Identification and characterization of genes involved in *Bacillus cereus* biofilm formation

by

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SUMMARY

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Bacillus cereus is a Gram-positive, spore-forming bacterium that is frequently identified as the causative agent of food-borne diseases and is also implicated in food spoilage of especially dairy products. The capacity of *B. cereus* to form biofilms on different substrata is of great concern in the food industry. Not only does biofilm formation cause economic loss by equipment failure, but contamination of food products via biofilm cells also raises safety concerns. Bacterial biofilms have been defined as structured multicellular communities that form through a complex developmental process. In contrast to Gram-negative bacteria, biofilm formation by Gram-positive bacteria has only recently been examined. Relatively few genes have been identified that are required for these bacteria to form biofilms and little is known about how they coordinate biofilm formation. In order to contribute to the advancement of knowledge regarding the process of biofilm formation in Gram-positive bacteria, the aim of this investigation was essentially to identify and characterize genes involved in *B. cereus* biofilm formation.

To investigate, *B. cereus* ATCC 14579 was subjected to transposon mutagenesis with the Tn*917*-LTV1 transposon. Screening of a collection of 3 500 insertional mutants for the ability

to form biofilms at the solid-liquid-air interface of glass surfaces led to the identification of eight biofilm-impaired mutants. Each of the mutants contained a single transposon insertion, and no significant differences were observed in the planktonic growth rate between the *B. cereus* wild-type and biofilm-impaired mutant strains. The chromosomal transposon insertion in three of the mutants mapped to genes involved in purine biosynthesis (*purA*, *purC* and *purL*), while the transposon insertion in two other mutants mapped to the *ftsE* gene and to the promoter region of the *motA* gene, respectively. In one of the mutants the transposon was located in the intergenic region between two divergently transcribed genes, which encodes a murein hydrolase exporter and nucleoside hydrolase, respectively. In the final two biofilm-impaired mutants the transposon was respectively mapped to genes encoding a putative membrane spanning protein and a putative protein of unknown function. Results obtained by quantitative real-time PCR assays indicated that expression of each of the identified *B. cereus* ATCC 14579 genes, with the exception of the *motA* gene, was up-regulated in the biofilm population. In the case of *motA*, expression of the gene was down-regulated 3.2-fold in the biofilm population and results obtained during the course of this investigation indicated that motility, rather than the presence of flagella, is required for *B. cereus* biofilm formation. Although this result is in agreement with that reported previously for *B. subtilis*, none of the other genes identified in this investigation have previously been implicated in biofilm formation by Gram-positive bacteria.

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LIST OF ABBREVIATIONS

A	Absorbance
AI-2	autoinducer-2
bp	base pair
bla	β-lactamase gene
ca.	approximately
cat	chloramphenicol acetyltransferase gene
°C	degrees Celsius
cDNA	complementary DNA
cfp	cyan fluorescent protein gene
cm	centimetre
CP	crossing point
CsCl	cesium chloride
CSF	competence and sporulation factor
CTAB	hexadecyltrimethylammonium bromide
d	day
DEPC	diethyl pyrocarbonate
dH_2O	distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside-5'-triphosphate
EDTA	ethylenediaminetetra-acetic acid
e.g.	for example
erm	erythromycin resistance gene
EtOH	ethanol
Fig.	figure
gfp	green fluorescent protein gene
× *g*	centrifugal force
h	hour
IPTG	isopropyl β-D-thiogalactoside
i.e.	that is
kb	kilobase pairs

KOAc	potassium acetate
lacZ	β-galactosidase gene
LB	Luria-Bertani
l	litre
M	molar
mg	milligram
min	minute
ml	millilitre
mM	millimolar
Mut	mutant
ng	nanogram
nM	nanomolar
nm	nanometer
nt	nucleotide
ORF	open reading frame
PCR	polymerase chain reaction
PVC	polyvinylchloride
RNA	ribonucleic acid
RT	reverse transcriptase
s	second
SDS	sodium dodecyl sulphate
TE	Tris-EDTA
2-DE	two-dimensional electrophoresis
U	units
µg	microgram
µl	microlitre
µM	micromolar
UV	ultraviolet
v.	version
V	volts
v/v	volume per volume
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
yfp	yellow fluorescent protein gene

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RESEARCH COMMUNICATIONS

Papers published:

1. Vilain, S., **Pretorius, J.M.**, Theron, J. and Brözel, V.S. (2009). DNA as an adhesin: *Bacillus cereus* requires extracellular DNA to form biofilm. Applied and Environmental Microbiology 75, 2861-2868.

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CHAPTER ONE
LITERATURE REVIEW

1.1 General introduction

1.1.1 *Bacillus cereus*

The *Bacillus cereus* group is a very homogenous cluster within the genus *Bacillus* and comprises six recognized species: *B. cereus*, *B. thuringiensis*, *B. anthracis*, *B. mycoides*, *B. pseudomycoides* and *B. weihenstephanensis* (Jensen *et al.*, 2003; Priest *et al.*, 2004). Of these, *B. cereus* and *B. anthracis* have received considerable attention due to their clinical importance, while *B. thuringiensis* has been used to control insect pests since the 1920s.

Bacillus cereus is an opportunistic pathogen commonly isolated from soil and causes two distinct types of food-poisoning syndromes, *i.e.* emesis and diarrhea (Granum and Lund, 1997). The emetic syndrome is characterized by nausea and vomiting, and is caused by cereulide, a small cyclic non-ribosomally synthesized heat-stable dodecadepsipeptide. The cereulide synthetase gene cluster is located on pCER270, a mega-virulence plasmid related to the pXO1 virulence plasmid of *Bacillus anthracis* (Hoton *et al.*, 2005; Ehling-Schulz *et al.*, 2006; Rasko *et al.*, 2007) and is restricted to a single evolutionary lineage of closely related emetic strains (Ehling-Schulz *et al.*, 2005). The emetic syndrome is usually relatively mild, but may occasionally have a fatal outcome (Mahler *et al.*, 1997; Dierick *et al.*, 2005). The diarrheal syndrome is caused by heat-labile enterotoxins produced during vegetative growth in the small intestine. Three different enterotoxins have been characterized, namely haemolysin BL (Hbl) (Beecher *et al.*, 1995), non-haemolytic enterotoxin (Nhe) (Granum *et al.*, 1999) and cytotoxin K (CytK) (Lund *et al.*, 2000).

Bacillus thuringiensis is mainly regarded as an insect pathogen (Jensen *et al.*, 2003). It produces large crystal protein inclusions (δ-endotoxins) during sporulation. The genes encoding these insecticidal proteins are located on large transmissible plasmids (Rasko *et al.*, 2005). *Bacillus anthracis* is the etiological agent of anthrax, an acute fatal disease of mammals in general but herbivores in particular (Mock and Fouet, 2001). Spores germinate within the host to produce vegetative cells, which multiply and synthesize plasmid-encoded virulence factors that ultimately kill the infected host. Virulent strains carry two large plasmids, *i.e.* pXO1 (181 kb) and pXO2 (96 kb). Whereas pXO1 harbours the genes encoding

the tripartite lethal toxin, pXO2 encodes the genes responsible for the regulation and synthesis of another virulence factor, namely the poly-γ-D-glutamic acid capsule (Rasko *et al.*, 2005).

Based on comparative genomic analyses of *B. cereus*, *B. anthracis* and *B. thuringiensis*, it was shown that these organisms carry different plasmids in a highly similar genetic background (Ivanova *et al.*, 2003; Read *et al.*, 2003; Hoffmaster *et al.*, 2004; Rasko *et al.*, 2004; Han *et al.*, 2006). Only subtle differences in gene content and protein similarities are observed when the chromosomes are compared. Based on the high genetic relatedness between these three *Bacillus* species, it has been suggested that they should be grouped together as members of a single species, namely *Bacillus cereus sensu lato* (Helgason *et al.*, 2000; Rasko *et al.*, 2005).

1.1.2 *Bacillus cereus* ecology

Bacillus species are commonly isolated from soil, with members of the *Bacillus cereus* group being the most prevalent (von Stetten *et al.*, 1999). Because of its prevalence in soil, *B. cereus* (*sensu stricto*) has traditionally been viewed as a saprophytic soil organism. However, *B. cereus* has also been observed in the gut microflora of various invertebrates (Margulis *et al.*, 1998; Luxananil *et al.*, 2001), leading to the suggestion that this might be its natural habitat (Jensen *et al.*, 2003). *B. anthracis* and *B. thuringiensis* are mammalian and insect pathogens, respectively, and do not normally grow in soil. In the case of *B. anthracis* the vegetative cells reach a serum concentration of greater than 10^7 cells/ml in an infected animal prior to death. The cells are subsequently out-competed by anaerobic bacteria from the gastrointestinal tract of the carcass, leading to sporulation of *B. anthracis* cells. These spores then lie dormant in the soil until they are ingested by grazers, reinitiating the cycle of infection. It is unclear whether *B. anthracis* spores are capable of germination and growth in the environment (Van Ness, 1971). Growth of *B. anthracis* outside its host often leads to the loss of the pXO2 virulence plasmid (Jensen *et al.*, 2003), indicating that vegetative growth might be restricted to infected hosts. *B. thuringiensis* has been reported to grow in soil under favourable conditions (Saleh *et al.*, 1970) and is thought to occur naturally as a member of the intestinal flora of various invertebrates (Jensen *et al.*, 2003).

A recent paper by Vilain *et al.* (2006) demonstrated that *B. cereus* is well adapted for growth as a saprophytic soil organism. It was shown that not only was *B. cereus* capable of vegetative

growth and eventual sporulation in a medium consisting of only soil-extracted solubilized organic matter, but spores inoculated into the same medium were also shown to be capable of germination and subsequent vegetative growth. Notably, *B. cereus* cells grown in this medium was shown to switch to a multicellular phenotype that is characterized by formation of filaments and aggregation into macroscopic clumps. This was in contrast to the single-celled growth observed in rich media. It was furthermore shown that *B. cereus* was capable of translocation through soil in a manner that is independent of flagellar motility. Rather, translocation appeared to occur as a result of extension of the multicellular filaments through growth and cell division. This data therefore provides strong support for *B. cereus* being primarily a soil saprophyte. It is interesting to note that *B. cereus* preferentially displays a multicellular phenotype when growing in its natural environment, be it as translocating bundled filaments in soil (Vilain *et al.*, 2006) or as filaments attached to the invertebrate intestine (Margulis *et al.*, 1998). It may therefore be possible that the single-celled growth of *B. cereus* in nutrient-rich liquid media is an artifact of the laboratory culturing conditions.

1.1.3 *Bacillus cereus* pathogenesis

In addition to its familiar role in food-borne infections and intoxications, *B. cereus* has long been known to be capable of causing opportunistic infections in individuals recovering from surgery and in immunocompromised individuals (Turnbull *et al.*, 1979). It has, however, not been regarded as a true infective pathogen of healthy individuals and the identification of *B. cereus* in the clinical laboratory is usually dismissed as either insignificant or as contamination (Miller *et al.*, 1997). Nevertheless, several recent reports have documented fatal and life-threatening *B. cereus* infections in apparently healthy individuals. In all cases the symptoms resembled those of pulmonary anthrax (Miller *et al.*, 1997; Hoffmaster *et al.*, 2004; Avashia *et al.*, 2007).

The first case of a life-threatening *B. cereus* infection was reported in 1994 in Louisiana and involved a welder by trade. The patient survived, but only after 44 days of mechanical ventilation, aggressive antimicrobial chemotherapy and a partial lobectomy. *B. cereus* G9241, the etiological agent in this near-fatal case, was characterized in 2004 after a retrospective analysis of *B. cereus* isolates that caused unusually severe human disease. The isolate was identified as *B. cereus* by traditional phenotypic characterization and differed from *B. anthracis* in all respects, except for the fact that it expressed a capsule, which is unusual for *B.*

cereus. Whole-genome sequence analysis revealed that the *B. cereus* strain was closely related to but distinct from *B. anthracis* (Hoffmaster *et al.*, 2004). The strain also harboured a plasmid that was closely related to *B. anthracis* pXO1 and contained a complete anthrax toxin biosynthetic complex. A second large plasmid of 218 kb, designated pBC218 and completely unrelated to *B. anthracis* pXO2, contained a novel polysaccharide capsule biosynthetic cluster that is presumably responsible for encapsulation of the *B. cereus* G9241 strain. Subsequent to this case, two fatal cases, also involving welders, were reported during 1997 in Louisiana (Miller *et al.*, 1997). In 2003, a further two fatalities were recorded in Texas, involving a welder and a metalworker, respectively (Avashia *et al.*, 2007). In all of these cases the etiological agents were identified as encapsulated *B. cereus* strains that harboured *B. anthracis* virulence genes (Hoffmaster *et al.*, 2006; Sue *et al.*, 2006).

More recently the sudden deaths of eight healthy wild chimpanzees in Côte d'Ivoire, and three chimpanzees and one gorilla in Cameroon were reported (Leendertz *et al.*, 2004; Leendertz *et al.*, 2006). The strains involved in these outbreaks differed significantly from classic *B. anthracis* in that they were motile, resistant to gamma phage and for the isolates from Cameroon, resistant to penicillin G. The strains also expressed a capsule in the absence of induction by CO_2 and displayed β-hemolytic activity after subculture. These strains did, however, harbour two large plasmids of sizes corresponding to those of *B. anthracis* pXO1 and pXO2, respectively, expressed a capsule and secreted protective antigen into the medium (Klee *et al.*, 2006). Whether these strains should be regarded as *B. cereus* or as *B. anthracis* is still controversial (Okinaka *et al.*, 2006). Nevertheless, it is interesting to note that these primate outbreaks were most probably due to ingestion of infected material as opposed to the above-mentioned human cases that were most likely due to inhalation of spores. This therefore suggests that these anthrax-like *B. cereus* strains are capable of causing a disease that resembles gastrointestinal anthrax, in addition to pulmonary anthrax. This might be cause for concern in the dairy industry since *B. cereus* is known for establishing biofilms in milk processing equipment, and contamination of milk and milk products due to the spores being able to survive the pasteurization process (Flint *et al.*, 1997).

1.2 Bacterial biofilms

Biofilms are defined as communities of microbial cells that are adherent to a surface, interface or each other and are encased in a self-produced exopolymeric matrix (Costerton *et al.*, 1995). Bacterial biofilm formation was first observed in 1933 by Arthur T. Henrici, who noted that marine bacteria grow for the most part on submerged surfaces, rather than being free-floating (Henrici, 1933). Subsequent pioneering research by Claude E. ZoBell showed that in aqueous environments dissolved nutrients tend to accumulate at solid surfaces (ZoBell, 1937; Stark *et al.*, 1938) and, as a result, surface-attached growth is favoured to such an extent that the majority of bacterial growth is surface-associated (ZoBell, 1943). In the same paper, ZoBell noted that the attachment of bacteria to surfaces appeared to be actively facilitated by the cells themselves rather than by passive adsorption. He also noted that the attachment of bacterial cells to surfaces was soon followed by the secretion of a "cementing substance" and subsequent growth of attached cells to form microcolonies (ZoBell, 1943).

The year following the paper by ZoBell was marked by the discovery by Oswald Avery and co-workers that DNA, and not proteins as many believed at the time, is the agent of heredity (Avery *et al.*, 1944). This discovery had a pronounced influence on microbiology research. The emphasis shifted from the study of bacteria and their interaction with their natural environment to focusing on the molecular genetics of bacteria and their phages. For these investigations, pure cultures of bacteria were typically grown as batch cultures in nutrient-rich liquid media. Although this culturing system proved to be very useful, it spawned a generation of bacteriologists that modeled the natural physiology and lifestyle of bacteria on exponentially growing planktonic cells. It has since been shown that bacteria growing as biofilms represent the predominant mode of growth in their natural environment (Geesey *et al.*, 1977).

Over the last decade, much progress has been made towards understanding the development of bacterial biofilms. This progress has been largely due to the focus of analyzing biofilms using genetic (O'Toole and Kolter, 1998; Branda *et al.*, 2004; Ren *et al.*, 2004), proteomic (Sauer and Camper, 2001; Oosthuizen *et al.*, 2002; Resch *et al.*, 2006) and molecular biological (Branda *et al.*, 2001; Klausen *et al.*, 2003; Vlamakis *et al.*, 2008) approaches. Current models, based largely on the Gram-negative bacterium *Pseudomonas aeruginosa*, depict biofilm formation commencing when planktonic bacterial cells attach irreversibly to a

surface. This attachment is followed by growth into a mature, structurally complex biofilm and culminates in the dispersion of detached bacterial cells into the bulk fluid (Sauer *et al.*, 2002). It should be noted, however, that biofilm development in different bacteria may differ due to the utilization of different pathways, reflective of an organism's specific environmental niches (Davey and O'Toole, 2000; O'Toole *et al.*, 2000).

1.2.1 Biofilm formation by *Bacillus* species

Bacillus subtilis has long been studied as a Gram-positive model organism for cellular differentiation and bacterial sporulation, and it is currently one of the best characterized bacterial species (Sonenshein *et al.*, 2001). The study of bacterial biofilms, however, has until recently focused largely on Gram-negative bacteria that are either of clinical importance, e.g. *P. aeruginosa*, or that have been well characterized, e.g. *Escherichia coli* (Davey and O'Toole, 2000; Hall-Stoodley *et al.*, 2004). It is therefore not surprising that when the first research groups started to investigate biofilm formation by *B. subtilis*, they attempted to adapt the methods used previously for the study of Gram-negative biofilms to those formed by Gram-positive bacteria.

A landmark paper by Branda *et al.* (2001) mentions that the work described in their paper started as an attempt to characterize the biofilm-forming capabilities of *B. subtilis*, but the research got side-tracked when they realized that *B. subtilis* biofilms differ fundamentally from those formed by *P. aeruginosa*. In contrast to the surface-associated submerged biofilms of *P. aeruginosa*, they found that *B. subtilis* preferentially formed a floating pellicle (biofilm) on the surface of the liquid culture medium and around the edge of the culture vessel at the air-liquid interface. They also noted a macroscopic difference between the floating biofilm formed by laboratory strains and natural (wild-type) strains of *B. subtilis*. The wild-type strains formed robust biofilms with intricate macroscopic web-like structures, while the laboratory strains formed flat undifferentiated biofilms. Microscopic examination of the surface of the colonies and biofilms formed by the wild-type *B. subtilis* strains revealed highly structured aerial projections that consisted of chains of cells bundled in parallel. The aerial structures were subsequently shown to be simple fruiting bodies that serve as preferential sites for sporulation within the context of biofilms. The development of biofilms followed a characteristic pattern. After inoculation of standing cultures, motile cells proliferated throughout the liquid medium as planktonic cells until they reached a density of *ca.* 3×10^7 colony forming units (cfu)/ml after 24 h at room temperature. At that point, the

vast majority of cells began to migrate to the air-liquid interface where they formed a floating biofilm on the surface of the medium. By 36-48 h, the cell density dropped to *ca.* 3×10^5 cfu/ml, and this drop coincided with substantial growth of the biofilm. In contrast to the planktonic cells, which retained their motility, the cells at the air-liquid interface became non-motile and formed long chains that were bound together by presumably an exopolysaccharide matrix. By 60 h, some cells within the chains began to sporulate and by 96 h, more than 50% of the viable cells within the biofilm sporulated. In contrast, less than 5% of the viable planktonic cells sporulated, even after 120 h of incubation (Branda *et al.*, 2001).

Biofilm formation by *B. cereus* has also recently been investigated, since biofilms produced by this bacterium are considered a potential health hazard in the dairy industry (Crielly *et al.*, 1994; Lindsay *et al.*, 2000). Initially, these studies focused on surface-associated submerged biofilms that were obtained by culturing of the bacteria in batch culture in the presence of glass wool as an attachment substratum (Oosthuizen *et al.*, 2001; Oosthuizen *et al.*, 2002). However, it has subsequently been shown that *B. cereus* biofilms form preferentially at air-liquid interfaces under static culturing conditions and that these biofilms function as a nidus for sporulation (Wijman *et al.*, 2007). It was reported that the amount of biofilm formed on submerged surfaces were *ca.* half of that formed at the air-liquid interface. Differences were also noted in the ability of different *B. cereus* strains to form biofilms. Of the two sequenced *B. cereus* strains included in the study, strain ATCC 10987 formed more than double the amount of biofilm formed by strain ATCC 14579 in LB medium after 48 h of growth at 30°C. It has previously been suggested that the formation of biofilms at the air-liquid interface may involve oxygen availability at the surface, causing aerotaxis of *B. cereus* towards oxygen (Laszlo *et al.*, 1984). In support of this notion, Wijman *et al.* (2007) reported that a *B. cereus* mutant with reduced motility was indeed severely impaired in its ability to form biofilms at the air-liquid interface.

1.2.2 Biofilm formation leads to modification of the bacterial transcriptome

Several studies have reported that the biofilm cells of Gram-positive bacteria express a distinct transcriptome when compared to their planktonic counterparts. A study by Beenken *et al.* (2004) showed that in *Staphylococcus aureus* biofilm cells the expression of 48 genes were induced by a factor of more than two, while expression of 84 genes were repressed by a similar factor. Two independent studies have also reported differential gene expression in *B.*

subtilis biofilm cells. Stanley *et al.* (2003) studied the gene expression of *B. subtilis* in the early stage of surface-attached submerged biofilm formation (8, 12 and 24 h after inoculation in batch culture), and reported that 519 of the *B. subtilis* genes were differentially expressed in at least one time point as the planktonic cells transitioned to a biofilm mode of growth. Many of the genes differentially expressed during biofilm formation are involved in motility and chemotaxis, phage-related functions, membrane bioenergetics and sugar catabolism. In contrast, Ren *et al.* (2004) studied gene expression in mature *B. subtilis* biofilms (5-d biofilms), and reported significant induction of 342 genes and repression of 248 genes in biofilm cells compared to planktonic cells. Genes that were highly expressed in the biofilm comprised sporulation genes, as well as genes that have functions for transport, metabolism and antibiotic production.

1.2.3 Biofilm formation leads to modification of the bacterial proteome

Proteomic analysis, using two-dimensional SDS-polyacrylamide gel electrophoresis (2-DE), has consistently shown differences between the proteomes of biofilm and planktonic cells. In an early study by Oosthuizen *et al.* (2001), it was reported that *B. cereus* DL-5 biofilm cells expressed at least 10 proteins as a result of surface attachment. Of these, four proteins were unique to the biofilm profile, while the other six proteins represented modified forms of proteins appearing in both the biofilm and planktonic proteome profiles. Moreover, seven proteins were reported to be expressed uniquely in planktonic cells (Oosthuizen *et al.*, 2001). Although the identity of the proteins were not determined in this study, it nevertheless provided experimental evidence indicating that the proteome of planktonic *B. cereus* cells changed in the biofilm mode of growth with certain proteins being expressed, repressed or modified. A subsequent study by the same group yielded more detailed information, as the proteome of both 2- and 18-h old biofilm cells were compared to that of planktonic cells (Oosthuizen *et al.*, 2002). The 2-h microcolony proteome revealed the expression of 15 unique proteins, while the mature 18-h biofilm expressed seven unique proteins. Moreover, differences in the proteomes of the microcolony and mature biofilm were largely due to up- and down-regulation of the expression of a multitude of proteins. Amongst proteins identified, it was suggested that YhbH, a member of the sigma-54 (σ^{54}) modulation protein family, may play an important role in regulation of the biofilm phenotype. A comprehensive proteomic analysis of *S. aureus* biofilm and planktonic cells revealed significant changes in the expression of 427 proteins (Resch *et al.*, 2006). Biofilm cells expressed higher levels of

proteins associated with cell attachment, peptidoglycan synthesis, fibrinogen-binding proteins, and enzymes involved in pyruvate and formate metabolism. Comparison of the proteomic data with transcriptomic data, produced by the same group (Resch *et al.*, 2006), showed qualitative agreement between the results, although the absolute values differed greatly.

1.2.4 Biofilm cells represent a distinct phenotype

Recently, statistical evidence was provided for a biofilm-specific phenotype of *B. cereus* (Vilain and Brözel, 2006). The total proteomes of 24-h old biofilm cells and their associated suspended populations were compared to those of exponential, transient and stationary phase cells. Variation in the quantities of all detectable protein spots was statistically analyzed using principal component analysis. The analysis revealed a unique biofilm phenotype that could not be the result of various mixtures of planktonic cells in different growth phases. The analysis also revealed that the biofilm-associated planktonic population possessed a unique phenotype that is distinct from that of the biofilm and planktonic populations, and that this phenotype was not the result of a mixture of the two bacterial populations.

1.3 Genes involved in biofilm formation by *B. subtilis*

Biofilm formation has been well studied using Gram-negative bacterial model systems. It has been reported that for these bacteria, structural components such as flagella, pili and adhesins, outer membrane proteins, as well as the production of extracellular polymeric substances play essential roles in biofilm formation and/or maturation (Hoyle *et al.*, 1993; O'Toole and Kolter, 1998; Vidal *et al.*, 1998; Hentzer *et al.*, 2001; Whiteley *et al.*, 2001). In contrast, biofilm formation by *B. subtilis* has only recently been examined (Branda *et al.*, 2001). Consequently, relatively few genes have been identified that are required for these bacteria to form biofilms, and little is known about how they coordinate biofilm formation. In the following sections, determinants of *B. subtilis* biofilm formation that have been identified thus far will be discussed in greater detail.

1.3.1 Regulatory and associated genes

Towards identifying genes that are involved in *B. subtilis* NCIB 3610 biofilm formation, Branda *et al.* (2001) used a "candidate-gene" approach in which genes known to be required

for sporulation were specifically targeted for disruption and the mutants analyzed with regard to pellicle (biofilm) morphology. It was reported that a *spo0A* mutant lacking Spo0A, the key transcriptional regulator that governs entry into sporulation (Grossman, 1995), failed to form a pellicle in standing cultures. Moreover, a *spo0H* (*sigH*) mutant lacking sigma-H (σ^H), a sigma factor that controls expression of proteins involved in the early stages of sporulation (Grossman, 1995), produced thin pellicles. In contrast, a *sigF* mutant lacking sigma-F (σ^F), the first compartment-specific sigma factor produced during sporulation (Stragier and Losick, 1996), formed pellicles that closely resembled those formed by the wild-type *B. subtilis* NCIB 3610 strain. Based on these results, it was concluded that both Spo0A and σ^H, in addition to being key regulators of the initial steps of sporulation, also play an important role in the formation of *B. subtilis* biofilms.

The involvement of Spo0A in the formation of surface-associated submerged biofilms by *B. subtilis* JH642 was also reported in an independent study performed by Hamon and Lazazzera (2001). It was shown that *spo0A* mutant cells adhered to a glass surface in a monolayer of cells and lacked a defined three-dimensional architecture. The requirement of *spo0A* for biofilm development was thought to be the result from its failure to repress expression of AbrB, a transcriptional repressor of several genes (Strauch and Hoch, 1993) that also represses biofilm formation (Hamon and Lazazzera, 2001). A subsequent genome-wide expression profiling study of biofilms formed by *spo0A abrB* or *sigH abrB* mutant strains led to the identification of two AbrB-regulated genes that were shown by mutation analysis to play a role in *B. subtilis* biofilm formation (Hamon *et al.*, 2004). Disruption of these genes, namely *sipW*, encoding a signal peptidase, and *yoaW*, which is predicted to encode a secreted protein of unknown function, resulted in a two-fold reduction in surface-associated biofilm formation by the respective mutant strains (Hamon *et al.*, 2004). Whereas the *yoaW* mutant exhibited a biofilm structure with reduced depth, the *sipW* mutant exhibited only surface-attached cells and did not form a mature biofilm. It has, however, recently been reported that a *yoaW*-null mutation in *B. subtilis* NCIB 3610 formed biofilms that were indistinguishable from those of the wild-type strain, both on solid and in liquid medium (Chu *et al.*, 2008).

1.3.2 Genes involved in flagellar synthesis

Genes required for *B. subtilis* biofilm formation has also been identified by screening of libraries that were generated by means of transposon mutagenesis. Using this approach, a

collection of 5 000 insertional mutants of *B. subtilis* JH642 were screened for the ability to form biofilm on polyvinylchloride (PVC) microtiter plates (Chagneau and Saier, 2004). Four insertional mutants were identified that were defective in surface-attached biofilm formation but not growth impaired. Two of these mutants had transposon insertions in genes involved in flagellar synthesis, namely *fliG* and *flhP*. The *fliG* gene is a member of the *fla/che* operon, which encodes a majority of the proteins involved in flagellar synthesis, as well as all but two of the general chemotaxis proteins (Aizawa *et al.*, 2001). The *flhP* gene is the second gene of the *flhOP* operon and encodes a protein similar to one in the flagellar hook-basal body complex of *Salmonella typhimurium* (Aizawa *et al.*, 2001). For the other two mutants identified in this study the transposon was respectively located in the *gltA* structural gene, encoding a subunit of glutamate synthetase, and in the promoter region of the *ampS* gene, which encodes a putative cytoplasmic aminopeptidase. However, the role of these enzymes in *B. subtilis* JH642 biofilm formation remains to be determined.

To characterize the effect of the *fliG* and *flhP* mutations on *B. subtilis* biofilm formation, the two flagellar mutants were compared to a *cheA* mutant that is flagellated and motile but incapable of chemotaxis, and to a *motA* mutant that synthesizes normal flagella but do not rotate. Whereas the *motA* mutant exhibited a phenotype similar to that of the biofilm-defective *fliG* and *flhP* transposon mutants, the *cheA* mutant exhibited only a moderate decrease in surface-attached biofilm formation. The results therefore indicated that motility, as opposed to the presence of flagella *per se*, is required for *B. subtilis* biofilm formation by possibly promoting the recruitment and initial attachment of *B. subtilis* to surfaces and other bacteria. In contrast, it was suggested that chemotaxis may play a less important role in biofilm formation by facilitating initial recognition of surfaces to which bacteria attach or, alternatively, it could mediate recognition of other cellular constituents in the biofilm community and thereby promote proper intercellular orientation and communication (Chagneau and Saier, 2004).

It is interesting to note that although the above study demonstrated that flagella are required for surface-attached biofilm formation, a DNA microarray analysis of surface-attached *B. subtilis* JH642 biofilms has shown that the expression of genes encoding flagella is repressed (Stanley *et al.*, 2003). In a report by Ren *et al.* (2004), in which *B. subtilis* JH642 was grown in continuous reactors to develop air-liquid biofilms, it was shown that there is no significant difference in the expression level of the gene encoding flagellin in biofilm cells when

compared with planktonic cells. Furthermore, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of membrane proteins demonstrated that flagellin was significantly produced by *B. subtilis* B-1 cells in a floating biofilm (Morikawa *et al.*, 2006). These apparent contradictions remain to be explained, but may be related to the different culturing systems used in the respective investigations.

1.3.3 Genes involved in the synthesis of biofilm matrix components

Since bacterial biofilm formation and maintenance are mediated by an extracellular matrix (Costerton *et al.*, 1999), Branda *et al.* (2001) specifically targeted *B. subtilis* NCIB 3610 genes for inactivation that may be involved in exopolysaccharide synthesis and of which the transcription is under the control of both Spo0A and σ^H. Two genes, namely *yveQ* and *yveR*, were identified that appear to encode EPS biosynthetic enzymes (Britton *et al.*, 2002). In standing cultures, the *yveQ* and *yveR* mutants formed thick but fragile pellicles that tended to break apart and sink to the bottom of the culture vessel (Branda *et al.*, 2001). Growth of the pellicle at the air-liquid interface were in both mutants characterized by a decrease in the length of the cell chains and the mature pellicle eventually consisted of short densely packed disordered chains of cells. It was thus suggested that an EPS matrix, produced through an unknown process involving the *yveQ* and *yveR* genes, may facilitate production of *B. subtilis* biofilms by possibly mediating cell-cell interactions. Ren *et al.* (2004), based on transcriptomic data indicating that expression of *yveR* is up-regulated in *B. subtilis* JH642 biofilm cells, also constructed a knockout of the *B. subtilis* JH642 *yveR* gene. The mutant was reported to display an identical biofilm phenotype to that described by Branda *et al.* (2001). The 15-gene *yveK-T - yvfA-F* operon, which includes the *yveQ* and *yveR* genes, has since been renamed to *epsA-O* (Kearns *et al.*, 2005). EpsA and B are similar to enzymes that regulate EPS chain length, EpsC is similar to nucleotide sugar synthesizing enzymes, EpsD, E, F, H (formerly known as *yveR*), J, L and M are all predicted to be glycosyl transferases, EpsK is similar to proteins involved in saccharide export, and EpsG (formerly known as *yveQ*) is similar to proteins involved in polymerization of EPS repeating units.

It was recently reported that EpsE, in addition to its putative enzymatic function, also acts as an inhibitor of motility in *B. subtilis* by interacting with the flagellum protein FliG (Blair *et al.*, 2008). FliG subunits polymerize into a wheel-like rotor attached to the flagellar basal body and transduce the energy of proton flux through the MotA-MotB proton channel into the

rotational energy of the flagellum (Lloyd *et al.*, 1996). It was thus suggested that EpsE may function as a type of molecular clutch that disengages the rotor from the power source. The clutch provided by EpsE was proposed to help stabilize biofilms in the environment by acting as a fail-safe mechanism to prevent flagella from rotating while the cells are bound by EPS (Blair *et al.*, 2008).

Based on results obtained from screening a *B. subtilis* Functional Analysis (BFA) mutant collection (see Section 1.3.4), two *B. subtilis* mutants harboring insertions in the *sipW* and *yqxM* genes, respectively, were identified that formed pellicles of inconsistent thickness and failed to colonize the entire available surface. The importance of these genes in *B. subtilis* biofilm formation was recently investigated in greater detail. The *yqxM* and *sipW* genes are the first and second in a three-gene operon whose third member is *tasA* (Stover and Driks, 1999). The *sipW* gene encodes a type I signal peptidase that is specifically required for the maturation and secretion of the proteins encoded by *yqxM* and *tasA* (Serrano *et al.*, 1999). In a study by Chu *et al.* (2006), it was demonstrated by targeted gene disruption that each of the three genes of the *yqxM-sipW-tasA* operon is needed for formation of robust biofilms by *B. subtilis* NCIB 3610. In a subsequent study, Branda *et al.* (2006) reported that the *tasA* gene product is a major protein component of the biofilm extracellular matrix and demonstrated that YqxM is important for the proper localization of TasA to the matrix. Notably, it was shown that a *tasA eps* double mutant failed to form a pellicle. This was in contrast to the *tasA* mutant that formed flat pellicles, and the *eps* mutant that formed flat fragile pellicles. These results therefore indicate that not only are TasA and EPS important components of the *B. subtilis* biofilm matrix, but also that TasA makes a contribution to the matrix that is different from and complementary to that made by EPS.

In a recent study, it was reported that the extracellular matrix produced by *B. subtilis* B-1, an environmental strain that forms robust floating biofilms, was composed predominantly of the exopolymer poly-γ-DL-glutamic acid (γ-PGA) (Morikawa *et al.*, 2006). It was subsequently shown that both biofilm formation and γ-PGA production by *B. subtilis* B-1 increased with increasing Mn^{2+} or glycerol concentration, indicating that there is a direct correlation between these two processes. Similarly, Stanley and Lazazzera (2005) also reported that γ-PGA enhances *B. subtilis* biofilm formation. Experimental evidence to this effect was obtained by transferring genetic determinants controlling formation of the exopolymer from a wild-type *B. subtilis* RO-FF-1 strain to *B. subtilis* JH642, an exopolymer-negative laboratory strain. The

latter was subsequently shown to produce enhanced biofilms. Moreover, the study also identified DegSU, a two-component system controlling expression of degradative enzymes (Mader *et al.*, 2002), DegQ, a pleiotropic activator of gene expression (Msadek *et al.*, 1991), and SwrA, a regulator of swarming motility in *B. subtilis* (Kearns and Losick, 2005), as novel regulators of γ-PGA production. The inability of *B. subtilis* JH642 to produce γ-PGA was subsequently mapped to two base pairs, *i.e.* a single base pair change in the promoter region of *degQ* and a single base pair insertion in the coding region of *swrA* (formerly known as *yvzD*).

Interestingly, deletion of *ywsC*, the product of which has been shown to be absolutely required for γ-PGA synthesis (Urushibata *et al.*, 2002), in the wild-type *B. subtilis* RO-FF-1 strain did not lead to a marked decrease in surface-associated biofilm formation (Stanley and Lazazzera, 2005), while attempts to generate a *B. subtilis* B-1-derived *ywsC* knockout mutant proved unsuccessful (Morikawa *et al.*, 2006). In this regard, it is important to note that *B. subtilis* NCIB 3610 does not produce γ-PGA, albeit capable of forming biofilms. Indeed, Branda *et al.* (2006) reported that γ-PGA does not contribute significantly to the extracellular matrix of *B. subtilis* NCIB 3610 and reported that its synthesis is normally repressed during biofilm development. Moreover, Chagneau and Saier (2004) generated a knockout mutant of the *ywsC* gene in *B. subtilis* JH642, which is the same strain used by Stanley and Lazazzera (2005), and reported that the mutant showed no difference in biofilm formation compared to the wild-type strain. These results therefore appear to suggest that the production of γ-PGA may not be essential for biofilm formation by *B. subtilis*. It does, however, not exclude the possibility that the apparent discordant data may result from differences between the investigative systems used.

1.3.4 Other genes

To further identify genes involved in *B. subtilis* biofilm formation, Branda *et al.* (2004) screened the *B. subtilis* Functional Analysis (BFA) mutant collection, which was constructed by a consortium of laboratories from Europe and Japan. This collection includes mutants in which most *B. subtilis* genes of unknown function were individually disrupted in the laboratory strain 168 (Vagner *et al.*, 1998; Yoshida *et al.*, 2000). Each of the 2 105 mutants, representing 51% of the genome, were cultured in microtiter plates and the wells were inspected for mutants that produced an atypical pellicle. Twenty-four mutants that produced a

pellicle with an unusual morphology and exhibited planktonic growth rates similar to that of the wild-type 168 strain were identified. The ability of these 24 insertional mutations to affect pellicle formation in a wild-type *B. subtilis* strain was subsequently investigated by introducing each of these mutations into the wild-type strain NCIB 3610. In instances where the genes identified during the course of this investigation formed part of an apparent operon, the possibility that the observed defects may be due to polar effects on downstream genes was excluded by deleting the immediate downstream gene and analyzing the mutant for its ability to form a pellicle. In all cases, these mutants formed pellicles that were indistinguishable from that formed by the parental strain, thus confirming that the genes discussed below were indeed responsible for the observed mutant phenotype. In addition to mutations in the *sipW* and *yqxM* genes, which were shown to affect *B. subtilis* biofilm formation (see above), four more genes were identified that are involved in the development of floating biofilms in the wild-type *B. subtilis* NCIB 3610 strain.

