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).

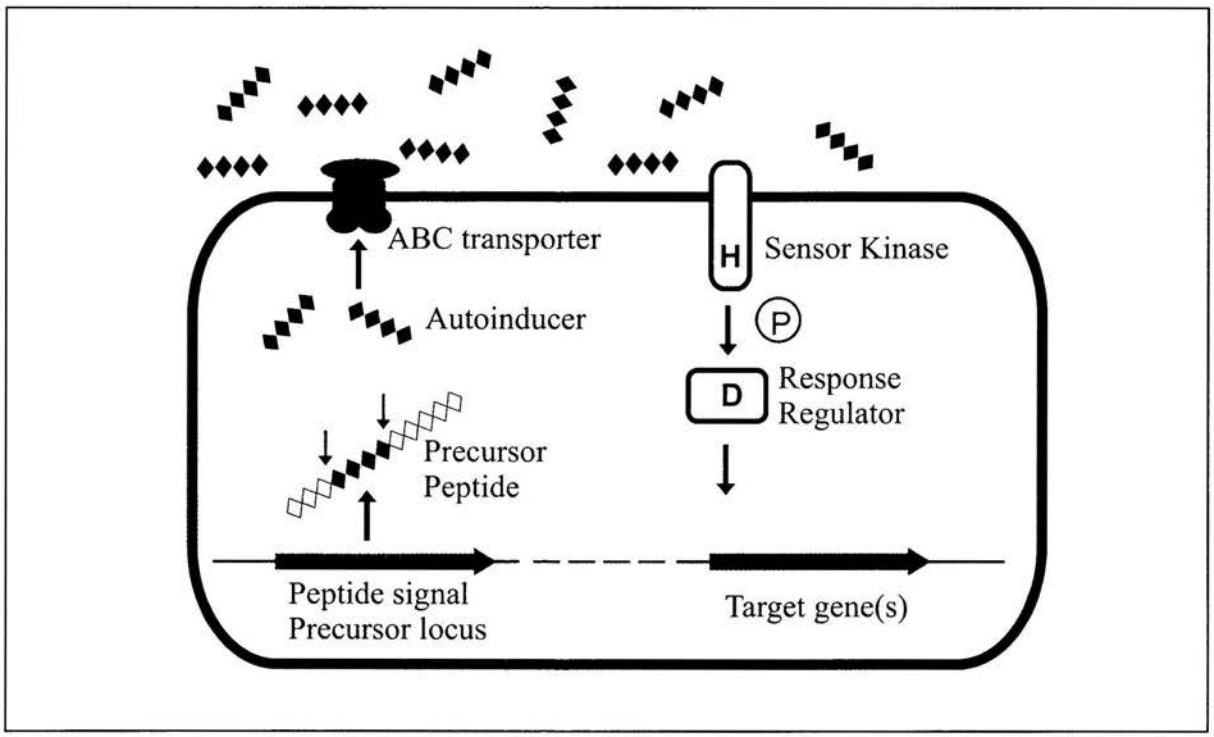

Figure 1.1: A general model for peptide-mediated quorum sensing in Gram-positive bacteria. In the figure, the precursor peptide (grey and black diamonds) is cleaved (arrows) to produce the processed autoinducer peptide (black diamonds). The P in a circle represents the phosphorylation cascade. (Adapted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

