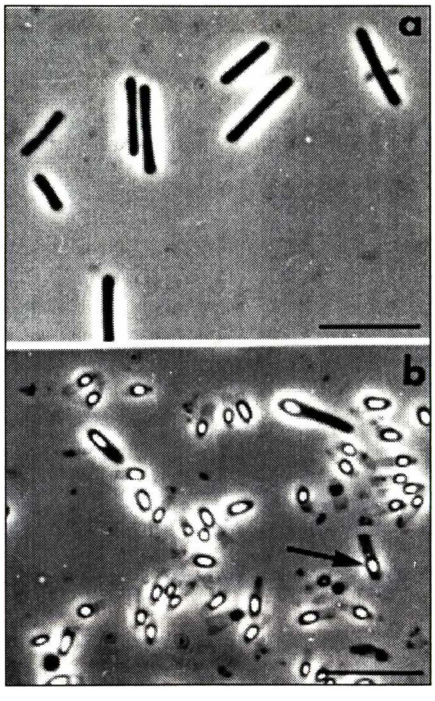

Figure 4.1. Phase-contrast micrographs of vegetative (a) and sporulated (b) cells of strain SPL77A^T. Bar length equals 10 μm for each panel. Subterminal position of spore in the cell is indicated with an arrow.

Vegetative cells of strains SPL77A^T and SPL77B were motile by means of 3 to 7 peritrichous flagella per cell (Fig. 4.2a).

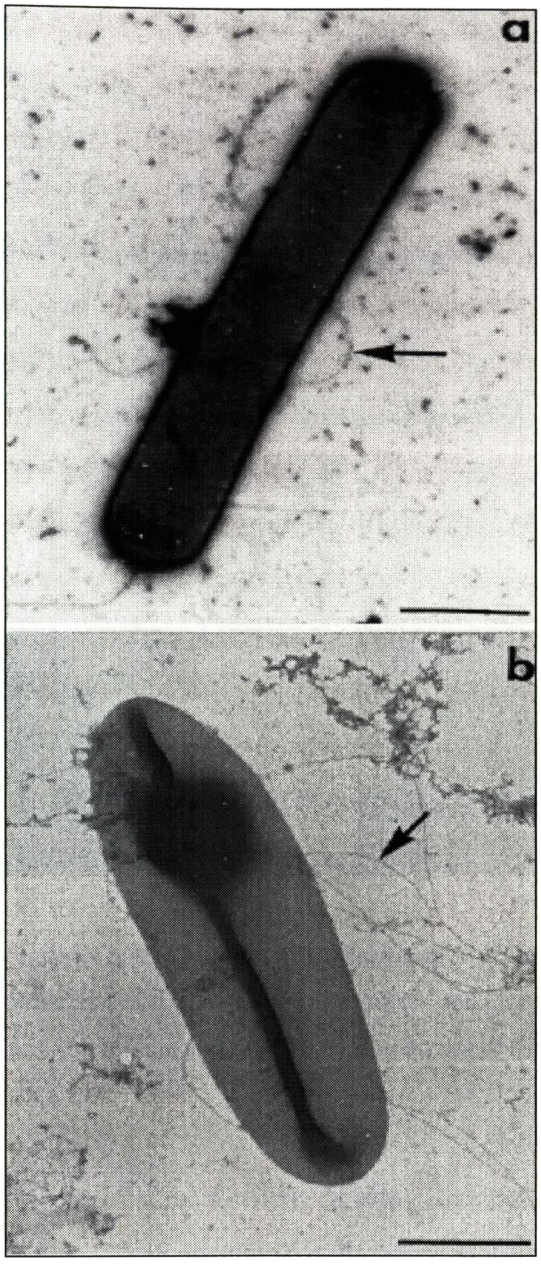

Figure 4.2. Electron micrographs of negatively stained cells of strain SPL77A^T showing peritrichous flagella in both a vegetative (a) and a sporulated (b) cell. Bar length equals 1.5 μm for each panel. A peritrichous flagellum is indicated with an arrow.

Electron micrographs of ultra thin sections from vegetative cells showed the organism was rod-shaped (Fig. 4.3a) and frequently produced excess membranes during cell division (Fig. 4.3b). The Gram positive type, single layer cell wall was observed in ultra thin sections of vegetative cells of both strains (Fig. 4.3c).

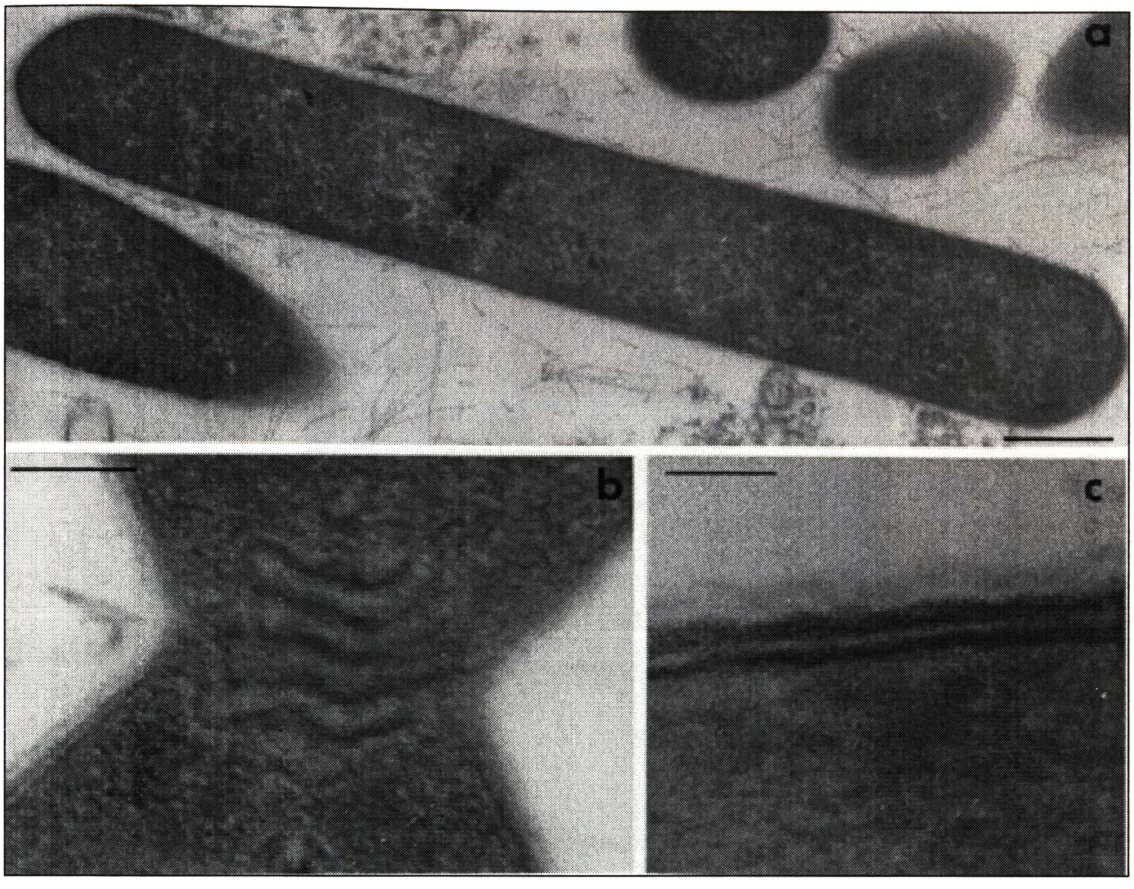

Figure 4.3. Electron micrographs of thin sections of vegetative cells of strain SPL77A^T showing cell shape (a), the excess of membranes at the site of cell division (b) and Gram positive type cell wall (c). Bar length equals (a) 0.5 μm, (b) 150 nm and (c) 50 nm. The excess of membranes at the site of cell division is indicated with an arrow.

Elliptical, subterminal spores were formed, starting during early stationary growth phase (Figs. 4.1b and 4.4a). The sporulated cells were motile and carried peritrichous flagella (Fig. 4.2b). The spores caused swelling of the maternal cells. As observed in electron micrographs, the mature spores had a fully developed endospore structure (Fig. 4.4b).

With both strains SPL77A^T and SPL77B, cells in exponential growth phase stained Gram positive. The KOH reaction was always negative (i.e. characteristic of Gram positive cells) irrespective of growth medium or age of the culture. This Gram positive

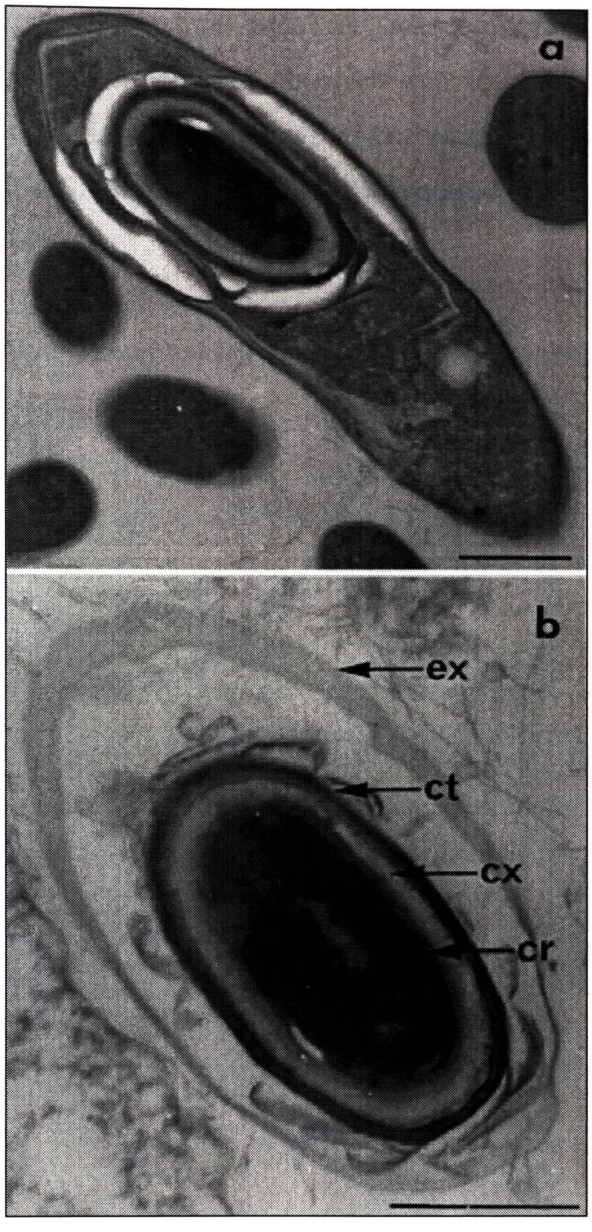

Figure 4.4. Electron micrographs of thin sections of a sporulated cell of strain SPL77A^T (a) and of a mature spore (b). Bar length equals 0.5 μm for each panel. ex, exosporium; ct, spore coat; cx, cortex and cr, spore core.

type was confirmed by single layer cell wall, as observed in electron micrographs of ultra thin sections (Fig. 4.3c).

Strains SPL77A^T and SPL77B grew optimally at 30°C to 38.5°C (Fig. 4.5a). The lowest temperature at which growth of strain SPL77A^T was observed was 3.8°C and the highest temperature at which growth was observed was 40.5°C. The optimal pH for growth of both strains was 6.4 to 7.2 and growth occurred at pH values between 4.7 and 9.5 (Fig. 4.5b). Strains SPL77A^T and SPL77B required anaerobic conditions for growth.

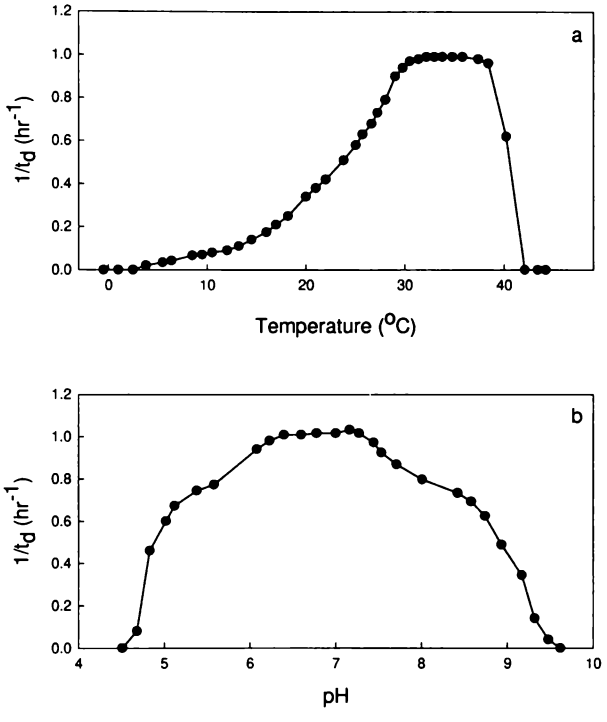

Figure 4.5. Growth of strain SPL77A^T at various incubation temperatures at pH 7.0 (a) and at various pH values at 30°C (b). t_d, doubling time.

The substrates utilised and fermented by strain SPL77A^T in the presence of 0.1 % w/v yeast extract were fructose, glucose, maltose, mannose, sorbitol and trehalose. Cellobiose was utilised but only weakly fermented. Gelatine was hydrolysed, lecithinase activity was present, casein and meat were digested, and ammonia and hydrogen sulphide were produced.

The milk reaction was positive for both digestion and curd formation. Addition of Tween 80 did not stimulate growth. The remaining substrate utilisation tests were negative.

With the Rapid ID 32 A, positive reactions were observed for β-N-acetyl-glucosaminidase and proline arylamidase and weak reactions were observed for arginine arylamidase and pyroglutamic acid arylamidase. Strain SPL77B was positive or negative for the same biochemical tests as strain SPL77A^T, with the exception that meat, casein and milk were only weakly digested and β-N-acetyl-glucosaminidase activity was not present in strain SPL77B. On medium containing 3 % w/v oat spelts xylan, colonies of strain SPL77A^T or SPL77B did not produce clear zones indicative of xylan degradation.

The major fermentation products formed by strain SPL77A^T in PYGS broth were: acetate (43 to 45 mM), ethanol (19 to 20 mM), butyrate (18 to 20 mM), isovalerate (6 to 7 mM), butanol (4 to 5 mM), isobutyrate (2 to 3 mM), oxalacetate (1 to 2 mM), lactate (1 mM), hydrogen and carbon dioxide. The major fermentation products formed by strain SPL77B were ethanol (22 to 26 mM), acetate (23 to 25 mM), butyrate (16 to 17 mM), isovalerate (2 to 3 mM), butanol (1 to 2 mM), isobutyrate (1 mM), oxalacetate (1 mM), lactate (1 mM), hydrogen and carbon dioxide.

The supernatant of strain SPL77A^T was non-toxic to mice.

Genotypic characterisation. The DNA base composition of meat strains was 27.3 mol% G+C for strain SPL77A^T and 28.4 mol% G+C for strain SPL77B.

The 16S rRNA gene sequences for strain SPL77A^T and strain SPL77B consisted of a continuous stretch of 1481 and 1486 nucleotides, respectively. This length of sequence indicates that approximately 96 % of the sequence of the 16S rRNA molecule was determined.

Pairwise alignment of the almost complete gene sequences of the two meat isolates showed them to be homogeneous (100 % 16S rRNA sequence similarity). Sequence searches of GenBank and the Ribosomal Database Project Libraries revealed that the unknown Gram positive rod was most closely associated with the *Clostridium* subphylum (data not shown) and in particular to Cluster I clostridia (*sensu* Collins *et al.*, 1994). Results of the sequence similarity calculations indicate that the nearest relatives of both strains are *C. fallax* (94.5 % sequence similarity), *C. algidicarnis* (94.1 %), *C. intestinale* (93.9 %), *C. cadaveris* (93.6 %) and *C. carnis* (93.5 %).

The sequence of strain SPL77A^T was deposited in the GenBank database under the accession number AF069742.

Phylogenetic characterisation. A tree depicting the phylogenetic affinity of the unknown bacterium within the Cluster I clostridia is shown in Fig. 4.6.

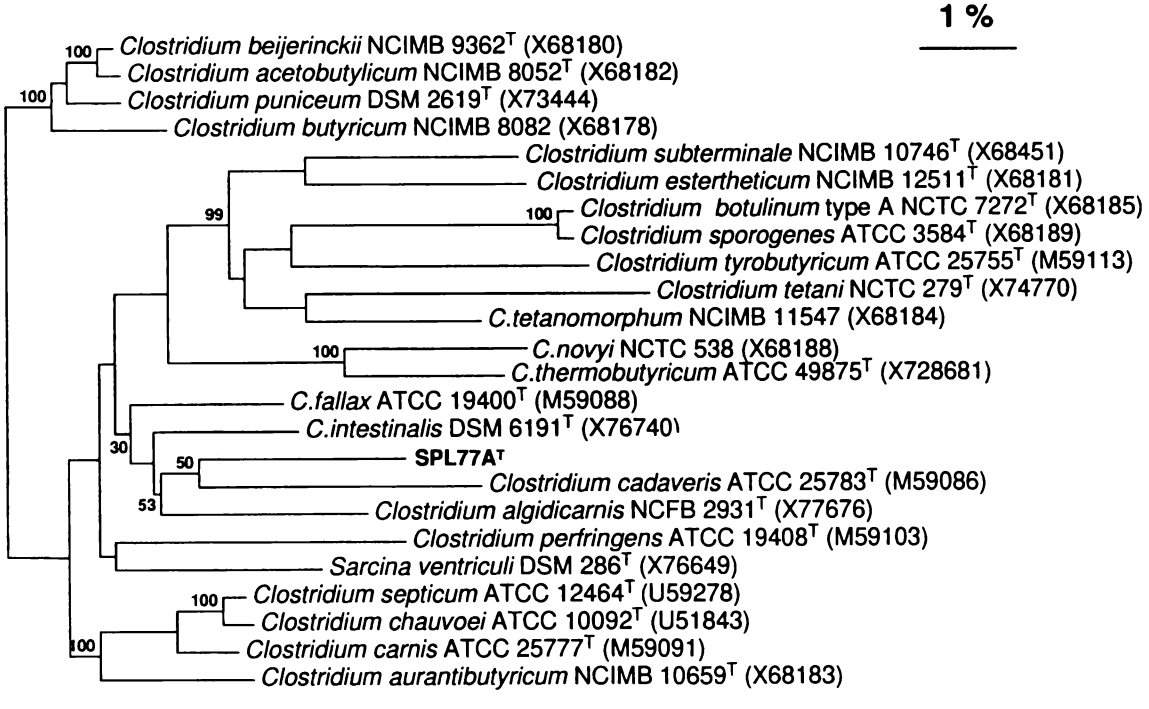

Figure 4.6. Phylogenetic tree showing the relationship of strain SPL77A^T within Cluster I of the genus *Clostridium* (Collins *et al.*, 1994). The scale bar represents 1 % sequence divergence. The values on the branches indicate the level of support derived from bootstrap analyses of 1000 replicates.

Detailed phylogenetic analysis not only confirmed the position of strains SPL77A^T and SPL77B within Cluster I of the genus *Clostridium*, but also the relatively low level of phylogenetic relatedness of both strains to other clostridia within this cluster.

