Identification of a novel gene, \textit{fimV}, involved in twitching motility in \textit{Pseudomonas aeruginosa}

Annalese B. T. Semmler, Cynthia B. Whitchurch, Andrew J. Leech and John S. Mattick

Transposon mutagenesis was used to identify a new locus required for twitching motility in \textit{Pseudomonas aeruginosa}. Four \textit{Tn}5-B21 mutants which lacked twitching motility and a fifth which exhibited impaired motility were found to map to the same \textit{KpnI} restriction fragment at approximately 40 min on the \textit{P. aeruginosa} genome. Cloning and sequencing studies showed that all five transposon insertions occurred within the same 2-8 \text{kb ORF}, which was termed \textit{fimV}. The product of this gene has a putative peptidoglycan-binding domain, predicted transmembrane domains, a highly acidic C terminus and anomalous electrophoretic migration, indicating unusual primary or secondary structure. The \textit{P. aeruginosa} genome also possesses a paralogue of \textit{fimV}.

Homologues of \textit{fimV} were also found in the sequenced genomes of the other type-IV-fimbriated bacteria \textit{Neisseria gonorrhoeae}, \textit{Neisseria meningitidis}, \textit{Legionella pneumophila} and \textit{Vibrio cholerae}, but not in those of other bacteria which lack type IV fimbriae. A \textit{fimV} homologue was also found in the genome sequence of \textit{Shewanella putrefaciens}, along with many other homologues of type IV fimbrial genes, indicating that this bacterium is also likely to produce type IV fimbriae. Wild-type twitching motility was restored to \textit{fimV} mutants by complementation in a dosage-dependent manner. Overexpression of \textit{fimV} resulted in an unusual phenotype where the cells were massively elongated and migrated in large convoys at the periphery of the colony. It is suggested that FimV may be involved in remodelling of the peptidoglycan layer to enable assembly of the type IV fimbrial structure and machinery.

\textbf{Keywords:} type IV pili, fimbriae, twitching motility, surface translocation, \textit{Pseudomonas aeruginosa}

\section*{INTRODUCTION}

\textit{Pseudomonas aeruginosa} is an opportunistic pathogen of animals and humans infecting immunocompromised hosts (Sato et al., 1988). Pathogenesis by this bacterium involves the production of a number of extracellular virulence determinants including lipases and phospholipases, proteases, exopolysaccharides, alkaline phosphatases, pyochelins and type IV fimbriae. Together, these factors contribute to the bacterium’s successful attachment to and colonization of the host epithelial tissues and its resistance to host defences (Bodey et al., 1983).

