CHAPTER FIVE

CONSTRUCTION AND CHARACTERISATION OF AN OprG-DEFICIENT MUTANT STRAIN OF *Pseudomonas aeruginosa* PAO1 (DSM 1707)

5.1 INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous gram-negative bacterium and an important opportunistic pathogen of humans, causing serious infections in immunocompromised patients such as those with cancer or AIDS, and patients suffering from cystic fibrosis and severe burns (Van Delden and Iglewski, 1998). The success of *P. aeruginosa* to grow in diverse environmental niches is attributed to its broad metabolic diversity and its many cell-associated and secreted virulence factors (Lazdunski *et al.*, 1990; Van Delden and Iglewski, 1998), as well as its ability to attach to both biotic and abiotic surfaces with the subsequent development of biofilms (Watnick and Kolter, 2000; 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 analysing biofilms using genetic (O'Toole and Kolter, 1998; 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 rate and extent of attachment of bacterial cells to a surface is influenced, amongst other, by cell surface hydrophobicity, presence of flagella, pili and adhesins, and production of extracytoplasmic polymeric substances (O'Toole and Kolter, 1998; DeFlaun *et al.*, 1999; Genevaux *et al.*, 1999; Espinosa-Urgel *et al.*, 2000). Furthermore, bacterial membrane proteins have been reported to influence attachment and may also play a role in early biofilm development. Mutations in surface and membrane proteins caused defects in the attachment of *P. putida* to corn (Espinosa-Urgel *et al.*, 2000), whilst attachment to abiotic surfaces was shown in *E. coli* to cause major changes in outer membrane composition (Otto *et al.*, 2001). Using a proteomic approach, Otto *et al.* (2001) demonstrated increased levels for 17 outer membrane proteins and decreased levels for 15. In *P. aeruginosa*, loss of porin OpdF has detrimental effects on the ability of the bacteria to form biofilms (Finelli *et al.*, 2003), whereas loss of the major outer membrane porin OprF results in poor biofilm formation under

anaerobic conditions (Yoon *et al.*, 2002). In addition to these two porins, several other outer membrane proteins (OMPs) have been reported to be differentially expressed in planktonic versus biofilm cells. Expression of OprE1 (Sauer *et al.*, 2002) and OprF (this study) has been shown to be up-regulated in *P. aeruginosa* biofilms, whilst expression of several other OMPs such as OprB, OpdT, OpmH and OpdP were down-regulated in the biofilm population (this study; Chapter 4).

The results obtained in the preceding Chapter indicated the presence of two isoforms of the outer membrane protein porin OprG of which the expression was up-regulated in the biofilm population and planktonic population, respectively. The appearance of OprG in the outer membrane is highly dependent on growth conditions. In particular, Yates *et al.* (1989) observed a direct relationship between the iron concentration in the medium and expression of OprG, and suggested that this OMP is involved in low-affinity iron uptake. Other conditions, including growth into the stationary phase, higher growth temperatures, Mg^{2+}-deficiency, certain lipopolysaccharide alterations and the presence of carbon sources, also result in the expression of varying concentrations of OprG (Hancock *et al.*, 1990; Hancock and Brinkman, 2002). Such apparently broad regulation of this OMP has hampered efforts to assigning a function to OprG.

Based on recent reports indicating a role for different OMPs in *P. aeruginosa* biofilm development (Yoon *et al.*, 2002; Finelli *et al.*, 2003), the aims of this study were to generate a *P. aeruginosa* PAO1 (DSM 1707) mutant strain deficient in OprG and to compare its ability to form biofilms on a glass wool substratum with that of the wild-type *P. aeruginosa* strain. OprG, encoded by PA4067, was selected, as it was the only other OMP that displayed up-regulation in biofilms, and, unlike OprF, has not yet been investigated for its involvement in biofilm formation.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial strains, plasmids and culture conditions

Bacterial strains, plasmids and primers used in this study are listed in Table 5.1. *Escherichia coli* and *Pseudomonas aeruginosa* strains were routinely cultivated at 37°C on a rotary shaker (200 rpm) in Luria Bertani (LB) broth (0.5% [w/v] yeast extract; 1% [w/v] tryptone; 1% [w/v]

NaCl, pH 7.4). For comparative studies, *P. aeruginosa* PAO1 (DSM 1707) and mutant strains were grown in a modified mineral salts medium with glucose and yeast extract (MSGY) of the following composition (in distilled water): 1.74 g/L $NaNH_4HPO_4.4H_2O$; 0.54 g/L $NaH_2PO_4.H_2O$; 0.2 g/L $MgSO_4.7H_2O$; 0.2 g/L yeast extract; 0.04 g/L KCl; 0.005 g/L $FeSO_4.7H_2O$; 5.0 g/L glucose and 1% (v/v) trace mineral solution (2.86 g/L H_3BO_3; 1.81 g/L $MnCl_2.4H_2O$; 0.22 g/L $ZnSO_4.7H_2O$; 0.08 g/L $CuSO_4.5H_2O$; 0.06 g/L $CoCl_2.6H_2O$; 0.025 g/L $Na_2MoO_4.2H_2O$) (Atlas, 1993). The following antibiotics were used to maintain the plasmid DNA and chromosomal insertions in *P. aeruginosa* strains: gentamicin at 50 µg/ml, tetracycline at 10 µg/ml and carbenicilin at 200 µg/ml. 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. All antibiotics were purchased from Sigma-Aldrich (St Louis, MO, USA).

5.2.2 Genomic DNA isolation

The genomic DNA of wild-type and mutant *P. aeruginosa* PAO1 (DSM 1707) strains was isolated using cetyltrimethylammonium bromide (CTAB), as described by Jansen (1995). Briefly, the cells from 500 µl of an overnight culture were collected by centrifugation at 8 000 × *g* for 3 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 the proteins were digested by 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 was added and incubation was continued for 10 min at 65°C. The CTAB-protein and -polysaccharide complexes were removed by extraction with an equal volume of chloroform/isoamyl alcohol (24:1), followed by centrifugation at 8 000 × *g* for 5 min. The supernatant, containing the genomic DNA, was recovered and transferred to a clean microfuge tube. The remaining CTAB was removed by addition of an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) followed by centrifugation (8 000 × *g*, 5 min). The chromosomal DNA was precipitated from the recovered supernatant by addition of 0.6 volume isopropanol. The precipitated chromosomal DNA was pelleted by brief centrifugation, rinsed with 70% ethanol, dried under vacuum and resuspended in 20 µl UHQ water. An aliquot of the DNA was analysed by electrophoresis on a 0.8% (w/v) agarose gel.

5.2.3 DNA amplification

5.2.3.1 Oligonucleotide primers

The oligonucleotide primers used in PCR assays to amplify the genomic region containing the PA4067 ORF from *P. aeruginosa* PAO1 (DSM 1707), are indicated in Table 5.1. The primers were designed on the basis of the complete genome sequence of *P. aeruginosa* strain PAO1 (Stover *et al.*, 2000). All primers were synthesised by Inqaba Biotechnical Industries (Pretoria, South Africa).

5.2.3.2 Polymerase chain reaction (PCR) amplification of DNA fragments

The reaction mixtures (50 µl) contained 100 ng of *P. aeruginosa* PAO1 (DSM 1707) genomic DNA as template, 20 pmol of each the FOR4067 and REV4067 primer, 1 × PCR buffer (50 mM KCl; 10 mM Tris-HCl [pH 9.0]; 0.1% [v/w] TritonX-100), $MgCl_2$ at 1 mM, each deoxynucleoside triphosphate (dNTP) at a concentration of 0.2 mM, dimethyl sulfoxide (DMSO) at 5% (v/v) and 1 U of *Taq* DNA polymerase (Southern Cross Biotechnology, Cape Town, South Africa). The tubes were placed in a Perkin-Elmer GeneAmp 2400 thermal cycler and following an initial denaturation at 94°C for 5 min, the reactions were subjected to 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C and elongation for 2 min at 72°C. After the last cycle, the reactions were kept for 5 min at 72°C to complete synthesis of all strands. For control purposes, an identical reaction mixture lacking template DNA was included. Aliquots of the PCR reaction mixtures were subsequently analysed by electrophoresis on a 0.8% (w/v) agarose gel in the presence of an appropriate DNA molecular weight marker.

5.2.4 Agarose gel electrophoresis

DNA was analysed by agarose gel electrophoresis (Sambrook *et al.*, 1989). For this purpose, horizontal 0.8% (w/v) agarose slab gels were cast and electrophoresed at 100 V in 1 × TAE buffer (40 mM Tris-HCl; 20 mM NaOAC; 1 mM EDTA, pH 8.5). The agarose gels were supplemented with ethidium bromide (0.5 µg/ml) in order to allow visualisation of the DNA on an UV transilluminator. Where appropriate, the DNA fragments were sized according to their migration in the gel as compared to that of standard DNA molecular weight marker, namely GeneRuler™ DNA Ladder Plus (Fermentas AB, Germany).