A mutant strain harbouring an *yhxB*-null mutation formed fragile pellicles that in the initial stages of pellicle formation proliferated as long chains of cells that formed loose aggregates. The *yhxB* gene, which was renamed to *pgcA* by Lazarevic *et al.* (2005), encodes a α-phosphoglucomutase enzyme (α-PGM), which is responsible for interconversion of glucose 6-phosphate and α-glucose 1-phosphate. Not only does glucose 6-phosphate hydrolysis yield energy, but it is also isomerized by the *pgcA*-encoded α-PGM into α-glucose 1-phosphate, the precursor of UDP-glucose. It was suggested that UDP-glucose may act as a metabolic signal regulating *B. subtilis* biofilm formation through an unknown pathway (Lazarevic *et al.*, 2005). Alternatively, Branda *et al.* (2004) suggested that *pgcA* may promote the synthesis of nucleotide sugars which, through a processes catalyzed by proteins encoded by the *epsA-O* operon, are incorporated into exopolysaccharides of the extracellular matrix of *B. subtilis* biofilms.

A mutant strain harbouring an *ecsB*-null mutation formed flat pellicles that were composed of long chains of cells that were bound together loosely. The *ecsB* gene is the second gene of the *ecsABC* operon, which encodes an ABC transporter referred to as Ecs (Leskela *et al.*, 1996) and is known to promote the Sec-dependant secretion of several degradative enzymes (Leskela *et al.*, 1999). Whereas the *ecsB* gene encodes the transmembrane subunit of the ABC transporter, the *ecsA* gene encodes the ATPase subunit. However, the *ecsC* gene encodes a protein that shows no significant sequence similarity to any protein of known function and,

unlike EcsA and EcsB, the EscC protein is not required for functioning of the ABC transporter (Kontinen and Sarvas, 1988; Leskela *et al.*, 1999). Although the results indicated that Ecs activity is important for the development of *B. subtilis* biofilms, the mechanism by which this occurs is not yet known. It was, however, proposed that Ecs could promote the maturation and secretion of protein components of the extracellular matrix and in this way contribute to the structure of pellicles (Branda *et al.*, 2004).

A mutant strain harbouring an *yqeK*-null mutation formed pellicles that were initially thin and flat but eventually became much thicker. The *yqeK* gene is located within a cluster of eight genes (*yqeG* to *yqeM*) that may constitute an operon of which the function is not yet known. The *yqeK* gene has not yet been characterized, but is predicted to encode a metal-dependant phosphatase based on the presence of a conserved HD sequence motif characteristic of this family of enzymes. The biological function of YqeK, as well as its role in *B. subtilis* biofilm development remains to be elucidated.

A mutant strain harbouring an *ylbF*-null mutant was initially defective in pellicle formation, but prolonged incubation resulted in the formation of pellicles that were heterogeneous in morphology. The *ylbF* gene, which appears to lie within a two-gene operon, encodes a protein that promotes translation and/or stability of ComK (Tortosa *et al.*, 2000), a transcription factor that plays a critical role in the induction of natural competence (Hahn *et al.*, 1996). Although this may suggest that YlbF might work in conjunction with ComK to coordinate *B. subtilis* biofilm development, a mutant strain from which *comK* was deleted formed wild-type pellicles, while deletion of both *ylbF* and *comK* yielded a mutant strain that formed pellicles indistinguishable from those formed by the *ylbF* mutant. These results therefore indicate that the mutant phenotype conferred by deletion of the *ylbF* gene was not due to misregulation of ComK. It was thus concluded that YlbF may control the development of *B. subtilis* biofilms via a pathway that does not include ComK or that is entirely separate from processes that govern competence (Branda *et al.*, 2004).

A mutant strain harbouring a *ymcA*-null mutant formed pellicles that exhibited a phenotype essentially identical to that of the *ylbF* mutants. The *ymcA* gene is the second in an apparent two-gene operon, *ymcBA*. The *ymcA* gene has not been characterized previously, but it is predicted to encode a protein whose sequence resembles that of YlbF (19% identity and 51% similarity). However, despite the similarity, subsequent bioinformatic analyses indicated that

YlbF and YmcA appear to have different domain structures and are unlikely to be paralogs (Branda *et al.*, 2004). It was suggested that *ylbF* and *ymcA* may work together to regulate biofilm development, but the manner and the pathway through which this may occur remain unknown.

1.4 Quorum sensing

Quorum sensing is the regulation of bacterial gene expression in response to fluctuations in cell-population density (Miller and Bassler, 2001; Waters and Bassler, 2005). Quorum sensing is mediated by signal molecules, called autoinducers, which increase in concentration as a function of cell density. Extracellular accumulation of the autoinducer to a minimal threshold stimulatory concentration leads to the detection of the signal by members of the population and subsequent alterations in gene expression. Using these signal-response systems, bacteria can synchronize particular behaviours on a population-wide scale and thus function as multicellular organisms. Quorum sensing-controlled behaviours include bioluminescence in *Vibrio harveyi* (Bassler *et al.*, 1994), virulence in *V. cholerae* (Miller *et al.*, 2002), biofilms in *P. aeruginosa* (Davies *et al.*, 1998) and competence in *B. subtilis* (Solomon *et al.*, 1995). Quorum sensing systems that regulate intraspecific behaviour can be divided into two types. These are the LuxI/LuxR-type quorum sensing systems in Gram-negative bacteria responsible for the production of *N*-acyl-*L*-homoserine lactone autoinducers, and the oligopeptide/two-component-type quorum sensing systems in Gram-positive bacteria responsible for the production of autoinducer peptides (Miller and Bassler, 2001; Sturme *et al.*, 2002; Waters and Bassler, 2005). In recent years, a third type of quorum sensing system has been characterized that not only appears to participate in intraspecific bacterial behaviour, but also seems to regulate the interspecific interactions among bacteria of different genera. This system, which is present in a wide variety of Gram-negative and Gram-positive bacteria, involves the production of autoinducer-2 (AI-2) signal molecules in response to cell density (Xavier and Bassler, 2003). In the discussion below reference will also be made to the PlcR regulon present in some members of *B. cereus* group, the expression of which appears to be dependent on the uptake of a peptide acting as a quorum sensing effector.

1.4.1 Intraspecies communication via autoinducing peptides

Peptide-mediated quorum sensing in Gram-positive bacteria (Fig. 1.1) commences when a peptide signal precursor locus is translated into a precursor protein. Following cleavage of the precursor protein to produce the processed autoinducer peptide, the latter is secreted out of the cell via a dedicated ABC transporter. When the extracellular concentration of the autoinducer peptide accumulates to the minimal stimulatory level, it is detected by a two-component signaling system of which the signaling mechanism is a phosphorylation/dephosphorylation cascade. In this system, a histidine sensor kinase detects the autoinducer peptide and then undergoes autophosphorylation on a conserved histidine residue. Subsequently, the phosphate is transferred to the cognate response regulator protein, which becomes phosphorylated on a conserved aspartate residue. The phosphorylated response regulator then activates transcription of the quorum sensing-controlled target genes (Miller and Bassler, 2001; Sturme *et al.*, 2002).

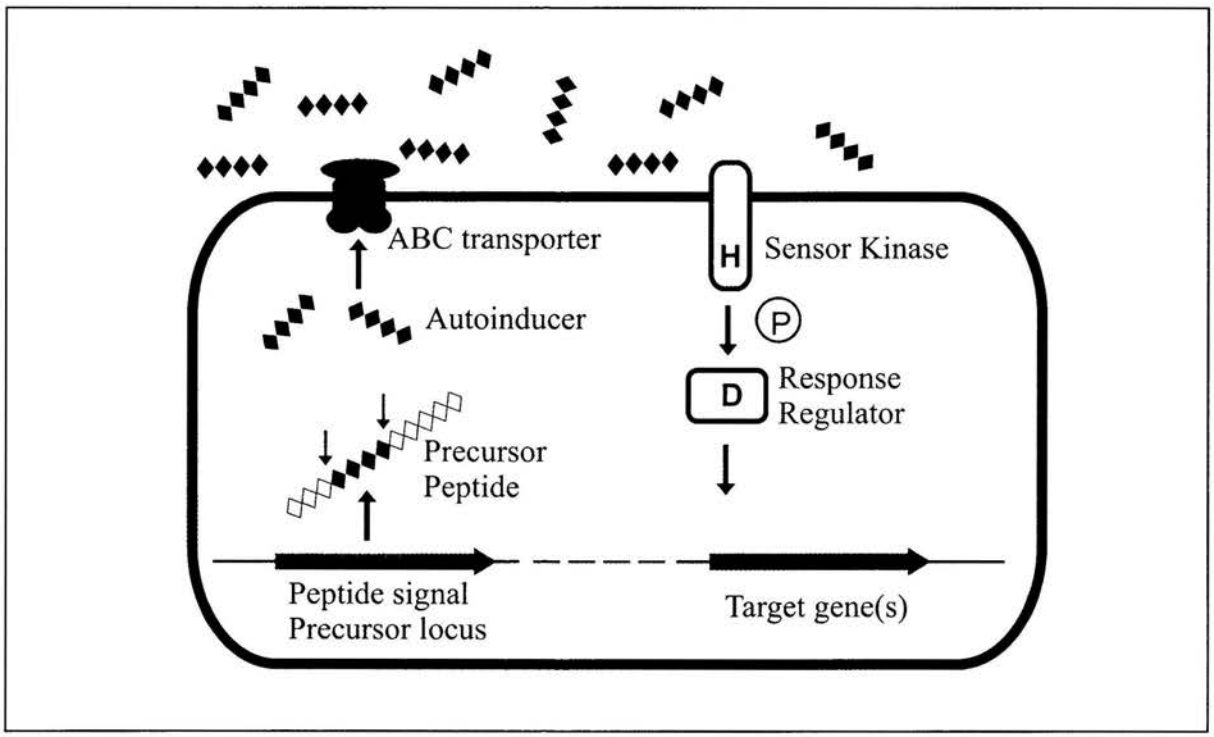

Figure 1.1: A general model for peptide-mediated quorum sensing in Gram-positive bacteria. In the figure, the precursor peptide (grey and black diamonds) is cleaved (arrows) to produce the processed autoinducer peptide (black diamonds). The P in a circle represents the phosphorylation cascade. (Adapted from Miller and Bassler, 2001).

An extensively studied peptide-mediated quorum sensing system in Gram-positive bacteria is the *B. subtilis* ComP/ComA competence and sporulation system (Solomon *et al.*, 1995; Hahn

et al., 1996; Perego, 1997). In any given population of *B. subtilis* cells, only *ca.* 10% of the cells become competent and this is achieved at the transition between the exponential growth phase and stationary phase, *i.e.* at high cell density. Sporulation also occurs preferentially under conditions of high cell density in a nutrient-depleted environment. Commitment to vegetative growth, competence or sporulation is irreversible, and therefore entry into these pathways needs to be tightly controlled (Miller and Bassler, 2001). Two autoinducer peptides, *i.e.* ComX and CSF (competence and sporulation factor), mediate quorum sensing control of competence and sporulation (Fig. 1.2). Both peptides are produced during cell growth and are secreted into the extracellular environment where they accumulate, as the cell density increases.

ComX is a 10-amino-acid peptide derived from a precursor peptide encoded by *comX* (Magnuson *et al.*, 1994) and is secreted by the ComQ protein (Bacon Schneider *et al.*, 2002). When the cell density is high, ComX is detected by the membrane-bound histidine sensor kinase ComP. ComX-binding stimulates ComP to autophosphorylate and transfer phosphate to the response regulator ComA (Solomon *et al.*, 1995). The phosphorylated ComA subsequently activates transcription of the *comS* gene, the product of which (ComS) inhibits the proteolytic degradation of ComK and thus results in an increased level of the ComK protein. ComK is a transcription factor that controls the expression of genes required to develop competence (van Sinderen *et al.*, 1995; Turgay *et al.*, 1997).

The CSF precursor peptide is encoded by the *phrC* gene and the five amino acids of the C-terminus of the precursor peptide are cleaved to form the pentapeptide CSF signal molecule (Solomon *et al.*, 1996). The extracellular CSF is imported into *B. subtilis* by the oligopeptide permease (Opp) transporter, an ABC-type oligopeptide transporter (Solomon *et al.*, 1996). At low intracellular concentrations, CSF binds to and inhibits RapC, a ComA-specific phosphatase, thus causing a net increase in the level of phosphorylated ComA. Since phosphorylated ComA is the response regulator controlling expression of genes required for competence, the development of competence is therefore favoured (Core and Perego, 2003). At high intracellular concentrations, CSF inhibits expression of ComS and this results in increased proteolysis of ComK, thereby inhibiting the development of competence. Moreover, a high intracellular concentration of CSF also promotes sporulation. In this case, CSF inhibits RapB, a phosphatase responsible for dephosphorylating the phosphorylated Spo0A response regulator, which is involved in promoting sporulation (Grossman, 1995). Therefore, inhibition

of the RapB phosphatase activity increases the levels of phosphorylated Spo0A, thus favouring a switch in commitment from competence to the sporulation pathway (Hoch, 1993; Perego, 1997).

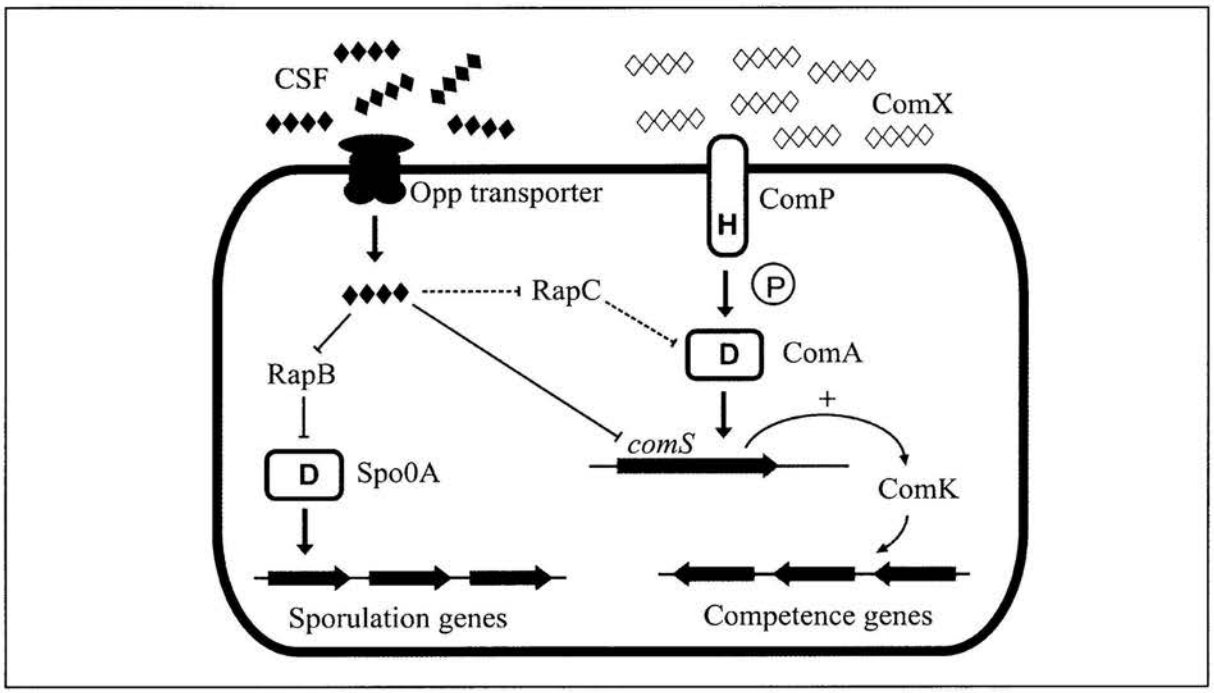

Figure 1.2: The *B. subtilis* ComP/ComA quorum sensing system for control of sporulation and competence. *B. subtilis* uses two processed autoinducer peptides, ComX (grey diamonds) and CSF (black diamonds), to regulate the competence and sporulation processes. Accumulation of the processed ComX peptide enables it to interact with the ComP sensor kinase, which autophosphorylates on a histidine residue (H) and the phosphate is subsequently transferred to an aspartate reside (D) on the ComA response regulator. Phospho-ComA activates expression of *comS*, resulting in an increased level of the ComK protein (+) by inhibiting ComK proteolysis. ComK is a transcription factor that activates the expression of genes required for development of the competent state. The second autoinducer peptide CSF is transported into cell via the Opp transporter (grey protein complex). At low intracellular concentrations, CSF inhibits the ComA-specific phosphatase RapC, thus increasing the level of phospho-ComA and leading to competence (dashed line). At high intracellular concentrations, CSF inhibits competence and promotes spore development (black lines). CSF inhibits ComS, thus reducing transcription of competence genes and promoting sporulation instead. Moreover, CSF inhibits the RapB phosphatase, thus increasing the level of phosphorylated Spo0A that induces sporulation. (Adapted from Miller and Bassler, 2001).

1.4.2 Regulation of the PlcR regulon via a cell-cell signalling peptide

PlcR, a pleiotropic regulator of extracellular virulence gene expression, was first identified in *B. thuringiensis* (Lereclus *et al.*, 1996). The *plcR* gene was subsequently reported to be present in both *B. cereus* and *B. anthracis*. However, in contrast to *B. cereus*, the polypeptide encoded by the *B. anthracis plcR* gene is truncated and thus not active as a transcriptional activator (Agaisse *et al.*, 1999). PlcR positively regulates its own expression (Lereclus *et al.*, 1996) and activates the transcription of several genes encoding degradative enzymes, cell

surface proteins and enterotoxins that are potentially involved in bacterial virulence (Agaisse *et al.*, 1999; Økstad *et al.*, 1999). Indeed, deletion of the *plcR* gene in *B. cereus* and *B. thuringiensis* reduced haemolytic activity and virulence of the respective bacteria in insect larvae (Salamitou *et al.*, 2000).

Analysis of the promoter region of PlcR-regulated genes revealed that the -10 regions of these promoters are similar to the Pribnow box (TATAAT) of *B. subtilis* promoters recognized by the major sigma factor of the vegetative growth phase, σ^A. However, the -35 regions differ from the TTGACA consensus sequence (Lereclus *et al.*, 1996; Agaisse *et al.*, 1999). The promoter region of PlcR-regulated genes also contains a highly conserved palindromic sequence (TATGNAN$_4$TNCATA), designated the PlcR box, that constitutes the PlcR recognition site (Agaisse *et al.*, 1999). In *B. thuringiensis* the centre of the palindromic PlcR box in the majority of promoters lies between position -41 and position -58 relative to the transcription start site, but may in some cases be more than 200 nucleotides upstream. The *B. cereus* ATCC 14579 genome contains 55 PlcR boxes of which 26 occur in the promoter region of genes and a further 24 in the promoter region of potential operons, which increases the number of genes potentially regulated by PlcR to more than a 100 genes (Ivanova *et al.*, 2003). The expression of *plcR* and PlcR-regulated genes is activated at the end of the vegetative phase in cells grown in rich medium (LB medium). In contrast, transcription is not activated in cells cultured in a sporulation-specific medium (Lereclus *et al.*, 2000). This was shown to be due to binding of phosphorylated Spo0A to two Spo0A boxes flanking the PlcR box, upstream of *plcR*, and repressing *plcR* expression, probably by preventing binding of the activator to its recognition sequence (Lereclus *et al.*, 2000; Liu *et al.*, 2003).

To identify genes involved in the activation of *plcR* transcription, transposon mutagenesis of *B. thuringiensis* was performed and three mutants were identified that displayed impaired *plcR* expression. The transposon insertions were mapped to a five-gene operon, encoding components of the oligonucleotide permease system (Opp) (Gominet *et al.*, 2001). As discussed in the previous section, the Opp system of *B. subtilis* is responsible for importing the CSF peptide that acts as a signal to control the development of genetic competence and sporulation. Disruption of the *B. thuringiensis oppB* gene yielded a phenotype that was similar to that of a *plcR*-null mutant strain, thus indicating that *plcR* expression may be activated by the uptake of a signaling peptide acting as a quorum sensing effector (Gominet *et al.*, 2001). Evidence to this effect was provided by Slamti and Lereclus (2002) who showed

that PlcR regulon expression was dependent on a small 48-amino-acid peptide, designated PapR. The *papR* gene, located 70 bp downstream from *plcR* in *B. cereus* (Fig. 1.3), belongs to the PlcR regulon and its inactivation abolished expression of the PlcR regulon in *B. thuringiensis*. Moreover, PapR appears to be processed intracellularly since it was shown that the C-terminal end of PapR, consisting of a minimum of five amino acids, activated the PlcR regulon by allowing PlcR to bind to its target DNA (Slamti and Lereclus, 2002). Notably, the activation system was found to be strain-specific, with the specificity determined by the first residue of the pentapeptide.

Based on the above results, the following model for PlcR-regulated gene expression has been proposed. It was proposed that the activity of PlcR is dependent on the secreted signaling peptide PapR, which is reimported into the cell via the Opp transporter system. When high bacterial densities are reached, there would be a concomitant increase in the intracellular concentration of PapR, thereby promoting its interaction with PlcR. The PapR:PlcR complex can then bind to its DNA recognition site, the palindromic PlcR box, thus triggering a positive feedback loop that up-regulates the expression of *plcR*, *papR* and various virulence factors (Slamti and Lereclus, 2002). Insights into the molecular basis of PlcR-mediated activation of gene expression were recently obtained when the crystal structure of the complex formed between PlcR and the C-terminal PapR pentapeptide, both from *B. thuringiensis*, was solved (Declerck *et al.*, 2007). It was concluded that two monomers, each comprising of a complexed PapR pentapeptide and PlcR polypeptide, dimerizes and subsequently polimerizes into higher order structures (tetramers, hexamers and higher oligomers) in a PapR-concentration dependent manner, which places the N-terminal helix-turn-helix (HTH) DNA-binding domains of the PlcR polypeptides in the correct conformation for binding to its target DNA. In contrast, the orientation of the HTH domains of dimers comprising of only apo-PlcR is incompatible with them being able to bind to the target DNA (Fig. 1.4).

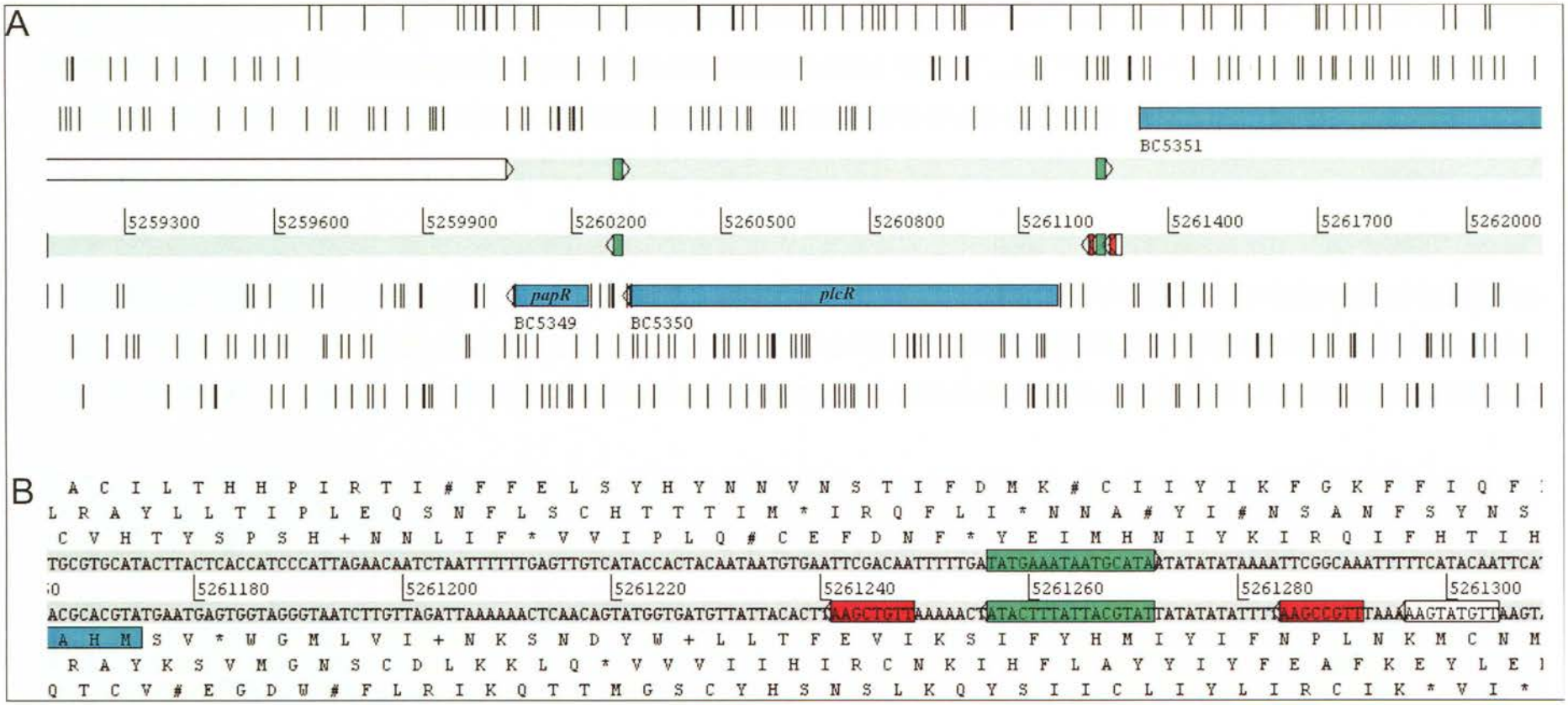

Figure 1.3: (A) Genomic region of *Bacillus cereus* ATCC 14579, containing the *plcR* and *papR* genes. The dark grey horizontal bars represent the two strands of the DNA double helix. The light grey horizontal bars represent the three possible reading frames in each strand. The vertical lines represent stop codons. Coding sequences are indicated in blue and the direction of transcription is indicated by an arrow head. PlcR boxes are indicated in green and the binding sites for phosphorylated Spo0A are indicated in red. (B) Close-up view of the *plcR* promoter region, showing individual nucleotides.

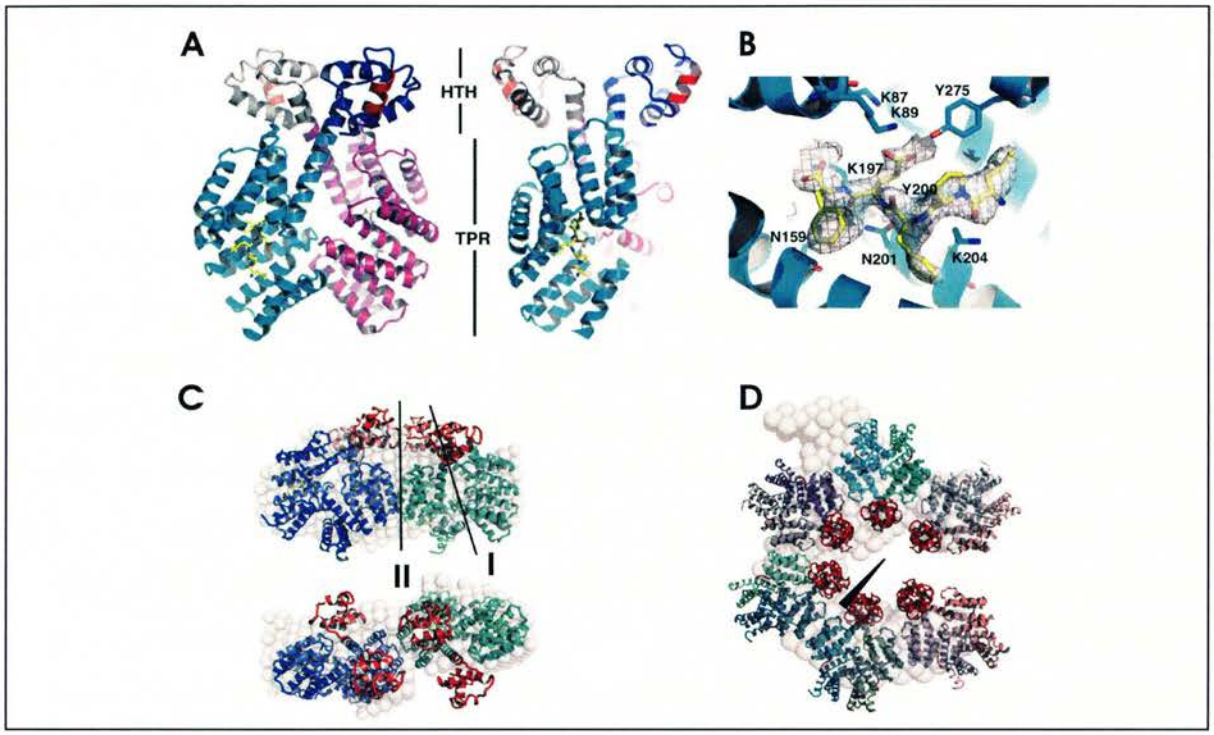

Figure 1.4: Structure of PapR5:PlcR and higher order structures formed in the presence of increasing concentrations of PapR5. The C-terminal 5-amino-acid peptide derived from PapR (PapR5) is shown in yellow. The two PlcR polypeptides are coloured in cyan and magenta, respectively, and their respective N-terminal helix-turn-helix (HTH) DNA-binding domains are indicated in blue and grey. Helix 3 of the HTH domains, capable of inserting into the major DNA groove, is coloured in red. The C-terminal of each PlcR polypeptide is composed of 11 helices, forming five tetratricopeptide repeats (TPR), and a capping C-terminal helix. (A) Ribbon presentation of the structure of PapR5:PlcR. The structures on the left and right represent 90° views. Two PlcR polypeptides, each complexed with PapR5 in the TPR domain, dimerize via the TPR domains. The DNA-binding surfaces of both HTH domains (helix 3, in red) of the dimer face in opposite directions. This conformation is incompatible with the HTH domains being able to simultaneously associate with the palindromic PlcR box. (B) Close-up view of the peptide-protein interactions between PapR5 and PlcR. (C) Ninety degree (90°) views of a crystallographic tetramer obtained by polymerization of two PapR5:PlcR dimers in the presence of PapR5 at a concentration of 1.2 mg/ml. The Type I and Type II dimer axes are indicated. (D) Modeled structure of a PapR5:PlcR hexa-(dimer) that is predicted to form as result of PapR5 binding at a concentration of 3.6 mg/ml. The structures presented in (C) and (D) serve to illustrate the formation of a right-handed spiral that places the HTH domains of the PlcR polypeptides in the correct conformation for binding to the target DNA. (Adapted from Declerck *et al.*, 2007).

The role of PlcR in biofilm development by *B. cereus* ATCC 14579 was recently investigated (Hsueh *et al.*, 2006). It was reported that when cultured in a low nutrient medium in polystyrene plates, a *plcR*-null mutant strain developed up to four times more surface-attached biofilm than the wild-type *B. cereus* strain. The increased biofilm formation by the mutant strain was associated with a ten-fold increase in production of a novel lipopeptide biosurfactant (Hsueh *et al.*, 2007). Coating of the polystyrene plates with surfactin, a biosurfactant from *B. subtilis*, was shown to rescue the relative deficiency in biofilm formation by the wild-type *B. cereus* strain to levels that were similar to those observed for the PlcR-deficient mutant strain. It is curious to note that although all of the genes belonging

to the PlcR regulon are positively regulated by PlcR (Lereclus *et al.*, 1996; Agaisse *et al.*, 1999), it appears that production of the novel lipopeptide biosurfactant in *B. cereus* ATCC 14579 is either directly or indirectly repressed by PlcR. Nevertheless, the requirement of a biosurfactant for biofilm formation by *B. cereus* is in agreement with results reported for other Gram-negative and Gram-positive bacteria. Rhamnolipid, a biosurfactant produced by *P. aeruginosa*, has been reported to be involved in the development (Lequette and Greenberg, 2005) and maintenance (Davey *et al.*, 2003) of biofilm architecture. Moreover, surfactin was shown to be required for *B. subtilis* biofilm formation in microtitre plates and on plant roots (Bais *et al.*, 2004), while the addition of surfactin to surfactin-deficient mutants of *B. subtilis* A1/3 was reported to rescue their ability to form pellicles at the air-liquid interface of standing cultures (Hofemeister *et al.*, 2004). It has recently been reported that potassium leakage across the cytoplasmic membrane of *B. subtilis*, caused by the action of surfactin, serves as a signal to activate a membrane histidine kinase protein, KinC. This kinase protein was subsequently shown to govern the expression of the *yqxM-sipW-tasA* operon, which is involved in the synthesis of the extracellular matrix of the biofilm (Lopez *et al.*, 2009). Interestingly, these results imply that the lowered intracellular potassium concentration, rather than the surface tension-lowering properties of surfactin, serves as a trigger for biofilm formation.

1.4.3 Interspecies signalling via the quorum sensing molecule AI-2

In contrast to acylated homoserine lactones and oligopeptide autoinducers, which are used by Gram-negative and Gram-positive bacteria, respectively, the autoinducer AI-2 is produced by a wide variety of Gram-negative and Gram-positive bacteria (Surette and Bassler, 1998; Xavier and Bassler, 2003). Clues to the widespread prevalence of AI-2 came from discoveries that an activity in the cell-free culture fluids of many bacterial species could be detected by AI-2-specific *Vibrio harveyi* reporter strains (Bassler *et al.*, 1997). Consequently, it has been proposed that AI-2 is a universal signal that functions in interspecies cell-to-cell communication (Xavier and Bassler, 2003). Moreover, it has been reported to control a variety of cellular processes, including the production of pathogenecity factors, toxin production, swarming motility and biofilm formation (Kim *et al.*, 2003; Merritt *et al.*, 2003; Wen and Burne, 2004; Merritt *et al.*, 2005; Auger *et al.*, 2006; Gonzalez Barrios *et al.*, 2006).

Since the discovery of AI-2 in the Gram-negative bacterium *V. harveyi* (Bassler *et al.*, 1994), much progress has been made in unraveling the mechanism of AI-2 detection and signal transduction (Waters and Bassler, 2005), as well as the biosynthetic pathway of AI-2 (Schauder *et al.*, 2001) in this bacterium. The AI-2 of *V. harveyi* is a furanosyl borate diester (Chen *et al.*, 2002), which is bound in the periplasm by the LuxP protein. The LuxP-AI-2 complex subsequently interacts with the inner membrane-bound sensor histidine kinase, LuxQ. The LuxQ protein is a two-component protein of the hybrid-sensor class since it contains a sensor kinase domain and a response regulator domain (Bassler *et al.*, 1994). At low cell density, in the absence of appreciable amounts of AI-2, LuxQ acts as a sensor kinase, autophosphorylates, and subsequently transfers the phosphate to the cytoplasmic protein LuxU, which then transfers the phosphate to the response regulator protein LuxO (Freeman and Bassler, 1999). The phosphorylated LuxO, in conjunction with a σ^{54} transcription factor, activates transcription of five small regulatory RNAs, designated Qrr1-5 (Lenz *et al.*, 2004). These small RNAs interact with the RNA chaperone Hfq to destabilize the mRNA transcript encoding LuxR, the transcriptional activator required for the expression of the luciferase operon *luxCDABE* (Swartzman *et al.*, 1992). Thus, at low cell density, the bacteria do not express bioluminescence. At high cell density, when the AI-2 accumulates to a minimal threshold stimulatory concentration, the LuxQ sensor switches from being a kinase to being a phosphatase and thus drains phosphate from LuxO via LuxU. Unphosphorylated LuxO cannot induce expression of the Qrr RNAs, thereby allowing transcription of *luxR* mRNA, production of LuxR and ultimately expression of bioluminescence (Waters and Bassler, 2005). The AI-2 density-dependent expression of bioluminescence in *V. harveyi* is shown in Fig. 1.5.

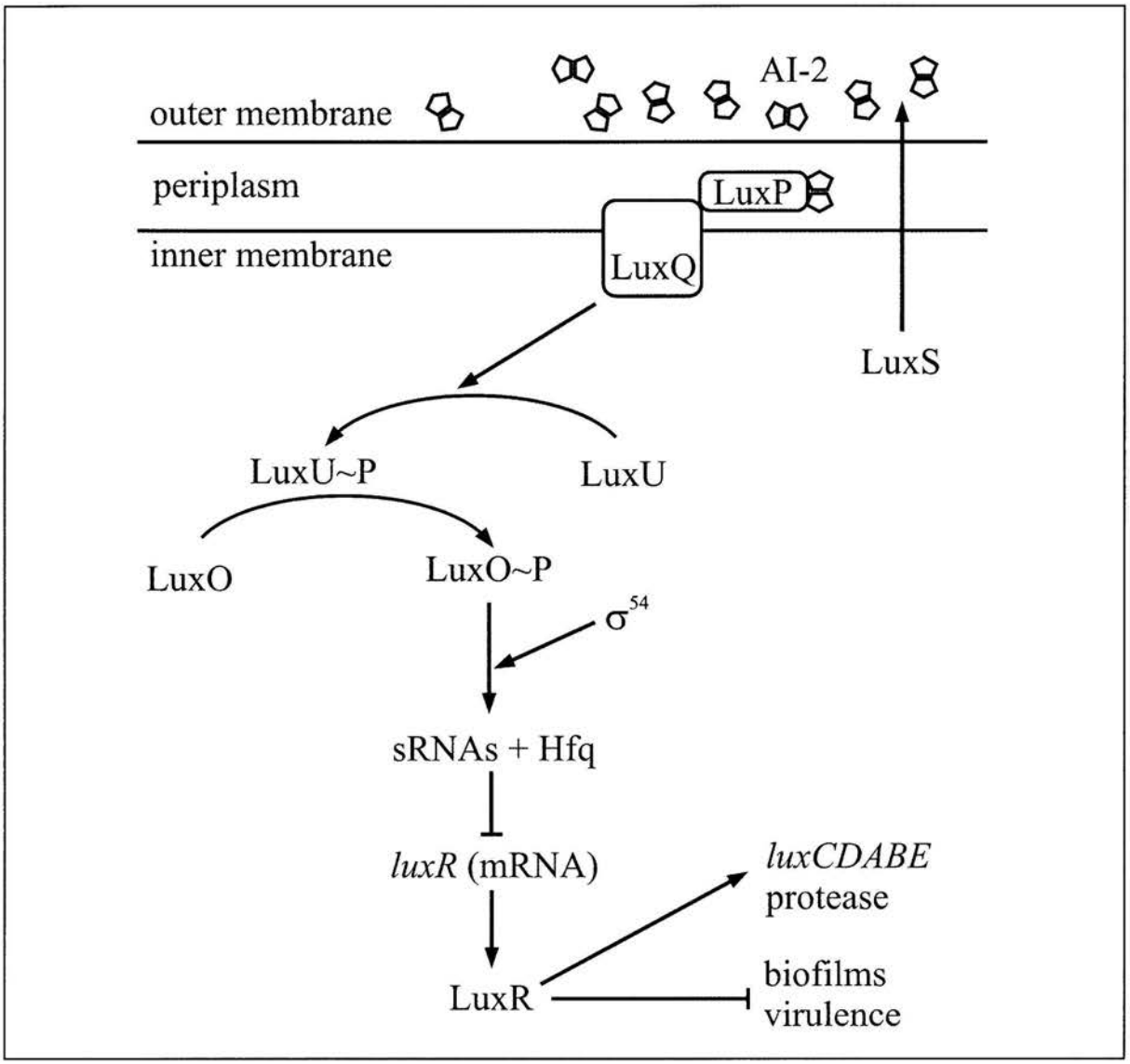

Figure 1.5: AI-2-dependant quorum sensing in *V. harveyi*. AI-2 (double pentagons) is synthesized in a reaction mediated by the LuxS enzyme. The autoinducer AI-2 is detected by LuxQ, in addition to a periplasmic binding protein, called LuxP. The LuxQ protein is a hybrid sensor kinase that contains a sensor kinase domain and an attached response regulator domain. Signalling from the LuxQ sensor is channelled to LuxU, a phosphotransferase protein that relays the information to the response regulator LuxO. At low cell density, LuxQ autophosphorylates and conveys phosphate through LuxU to LuxO. Phosphorylated LuxO indirectly suppresses *luxCDABE* expression. At high cell density, when LuxQ interact with AI-2, it changes from a kinase to phosphatase that drains away phosphate from LuxO via LuxU. Unphosphorylated LuxO is inactive, thus LuxR binds to the *luxCDABE* promoter and activates its transcription. In *V. cholerae*, the LuxS/AI-2 system functions analogously to that of *V. harveyi*, but controls virulence and biofilm formation instead of regulating bioluminescence. In *V. cholerae*, AI-2-mediated quorum sensing promotes virulence factor expression and biofilm formation at low cell density and represses these traits at high cell density. (Adapted from Waters and Bassler, 2005).

