CHAPTER FOUR
DISCUSSION

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

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

4.1.1 *B. cereus ΔmotAB* is biofilm-impaired

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

4.1.2 The motor complex of bacterial flagella

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

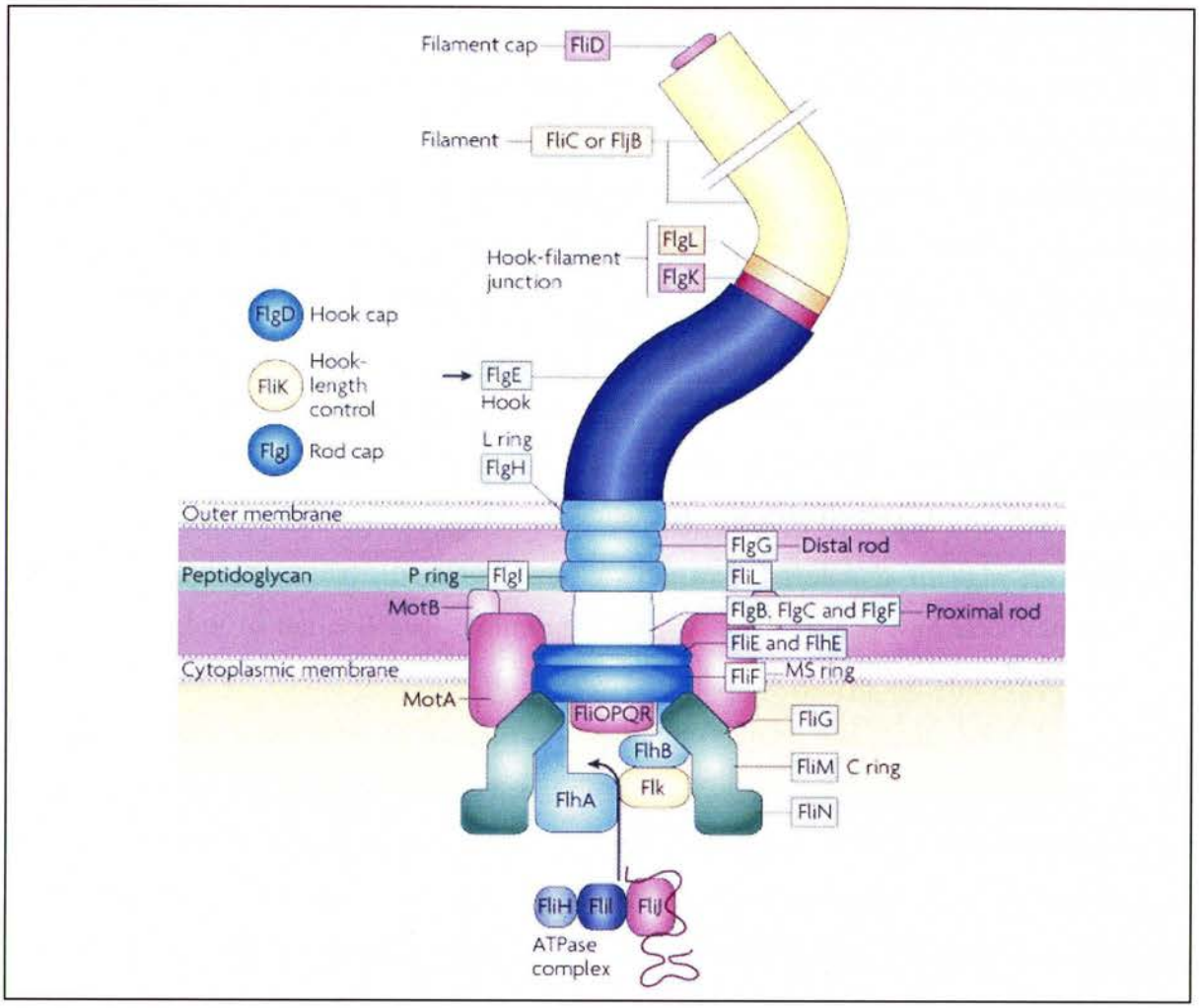

Figure 4.1: Schematic representation of the structure and components of the *Salmonella enterica* serovar Typhimurium flagellum. Genes whose inactivation results in non-flagellated mutants are called *flg*, *flh*, *fli* and *flj*, depending on their location on the genetic map, and genes whose inactivation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

