UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Transcriptional analysis and mutagenesis of the *htp* fimbrial gene cluster from *Pseudomonas aeruginosa* PAO1

by

AMANDA SWANEPOEL

Submitted in partial fulfillment of the requirements for the degree
Master of Science
in the Faculty of Natural and Agricultural Sciences
Department of Microbiology and Plant Pathology
University of Pretoria
Pretoria

May 2006

© University of Pretoria

ACKNOWLEDGEMENTS

Firstly thanks to my Heavenly Father for giving me the ability and strength to pursue this study

Thank you Jay my love, you have truly been an inspiration as well as my pillar of strength during all of this

Mom, Dad and family. Your support during this MSc has been incredible. I truly appreciate it and love you all dearly for it

Marthi, Joleen and Liesel, you guys have absolutely been great! Thanks for all your support and all the great laughs we had in 9-38

Professor J. Theron. Thank you for leading me during this study and having an open office door when problems and questions were attacking from all sides. It has been a great journey and I have learned a lot from you, which I will always remember

Thank you to the National Research Foundation for granting me a scolarship to be able to finance this MSc

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Transcriptional analysis and mutagenesis of the *htp* fimbrial gene cluster from *Pseudomonas aeruginosa* PAO1

by

AMANDA SWANEPOEL

Supervisor: Prof. J. Theron
Department of Microbiology and Plant Pathology
University of Pretoria

for the degree M.Sc

Pseudomonas aeruginosa, a ubiquitous environmental bacterium and an opportunistic human pathogen, is one of the most and best studied biofilm-forming organisms and has emerged as a model organism in the study of surface- and biofilm-induced gene expression. *P. aeruginosa* forms biofilms through a series of interactions between the cells and adherence to surfaces, which is mediated by surface appendages such as flagella and type IV pili. A gene cluster, designated *htpABCDEFGHI*, which appears to encode protein products with homology to those encoded by recently described novel pilus biogenesis and assembly systems, has been identified in *P. aeruginosa* PAO1. Since the pili produced by these systems, designated Flp, are associated with the ability of the bacteria to bind non-specifically to inert surfaces, the aims of this study were to characterize the transcriptional organization of the putative *P. aeruginosa* PAO1 *htp* gene cluster and to determine the functional importance of the *htp* gene cluster in the ability of *P. aeruginosa* PAO1 to adhere to surfaces.

In silico evidence has suggested that the pilin subunit gene *flp* is not part of the *P. aeruginosa htp* gene cluster thought to encode proteins involved in the synthesis, assembly and export of these pili. To determine the transcriptional organization of this gene cluster, total RNA from *P. aeruginosa* PAO1 was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). Primers designed to amplify regions spanning gene junctions yielded amplicons at each individual gene junction from *htpA* to *htpI*, as well as an amplicon for *flp*. Moreover,

corresponding sigma 70 (σ^{70}) consensus sequences were identified in the intergenic region between the *htpA* and *flp* genes and promoter function of the *flp* and *htpA* upstream region was subsequently confirmed using *lacZ* reporter gene constructs transformed into *P. aeruginosa* PAO1. The results therefore indicated that the *htp* gene cluster is an operon transcribed as a polycistronic message, whilst the *flp* gene is transcribed independently as a monocistronic message.

To determine the functional importance of the *htp* gene cluster in *P. aeruginosa* PAO1, the *htpD* gene, encoding a putative NTPase, was inactivated by *in vivo* homologous recombination with an appropriately constructed allelic exchange vector to generate the isogenic mutant strain PAOHtpD. Comparative analysis of the wild-type *P. aeruginosa* PAO1 and mutant PAOHtpD strain revealed that the mutant strain was impaired in its ability to attach to a glass wool substratum and also in its ability to grow as a biofilm. Since the mutant PAOHtpD strain was not growth-impaired, these results indicate that the *htp* gene cluster plays a role in *P. aeruginosa* PAO1 biofilm development under the culturing conditions used in this study. Thus, it can be proposed that the *flp* and *htp* gene cluster of *P. aeruginosa* PAO1 may play a role in its ability to successfully colonize abiotic surfaces.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS i
SUMMARY ii
LIST OF ABBREVIATIONS viii
LIST OF FIGURES x
LIST OF TABLES xii

CHAPTER ONE: LITERATURE REVIEW 1

1.1 GENERAL INTRODUCTION 2
1.2 BIOFILM FORMATION BY *P. aeruginosa* 3
1.2.1 Steps in biofilm development 4
1.2.1.1 Reversible attachment 4
1.2.1.2 Irreversible attachment 6
1.2.1.3 Biofilm maturation 7
1.2.1.4 Detachment 8
1.3 BACTERIAL SURFACE APPENDAGES REQUIRED FOR BIOFILM FORMATION 9
1.3.1 Importance of flagella 9
1.3.2 Importance of pili and adhesins 10
1.4 PROTEIN SECRETION PATHWAYS 11
1.4.1 The general secretion pathway (Gsp) 11
1.4.1.1 Secretion across the inner membrane 11
1.4.1.2 Protein folding in the periplasmic space 14
1.4.1.3 Secretion across the outer membrane 14
1.5 TYPE IV PILUS EXPRESSION AND ASSEMBLY 14
1.5.1 Type IV pili 15
1.5.1.1 Characteristics 15
1.5.1.2 Type IV pilus biogenesis genes 16
1.5.2 Model for type IV pilus assembly and secretion 18
1.5.3 Relationship between type II and type IV secretion systems 19
1.6 NOVEL FIMBRIAL EXPRESSION AND ASSEMBLY SYSTEMS 21
1.7 AIMS OF THIS INVESTIGATION 25

CHAPTER TWO: TRANSCRIPTIONAL ANALYSIS OF A NOVEL PILIN-ENCODING SYSTEM IN *Pseudomonas aeruginosa* PAO1 **26**

2.1 INTRODUCTION 27
2.2 MATERIALS AND METHODS 29
2.2.1 Bacterial strains, plasmids and culture conditions 29
2.2.2 Genomic DNA extraction 29
2.2.3 DNA amplification 30
2.2.3.1 Oligonucleotide primers 30
2.2.3.2 Polymerase chain reaction (PCR) 30
2.2.4 Agarose gel electrophoresis 30
2.2.5 Purification of DNA fragments from agarose gels 33
2.2.6 Cloning of DNA fragments into plasmid vectors 33
2.2.6.1 Ligation reactions 33
2.2.6.2 Preparation of competent *E. coli* DH5α cells 33
2.2.6.3 Transformation of the *E. coli* DH5α cells 34
2.2.7 Screening of transformants 34
2.2.7.1 Colony-PCR for screening of putative recombinant plasmids 34
2.2.7.2 Plasmid DNA extraction 35
2.2.8 Restriction enzyme digestion 35
2.2.9 Nucleotide sequencing 36
2.2.10 Plasmid constructions 36
2.2.11 Expression and purification of the recombinant GST-Flp fusion protein 37
2.2.11.1 Expression of the *P. aeruginosa* Flp protein in *E. coli* 37
2.2.11.2 Purification of the GST-Flp protein by glutathione affinity chromatography 40
2.2.11.3 Thrombin cleavage of the purified GST-Flp protein 40
2.2.12 Characterization of the recombinant GST-Flp protein 41
2.2.12.1 SDS-PAGE 41
2.2.12.2 Visualization of proteins on SDS-polyacrylamide gels 41
2.2.12.3 Western blot analysis 42
2.2.13 Determination of β-galactosidase activity of the recombinant pALacZsd reporter vectors in *P. aeruginosa* PAO1 43
2.2.13.1 Preparation of *P. aeruginosa* PAO1 competent cells 43
2.2.13.2 Transformation of competent *P. aeruginosa* PAO1 cells 43

2.2.13.3 Culturing conditions 44
2.2.13.4 β-galactosidase assays 44
2.2.13.5 Total protein concentration 44
2.2.14 Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of adjacent genes in the putative *htp* operon of *P. aeruginosa* PAO1 45
2.2.14.1 Oligonucleotide primers 45
2.2.14.2 Total RNA extraction 45
2.2.14.3 RT-PCR 46
2.3 RESULTS 46
2.3.1 Reverse transcriptase-polymerase chain reaction (RT-PCR) 46
2.3.2 Identification of potential promoter sequences 47
2.3.3 Functional analysis of the potential promoter regions 49
2.3.3.1 Construction of recombinant pALacZsd$_{(PAO1)}$IN1 and pALacZsd$_{(PAO1)}$IN2 reporter vectors 49
2.3.3.2 β-Galactosidase activity assays 52
2.3.4 Expression of the *P. aeruginosa* Flp protein as a GST fusion protein in *E. coli* 54
2.3.4.1 Construction of recombinant bacterial expression vector pGEX-Flp 54
2.3.4.2 Expression and purification of recombinant GST-Flp fusion protein 57
2.3.5 Protease cleavage of purified GST-Flp fusion proteins 60
2.4 DISCUSSION 60

CHAPTER THREE: CONSTRUCTION AND CHARACTERIZATION OF AN HtpD (PA4302)-DEFICIENT MUTANT STRAIN OF *Pseudomonas aeruginosa* PAO1 **66**

3.1 INTRODUCTION 67
3.2 MATERIALS AND METHODS 69
3.2.1 Bacterial strains, plasmids and culture conditions 69
3.2.2 Construction of allelic exchange vector pUCori-HtpD-Gent 69
3.2.2.1 DNA amplification 69
3.2.2.1.1 Oligonucleotide primers 70
3.2.2.1.2 Polymerase chain reaction (PCR) amplification 70
3.2.2.2 Construction of allelic exchange vector pUCori-HtpD-Gent 72
3.2.3 Generation of *P. aeruginosa* PAO1 mutant strains 72

3.2.4 Characterization of *P. aeruginosa* PAO1 mutant strains 72
3.2.4.1 PCR analyses 74
3.2.4.2 Southern blot analysis 74
3.2.4.2.1 Preparation of labelled probe 74
3.2.4.2.2 Preparation of the membrane 74
3.2.4.2.3 Nucleic acid hybridization 75
3.2.4.2.4 Detection of hybridized probe DNA 76
3.2.5 Complementation analysis 76
3.2.5.1 Construction of complementation plasmid pJB-IP-HtpD 76
3.2.5.2 Complementation of the PAOHtpD mutant strain 77
3.2.6 Batch assays of the *P. aeruginosa* PAOHtpD mutant strain 77
3.2.6.1 Determination of bacterial growth curves 77
3.2.6.2 Biofilm formation 77
3.2.7 Transmission electron microscopy 79
3.3 RESULTS 80
3.3.1 Construction of allelic exchange vector pUCori-HtpD-Gent 80
3.3.1.1 Construction of plasmids pGEM-Gent and pUC-HtpD 80
3.3.1.2 Construction of pUCori-HtpD-Gent 81
3.3.2 Engineering of an HtpD-deficient *P. aeruginosa* PAO1 strain 82
3.3.2.1 Generation of mutant strains 82
3.3.2.2 Southern blot analysis of *P. aeruginosa* mutant strain PAOHtpD 82
3.3.2.3 PCR analysis of *P. aeruginosa* mutant strain PAOHtpD 84
3.3.3 Construction of complementation plasmid pJB-IP-HtpD 87
3.3.4 Characterization of the PAOHtpD mutant strain 88
3.3.4.1 Growth curves 88
3.3.4.2 Biofilm development on glass wool 88
3.3.4.3 Electron microscopic characterization of cellular morphology 95
3.4 DISCUSSION 95

CHAPTER FOUR: CONCLUDING REMARKS 101

REFERENCES 107

LIST OF ABBREVIATIONS

%	percentage
°C	degrees Celsius
µg	microgram
µl	microlitre
µm	micrometre
A	absorbance
Å	Angstrom
Amp^r	ampicillin resistance
ATP	adenosine triphosphate
BAC	biofilm-associated cells
bp	base pair
C	carboxy
ca.	approximately
cfu	colony forming units
cm	centimetre
cm^2	square centimetre
CTAB	cetyltrimethylammonium bromide
ddH_2O	deionized distilled water
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleoside-5'-triphosphate
EDTA	ethylenediaminetetra-acetic acid
e.g.	for example
EPS	exopolysaccharides
Fig.	figure
Gm^r	gentamicin resistance
Gsp	general secretion pathway
GST	glutathione-*S*-transferase
h	hour
IPTG	isopropyl β-D-thiogalactoside
kb	kilobase pairs
kDa	kilodalton
kV	kilovolt
L	litre

LB broth	Luria-Bertani broth
LPS	lipopolysaccharide
M	molar
mA	milliampere
MCS	multiple cloning site
min	minute
ml	millilitre
mM	millimolar
Mr	molecular mass
N	amino
nm	nanometer
nt	nucleotide
NH_4OAc	ammonium acetate
OD	optical density
ONPG	2-nitrophenyl-β-D-galactopyranoside
ORF	open reading frame
ori	origin of replication
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pmol	picomole
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcriptase-polymerase chain reaction
s	second
SDS	sodium dodecyl sulphate
T	translational terminator
t	transcription terminator
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
U	units
UHQ	ultra high quality
V	volts
v.	version
v/v	volume per volume
W	Watt
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

LIST OF FIGURES

Fig. 1.1 Model of biofilm development. 5

Fig. 1.2 Sec secretion process across the inner membrane. 13

Fig. 1.3 Models for type II secretion and type IV pilus biogenesis. 20

Fig. 1.4 Organization of the *htp* gene cluster in *P. aeruginosa* compared to the *flp-tad* gene cluster in *A. actinomycetemcomitans* and *H. ducreyi*, and the *pilA-cpa* gene cluster in *C. crescentus*. 24

Fig. 2.1 Construction of the bacterial expression vector pGEX-Flp. 38

Fig. 2.2 Construction of the recombinant reporter vectors pALacZ-IN1 and pALacZ-IN2. 39

Fig. 2.3 RT-PCR analysis of the *P. aeruginosa* PAO1 *htp* gene cluster and *flp* ORF. 48

Fig. 2.4 Sequence of the predicted *flp* (PA4306) and *htp* operon (PA4305-PA4297) promoters. 49

Fig. 2.5 Plasmid map of recombinant pALacZsd reporter plasmids (a) and agarose gel electrophoresis indicating amplification of the cloned DNA fragments in the recombinant pALacZsd reporter vector constructs (b). 51

Fig. 2.6 Graph displaying the β-galactosidase activity per total cellular biomass of the different promotor-*lacZ* constructs in planktonic (P) cells and biofilm (B) cells. 53

Fig. 2.7 Plasmid map of recombinant expression vector pGEX-Flp (a) and agarose gel electrophoretic analysis of the recombinant bacterial expression vector pGEX-Flp (b). 56

Fig. 2.8 Expression of the recombinant GST-Flp fusion protein in *E. coli*. 58

Fig. 2.9 SDS-PAGE and Western blot analyses following expression and purification of the recombinant GST-Flp fusion protein and GST protein. 59

Fig. 2.10 Thrombin cleavage of purified GST-Flp. 61

Fig. 3.1 Construction of the recombinant allelic exchange vector pUCori-HtpD-Gent. 73

Fig. 3.2 Construction of the complementation plasmid pJB-IP-HtpD. 78

Fig. 3.3 Plasmid map of the recombinant allelic exchange vector pUCori-HtpD-Gent (a) and agarose gel electrophoretic analysis of the recombinant plasmid pUCori-HtpD-Gent (b). 83

Fig. 3.4 Southern blot analysis of genomic DNA extracted from the *P. aeruginosa* mutant strain PAOHtpD, containing an insertionally inactivated *htpD* gene. 85

Fig. 3.5a Schematic presentation of specific primer annealing positions and direction of amplification in the mutant PAOHtpD strain. 86

Fig. 3.5b Agarose gel electrophoretic analysis of the amplification products obtained, following PCR analysis of *P. aeruginosa* PAO1 and PAOHtpD. 86

Fig. 3.6 Plasmid map of the complementation plasmid pJB-IP-HtpD (a) and agarose gel electrophoretic analysis of the complementation plasmid pJB-IP-HtpD (b). 89

Fig. 3.7 Growth curves of wild-type *P. aeruginosa* PAO1, mutant PAOHtpD and PAOHtpD containing plasmid construct pJB-IP-HtpD. 90

Fig. 3.8 Photomicrographs showing biofilm development of *P. aeruginosa* wild-type and mutant strains on glass wool over time. 92

Fig. 3.9 Propensity of PAOHtpD cells to occur as a biofilm. 94

Fig. 3.10 Representative electron micrographs of thin sections through resin-embedded cells of the *P. aeruginosa* wild-type and mutant strains. 96

LIST OF TABLES

Table 2.1 Bacterial strains and plasmids used in this study 31

Table 2.2. Oligonucleotides used in this study 32

Table 3.1 Bacterial strains, plasmids and oligonucleotides used in this study 71

CHAPTER ONE

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium and highly successful in colonizing a diversity of environments (Palleroni, 1992a; 1992b). Not only is it an opportunistic pathogen of humans, causing infections in immunocompromised patients such as those with cancer or AIDS and those suffering from cystic fibrosis and burns (Van Delden and Iglewski, 1998; Ramsey and Wozniak, 2005), but it has also been shown to infect plants and insects (Schroth *et al.*, 1992; Rahme *et al.*, 1995). The success of *P. aeruginosa* in colonizing these diverse environments is attributed to its ability to synthesize a large number of different virulence factors such as alginate, pili and lipopolysaccharides, and secreted virulence factors, including toxins, proteases and haemolysins (Lazdunski *et al.*, 1990; Van Delden and Iglewski, 1998; Ramsey and Wozniak, 2005).

In the vast majority of ecological niches, *P. aeruginosa* can grow in association with surfaces, which leads to the formation of biofilms (Zobell, 1943; Costerton *et al.*, 1995). Biofilms have been defined as structured communities of bacterial cells that are enclosed in a self-produced polymeric matrix and adhere to biotic and abiotic surfaces, an interface or each other (Costerton *et al.*, 1995). The ability of bacteria to form biofilms provides its members with a number of benefits. In addition to the advantage of resistance to environmental changes (Donlan and Costerton, 2002; Jefferson, 2004), the biofilm bacteria may benefit from a number of properties of a communal existence, including division of metabolic burden (Geesey, 2001; Yarwood *et al.*, 2004), gene transfer (Ghigo, 2001; Molin and Tolker-Nielsen, 2003) and altruistic behaviour (Rice and Bayles, 2003).

Biofilm formation occurs in response to a variety of environmental signals (Davey and O'Toole, 2000; Stanley and Lazazzera, 2004) that leads to a number of changes in gene regulation that cause the adhering cells to become phenotypically (Costerton *et al.*, 1995; Davies and Geesey, 1995; Sauer *et al.*, 2002) and metabolically (Costerton *et al.*, 1999; Davey and O'Toole, 2000) distinct from their planktonic counterparts. The complex biofilm architecture also provides an opportunity for metabolic cooperation, and niches are formed within the spatially well-organized systems. Consequently, the bacteria are exposed to an array of distinct physicochemical conditions within a biofilm that can result in differential gene expression (Davey and O'Toole, 2000; O'Toole *et al.*, 2000a; Sauer and Camper, 2001; Whiteley *et al.*, 2001; Sauer *et al.*, 2002).

Recent studies have suggested that biofilm formation occurs as a sequential, developmental process (O'Toole *et al.*, 2000a; Sauer *et al.*, 2002; Stoodley *et al.*, 2002). Current models, based largely on *P. aeruginosa*, depict biofilm formation commencing when planktonic bacterial cells attach irreversibly to a surface. This attachment is followed by growth into a mature, structurally complex biofilm and culminates in the dispersion of detached bacterial cells into the bulk fluid (Sauer *et al.*, 2002). Notably, the bacteria within each of the stages of biofilm development are believed to be physiologically distinct from cells in the other stages (Sauer *et al*., 2002). The phenotypic heterogeneity within the biofilm has been interpreted as a specialization or division of labour, similar to cellular differentiation seen in multicellular organisms (O'Toole *et al.*, 2000a; Webb *et al.*, 2003a).

Of the processes leading to the formation of biofilms, bacterial surface appendages required for initial attachment, amongst them type IV pili, have been best characterized. As the role of pili in the biofilm formation process is closely related to the aims of this investigation (Section 1.7), information pertinent to their role in *P. aeruginosa* biofilm development, as well as their biogenesis and secretion from Gram-negative bacteria will be discussed in this review of the literature.

1.2 BIOFILM FORMATION BY *P. aeruginosa*

As a consequence of analyzing biofilms using genetic (O'Toole and Kolter, 1998a; 1998b; Whiteley *et al.*, 2001; Finelli *et al.*, 2003), proteomic (Sauer and Camper, 2001; Sauer *et al.*, 2002) and molecular biological (Tolker-Nielsen *et al.*, 2000; De Kievit *et al.*, 2001; Klausen *et al.*, 2003) approaches, much progress has been made towards understanding the development of bacterial biofilms. In addition, extensive biophysical, structural and chemical analysis of bacterial biofilms has led to a basic model for biofilm structure (Costerton *et al.*, 1995; Tolker-Nielsen *et al.*, 2000). In this model, bacteria form microcolonies surrounded by copious amounts of exopolysaccharide and between the microcolonies are water-filled channels (Costerton *et al.*, 1995). It has been suggested that these channels serve to promote the influx of oxygen, organic substrates and nutrients, and the efflux of carbon dioxide and metabolic by-products (DeBeer *et al.*, 1994; Costerton *et al.*, 1995; 1999).

1.2.1 Steps in biofilm development

It has been proposed that microbial biofilm formation may be a further example of a bacterial developmental process (Davey and O'Toole, 2000; O'Toole *et al.*, 2000a; Stoodley *et al.*, 2002), not unlike that observed in cell cycle-controlled swarmer-to-stalk cell transition in *Caulobacter crescentus* (Dworkin, 1999), sporulation in *Bacillus subtilis* (Branda *et al.*, 2001) and fruiting body formation in *Myxococcus xanthus* (Shimkets, 1999). Similar to these developmental systems, building a biofilm requires a series of discreet and well-regulated steps. While the exact molecular mechanisms may differ from organism to organism, the stages of biofilm development appear to be conserved among a wide range of microbes (Fig. 1.1). These stages include attachment of free-floating bacterial cells to a surface, the growth and aggregation of cells into microcolonies followed by growth into mature, structurally complex biofilm (maturation), and the dispersal of detached bacterial cells into the bulk fluid (O'Toole *et al.*, 2000a; Sauer *et al.*, 2002).

1.2.1.1 Reversible attachment

Prior to surface colonization, a preconditioning film, composed of proteins, glycoproteins and organic nutrients, is believed to form on the attachment surface, thus resulting in a nutritionally rich zone that is metabolically favourable for bacterial cells (Marshall *et al.*, 1971; 1985; Beveridge *et al.*, 1997). Once a surface has been conditioned, its properties are permanently altered so that the affinity of an organism for a native or a conditioned surface can be quite different (Hermansson and Marshall, 1985; Boland *et al.*, 2000). Planktonic bacteria may be brought into close approximation of the conditioned surface by either a random (*e.g.* sedimentation and liquid flow) or in a directed fashion (*e.g.* chemotaxis and active motility) (Quirynen *et al.*, 2000). Initial attachment of the bacteria to the conditioned surface is then facilitated by van der Waals forces, electrostatic and hydrophilic interactions and specific interactions, or by a combination of these, depending on the proximity of the organism to the attachment surface (Van Loosdrecht *et al.*, 1990; An *et al.*, 2000). The individual adherent cells that initiate biofilm formation on a surface are surrounded by only small amounts of exopolymeric material and many are capable of independent movement by means of pilus-mediated twitching or gliding (O'Toole and Kolter, 1998a). These cells are, however, not yet committed to the process of biofilm formation and many may leave the surface to resume the planktonic lifestyle (Sauer *et al.*, 2002).

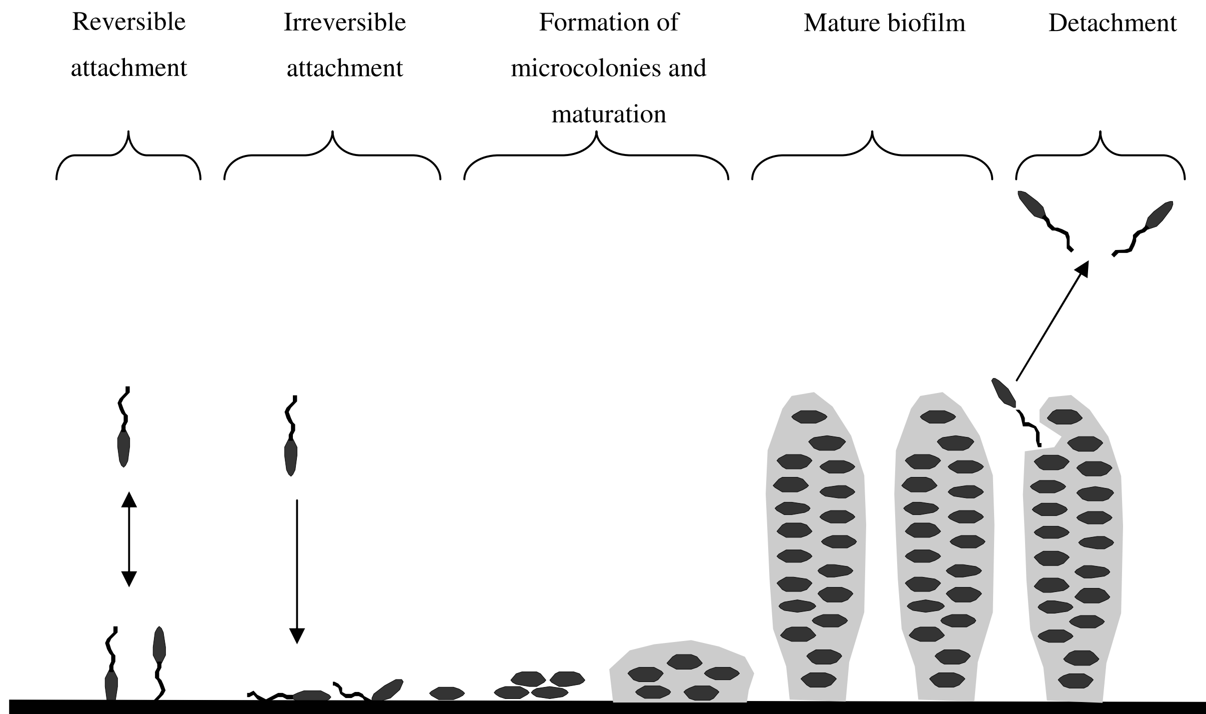


Fig. 1.1 Model of biofilm development. In response to environmental cues, planktonic cells initiate cell-to-surface and cell-to-cell contacts resulting in the formation of microcolonies. In response to developmental signals, microcolonies undergo differentiation to form a well-developed biofilm characterized by pillar- or mushroom-like structures surrounded by bacterial exopolysaccharides (EPS) and interspersed with fluid-filled channels. Once the biofilm has reached critical mass, some of the biofilm cells may detach to colonize other surfaces (Modified from O'Toole *et al.*, 2000a and Stoodley *et al.*, 2002).

1.2.1.2 Irreversible attachment

Following the initial reversible attachment to a surface, the bacteria must not only maintain contact with the substratum but also grow in order to develop a mature biofilm. Thus, the stage of reversible attachment is followed by a phase during which production of bacterial exopolysaccharides (EPS) results in more stable attachment by forming organic bridges between the cells and substratum (Notermans *et al.*, 1991). Reporter gene studies have established that expression of the *P. aeruginosa* alginate biosynthetic genes *algC* (Davies and Geesey, 1995) and *algD* (Hoyle *et al.*, 1993) are up-regulated within 15 min following initial attachment to a surface, with a concomitant increase in alginate production. Although the production of alginate has been considered to form the structural and mechanical framework required for biofilm formation (Stoodley *et al.*, 2002), recent reports, however, have indicated that EPS rich in mannose or glucose is essential for *P. aeruginosa* biofilm formation (Friedman and Kolter, 2004; Jackson *et al.*, 2004; Matsukawa and Greenberg, 2004).

Transition from reversible to irreversible attachment is also mediated by pili, fimbriae and fibrillae (Jacob-Dubuisson *et al.*, 1993; Rudel *et al.*, 1995; Pratt and Kolter, 1998). Whereas flagellar-mediated motility is important in establishing initial cell-surface contacts, twitching motility has been shown to be required for maturation of *P. aeruginosa* biofilms under quiescent conditions (O'Toole and Kolter, 1998a). Twitching motility refers to a mode of surface translocation mediated by type IV pili (Wall and Kaiser, 1999) in which the pili are believed to extend and retract, thus propelling the cells along the surface (Palmer, 1999). Specifically, twitching motility is required for the formation of microcolonies within the biofilm by facilitating interactions of bacteria with one another at the surface, forming groups of cells, thereby helping to strengthen the degree of attachment to a surface (O'Toole and Kolter, 1998a).

Microscopy observations have shown that initial surface attachment in *P. aeruginosa* proceeds from transient cell pole-mediated interactions (reversible attachment) to stable surface interactions that occur via the long axis of the cell body (irreversible attachment) (Sauer *et al.*, 2002). Recently, a new class of *P. aeruginosa* biofilm mutant was described that was able to initiate surface attachments but failed to form microcolonies in flow cell-grown biofilms, despite being proficient in twitching and swimming motility (Caiazza and O'Toole, 2004). The transposon insertion was subsequently mapped to open reading frame

PA5346, which encodes a protein of unknown function, and was designated *sadB*. Since the mutant cells were arrested at reversible attachment, it was proposed that *sadB* may be required for the transition from reversible to irreversible attachment, but the exact mechanism by which SadB promotes this transition is not yet known.

1.2.1.3 Biofilm maturation

Once the bacteria have irreversibly attached to a surface, the process of biofilm maturation begins. During this process, binary division of irreversibly attached cells causes the daughter cells to spread outward and upward from the attachment point to form microcolonies or cell clusters (Heydorn *et al.*, 2000; Tolker-Nielsen *et al.*, 2000). Alternatively, the attached cells can be redistributed by surface motility (O'Toole and Kolter, 1998a; 1998b; Klausen *et al.*, 2003) and/or single cells may be recruited from the bulk fluid to the developing biofilm (Tolker-Nielsen *et al.*, 2000). The nature of the surface that is being colonized and the physical and chemical conditions of the environment will determine which of the mechanisms of biofilm formation will dominate (Hall-Stoodley and Stoodley, 2002; Stoodley *et al.*, 2002). Maturation of the biofilm results in the generation of mushroom- or pillar-like structures interspersed with fluid-filled channels (Costerton *et al.*, 1995; Tolker-Nielsen *et al.*, 2000), and once fully developed, a biofilm generates altered patterns of bacterial growth, physiological cooperation and metabolic efficiency (Costerton *et al.*, 1999; Rice *et al.*, 2000; Geesey, 2001; Werner *et al.*, 2004).

Notably, the biofilm cells display altered behaviour in gene expression. In a recent study, mature biofilms of *P. aeruginosa* were shown to have a radically different protein profile from planktonic bacteria grown in chemostats (Sauer *et al.*, 2002). As much as 50% of the detectable proteome (over 800 proteins) was shown to have a six-fold or greater difference in expression. Of these, more than 300 proteins were detectable in mature biofilm samples that were undetectable in planktonic bacteria. The identified proteins fell into five major classes, *i.e.* metabolism, phospholipid and lipopolysaccharide (LPS)-biosynthesis, membrane transport and secretion, as well as adaptation and protective mechanisms (Sauer *et al.*, 2002). By making use of DNA microarrays to compare gene expression of biofilm and planktonic *P. aeruginosa* PAO1 grown either in chemostats or in once-flow through tubing, Whiteley *et al.* (2001) reported that 73 genes displayed alterations in expression. The genes identified to be up-expressed in mature biofilms were genes encoding proteins involved in translation,

metabolism, gene regulation and membrane transport and/or secretion, whilst flagella and pilin genes were down-regulated, as was the gene encoding RpoS.

1.2.1.4 Detachment

The growth potential of the bacterial biofilm is ultimately limited by the availability of nutrients in the immediate environment, the expression of quorum-sensing molecules released in response to nutrient limitation, accumulation of toxic by-products and other factors, including pH, oxygen perfusion, carbon source availability and osmolarity (Puskas *et al.*, 1997; Allison *et al.*, 1998; Davies *et al.*, 1998; O'Toole and Kolter, 1998b; O'Toole *et al.*, 2000b; Prigent-Combaret *et al.*, 2001; Sauer *et al.*, 2004). At some point, the biofilm reaches critical mass and a dynamic equilibrium is reached at which the cells may detach and together with progeny of other biofilm cells may colonize other surfaces (Korber *et al.*, 1989).

Although it has generally been believed that bacterial cells leave the biofilm after division of cells in the outer layers of the biofilm matrix, recent studies have suggested that the detachment process may be more complex than originally thought. Several recent studies have reported pronounced activity and cellular differentiation localized to the center of mature biofilm structures, which led to the dispersal of cells from inside the structure, leaving behind large transparent cavities, or hollow "shells" made up of non-motile cells (Tolker-Nielsen *et al.*, 2000; Sauer *et al.*, 2002). Several mechanisms for biofilm dissolution and consequently, cell dispersal have been proposed. Enzymes such as polysaccharide lyases that degrade the extracellular polysaccharide matrix have been reported to play a role in biofilm dissolution in several organisms (Sutherland, 1999; Kaplan *et al.*, 2003). Boyd and Chakrabarty (1994) reported that induction of alginate lyase expression in *P. aeruginosa* substantially decreased the amount alginate produced, which corresponded with a significant increase in the number detached cells. It was thus suggested that the role of alginate lyase in wild-type *P. aeruginosa* might be to cause the release of cells from solid surfaces or biofilms, thereby aiding in the dispersal of these organisms. Recently, death of a subpopulation of cells has also been observed as a normal feature of biofilm development in *P. aeruginosa* (Webb *et al.*, 2003b). Cell death occurred inside microcolony structures, and killed only a subpopulation of cells within the biofilm. *P. aeruginosa* cell death was linked to the expression of a Pf1-like filamentous prophage of *P. aeruginosa* (Webb *et al.*, 2003b). It was proposed that prophage-mediated cell death might be an important mechanism of differentiation inside *P. aeruginosa*

microcolonies, which facilitates subsequent dispersal of a subpopulation of surviving cells (Webb *et al.*, 2003b).

1.3 BACTERIAL SURFACE APPENDAGES REQUIRED FOR BIOFILM FORMATION

Transposon and site-directed mutageneses analyses have greatly facilitated the identification and characterization of bacterial structural components required for initial attachment. The rate and extent of attachment of bacterial cells to a surface is influenced by cell surface hydrophobicity, presence of flagella, pili and adhesins, outer membrane proteins and production of extracellular polymeric substances (EPS) (O'Toole and Kolter, 1998a; 1998b; DeFlaun *et al.*, 1999; Genevaux *et al.*, 1999; Espinosa-Urgel *et al.*, 2000; Finelli *et al.*, 2003; Jackson *et al.*, 2004). In addition, evidence suggests that the primary development of a biofilm might be regulated at the level of population density-dependent gene expression controlled by cell-to-cell signaling molecules such as acylated homoserine lactones (McLean *et al.*, 1997; Allison *et al.*, 1998; Davies *et al.*, 1998). In the following sections, the role(s) of flagella and pili in adhesion of bacteria to surfaces will be addressed specifically.

1.3.1 Importance of flagella

Although earlier studies have suggested that simple chemical models could account for the bacterial behaviour during the initial stages of attachment (Marshall *et al.*, 1971; McEldowney and Fletcher, 1986), subsequent studies, mainly through transposon mutagenesis, have shown that structural components such as flagella, pili and adhesins play an important role in bacterial interaction with the surface. The primary function of flagella in biofilm formation is believed to be in transport and in initial cell-to-surface interactions. This has been based on observations that the absence of flagella impaired the ability of *P. fluorescens* and *P. putida* to colonize potato and wheat roots (De Weger *et al.*, 1987; DeFlaun *et al.*, 1994), and reduced cellular adhesion of *P. aeruginosa* and *P. fluorescens* to a polystyrene surface (O'Toole and Kolter, 1998a; 1998b). Similarly, the absence of flagella in *Vibrio cholerae* (Watnick *et al.*, 1999) and in *Escherichia coli* (Genevaux *et al.*, 1996; Pratt and Kolter, 1998) prevented the mutant strains in forming biofilms resembling those formed by the wild-type bacteria on polyvinylchloride (PVC).

More recent evidence obtained though proteomic analysis has indicated that gene expression of flagellar components (*fleN* and *flgG*) was down-regulated in 6-h biofilms of *P. putida*

(Sauer and Camper, 2001). In addition, expression of the *flgD*, *fliD* and *flgE* genes was reported to be repressed in 24-h biofilms of *P. aeruginosa* (Whiteley *et al.*, 2001). These observations seem to indicate that after initial cell-to-surface contact, the flagella become dispensable for further biofilm development. However, several studies have subsequently shown that the initial down-regulation of flagella is transitory and motile bacteria are present in biofilms at later stages of biofilm development, suggesting a role for flagella in the detachment of cells from the biofilm (Sauer and Camper, 2001; Sauer *et al.*, 2002).

1.3.2 Importance of pili and adhesins

Pili and pilus-associated adhesins have also been shown to be important for the adherence to and colonization of surfaces. Expression of *sfaA*, the gene encoding S-fimbrial adhesins, of a pathogenic strain of *E. coli* has been reported to be up-regulated upon attachment (Schmoll *et al.*, 1990). In *E. coli*, attachment is reduced by mutations in the curlin biosynthetic gene *csgA* (Vidal *et al.*, 1998; Dorel *et al.*, 1999), and in the type I pili biosynthetic gene *fimH*, which encodes the mannose-specific adhesin (Pratt and Kolter, 1998). Similarly, mutations in the mannose-sensitive hemagglutinin pilus of *V. cholerae* also resulted in a reduction of adhesion to surfaces (Watnick *et al.*, 1999). In *P. aeruginosa*, type IV pili mutants have been shown to be impaired in their ability to form microcolonies (O'Toole and Kolter, 1998a), whilst Vallet *et al.* (2001) reported that a *cupA* mutant was defective in biofilm formation in *P. aeruginosa*. The *cupA* gene cluster encodes the components of a chaperone/usher pathway that is involved in assembly of fimbrial subunits such as P pili and type I pili, but not type IV pili (Soto and Hultgren, 1999), suggesting that pili other than type IV pili may be involved in initial attachment of *P. aeruginosa* to surfaces.

The importance of pili in the initial stages of biofilm formation has been supported by proteomic analysis indicating the up-regulation of gene expression of type IV pili components (*pilR*, *pilC* and *pilK*) in 6-h old biofilms of *P. putida* (Sauer and Camper, 2001). In mature biofilms of *P. aeruginosa*, expression of the *pilA* gene, which encodes the type IV pilin subunit, was repressed. This may indicate that although type IV pili are involved in the initial steps of biofilm formation, they may not be required for maintenance of the mature biofilm (Whiteley *et al.*, 2001). In addition to a role for type IV pili in the initial phase of biofilm development (O'Toole and Kolter, 1998a), a model for *P. aeruginosa* biofilm development has recently been proposed in which type IV pili-driven bacterial migration plays a key role in

structural formation in the late stage of biofilm development (Klausen *et al.*, 2003). According to the model, the formation of mushroom-shaped structures in *P. aeruginosa* biofilms occurs through stalk formation by proliferation of bacteria that have down-regulated twitching motility and cap formation by bacteria that climb the microcolony stalks by the use of type IV pili and aggregate on top.

1.4 PROTEIN SECRETION PATHWAYS

Due to the apparent important role that pili play during the early stages of biofilm formation, the emphases in the next part of the literature review will be placed on type IV pili and related novel pilus pathways used in the secretion of pili and adhesins. In Gram-negative bacteria, secretion of extracellular proteins involves passing through the periplasm in addition to two membranes, the inner or cytoplasmic membrane and the outer membrane (Lengeler *et al.*, 1999). Since both the inner and outer membranes are quite complex (Lengeler *et al.*, 1999), Gram-negative bacteria possess at least five different pathways for protein secretion. Their classification is broadly based on sequence, structure and function (Lory, 1998; Thanassi and Hultgren, 2000). Most of the export pathways are dependent on the general secretory (Sec) pathway to export proteins across the inner membrane prior to secretion across the outer membrane. In contrast to type II, IV and V pathways, which export proteins with cleavable N-terminal signal sequences, and require the Sec translocase system for translocation of the proteins across the inner membrane, the type I and III pathways are capable of exporting proteins directly from the cytoplasm to the external environment. Since the secretion of pilin and adhesins are mostly dependent of the Sec translocation system, this section will be introduced first by a discussion of the general secretion pathway (Gsp).

1.4.1 The general secretion pathway (Gsp)

1.4.1.1 Secretion across the inner membrane

The Sec secretion system of *E. coli* (Fig. 1.2) has been studied extensively and consists of a cytoplasmic secretion-specific chaperone (SecB), a protein translocation ATPase (SecA) and an integral membrane protein complex formed by at least six different protein subunits (SecY, SecE, SecD, SecF, SecG and YajC) (Pugsley, 1993; Pugsley *et al.*, 1997).

The SecB chaperone maintains presecretory proteins in an unfolded and translocation-competent state and is also responsible for targeting them to SecA. SecA then targets the

presecretory protein-SecB complex to the Sec complex in the membrane for export across the inner membrane. Translocation of the presecretory protein is initiated by the binding of ATP to SecA, which promotes insertion of the signal peptide and part of the mature protein sequence, together with a SecA domain, into a translocation channel consisting of SecYEG (Duong and Wickner, 1999). SecA translocates *ca.* 20-30 amino acids of the exported protein upon ATP binding (Driessen *et al.*, 1998) and subsequent ATP hydrolysis results in the release of SecA from the partially translocated protein and the Sec complex in the inner membrane. This process is then repeated by SecA binding to another region of the exported protein, thereby promoting translocation of another 20-30 amino acids. The translocation intermediates are driven forward by the proton-motive force (PMF), which also promotes the release of SecA from the Sec complex (Economou *et al.*, 1998).

