CHAPTER 4

INVOLVEMENT OF THE *htp* GENE CLUSTER OF *P. aeruginosa* DSM1707 IN BIOFILM FORMATION AND PROTEIN SECRETION

4.1   INTRODUCTION

Pseudomonas aeruginosa has been shown to assume a biofilm mode of growth, embedding itself in a gelatinuos organic polymer matrix composed of alginate (Costerton *et al.*, 1995; 1999). The biofilm bacteria display particular phenotypes that distinguish them from their planktonic counterparts (Steyn *et al.*, 2001; Sauer *et al.*, 2002). Biofilms are believed to be initiated by the attachment of a single planktonic cell on a surface followed by multiplication and the development of microcolonies separated by water-filled channels (Costerton *et al.*, 1995; 1999). The ability to form biofilms endows the bacteria with several important characteristics, including an enhanced tolerance towards stresses and a marked increase in resistance to antibiotics (Nickel *et al.*, 1985; Cochran *et al.*, 2000).

Motility is often recognized as a factor contributing to adhesion and colonization of both biological (Piette and Odziak, 1992; Scharfman *et al.*, 1996) and abiotic surfaces (Korber *et al.*, 1994; O'Toole and Kolter, 1998a; 1998b). It has been proposed that motility may play an important role to overcome electrostatic repulsive forces between the substratum surface and bacterial envelope, both negatively charged, as well as to reduce the effective radius of interaction between the surface and the cell, thereby lowering the energy barrier (Van Loosdrecht *et al.*, 1990; Marshall, 1992). Fimbriae such as thin aggregative fimbriae in *Salmonella enteritidis* (Austin *et al.*, 1998), type I pili (Pratt and Kolter, 1998) and curli (Prigent-Combaret *et al.*, 1999) in *Escherichia coli*, mannose-sensitive haemaglutinin (MshA) type IV pili in *Vibrio cholerae* El Tor (Watnick and Kolter, 1999; Watnick *et al.*, 1999) and type IV pili in *P. aeruginosa* (O'Toole and Kolter, 1998a) have been described as major structures required for either stable cell-to-surface attachment and/or cell-to-cell interactions required in the formation of microcolonies.

Over the past few years, *P. aeruginosa* has become the preferred model bacterium for studies on type IV pili (Darzins, 1994; Alm and Mattick, 1997). In addition to being considered virulence factors, the polar type IV pili of *P. aeruginosa* are multifunctional structures which play a role in adherence to, and colonization of, mucosal surfaces (Doig *et al.*, 1988; Hahn, 1997; Kang *et al.*, 1997), the initial stages of infection by bacteriophages (Rehmat and Shapiro, 1983; Roncero *et al.*, 1990; Mattick *et al.*, 1996) and twitching motility (Bradley, 1980; Darzins, 1993; Darzins, 1994; Wall and Kaiser, 1999). The type IV pili also appear to be important for adherence to abiotic surfaces, because mutations in three genes associated

with pilus formation (*pilB*, *pilC* and *pilY1*) yielded *P. aeruginosa* strains defective in attachment to polyvinyl chloride (PVC) (O'Toole and Kolter, 1998a). However, Vallet *et al.* (2001) have reported the existence of a novel cluster of genes (*cup*) specifying the components of a chaperone-usher pathway and furthermore showed that mutants devoid of a functional CupA protein are defective in the formation of biofilm, in a manner that is independent of the presence of type IV pili. These results would thus suggest that other, as yet unidentified, factors are available to *P. aeruginosa* to facilitate its binding to various surfaces.

Recently, Kachlany *et al.* (2000) identified seven *tad* genes (*tadABCDEFG*) of *Actinobacillus actinomycetemcomitans* that are required for the secretion and assembly of Flp fibrils required for tight nonspecific adherence of the bacteria to surfaces. The *flp-1* gene located upstream from *tadA* is thought to be the first gene of an apparent operon that includes the *tad* gene cluster. Non-polar mutations in the *flp-1* gene and in any of the *tad* genes resulted in a defect in adherence and failure to produce fibrils. Similarly, Nika *et al.* (2002) reported that mutations within the homologous *flp* gene cluster of *Haemophilus ducreyi* resulted in mutants that were defective in their ability to attach to both plastic and human foreskin fibroblast cells *in vitro*. A related locus in *Caulobacter crescentus*, with genes corresponding to those of the *flp-1-tadC* region, was found to be responsible for the production of novel pili of unknown function (Skerker and Shapiro, 2000). Using the available genome sequence of *P. aeruginosa* PAO1, a cluster of nine genes (*htpABCDEFGHI*) showing homology to those described above has been identified (Chapter 2), but the functional significance of this gene cluster in *P. aeruginosa* remains to be determined.

Since fimbriae other than type IV pili may play a role in attachment and biofilm formation of *P. aeruginosa*, and considering that the Flp pilus biogenesis system of both *A. actinomycetemcomitans* and *H. ducreyi* has been shown to play a role in adherence of these bacteria to abiotic surfaces, the aims of this part of the investigation were (i) to determine the role of the *P. aeruginosa htp* system in the ability of the organism to form biofilms and (ii) to determine whether the *P. aeruginosa htp* system is involved in the secretion of proteins other than the putative HtpP pili by using high resolution 2-dimensional gel electrophoresis (2DE) of extracellular protein samples.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this part of the study are listed in Table 4.1. The *P. aeruginosa* strains were maintained either on *Pseudomonas* Isolation Agar (PIA; Difco) or on *Pseudomonas* Agar Base (PAB; Sigma-Aldrich) and routinely cultivated at 37°C with shaking at 250 rpm in Luria-Bertani (LB) broth (0.5% [w/v] yeast extract, 1% [w/v] tryptone, 1% [w/v] NaCl; pH 7.4). The following antibiotics were used to maintain the plasmid DNA and chromosomal insertions in *P. aeruginosa* wild-type and mutant strains: neomycin at 150 µg/ml, carbenicillin at 150 µg/ml and gentamicin at 50 µg/ml. All antibiotics were purchased from Sigma-Aldrich.

Table 4.1 Bacterial strains, plasmids and primers used in this study

Strain, plasmid or primer	Relevant properties	Reference
Strains:		
P. aeruginosa		
DSM1707	Wild-type, Prototroph (PAO1)	DSM[#]
DSMHtpD	DSM1707*htpD*::Gm^r	This study, Chapter 3
DSMHtpE	DSM1707*htpE*::Gm^r	This study, Chapter 3
Plasmids:		
pJB-DEF-Kan	pJB3Tc20 harbouring a *lac-htpDEF* DNA fragment and a kanamycin gene as selectable marker	This study, Chapter 3
Primers:		
fD1	5' - AGAGTTTGATCCTGGCTCAGT - 3'	Weisburg *et al.* (1991)
rP2	5' - AGGGCTACCTTGTTACGACTT - 3'	Weisburg *et al.* (1991)

[#] DSM - Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany

4.2.2 Batch assays

Exponentially growing cultures of *P. aeruginosa* DSM1707, DSMHtpD and DSMHtpE were inoculated to an OD_{540} of 0.05 into 2 ml LB-broth in 28-ml McCartney bottles with or without glass wool (0.05 g; mean diameter 15 µm; Merck). The optical density at 540 nm,

culturable count and total cellular protein concentration were determined every 2 h for 12 h, and after 16 h and 26 h. For these assays, planktonic cells were obtained from cultures grown in the absence of glass wool, while cultures grown in the presence of glass wool were used as a source of biofilm and biofilm-associated cells. The culture medium of the latter cultures was carefully aspirated, transferred to a new microfuge tube and referred to as planktonic cells grown in the presence of glass wool (PGW). Following careful rinsing of the glass wool with a small volume of LB-broth, the attached (biofilm) cells were then removed from the glass wool by adding 2 ml sterile LB-broth and vortexing for 1 min. The supernatant was recovered and subsequently transferred to a new microfuge tube, and the removal of the biofilms cells from the glass wool substratum was verified by light microscopy. All of the assays were performed in triplicate.

4.2.2.1   Culturable counts

The culturable count of planktonic, PGW and attached populations was determined by plating 100 µl aliquots of serial 10-fold dilutions, prepared in ddH$_2$O, of each population onto triplicate plates of LB-agar containing the appropriate antibiotic and incubating overnight at 37°C. The colonies were counted on plates containing between 30 to 300 colonies, and then averaged.

4.2.2.2   Total protein concentration

The total protein concentration of the planktonic, PGW and attached populations was determined using a commercial kit (Coomassie plus Protein Assay Reagent; Pierce) and bovine serum albumin (BSA) as standard. The bacterial cells from the planktonic, PGW and attached samples, prepared as described above (Section 4.2.2), were collected by centrifugation at 10 000 rpm for 5 min and then suspended in 500 µl UHQ water. The cell suspensions were subsequently sonicated by three 20-s pulses using a 4710 Series Ultrasonic Homogenizer (Cole-Palmer) at an output of 40% and then heated to 95°C for 10 min. An aliquot (50 µl) of each sample was added to 1.5 ml Protein Assay Reagent, mixed well and the absorbance at 590 nm determined. UHQ water was used as a blank to zero the absorbency readings and the protein concentration (µg/ml) was then determined from the prepared standard curve.

4.2.3   Light microscopy

The ability of the *P. aeruginosa* wild-type and mutant strains to form biofilms was assayed using glass wool as an attachment substratum (Steyn *et al.*, 2001; Oosthuizen *et al.*, 2002). A single colony of each *P. aeruginosa* strain was inoculated into 10 ml LB-broth supplemented with the appropriate antibiotics and incubated at 37°C to an OD_{540} of 0.3. An aliquot of the exponentially growing cultures were then inoculated into 2 ml LB-broth in 28-ml McCartney bottles with or without glass wool (0.05 g; mean diameter 15 µm; Merck). The cultures were incubated for 16 h at 37°C with shaking and then prepared for microscopy. For microscopic analysis of planktonic cells, 15 µl of the cultures were transferred to clean microscope slides and spread evenly over the surface of the slides. For microscopic analysis of attached (biofilm) cells, sections of the glass wool were carefully transferred to microscope slides using tweezers and pryed apart carefully, taking care not to disrupt the biofilm. The cells were then stained with 0.1% (w/v) crystal violet. Coverslips were mounted onto the glass slides and sealed with silicone to prevent the samples from drying out. Analysis of the slides was performed by bright-field microscopy using a Zeiss Axiovert 200 fluorescent microscope (Carl Zeiss) and images were captured using a Nikon charge-coupled device (CCD) camera.

4.2.4   Transmission and scanning electron microscopy

For transmission electron microscopy, the bacterial cells of early exponential (2 h), mid-exponential (8 h) and stationary (24 h) phase cultures of the *P. aeruginosa* wild-type and mutant strains were collected by centrifugation at 7 000 rpm for 3 min and then fixed for 1 h at room temperature in 0.1 mM sodium cacodylate-phosphate buffer containing 0.1% gluteraldehyde. The cell pellets were washed three times, 15 min each wash, in the above buffer and post-fixed in 1% osmium tetroxide for 1 h. The pellets were washed once more with 0.1 M sodium cacodylate-phosphate buffer before being dehydrated by sequential treatment for 15 min each in 50%, 70%, 90% and 100% ethanol. The treatment with 100% ethanol was repeated twice to ensure complete dehydration of the samples. The samples were subsequently embedded in Quetol resin. This involved incubation of the cells (in fresh 100% ethanol) and resin, in a ratio of 1:2, for 1 h at room temperature, followed by the addition of an equal volume of resin and incubation for a further 1 h. After incubation in 100% resin overnight, the samples were transferred to fresh resin and mounted in embedding molds before being polymerized at 60°C for 48 h. For microscopic analysis, ultra-thin cell sections

were collected on copper grids, stained for 30 min in 5% uranyl acetate, washed in ddH_2O and counter-stained for 3 min in 3% lead citrate. The samples were then viewed with a JEOL transmission electron microscope at 60 kV.

