ENUMERATION AND DISCRIMINATION OF THERMOPHILIC BACILLI IN MILK POWDER

A thesis

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by

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Abstract

A survey of milk powders from eighteen different countries has established that the same cohorts of strains of thermophilic bacilli are the major contaminants regardless of source. In particular, *Anoxybacillus flavithermus* strain C emerges as the most commonly encountered contaminant especially in powders with a medium- to high thermophile count. *Bacillus licheniformis* strain F was the next most common contaminant overall which dominated low- to medium count powders. This organism was also the broadest distributed being present in 96% of the milk powders screened. The thermophilic *Geobacillus stearothermophilus* strain A represented the third most often occurring milk powder isolate. The presence of *B. licheniformis* strain G, *Bacillus subtilis* and *A. flavithermus* strain D as low-level contaminants of milk powder was reconfirmed.

Methods have been developed for the rapid and efficient extraction of bacteria from milk powder and their enumeration using real-time PCR methodology based on the 16S rRNA gene or the *spo0A* gene. Further modifications to these methods allow discrimination between live and dead cells and between spores and vegetative cells. The former is important because the majority of vegetative cells in milk powder are dead as a result of processing stresses, yet their DNA remains available for amplification. Limits of detection of these methods for viable cells and spores are less than 1000 thermophiles per gram of milk powder. These methods can yield results within a time period of 90 minutes and thus are amenable to real-time monitoring of factory contamination *in situ.*

A range of other methodologies and approaches to detecte and enumerate thermophilic bacilli were investigated but were not regarded as applicable. The use of antibodies raised against thermophilic bacilli did not produce sufficient discrimination between strains and did not allow enumeration of different strains with the same efficiency of detection, and could not differentiate between live and dead cells. DGGE-PCR performed on the highly conserved region of the 16S rRNA gene also exposed the presence of organisms other than thermophilic bacilli in milk powder compromising the resolution of the detection method. RAPD-PCR provided a high

discrimination between the strains but was not applicable for the use in a factory setting.

Overall, the discovery that virtually all milk powders produced internationally and in New Zealand are dominated by thermophilic strains representing four species is a fundamental finding regarding factory-derived contamination. This will have long-term effects on the future operation and design of evaporator lines, and has important economic implications with respect to optimizing day-to-day factory operation and storage of milk powders. In addition, the quantitative real-time PCR assays from this study should allow for a robust control of the drying process during extended run times and thus, are of important economical benefices to milk powder manufacturer.

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Abbreviations

A	ampere
A	adenine
ATCC	American type culture collection
bp	base pairs
BSA	bovine serum albumin
C	cytosine
CIP	cleaning-in-place
cfu	colony forming unit
C_t	threshold cycle number
dNTP	deoxynucleotide triphosphate (dATP, dTTP, dGTP and dCTP)
DSI	direct steam injection
DSM	Deutsche Sammlung von Mikroorganismen
EDTA	Ethylenediaminetetraacetic acid
f	Femto [10^{-15}]
g	gram
g	gravitational force
G	guanosine
I	inosine
IgG	immunoglobulin G
λ	Lambda (wave length)
m	Milli [10^{-3}]
m	meter
M	molarity
ml	thousandth part of a liter
µ	Micro [10^{-6}]
n	Nano [10^{-9}]
N	normality
N	any nucleotide (adenine, thymine, guanosine and cytosine)
nm	nanometer
p	Pico [10^{-12}]
PCR	polymerase chain reaction
PBS	phosphate buffered saline buffer

r	correlation coefficient
rDNA	ribosomal DNA
rpm	revolutions per minute
T	temperature
T	thymine
T_m	melting point temperature
TAE	tris acetic acid EDTA buffer
TE	Tris EDTA buffer
Tris	tris-(hydroxymethyl)-aminomethane
tRNA	transfer RNA
TRU	Thermophile Research Unit
TSA	tryptic soy agar
TSB	tryptic soy broth
UV	ultra violet
V	volts
V	volume
v/v	volume per volume
w/v	weight per volume

Literature review

1.1. Milk Composition and Properties

Bovine milk is a complex nutritional fluid composed of water, lipids, carbohydrates, proteins, minerals and vitamins. Approximately 87% of fresh bovine milk consists of water (Jenness and Sloan, 1970) and thus, the physical properties of milk are primarily those of an aqueous system. All other constituents, either polar or apolar are dissolved, dispersed or emulsified. Milk contains an average of 4.8% α-lactose (β-D-galactopyranosyl-(1-4)-β-D-glucopyranose) as the primary carbohydrate which is regarded as the principle carbon source for the growth of contaminating micro-organisms. Further, free glucose and galactose can be found at low concentrations of approximately 0.1 mM (Jenness and Sloan, 1970).

Lipids contribute approximately 3.9% of the milk components, which are mainly present as triglycerides (98%) (Walstra and Jenness, 1984). The remaining lipids include mono- and diglycerides, free fatty acids, phospholipids and sterols. The fat in milk occurs nearly entirely as globules of triglyceride which are surrounded by a lipid bilayer membrane similar to the structure of a typical cell membrane, with the polar groups on the outside stabilizing the fat globules, helping to form an emulsion.

Proteins in bovine milk make up approximately 3.25% of the milk of which approximately 80% consists of caseins. There are four types of casein and they are classified by their solubility upon acidification to pH 4.6 which include the α_{s1}-, α_{s2}-, β-, and κ-caseins. Principally, caseins are proteins which are conjugated by ester-bound phosphate and serine residues. Most of the caseins exist as multi-colloidal particles, also known as casein micelles, which are linked to each other mediated by interactions involving calcium and phosphorous (Walstra and Jenness, 1984; Walstra, 1990). The remaining 20% of the protein component are the so called "whey proteins" including mainly β-lactoglobulin, α-lactoglobulin, bovine serum albumin and immunoglobulins.

Milk also contains several minerals, particularly calcium, phosphorous, potassium and zinc. Furthermore, all B vitamins and all fat soluble vitamins (A, D, E and K) can also be found in milk.

1.2. Micro-organisms associated with milk

The highly nutritious properties of milk can allow for extensive growth of a large variety of micro-organisms including Gram-negative and Gram-positive bacteria, yeast and fungi (Phillips and Griffiths, 1990; Gilmour and Rowe, 1990). In general, micro-organisms in milk are not classified by their Gram stain but with reference to their capability to grow and multiply at various temperature ranges. Accordingly, organisms which occur in milk products and elsewhere are divided into psychrophiles, psychrotrophs, thermodurics, mesophiles and thermophiles. Psychrophiles are micro-organisms whose cardinal growth temperatures (minimum, optimum and maximum) are at or below 0, 15 and 20°C, respectively. Micro-organisms which grow at psychrophilic conditions but also at higher temperatures are termed "psychrotrophs" (Morita, 1975; Helmke and Weyland, 2004). The optimum temperature of growth for psychrophiles and psychrotrophs is not well defined due to the great diversity of such organisms that can occur in milk. However, most of these organisms will grow rapidly at 21°C (Cousins et al., 1977), whereas many psychrophiles are also able to grow under refrigeration conditions (Muir and Phillips, 1984; Greene and Jezeski, 1954; Richard, 1981). Psychrophiles and psychrotrophs are essentially equivalent in regards to their sensitivity to heat, with these organisms being destroyed by pasteurisation. Mesophiles are classified as the group of organisms with an optimal growth temperature range of 20°C to 45°C whereas thermophiles grow above 45°C (Singleton and Sainsbury, 1987). Thermoduric organisms survive pasteurisation and heat-treatments of 63°C for 30 minutes (Phillips and Griffiths, 1990) but do not grow at these temperatures.

Psychrophilic and psychrotrophic contaminants typically include genera of *Achromobacter*, *Acinetobacter*, *Aeromonas*, *Chromobacterium*, *Enterobacter*, *Escherichia*, *Flavobacterium*, *Klebsiella*, *Pseudomonas*, *Bacillus* and *Serratia* (Phillips and Griffiths, 1990; Bramley and McKinnon, 1990). This group of contaminants also includes pathogenic bacteria such as *Bacillus cereus*,

Staphylococcus aureus, *Salmonella typhii/typhimurium*, *Escherichia coli*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Listeria monocytogenes* and some *Clostridial* species. Phillips and Griffiths (1990) also reported that about 86% of the psychrotrophic bacteria isolated from raw milk were *Bacillus* species such as *B. cereus*, *Bacillus circulans*, *Bacillus coagulans*, *Bacillus subtilis* and *Bacillus licheniformis* (Phillips and Griffiths, 1986; Mikolajcik, 1979).

Bacteria which are able to survive pasteurisation of 63°C for 30 minutes or 72°C for 15 seconds are classified as thermoduric organisms. Thermoduric genera associated with milk are typically positive to the Gram strain and include members of *Bacillus* and *Clostridium*. *Alcaligenes tolerans* is the only Gram-negative organism associated with milk processing which is reported to survive pasteurization procedures. However, the most important thermoduric bacteria in milk processing are aerobic spore-formers of the genus *Bacillus*. Spores of bacilli are ubiquitously distributed and occur in many environments such as soil, sediments and natural water sources. For example, spores of *Bacillus* have been found in grass and silage ranging from 10^2 to 10^6 colony-forming units per gram (cfu g^{-1}) (Te Giffel et al., 2002). These spores pass unaffected through the gastrointestinal tracts of cows, from which they can be excreted in the faeces, thus, contaminating the udder and animal bedding. Silage has also been shown to contribute substantially to the contaminating load of raw milk (Te Giffel et al., 2002).

1.3. Thermophilic bacilli in milk powder processing

The duration and temperature conditions applied during the manufacture of milk powders are especially suited for the growth of thermophilic bacilli (Stadhouders et al., 1982; Kwee et al., 1986; Murphy et al., 1999). Due to the rather unique and restrictive thermophilic environment during milk production the diversity of bacterial species growing during the drying process is restricted to thermophilic bacilli only. However, other groups of microorganisms such as psychrotrophs and mesophiles can also compromise the quality of milk powders due to poor milk handling prior to milk processing. In particular the transport and storage of the raw milk between the farms and the factory site under non-refrigerated conditions can cause significant growth of non-thermophiles. Poor milk handling can cause deterioration in protein structure,

which can increase biofouling when subjected to temperature stress, and rupturing of fat micelles and membranes can influence the lipid distribution, which can in turn affect the microbial flora that can develop. Whereas previously all thermophilic isolates from powder were commonly assigned to the ubiquitously distributed *Bacillus stearothermophilus*, *Bacillus licheniformis* and *Bacillus subtilis*, modern molecular genetic approaches can now be used to obtain more accurate results, which help to distinguish species on strain and even on sub-strain level. Extensive investigations of thermophilic contaminants being present in milk powder indicated that one or more of four species of thermophilic bacilli normally dominate powders. Furthermore, these contaminating species seem to be ubiquitous in milk powder processing facilities throughout the world, and in an extensive survey these thermophiles were found to constitute over 96% of all contaminants in milk powders (Rueckert et al., 2004). These four species and their closely related sub-strains are *Geobacillus stearothermophilus* (strain A), *Anoxybacillus flavithermus* (strains B, C and D), *B. licheniformis* (strains F and G) and *B. subtilis* (strain BS), with strains C, F and A being the predominant contaminants (Ronimus et al., 2003; Rueckert et al., 2004). In both studies, the strain identification was performed by the molecular procedure of Randomly Amplified Polymorphic DNA or RAPD profiling.

The typical number of bacilli (psychrotrophs, mesophiles and thermophiles) in raw milk, usually as spores, is generally low, in the range of ≤ 50 colony-forming units per ml (Martin, 1974; Phillips and Griffiths, 1986; McGuiggan, et al., 1994; Cook and Sandeman, 2001). Typically, *B. licheniformis*, *Bacillus pumilus*, *Bacillus brevis*, *Bacillus megaterium* and *B. subtilis* are purportedly the most common species (Martin, 1981; Phillips and Griffiths, 1986; Waes, 1976, Cook and Sandeman, 2001). These thermo-resistant spores are introduced into the processing line with the raw milk, and most are able to withstand the heat-treatment applied during processing to milk powder, as was shown in laboratory experiments by Janštová et al. (2001). Moreover, thermophilic strains of bacilli are capable to adhere and colonize on surfaces (Biofilm) within the stainless steel tubes of the pre-heaters and evaporators in areas where temperatures and viscosities allow for their growth. In general, biofilms consist of a variety of different microorganisms (communities) colonizing environmental surfaces. The mechanisms involved in biofilm formation are only poorly understood and the simplified theories of adhesion suggest two stages in the

process, involving van der Waals's attractive forces, electrostatic forces, hydrophobic and steric forces. In the second stage, bacteria attached to the surface produce, and release, extra-cellular polysaccharides which provide a strong permanent adhesion (Flint et al., 1997). Hausmark and Rönner (1992) observed that spores attach and colonize more readily than vegetative cells on stainless steel surfaces, presumably due to their relatively high hydrophobicity. These findings were supported by similar experimentation by Flint et al. (2001). The effect of hydrophobicity on the efficiency of adherence to surfaces was also investigated by Weincek et al. (1991) using two strains of *B. subtilis* spores, which differed in hydrophobicity properties. The study also reported that the more hydrophobic strain attached in greater numbers and that exopolysaccharides secreted by the bacteria may have played a determinative role in the formation and development of biofilms. The polymer matrixes produced by these bacteria served as a protective matrix for the embedded cells from environmental stress such as chemicals, turbulent flow or nutrient limitation (Davey and O'Toole, 2000). In addition, bacteria in biofilms are thought to have greater protection from biocides and cleaning agents commonly used during CIP regimes in milk processing factories than free-living planktonic cells. This protection has been attributed to diffusion limitation or the neutralization of biocides through the exopolymer matrix (Davey and O'Toole, 2000).

Parkar et al. (2003) could show that thermophilic bacilli readily attached to stainless steel surfaces and formed mature biofilms within six hours of incubation in pasteurized skim milk at 55°C. In addition, stainless steel surfaces covered with a fouling layer after contact with pasteurized skim milk tended to attract 10 to 100 times more vegetative cells and spores of *G. stearothermophilus* than clean surfaces. Similarly, foulant in suspension also promoted up to 90% of planktonic *G. stearothermophilus* cells to be adsorbed (Flint et al., 2001). Fouling in the plant is due to the mechanical and thermal denaturing of milk proteins, which adsorb to surfaces (particularly hot surfaces) more readily and can form an accumulation (film) many layers thick. Fouling is also thought to be the predominant mechanisms behind bacterial attachment and colonization to stainless steel during processing in dairy plants.

Barnes et al. (1999) reported that skim milk or individual milk proteins such as α-casein, β-casein and κ-casein adsorbed to stainless steel substantially reduced the

number of attached bacteria compared to clean surfaces due to blockage of adhesion sites for cell attachment. Thus, according to Barnes et al. (1999) the conditioning of stainless steel with proteins could provide a short-term solution to reduce the attachment of microorganisms to the surface of stainless steel. However, biofilm development can be difficult to control, even with proper cleaning procedures such as CIP. Within the working environment of a milk powder processing line it is generally the case that biofilm formation of thermophilic bacilli on stainless steel surfaces will establish and mature, usually within a period of 18 to 24 hours.

Biofilms are thought to represent the major cause of product contamination in processing due to biotransfer of bacteria from the biofilm into the process stream. Where thermophilic bacilli constitute the biofilm organism then both spores and vegetative cells of the species can be transferred in this manner. Since sporulation is normally a response to stress or nutrient limitation, the presence of spores might not be expected in early development of biofilm. Flint et al (2001) reported that within the first 18 hours of biofilm formation, vegetative cells exceeded spore-forms both in the biofilm and in the processing stream passing over the biofilm. Presumably, premature biofilms in the first 18 hours provide for sufficient nutrition to cells embedded, whereas in mature biofilms, cells located below the surface layers are likely to experience nutrient limitation and thus sporulate.

1.4. A general overview of milk processing for powder production

Fresh bovine milk as it is received from the cow is usually regarded as sterile and contaminating bacteria are introduced into the milk by post-milking handling when the milk comes in contact with environmental sources such as soil on contaminated teats and/or the numerous surfaces of the milking equipment during transport and processing (Muir, 1990; Christiansson et al., 1999). Silage as mentioned previously, is also considered to be a source of contamination (Te Giffel et al., 2002; Christiansson et al., 1999).

A typical milk powder processing line is illustrated schematically in Figure 1.1. Immediately after the cow has been milked, the milk is cooled to 5°C or below and stored under refrigeration until delivered to the milk plant. On arrival at the plant, the raw milk is collected and stored at 8 to 10°C in silos, usually for no more than 16 to 24 hours, but for no longer than 72 hours. During this period of storage the milk is

pasteurized at 72°C for 15 seconds and subsequently its butter-fat content is measured and the milk brought to a constant butter-fat content so that a powder with consistent characteristics is produced. The butter-fat content of raw milk can vary according to season and feed supply and is normally adjusted to approximately 3.5% (w/v) for whole powder manufacture. This involves heating the milk to approximately 50°C, removing the fat by centrifugation and adding back fat to the required amount from the cream. For skim milk powders no addition of removed fat occurs. Following butter-fat standardization the milk is stored under refrigeration until processing begins (Figure 1.1). In many plants the pasteurization step follows standardization in the process line, though the position and timing of this treatment varies with the plant set-up. Processing is initiated by passing the milk through the pre-heat section of the evaporators, where it is heated using the waste heat of the evaporation line. The large surface area of the heat exchanger and the temperature range from 45 to 75°C used in these pre-heaters offer good growth conditions for thermophilic bacteria which can colonize the surfaces and form biofilms (Flint at al., 2001; Parkar et al., 2003). As processing time continues the bacilli growing in these biofilms can be transferred into the product stream. At the end of the pre-heat section the milk will have reached a temperature of approximately 72°C before being passed through the DSI (direct steam injector), which raises the milk to the temperature specified for processing. Temperatures of between 85°C to 92°C are most common and the chosen temperature relates to the desired properties required for the powder end-use. These heat treatments impart desired functional properties to the powder such as powder solubility, the inactivation of lipases and proteases responsible for lipolysis and proteolysis during powder storage (Chen et al., 2002), or the activation of natural antioxidants, i.e. -SH groups originating from cysteine and methionine (GEA Niro A/S; Denmark). Steam injection may be applied for periods of up to 45 seconds in order to raise the milk to the specified temperature and this time/temperature exposure will undoubtedly result in the death of a large number of the vegetative cells of thermophilic bacilli formed in biofilms in the pre-heat section, and of any non-thermophilic microbes present in the raw milk. Spores of thermophilic bacilli are most likely to survive this heat exposure and are thought to be the major source of contamination for the rest of the process line.

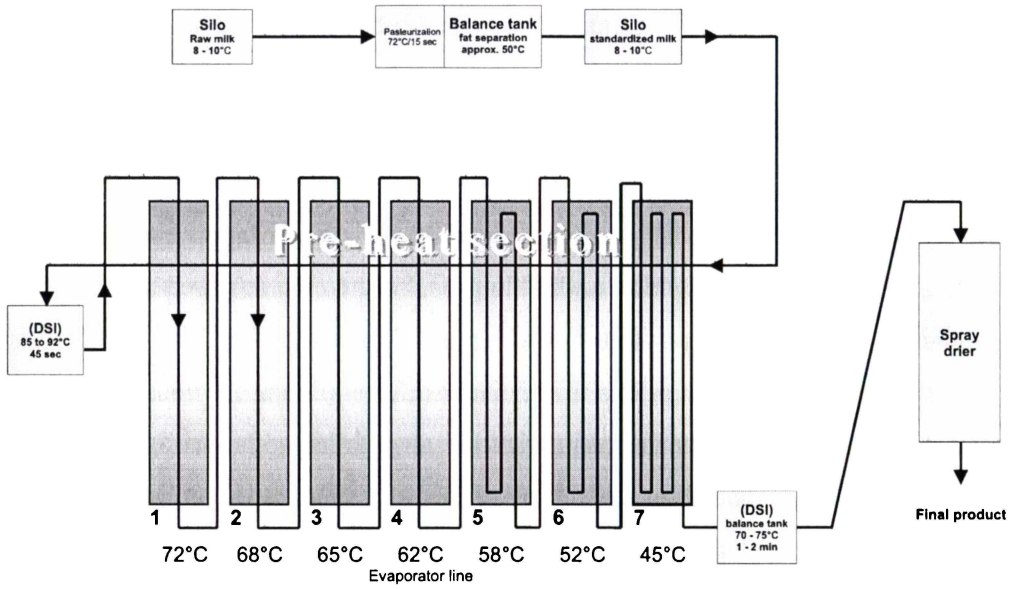

Figure 1.1. Schematic flow diagram of a typical milk powder processing plant.

After heat-treatment the milk undergoes a two-step water-removal process, which ultimately removes approximately 95% of the total water. The first step involves the milk being pumped through the evaporation line operating typically from 72 to 46°C under vacuum in order to lower the boiling temperature of water. Conventionally, a series of falling-film calandria are used whereby the milk is distributed into steel tubes (up to 200 per calandria) so that a film of milk under vacuum falls a distance of approximately 20 meters while in contact with the hot steel surface (heated on the other side by hot water). As water is lost through evaporation the milk cools, is collected at the base of the calandria and pumped to the top of the next calandria in the series. Between 3 to 7 calandria are used in a processing line frequently with more than one pass of the milk over a calandria in some circumstances. After evaporation the milk will have been concentrated to approximately 50 to 52% total milk solids and its temperature dropped to around 42 to 45°C. Milk concentrate is held at this temperature in a balance tank (typically for 1 to 2 hours) to ensure adequate volumes are available to maintain the operation of the spray-drier for the desired run time. Prior to entry to the spray-drier the temperature of the concentrate is again raised by DSI to temperatures of typically 70 to 75°C for 1 to 2 minutes in order to increase the efficiency of spray drying. The final step, spray drying, involves the dispersion of the atomized milk concentrate into a stream of hot air, typically at 180 to 200°C, where almost all moisture is removed from the concentrate after a very short residence time of a few seconds. The atomizer can

either be a pressure nozzle or a centrifugal disc. Spray-drying yields the final milk powder. Following powder formation, the product immediately enters a system of cyclones to cool and collect the powder. Finally, the product is packaged and stored, in some cases in plastic impermeable layers under a nitrogen atmosphere. At the end of milk processing the bacterial level of contaminants (thermophiles, etc.) is routinely determined by plate counting to grade the final milk powder. The time is takes from the point of entry into the evaporator pre-heat to the production of a bagged powder will take no more than 30 minutes. In other words, a thermophilic bacterium suspended in the milk entering the line would be unable to achieve more than one cell division. Even this is unlikely since growth in many parts of the process line will be slow or inhibited due to temperatures either being too high or low, or the water activity of the milk being restrictive during the later stages of evaporation. Thus increases in numbers of thermophilic bacteria in the powder reflect growth of biofilms of thermophilic bacteria at particular parts of the plant suited to their growth, and the shedding of organisms into the milk flow as biofilms develop and mature. The parts of the plant that are implicated in major growth are the pre-heat line between 45 to 70°C, and those evaporators operating at temperatures at and above 55°C, and where the water activity of milk is not restrictive (usually evaporators number 2 and 3 are commonly implicated in a seven calandria line). The final thermophile count in the powder will reflect the number of viable cells and spores shed into the line during processing and the numbers killed during the DSI stages and the spray-drying. Commonly, numbers of thermophiles are very low (<100 cfu g^{-1}) when processing proceeds after cleaning in place (where biofilms are reduced or removed) and remain low for periods of up to 12 hours, and thereafter often exhibit a steep increase such that by 18 to 24 hours more than 30,000 cfu g^{-1} can often be present.

1.5. Methods for detection and identification of contaminants in milk and other foods

There are a variety of molecular, biochemical and microbiological methods available to detect and identify micro-organisms associated with milk and food stuffs. Microbiological methods include physiological and biochemical test kits such as, for example, the API 20E (API, bioMerieux Vitek, Hazelwood, Mo.), the Vitek GNI card (Vitek; bioMerieux Vitek), and the Becton Dickinson Cobas Micro ID-E/NF (Cobas; Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.). These kits are

designed for a wide variety of micro-organisms covering nearly all bacterial groups. In addition, these test systems are easy-to-use, not very labor intensive and results can be gained after approximately 2 to 48 hours of incubation. However, physiological tests have limitations and the results obtained are not always reproducible or well correlated, for example, to molecular biological methods based on PCR. In addition, many methods only allow identification of micro-organisms to the genus level (O'Hara et al., 1993; Juang and Morgan, 2001). However, from the general perspective of a dairy factory the enumeration of bacteria is of higher importance than their identification at species- or strain-level. Commonly, groups of organisms either psychrotrophs, mesophiles or thermophiles are assessed using selective media, which are incubated under particular temperature conditions. On the other hand, the detection and identification of food-borne microorganisms is sometimes required in order to ascertain the absence of pathogenic or toxin-producing organisms in special foods such as baby-food or in food for immuno-compromised patients. In addition, the identification of thermophilic contaminants can also help in the understanding of the distribution of thermophiles within the process run, especially, when new processing technologies are applied. For example, the more heat-resistant and osmoto-intolerant *G. stearothermophilus* will be found at locations with higher temperature and water-activity such as those occurring after DSI prior to evaporation. On the other hand, the relatively osmotolerant and more heat-sensitive *B. lichenifomis* will be found further downstream in the evaporation line at lower operating temperatures and water-activity.

Some molecular biological techniques used in scientific laboratories are capable of assessing both, the number and types of organisms. However, their routine application in a factory setting has to be validated for each individual technique regarding complexity and cost.. These techniques are mostly based on the amplification of single or multiple DNA segments, e.g. randomly amplified polymorphic DNA-PCR (RAPD-PCR), arbitrarily primed PCR (AP-PCR), repetitive extragenetic palindromic PCR (REP-PCR), restriction fragment length polymorphism PCR (RFLP-PCR), PCR ribotyping, real-time PCR and denaturing gradient gel electrophoresis PCR (PCR-DGGE).

REP-PCR, for instance, targets the repetitive extra-genetic interspersed sequence elements whose size and distribution within the genome is unique and conserved for individual bacterial strains (Malathum et al., 1998; Cherif et al., 2003).

RFLP-PCR is based on the digestion of DNA with a restriction endonuclease producing DNA fragment fingerprints which often differ between organisms. In order to obtain sufficient DNA for restriction digest analysis a specific DNA sequence can be amplified using PCR prior to RFLP. For instance, this technique has been successfully use to distinguish the A, B and O alleles of the ABO blood group polymorphisms in humans, demonstrating the discriminating power of this technique (Mifsud et al., 1996). PCR-ribotyping is a technique amplifying the 16S-23S intergenic spacer region of bacterial rRNA operons by using primers targeting the highly conserved regions at the 3'- and 5'-end of the 16S and 23S rRNA gene, respectively (Dasen et al., 1994; Bidet et al., 2000). The sequence heterogeneity and sequence length polymorphism within the spacer region is reflected by a unique PCR product fingerprint similar to those obtained by RAPD-PCR. Similarity or dissimilarity of the amplicon patterns generated can be used to differentiate species. Unfortunately, DNA fingerprinting methods are only useful in the determination of unidentified organisms when appropriate reference fingerprint patterns are available which can be used for comparative identification. In order to classify and identify new or unidentified organisms 16S rRNA gene amplification and sequencing is then the method of choice. When the sequence of an organism is obtained, it can be used in a BLASTN search and sequence alignment against a large database of DNA sequences (NCBI) to find its closest homologue by sequence alignment.

A non-PCR-based method such as flow cytometry has also been employed in food microbiology, for example, to determine the total bacterial content in milk with a detection limit of less then 10^4 bacteria ml^{-1} (Gunasekera et al., 2000). This technology employs instruments that are able to scan individual cells flowing through an excitation source in a liquid medium. The technique can be used to distinguish between viable and dead cells similar to the technique of fluorescence microscopy with systems similar to live/dead staining techniques (Chapter 5). Accordingly, viable cells have intact cell membranes impermeable to fluorescence dyes such as propidium iodide, which only enters dead cells. SYTO BC or thiazole orange, for example, are permeable dyes entering both, live and dead cells. Thus, the combination of these different dyes provides for the discrimination of live and dead bacteria.

Three molecular methods have been used extensively in this thesis for the identification and the enumeration of contaminating thermophilic bacilli in milk powders. These methods are RAPD-PCR, real-time PCR using TaqMan probes and

SYBR Green I protocols, and DGGE-PCR. The principles and basis for using these methods for this project are described in some detail.

In this thesis, RAPD-PCR has been used extensively as the method of choice for identifying contaminating strains of thermophilic bacilli in milk powder. RAPD-PCR was first described by Williams et al. in 1990 and is based on the variable distribution of priming sites within the genome between different individuals. The RAPD technique utilizes a single primer for random amplification of DNA segments. A typical RAPD primer is usually 10 nucleotides long with 60 to 70% G + C content (Péres et al., 1998) and can be designed either specifically or arbitrarily (AP-PCR). The principle of this technique is that, according to randomly distributed sequence homologies within the genome, e.g. so called sequence polymorphisms, amplification under low-stringency annealing conditions occurs at multiple genomic loci generating a defined set of PCR products. These PCR products can be separated into distinct bands dependent on molecular weight by agarose gel electrophoresis producing RAPD profiles specific to the strain or species. In general, sequence-polymorphisms between different strains can be determined by RAPD-PCR through the presence or absence of RAPD-marker bands which are either caused by priming-failure due to sequence diversities in the priming site of different strains or because of insertions or deletions within the amplification fragment located between the two priming sites.

RAPD-PCR for the identification and comparison of known and unknown strains has advantages over 16S rDNA sequencing or physiological tests. In contrast to the latter approaches, RAPD-PCR is relatively quick to perform and large numbers of anonymous genomes can be screened simultaneously (Hadrys et al., 1992; Ronimus et al., 1997) and thus, the method has found its application in numerous studies (Welsh and McCelland, 1990; Gang and Weber, 1996; Klijn et al., 1997; Christiansson et al., 1999; Te Giffel et al., 2002). However, RAPD-PCR has also limitations. For instance, the generation of RAPD markers depends on the PCR conditions applied and small changes in the protocol, such as temperature cycling conditions and/or the concentration of magnesium, the primer and the *Taq* DNA polymerase have been shown to affect the RAPD profile obtained (Péres et al., 1998; Ronimus et al., 1997). Changes of these kinds can influence the reliability and reproducibility and may result in misinterpretation of RAPD profiles especially when the method is used in different laboratories. Thus, in order to maintain the accuracy and reproducibility of the technique it is crucial to perform RAPD-PCR strictly under

the same optimized protocol. On the other hand, Ronimus et al. (1997) have shown that a RAPD-PCR protocol targeting thermophilic and mesophilic *Bacillus* species was quite robust and that small changes in the PCR conditions did not affect the result obtained. For instance, increasing the concentration of *Taq* DNA Polymerase resulted in an enhancement in both the sensitivity and resolution of the RAPD-PCR with a higher banding pattern density, but the characteristic RAPD profile was still evident. The RAPD protocol of Ronimus et al. (1997) has been used in two independent studies to determine and identify thermophilic bacilli in milk powders of various sources (Ronimus et al., 2003; Rueckert et al., 2004).

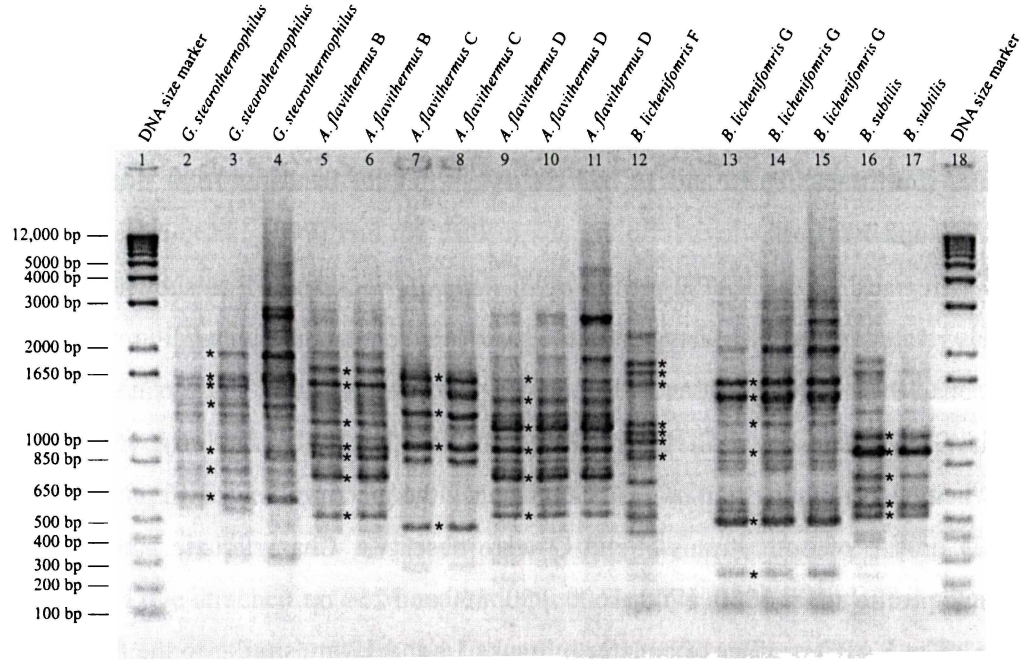

Figure 1.2. Randomly amplified polymorphic DNA (RAPD) PCR of the seven dairy strains isolated from New Zealand milk powders. The track numbers and the corresponding type strains are: (2 to 4) *G. stearothermophilus* strain A; (5 and 6) *A. flavithermus* strain B; (7 and 8) *A. flavithermus* strain C; (9 to 11) *A. flavithermus* strain D; (12) *B. licheniformis* strain F; (13 to 15) *B. licheniformis* strain G and (16 and 17) *B. subtilis*. DNA Ladder (track 1 and 18) denotes the molecular size marker. Amplicon size is indicated in the left margin.

A total of nearly 2400 isolates of thermophilic bacilli from milk powder were compared by their RAPD profiles with a high degree of reliability and reproducibility. As a result of the analysis about 98% of the isolate were assigned by RAPD profile comparison to seven profile types (A, B, C, D, F, G and BS) belonging to four species of bacilli. 16S rDNA gene sequencing and RAPD marker comparison with reference strains revealed that three profiles (B, C and D) belong to the species *A. flavithermus*, and another two RAPD profiles (F and G) to the species *B. licheniformis,* illustrating

the sensitivity of this method below the species level. Profiles A and BS were closely related to the type-strains *G. stearothermophilus* (DSMZ 22) and *B. subtilis* (DSMZ 345), respectively.

Figure 1.2 demonstrates the RAPD fingerprints of the seven profiles in which the most characteristic and highly reproducible bands of the profile are indicated with an asterisk. In tracks 2 to 4 of Figure 1.2 *G. stearothermophilus* strain A is shown. This isolate shared common RAPD markers with type strain DSMZ 22 at 1950, 1600, 1520, 1340, 900, 780 and 630 bp.

Tracks 5 to 11 show *A. flavithermus* strains. The profiles from track 5 and 6 could routinely be identified as the B isolate (Ronimus et al., 2003) using the amplification products at 1700, 1600, 1200, 1000, 870, 750 and 500 bp. Track 7 and 8 show strain C, which lacks the bands between 450 and 950 bp but has characteristic bands at approximately 450, 1000, 1250 and 1600 bp. The *A. flavithermus* strain D profiles from tracks 9, 10 and 11 had consistent marker bands at 1600, 1400, 1150, 950, 750 and 500 bp.

In track 12 the RAPD profile of *B. licheniformis* strain F is shown which is closely related to the DSMZ type strain 13 with two characteristic banding clusters at positions 1850, 1700, 1600 and 1150, 1050, 1000 and 850 bp. The *B. licheniformis* strain G isolates shown in tracks 13 to 15 has been classified by 16S rDNA sequencing as another strain *of B. licheniformis* although significant differences in the RAPD profile of both strains F and G were observed. Characteristic bands in the strain G profile are at 1850, 1700, 1600, 950, 500 and 250 bp.

The RAPD profiles of isolates in tracks 16 and 17 are similar to the DSM type strains of *B. subtilis* 347 and 10, with shared bands at 1050, 950, 750, 550 and 500 bp with the amplicon at 950 bp being particularly prominent.

However, culture-dependent methods are relatively labor-intensive and time-consuming (often requiring a period of 2 to 3 days to obtain a result), since they are based on the screening of individual bacterial colonies, which need to be isolated and separately grown to acquire enough biomass for DNA extraction. In addition, profiling these isolates is also relatively expensive, as they require growth media and various plastic wares such as sterile petri dishes, micro-centrifuge tubes, aero-resistant filter tips for pipetting and labour. A method which can be applied directly to milk powder without the requirement for prior isolation and culturing of contaminants, yet still retaining specificity would obviously have advantages.

A major hurdle in being able to apply PCR methods directly to milk contaminants without first culturing them is interference with the PCR by milk constituents. For PCR to succeed it is essential that the bacterial DNA can be obtained free from the huge relative quantities of milk proteins, lipids and carbohydrates. DNA extraction methods have been described by numerous authors using either cell culturing or enzymatic DNA extraction techniques (Lipkin et al., 1993; Rijpens et al., 1996; Herman et al., 1997; Cornejo et al., 1998; Romero et al., 1999; Gunasekera et al., 2000; Nogva et al., 2000; Timisjärvi and Alatossava, 2004), but are long and/or expensive and not suitable for a quick routine method. A more recent study described a rapid and inexpensive DNA isolation procedure and was based on the extraction of milk by tri-sodium citrate and *n*-decane to separate bacterial cells and spores from milk constituents, followed by DNA release of bacterial cells and spores by ultra-sonication (Rueckert et al., 2005a; Chapter 3). Trisodium-citrate chelates divalent cations such as calcium causing the dissociation of the casein micelles (Walstra, 1990; Walstra et al., 1999) and the milk lipids are effectively separated by *n*-decane extraction and centrifugation.

Any PCR assay targeting different species or strains of contaminating bacteria requires that primers specific to the target sequences are available in order to retain a high degree of discrimination against background DNA. The specificity of such an assay can be increased by applying TaqMan-PCR (also known as 5' nuclease PCR). TaqMan-PCR employs an additional short oligonucleotide probe which has a fluorescent dye attached at the 5'-end and a quencher dye at the 3'-end. The so-called TaqMan probe anneals between the forward and reverse primer sites illustrated in Figure 1.3 (Heid et al., 1996). As long as both dyes are attached to the hybridization probe the fluorescence energy emitted by the reporter dye is suppressed by the quencher similar to the Förster-type energy transfer (Förster, 1948; Lakowics 1983).

Once the TaqMan probe is hybridized to the target sequence and the PCR reaction is in the extension phase, the 5'- to 3'-endonuclease activity of the *Taq* DNA polymerase cleaves the hybridization probe if it is hybridized to the target sequence, separating the reporter dye from the quencher which results in a higher fluorescence signal of the reporter dye. This increase in fluorescence associated with the increasing cycle number of the reaction is directly proportional to the amount of PCR product generated. The PCR cycle at which the growth curve crosses a specified threshold cycle can be utilized to estimate the initial quantity of DNA template in the reaction

(Heid et al., 1996). The TaqMan technique can be extended to multiplex PCR (Chamberlain et al., 1988; Henegariu et al., 1997) allowing the simultaneous amplification and detection of different DNA templates by the action of more than one set of primers and hybridization probes in the same reaction (Courtney and Massung, 2003). There is a broad range of reporter dyes and quenchers available for the simultaneous detection of multiple fluorophores in a single tube.

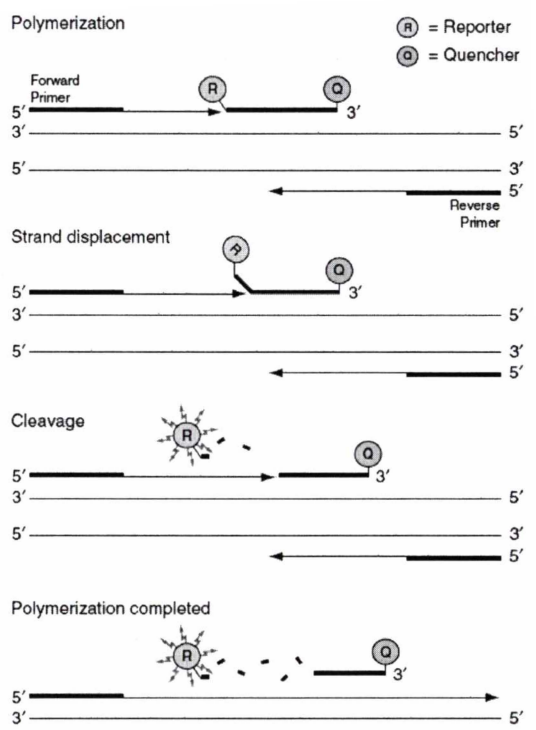

Figure 1.3. Principle of TaqMan real-time PCR method. During PCR, the TaqMan probe anneals between both primer sites within the sequence of interest. The nucleolytic activity of the Taq polymerase cleaves the probe distorting the Förster energy transfer which results in an increase in fluorescence. The probe fragments are then displaced and polymerisation of the target continues. (Sources of the image: Applied Biosystems).

Alternatively, real-time PCR can also be performed using the DNA intercalating dye SYBR Green. The SYBR Green chemistry provides a convenient and inexpensive way to substitute the traditional TaqMan PCR, while maintaining the accuracy of the qualitative and quantitative detection of PCR products. SYBR Green in solution emits minimal fluorescence giving a low background signal during the first PCR cycles. When SYBR Green intercalates with the double-stranded PCR product during the extension step of the polymerization the fluorescence increases significantly. Similar to the TaqMan technology, the SYBR Green fluorescence increases proportionally to the amount of amplification product generated. The principle of the SYBR Green chemistry is demonstrated in Figure 1.4.

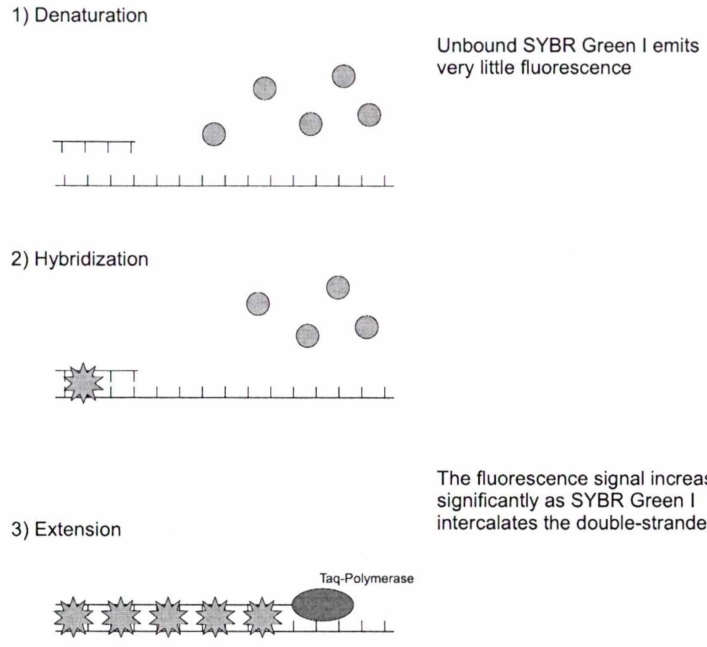

Figure 1.4. SYBR Green I chemistry. The dye only intercalates to double-stranded DNA associated with an increasing fluorescence output.

The PCR-DGGE technique exploits the fact that otherwise identical DNA amplicons, which may differ by only a single nucleotide will have different melting properties when separated through a gradient of increasing chemical denaturant (Fischer and Lerman; 1983). The method has recently been applied to food and food-related samples (Ampe and Miambi, 2000; Dewettinck et al., 2001; Fasoli et al., 2003) and a detailed description of the DGGE-PCR method is reviewed in Chapter 6.

A RAPD-based survey of thermophilic bacilli in milk powders from different countries

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2.1. Abstract

Twenty-eight milk powders from 18 different countries were examined for the number and type of contaminating thermophilic bacilli. Of 742 isolates examined, 96.8% were assigned to the same strains of bacilli as previously found in New Zealand powders. The dominant isolate was *Anoxybacillus flavithermus* strain C followed by *Bacillus licheniformis* strain F. The former was also prevalent in New Zealand powders and the results demonstrate that *A. flavithermus* represents a widespread contaminant, seemingly ubiquitous in factories producing milk powder. The presence of thermophilic strains of *Geobacillus stearothermophilus* and to a lesser extent of *Bacillus subtilis* in milk powders was reconfirmed.

Keywords: Milk Powder; Thermophiles; *Anoxybacillus flavithermus*; *Bacillus licheniformis*.

2.2. Introduction

Thermophilic bacilli are widely recognised as industrially important contaminants during the production of milk powders (Stadhouders et al., 1982; Kwee et al., 1986; Murphy et al., 1999; Ronimus et al., 2003). Fresh bovine milk is usually regarded as sterile and bacteria are introduced into the milk due to udder infections or from environmental sources during milking and processing (Phillips and Griffiths, 1990). During processing the milk is heated; usually a pasteurisation step is followed by either a low-, medium- or high temperature regime, depending on the end-use application of the powder. Regardless, these heat treatments will influence the survival of vegetative cells and spores entering the evaporation stage of processing.

The milk is then homogenised and its water content is typically reduced by thermally-enhanced evaporation in falling-film evaporators. During evaporation the milk is exposed to temperatures between 75°C to 45°C, which is a range suitable for the growth of thermophiles. In powder processing, the transit time for milk from the storage tank to the spray dryer usually does not exceed 20 to 30 minutes, and processing run times of up to 16 to 20 hours are typical before cleaning-in-place (CIP) regimens are applied. After 16 hours processing, thermophile numbers are typically increasing exponentially and can reach levels where the product is downgraded. Although thermophilic bacilli are not a potential health hazard to the consumer, they are used as an indicator of the processing plant hygiene and for good manufacturing practice. Furthermore, numbers of thermophilic bacilli are a factor in grading the quality of the milk powder and they can be of concern in spoilage of reconstituted milk powder (Kwee et al., 1986) and in ultra-high temperature (UHT) milk (Mostert et al., 1979; Westhoff and Dougherty, 1981). In addition, hydrolytic enzymes, e.g. proteases and lipases, possibly derived from thermophilic bacteria may play a detrimental role during short-to-medium term storage (Chen et al., 2003).

Thermophilic Gram-positive aerobic bacilli produce extremely heat-resistant spores, which can result in both pre- and post-heat treatment contamination (Phillips and Griffiths, 1990; White et al., 1993). Discounting any significant post-spray drying contamination of the powder, the occurrence of thermophilic bacilli in milk powders can only be derived from two sources; either from the raw milk as it is received from the suppliers or from growth within the milk processing plant. The typical number of thermophilic bacilli in raw milk, usually as spores, is generally small in the range of ≤ 50 colony forming units per mL (cfu mL^{-1}) (Martin, 1974; Phillips and Griffiths, 1986; McGuiggan, et al., 1994; Cook and Sandeman, 2001). During processing, the raw milk is concentrated approximately 10-fold to form a powder, so that the expected number of thermophilic bacilli in the final product derived from the milk itself would result in a maximum of 500 cfu g^{-1}, provided that no significant growth occurred within the process stream. The short transit time of the milk does not allow for substantial growth of thermophilic bacteria within the process stream, thus growth is thought to occur as bio-film within the plant resulting in bio-transfer to the product stream (Stadhouders et al., 1982; Wirtanen et al., 1996; Flint et al., 2001). Cell counts for thermophilic bacilli above the threshold of 500 cfu g^{-1} are thus indicative of

factory-derived growth within the processing lines. Additionally, if it can be shown that the proportion of species isolated from milk powders are significantly different from those commonly found as contaminants in raw milk then factory-associated growth is implicated.

Processing factories will have many different combinations of initial heat treatment, evaporation and spray drying, and these might be expected to create conditions that favour the growth of different strains of thermophilic contaminants. In a previous study, 1470 thermophilic isolates from milk powders produced in six different New Zealand factories and over five milking seasons were identified using a random amplified polymorphic DNA (RAPD) method (Ronimus et al., 2003). The results demonstrated the presence of seven strains representing four species of thermophilic bacilli, which accounted for over 98% of all isolates. The two most prevalent isolates were strains of *Geobacillus stearothermophilus* and *Anoxybacillus flavithermus*. The *G. stearothermophilus* strain was very similar to the type strains DSM 22 and DSM 1550 (equivalent to ATCC 12980) while three variants of the *A. flavithermus* RAPD pattern (B, C and D) were found to be related to the *A. flavithermus* strain DSM 2641 with pattern C being the most common. Two groups of isolates (F and G) were related to *Bacillus licheniformis* DSM 13 and DSM 8785. Further, *Bacillus subtilis* isolates similar to the common *B. subtilis* type strains DSM 10 (*B. subtilis* subspecies *subtilis*) and DSM 347 (*B. subtilis* subspecies *spizizenii*) (Nakamura et al., 1999) were also isolated.

Although there are some variations in the manufacturing processing regimes of the New Zealand factories which have been investigated (unpublished), there was no simple correlation between factory type and the spectrum of isolated *Bacillus* strains found. Indeed, close scrutiny of one factory showed that the composition of dominant strains could change over the milking season, and in some instances even between process runs. Thus, subtle changes in the process regime, milk composition through the season, number and types of bacteria in the raw milk or other factors, including CIP treatments, pre-heat temperatures, plant hygiene and dryer operating conditions (Varnam and Sutherland, 1994a), might affect the occurrence of a particular strain within the process line, altering the composition of the contaminants. This publication extends the approach taken with New Zealand milk powders to powders obtained from 18 different countries covering a very wide geographical spread. The aim of this research was to examine whether the strains of thermophilic

bacilli found to dominate powders produced in NZ (Ronimus et al., 2003) were also dominant in powders produced elsewhere.

2.3. Materials and methods

Powders were obtained from normal retail outlets and wherever possible from companies which stated they had been produced in the country of origin. Given the commodity nature of the milk powder market there is no absolute guarantee that these powders were derived from milk originating from the country indicated, but might represent repackaged or re-processed powder obtained from the world market. For some powders, which were obtained from domestic factories (Germany B, Switzerland, France A, B and C) we are confident that the powders represent the product of locally processed milk.

Powders were stored at room temperature until required and care was taken to obtain samples from the packages in an aseptic manner and within the product use by date. Measures to eliminate laboratory contamination included incorporation of negative control plates, aerosol-resistant tips for pipetting and disposable spreaders for cell harvesting. Powders were enumerated for thermophilic contaminants by plate counting and isolates identified by RAPD-PCR as described previously (Ronimus et al., 1997). Spore counts were obtained by heating reconstituted powders at 80°C for 10 minutes then plating (Falk Warnecke, personal communication). In general, after enumeration twenty-eight individual colonies showing growth at the lowest dilution for each powder were aseptically streaked to fresh tryptic soy-starch plates for RAPD analysis. For some powders additional colonies were assessed by RAPD analysis and for some low count powders less than 28 colonies were obtained on dilution plates. For isolates which could not be unequivocally identified from their RAPD pattern, a partial sub-unit of the 16S rDNA gene was PCR amplified and sequenced (Ronimus et al., 2003). The DNA sequences were aligned to the NCBI BLASTN database (Altschul et al., 1997) to find their closest homologues.

The RAPD patterns of individual isolates were identified by comparison to *Bacillus* reference strains obtained from either the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) or the ATCC (American Type Culture Collection). These included *G. stearothermophilus* (DSM 22 and DSM 1550) *Geobacillus thermocatenulatus* (DSM 730), *Geobacillus kaustophilus* (DSM 7263),

Bacillus caldolyticus (DSM 405), *Bacillus caldotenax* (DSM 406), *Bacillus caldovelox* (DSM 411), *A. flavithermus* (DSM 2641), *B. licheniformis* (DSM 13 and DSM 8785), *B. subtilis* subspecies *subtilis* (DSM 10) and *B. subtilis* subspecies *spizizenii* (DSM 347). Conditions for growth of all cultures followed recommendations of the supplier. The seven thermophilic dairy cultures used as reference strains, e.g. *G. stearothermophilus* strain A, *A. flavithermus* (strains B, C and D), *B. licheniformis* (strains F and G) and *B. subtilis* were derived from New Zealand milk powder samples (Ronimus et al., 2003) and are deposited in the Thermophile Research Unit culture collection.

2.4. Results

Results for viable plate counts, spore counts and RAPD pattern distribution are summarised in Table 2.1. The viable counts of the milk powders varied from the lowest with 7 cfu g^{-1} of Germany (A) to the highest with 2.2×10^5 cfu g^{-1} of USA (A). The proportions of spores to vegetative cells varied from practically none to 100%, with no relationship to the viable plate count or powder origin. There are no obvious correlations that can be drawn relating RAPD distribution to the source of the powder, powder type (skim or whole milk), viable count or spore count. The conclusion is that three strains of thermophilic bacilli are the most common contaminants of the milk processing operation, contributing 91.9% of the RAPD profiles screened. These three strains are *G. stearothermophilus* strain A, *A. flavithermus* strain C and *B. licheniformis* strain F which were also the most frequently encountered in the extensive longitudinal survey conducted on New Zealand milk processing factories (Ronimus et al., 2003). The other four strains encountered in the previous survey constituted a second ranking of strains detected in this survey (4.8% of RAPD patterns), and less than 3.2% of all 742 RAPD isolates were of novel strains of bacilli. For these isolates a partial 16S rDNA sequence was determined from which they were identified (Altschul et al., 1997) as *Bacillus circulans*, *Ureibacillus thermosphaericus*, *Bacillus coagulans* and *Bacillus pumilus* (Table 2.2).

The overall distribution of RAPD profiles is summarised in Table 2.3 and shows that *A. flavithermus* strain C was the most commonly isolated profile constituting 43.4% of all contaminants and was isolated from 20 of the 28 powders.

Strain C tended to be the dominant isolate in milk powders with thermophile counts above 500 cfu g^{-1}. For example, it was the dominant organism in twelve milk powder samples, ten of these with thermophile loads ranging from 1.1×10^3 to 2.2×10^5 cfu g^{-1}.

Table 2.1. Summery of the distribution [%] of RAPD profile types of thermophilic isolates found in milk powders, total plate counts and spore counts

Country	Powder type	Number of isolates	A	B	C	D	F	G	B. sub	Other	Total plate count [cfu g^{-1}][a]	Spore count [cfu g^{-1}][b, a]
Poland (A)	whole	24	-	-	-	-	100	-	-	-	2.8×10^2	1.4×10^2
Poland (B)	skim	26	23.1	-	53.8	-	19.2	-	3.8	-	**2.4×10^3**	**1.5×10^3**
Poland (C)	whole	48	14.6	-	43.7	-	31.3	-	-	10.4	**5.7×10^3**	**9.4×10^2**
Germany (A)	whole	8	-	-	50	-	37.5	-	12.5	-	6.7×10^0	8.0×10^0
Germany (B)	skim	42	7.1	-	47.6	-	33.3	7.1	2.4	2.4	**5.2×10^3**	N.D.
Switzerland	skim	7	-	-	-	-	28.6	42.8	28.6	-	8.8×10^1	4.2×10^1
France (A)	whole	28	-	-	3.6	-	89.3	7.1	-	-	3.8×10^2	2.4×10^2
France (B)	whole	27	-	-	85.2	-	14.8	-	-	-	**3.5×10^4**	**2.7×10^4**
France (C)	whole	9	-	-	-	-	88.9	-	11.1	-	4.2×10^2	3.8×10^2
Portugal	skim	19	-	-	-	5.3	36.8	5.3	26.3	26.3	7.5×10^1	4.2×10^1
Finland	skim	10	-	-	-	-	50	10	30	10	2.7×10^1	2.0×10^1
Netherlands	whole	4	25	-	-	-	75	-	-	-	1.4×10^1	1.2×10^1
Great Britain (A)	skim	11	45.4	-	36.4	-	18.2	-	-	-	5.2×10^1	2.5×10^1
Great Britain (B)	skim	26	-	-	76.9	-	19.2	-	-	3.8	**4.0×10^4**	**3.0×10^4**
Ireland	skim	28	-	-	7.1	7.1	75	-	3.6	7.1	**1.0×10^3**	**7.2×10^2**
Canada	skim	31	-	-	80.6	-	19.4	-	-	-	**3.8×10^4**	**2.7×10^4**
USA (A)	skim	36	5.5	-	91.7	-	2.8	-	-	-	**2.2×10^5**	**5.0×10^4**
USA (B)	skim	35	-	-	45.7	-	51.4	-	2.9	-	**4.4×10^3**	**1.1×10^3**
Mexico	whole	42	4.8	-	92.8	-	-	-	2.4	-	**1.3×10^3**	8.0×10^0
Chile	whole	32	37.5	-	3.1	-	50	-	-	9.4	**6.8×10^2**	9.2×10^1
Brazil	whole	16	-	-	-	-	87.5	-	-	12.5	1.4×10^1	1.7×10^1
South African	skim	29	3.4	-	41.4	-	37.9	-	3.4	13.8	2.7×10^2	1.0×10^2
Thailand	whole	41	-	-	97.6	-	2.4	-	-	-	**5.9×10^3**	**1.5×10^3**
Australia (A)	whole	28	-	-	3.6	-	85.7	3.6	7.1	-	**8.6×10^3**	**8.5×10^2**
Australia (B)	whole	9	11.1	-	-	-	88.9	-	-	-	3.7×10^1	4.2×10^1
New Zealand (A)	whole	20	5	-	10	-	80	-	5	-	**7.2×10^2**	4.3×10^2
New Zealand (B)	whole	55	56.4	-	40	-	1.8	-	1.8	-	**2.3×10^3**	4.8×10^2
New Zealand (C)	skim	51	15.7	-	43.2	-	41.1	-	-	-	**1.1×10^3**	1.7×10^2
Number of isolates		742	80	0	322	3	280	11	22	24		
Number of milk powders		28	13	0	20	2	27	6	14	9		

Plate counts above the threshold ≥500 cfu g^{-1} are indicated in bold. N.D.: Not determined.
A=*G. stearothermophilus*; B, C and D=*A. flavithermus*; F and G=*B. licheniformis*; B. sub=*B. subtilis*
[a] Incubation at 55°C for 16 hours.
[b] Activation by heat treatment at 80°C for 10 min.

The second most common thermophilic isolate was *B. licheniformis* strain F, which represented 37.7% of the 742 isolates overall (Table 2.3) and was present in all but one of the powders investigated. The common occurrence of this organism in milk powders regardless of the country of origin is possibly a reflection of it being a common thermophilic isolate in soil (Cook and Sandeman, 2001; unpublished results). Strain F was the dominant strain in 13 milk samples, which were mainly low count whole milk powders (≤ 500 cfu g^{-1}). However, five medium count powders (500 ≤ 30,000 cfu g^{-1}) contained strain F as the dominant contaminant, e.g. Ireland,

USA (B), Chile, Australia (A) and New Zealand (A) suggesting that growth had occurred within the process line in at least some factories.

Table 2.2. 16S rDNA sequence alignments

Organism	Isolate and country of origin	Length [bp]	Alignment identity [%]	Accession number
B. circulans	Isolate form Finland	789	99	AY294318
	Isolate B from South Africa	470	99	AY294321
	Isolate B from Poland	718	99	AY294319
U. thermosphaericus	Isolate C from Poland	492	98	AY294317
	Isolate A from Poland	723	97	AY294316
	Isolate from Ireland	686	99	AY299517
	Isolate A from Portugal	514	95	AY294315
	Isolate B from Chile	677	99	AY294314
B. coagulans	Isolate A from Chile	649	98	AY294322
	Isolate C from South Africa	408	96	AY294323
	Isolate A from South Africa	548	97	AY294324
B. pumilus	Isolate from Germany	784	99	AY294325

G. stearothermophilus strain A was the third most common isolate representing 10.8% of the overall isolates tested and was present in approximately half of the milk powders analysed. It was the dominant isolate in only one medium count powder from New Zealand (B), but accounted for relatively high levels in two other powders (45.4% of Great Britain (A) and 37.5% of the powder from Chile). *G. stearothermophilus* strain A was not restricted to medium count powders being present in several low thermophile count powders as well.

Table 2.3. Occurrence (number of isolates with stated strains)

Rank	Organism	Occurrence [%]	Number of isolates
1	*Anoxybacillus flavithermus* C	43.4	322
2	*Bacillus licheniformis* F	37.7	280
3	*Geobacillus stearothermophilus* A	10.8	80
4	*Bacillus subtilis*	2.9	22
5	*Bacillus licheniformis* G	1.5	11
6	*Bacillus circulans*	1.2	9
8	*Ureibacillus thermosphaericus*	1.2	9
7	*Bacillus coagulans*	0.7	5
9	*Anoxybacillus flavithermus* D	0.4	3
10	*Bacillus pumilus*	0.1	1

In agreement with the previous findings (Ronimus et al., 2003), *B. licheniformis* strain G and *B. subtilis* constituted a minor proportion of the contaminating isolates, but were distributed widely through the milk powder samples. The remaining thermophilic strains occurred in insignificant numbers. For example, *A. flavithermus* strain D accounted for only three individual isolates and was present in only two milk powders (Portugal and Ireland). *A. flavithermus* strain B was not found in any sample.

Figures 2.1a and 2.1b show a comparison of RAPD profiles of type strains with milk powder isolates from different geographical sources. Tracks 2 to 9 show (Figure 2.1a) that there is only a small degree of variation between the *G. stearothermophilus* milk powder strains from Poland (C), New Zealand (B), Chile, Australia (B) and the control New Zealand milk powder profile in track 3, despite their different origins. However, strain A lacks the convincing 440 base pairs (bp) marker band and possesses an additional band around 1390 bp (indicated with asterisks in track 3 of Figure 2.1a). The isolate from Germany B (track 4) was the most different, but still easily matched with those from New Zealand and DSM 1550. In tracks 10 to 19 of Figure 2.1a the RAPD profiles of *A. flavithermus* strain C isolates are shown. In the large scale New Zealand investigation four bands were universally present at approximately 460, 1000, 1250 and 1600 bp (indicated with asterisks in track 10 of Figure 2.1a). Only the France B profile (track 13) lacks the convincing 1000 bp band.

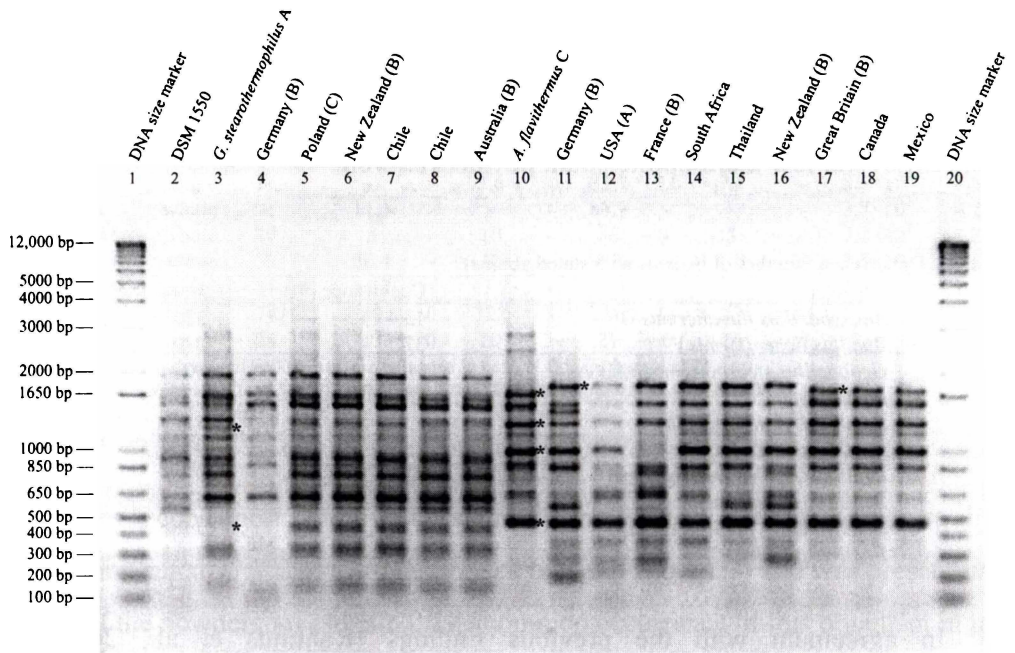

Figure 2.1a. RAPD fingerprint profiles of selected isolates of *G. stearothermophilus* A (tracks 4 to 9) and *A. flavithermus* C (tracks 11 to 19) derived from milk powders. The countries of origin are indicated above each track. Tracks 2 and 3 show the *G. stearothermophilus* reference strain DSM 1550 and A, respectively. The *A. flavithermus* isolate C from New Zealand is shown in track 10. DNA Ladder (track 1 and 20) denotes the molecular size marker. The sizes of the bands are indicated in the left margin. The Asterisks indicate the bands mentioned previously in the text.