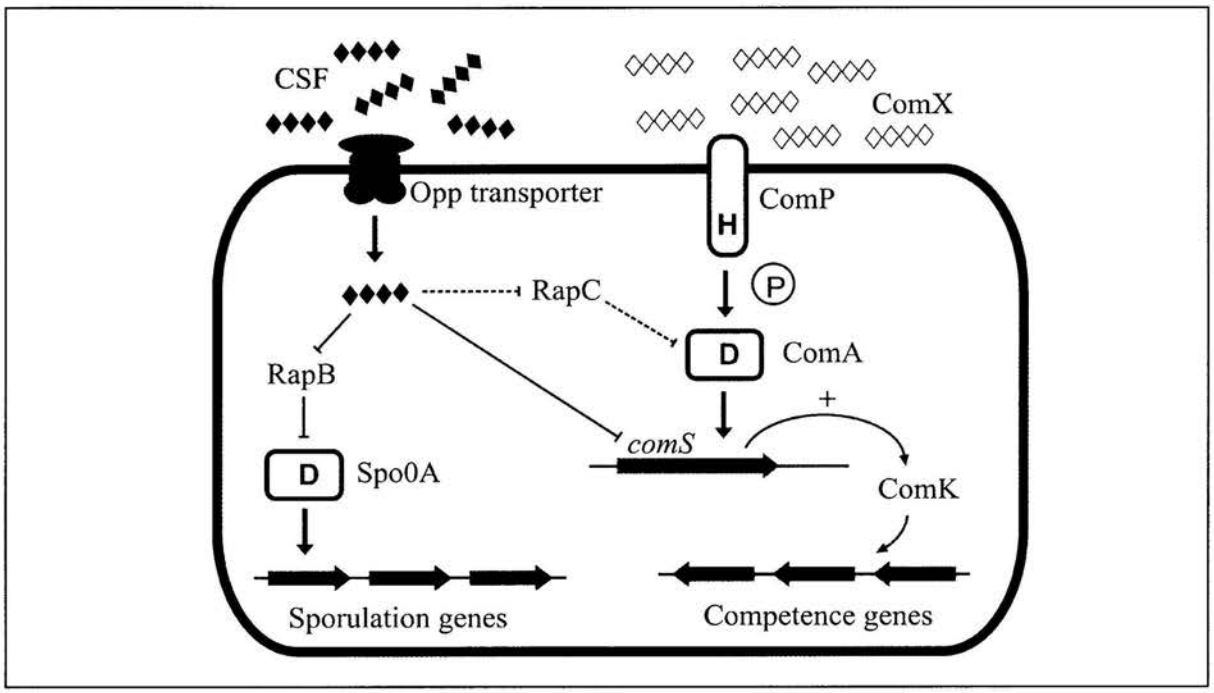

Figure 1.2: The *B. subtilis* ComP/ComA quorum sensing system for control of sporulation and competence. *B. subtilis* uses two processed autoinducer peptides, ComX (grey diamonds) and CSF (black diamonds), to regulate the competence and sporulation processes. Accumulation of the processed ComX peptide 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).

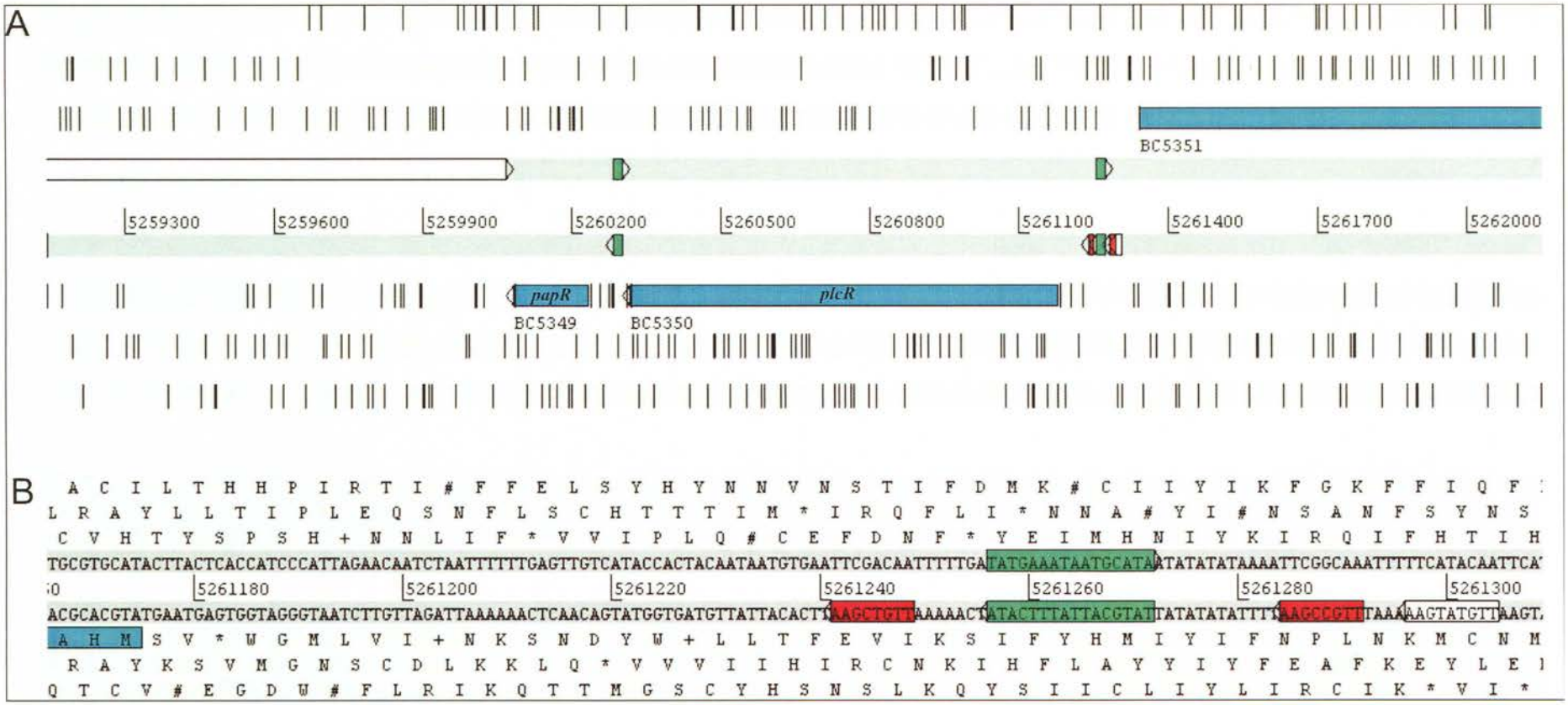

Figure 1.3: (A) Genomic region of *Bacillus cereus* ATCC 14579, containing the *plcR* and *papR* genes. The dark grey horizontal bars represent the two strands of the DNA double helix. The light grey horizontal bars represent the three possible reading frames in each strand. The vertical lines represent 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.