For scanning electron microscopy, cells, fixed as described above, were filtered through a 0.2 μm filter (Millipore) and washed with 0.1 M sodium cacodylate-phosphate buffer prior to being dehydrated in ethanol (as above). The filter was then placed onto an iron grid, scatter-coated with gold and observed on a JEOL 5800LV scanning electron microscope at 25 kV.

4.2.5 Characterization of mutant cultures

In addition to performing a Gram stain and culturing of the *P. aeruginosa* wild-type and mutant strains on selective *Pseudomonas* Isolation Agar (PIA), the DSMHtpD and DSMHtpE strains were furthermore characterized by means of fluorescent *in situ* hybridization (FISH) and 16S rDNA gene analysis to confirm their identity.

4.2.5.1 Gram staining

Gram staining was performed as described by Gerhardt *et al.* (1994). Briefly, cells fixed on a clean microscope slide were stained for 1 min with crystal violet and then thoroughly rinsed under running water. The procedure was repeated once more using an iodide solution before destaining the cells by incubating the fixed cells for 30 s with acetone-alcohol. After removal of the acetone-alcohol, the cells were counter-stained for 2 min with safranin, rinsed under running water, air-dried and then examined by light microscopy.

4.2.5.2 Fluorescent *in situ* hybridization

4.2.5.2.1 Preparation of glass slides

Glass slides were prepared according to the procedure described by Amann *et al.* (1990). The microscope slides were soaked for 1 h in a 10% KOH solution prepared in ethanol after which they were thoroughly washed in distilled water and ethanol. The slides were finally rinsed in distilled water, air-dried and then coated with gelatin by soaking the slides for 30

min at 70°C in a solution containing 0.1% gelatin and 0.01% KCr(SO$_4$)$_2$. The slides were used once they were air dry.

4.2.5.2.2 Oligonucleotide probe

Probe Pseudo (5'-GCTCCGGTAGCCTTC-3'), which is covalently linked to tetramethylrhodamine at the 5' end (MWG Biotech, Germany), was used in this investigation. The probe is complementary to a region spanning nucleotides 1432-1446 on the 23S rDNA gene in *P. aeruginosa* (Amann *et al.*, 1996; MacDonald and Brözel, 2000).

4.2.5.2.3 Hybridization of whole cells

Whole cell *in situ* hybridization was performed according to the method of DiChristina and DeLong (1993). A culture of *E. coli* DH5α cells was included in these assays to serve as a negative control. Cultures of the *P. aeruginosa* wild-type and mutant strains were grown to mid-exponential phase in LB-broth supplemented with the appropriate antibiotics, after which the cells from 1.5 ml of each culture was collected by centrifugation at 7 000 rpm for 5 min. The cells were suspended in 100 µl Phosphate-buffered saline (PBS: 130 mM NaCl, 10 mM Na$_2$HPO$_4$; pH 7.2) and then fixed in 0.1 volume 35% (v/v) formaldehyde for 3 h at room temperature. A volume of 4 µl of each fixed cell suspension was applied to a gelatin-coated microscope slide and allowed to air dry prior to dehydrating the cells by sequential washes in 50%, 75% and 90% ethanol (3 min each). To facilitate subsequent hybridization procedures, gene frames (Advanced Biotechnologies), consisting of adhesive frames and polyester covers, encasing 100 mm^2 (20 µl) cell spot area on the slide, were used to prevent evaporative loss of the hybridization solution during fluorescent *in situ* hybridization. For hybridization, 10 µl of prewarmed (46°C) hybridization mixture (5 × SET, 0.01% [w/v] SDS; pH 7.8) containing 300 ng of the fluorescent-labeled probe (50 ng/µl) was pipetted onto the polyester cover (1 × SET buffer contained 0.15 M NaCl, 1 mM EDTA, 20 mM Tris; pH 7.8). The slide with the adhesive frame facing down was pressed on top of the cover. The slide was incubated with the hybridization solution for 18 h at 46°C in a 50-ml polypropylene tube floating in a temperature-regulated water bath. After hybridization, the microscope slide was washed to remove unhybridized probe by submersing the slide in 30 ml of prewarmed washing buffer (0.03 M NaCl, 0.2 mM EDTA, 4 mM Tris; pH 7.8) for 30 min at 48°C. The

hybridized samples were analyzed by epifluorescence microscopy using a Zeiss Axiovert 200 fluorescent microscope, fitted with a 63x/1.4 Zeiss Neofluor objective, and the no. 10 Zeiss filter set (excitation, 470 to 490 nm; emission, 515 to 565 nm; dichroic, 510 nm). The images were captured using a Nikon charge-coupled device (CCD) camera.

4.2.5.3   16S rDNA analysis

4.2.5.3.1 PCR amplification of the 16S rDNA gene

Chromosomal DNA of the *P. aeruginosa* wild-type and mutant strains were extracted as previously described (Section 3.2.2) and a region of the 16S rDNA gene was then amplified using universal primers fD1 and rP2 (Weisburg *et al.*, 1991) (Table 4.1). The PCR reaction mixture (50 µl) contained 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% [v/v] TritonX-100), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 25 pmol of each primer and 1 U of *Taq* DNA polymerase (Southern Cross Biotechnology). Following an initial denaturation at 94°C for 3 min, the samples were subjected to 30 cycles of amplification in a Perkin-Elmer GeneAmp 2400 thermal cycler using the following cycle conditions: denaturation at 94°C for 45 s, primer annealing at 55°C for 30 s and extension at 72°C for 1 min followed by a final extension step at 72°C for 4 min. As a control, a reaction mixture containing distilled water and all other reagents but no template DNA was included in the analysis. Following PCR amplification, aliquots of the reaction mixtures were analyzed in the presence of an appropriate molecular size marker by 1% (w/v) agarose gel electrophoresis (Section 3.2.4).

4.2.5.3.2 Nucleic acid sequencing and analysis

The amplicons were purified from the agarose gel using a silica suspension (Section 3.2.5) and the nucleotide sequence of the purified amplicons were determined using an ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Perkin-Elmer) with a Model 377 automated DNA sequencer (Perkin-Elmer), as previously described (Section 3.2.9.1). The amplified DNA fragments were sequenced in both directions using 12.5 pmol of the fD1 or rP2 primer. The identities of the strains were determined by searching known sequences in the GenBank Database using a BLASTN

homology search (Altschul *et al.*, 1997) available at the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/).

4.2.6 Two-dimensional gel electrophoretic analyses of extracellular proteins

4.2.6.1 Preparation of extracellular protein samples

Flasks containing 100 ml of LB-broth supplemented with the appropriate antibiotics were inoculated with the wild-type *P. aeruginosa* DSM1707 and mutant DSMHtpD strains and grown with shaking for 16 h at 37°C. Following incubation, the bacterial cells were removed from each culture supernatant by centrifugation at 10 000 rpm for 10 min followed by filtration of the supernatant through a 0.2 μm filter (Millipore). The extracellular proteins were then precipitated by mixing the cell-free filtrate with 4 ml of 50% [w/v] TCA (Merck) followed by incubation on ice for 30 min. The precipitate was collected by centrifugation as above, washed three times with ice-cold 70% ethanol, dried and dissolved in 30 μl sample buffer (8 M urea, 1% [v/v] TritonX-100, 15 mM DTT, 5 mM PMSF) containing SDS (9:1) (Hirose *et al.*, 2000).

4.2.6.2 Concentration of protein samples

Prior to electrophoresis, the respective protein samples were concentrated using the method of Wessel and Flugge (1984). An aliquot (20 μl) of the prepared extracellular protein samples was transferred to new microfuge tubes, mixed with 80 μl methanol and then immediately centrifuged at 5 000 rpm for 1 min. After centrifugation, 20 μl chloroform was added to each tube and briefly vortexed before centrifugation at 5 000 rpm for 30 s. The pellets were rinsed with 60 μl ddH$_2$O by vigorous vortexing of the tubes followed by brief centrifugation (9 000 rpm, 10 s). The supernatants were carefully aspirated and the tubes were incubated at room temperature for 3 min to allow all traces of the chloroform to evaporate. Following incubation, 60 μl methanol was added to each tube and the samples were centrifuged at 9 000 rpm for 4 min to collect the white protein pellet. The supernatant was discarded and the pellet dried under vacuum before being solubilized in 20 μl loading buffer (9 M urea, 65 mM DTE, 65 mM CHAPS, 5% [v/v] ampholytes; pH 3.0 - 10.0). The protein content of each sample was determined as described earlier (Section 4.2.2.2) and standardized to 10 μg/μl.

4.2.6.3   Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis (2DE) was performed according to the method of O'Farrell (1975). For 2DE electrophoresis, nonlinear isoelectric focussing (IEF) tube gels, containing 6.7% (v/v) polyacrylamide, 15 M urea and 2% (v/v) ampholytes (Ampholine pH 5.0 - 7.0 and Pharmalyte pH 3.0 - 10.0; Amersham-Pharmacia Biotech) in a 4:1 ratio, were cast in 12-cm-long glass tubes (1 mm in diameter). The gels were pre-electrophoresed at 200 V for 15 min, 300 V for 30 min and 400 V for 30 min. After pre-focusing, 20 µl of protein extract suspended in 5 µl sample buffer (9.5 M urea, 2% [v/v] Nonidet-P40, 2% [v/v] ampholytes, 5% [v/v] 2-mercaptoethanol) was loaded per gel. Gels were electrophoresed at 400 V for 16 h and then for an additional 1 h at 800 V. Upon completion of the focussing time, the tube gels containing focussed proteins were equilibrated immediately for 20 min in 5 ml treatment buffer (62.5 mM Tris-HCl, 2% [w/v] SDS, 10% [v/v] glycerol, 5% [v/v] 2-mercaptoethanol; pH 6.8). The isoelectric focusing gels were embedded onto uniform 10% SDS-polyacrylamide separating gels using a sealing solution (0.5 M Tris-HCl, 1% [w/v] SDS, 1% [w/v] agarose; pH 6.8). The second dimensional separation was performed using a Protean II electrophoresis unit (BioRad) at 5 Watt for 15 min, followed by 10 Watt for 5 h 35 min. The buffer used was the standard Laemmli buffer used for SDS-PAGE (25 mM Tris-HCl, 250 mM glycine, 0.1% [w/v] SDS; pH 8.3) (Laemmli, 1970) and a constant temperature of 18°C was maintained during electrophoresis. The pH gradient (pH 4.0 - 7.0) was determined experimentally by using the 2-D SDS-PAGE Standard from BioRad (Cat. 161-0320). The molecular masses were determined by electrophoresis of a premixed molecular weight marker (Roche), covering the 14 - 98 kDa range, in the second dimension.

4.2.6.4   Staining of 2DE gels

To allow for comparative analysis, the gels were stained with silver nitrate. For silver staining, the proteins were fixed for 3 h in a solution containing 10% acetic acid and 30% ethanol. The sensitizing step was carried out by incubating the gels twice in 10% ethanol for 10 min each time, with gentle shaking. After washing the gel three times for 10 min each in ddH$_2$O, the gels were placed in 0.02% sodium thiosulfate for 1 min and again washed thoroughly with ddH$_2$O. The silver staining reaction was performed with a 0.1% silver nitrate solution for 30 min. After thorough washing of the gel, protein spots were developed in developer solution (0.01% [v/v] formaldehyde, 1.2% [w/v] sodium carbonate, 0.008% [w/v]

sodium thiosulfate) for 20 min. For N-terminal sequencing of selected protein spots, gels were stained in a 0.3% Coomassie Brilliant Blue R-250 (Sigma) stain for 2 h and then destained overnight with a destaining solution (25% methanol, 10% acetic acid).