Despite the high degree of conservation of these distinguishing markers, there are noticeable variations amongst the profiles shown. For example, the Germany (B), USA (A), France (B), South Africa, Thailand and New Zealand (B) profiles (tracks 11

to 16) contain a prominent band at 1780 bp (indicated with an asterisk in track 11 of Figure 2.1a) while the control strain C profile (track 10) and those from Great Britain (B), Canada and Mexico (tracks 17 to 19) lack this marker while containing a band at 1715 bp (indicated with an asterisk in track 17 of Figure 2.1a). In tracks 2 to 12 of Figure 2.1b the *B. licheniformis* F profiles are compared. Five bands at 677, 850, 1000, 1042 and 1147 bp are common to all of the isolates (indicated with asterisks in track 12, Figure 2.1b). In tracks 9 and 11, a prominent band at 592 bp is present (indicated with asterisks, Figure 2.1b), giving a profile that was also found in the previous study. Although only a small number of *B. licheniformis* strain G and *B. subtilis* isolates were found, overall the profiles of the isolates shown are nearly identical to those from New Zealand and in the case of *B. subtilis*, to the type strain DSM 10.

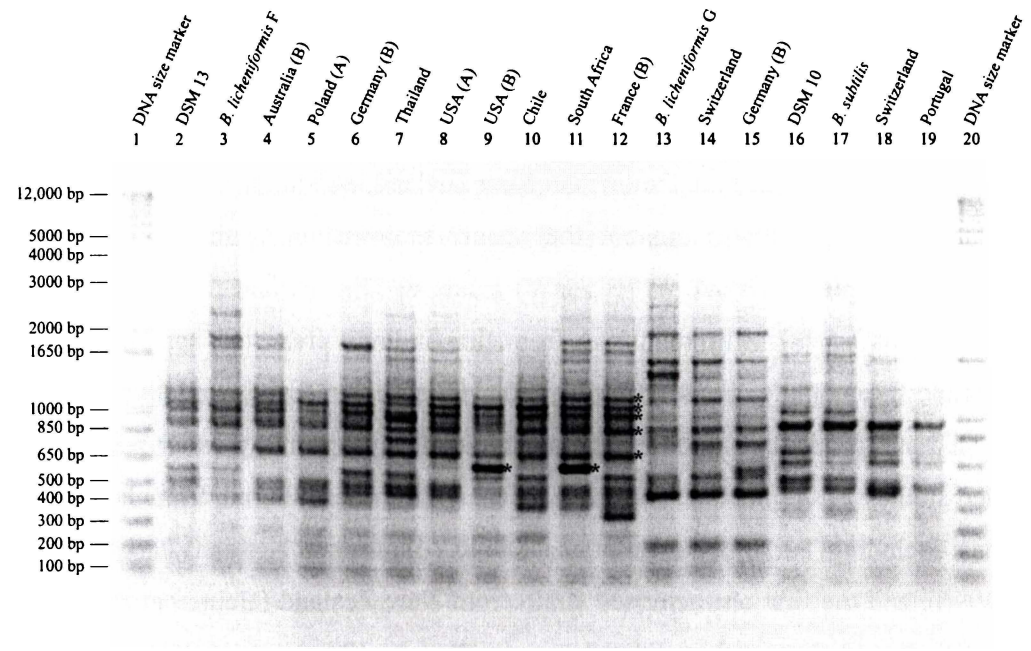

Figure 2.1b. RAPD fingerprint profiles of selected isolates of *B. licheniformis* type F (tracks 4 to 12), *B. licheniformis* G (track 14 and 15) and *B. subtilis* (track 18 and 19) derived from milk powders. The countries of origin are indicated above each track. Tracks 2 and 3 show the *B. licheniformis* reference strain DSM 13 and F, respectively. *B. licheniformis* G and *B. subtilis*, formerly isolated from New Zealand milk powders, are shown in tracks 13 and 17, respectively. *B. subtilis* reference strain DSM 10 is shown in track 16. DNA Ladder (track 1 and 20) denotes the molecular size marker. The sizes of the bands are indicated in the left margin. The Asterisks show the bands mentioned previously.

2.5. Discussion

This survey re-enforces that a limited number of strains of thermophilic bacilli are responsible for the contamination of milk powders during processing. In this

study, 96.8% of the 742 isolates could be identified by their RAPD profiles to be similar to isolates identified in a previous study of 1470 thermophilic isolates from New Zealand milk powders (Ronimus et al., 2003). The dominant profiles represent four species, i.e. *G. stearothermophilus* (strain A), *A. flavithermus* (strains B, C and D), *B. licheniformis* (strains F and G) and *B. subtilis*.

A direct comparative analysis of the data derived from this study and that from the New Zealand investigation is constrained by several factors. The most important is that the New Zealand study analysed "problem milk powders" that were near or higher than the national specification limit of 30,000 cfu g^{-1} for viable plate counts for thermophiles. In contrast, this study investigated mainly powders available for retail sale and that had presumably met national criteria for specification limits prior to sale. Furthermore, it was not the intention of this study to assess the overall impact of subtle differences in factory design, seasonal variation in spore numbers and type, the different end-use specifications of milk powders, e.g. high, medium and low heat-treatment (Kwee et al., 1986; Varnam and Sutherland, 1994b; Murphy et al., 1999), evaporator number, operating temperatures and spray drier conditions. These variations exist even between factories in New Zealand, and the results of this survey indicate that such features are not the major determinants of the contaminating species.

An important result stemming from the findings presented here is that the facultative thermophile *A. flavithermus* strain C is shown to be a relatively newly described and particularly problematic contaminant during milk powder production. Earlier studies of *A. flavithermus* have been with those cultured from hot spring habitats including Turkey (Beldüz et al., 2000), Yellowstone National Park (Nold et al., 1996), and the first characterised strain from New Zealand (Heinen et al., 1982). *A. flavithermus* was recently validated as a new species (Pikuta et al., 2000), formerly named *Bacillus flavothermus* (founding type strain is DSM 2641). This strain was prevalent in powders with higher thermophilic counts, consistent with growth in the process line and possibly indicates that contamination may be factory derived rather than introduced with the milk.

B. licheniformis strain F has been demonstrated to represent an ubiquitously distributed organism of milk powder, being present in 27 of 28 powders examined. *B. licheniformis* is normally the most numerous mesophilic isolate in raw milk (Waes, 1976; Phillips and Griffiths, 1986; Bramley and McKinnon, 1990; Crielly et al., 1994;

Cook and Sandeman, 2001) and thermophilic strains have also been found to be prevalent in milk (Chopra and Mathur, 1984; Phillips and Griffiths, 1986; Muir, 1990; Crielly et al., 1994; Murphy et al., 1999). In this study, *B. licheniformis* strain F is shown to be both a low level isolate possibly derived from factory-external sources and also a likely source of factory-derived contamination in milk powders.

G. stearothermophilus strain A, which is similar to the type strains *G. stearothermophilus* DSM 1550 and DSM 22, has been reconfirmed as a milk powder isolate. In conjunction with the findings on New Zealand powders these strains may be encountered more often in high-count milk powders. Significantly, *G. stearothermophilus* strain A isolates are able to grow on 316L stainless steel immersed in pasteurised skim milk with a calculated doubling time of only 19 minutes at 55°C (Flint et al., 2001) and thus show the potential to form bio-films in milk processing factories (Langeveld et al., 1995). *G. stearothermophilus* is considered to be an obligate thermophile unable to grow at 37°C. This is in contrast to *A. flavithermus* strain C strains which is a facultative thermophile growing between 37°C and 75°C (Ronimus et al., 2003).

A total of 2.9% of the isolates examined were identified as *B. subtilis* related to DSM 10. Thermophilic strains of *B. subtilis* are common in the environment (Deák and Timár, 1988) and in milk products (Waes, 1976; Norris et al., 1981; Martin, 1981; Crielly et al., 1994). Some strains can grow in milk at 55°C (Basappa et al., 1974). The occurrence of the other lower level isolates such as *U. thermosphaericus*, *B. coagulans, B. circulans* and *B. pumilus* suggests these species are unlikely to represent factory-derived organisms.

The low genetic variation of the isolated strains shown in Figures 1a and 1b supports the ubiquitous prevalence of the strains. For example, the RAPD fingerprints of *G. stearothermophilus* strain A isolates were identical in the Polish, New Zealand, Chilean and Australian (B) powders. A similar situation was found for the profiles of strain G of *B. licheniformis* for the isolates from Switzerland and Germany (B). Further, the New Zealand strain C profile (track 10, Figure 2.1a) was virtually identical to those from Great Britain (B), Canada and Mexico. The profile with most variation was that for *B. licheniformis* strain F. This is possibly because they were more representative of the diversity of soil, compost and silage origins (Bramley and McKinnon, 1990; Phillips and Griffiths, 1990; Gilmour and Rowe, 1990), than to different milk powder processing conditions.

The presence of high spore counts in the powder samples is intriguing. Conventionally, all milk is pasteurised prior to processing and this, and additional heat treatments prior to evaporation, should inactivate vegetative forms of thermophilic bacilli. If pasteurisation and heat-treatment do not induce germination of spores coming from the bulk milk, then the powder should only contain spore forms. This is usually not the case and suggests either that large numbers of vegetative cells survive pasteurisation and subsequent milk heating or that spore germination post-heating occurs and the growth of vegetative cells ensues. The former can be discounted because the previous study has shown (Ronimus et al., 2003) that following CIP the thermophile count is very low and typically increases through the processing run, provided that the milk supply is from the same bulk storage tank where the number of contaminants entering the process line throughout the run is essentially stable. Additionally, we have shown that mid-log cultures of *A. flavithermus* strain C and *B. licheniformis* strain F are unable to survive heat-treatment at 80°C for 10 minutes in tryptic soy-starch medium (results not shown), which supports the growth of these strains within the plant. Where high spore numbers are present it is likely that these have been formed within the process line; this is interesting because superficially these bacteria exist in a rich medium supporting vegetative growth. Presumably, the location of growth, either bio-film or elsewhere, might not be as supportive of growth and there are advantages to the organism to sporulate. Direct microscopic counts (results not shown) on re-suspended powders indicates that all contained between one and several orders of magnitude more bacterial cells than enumerated by viable counts (therefore presumed to be dead cells), so that differences in spore percentage might simply reflect the difference in survival of vegetative cells during stages of processing that are inhibitory such as spray drying.

2.6. Conclusions

In conclusion, the main groups of thermophilic bacilli described here represent a worldwide source of contamination with important economic dimensions regardless of the geographic location, or the milk processing regimes and practices under which the powders were produced. Medium and high count powders are deemed to reflect factory-derived thermophile growth in the processing plant rather than concentration

from the raw milk, and the evidence from this survey establishes the relatively unknown *A. flavithermus* strain C within the dairy industry as a major contaminant on a global scale. It also reconfirms the status of *G. stearothermophilus*, *B. licheniformis*, and to a lesser extent of *B. subtilis*, as contaminants important to the manufacturing of milk powders.

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Development of a rapid detection and enumeration method for thermophilic bacilli in milk powders

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3.1. Abstract

Thermophilic strains of *Geobacillus*, *Anoxybacillus* and *Bacillus* that are able to grow at 55°C and above are recognized as commonly occurring contaminants during the production of milk powders. In particular, *Anoxybacillus flavithermus* strain C and *Bacillus licheniformis* strain F are often the most prevalent. We describe here the development of a TaqMan-based real-time-PCR assay using a small amplicon of the ribosomal 16S rRNA gene for the selective and quantitative detection of thermophilic bacilli in milk powders. We further present an effective, rapid and inexpensive method for the isolation of total bacterial DNA from milk powder for quantitative PCR analysis within 20 minutes. With this method, the detection of thermophilic bacilli in milk powder can be accomplished within 1 hour. The detection limit for reconstituted and inoculated milk was 8 vegetative cfu ml^{-1} and 64 spores ml^{-1}, respectively.

Keywords: *Anoxybacillus*; *Bacillus*; *Geobacillus*; Milk powder; Real-time PCR, Spores, Thermophiles.

3.2. Introduction

Thermophilic bacilli which grow at 55°C, or above, are the bacterial contaminants that are most commonly found in milk powders. RAPD-PCR studies on skim, whole and buttermilk powders produced in factories of the major milk producing countries have shown that over 96% of thermophilic contaminants could be assigned to four species of bacilli, e.g., *Geobacillus stearothermophilus* (strain A), *Anoxybacillus flavithermus* (strains B, C and D), *Bacillus licheniformis* (strains F and

G) and *Bacillus subtilis* (Ronimus et al., 2003; Rueckert et al., 2004). Two strains, in particular, were exceedingly prevalent and dominated the flora of the majority of milk samples analysed. These were *A. flavithermus* strain C which contributed over 43% and *B. licheniformis* strain F which contributed for 38% of the 742 milk powder contaminating isolates subjected to RAPD analysis (Rueckert et al., 2004).

Although these contaminants do not constitute a health risk to the consumer they are used as an index of plant hygiene. Accordingly, dairy plants adopt specification limits for thermophile counts in their products, and practice strict hygiene standards to meet them, in order to guarantee the marketability of their products. In a typical powder processing run the product is formed continuously over an 18 to 24 hours processing period. During this time period the increase in the number of thermophilic bacilli in the powder formed can appear to be similar to a typical bacterial growth curve, with numbers increasing exponentially by the end of the operation. Factors such as product type (whole or skim milk), the microbiological quality of the raw milk, operating conditions in the plant (low-, medium- and high-heat treatment products) and the plant hygiene can all affect the growth of thermophilic contaminants. Consequently, it is difficult to predict a processing time whereby assurance that thermophile numbers will be below specification can be guaranteed. Common practice is to end processing runs with the expectation that they will not exceed specification limits and the plant subjected to a cleaning-in-place (CIP) cycle. The transit time of the milk during processing is usually less than 30 minutes, and growth of thermophilic bacilli is not possible in all sections of the processing line due to restrictive temperatures and/or viscosities of the product. Thus, significant growth in thermophile numbers cannot occur in the milk being processed but is thought to originate from bio-films (Langeveld et al., 1995; Parkar et al., 2003; Parkar et al., 2004) in the process line in area suitable for growth – hence the emphasis on CIP. The currently used method of monitoring thermophile contaminants is based on plate counting on powder sample which produces results 24 to 48 hours after the processing runs have been completed, i.e. it is retrospective. An accurate and reliable real-time monitoring method for determining contamination levels would allow processing to continue until specification limits were close to being met and would maximise plant efficiency and reduce the amount of cleaning chemicals used.

In the present paper we describe the development and evaluation of a real-time PCR assay for the selective, rapid and quantitative detection of the two major

thermophilic milk powder bacilli. The quantitative PCR method is based on the TaqMan technology using specific primers and probe which were designed to target 16S rRNA genes. Furthermore, the PCR assay was adapted to a field-portable Smart Cycler II system (Cepheid, USA) allowing flexible and efficient application in non-research laboratory environments. We further describe a rapid and inexpensive method for total bacterial extraction from milk samples based on trisodium citrate and *n*-decane extraction. This method, when combined with ultrasonic cell disruption, previously described by Fykse et al. (2003) and Belgrader et al. (1999), gives a rapid and efficient DNA isolation method suitable for quantitative PCR analysis.

3.3. Materials and methods

3.3.1. Bacterial strains and culture preparation

Cultures of *A. flavithermus* C and *B. licheniformis* F, previously isolated from milk powders (Ronimus et al., 1997), were grown at 55°C in tryptic soy broth (TSB) supplemented with 0.2% (w/v) soluble potato-starch. Spores of *A. flavithermus* C and *B. licheniformis* F were produced by incubation of cultures at 55°C for 48 hours in liquid Castenholz medium (DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). The extent of sporulation was checked by phase-contrast microscopy (1000× magnification) and the spores harvested with sterile deionised water and centrifuged at 16,100 *g* (5415 D, Eppendorf). The spore suspensions were purified from contaminating DNA and vegetative cells in the following way. Lysozyme (2 mg ml^{-1}) was added and the suspensions incubated for 30 minutes at 37°C. The spores were then washed twice in sterile deionised water and sonicated for 125 seconds (XL-2020, Misonix). The power output was 120 W at 20 kHz. The spore suspensions were then further processed and stored as described by Ryu et al. (2003) including washing with 1 M NaCl, 0.1 M NaCl, 0.1% SDS and sterile deionised water and storage at 4°C. Spore and vegetative cell counts were conducted by phase-contrast microscopy using a Thoma counting Chamber and by plate counting in tryptic soy agar (TSA) with 0.2% (w/v) soluble potato-starch at 55°C.

The growth condition for the non-thermophilic bacterial cultures e.g., *Lactobacillus plantarum* DSM 20205 (NCIB 6376, ATCC 8014), *Pseudomonas*

aeruginosa DSM 5168, *Streptococcus thermophilus* DSM 20479 (NCIB 8779), *Streptococcus uberis* NZRM 2266, *Escherichia coli* DSM 301 (ATCC 4157), *Clostridium paradoxum* (DSM 7308), *Clostridium perfringens* (*Clostridium welchii*) (Sigma-Aldrich), *Micrococcus luteus* (DSM 2786), and *Bacillus megaterium* (DSM 32) followed recommendations of the suppliers.

3.3.2. DNA preparation

DNA was extracted from all cultures by a modification of the procedure described by Sambrook and Russel (2001). Following growth, 10 ml of each cell suspension was harvested by centrifugation and re-suspended in 1 ml of 50 mM Tris HCl (pH 8.0), 100 mM NaCl and 100 mM EDTA. A total of 2 mg ml^{-1} lysozyme was added and the samples incubated at 37°C for 30 minutes. SDS and proteinase K were added to 1% (w/v) and 200 µg ml^{-1} respectively, and the samples further incubated at 55°C for 1 hour. Subsequently, the samples were extracted with equal volumes of phenol:chloroform (1:1) followed by chloroform and chloroform:isoamyl alcohol (24:1). The DNA was then precipitated by addition of 0.1 (v/v) of 3 M sodium acetate and 0.6 (v/v) of isopropanol and incubation at -20°C for 15 min. The DNA pellets were washed twice with ice chilled 80% ethanol, air dried and re-suspended in 200 µl of 1× TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). RNase was added (50 µg ml^{-1}) and the DNA samples incubated at 37°C for 30 minutes. The DNA was then purified and recovered as described above including phenol:chloroform, chloroform and chloroform:isoamyl alcohol extraction and sodium acetate and isopropanol precipitation. The DNA pellet was re-suspended in 100 µl of 1× TE (pH 8.0), quantified by spectrophotometer (Pharmacia Biotech, Ultraspec 3000) readings at A_{260}, A_{280} and A_{320} and stored at -20°C. The DNA preparations were used as positive controls and as quantification standards.

3.3.3. Ultrasonic treatment of vegetative cells

DNA release from vegetative cultures was accomplished by ultra-sonication treatment using a modification of the method described by Fykse et al. (2003) and Belgrader et al. (1999). Cultures of *A. flavithermus* C and *B. licheniformis* F were

grown in TSB-starch at 55°C until an optical density at 600 nm (OD_{600}) of 1.2 and chilled on ice for twenty minutes. The cells were then washed twice in sterile deionised water and their numbers determined by microscopic counting. The cell suspension samples were kept in ice-water until ultrasonic treatment was conducted. Sonication was performed with the ultrasonic liquid processor XL-2020 (Misonix) using a 3.2 mm microtip disruptor horn in a 2 ml Eppendorf tube with a final volume of 1 ml of bacterial suspension. Sonication was accomplished at 120 W at 20 kHz. After sonication, the samples were briefly centrifuged at 16,100 *g* for 2 minutes in order to remove the cell debris and 10 µl of the supernatant were used for PCR analysis. The optimal sonication time for DNA release was determined in duplicate with a 4.8×10^8 cfu ml^{-1} culture of *A. flavithermus* C using time periods between 5 and 250 seconds. After the optimal sonication time for DNA release had been determined, a standard curve was constructed for the DNA released from cell concentrations over the range 10^2 to 10^8 cfu ml^{-1} and the threshold cycle (C_t) values obtained by quantitative PCR. All assays were run in triplicate at each cell concentration.

3.3.4. Ultrasonic treatment of spores

Spores of *A. flavithermus* C and *B. licheniformis* F were disrupted using a modification of the protocol described by Belgrader et al. (1999). Ultrasonic treatment of spores was conducted with the liquid processor XL-2020 (Misonix) using a 3.2 mm microtip disruptor horn in 2 ml tubes. The optimal sonication time for DNA release from *B. licheniformis* F spores was performed in duplicate using 6.7×10^7 spores ml^{-1} in the presence of 50 mg of 0.1 mm glass beads (Biospec Products) at 120 W at 20 kHz in a final volume of 1 ml sterile deionised water. Ultra-sonication was performed for different periods of time ranging from 5 to 600 seconds. Subsequently, triplicates from 10-fold serial dilutions between 10^2 to 10^7 spores ml^{-1} were sonicated using the optimised sonication time following the protocol outlined for vegetative cells. After ultrasonic treatment was accomplished the samples were centrifuged for 2 minutes at 16,100 *g* and PCR analysis performed on the supernatants.

3.3.5. Extraction of vegetative cells and spores from milk powder for DNA preparation

A whole milk powder which contained less than 300 cfu g^{-1} of thermophilic bacilli and no detectable thermophilic spores was reconstituted to 1× milk and sonicated for 20 minutes at 120W output power at 20 kHz in order to release DNA form vegetative cells and shear it below the level of detection of the PCR assay. Equal numbers of *A. flavithermus* C and *B. licheniformis* F vegetative cells or spores were then inoculated into the re-constituted milk to give concentrations of 8×10^7 cfu ml^{-1} for the vegetative cell sample and 6.4×10^7 cfu ml^{-1} for the spore sample. The inoculated milk samples were then 10-fold serially diluted into sterile milk to final concentrations of 8×10^2 and 6.4×10^3 cfu ml^{-1}, respectively. Triplicates of 1 ml of each milk dilution were used to recover the bacterial cells. A total of 200 µl of 1.2 M trisodium citrate and 200 µl of *n*-decane (Merk-Schuchardt) were then added to 1 ml of each milk sample followed by vortexing for 5 seconds and centrifugation at 16,100 *g* for 10 minutes. The upper layer of cream was carefully removed from the tube wall using a sterile 200 µl tip and the supernatant poured off carefully by gently inverting the tube. The samples were re-centrifuged for 1 minute to collect any remaining liquid which was then carefully removed by pipetting while avoiding disrupting the cell pellet. The pellet was re-suspended in 1 ml of sterile deionised water and the DNA released by sonication as described above for vegetative cells and spores.

3.3.6. Probe and primer design

Partial 16S rRNA genes of all seven thermophilic bacilli found in New Zealand milk powders including *G. stearothermophilus* (strain A), *A. flavithermus* (strains B, C and D), *B. licheniformis* (strains F and G) and *B. subtilis* were obtained by sequencing. The accession numbers are listed in Table 3.1. The genes were amplified in an Eppendorf Mastercycler (Gradient) with forward primer 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse primer 1522r (5'-TGC GGT GGA TCA CCT CCT T-3') (Johnson, 1994) using the following PCR protocol: 96°C for 225 seconds then 35 cycles of 92°C for 45 seconds, 45°C for 30 seconds, 72°C for 90 seconds and finally a 4 minutes extension step at 72°C.

In order to define primer sequences for the real-time PCR assay the NCBI database was searched for relevant 16S rRNA sequences. The 16S rRNA gene sequences of 69 species were aligned incorporating those bacteria commonly isolated from milk (Gilmour and Rowe, 1990) and sequences from representative thermophilic bacterial (Ronimus et al., 2003) and archaeal genera. The sequences were aligned using the CLUSTAL W multiple sequence alignment software provided by European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw/).

Figure 3.1. Partial 16S ribosomal sequence alignment for the selection of the TaqMan probe and primers for *A. flavithermus* strain C and *B. licheniformis* strain F.

Primers were designed using the online Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Forward and reverse primers were synthesized by Invitrogen, New Zealand, with the following sequences: forward primer, 5'-AGG AAC ACC AGT GGC GAA G-3' and reverse primer, 5'-GGA TGT CAA GAC CTG GTA AGG-3'.

The primers were designed to target a small region within the 16S rRNA genes at position 711-998 according to the numbering of Brosius et al. (1980) (Figure 3.1). Due to the conserved nature of the 16S rRNA genes among prokaryotes a TaqMan probe specific to milk bacilli contaminants was designed and its efficiency to discriminate bacilli verified by BLASTN search (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1997). The dual-labelled TaqMan probe contained the Cy™3 reporter dye at the 5'-end and the BHQ2 Dark

Quencher at the 3'-end. The sequence of the probe was 5'-ACG ATG AGT GCT AAG TGT TAG AGG G-3'. The probe was synthesised by PROLIGO, Australia.

Table 3.1. 16S rRNA sequences

Organism	strain	sequence length [bp]	Completion	NCBI accession number
Geobacillus stearothermophilus	A	1527	partial	AY672761
Anoxybacillus flavithermus	B	1526	partial	AY672762
Anoxybacillus flavithermus	C	1523	partial	AY643748
Anoxybacillus flavithermus	D	1496	partial	AY672763
Bacillus licheniformis	F	1286	partial	AY751776
Bacillus licheniformis	G	1507	partial	AY672764
Bacillus subtilis	BS	1510	partial	AY672765

3.3.7. Real-time PCR

Real-time PCR analysis was performed with a Smart Cycler II system (Cepheid, USA) in 25 µl Smart Cycler reaction tubes. The amplification reactions were performed with 4 mM $MgCl_2$, 0.2 mM dNTPs, 10× *Taq* PCR reaction buffer, 1.25 units of Ampli*Taq* DNA polymerase (Roche), 600 nM of forward and reverse primer and 300 nM of TaqMan probe. The amplification reaction for the DNA standard curves contained 1 µl of DNA sample diluted between 15 fg and 15 ng, i.e. DNA purified from pure cultures of *A. flavithermus* (strain C) and/or *B. licheniformis* (strain F). PCR reactions from sonicated vegetative cells and spores contained 10 µl of sample. The PCR reaction was cycled once at 95°C for 120 seconds, 42 cycles at 95°C for 5 seconds and at 62°C for 20 seconds. Furthermore, negative controls (no DNA template) and positive controls (0.1 ng of *A. flavithermus* C DNA) were included in each experiment. The threshold cycle number C_t was determined using the primary curve analysis of the Smart Cycler software (Cepheid Smart Cycler Version 2.0b). The threshold cycle number C_t was determined for each reaction by setting a fixed manual threshold of 4.5 fluorescence units. The C_t values were then plotted against the log amount of DNA or colony forming units per ml using the Microsoft Excel software and the equation, the correlation coefficient (R^2) and standard deviations of the corresponding triplicates determined.

3.4. Results

3.4.1. Real-time PCR optimization

The quantitative real-time PCR assay was optimized to minimise the threshold cycle number (C_t) while yielding the highest fluorescence intensity of the reporter dye (Pfaffl, 2002; Teo et al., 2002; Decker et al., 2002; Ball et al., 2002; Vandesompele et al., 2002). This included the titration of $MgCl_2$, TaqMan probe and primer concentrations. Primer concentrations between 100 nM to 1100 nM were tested. The mean value for the threshold cycle number (C_t) for all primer concentrations was 14.92 ± 0.25 for 15 ng target DNA samples, while the formation of primer-dimers increased gradually with increasing ratio of primer to target DNA concentration. The best primer pair performance was observed using 600 nM for both forward and reverse primer (Appendix 12.2.1.). The $MgCl_2$ concentration was varied between 1.5 and 5 mM with no significant effect on the PCR amplification efficiency or formation of non-specific products or primer-dimers. The concentration of the TaqMan probe was titrated between 50 and 1000 nM using 15 ng of target DNA. The maximum fluorescence reporter signal was obtained using 300 to 1000 nM of the probe (Appendix 12.2.3.). The 16S rRNA PCR primers and TaqMan probe worked well in a range between 58°C to 62°C using a combined annealing-extension step of 20 seconds (Appendix 12.2.2.). The final annealing temperature for quantitative PCR of 62°C was chosen in order to reduce partial primer annealing due to single-base mismatches in the priming sequence of species other than *Bacillus*, *Geobacillus* and *Anoxybacillus*.

3.4.2. Primer and probe selectivity

Specific primers and a hybridisation probe were designed for all seven thermophilic bacilli found in New Zealand milk powders (Ronimus et al., 2003) including *G. stearothermophilus* (strain A), *A. flavithermus* (strains B, C and D), *B. licheniformis* (strains F and G) and *B. subtilis*. Both primers were then tested for their selectivity by NCBI (BLAST) search, multiple 16S rRNA alignments and real-time PCR. Due to the highly conserved nature of the 16S rRNA genes the designed primers were also specific for some non-*Bacillus* species.

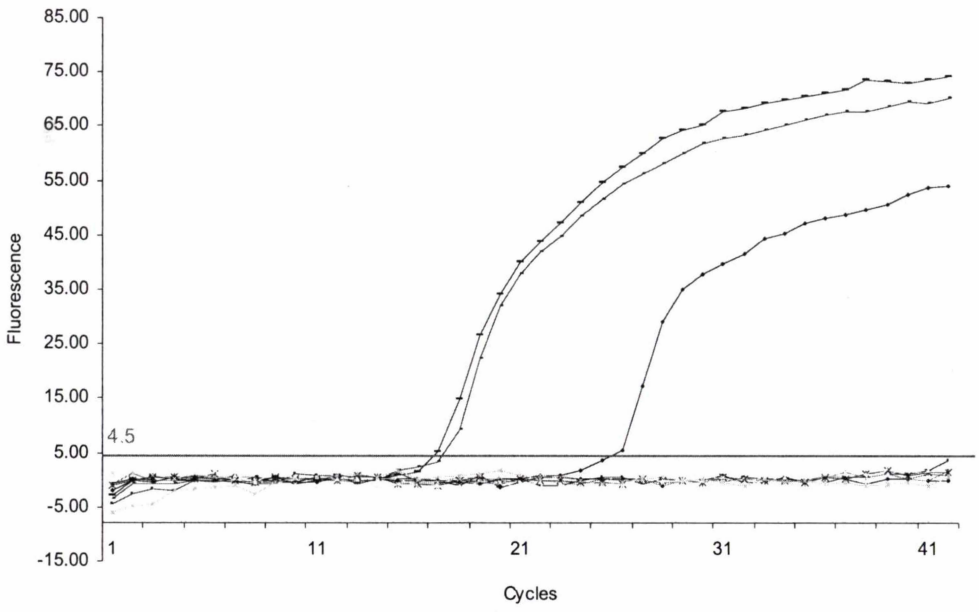

Figure 3.2a. Amplification plot of quantitative real-time PCR using the Smart Cycler II detection system. The primers and probe were tested for selectivity against 1 ng of initial genomic DNA of *A. flavithermus* C, *B. licheniformis* F, *B. megaterium* and against non-*Bacillus* species mentioned in the text. Both, *A. flavithermus* C and *B. licheniformis* F crossed the manual threshold line at 16.83 and 17.19, respectively. The mesophilic *B. megaterium* responded with a C_t of 25.53. The PCR reactions for non-*Bacillus* species showed no increase of the reporter dye during amplification and thus were negative for the primers and probe combination.

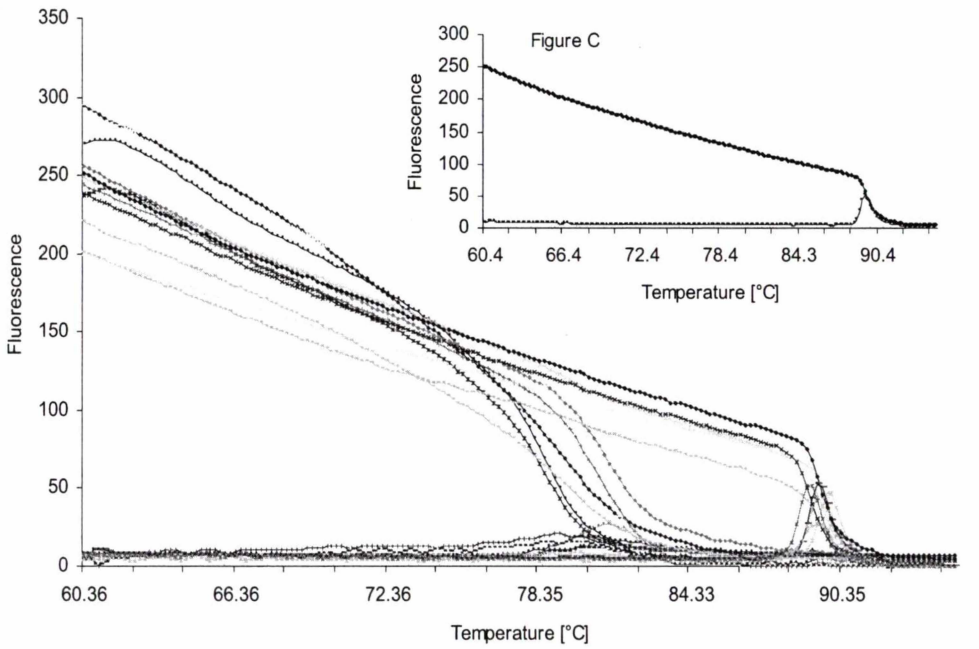

Figure 3.2b. Melting point analysis of 16S rRNA amplicons. The graphs show the melting curves and the corresponding bell-shaped 1st derivatives. The PCR products were identified using melting point analysis by adding 0.25× SYBR Green I to the completed PCR reaction (60°C to 95°C; 0.2°C sec^{-1}). Melting points were recorded for *A. flavithermus* C (89.58°C), *B. licheniformis* F (89.99°C), *B. megaterium* (89.14°C), *L. plantarum* (89.38°C) and *P. aeruginosa* (89.58°C). The PCR amplification of the non-*Bacillus* species resulted in the limited formation of unspecific amplification product at approximately 80°C as also determined by agarose gel electrophoresis. Nonspecific PCR products were not observed for reactions where the correct product was formed as shown in (C) for *A. flavithermus* C.

Multiple 16S rRNA alignments showed some degree of primer compatibility, for instance to *Lactobacillus, Enterobacter, Leptospira, Pseudomonas, Staphylococcus* and *Streptococcus*, while the probe-sequence was chosen to exclusively target milk-derived thermophilic bacilli.

The specificity of the primers and probe were further investigated by real-time PCR using 1 ng of target DNA against eight non-*Bacillus* stains (*Lactobacillus plantarum*, *Pseudomonas aeruginosa*, *Streptococcus thermophilus*, *Streptococcus uberis*, *Escherichia coli*, *Clostridium paradoxum*, *Clostridium perfringens* (*Clostridium welchii*) (Sigma-Aldrich) and *Micrococcus luteus*, the thermophilic milk-bacilli (*A. flavithermus* C and *B. licheniformis* F), one mesophilic *Bacillus* strain (*Bacillus megaterium*) and against calf thymus DNA (Sigma-Aldrich).

The milk-derived thermophilic strains were positive for the primer and probe combination while the other species were negative. The mesophilic *B. megaterium* DNA had a C_t of 25.53, approximately 5 amplification cycles later than the dairy strains, which indicates either poor priming due to sequence diversity and/or a lower ribosomal operon number (Figure 3.2a). Under the optimised PCR conditions some of the non-*Bacillus* species e.g. *L. plantarum* and *P. aeruginosa* (Figure 3.2b) produced an amplified product which could be detected by melting point analysis in the presence of 0.25× SYBR Green I and by agarose gel electrophoresis. Similarly, lowering the annealing temperature of the PCR assay also resulted in amplified product formation for some of the non-target species. However, although the TaqMan probe was present in these reactions no corresponding increase in fluorescence was obtained indicating the high degree of specificity of the probe for the target species (Figure 3.2a).

3.4.3. DNA standard curve and PCR reproducibility

Standard curves for 16S rRNA genes were established using triplicates of 10-fold dilution series of *A. flavithermus* C and *B. licheniformis* F genomic DNA. The plots of the C_t values and the log quantities of standard DNA were linear between 15 fg and 15 ng of initial target DNA per reaction. The correlation coefficient (R^2) of both standard curves were greater than 0.99 (Figure 3.3).

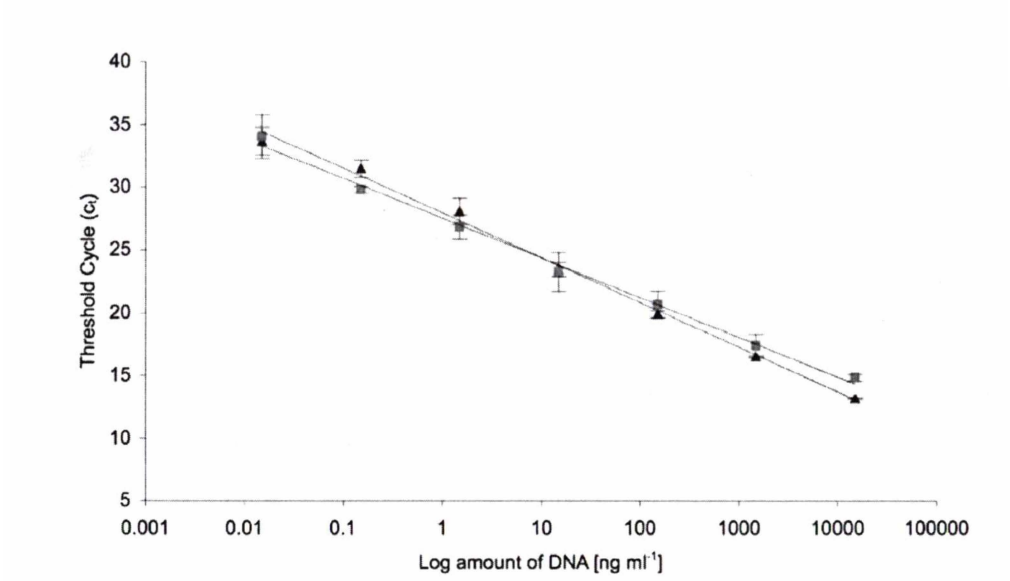

Figure 3.3. Standard curves for (▲) *A. flavithermus* C (y = -1.5448ln(x) + 27.934) and (■) *B. licheniformis* F (y = -1.3753ln(x) + 27.542) genomic DNA for 16S rRNA quantitative real-time PCR. The DNA was serially diluted 10-fold and the PCR reactions performed in triplicate. The mean values of the threshold cycle number C_t were then plotted against the log amount of initial DNA template and the equation and linear correlation coefficient (R^2) determined in Microsoft Excel. One microlitre of standard DNA was used in a 25 µl PCR reaction. The error bars are the standard deviation for three replicates.

3.4.4. Optimisation of DNA release from vegetative cells and spores by ultrasonic treatment

Genomic DNA extraction from vegetative cells and spores was performed by ultrasonic treatment. Ten micro-litres of the sonicated samples was used for 16S rRNA quantitative PCR amplification in the Smart Cycler II and the relative yield of released DNA quantified using the DNA standard curve for 16S rRNA genes. The maximum DNA recovery for vegetative cells was achieved by sonication for 125 seconds (Figure 3.4). Exposure times beyond 125 seconds led to higher C_t values, presumed due to excessive DNA fragmentation. The extent of spore disruption by sonication was similarly determined by 16S rRNA PCR analysis and OD_{450} measurement of the sonicated samples. The maximum yield of DNA released from spores occurred after 6 minutes of sonication (Figure 3.5).

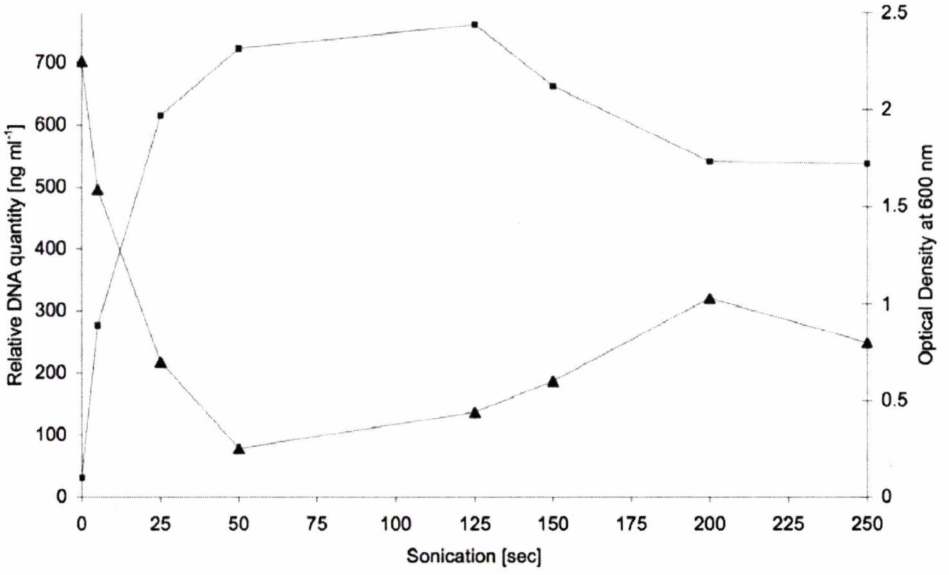

Figure 3.4. Effect of sonication on cell lysis and DNA released from a 16 hour culture of 4.8×10^8 *A. flavithermus* C vegetative cells in 1 ml of sterile deionised water. Samples were prepared in duplicate and the efficiency of cell lysis assessed by (■) 16S rRNA PCR and (▲) OD_{600} readings. Relative quantities of DNA released were determined using the 16S rRNA standard curve for *A. flavithermus* C (Figure 3.3) and mean values plotted against ultra-sonication time.

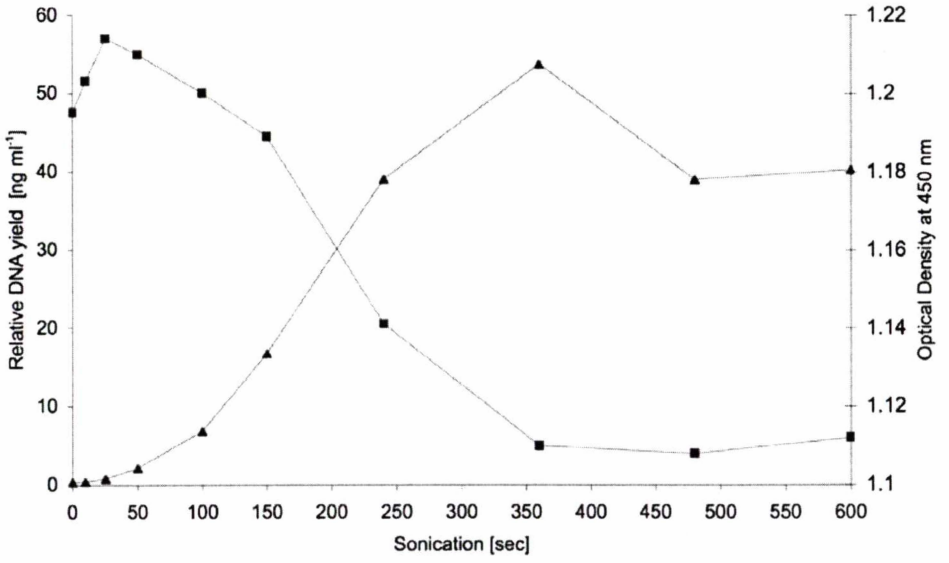

Figure 3.5. Effect of ultra-sonication on spore disruption (■) and DNA release (▲) for a 6.7×10^7 spore ml^{-1} suspension of *B. licheniformis* F in 1 ml of sterile deionised water. The relative DNA yield was determined using the 16S rRNA standard curve for *B. licheniformis* F (Figure 3.3). The maximum DNA yield was obtained after 360 seconds of sonication. The unsonicated spore suspensions had a mean relative DNA yield of 0.4 ng ml^{-1}.

However, the efficiency of spore disruption was 14 times less than for vegetative cells based on the numbers of vegetative cells and spores and the respective C_t values. This may be due to two reasons: first, any spore disrupted early

in the sonication treatment might have its DNA sheared as sonication continued, to a point below the detection level. Second, spore disruption is inefficient due to their resistance to mechanical shear forces resulting in poor spore lysis. The latter scenario was investigated by plate counting spore suspensions containing 10^7 cfu ml^{-1} after having been sonicated for 6 minutes. The sonicated spores were heat-activated for 20 minutes at 80°C and poured into TSA-starch agar plates followed by incubation at 55°C for 36 hours. A mean of 88% of sonicated spores formed colonies suggesting that the drop in the relative DNA yield beyond 6 minutes of sonication was due to fragmentation of DNA of those few spores already fractured.

3.4.5. Correlation of C_t value with number of vegetative cells

Standard curves were established for vegetative cells of separate cultures of *A. flavithermus* C and *B. licheniformis* F in sterile deionised water and for a mixed culture containing equal numbers of both organisms in reconstituted milk powder (Figure 3.6).

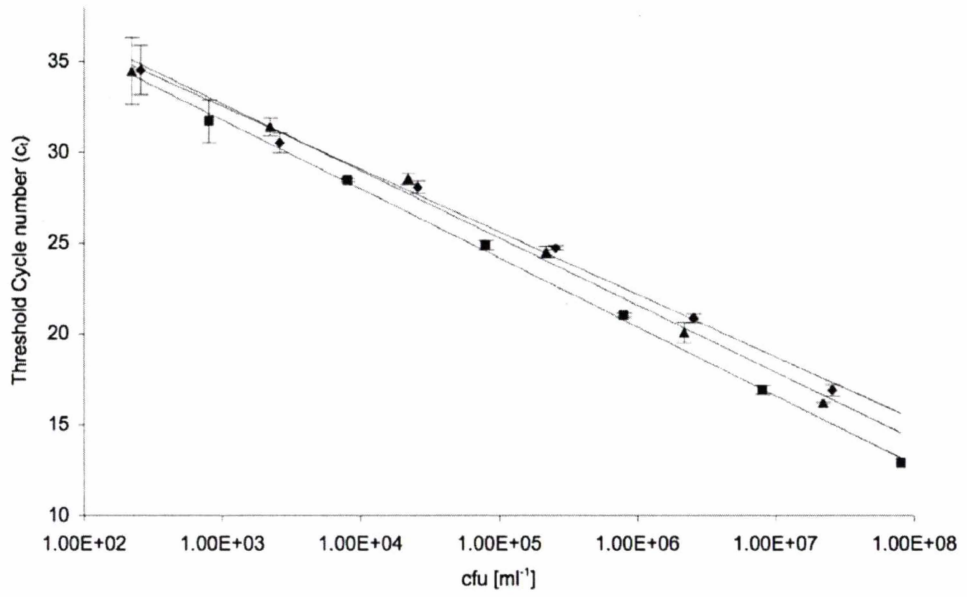

Figure 3.6. Semi-logarithmical plot of C_t value against vegetative cell concentration of (■) *A. flavithermus* C (y = -1.605ln(x) + 43.766), (♦) *B. licheniformis* F (y = -1.4963ln(x) + 42.855) and (▲) a mixed culture (y = -1.6462ln(x) + 43.131) in milk. An aliquot of 10 µl of sonicated sample was used in a 25 µl PCR reaction. The error bars are the standard deviation for three sonicated replicates.

The Smart Cycler PCR amplifications for pure cultures were linear over a range of 2×10^7 to 2×10^2 cfu ml^{-1} and 8×10^7 to 8×10^2 cfu ml^{-1} for the mixed culture of both organisms recovered from milk. The correlation coefficient (R^2) for all standard curves were greater than 0.99.

3.4.6. Correlation of C_t value with number of spores

The standard curves for spores used 10-fold diluted spore suspensions of *A. flavithermus* C and *B. licheniformis* F in sterile deionised water and from a mixed spore suspension of both organisms in reconstituted milk. The 16S rRNA PCR assays were linear for pure cultures in a range between 1.15×10^8 to 1.3×10^4 spores ml^{-1} and 6.4×10^7 to 6.4×10^3 spores ml^{-1} for mixed cultures of both organisms in milk (Figure 3.7).

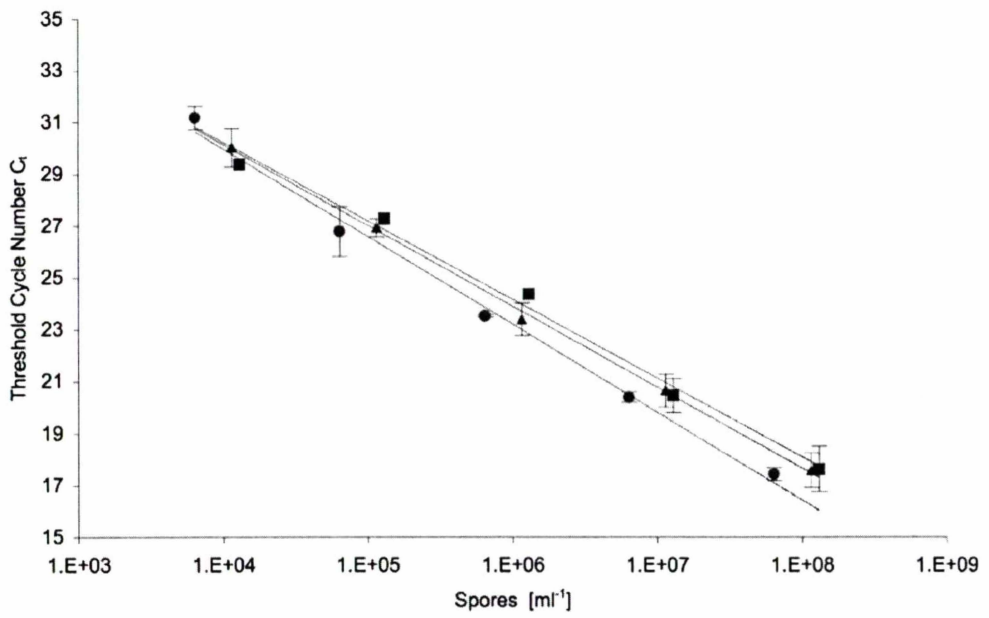

Figure 3.7. Semi-logarithmical plot of C_t value against spore concentration for (▲) *A. flavithermus* C (y = -1.3554ln(x) + 42.647; R^2 = 0.99), (■) *B. licheniformis* F (y = -1.3185ln(x) + 42.404; R^2 = 0.98) and a mixed spore culture of (●) both organisms in milk (y = -1.4723ln(x) + 43.563; R^2 = 0.99). The error bars are the standard deviation for three sonicated replicates.

3.5. Discussion

Thermophilic strains of *A. flavithermus* C and *B. licheniformis* F can have a significant impact on the overall economy of the milk powder processing operations

(Ronimus et al., 2003; Rueckert et al., 2004). Thus, there is a need for a rapid and quantitative detection method for the real-time monitoring of these organisms within the plants to overcome unnecessary plant down time. Real-time PCR with specific primers and TaqMan probe can provide a highly selective and sensitive method for the accurate quantification of individual species from a background of diverse DNA species. However, real-time PCR detection of milk powder contaminants requires a rapid bacterial and DNA extraction method to remove fat, proteins and minerals and yet be still sensitive and robust enough for quantitative analysis. DNA and bacterial cell extraction methods of milk contaminants have been described (Cornejo et al., 1998; Herman et al., 1997; Rijpens et al., 1996; Gunasekera et al., 2000; Nogva et al., 2000) allowing in at least one case the detection of 5 to 50 cfu ml^{-1} (Romero et al., 1999). Unfortunately, these procedures employ either time consuming bacteriological isolations or complex DNA extraction methods to obtain amplifiable PCR targets. In this investigation, a rapid and inexpensive DNA extraction method from milk, based on trisodium citrate and *n*-decane extraction of vegetative cells and spores combined with ultrasonic treatment, was developed which produces DNA samples suitable for quantitative PCR in less than 20 minutes. Trisodium citrate is known for its ability to chelate traces of divalent cations, including calcium ions. The removal of calcium ions associated with casein micelles of the milk leads to the dissociation of the ß-casein (Walstra, 1990; Walstra et al., 1999) and milk clearance. Furthermore, milk lipids can be easily separated using *n*-decane.

Quantitative real-time analysis was accomplished using specific primers and a TaqMan probe targeting the ribosomal 16S rRNA genes of thermophilic milk powder bacilli. The majority of prokaryotes contain multiple ribosomal operons which can vary from one to 15 copies per genome (Klappenbach et al., 2001; Shaver et al., 2001). *B. subtilis* strain 168, for instance, contains 10 ribosomal 16S rRNA copies in its genome (Bott et al., 1984) and some laboratory *B. subtilis* strains were reported to contain nine rather than ten ribosomal operons due to deletion (Widom et al., 1988). In addition, analysis of the 16S-23S rRNA intergenic transcribed spacer regions from six species of the *Bacillus cereus* group have indicated that the number of ribosomal operons varied between 8 to 12 (Cherif et al., 2003; Daffonchio et al., 2000). The number of 16S rRNA copies from *A. flavithermus* C and *B. licheniformis* F was investigated by southern hybridization of restricted genomic DNA, and it could be shown that at least 8 copies of the 16S rRNA gene occur in both target organisms

(Chapter 7). The ratio of 16S rRNA copies for both organisms was also examined by 16S rRNA quantitative PCR using 1 ng of genomic DNA. The fluorescence profiles for amplification product for both organisms crossed the threshold base line at a mean C_t of 17.01 ± 0.24 supporting the notion that near equivalent copies of 16S ribosomal RNA genes were likely to be located on the genomes (Figure 3.2A). In addition, the detection of the other contaminating strains of thermophilic bacilli, e.g., *G. stearothermophilus* (strain A), *A. flavithermus* (strain B and D), *Bacillus licheniformis* (strain G) and *B. subtilis* was also checked using 9 ng of initial genomic target DNA in comparison to *A. flavithermus* (strain C) and *B. licheniformis* (strain F). The mean C_t value for all bacilli was 14.75 ± 0.41 (section 7.3.4.).

The quantitative PCR analysis of small regions of the 16S rRNA genes has the potential to detect genomic DNA in a linear range from 15 ng to 15 fg (Figure 3.3). This indicates a lower detection limit of approximately 2 to 10 cells for the current method assuming an average DNA content of 2 to 8.9 fg per cell (Bakken and Olsen, 1989). However, the sensitivity and reproducibility of the quantitative PCR assay can be limited by the effectiveness of the DNA isolation method applied, especially for very low DNA target samples.

Sonication using 120 W at 20 kHz of a 16 hour 4.8×10^8 cfu ml^{-1} vegetative culture of *A. flavithermus* C in sterile deionised water for 125 seconds was sufficient to obtain the highest yield of PCR amplifiable genomic DNA. Ultra-sonication beyond this threshold led to a progressive degradation of the DNA template similar to that reported by Bakken and Olsen (1989) and Fykse et al. (2003). DNA release by boiling cultures in a water bath for 10 minutes was also investigated but was deemed to be unsuitable for quantitative analysis. The relative yield of DNA for boiled samples of a 4.8×10^8 cfu ml^{-1} culture of *A. flavithermus* C was 39.5 ng ± 1.13 ng ml^{-1} and not significantly different from an untreated control sample (31.13 ng ± 3.51 ng ml^{-1}) whereas the 125 second sonicated sample contained twenty times more DNA (761.4 ng ± 10.74 ng ml^{-1}).

Spores are extremely resistant to the sonication disruption method (Levi et al., 2003; Berger and Marr, 1960) and need extended sonication times in the presence of glass beads (Belgrader et al., 1999). Glass beads can help to increase the energy release by implosion and facilitate the crushing of the spores. However, the maximum DNA yield for spores was still fourteen times lower compared to equivalent numbers of vegetative cells. The reasons are due to the extreme resistance of spores against

mechanical forces. This was confirmed by spore counts in which 88% of the spores survived the sonication procedure and were still able to form viable colonies. Another reason for the poor DNA yield from spores could be that the prolonged sonication time of 6 minutes could have caused excessive fragmentation of DNA released earlier in the sonication process. Thus, with spore suspensions, DNA release is a compromise between extending the sonication time to try and break open as many spores as possible weighed against the rapid fragmentation of DNA once it is released. The maximum DNA yield for 6.7×10^7 spore ml^{-1} cultures of *B. licheniformis* F in the presence of 50 mg of 0.1 mm glass beads was determined to be 53.75 ng ml^{-1} after 6 minutes of ultrasonic disruption. Surprisingly, the effect of sonication beyond 6 minutes did not cause progressively more spores to be lysed as shown by the OD_{450} reading, indicating that the rate of spore disruption is not constant to the sonication time (Figure 3.5). The unsonicated spore suspension responded with a mean value equivalent to a DNA concentration of 0.4 ng ml^{-1} which we assume reflects extraneous DNA (possibly derived from lysed mother cells) still adhering to the outside of the spore (Belgrader et al., 1999).

In conclusion, the methods described in this study can help to optimize the plant running time for milk powder production, as it enables the quantitative and selective real-time assessment of thermophilic contaminants in milk powders in less than 1 hour. The quantitative PCR assay has a lower detection limit of 8 vegetative cell ml^{-1} and 64 spores ml^{-1} of reconstituted milk and is linear over six and five orders of magnitude, respectively. The next logical step is the application of the current method to the analysis of contamination in a factory setting during processing runs. It is envisaged that the cumulative data from multiple sampling times during processing runs, starting soon after CIP treatment and continuing to sub-specification thresholds, would aid in generating an accurate and reliable enumeration and identification of the thermophilic flora.

3.6. References

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Development of a real-time PCR assay targeting the sporulation gene, *spo0A*, for the enumeration of thermophilic bacilli in milk powder

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4.1. Abstract

Thermophilic bacilli, such as *Anoxybacillus*, *Geobacillus* and *Bacillus*, are common contaminants growing within the processing lines of milk powder producing factories. These contaminants are used as indicator organisms for plant hygiene and specification limits based on their numbers have been implemented to ensure milk powder quality. In this study, we present a SYBR Green-based real-time PCR assay for the rapid detection and enumeration of these thermophilic bacilli in milk powder using the *spo0A* sporulation gene as quantification target. With this method the detection of thermophilic bacilli in milk powder can be accomplished within 1 h. The detection limit for reconstituted and inoculated milk was 80 vegetative cfu ml^{-1} and 640 spores ml^{-1}, respectively.

Keywords: *Anoxybacillus*; *Bacillus*; *Geobacillus*, Milk powder; Real-time PCR; SYBR Green; *spo0A* sporulation gene, Thermophiles.

4.2. Introduction

Seven strains of thermophilic bacilli able to grow at 55°C or above have been recognized as the major contaminants growing in the processing lines of New Zealand milk powder plants (Ronimus et al., 2003). These are *Geobacillus stearothermophilus* (strain A), *Anoxybacillus flavithermus* (strains B, C and D), *Bacillus licheniformis* (strains F and G) and *Bacillus subtilis* (strain BS). Furthermore, a second investigation on milk powders derived from eighteen different countries demonstrated that *A. flavithermus* strain C, *B. licheniformis* strain F and *G. stearothermophilus*

strain A represent world-wide sources of contamination being near-ubiquitously present in milk powders, and are thus of economic importance in processing (Rueckert et al., 2004). Although it is universally accepted that these thermophilic bacilli do not constitute a health risk to the consumer, they have been deemed to be an indicator of plant hygiene during processing, and specification limits have been implemented based on numbers of thermophilic bacilli in products (Stadhouders et al., 1982; Murphy et al., 1999; Kwee et al., 1986; Ronimus et al., 2003). Typically, milk powder is produced continuously over an 18 to 24 hour processing period during which the number of thermophilic bacilli in the product often mirrors that of a typical bacterial growth curve. Thus, with increased processing time, the number of thermophiles increases until specification limits are reached and the process run is terminated to prevent product downgrading. Many factors, such as the microbiological quality of the raw milk or the thermal operating conditions in the plant (low-, medium or high heat treatments) or the plant hygiene can all affect the growth of thermophiles in the processing line, making it difficult to predict a processing time that ensures thermophile numbers at the end of the run are below specified limits. In practice, actual numbers of contaminants in milk powders are determined retrospectively by plate counting, providing results commonly after 16 to 48 hours. Due to these microbiological constraints, it is common practice to terminate processing runs by initiating CIP (cleaning in place) with the expectation that the numbers of thermophilic bacilli will be below their specification limits. A real-time method for monitoring contamination level during production would allow processing to continue until specification limits were close to being met, could maximise plant efficiency and reduce the amount of cleaning chemicals used.

In the present study we describe the development of a rapid method for the detection and enumeration of the seven major thermophilic bacilli in milk powder. For this purpose, the complete and near-complete nucleotide gene sequences of the sporulation transcription factor *spo0A* were obtained and used to develop a quantitative real-time PCR assay based on SYBR green technology. The current assay, combined with previously described methods for total bacterial extraction from reconstituted milk and DNA preparation from vegetative cells and spores (Belgrader et al., 1999; Fykse et al., 2003; Rueckert et al., 2005a), represents an effective

protocol to enumerate these bacilli during milk processing within 60 minutes of sampling.

4.3. Material and methods

4.3.1. Bacterial strains and culture preparation

Geobacillus stearothermophilus (strain A), *A. flavithermus* (strains B, C and D), *B. licheniformis* (strains F and G) and *B. subtilis* (BS) were isolated from New Zealand milk powders by Ronimus et al. (2003). Strains of *Ureibacillus thermosphaericus*, *Bacillus circulans*, *Bacillus coagulans* and *Bacillus pumilus* were isolated from milk powders and their origin listed in Table 4.3 (Rueckert et al., 2004). All cultures were grown at 55°C in tryptic soy broth (TSB) supplemented with 0.2% (w/v) soluble potato-starch (Sigma; S2004). *Bacillus cereus* DSM 31, *Bacillus smithii* DSM 459, *Bacillus megaterium* DSM 32, *Clostridium paradoxum* DSM 7308, *Streptococcus thermophilus* DSM 20479, *Escherichia coli* DSM 301, *Micrococcus luteus* DSM 2786 and *Lactobacillus plantarum* DSM 20205 were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and cultured according to the supplier's recommendation. Genomic DNA from *Clostridium perfringens* (*Clostridium welchii*) was purchased from Sigma-Aldrich (D5139).

Spores of *A. flavithermus* C and *B. licheniformis* F were produced in liquid Castenholz medium (DSMZ) by incubation at 55°C for 72 hours of an inoculum grown in TSB-starch. On completion of sporulation the spores were harvested by centrifugation at 16,100 *g* (5415 D, Eppendorf) and the pellet re-suspended in sterile deionised water. The suspensions were purified of vegetative cells by lysozyme-treatment (2 mg ml^{-1}) for 30 minutes at 37°C, followed by sonication for 125 seconds (XL-2020, Misonix) at 120 W at 20 kHz and washing in two changes of sterile deionised water. The spores were then washed successively with 1 M NaCl, 0.1 M NaCl, 0.1% SDS and sterile deionised water before storing at 4°C. Spore and vegetative cell counts were conducted by phase-contrast microscopy using a Thoma counting chamber and by plate counting on tryptic soy agar (TSA) with 0.2% (w/v) soluble potato-starch at 55°C.

4.3.2. Ultra-purification of genomic DNA

DNA was extracted from all cultures by a modification of the procedure described by Sambrook and Russel (2001). Following growth, 10 ml of cell suspension were harvested and re-suspended in 1 ml of 50 mM Tris HCl (pH 8.0), 100 mM NaCl and 20 mM EDTA. A total of 2 mg ml^{-1} lysozyme and 50 µg ml^{-1} RNase was added and the samples incubated at 37°C for 45 minutes. SDS and proteinase K were added to 1% (w/v) and 200 µg ml^{-1}, respectively, and the samples incubated at 55°C for 1 hour. Subsequently, the samples were extracted with equal volumes of phenol:chloroform (1:1) followed by chloroform and chloroform:isoamyl alcohol (24:1). The DNA was then precipitated by the addition of 1/10 volumes of 3 M sodium acetate and 0.6 volumes of isopropanol and incubating at -20°C for 15 minutes. The DNA pellets were washed twice with ice chilled 80% ethanol, air dried and the DNA pellets re-suspended in 500 µl of 1× TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The samples were quantified by spectrophotometer (Pharmacia Biotech, Ultraspec 3000) readings at A_{260} for DNA, A_{280} for contaminating proteins (containing tryptophan, tyrosine and/or phenylalanine) and at A_{320} to compensate for background effects. The samples were stored at -20°C. The DNA preparations were used as quantification standards and positive controls.