Table 5.1 Bacterial strains, plasmids and oligonucleotide primers used in this study

Strain, plasmid or primer	Relevant properties	Reference
Strains:		
E. coli		
INFαF'	F´ *end*A1 *rec*A1 *hsd*R17 (r_k-, m_k+) *sup*E44 *thi*-1 *gyr*A96 *rel*A1 φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 λ-	Invitrogen
HB 101	F–, *thi*-1, *hsd*S20 (r_B–, m_B–), *sup*E44, *rec*A13, *ara*-14, *leu*B6, *pro*A2, *lac*Y1, *gal*K2, *rps*L20 (str^r), *xyl*-5, *mtl*-1	Promega
P. aeruginosa		
DSM 1707	Wild-type, Prototroph (PAO1)	DSM*
DSMOprG	DSM1707OprG::Gm^R	This study
Plasmids:		
pGEM®-T Easy	ColE1, Amp^R, LacZα peptide, cloning vector for PCR amplicons	Promega
pGEM-OprG	pGEM®-T Easy vector containing PA4067 amplicon	This study
pGEM-OprG-Gent	pGEM-OprG with a Gm^R cassette inserted at the *Kpn*I sites of the OprG-encoding ORF	This study
pSS125	pUC19 containing *oriT*	S.J. Suh (unpublished)
pGori-OprG-Gent	pGEM-OprG-Gent with *oriT*	This study
pU8G	Gm^R cassette cloned into pUC4K	J.B. Weyers (unpublished)
pRK600	Cm^R, ColE1, RK2-Mob^+, RK2-Tra^+, helper plasmid in triparental conjugations	Kessler *et al.* (1992)
Primers:		
FOR4067	5'-GGTTTAAAATGCTGCGCTCCCTGAC-3'	
REV4067	5'-CCAAATTTGCATGTTTGAACGCCCC-3'	
GenomicA	5'-CGAGCGACTATGAGTGGAGC-3'	
GenomicB	5'-CAGCACGCGGTGATGGTCTG-3'	
IntRp4067	5'-CGCGTGCTCGTTGAGCATG-3'	
Gentp1-1	5'-GCGGCGTTGTGACAATTTAC-3'	

*DSM – Deutche Sammlung von Mikroorganismen, Braunschweig, Germany

5.2.5 Purification of DNA fragments from agarose gels

DNA fragments were purified from 0.8% (w/v) agarose gels using a silica suspension, as described by Boyle and Lew (1995). Briefly, the DNA band of interest was excised from the agarose gel and mixed with 400 µl of a 6 M NaI solution. The agarose was dissolved by incubation at 55°C for 10 min, whereafter 8 µl of the silica suspension was added to the sample. The DNA was allowed to bind to the silica by incubation of the samples on ice for 30 min with intermittent vortexing. The DNA-silica complex was pelleted by centrifugation (8 000 × *g* for 30 s) and washed four times with Wash buffer (50 mM NaCl; 10 mM Tris-HCl [pH 7.5]; 2.5 mM EDTA; 50% [v/v] ethanol). The DNA was eluted from the silica matrix in a final volume of 7 µl UHQ water by incubation at 55°C for 10 min. The purified DNA fragments were analysed on a 0.8% (w/v) agarose gel to assess both their purity and concentration.

5.2.6 Nucleotide sequencing and sequence analysis

The nucleotide sequence of the gel-purified amplicon was determined using an ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The primers used for PCR amplification were used in the nucleotide sequencing reactions (Table 5.1). Each reaction mixture contained 80-100 ng of gel-purified amplicon, 12 pmol of sequencing primer, 2 µl Terminator Ready Reaction Mix and water in a final volume of 5 µl. Cycle sequencing reactions were performed in a Perkin-Elmer GeneAmp 2400 thermal cycler with 25 of the following cycles: denaturation at 96°C for 10 s, primer annealing at 50°C for 5 s and extension at 60°C for 4 min. Following brief centrifugation, the extension products were precipitated by the addition of 4 µl UHQ water and 16 µl absolute ethanol and incubated at room temperature for 30 min in the dark. The tubes were then centrifuged at 13 000 × *g* for 30 min and the supernatants carefully aspirated. The pellets were rinsed with 50 µl of 70% ethanol, dried under vacuum for 10 min and stored at 4°C. Prior to electrophoresis, the purified extension products were resuspended in 3.5 µl Blue dextran/EDTA loading buffer, denatured for 2 min at 90°C and loaded onto a model 377 automated DNA sequencer (Perkin-Elmer). 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 and BLASTP (Altschul *et al.*, 1997).

5.2.7 Restriction endonuclease digestions

All restriction endonuclease digestions were performed in clean microfuge tubes and contained the appropriate concentration of salt (using the 10× buffer supplied by the manufacturer) for the specific enzyme and 5-10 U of enzyme per µg of plasmid DNA. The reaction volumes were small (10-20 µl) and incubation was typically for 1-1.5 h at 37°C. All restriction enzymes were supplied by Roche or Promega (Promega Corp., Madison, WI, USA). The digestion products were typically analysed on a 0.8% (w/v) agarose gel in the presence of appropriate DNA molecular weight markers.

5.2.8 Cloning of DNA fragments into plasmid vectors

The pGEM®-T Easy vector system (Promega) was used for cloning of PCR amplicons. Ligation of the gel-purified amplicon and pGEM®-T Easy vector was performed at 4°C overnight in a final reaction volume of 10 µl. The reaction mixture contained 50 ng of vector DNA, 250 ng of insert DNA, 3 U of T4 DNA ligase (3 U/µl; Promega), 5 µl of a 2 × DNA ligase buffer and UHQ water. Ligation of specific DNA fragments and vector DNA was performed at 16°C overnight in a final reaction volume of 10 µl, which contained 1 µl of a 10 × DNA ligase buffer (660 mM Tris-HCl [pH 7.5]; 10 mM DTT; 50 mM $MgCl_2$; 10 mM ATP), 1 U of T4 DNA ligase (1 U/µl; Roche) and the ratio of vector to insert was typically in excess of 1:5.

5.2.9 Transformation of competent *E. coli* cells

Competent *E. coli* INFαF' cells (Invitrogen, Carlsbad, CA, USA) were transformed according to the manufacturer's instructions. Briefly, competent cells (50 µl) were mixed with 2 µl of the ligation reaction mixture in a sterile microfuge tube and incubated on ice for 30 min. The cells were subsequently incubated at 42°C for 2 min and then chilled on ice for 2 min. Following the addition of 250 µl of LB medium, the cells were allowed to recover by incubation at 37°C for 1 h. The transformed cells were selected by plating the transformation mixtures on LB agar supplemented with the appropriate antibiotic and incubated at 37°C overnight. When appropriate, the cells were plated together with 10 µl IPTG (100 mM stock solution) and 50 µl X-gal (2% [w/v] stock solution) to allow for blue/white colour selection, based on insertional inactivation of the *lacZ'* marker gene in the pGEM®-T Easy vector (Promega).

5.2.10 Extraction and purification of plasmid DNA

Plasmid DNA was isolated from transformants using the alkaline lysis method, as described by Sambrook *et al.* (1989). Single colonies were inoculated into 5 ml of LB broth containing the appropriate antibiotic and incubated at 37°C overnight. The cells from 3 ml of the cultures were then collected by centrifugation at 13 000 × *g* for 2 min. The bacterial cell pellets were suspended in 100 µl of Solution 1 (50 mM glucose; 25 mM Tris-HCl [pH 8.0]; 10 mM EDTA; 10 mg/ml lysozyme) and incubated at room temperature for 5 min. The resultant spheroplasts were lysed by the addition of 200 µl of freshly prepared Solution 2 (0.2 N NaOH; 1% [w/v] SDS), followed by incubation on ice for 5 min. Subsequently, 150 µl of Solution 3 (3 M sodium acetate, pH 4.8) was added and incubation was continued on ice for a further 10 min. The cell debris was removed by centrifugation at 13 000 × *g* for 10 min and the supernatant transferred to a clean microfuge tube. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, mixed and the organic and aqueous phases separated by centrifugation at 13 000 × *g* for 5 min. The upper aqueous phase was recovered and extracted with an equal volume of chloroform. The plasmid DNA was finally precipitated from the aqueous phase by the addition of 2.5 volumes absolute ethanol, followed by incubation for 30 min at -70°C. The plasmid DNA was recovered by centrifugation, washed with 70% ethanol and dried under vacuum before being suspended in 30 µl UHQ water. The plasmid DNA was incubated with 1 µl RNase A (10 mg/ml) for 1 h at 37°C to remove any contaminating RNA, and stored at -20°C until needed.

5.2.11 Construction of allelic exchange vector

All molecular cloning techniques employed in the construction of the allelic exchange vector were performed according to the procedures described in the preceding sections. All plasmid constructs were confirmed by restriction endonuclease digestion using agarose gel electrophoresis and by nucleotide sequencing. The cloning strategy employed in the construction of the allelic exchange vector pGori-OprG-Gent is indicated diagrammatically in Fig. 5.1.