4.2.6.5   Image analysis

To account for experimental variations, two separate gels were prepared for each protein sample. Gel images were obtained with an Agfa T1200 scanner, re-sized and matched onto a grid similar to the system of Pederson *et al.* (1978). The number of protein spots was determined and distinct differences between patterns were noted.

4.2.6.6   N-terminal amino acid sequencing and protein identification

The regions of the Coomassie blue-stained gels containing protein spots of interest were excised and electroblotted onto Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore). For this purpose, the gel and membrane, cut to the same size as the gel, were soaked in transfer buffer (120 mM Tris, 40 mM glycine) for 15-30 min after which the proteins were transferred to the membrane at 120 mA for 2 h at 4°C, using a Trans-blot cell (BioRad). The N-terminal sequence was determined using automated Edman degradation on a Procise 492 automatic sequencer (Applied Biosystems, Courtabeuf, France). The proteins were identified using the BLASTP and TBLASTN programmes to search for homologous amino acid sequences in the *P. aeruginosa* genome database (http://www.pseudomonas.com) and in the Nonredundant Database at the National Centre for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/).

4.3   RESULTS

To investigate the functional importance of a novel putative pilus assembly and secretion system in *P. aeruginosa*, mutant strains of the wild-type *P. aeruginosa* DSM1707 strain were constructed with mutations into the *htpD*, *htpE* and *htpDEF* ORFs. The mutations were introduced by disrupting the targeted ORFs using pUC18-based allelic exchange vectors (Chapter 3). The mutant DSMHtpDEF strain was found to be severely growth-impaired and

consequently excluded from all further analyses. In order to confirm that the phenotypes of the mutant DSMHtpD and DSMHtpE strains were indeed caused by the mutation in the *htpD* and *htpE* ORFs, respectively, complementation analysis would also have to be performed. A broad-host-range plasmid, pJB-DEF-Kan, was thus constructed which would provide the mutant strains with a wild-type copy of each of the *htpDEF* ORFs under the transcriptional control of the constitutive *lac*I promoter (Chapter 3). In this part of the investigation, both the mutant DSMHtpD and DSMHtpE strains, together with the complement strains, were tested for their growth properties and capacity to form biofilms. As the Htp system of *P. aeruginosa* is proposed to be a novel secretion system, the extracellular protein profiles of the wild-type *P. aeruginosa* DSM1707 and mutant DSMHtpD strains were also compared by 2-dimensional gel electrophoresis followed by the N-terminal sequencing of selected protein spots.

4.3.1   Growth curves

Since several reports have noted that insertion mutagenesis may influence the growth properties of a particular mutant strain (Kadurugamuwa *et al.*, 1993; Hoang *et al.*, 2000; DSMHtpDEF in this study), it is possible that the observed effect following mutagenesis may be due to growth impairment of the strain rather than inactivation of a specific gene. Thus, to investigate whether the introduced mutations influenced the growth properties of the mutant strains, the wild-type *P. aeruginosa* DSM1707 as well as mutant DSMHtpD and DSMHtpE strains were cultured in LB-broth and their growth was followed by taking optical density readings at 540 nm every 2 h over a period of 12 h and then at 16 and 26 h. In addition, the total cellular protein concentration of each strain was also determined at these time intervals as a measure of their growth yield. The results indicated that the DSMHtpE strain displayed a growth rate (Fig. 4.1A) and growth yield (Fig. 4.1B) very similar to the wild-type DSM1707 strain, while the DSMHtpD strain is slightly impaired in both its growth rate (Fig. 4.2A) and growth yield (Fig. 4.2B). Providing the mutant DSMHtpE strain with a wild-type copy of each of the *htpDEF* ORFs on pJB-DEF-Kan resulted in a growth rate and yield similar to that previously observed (Fig. 4.1), but it failed to completely restore the impaired growth rate of the mutant DSMHtpD strain (Fig. 4.2).
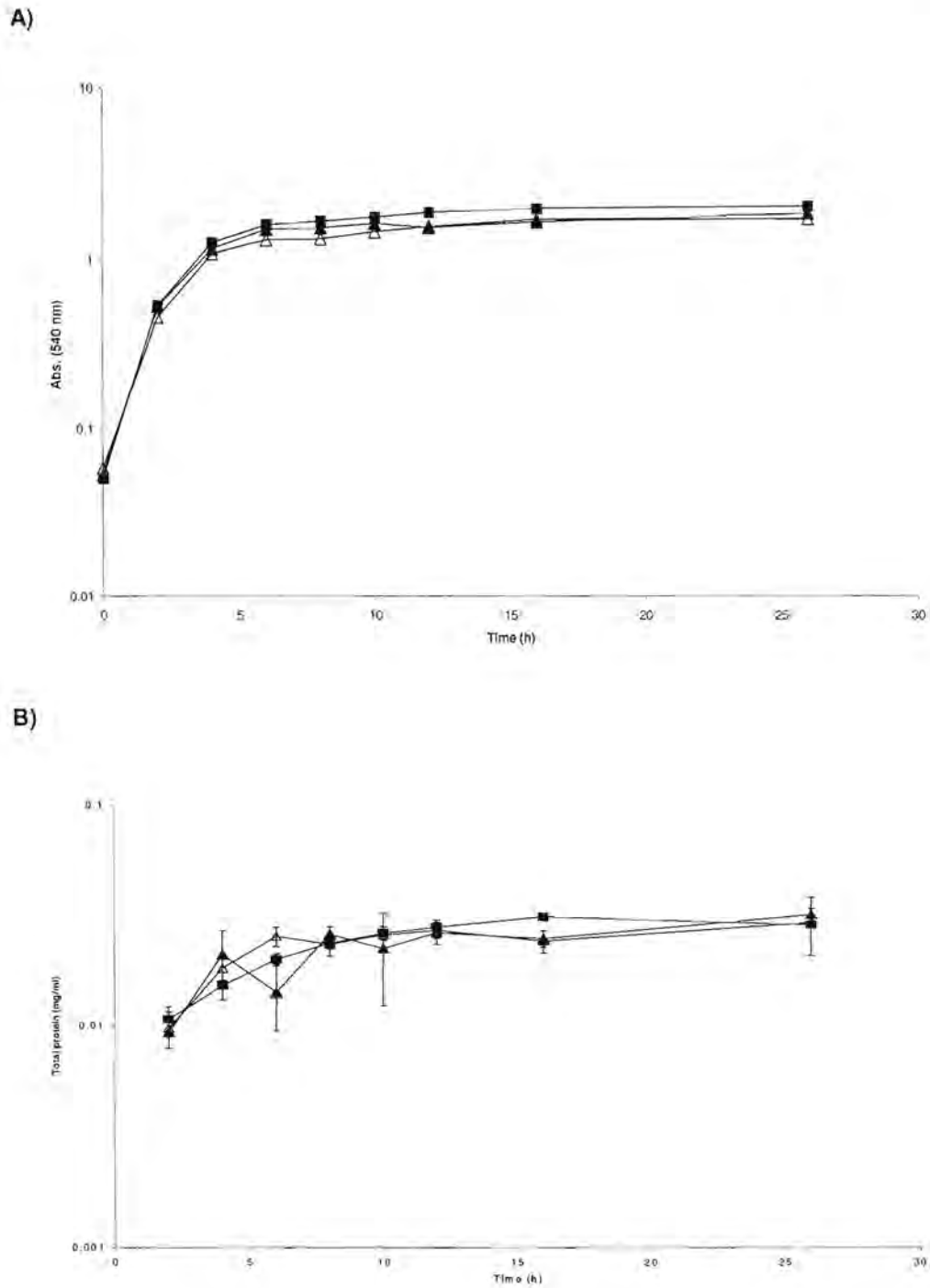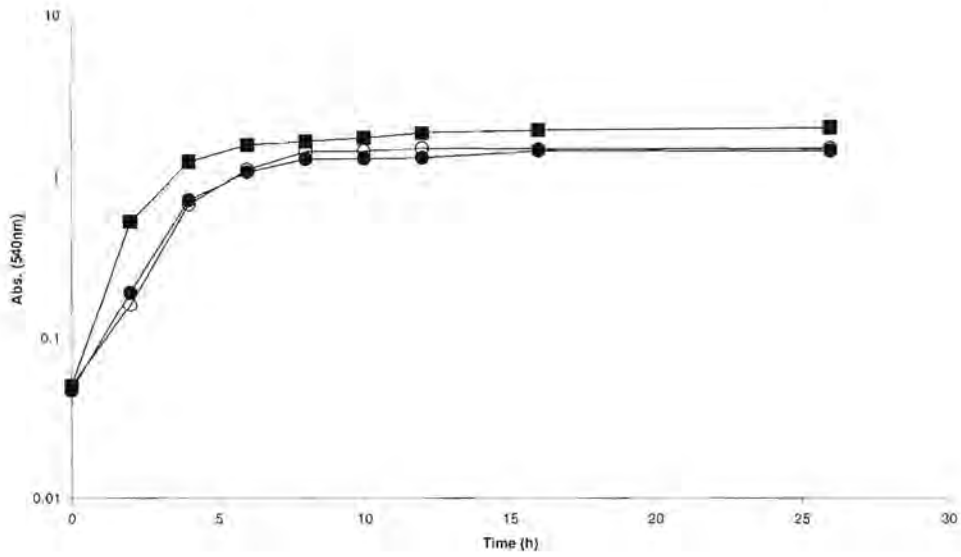
A)

B)

Fig. 4.1 Optical density (A) and total cellular protein concentration (B) of wild-type *P. aeruginosa* DSM1707 (■), mutant DSMHtpE (▲) and DSMHtpE containing plasmid construct pJB-DEF-Kan (△). Error bars denote one standard deviation of the mean.
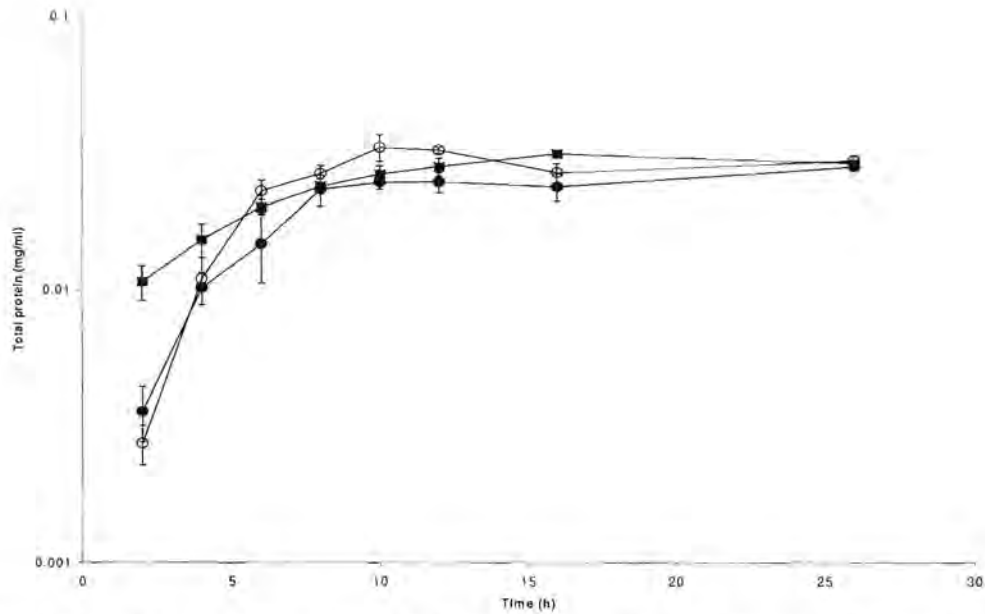
A)

B)

Fig. 4.2 Optical density (A) and total cellular protein concentration (B) of wild-type *P. aeruginosa* DSM1707 (■), mutant DSMHtpD (●) and DSMHtpD containing plasmid construct pJB-DEF-Kan (○). Error bars denote one standard deviation of the mean.