Type IV fimbriae are flexible, filamentous surface appendages produced at the poles of the bacterial cell which mediate attachment to the host epithelial tissue and a form of surface translocation termed twitching motility. They also appear to act as receptors for certain bacteriophages. The mechanism of twitching motility has been proposed to be fimbrial retraction and extension (Bradley, 1980). Bacteria that exhibit twitching motility can be seen as rough, spreading colonies on agar plates under humid conditions, and as very fine zones of rapid colony expansion on smooth surfaces (Semmler et al., 1999). This phenotype has been used to distinguish...
Table 1. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description*</th>
<th>Reference</th>
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<tbody>
<tr>
<td>pBluescript II KS/SK(+)</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt; cloning vectors</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pUCP18</td>
<td>E. coli/P. aeruginosa shuttle vector</td>
<td>Schweizer (1991)</td>
</tr>
<tr>
<td>pUCPKS/SK</td>
<td>P. aeruginosa T7 expression vectors</td>
<td>Watson &lt;i&gt;et al.&lt;/i&gt; (1996a)</td>
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<tr>
<td>pUK21</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt; cloning vector</td>
<td>Vieira &amp; Messing (1991)</td>
</tr>
<tr>
<td>pRIC380</td>
<td>P. aeruginosa suicide vector</td>
<td>Alm &amp; Mattick (1996)</td>
</tr>
<tr>
<td>pMMB207</td>
<td>Chlor&lt;sup&gt;R&lt;/sup&gt; cloning vector with inducible tac promoter</td>
<td>Morales &lt;i&gt;et al.&lt;/i&gt; (1991)</td>
</tr>
<tr>
<td>pSM-TET</td>
<td>Source of Tc&lt;sup&gt;R&lt;/sup&gt; cassette</td>
<td>Mongkolsuk &lt;i&gt;et al.&lt;/i&gt; (1993)</td>
</tr>
<tr>
<td>pAB1</td>
<td>Source of pilZ probe</td>
<td>Alm &lt;i&gt;et al.&lt;/i&gt; (1996)</td>
</tr>
<tr>
<td>pUS1</td>
<td>Source of xcpY–Z probe, kindly provided by M. Bally, CNRS, Marseille, France</td>
<td>Filloux &lt;i&gt;et al.&lt;/i&gt; (1990)</td>
</tr>
<tr>
<td>pUS13</td>
<td>Source of xcpQ probe, kindly provided by M. Bally, CNRS, Marseille, France</td>
<td>Akrim &lt;i&gt;et al.&lt;/i&gt; (1993)</td>
</tr>
<tr>
<td>pMO010323, pMO011618, pMO012140</td>
<td>pLA2917 containing partial Sau3A PAO1 chromosomal DNA fragments, kindly provided by B. Holloway, Monash University, Melbourne, Australia</td>
<td>Ratnaningsih &lt;i&gt;et al.&lt;/i&gt; (1990)</td>
</tr>
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<td>pASP6</td>
<td>6 kb EcoRI fragment from pMO011618 in pUCP18</td>
<td>This study</td>
</tr>
<tr>
<td>pASB351</td>
<td>3.5 kb HindIII fragment from pMO011618 in pBluescript II KS(+)</td>
<td>This study</td>
</tr>
<tr>
<td>pASB1</td>
<td>1 kb EcoRI fragment from pASB351 in pBluescript II KS(+)</td>
<td>This study</td>
</tr>
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<td>pASE281</td>
<td>2.8 kb Ppu101/BspHI fragment from pMO011618 in pUCPKS (fimV in direction of lac promoter)</td>
<td>This study</td>
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<td>pASE280</td>
<td>2.8 kb Ppu101/BspHI fragment from pMO011618 in pUCPKS (fimV in direction of T7 promoter)</td>
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</tr>
<tr>
<td>pASE230</td>
<td>2.4 kb EcoRI/KpnI fragment from pASE281 in pUCPSK (truncated fimV in direction of T7 promoter)</td>
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<td>pASE18a</td>
<td>2.2 kb NruI fragment from pASE280 in EcoRV site of pUCPKS (truncated fimV in direction of T7 promoter)</td>
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<td>pAS36</td>
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<td>This study</td>
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<td>pAST36</td>
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<td>This study</td>
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<td>pRIC380 carrying usg-1::Tc&lt;sup&gt;R&lt;/sup&gt; on SpeI fragment</td>
<td>This study</td>
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<td>pAST14</td>
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<td>pASS14</td>
<td>pRIC380 carrying bisT::Tc&lt;sup&gt;R&lt;/sup&gt; on SpeI fragment</td>
<td>This study</td>
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<tr>
<td>pASM281</td>
<td>2.8 kb XbaI/KpnI fragment from pASE281 in pMMB207 (fimV in direction of tac promoter)</td>
<td>This study</td>
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*<sup>Amp</sup><sup>R</sup>, ampicillin resistance; <sup>Tc</sup><sup>R</sup>, tetracycline resistance; <sup>Kan</sup><sup>R</sup>, kanamycin resistance; <sup>Chlor</sup><sup>R</sup>, chloramphenicol resistance.
between bacteria which have functional fimbriae and those which possess a mutation in genes affecting fimbrial biogenesis or function (Hobbs et al., 1993; Alm & Mattick, 1997). Twitching motility also appears to be an important virulence factor as mutants which lack functional type IV fimbriae have reduced infectivity (Hazlett et al., 1991; Comolli et al., 1999). Twitching motility has also been shown to be involved in biofilm formation (O’Toole & Kolter, 1998), which may be important during infection (Potera, 1999; Costerton et al., 1999).

Twitching motility and type IV fimbriae have been described in a wide range of bacteria, including *P. aeruginosa*, *Neisseria gonorrhoeae*, *Neisseria meningitidis* and other *Neisseriaceae*, various *Moraxella* species, *Dichelobacter nodosus*, *Branhamella catarrhalis*, *Suttonella indologenes*, *Alteromonas putrefaciens*, *Pasteurella multocida*, *Xanthomonas maltophilia*, *Kingella denitrificans* and many others (Mattick et al., 1993). Related genes encoding the type IV fimbrial subunit and other components have also been found in a number of bacteria not previously recognized to possess type IV fimbriae, including *Aeromonas* spp. (Pepe et al., 1996; Barnett et al., 1997), *Legionella pneumophila* (Liles et al., 1998; Stone & Abu Kwaik, 1998), *Pseudomonas syringae* (Roine et al., 1998) and *Azoarcus* spp. (Dorr et al., 1998), the last two indicating that type IV fimbriae are important in bacterial colonization not only of animals but also of plants, fungi and protozoa. Type IV fimbriae have also been found in *Myxococcus xanthus*, where they have been shown to be required for social gliding motility, a process which appears to be functionally equivalent to twitching motility (Wu & Kaiser, 1995; Semmler et al., 1999).

Type IV fimbriae are filaments of about 6 nm in diameter which range up to several µm in length. They are composed of a small (145–160 aa) structural subunit (pilin or PilA in *P. aeruginosa*) with a characteristic highly conserved and highly hydrophobic amino-terminal region. This forms the core of the helical structure, whose outer face is comprised of the more hydrophilic and more variable domains of the subunit (Folkhard et al., 1981; Paranchych & Frost, 1988; Dalrymple & Mattick, 1987; Parge et al., 1990; Forest & Tainer, 1997).