4.3.3. Sequencing of the spo0A genes

Degenerate oligonucleotide primers targeting small regions of the *spo0A* genes of *Bacillus* sp. were synthesized (Invitrogen, New Zealand) as previously described by Brill et al. (1997). The primers were used to amplify a region of 300 bp of the *spo0A* gene of *G. stearothermophilus* strain A, *A. flavithermus* (strains B, C and D), *B. licheniformis* (strains F and G) and *B. subtilis* strain BS according to the protocol listed (Brill et al. 1997). Following PCR, the amplification reactions were electrophoresed through a 1.5% (w/v) agarose gel (SeaKem, LE Agarose) at 55 or 98 Volts (Power Pack Model 250; Life Technologies, GIBCO BRL) using a HORIZON 11·14 or 20·25 electrophoresis box (Life Technologies, GIBCO BRL), respectively. Gels were stained with ethidium bromide (20 µg ml^{-1}) and de-stained with sterile distilled water for 10 minutes. The gels were then viewed on a UV transilluminator

and the *spo0A* amplification products excised with a sterile razor blade. The excised bands were wrapped between sheets of parafilm and frozen at -20°C. Subsequently, a firm and constant pressure was applied to the wrapped gels and the exudates collected into 1.5 ml Eppendorf tubes. These DNA samples were extracted with phenol:chloroform, chloroform and chloroform:isoamyl alcohol, then further precipitated with sodium acetate/isopropanol and quantified as described. The PCR amplified *spo0A* products were sequenced in order to design eight strain-specific inverse PCR oligonucleotide primers (Table 4.1). The PCR products were additionally used as oligonucleotide probes for southern hybridization in order to find suitable restriction enzymes for inverse-PCR (Ochman et al., 1988). For this purpose, aliquots of 3 µg of genomic DNA of *G. stearothermophilus* A, *A. flavithermus* (strains B, C and D), *B. licheniformis* (strains F and G) and *B. subtilis* were used for total restriction digestion employing *Eco* RI, *Cla* I, *Eco* RV, *Hae* II and *Pst* I (Roche). Restriction digestion was performed according to the manufacturers recommendations and the restricted DNA samples purified by phenol, chloroform and isoamyl alcohol extraction and precipitation. An aliquot of 1 µg of the restricted DNA was then separated though a 0.7% (w/v) agarose gel by electrophoresis and southern lifts performed using the alkali transfer method (Sambrook and Russel, 2001). Southern hybridization employed α-^{32}P-dCTP incorporation method described in Sambrook and Russel, (2001) (section 7.2.5, 7.2.6. and 7.2.7). Restriction digested samples, which produced hybridization signals in the range of 700 to 2000 bp were used for ligation and inverse PCR. For this purpose, an aliquot of 0.5 µg of restricted DNA was used in a 100 µl reaction using 1.5 units T4 Ligase and 20 µl of 5× ligase buffer (Invitrogen). The reactions were incubated over night at 15°C and an aliquot of 10 ng of circular DNA used to carry out inverse PCR on a Mastercycler (Gradient; Eppendorf) using 4 mM $MgCl_2$, 0.2 mM dNTPs, 10× *Taq* PCR reaction buffer, 1.25 units of Ampli*Taq* DNA polymerase (Roche), 500 nM of forward and reverse inverse primer. The PCR amplifications were cycled once at 95°C for 120 seconds and 35 cycles at 92°C for 30 seconds, 48°C for 30 seconds and at 72°C for 90 seconds. The PCR included a final extension step of 5 minutes at 72°C. Subsequently, the PCR products were separated by electrophoresis through a 1.5% (w/v) agarose gel and the PCR bands of interest purified from the gel as described above and used for sequencing. Additionally, two conventional PCR primer pairs were designed from the

spo0A gene sequences of *G. stearothermophilus* (Muchovà, et al., 1999) and *B. subtilis* (Ferrari et al., 1985) (Table 4.1) to obtain sequence information for DNA samples which could not be sequenced by inverse PCR. These amplification reactions contained 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 10× *Taq* PCR reaction buffer, 1.25 units of Ampli*Taq* DNA polymerase (Roche), 500 nM of both primers and 10 ng of genomic DNA. Both amplification reactions were cycled as follows: 95°C for 120 seconds and 35 cycles at 95°C for 30 seconds, 47°C for 30 seconds, 72°C for 90 seconds and finally, a 5 minutes extension step at 72°C.

Multiple sequence alignments were performed on-line with CLUSTAL W 1.82 (European Bioinformatics Institute; http://www.ebi.ac.uk/clustalw/). Phylogenetic analysis employed CLUSTAL X using the neighbour-joining algorithm while the analysis was statistically validated using 100 bootstraps (Thompson et al., 1994). The phylogenetic cladogram was illustrated using NJPLOT provided by CLUSTAL X.

Table 4.1 Primers

Primer	Organism	Strain	Orientation	Oligonucleotide sequences (5′-3′)*
Inverse PCR	*G. stearothermophilus*	A	forward	ACTTAGCCCACCATATTC
			reverse	ACTTGGACGCGAGCATTA
	A. flavithermus	B, C, D	forward	AGCAGTTGAACTTGGMGC
			reverse	CCAATTCGTGACGGAAA
	B. licheniformis	F, G	forward	TGACATGGAGAAYTTAGT
			reverse	ATCACGACCATCATCCA
	B. subtilis	BS	forward	TACTTTATTCTCAAACCGT
			reverse	AAAGCAGTATTATACGCA
Conventional PCR	*G. stearothermophilus*	B4419	forward	AGCATTAAAGTGTGTATTGCCGA
			reverse	TTATGACGCTTTATGCTCAA
	B. subtilis	W168	forward	GAGAAAATTAAAGTTTGTGTTGCT GA
			reverse	TTAAGAAGCCTTATGCTCTAACCT
Real-Time PCR	*Geobacillus*, *Bacillus*,		forward	ATYATGYTVACRGCVTTYGGBCAR GAAGA
	Anoxybacillus		reverse	TAKCCTTTWATRTGIGCDGGIACR CCGATTTC

* Nucleotide substitution according to the universal degenerate code (Invitrogen; https://catalog.invitrogen.com/): M=(A/C), R=(A/G), W=(A/T), Y=(C/T), K=(G/T), V=(A/G/C) B=(T/C/G), D=(A/G/T) and I=(A/G/C/T). R=(A/G), W=(A/T), Y=(C/T), K=(G/T), V=(A/G/C) B=(T/C/G), D=(A/G/T) and I=(A/G/C/T).

4.3.4. Sequencing

DNA sequencing was carried out by the Waikato DNA Sequencing Facility on a MegaBACE capillary DNA Analysis System.

4.3.5. Extraction of vegetative cells and spores from reconstituted milk

Bacterial cells and spores were separated from milk as described in Rueckert et al. (2005a). This included the addition of 200 µl of 1.2 M trisodium citrate and 200 µl of *n*-decane (Merk-Schuchardt) to 1 ml of reconstituted milk powder in sterile water (0.1 g ml^{-1}). The sample was vortexed for 10 seconds and centrifuged at 16,100 *g* for 10 minutes. The upper layer of cream was carefully detached from the tube wall using a sterile 200 µl pipettor tip and the supernatant poured off carefully by gently inverting the tube. The samples were re-centrifuged for 2 minutes to collect any remaining liquid, which was then carefully removed by pipetting while avoiding disrupting the cell pellet. The bacterial pellet was re-suspended in 1 ml of sterile deionised water and the DNA released by sonication.

4.3.6. DNA preparation from vegetative cells and spores

DNA extraction from cells and spores for quantitative PCR analysis was accomplished by sonication as described in Rueckert et al. (2005a). Sonication was performed with the ultrasonic liquid processor XL-2020 (Misonix) at 120 W at 20 kHz using a 3.2 mm microtip disruptor horn on 1ml of bacterial suspension in a 2 ml Eppendorf micro-tube. DNA from vegetative cells was released by sonication for 125 seconds. Spores were sonicated in the presence of 50 mg of 0.1 mm glass beads (Biospec Products) for 360 seconds. After sonication, the samples were centrifuged at 16,100 *g* for 2 minutes and a 10 µl aliquot of the supernatant subjected to quantitative PCR analysis.

4.3.7. Real-time PCR

PCR analysis was performed with a Smart Cycler II system (Cepheid, USA) in 25 µl optical reaction tubes. The reactions were performed with 4 mM $MgCl_2$, 0.2

mM dNTPs, 10× *Taq* PCR reaction buffer, 1.5 units of *TaKaRa Taq*^{TM} Hot Start enzyme (TaKaRa Bio INC.), 0.25× SYBR Green I (Sigma-Aldrich; S9430) and 600 nM of forward and reverse primer. The amplification reactions for DNA standard curves utilised 1 µl of a 10-fold dilution series of template DNA, i.e. ultra-purified DNA from *A. flavithermus* strain C and *B. licheniformis* strain F. Quantitative PCR analysis used a 10 µl aliquot of the DNA preparations of sonicated cells or spores extracted from reconstituted milk samples. The real-time PCR was cycled once at 95°C for 120 seconds, 40 repetitions at 95°C for 5 seconds, 57°C for 5 seconds, 68°C for 20 seconds and fluorescence acquisition at 83°C for 6 seconds. On completion of cycling, amplicons were directly identified using the melting point analysis protocol of the Smart Cycler instrument (60°C to 95°C; 0.2°C sec^{-1}). Quantitative real-time PCR was performed in triplicate for each sample and negative controls (no DNA template) and positive controls (0.1 ng of ultra-purified *A. flavithermus* C genomic DNA) were included in each experiment.

4.4. Results

4.4.1. Sequencing of the spo0A genes and primer design

Complete and near-complete DNA sequences of the *spo0A* genes were obtained by inverse PCR for restriction digested and circularized genomic DNA of *G. stearothermophilus* A (*Eco* RI), *A. flavithermus* C (*Cla* I), *B. licheniformis* (strains F and G) (*Eco* RV) and *B. subtilis* (BS) (*Eco* RI). Inverse PCR did not produce informative sequence data for the upstream regions starting from the *spo0A* hybridization probe for *B. subtilis* and *A. flavithermus* strain C and thus, these sequence data were acquired by conventional PCR using the PCR primers designed from the *spo0A* gene sequences of *G. stearothermophilus* (Muchovà, et al., 1999) and *B. subtilis* (Ferrari et al., 1985), respectively (Table 4.1). Similarly, due to the presence of an *Eco* RV restriction site downstream from the probe hybridization site within the *spo0A* gene sequences of *B. licheniformis* strain F and G, inverse PCR was not capable of providing full gene sequences for these strains. However, these sequences were acquired using the *B. subtilis* specific primer pair (Table 4.1). Southern hybridization did not produce a hybridization signal within the expected

molecular size range for *A. flavithermus* B and D. The near-complete *spo0A* sequences of both strains were obtained using the *G. stearothermophilus* specific PCR primer pair (Table 4.1).

The *spo0A* protein alignment of the translated nucleotide sequences is shown in Figure 4.1 and the NCBI accession numbers are listed in Table 4.2. From the alignment of the seven new *spo0A* nucleotide sequences, a degenerate oligonucleotide primer pair for real-time PCR was designed targeting the phosphor-acceptor and effector-domain of the encoding *spo0A* protein (Table 4.1; Figure 4.1). The protein sequences of both regions are highly conserved and identical for all bacilli from Figure 4.2. A contrary situation was found for the internal domain, the so-called connector-segment that exhibits a high degree of sequence divergence (Figure 4.1).

Table 4.2 NCBI accession numbers of *spo0A* sequences

Organism	strain	Sequence length [bp]	Completion	NCBI accession number
Geobacillus stearothermophilus	A	780	full	AY672766
Anoxybacillus flavithermus	B	720	partial	AY672767
Anoxybacillus flavithermus	C	743	partial	AY672771
Anoxybacillus flavithermus	D	714	partial	AY672768
Bacillus licheniformis	F	772	partial	AY672770
Bacillus licheniformis	G	772	partial	AY672769
Bacillus subtilis	BS	773	partial	AY672772