4.3.2   Microscopic analysis of biofilm phenotypes

To determine whether the DSMHtpD and DSMHtpE mutant strains were able to attach and form biofilms on glass wool as an attachment substratum, exponentially growing cultures of these strains were inoculated into LB-broth in McCartney bottles containing glass wool. Whereas the mutant strains were cultured in the presence of gentamicin, the wild-type *P. aeruginosa* DSM1707, included as a control in the analyses, was cultured in the absence of gentamicin. The glass wool was carefully recovered from cultures grown for 16 h and then analyzed by bright-field microscopy after staining with crystal violet.

The obtained results indicated that the *P. aeruginosa* wild-type and mutant strains attached to glass wool, forming biofilms within 16 h (Fig. 4.3). However, some phenotypic differences between the biofilm structures could be observed. The wild-type DSM1707 strain showed a uniform colonization of the glass wool surface punctuated with dense, thick multilayered structures (Fig. 4.3A,B). The biofilm formed by the DSMHtpE strain (Fig. 4.3C,D) resembled that of the wild-type DSM1707 strain in so far as the cells colonized the glass wool surface uniformly, but the biofilm structures were not as dense or as well defined as the biofilm structures of the wild-type DSM1707 strain. In contrast, the mutant DSMHtpD strain showed sparse colonization of the glass wool surface and the biofilm structures had the appearance of mats of cells interspersed with occasional cell clumps (Fig. 4.3G,H). Notably, the DSMHtpD cells appeared to be longer than either the wild-type DSM1707 or mutant DSMHtpE cells. Complementation of the mutant DSMHtpE (Fig. 4.3E,F) and DSMHtpD (Fig. 4.3I,J) strains with plasmid pJB-DEF-Kan *in trans* did not result in restoration of the mutant biofilm phenotypes to wild-type levels.

4.3.3   Characterization of the mutant cultures

To exclude the possibility that the observed difference in cell size between the DSMHtpD and wild-type DSM1707 strains was due to contamination of the DSMHtpD culture, the cells of wild-type DSM1707 and the mutant DSMHtpD strains were subjected to whole cell fluorescent *in situ* hybridization. The cells from the respective strains all stained Gram-negative (results not shown), and epifluorescence microscopy of the hybridized samples indicated that the fluorescent *Pseudomonas*-specific Pseudo probe hybridized to the wild-type DSM1707 and DSMHtpD cells. No fluorescence was detected in the control sample

consisting of *E. coli*, thereby confirming the probe specificity (Fig. 4.4). In order to furthermore confirm the identity of the respective mutant DSMHtpD and DSMHtpE strains, a partial 16S rDNA sequence of each strain was determined. The partial 16S rDNA sequence of the DSMHtpD (836 bp) and DSMHtpE (604 bp) strains displayed 97 and 99% identity, respectively, to the 16S rDNA sequence of *P. aeruginosa* PAO1. These results thus confirmed that the mutant DSMHtpD and DSMHtpE strains were indeed derived from *Pseudomonas aeruginosa* and that the difference in cell size of the DSMHtpD strain may have been a direct or indirect consequence of the mutation being introduced into the *htpD* ORF.

4.3.4   Biofilm development on glass wool

To further investigate the propensity of the DSMHtpD and DSMHtpE mutant cells to attach and grow as biofilms, the ratio of attached (biofilm) to suspended (PGW) biomass was calculated. To investigate, the optical density at 540 nm and total cellular protein concentration of the attached (biofilm) and suspended (PGW) cells of exponentially growing cultures in McCartney bottles with glass wool were determined every 2 h for 12 h and after 16 and 26 h of culturing. Whereas the PGW cells were obtained by careful aspiration of the culture fluid, the attached (biofilm) cells were recovered from the glass wool by vortexing as described in Section 4.2.2.

The obtained results indicated that cells of the mutant DSMHtpE strain was impaired in its ability to attach to the glass wool substratum, as determined by optical density (Fig. 4.5A) and total cellular protein concentration (Fig. 4.5B) measurements. In contrast to cells of the wild-type DSM1707 strain, which were capable of attaching to the glass wool substratum after 2 h of culturing, cells of the DSMHtpE strain attached to the glass wool substratum only after 4 h of culturing. However, following attachment, cells of both the wild-type DSM1707 and mutant DSMHtpE strains displayed a propensity to grow as biofilms. This is evidenced by an increase in the ratio of attached to suspended biomass of both strains, albeit to a slightly lesser degree in the case of the DSMHtpE strain compared to the DSM1707 strain.

The ratio of attached to suspended biomass was greater for the wild-type DSM1707 strain than for the mutant DSMHtpD strain, as measured by optical density (Fig. 4.6A) and total cellular protein concentration (Fig. 4.6B). However, similar ratios of attached to suspended

DSM1707

A)

B)

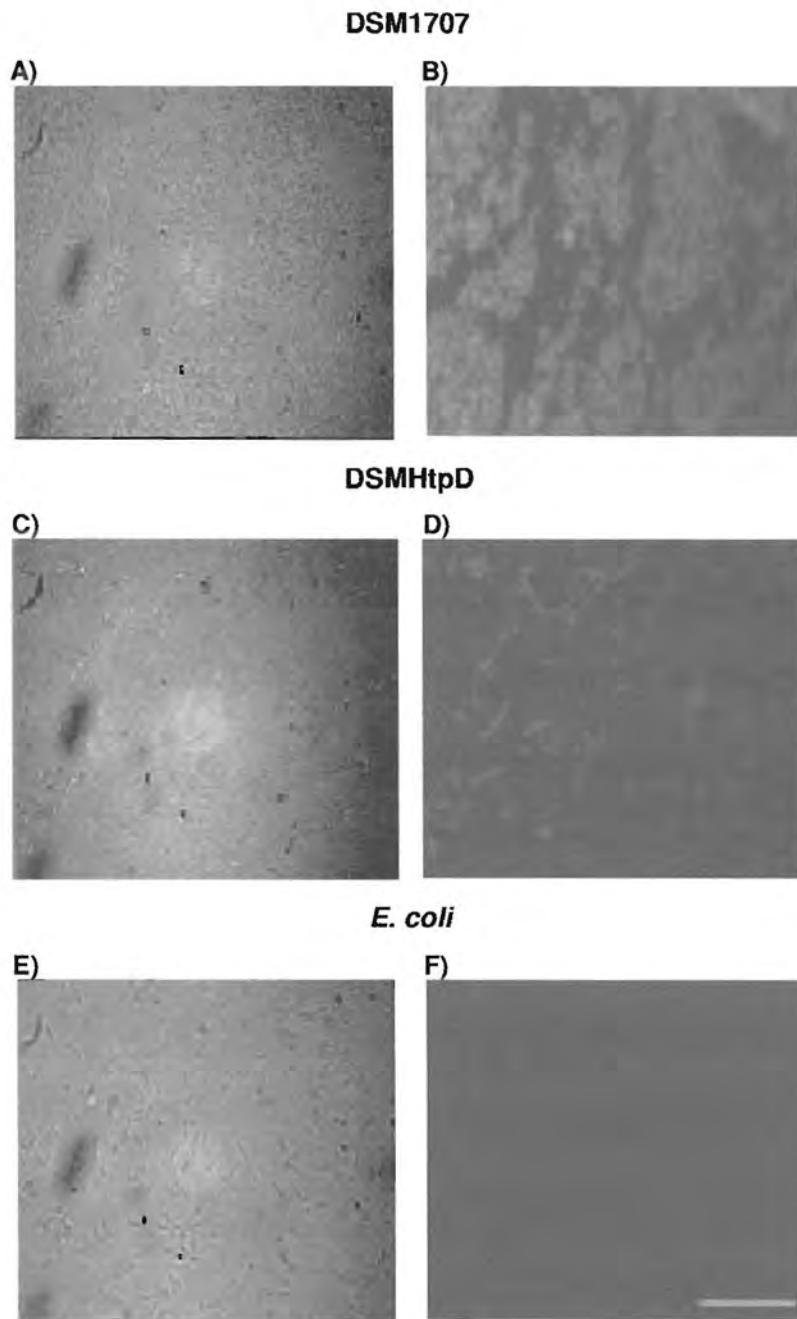**DSMHtpD**

C)

D)

E. coli

E)

F)

Fig. 4.4   *In situ* hybridization of cells of wild-type *P. aeruginosa* DSM1707 and mutant strains. Cells of DSM1707 (A,B), DSMHtpD (C,D) and *E. coli* (E,F) were hybridized with the tetramethylrhodamine-labeled Pseudo probe. The left panel shows phase contrast images and the right panel shows epifluorescence micrographs of the corresponding fields. The bar represents 20 µm and applies to all of the images.

biomass were observed for both strains after 2 h of culturing, indicating that the cells of the DSMHtpD strain were capable of attaching efficiently to the glass wool substratum. The cells were, however, less prone to occur in a biofilm when compared to the wild-type DSM1707 cells. Although a similar trend was observed based on total cellular protein concentration measurements, these results indicated much less of the mutant cells attached to glass wool substratum compared to the DSM1707 cells. This discrepancy between the optical density and protein concentration data may be due to the longer length of the cells yielding higher protein concentration values although there are fewer cells present. From these results it was concluded that the DSMHtpE strain is impaired in its ability to attach to the glass wool surface but once attached, is capable of growing as a biofilm. In contrast, the DSMHtpD strain was capable of attaching to the glass wool surface, but was impaired in its ability to grow as a biofilm.

4.3.5 Electron microscopic characterization of cellular morphology

4.3.5.1 Scanning electron microscopy

The results obtained by bright-field microscopy regarding the ability of the *P. aeruginosa* wild-type and mutant strains to form biofilms on glass wool indicated that the DSMHtpD cells displayed an altered cellular morphology, being longer than the cells from the wild-type DSM1707 and mutant DSMHtpE cultures (Fig. 4.3). To investigate, cells from 16-h old cultures of the wild-type *P. aeruginosa* DSM1707, as well as mutant DSMHtpD and DSMHtpE strains, were examined by scanning electron microscopy as described under Materials and Methods (Section 4.2.4).