In *V. harveyi*, AI-2 is produced from *S*-adenosylmethionine (SAM) in three enzymatic steps (Fig. 1.6). SAM is an essential cofactor for anabolic processes such as DNA, RNA and protein synthesis (Berg *et al.*, 2002). The use of SAM as a methyl donor in these metabolic processes produces the intermediate product *S*-adenosylhomocysteine (SAH), which is hydrolyzed by the nucleosidase enzyme Pfs to adenine and *S*-ribosylhomocysteine (SRH). LuxS catalyzes the cleavage of SRH to homocysteine and 4,5-dihydroxy 2,3-pentanedione (DPD) (Schauder *et al.*, 2001). DPD is the precursor of AI-2 and is able to undergo spontaneous cyclization to different interconverting furanones, the identity of which depends on the chemical environment in which the reaction takes place (Vendeville *et al.*, 2005; Xavier *et al.*, 2007). One or all of these products is believed to be the AI-2 signal and different bacterial species appear to recognize various forms of DPD as the AI-2 signal. Because the AI-2s interconvert, bacteria that detect distinct DPD derivatives are thus able to communicate with one another (Waters and Bassler, 2005).

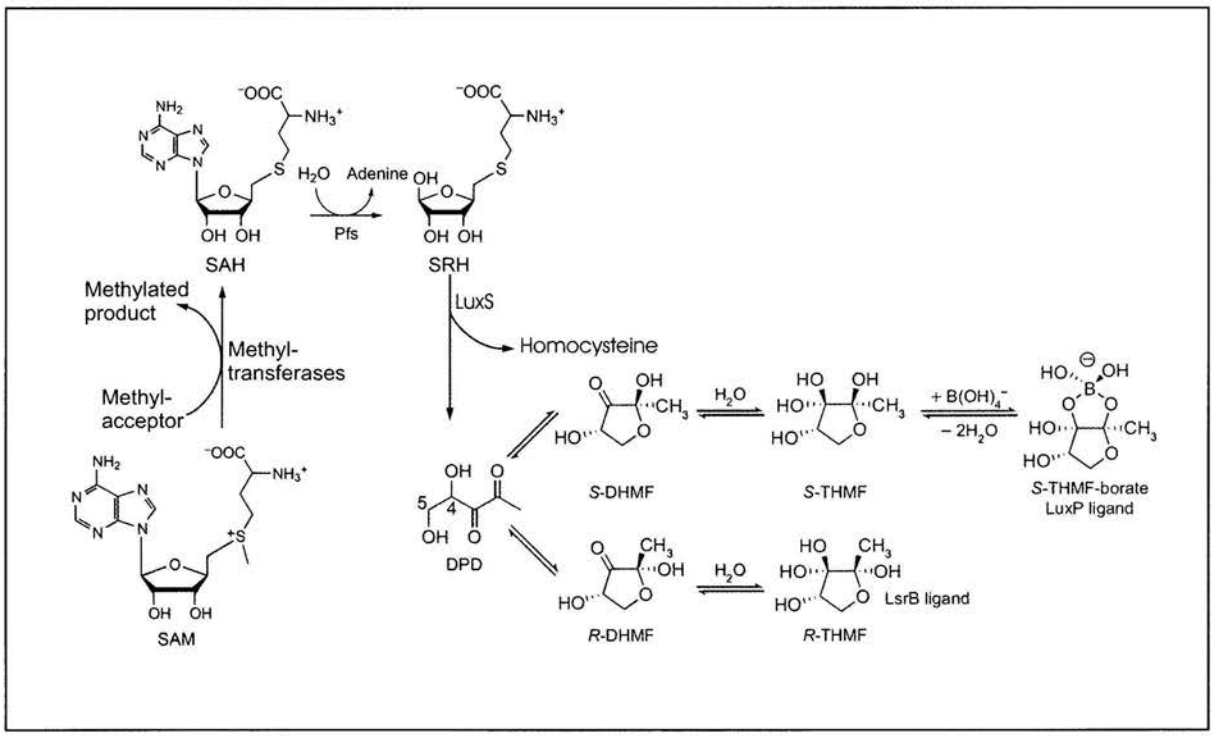

Figure 1.6: Biosynthesis of AI-2 from the precursor *S*-adenosylmethionine (SAM). Many methyltransferases act on SAM and transfer a methyl group to various substrates. These reactions also produce *S*-adenosylhomocysteine (SAH). Pfs hydrolyzes adenine from SAH to form *S*-ribosylhomocysteine (SRH). LuxS acts on SRH to produce 4,5-dihydroxy 2,3-pentanedione (DPD) and homocysteine. DPD is able to undergo spontaneous cyclization to either of the two forms (*R* or *S*) of 2,4-dihydroxy-2-methylhydro-3-furanone (DHMF). Hydration of *S*-DHMF yields *S*-TMHF that subsequently forms a diester with boric acid to generate the active form of AI-2 in *V. harveyi*. On the other hand, *R*-DHMF hydrates to form *R*-THMF, the active form of enteric AI-2. (Adapted from Xavier and Bassler, 2003 and Xavier *et al.*, 2007).

In contrast to Gram-negative bacteria, only a limited number of studies regarding LuxS/AI-2-dependent quorum sensing in Gram-positive bacteria have been undertaken. In one of the first such studies, it was shown that a *luxS* homologue present in the genome of *Streptococcus gordonii* was responsible for the synthesis of functional AI-2, as was evidenced by its ability to induce bioluminescence expression in an AI-2 reporter strain of *V. harveyi* (McNab *et al.*, 2003). Although a *luxS* mutant, generated by insertional inactivation of the *luxS* gene, was not affected in its ability to form biofilms on a polystyrene surface, the mutant *S. gordonii* strain was unable to form a mixed species biofilm with a *luxS*-null mutant strain of *Porphyromonas gingivalis*. However, complementation of the *luxS* mutation in *S. gordonii* restored normal biofilm formation with the *luxS*-deficient *P. gingivalis*, suggesting that LuxS-dependent intercellular communication is essential for mixed-species biofilm formation between cells of these two bacterial species (McNab *et al.*, 2003).

More recently, it was reported that *B. subtilis* JH642 also harbours an active *luxS* gene that mediates production of functional AI-2 capable of inducing bioluminescence expression in a *V. harveyi* AI-2 reporter bioassay (Lombardía *et al.*, 2006). In contrast to enteric bacteria for which *luxS* expression is constitutive (Beeston and Surette, 2002; Xavier and Bassler, 2005), it was reported that expression of the *luxS* gene of *B. subtilis* was down-regulated at the end of the exponential growth phase through an apparent AI-2-dependant negative autoregulatory feedback loop. It was furthermore reported that *luxS* expression was negatively regulated by SinR and Spo0A, both of which had previously been reported to regulate biofilm formation and swarming migration (Branda *et al.*, 2001; Hamon and Lazazzera, 2001; Kearns *et al.*, 2005; Chu *et al.*, 2006). In contrast, AbrB had no effect on *luxS* expression. Subsequent analysis of LuxS-proficient and LuxS-deficient wild-type *B. subtilis* RG4365 cells indicated that the LuxS-dependent quorum sensing system was indeed required to form robust and highly structured floating biofilms (pellicles) in standing liquid cultures, as well as to swarm on solid surfaces. Moreover, although LuxS did not affect spore development, LuxS activity was shown to be required for the formation of fruiting bodies where AI-2 production and spore morphogenesis were spatially regulated at different sites of the developing fruiting body (Fig. 1.7).

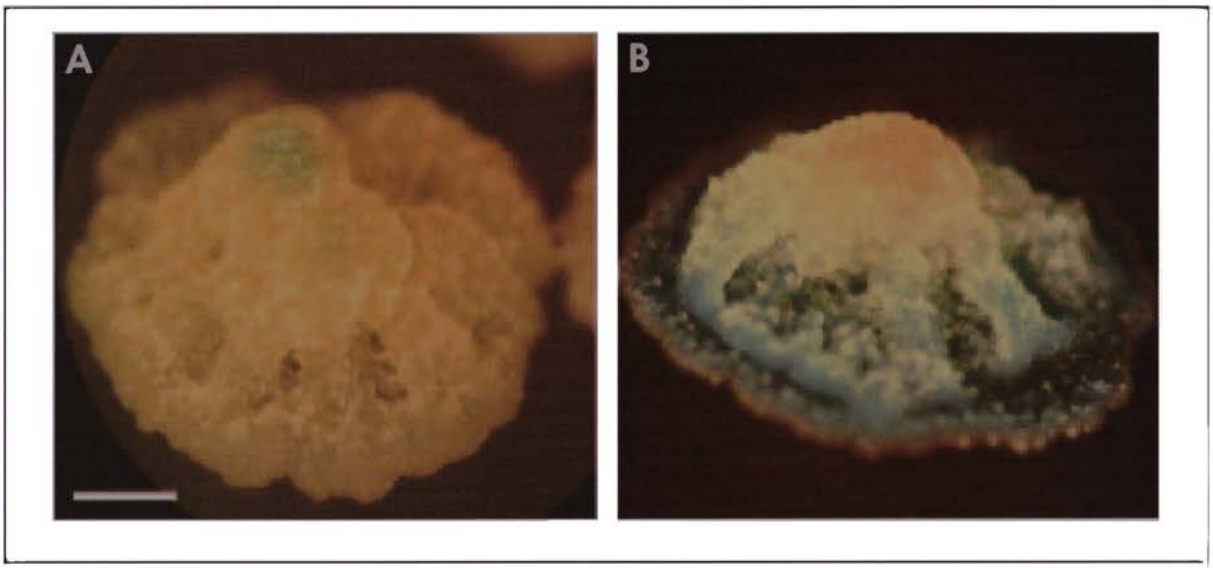

Figure 1.7: Expression of a *sspB-lacZ* fusion (A) and a *luxS-lacZ* fusion (B) by *B. subtilis* colonies grown on agar containing X-Gal. The *sspB* gene is expressed late during sporulation and the localized development of the blue colour indicates that spore morphogenesis is restricted to the older cells localized at the pinnacle of the colony. The *luxS* activity forms a gradient from the bottom part of the colony towards the top, suggesting an AI-2 concentration gradient. (From Lombardía *et al.*, 2006).

It has also been reported that genes encoding Pfs- and LuxS-like enzymes are present in the recently sequenced genomes of *B. cereus*, *B. thuringiensis* and *B. anthracis* (Jones and Blaser, 2003). For *B. anthracis*, it was shown that the predicted *luxS* ortholog encodes a LuxS enzyme that is responsible for the production of functional AI-2 (Jones and Blaser, 2003). In a more extensive study, synthesis of functional AI-2 signal molecules by *B. cereus* ATCC 10987 was demonstrated through induction of luminescence in a *Photorhabdus luminescens* AI-2 reporter bioassay (Auger *et al.*, 2006). The level of light induction exhibited growth-phase dependence with a maximum corresponding to a late-exponential phase culture, as was also observed in *B. subtilis* JH642 (Lombardía *et al.*, 2006). The exogenous addition of *in vitro*-synthesized AI-2 was shown to have an inhibitory effect on biofilm formation by *B. cereus* ATCC 10987 in polyvinylchloride microtiter plates. Moreover, the addition of AI-2 to preformed biofilms reduced its density, suggesting that AI-2 may play a role in eliciting the release of cells from a preformed biofilm (Auger *et al.*, 2006). Bioinformatic analyses indicated that the genome of *B. cereus* ATCC 10987 lacked homologs of the *V. harveyi luxP* and *luxQ* genes (Auger *et al.*, 2006). However, the genome does contain genes encoding a putative Lsr ABC transporter, which is similar to that involved in the uptake and processing of AI-2 in enteric bacteria (Taga *et al.*, 2003; Xavier and Bassler, 2005; Xavier *et al.*, 2007)

(Fig. 1.8). It is interesting to note that the Lsr-like system was not found in any other sequenced Gram-positive bacterium, including *B. subtilis*, and thus appears to be unique to *B. cereus*.

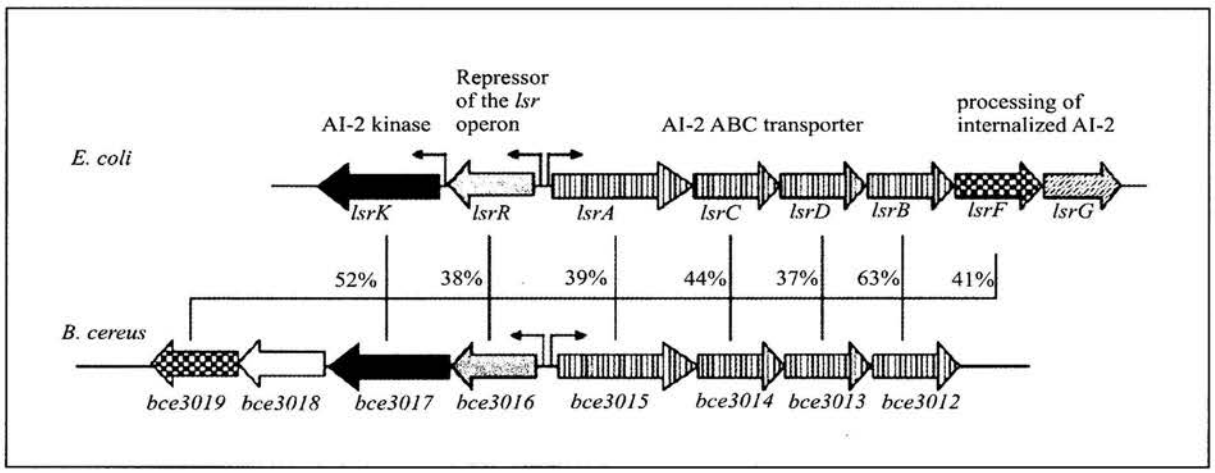

Figure 1.8: Genetic organization and sequence analysis of the *lsr* region in *B. cereus* ATCC 10987 and *E. coli* K-12. Putative transcription start sites are indicated by arrows. For each gene product, the similarity between the *B. cereus* and *E. coli* proteins is indicated as a percentage of identity. The genes encoding the ABC transporter are represented by striped boxes, LsrR-like regulators are represented by gray boxes, and processing enzymes are represented by checkered boxes (*lsrF*-like) or a diagonally striped box (*lsrG*). (From Auger *et al.*, 2006).

The LuxS regulated (Lsr) transporter system of *E. coli* is encoded by genes in the *lsr* operon (*lsrACDBFGE*) (Xavier and Bassler, 2005; Xavier *et al.*, 2007). Whereas the first four genes encode components of the AI-2 transport apparatus, the distal genes are required for processing of AI-2 following internalization. Adjacent to, but transcribed divergently from the *lsr* operon, is *lsrR*, which encodes a repressor of *lsr* transcription, and *lsrK*, which encodes a kinase that phosphorylates intracellular AI-2 following import. Phosphorylation of internalized AI-2 has been shown to induce transcription of the *lsr* operon, indicating that phosphorylated AI-2 is the inducer of the system (Xavier and Bassler, 2005). The phosphorylated AI-2 is proposed to bind to the LsrR repressor, thereby inactivating it and thus resulting in derepression of *lsr* transcription (Fig. 1.9). Although the function of the LsrF and LsrG proteins have yet to be clarified, it was recently reported that LsrG cleaves phosphorylated DPD (Xavier *et al.*, 2007). Degradation of phosphorylated DPD was shown to terminate the induction of the *lsr* operon, which, in turn, closed the AI-2 signalling cycle. Whether the Lsr system of *B. cereus*, which appears to lack a homologue of the *lsrG* gene (Fig. 1.8), is indeed used for the internalization, phosphorylation and processing of the AI-2 signal remains to be determined.

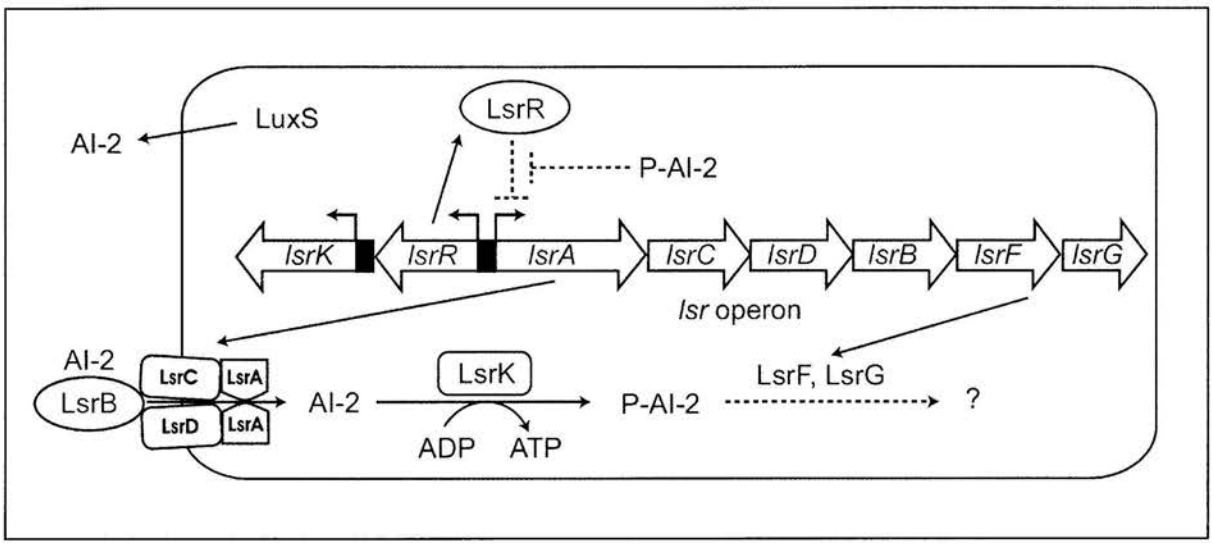

Figure 1.9: Model for Lsr-mediated internalization and processing of AI-2 in *E. coli*. AI-2 is synthesized in a reaction mediated by LuxS and accumulates extracellularly. AI-2 is bound by the periplasmic protein LsrB and internalized by the Lsr ABC-type transporter, and intracellular AI-2 is phosphorylated by the LsrK kinase. Phosphorylated AI-2 induces expression of the *lsr* operon, thereby inactivating it. Induction of *lsr* expression causes rapid Lsr-dependent AI-2 internalization. The LsrF and LsrG may be required for processing of internalized AI-2, as indicated by the dotted lines. (From Xavier *et al.*, 2007).

1.5 Regulation of biofilm formation by *B. subtilis*

In standing liquid medium, cells of *B. subtilis* switch from a submerged highly motile planktonic state in which the bacteria swim as single cells, to a non-motile state in which the cells grow as bundled chains that rise to the surface and form a robust pellicle (Branda *et al.*, 2001). The bundled chains of cells are held together by an extracellular matrix that is primarily composed of EPS and protein (Branda *et al.*, 2001; Branda *et al.*, 2006). Consequently, matrix production and assembly is regarded as the critical determinant for transition to the biofilm lifestyle. As discussed previously (Section 1.3.3), *B. subtilis* biofilm formation requires the expression of two operons that encode structural components of the extracellular matrix. One operon is the 15-gene *epsA-O* operon (henceforth *eps*) that encodes the biosynthetic machinery required to produce EPS (Branda *et al.*, 2001), while the other operon is the three-gene *yqxM-sipW-tasA* operon (henceforth *yqxM*) that encodes the secreted matrix protein TasA (Branda *et al.*, 2006; Chu *et al.*, 2006). Due to the importance of the extracellular matrix to biofilm formation, much focus has been placed on elucidation of the regulatory circuitry that governs expression of the *eps* and *yqxM* operons.

1.5.1 Regulation of the *eps* operon

Towards identifying regulators for *B. subtilis* biofilm formation, initial studies performed by Kearns *et al.* (2005) focused on the two regulatory genes *sinR*, which is known to encode a DNA binding protein (Gaur *et al.*, 1991), and *sinI*, which is known to encode an antagonist of *SinR* with which it forms a complex (Bai *et al.*, 1993). It had previously been reported that cells of a *sinR* mutant causes the formation of rugose colonies in which cells grow as chains of non-motile cells, while cells of a *sinI* mutant are always motile and do not form chains (Gaur *et al.*, 1991; Bai *et al.*, 1993). Consequently, the effect of mutations in these genes on biofilm formation and swarming motility was investigated by introducing null mutations of *sinI* and/or *sinR* into the wild-type *B. subtilis* NCIB 3610 strain. The results indicated that although the *sinI* mutant did not produce a pellicle, it did swarm as readily as the wild-type strain. In contrast, the *sinR* mutant grew as bundled chains and formed robust rugose pellicles but was unable to swarm. Consistent with the idea that SinI is an antagonist of SinR, the *sinI sinR* double mutant displayed phenotypes indistinguishable from those of the *sinR* mutant (Fig. 1.10). Since cells containing mutations in the *eps* operon had previously been reported to be defective in bundling of cell chains and to produce fragile pellicles (Branda *et al.*, 2001), the relationship of *eps* genes to *sinR* was also investigated by introducing a mutation of *epsH* (formerly known as *yveR*) into the *sinR* mutant. The *epsH sinR* double mutant was found to be epistatic to the *sinR* mutation in that the double mutant resembled the *epsH* single mutant with regards to its inability to form bundled cell chains and production of fragile pellicles (Fig. 1.10). Notably, the *epsH* mutation did not restore swarming to the *sinR* mutant, thus indicating that the motility defect caused by the absence of SinR was not a consequence of tethering the cells to each other by EPS.

The results presented above suggested that SinR is a negative regulator of the *eps* operon. To confirm, a transcriptional fusion of *lacZ* to the promoter region upstream of the first gene in the *eps* operon (P_{epsA}) was constructed and introduced into the *sinI* and *sinR* mutant strains, respectively. Whereas the *sinR* mutation up-regulated expression of the P_{epsA}-*lacZ* reporter construct by *ca.* 20-fold, the *sinI* mutation down-regulated its expression by *ca.* 30-fold. Subsequent electrophoretic mobility shift assays (EMSAs) and DNase I footprinting experiments indicated that SinR binds to several sites (GTTCTYT) within the regulatory region of the *eps* operon (Fig. 1.11A). During the course of these experiments, it was also shown that the addition of SinI as a competitor in the EMSAs diminished the ability of SinR

to bind to its target DNA, thus confirming that SinI is an antagonist of SinR that inhibits the capacity of the repressor protein to bind to its target DNA (Kearns *et al.*, 2005).

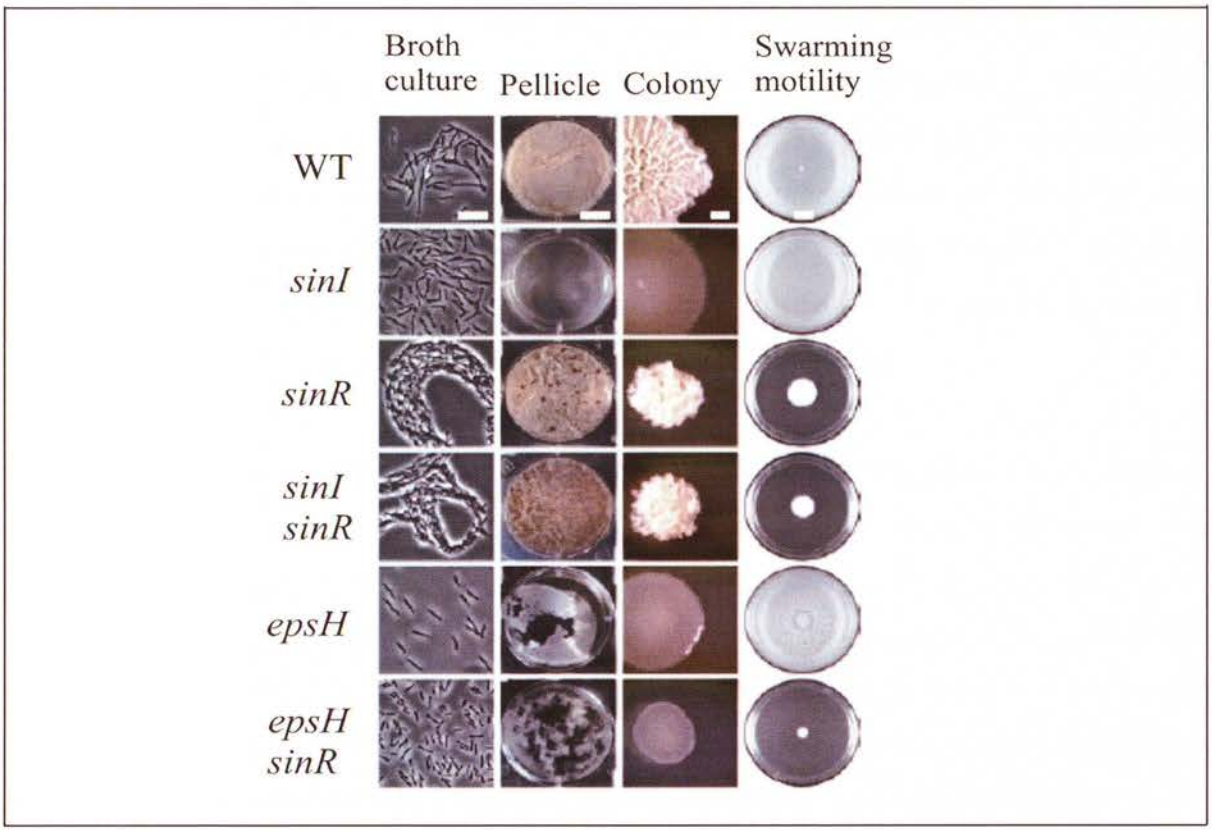

Figure 1.10: Effect of mutations in *sinR*, *sinI* and *epsH* on chain bundling, pellicle formation, colon morphology and swarming motility. The broth culture cells were grown to mid-exponential phase in LB broth while pellicles were obtained by culturing cells for 3 days in 6-well culture plates. (From Kearns *et al.*, 2005).

Cumulatively, these results therefore not only showed that transcription of the *eps* operon is under the negative regulation of SinR and that derepression is achieved by the action of the anti-repressor SinI, but, importantly, it also showed that *B. subtilis* exists as two mutually exclusive physiological states in which cells either grow as bundled chains or as single motile cells. Each state is associated with an alternative multicellular behaviour in that growth in bundled chains promoted biofilm formation, whereas growth as single cells promoted swarming. Consequently, it was proposed that SinR serves as a master regulator for biofilm formation that governs the state in which cells are capable of swimming or swarming to a sessile state characteristic of biofilms in which long chains of cells are bound to each other by EPS in tight bundles (Kearns *et al.*, 2005).

1.5.2 Regulation of the *yqxM* operon

In an extension of the studies performed by Kearns *et al.* (2005), another group (Chu *et al.*, 2006) adopted a similar experimental approach and reported that SinR also controls the transcription of the *yqxM* operon. SinR was shown to bind to multiple sites within the promoter region for the operon (Fig. 1.11), thereby repressing its transcription. Furthermore, microarray analysis with RNA from cells of a *sinI* mutant and a *sinR* mutant indicated that members of the *eps* and *yqxM* operons represented a high proportion of the genes that were most strongly controlled by SinR. These findings therefore not only provided supporting evidence for SinR acting as a master regulator of biofilm formation, but also indicated that it is likely to be one of its principal biological functions (Chu *et al.*, 2006).

The AbrB repressor protein had previously been implicated in the formation of surface-attached biofilms by *B. subtilis* JH642 and microarray analysis showed that AbrB also represses transcription of the *yqxM* operon (Hamon *et al.*, 2004). To elucidate the relative contributions of AbrB and SinR to control expression of the *yqxM* operon, Chu *et al.* (2008) introduced a null mutation of *abrB* in *B. subtilis* NCIB 3610 and reported that the mutant strain formed very thick pellicles in standing cultures that resembled the pellicles formed by a *sinR* mutant. By making use of a transcriptional fusion between *lacZ* and the promoter for the *yqxM* operon (P_{yqxM}), it was shown that reporter gene expression was up-regulated 8-fold and 5-fold in the *abrB* mutant and *sinR* mutant, respectively, while the level of expression in a *abrB sinR* double mutant was almost equal to the sum of the levels for the single mutants. These results therefore indicated that the two repressor proteins acted independently in repressing the *yqxM* operon, with AbrB repressing the operon to a greater extent than SinR. The results of EMSAs and DNase I footprinting experiments indicated that the promoter region of the *yqxM* operon contained two binding sites for AbrB (Fig. 1.11), in addition to the binding sites for SinR. Analysis of the upstream regulatory region of the promoter of the *yqxM* operon furthermore indicated the presence of two direct repeat sequences (TGAGCAA), the deletion of which from the P_{yqxM}-*lacZ* reporter construct resulted in a dramatic decrease in *lacZ* expression (60-fold) in the *B. subtilis* NCIB 3610 strain. This result therefore indicated that P_{yqxM} may be under the positive control of an activator protein. The Slr protein, encoded by the *slr* gene located immediately adjacent to the *eps* operon, was subsequently shown to promote transcription of the *yqxM* operon. Not only did a *slr*-null

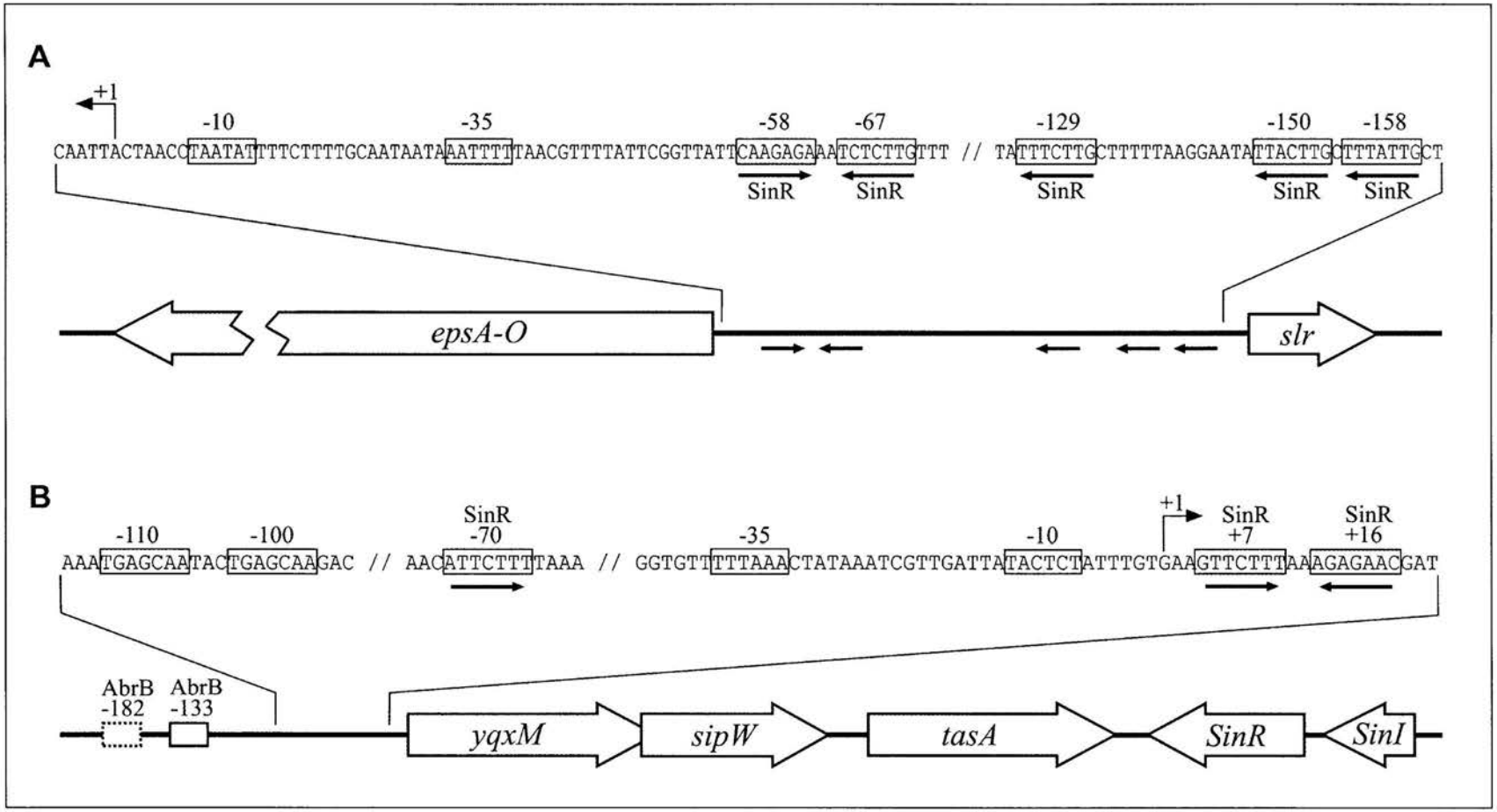

Figure 1.11: Structure and organization of SinR and AbrB recognition sequences in the promoter regions of the *epsA-O* and *yqxM-sipW-tasA* operons, as determined by DNase I footprinting experiments. (Kearns *et al.*, 2005; Chu *et al.*, 2006; Strauch *et al.*, 2007; Chu *et al.*, 2008).

mutant strain of *B. subtilis* NCIB 3610 form thin flat pellicles, but expression of the P_{yqxM}-*lacZ* reporter construct was impaired in the absence of Slr. Indeed, overexpression of the *yqxM* operon from an IPTG-inducible promoter in a Slr mutant background largely restored pellicle formation to the *slr* mutant, indicating that the *yqxM* operon is the major, if not the only, target of the Slr activator protein. Analysis of a P_{slr}-*lacZ* transcriptional fusion in a *sinR* mutant and an *abrB* mutant indicated an increase in *lacZ* expression (25-fold and 2-fold, respectively), suggesting that the *slr* gene is under the negative control of SinR and AbrB (Chu *et al.*, 2008). It should be noted that in a parallel series of experiments, it was shown that the *eps* operon was only slightly repressed by AbrB and that the Slr activator protein was not needed for expression of the *eps* operon (Chu *et al.*, 2008).

Cumulatively, these results show that TasA protein production by the *yqxM* operon is governed by an intricate network involving the interplay of negatively (SinR and AbrB) and positively acting (Slr) regulatory proteins. Moreover, although Slr promotes transcription of the *yqxM* operon, the *slr* gene is itself under the negative control of SinR and AbrB. This high level of regulation has been proposed to ensure tight regulation of the operon so that the TasA protein is produced only under appropriate conditions (Chu *et al.*, 2008).

1.5.3 Derepression of the *eps* and *yqxM* operons by SinI

It has been reported that SinI binds to the SinR repressor protein in a 1:1 stoichiometry (Lewis *et al.*, 1996). It can therefore be expected that the cellular concentration of SinI should be at least as great as that of SinR under the conditions in which SinR-controlled genes are derepressed. Indeed, Kearns *et al.* (2005) reported that the concentration of SinI must be equal to, or in excess of, that of SinR to displace the repressor from its operator in EMSAs. However, analysis of transcriptional fusions of *lacZ* with the promoters of *sinI* (P_{sinI}) and *sinR* (P_{sinR}) indicated that reporter gene expression from P_{sinI}-*lacZ* was *ca.* 15-fold lower than that of P_{sinR}-*lacZ* in *B. subtilis* NCIB 3610 cells when growing in a medium that promotes biofilm formation (Chai *et al.*, 2007). This unexpected result was confirmed by quantitative immunoblot analysis using antibodies directed against SinI or SinR. The results showed that the cellular concentration of SinI was 18-fold lower than that of SinR in cells reaching early stationary phase, the time at which derepression of SinR-controlled genes commences (Kearns *et al.*, 2005). These results therefore suggest that the concentration of SinI may be too low to counteract SinR effectively.

To resolve how SinI could override SinR under these conditions, Chai *et al.* (2007) monitored expression of the *sinI* gene using a green fluorescent reporter gene (*gfp*) fused to the promoters of *sinI* (P_{sinI}) and *sinR* (P_{sinR}). Fluorescence microscopy of early stationary phase cultures harboring chromosomally integrated copies of the respective reporter constructs indicated that P_{sinR}-*gfp* was expressed in nearly all cells. In contrast, P_{sinI}-*gfp* was highly expressed in only a small minority (*ca.* 2%) of the cells. In this sub-population the ratio of SinI to SinR was estimated to be 2.8:1, thus indicating that SinI accumulated to sufficient levels to counteract the uniformly present SinR. Fluorescent microscopy experiments using cells that harboured a *gfp* fusion to the promoters for the *eps* (P_{epsA}) and *yqxM* (P_{yqxM}) operons showed that both reporter constructs were expressed in only a subset of cells. Using a dual-labelling experiment (P_{sinI}-*cfp* and P_{yqxM}-*yfp*), it was subsequently shown that those cells that expressed SinI also expressed the *yqxM* operon. These results therefore suggest that at the early stages of biofilm formation only a sub-population of cells (those in which the concentration of SinI reaches or exceeds that of SinR) are specialized for the production of the biofilm matrix components (Chai *et al.*, 2007).

Since matrix production is energetically costly, it is thought that this strategy might relegate the cost to a sub-population that provides protection for the entire community (Chai *et al.*, 2007). In this regard, Branda *et al.* (2006) reported that although *eps* and *tasA* mutants were unable to form architecturally complex biofilms individually, they could assemble a biofilm that resembled that of the wild-type *B. subtilis* strain when mixed together in co-culture. These results therefore appears to support the notion that biofilms can be assembled when only a minority of cells in the population produces the matrix components, and that the matrix can be shared.