After translocation, the presecretory protein remains bound to the inner membrane by its amino (N)-terminal signal peptide and the signal peptide is cleaved by the appropriate membrane-bound signal peptidase. A typical signal sequence contains a stretch of *ca.* 18-30 amino acids that can be divided into three general domains: a N domain containing positively-charged amino acids that associates the presecretory protein with the inner membrane and correctly orientates the protein for translocation, a H domain containing a core of hydrophobic amino acids that inserts the signal sequence into the inner membrane, and a C domain containing a cleavage site recognized by the signal peptidase (Pugsley, 1993; Salmond and Reeves, 1993). There are three types of signal peptidases and each type possesses different substrate specificity (Pugsley, 1993; Peatzel *et al.*, 2000). Whereas the type I signal peptidases are responsible for proteolytic processing of the N-terminal signal peptides of secreted proteins, the type II signal peptidases are responsible for proteolytic processing of secreted lipoproteins only (Peatzel *et al.*, 2000). The type III signal peptidases are responsible for proteolytic processing of type IV prepilins and possess two enzymatic activities: an endoproteolytic activity, which is required for removal of the signal peptides of type IV pilin subunits, and a N-methylation activity for posttranslational modification of type IV pilins (Lory and Strom, 1997; Peatzel *et al.*, 2000). After proteolytic processing by the appropriate signal peptidase, the mature potein is usually released into the periplasm, whereas its signal peptide is further degraded by protease IV (Ichihara *et al.*, 1986).

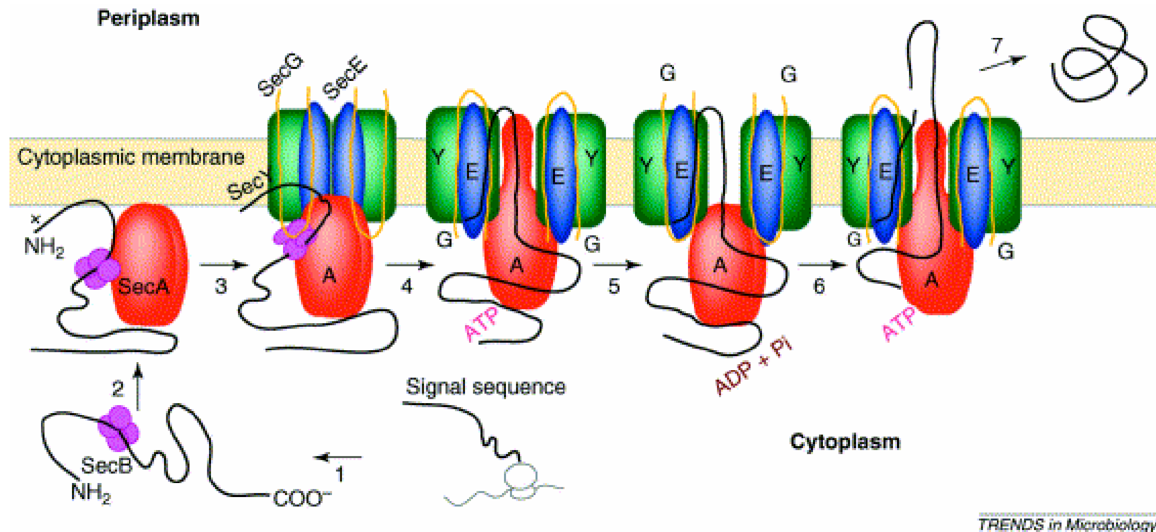


Fig. 1.2 Sec secretion process across the inner membrane. The precursor protein is represented by a black line and the signal sequence is represented by the gray region. Steps 1-3 is targeting of the precursor protein: The signal sequence and its immediate C-terminal region comprise an initiation sequence that is recognized by the Sec machinery. SecB, the Sec-specific chaperone, channels the precursor protein to the Sec translocation pathway where it targets its precursor to the translocase by binding to SecA. The precursor protein-SecA complex then binds to the membrane at a high affinity SecA binding site. SecY, E, and G forms a hetero-trimeric complex, SecYEG, which provides the channel for the protein to cross the inner membrane. Steps 4 and 5 is translocaton initiation and requires ATP, but not hydrolysis. Step 6 is continuation of the translocation and requires ATP hydrolysis and/or proton-motive force. Translocation is thought to occur in a stepwise fashion involving 20-30 amino acid residues. Step 7 is completion of translocation where the protein is released into the periplasmic space (Mori and Ito, 2001).

1.4.1.2 Protein folding in the periplasmic space

Secreted proteins exposed temporarily to the periplasmic environment may need to remain partially unfolded before they are transported across the outer membrane. Periplasmic proteins, such as DsbA and DsbC, are required for the formation of disulphide bonds in proteins that are present in the periplasm (Bardwell, 1994), whilst at least four periplasmic proteins (SurA, PpiA, PpiD and FkpA) catalyzes isomerization of peptidyl-proline bonds in proline-containing proteins, which may be required for their proper folding (Lazar *et al.*, 1996; Dartigalongue *et al.*, 1998).

In addition to enzymes required for proper folding of proteins, the periplasm also contains chaperones that can prevent secreted proteins from folding into their final conformation or can assist proteins in folding correctly. Known periplasmic chaperones include the P pilus-specific chaperone PapD (Holmgren and Branden, 1989) and LolA, a chaperone that is required for proper localization of outer membrane lipoproteins (Tajima *et al.*, 1998). The periplasm also contains several proteases that activate turnover of damaged or misfolded proteins. Amongst these, the protease DegP is considered to be one of the primary proteases responsible for turnover of misfolded proteins in the periplasm (Pallen *et al.*, 1997).

1.4.1.3 Secretion across the outer membrane

The final step of the general secretion pathway is translocation of proteins to be secreted across the outer membrane. Whereas all proteins secreted by the Gsp traverse the inner membrane via the Sec translocation complex, the terminal step can take several different routes, referred to as terminal branches. The main terminal branch of the Gsp is referred to as the type II secretion pathway, while the chaperone/usher, the autotransporter and type IV secretion pathways each represent alternate terminal branches of the Gsp (Thanassi *et al.*, 1998; Thanassi and Hultgren, 2000).

1.5 TYPE IV PILUS EXPRESSION AND ASSEMBLY

As discussed previously, adherence of bacteria to surfaces is often mediated by proteinaceous appendages (flagella, pili, fimbriae) protruding from the cell envelope. Among the best-characterized surface appendages are the type 1 and P pili of *Escherichia coli* (Orndorf and Bloch, 1990; Saulino *et al.*, 1998), type IV pili of *P. aeruginosa* (Strom and Lory, 1993; Hahn, 1997), and curli of *E. coli* and *Salmonella enteritidis* (Olsen *et al.*, 1989; Romling *et*

al., 1998). These pili have all 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. Fimbriae have been classified based on morphological, serological, biochemical or functional criteria (Sauer *et al.*, 2000), and details on the molecular mechanisms of fimbrial biosynthesis have been worked out to varying degrees in different systems. Whereas type IV pili in *P. aeruginosa* are assembled via the type II secretion system (Nunn, 1999), type 1 and P pili in *E. coli* are translocated to the cell surface by a chaperone-usher pathway (Sauer *et al.*, 2000) and curli fimbriae, in *E. coli* and *Salmonella* spp., are assembled by an extracellular nucleation-precipitation pathway (Romling *et al.*, 1998). Since type IV pili play an important role in the ability of *P. aeruginosa* to attach to surfaces, their properties and biogenesis merit further discussion.

1.5.1 Type IV pili

The pili from a broad spectrum of Gram-negative bacteria are grouped as type IV pili on the basis of amino acid sequence similarities among their major pilin subunit. The homology between different type IV pilins is highest at their N-terminus, but also extends to some areas of the C-terminus, where other conserved features, like a pair of cysteine residues that form a disulfide bridge in the mature protein, can be identified (Strom and Lory, 1993). The type IV pili are regarded as important virulence factors and occur in many different pathogens, including *P. aeruginosa* (Strom and Lory, 1993). In *P. aeruginosa*, type IV pili have been implicated in a variety of functions, including adhesion to biotic and abiotic surfaces (Hahn, 1997; O'Toole and Kolter, 1998b), modulation of target cell specificity (Bieber *et al.*, 1998), bacteriophage adsorption (Mattick *et al.*, 1996) and twitching motility (Wall and Kaiser, 1999).

1.5.1.1 Characteristics

The major subunit of the type IV pilus is type IV pilin, which possesses a number of unique properties. It contains a short (6-7 amino acids) basic N-terminus leader peptide, a modified amino acid residue (N-methylophenylalanine) at the N-terminus of mature pilin, a highly hydrophobic N-terminal domain, and a disulfide-bonded C-terminal domain (Hobbs and Mattick, 1993; Pugsley, 1993; Alm and Mattick, 1997). A specialized inner membrane signal peptidase is required to remove the leader peptide and for maturation of the pilin subunit (Nunn and Lory, 1991; Strom and Lory, 1992). Based on immunologic and crystallographic

data, the type IV pili structures have a diameter of 60 Å and are typically up to 4 000 nm long, with a pitch distance of *ca.* 40 Å and about 5 subunits per turn (Parge *et al.*, 1995). Although most type IV pili are flexible rod-like structures and arranged in a helical manner, the toxin-coregulated pili (Tcp) of *V. cholerae* (Taylor *et al.*, 1987) and the bundle-forming pili (Bfp) of enteropathogenic *E. coli* (Girón *et al.*, 1991) have some unique characteristics among type IV pili. The TcpA and BfpA pilins assemble into straight fibers of variable length that have a strong tendency to aggregate laterally (Bieber *et al.*, 1998). In addition, whereas most type IV prepilin proteins are characterized by a short basic leader sequence and a conserved phenylalanine residue that follows the cleavage site (referred to as type IVa pili), the latter prepilins are characterized by larger leader peptides and the absence of phenylalanine in the position immediately after the conserved leader peptide cleavage site (referred to as type IVb pili). At present, only two tip adhesins have been identified among type IV pili. These are PilC of *Neisseria gonorrhoeae* (Rudel *et al.*, 1995) and *N. meningitides* (Ryll *et al.*, 1997), respectively. Although there is a homologue of *Neisseria pilC* in *P. aeruginosa*, termed *pilY* (Alm *et al.*, 1996a), the role of PilY is, however, unclear since the main adhesion determinant of *P. aeruginosa* type IV pili is found in their major pilin subunit (Farinha *et al.*, 1994; Lee *et al.*, 1994).

1.5.1.2 Type IV pilus biogenesis genes

In recent years, *P. aeruginosa* has been used as the primary model for studying fimbrial biology. Characterization of *P. aeruginosa* mutants, which lack twitching motility, has led to the identification of a large number of genes, located in six chromosomal clusters, that are involved in fimbrial biogenesis and function. Many of these genes have homology to other gene/protein sets involved in protein secretion and DNA uptake in various bacteria (Hobbs and Mattick, 1993; Alm and Mattick, 1997). These genes can be divided into four groups: (i) four transcriptional regulators (*pilS*, *pilR*, *fimS*, *algR*); (ii) eight *che*-like genes that control flagellar-based swimming and type IV pilus-based twitching (Darzins and Russell, 1997) (*pilG*, *H, I, J, K, L*, *chpA* and *chpB*); (iii) 19 type IV pilus biogenesis genes (*pilA, B, C, D, E, F, M, N, O, P, Q, V, W, X, Y1, Y2, Z, fimT* and *fimU*); and (iv) two pilus function genes (*pilT* and *pilU*) (Alm and Mattick, 1997; Wall and Kaiser, 1999; Chiang *et al.*, 2005).

Several of the *P. aeruginosa* chromosomal gene clusters contain genes directly involved in fimbrial assembly. The *pilABCD* locus encodes the major pilin (PilA) and three ancilliary

proteins, namely: PilB, a cytoplasmic protein possessing an ATP-binding motif; PilC, an inner membrane protein; and PilD, a prepilin peptidase that performs both cleavage and methylation of the pilin subunit (Nunn *et al.*, 1990; Nunn and Lory, 1991; 1992). Mutations within PilC have been reported to cause the loss of surface fimbriae (Mattick *et al.*, 1996). Similarly, inactivation of the *pilB* gene or mutagenesis of the ATP-binding motif results in the inability to assemble fimbriae on the cell surface (Turner *et al.*, 1993). It has therefore been suggested that PilB plays a role in providing energy for the assembly and function of the type IV pilus export apparatus (Chiang *et al.*, 2005). Notably, both the PilT and PilU proteins, like the PilB protein, contain nucleotide-binding motifs, suggesting that they also have NTPase activity (Whitchurch *et al.*, 1991; Whitchurch and Mattick, 1994). However, in contrast to *pilB* mutants, both *pilT* and *pilU* mutants express structurally normal pili, but lack motility (Whitchurch *et al.*, 1991; Whitchurch and Mattick, 1994; Wu *et al.*, 1997). Recently, it was reported that these proteins are essential for twitching motility. Since *pilT* and *pilU* mutants are hyperpiliated, it may indicate that these mutants are unable to retract their type IV pili. It has been proposed that PilT, specifically, serves as a biological motor required for the retraction of type IV pili (Forest *et al.*, 2004; Chiang *et al.*, 2005). Conversely, PilB may therefore provide energy for pilus extension by polymerization of the type IV pilin subunits (Wall and Kaiser, 1999). Located 25 kb from *pilABCD*, the genes *fimT*, *fimU*, *pilV* and *pilE* encode products of which the function(s) is not yet known, but they contain prepilin-like N-terminal hydrophobic domains (Russell and Darzins, 1994; Alm and Mattick, 1995; 1996). Whereas *pilV*, *pilE* and *fimU* mutants are unable to produce extracellularly assembled fimbriae and accumulate PilA within the membrane fraction, a *fimT* mutant resembles the wild-type phenotype (Alm and Mattick, 1996).

The operon *pilMNOPQ* encodes components of pilus assembly located at the inner membrane and outer membrane, and mutants in these genes are non-fimbriated (Martin *et al.*, 1995). PilQ appears to be the sole protein of the assembly system that is an integral outer membrane component (Martin *et al.*, 1993) and it is capable of oligomerizing to form a gated channel with a central cavity of *ca.* 55 Å (Tonjum and Koomey, 1997; Bitter *et al.*, 1998). The size of the central pore formed by PilQ oligomers is in agreement with the calculated outer diameter (52 Å) of the type IV pili (Folkhard *et al.*, 1981). These properties make PilQ the ideal candidate for assembly of the channel through which the pilus rod is exported to the extracellular milieu (Alm and Mattick, 1997; Collins *et al.*, 2005). Apart from PilQ, the

specific components of type IV pilus secretion systems are inner membrane or cytoplasmic proteins.

With reference to the other proteins involved in fimbrial biogenesis, there are a number of other genes whose products are involved in the export of the fimbrial subunit, as mutations in these genes are non-fimbriated. These include the *pilW* and *pilX* genes, which encode prepilin-like proteins, the *pilY1* gene, which encodes a homologue of the gonococcal PilC adhesin and the *pilY2* gene, which encodes a novel small protein of unknown function (Alm *et al.*, 1996a). In addition, both *pilF* and *pilZ* mutants are blocked in the export/assembly of fimbrial biogenesis, with the processed subunit accumulating in the membrane fraction (Alm *et al.*, 1996b; Watson *et al.*, 1996a; 1996b).

1.5.2 Model for type IV pilus assembly and secretion

Although knowledge regarding type IV pilus biogenesis remains incomplete, the following working model for type IV pilus assembly of *P. aeruginosa* (Fig. 1.3) has been proposed by Alm and Mattick (1997) and Hahn (1997). Following translocation of the pre-PilA precursor subunits into the periplasmic compartment by the general secretory pathway, these molecules are transitorily anchored into the inner membrane, at the periplasmic side, by a conserved hydrophobic domain located at the N-terminus, immediately after the signal peptide, while the hydrophilic C-terminal domains are oriented towards the periplasm (Kaufman *et al.*, 1991; Pugsley, 1996). The hydrophobic N-terminus is subsequently cleaved at the cytoplasmic side of the inner membrane by prepilin peptidase, PilD, to generate mature PilA (Nunn and Lory, 1991). The mature pilins pack into a pilus fiber in a helical structure with its hypervariable regions exposed. The core of the pilus forms a continuous hydrophobic layer with the inner membrane. This hydrophobic continuum facilitates the low-energy-requiring polymerization and depolymerization of the pili that is under control of the inner membrane-associated protein complex. PilT may function as an ATPase and transduce energy for depolymerization (Chiang *et al.*, 2005), whilst PilB, another nucleotide-binding motif containing protein, may transduce energy for pilus polymerization (Wall and Kaiser, 1999). The assembled pilin is thought to be translocated across the outer membrane via a gated channel formed by a multimeric complex of PilQ. PilP, a probable lipoprotein (Martin *et al.*, 1995), may function in stabilizing the PilQ complex. In the case of *Neisseria* spp., PilC facilitates the translocation

of the pili through the pore and thereby presents itself to the pilus tip, where it functions as a specific adhesin (Nassif *et al.*, 1994; Rudel *et al.*, 1995).

1.5.3 Relationship between type II and type IV secretion systems

All type IV pili use a similar secretion and assembly machinery, which is functionally and evolutionary related to components of the type II secretion system of Gram-negative bacteria (Pugsley, 1993; Russel, 1998) (Fig. 1.3). Consequently, the type IV pilus assembly machinery has been grouped as part of the type II secretion pathway. In *P. aeruginosa*, PilD is shared between the type II (Xcp) export system (Filloux *et al.*, 1998) and the type IV pilus biogenesis system (Strom and Lory, 1993). PilD (XcpA) is a bifunctional enzyme carrying out both cleavage and N-methylation of the PilA pilin subunit, as well as of four other proteins, *i.e.* PilE and PilV-X (XcpT-W) (Nunn and Lory, 1992; Strom and Lory, 1992) that are part of the export machinery. The XcpTUVW proteins are referred to as pseudopilins, *i.e.* proteins homologous to the major subunit of type IV pili in the amino-terminal moiety (Filloux *et al.*, 1998), and they are involved in type IV pilus biogenesis, probably as components of the type II secretion apparatus (Filloux *et al.*, 1998). This suggests that these pseudopilin subunits may form a pilus-like fiber similar to that of type IV pili.

In addition to the central requirement for PilD and the sequence similarities of the pilin and pseudopilin proteins, the similarity of the two systems also extends to the presence of at least three additional sets of proteins whose homologues can be seen in both: PilB and XcpR, PilC and XcpS, and PilQ and XcpQ. PilB and XcpR are characterized by their nucleotide-binding motif and peripheral membrane localization, and it is likely that these proteins play a role in providing energy for the assembly and function of the type IV pilus and type II export apparatus (Turner *et al.*, 1993; Wall and Kaiser, 1999). PilC and XcpS are integral cytoplasmic membrane proteins and it has been postulated that this component interacts at the cytoplasmic face of the inner membrane with the corresponding nucleotide-binding PilB homologue (XcpR) (Bally *et al.*, 1992). Of all the components of the type II and type IV pilus biogenesis systems, only one protein in each system is localized within the outer membrane. PilQ and its homologue in the type II export apparatus, XcpQ, form multimers of 12-14 subunits (Tonjum and Koomey, 1997; Bitter *et al.*, 1998), suggetsing that the protein is able to form a large channel in the outer membrane.

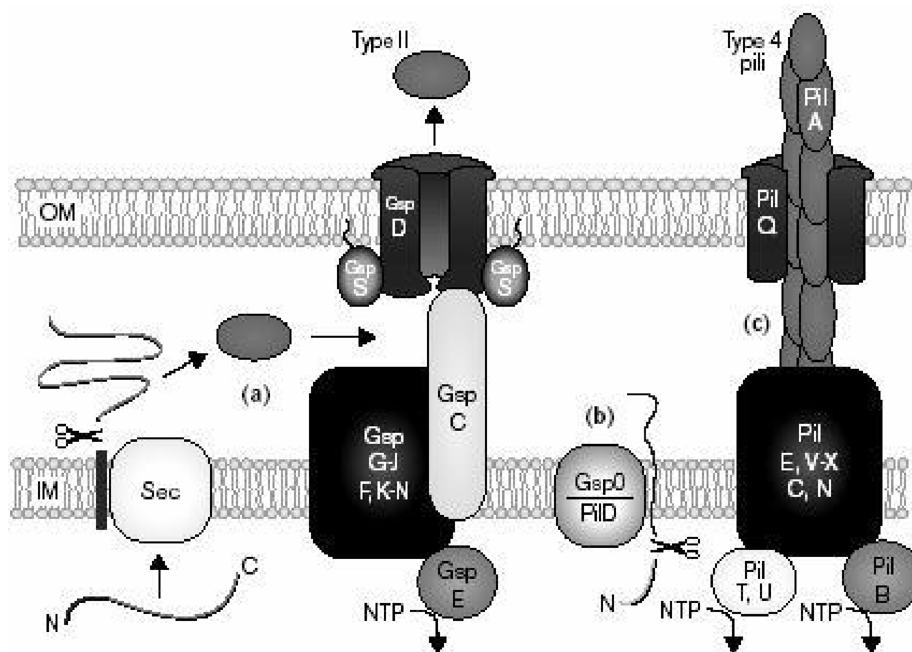


Fig. 1.3 Models for type II secretion and type IV pilus biogenesis. Components of the type II secretion are indicated using the general secretory pathway (Gsp) nomenclature, and type IV pilus proteins are labeled according to the *P. aeruginosa* Pil system. Similar shading and location indicates homologous components. (a) Type II substrates cross the inner membrane (IM) via the Sec system followed by signal-sequence cleavage and protein folding in the periplasm. The GspD secretin, indicated as a complex with the GspS lipoprotein, serves as a gated channel for secretion of substrates to the cell surface. GspC may transmit energy from the inner membrane, presumably generated by the cytoplasmic GspE nucleotide-binding protein, to the outer membrane (OM) complex. GspG-J exhibit homology to the pilin subunit PilA and are processed by the GspO prepilin peptidase. (b) GspO cleaves their amino-terminal leader sequence on the cytoplasmic face of the inner membrane. In *P. aeruginosa*, the type IV prepilin peptidase PilD is the same protein as GspO. (c) Type IV pilus biogenesis requires the outer membrane secretin PilQ. The type IV pilus system contains at least four pilin-like components (PilE and PilV-X), in addition to PilA, which are processed by PilD. Additional nucleotide-binding proteins (PilT, PilU) are present and are involved in twitching motility (Thanassi and Hultgren, 2000).

1.6 NOVEL FIMBRIAL EXPRESSION AND ASSEMBLY SYSTEMS

Novel pili and pilus biogenesis/secretion systems have been described in *Actinobacillus actinomycetemcomitans* (Kachlany *et al.*, 2000), *Haemophilus ducreyi* (Nika *et al.*, 2002) and *Caulobacter 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 *flp-tad* fimbrial gene cluster in *Actinobacillus actinomycetemcomitans***

A. actinomycetemcomitans, a Gram-negative coccobacillus, is the etiological agent of localized periodontitis, infective endocarditis and brain abscesses (Slots and Ting, 1999; Fives-Taylor *et al.*, 2000). Fresh clinical isolates have been reported to be able to adhere tightly to solid abiotic surfaces, such as glass, plastic and hydroxyapatite, and to form a tenacious biofilm (Fine *et al.*, 1999; Kachlany *et al.*, 2000). Electron microscopy has revealed that these clinical isolates express long, thick fibrils (Holt *et al.*, 1980; Inouye *et al.*, 1990), each of which consists of a parallel array of individual pili of *ca.* 5 to 7 nm in diameter (Inouye *et al.*, 1990; Kachlany *et al.*, 2001). The fibrils are often several microns long and up to 100 nm thick (Kachlany *et al.*, 2001). Biochemical analysis has indicated that the pili of *A. actinomycetemcomitans* are composed of a 6.5-kDa polypeptide, which has been designated Flp (for fimbrial low-molecular-weight protein) (Inoue *et al.*, 1998). Subsequent amino acid sequence analysis of Flp has led to the identification of the *flp-1* gene, and indicated that the Flp-1 precursor has a signal peptide sequence at its N-terminus that is likely to be cleaved upon export from the bacterial cell (Inoue *et al.*, 1998; Kachlany *et al.*, 2001). Insertion mutagenesis studies have indicated that *flp-1* mutants fail to adhere to surfaces and do not express pili (Kachlany *et al.*, 2001). Immediately downstream from *flp-1* is a second homologous gene, *flp-2*, of which the functional significance is not known since the gene does not appear to be expressed in *A. actinomycetemcomitans* (Kachlany *et al.*, 2001). Nevertheless, phylogenetic analysis of Flp-2 has indicated that not only is it a homologue of Flp-1, but that both Flp-1 and Flp-2 belong to a distinct subfamily of the type IV pilin subunits (Kachlany *et al.*, 2001).

The *flp* genes of *A. actinomycetemcomitans* are located upstream of a cluster of seven novel genes, which have been termed *tadABCDEFG* (for tight adherence) (Kachlany *et al.*, 2000).

Like *flp-1* mutants, mutations in any of the *tad* genes resulted in a defect in adherence and failure to produce fibrils (Kachlany *et al.*, 2000). Analysis of the amino acid sequences of the proteins encoded by the *tadABCDEFG* genes revealed that only TadA is similar to proteins of known function and it was reported that the TadA protein is an ATPase required for fibril production and tenacious adherence (Bhattacharjee *et al.*, 2001). Subsequent phylogenetic analysis revealed that TadA is the first representative of a distinct subfamily of potential type IV secretion NTPase genes (Planet *et al.*, 2001). Based on the phylogenies of the *tadA* and *flp* genes (Kachlany *et al.*, 2000; Planet *et al.*, 2001), it was suggested that they have experienced an evolutionary history that is independent of other secretion systems. Consequently, it was proposed that the *flp-tad* region specifies a novel system for the assembly and secretion of Flp pili that is distinct from the type II and type IV secretion systems (Bhattacharjee *et al.*, 2001).

- **The *flp-tad* fimbrial gene cluster in *Haemophilus ducreyi***

A 15-gene cluster has been identified in *H. ducreyi*, the etiological agent of the sexually transmitted disease chancroid, which encodes predicted proteins with significant homology to those encoded by the *A. actinomycetemcomitans flp-tad* locus (Nika *et al.*, 2002). Mutations within the *flp-1* and *flp-2* genes of *H. ducreyi* were found to significantly reduce the ability of the bacteria to attach and to form microcolonies when cultured *in vitro* with human foreskin fibroblasts. Furthermore, a mutant *H. ducreyi* strain with an inactivated *tadA* gene exhibited a decrease in virulence in a rabbit model for experimental chancroid (Nika *et al.*, 2002) and in humans (Spinola *et al.*, 2003).

- **The *pilA-cpa* pilus sytem in *Caulobacter crescentus***

A *pilA-cpa* locus (for *Caulobacter* pilus assembly) in *C. crescentus*, a non-pathogenic bacterium, was found to be responsible for the production of pili of unknown function, although it appears that the pili are used as receptors by bacteriophage ΦCbK (Skerker and Shapiro, 2000). The *C. crescentus* pilus-encoding region contains the pilin gene *pilA*, which is a member of the *flp* superfamily, as well as several homologues of genes present in the *flp-tad* locus of *A. actinomycetemcomitans* (Skerker and Shapiro, 2000). 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, suggesting that may function in pilus biogenesis and as a secretion pathway for the pili (Skerker and Shapiro, 2000).

- **The *flp-htp* gene cluster in *Pseudomonas aeruginosa***

The widespread existence of the TadA secretion ATPase and Flp pilin protein (Planet *et al.*, 2001), together with our interest in identifying factors mediating adhesion of *P. aeruginosa* to surfaces, prompted an earlier 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, a 9.172-kb region of the *P. aeruginosa* DNA, located at nucleotides 482 1381 - 483 0553, was identified that contained nine open reading frames (ORFs) encoding predicted proteins with homology to some of the Tad and/or Cpa proteins. These ORFs (PA4297 through PA4305) appear to be organized in a single transcribed operon. Furthermore, a protein encoded by the PA4306 ORF, located immediately upstream from this gene cluster, was found to display significant sequence similarity to the Flp-1 and PilA proteins of *A. actinomycetemcomitans* and *C. crescentus*, respectively (Fig. 1.4). The sequence similarities and the order of the homologous genes in *P. aeruginosa*, with the exception of the orientation of the *flp* gene, more closely resembles that of the *pilA-cpa* system of *C. crescentus* than the *flp-tad* systems of *A. actinomycetemcomitans* and *H. ducreyi*. Based on the above results, the *P. aeruginosa* gene cluster has been termed *htp*; the PA4305 through PA4297 genes were termed *htpABCDEFGHI*, and the upstream PA4306 gene, encoding a putative pilus monomer, was named *flp* (van Schalkwyk, 2003). Based on the available *in silico* data, a model was also proposed whereby this system may function. In this model, HtpD is proposed to act as an energizing (NTPase) protein, while HtpB, which displays similarity to the pulD/Type IV family of secretins, may form channels in the bacterial outer membrane to allow for export of the Flp prepilin subunits. The prepilin is proposed to be processed by an as-yet-unidentified prepilin peptidase. 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 (van Schalkwyk, 2003).

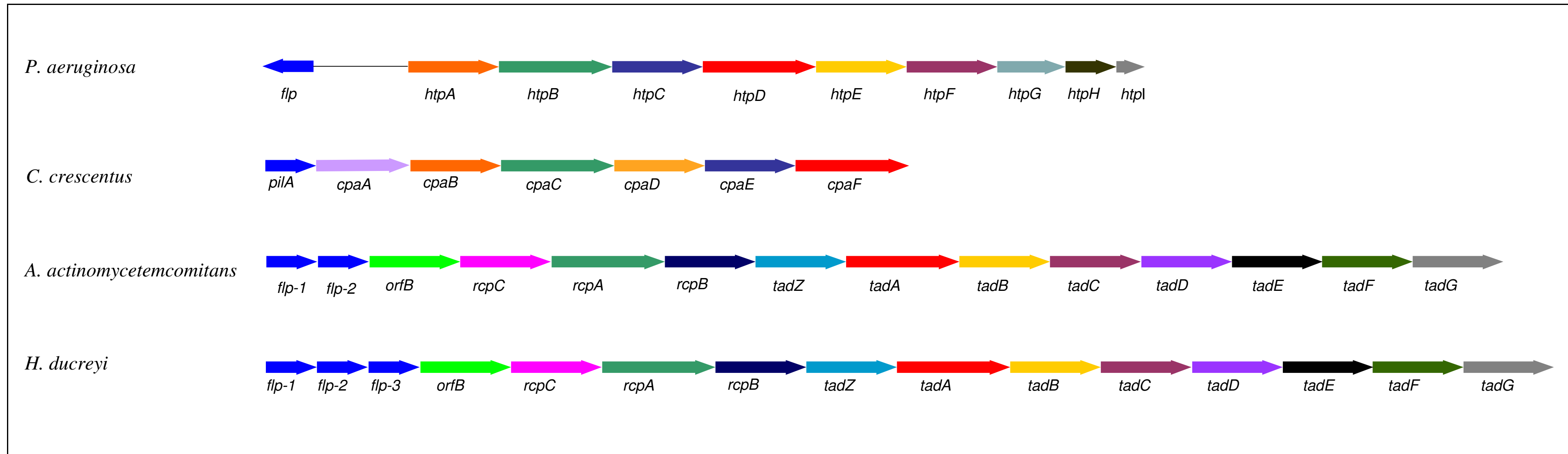


Fig. 1.4 Organization of the *flp-htp* gene cluster in *P. aeruginosa* compared to the *flp-tad* gene cluster in *A. actinomycetemcomitans* (GenBank accession no. AF152598) and *H. ducreyi* (GenBank accession no. AY083157), and the *pilA-cpa* gene cluster in *C. crescentus* (GenBank accession no. AAF40193). Open reading frames with similar predicted protein products (as determined by BLAST analysis) are indicated in the same colour. Designations below the ORFs indicate the ORF name given in the corresponding genome sequencing projects (Adapted from Van Schalkwyk, 2003).

1.7 AIMS OF THIS INVESTIGATION

As early as 1933, Henrici recognized the phenomenon that marine bacteria grow for most part on submerged surfaces, rather than being free-floating. With the re-discovery that bacteria are found predominantly attached to surfaces in aquatic systems (Geesey *et al.*, 1977; Costerton, 1995), much attention has been paid to unravelling the molecular mechanisms underlying the formation and regulation of biofilms (Costerton *et al.*, 1995; Davey and O'Toole, 2000; O'Toole *et al.*, 2000; Stoodley *et al.*, 2002). However, a novel pilus biogenesis/secretion system has been described in *A. actinomycetemcomitans* (Kachlany *et al.*, 2000; 2001) and homologues of this system have since been identified in the genomes of a wide variety of Gram-negative and Gram-positive bacteria (Planet *et al.*, 2001). It has subsequently been reported that the novel Flp pili may play a role in the attachment of *A. actinomycetemcomitans* (Kachlany *et al.*, 2000; 2001) and *H. ducreyi* to surfaces (Nika *et al.*, 2002). In *P. aeruginosa*, open reading frames (ORFs) that display homology to the novel pilus biogenesis genes have been designated *htp* and the ORF encoding the major fimbrial subunit was designated *flp* (Van Schalkwyk, 2003). Although the flagellum and type IV pili of *P. aeruginosa* have been implicated as playing a role during the early stages of biofilm formation by this bacterium (O'Toole and Kolter, 1998a), the involvement of pili other than type IV pili in this process has not yet been considered. Consequently, the primary aims of this investigation were the following:

- To investigate the transcriptional organization of the putative *flp-htp* fimbrial gene cluster of *P. aeruginosa* PAO1.

- To generate a *P. aeruginosa* PAO1 mutant strain in which the *htp* gene cluster has been inactivated and to compare the capability of the *P. aeruginosa* wild-type and mutant strains to form biofilm on glass wool.

CHAPTER TWO

TRANSCRIPTIONAL ANALYSIS OF A NOVEL PILIN-ENCODING SYSTEM IN *Pseudomonas aeruginosa* PAO1

2.1 INTRODUCTION

It has long been recognized that bacteria can switch from free-living unicellular organisms to sessile multicellular communities known as biofilms (Costerton *et al.*, 1987; Costerton *et al.*, 1995). *Pseudomonas aeruginosa*, an opportunistic pathogen primarily infecting immuno-compromised persons (Lazdunski *et al.*, 1990; Van Delden and Iglewski, 1998; Ramsey and Wozniak, 2005), preferentially assumes a biofilm mode of growth by embedding itself in a gelatinous organic polymer matrix composed of alginate (Costerton *et al*., 1995). Biofilm formation occurs in response to a variety of environmental signals (Davey and O'Toole, 2000; Stanley and Lazazzera, 2004) and the transition to surface-attached growth has been reported to result in diverse changes in gene expression, which causes the attaching cells to become phenotypically (Costerton *et al.*, 1995; Davies and Geesey, 1995; Sauer *et al.*, 2002) and metabolically (Costerton *et al.*, 1999; Davey and O'Toole, 2000) distinct from their planktonic counterparts. Most notably, biofilms are resistant to antimicrobial agents and it has been reported that bacteria existing in a biofilm can become up to 1000-times more resistant to antimicrobial agents than planktonic cells (Nickel *et al.*, 1985; Mah and O'Toole, 2001).

In *P. aeruginosa*, both flagellar motility and type IV pilus-based twitching motility have been proposed to play an important role during the initial attachment of the cells to surfaces (De Weger *et al.*, 1987; DeFlaun *et al.*, 1999; O'Toole and Kolter, 1998a; 1998b). The type IV pili are presumably the principal adhesins mediating adherence to eukaryotic cell surfaces (Kang *et al.*, 1997; Hahn, 1997), as well as abiotic surfaces (O'Toole and Kolter, 1998b). They appear to be required for initiation of biofilm formation by promoting cell aggregation and the formation of microcolonies. 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, a novel class of pili was identified in *Actinobacillus actinomycetemcomitans* (Kachlany *et al.*, 2000), *Caulobacter crescentus* (Skerker and Shapiro, 2000) and *Haemophilus ducreyi* (Nika *et al.*, 2002). Pili purified from *A. actinomycetemcomitans* (Inoue

et al., 1998) and *C. crescentus* (Skerker and Shapiro, 2000) comprised of a single major subunit with an apparent molecular mass of 6.5 and 4.8 kDa, respectively. The amino acid sequences of these small proteins display similarity to known type IV prepilin proteins, and the *A. actinomycetemcomitans* protein was designated Flp (Inoue *et al.*, 1998). The gene encoding the major fimbrial subunit proved to be part of a large gene cluster in all three organisms that constitute a polycistronic operon encoding a novel secretion system for the assembly and release of the pili (Skerker and Shapiro, 2000; Nika *et al.*, 2002; Haase *et al.*, 2003). Albeit that the role of these novel pili in *C. cresenctus* is not known, it has been reported that they are required by *A. actinomycetemcomitans* for adherence since cells containing mutations in a *flp-1* gene do not express pili and fail to adhere to glass (Kachlany *et al.*, 2000; 2001). Moreover, it has been reported that mutations within the *flp-1* and *flp-2* genes of *H. ducreyi* significantly reduced the ability of the bacteria to attach and to form microcolonies when cultured *in vitro* with human foreskin fibroblasts (Nika *et al.*, 2002).

Using the available sequence of the *P. aeruginosa* PAO1 genome, homologues to several of the previously reported *tad* and *cpa* genes have been identified by *in silico* analyses (Van Schalkwyk, 2003). The *P. aeruginosa* gene cluster was subsequently termed *htp* (for homologous to type IV pilus biogenesis proteins). However, the genetic organization of the *P. aeruginosa* gene cluster more closely resembles that of the *pilA-cpa* system of *C. crescentus* than the *flp-tad* systems of *A. actinomycetemcomitans* and *H. ducreyi* (see Fig. 1.4). Despite these similarities, the *P. aeruginosa* PAO1 gene cluster has several unique features. Most notably, although a single homologue of the *flp* prepilin gene could be identified in the *P. aeruginosa* genomic sequence (PA4306 [*flp*]), it does not appear to form part of the putative operon structure, but rather appears to be transcribed in the opposite direction compared to the *htp* gene cluster (PA4305 through PA4297).

Based on a report indicating that fimbriae other than type IV pili may play a role in attachment and biofilm formation of *P. aeruginosa* (Vallet *et al.*, 2001) and on reports indicating that the Flp pili of both *A. actinomycetemcomitans* and *H. ducreyi* play a role in adherence of these bacteria to abiotic surfaces (Kachlany *et al.*, 2001; Nika *et al.*, 2002), the functional significance of the *P. aeruginosa htp* gene cluster warrants further investigation. Consequently, the aims of this part of the investigation were (i) to analyze the transcriptional organization of the *P. aeruginosa* PAO1 *htp* gene cluster, (ii) to investigate the transcriptional activity of putative promoter elements under different growth conditions using *lacZ*

transcriptional fusions; and (iii) to express the Flp pilin-encoding gene of *P. aeruginosa* PAO1 in *Escherichia coli* to allow for its characterization.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this part of the study are listed in Table 2.1. *P. aeruginosa* and *Escherichia coli* strains were routinely cultured in LB broth (1% [w/v] tryptone, 1% [w/v] NaCl, 0.5% [w/v] yeast extract; pH 7.4) (Sambrook *et al*., 1989) at 37°C with shaking at 200 rpm, and maintained at 4°C on LB agar (LB broth containing 1.2% [w/v] bacteriological agar) or at -70°C as glycerol cultures. In *P. aeruginosa* PAO1, tetracycline at 100 µg/ml was used for plasmid DNA selection and maintenance. For plasmid DNA selection and maintenance in *E. coli*, the following concentrations of antibiotics were used: 100 µg/ml for ampicillin and 20 µg/ml for tetracycline. All antibiotics were obtained from Roche Diagnostics.

2.2.2 Genomic DNA extraction

Genomic DNA was isolated from *P. aeruginosa* PAO1 using cetyltrimethylammonium bromide (CTAB), as described by Jansen (1995). The cells from 1.5 ml of an overnight culture were pelleted by centrifugation at 14 000 rpm for 5 min and suspended in 567 µl of 1 × TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). The cells were lysed by the addition of SDS to a final concentration of 0.5% (v/v) and proteins were digested by the addition of Proteinase K to a final concentration of 100 µg/ml in a total volume of 600 µl. Following incubation at 37°C for 1 h, 100 µl of 5 M NaCl and 80 µl of a CTAB/NaCl solution (10% [w/v] CTAB in 0.7 M NaCl) was added, mixed thoroughly and incubated at 65°C for 10 min. The suspension was extracted with an equal volume of chloroform:isoamyl alcohol (24:1), followed by centrifugation at 10 000 rpm for 5 min. The supernatant, containing the genomic DNA, was transferred to a clean microfuge tube. The residual CTAB was removed by addition of an equal volume of phenol:choroform:isoamyl alcohol (25:24:1), followed by centrifugation at 10 000 rpm for 5 min. The genomic DNA was precipitated from the recovered aqueous supernatant by addition of 0.6 volume isopropanol. The precipitated genomic DNA was pelleted by centrifugation at 14 000 rpm for 2 min, washed with 70% ethanol, dried under vacuum and suspended in 30 µl 1 × TE buffer. An aliquot of the genomic DNA was analyzed by agarose electrophoresis.

2.2.3 DNA amplification

2.2.3.1 Oligonucleotide primers

The oligonucleotide primers used in PCR to amplify the putative *flp* (PA4306) ORF and intergenic region between *htpA* (PA4305) and *flp* (PA4306) from the extracted *P. aeruginosa* PAO1 genomic DNA are indicated in Table 2.2. The oligonucleotides were designed based on the published *P. aeruginosa* PAO1 genome sequence (Stover *et al*., 2000), available at http://www.pseudomonas.com, using the DNAMAN v.4.13 (Lynnon Biosoft) software program. To facilitate cloning of the amplicons, unique restriction endonuclease recognition sites were incorporated at the 5' or 3' terminus of the respective oligonucleotides. The oligonucleotides were synthesized by Inqaba Biotech.