The results, presented in Fig. 4.7, revealed distinct morphological differences between the cells of strain DSMHtpD and those of the wild-type DSM1707 and mutant DSMHtpE strains. The cells of the wild-type DSM1707 strain (Fig. 4.7 A,B), as well the cells of the mutant DSMHtpE (Fig. 4.7C,D) and complemented (Fig. 4.7E,F) strains, were short straight rods similar in size to each other (1.5 µm in length and 0.4 µm in width). In contrast, cells from the mutant DSMHtpD culture (Fig. 4.7G,H) consisted mostly of cells that were typically at least twice the length of the wild-type DSM1707 cells, despite displaying a similar width (0.5 µm). The length of these rod-shaped cells varied between 3 to 17 µm. Complementation of the mutant DSMHtpD strain with the recombinant plasmid pJB-DEF-Kan did not result in
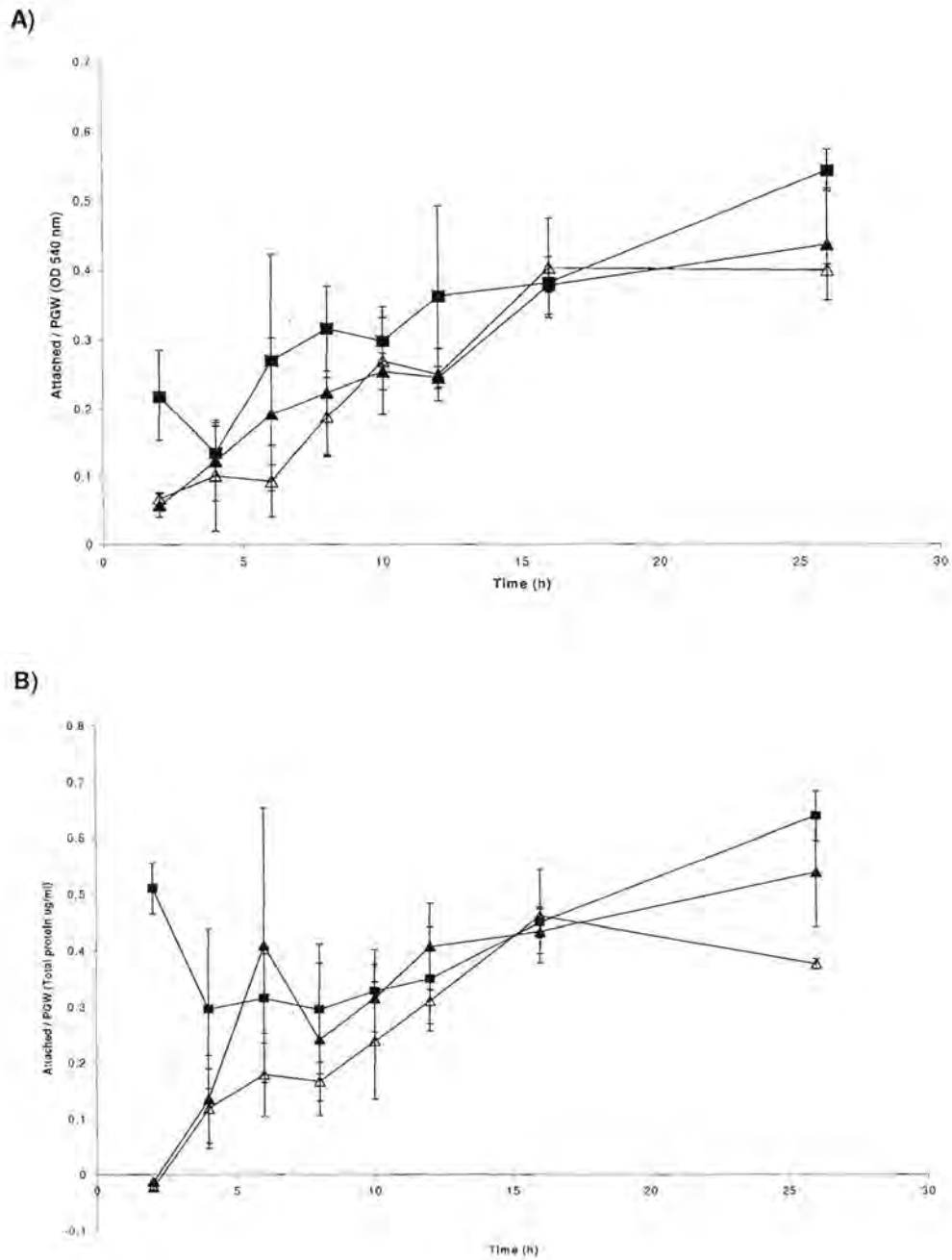
A)

B)

Fig. 4.5   Propensity of DSMHtpE cells to occur as a biofilm. The ratio of attached (biofilm) over planktonic cells grown in the presence of glass wool (PGW) is indicated for wild-type *P. aeruginosa* DSM1707 (■), mutant DSMHtpE (▲) and DSMHtpE containing the plasmid construct pJB-DEF-Kan (△), as measured by optical density (A) and total cellular protein concentration (B). Error bars denote one standard deviation of the mean.
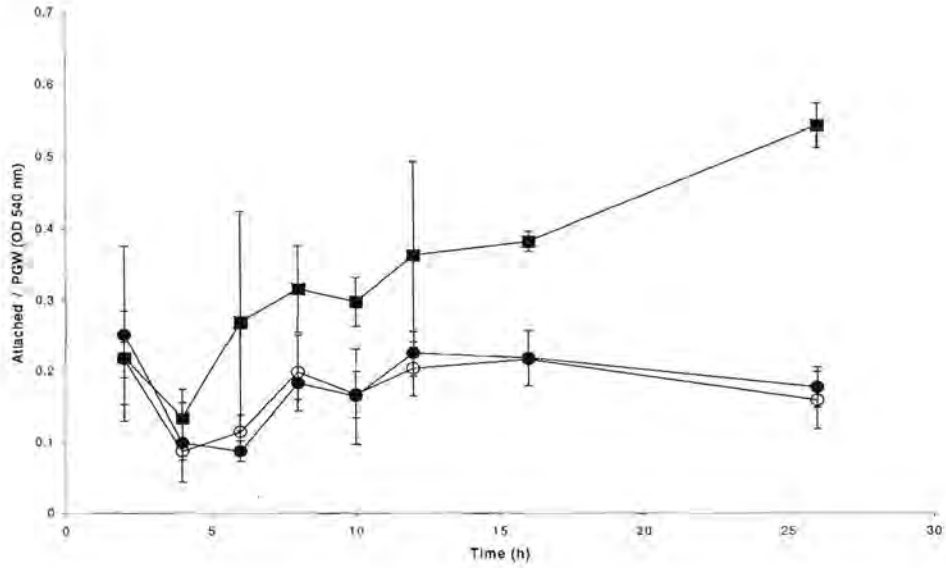
127

A)

B)

Fig. 4.6 Propensity of DSMHtpD cells to occur as a biofilm. The ratio of attached (biofilm) over planktonic cells grown in the presence of glass wool (PGW) is indicated for wild-type *P. aeruginosa* DSM1707 (■), mutant DSMHtpD (●) and DSMHtpD containing the plasmid construct pJB-DEF-Kan (○), as measured by optical density (A) and total cellular protein concentration (B). Error bars denote one standard deviation of the mean.
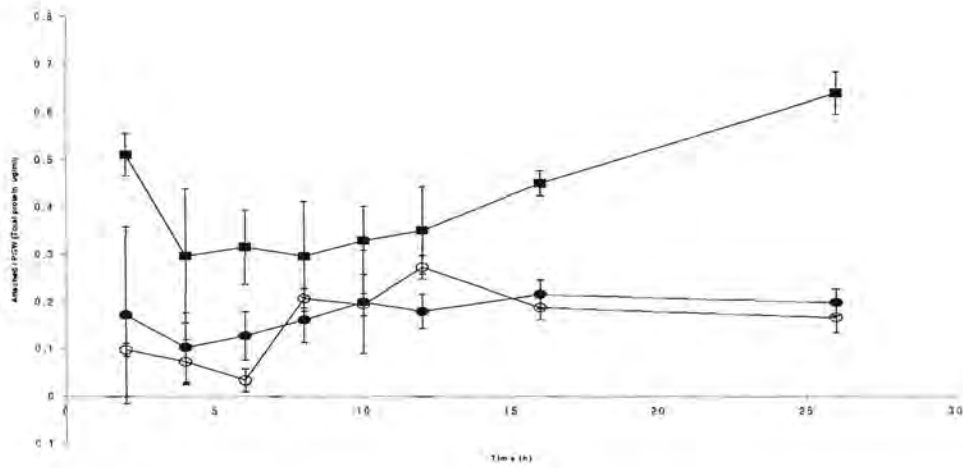
restoration of the cellular morphology to the wild-type phenotype (Fig. 4.7I,J). The cells of the complemented strain were still longer than those of the wild-type DSM1707 and mutant DSMHtpE strains.

A compound appearing as amorphous foamy white material, present on both the bacterial cell surface and filter, was detected in DSM1707, DSMHtpE and DSMHtpD cells (Fig. 4.7B,D and H, respectively). Treatment of the samples with 20 µg/ml Proteinase K or 20 units of DNase I did not result in removal of the material (results not shown). The possibility that the material may be extracellular polysaccharide substances (EPS) remains to be determined. In addition, structures appearing as "blebs" or vesicles were observed on cell surfaces of the wild-type DSM1707, as well as mutant DSMHtpE and DSMHtpD strains (Fig. 4.7K,L and M, respectively).

4.3.5.2 Transmission electron microscopy

It was thought that the difference in cell size between the DSMHtpD and wild-type DSM1707 as well as mutant DSMHtpE cells might, in some way, be related to an inability of the DSMHtpD cells to secrete HtpP pili and/or other proteins. The cells from early exponential, mid-exponential and stationary phase cultures of the *P. aeruginosa* wild-type and mutant strains, as well as complemented mutant strains, were therefore processed for electron microscopy and then viewed with a JEOL transmission electron microscope.

Although the cells from early exponential phase cultures of the *P. aeruginosa* wild-type and mutant strains displayed a similar cellular morphology and size (not shown), the cells from mid-exponential and stationary phase cultures of the DSMHtpD strain differed notably from the other two strains (Fig. 4.8A). The cells appeared to increase in length, and a granular substance could be seen accumulating in the cytoplasm of DSMHtpD cells from a mid-exponential culture, which become more electron-dense and compact in cells of the stationary phase culture. This granular material appeared to be absent at either one or both poles of the cells and the double-layered membrane structure remained intact, indicating an absence of the material in the periplasmic space (Fig. 4.8B). No similar electron-dense material could be observed in cells of the wild-type DSM1707 or mutant DSMHtpE strains. Complementation of the mutant strains with plasmid pJB-DEF-Kan *in trans* did not result in altered phenotypes and the cells resembled the mutant cells.

4.3.6 Two-dimensional gel electrophoresis of extracellular proteins

To characterize the extracellular protein differences between wild-type *P. aeruginosa* DSM1707 and the mutant strain DSMHtpD, high-resolution 2D gel electrophoresis was performed on 16-h old cultures (Fig. 4.9). The reproducibility of separation was high, and 360 distinct protein spots and 349 distinct protein spots for wild-type *P. aeruginosa* DSM1707 and DSMHtpD, respectively, were observed in the pH range from 4 to 7 after silver staining. By matching and comparing the respective 2-DE protein patterns, 20 protein spots were uniquely present in DSMHtpD (*i.e.* not present or could not be detected by silver stain in the DSM1707 pattern), while the wild-type *P. aeruginosa* DSM1707 strain displayed 28 unique protein spots. Five protein spots were selected for N-terminal protein sequencing. Protein spots DM1 and DM2 were obtained from the 2D gel of extracellular proteins from DSMHtpD, while protein spots WT1, WT2 and WT3 were obtained from the 2D gel of the wild-type DSM1707 strain. The obtained amino acid sequences were subsequently subjected to database searches as described under Materials and Methods (Section 4.2.6.6). The results revealed that 4 spots displayed 100% homology with previously identified proteins, while the identity of 1 spot (WT2) could not be determined, as an unambiguous amino acid sequence could not be obtained due to contamination of the excised protein spot with other proteins. The results of this analysis are summarized in Table 4.2.

