CHAPTER TWO
MATERIALS AND METHODS

2.1 General procedures

2.1.1 Bacterial strains

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

2.1.2 Plasmid and transposon

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

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

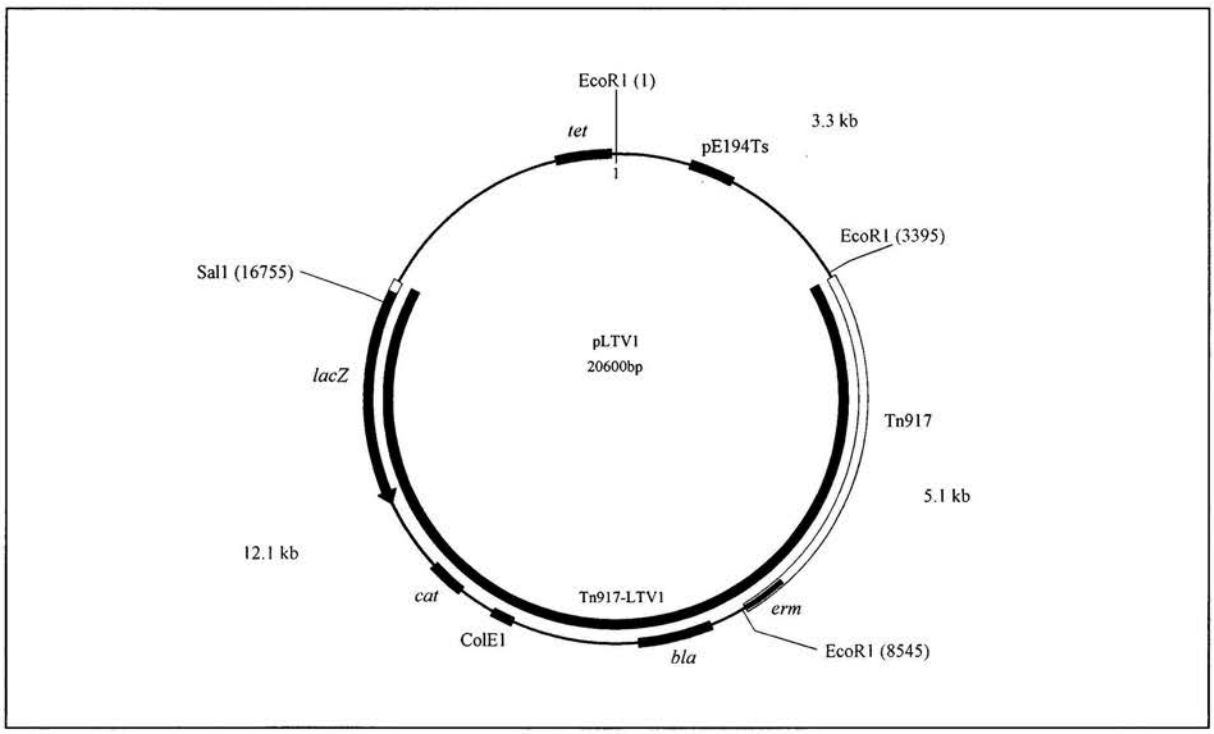

Figure 2.1: Restriction map of pLTV1. Tn*917*-LTV1 is indicated in blue and the original Tn*917* is indicated in yellow. The three *Eco*RI sites and the sizes of the restriction fragments are indicated in red.

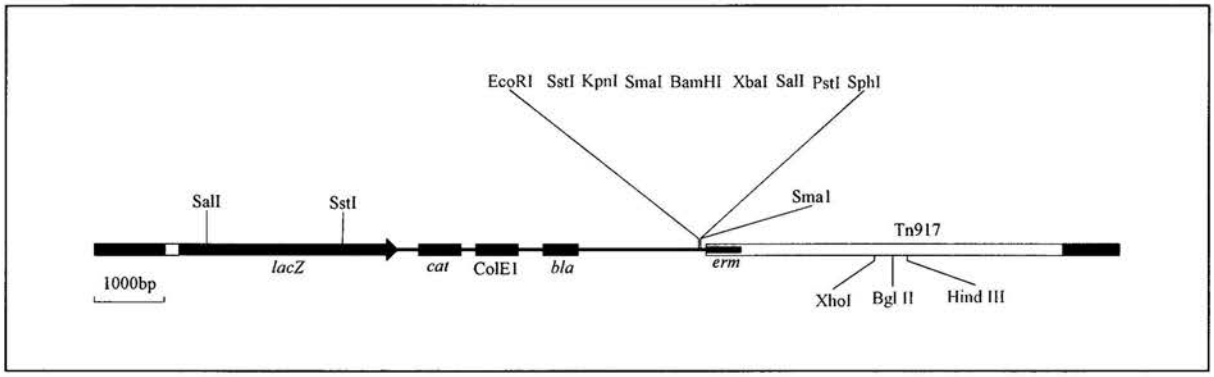

Figure 2.2: Hypothetical chromosomal insertion of Tn*917*-LTV1. Chromosomal flanking DNA is shown in green. Unique restriction sites that may be used to clone DNA flanking the *lacZ* proximal end is shown in red.