Relatedness of meat strains SPL77A^T and SPL77B to their taxonomic neighbours. Gel electrophoresis of soluble cell proteins demonstrated that strains SPL77A^T and SPL77B had indistinguishable profiles (Fig. 4.7). In the protein profiles of both strains major bands occurred at approximately 130, 45, 40, 19 and 16 kD. However, as shown in Fig. 4.7, there were distinct differences in the protein profiles, as evidenced by changes in the concentration and positions of major and minor protein bands between profiles of these two meat isolates and their phylogenetic relatives *C. fallax* DSM 2631^T, *C. algidicarnis* NCFB 2931^T, *C. intestinale* DSM 6191^T, *C. cadaveris* DSM 1284^T and *C. carnis* DSM 1293^T.

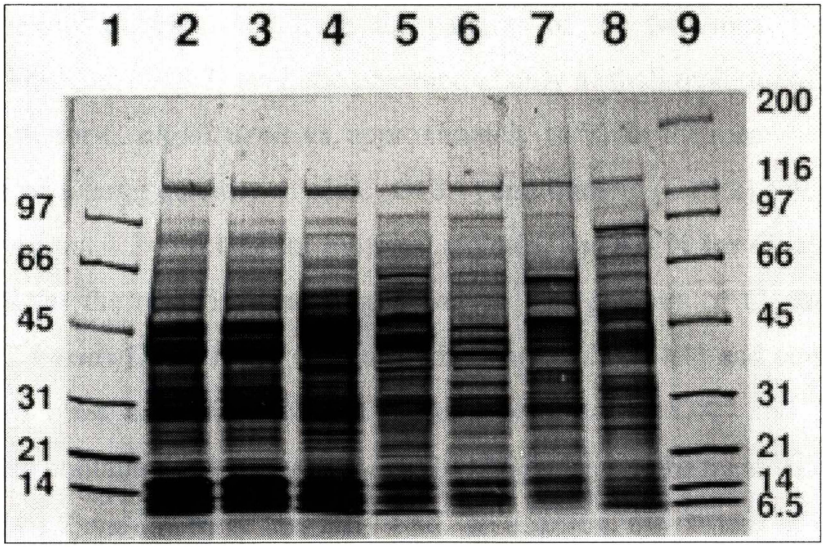

Figure 4.7. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) of soluble cell proteins from strains SPL77A^T and SPL77B and other phylogenetically related clostridia. Lane 1, low range protein standard; lane 2, strain SPL77A^T; lane 3, strain SPL77B; lane 4, *C. fallax* DSM 2631^T; lane 5, *C. algidicarnis* NCFB 2931^T; lane 6, *C. intestinale* DSM 6191^T; lane 7, *C. cadaveris* DSM 1284^T; lane 8, *C. carnis* DSM 1293^T and lane 9, broad range protein standard. The values on the left of lane 1 and on the right of lane 9 indicate the positions of protein standards in kilodaltons.

The major CFA identified in cell extracts of strain SPL77A^T were myristic (C14:0, 14.7 %), palmitic (C16:0, 11.6 %) and oleic (C18:1, 6.1 %) acids. A number of minor fatty acids were also detected (Table 4.2). Strain SPL77B had a CFA pattern very similar to that of strain SPL77A^T. However, some differences were observed in the presence and proportions of minor fatty acids. In comparison to strain SPL77A^T, strain SPL77B lacked *cis*-9-elaidic acid (C18:1), as well as two unknown compounds (equivalent chain length 15.350 and 17.538). The CFA patterns of *C. fallax* DSM 2631^T and *C. intestinale* DSM 6191^T differed from the patterns of strains SPL77A^T and SPL77B by the presence of lauric acid (C12:0) and absence of myristoleic acid (C14:1). In addition, the CFA composition of *C. fallax* DSM 2631^T had almost double the quantity of palmitic acid (C16:0) and nearly ten times the quantity of linoleic acid (C18:2) than for the two meat strains, but only half the amount of myristic acid (C14:0). The CFA pattern of *C. intestinale* DSM 6191^T differed also in the proportion of palmitolic (C16:1) and oleic (C18:1) acids formed. The CFA pattern of *C. algidicarnis* NCFB 2931^T was easily distinguished from the patterns of the two meat isolates by the presence of linolenic (C18:3) acid, the presence of only a small quantity of myristic acid (C14:0) (1.7 % for *C. algidicarnis* vs. approximately 14 % for the meat isolates), and by the presence of a large quantity of oleic (C18:1) and linoleic (C18:2) acids (11.0 % for *C. algidicarnis* vs. 4.2 to 6.1 % for the meat isolates, and 6.4 % for *C. algidicarnis* vs. 1.2 to 1.7 % for the meat isolates, respectively). The patterns of *C. cadaveris* DSM 1284^T and *C. carnis* DSM 1293^T lacked myristoleic acid (C14:1) and contained higher proportion of oleic acid (C18:1) than those of strains SPL77A^T and SPL77B. *C. cadaveris* also contained 3.8 % linoleic acid (C18:2) whereas the meat strains contained only between 1.2 and 1.7 % of this fatty acid. In addition, the pattern of *C. carnis* was distinguished from the pattern of the two meat strains by a lack of myristic acid (C14:0), which formed approximately 14 % of total CFA in the meat isolates, and the presence of heptadecanoic (C17:0) and *cis*-10-heptadecanoic (C17:1) acids, which were absent from the patterns of meat isolates.

Table 4.2. Differences in cellular fatty acid composition of strains SPL77A^T and PL77B, and phylogenetically related clostridia (values are in % of total peak area).

Equivalent chain length	FAME*	SPL77A^T	SPL77B	*C. fallax* DSM 2631^T	*C. algidicarnis* NCFB 2931^T	*C. intestinale* DSM 6191^T	*C. cadaveris* DSM 1284^T	*C. carnis* DSM 1293^T
12.000	12:0	-	-	1.0	-	1.2	-	-
12.206	Unknown	-	-	-	2.6	-	-	-
14.000	14:0	14.7	13.6	6.7	1.7	5.2	12.5	-
14.527	14:1	2.8	2.0	-	2.5	-	-	-
14.716	Unknown	2.0	1.4	-	-	-	-	-
15.350	Unknown	1.4	-	5.1	6.7	7.6	2.4	2.0
15.525	15:1	-	-	1.0	-	1.0	-	-
15.672	Unknown	3.8	2.3	1.1	-	-	-	-
15.868	Unknown	-	-	2.3	3.2	3.7	1.1	-
16.000	16:0	11.6	10.2	21.3	9.2	11.7	13.0	9.5
16.288	Unknown	-	-	2.2	-	2.0	1.6	1.8
16.336	Unknown	1.7	1.8	-	-	-	-	-
16.398	16:1	2.9	3.6	1.3	1.5	5.6	1.4	2.2
17.000	17:0	-	-	-	-	-	-	1.4
17.411	17:1 *cis-10*	-	-	-	-	-	-	1.0
17.538	Unknown	2.8	-	2.2	1.8	3.9	1.5	-
18.000	18:0	3.5	3.0	5.1	1.3	4.7	5.6	4.7
18.207	18:1 *cis-9*	3.0	-	-	-	-	1.0	2.2
18.402	18:1	6.1	4.2	6.5	11.0	8.0	9.6	10.6
18.446	Unknown	-	-	3.1	-	3.1	-	-
19.031	18:2	1.2	1.7	11.2	6.4	1.8	3.8	1.1
19.426	18:3	-	-	-	1.6	-	-	-

*Only fatty acids occurring in concentration over 1 % are listed.

Characterisation of meat strains DB1A^T and R26

Phenotypic characterisation. Colonies of strains DB1A^T and R26 on CBA agar were circular with an entire margin, raised, convex, shiny, smooth and β-haemolytic. The colonies of these meat strains were 0.7 to 3.0 mm in diameter. The colonies of strain DB1A^T were grey-white and opaque with translucent edges; colonies of strain R26 were grey and semi-translucent. With both strains, cells in exponential growth phase were relatively thin, straight rods 2.0 to 7.5 μm long and 0.4 μm wide (Fig. 4.8a). These rods occurred singly or in pairs.

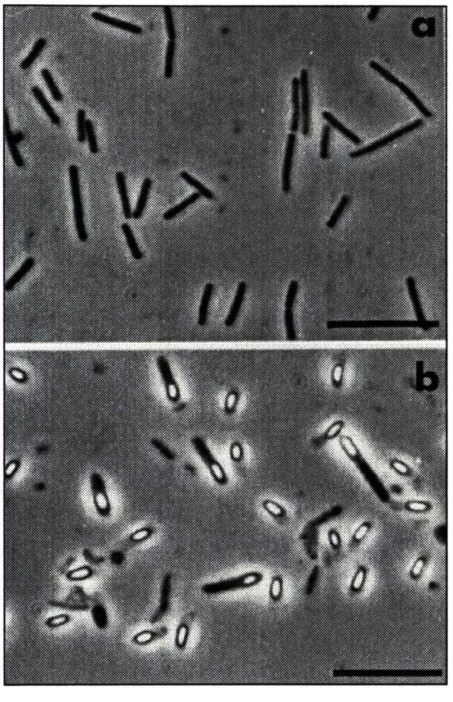

Figure 4.8. Phase-contrast micrographs of vegetative (a) and sporulated (b) cells of strain DB1A^T. Bar length equals 10 μm for each panel.

Vegetative cells of strains DB1A^T and R26 were motile by means of peritrichous flagella (Fig. 4.9).

Electron micrographs of ultra thin sections of vegetative cells of strains DB1A^T and R26 showed the organisms were round-ended rods (Fig. 4.10a), and that cell division may involve both a septation and a pinching mechanism (Fig. 4.11a and b).

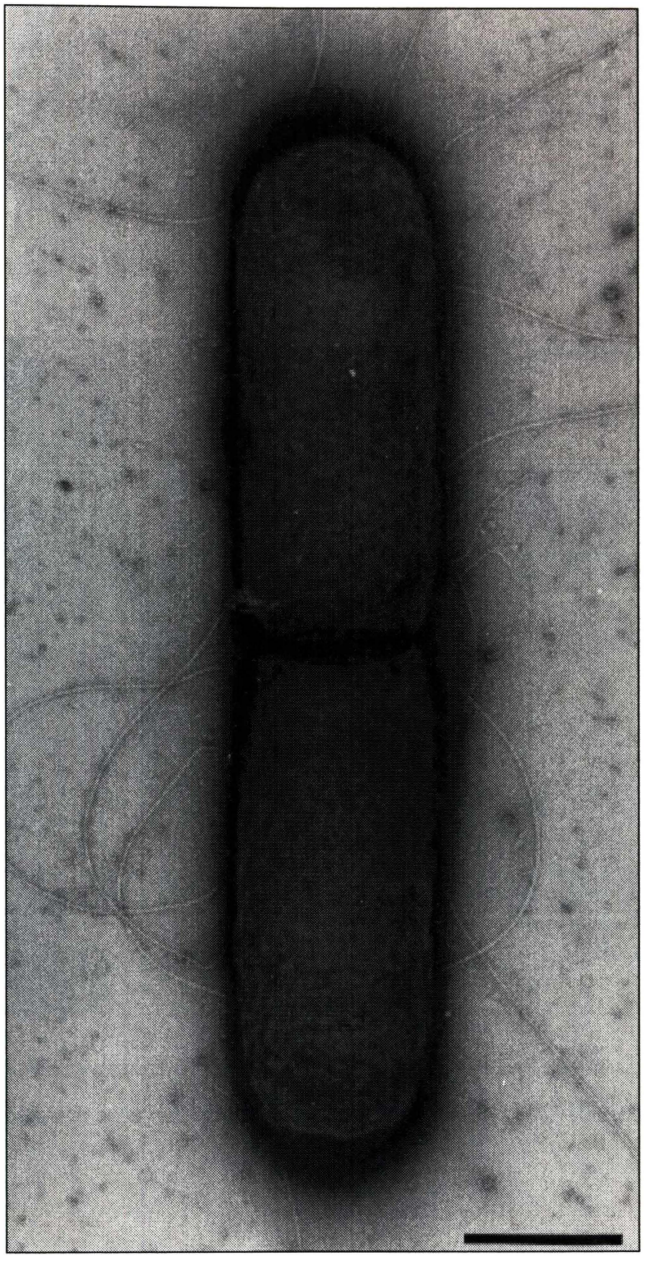

Figure 4.9. Electron micrographs of a negatively stained cell of strain DB1A^T showing peritrichous flagella. Bar length equals 0.3 μm.

A Gram positive type, single layer cell wall was observed in ultra thin sections of vegetative cells of both strains (Fig. 4.10b).

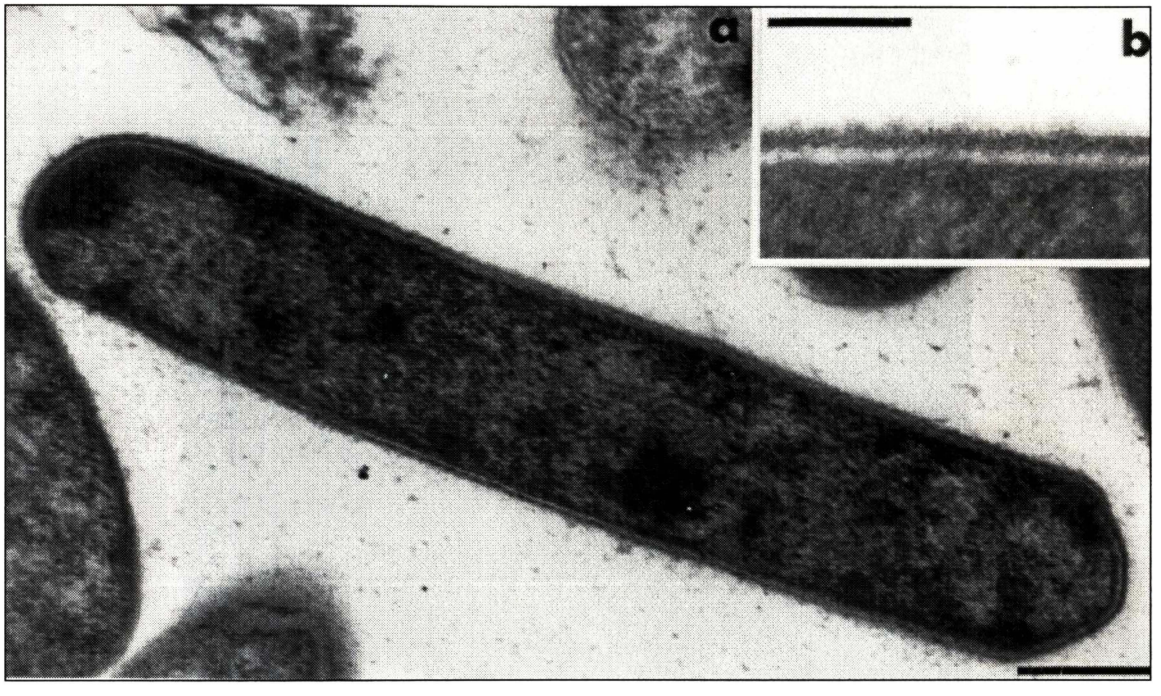

Figure 4.10. Electron micrographs of thin sections of vegetative cells of strain DB1A^T showing cell shape (a) and Gram positive type cell wall (b). Bar length equals (a) 0.3 μm and (b) 0.1 μm.

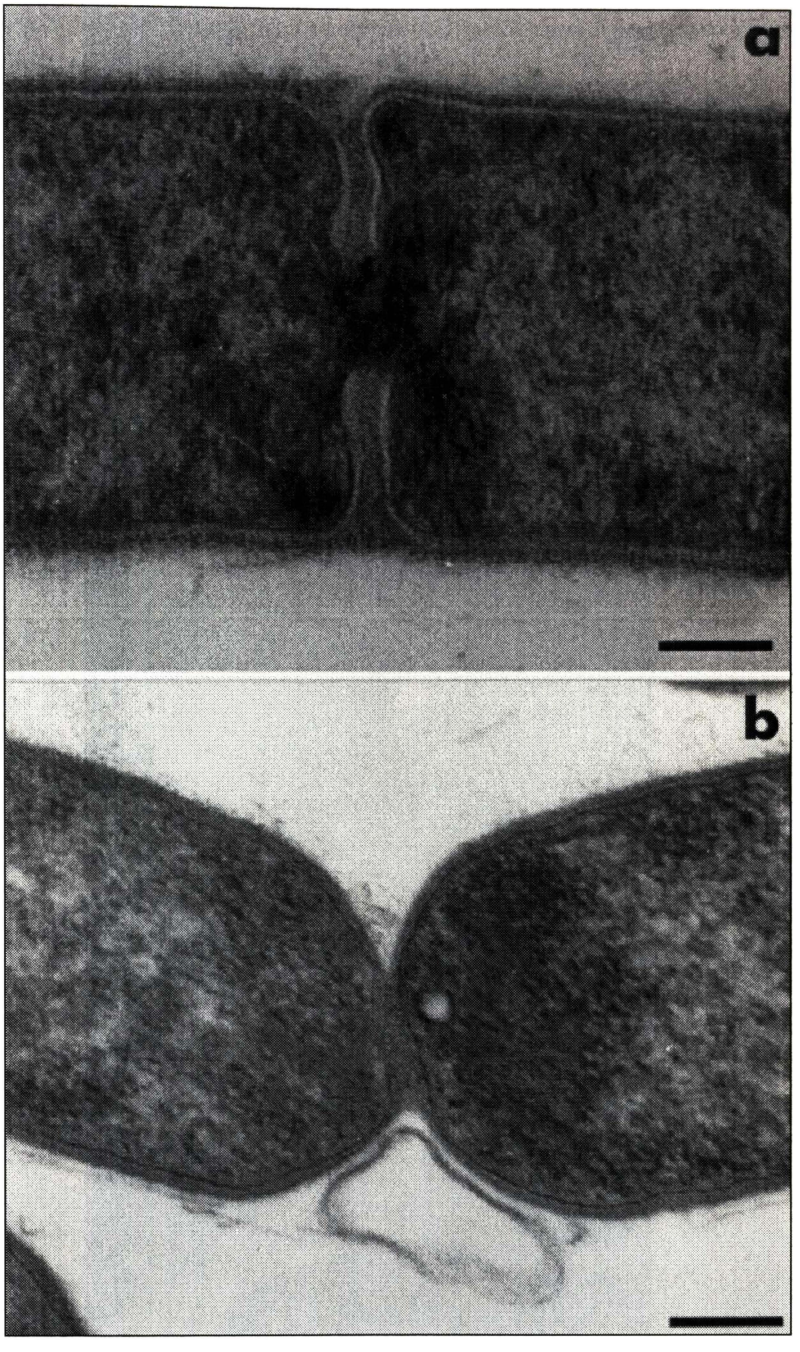

Figure 4.11. Electron micrographs of thin sections of a dividing cell of strain DB1A^T indicating a septation (a) or pinching (b) mechanism of cell division. Bar length equals 0.1 μm for each panel.

Elliptical, subterminal spores were formed in late stationary growth phase (Figs. 4.8b and 4.12a). The spores caused slight swelling of the maternal cells. The mature spores had a fully developed endospore structure (Fig. 4.12b).