The amino acid sequence derived from protein spot DM1 corresponded to the sequence of β-lactamase. This enzyme catalyzes the hydrolysis of an amide bond in the β-lactam ring of antibiotics belonging to the penicillin/cephalosporin family (Bush, 1989). There are four groups of β-lactamase enzymes, classed A, B, C and D according to sequence, substrate specificity and kinetic behavior (Knox and Moews, 1991). The class A (penicillinase-type) is the most common and the genes for class A β-lactamases are widely distributed in bacteria, frequently located on transmissible plasmids in Gram-negative organisms, although an equivalent chromosomal gene has been found in a few species, *e.g. Rhodopseudomonas capsulata* (Scahill *et al.*, 1989).

The amino acid sequence derived from protein spot DM2 was determined to be the sequence of the alpha chain (RpoA) (PA4238) of the DNA-dependent RNA polymerase enzyme (RNAP). This enzyme catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphatases as substrates and the RNAP catalytic core consists of two alpha (α), one beta (β) and one beta prime (β') subunit. The alpha (α) subunit consists of two independently folded domains, referred to as amino-terminal and carboxy-terminal domains. Whereas the amino-terminal domain is involved in the interaction with the other subunits of the RNA polymerase, the carboxyl-terminal domain interacts with the DNA and transcriptional activators (Busby and Ebright, 1995; Jeon *et al.*, 1995; Darst and Zhang, 1998).

The amino acid sequence derived from protein spot WT1 corresponded to GroEL (PA4385). The oligomeric GroEL protein is a member of a family of molecular chaperones with ATPase activity that prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides synthesized under stress conditions (Zeilstra-Ryalls *et al.*, 1991). Generally, GroEL is located in the cytoplasm of cells, but its distribution seems to vary with the organism. The GroEL protein of *H. ducreyi* has been shown to be present on the cell surface (Frisk *et al.*, 1998), while it has been reported to be associated with the outer membrane surface of *Helicobacter pylori* (Phadnis *et al.*, 1996) and *Salmonella typhimurium* (Ensgraber and Loos, 1992). In *A. actinomycetemcomitans*, most of the GroEL protein in stressed (heat-shocked) cells has been shown to be present in the extracellular medium (Goulhen *et al.*, 1998). In *H. ducreyi*, decreased adherence of the bacterial cells to human genital cell lines was shown by lowering of the level of GroEL, and it was suggested that GroEL directly or indirectly affects adherence (Parsons *et al.*, 1997). In *S. typhimurium*, a role for GroEL involved in interaction with intestinal mucus has been suggested (Ensgraber and Loos, 1992), and in *A. actinomycetemcomitans*, the GroEL protein was reported to be toxic for periodontal ligament epithelial cells, suggesting that the protein may play a role in disease initiation (Goulhen *et al.*, 1998).

The amino acid sequence derived from protein spot WT3 was determined to be the sequence of RpsA (PA3162), which is identical to the S1 protein of the 30S subunit of prokaryotic ribosomes. The ribosomes catalyze mRNA-directed protein synthesis in all organisms. Approximately $^2/_3$ of the mass of the ribosome consists of RNA and $^1/_3$ of protein. The proteins are named in accordance with the subunit of the ribosome that they belong to - the

small (S1 to S31) and the large (L1 to L44) - and they usually decorate the rRNA cores of the subunits (Chandrasanyal and Liljas, 2000; Maguire and Zimmermann, 2001). While the crucial activities of decoding and peptide transfer are RNA-based, the proteins play an active role in functions that may have evolved to streamline the process of protein synthesis (Maguire and Zimmermann, 2001). The ribosomal protein S1 is organized into at least two distinct domains; a ribosome-binding domain at the N-terminal region and a nucleic acid-binding domain at the C-terminal region, which is referred to as the S1 domain and has been found in a large number of RNA-associated proteins (Subramanian, 1983). It has consequently been suggested that S1 is a RNA-binding protein helping polynucleotide phosphorylase (PNPase) to degrade mRNA, or it may serve as a helper molecule involved in other RNase activities (Danchin, 1997).

4.4 DISCUSSION

In this study, a cluster of genes (*htp*) was identified in *P. aeruginosa* that encodes products with homology to proteins involved in the formation of novel pili in other bacteria, amongst them *A. actinomycetemcomitans*, *H. ducreyi* and *C. crescentus* (Chapter 2). Whereas these pili are associated with the ability of *A. actinomycetemcomitans* to bind non-specifically to inert surfaces (Haase *et al.*, 1999; Kachlany *et al.*, 2000), inactivation of any one of the genes encoding either the pilus monomer or assembly/secretion proteins resulted in a reduced ability of *H. ducreyi* to attach to both biotic and abiotic surfaces (Nika *et al.*, 2002).

To determine whether inactivation of ORFs in the *htp* gene cluster of *P. aeruginosa* DSM1707 had an effect(s) similar to that observed with the *tad* loci of *A. actinomycetemcomitans* and *H. ducreyi*, the mutant *P. aeruginosa* DSMHtpD and DSMHtpE strains were tested for their ability to bind to an abiotic surface using glass wool as an attachment substratum. Although both of the mutant strains were found to be capable of forming biofilms on glass wool within 16 h of culturing, inspection of the biofilm phenotype revealed that the wild-type *P. aeruginosa* DSM1707 and the mutant DSMHtpE strains were both able to form copious amounts of structured biofilms, while the DSMHtpD mutant displayed sparse colonization of the glass wool surface and the cells were localized in clusters. More detailed analysis of the defects conferred by the mutations in the *htpD* and *htpE* ORFS was obtained through determining the ratio of attached (biofilm) to suspended

Table 4.2 Summary of the extracellular proteins identified from 2DE gels and unique to either *P. aeruginosa* DSM1707 and mutant DSMHtpD

Strain	Protein spot	Protein identity	Function	N-terminal sequence	Estimated from gel		Calculated from sequence		Accession nr.*
					Size (kDa)	pI	Size (kDa)	pI	
DSMHtpD	DM1	Beta-lactamase	Hydrolysis of amide bond in β-lactam ring of certain antibiotics	HPETLVKVKD	27	5.6	28.9	5.6	P00810
DSMHtpD	DM2	α-subunit of RNA polymerase (RpoA)	Facilitates interaction with RNA polymerase subunits, and binding to DNA and transcriptional factors	MQGSVTEFL	41	4.8	39.5	5.1	PA4238
DSM1707	WT1	GroEL	Prevents misfolding and promotes the refolding and proper assembly of unfolded proteins	KEVKFGDSAR	62	4.9	57	5.0	PA4385
DSM1707	WT2	Unknown		[FTL][IP][HA][WV][VTL][PTW][FVG]T	44	5.3	-	-	
DSM1707	WT3	S1 protein of the 30S ribosomal subunit (RpsA)	Binds to ribosome and unwinds mRNA structures before entry of the mRNA into ribosome	ESFAELFEE	69	4.6	62	4.8	PA3162

* *P. aeruginosa* database (http://www.pseudomonas.com)

(PGW) biomass over a period of 26 h of culturing. Notably, the attached cells of the *P. aeruginosa* wild-type and mutant cells experienced an apparent lag phase in growth following their initial attachment to the glass wool substratum. This lag phase was shorter (4 h) for wild-type DSM1707 compared to DSMHtpE (6 h) and DSMHtpD (6 h). The lag phase may represent a period of time whereby the primary colonizing cells adapt to a sessile environment before they divide (Rice *et al.*, 2000), and this phenomenon has also been observed for *P. putida* growing as biofilm on silicon tubing (Sauer and Camper, 2001). However, after this initial lag the ratio of attached to suspended (PGW) cells of DSMHtpE increased over time, as measured by optical density and total cellular protein concentration, indicating bacterial growth on the surface of the glass wool. In contrast, DSMHtpD did not display such an increase following attachment and the ratio of attached to suspended (PGW) cells were much lower compared to wild-type DSM1707. Cumulatively, the obtained results indicate that whereas the mutant DSMHtpE strain is impaired only in its ability to attach glass wool, the mutant DSMHtpD strain is biofilm-deficient.

Since both strains were still able to attach to the glass wool surface, the DSMHtpD and DSMHtpE mutant strains were tested for their twitching motility phenotypes by a subsurface agar assay (Darzins, 1993; Beatson *et al.*, 2002). The wild-type *P. aeruginosa* DSM1707 and mutant strains displayed twitching motility, although the twitch zone of DSMHtpD, *i.e.* the area containing cells that have used type IV pilus-mediated twitching motility to move away from the point of inoculation, was consistently smaller for this strain than that of the wild-type DSM1707 and DSMHtpE mutant strains (results not shown). However, the surface colony also exhibited reduced spreading, which is in agreement with the observed impaired growth rate of the mutant DSMHtpD strain compared to that of the other strains (Fig. 4.2). Consequently, it can be proposed that the ability of the DSMHtpE and DSMHtpD mutant strains to interact with the glass wool surface may be due to the presence of functional type IV pili, but it does not totally exclude the possibility of an auxiliary role played by other pili such as HtpP. The impaired ability of DSMHtpD to grow as a biofilm could have been due to a lower growth rate.

During the course of the above investigations, the cells of the DSMHtpD strain were consistently found to be larger than cells from both the wild-type DSM1707 and mutant DSMHtpE strains. This was unexpected, as reports by Kachlany *et al.* (2000; 2001) and Nika *et al.* (2002) did not mention any alterations in the cellular morphology of mutant *A.*

actinomycetemcomitans or *H. ducreyi* strains. Nevertheless, scanning and transmission electron microscopic examination of cells from wild-type DSM1707 and the mutant DSMHtpE and DSMHtpD cultures confirmed that the DSMHtpD cells presented an altered morphology, the cells being at least twice as long as those of either the wild-type DSM1707 or mutant DSMHtpE strains. Due to the novelty and unexpected nature of these results, the purity of the DSMHtpD strain was confirmed by two independent methods, namely fluorescence *in situ* hybridization using a *Pseudomonas*-specific probe and 16S rDNA gene analysis. The obtained results confirmed DSMHtpD to be *P. aeruginosa*. Thus, the phenotypic differences between the *P. aeruginosa* wild-type and mutant strains could be attributed to the inactivation of the *htpD* ORF rather than it being the result of contaminated cell cultures.

Using SEM, a white foamy material surrounding and associated with the *P. aeruginosa* wild-type and mutant cells was observed, which was more evident on cells from older cultures (stationary phase cultures). Since both Proteinase K and treatment with DNase I did not lead to removal of this material, the possibility that it may be exopolysacharides cannot be excluded. Exopolysaccharides may play a role in *P. aeruginosa* adherence and biofilm formation by serving to overcome electrostatic repulsive forces between the substratum surface and the bacterial envelope (Van Loosdrecht *et al.*, 1990; Marshall, 1992). In recent reports, characterized non-mucoid mutants have been used to demonstrate the involvement of capsular polysaccharides in promoting the adhesion process in *S. epidermidis* (Muller *et al.*, 1993) or stabilizing the three-dimensional biofilm structure in *V. cholerae* (Watnick and Kolter, 1999) and *P. aeruginosa* (Nivens *et al.*, 2001). In addition, membrane vesicles ("blebs") could be observed by SEM on the surfaces of wild-type *P. aeruginosa* DSM1707 and mutant DSMHtpE and DSMHtpD cells. The existence and release of membrane vesicles during normal growth in culture has been previously reported for *P. aeruginosa* (Kadurugamuwa and Beveridge, 1995), as well as some other Gram-negative bacteria such as *Bacteroides* spp. (Mayrand and Holt, 1988), *Borrelia burgdorferi* (Whitmire and Garon, 1993) and *H. influenzae* (Wispelwey *et al.*, 1989). In *P. aeruginosa*, the natural release of membrane vesicles was increased 3-fold on exposure of the organism to the antibiotic gentamicin and it was proposed that the membrane vesicles may serve as a means whereby proteins, DNA and enzymes are excreted from the cells (Kadurugamuwa and Beveridge, 1995).