The biogenesis and function of type IV fimbriae in *P. aeruginosa* is dependent on at least 35 genes which are located in several clusters on the chromosome. These include genes encoding the fimbrial subunit (PilA), a leader peptidase (PilD), ancillary proteins with pre-pilin-like leader sequences (PilE, PilV, PilW, PilX, FimT, FimU), inner and outer-membrane proteins (PilC, PilQ), nucleotide-binding proteins (PilB, PilT, PilU), other proteins whose functions are not clear (PilM-P, PilF, PilY1, PilY2, PilZ), the RpoN sigma factor, 2 two-component sensor-regulator pairs (PilS/PilR and FimS/AlgR) and a complex chemosensory signal transduction system (PilG-L, ChpA-C) (for a recent review see Alm & Mattick, 1997). Here, we report the identification and characterization of a novel gene, fimV, which is also required for twitching motility.

**METHODS**

**Bacterial strains, plasmids and media.** The *Escherichia coli* strain DH5α (recA endA1 gyrA96 bsdR17 thi-1 supE44 relA1 Δ80dlacZAM15) was used in all genetic manipulations and in the preparation of DNA sequencing templates; *E. coli* S17-1 was used as the donor strain in the bacterial conjugation (Simon et al., 1983). The *P. aeruginosa* strains used were PAK (D. Bradley, Memorial University of Newfoundland, Canada), Tn3-B21 mutants of this strain (Hobbs et al., 1993), PAKpilA::Tc (previously referred to as AWK; Watson et al., 1996b) and Add1976 (Brunschwig & Darzins, 1992). A PAO1 cosmid library (Ratnamanesh et al., 1990) was used in the subcloning and sequence analysis of the fimV region. Details of plasmid construction are given in Table 1. *P. aeruginosa* competent cells and transformations were prepared as described previously (Mattick et al., 1987). *E. coli* and *P. aeruginosa* liquid cultures were maintained in Luria–Bertani (LB) broth (Sambrook et al., 1989) and solid media was prepared by adding 1.0–1.5% Select agar (Gibco-BRL). Light microscopy was performed using nutrient media (4 g tryptone f-1, 2 g yeast extract f-1 and 2 g NaCl f-1) solidified with 8 g GelGro (ICN) f-1 for greater optical clarity. The following antibiotic concentrations were used for the selection of *E. coli*: 12.5 µg tetracycline ml-1 for plasmid selection and 40 µg tetracycline ml-1 for cosmid selection; 100 µg ampicillin ml-1, 25 µg chloramphenicol ml-1 and 50 µg kanamycin ml-1. The concentrations of antibiotics for the selection of *P. aeruginosa* were 500 µg carbenicillin ml-1, 250 µg chloramphenicol ml-1, 20 µg rifampicin ml-1 and 200 µg tetracycline ml-1.

The *ptac* expression studies involved cloning the *fimV* gene into pMMP207 (Morales et al., 1991), a chloramphenicol-resistant *tac* promoter expression shuttle vector that is inducible with IPTG. Following transformation of the construct into wild-type PAK and the Tn3-B21 mutants, the transformants were exposed to varying concentrations (0–100 mM) IPTG with 250 µg chloramphenicol ml-1 on 1% agar plates.

**Construction of isogenic mutants.** Allelic exchange mutants were constructed of the genes *usg-1* and *bisT*, which lie adjacent to *fimV*, using the sucrose selection system described previously (Schweizer, 1992; Alm & Mattick, 1996). Briefly, these genes were subcloned into the vector pUK21 (forming pAS36 and pAS14, respectively). The tetracycline gene cartridge from pSM-TET was cloned into the *PstI* site within *usg-1* and into a blunted *NcoI*/*Ndel* site in *bisT* to disrupt the genes. The resulting clones were then digested with *SpeI*, whose sites span the multiple cloning site of pUK21, and the disrupted genes were inserted into the suicide vector pRJC380. This vector carries the genes *sacBR*, which promote sensitivity to sucrose, and *oriT*, enabling conjugal transfer. The constructs were then transformed into the *E. coli* donor strain S17-1 in preparation for mating into *P. aeruginosa*. Following conjugation, the transconjugates were selected on 5% sucrose media containing tetracycline. This forces the excision of the plasmid whilst leaving the homologously recombined mutated gene in the chromosome. Mutants were confirmed using Southern analysis and examined using the subsurface twitching assay (see below).
Recombinant DNA techniques. The preparation of plasmid DNA, restriction endonuclease digestion (New England Biolabs), ligation reactions, Southern blotting and radiolabelling of probe were carried out using standard protocols (Sambrook et al., 1989).

Sequence analysis. Sequence templates were generated by a combination of subcloning and shotgun cloning strategies. The cDNA was prepared for sequencing using a modified alkaline lysis method involving PEG precipitation (Applied Biosystems). Sequencing was performed using the Applied Biosystems PRISM system on a 373A automated sequencer. Nucleotide and predicted protein sequences were analysed using gapped BLAST (Altschul et al., 1997), SMART (Schultz et al., 1998; Ponting et al., 1999) and PSORT (Nakai & Horton, 1999) programs.