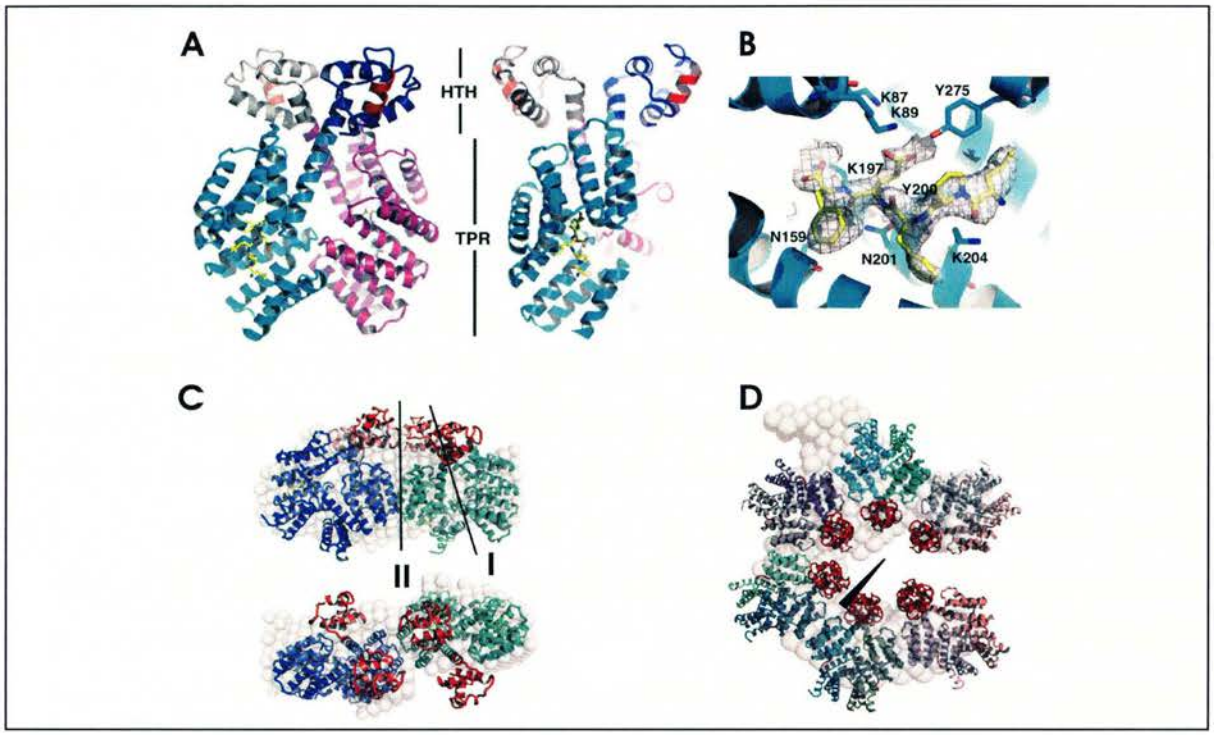

Figure 1.4: Structure of PapR5:PlcR and higher order structures formed in the presence of increasing concentrations of PapR5. The C-terminal 5-amino-acid peptide derived from PapR (PapR5) is shown in yellow. The two PlcR polypeptides are coloured in cyan and magenta, respectively, and their respective 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.