2.2.3.2 Polymerase chain reaction (PCR)

The PCR reaction mixture (50 µl) contained 150 ng of genomic DNA as template, 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 0.1% [v/v] TritonX-100), 1.5 mM $MgCl_2$, 5% (v/v) DMSO, 200 µM of each dNTP, 25 pmol of each oligonucleotide and 1 U of *Taq* DNA polymerase (Southern Cross Biotechnology). The PCR reaction mixtures were placed in a Perkin-Elmer GeneAmp® 2700 thermal cycler. Following incubation at 94°C for 5 min, the samples were subjected to 25 cycles of denaturation at 94°C for 30 s, oligonucleotide annealing at 60°C for 40 s and elongation at 72°C for 40 s. After the last cycle, a final elongation step was performed at 72°C for 5 min to complete synthesis of all DNA strands. For control purposes, a reaction mixture containing all reagents except template DNA was included. The PCR reaction mixtures were analyzed by electrophoresis on a 1% (w/v) agarose gel in the presence of an appropriate DNA molecular weight marker.

2.2.4 Agarose gel electrophoresis

DNA was analyzed on horizontal 1% (w/v) agarose slab gels supplemented with 0.5 µg/ml ethidium bromide (Sambrook *et al*., 1989). The agarose gels were electrophoresed at 90 V in 1 × TAE buffer (40 mM Tris-HCl, 20 mM NaOAc, 1 mM EDTA; pH 8.5) and the DNA visualized on a UV transilluminator. The DNA fragments were sized according to their migration in the gel as compared to that of a standard DNA molecular marker, namely the GeneRuler™ 100-bp DNA ladder Plus (Fermentas AB).

Table 2.1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Reference
Strains:		
E. coli DH5α	F^- *recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1* Δ(*lacZYA-argF*) U169 λ^- [Φ80d*lac*ZΔM15]	Promega
P. aeruginosa PAO1	Wild-type	Holloway *et al.* (1979)
Plasmids:		
pGEM®-T Easy	Cloning vector for PCR products, ColE1, Amp^r, LacZα peptide	Promega
pGEX-2T	Prokaryotic GST fusion protein expression vector, ColE1, Amp^r, $laqI^q$, *tac* promoter, thrombin cleavage site	Smith and Johnson, 1989
pALacZsd	Tet^r, IncQ/RSF1010, t, T, promoterless *lacZ* gene	Weyers, 1999
pALacZsdIP	pALacZsd containing the *lac* promoter from pBluescript (co-ordinates 719-1719)	Weyers, 1999
pGEM-Flp	pGEM®-T Easy containing the 220-bp *flp* ORF amplification product	This study
pGEX-Flp	pGEX-2T with the *flp* ORF cloned into the *Bam*HI and *Eco*RI sites	This study
pGEM-IN1	pGEM®-T Easy containing the 410-bp IN-1F - IN-1R PCR amplification product	This study
pGEM-IN2	pGEM®-T Easy containing the 410-bp IN-2F - IN-2R PCR amplification product	This study
$pALacZsd_{(PAO1)}IN1$	pALacZsd containing the intergenic region cloned as a *Pst*I - *Bam*HI fragment in the 5'-to-3'orientation	This study
$pALacZsd_{(PAO1)}IN2$	pALacZsd containing the intergenic region cloned as a *Pst*I - *Bam*HI fragment in the 3'-to-5'orientation	This study

Table 2.2 Oligonucleotides used in this study

Oligonucleotide primer	Nucleotide sequence		
PCR amplification*:			
Flp-F	5' - CGTGTATTGCAAAGTTCGC**ggatcc**C - 3';	*Bam*HI site incorporated	
Flp-R	5' - GCGACCGG**gaattc**GCTCGCGTC - 3';	*Eco*RI site incorporated	
IN-1F	5' - GCG**ctgcag**ACCCCTGCTATGCGTCCGG - 3';	*Pst*I site incorporated	
IN-1R	5' - CGC**ggatcc**CACGAACAGGGTCAGGTTC - 3';	*Bam*HI site incorporated	
IN-2F	5' - GCG**ggatcc**ACCCCTGCTATGCGTCCGG - 3';	*Bam*HI site incorporated	
IN-2R	5' - GCG**ctgcag**CACGAACAGGGTCAGGTTC - 3';	*Pst*I site incorporated	
Nucleotide sequencing:			
pUC/M13 Forward	5' - CCCAGTCACGACGTTGTAAAACG - 3'		
pUC/M13 Reverse	5' - CAGGAAACAGCTATGAC - 3'		
RT-PCR analyses:			
		Location	**Size (bp)**
4297-F	5' - CCACCAGCAACAGGCAGAT - 3'	*htpI*	203
4298-R	5' - TGCCTACCAGCGCTACCTGA - 3'	*htpH*	
4298-F	5' - CAGCAAGACGCCGAACA - 3'	*htpH*	252
4299-R	5' - AAGGCGACCGGGAAGA - 3'	*htpG*	
4299-F	5' - TGTCGCGGACCATGTTCA - 3'	*htpG*	267
4300-R	5' - CAGGAACGCATCGGCAA - 3'	*htpF*	
4300-F	5' - AAGCTGTCGTCCATCC - 3'	*htpF*	247
4301-R	5' - TGCAACTGCTCGGCTGCTA - 3'	*htpE*	
4301-F	5' - CAGCCGTTCAACCACCAGT - 3'	*htpE*	328
4302-R	5' - CCTTGCGCCTGATGATTTG - 3'	*htpD*	
4302-F	5' - TGCCGTCTTCGTCGATCT - 3'	*htpD*	377
4303-R	5' - AGCGCTACCTGCCCAATGT - 3'	*htpC*	
4303-F	5' - TTTGCCGAGGCTGATGA - 3'	*htpC*	404
4304-R	5' - GCATCCTCGGTGCGTTC - 3'	*htpB*	
4304-F	5' - ATGGCCAGCCGTTCGAT - 3'	*htpB*	416
4305-R	5' - TCCGCAGCAAGGACGA - 3'	*htpA*	
4306-F	5' - TCCTGGCGGACGAAGA - 3'	*flp*	133
4306-R	5' - CCGTCGAAGAAGGCTTTCA - 3'	*flp*	

* In oligonucleotide sequences, the restriction endonuclease sites are indicated in bold lower case letters

2.2.5 Purification of DNA fragments from agarose gels

DNA fragments were purified from agarose gel slices, as described by Boyle and Lew (1995). The DNA fragments were excised from the agarose gel with a scalpel blade, mixed with 400 µl of a 6 M NaI solution, and the agarose was melted by incubation at 55°C for at least 10 min. Following complete dissolution of the agarose, 10 µl of a silica suspension was added to the sample, vortexed for 10 s and then incubated on ice for 30 min. The silica-bound DNA was pelleted by centrifugation at 14 000 rpm for 30 s and the pellet washed three times with 500 µl of ice-cold New Wash (50 mM NaCl, 10 mM Tris-HCl [pH 7.5], 25 mM EDTA, 50% [v/v] ethanol). The DNA was eluted from the silica matrix at 55°C for 10 min in a final volume of 15 µl of 1 × TE buffer. Following brief centrifugation to pellet residual silica, an aliquot of the recovered supernatant was analyzed by electrophoresis on a 1% (w/v) agarose gel to assess the purity and concentration of the DNA.

2.2.6 Cloning of DNA fragments into plasmid vectors

2.2.6.1 Ligation reactions

For cloning of PCR amplicons, the pGEM®-T Easy vector system (Promega) was used. The ligation reaction mixtures contained 10 µl of a 2 × Rapid ligation buffer, 50 ng of pGEM®-T Easy vector DNA, 150 ng of purified amplicon, 1 µl of T4 DNA ligase (3 U/µl) and UHQ water to a final volume of 20 µl. Ligation of specific DNA fragments and vector DNA was performed in a 20-µl reaction volume containing 1 × DNA ligase buffer (2 mM Tris-HCl, 0.1 mM EDTA, 0.5 mM DTT, 6 mM KCl, 5% [v/v] glycerol; pH 7.5) and 1 U T4 ligase (Roche Diagnostics). The ratio of insert to vector was typically in excess of 5:1. All of the ligation reactions were incubated at 16°C overnight.

2.2.6.2 Preparation of competent *E. coli* DH5α cells

Competent *E. coli* DH5α cells were prepared by the $CaCl_2$ method according to Sambrook *et al.* (1989). A single colony of a freshly streaked culture of *E. coli* DH5α was inoculated into 10 ml of LB broth and cultured at 37°C overnight with shaking. Following incubation, 1 ml of the culture was inoculated into 100 ml pre-warmed (37°C) sterile LB broth and incubated at 37°C until an optical density of 0.4 at 550 nm was reached. The flask was then incubated on ice for 15 min to inhibit further growth, after which the cells from 30 ml of the culture were collected by centrifugation at 4 000 rpm for 10 min at 4°C. The cell pellet was

suspended in 10 ml ice-cold 100 mM $CaCl_2$, incubated on ice for 1 h and then pelleted as above. The pellet was suspended in 1 ml of the $CaCl_2$ solution. The competent cells were incubated on ice for at least 1 h prior to transformation or, alternatively, the cells were aliquoted (100 µl) and incubated at 4°C overnight prior to transformation.

2.2.6.3 Transformation of the *E. coli* DH5α cells

Transformation was performed by the heat shock method, as described by Sambrook *et al.* (1989). The competent *E. coli* DH5α cells (100 µl) and the ligation reaction mixture (10 µl) was mixed in a pre-cooled microfuge tube and incubated on ice for 1 h. The cells were heat-shocked at 42°C for 90 s and immediately placed on ice for 2 min. Subsequently, 900 µl of pre-heated (37°C) LB broth was added and the cells incubated at 37°C for 1 h. For cloning procedures involving pGEM®-T Easy, the cells were plated on LB agar containing ampicillin in the presence of 40 µl X-gal (2% [w/v] stock solution) and 10 µl IPTG (100 mM stock solution) to allow for blue-white selection. The plates were incubated at 37°C overnight and examined for the presence of recombinant transformants with a Gal^- phenotype. A positive control (10 ng of pUCl8 plasmid DNA) and negative control (competent cells only) were included to determine the competency of the *E. coli* DH5α cells and to test for contamination, respectively.

2.2.7 Screening of transformants

2.2.7.1 Colony-PCR for screening of putative recombinant plasmids

To verify that insert DNA was cloned successfully into the pALacZsd reporter vector, which lacks a readily selectable marker inactivation system, transformants were randomly selected and screened by performing a colony-PCR. The transformants were inoculated into 5 ml LB broth containing the appropriate antibiotic and cultured at 37°C overnight. The cells were collected by centrifugation at 14 000 rpm for 5 min and washed with ddH_2O. The pellet was suspended in 500 µl ddH_2O and the cells lysed by heating the suspension to 100°C for 10 min. After centrifugation at 14 000 rpm for 4 min to remove the cellular debris, the supernatant fluid was used in the PCR immediately. The PCR reactions were performed using 5 µl of the supernatant and the appropriate oligonucleotides, as described previously (Section 2.2.3.2). For control purposes, reaction mixtures from which template DNA was omitted or that contained the parental vector as template DNA were included. Aliquots of the

PCR reaction mixtures were subsequently analyzed by electrophoresis on 1% (w/v) agarose gels in the presence of an appropriate DNA molecular weight marker.

2.2.7.2 Plasmid DNA extraction

Plasmid DNA was isolated from selected transformants using a modified alkaline lysis method that yielded highly purified plasmid DNA (Sambrook *et al.*, 1989). Single colonies were each inoculated into 10 ml LB broth supplemented with the appropriate antibiotic and incubated at 37°C overnight with shaking. The cells from 3 ml of an overnight culture were collected by centrifugation at 14 000 rpm for 5 min. The cell pellet was suspended in 100 µl of Solution 1 (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, 10 mg/ml lysozyme; pH 8.0) and incubated at room temperature for 10 min. The cells were lysed following the addition of 200 µl of Solution 2 (0.2 N NaOH, 1% [w/v] SDS). After incubation on ice for 5 min, 150 µl of ice-cold 7.5 M NH_4OAc (pH 7.6) was added and incubation was continued on ice for a further 10 min. The insoluble aggregate that formed was collected by centrifugation at 14 000 rpm for 10 min, after which the plasmid DNA was precipitated from the recovered supernatants by the addition of 650 µl isopropanol and incubation at room temperature for 10 min. The precipitated plasmid DNA was collected by centrifugation at 14 000 rpm for 10 min and suspended in 200 µl of 2 M NH_4OAc (pH 7.4). After incubation on ice for 10 min, the proteins were removed by centrifugation at 14 000 rpm for 10 min. To precipitate the plasmid DNA, 110 µl isopropanol was added to the recovered supernatant and incubated at room temperature for 10 min. The plasmid DNA was pelleted by centrifugation, as above, washed with 70% ethanol, dried under vacuum and then resuspended in 30 µl 1 × TE buffer. To remove contaminating RNA, extracted plasmid DNA was incubated with 1 µl RNase A (10 mg/ml) at 37°C for 30 min.

2.2.8 Restriction enzyme digestion

Approximately 1 µg of plasmid DNA was digested with 3 U of enzyme in the appropriate concentration of salt, using the 10× buffer supplied by the manufacturer. The final reaction volumes were typically 20 µl and incubation was at 37°C for 2 h, after which the enzymes were inactivated by heating to 65°C for 5 min. For digestions involving two enzymes of which the salt concentrations differed for optimal activity, the plasmid DNA was first digested with the enzyme requiring the lowest salt concentration. The salt concentration was then adjusted and the second enzyme added. All restriction enzymes were supplied by

Promega. The digestion products were analyzed on a 1% (w/v) agarose gel in the presence of an appropriate DNA molecular weight marker.

2.2.9 Nucleotide sequencing

Nucleotide sequencing of cloned insert DNA was performed using the BigDye™ Terminator v.3.1 Cycle Sequencing Ready Reaction kit (Perkin-Elmer Applied Biosystems), and the universal pUC/M13 forward or reverse oligonucleotides (Table 2.2). Each sequencing reaction contained 2 µl Ready Reaction mix, 1 × sequencing buffer, 3.2 pmol sequencing oligonucleotide, 100 ng of purified template DNA and UHQ water to a final volume of 10 µl. Cycle sequencing reactions were performed in a Perkin-Elmer GeneAmp® 2700 thermal cycler with 25 of the following cycles: denaturation at 96°C for 10 s, oligonucleotide annealing at 50°C for 5 s and elongation at 60°C for 4 min. The reaction volume was adjusted to 20 µl with UHQ water, after which the extension products were precipitated by the addition of 48 µl of 100% ethanol and 2 µl of 3 M NaOAc (pH 4.6). The tubes were vortexed briefly, incubated at room temperature for 30 min in the dark, centrifuged at 14 000 rpm for 30 min and the supernatant carefully aspirated. The pellets were rinsed with 100 µl 70% ethanol, vacuum-dried and suspended in 3.5 µl Blue dextran/EDTA loading buffer. Prior to electrophoresis, the extension products were denatured by heating to 90°C for 2 min and loaded onto an ABI PRISM™ 3100 Genetic Analyzer. The nucleotide sequences obtained were compared against the *P. aeruginosa* PAO1 genome database (http://www.pseudomonas.com), as well as against sequences in the GenBank Database (http://www.ncbi.nlm.gov) by using BLASTN (Altschul *et al.*, 1997).

2.2.10 Plasmid constructions

All molecular cloning techniques employed in the construction of the respective vectors were performed according to the procedures described in the preceding sections. All plasmid constructs were confirmed by restriction endonuclease digestions and by nucleotide sequencing.

- **pGEM-Flp**

Oligonucleotides Flp-F and Flp-R were used with chromosomal DNA from *P. aeruginosa* PAO1 as template DNA to yield a 220-bp amplicon, which was cloned into pGEM®-T Easy to generate pGEM-Flp.

- **pGEX-Flp**

To construct the recombinant prokaryotic expression vector pGEX-Flp, the *flp* ORF was excised from plasmid pGEM-Flp by digestion with both *Bam*HI and *Eco*RI, and then cloned into pGEX-2T that had been prepared in an identical manner. The cloning strategy employed in the construction of pGEX-Flp is indicated in Fig. 2.1.

- **Recombinant pALacZsd reporter vector constructs**

Towards construction of the recombinant reporter vector $pALacZsd_{(PAO1)}IN1$, the intergenic region between the *flp* (PA4306) and *htpA* (PA4305) ORFs was PCR-amplified using *P. aeruginosa* PAO1 chromosomal DNA as template and oligonucleotides IN-1F and IN-1R. The amplicon was cloned into pGEM®-T Easy vector DNA to generate recombinant plasmid pGEM-IN1. The insert DNA was subsequently recovered by digestion with both *Pst*I and *Bam*HI, and cloned into the same sites of pALacZsd to yield $pALacZsd_{(PAO1)}IN1$. A similar approach was adopted in the construction of the recombinant reporter vector $pALacZsd_{(PAO1)}IN2$, except that oligonucleotides IN-2F and IN-2R were used in the PCR. The amplicon was cloned into pGEM®-T Easy vector DNA to generate pGEM-IN2, after which the insert DNA was recovered by digestion with both *Pst*I and *Bam*HI and cloned into identically digested pALacZsd to complete construction of $pALacZsd_{(PAO1)}IN2$. The cloning strategy employed in the construction of the respective reporter vectors, containing the cloned intergenic region in opposite transcriptional orientations, is indicated in Fig. 2.2.

2.2.11 Expression and purification of the recombinant GST-Flp fusion protein

2.2.11.1 Expression of the *P. aeruginosa* Flp protein in *E. coli*

Single colonies of *E. coli* DH5α transformed with either recombinant pGEX-Flp or parental pGEX-2T plasmid DNA were inoculated into 1 ml LB broth containing ampicillin and incubated at 37°C overnight with shaking. Expression of the GST fusion protein was induced with IPTG, as described by Smith and Johnson (1988). Briefly, the overnight cultures were diluted 1:10 in 1 ml of fresh LB broth and incubated at 37°C for 2 h. To each culture, IPTG was then added to a final concentration of 0.5 mM and the cultures were incubated at 37°C for an additional 6 h. The bacterial cells were then harvested by centrifugation at 14 000 rpm for 5 min and directly suspended in 200 µl of 2 × Protein Solvent Buffer (PSB: 125 mM Tris [pH 6.8], 4% [w/v] SDS, 20% [v/v] glycerol, 10% [v/v] 2-mercaptoethanol, 0.002% [w/v] bromophenol blue). The samples were heated to 94°C for 10 min and sonicated by three 15-s

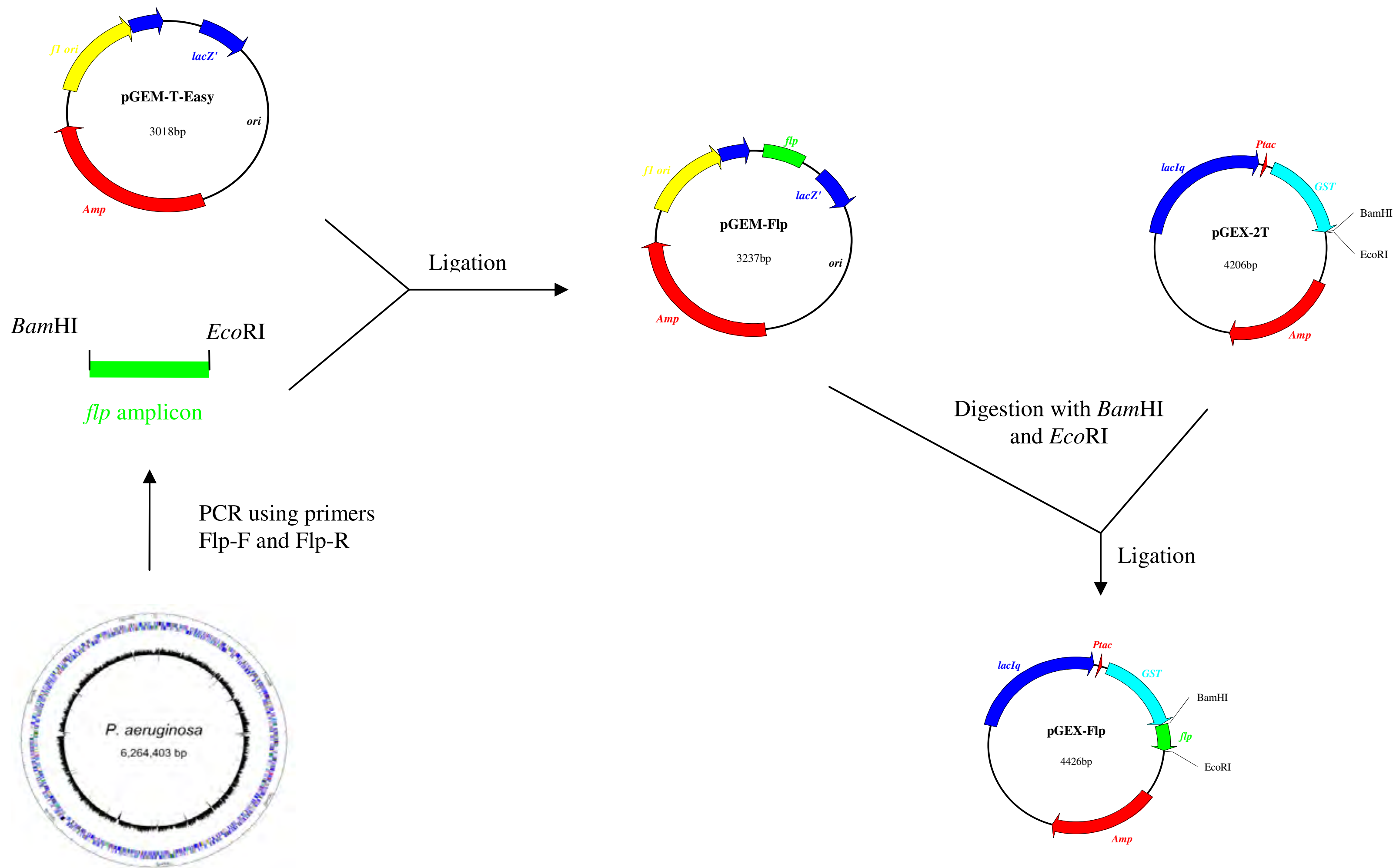


Fig. 2.1 Construction of the bacterial expression vector pGEX-Flp.

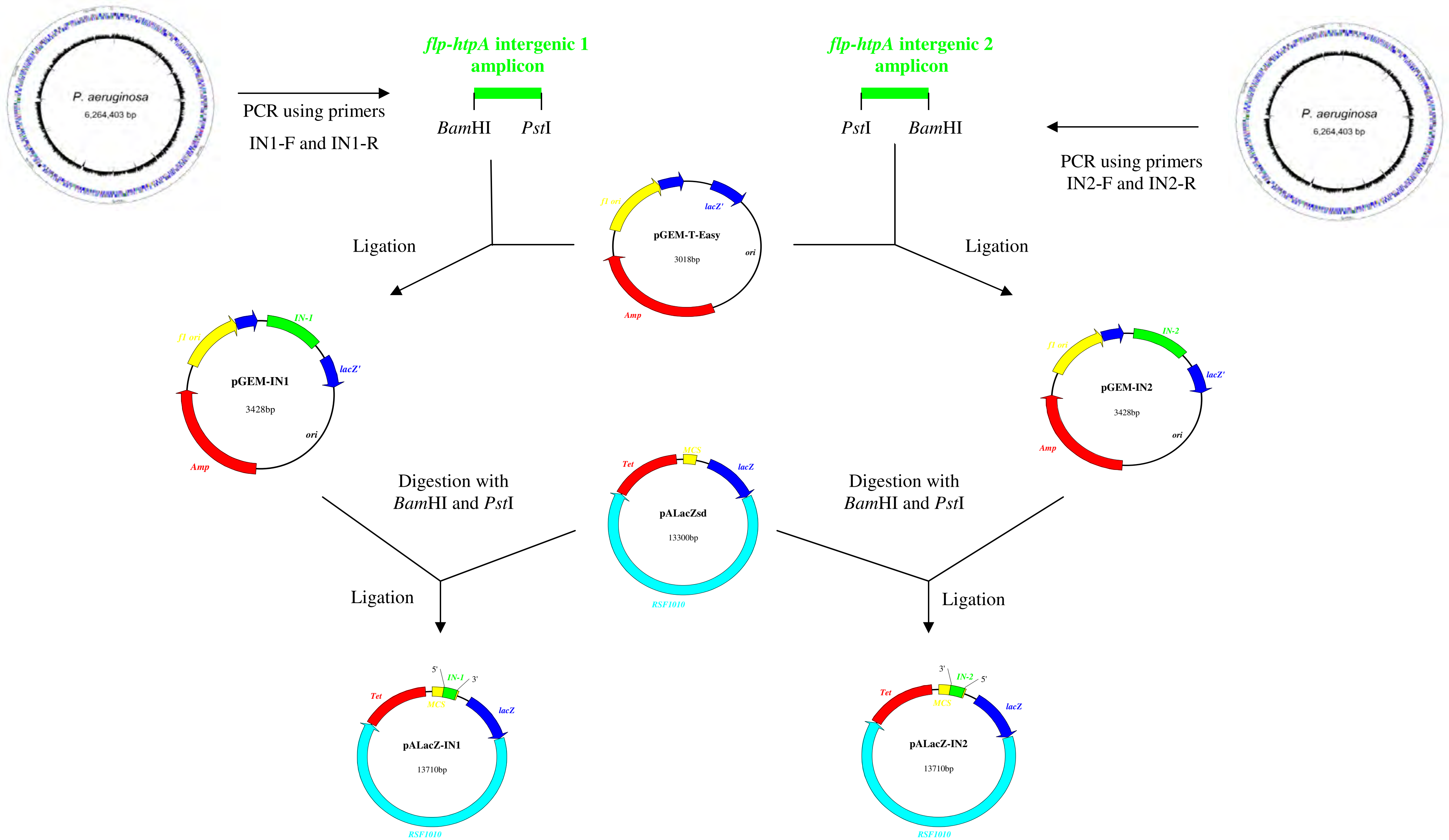


Fig. 2.2 Construction of the recombinant reporter vectors pALacZsd$_{(PAO1)}$IN1 and pALacZsd$_{(PAO1)}$IN2, respectively.

pulses, using a 4710 Series Ultrasonic Homogenizer (Cole-Palmer Instruments) at an output of 50%, prior to SDS-PAGE analysis.

2.2.11.2 Purification of the GST-Flp protein by glutathione affinity chromatography

The recombinant GST-Flp protein was expressed, as described above, except that 10-ml cultures of *E. coli* DH5α containing the recombinant pGEX-Flp plasmid were induced with IPTG. The bacterial cells were harvested by centrifugation at 5 000 rpm for 10 min and then lysed according to the procedures described by Theron *et al.* (1994). Briefly, the cell pellets were suspended in 1 ml of ice-cold lysis buffer (20% [w/v] sucrose, 100 mM Tris-HCl [pH 8.0], 10 mM EDTA), 8.4 µl lysozyme (5 mg/ml) was added and the samples were incubated at room temperature for 15 min and then on ice for 30 min. Following addition of DTT to 10 mM, TritonX-100 and Tween-20 to 1% (v/v), and PBS to a final concentration of 1× (13.7 mM NaCl, 0.27 mM KCl, 0.43 mM $Na_2HPO_4.7H_2O$, 0.14 mM KH_2PO_4; pH 7.3), the samples were incubated at 4°C overnight. The cellular debris was removed by centrifugation at 20 000 rpm for 20 min and the cytoplasmic (supernatant) fractions were recovered and stored at -20°C until needed.

The recombinant GST-Flp fusion protein was purified directly from the cleared supernatants by affinity chromatography with glutathione agarose (sulphur linkage; Sigma-Aldrich), as described by Smith and Johnson (1988). The glutathione was pre-swollen in MTPBS buffer (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4; pH 7.3), washed twice in the same buffer and stored in MTPBS at 4°C as a 50% (v/v) slurry. The cleared supernatant fractions (500 µl) were mixed with 125 µl of glutathione agarose and incubated at room temperature for 45 min with gentle agitation to allow adsorption of the fusion protein to the glutathione agarose. After incubation, the agarose was collected by centrifugation at 2 000 rpm for 1 min and washed three times with 1 ml of MTPBS. The recombinant GST-Flp protein was eluted by competition with free glutathione in two 2-min washes with 125 µl of 50 mM Tris-HCl (pH 8.0) containing 5 mM reduced glutathione (final pH 7.5, freshly prepared). The proteins were subsequently analyzed by SDS-PAGE and Western blot analyses, as described below.

2.2.11.3 Thrombin cleavage of the purified GST-Flp protein

The GST carrier was proteolytically cleaved from the GST-Flp fusion protein using thrombin (Sigma-Aldrich) according to the manufacturer's instructions. The purified GST-Flp fusion

protein was incubated for 16 h at room temperature with 10 U of thrombin per mg of protein. The Flp protein was subsequently purified away from the GST carrier by glutathione affinity chromatography, as described above, except that after adsorption the agarose containing the GST carrier was collected by centrifugation at 2 000 rpm for 1 min and discarded, whilst the supernatant containing the purified Flp protein was recovered. The Flp protein was also concentrated from samples of pooled supernatants using the method of Wessel and Flügge (1984) or by precipitation with acetone, as follows. One volume of the supernatant was mixed with four volumes of ice-cold acetone and incubated at -70°C for 1 h. The precipitated proteins were collected by centrifugation at 14 000 rpm for 30 min at 4°C, the supernatant discarded and the protein pellet was then suspended in 20 µl of 2 × PSB. The native Flp protein was subsequently analyzed by SDS-PAGE.

2.2.12 Characterization of the recombinant GST-Flp protein

2.2.12.1 SDS-PAGE

Protein samples were electrophoresed on discontinuous SDS-polyacrylamide gels, as described by Laemmli (1970). The 5% stacking gel (5% [w/v] acrylamide, 0.17% [w/v] bis-acrylamide, 125 mM Tris-HCl, 0.1% [w/v] SDS; pH 6.8) and 12% separating gel (12% [w/v] acrylamide, 0.34% [w/v] bis-acrylamide, 0.375 mM Tris-HCl, 0.1% [w/v] SDS; pH 8.8) were polymerized by the addition of 0.1% (w/v) ammonium persulfate and 100 µl TEMED. Electrophoresis was performed using a Hoefer Mighty Small™ electrophoresis unit at 120 V in 1 × TGS buffer (0.025 M Tris [pH 8.3], 0.192 M glycine, 0.1% [w/v] SDS). Alternatively, purified Flp protein samples were electrophoresed on 20% SDS-polyacrylamide gels, as described above, or on Tris-tricine 20% SDS-polyacrylamide gels using 1 × anode buffer (0.2 M Tris [pH 8.9]) and 1 × cathode buffer (0.1 M Tris [pH 8.9], 0.1 M tricine, 1% [w/v] SDS) as the electrophoresis buffer (Schagger and von Jagow, 1987). Following electrophoresis, the proteins were visualized by staining the SDS-polyacrylamide gels with either Coomassie Brilliant Blue or silver nitrate, as described below.

2.2.12.2 Visualization of proteins on SDS-polyacrylamide gels

- **Coomassie Brilliant Blue staining**

After electrophoresis, the gels were stained with 0.125% (w/v) Coomassie Brilliant Blue (prepared in 50% [v/v] methanol; 10% [v/v] glacial acetic acid) and the proteins were

visualized by counterstaining the gels in a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol. The protein sizes were determined by comparison to a broad range (6-175 kDa) protein molecular weight marker (New England Biolabs).

- **Silver nitrate staining**

After electrophoresis, proteins were also visualized by silver nitrate staining, as described by Switzer *et al.* (1979). The proteins were fixed by incubating the gels overnight in 200 ml of a fixing solution consisting of 30% (v/v) ethanol and 10% (v/v) acetic acid. Following incubation, the gels were washed twice for 10 min each wash in 10% (v/v) ethanol and then three times for 10 min each wash in ddH$_2$O. The gels were sensitized in 0.02% (w/v) sodium thiosulfate for 1 min. After washing the gels three times for 1 min each wash in ddH$_2$O, the silver staining reaction was performed by soaking the gels in a freshly prepared 0.1% (w/v) silver nitrate solution for 30 min. After thorough washing of the gels in ddH$_2$O, protein bands 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 or until they were clearly visible. Development of protein bands was stopped by washing the gels twice for 5 min each wash in 10% (v/v) acetic acid, after which the gels were rinsed thoroughly with ddH$_2$O.

2.2.12.3 Western blot analysis

After SDS-PAGE, the unstained gel and Hybond™-C$^+$ nitrocellulose membrane (Amersham Bioscience), cut to the same size as the gel, were equilibrated for 30 min in transfer buffer (20 mM Tris, 1 M glycine) (Sambrook *et al.*, 1989). The proteins were transferred from the gel onto the nitrocellulose membrane at 28 V, 120 mA for 90 min using a Mighty Small™ Transphor Electrophoresis unit (Hoefer). Following transfer, the membrane was rinsed once for 5 min in 1 × PBS, after which non-specific binding sites were blocked by incubating the membrane in blocking solution (1 × PBS containing 3% [w/v] gelatin) at 4°C overnight. The primary antibody, polyclonal anti-GST (Santa Cruz Biotechnology, Inc.), was diluted 1:1000 in fresh blocking solution and added to the membrane. Following incubation for 2 h at room temperature with gentle agitation, the unbound primary antibody was removed by washing the membrane three times for 5 min in wash buffer (0.05% [v/v] Tween-20 in 1× PBS). The secondary antibody, protein A conjugated to horseradish peroxidase, was diluted 1:1000 in fresh blocking solution, added to the membrane and then incubated at room temperature for 1 h with gentle shaking. The membrane was washed three times for 5 min each in wash buffer

and then once for 5 min in 1 × PBS. The antigen-antibody complexes were detected calorimetrically by immersing the membrane in the peroxidase enzyme substrate (60 mg of 4-chloro-1-naphtol in 20 ml ice-cold methanol and 100 ml of 1 × PBS containing 60 µl H_2O_2, mixed just prior to use) until bands became visible, after which the membrane was rinsed with water and air-dried.

2.2.13 Determination of β-galactosidase activity of the recombinant pALacZsd reporter vectors in *P. aeruginosa* PAO1

2.2.13.1 Preparation of *P. aeruginosa* PAO1 competent cells

Competent *P. aeruginosa* cells were prepared and transformed essentially by the procedures described by Olsen *et al*. (1982). A single colony of *P. aeruginosa* PAO1, cultured overnight at 37°C on Pseudomonas Isolation Agar (4.5% [w/v] PIA agar, 2% [v/v] glycerol), was inoculated into 10 ml TN broth (0.5% [w/v] tryptone, 0.1% [w/v] dextrose, 0.25% [w/v] yeast extract, 0.4% [w/v] $NaNO_3$) (Olsen and Shipley, 1973) and incubated at 37°C overnight. Following incubation, 500 µl of the overnight culture was inoculated into 50 ml TN broth and grown to an OD_{540} of 0.5. The culture was then incubated on ice for 20 min to inhibit further growth of the cells. The cells from 4 ml of the culture were collected in 2-ml Eppendorf tubes by centrifugation at 7 000 rpm for 10 min at 4°C. The pellet was suspended in 2 ml ice-cold filter-sterilized 0.15 M $MgCl_2$, incubated on ice for 5 min, pelleted as before and gently resuspended in 1 ml of the ice-cold $MgCl_2$ solution. After incubation on ice for 20 min, the cells were collected by centrifugation and the pellet resuspended in 100 µl of ice-cold $MgCl_2$.

2.2.13.2 Transformation of competent *P. aeruginosa* PAO1 cells

The competent *P. aeruginosa* PAO1 cells were transformed by addition of 500 ng plasmid DNA ($pALacZsd_{(PAO1)}IN1$, $pALacZsd_{(PAO1)}IN2$, pALacZsd and pALacZsdIP) to 100 µl of the competent cells in an Eppendorf tube. Following incubation on ice for 1 h, the cells were incubated at 37°C for 3 min and chilled on ice for 5 min. After the addition of 900 µl LB broth, the transformation mixtures were incubated at 37°C for 3 h. The cells were plated on LB agar supplemented with 100 µg/ml tetracycline and the agar plates were incubated at 37°C overnight.

2.2.13.3 Culturing conditions

A single colony of freshly streaked cultures of each transformant, as well as *P. aeruginosa* PAO1 was inoculated into 50 ml LB broth and incubated at 37°C for 7 h with shaking. All cultures, except *P. aeruginosa* PAO1, were supplemented with 100 µg/ml tetracycline. These exponentially growing cultures were then inoculated to an OD_{540} of 0.05 into 2 ml LB broth in 28-ml McCartney bottles with or without 0.05 g glass wool (mean diameter 15 µm; Merck). The cultures were incubated at 37°C for 16 h with shaking. Planktonic cells were obtained from cultures grown in the absence of glass wool, whilst cultures grown in the presence of glass wool were used as a source of biofilm cells. The supernatant of these cultures was carefully aspirated and the glass wool was gently rinsed with 1 ml LB broth. Following the addition of 2 ml LB broth to the glass wool, the samples were vortexed for 1 min to remove the biofilm biomass from the glass wool substratum. The supernatants were recovered and transferred to new microfuge tubes to be used in β-galactosidase assays and protein concentration determinations, respectively.

2.2.13.4 β-galactosidase assays

β-galactosidase activity was assayed using ONPG (Roche Diagnostics) as chromogenic substrate according to the procedure described by Miller (1972). Samples of the planktonic and biofilm biomass (500 µl) were incubated on ice for 20 min to inhibit further growth of the cells. For activity assays, 500 µl of Z-buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$, 0.5 M β-mercaptoethanol) was added to each sample, followed by the addition of 10 µl of 0.1% (w/v) SDS and 20 µl chloroform to lyse the bacterial cells. Following incubation at 28°C for 10 min, 200 µl of the ONPG substrate (4 mg/ml in Z-buffer) was added to each sample and mixed to initiate the colour reaction. The colour reactions were terminated by the addition of 500 µl of 1 M Na_2CO_3 after 10 min and the samples were briefly centrifuged to collect the cellular debris and remaining glass wool. Hydrolysis of the ONPG substrate was quantified by transferring the supernatant (1 ml) to plastic cuvettes and measuring the absorbance at 414 nm in a Spectronic® 20 spectrophotometer (Spectronic Instruments).

2.2.13.5 Total protein concentration

The total protein concentration of the planktonic and biofilm biomass 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 and attached samples, prepared as described above (Section 2.2.15.3), were collected by centrifugation at 14 000 rpm for 5 min and then suspended in 500 µl UHQ water. The cell suspensions were subsequently sonicated by three 15-s pulses, using a 4710 Series Ultrasonic Homogenizer (Cole-Palmer Instruments) at an output of 50%, and then heated to 95°C for 10 min. An aliquot (50 µl) of each sample was added to 1.5 ml of the Coomassie Plus™ Reagent, mixed well and after incubation at room temperature for 10 min the absorbance at 595 nm was determined. UHQ water was used as a blank to zero the absorbency readings and the protein concentration was then determined from the prepared standard curve. The β-galactosidase activity per total cellular biomass was determined by dividing the β-galactosidase values by the protein concentration to ensure that the difference in β-galactosidase activity was due to differences in promoter activity, and not due to variations in the yield of biomass. All of the above assays were performed at least twice and on separate occasions.

2.2.14 Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of adjacent genes in the putative *htp* operon of *P. aeruginosa* PAO1

2.2.14.1 Oligonucleotide primers

For RT-PCR amplification of each contiguous gene junction, a forward oligonucleotide, annealing to the 3' region of each ORF, and a reverse oligonucleotide, annealing to the 5' region of the adjacent ORF, were designed based on the *P. aeruginosa* PAO1 *htpA* through *htpI* (PA4297 - PA4305) sequences. In addition, oligonucleotides annealing to the 5' and 3' regions of the *flp* ORF was also designed. The RT-PCR amplicons were between 133 to 416 bp in size. The oligonucleotides were designed using DNAMAN v.4.13 (Lynnon Biosoft), whilst optimal oligonucleotide pairs were analyzed by PerlPrimer v.1.1.6 (Owen and Marshall, 2003-2005). To verify the target sequence specificity of each newly designed oligonucleotide pair, the oligonucleotide sequences were subjected to a BLASTN analysis (available at http://www.ncbi.nlm.nih.gov/BLAST). The oligonucleotide sequences are indicated in Table 2.2 and were synthesized by Integrated DNA Technologies, Inc.

2.2.14.2 Total RNA extraction

Prior to RT-PCR, total RNA was extracted from *P. aeruginosa* PAO1 cultures, using the Aurum™ Total RNA extraction kit (BioRad) according to the manufacturer's instructions.

Briefly, the cells from 1 ml of an overnight culture of *P. aeruginosa* PAO1 were collected by centrifugation at 13 000 rpm for 1 min. The cell pellets were suspended in 100 µl of 1 × TE buffer containing 500 µg/ml lyzozyme and incubated at room temperature for 5 min, after which 350 µl of the supplied lysis solution was added to the suspension and the cells lysed by vigorous pipetting. Following addition of 250 µl of isopropanol, the cell lysate was centrifuged through a RNA-binding column at 15 000 rpm for 30 s, after which the column was rinsed with wash solution, treated with DNase I to remove contaminating genomic DNA (15 min at room temperature) and the RNA was then eluted from the column using the supplied elution buffer.

2.2.14.3 RT-PCR

The RT-PCR reactions were performed using the iScript™ One-Step RT-PCR Kit with SYBR® Green (BioRad) according to the manufacturer's instructions. Each reaction mixture contained 50 ng RNA, 25 µl of 2 × SYBR® Green RT-PCR reaction mix (containing 0.4 mM of each dNTP, $MgCl_2$, iTaq DNA polymerase, 20 nM fluorescein, SYBR® Green I dye and stabilizers), 50 pmol of each forward and reverse oligonucleotides and 5 µl of iScript Reverse Transcriptase in a final volume of 50 µl. The reaction mixtures were incubated in a Perkin-Elmer GeneAmp® 2700 thermal cycler at 50°C for 10 min to reverse-transcribe the RNA, followed by incubation at 95°C for 5 min to inactivate the Reverse Transcriptase. Subsequently, the reaction mixtures were subjected to 30 cycles of denaturation at 95°C for 10 s, oligonucleotide annealing at 60°C for 30 s and extension at 72°C for 45 s. Aliquots of each reaction mixture were analyzed by 1% (w/v) agarose gel electrophoresis in the presence of an appropriate DNA molecular weight marker.