Protein expression and analysis. The FimV protein was subcloned into a P. aeruginosa expression plasmid, pUCPKS (Watson et al., 1996a) and transformed into P. aeruginosa ADD1976, which contains a chromosomal T7 RNA polymerase gene under the control of an inducible $\beta$-galactosidase promoter (Brunschwig & Darzins, 1992). Protein expression was induced in the presence of $[^{35}S]$methionine and analysed on 7.5% SDS-polyacrylamide gels as described previously (Alm & Mattick, 1995).

Western blotting. Bacterial cells from plates were resuspended to an OD$_{600}$ of 1.0 in 50 mM sodium carbonate buffer pH 9.6. Samples (1 ml) were centrifuged and the cell pellet was resuspended in 100 μl sample buffer (60 mM Tris/HCl pH 6.8, 2% SDS, 10% glycerol, 5% $\beta$-mercaptoethanol, 0.001% bromophenol blue). To remove DNA, the samples were centrifuged at 45000 r.p.m. for 90 min and the supernatant was heated to 100 °C for 5 min. Proteins in the samples were then separated by SDS-PAGE using a 15% polyacrylamide gel and a 5% stacking gel as described by Laemmli (1970) and transferred electrophoretically to Hybond-C nitrocellulose membranes (Amersham) in the Tris/glycine system described by Towbin et al. (1979). Proteins were detected with anti-PilA antiserum (1:5000) followed by goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (1:5000; Boehringer Mannheim).

ELISA. This was based on a method described by Engvall & Perlmann (1972). The cells were resuspended in 50 mM sodium carbonate buffer pH 9.6 at an OD$_{600}$ of 1.0 and 200 μl of suspension was loaded into wells of a 96-well ELISA plate. After overnight incubation at 4 °C, the wells were washed with PBS (137 mM NaCl, 2 mM KCl, 10 mM NaHPO$_4$, pH 7.4) containing 0.1% Tween 20, blocked with 3% BSA for 1 h and then exposed to an anti-PilA antibody at a starting dilution of 1:500 for 2 h at 37 °C. After removal of antisera, the wells were washed again with PBS containing 0.1% Tween 20. Goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase was then added (1:5000) and the mixture incubated for 2 h at 37 °C. Detection was carried out using 20 mg $\beta$-nitrophenyl phosphate (Sigma) ml$^{-1}$ in 1 M Tris buffer pH 8.0 and the plate was read at 405 nm using an ELISA reader (Bio-Rad).

Elastase assay. Aliquots (2 μl) of overnight broth cultures were inoculated onto the surface of LB agar plates containing 0.1% elastin (Sigma). After incubation at 37 °C for 2–3 d, plates were examined for zones of proteolytic clearing surrounding the colonies.

Twitching motility assay. Twitching motility was assayed as described previously (Alm & Mattick, 1995). Briefly, the P. aeruginosa strain to be tested was stab-inoculated through a 1% agar plate, and after overnight growth at 37 °C the zone of twitching motility between the agar and Petri dish interface was visualized by staining with Coomassie brilliant blue R250.

Light still and video microscopy. Light microscopy was performed as described previously (Semmler et al., 1999). Sterile microscope slides were submerged in molten GelGro media to obtain a thin layer of media coating the slide. The slides were allowed to set in a horizontal position and air-dried briefly prior to use. The slides were then inoculated with a small loopful of bacteria taken from an overnight plate culture. A sterile glass coverslip was placed over the point of inoculation and the slide transferred to a large Petri dish containing a moist tissue and sealed with Nescofilm (Bando Chemical Industries) to maintain humid conditions. Incubation times ranged from 2–6 h at 37 °C.

Slide cultures were examined using a Zeiss Axioskop 50 microscope with Nomarski facilities at ×200 to ×400 magnification. Video microscopy was performed in a room heated to 30 °C. Video images were recorded over a period of 2–4 h at speeds of either 1 field per 3-22 s, 1 field per 0.66 s or real time (1 field per 1/50 s) using a JVC TK-138IEG video camera connected to a Sanyo TLS-S2500P time-lapse video recorder. Video images were edited and converted to Quicktime movies using Avid Videoshop version 3.0 and can be viewed at http://www.cmcb.uq.edu.au/cmcb/PUBS/twitch.html.

RESULTS

Initial characterization of the transposon mutants

We have previously generated a library of twitching motility mutants through transposon mutagenesis of the P. aeruginosa strain PAK genome (Hobbs et al., 1993). The mutants were then classified according to their sensitivity or resistance to infection by the fimbiae-specific bacteriophage PO$_4$ and further subgrouped according to the size of the KpnI restriction fragments into which the transposon had inserted. Five of these mutants (S4, S76, S125, S359 and S361) that retained phage sensitivity were found to have transposon insertions in the same large (> 25 kb) KpnI fragment.