2.1.3 Media and growth conditions

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

2.1.4 Statistical analysis

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

2.2 Isolation of pLTV1

2.2.1 Plasmid extraction

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

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

2.2.2 Agarose gel electrophoresis

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

2.2.3 Restriction enzyme digestion

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

2.2.4 CsCl-EtBr density gradient centrifugation

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

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

2.3 Transformation of *Bacillus cereus* ATCC 14579

2.3.1 Preparation of electrocompetent cells

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

2.3.2 Dialysis of plasmid DNA

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

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

2.3.3 Electroporation

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

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

2.4.1 Transposon mutagenesis

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

2.4.2 Identification of biofilm mutants

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

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

2.4.3 Quantification of biofilm deficiency

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

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

2.5.1 Growth curves of biofilm-impaired mutants

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

2.5.2 Stereomicroscopy of biofilm-impaired mutants

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

2.5.3 Motility assay

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

2.5.4 Transmission electron microscopy

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

2.5.5 Laser scanning confocal microscopy

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

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

2.6.1 Isolation of chromosomal DNA from biofilm-impaired mutants

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

2.6.2 Preparation of competent *E. coli* HB101 cells

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

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

2.6.3 Cloning of the DNA flanking transposon insertions

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

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

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

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

2.6.5 Sequencing of flanking DNA

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

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

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

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

2.7.1 Preparation of the labelled probe

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

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

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

2.7.2 Preparation of the membrane

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

2.7.3 Hybridization

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

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

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

2.7.4 Detection of the hybridized DNA probe

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

2.8 Relative expression of selected *B. cereus* genes

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

2.8.1 Primers

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

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

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

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

2.8.2 RNA isolation

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

2.8.3 cDNA synthesis

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

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

2.8.4 Reverse transcriptase (RT) negative control PCR

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

2.8.5 Quantitative real-time PCR

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

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

CHAPTER THREE
RESULTS

3.1 Isolation of pLTV1

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

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

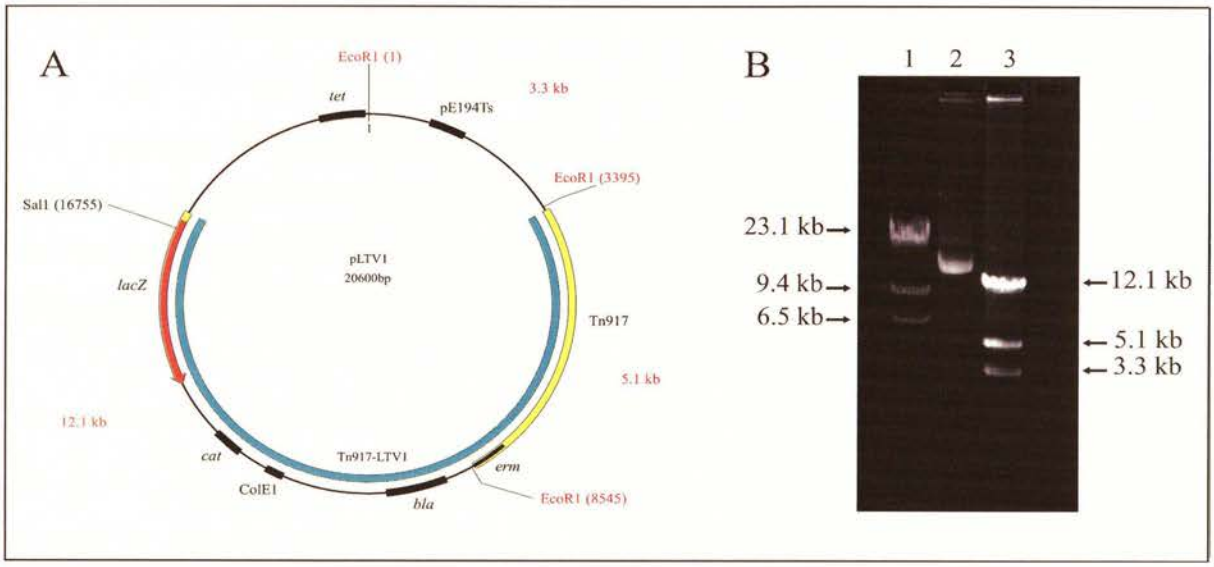

Figure 3.1: Characterization of purified pLTV1 plasmid DNA. (A) Plasmid map of pLTV1 harboring the Tn*917*-LTV1 transposon. The *Eco*RI restriction enzyme sites and sizes of the digestion products are indicated in red. (B) Agarose gel electrophoretic analysis of pLTV1. Lane 1, DNA molecular weight marker;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

