## Title

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# *Pseudomonas aeruginosa fimL* regulates multiple virulence functions by intersecting with Vfr-modulated pathways

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## Summary

**Virulence of *Pseudomonas aeruginosa* involves the co-ordinate expression of a range of factors including type IV pili (tfp), the type III secretion system (TTSS) and quorum sensing. Tfp are required for twitching motility, efficient biofilm formation, and for adhesion and type III secretion (TTS)-mediated damage to mammalian cells. We describe a novel gene (*fimL*) that is required for tfp biogenesis and function, for TTS and for normal biofilm development in *P. aeruginosa*. The predicted product of *fimL* is homologous to the N-terminal domain of ChpA, except that its putative histidine and threonine phosphotransfer sites have been replaced with glutamine. *fimL* mutants resemble *vfr* mutants in many aspects including increased autolysis, reduced levels of surface-assembled tfp and diminished production of type III secreted effectors. Expression of *vfr in trans* can complement *fimL* mutants. *vfr* transcription and production is reduced in *fimL* mutants whereas cAMP levels are unaffected. Deletion and insertion mutants of *fimL* frequently revert to wild-type phenotypes suggesting that an extragenic suppressor mutation is able to overcome the loss of *fimL*. *vfr* transcription and production, as well as cAMP levels, are elevated in these revertants, while *Pseudomonas* quinolone signal (PQS) production is reduced. These results suggest that the site(s) of spontaneous mutation is in a gene(s) which lies upstream of *vfr* transcription, cAMP, production, and PQS synthesis. Our studies indicate that Vfr and FimL are components of intersecting pathways that control twitching motility, TTSS and autolysis in *P. aeruginosa*.**

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## Introduction

*Pseudomonas aeruginosa* is a ubiquitous Gram-negative bacterium found throughout the environment. This opportunistic pathogen causes serious and often life-threatening infections in immunocompromised humans such as those who are suffering from severe burns, cystic fibrosis or AIDS, who are undergoing cancer chemotherapy, or who are recovering from major surgery (Giamarellou, 2000). *P. aeruginosa* can infect many eukaryotes including mice, insects, nematodes and plants (Jander *et al*., 2000; Rahme *et al*., 2000; Tan and Ausubel, 2000). *P. aeruginosa* is equipped with a large arsenal of secreted and cell-associated virulence factors which provide nutrients for bacterial growth, enhance invasive potential and/or directly damage host tissue (Engel, 2003). The secretion of some of these virulence factors requires the type III secretion system (TTSS) that allows translocation of a set of toxins, termed effector proteins, directly into the eukaryotic host cell where they modulate host signal transduction pathways causing, among other things, cytotoxicity and disruption of the host cell cytoskeleton (Engel, 2003).

The major adhesins that *P. aeruginosa* utilizes to facilitate colonization of host epithelial cells are type IV pili (tfp) which are flexible, thin filaments up to several microns in

length located at the poles of the bacterial cell (for recent review, see Mattick, 2002). These organelles have been demonstrated to play a role in adherence to epithelial cells in culture (Woods *et al*., 1980; Doig *et al*., 1988; Chi *et al*., 1991; Comolli *et al*., 1999a) and in virulence in several different animal models of infection (reviewed in Hahn, 1997). Tfp have additional roles in *P. aeruginosa*, functioning as receptors for the binding and entry of bacteriophages (Bradley, 1980) and mediating bacterial movement on a solid surface in a process called twitching motility (Henrichsen, 1975; Bradley, 1980). Twitching motility occurs in a wide variety of pathogenic bacteria and is mediated by fimbrial extension and retraction (Merz *et al*., 2000; Skerker and Berg, 2001; Mattick, 2002). In the case of *P. aeruginosa*, tfp and twitching motility are also involved in the formation and development of biofilms (O'Toole and Kolter, 1998; Klausen *et al*., 2003a,b).

*Pseudomonas aeruginosa* tfp are polymers composed of the PilA protein (Sastry *et al*., 1985), referred to as pilin. The biosynthesis of the intact pilus on the bacterial surface and its proper function require the products of up to 40 genes arranged in multiple loci dispersed throughout the *P. aeruginosa* chromosome (Mattick, 2002). These genes fall into two broad categories: those that encode proteins involved in the structure and assembly of these organelles and those that encode regulatory proteins that control both the production of tfp (and other virulence determinants) and twitching motility in response to environmental stimuli. Included in the latter is Vfr (Beatson *et al*., 2002a), a cAMP-binding protein that has been implicated in the global regulation of numerous virulence determinants of *P. aeruginosa* including exotoxin A production, quorum sensing, expression of numerous proteins secreted by the type II general secretion pathway, repression of flagellar biosynthesis, tfp biogenesis and twitching motility, and expression of the TTSS apparatus and effector proteins (West *et al*., 1994; Albus *et al*., 1997; Beatson *et al*., 2002a; Dasgupta *et al*., 2002; Wolfgang *et al*., 2003).

Here we describe a novel *P. aeruginosa* gene, *fimL*, which is required for multiple virulence functions including tfp biogenesis, twitching motility, type III secretion (TTS)-mediated cytotoxicity towards epithelial cells *in vitro* and autolysis. Our studies indicate that FimL controls these activities, at least in part, through regulation of *vfr* expression under specific culture conditions. We have also found that *fimL* mutants frequently revert to wild-type phenotypes through acquisition of extragenic suppressor mutation(s) that result in elevated production of cAMP and Vfr.

## Results

### *Identification of* P. aeruginosa *transposon mutants of gene PA1822*

Fifteen non-twitching transposon mutants of the cytotoxic *P. aeruginosa* strain PA103 were identified in a screen for loss of cytotoxicity on Manin Darby Canine Kidney (MDCK) epithelial cell monolayers (Kang *et al*., 1997), suggesting that pili were necessary for killing epithelial cells. Sequence analysis of inverse polymerase chain reaction (PCR)-generated regions adjacent to the transposon insertions in the non-twitching mutants revealed that 12 of the mutants contained insertions into previously characterized genes. One of the remaining three mutants, PA103 mutant I, was found to harbour a transposon insertion within a novel open reading frame (ORF).

In order to isolate the genomic region containing the site of transposon insertion of PA103 mutant I for sequence analysis, a PA103 genomic fragment able to complement the twitching defect of PA103 mutant I was identified from a cosmid library by screening for the ability to restore twitching motility. Of 1000 cosmids tested, pCos5-9B was the sole cosmid able to rescue the twitching motility defect of PA103 mutant I (data not shown). Southern blot hybridization, using a probe corresponding to the PA103 mutant I inverse PCR product, was used to identify a 3.3 kb *Kpn*I fragment of pCos5-9B that contained the site of transposon insertion. The complete nucleotide sequence of the PA103 3.3 kb *Kpn*I fragment from pCos5-9B was determined for both strands (GenBank Accession No. AF083252). The sequence for the entire genome of *P. aeruginosa* PAO1 has since been determined (Stover *et al*., 2000) and the sequence of the corresponding regions are almost identical to that determined here for PA103.