2.3 RESULTS

2.3.1 Reverse transcriptase-polymerase chain reaction (RT-PCR)

A novel pilus biogenesis/secretion system has been identified in *P. aeruginosa* through *in silico* analyses (Van Schalkwyk, 2003) that displays homology to those described previously in *A. actinomycetemcomitans* (Kachlany *et al.*, 2000), *H. ducreyi* (Nika *et al.*, 2002) and *C. crescentus* (Skerker and Shapiro, 2000). However, in contrast to the *flp-tad* (Nika *et al.*, 2002; Haase *et al.*, 2003) and *pilA-cpa* (Skerker and Shapiro, 2000) gene clusters of these bacteria, which are organized in a single transcribed operon, the putative *htp* pilus

biogenesis/secretion system of *P. aeruginosa* PAO1 appears to consist of at least two distinct transcriptional units. Whereas the *htpA-I* (PA4305 through PA4297) ORFs are organized as a single transcribed operon, the *flp* (PA4306) ORF is located upstream from the *htpA-I* ORFs and appears to be transcribed divergently and in the opposite direction.

Thus, to determine whether the *htpA-I* gene cluster might comprise a single polycistronic operon, transcription was assessed at each individual gene junction from *htpA-htpI*. Oligonucleotide primers were therefore designed for the 3' region of each ORF and the 5' region of the adjacent ORF for all ORFs in the *htpA-I* gene cluster so that a product would result only if the two ORFs are co-transcribed. To determine whether the *flp* ORF is indeed transcribed, oligonucleotides were also designed for the 5' and 3' regions of the ORF. All the oligonucleotide sets were then used in RT-PCR on total RNA isolated from *P. aeruginosa* PAO1, as described under Material and Methods (Section. 2.2.14), and the results are presented in Fig. 2.3.

Amplicons were obtained from consecutive gene junctions *htpA-I*, indicating that all nine of the ORFs in the *htpA-I* gene cluster are co-transcribed in the form of a polycistronic message (Fig. 2.3b, lanes 2 through 9). Furthermore, evidence was obtained indicating that the *flp* ORF, encoding the putative prepilin protein, is also transcribed (Fig. 2.3b, lane 10). In each assay where a cDNA amplicon was obtained, the corresponding control RNA template (without reverse transcription) was negative, confirming the lack of DNA contamination in the respective samples (Fig. 2.3a).

2.3.2 Identification of potential promoter sequences

Based on the results obtained in the preceding section, *flp* is likely not part of the *htpA-I* gene cluster and appears to be transcribed in the opposite direction to the *htp* gene cluster. Between *htpA* (PA4305) and *flp* (PA4306) is an intergenic region of *ca*. 410 bp and it was thus hypothesized that the promoters for *flp* and the polycistronic *htp* gene cluster would be located within the intergenic region. Consequently, the intergenic region between the *flp* and *htpA* genes was analyzed, using the NEURAL NETWORK PROMOTER PREDICTION (at http://www-hgc-lbl.gov/projects/promoter.html) program, in order to identify consensus promoter sequences.

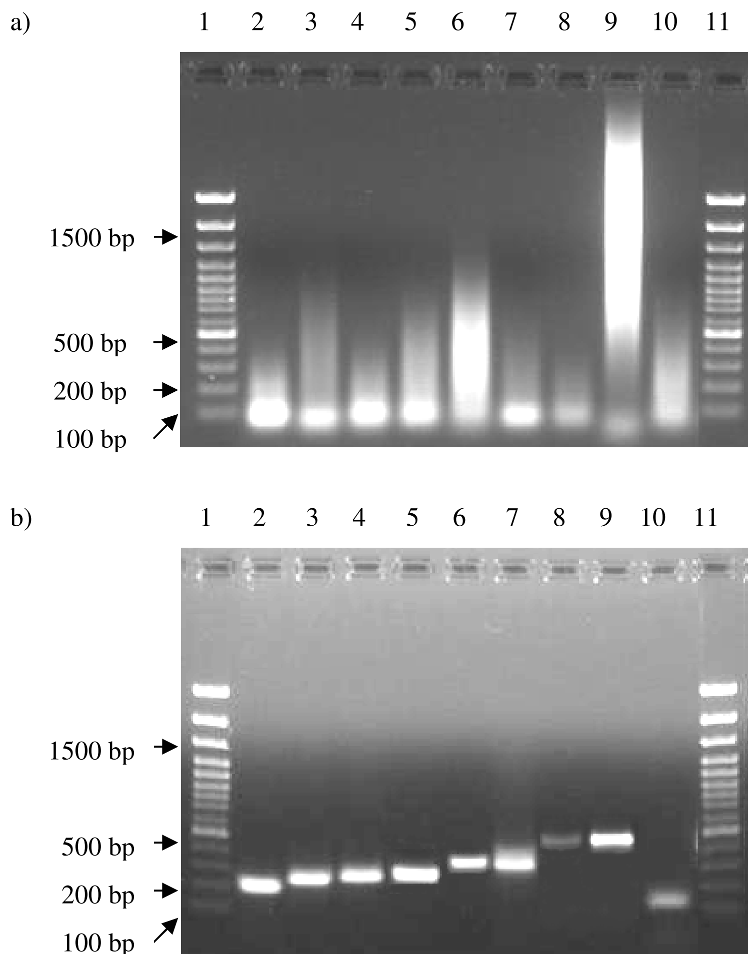


Fig. 2.3 RT-PCR analysis of the *P. aeruginosa* PAO1 *htp* gene cluster and *flp* ORF (Refer to Table 2.1 for the annealing position for each oligonucleotide). The reaction conditions were as follows (a) PCR using total RNA isolated from *P. aeruginosa* PAO1 without RT and (b) RT-PCR using total RNA isolated from *P. aeruginosa* PAO1. In each of the figures, the lanes are as follows: Lanes 1 and 11, DNA molecular weight marker; lanes 2 contained oligonucleotides 4297-F and 4298-R, which amplify a 203-bp product that spans from *htpI* to *htpH*; lanes 3 contained oligonucleotides 4298-Fand 4299-R, which amplify a 252-bp product that spans from *htpH* to *htpG*; lanes 4 contained oligonucleotides 4299-F and 4300-R, which amplify a 267-bp product that spans from *htpG* to *htpF*; lanes 5 contained oligonucleotides 4300-F and 4301-R, which amplify a 247-bp product that spans from *htpF* to *htpE*; lanes 6 contained oligonucleotides 4301-F and 4302-R, which amplify a 328-bp product that spans from *htpE* to *htpD*; lanes 7 contained oligonucleotides 4302-F and 4303-R, which amplify a 377-bp product that spans from *htpD* to *htpC*; lanes 8 contained oligonucleotides 4303-F and 4304-R, which amplify a 404-bp product that spans from *htpC* to *htpB*; lanes 9 contained oligonucleotides 4304-F and 4305-R, which amplify a 416-bp product that spans from *htpB* to *htpA*; lanes 10 contained oligonucleotides 4306-F and 4306-R, which amplify the 133-bp *flp* ORF. The sizes of the DNA molecular weight marker, GeneRuler™ 100-bp DNA Ladder Plus (Fermentas), are indicated to the left of the figure.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Two putative promoter sequences could be identified that are located on the two different strands in the intergenic region (Fig. 2.4). Whereas the putative promoter of *flp* is located at positions -90 to -45 relative to the translational start site of Flp on the positive strand, the putative promoter of the *htp* operon is located on the reverse strand at positions -252 to -207 relative to the translational start site of HtpA. The sequences of the putative *flp* promoter (TTGCAA - 16 bp - TAGCCT) and *htp* operon promoter (TTGACA - 16 bp - TAGATT) resembles the consensus sequences of the promoters (TTGACA - 15 to 17 bp - TATAAT) recognized by the *E. coli* sigma 70 (σ^{70})-RNA polymerase holoenzyme (Harley and Reynolds, 1987). No other known promoter sequences could be identified in either the intergenic region or the upstream regions of the genes comprising the *htp* gene cluster, thus providing supporting evidence for transcription of the *htp* gene cluster as a polycistronic message.

Fig. 2.4 Sequence of the predicted *flp* (PA4306) and *htp* operon (PA4305 - PA4297) promoters. The predicted -10 and -35 hexamers for these promoters are shown in green and red boxes, respectively. The bent arrows indicate the predicted transcriptional start sites.

2.3.3 Functional analysis of the potential promoter regions

2.3.3.1 Construction of recombinant pALacZsd$_{(PAO1)}$IN1 and pALacZsd$_{(PAO1)}$IN2 reporter vectors

To investigate whether the intergenic region has promoter activity, pALacZsd was used as the source for reporter vector construction. The pALacZsd vector contains a Tet^r gene, a multiple cloning site (MCS) upstream of a promoterless *lacZ* reporter gene and transcriptional and translational terminators upstream of the reporter gene to reduce basal levels of reporter gene expression. The broad host range of the vector permits replication of the plasmid in a wide variety of Gram-negative bacteria, including *E. coli* and *P. aeruginosa* (Weyers, 1999).

Towards construction of the desired reporter vectors, the intergenic region between *flp* and *htpA* was obtained by PCR amplification using *P. aeruginosa* PAO1 genomic DNA as template and different sets of oligonucleotides (containing *Pst*I and *Bam*HI sites, respectively) to allow for directional cloning of the intergenic region in both transcriptional orientations. Following PCR, the 410-bp amplicons were cloned into pGEM®-T Easy to generate pGEM-IN1 and pGEM-IN2, respectively. Restriction of the derived recombinant plasmids with *Pst*I and *Bam*HI resulted in the excision of 410-bp DNA fragments, indicating that the amplicons were cloned successfully into the pGEM®-T Easy vector, and the integrity of the cloned insert DNAs was subsequently verified by nucleotide sequencing. To complete construction of the reporter vectors, DNA fragments containing the intergenic region were recovered from the respective recombinant pGEM®-T Easy plasmids by digestion with both *Pst*I and *Bam*HI and then cloned into identically prepared pLacZsd vector DNA, to generate pALacZsd$_{(PAO1)}$IN1 and pALacZsd$_{(PAO1)}$IN2 (Fig. 2.5a).

Due to the small size of the insert DNA (410 bp) and the large size of the vector DNA (12.3 kb), coupled with the fact that pALacZsd is a low-copy-number plasmid, screening of putative recombinants by means of restriction enzyme digestion proved to be inefficient. Thus, as the DNA fragments were cloned directionally into the reporter plasmid, recombinant transformants were rather identified by colony PCR analysis using the intergenic region-specific oligonucleotides. Using pALacZsd$_{(PAO1)}$IN1 and pALacZsd$_{(PAO1)}$IN2 as template DNA and the appropriate oligonucleotides, PCR was carried out as described under Materials and Methods (Section 2.2.7.1). An aliquot of the respective reaction mixtures was analyzed by agarose gel electrophoresis and a single discreet DNA fragment of *ca.* 410 bp was observed (Fig. 2.5b, lanes 2 and 3, respectively). As expected, no amplification products were obtained when the parental pALacZsd vector was used as template DNA in the PCR reactions (Fig. 2.5b, lanes 4 and 5, respectively) or from control reactions that lacked template DNA (Fig. 2.5b, lane 6). These results therefore served to confirm that the intergenic region had been cloned successfully into the pALacZsd reporter vector, yielding two recombinant vectors in which the orientation of the putative promoters relative to the reporter gene differed from each other. Whereas pALacZsd$_{(PAO1)}$IN1 contained the putative *flp* promoter upstream of the reporter gene, pALacZsd$_{(PAO1)}$IN2 contained the putative *htpA* promoter upstream of the reporter gene.

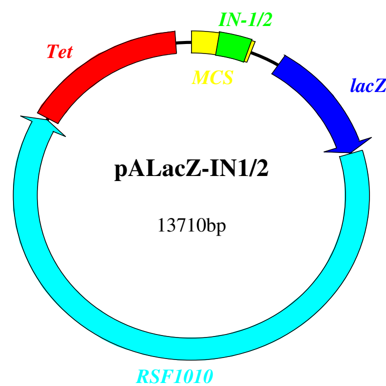


Fig. 2.5a Plasmid map of recombinant pALacZsd reporter plasmids, harbouring the 410-bp *flp-htpA* intergenic region in a 5'-to-3' (IN1) and 3'-to-5' (IN2) transcriptional orientation.

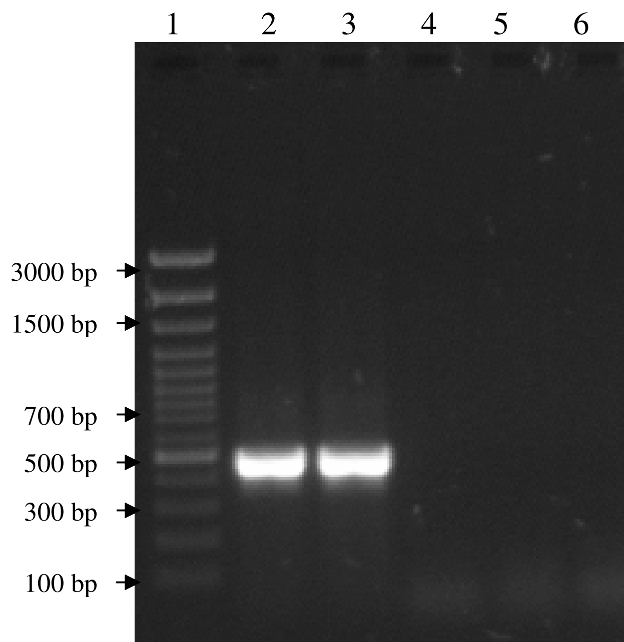


Fig. 2.5b Agarose gel electrophoresis indicating amplification of the cloned DNA fragments in the recombinant pALacZsd reporter vector constructs, using intergenic region-specific oligonucleotide primer pairs. Lane 1, DNA molecular weight marker; lane 2, amplified insert DNA from pALacZsd$_{(PAO1)}$IN1; lane 3, amplified insert DNA from pALacZsd$_{(PAO1)}$IN2. As controls, aliquots of the PCR reaction mixtures containing pALacZsd as template DNA and oligonucleotides IN-1F and IN-1R (lane 4) or IN-2F and IN-2R (lane 5) were included, whilst a control reaction lacking DNA template is indicated in lane 6. The sizes of the DNA molecular weight marker, GeneRuler™ 100-bp DNA Ladder Plus (Fermentas), are indicated to the left of the figure.

2.3.3.2 β-Galactosidase activity assays

For β-galactosidase activity assays, the recombinant and control pALacZsd reporter vector constructs were transformed into competent *P. aeruginosa* PAO1 cells and β-galactosidase expression was assayed by using ONPG as a chromogenic substrate. *P. aeruginosa* PAO1 was included in these assays as a control to determine the residual β-galactosidase activity in the cells, whilst *P. aeruginosa* PAO1 cells transformed with pALacZsdIP, containing the *lac* promoter from plasmid pBluescript SKII (+), served as a positive control. To assay the promoter activity in both planktonic and biofilm cell populations, the cultures were incubated in the presence and absence of glass wool as an attachment substratum. Following overnight incubation of the cultures, 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 (attached) cells. All assays were performed at least twice and on separate occasions, and the results are presented in Fig. 2.6.

Analysis of the results obtained for *P. aeruginosa* PAO1 cells indicated a lack of β-galactosidase expression. These results served to confirm that *P. aeruginosa* is a β-galactosidase-negative host and thus suitable for use with the pALacZsd reporter vector. Similarly, cells containing the promoterless control vector pALacZsd also displayed no β-galactosidase activity. As expected, the cells containing the positive control vector, pALacZsdIP, displayed β-galactosidase activity under all the growth conditions investigated. Analysis of the results obtained for *P. aeruginosa* PAO1 transformed with the recombinant reporter vectors indicated β-galactosidase activity in both the planktonic and biofilm cells. However, comparison of the results obtained for cultures transformed with the pALacZsd$_{(PAO1)}$IN1 reporter construct, containing the putative *flp* promoter, indicated that expression of the *lacZ* reporter gene was up-regulated 2.2-fold in biofilm cells when compared to planktonic cells. Similarly, reporter gene expression from pALacZsd$_{(PAO1)}$IN2, containing the putative *htpA* promoter, was up-regulated 5.3-fold in biofilm cells when compared to planktonic cells. The results also indicated that expression of the *lacZ* gene in biofilm cells was 1.7-fold higher in the case of cells containing pALacZsd$_{(PAO1)}$IN2 compared to cells containing pALacZsd$_{(PAO1)}$IN1. These results therefore confirmed the presence of DNA sequences in the upstream region of *flp* and *htpA-I* that are able to function as promoters in *P. aeruginosa* PAO1, and also indicated that the putative promoters are responsible for up-regulated expression of the *lacZ* reporter gene following attachment *of P. aeruginosa* PAO1 cells to a surface.

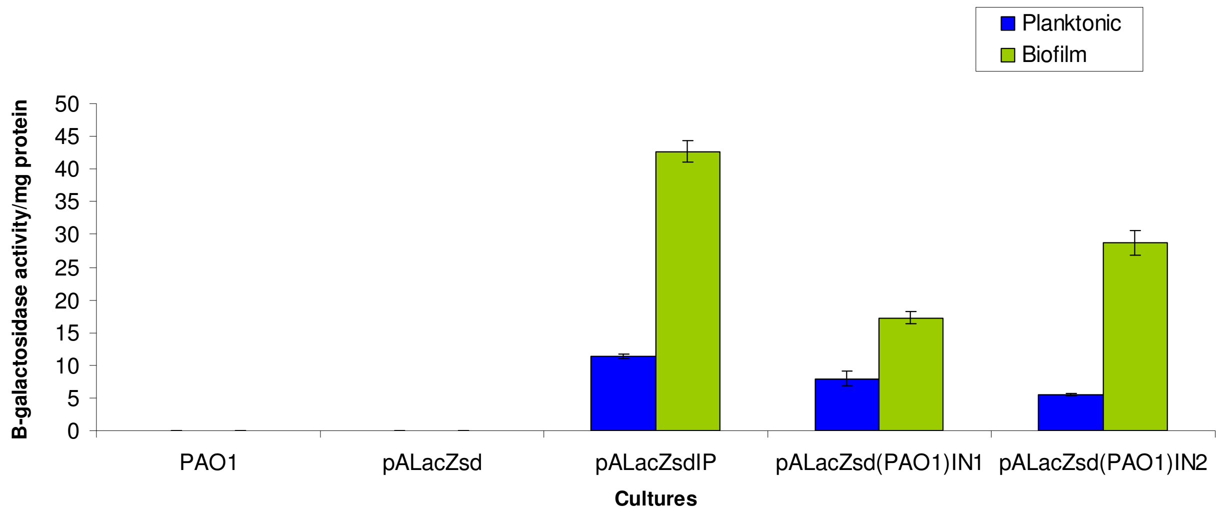


Fig. 2.6 Graph displaying the β-galactosidase activity per total cellular biomass of the different promoter-*lacZ* constructs in planktonic cells and biofilm cells. pALacZsdIP was used as a positive control vector, whereas pALacZsd was used as a negative control vector. Error bars denote one standard deviation of the mean.

2.3.4 Expression of the *P. aeruginosa* Flp protein as a GST fusion protein in *E. coli*

The putative Flp polypeptide of *P. aerginosa* PAO1 consists of 72 amino acids and has a predicted molecular mass of 7.3 kDa. However, *in silico* analysis of the polypeptide, using SignalP (at http://www.cbs.dtu.dk/services/SignalP), indicated that the first 22 amino acids of the polypeptide possess characteristics of a signal peptide, thus yielding a mature protein with a theoretical molecular mass of 4.9 kDa. However, such a low-molecular-mass native protein might not be readily detected in a background of endogenous *E. coli*-expressed cellular proteins and purification of the protein would be complicated. Consequently, it was elected to rather express the *P. aeruginosa* Flp polypeptide as a GST fusion protein in *E. coli* by making use of the pGEX vector system (Fig. 2.7a), which consists of three vectors, namely pGEX-1, pGEX-2T and pGEX-3X (Smith and Johnson, 1989). Linkers encoding the cleavage recognition site of thrombin or factor Xa were introduced immediately 5' to the multiple cloning site of pGEX-1, thereby generating the plasmids pGEX-2T and pGEX-3X in which the reading frame at the multiple cloning site is shifted by either one or two nucleotides, respectively. These pGEX expression vectors contain an IPTG-inducible *tac* promoter, which directs the high-level cytoplasmic synthesis of heterologous proteins as fusions with the C-terminus of Sj26, a 27-kDa glutathione *S*-transferase (GST) protein encoded by the parasitic helminth *Schistosoma japonicum*. Furthermore, the recombinant fusion proteins may be purified from crude bacterial cell lysates by means of glutathione affinity chromatography under non-denaturing conditions and the protease cleavage sites can potentially facilitate proteolysis of the recombinant fusion proteins whereby the GST carrier can be removed to yield the native protein.

2.3.4.1 Construction of the recombinant bacterial expression vector pGEX-Flp

Since we were interested in characterizing the ultrastructure of the mature Flp pili, only the coding sequence of the mature Flp polypeptide, excluding a putative signal peptide sequence, was PCR-amplified, cloned and expressed. Using this approach, the possibility was also excluded that the Flp pre-protein may be cleaved by an *E. coli* signal peptidase, thereby preventing subsequent purification of the recombinant protein due to the removal of the GST carrier. To obtain the coding sequence of the mature Flp polypeptide, the 5'-specific oligonucleotide used in the PCR was thus designed to exclude the nucleotides encoding the first 22 amino acids of the polypeptide that comprise the signal peptide sequence. To allow

for stable and specific annealing of the 3'-specific oligonucleotide, the oligonucleotide was designed to anneal to a target region upstream of the *flp* gene. Thus, the size of the amplicon, containing the mature Flp-encoding region, was larger (220 bp) than that of the mature *flp* gene only (150 bp). Nevertheless, translation of the GST-Flp fusion protein would be expected to terminate at the stop codon of Flp, thus yielding a mature Flp protein of 4.9 kDa.

The *flp* coding region was PCR-amplified using chromosomal DNA of *P. aeruginosa* PAO1 as template and oligonucleotides Flp-F (containing a *Bam*HI site) and Flp-R (containing a *Eco*RI site), as described under Materials and Methods (Section 2.2.3). An aliquot of the reaction mixture was analyzed by agarose gel electrophoresis and a single discreet amplicon of the expected size (*ca.* 220 bp) was observed (Fig. 2.7b, lane 2). In contrast, no amplification product was observed in the negative control in which template DNA was omitted. The amplicon was purified from the agarose gel, cloned into pGEM®-T Easy to generate pGEM-Flp and the integrity of the cloned insert DNA was verified by nucleotide sequencing prior to it being used in subsequent cloning procedures.

The Flp-encoding region was subsequently cloned into the pGEX-2T expression vector to position it in the correct reading frame for translation of the GST-Flp fusion protein. To construct plasmid pGEX-Flp, the Flp-encoding region was recovered from pGEM-Flp by digestion with both *Bam*HI and *Eco*RI, purified from an agarose gel and then cloned into pGEX-2T vector DNA, which had been prepared similarly to allow for directional cloning of the insert DNA. Following transformation of competent *E. coli* DH5α cells, the plasmid DNA extracted from randomly selected ampicillin-resistant transformants was characterized by agarose gel electrophoresis. Plasmid DNA migrating slower than the parental pGEX-2T vector DNA on agarose gels were selected and analyzed for the presence of a cloned insert DNA by digestion with both *Bam*HI and *Eco*RI, which flank the insert DNA in the multiple cloning site of pGEX-2T. In contrast to the non-recombinant plasmid DNA, which was linearized (Fig. 2.7b, lane 5), digestion of the recombinant plasmid DNA excised a 220-kb DNA fragment (Fig. 2.7b, lane 6), indicating that the *flp* gene had been cloned successfully. A recombinant clone, designated pGEX-Flp, was selected and used in all subsequent experiments.

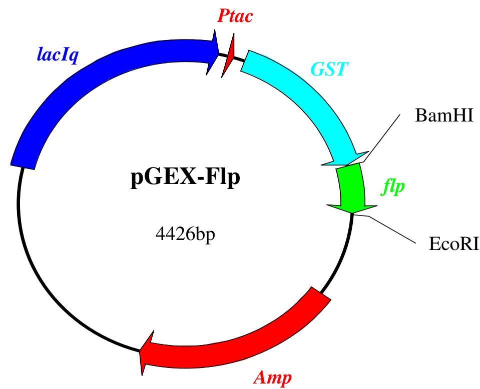


Fig. 2.7a Plasmid map of recombinant expression vector pGEX-Flp, constructed by cloning the 220-bp Flp-encoding region into the *Bam*HI and *Eco*RI sites of pGEX-2T.

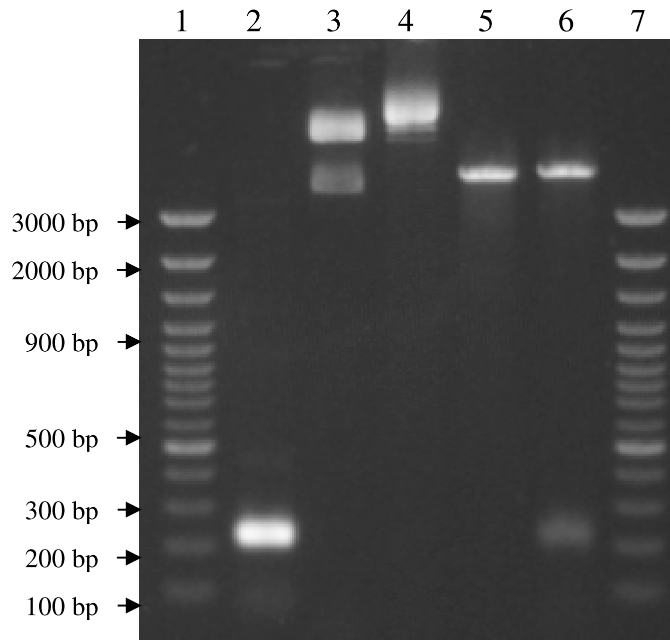


Fig. 2.7b Agarose gel electrophoretic analysis of the recombinant bacterial expression vector pGEX-Flp. Lanes 1 and 7, DNA molecular weight marker; lane 2, sample of the reaction mixture following PCR using *P. aeruginosa* PAO1 chromosomal DNA as template and oligonucleotides Flp-F and Flp-R; lane 3, uncut parental pGEX-2T vector DNA; lane 4, uncut recombinant plasmid pGEX-Flp; lane 5, pGEX-2T vector DNA digested with both *Eco*RI and *Bam*HI; lane 6, recombinant plasmid pGEX-Flp digested with both *Eco*RI and *Bam*HI. The sizes of the DNA molecular weight marker, GeneRuler™ 100-bp DNA Ladder Plus (Fermentas), are indicated to the left of the figure.

2.3.4.2 Expression and purification of the recombinant GST-Flp fusion protein

For bacterial expression, overnight cultures of *E. coli* DH5α cells containing either the non-recombinant pGEX-2T or recombinant pGEX-Flp vector were diluted in fresh LB broth and grown in the presence of IPTG to induce high-level protein expression. Following incubation, cell lysates were prepared from the respective cultures and analyzed by SDS-PAGE. Analysis of the Coomassie blue-stained SDS-polyacrylamide gel indicated that expression of the 27-kDa GST protein was induced only in cells containing the non-recombinant pGEX-2T vector (Fig. 2.8, lane 5). In contrast to control cell lysates prepared from untransformed *E. coli* DH5α cells, an over-expressed protein of *ca.* 32 kDa was clearly visible in the cell lysate preparation from induced cells containing the recombinant pGEX-Flp plasmid (Fig. 2.8, lane 7). The size of the 32-kDa protein corresponds with the calculated size for the GST-Flp fusion protein (4.9 kDa for the Flp polypeptide and 27 kDa for the GST protein).

The GST-Flp fusion protein was subsequently purified from bacterial extracts by glutathione agarose affinity chromatography and the purity was assessed by SDS-PAGE. Purification of the highly soluble GST protein was included as a control in this assay. Appropriately sized fusion proteins were found to be the major component in the cell lysate preparations (Fig. 2.9a, lanes 2 and 5, respectively) and, after elution from glutathione agarose beads, the GST and GST-Flp proteins were purified to near homogeneity, as evidenced by the presence of only one band by Coomassie Brilliant Blue staining of the gel following SDS-PAGE analysis (Fig. 2.9a, lanes 4 and 7, respectively). The identity of the purified recombinant GST-Flp protein was subsequently verified by subjecting an unstained SDS-polyacrylamide gel to Western blot analysis using a polyclonal anti-GST antibody (Fig. 2.9b). The anti-GST antibody reacted specifically with the 32-kDa protein purified from the cell lysate of IPTG-induced *E. coli* DH5α cells transformed with pGEX-Flp, as well as with the control 27-kDa GST protein. These results therefore confirmed that the Flp polypeptide of *P. aeruginosa* PAO1 was expressed successfully in *E. coli* and that the protein could be purified to near homogeneity by glutathione affinity chromatography.

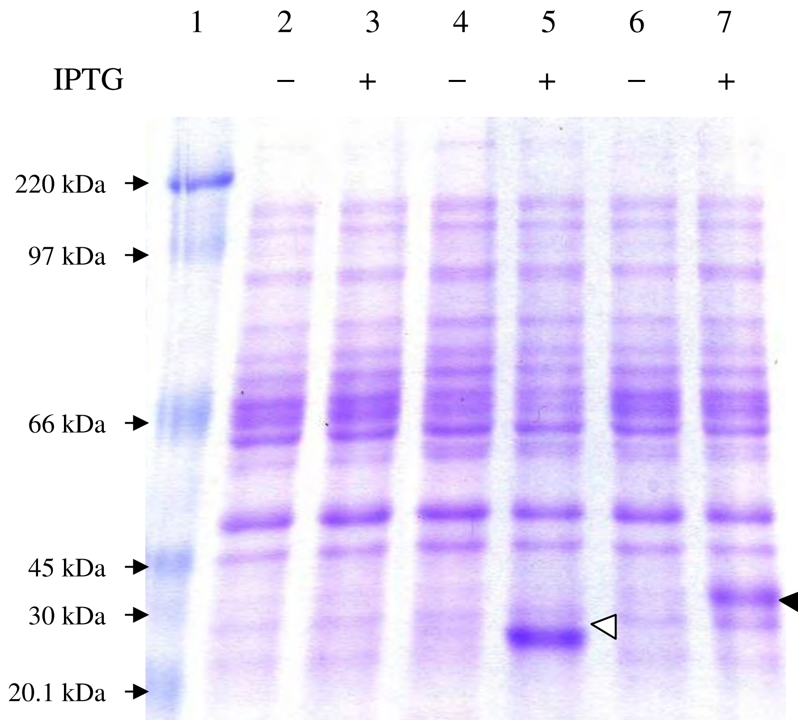


Fig. 2.8 Expression of the recombinant GST-Flp fusion protein in *E. coli*. *E. coli* DH5α cells were transformed with the non-recombinant pGEX-2T plasmid (lanes 4 and 5) or with the recombinant pGEX-Flp plasmid (lanes 6 and 7). The cultures were either induced by the addition of IPTG (+) or left uninduced (-), and harvested 6 h post-induction. Uninduced (lane 2) and IPTG-induced *E. coli* DH5α cells (lane 3) were included as controls. The different bacterial extracts were resolved by 12% SDS-PAGE, after which the gel was stained with Coomassie Brilliant Blue. The position of the GST-Flp fusion protein (closed arrow) and the GST protein (open arrow) is indicated. The sizes of the protein molecular weight markers (lane 1) are indicated to the left of the figure.

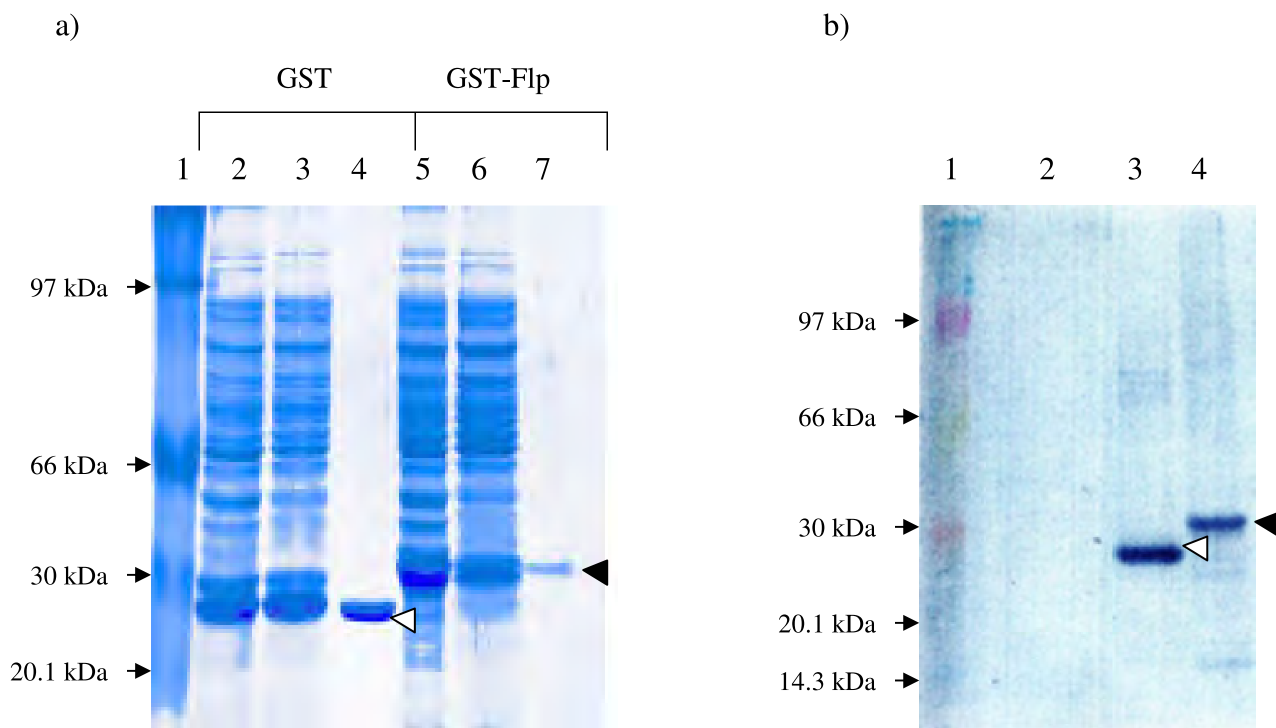


Fig. 2.9 SDS-PAGE and Western blot analyses following expression and purification of the recombinant GST-Flp fusion protein and GST protein. (a) Following expression of these proteins in IPTG-induced recombinant *E. coli* DH5α cultures, bacterial extracts were prepared and the GST-Flp fusion protein and GST protein purified by glutathione affinity chromatography. Proteins in different fractions were resolved by 12% SDS-PAGE and visualized by staining the gel with Coomassie Brilliant Blue. Lanes 2 and 5, cell lysates of IPTG-induced cultures; lanes 3 and 6, cytoplasmic (supernatant) fractions obtained following cell lysis and centrifugation to pellet cellular debris; lanes 4 and 7, purified proteins following elution from the glutathione agarose. The position of the GST-Flp fusion protein (closed arrow) and GST protein (open arrow) is indicated. The sizes of the protein molecular weight markers (lane 1) are indicated to the left of the figure.

(b) Western blot analysis of the recombinant GST-Flp fusion protein using a polyclonal anti-GST antibody as primary antibody. In order to confirm the identity of the over-expressed purified 32-kDa protein, the purified protein was resolved by SDS-PAGE and then subjected to Western blot analysis. Lane 1, Protein molecular weight marker; lane 2, IPTG-induced *E. coli* DH5α culture; lane 3; purified GST protein; lane 4, purified recombinant GST-Flp fusion protein. The position of the GST-Flp fusion protein (closed arrow) and GST protein (open arrow) is indicated. The sizes of the protein molecular weight markers are indicated to the left of the figure.

2.3.5 Protease cleavage of purified GST-Flp fusion proteins

To allow for ultrastructure characterization of mature *P. aeruginosa* PAO1 Flp pili, the GST carrier was removed from the GST-Flp fusion protein by incubation of the purified recombinant fusion protein with thrombin. Since thrombin does not cleave the GST carrier (Smith and Johnson, 1989), it is therefore possible to remove the GST carrier and any uncleaved GST-Flp fusion protein from the cleavage reaction by absorption on glutathione agarose, thereby leaving only the purified native Flp protein behind. Analysis of a Coomassie blue-stained 20% SDS-polyacrylamide gel indicated that although incubation of the GST-Flp protein with thrombin resulted in the proteolytic cleavage of the fusion protein, as evidenced by a shift in the migration of treated versus untreated protein samples (Fig. 2.10a, lanes 4 and 3, respectively), the native Flp protein (4.9 kDa) could not be visualized on the stained gel. Similar results were obtained despite using a Tris-tricine 20% SDS-polyacrylamide gel, which has been reported to result in much improved separation and resolution of small proteins and peptides (Schaegger and von Jagow, 1987), or by staining the 20% SDS-polyacrylamide gel with silver nitrate, which increases the sensitivity of protein detection from 10- to 100-fold compared to Coomassie Brilliant Blue staining (Switzer *et al.*, 1979) (Fig. 2.10b and c, respectively). Since it is possible that the Flp protein may not have stained properly due to its small size, a sample of the purified native GST-Flp protein was viewed under a transmission electron microscope following negative staining of the proteins. However, due to the presence of contaminating GST protein in the thrombin-cleaved GST-Flp protein samples (Fig. 2.10, lanes 4), structures that may resemble pili could not be identified (Fig. 2.10, insert). These results precluded further analyses regarding the *P. aeruginosa* PAO1 Flp pilus structure.

2.4 DISCUSSION

The ability of diverse bacteria to adhere to surfaces is a property important for the colonization of environmental niches and the production of biofilms (Costerton *et al.*, 1995). Adherence is often mediated by proteinaceous appendages (pili, fimbriae) present on the cell surface (Abraham *et al.*, 1998; O'Toole and Kolter, 1998a; Pratt and Kolter, 1998; Watnick *et al.*, 1998; Vidal *et al.*, 1998; Prigent-Combaret *et al.*, 1999). Among the best characterized are the type I (Orndorff and Bloch, 1990; Saulino *et al.*, 1998; Schilling *et al.*, 2001) and type IV (Strom and Lory, 1993; Parge *et al.*, 1995; Alm and Mattick, 1997) pili, the latter having

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

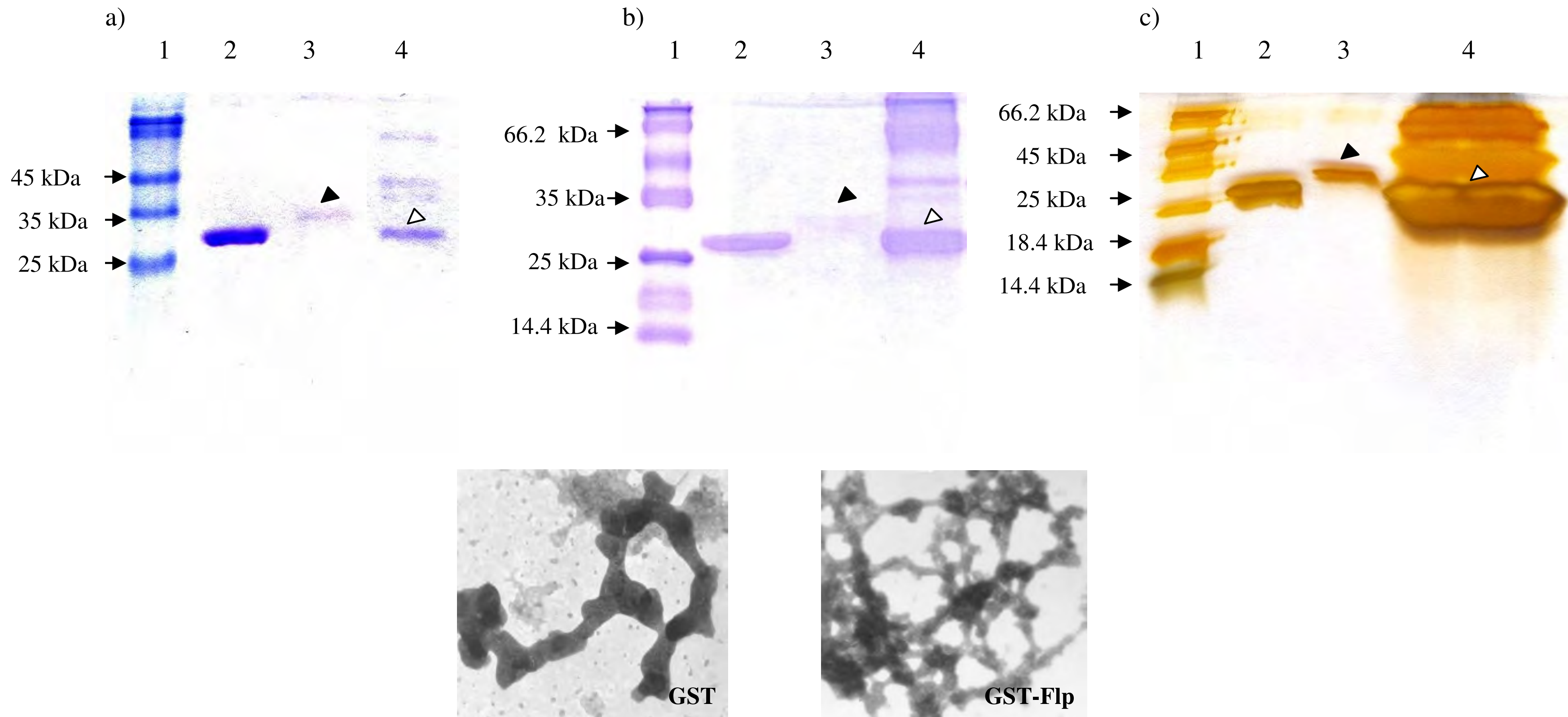


Fig. 2.10 Thrombin cleavage of purified GST-Flp. Purified GST-Flp protein was incubated with thrombin at room temperature for 16 h. The samples were purified by glutathione affinity chromatography and analyzed by electrophoresis through a (a) 20% SDS-polyacrylamide gel stained with Coomassie Brilliant Blue, (b) Tris-tricine 20% SDS-polyacrylamide gel stained with Coomassie Brilliant Blue and (c) 20% SDS-polyacrylamide gel stained with silver nitrate. In each of the figures, the lanes are as follows: Lanes 1, protein molecular weight marker; lanes 2, purified GST protein; lanes 3, purified GST-Flp protein; lanes 4, thrombin cleavage reaction of GST-Flp. The position of the GST-Flp fusion protein (closed arrow) and GST protein (open arrow) is indicated. The sizes of the protein molecular weight markers are indicated to the left of the figures. Shown below the SDS-polyacrylamide gels are electron micrographs of the purified GST protein and a sample of the purified GST-Flp protein.

been identified in a broad spectrum of Gram-negative bacteria, including *P. aeruginosa* PAO1 (Smyth *et al.*, 1996). Recently, a novel class of fimbriae (pili) was identified in *C. crescentus* (Skerker and Shapiro, 2000), *A. actinomycetemcomitans* (Inoue *et al.*, 1998; Haase *et al.*, 1999; Kachlany *et al.*, 2000; 2001) and *H. ducreyi* (Nika *et al.*, 2002), and the gene encoding the major fimbrial subunit proved to be part of a larger gene cluster in these bacteria that constitute a polycistronic operon. Although a homologous gene cluster has been identified in *P. aeruginosa*, *in silico* evidence suggests that the fimbrial subunit gene *flp* is not part of a cluster of nine genes (*htpA-I*) thought to encode proteins involved in the assembly and export of these fimbrae (Van Schalkwyk, 2003). Towards investigating the functional importance of the putative *flp-htp* fimbrial biogenesis/secretion system, the transcription of the *htp* gene cluster in *P. aeruginosa* PAO1 was investigated in this part of the investigation, and an attempt was also made to characterize the Flp pili.