3.2 Transposon mutagenesis of *B. cereus* ATCC 14579

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

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

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

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

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

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

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

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

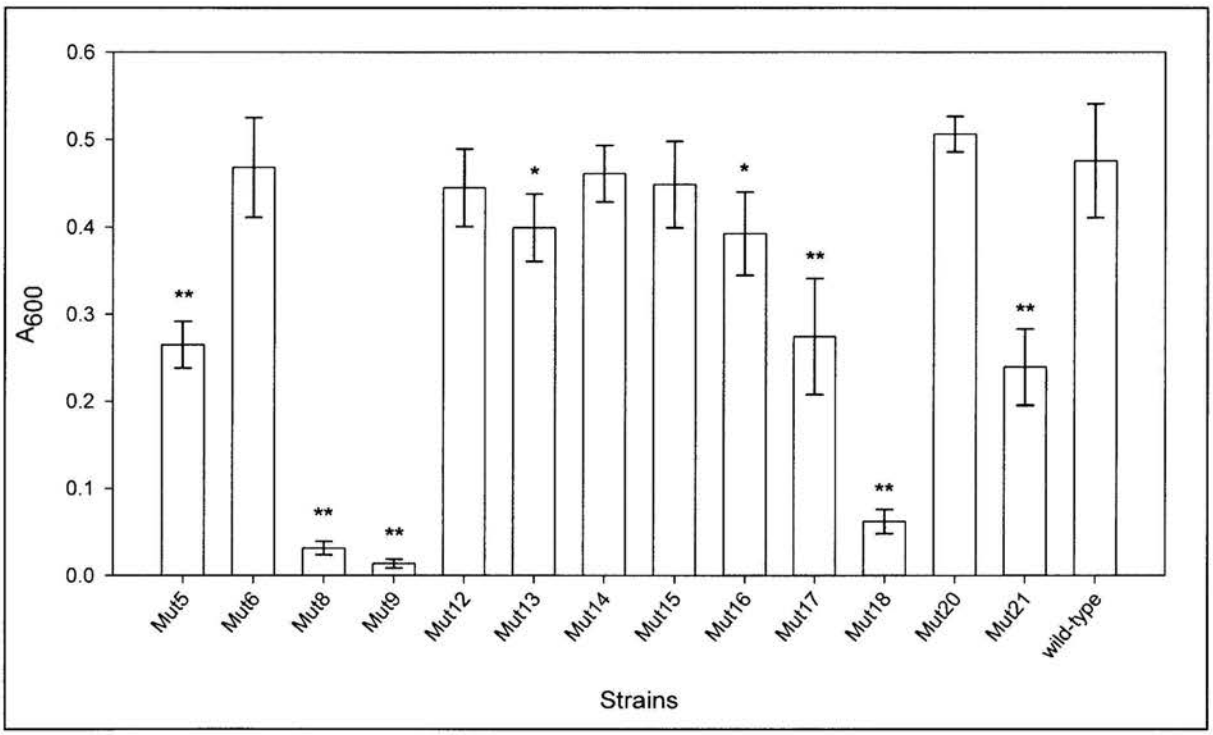

Figure 3.2: Biofilm formation by wild-type *B. cereus* ATCC 14579 and the derived Tn*917*-LTV1 transposon mutants (Mut). Biofilm formation was quantified by determining the A_{600} of the biofilm biomass after growth of the biofilm for 72 h in glass beakers without shaking. The data shown are the averages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

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

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

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

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

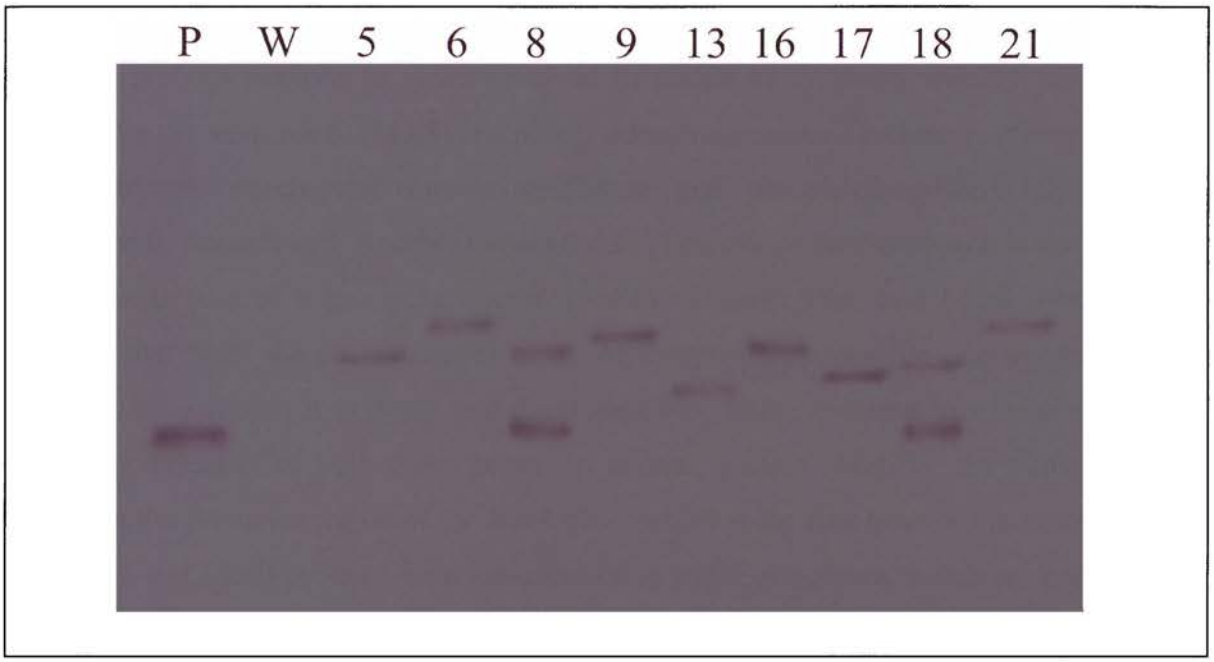

Figure 3.3: Southern blot analysis of chromosomal DNA extracted from *B. cereus* biofilm-impaired mutants. Chromosomal DNA extracted from wild-type *B. cereus* ATCC 14579 (lane W) and the respective *B. cereus* mutants (lanes 5 through 21) were digested with *Eco*RI, resolved by agarose gel electrophoresis,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