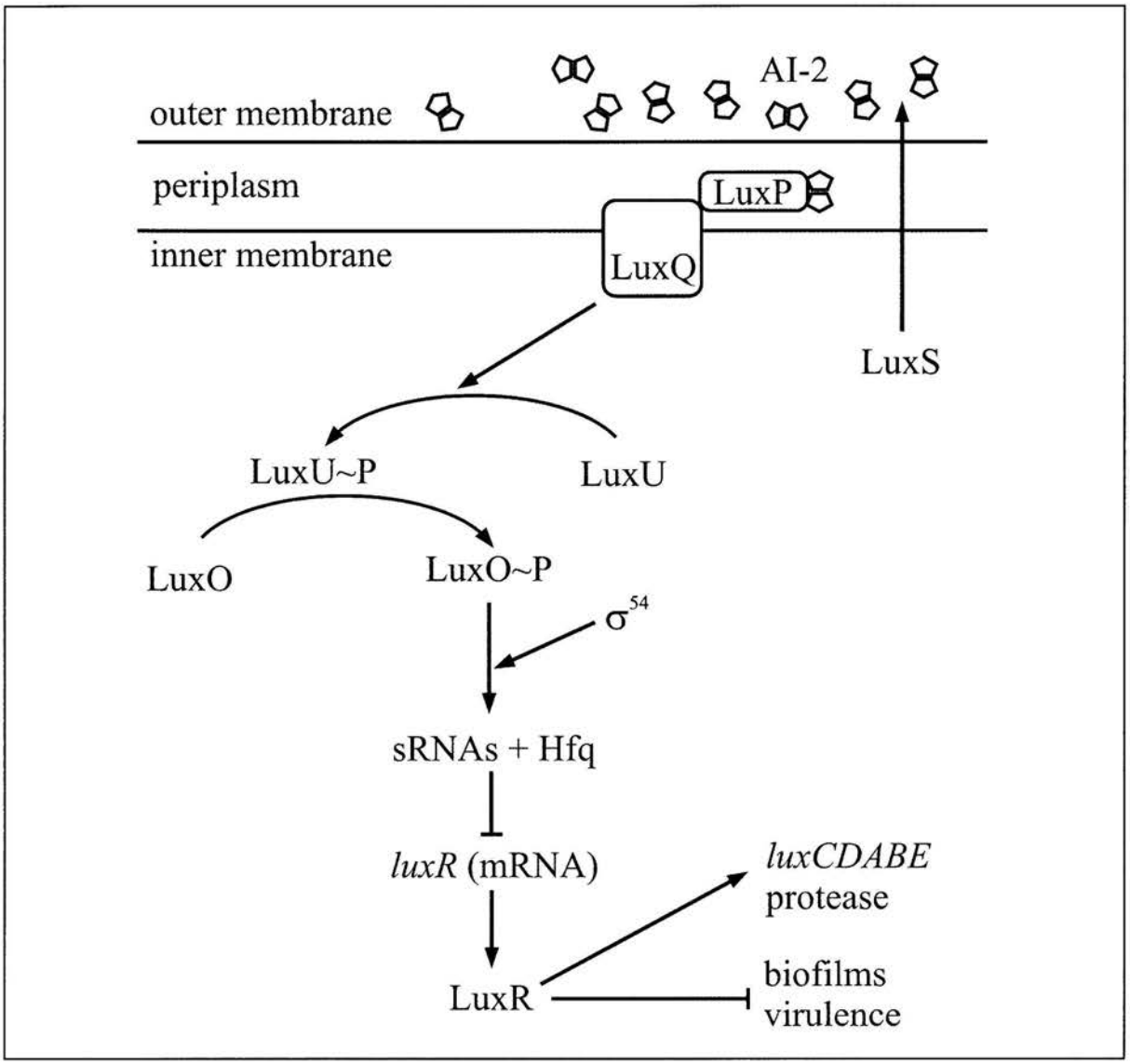

Figure 1.5: AI-2-dependant quorum sensing in *V. harveyi*. AI-2 (double pentagons) is synthesized in a reaction mediated by the LuxS enzyme. The autoinducer AI-2 is detected by LuxQ, in addition to a periplasmic binding protein, called LuxP. The LuxQ protein is a hybrid sensor kinase that contains a 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).