1.5.4 Control of biofilm formation

Based on the preceding sections, it follows that biofilm formation by *B. subtilis* is essentially governed by two parallel pathways of repression and antirepression (Fig. 1.12). The first of these is the SinR/SinI pathway. During exponential growth the *eps* and *yqxM* operons are repressed in all cells by the SinR repressor protein, which is expressed constitutively from a promoter dependent on the major housekeeping sigma factor σ^A (Gaur *et al.*, 1988; Shafikhani *et al.*, 2002). SinI, the antagonist of SinR, is expressed from two σ^A-dependent promoters, the

major one being under the positive control of the sporulation regulatory protein Spo0A (Shafikhani *et al.*, 2002). Although it has been shown that σ^H is also required for biofilm formation (Branda *et al.*, 2001), its effect may be limited to stimulating expression of Spo0A since *spo0A* itself contains a σ^H-dependent promoter (Predich *et al.*, 1992). Nevertheless, both Spo0A and σ^H can be proposed to contribute to biofilm formation in part by stimulating the synthesis of the SinR antagonist. It has furthermore been reported that SinI is expressed in only a sub-population of the biofilm cells (Chai *et al.*, 2007). This heterogonous nature of *sinI* gene expression is believed to be governed by a bistable switch. Bistable switches produce polarized expression states and result in a sub-population of specialized cell types that are either "ON" or "OFF" for gene expression (Veening *et al.*, 2005; Dubnau and Losick, 2006). Spo0A is known to be distributed in a bimodal manner amongst cells of the population with some cells attaining high levels of Spo0A activity (Spo0A-ON cells) and other cells a relatively low level (Spo0A-OFF cells) (Veening *et al.*, 2005). This is, however, not a simple ON/OFF switch since the level of phosphorylated Spo0A rises gradually in cells that ultimately attain a high level of Spo0A activity. As a consequence of being regulated by Spo0A, SinI expression and activity is therefore also bimodal in cultures. Relief from SinR-mediated repression is achieved through binding of SinI to SinR, thereby preventing the repressor from binding to its target DNA and thus allowing expression of the *eps* and *yqxM* operons.

In a parallel pathway to the SinR/SinI pathway, *B. subtilis* biofilm formation is also controlled by a Spo0A/AbrB pathway (Hamon and Lazazzera, 2001). AbrB is a transcriptional repressor protein that is abundant in vegetatively growing cells, and represses transcription of many genes that are active in the stationary phase (Strauch *et al.*, 1990). Repression of *abrB* transcription appears to be a major role for Spo0A in biofilm formation, as an *abrB* mutation can bypass the requirement for Spo0A for biofilm formation (Hamon and Lazazzera, 2001). Moreover, σ^H may indirectly repress AbrB expression since σ^H is known to activate the expression of *spo0A* (Predich *et al.*, 1992). AbrB-regulated loci that are required for biofilm formation include the *yqxM* operon (Hamon *et al.*, 2004; Chu *et al.*, 2008), although the *eps* operon has also been reported to be under the negative control of AbrB (Chu *et al.*, 2008). It was recently reported that AbrB is inactivated at the end of the exponential growth phase by the product of an uncharacterized gene, *abbA* (formerly known as *ykzF*), the transcription of which is under the direct positive control of Spo0A (Banse *et al.*, 2008). Relief from AbrB-mediated repression is proposed to be mediated by AbbA, which acts as an antirepressor by

binding to AbrB, and thus prevents AbrB from binding to DNA, thereby allowing expression of the *eps* and *yqxM* operons. In the case of the *yqxM* operon, its expression has furthermore been shown to be under the positive control of the Slr activator protein, albeit that the *slr* gene is under the negative control of both SinR and AbrB (Chu *et al.*, 2008).

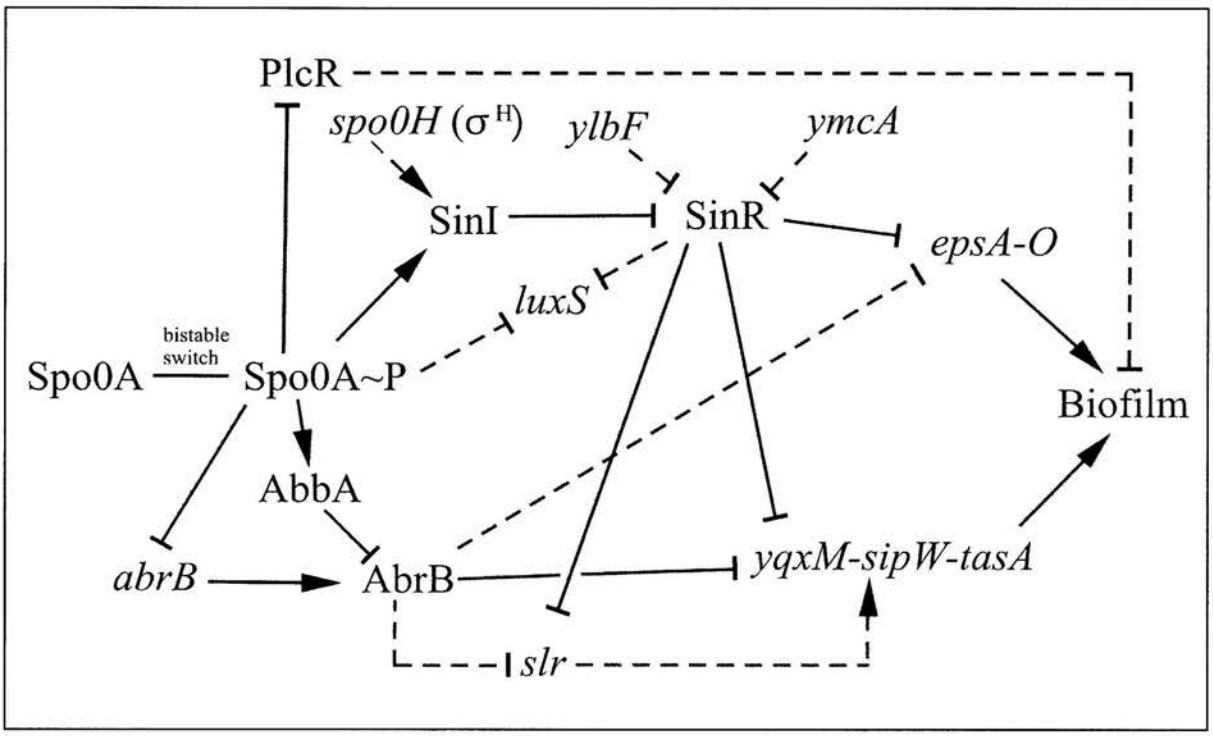

Figure 1.12: Regulation of *Bacillus* multicellular behaviour. Green arrows indicate activation of gene expression and red T bars indicate repression. Solid lines denote direct regulation and dashed lines denote unknown mechanisms. The pathway indicated in blue occurs only in *B. cereus*. SinR and AbrB represent parallel pathways, both controlled by the response regulator Spo0A. Thus, activation of Spo0A sets in motion events (induction of the antirepressor genes *sinI* and *abbA*) that relieve SinR- and AbrB-mediated repression. The ultimate targets of the repression are the *epsA-O* and *yqxM-sipW-tasA* operons, which govern extracellular matrix production required for biofilm formation. Also indicated is the *slr* gene, the product of which promotes transcription of the *yqxM-sipW-tasA* operon. In *B. subtilis* the LuxS-dependent quorum system was shown to be required for biofilm formation and that expression of the *luxS* gene is negatively regulated by both SinR and Spo0A (Lombardía *et al.*, 2006). In the case of *B. cereus*, biofilm formation is impaired via direct or indirect repression of the production of a lipopeptide biosurfactant by the pleiotropic regulator PlcR (Hsueh *et al.*, 2006).

1.6 Aims of this investigation

Many bacteria can assume a communal lifestyle, forming biofilms. Indeed, in the microbial world, existence within surface-associated structured multicellular communities may be the rule rather than the exception (Davey and O'Toole, 2000; O'Toole *et al.*, 2000; Hall-Stoodley *et al.*, 2004). Biofilms are sessile microbial populations, often embedded in an extracellular polymeric matrix, localized to surfaces and interfaces (Costerton *et al.*, 1995). In the Gram-positive bacterium *B. subtilis*, biofilm formation has been examined on solid surfaces (Hamon

and Lazazzera, 2001; Hamon *et al.*, 2004) and at air-liquid interfaces (Branda *et al.*, 2001; Branda *et al.*, 2004). Based on these studies, a number of determinants of biofilm formation have been identified that include, amongst other, transcriptional regulators, proteins involved in extracellular matrix biosynthesis, flagellar proteins and a number of proteins of largely unknown function. Despite the progress that have been made, elucidation of genes, proteins and molecular mechanisms involved in biofilm formation by *B. subtilis* continues (Chai *et al.*, 2009). Clearly, much is still to be learned regarding the development, maintenance and dissolution of *B. subtilis* biofilms.

To further our understanding of biofilm formation by Gram-positive bacteria, we have focused our attention on *B. cereus*; a pathogen that causes two distinct types of food poisoning, the diarrheal and emetic syndromes, as well as a variety of local and systemic infections (Turnbull *et al.*, 1979; Granum and Lund, 1997). It is regarded as one of the common organisms that impair the quality of dairy products. *B. cereus* has been shown to form biofilms on a variety of surfaces, including plastic, glass wool and stainless steel (Oosthuizen *et al.*, 2002; Auger *et al.*, 2006; Wijman *et al.*, 2007). This is of great concern since biofilm accumulation in food processing environments can lead to decreased food quality and safety, which impacts public health and the economy (Flint *et al.*, 1997). Identification and characterization of genetic determinants involved in *B. cereus* biofilm formation will not only aid in developing strategies for its control and thus minimize the potential for food contamination, but it would also contribute to the advancement of knowledge regarding the mechanisms of biofilm formation in Gram-positive bacteria.

Therefore, the aims of this investigation were the following:

- To identify genes involved in biofilm formation by *B. cereus* ATCC 14579 through the construction and screening of transposon mutant libraries for biofilm-impaired mutants, and cloning and sequencing of chromosomal DNA flanking the transposon insertions.

- To characterize selected *B. cereus* ATCC 14579 biofilm-impaired mutants with regards to their phenotypic properties.

- To compare the transcriptional activity of selected *B. cereus* ATCC 14579 genes when growing planktonically and in a biofilm through quantitative real-time PCR analysis.

CHAPTER TWO
MATERIALS AND METHODS

2.1 General procedures

2.1.1 Bacterial strains

Bacillus subtilis PY177, harbouring plasmid pLTV1, was obtained from Prof. A. Moir at the University of Sheffield, United Kingdom. *Bacillus cereus* ATCC 14579, used as the type strain in transposon mutagenesis studies, was obtained from the BCCM/LMG Bacteria Collection (LMG 6923). *Escherichia coli* HB101 (Sambrook and Russel, 2001) was used as the general cloning host for the recovery of *B. cereus* genomic DNA flanking the chromosomally inserted transposons.

2.1.2 Plasmid and transposon

Plasmid pLTV1 (Camilli *et al.*, 1990), of which a plasmid map is provided in Fig. 2.1, served as the vector for delivery of the Tn*917* transposon. The Tn*917* transposon from *Streptococcus faecalis* is a 5.266-kb transposon that is closely related to the Tn*3* family of transposons of Gram-negative bacteria (Perkins and Youngman, 1984; Shaw and Clewell, 1985). It confers on its host resistance to the macrolide-lincosamide-streptogramin B (MLS) antibiotics and undergoes enhanced transposition upon exposure to erythromycin (*erm*). Tn*917* possesses two non-identical 38-bp inverted terminal repeats that differ by 4 bp and has been shown to generate a 5-bp duplication upon insertion. It has been demonstrated that foreign DNA may be inserted into the *Hpa*I site between the left terminal repeat and the *erm* open reading frame (ORF), without affecting transposition or expression of *erm* in *B. subtilis* (Youngman *et al.*, 1984). This observation has subsequently been used to construct a variety of recombinant Tn*917* derivatives that are useful for transposon mutagenesis in different Gram-positive hosts. One such derivative, designated Tn*917*-LTV1, has been constructed with the aim of facilitating the characterization of disrupted genes (Fig. 2.2). Not only does this modified transposon allow for cloning of chromosomal DNA flanking transposon insertions into *E. coli*, but it also transposes at a significantly elevated frequency in Gram-positive hosts. In addition to inclusion of the chloramphenicol acetyltransferase (*cat*) antibiotic resistance marker that confers chloramphenicol resistance in Gram-positive hosts, Tn*917*-LTV1 has furthermore been modified by insertion of the ColE1 origin of replication and the β-lactamase

(*bla*) antibiotic resistance marker to allow for replication and selection, respectively, in *E. coli* (Camilli *et al.*, 1990). Replication of pLTV1 in Gram-positive hosts is facilitated by the temperature-sensitive pE194Ts origin of replication, which ensures replication of pLTV1 at the permissive temperature of 30°C. At non-permissive temperatures, *i.e.* temperatures exceeding 42°C, plasmid replication is inhibited and thus facilitates curing of the plasmid from the transformed Gram-positive host (Villafane *et al.*, 1987). The strategy for transposon mutagenesis with Tn*917*-LTV1 comprises transformation of the strain of interest with pLTV1, followed by culturing of the cells at the permissive temperature (30°C) in the presence of at least tetracycline. To produce a mutant library the cells are diluted into broth supplemented with chloramphenicol and erythromycin, and cultured at the non-permissive temperature (at least 42°C).

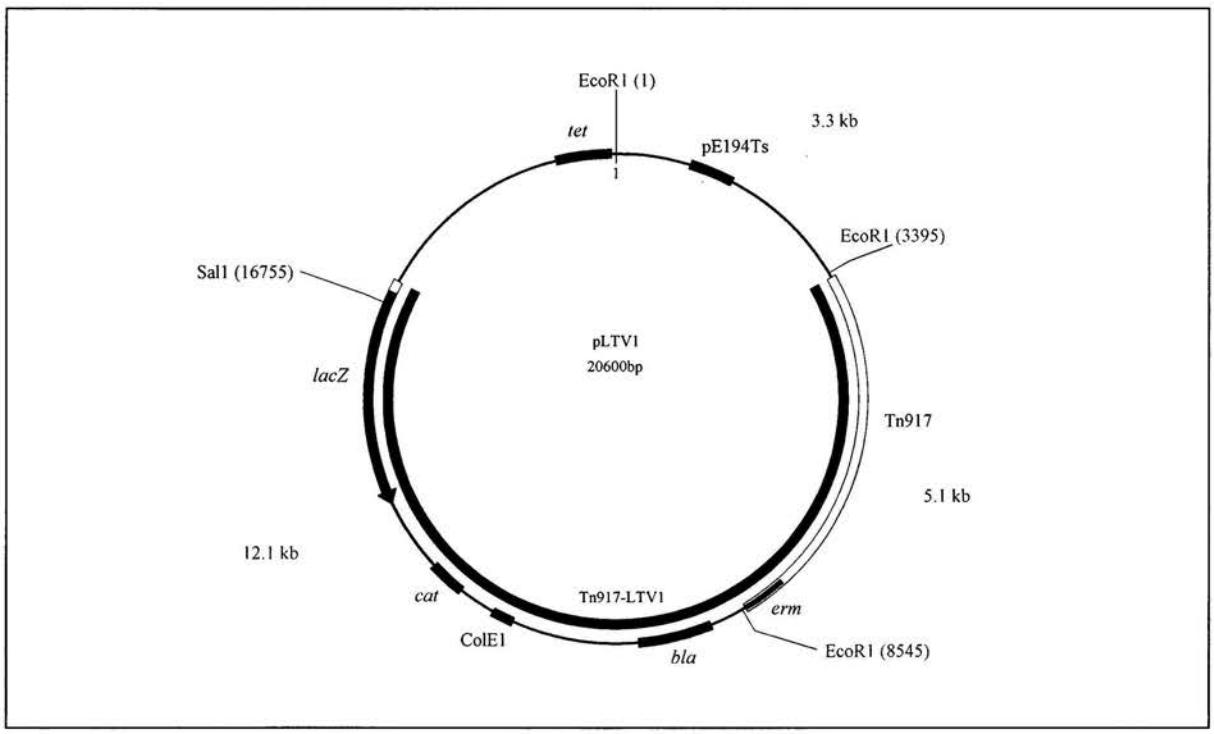

Figure 2.1: Restriction map of pLTV1. Tn*917*-LTV1 is indicated in blue and the original Tn*917* is indicated in yellow. The three *Eco*RI sites and the sizes of the restriction fragments are indicated in red.

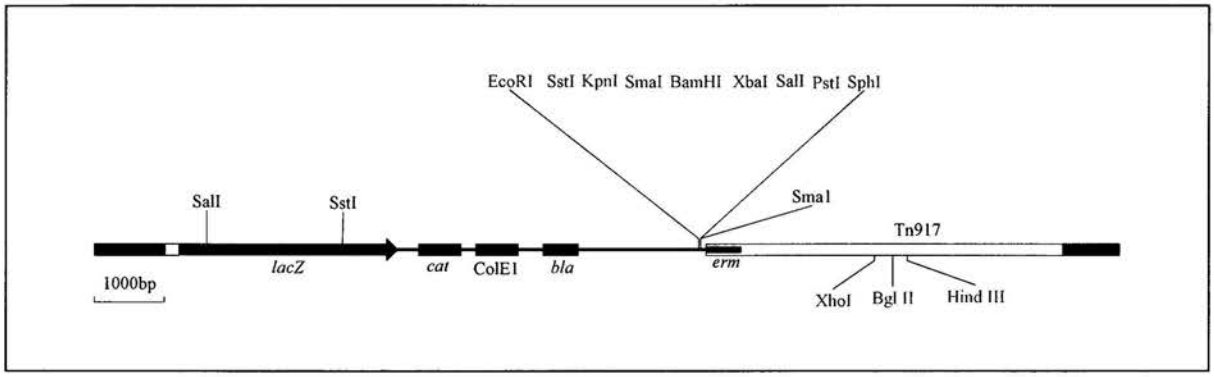

Figure 2.2: Hypothetical chromosomal insertion of Tn*917*-LTV1. Chromosomal flanking DNA is shown in green. Unique restriction sites that may be used to clone DNA flanking the *lacZ* proximal end is shown in red.

2.1.3 Media and growth conditions

B. cereus ATCC 14579 was routinely cultured at 37°C in Luria-Bertani broth (LB: 1% [w/v] tryptone, 0.5% [w/v] yeast extract, 0.5% [w/v] NaCl; pH 7.2). *B. subtilis* PY177 and *B. cereus*(pLTV1) were cultured at 30°C in LB broth containing tetracycline (50 µg/ml), erythromycin (1 µg/ml) and chloramphenicol (10 µg/ml). *E. coli* HB101 was cultured at 37°C in LB broth and ampicillin (100 µg/ml) was used to select for the *bla* marker on Tn*917*-LTV1. When needed, agar was added to the respective culture media to a final concentration of 1.2% (w/v).

2.1.4 Statistical analysis

Experimental data were presented graphically by plotting means and reporting the standard deviation of the mean via error bars using SigmaPlot® v 10.0 (Systat Software). The statistical significance of differences in two sample comparisons were calculated using the GenStat® discovery edition 3 (VSN International) statistical software by means of hypothesis testing using the two sample unpaired Student's *t*-test.

2.2 Isolation of pLTV1

2.2.1 Plasmid extraction

Large-scale plasmid extractions were performed using a modified alkaline lysis method (Birnboim and Doly, 1979). The cells from an overnight culture (1 litre) of *B. subtilis* PY177 were harvested in 500-ml bottles by centrifugation at 4000 × *g* for 15 min at 4°C in a Sorvall GS-3 rotor. The cell pellets were suspended in 76 ml of ice-cold STE buffer (0.1 M NaCl, 1 mM EDTA, 10 mM Tris; pH 8). The cell suspension was divided into two equal aliquots and

harvested in 45-ml polypropylene tubes by centrifugation at 8000 × *g* for 5 min at 4°C in a Sorvall HB-4 rotor. Each cell pellet was suspended in 5.1 ml of ice-cold Solution 1 (50 mM glucose, 10 mM EDTA, 25 mM Tris; pH 8) containing 2 mg/ml lysozyme. After incubation on ice for 2 h, 10.2 ml of Solution 2 (0.2 M NaOH, 1% SDS) was added and the contents were mixed thoroughly by inverting the tubes several times. After incubation on ice for 5 min, 7.7 ml of Solution 3 (3 M KOAc; pH 4.8) was added and the contents mixed gently by repeatedly inverting the tubes. Following incubation on ice for 10 min the tubes were centrifuged at 16 000 × g for 10 min at 4°C. The plasmid DNA-containing supernatants were decanted into clean tubes and 1 volume of 2-propanol was added to each tube, followed by incubation at room temperature for 20 min. The precipitated plasmid DNA was collected by centrifugation at 16 000 × *g* for 15 min at room temperature. The DNA pellets were washed twice with 70% EtOH, dried under vacuum and then suspended in 1.5 ml of TE buffer (1 mM EDTA, 10 mM Tris; pH 8).

2.2.2 Agarose gel electrophoresis

An aliquot of each plasmid DNA preparation was analyzed by agarose gel electrophoresis (Sambrook and Russel, 2001). Horizontal 0.8% (w/v) agarose slab gels were cast and ethidium bromide (EtBr) was added to a final concentration of 0.2 ng/µl. Gels were electrophoresed at 100 V in 1 × TAE buffer (40 mM Tris acetate, 1 mM EDTA; pH 8.5). The DNA was visualized by UV-induced fluorescence at 312 nm on a transilluminator.

2.2.3 Restriction enzyme digestion

The identity of pLTV1 was confirmed by means of restriction enzyme digestion. The 15-µl reaction consisted of 1 µg of the plasmid DNA, 0.5 µl of *Eco*RI (10 U/µl), 1.5 µl of the supplied 10× restriction buffer and 9 µl of dH_2O. The reaction was incubated at 37°C for 1 h and analyzed by agarose gel electrophoresis in the presence of an appropriate DNA molecular weight marker.

2.2.4 CsCl-EtBr density gradient centrifugation

For purification of pLTV1 by CsCl-EtBr density gradient centrifugation (Sambrook and Russel, 2001), 3 ml of the plasmid DNA solution was transferred to a 15-ml Falcon® tube and exactly 3.0 g of CsCl was added. The tube was incubated at 30°C in a water bath until all CsCl had dissolved and 240 µl of a 10 mg/ml EtBr solution was added. The DNA solution

was transferred to Beckman Ultra Clear tubes, which were then filled to the brim with light paraffin oil. The gradients were obtained by centrifugation at 173 500 × *g* (38 000 rpm) for 40 h at 20°C in a SW50.1 swing-bucket rotor, using a Beckman L5-50 ultracentrifuge. At the end of the run the rotor was allowed to decelerate without the use of the brake. The tube was viewed under UV light and the covalently closed circular plasmid DNA band was removed by inserting a hypodermic needle, connected to a syringe, into the tube just below the DNA band. The plasmid DNA-containing solution was transferred to a 15-ml Falcon® tube and 3 volumes of TE buffer were added. The solution was then extracted with an equal volume of water-saturated 1-butanol. The phases were mixed by vortexing and separated by centrifugation at 2500 × *g* for 5 min at 25°C. The extraction was repeated until all traces of EtBr were removed. The plasmid DNA was subsequently precipitated with 2 volumes of absolute EtOH and incubation at 4°C for 1 h. The precipitated plasmid DNA was recovered by centrifugation at 15 000 × *g* for 15 min, washed twice with 70% EtOH and dried under vacuum before being suspended in 200 µl of TE buffer.

2.3 Transformation of *Bacillus cereus* ATCC 14579

2.3.1 Preparation of electrocompetent cells

Electrocompetent *B. cereus* cells were prepared, as described previously (Bone and Ellar, 1989; Tremblay and Archibald, 1993). A flask containing 200 ml of LB broth was inoculated with 0.4 ml from an overnight *B. cereus* culture and incubated at 37°C with shaking at 200 rpm. Once the culture had reached an A_{600} of 0.58 (after *ca.* 3 h), the flask was chilled in an ice water bath to inhibit further bacterial growth. The cells were harvested by centrifugation at 4000 × *g* for 10 min at 4°C in a pre-cooled Sorvall GSA rotor, and the cell pellet was suspended in 50 ml of ice-cold 1 mM HEPES (pH 7). The cell suspension was divided into two equal aliquots and harvested in 45-ml polypropylene tubes by centrifugation at 5000 × *g* for 5 min in a pre-cooled Sorvall SS-34 rotor. Each cell pellet was washed three times with 20 ml of ice-cold 1 mM HEPES, 10% glycerol (pH 7.2). After the third wash the cell pellets were suspended in 1 ml of ice-cold 10% glycerol. The cells were divided into aliquots of 80 µl and frozen at -70°C.

2.3.2 Dialysis of plasmid DNA

Dialysis of the purified pLTV1 plasmid DNA was performed to remove any residual salts, especially traces of CsCl, which may cause arcing during electroporation. Dialysis was

performed in a disposable 10-100 µl Pierce Slide-A-lyzer® dialysis cassette with a 10 000 Dalton molecular weight cut-off limit against 10 000 volumes of TE buffer for 24 h.

2.3.3 Electroporation

For electroporation the cells were thawed on ice and *ca.* 0.9 µg of dialysed pLTV1 plasmid DNA was added to 80 µl of electrocompetent *B. cereus* cells. The cells were transferred into an 0.1-cm inter-electrode gap electroporation cuvette (Eppendorf) and exposed to a single electrical pulse using an Eppendorf Multiporator® set at 600–900V. Immediately following the electrical discharge, 1 ml of LB broth was added to the electroporation cuvette and incubated on the bench for 20 min. The contents of the cuvette was subsequently transferred to a sterile test tube and incubated at 30°C for 2 h with gentle agitation. Aliquots of 100 µl of the electroporated cells were plated onto LB agar containing 1 µg/ml erythromycin. The agar plates were incubated at 30°C for 48 h.

2.4 Construction and screening of *B. cereus* transposon mutant libraries

2.4.1 Transposon mutagenesis

Transposon mutagenesis was performed, as described previously (Camilli *et al.*, 1990; Clements and Moir, 1998), with the following modifications. A single colony of *B. cereus*(pLTV1) was inoculated into 50 ml of LB broth containing tetracycline (50 µg/ml), erythromycin (1 µg/ml) and chloramphenicol (10 µg/ml), and cultured overnight at 30°C. The stationary phase culture was then diluted 1:800 in pre-warmed (43°C) LB broth containing erythromycin (1 µg/ml) and chloramphenicol (5 µg/ml), and incubated at 43°C for 24 h with shaking at 200 rpm. Following incubation the culture was again diluted and incubated as described above. The cells were plated onto LB agar containing erythromycin (1 µg/ml) and chloramphenicol (5 µg/ml), and incubated at 43°C to obtain *B. cereus* ATCC 14579::Tn*917*-LTV1 mutant colonies.

2.4.2 Identification of biofilm mutants

A total of 3 500 mutants from five independent libraries were screened. Individual mutants were cultured to stationary phase in the wells of polyvinylchloride (PVC) 96-well microtiter plates (Greiner) and then 5 µl of the culture was inoculated into acid-washed Durham tubes (SACO; 25 × 6.5 mm) containing 200 µl of LB broth. The tubes were incubated statically at

25°C for 32 h, after which the tubes were inspected visually for the presence of biofilm-impaired mutants by comparison of the amount of growth at the air-liquid interface to that of the wild-type *B. cereus* biofilm.

2.4.3 Quantification of biofilm deficiency

The wild-type *B. cereus* and individual biofilm-impaired mutants were cultured at room temperature for 72 h without agitation in 25-ml acid-washed glass beakers containing 15 ml of LB broth. The culture media containing the planktonic cells were carefully aspirated, and the biofilm cells were rinsed from the walls of the beakers with 15 ml of PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4; pH 7.4). The mutant biofilm biomass was quantified relative to that of wild-type *B. cereus* biofilms by measuring the absorbance (A) at 600 nm with a Spectronic 20 GeneSys spectrophotometer. The assays were repeated five times.

2.5 Characterization of *B. cereus* biofilm-impaired mutants

2.5.1 Growth curves of biofilm-impaired mutants

The wild-type *B. cereus* and biofilm-impaired mutants were cultured overnight at 37°C in LB broth and subsequently inoculated to an A_{600} of 0.01 into 250-ml Erlenmeyer flasks containing 100 ml of LB broth. The cultures were incubated at 37°C with shaking at 200 rpm and the A_{600} was determined every 2 h for 12 h and after 24 h. For this purpose, a 1-ml aliquot of the culture was transferred to an Eppendorf tube and then chilled on ice to inhibit further bacterial growth. This made it possible to return all the cultures to the incubator in only a few minutes, thereby disrupting the incubation as little as possible. The assays were repeated three times.

2.5.2 Stereomicroscopy of biofilm-impaired mutants

The wild-type *B. cereus* and biofilm-impaired mutants were cultured overnight at 37°C in LB broth and subsequently inoculated to an A_{600} of 0.01 into 100-ml glass beakers containing 50 ml of LB broth. A microscope slide was suspended halfway into the broth perpendicular to the surface of the broth. The cultures were incubated at room temperature for 40 h without agitation, after which the microscope slides were carefully removed from the broth and examined under a Zeiss stereomicroscope.

2.5.3 Motility assay

Motility of selected *B. cereus* strains were evaluated using the stab inoculation technique (Ederer *et al.*, 1975). For this purpose, 8 ml of motility medium (0.5% [w/v] tryptone, 0.25% [w/v] yeast extract, 1% [w/v] NaCl, 0.4% [w/v] agar) was dispensed into test tubes. The *B. cereus* ATCC 14579 wild-type and biofilm-impaired mutant (Mut21) strains were cultured overnight at 30°C on LB agar. Following incubation, the bacterial growth was stab inoculated with an inoculation loop into the center of the agar column. The test tubes were incubated overnight at 30°C and motility was manifested as clouding of the growth medium that progressed laterally and downwards from the stab inoculation line.

2.5.4 Transmission electron microscopy

The *B. cereus* ATCC 14579 wild-type and biofilm-impaired mutant (Mut21) strains were cultured overnight at 37°C in LB broth. Following incubation, a drop (5 µl) of each liquid culture was pipetted into a sterile Petri dish and the bacterial cells were allowed to adsorb onto copper-coated grids by placing the grids on the surface of each drop for 30 s. The grids were removed, blotted dry with filter paper and then stained for 30 s with a solution of 1% phosphotungstic acid (pH 6.9). The negatively stained cells were viewed on a JEOL JEM-2100F transmission electron microscope.

2.5.5 Laser scanning confocal microscopy

The *B. cereus* ATCC 14579 wild-type and biofilm-impaired mutant (Mut17) strains were cultured for 12 h at 37°C in LB broth. Following incubation, the bacterial cells from 15 ml of the culture were harvested by centrifugation at 2500 × *g* for 5 min and suspended in 0.5 volume of fresh LB broth. The bacterial cell membranes were stained with the fluorescent dye FM® 4-64 (Molecular Probes), which was dissolved in dH_2O to a concentration of 200 µg/ml. The bacterial cells were prepared for microscopy by cooling the tubes on ice, followed by addition of FM® 4-64 to a final concentration of 5 µg/ml to the cells. The samples were viewed with a Zeiss LSM 510 META laser scanning confocal microscope, using the 543-nm green helium neon laser for excitation of the fluorescent dye.

2.6 Recovery of *B. cereus* chromosomal DNA flanking the transposon insertions

2.6.1 Isolation of chromosomal DNA from biofilm-impaired mutants

Chromosomal DNA was isolated with hexadecyltrimethylammonium bromide (CTAB), as described by Wilson (2001). Biofilm-impaired mutants of *B. cereus* were cultured overnight at 37°C in 20 ml of LB broth with shaking at 200 rpm. The cells from 1.5 ml of the overnight cultures were harvested in Eppendorf tubes by centrifugation at 15 000 × *g* for 2 min in a microcentrifuge and washed once in 800 µl of ice-cold STE buffer. The cell pellets were suspended in 567 µl of TE buffer containing 5 mg/ml lysozyme and incubated on ice for 2 h. Subsequently, 30 µl of a 10% SDS solution was added and, following incubation at 37°C for 1 h, 3 µl of a 20 mg/ml Proteinase K solution was added. The tubes were incubated overnight at 50°C, after which 100 µl of 5 M NaCl and 80 µl of a CTAB solution (10% CTAB in 0.7 M NaCl) were added, and the tubes were incubated at 65°C for 10 min. The CTAB-protein/polysaccharide complexes were removed by extraction with an equal volume of phenol:chloroform, followed by centrifugation (15 000 × *g*, 5 min). The upper aqueous phase, containing the chromosomal DNA, was recovered and transferred to a new tube. The remaining CTAB was removed by addition of an equal volume of chloroform, followed by centrifugation at 15 000 × *g* for 5 min. The chromosomal DNA was precipitated from the recovered aqueous phase by addition of 1 volume of 2-propanol and then pelleted by centrifugation at 15 000 × *g* for 15 min. The DNA pellets were washed with 70% EtOH, dried under vacuum and then suspended in 30 µl of TE buffer.

2.6.2 Preparation of competent *E. coli* HB101 cells

Chemically competent *E. coli* HB101 cells were prepared by the method of Inoue (Inoue *et al.*, 1990; Sambrook and Russel, 2001). A single colony of *E. coli* HB101 was inoculated into 20 ml of LB broth and incubated at 37°C with shaking at 200 rpm. After 8 h, this starter culture was used to inoculate 500 ml of SOB broth (2% [w/v] tryptone, 0.5% [w/v] yeast extract, 0.05% [w/v] NaCl, 10 mM $MgCl_2$, 2.5 mM KCl) to an A_{600} of 0.01. The culture was incubated overnight at 18°C with moderate shaking, after which the A_{600} was measured at 30-min intervals. Once an A_{600} of 0.55 had been reached, the flask was chilled in an ice water bath to inhibit bacterial growth. The cells were harvested in 250-ml bottles by centrifugation at 2500 × *g* for 10 min at 4°C in a Sorvall GSA rotor. The supernatant was discarded and the bottle was placed upside-down on paper towels to drain away excess fluid. The cell pellet was suspended gently in 80 ml of filter-sterilized ice-cold transformation buffer (55 mM

$MnCl_2 \cdot 4H_2O$, 15 mM $CaCl_2 \cdot 2H_2O$, 250 mM KCl, 10 mM PIPES [pH 6.7]) and incubated on ice for 10 min. The cells were harvested, as above, and then suspended in 20 ml of transformation buffer. Following addition of 1.5 ml of DMSO and incubation on ice for 10 min, 200-µl aliquots were transferred to 1.5-ml Eppendorf tubes and frozen in liquid nitrogen prior to storage in a -70°C freezer.

2.6.3 Cloning of the DNA flanking transposon insertions

Chromosomal DNA from *B. cereus* biofilm-impaired mutants was digested with either *Eco*RI or *Bam*HI, followed by self-ligation and transformation into *E. coli* HB101. The individual digestion reactions consisted of 1 µg of chromosomal DNA, 5 U of restriction enzyme, 3 µl of the appropriate 10× restriction buffer and dH_2O to a final reaction volume of 30 µl. Following incubation at 37°C for 1 h, the reactions were diluted with TE buffer to a final volume of 200 µl. A phenol:chloroform extraction was performed and the digested DNA fragments were recovered by ethanol precipitation, as described previously (Section 2.6.1). Following precipitation, the restriction fragments were suspended in 20 µl of TE buffer. The DNA fragments were self-ligated by addition of 1 µl of T4 DNA ligase (5U/µl; Fermentas), 20 µl of ligation buffer and dH_2O to a final volume of 200 µl. The reactions were incubated overnight at 15°C. Following incubation, the DNA was recovered by ethanol precipitation and suspended in 15 µl of TE buffer. Prior to transformation the *E. coli* HB101 competent cells were thawed on ice and the self-ligated products were then added to the cells. Following incubation on ice for 30 min, the cells were subjected to a heat shock at 42°C for 90 s in a pre-heated circulating water bath and again incubated on ice for 2 min. Subsequently, 800 µl of SOC broth (identical to SOB, except for the addition of 20 mM glucose prior to use) was added and the cells were transferred to 15-ml Falcon® tubes. The cells were allowed to recover at 37°C for 30 min and were subsequently plated onto LB agar containing 100 µg/ml ampicillin. A positive control (20 ng of pGEM®-3Zf(+) plasmid DNA) and negative control (competent cells only) were also included to determine the competency of the *E. coli* HB101 cells and to test for contamination, respectively. The agar plates were incubated at 37°C for 16 h.

2.6.4 Isolation of plasmid DNA from *E. coli* HB101

The recombinant plasmids containing flanking chromosomal DNA from *B. cereus* biofilm-impaired mutants was isolated from *E. coli* HB101 using a modified alkaline lysis method

(Birnboim and Doly, 1979). Single colonies were inoculated into 5 ml of LB broth containing 100 µg/ml ampicillin and incubated overnight at 37°C with shaking. The cells from 1.5 ml of the overnight cultures were harvested in 1.5-ml Eppendorf tubes by centrifugation at 15 000 × *g* for 2 min in a microcentrifuge. The cell pellets were suspended in 100 µl of ice-cold Solution 1 (50 mM glucose, 10 mM EDTA, 25 mM Tris; pH 8), followed by incubation on ice for 10 min. Following incubation, 200 µl of freshly prepared Solution 2 (0.2 M NaOH, 1% SDS) was added to each tube, mixed gently and incubated at room temperature for 5 min. After incubation, 150 µl of ice-cold Solution 3 (3 M KOAc; pH 4.8) was added and incubation was continued on ice for 10 min. The precipitated chromosomal DNA and cell debris were removed by centrifugation at 15 000 × *g* for 10 min and the supernatant was transferred to a new tube. The plasmid DNA was precipitated from the recovered supernatant by addition of 2 volumes of absolute ethanol and incubation on ice for 20 min. The plasmid DNA was pelleted by centrifugation at 15 000 × *g* for 15 min, washed with 70% EtOH, dried under vacuum and suspended in 50 µl of 10 mM Tris (pH 8).

2.6.5 Sequencing of flanking DNA

Nucleotide sequencing of the flanking DNA was performed using the Applied Biosystems BigDye™ Terminator v.3.1 Cycle Sequencing Ready Reaction kit according to the manufacturer's instructions. The sequencing reactions contained 2 µl of Ready Reaction mix, 10 pmol of the *917*S sequencing primer (5'-CTCACAATAGAGAGATGTCACC-3') and 200 ng of plasmid DNA in a final reaction volume of 7 µl. Cycle sequencing reactions were performed in an Applied Biosystems GeneAmp® 2700 thermal cycler with 25 cycles of denaturation at 96°C for 10 s, primer annealing at 50°C for 5 s and elongation at 60°C for 4 min. The extension products were precipitated by addition of 2 µl of dH_2O, 1 µl of 3 M NaOAc (pH 5.2) and 20 µl of absolute ethanol, followed by incubation at -20°C. The tubes were centrifuged at 15 000 × *g* for 15 min at 4°C and the supernatant carefully aspirated. The pellets were washed with 70% EtOH, dried under vacuum and stored at 4°C before being submitted to the University of Pretoria's DNA Sequencing Core Facility. Prior to electrophoresis, the purified extension products were suspended in 3.5 µl of Blue dextran/EDTA loading buffer, denatured at 90°C for 2 min and loaded onto an ABI PRISM™ 3100 Genetic Analyzer. Nucleotide sequences were analyzed with BioEdit Sequence Alignment Editor v.7.0.4.1 (Hall, 1999), and identified by NCBI BLASTn searches (Altschul

et al., 1990) against the GenBank *B. cereus* ATCC 14579 database (accession number: NC_004722) available at www.ncbi.nlm. nih.gov/BLAST.