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

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

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

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

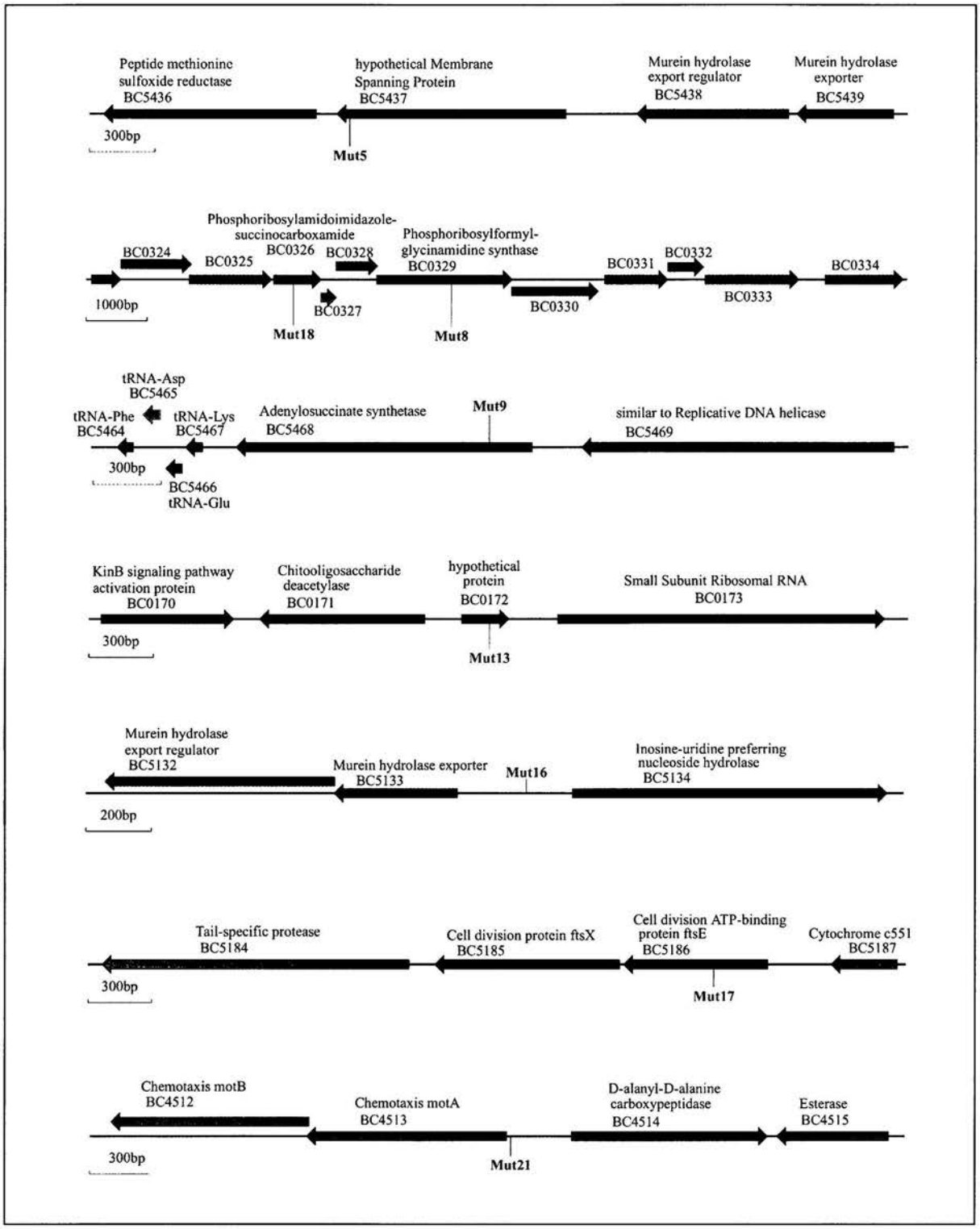

Figure 3.4: Locations of the Tn*917*-LTV1 insertions in the *B. cereus* biofilm-impaired mutants isolated in this study. The position of transposon insertions are depicted by a vertical line bearing the name of the mutant. Directions of transcription are indicated by arrowheads. Gene designations are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

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

3.6.1 Growth curves

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

3.6.2 Microscopic analysis of biofilm phenotypes

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

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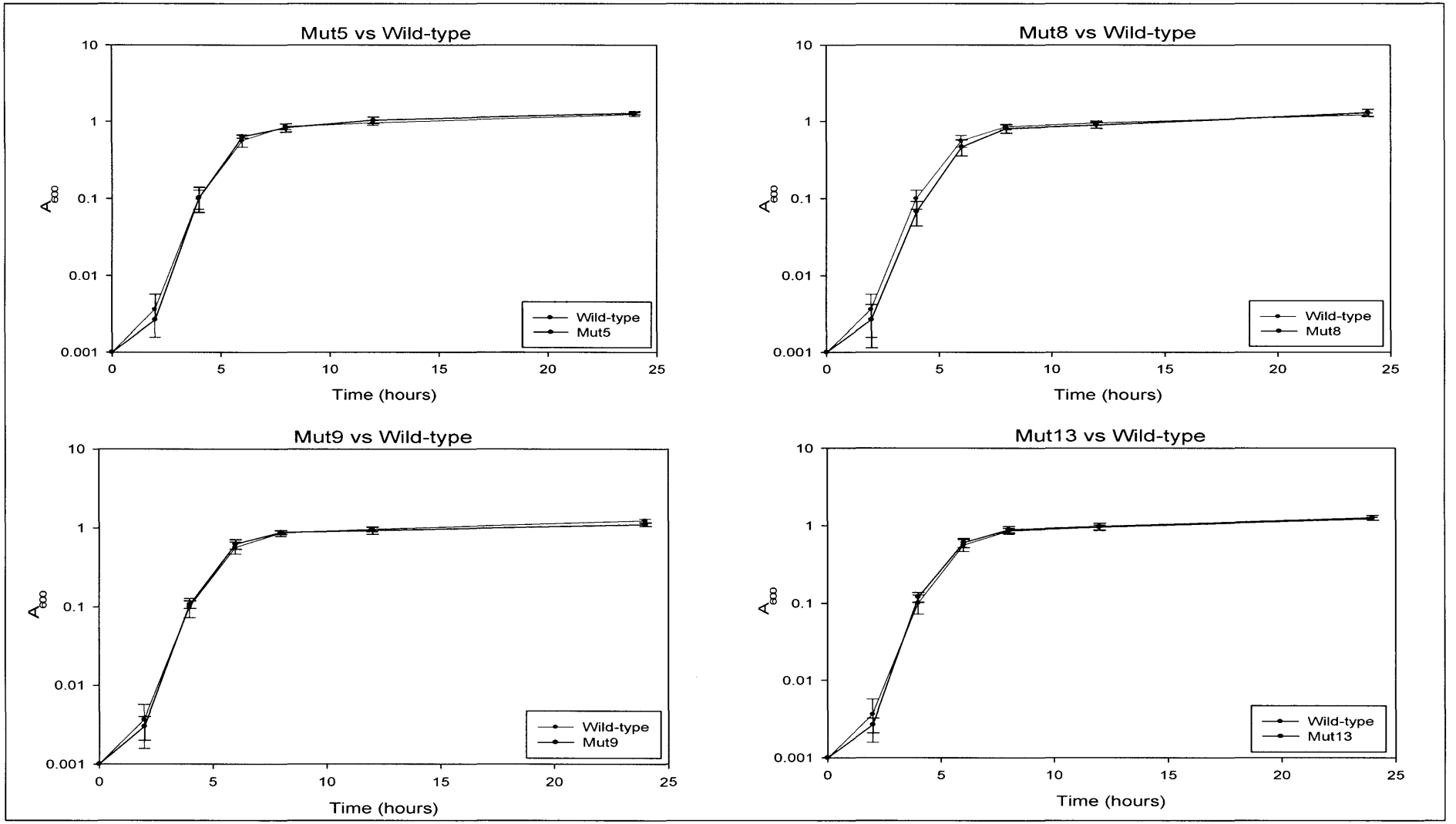