4.4.2 Real-time PCR optimization

The quantitative real-time PCR assay was optimized following recommendations by Cepheid, Pfaff (2002), Teo et al. (2002), Decker et al. (2002), Ball et al. (2002) and Vandesompele et al. (2002). This included primer titration between 100 nM to 1100 nM against low, medium and high DNA target concentrations (0.01 ng, 0.1 ng and 1 ng) with the primer performance being rated by threshold cycle number (C_t) response, end-point fluorescence and primer-dimer formation. A concentration of 600 nM for both forward and reverse primer performed with the highest efficiency; at higher concentrations the formation of primer-dimers became more evident while at lower concentrations, less product formation was observed (Appendix 12.3.4). The $MgCl_2$ concentration was titrated between 1.5 and 8.5 mM; concentrations of 2.5 mM $MgCl_2$ or less gave poor amplification on the Smart Cycler II and resulted in the formation of primer-dimers (Appendix 12.3.1).

```
A. flavithermus D          ----------SGIVHLLEEYITSQGDMEVIGVAYNGQDCLQLLRERDPDVLVLDIIMP 48
A. flavithermus B          ----------QXLGIVHLLEEYITSQGDMEVIGVAYNGQDCLQLLRERDPDVLVLDIIMP 50
A. flavithermus C          ----------VVHLLEEYITSQGDMEVIGVAYNGQDCLQLLRDRDPDVLVLDIIMP 46
G stearothermophilus A     -MSIKVCIADDNRELVSLLDEYISSQPDMEVIGTAYNGQDCLQMLEEKRPDILLLDIIMP 59
B. licheniformis F         MEKIKVCVADDNRELVGLLSEYIEGQSDMEVLGVAYNGQECLSLFKDKEPDVLVLDIIMP 60
B. licheniformis G         MEKIKVCVADDNRELVGLLSEYIEGQSDMEVLGVAYNGQECLSLFKDKEPDVLVLDIIMP 60
B. subtilis BS             ----------YRELVSLLSEYIEGQEDMEVIGVAYNGQECLSLFKEKDPDVLVLDIIMP 49
                                     :* **.*** .* ****:*.*****:**.::.:: **:*:******

A. flavithermus D          HLDGLAVLEKLR-MFKQPLPNVIMLTAFGQEDVTKKAVELGASYFILKPFDIEHLVNQIR 107
A. flavithermus B          HLDGLAVLEKLR-MFKQPLPNVIMLTAFGQEDVTKKAVELGASYFILKPFDIEHLVNQIR 109
A. flavithermus C          HLDGLAVLEKLR-MIKQPLPNVIMLTAFGQEDVTKKAVELGASYFILKPFDIEYLVNQIR 105
G. stearothermophilus A    HLDGLAVLERIR-AGFEHQPNVIMLTAFGQEDVTKKAVELGASYFILKPFDMENLAHHIR 118
B. licheniformis F         HLDGLAVLERLRESQLKKQPNVIMLTAFGQEDVTKKAVDLGASYFILKPFDMENLVGHIR 120
B. licheniformis G         HLDGLAVLERLRESQLKKQPNVIMLTAFGQEDVTKKAVDLGASYFILKPFDMENLVGHIR 120
B. subtilis BS             HLDGLAVLERLRESDLKKQPNVIMLTAFGQEDVTKKAVDLGASYFILKPFDMENLVGHIR 109
                           *********::*    :  ******************:************:* *. :**

A. flavithermus D          HVCGKKPALLKRP------LLSP-IRDGKPKNLDASITNIIHEIGVPAHIKCYLYLREAI 160
A. flavithermus B          HVCGKKPALLKRP------LLSP-IRDGKPKNLDASITNIIHEIGVPAHIKCYLYLREAI 162
A. flavithermus C          HVCGKKPAVFKRP------ILAP-IRDGKPKNLDASITSIIHEIGVPAHIKCYLYLREAI 158
G. stearothermophilus A    QVYGKTTPVVRKA------APAPQVRDNKPKNLDASITSIIHEIGVPAHIKCYLYLREAI 172
B. licheniformis F         QVSGNGSQLTHRAPSSQSSVLRP-QPESPKKNLDASITTIIHEIGVPAHIKCYLYLREAI 179
B. licheniformis G         QVSGNGSQLTHRAPSSQSSVLRP-QPESPKKNLDASITTIIHEIGVPAHIKCYLYLREAI 179
B. subtilis BS             QVSGNASSVTHRAPSSQSSIIRSSQPEPKKKNLDASITSIIHEIGVPAHIKCYLYLREAI 169
                           :* *: . : ::.          .   :   ********.********************

A. flavithermus D          AMVYNDVELLGSITKVLYPDIAKKYNTTASRVERAIRHAIEVAWSRGNLESISSLFGHTV 220
A. flavithermus B          AMVYNDVELLGSITKVLYPDIAKKYNTTASRVERAIRHAIEVAWSRGNLESISSLFGHTV 222
A. flavithermus C          AMVYNDVELLGSITKVLYPDIAKKYNTTASRVERAIRHAIEVAWSRGNLESISSLFGHTV 218
G. stearothermophilus A    AMVYHDIELLGSITKVLYPDIAKKYNTTASRVERAIRHAIEVAWSRGNLDSISSLFGYTV 232
B. licheniformis F         SMVYNDIELLGSITKVLYPDIAKKFNTTASRVERAIRHAIEVAWSRGNIDSISSLFGYTV 239
B. licheniformis G         SMVYNDIELLGSITKVLYPDIAKKFNTTASRVERAIRHAIEVAWSRGNIDSISSLFGYTV 239
B. subtilis BS             SMVYNDIELLGSITKVLYPDIAKKFNTTASRVERAIRHAIEVAWSRGNIDSISSLFGYTV 229
                           :***:*:*****************:**********************::*******:**

A. flavithermus D          SMSKAKPT-TQSYAMC----------- 235
A. flavithermus B          SMSKAKPT-TQSYAMC----------- 237
A. flavithermus C          SMSKAKPTNSEFIAMVADRLRLEHKAS 245
G. stearothermophilus A    SVSKAKPTNSEFIAMVADKLRLEHKAS 259
B. licheniformis F         SMSKAKPTNSEFVAMSQQ--------- 257
B. licheniformis G         SMSKAKPTNSDGRDVADK--------- 257
B. subtilis BS             SMTKAKPTNSEFIAMVADKLRLEHKGS 256
                           *::***** ::   :
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Figure 4.1. CLUSTAL W (1.82) multiple sequence alignment of *spo0A* protein sequences derived from the seven thermophilic milk powder bacilli. Reference numbers at the right margin correspond to the position of amino acid in each sequence; "*" means that the residues are identical; ":" means that conserved substitutions have been observed; "." means that semi-conserved substitutions are observed and sequence gaps are indicated by "-". According to Brown et al. (1997), the location of the phosphor-acceptor domain, the connector-segment and the effector-domain are approximately at residue positions 0-122, 123-142 and 143-259, respectively, referring to the numbering of *G. stearothermophilus* strain A. The primer annealing sites for PCR amplification are highlighted in grey at positions 80-89 (forward primer) and 164-174 (reverse primer).

Magnesium concentrations between 3.5 and 8.5 mM $MgCl_2$ amplified equally well, while end-point fluorescence readings were highest between 3.5 and 5.5 mM $MgCl_2$ (Appendix 12.3.1). Thermal cycling was optimized for template denaturing, annealing, and extension following recommendation from Cepheid (Getting Started with Smart Cycler® Real Time PCR; SYBR® Green I Assays on the Smart Cycler® System). Primer pair annealing was optimized between 45°C and 63°C using a 20 second elongation step (Appendix 12.3.2).

The fluorescence acquisition temperature was 83°C for a period of 6 seconds subsequent to elongation to reduce the detection of non-specific amplification products (Vandesompele et al., 2002). The SYBR Green I titration was performed between dilutions of 0.125× and 1× of reporter dye. The reproducibility of the

reactions were consistent using 0.125 to 0.25× SYBR Green I. A concentration of 0.5× led occasionally to PCR inhibition, especially at low template number while concentrations above 0.75× resulted in PCR failure (Appendix 12.3.3).

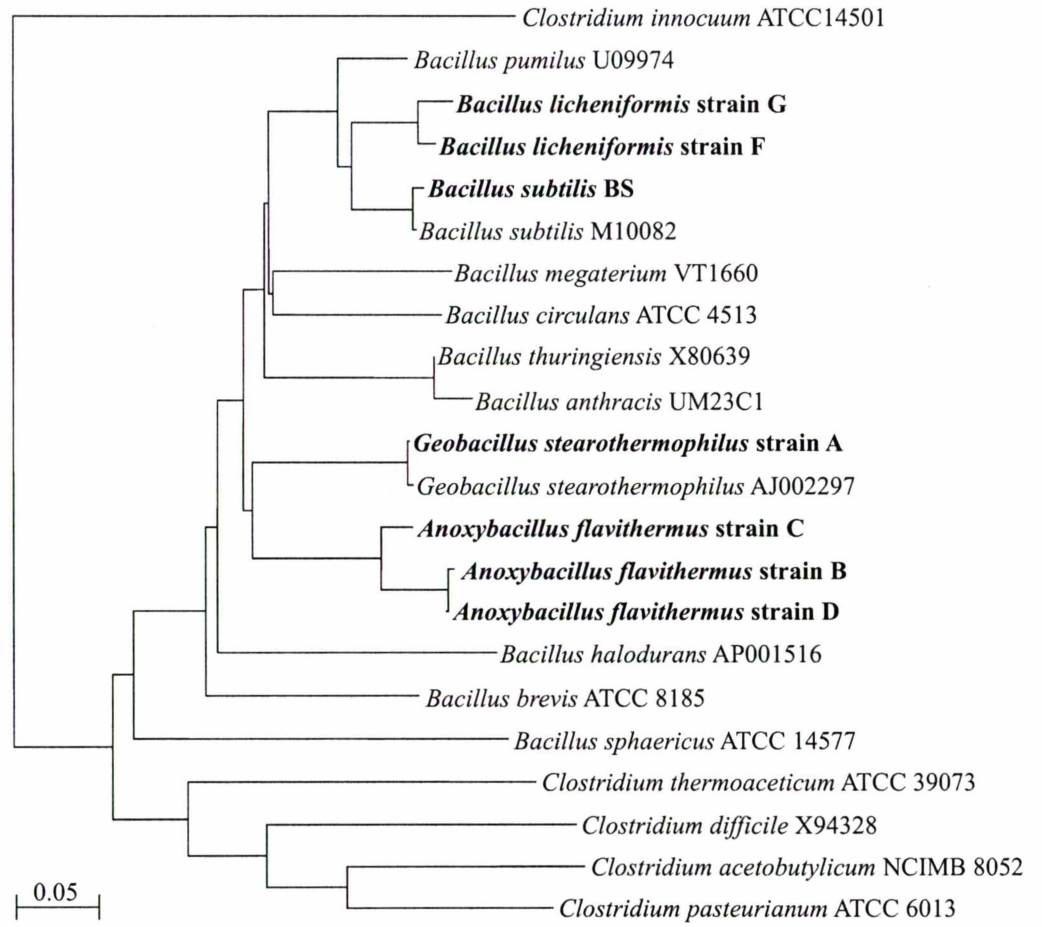


Figure 4.2. Phylogenetic cladogram of complete and near-complete *spo0A* protein sequences using the neighbor-joining method. The scale bar length of 0.05 denotes the number of amino acid replacements per site validated with 100 bootstraps. The NCBI accession numbers or type strain numbers are indicated after the species nomenclature. The new *spo0A* sequences are indicated in bold and their accession numbers are shown in Table 4.2.

4.4.3. Primer selectivity

The real-time PCR primers were tested against 13 bacilli, two clostridial strains and four non-spore-formers using 5 ng of genomic DNA per 25µl reaction. All the bacilli produced an amplicon of the expected size although slight variations in amplicon-length were observed (Figure 4.3a). Melting point analysis performed on the PCR amplicons showed that the melting point temperatures (T_m) varied between 81.93 to 89.02°C for the *Bacillus* strains examined. Melting analyses were performed in triplicate for each sample and the mean values and standard deviations are listed in

Table 4.3. The clostridial strains, the non-spore-formers and the negative controls produced no amplicon with the primer combination used (Figure 4.3b).

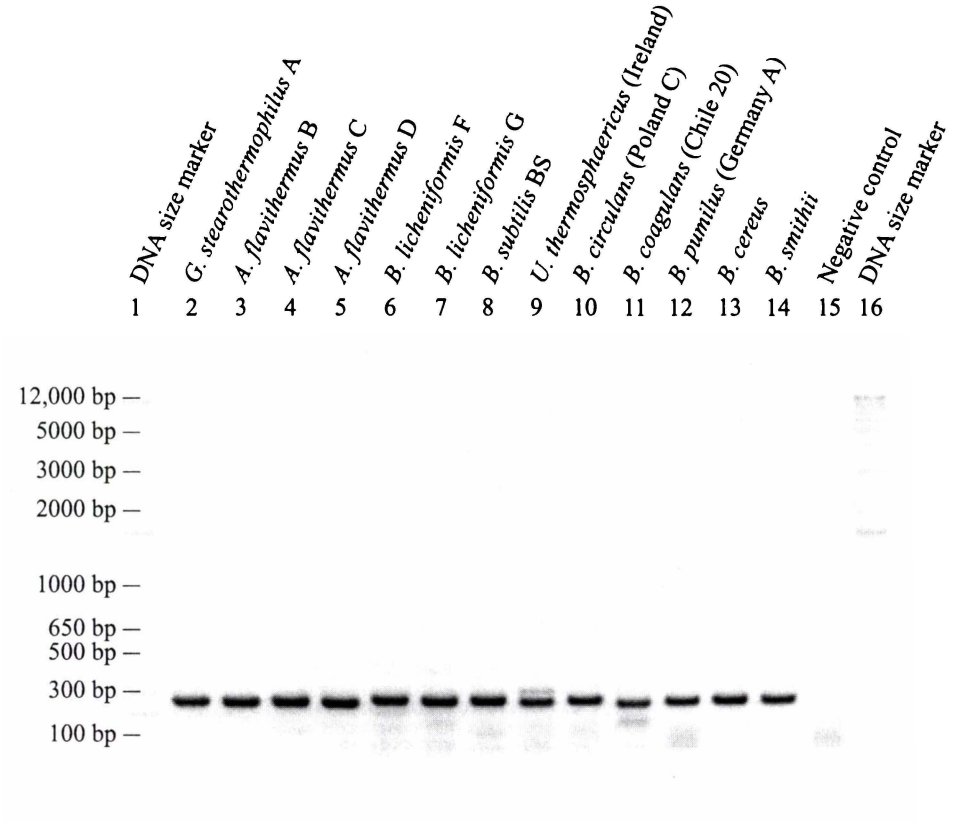


Figure 4.3a. Agarose gel electrophoresis of amplification products of a small region of *spo0A* genes from diverse bacilli using the real-time PCR primers. DNA size marker: 1 Kb Plus DNA Ladder (Invitrogen); Negative control: PCR reaction did not contain any DNA template.

Table 4.3. Melting point analysis of *spo0A* amplicons

Organism	T_m [°C]
B. licheniformis strain G	89.0 ± 0.1
B. licheniformis strain F	88.7 ± 0.1
G. stearothermophilus strain A	88.7 ± 0.2
B. subtilis BS	87.9 ± 0.1
B. coagulans Chile isolate 20	85.9 ± 0.2
B. pumilus Germany B isolate 6	85.3 ± 0.1
A. flavithermus strain B	84.6 ± 0.1
A. flavithermus strain C	84.6 ± 0.1
A. flavithermus strain D	84.6 ± 0.1
B. megaterium	83.9 ± 0.1
B. smithii	83.5 ± 0.1
B. cereus	83.3 ± 0.1
B. circulans Finland isolate 9	82.5 ± 0.1
U. thermosphaericus Ireland isolate 6	82.5 ± 0.1
U. thermosphaericus Chile isolate 8	82.5 ± 0.1
B. circulans Poland C isolate 14	81.9 ± 0.1

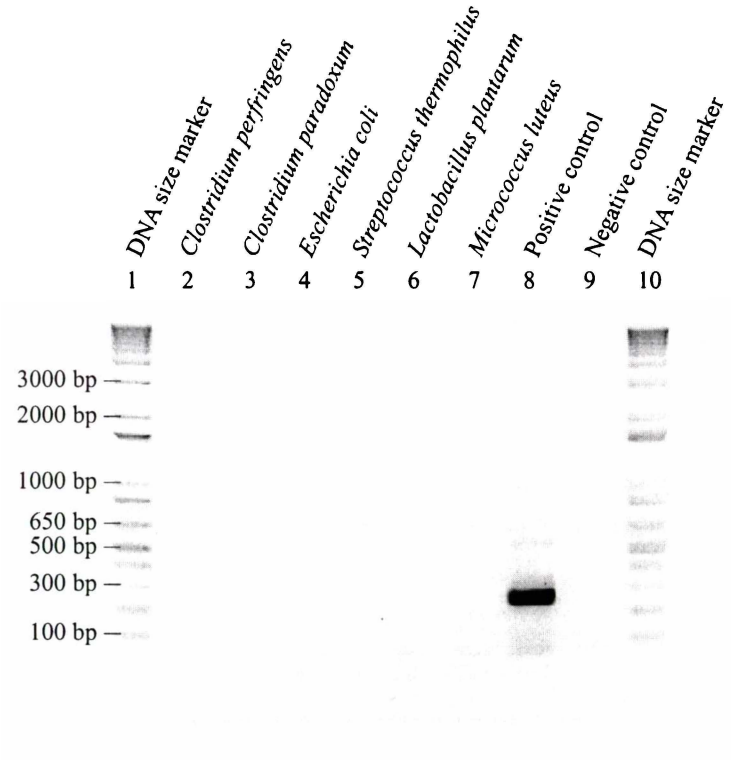


Figure 4.3b. Agarose gel electrophoresis of amplification products of a small region of the *spo0A* genes from clostridial strains and non-spore-formers using the real-time PCR primers. DNA size marker: 1 Kb Plus DNA Ladder (Invitrogen); Positive control: *A. flavithermus* strain C; Negative control: PCR reaction did not contain any DNA template.

4.4.4. Real-time PCR specificity and sensitivity

The specificity of the real-time PCR assay was investigated using 10 ng of genomic DNA from each of the seven milk powder derived bacilli (Ronimus et al., 2003). The results are shown in Figure 4.4 (a, b and c). All strains produced positive results with amplicon density producing a fluorescent response which exceeded the threshold baseline at a mean C_t of 16.95 ± 0.53 indicating near identical amplification rates achieved with the primer pair.

The sensitivity range of the PCR assay was assessed in triplicate using 10-fold dilution series of *A. flavithermus* C and *B. licheniformis* F ultra-purified genomic DNA. The standard curve obtained for *A. flavithermus* C was $C_t = -1.8022 \times \ln(\text{DNA [ng]}) + 21.266$ and for *B. licheniformis* F was $C_t = -1.6521 \times \ln(\text{DNA [ng]}) + 21.607$ (Figure 4.5). The assays were linear over a DNA concentration of six orders of magnitude from 15 ng to 150 fg of initial amplification target. The correlation coefficients were greater than 0.99.

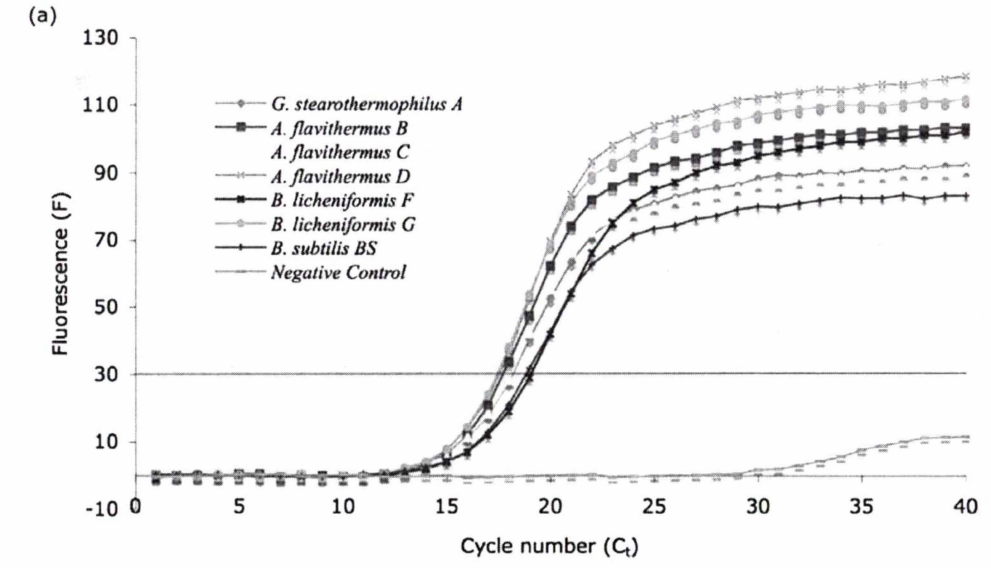
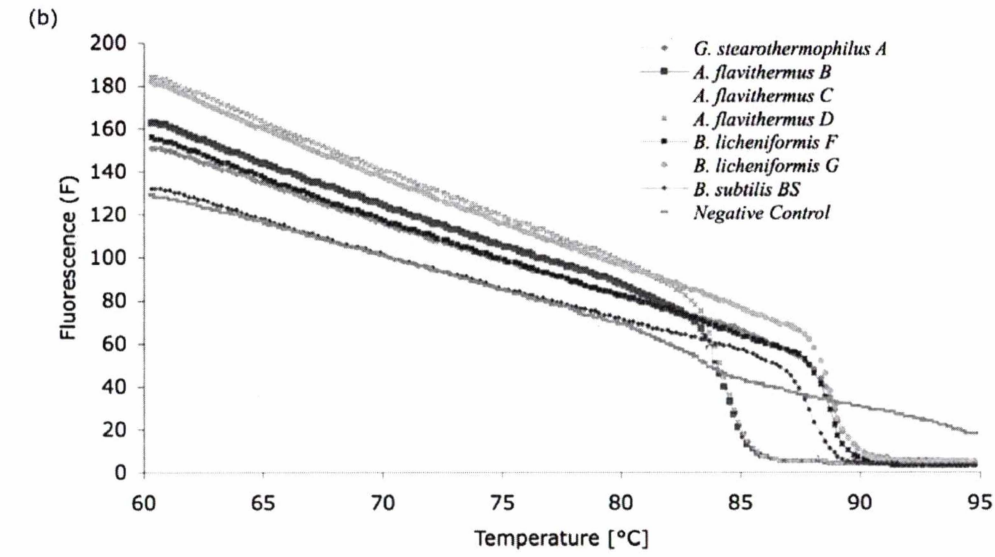


Figure 4.4a. Amplification plot of quantitative real-time PCR on the Smart Cycler II targeting the *spo0A* sporulation genes of *G. stearothermophilus* A, *A. flavithermus* (strains B, C and D), *B. licheniformis* (strains F and G) and *B. subtilis* BS. The PCR reactions were performed under identical conditions using 10 ng of genomic DNA as initial template. The bacilli responded with a mean C_t of 16.95 ± 0.53 indicating near identical amplification rates. The negative control (no template) did not cross the manual threshold base line at 30 within the set 40 amplification cycles.



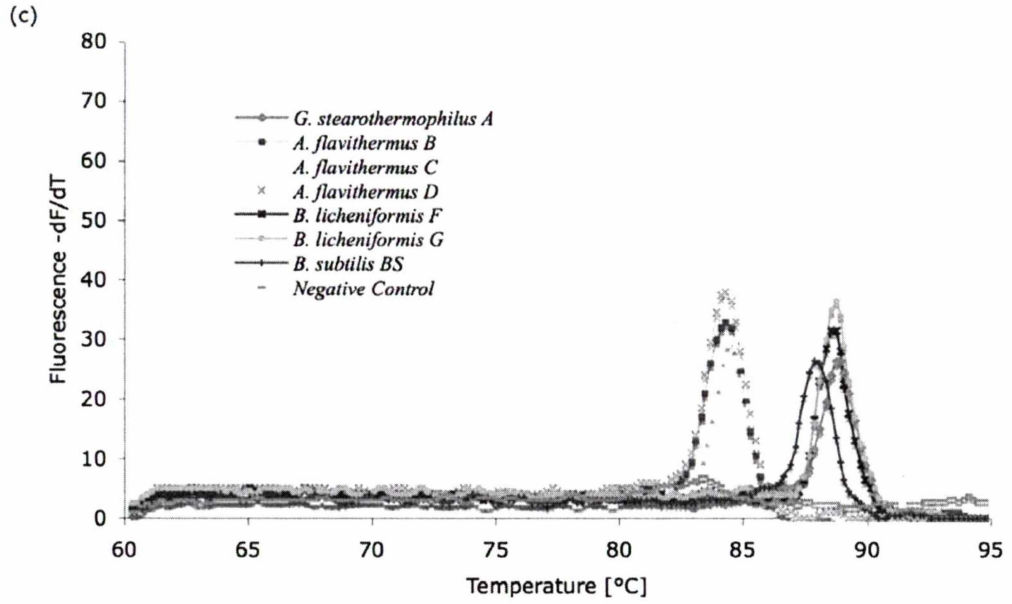


Figure 4.4 b and c. Melting curve analysis of *spo0A* amplification products. The graph (b) shows the melting curves derived by increasing temperature over the range 60°C to 95°C with a temperature transition of 0.2°C s^{-1}. The melting temperatures (T_m) of the amplification products can be seen in the points were the fluorescence signal experiences its most rapid decrease. This is the temperature at which 50% of the double stranded DNA of a reaction is dissociated. Figure (c) illustrates the corresponding melting peaks derived from the negative first derivative of the melting curve fluorescence plotted over the temperature (- dF/dT versus T).

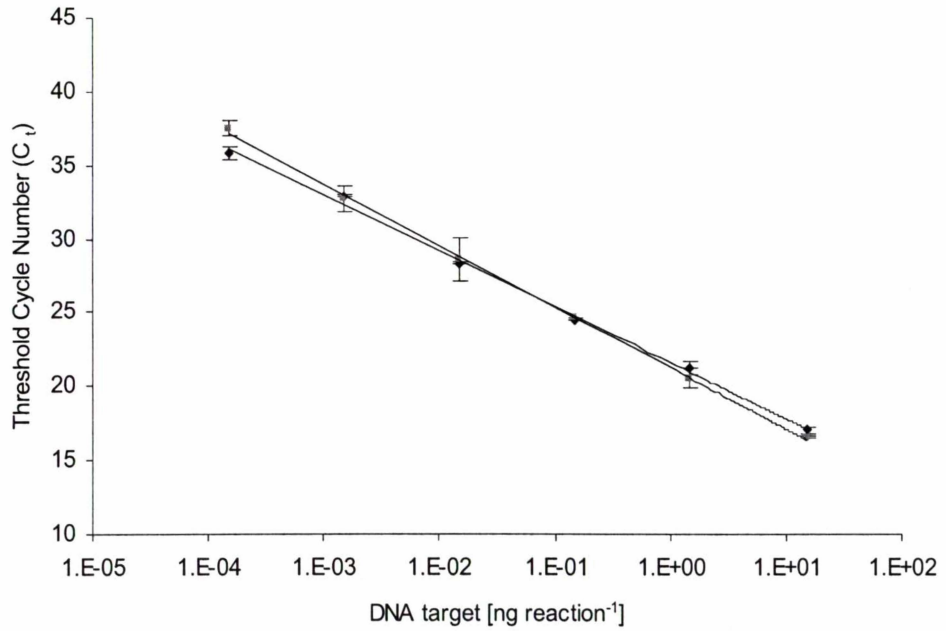


Figure 4.5. DNA standard curves for quantitative real-time amplifications of a small region of the *spo0A* gene of *A. flavithermus* C (■) and *B. licheniformis* F (♦). One microlitre of standard DNA was used in a 25 µl PCR reaction. The reaction for each DNA concentration was performed in triplicate. The mean values of the primary C_t were then plotted against the log amount of initial DNA template and the linear correlation coefficient (R) determined in Microsoft Excel. The target DNA ranged from 150 fg to 15 ng per reaction. The error bars are the standard deviations for three replicates.

4.4.5. Correlation of C_t value with number of vegetative cells

Standard curves for vegetative cells were obtained in triplicate for *A. flavithermus* strain C and *B. licheniformis* strain F in sterile deionised water. For this purpose, a 10-fold dilution series of each strain in water was subjected to the DNA extraction regime and quantitative PCR performed on the supernatant. The assays were linear over a range of 10^7 to 10^3 cfu ml^{-1} (colony forming units per milliliter) for both strains with a lower detection limit of approximately 30 cfu ml^{-1}. The standard curve obtained for *A. flavithermus* C was $C_t = -1.7776 \times \ln(\text{cfu ml}^{-1}) + 49.735$ and for *B. licheniformis* F $C_t = -1.6803 \times \ln(\text{cfu ml}^{-1}) + 48.989$. The correlation coefficient for both curves was greater than 0.99 (Figure 4.6).

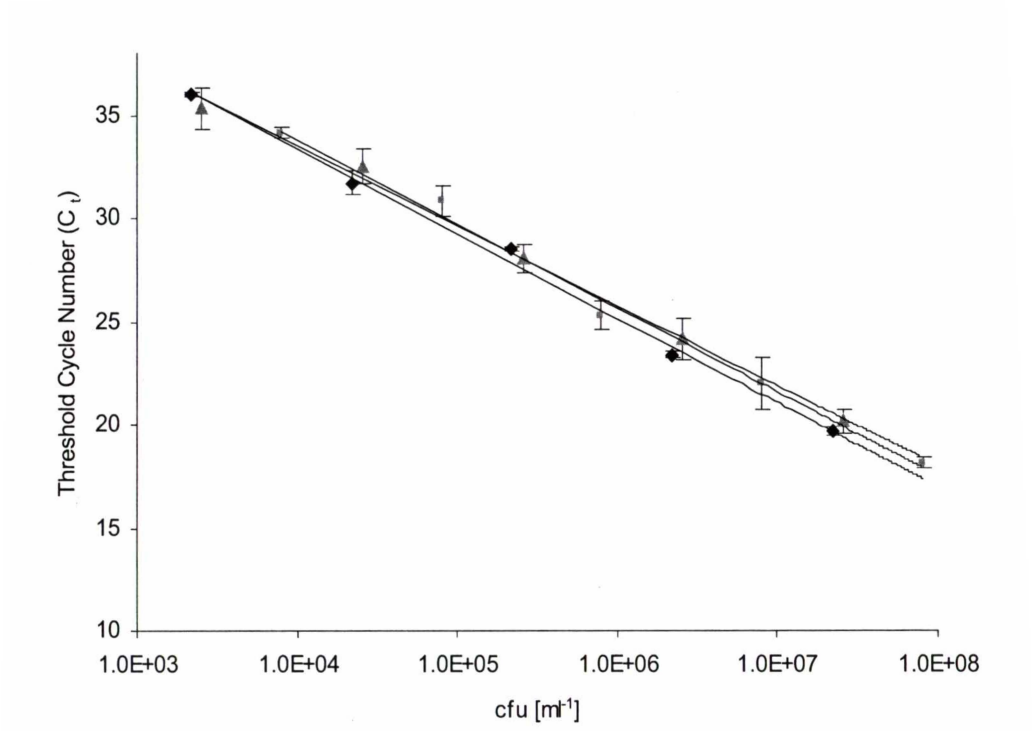


Figure 4.6. Semi-logarithmical plot of standard curves derived from Smart Cycler II amplifications of vegetative cells of (♦) *A. flavithermus* C, (▲) *B. licheniformis* F and (■) of a mixed culture recovered from milk. Ten microlitres of sonicated sample were used in a 25 µl reaction for quantitative PCR and the mean values for C_t plotted against the cell quantities. The error bars are the standard deviations for three sonicated replicates.

Furthermore, a standard curve was constructed in triplicate where an equal number of vegetative cells of both strains was added to sterile reconstituted milk. Subsequently, the samples were 10-fold serially diluted in sterile milk and the cells recovered by trisodium citrate and *n*-decane extraction, the DNA released by

sonication and quantitative PCR performed on the supernatants. The assay was again linear over a range of five orders of magnitude (from 10^7 to 10^3 cfu ml^{-1}) with a lower detection limit of 80 cfu ml^{-1} of reconstituted milk. The standard curve was calculated to $C_t = -1.7734 \times \ln(\text{cfu ml}^{-1}) + 50.187$ with a correlation coefficient of 0.99.

4.4.6. Correlation of C_t value with number of spores

Standard curves for spores were obtained following the procedure outlined for vegetative cells in paragraph 3.5. The correlations of C_t values to decimal dilutions of individual spore suspensions of both organisms in sterile water were linear in a range of 10^8 to 10^4 spores ml^{-1}. The standard curves for *A. flavithermus* C and *B. licheniformis* F were $C_t = -1.2403 \times \ln$ (spores ml^{-1}) + 45.173 and $C_t = -1.4309 \times \ln$ (spores ml^{-1}) + 49.673, respectively. The correlation of threshold cycle numbers to decimal dilutions of equally mixed spores of both strains in reconstituted milk was $C_t = -1.4258 \times \ln(\text{spores ml}^{-1}) + 48.222$ and linear between 10^8 to 10^4 spores ml^{-1} with a lower detection limit of approximately 640 spores ml^{-1}. The correlation coefficient for all standard curves was greater than 0.99 (Figure 4.7).

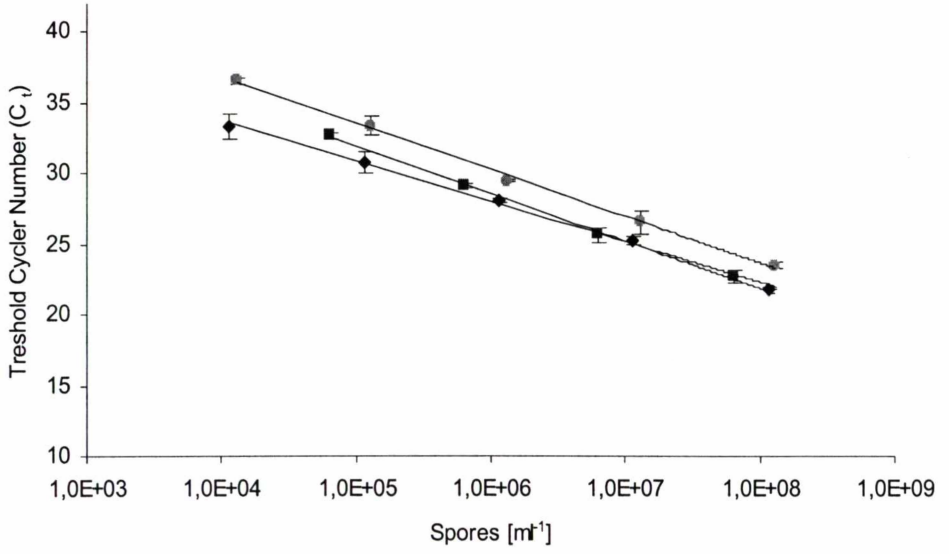


Figure 4.7. Semi-logarithmical plot of 10-fold serial dilutions of (•) *B. licheniformis* F and (♦) *A. flavithermus* C spores in sterile water and (■) the mixed spore culture of both organisms in reconstituted milk. Ten microlitres of sonicated sample were used in a 25 µl reaction for quantitative PCR and the mean values for C_t plotted against the spore quantities. The error bars are the standard derivations for three sonicated replicates.

4.5. Discussion

This study describes the development of a real-time PCR assay for the rapid quantification of thermophilic bacilli in milk powder. These thermophilic bacilli are of hygienic concern to the manufacturers and processors of milk powder, and the ability to monitor these contaminants during milk processing would have economic benefits.

The use of the sporulation transcription factor *spo0A* as a PCR detection and quantification target has several advantages and the decision to target this marker was based on the studies by Brown et al. (1994) and Brill et al. (1997). Both authors concluded that the *spo0A* gene is present exclusively in endospore-forming bacteria such as *Bacillus* and *Clostridium* since attempts to identify homologous *spo0A* sequences in non-sporulating or asporogenic bacteria employing PCR and southern hybridization were negative. Brown et al. (1994) also proposed the gene being present as a unique sequence with only a single copy in the genome of endospore forming bacteria. This provides a marker particularly suitable for PCR quantification of different target organisms since ambiguities in results related to multiple target copies, as occurs with the 16S rRNA gene can be avoided (Rueckert et al., 2005a). Although a TaqMan probe confers additional specificity to a real-time PCR protocol, we preferred the utilization of the SYBR Green I chemistry for the current study as the dye offers two advantages; firstly, in terms of cost, the assays are cheaper to set up and secondly it provides the ability to differentiate amplification products through DNA amplicon melting-curve analysis (and thereby confirm the identity of the contaminants) due to sequence heterogeneity within the priming region (Ririe, 1997). Nevertheless, when using SYBR Green I precaution must be observed when obtaining threshold cycle numbers in real-time as the dye binds non-specifically to any double-stranded DNA and false positive signals can be generated derived from non-specific amplification and primer-dimers. Thus, melting point analysis on the amplification products is essential to confirm the veracity of the amplicon.

During the course of this study Onyenwoke et al. (2004) found experimental evidence of homologous *spo0A* sequences in asporogenic bacteria such as *Thermoanaerobacter*, *Thermobrachium*, *Thermosyntropha*, *Megasphaera*, *Veillonella*, *Selenomonas*, *Tissierella* and *Eubacterium*. Contrarily, when Onyenwoke et al. (2004) applied the same detection regimes to non-spore-forming species

routinely associated with milk (Gilmour and Rowe, 1990) such as *Leuconostoc*, *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Enterococcus*, *Staphylococcus* and *Listeria*, no indications of *spo0A* homologues were found in any of these species. On the other hand, assignments of sequence similarity based on *e*-values from BLAST alignments of the *B. subtilis spo0A* gene against complete genomes of some non-spore-forming bacteria indicated the presence of sequences with low homology to *spo0A*. However, the scores evaluated for these homologues were so low that a definitive identification could not be justified (Onyenwoke et al., 2004). When the real-time PCR protocol developed in this study was applied to the non-sporulating control species used in this study no amplification product was formed within the set 40 amplification cycles and thus these organisms were *spo0A* negative.

There are only four endospore-forming genera of bacteria which have been routinely associated with dairy products, i.e. *Anoxybacillus*, *Geobacillus*, *Bacillus* and *Clostridium* (Gilmour and Rowe, 1990; Bramley and McKinnon, 1990; Ronimus et al., 2003; Rueckert et al., 2004). Although thermophilic species of clostridia are prevalent in many ecosystems they do not appear as contaminants during the milk powder production due to the aerobic nature of the process, and therefore they can be discounted for this study. Additionally, when the current real-time PCR assay was applied to *C. paradoxum* and *C. perfringens* there was no amplification of the clostridial *spo0A* genes observed. This was not unexpected as the *spo0A* sequence alignment and phylogenetic analysis showed that the clostridial species possessed sequences, which differed extensively from those of *Anoxybacillus*, *Geobacillus* and *Bacillus* (Figure 4.2). For these reasons we did not extensively test our protocol to a greater number of species commonly found in milk. We envisage that in the factory the test would be applied to raw milk at the start of processing (when thermophile numbers are low) to give a background reading for all contaminants. Subsequent tests throughout the processing run would monitor the increase in thermophiles only (since conditions would not be conducive for growth of other contaminants). We have established that the only thermophiles able to grow under processing conditions are the seven strains mentioned and which are all quantitatively detected by our protocol (Ronimus et al., 2003; Rueckert et al., 2004).

An added advantage of using the *spo0A* sequence selected is that it lies in the so-called connector segment of the gene. This region displays the highest degree of sequence diversity, either in sequence length or nucleotide substitution. The highly

divergent nature of this section of the gene is reflected in the acquisition of PCR amplicons with different melting point properties. As reported by Ririe (1997), the melting temperature of a PCR product is a function of the GC-content, amplicon length and sequence and thus, PCR products can be distinguished by their melting curves. This feature can be exploited to distinguish between the contaminating milk powder bacilli. For instance, the two major thermophilic contaminants *A. flavithermus* C and *B. licheniformis* F (Rueckert et al., 2004) possess melting points which differ by more than 4°C, and we can easily distinguish these strains (Figure 4.4 b, c and Table 4.3). Both organisms were also distinguishable when a mixture of purified DNA, vegetative cell or spores was used for amplification, resulting in the acquisition of two composite melting peaks (Appendix 12.3.5). However, the melting points of the amplicons of *B. licheniformis* and *G. stearothermophilus* were not sufficiently different to allow unequivocal discrimination of these strains. Furthermore, melting curve analysis did not distinguish bacilli at strain-level as the melting points of all three *A. flavithermus* strains and both *B. licheniformis* strains were nearly identical (Table 4.3; Figure 4.4 b and c). Another limitation of melting curve analysis was found in the inconsistency of the absolute positions of the melting temperatures (T_m) for a given organisms throughout different PCR master-mix preparations and variations of up to 1°C were common (data not shown). Shifts in the melting point temperature are likely due to different SYBR Green preparations or inconsistent temperature transitions during denaturing (Ririe, 1997).

There is one further consideration in equating plate count enumeration with the corresponding quantitative PCR response. Colony forming units (cfu) might under-represent the equivalent PCR response for several reasons. The correlation of gene and cell number varies depending on the growth phase of a culture (Ludwig and Schleifer, 2000). For instance, cellular DNA content of a single cell doubles just prior to cell division so a culture in the exponential phase of growth with a high rate of cell division will have an earlier threshold cycle than a corresponding stationary culture. DNA in dead cells might still be amplified and contribute to the cell number determined by PCR. We have already described a methodology using DNase to overcome this problem (Rueckert et al., 2005b).

Using purified genomic DNA the quantitative real-time PCR method produced a linear response over a DNA concentration of 150 fg to 15 ng. This equates to a lower detection limit of between 17 to 75 individual cells for the current assay

assuming an average DNA content of 2 to 8.9 fg per cell (Bakken and Olsen, 1989). Vegetative cells and spores extracted from milk powders were detected to a lower limit of 800 cfu g^{-1} and 6400 spores g^{-1}, respectively. A common specification limit for milk powder is set at 30,000 thermophiles g^{-1} of powder (Ronimus et al., 2003; Rueckert et al., 2004) and the current method meets the requirements for contaminant analysis to allow optimization of the process run. Quantification of contaminant levels in milk powder were easily accomplished in less than one hour. We envisage that cumulative data from samples taken throughout the process run, starting soon after CIP treatment, will generate a robust real-time enumeration of thermophilic contaminants so that a real-time growth curve can be integrated into process management.

4.6. References

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Rapid differentiation and enumeration of the total-, viable vegetative cell and spore content of thermophilic bacilli in milk powders with reference to *Anoxybacillus flavithermus*

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5.1. Abstract

Aims: The development of a rapid method for the selective detection and enumeration of the total and viable vegetative cell and spore content of thermophilic bacilli in milk powder by PCR.

Methods and Results: Quantitative PCR and microscopy indicate the presence of up to 2.9 log units more cells in milk powder than accounted for by plate counting due to the majority of cells being killed during milk processing. Two approaches for viable and dead cell differentiation of thermophilic bacilli by quantitative PCR were evaluated, these being the nucleic binding dye ethidium monoazide (EMA) and DNase I digestion. The former agent exposed to a viable culture of *Anoxybacillus flavithermus* caused considerable cell inactivation. In contrast, DNase I treatment had no effect on cell viability and was utilised to develop DNA extraction methods for the differential enumeration of total-, viable vegetative cells and spores in milk powder. Moreover, the methods were further applied and evaluated to 41 factory powder samples taken throughout eight process runs to assess changes in numbers of vegetative cells and spores with time. DNase I treatment reduced vegetative cell numbers enumerated with PCR by up to 2.6 log units. The quantification of spores in the factory milk powders investigated indicates on average the presence of 1.2 log units more spores than determined by plate counting.

Conclusions: The method presented in this study provides the ability to selectively enumerate the total and viable cell and spore content of reconstituted milk.

Significance and Impact of Study: The current study provides a tool to monitor the extent of thermophilic contamination during milk powder manufacturing 60 to 90 min after sampling.

Keywords: Milk powder; Thermophilic bacilli; Cell enumeration; Live/dead cell differentiation; Ethidium monoazide bromide; DNase I; Spores; Quantitative PCR.

5.2. Introduction

The contaminating role of thermophilic *Bacillus* species during the production of milk powder has been well documented (Stadhouders *et al.* 1982; Kwee *et al.* 1986; Murphy *et al.* 1999; Ronimus *et al.* 2003). In particular, strains of *Anoxybacillus flavithermus* strain C, *Bacillus licheniformis* strain F and *Geobacillus stearothermophilus* strain A have been found predominantly and nearly ubiquitously distributed in milk powders despite the country of origin (Rueckert *et al.* 2004). These thermophilic contaminants can have significant economic consequences when they exceed specification limits and may result in downgrading of the product. Typically, the number of contaminating thermophiles is determined by plate counting, producing results at least 16 hours after milk processing has been terminated. With this testing regime, it is difficult to predict the optimum length of a process run yet still ensure that thermophile numbers are below specified limits. Real-time monitoring of thermophiles throughout the process run would offer a flexible and economic management strategy which allows processing to be continued until specified limits are reached.

A method for the quantitative detection of the seven most commonly occurring thermophilic milk powder bacilli including *G. stearothermophilus* (strain A), *A. flavithermus* (strains B, C and D), *B. licheniformis* (strains F and G) and *Bacillus subtilis* (Ronimus *et al.* 2003) has recently been developed (Rueckert *et al.* 2005). However, although PCR methods are extremely selective and accurate with regard to the amplification of a specific DNA target sequence, the major disadvantage arises in the inability to differentiate between DNA originating from viable or dead cells. This is problematic, in particular, with milk powder as a PCR sample. Growth of thermophiles primarily occurs in the pre-heaters, evaporators and heat exchangers and the majority of cells are killed by subsequent direct stream injection (DSI),

concentrate formation and final spray drying (Murphy *et al.* 1999; Thompson *et al.* 1978). These dead cells still contain amplifiable DNA and would cause an overestimation of thermophile contaminant level by quantitative PCR. If no account is taken of the numbers of dead cells in any estimation of contamination, then processing runs would be terminated well before specified allowable numbers of thermophiles are met.

Nucleic acid-based methods for the differentiation of viable and dead cells have been applied using reverse transcription PCR (Herman 1997; Norton and Batt 1999) to detect viable *Listeria monocytogenes*. Another strategy employed by Nogva *et al.* (2003) and Rudi *et al.* (2005) uses the nucleic acid binding dye ethidium monoazide bromide (EMA) to discriminate between living and dead cells of *Escherichia coli*, *Salmonella* sp., *L. monocytogenes* and *Campylobacter jejuni* by PCR. EMA is a DNA intercalating agent (Waring 1965; Bolton and Kearns 1978) which is reported to selectively penetrate the membrane of dead cells, but is purportedly unable to penetrate the intact membranes of live cells (Nogva *et al.* 2003; Rudi *et al.* 2005). Once photolysed, EMA covalently links to DNA and prevents PCR amplification. In addition, the utilisation of DNase has been used in the assessment of mammalian cell viability by flow cytometric analysis (Frankfurt 1983) and is based on the assumption that dead cells lose membrane integrity, making their DNA accessible to DNase degradation.

The goal of this study was to adapt and apply the previously developed quantitative PCR method for enumeration of thermophilic bacilli (Rueckert *et al.* 2005) to milk powder samples throughout process runs to assess changes in the numbers of vegetative cells and spores of thermophilic bacilli with time, and to determine the contribution of dead cells to the PCR quantification. As a consequence, methods have been developed which allow for the selective enumeration of both viable cells and spores. The current study provides the ability to monitor the extent of thermophilic contamination during milk powder manufacturing 60 to 90 min after sampling.

5.3. Materials and methods

5.3.1. Bacterial strains and culture preparation

G. stearothermophilus strain A, *A. flavithermus* strain C and *B. licheniformis* strain F were derived from New Zealand milk powder samples (Ronimus *et al.* 2003). The organisms were routinely grown at 55°C in either tryptic-soy broth (Bacto™) (TSB) under agitation or in tryptic-soy agar (TSA) supplemented with 0.2% (w/v) soluble potato-starch (Sigma; S2004). Spores of *A. flavithermus* C and *B. licheniformis* F were produced in liquid Castenholz medium (DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) by inoculating 1 ml of the TSB-starch stationary grown pre-cultures to 1 liter of Castenholtz medium. The cultures were grown at 55°C for 72 hours and the spores harvested and purified as described by Rueckert *et al.* (2005).

5.3.2. Factory powders

A total of forty-one individual whole milk powder samples were derived from eight milk powder processing runs from a New Zealand dairy factory in the seasonal period between January and February 2004. The factory runs GO10, GO11, GO12, GO13, GO14, GO15 and EN22 and EN23 are successive and constitute continuous processing of 17 to 19 hours separated by a cleaning in place (CIP) regime. Inconsistencies in the CIP regime applied prior to EN23 were reported from the plant management. The samples were collected from the powder drying belt immediately after formation and stored at room temperature until required. When samples were used for enumeration care was taken to ensure that contamination was minimised. This included processing under a laminar flow cabinet and using aerosol-resistant filter tips. The total and viable vegetative cell and spore content for each powder was determined by plate counting, microscopy and quantitative PCR (Rueckert *et al.* 2005).

Total plate counts and spore counts were obtained on TSA-starch by pouring duplicates of decimal dilution series of reconstituted milk into warm liquid medium. The medium was allowed to solidify and the plates incubated at 55°C for 16 to 48

hours. Spore counts were obtained by heat-treatment of reconstituted milk samples in a water bath at 80°C for 20 min prior to plating. The number of vegetative cells was calculated as the difference between total count and spore count.

5.3.3. Ethidium monoazide bromide treatment

Five milligrams of solid ethidium monoazide bromide (EMA) were purchased from Biotium, Inc. (USA) and according to the manufacture's recommendation dissolved in *N,N*-dimethylformamide in absence of light. Aliquots of 50 µl of the 10 mg ml^{-1} EMA stock solution were stored at -20°C in light-impermeable micro-tubes.

The effect of EMA on cell viability was investigated using a modification of the standardised protocol described by Nogva *et al.* (2003). Accordingly, a culture of *A. flavithermus* C diluted in sterile 0.9% NaCl was exposed for 5 min to 0.1, 1, 10 and 100 µg ml^{-1} EMA on ice, respectively. The samples were prepared in two sets of duplicates for each EMA concentration in the absence of light in clear 1.5 ml Eppendorf micro-tubes using a final volume of 1 ml. Subsequently, the samples were exposed for 1 min to a 500 W halogen light source (Osram, T3Q clear halogen) which was 20 cm distant from the sample tubes. Duplicates of each EMA concentration were then immediately diluted 10-fold in sterile deionised water and poured into warm liquid TSA-starch. Similarly, duplicates of control samples treated in the same way using the equivalent amounts of *N,N*-dimethylformamide as was used for the EMA treated samples were also included. Furthermore, duplicates of positive control cultures containing no additives but treated as outlined above were used as reference to determine the survival rates. The recovery of cell numbers was determined by colony counting. In addition, duplicates of the same experimental batches, e.g. EMA, control and positive samples were sonicated for 125 seconds at 120 W at 20 kHz (Liquid Processor XL-2020, Misonix) as described by Rueckert *et al.* (2005). The samples were then centrifuged for 2 min at 16,100× *g* and PCR analysis performed on the supernatant.

5.3.4. DNase I treatment

The effect of DNase I (Sigma, DN25) treatment on the viability of *A. flavithermus* C was investigated in duplicate experiments by plate counting and

quantitative PCR using a 1.1×10^5 cfu ml^{-1} culture diluted to this concentration in sterile 0.9% NaCl. Aliquots of 1 ml of culture were exposed for 15 min at 37°C to 0 (positive control), 50, 100, 150 and 200 Kunitz units of DNase I, respectively. The samples contained additionally 100 µl 10× DNase buffer (10 mmol l^{-1} EDTA, 75 mmol l^{-1} $MgCl_2$ and 200 mmol l^{-1} Tris-HCl, pH 7.5). Following incubation, duplicates of the samples were serially diluted in sterile deionised water and immediately poured into warm liquid TSA-starch medium followed by incubation at 55°C for 16 to 24 hours to determine their colony counts. Additionally, aliquots of each treatment were centrifuged at 16,000× *g* for 10 min and the cell pellets resuspended in 1ml of sterile deionised water. The samples were then boiled for 10 min and sonicated for 125 seconds at 20 kHz at 120 W as described and quantitative PCR performed on the supernatants. All treatments were performed in duplicate.

The effect of DNase I on the viability and growth of thermophilic bacilli was extended to include *G. stearothermophilus* strain A and *B. licheniformis* strain F. For this purpose, the organisms were grown in TSB-starch until the cultures reached early-mid exponential growth phase at an OD_{600} of approximately 0.5 (Pharmacia Biotech, Ultraspec 3000). Subsequently, the cultures were stored over night at 4°C and on the following morning 100 µl of each cultures was used to inoculate duplicate flasks of the following three media: 1) TSB-starch, 2) TSB-starch supplemented with 5 ml of 10× DNase buffer and 3) TSB-starch supplemented with 5 ml of 10× DNase buffer and 200 Kunitz units ml^{-1} of DNase I. The final volume of each flask was 50 ml. The OD_{600} of the cultures were monitored hourly over an incubation period of 12 hours at 55°C using 500 µl of culture for spectrometer reading.

5.3.5. DNA preparation for the enumeration of total and viable vegetative cells

Total bacterial DNA extraction from milk was performed by the method described in Rueckert *et al.* (2005). This included the addition of 200 µl of 1.2 mol l^{-1} tri-sodium citrate and 200 µl of *n*-decane to 1 ml of reconstituted milk (0.1 g ml^{-1}), followed by brief vortexing and centrifugation at 16,100× *g* (5415 D, Eppendorf) for 10 min. The top layer of cream was carefully extracted from the microcentrifuge tube using a sterile 200 µl pipette-tip and the supernatant poured off by gently inverting the tube. The samples were re-centrifuged for 2 min at 16,100× *g* and the remaining supernatant removed with a pipettor, while avoiding the disruption of the cell pellet.

The pellet was re-suspended in 1 ml of sterile deionised water and the sample subjected to ultra-sonication (Liquid Processor XL-2020, Misonix) for 125 seconds at 120 W at 20 kHz. The samples were then centrifuged for 2 min at 16,100× *g* and PCR analysis performed on the supernatant.

DNA from viable vegetative cells only was obtained by adding DNase I to the reconstituted milk, before the extraction protocol was applied. This removed contaminating DNA from dead cells, but had no effect on DNA in viable cells. For this method, 100 µl of 10× DNase buffer and 200 Kunitz units of DNase I were added to 1 ml reconstituted milk, followed by brief vortexing and incubation at 37°C for 15 min in a water bath. Subsequently, the samples were tri-sodium citrate and *n*-decane extracted, re-suspended in 1 ml sterile deionised water and boiled for 10 min to denature the DNase. The DNA of viable vegetative bacteria was then released by ultra-sonication as described above.

5.3.6. DNA preparation for the enumeration of total spores

DNA purification from spores was accomplished by adding 100 µl of 10× DNase buffer and 200 Kunitz units of DNase I to 1 ml of reconstituted milk. The sample was then sonicated for 125 seconds at 120 W at 20 kHz and further incubated for 15 min at 37°C in order to release and degrade DNA of both live and dead vegetative cells. Subsequently, the sample was tri-sodium citrate and *n*-decane extracted and the spores re-suspended in 1 ml of sterile deionised water. Residual DNase I was inactivated by boiling the sample for 10 min and the spore DNA released by sonication for 6 min in the presence of 50 mg of 0.1 mm glass beads (Biospec Products) at 120 W at 20 kHz (Rueckert *et al.* 2005). Prior to use, the glass beads were baked for 8 hours at 250°C to eliminate any contaminating DNA. The samples were centrifuged for 2 min at 16,100× *g* and 10 µl of the supernatant used for quantitative PCR analysis.

In addition, negative controls were performed on the DNase-treated spore samples prior to heat-treatment. For this purpose, the samples were centrifuged for 10 min at 16,000× *g* and an aliquot of 50 µl spore-free supernatant boiled and 10 µl subjected to quantitative PCR.

5.3.7. Evaluation of the DNA extraction methods for total-, viable vegetative cells and spores

The methods for the separate detection and quantification of total-, viable vegetative cells and spores in milk were evaluated in triplicate by adding three dilutions of known numbers of viable and dead cells and spores of *A. flavithermus* C to both reconstituted sterile milk (EN22, 1st hour) and sterile deionised water, respectively. The cell and spore numbers added were chosen to simulate the bacteriological load of low-, medium- and high count milk powders (Ronimus *et al.* 2003; Rueckert *et al.* 2004). Viable vegetative cell and spore numbers were determined by plate counting and microscopy. Dead vegetative cells were obtained by heat-killing of an aliquot of the viable cell culture at 95°C for 10 min in a water bath. The extent of cell death was confirmed by plate counting. The different cell fractions from both media were then recovered and their DNA released as described above.

5.3.8. Quantitative real-time PCR

Quantitative real-time PCR analysis amplifying small regions of the 16S rRNA genes was performed with a Smart Cycler II system (Cepheid, USA) in 25 µl Smart Cycler reaction tubes. The amplifications were performed using a modified protocol as described by Rueckert *et al.* (2005) with 1.5 units of the *TaKaRa Taq*^{TM} Hot Start enzyme (TaKaRa Bio INC.), 4 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} dNTPs (TaKaRa Bio INC.), 10× *TaKaRa Taq* PCR reaction buffer, 600 nmol l^{-1} of forward and reverse primer, 150 nmol l^{-1} of TaqMan probe and 10 µl of sample. The PCR reaction was cycled once at 95°C for 90 seconds followed by 42 repetitions at 95°C for 5 seconds and at 62°C for 20 seconds. DNA quantities of the samples were calculated using the standard curves for vegetative cells and spores (Rueckert *et al.* 2005).

5.3.9. Phase-contrast and fluorescence microscopy

The total number (viable and dead) of bacterial rod forms obtained after extraction from reconstituted milk powder was determined by phase-contrast microscopy (Olympus, BH2, 1000× magnification) using a Thoma counting chamber. In order to detect spores a malachite green and safranin red (Sigma; M9015, M323950) staining protocol was employed as outlined by Clark (1973). The staining protocol was used on spores and cells extracted from reconstituted milk powder.

The differentiation of viable and dead cells of selected samples was assessed using fluorescence microscopy (Leica DMRE, 50W Hg burner, I3 block, excitation filter BP 450-490 nm, dichroic mirror at 510 nm and long pass filter at 515 nm) with SYTO BC (Molecular Probes; S-34855) and propidium iodide (Sigma; P4170). An exponential culture of *A. flavithermus* C grown in TSB-starch was used as a positive control and included in the SYTO BC and propidium iodide two-colour staining assay. A control culture was additionally subjected to the extraction regime used for concentrating bacteria from milk constituents to assess the effect these treatments had on cell viability. The staining procedures were then performed according to protocols outlined by Green (1990).

5.4. Results

5.4.1. Effect of EMA treatment on cell viability

The effect of EMA on the viability of *A. flavithermus* strain C cells diluted to 3.6×10^5 cfu ml^{-1} was investigated in the presence of 0.1, 1, 10 and 100 μg ml^{-1} of EMA. In addition, the same culture was also exposed to 0.1 and 1% (v/v) of *N,N*-dimethylformamide which corresponds to the amount of solvent used for delivering 10 and 100 μg ml^{-1} of EMA. The results are shown in Table 5.1. Significantly, EMA concentrations of 10 and 100 μg ml^{-1} killed all cells. Based on plate count data the survival rates of cultures exposed to 1 and 0.1 μg ml^{-1} of EMA were 0.75 and 58% relative to the positive control, respectively. In contrast, the viability of the control cultures incubated with *N,N*-dimethylformamide was not affected by the solvent and quantitative PCR on these samples showed high correlations to the positive control. However, PCR inhibition was observed for the EMA-treated cultures indicating that

the agent must have penetrated the membrane of viable cells and covalently cross-linked with the DNA during photolysis. Based on quantitative PCR, the signal log value reductions of the 0.1, 1, 10 and 100 μg ml^{-1} EMA-treated samples to the positive control were 0.8, 2, 2.3 and 4.

Table 5.1. Effect of EMA and *N,N*-dimethylformamide only treatment on viability of *A. flavithermus* strain C

	Ethidium monoazide bromide [μg ml^{-1}]				Dimethylformamide [%]		positive
	0.1	1	10	100	0.1	1	control
Plate count [a, b]	2.1×10^5	2.7×10^3	0	0	3.9×10^5	3.8×10^5	3.6×10^5
PCR [a, c]	9.8×10^4	6.2×10^3	3.0×10^3	6.2×10^1	6.7×10^5	7.4×10^5	6.1×10^5

[a] cfu ml^{-1}; [b] mean of six replicates; [c] mean of duplicate experiments

5.4.2. Effect of DNase I treatment on cell viability

The effect of DNase treatment on the viability of *A. flavithermus* C was investigated by plate count and quantitative PCR. The results are shown in Table 5.2. The plate counts of the DNase-treated cultures exceeded those of the untreated positive control by 0.13 to 0.7 log units with the recovery rate having its maximum using 200 Kunitz unit of enzyme. This was somewhat in contrast to the results obtained by PCR where the positive control contained approximately two-times more cells as was found for DNase-treated cultures.

We further investigated the effect of 1× DNase buffer and DNase I to 200 Kunitz units ml^{-1} in TSB-starch on the growth properties of *G. stearothermophilus* A, *A. flavithermus* C and *B. licheniformis* F. All three cultures showed similar growth in terms of curve progression and OD readings to the positive control of the same culture in TSB-starch without additives (results not shown).

Table 5.2. Effect of DNase I treatment on viability of *A. flavithermus* strain C

	DNase I [Kunitz units]				positive
	50	100	150	200	control
Plate count [a, b]	1.5×10^5	3.2×10^5	2.7×10^5	5.8×10^5	1.1×10^5
PCR [a, c]	2.0×10^5	1.5×10^5	2.0×10^5	2.3×10^5	4.6×10^5

[a] cfu ml^{-1}; [b] mean of six replicates; [c] mean of duplicate experiments

5.4.3. Evaluation of differential DNA extraction for total-, viable vegetative cells and total spores

The results of the evaluation of the three differential extraction methods are shown in Table 5.3 and 5.4. The correlation coefficients (r) of the dilution samples (high, medium and low count samples) between plate count and quantitative PCR for total vegetative cells were greater than 0.99 for both the reconstituted milk and water suspension. The average PCR signal reduction between total and viable vegetative cells was 0.34 log units for both preparations indicating that a mean of 56% of the total DNA content had been DNase I degraded. Further, the correlation coefficients for viable cells determined by plate count and quantitative PCR were 0.96 for the water and 0.97 for the milk preparation, respectively.

Similarly, the correlations of spore preparations between plate count and PCR was r = 0.97 for the milk and r = 0.96 for the water suspension, respectively. PCR performed on the negative controls did not reach the threshold set within 42 cycles.

Table 5.3. Recovery of *A. flavithermus* C added to deionised water by colony counting and quantitative PCR

	Plate count [cfu ml^{-1}] [(b)]			16S rDNA PCR [cfu ml^{-1}] [(c)]		
	viable	dead [(a)]	spores	total cells	viable cells	total spores
High	3.7×10^{6}	3.7×10^{6}	7.0×10^{6}	$7.1\times10^{6} \pm 6.6\times10^{4}$	$1.9\times10^{6} \pm 4.3\times10^{5}$	$3.8\times10^{6} \pm 1.3\times10^{6}$
Medium	3.7×10^{4}	3.7×10^{4}	7.0×10^{4}	$7.5\times10^{4} \pm 1.4\times10^{4}$	$3.0\times10^{4} \pm 2.9\times10^{3}$	$3.2\times10^{4} \pm 3.4\times10^{3}$
Low	3.7×10^{2}	3.7×10^{2}	7.0×10^{2}	$1.1\times10^{3} \pm 9.7\times10^{1}$	$8.6\times10^{2} \pm 1.6\times10^{2}$	$1.5\times10^{3} \pm 8.8\times10^{1}$

[(a)] Vegetative cell count determined prior to heat-kill; [(b)] mean of six replicates; [(c)] mean of triplicate experiments

Table 5.4. Recovery of *A. flavithermus* C added to reconstituted milk by colony counting and quantitative PCR

	Plate count [cfu ml^{-1}] [(b)]			16S rDNA PCR [cfu ml^{-1}] [(c)]		
	viable	dead [(a)]	spores	total cells	viable cells	total spores
High	3.7×10^{6}	3.7×10^{6}	7.0×10^{6}	$5.9\times10^{6} \pm 3.2\times10^{5}$	$2.9\times10^{6} \pm 7.8\times10^{5}$	$5.2\times10^{6} \pm 3.0\times10^{5}$
Medium	3.7×10^{4}	3.7×10^{4}	7.0×10^{4}	$8.3\times10^{4} \pm 8.2\times10^{3}$	$5.6\times10^{4} \pm 4.3\times10^{3}$	$2.4\times10^{4} \pm 5.1\times10^{3}$
Low	3.7×10^{2}	3.7×10^{2}	7.0×10^{2}	$1.0\times10^{3} \pm 2.6\times10^{2}$	$7.9\times10^{2} \pm 1.7\times10^{1}$	$6.1\times10^{2} \pm 1.2\times10^{2}$

[(a)] Vegetative cell count determined prior to heat-kill; [(b)] mean of six replicates; [(c)] mean of triplicate experiments

5.4.4. Enumeration of thermophiles in factory milk powder samples

Results for plate counts, microscopic counts and quantitative real-time PCR of thermophilic bacilli in the factory samples are shown in Table 5.5. Plate counts for vegetative cells and spores during the first hour of processing were typically in the

range between 50 and 890 cfu g^{-1} for six of the eight factory runs presented. However, the initial plate counts for GO10 and EN23 were higher with between 1.0×10^3 to 3.9×10^3 thermophiles per gram of powder. The number of cultivable thermophilic bacilli increased with processing time commonly by one to two orders of magnitude over the initial count. Based on the number of thermophiles in the last sample taken in each run, GO12 would be regarded as a low count powder with thermophile counts $\leq$ 550 g^{-1}. Accordingly, GO10, GO11, GO13, GO14, GO15 and EN22 are medium count powders with thermophile counts below 3×10^4 cfu g^{-1} and EN23 is a high count powder with numbers exceeding 3×10^4 cfu g^{-1} during the last four hours of processing (Ronimus *et al.* 2003; Rueckert *et al.* 2004).

The enumeration of total thermophilic bacilli by quantitative PCR (Rueckert *et al.* 2005) indicated thermophile numbers exceeded those determined by plate counts (Table 5.5). On average, PCR quantification overestimated the plate counts for vegetative cells and spores by 2.2 and 1.2 log units, respectively. As expected, when the contribution of dead cells was removed by the use of the DNase I treatment, the apparent number of vegetative cells more closely correlated with the plate count (reductions of between 0.8 to 2.6 log units were achieved). For milk powder samples with thermophilic loads below 420 cfu g^{-1} no enumeration was achieved by PCR since any amplification of signal did not reach the threshold set within 42 PCR cycles.

Table 5.5. Summary of plate counts [a], microscopic counts [b] and 16S rDNA quantitative real-time PCR counts [c] of thermophilic bacilli in milk factory powders.

		Vegetative cells [cfu g^{-1}]				Spores [cfu g^{-1}]	