5.2.11.1 Construction of recombinant plasmid pGEM-OprG

PCR using oligonucleotide primer pair FOR4067 and REV4067, together with chromosomal DNA from *P. aeruginosa* PAO1 (DSM 1707), resulted in the amplification of a 2.734-kb

DNA fragment containing the full-length *oprG* gene. The amplicon was purified from an agarose gel and cloned into pGEM®-T Easy, resulting in the generation of the recombinant plasmid pGEM-OprG.

5.2.11.2 Construction of allelic exchange vector pGori-OprG-Gent

Recombinant plasmid pGEM-OprG was digested with *Kpn*I, which cuts twice in *oprG* only, thus excising a DNA fragment of 207 bp. The 5' protruding ends of the restricted vector DNA were subsequently blunt-ended at 37°C for 30 min after the addition of 2 U Klenow DNA polymerase (2 U/µl; Roche) and 1 µl of a dNTP mixture containing 250 µM of each dNTP. The gentamicin resistance cassette was recovered from plasmid pU8G by digestion with *Eco*RI and blunt-ended, as described above, before being ligated into the deletion site of plasmid pGEM-OprG to yield pGEM-OprG-Gent. To complete the construction of allelic exchange vector pGori-OprG-Gent, an *oriT*-containing DNA fragment was recovered from plasmid pSS125 by digestion with *Xba*I and cloned into pGEM-OprG-Gent, which had been linearised by digestion with *Spe*I.

5.2.12 Generation of mutant *P. aeruginosa* PAO1 (DSM 1707) strains

The allelic exchange vector pGori-OprG-Gent was introduced into *P. aeruginosa* PAO1 (DSM 1707) by triparental conjugation, as described by Kessler *et al*. (1992). A single colony of freshly streaked cultures of donor (*E. coli* INFαF' containing the allelic exchange vector), helper (*E. coli* HB 101 containing pRK600) and recipient (*P. aeruginosa*) 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 onto LB agar supplemented with 50 µg/ml gentamicin and 10 µg/ml tetracycline. The agar plates were then incubated at 37°C for a further 24 to 48 h. Single colonies were subsequently replica-plated onto LB agar plates supplemented with 200 µg/ml carbenicilin and incubated at 37°C for 24 h. Colonies that were unable to grow on plates containing 200 µg/ml carbenicilin were selected from the replica plates and maintained on LB agar plates containing 50 µg/ml gentamicin. One of these, designated DSMOprG, was selected and used in subsequent investigations.

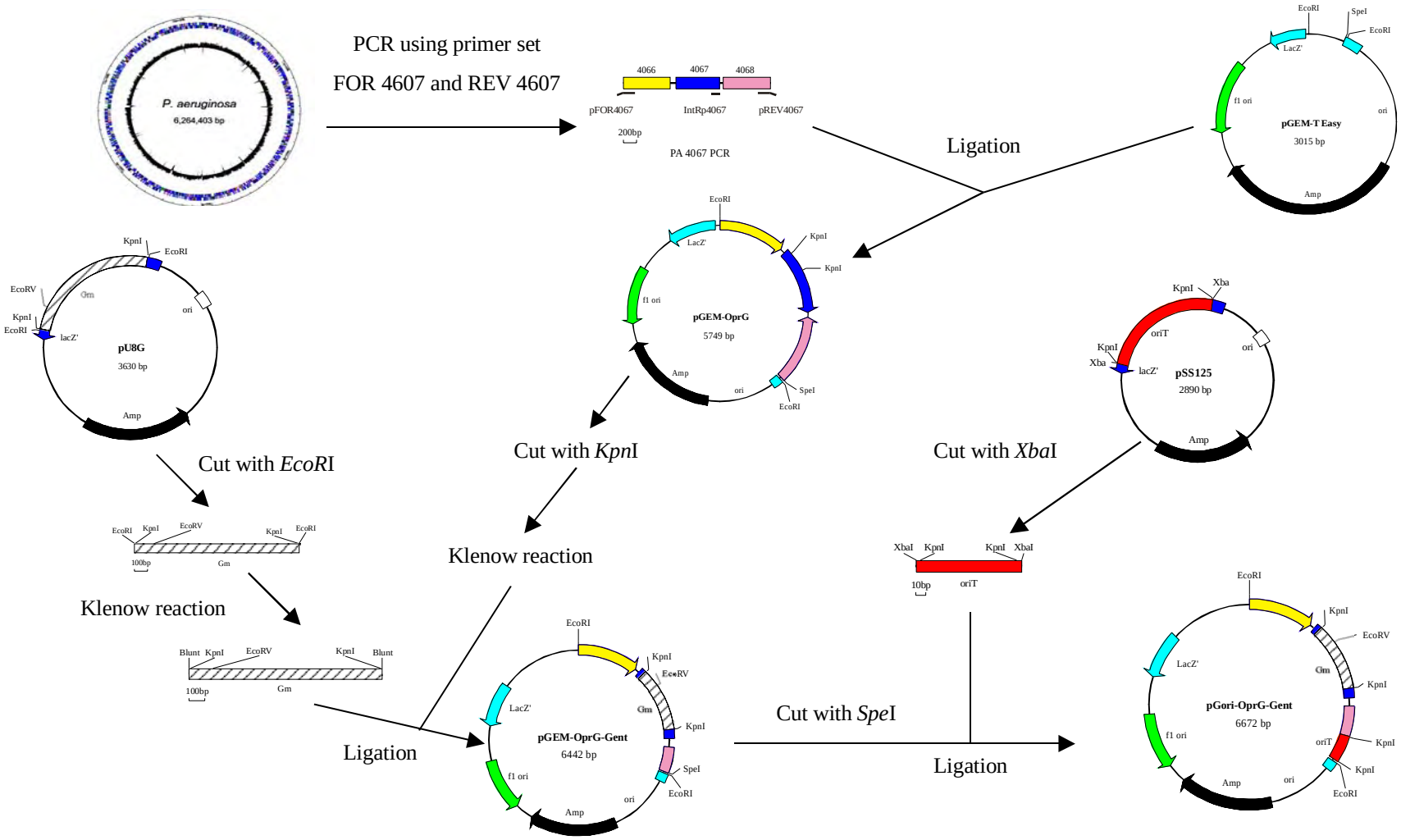


Fig. 5.1 Diagrammatic representation of the cloning strategy used to construct the allelic exchange vector pGori-OprG-Gent.

5.2.13 Characterisation of mutant *P. aeruginosa* PAO1 (DSM 1707) strains

5.2.13.1 Oligonucleotide primers

The DSMOprG mutant strain was analysed for the presence of the gentamicin resistance cassette within the *oprG* gene by PCR analyses. Oligonucleotide primers GenomicB and Gentp1-1 (Table 5.1) were used to amplify a hybrid amplicon consisting of the 5' end of the gentamicin resistance cassette and the 3' end of the interrupted *oprG* gene, whereas oligonucleotide primers GenomicA and IntRp4067 (Table 5.1) were used to amplify a hybrid amplicon containing the 3' end of the gentamicin resistance cassette and the 5' end of the interrupted *oprG* gene. Oligonucleotide primer pair GenomicA and GenomicB (Table 5.1) were also used to amplify the *oprG* gene interrupted by the gentamicin resistance cassette in mutant chromosomal DNA.

5.2.13.2 PCR amplification

The PCR reaction mixtures (50 µl) contained 100 ng of chromosomal DNA, 1 × PCR buffer (50 mM KCl; 10 mM Tris-HCl [pH 9.0]; 0.1% [v/v] TritonX-100), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 25 pmol of each the sense and antisense primer and 1 U of *Taq* DNA polymerase (Southern Cross Biotechnology). The PCR reaction mixtures were placed in a Perkin-Elmer GeneAmp 2400 thermocycler using the following temperature profile: initial denaturation for 5 min at 94°C, followed by 30 cycles of denaturation for 1 min at 94°C, primer annealing for 60 s at 55°C (primers GenomicB - Gentp1-1 as well as primers GenomicA - IntRp4067) or 60°C (primers GenomicA - GenomicB), and elongation for 3 min at 72°C. After the last cycle, the reactions were kept at 72°C for 5 min to complete synthesis of all strands. 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 analysed by electrophoresis on 0.8% (w/v) agarose gels.

5.2.14 Batch assays of the *P. aeruginosa* DSMOprG mutant strain

5.2.14.1 Determination of bacterial growth curves

The *P. aeruginosa* wild-type PAO1 (DSM 1707) and mutant DSMOprG strains were cultured at 37°C overnight on a rotary shaker (200 rpm) in MSGY broth. The overnight cultures were diluted in fresh broth and incubated until mid-exponential phase was reached (OD_{600} = 0.1).