Figure 3.5: Growth curves of wild-type *B. cereus* ATCC 14579 and biofilm-impaired mutants in LB broth at 37°C. The data shown are the averages of three independent assays, and error bars indicate the standard deviation of the mean.

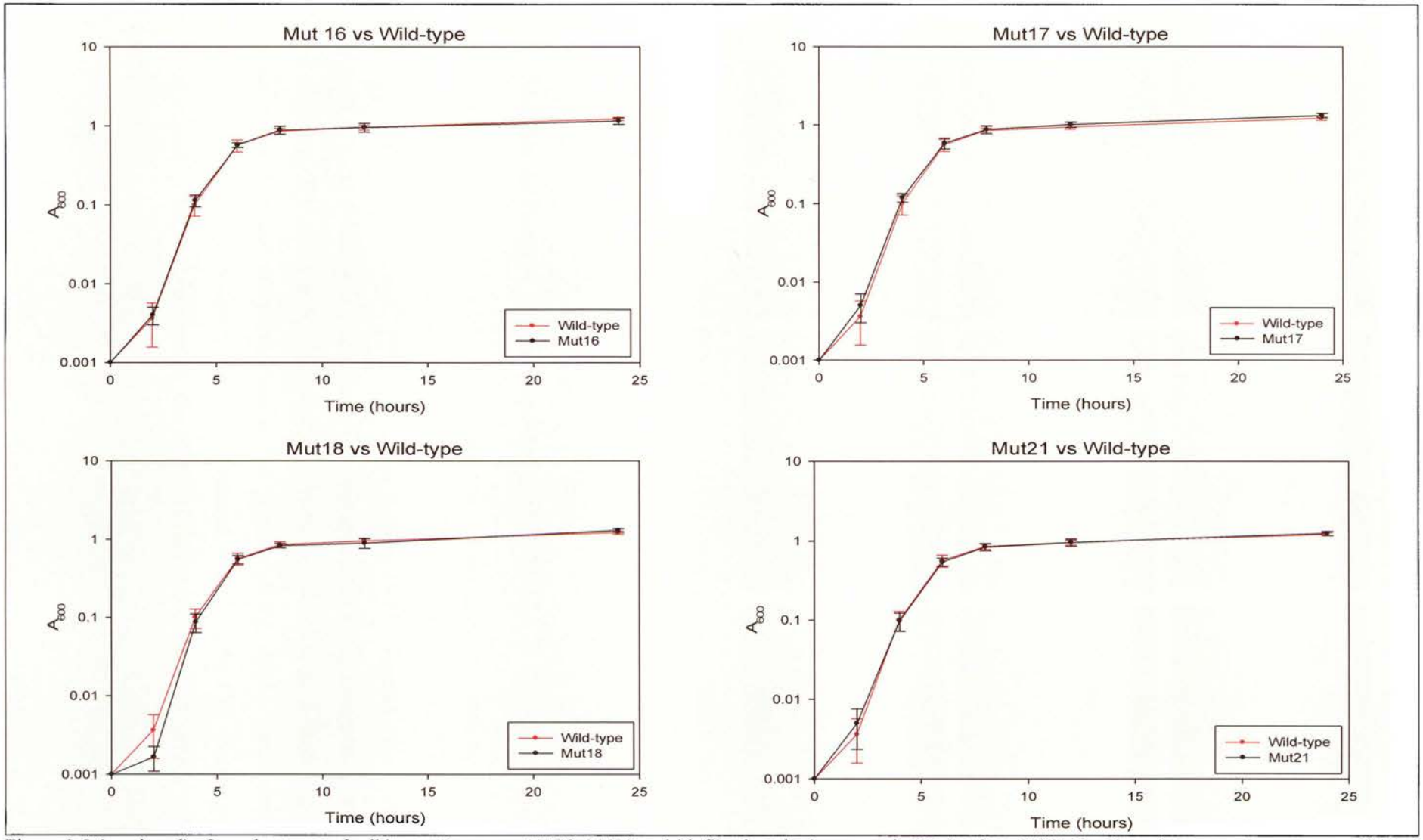

Figure 3.5 (continued): Growth curves of wild-type *B. cereus* ATCC 14579 and biofilm-impaired mutants in LB broth at 37°C. The data shown are the averages of three independent assays, and error bars indicate the standard deviation of the mean.