The transcriptional analysis focused initially on establishing whether the *P. aeruginosa* PAO1 *htpA-I* gene cluster forms a single transcriptional unit. Since it was not possible to amplify the full-length 9.2-kb transcript using standard RT-PCR conditions, the *htp* gene cluster was subsequently examined by assaying each gene junction by RT-PCR. The gene junction transcription analysis indicated that all the genes are co-transcribed and it was thus concluded that the entire gene cluster from *htpA* through *htpI* is transcribed as a polycistronic message. Furthermore, RT-PCR of the *flp* ORF, which is orientated in the opposite direction to the *htp* gene cluster, confirmed that this ORF is indeed transcribed. Since transcription occurs from a promoter proximal to a gene, the 410-bp intergenic region between *flp* and *htpA* was analyzed for conserved promoter sequences, the results of which revealed two potential *E. coli* σ^{70} promoter sequences. Subsequent analysis of *lacZ* reporter gene fusion constructs containing the intergenic region in both transcriptional orientations resulted in β-galactosidase activity, indicating the presence of DNA sequences that could function as promoters in *P. aeruginosa* PAO1. Notably, up-expression of the *lacZ* reporter gene was observed following attachment of cells to a glass wool substratum, albeit that up-regulation for the reporter construct containing the *htp* operon promoter appeared to be higher than that of the construct containing the potential *flp* promoter. This may be related to the difference in promoter sequences, since the potential σ^{70} promoter sequence of the *htp* operon resembles the *E. coli* consensus σ^{70} promoter sequences more closely than does that of the potential *flp* promoter (Fig. 2.4).

Similar to the results obtained above, several studies regarding gene expression have also reported differential expression of numerous genes in biofilm cells when compared to their planktonic counterparts (Prigent-Combaret *et al.*, 1999; Steyn *et al.*, 2001; Sauer and Camper, 2001; Whiteley *et al.*, 2001; Sauer *et al.*, 2002). In *P. aeruginosa* expression of genes involved in biofilm formation and development, amongst other, *algC* (Davies and Geesey, 1995), *algD* (Hoyle *et al.*, 1993) and *pilA* (O'Toole *et al.*, 2000b) is up-regulated in biofilm-growing cells. However, the regulation of only a few of these genes has been reported. Notably, transcription of the above genes is dependent on the alternative sigma factor σ^{54} (Zielinski *et al.*, 1992; Arora *et al.*, 1997; Boucher *et al.* 2000). Furthermore, genes encoding proteins that aid in biofilm development such as those required for flagellar assembly (Kinsella *et al.*, 1997) and type IV pilin synthesis (Arora *et al.*, 1997) have all been reported to be σ^{54} dependent. These results therefore suggest that σ^{54} rather than σ^{70} plays an important role in the transcription of genes required for biofilm formation (Studholme *et al.*, 2000). The finding that the potential *flp* and *htp* operon promoters resemble *E. coli* σ^{70} promoter sequences is therefore surprising. Nevertheless, these results are in agreement with those reported regarding transcription of the *flp*-*tad* fimbrial gene cluster of *A. actinomycetemcomitans* for which σ^{70} consensus sequences were located at -10 and -35 relative to the transcription start site (Haase *et al.*, 2003). In addition, transcription of the *rhlAB* operon of *P. aeruginosa*, which is responsible for production of rhamnolipid required for maintenance of the three-dimensional structure of biofilms (Davey *et al.*, 2003), has also been reported to be dependent on σ^{70} and not on σ^{54} (Medina *et al.*, 2003).

The unique transcriptional organization of the *P. aeruginosa flp-htp* fimbrial gene cluster may reflect on a complex regulation system whereby the Flp pili are expressed, processed and secreted. For *C. crescentus* it has been reported that transcription of the major pilin subunit gene (*pilA*) is regulated by the global two-component response regulator CtrA, which is essential for the expression of multiple cell cycle events such as DNA replication, DNA methylation and flagellar biogenesis (Skerker and Shapiro, 2000). Transcriptome analysis of *P. aeruginosa* of quorum sensing-controlled genes indicated that expression of the *htpA-I* (PA4297-4305) and *flp* (PA4306) genes is induced by quorum sensing via acyl-homoserine lactone (acyl-HSL) signals. Whereas expression of the *P. aeruginosa flp* and *htp* genes was induced by N-(3-oxododecanoyl)-L-HSL (3O-C_{12}-HSL) (Wagner *et al.*, 2003), a greater

response was observed with both 3O-C_{12}-HSL and N-butyryl-L-HSL (C_4-HSL) (Schuster *et al.*, 2003). However, in a subsequent transcriptome analysis of *P. aeruginosa*, Wagner *et al.* (2004) reported that expression of only the *htpB* (PA4304) and *htpD* (PA4302) genes was up-regulated by both 3O-C_{12}-HSL and C_4-HSL. The significance of these findings is unclear. Not only are these results contradictory to those reported previously, but the results obtained in this study indicated that these genes form part of an operon and it would therefore be expected that expression of all genes forming part of the operon would be up-regulated. It has also been reported that the *las* and *rhl* quorum sensing system transcription activators, LasR and RhlR, respectively, bind to a specific palindromic sequence in the promoter region of quorum sensing-controlled genes. This sequence, designated the las-rhl box-like sequence, has a minimal consensus sequence defined as NNCT-N12-AGNN (Whiteley and Greenberg, 2001). However, in this study, *in silico* analysis of the intergenic region and upstream sequences of the respective *flp* and *htp* genes indicated that none of these genes contained a las-rhl box-like sequence (results not shown). Note should also be taken that although quorum sensing has been reported to be important for biofilm formation with *P. aeruginosa* (McLean *et al.*, 1997; Davies *et al.*, 1998), it is still a controversial issue as several reports have disputed the importance of quorum sensing in biofilm formation in Gram-negative strains (Sauer and Camper, 2001; Heydorn *et al.*, 2002; Purevdorj *et al.*, 2002; Stoodley *et al.*, 2002). These apparent contradictory and discordant data necessitates that investigations are undertaken in future to determine the precise manner and by which (other) factors expression of the *flp* gene and *htp* operon may be controlled.

Unlike *A. actinomycetemcomitans* and *C. crescentus* for which spontaneous Flp hyperpiliated mutants are available, thus greatly facilitating their purification and characterization (Kachlany *et al.*, 2001; Skerker and Shapiro, 2002), no such mutants are available for *H. ducreyi* or *P. aeruginosa*. Indeed, the Flp fimbriae of *H. ducreyi* have not yet been detected on the surface of cells (Nika *et al.*, 2002). To characterize the structure of *P. aeruginosa* Flp pili, a strategy was therefore adopted whereby the Flp protein was to be overexpressed in *E. coli* in order to obtain sufficient amounts of the native protein to allow for its characterization. Multiple sequence alignment analyses of the Flp proteins have indicated that they all contain a positively charged leader sequence, followed by a region of *ca.* 20 hydrophobic amino acids and a C-terminal variable region (Kachlany *et al.*, 2001). Notably, a glycine (Gly) residue is invariably conserved immediately preceding the cleavage site between the leader sequence and the hydrophobic domain. In the case of *C. crescentus*, N-terminal sequence data has

indicated that the N-terminal residue of the mature PilA pilin protein is an alanine (Ala) (Skerker and Shapiro, 2000). Similarly, sequence analysis of the *P. aeruginosa* PAO1 Flp polypeptide indicated the presence of an N-terminus signal peptide sequence with the predicted cleavage site between Gly_{21} and Ala_{22}. Thus, the region encoding the mature Flp polypeptide of *P. aeruginosa* PAO1 was PCR-amplified, cloned and expressed as a GST fusion protein in *E. coli*. SDS-PAGE analysis of cell lysates indicated that the GST-Flp polypeptide was expressed to a high level and the identity of the purified fusion protein was confirmed by Western blot analysis using an anti-GST antibody. To enable characterization of the Flp ultrastructure, the purified GST-Flp fusion protein was proteolyticaly cleaved with thrombin in an attempt to obtain mature native Flp protein. Although the 32-kDa GST-Flp protein was cleaved, yielding a clearly visible 27-kDa GST protein, the 4.9-kDa mature Flp polypeptide could, however, not be visualized on SDS-polyacrylamide gels, despite using different electrophoresis systems and staining methods. The inability to visualize these proteins may not be surprising, since studies on the pili of *A. actinomycetemcomitans* and *C. crescentus* both relied on the use of antibodies for their detection on SDS-polyacrylamide gels (Kachlany *et al.*, 2001; Skerker and Shapiro, 2000). The inability to obtain pure Flp, without GST protein contamination, however, precluded further characterization of the *P. aeruginosa* PAO1 Flp pilin protein.

In conclusion, the results presented here indicate that the *htp* gene cluster of *P. aeruginosa* is transcribed as a polycistronic message, whilst the *flp* gene is transcribed independently. Putative *flp* and *htp* operon promoters were identified in the intergenic region upstream of *flp* and *htpA*, respectively. Using reporter assays, transcription of both the *flp* and *htp* operon was found to be greater in biofilms than in planktonic cells, suggesting that the Flp pili may play a role in *P. aeruginosa* PAO1 biofilm development. This warranted further investigation and the details of these investigations are presented in the following Chapter.

CHAPTER THREE

CONSTRUCTION AND CHARACTERIZATION OF AN HtpD (PA4302)-DEFICIENT MUTANT STRAIN OF *Pseudomonas aeruginosa* PAO1

3.1 INTRODUCTION

In natural and artificial habitats, most bacteria, including *Pseudomonas aeruginosa*, have a strong tendency to adhere to surfaces within microbial consortia called biofilms (Costerton *et al*., 1995). The formation of a well-developed biofilm is believed to occur in a sequential process of transport of microorganisms to a surface, initial attachment of the microorganisms to the surface, formation of microcolonies and formation of well-developed biofilms (O'Toole *et al*., 2000a; Sauer *et al*., 2002; Stoodley *et al*., 2002). Over the past few years, much progress has been made towards understanding the development of *P. aeruginosa* biofilms. This progress has been largely due to the recent focus of analyzing biofilms using genetic (O'Toole and Kolter, 1998a; 1998b; Whiteley *et al*., 2001; Finelli *et al*., 2003), proteomic (Steyn *et al*., 2001; Sauer *et al*., 2002) and molecular biological (Tolker-Nielsen *et al*., 2000; Heydorn *et al*., 2002; Klausen *et al*., 2003) approaches. The results of these analyses have indicated that bacterial attachment to a surface is dictated by several factors, including surface composition (Marshall, 1985; Van Loosdrecht *et al*., 1990), environmental factors (O'Toole *et al*., 2000b; Stoodley *et al*., 2002; Stanley and Lazazzera, 2004) and several different gene products (Davies and Geesey, 1995; Allison *et al*., 1998; O'Toole and Kolter, 1998a; DeFlaun *et al*., 1999; O'Toole *et al*., 2000a; Parkins *et al*., 2001; Vallet *et al*., 2001; Finelli *et al*., 2003; Caiazza and O'Toole, 2004).

Motility is often recognized as a factor contributing to adhesion and colonization of both biotic (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). In addition to flagella, fimbriae such as 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. The polar type IV pili of *P. aeruginosa* are multifunctional structures that not only play a role in adherence to and colonization of biotic (Kang *et al*., 1997; Hahn, 1997) and abiotic (O'Toole and Kolter, 1998a) surfaces, but it also plays a role in the initial stages of infection by bacteriophages (Rehmat and Shapiro, 1983; Mattick *et al*., 1996) and twitching motility (Darzins, 1994; Wall and Kaiser, 1999).

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 non-specific adherence of the bacteria to surfaces. Mutations in any of the *tad* genes (Kachlany *et al.*, 2000) and in the *flp-1* gene (Kachlany *et al.*, 2001) resulted in a defect in adherence and failure to produce fibrils. The *flp-1* gene located upstream from *tadA* is the first gene of an operon that includes the *tad* gene cluster (Haase *et al.*, 2003). 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*. Using the available genome sequence of *P. aeruginosa* PAO1, a cluster of nine genes, designated *htpABCDEFGHI*, has been identified in the genomic DNA of *P. aeruginosa*. Most of these genes were found to encode predicted protein products that display homology to the proteins involved in the formation and release of fimbriae in other bacteria, including *A. actinomycetemcomitans*, *C. crescentus* and *H. ducreyi* (Van Schalkwyk, 2003). The *htp* genes of *P. aeruginosa* have been shown to comprise a polycistronic operon that excludes the putative pilus subunit-encoding gene *flp*, and it was also shown that expression of the *htp* operon and *flp* gene is up-regulated by attachment of *P. aeruginosa* PAO1 to a substratum (Chapter 2).

All known secretion systems have at least one protein that is thought to use NTP hydrolysis to provide energy for secretion complex assembly or the movement of macromolecules across membranes (Burns, 1999; Christie, 2001; Mori and Ito, 2001; Thanassi and Hultgren, 2001). The TadA protein of *A. actinomycetemcomitans* has been reported to have ATPase activity and is required to energize the assembly or secretion of Flp pili for tight non-specific adherence of *A. actinomycetemcomitans* (Bhattacharjee *et al.*, 2001). Although the TadA protein of *H. ducreyi* has not yet been characterized at a biochemical level, inactivation of *tadA* by insertional mutagenesis (Nika *et al.*, 2002) resulted in a phenotype similar to that of an *A. actinomycetemcomitans tadA* mutant (Kachlany *et al.*, 2000); that is the bacteria exhibited a greatly reduced ability to attach to inert surfaces. Notably, sequence analysis of the HtpD protein (PA4302) of *P. aeruginosa* PAO1 has revealed that it contains the highly conserved Walker A and B motifs (Van Schalkwyk, 2003). These motifs are characteristic of NTPases and are required for nucleotide binding and hydrolysis (Walker *et al.*, 1982), suggesting that HtpD may function as an energizing protein of the putative Flp biogenesis/secretion system of *P. aeruginosa*.

Towards determining the functional importance of the *P. aeruginosa htp* gene cluster in the ability of *P. aeruginosa* to attach to surfaces, the *htpD* (PA4302) gene was thus targeted for insertional inactivation in this study, as it was expected that inactivation of this gene would severely impair the functioning of the *htp* operon and therefore result in a drastic change in phenotype. Consequently, the aims of this part of the investigation were (i) to generate a *P. aeruginosa* PAO1 mutant strain deficient in HtpD and (ii) to compare its ability to form biofilms on a glass wool substratum with that of the wild-type *P. aeruginosa* PAO1 strain.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this part of the study are listed in Table 3.1. *P. aeruginosa* and *E. coli* strains were routinely cultured in LB broth (1% [w/v] tryptone, 1% [w/v] NaCl, 0.5% [w/v] yeast extract; pH 7.4) (Sambrook *et al*., 1989) at 37°C with shaking at 200 rpm, and maintained at 4°C on LB agar (LB broth containing 1.2% [w/v] bacteriological agar) or at -70°C as glycerol cultures. For plasmid DNA selection and maintenance in *E. coli,* the concentration of antibiotics used was: 100 μg/ml for ampicillin and 10 μg/ml for gentamicin. The following antibiotics were used to maintain the plasmid DNA and chromosomal insertions in *P. aeruginosa* strains: gentamicin at 50 μg/ml and tetracycline at 100 μg/ml. All antibiotics were purchased from Roche Diagnostics.

3.2.2 Construction of allelic exchange vector pUCori-HtpD-Gent

All molecular cloning techniques employed in the construction of a recombinant allelic exchange vector pUCori-HtpD-Gent and complementation plasmid pJB-IP-HtpD (Section 3.2.5) was performed according to the procedures described in Chapter 2 (Sections 2.2.5 through 2.2.9). All plasmid constructs were confirmed by restriction endonuclease digestions using agarose gel electrophoresis and by nucleotide sequencing.

3.2.2.1 DNA amplification

To generate an isogenic mutant strain of *P. aeruginosa* PAO1 in which the wild-type *htpD* (PA4302) gene on the *P. aeruginosa* genome was replaced with an *in vitro*-modified allele, an allelic exchange vector was constructed in which the *htpD* ORF was disrupted by the insertion of a cassette encoding gentamicin resistance. Consequently, both the *htpD* gene and

gentamicin resistance cassette was obtained by PCR amplification and then used in the construction of the allelic exchange vector, as described below.

3.2.2.1.1 Oligonucleotide primers

The oligonucleotide primers used in PCR to amplify the *htpD* gene was designed based on the published genome sequence of *P. aeruginosa* strain PAO1 (Stover *et al.*, 2000), available at http://www.pseudomonas.com. Oligonucleotide primers used to PCR amplify a gentamicin resistance cassette was designed based on the sequence of plasmid pCM351 (Marx and Lidstrom, 2002). To facilitate cloning of the amplicons, unique restriction endonuclease recognition sites were incorporated at the 5' terminus of the respective primers. The oligonucleotides were designed using the DNAMAN v.4.13 (Lynnon Biosoft) software program and are indicated in Table 3.1. The oligonucleotides were synthesized by Inqaba Biotech.

3.2.2.1.2 Polymerase chain reaction (PCR) amplification

Genomic DNA from *P. aeruginosa*, extracted as described previously (Section 2.2.2), was used as template DNA in the PCR to amplify the *htpD* gene, whilst plasmid pCM351 which harbours a gentamicin resistance cassette on the genetic backbone, was used as template DNA in the PCR to amplify the gentamicin resistance cassette. The PCR reaction mixtures (50 µl) contained either 100 ng of genomic DNA or 20 ng of plasmid DNA as template, 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 0.1% [v/v] TritonX-100), 1.5 mM $MgCl_2$, 5% (v/v) DMSO, 200 µM of each dNTP, 25 pmol of each of the appropriate oligonucleotides and 1 U of *Taq* DNA polymerase (Southern Cross Biotechnology). Following incubation at 94°C for 5 min, the reaction mixtures were subjected to 30 of the following cycles using a Perkin-Elmer GeneAmp® 2700 thermal cycler: denaturation at 94°C for 1 min, oligonucleotide annealing at 70°C (4302-F and 4302-R) or 55°C (Gent-F and Gent-R) for 1 min and elongation at 72°C for 1 min, followed by elongation for 5 min at 72°C in the final cycle to complete synthesis of all DNA strands. For control purposes, reaction mixtures containing UHQ water and all other reagents but no template DNA were included. Aliquots of the PCR reaction mixtures were subsequently analyzed by agarose gel electrophoresis on 1% (w/v) agarose gels in the presence of an appropriate DNA molecular weight marker.

Table 3.1 Bacterial strains, plasmids and oligonucleotides used in this study

Strain, plasmid or primer	Relevant properties	Reference
Strains:		
E. coli		
DH5α	F⁻ *recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1* Δ(*lacZYA-argF*) U169 λ⁻ [Φ80d*lac*ZΔM15]	Promega
P. aeruginosa		
PAO1	Wild-type	Holloway *et al.* (1979)
PAO1HtpD	PAO1HtpD::Gm^r	This study
Plasmids:		
pUC18	Cloning vector, ColE1, Amp^r, LacZα peptide	Stratagene
pGEM®-T Easy	Cloning vector for PCR products, ColE1, Amp^r, LacZα peptide	Promega
pJB3cT20	Derivative of pJB3, *oriV*, *oriT*, Tc^r, Amp^r	Blatney *et al.* (1997)
pRK600	Cm^r, ColE1, RK2-Mob$^+$, RK2-Tra$^+$, helper plasmid in triparental conjugations	Kessler *et al.* (1992)
pSS125	pUC19 containing *oriT*	S.J. Suh (unpublished)
pGEM-Gent	pGEM®-T Easy vector containing gentamicin resistance cassette amplicon	This study
pGEM-HtpD	pGEM®-T Easy vector containing *htpD* (PA4302) amplicon	This study
pUC-HtpD	pUC18 vector containing *htpD* amplicon cloned into the *Hind*III and *Xba*I sites	This study
pUC-HtpD-Gent	pUC-HtpD with a Gm^r cassette inserted at the *Pst*I and *Xho*I sites of the HtpD ORF	This study
pUCori-HtpD-Gent	pUC-HtpD-Gent with *oriT*	This study
pGEM-IP	pGEM®-T Easy vector containing the *lac* promoter from pBluescript SKII (+)	P. Medronho (unpublished)
pGEM-plac	pGEM®-T Easy vector containing the *lac* promoter amplicon	This study
pUC-IP-HtpD	pUC-HtpD containing the *lac* promoter cloned upstream of *htpD* as a *Kpn*I - *Xba*I DNA fragment	This study
pJB-IP-HtpD	The *lac-htpD* DNA fragment cloned into the *Kpn*I and *Hind*III sites of pJB3cT20	This study
Oligonucleotides:		
PCR amplification*:		
4302-F	5' - CGC**tctaga**ATGAGCACGGGATTCGGCGCGCG - 3';	*Xba*I site incorporated
4302-R	5' - GCG**aagctt**GGTTGCGCGCCAGGGTGAAGATG - 3';	*Hind*III site incorporated
Gent-F	5' - GCG**ctgcag**GGTACCATGGATGCATATGGC - 3';	*Pst*I site incorporated
Gent-R	5' - GCG**ctcgag**TTAGGTGGCGGTACTTGGGTCG - 3';	*Xho*I site incorporated
IP-F	5' - **ggtacc**CAGGTATCCGGTAAGC - 3';	*Kpn*I site incorporated
IP-R	5' - **tctaga**AGCTGTTTCCTGTGTG - 3';	*Xba*I site incorporated
4302-SF	5' - GATGGTCGACCAGAGCGTGCCG - 3'	
4302-SR	5' - GATGAGCTTGACGATGCTGTCC - 3'	
Nucleotide sequencing:		
pUC/M13 Forward	5' - CCCAGTCACGACGTTGTAAAACG - 3'	
pUC/M13 Reverse	5' - CAGGAAACAGCTATGAC - 3'	

* In oligonucleotide sequences, the restriction endonuclease sites are indicated in bold lower case letters

3.2.2.2 Construction of allelic exchange vector pUCori-HtpD-Gent

Oligonucleotides Gent-F and Gent-R were used with plasmid pCM351 as template DNA to PCR amplify the 840-bp gentamicin resistance cassette, which was cloned into pGEM®-T Easy to generate pGEM-Gent. Oligonucleotides 4302-F and 4302-R were used with chromosomal DNA from *P. aeruginosa* PAO1 to PCR amplify the 1.762-kb full-length *htpD* gene (PA4302), which was cloned into pGEM®-T Easy to generate pGEM-HtpD, and subsequently recloned into pUC18 vector DNA as a *Hind*III - *Xba*I DNA fragment to generate pUC-HtpD. To inactivate the *htpD* ORF, plasmid pUC-HtpD was digested with *Pst*I and *Xho*I, which cut in *htpD* only, and the gentamicin resistance cassette was ligated into the deletion site of plasmid pUC-HtpD as a *Pst*I - *Xho*I DNA fragment to yield pUC-HtpD-Gent. To complete the construction of allelic exchange vector pUCori-HtpD-Gent, an *oriT*-containing DNA fragment was recovered from plasmid pSS125 by digestion with *Bam*HI and cloned into pUC-HtpD-Gent, which had been linearized by digestion with *Bam*HI. The cloning strategy employed in the construction of the allelic exchange vector pUCori-HtpD-Gent is indicated diagrammatically in Fig. 3.1.

3.2.3 Generation of *P. aeruginosa* PAO1 mutant strains

The allelic exchange vector pUCori-HtpD-Gent was introduced into *P. aeruginosa* PAO1 by triparental mating, as described by Kessler *et al*. (1992). A single colony of freshly streaked cultures of donor (*E. coli* DH5α containing the allelic exchange vector), helper (*E. coli* DH5α containing pRK600) and recipient (*P. aeruginosa* PAO1) strains were mixed on LB agar with a sterile inoculation needle and then incubated at 37°C overnight. Following incubation, the mixed growth was streaked on LB agar supplemented with 50 µg/ml gentamicin and 10 µg/ml tetracycline (to counterselect against *E. coli* donor cells). The agar plates were then incubated at 37°C for a further 24 to 48 h. Single colonies were selected from the plates and maintained on LB agar containing 50 µg/ml gentamicin. One of these, designated PAOHtpD, was selected and used in subsequent investigations.

3.2.4 Characterization of *P. aeruginosa* PAO1 mutant strains

The replacement of the wild-type *htpD* ORF with the mutant null allele in the putative *P. aeruginosa* mutant strains was verified by PCR analyses and Southern blot hybridization (Southern, 1975).

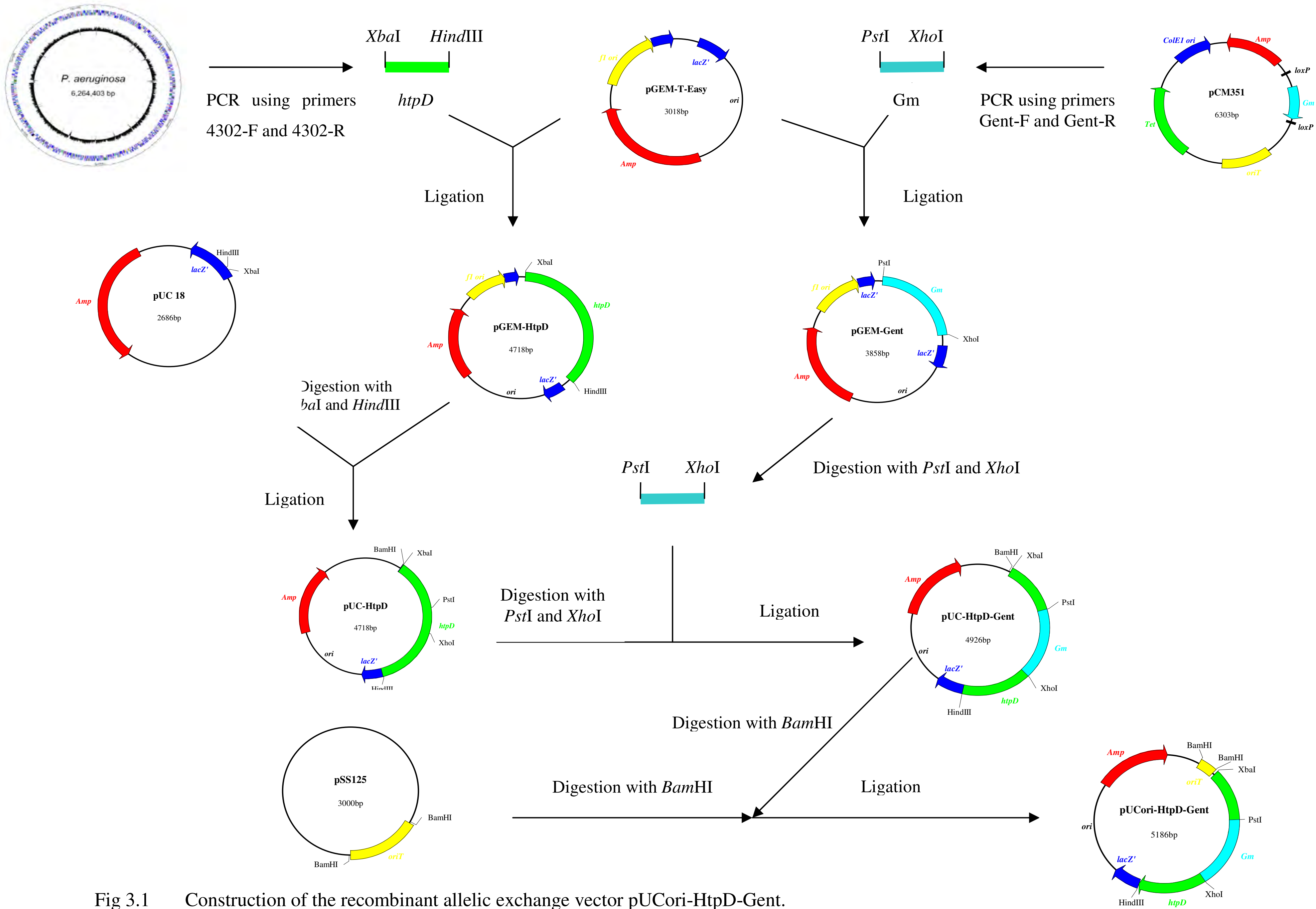


Fig 3.1 Construction of the recombinant allelic exchange vector pUCori-HtpD-Gent.

3.2.4.1 PCR analyses

The PAOHtpD mutant strain was analyzed for the presence of the gentamicin resistance cassette within the *htpD* ORF by PCR analyses. Oligonucleotide primers 4302-SF and Gent-F were used to amplify a hybrid amplicon consisting of the 5'-end of the gentamicin resistance cassette and the 5'-end of the interrupted *htpD* gene, whereas oligonucleotide primers 4302-SR and Gent-R were used to amplify a hybrid amplicon containing the 3'-end of the gentamicin resistance cassette and the 3'-end of the interrupted *htpD* gene. Oligonucleotide primer pair 4302-SF and 4302-SR (Table 3.1) were also used to amplify the *htpD* gene interrupted by the gentamicin resistance cassette in mutant chromosomal DNA. The PCR reactions were performed, as described previously (Section 3.2.2.1.2), except that oligonucleotide annealing was performed at 70°C for 60 s for all oligonucleotide pairs. For all of the analyses, UHQ water served as a negative control, while chromosomal DNA extracted from the parental and mutant strains provided sample template DNA. Following PCR amplification, aliquots of the respective reaction mixtures were analyzed by electrophoresis on 1% (w/v) agarose gels in the presence of an appropriate DNA molecular weight marker.

3.2.4.2 Southern blot analysis

3.2.4.2.1 Preparation of labelled probe

For Southern blot analysis, the gentamicin resistance cassette was used as probe and labelled with digoxigenin-dUTP (DIG-dUTP) during the PCR reaction, using the DIG PCR Probe Synthesis kit (Roche Diagnostics) according to the manufacturer's instructions. The gentamicin resistance cassette was PCR-amplified using plasmid pGEM-Gent as template DNA and oligonucleotides Gent-F and Gent-R, as described previously (Section 3.2.2.1.2). The concentration and labelling efficiency of the probe DNA were subsequently determined, using the control labelled DNA supplied with the kit according to the manufacturer's instructions.

3.2.4.2.2 Preparation of the membrane

Aliquots of extracted genomic DNA of the *P. aeruginosa* wild-type PAO1 and PAOHtpD mutant strains were digested with *Asc*I overnight at 37°C (10 U of *Asc*I per 800 ng of genomic DNA). Plasmids pGEM-Gent, digested with *Pst*I and *Xho*I, and pUC-HtpD-Gent,

digested with *Xba*I and *Hind*III, were included in the analysis as controls to confirm probe specificity. The genomic and plasmid DNA samples were separated by electrophoresis on a 0.7% (w/v) agarose gel and then transferred from the agarose gel to a Hybond™-N nylon membrane (Amersham Bioscience) by capillary blotting. For this purpose, the DNA was first denatured by soaking the gel for 30 min with constant agitation in denaturing solution (1.5 M NaCl, 0.5 M NaOH), rinsed in ddH_2O and then neutralized, as above, in neutralization solution (1 M Tris-HCl [pH 7.2], 1.5 M NaCl). Six pieces of filter paper, soaked in 20 × SSC (3 M NaCl, 0.3 M sodium citrate; pH 7.0) were stacked on a piece of Glad Wrap™, folded so as to surround the gel. The inverted gel (with the orientation marked by cutting one corner) was placed onto the filter paper followed by the nylon membrane and then two pieces of filter paper, all of which were pre-wet in 2 × SSC. Two additional dry filter papers and several paper towels were stacked on top of the wet filter papers and weighed down by a light weight. Transfer was allowed to proceed at room temperature for at least 18 h, after which the membrane was rinsed in 2 × SSC and the DNA fixed to the membrane by UV irradiation for 5 min each side.

3.2.4.2.3 Nucleic acid hybridization

The hybridization temperature was calculated using the following formula (Roche Diagnostics): $T_m = 49.82 + 0.41(\%G+C) - (600/l)$, where l is the length of the hybrid in bp. The optimum hybridization temperature was then calculated using $T_{opt} = T_m - 25°C$. The optimal hybridization temperature for the gentamicin probe was calculated at 47°C. The membrane was incubated in DIG-Easy Hyb buffer, pre-warmed to 47°C, and pre-hybridized for 30 min with gentle agitation. The pre-hybridization buffer was then decanted and replaced with 10 ml of hybridization buffer so as to cover the membrane, after which 25 ng/ml of the denatured labelled probe DNA was added to the buffer. The probe DNA was denatured by heating in a boiling water bath for 5 min and placed immediately on ice prior to being added to the membrane. Hybridization was allowed to proceed overnight at 47°C. After hybridization, the membrane was washed twice for 5 min each wash in 2 × SSC; 0.1% (v/v) SDS at room temperature, and then twice for 15 min each wash in 0.5 × SSC; 0.1% (v/v) SDS at 68°C.

3.2.4.2.4 Detection of hybridized probe DNA

The hybridized probe was subsequently detected using the DIG-High Prime Labelling and Detection kit (Roche Diagnostics). The membrane was thus rinsed for 5 min in washing buffer (0.1 M Maleic acid, 0.15 M NaCl [pH 7.5], 0.3% [v/v] Tween-20), followed by incubation for 30 min at room temperature in 1 × blocking solution (prepared by diluting the supplied blocking solution 10-fold in Maleic acid buffer [0.1 M Maleic acid, 0.15 M NaCl; pH 7.5]). The membrane was then incubated for 30 min at room temperature with gentle agitation in 20 ml of the anti-digoxigenin alkaline phosphatase-conjugated antibody solution (diluted 1:5000 [150 mU/ml] in blocking solution). The unbound antibodies were removed by washing the membrane twice for 15 min each wash in washing buffer at room temperature with gentle agitation and the membrane was then equilibrated for 5 min in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl; pH 9.5). The membrane was immersed in 10 ml of the enzyme substrate solution (NBT/BCIP stock diluted 1:50 in detection buffer). Once the bands became visible, the colour reaction was stopped by rinsing the membrane with ddH_2O.

3.2.5 Complementation analysis

To prove that any altered phenotypes that may be displayed by the *P. aeruginosa* mutant strain was due to the disruption of the *htpD* gene, a complementation plasmid was constructed whereby the mutant strain could be complemented. The strategy used for construction of the pJB-IP-HtpD complementation plasmid is indicated diagrammatically in Fig. 3.2.

3.2.5.1 Construction of complementation plasmid pJB-IP-HtpD

The *lac* promoter, originating from pBluescript SKII (+), had been previously cloned into pGEM®-T Easy and designated pGEM-IP (Ms P. Medronho, unpublished results). Due to the cloning strategy to be used in this study and the lack of appropriate restriction enzyme cut sites flanking the insert DNA, it was not possible to recover the *lac* promoter DNA fragment from pGEM-IP by restriction endonuclease digestion. Consequently, the *lac* promoter was obtained by PCR amplification using pGEM-IP as template DNA together with oligonucleotides IP-F and IP-R (Table 3.1). Following PCR, the 500-bp amplicon was cloned into pGEM®-T Easy to generate pGEM-plac. The *lac* promotor DNA fragment was subsequently recovered by digestion with both *Kpn*I and *Xba*I and then cloned into identically prepared pUC-HtpD (Section 3.2.2.2) to yield plasmid pUC-IP-HtpD. Following digestion of pUC-IP-HtpD with both *Kpn*I and *Hind*III, the excised *P. aeruginosa*-specific *htpD* DNA

insert together with the upstream *lac* promoter was cloned into the broad-host-range pJB3Tc20 vector (Blatney *et al.*, 1997), which had been digested with the same restriction enzymes, to generate the complementation plasmid pJB-IP-HtpD.

3.2.5.2 Complementation of the PAOHtpD mutant strain

The complementation plasmid pJB-IP-HtpD plasmid was introduced into the *P. aeruginosa* PAOHtpD mutant strain by triparental mating, as described previously (Section 3.2.3), with the following modifications. Following streaking of the respective donor, helper and recipient strains on LB agar, the resultant mixed growth was streaked on LB agar supplemented with 50 µg/ml gentamicin (to select for the PAOHtpD mutant strain) and 100 µg/ml tetracycline (to select for the pJB-HtpD-IP plasmid and to counterselect against *E. coli* donor cells). The agar plates were incubated at 37°C overnight. A strain that displayed resistance to both gentamicin and tetracycline was consequently selected and used in subsequent investigations.

3.2.6 Batch assays of the *P. aeruginosa* PAOHtpD mutant strain

3.2.6.1 Determination of bacterial growth curves

The *P. aeruginosa* wild-type PAO1, mutant PAOHtpD and complemented mutant strains were cultured at 37°C overnight with shaking in 100 ml of LB broth supplemented with the appropriate antibiotics. The overnight cultures were diluted in fresh broth and incubated until mid-exponential phase was reached (OD_{540} = 0.5). The cultures were subsequently used to inoculate 200 ml of LB broth to an optical density of 0.05. The flasks were incubated at 37°C with shaking and the optical density at 540 nm of each culture was determined every 2 h for the first 12 h, and then at 16 h and 30 h using a Spectronic® 20 Genesys™ spectrophotometer (Spectronic Instruments). The growth curves were all performed in triplicate.

3.2.6.2 Biofilm formation

The ability of the *P. aeruginosa* wild-type PAO1, mutant PAOHtpD and complemented mutant strains to form biofilms was assayed using glass wool as an attachment substratum (Steyn *et al.*, 2001; Oosthuizen *et al.*, 2001). Preculture was performed in 100-ml Erlenmeyer flasks containing 10 ml of LB broth. The flasks were incubated at 37°C with shaking until mid-exponential phase was reached (OD_{540} = 0.5) and then used to inoculate 2 ml of LB broth

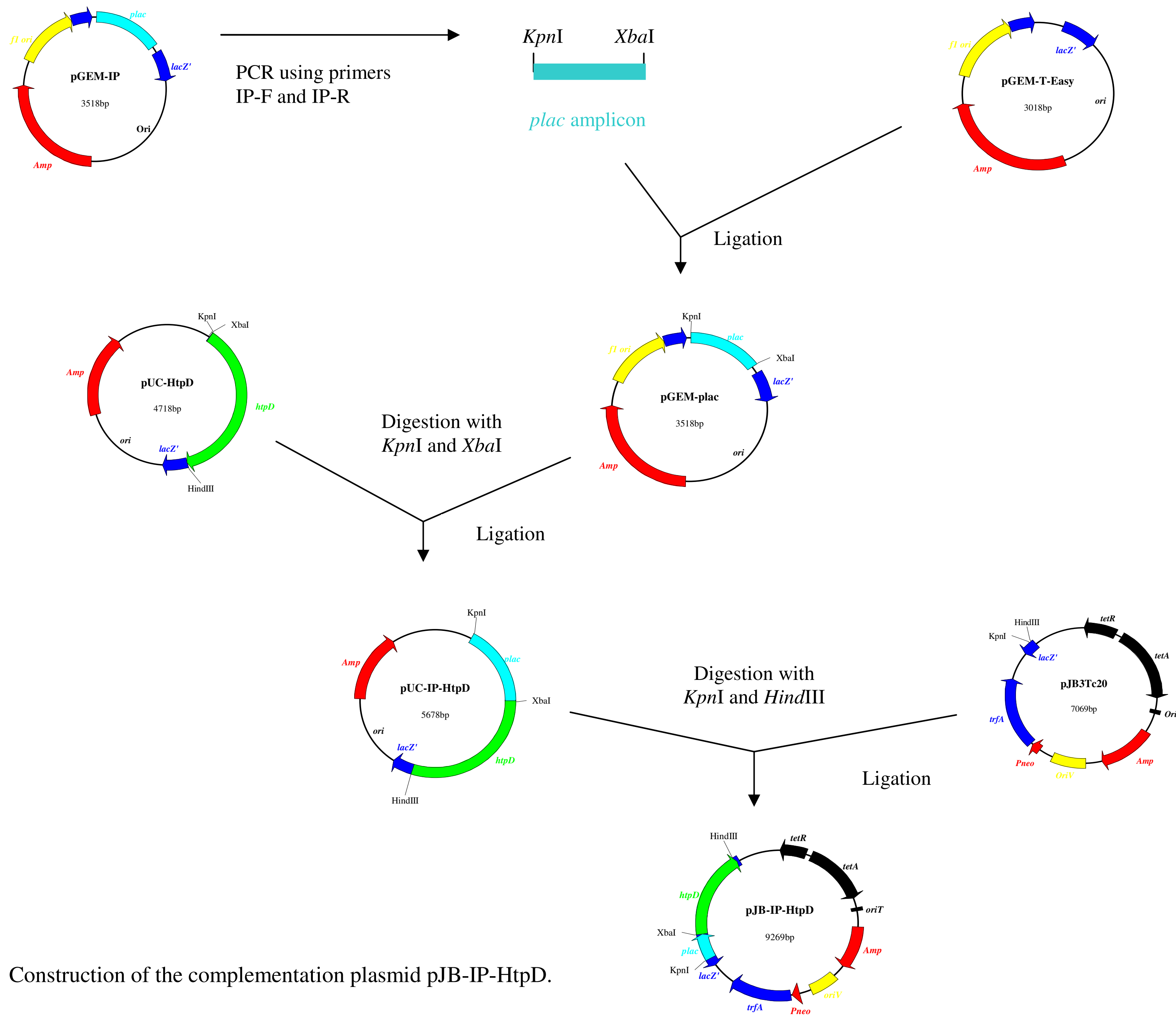


Fig. 3.2 Construction of the complementation plasmid pJB-IP-HtpD.

in 28-ml McCartney bottles, with and without 0.05 g glass wool (mean diameter 15 μm; Merck), to an optical density of 0.05. Biofilm formation was monitored on various samples of glass wool obtained at times 0 h, 2 h, 4 h, 8 h, 12 h, 16 h and 24 h after inoculation. The samples were transferred to clean microscope slides and subsequently stained with 0.01% (w/v) crystal violet prior to being viewed by bright-field microscopy using a Zeiss Axiovert 200 fluorescent microscope. Images were captured using a Nikon DXM 1200 digital camera. In addition, the propensity of the wild-type, mutant and complemented *P. aeruginosa* PAO1 strains to form biofilms on glass wool in LB broth was also investigated over a period of 24 h of culturing. Preculture was performed as above and then used to inoculate 25 ml of LB broth in 50-ml Erlenmeyer flasks, with 0.625 g glass wool, to an optical density of 0.05. For these assays, the culture medium of cultures grown in the presence of glass wool was aspirated, transferred to a new flask and referred to as biofilm-associated cells (BAC). Following careful rinsing of the glass wool with LB broth, the attached (biofilm) cells were then removed from the glass wool by adding 25 ml sterile LB broth and vortexing for 5 min. The supernatant was recovered and the removal of the biofilm cells from the glass wool substratum was verified by light microscopy. The optical density at 540 nm of both the attached (biofilm) and BAC populations was subsequently determined. These assays were performed in triplicate.