The analysis of thin sections of the cells from P. aeruginosa wild-type and mutant strains by TEM revealed the presence of granular electron-dense material in the cells of DSMHtpD only, which became more dense in stationary phase cultures compared to mid-exponential phase cultures (Fig. 4.8). It is tempting to speculate that the more drastic effect caused by inactivation of the htpD ORF, compared to the inactivation of the htpE ORF, could, in some way, have resulted in the intracellular accumulation of the proteins, amongst them HtpP prepilin, and consequently contributed to the cells becoming larger. However, such a conclusion awaits the production of anti-HtpP antibodies, which could be used in immuno-electron microscopical analysis to determine whether HtpP prepilin proteins are present in the electron-dense intracellular material. Alternatively, it may be that inactivation of the htpD ORF could have disturbed cell division to such an extent that the morphogenesis of the bacteria was affected, thereby resulting in larger cells. Cell division follows chromosome replication and involves separation of the daughter chromosomes and movement to the centre of the prospective daughter cells (partitioning), followed by septum formation and cell separation (Schmid and von Freiesleben, 1996). The lack of cell envelope invaginations and/or septa (Fig. 4.8) suggests that the cell division cycle might have been affected at the partitioning phase. Thus, the granular material observed in the cells may also contain unsegregated genomic DNA. Additionally, a specific structure, the periseptal annulus, has been found in the middle of cells that might define a compartment embracing the region where invagination of the cell envelope takes place (Schmid and von Freiesleben, 1996). In this regard, it is interesting to note that the PA4303 (htpC) gene displays homology to a probable septum site-determining protein from Mesorhizobium loti (GenBank accession no. NP102599.1; 47% amino acid similarity over 117 amino acids). Taken together, these results may be reminiscent of those obtained with Par- mutants, which are characterized by aberrant nucleoid morphology and difficulty in separating the daughter nucleoids (Schmid and von Freiesleben, 1996). The possibility that the granular material observed in DSMHtpD cells may represent unsegregated genomic DNA could be investigated by staining unfixed cells with DAPI (4',6-diamidino-2-phenylindole) for visualization of the DNA

In this study, no pili on the surface of DSM1707, DSMHtpD or DSMHtpE cells could be detected through electron microscopy. There may be several possibilities that could explain the inability to detect fibrils on the cells. First, the HtpP fibrillar structure encoded upstream from the *htp* gene cluster in *P. aeruginosa* is smaller than those previously described for *A. actinomycetemcomitans* and *H. ducreyi* (Table 2.1) so that it may not be detectable by TEM

and SEM methods utilized in the present study. Secondly, the number of fibrils formed by *P. aeruginosa* may be very small, thus making detection of these structures more difficult. Thirdly, any *htpP*-encoded fibrils formed by *P. aeruginosa* may be so fragile that they were destroyed during sample preparation. It is also not clear why complementation of the DSMHtpD and DSMHtpE mutant strains with the wild-type *htpDEF* genes *in trans* did not allow these mutants to attach to glass wool at levels approximately those obtained with the wild-type parent strain, or resulted in restoration of the cellular morphology of cells of the DSMHtpD strain. It is possible that these genes were not expressed from the complementation plasmid pJB-DEF-Kan or, alternatively, that the proteins were indeed expressed but at very low levels and thus not able to fully complement the inactivated proteins. Additional experimentation, involving Northern blot analysis, would be required to determine whether the genes are indeed transcribed and to what level.

The ability of *P. aeruginosa* to bind to surfaces is thought to also depend, in addition to flagella and type IV pili, on the interaction of membrane and secreted proteins with surfaces. Thus, in this preliminary study, the extracellular proteins of stationary phase planktonic growth cultures from wild-type *P. aeruginosa* DSM1707 and mutant DSMHtpD strains were isolated and separated by 2D gel electrophoresis over a pI range between 4 and 7, which is the pI range of the majority of secreted proteins in *P. aeruginosa* (Nouwens *et al.*, 2002). Although the expression (presence or absence of a given protein spot), position (pI and mass), and abundance of proteins were very similar between the DSM1707 and DSMHtpD strains, a small collection of unique proteins were excised from the respective gels and examined by N-terminal sequencing to determine their identities.

Interestingly, the identifications generated from the extracellular DSM1707 and DSMHtpD proteins (Table 4.2) did not agree with their subcellular locations predicted from computational analysis of the *P. aeruginosa* genome. Extracellular proteins characterized here, which have been regarded as being localized to the cytoplasm, included the chaperonin GroEL, the ribosomal protein S1 (both from DSM1707) and the α subunit of RNA polymerase (from DSMHtpD). In addition to these proteins, a highly expressed protein identified as β-lactamase was uniquely present in the extracellular proteomic profile of the mutant DSMHtpD strain. It should, however, be noted that the *bla* gene encoding β-lactamase was shown by Southern blot analysis, using pUC18 plasmid DNA as labeled probe (Fig. 3.12, Chapter 3), to be present on the genome of the DSMHtpD strain. Its presence

resulted from the genomic integration of the pUC18-based allelic exchange vector used during the construction of the mutant strain. Thus, the presence of β-lactamase in the extracellular proteomic profile of DSMHtpD could have been due to expression of the genomically integrated *bla* gene of the pUC18 vector DNA.

With reference to the GroEL protein, although it is generally considered to be a cytoplasmic protein in prokaryotes (Scorpio *et al.*, 1994; Taguchi *et al.*, 1996), many reports have suggested that this protein may also be associated with the outer membrane (Phadnis *et al.*, 1996; Taguchi *et al.*, 1996). For example, the GroEL protein of *A. actinomycetemcomitans* is present both intra- and extracellularly, and is proposed to more likely be within the extracellular material surrounding the bacteria than on the outer membrane. Notably, even in the absence of stress *A. actinomycetemcomitans* cells produce a detectable amount of GroEL protein (Goulhen *et al.*, 1998). The latter has also been reported in myxobacterial species (Esaguy and Aguas, 1997). In the case of *H. ducreyi*, the ability of GroEL to bind directly to HEp-2 cells, HeLa cells and human fibroblasts suggest that it may play a role in attachment of the bacteria to host cells (Frisk *et al.*, 1998). The presence of GroEL in the culture supernatant of wild-type *P. aeruginosa* DSM1707, as detected in this study, is in agreement with a recent report by Nouwens *et al.* (2002) whom also detected GroEL and GroES in the extracellular fraction. The GroEL-like proteins may function as chaperones that help in transporting proteins across cell membranes, and assist in protein folding (Flynn *et al.*, 1989; Watson, 1990; Zeilstra-Ryalls *et al.*, 1991) or play a key role in the assembly of cell surface components such as fimbriae (Van Rosmalen and Saier, 1993). Thus, it is tempting to speculate that the *P. aeruginosa* GroEL protein may play a similar role in the assembly of fimbriae, *e.g.* type IV pili or the novel HtpP pili, and/or in facilitating attachment of the organism to biotic surfaces.

Except for the GroEL protein, it is unclear why proteins that have been annotated as being localized to the cytoplasm were detected in the extracellular proteomes of DSM1707 and DSMHtpD. The presence of these proteins, however, can be explained in one of two ways. The proteins could have been released into the culture supernatant as a result of cell lysis with subsequent leakage of the proteins from the lysed cells. Proteins resulting from cellular turnover and lysis would not have been excluded by the filtration process through the 0.2 μm filter prior to TCA/methanol precipitation. Alternatively, the presence of the intracellular proteins in the extracellular environment may also have been due to exocytosis, in which

small portions of the outer membrane and periplasm form vesicles that are released in the extracellular environment (Kadurugamuwa and Beveridge, 1995; Beveridge, 1999). The role of membrane vesicles secreted by bacteria has not been well established, but such vesicles have been shown to package proteins, proteases (*e.g.* alkaline phosphatase and hydrolases) and DNA (Kadurugamuwa and Beveridge, 1995; Li *et al.*, 1996; Fernandez *et al.*, 2000). Thus, proteins which may either form part of the vesicle or that are packaged in such vesicles could be present in the extracellular protein fraction, as these vesicles (50-150 nm) are small enough to pass through the 0.2 µm filter used in this study. Such a scenario is not entirely unlikely, as vesicles on the cell surface of the *P. aeruginosa* wild-type and mutant cells was clearly visible by scanning electron microscopy (Fig. 4.7 K,L,M).

In conclusion, the results obtained in this part of the investigation indicated that the phenotypic properties of the mutant DSMHtpE cells resembled that of the wild-type *P. aeruginosa* DSM1707 cells. In contrast, the mutant DSMHtpD strain was clearly different with regard to its cellular morphology and capacity to grow as a biofilm. Since the use of different adhesins may provide *P. aeruginosa* with high adaptive advantages to colonize different surfaces, further detailed characterization of the *htp* gene cluster may provide new insight into *P. aeruginosa* strategies for attachment to surfaces. Although the impaired biofilm growth of the DSMHtpD strain could not be directly attributed to the inability of the cells to synthesis or secrete HtpP pili, the results do, however, suggest that *htpD* plays an important role in the putative *P. aeruginosa htp* pilus biogenesis/secretion system. This was evidenced by inactivation of the *htpD* ORF having a more severe effect on the properties of the bacterial cells than did inactivation of the *htpE* ORF.

CHAPTER 5

CONCLUDING REMARKS

In both natural and artificial habitats, most bacteria, including *P. aeruginosa*, have a strong tendency to adhere to surfaces within microbial consortia called biofilms (Costerton *et al.*, 1995). Bacterial surface appendages have been proposed to play a key role for attachment to surfaces. In *Pseudomonas* spp., flagellar motility has often been associated with the initial step(s) in biofilm development, while fimbriae (type IV pili) have been described as major structures required for either stable cell-to-surface attachment and/or for cell-to-cell interactions required for biofilm development (O'Toole and Kolter, 1998a; 1998b). However, identification of new genes involved in biofilm formation and the understanding of the complete array of adhesive mechanisms used by *P. aeruginosa* to colonize surfaces is fundamental in understanding the molecular basis of biofilm formation.

Novel pili and pilus biogenesis/secretion systems have recently been described in *A. actinomycetemcomitans* (Kachlany *et al.*, 2000), *H. ducreyi* (Nika *et al.*, 2002) and *C. crescentus* (Skerker and Shapiro, 2000). Not only do the pili proteins share conserved features with known type IV pili, but the Flp pili of *A. actinomycetemcomitans* and *H. ducreyi* have been reported to be important for the colonization properties of these bacterial species. The above pilus biogenesis/secretion systems have in common a putative peptidase (OrfB; CpaA) that processes the signal peptide found on the prepilin proteins (Flp; PilA), an NTPase (TadA; CpaF) that provides energy by ATP hydrolysis for transport of the pilin across the inner membrane and a specialized outer membrane protein (RcpA; CpaC) that forms a channel that allows the pilin subunit to reach the cell surface (Kachlany *et al.*, 2000; 2001; Bhattacharjee *et al.*, 2001; Skerker and Shapiro, 2000). The widespread existence of the TadA secretion ATPase (Planet *et al.*, 2001), together with our interest in identifying factors mediating adhesion of *P. aeruginosa* to surfaces, prompted this investigation to determine whether similar fimbriae and/or biogenesis/assembly proteins are present in *P. aeruginosa*. Using the available sequence of the *P. aeruginosa* PAO1 genome, genes have been identified that display homology to the previously characterized genes comprising the *flp-rcp-tad* and *pilA-cpa* pilus biogenesis/secretion systems of *A. actinomycetemcomitans* and *C. crescentus*, respectively. The *P. aeruginosa* gene cluster was termed *htp* for homologous to type IV pilus biogenesis proteins (Chapter 2). Although the properties of the individual proteins have been discussed, several aspects of the *P. aeruginosa htp* system merit further discussion.