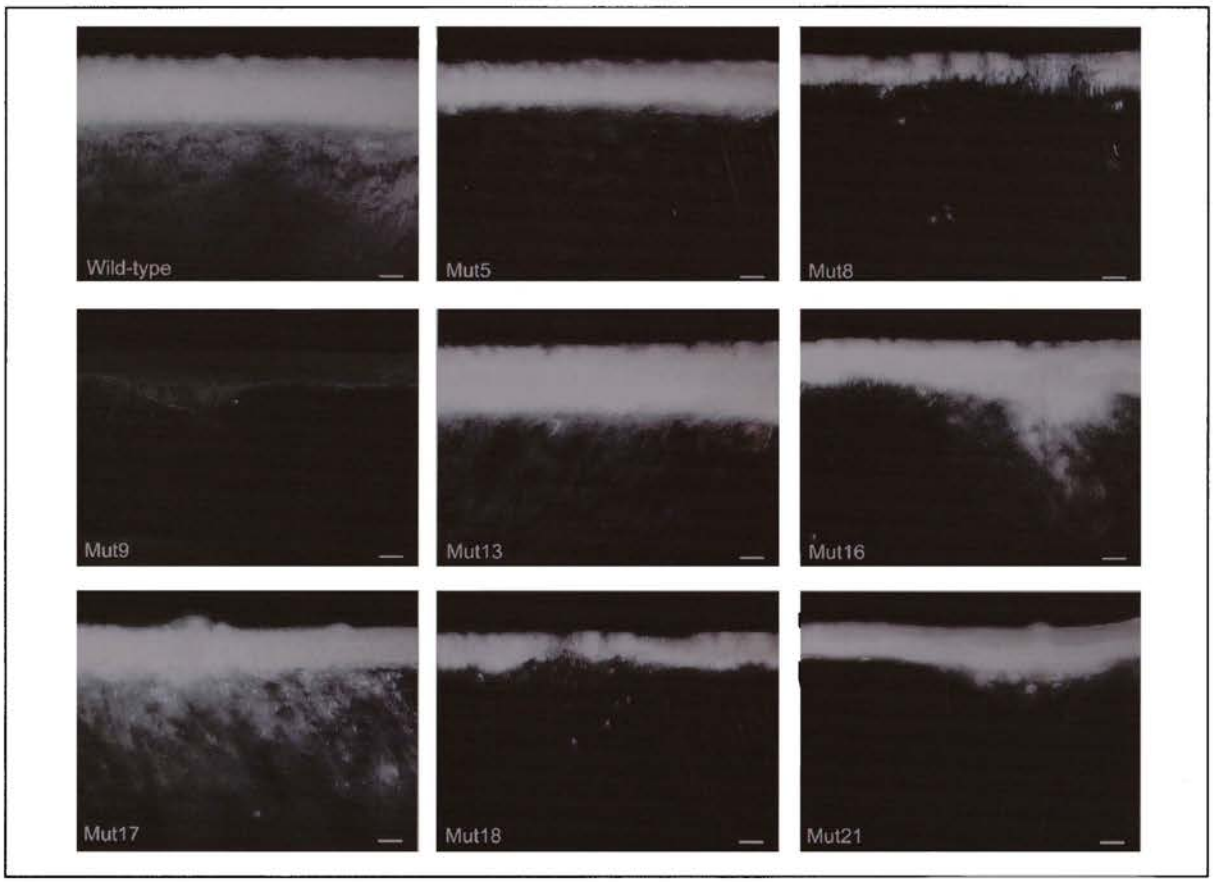

Figure 3.6: Phenotypes of the biofilms formed at the air-liquid interface on glass slides by wild-type *B. cereus* ATCC 14579 and the biofilm-impaired mutants (Mut). To assay biofilm formation, the wild-type and each mutant was inoculated into LB broth containing a microscope slide and the cultures were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