3.2.7 Transmission electron microscopy

For transmission electron microscopy, the bacterial cells of early exponential, mid-exponential and stationary phase cultures of the wild-type, mutant and complemented *P. aeruginosa* PAO1 strains were collected by centrifugation at 10 000 rpm for 3 min. The cells were then fixed for 1 h at room temperature in 0.075 M phosphate buffer (pH 7.4) containing 2.5% gluteraldehyde. The cell pellets were washed three times, 10 min each wash, in 0.075 M phosphate buffer (pH 7.4) and post-fixed in 1% osmium tetroxide for 1 h. The pellets were washed, as above, with ddH_2O before being dehydrated by sequential treatment for 10 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. For microscopic analysis, ultra-thin cell sections were collected on copper grids, stained for 10 min in 4% uranyl acetate, washed in ddH_2O and counter-stained for 2 min in Reynold's lead citrate. The samples were then viewed with a Phillips 301 transmission electron microscope at 36 kV.

3.3 RESULTS

3.3.1 Construction of allelic exchange vector pUCori-HtpD-Gent

The construction of mutations in bacteria, where a gene is replaced by an *in vitro*-modified or an *in vitro*-inactivated allele, followed by analysis of the resulting effects on the microorganism, is frequently used in microbiology research for the identification and study of gene function (Heilmann *et al.*, 1996; Loo *et al.*, 2000; Nika *et al.*, 2002). Allelic exchange methods, especially, represent a powerful approach to specifically mutate or inactivate a gene of interest. The classical method of allelic exchange relies on the use of so-called "suicide plasmids" that are unable to replicate in the bacterial strain under investigation (Goldberg and Ohman, 1987; Suh *et al.*, 1999; Finelli *et al.*, 2003). In such instances, a recombinant plasmid that contains a cloned copy of a disrupted chromosomal gene is introduced into a recipient strain. Since the plasmid cannot replicate, selection for some property on the plasmid results in isolates that have integrated the disrupted DNA fragment into the host chromosome via homology between the DNA fragment and the corresponding region of the recipient chromosome. Since such double crossover events are rare, it is not feasible to screen for such events if the cloned gene cannot be directly selected. However, by inactivating the cloned gene with a readily selectable marker, such as an appropriate antibiotic resistance gene, it is possible to directly screen for potential mutants based on their newly acquired resistance to the antibiotic. The gentamicin resistance cassette that was used in this study to disrupt the *htpD* gene of *P. aeruginosa* PAO1 was obtained from the genetic backbone of plasmid pCM351. The cassette consists of the gene *aacC1*, which encodes the enzyme 3-*N*-aminoglycoside acetyltransferase, and is flanked by transcriptional and translational stop signals (Marx and Lidstrom, 2002). By making use of the *aacC1* gene, it would thus be possible to rapidly and directly screen for *P. aeruginosa* PAO1 mutant strains based on their newly acquired resistance to gentamicin.

3.3.1.1 Construction of plasmids pGEM-Gent and pUC-HtpD

To obtain the gentamicin resistance cassette, PCR was performed by making use of oligonucleotides Gent-F (containing a *Pst*I site) and Gent-R (containing a *Xho*I site) and plasmid pCM351 as template DNA (Section 3.2.2). The *ca.* 840-bp amplicon (Fig. 3.3b, lane 2) was cloned into pGEM®-T Easy vector DNA and a recombinant plasmid from which an insert of the expected size was excised by digestion with both *Pst*I and *Xho*I was designated

pGEM-Gent. The integrity of the cloned insert DNA was verified by nucleotide sequencing prior to it being used in further DNA manipulations.

To obtain the *htpD* gene, oligonucleotide primers 4302-F (containing a *Xba*I site) and 4302-R (containing a *Hind*III site) were used in a PCR with chromosomal DNA of *P. aeruginosa* PAO1 (Section 3.2.2). The resultant amplicon of *ca.* 1.762 kb (Fig. 3.3b, lane 3), which corresponded in size to the full-length *htpD* (PA4302) gene, was subsequently cloned into pGEM®-T Easy vector DNA and restriction endonuclease digestion of the derived recombinant plasmid DNAs with *Xba*I and *Hind*III resulted in the excision of DNA fragments of the expected size. A recombinant clone, designated pGEM-HtpD, was selected and the integrity of the cloned insert DNA was verified by nucleotide sequencing of both terminal ends. To facilitate construction of the desired allelic exchange vector, the insert DNA was recovered from pGEM-HtpD by digestion with both *Xba*I and *Hind*III and cloned into similarly prepared pUC18 vector DNA, which is unable to replicate in *P. aeruginosa*, to generate pUC-HtpD.

3.3.1.2 Construction of pUCori-HtpD-Gent

Recombinant plasmids pGEM-Gent and pUC-HtpD served as sources for the construction of the allelic exchange vector pUCori-HtpD-Gent (Fig. 3.3a). Digestion of recombinant plasmid pUC-HtpD with *Pst*I and *Xho*I, which each cuts singularly in *htpD* and flank the Walker A and B motifs of HtpD, yielded two DNA fragments corresponding in size to 4.135 kb and 313 bp. The larger of the two DNA fragments was excised from the agarose gel and purified using a silica suspension. The full-length 840-bp gentamicin resistance cassette was recovered from plasmid pGEM-Gent by digestion with both *Pst*I and *Xho*I. The gel-purified DNA fragments were subsequently ligated and following transformation of competent *E. coli* DH5α cells, plasmid DNA from gentamicin-resistant transformants were characterized by agarose gel electrophoresis and by restriction enzyme digestion. Digestion of the recombinant plasmid DNA with *Pst*I and *Xho*I excised a DNA fragment of 840 bp (Fig. 3.3b, lane 6). These results indicated that the gentamicin resistance cassette had been cloned successfully. One of the recombinant clones was selected for further use and designated pUC-HtpD-Gent.

To facilitate the conjugative transfer of the allelic exchange vector from *E. coli* to *P. aeruginosa*, an *oriT*-containing DNA fragment was cloned onto the pUC-HtpD-Gent vector

backbone. Consequently, the recombinant vector was linearized by digestion with *Bam*HI and the *oriT* was cloned from plasmid pSS125 as a 230-bp *Bam*HI DNA fragment. The plasmid DNA from randomly selected gentamicin-resistant transformants was screened by restriction endonuclease digestion. Digestion of the recombinant plasmid DNA containing the cloned *oriT* with *Bam*HI yielded DNA fragments corresponding to 4.975 and 0.230 kb (Fig. 3.3b, lane 7). In contrast, recombinant plasmid DNA lacking the cloned *oriT* yielded a single DNA fragment 4.975 kb, corresponding in size to the recombinant vector backbone and the cloned gentamicin resistance cassette (Fig. 3.3b, lane 8). These results therefore confirmed that the *oriT* was successfully cloned. The allelic exchange vector pUCori-HtpD-Gent was subsequently used in the engineering of *P. aeruginosa* PAO1 mutant strains.

3.3.2 Engineering of an HtpD-deficient *P. aeruginosa* PAO1 strain

3.3.2.1 Generation of mutant strains

Mutant strains of the wild-type *P. aeruginosa* PAO1 strain were generated by introducing the allelic exchange vector pUCori-HtpD-Gent into the wild-type PAO1 strain by triparental mating, and selecting for subsequent homologous recombination events between the *P. aeruginosa* DNA flanking the gentamicin resistance cassette in the vector and the wild-type locus on the genome. Recipient *P. aeruginosa* strains harbouring an integrated copy of the *htpD*::*Gm*^{r} null allele were selected by plating onto selective medium, as described under Materials and Methods (Section 3.2.3). A gentamicin-resistant *P. aeruginosa* strain was selected and designated PAOHtpD.

3.3.2.2 Southern blot analysis of *P. aeruginosa* mutant strain PAOHtpD

To determine whether the gentamicin resistance cassette was present in the genome of the mutant PAOHtpD strain, Southern blot analysis was performed. The genomic DNA of strain PAOHtpD was isolated, digested with *Asc*I 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 - labeled DNA probe specific for the gentamicin gene. In this analysis, recombinant plasmids pGEM-Gent and pUC-HtpD-Gent, digested with the appropriate enzymes to excise the cloned gentamicin-containing DNA fragments, were included as positive controls, while *Asc*I-digested genomic DNA of the wild-type *P. aeruginosa* PAO1 strain was included as a negative hybridization control.

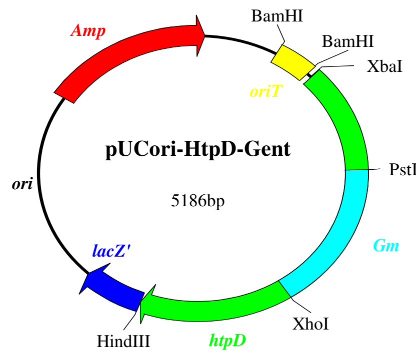


Fig. 3.3a Plasmid map of the recombinant allelic exchange vector pUCori-HtpD-Gent.

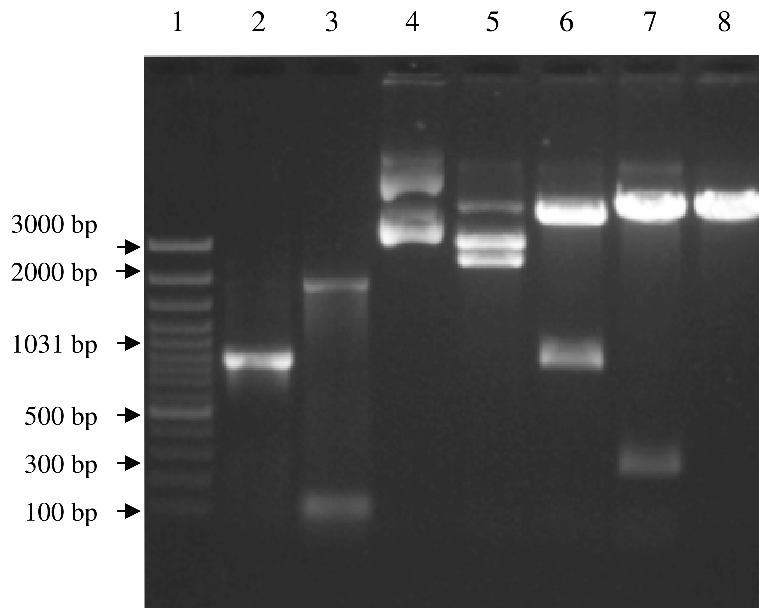


Fig. 3.3b Agarose gel electrophoretic analysis of the recombinant plasmid pUCori-HtpD-Gent. Lane 1, DNA molecular weight marker; lane 2, sample of the reaction mixture following PCR using pCM351 plasmid DNA as template and primers Gent-F and Gent-R; lane 3, sample of the reaction mixture following PCR using *P. aeruginosa* chromosomal DNA as template and primers 4302-F and 4302-R; lane 4, uncut recombinant allelic exchange vector pUCori-HtpD-Gent; lane 5, plasmid pUCori-HtpD-Gent digested with *Xba*I and *Hind*III; lane 6, recombinant allelic exchange vector pUCori-HtpD-Gent digested with *Xho*I and *Pst*I; lane 7, recombinant allelic exchange vector pUCori-HtpD-Gent digested with *Bam*HI; lane 8, plasmid pUC-HtpD-Gent digested with *Bam*HI. The sizes of the DNA molecular weight marker, GeneRuler^{TM} DNA Ladder Plus (Fermentas), are indicated to the left of the figure.

The results indicated that the probe specific for the gentamicin gene (Fig. 3.4) hybridized with the gentamicin resistance cassettes excised from plasmids pGEM-Gent and pUC-HtpD-Gent, as well as with a DNA restriction fragment from the PAOHtpD chromosomal DNA. The probe, however, did not hybridize with the *Asc*I-digested chromosomal DNA of the *P. aeruginosa* PAO1 strain. From these results it was thus concluded that a single copy of the mutant *htpD*::Gm^r allele was integrated into the chromosomal DNA of PAOHtpD.

3.3.2.3 PCR analysis of *P. aeruginosa* mutant strain PAOHtpD

To determine whether integration of the mutant allele in the PAOHtpD strain occurred by means of a single or double crossover event, PCR analyses was performed using different pairs of oligonucleotide primers (Fig. 3.5a). The amplified hybrid products occurred only if the gentamicin resistance cassette was located within the chromosomal-borne inactivated *htpD* gene. Moreover, oligonucleotides were also used that annealed to genomic sequences flanking the region in which the mutant allele was integrated.

Primers 4302-SF and Gent-F, as well as 4302-SR and Gent-R, were used to amplify a 1.673-kb and a 1.456-kb hybrid product, respectively, if the gentamicin resistance cassette was located within the disrupted *htpD* gene. The respective products were produced when PAOHtpD chromosomal DNA was used as template (Fig. 3.5b, lanes 6 and 7, respectively). As expected, when wild-type *P. aeruginosa* PAO1 chromosomal DNA was used as template in the PCR reactions no products were amplified using these oligonucleotide pairs (Fig. 3.5b, lanes 3 and 4, respectively). In the final analysis, primers were used that annealed to genomic sequences upstream and downstream of the *htpD* gene in which the mutant allele was integrated. Thus, primers 4302-SF and 4302-SR were used to amplify either a 2.315-kb product in the absence of the gentamicin resistance cassette, or a 2.842-kb product in the presence of the 840-kb gentamicin resistance cassette. As expected, a 2.842-kb product was produced when PAOHtpD chromosomal DNA was used as template (Fig. 3.5b, lane 5). Template DNA from wild-type PAO1 generated the 2.315-kb product, indicative of the absence of the gentamicin cassette within the *htpD* gene (Fig. 3.5b, lane 2). These results therefore confirmed that a single copy of the mutant *htpD*::Gm^r allele was integrated into the chromosomal DNA of PAOHtpD and that it occurred by means of a double crossover event.

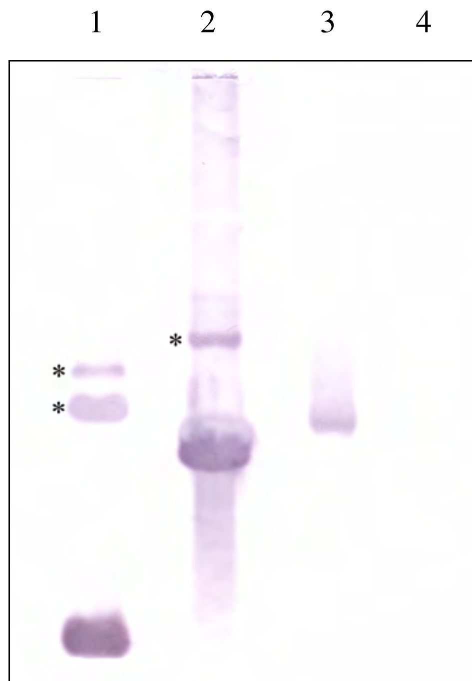


Fig. 3.4 Southern blot analysis of genomic DNA extracted from the *P. aeruginosa* mutant strain PAOHtpD, containing an insertionally inactivated *htpD* gene. Genomic DNA extracted from the mutant PAOHtpD (lane 3) and wild-type PAO1 (lane 4) strains were digested with *Asc*I, resolved by agarose electrophoresis and transferred to a nylon membrane. Recombinant plasmid pGEM-Gent, digested with *Pst*I and *Xho*I (lane 1), and pUC-HtpD-Gent, digested with *Hind*III and *Xba*I (lane 2) were included as positive hybridization controls. The membrane was probed with a DIG-labeled gentamicin resistance cassette. The asterisks (*) indicate hybridization of the probe to uncut and partially digested recombinant plasmid DNA.

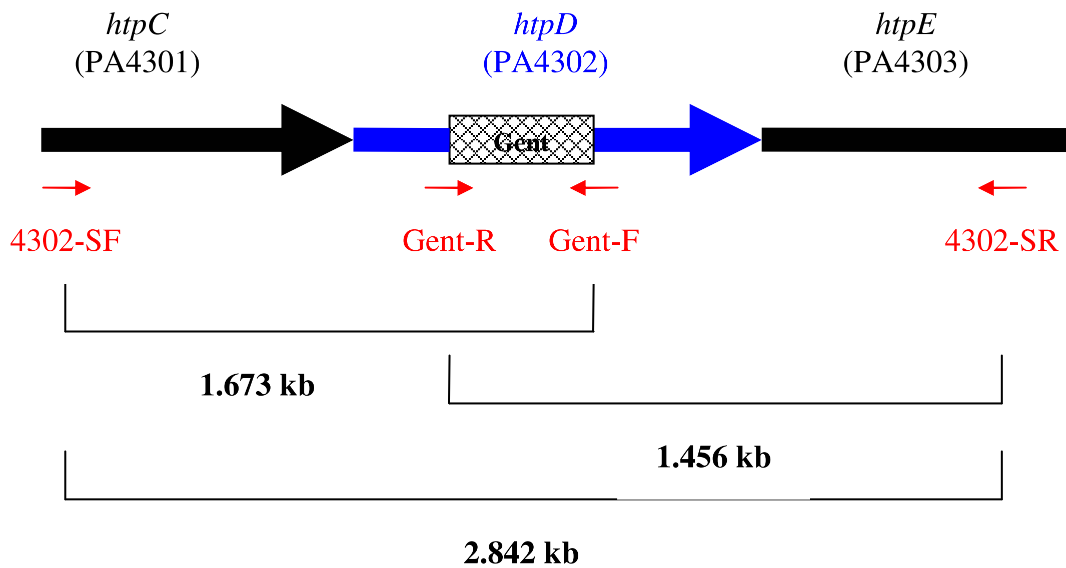


Fig. 3.5a Schematic presentation of specific primer annealing positions and direction of amplification in the mutant PAOHtpD strain. The sizes of the different amplicons, as expected for a double crossover event, are indicated by brackets.

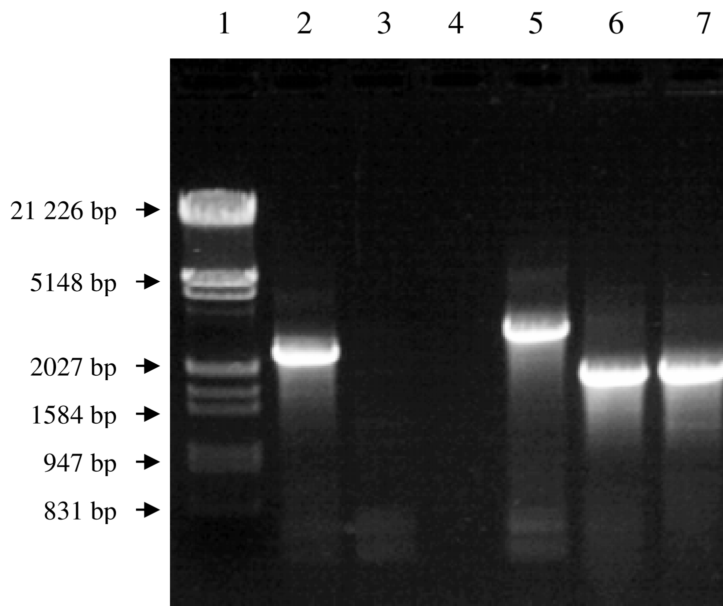


Fig. 3.5b Agarose gel electrophoretic analysis of the amplification products obtained, following PCR analysis of *P. aeruginosa* PAO1 and PAOHtpD using primers 4302-SF and 4302-SR (lanes 2 and 5), 4302-SF and Gent-F (lanes 3 and 6), and 4302-SR and Gent-R (lanes 4 and 7). Lanes 2, 3 and 4 represent genomic DNA from wild-type *P. aeruginosa* PAO1, while lanes 5, 6 and 7 represent genomic DNA from mutant strain PAOHtpD. The sizes of the molecular weight marker, phage λ digested with *Hind*III, are indicated to the left of the figure.

3.3.3 Construction of complementation plasmid pJB-IP-HtpD

To investigate whether any altered phenotypes that may be displayed by the *P. aeruginosa htpD* mutant strain was due to the disruption of the wild-type *htpD* gene, a complementation plasmid, containing an intact copy of the *htpD* gene under transcriptional control of a *lac* promoter, was constructed whereby the mutant strain could be complemented. Since complementation studies would require that the plasmid DNA is capable of replicating in *P. aeruginosa*, the broad-host-range plasmid pJB3Tc20 was selected for construction of the complementation plasmid. This plasmid contains both a vegetative origin of replication (*oriV*) that permits replication in wide variety of Gram-negative bacteria, including *E. coli* and *P. aeruginosa*, and an *oriT* that permits introduction of recombinant DNA into *P. aeruginosa* by triparental mating procedures. In addition, the plasmid also harbours tetracycline and ampicillin resistance markers to allow for plasmid selection and maintenance in these bacterial hosts (Blatney *et al.*, 1997).

The *lac* promoter was obtained by PCR amplification using oligonucleotides IP-F (containing a *Kpn*I site) and IP-R (containing a *Xba*I site) and recombinant pGEM-IP plasmid DNA as template, which contains a cloned copy of the *lac* promoter from plasmid pBluescript SKII (+). Following PCR, the 500-bp amplicon was cloned into pGEM®-T Easy vector DNA to generate pGEM-plac and the integrity of the cloned insert DNA was verified by nucleotide sequencing. The insert DNA was subsequently recovered by digestion with both *Kpn*I and *Xba*I and then cloned into pUC-HtpD, which had been prepared identically, to generate pUC-IP-HtpD, in which the *lac* promoter was cloned in the correct transcriptional orientation relative to the *htpD* gene. To complete construction of the complementation plasmid, plasmid pUC-IP-HtpD was digested with *Kpn*I, which cuts at the 5'-end of the *lac* promoter, and *Hind*III, which cuts at the 3'-end of the cloned *htpD* gene. The DNA fragment was purified from the agarose gel and cloned into pJB3Tc20, which had been digested with both *Kpn*I and *Hind*III. The derived plasmid DNA, designated pJB-IP-HtpD (Fig. 3.6a), was subsequently characterized by restriction endonuclease digestion.

To verify the presence of the *lac-htpD* insert DNA, plasmid pJB-IP-HtpD was digested with both *Kpn*I and *Hind*III and yielded expected bands corresponding to *ca.* 7.069 kb and 2.262 kb (Fig. 3.6b, lane 5). The recombinant plasmid pJB-IP-HtpD was furthermore characterized by digestion with *Xba*I and *Kpn*I or *Hind*III. Whereas digestion of the recombinant plasmid

with *Xba*I and *Kpn*I yielded two DNA fragments corresponding to the size of the pJB-HtpD vector DNA (8.831 kb) and the cloned *lac* promoter (500 bp) (Fig. 3.6b, lane 6), digestion with *Xba*I and *Hind*III resulted in the excision of a 1.762-kb DNA fragment corresponding in size to the *htpD* gene (Fig. 3.6b, lane 7), thus confirming successful cloning of the *lac-htpD* DNA fragment. The complementation plasmid pJB-IP-HtpD plasmid was introduced into the constructed *P. aeruginosa* PAOHtpD mutant strain by triparental mating, as described under Materials and Methods (Section 3.2.5.2), and a strain that displayed resistance to both gentamicin and tetracycline was consequently selected for further use.

3.3.4 Characterization of the PAOHtpD mutant strain

3.3.4.1 Growth curves

Since it has been noted previously that insertion mutagenesis may influence the growth properties of the mutant strain (Kadurugamuwa *et al.*, 1993; Hoang *et al.*, 2000), 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 mutation influenced the growth properties of the mutant strain, the wild-type *P. aeruginosa* PAO1 and mutant PAOHtpD 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 24 h. The results obtained (Fig. 3.7) indicated that the PAOHtpD strain displayed a growth very similar to the wild-type PAO1 strain. In contrast, the mutant PAOHtpD strain complemented with a wild-type copy of the *htpD* gene on pJB-IP-HtpD *in trans* displayed impaired growth (Fig. 3.7). However, the impaired growth of this strain may have been due to the selective pressure exerted by culturing of the strain in LB broth containing both gentamicin and tetracycline antibiotics.

3.3.4.2 Biofilm development on glass wool

To determine whether the mutant PAOHtpD strain was able to attach and grow as a biofilm, an exponentially growing culture of the strain was inoculated into LB broth in McCartney bottles containing glass wool as attachment substratum. Whereas the mutant strain was cultured in the presence of gentamicin, the wild-type *P. aeruginosa* PAO1, included as a control in the analyses, was cultured in the absence of gentamicin. Biofilm development on glass wool was subsequently monitored at various time intervals by bright-field microscopy after staining of the glass wool with crystal violet. The results are presented in Fig. 3.8.

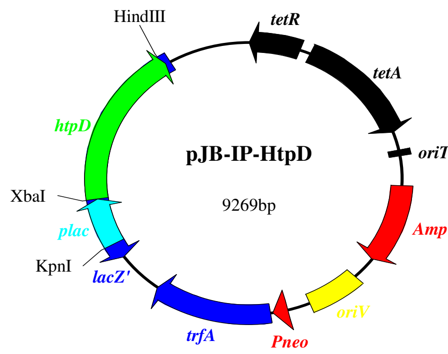


Fig. 3.6a Plasmid map of the complementation plasmid pJB-IP-HtpD.

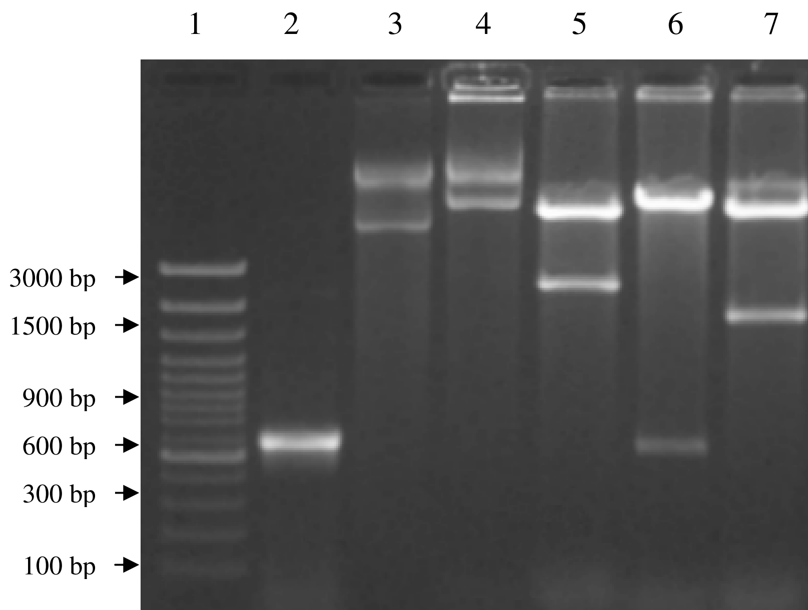


Fig. 3.6b Agarose gel electrophoretic analysis of the complementation plasmid pJB-IP-HtpD. Lane 1, DNA molecular weight marker; lane 2, sample of the reaction mixture following PCR using pGEM-IP plasmid DNA as template and primers IP-F and IP-R; lane 3, uncut plasmid pJB3Tc20; lane 4, uncut complementation plasmid pJB-IP-HtpD; lane 5, complementation plasmid pJB-IP-HtpD digested with *Kpn*I and *Hind*III; lane 6, complementation plasmid pJB-IP-HtpD digested with *Xba*I and *Kpn*I; lane 7, complementation plasmid pJB-IP-HtpD digested with *Xba*I and *Hind*III**.** The sizes of the DNA molecular weight marker, GeneRuler™ DNA Ladder Plus (Fermentas), are indicated to the left of the figure.

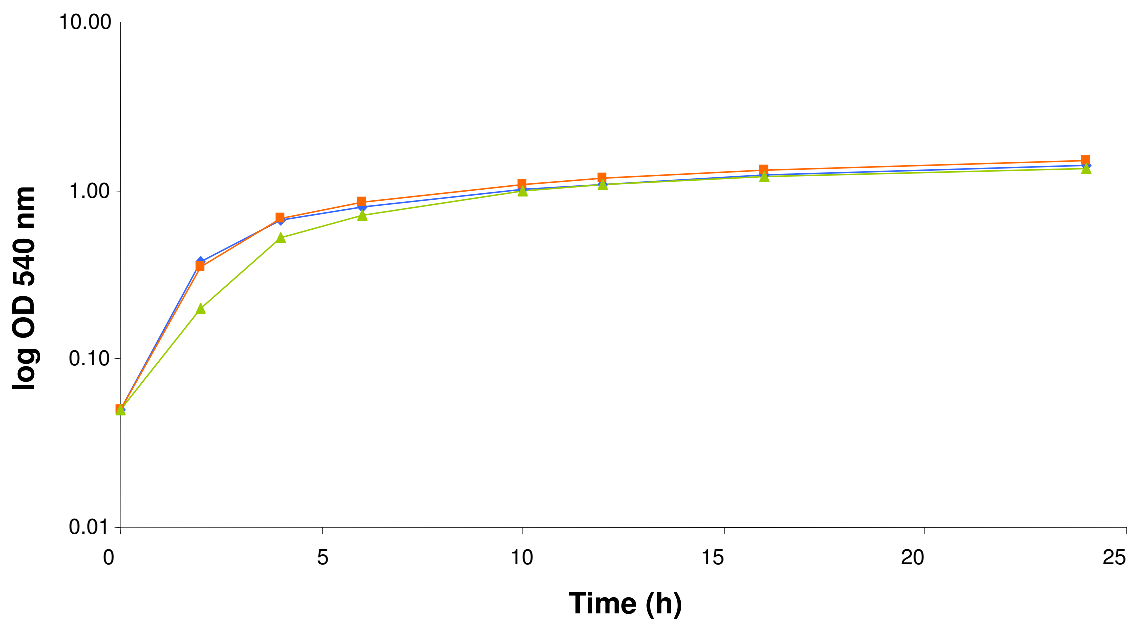
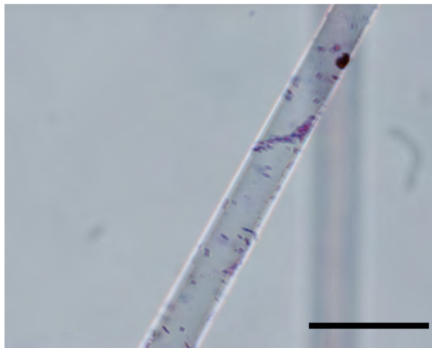
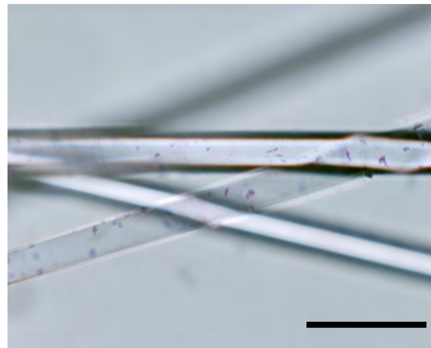
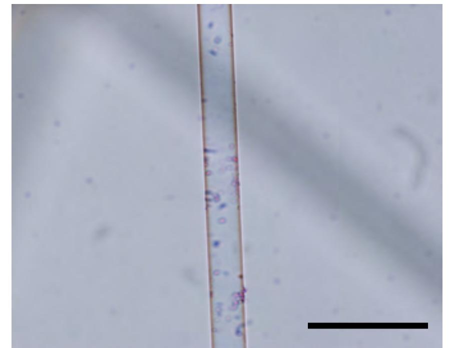
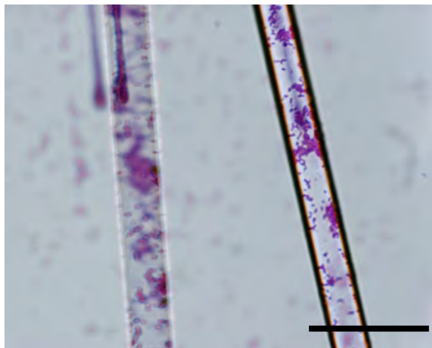
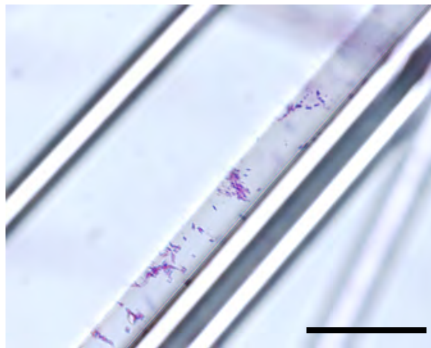
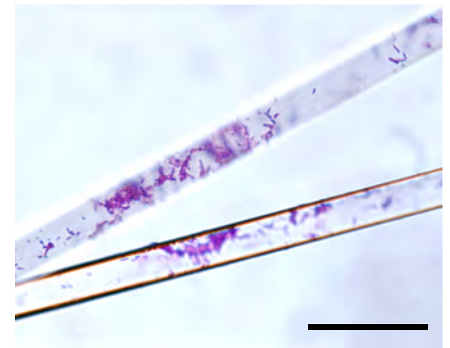
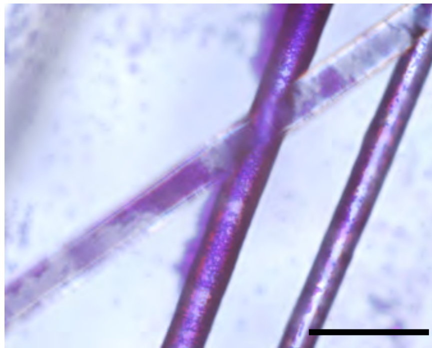
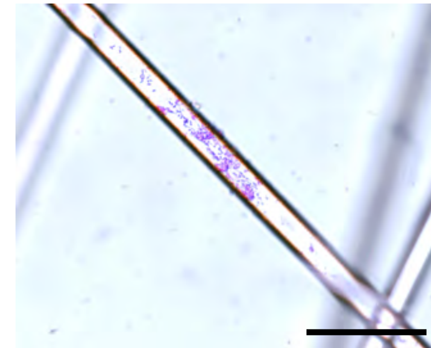
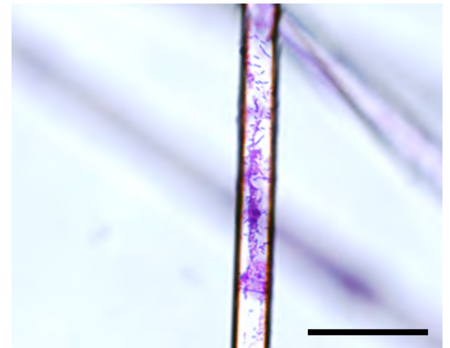
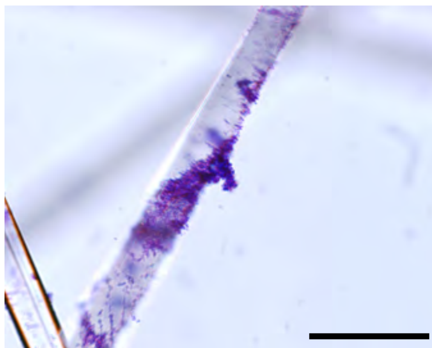
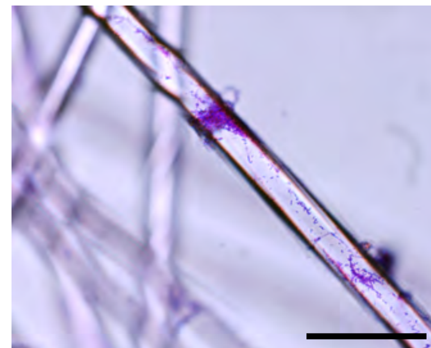
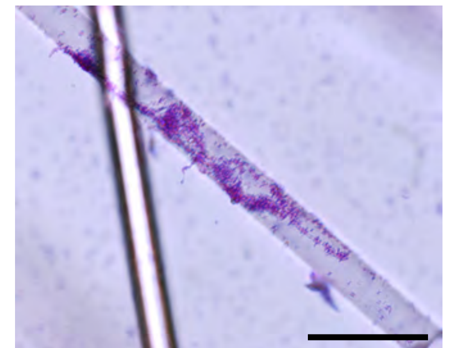


Fig. 3.7 Growth curves wild-type *P. aeruginosa* PAO1 (♦), mutant PAOHtpD (■) and PAOHtpD containing plasmid construct pJB-IP-HtpD (∆) in LB broth.

Although few cells of both the wild-type PAO1 and mutant PAOHtpD strains were visible on the glass wool 2 h after inoculation, more cells became visible on the glass wool after 4 h and microcolonies were visible. In contrast to the wild-type PAO1 strain, which showed a uniform colonization of the glass wool surface punctuated with dense, thick multi-layered structures from 8 h onwards, the mutant PAOHtpD strain showed sparse colonization of the glass wool surface and the biofilm structures had the appearance of cell clumps. Nevertheless, biofilm structures formed within 16 h and these became denser after 24 h of incubation, albeit that the PAOHtpD biofilm structures were not as dense or as well defined as the biofilm structures of the wild-type PAO1 strain. Complementation of the mutant PAOHtpD strain with plasmid pJB-IP-HtpD *in trans* did not result in restoration of the mutant biofilm phenotypes to wild-type levels, but rather resembled the biofilms formed by the mutant PAOHtpD strain (Fig. 3.8).

To further investigate the propensity of the PAOHtpD mutant cells to attach and grow as biofilms, the ratio of attached (biofilm) to biofilm-associated (BAC) biomass was calculated at various time intervals of culturing from cultures grown in LB broth with glass wool. Whereas the BAC cells were obtained by careful aspiration of the culture fluid, the attached (biofilm) cells were recovered from the glass wool substratum by vortexing. The results indicated that cells of both the wild-type PAO1 and mutant PAOHtpD strains were capable of attaching to the glass wool substratum after 2 h of culturing, albeit that cells of the mutant strain was less efficient in attaching to the glass wool substratum. This was evidenced by a lower ratio of biofilm to BAC biomass (Fig. 3.9a). Following attachment, cells of the PAOHtpD strain also appeared to be less prone to occur in a biofilm when compared to the wild-type PAO1 cells. After an initial decrease in the ratio of biofilm to BAC biomass of the PAOHtpD strain the ratio increased over time, but was lower than that of the wild-type PAO1 strain. Notably, after 12 h the ratio of biofilm to BAC biomass of the wild-type PAO1 cells, but not mutant PAOHtpD cells, declined sharply and may be representative of cells actively detaching from the biofilm. From the results obtained it was therefore concluded that the PAOHtpD strain was impaired in both its ability to attach to the glass wool surface and in its ability to grow as a biofilm. Moreover, comparative analyses of growth curves obtained for the biofilm and biofilm-associated (BAC) populations of the wild-type PAO1 and mutant PAOHtpD strains indicated that these deficiencies were not due to a general growth impairment of the PAO1HtpD strain, since the mutant strain displayed exponential growth in both the biofilm and BAC growth phases (Fig. 3.9b).