2.7 Characterization of *B. cereus* biofilm-impaired mutants by Southern blot hybridization

Southern blot analysis (Southern, 1975) was performed to confirm integration of Tn*917*-LTV1 into the genome of *B. cereus* ATCC 14579 mutants. For this purpose, chromosomal DNA from the biofilm-impaired mutants was digested with *Eco*RI and the resultant fragments were resolved by agarose gel electrophoresis and transferred to a nylon membrane. The 5.1-kb *Eco*RI restriction fragment of pLTV1 (Fig. 2.1) was purified from an agarose gel and used as a template for random primed DNA labelling with digoxigenin-dUTP. The DNA probe was subsequently hybridized to the immobilized DNA fragments and detected using the Roche DIG-High Prime DNA Labelling and Detection Starter kit (Roche Diagnostics).

2.7.1 Preparation of the labelled probe

An *Eco*RI digest of pLTV1 was resolved on an 1% (w/v) agarose gel and the 5.1-kb DNA fragment was purified from the gel with a silica suspension, as described by Boyle and Lew (1995). A gel slice containing the 5.1-kb DNA fragment was placed in a pre-weighed Eppendorf tube and, following addition of 2.5 volumes of 6 M NaI, the tube was incubated at 55ºC until the agarose had completely melted. Subsequently, 7 µl of a 50% (w/v) silica (Sigma S5631; 0.5-10 µm particles) suspension in TE buffer was added to the sample. The tube was incubated on ice for 15 min with occasional vortexing. After incubation the silica particles were collected by centrifugation (15 000 × *g*, 30 s) and washed four times with Wash buffer (50 mM NaCl, 2.5 mM EDTA, 10 mM Tris [pH 7.5], 50% [v/v] EtOH). For elution of the DNA, the pellet was suspended in 7 µl of TE buffer and incubated at 55ºC for 2 min. The tube was briefly centrifuged at 15 000 × *g* for 30 s and the supernatant containing the purified DNA fragment was collected, and analyzed by agarose gel electrophoresis in the presence of an appropriate DNA molecular weight marker.

To prepare the DIG-dUTP labelled probe, 500 ng of the purified restriction fragment was diluted with dH_2O to a final volume of 16 µl. The DNA was denatured by boiling in a water bath for 10 min and then chilled in an ice water bath. The labelling reaction was initiated by addition of 4 µl of DIG-High Prime labelling mix (containing random primers, nucleotides, DIG-dUTP, Klenow polymerase and buffer components) and then incubated at 37ºC for 20 h.

Following incubation the reaction was terminated by addition of 2 µl of 0.2 M EDTA (pH 8) and heating to 65°C for 10 min. The DNA probe concentration was quantified using the labelled control DNA provided with the kit according to the manufacturer's instructions.

2.7.2 Preparation of the membrane

Chromosomal DNA of *B. cereus* biofilm-impaired mutants were isolated using the CTAB method, as described in Section 2.6.1, and digested with *Eco*RI before being resolved by electrophoresis on a 1% (w/v) agarose gel. Following electrophoresis the gel was soaked in a depurination solution (0.2 M HCl) for 15 min with gentle agitation. The gel was rinsed three times with dH_2O and then incubated in denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 30 min. Following denaturation and hydrolysis of the phosphodiester backbone at the sites of depurination, the gel was neutralized by soaking in neutralization solution (1 M Tris-HCl, 1.5 M NaCl) for 30 min. The DNA fragments were transferred to a Hybond-N+™ nylon membrane (Amersham) by capillary blotting. For this purpose, pieces of filter paper (Whatman no. 3) were cut to the same size as the gel and stacked to a height of 1.5 cm in a shallow container. The stack of filter papers was soaked with 20 × SSC (3 M NaCl, 0.3 M sodium citrate) and the gel placed on top of the filter paper stack. The membrane was briefly soaked in dH_2O and placed on top of the gel. Four sheets of filter paper, saturated with 2 × SSC, were placed on top of the membrane. A 10-cm stack of paper towels was cut to the same size as the gel and placed on top of the filter papers. A glass plate was placed on top of the paper towel stack and weighed down with a bottle containing *ca.* 500 ml of water. Transfer was allowed to proceed overnight at room temperature. The DNA fragments were cross-linked to the membrane by UV irradiation for 5 min on each side on a 312-nm transilluminator, followed by washing in 2 × SSC for 5 min. The wet membrane was placed between two sheets of dry filter paper and dried at 60°C in an oven. The membrane was stored in an air-tight container at 4°C until needed.

2.7.3 Hybridization

The melting temperature (T_m) for the probe was calculated according to the following equation (Roche Diagnostics) for hybridization solutions containing 50% formamide.

$$T_m = 49.82 + 0.41(\%G + C) - \frac{600}{\lambda} \quad [where\ \lambda = length\ of\ hybrid\ in\ base\ pairs]$$

The optimum hybridization temperature (T_{opt}) for hybridization in DIG-Easy Hyb is 20-25°C below the calculated T_m value. Tn*917* has a (%G+C) content of 33.51% and the length of the DIG-dUTP labelled extension products vary typically between 200-1000 nt. The theoretical optimum hybridization temperature range was thus calculated to be 38-43°C for the probe. The membrane was incubated for 30 min in 10 ml of DIG-Easy Hyb, pre-heated to 42°C, with gentle agitation in a sealed hybridization bag. The pre-hybridization buffer was subsequently poured off and replaced with 7 ml of DIG-Easy Hyb containing the labelled DNA probe at a concentration of 25 ng/ml. The DNA probe was denatured prior to its addition to the hybridization buffer by boiling in a water bath for 5 min and rapidly cooling in an ice water bath. Hybridization was performed at 42°C for 16 h, after which the membrane was washed twice for 5 min each time in 2 × SSC, 0.1% SDS at room temperature, followed by two washes of 15 min each in 0.5 × SSC, 0.1% SDS at 68°C.

2.7.4 Detection of the hybridized DNA probe

To detect the hybridized DNA probe the membrane was briefly rinsed in washing buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3% [v/v] Tween-20; pH 7.5), followed by a 30-min incubation in blocking solution (supplied in the kit and prepared according to the manufacturer's instructions). The membrane was then incubated at room temperature for 30 min in 20 ml of an antibody solution, comprising a 1:5 000 dilution of the alkaline phosphatase-conjugated anti-digoxygenin antibody in fresh blocking solution. The membrane was washed twice for 15 min each wash in washing buffer, followed by equilibration for 2 min in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl; pH 9.5). The detection buffer was discarded and the membrane was placed face-up in an empty container in the dark. The membrane was then immersed in 10 ml of freshly prepared alkaline phosphatase enzyme substrate solution (NBT/BCIP stock diluted 1:50 in detection buffer). The colour reaction was stopped once the desired band intensity was reached by rinsing the membrane in TE buffer.

2.8 Relative expression of selected *B. cereus* genes

All solutions and buffers for RNA work were prepared with dH_2O treated with diethyl pyrocarbonate (DEPC) in the following way. DEPC was added to the dH_2O to a final concentration of 0.05% (v/v) and the solution was incubated overnight at 37°C with shaking. Following incubation the DEPC was destroyed by autoclaving the solution for 45 min at 121°C.

2.8.1 Primers

Primers used in quantitative real-time PCR were designed based on sequence data obtained in Section 2.6.5, using Primer3 v.0.4.0 (Rozen and Skaletsky, 2000). The primers were designed in such a way that the amplicon size would be in the range of 100-200 bp, since amplification efficiency drops significantly with increasing amplicon size. No mismatches were allowed and only primer pairs ending in a G or C at the 3' end were considered. The lengths of primers were restricted to 18-23 nt with a theoretical T_m of at least 63°C. Primers that were predicted by PerlPrimer v.1.1.13 (Marshall, 2004; Marshall, 2007) to be capable of forming extendable 3' dimers were excluded. The selected primers were synthesized commercially by Operon Biotechnologies and are indicated in Table 2.1. The supplied lyophilized primers were dissolved in 10 mM Tris (pH 8) to a final concentration of 100 µM. Working stock solutions were subsequently prepared by diluting the primers in dH_2O to a final concentration of 10 µM.

Table 2.1: Real-time PCR primers used for relative quantification of gene expression in biofilm versus planktonic cells

*Target RNA	Amplicon size	Forward Primer (5'→3')	Reverse Primer (5'→3')
16S	162 bp	16SF: TAGGTGGCAAGCGTTATCCG	16SR: GCATTTCACCGCTACACATGG
BC5437	154 bp	5F: TTGCACGTTCATTTGGAAAGG	5R: CCCGCTACTGGAATTAAGCC
BC0329	162 bp	8F: AAGCACAGTTGTTACGCCAG	8R: CATACGATATTACGCGCTGCC
BC5468	169 bp	9F: CCCAATTGCTGGTGGTGTAAC	9R: TCCATACTCGCGACCAACTTC
BC0172	101 bp	13F: GATGAGCGTATTCATGAAGTTG	13R: CGAATACATTCTTCTGTGTTAGC
BC5133	110 bp	16AF: TCCAGGAAGTTTGATTGGGATG	16AR: ACGGCATAATAGCAACGAGTG
BC5134	108 bp	16BF: TTGCTGATTACGGCAATGTTCC	16BR: GACGTTCTGAACCACCGAATATC
BC5186	160 bp	17F: CCAAGTGAGCTTTCGGGC	17R: TGCCGCGCTCATTAATGC
BC0326	165 bp	18F: TTTCGCAAGCTGTCGTGTAAG	18R: CCAAGATCACGACGGAATACG
BC4513	184 bp	21F: TGCGCGGTATGAAATTTGTG	21R: CCAAGCGCAGCTACAAGAC

* The target RNA refers to the ORF number of the gene on the annotated *B. cereus* ATCC 14579 genome.

2.8.2 RNA isolation

Biofilm-impaired mutants of *B. cereus* were cultured at 25°C for 72 h without agitation in 25-ml acid-washed glass beakers containing 15 ml of LB broth. The culture medium was carefully aspirated, taking care not to disturb the biofilm. Planktonic cells from 1.5 ml of the culture medium were harvested in Eppendorf tubes by centrifugation at 15 000 × *g* for 2 min and suspended in 1 ml of EZ RNA reagent (Bio Basic), a mono-phasic solution of phenol and guanidinium isothiocyanate (Chomczynski and Sacchi, 1987). The biofilm cells were rinsed from the wall of the glass beaker with 5 ml of EZ RNA reagent and 1.5-ml aliquots were transferred to Eppendorf tubes. Following incubation at 30°C for 10 min, 200 µl of chloroform was added to each tube. The contents of the tubes were mixed by vortexing for 15 s, incubated at room temperature for 3 min and then centrifuged at 12 000 × *g* for 15 min at 4°C. The upper aqueous phases were transferred to new tubes, followed by addition of 500 µl of 2-propanol and incubation at room temperature for 10 min. The precipitated RNA was pelleted by centrifugation at 12 000 × *g* for 10 min at 4°C. The RNA pellets were washed by adding 1 ml of 75% EtOH, vortexing and centrifuging at 7500 × *g* for 5 min at 4°C. The RNA pellets were dried under vacuum for 5 min and suspended in 10 µl of DEPC-treated dH_2O. Contaminating DNA was removed by pooling the contents from four tubes and then adding 5 µl of 10× reaction buffer (100 mM Tris-HCl [pH 7.5], 25 mM $MgCl_2$, 1 mM $CaCl_2$), 2 µl of RNase-free DNase I (1 U/µl; Fermentas) and 3 µl of dH_2O. The tubes were incubated at 37°C for 30 min, after which the reaction was terminated by addition of 5 µl of 25 mM EDTA and incubation at 65°C for 10 min. The purified RNA was immediately reverse transcribed using the Quantitect™ Reverse Transcription kit (Qiagen).

2.8.3 cDNA synthesis

Total cDNA was synthesized using the Quantitect™ Reverse Transcription kit (Qiagen) according to the manufacturer's protocol. Prior to reverse transcription, each RNA preparation (12 µl) was incubated at 42°C for 2 min with 2 µl of gDNA wipeout buffer (supplied in the kit) to ensure complete removal of contaminating genomic DNA. To reverse transcribe the RNA, 4 µl of Quantiscript™ RT buffer (containing an optimized blend of buffer components and dNTPs), 1 µl of RT primer mix (containing an optimized blend of oligo-dT and random primers) and 1 µl of Quantiscript™ Reverse Transcriptase were added to the RNA preparation in a final volume of 20 µl. The reactions were incubated at 42°C for 15 min, after which the enzyme was inactivated by heating to 95°C for 3 min. A reverse transcriptase (RT) negative

control was prepared by adding 1 µl of dH_2O to a reaction instead of the reverse transcriptase. The cDNA was stored at -70°C until needed.

2.8.4 Reverse transcriptase (RT) negative control PCR

To verify the absence of contaminating genomic DNA in the samples, the cDNA and RT negative control samples, as described above, were serially diluted from 10^{-2} to 10^{-8} and used as template in conventional PCR reactions. The reactions contained 400 nM of each the forward and reverse primer, 200 µM of each dNTP, 1 µl of the appropriate dilution of cDNA or RT negative control, 0.5 U of GoTaq® Flexi DNA polymerase (Promega), 2.5 µl of the supplied reaction buffer, 1.5 mM $MgCl_2$ and dH_2O to a final volume of 25 µl. Thermal cycling was performed in a GeneAmp® 2700 thermal cycler with the following cycling parameters: initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s and extension at 72°C for 30 s. After a final extension at 72°C for 7 min, the reaction mixtures were analyzed by agarose gel electrophoresis in the presence of an appropriate DNA molecular weight marker.

2.8.5 Quantitative real-time PCR

Quantitative real-time PCR amplification of selected *B. cereus* cDNAs was performed in quadruplicate reactions on a LightCycler instrument (Roche Diagnostics), using the QuantiTect™ SYBR® Green PCR Kit (Qiagen) according to the manufacturer's instructions. Each reaction mixture (15 µl) contained 1.5 µl of the 10^{-1} dilution of cDNA, 400 nM of each the forward and reverse primers, 7.5 µl of Quantitect™ SYBR® Green PCR master mix (containing HotStar Taq®, dNTP mixture inclusive of dUTP, SYBR® Green I, ROX passive reference dye and 5 mM $MgCl_2$), and 4.8 µl of dH_2O. The reaction mixtures were incubated at 95°C for 15 min to activate the HotStar Taq® DNA polymerase and then subjected to 45 cycles of denaturation at 94°C for 15 s, primer annealing at 60°C (57°C for *purA*) for 30 s and extension at 72°C for 20 s. A non-template negative control with 16S primers was included to detect DNA contamination. During each cycle, data acquisition was performed during the extension step and analyzed using LightCycler® software v.3.5 (Roche Diagnostics). To confirm specific amplification, melt-curve analysis of the amplicons was performed by decreasing the temperature from 95°C to 65°C with a temperature transition rate of 20°C/s and then increasing the temperature to 95°C at a rate of 0.1°C/s with continuous fluorescence measurement. For Crossing Point (CP) determination, the Second Derivate Maximum Method

was used, which is included in the LightCycler® software. The PCR efficiency for each reaction was calculated from the exported fluorescence data using LinRegPCR v.11.1 (Ramakers *et al.*, 2003; Ruijter *et al.*, 2009) and expression of each gene relative to the 16S rRNA was quantified using REST© 2005 (Pfaffl *et al.*, 2002).

CHAPTER THREE
RESULTS

3.1 Isolation of pLTV1

Plasmid DNA purification from *Escherichia coli* strains harbouring vectors used for cloning is commonly performed in a small (1.5-3 ml of the recombinant culture) and relatively crude mini-preparation format, and typically yields DNA in sufficient quantity and purity for cloning purposes. However, these cloning vectors are mostly based on mutated pMB1 replicons that are capable of replicating to very high copy numbers in *E. coli*. Commonly used vectors such as pGEM and pBluescript reach copy numbers of 300-400 per cell, while vectors such as pUC18/pUC19 are present at 500-700 copies per cell (Ausubel *et al.*, 2002). In contrast, the replication of pLTV1 in Gram-positive hosts relies on the origin of replication from the thermo-sensitive plasmid pE194Ts, which has a copy number of *ca.* 10 in *Bacillus subtilis*. As a result, the yield of pLTV1 from a plasmid preparation is low and is further exacerbated by the fact that the efficiency of lysis tends to be lower for Gram-positive compared to Gram-negative bacteria. This is most likely due to the occurrence of *N*-nonsubstituted glucosamine residues in the peptidoglycan of cell walls of bacilli, resulting in lysozyme-resistance and thus inefficient cell lysis during DNA extraction procedures (Araki *et al.*, 1972; Hayashi *et al.*, 1973). Moreover, the introduction of plasmid DNA into Gram-positive bacterial hosts by electroporation procedures requires highly purified DNA preparations that are free of residual salts. The latter may cause arcing during electroporation that results in greatly diminished transformation efficiencies.

Based on the above considerations, the pLTV1 plasmid DNA was thus extracted from 1-litre cultures of *B. subtilis* PY177 and subsequently purified by CsCl-EtBr density gradient centrifugation. Using this approach, a high yield of covalently closed circular plasmid DNA free from salts, protein, RNA and chromosomal DNA contaminants was obtained (Fig. 3.1B, lane 2). Prior to using pLTV1 as a vector to deliver the Tn*917*-LTV1 transposon into the genome of *B. cereus* ATCC 14579 during transposon mutagenesis, its identity was confirmed by restriction enzyme digestion using agarose gel electrophoresis. Digestion of the purified pLTV1 plasmid DNA with *Eco*RI yielded three DNA fragments of 12.1 kb, 5.1 kb and 3.3 kb (Fig. 3.1B, lane 3), which, based on the plasmid map of pLTV1 (Fig. 3.1A), is in agreement with the expected number and size of the restriction fragments.

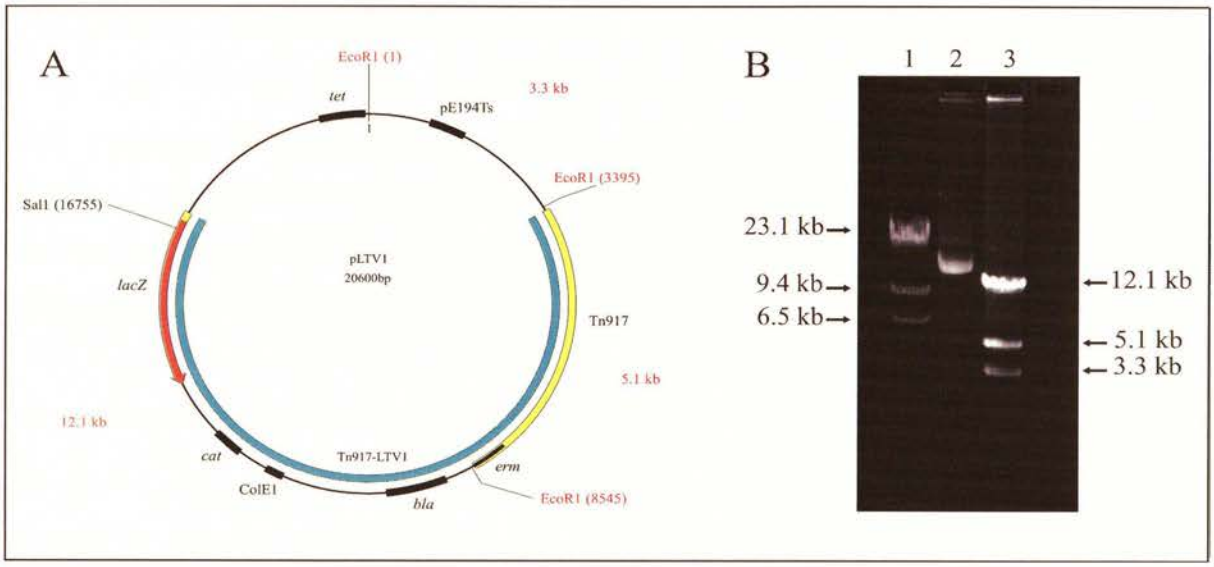

Figure 3.1: Characterization of purified pLTV1 plasmid DNA. (A) Plasmid map of pLTV1 harboring the Tn*917*-LTV1 transposon. The *Eco*RI restriction enzyme sites and sizes of the digestion products are indicated in red. (B) Agarose gel electrophoretic analysis of pLTV1. Lane 1, DNA molecular weight marker; lane 2, purified uncut pLTV1 plasmid DNA; lane 3, pLTV1 plasmid DNA digested with *Eco*RI. The sizes of the DNA molecular weight marker, phage λ DNA digested with *Hin*dIII, are indicated to the left of the figure

3.2 Transposon mutagenesis of *B. cereus* ATCC 14579

The construction of mutant strains from which specific functions have been eliminated is central to the analysis of various questions in microbiology. This approach has been used successfully to determine the genetics of biofilm formation in different bacterial species (Heilmann *et al.*, 1996; Chagneau and Saier, 2004) and to determine the function of undefined ORFs (Branda *et al.*, 2004). To generate such loss-of-function mutations, random transposon insertion mutagenesis methods have often been used. Transposons, being mobile genetic elements, have the capability of inserting themselves into the ORFs or promoters of genes on a bacterial chromosome, thereby disrupting the gene itself and sometimes additional genes that are encoded downstream of the mutated gene. This therefore represents a powerful approach towards identifying genes involved in a specific function, provided that an appropriate high-throughput screen is available. Using such an approach, various genes involved in biofilm formation by *B. subtilis* have been identified (Branda *et al.*, 2004; Chagneau and Saier, 2004).

Towards identifying genes that may be involved in biofilm formation by *B. cereus*, strain ATCC 14579 was subjected to transposon mutagenesis with pLTV1-Tn*917*. For transposon mutagenesis, pLTV1-Tn*917* was introduced into *B. cereus* ATCC 14579 by electroporation and transformants were selected on selective agar medium (LB agar supplemented with

erythromycin) at the permissive temperature of 30°C. To allow for chromosomal integration of the Tn*917*-LTV1 transposon, transformants were inoculated into selective LB medium (LB broth supplemented with erythromycin and chloramphenicol) and incubated at the non-permissive temperature of 43°C. The cultures were then plated onto the same selective agar medium and incubated overnight at 43°C. Using this approach, five independent *B. cereus* transposon mutant libraries were constructed that yielded a total of 3 500 mutants.

3.3 Screening for *B. cereus* biofilm-impaired mutants

Previous studies aimed at the identification of biofilm-impaired transposon mutants in bacterial species such as *E. coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* have employed a screening method based upon the use of the wells of microtiter plates as attachment surfaces, followed by the removal of the planktonic cells and staining of the adherent biofilm cells with crystal violet (O'Toole *et al.*, 1999; Stepanovic *et al.*, 2000). Preliminary experiments in which biofilms of wild-type *B. cereus* ATCC 14579 were cultured by the above standard approach in polystyrene and polyvinylchloride (PVC) 96-well microtiter plates yielded highly variable quantities of biofilm (data not shown). The biofilms of the wild-type did not appear to adhere well to the plastic walls, leading to variable loss of biofilm during removal of the planktonic phase and the subsequent staining with crystal violet. A recent report regarding the development of biofilms by strains of *B. cereus* is in agreement with these observations (Wijman *et al.*, 2007). It was reported that *B. cereus* forms minimal amounts of biofilm on submerged surfaces and that the biofilms develop preferentially at the air-liquid interface.

A novel method was thus developed during the course of this investigation in an effort to facilitate high-throughput screening of the large number of *B. cereus* ATCC 14579 transposon mutants. For this purpose, cultures were grown in 96-well microtiter plates and then inoculated into Durham tubes, which were kept upright in the wells of a microtiter plate. Following incubation at 25°C without shaking, the biofilm formed at the solid-liquid-air interface was scored visually without any sample handling and compared to that of the wild-type *B. cereus* ATCC 14579. Putative biofilm-impaired mutants were selected and then re-screened using the same assay. This led to the identification of 13 transposon mutants that were repeatedly deficient in biofilm formation. As this initial screen is only semi-quantitative, the mutants were subsequently assayed by culturing in 25-ml glass beakers and the biofilm

biomass formed at the glass surface was quantified by spectrophotometry. For this purpose, the liquid phase was carefully removed by aspiration and the biofilm was rinsed from the glass surface and suspended in buffer.

The results indicated that of the thirteen Tn*917*-LTV1 mutants of *B. cereus* ATCC 14579 analyzed, only eight mutants were significantly impaired in their ability to form biofilm at the air-liquid interface when compared to the wild-type *B. cereus* (Fig. 3.2). In contrast, five mutants (Mut6, Mut12, Mut14, Mut15 and Mut20) were found not to be impaired in biofilm production, despite having been scored as biofilm-impaired in the semi-quantitative Durham tube-based assay. These mutants may have appeared biofilm-impaired in the initial assay because the biofilms growing at the air-liquid interface tend to become fragile with increasing age, and eventually break off and sink to the bottom of the vessel. Notably, these results do, however, suggest that the presence of the transposon-itself is not a contributing factor to the biofilm deficiency observed in the remainder of the biofilm-impaired mutants.

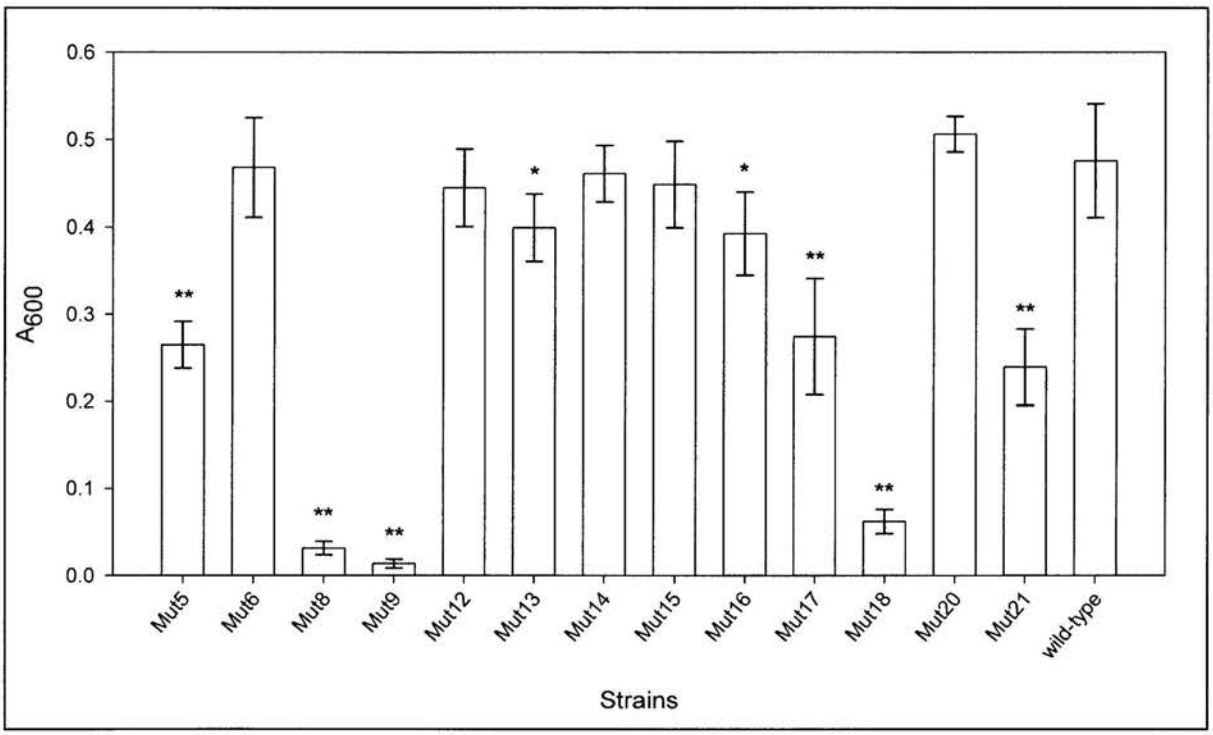

Figure 3.2: Biofilm formation by wild-type *B. cereus* ATCC 14579 and the derived Tn*917*-LTV1 transposon mutants (Mut). Biofilm formation was quantified by determining the A_{600} of the biofilm biomass after growth of the biofilm for 72 h in glass beakers without shaking. The data shown are the averages of five independent assays. Error bars indicate the standard deviation of the mean. Biofilm-impaired mutants, indicated by the shaded bars, were selected for further analyses. (* $p < 0.05$, ** $p < 0.01$, $n = 5$)

Only those *B. cereus* transposon mutants that were significantly impaired in biofilm formation in both the semi-quantitative and quantitative biofilm assays were subsequently selected for further study.

3.4 Characterization of *B. cereus* biofilm-impaired mutants by Southern blot analysis

To confirm the presence of the Tn*917*-LTV1 transposon in the genome of the *B. cereus* biofilm-impaired mutants, Southern blot hybridization was performed. The chromosomal DNA from the mutants was thus isolated, digested with *Eco*RI and separated by agarose gel electrophoresis. The DNA fragments were transferred to a nylon membrane by capillary blotting and the membrane was then hybridized with a DIG-dUTP labelled DNA probe that corresponds to a 5.1-kb fragment of the Tn*917*-LTV1 transposon (Fig. 3.1A). In this analysis, *Eco*RI-digested pLTV1 plasmid DNA was included as a positive hybridization control, while *Eco*RI-digested chromosomal DNA from wild-type *B. cereus* ATCC 14579 was included as a negative hybridization control.

The results, presented in Fig. 3.3, indicated that the probe did not hybridize with the digested chromosomal DNA of the wild-type *B. cereus* ATCC 14579. The probe did, however, hybridize with the 5.1-kb pLTV1 restriction fragment, as well as with chromosomal restriction fragments from the selected transposon mutants. These results therefore confirmed that a single copy of the Tn*917*-LTV1 transposon had integrated into the genome of the respective mutants. In the case of chromosomal DNA extracted from mutants Mut8 and Mut18, the probe DNA hybridized with two DNA restriction fragments (Fig. 3.3). Interestingly, the smallest band on the membrane for both of these mutants appeared to be similar in size to the 5.1-kb pLTV1 restriction fragment. This is the expected minimum size for a chromosomal *Eco*RI-restriction fragment containing a transposon-derived sequence, provided that the *Eco*RI site is immediately adjacent to the inserted transposon. It is, however, unlikely that these smaller DNA fragments were derived from a chromosomal insertion of the transposon. The most plausible explanation for these results is that a single copy of the transposon had integrated into the chromosomal DNA, but that an extrachromosomal copy of pLTV1 is also present in these two mutants. Similarly, the presence of pLTV1 in Tn*917*-LTV1 transposon mutants of *Bacillus* sp. strain SG-1 has been reported previously (van Waasbergen *et al.*, 1993).

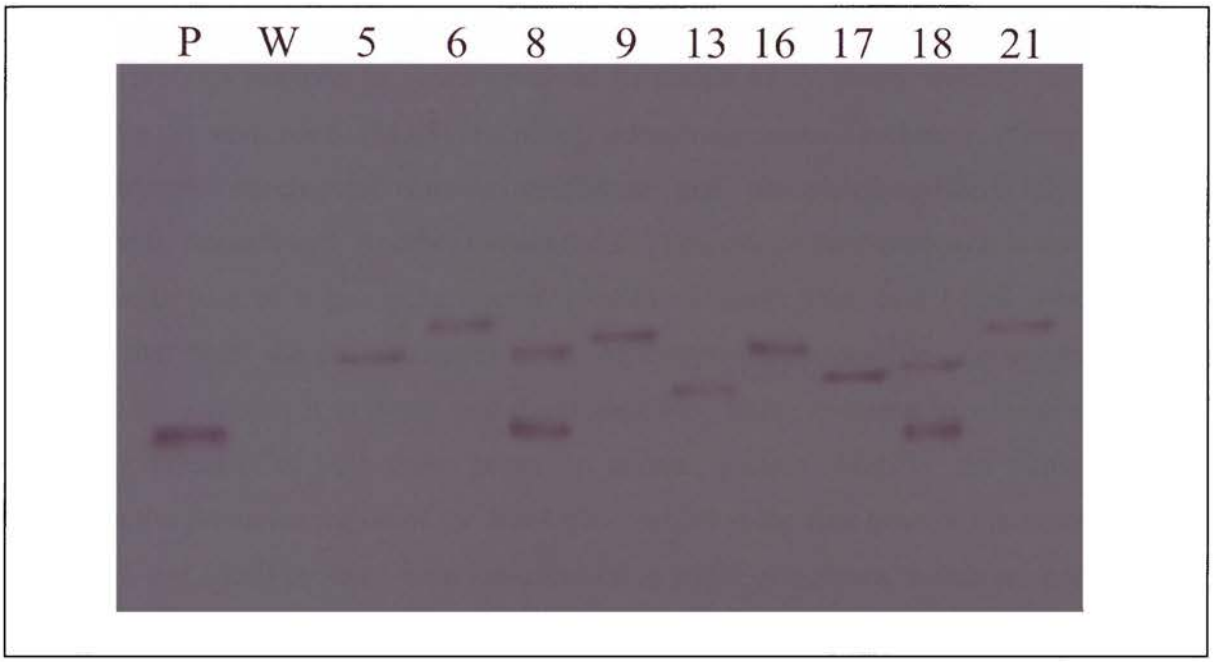

Figure 3.3: Southern blot analysis of chromosomal DNA extracted from *B. cereus* biofilm-impaired mutants. Chromosomal DNA extracted from wild-type *B. cereus* ATCC 14579 (lane W) and the respective *B. cereus* mutants (lanes 5 through 21) were digested with *Eco*RI, resolved by agarose gel electrophoresis, transferred to a nylon membrane and then hybridized with a DIG-dUTP labelled probe derived from pLTV1. *Eco*RI-digested pLTV1 plasmid DNA (lane P) was included as a positive control. A non biofilm-impaired transposon mutant (Mut6) was included to demonstrate that the presence of the transposon itself was not responsible for the observed mutant phenotype.

3.5 Identification of disrupted genes in *B. cereus* biofilm-impaired mutants

To determine the position of the chromosomal Tn*917*-LTV1 transposon insertions in the *B. cereus* biofilm-impaired mutants, the chromosomal DNA flanking the sites of transposon insertion was cloned directly into *E. coli* by making use of the ColE1 replicon within Tn*917*-LTV1. For this purpose, chromosomal DNA was isolated from the respective biofilm-impaired mutants, digested with either *Eco*RI or *Bam*HI and then self-ligated. Following transformation of competent *E. coli* HB101 cells, plasmid DNA was extracted from ampicillin-resistant transformants and characterized by agarose gel electrophoresis, as well as by nucleotide sequencing of the rescued chromosomal DNA. In order to map the positions of the transposon insertions, the nucleotide sequences of the rescued chromosomal DNA fragments were subjected to homology searches against the *B. cereus* ATCC 14579 genome sequence (GenBank accession no. NC_004722) using the NCBI BLASTn program. The results are presented graphically in Fig. 3.4.

In two of the biofilm-impaired mutants the transposon was mapped to a gene encoding a putative membrane spanning protein (Mut5) and to a gene encoding a putative protein of

unknown function (Mut13), respectively. The transposon insertions in three of the biofilm-impaired mutants mapped to genes involved in purine biosynthesis, namely *purA* (Mut9), *purC* (Mut18) and *purL* (Mut8) encoding adenylosuccinate synthetase, phosphoribosyl-aminoimidazole succinocarboxamide synthetase and phosphoribosylformylglycinamidine synthetase II, respectively. Another mutant (Mut17) contained the transposon in the *ftsE* gene. This gene is part of a two-gene operon (*ftsEX*) encoding FtsE and FtsX, which form a complex that bears the characteristics of an ABC-type transporter (de Leeuw *et al.*, 1999). Owing to its location, it is likely that the transposon, which contains terminator sequences, affected expression of both these genes. In another mutant (Mut21), the transposon was located in the promoter region of the *motA* gene, which is the first gene of the *motAB* operon. The MotA and MotB proteins form ion-conducting stator complexes, which are necessary for flagellar motor rotation (Terashima *et al.*, 2008). Insertion of the transposon into the promoter of *motA* could have a polar effect on the expression of the downstream *motB* gene, as it probably introduces premature termination in the *motAB* operon. In the final biofilm-impaired mutant (Mut16), the transposon was located in the intergenic region between two genes that are divergently transcribed. The gene upstream of the inserted transposon (BC5133) is the first of an apparent two-gene operon encoding proteins involved the export of murein hydrolase. Murein hydrolases are a family of enzymes that specifically cleave structural components of the bacterial cell wall (Rice and Bayles, 2008). The gene downstream of the transposon insertion (BC5134) encodes a nucleoside hydrolase that, despite its preference for inosine and adenosine, catalyzes the hydrolysis of purine and pyrimidine nucleosides into ribose and the associated base (Parkin *et al.*, 1991b).

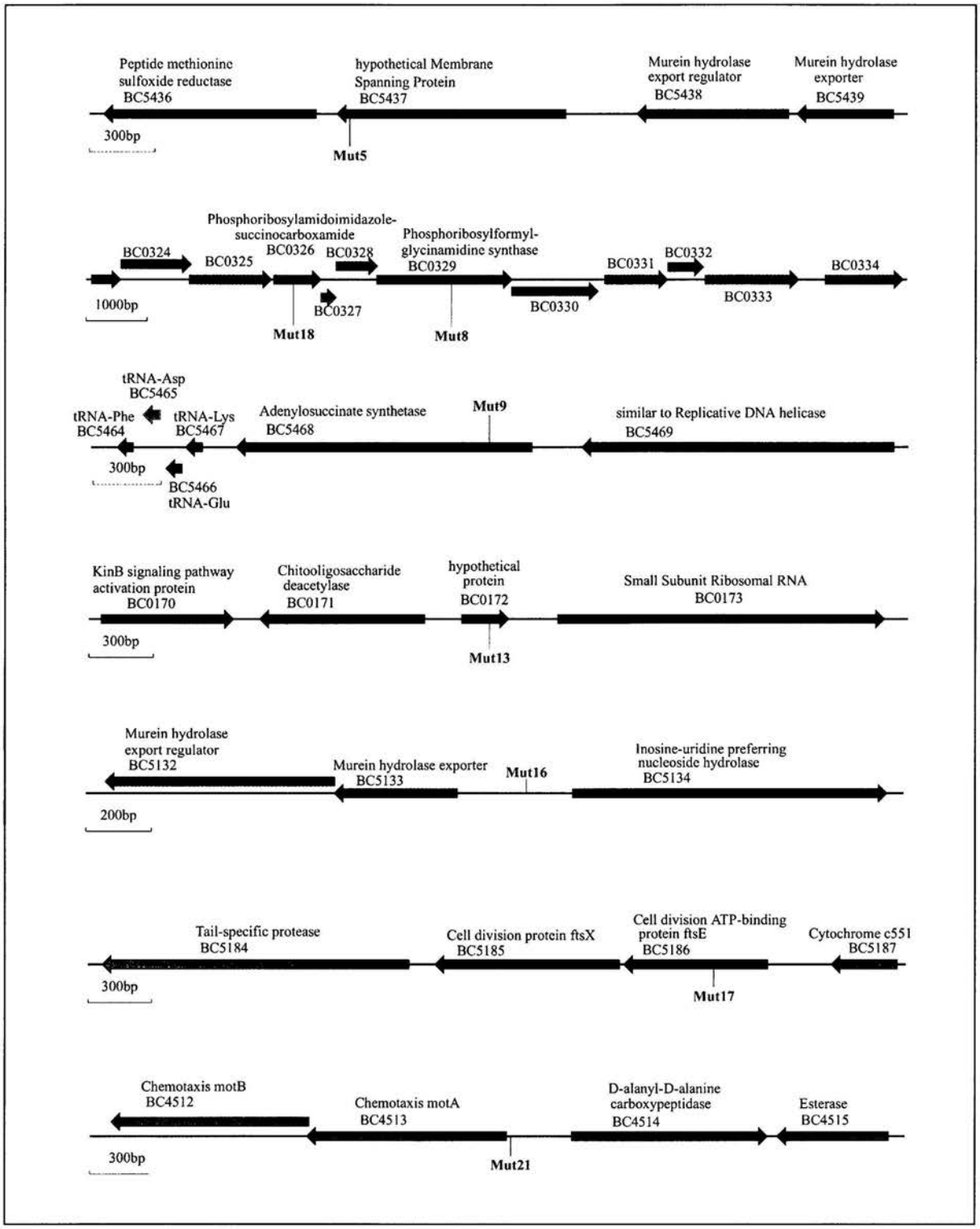

Figure 3.4: Locations of the Tn*917*-LTV1 insertions in the *B. cereus* biofilm-impaired mutants isolated in this study. The position of transposon insertions are depicted by a vertical line bearing the name of the mutant. Directions of transcription are indicated by arrowheads. Gene designations are presented above the genes and correspond to that of the annotated *B. cereus* ATCC14579 genome sequence (GenBank accession no. NC_004722).