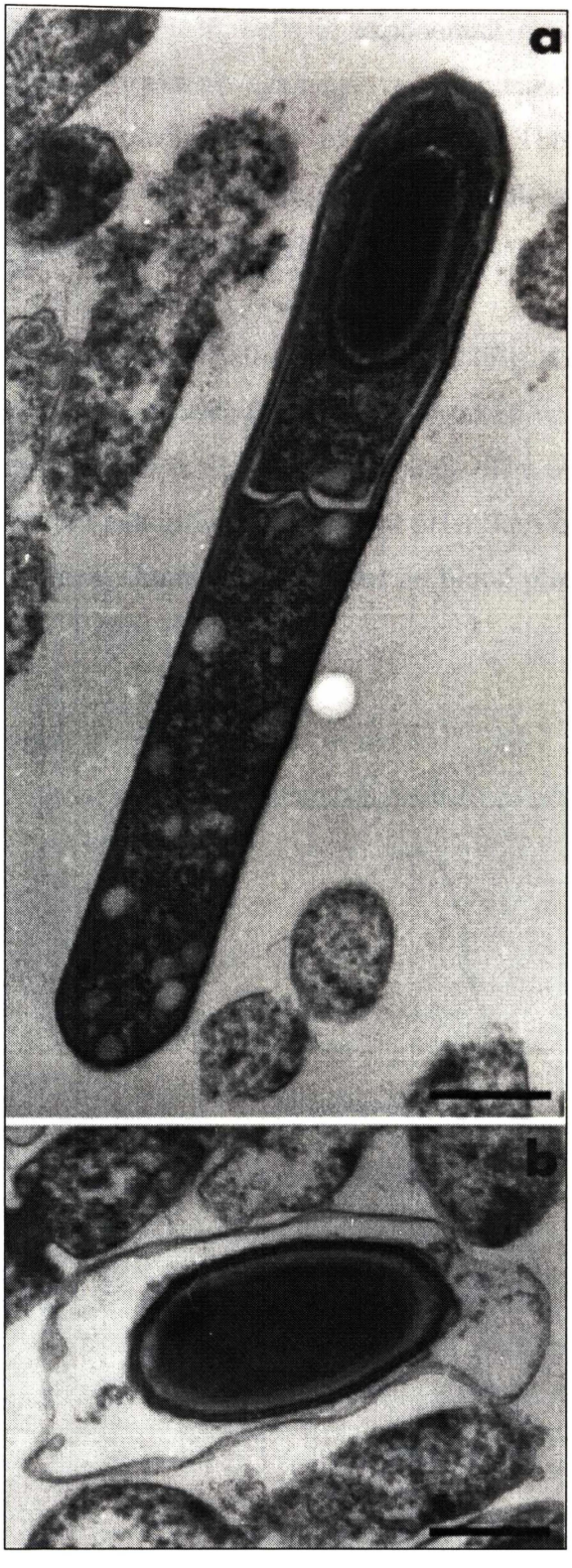

Figure 4.12. Electron micrographs of thin sections of a sporulated cell of strain DB1A^T (a) and of a mature spore (b). Bar length equals 0.5 μm for each panel.

With both strains DB1A^T and R26, cells in exponential growth phase stained Gram positive. The KOH reaction was always negative (i.e. characteristic of Gram positive cells) irrespective of growth medium or age of the culture. This Gram positive type was confirmed by the presence of a single layer cell wall, as observed in electron micrographs of ultra thin sections (Fig. 4.10b).

Strains DB1A^T and R26 grew optimally at 20 to 22°C (Fig. 4.13a). The lowest tested temperature at which growth of strain DB1A^T was observed was –1.5°C and the highest temperature at which growth was observed was 26°C. The optimal pH for growth of both strains was 6.2 to 8.6 and growth occurred at pH values between 5.4 and 8.9 (Fig. 4.13b). No growth of meat strains was observed on blood plates incubated aerobically at 20°C for 14 days.

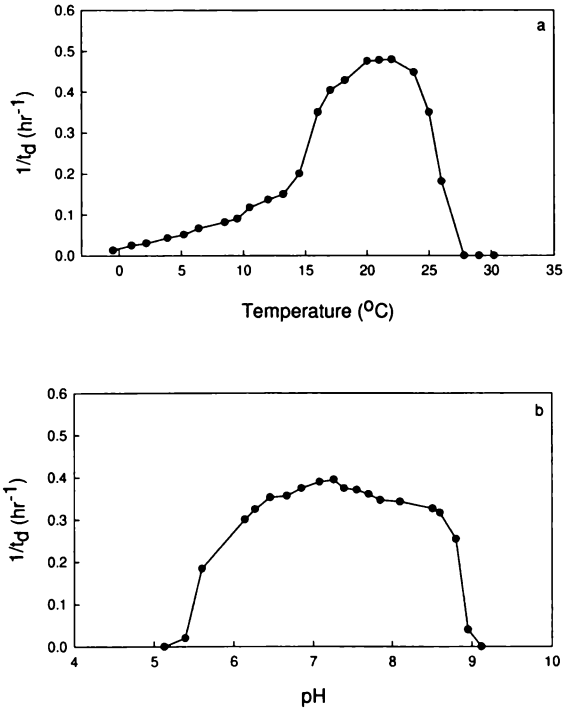

Figure 4.13. Growth of strain DB1A^T at various incubation temperatures at pH 7.0 (a) and at various pH values at 20°C (b). t_d, doubling time.

Very little or no growth of either meat strain occurred in the PY broth containing 0.1 % w/v yeast extract in the absence of fermentable carbohydrate. The substrates fermented by strain DB1A^T in this broth were: cellobiose, fructose, glucose, inositol, maltose, mannose, salicin and trehalose. The substrates not fermented were: adonitol, arabinose, dextran, galactose, inulin, lactose, mannitol, rhamnose, raffinose, sorbitol, sorbose, sucrose and xylose. Gelatine, starch and esculin were hydrolysed. The milk reaction was positive for curd formation. Addition of Tween 80 did not stimulate growth. The remaining tests were negative. Strain R26 was positive or negative for the same biochemical tests as strain DB1A^T, with the exception that sucrose was fermented, galactose was weakly fermented and inositol was not fermented. On medium containing 3 % oat spelts xylan, colonies of strain DB1A^T or R26 did not produce clear zones indicative of xylan degradation.

The major fermentation products formed by strains DB1A^T and R26 in PYGS broth were: ethanol (26.1 and 20.9 mM, respectively), acetate (8.9 mM for each strain), butyrate (6.5 and 6.6 mM, respectively), lactate (5.1 and 5.6 mM, respectively), butanol (1.2 and 0.3 mM, respectively), carbon dioxide and hydrogen.

The supernatant of strain DB1A^T was non-toxic to mice.

Strains DB1A^T and R26 produced a headspace gas of similar composition (Table 4.3). The major gases produced were carbon dioxide and hydrogen, and the major volatiles were 1-butanol, butyl butyrate and butyric acid. There were differences, however, in relative proportions of some volatiles produced by the two strains. The headspace volatiles of packs inoculated with strain DB1A^T contained almost twice the concentration of butanol and three times the concentration of butyl butyrate compared with packs inoculated with strain R26. Acetic acid and traces of butanol were the only volatiles detected in the artificially created headspace of uninoculated control packs.

Table 4.3. Concentrations of some major headspace volatile compounds in vacuum-packed meat inoculated with pure cultures of strains DB1A^T and R26, and *C. estertheticum*, compared with uninoculated control packs, after storage for 84 days at 2°C.

Compound*	DB1A^T‡	R26‡	*C. estertheticum*†	Uninoculated control‡
Acetic acid	0.4	0.3	0.5	0.8
Butyric acid	1.8	2.5	2.5	-
1-Butanol	224	123	38	t
1-Pentanol	0.3	0.2	t	-
3-Methylbutanal	2.1	4.0	t	-
1-Butyl acetate	0.4	0.4	4.5	-
1-Butyl butyrate	4.4	1.4	20.5	-
Carbon dioxide (%)§	68	69	68.5	-
Hydrogen (%)§	30	30	28.5	-

*, concentrations in ng ml^{-1} of gas, unless otherwise stated; †, data from Dainty *et al.* (1989), means of duplicate tests; ‡, with packs inoculated with pure cultures of strains DB1A^T and R26, and with uninoculated control packs, 1-hexanol, 1-octen-3-ol, hexanal, benzaldehyde, ethyl butyrate, 1-butyl formate, 1-butyl propionate and oxygen were not detected, and the presence of nitrogen was not determined; these compounds, however, were present in vacuum packs inoculated with the pure culture of *C. estertheticum* (Dainty *et al.*, 1989); §, ± 1 %; t, trace; -, none detected.

Genotypic characterisation. DNA base composition of DNA was 29.4 mol% G+C for strain DB1A^T, 28.3 mol% G+C for strain R26.

Similarity analysis of the almost complete 16S rRNA gene sequences of the two meat isolates showed them to be nearly identical. Pairwise comparison of the sequences from strains DB1A^T and R26 demonstrated three mismatches within the 1483 nucleotides used in the comparison, corresponding to a 99.8 % 16S rRNA gene sequence similarity between the two meat isolates. Sequence searches of GenBank database revealed that the unknown Gram positive rod was most closely associated with the *Clostridium* subphylum (data not shown) and in particular to Cluster I clostridia (*sensu* Collins *et al.*, 1994). Results of the sequence similarity calculations indicate that the nearest relatives of both strains are *C. vincentii* (95.5 % sequence similarity), *C. septicum* (95.4 %), *C.*

chauvoei (95.2 %), non-proteolytic *C. botulinum* types B and E (95.0 %) and *C. carnis* (94.7 %).

The sequences of strains DB1A^T and R26 were deposited in the GenBank database under the accession numbers AF092548 and AF143692, respectively.

Phylogenetic characterisation. Phylogenetic analysis confirmed that the unknown Gram positive rod was most closely associated with the *Clostridium* subphylum and Cluster I clostridia (*sensu* Collins *et al.*, 1994). Within Cluster I, strains DB1A^T and R26 were placed in the monophyletic unit containing sub-lines represented by *C. carnis*, *C. chauvoei, C. septicum* and *C. quinii* (Fig. 4.14). Within this unit, however, strain DB1A^T branched deeply, indicating a relatively low level of phylogenetic relatedness to other clostridia within this phylogenetic clade.

Relatedness of meat strains DB1A^T and R26 to their taxonomic neighbours. Gel electrophoresis of soluble cell proteins demonstrated that strains DB1A^T and R26 had similar profiles of major protein bands. In the protein profiles, the major bands for these strains occurred at approximately 120, 46-38, 35, 33-27 and 16 kD. However, as shown in Fig. 4.15, strain R26 produced additional minor bands at approximately 34 and 47 kD, whereas these bands were not detected in the pattern of strain DB1A^T. There were distinct differences in the concentrations and positions of major and minor protein bands, between profiles of these two meat isolates and those of their phylogenetic relatives *C. vincentii* DSM 10228^T, *C. septicum* NZRM 18^T, *C. chauvoei* NZRM 99^T, non-proteolytic *C. botulinum* types B (17B) and E (Beluga), and *C. carnis* DSM 1293^T.

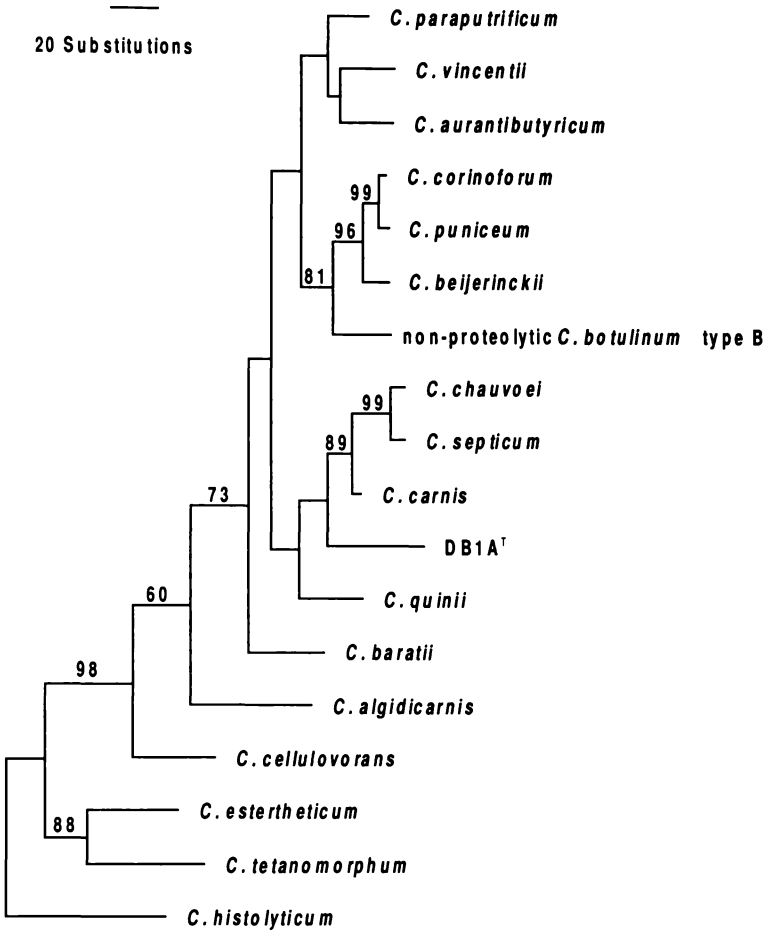

Figure 4.14. Most parsimonious tree showing position of strain DB1A^T within Cluster I of the genus *Clostridium* (Collins *et al.*, 1994). The tree was inferred from aligned 16S rRNA gene sequences using a heuristic search under the criterion of maximum parsimony. The values on the branches indicate the level of support derived from bootstrap analyses of 2000 replicates. Where no number is shown, bootstrap values are less than 50 %. The following reference strains were used in phylogenetic analyses: *C. algidicarnis* NCFB 2931^T (X77676), *C. aurantibutyricum* NCIMB 10659^T (X68183), *C. baratii* ATCC 43756 (X68175), *C. beijerinckii* DSM 791^T (X68179), non-proteolytic *C. botulinum* type B (17B) ATCC 25765 (X68173), *C. carnis* ATCC 25777^T (M59091), *C. cellulovorans* DSM 3052^T (X73438), *C. chauvoei* ATCC 10092^T (U51843), *C. corinoforum* DSM 5906 (X76742), *C. estertheticum* NCIMB 12511^T (X68181), *C. histolyticum* ATCC 19401^T (M59094), *C. paraputrificum* DSM 2630^T (X73445), *C. puniceum* DSM 2619^T (X71857), *C. quinii* DSM 6736^T (X57262), *C. septicum* ATCC 12464^T (U59278), *C. tetanomorphum* NCIMB 11547 (X68184) and *C. vincentii* DSM 10228^T (X97432). The sequence of strain R26 was excluded from the analysis because of its close similarity to strain DB1A^T. The *Clostridium* Cluster II species *C. histolyticum* was used as an outgroup.

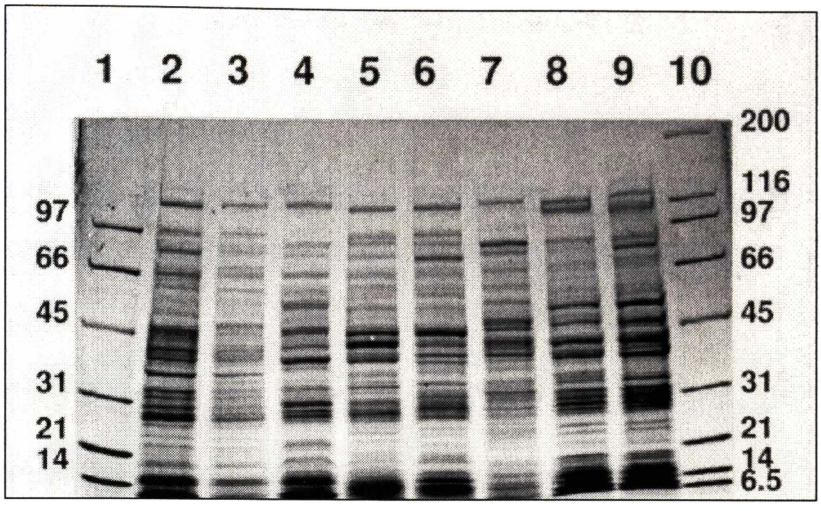

Figure 4.15. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) of soluble cell proteins from strains DB1A^T and R26 and their nearest relatives. Lane 1, low range protein standard; lane 2, strain DB1A^T; lane 3, strain R26; lane 4, *C. vincentii* DSM 10228^T; lane 5, *C. septicum* NZRM 18^T; lane 6, *C. chauvoei* NZRM 99^T; lane 7, *C. carnis* DSM 1293^T; lane 8, non-proteolytic *C. botulinum* type B 17B; lane 9, non-proteolytic *C. botulinum* type E (Beluga) and lane 10, broad range protein standard. The values on the left of lane 1 and on the right of lane 9 indicate the positions of protein standards in kilodaltons.

The major CFA identified in cell extracts of both meat strains were palmitic, oleic, myristic and palmitoleic acids (Table 4.4). The CFA pattern of *C. vincentii* DSM 10228^T was easily distinguished from the patterns of the two meat isolates by the absence of lauric acid (C12:0) and the presence of only a small quantity of myristic (C14:0) and oleic (C18:1) acids. The CFA patterns of *C. septicum* NZRM 18^T and *C. chauvoei* NZRM 99^T differed from the patterns of strains DB1A^T and R26 by the absence of lauric acid. In addition, the CFA composition of *C. septicum* NZRM 18^T had only 1.3 % of myristic acid, while the two meat strains had 5.6 and 6.7 % of this acid. The patterns of *C. botulinum* type B (17B) and *C. botulinum* type E (Beluga) contained pentadecanoic acid (C15:1) and lower proportions of palmitoleic (C16:1), oleic (C18:1) and linoleic (C18:2) acids than those of strains DB1A^T and R26. The pattern of *C. carnis* DSM 1293^T was distinguished from the patterns of the two meat strains by lack of lauric and myristic acids, which were present in the patterns of meat isolates; and the presence of heptadecanoic (C17:0) and *cis*-10-heptadecanoic (C17:1) acids, which were absent from the patterns of meat isolates.

Table 4.4. Differences in cellular fatty acid composition of strains DB1A^T and R26, and phylogenetically related clostridia (values are in % of total peak area).

Equivalent chain length	FAME*	DB1A^T	R26	*C. vincentii* DSM 10228^T	*C. septicum* NZRM 18^T	*C. chauvoei* NZRM 99^T	*C. botulinum* type B (17B)	*C. botulinum* type E (Beluga)	*C. carnis* DSM 1293^T
12.000	12:0	1.0	1.2	-	-	-	-	1.0	-
14.000	14:0	5.6	6.7	2.3	1.3	5.1	5.7	7.5	-
15.525	15:1	-	-	-	-	-	1.2	1.5	-
15.672	Unknown	-	-	2.1	-	-	1.2	1.6	-
16.000	16:0	8.8	9.7	9.1	11.7	10.9	5.8	8.3	9.5
16.288	Unknown	3.5	4.0	2.8	-	-	-	1.6	-
16.398	16:1	5.5	6.0	3.1	-	0.8	1.2	1.0	2.2
17.000	17:0	-	-	-	-	-	-	-	1.4
17.411	17:1 *cis-10*	-	-	-	-	-	-	-	1.0
17.538	Unknown	-	-	3.8	-	-	-	2.0	-
18.000	18:0	3.2	3.4	1.8	8.8	5.9	2.9	3.1	4.7
18.207	18:1 *cis-9*	1.8	2.2	1.5	1.2	1.0	1.9	2.7	2.2
18.402	18:1	7.7	9.1	3.4	10.8	11.6	3.3	2.6	10.6
18.410	Unknown	-	-	2.1	1.0	-	-	-	-
18.446	Unknown	2.5	2.6	1.7	-	-	-	-	-
19.031	18:2	2.4	2.9	2.2	3.1	2.4	1.0	1.0	1.1

*Only C10 to C20 fatty acids occurring in concentrations over 1 % are listed. Concentrations of individual fatty acids are expressed as % of total peak area that might have included peaks of straight chain and/or branched fatty acids with an effective chain length of less than 10, as well as some unknown compounds. -, not detected or less than 1 %.