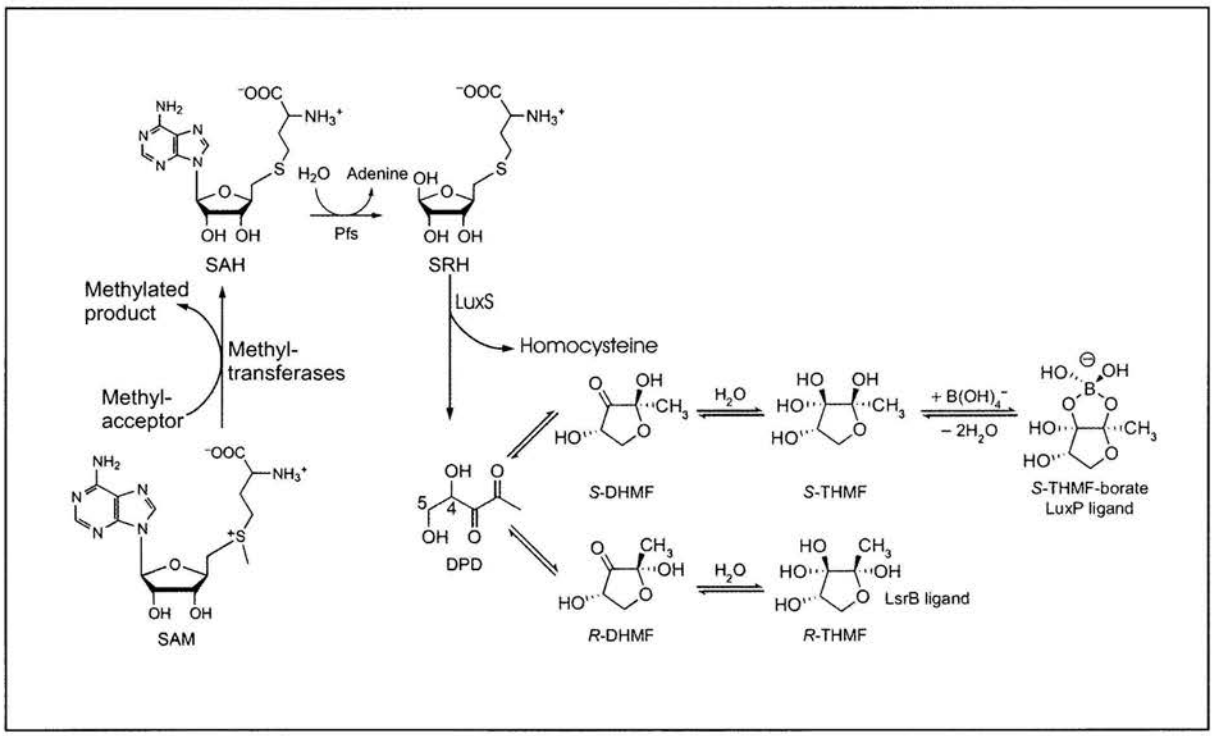

Figure 1.6: Biosynthesis of AI-2 from the precursor *S*-adenosylmethionine (SAM). Many methyltransferases act on SAM and transfer a methyl group to various substrates. These reactions also produce *S*-adenosylhomocysteine (SAH). Pfs hydrolyzes adenine from SAH to form *S*-ribosylhomocysteine (SRH). LuxS 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).

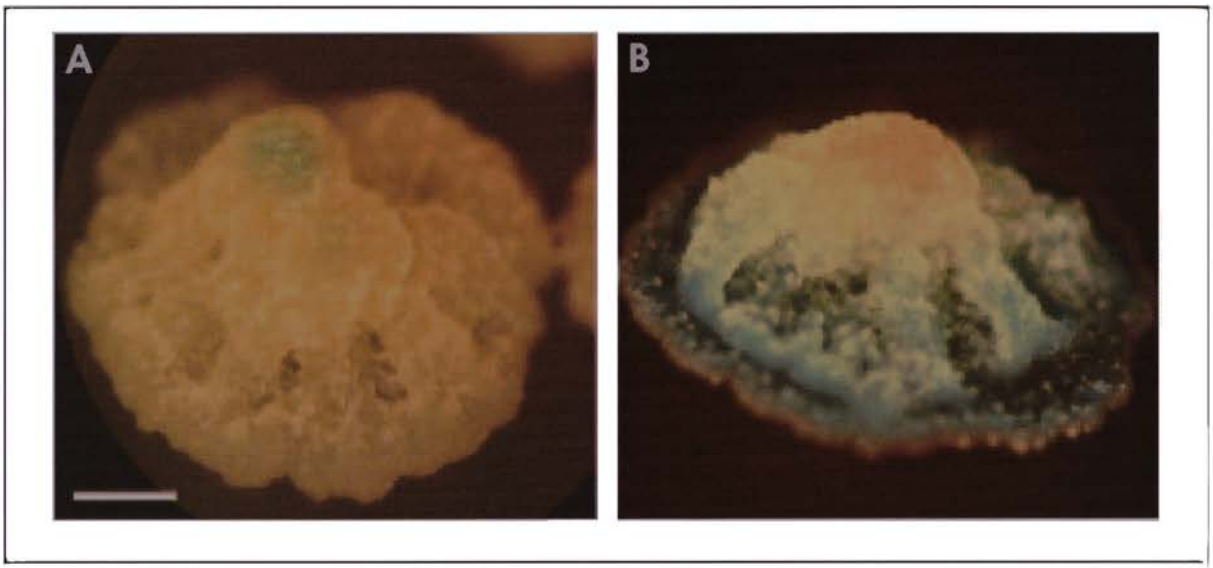

Figure 1.7: Expression of a *sspB-lacZ* fusion (A) and a *luxS-lacZ* fusion (B) by *B. subtilis* colonies grown on agar containing X-Gal. The *sspB* gene is expressed late during sporulation and the localized development of the blue colour indicates that spore morphogenesis is restricted to the older 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*.