Factory run	Sampling hour	Plate count	Microscope count	Total count 16S rRNA	Viable count 16S rRNA	Plate count	Total count 16S rRNA
EN22	1	0	N.D.	N.D.	N.D.	0	N.D.
	12	1.5×10^{3}	1.2×10^{5}	1.9×10^{5}	2.9×10^{4}	1.8×10^{3}	1.6×10^{4}
	16	2.5×10^{4}	4.0×10^{5}	2.6×10^{6}	1.4×10^{5}	1.5×10^{4}	1.6×10^{5}
	17	1.9×10^{4}	8.0×10^{5}	9.4×10^{5}	9.7×10^{4}	5.9×10^{3}	4.3×10^{4}
	18	2.5×10^{4}	4.8×10^{5}	1.2×10^{6}	6.7×10^{4}	9.6×10^{3}	2.5×10^{4}
	19	1.4×10^{4}	1.6×10^{6}	1.8×10^{6}	9.4×10^{4}	1.0×10^{4}	8.9×10^{4}
EN23	1	3.9×10^{3}	7.0×10^{5}	8.7×10^{5}	5.5×10^{4}	2.0×10^{3}	1.0×10^{5}
	16	1.8×10^{5}	2.2×10^{6}	1.8×10^{7}	7.1×10^{4}	6.8×10^{4}	3.2×10^{5}
	17	1.6×10^{5}	3.1×10^{6}	2.4×10^{7}	1.8×10^{5}	1.5×10^{5}	7.1×10^{5}
	18	2.4×10^{5}	2.8×10^{6}	2.2×10^{7}	1.2×10^{5}	2.2×10^{5}	2.5×10^{5}
	19	2.7×10^{5}	3.6×10^{6}	2.1×10^{7}	1.6×10^{5}	1.9×10^{5}	3.7×10^{5}
GO10	1	1.0×10^{3}	1.6×10^{5}	1.1×10^{5}	4.9×10^{3}	1.1×10^{3}	3.7×10^{4}
	12	4.7×10^{2}	1.2×10^{5}	9.0×10^{4}	3.3×10^{3}	4.0×10^{2}	4.9×10^{3}
	16	3.3×10^{3}	7.6×10^{5}	5.0×10^{5}	5.4×10^{3}	2.3×10^{3}	2.4×10^{4}
	17	1.4×10^{4}	8.8×10^{5}	6.9×10^{5}	6.6×10^{3}	9.5×10^{3}	6.5×10^{4}
	18	1.1×10^{4}	8.5×10^{5}	8.9×10^{5}	1.2×10^{4}	1.9×10^{4}	6.1×10^{4}
GO11	1	8.9×10^{2}	1.2×10^{5}	6.3×10^{4}	5.4×10^{2}	4.8×10^{2}	N.D.
	12	2.0×10^{2}	1.2×10^{5}	1.9×10^{4}	N.D.	3.0×10^{2}	N.D.
	16	5.2×10^{2}	2.4×10^{5}	1.8×10^{5}	1.6×10^{3}	3.8×10^{2}	1.5×10^{3}
	17	1.0×10^{3}	5.2×10^{5}	4.6×10^{5}	7.6×10^{3}	1.3×10^{3}	4.0×10^{4}
	18	3.7×10^{3}	1.0×10^{6}	4.1×10^{6}	2.5×10^{4}	3.3×10^{3}	6.4×10^{4}
GO12	1	5.0×10^{1}	N.D.	1.5×10^{4}	N.D.	1.0×10^{2}	2.3×10^{4}
	12	2.9×10^{2}	N.D.	5.6×10^{4}	N.D.	2.5×10^{2}	1.8×10^{4}
	16	1.0×10^{2}	4.0×10^{4}	5.9×10^{4}	1.7×10^{3}	2.0×10^{2}	1.7×10^{4}
	17	5.5×10^{2}	2.0×10^{5}	8.1×10^{4}	2.2×10^{3}	3.0×10^{2}	1.5×10^{4}
GO13	1	6.0×10^{1}	N.D.	7.1×10^{4}	N.D.	1.5×10^{2}	1.8×10^{4}
	12	1.5×10^{2}	2.0×10^{4}	1.0×10^{4}	N.D.	3.0×10^{2}	N.D.
	16	9.6×10^{2}	1.2×10^{5}	9.6×10^{4}	5.2×10^{2}	4.6×10^{2}	8.1×10^{3}
	17	5.3×10^{2}	2.4×10^{5}	9.7×10^{4}	4.1×10^{2}	6.0×10^{2}	1.1×10^{4}
	18	9.7×10^{2}	2.0×10^{5}	1.6×10^{5}	6.7×10^{2}	1.7×10^{3}	9.4×10^{3}
GO14	1	7.7×10^{1}	N.D.	2.6×10^{4}	N.D.	6.5×10^{1}	3.4×10^{3}
	12	2.4×10^{2}	4.0×10^{4}	1.5×10^{5}	N.D.	1.6×10^{2}	4.6×10^{4}
	16	7.0×10^{2}	6.0×10^{5}	4.1×10^{5}	3.2×10^{3}	1.4×10^{3}	2.0×10^{4}
	17	5.6×10^{3}	7.6×10^{5}	7.7×10^{5}	1.1×10^{4}	4.5×10^{3}	6.5×10^{4}
	18	9.8×10^{3}	2.0×10^{5}	6.1×10^{5}	1.6×10^{4}	9.2×10^{3}	4.4×10^{4}
GO15	1	4.5×10^{2}	N.D.	6.9×10^{4}	1.6×10^{2}	7.5×10^{2}	6.9×10^{3}
	12	4.2×10^{2}	4.0×10^{4}	1.0×10^{5}	N.D.	5.5×10^{2}	1.9×10^{4}
	16	2.5×10^{3}	1.2×10^{5}	2.5×10^{5}	1.8×10^{3}	9.8×10^{3}	4.4×10^{4}
	17	4.6×10^{3}	2.4×10^{5}	7.5×10^{5}	3.5×10^{3}	2.8×10^{3}	4.1×10^{4}
	18	1.6×10^{4}	6.4×10^{5}	9.2×10^{5}	3.7×10^{3}	1.0×10^{4}	9.1×10^{4}
	19	2.8×10^{4}	6.0×10^{5}	1.5×10^{6}	3.4×10^{3}	2.9×10^{4}	1.1×10^{5}

[a] mean of six replicates; [b] mean of eight replicates; [c] one observation contributed to each data point; N.D.: Not detectable

5.4.5. Phase-contrast and fluorescence microscopy

The total number of *Bacillus* rod forms in each milk powder was also determined by phase-contrast microscopy, and results are shown in Table 5.5. Typically, direct microscopic counts exceeded plate counts for vegetative cells between 1.1 and 2.9 log units and differed from quantitative PCR for total vegetative count between -0.8 and 0.9 log units. Although rod morphologies could easily be differentiated by phase-contrast, spores were indistinguishable from milk colloids similar size and refraction. In order to observe spores and vegetative forms a malachite green and safranin red counterstaining was employed on tri-sodium citrate and *n*-decane extracted milk samples (Figure 5.1D).

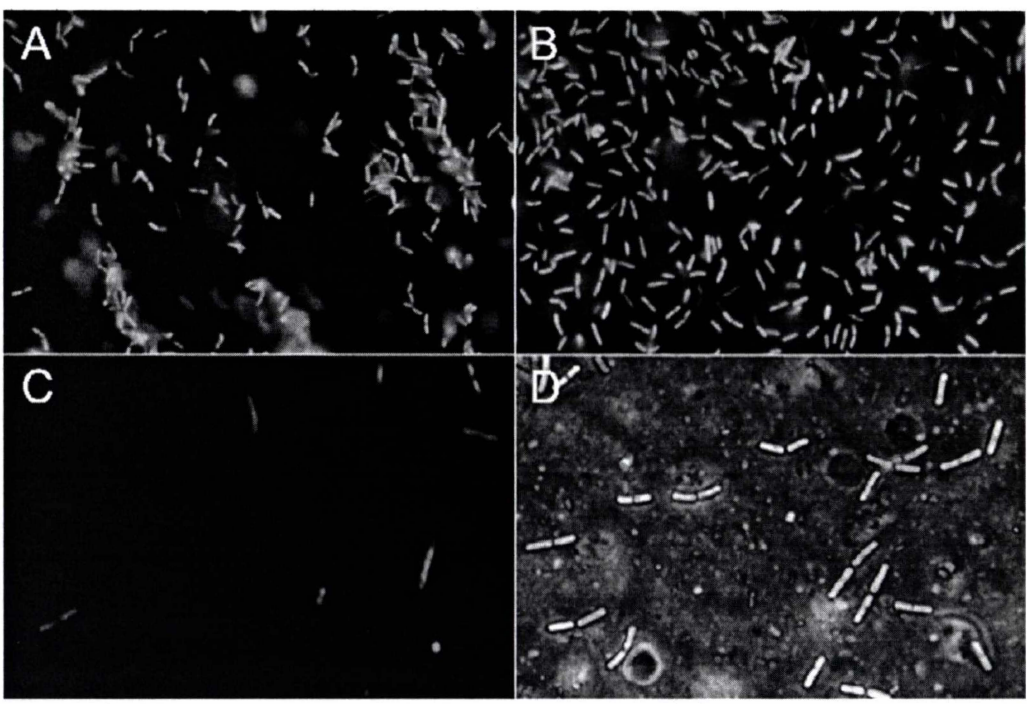

Figure 5.1. The image (A) shows the live/dead stain of the TSB control culture of *A. flavithermus* C. (B) Live/dead stain of the control culture after tri-sodium citrate and *n*-decane treatment. (C) Live/dead stain of an extracted milk powder. (D) Malachite green and safranin red stain of tri-sodium citrate and *n*-decane extracted reconstituted milk.

When milk powder samples were subjected to the live/dead differentiating fluorescence dyes SYTO BC and propidium iodide only dead cells were observed, i.e. all cells stained red (Figure 5.1C). Further, when the test was performed on an exponentially grown culture of *A. flavithermus* strain C all cells were alive and stained green (Figure 5.1A). The same culture, subjected to tri-sodium citrate treatment, showed that only a small proportion (< 5%) of cells were either killed or

their membranes partially permeablised during this procedure. The tri-sodium citrate treatment also increased the number of colonies obtained on subsequent plating, as the agent disrupts cell aggregates formed during culturing (Figure 5.1B, data not shown).

5.5. Discussion

The rapid enumeration of thermophilic contaminants during milk processing is advantageous to the manufacturer as it enables the optimisation of the plant running time to a specified upper limit of contamination. Crucially, before quantitative PCR can be applied on powders, DNA originating from dead cells needs to be removed to avoid overestimation of contaminants. One approach undertaken in this study to discriminate viable and dead cells was the utilization of the nucleic acid binding dye EMA. However, this agent applied under the standardised protocol of Nogva *et al.* (2003) was not suitable for the purpose of this study, as the dye was cytotoxic towards the most commonly occurring milk powder contaminant (Rueckert *et al.* 2004). Additionally, quantitative PCR performed on the same batch of samples resulted in a signal reduction of up to 4 log units relative to the untreated positive control indicating that the dye had penetrated the membrane of viable cells and covalently cross-linked their DNA.

DNase from bovine pancrease is unable to penetrate the membrane of viable cells (Frankfurter 1983; Fischer 1982). Not unexpectedly therefore DNase concentrations of up to 200 Kunitz units ml^{-1}, which corresponds to 0.33 mg ml^{-1} of protein, had no effect on either viability or growth properties of *A. flavithermus* C, *B. licheniformis* F and *G. stearothermophilus* A. Fischer (1982) reported that DNase in concentrations of up to 1 mg ml^{-1} added to the growth medium was not cytotoxic during mammalian cell culturing and prevented the culture from forming unwanted cell-clumping. Similar findings were observed in this study with *A. flavithermus* C where cell numbers from cultures exposed to DNase exceeded those of the positive control by up to 0.7 log units which we attribute to the disaggregation of cell clumps by the enzyme. Quantitative PCR on the same batch of samples, however, showed a reverse effect with cell numbers halved for the DNase-treated cultures (Table 5.2). This discrepancy can possibly be explained by the removal of either DNA from dead cells and/or DNA adhering to the surface of viable cells from the DNase-treated

cultures. Disaggregation of cell clumps was also observed following tri-sodium citrate treatment of *A. flavithermus* C grown in TSB (Figure 5.1A and B).

The enumeration of the total and viable vegetative cell and spore content by quantitative PCR in milk powders requires three separate DNA preparations. The most rapid is DNA extraction for the total vegetative cell content, i.e. live and dead cells which requires milk reconstitution, dissolution of milk casein aggregates and DNA release by sonication. This can be completed within 30 min. The extraction of DNA originating from viable cells can be completed in approximately 50 min. This requires an additional DNase-treatment of reconstituted milk to degrade DNA of dead cells, followed by milk extraction, DNase inactivation and DNA release by sonication. The extraction of spore DNA requires initial sonication of reconstituted milk to release DNA from vegetative cells, followed by DNase-treatment. The milk sample is then extracted in order to concentrate the spores, any residual DNase is inactivated by heat and the spore DNA released by sonication. DNA samples ready-to-use for spore enumeration by PCR were typically obtained after 55 min.

In general, DNase I concentrations above 100 Kunitz units ml^{-1} were essential for the rapid and rigorous elimination of unwanted DNA from the samples. However, due to the diversity of milk powders with respect to cell number or heat-treatment applied to produce the powder, e.g. high-, medium or low heat, the proportions of viable and dead cells can vary significantly (Murphy *et al.* 1999; Thompson *et al.* 1978). To ensure that DNA from either dead cells or total cells is removed rigorously under all circumstances we regard the use of 200 Kunitz units ml^{-1} of DNase as advisable. The enzyme was completely inactivated by both, tri-sodium citrate extraction which chelates divalent magnesium ions and subsequent boiling of the samples for 10 min.

The methods established for the selective and quantitative detection of viable vegetative cells and spores in reconstituted milk were evaluated by adding defined numbers of viable and dead vegetative cells and spores of *A. flavithermus* C to both sterile milk and sterile deionised water. The different DNA fractions were then extracted and subjected to quantitative PCR. The results from these experiments show that in either suspending medium good correlations were obtained between enumeration based on plate count data and quantitative PCR for low, medium and high count samples.

DNase treatment for the enumeration of viable vegetative cells from the factory samples reduced cell numbers by up to 2.6 log units indicating that the vast majority of cells in milk powder are dead. This fact was confirmed by comparing total counts derived by phase-contrast microscopy and plate counting, in which cell numbers determined by the former method exceeded colony counting by up to 2.9 log units. Microscopic counting cannot differentiate between psychrotrophic, mesophilic or thermophilic rod forms, but only the latter would be expected to increase during milk processing, and numbers of psychrophiles and mesophiles in the milk supply were always low or non-existent (results not shown), so their contribution can be discounted. This supports the contention that the majority of thermophilic bacilli in the powder have grown in the processing line and been killed by the processing conditions. However, their DNA is still largely intact and detectable by PCR. Furthermore, the assessment of the proportions of viable and dead cells in reconstituted milk using fluorescence microscopy was limited by the sensitivity of the method due to the fact that numbers of viable cells were under the threshold of the detection limit of this method. This infers that processing conditions inactivate the vast majority of cells formed in the process line.

The quantification of thermophilic spores by PCR for the milk powders investigated indicates on average the presence of 1.2 log units more spores than determined by plate counting. The excess of spores in milk powders was indirectly supported by the numbers of total cells enumerated by microscopy and PCR as spores evolve from vegetative cells. Surprisingly, all attempts to increase the efficacy of germination by varying the heat-activation conditions of 80°C and 100°C for 10, 20 or 30 min or inducing germination by the addition of amino acids, carbohydrates (Thrane *et al.* 2000) or calcium chelates of dipicolinic acid (Lewis 1972; Donnellan *et al.* 1964) did not increase the germination of selected milk powders (Appendix 12.6.2). The highest recovery of thermophilic spores was obtained by heat activation at 80°C for 20 min, which concurs with the findings of McGuiggan *et al.* (2002). It is possible that no single germination regime will be optimal for all thermophilic strains present in milk powders, and that optimum conditions will vary between species or even strains (McGuiggan *et al.* 2002). For example, the thermophilic milk powder isolate *B. licheniformis* strain F requires no heat-activation to germinate efficiently and outgrowth occurs within 40 min after incubation in TSB-starch at 55°C, whereas *A. flavithermus* C has an obligate requirement for heat-activation for maximal

germination and cell recovery (Appendix 12.6.1). The findings made in this study suggest that spore germination is not only influenced by germination conditions. It is possible that conditions during spore formation might also affect germination. Spores of *A. flavithermus* C produced in Castenholz medium were enumerated equally well by phase-contrast microscopy, conventional plate counting and quantitative PCR indicating near complete germination rates (Table 5.3 and 5.4). Thus, the discrepancies noted when these methods were applied to factory milk powder samples must be related directly to the processing conditions when the powder is formed.

In conclusion, the methods presented in this study provide the ability to selectively enumerate the total and viable vegetative cell and total spore content of reconstituted milk, and can be achieved within 90 min of sampling. The total vegetative bacterial load, i.e. live and dead cells can be achieved even more rapidly, within 60 min of sampling, and although this value may not have a direct bearing on the specified limits for viable thermophiles in milk powder, it can be useful as an indicator of overall plant hygiene. If both viable numbers and dead cell numbers are low then plant hygiene is satisfactory, whereas powders with a low viable count but a high dead cell count indicate a low-hygienic operation, which is being masked by the final heat-treatment and spray-drying. It is envisaged that monitoring of production runs in factories would be performed continuously so that the trends in the counts throughout the entire runs can be assessed and ultimately lead to a reliable accounting of the overall level of thermophilic contaminants.

5.6. References

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Identification of thermophilic bacilli in milk powder using denaturing gradient gel electrophoresis (DGGE)

6.1. Introduction

Denaturing gradient gel electrophoresis (DGGE) is a molecular fingerprinting technique for the separation of a complex and diverse mixture of DNA species that are generated by PCR. The theoretical aspects of DGGE have been initially described by Fischer and Lerman, (1983). Accordingly, PCR products derived from a diverse pool of DNA of a single amplification reaction are separated through a poly-acrylamide gel with an increasing gradient of denaturant. DNA sequences with different nucleotide sequences possess different melting properties in discrete segments, the so called "melting domains". The melting temperatures (T_m) of these domains is sequence specific and depends on both the hydrogen bonds between complimentary base pairs and on the attraction of neighbouring bases of the same strand. Consequently, AT-rich domains tend to melt at low denaturant strength whereas domains rich in GC melt at higher denaturant strength. When the DNA is separated through a denaturing electrophoresis gel and the melting point of the lowest melting domain is reached then the DNA becomes partially melted, creating branches which slow its diffusion. As the DNA molecule continues its migration into higher concentration of denaturant in the gel, domains with higher melting points undergo additional dissociation until the final and most stable melting domain of the molecule is dissociated. Further, single-stranded DNA retards the mobility of the molecule at a given concentration of denaturant until equilibrium between molecule mobility and the degree of dissociation occurs, halting migration through the gel. However, although fully dissociated, the complete run out of the DNA molecule through the gel at a given gradient can be prevented by incorporation of an artificial high melting domain at one end of the molecule (Myers et al., 1985a). This can be accomplished by PCR using a primer with a 5'-end tailed GC-rich clamp of approximately 30 to 50 nucleotides. The use of the clamp is advantageous as 99% of all single base substitutions can be detected when the entire target sequence is allowed to dissociate

while the GC-clamp remains in the duplex configuration (Myers et al., 1985b). Theoretically, the sensitivity of the method should allow discrimination of sequence differences at the level of a single base substitution throughout the entire molecule.

One of the main fields where the technique of denaturing gradient gel electrophoresis finds its application is in determining the structural biodiversity in an environmental community (Fujimoto et al., 2003). Nowadays, it is commonly accepted that the vast majority of micro-organisms in the environment are not yet cultivated, characterised or isolated due to the lack of knowledge about their natural habits or growth requirements. Traditional culturing techniques often fail to reflect the complex ecosystem of an environment with micro-organisms often dependent on the presence of other members of the community. Thus, culture-independent techniques such as DGGE represent a new approach in the understanding and identification of complex microbial communities in the environment. The most common genetic marker for the DGGE method is the 16S rRNA gene. Due to the constraints of functional properties of the ribosome, certain domains of the gene remain strictly conserved throughout all evolutionary lineages, while in other regions the nucleotide sequence is species-specific. Areas of the gene with high levels of sequence conservation from diverse organisms allow for the design of primer pairs which can be specific for either an entire phylogenetic domain or even only for one genus.

The great potential of the culture-independent DGGE technique has recently been applied to food microbiology, for example, for the detection of the dominant species in mixed probiotic cultures from yoghurt and lyophilised products (Fasoli et al., 2003). For instance, food with a defined microbial flora used as a starter culture can easily be displayed by DGGE as each DGGE band theoretically corresponds to the species expected to be in the product. Fasoli et al., (2003) could show that it was possible to detect and identify the dominant bacterial components of probiotic products. Furthermore, all yoghurts screened were found to contain *Bifidobacterium lactis*, although not declared on the product label, in addition to *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. The author also observed discrepancies with some lyophilised products, i.e. the incorrect notification of some organisms (for instance, *Bifidobacterium* and some *Bacillus* species) which could not be identified in the product, and the presence of non-declared organisms. DGGE analysis has also been used to investigate the bacterial communities of different fermented maize foods (pozol, poto-poto and ogi) from Mexico, Congo and

Benin (Ampe and Miambi, 2000). The DGGE bands obtained from fermented maize products from different geographical origins suggested that *Lactobacillus plantarum*, *Lactobacillus delbrueckii* and *Lactobacillus fermentum* are well adapted to the fermentation of maize. In another study, Dewettinck et al. (2001) applied the DGGE method using total DNA extracts from bacterial populations in ground water and bottled mineral water and compared the results with bacterial isolates from plate counts. The authors showed that a comparison of the DGGE-fingerprints from colonies obtained on plated samples and DNA extracted from samples indicated the presence of bacteria in the water, which could not be cultivated.

In the current study DGGE-PCR was used to investigate its suitability to detect and identify thermophilic bacteria in milk powder. For this purpose, the total bacterial DNA content of milk powders was extracted and subjected to DGGE and compared to reference profiles of the most commonly occurring thermophilic bacilli found in milk powders (Ronimus et al., 2003).

6.2. Method and materials

6.2.1. DNA extraction from milk powder for DGGE-PCR

DNA was extracted from milk powder samples by a modification of the procedure described by Sambrook and Russel (2001). An aliquot of 1 gram milk powder was re-suspended in 8 ml of sterile deionised water. A total of 2 mg ml^{-1} lysozyme and 50 µg ml^{-1} of RNAse were added and the samples incubated at 37°C for 2 hours. SDS and proteinase K were added to 1% (w/v) and 200 µg ml^{-1}, respectively, and the samples further incubated at 55°C for 90 minutes. Subsequently, the samples were extracted with 4 ml of phenol:chloroform (1:1) followed by extraction with chloroform and chloroform:isoamyl alcohol (24:1). The DNA was then precipitated by addition of 1/10 (v/v) of 3.0 M sodium acetate and 0.6 volumes of isopropanol, followed by incubation at -20°C for 15 minutes. The DNA pellets were washed twice with 3 ml of chilled 80% ethanol, air-dried and the DNA pellets re-suspended in 200 µl of sterile deionised water. The DNA samples were quantified by spectrophotomer (Pharmacia Biotech, Ultraspec 3000) readings at A_{260}, A_{280} and A_{320} and stored at -20°C until required for DGGE-PCR analysis.

6.2.2. DNA preparation from Geobacillus, Anoxybacillus and Bacillus

DNA preparation from *Geobacillus*, *Anoxybacillus* and *Bacillus* was performed as described in Chapter 4 (section 4.3.2).

6.2.3. DGGE-PCR analysis

The DGGE-PCR was performed on the variable V3 region of the 16S rRNA gene with modified primers previously designed by Muyzer at al. (1993). The nucleotide sequence was 5′-CCT ACG GGA GGC AGC AG-3′ for the forward primer RR71-357f and 5′-ATT ACC GCG GCT GCT GG-3′ for the reverse primer 519r. The forward primer contained additionally a 57-nucleotide GC-rich sequence (GC clamp) 5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3′. The amplification of DNA was performed with the Mastercycler (Gradient; Eppendorf) in 50 µl reaction volumes containing 1.25 mM $MgCl_2$, 0.2 mM dNTPs, 10× *Taq* PCR reaction buffer, 1.25 units of JumpStart *Taq* DNA polymerase (Sigma), 200 nM of forward and reverse primer and approximately 20 ng of target DNA. The PCR reactions were amplified using a touchdown protocol as follows: 5 minutes at 94°C for initial denaturing and *Taq* polymerase activation; 94°C for 30 seconds for amplicon denaturing, primer annealing at 65°C for 1 minute in the first cycle followed by a decrease of 0.5°C for the next 21 cycles of amplification. The annealing temperature for the remaining eight cycles of the amplification was 55°C. The extension steps during PCR were carried out at 72°C for 1 minute including a final extension step at 72°C for 5 minutes at the end of the amplification.

An aliquot of 5 µl of the DGGE-PCR reaction product was loaded onto a 1.5% agarose gel together with 5 µl of gel loading buffer (0.025% bromophenol blue, 0.025% xylene cyanol FF, 30% glycerol in 1× TAE), electrophoresed, stained with ethidium bromide (20 µg ml^{-1} in distilled water) and visualized with the Eagle Eye II system (Stratagene Corp., USA) to verify successful amplification.

6.2.4. Denaturing Gradient Gel Electrophoresis

DGGE was performed with a DCode™ Universal Mutation Detection System Gradient Delivery System from Bio-Rad (Richmond, USA) using 16 cm × 16 cm × 1 mm poly-acrylamide gels. The setting up of the DGGE apparatus and the pouring of the poly-acrylamide gel followed suggestions of the manufacturer described in the instruction manual of the Gradient Delivery System Model 475. Electrophoresis was performed in 1× TAE (40 mM Tris-HCl, pH 8.3; 20 mM glacial acetic acid and 1 mM EDTA) with an 8% (w/v) acrylamide gel (acrylamide-N,N-methylenebisacrylamide, 37.5:1). The acrylamide gel was formed with the 0% (8% (w/v) acrylamide in 1× TAE) and 100% denaturing solution (8% (w/v) acrylamide, 7,0 M urea, 40% formamide, 0.003% bromophenol blue, 0.003% xylene cyanol in 1× TAE) to give a final denaturing gradients of either 25 to 60% or 30 to 60%. Polymerisation was initiated by adding 144.4 µl of a 10% (w/v) solution of ammonium persulphate (Sigma, A-3678) and 14.4 µl TEMED (N,N,N,N-tetramethylethylene diamine) to both gel solutions.

An aliquot of 20 µl PCR sample and 20 µl of 2× DGGE loading buffer (2% (w/v) bromophenol blue, 2% (w/v) xylene cyanol and 70% (v/v) glycerol in sterile deionised water) were used to load the acrylamide gel. Electrophoresis was performed with a constant voltage of 140 V at 60°C for approximately 4.5 to 5 hours. Subsequent to electrophoresis the acrylamide gel was gently removed by binding to chromatography paper and stained for 10 minutes with ethidium bromide (10 mg ml^{-1} in 1× TAE) and de-stained in 1× TAE for a further 10 minutes. Alternatively, silver staining was performed on the gel (section 6.2.5).

6.2.5. Silver staining of DGGE poly-acrylamide gels

Silver staining of poly-acrylamide gels was performed according to Blum et al. (1987), modified according to Rabilloud et al. (1988). All solutions used for staining were filtered through a 0.45 µl pore filter in order to remove insoluble impurities and gloves were worn at all times when handling the gels. The solutions used for staining were at least 10-times the volume of the gel.

Fixation of DNA bands in the gel was performed for at least 60 minutes with 0.5 ml l^{-1} of 37% (w/v) *p*-formaldehyde in 50% (v/v) methanol and 12% (v/v) acetic

acid. The gel was then washed once with 50% (v/v) ethanol for 20 minutes and twice for 20 seconds in deionised water. Subsequently, the gel was sensitised for 1 minute in 1 mM sodium thiosulfate ($Na_2S_2O_3 \times 5\ H_20$). The gel was then washed three times in deionised water for 20 seconds each before impregnation was carried out for 20 minutes with a 12 mM silver nitrate solution ($AgNO_3$) containing 0.75 ml l^{-1} of 37% (w/v) *p*-formaldehyde. The gel was immersed twice in deionised water for 20 seconds followed by developing for 10 to 20 minutes in a solution of 0.5 ml l^{-1} of 37% (w/v) p-formaldehyde, 0.6 M sodium carbonate (Na_2CO_3) and 16 µM sodium thiosulfate. Washing was repeated twice with deionised water for 2 minutes and the staining process stopped by incubation of the gel for 10 minutes in 50% (v/v) methanol and 12% (v/v) acetic acid. Final washing was performed in 50% (v/v) methanol for at least 20 minutes. The gels were stored in the same solution at 4°C.

6.3. Results and discussion

Thermophilic species of *Geobacillus*, *Anoxybacillus* and *Bacillus* previously isolated from New Zealand milk powders (Ronimus et al., 2003) were subjected to DGGE analysis and their profiles used as molecular markers to describe the microbial diversity of milk powder samples. The DGGE fingerprint profiles of *G. stearothermophilus* (strain A), *A. flavithermus* (strains B, C and D), *B. licheniformis* (strains F and G) and *B. subtilis* are shown in Figures 6.1 and 6.2. The differences in appearance of banding pattern between Figures 6.1 and 6.2 is probably the result of different denaturing gradients being used to separate the samples. As a consequence, DNA samples electrophoresed through the lower strength gradient gel migrated further into the gel until they underwent complete denaturation. In contrast, although the DNA samples from the higher strength gradient gel possess the same melting points, these points will be reached closer to the origin of the gel due to the higher denaturant concentration. In addition, the differences in band numbers and densities for some samples are likely due to different PCR master mix preparations and different ethidium bromide staining solutions being used. The use of a DGGE DNA standard commercially available would have strengthened the evaluation of DNA markers between both DGGE gels.

G. stearothermophilus showed 5 major DGGE bands as seen in lane 1 of Figure 6.2. Remarkably, the majority of amplification bands of *G. stearothermophilus*

appear well below the DNA bands of the other bacilli which must reflect higher melting point temperatures. This is due to the higher GC content of the *G. stearothermophilus* DNA amplicon (63.08%) compared with other members of the *Bacillus* genus (*B. licheniformis* (strain F 53.86%; strain G 53.33%), *A. flavithermus* strain B, C and D (55.67%) and *B. subtilis* (54.36%)). The GC contents were determined from the 16S rDNA sequences using the BioMath calculator from Promega (http://www.promega.com).

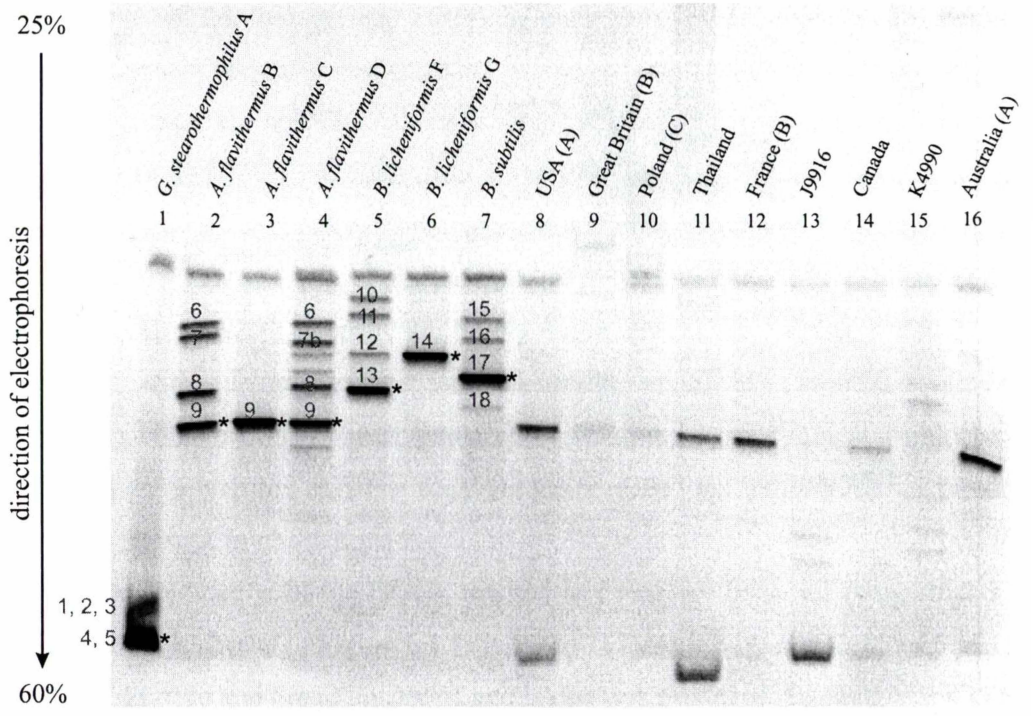

Figure 6.1. DGGE analysis of PCR-amplified 16S rDNA from reference strains of thermophilic bacilli (lanes 1 to 7) and on DNA extracted from milk powders of different geographical origin (lanes 8 to 16). The arrow on the left margin indicates the direction of electrophoresis and the 25 to 60% separation gradient. The origin of the samples is indicated above each track. The numbering of selected bands follows the explanation in the text. In addition, asterisks indicate primary DGGE markers mentioned in the text. The gel was ethidium bromide stained.

The three thermophilic *A. flavithermus* strains B, C and D share a common primary band (band 9 in Figure 6.1, lanes 2 to 4). The fingerprints of strains B and D are almost identical with the exception of band 7b of *A. flavithermus* D (Figure 6.1, lane 4) which appears to have a slightly higher melting point than band 7 of strain B (lane 2). In contrast, the DGGE profiles from both strains of *B. licheniformis* (F and G) are quite different (Figure 6.1, lanes 5 and 6) with the exception of bands 12 (strain F) and 14 (strain G).

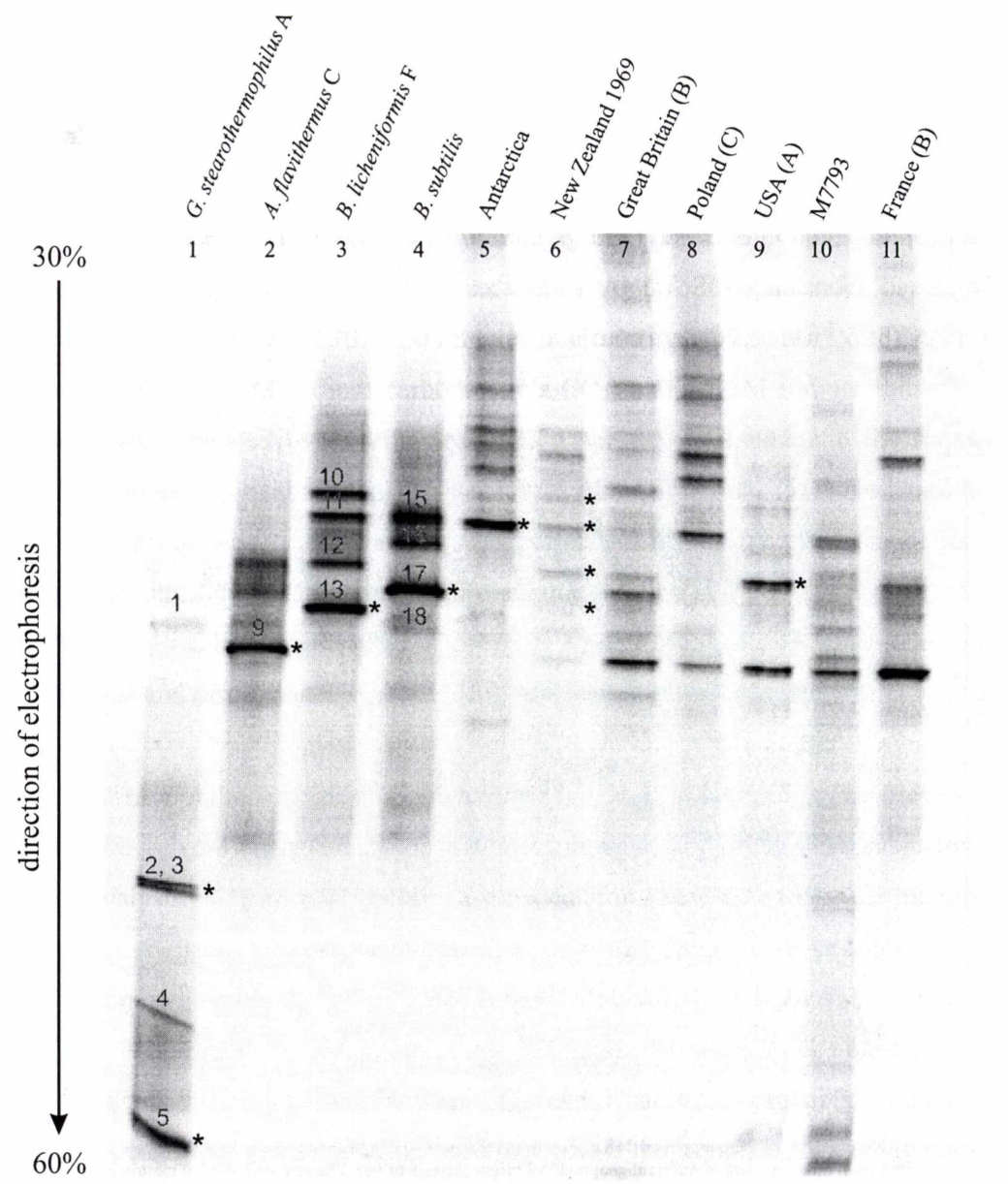

Figure 6.2. DGGE analysis of PCR-amplified 16S ribosomal DNA of *G. stearothermophilus* A, *A. flavithermus* C, *B. licheniformis* F and *B. subtilis* (lanes 1 to 4) and on total DNA derived from milk powders of different geographical origin (lanes 5 to 11). The arrow on the left margin indicates the direction of electrophoresis and the 30 to 60% separation gradient. The origin of the samples is indicated above each track. The numbering of selected bands follows the explanations in the text. In addition, asterisks indicate primary DGGE markers. The gel was ethidium bromide stained.

The DGGE profile of *B. licheniformis* strain F suggests that at least four different V3 sequence variations of the 16S rRNA gene exist (Figures 6.1 and 6.2, bands 10, 11, 12 and 13) with band 13 as the primary amplicon. The DGGE analysis performed on *B. licheniformis* G comprised one single primary amplicon (Figure 6.1, band 14 from lane 6) which seems to be similar to the band 12 of strain F (Figure 6.1, lane 5). *B. subtilis* showed four structurally different markers with band 17 as primary amplicon.

An expectation with DGGE profiling is that a single bacterial species will produce a single amplicon, the position of which on a DGGE gel would enable its identification. In practice this was seldom the case and only observed for the DGGE samples from *A. flavithermus* strain C and *B. licheniformis* strain G (Figure 6.1; band 9 in lane 3 and band 14 in lane 6). Nevertheless, this was not an entirely robust result, as strain C produced multiple amplicons for the amplification reaction shown in Figure 6.2 (lane 2). The reason for multiple bands is likely due to multiple copies of the 16S rRNA gene being present on the genome, and different band densities for the same strain would then reflect either the number of gene copies with identical sequence for the region amplified. This problem cannot be fully resolved with the current approach; the best that can be achieved is to use the dominant bands for each of the reference strains as an indication of the likely contaminants being present in an unknown sample. Fortunately, a dominant band is observed for each reference strain, and these are indicated by asterisks on the right of the appropriate band in each of the figures. Bands of lower intensity are highly dependent on the staining protocol used, the staining and development time, template concentration and so are of less value for identifying contaminants in milk powders. A solution to this problem would be to choose a gene which has only one copy per genome, and the *spo0A* gene is a possible candidate for such investigation.

The application of the DGGE method to determine bacterial contaminants of milk powders is shown in Figure 6.1 (lanes 8 to 16) and in Figure 6.2 (lanes 5 to 11). Overall, a diverse and broad cluster of amplicons was produced. In comparison to the reference profiles, those from milk powders show greater variations in signal strength. There are two likely reasons for this; first, the DNA extracted reflects the occurrence of several different species present. Second, there is the possibility of primer bias towards particular versions of the V3 variable region between species resulting in different end-point amplicon quantities.

However, some information can be derived from the results obtained. For example, the milk powder USA (A) contains two primary markers identical to the marker 5 of *G. stearothermophilus* strain A and marker 9 of *A. flavithermus* strain C (Figure 6.1 lane 8 and Figure 6.2 lane 9). Both organisms were present in the powder as identified by RAPD analysis (Rueckert et al., 2004) in the proportion 6% of *G. stearothermophilus* strain A and 91% *A. flavithermus* strain C. Interestingly, the RAPD analysis also detected low numbers (3%) of *B. licheniformis* strain F whereas

the DGGE profile did not detect its presence. Possibly the number of cells of strain F was below the detection threshold of the DGGE technique (reported to be 10^4 to 10^5 cfu ml^{-1} Dewettinck et al., 2001) or cells of this strain exist mainly as spores which are not detected, as DNA was only extracted from vegetative cells. This powder contained a third primary DGGE marker (indicated with an asterisk) and some lower intensity markers (Figure 6.2 lane 9) which could not be assigned to any of the reference species. However, the presence of a DGGE marker cannot unequivocally be correlated with culture-dependent RAPD analysis. The majority of vegetative cells which develop in the process line are killed by the processing stresses, e.g. pasteurisation, direct-stream injection, heating prior to evaporation, post evaporator heat-treatment and spray-drying; such cells would not be detected by RAPD (which requires culturing of viable cells) but their DNA would be detected by DGGE. This was particularly evident with *G. stearothermophilus* strain A which was positively identified by DGGE analysis in powders from Great Britain (B), Poland (C), Thailand, J9916, M7793, Canada, and Australia (A) (Figures 6.1 and 6.2). However, the PCR amplification of strain A markers was not consistent as can be seen in both figures. The powders from Great Britain (B) and Poland (C) produced visible bands only for those reactions separated in Figure 6.2. Viable counts and RAPD analysis confirmed the presence of strain A only for the powders from Poland (C), USA (A), J9916 and M7793.

The dominant milk powder isolate *A. flavithermus* C was positively identified by DGGE analysis in powders from USA (A), Great Britain (B), Thailand, France (B), Canada and Australia (A) and this is in agreement with RAPD analysis (Rueckert et al., 2004), where viable forms of *A. flavithermus* C were detected in high numbers. Presumably, this organism is better able to tolerate the conditions applied in the milk powder production process compared to *G. stearothermophilus* A.

B. licheniformis strain F is a widely distributed contaminant in milk powders and the isolate was present in all but one milk powder sample subjected to RAPD analysis at levels between 14 cfu ml^{-1} (Antarctic) and 7680 cfu ml^{-1} (Great Britain (B)). However, none of the milk powders subjected to DGGE analysis were convincingly positive for *B. licheniformis* F, with the exception of the New Zealand milk powder from 1966, suggesting either a bias in the amplification or more likely that cell numbers of strain F were below the level of detection (Dewettinck et al., 2001). RAPD analysis of the 100 year old milk powder sample returned from

Antarctica and the 39 year old powder from New Zealand (1966) both contained thermophilic and mesophilic bacilli in low numbers (<360 cfu g^{-1}) with *B. subtilis* and *B. licheniformis* strain F the most prevalent isolates (Chapter 10). These levels should be below the detection level of the current DGGE protocol which proved true for the Antarctic powder with no characteristic DGGE marker being present for strain F and BS. However, the New Zealand milk powder sample produced a profile pattern with similarities to the standard *B. licheniformis* strain F (indicated with asterisks in Figure 6.2 lane 6) although it could not unequivocally be assigned to the DGGE marker of strain F (Figure 6.2, lane 3). Interestingly, the Antarctic powder produced a strong band (indicated with an asterisk in Figure 6.2 lane 5) with no significant correlation to bands for *B. licheniformis* F, *B. subtilis* or any of the other thermophilic milk powder isolates. The same powder, subjected to PCR specific for the amplification of a small region of the sporulation gene *spo0A,* produced an amplicon with a melting point temperature (T_m) unlike any of the other seven known milk powder derived *Bacillus* species (Figure 6.3). In Figure 6.3 the T_m values of 82.21°C, 85.04°C and 88.93°C correspond to the Antarctic, France (B) and the M7793 powder samples, respectively. The latter sample, a high count milk powder from the study of Ronimus et al. (2003) contained *G. stearothermophilus* strain A (T_m 88.73°C) as dominant isolate (Figure 6.2; band 10). The sample from France (B) was shown by DGGE (Figure 6.1 lane 12) and RAPD (Rueckert et al., 2004) to contain *A. flavithermus* strain C (T_m 85.04°C) as dominant isolate, which was also confirmed by real-time PCR. The amplicon from the Antarctic powder has a T_m of 82.21°C, 3.5°C below the lowest T_m of the seven strains of dairy bacilli, and thus, must be a novel unknown contaminant.

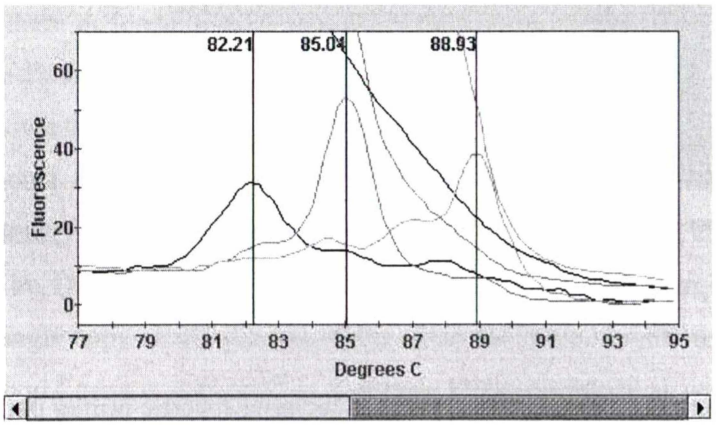

Figure 6.3. Melting point analysis of *spo0A*-real-time PCR amplicons on the Smart Cycler II detection system for milk powder samples from Antarctica (82.21°C), France (B) (85.04°C) and M7793 (88.93°C), respectively.

Figure 6.4 shows the silver stain of the poly-acrylamide gels from Figure 6.1. Staining nucleic acids with silver ions has been described and improved by several authors including Boulikas and Hancock (1981), Blum et al. (1987), Guillemette and Lewis (1983), Kolodny (1984), Beidler et al. (1982) and Bassam et al. (1991). The staining method is reported to be highly sensitive with about 1000 times more sensitivity than ethidium bromide staining. However, when the method by Blum et al. (1987) was applied to poly-acrylamide gels the sensitivity was not significantly increased over ethidium bromide staining. Taking into account that the silver stain method is complex, expensive and time consuming, DGGE gels in this study were routinely ethidium bromide stained. Although the silver stain from Figure 6.4 does not provide any improvement over the ethidium bromide strain from Figure 6.1 it was included into the report to demonstrate the effect of the procedure to following researcher.

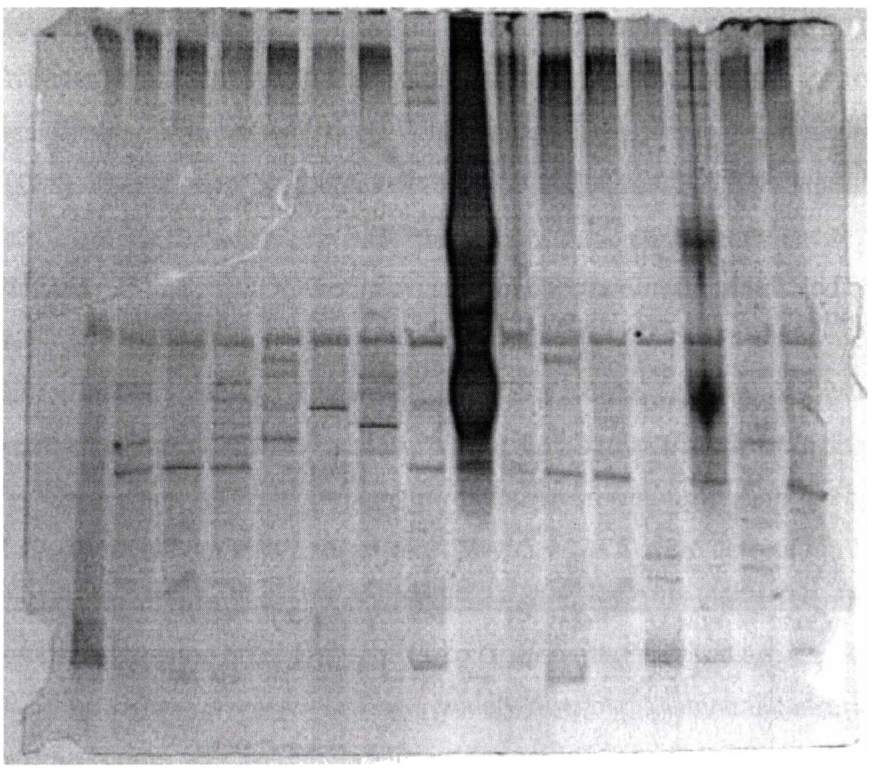

Figure 6.4. Silver staining of the poly-acrylamide gel from Figure 6.1.

6.4. Conclusion

Denaturaning gradient gel electrophoreses with a bacterial specific primer pair targeting the variable V3 region of the multi-copy 16S rRNA gene (Muyzer at al.,

1993) was applied to individual milk powder isolates and to milk powders in order to investigate the suitability of the method to detect and identify thermophilic bacilli. In practice because the primers target sequences, which are highly conserved among all prokaryotes, the method will detect any prokaryote organism present in the powder. When applied to DNA from individual isolates of thermophilic milk powder bacilli, unique profiles were obtained which allowed differentiation of the strains used. When applied to DNA extracted from milk powders, the method produced results which were not as easy to interpret. Because of multiple banding patterns, DGGE marker affiliations for individual strains in milk powder samples was somewhat complex and frequently more difficult to interpret than for pure cultures. On the other hand, the presence of dominant bands for some milk samples provided a good basis for identification of individual strains. Even so, these results did not always correlate with those obtained by RAPD analysis conducted on the same powders, which indicated different dominant organisms being present. This discrepancy can be explained by the different targets for each procedure. RAPD analysis can only be conducted on the viable cells in the powder, whereas DGGE analysis is based on the amplification of DNA from living cells and those killed during processing but where the DNA remains intact. Therefore the DGGE method can provide an insight into the "history" of the powder, i.e. contaminants which increased during processing but which were eliminated by heat treatments. However, the main restriction of DGGE as a routine milk powder screening method is the low detection limit of 10^4 to 10^5 cfu ml^{-1} (Dewettinck et al., 2001) as was confirmed with low level contaminants such as *B. licheniformis* and *B. subtilis*. The limit of detection may even depend on the DNA extraction method applied, the template concentration or on the PCR reaction itself where different templates compete for amplification. Further, the DNA purification method used in this survey is incapable of extracting DNA from spores. The advantage of the method is that it can produce an assessment of thermophilic food-borne contaminants to strain level suitable for milk powder analysis, and provide a history of contaminants of the powder not available to culture-dependent methods. The DGGE method could possibly be improved by targeting a gene present as a single copy in the genome, rather than the more ubiquitous 16S rRNA gene, and the *spo0A* gene is suggested as a suitable target for bacilli as was discussed in section 4.5.

Estimation of the copy number of 16S ribosomal RNA genes of thermophilic bacilli

7.1. Introduction

The 16S rRNA gene has been used by numerous workers (Hendolin et al., 1997; Klausegger, 1999; Wilson et al., 1990; Corless et al., 2000) as a PCR amplification target for non-culture-based molecular detection methods of bacteria. In particular, the gene has been used widely to determine the relatedness of organisms in phylogenetic analyses and more recently, the gene has also become important as a means of identifying unknown organisms. The 16S rRNA has a conserved structural function in the ribosome and in contrast to a typical functional gene, which is transcribed and translated into a protein, the 16S rRNA is subjected to a relatively low evolutionary pressure. The large and increasing number of full-length and near full-length 16S rRNA sequences indicates the gene is highly conserved across the eubacterial kingdom, although there are still sufficient regions of sequence variation to allow for inter-species discrimination employing PCR. An important fact with respect to the quantitative real-time PCR method from Chapter 3 is that the 16S rRNA gene is often present in multiple copies in the genomes of prokaryotes and can vary from one to 15 copies per genome (Klappenbach et al., 2001; Shaver et al., 2001). *B. subtilis* strain 168, for instance, is reported to contain 10 copies in its genome, and some *B. subtilis* laboratory strains contain nine ribosomal operons due to deletion within the rrnJ-rrnW or rrnI-rrnH-rrnG gene cluster (Widom et al., 1988, Rudner et al., 1994). In addition, members of the *Bacillus cereus* group are shown to possess between 8 to 12 copies of this gene (Cherif et al., 2003; Daffonchio et al., 2000). The fact that this favoured PCR marker is present in multiple copies and in unequal numbers in the genome of prokaryotes is only of minor importance in the qualitative studies on biodiversity, provided that all amplification target regions of the gene have the same sequence. In contrast, if the gene is used as quantification PCR marker than this will have influence on the assay and ambiguities in the results due to different copy numbers of different target organisms can cause over- and/or underestimations

of cell quantities. On the other hand, a target gene present in multiple copies increases the sensitivity of detection during quantitative analysis, particularly when low numbers of the target cells are present (Corless at al., 2000). Indeed, the quantitative PCR assay for the 16S rRNA gene (Rueckert et al., 2005a; Chapter 3) was 10-fold more sensitive than was achieved with the PCR assay targeting the sporulation gene *spo0A* (Rueckert et al., 2005c; Chapter 4), which is reported as being present only as a single copy in the genome of endospore-formers (Brown et al., 1997). However, if the copy number of species present in a sample varies, then it is difficult to directly correlate cell numbers with quantitative PCR results, which was the intention of this study. The problem of potential variability can also be compounded when the multiple copies of the 16S rRNA gene in an organism do not have the same sequence (Acinas et al., 2004). This might offer two possibilities; either that primer bias is becoming a feature or that a quantitative assay might be undermined by a TaqMan probe which does not detect all sequence variants.

The purpose of this investigation was to estimate the number of 16S rRNA genes in each of the seven strains of thermophilic bacilli commonly found in milk powder (Ronimus et al., 2003) in order to determine if the quantitative PCR assay from Chapter 3 requires any kind of correction to compensate for intergenomic 16S rRNA copy number variation.

7.2. Materials and Methods

7.2.1. Bacterial strains and culture preparation

Geobacillus stearothermophilus (strain A), *A. flavithermus* (strains B, C and D), *B. licheniformis* (strains F and G) and *B. subtilis* (BS) were isolated from New Zealand milk powders by Ronimus et al. (2003) and the cultures were grown at 55°C in tryptic soy broth (TSB) supplemented with 0.2% (w/v) soluble potato-starch.

7.2.2. Ultra-purification of genomic DNA

DNA preparation from all cultures was performed as described in section 4.3.2.

7.2.3. Restriction digests of genomic DNA

Restriction enzyme digestions were performed on genomic DNA in 25 µl reaction volumes using the following restriction enzymes and reaction buffers (Roche): *Eco* RV/REact 2, *Hind* III/REact 2, *Hae* II/REact 2 and *Cla* I/REact 2. The reactions contained 1 µg of genomic DNA, 1.5 units of restriction enzyme and one-tenth of volume of 10× reaction buffer. The samples were incubated for 2 hours at 37°C and the restricted DNA electrophoresed through a 0.7% (w/v) agarose (Seakem; LE agarose) gel in 1× TAE buffer (0.04 M Tris, 0.04 M acetic acid, 1 mM EDTA, pH 8.3).

7.2.4 Southern hybridization

Southern lifts were performed using the alkali transfer method as described by Sambrook and Russel (2001). Following electrophoresis the gel was stained, de-stained and digitally photographed (Eagle Eye, Stratagene, USA) as described in section 4.3.3. The gel was then incubated in two changes of 0.25 N HCl for 5 minutes with agitation, followed by immersion in at least 3 gel volumes of transfer buffer (0.4 N NaOH) for 15 minutes. This step was repeated and the gel placed upside down on a clean and disinfected bench top on top of a plastic sheeting. A piece of Hybond N^+ membrane was cut to the dimension of the gel, marked with a pencil in one corner and immersed in MQ water for 10 minutes. The membrane was placed on the gel while avoiding any air bubbles between of Hybond N^+ membrane and the surface of the gel. The following items were placed in sequence on the top of the membrane: 3 sheets of transfer buffer-saturated 3MM chromatography paper, 3 sheets of dry 3MM paper and a stack of paper towels at least 6 cm high, a glass plate and a 0.5 kg weight. The transfer of the DNA to the Hybond N^+ membrane was left to proceed overnight. After the transfer was complete the membrane was soaked in 0.4 N NaOH for at least 20 minutes. Subsequently, the membrane was briefly rinsed in 0.5× SSC and allowed

to air-dry on 3MM paper. The DNA was cross-linked to the membrane by exposure for 30 seconds to UV light (120 joules cm^{-2}, BLX-254, Life Technologies).

7.2.5. PCR amplification and ^{32}P-labelling of the hybridization probe

The 243 bp ribosomal amplicon which was used as a DNA probe for southern hybridisation was amplified by PCR using forward primer RR132 (5′-GGA GGG TCA TTG GAA ACT GG-3′) and reverse primer RR133 (5′-GCG TTA GCT GCA GCA CTA AAG GG-3′). The primers primed at position 626 to 646 bp and 847 to 870 bp according to the 16S rRNA gene of *Escherichia coli* (Brosius et al., 1980). Preparative PCR amplifications were performed in 25 µl reactions containing 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 10× *Taq* PCR reaction buffer, 1.25 units of Ampli*Taq* DNA polymerase (Roche), 600 nM of forward and reverse primer and 10 ng of both *G. stearothermophilus* A and *B. licheniformis* F genomic DNA. The PCR amplifications were cycled once at 95°C for 120 seconds and 35 cycles at 92°C for 30 seconds, 55°C for 30 seconds and at 68°C for 60 seconds. The final extension was at 72°C for 10 minutes. The amplified DNA was separated by gel electrophoresis and the band of interest recovered and purified from the gel as described in section 4.3.3. The standard method for radioactive labelling was carried out with the Rediprime™ II DNA Labelling System (Amersham Biosciences) and the protocol performed according to the Rediprime manual user handbook. The DNA sample (25 ng) was diluted in TE (pH 8.0) to give a final volume of 45 µl. The sample was denatured at 95°C for 5 minutes and placed immediately in ice-water for a further 5 minutes. Subsequently, the sample was added to a Rediprime tube and mixed with an aliquot (5 µl) of α-^{32}P-dCTP. The reaction was incubated for 1 hour at 37°C. On completion, 5 µl of 0.2 M EDTA was added to the reaction mixture. The radioactively labelled probe was denatured by heating at 96°C for 5 minutes in a PCR instrument and the denatured probe finally added to the pre-hybridization solution.

7.2.6. Preparation and execution of hybridization