Several putative ORFs were identified in the region, including PA1822 which contained the site of transposon insertion in PA103 mutant I (at nucleotide 792 of PA1822; Fig. 1A). PA1822 is a 1686 nucleotide ORF predicted to encode a 61 kDa protein of 562 amino acids. In a separate screen of a mTn*5*-Tc transposon library of PAO1293 for mutants defective in twitching motility (Beatson *et al*., 2002a), we identified a transposon mutant (31E6) that also contained an insertion in PA1822 (at nucleotide 891; Fig. 1A). Because of its role in twitching motility and tfp biogenesis (see below), we refer to PA1822 as *fimL*.

To confirm the predicted ORF for *fimL* (PA1822), we tested the ability of *Escherichia coli* to synthesize the predicted *fimL* gene product. Plasmids pJC207+ and pJC207– containing the *fimL* gene cloned in both directions relative to the T7 promoter in pGEM-7 and the vector control were transformed into *E. coli* strain BL21(DE3) which expresses T7 polymerase under control of the *lac* promoter. When transcription was induced with isopropyl β-D-thiogalactoside (IPTG), only the clone pJC207+ which contains the full-length *fimL* gene in the same direction relative to the T7 promoter produced an approximately 60 kDa protein (Fig. 1B) which is consistent with the predicted molecular weight (61 kDa) of the *fimL* gene product.

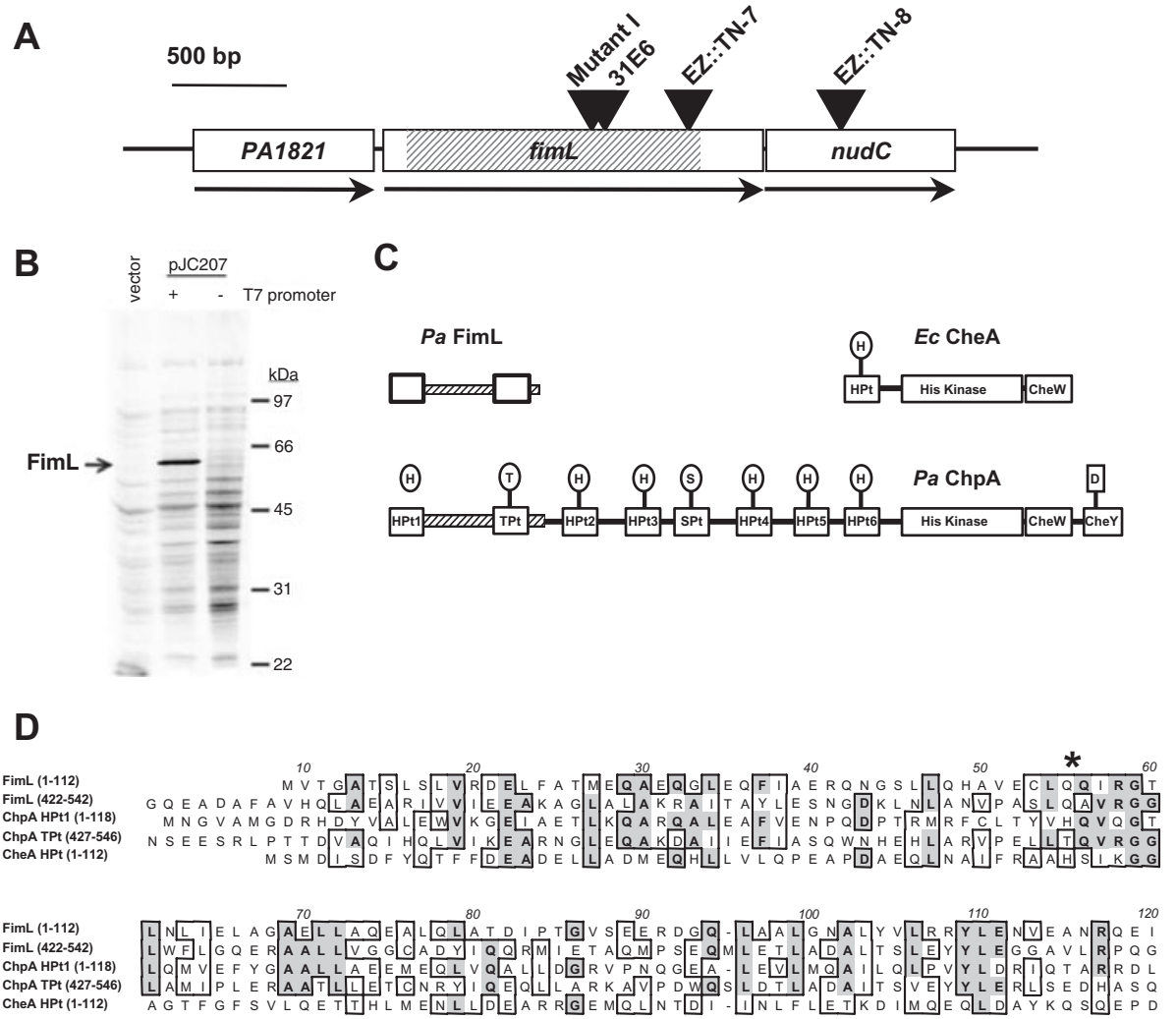


**Fig. 1.** A. Genetic organization of the *fimL* locus. Arrows represent the direction of transcription of the genes. The location of each transposon insertion is indicated by a filled triangles. The shaded region of *fimL* indicates the region deleted in the in-frame deletion mutants of *fimL*.
B. Expression of the *fimL* gene in *E. coli*. Products of pJC207+/– which contain only the *fimL* gene cloned in pGem-7 in both directions relative to the T7 promoter (+, in the same direction; –, in the opposite direction) are shown in lanes 2 and 3. The products of expression of pGEM-7 lacking insert are displayed in lane 1. Protein molecular weight markers are indicated to the right of the figure. The arrow indicates the *fimL* product produced by pJC207+.
C. Schematic representations of relevant domains and features of *P. aeruginosa* (*Pa*) FimL, *P. aeruginosa* ChpA and *E. coli* (*Ec*) CheA. 'HPt', 'TPt' and 'SPt' indicate predicted histidine-, threonine- or serine-containing phosphotransfer domains, respectively; 'His Kinase' denotes an autocatalytic histidine kinase domain which incorporates the CheY docking domain (P2) and the N, G1, F, and G2 boxes which are required for ATP binding; 'CheW' indicates a CheW-like domain which is involved in mediating CheW interactions with CheA; and 'CheY' denotes a CheY-like response regulator domain.
D. Alignment of the 'HPt-like' and 'TPt-like' domains of *P. aeruginosa* (*Pa*) FimL with the putative HPt1 and TPt domains of *Pa* ChpA and the HPt domain of *E. coli* (*Ec*) CheA. Similar residues are boxed; identical residues are indicated in bold and are shaded. * denotes the position of the predicted site of phosphorylation in the HPt/TPt domains of ChpA and CheA. The alignment was generated using the CLUSTALW feature in MacVector (Oxford Molecular Group).