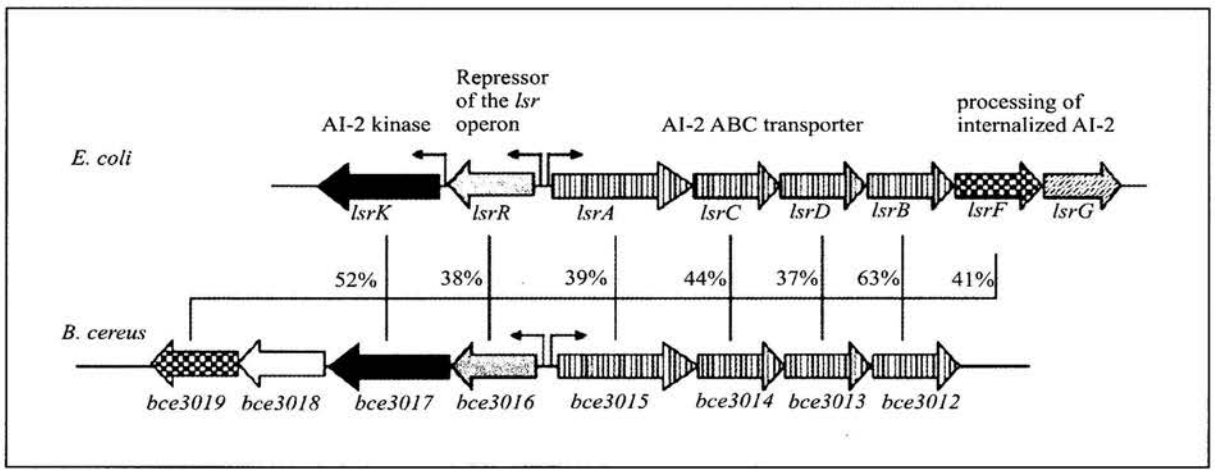

Figure 1.8: Genetic organization and sequence analysis of the *lsr* region in *B. cereus* ATCC 10987 and *E. coli* K-12. Putative transcription start sites are indicated by arrows. For each gene product, the similarity between the *B. cereus* and *E. coli* proteins is indicated as a percentage of identity. 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.

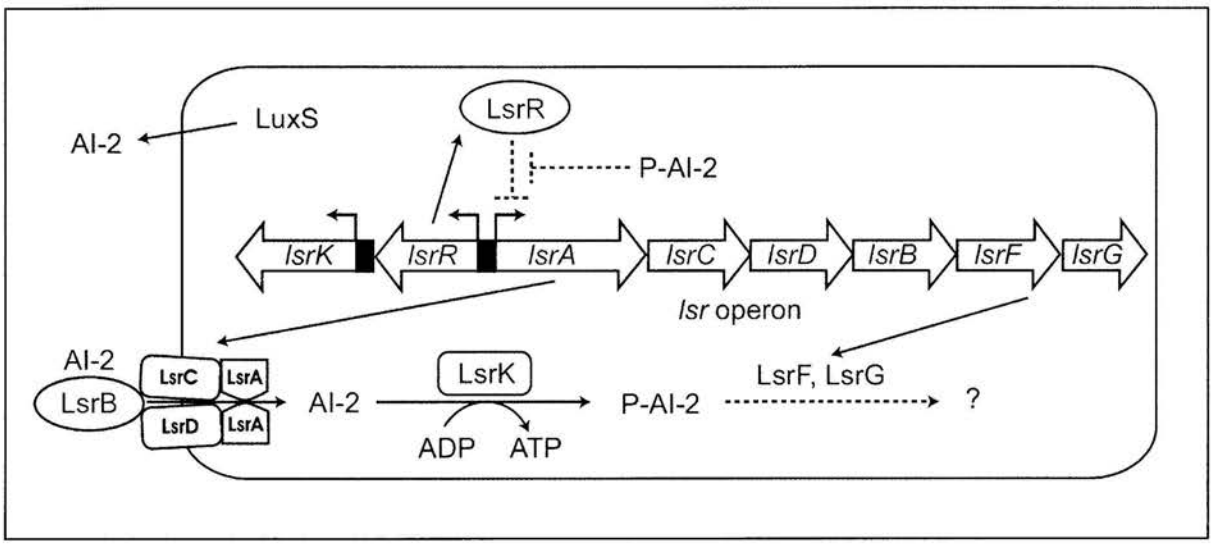

Figure 1.9: Model for Lsr-mediated internalization and processing of AI-2 in *E. coli*. AI-2 is synthesized in a reaction mediated by LuxS and accumulates extracellularly. AI-2 is bound by the periplasmic protein LsrB and internalized by the Lsr ABC-type transporter, and intracellular AI-2 is phosphorylated 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).