The cultures were subsequently used to inoculate 100 ml of MSGY broth in 250-ml Erlenmeyer flasks to an optical density of 0.03. The flasks were incubated at 37°C on a rotary shaker (200 rpm) and the optical density at 600 nm of each culture was determined at different time intervals.

5.2.14.2 Biofilm formation

The ability of the *P. aeruginosa* wild-type PAO1 (DSM 1707) and mutant DSMOprG 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 50 ml of MSGY broth. The flasks were incubated at 37°C on a rotary shaker (200 rpm) until mid-exponential phase was reached (OD_{600} = 0.1) and then used to inoculate 50 ml of MSGY broth in 100-ml Erlenmeyer flasks, with and without 1.25 g glass wool (mean diameter 15 µm, total surface area 1 725 cm^2) (Merck, Darmstadt, Germany), 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, 18 h and 26 h after inoculation. The glass wool was stained with 0.01% (w/v) crystal violet and immediately viewed by bright-field microscopy using a Zeiss Axiovert 200 fluorescent microscope (Zeiss Axioskop, Zeiss, Oberkochen, Germany). Images were captured using a Nikon charge-coupled device (CCD) camera (Nikon Instech Co., Kanagawa, Japan). In addition, the propensity of the wild-type and mutant *P. aeruginosa* strains to form biofilms on glass wool in MSGY broth was also investigated over a period of 26 h of culturing. 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 surface influenced planktonic (SIP) cells. Following careful rinsing of the glass wool with a small volume of MSGY broth, the attached (biofilm) cells were then removed from the glass wool by adding 50 ml sterile MSGY broth and vortexing for 5 min. The supernatant was recovered and the removal of the biofilms cells from the glass wool substratum was verified by light microscopy. The optical density at 600 nm of both the attached (biofilm) and SIP populations was subsequently determined. These assays were performed in triplicate.

5.3 RESULTS

5.3.1 Construction of allelic exchange vector pGori-OprG-Gent

5.3.1.1 Construction of plasmid pGEM-OprG

Oligonucleotide primers FOR4067 and REV4067 were used in a PCR with chromosomal DNA of *P. aeruginosa* PAO1 (DSM 1707), as described under Materials and Methods (Section 5.2.3). To allow for a sufficient amount of sequence similarity with the chromosomal DNA that would permit homologous recombination between the *oprG* gene, following its inactivation by a marker gene, and chromosomal gene, primers FOR4067 and REV4067 were designed to anneal at the 5' ends of PA4066 (encoding a hypothetical protein) and PA4068 (encoding an epimerase), respectively. Following PCR, an aliquot of the reaction mixture was analysed by agarose gel electrophoresis and a single discreet amplicon of *ca.* 2.734 kb was observed (Fig. 5.2, lane 2). This corresponded in size to the full-length PA4066 (0.518 kb), *oprG* (0.698 kb) and PA4068 (0.929 kb) genes, together with intergenic regions totalling 0.589 kb. In contrast, no amplification product was observed in the negative control in which template DNA was omitted (Fig. 5.2, lane 3). The gel-purified amplicon was subsequently cloned into pGEM®-T Easy vector DNA and restriction endonuclease digestion of the derived recombinant plasmid DNA with *Eco*RI, which flanks the cloning site of the vector, resulted in the excision of a 2.734-kb DNA fragment (not shown). A recombinant clone, designated pGEM-OprG, was selected and the integrity of the cloned amplicon was verified by nucleotide sequencing of both terminal ends prior to it being used in subsequent DNA manipulations.

5.3.1.2 Construction of the allelic exchange vector pGori-OprG-Gent

Since double crossover events that incorporate a cloned gene from a plasmid into the chromosome of an organism 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 *oprG* gene of *P. aeruginosa* consists of the gene *aacC1*, which encodes the enzyme 3-*N*-aminoglycoside acetyltransferase, and is flanked by transcriptional and translational stop signals (Luckow *et al.*, 1993). By making use of the *aacC1* gene, it would thus be possible to rapidly and directly

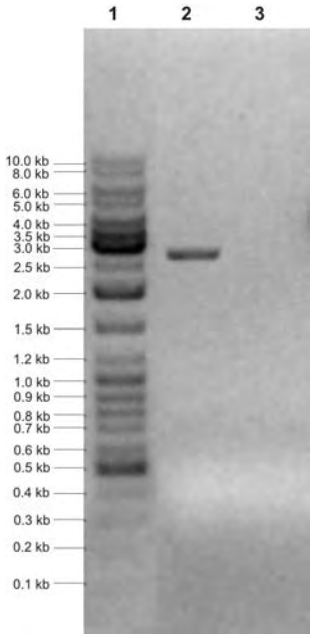


Fig. 5.2 **Agarose gel electrophoretic analysis of the amplicon obtained by PCR amplification using *P. aeruginosa* chromosomal DNA as template and primers FOR4067 and REV4068. Lane 1, DNA molecular weight marker; lane 2, sample of the reaction mixture following PCR; lane 3, negative control PCR reaction mixture lacking template DNA. The sizes of the DNA molecular weight marker, GeneRuler™ DNA Ladder Plus, are indicated to the left of the figure.**

screen for *P. aeruginosa* mutant strains based on their newly acquired resistance to gentamicin.

Recombinant plasmids pGEM-OprG and pU8G served as sources for the construction of the allelic exchange vector pGori-OprG-Gent (Fig. 5.3a). Digestion of recombinant plasmid pGEM-OprG with *Kpn*I, which cuts twice in *oprG*, yielded two DNA fragments corresponding in size to 5.543 and 0.207 kb. The larger of the two DNA fragments was excised from the agarose gel and purified using a silica suspension. The full-length 900-bp gentamicin resistance cassette was recovered from plasmid pU8G by digestion with *Eco*RI. Since restriction of the vector and insert DNA yielded incompatible termini, the DNA fragments were converted to blunt-ended DNA fragments by treatment with Klenow DNA polymerase prior to ligation. The gel-purified DNA fragments were subsequently ligated and following transformation of competent *E. coli* INFαF' cells, plasmid DNA from gentamicin-resistant transformants were characterised by agarose gel electrophoresis and by restriction enzyme digestion. Digestion of the recombinant plasmid DNA with *Eco*RV, which cuts once at the 5' end of the gentamicin resistance cassette only, yielded a linear DNA fragment of 6.442 kb (Fig. 5.3b, lane 4). These results indicated that the gentamicin resistance cassette had been cloned successfully. One of the recombinant clones was selected for further use and designated pGEM-OprG-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 pGEM-OprG-Gent vector backbone. Consequently, the recombinant vector was linearised by digestion with *Spe*I and the *oriT* was cloned from plasmid pSS125 as a 230-bp *Xba*I DNA fragment. Although *Spe*I and *Xba*I generate compatible termini, the resultant hybrid site does not constitute a target for either of the restriction endonuleases. Therefore, plasmid DNA from randomly selected gentamicin-resistant transformants was screened by digestion with *Kpn*I, which cuts twice in *oprG* and twice in the cloned *oriT* DNA fragment. Digestion of the recombinant plasmid DNA containing the cloned *oriT* yielded DNA fragments corresponding to 3.859, 1.683, 0.9 and 0.230 kb (Fig. 5.3b, lane 7). In contrast, recombinant plasmid DNA lacking the cloned *oriT* yielded two DNA fragments of 5.542 and 0.9 kb, corresponding in size to the recombinant vector backbone and the cloned gentamicin resistance cassette, respectively (Fig. 5.3b, lane 6). These results therefore confirmed that the *oriT* was successfully cloned. One of the recombinant clones was selected for further use and designated pGori-OprG-Gent.

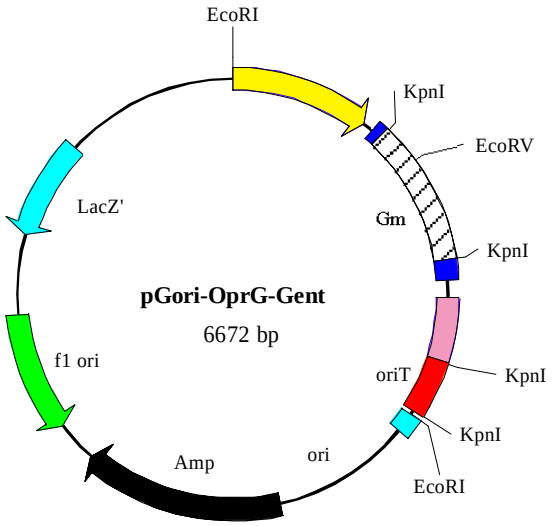


Fig. 5.3a Plasmid map of the recombinant allelic exchange vector pGori-OprG-Gent.