DNA flanking the site of insertion of the transposon in each of these mutants was cloned using a 'marker rescue' approach that involved digestion of mutant chromosomal DNA with restriction enzymes (either EcoRI or HindIII) which cut once within the transposon beyond the tetracycline resistance marker, ligation into pBluescript II KS(–) and recovery of tetracycline-resistant *E. coli* colonies. The DNA adjacent to the transposon insertion in each of the mutants was then sequenced using a primer which is specific for the inverted repeats of Tn5-B21 (Hobbs et al., 1993). Database searches with these sequences revealed that the transposon mutants were unique and not located in previously characterized genes.

Cloned chromosomal DNA flanking the site of transposon insertion in S76 was used for Southern analysis of mutant genomic DNA. This analysis demonstrated that the transposon insertions in mutants S4, S76, S359 and...
S361 were located in the same 10 kb HindIII and 6 kb EcoRI restriction fragments, whereas the S125 insertion site was not located within these fragments. The cloned DNA from S76 was also used to screen a reference PAO1 cosmid library (Ratnaningsih et al., 1990), from which we identified three cosmids (pMO010323, pMO011618 and pMO012140) covering the region. Restriction mapping of these cosmids and further Southern analysis showed that the transposon insertion site of the S125 mutant was located in 3–5 kb HindIII and 1 kb EcoRI fragments contained within these cosmids, adjacent to the restriction fragments containing the other four insertions (Fig. 1).

Cosmids pMO011618 (leu-10 trpF pur-9013 met-9011) and pMO012140 (leu-10 trpF) have been previously mapped to approximately 40 min on the P. aeruginosa chromosome by the presence of metabolic markers (Ratnaningsih et al., 1990). This region of the P. aeruginosa genome is known to contain the fimbrial gene pilZ (Alm et al., 1996) and a cluster of genes (xcpQ–Z) which are required for protein secretion in this organism (Filloux et al., 1990; Bally et al., 1992; Akrim et al., 1993). Southern analysis of the cosmids with probes specific for pilZ, xcpQ and xcpY–Z (see Table 1) demonstrated that the transposon insertions were not in the vicinity of these genes (data not shown). The mutants were also found to have no defect in their ability to secrete elastase, an enzyme exported via the Xcp pathway, indicating that the gene(s) into which the transposons had inserted is not involved in this system (data not shown).

Cloning and sequence analysis of the fimV region

The 6 kb EcoRI (pASP6) and 3.5 kb HindIII (pASB351) fragments (Fig. 1) were subcloned from pMO011618 and sequenced. The complete sequence of 8 kb of this region (GenBank accession no. U93274) identified six ORFs (Fig. 1), three of which were previously characterized genes from P. aeruginosa: leuB (3-isopropylmalate dehydrogenase); asd (aspartate-β-semialdehyde dehydrogenase); and orfA (Hoang et al., 1997; Hoang & Schweizer, 1997). Our sequence analysis of this region revealed a frameshift error in the previously reported sequence of orfA. The revised sequence shows that this putative gene encodes a protein which shows significant homology to the product of the unknown genes termed usg-1 from Azotobacter vinelandii (50% identity and 66% similarity over 333 aa) and E. coli (36% identity and 53% similarity over 339 aa). In light of this we have renamed orfA as usg-1. Interestingly, the Usg-1 proteins are also predicted to belong to the family of aspartate-β-semialdehyde dehydrogenases and in fact are homologous to the asd gene products from Vibrio cholerae and Vibrio mimicus, as well as Shewanella sp. and L. pneumophila. These enzymes catalyse the second step in the common biosynthetic pathway leading from aspartate to the cell wall precursor meso-diaminopimelate, lysine, methionine, isoleucine and threonine. Two of the remaining ORFs, which we have termed orfB and hisT, showed significant homology to genes characterized in other bacteria. The product of orfB (187 aa) shows close strong similarity to the hypothetical proteins YafE from E. coli (58% identity and 68%
similarity over 182 aa) and YcgJ from Bacillus subtilis (37% identity and 54% similarity over 166 aa), and to putative methyltransferases from a number of bacterial species including Lactococcus lactis, Bacillus steator- thermophilus, Streptomyces hygroscopicus, Micrococcus luteus and E. coli. HisT shows strong homology to pseudouridylate synthetases (involved in tRNA modification) from a broad spectrum of bacterial species (Arps & Winkler, 1987). Sequence analysis of the region downstream of hisT indicated that the previously characterized gene trpF (phosphoribosyl anthranilate isomerase) (Murata, 1996) is located immediately downstream of hisT.

The remaining ORF in this region was found to contain all five transposon insertions (Fig. 1). This ORF, designated fimV, is 2.8 kb in size and has an overall G + C content of 67.5 mol%, in agreement with the estimated 67 mol% for the P. aeruginosa genome as a whole (West & Iglewski, 1988). Further analysis of the fimV sequence showed a decrease in rare codon usage within the ORF and a high G + C bias (81.7%) in the third codon position, suggestive of a likely coding region (West & Iglewski, 1988).