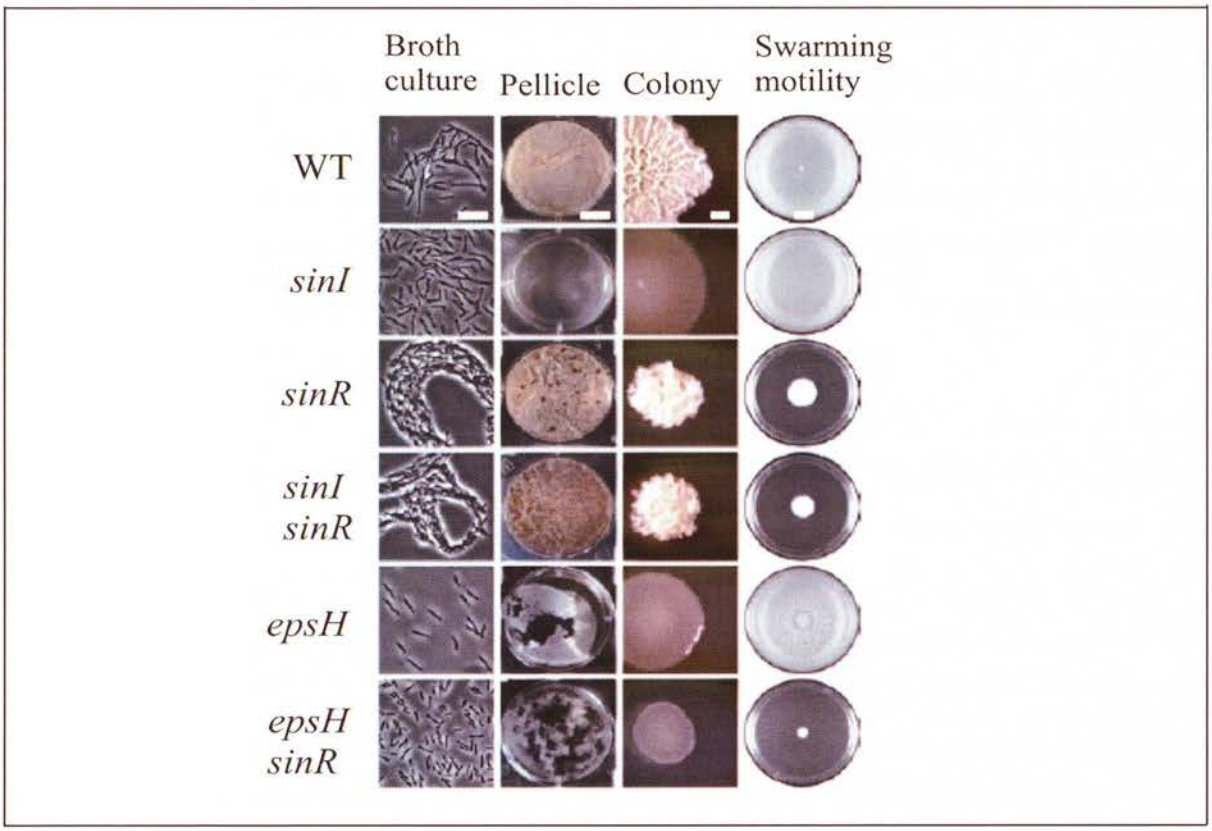

Figure 1.10: Effect of mutations in *sinR*, *sinI* and *epsH* on chain bundling, pellicle formation, colon morphology and swarming motility. The broth culture cells were grown to mid-exponential phase in LB broth while pellicles were obtained by culturing cells for 3 days in 6-well culture plates. (From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