3.6.3 Motility assay of the biofilm-impaired mutant Mut21

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

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

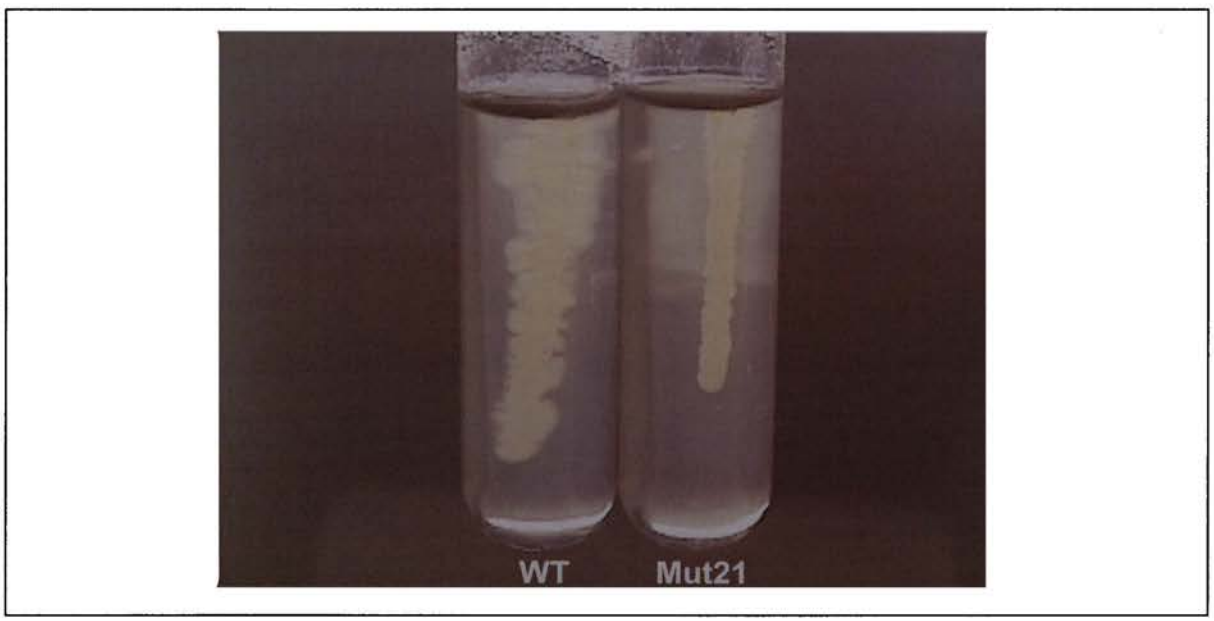

Figure 3.7: Motility assays for wild-type *B. cereus* ATCC 14579 (WT) and the *motAB* mutant strain Mut21. The cultures were stab inoculated into soft agar and the tubes were incubated overnight at 30°C.

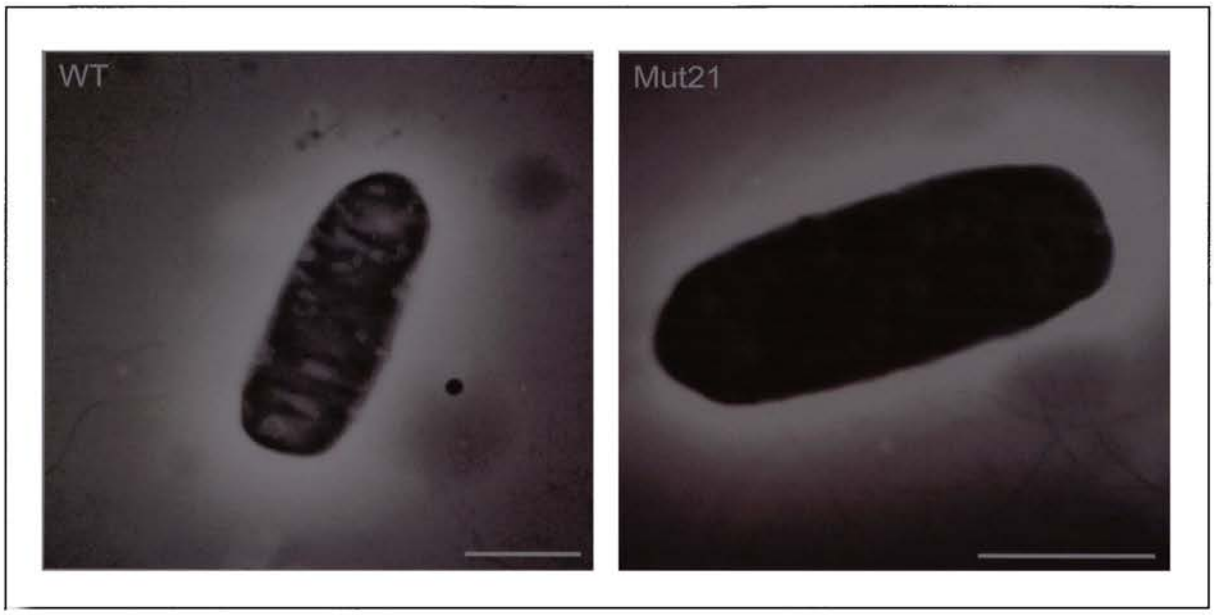

Figure 3.8: Representative transmission electron micrographs of negatively-stained cells from wild-type *B. cereus* ATCC 14579 (WT) and the *motAB* mutant strain Mut21. The bacteria were cultured for 16 h in LB broth and cells were adsorbed onto carbon-coated copper grids prior to negative staining with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