3.6 Characterization of *B. cereus* biofilm-impaired mutants

3.6.1 Growth curves

It has been noted that insertion mutagenesis may influence the growth properties of a particular mutant strain (Day *et al.*, 2007). Therefore, it is possible that the impaired biofilm formation observed for the different *B. cereus* transposon insertion mutants may be due to growth impairment rather than inactivation of a specific gene. To investigate whether the introduced mutations influenced the growth properties of the mutants, the wild-type *B. cereus* ATCC 14579 and biofilm-impaired mutants were cultured in LB broth, and their growth was followed by taking A_{600} readings every 2 h over a period of 12 h and again at 24 h. The results indicated that the biofilm-impaired mutants displayed growth rates very similar to wild-type *B. cereus* and no statistically significant differences between the mutants and the wild-type strain were detected at any of the time intervals (Fig. 3.5). From these results, it could thus be concluded that the mutations identified in the preceding section were specific to *B. cereus* biofilm development.

3.6.2 Microscopic analysis of biofilm phenotypes

Biofilms formed by the different *B. cereus* transposon insertion mutants on microscope glass slides in 40-h standing cultures were examined and compared by stereomicroscopy. Although the transposon insertions did not abrogate biofilm formation, notable differences were nevertheless observed with regards to the biofilm morphology (Fig. 3.6). All of the mutants, except for Mut13, formed biofilms at the air-liquid interface on the glass slide that were noticeably thinner than that formed by wild-type *B. cereus* ATCC 14579. The wild-type *B. cereus* formed a thick biofilm, and chains of cells that resembled a dense curtain could be seen to descend from the biofilm into the culture medium. A similar curtain of descending cell chains was also observed for biofilms formed by Mut13 and Mut17, and to lesser extent for the biofilm formed by Mut16 (Fig. 3.6).

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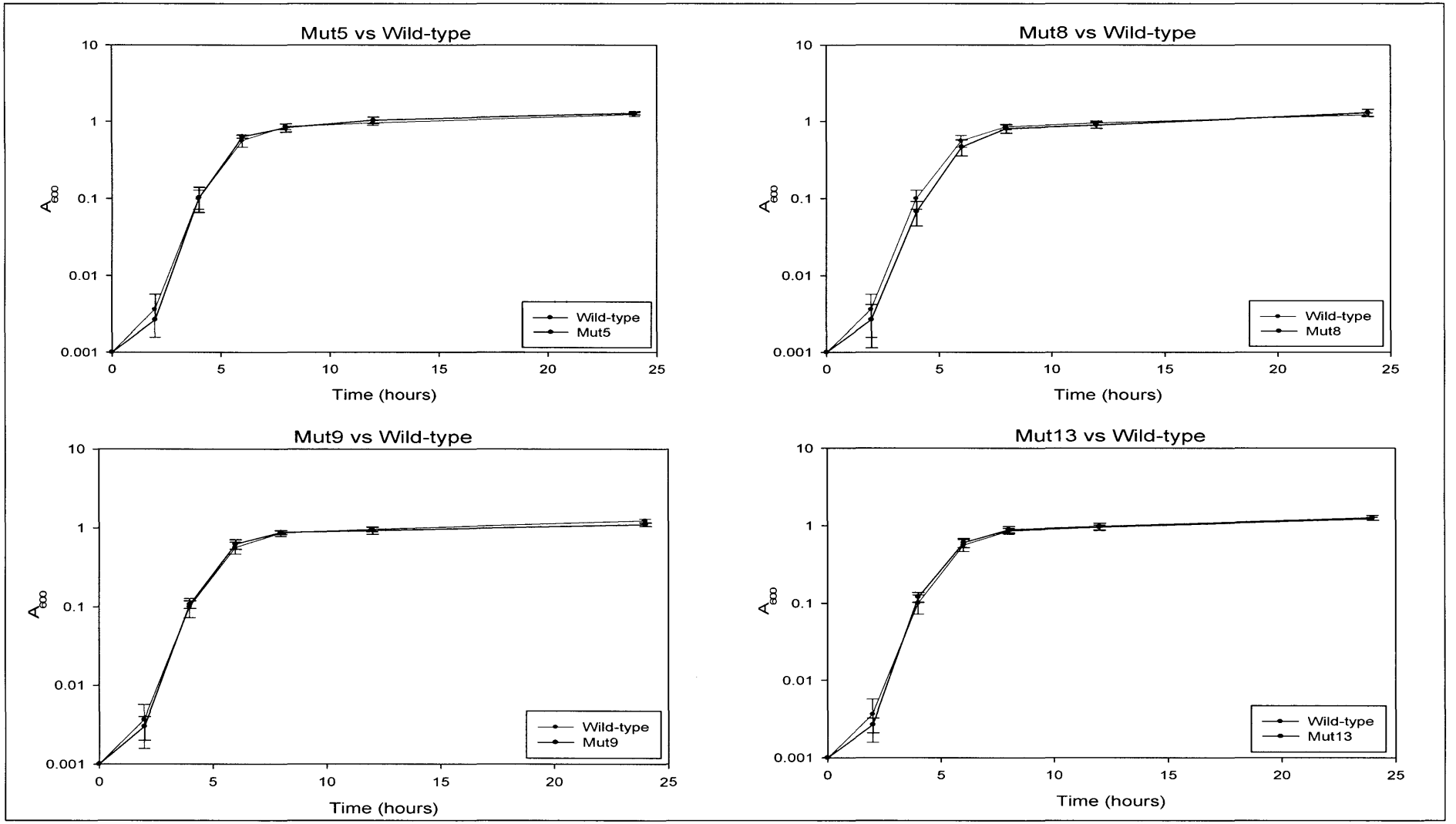

Figure 3.5: Growth curves of wild-type *B. cereus* ATCC 14579 and biofilm-impaired mutants in LB broth at 37°C. The data shown are the averages of three independent assays, and error bars indicate the standard deviation of the mean.

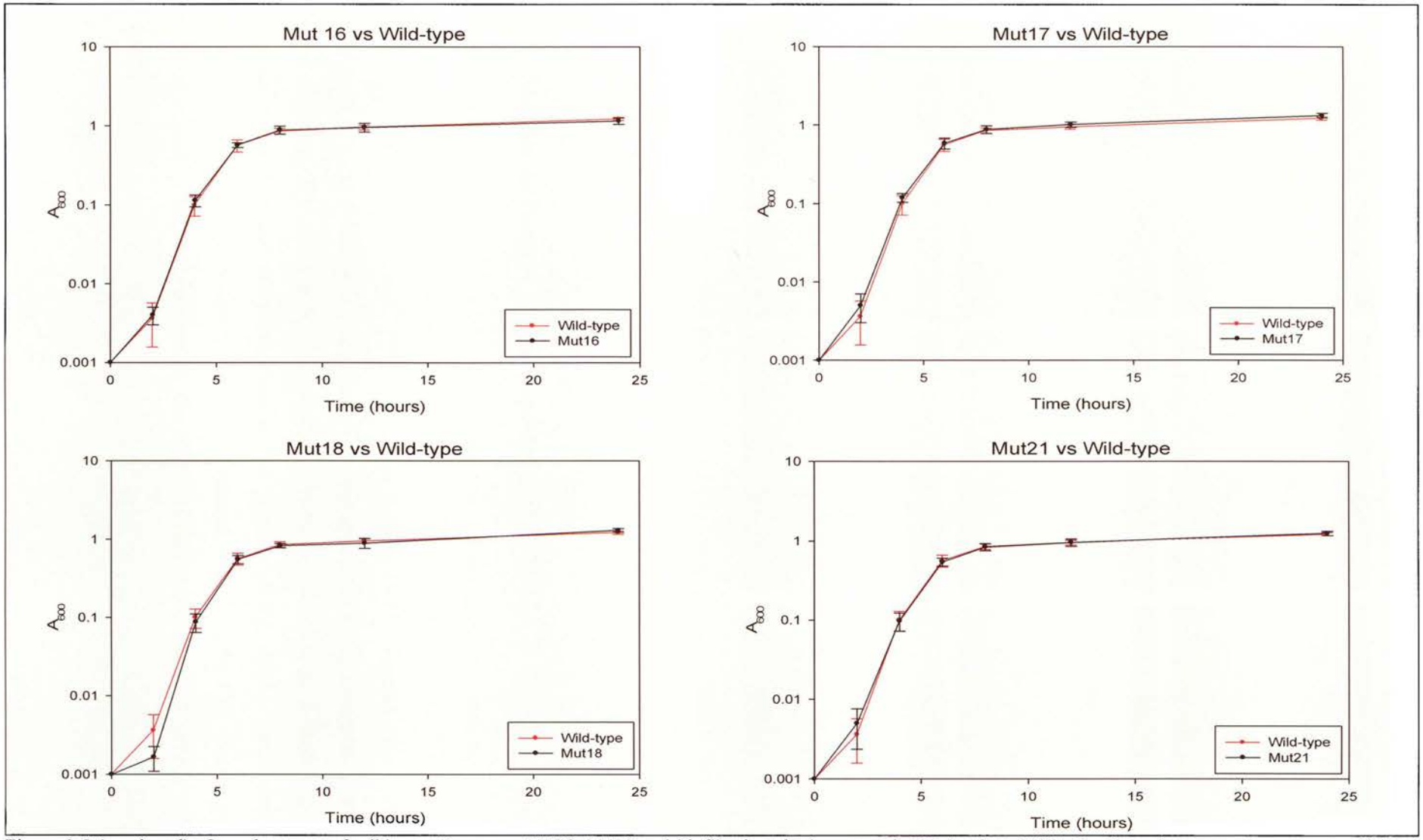

Figure 3.5 (continued): Growth curves of wild-type *B. cereus* ATCC 14579 and biofilm-impaired mutants in LB broth at 37°C. The data shown are the averages of three independent assays, and error bars indicate the standard deviation of the mean.

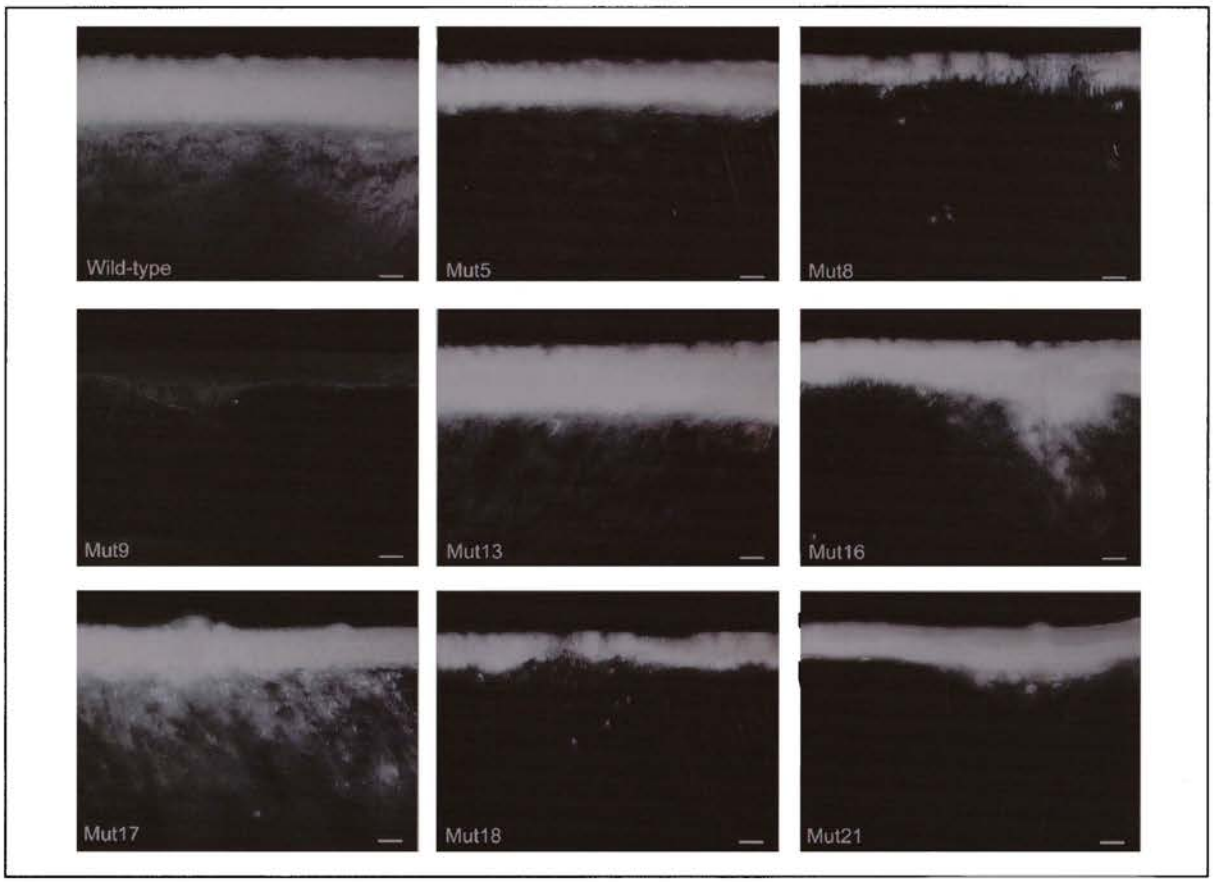

Figure 3.6: Phenotypes of the biofilms formed at the air-liquid interface on glass slides by wild-type *B. cereus* ATCC 14579 and the biofilm-impaired mutants (Mut). To assay biofilm formation, the wild-type and each mutant was inoculated into LB broth containing a microscope slide and the cultures were incubated at room temperature for 40 h without shaking. The microscope slides were then carefully withdrawn and examined by stereomicroscopy. Bars = 1 mm.

3.6.3 Motility assay of the biofilm-impaired mutant Mut21

Mechanisms of biofilm formation have been well established in Gram-negative bacterial model systems and motility is often reported to be involved in the initial stages of biofilm formation (O'Toole and Kolter, 1998; Pratt and Kolter, 1999; Watnick *et al.*, 2001). In *B. subtilis* the motility function is conferred by the presence of cell surface peritrichous flagella (Mirel *et al.*, 1992), and the MotA and MotB proteins function as a stator complex that generates the torque that drives rotation of the flagella (Berg, 2003). Since one of the *B. cereus* biofilm-impaired mutants, Mut21, contained the Tn*917*-LTV1 transposon in the promoter region of the *motAB* operon, the effect of this mutation on *B. cereus* motility was thus explored.

To investigate, the wild-type and mutant *B. cereus* strains were stab inoculated into a motility medium and the tubes incubated overnight. When stabbed into agar of sufficiently low concentration, motile anaerobic and facultatively anaerobic bacteria are capable of spreading into the agar by means of flagella-mediated motility causing cloudy turbidity in the agar surrounding the stab line. Non-motile bacteria however, only grow along the stab line. The results indicated that, in contrast to the wild-type *B. cereus* strain, the *motAB* mutant strain Mut21 was deficient in motility in the soft agar stabs (Fig. 3.7). The lack of motility in the *motAB* mutant strain Mut21 could be due to absence of functional flagella or to the inability of the flagellar motor to rotate. To distinguish between these possibilities, the wild-type and mutant *B. cereus* cells were negatively stained and examined. Transmission electron microscopy showed that the mutant cells had peritrichous flagella resembling that of the wild-type strain (Fig. 3.8). These results therefore suggested that the *motAB* mutation resulted in paralyzed flagella, as had also been observed for *B. subtilis* (Mirel *et al.*, 1992).

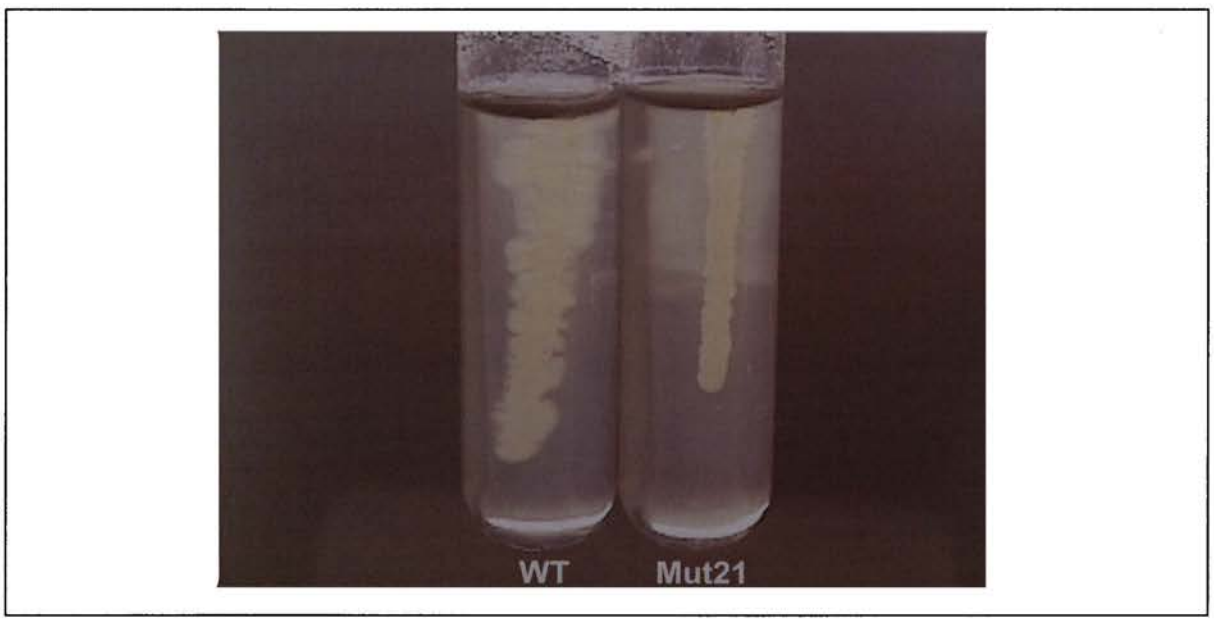

Figure 3.7: Motility assays for wild-type *B. cereus* ATCC 14579 (WT) and the *motAB* mutant strain Mut21. The cultures were stab inoculated into soft agar and the tubes were incubated overnight at 30°C.

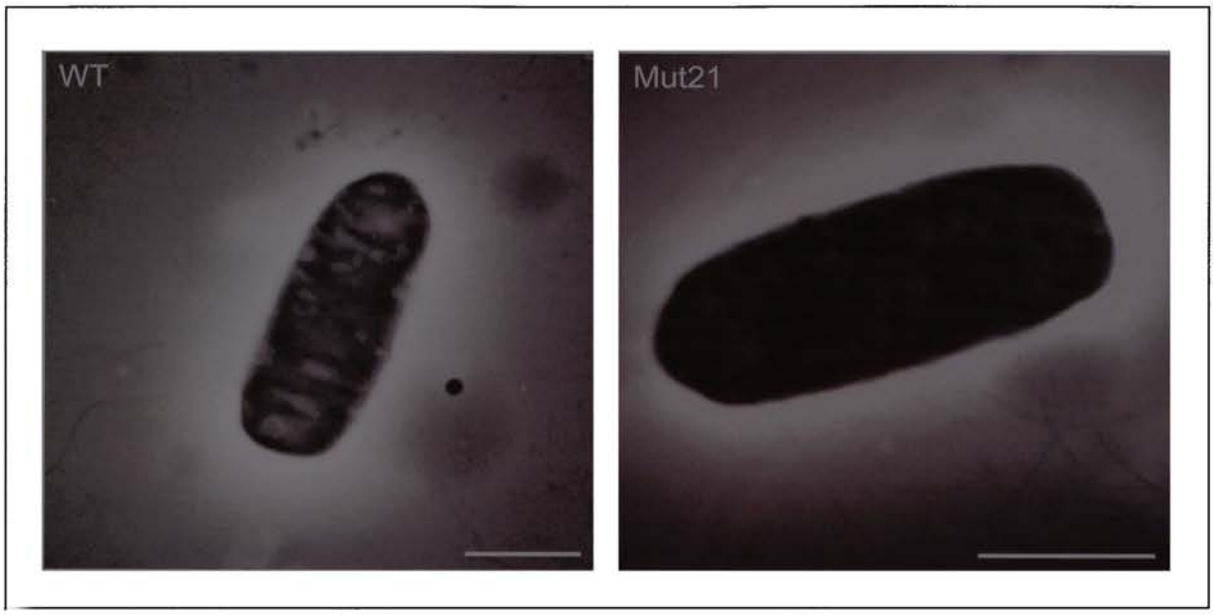

Figure 3.8: Representative transmission electron micrographs of negatively-stained cells from wild-type *B. cereus* ATCC 14579 (WT) and the *motAB* mutant strain Mut21. The bacteria were cultured for 16 h in LB broth and cells were adsorbed onto carbon-coated copper grids prior to negative staining with phosphotungstic acid. Bars = 1 µm.

3.6.4 Laser scanning confocal microscopy of the biofilm-impaired mutant Mut17

The FtsE and FtsX proteins, which are widely conserved among Gram-negative and Gram-positive bacteria (de Leeuw *et al.*, 1999), constitute an apparent ABC transporter that localizes to the septal ring in *E. coli* (Schmidt *et al.*, 2004). Several reports have indicated that *ftsE* and/or *ftsX* mutants exhibit morphological defects suggestive of impaired cell division in *E. coli*, *Neisseria gonorrhoeae* and *Aeromonas hydrophila* (Merino *et al.*, 2001; Ramirez-Arcos *et al.*, 2001; Schmidt *et al.*, 2004). In the preceding section, a *B. cereus ftsE* mutant (Mut17) was isolated in which the transposon is located inside the coding region of *ftsE*, the first gene of the *ftsEX* operon (Fig. 3.5). To determine whether the mutation causes similar morphological defects as those previously described, the *B. cereus* wild-type and *ftsEX* mutant strain Mut17 were stained with the lipophilic styryl dye FM® 4-64. This water-soluble FM® dye, which is non-toxic to cells and virtually non-fluorescent in aqueous media, is believed to insert into cell membranes where it becomes intensely fluorescent upon excitation. When the stained cells were viewed by laser scanning confocal microscopy, no noticeable differences in the cell morphology of wild-type and mutant cells were observed and septal rings in both these *B. cereus* strains were clearly visible (Fig. 3.9). These results therefore suggest that interruption of *ftsEX* has no apparent effect on cell morphology.

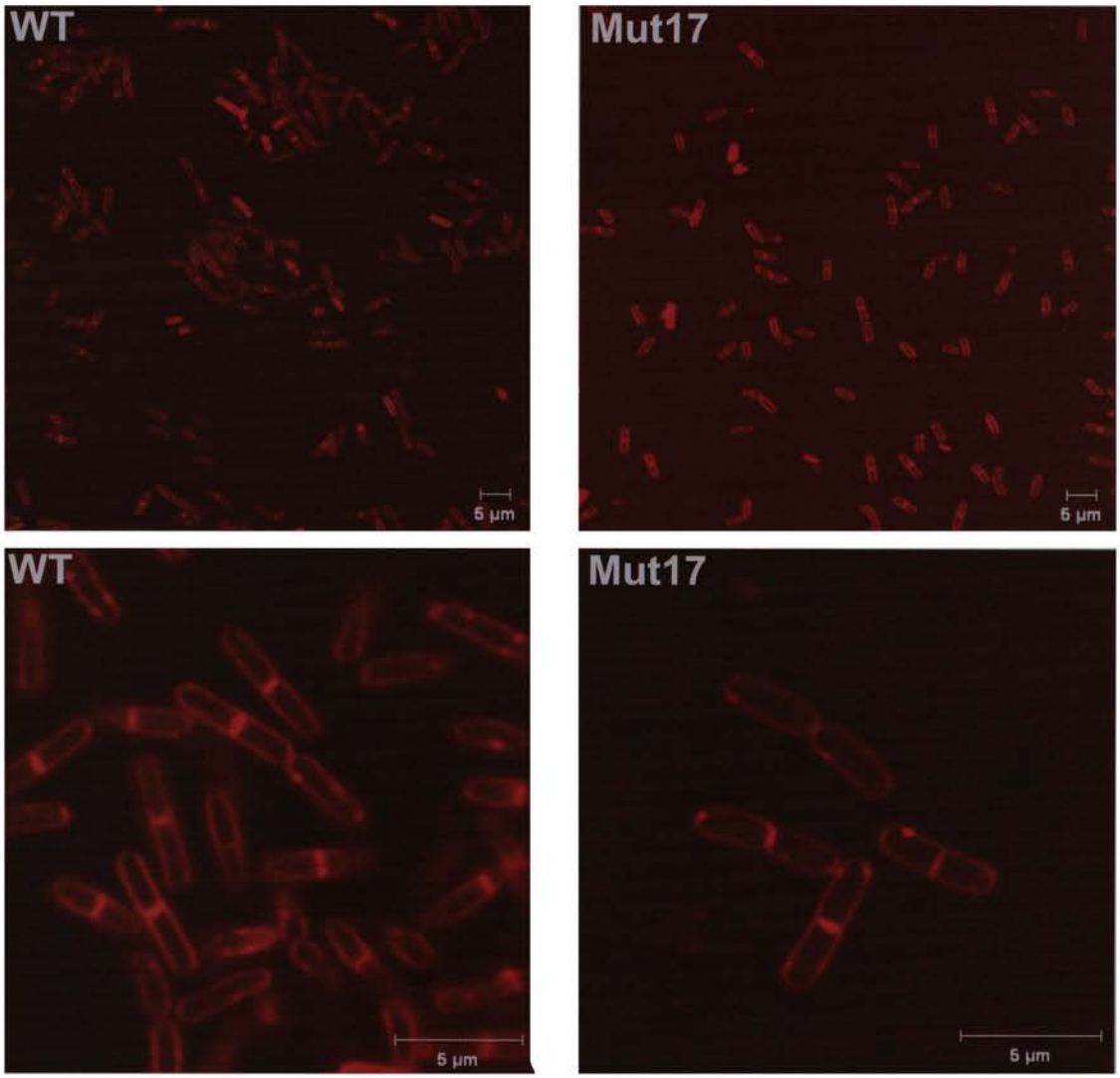

Figure 3.9: Representative laser scanning confocal micrographs of planktonic cells from wild-type *B. cereus* ATCC 14579 (left panel) and the *ftsEX* mutant strain Mut17 (right panel). The bacteria were grown for 12 h in LB broth and the cells were then stained with FM® 4-64, a lipophilic styryl dye, which inserts into cell membranes and subsequently fluoresces intensely at 734 nm upon excitation with green light at 558 nm. The bottom panel depicts the cells at higher magnification. Bars = 5µm in all cases.

3.7 Relative expression of selected genes in *B. cereus* biofilms

Mapping of the transposon insertions in the *B. cereus* biofilm-impaired mutants led to the identification of several genes that are implicated in biofilm formation (Section 3.5, Fig. 3.6). Thus, the differential expression of these genes in cells growing planktonically and in a biofilm was subsequently investigated. For this purpose, biofilms of the wild-type *B. cereus* ATCC 14579 and biofilm-impaired mutants were cultured in glass beakers for 72 h without shaking. Total RNA was isolated from both biofilm and planktonic populations, treated extensively with DNase I and subjected to reverse transcription, followed by real-time PCR.

The expression of each gene relative to that of 16S rRNA was quantified with REST© 2005 (Pfaffl *et al.*, 2002).

The results, presented in Fig. 3.10 and Table 3.1, indicated that expression of all but one of the genes implicated in *B. cereus* ATCC 14579 biofilm formation was significantly up-regulated in the biofilm compared with planktonic populations. Expression of the *motA* gene, which is required for rotation of the flagellar motor, was down-regulated 3.2-fold in the biofilm cells and thus suggests that motility becomes dispensable in the mature biofilm. There appears to be an elevated requirement for purines in biofilm cells, as was evidenced by the up-regulated expression (at least 1.5-fold) of the three *pur* genes *purA*, *purC* and *purL* in the biofilm. There also appears to be an enhanced requirement in biofilm cells for the putative ABC transporter encoded by the *ftsXE* operon, as expression of the *ftsE* gene was up-regulated 4.1-fold in the biofilm. In the case of Mut16, for which the transposon insertion was mapped to the intergenic region between two divergently transcribed genes, the expression of BC5133 encoding a murein hydrolase exporter and that of BC5134 encoding a nucleoside hydrolase was up-regulated 3.7- and 4.5-fold, respectively, in the biofilm. Likewise, expression of two uncharacterized genes was also significantly up-regulated in the biofilm. Whereas expression of BC5437 encoding a hypothetical membrane-spanning protein was up-regulated 4.3-fold, expression of BC0172 encoding a conserved hypothetical protein was up-regulated 3.5-fold.

The possibility of DNA contamination in the RNA preparations used above was eliminated by performing DNase I treatments and verified by subjecting the samples to PCR amplification, using *Taq* DNA polymerase and the gene-specific primer pairs (Table 2.1). No amplicons were obtained from control reaction mixtures lacking template or from the RNA preparations that were subsequently used for cDNA synthesis. In addition, the amplification specificity of the real-time PCR was verified by agarose gel electrophoresis and a single amplicon of the expected length for each target was obtained (results not shown). Furthermore, to confirm its reproducibility, melt curve analysis was performed and the efficiency of each individual real-time PCR reaction was calculated, as described under Materials and Methods (Section 2.8.5). The data obtained indicated that the real-time PCR was reproducible and sensitive (Table 3.1). The amplification plots and melting curves of the respective amplicons are provided in the Appendix to this dissertation.

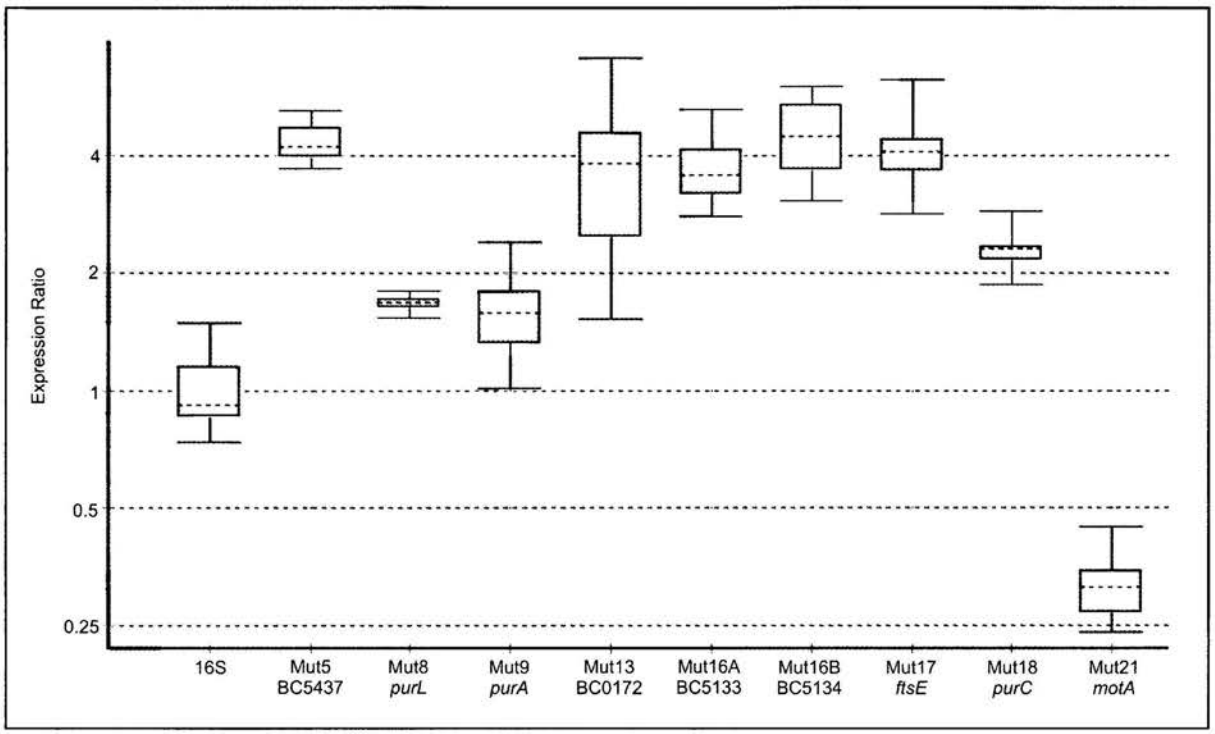

Figure 3.10: Whisker-box plot indicating the ratio of gene transcripts in a biofilm to the gene transcripts in the planktonic population quantified by quadruplicate real-time PCR reactions using the 16S rRNA gene as endogenous control. Boxes represent the interquartile range and the dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

Table 3.1: Descriptive statistics of real-time PCR reactions

Mutant	Gene	PCR Efficiency	*Mean CP (biofilm)	*Mean CP (planktonic)	#P(H1)	†Expression
	16S rRNA	0.689	10.12	9.97	1.000	1.000
Mut5	BC5437	0.562	27.74	30.85	0.004	4.321
Mut8	BC0329 (*purL*)	0.685	29.78	30.62	0.000	1.677
Mut9	BC5468 (*purA*)	0.642	27.62	28.37	0.048	1.571
Mut13	BC0172	0.686	36.39	38.66	0.012	3.546
Mut16A	BC5133	0.692	30.13	32.46	0.000	3.675
Mut16B	BC5134	0.552	35.58	38.8	0.000	4.465
Mut17	BC5186 (*ftsE*)	0.636	26.32	29.02	0.004	4.087
Mut18	BC0326 (*purC*)	0.577	34.02	35.67	0.004	2.289
Mut21	BC4513 (*motA*)	0.602	31.08	28.43	0.007	0.310

* CP is the crossing point or cycle at which the amplification plot crosses the threshold.
\# P(H1) is the probability of the alternate hypothesis, namely that the difference in expression of a gene in biofilm versus planktonic cells is due only to chance.
† Expression is the fraction of the expression of the selected genes in biofilm versus planktonic cells.

CHAPTER FOUR
DISCUSSION

During the course of this study, several genes were identified as being important for biofilm formation by *Bacillus cereus* ATCC 14579. Many of these genes have not yet been characterized with regards to a role in biofilm formation in Gram-positive bacteria and consequently, the information provided here will be supplemented with that obtained from applicable studies undertaken on Gram-negative bacteria. Each of the sections presented in this Chapter will be introduced with a brief summary regarding the properties of the *B. cereus* biofilm-impaired mutants and therefore a certain degree of redundancy may exist between the information presented here and that presented previously in Chapter 3.

4.1 Flagella-mediated motility is required in *B. cereus* biofilm formation

4.1.1 *B. cereus ΔmotAB* is biofilm-impaired

The biofilm-impaired mutant strain Mut21, in which the transposon was located in the promoter region of the *motAB* operon, displayed a growth rate similar to wild-type *B. cereus*, but formed *ca.* 50% of the wild-type biofilm biomass (Fig. 3.2). Although biofilm formation was not completely abrogated in this mutant strain, the biofilm was noticeably thinner and lacked chains of cells descending from the biofilm into the culture medium when compared to biofilm of the wild-type *B. cereus* strain (Fig. 3.6). Expression of the *motA* gene was down-regulated 3.2-fold in the biofilm compared with planktonic populations (Fig. 3.10). Phenotypic assays indicated that the Mut21 strain was deficient in motility in soft agar stabs, despite containing peritrichous flagella.

4.1.2 The motor complex of bacterial flagella

Bacterial flagella are helical structures that function as propellers to drive cell locomotion. They thrust the cells in liquids (swimming) or on surfaces (swarming) so that cells can move to favourable environments (Berg, 2003; Macnab, 2003; Terashima *et al.*, 2008). Flagella from Gram-positive and from Gram-negative bacteria are essentially identical, except that flagella from Gram-negative bacteria extend through a second, outer membrane that is absent from Gram-positive bacteria (Chevance and Hughes, 2008). The structure of a typical Gram-negative flagellum and its membrane-embedded motor is depicted in Fig. 4.1.