### fimL *is part of a cluster of three genes*

Two ORFs which are predicted to be transcribed in the same direction flank the *fimL* gene, suggesting that it may be part of a small operon (Fig. 1A). The upstream ORF (PA1821) is predicted to encode a 270-amino-acid protein (30 kDa) which has been annotated in the PAO1 genome sequence to be a probable enoyl-CoA hydratase/ isomerase. This family of proteins includes biosynthetic enzymes involved in fatty acid elongation. BLASTP analysis indicates that this protein has highest identity (74–80%) to homologues from other *Pseudomonas* species whereas the next closest homologues (46–48% identity) are mammalian peroxisomal and mitochondrial enzymes rather than enoyl-CoA hydratases from other Gram-negative bacteria. The stop codon of PA1821 is separated from the putative start codon of *fimL* by 40 nucleotides. The third

ORF (PA1823; *nudC*) in this gene cluster has a start codon that overlaps the *fimL* stop codon. PA1823 (*nudC*) encodes a putative 278-amino-acid protein (31 kDa) that is predicted by SWISS-PROT to be a NADH pyrophosphatase and as such belongs to the NudC subfamily of the Nudix hydrolase family, which is a class of proteins that catalyse the hydrolysis of nucleoside diphosphates from a variety of substrates (Bessman *et al*., 1996).

BLASTP analysis shows that *P. aeruginosa* FimL is homologous (35% identity, 56% similarity) to a 576-amino-acid hypothetical protein from *Microbulbifer degredans* (ZP_00065238). The FimL homologue of *M. degredan*s shares a similar genetic arrangement to that of *P. aeruginosa fimL*. In both bacteria, the gene encoding the FimL homologue is followed by a gene encoding a NudC homologue, although in *M. degredans* a gene encoding a PA1821 homologue is not located upstream.

The location of *fimL* upstream of *nudC* raises the possibility that the twitching motility defects observed in PA103 mutant I and PAO1293 mutant 31E6 could result from polar effects of *fimL* transposon insertions on *nudC* transcription. We undertook several approaches to address this possibility. First, we constructed an isogenic in-frame deletion mutant of *fimL* and an EZ::TN<TET-1> insertion mutant of *nudC* in both PA103 and PAO1 (Fig. 1A). Twitching motility stab assays (in which twitching motility results in colony expansion at the agar/plastic interface) demonstrate that these *fimL* deletion mutants of PA103 and PAO1 behaved identically to that observed with the original transposon insertion mutants of PA103 (mutant I), and PAO1293 (mutant 31E6) (Fig. 2A; data not shown) whereas the *nudC* mutants showed no defects in twitching motility (data not shown). These observations indicate that the observed twitching motility defects of *fimL* mutants result from the loss of *fimL* and not from polar influences on *nudC* expression nor from a secondary mutation elsewhere in the genome.

Next we determined that FimL alone was sufficient to complement the twitching defects observed in *fimL* mutants. An 8 kb *Bam*HI fragment containing *fimL* and its surrounding ORFs (PA1821 and *nudC*) was subcloned from the complementing cosmid pCos5-9B into pUCPSK to create pCW32. This plasmid was subjected to *in vitro* transposon mutagenesis with EZ::TN<TET-1> and plasmids containing transposon insertions in *fimL* (pCW32-EZ::TN-7) or *nudC* (pCW32-EZ::TN-8) were isolated (Fig. 1A). pUCPSK, pCW32, pCW32-EZ::TN-7 and pCW32-EZ::TN-8 were transformed into wild-type PA103, mutant I and PA103Δ*fimL* and twitching motility was assayed (data not shown). Both pCW32 and pCW32-EZ::TN-8 (*nudC* insertion) but not pCW32-EZ::TN-7 (*fimL* insertion) restored twitching motility to the PA103 *fimL* mutants indicating that *fimL* is required for the ability to complement twitching motility to *fimL* mutants of PA103. Together, these results convincingly demonstrate that mutation of *fimL* (and not *nudC*) is responsible for the defects in twitching motility observed with *P. aeruginosa fimL* mutants.

Interestingly, when we tried complementation of twitching motility with pCW32 we found that this plasmid did not restore twitching motility to PAO1Δ*fimL* and completely abrogated twitching motility in wild-type PAO1, whereas inhibition of twitching motility by pCW32 was not observed with wild-type PA103 (see above). Repression of twitching motility in PAO1 did not occur with pCW32-EZ::TN-7 (*fimL* insertion) but did with pCW32-EZ::TN-8 (*nudC* insertion) suggesting that the presence of *fimL* on pCW32 was responsible for the loss of twitching motility in PAO1 (data not shown). To examine this further we subcloned *fimL* from pCW32-EZ::TN-8 (to utilize convenient cloning sites located in the EZ::TN) into the low-copy-number, IPTG-inducible expression vector pMMB208 in both orientations relative to the *Ptac* promoter (pJB23-1 and pJB23-2). Both orientations of *fimL* relative to *Ptac* could restore twitching motility in PAO1Δ*fimL* (data not shown), indicating that the defect in twitching motility in this mutant results from deletion of *fimL*. Titration of FimL expression from pJB23-1 (*fimL* with *Ptac*) with 0–10 mM IPTG shows that higher levels of expression of FimL causes a reduction in twitching motility zone size in PAO1 and PAO1Δ*fimL* (data not shown). The observed repression of twitching motility in PAO1 with the higher-copy-number pUCPSK clone pCW32 and with high IPTG concentrations when expressing FimL from pJB23-1 suggests that FimL levels within PAO1 need to be tightly regulated.

### *FimL is homologous to the N-terminal domain of ChpA*

Twitching motility in *P. aeruginosa* is controlled by a complex chemosensory pathway comprised of the proteins PilG, PilH, PilI, PilJ, PilK, ChpA, ChpB and ChpC (D'Argenio *et al*., 2002; Wolfgang *et al*., 2003; Whitchurch *et al*., 2004). The central component of the pathway, ChpA, is a hybrid of homologues of the histidine kinase CheA and the response regulator CheY (Fig. 1C; Whitchurch *et al*., 2004). *P. aeruginosa* ChpA (2477 amino acids) is a highly complex protein possessing nine potential sites of phosphorylation: six histidine-containing phosphotransfer (HPt) domains, two novel serine- and threonine-containing phosphotransfer domains (SPt, TPt) and a CheY-like receiver domain at its C-terminus (Fig. 1C; Whitchurch *et al*., 2004). BLASTP analysis shows that *P. aeruginosa* FimL is homologous (33% identity, 51% similarity) to the N-proximal domains of *P. aeruginosa* ChpA and its homologues found in *M. degredans*, *Pseudomonas fluorescens*, *Pseudomonas syringae*, *Pseudomonas putida*, *Xyella fastidiosa*, *Xanthomonas axonopodis*, *Xanthomonas campestris* and *Nitrosomonas europaea.* FimL