Characterisation of meat strain SPL73^T

Phenotypic characterisation. The colonies of strain SPL73^T grown on the surface of CBA containing 5 % v/v sheep blood were circular, 0.8 to 2.5 mm in diameter, entire margin, grey-white, translucent, raised, convex, shiny and β-haemolytic. Cells in the exponential growth phase occurred singly as tapered straight to slightly bent rods, 1.8 to 2.8 μm long and 0.5 to 0.8 μm wide. Both non-sporulated and sporulated cells were motile by means of peritrichous flagella (Figs. 4.16a and b).

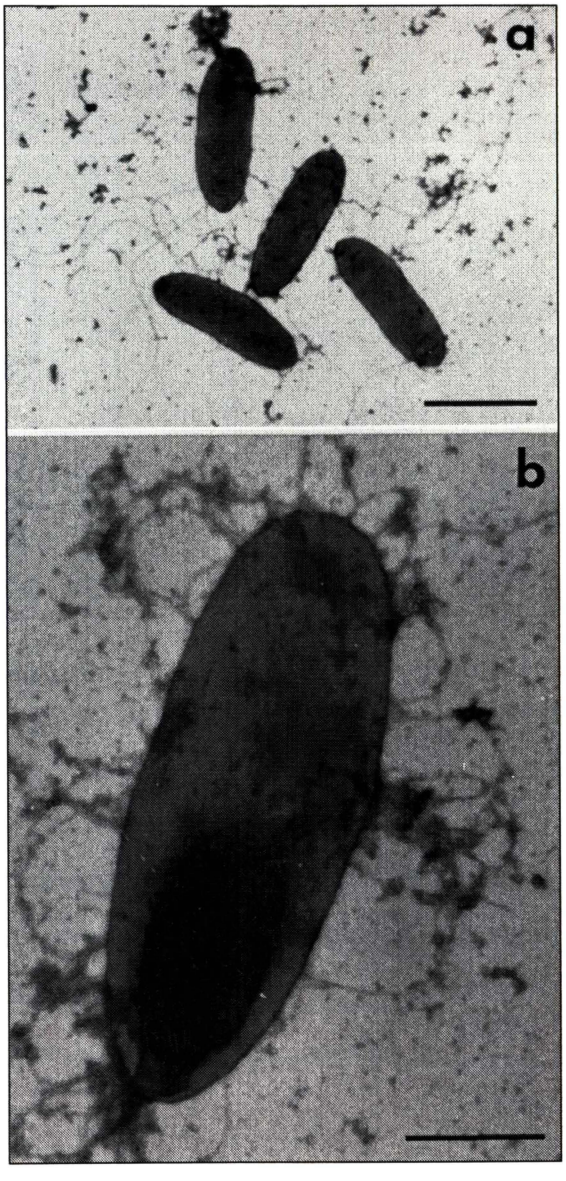

Figure 4.16. Electron micrographs of negatively stained cells of strain SPL73^T showing peritrichous flagella in both a vegetative (a) and a sporulated (b) cell. Bar length equals (a) 2 μm, (b) 0.5 μm.

Electron micrographs of ultra thin sections of vegetative cells confirmed that the organism was rod-shaped (Fig. 4.17a). The multi layer cell wall was observed in ultra thin sections of vegetative cells of the strain (Fig. 4.17b). Conventional Gram staining and the KOH reaction were indicative of Gram negative cell wall type.

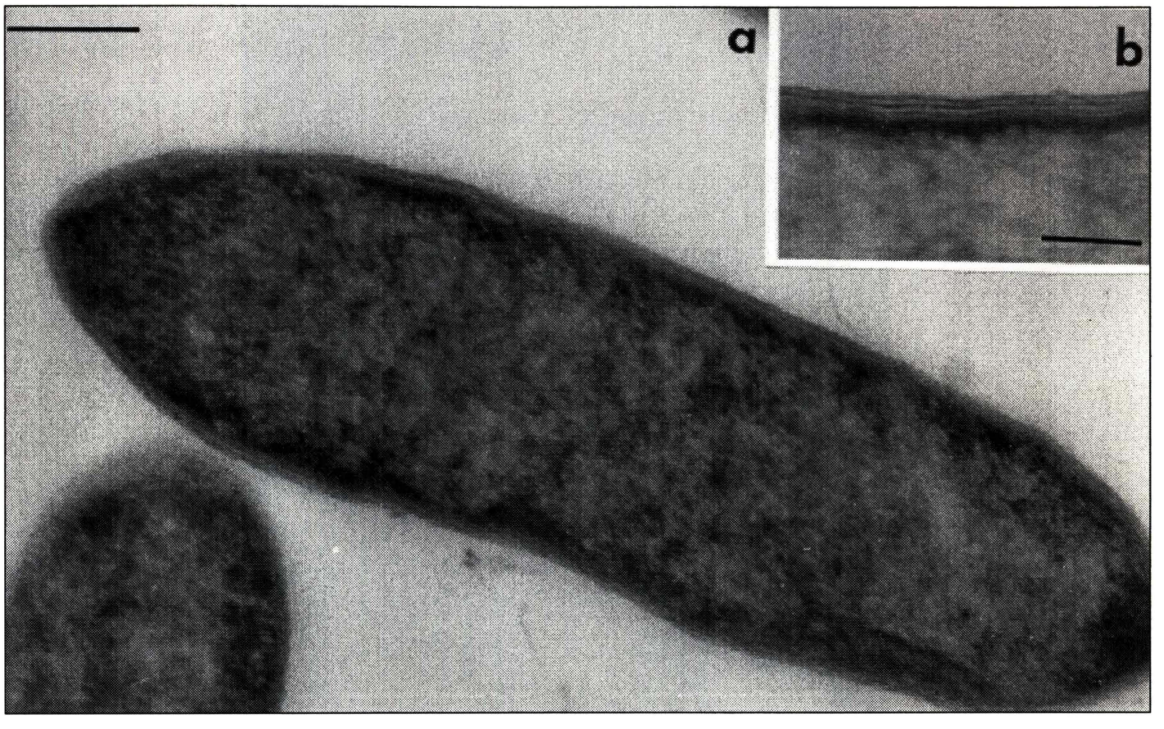

Figure 4.17. Electron micrographs of ultra thin sections of vegetative cells of strain SPL73^T showing cell shape (a) and multi layer cell wall (b). Bar length equals (a) 0.2 μm, (b) 0.1 μm.

During late stationary growth phase, elliptical, subterminal spores were formed (Fig. 4.18a). The mature spores had a fully developed endospore structure (Fig. 4.18b). Spores did not swell the maternal cells.

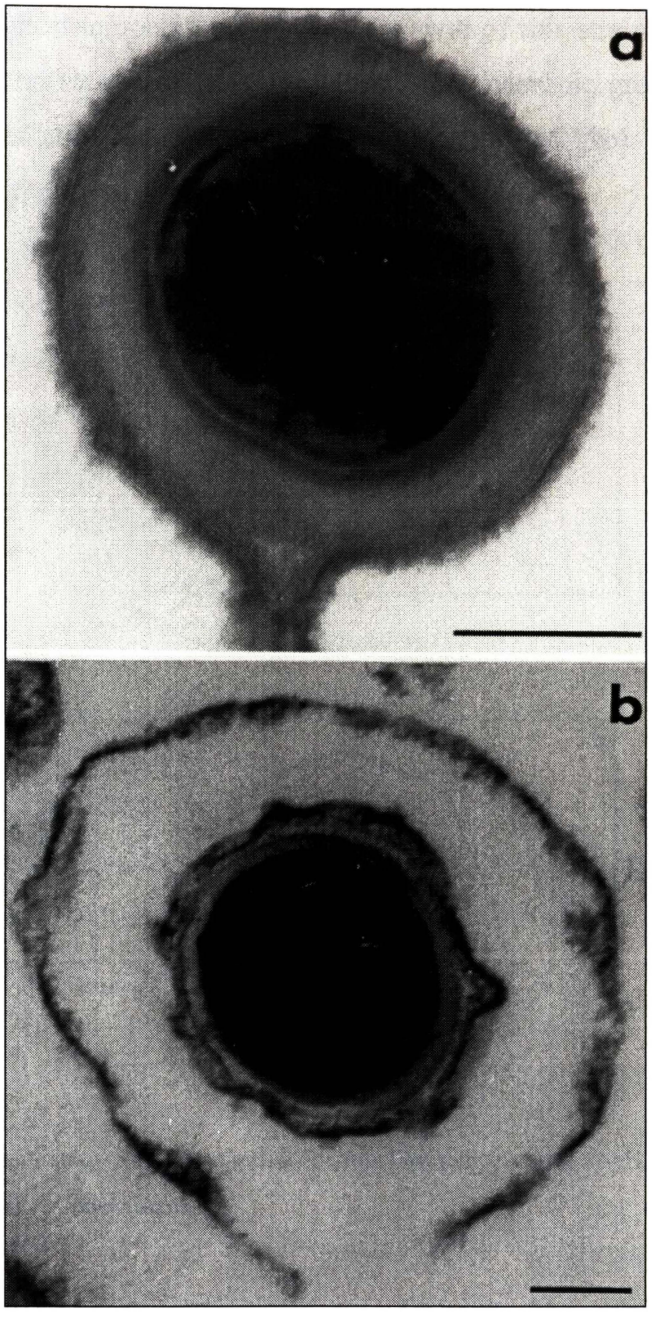

Figure 4.18. Electron micrographs of ultra thin sections of a sporulated cell of strain SPL73^T (a) and of a mature spore (b). Bar length equals 0.3 μm for each panel.

No growth of strain SPL73^T occurred on aerobic CBA containing 5 % v/v sheep blood. Strain SPL73^T grew optimally anaerobically between 25.5°C and 30.0°C (Fig. 4.19a). The lowest and highest temperatures at which growth of this strain was observed were 2.5°C and 32.2°C, respectively. The optimal pH for anaerobic growth was 6.8 to 7.0, and growth occurred at pH values between 4.7 and 9.1 (Fig. 4.19b).

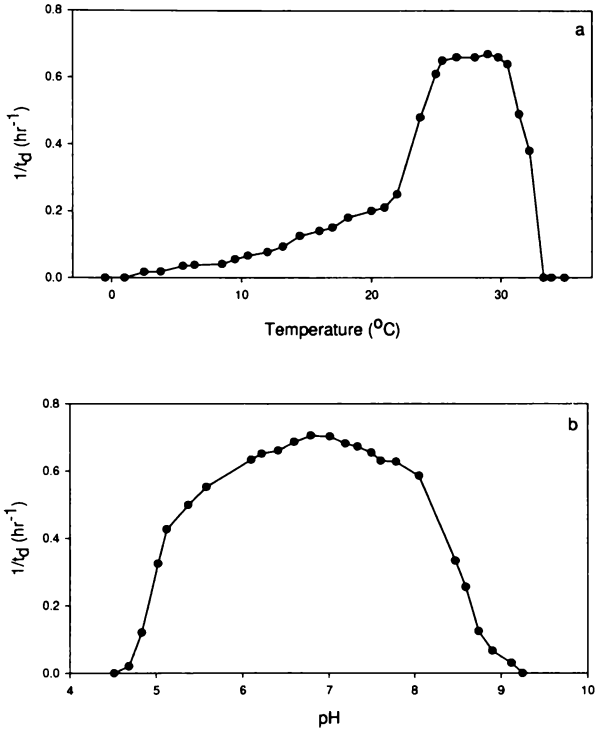

Figure 4.19. Growth of strain SPL73^T at various incubation temperatures at pH 7.0 (a) and at various pH values at 26°C (b). t_d, doubling time.

Strain SPL73^T was saccharoclastic, but not proteolytic. The substrates fermented by strain SPL73^T in PY broth were arabinose, cellobiose, fructose, galactose, glucose, inulin, lactose, maltose, mannose, rhamnose, raffinose, salicin, sucrose and xylose. The substrates not fermented were adonitol, dextran, inositol, mannitol, sorbitol, sorbose and trehalose. Starch was hydrolysed and hydrogen sulphide was produced. Gelatine and esculin were not hydrolysed, lecithinase and lipase activities were absent, meat and

casein were not digested and the milk reaction was negative. Neither indole nor ammonia was produced, nitrate was not reduced, oxidase and catalase activity was absent and urea was not hydrolysed. No growth was observed in the presence of bile. Addition of Tween 80 did not stimulate growth. The major fermentation products formed by strain SPL73^T in PYGS broth were acetate, formate, lactate, ethanol, butyrate, butanol, hydrogen and carbon dioxide.

On medium containing 3 % oat spelts xylan, colonies of strain SPL73^T were surrounded by clear zones indicative of xylan degradation. At 26°C xylanase activity of this strain was 0.110 U ml^{-1} at pH 7.0 and 0.073 U ml^{-1} at pH 5.6.

The supernatant of the broth culture of strain SPL73^T was not toxic to mice.

Genotypic characterisation. The DNA base composition for strain SPL73^T was 38.4 mol% G+C.

The 16S rRNA gene sequence of strain SPL73^T containing a continuous stretch of 1502 nucleotides was used to search GenBank and the Ribosomal Database Project Libraries. Sequence searches showed that strain SPL73^T was most closely associated with the *Clostridium* subphylum (data not shown). Results of the sequence similarity calculations indicated that the nearest sequence similarity relatives of strain SPL73^T are *C. aerotolerans* (97.7 %), *C. xylanolyticum* (97.6 %), *C. celerecrescens* (97.0 %) and *C. sphenoides* (96.3 %).

The sequence of strain SPL73^T was deposited in the GenBank database under the accession number AF092549.

Phylogenetic characterisation. Detailed phylogenetic analyses placed strain SPL73^T within Cluster XIVa of the genus *Clostridium* (*sensu* Collins *et al.*, 1994). In this cluster strain SPL73^T was located within a unit composed of *C. xylanolyticum* and *C. aerotolerans* (Fig. 4.20).

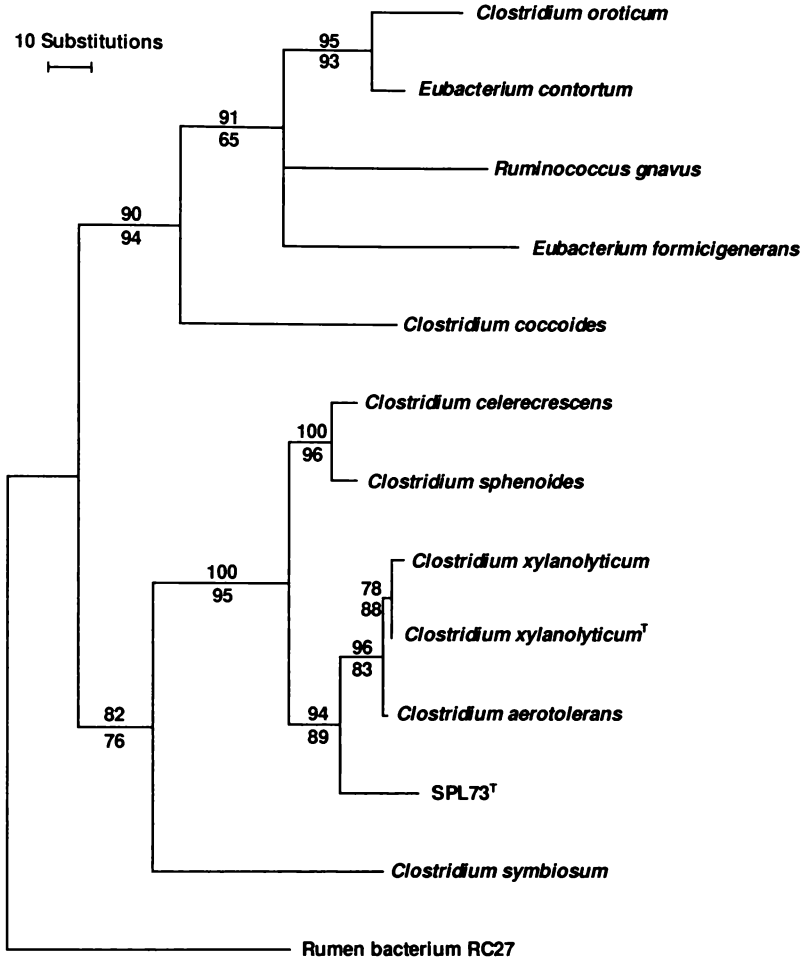

Figure 4.20. Consensus maximum parsimony and maximum likelihood tree generated from aligned 16S rRNA gene sequences, showing position of strain SPL73^T within Cluster XIVa of the genus *Clostridium* (Collins *et al.*, 1994). The values above branches indicate the level of support derived from maximum parsimony bootstrap analyses of 2000 replicates. The values below branches indicate the level of support derived from maximum likelihood bootstrap analyses of 100 replicates. The following reference strains were used in phylogenetic analyses: *C. aerotolerans* DSM 5434^T (X76163), *C. celerecrescens* DSM 5628^T (X71848), *C. coccoides* DSM 2088 (M59090), *C. oroticum* ATCC 13619^T (M59109), *C. sphenoides* DSM 632^T (X73449), *C. symbiosum* ATCC 14940^T (M59112), *C. xylanolyticum* DSM 6555^T (X71855), *C. xylanolyticum* ATCC 4963 (X76739), *Eubacterium contortum* ATCC 25540^T (L34615), *Eubacterium formicigenerans* ATCC 27755^T (L34619) and *Ruminococcus gnavus* ATCC 29149^T (L76597). Unidentified rumen bacterium RC27 (AF001716) was used as an outgroup. Bar represents 10 nucleotide substitutions.

Within this unit, however, strain SPL73^T represented a distinct, deeply branching line. The robustness of this grouping was well supported by the results of both MP and ML bootstrap analyses (94 % and 89 %, respectively).

Relatedness of meat strain SPL73^T to its taxonomic neighbours. Gel electrophoresis of soluble cell proteins demonstrated that there were distinct differences between the profiles of meat strain SPL73^T and its phylogenetic relatives *C. xylanolyticum* DSM 6555^T, *C. aerotolerans* DSM 5434^T, *C. sphenoides* DSM 632^T and *C. celerecrescens* DSM 5628^T. The main dissimilarities between these profiles are differences in the concentrations and positions of major protein bands at approximately 45 kD and of minor bands (Fig. 4.21).