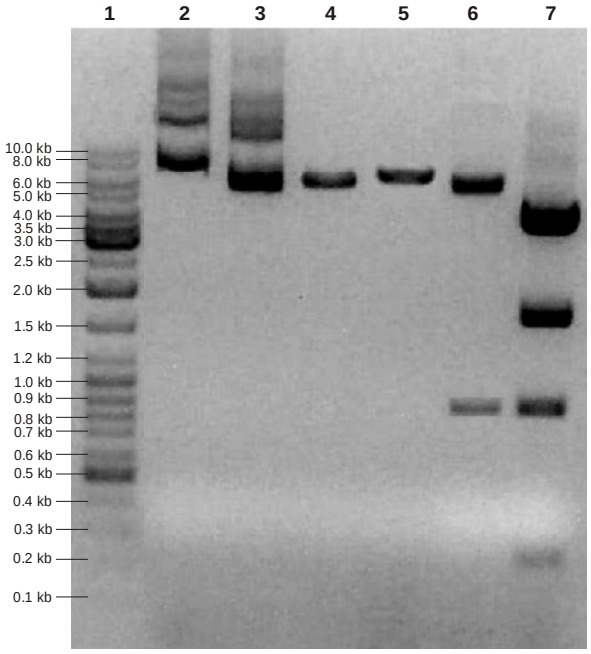


Fig. 5.3b Agarose gel electrophoretic analysis of the recombinant plasmid pGori-OprG-Gent. Lane 1, DNA molecular weight marker; lane 2, uncut recombinant allelic exchange vector pGori-OprG-Gent; lane 3, uncut plasmid pGEM-OprG-Gent; lane 4, plasmid pGEM-OprG-Gent digested with *Eco*RV; lane 5, recombinant allelic exchange vector pGori-OprG-Gent digested with *Eco*RV; lane 6, plasmid pGEM-OprG-Gent digested with *Kpn*I; lane 7, recombinant allelic exchange vector pGori-OprG-Gent digested with *Kpn*I. The sizes of the DNA molecular weight marker, GeneRuler™ DNA Ladder Plus, are indicated to the left of the figure.

5.3.2 Engineering of an OprG-deficient *P. aeruginosa* PAO1 (DSM 1707) strain

5.3.2.1 Generation of mutant strains

Mutant strains of the wild-type *P. aeruginosa* PAO1 (DSM 1707) strain were generated by introducing the allelic exchange vector pGori-OprG-Gent into the wild-type PAO1 (DSM 1707) 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 *oprG*::Gm^R null allele were selected by plating onto selective medium, as described under Material and Methods (Section 5.2.13). A gentamicin-resistant *P. aeruginosa* strain was selected and designated DSMOprG.

5.3.2.2 PCR analysis of *P. aeruginosa* mutant strain DSMOprG

The presence of an integrated copy of the mutant allele in the DSMOprG strain was verified by PCR analyses using different pairs of oligonucleotide primers (Fig. 5.4a) to amplify hybrid products only if the gentamicin resistance cassette was located within the chromosomal-borne inactivated *oprG*. Moreover, primers were also used that annealed to genomic sequences flanking the region in which the mutant allele was integrated.

Primers GenomicA and IntRp4067 as well as GenomicB and Gentp1-1 were used to amplify a 2.101-kb and 2.714-kb hybrid product, respectively, if the gentamicin resistance cassette was located within the disrupted *oprG* gene. The respective products were produced when DSMOprG chromosomal DNA was used as template (Fig. 5.4b, lanes 3 and 4, respectively). As expected, when wild-type *P. aeruginosa* PAO1 (DSM 1707) chromosomal DNA was used as template in the PCR reactions a 1.201-kb product was amplified using primers GenomicA and IntRp4067 (Fig. 5.4b, lane 6), but no product was amplified using primers GenomicB and Gentp1-1 (Fig. 5.4b, lane 7). In the final analysis, primers were used that annealed to genomic sequences upstream and downstream of the PA4067 ORF in which the mutant allele was integrated. Thus, primers GenomicA and GenomicB were used to amplify either a 2.798-kb product in the absence of the gentamicin resistance cassette, or a 3.698-kb product in the presence of the 0.9-kb gentamicin resistance cassette. As expected, a 3.7-kb product was produced when DSMOprG chromosomal DNA was used as template (Fig. 5.4b, lane 2). Template DNA from wild-type PAO1 (DSM 1707) generated the 2.8-kb product indicative of the absence of the gentamicin cassette within the *oprG* gene (Fig. 5.4b, lane 5).

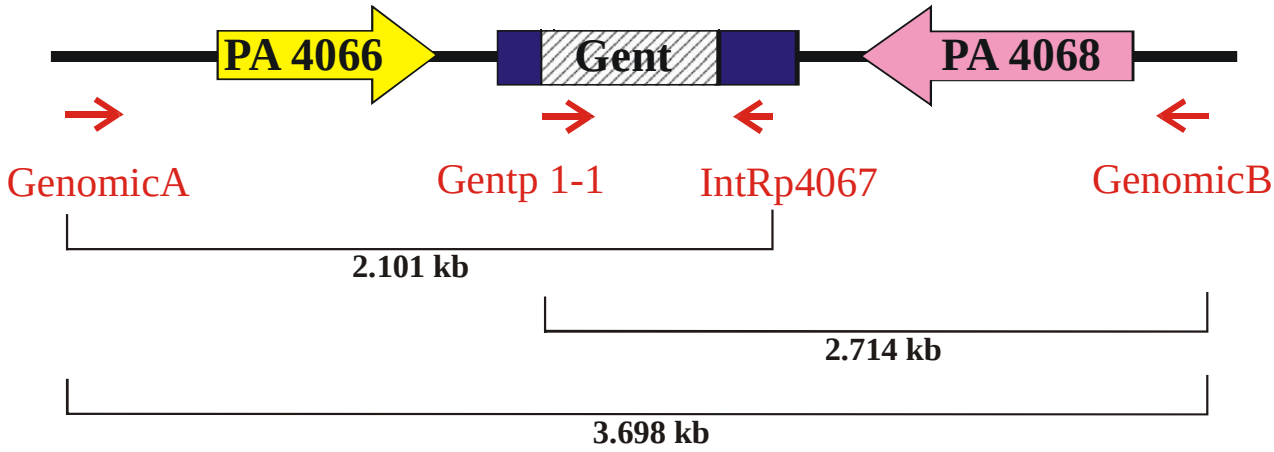


Fig. 5.4a **Schematic presentation of specific primer annealing positions and direction of amplification in the mutant DSMOprG strain. The expected sizes of the different amplicons are indicated by brackets.**

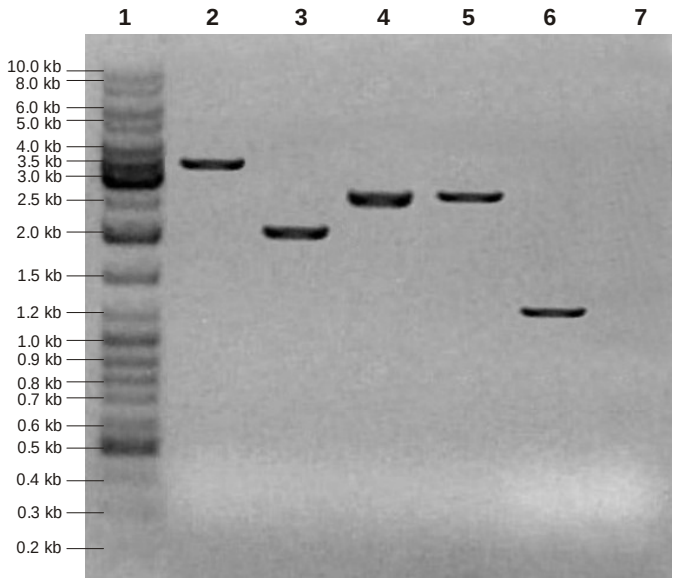


Fig. 5.4b **Agarose gel electrophoretic analysis of the amplification products obtained following PCR analysis of DSMOprG and *P. aeruginosa* PAO1 (DSM 1707) using primers GenomicA and GenomicB (lanes 2 and 5), GenomicA and IntRp4067 (lanes 3 and 6), and GenomicB and Gentp1-1 (lanes 4 and 7). Lanes 2, 3 and 4 represent genomic DNA from mutant strain DSMOprG, while lanes 5, 6 and 7 represent genomic DNA from wild-type *P. aeruginosa* PAO1 (DSM 1707). The sizes of the molecular weight marker, GeneRuler™ DNA Ladder Plus, are indicated to the left of the figure.**

5.3.3 Characterisation of the DSMOprG mutant strain

5.3.3.1 Growth curves

Since reports have noted that insertion mutagenesis may influence the growth properties of a particular 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 (DSM 1707) and mutant DSMOprG strains were cultured in MSGY broth and their growth was followed by taking optical density readings at 600 nm over a period of 26 h. The results that were obtained (Fig. 5.5) indicated that culturing of the wild-type PAO1 (DSM 1707) and mutant DSMOprG strains in MSGY broth yielded generation times of 130.60 min and 150.38 min, respectively. These results thus indicated that the mutant DSMOprG was slightly growth-impaired when compared to the wild-type strain.