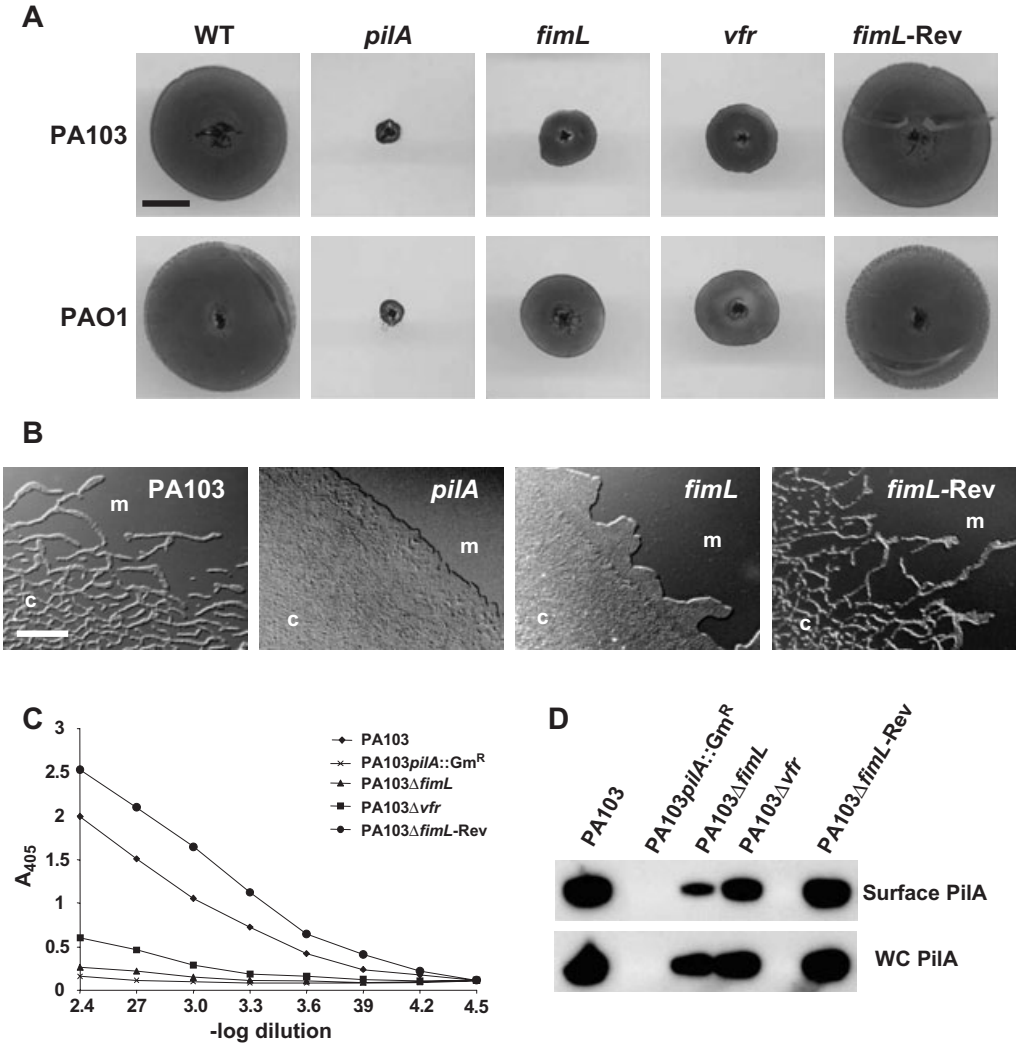


**Fig. 2.** A. Subsurface twitching motility at agar/plastic interface of tissue culture-treated polystyrene dishes after 16 h incubation at 37°C. Top row: PA103, PA103*pil*A::Gm$^R$, PA103Δ*fimL*, PA103Δ*vfr* and PA103Δ*fimL*-Rev. Bottom row: PAO1, PAO1Δ*pilA*, PAO1Δ*fimL*, PAO1Δ*vfr* and PAO1Δ*fimL*-Rev. Bar represents 1 cm.

B. Light microscopy of zones of twitching motility showing typical colony expansion zones obtained at the interstitial surface between the glass coverslip and GelGro (ICN) media for PA103, PA103*pil*A::Gm$^R$, PA103Δ*fimL* and PA103Δ*fimL*-Rev. These are representative of phenotypes observed with corresponding PAO1 strains. Micrographs were taken after 3 h incubation at 37°C. Bar represents 50 μm; 'm' denotes the media and 'c' denotes the colony.

C. ELISA of whole cells from 8 h plate cultures of *Pseudomonas* strains PA103 (◆); PA103*pilA*::Gm$^R$ (×); PA103Δ*fimL* (▲); PA103Δ*vfr* (■); PA103Δ*fimL*-Rev (●). PilA was detected with anti-PilA serum and is indicative of the levels of surface pili in these strains. ELISA shown is representative of multiple experiments.

D. Immunoblot of PilA found in sheared surface pili preparations (top) and in whole cell (WC) preparations after surface pili have been sheared from 8 h plate cultures (bottom) of strains PA103 (lane 1); PA103*pilA*::Gm$^R$ (lane 2); PA103Δ*fimL* (lane 3); PA103Δ*vfr* (lane 4); and PA103Δ*fimL*-Rev (lane 6). Lane 5 is a blank lane. Immunoblots shown are representative of multiple experiments.

is also homologous to the N-terminal domain of the 991-amino-acid *Xanthomonas oryazae* PilL protein which has been shown to have a possible interaction with flagellar biosynthetic protein FlhF (Shen *et al*., 2001). *X. oryazae* PilL is significantly longer than FimL and is 90% identical to the first 992 amino acids of the 2422-amino-acid *X. axonopodis* ChpA homologue, and 82% identical to the first 984 amino acids of the 2345-amino-acid *X. campestris* ChpA homologue. As such it appears to be either a truncated version of the *X. axonopodis* and *X. campestris* PilL proteins (ChpA homologues) or (more likely) a sequencing error, as originally occurred in the case of *P. aeruginosa* ChpA which has a domain homologous to FimL (and which was originally referred to as PilL) (Whitchurch *et al*., 2004). Only *P. aeruginosa* and *M. degredans* encode both ChpA and FimL homologues.

The N-terminal 563 amino acids of *P. aeruginosa* ChpA are homologous to FimL across the entire length of FimL.

Interestingly, this 'FimL-like' domain of ChpA contains two regions that are highly homologous to HPt and TPt domains, respectively, except that the predicted phosphorylatable residues of these domains have been replaced with glutamine (H–Q and T–Q substitutions respectively) (Fig. 1C and D). These glutamine substitutions would remove the ability of FimL to participate in phosphorelay reactions, but are otherwise structurally conservative substitutions. Therefore, although FimL possesses two domains that are homologous to potential phosphotransfer domains, we would predict that FimL is incapable of phosphorylation in these regions and that it may act as a competitive inhibitor of phosphotransfer interactions involving ChpA and its interacting partners. Similarly, the *M. degredans* FimL homologue is highly conserved across the entire FimL-like domain of *M. degredans* ChpA protein, including the HPt and TPt domains. The *M. degredans* FimL protein contains a histidine–arginine substitution in the HPt domain but retains the threonine residue in the TPt domain. There were no other identifiable functional domains in FimL according to SMART or Pfam domain analysis.