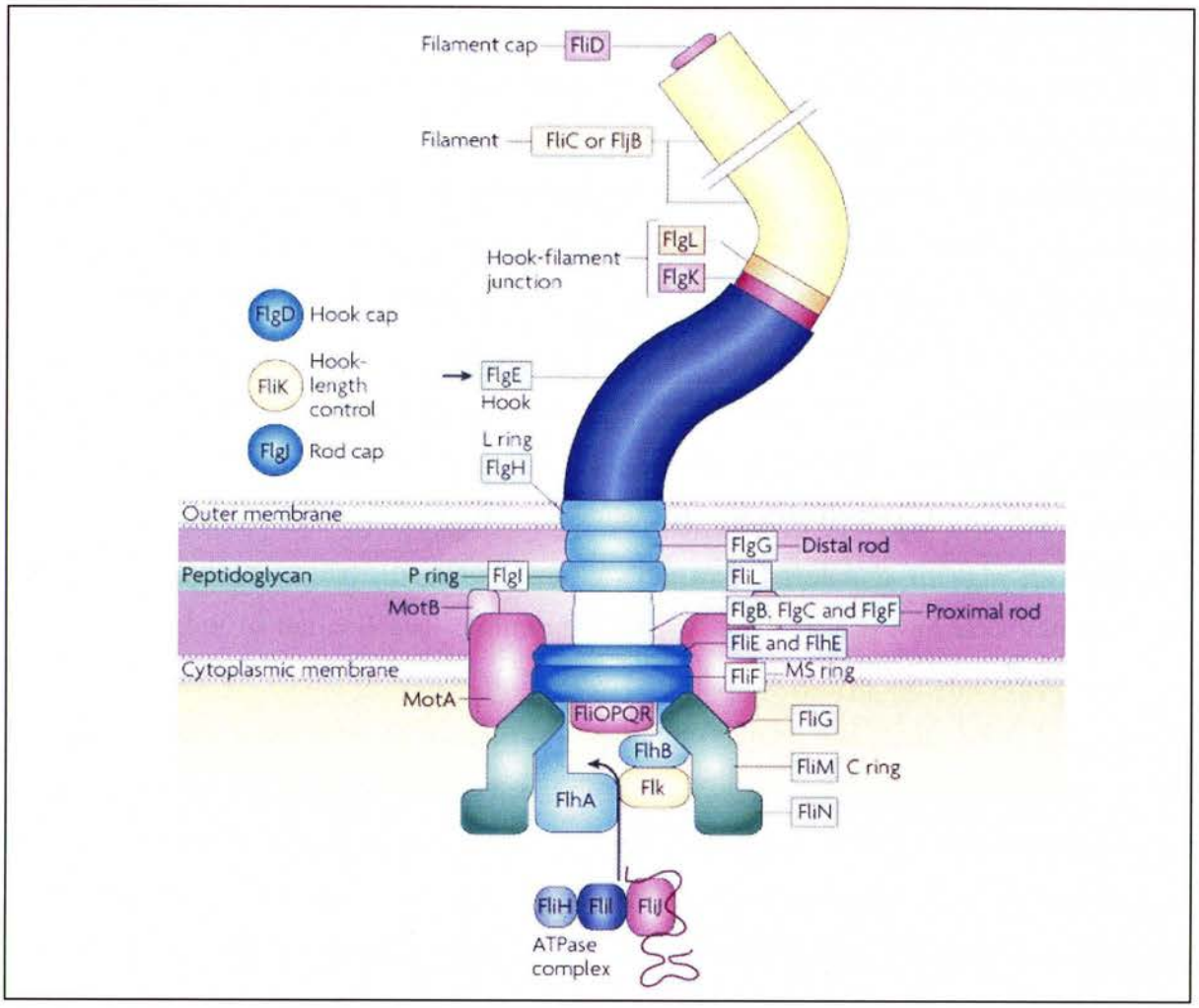

Figure 4.1: Schematic representation of the structure and components of the *Salmonella enterica* serovar Typhimurium flagellum. Genes whose inactivation results in non-flagellated mutants are called *flg*, *flh*, *fli* and *flj*, depending on their location on the genetic map, and genes whose inactivation results in paralyzed flagella are called *mot* (Iino *et al.*, 1988). The FliC flagellin subunits self-assemble to form a hollow double-tubular structure, consisting of as many as 20 000 subunits, which is capped by a structure composed of FliD that directs the polymerization of exported FliC subunits. The hook structure consists of 120 copies of a single protein, FlgE, and the junction between the hook and the filament, which serves as a structural adapter connecting the two, consists of the proteins FlgK and FlgL (from Chevance *et al.*, 2008).

The bacterial flagellum is powered by a transmembrane proton (H^+) gradient (Manson *et al.*, 1977; Blair, 2003) or, in some cases, by a sodium ion (Na^+) gradient (Imae and Atsumi, 1989; Asai *et al.*, 1997; Ito *et al.*, 2005). The flagellar motor has been studied extensively in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (Yamaguchi *et al.*, 1986; Kojima and Blair, 2004; Terashima *et al.*, 2008), and can be divided in two substructures, namely the rotor and stator. The rotor is composed of the FliG, FliM and FliN proteins and acts as a switch, determining the clockwise or counterclockwise rotation of the flagellum (Silverman and Simon, 1974; Francis *et al.*, 1994). The second structure is the stator, which is the stationary component of the motor within which the motor turns. The stator is composed of two integral membrane proteins, MotA and MotB, which form a complex with a ratio of

four MotA to two MotB proteins (Braun and Blair, 2001; Kojima and Blair, 2004). MotA has four transmembrane domains and the rest of the molecule is localized in the cytoplasm. A large cytoplasmic loop between the second and third transmembrane domains contains conserved charged residues that have been shown to interact with conserved charged residues in the rotor protein FliG, and their electrostatic interaction are important for torque generation (Zhou *et al.*, 1995; Lloyd and Blair, 1997; Zhou and Blair, 1997). The MotB protein has a single transmembrane domain at its N-terminus. This transmembrane domain contains an absolutely conserved negative charge residue (Asp32 in MotB of *E. coli*), which is critical for motor rotation and predicted to be the ion-binding site in the stator complex (Zhou *et al.*, 1998). Most of the MotB protein is located in the periplasmic space and the C-terminal portion of MotB contains a peptidoglycan-binding motif, which is thought to anchor the MotAB complex to the cell wall (Chun and Parkinson, 1988; De Mot and Vanderleyden, 1994). The MotAB complex is believed to conduct protons across the inner membrane and couples this proton transport to rotation of the motor (Kojima and Blair, 2001; Blair, 2003). Mutational analyses suggest that when protons cross the membrane, they bind to the conserved aspartate (Asp32) residue within MotB, causing a conformational change in the stator that drives the rotor through an elementary rotational step. This is followed by deprotonation of the Asp residue with release of the proton into the cytoplasm, and restoration of the stator to its original conformation (Zhou *et al.*, 1998; Kojima and Blair, 2001). It is estimated that there are at least 11 stator complexes associated with each motor (Reid *et al.*, 2006), but each complex apparently can act independently to generate torque (Block and Berg, 1984; Blair and Berg, 1988).

As mentioned above, flagellar rotation is powered by electrochemical gradients of either protons or Na^+ ions across the cytoplasmic membrane. *Bacillus subtilis*, however, possesses both a proton-driven MotAB stator and an Na^+-driven MotPS stator, but possesses only one set of flagellar genes so that both stators would have to interact with a single form of FliG (Ito *et al.*, 2004). In a subsequent study, Ito *et al.* (2005) assayed motility of wild-type *B. subtilis* and of mutant strains that expressed only one stator, either MotAB or MotPS, or that lacked both stators. *B. subtilis* mutant strains expressing a single stator were shown to be both flagellated and capable of motility under certain conditions. Flagellar motility of strains expressing only MotPS was stimulated by elevated pH and Na^+ concentrations, whereas strains expressing MotAB were motile at neutral pH and a low concentration of Na^+. Moreover, strains expressing MotAB or MotPS had an average of 11 flagella/cell. In contrast,

a statorless *motAB motPS* double mutant was non-motile, despite having an average of four flagella/cell. These results therefore demonstrate that both MotAB and MotPS of *B. subtilis* support motility under different conditions, and that the presence of Mot stators influences the number of flagella/cell.

Based on the above, it was therefore of importance to determine whether *B. cereus* ATCC 14579 also contained two different stators and, if so, which of these stators were in fact inactivated in the biofilm-impaired Mut21 strain. Subsequent analysis of the genome sequence of *B. cereus* ATCC 14579, available at both NCBI (GenBank: AE016877.1 GI:29899096) and the CereList webserver (http://bioinfo.hku.hk/GenoList), revealed that it may indeed possess a dual motility system. However, both BC4513 and BC1625 are annotated as encoding the chemotaxis MotA protein, while both BC4512 and BC1626 are annotated as encoding the chemotaxis MotB protein. Therefore, to determine whether the transposon that was mapped in the Mut21 strain resulted in the inactivation of the MotAB or MotPS stator, the above-mentioned four encoded Mot proteins of *B. cereus* ATCC 14579, as well as the MotA, MotB, MotP and MotS proteins of *B. subtilis* were analyzed by multiple sequence alignment using ClustalX (Higgins and Sharp, 1988; Larkin *et al.*, 2007). Based on the results obtained (Fig. 4.2), it appears that BC4513 and BC4512 encode a *B. subtilis* MotAB ortholog, while BC1626 and BC1625 encode a *B. subtilis* MotPS ortholog. Furthermore, NCBI BLASTp analysis of the FliG rotor protein of *B. subtilis* against the *B. cereus* proteome (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) revealed only one candidate for a rotor protein. It therefore appears that *B. cereus* has a dual motility system, similar to that of *B. subtilis*, where a single set of flagellar rotor proteins can interact with the two distinct stator-force generators to generate rotation under different ionic conditions.

To furthermore confirm that BC4513 and BC4512 encode a MotAB stator, the transcriptional organization of the *motAB* operon in *B. cereus* ATCC 14579 was compared to that of *B. subtilis*. The *B. subtilis* 168 *motAB* operon has been well-characterized and is known to be part of the σ^D regulon (Mirel *et al.*, 1992). In *motA* of *B. subtilis* the translational start codon is 34 nucleotides (nt) downstream from the transcription start site, and the ribosome binding site is located at position +16 to +21. The MotA and MotB proteins are present in two different reading frames that overlap by eight amino acids. The ribosome binding site of *motB* is located 10 nt upstream from the translational start codon, within the MotA open reading frame (ORF). A rho-independent termination signal is located nine nt downstream from the

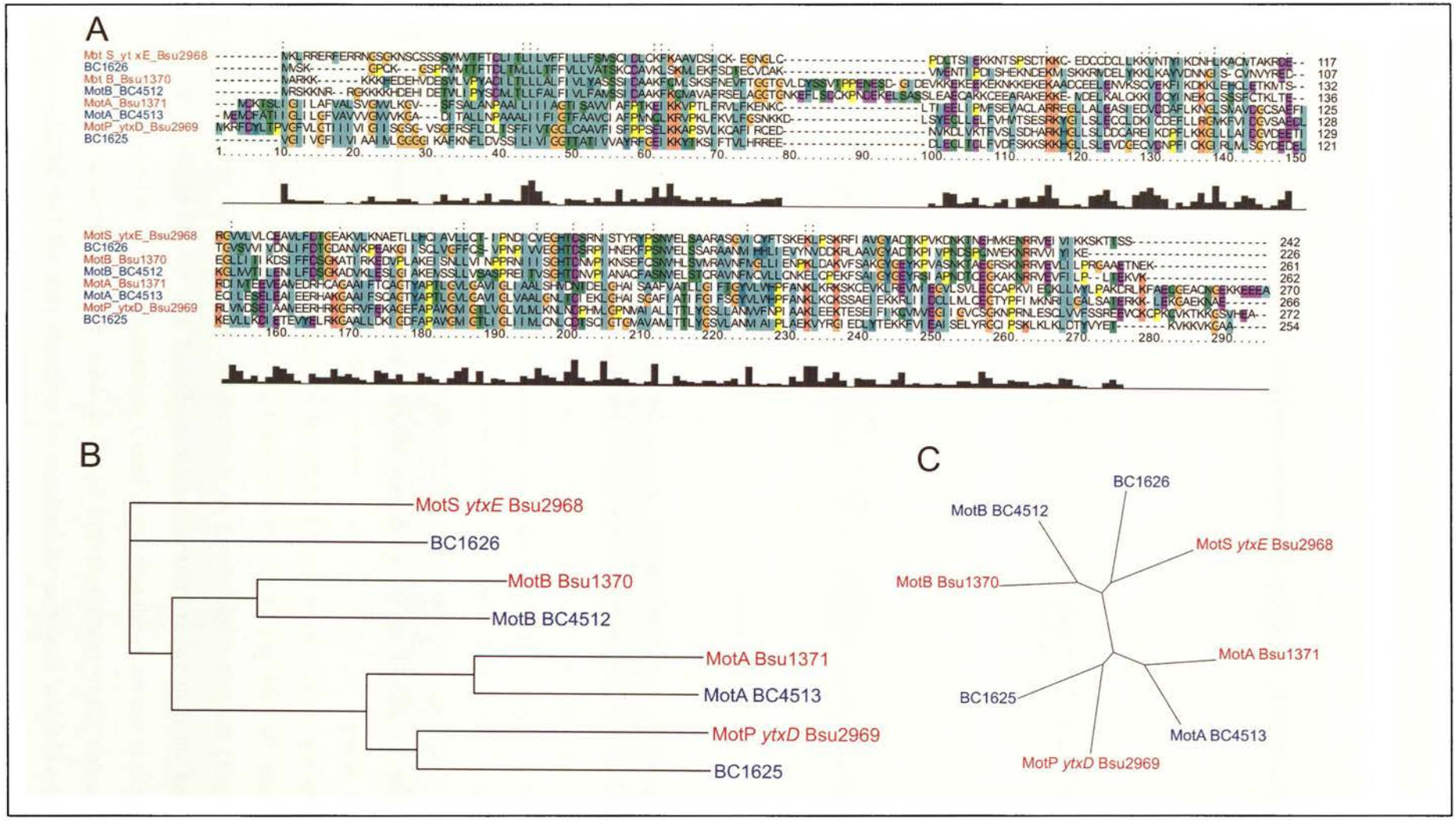

Figure 4.2: Alignment of amino acid sequences of the *B. cereus* ATCC 14579 stator components with the amino acid sequence of MotA, MotB, MotP and MotS of *B. subtilis*. Neighbor-joining bootstrap analysis with 1000 trials provided ≥99% bootstrap support for the branches indicated in both the phylogram shown in B, and the radial tree shown in C. The alignment, shown in A, was maximized by introducing gaps that are indicated by dashes. Abbreviations: MotS_ytxE_Bsu2968 is MotS, MotB_Bsu1370 is MotB, MotA_Bsu1371 is MotA and MotP_ytxD_Bsu2969 is MotP from *B. subtilis* 168 (GenBank accession number: NC_000964)