5.3.3.2 Biofilm development on glass wool

To determine whether the DSMOprG mutant strain was capable of forming biofilms, biofilm development on glass wool in MSGY broth was monitored at various times by bright-field microscopy after staining of the glass wool with crystal violet. For this analysis, the wild-type *P. aeruginosa* PAO1 (DSM 1707) strain was included as a control. Although very few cells of both strains were visible on the glass wool up to 4 h after inoculation (Fig. 5.6a and b), more cells became visible on the glass wool after 8 h (Fig. 5.6c) and dense biofilm structures were formed within 18 h (Fig. 5.6d) that became denser after 26 h (Fig. 5.6e). However, some phenotypic differences between the biofilm structures of the wild-type PAO1 (DSM 1707) and mutant DSMOprG strain could be observed. Whereas the wild-type strain showed uniform colonisation of the glass wool surface punctuated with dense, thick multilayered structures, the DSMOprG strain showed sparser colonisation of the glass wool surface and the biofilm structures had the appearance of cell clumps (Fig. 5.6d and e).

To further investigate the propensity of the DSMOprG mutant cells to attach and grow as biofilms, the ratio of attached (biofilm) to surface influenced planktonic (SIP) biomass was calculated at various time intervals of culturing from cultures grown in MSGY broth with glass wool. Whereas the SIP cells were obtained by careful aspiration of the culture fluid, the

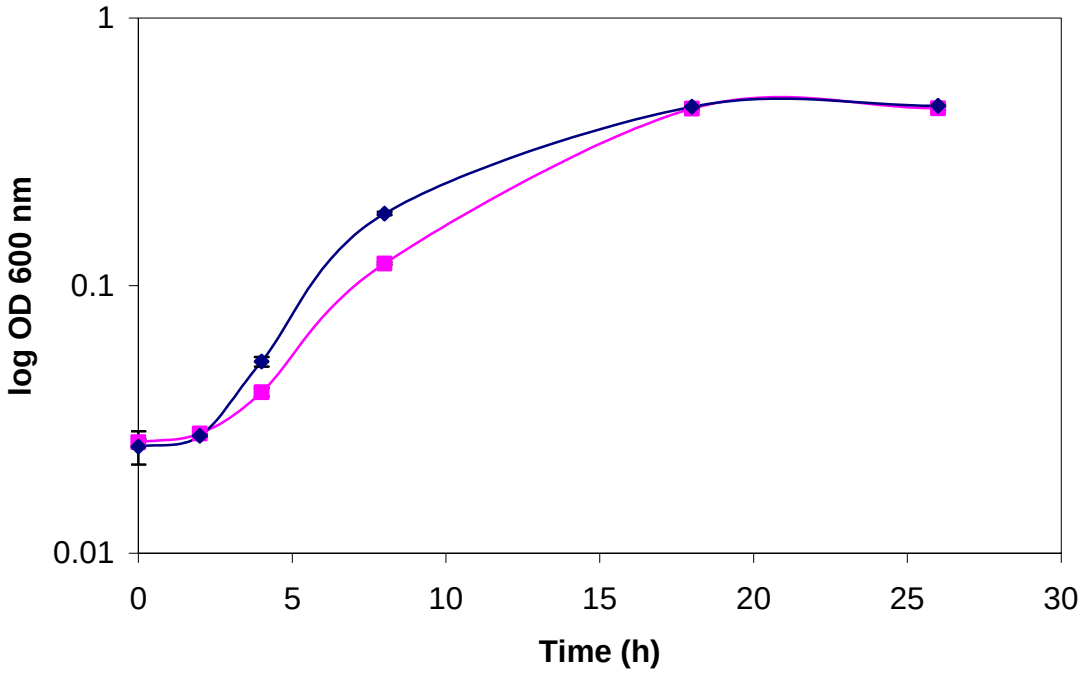


Fig. 5.5 **Growth curves of the wild-type *P. aeruginosa* (♦) and mutant DSMOprG strain (■) in MSGY broth.**

attached (biofilm) cells were recovered from the glass wool substratum by vortexing. The results obtained are presented in Fig. 5.7.

Comparative analyses of growth curves obtained for the planktonic, biofilm and planktonic surface influenced planktonic (SIP) populations of the wild-type PAO1 (DSM 1707) and mutant DSMOprG strains indicated that DSMOprG was impaired in its growth (Fig. 5.7a), thus confirming earlier results (Fig. 5.5). Regarding the propensity of DSMOprG to attach and grow as a biofilm, the results indicated that cells of both the wild-type PAO1 (DSM 1707) and mutant DSMOprG strains were capable of attaching to the glass wool substratum after 2 h of culturing, albeit that cells of the DSMOprG strain was more efficient in attaching to the glass wool substratum as was evidenced by a higher ratio of biofilm to SIP biomass (Fig. 5.7b). However, following attachment, cells of the DSMOprG strain were less prone to occur in a biofilm when compared to the wild-type PAO1 (DSM 1707) cells. This was evidenced by a decrease in the ratio of biofilm to SIP biomass of the DSMOprG strain compared to the wild-type PAO1 (DSM 1707) strain. From these results it was concluded that the DSMOprG strain was capable of attaching to the glass wool surface, but was impaired in its ability to grow as a biofilm.

5.4 DISCUSSION

Although *P. aeruginosa* encodes in excess of 160 known or predicted outer membrane proteins (Stover *et al.*, 2000), the functions of only a very few is known (Hancock and Brinkman, 2002). Outer membrane proteins (OMPs) have been reported to play an important role in antibacterial resistance, transport of nutrients, facilitation of cell-to-cell signalling and virulence in pathogenic bacteria (Ito *et al.*, 1999; Ochs *et al.*, 1999; Pearson *et al.*, 1999). In addition, several different OMPs have been reported to play a role in biofilm formation and development (Espinosa-Urgel *et al.*, 2000; Otto *et al.*, 2001; Yoon *et al.*, 2002). In this study, the role of outer membrane protein porin OprG in *P. aeruginosa* biofilm development was specifically investigated, since its expression was found to be up-regulated in an 18-h old *P. aeruginosa* biofilm population (Chapter 4). 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, 1998; Pratt and Kolter, 1998; Loo *et al.*, 2000). To generate such loss-of-function