Given the similarity of FimL to the N-proximal domain of the complex chemosensory protein ChpA, we considered the possibility that FimL participates in signal transduction control of twitching motility in *P. aeruginosa* by intersecting with this system. We expressed ChpA (pUCPChpA, pMMBChpA), the FimL-like N-proximal domain of ChpA (pJEN54, pJB24) and a clone containing all of *pilG-pilK* (pJEN53a) in *fimL* mutants of PA103 and PAO1 and assayed the ability of these clones to complement twitching motility to determine whether provision of any of these signalling components provided *in trans* could bypass the *fimL* mutation. None of the clones were able to restore twitching motility to *fimL* mutants (data not shown). Of note, expression of pUCPChpA in PAO1 suppresses twitching motility in the wild-type strain (Whitchurch *et al*., 2004).

### fimL *mutants have reduced surface-assembled tfp and twitching motility*

The twitching motility phenotypes of the *fimL* mutants were examined via the subsurface stab assay. In this assay, normal twitching motility results in rapid colony expansion at the agar–Petri dish interface (Fig. 2A); non-twitching mutants such as *pilA* mutants produce no such zone of expansion (Fig. 2A). Whereas PAO1 elicits 2–3 cm twitching motility zones under these conditions, PA103 demonstrates very poor twitching motility at the agar–Petri dish interface when standard polystyrene Petri dishes are used, routinely producing zones of colony expansion $<1$ cm in diameter after 24 h incubation at 37°C. We have found, however, that tissue culture-treated polystyrene dishes promote extensive twitching motility in PA103 (Fig. 2A) possibly due to enhanced binding of the tfp to the treated surface. Twitching motility stab assays using tissue culture-treated dishes show that *fimL* mutants of both PA103 and PAO1 are capable of some twitching motility. *fimL* mutants produce zones of colony expansion which are very reduced relative to wild type but which are significantly larger than that produced by the non-twitching *pilA* mutants (Fig. 2A).

Studies using time-lapse video microscopy to examine the dynamics of twitching motility at a cellular level have revealed that twitching motility is a complex social process. Wild-type bacteria move in a highly co-ordinated fashion, initially forming rafts of cells which move away from the colony edge, behind which an intricate lattice-like network of cells is formed (Semmler *et al*., 1999; see Fig. 2B). Non-twitching mutants (such as *pilA* mutants) demonstrate no differentiation of the colony edge (Semmler *et al*., 1999; see Fig. 2B). We used this slide microscopy technique to more closely examine the twitching motility of *fimL* mutants in both PA103 and PAO1 strain backgrounds. When examined after 2–4 h incubation at 37°C, *fimL* mutants of both strains behaved similarly and appear capable of the early stages of twitching motility forming large, exaggerated rafts of cells which move away from the colony edge but lack the development of the characteristic lattice network behind these rafts (Fig. 2B).

ELISA and Western blots using anti-PilA anti-serum were used to examine the degree of surface-assembled tfp and pilin production in *fimL* mutants. As we only have anti-sera specific for the PA103 PilA subunit, PAO1 strains were not included in these ELISA and Western analyses. We assayed tfp production and surface assembly from 8 h plate cultures to avoid problems with autolysis of PA103 strains (see below). ELISA of whole cells and Westerns of sheared tfp demonstrate that *fimL* mutants produce very small amounts of surface-assembled tfp (Fig. 2C and D), which are presumably sufficiently functional to facilitate the small amount of twitching motility observed in *fimL* mutants. Levels of cell-associated pilin subunit were assayed by Western analysis of lysed whole cells. These studies reveal that *fimL* mutants remain capable of producing pilin subunit (Fig. 2D). Thus, the reduction in levels of surface-assembled tfp seen in strains lacking *fimL* is not likely to result from a defect in pilin production *per se*, but possibly results from an inability to properly co-ordinate the biogenesis of the tfp structures either through defects in assembly or due to increased retraction.

Other tfp-related phenotypes of *P. aeruginosa* include sensitivity to certain bacteriophage and swarming motility. We assayed sensitivity to the tfp-specific bacteriophage PO4 (Bradley and Pitt, 1974) and found that the *fimL* mutants had wild-type levels of sensitivity to this bacteriophage (data not shown; Kang *et al*., 1997). Wild-type sensitivity of *fimL* mutants to phage PO4 is consistent with

the ability of these strains to produce at least a small amount of functional surface-assembled tfp.

*Pseudomonas aeruginosa* is unique among Gram-negative bacteria that demonstrate flagella-mediated swarming motility in that *P. aeruginosa* swarming also requires tfp (Kohler *et al*., 2000). As strain PA103 is non-flagellated (Montie *et al*., 1982), we assayed swarming motility using PAO1 and its isogenic mutants on 0.8% nutrient broth (Oxoid) containing 0.5% glucose solidified with 0.5% agar as described previously (Deziel *et al*., 2001). Under these conditions PAO1Δ*fimL* demonstrated wild-type swarming motility (data not shown) whereas the isogenic *pilA* mutant demonstrated only a small zone of swarming motility (data not shown). We also examined flagella-mediated swimming motility through Luria broth (LB) set with 0.3% agar and found swimming to be normal for the isogenic PAO1 *fimL* and *pilA* mutants (data not shown).

### fimL *mutants form mature but irregularly shaped biofilms*

The role of tfp and twitching motility in biofilm development by *P. aeruginosa* has recently been carefully examined using time-lapse confocal laser scanning microscopy (CLSM) of colour-coded bacteria (Klausen *et al*., 2003a,b). In these studies it was observed that in glucose media *P. aeruginosa* PAO1 biofilms develop by initially forming microcolonies by clonal growth of a sessile subpopulation (in which twitching motility has been downregulated) and thereafter migrating bacteria accumulate on top of the microcolonies (stalks) to form mushroom caps. This migration was found to be dependent on tfp (Klausen *et al*., 2003a). Given the markedly reduced twitching motility and levels of surface-assembled tfp seen in *fimL* mutants, we were interested in determining the influence that *fimL* mutation would have on biofilm development by *P. aeruginosa*. As PA103 forms poor biofilms (T. Tolker-Nielsen, unpubl. obs.), we assayed biofilm development in PAO1.