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

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

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

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

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

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

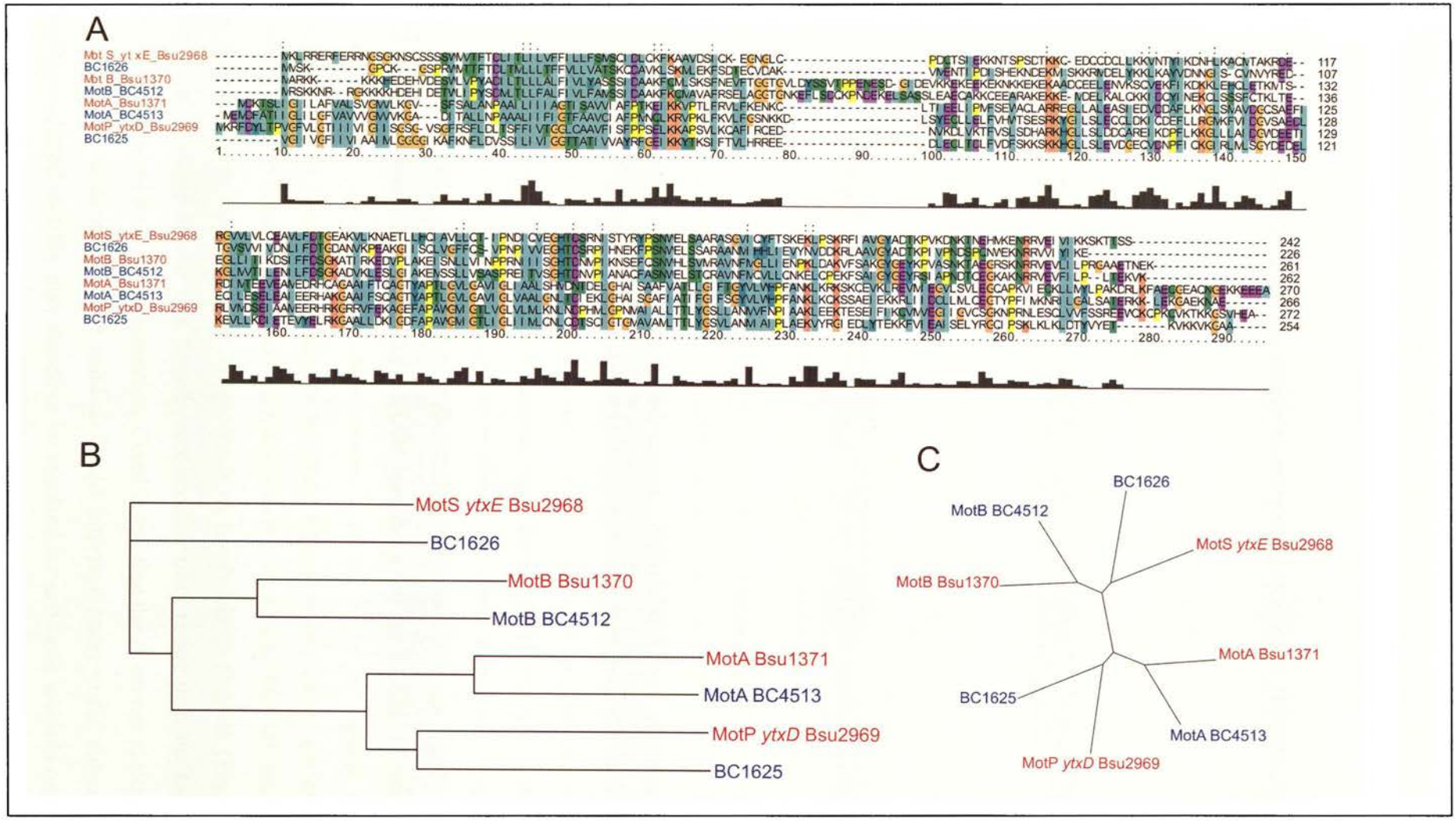

Figure 4.2: Alignment of amino acid sequences of the *B. cereus* ATCC 14579 stator components with the amino acid sequence of MotA, MotB, MotP and MotS of *B. subtilis*. Neighbor-joining bootstrap analysis with 1000 trials provided ≥99% bootstrap support for the branches indicated in both the phylogram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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

4.3.1 *B. cereus ΔftsEX* is biofilm-impaired

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

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

4.3.2 FtsEX is required for divergent activities in different bacteria

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

4.4.2 Murein hydrolases are involved in eDNA release and biofilm formation

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

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

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

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

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

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

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

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

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

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

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

CidA            HKHRKGVDAYE 131
BC5133          KERESR----- 124
                ::::..
```

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

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

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

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

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

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

CHAPTER FIVE
CONCLUDING REMARKS

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

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

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

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

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

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

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

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

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