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

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

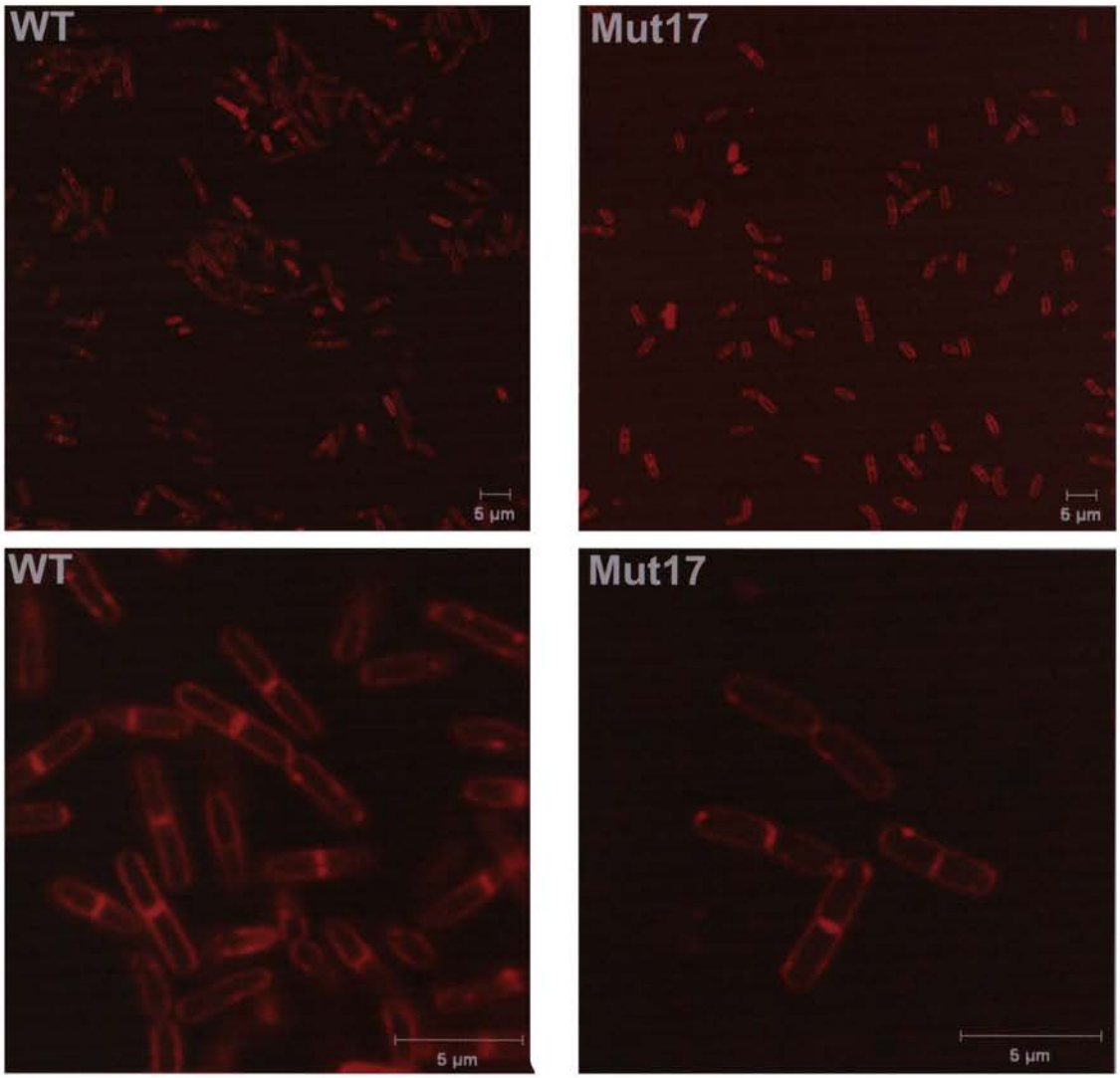

Figure 3.9: Representative laser scanning confocal micrographs of planktonic cells from wild-type *B. cereus* ATCC 14579 (left panel) and the *ftsEX* mutant strain Mut17 (right panel). The bacteria were grown for 12 h in LB broth and the cells were then stained with FM® 4-64, a lipophilic styryl dye,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

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

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

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

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

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

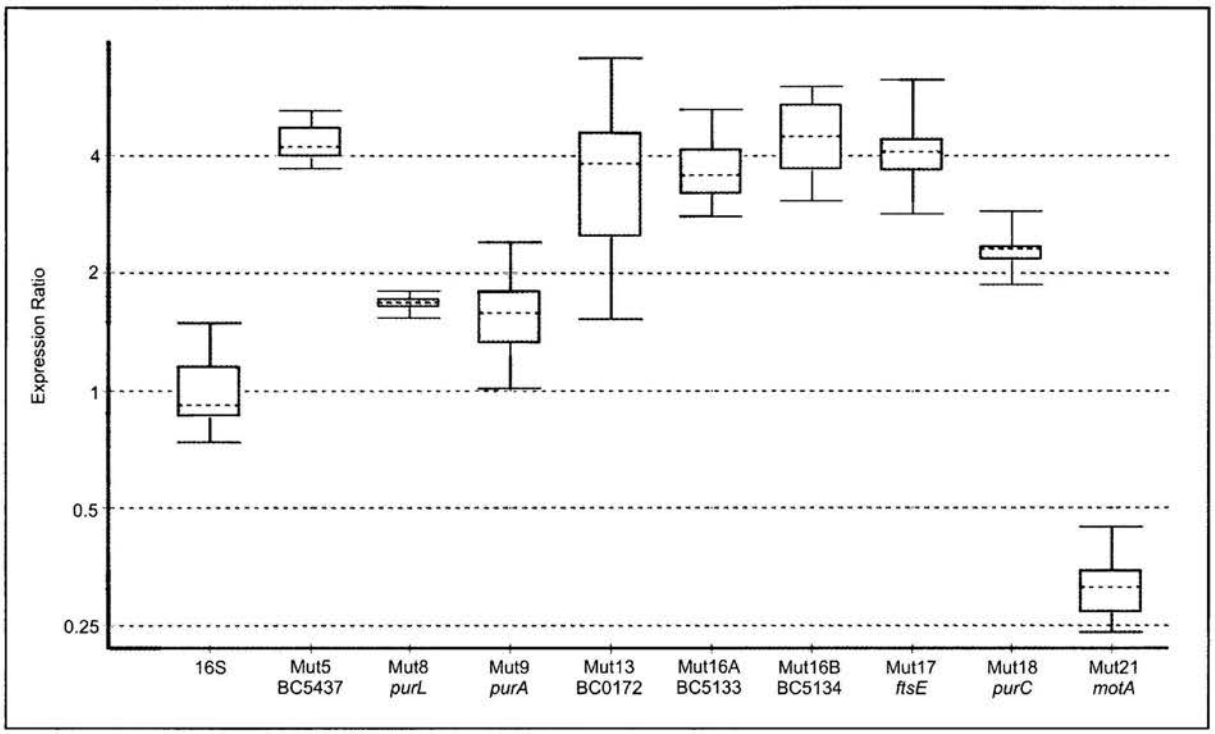

Figure 3.10: Whisker-box plot indicating the ratio of gene transcripts in a biofilm to the gene transcripts in the planktonic population quantified by quadruplicate real-time PCR reactions using the 16S rRNA gene as endogenous control. Boxes represent the interquartile range and the dotted line represents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

Table 3.1: Descriptive statistics of real-time PCR reactions

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

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