Initial examination of 4-day-old biofilms formed by green fluorescent protein (GFP)-tagged *fimL* mutants in glucose minimal media shows that these mutants form more irregular and asymmetric mushroom structures than wild-type PAO1 (Fig. 3A). To determine whether biofilm development by PAO1Δ*fimL* solely results from clonal growth (as is observed for *pilA* mutants) (Klausen *et al*., 2003a); we

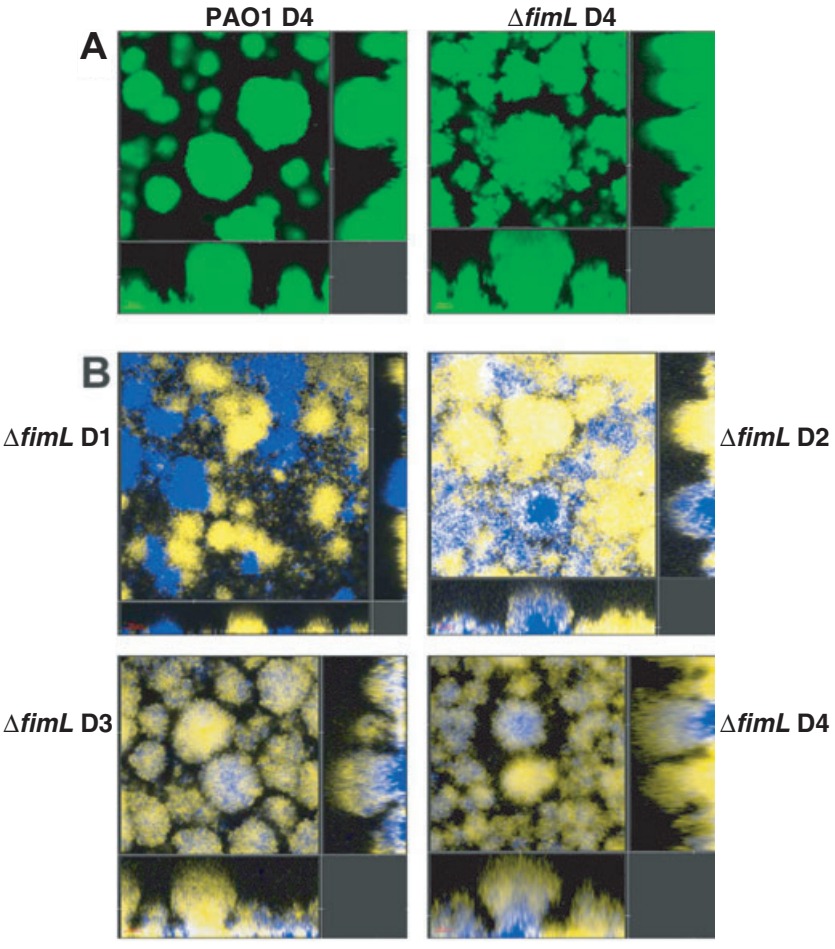


**Fig. 3.** FimL biofilm structure.
A. Confocal laser scanning microscopy (CLSM) micrographs of GFP-tagged *P. aeruginosa* PAO1 and PAO1Δ*fimL* 4-day-old biofilms grown on glucose minimal medium showing the more irregular and asymmetric mushroom structures produced by PAO1Δ*fimL*.
B. Time-lapse CLSM in a colour-coded *P. aeruginosa* PAO1Δ*fimL* biofilm. The biofilm was initiated with a 1:1 mixture of yellow fluorescent and cyan fluorescent PAO1Δ*fimL*. The CLSM micrographs were acquired after 1, 2, 3 and 4 days of biofilm development in glucose minimal medium. Days 1 and 2 show development of yellow or cyan stalks. The presence of mixed colour caps on either yellow or cyan stalks are visible in Days 3 and 4. The central pictures show horizontal projections, and the flanking pictures show vertical sections. Bars represent 20 mm.

examined biofilm development by PAO1Δ*fimL* mutants more closely, by initiating biofilms with a 1:1 mixture of yellow fluorescent protein (YFP)- or cyan fluorescent protein (CFP)-tagged PAO1Δ*fimL*. Biofilm development was observed by CSLM over a 4 day period (Fig. 3B). Again, the 4-day-old biofilms formed more irregularly shaped structures than is usually observed for wild-type PAO1 (Fig. 3B). Surprisingly, however, these assays show that PAO1Δ*fimL* biofilm development occurs through a similar developmental procedure as observed for wild-type PAO1. The mixed colour caps atop the monocoloured stalks are indicative that the bacteria migrated to the top of stalks formed by a sessile subpopulation to form the mushroom caps (Fig. 3B; Klausen *et al*., 2003a). Time-lapse video microscopy of initial biofilm development confirms that *fimL* mutants migrate (twitch) on the surface in a manner indistinguishable from wild-type PAO1. It is possible that the magnitude of the tfp defect in the *fimL* mutant may depend on environmental conditions and may be less severe in the setting of biofilm growth than during growth on agar plates.

### fimL *mutants show increased autolysis*

Many strains of *P. aeruginosa*, in particular PA103, demonstrate 'plaque-like' clearings associated with iridescent autolysis when grown on agar plates (Warner, 1950; Zierdt, 1971; D'Argenio *et al*., 2002). The mechanism of autolysis is unknown but is associated with *Pseudomonas* quinolone signal (PQS) production (D'Argenio *et al*., 2002), a secreted quinolone whose production is induced during stationary-phase growth and that interfaces with the Las and Rhl quorum-sensing systems (Diggle *et al*., 2003). Although plate-grown PAO1 does not exhibit significant autolysis, PQS-overproducing strains of PAO1 are highly autolytic. This phenotype can be suppressed by mutation in PQS biosynthetic genes (D'Argenio *et al*., 2002).

As plate-grown PA103 is visibly autolytic, we examined PA103Δ*fimL* for this property. As shown in Fig. 4A, PA103 *fimL* mutants show increased autolysis when cultured on LB agar plates. Given the association between PQS production and autolysis, we measured levels of cell-