**BLAST** search analyses at NCBI revealed that FimV shows regions of homology with the recently described protein TspA of N. meningitidis (Kizil et al., 1999). Analysis of the Unfinished Microbial Genomes databases at NCBI also identified homologies between FimV and predicted proteins from N. gonorrhoeae (TspA equivalent), L. pneumophila, V. cholerae and Shewanella putrefaciens (Fig. 2). FimV also shows significant homology with the predicted product of a second P. aeruginosa gene identified in the unfinished genome sequences (Fig. 2). Interestingly, except for Shewanella putrefaciens, these bacterial species are all known to possess type IV fimbriae. Further analysis of the Shewanella putrefaciens genome sequences indicated that this organism should also be capable of producing type IV fimbriae as it possesses many close homologues of P. aeruginosa proteins required for type IV fimbrial biogenesis (including homologues of PilA-F, PilM-Q, PilT and PilU). It appears therefore that fimV is specific to type-IV-fimbriate bacteria.

FimV and its homologues show a number of features in common. Each of these proteins is highly acidic with an estimated pH ranging from 3.24 to 4.64. Acidic residues are located across the entire length of these proteins, with large numbers clustered in the carboxy termini (Fig. 2). All of these proteins are predicted to possess at least one transmembrane spanning region (Fig. 2) and, except for TspA and the L. pneumophila FimV homologue, have predicted signal sequences. It is expected therefore that these proteins are integral cytoplasmic membrane proteins. SMART analysis at EMBL also predicted that each of these proteins possesses a putative peptidoglycan-binding domain in their N-terminus (Fig. 2). This motif has been found in a variety of enzymes involved in bacterial cell wall degradation (Joris et al., 1992). Sequence homology between these proteins is strongest in the region surrounding this domain.

In P. aeruginosa, fimV is situated between the genes usg-1 and hisT (Fig. 1). Interestingly, in E. coli usg-1 is known to be located directly upstream of and within an operon with hisT (Arps & Winkler, 1987). The difference therefore between the E. coli and P. aeruginosa genomic organization is the presence of fimV between usg-1 and hisT. A search of the E. coli genome shows that it does not contain a homologue of FimV. We constructed allelic exchange mutants of the P. aeruginosa genes usg-1 and hisT to determine whether
C terminus of FimV, by removing 455 bp (pASE230) and the anomaly we then generated truncations of the acidic and end sequence analysis. To try to locate the source of (98 kDa). The fidelity of the insert including the stop than that predicted from the sequence for FimV was estimated to be 145 kDa, which was 47 kDa greater found in the other samples (Fig. 3). The size of this band observed in the pASE280 expressed lane that was not under

Fig. 3. Expression of the fimV gene in P. aeruginosa ADD1976. Proteins encoded in plasmids under the direction of the external T7 promoter were labelled with [35S]methionine and separated on a 7.5% polyacrylamide gel. Rifampicin (200 µg ml−1) was added to inhibit host RNA polymerase. The plasmids used were pUCPKS (lane 1), pASE280 (lane 2), pASE281 (lane 3), pASE230 (lane 4) and pASE18a (lane 5). The mobility of size markers is indicated on the left.

...tion system. fimV was cloned as a 3,161 bp Ppu10I/BspHI fragment covering the predicted coding region of fimV (from −86 bp to the stop codon) into the broad-host-range T7 expression vectors pUCPKS/SK (Fig. 1). The gene was cloned both in the forward and reverse direction (pASE280 and pASE281, respectively) relative to the T7 promoter. These constructs were transformed into P. aeruginosa ADD1976, which contains a chromosomal T7 RNA polymerase gene under lac promoter control. A unique band was observed in the pASE280 expressed lane that was not found in the other samples (Fig. 3). The size of this band was estimated to be 145 kDa, which was 47 kDa greater than that predicted from the sequence for FimV (98 kDa). The fidelity of the insert including the stop codon was confirmed by restriction enzyme profiling and end sequence analysis. To try to locate the source of the anomaly we then generated truncations of the acidic C terminus of FimV, by removing 455 bp (pASE230) and 1005 bp (pASE18a) from the end of the fimV gene, with in-frame stop codons close downstream in the vector.

T7 expression of FimV

The protein encoded by fimV was examined using a T7 expression system. fimV was cloned as a 3,161 bp Ppu10I/BspHI fragment covering the predicted coding region of fimV (from −86 bp to the stop codon) into the broad-host-range T7 expression vectors pUCPKS/SK (Fig. 1). The gene was cloned both in the forward and reverse direction (pASE280 and pASE281, respectively) relative to the T7 promoter. These constructs were transformed into P. aeruginosa ADD1976, which contains a chromosomal T7 RNA polymerase gene under lac promoter control. A unique band was observed in the pASE280 expressed lane that was not found in the other samples (Fig. 3). The size of this band was estimated to be 145 kDa, which was 47 kDa greater than that predicted from the sequence for FimV (98 kDa). The fidelity of the insert including the stop codon was confirmed by restriction enzyme profiling and end sequence analysis. To try to locate the source of the anomaly we then generated truncations of the acidic C terminus of FimV, by removing 455 bp (pASE230) and 1005 bp (pASE18a) from the end of the fimV gene, with in-frame stop codons close downstream in the vector.