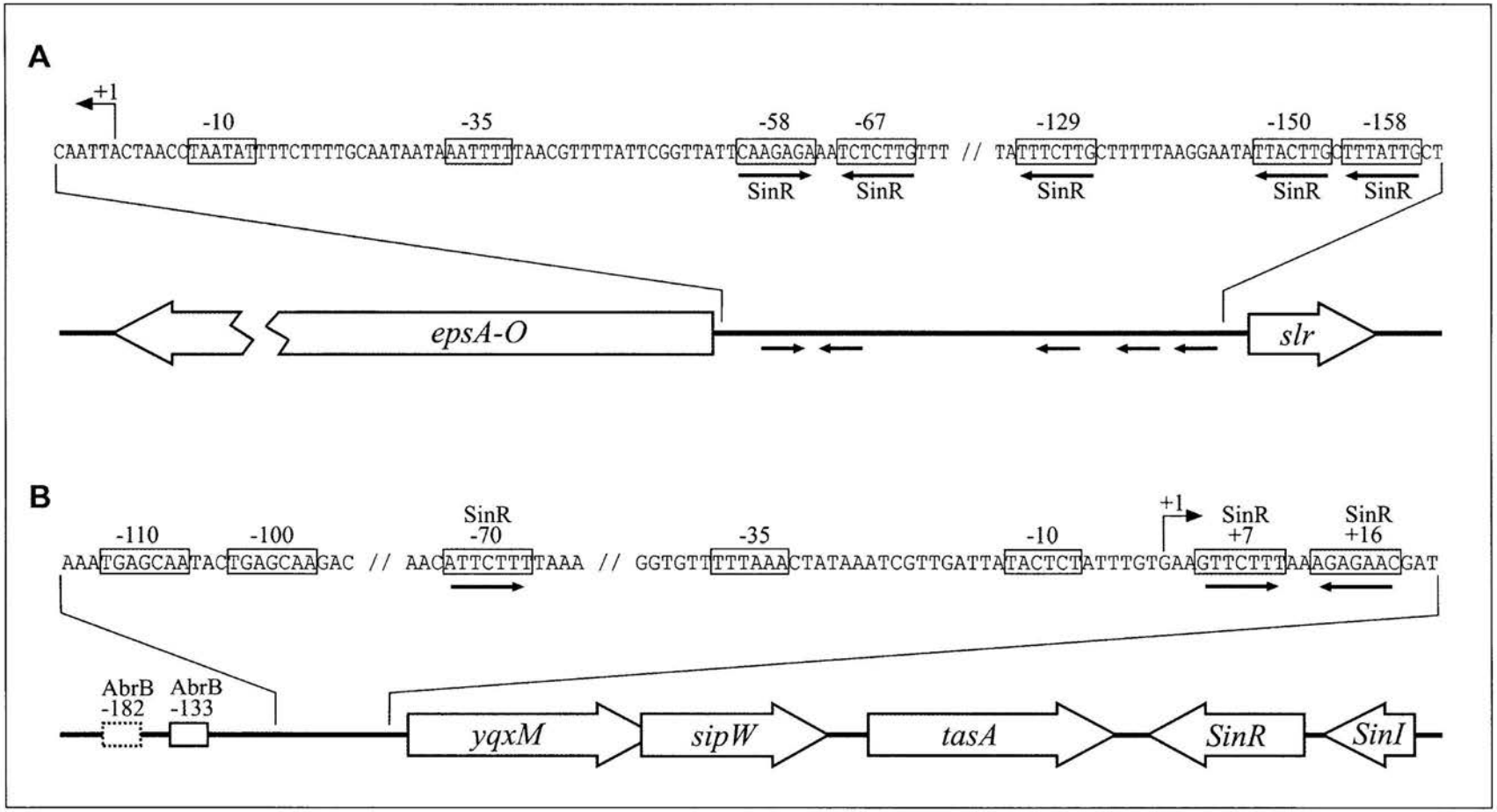

Figure 1.11: Structure and organization of SinR and AbrB recognition sequences in the promoter regions of the *epsA-O* and *yqxM-sipW-tasA* operons, as determined by DNase I footprinting experiments. (Kearns *et al.*, 2005; Chu *et al.*, 2006; Strauch *et al.*, 2007; Chu *et al.*, 2008).

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).

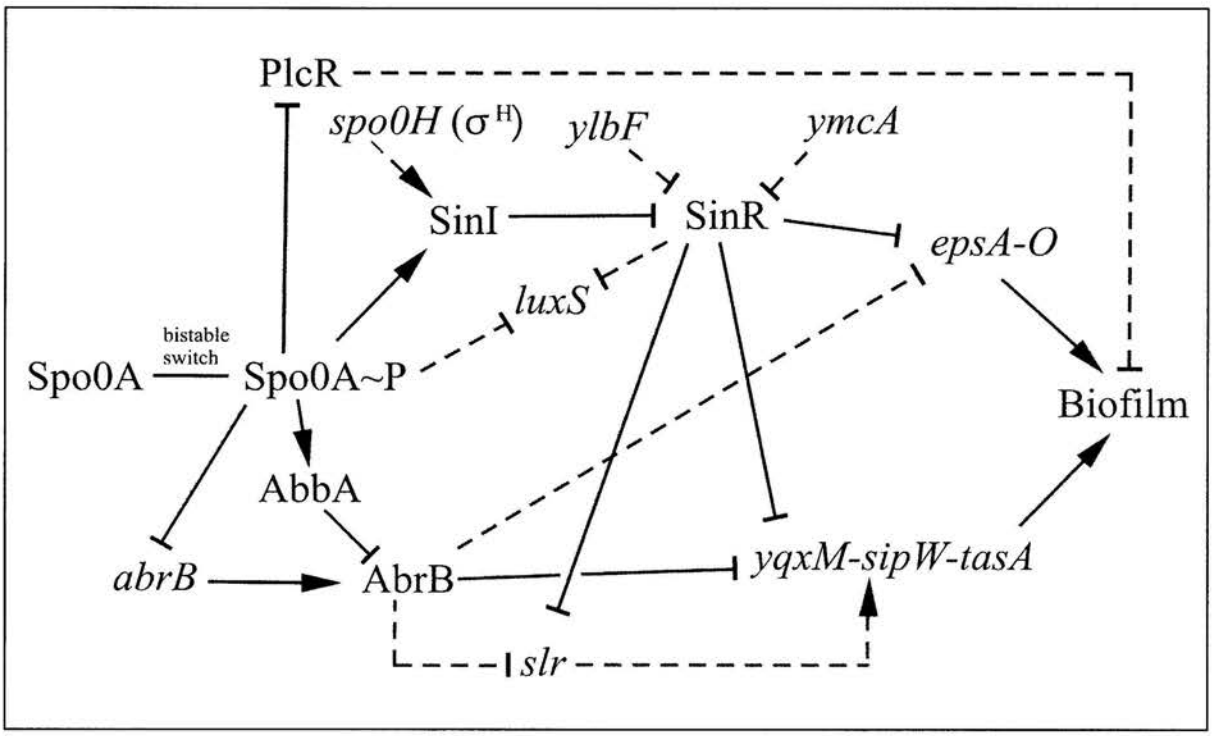

Figure 1.12: Regulation of *Bacillus* multicellular behaviour. Green arrows indicate activation of gene expression and red T bars indicate repression. Solid lines denote direct regulation and dashed lines denote unknown mechanisms. The pathway indicated in blue occurs only in *B. cereus*. SinR and AbrB 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.