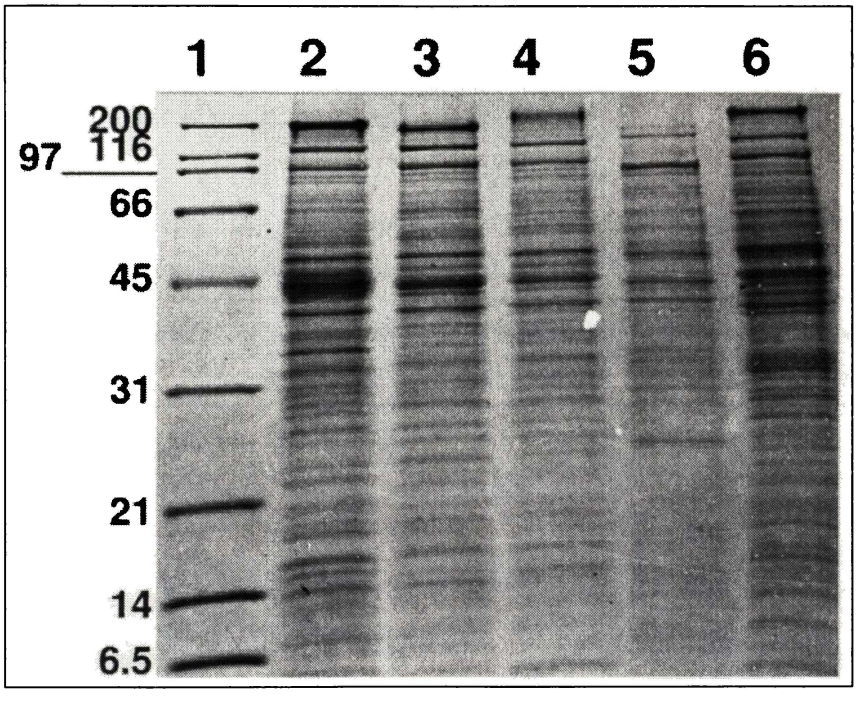

Figure 4.21. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of soluble cell proteins from strain SPL73^T and phylogenetically related clostridia. Electrophoresis was carried out in 8-16 % gradient Tris-HCl polyacrylamide gel. Lane 1, broad range protein standard; lane 2, strain SPL73^T; lane 3, *C. xylanolyticum* DSM 6555^T; lane 4, *C. aerotolerans* DSM 5434^T; lane 5, *C. celerecrescens* DSM 5628^T; lane 6, *C. sphenoides* DSM 632^T. The values on the left of lane 1 indicate the positions of protein standards in kilodaltons.

The major fatty acids detected in the cell extract of strain SPL73^T were myristic (C14:0, 3.9 %), palmitic (C16:0, 17.8 %), palmitoleic (C16:1, 6.6 %) and oleic (C18:1, 5.0 %) acids. Distinct differences were observed between the CFA pattern of the meat strain and the patterns of its phylogenetic neighbours (Table 4.5). The CFA pattern of *C. xylanolyticum* DSM 6555^T differed from the pattern of strain SPL73^T by the presence of stearic acid (C18:0) and three unknown compounds (equivalent chain lengths 15.672, 17.633 and 18.475). In addition, the CFA composition of *C. xylanolyticum* DSM 6555^T differed from that of the meat strain in the proportion of palmitic (16:0) and palmitoleic (C16:1) acids formed. The CFA pattern of *C. aerotolerans* DSM 5434^T contained only two fatty acids, contrasting with the eight associated with the meat strain. The pattern of *C. sphenoides* DSM 632^T contained a higher proportion of palmitic acid (C16:0) and a lower proportion of palmitoleic acid (C16:1) than the pattern of strain SPL73^T. The pattern of *C. sphenoides* also contained stearic acid (C18:0) and three unknown compounds (equivalent chain lengths 15.672, 17.633 and 18.475), which were absent from the pattern of the meat strain.

Table 4.5. Differences in cellular fatty acid composition of strain SPL73^T and phylogenetically related clostridia (values are in % of total peak area).

Equivalent chain length	FAME*	SPL73^T	*C. xylanolyticum* DSM 6555^T	*C. aerotolerans* DSM 5434^T	*C. sphenoides* DSM 632^T	*C. celerecrescens* DSM 5628^T
14.000	14:0	3.9	4.3	-	2.4	9.2
15.350	Unknown	3.5	-	-	1.9	–
15.672	Unknown	-	3.5	-	1.8	1.2
16.000	16:0	17.8	3.0	19.6	30.3	14.0
16.398	16:1	6.6	1.7	-	3.9	1.0
17.538	Unknown	9.2	12.6	8.3	6.8	3.2
17.633	Unknown	-	5.5	-	6.1	–
18.000	18:0	-	4.1	-	4.5	5.5
18.362	18:1	5.0	3.1	-	6.0	5.6
18.404	Unknown	6.3	5.4	-	2.7	–
18.493	Unknown	3.0	-	-	6.8	–
18.475	Unknown	-	2.6	-	2.9	1.9

*Only fatty acids occurring in concentrations over 1 % are listed.

Characterisation of meat strains K21 and K24

Phenotypic characterisation. The colonies of strains K21 and K24 grown on the surface of CBA containing 5 % v/v sheep blood were circular, 0.7 to 2.6 mm in diameter, lobate margin, semi-opaque, raised, convex, shiny and weakly β-haemolytic. The colonies of strain K21 were grey-cream whereas colonies of strain K24 were dirty yellow. Cells in the exponential growth phase occurred most frequently singly as long straight to slightly bent rods (Fig. 4.22a), 6.3 to 12.2 μm long and 0.9 to 1.3 μm wide. Vegetative cells were motile by means of peritrichous flagella (not shown).

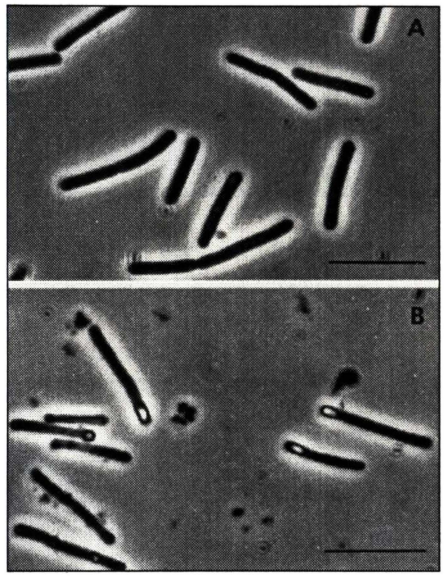

Figure 4.22. Phase-contrast micrographs of vegetative (a) and sporulated (b) cells of strain K21. Bar length equals 3.5 μm for each panel.

Electron micrographs of ultra thin sections of vegetative cells confirmed that the organism was rod-shaped (Fig. 4.23a). The Gram positive type cell wall was observed in ultra thin sections of vegetative cells of the strain (Fig. 4.23b). Gram positive type was confirmed with both conventional Gram staining and the KOH reaction. Elliptical, subterminal spores (Fig. 4.22b) were formed only after prolonged incubation. Formation of mature spores was preceded by formation of a spore-like structure in subterminal to terminal position of the maternal cell (Fig. 4.24a), followed by formation of an immature spore (Fig. 4.24b). Spores caused only little swelling of the maternal cells.

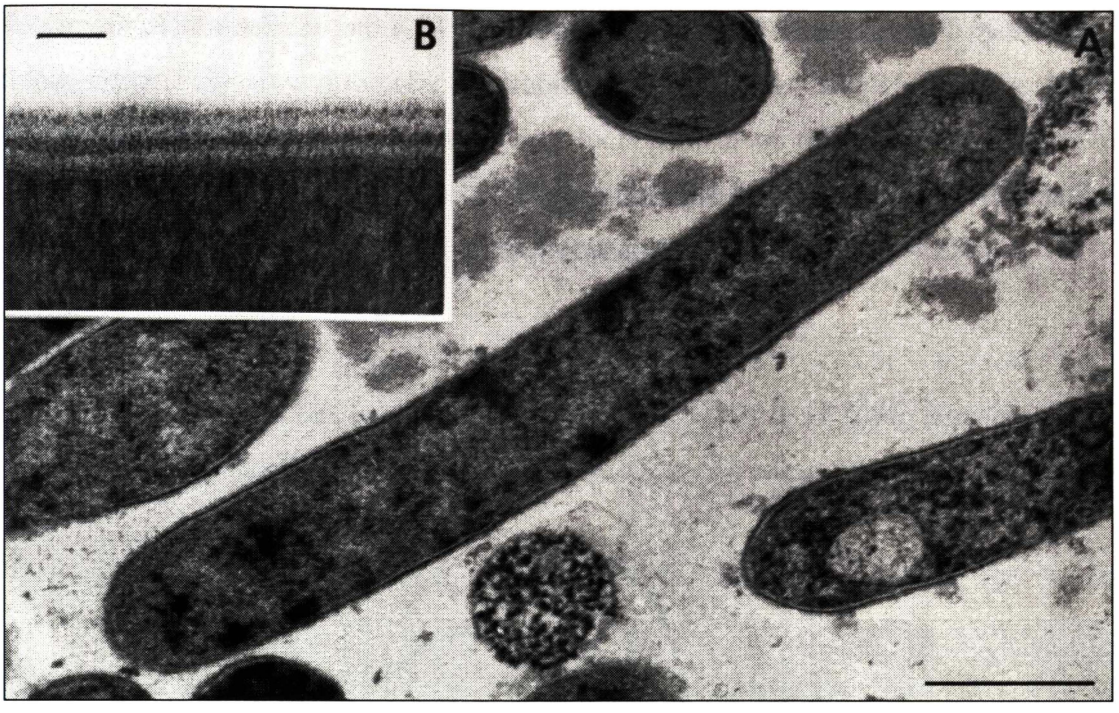

Figure 4.23. Electron micrographs of thin sections of vegetative cells of strain K21 showing cell shape (a) and Gram positive type cell wall (b). Bar length equals (a) 1.0 μm and (b) 0.1 μm.

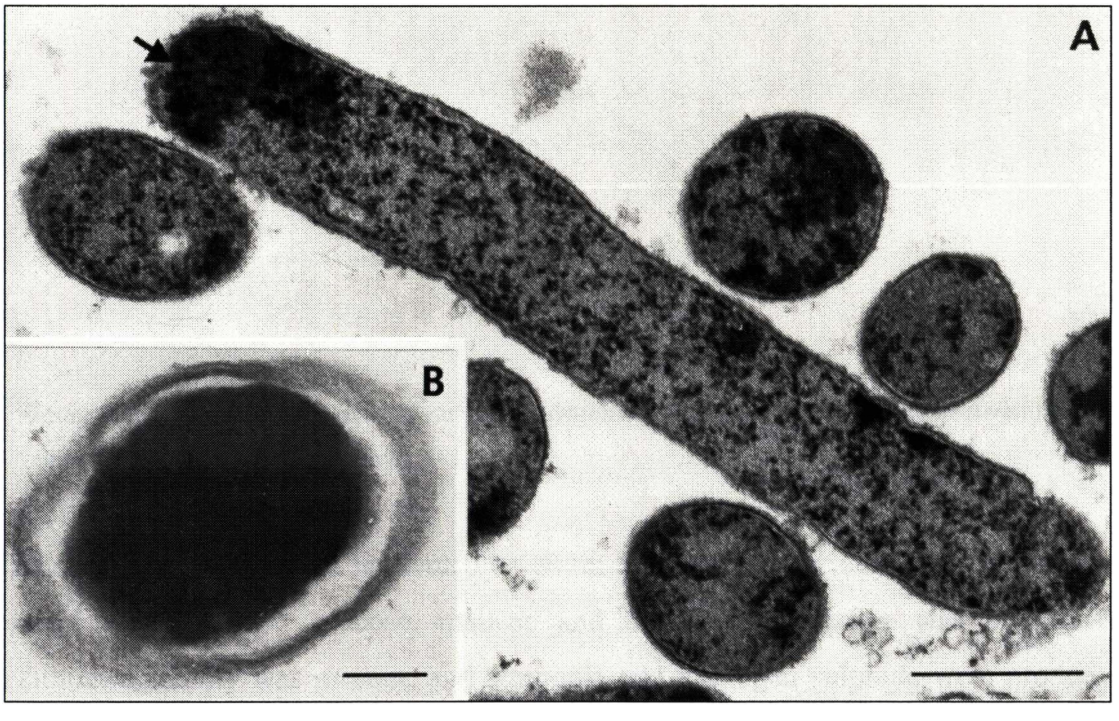

Figure 4.24. Electron micrographs of thin sections of a cell of strain K21 that begins sporulation (a) and of an immature spore (b). Bar length equals 1.0 μm (a) and 0.2 μm (b). Arrow indicates the formation of pre-spore structure.

No growth of strains K21 and K24 occurred on aerobic CBA containing 5 % v/v sheep blood. Strain K21 grew optimally anaerobically between 14.5 and 21.0°C (Fig. 4.25a). The lowest and highest temperatures at which growth of this strain was observed were –1.5°C and 25.0°C, respectively. The optimal pH for anaerobic growth was 7.03 to 7.39, and growth occurred at pH values between 5.54 and 8.81 (Fig. 4.25b).

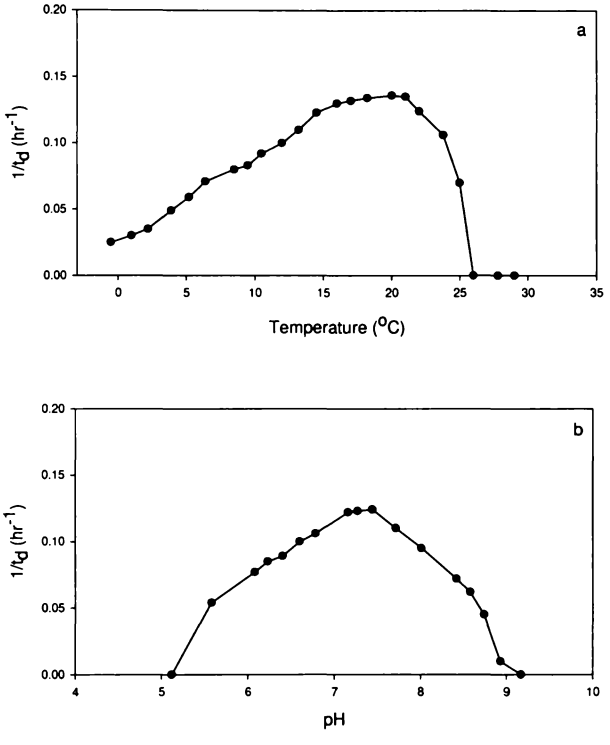

Figure 4.25. Growth of strain K21 at various incubation temperatures at pH 7.0 (a) and at various pH values at 15°C (b). t_d, doubling time.

The substrates utilised and fermented by strain K21 in the presence of 0.1 % w/v yeast extract were fructose, glucose, maltose and trehalose. Gelatine was hydrolysed, lecithinase activity was present, and ammonia and hydrogen sulphide were produced. The milk reaction was positive for curd formation. Addition of Tween 80 did not stimulate growth. The remaining substrate utilisation tests were negative. With the Rapid ID 32 A, weak positive reaction was observed for α-glucosidase.

Strain K24 was positive or negative for the same biochemical tests as strain K21, with the exception that milk reaction was negative, cellobiose and mannose were weakly fermented, and proline arylamidase and pyroglutamic acid arylamidase activities were present. On medium containing 3 % oat spelts xylan, colonies of strain K21 or K24 did not produce clear zones indicative of xylan degradation.

The major fermentation products formed by strains K21 and K24 in PYGS broth were ethanol (15 to 21 mM), acetate (14 to 16 mM), butyrate (9 to 11 mM), isocaproate (5 to 7 mM), lactate (4 mM), pyruvate (3 mM), hydrogen and carbon dioxide.

The supernatant of the broth culture of strain K21 was not toxic to mice.

Genotypic characterisation. The DNA base composition for strain K21 was 29.2 mol% G+C and for strain K24 was 29.6 mol% G+C.

Similarity analysis of the almost complete gene sequences of the two meat isolates showed them to be nearly identical. Pairwise comparison of the sequences from strains K21 and K24 demonstrated five mismatches within the 1507 nucleotides used in the comparison, corresponding to a 99.7 % 16S rRNA gene sequence similarity between the two meat isolates. Sequence searches of GenBank database revealed that the unknown Gram positive rod was most closely associated with the *Clostridium* subphylum (data not shown) and in particular to Cluster I clostridia (*sensu* Collins *et al.*, 1994). Results of the sequence similarity calculations indicate that the nearest relatives of both strains are *C. estertheticum* (97.9 % sequence similarity), *C. subterminale* (95.1 %), *C. tetanomorphum* (95.1 %), *C. argentinense* (95.7 %), proteolytic *C. botulinum* type A (93.0 %) and *C. sporogenes* (93.1 %).

Phylogenetic characterisation. Detailed phylogenetic analyses placed strain K21 within Cluster I of the genus *Clostridium* (*sensu* Collins *et al.*, 1994). In this cluster strain K21 was located within a unit composed of *C. estertheticum*, *C. subterminale* and *C. argentinense*, branching together with *C. estertheticum* (Fig. 4.26).

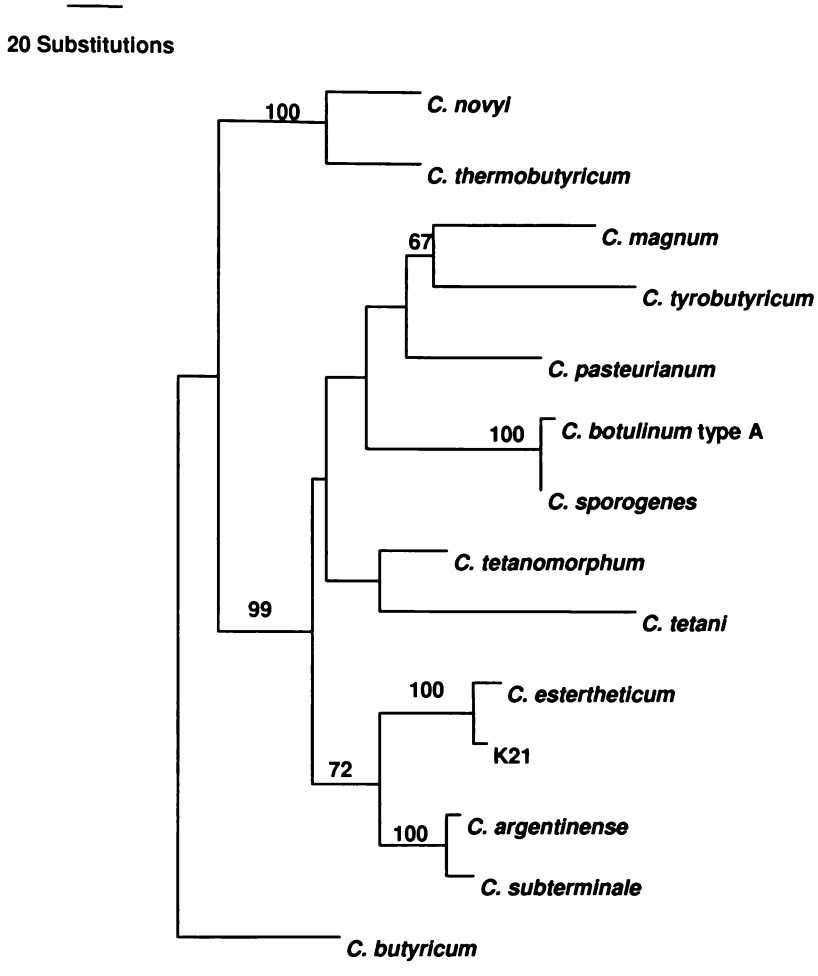

Figure 4.26. Maximum likelihood tree generated from aligned 16S rRNA gene sequences, showing phylogenetic position of strain K21 within Cluster I of the genus *Clostridium* (Collins *et al.*, 1994). The values above branches indicate the level of support derived from maximum parsimony bootstrap analyses of 2000 replicates. When no value is shown, bootstrap support is less than 50 %. The following reference strains were used in phylogenetic analyses: *C. argentinense* ATCC 27322^T (X68316), *C. botulinum* type A NCTC 7272^T (X68185), *C. estertheticum* NCIMB 12511^T (X68181), *C. magnum* DSM 2767^T (X77835), *C. novyi* type A NCTC 538 (X68188), *C. pasteurianum* ATCC 6013^T (M23930), *C. sporogenes* ATCC 3584^T (X68189), *C. subterminale* NCIMB 10746^T (X68451), *C. tetanomorphum* NCIMB 11547 (X68184), *C. tetani* NCTC 279^T (X74770), *C. thermobutyricum* ATCC 49875 (X72868) and *C. tyrobutyricum* ATCC 25755^T (M59113). *C. butyricum* NCIMB 8082 (X68178) was used as an outgroup. Bar represents 20 nucleotide substitutions.