Phenotypic characterization of the mutants

As previously described, the identification of transposon insertions in fimbrial genes was initially based on alterations to colony morphology on the agar surface. We examined the twitching motility of these mutants in more detail using the more sensitive subsurface stab assay where twitching motility results in a zone of colony expansion at the interstitial surface between the agar and Petri dish. Four of the fimV mutants (S4, S76, S359, S361) had the same phenotype, all showing a loss of twitching motility in comparison to wild-type. S4 was used as the representative strain for further studies on this group. The fifth mutant, S125, was found to have a small irregular twitching zone (Fig. 4).

We examined whether the fimbrial subunit (PilA or pilin) is produced in the mutants and if so, if it is assembled on the cell surface into fimbriae. Whole-cell Western analysis, using an anti-PilA antiserum, revealed that all of the transposon mutants produced the fimbrial subunit. In S4, S76, S359 and S361 there was no detectable pilin on the surface of the cells shown by ELISA, whereas S125 had a small amount of the protein localized on the surface. This amount was greater than in the PAKpilA::TcR mutant but significantly less than in the wild-type (data not shown).

We assessed quantitatively the degree of sensitivity of each of the fimV mutants for infection by the type-IV-
fimbriae-specific bacteriophage PO₄. All mutants demonstrated wild-type titres for this phage (data not shown). These data suggest that the S4 group of fimV mutants may fall into the same class as mutants of pilG, pilI and pilJ, which also appear incapable of producing assembled pilus filaments but remain sensitive to fimbriae-specific bacteriophage. It has been proposed that these strains form a preliminary structure or prepilus complex consisting of an exposed pilus tip at the cell surface to which the phage can bind and subsequently infect (Darzins, 1993, 1994).

**Complementation of fimV**

The plasmid pASE281 containing fimV cloned downstream of the lac promoter (which is constitutively active in *P. aeruginosa*) was used for complementation studies of the fimV mutants. Subsurface twitching assays showed that the non-motile S4 mutant had twitching motility restored by pASE281 but not to wild-type levels. Instead, the zones remained small and irregular (Fig. 4c). S125 was also not complemented to wild-type twitching motility but appeared to exhibit exaggerated medusa-like structures erupting from the centre of the colony (Fig. 4f). PAK containing this construct also resulted in a reduction and aberration of the wild-type twitching zone (Fig. 4d). These results suggested that the overexpression of fimV may be interfering with normal twitching motility both in the mutants and in wild-type PAK.

To test this possibility, fimV was cloned into pMMB207, a vector that has an inducible tac promoter (carries the repressor lacI), to generate plasmid pASM281 (Fig. 1). By varying the concentrations of IPTG, the levels of expressed FimV could therefore be altered. Using a concentration range of 0–10 mM IPTG, the twitching zones from the S4 and S125 mutants and PAK were observed. The results showed that between 0.01 mM and 0.03 mM IPTG there was complete restoration of normal twitching motility in the mutants and the wild-type was unaffected (Fig. 5). (We presume that the twitching motility observed in the mutants at 0 mM IPTG was due to leakage of the tac promoter.) Levels of IPTG above 0.03 mM resulted in reduced and aberrant twitching zones in both wild-type and the mutants (Fig. 5). These data confirm that the loss of fimV is responsible for the loss of twitching motility in the mutants, that overexpression of fimV causes aberrant twitching motility and that a specific level of FimV expression is required for normal motility.

**Microscopic analysis of fimV mutants**

We have recently examined twitching motility in wild-type *P. aeruginosa* using video microscopy and have shown that this process involves leading edge rafts of aggregated cells in tight cell–cell contact leading out from the colony edge. These rafts appear to move primarily in an outward manner with occasional limited cell reversal. Behind these rafts the cells break up and form a network which resembles a lattice structure (Fig. 6a), wherein the cells move bidirectionally with frequent cell reversal. They are also capable of moving quite long distances within the lattice network but always within close contact of neighbouring cells. In comparison, the PAKpilA::TcR mutant has a uniform leading edge where no cells are observed moving away from the colony (Semmler et al., 1999).