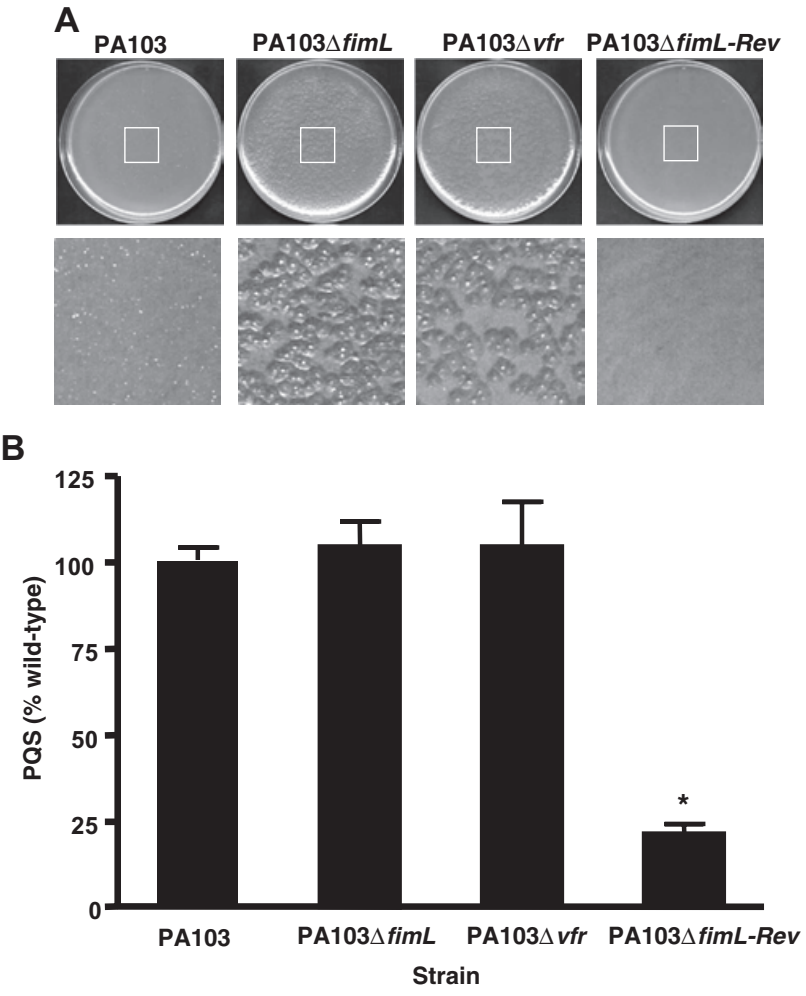


**Fig. 4.** A. Autolysis of PA103 strains. PA103, PA103Δ*fimL*, PA103Δ*vfr* and PA103Δ*fimL*-Rev were grown on confluent LB agar plate cultures at 37°C for 24 h. An enlarged portion of the boxed area in each plate in the upper portion of the panel is shown below each plate. Extensive autolysis is clearly visible in the PA103Δ*fimL* and PA103Δ*vfr* plates. PA103 shows a small degree of autolysis. No autolysis is visible in PA103Δ*fimL*-Rev.
B. Cell-associated PQS levels. PA103, PA103Δ*fimL*, PA103Δ*vfr* and PA103Δ*fimL*-Rev were grown as confluent LB agar plate cultures at 37°C for 16 h. The bacteria were collected by suspension in PBS, and PQS was extracted and separated by thin layer chromatography. The PQS spot was visualized by UV illumination and quantified on a Bio-Rad gel doc station using Quantity One software (version 4.5.0). The experiment was repeated three times with three biological replicates in each experiment. The mean ± SEM (N-9) is shown. $*P < 0.0001$ compared with wild type (Student's two-tailed *t*-test).

associated PQS in plate-grown cultures of wild-type PA103 and PA103Δ*fimL* at 8 and 16 h. PQS was not detectable in 8 h plate cultures for any of the PA103 strains (data not shown) but was measurable in PA103 after 16 h of plate growth (Fig. 4B). This may correspond to the onset of stationary-phase growth. No changes in cell-associated PQS levels were detected in the FimL mutant after 16 h of plate growth (Fig. 4B). It is possible that very small changes in PQS may be sufficient to trigger autolysis or that autolysis in fimL and *vfr* mutants is triggered via a mechanism independent of PQS production.

### fimL *is required for TTSS and for adhesion to epithelial cells*

PA103 is a highly cytotoxic strain of *P. aeruginosa* that uses the TTSS to translocate effector proteins directly into epithelial cells. The transposon insertion mutant of *fimL* PA103 mutant I was originally isolated in a genetic screen to identify mutants that were non-cytotoxic towards epithelial cells (Kang *et al*., 1997). We confirmed the role of *fimL* in cytotoxicity using the in-frame deletion mutant PA103Δ*fimL*. Bacteria were added to the apical medium of HeLa cell monolayers and the amount of cell death after 5 h was assayed by LDH release (Fig. 5A). These studies found that PA103Δ*fimL* shows cytoxicity levels similar to the isogenic *pilA* mutant, to the TTSS mutant MutN (*pscJ*) and to uninfected control [$P$ = not significant (NS)].

We also tested the ability of the cloned *fimL* gene to restore cytotoxic activity to PA103Δ*fimL*. As expected, we found that addition of PA103Δ*fimL* transformed with the vector control (pUCPSK) to HeLa monolayers resulted in