PAO1	PAOHtpD	Complemented PAOHtpD	
			2 h
			4 h
			8 h
			12 h

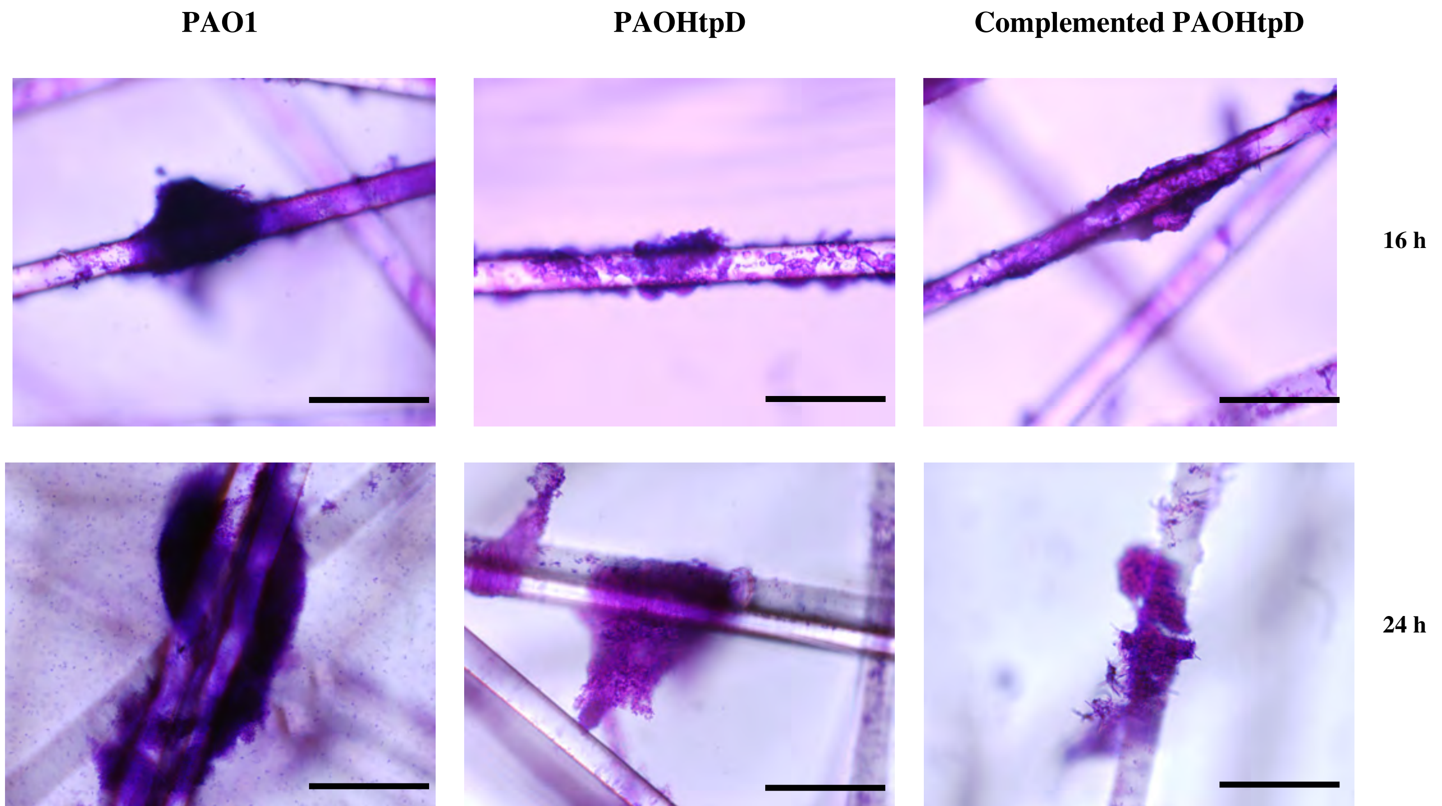
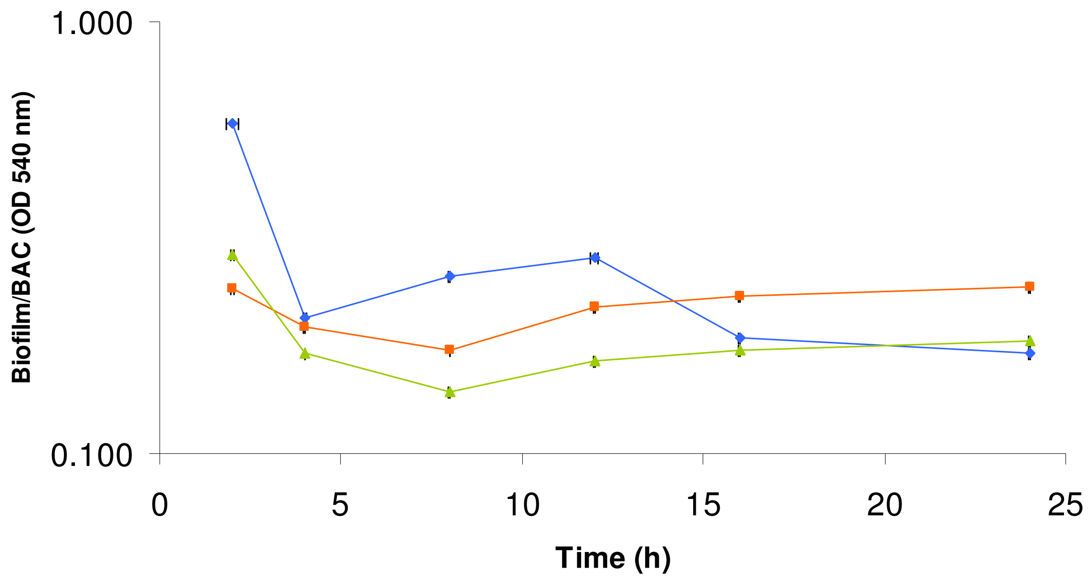


Fig. 3.8 Photomicrographs showing biofilm development of *P. aeruginosa* wild-type and mutant strains on glass wool over time. Two milliliters of LB broth, containing 0.05 g of glass wool, was inoculated with either the *P. aeruginosa* wild-type PAO1 and mutant PAOHtpD strain. The PAOHtpD strain containing plasmid construct pJB-IP-HtpD is also shown for comparative purposes. Bright-field microscopy was performed on samples of glass wool following staining with 0.01% (w/v) crystal violet at times 2 h, 4 h, 8 h, 12 h, 16 h and 24 h after inoculation, as indicated to the right of the figure. The size bars in all of the figures represents 50 µm.

a)



b)

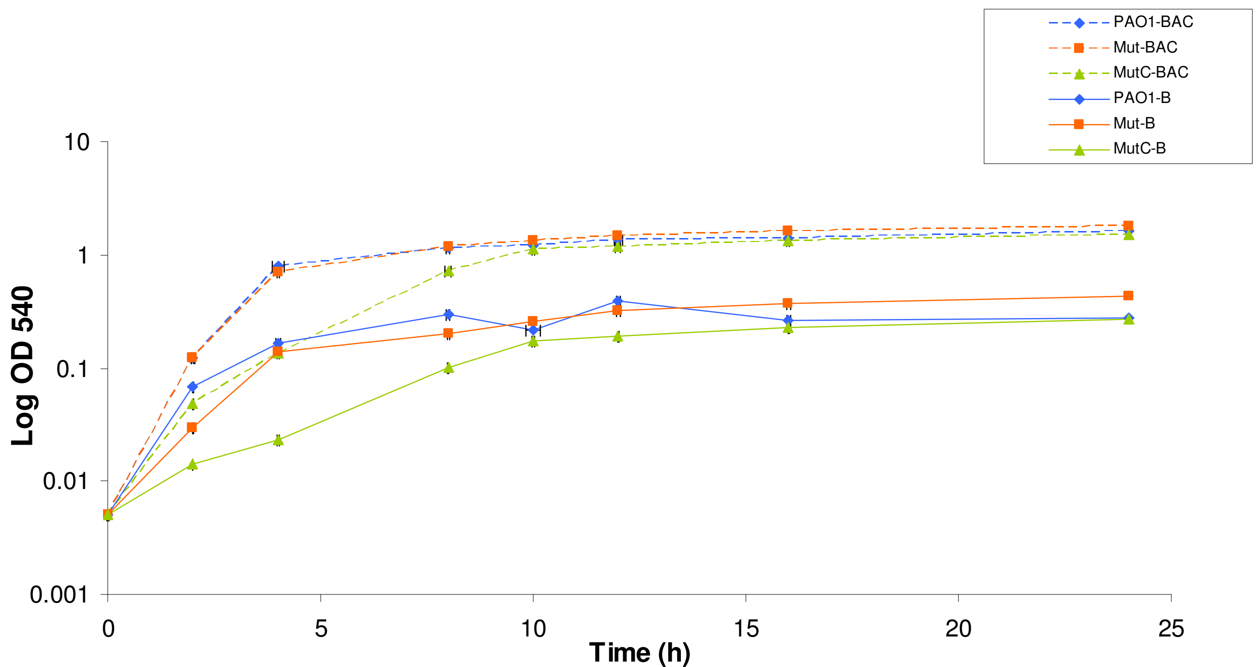


Fig. 3.9 Propensity of PAOHtpD cells to occur as a biofilm. (a) The ratio of attached (biofilm) to biofilm associated (BAC) cells grown in LB broth in the presence of glass wool is indicated for wild-type *P. aeruginosa* PAO1 (♦), mutant PAOHtpD (■) and complemented mutant strain (Δ), as measured by optical density. (b) Optical density at 540 nm of biofilm associated (BAC) and attached (B) populations of the wild-type *P. aeruginosa* PAO1 and mutant PAOHtpD strains. The PAOHtpD strain containing plasmid construct pJB-IP-HtpD is indicated for comparative purposes. Error bars denote standard error of the mean.

3.3.4.3 Electron microscopic characterization of cellular morphology

During bright-field microscopy analyses regarding the ability of the *P. aeruginosa* wild-type and mutant strains to form biofilms on glass wool, it was noted that the PAOHtpD cells displayed an altered cellular morphology and appeared to be longer than the cells from the wild-type PAO1 strain. To investigate, cells from early exponential, mid-exponential and stationary phase cultures of the *P. aeruginosa* wild-type and mutant strains, as well as complemented mutant strain, were processed for transmission electron microscopy and then viewed with a Phillips 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 PAOHtpD strain differed notably from those of the wild-type PAO1 strain. The results, presented in Fig. 3.10, revealed distinct morphological differences between the cells of strain PAOHtpD and those of the wild-type PAO1 strain. The cells of the wild-type PAO1 strain were short straight rods similar in size to each other (1 µm in length and 0.5 µm in width). In contrast, cells from the mutant PAOHtpD culture consisted mostly of cells that were typically at least twice the length of the wild-type PAO1 cells, despite displaying a similar width (0.5 µm). The length of these rod-shaped cells varied between 1.5 to 2.3 µm. Complementation of the mutant PAOHtpD strain with the recombinant plasmid pJB-IP-HtpD did not result in restoration of the cellular morphology to the wild-type phenotype, as evidenced by cells of the complemented strain being longer than those of the wild-type PAO1 strain (Fig. 3.10).

3.4 DISCUSSION

A cluster of nine genes, designated *htpA-I*, has been identified previously in *P. aeruginosa* that encodes products with homology to proteins involved in the biogenesis or secretion of novel pili in, amongst other, *A. actinomycetemcomitans*, *H. ducreyi* and *C. crescentus* (Van Schalkwyk, 2003). Inactivation of the genes encoding either the pilus monomer (*flp*) or the assembly/secretion proteins (*tadA-G*) has been reported to reduce the ability of *A. actinomycetemcomitans* (Haase *et al.*, 1999; Kachlany *et al.*, 2000; 2001) and *H. ducreyi* (Nika *et al.*, 2002) to bind to biotic and abiotic surfaces. Moreover, it has been suggested that the ATPase activity of TadA is required to energize the assembly or secretion of Flp pili for

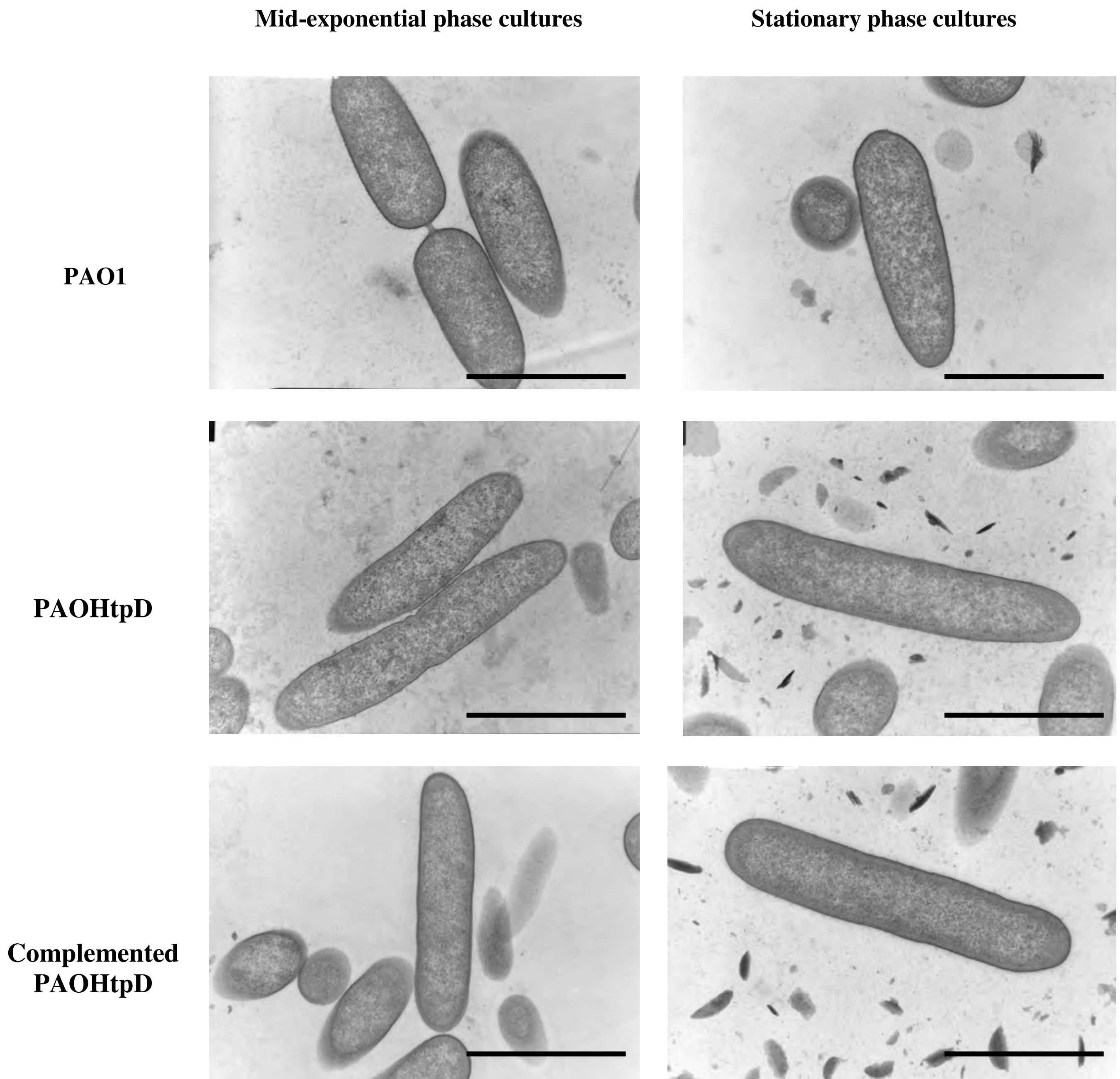


Fig. 3.10 Representative electron micrographs of thin sections through resin-embedded cells observed by transmission electron microscopy (TEM). The *P. aeruginosa* wild-type PAO1 and mutant PAOHtpD strains were cultured to mid-exponential phase and to stationary phase in LB broth prior to preparation for electron microscopic analysis. Electron micrographs of the PAOHtpD strain containing plasmid construct pJB-IP-HtpD are shown for comparative purposes. The size bar in each transmission electron micrograph represents 1 µm.

tight adherence (Bhattacharjee *et al.*, 2001). Consequently, in this study, the *htpD* gene, a putative energizing protein of the *P. aeruginosa* biogenesis/secretion system, was specifically targeted for mutagenesis in an effort to determine whether inactivation of the *P. aeruginosa* PAO1 *htp* gene cluster may result in a similar attachment-deficient phenotype.

A frequently used approach whereby the role of proteins in biofilm formation has been determined relies on the use of isogenic mutant strains from which specific functions have been eliminated (Heilmann *et al.*, 1996; O'Toole and Kolter, 1998a; Pratt and Kolter, 1998; Loo *et al.*, 2000). To generate such loss-of-function mutations, both random transposon insertion mutagenesis (O'Toole and Kolter, 1998a; Kachlany *et al.*, 2000; Hayes, 2003; Caiazza and O'Toole, 2004) and allelic exchange methods (Toder, 1994; Suh *et al.*, 1999; Dasgupta *et al.*, 2000; Finelli *et al.*, 2003) have been useful. Since transposons are capable of catalyzing their own movement to alternate sites within a chromosome, they are not ideally suited for constructing mutant strains with specific targeted mutations. In contrast, allelic exchange involves using plasmids that are conditional for their replication in the studied strain ("suicide plasmids") to deliver an *in vitro*-inactivated or an *in vitro*-modified allele of a gene of interest in the chromosome. Mutations made by allelic exchange are thus targeted, making it a more attractive method of mutagenesis than random transposon insertion mutagenesis (Toder, 1994). Towards determining the importance of the *htp* gene cluster and Flp pili in *P. aeruginosa* PAO1 biofilm development, allelic exchange was therefore preferred in this study for constructing a mutant strain. Consequently, an allelic exchange vector harbouring a cloned copy of the *htpD* gene that had been inactivated through insertion of a gentamicin resistance cassette was constructed and introduced into *P. aeruginosa* PAO1, after which presumptive mutant strains were characterized by Southern blot hybridization and by PCR analyses. The results obtained from these analyses indicated that insertional inactivation of the *htpD* gene in the mutant PAOHtpD strain occurred by means of a double crossover event and consequently resulted in the integration of only the mutant *htpD*::Gm^r allele. Note should be taken that polar mutations resulting from inactivation of the *htpD* gene would have no effect on the outcome tested in this study, *i.e.* determining the importance of the *htp* gene cluster in *P. aeruginosa* PAO1 biofilm formation by making use of a mutant strain that lacks a functional *htp* system.

To determine whether inactivation of the *htp* gene cluster of *P. aeruginosa* PAO1 influenced its ability to form and grow as biofilms, the mutant *P. aeruginosa* PAOHtpD strain was tested

for its ability to bind to an abiotic surface using glass wool as an attachment substratum. Although the PAOHtpD mutant strain was capable of forming biofilms on glass wool within 16 h of culturing, inspection of the biofilm phenotype revealed that in contrast to wild-type *P. aeruginosa* PAO1, the mutant PAOHtpD strain displayed sparser colonization of the glass wool surface and the cells were localized in clusters. More detailed analysis of the defects conferred by inactivation of the *htpD* gene was obtained through determining the ratio of attached (biofilm) to biofilm-associated cell (BAC) biomass over a period of 24 h of culturing. Compared to the wild-type PAO1 strain, cells of the PAOHtpD strain not only attached less efficiently to the glass wool after 2 h, but the ratio of biofilm to BAC cells also dropped until 8 h after inoculation. Similar to results reported by Rice *et al*. (2000), these results may indicate that a greater proportion of the attached PAOHtpD cells detached following the first and second division events compared to the wild-type PAO1 strain. The ratio of PAOHtpD biofilm to BAC biomass subsequently increased over time, albeit lower than the wild-type strain, indicating bacterial growth on the surface of the glass wool. However, after 12 h the ratio of biofilm to BAC biomass of the wild-type PAO1 strain, but not the mutant PAOHtpD strain, declined sharply. Although it was beyond the scope of this study to determine the factors responsible for affecting the ratio of biofilm to biofilm-associated cells (BAC), it may be that starvation could have resulted in increased detachment of the wild-type PAO1 biofilm cells to allow bacteria to search for nutrient-rich environments (O'Toole *et al.*, 2000a; Sauer *et al.*, 2004). The biofilm formed by PAOHtpD was less dense than that of the wild-type PAO1 strain and also had the appearance of cell clumps. Therefore, it may have been that the environmental conditions were more favourable such that rather than actively detaching from the biofilm, an equilibrium was reached between cells leaving and joining the biofilm (Korber *et al.*, 1989). Cumulatively, the obtained results indicate that the mutant PAOHtpD strain is both attachment- and biofilm-impaired. Since these abilities were impaired, but not abrogated, it can be proposed that the ability of the mutant PAOHtpD strain to interact with the glass wool surface may be associated with 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 Flp.

Although *A. actinomycetemcomitans* uses its *flp-tad* gene cluster to synthesize proteins that form fibrils, no similar structures could be detected on the surface of the wild-type *P. aeruginosa* PAO1 or mutant PAOHtpD cells through transmission electron microscopy (TEM). These results are in agreement with those reported for *H. ducreyi* (Nika *et al.*, 2002).

The inability to detect fibrils on the cells may be due to the fibrillar structure encoded by the *flp-htp* gene cluster in *P. aeruginosa* PAO1 being smaller than that described for *A. actinomycetemcomitans* so that it was not detectable by the TEM methods utilized in this study. Moreover, the number of fibrils formed by *P. aeruginosa* PAO1 may be very small, thus making detection of these structures more difficult. It is also possible that any *flp*-encoded fibrils formed by *P. aeruginosa* PAO1 may be so fragile that they were destroyed during sample preparation. This is supported by the observation that flagella were also not visible on cells from both the wild-type and mutant strains. However, despite the apparent lack of detectable surface structures, a phenotype similar to that of *A. actinomycetemcomitans* and *H. ducreyi tadA* and *flp* mutants was obtained; that is, the *P. aeruginosa* PAO1 became less adherent to an abiotic surface.

In addition to the defects caused in the propensity of *P. aeruginosa* PAO1 to attach to glass wool and grow as a biofilm, the mutant cells also exhibited a striking difference in their morphology. The cells from the isogenic mutant PAOHtpD cultures were typically twice as long as those of the wild-type PAO1 strain. This feature appears to be unique to *P. aeruginosa*, 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. It is tempting to speculate that inactivation of the *htpD* gene could, in some way, have resulted in the intracellular accumulation of proteins, among them the Flp prepillin, and consequently caused the cells to become longer. However, such a conclusion awaits the production of anti-Flp antibodies, which could be used in immuno-electron microscopy analysis to determine whether Flp prepilin proteins are present intracellularly. Alternatively, it may be that inactivation of the *P. aeruginosa* PAO1 *htp* gene cluster 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). In this regard, it is interesting to note that the *htpC* (PA4303) gene displays homology to a probable septum site-determining protein from *Mesorhizobium loti* (GenBank accession no. NP102599.1; 47% amino acid similarity). Thus, it may be possible that the perturbations in the *P. aeruginosa* PAO1 *htp* gene cluster could have affected the cell division cycle at the partitioning phase since cell envelope invaginations and/or septa appeared to be absent from the PAOHtpD cells (Fig. 3.10). These results also suggest that the

production of the Flp pili might be linked to both cell shape and division. Such a link has already been established for *C. crescentus* and it was reported that transcription of the *pilA* gene is cell cycle-regulated and occurs only in late predivisional cells (Skerker and Shapiro, 2000).

In conclusion, the results presented here suggest a role for the *htp* gene cluster, and indirectly for Flp, in *P. aeruginosa* PAO1 biofilm development under the culturing conditions used in this study. This was evidenced by cells of the mutant PAOHtpD strain being attachment-impaired and also less prone to occur in a biofilm when compared to the wild-type PAO1 cells. 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.

CHAPTER FOUR

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). Microbial biofilm formation is believed to represent a sequential bacterial development process (O'Toole *et al.*, 2000a; Stoodley *et al.*, 2002) and a series of genetic and phenotypic determinants involved in the different stages of biofilm development have been identified (O'Toole *et al.*, 2000b; Stoodley *et al.*, 2002; Sauer *et al.*, 2002; Davey *et al.*, 2003). 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) (O'Toole and Kolter, 1998a; 1998b) and, more recently, Cup adhesins (Vallet *et al.*, 2001), 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. 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 pilin proteins share conserved features with known type IV pili, but the novel Flp pili of *A. actinomycetemcomitans* and *H. ducreyi* have been reported to be important for the colonization properties of these bacterial species (Kachlany *et al.*, 2000; 2001; Nika *et al.*, 2002). *In silico* evidence has subsequently been obtained indicating that *P. aeruginosa* contains a homologous pilus biogenesis/secretion system, which was designated *htp*, and the major pilin-encoding gene was termed *flp* (Van Schalkwyk, 2003). In this study, the *htp* fimbrial gene cluster of *P. aeruginosa* PAO1 was subjected to transcriptional and mutational analyses in an effort to determine its functional significance in biofilm formation. The new information that has evolved during the course of this study is summarized briefly in the sections below and some suggestions regarding future research will be made.

In contrast to the *flp-tad* fimbrial gene clusters of *A. actinomycetemcomitans* and *H. ducreyi*, which are organized in a single transcribed operon (Nika *et al.*, 2002; Haase *et al.*, 2003), the putative *htp* pilus biogenesis/secretion system of *P. aeruginosa* appears to consist of at least two distinct transcriptional units. Whereas the *htpA* through *htpI* open reading frames (ORFs), encoding components that may be required for secretion of the pilin subunits, are organized as a single transcribed operon, the *flp* ORF, encoding the putative pilin subunit, is located upstream from the *htpA-I* ORFs and appears to be divergently transcribed. Gene junction transcription analyses of the putative *htp* operon indicated that the *htp* gene cluster constitutes an operon and evidence was obtained indicating that the *flp* gene is indeed

transcribed. Similar to the *flp-tad* fimbrial gene cluster of *A. actinomycetemcomitans* (Haase *et al.*, 2003), canonical σ^{70} promoters were identified *in silico* in the intergenic region upstream of the *flp* and *htpA* genes, respectively (Chapter 2). The presence of DNA sequences in the upstream regions of these genes that function as promoters was confirmed in β-galactosidase activity assays using promoter-probe constructs in which the intergenic region was cloned in both transcriptional orientations relative to the *lacZ* reporter gene. In these experiments, the respective clones were assayed in batch culture following growth of the recombinant *P. aeruginosa* PAO1 strains in the absence or presence of glass wool. The results obtained indicated that both the promoters were up-regulated following attachment of the bacterial cells to a surface, suggesting that the *flp* and the *htp* gene cluster may play a role during the initial stages of biofilm formation by *P. aeruginosa* PAO1. To provide further insights into the regulation of Flp expression, the exact location of the respective promoters may in future be determined through primer extension assays or S1 nuclease protection assays.

Towards characterizing the ultrastructure of the Flp polypeptide of *P. aeruginosa* PAO1, the protein was expressed as a GST-Flp fusion protein in *E coli* and purified to near homogeneity by glutathione affinity chromatography (Chapter 2). However, it was not possible to purify the mature native Flp protein following thrombin cleavage of the fusion protein to remove the GST carrier. Although proteolytic cleavage did occur, the yield of mature Flp appeared to be very low and could not be detected in either Coomassie- or silver-stained SDS-polyacrylamide gels, thus precluding ultrastructural analysis of the Flp polypeptide. In future, an alternative approach may be adopted whereby the thrombin-liberated mature Flp polypeptide can be purified directly from reverse-stained SDS-polyacrylamide gels (Fernandez-Patron *et al.*, 1992). Reverse staining procedures are purportedly very sensitive and exploit the ability of proteins and protein-SDS complexes to bind Zn^{2+} and that of imidazole to react with unbound Zn^{2+} to produce insoluble zinc imidazolate ($ZnIm_2$). Thus, deposition of $ZnIm_2$ along the gel surface results in the formation of a deep white-stained background, against which unstained protein bands contrast, thereby facilitating their detection and subsequent excision from the gel (Fernandez-Patron *et al.*, 1995; Castellanos-Serra *et al.*, 1996). Alternatively, a different expression system may in future be used for expression of the *P. aeruginosa* PAO1 Flp polypeptide. Rather than expressing Flp (4.9 kDa) as a fusion with the 27-kDa GST protein and then attempting to recover the native protein following protease cleavage, it may be possible to express the mature Flp protein as a fusion

with a smaller carrier protein, *e.g.* as a hisidine-tagged protein. The smaller carrier protein would not be expected to drastically alter the structure of the Flp protein and it should therefore be possible to characterize the purified protein directly by electron microscopy procedures.

To determine the functional importance of the *P. aeruginosa* PAO1 *htp* gene cluster in biofilm development, an isogenic mutant strain containing an inactivated *htpD* allele was generated and its ability to form biofilms on a glass wool substratum was compared to that of the wild-type *P. aeruginosa* PAO1 strain (Chapter 3). HtpD, encoded by PA4302, displays homology to a subfamily of secretion NTPases (Planet *et al.*, 2001) and was selected for insertional inactivation in this investigation, since results of studies on the 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. Although it is thought that the ATPase might provide energy for polymerization of the pili or structures that function to extrude proteins through the outer membrane, it might also serve to 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 (Bhattacharjee *et al.*, 2001). Interestingly, the cells from the isogenic mutant strain PAOHtpD were longer than the wild-type *P. aeruginosa* PAO1 cells (Chapter 3). The intracellular accumulation Flp prepilin proteins, together with a defective cell division mechanism, might have contributed to the observed increase in cell length. The defect in cell division may be a consequence of the presence of unsegregated genomic DNA, which, in turn, may have prevented septum formation and cell division. These hypotheses are, however, subject to verification by Western blot analysis using an anti-Flp antibody and staining of the cells with DAPI to visualize the location of DNA within the cells.

The results obtained during the course of this investigation indicated that the mutant PAOHtpD strain was capable of forming biofilms on glass wool within 16 h of culturing in LB broth. However, inspection of the biofilm phenotype revealed that, in contrast to wild-type *P. aeruginosa* PAO1, the mutant strain displayed sparser colonization of the glass wool surface and the cells were localized in clusters. More detailed analysis of the altered biofilm phenotype conferred by inactivation of the *htpD* gene was obtained through determining the ratio of biofilm (attached) to biofilm-associated cell (BAC) biomass over a period of 24 h of culturing. Compared to the wild-type PAO1 strain, cells of the mutant PAOHtpD strain was

both attachment- and biofilm-impaired. This was not the result of impaired growth, as growth curves of planktonic, biofilm-associated (BAC) and biofilm cultures of the *P. aeruginosa* wild-type PAO1 and mutant PAOHtpD strains were near identical (Chapter 3). The ability of mutant PAOHtpD cells to attach to a substratum could have been due to the expression of functional type IV pili, which have been reported to play an essential role during the early stages of biofilm formation (O'Toole and Kolter *et al.*, 1998a). Although the Flp pili therefore do not appear to be directly involved in surface attachment by *P. aeruginosa* PAO1, the results do not exclude an auxiliary role for Flp 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* PAO1 mutant strain deficient in Flp pili would have been useful in this respect, several attempts during the course of this study aimed at generating such a mutant strain and using different approaches were unsuccessful. The apparent refractoriness of some *P. aeruginosa* genes to mutagenesis has also been observed by other members of our research group. Attempts to inactivate PA3731, which encodes a conserved hypothetical protein that is uniquely expressed in biofilms of *P. aeruginosa* (B. Steyn, personal communication) and PA4462, which encodes the alternative sigma factor σ^{54} (rpoN) (J.B. Weyers, personal communication), has also not been successful.

Notably, complementation of the PAOHtpD mutant strain with the wild-type *htpD* gene *in trans* did not restore the ability of the complemented mutant strain to attach to glass wool at levels approximating those obtained with the wild-type parent strain, and also did not restore the cellular morphology. It is possible that the *htpD* gene was not expressed from the complementation plasmid pJB-IP-HtpD or, alternatively, that the protein was expressed but at very low levels and thus not able to fully complement the inactivated protein. However, it is most likely that insertional inactivation of *htpD* influenced downstream gene expression and the mutant PAOHtpD strain should therefore in future rather be complemented with a cloned copy of the full-length *htp* operon *in trans*. Nevertheless, these results provide further supporting evidence for the polycistronic nature of the *htp* operon transcript and that inactivation of *htpD* resulted in the inactivation or, at least, severe impairment of the functioning of the *htp* gene cluster.

In summary, a cluster of genes (*htp*) in *P. aeruginosa* PAO1 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 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 Flp fibrils, and how these components mediate attachment to not only biotic and abiotic surfaces, but interbacterial adhesion as well. Furthermore, dissection of the factors or networks controlling expression of the *flp* gene and *htp* operon may provide a better understanding regarding the complexity and specificity of this adherence mechanism. In addition to investigating the importance of the *flp*-*htp* fimbrial biogenesis/secretion system in mediating adherence of *P. aeruginosa* PAO1 to surfaces, future studies may also be directed towards investigating the importance of this system in virulence of *P. aeruginosa* PAO1. Mutations in the *tadA* gene of *A. actinomycetemcomitans* and in the homologous *tadD* gene of *Pasteurella multocida* have been reported to reduce the virulence of these bacteria in their respective animal models (Fuller *et al.*, 2000; Schreiner *et al.*, 2003), while full expression of virulence by *H. ducreyi* in humans has been reported to require an intact *flp*-*tad* gene cluster (Spinola *et al.*, 2003). Such studies may provide new insight not only into the role of the *flp*-*htp* system of *P. aeruginosa* PAO1 in attachment to surfaces, but also into its virulence properties.

REFERENCES

Abraham, S.N., Jonsson, A.B. and Normark, S. (1998). Fimbriae-mediated host-pathogen cross-talk. *Curr. Opin. Microbiol.* 1: 75-81.

Allison, D.G., Ruiz, B., San Jose, C., Jaspe, A. and Gilbert, P. (1998). Extracellular products as mediators of the formation and detachment of *Pseudomonas fluorescens* biofilms. *FEMS Microbiol. Lett.* 167: 179-184.

Alm, R.A. and Mattick, J.S. (1995). Identification of a gene, *pilV*, required for type IV fimbrial biogenesis in *Pseudomonas aeruginosa*, whose product possesses a pre-pillin-like leader sequence. *Mol. Microbiol.* 16: 485-496.

Alm, R.A. and Mattick, J.S. (1996). Identification of two genes with prepilin-like leader sequences involved in type IV fimbrial biogenesis in *Pseudomonas aeruginosa*. *J. Bacteriol.* 178: 3809-3817.

Alm, R.A. and Mattick, J.S. (1997). Genes involved in the biogenesis and function of type 4 fimbriae in *Pseudomonas aeruginosa*. *Gene* 192: 89-98.

Alm, R.A., Hallinan, J.P., Watson, A.A. and Mattick, J.S. (1996a). Fimbrial biogenesis genes of *Pseudomonas aeruginosa: pilW* and *pilX* increase the similarity of type 4 fimbriae to the GSP protein secretion systems and *pilY1* encodes a gonococcal PilC homologue. *Mol. Microbiol.* 22: 161-173.

Alm, R.A., Bandero, A.J., Free, P.D. and Mattick, J.S. (1996b). Identification of a novel gene, *pil*Z, essential for type 4 fimbrial biogenesis in *Pseudomonas aeruginosa*. *J. Bacteriol.* 178: 46-53.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucl. Acids Res.* 25: 3389-3402.

An, Y.H., Dickinson, R.B. and Doyle, R.J. (2000). Mechanisms of bacterial adhesion and pathogenesis of implant and tissue infections. In: *Handbook of Bacterial Adhesion: Principles, Methods and Applications*, pp. 1-27. An, Y.H. and Friedman, R.J. (eds). Humana Press Inc., Totowa, NJ, USA.

Arora, S.K., Ritchings, B.W., Almira, E.C., Lory S. and Ramphal R. (1997). A transcriptional activator, FleQ, regulates mucin adhesion and flagellar gene expression in *Pseudomonas aeruginosa* in a cascade manner. *J. Bacteriol.* 179: 5574-5581.

Bally, M., Filloux, A., Akrim, M., Ball, G., Lazdunski, A. and Tommassen, J. (1992). Protein secretion in *Pseudomonas aeruginosa*: Characterization of seven *xcp* genes and processing of secretory apparatus components by prepilin peptidase. *Mol. Microbiol.* 6: 1121-1131.

Beveridge, T.J., Makin, S.A., Kadurugamuwa, J.L. and Li, Z. (1997). Interactions between biofilms and the environment. *FEMS Microbiol. Rev.* 20: 291-303.

Bhattacharjee, M.K., Kachlany, S.C., Fine, D.H. and Figurski, D.H. (2001). Nonspecific adherence and fibril biogenesis by *Actinobacillus actinomycetemcomitans*: TadA protein is an ATPase. *J. Bacteriol.* 183: 5927-5936.

Bieber, D., Ramer, S.W., Wu, C.Y., Murray, W.J., Tobe, T., Fernandez, R. and Schoolnik, G.K. (1998). Type IV pili, transient bacterial aggregates and virulence of enteropathogenic *Escherichia coli. Science* 280: 2114-2118.

Bitter, W., Koster, M., Latijnhouwers, M., de Cock, H. and Tommassen, J. (1998). Formation of oligomeric rings by XcpQ and PilQ, which are involved in protein transport across the outer membrane of *Pseudomonas aeruginosa. Mol. Microbiol.* 27: 209-219.

Blatney, J.M., Brauteset, T., Winther-Larson, H.C., Haugan, K. and Valla, S. (1997). Construction and use of a versatile set of broad-host-range cloning and expression vectors based on the RK2 replicon. *Appl. Environ. Microbiol.* 63: 370-379.

Boland, T., Dufrene, Y., Barger, B. and Lee, G. (2000). Molecular basis of cell adhesion to polymers characterized by AFM. *Crit. Rev. Biomed. Eng.* 28: 195-196.

1. Boucher, J.C., Schurr, M.J. and Deretic, V. (2000). Dual regulation of mucoidy in *Pseudomonas aeruginosa* and sigma factor antagonism. *Mol. Microbiol.* 36: 341-351.

Boyd, A. and Chakrabarty, A.M. (1994). Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* 60: 2355-2359.

Boyle, J.S. and Lew, A.M. (1995). An inexpensive alternative to glassmilk for DNA purification. *Trends Genet.* 11: 8.

Branda, S.S., Gonzalez-Pastor, J.E., Ben-Yehuda, S., Losick, R. and Kolter, R. (2001). Fruiting body formation by *Bacillus subtilis*. *Proc. Natl. Acad. Sci., USA* 98: 11621-11626.

Burns, D.L. (1999). Biochemistry of type IV secretion. *Curr. Opin. Microbiol.* 2: 25-29.

Caiazza, N.C. and O'Toole, G.A. (2004). SadB is required for the transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas aeruginosa* PA14. *J. Bacteriol.* 186: 4476-4485.

Castellanos-Serra, L.R., Fernandez-Patron, C., Hardy, E. and Huerta, V. (1996). A procedure for protein elution from reverse-stained polyarcylamide gels applicable at the low picomole level: An alternative route to the preparation of low abundance proteins for microanalysis. *Electrophoresis* 17: 1564-1572.

Chiang, P., Habash, M. and Burrows, L.L. (2005). Disparate subcellular localization patterns of *Pseudomonas aeruginosa* type IV pilus ATPases involved in twitching motility. *J. Bacteriol.* 187: 829-839.

Christie, P.J. (2001). Type IV secretion: Intracellular transfer of macromolecules by systems ancestrally related to conjugation machines. *Mol. Microbiol.* 40: 294-305.

Collins, R.F., Frye, S.A., Balasingham, S., Ford, R.C., Tonjum, T. and Derrick, J.P. (2005). Interaction with type IV pili induces structural changes in the bacterial outer membrane secretin PilQ. *J. Biol. Chem.* 280: 18923-18930.

Costerton, J.W., Cheng, K-J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M. and Marrie, T.J. (1987). Bacterial biofilms in nature and disease. *Annu. Rev. Microbiol*. 41: 435-464.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. and Lappin-Scott, H.M. (1995). Microbial biofilms. *Annu. Rev. Microbiol.* 49: 711-745.

Costerton, J.W., Stewart, P.S. and Greenberg, E.P. (1999). Bacterial biofilms: A common cause of persistent infections. *Science* 284: 1318-1322.

Dartigalongue, C. and Raina, S. (1998). A new heat-shock gene, *ppiD*, encodes a peptidyl-prolyl isomerase required for folding of outer membrane proteins in *Escherichia coli*. *EMBO J.* 17: 3968-3980.

Darzins, A. (1994). Characterisation of a *Pseudomonas aeruginosa* gene cluster involved in pilus biosynthesis and twitching motility: Sequences similarity to the chemotaxis proteins of enterics and the gliding bacterium *Myxococcus xanthus*. *Mol. Microbiol.* 11: 137-153.

Darzins, A. and Russell, M.A. (1997). Molecular genetic analysis of type-4 pilus biogenesis and twitching motility using *Pseudomonas aeruginosa* as a model system - a review. *Gene* 192: 109-115.

Dasgupta, N., Arora, S.K. and Ramphal, R. (2000). *fle*N, a gene that regulates flagellar numbers in *Pseudomonas aeruginosa*. *J. Bacteriol.* 182: 357-364.

Davey, M.E., Caiazza, N.C. and O'Toole, G.A. (2003). Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa*. *J. Bacteriol.* 185: 1027-1036.

Davey, M.E. and O'Toole, G.A. (2000). Microbial biofilms: From ecology to molecular genetics. *Microbiol. Mol. Biol. Rev.* 64: 847-867.

Davies, D.G. and Geesey, G.G. (1995). Regulation of the alginate biosynthesis gene *algC* in *Pseudomonas aeruginosa* during biofilm development in continuous culture. *Appl. Environ. Microbiol.* 61: 860-867.

Davies, D.G., Parsek, M.R., Pearson, J.P., Iglewski, B.H., Costerton, J.W. and Greenberg, E.P. (1998). The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* 280: 295-298.

De Kievit, T.R., Gillis, R., Marx, S., Brown, C. and Iglewski, B.H. (2001). Quorum sensing in *Pseudomonas aeruginosa* biofilms: Their role and expression patterns. *Appl. Environ. Microbiol.* 67: 1865-1873.

De Weger, L.A., Van der Vlught, C.I.M., Wijfjes, A.H.M., Bakker, P.A.H.M., Schippers, B. and Lugtenberg, B. (1987). Flagella of a plant growth-stimulating *Pseudomonas fluorescens* strain are required for colonization of potato roots. *J. Bacteriol.* 169: 2769-2773.

DeBeer, D., Stoodley, P., Roe, F. and Lewandowski, Z. (1994). Effects of biofilm structure on oxygen distribution and mass transport. *Biotech. Bioeng.* 43: 1131-1138.

DeFlaun, M.F., Marshall, B.M., Kulle, E-P. and Levy, S.B. (1994). Tn5 insertion mutants of *Pseudomonas fluorescens* defective in adhesion to soil and seeds. *Appl. Environ. Microbiol.* 60: 2637-2642.

DeFlaun, M.F., Oppenheimer, S.R., Streger, S., Condee, C.W. and Fletcher, M. (1999). Alterations in adhesion, transport, and membrane characteristics in an adhesion-deficient pseudomonad. *Appl. Environ. Microbiol.* 65: 759-765.

Donlan, R.M. and Costerton, J.W. (2002). Biofilms: Survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* 15: 167-193.