Relatedness of meat strains K21 and K24 to their taxonomic neighbours. Gel electrophoresis of soluble cell proteins demonstrated profiles of strain K21 and K24 were very similar. As shown in Fig. 4.27, there were distinct differences, however, between the profiles of meat strains and those of their close relatives *C. estertheticum* DSM 8809^T and *C. subterminale* DSM 6970^T. These differences were evidenced by changes in the concentration and positions of major and minor protein bands, especially at approximately 66, 45 and 31 kD (Fig. 4.27).

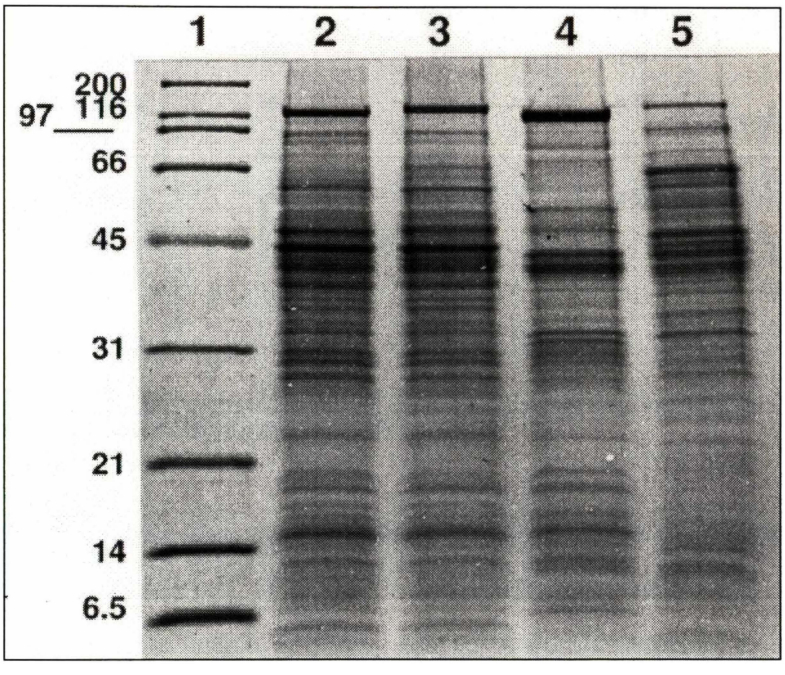

Figure 4.27. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) of soluble cell proteins from strains K21 and K24 and their nearest relatives. Lane 1, low range protein standard; lane 2, strain K21; lane 3, strain K24; lane 4, *C. estertheticum* DSM 8809; lane 5, *C. subterminale* DSM 6970^T. The values on the left of lane 1 indicate the positions of protein standards in kilodaltons.

The major fatty acids detected in the cell extract of strains K21 and K24 were myristic (C14:0), palmitic (C16:0) and palmitoleic (C16:1) acids. A major proportion of an unknown compound with an effective chain length of 15.672 was also present in the CFA patterns of both meat strains. Distinct differences were observed between the CFA pattern of the meat strains and the patterns of their neighbours (Table 4.6).

Table 4.6. Differences in cellular fatty acid composition of strains K21 and K24, and related clostridia (values are in % of total peak area).

Equivalent chain length	FAME*	K21	K24	*C. estertheticum* DSM 5434^T	*C. subterminale* DSM 632^T
12.206	Unknown	3.8	3.3	–	8.0
14.000	14:0	10.0	8.6	20.0	2.8
15.000	15:0	1.1	1.4	–	–
15.525	15:1	1.3	1.3	–	–
15.672	Unknown	8.4	8.2	10.9	–
16.000	16:0	11.0	12.6	3.0	8.6
16.288	Unknown	2.0	2.0	2.8	–
16.336	Unknown	3.9	3.6	5.0	–
16.398	16:1	6.0	6.3	14.4	–
16.491	Unknown	1.1	1.2	3.8	–
17.538	Unknown	–	–	–	1.5
18.000	18:0	–	–	–	3.6
18.207	18:1 *cis-9*	–	–	–	3.6
18.362	18:1	2.0	1.9	–	6.3

*Only fatty acids occurring in concentrations over 1 % are listed.

The CFA pattern of *C. estertheticum* DSM 8809^T differed from the patterns of strains K21 and K24 by the absence of C15:0, C15:1 and C18:1 fatty acids, and one unknown compound (equivalent chain length 12.206). The CFA composition of *C. estertheticum* contained a higher proportion of myristic (C14:0) and palmitoleic acid (C16:1), and a lower proportion of palmitic acid (16:0) than the patterns of strains K21 and K24. The pattern of *C. subterminale* DSM 6970^T differed from those of the meat strains in the proportion myristic (C14:0) and oleic (C18:1) acids formed. In contrast to CFA compositions of meat strains K21 and K24, the CFA composition of *C. subterminale* lacked C15:0, C15:1 and C16:1 acids, as well as four unknown compounds, in particular one with an effective chain length of 15.672. The pattern of *C. subterminale* also contained C18:0 and C18:1 *trans-9* acids, which were absent from the patterns of the meat strains .

Discussion

Taxonomic status of meat strains SPL77A^T and SPL77B

Polyphasic differentiation of strains SPL77A^T and SPL77B from their taxonomic neighbours. When strains SPL77A^T and SPL77B were initially isolated from spoiled vacuum-packed meat, their taxonomic status was uncertain. According to the phenotypic criteria and identification keys of Cato *et al.* (1986) and Holdeman *et al.* (1977), these strains could not be assigned to any of the clostridial species described at the time. Similarly, the Rapid ID 32A system designed for identification of medically important anaerobes proved unsatisfactory with these microorganisms. Strains SPL77A^T and SPL77B were unable to produce abundant gas in artificially inoculated chill-stored packs and did not seem to resemble known psychrophilic 'blown pack'-causing clostridia. In addition, the phenotypic characteristics for other psychrophilic and psychrotrophic clostridial species discovered between 1986 and 1998 differed from those described here for strains SPL77A^T and SPL77B (Collins *et al.*, 1992; Kalchayanand *et al.*, 1993; Lawson *et al.*, 1994; Kotsyurbenko *et al.*, 1995; Mountfort *et al.*, 1997). Consequently, in phenotypic terms, the meat strains were either a new species or were previously described clostridial species that had been phenotypically modified by growth conditions in the laboratory.

The results of 16S rRNA gene sequence similarity and treeing analysis conducted in this study demonstrated that strains SPL77A^T and SPL77B are phylogenetically related to microorganisms in Cluster I of the genus *Clostridium* (Collins *et al.*, 1994). The nearest phylogenetic relatives of these meat strains are *C. fallax* DSM 2631^T, *C. algidicarnis* NCFB 2931^T, *C. intestinale* DSM 6191^T, *C. cadaveris* DSM 1284^T and *C. carnis* DSM 1293^T. Sequence divergence values of the meat strains SPLL77A^T and SPL77B and their phylogenetic relatives of approximately 5.5 to 6.4 % indicate that these meat strains should be assigned to a new species (Stackebrandt and Goebel, 1994).

Strains SPL77A^T and SPL77B were easily differentiated from their phylogenetic neighbours by soluble protein profiles, cellular fatty acid profiles and phenotypic properties (Table 4.7). During growth in PYGS broth, both strains produced a complex mixture of eight alcohols and volatile and nonvolatile fatty acids, including ethanol, butanol, isovalerate, isobutyrate and oxalacetate. None of their near phylogenetic relatives are known to produce this combination of compounds (Cato *et al.*, 1986; Lee *et al.*, 1989; Lawson *et al.*, 1994). The meat strains were lecithinase-positive, whereas their relatives are lecithinase-negative. Of five reference strains used in the comparison, only *C. cadaveris* DSM 1284^T hydrolyses gelatine and digests meat. This reference strain, however, produces indole, whereas strains SPL77A^T and SPL77B do not, and it is unable to ferment mannose, sorbitol and trehalose – three carbohydrates utilised and fermented by the two meat strains.

Strains SPL77A^T and SPL77B were able to grow at 4°C. Therefore, by the definition of Eddy (1960) they can be described as psychrotrophic. However, the phenotypic characteristics of these strains differed from those of the other psychrophilic and psychrotrophic clostridia (Table 4.8). Of all the psychrophilic and psychrotrophic strains used for comparison in this study, only the meat strains formed ethanol, butanol, isovalerate, isobutyrate and oxalacetate as fermentation products. These strains were readily distinguished from *C. arcticum*, *C. estertheticum*, *C. laramiense* and *C. vincentii* by their maximum and optimum growth temperature and substrate utilisation pattern (Cato *et al.*, 1986; Collins *et al.*, 1992; Kalchayanand *et al.*, 1993; Mountfort *et al.*, 1997). In addition, *C. laramiense* exhibits lipase but not lecithinase activity, reduces nitrate but does not hydrolyse gelatine (Kalchayananad *et al.*, 1993). Unlike *C. putrefaciens*, strains SPL77A^T and SPL77B were both proteolytic and saccharolytic. With respect to the optimum growth temperature, the meat strains showed closest similarity with *C. algidicarnis* and non-proteolytic *C. botulinum* types B, E or F. However, *C. algidicarnis* cannot hydrolyse gelatine or digest meat and utilises a different range of substrates than strains SPL77A^T and SPL77B (Lawson *et al.*, 1994). Non-proteolytic *C. botulinum* types B, E or F exhibit lipase but not lecithinase activity, can hydrolyse starch but do not digest meat or milk (Cato *et al.*, 1986).

Table 4.7. Some properties which differentiate strain SPL77A^T from phylogenetically related clostridia.

Phenotypic property	SPL77A^T	*C. fallax**	*C. algidicarnis*†	*C. intestinale*‡	*C. cadaveris**	*C. carnis**
Fermentation products	A,B,ib,iv,l,o,2,4	A,B,L (s,2,4)	A,B	A,B (l,f,s)	B,A,2,4 (f,p,l,s)	B,A,L,f (s)
Gelatine hydrolysis	+	-	-	-	+	-
Meat digestion	+	-	-	NR	+	-
Indole production	-	-	-	-	+	-
Lecithinase activity	+	-	-	-	-	-
Esculin hydrolysis	-	+	-	+	-	+
Fermentation of						
Mannose	+	+	+	+	- (w)	+ (w)
Sorbitol	+	– (w)	-	+	-	-
Trehalose	+	-	-	+	-	- (w)

*, results from Cato *et al.* (1986); †, results from Lawson *et al.* (1994); ‡, results from Lee *et al.* (1989). Results in parentheses are for some strains of the species. +, positive; -, negative; w, weak reaction (pH from 5.5 to 5.9); NR, not reported; A, acetate; B, butyrate; L or l, lactate; ib, isobutyrate; iv, isovalerate; f, formate; p, propionate; py, pyruvate; s, succinate; o, oxalacetate; 2, ethanol; 4, butanol. Upper case letters indicate major and lower case letters indicate minor fermentation product.

Table 4.8. Some properties which differentiate strain SPL77A^T from other psychrophilic and psychrotrophic clostridia.

Phenotypic property	SPL77A^T	*C. arcticum**	*C. algidicarnis*†	*C. botulinum** non-proteolytic types B, E, F	*C. estertheticum*‡	*C. fimetarium*§	*C. laramiense*¶	*C. putrefaciens**	*C. vincentii*#
Maximum growth temperature (°C)	40.5	37	37	<45	15	30	20	<37	20
Optimum growth temperature (°C)	30-38.5	22-25	25-30	25-30	10-12	20-25	15	15-22	12
Fermentation products	A,B,ib,iv,l,o, 2,4	P,A (b)	A,B	B,A (l)	B,A	A,F,L,2	A,B,ib,p	a,f,l (s)	A,F,B
Gelatine hydrolysis	+	-	-	+	NR	NR	-	-	NR
Meat digestion	+	-	-	-	NR	NR	+	-	NR
Lipase activity	-	NR	NR	+	NR	NR	+	-	NR
Lecithinase activity	+	NR	-	-	NR	NR	-	-	NR
Starch hydrolysis	-	-	-	+	+	-	+	-	-
Fermentation of									
Cellobiose	-	w	-	-	+	+	-	-	+
Galactose	-	NR	NR	-	+	+	+	-	+
Lactose	-	-	-	-	NR	-	-	-	+
Maltose	+	-	-	+	+	+	+	-	+
Mannitol	-	w	-	-	+	-	+	-	NR
Sorbitol	+	-	-	+	+	-	NR	-	-
Trehalose	+	-	-	+	NR	-	-	-	NR
Xylose	-	+	+	-	+	+	-	-	+

*, results from Cato *et al.* (1986); †, results from Lawson *et al.* (1994); ‡, results from Collins *et al.* (1992); §, results from Kotsyurbenko *et al.* (1995); ¶, results from Kalchayanand *et al.* (1993); #, results from Mountfort *et al.* (1997). For abbreviation and symbols, see Table 4.7.

In contrast, strains SPL77A^T and SPL77B showed lecithinase but not lipase activity, did not hydrolyse starch but digested meat and milk.

Only small differences in colony morphology, physiological and biochemical reactions, DNA base composition and minor cellular fatty acids were found between strains SPL77A^T and SPL77B, suggesting that the two isolates are different strains of the same species.

Based on both phenotypic and phylogenetic findings strains SPL77A^T and SPL77B merit classification as a new species within the genus *Clostridium*, for which the name *Clostridium frigidicarnis* has been proposed (Broda *et al.*, 1999).

Description of Clostridium frigidicarnis *sp. nov.* *Clostridium frigidicarnis* (fri.gi.di. car'nis L. adj. *frigidus* cool; L. n. *caro* meat; N. L. gen. n. *frigidicarnis* of cool meat). Colonies on sheep blood agar measure from 2.2 to 7.2 mm in diameter and are circular to irregular; with undulate, lobate or erose margin; creamy grey to grey; opaque or semi-opaque to translucent and β-haemolytic. Cells are Gram positive, motile rods (4.5 to 9.4 μm long and 1.3 to 1.6 μm wide) occurring singly or in pairs. Elliptical spores are produced in early-stationary growth phase. The microorganism is psychrotrophic and grows between 3.8°C and 40.5°C (the optimum growth temperature is 30°C to 38.5°C). At 30°C it grows between pH 4.7 and 9.5 and its optimum pH for growth is 6.4 to 7.2. Fructose, glucose, mannose, mannitol and trehalose are fermented, lecithinase activity is present, gelatine is hydrolysed. The fermentation products formed in PYGS broth are acetate, ethanol, butyrate, isovalerate, butanol, isobutyrate, oxalacetate, lactate, hydrogen and carbon dioxide. The major cellular fatty acids are myristic, palmitic and oleic. The DNA base composition is 27.3 to 28.4 mol% G+C. Isolated from vacuum-packed temperature-abused beef and from faeces, hides and tonsils of bovine slaughter stock. Probably forms a part of a normal flora of vacuum-packed chilled meats. The type strain is strain SPL77A^T, this strain has been deposited in the Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH, Braunschweig, Germany, under the culture collection number DSM 12271.

Taxonomic status of meat strains DB1A^T and R26

Analysis of headspace volatiles as a tool for detection of clostridial 'blown pack' spoilage. Psychrotolerant clostridia have been associated with instances of chilled meat spoilage where initial microbiological examination failed to detect a causative microorganism. Difficulties with the isolation of the causative clostridia have been reported (Dainty *et al.*, 1989; Kalchayanand *et al.*, 1989; Broda *et al.*, 1996b). It has, therefore, been suggested that the composition of headspace volatiles of 'blown packs', as analysed by gas chromatography and mass spectrometry, may indicate the bacterial species likely to be involved in the spoilage, prior to lengthy and unreliable isolation of causative psychrotolerant clostridia (Dainty *et al.*, 1989; Broda *et al.*, 1996b). Butanol, butyric acid, carbon dioxide and hydrogen are products of butyric type fermentative metabolism, typical of mesophilic saccharolytic clostridia (Jones and Woods, 1986; Jones and Woods, 1989). Similar products with a unique range of butyl esters were found in the headspace of vacuum packs, both naturally spoiled and artificially contaminated by psychrophilic *C. estertheticum* (Dainty *et al.*,1989), and in those naturally spoiled by other psychrotolerant clostridia (Broda *et al.*, 1996b). In this study a similar composition of volatiles, including butyl esters, was detected in the headspace of 'blown' lamb packs inoculated with pure cultures of psychrophilic strains DB1A^T and R26 (Table 4.3). This finding confirms that the ability to produce butanol, butyric acid, butyl esters, carbon dioxide and hydrogen may be considered a property of many clostridial species, including psychrophilic strains. Moreover, this fingerprint of headspace volatiles may serve as a useful indicator that clostridial species are responsible for 'blown pack' spoilage.

Polyphasic differentiation of strains DB1A^T and R26 from their taxonomic neighbours. The results of 16S rRNA gene sequence similarity and phylogenetic analyses conducted in this study demonstrate that although strain DB1A^T is phylogenetically related to microorganisms in Cluster I of the genus *Clostridium* (Collins *et al.*, 1994), it represents a distinct branch within this cluster. Within Cluster I, strain DB1A^T was firmly placed in the monophyletic unit that also contains *C. carnis*, *C. chauvoei, C. septicum* and *C. quinii* (Fig. 4.14).

The robustness of this phylogenetic unit was supported by results of maximum parsimony and neighbour joining. Sequence divergence values of 4.5 to 5 % between the meat strain and its nearest relatives *C. vincentii*, *C. septicum*, *C. chauvoei*, non-proteolytic *C. botulinum* types B and E and *C. carnis*; the distinct phylogenetic position of the meat strain in Cluster I, together with its relatively low level of phylogenetic relatedness to Cluster I clostridia indicate that strain DB1A^T should be assigned to a new species (Stackebrandt and Goebel, 1994).