Hybridization was performed with a PCR amplified region of the 16S rRNA genes of *G. stearothermophilus* strain A and *B. licheniformis* strain F. The hybridization process was divided into three steps and was carried out in

hybridization bottles with rotating movement in an incubator. These steps included: pre-hybridization, hybridization with the α-^{32}P-dCTP-labelled probe and washing. Firstly, the membrane was pre-hybridised in 20 ml of Church-Gilbert solution (1 mM EDTA, 0.5 M Na_2HPO_4, 7% SDS (w/v), 1% bovine serum albumin (w/v), pH 7.2) at 65°C for at least one hour. Secondly, the pre-hybridization solution was discarded and 12 ml of fresh 65°C Church-Gilbert solution containing the labelled probe added to the bottle. Hybridization was carried out for at least 16 hours at 65°C. Thirdly, after hybridization, washing was carried out with at least three changes of 80 ml of phosphate buffer solution I (40 mM Na_2HPO_4, 1 mM EDTA, 5% SDS (w/v), 0.5% bovine serum albumin (w/v), pH 7.2) for 1 hour at 65°C. The membrane was then washed with eight changes of 80 ml of phosphate buffer solution II (40 mM Na_2HPO_4, 1 mM EDTA, 1% SDS (w/v), pH 7.2) for 1 hour at 65°C (Sambrook and Russel, 2001).

7.2.7. Autoradiography and imaging

Audioradiography was carried out with the Fujifilm BSA-1800 II Storage Phosphor Imager system (Fujifilm). For this purpose the membrane was covered with layers of Handy Wrap Foil and exposed in a cassette for 90 minutes to a phosphor imager plate. The imager plate was subsequently used to create an image with the BAS-8000 as described in the user manual, and the visualized membrane archived as a TIF-image file. Subsequently, the membrane was placed in a stainless steel, black velvet-lined cassette (Kodak) equipped with two Kodak X-Omatic regular intensifying screens on both sides. In complete darkness a 20.3 cm × 25.4 cm sheet of Kodak film (Scientific Imaging Film, X-Omat™ AR) was placed on top of the membrane. The cassette was closed and left overnight (16 to 24 hours) at -70°C before the film was developed. After autoradiography, the cassette was transferred to the darkroom and the film developed in the following way: in total darkness, the film was removed from the cassette and developed with Kodak GBX developer and replenisher for 5 minutes. During development the film was gently moved by agitation. The film was then transferred to a water bath for approximately 1 minute with gentle agitation. The film was fixed in Kodak GBX fixer and replenisher for 5 minutes followed by extensive rinsing with tap water for 15 minutes and air drying.

7.2.8. Quantitative real-time PCR

Quantitative real-time PCR analysis was performed with a Smart Cycler II system (Cepheid, USA) using the protocol described in Rueckert *et al.* (2005a) (section 3.3.7). The only modification was the TaqMan probe (Rueckert *et al.*, 2005a), which employed the VIC-dye as reporter and TAMRA as quencher.

7.3. Results

7.3.1. Amplification of the hybridization probe

A nucleotide probe for Southern hybridization was generated with primers RR132 and RR133 using genomic DNA of *G. stearothermophilus* strain A and *B. licheniformis* strain F as target (Figure 7.1A). The probe was 243 bp in length and the corresponding band from the electrophoresis gel was recovered for DNA purification (Figure 7.1B).

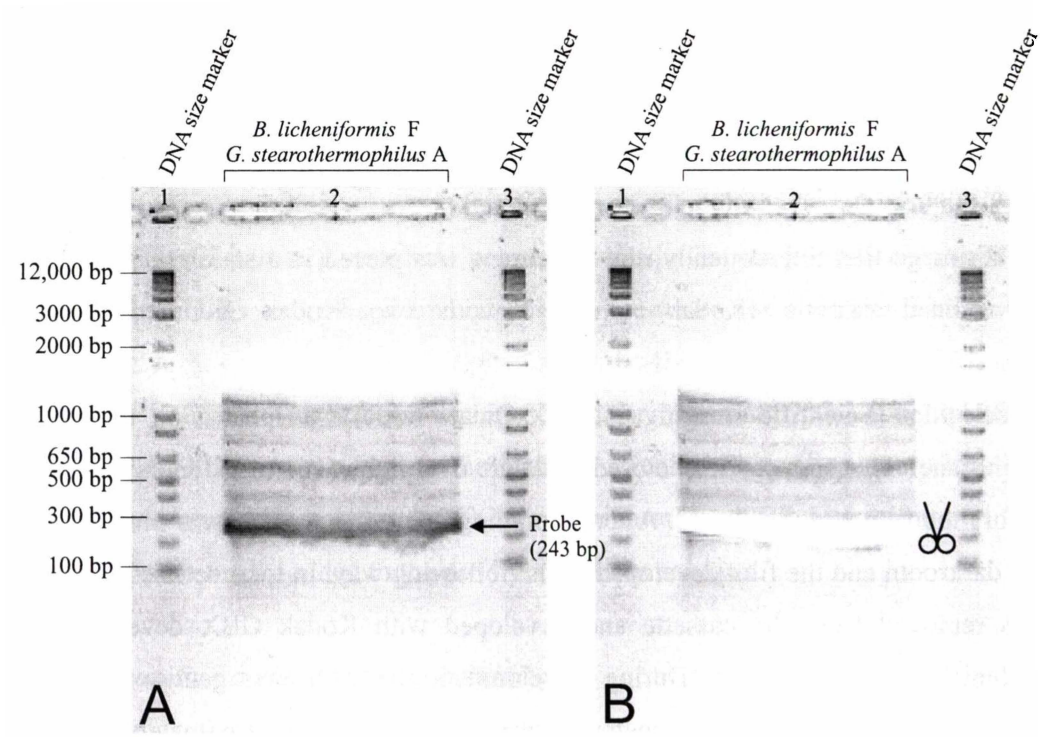

Figure 7.1 A and B. PCR amplification of a small region of the 16S rRNA genes from strains of *G. stearothermophilus* and *B. licheniformis*. A) Preparative agarose-gel electrophoresis of the amplification reaction. The amplification band of interest is indicated with an arrow at the right margin. B) The amplification band of interest was excised from the gel for DNA purification and Southern hybridization.

7.3.2. Restriction digests of genomic DNA

There are two requirements for the restriction enzymes used in this investigation: firstly, they should cause the complete restriction of genomic DNA for all seven strains of bacilli, and secondly, they should recognize at least one restriction site situated within each of the ribosomal operons in order to create a physical map which after Southern hybridization would create a hybridization pattern that will allow the enumeration of the number of operons in the genome. Additionally, the restriction must not occur within the sequence of the hybridization probe, i.e. between positions 626 and 870 bp according to Brosius et al. (1980), in order to prevent overestimation in 16S ribosomal RNA gene numbers.

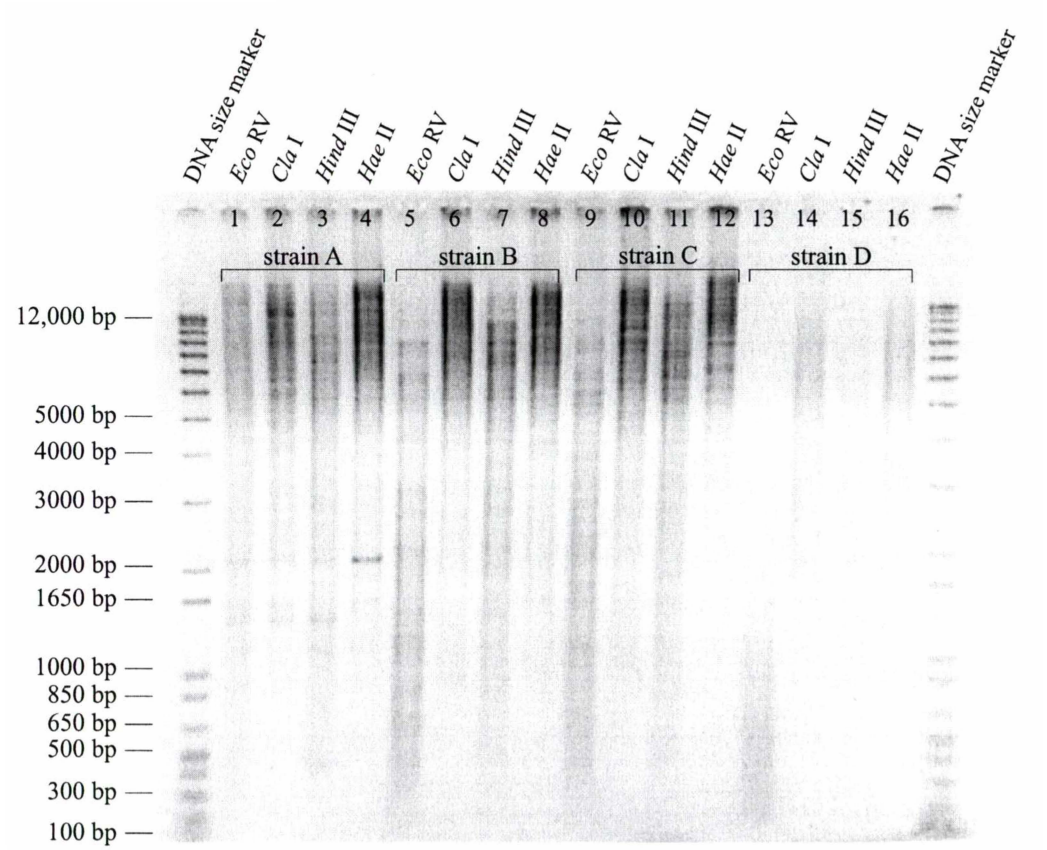

Figure 7.2. Restriction digest of genomic DNA of *G. stearothermophilus* (strain A) and *A. flavithermus* (strains B, C and D). DNA size marker is indicated in the left margin: 1 Kb Plus DNA Ladder (Invitrogen). The restriction enzymes used for digestion are indicated above each track.

In pursuit of this purpose, a virtual restriction map of all ten ribosomal operons (16S, 23S and 5S rRNA genes) of *B. subtilis* (Z99119 and AL009126) and the seven dairy bacilli was generated using the online software program from the REB-site

(http://tools.neb.com/REBsites/index.php3). Four enzymes were chosen that met both of the above criteria, i.e. *Hind* III recognizing six sites, *Hae* II and *Eco* RV recognizing two sites and *Cla* I which recognizes one site within the ribosomal operons of *B. subtilis*. Furthermore, *Hind* III also cleaved the 16 rRNA genes of *A. flavithermus* (strains B, C and D) at position 80 according to Brosius et al., (1980), and the 16S rDNA of *G. stearothermophilus* strain A and *B. subtilis* were also cleaved by *Hae* II at positions 440 (strain A and BS) and 477 (strain BS). Finally, sequence analysis with the 16S rRNA genes of the seven thermophilic milk powder bacilli indicated that none of the restriction enzymes employed cleaved the sequence of the hybridization probe within positions 626 and 870 (Brosius et al., 1980). The restricted and electrophoresed DNA samples from *G. stearothermophilus* (strain A), *A. flavithermus* (strains B, C and D), *B. licheniformis* (strains F and G) and *B. subtilis* (strain BS) are shown on Figure 7.2 and 7.3.

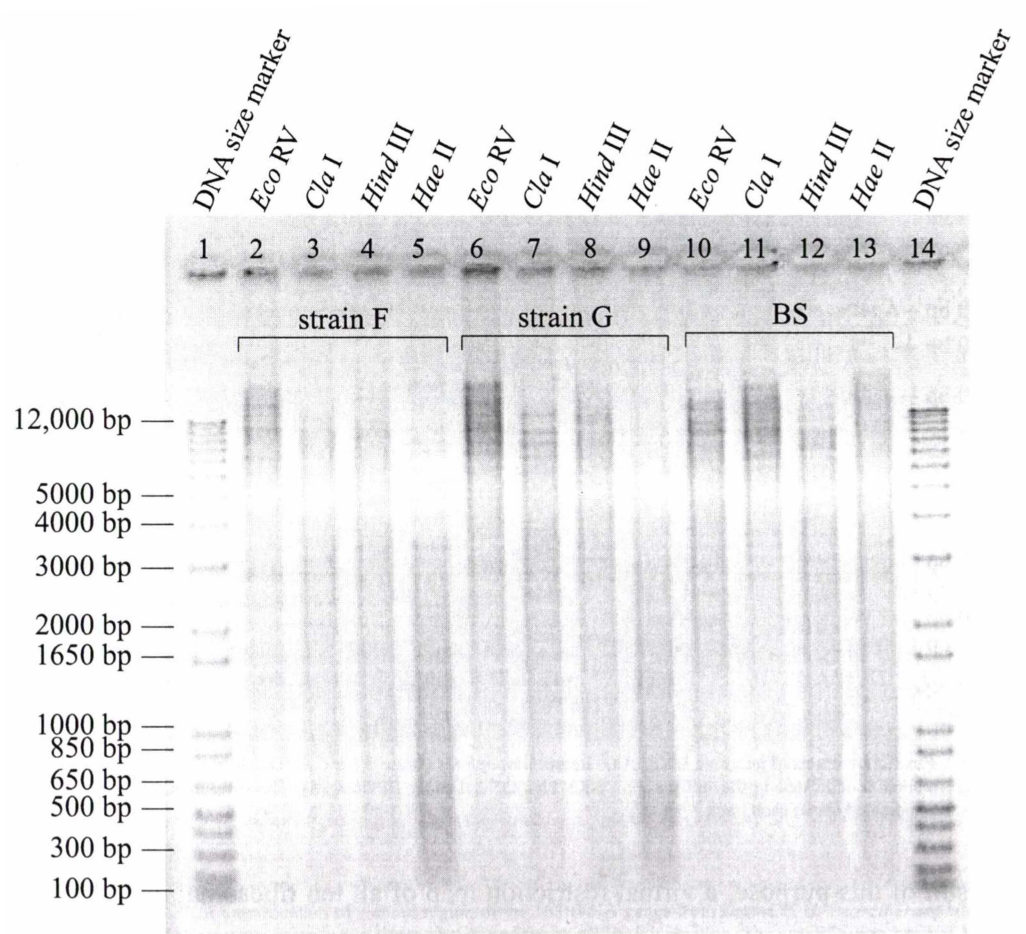

Figure 7.3. Restriction digest of genomic DNA of *B. licheniformis* (strains F and G) and *B. subtilis* (strain BS). DNA size marker is indicated in the left margin: 1 Kb Plus DNA Ladder (Invitrogen). The restriction enzymes used for digestion are indicated above each track.

7.3.3. Southern hybridization

Genomic DNA from all seven thermophilic bacilli was digested with the four restriction enzymes, the restricted DNA was separated by electrophoresis and southern blotted onto nylon-cellulose membranes (Hybond-N^+). The membranes were then hybridized with the radioactively labelled probe and the blots processed with the phosphor-imager system and subsequent with the Kodak film to obtain a visual image resulting from the hybridization. The phosphor images are shown in Figures 7.4, 7.6 and 7.7 and the corresponding Kodak images in Figure 7.5 and 7.8 where black bands indicate locations where the probe hybridized to its complementary sequences.

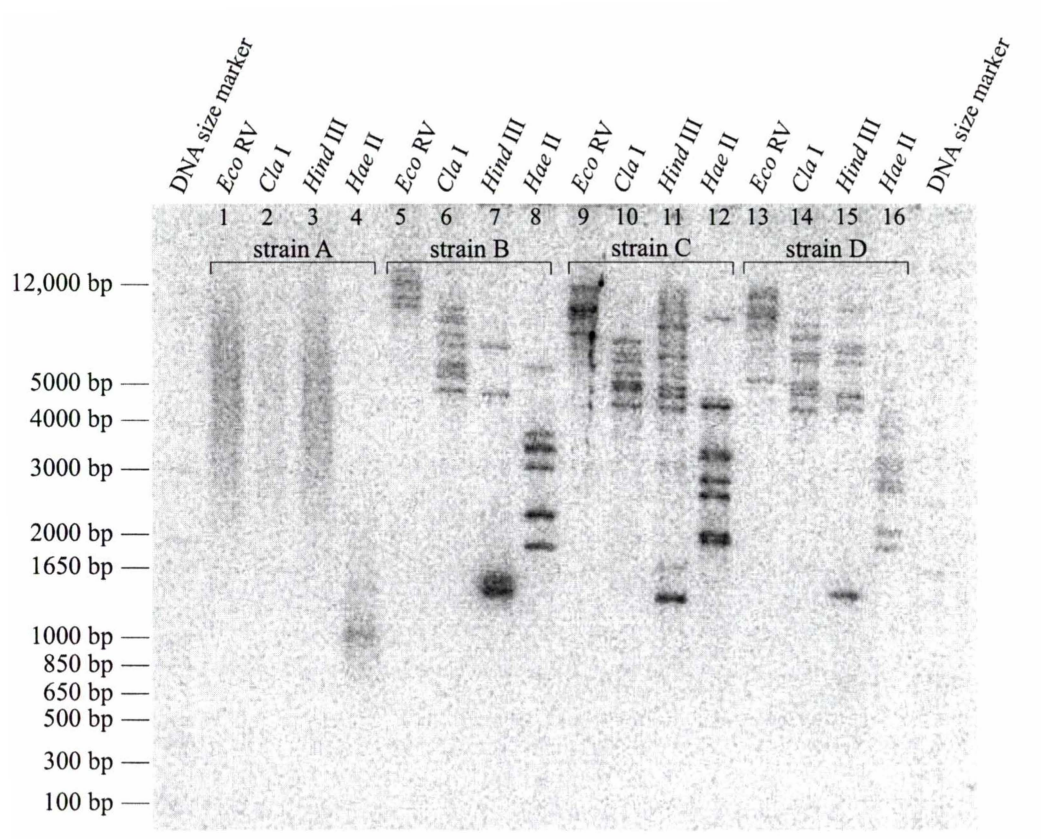

Figure 7.4. Phosphor image of hybridization of the *G. stearothermophilus* (strain A) and *A. flavithermus* (strains B, C and D) using the radioactively labelled probe. DNA size marker is indicated in the left margin: 1 Kb Plus DNA Ladder (Invitrogen). The restriction enzymes used for digestion are indicated above each track.

The number of hybridization signals for each sample was used to estimate the number of 16S rRNA gene copies in the genome. Accordingly, the hybridization probe produced multiple hybridization signals for each restriction digested DNA sample confirming the presence of multiple 16S rRNA copies in the genome of

Geobacillus, *Anoxybacillus* and *Bacillus*. Furthermore, the number of hybridization sites varied between the strains investigated and also within individual strains depending on the restriction enzyme used for DNA digestion. For instance, although the hybridization patterns of all three strains of *A. flavithermus* showed some similarities when digested with a particular enzyme (Figure 7.4 and 7.5), the number of enumerated hybridization signals differed (Table 7.1). For example, when DNA of strains B, C and D was digested with *Eco* RV characteristic hybridization clusters of 5 to 7 individual bands appeared in the molecular size area between 5000 to 12,000 bp for all three samples. The same samples digested with *Cla* I produced clusters of 7 to 9 signals between 4000 and 10,500 bp according to the DNA size marker. A more differentiated hybridization pattern was achieved for these strains using *Hind* III and *Hae* II with signal clusters in the range of approximately 1500 to 12,000 bp and 1800 to 10,000 bp, respectively. The experiment with *G. stearothermophilus* strain A from Figure 7.4 in lanes 1 to 4 could not be evaluated due to failure in the acquisition of differentiated hybridization signal. This experiment was repeated and the results are shown in Figure 7.6.

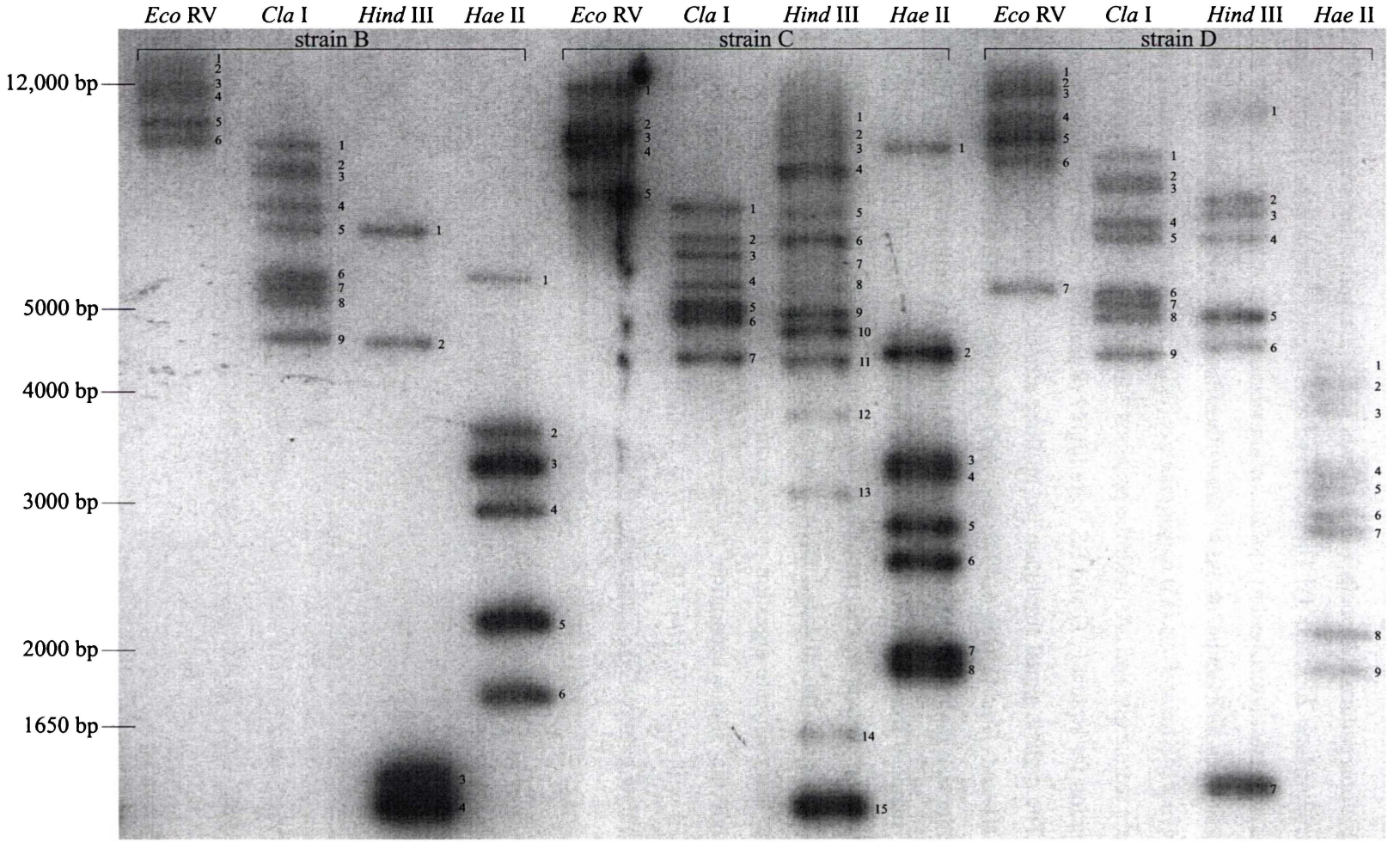

Figure 7.5. Kodak film image of the Southern hybridization of *A. flavithermus* (strains B, C and D) according to Figure 7.4. *G. stearothermophilus* (strain A) was omitted from the image. The 16S rRNA copy number was enumerated from both the phosphor images and the Kodak films. The corresponding counts are shown at the right of each hybridization site. DNA size marker is indicated in the left margin: 1 Kb Plus DNA Ladder (Invitrogen). The restriction enzymes used for digestion are indicated above each track.

Only a insufficient differentiation of the hybridization signals was obtained with *Eco* RV, *Cla* I and *Hind* III digested DNA of *G. stearothermophilus* strain A producing a hybridization pattern clustering 3 to 4 signals above the molecular weight of 7000 bp (Figure 7.6; lane 2, 3 and 4). The DNA sample digested with *Hae* II from lane 5 generated at least 8 resolved hybridization signals.

Figures 7.7 and 7.8 both show the Southern hybridization blot of *B. licheniformis* (strains F and G) and *B. subtilis* (BS) from which the ribosomal operon numbers were estimated. As with the previous four strains, the signal separation and distribution for strains F, G and BS were highly dependent on the restriction enzyme used. The highest signal resolution for all three strains was achieved using *Eco* RV, *Hind* III and *Hae* II (Figure 7.7 and 7.8) yielding hybridization signals in the molecular size range of between 2000 and >12,000 bp. In common, the *Cla* I digested DNA samples from strains F, G and BS produced a insufficient separation of hybridization signals in the molecular size range of 5000 to 8000 bp. This was unexpected since the digested DNA samples from Figure 7.3 (lanes 3, 7 and 11) indicate that complete digestion of the genomic DNA was evident to have occurred. Although the signal separation for these samples was less distinguishable than obtained with *Eco* RV, *Hind* III and *Hae* II, their signal resolution was sufficient to differentiate between 5 to 7 signals.

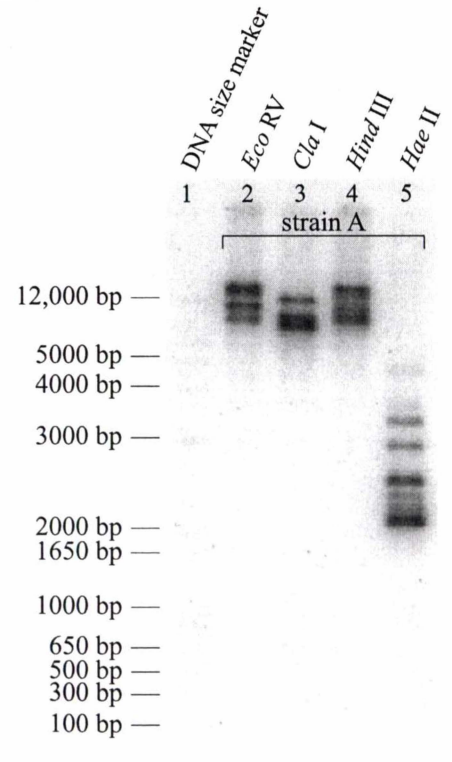

Figure 7.6 Phosphor image of hybridization of the *G. stearothermophilus* (strain A) using the radioactively labelled probe. DNA size marker is indicated in the left margin: 1 Kb Plus DNA Ladder (Invitrogen). The restriction enzymes used for digestion are indicated above each track.

A summary of the hybridization signals as enumerated from the phosphor images and the Kodak films (Figures 7.4 to 7.8) is listed in Table 7.1. In some instances, hybridization signals could not be unequivocally enumerated for two reasons. First, insufficient signal separation caused by multiple signal transitions which did not allow for a clear discrimination. Secondly, some hybridization signals with distinctively brighter signal intensity are likely to be derived from multiple hybridization sites located on one restriction fragment. For these samples, the number of 16S ribosomal copies is presumed to represent a minimum number, and is probably greater than that shown in Table 7.1.

Table 7.1. 16S rRNA copy number estimation from the phosphor image and Kodak film

Organism	*Eco* RV	*Cla* I	*Hind* III	*Hae* II
G. stearothermophilus strain A	4+	3+	4+	8+
A. flavithermus strain B	6+	9	4+	6+
A. flavithermus strain C	5+	7+	15	8+
A. flavithermus strain D	7+	9	7+	9
B. licheniformis strain F	6+	5+	6+	5+
B. licheniformis strain G	7+	6+	5+	8+
B. subtilis strain BS	7+	7+	8+	7+

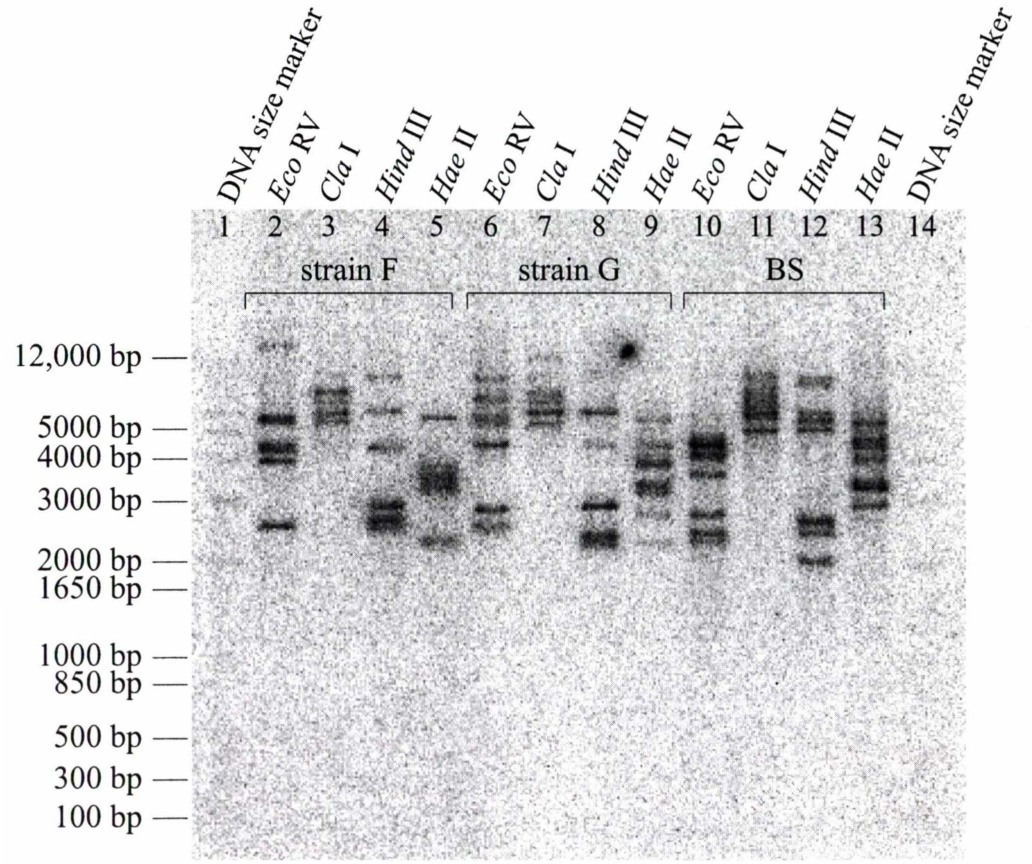

Figure 7.7. Phosphor image of hybridization of the *B. licheniformis* (strains F and G) and *B. subtilis* using the radioactively labelled probe. DNA size marker is indicated in the left margin: 1 Kb Plus DNA Ladder (Invitrogen). The restriction enzymes used for digestion are indicated above each track.

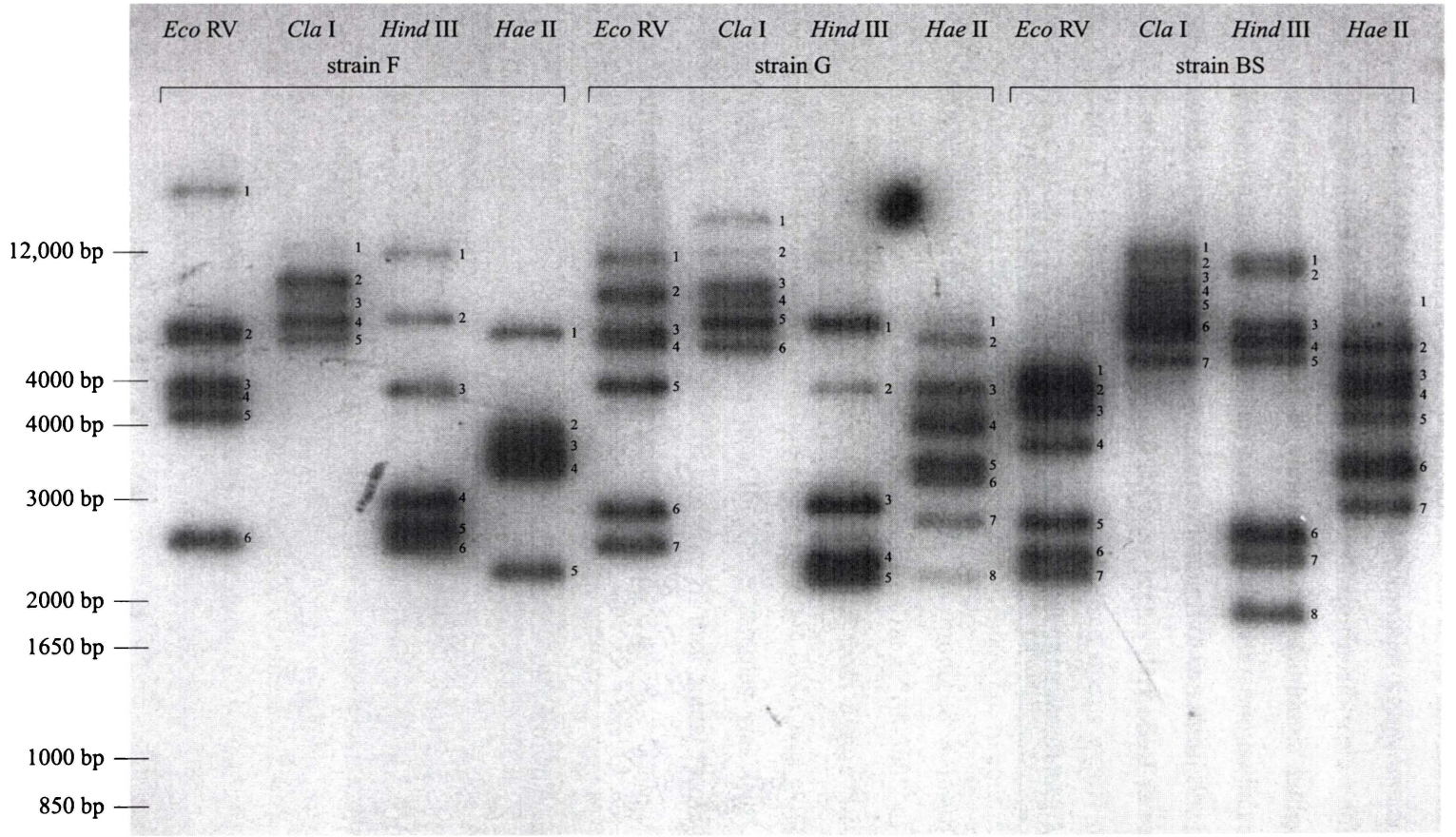

Figure 7.8. Kodak film image of the Southern hybridization of *B. licheniformis* (strains F and G) and *B. subtilis* (strain BS) according to Figure 7.7. The 16S rRNA copy number was enumeration from both the phosphor images and the Kodak films. The corresponding counts are shown at the right of each hybridization site. DNA size marker is indicated in the left margin: 1 Kb Plus DNA Ladder (Invitrogen). The restriction enzymes used for digestion are indicated above each track.

7.3.4. Quantitative real-time PCR for the estimation of the intergenomic ratio of ribosomal operon number within the dairy bacilli

Quantitative PCR was performed on 10 ng of genomic DNA per amplification reaction for each of the seven dairy strains. The primary amplification plot and threshold cycle numbers are shown in Figure 7.9 and Table 7.2, respectively. The lowest C_t of the seven strains was obtained with *G. stearothermophilus* A, which differed from the highest C_t produced by *B. subtilis* by one PCR cycle.

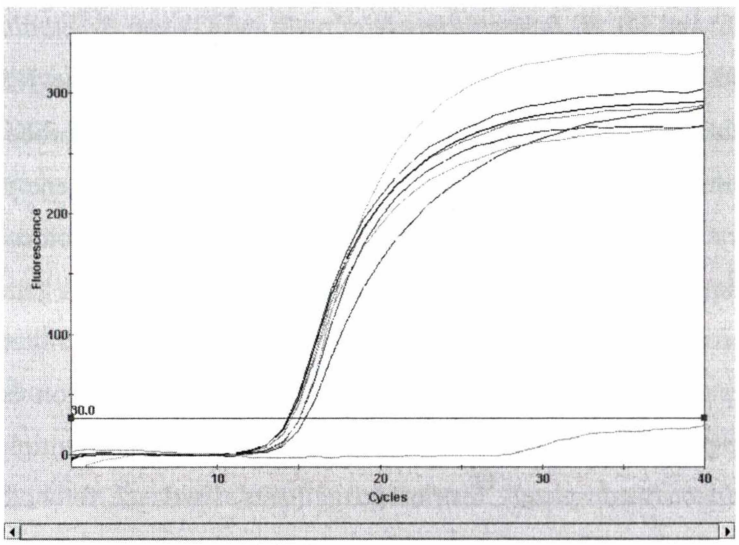

Figure 7.9. Primary amplification plot of small regions of the 16S rRNA genes of the seven strains of thermophilic dairy bacilli.

Table 7.2. Threshold cycle number (C_t) and theoretical 16S rRNA copy number per cell

	Bacillus strain						
	A	B	C	D	F	G	BS
Threshold cycle number (C_t)	14.36	14.55	14.54	15.06	15.05	14.30	15.40
Relative amount of initial target DNA [ng] [(a)]	6.544	5.786	5.824	4.159	4.186	6.803	3.337
Estimated number of target copies in 10 ng DNA [(b), (c)]	1.2×10^7	1.0×10^7	1.0×10^7	7.5×10^6	7.5×10^6	1.2×10^7	6.0×10^6
Calculated number of 16S rRNA genes per genome	14.13	12.5	12.6	9	9	14.72	7.1

[(a)] Relative amount of initial target DNA using the DNA standard curve of *A. flavithermus* (section 3.4.3)
[(b)] Initial amount of gene copies for strain D calculated assuming 5 fg DNA and 9 16S rRNA genes per cell, respectively
[(c)] Gene copy number calculated based on the ratios of the "initial amount of template DNA" referring to strain D

From this experiment, the estimated 16S rRNA copy number of the seven strains was calculated on the basis that a single cell contains a total of approximately 5 fg chromosomal DNA (Bakken and Olsen, 1989) and using *A. flavithermus* strain D as the reference strain containing nine 16S rRNA gene copies per cell (section 7.3.3). In addition, the assumption was made that PCR efficiency and the initial amounts of

template DNA were identical for all seven strains so that the variations in C_t directly correlate to different numbers of the 16S rRNA gene within the genome. The results from Table 7.2 demonstrate that a C_t difference of one PCR cycle theoretically reflects a two-fold variation in copy number as occurs, for example, for strain A and BS.

7.4. Discussion

The number of 16S rRNA copies from *G. stearothermophilus* (strain A), *A. flavithermus* (strains B, C and D), *B. licheniformis* (strains F and G) and *B. subtilis* (strain BS) was estimated by southern hybridization using a small region of the 16S rRNA gene of *B. licheniformis* F and *G. stearothermophilus* A as hybridization probe.

Although the results of this investigation confirm that the 16S rRNA gene is present in multiple copies in the genomes of the seven *Bacillus* strains, the protocol applied was not able to determine unequivocally the exact number of ribosomal gene copies (Table 7.1) for two reasons: firstly, the enumeration was in some cases limited by inadequate signal separation and secondly, insufficient restriction digestion of genomic DNA offers the possibility that several signals were derived from multiple hybridization sites located on incompletely restricted fragments. Evidence for both scenarios arose in the acquisition of hybridization signals of different intensities, with signals of higher intensity likely to be due to multiple signals overlapping. Multiple signals derived from adjacent ribosomal operons located on a DNA fragment are unlikely where the fragment size is below 5000 bp since one ribosomal operon (including 16S-, 23S- and 5S rRNA genes) of *B. subtilis*, for example, stretches over an average length of approximately 5000 bp. On a theoretical basis, this would also be valid for two adjoining ribosomal operons as the closest distance between two hybridization sites is greater than 5000 bp. Thus, signals with distinctively higher intensities, which appear below 5000 bp, are due to insufficient signal separation of the hybridization site located on two different DNA fragments of similar size. In contrast, the probability of two adjacent hybridization sites located on one DNA fragment increases with increasing molecular size of the DNA fragment.

The maximal number of hybridization signals determined by Southern hybridization was eight for *G. stearothermophilus* (strain A), *B. licheniformis* (strain G) and *B. subtilis*, nine for *A. flavithermus* (strain B and D), fifteen for *A.*

flavithermus strain C and six for *B. licheniformis* (strain F). The relatively low ribosomal operon number of six for strain F can be explained by the overlapping of multiple hybridization signals discussed above. For *A. flavithermus* strain C there are three possible explanations for the relative high number of hybridization sites obtained when its genomic DNA was restricted with *Hind* III. Firstly and most likely, some of the 16S rRNA genes located in the genome of strain C possess an unrecognised *Hind* III restriction site within the sequence of the DNA probe, which was not present in the 16S rRNA gene sequenced and used for sequence analysis. Secondly, non-specific hybridization of the probe occurred to targets other than the 16S rRNA gene. This may be the explanation for the hybridization signals of lower intensity such as the bands 5, 7, 8, 12, 13 and 14 of the *Hind* III digested strain C DNA from Figure 7.5. But the possibility of non-specific binding can be discounted for two reasons: firstly, it was not observed for any of the other samples blotted onto the membrane in Figure 7.5 and secondly, the hybridization was executed under highly stringent conditions, which should minimize non-specific hybridization. The third explanation is that strain C, indeed, contains a significantly higher number of ribosomal operons compared to the other strains.

The attempt to estimate the relative number of 16S rRNA gene copies between the seven thermophiles by quantitative PCR was also inconclusive. In this experiment the approximate gene copy number of each strain was calculated from the threshold crossing points (C_t) with the assumption that the amount of template DNA in each reaction was ideally the same. However, the assumptions on which this premise is based mean that a threshold crossing discrepancy of one PCR cycle, as was observed between *G. stearothermophilus* strain A and *B. subtilis* strain BS, reflects twice as many gene copies existing in strain A. The results obtained from this experiment do not confirm the results from southern hybridization. For example, the two *A. flavithermus* samples of strain B and D digested with *Cla* I Figure 7.4 and 7.5 produced nine well separated hybridization signals with similar signal intensities, supporting that each signal was derived from a single hybridization site located on a single restriction fragment. Thus, southern hybridization suggests that both strains are likely to possess identical numbers of 16S rRNA gene copies whereas quantitative PCR recognised an excess of 3 to 5 copies per genome in strain B compared to strain D.

However, the quantitative PCR experiment is unlikely to precisely measure gene copy numbers due to restricted factors such as pipetting errors, DNA quantitation errors or primer bias. In fact, these limitations were also evident for triplicate repetitions of chromosomal DNA amplifications performed to generate the standard curves (section 3.4.3 and 4.4.4) and C_t discrepancies between 0.1 and 0.65 cycle were common for a single DNA dilution. Consequently, any 16S rRNA gene copy discrepancies within the genomes of the seven bacilli will be outbalanced by this limitation. In order to demonstrate this the following is an example of a calculation supplied by Randy Rasmussen (http://docs.appliedbiosystems.com/pebiodocs).

The equation (1) described the amplification of a PCR reaction during the exponential growth phase where T_n is the amount of target sequence at PCR cycle n, T_o is the initial amount of target and E described the efficiency of the reaction (2). The latter term is calculated from the slope of the standard curve; in this case, from the DNA standard curve derived from *B. licheniformis* strain F (section 3.4.3). The equation (3) describes the linearization of (1).

(1) $T_n = T_o(E)^n$

(2) $E = 10^{(-1/slope)}$

(3) $\log T_n = \log T_o + n*\log E$

The amount of PCR product needed to produce a signal above the background (T_n) is unknown for the Smart Cycler and each PCR machine may have a different sensitivity. However, according to Randy Rasmussen, it requires approximately 10^{10} copies of a PCR product to produce a signal above background. For example, for a reaction starting with 9 copies and an efficiency of 1.96 equation (3) predicts the first signal at C_t 30.95. The same reaction containing 10 copies at the start of the amplification would produce a threshold crossing at 30.79 shifting the threshold crossing by 0.16 PCR cycles. This calculation demonstrates that for a probable case of one copy difference between different strains of bacilli the resulting quantification would still be below the above-mentioned errors involved in setting up a reaction. However, the quantification error becomes more evident the greater the gene copy discrepancies between strains. For instance, if the gene copy number of a strain exceeds the copy number of a reference strain by a factor of 2 then, according to

equation (3) both samples would respond with one PCR cycle difference. And although threshold crossing of *G. stearothermophilus* A and *B. subtilis* differed by one cycle (Table 7.2) this is more likely to be due to inadequate DNA template delivery by imprecise DNA target quantification and pipetting errors.

In conclusion, although the presence of multiple 16S rRNA gene copies in the genome of the seven *Bacillus* strains could be confirmed by southern hybridization, the method did not allow for the conclusive determination of the exact copy number due to low resolution. In addition, attempts to establish copy number discrepancy using quantification PCR did not have sufficient accuracy since errors in replicate samples of the same target exceeded differences found between strains. In other words, the 16S rRNA gene can be used as quantification marker when the copy number variation of this gene among the dairy strains is small, i.e. less than factor two. In this case any enumeration error associated with different gene numbers will be smaller than the error associated with setting up the amplification reaction.

16S-23S ribosomal intergenic spacer region - a sequence analysis

8.1. Introduction

Although the ribosomal RNA genes (16S, 23S and 5S) are highly conserved among prokaryotes, a different situation occurs for the intergenic spacer region between the 16S and 23S ribosomal genes. The 16S-23S intergenic spacer has inherently much greater variation in both sequence and nucleotide length than the adjacent ribosomal genes (Shaver et al., 2001). Because many prokaryotes possess more than a single ribosomal operon per genome the intergenic region between different operons can exhibit an even greater variation in nucleotide sequence and length. For this reason the intergenic spacer region is often a favoured target to distinguish between species and strains (Klappenbach et al., 2001; Shaver et al., 2001; Bott et al., 1984; Widom et al., 1988; Cherif et al., 2003; Daffonchio et al., 2000; Nagpal et al., 1998). Accordingly, the analysis of the 16S-23S ribosomal intergenic spacer in prokaryotes has become a popular tool to determine the relatedness between species, especially, for the comparison of closely related organisms when 16S sequencing alignments are too similar to provide for an adequate discrimination (Clayton et al., 1995; Nagpal et al., 1998).

The genome of *B. subtilis* strain 168 contains ten ribosomal operons, and sequence alignment analysis revealed that there exist at least three distinct types of 16S-23S ribosomal spacer regions as reported by Nagpal et al. (1998). One type including rrnA and rrnO are of identical sequence (346 bp) and contain the sequences for isoleucine and alanine tRNA (Loughney et al., 1982). The remaining 16S-23S intergenic spacers do not encode for any tRNA and appear in two distinct sizes of 167 bp (rrnG, rrnH, rrnI, rrnW and rrnJ) and 170 bp (rrnB, rrnD and rrnE) (Nagpal et al., 1998). The sequences of rrnG, rrnH and rrnI are identical and the spacer sequences of rrnW and rrnJ are 99.4% identical. The three 170 bp intergenic spacers possessed nearly identical sequences with 99.4 to 97.6% homology (Nagpal et al., 1998).

The significant degree of sequence and length variation within the intergenic spacer among prokaryotes and even within a single genome commonly results in multiple PCR amplifications of characteristic size distribution, which can be useful for discrimination at the species-level (Jensen et al., 1993). Intergenic sequence comparisons even allowed the discrimination of several species within the genus *Bacillus*. For example, Wunschel et al. (1995) differentiated *B. subtilis* from the *B. cereus* group including *B. anthracis*, *B. thuringiensis* and *B. cereus* based on the PCR amplification of the 16S-23S ribosomal spacers. Nagpal et al. (1998) confirmed the close relatedness of *B. subtilis* strain 168 and W23 by intergenic spacer sequence alignment analysis with 99.9 to 100% conservation between both strains. Furthermore, the sequence differences between both strains were less than between the ten operons within strain 168. The same study also revealed that *Bacillus atrophaeus* var. *niger*, a member *B.* subtilis group, possessed quite distinct 16S-23S intergenic spacer sequences compared to strain 168 and W23. The sensitivity of discrimination between species or even between strains can be improved using heteroduplex cross-hybridization of 16S-23S PCR amplicons as was described by Jensen et al. (1996). This procedure amplifies the heterologous intergenic spacer region starting from the 16S and 23S ribosomal genes producing amplicons that are homologous at the 3' and 5' sequences ends. These PCR products will cross-hybridize due to the homology at the 3'- and 5'-ends to form heteroduplex DNA structures. These structures have been shown to possess a reduced electrophoretic mobility compared to homologous double-stranded DNA. The reduced mobility during electrophoresis depend will on the amount of single-stranded DNA present in the heteroduplex DNA as well on the degree of secondary structure formed within these single-stranded regions (Jensen et al., 1993; Jensen et al., 1996).

The aim of this study was to analyse the differences of the 16S-23S intergenic spacer regions of the seven milk powder bacilli in order to establish if this marker can be used to devise a real-time PCR protocol for quantification and differentiation at the strain level. For this purpose, the 16S-23S intergenic spacer regions of all seven dairy bacilli were amplified by PCR and for each strain the complete sequence of at least one 16S-23S intergenic spacer was obtained by amplicon sequencing. These sequences were compared for nucleotide sequence diversity and length by CLUSTAL W (1.83) alignment analysis and the suitability of the ribosomal spacer region as real-time PCR target discussed.

8.2. Material and methods

8.2.1. Bacterial strains and culture preparation

Geobacillus stearothermophilus (strain A), *A. flavithermus* (strains B, C and D), *B. licheniformis* (strains F and G) and *B. subtilis* (BS) were isolated from New Zealand milk powders by Ronimus et al. (2003). The cultures were grown at 55°C in tryptic soy broth (TSB) supplemented with 0.2% (w/v) soluble potato-starch.

8.2.2. Ultra-purification of genomic DNA

DNA preparation from *Geobacillus*, *Anoxybacillus* and *Bacillus* was performed as described in Chapter 4 (section 4.3.2).

8.2.3. Amplification of the 16S-23S ribosomal intergenic spacer region and agarose-gel purification of amplicons

The 16S-23S ribosomal intergenic spacers of all seven thermophilic bacilli were amplified and sequenced using the 16S rDNA flanking forward primer TN114 (5′-TGT ACA CAC CGC CCG TC-3′) (De Silva et al., 1998) and the 23S rDNA flanking reverse primer TN116 (5′-ACG GTG GAT GCC TTG-3′) (Daffonchio et al., 1998a, 1998b). The polymerase chain reactions were performed with the MasterCycler (Eppendorf) with the reactions containing 4 mM $MgCl_2$, 0.2 mM dNTPs, 10× *Taq* PCR reaction buffer, 1.25 units of Ampli*Taq* DNA polymerase (Roche), 500 nM of both primers and 30 ng of genomic DNA. The amplification reactions were cycled as follows: 94°C for 165 seconds and 38 cycles at 94°C for 15 seconds, 58°C for 15 seconds, 72°C for 120 seconds and finally, a 4 minutes extension step at 72°C. Subsequent to PCR amplification the reaction mixtures were electrophoresed through a 1.5% (w/v) agarose gel (SeaKem). The agarose gels were stained with ethidium bromide (20 $\mu g\ ml^{-1}$) and de-stained with water for 10 minutes each. The gels were then placed onto an UV transilluminator and the bands of interest excised using a sterile razor blade. The DNA was then recovered from the excised bands and purified as described in Chapter 4 (section 4.3.3). The sequencing reactions

of the purified ribosomal intergenic spacers were carried out by the Waikato DNA Sequencing Facility (University of Waikato).

From the 16S-23S ribosomal intergenic spacer sequences a multiple sequence alignment was performed on-line with CLUSTAL W 1.82 (European Bioinformatics Institute; http://www.ebi.ac.uk/clustalw/). Phylogenetic analysis employed CLUSTAL X using the neighbour-joining algorithm while the analysis was statistically validated using 100 bootstraps (Thompson et al., 1994). The phylogenetic cladogram was illustrated using NJPLOT provided by CLUSTAL X. The aligned sequences were then used to select for a conserved region, which was used to design a primer pair for a potential quantitative PCR. These primers were: forward primer TN115 5′-TGC GGR TGG ATC ACC TCC TTT CT-3′ (Johnson, 1994) and reverse primer RR167 5′-GTT ATC TAG TTT TCA ARG A-3′. The amplification of a short region of the ribosomal intergenic spacer of the seven dairy bacilli was performed with the MasterCycler (Eppendorf) using 4 mM $MgCl_2$, 0.2 mM dNTPs, 10× *Taq* PCR reaction buffer, 1.25 units of Ampli*Taq* DNA polymerase (Roche), 500 nM of both primers and 15 ng of genomic DNA. The amplification reactions were cycled as follows: 94°C for 120 seconds and 40 cycles at 94°C for 15 seconds, 45°C for 15 seconds and elongation at 68°C for 20 seconds. The amplification reaction products were subjected to electrophoresis as described above.

8.2.4. Quantitative real-time PCR

PCR analysis was performed with a Smart Cycler II system (Cepheid, USA) in 25 µl optical reaction tubes. The reactions were performed with 4 mM $MgCl_2$, 0.2 mM dNTPs, 10× *Taq* PCR reaction buffer, 1.25 units of Ampli*Taq* DNA polymerase (Roche), 0.25× SYBR Green I (Sigma-Aldrich; S9430) and 600 nM of primers TN115 and TN116. The amplification reactions contained 1.5 ng of genomic template DNA, i.e. ultra-purified DNA from all seven bacilli. The real-time PCR was cycled once at 95°C for 120 seconds, 40 repetitions at 95°C for 5 seconds, 45°C for 5 seconds, 68°C for 30 seconds. The latter elongation step was used for fluorescence acquisition. On completion of amplification, PCR products were directly identified using the melting point analysis protocol of the Smart Cycler instrument (60°C to 95°C; 0.2°C sec^{-1}).

8.3. Results

8.3.1. 16S-23S intergenic spacer amplification and sequencing

The ribosomal intergenic sequences between 16S and 23S rRNA gene were amplified with primers TN114 and TN116, separated though agarose electrophoresis and the results are illustrated in Figures 8.1, 8.2 and 8.3. For all strains the polymerase chain reactions produced multiple amplification products of different sizes, and band densities. The bands of interest (those of greatest density) are numbered in Figures 8.1, 8.2 and 8.3. These bands were excised from the agarose-gel, the DNA recovered and used for sequencing.

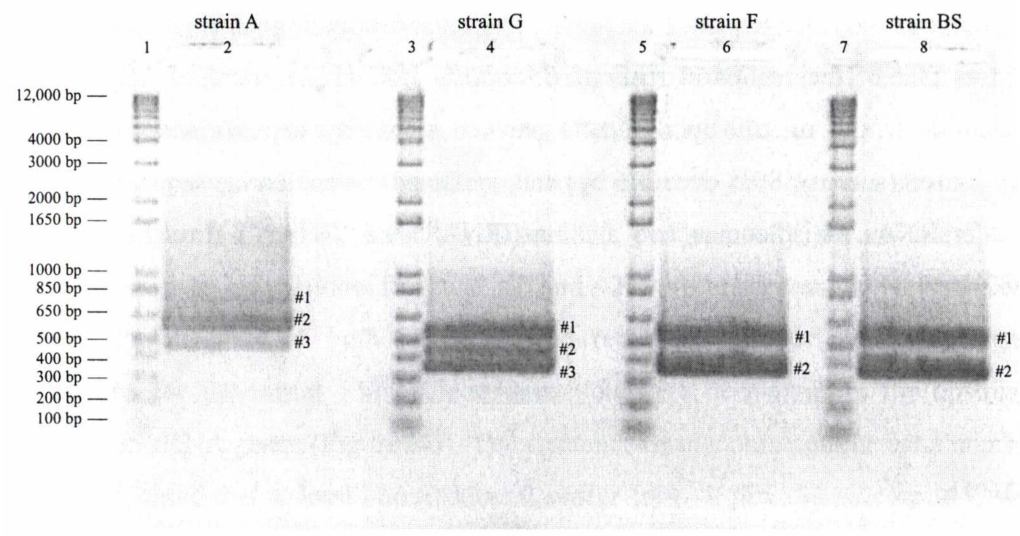

Figure 8.1. PCR amplification of the 16S-23S intergenic spacer regions of *G. stearothermophilus* (strain A), *B. licheniformis* (strains F and G) and *B. subtilis*. Primary amplification products, which were used for sequencing, are numbered at the right margin of each band of interest. DNA ladder (1 kb Plus DNA Ladder; GIBCO BRL, Life Technologies) in tracks 1, 3, 5 and 7 denotes the molecular size marker. The marker sizes are indicated in the left margin.

G. stearothermophilus (strain A) produced three visual distinguishable bands (#1, #2 and #3; track 2, Figure 8.1) of which band #3 did not produce usable sequence results. The intergenic spacer sequences derived from band #1 and #2 were 33 bp and 396 bp, respectively (Figure 8.5). Both sequences were homologous over the entire range of sequence #1 and scored to 100% and 99%, respectively, with an unspecified ribosomal spacer of *G. stearothermophilus* (EM-2_F) when subjected to blastn (NCBI). Furthermore, from the electropherogram of sequence #1 it became apparent that the signal peaks of the initial 33 nucleotides were well separated and that

sequence calling stopped abruptly due to multiple signal interactions during sequencing (data not shown). This kind of signal profile is characteristic of cross amplicon interference due to heterogeneous DNA sequences being present in the sequence reaction. Presumably, band #1 includes the amplification products of ribosomal spacer regions derived from various operons with the first thirty nucleotides being highly conserved whereas the subsequent sequences show heterogeneity.

B. licheniformis (strain G) DNA produced three distinct amplification bands during PCR (bands #1, #2 and #3 from track 4 in Figure 8.1) which all matched the ribosomal spacer regions of *B. licheniformis* ATCC 14580 when subjected to a blastn using default parameters (NCBI). The sequences derived from bands #1, #2 and #3 were 346 bp, 89 bp and 190 bp in length, respectively, with bands #2 and #3 being homologous over the sequence available. The closest homologous sequence to band #3 was that of the rrnD and rrnE of *B. subtilis* 168 with a score of 91% over a nucleotide length of 169 bp. Band #1 showed similarity to rrnA and rrnO of *B. subtilis* 168 (score of 89% over 346 bp) and contained the nucleotide sequences of the transfer RNAs of isoleucine and alanine ($tRNA^{Ile}$ and $tRNA^{Ala}$) (Loughney et al., 1982).

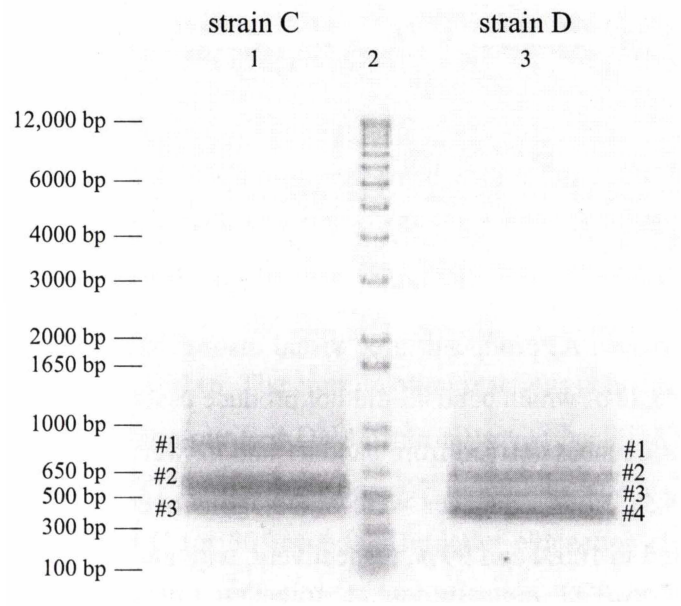

Figure 8.2. PCR amplification of the 16S-23S intergenic spacer regions of *A. flavithermus* (strains C and D). Primary amplification products, which were used for sequencing, are numbered on the right margin of each band of interest. DNA ladder (1 kb Plus DNA Ladder; GIBCO BRL, Life Technologies) in track 2 denotes the molecular size marker. The marker sizes are indicated in the left margin.

Two nucleotide sequences were derived from *B. licheniformis* strain F (Figure 8.1; track 6). Both bands #1 and #2 are identical over 88 bp and 171 bp to the internal

transcribed spacer of *B. licheniformis* EI-4N_F (NCBI), respectively. In addition, sequence #2 aligned to *B. subtilis* suggesting that rrnG, rrnH and rrnI are their closest homologs, scoring 89% over the entire range of the ribosomal spacer. The sequence result obtained from band #1 was interrupted after 88 bp due to multiple signals interfering, offering the probability that this band contains more than one intergenic spacer amplicon.

The two 16S-23S intergenic spacer sequences which were derived from amplifying the flanking regions of the 16S and 23S ribosomal genes of *B. subtilis* (track 8 from Figure 8.1) were 74 (band #1) and 163 nucleotides in length (band #2), respectively. Both sequences were identical when ClustalW aligned with the 163 bp sequence accounting for a complete 16S-23S intergenic spacer region. According to ClustalW analysis, rrnG, rrnH and rrnI were the closest homologous sequences being 93% identical when an alignment of all ten 16S-23S intergenic spacer regions of *B. subtilis* 168 was compiled. The electropherogram of sequence #1 could not be evaluated due to multiple signals overlapping after 74 nucleotides.

The three amplification products derived from *A. flavithermus* strain C are shown in Figure 8.2 (track 1, band #1, #2 and #3). Unfortunately, several attempts to acquire sequence information for band #1 and #2 were unsuccessful. Nevertheless, the electropherogram of both bands showed repeatedly proper nucleotide signal separations for the initial 130 nucleotides which were derived from the upstream located 16S rRNA genes (Figure 8.5). The sequence results obtained for band number #3 demonstrated that at least one ribosomal spacer from *A. flavithermus* strain C was successfully sequenced over a length of 248 bp. Interestingly, although the length of this sequence suggests it being of type rrnA or rrnO of *B. subtilis* 168, a sequence alignment (ClustalW) showed only a low score of 6% identity. The sequence results of *A. flavithermus* strain D derived from DNA recovered from bands #1, #2, #3 and #4 (Figure 8.2; track 3) were similar to those of *A. flavithermus* strain C. The sequence acquisition originating from bands numbered #1, #2 and #3 were successful only for a short segment of the 16S rRNA 5'-end (Figure 8.5). The nucleotide sequence derived from band number #4 was the only one to contain a complete intergenic spacer region and had a score to 90% (248 bp) with sequence #3 of *A. flavithermus* strain C.

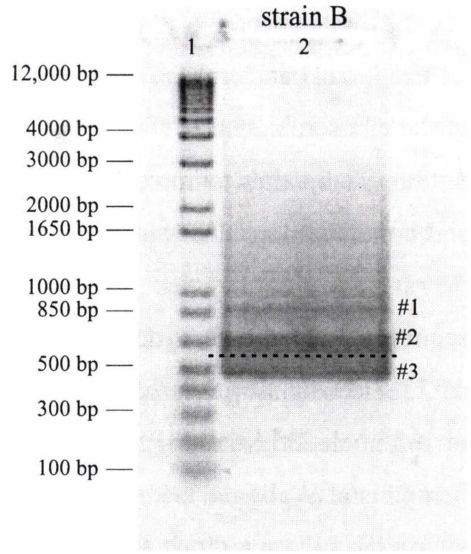

Figure 8.3. PCR amplification of the 16S-23S intergenic spacer regions of *A. flavithermus* (strain B). Primary amplification products, which were used for sequencing, are numbered at the right margin of each band of interest. DNA ladder (1 kb Plus DNA Ladder; GIBCO BRL, Life Technologies) in track 1 denotes the molecular size marker. The marker sizes are indicated in the left margin. The dashed line indicates the separation between band 2 and 3.

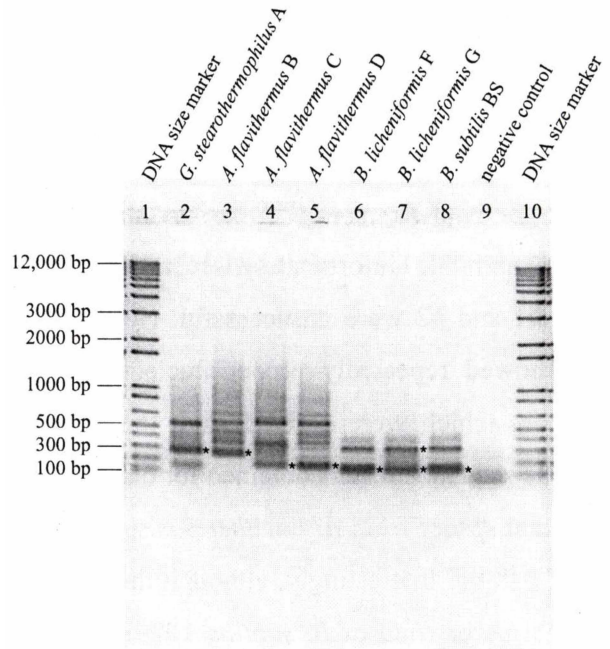

Figure 8.4. Amplification of small regions of the 16S-23S intergenic spacer region of the dairy bacilli. The amplicons which were expected according to the sequence results (Table 8.1) are indicated with an asterisk on the right side of each band. DNA ladder (1 kb Plus DNA Ladder; GIBCO BRL, Life Technologies) in tracks 1and 10 denotes the molecular size marker. The marker sizes are indicated in the left margin.

The amplification of *A. flavithermus* strain B produced three distinct primary bands (Figure 8.3; #1, #2 and #3; track 2) of approximately 850 bp, 650 bp and 500 bp, respectively. As with the previously described sequences, attempts to obtain sequence from the samples numbered #1 and #2 failed due to multiple sequence interference occurring for sequences following the 16S rRNA gene at the 5'-end.

Figure 8.5. ClustalW (1.83) multiple sequence alignment of 16S-23S intergenic spacer sequences of (A) *G. stearothermophilus*, (B, C and D) *A. flavithermus*, (F and G) *B. licheniformis* and (BS) *B. subtilis*. The strain nomenclature and the number of sequences correlating to the bands from Figure 8.1, 8.2 and 8.3 are indicated in the left margin. Sequences in grey letters belong to the 16S rRNA gene. The primer annealing sites for forward primer TN115 located at the 5'-end of the 16S rRNA gene and reverse primer RR167 for the designated quantitative PCR assay are highlighted in grey. "*" means that the residues are identical; "." means that semi-conserved substitutions are observed and sequence gaps are indicated by "-", primer orientations are indicated by ">" and "<", respectively.

Band number #3 of strain B was the only sample which produced valuable sequence information over 342 nucleotides revealing an intergenic spacer differing substantially in length from the 248 bp spacer of *A. flavithermus* strains C and D. Interestingly, these additional 94 nucleotides seems to constitute an extended segment at the 3'-end of the intergenic spacers of *A. flavithermus* strain B with the remaining 248 nucleotides well conserved towards strains C and D scoring 83 and 94%, respectively (Figure 8.5).

8.3.2. TaqMan probe design for real-time based PCR assay

The multiple sequence alignment from Figure 8.5 was used to design reverse primer RR167 that could theoretically serve as a TaqMan probe for quantitative PCR analysis. Within the newly acquired intergenic spacer sequences only one region constituted sufficiently high sequence conservation to be considered as a priming site. This site (RR167) is 19 nucleotides in length and contains one cytosine/thymine substitution on the third position at the 5'-prime end (Figure 8.5).

8.3.3. Amplification of small regions of the 16S-23S intergenic spacer region

The primer pair TN115 and RR167 was used to amplify small regions of the 16S-23S intergenic spacer in order to determine if both primers could be used to establish a quantitative real-time PCR. Table 8.1 shows the theoretical molecular sizes of the intergenic spacer amplicons for each *Bacillus* strain according to the sequencing results from Figure 8.5. The results obtained with both primers on DNA from the seven strains are illustrated in the agarose gel in Figure 8.4. The primer pair amplified multiple PCR products for all samples. In addition, the amplicons anticipated in Table 8.1 were also amplified as shown Figure 8.4. These amplicons are indicated with an asterisk on the right side of each predicted band.

Table 8.1. Expected 16S-23S intergenic spacer PCR amplification bands (bp) according to sequence results

	Bacillus strain						
Amplification band	A	B	C	D	F	G	BS
1	269	254	159	159	136	136	135
2	-	312	-	-	-	-	-

8.3.4. Quantitative real-time PCR with SYBR Green

A quantitative real-time PCR experiment was performed with primers TN115 and TN116 amplifying the ribosomal intergenic spacer region between 16S- and 23S rRNA gene. The amplification plot and melting point analysis are shown in Figures 8.7, 8.8 and 8.9. The numerical results are shown in Table 8.2.

Table 8.2. Quantitative real-time PCR amplifying the 16S-23S intergenic spacer region

Sample	Strain	Threshold cycle number	End-point-fluorescence [units]	Melting Temperature [°C] 1	2	3	4
1	A	13.70	303.78	90.69	-	-	-
2	B	13.84	287.47	85.25	86.66	91.64	-
3	C	13.32	248.80	85.11	87.05	89.73	91.97
4	D	13.90	321.47	84.58	89.87	91.27	-
5	F	13.41	256.75	82.64	83.91	90.40	-
6	G	13.57	303.78	82.02	83.32	84.08	89.93
7	BS	14.20	286.42	81.79	82.90	90.08	-

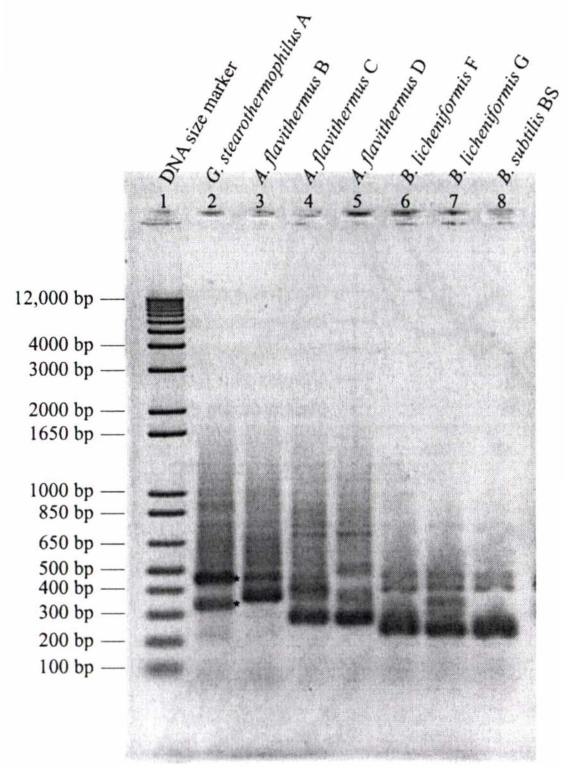

Figure 8.6. Agarose gel electrophoresis of 16S-23S intergenic PCR products amplified with the Smart Cycler II. Although *G. stearothermophilus* strain A produced at least two dominate amplification products (indicated with an asterisk) melting point analysis indicated that both bands possess near-identical melting point temperatures (Figure 8.9).

The amplification reaction of the seven thermophilic bacilli crossed the manual threshold line (set at 30) at a mean C_t of 13.71±0.3. The greatest threshold cycle discrepancy (delta C_t=0.88) was observed between *B. subtilis* and *A. flavithermus* strain C. Melting curve analysis performed on the completed PCR reactions resulted

in the acquisition of three or four melting peaks for each of the samples except for *G. stearothermophilus* strain A for which only one single melting peak was obtained at 90.69°C (Figure 8.9).

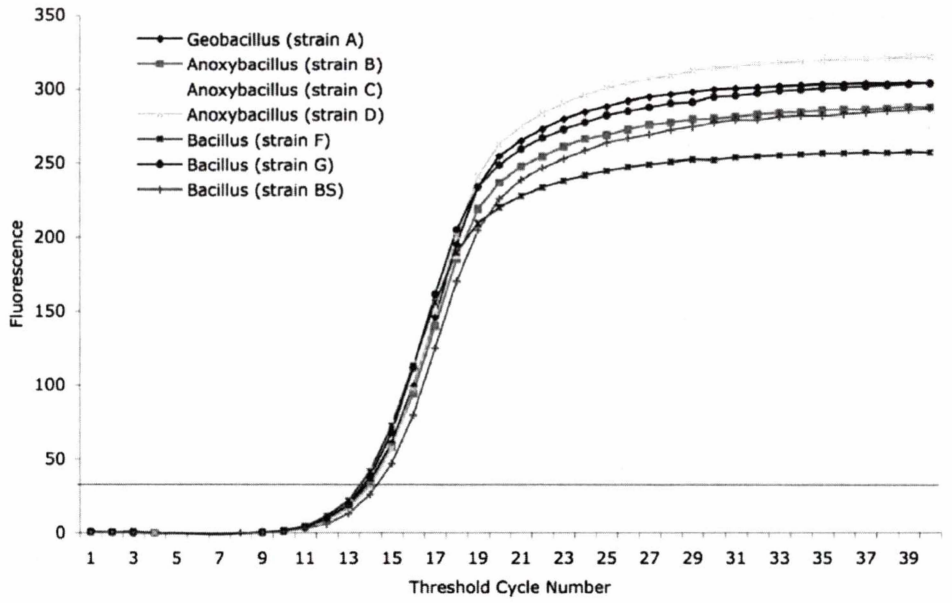

Figure 8.7. Amplification plot of quantitative real-time PCR on the Smart Cycler II targeting the intergenic spacer region of *G. stearothermophilus* A, *A. flavithermus* (strains B, C and D), *B. licheniformis* (strains F and G) and *B. subtilis* BS. The PCR reactions were performed under identical conditions using 1.5 ng of genomic DNA as initial template.

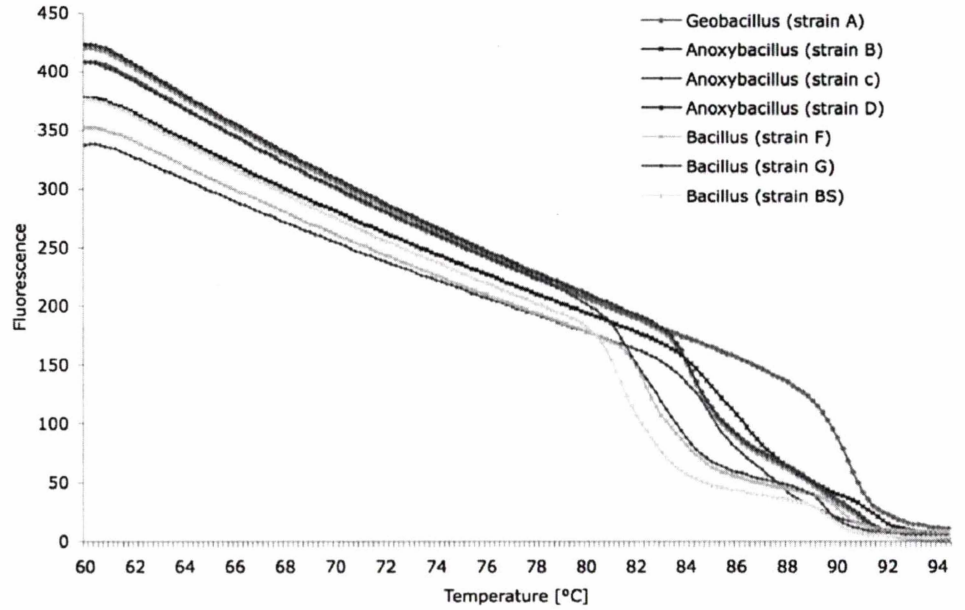

Figure 8.8 Melting curve analysis of the 16S-23S intergenic spacer amplification products. The graph shows the melting curves derived by increasing temperature over the range 60°C to 95°C with a temperature transition of 0.2°C s^{-1}. The curves contain more than one melting domain due to multiple amplification products generation different size and sequence.

Figure 8.6 shows the amplification reactions from Figure 8.7 and Table 8.2 separated through agarose gel-electrophoresis. The gel shows that all reactions contain multiple amplification products including strain A.

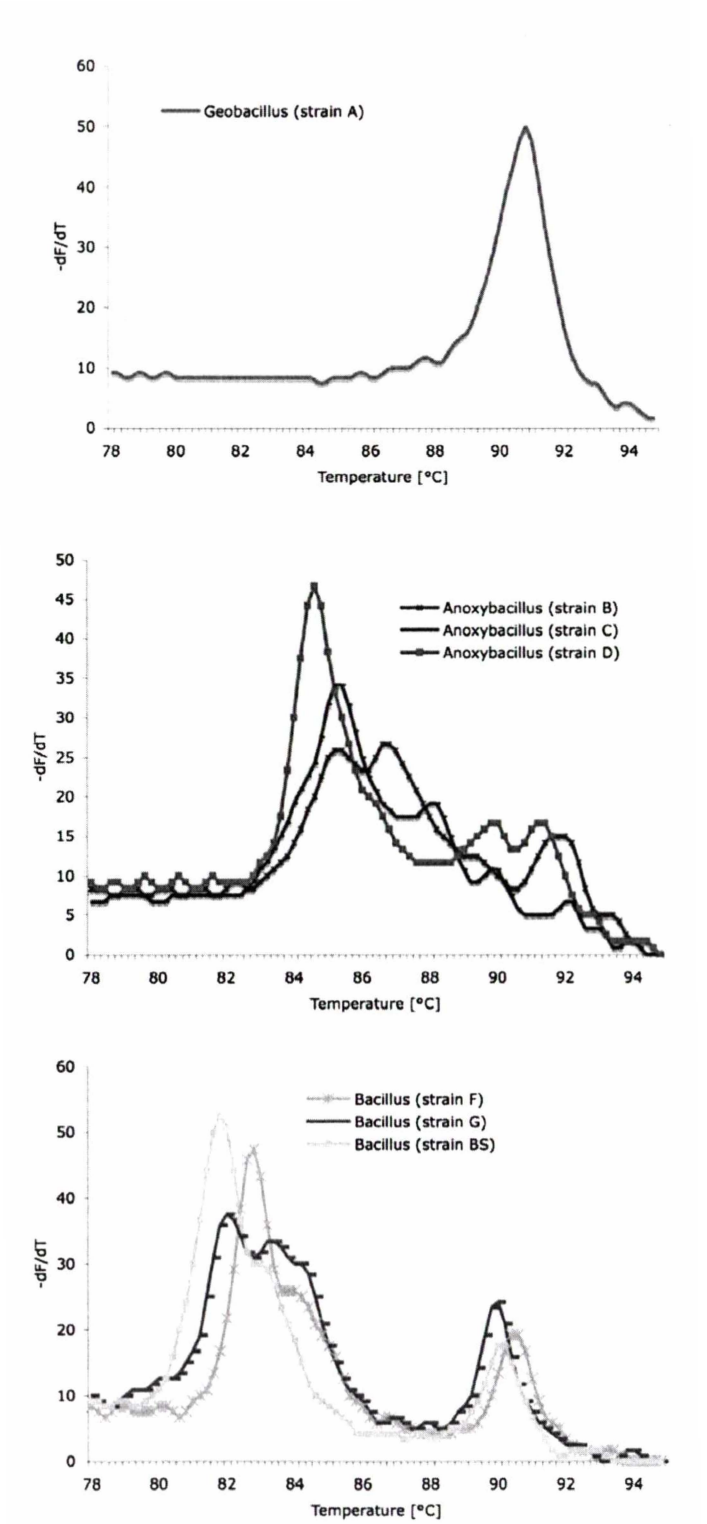

Figure 8.9. Melting peak analysis of the 16S-23S intergenic spacer amplicons derived from the negative first derivative of the melting curve fluorescence plotted over the temperature (-dF/dT versus T).

This was surprising since strain A produced only one melting peak (Figures 8.8, 8.9 and Table 8.2), despite of the two prominent PCR products indicated with asterisks in Figure 8.6. The only explanation that can be drawn from this result is that both PCR products possess near-identical melting temperatures resulting in one melting peak.
The melting point analysis on the 16S-23S intergenic amplicons of the three strains of *Anoxybacillus* showed near similar melting profiles (Figure 8.9; Table 8.2). Interestingly, the banding profile of strain B in Figure 8.6 (lane 3) shows two major PCR products at approximately 390 bp and 480 bp. In contrast, the corresponding melting profile of *Anoxybacillus* strain B in Figure 8.9 shows at least three distinctive peaks. Thus, one of the two bands in Figure 8.6 (lane 3) must consist of two different amplicons of similar molecular size but distinctively different melting temperatures. Similar observations were made for *B. licheniformis* (strains F and G) and *B. subtilis*, which had nearly identical profiles.

8.4. Discussion

The aim of the present study was two-fold, first to corroborate the RAPD-based strain groupings and more importantly to examine if the internal ribosomal spacer region was suitable to establish a quantitative real-time PCR assay for the discrimination and enumeration of the seven thermophilic milk powder bacilli. For these purposes, the 16S-23S intergenic spacers of all strains were amplified using a primer pair targeting the up- and downstream located 16S- and 23S rRNA genes. Priming these conserved regions will consequently result in the generation of amplicons including all possible ribosomal spacers located in the genome. However, agarose gel electrophoresis is only capable of separating amplicons by molecular size and is incapable of distinguishing sequence diversities as can be achieved by DGGE (Chapter 6). Thus, heterogeneous amplicons with similar size will appear as one individual band on the agarose gel and these amplicons will be recovered and purified simultaneously from the agarose gel. Consequently, when a sample containing multiple amplification products is subjected to sequencing it will generate raw data with multiple signal peaks overlapping and/or signal peaks, which are not evenly spaced as was observed for the majority of samples. However, two observations made in this investigation showed that the intergenic spacer of the seven thermophilic *Bacillus* strains occurs with substantial differences in both sequence and length.

Firstly, the gels from Figures 8.1, 8.2, 8.3 and 8.4 demonstrate that multiple amplification products have been generated for all strains indicative of the presence of internal ribosomal spacers with different sizes. Secondly, most of the samples produced good sequence reads for the first 130 nucleotides, which belong to the 16S rRNA gene, with the sequencing signals subsequently being interrupted abruptly for sequence down-stream indicating heterogeneous sequences.

Despite the reported length and sequence diversity within the 16S-23S intergenic spacers there was one well-conserved region of 19 nucleotides found in the new sequences, which appeared in all seven strains (Figure 8.5; priming site of RR167). Although only a limited number of intergenic spacers were sequenced there was evidence that this segment of conservation was ubiquitously present in many *Bacillus* species with, however, some degree of degeneracy (data not shown). For instance, 16S-23S intergenic spacer sequence alignments showed that this short region is identical in *B. licheniformis* (strains F and G), *B. subtilis* (BS), *Bacillus atrophaeus*, *Bacillus megaterium*, *Bacillus mojavensis*, *Bacillus pumilus*, and in addition, to all ten ribosomal spacers of *B. subtilis* strain 168. There was a single nucleotide substitution observed for sequence of *G. stearothermophilus* (strain A) and *A. flavithermus* (strains B, C and D) taking place at the third position at the 3'-end. Single and multiple nucleotide substitution were also found for *Bacillus sphaericus*, *Bacillus lentus*, *Bacillus flexus, Bacillus badius*, *Bacillus macroides* and *Bacillus circulans*.