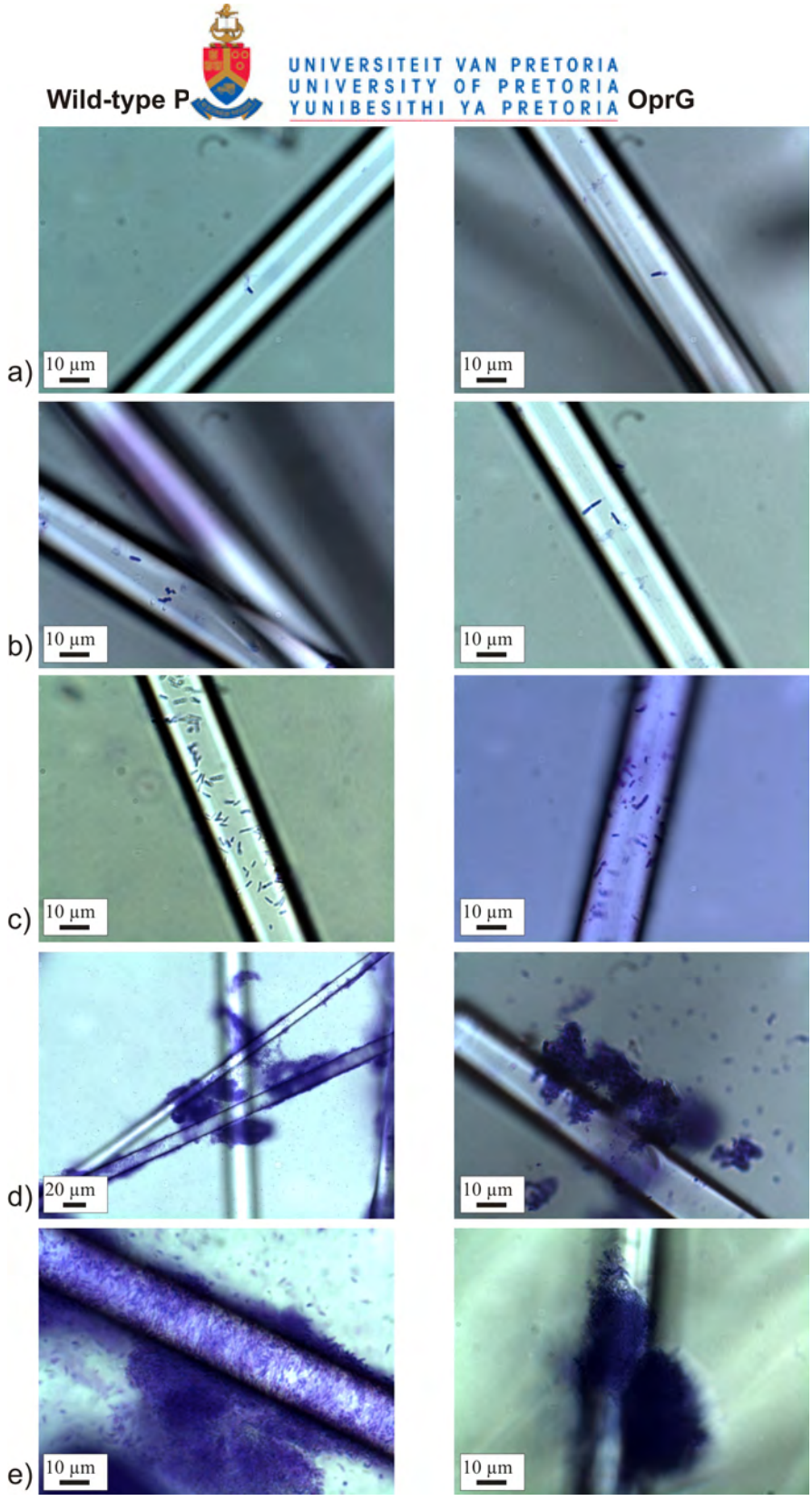
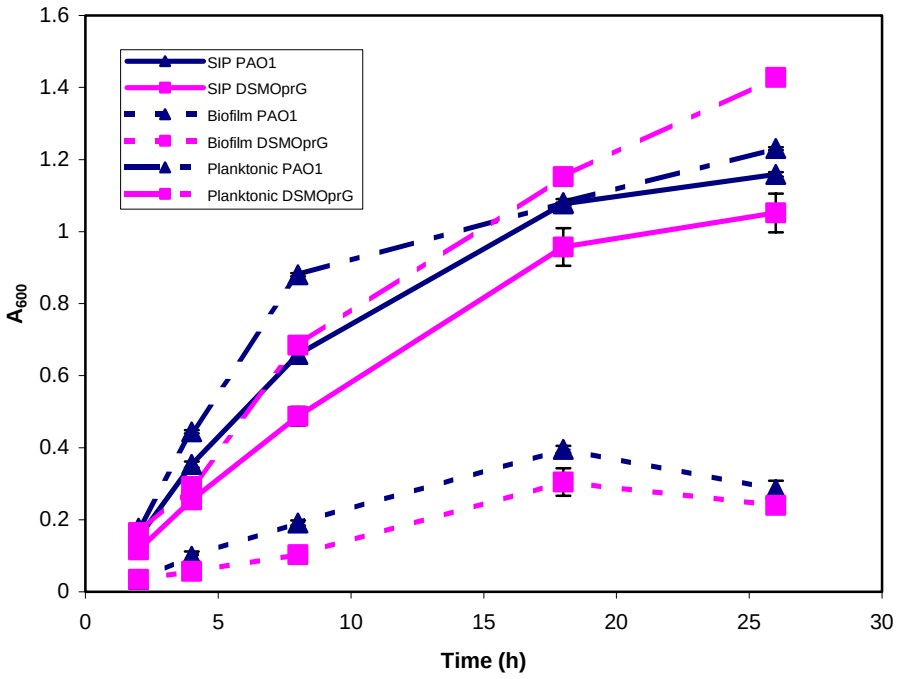


Fig. 5.6 **Biofilm development of *P. aeruginosa* wild-type and mutant strains on glass wool over time. Fifty milliliters of MSGY broth, containing 1.25 g of glass wool, was inoculated with either the *P. aeruginosa* wild-type PAO1 (DSM 1707) or mutant DSMOprG strain. Bright-field microscopy was performed on samples of glass wool following staining with 0.01% (w/v) crystal violet at times 2 h (a); 4 h (b); 8 h (c); 18 h (d) and 26 h (e) after inoculation.**

(a)



(b)

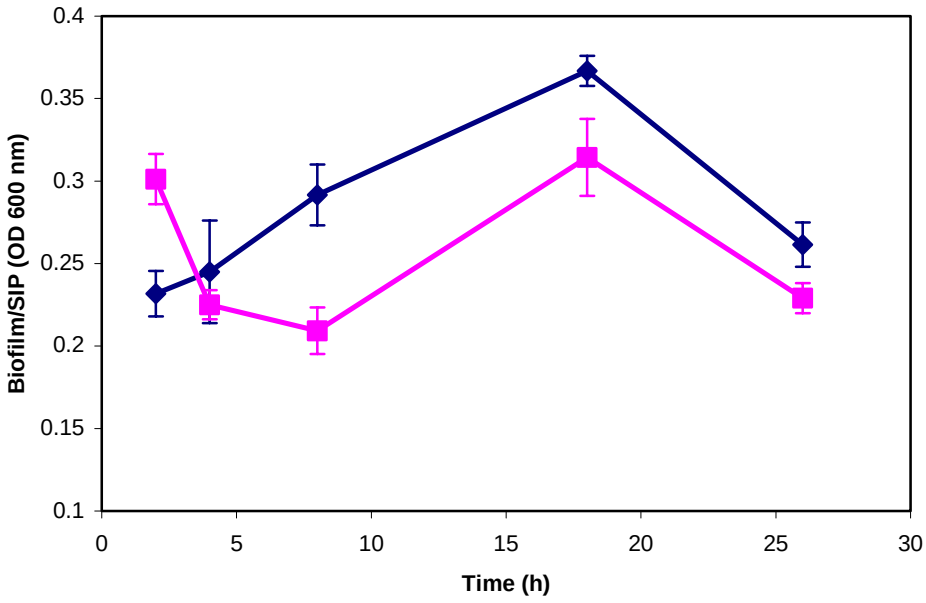


Fig. 5.7 **Propensity of DSMOprG cells to occur as a biofilm. (a) Optical density at 600 nm of planktonic, surface influenced planktonic (SIP) and attached (biofilm) populations of both wild-type *P. aeruginosa* PAO1 (DSM 1707) and mutant DSMOprG strains. (b) The ratio of attached (biofilm) to surface influenced planktonic (SIP) cells grown in MSGY broth in the presence of glass wool is indicated for wild-type *P. aeruginosa* PAO1 (DSM 1707) (♦) and mutant DSMOprG (■), as measured by optical density. Error bars denote standard error of the mean.**

mutations, both random transposon insertion mutagenesis and allelic exchange methods have been useful.

Transposons, being mobile genetic elements, have the capability of inserting themselves into genes on a bacterial chromosome or plasmid, thereby disrupting the gene itself and sometimes additional genes that are encoded downstream of the mutated gene. Nevertheless, this represents a powerful approach towards identifying genes involved in a specific function provided that an appropriate high-throughput screen is available (Hayes, 2003). Using such an approach, O'Toole and Kolter (1998) identified both pili and flagella as being important for the early stages of biofilm development. In addition, Espinosa-Urgel *et al.* (2000) also used this mutagenesis approach to identify several membrane and membrane-associated proteins that are required for attachment of *P. putida* to corn. In contrast to transposon insertion mutagenesis, 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 (Toder, 1994; Suh *et al.*, 1999; Dasgupta *et al.*, 2000). Mutations made by allelic exchange are thus targeted, making it a more attractive method of mutagenesis than random transposon insertion mutagenesis. Towards determining the importance of OprG in *P. aeruginosa* biofilm development, allelic exchange was therefore preferred in this study for constructing an OprG-deficient mutant strain. Consequently, an allelic exchange vector harbouring a cloned copy of the *oprG* gene that had been inactivated through insertion of a gentamicin resistance cassette was constructed. The vector was subsequently introduced into *P. aeruginosa* PAO1 (DSM 1707) and presumptive mutant strains were characterised by PCR analyses. The results obtained from these analyses indicated that the insertional inactivation of the *oprG* ORF in the mutant DSMOprG strain occurred by means of a double crossover event and consequently resulted in the integration of only the mutant *oprG*::Gm^R allele.