Strains DB1A^T and R26 were easily differentiated from their nearest relatives by soluble protein profiles, cellular fatty acid profiles and phenotypic properties (Table 4.9). The two meat strains were the only clostridia in this group to produce both ethanol and butanol and to hydrolyse both esculin and starch. *C. vincentii*, the only psychrophile among the nearest relatives of the meat strains, ferments lactose and xylose (Mountfort *et al.*, 1997). Similarly, *C. septicum* is lactose positive (Cato *et al.*, 1986). In contrast, strains DB1A^T and R26 did not utilise or ferment these two carbohydrates. The two meat isolates were readily distinguished from *C. chauvoei* and non-proteolytic *C. botulinum* types B and E by their ability to ferment cellobiose and salicin. In addition, *C. chauvoei* is unable to ferment trehalose, which was fermented by the meat strains, and *C. botulinum* types B and E are the only reference strains which are lipase-positive. Of the five reference strains used in the comparison, only *C. carnis* cannot hydrolyse gelatine. In addition, this reference strain is unable to ferment trehalose, a carbohydrate utilised and fermented by the two meat strains.

The two meat strains differed from other psychrophilic clostridia in their physiological and biochemical characteristics (Table 4.10). Of all the psychrophilic strains used for comparison in this study, only the meat strains formed both ethanol and butanol as their fermentation products. These strains differed from *C. estertheticum*, *C. laramiense* and *C. vincentii* by their maximum and optimum growth temperatures and substrate fermentation pattern (Collins *et al.*, 1992; Kalchayanand *et al.*, 1993 Mountfort *et al.*, 1997). With respect to their physiological characteristics, the meat strains showed closest similarity with *C. fimetarium* (Kotsyurbenko *et al.*, 1995).

Table 4.9. Some properties that differentiate strains DB1A^T and R26 from their nearest relatives.

Phenotypic property	DB1A^T/R26	*C. vincentii**	*C. septicum*†	*C. chauvoei*†	*C. botulinum*† non-proteolytic B, E, F	*C. carnis*†
Fermentation products	A,B,L,2,4	A,F,B	B,A,(F,p,l,2)	A,B,F,4,(l,s)	B,A,(l)	B,A,L,f,(s)
Esculin hydrolysis	+	+	+	+	-	+
Starch hydrolysis	+	-	-	-	±	-
Fermentation of						
Cellobiose	+	+	+(w)	-	-	+(w)
Lactose	-	+	+	+(w)	-	d
Salicin	+	NR	d	-	-	w
Trehalose	+	NR	+(w)	-	w(+)	-
Xylose	-	+	-	-	-	-

*, results from Mountfort *et al.* (1997); †, results from Cato *et al.* (1986). Results in parentheses are for some strains of the species. +, positive; -, negative; w, weak reaction (at pH 5.5-5.9); d, 40 to 60 % of strains positive; ±, 61 to 89 % of strains positive; NR, not reported; A, acetate; B, butyrate; F or f, formate; L or l, lactate; p, propionate; s, succinate; 2, ethanol; 4, butanol. Upper case letters indicate major and lower case letters indicate minor fermentation product.

Table 4.10. Some properties that differentiate strains DB1A^T and R26 from other psychrophilic clostridia and *C. putrefaciens*.

Phenotypic property	DB1A^T/R26	*C. vincentii**	*C. estertheticum*†	*C. fimetarium*‡	*C. laramiense* §	*C. putrefaciens*
Maximum growth temperature (°C)	26	20	15	30	20	<37
Optimum growth temperature (°C)	20-22	12	10-12	20-25	15	15-22
Fermentation products	A,B,L,2,4	A,F,B	B,A	A,F,L,2	A,B,ib,p	a,f,l,(s)
Esculin hydrolysis	+	+	+	NR	-	-
Starch hydrolysis	+	-	+	-	+	-
Fermentation of						
Arabinose	-	NR	+	+	-	-
Cellobiose	+	+	+	+	-	-
Mannitol	-	NR	+	-	+	-
Raffinose	-	NR	NR	-	+	-
Sorbitol	-	-	+	-	NR	-
Xylose	-	+	+	+	-	-

*, results from Mountfort *et al.* (1997); †, results from Collins *et al.* (1992); ‡, results from Kotsyurbenko *et al.* (1995); §, results from Kalchayanand *et al.* (1993); ¶, results from Cato *et al.* (1986). ib, isobutyrate. For remaining abbreviation and symbols, see Table 4.9.

However, *C. fimetarium* cannot hydrolyse starch and produces acid from arabinose and xylose, two carbohydrates that are not utilised or fermented by strains DB1A^T and R26. Unlike *C. putrefaciens*, which cannot ferment any carbohydrates, strains DB1A^T and R26 were obligately saccharolytic.

A high level of 16S rRNA gene homogeneity and only small differences in phenotypic characteristics were found between strains DB1A^T and R26, suggesting the two isolates should be classified as the same species. The results of phenotypic characterisation, as well as 16S rRNA gene sequence similarity, SDS-PAGE and CFA analyses conducted in this study demonstrate, that strains DB1A^T and R26 represent a new species within the genus *Clostridium*, for which the name *Clostridium gasigenes* has been proposed (Broda *et al.*, *in press* c).

Description of Clostridium gasigenes *sp. nov.* *Clostridium gasigenes* (ga.si'ge.nes. L. n. *gasum* gas; Gr. v. *gennaio* produce; N. L. gen. n. *gasigenes* gas producing). Colonies on sheep blood agar measure from 0.7 to 3.0 mm in diameter and are circular; with entire margin; grey-white to grey; convex, shiny and β-haemolytic. Cells are Gram positive motile rods producing elliptical subterminal spores during late-stationary growth phase. The microorganism is psychrophilic and grows between -1.5°C and 26°C (the optimum growth temperature is 20°C to 22°C). At 20°C the pH range for growth is 5.4 to 8.9 and the optimum pH for growth is 6.2 to 8.6. In Peptone Yeast Extract (PY) broth with 0.1 % w/v yeast extract the organism grows little or not at all in the absence of fermentable carbohydrate. Cellobiose, fructose, glucose, inositol, maltose, mannose, salicin and trehalose are fermented. Gelatine, esculin and starch are hydrolysed. The fermentation products formed in Peptone Yeast Extract Glucose Starch (PYGS) broth are ethanol, acetate, butyrate, lactate, butanol, carbon dioxide and hydrogen. The DNA base composition is from 28.3 to 29.4 mol% G+C. Isolated from an incident of 'blown pack' spoilage of vacuum-packed chilled lamb. Causes gas production and pack distension in vacuum-packed chilled lamb. The type strain is DB1A^T. This strain has been deposited in the DSMZ under the culture collection number DSM 12272.

Taxonomic status of meat strain SPL73^T

Polyphasic differentiation of strain SPL73^T from its taxonomic neighbours. Results of 16S rRNA gene sequence similarity analysis and database searches show that strain SPL73^T was most similar to *C. aerotolerans* DSM 5434^T, *C. xylanolyticum* DSM 6555^T, *C. celerecrescens* DSM 5628^T and *C. sphenoides* DSM 632^T. The results of phylogenetic analyses demonstrate, however, that the meat strain represents a distinct deep branch within a phylogenetic unit containing two strains of *C. xylanolyticum* and *C. aerotolerans* (Fig. 4.20). The meat strain characterised in this study was readily distinguished from its phylogenetic neighbours by its soluble protein profiles and cellular fatty acid profiles, as well as phenotypic properties (Table 4.11). The phylogenetic relatives of strain SPL73^T are mesophiles and grow optimally at temperatures between 35°C and 38°C (Cato *et al.*, 1986; van Gylswyk and van der Toorn, 1987; Palop *et al.*, 1989; Rogers and Baecker, 1991), whereas the meat strain is unable to grow at temperatures above 32.2°C and grows optimally between 25.5°C and 30.0°C. In contrast to *C. aerotolerans*, strain SPL73^T was obligately anaerobic. Additional phenotypic properties that differentiate strain SPL73^T from *C. xylanolyticum* were fermentation of arabinose and lactose (the meat strain ferments these two carbohydrates, whereas *C. xylanolyticum* does not) and from *C. aerotolerans* was fermentation of inulin and trehalose (in contrast to *C. aerotolerans* the meat strain ferments inulin, but not trehalose) (van Gylswyk and van der Toorn, 1987; Rogers and Baecker, 1991). Unlike strain SPL73^T, its two other nearest phylogenetic neighbours, *C. sphenoides* and *C. celerecrescens*, produce indole and ferment a different range of carbohydrates (Cato *et al.*, 1986; Palop *et al.*, 1989).

The results of detailed phylogenetic analyses conducted in this study placed strain SPL73^T in Cluster XIVa of the genus *Clostridium* (*sensu* Collins *et al.*, 1994). Members of Cluster XIVa are phylogenetically distinct from *sensu stricto* clostridia belonging to Cluster I (Collins *et al.*, 1994), i.e. rRNA homology group I (Johnson and Francis, 1975).

Table 4.11. Some properties which differentiate strain SPL73^T from phylogenetically related clostridia.

Phenotypic property	SPL73^T	*C. xylanolyticum* DSM 6555^T*	*C. aerotolerans* DSM 5434^T†	*C. sphenoides* DSM 632^T‡	*C. celerecrescens* DSM 5628^T§
Maximum growth temperature (°C)	32.2	NR	<45	<45	NR
Optimum growth temperature (°C)	25.5-30.0	35	38	30-37	35
Fermentation products	A,F,L,b,2,4	F,L,A,2	A,F,L,2	A,2 (F,s,l)	A,F,B,ib,iv,c,l,s,2
Gelatine hydrolysis	–	–	–	–	+
Indole production	–	–	–	+	+
Nitrate reduction	–	–	–	±	–
Fermentation of					
Arabinose	+	–	+	–w	+
Inulin	+	NR	–	–	–
Lactose	+	–	+	+w	+w
Trehalose	–	–	d	d	+

*, results from Rogers & Baecker (1991); †, results from van Gylswyk & van der Toorn (1987); ‡, results from Cato *et al.* (1986); §, results from Palop *et al.* (1989). Results in parentheses are for some strains of the species. +, positive; , negative; w, weak reaction (at pH 5.5-5.9); d, 40 to 60 % of strains positive; ±, 61 to 89 % of strains positive; NR, not reported; A, acetate; B or b, butyrate; c, caproate; F or f, formate; ib, isobutyrate; iv, isovalerate; L or l, lactate; s, succinate; 2, ethanol; 4, butanol. Upper case letters indicate major and lower case letters indicate minor fermentation product.

Cluster XIVa contains phenotypically and phylogenetically heterogenous microorganisms which include non-sporeforming cocci (e.g. ruminococci), aerotolerant rods that stain Gram negative (e.g. *C. aerotolerans*), and other organisms that frequently have one or more characteristics conflicting with those defined for members of the genus *Clostridium* (Cato *et al.*, 1986). Future taxonomic reclassification of this cluster is, therefore, likely to define a number of new genera and may result in nomenclatural changes for clostridial species currently contained within the cluster. However, until a taxonomic review of the genus *Clostridium* is undertaken, strain SPL73^T can be assigned to the genus *Clostridium*.

The majority of known psychrophilic and psychrotrophic *Clostridium* spp. appear to form a very diverse group of dominantly Gram positive, low-G+C-content organisms belonging to Cluster I clostridia (Collins *et al.*, 1994). Strain SPL73^T, with its multi layer thin section profile of cell wall type, G+C content of 38.4 mol% and phylogenetic placement in Cluster XIVa, can be readily differentiated from non-proteolytic *C. botulinum* types B, E and F (Cato *et al.*, 1986), *C. estertheticum* (Collins *et al.*, 1992), *C. algidicarnis* (Lawson *et al.*, 1994), *C. vincentii* (Mountfort *et al.*, 1997), *C. frigidicarnis* (Broda *et al.*, 1999) and *C. gasigenes* (Broda *et al.*, *in press* c). This meat strain differs from *C. arcticum* (which stains Gram negative and is not yet characterised with respect to G+C content and phylogenetic placement) and from *C. fimetarium* (which has a G+C content of 35.6 % and also lacks phylogenetic characterisation data) by its ability to hydrolyse starch and ferment lactose, maltose, raffinose, rhamnose and sucrose (Jordan and McNicol, 1979; Kotsyurbenko *et al.*, 1995). In addition, strain SPL73^T is unable to produce indole, whereas *C. arcticum* is able to do so, and strain SPL73^T ferments arabinose, whereas *C. arcticum* does not. Unlike strain SPL73^T, *C. putrefaciens* is non-saccharolytic (Cato *et al.*, 1986) and *C. laramiense* reduces nitrate, has lipase activity and ferments a different range of carbohydrates (Kalchayanand *et al.*, 1993).

The common sources of xylan-fermenting clostridia are farm soil, animal feeds and wood chips (Lamed and Zeikus, 1980; van Gylswyk and van der Toorn, 1987; Rogers and Baecker, 1991). Xylanolytic clostridia (e.g. *C. polysaccharolyticum*, *C. aerotolerans*) may also be present in the rumen. However, since strain SPL73^T cannot proliferate at temperatures above 33°C, well below a ruminant's body temperature, the source of carcass contamination with this meat strain appears to be extrinsic. Such contamination may occur as a result of contact with spores that either survive passage through the digestive system of slaughter animals or those from environmental sources carried on the coats of slaughter stock. Alternatively, contamination may have occurred from a secondary source located within the meat plant, such as the ventilation or drainage systems.

Both the phenotypic characterisation and a unique phylogenetic position of strain SPL73^T demonstrate that the meat strain should be classified as a new species within the genus *Clostridium*. For this species the name *Clostridium algidixylanolyticum* has been proposed (Broda *et al.*, *in press* b).

Description of Clostridium algidixylanolyticum *sp. nov.* *Clostridium algidixylanolyticum* (al.gi.di.xy.la.no.ly'ti.cum L. adj. *algidus* cold; Gr. derived M.L. *xylanum* xylan; Gr. adj. *lyticus* dissolving; N. L. gen. n. *algidixylanolyticum* cold xylan dissolving). Colonies on sheep blood agar are 0.8 to 2.5 mm in diameter, circular with entire margin, grey-white, translucent and β-haemolytic. Cells are single tapered motile rods (1.8 to 2.8 μm long and 0.5 to 0.8 μm wide). Cell wall type is Gram negative. Elliptical spores do not swell maternal cells and are produced in late stationary growth phase. The microorganism is obligately anaerobic. At pH 7.0 the microorganism grows optimally between 25.5°C and 30.0°C. At 26°C it grows optimally at pH 6.8 to 7.0. The temperature range for growth is 2.5°C to 32.2°C. The pH range for growth is 4.7 to 9.1. The microorganism is saccharoclastic, and it ferments arabinose, cellobiose, fructose, galactose, glucose, inulin, lactose, maltose, mannose, raffinose, rhamnose, salicin, sucrose and xylose. Starch is hydrolysed and xylan is degraded.

The fermentation products formed in PYGS broth are acetate, formate, lactate, ethanol, butyrate, butanol, hydrogen and carbon dioxide. The supernatant of the broth culture of the microorganism is non-toxic to mice. The DNA base composition is 38.4 mol% G+C. Isolated from vacuum-packed temperature-abused raw lamb. The type strain is strain SPL73^T. This strain has been deposited in the Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH, Braunschweig, Germany, under the culture collection number DSM 12273.

Taxonomic status of meat strains K21 and K24

The results of 16S rRNA gene sequence similarity and treeing analysis conducted in this study demonstrated that strains K21 and K24 are taxonomically related to microorganisms in Cluster I of the genus *Clostridium* (Collins *et al.*, 1994), and in particular to *C. estertheticum* DSM 8809^T and *C. subterminale* DSM 6970^T. *C. argentinense*, *C. tetanomorphum*, *C. sporogenes* and proteolytic *C. botulinum* type A were indicated as more distant taxonomic neighbours of the two meat strains. While sequence divergence values of strains K21 and K24, and *C. estertheticum* of approximately 2.1 % indicate that these meat strains may or may not be assigned to a new species (Stackebrandt and Goebel, 1994), sequence divergences of the meat strains and their other neighbours of approximately 4.3 to 7.0 % indicate that these microorganisms are probably related at inter-species level. The meat strains characterised in this study were readily distinguished from *C. estertheticum* and *C. subterminale* by their soluble protein profiles and cellular fatty acid profiles. With the exception of *C. estertheticum*, the taxonomic relatives of strains K21 and K24 are mesophiles and grow optimally at temperatures between 35°C and 38°C (Cato *et al.*, 1986; Collins *et al.*, 1992). In contrast, the meat strains characterised in this study are unable to grow at temperatures above 25°C and grow optimally between 14.5 and 21.0°C. Additional phenotypic characteristics that differentiate strains K21 and K24 from their taxonomic neighbours are presented Table 4.12.

The phenotypic characteristics of strains K21 and K24 also differed from those of the other psychrophilic clostridia (Table 4.13).

Table 4.12. Some properties that differentiate strains K21 and K24 from taxonomically related clostridia excluding *C. estertheticum**.

Phenotypic property	K21/K24	*C. subterminale*†	*C. argentinense*†	*C. tetanomorphum*‡	*C. sporogenes*†	*C. botulinum*† proteolytic type A
Fermentation products	A,B,ic,l,py,2	A.B,iV,ib (f,p,ic,l,s,2)	A,B,iv,ib (l,2,4)	A,B,2,4	A,B,iv,ib,2 (p,ic,v,l,s,4)	A,B,iV,ib (ic,v,p,2,3,4)
Gelatine hydrolysis	+	+	+	–	+	+
Meat digestion	–	±	±	–	+	+
Lipase activity	–	–	–	+	+	+
Lecithinase activity	+	±	–	–	–	–
Esculin hydrolysis	–	–	–	NR	+	+
Fermentation of						
Fructose	+	–	–	±	–(w)	–(w)
Glucose	+	–	–	+	+	+
Maltose	+	–	–	+	–	–(w)
Trehalose	+	–	–	NR	–	–
Xylose	–	–	–	–	–	–

*, comparison of properties of strains K21 and K14, and *C. estertheticum* is included in Table 4.12; †, results from Cato *et al.* (1986); ‡, results from Wilde *et al.* (1989). Results in parentheses are for some strains of the species. +, positive; –, negative; w, weak reaction (pH from 5.5 to 5.9); NR, not reported; A, acetate; B, butyrate; L or l, lactate; ib, isobutyrate; ic, isocaproate; iV or iv, isovalerate; F or f, formate; o, oxalacetate, p, propionate; py, pyruvate; s, succinate; v, valerate; 2, ethanol; 4, butanol. Upper case letters indicate major and lower case letters indicate minor fermentation product.

Table 4.13. Some properties that differentiate strains K21 and K24 from other psychrophilic clostridia and *C. putrefaciens*.

Phenotypic property	K21/K24	*C. vincentii**	*C. estertheticum*†	*C. fimetarium*‡	*C. laramiense* §	*C. putrefaciens*
Maximum growth temperature (°C)	25	20	15	30	20	<37
Optimum growth temperature (°C)	14.5-21	12	10-12	20-25	15	15-22
Fermentation products	A,B,ic,l,py,2	A,F,B	B,A	A,F,L,2	A,B,ib,p	a,f,l,(s)
Starch hydrolysis	–	–	+	–	+	–
Fermentation of						
Arabinose	–	NR	+	+	–	–
Galactose	–	+	+	+	+	–
Mannitol	–	NR	+	–	+	–
Xylose	–	+	+	+	–	–

*, results from Mountfort *et al.* (1997); †, results from Collins *et al.* (1992); ‡, results from Kotsyurbenko *et al.* (1995); §, results from Kalchayanand *et al.* (1993); ¶, results from Cato *et al.* (1986). For abbreviation and symbols, see Table 4.12.