Nevertheless, the primary goal of this study was to investigate whether the 16S-23S ribosomal spacer region could be utilized to quantitatively target the thermophilic milk powder bacilli. The answer to this question, unfortunately, was not pursued to its full extent due to insufficient time and funding restrictions. However, based on the data obtained, the 16S-23S rRNA intergenic spacer region would theoretically be well suited to develop a TaqMan-based assay using the described interior conserved sequence as a target for a TaqMan-probe with primers targeting the 16S- and 23S rRNA genes. And although this TaqMan-assay would generate PCR products of different lengths each amplification cycle would produce only one fluorescence unit per intergenic region, in the same way that, other assays produce identical sized amplicons. Furthermore, it could be shown that despite the generation of multiple amplification products for each strain during PCR the 16S-23S intergenic spacer may also be well suited as a target for a SYBR Green-based assay (Figure 8.6,

8.7 and 8.8). When 1.5 ng of genomic DNA from all seven thermophiles was used for quantitative PCR analysis then all samples responded at similar threshold cycle numbers. This was somewhat surprising, as the 16S-23S intergenic amplification products of all seven thermophiles are quite diverse with respect to their length, and since the amount of SYBR Green that can intercalate to DNA is a function of the amplicon's length (Ririe, 1997) PCR products of different length should produce different amounts of fluorescence emission. However, this was not observed and probably, the absolute amount of DNA generated during PCR was of similar magnitude among all seven strains. The current SYBR Green based-assay has another advantage as it can assist to discriminate *B. licheniformis* strain F from *G. stearothermophilus* strain A. Both strains were indistinguishable by *spo0A* quantitative PCR-assay (Chapter 4) due to near-similar melting temperatures. Thus, the 16S-23S intergenic spacer PCR assay and the *spo0A* PCR assay in conjunction can help to distinguish the three major thermophilic contaminants, e.g. *A. flavithermus* strain C, *B. licheniformis* strain F and *G. stearothermophilus* strain A (Chapter 2).

An Enzyme-linked immunosorbent assay for the detection of thermophilic bacilli

9.1. Introduction

Immunochemical methods have been used in various applications in basic research, veterinary and human medicine, agricultural areas and also for environmental and food analysis (Franek and Hruska, 2005). Generally, immunoassays such as the Enzyme Linked Immunosorbent Assay (ELISA) make use of the principal function of the immune system, specificity for particular antigens, to protect animals from infectious organisms or foreign molecules. This system produces antibodies, which travel constantly through the blood and lymphatic system of mammals in order to bind and destroy antigens. Two types of antibodies are used for research and diagnostic analyses, i.e. polyclonal and monoclonal antibodies. Polyclonal antibodies are generally produced by injecting a suitable animal (horse, rabbit, goat, sheep or pig) with the target antigen initiating an immune-response in the animal. However, polyclonal antibodies consist of many different clones of lymphocyte populations each of them carrying a different antigen receptor directed against a particular part (epitope) of the antigen. Monoclonal antibodies are derived from a homogeneous population of differentiated lymphocyte cells, which were fused to immortal myeloma cells to form hybridoma cells which can be grown indefinitely *in vitro* (Köhler and Milstein, 1975). Myeloma is a type of a cancer that leads to the uncontrolled and permanent cell division of the immunoglobulin producing plasma cells located in the bone marrow. Myelomas used to produce hybridomas have been selected from cells which have lost the cellular machinery necessary for the secretion of immunoglobulins (Harlow and Lane, 1988). Hybridomas produce a monoclonal antibody with specificity for only a single antigen epitope.

Several immunosorbent assays using either polyclonal or monoclonal antibodies have been used for the detection and quantification of micro-organisms in dairy food systems either targeting vegetative cells and/or spores. In particular the

psychrotrophic and endospore-forming pathogenic *B. cereus* has been used in several food-related studies as a target organism (Koo et al., 1998; Torkar and Mozina, 2000). For instance, Charni et al. (2000) produced two monoclonal antibodies against vegetative cells of *B. cereus*, which were used for ELISA. The monoclonal antibodies did not recognize spores from the same strain but showed high specificity against vegetative cells of *B. cereus*, *Bacillus thuringiensis* subsp. *berliner*, *B. thuringiensis* subsp. *kurstaki*, *B. thuringiensis* DSM 2045, *B. thuringiensis* subsp. *israelensis* and *Bacillus mycoides* originating from food and environmental samples. Quinlan and Foegeding (1997) produced several monoclonal antibodies against bacterial spores of *B. cereus* T and *Clostridium sporogenes* with quite different specificities. Some of the antibodies were highly selective against spores only whereas others showed cross-reactivity against the vegetative forms and spores of *B. subtilis*, *B. megaterium*, *B. stearothermophilus*, *Clostridium perfringens* and *Desulfotomaculum nigrificans*. Polyclonal antibodies have also been produced for the detection of a broad range of bacterial spores by enzyme-linked immunosorbent assays (Foegeding and Chang, 1993). Polyclonal and monoclonal antibodies raised against *Bacillus globigii* and/or *Bacillus anthracis* spores were as selective as the studies mentioned above in that no single antibody either poly- or monoclonal was completely specific in recognizing only the target organism, and all antibodies produced showed cross-reaction to surface epitopes of other *Bacillus* species (Longchamp and Leighton, 1999 and 2000). Blake and Weimer (1997) employed polyclonal antibodies attached to magnetic beads to quantify *Bacillus stearothermophilus* spores in complex food and environmental samples with an upper and lower limit of detection of 8×10^5 and 8×10^3 spores ml^{-1}, respectively.

In the present study, four polyclonal antibodies were produced which were raised against vegetative cells of the dominant thermophilic bacilli commonly found to contaminate milk powder. The antibodies were used to develop ELISA assays for the selective detection and enumeration of these organisms by Western dot blotting. Furthermore, the feasibility of the immunoassays for the detection, identification and enumeration of the thermophilic flora in milk powders was investigated and the results compared to other molecular methods such as RAPD-PCR and DGGE-PCR.

9.2. Materials and Method

9.2.1. Bacterial strains and preparation of cultures

The strains of thermophilic bacilli used in this investigation were previously isolated by Ronimus et al. (2003) from factory milk powder samples in New Zealand. These strains included *Geobacillus stearothermophilus* (strain A), *Anoxybacillus flavithermus* (strains B, C and D), *Bacillus licheniformis* (strains F and G) and *Bacillus subtilis* (BS). The non-thermophilic bacterial cultures, e.g., *Lactobacillus plantarum* DSM 20205 (NCIB 6376, ATCC 8014), *Pseudomonas aeruginosa* DSM 5168, *Streptococcus thermophilus* DSM 20479 (NCIB 8779), *Streptococcus uberis* NZRM 2266, *Escherichia coli* DSM 301 (ATCC 4157) and *Micrococcus luteus* (DSM 2786) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ-GmbH, Braunschweig, Germany). All cultures were grown overnight in tryptic soy broth (TSB) supplemented with 0.2% (w/v) soluble potato-starch with all seven strains of thermophilic bacilli incubated at 55°C and all non-thermophilic strains incubated at 37°C with exception of *L. plantarum* which was grown at 30°C.

After overnight growth, the cultures were harvested by centrifugation at 9,000 *g* (5415 D, Eppendorf) and washed in 0.9% NaCl by two repetitions of re-suspension and centrifugation. During these procedures the bacterial cultures were kept cold using an ice bath and refrigerated centrifuge.

Spores of thermophilic bacilli were produced in liquid Castenholz medium (Appendix 12.1.) by incubation of cultures at 55°C for at least 48 hours. The extent of sporulation was checked by phase-contrast microscopy (1000× magnification) and the spores harvested by centrifugation. The cell pellet was washed with sterile deionised water followed by centrifugation at 16,100 *g*. The spore suspensions were further washed twice with 0.9% NaCl by vortexing and centrifugation at 10,000 *g* and the supernatant discarded. The samples were then treated with 2 mg ml^{-1} of lysozyme (Sigma, L7001) at 37°C for 30 minutes to lyse vegetative cells. After lysis, washing with 0.9% NaCl was repeated with two repetitions. The spore preparations were stored in sterile deionised water at 4°C. Spore and vegetative cell counts were conducted by phase-contrast microscopy using a Thoma counting Chamber.

9.2.2. Immunodetection of thermophilic bacilli in milk powder

Bacterial cells were extracted from milk powder by the method described in Rueckert et al. (2005a) (Chapter 3). After extraction, the cell pellets were resuspended in 50 µl of sterile deionized water and the sample immediately used for dot blotting.

9.2.3. Polyclonal Antibody production

Polyclonal antibodies were separately produced at AgResearch (Hamilton, New Zealand) against vegetative cells of *G. stearothermophilus* strain A (F13), *A. flavithermus* strain C (G37), *B. licheniformis* strain F (C45) and a mixture of *B. licheniformis* (strains F and G) and *B. subtilis* (C55). For this purpose, laboratory rabbits were given injection using 10^7 cells of each preparation (in one ml volume) three-times at 15 day intervals. The antibodies were supplied as serum in 15 ml tubes.

9.2.4. Purification and desalting of immunoglobulin from rabbits

The antibodies were desalted and concentrated by a modified method as described by Harlow and Lane, (1988). For this purpose, sera containing immunoglobulin G (IgG) were centrifuged at 4°C at 3000 *g* for 30 minutes (Sorvall® RC 26 Plus) to separate any precipitates. Subsequently, the supernatants were transferred to sterile tubes with each tube containing a sterile magnetic stirrer. Slowly and consistently a saturated ammonium sulfate ($(NH_4)_2SO_4$) solution at 4°C was added to the serum while stirring to obtain a 25% (v/v) saturated salt solution. The samples were then stored at 4°C overnight to allow precipitation. The following day, the precipitate was separated from the serum by centrifugation at 3000 *g* for 30 minutes and the supernatant transferred to a new tube. The immunoglobulin solutions were then precipitated by adding saturated ammonium sulfate to the sera to obtain a final salt saturation of 45% (v/v). Precipitation and separation of the antibodies was carried out as previously. The precipitate was resuspended in phosphate-buffered saline (PBS; 140 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4; pH 7.2) and stored at 4°C.

The concentrated immunoglobulin solutions were desalted by dialysis using two separated changes of 1000 ml PBS at 4°C for at least 12 hours under gentle

agitation. For this purpose, the sera were transferred into dialysis bags which were sealed with a plastic clip and dipped into 5 liter beakers containing the PBS solution. Following dialysis the immunoglobulin solutions were transferred to sterile tubes and stored at 4°C.

The protein concentration of the second immunoglobulin fraction was determined by spectrophotometer (Pharmacia Biotech, Ultraspec 3000) readings as described by Bradford et al. (1976). For this purpose, 100 µl of protein solution was mixed to 1000 µl of 1:5 Bradford solution (50 mg Coomassie Blue G-250, 100 ml 85% H_3PO_4, 850 ml sterile deionized water and 50 ml ethanol) and the absorption measured at λ=595 nm. A standard curve of two-fold dilutions of bovine serum albumin (BSA) ranging from 1.0×10^{-1} to 3.12×10^{-3} mg ml^{-1} was used to calculate the protein concentration of the immunoglobulin fractions.

9.2.5. Dot blotting and immunoassay detection

Dot blotting and immunoassay detection employed the ECL Plus Western blotting kit (Amersham Pharmacia Biotech; RPN 2132). Vegetative cell suspensions were fixed onto a Hybond™ ECL™ nitrocellulose membrane (Amersham Pharmacia Biotech; RPN303D) and the cells labelled with the primary antibodies, e.g. either F13, G37, C45 and/or C55. The kit contains a secondary antibody immunoreaction conjugated with Lumigen PS-3, which is oxidized by horseradish peroxidase, generating an acridinium ester which further reacts with peroxide to produce a sustained chemiluminescence with maximum emission at a wavelength of 430 nm. Accordingly, the intensity of chemiluminescence released by a dot blot for a given antibody and strain is direct proportional to the cell quantities used for blotting.

Dot blotting of was carried out using the following protocol: bacterial strains used in this investigation were grown overnight, harvested, washed and the cell density determined as described above. Subsequently, the cultures were usually $1:10^{½}$ serially diluted (changes otherwise indicated in the text) and 2 µl of each dilution blotted onto the Hybond nitrocellulose membrane, and the membrane allowed to dry onto 3MM chromatography paper (Whatman). Prior to blotting the membrane had been pre-wetted by submersing in distilled water and equilibrating in PBS buffer (10 minutes each). The membranes were then immersed and incubated at room temperature for 1 hour on an orbital shaker in 20 ml of 5% (w/v) reconstituted non-fat

dried milk containing 0.1% (v/v) Tween 20 in PBS buffer to block all non-specific binding sites. The membranes were then rinsed twice for 2 minutes with several hundred milliliters of PBS-Tween 20 buffer (0.1% Tween 20 (v/v) in PBS buffer). Following washing the membranes were incubated with the primary antibodies diluted to 1:200 (unless otherwise stated in the text) in PBS-Tween 20 for 1 hour at room temperature on an orbital shaker using sufficient PBS-Tween 20 buffer to guarantee that the blots were completely covered. Subsequently, the membranes were rinsed with two changes of PBS-Tween 20 buffer, washed in the same buffer for 15 minutes and again rinsed with three changes of PBS-Tween 20 buffer for 5 minutes at room temperature. The membranes were then incubated with the secondary antibody (anti-rabbit Ig, horseradish peroxidase linked whole antibody from donkey; Life Science; NA 934) diluted 1:3000, and following the same procedure used as was applied for the primary antibodies. After dot blotting the membranes were placed onto a 3M chromatography paper sheet with the bacterial spots in the up position.

Immunodetection was carried out immediately after dot blotting according to the instruction manual of the ECL Plus Western blotting kit (Amersham Pharmacia Biotech) in a completely dark room. The detection reagents stored at 4°C were allowed to equilibrate to room temperature and the detection solutions A and B mixed in a ratio 40:1. The final volume of detection reagent required was at least 0.1 ml cm^{-2} of membrane. The membranes were placed cell side up onto foil wrap and the detection solution uniformly pipetted to cover the entire surface of the membrane, followed by incubation for 5 minutes at room temperature. Excess detection solution was then gently drained off by lifting a corner of the membrane with tweezers and the membranes placed in the luminescent image analyzer system (Fujifilm LAS-1000). The chemo-luminescence of the blots was captured using the automatic exposure time of the LAS-1000 system and the generated image stored as a digital computer file.

9.2.6. Evaluation of Dot-blots

Quantitative evaluation of the digitalized dot blots was performed on colour inversed computer images with the ImageJ 1.32j software package from Wayne Rasband (National Institute of Health, USA) available online at http://rsb.info.nih.gov/ij/. This software calculates the pixel density of a selected area in an image and creates a density histogram, which was used to determine the

antibody binding specificity towards selected microorganisms. Accordingly, the pixel density is a measure of the chemiluminescence released with areas of high pixel density correlating to high chemiluminescence intensity whereas a low pixel density correlates to low chemiluminescence output. The image files (TIF-format) of the immuno-blots were imported into the ImageJ software and the dilution serie of each single strain selected and analysed individually. The reverse dot plot values of each strain screened with a particular antibody were then retrieved from ImageJ and imported into Microsoft Excel 2002 from where they were joined to a data series. Subsequently, the data were then plotted with the line chart option of Excel whereas the values of the ordinate were plotted in reverse order against the dot blot positions.

9.3. Results

9.3.1. Antibody concentration and optimization

The protein content of the immunoglobulin fractions was determined by the Bradford method (Bradford et al., 1976). Total protein concentrations were 0.795 mg ml^{-1}, 0.925 mg ml^{-1}, 0.66 mg ml^{-1} and 0.56 mg ml^{-1} for antibody F13, G35, C55 and F45, respectively.

Three different antibody dilutions, e.g. 1:200, 1:2000 and 1:20000 were used to optimize the immuno-assay. Dot blotting was performed with serial dilutions of individual strains, i.e. *G. stearothermophilus* strain A (F13), *A. flavithermus* strain C (G37) and *B. licheniformis* strain F (C55 and F45). All three antibody dilutions produced positive immuno-responses for all strains of thermophilic bacilli at cell concentrations $\geq 10^5$ cells ml^{-1}. The lower limit of detection for antibodies varied significantly, with the concentration of 1:200 having the highest sensitivity and thus, this concentration was routinely used for all further antibody-assays.

9.3.2. Antibody specificity and sensitivity against vegetative cells of thermophilic bacilli from milk powder

The specificity and detection sensitivity of the antibodies was determined for each single antibody against serial dilutions of the seven strains of thermophilic

bacilli. The calculated cell numbers (from direct microscopic counts) used in the dot blotting are shown in Table 9.1.

Table 9.1. Cell numbers used for dot blotting (cell number dot^{-1})

Position	A	B	C	D	F	G	BS
1	6000	3800	2400	2800	4600	3000	3800
2	1900	1200	760	885	1450	950	1200
3	600	380	240	280	460	300	380
4	190	120	76	88	145	95	120
5	60	38	24	28	46	30	38
6	19	12	8	9	14	10	12
7	6	4	2	3	5	3	4
8	2	1	1	1	1	1	1

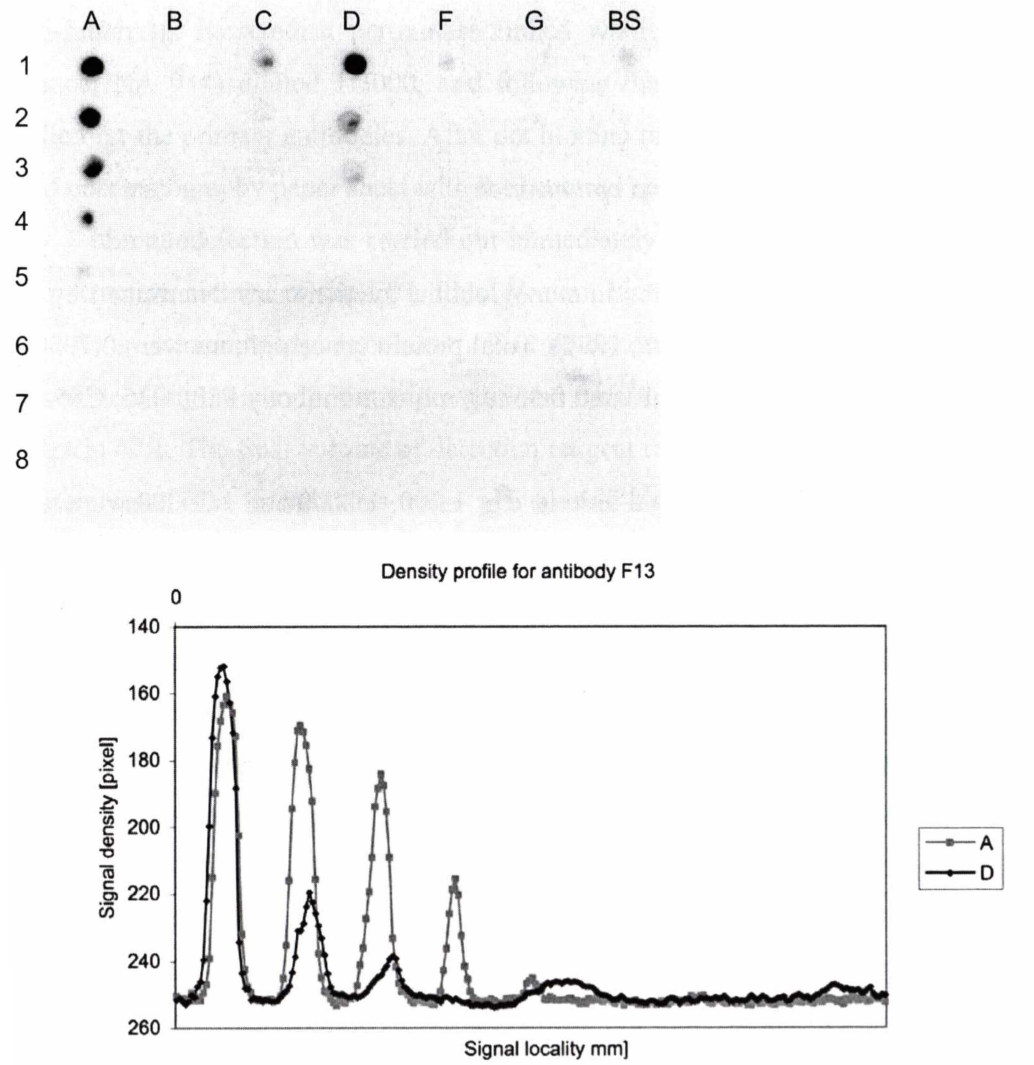

Figure 9.1. Dot blot and immuno-detection using the polyclonal antibody F13 produced against vegetative cells of *G. stearothermophilus* strain A. The specificity and sensitivity of F13 was checked against all seven milk powder bacilli. The density profile shows the evaluation of the dot blots using the ImageJ and Excel software.

The polyclonal antibody F13 produced against *G. stearothermophilus* strain A showed a high specificity towards its target strain with only *A. flavithermus* strain D also being detected to some extent (Figure 9.1). The immuno-detection assay using F13 as primary antibody was able to detect cells of strain A over a linear range of four dilutions with a lower detection limit of 9.5×10^4 cells ml^{-1} and an upper limit of 3.0×10^6 cells ml^{-1} (r = 0.977). *A. flavithermus* strain D was recognized over three dilutions covering the range from 1.4×10^5 to 1.4×10^6 cells ml^{-1}, but with a linear correlation coefficient (r) of 0.854. *A. flavithermus* strain C, *B. licheniformis* strain F and *B. subtilis* were also detected, however, the detection limit was more than an order of magnitude greater.

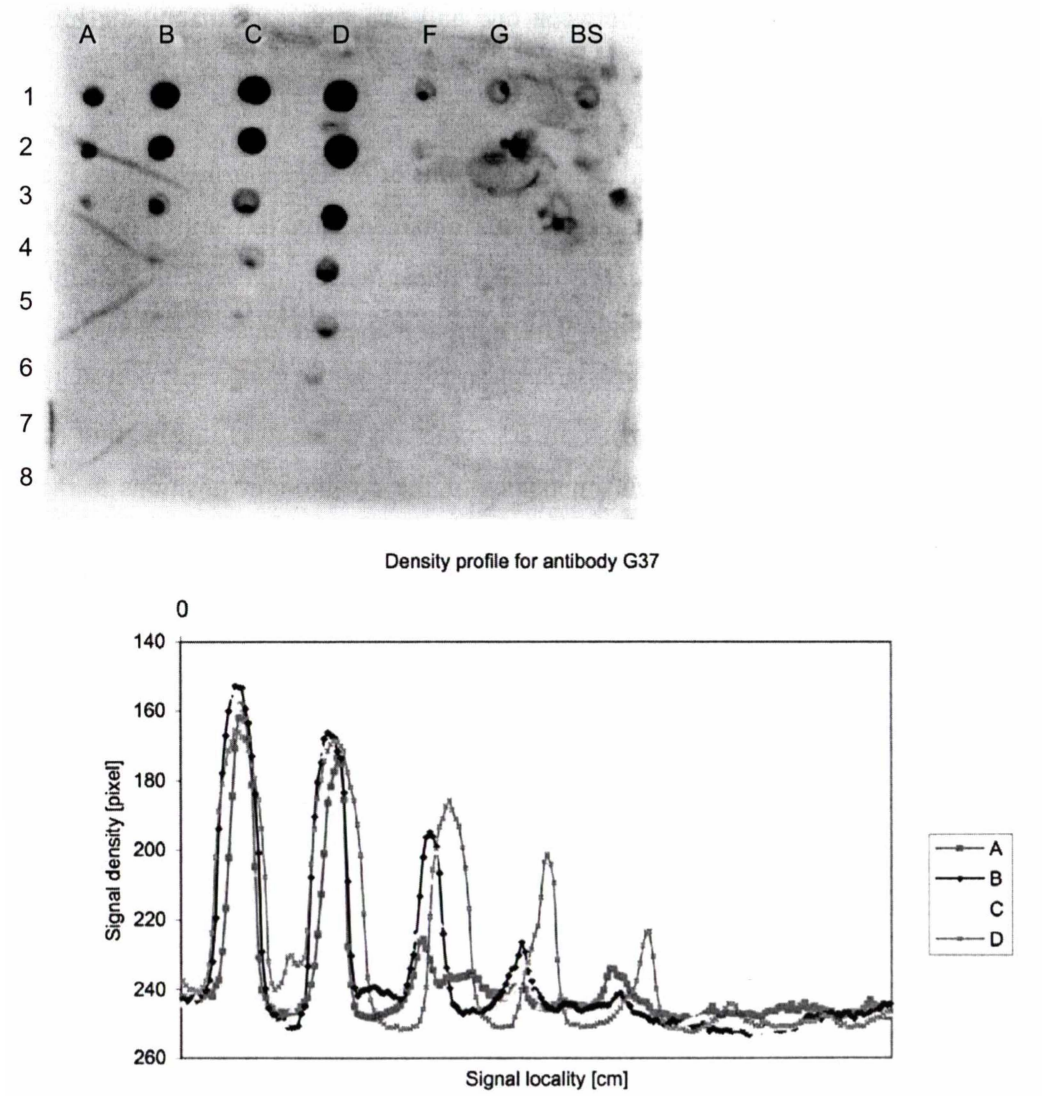

Figure 9.2. Dot blot and immuno-detection using the polyclonal antibody G37 produced against vegetative cells of *A. flavithermus* strain C. The specificity and sensitivity of G37 was checked against all seven strains of milk powder bacilli. The density profile shows the evaluation of the dot blot using the ImageJ and Excel software.

The antibody G37 produced against *A. flavithermus* strain C was quite specific for all three *Anoxybacillus* strains (B, C and D) as well as *G. stearothermophilus* strain A (Figure 9.2). The cell concentrations of strain D blotted onto the Hybond nitrocellulose membrane were linearly correlated to chemo-luminescence (r = 0.999) with a lower detection limit of 4.4×10^3 cells ml^{-1} and an upper limit of detection of 1.4×10^6 cells ml^{-1} between position 2 to 6 (Figure 9.2). The correlation for strain C was also linear (r = 0.989) with G37 over four dilutions from 4.1×10^4 cells ml^{-1} to 1.2×10^6 cells ml^{-1} (position 1 to 4) and strain B over five dilutions (r = 0.994) between 1.9×10^4 cells ml^{-1} to 1.9×10^6 cells ml^{-1} (position 1 to 5), respectively. *Geobacillus* strain A was positively detected by G37 over three dilutions ranging (r = 0.897) from 3.0×10^5 cells ml^{-1} to 3.0×10^6 cells ml^{-1}. *B. licheniformis* and *B. subtilis* were also detected but at levels between one and two orders of magnitude higher (Figure 9.2).

The antibody C55 was produced against cells of *B. licheniformis* (strains F and G) and *B. subtilis* BS. The immunoreactions obtained with this antibody showed highest specificity towards *B. subtilis* with a linear correlation of cell density to chemo-luminescence of r = 0.869. The detection ranged over three orders of magnitude from 1.9×10^3 cells ml^{-1} to 1.9×10^6 cells ml^{-1} (Figure 9.3). However, according to the density profile of strain BS some inconsistencies in pipetting have likely to be occurred as the signal intensities of the dot blots at positions 5 and 6 appear to be higher than those at positions 2 and 3 where the cell quantities are expected to be higher. This discrepancy was not perused further.

B. licheniformis strain F could be detected linearly (r = 0.988) with C55 over three dilutions from 2.3×10^5 to 2.3×10^6 cells ml^{-1} corresponding to position 1 to 3 in Figure 9.3.

The immuno-reaction of C55 towards *B. licheniformis* strain G was less specific compared to strains F and BS with only 1.5×10^6 cells ml^{-1} at position 1 in Figure 9.3 giving a detectable signal. Similarly, strain D of the *A. flavithermus* species was also found to bind to some degree with antibody C55 for cell densities of approximately 4.4×10^6 cells ml^{-1}. The remaining three strains, i.e. A, B and C, all produced a low level chemiluminescence at the highest cell density (position 1) possibly due to non-specific binding.

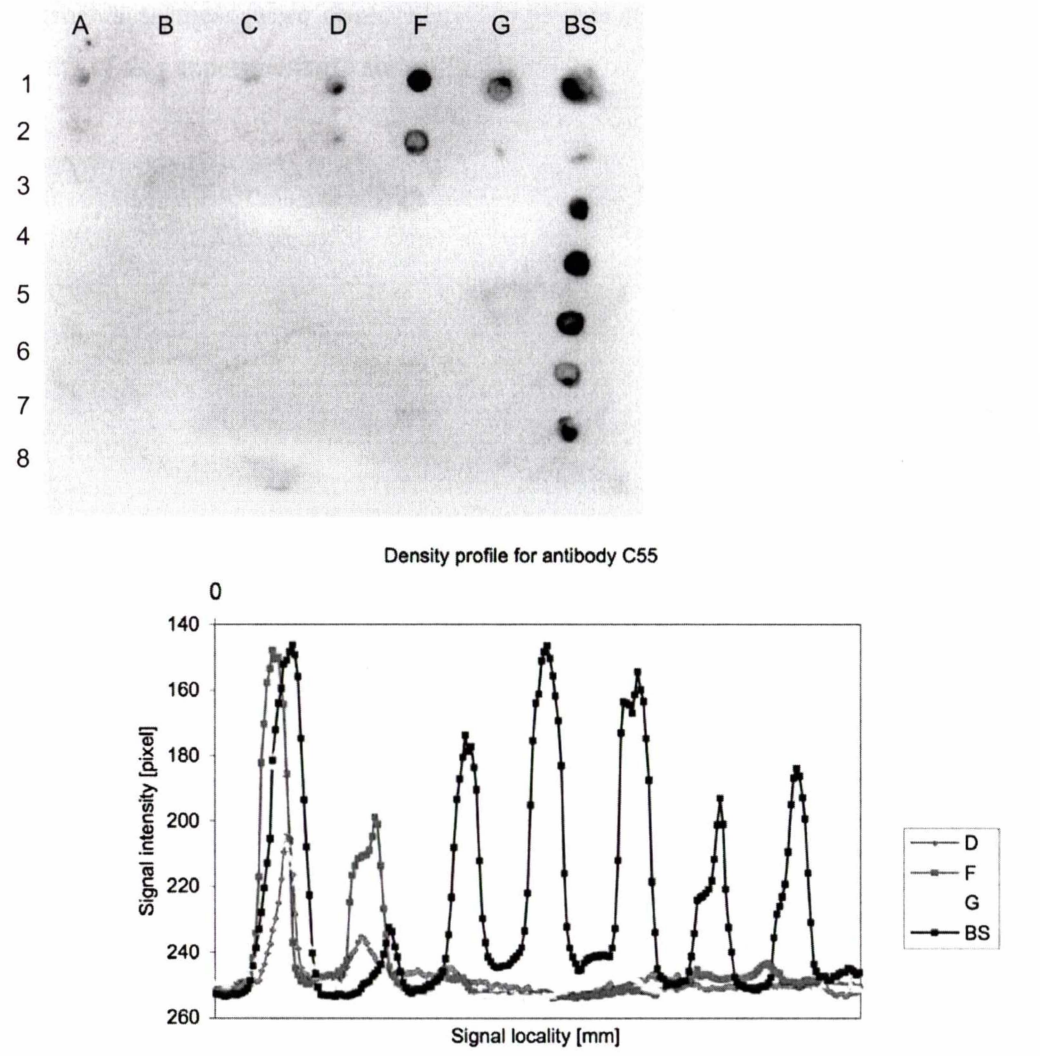

Figure 9.3. Dot blot and immuno-detection using the polyclonal antibody C55 produced against vegetative cells of *B. licheniformis* (strains F and G) and *B. subtilis* BS. The specificity and sensitivity of antibody C55 was checked against all seven strains of milk powder bacilli. The density profile shows the evaluation of the dot blot using the ImageJ and Excel software.

Figure 9.4 shows the immuno-blot performed with antibody F45 produced against vegetative cells of *B. licheniformis* F. Accordingly, F45 yielded highest sensitivity with strain F over three dilutions with a linear correlation of signal intensity to cell density of $r = 0.979$ ranging from 2.3×10^5 cells ml^{-1} to 2.3×10^6 cells ml^{-1} (position 1 to 3 in Figure 9.4). The lower limits of detection for *B. licheniformis* strain G and *B. subtilis* were 4.7×10^5 cells ml^{-1} and 6.0×10^5 cells ml^{-1}, respectively. According to the ImageJ software *A. flavithermus* D was the only organism of the remaining strains (strain A, B and C) giving a valid signal for 1.4×10^6 cells ml^{-1}.

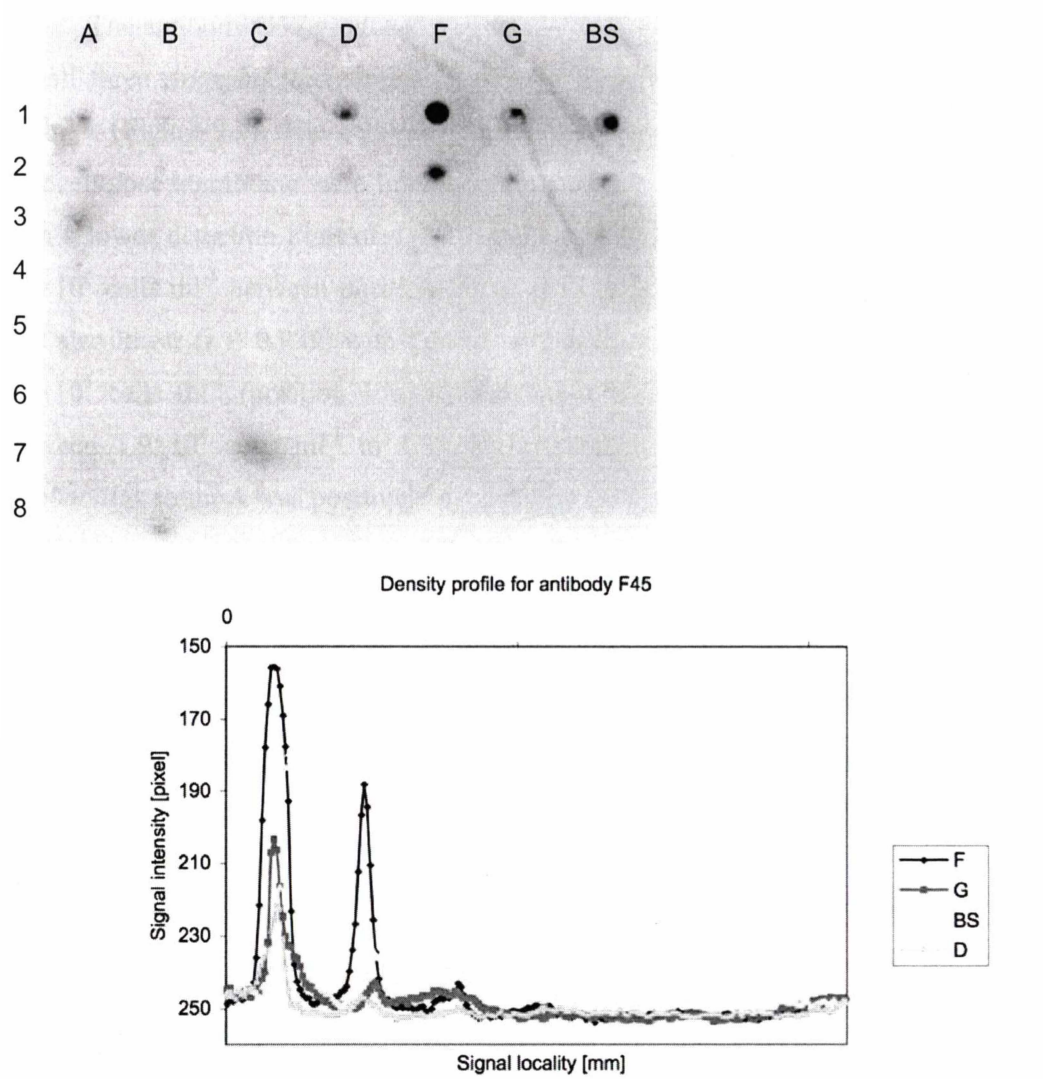

Figure 9.4. Dot blot and immuno-detection using the polyclonal antibody F45 produced against vegetative cells of *B. licheniformis* strain F. The specificity and sensitivity of the antibody was checked against all seven strains of milk powder bacilli. The density profile shows the evaluation of the dot blot using the ImageJ and Excel software.

9.3.3. Antibody specificity and sensitivity against spores of thermophilic milk powder bacilli

Spore preparations of all seven thermophilic bacilli were additionally exposed to an equally mixed suspension of all four antibodies, i.e. F13, G37, C55 and F45 in order to investigate their immuno-responses. Pure spore preparations were obtained for *G. stearothermophilus* strain A, *B. licheniformis* (strains F and G) and *B. subtilis*. However, the production of spores was incomplete for *A. flavithermus* strains B and C and failed completely for *A. flavithermus* strain D as assessed by microscopy. Furthermore, the protocol used was not capable to remove properly vegetative cell

fragments as these were observed under phase-contrast microscopy. However, the results of this experiment are shown in Figure 9.5.

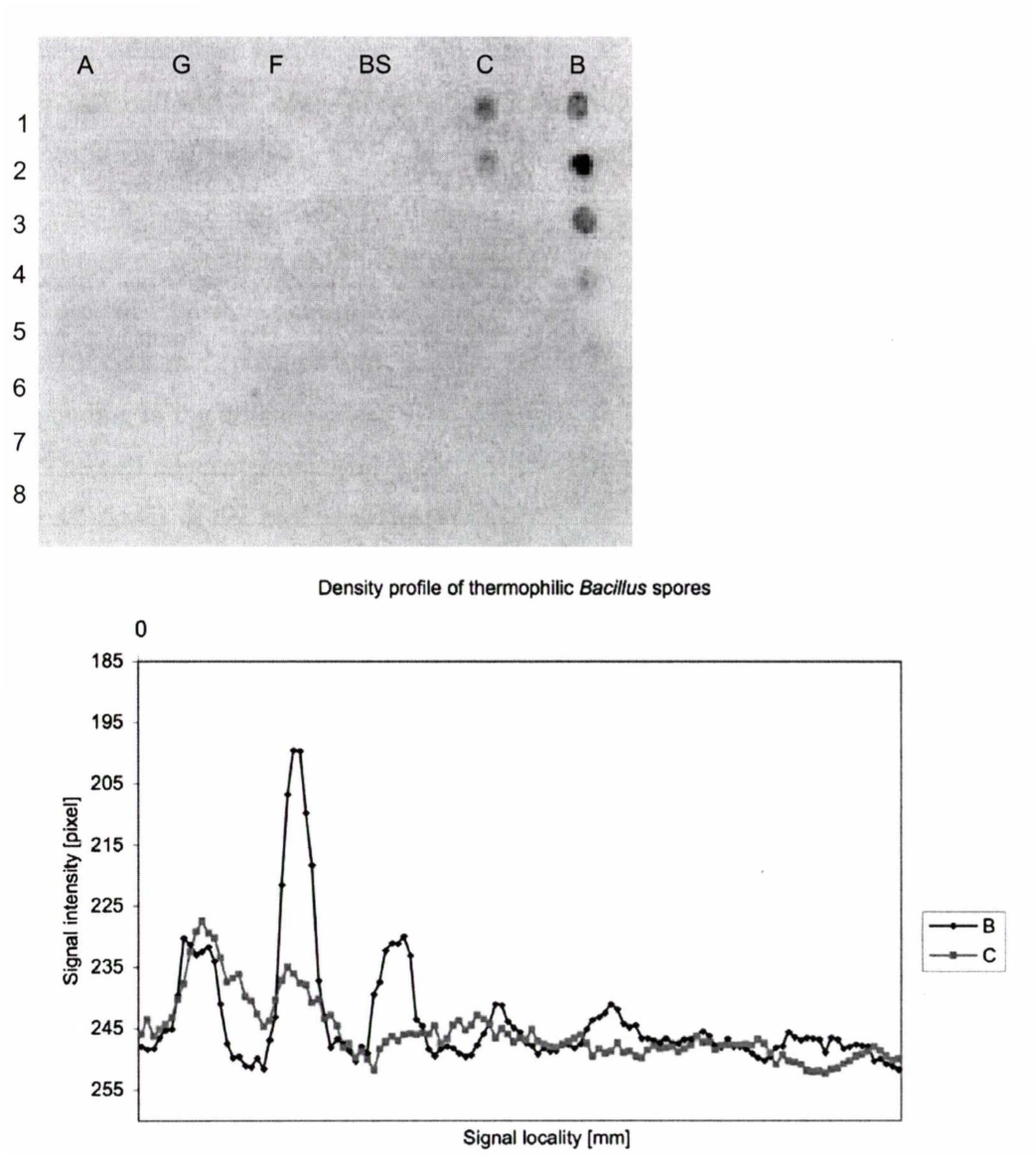

Figure 9.5. Dot blot and immuno-detection using all polyclonal antibodies produced against vegetative cells of thermophilic milk powder bacilli. The specificity and sensitivity of the immuno-reaction was checked against spores of the same milk powder bacilli. The density profile shows the evaluation of the dot blot using the ImageJ and Excel software.

The pure spore suspensions of *G. stearothermophilus* strain A, *B. licheniformis* (strains F and G) and *B. subtilis* BS were all negative for the immuno-assay with no chemiluminescence being recorded. A different situation was found for *A. flavithermus* strains B and C with both organisms producing positive signals. According to the dot blot result in Figure 9.5 the lower limit of detection for strains B and C was 2.8×10^{4} spores ml^{-1} and 9.4×10^{6} spores ml^{-1}, respectively. However, it

cannot be excluded that the positive immuno-response of these samples was rather due to the presence of vegetative cell fragments than due to spores.

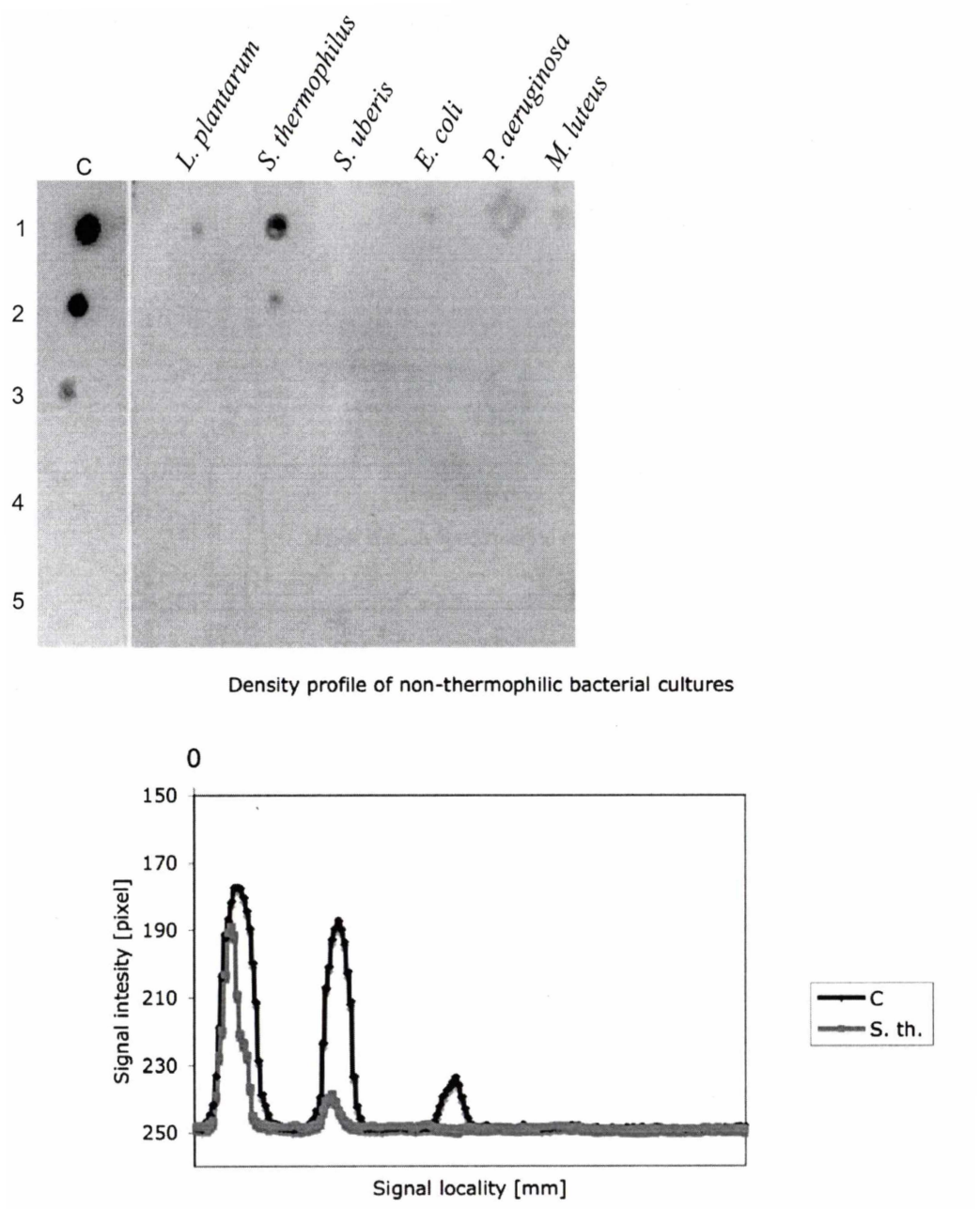

Figure 9.6. Dot blot and immuno-detection using all polyclonal antibodies produced against vegetative cells of selected thermophilic milk powder bacilli. The specificity and sensitivity of the immuno-reaction was checked against non-*Bacillus* cultures and *A. flavithermus* strain C as positive control. The density profiles show the evaluation of the dot blot using the ImageJ and Excel software.

9.3.4. Antibody specificity and sensitivity against cultures other than bacilli

The four antibodies were further tested for their recognition capacity against cultures other than bacilli, e.g. *L. plantarum*, *P. aeruginosa*, *S. thermophilus*, *S. uberis*, *E. coli* and *M. luteus* and the results are shown in Figure 9.6. For this purpose, the antibodies F13, G37, C55 and F45 were equally mixed and dot blotting and immuno-detection performed on 10-fold dilutions of cultures blotted onto the Hybond membrane as described in the method and materials section. *A. flavithermus* strain C was used as a positive control which could be detected at concentrations of 6.6×10^5 to 6.6×10^7 cells ml^{-1} (position 1 to 3). *S. thermophilus* was the only non-*Bacillus* species responding to the immuno-assay for cell concentrations $\geq 8.0\times10^6$ cells ml^{-1} (position 2). The cell concentrations (ml^{-1}) for *L. plantarum*, *P. aeruginosa*, *S. uberis*, *E. coli* and *M. luteus* at dot blot positions 1 were 1.38×10^7, 9.6×10^7, 4.0×10^7, 2.4×10^7 and 4.6×10^7, respectively.

9.3.5. Practical application of the immunoassays on milk powders

Dot blotting and immunodetection was applied to three high count skim milk powders (previously used in Rueckert et al. 2004, Chapter 2) to investigate the potential of the assay to detect the native flora of bacilli in powders. These powder samples were from USA (A), Great Britain (B) and Canada. The milk powders were investigated in two ways: firstly, the milk powders were reconstituted and dot blotting directly performed on $1:10^{½}$ serial dilutions. Secondly, the reconstituted powder samples were tri-sodium citrate and *n*-decane extracted and re-suspended in 50 µl of sterile water so that the final cell concentration was 20-fold higher than the initial non-extracted sample. The concentrated samples were then diluted and assayed as described above. The cell quantities used for dot blotting are listed in Table 9.2 and the corresponding immuno-blots are shown in Figure 9.7.

The polyclonal antibody F13 produced against *G. stearothermophilus* strain A resulted in a positive immuno-response with the milk powder samples USA (A) and Great Britain (B). The former sample was positively detected over several dilutions for both milk preparations, the extracted and the non-extracted, respectively. In contrast, recognizable signals for the Great Britain sample were obtained only for the concentrated dilutions suggesting that the cell concentration of the non-extracted

sample was below the detection limit of the assay. These results indicate the presence of *G. stearothermophilus* strain A in both powders, which was confirmed for USA (A) sample using RAPD-PCR (Rueckert et al., 2004 Chapter 2) and DGGE (Chapter 6). The Great Britain (B) powder, however, produced only a weak signal when it was incubated with the strain A specific polyclonal antibody. This sample was negatively tested for the presence of strain A when screened with DGGE. Similarly, the RAPD result is consistent with there being no viable cells of strain A present in the powder. The Canadian powder sample which had been positively tested for strain A by DGGE (Chapter 6) but found to be negative for this organism according to RAPD screening (Rueckert et al., 2004) gave no immuno-response when assayed with F13. Thus, it is very likely that *G. stearothermophilus* strain A grew to a small proportion in the process line but was subsequently killed by inhibitory processing stages. This would explain why the DGGE was capable and RAPD incapable to detect this organisms.

Table 9.2. Cell quantities used for dot blotting of retail milk powders from Figure 9.7 [cells dot^{-1}]

	USA (A)				Canada				Great Britain (B)			
	Total[(i)]		Viable[(ii)]		Total[(i)]		Viable[(ii)]		Total[(i)]		Viable[(ii)]	
position	A	B	A	B	A	B	A	B	A	B	A	B
1	4.3×10^{3}	8.6×10^{4}	4.4×10^{1}	8.8×10^{2}	1.7×10^{3}	3.4×10^{4}	8.0×10^{0}	1.5×10^{2}	8.4×10^{3}	1.7×10^{5}	8.0×10^{0}	1.6×10^{2}
2	1.4×10^{3}	2.7×10^{4}	1.4×10^{1}	2.8×10^{2}	5.4×10^{2}	1.1×10^{4}	2.5×10^{0}	4.8×10^{1}	2.6×10^{3}	5.3×10^{4}	2.5×10^{0}	5.0×10^{1}
3	4.3×10^{2}	8.6×10^{3}	4.4×10^{0}	8.8×10^{1}	1.7×10^{2}	3.4×10^{3}	0	1.5×10^{1}	8.4×10^{2}	1.7×10^{4}	0	1.6×10^{1}
4	1.4×10^{2}	2.7×10^{3}	1.4×10^{0}	2.8×10^{1}	5.4×10^{1}	1.1×10^{3}	0	4.8×10^{0}	2.6×10^{2}	5.3×10^{3}	0	5.0×10^{0}
5	4.3×10^{1}	8.6×10^{2}	0	8.8×10^{0}	1.7×10^{1}	3.4×10^{2}	0	1.5×10^{0}	8.4×10^{1}	1.7×10^{3}	0	1.6×10^{0}
6	1.4×10^{1}	2.7×10^{2}	0	2.8×10^{0}	5.4×10^{0}	1.1×10^{2}	0	0	2.6×10^{1}	5.3×10^{2}	0	0
7	4.3×10^{0}	8.6×10^{1}	0	0	1.7×10^{0}	3.4×10^{1}	0	0	8.4×10^{0}	1.7×10^{2}	0	0
8	0	2.7×10^{1}	0	0	0	1.1×10^{1}	0	0	2.6×10^{0}	5.3×10^{1}	0	0
9	0	8.6×10^{0}	0	0	0	3.4×10^{0}	0	0	0	1.7×10^{1}	0	0
10	0	2.7×10^{0}	0	0	0	1.1×10^{0}	0	0	0	5.3×10^{0}	0	0

(i) = Cell count determined by phase-contrast microscopy using a Thoma chamber including dead and viable cells
(ii) = Cell count determined by plate counting (Rueckert et al., 2004)
A = reconstituted milk
B = reconstituted, tri-sodium citrate and *n*-decane concentrated milk sample

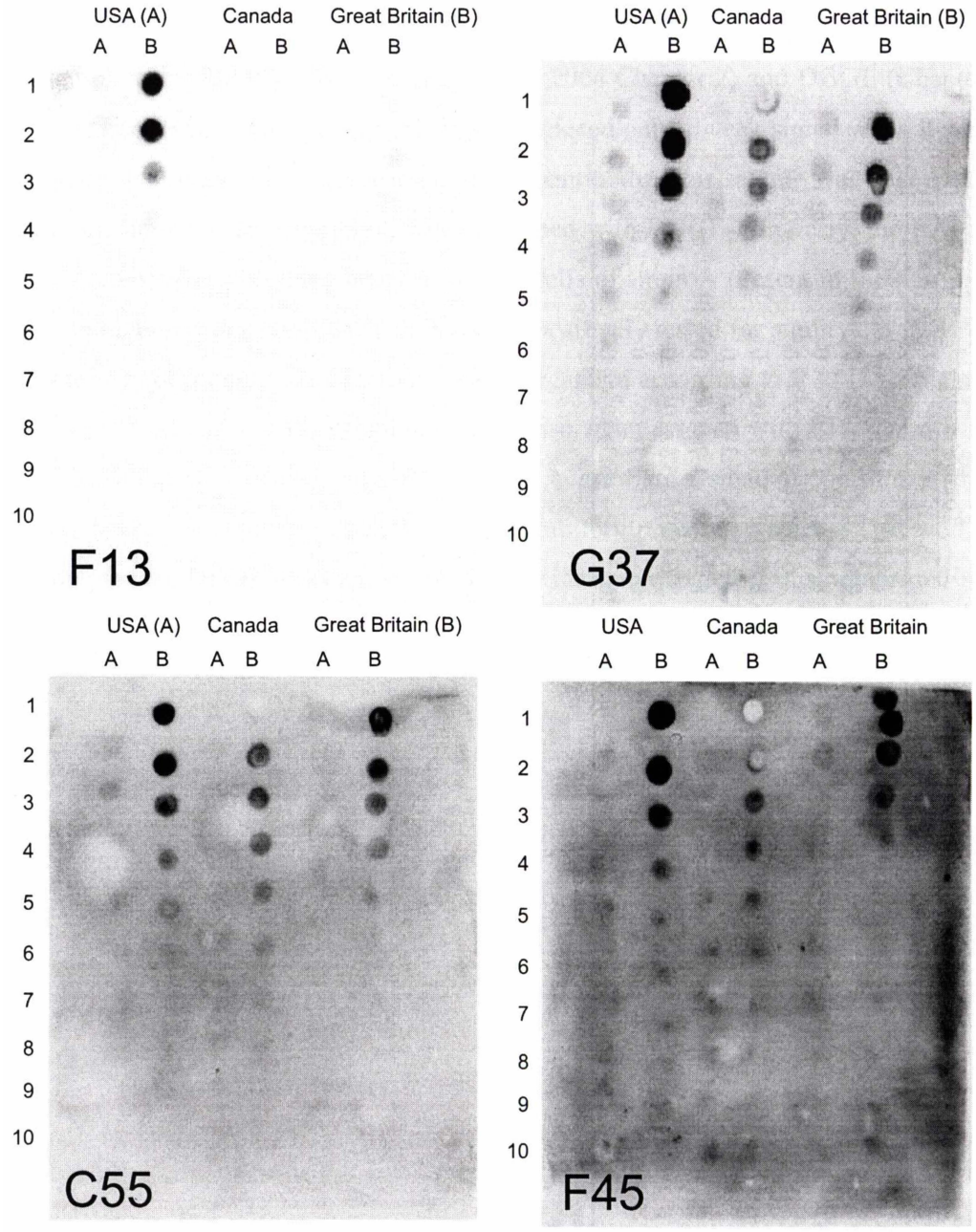

Figure 9.7. Dot blot and immunodetection of the milk powder samples USA (A), Canada and Great Britain (B) using the polyclonal antibody F13, G37, C55 and F45. (A) The reconstituted milk was serial diluted and 2 µl used for dot blotting. (B) The reconstituted milk was tri-sodium citrate extracted prior to dot blotting, resuspended in 50 µl of sterile water and 2 µl of each dilution used for dot blotting.

The immuno-assay performed with G37 was positive for all three powder samples including both cell preparations, which concurs with the findings made with the DGGE and RAPD analyses. It also supports the notion that *A. flavithermus* strain C was the dominant contaminant being present in high numbers in milk powders. On a theoretical basis, the un-extracted dilution samples (A) from USA (A) (position 5

and 6) and Great Britain (B) (position 3 and 4) should not contain any vegetative cell forms at this dilution, either viable or dead, and thus should not give any immuno-response (Table 9.2; Figure 9.7). However, positive signals were recorded for these dilutions which we attribute to cell debris being present in powders which are unrecognizable by phase-contrast microscopy.

All three powders responded positively with antibody F45 indicating that *B. licheniformis* strain F was present. Indeed, the culture-depended RAPD technique identified strain F in all three samples with an approximate viable proportion of 616 cfu g^{-1}, 760 cfu g^{-1} and 737 cfu g^{-1} for the USA (A), Canada and Great Britain (B) powder, respectively (Rueckert et al., 2004, Chapter 2). Interestingly, the results obtained with the immuno-assay suggested up to 10-fold more vegetative cell of *B. licheniformis* F than detected by plate-counting and RAPD. This supports the presence of additional inactive cells in the powders, which were killed during processing. The DGGE method employed on the powders did not allow for an unequivocal determination about the presence of strain F (Chapter 6).

Similar to the immuno-detection of antibody F45 were the results obtained with antibody C55. This antibody was produced against a mixture of vegetative cells of *B. licheniformis* (strain F and G) and *B. subtilis* BS and the results from this experiment are not conclusive enough to allow for a concrete answer about the origin of the positive immuno-response (Figure 9.7). It might be the result of either strain, although both RAPD and DGGE were not indicative for strain F and BS in either powder so that it is more likely that the positive immuno-response was due to strain F being present in these samples.

9.4. Discussion

In this study, four polyclonal antibodies were produced in order to develop immunoassays for the selective and quantitative detection of thermophilic bacilli in milk powder. These polyclonal antibodies were derived from rabbits injected with vegetative cell forms of either *G. stearothermophilus* strain A, *A. flavithermus* strain C, *B. licheniformis* strain F or a mixture of *B. licheniformis* (strains F and G) and *B. subtilis*. Although each individual polyclonal antibody preparation was specific towards its target organism, their sensitivities varied significantly and all antibodies also some showed cross-reactivity to bacilli other than the target organism. For

instance, the antibody G37 produced against *A. flavithermus* strain C showed its highest specificity towards strain D and the sensitivity of this antibody to other bacilli, including strain C, was reduced by one to two orders of magnitude. Similarly, antibody C55 raised against *B. licheniformis* (strains F and G) and *B. subtilis* showed preferential binding to the latter organism and its recognition towards strains F and G was also reduced by up to three orders of magnitude. The maximum sensitivity achieved with antibody F13 for the detection of *G. stearothermophilus* A was 9.5×10^4 cells ml^{-1}, and this antibody cross-reacted more readily with *A. flavithermus* D. The immunoassay of antibody F45 showed the lowest sensitivity of all antibodies used in this study with only 2.3×10^5 cells ml^{-1} of strain F being detected. Thus, the relatively low sensitivity obtained with the latter two polyclonal antibody assays makes their application to the analysis of milk powder problematic as these assays just meet the requirements to detect thermophiles at the current specification regulations of 3.0×10^4 cells per gram of powder. In addition, none of the immunoassays were capable of distinguishing between antigens derived from viable or dead cells. In fact, we found evidence that even cell debris is recognized by the assays, and furthermore, spores remain completely undetected due to their different surface antigens. Of significant concern is that spores can germinate and multiply in the downstream food processing line and thus, they should also be included in the estimation of thermophilic contaminants. The study of Rueckert et al. (2005b) (Chapter 5) demonstrated that dead cells contribute to the vast majority of the bacterial flora constituting on average approximately 98% of the total vegetative cell content in milk powders. And although these dead cells can be used as an indicator for the overall plant hygiene, they are microbiological irrelevant for downstream processing of food and thus are of little concern to the manufacturer.

In conclusion, the polyclonal antibodies produced in this study were able to detect the dominant proportions of thermophilic bacilli in milk powders. In general, the results obtained on selected milk powders correlated reasonably well with the results derived from DGGE-PCR and RAPD-PCR with the dominant thermophiles being detected by either method. In some instances, however, the PCR methods did not detect lower-level contaminants due to the following reasons: The culture-dependent RAPD method was incapable of detecting the dead cell proportion but these inactive cells are still present in the samples from where they are detected by the immunoassay. Further, the detection of thermophilic bacilli with the current DGGE-

PCR method was sometimes limited to the detection of medium- to high-count contaminants due to amplification bias against low-level contaminants. However, the major advantage of the presented ELISA immunoassays were their rapid and facile application to the quantitative analysis of thermophilic bacilli in milk powder without the need of expensive laboratory equipment or other investments. The sensitivity of the current assays can also be improved when the concentration of antibody detection reagent is increased.

Survival of thermophilic spore-forming bacteria in a 90^{+} year old milk powder from Ernest Shackelton's Cape Royds Hut in Antarctica

Published in the *Journal of Dairy Research* (*in press*).

10.1. Abstract

Milk powder taken to Antarctica on Shackelton's British Antarctic Expedition in 1907 was produced in New Zealand by a roller drying process in the first factory in the world dedicated to this process. Thermophilic bacilli are the dominant contaminants of modern spray-dried milk powders and the 1907 milk powder allows a comparison to be made of contaminating strains in roller-dried and spray-dried powders. Samples of milk powder obtained from Shackelton's Hut at Cape Royds had low levels of thermophilic contamination (< 500 cfu ml^{-1}) but the two dominant strains (*Bacillus licheniformis* strain F and *Bacillus subtilis*) were typical of those found in spray-dried powders. Soil samples from the floor of the hut also contained these strains, whereas soils distant from the hut did not. Differences in the RAPD profiles of isolates from the milk powder and the soils suggest that cross contamination between these sources was unlikely. It is significant that the most commonly encountered contaminant strain in spray-dried milk (*Anoxybacillus flavithermus* strain C) was not detected in the 1907 sample.

Keywords: *Bacillus licheniformis*, *Bacillus subtilis*, RAPD-PCR, thermophilic, *Anoxybacillus flavithermus*, *Geobacillus stearothermophilus*; Milk powder; Antarctica.

10.2. Introduction

Due to the vital role in human nutrition that milk plays, milk products are routinely analyzed for a number of potential microbial contaminants. Thermophilic spore-forming bacterial contamination is routinely monitored despite there being no evidence of their involvement in human disease; but they are a useful indicator organism of good hygiene practices within milk powder producing factories and for downstream food processing requirements (Stadhouders et al. 1982; Kwee et al. 1986; Murphy *et al,* 1999). The milk industry is usually required to store samples of powder products for a number of years (commonly 2 to 3), after which they are usually destroyed, thus precluding the analysis of the survival of contaminants in powder stored for a long period. To our knowledge, in no published cases have the number of surviving thermophilic bacilli been assessed in very old milk powders (even over a few years old), although several studies have shown that bacterial spores can survive for extensive time-periods (Sneath 1962; Setlow 1994; Cano and Borucki, 1995; Dombrowski 1963; Wilson and Shipp 1938; Bartholomew and Paik 1966).

Studies on the effect of storage conditions on the longevity of microorganisms in milk powder, or their spores in the case of bacilli, have shown that the storage temperature, total solids content and the relative humidity are important factors (McDonough and Hargrove 1968; Higginbottom 1969; Reddy et al. 1975; Mercurio and Tadjalli 1979). With regard to possible freeze-thaw cycles affecting the long-term survival, *Bacillus* endospores are known to possess both extreme resistance to long-term desiccation and to multiple cycles of freezing and thawing (Nicholson et al. 2000).

Recently, two large-scale molecular-based surveys using randomly amplified polymorphic DNA-PCR (RAPD-PCR) were undertaken in which nearly 2400 isolates of thermophilic bacterial contaminants of milk powders were analysed, one using powders solely from New Zealand, and the other from a wide-geographic spread of 18 countries (Ronimus et al. 2003; Rueckert et al. 2004). The results show that seven strain groups from four species of bacilli are the major cause of contamination. These are *Geobacillus stearothermophilus* strain A, *Anoxybacillus flavithermus* (strains B, C and D), *Bacillus licheniformis* (strains F and G) and *Bacillus subtilis*. The presence of thermophilic strains of *G. stearothermophilus*, *B. licheniformis* and *B. subtilis* has been confirmed in both raw milk and milk powders in numerous instances (Reddy et

al. 1975; Stadhouders et al. 1982; Chopra and Mathur 1984; Kwee et al. 1986; Phillips and Griffiths 1986; Phillips and Griffiths 1990; Crielly et al. 1994; Murphy et al. 1999). Significantly, strains of *A. flavithermus,* which was originally isolated from a hot spring in New Zealand (Heinen et al. 1982) and subsequently from Yellowstone (Nold et al. 1996) and Turkey (Beldüz et al. 2000), have recently been conclusively identified in milk powders (Flint et al. 2001; Ronimus et al. 2003; Rueckert et al. 2004). The *A. flavithermus* strains (groups B, C and D) have no doubt over the years been routinely classified by the dairy industry as *Bacillus stearothermophilus* (*G. stearothermophilus*).

Prior to spray drying, milk powder was produced by roller drying and although thermophilic bacilli were recognized as major contaminants it is unclear whether the strains that predominate in modern spray drying were also those found in roller-dried powder. Recently, we were able to obtain a milk powder sample from one of the earliest roller-dried powders produced, and this allowed analysis of the thermophilic contaminants.

Ernest Shackelton's Nimrod of the British Antarctic Expedition left New Zealand on January 1, 1908, from Lyttelton Harbour, Christchurch, bound for Antarctica. On board were provisions obtained from both the UK and New Zealand. Of particular relevance to this communication were supplies of dried whole milk powder obtained from the LD Nathan Company packaged under the local name Defiance Brand, but registered in 1906 in the UK under the name Glaxo (Shackelton 1910). Shackelton's Hut at Cape Royds still has containers of this milk powder and we were able to obtain samples of powder from a previously opened canister (presumably by members of Shackelton's team in 1908). The prospect of being able to analyse a milk powder produced at the start of the 1900s in New Zealand, by roller drying and stored in very favourable conditions (cold and dry), represented a remarkable opportunity. Results could aid in our understanding of the source of present day factory-derived contamination in the New Zealand milk powder industry. Additionally, we were able to obtain a sample of a spray-dried powder produced by the NZ Dairy Company in 1966, and stored at ambient temperature since that time which was included in this survey.

10.3. Materials and Methods

10.3.1. Milk powders

The whole milk powder from Shackelton's Hut at Cape Royds was produced in New Zealand, circa 1907, under the Defiance Brand for the Imperial Dry Milk Company, Ltd. of the UK (28 Gracechurch Road, London; see Figure 10.1). The Imperial Dry Milk Company was registered in 1905 (Millen 1991), which subsequently was registered as Glaxo in the UK on October 27, 1906 (Jephcott 1969). The compositional analysis on the 10 kg metal storage box was as follows: 4.9% moisture, 5.6% mineral matter, 26.2% proteins, 27% fat, 36.3% milk sugar. The storage tin had been opened leaving the possibility of some contamination, although the vast bulk of the milk powder was still in place. The powder had a flaky appearance and was slow to reconstitute after incubation for 15 minutes in 50°C warm water and vigorous vortexing. The 1966 whole milk powder was produced by Anchor Products, New Zealand, for the New Zealand Co- operative Dairy Co., Ltd., Hamilton, New Zealand and had an expiry date of January, 1967. The compositional analysis was: 26.5% butterfat, 28% protein, 6.0% mineral salts, 35.5% lactose and 3.0% moisture, 1.0% minor constituents (100 calories per 100 g). Thus the two whole milk powders were essentially identical with respect to their compositions. The 1966 whole milk powder had a fine powder appearance and it was reconstituted only after incubation for 15 minutes in 50°C warm water and vigorous vortexing.

Figure 10.1. Digital image of the original container of milk powder at Shackelton's Cape Royds hut used for sampling. Note finger on left for size perspective.

10.3.2. Soil samples

Nine soil samples were derived from different geographical locations. The three samples "Mainway", "Hallway" and "Entrance" were collected within the hut domain while four soil samples were derived from the close hut surrounding with local distances of approximately 0, 10, 50 and 2500 meters from the hut, respectively while two soils were derived from the Antarctic continent approximately 120 and 400 km away from the hut. Soil samples were collected in sterile 50 ml Falcon tubes using sterilized spatulas. The samples were stored at -20°C until culturing was performed.

10.3.3. Culturing techniques

The powder samples were reconstituted and diluted in sterile deionised water. For total counts of bacilli, aliquots were pour plated with TSA medium with 0.2 soluble potato starch and incubated at 55°C for 16 to 24 hours. The number of spores was determined after heat-treatment of the reconstituted milk at 80°C for 20 min, followed by incubation at 55°C in TSA-starch. Individual colonies from the dilution plates were then re-streaked to TSA-starch plates and re-incubated at either 35°C or 55°C (dependent on the colonies previous isolation temperature) for 16 hours to obtain sufficient bio-mass for DNA preparation. The cells were harvested with sterile disposable spreaders and DNA isolated as described by Ronimus et al. (1997).

The thermophilic load of the soil samples was determined by resuspending 4 gram of soil into 10 ml of sterile water and plating as described for the milk powder samples.

10.3.4. RAPD analyses

RAPD analyses employed Operon Technologies 10-mer primer OPR13 (GGACGACAAG) in a 35 cycle PCR reaction with an annealing temperature of 36°C, again as described by Ronimus et al. (1997). PCR products were analysed by agarose gel electrophoresis using 1.5 % LE SeaPlaque agarose gels (FMC Corp., San Diego, USA) followed by image capture using an Eagle Eye System (Stratagene Corp., USA).

10.4. Results

A total count and spore count, together with the composition of strains or species identified is shown in Table 10.1. Total plate counts for thermophilic bacilli were all low, with the New Zealand milk samples from 1966 having a maximum of 360 per gram. For the Shackelton Hut samples the highest total count was found in the milk powder itself, soil samples from within the Hut had only slightly lower total counts but the ramp outside the Hut and more distant soil samples recovered no thermophilic bacilli. This result would be consistent with the soil in the Hut having been contaminated with organisms from the milk powder. Spore counts do not follow this trend, with the exception that spore numbers on the ramp sample were barely countable and the more distant soils contained no thermophilic spores. However, spore numbers in all soil samples in the Hut exceeded numbers of spores in the milk powder, sometimes by over an order of magnitude and it is difficult to explain this result as contamination of these samples directly by milk powder (where the ratio of spores to total count would have been expected to be relatively constant). A total of 260 isolates from the various samples were subjected to RAPD profiling, 66 from the roller-dried Shackelton Hut powder, 113 soil-derived isolates from the floor and the adjacent surroundings of Hut and 81 from the 1966 spray-dried powder. Two species dominated all these samples viz *B. licheniformis* followed by *B. subtilis*. The cell density of each strain present in any sample can be derived from the proportion of RAPD fingerprints obtained from the isolates subjected to RAPD analysis for that sample. The distribution of these RAPD fingerprints differed between the Shackelton milk sample and the Hut soil samples. For example, in the former, the total count was clearly dominated by *B. subtilis*, whereas the spore count was largely constituted of *B. licheniformis* strain F (Table 10.1). This distribution was quite different in the soil samples where generally both strains were present in more equal proportions.

A more detailed analysis of the RAPD patterns of isolates from milk powder and Hut soil sources is shown in Figures 10.2 to 10.5. When the RAPD fingerprints for *B. subtilis* were examined for variations between the milk and soil samples (Figure 10.2), the patterns obtained for all milk powder isolates were identical (tracks 13 to 16) and consistent with the RAPD pattern of both the DSM type strain for *B. subtilis* (track 2) and our own reference strain of *B. subtilis*, which was isolated from a spray-dried powder (track 3).