In silico analysis of the respective proteins indicated that HtpD (PA4302) is a probable cytoplasmic membrane-associated NTPase protein, while HtpB (PA4304) is proposed to be a secretin and the putative fimbrial subunit protein, HtpP (PA4306), contains a type IV-like leader sequence and belongs to the Flp subfamily of type IV pili. However, none of the genes in the *htp* gene cluster encoded a putative prepilin peptidase. In fact, there is only one such gene on the whole *P. aeruginosa* PAO1 genome, i.e. *pilD*. It has been shown that this prepilin peptidase is involved in pilin and pseudopilin processing (Nunn and Lory, 1992; Strom *et al.*, 1993). Nevertheless, a putative peptidase-encoding gene (PA4295) was identified downstream of the *htpA-I* gene cluster, but in the opposite transcriptional orientation, which displayed 23% amino acid identity with the proposed peptidase of the *flp-rcp-tad* system in pairwise alignments. Based on the available *in silico* data, a model has been proposed whereby this system functions (Chapter 2). In this model, HtpD is proposed to act as an energizing protein, while HtpB may form channels in the bacterial outer membrane to allow for export of the HtpP prepilin subunits. The prepilin is proposed to be processed by an as-yet-unidentified prepilin peptidase, but possibly the PA4295-encoded protein. The remainder of the Htp proteins (HtpA, C, E through I) are integral membrane proteins, which may aid in the assembly and stabilization of the export apparatus.

By contrast to the *flp-rcp-tad* and *pilA-cpa* gene clusters, which are organized in a single transcribed operon, the putative *htp* pilus biogenesis/secretion system of *P. aeruginosa* consists of at least three distinct transcriptional units. The *htpA* through *htpI* genes are organized as a single transcribed operon, while the *htpP* and PA4295 genes are located upstream and downstream, respectively, from the *htpA-I* ORFs and appear to be divergently transcribed. This unique organization of the *htp* system may also reflect on a complex regulation system whereby the HtpP pili are expressed, processed and secreted. In this regard, the transcription profile of all the genes in the *pilA-cpa* gene cluster of *C. crescentus* has been determined using DNA microarray analysis. The genes *cpaB - cpaF* are co-induced 15 min before the *pilA* gene, and the gene encoding the putative prepilin peptidase, *cpaA*, is induced slightly after the *cpaB - cpaF* group. These genes are thus all induced prior to pilin gene transcription and pilus assembly (Skerker and Shapiro, 2001). By analogy to the *C. crescentus* system, it can be envisaged that the genes encoding components of the pilin secretion apparatus (*htpA* through *htpI*) are transcribed first, followed by transcription of the PA4295 gene and then the pilin-encoding gene *htpP*. Recently, Schuster *et al.* (2003) analyzed the transcriptome of *P. aeruginosa* PAO1 using Affymetrix GeneChip genome

arrays. The expression of 315 genes, including the *htpP* (PA4306) and *htpA-I* (PA4305-PA4297) genes, was reported to be induced by quorum sensing via acyl-homoserine lactone (acyl-HSL) signals. Although expression of the *htp* genes were induced by N-(3-oxododecanoyl)-L-HSL ($3O_{12}$-HSL), a slightly greater response was observed with both $3O_{12}$-HSL and N-butyryl-L-HSL (C_4-HSL). Furthermore, maximum levels of induction were observed in stationary phase cultures. Notably, *in silico* analysis of the upstream sequences of the respective *htp* genes indicated that none of the *htp* genes contained a *las-rhl* box-like sequence (NNCT-N_{12}-AGNN), which had previously been identified by Whiteley and Greenberg (2001) in promoters of several quorum sensing-controlled genes (results not shown). Thus, it might be that the expression of the *htp* genes is controlled indirectly by quorum sensing. However, further investigations are required to determine the precise manner and by which (other) factors expression of the *htp* genes are controlled.

Based on reports indicating that mutations in the *flp-rcp-tad* gene clusters in *A. actinomycetemcomitans* and *H. ducreyi* correlated with decreased adherence *in vitro* (Kachlany *et al.*, 2000; Nika *et al.*, 2002), the functional importance of the putative *htp* gene cluster in *P. aeruginosa* was investigated. Thus, to determine whether a similar phenotype could be obtained in *P. aeruginosa*, strains were constructed with specific mutations in three open reading frames (ORFs) within the *htp* gene cluster (Chapter 3). These were chosen on the basis of being conserved in all three of the described novel pilus biogenesis/secretion systems. A gentamicin resistance cassette, which is flanked by transcriptional terminators on both ends (Luckow *et al.*, 1993), was inserted via allelic exchange into the *htpD*, *htpE* and *htpDEF* ORFS, to construct *P. aeruginosa* mutant strains DSMHtpD, DSMHtpE and DSMHtpDEF, respectively. Insertional frameshift or nonsense disruption of an ORF within an operon can affect downstream gene expression in addition to the targeted gene and the resultant polar effects could therefore confuse the assignment of a mutant phenotype to the disrupted gene. In this study, a polar mutation, should it occur, would have no effect on the outcome tested, as the primary aim of this investigation was to determine the importance of the *htp* gene cluster in the ability of *P. aeruginosa* to attach to a surface.

The newly constructed DSMHtpD and DSMHtpE mutant strains were subsequently characterized to determine the functional importance of the *htp* gene cluster of *P. aeruginosa* (Chapter 4). Compared to the DSMHtpE strain, which resembled the wild-type *P. aeruginosa* DSM1707 strain, the DSMHtpD strain was found to differ substantially. Most notably, the

DSMHtpD strain was severely impaired in its ability to grow as a biofilm and the cells were larger than the wild-type *P. aeruginosa* DSM1707 cells. These results may be explained as follow. Due to the strategy used for insertional inactivation of the *htpD* and *htpE* ORFs, it is likely that polar mutations would have been introduced downstream from the insertion sites. Thus, whereas the DSMHtpE strain may be capable of transcribing the *htpABCD* genes, the DSMHtpD strain may only transcribe the *htpABC* genes. As mentioned earlier, all pilus biogenesis/secretion systems contain a signal peptidase, NTPase and outer membrane secretin. Based on the results obtained during the *in silico* analysis of the *P. aeruginosa htp* gene cluster (Chapter 2), it has been proposed that the *P. aeruginosa* PA4295 gene may encode a putative prepilin peptidase. However, the PA4295 gene, as is the putative pilin-encoding gene *htpP*, appears to be divergently transcribed from the putative *htp* operon and its expression would therefore not be expected to be influenced by polar mutations arising from inactivation of either the *htpD* or *htpE* ORFs. Furthermore, both the mutant DSMHtpD and DSMHtpE strains would be expected to express the *htpB* gene encoding a probable secretin (HtpB). Consequently, these two strains may differ from each other only in their ability (DSMHtpE) or inability (DSMHtpD) to express HtpD, a probable NTPase. Since the DSMHtpE strain is therefore likely to contain all three the essential components of pilus biogenesis/secretion systems, it may have been able to synthesize and secrete the HtpP pili. By contrast, the DSMHtpD strain encoded only two of these essential components, and the inability of DSMHtpD cells to grow as a biofilm may thus be a consequence of its inability to produce or secrete HtpP pili. Furthermore, the intracellular accumulation of HtpP, if indeed synthesized, may have resulted in the observed increase in cell size of cells from the mutant DSMHtpD strain. These proposed hypotheses are, however, subject to verification by Northern blot analysis using appropriate *htp* gene probes and/or by Western blot analyses using an anti-HtpP antibody. Alternatively, the inability of the DSMHtpD strain to grow as a biofilm may be due to its impaired growth rate. Inactivation of the *htpD* ORF resulted in a lower growth rate of the DSMHtpD strain compared to the wild-type *P. aeruginosa* DSM1707 and mutant DSMHtpE strains.

The more drastic perturbations observed when *htpD* was insertionally inactivated is furthermore suggestive of the view that the *htpD* ORF plays an important role in the functioning of the putative *htp* pilus biogenesis/secretion system. The results of studies on the toxin-coregulated pilus (TCP) of *V. cholerae* (Iredell and Manning, 1997) and Flp pilus of *A. actinomycetemcomitans* (Bhattacharjee *et al.*, 2001) suggest that the presence of the putative

ATPase is required for these pilus biogenesis/secretion systems to function. However, the role of the ATPase in the pilus and protein secretion systems is not yet understood. The putative ATPase might provide energy for polymerization of pili or structures that function to extrude proteins through the outer membrane. Alternatively, it might provide energy to the outer membrane that is necessary to translocate the exoproteins or pilins across the membrane or to modulate a gating mechanism for the outer membrane channel.

Despite inactivation of the *htpD* ORF, cells of the DSMHtpD strain was capable of attaching efficiently to the glass wool substratum. This may have been due to the expression of functional type IV pili, as evidenced in twitching motility assays, which have been reported to play an essential role during the early stages of biofilm formation by mediating attachment of the bacterial cells to a surface (O'Toole and Kolter *et al.*, 1998a; 1998b). The HtpP pili therefore appear not to be directly involved in surface attachment by *P. aeruginosa*, but the results do not exclude an auxiliary role for HtpP pili in this process. This auxiliary role may be related to cell-to-cell interactions since the biofilm phenotypes of the respective strains differed from each other. Although the construction of a *P. aeruginosa* DSM1707 mutant deficient in HtpP pili may prove valuable in this respect, it was not attempted in this investigation as this forms part of a separate investigation currently being undertaken in our laboratory.

In this study, 2-D gel electrophoresis followed by N-terminal sequencing was also used to detect differences in extracellular proteins between the *P. aeruginosa* DSM1707 and mutant DSMHtpD strains (Chapter 4). The results obtained from this preliminary study indicated that the identified proteins are usually found intracellularly and their localization in the extracellular proteome of DSMHtpD and wild-type DSM1707 is most likely to be due to cell autolysis or exocytosis, in which vesicles packaging membrane and intracellular proteins, are released into the extracellular environment. Since stationary phase cultures were used in this analyses, more conclusive data may be obtained by making use of early and/or mid-exponential growth phase cultures. Furthermore, membrane proteins have been reported to have a substantial influence on attachment and may also play a role in early biofilm development. These investigations should therefore also be extended to the analysis of membrane protein fractions of the *P. aeruginosa* wild-type and mutant strains by 1-D gel electrophoresis.

In summary, a cluster of genes (*htp*) in *P. aeruginosa* that may specify the components of a putative pilus biogenesis/secretion system, which is involved in the assembly of fimbrial subunits in other microorganisms, was identified and characterized. Of future importance would be to determine how the Htp proteins expressed by the *htp* gene cluster result in synthesis, assembly and secretion of HtpP fibrils, and how these components mediate attachment to not only biotic and abiotic surfaces, but interbacterial adhesion as well. In addition, dissection of the networks controlling expression of the *htp* gene cluster may lead to a better understanding regarding the complexity and specificity of this adherence mechanism. Such studies are currently being undertaken in our laboratory and should bring a new insight into the *P. aeruginosa* strategies for attachment to surfaces.

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