During growth in PYGS broth, the meat strains produced a complex mixture of five volatile and nonvolatile fatty acids and one alcohol, including isocaproic and pyruvic acids. None of the previously described psychrophilic clostridia are known to produce this combination of compounds (Cato *et al.*, 1986; Collins *et al.*, 1992; Kalchayanand *et al.*, 1993; Kotsyurbenko *et al.*, 1995; Mountfort *et al.*, 1997). The meat strains were readily distinguished from *C. estertheticum*, *C. fimetarium*, *C. laramiense*, *C. putrefaciens* and *C. vincentii* by their maximum and optimum growth temperature and substrate utilisation pattern (Table 4.13). In addition, *C. estertheticum* can hydrolyse esculin and ferment salicin and sorbose, while strains K21 and K24 cannot utilise these three substrates. *C. laramiense* exhibits lipase but not lecithinase activity, ferments inositol, raffinose and sucrose and reduces nitrate but does not hydrolyse gelatine (Kalchayananad *et al.*, 1993). In contrast, the meat strains are lipase negative, lecithinase positive, cannot ferment inositol, raffinose and sucrose, do not reduce nitrate and can hydrolyse gelatine. With respect to the optimum and maximum growth temperatures, the meat strains showed closest similarity with *C. putrefaciens*. However, unlike *C. putrefaciens*, strains K21 and K24 were saccharolytic. These strains also differed from *C. fimetarium* and *C. vincentii* in a range of utilisable substrates (Table 4.13).

A high level of 16S rRNA gene homogeneity and only small differences in phenotypic characteristics were found between strains K21 and K24, suggesting the two isolates should be classified as the same species. The results of phenotypic characterisation and chemotaxonomic analyses conducted in this study provided consistent evidence that strains K21 and K24 may represent a new species within the genus *Clostridium*. However, a high degree of 16S rRNA gene homogeneity between these strains and *C. estertheticum*, as well as some degree of similarity with respect to growth temperatures, ecological niche and meat spoilage potential indicate that DNA-DNA hybridisation studies may be required to confirm genomic distinctiveness of strains K21 and K24 from *C. estertheticum*, another 'blown pack' spoilage causing bacterium.

Deposition of the 16S rRNA gene sequence for strain K21 in the GenBank database, and deposition of this strain in the DSMZ culture collection have been delayed until its genomic distinctiveness has been established.

Conclusions

Seven meat strains of psychrophilic and psychrotrophic *Clostridium* spp. from vacuum-packed meats were characterised using a polyphasic approach. Phylogenetic analyses indicated that all the strains belong to the genus *Clostridium* (*sensu* Collins *et al.*, 1994). The meat strains differed from taxonomically related clostridia in cellular fatty acid composition, soluble protein profiles and phenotypic properties. On the basis of 16S rDNA similarity analysis, and phylogenetic and phenotypic characterisation five meat strains were assigned to one of three new species, for which the names *Clostridium frigidicarnis*, *Clostridium gasigenes* and *Clostridium algidixylanolyticum* are proposed. DNA-DNA hybridisation studies may be required to confirm genomic distinctiveness of the remaining two meat strains from previously described clostridial species.

General discussion

Cause of 'blown pack' spoilage: the search for a spoilage agent

Historically, spoilage of meat due to growth of clostridia has been attributed to a compromised chilling regime and/or processing hygiene and, with modern processing practices, this type of spoilage has been thought to be a matter only of historical interest (Roberts and Mead, 1986; Gill, 1991). Meat spoilage by psychrophilic and/or psychrotrophic species of clostridia had not been anticipated. Until 1989, only three of over 100 clostridial species were known to be able to grow at refrigeration temperatures (Cato *et al.*, 1986) and these had not been found in high numbers on vacuum-packed chilled meat. Consequently, when incidents of 'blown pack' spoilage of vacuum-packed chilled meats were initially reported (Dainty *et al.*, 1989; Kalchayanand *et al.*, 1989), it appeared that these might be scientific curios, and that populations of psychrophilic and/or psychrotrophic *Clostridium* spp. may be considered an ecological rarity, rather than a serious processing threat. Surprisingly, however, more incidents of clostridial spoilage of vacuum-packed meats have been reported and, in the studies that followed (Boerema *et al.*, 1997; Broda *et al.*, 1997), hundreds of psychrophilic and psychrotrophic clostridial isolates have been obtained from meat, slaughter stock and the meat plant environment.

Typically, searches for obligately anaerobic cold-loving microorganisms were conducted in anaerobic, permanently cold environments, such as deep marine sediments of the polar regions, and some of these searches have seen the successful isolation of psychrophilic and psychrotrophic clostridia (Starka and Stokes, 1960; Roberts and Hobbs, 1968; Jordan and McNicol, 1979). These microorganisms were thought to be less likely to occur in seasonally cold environments of varying anaerobiosis, such as a farm or meat processing plant. Furthermore, searches for psychrophilic clostridia in warm-blooded animals would not appear logical; these being long recognised as reservoirs of mesophilic clostridia associated with animal disease.

It is likely that the use of culture at temperatures suitable for the growth of mesophiles, as well as the use of media that selected for pathogenic clostridial species, might have prevented earlier detection of psychrophilic and psychrotrophic clostridia that survived as spores during passage through the digestive system of slaughter animals. The widespread presence of psychrophilic and psychrotrophic clostridia in slaughter animals may have been overlooked because this specific environment had been screened with inappropriate methods.

In the past, the spoilage status or shelf life expectancy assessments of meat have been strongly enumeration based. The propensity for spoilage was thought to correlate positively with the initial bacterial numbers present on the meat surface. Similarly, specific organisms needed to be present on meat in high numbers to be considered as primary causative agents of meat spoilage. Because the majority of bacteria associated with vacuum-packed meats were thought to be aerobic, facultatively anaerobic or microaerophilic species, these bacterial populations are routinely enumerated using aerobic methods (Bell *et al.*, 1997). It has been shown, however, that spoilage bacteria constitute only a minor proportion of the initial microflora present on meat and, consequently, total number of contamination microorganisms often provides a poor indication of an expected shelf life of a product (Mäkelä and Korkeala, 1987; Borch *et al.*, 1988). Similarly, 'blown pack' spoilage of clostridial aetiology, low or moderate counts of common spoilage microorganisms together with an absence of other identifiable spoilage agent(s) may not reflect the actual sensory attributes and spoilage status of a product.

Some microorganisms, e.g. *Shewanella putrefaciens*, are able to spoil meat when present in only moderate numbers. Others, including *Clostridium* spp., while being metabolically active on meat, may compete poorly in laboratory enrichment or even be inhibited on routine microbiological media. It is also possible for spoilage to be caused by novel microorganisms, such as psychrophilic or psychrotrophic clostridia, whose growth has been promoted in an ecological niche created by the changing meat processing system. It appears, therefore, that traditional, enumeration driven

assessment of microbiological quality of meat may need to be modified to allow the role of microorganisms that have high spoilage potential, but are difficult to cultivate or those associated with a specific niche in meat spoilage to be recognised. It is expected that the development of an alternative base for this assessment may be greatly helped by the use of molecular methods.

Our entire knowledge of the composition of meat microflora and its role in meat spoilage has been derived from culturing on laboratory media. It has been assumed that the nutritional and environmental requirements of spoilage associated microorganisms are known and are reproducible under laboratory conditions. However, as demonstrated with 'blown pack' causing clostridia, some bacterial species cannot be readily cultured on routine laboratory media and others may even be permanently non-culturable. Recently, it has been postulated that in some ecosystems as much as 99 % of the total microbial populations may have a viable but non-culturable status (Amann *et al.*, 1995; Pace, 1997; Ward *et al.*, 1998; Hugenholtz *et al.*, 1998). The diversity of microbial populations that occur naturally on meat, in slaughter stock and/or in farm and meat plant environments may be studied with the analysis of cloned rRNA genes (Amann *et al.*, 1994). It is expected that molecular characterisation of these naturally occurring microbial populations may give a new perspective on the spoilage and pathogenicity potential of meat associated bacteria.

In the present study the traditional approach to determining the cause of meat spoilage has been explored. With this approach, bacterial isolation, identification and typing are necessary to confirm that a microorganism causes specific spoilage symptoms and to determine the source of that spoilage agent. However, recent developments in molecular ecology and epidemiology appear to question this traditional approach. It is now recognised that with some (e.g. non-culturable) microorganisms it may not be possible to satisfy the Koch's third postulate in its traditional form, i.e. under experimental conditions to reproduce disease/spoilage symptoms identical to those observed originally. Similarly, with psychrophilic and psychrotrophic clostridia that did not caused 'blown pack' spoilage symptoms when inoculated in pure cultures, future

studies may determine whether synergy between several strains or the density of clostridial populations on meat are significant determinants in the occurrence of 'blown pack' spoilage.

Low temperature is increasingly being used to extend shelf life of fresh meat. Between meat production and its consumption, numerous opportunities are presented to low-temperature-growing organisms to colonise this habitat. The changes to food processing and preservation inevitably mean new ecological niches are being created. The different conditions select microbial populations that differ from those traditionally associated with products. These are likely to be either opportunistic psychrophilic and psychrotrophic bacteria that are in the right place at the right time or bacteria able to adapt to cold habitats (Palumbo, 1986; D'Aoust, 1991; Gounot, 1991). It is expected that the role of both these microbial groups in meat spoilage and food-borne disease will increase in coming years.

Cause of 'blown pack' spoilage: taxonomic considerations

In the last decade, bacterial systematics has been strongly shaped by phylogenetic data. Many bacterial groups have been renamed and reclassified to reflect 'natural' relationships of their members. In recent years, description of many previously unrecognised, as well as novel, species of spoilage microorganisms has usually employed the polyphasic approach that, as currently used, is directed by phylogenetic data. To fulfil the requirements of this approach, considerable effort has been made in the present study to describe phylogenetic placement of the newly described species *C. frigidicarnis*, *C. gasigenes* and *C. algidixylanolyticum* among their closest relatives within the genus *Clostridium*.

The intellectual desire to classify organisms according to the 'natural order' is an ancient one. Early cladists considered taxonomy to be the highest science because the insight into arrangement of God-made species was thought to allow insight into God's mind (Gould, 1998). It was through the use of molecular methods that it was thought that, for the first time in history, 'natural' relationships between microorganisms could

be deduced. In a relatively short time cladistic data have become predominant in the classification and delineation of microbial taxa. However, the role that lateral transfer of genes between both closely or distantly related organisms has been and is playing in confounding the cladistic approach is increasingly recognised (Doolittle, 1999).

The widespread exchange of microbial genes may mean that the gene hierarchy cannot be treated as a synonym for hierarchy of whole organisms and that, with gene data, the construction of an inclusive hierarchy of microorganisms may not be possible. In systematics, lateral transfer of microbial genes changes the way various taxonomic groups are delineated, while they can be described by a proportion of shared genes, these groups no longer can be described by shared ancestry. Consequently, phylogenetic analysis for classification and delineation of microbial taxa can be considered redundant.

In contrast to early, artificial classifications of microorganisms, the use of molecular sequence data for constructing phylogenetic classifications has promised to give a coherent, objective base to microbial systematics. However, genotypic approaches have failed to derive a precise definition of the basic taxonomic rank, the microbial species, and a basis for its recognition has remained empirical and subjective. Throughout history, microbiologists were free to recognise as a species any group of strains that had some common characteristics that could be satisfactorily distinguished from other groups of strains. Today, microbial species may be viewed as a group of strains that share a basic genome structure but to increase adaptability to various environments, commonly exchange genes with other microbial groups. It, perhaps, should come as no surprise that classification of phenotypically and genotypically disparate entities lacks academic coherency.

The majority of microbiologists working in the area of food-borne disease and food spoilage recognise sufficient stability of clonal populations of bacteria for bacterial species to be recognised as more than a purely artificial concept. Notwithstanding the ongoing discussions concerning natural versus artificial classifications, simple

classification schemes that enable ready recognition of an unknown microorganism need to be available to bacteriologists for the purpose of disease/spoilage diagnosis. Although with molecular approaches individual bacterial strains can be characterised with great precision, their identification had become increasingly difficult. The present study demonstrated that neither a phenotypic nor a genotypic approach enabled the allocation of some meat strains of 'blown pack' associated clostridia to known clostridial species. It is thought that the transformation of the data gathered in this study into a practical identification system that enables recognition of psychrophilic and psychrotrophic clostridia should take precedence in future taxonomic studies.

In the present study, attempts to identify some of the NZ psychrotolerant *C. botulinum*-like meat strains accentuated the conflict of the academic and diagnostic approaches to the taxonomy of these microorganisms. With the 16S rRNA gene approach, these meat strains would have been identified as *C. botulinum*. Traditionally, however, the 'botulinum' epithet has been assigned solely on the basis of a diagnostically important property, botulinal neurotoxin production. The New Zealand clostridial isolates have been found unable to produce neurotoxin and, *sensu stricto*, these isolates are not *C. botulinum*. Furthermore, the presence of these isolates on vacuum-packed meat will not compromise microbiological safety of the product. For practical reasons, taxonomy for diagnosis requires that bacterial nomenclature reflects the connection between a microorganisms and disease. With human and animal pathogens, this pragmatic taxonomy overrides taxonomy that is developed for academic purposes. Species epithets of New Zealand *C. botulinum*-like isolates derived from the 16S rRNA gene sequence comparisons would not reflect their non-toxigenic status and would incorrectly imply that food safety hazard exists when one does not exist. Consequently, it is believed that these isolates should be renamed.

Recent developments in microbial systematics have certainly elucidated the extent of the complexity of bacterial relationships. The occurrence of lateral gene transfer between microbial species has recalled the worst horror of early cladists: "Were these units capable of blending with one another indefinitely, they would no longer be units,

and species could not be recognised. The system of life would be a maze of complexities (...) it would be unintelligible chaos to man" (Dana, 1857; in Gould, 1998). As shown with molecular approaches to classification and delineation of taxa, chaos may actually be the natural state of microbial relationships. Furthermore, lateral gene transfer that, with microorganisms, occurs by natural mechanisms, has become a reality for higher organisms by means of human intervention, genetic engineering.

Control of 'blown pack' spoilage: possibilities for intervention

Research into the cause of 'blown pack' spoilage has been undertaken in the present study because it was believed that knowledge of the causative agent(s) would enable modification of processing conditions in the way that growth of these agents on vacuum-packed meat is prevented.

With at least some species, spores of psychrophilic and psychrotrophic clostridia causing 'blown pack' spoilage appear to be brought into the meat processing environment by the slaughter animals. The route for carcass contamination with these organisms appears to be extrinsic and contamination is likely to occur by contact with the slaughter animals' own faeces or hides. Control of 'blown pack' spoilage of vacuum-packed meat can, thus, be approached in a manner that includes (1) preventing the initial contamination, (2) removing clostridia from carcasses or (3) preventing their germination and growth on meat.

Prevention of carcass contamination in the first place would concern general measures to improve plant sanitation and dressing hygiene. It is possible that the initial load of 'blown pack' spoilage causing clostridia on the carcass can be reduced by limiting the direct access to the slaughter floor of poorly presented/dirty animal stock, or by applying a different dressing system. Removal from the abattoir of in-plant sources of psychrophilic and psychrotrophic clostridia would minimise re-contamination of the product. However, due to the high prevalence of these microorganisms in slaughter stock (Boerema *et al.*, 1997) the limitation rather than absolute prevention of transfer of clostridia onto carcasses may only be feasible. Plant sanitation and process hygiene

would remain paramount in containing, if not controlling, meat plant spread of psychrophilic and psychrotrophic *Clostridium* spp.

Because 'blown pack' spoilage clostridia are likely to be present on carcasses as spores, extreme carcass/cut decontamination procedures, such as γ-irradiation of the packed product, would be required to ensure their elimination. Such decontamination treatments are currently unacceptable in New Zealand meat processing.

An alternative approach to controlling 'blown pack' spoilage may be prevention of the outgrowth of clostridial spores present on meat cuts by inhibition of spore activation and/or germination. Heat-shock is often required to activate clostridial spores. To clostridial spores present on the meat surface this requirement may be satisfied during the heat shrinking procedure applied to the meat cut after vacuum packaging. This procedure consists of brief immersion in, or exposure of the packed product to, hot water or steam, respectively. On the other hand, clostridia are renowned for their exact germination requirements. Little is known, however, about conditions under which the germination of psychrophilic and psychrotrophic clostridial species may occur in vacuum-packed meat. It has been suggested that storage temperature, meat pH, glucose/lactate availability, interactions between clostridia and competing microflora, and combinations of these factors may be involved. Whether inhibition of the outgrowth of clostridial spores may be achieved through judicious selection of appropriate combinations of the above factors remains to be determined.

Although this study did not deliver an instant answer concerning control of 'blown pack' spoilage, it has added to our understanding of the aetiology of this type of spoilage. It is hoped that this knowledge will advance the process of developing efficient measures to control meat spoilage caused by the growth of psychrophilic and psychrotrophic clostridia.

Summary of Conclusions

- Conventional phenotype-based methods were found to be ineffective for the identification of psychrophilic and psychrotrophic clostridia associated with the spoilage of chilled meats.
- 16S rDNA based molecular methods were shown to effect differentiation of psychrophilic and psychrotrophic clostridia at a genotypic species level and below. The use of these methods for the identification of unknown clostridial isolates needs to be approached with care.
- With genomic DNA- and/or the 16S-23S rDNA internal transcribed spacer-based methods, strains of psychrophilic and psychrotrophic clostridia from their natural sources and from spoiled meat can be differentiated at intra-species or inter-strain levels.
- Hides and faeces of slaughter animals were identified as a common source of many species of psychrophilic and psychrotrophic clostridia. One of these species was confirmed as a causative agent of 'blown pack' spoilage.
- Eight local isolates of psychrotrophic clostridia were identified with genotypic methods as non-toxigenic non-proteolytic *C. botulinum* type B-like strains.
- Five local isolates of psychrophilic and psychrotrophic clostridia were found to represent three previously undescribed clostridial species; these new species have been characterised and named.

Recommendations for Future Research

- Molecular methods should be applied to determine qualitative composition of naturally occurring microbial populations on slaughter animals, dressed carcasses and in the meat plant environment.
- *C. botulinum*-like isolates should be renamed to reflect the non-toxigenic status of New Zealand derived meat strains.
- The effect of meat processing procedures, such as heat shrinking of vacuum-packed product, on the germination of spores of psychrophilic and psychrotrophic clostridia should be determined.
- DNA-DNA homology studies should be employed to establish whether isolates K21 and K24 belong to known or previously undescribed clostridial species
- Molecular highly discriminatory methods should be used to assess clonality of source and spoiled product derived isolates of psychrophilic clostridia.
- A routine system, based on available phenotypic and genotypic information, should be developed to enable ready recognition of psychrophilic and psychrotrophic clostridia associated with 'blown pack' spoilage of vacuum-packed meats.

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