Table 10.1. Numbers of thermophilic cells and spores in milk powders and soils and distribution of RAPD profiles in each sample

Sample	Sample type	Total count [cfu g^{-1}]	Spore count[a] [cfu g^{-1}]	Number of isolates	Thermophiles [cfu g^{-1}]				
					A	F	G	*B. subtilis*	Other
Shackelton Hut	Milk powder	170	-	36	-	14	-	156	-
Shackelton Hut	Milk powder	-	17	30	0.5	14	2.5	-	-
Mainway	soil	71	-	18	-	24	3	44	-
Mainway	soil	-	320	14	-	68	-	252	-
Hallway	soil	140	-	18	-	78	-	54	8[b]
Hallway	soil	-	44	18	-	42	-	-	2[c]
Entrance	soil	120	-	18	-	73	-	47	-
Entrance	soil	-	160	17	-	66	-	94	-
Ramp	soil	0	-	0	-	-	-	-	-
Ramp	soil	-	4	10	-	3.6	-	0.4	-
Antarctic soils	soil	0	0	0	-	-	-	-	-
NZ 1966	Milk powder	360	-	53	34	306	13	7	-
NZ 1966	Milk powder	-	330	28	12	236	-	12	70[d]

A = *G. stearothermophilus*; F and G = *B. licheniformis*;

[a] Activation by heat treatment at 80°C for 20 minutes and incubation at 55°C for >16 hours

[b] Unidentified profile; [c] *Ureibacillus thermosphaericus*; [d] *B. coagulans* (58) unidentified (12)

Some of the patterns obtained from soil isolates also had identical RAPD profiles to that of the milk isolates, e.g. track 12, while others were very similar, e.g. tracks 10 and 11. However, the majority of soil profiles were clearly different from those of the milk isolates. In particular, they contained a prominent distinguishing band at 1165 bp, (shown by an asterisk in Figure 10.2), and other less prominent bands in some of the isolates reinforce this difference.

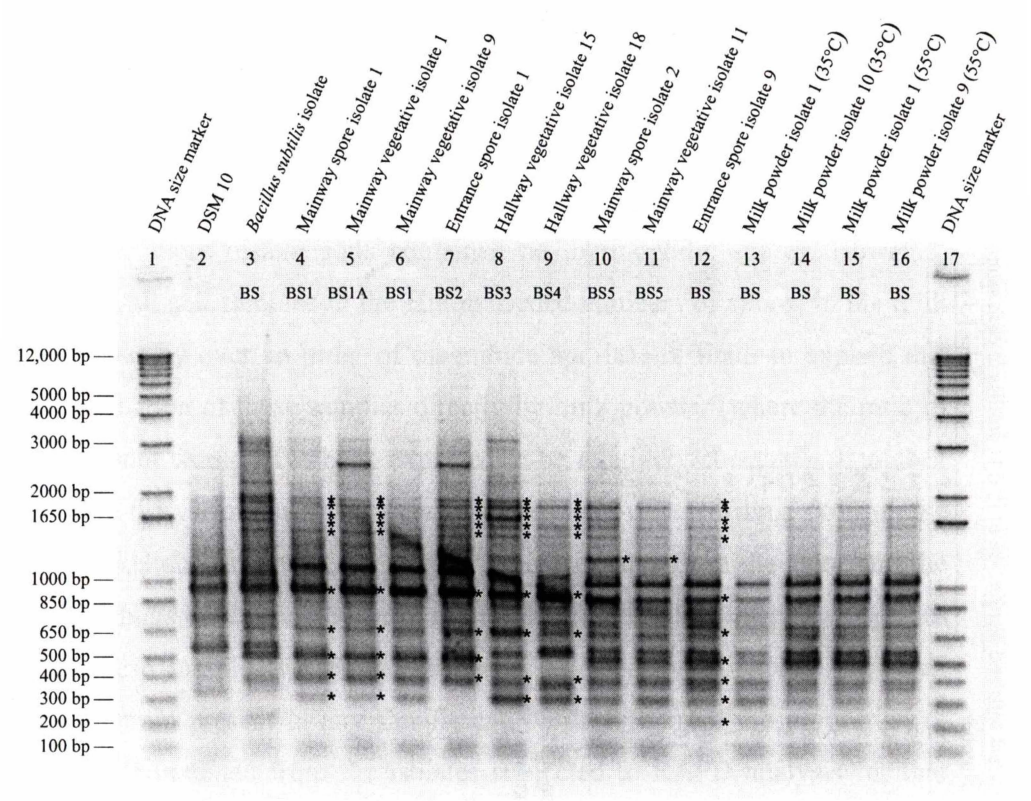

Figure 10.2. RAPD-fingerprint profiles of selected *Bacillus subtilis* soil (tracks 4 to 12) and Shackelton Hut milk powder isolates (tracks 13 to 16). Track 2 shows the fingerprint of the reference strain DSM 10 and track 3 the common *B. subtilis* isolate found in New Zealand factory powders (Ronimus et. al., 2003). Asterisks identify inter-sample distinguishing bands between milk-derived and soil-derived RAPD profile patterns.

Isolates identified as *B. licheniformis* strain F all have common bands at 680, 850, 1000, 1040 and 1150 bp as indicated with asterisks in Figure 10.3 track 2, but these isolates display a greater degree of genetic diversity than shown with *B. subtilis* (Figures 10.3 and 10.4). While all profiles are clearly of the *B. licheniformis* type (tracks 1 and 2 in each Figure are of the DSM type strain and our reference strain isolated from spray-dried milk powder, respectively), consistent sub-strain variations are observed. Profiles, which are characteristic of the type strain of *B. licheniformis* F are found in milk isolates (Figure 10.4, tracks 6 and 7) and soils from the Hut (Figure

10.3 tracks 7 to 9, 17 and 18). A variant of this profile (which we term F1) is characterised by a prominent band at approximately 640 bp and was present in isolates from milk (Figure 10.4 tracks 9 and 10) and soils (Figure 10.3 tracks 3, 4 and 12 to 16). Variant profile F2 was again found in soil and milk isolates (Figure 10.3 tracks 5, 6, 10, 11 and 19; Figure 10.4 tracks 3 to 5 and 8).

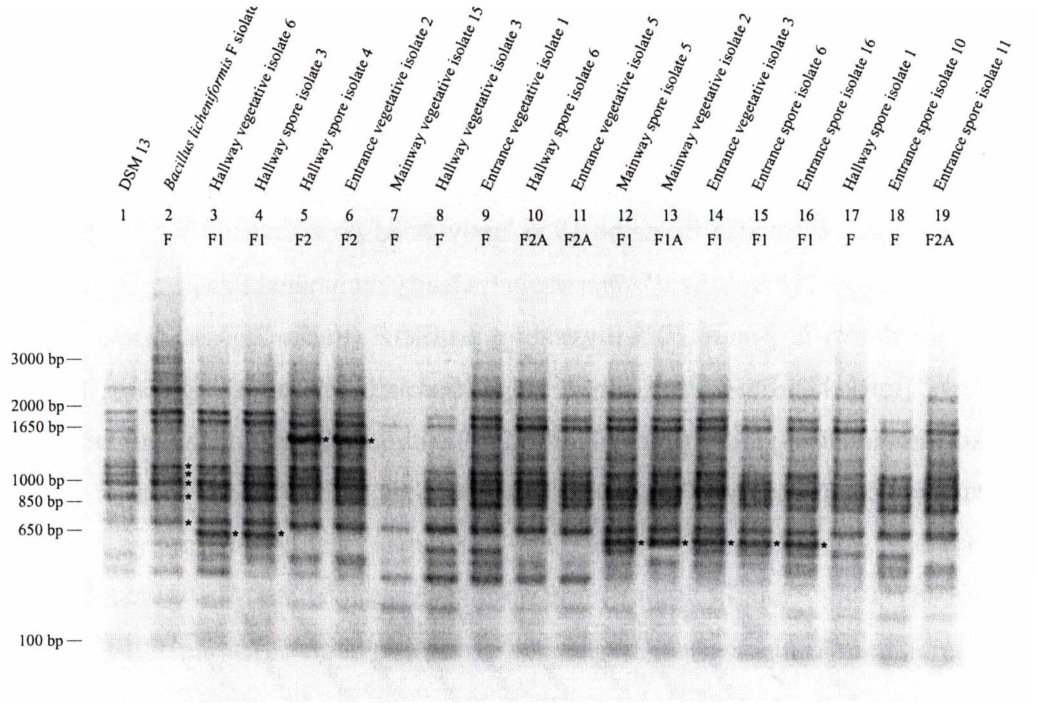

Figure 10.3. RAPD-fingerprint profiles of thermophilic *B. licheniformis* strain F isolates from Antarctic soil samples. Asterisks identify inter-sample distinguishing bands between milk-derived and soil-derived RAPD profiles pattern.

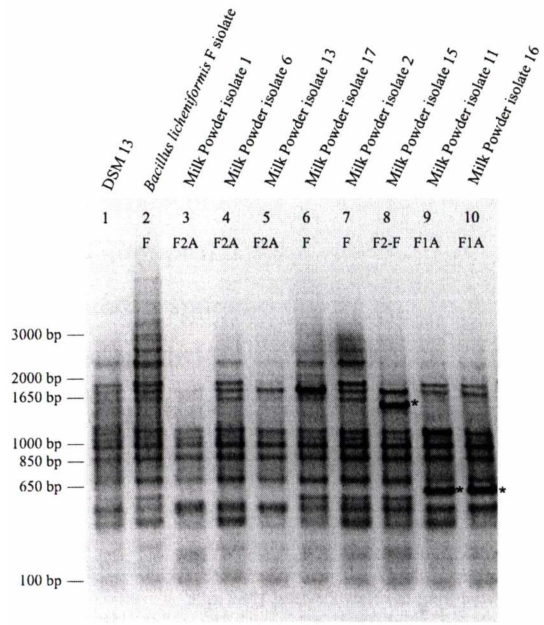

Figure 10.4. RAPD-fingerprint of thermophilic *B. licheniformis* strain F isolates from the Shackelton Hut milk powder. Asterisks identify inter-sample distinguishing bands between milk-derived and soil-derived RAPD profile patterns.

Of the 179 RAPD profiles that were run for the milk and soil samples from the Hut 171 were easily identified as *B. subtilis* or *B. licheniformis* strain F. Of the remaining 8 profiles, four were identified as *B. licheniformis* strain G, one as *Ureibacillus thermosphaericus*, one was unidentified and one as *G. stearothermophilus* strain A. The latter was only isolated as a spore from the milk powder and is significant since this profile is common in surveys of present day spray-dried milk powders (Ronimus et al. 2003; Rueckert et al. 2004). We have previously reported the occurrence of *U. thermosphaericus* in spray-dried milk, but only at very low levels, where it is considered to indicate contamination present in the raw milk from soil.

Eighty-one isolates from the 1966 spray-dried powder were RAPD profiled and contamination by *B. licheniformis* strain F clearly dominated this powder. Typical results are shown in Figure 10.5 illustrating profile F (tracks 2, 3, 4, 6 and 8) and profile F2 (track 11). In addition, profiles identified as *G. stearothermophilus* strain A (track 5) *Bacillus coagulans* (tracks 7, 9 and 10) and *B. subtilis* (not shown) were less frequently encountered.

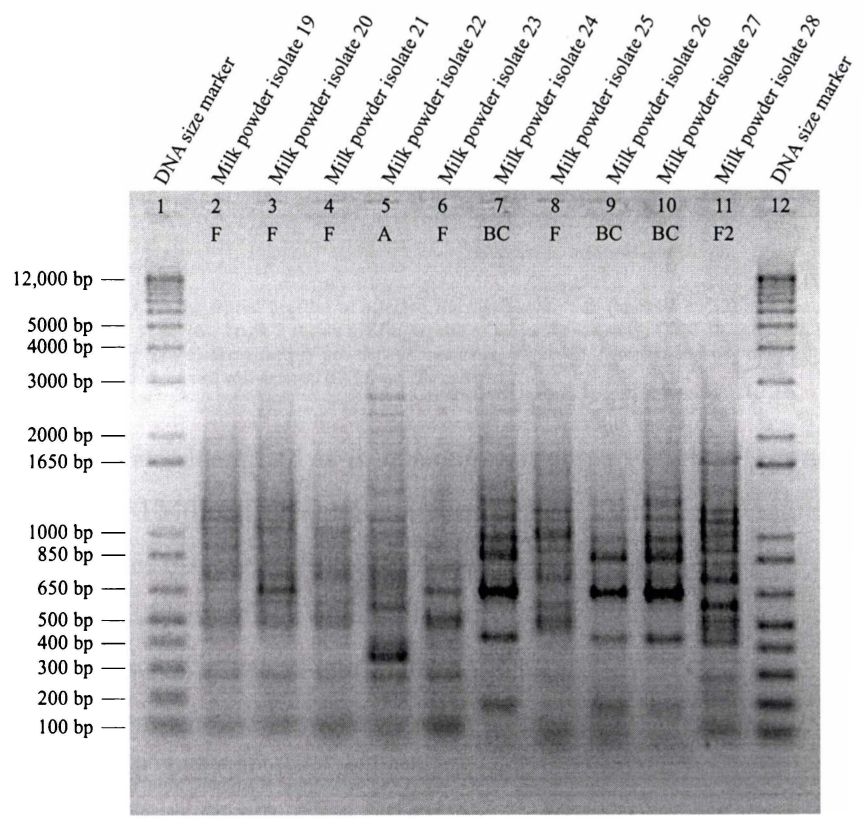

Figure 10.5. RAPD-fingerprint profiles of thermophilic bacilli isolated from the New Zealand 1966 milk powder (F and F2 = *B. licheniformis*; A = *G. stearothermophilus*; BC = *B. coagulans*).

10.5. Discussion

The three critical storage parameters enhancing the survival of microorganisms following milk powder production and long-term storage are the water activity, relative humidity and the temperature (Higginbottom, 1969; Thompson et al. 1978; Stapelfeldt et al. 1997). Extremely dry conditions can lead to morphological changes in cells and generally, a slow reduction of the microbial population (Troller, 1991). Additional contributing factors include the preheat-treatment of the powder (Findlay et al. 1946), the milk powder structure, storage conditions (particularly the lack of oxygen), the type of organisms present (Thompson et al. 1978; Kieseker & Aitken, 1993; Celestino et al. 1997) and type and concentration of milk solids, i.e. skim or whole milk (Mazas et al. 1999). Recovery of growth after storage will be affected by the aeration (Long & Williams, 1959), incubation temperature, growth medium, pH and any spore activation procedures (Daemen, 1981; McGuiggan et al. 1994), although many bacilli do not necessarily require an activation step for germination to proceed (Sneath, 1962). Spores can be activated by the factory heat treatments (Lane, 1988), and at the same time can also be protected by milk solids themselves from heat (Raju & Kumar, 1989). The modern day shelf-life of whole milk powder is considered to be about 6 months at room temperature (Celestino et al. 1997), but when stored under vacuum or with an inert gas then it is 12 months (Kieseker & Aitken, 1993). Without protection from oxygen, auto-oxidation products might be expected to have a deleterious effect on the longevity of microorganisms (Celestino et al. 1997). Overall, a decrease in the total microbial content occurs after prolonged storage of dried milk powders, with endospore-forming bacilli being able to survive both the heat-treatments during powder production and the resulting desiccation, and are thus, the most likely to be isolated after prolonged storage (Keogh, 1966). Indeed, in a study using pure cultures of *B. subtilis* spores added to milk before drying no change in the viable count was observed up to 110 weeks at relative humidities between 5 and 50% (Higginbottom, 1969). Long-term studies dealing with the numbers of thermophiles with time of storage are relatively few in number. In a study using mesophilic recovery of *B. subtilis* at 32°C by Thompson et al. (1978), the authors studied the effect of varying temperatures (71.1°C to 93.3°C) during spray drying on the survival of cultures spiked into non-fat milk powder and found the survival rate ranged between 32.2 and

40.4 % after 36 weeks. Of those species that did survive *B. subtilis* was the most resistant to drying and recovery after storage (Thompson et al. 1978). In another investigation performed by Celestino et al. (1997) a medium-heat whole-milk powder (which was neither stored under an inert gas or vacuum) showed no significant difference between the total plate counts of aerobic thermophiles and anaerobic thermophiles over an eight months period. In contrast, in a study looking at the survival of *Salmonella* in skim milk powders stored at ambient temperature with 4.4% moisture content there was an approximate 90% die off after 15 weeks (McDonough & Hargrove, 1968). The total mesophilic plate count decreased significantly, by approximately 80%, attributed to low water activity. In a New Zealand-based study no significant decrease in thermophile count was observed after 5 months storage at ambient temperature (Lane, 1988). As most of these studies are "short-term" with respect to the powders reported here it is difficult to extrapolate the findings to that of a powder nearly 100 years old.

We must assume that the container of milk powder in Shackelton's Hut had been opened by members of Shackelton's party, and thereafter remained covered. It is also possible that Hut visitors since the late 1950's may have opened and disturbed some of the contents, and therefore contamination of the powder from the Hut surroundings cannot be excluded. Given the prevailing low temperature in the Hut throughout the year, it is likely that the temperature inside the Hut never exceeded 20°C throughout the nearly 100 years, and it is reasonable to assume that allied with the low humidity and water content of the powder that no growth of vegetative cells of thermophilic bacilli or germination of their spores would likely occur. Similar assumptions can be made about the soil environment within the Hut. Therefore the occurrence of thermophilic cells and spores reflects their original distribution and not growth.

Our analysis of many spray-dried powders from NZ factories shows that the most common RAPD profiles encountered are *B. licheniformis* strain F, *G. stearothermophilus* strain A and *A. flavothermus* strain C (Ronimus et al. 2003; Rueckert et al. 2004). The latter is completely absent from the Antarctic powder, but was also not detected in the 1966 spray-dried powder so possibly this strain either does not survive long-term or was never present in either powder originally. It is of interest that of all the samples examined from Antarctica only the milk powder contained a representative of *G. stearothermophilus* strain A, albeit only a single

spore from the 94 isolates profiled. In the 1966 spray dried powder 6 of the 81 isolates examined were of this strain. Thus, the two most typical profiles of modern day powders are absent or did not survived long-term storage.

Based on the proportions of the profiles of the two most common strains present in the Antarctic samples it would appear unlikely that the milk powder has been contaminated by soil organisms. Firstly, the total count (consisting largely of vegetative cells) of the milk powder sample is dominated by *B. subtilis,* with spores only of *B. licheniformis* strain F. In the soil samples, apart from the Hallway, soils contain spores of *B. subtilis*, sometimes as the dominant form, e.g. the Mainway (Table 10.1). Additionally, all profiles of isolates of *B. subtilis* from the milk were identical, whereas those from the soil were varied and different (Figure 10.2). Secondly, the total counts of soil samples contained a far greater proportion of *B. licheniformis* strain F cells as a percentage of the total compared to the milk powder. Since growth or spore germination is unlikely in these habitats, these differences can only arise from different sources of contamination. It would appear unlikely that the milk had been contaminated with thermophilic bacilli from the soil or other habitats within the Hut; if so, a greater proportion of *B. subtilis* spores would have been predicted in the powder sample, and relatively more *B. licheniformis* strain F cells contributing to the total count. In summary the results obtained are consistent with the milk powder containing the remnants of a population of thermophilic bacilli present in the powder at the time of formation, whereas the soil samples have derived their thermophilic bacilli from a different source. While it is not possible to discount the milk powder as the source of some of these contaminants in the soil, other strains are more likely to have derived from other sources. The hay taken as animal fodder, the animals themselves and human activities resulting from Hut occupancy are all possible sources of these organisms.

Conclusive support for this hypothesis can most readily come from analysis of milk powders in canisters with their seals intact. These are still present in the Hut, but access to such samples is restricted and unlikely. Meanwhile, these results form a base, which can be compared over time with further samples.

10.6. Acknowledgements

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10.7. References

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Conclusion

The results from this study have highlighted that thermophilic bacilli are important contaminants with immense economic impact on milk powder producing factories worldwide. In order to minimize the economic damage inflicted by these contaminants, while simultaneously producing high-grade milk powder in every processing run, a new technique monitoring thermophiles in milk powder is required that would allow for flexible management of the plant processing time. The current study, which is based on quantitative real-time PCR, can provide this tool. This technology offers advantages over other methodologies when it comes to real-time monitoring of thermophiles in milk powder. Significantly, quantitative PCR analysis is rapid with the enumeration of thermophiles able to be completed within 1.5 hours, providing the opportunity of screening up to 13 samples during an average-length milk powder processing run. At the same time, only 30 percent of the total analysis time requires labour, which is mainly occupied with DNA extraction and PCR setup. Furthermore, the development of the differential DNA extraction procedures for total-, viable cells and spores facilitates the analysis of the viable and spore fraction. For example, the viable cell and spore counts are important in the microbiological grading of milk powders, whereas the total thermophilic load (which includes dead cells) might be a better indicator of the overall hygiene of a processing run. The technical aspects of real-time PCR have been improved over the past years and now user-friendly real-time PCR machines are available that are specifically designed for portability and convenient application in non-research environments. Similar advancement has been made in the field of sonication and the rather laboured sonication apparatus used in this study could be substituted by a portable mini-sonicator such as was developed by Belgrader at al. (1999) specifically designed for the disruption of bacterial endospores. However, the increasing range of real-time PCR machines available on the market also allows the possibility of avoiding a system like the Smart Cycler for several reasons. First, the optical reaction tubes are expensive (NZ$ 1.50 per piece) and operate only in the Smart Cycler increasing the cost per reaction exorbitantly. Second, the flat design of the tubes is dimensioned in a

way where a small volume faces a large surface in order to achieve rapid heat and cooling transition cycles. This unique design, unfortunately, could potentially be linked to the differential absorption of PCR reaction components to the tube surface, which in many cases severely affected the reliable and reproducible quantification of samples with low template concentrations. In support of these potential problems, the study of Teo et al. (2002) on the Light Cycler from Roche found surface-abstraction of *Taq*-polymerase, magnesium and target DNA as the main cause for PCR failure. This problem did not occur when the PCR assays developed in this study were performed with the ABI Prism 7700 (Applied Biosystems) or with the Master Cycler (Eppendorf) which both use conventional PCR tubes. The failure in the reliable amplification of low target copy numbers on the Smart Cycler could partially be compensated by the use of the high-fidelity *Taq*-Polymerase from *TaKaRa*. However, this enzyme is very expensive (NZ$ 2.60 per unit) compared to the Roche variant (NZ$ 0.55 per unit) increasing the cost of the assay.

The use of SYBR Green I as a reporter dye for monitoring the *spo0A* amplification offered a two-fold advantage over the use of the TaqMan-probe in the 16S rRNA assay. First, it was approximately NZ$ 0.50 less expensive per reaction and second, it allowed the identification of the dominant contaminants by temperature melting point analysis. On the other hand, the 16S rRNA assay could not have been performed with SYBR Green due to the inability of the primers alone to discriminate specifically only thermophilic bacilli. In addition, it was also impossible to distinguish the 16S rRNA amplicons using melting point analysis. The TaqMan probe used for targeting the ribosomal 16S gene provided the assay with its high specificity and would further permit the assay to be applied in a multiplex PCR setting. Theoretically, multiplex PCR would also be conceivable using the *spo0A* sporulation genes. Due to the high degree of sequence diversity of the *spo0A* target region used for quantification, multiple TaqMan probes, specific only for individual species of thermophiles, e.g. *Anoxybacillus*, *Bacillus* and *Geobacillus* could be designed to increase the discriminating power of the assay.

The evaluation of the DNA extraction and quantification methods was performed only for the two broadest and most frequently encountered organisms, e.g. *A. flavithermus* strain C and *B. licheniformis* strain F due to time and funding restrictions. However, from a dairy industry point of view, *Geobacillus* species are of particular interest due to the extreme heat-resistance of their spores. And although the

efficiency of the DNA isolation for vegetative cells and spores of *A. flavithermus* C and *B. licheniformis* F could be shown to be of the same magnitude the application of the methodologies to *Geobacillus* might require some adaptations in order to consider the unique physiology of this organism.

In addition, the DNA extraction techniques and quantitative assays have to be validated regarding critical factors such as their suitability and applicably in non-research environments, their reproducibility through different operators and different laboratory equipment and chemicals.

An alternative method to quantitative PCR, which can be applied to the quantitative analysis of thermophilic bacilli in milk powders, is immuno-detection by ELISA. This method can be accomplished within 6 hours of sampling allowing the analysis of several samples during a common processing run. Although the method was not thoroughly capable of inter-strain differentiation it can provide a tool to assess the trends of total thermophilic bacilli levels in milk powder during processing. Theoretically, the method can also be extended to the analysis of spores of thermophilic bacilli, when appropriate polyclonal antibodies are used. Significantly, the method has the advantage that it does not require expensive laboratory equipment. The down side of the ELISA assay, however, is that the technique was labour intensive, it had a lower sensitivity and it was also incapable of differentiating between viable and dead cells.

The culture-dependent randomly amplified polymorphic DNA-PCR method has been proven to be a reliable and highly specific molecular diagnostic tool to obtain an overall insight into the bio-diversity of milk powders. The advantage of this technique lies in the ability to quantitatively survey the proportions of thermophiles, either as vegetative cells or spores, with the potential to detect the dominant as well as the low-level contaminants. Moreover, RAPD-PCR offers a very high resolution enabling the differentiation between strains and even sub-strains as was shown in Chapter 2. Thus, RAPD-PCR was the method of choice to investigate the very basic composition of thermophilic contaminants isolated from milk powders derived from many different countries. Other methods employed in this study were dependent on the results gained with RAPD-PCR to varying degrees highlighting the importance of this molecular technical tool. However, with respect to the goals of this thesis, the

method has two significant limitations preventing its application to the analysis of thermophilic milk powder bacilli in a factory setting. First, the method is labour intensive due to the screening of individual isolates, and second, RAPD-PCR is also very time consuming with results becoming available at the earliest 48 hours after sampling. The time for the assessment of thermophilic contaminants in milk powders can be reduced when the non-quantitative and culture-independent denaturant gradient gel-electrophoresis (DGGE) is employed. However, the benefit in gaining a rapid result is partially over-shadowed by the loss in screening resolution, and although the method produced distinctive DGGE markers for each individual *Bacillus* strain the situation in milk powder was somewhat complex. The current DGGE-PCR targeting the highly conserved V3 region cannot distinguish between bacterial organisms and thus, the assay detected the entire bacterial community of milk powders, e.g. psychrophiles, mesophiles and thermophiles compromising the utility of the method. Theoretically, this problem could be bypassed by using the sporulation gene (*spo0A*) as DGGE marker, which is known to be present in endospore-formers only. Furthermore, DGGE could also be used to differentiate viable and dead cells, as well as spores in milk powders, if the newly developed extraction methods from Chapter 5 are used. In order to increase the detection limit of DGGE it is possible to concentrate the DNA by precipitation prior to PCR. Overall, the DGGE method is non-quantitative and thus limited only to the detection of bacteria in milk powder.

Appendix

12.1. Castenholz Medium (CMD)

Castenholz Medium was used in this study to induce sporulation of the thermophilic milk powder bacilli at 55°C. This medium consists of the salt solution (0.1 g l^{-1} nitrilotriacetic acid; 0.06 g l^{-1} $CaSO_4 \cdot 2H_2O$; 0.1 g l^{-1} $MgSO_4 \cdot 7H_2O$; 0.008 g l^{-1} NaCl; 0.103 g l^{-1} KNO_3; 0.0689 g l^{-1} $NaNO_3$; 0.111 g l^{-1} Na_2HPO_4), 1 ml of Nitsch's trace element solution (2.2 g l^{-1} $MnSO_4 \cdot H_2O$; 0.5 g l^{-1} $ZnSO_4 \cdot 7H_2O$; 0.5 g l^{-1} H_3BO_3; 0.016 g l^{-1} $CuSO_4$; 0.025 g l^{-1} $Na_3MoO_4 \cdot 2H_2O$; 0.046 g l^{-1} $CoCl_2 \cdot 6H_20$), 3 g l^{-1} trypticase peptone, 3 g l^{-1} yeast extract and 0.28 mg l^{-1} $FeCl_3$. The iron solution was added separately after filter-sterilisation with an Acrodisc 32 Syringe Filter (0.2 µm Supor Membrane; Pall Corporation; Gelman Laboratory; Ref 4652) after autoclaving of the other constituents to prevent iron precipitation during sterilization. Optionally, additional $MnSO_4$ can be added to 5 mg l^{-1} to facilitate sporulation. The pH of the medium was adjusted with NaOH to 7.8 prior to autoclaving.

12.2. 16S rRNA real-time PCR optimization

12.2.1. Primer optimization

The primer concentration of the quantitative PCR assay targeting the 16S rDNA was optimized on the Smart Cycler II using primer concentrations in the range between 100 and 1100 nM (Figure 12.1, Figure 12.2 and Table 12.1). The mean threshold cycle number (C_t) for all primer concentrations was 14.92 ± 0.25 using 15 ng of genomic target DNA. The primer concentration of 600 nM for forward and reverse primer yielded highest end-point-fluorescence (Figure 12.2, Table 12.1). Subsequent to the amplification reaction the samples were the loaded onto a 1.5% agarose and electrophoresed to visualize the PCR products and any other possible secondary amplicons (Figure 12.3).

Table 12.1. 16S rRNA primer optimization

Sample	Primer concentration [nM]		Threshold cycle number	End-point-fluorescence [units]
	Forward	Reverse		
1	100	100	14.87	517.43
2	200	200	14.94	473.57
3	300	300	15.08	508.00
4	400	400	15.32	493.00
5	500	500	14.70	552.57
6	600	600	14.38	651.71
7	700	700	14.94	514.71
8	800	800	14.80	584.14
9	900	900	15.14	462.75
10	1000	1000	15.05	560.25
11	1100	1100	14.95	536.71

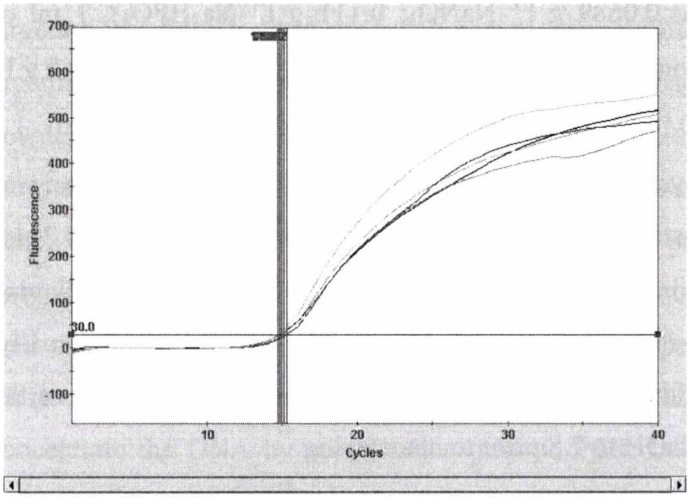


Figure 12.1. Primer optimization of the 16S rDNA PCR assay. The graph shows the primary amplification plots of primer concentrations ranging from 100 to 500 nM for both the forward and reverse primer, respectively.

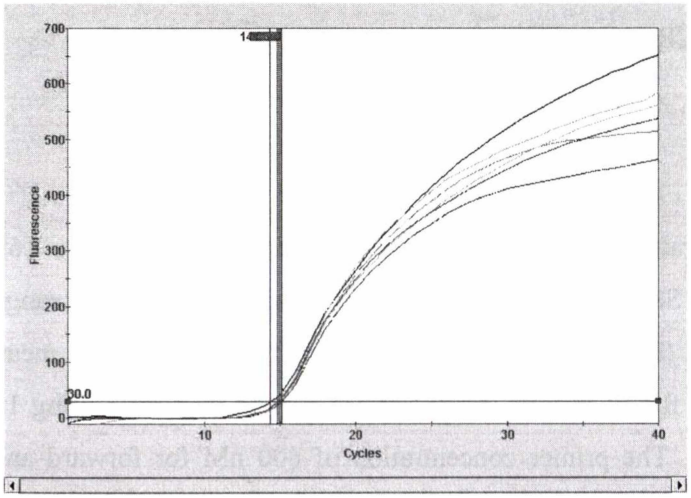


Figure 12.2. Primer optimization of the 16S rRNA PCR assay. The graph shows the primary amplification plots of primer concentrations ranging from 600 to 1100 nM for both the forward and reverse primer, respectively.

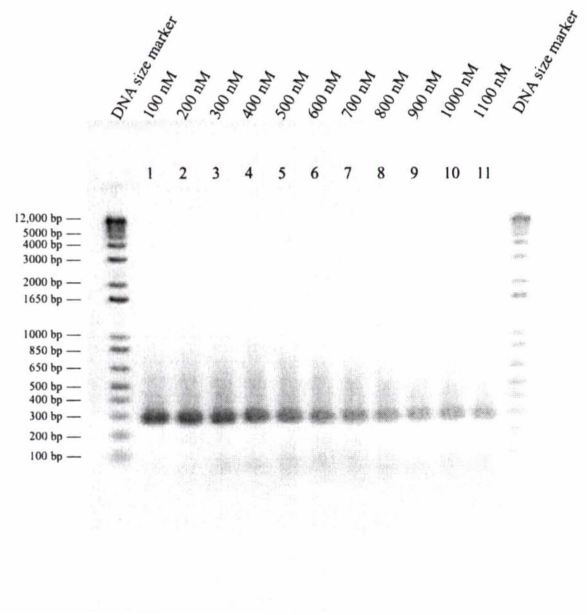


Figure 12.3. Agarose gel electrophoresis of the 16S rDNA PCR amplicons from Figure 12.1 and 12.2. The image demonstrates that the assay cleanly produces one product with a limited formation of primer dimers (<100 bp).

12.2.2. Optimization of the primer annealing temperature

The annealing temperature of the 16S rDNA primers including TaqMan probe was optimized between 58 and 62°C for 20, 30 and 40 seconds using a combined annealing-elongation step. The final annealing-elongation temperature and duration was chosen to be 62°C for 20 seconds (Figure 12.4).

12.2.3. Optimization of the Cy3-BHQ TaqMan probe concentration

The concentration of the TaqMan probe was titrated between 50 and 1000 nM amplifying 15 ng of initial genomic target DNA. Highest end-point fluorescences were obtained using 300 to 1000 nM of probe (Figures 12.5 and 12.6). A final TaqMan probe concentration of 300 nM (Chapter 3) was used for the standard protocol of the 16S rDNA PCR assay, as this was the most cost-effective concentration yielding high end-point fluorescence. However, the concentration of the TaqMan probe was reduced in Chapter 5 to 150 nM to further reduce the cost of the assay. For further research, labelling of the TaqMan probe using the VIC-TAMRA constellation is recommended to yield overall higher end-point fluorescence (Figure 12.4).

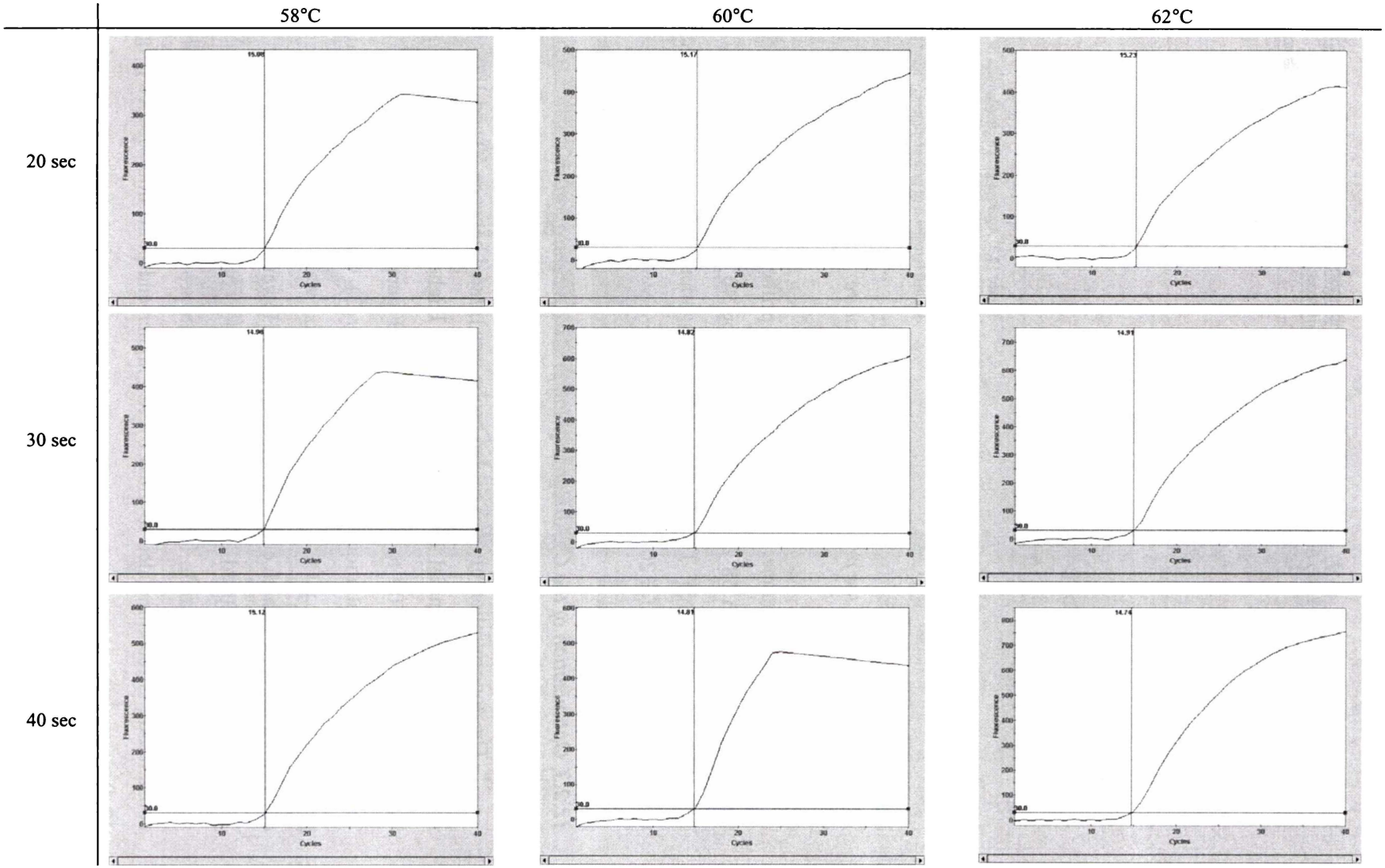


Figure 12.4. 16S rDNA PCR amplification plots for the optimization of primer pair annealing and elongation regarding temperature and duration. The vertical red lines and numbers indicate the threshold crossing and cycle number (C_t). The optimization experiment was performed with the VIC-TAMRA labelled TaqMan probe using 100 nM.

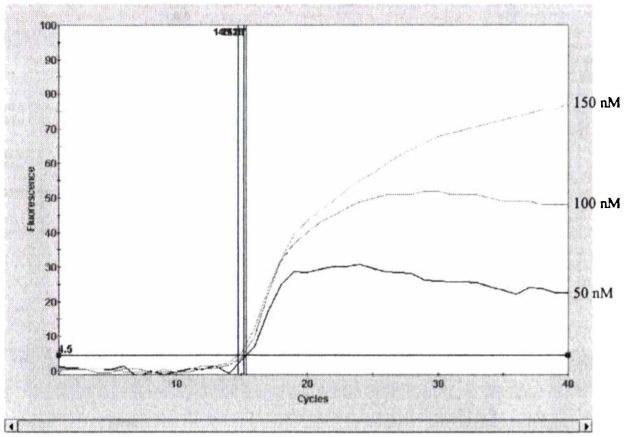


Figure 12.5. Cy3-BHQ TaqMan probe titration employing 50 to 150 nM of probe.

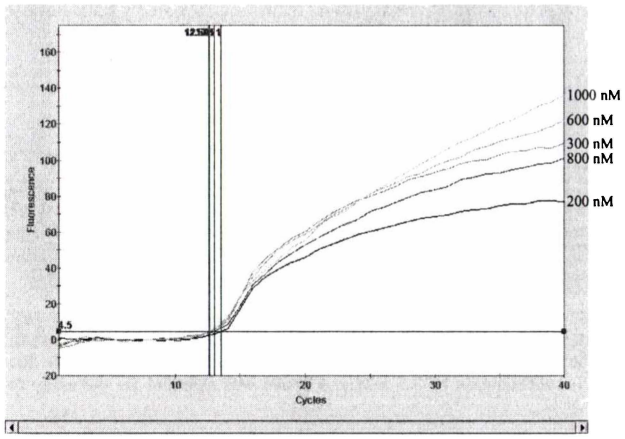


Figure 12.6. Cy3-BHQ TaqMan probe titration employing 200 to 1100 nM of probe.

12.3. *Spo0A* real-time PCR optimization

12.3.1. Titration of $MgCl_2$

The $MgCl_2$ concentration was titrated between 1.5 and 8.5 mM; concentrations of 2.5 mM $MgCl_2$ or less gave no amplification on the Smart Cycler II (Figure 12.7, Figure 12.8, Table 12.2). In contrast, with the Master Cycler (Eppendorf) the PCR reactions amplified well under similar conditions using magnesium chloride concentrations of 1.5 and 2.5 mM (data not shown). Magnesium concentrations between 3.5 and 8.5 mM $MgCl_2$ supported the amplification on the Smart Cycler II equally well while end-point fluorescence readings were highest using between 3.5 and 6.5 mM $MgCl_2$ (Figure 12.7 and 12.8).

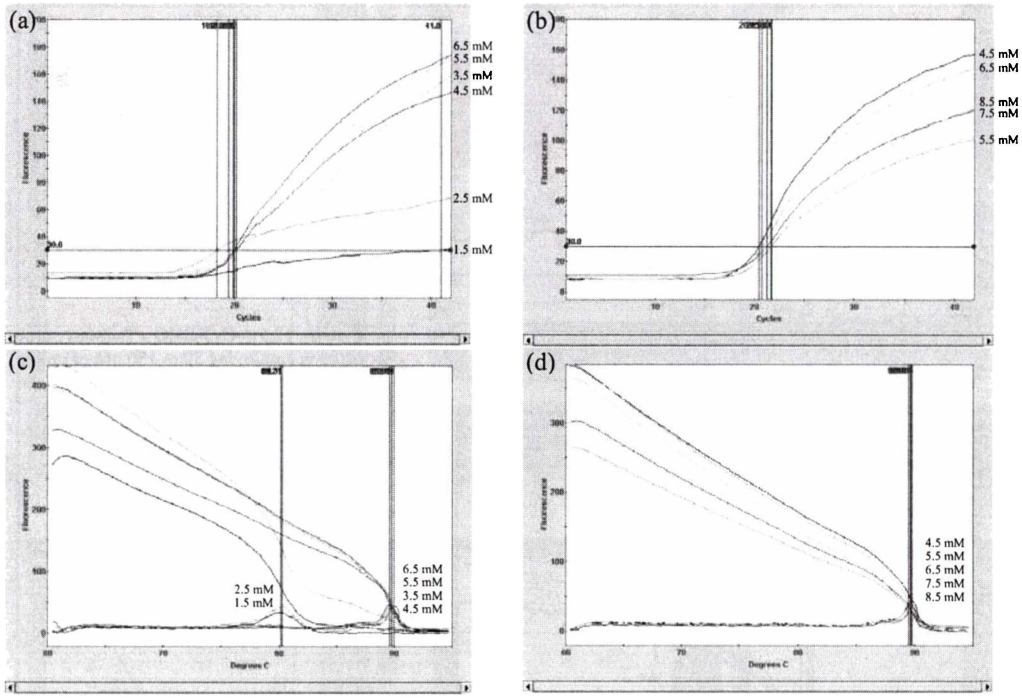
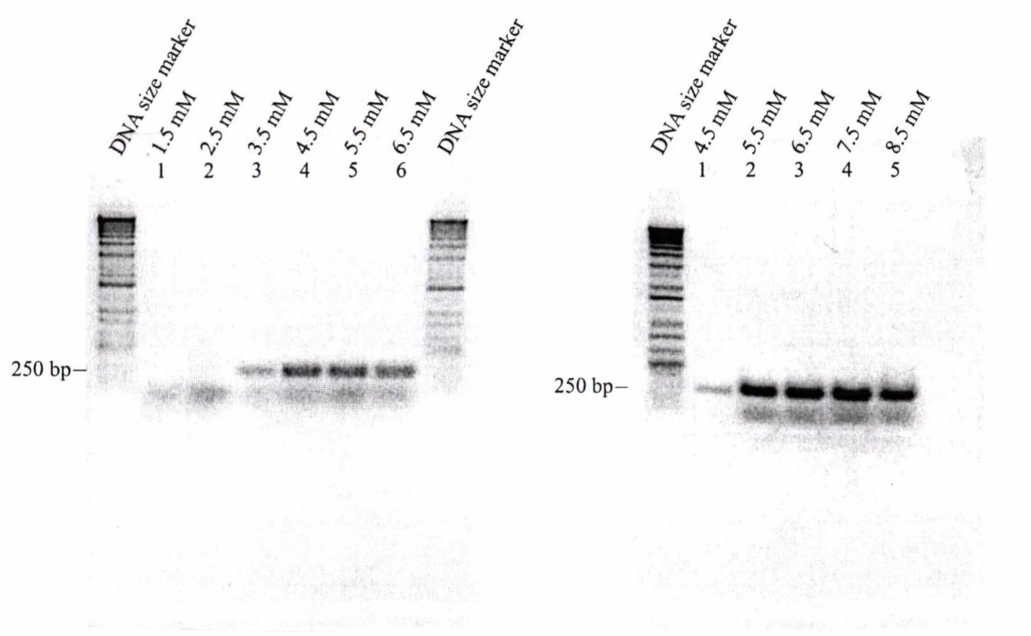


Figure 12.7. Optimization of the $MgCl_2$ concentration of the *spo0A* PCR assay using the Smart Cycler II. The primary amplification plot are shown in (a) and (b) and the corresponding melting curve analyses in (c) and (d). Magnesium concentrations of 1.5 and 2.5 mM failed to amplify the *spo0A* gene. However, the corresponding amplification plots are due to the formation of primer dimers (c). A final $MgCl_2$ concentration of 3.5 mM and above was essential for the assay to amplify the correct amplicon (c and d).

Table 12.2. Optimization of the $MgCl_2$ concentration of the *spo0A* assay

Sample	$MgCl_2$ [mM]	Threshold cycle number	End-point-fluorescence [units]	Melting Temperature [°C]
1	1.5	41.00	31.00	80.21
2	2.5	18.20	69.00	80.31
3	3.5	20.00	159.00	89.76
4	4.5#1	20.20	157.00	90.01
5	4.5#2	20.45	147.00	89.75
6	5.5#1	19.38	101.00	89.79
7	5.5#2	21.67	172.00	89.76
8	6.5#1	19.90	148.00	89.61
9	6.5#2	20.80	174.00	89.64
10	7.5	21.25	120.00	89.61
11	8.5	21.71	121,00	89.43

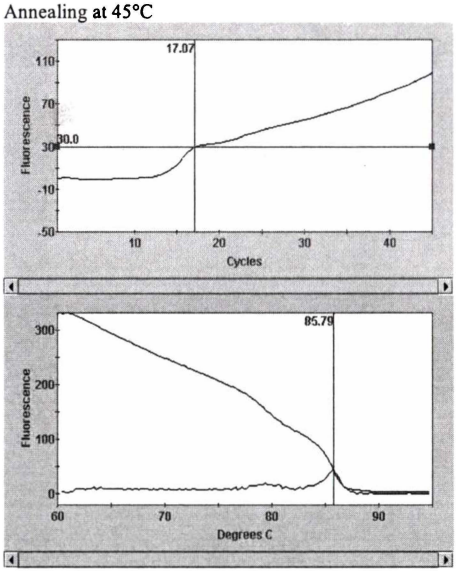


12.8. Agarose gel-electrophoresis of *spo0A* amplicons generated as a function of different $MgCl_2$ concentrations. These reactions are the same as shown in Figure 12.7. In the presence of 2.5 mM magnesium chloride and below there was no PCR amplification of the *spo0A* gene observed. The different banding intensities between gels is due to inconsistent staining with ethidium bromide.

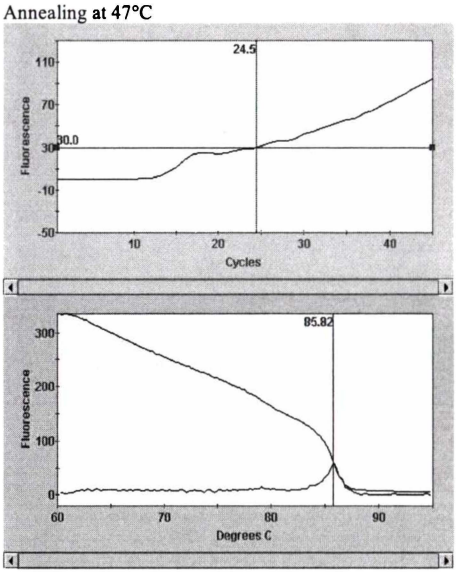
12.3.2. Primer annealing temperature optimization

The primer annealing temperature of the primers targeting the *spo0A* gene was optimized in the range from 45 to 63°C for a holding time of 5 seconds. The experiment was performed using 600 nM of forward and reverse primer amplifying 15 ng of genomic *B. licheniformis* F DNA. The results obtained are shown in Figure 12.9. Accordingly, the threshold cycle changed over the annealing temperature range by approximately one C_t.

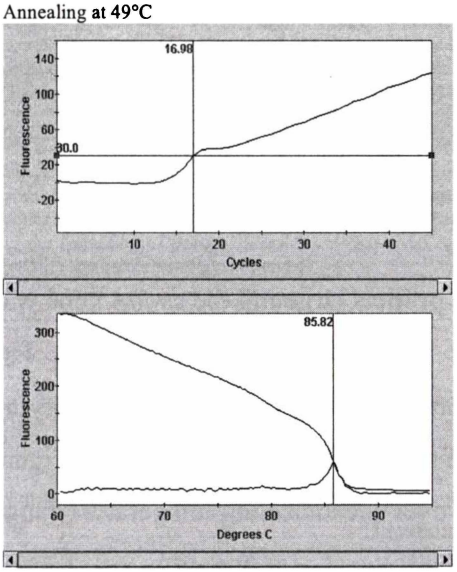
Annealing at 45°C



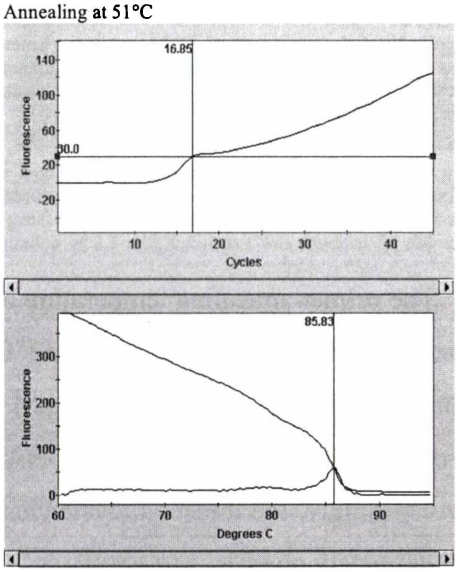
Annealing at 47°C



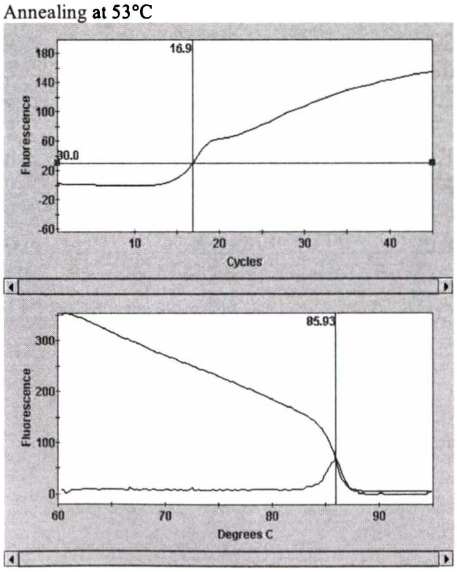
Annealing at 49°C



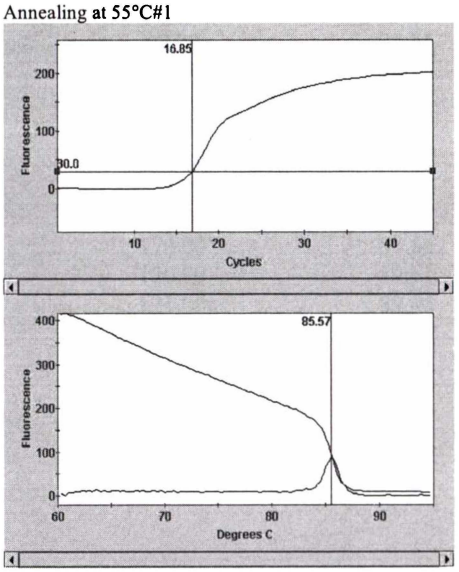
Annealing at 51°C



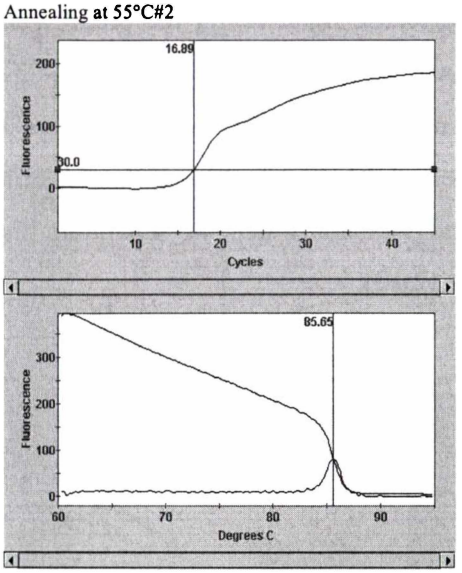
Annealing at 53°C



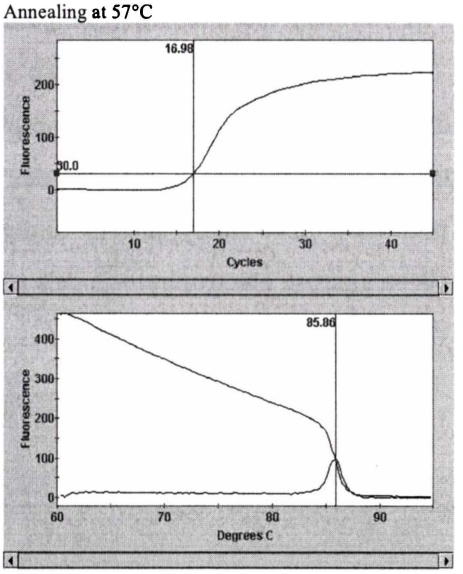
Annealing at 55°C#1



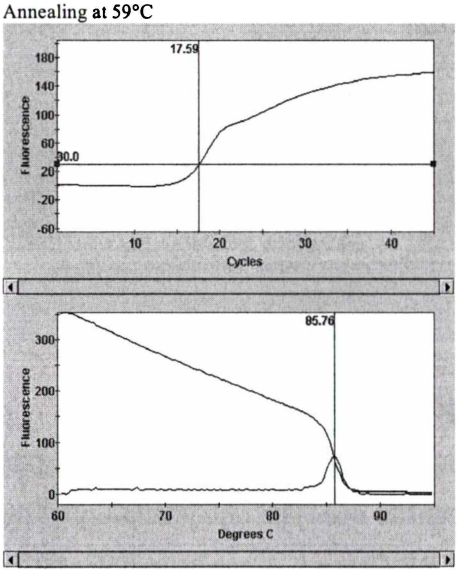
Annealing at 55°C#2



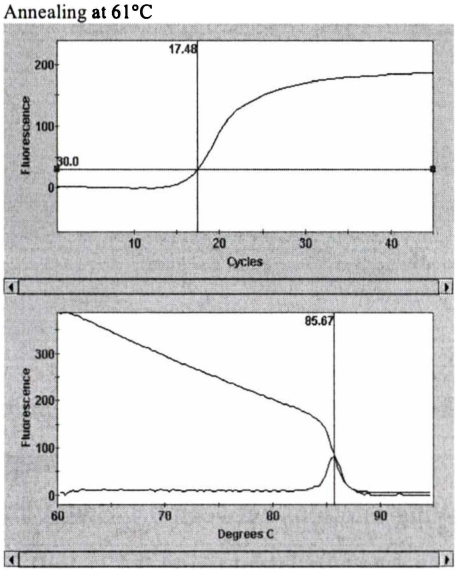
Annealing at 57°C



Annealing at 59°C



Annealing at 61°C



Annealing at 63°C

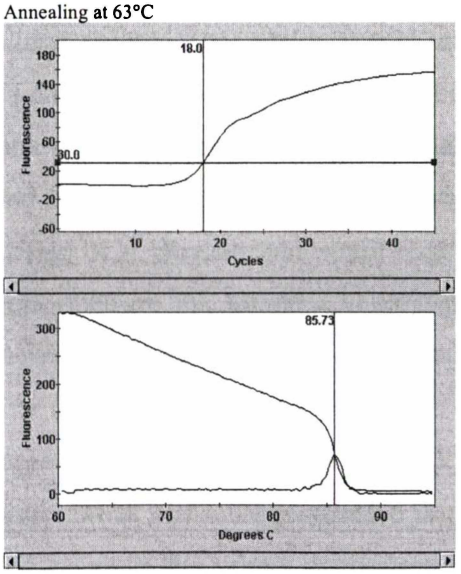


Figure 12.9. Optimization of the primer annealing temperature of the *spo0A* assay on the Smart Cycler II. The images show the primary amplification plots with the corresponding melting curve analyses. PCR reactions with annealing temperatures below 53°C resulted in the generation of primer-dimers, which are represented by atypical amplifications as shown by the irregular amplification curves. More regular amplification curves are exhibited at annealing temperatures of 55°C or greater.

In contrast, the annealing temperature affected significantly the progression curve of the amplification plots (Figure 12.9) and below temperatures of 53°C this was noticeable as a biphasic curve; the biphasic nature being indicative of a change in the order of the amplification reaction due to the increased generation of non-specific PCR products, most likely primer dimers, and was confirmed by separating the products on agarose electrophoresis gels (Figure 12.10).

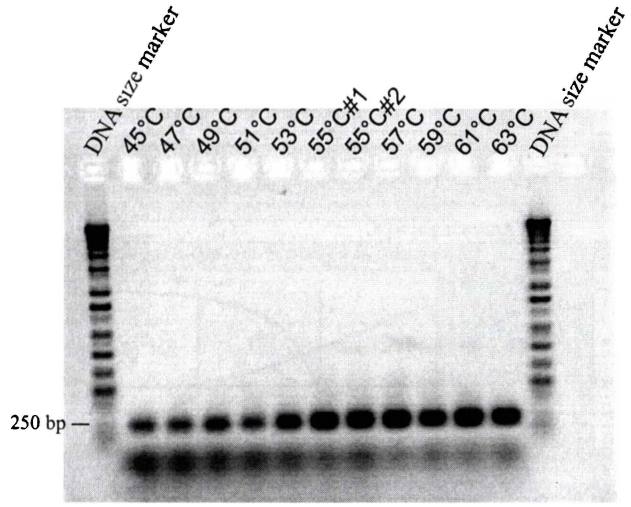


Figure 12.10. Agarose gel electrophoresis of reaction performed to optimize the primer annealing temperature for the *spo0*A PCR assay on the Smart Cycler II. The formation of primer dimers becomes more evident with lower annealing temperatures while product formation was suppressed.

12.3.3. SYBR Green optimization

The SYBR Green I titration was performed using the 16S rDNA PCR assay employing dilutions between 0.125 and 1× of reporter dye. The reproducibility of the reactions was consistent using 0.125 to 0.25× SYBR Green I. A concentration of 0.5× led occasionally to PCR inhibition, especially at low template concentration, while concentrations above 0.75× resulted in PCR failure (data not shown). Figure 12.11 and Table 12.3 show the results obtained titrating SYBR Green between 0.125× and 1× of dye amplifying 1.5 ng of *B. licheniformis* strain F DNA.

Table 12.3. Optimization of SYBR Green I concentration

Sample	SYBR Green	Threshold cycle number	End-point-fluorescence [units]	Melting Temperature [°C]
1	0.125×	12.09	218.80	90.38
2	0.25×	12.23	229.00	90.44
3	0.5×	12.16	660.90	90.63
4	0.75×	29.70	888.60	82.33
5	1.0×	28.73	894.97	82.14

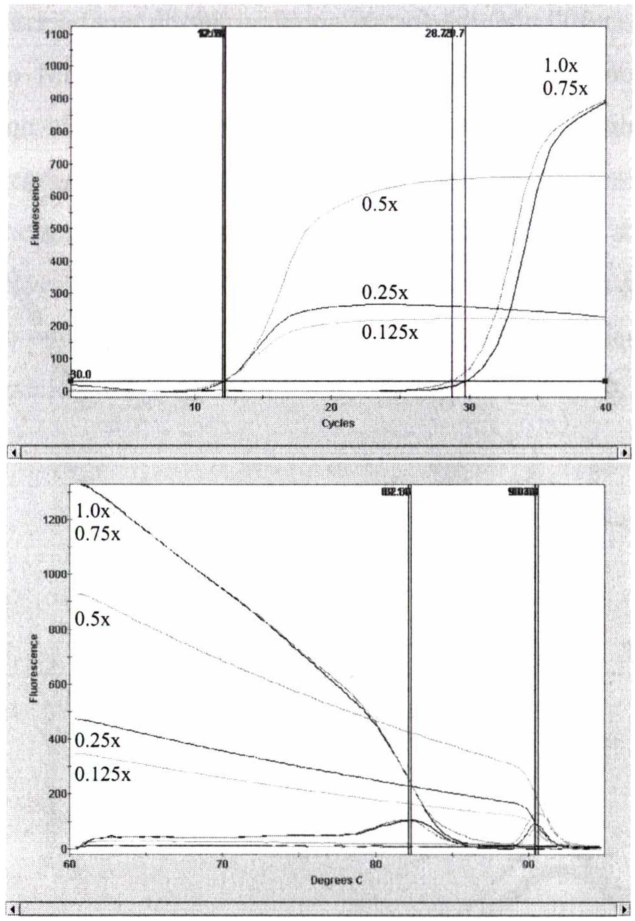


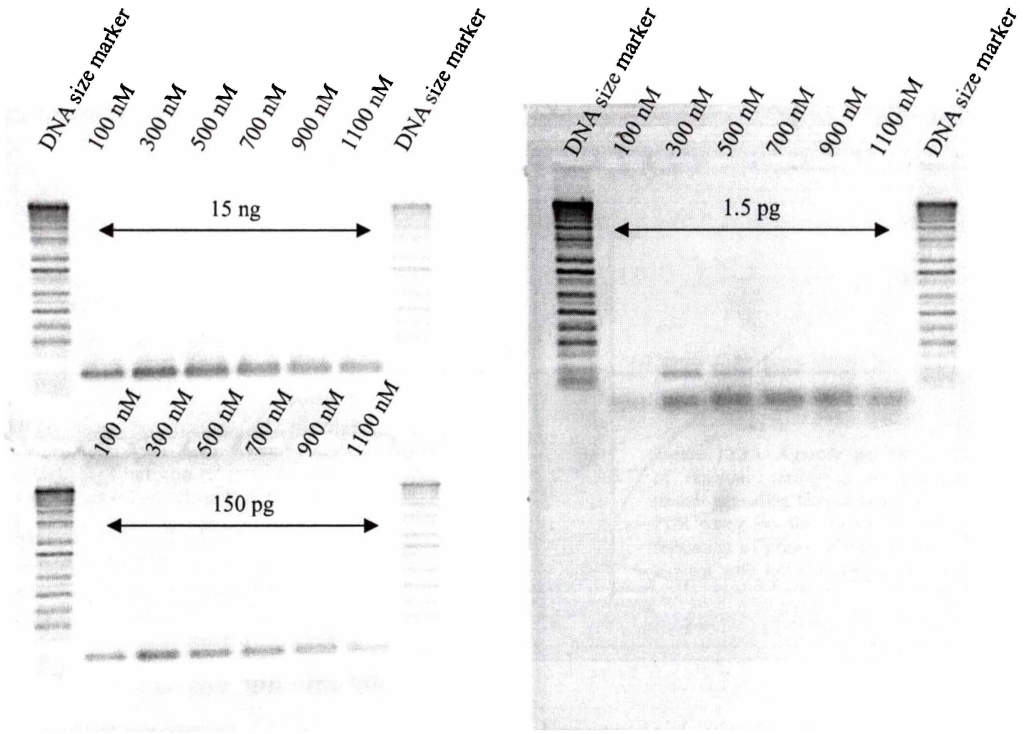
Figure 12.11. Optimization of the SYBR Green I concentration. The experiment was performed employing the 16S rDNA quantitative PCR assay amplifying 1.5 ng of genomic DNA from *B. licheniformis* F. SYBR Green concentrations of up to 0.5× did not affect the amplification. In contrast, reaction containing SYBR Green containing 0.75× or above generated non-specific amplification products.

In order to obtain highest end-point fluorescence while guaranteeing the unhindered amplification with low template concentrations, SYBR Green was generally standardized to 0.25× for quantitative PCR.

12.3.4. Optimization of primer concentration for the spo0A assay

The primer concentration of the *spo0A* PCR assay was titrated between 100 and 1100 nM using low, medium and high target concentrations (1.5 pg, 150 pg and 15 ng) of *A. flavithermus* strain C genomic DNA. The amplification reactions were performed on the Master Cycler (Eppendorf) using the optimized PCR conditions regarding primers, annealing temperature and magnesium concentration. The primer titer reactions amplified well over the entire titration range for high and medium DNA target concentrations (Figure 12.12). In contrast, the amplification from 1.5 pg of target DNA was more responsive to primer concentration as shown in Figure 12.12. For example, the primer concentration of 100 nM failed to amplify while at higher

primer concentrations (greater than 300 nM) the formation of primer-dimers became evident. The experiment was confirmed on the Smart Cycler II and 600 nM of forward and reverse primer amplified best over the DNA template range (data not shown).



12.12. *Spo0A* primer titration performed on high-, medium and low template DNA on the Master Cycler from Eppendorf.

12.3.5. *Spo0A* PCR with mixed populations of thermophilic bacilli

The simultaneous detection of *A. flavithermus* C and *B. licheniformis* F genomic DNA resulted in the acquisition of two composite melting peaks as shown in the example of a mixed spore population of both stains (Figure 12.13). However, at lower target DNA concentrations priming bias was observed, resulting in either a bias to one of the strains or of an undifferentiated wide melting peak resembling neither species.

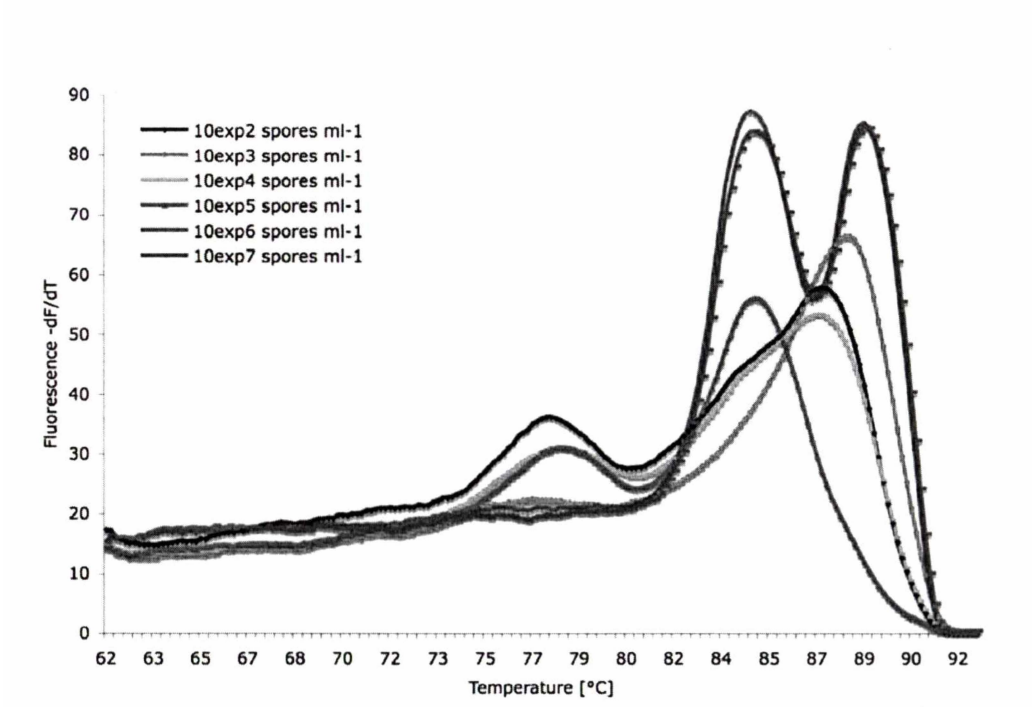


Figure 12.13. Melting curve analysis of *spo0A* amplification products derived from purified genomic DNA of a mixed spore suspension of *B. licheniformis* F and *A. flavithermus* C. The simultaneous amplification of both DNA species resulted in the acquisition of two composite melting peaks, which became more evident the more initial template DNA was used for amplification. The melting peaks appearing at approximately 77 to 78°C are the result of primer-dimers.

12.4. Detection of high, medium and low DNA target numbers with the Smart Cycler II System for two separate quantitative PCR assays

The following two examples demonstrate the linear detection range of the Smart Cycler II, determined for the two quantitative PCR assays using decimal dilution series of *A. flavithermus* strain C genomic DNA. The assays were performed under the optimized conditions described in Chapters 3 and 4. The upper limit of detection for both assays was approximately 15 ng. Initial DNA target concentrations much greater were still well amplified although they were out of the linear range of

the approximation curves (data not shown). The lower limits of detection were 150 fg for the *spo0A* and 15 fg for the 16S rRNA assay, respectively. Furthermore, there was a direct correlation observed between the quantity of initial target DNA and end-point fluorescence value, which was almost entirely independent of the reporter dye used, e.g. SYBR Green or Cy3 (Figure 12.14, 12.15 and 12.16 and Table 12.4 and 12.5).

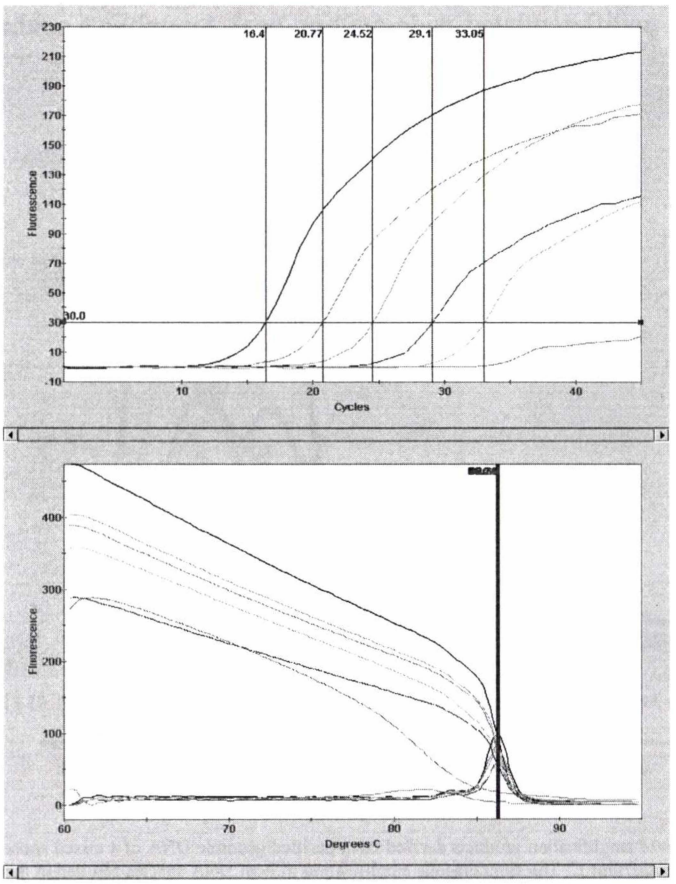
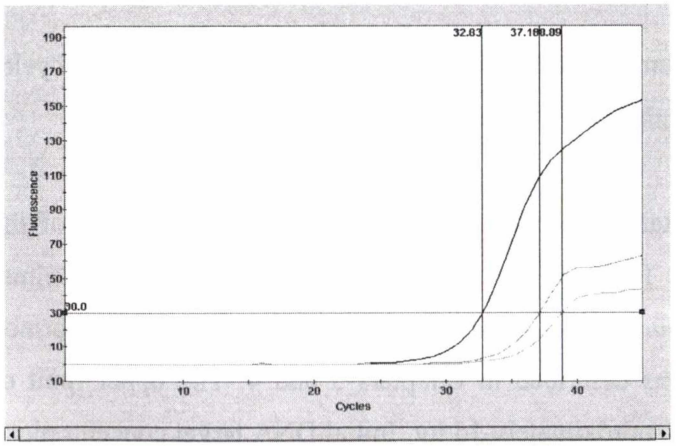


Figure 12.14. Primary amplification plot with corresponding melting point analysis of *spo0A* PCR amplicons using 1.5×10^{1}, 1.5×10^{0}, 1.5×10^{-1} and 1.5×10^{-2}, 1.5×10^{-3} ng of *A. flavithermus* C genomic DNA per reaction. The negative control containing no DNA template did not cross the threshold base line within 45 amplification cycles (Table 12.4).



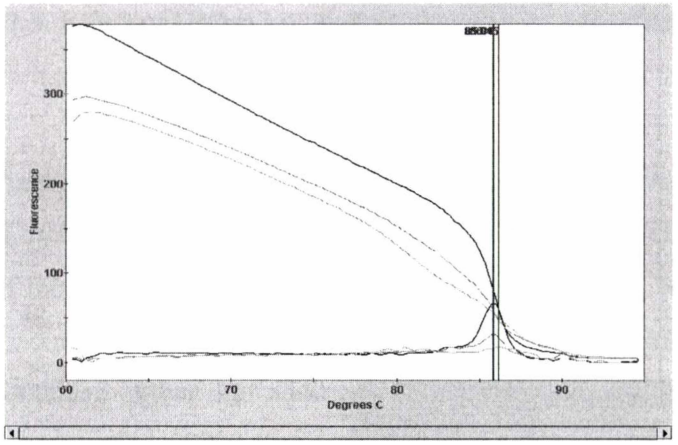
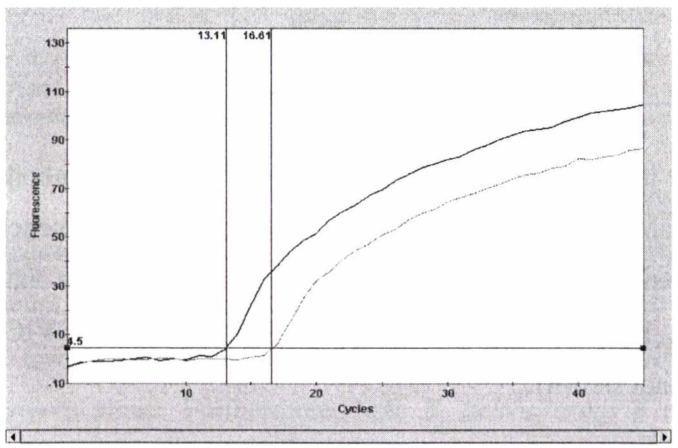


Figure 12.15. Primary amplification plot with corresponding melting point analysis of *spo0A* PCR amplicons using 1.5×10^{-3}, 1.5×10^{-4} and 1.5×10^{-5} ng of *A. flavithermus* C genomic DNA.

Generally, the amount of template DNA was directly proportional to the end-point fluorescence with high initial template numbers producing high end-point fluorescence readings while low initial templates numbers produced low end-point fluorescence readings (Figures 12.14 and 12.15; Table 12.4 and 12.5).

Table 12.4. Detection range of the *spo0A* PCR assay on the Smart Cycler II

Sample	DNA [ng reaction^{-1}]	Threshold cycle number	End-point-fluorescence [units]	Melting Temperature [°C]
1	1.5×10^{1}	16.40	213.00	86.26
2	1.5×10^{0}	20.77	171.00	86.21
3	1.5×10^{-1}	24.52	177.24	86.24
4	1.5×10^{-2}	29.10	115.67	86.26
5a	1.5×10^{-3}	33.05	112.52	86.40
5b	1.5×10^{-3}	32.83	153.00	85.86
6	1.5×10^{-4}	37.18	62.60	85.84
7	1.5×10^{-5}	38.89	43.00	86.15
8	0	-	21.00	-



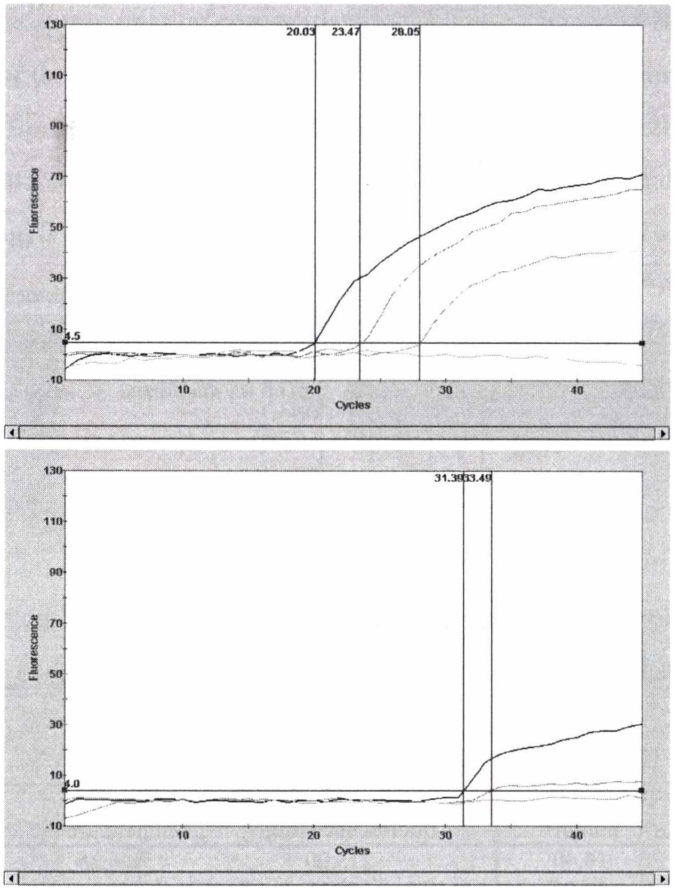


Figure 12.16. Primary amplification plot of 16S rDNA PCR amplicons using 1.5×10^{1}, 1.5×10^{0}, 1.5×10^{-1}, 1.5×10^{-2}, 1.5×10^{-3}, 1.5×10^{-4} and 1.5×10^{-5} ng of *A. flavithermus* C genomic DNA. The negative control containing no DNA template did not cross the threshold base line within 45 amplification cycles (Table 12.5).

Table 12.5. Detection range of the 16S rRNA PCR assay on the Smart Cycler II

Sample	DNA [ng reaction^{-1}]	Threshold cycle number	End-point-fluorescence [units]
1	1.5×10^{1}	13.11	104.81
2	1.5×10^{0}	16.61	86.64
3	1.5×10^{-1}	20.03	70.91
4	1.5×10^{-2}	23.47	64.92
5	1.5×10^{-3}	28.05	40.51
6	1.5×10^{-4}	31.39	30.28
7	1.5×10^{-5}	33.49	7.81
8	0	0	1.15

For the 16S rRNA PCR assay it was found that higher TaqMan probe concentrations (greater than or equal to 300 nM) were unable to increase the end-point fluorescence of the very low template concentrations (data not shown). However, the absolute end-point fluorescence could be increased approximately by 500% using the VIC-TAMRA labelled probe (data not shown).

12.5. Effect of DNase I on the viability and growth of thermophilic bacilli

The effect of DNase I (200 Kunitz units ml^{-1}) and 1× DNase buffer on the viability and growth of *G. stearothermophilus* strain A, *A. flavithermus* strain C and *B. licheniformis* strain F was investigated in TSB-starch at 55°C. The optical density of this medium inoculated with one of the test strains was monitored at 600 nm with readings taken every 30 minutes over a time period of 12.5 hours. Replicates contained either no addition, addition of DNase buffer or DNase at a final concentration of 200 Kunitz units ml^{-1}. The results, which are shown in Figure 12.17 indicated that the addition of 1× DNase I buffer and DNase I had no influence on the growth rate of these cultures which were similar to those grown in TSB-starch.

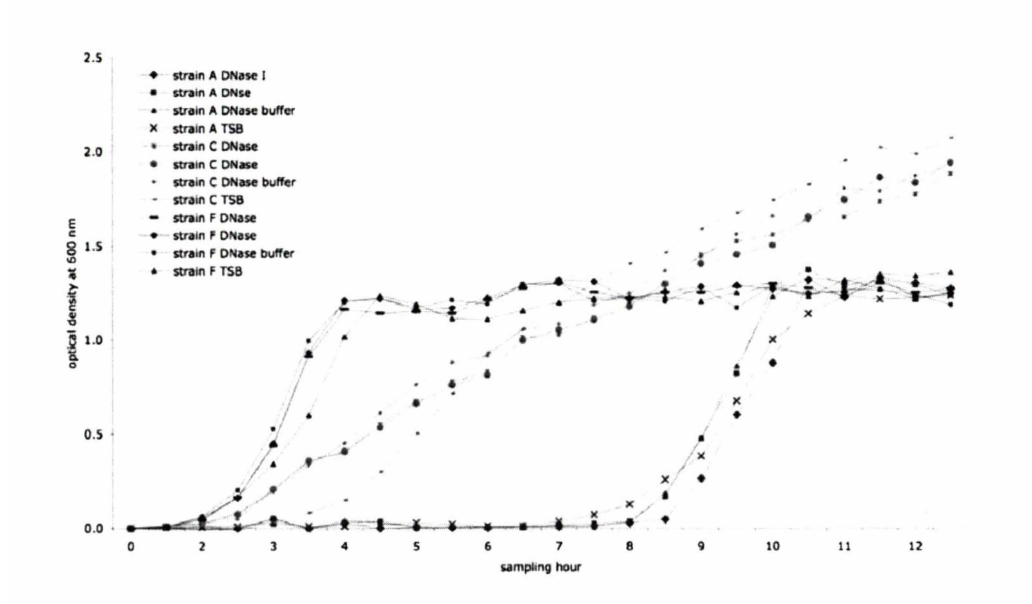


Figure 12.17 demonstrate that the addition of 1× DNase 1 buffer or 200Kunitz units of DNase per ml had no effect of the growth rate of any of the three organisms tested.

The growth properties of the investigated stains differed from each other. *B. licheniformis* F and *A. flavithermus* C completed the lag phase after approximately 2 hours after incubation at 55°C. In contrast, *G. stearothermophilus* A showed an extended lag phase of approximately eight hours before entering the exponential growth phase. Furthermore, both *B. licheniformis* F and *G. stearothermophilus* A reached the stationary phase approximately 2 hours after they had entered the early exponential growth phase. This was substantially different from the observation made for *A. flavithermus* C, as this organism did not possess an exponential growth pattern.

Instead, the exponential growth phase was replaced by a near-linear increase of cell density, which had not yet been completed after 12.5 hours of incubation. Significantly, the end-point OD_{600} reading of *B. licheniformis* and *G. stearothermophilus* was approximately 1.27, whereas the OD_{600} of *A. flavithermus* in the 13th hour of growth was approximately one OD_{600} unit higher (2.22) and was still increasing at the final sampling point (Figure 12.17).

12.6. Germination of thermophilic spores

12.6.1. Germination of B. licheniformis F and A. flavithermus C spores under different conditions

The ability of *A. flavithermus* C and *B. licheniformis* F spores to germinate was investigated in different media. These media were 1× TSB, Buffer A (10 mM L-alanine, 10 mM $CaCl_2$ and Tris-HCl, pH 8.0) and Buffer B (50 mM L-alanine, 50 mM Glucose, 4 mM KCl at pH 8.0). In addition, germination of *B. licheniformis* F spores was also investigated for a spore suspension, which was incubated for 20 minutes in 0.2 M tri-sodium citrate prior to incubation in 1× TSB at 55°C to study if tri-sodium citrate affected the germination rate. Furthermore, germination of strain F spores was also performed in 0.1× TSB and sterilised distilled water.

The germination experiments were performed in the following way: a total of 10^9 spores (microscopic count) were re-suspended in 20 ml of germination medium and the suspensions incubated in a water bath at 55°C for up to 210 minutes in 50 ml Falcon tubes. In addition, one spore sample of each strain was heat-activated at 80°C for 20 minutes in sterile water before the activated spores were re-suspended in 1× TSB for germination. The remaining samples were not heat-activated. The extent of germination and cell growth was assessed by OD readings at 600 nm and microscopy every 10 minutes (Figures 12.18 and 12.19; Table 12.6 and 12.7). The visual observation of germination under the microscope was recorded using the following terms:

1. Core spore: endured spore form with no noticeable visual changes of the spore morphology, size and refraction.

2. Spore swelling: noticeable increase of spore size attended with a decrease of spore refraction
3. Outgrowth: some spores grow out by releasing a vegetative cell form
4. Outgrown: the majority of spores are outgrown
5. Cell division: vegetative cell proliferation

The three spore suspensions of *B. licheniformis* F grown in 1× TSB showed similar germination rates and their growth curves were nearly identical despite the pre- treatments applied, e.g. no-treatment, heat-treatment or tri-sodium citrate treatment. The sample grown in 0.1× TSB germinated as efficiently as the 1× TSB samples (Table 12.6) although the culture possessed a reduced growth rate during cell division probably due to nutrient deficiency. The OD_{600} of the samples incubated in Buffer A and B did not increased during incubation at 55°C.

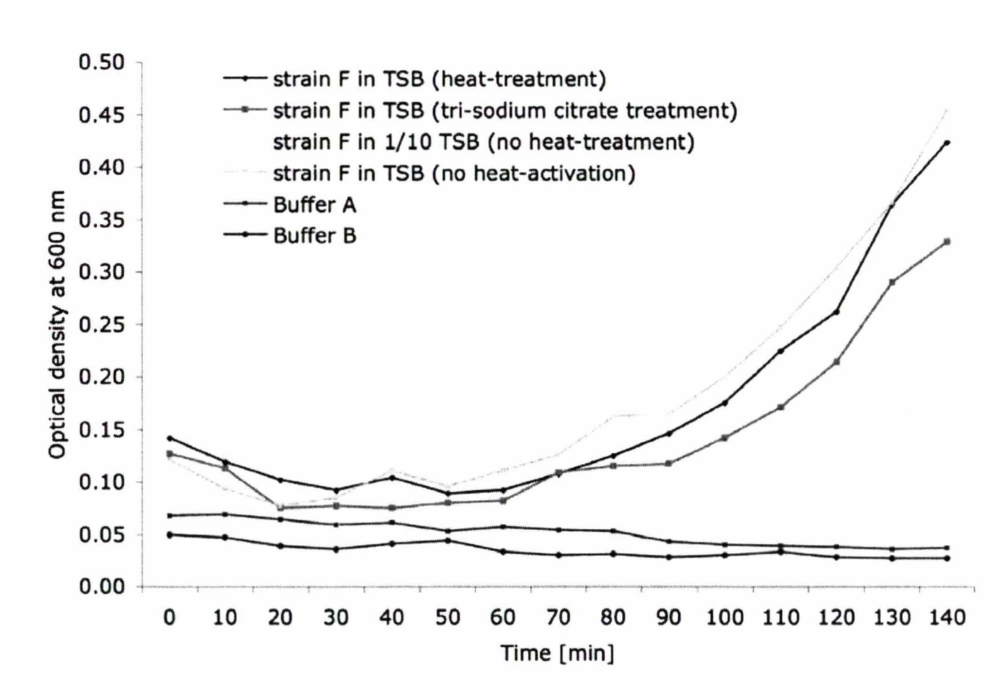


Figure 12.18. Germination efficiencies of *B. licheniformis* F in different media.

However, the spore suspension of strain F incubated in Buffer B showed rapid spore swelling during the first 10 minutes of incubation similar to that observed with the TSB samples. However, germination did not extend past the stage of spore swelling.

Table 12.6. Microscopical observation of spore germination and cell division of *B. licheniformis* strain F in different media at 55°C

Time	Germination medium					
[min]	TSB[a]	TSB[b]	1/10 TSB[a]	TSB[c]	Buffer A[a]	Buffer B[a]
0	core spore	core spore	core spore	core spore	core spore	core spore
10	spore swelling	spore swelling	spore swelling	spore swelling	core spore	spore swelling
20	spore swelling	spore swelling	spore swelling	spore swelling	core spore	spore swelling
30	spore swelling	spore swelling	spore swelling	spore swelling	core spore	spore swelling
40	outgrowth	outgrowth	outgrowth	outgrowth	core spore	spore swelling
50	outgrowth	outgrowth	outgrowth	outgrowth	core spore	spore swelling
60	outgrown	outgrown	outgrowth	outgrown	core spore	spore swelling
70	outgrown	outgrown	outgrown	outgrown	core spore	spore swelling
80	cell division	cell division	cell division	cell division	core spore	spore swelling
90	cell division	cell division	cell division	cell division	core spore	spore swelling
100	cell division	cell division	cell division	cell division	core spore	spore swelling
110	cell division	cell division	cell division	cell division	core spore	spore swelling
120	cell division	cell division	cell division	cell division	core spore	spore swelling
130	cell division	cell division	cell division	cell division	core spore	spore swelling

[a] No spore activation by heat.
[b] Spores incubated in 0.2M tri-sodium citrate prior to incubation in TSB.
[c] Spores activation by heat at 80°C for 20 min in sterilized water.

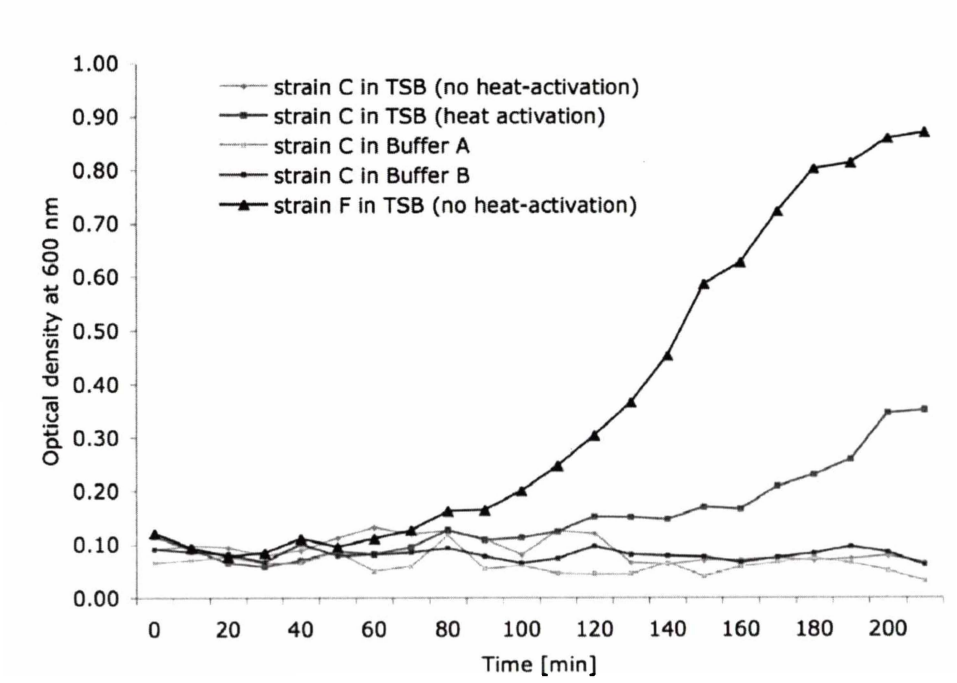


Figure 12.19. Germination efficiencies of *A. flavithermus* strain C in different media. In addition, a spore culture of B. licheniformis strain F was also included in order to demonstrate the different germination and growth properties,

The heat-activated spore suspension of *A. flavithermus* C that was grown in 1× TSB completed outgrowth after 40 minutes and entered the exponential phase of growth (which is rather linear) after 90 minutes of incubation at 55°C. In contrast, the

spore suspension, which was not exposed to the heat-treatment regime, showed no indication of germination or spore swelling within 210 minutes of incubation. Similarly, the spores suspension incubated in Buffers A and B showed no indication of induction of germination. Furthermore, a *B. licheniformis* F spore culture incubated in 1× TSB with no heat-activation was included to demonstrate the different progressions of growth from Figure 12.17.

Table 12.7. Microscopical observation of spore germination and cell division of *A. flavithermus* strain C in different media at 55°C

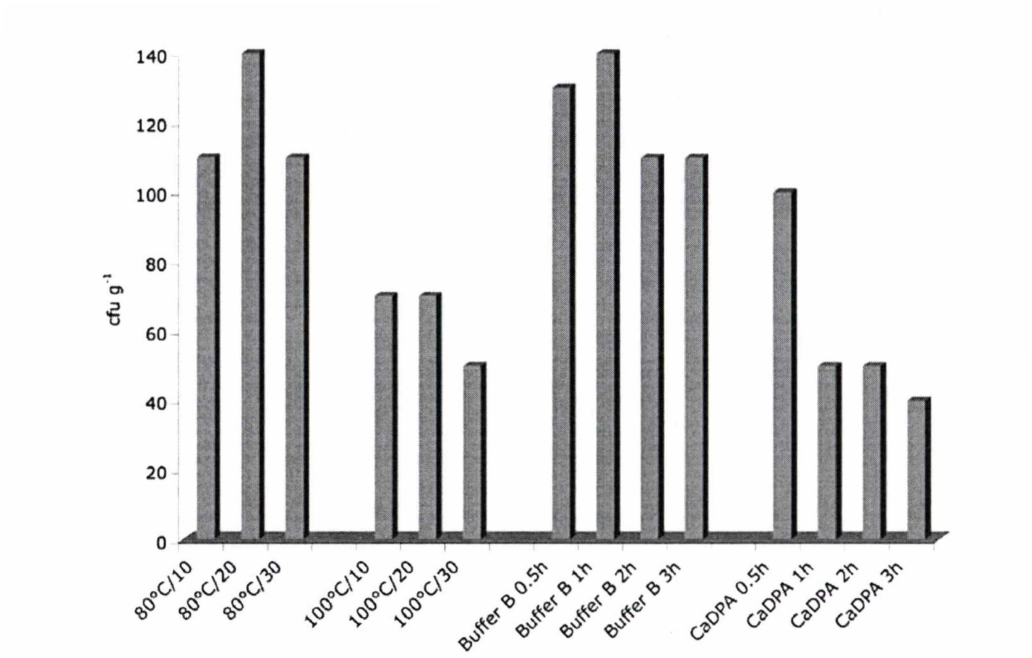
Sample number	Time [min]	Germination medium			
		TSB[a]	TSB[b]	Buffer A[a]	Buffer B[a]
1	0	core spore	core spore	core spore	core spore
2	10	core spore	spore swelling	core spore	core spore
3	20	core spore	spore swelling	core spore	core spore
4	30	core spore	outgrowth	core spore	core spore
5	40	core spore	outgrown	core spore	core spore
6	50	core spore	cell division	core spore	core spore
7	60	core spore	cell division	core spore	core spore
8	70	core spore	cell division	core spore	core spore
9	80	core spore	cell division	core spore	core spore
10	90	core spore	cell division	core spore	core spore
11	100	core spore	cell division	core spore	core spore
12	110	core spore	cell division	core spore	core spore
13	120	core spore	cell division	core spore	core spore
14	130	core spore	cell division	core spore	core spore
15	140	core spore	cell division	core spore	core spore
16	150	core spore	cell division	core spore	core spore
17	160	core spore	cell division	core spore	core spore
18	170	core spore	cell division	core spore	core spore
19	180	core spore	cell division	core spore	core spore
20	190	core spore	cell division	core spore	core spore
21	200	core spore	cell division	core spore	core spore
22	210	core spore	cell division	core spore	core spore

[a] No spore activation by heat.
[b] Spores activation by heat at 80°C for 20 min in sterilized water.

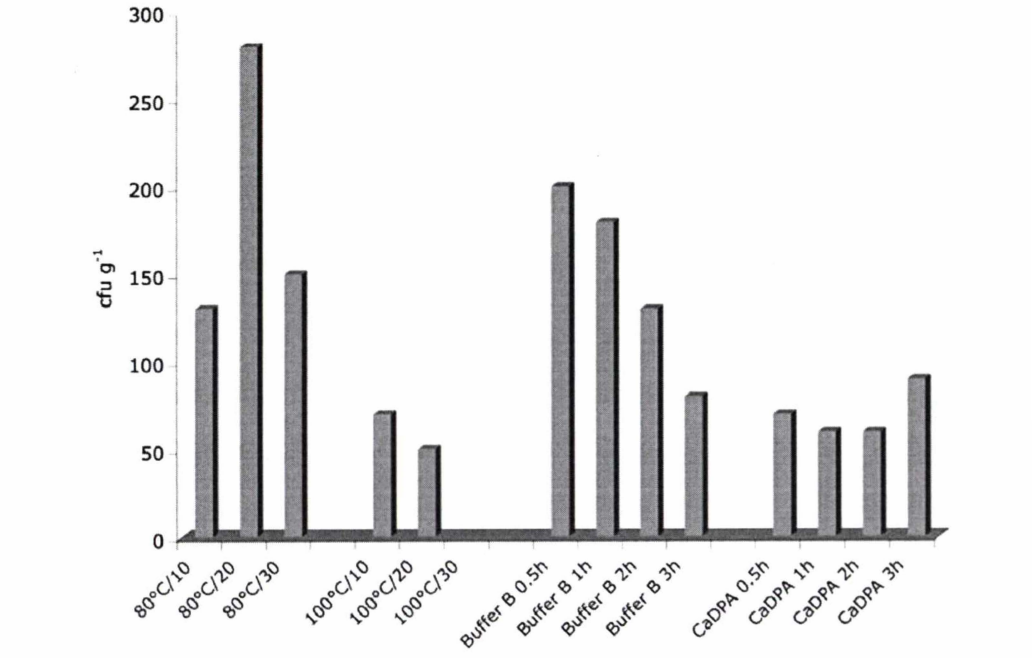
12.6.2. Germination of thermophilic bacilli in reconstituted milk powder using different germination conditions

The findings made in Chapter 5 indicate an excess of spores in some of the milk powders screened, which could not be activated by the heat-treatment at 80°C for 20 minutes. For example, quantitative PCR performed on the factory powder GO12 (12^{th} hour) and in the Mexican powder overestimated spore numbers by 1 and 3 orders of magnitude, respectively. In an attempt to increase the germination rate in these milk powders different activation regimes were investigated in duplicate

including heat-treatments of reconstituted milk at 80 and 100°C (for 10, 20 and 30 minutes) and germination buffers prior to plating in TSA-starch and incubation at 55°C for 16 to 24 hours. The germination buffers were Buffer B (section 12.6.2) and CaDPA buffer (0.04 M $CaCl_2$, 0.04 M dipicolinic acid, 0.08 M NaCl, 0.01 M Tris-HCl at pH 7.7). The results are shown in Figure 12.20 and 12.21.



12.20. Germination efficiency of the Mexican milk powder sample using different activation protocols



12.21. Germination efficiency of milk powder sample GO12 (12^{th} hour) using different activation protocols.

The highest germination rate for both milk samples was obtained by heat-treatment at 80°C for 20 minutes. Temperature holding times of 10 or 20 minutes yielded less spores to germinate. Similarly, heat-activation at 100°C was inhibitory to the germination process with holding times for 30 minutes yielding lowest cell recoveries. The second most effective germination regime was incubation of the milk samples in Buffer B with the tendency that prolonged incubation cause fewer spores to germinate. Inducing germination by dipicolinic acid caused only a small proportion of the spores to form colonies.

In conclusion, this limited experiment demonstrated that the efficiency of germination depends strongly on the germination activation regime applied. As shown in Chapter 2, milk powders can contain different species of thermophilic bacilli, which may require different germination conditions for maximum germination, as was shown for *A. flavithermus* strain C and *B. licheniformis* strain F (section 12.5). Under these circumstances, i.e. without knowing the strain distribution of the spores in powders, it is difficult to establish a germination regime that is optimal for all species of bacilli spores. Interestingly, spore activation at 100°C or prolonged duration at 80°C was inhibitory to the germination process. It is conceivable that the spores that are generated in the preheat have different properties to those generated in the calandria, and these effects will also be dependent on species, the type of heat-treatments used (low, medium and high) and the powder itself, e.g. skim-, whole-, butter- or goat milk powder.

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