To determine whether inactivation of the *oprG* gene of *P. aeruginosa* PAO1 (DSM 1707) affected its ability to form biofilm, the OprG-deficient mutant strain was tested for its ability to attach to and develop into a biofilm using glass wool as attachment substratum. Although the DSMOprG mutant strain was capable of forming biofilms on glass wool within 18 h of culturing, inspection of the biofilm phenotype revealed that in contrast to wild-type *P. aeruginosa* PAO1 (DSM 1707), the mutant DSMOprG strain displayed sparser colonisation of the glass wool surface and the cells were localised in clusters. More detailed analysis of

the defects conferred by inactivation of the *oprG* gene was obtained through determining the ratio of attached (biofilm) to surface influenced planktonic (SIP) biomass over a period of 26 h of culturing. Compared to the wild-type strain, cells of the DSMOprG strain attached more efficiently to the glass wool after 2 h, but the ratio of biofilm to SIP cells then 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 DSMOprG cells detached following the first and second division events compared to the wild-type PAO1 (DSM 1707) strain. The ratio of biofilm to SIP DSMOprG cells subsequently increased over time, albeit lower than the wild-type strain, indicating bacterial growth on the surface of the glass wool. After 18 h, the ratio of biofilm to SIP cells of both the wild-type and mutant *P. aeruginosa* strains declined sharply. These results coincide with results obtained earlier in this investigation by 2-DE analysis and those reported by Sauer *et al.* (2004), indicating that FliC is expressed in 18-h old *P. aeruginosa* biofilm populations (Sauer *et al.*, 2002; Sauer *et al.*, 2004; Chapter 4 of this study). Therefore, cells of both strains may have been actively detaching from the biofilm by reverting to a planktonic mode of growth. It is evident from these results that a common signal must have been responsible for detachment of the wild-type and mutant cells from the biofilm. Although it was beyond the scope of this study to determine the factors responsible for affecting the ratio of biofilm to surface influenced planktonic (SIP) cells, it may be that the onset of stationary phase or extended incubation could have resulted in increased detachment (Lamed and Bayer, 1986; Allison *et al.*, 1998). Alternatively, starvation could have resulted in increased detachment by an unknown mechanism to allow bacteria to search for nutrient-rich environments (O'Toole *et al.*, 2000; Sauer *et al.*, 2004).

Although the function of OprG is not known, further evidence supporting a role for this outer membrane protein in the *P. aeruginosa* biofilm developmental cycle has been provided by Sauer *et al.* (2004). Using microarray analysis, expression of OprG in four day-old biofilms was reported to be up-regulated 2.7-fold compared to dispersed cells. To identify homologues of the *P. aeruginosa* PAO1 OprG protein, a BLASTP search of the protein sequences in the GenBank Database was conducted. OprG displays 54% amino acid sequence similarity with the outer membrane protein OmpW of *E. coli*. The OmpW protein of *E. coli* has been reported to be a minor outer membrane protein that is localized to the poles of the cell (Lai *et al.*, 2004) and it serves as a receptor for colicin S4 (Pilsl *et al.*, 2004). Notably, colicin receptors have been reported to participate in other cellular functions such as the uptake of nutrients, *e.g.* iron complexes (Braun *et al.*, 1976), vitamin B_{12} (Di Masi *et al.*,

1973) and nucleosides (Hantke, 1976). Moreover, expression of Omp21 of *Comamonas acidovorans*, which displays 30% amino acid sequence identity to OmpW of *E. coli*, is induced by oxygen limitation (Baldermann *et al.*, 1998). Since a large portion of the mature *P. aeruginosa* biofilm population is under oxygen limitation (DeBeer *et al.*, 1994; Sauer *et al.*, 2002; Yoon *et al.*, 2002; Walters *et al.*, 2003; Werner *et al.*, 2004), it is tempting to speculate that OprG may be required for nutrient uptake, but under conditions of oxygen limitation, such as those experienced in a biofilm, its expression is up-regulated to enhance uptake of nutrients required for growth of the biofilm cells.

In conclusion, the results presented here suggest a role for the outer membrane protein OprG in *P. aeruginosa* biofilm development under the culturing conditions used in this study. This was evidenced by cells of an OprG-deficient *P. aeruginosa* strain, despite being attachment-proficient, were less prone to occur in a biofilm when compared to the wild-type PAO1 (DSM 1707) cells. It is as yet unclear whether OprG is indirectly, *e.g.* through structural alterations in the outer membrane, or directly responsible for this phenotype. Complementation studies, using a recombinant plasmid to provide the wild-type *oprG* gene *in trans*, may aid in clarifying the role of OprG in *P. aeruginosa* biofilm development. However, elucidating the role played by OprG during biofilm formation might not be a trivial task as it is also likely that the slower growth rather than inactivation of *oprG* may account for the altered phenotype displayed by the mutant DSMOprG strain.

5.5 ACKNOWLEDGEMENTS

The author thanks J.B. Weyers for excellent technical assistance in the laboratory.

5.6 REFERENCES

Allison, D.G., Ruiz, B., SanJose, 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.

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.

Atlas, R.M. (1993). *Handbook of Microbiological Media*, p. 607. CRC Press Inc., Boca Raton, Florida, USA.

Baldermann, C., Lupas, A., Lubieniecki, J. and Engelhardt, H. (1998). The regulated outer membrane protein Omp21 from *Comamonas acidovorans* is identified as a member of a new family of eight-stranded beta-sheet proteins by its sequence and properties. *J. Bacteriol. 180*, 3741-3749.

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

Braun, V., Hancock, R.E., Hantke, K., Hartmann, A. (1976). Functional organization of the outer membrane of *Escherichia coli*: Phage and colicin receptors as components of iron uptake systems. *J. Supramol. Struct. 5*, 37-58.

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

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., 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.

Di Masi, D.R., White, J.C., Schnaitman, C.A. and Bradbeer, C. (1973). Transport of vitamin B12 in *Escherichia coli*: Common receptor sites for vitamin B12 and the E colicins on the outer membrane of the cell envelope. *J. Bacteriol. 115*, 506-513.

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.

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.

Genevaux, P., Bauda, P., DuBo, 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.

Hancock, R.E. and Brinkman, F.S. (2002). Function of *Pseudomonas* porins in uptake and efflux. *Annu. Rev. Microbiol. 56*, 17-38.

Hancock, R.E.W., Siehnel, R. and Martin, N. (1990). Outer membrane proteins of *Pseudomonas. Mol. Microbiol. 4*, 1069-1075.

Hantke, K. (1976). Phage T6-colicin K receptor and nucleoside transport in *Escherichia coli*. *FEBS Lett. 70*, 109-112.

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.

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.

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.

Ito, K., Matsuo, E. and Akiyama, Y. (1999). A class of integral membrane proteins will be overlooked by the 'proteome' study that is based on two-dimensional gel electrophoresis. *Mol. Microbiol. 31*, 1600-1610.

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

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

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.

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.

Lai, E-M., Nair, U., Phadke, N.D. and Maddock, J.R. (2004). Proteomic screening and identification of differentially distributed membrane proteins in *Escherichia coli*. *Mol. Microbiol. 52*, 1029-1044.

Lamed, R. and Bayer, E.A. (1986). Contact and cellulolysis in *Clostridium thermocellum* via extensive surface organelles. *Experientia 42*, 72-73.

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

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.

Luckow, V.A., Lee, S.C., Barry, G.F. and Olins, P.O. (1993). Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. *J. Virol. 67*, 4566-4579.

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

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

Ochs, M.M., Lu, C-D., Hancock, R.E.W. and Abdelal, A.T. (1999). Amino acid-mediated induction of the basic amino acid-specific outer membrane porin OprD from *Peudomonas aeruginosa*. *J. Bacteriol. 181*, 5426-5432.

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.

Otto, K., Norbeck, J., Larsson, T., Karlsson, K.A. and Hermansson, M. (2001). Adhesion of type 1-fimbriated *Escherichia coli* to abiotic surfaces leads to altered composition of outer membrane proteins. *J. Bacteriol. 183*, 2445-2453.

Pearson, J.P., Van Delden, C. and Iglewski, B.H. (1999). Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. *J. Bacteriol. 181*, 1203-1210.

Pilsl, H., Smajs, D. and Braun, V. (2004). Characterization of colicin S4 and its receptor, OmpW, a minor protein of the *Escherichia coli* outer membrane. *J. Bacteriol. 181*, 3578-3581.

Pratt, L.A. and Kolter, R. (1998). Genetic analysis of *Escherichia coli* biofilm formation: Roles of flagella, motility, chemotaxis and type 1 pili. *Mol. Microbiol. 30*, 285-293.

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.

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, 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.

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.

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.

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. biofilm. *J. Bacteriol. 182*, 6482-6489.

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

Walters, M.C., Roe, F., Bugnicourt, A., Franklin, M.J. and Stewart, P.S. (2003). Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. *Antimicrob. Agents Chemother. 47*, 317-323.

Watnick, P.I. and Kolter, R. (2000). Biofilm, city of microbes. *J. Bacteriol. 182*, 2675-2679.

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.

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.

Yates, J.M., Morris, G. and Brown, M.R. (1989). Effect of iron concentration and growth rate on the expression of protein G in *Pseudomonas aeruginosa. FEMS Microbiol. Lett. 49*, 259-262.

Yoon, S.S., Hennigan, R.F., Hilliard, G.M., Ochsner, U.A., Parvatiyar, K., Kamani, M.C., Allen, H.L., DeKievit, T.R, Gardner, P.R., Schwab, U., Rowe, J.J., Iglewski, B.H., McDermott, T.R., Mason, R.P., Wozniak, D.J., Hancock, R.E., Parsek, M.R., Noah, T.L., Boucher, R.C. and Hassett D.J. (2002). *Pseudomonas aeruginosa* anaerobic respiration in biofilms: Relationships to cystic fibrosis pathogenesis. *Dev. Cell 3*, 593-603.