Dorel, C., Vidal, O., Prigent-Combaret, C., Vallet, I. and Lejeune, P. (1999). Involvement of the Cpx signal transduction pathway of *E. coli* in biofilm formation. *FEMS Microbiol. Lett.* 178: 169-175.

Driessen, A.J., Fekkes, P. and Van Der Wolk, J.P. (1998). The Sec system. *Curr. Opin. Microbiol.* 1: 216-222.

Duong, F. and Wickner, W. (1999). The PrlA and PrlG phenotypes are caused by a loosened association among the translocase SecYEG subunits. *EMBO J.* 18: 3263-3270.

Dworkin, M. (1999). Fibrils as extracellular appendages of bacteria: Their role in contact-mediated cell-cell interactions in *Myxococcus xanthus*. *Bioessays* 21: 590-595.

Economou, A. (1998). Bacterial preprotein translocase: Mechanism and conformational dynamics of a processive enzyme. *Mol. Microbiol.* 27: 511-518.

Espinosa-Urgel, M., Salido, A. and Ramos, J.L. (2000). Genetic analysis of functions involved in adhesion of *Pseudomonas putida* to seeds. *J. Bacteriol.* 182: 2363-2369.

Farinha, M.A., Conway, B.D., Ellert, L.M.G., Irvin, N.W., Sherburne, R. and Paranchych, W. (1994). Alteration of the pilin adhesin of *Pseudomonas aeruginosa* PAO results in normal pilus biogenesis but a loss of adherence to human pneumocyte and decreased virulence in mice. *Infect. Immun.* 62: 4118-4123.

Fernandez-Patron, C., Calero, M., Collazo, P.R., Garcia, J.R., Madrazo, J. and Musacchio, A. (1995). Protein reverse staining: High-efficiency microanalysis of unmodified proteins detected on electrophoresis gels. *Anal. Biochem.* 224: 203-211.

Fernandez-Patron, C., Castellanos-Serra, L. and Rodriguez, P. (1992). Reverse staining of sodium dodecyl sulfate polyacrylamide gels by imidazole-zinc salts: Sensitive detection of unmodified proteins. *BioTechniques* 12: 564-573.

Filloux, A., Michel, G. and Bally, M. (1998). GSP-dependant protein secretion in Gram-negative bacteria: The Xcp system of *Pseudomonas aeruginosa*. *FEMS Microbiol. Rev.* 22: 177-198.

Fine, D.H., Furgang, D., Kaplan, J., Charlesworth, J. and Figurski, D.H. (1999). Tenacious adhesion of *Actinobacillus actinomycetemcomitans* strain CU1000 to salivary-coated hydroxyapatite. *Arch. Oral Biol.* 44: 1063-1076.

Finelli, A., Gallant, C.V., Jarvi, K. and Burrows, L.L. (2003). Use of in biofilm expression technology to identify genes involved in *Pseudomonas aeruginosa* biofilm development. *J. Bacteriol.* 185: 2700-2710.

Fives-Taylor, P.M., Meyer, D.H., Mintz, K.P. and Brissette, C. (2000). Virulence factors of *Actinobacillus actinomycetemcomitans. Periodontol.* 20: 136-167.

Folkhard, W., Marvin, D.A., Watts, T.H. and Parenchych, W. (1981). Structure of polar pili from *Pseudomonas aeruginosa* strain K and O. *J. Mol. Biol.* 149: 79-93.

Forest, K.T., Satyshur, K.A., Worzalla, G.A., Hansen, J.K. and Herdendorf, T.J. (2004). The pilus-retraction protein PilT: Ultrastructure of the biological assembly. *Acta Crystallogr. D Biol. Crystallogr.* 60: 978-982.

Friedman, L. and Kolter, R. (2004). Two genetic loci produce distinct carbohydrate-rich structural components of the *Pseudomonas aerguinosa* biofilm matrix. *J. Bacteriol.* 186: 4457-4465.

Fuller, T.E., Kennedy, M.J. and Lowery, D.E. (2000). Identification of *Pasteurella multocida* virulence genes in a septicemic mouse model using signature-tagged mutagenesis. *Microb. Pathog.* 29: 25-38.

Geesey, G.G. (2001). Bacterial behaviour at surfaces. *Curr. Opin. Microbiol.* 4: 296-300.

Geesey, G.G., Richardson, W.T., Yeomans, H.G., Irvin, I.T. and Costerton, J.W. (1977). Microscopic examination of natural sessile bacterial populations from an alpine stream. *Can. J. Microbiol.* 23: 1733-1736.

Genevaux, P., Bauda, P., DuBow, M.S. and Oudega, B. (1999). Identification of Tn10 insertions in the *rfaG*; *rfaP* and *galU* genes involved in lipopolysaccharide core biosynthesis that affects *Escherichia coli* adhesion. *Arch. Microbiol.* 172: 1-8.

Genevaux, P., Muller, S. and Bauda, P. (1996). A rapid screening procedure to identify mini-Tn10 insertion mutants of *Escherichia coli* K-12 with altered adhesion properties. *FEMS Microbiol. Lett.* 142: 27-30.

Ghigo, J.M. (2001). Natural conjugative plasmids induce bacterial biofilm development. *Nature* 412: 442-445.

Girón, J.A., Ho, A.S. and Schoolnik, G.K. (1991). An inducible bundle-forming pilus of enteropathogenic *Escherichia coli. Science* 254: 710-713.

Goldberg, J.B. and Ohman, D.E. (1987). Construction and characterization of *Pseudomonas aeruginosa algB* mutants: Role of *algB* in high-level production of alginate. *J. Bacteriol.* 169: 1593-1602.

Haase, E.M., Stream, J.O. and Scannapieco, F.A. (2003). Transcriptional analysis of the 5' terminus of the *flp* fimbrial gene cluster from *Actinobacillus actinomycetemcomitans*. *Microbiol.* 149: 205-215.

Haase, E.M., Zmuda, J.L. and Scannapieco, F.A. (1999). Identification and molecular analysis of rough-colony-specific outer membrane proteins of *Actinobacillus actinomycetemcomitans*. *Infect. Immun.* 67: 2901-2908.

Hahn, H.P. (1997). The type-4 pilus is the major virulence-associated adhesin of *Pseudomonas aeruginosa* - a review. *Gene* 192: 99-108.

Hall-Stoodley, L. and Stoodley, P. (2002). Developmental regulation of microbial biofilms. *Curr. Opin. Biotechnol.* 13: 228-233.

Harley, C.B. and Reynolds, R.P. (1987). Analysis of *E. coli* promoter sequences. *Nucl. Acids Res.* 15: 2343-2361.

Hayes, F. (2003). Transposon-based strategies for microbial functional genomics and proteomics. *Annu. Rev. Genet.* 37: 3-29.

Heilmann, C., Gerke, C., Perdreau-Remington, F. and Gotz, F. (1996). Characterization of Tn917 insertion mutants of *Staphylococcus epidermidis* affected in biofilm formation. *Infect. Immun.* 64: 277-282.

Henrici, A.T. (1933). Studies of fresh water bacteria. I. A direct microscopic technique. *J. Bacteriol.* 25: 277-287.

Hermansson, M. and Marshall, K.C. (1985). Utilization of surface localized substrate by non-adhesive marine bacteria. *Microb. Ecol*. 11: 91-105.

Heydorn, A., Ersball, B., Kato, J., Hentzel, M., Parsek, M.R., Tolker-Nielsen, T., Givskov, M. and Molin, S. (2002). Statistical analysis of *Pseudomonas aeruginosa* biofilm development: Impact of mutations in genes involved in twitching motility, cell-to-cell signaling, and stationary phase sigma factor expression. *Appl. Environ. Microbiol.* 68: 2008-2017.

Heydorn, A., Nielsen, A.T., Hentzer, M., Sternberg, C., Givskov M., Ersbøll, B.K. and Molin, S. (2000). Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiol.* 146: 2395-2407.

Hoang, T.T., Kutchma, A.J., Becher, A. and Schweizer, H.P. (2000). Integration-proficient plasmid for *Pseudomonas aeruginosa*: Site-specific integration and use for engineering of reporter and expression strains. *Plasmid* 43: 59-72.

Hobbs, M. and Mattick, J.S. (1993). Common components in the assembly of type 4 fimbriae, DNA transfer systems, filamentous phage and protein-secretion apparatus: A general system for the formation of surface-associated protein complexes. *Mol. Microbiol*. 10: 233-243.

Holloway, B.W., Krishnapillai, V. and Morgan, A.F. (1979). Chromosomal genetics of *Pseudomonas*. *Microbiol. Rev.* 43: 73-102.

Holmgren, A. and Branden, C.I. (1989). Crystal structure of chaperone protein PapD reveals an immunoglobulin fold. *Nature* 342: 248-251.

Holt, S.C., Tanner, A.C. and Socransky, S.S. (1980). Morphology and ultrastructure of oral strains of *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus. Infect. Immun.* 30: 588-600.

Hoyle, B.D., Williams, L.J. and Costerton, J.W. (1993). Production of mucoid exopolysaccharide during development of *Pseudomonas aeruginosa* biofilms. *Infect. Immun.* 61: 777-780.

Ichihara, S., Suzuki, T., Suzuku, M. and Mizushima, S. (1986). Molecular cloning and sequencing of the *sppA* gene and characterization of the encoded protease IV, a single peptide peptidase, of *Escherichia coli. J. Biol. Chem.* 261: 9405-9411.

Inoue, T., Tanimoto, I., Ohta, H., Kato, K., Murayama, Y. and Fukui, K. (1998). Molecular characterization of low-molecular-weight component protein, Flp, in *Actinobacillus actinomycetemcomitans* fimbriae. *Microbiol. Immunol.* 42: 253-258.

Inouye, T., Ohta, H., Kokeguchi, S., Fukui, K. and Kato, K. (1990). Colonial variation and fimbriation of *Actinobacillus actinomycetemcomitans. FEMS Microbiol. Lett.* 57: 13-17.

Jackson, K.D., Starkey, M., Kremer, S., Parsek, M.R. and Wozniak, D.J. (2004). Identification of *psl*, a locus encoding a potential exopolysaccharide that is essential for *Pseudomonas aeruginosa* PAO1 biofilm formation. *J. Bacteriol.* 186: 4466-4475.

Jacob-Dubuisson, F., Heuser, J., Dodson, K., Normark, S. and Hultgren, S. (1993). Initiation of assembly and association of the structural elements of a bacterial pilus depend on two specialized tip proteins. *EMBO J.* 12: 837-847.

Jansen, K. (ed). (1995). Current Protocols in Molecular Biology. John Willey & Sons Inc., USA.

Jefferson, K.K. (2004). What drives bacteria to produce a biofilm? *FEMS Microbiol. Lett.* 236: 163-173.

Kachlany, S.C., Planet, P.J., Bhattacharjee, M.K., Kollia, E., DeSalle, R., Fine, D.H. and Figurski, D.H. (2000). Nonspecific adherence of *Actinobacillus actinomycetemcomitans* requires genes widespread in *Bacteria* and *Archaea. J. Bacteriol.* 182: 6169-6176.

Kachlany, S.C., Planet, P.J., DeSalle, R., Fine, D.H., Figurski, D.H. and Kaplan, J. (2001). *flp*-1, the first representative of a new pilin gene subfamily, is required for non-specific adherence of *Actinobacillus actinomycetemcomitans. Mol. Microbiol.* 40: 542-554.

Kadurugamuwa, J.L., Clarke, A.J. and Beveridge, T.J. (1993). Surface action of gentamicin on *Pseudomonas aeruginosa. J. Bacteriol.* 175: 5798-5805.

Kang, P.J., Hauser, A.R., Apodaca, G., Fleizig, S.M., Wiener-Kronish, J., Mostov, K. and Engel, J.N. (1997). Identification of *Pseudomonas aeruginosa* genes required for epithelial cell injury. *Mol. Microbiol*. 24: 1249-1262.

Kaplan, J.B., Ragunath, C., Ramasubbu, N. and Fine, D.H. (2003). Detachment of *Actinobacillus actinomycetemcomitans* biofilm cells by an endogenous beta-hexosaminidase activity. *J. Bacteriol.* 185: 4693-4698.

Kaufman, M.R., Seyer, J.M. and Taylor, R.T. (1991). Processing of Tcp pilin by TcpJ typifies a common step intrinsic to a newly recognized pathway for extracellular protein secretion by Gram-negative bacteria. *Genes Dev.* 5: 1834-1846.

Kessler, B., de Lorenzo, V. and Timmis, K.N. (1992). A general system to integrate *lacZ* fusions into the chromosomes of Gram-negative eubacteria: Regulation of the *Pm* promoter of the TOL plasmid studied with all controlling elements in monocopy. *Mol. Gen. Genet.* 233: 293-301.

Kinsella, N., Guerry, P., Cooney, J. and Trust, T.J. (1997). The *flgE* gene of *Campylobater coli* is under the control of the alternative sigma factor σ^{54}. *J. Bacteriol.* 179: 4647-4653.

Klausen, M., Aaes-Jorgensen, A., Molin, S. and Tolker-Nielsen, T. (2003). Involvement of bacterial migration in the development of complex multicellular structures in *Pseudomonas aeruginosa* biofilms. *Mol. Microbiol.* 50: 61-68.

Korber, D., Lawrence, J.R. and Caldwell, D.E. (1994). Effect of motility on surface colonisation and reproductive success of *Pseudomonas fluorescens* in dual-dilution continuous culture and batch culture systems. *Appl. Environ. Microbiol.* 60: 1663-1669.

Korber, D.R., Lawrence, J.R., Sutton, B. and Caldwell, D.E. (1989). Effects of laminar flow velocity on the kinetics of surface colonization by mot^+ and mot^- *Pseudomonas fluorescens. Microb. Ecol.* 18: 1-19.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.

Lazar, S.W. and Kolter, R. (1996). SurA assists the folding of *Escherichia coli* outer membrane proteins. *J. Bacteriol.* 178: 1770-1773.

Lazdunski, A., Guzzo, J., Filloux, A., Bally, M. and Murgier, M. (1990). Secretion of extracellular proteins by *Pseudomonas aeruginosa*. *Biochem.* 72: 147-156.

Lee, K.K., Sheth, H.B., Wong, W.Y., Sherburne, R., Paranchych, W., Hodges, R.S., Lingwood, C.A., Krivan, H. and Irvin, R.T. (1994). The binding of *Pseudomonas aeruginosa* pili to glycoshingolipids is a tip-associated event involving the C-terminal region of the structural pilin subunit. *Mol. Microbiol.* 11: 705-713.

Lengeler, J.W., Drews, G. and Schlegel, H.G. (1999). Biology of the Prokaryotes. Blackwell Science, Stuttgart, Germany.

Loo, C.Y., Corliss, D.A. and Ganeshkumar, N. (2000). *Streptococcus gordonii* biofilm formation: Identification of genes that code for biofilm phenotypes. *J. Bacteriol.* 182: 1374-1382.

Lory, S. (1998). Secretion of proteins and assembly of bacterial surface organelles: Shared pathways of extracellular protein targeting. *Curr. Opin. Microbiol.* 1: 27-35.

Lory, S. and Strom, M.S. (1997). Structure-function relationship of type-IV prepilin peptidase of *Pseudomonas aeruginosa* - a review. *Gene* 192: 117-121.

Mah, T.F. and O'Toole, G.A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol.* 9: 34-39.

Marshall, K.C. (1985). Mechanisms of bacterial adhesion at solid-water interfaces. In: *Bacterial Adhesion: Mechanisms and Physiological Significance*, pp. 133-161. Savage, D.C. and Fletcher, M. (eds). Plenum Press, New York, USA.

Marshall, K.C. (1992). Biofilms: An overview of bacterial adhesion, activity and control at surfaces. *ASM News* 58: 202-207.

Marshall, K.C., Stout, R. and Mitchell, R. (1971). Mechanisms of the initial events in the sorption of marine bacteria to surfaces. *J. Gen. Microbiol.* 68: 337-348.

Martin, P.R., Hobbs, M., Free, P.D., Jeske, Y. and Mattick, J.S. (1993). Characterization of *pilQ*, a new gene required for the biogenesis of type 4 fimbriae in *Pseudomonas aeruginosa*. *Mol. Microbiol*. 9: 857-868.

Martin, P.R., Watson, A., McCaul, T. and Mattick, J.S. (1995). Characterization of a five-gene cluster required for the biogenesis of type 4 fimbriae in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 16: 497-508.

Marx, C.J. and Lidstrom, M.E. (2002). Broad-host-range *cre-lox* system for antibiotic marker recycling in Gram-negative bacteria. *BioTechniques* 33: 1062-1067.

Matsukawa, M. and Greenberg, E.P. (2004). Putative exopolysaccharide synthesis genes influence *Pseudomonas aeruginosa* biofilm development. *J. Bacteriol.* 186: 4449-4456.

Mattick, J.S., Whitchurch, C.B. and Alm, R.A. (1996). The molecular genetics of type IV fimbriae in *Pseudomonas aeruginosa* - a review. *Gene* 179: 147-155.

McEldowney, S. and Fletcher, M. (1986). Variability of the influence of physiological factors affecting bacterial adhesion to polystyrene substrata. *Appl. Environ. Microbiol.* 52: 460-465.

McLean, R.J., Whiteley, M., Stickler, D.J. and Fuqua, W.C. (1997). Evidence of autoinducer activity in naturally occurring biofilms. *FEMS Microbiol. Lett.* 154: 259-263.

Medina, G., Juárez, K. and Soberón-Chávez, G. (2003). The *Pseudomonas aeruginosa rhlAB* operon is not expressed during the logarithmic growth phase of growth even in the presence of its activator RhlR and the autoinducer N-butyryl-homoserine lactone. *J. Bacteriol.* 185: 377-380.

Miller, J. H. (1972). Experiments in Molecular Genetics. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory.

Molin, S. and Tolker-Nielsen, T. (2003). Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilization of the biofilm structure. *Curr. Opin. Biotechnol.* 14: 255-261.

Mori, H. and Ito, K. (2001). The Sec protein-translocation pathway. *Trends Microbiol.* 9: 494-500.

Nassif, X., Beretti, J.L., Lowy, J., Stenberg, P., O'Gaora, P., Pfeifer, J., Normark, S. and So, M. (1994). Roles of pilin and PilC in adhesion of *Neisseria meningitidis* to human epithelial and endothelial cells. *Proc. Natl. Acad. Sci., USA* 91: 3769-3773.

Nickel, J.C., Ruseska, I., Wright, J.B. and Costerton, J.W. (1985). Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. *Antimicrob. Agents Chemother.* 27: 619-624.

Nika, J.R., Latimer, J.L., Ward, C.K., Blick, R.J., Wagner, N.J., Cope, L.D., Mahairas, G.G., Munson, R.S. and Hansen, E.J. (2002). *Haemophilus ducreyi* requires the *flp* gene cluster for microcolony formation *in vitro*. *Infect. Immun.* 70: 2965-2975.

Notermans, S., Doormans, J.A. and Mead, G.C. (1991). Contribution of surface attachment to the establishment of microorganisms in food processing plants: A review. *Biofouling* 5: 1-6.

Nunn, D. (1999). Bacterial Type II protein export and pilus biogenesis: More than just homologies? *Trends. Cell. Biol.* 9: 402-408.

Nunn, D. and Lory, S. (1991). Product of the *Pseudomonas aeruginosa* gene *pilD* is a prepilin leader peptidase. *Proc. Natl. Acad. Sci., USA* 89: 47-51.

Nunn, D. and Lory, S. (1992). Components of the protein excretion apparatus of *Pseudomonas aeruginosa* are processed by the type IV prepilin peptidase. *Proc. Natl. Acad. Sci., USA* 89: 47-51.

Nunn, D., Bergman, S. and Lory, S. (1990). Products of three accessory genes *pilB, pilC* and *pilD*, are required for biogenesis of *Pseudomonas aeruginosa* pili. *J. Bacteriol.* 172: 2911-2919.

O'Toole, G.A. and Kolter, R. (1998a). Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol. Microbiol.* 30: 295-304.

O'Toole, G.A. and Kolter, R. (1998b). Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: A genetic analysis. *Mol. Microbiol.* 28: 449-461.

O'Toole, G.A., Kaplan, H.B. and Kolter, R. (2000a). Biofilm formation as microbial development. *Annu. Rev. Microbiol.* 54: 49-79.

O'Toole, G.A., Gibbs, K.A., Hager, P.W., Phibbs, P.V. and Kolter, R. (2000b). The global carbon metabolism regulator Crc is a component of a signal transduction pathway required for biofilm development by *Pseudomonas aeruginosa*. *J. Bacteriol.* 182: 425-431.

Olsen, A., Jonsson, A. and Normark, S. (1989). Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli*. *Nature* 338: 652-655.

Olsen, R.H. and Shipley, P. (1973). Host range and properties of the *Pseudomonas aeruginosa* R factor R1822. *J. Bacteriol.* 113: 772-780.

Olsen, R.H., DeBusscher, G. and McCombie, W.R. (1982). Development of broad range and gene banks: Self-cloning of the *Pseudomonas aeruginosa* PAO chromosome. *J. Bacteriol*. 150: 60-69.

Oosthuizen, M.C., Steyn, B., Theron, J., Cosette, P., Lindsay, D., Von Holy, A. and Brözel, V.S. (2002). Proteomic analysis reveals differential protein expression by *Bacillus cereus* during biofilm development. *Appl. Environ. Microbiol.* 68: 2770-2780.

Orndorff, P.E. and Bloch, C.A. (1990). The role of type 1 pili in the pathogenesis of *Escherichia coli* infections: A short review and some new ideas. *Microb. Pathog*. 9: 75-79.

Pallen, M.J. and Wren, B.W. (1997). The HtrA family of serine proteases. *Mol. Microbiol*. 26: 209-221.

Palleroni, N.J. (1992a). Introduction to the family *Pseudomonadaceae*. In: *The Prokaryotes*, pp. 3071-3085. Balows, A., Truper, H.G., Dworkin, M., Harder, W. and Schleifer K-H. (eds). Springer-Verlag, New York, USA.

Palleroni, N.J. (1992b). Human- and animal-pathogenic Pseudomonads. In: *The Prokaryotes*, pp. 3086-3103. Balows, A., Truper, H.G., Dworkin, M., Harder, W. and Schleifer K-H. (eds). Springer-Verlag, New York, USA.

Palmer, R. (1999). Microscopy flow cells: Perfusion chambers for real-time study of biofilms. In: *Biofilms*. Doyle, R. (ed). Academic Press, Orlando, Fl., USA.

Parge, H.E., Forest, K.T., Hickey, M.J., Christensen, D.A., Getzoff, E.D. and Tainer, J.A. (1995). Structure of the fibre-forming protein pilin at 2.6 Å resolution. *Nature* 378: 32-38.

Parkins, M.D., Ceri, H. and Storey, D.G. (2001). *Pseudomonas aeruginosa* GacA, a factor in multihost virulence, is also essential for biofilm formation. *Mol. Microbiol.* 40, 1215-1226.

Peatzel, M., Dalbey, R.E. and Strynadka, C.J. (2000). The structure and mechanism of bacterial type I signal peptidases: A novel antibiotic target. *Pharm. Therap.* 87: 27-49.

Piette, J.P. and Odziak, E.S. (1992). A model study of factors involved in adhesion of P*seudomonas fluorescens* to meat. *Appl. Environ. Microbiol.* 58: 2783-2791.

Planet, P.J., Kachlany, S.C., DeSalle, R. and Figurski, D.H. (2001). Phylogeny of genes for secretion NTPases: Identification of the widespread *tadA* subfamily and development of a diagnostic key for gene classification. *Proc. Natl. Acad. Sci., USA* 98: 2503-2508.

Pratt, L.A. and Kolter, R. (1998). Genetic analysis of *Escherichia coli* biofilm formation: Defining the roles of flagella, motility, chemotaxis and type I pili. *Mol. Microbiol.* 30: 285-294.

Prigent-Combaret, C., Brombacher, E., Vidal, O., Ambert, A., Lejeune, P., Landini, P. and Dorel, C. (2001). Complex regulatory network controls initial adhesion and biofilm development in *Escherichia coli* via regulation of the *csgD* gene. *J. Bacteriol.* 183: 7213-7223.

Prigent-Combaret, C., Vidal, O., Dorel, C. and Lejeune, P. (1999). Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli. J. Bacteriol*. 181: 5993-6002.

Pugsley, A.P. (1993). The complete general secretory pathway in Gram-negative bacteria. *Microbiol. Rev*. 57: 50-108.

Pugsley, A.P. (1996). Multimers of the precursor of a type IV pillin-like component of the general secretory pathway are unrelated to pili. *Mol. Microbiol.* 20: 1235-1245.

Pugsley, A.P., Francetic, O., Possot, O.M., Sauvonnet, N. and Hardie, K.R. (1997). Recent progress and future directions in studies of the main terminal branch of the general secretory pathway in Gram-negative bacteria – a review. *Gene* 192: 13-19.

Purevdorj, B., Costerton, J.W. and Stoodley, P. (2002). Influence of hydrodynamics and cell signaling on the structure and behavior *of Pseudomonas aeruginosa* biofilms. *Appl. Environ. Microbiol.* 68: 4457-4464.

Puskas, A., Greenberg, E.P., Kaplan, S. and Schaefer, A.L. (1997). A quorum-sensing system in the free-living photosynthetic bacterium *Rhodobacter sphaeroides*. *J. Bacteriol.* 179: 7530-7537.

Quirynen, M., Dierickx, K. and Van Steenberghe, D. (2000). Effects of surface roughness and free energy on oral bacterial adhesion. In: *Handbook of Bacterial Adhesion: Principles, Methods and Applications*, pp. 91-102. An, Y.H. and Friedman, R.J. (eds). Humana Press. Inc., Totowa, NJ, USA.

Rahme, L.G., Stevens, E.J., Wolfort, S.F., Shao, J., Tompkins, R.G. and Ausubel, F.M. (1995). Common virulence factors for bacterial pathogenicity in plants and animals. *Science* 268: 1899-1902.

Ramsey, D.M. and Wozniak, D.J. (2005). Understanding the control of *Pseudomonas aeruginosa* alginate synthesis and the prospects for management of chronic infections in cystic fibrosis. *Mol. Microbiol.* 56: 309-322.

Rehmat, S. and Shapiro, J.A. (1983). Insertion and replication of the *Pseudomonas aeruginosa* mutator phage D3112. *Mol. Gen. Genet*. 192: 416-423.

Rice, A.R., Hamilton, M.A. and Camper, A.K. (2000). Apparent surface associated lag time in growth of primary biofilm cells. *Microb. Ecol*. 41: 8-15.

Rice, K.C. and Bayles, K.W. (2003). Death's toolbox: Examining the molecular components of bacterial programmed cell death. *Mol. Microbiol.* 50: 729-738.

Romling, U., Brian, Z., Hammar, M., Sierralta, W.D. and Normark, S. (1998). Curli fibres are highly conserved between *Salmonella typhimurium* and *Escherichia coli* with respect to open structures and regulation. *J. Bacteriol.* 180: 722-731.

Rudel, T., Scheurepflug, I. and Meyer, T.F. (1995). *Neisseria* PilC protein identified as type-4 pilus tip-located adhesin. *Nature* 373: 357-359.

Russel, M. (1998). Macromolecular assembly and secretion across the bacterial cell envelope: Type II protein secretion systems. *J. Mol. Biol.* 279: 485-499.

Russell, M.A. and Darzins, A. (1994). The *pilE* gene product of *Pseudomonas aeruginosa*, required for pilus biogenesis, shares amino acid sequence identity with the N-terminal of type 4 prepilin proteins. *Mol. Microbiol.* 13: 973-985.

Ryll, R.R., Rudel, T., Scheuerpflug, I., Barten, R. and Meyer, T.F. (1997). PilC of *Neisseria meningitides* is involved in class II pilus formation and restores pilus assembly, natural transformation competence and adherence to epithelial cells in PilC-deficient gonococci. *Mol. Microbiol.* 23: 879-892.

Salmond, G.P.C. and Reeves, P.J. (1993). Membrane traffic wardens and protein secretion in Gram-negative bacteria. *Trends Biochem. Sci.* 18: 7-12.

Sambrook, J., Fritsch, E.F. and Maniates, T. (1989). Molecular cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.

Sauer, F.G., Mulvey, M.A., Schilling, J.D., Martinez, J.J. and Hultgren, S.J. (2000). Bacterial pili: Molecular mechanisms of pathogenesis. *Curr. Opin. Microbiol.* 10: 548-556.

Sauer, K. and Camper, A.K. (2001). Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. *J. Bacteriol.* 183: 6579-6589.

Sauer, K., Camper, A.K., Ehrlich, G.D., Costerton, J.W. and Davies, D.G. (2002). *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J. Bacteriol.* 184: 1140-1154.

Sauer, K., Cullen, M.C., Rickard, A.H., Zeef, L.A., Davies, D.G. and Gilbert, P. (2004). Characterization of nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm. *J. Bacteriol.* 186: 7312-7326.

Saulino, E.T., Thanassi, D.G., Pinker, J.S. and Hultgren, S.J. (1998). Ramifications of kinetic partitioning on usher-mediated pilus biogenesis. *EMBO J.* 17: 2177-2185.

Schagger, H. and von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166: 368-379.

Scharfman, A., Kroczynski, H., Carnoy, C., Van Brussel, E., Lamblin, G., Ramphal, R. and Roussel, P. (1996). Adhesion of *Pseudomonas aeruginosa* to respiratory mucins and expression of mucin-binding proteins are increased by limiting iron during growth. *Infect. Immun*. 64: 5417-5420.

Schilling, J.D., Mulvey, M.A. and Hultgren, S.J. (2001). Structure and function of *Escherichia coli* type 1 pili: New insight into pathogenesis of urinary tract infections. *J. Inf. Dis.* 183(Suppl 1): S36-40.

Schmid, M.B. and von Freiesleben, U. (1996). Nucleoid segregation. In: *Escherichia coli and Salmonella: Cellular and Molecular Biology*, Vol. 2, pp. 1662-1671. Neihardt, F.C. (ed.). ASM Press, Washington, D.C., USA.

Schmoll, T., Ott, M., Oudega, B. and Hacker, J. (1990). Use of a wild-type gene fusion to determine the influence of environmental conditions on expression of the S fimbrial adhesin in an *Escherichia coli* pathogen. *J. Bacteriol.* 172: 5103-5111.

Schreiner, H.C., Sinatra, K., Kaplan, J.B., Furgang, D., Kachlany, S.C., Planet, P.J., Perez, B.A., Figurski, D.H. and Fine D.H. (2003). Tight-adherence genes *of Actinobacillus actinomycetemcomitans* are required for virulence in a rat model. *Proc. Natl. Acad. Sci., USA* 100: 7295-7300.

Schroth, M.N., Hildebrand, D.C. and Panopoulos, N. (1992). Phytopathogenic pseudomonads and related plant-associated pseudomonads. In: *The Prokaryotes*, pp. 3104-3131. Balows, A., Truper, H.G., Dworkin, M., Harder, W. and Schleifer K-H. (eds). Springer-Verlag, New York, USA.

Schuster, M., Lostroh, C.P., Ogi, T. and Greenberg, E.P. (2003). Identification, timing and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: A transcriptome analysis. *J. Bacteriol.* 185: 2066-2079.

Shimkets, L.J. (1999). Intercellular signalling during fruiting-body development of *Myxococcus xanthus*. *Annu. Rev. Microbiol.* 53: 525-549.
Skerker, J.M. and Shapiro, L. (2000). Identification and cell cycle control of a novel pilus system in *Caulobacter crescentus*. *EMBO J.* 13: 3223-3234.

Slots, J. and Ting, M. (1999). *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in human periodontal disease: Occurrence and treatment. *Periodontol.* 20: 82-121.

Smith, D.B. and Johnson, K.S. (1989). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase. *Gene* 67: 31-39.

Smyth, C.J., Marron, M.B., Twohig, J.M. and Smith, G.J. (1996). Fimbrial adhesins: Similarities and variations in structure and biogenesis. *FEMS Immun. Med. Microbiol.* 16: 127-139.

Soto, G.E. and Hultgen, S.J. (1999). Bacterial adhesions: Common themes and variations in architecture and assembly. *J. Bacteriol.* 181: 1059-1071.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98: 503-517.

Spinola, S.M., Fortney, K.R., Katz, B.P., Latimer, J.L., Mock, J.R., Vakevainen, M. and Hansen, E.J. (2003). *Haemophilus ducreyi* requires an intact *flp* gene cluster for virulence in humans. *Infect. Immun.* 71: 7178-7182.

Stanley, N.R. and Lazazzera, B.A. (2004). Environmental signals and regulatory pathways that influence biofilm formation. *Mol. Microbiol.* 52: 917-924.

Steyn, B., Oosthuizen, M.C., MacDonald, R., Theron, J. and Brözel, V.S. (2001). The use of glass wool as an attachment surface for studying phenotypic changes in *Pseudomonas aeruginosa* biofilms by two-dimensional gel electrophoresis. *Proteomics* 1: 871-879.

Stoodley, P., Sauer, K., Davies, D.G. and Costerton, J.W. (2002). Biofilms as complex differentiated communities. *Annu. Rev. Microbiol.* 56: 187-209.

Stover K.C., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Hichey, M.J., Brinkman, F.S.L., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., Garber, R.L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L.L., Coulter, S.N., Folger, K.R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G.K.-S., Wu, Z., Paulsen, I., Reizer, J., Saier, M.H., Hancock,

R.E.W., Lory, S. and Olson, M.V. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1: An opportunistic pathogen. *Nature* 406: 959-964.

Strom, M. and Lory, S. (1992). Kinetics and sequence specificity of processing of prepilin by PilD, the type IV leader peptidase of *Pseudomonas aeruginosa*. *J. Bacteriol.* 174: 7345-7351.

Strom, M. and Lory, S. (1993). Structure-function and biogenesis of type IV pili. *Annu. Rev. Microbiol.* 47: 565-596.

Studholme, D.J. and Buck, M. (2000). The biology of enhancer-dependent transcriptional regulation in bacteria: Insights from genomic sequences. *FEMS Microbiol. Lett.* 186: 1-9.

Suh, S.J., Silo-Suh, L., Woods, D.E., Hassett, D.J., West, S.E. and Ohman, D.E. (1999). Effects of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. *J. Bacteriol.* 181: 3890-3897.

Sutherland, I.W. (1999). Biofilm exopolysaccharides. In: *Microbial Extracellular Polymeric Substances: Characterization, Structure and Function*, pp. 73-92. Wingender, J., Neu, T.R. and Flemming, H-C. (eds). Springer-Verlag, Berlin, Germany.

Switzer, R.C., Merril, C.R. and Shifrin, S. (1979). A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. *Anal. Biochem.* 98: 231-237.

Tajima, T., Yokota, N., Matsuyama, S. and Tokuda, H. (1998). Genetic analyses of the *in vivo* function of LolA, a periplasmic chaperone involved in the outer membrane localization of *Escherichia coli* lipoproteins. *FEBS Lett.* 439: 51-54.

Taylor, R.K., Miller, V.L., Furlong, D.B. and Melakanos, J.J. (1987). Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. *Proc. Natl. Acad. Sci., USA* 84: 2833-2837.

Thanassi, D.G. and Hultgen, S.J. (2000). Multiple pathways allow protein secretion across the bacterial outer membrane. *Curr. Opin. Cell. Biol.* 12: 420-430.

Thanassi, D.G., Saulino, E.T. and Hultgren, S.J. (1998). The chaperone/usher pathway: A major terminal branch of the general secretory pathway. *Curr. Opin. Microbiol.* 1: 223-231.

Theron, J., Uitenweerde, J.M., Huismans, H. and Nel, L.H. (1994). Comparison of the expression and phosphorylation of the nonstructural protein NS2 of three different Orbiviruses: Evidence for the involvement of an ubiquitous cellular kinase. *J. Gen. Virol.* 75: 3401-3411.

Toder, D.S. (1994). Gene replacement in *Pseudomonas aeruginosa*. *Methods Enzymol.* 235: 466-474.

Tolker-Nielsen, T., Brinch, U.C., Ragas, P.C., Andersen, J.B., Jacobsen, C.S. and Molin, S. (2000). Development and dynamics of *Pseudomonas* sp. biofilms. *J. Bacteriol.* 182: 6482-6489.

Tommassen, J., Filloux, A., Bally, M., Murgier, M. and Lazdunski, A. (1992). Protein secretion in *Pseudomonas aeruginosa*. *FEMS Microbiol. Rev.* 103: 73-90.

Tonjum, T. and Koomey, M. (1997). The pilus colonization factor of pathogenic neisserial species: Organelle biogenesis and structure/function relationships - a review. *Gene* 192: 155-163.

Turner, L.R., Lara, J.C., Nunn, D.N. and Lory, S. (1993). Mutation in the consensus ATP-binding sites of XcpR and PilB eliminate extracellular protein secretion and pilus biogenesis in *Pseudomonas aeruginosa*. *J. Bacteriol.* 175: 4962-4969.

Vallet, I., Olson, J.W., Lory, S., Lazdunski, A. and Filloux, A. (2001). The chaperone/usher pathways of *Pseudomonas aeruginosa*: Identification of fimbrial gene clusters (cup) and their involvement in biofilm formation. *Proc. Natl. Acad. Sci., USA* 98: 6911-6916.

Van Delden, C. and Iglewski, B.H. (1998). Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. *Emerg. Infect. Dis.* 4: 551-560.

Van Loodsdrecht, M.C.M., Lyklema, J., Norde, W. and Zehnder, A.J.B. (1990). Influence of interfaces on microbial activity. *Microbiol. Rev.* 54: 75-87.

Van Schalkwyk, A. (2003). Characterization of a putative pilus assembly and secretion system in *Pseudomonas aeruginosa* DSM1707. M.Sc Thesis, Department of Microbiology and Plant Pathology, University of Pretoria, South Africa.

Vidal, O., Longin, R., Prigent-Combaret, C., Dorel, C., Hooreman, M. and Lejeune, P. (1998). Isolation of an *Escherichia coli* K-12 mutant strain able to form biofilms on inert surfaces: Involvement of a new *ompR* allele that increases curli expression. *J. Bacteriol.* 180: 2442-2449.

Wagner, E.V., Bushnell, D., Passador, L., Brooks, A.I. and Iglewski, B.H. (2003). Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: Effects of growth phase and environment. *J. Bacteriol.* 185: 2080-2095.

Wagner, E.V., Gillis, R.J. and Iglewski, B.H. (2004). Transcriptome analysis of quorum-sensing regulation and virulence factor expression in *Pseudomonas aeruginosa*. *Vaccine* 22S: S15-20.

Walker, J.E., Saraste, M., Runswick, M.J. and Gay, N.J. (1982). Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* 1: 945-951.

Wall, D. and Kaiser, D. (1999). Type IV pili and cell motility. *Mol. Microbiol.* 32: 1-10.

Watnick, P.I., Fulner, K.J. and Kolter, R. (1999). A role for the mannose-sensitive hemagglutinin in biofilm formation by *Vibrio cholera* El Tor. *J. Bacteriol.* 181: 3606-3609.

Watson, A., Alm, R.A. and Mattick, J.S. (1996a). Identification of a gene, *pilF*, that is required for type 4 fimbrial biogenesis and twitching motility in *Pseudomonas aeruginosa. Gene* 180: 49-56.

Watson, A., Alm, R.A. and Mattick, J.S. (1996b). Functional expression of heterologous type 4 fimbriae in *Pseudomonas aeruginosa. Gene* 175: 143-150.

Webb, J.S., Givskov, M. and Kjelleberg, S. (2003a). Bacterial biofilms: Prokaryotic adventures in multicellularity. *Curr. Opin. Microbiol.* 6: 578-585.

Webb, J.S., Thompson, L.S., James, S., Charlton, T., Tolker-Nielsen, T., Koch, B., Giskov, M. and Kjelleberg, S. (2003b). Cell death in *Pseudomonas aeruginosa* biofilm development. *J. Bacteriol.* 185: 4585-4592.

Werner, E., Roe, F., Bugnicourt, A., Franklin, M.J., Heydorn, A., Molin, S., Pitts, B. and Stewart, P.S. (2004). Stratified growth in *Pseudomonas aeruginosa* biofilms. *Appl. Environ. Microbiol.* 70: 6188-6196.

Wessel, D. and Flügge, U.I. (1984). A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* 138: 141-143.

Weyers, J.B. (1999). Isolation and charactrization of attachment-induced regulatory elements in *Pseudomonas aeruginosa* PAO1. M.Sc Thesis, Department of Microbiology, University of the Western Cape, South Africa.

Whitchurch, C.B. and Mattick, J.S. (1994). Characterization of a gene *pilU* required for twitching motility but not phage sensitivity in *Pseudomonas aeruginosa. Mol. Microbiol.* 13: 1079-1091.

Whitchurch, C.B., Hobbs, M., Livingston, S.P., Krishnapillai, V. and Mattick, J.S. (1991). Characterization of a *Pseudomonas aeruginosa* twitching motility gene and evidence for a specialized protein export system widespread in eubacteria. *Gene* 101: 33-44.

Whiteley, M., Bangera, M.G., Bumgarner, R.E., Parsek, M.R., Teitzel, G.M., Lory, S. and Greenberg, E.P. (2001). Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* 413: 860-864.

Whiteley, M. and Greenberg, E.P. (2001). Promoter specificity elements in *Pseudomonas aeruginosa* quorum sensing-controlled genes. *J. Bacteriol.* 183: 5529-5534.

Wu, S.S., Wu, J. and Kaiser, D. (1997). The *Myxococcus xanthus pilT* locus is required for social gliding motility although pili are still produced. *Mol. Microbiol.* 23: 109-121.

Yarwood, J.M., Bartels, D.J., Volper, E.M. and Greenberg, E.P. (2004). Quorum sensing in *Staphylococcus aureus* biofilms. *J. Bacteriol.* 186: 1838-1850.

Zielinski, N.A., Maharaj, R., Roychoudhury, S., Danganan, C.E., Hendrickson, W. and Chakrabarty, A.M. (1992). Alginate synthesis in *Pseudomonas aeruginosa*: Environmental regulation of the *algC* promoter. *J. Bacteriol.* 174: 7680-7688.

Zobell, C.E. (1943). The effect of solid surfaces on bacterial activity. *J. Bacteriol.* 46: 39-56.