We examined the micromorphological details of the twitching zones produced by the fimV mutants. The mutant S4 (and the other non-motile mutants S76, S359, S361) resembled the PAKpilA::TcR mutant (Fig. 6b). In comparison, mutant S125 formed large rafts leading out from the centre of the colony, but lacked the lattice network of cells which is characteristic of wild-type (Fig. 6c). These rafts also appeared to be larger than those exhibited by PAK. Individual cells were visible within these rafts but, unlike PAK, these cells appeared to be simply moving back and forth, resulting in a reduction in the net forward movement of the rafts. It appears therefore that S125, with its reduced fimbriae production, is capable of exhibiting the first stage of twitching motility, raft formation. It is clear that for this stage to occur at least a small number of fimbriae are required as mutants lacking surface fimbriae (PAKpilA::TcR, S4, S76, S359, S361) are incapable of exhibiting any raft formation or cellular movements. Time-lapse videos of these experiments may be viewed at our web site (http://www.cmcb.uq.edu.au/cmcb/PUBS/twitch.html).
In view of the complementation data, which suggested that *fimV* function is controlled by gene dosage, the effect of overexpressing *fimV* in the wild-type, in the non-motile *fimV* mutant S4 and in S125 was also examined. Transformation of pASE281 into the mutants partially restored the raft and lattice-type structures typical of twitching motility, but also resulted in dramatic cell elongation (Fig. 6d), which was also observed when *fimV* was overexpressed in the wild-type (not shown). The most spectacular phenotype was observed in S125 with pASE281 which exhibited grossly elongated cells, and a medusa-like phenotype at the colony level (see Fig. 4f). These effects were not observed in controls with vector alone, indicating that the elongated phenotype was not an artefact of carbenicillin selection (data not shown).

Electron microscopy of cells taken from the colony edges confirmed that overexpression of *fimV* in PAK, S4 and S125 produces cells that are grossly elongated, often with lengths up to 50–100 times normal (data not shown). Interestingly, only a small percentage of the population demonstrated this abnormality, and cells at the centre of the colony, away from the twitching edge, appeared largely normal. This suggests that only those cells which are actively in twitching mode (see Semmler et al., 1999) are affected by abnormally high levels of FimV.

**DISCUSSION**

In this report we have identified a novel gene, *fimV*, which is required for twitching motility, bringing the total now to 36 genes required for the biogenesis and function of type IV fimbriae in *P. aeruginosa*. Searches of the Unfinished Microbial Genomes database at NCBI indicates that homologues of FimV may be found in *N. meningitidis*, *N. gonorrhoeae*, *L. pneumophila*, *V. cholerae* and *Shewanella putrefaciens* as well as a second homologue in *P. aeruginosa*. No homologues have yet been identified in any other genome. As far as we can tell, *fimV* only occurs in type-IV-fimbriate bacteria. It therefore appears that FimV is specific for the biogenesis and/or function of type IV fimbriae.

Although the precise role of FimV has not yet been determined, the examination of the mutant phenotypes has revealed that this protein is essential for twitching motility. Four of the five *fimV* mutants (S4, S76, S359, S361) are incapable of twitching motility. Western analysis and ELISA data showed that these mutants
continue to produce the PilA subunit but do not produce surface-assembled fimbriae, suggesting a lesion in the process of fimbrial assembly. The fifth mutant, S125, produces a small, irregular twitching zone, demonstrates impairment of cellular motility during twitching and expresses a reduced number of fimbriae on the cell surface. The differences observed between S125 and the other mutants is likely to be due to the sites of transposon insertions, which in S125 is located ~230 bp from the stop codon of \textit{fimV}. We predict that this mutant produces a truncated form of \textit{FimV}, presumably with partial function, whilst the others do not produce a stable \textit{fimV} product.

Our complementation data indicates that a precise level of \textit{FimV} is required for normal twitching motility. Microscopic examinations show that overexpression of \textit{fimV} results in the formation of dramatically elongated cells, probably accounting for the observed defects in twitching motility when \textit{FimV} is produced at high levels. The presence of a putative peptidoglycan-binding domain in the \textit{N}-terminus of \textit{FimV} and its homologues may give some clue as to the function of these proteins. The elongated cell phenotype obtained when \textit{fimV} is overexpressed may be due to interference by high levels of \textit{FimV} during the remodelling of the peptidoglycan layer in the process of cell division and/or fimbrial biogenesis, perhaps indicating some direct association of \textit{FimV} with peptidoglycan components. It is also of interest to note that \textit{fimV} is located downstream of the genes \textit{asd} and \textit{usg-1}, the products of which are predicted to be involved in the production of the cell wall precursor \textit{meso}-diaminopimelate. We propose that \textit{FimV} may be involved in remodelling of the peptidoglycan layer to enable assembly of the type IV fimbrial structure and associated machinery. Interestingly, upstream of the pilM-\textit{Q} operon of \textit{P. aeruginosa} is a gene, \textit{ponA}, encoding a high-molecular-mass penicillin-binding protein (PBP-1A), one of a class of proteins involved in the formation and maintenance of the peptidoglycan layer (Martin \textit{et al.}, 1993). A related gene (\textit{fimD}) is also observed in the same operon as the fimbrial subunit genes (\textit{fimA} and \textit{fimZ}) in class II strains of \textit{D. nodosus} (Hobbs \textit{et al.}, 1991). Taken together, these observations suggest that cell wall structure and local remodelling is important for type IV fimbrial biogenesis and/or function at the pole of the cells.

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