motB stop codon. Analysis regarding the transcriptional organization of the *motAB* operon of *B. cereus* ATCC 14579 indicated that the predicted MotA and MotB ORFs overlap by three amino acids in two different reading frames, and is followed by a rho-independent transcription termination signal immediately after the *motB* stop codon (Fig. 4.3). These results therefore not only provide further supporting evidence that BC4513 and BC4512 of *B. cereus* are organized in a single *motAB* operon similar to that found in *B. subtilis*, but also indicate that both MotA and MotB would be inactivated through insertion of the transposon into the promoter region of the *motAB* operon.

```
                                                        ***Lys
3'- aCUACAUUUUauuuucuccuaccguaagcgguaggagaaaauaaa -5'
    ..........((((((((((((((....))))))))))))))...
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Figure 4.3: Transcriptional organization of the *motAB* operon of *B. cereus* ATCC 14579. In the mRNA sequence depicted above, Lys is the C-terminal amino acid of MotB and the three asterisks represent the stop codon. The inverted repeat sequence is predicted to basepair to form a stem-loop structure and is indicated by brackets. The uracil-rich sequence, following the stem-loop structure, is indicated by uppercase characters. The analysis was performed using the FindTerm program available at www.softberry.com.

4.1.3 *B. cereus* requires flagella-mediated motility for biofilm formation

The requirement for flagella has emerged as a common theme in biofilm formation in several Gram-negative bacteria subjected to genetic analysis, e.g. *E. coli* (Pratt and Kolter, 1998), *Pseudomonas aeruginosa* (O'Toole and Kolter, 1998) and *Vibrio cholerae* (Watnick and Kolter, 1999). For each of these bacteria, mutations in genes involved in flagella-mediated motility hindered biofilm formation. There is some evidence to suggest that the same may be true for Gram-positive bacteria. Chagneau and Saier (2004) reported that mutants of *B. subtilis* that were defective in flagellar synthesis contained transposon insertions in genes involved in flagellar synthesis, namely *fliG* and *flhP*. Moreover, it has also been reported that a *B. subtilis* mutant strain lacking MotAB was defective in biofilm formation (Ito *et al.*, 2004). Notably, such MotAB mutant strains were shown to be capable of synthesizing flagella, but the flagella were unable to rotate (Mirel *et al.*, 1992; Ito *et al.*, 2004). In accordance with these results, the biofilm-impaired *B. cereus* MotAB mutant strain was shown to be non-motile, despite the presence of perithrichous flagella (Figs. 3.7 and 3.8). Therefore, it would appear that flagella-mediated motility rather than the mere presence of flagella is required for biofilm formation. Considering that the *B. cereus* biofilms in this study were allowed to develop at the solid-air-liquid interface under static culturing conditions, flagella-mediated motility may therefore be required for aerotaxis towards oxygen and/or for

establishing cell-surface contacts. Furthermore, quantitative real-time PCR experiments indicated that expression of the *motAB* operon was repressed at an early stage of *B. cereus* biofilm development (72 h after inoculation), indicating that once a biofilm phenotype has been established then high-level expression of *motAB* is no longer required and the operon is down-regulated.

4.2 Purine biosynthesis is required in *B. cereus* biofilm formation

4.2.1 *B. cereus pur* mutants are biofilm-impaired

Screening of the *B. cereus* ATCC 14579 transposon library yielded three mutants that were deficient in purine biosynthesis genes, *i.e.* Mut8, Mut9 and Mut18 (see below). Although the mutants were not growth-impaired, they did display severe biofilm-impairment. The Mut18 strain formed less than 15% of the biofilm biomass formed by the wild-type strain, whilst the Mut8 and Mut9 strains formed *ca.* 6.5% and 2.8% of the wild-type biofilm biomass, respectively (Fig. 3.2). The biofilms formed by the Mut8 and Mut18 strains had a similar appearance, but was distinct from that of the biofilm formed by the Mut9 strain, albeit that all three mutants formed much thinner biofilms than the wild-type strain (Fig. 3.6). The three purine biosynthesis genes were all up-regulated *ca.* 1.5-fold in the biofilm compared with planktonic populations (Fig. 3.10).

The transposon insertion in Mut8 mapped to *purL*, which encodes phosphoribosylformylglycinamidine synthase (FGAM synthetase). This enzyme catalyzes the formation of 2-(formamido)-N_1-(5-phospho-D-ribosyl)acetamidine from N_2-formyl-N_1-(5-phospho-D-ribosyl)glycinamide and L-glutamine, which is the fourth step in *de novo* purine biosynthesis (Hartman and Buchanan, 1959; Schendel *et al.*, 1989). In *B. subtilis* the FGAM synthetase comprises a complex of three proteins, *i.e.* PurS, PurL and PurQ (Ebbole and Zalkin, 1987; Saxild and Nygaard, 2000), and deletion of any one of the three corresponding genes results in a purine-auxotrophic phenotype. Mut18 contained the transposon insertion in *purC*, which encodes 5-aminoimidazole-4-(N-succinylcarboxamide) ribonucleotide (SAICAR) synthase. This enzyme catalyzes the formation of (S)-2-(5-amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxamido)succinate from 5-amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxylate and L-aspartate in purine biosynthesis (Hartman and Buchanan, 1959; Ebbole and Zalkin, 1987). Finally, the transposon insertion in Mut9 mapped to *purA*, which encodes adenylosuccinate synthetase that catalyzes the first committed step in the *de novo* biosynthesis

of AMP (Mantsala and Zalkin, 1992). Because of its essential role in purine biosynthesis and nucleotide salvage pathways, adenylosuccinate synthetases are present in all known organisms and tissues, with the exception of mature red blood cells (Mantsala and Zalkin, 1992; Honzatko and Fromm, 1999).

4.2.2 *B. cereus* requires extracellular DNA for biofilm formation

Extracellular polymeric substances (EPS) are biopolymers synthesized by microbes in which their biofilms are encased (Sutherland, 2001a; Flemming *et al.*, 2007). The EPS has various roles in biofilm physiology, including adhesion of biofilms to surfaces, sequestering of nutrients from the environment, and protection from environmental factors and predators (Allison *et al.*, 1998; Stewart and Costerton, 2001; Teitzel and Parsek, 2003). In the case of *Bacillus* spp., the composition of the extracellular polymeric matrix is not known, but studies of other biofilm bacteria have shown that polysaccharides are a major component of the EPS (Sutherland, 2001b; Allison, 2003; Vuong and Otto, 2008). Exopolysaccharide biosynthesis in *B. subtilis* biofilms is encoded by the 15-gene *epsA-O* operon, although the chemical nature of the polymer is not known (Kearns *et al.*, 2005). The only genes predicted to encode polysaccharide biosynthesis in *B. cereus* ATCC 14579 occur in a 17-gene operon (BC5263 to BC5279) and produce a putative galactose-containing polymer (Ivanova *et al.*, 2003). In addition to polysaccharides, biofilms may also contain proteins and nucleic acids (Allison, 2003; Tsuneda *et al.*, 2003; Allesen-Holm *et al.*, 2006; Flemming *et al.*, 2007). *B. subtilis* secretes the TasA protein, which occurs in the EPS and is required for biofilm formation (Branda *et al.*, 2006). The nucleic acid present in the EPS matrix has been termed extracellular DNA (eDNA), and was first shown to occur in the EPS of *P. aeruginosa* biofilms (Whitchurch *et al.*, 2002). It has since been shown that eDNA is required for the structural integrity of biofilms of Gram-positive pathogens such as *Staphylococcus epidermidis*, *Streptococcus pneumoniae* and *Staphylococcus aureus* (Moscoso *et al.*, 2006; Qin *et al.*, 2007; Izano *et al.*, 2008). To investigate the link between purine biosynthesis and biofilm formation, a collaborative study was undertaken with the group of Prof V.S. Brözel in which the *B. cereus* ATCC 14579 *purA*, *purC* and *purL* mutants were characterized in greater detail. The results of these studies have been published elsewhere (Vilain *et al.*, 2009), and are briefly summarized below.

Although purines are essential anabolites for DNA and RNA synthesis (Switzr *et al.*, 2002) and despite the *pur* mutants being auxotrophic for purines, the three *pur* mutants nevertheless grew planktonically in LB broth, without supplemental nucleotides, at a rate similar to that of the wild-type *B. cereus* strain. This result can be explained by the inclusion of yeast extract in the LB broth. Since yeast extract is an autolysate of *Saccharomyces cerevisiae* that contains ribonucleotides (Sezonov *et al.*, 2007), it is therefore the most likely source of purines that supplied the needs of these auxotrophic mutants. These results thus indicate that the exogenous nucleotide source in LB broth was sufficient for planktonic growth, but was not sufficient to support biofilm development of the respective *pur* mutants. Laser scanning confocal microscopy of *B. cereus* biofilms on glass wool, which had been stained with the nucleic acid stains Syto9 and propidium iodide (BacLight™), indicated that there were nucleic acids present in the cell-associated matrix. This was subsequently confirmed by the release of high-molecular-weight ethidium bromide-stained material when suspensions of biofilm cells were loaded into the wells of an agarose gel. The material was shown to be a substrate for DNases and RNases, indicating that it consisted mainly of DNA with some RNA also being present. In contrast to stationary-phase planktonic cells, which released very little eDNA, the early logarithmic-phase planktonic cultures released copious amounts of the eDNA. Indeed, glass wool exposed to exponentially growing planktonic populations of *B. cereus* acquired a DNA-containing conditioning film. The eDNA of biofilm and exponential-phase planktonic populations was found to be similar to chromosomal DNA by Southern blotting hybridization experiments. Notably, glass wool exposed to exponential- or stationary-phase cultures of the *purA* mutant, which was severely biofilm-impaired, did not contain detectable eDNA nor did they convey a DNA-containing conditioning film to the glass wool surface (Vilain *et al.*, 2009). Cumulatively, these results therefore indicate that not only are exponential-phase cells of *B. cereus* decorated with eDNA and that biofilm formation requires DNA as part of the EPS matrix, but also that adenylosuccinate synthetase (PurA) activity is required for biofilm formation.

4.3 A predicted ABC transporter, FtsEX, is required in *B. cereus* biofilm formation

4.3.1 *B. cereus ΔftsEX* is biofilm-impaired

The biofilm-impaired mutant strain Mut17 contained the transposon in the *ftsE* gene. However, *ftsE* is organized with *ftsZ* in one operon, and therefore the insertion in *ftsE* is polar onto *ftsX*. The mutant strain displayed a growth rate similar to wild-type *B. cereus*, but formed

ca. 58% of the wild-type biofilm biomass (Fig. 3.2). The morphology of the mutant biofilm resembled that of the wild-type, except that it was thinner and more fragile than the wild-type biofilm (Fig. 3.6). Expression of the *ftsE* gene was up-regulated 4.1-fold in the biofilm compared with planktonic populations (Fig. 3.10). Phenotypic assays indicated no noticeable differences between the cell morphology of mutant and wild-type *B. cereus* cells.

4.3.2 FtsEX is required for divergent activities in different bacteria

- **FtsEX is needed for cell division in *E. coli***

In *E. coli*, cell division involves the formation and subsequent constriction of a septum in the middle of the cell. Genetic studies have identified several conditional lethal mutants that fail to septate at the non-permissive temperature, thus resulting in elongated cells that filament and die. These mutants have been designated *fts* (filamentation temperature sensitive phenotype) (Donachie, 1993). Among the *fts* genes, the *ftsA*, *ftsQ* and *ftsZ* genes have been studied the most extensively and were reported to play an essential role in septation and cell division (Donachie, 1993; Lutkenhaus and Addinall, 1997; Errington *et al.*, 2003). In addition to the above *fts* gene cluster, a second *fts* gene cluster is located at a different position in the *E. coli* genome and consist of three genes, i.e. *ftsY*, *ftsE* and *ftsX*, which are organized in one operon (Gill *et al.*, 1986; de Leeuw *et al.*, 1999). FtsE and FtsX can interact and form a complex in the inner membrane that displays characteristics common to ATP-binding cassette (ABC) transporters (Gill and Salmond, 1987; de Leeuw *et al.*, 1999). The ABC transporters are integral membrane proteins that derives energy from the hydrolysis of ATP to actively transport a wide variety of substrates either into or out of the cells (Moussatova *et al.*, 2008). In contrast, FtsY functions in the targeting of proteins to the *E. coli* inner membrane as the receptor of the signal recognition particle (SRP) (Luirink *et al.*, 1994; Egea *et al.*, 2005). It should, however, be noted that it is only in *E. coli* and *Haemophilus influenzae* that the *ftsEX* gene cluster is preceded by the *ftsY* gene. In all other bacterial species of which the genome sequences are available, the *ftsY* homologues are present but encoded by separate genes (de Leeuw *et al.*, 1999; Ivanova *et al.*, 2003).

Initial reports have characterized *ftsE* and *ftsX* as essential genes involved in *E. coli* cell division (Salmond and Plakidou, 1984; Gill *et al.*, 1986; Taschner *et al.*, 1988). However, the involvement of FtsEX in cell division appears controversial, in part because the mutants did not show extensive filamentation in minimal medium, and also because the division defects in

ftsEX mutants are often salt remedial (de Leeuw *et al.*, 1999; Schmidt *et al.*, 2004) and osmoremedial (Reddy, 2007). Considering the homology of FtsEX to ABC transporters, attempts have been made to identify potential FtsEX substrates. An insertion mutant of *ftsE* was shown to be filamentous and required high concentrations of salt (1% [w/v]) for its viability. Therefore, *ftsE* was considered a conditional salt-dependent essential gene and was implicated in cell division and/or salt transport (de Leeuw *et al.*, 1999). In this regard, it is also interesting to note that *ftsE*(Ts) mutants were shown to be defective in translocating potassium pump proteins into the cytoplasmic membrane at the restrictive temperature, leading to the suggestion that FtsE could participate in protein translocation (Ukai *et al.*, 1998). However, Arends *et al.* (2009) was of the opinion that in both of these reports "the data were not compelling and that a connection to cell division, if any, was not obvious".

In an attempt to clarify the role of the FtsE and FtsX proteins in *E. coli* cell division, Schmidt *et al.* (2004) performed a comprehensive study in which it was shown that FtsE and FtsX localize to the septal ring and thus indicates that they may participate directly in the division process. Moreover, septal ring assembly in a *ftsEX* mutant was shown to be normal in LB medium that contained 1% NaCl, but was defective upon a shift to LB medium that lacked NaCl. It was also shown that in cells depleted of FtsEX, septal ring assemblies contained the early division proteins FtsZ, FtsA and ZipA, but lacked the late proteins FtsK, FtsQ, FtsL, FtsJ and FtsN (Schmidt *et al.*, 2004). Although these results therefore implicate FtsEX as contributing to cytokinesis by improving the assembly and/or stability of the septal ring, the mechanism by which these proteins contribute to septal ring assembly has not yet been elucidated. Subsequently, it was proposed by the same group that it probably involves protein-protein interactions (Arends *et al.*, 2009). This was based on previous studies indicating that FtsX interacts with FtsA and FtsQ in a bacterial two-hybrid system (Karimova *et al.*, 2005), and that FtsE interacts with FtsZ in a co-precipitation assay (Corbin *et al.*, 2007). Interestingly, based on the characterization of mutant FtsE proteins with site-directed mutations in the ATP-binding site and topology analysis of the FtsX protein, Arends *et al.* (2009) furthermore noted that FtsEX might not actually be a transporter at all. It was demonstrated that although the mutant FtsE proteins supported septal ring assembly (when produced together with FtsX) the rings constricted poorly, thus suggesting that FtsEX uses ATP to facilitate constriction rather than assembly of the septal ring. Moreover, topography analysis revealed that FtsX has four transmembrane domains. However, none of these domains contain a charged amino acid and is therefore inconsistent with the idea that FtsEX

could function as a transporter of ions or charged molecules. These results led Arends *et al.* (2009) to conclude that FtsEX is not really a transporter, but that it probably hydrolyzes ATP to support cell division. Cumulatively, the results from these different studies indicate that FtsEX, in the case of *E. coli*, could be important for improving constriction rather than, or in addition to, septal ring assembly.

- **FtsEX is needed for cellular differentiation in *B. subtilis***

During the course of sporulation, the *B. subtilis* cells undergo morphological changes of which the most characteristic is the formation of an asymmetrically positioned (polar) septum that divides the developing cell (sporangium) into the forespore and the mother cell (Stragier and Losick, 1996). Spo0A is the key transcriptional regulator of sporulation (Grossman, 1995) and the activated form of Spo0A (Spo0A~P) directly controls the transcription of more than 120 genes (Liu *et al.*, 2003; Molle *et al.*, 2003; Fujita *et al.*, 2005). Despite a wealth of knowledge regarding the process of sporulation in *B. subtilis* (Stragier and Losick, 1996; Errington, 2003; Setlow, 2003; Piggot and Hilbert, 2004), very little is known regarding the specific signals required for its initiation or about the cellular components channeling them.

Recently, Garti-Levi *et al.* (2008) reported on the characterization of a *B. subtilis* FtsEX mutant, which was shown to form a medial septum instead of a polar septum upon a shift to sporulation conditions. Nevertheless, a polar septum was eventually formed by the FtsEX mutant sporangia, which were capable of generating mature and functional spores. These results therefore suggest that FtsEX ensures accurate temporal polar division at the onset of sporulation. Data was subsequently presented that indicated that FtsEX is required for efficient activation of Spo0A at the onset of sporulation. Specifically, FtsEX was shown to act upstream of the sporulation phosphorelay that ultimately leads to the phosphorylation of Spo0A. These results suggested that FtsEX may therefore form an ABC transporter that stimulates the sporulation phosphorelay either by importing or secreting an extracellular signal. Subsequent localization studies indicated that both FtsE and FtsX localized to the cell membrane and that its localization was neither restricted to the division septum nor dependent on the FtsZ cell division protein. Furthermore, co-culturing of the mutant FtsEX cells with wild-type *B. subtilis* cells indicated that the FtsEX mutant phenotype could not be complemented extracellularly, suggesting that FtsEX could function as an importer, rather than an exporter, of an as yet unidentified sporulation signal (Garti-Levi *et al.*, 2008).

Therefore, in contrast to *E. coli*, FtsEX of *B. subtilis* does not appear to play a role in cell division but rather appears to regulate entry into sporulation through its action as an ABC transporter that is responsible for importing a extracellular sporulation signal.

4.3.3 *B. cereus* biofilm formation is facilitated by FtsEX

Based on the preceding sections, it appears that FtsEX mediates divergent activities in *E. coli* and *B. subtilis*. Whether FtsEX of *B. cereus* functions in manner similar to that in *E. coli* or *B. subtilis* remains to be determined, but the available evidence suggests the latter. Similar to *B. subtilis*, but in contrast to *E. coli*, inactivation of FtsEX of *B. cereus* did not result in inhibition of cell division and growth, and also did not cause the formation of filamentous cells. In fact, the *B. cereus* FtsEX mutant cells were viable, and displayed a growth rate similar to that of the wild-type strain and grew to the same density as the wild-type (Fig. 3.5, Mut 17). Moreover, the mutant cells did not have any morphological defects that could be suggestive of impaired division (Fig. 3.9).

Since the emphasis of this study was on the identification of genes involved in biofilm formation by *B. cereus*, the effect of FtsEX on sporulation was not investigated. Nevertheless, based on the similarities between FtsEX mutants of *B. cereus* and *B. subtilis*, it is likely that FtsEX of *B. cereus* could function as an importer of an extracellular signal that ultimately results in phosphorylation of Spo0A, as has been reported for *B. subtilis* (Garti-Levi *et al.*, 2008). This is of particular interest, as previous studies have indicated that *spo0A* mutants of *B. subtilis* failed to form biofilms (Branda *et al.*, 2001; Hamon and Lazazzera, 2001). Of special significance, however, is that both SinR and AbrB are controlled by Spo0A (Chu *et al.*, 2008). SinR and AbrB act in parallel to control expression of the *epsA-O* and *yqxM-sipW-tasA* operons, which, respectively, encode the exopolysaccharide and TasA protein components of the extracellular matrix of *B. subtilis* biofilms (Kearns *et al.*, 2005; Branda *et al.*, 2006). Notably, cells lacking both these matrix components are unable to form biofilms (Branda *et al.*, 2006). Activation of Spo0A, however, has been reported to set in motion events (induction of the antirepressor gene *sinI* and repression of *abrB*) that relieve SinR- and AbrB-mediated repression of the *epsA-O* and *yqxM-sipW-tasA* operons, thus resulting in their expression and the formation of biofilms (Chu *et al.*, 2008). Therefore, a consequence of FtsEX importing an extracellular signal that results in activation of Spo0A (Garti-Levi *et al.*,

2008), is that it may indirectly affect biofilm formation by allowing expression of the extracellular matrix components required for biofilm formation.

If FtsEX functions by importing an extracellular signal that leads to activation of Spo0A, it would then be expected that in the absence of FtsEX, biofilm formation would be abrogated. However, the *B. cereus* FtsEX mutant was capable of forming a biofilm, albeit thinner and more fragile than the biofilm of the wild-type strain. This raises the possibility that multiple transporters exist, which import different extracellular signals that are capable of leading to activation of Spo0A. In support of this, mutation in the *ybdA* gene encoding the ATPase subunit of a putative *B. subtilis* ABC transporter has been reported to reduce Spo0A activation (Isezaki *et al.*, 2001), whilst the *B. subtilis* ABC transporter composed of YheH and YheI proteins has been shown to bind to KinA and to affect Spo0A activation (Fukushima *et al.*, 2006). Taken together, these studies corroborate that several ABC transporters (and accordingly, probably an assortment of signals) are involved in activating Spo0A via induction of phosphorelay components. The increased fragility of the *B. cereus* FtsEX biofilm may thus be due to reduced production of extracellular matrix components, which are required to form and maintain the structural integrity of the biofilm (Branda *et al.*, 2001; Branda *et al.*, 2006). Intriguingly, this would suggest that the extracellular signals imported by ABC transporters other than FtsEX are not as efficient in activating Spo0A compared to that imported by FtsEX. This lower level of Spo0A activation would be expected to relieve SinR- and AbrB-mediated repression of the *epsA-O* and *yqxM-sipW-tasA* operons to a lesser extent, thus resulting in the synthesis of reduced amounts of the extracellular matrix components, and therefore resulting in more fragile biofilms.

4.4 Different hydrolase enzymes may be required in *B. cereus* biofilm formation

Screening of the *B. cereus* ATCC 14579 transposon library yielded a mutant, namely Mut16, that formed a slightly thinner biofilm than the wild-type strain and a curtain of cells descending from the biofilm, which characterized the wild-type biofilm, was largely absent in the mutant biofilm (Fig. 3.6). The mutant strain formed *ca.* 18% less biofilm biomass than the wild-type *B. cereus* strain (Fig. 3.2), but displayed a growth rate similar to the wild-type strain. Cloning and sequencing of the chromosomal DNA flanking the transposon insertion in the Mut16 strain revealed that the transposon was inserted between two divergently transcribed genes, *i.e.* BC5133 encoding a murein hydrolase exporter and BC5134 encoding

an inosine-uridine preferring nucleoside hydrolase (Fig. 3.4). Expression of both these genes was up-regulated *ca.* 4-fold in the biofilm compared with planktonic populations (Fig. 3.10).

4.4.1 Inosine-uridine preferring nucleoside hydrolases play a role in purine salvage in protozoan parasites, and spore germination in *B. thuringiensis*

The BC5134 encoded protein of *B. cereus* ATCC 14579 has been annotated as an inosine-uridine preferring nucleoside hydrolase (Ivanova *et al.*, 2003). Subsequent NCBI BLASTp analysis revealed that it displayed highest homology to protozoan nucleoside hydrolases, notably a non-specific nucleoside hydrolase of *Leishmania major* (49% similarity, 26% identity) and an inosine-uridine preferring nucleoside hydrolase of *Tetrahymena thermophila* (46% similarity, 26% identity). Protozoan parasites lack a *de novo* biosynthetic pathway for purine synthesis and therefore rely exclusively on purine salvage from the host for DNA and RNA synthesis (Degano *et al.*, 1996; Gopaul *et al.*, 1996). The inosine-uridine preferring nucleoside hydrolase is the most abundant nucleoside hydrolase present in these organisms. Although it prefers inosine and uridine as substrate, the enzyme is also capable of catalyzing the hydrolysis of both purine and pyrimidine nucleosides (Parkin *et al.*, 1991a).

More recently, based on N-terminal sequencing of proteins from purified exosporium, it has been reported that a nucleoside hydrolase is tightly adsorbed to the exosporia of *B. cereus* and *B. anthracis* (Steichen *et al.*, 2003; Todd *et al.*, 2003; Redmond *et al.*, 2004). The exosporium is the outermost layer of spores of these pathogens and it represents the surface layer that makes initial contact with the host (Desrosier and Lara, 1984). In contrast, *B. subtilis* has no clearly defined exosporial layer and consequently, its exosporium has not been studied in great detail (Errington, 2003; Setlow, 2003). In nature, spores germinate in response to nutrient germinants that are typically amino acids, sugars or purine nucleosides (Setlow, 2003). Amongst nutrient germinants, inosine has been reported to be the best germinant for *B. cereus* and *B. thurigiensis* spores. Inosine is also an important co-germinant in *B. cereus*, *B. anthracis* and *B. thurigiensis* spore germination (Foerster and Foster, 1966; Barlass *et al.*, 2002; Hornstra *et al.*, 2005). Inosine-initiated spore germination starts with the interaction of inosine with its corresponding receptors, *i.e.* GerI, GerQ and GerR. The spores then undergo the release of dipicolinic acid and cations, hydrolysis of the peptidoglycan cortex, germ-cell wall expansion and finally, resumption of vegetative growth (Setlow, 2003; Hornstra *et al.*, 2006).

Recently, the importance of an exosporium-associated uridine preferring nucleoside hydrolase with regards to spore germination was investigated (Liang *et al.*, 2008). A mutant *B. thurigiensis* strain in which a homologous gene (designated *iunH*) to the putative nucleoside hydrolase previously identified from the exosporium of *B. cereus* and *B. anthracis*, was insertionally disrupted. Disruption of the *iunH* gene did not affect vegetative growth and sporulation of the mutant strain. However, it did result in an increase in the inosine- or adenosine-induced germination rate, whereas overexpression in a complemented strain resulted in a significant decrease of its germination-triggering capacity (Liang *et al.*, 2008). Therefore, these results indicate that the amount of inosine or adenosine acting as germinant can be modulated by the activity of IunH, thus allowing for modulation of the inosine- or adenosine-induced germination of *B. thurigiensis* spores.

4.4.2 Murein hydrolases are involved in eDNA release and biofilm formation

The BC5133 encoded protein of *B. cereus* ATCC 14579 has been annotated as a murein hydrolase exporter (Ivanova *et al.*, 2003). Murein hydrolases, also referred to as autolysins, are a unique family of enzymes that specifically cleave structural components of the bacterial cell wall. They have been shown to participate in a number of important biological processes during cell growth, including cell wall growth, as well as peptidoglycan recycling and turnover (Rice and Bayles, 2008). Moreover, the simultaneous disruption of multiple murein hydrolase genes in *B. subtilis* 168 has been shown to have little effect on bacterial growth, but resulted in the inability of the cells to separate, thus indicating that murein hydrolases are required for daughter cell separation after completion of the newly formed septum (Blackman *et al.*, 1998).

Murein hydrolases have been implicated in biofilm formation of different Gram-positive bacteria. It has been reported that disruption of the *atlE* and *atlA* genes, which encode the primary murein hydrolase of *S. epidermidis* and *S. aureus*, respectively, causes a dramatic decrease in their abilities to form biofilm on polystyrene surfaces (Heilmann *et al.*, 1997; Biswas *et al.*, 2006). Likewise, inactivation of the gene encoding an Atl homologue in *S. mutans*, namely AtlA, also reduced its biofilm-forming ability (Shibata *et al.*, 2005; Ahn and Burne, 2006). The mutant biofilms were reported to have a sponge-like architecture with large gaps and contained 30% less biomass than those formed by the wild-type strain (Shibata *et al.*, 2005). It was subsequently reported that the number of proteins and amount of protein

extractable from the surface of the *S. mutans* AtlA-deficient strains were dramatically reduced, suggesting that the presence of AtlA results in modifications of the cell surface that enhance the capacity to form biofilms (Ahn and Burn, 2006). Recently, the major autolysin (Atn) of *Enterococcus faecalis* was also reported to be required for biofilm formation under static and hydrodynamic conditions (Guiton *et al.*, 2009). Specifically, Atn-deficient mutants were shown to be delayed in biofilm development due to defects in primary adherence and DNA release. The latter was also shown to be an important structural component of the *E. faecalis* biofilms and to be crucial for the growth, maturation and structural stability of the biofilms (Guiton *et al.*, 2009).

Analysis of the BC5133 encoded protein of *B. cereus* with the NCBI conserved domain database tool (Marchler-Bauer and Bryant, 2004) identified the protein as a holin-like protein, similar to the CidA protein of *S. aureus*. In the case of *S. aureus*, the *cidA* gene has been shown to encode a holin protein that is an effector of murein hydrolase activity and cell lysis (Rice *et al.*, 2003). Recently, the *cidA* gene was shown to promote cell lysis and the release of DNA during the development of a biofilm (Rice *et al.*, 2007; Mann *et al.*, 2009). In both static and flow-cell biofilm assays, the biofilm produced by a *S. aureus* CidA mutant was more loosely compacted and less adherent to the substrate compared to the wild-type strain. Treatment of wild-type *S. aureus* biofilm with DNase I caused extensive cell detachment that resulted in its destabilization, while a similar treatment of the lysis-defective CidA mutant biofilm had a minimal effect. Furthermore, quantitative real-time PCR experiments demonstrated the presence of 5-fold less genomic DNA in the mutant biofilm relative to the wild-type biofilm. These results therefore indicate that CidA-mediated cell lysis, with the concomitant release of genomic DNA, plays a significant role during *S. aureus* biofilm development.

4.4.3 Additional evidence for a link between *B. cereus* eDNA and biofilm formation

The transposon in the *B. cereus* biofilm-impaired mutant strain Mut16 was inserted between two divergently transcribed genes, which, respectively, encode an inosine-uridine preferring nucleoside hydrolase and murein hydrolase exporter. Due to the location of the transposon it is not possible to determine whether the biofilm-impaired phenotype is the result of inactivation of a single gene or both genes. To distinguish, knockout mutants for each of these two genes would be required to clarify the role of these proteins in *B. cereus* biofilm

formation. Nevertheless, it appears that both of these proteins are required in the *B. cereus* biofilm population as their expression was up-regulated *ca.* 4-fold relative to the planktonic populations.

Despite having been implicated in modulating inosine-induced spore germination (Liang *et al.*, 2008), it is tempting to speculate that the nucleoside hydrolase may play a role in nucleotide salvage during biofilm development. As indicated above, expression of the *B. cereus purA*, *purC* and *purL* genes were up-regulated in biofilm populations, indicating an elevated requirement for purines in the biofilm population. Moreover, a *B. cereus purA* mutant biofilm lacked eDNA and it did not only show a diminished capacity to form biofilms, but also displayed an altered biofilm morphology compared to the biofilm of the wild-type strain. The biofilm-deficiency seen in the Mut16 strain may thus point to the possibility that nucleotide salvage from the LB broth, which contains ribonucleotides derived from a yeast autolysate, is involved in eDNA synthesis and thus biofilm formation.

The transposon present in the genome of the *B. cereus* mutant strain Mut16 may also have influenced expression of the BC5133 gene. This gene, together with the downstream BC5132 gene, form an apparent operon of which the encoded proteins have been annotated as a murein hydrolase exporter and murein hydrolase regulator, respectively (Ivanova *et al.*, 2003). Despite having been annotated as an exporter of murein hydrolase, the NCBI conserved domain database tool identified the BC5133 encoded protein as a holin-like protein. Holins are phage-encoded small integral membrane proteins that control the activity of murein hydrolases and timing of host cell lysis during bacteriophage infection (Rice and Bayles, 2008). Based on structural analyses, sequence homology and gene organization, the putative BC5133-5132 operon appears to be an ortholog of the *S. aureus cidAB* operon (Fig. 4.4). Not only does the *B. cereus* BC5133 encoded protein display 25% sequence identity and 62% sequence similarity to the CidA protein of *S. aureus* MW2, but it also has the structural features of a holin. These include a small size (124 amino acids), four membrane-spanning domains, a hydrophobic N-terminus, and a highly polar, charge-rich C-terminal domain (Fig. 4.4) (Young and Blasi, 1995). Recent studies in *S. aureus* have shown that CidA functions as a holin (Rice *et al.*, 2003), and promotes cell lysis and the release of DNA during the

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CidA            MHKVQLIIKLLLQLGIIIVITYIGTEIQKIFHLPLAGSIVGLFLFYLLLQFKIVPLTWVE 60
BC5133          --MNMKFTKILVQIAALYVFYMVGTWVQEMLNIPIPGSLIGMFLLLVLLSLKVLPVKWFD 58
                      : *:*:*:. : *:  :** :*::::*:.**::*:**: :**.:*::*:.*.:

CidA            DGANFLLKTMVFFFIPSVVGIMDVASEITLNYILFFAVIIIGTCIVALSSGYIAEKMSVK 120
BC5133          LGAETLVAIMPFLLIPPTLGLMNYGAFFMSKGISLFITVVASTFLIIIVAGHTGQYLANR 118
                 **: *:  * *::**..:*:*: .: :  : * :* .:: .* :: : :*: .: :: :

CidA            HKHRKGVDAYE 131
BC5133          KERESR----- 124
                ::::..
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Figure 4.4: Clustal (Larkin *et al.*, 2007) alignment of amino acid sequences of *B. cereus* ATCC 14579 BC5133 and *S. aureus* MW2 CidA. The shaded sequences represent putative transmembrane helixes predicted by TMpred (Hofmann and Stoffel, 1993). Amino acids depicted in red are small and hydrophobic. Blue indicates acidic amino acids; magenta denotes basic amino acids while green denotes hydroxyl, amine and basic amino acids. Identical amino acids are indicated by a "*" and similar amino acids are marked by a ":", while semi-conserved substitutions resulting in amino acids with a similar steric conformation are indicated by a ".".

development of a biofilm (Rice *et al.*, 2007). In these studies, cell lysis and DNA release were found to be critical for biofilm attachment during the initial stages of development and the released DNA remained an important matrix component during biofilm maturation. Since biofilm formation by *B. cereus* has been shown to require eDNA as part of the EPS matrix (see above; Vilain *et al.*, 2009), it is tempting to speculate that the BC5133 encoded protein may have a function analogous to CidA, *i.e.* mediating cell lysis and thereby providing a source of eDNA for use in the biofilm matrix. However, before such a conclusion can be reached, further research involving the construction and characterization of mutant strains with inactivated BC5133 and BC5132 genes are required.

4.5 Proteins of unknown function are required in *B. cereus* biofilm formation

During screening of the *B. cereus* ATCC 14579 transposon library, two biofilm-impaired mutants were identified in which the transposon was mapped to different genes encoding putative proteins. In the first of these mutants, Mut13, the transposon was inserted in a gene encoding a small conserved hypothetical protein (BC0172) that is only found in *Bacillus* spp. The predicted protein comprises 72 amino acids and has a theoretical molecular mass of 9.1 kDa. The Mut13 strain formed *ca.* 16% less biofilm biomass than the wild-type strain (Fig. 3.2), despite the mutant biofilm being very similar in appearance to that formed by the wild-type *B. cereus* strain (Fig. 3.6). Expression of the gene was up-regulated 3.5-fold in the biofilm compared with planktonic populations (Fig. 3.10).

With regards to the second mutant, Mut5, the transposon was inserted in a gene encoding a hypothetical membrane spanning protein (BC5437). Subsequent NCBI BLASTp analysis indicated that the putative protein is widely conserved in Gram-positive bacteria, especially amongst members of the genera *Bacillus* and *Listeria*. The NCBI conserved domain database tool (Marchler-Bauer and Bryant, 2004) revealed the presence of a transmembrane domain in the *B. cereus* BC5437 encoded protein. Although this domain is found in putative permeases and predicted transmembrane proteins, it has no known function. Nevertheless, the putative protein appears to be required for *B. cereus* biofilm formation as evidenced by the mutant strain forming *ca.* 44% less biofilm biomass than the wild-type (Fig. 3.2). Moreover, a curtain of cells descending from the biofilm, which characterized the wild-type biofilm, was almost completely absent from the mutant biofilm (Fig. 3.6). Expression of this gene was up-regulated *ca.* 4.3-fold in biofilm compared with planktonic populations.

The lack of studied homologues to the above-mentioned two *B. cereus* genes precluded further characterization of these proteins with regards to a possible function in *B. cereus* biofilm formation. Consequently, their role in *B. cereus* biofilm formation remains to be elucidated.

CHAPTER FIVE
CONCLUDING REMARKS

Bacillus cereus is best known as the etiological agent of two distinct food poisoning syndromes (Granum and Lund, 1997). More recently, it has emerged as a potentially important pathogen, causing a variety of rare but severe infections that resemble pulmonary and gastrointestinal anthrax (Hoffmaster *et al.*, 2006; Klee *et al.*, 2006). *B. cereus* is capable of forming biofilms on various surfaces and is of great concern in food processing environments where it can lead to decreased food quality and safety (Lindsay *et al.*, 2000; Flint *et al.*, 1997). Towards a more comprehensive understanding of biofilm formation by *B. cereus*, the aim of this investigation was essentially to identify genes that may play a role in this developmental process. The new information that has evolved during the course of this investigation is summarized briefly in the sections below and some suggestions regarding future research will be made.

In order to identify candidate-biofilm genes, *B. cereus* ATCC 14579 was subjected to transposon mutagenesis and a collection of 3 500 mutants from five independent libraries was screened for their ability to form a biofilm. During the course of this study, a novel screening method was developed that greatly facilitated high-throughput screening of the large number of *B. cereus* transposon mutants. This approach overcomes many of the inherent limitations associated with the conventional approach in which biofilms are allowed to form in the wells of microtitre plates (O'Toole and Kolter, 1998; O'Toole *et al.*, 1999). The most significant of these limitations are that the conventional method does not take into account that not all bacterial biofilms develop preferentially on submerged surfaces (Wijman *et al.*, 2007), and the method-itself results in variable loss of the biofilm during the washing and fixing steps that may lead to false positive results. Using the screening assay described in this study, eight biofilm-impaired mutants of *B. cereus* were identified. This represents an improvement over the study of Chagneau and Sair (2004) in which only four *B. subtilis* biofilm-defective mutants were identified from a collection 5 000 transposon insertion mutants using the traditional microtitre plate-based assay.

Amongst the eight biofilm-impaired mutants of *B. cereus* identified in this study, two contained transposon insertions in genes (BC0172 and BC5437) encoding putative proteins of unknown function. Interestingly, results of quantitative real-time PCR assays indicated that of

the different genes analyzed, these two genes were of the most highly up-regulated genes in the *B. cereus* biofilm population. Furthermore, their requirement in biofilm formation appears to be limited to Gram-positive bacteria only, as both proteins were found to occur in primarily *Bacillus* spp. However, due to the lack of characterized homologues, their function in *B. cereus* biofilm formation remains to be clarified. In a third biofilm-impaired mutant the transposon was inserted between two divergently transcribed genes and consequently, it is unclear whether the biofilm-impaired phenotype is due to inactivation of one or both of these genes. Further experiments involving the use of mutant strains from which the individual genes have been inactivated is required to distinguish between these different possibilities. Nevertheless, it is interesting to note that both genes, encoding a nucleoside hydrolase and a putative regulator of murein hydrolase activity (based on structural and sequence homology analyses), respectively, were highly up-regulated (*ca.* 4-fold) in biofilm populations, indicating a need for both proteins in the biofilm population.

Results obtained during the course of this study indicated that flagella-mediated motility is important during the early stages of *B. cereus* biofilm development. In this study, a *B. cereus motAB* mutant was shown to produce paralyzed flagella, thus resulting in impaired biofilm formation. Based on bioinformatic analyses, it was, however, also shown that *B. cereus* possesses a MotPS stator, in addition to the MotAB stator. Consequently, since only the *motAB* operon was inactivated, the contribution of MotPS, if any, in *B. cereus* biofilm development has yet to be determined. In this regard, it is important to note that the *B. cereus* biofilms were allowed to form in LB broth, which had a pH of 7.2 and > 86 mM NaCl. Under this Na^+ and pH profile, it has been reported that *B. subtilis* MotAB forms the torque-generating unit of a H^+-driven flagellar motor, whereas the opposite optima (high pH and high Na^+) allows formation of the Na^+-driven flagellar motor (Ito *et al.*, 2005). Therefore, it is possible that only MotAB-dependent biofilm formation was assayed in this study. This may be of particular interest since it would imply that the contribution of MotPS-dependent biofilm formation may be modest upon mutational loss of MotAB from the wild-type *B. cereus* strain. However, to determine whether this is indeed the case, further experiments using *B. cereus* strains with targeted mutations in *motAB* and *motPS*, and cultured under different conditions (pH and NaCl) are required.

In this study, it was shown that *B. cereus* biofilm formation is dependent on the presence of purine biosynthesis genes, as was evidenced by the impaired biofilm formation of three

mutants deficient in the *purA*, *purC* and *purL* purine biosynthesis genes. Moreover, these three genes were up-regulated in biofilm relative to planktonic cells, indicating an elevated requirement for purines in biofilm cells. In an extension of these studies, it was also shown that extracellular DNA (eDNA) is an integral component of the EPS of *B. cereus* biofilms (Vilain *et al.*, 2009). These results are in agreement with previous reports indicating that eDNA is present in the EPS matrix of biofilms of different Gram-negative bacteria (Whitchurch *et al.*, 2002; Allesen-Holm *et al.*, 2006; Thomas *et al.*, 2008), as well as Gram-positive cocci (Moscoso *et al.*, 2006; Qin *et al.*, 2007; Izano *et al.*, 2008). However, the report on *B. cereus* constitutes the first report of a Gram-positive rod whose biofilms contain eDNA. Despite its importance in *B. cereus* biofilm formation, the origin and mechanism of eDNA release remains to be determined. In *P. aeruginosa*, a paradigm organism for the study of Gram-negative bacterial biofilms, eDNA release depends on quorum sensing (Allesen-Holm *et al.*, 2006), and there is evidence to suggest that cell lysis of a sub-population of the biofilm through prophage induction within the biofilm may contribute to the release of eDNA (Webb *et al.*, 2003; Allesen-Holm *et al.*, 2006). Moreover, the presence of eDNA in the EPS matrix may also be as a consequence of the release of membrane vesicles that contain DNA (Kadurugamuwa and Beveridge, 1995; Renelli *et al.*, 2004). In *S. aureus* and *S. pneumoniae*, DNA is released from a lysing sub-fraction of the bacterial population and the actions of both bacteriocins and autolysins have been implicated in the release of genomic DNA (Kreth *et al.*, 2005; Rice *et al.*, 2007; Mann *et al.*, 2009). In contrast, several lines of evidence argue against cell lysis as being responsible for the presence of eDNA in the biofilm matrix of *B. cereus* biofilms. In the case of *B. cereus*, the experiments that were performed relied on the use of exponentially growing populations that were prepared by washing cells from an exponentially growing culture in order to eliminate the possibility that eDNA that originated from previously lysed cells was transferred with the inoculum. Also, biofilm cells showed green fluorescence in the cytosol upon staining with BacLight™, indicating that they were alive. Furthermore, protein gel electrophoresis of concentrated culture supernatant did not indicate detectable levels of cytosolic proteins, thus also arguing against lysis. Moreover, the bulk of eDNA detected was not free-floating in the culture broth, as would be expected in the case of lysed cells, but was rather cell-associated. Finally, the *B. cereus purA* mutant strain did not show any cell-associated or free DNA in planktonic cultures (Vilain *et al.*, 2009). Therefore, in the case of *B. cereus* eDNA, which was shown to be similar to chromosomal DNA, it may be that the eDNA is released through a novel as yet unknown mechanism, and thus warrants further investigation.

During the course of this study, a *B. cereus* FtsEX mutant was shown to be impaired in biofilm formation. These results are novel, as FtsEX has not yet been implicated in biofilm formation in either Gram-negative or Gram-positive bacteria. Although FtsE and FtsX, which display homology to ABC transporters, are widely conserved among Gram-negative and Gram-positive bacteria (de Leeuw *et al.*, 1999), evidence suggests that they mediate divergent activities. In *E. coli*, FtsEX is required in septal ring assembly and/or improving constriction of the septal ring during cytokinesis (Schmidt *et al.*, 2004; Arends *et al.*, 2009), whereas the *B. subtilis* homologue appears to be responsible for importing an inducer of sporulation (Garti-Levi *et al.*, 2008). The greater similarity between the properties of FtsEX mutants of *B. cereus* and *B. subtilis*, as opposed to that of *E. coli* FtsEX mutants, suggest that FtsEX of *B. cereus* may have a function analogous to that of *B. subtilis*. Data obtained for *B. subtilis* indicated that FtsEX imports an as yet unknown extracellular signal that acts at the top of a hierarchical cascade of events responsible for initiation of sporulation (Garti-Levi *et al.*, 2008). This therefore implies that FtsEX functions by importing a signal that activates the histidine kinases (KinA, KinB and KinC), which subsequently transfer phosphate to the phosphotransferases Spo0F and Spo0B, and finally to Spo0A (Jiang *et al.*, 2000). The activation of Spo0A has particular significance for the process of biofilm formation, as Spo0A controls regulatory proteins governing biofilm formation in *B. subtilis* (Branda *et al.*, 2006; Chu *et al.*, 2008). Activation of Spo0A relieves repression of the *epsA-O* and *yqxM-sipW-tasA* operons and thus results in the production of extracellular matrix components required for biofilm formation by *B. subtilis* (Branda *et al.*, 2001; Branda *et al.*, 2006). Although the results obtained in this study indicated that FtsEX is important for biofilm formation by *B. cereus*, the mechanism by which this occurs is not known.

In conclusion, biofilm formation is a complex and highly regulated process that involves a combination of several genetic and environmental factors acting at different stages. This study has led to the identification of several genes that have not previously been implicated in biofilm formation by *B. cereus*. Continued studies of these genes should be important for gaining a full understanding of their specific roles in the biofilm formation process. This may ultimately provide new insights into the biofilm forming capacity of not only *B. cereus*, but also Gram-positive bacteria in general.

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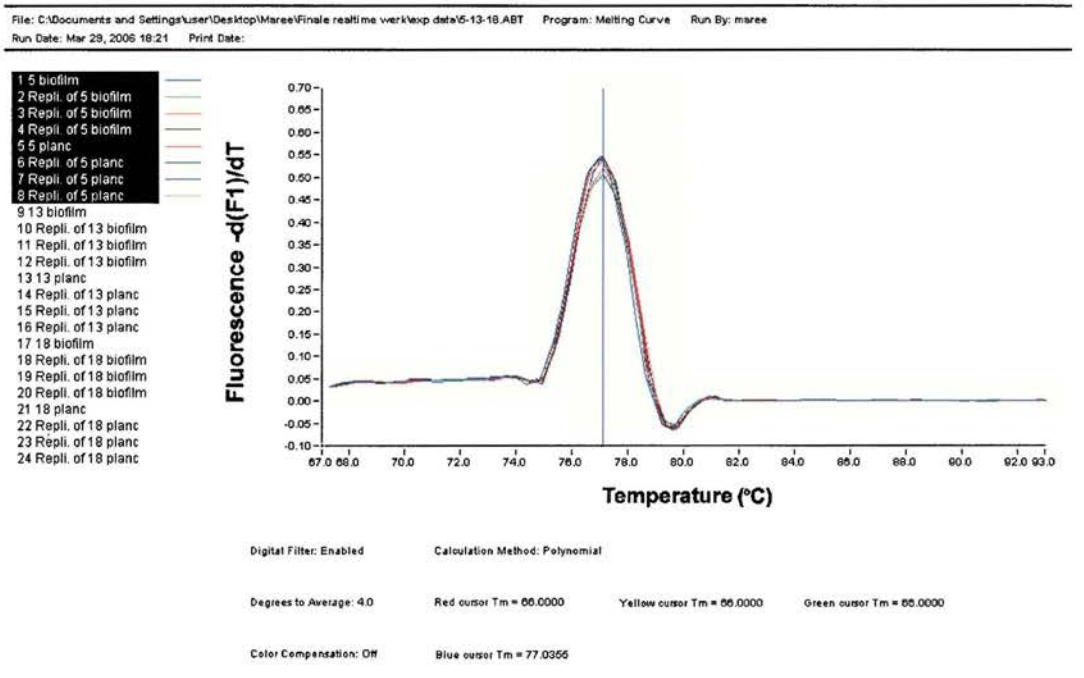
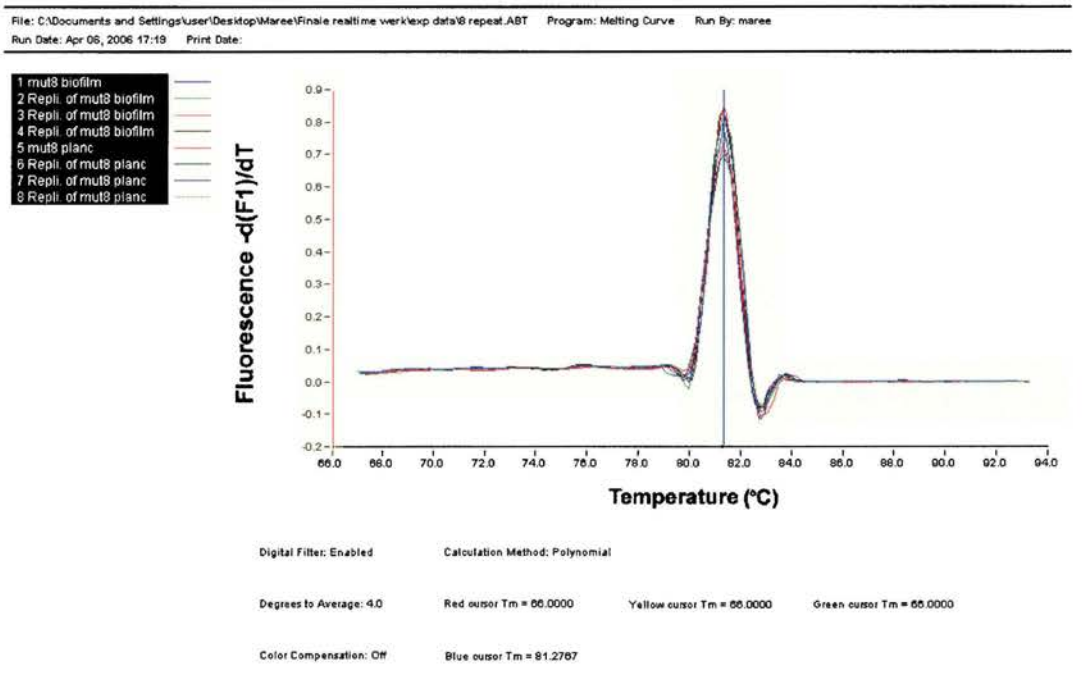
APPENDIX 1

Melting curve analysis for each individual primer pair. Data was obtained following the last amplification cycle by continuous measurement of fluorescence between 65°C and 95°C with a temperature transition rate of 0.1°C/s.





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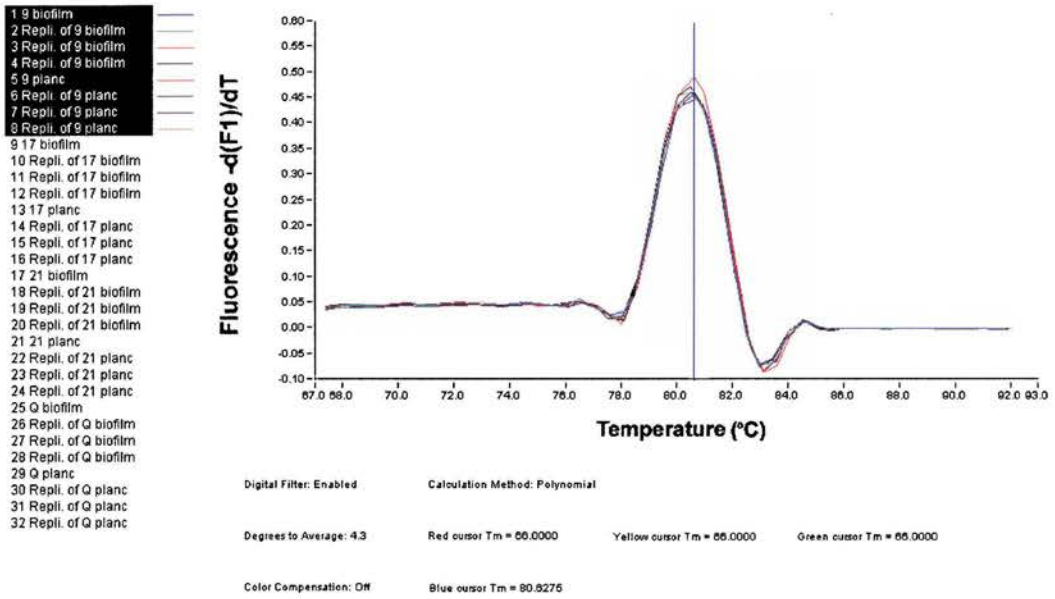

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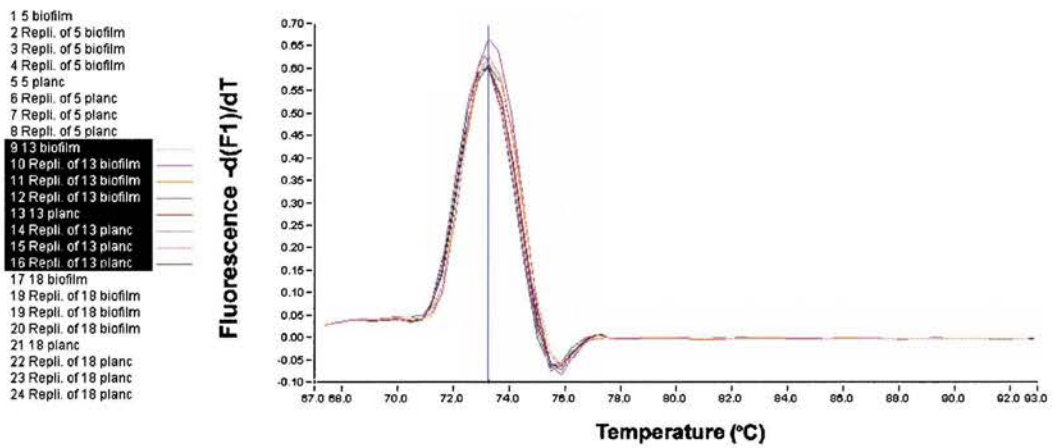

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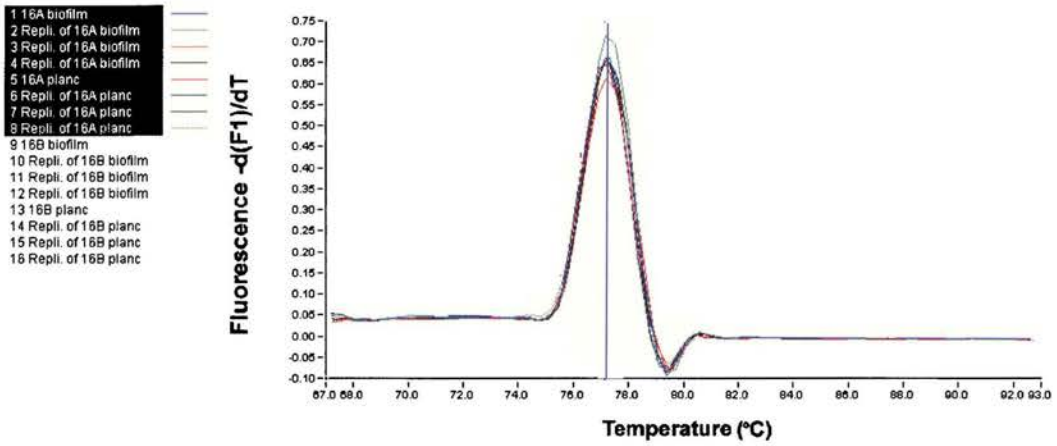

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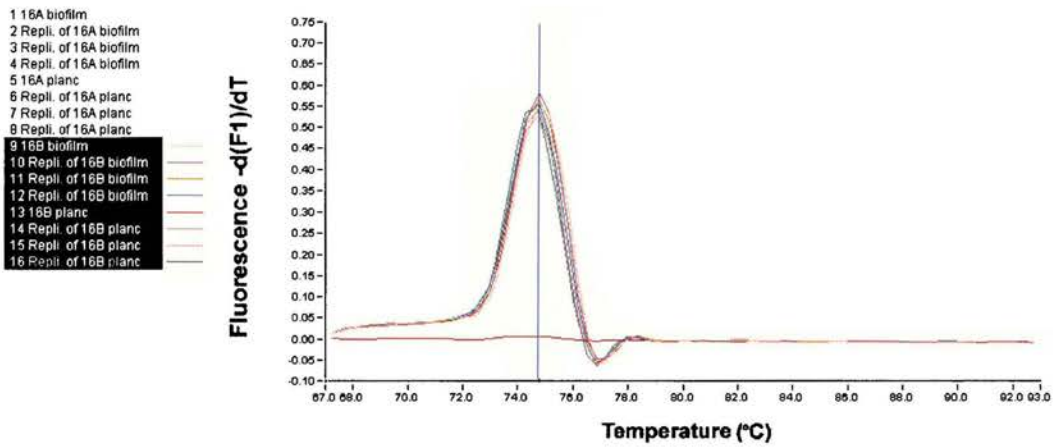

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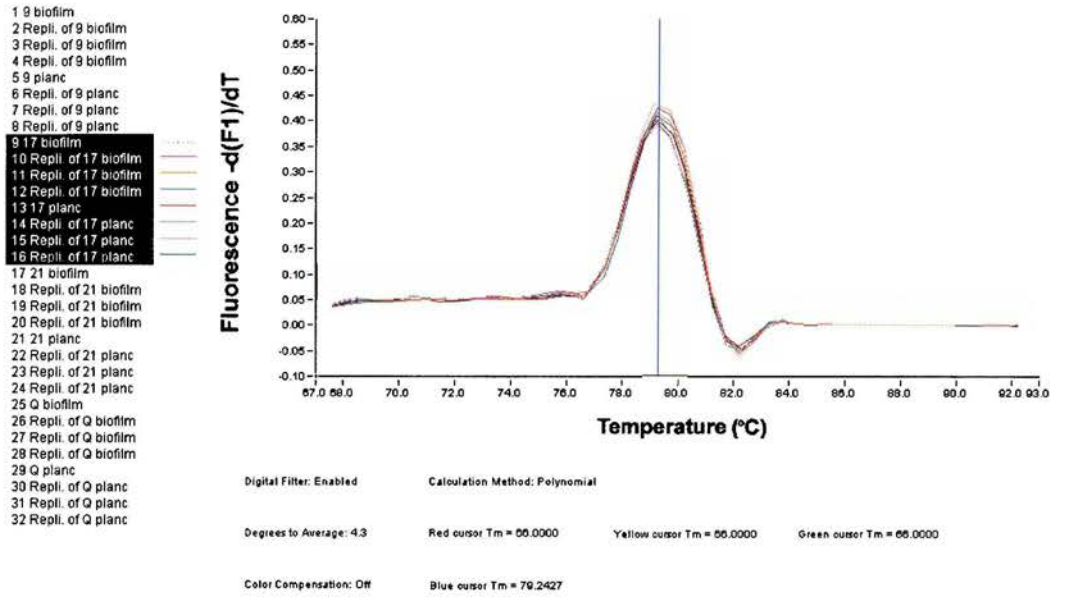

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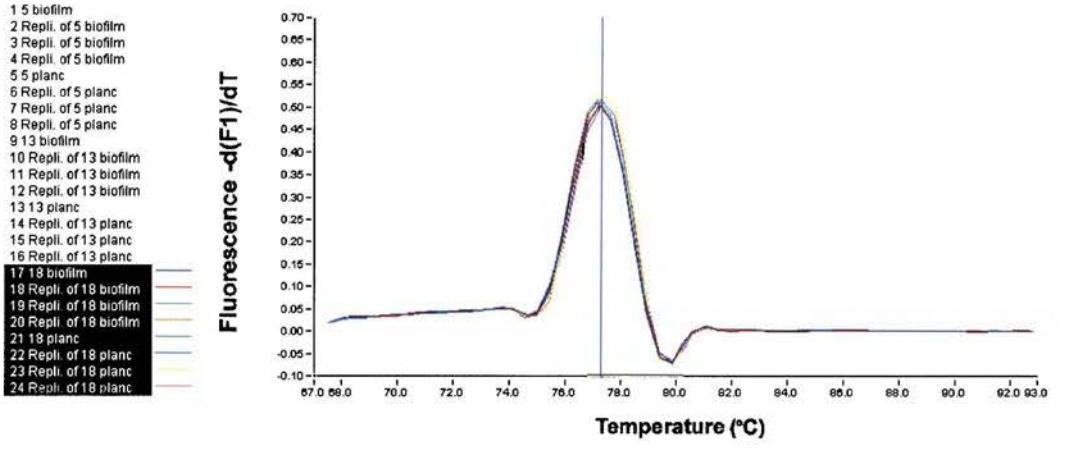

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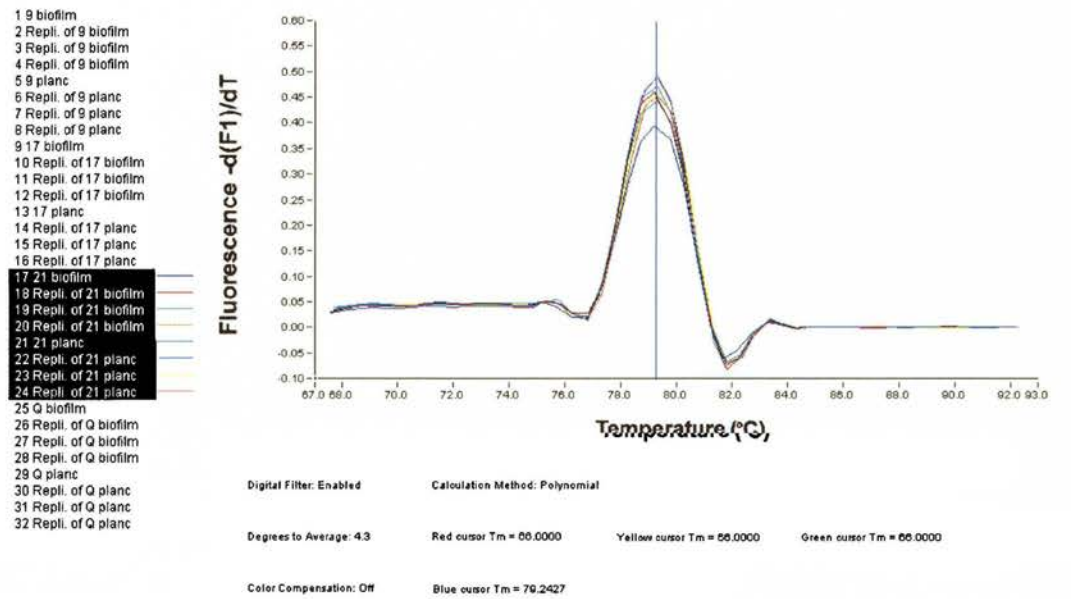

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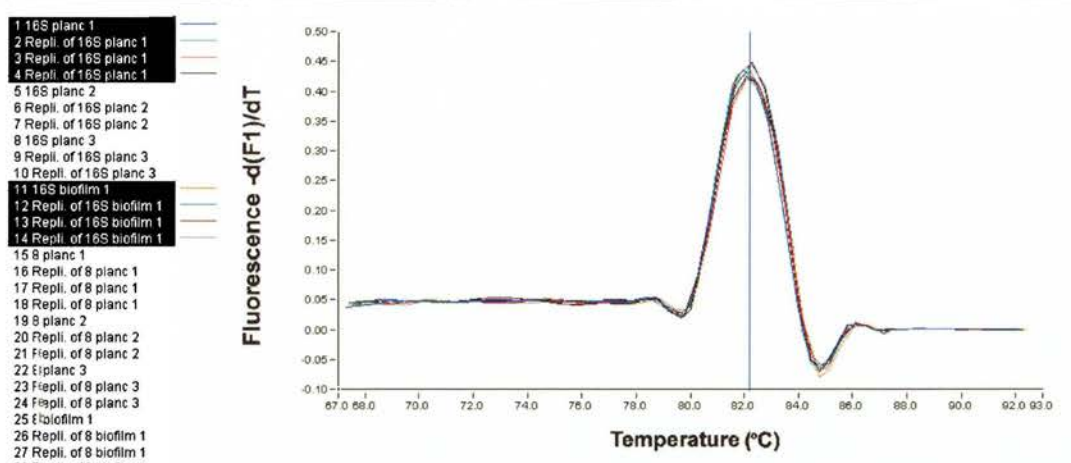

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