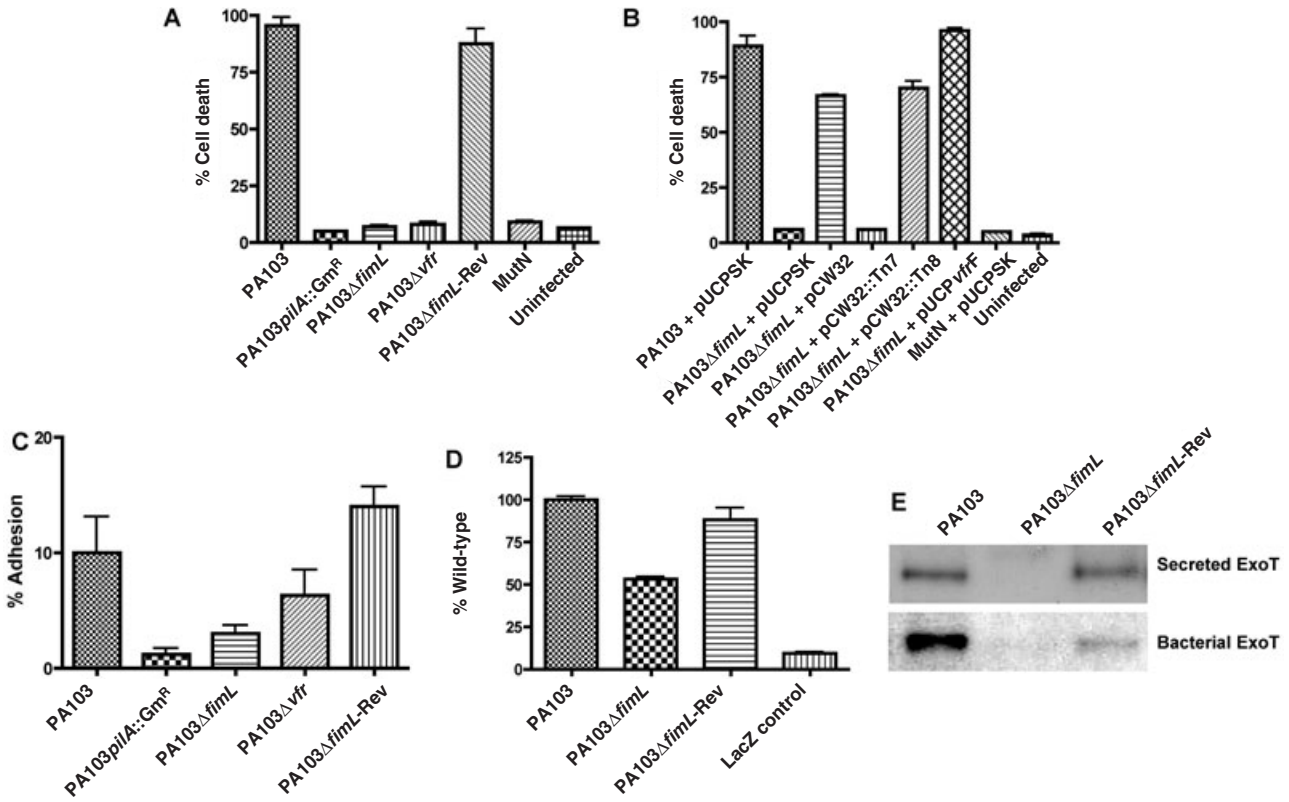


**Fig. 5.** A and B. *P. aeruginosa* cytotoxicity of epithelial cell monolayers. (A) PA103, PA103*pilA*::Gm$^R$, PA103Δ*fimL*, PA103Δ*vfr*, PA103Δ*fimL*-Rev, MutN (*pscJ*) and (B) PA103, PA103Δ*fimL* and MutN transformed with indicated plasmids were added to the apical surface of HeLa cell monolayers (moi = 50) and co-cultivated for 5 h. Cell death was quantified by LDH release and is normalized to LDH release caused by triton lysis. Assays were performed in triplicate and data are presented as means ± SD.

C. *P. aeruginosa* adhesion to epithelial cell monolayers. PA103, PA103*pilA*::Gm$^R$, PA103Δ*fimL*, PA103Δ*vfr* and PA103Δ*fimL*-Rev were added to the apical surface of HeLa cell monolayers (moi = 250) and co-cultivated for 1 h. Cfu of adherent bacteria were quantified and normalized to the cfu of the input inoculum (% Adhesion). Multiple assays were performed ($n$ = 6) and data are presented as means ± SD.

D. *vfr* promoter activity. The indicated strains harbouring the *Pvfr–lacZ* fusion integrated into the chromosome at the CTX site were co-cultivated with HeLa cell moloayers for 1.5 h. The β-galactosidase activities of samples were measured, specific activities (relative to $OD_{600}$) calculated and results normalized to PA103. Multiple samples were assayed ($n$ = 6) and data are presented as means ± SD.

E. ExoT production and secretion. The indicated strains were grown in the presence of HeLa cells for 1.5 h. The bacteria were collected by centrifugation and lysed in Laemmli buffer. The media were filtered and TTS-secreted proteins concentrated by ammonium sulphate precipitation. The bacterial pellets and media supernatants were electrophoresed on 10% SDS-PAGE and immunoblotted with anti-ExoT. Results shown are representative of those obtained from multiple experiments.

cell death similar to HeLa cells exposed to media alone or to MutN containing pUCPSK (Fig. 5B). The *fimL* clone pCW32 and pCW32-EZ::TN8, which carries a Tet insertion in *nudC* (Fig. 1A), restored the ability of PA103Δ*fimL* to kill HeLa cells whereas pCW32-EZ::TN7, which has a Tet insertion in *fimL* (Fig. 1A), did not restore cytotoxicity (Fig. 3B). These results confirm that *fimL* and not *nudC* or some other secondary mutation elsewhere on the genome is mediating the defect in cytotoxicity observed with PA103 *fimL* mutants.

As tfp are the major adhesin used by *P. aeruginosa* for attachment to epithelial cells, one role of tfp in cytotoxocity is to mediate the initial stages of attachment of bacteria to epithelial cells. Indeed, we have previously demonstrated that functional tfp are required for cytotoxicity towards epithelial cells (Kang *et al*., 1997; Comolli *et al*., 1999a). Given the reduced levels of surface-assembled tfp in *fimL* mutants (Fig. 2C and D), we predicted that *fimL* mutants would be severely defective in their ability to attach to epithelial cells. As shown in Fig. 5C, PA103Δ*fimL* shows very poor adhesion to HeLa cells similar to that observed with the non-piliated *pilA* mutant ($P$ = NS). Therefore, the loss of cytotoxicity in *fimL* mutants at least in part results from defective adhesion.

### fimL *regulates* Vfr

*vfr* mutants have been reported to be deficient in twitching motility and TTS (Beatson *et al*., 2002b; Wolfgang *et al*., 2003). In particular, the twitching motility and tfp phenotypes of *vfr* mutants are very reminiscent of what we have observed for *fimL* mutants, suggesting that *fimL* and *vfr* mutants may function in a common pathway. Interestingly, it was also reported that PAO1 *vfr* mutants demonstrate mild autolysis although the role of this cAMP-binding transcriptional regulator in autolysis is unknown (D'Argenio *et al*., 2002). To more rigorously compare the similarities between *fimL* and *vfr* mutants, we generated isogenic in-frame deletion mutants of *vfr* in PAO1 and PA103 and assessed the twitching motility, tfp production, autolysis and PQS production in these mutants in the appropriate strain background. All of the mutants had similar growth rates when grown in LB with vigorous aeration (data not shown). We found that both *fimL* and *vfr* mutants of PAO1 and PA103 show similar defects in twitching motility by stab assay (Fig. 2A), and PA103Δ*vfr* assembles small amounts of surface tfp (Fig. 2C and D), although PA103Δ*fimL* has a more severe reduction in surface-assembled tfp than PA103Δ*vfr* (Fig. 2C and D; recall that the anti-pilin antibody does not recognize PAO1 pilin, which precludes direct quantification of PAO1 pilin). Similar to PA103Δ*fimL*, plate-grown PA103Δ*vfr* exhibits increased autolysis and wild-type level PQS production relative to wild type (Fig. 4A and B). It should be noted that in the culture conditions under which we performed these assays, autolysis of PA103 is minimized and autolysis is not observed in any of the PAO1 strains (including PAO1Δ*vfr*) even after 10 days of incubation at 30°C (data not shown). The PA103 *fimL* and *vfr* mutants also show similar defects in cytotoxicity of epithelial cells *in vitro* ($P$ = NS; Fig. 5A and C) and are both defective in adhesion to epithelial cells relative to wild type ($P < 0.05$; Fig. 5C). Interestingly, PA103Δ*vfr* shows increased adherence to epithelial cells relative to PA103Δ*fimL* ($P < 0.05$). This is consistent with our observations that PA103Δ*vfr* produces more surface-assembled tfp than PA103Δ*fimL*.

Given the phenotypic similarities between *vfr* and *fimL* mutants, we tested whether cloned *vfr* could complement *fimL* mutants. The clones pUCP*vfr*F and pUCP*vfr*A contain *vfr* subcloned into pUCPSK in both orientations relative to the *Plac* promoter and both can complement *vfr* mutants of PAK and PAO1 (Beatson *et al*., 2002a). We transformed both pUCP*vfr*F (*vfr* with *Plac*) and pUCP*vfr*A (*vfr* against *Plac*) into wild type and *fimL* mutants of PA103 and PAO1 and examined twitching motility via stab assay. Neither plasmid altered the twitching motility phenotypes of wild-type strains (data not shown). pUCP*vfr*F but not pUCP*vfr*A was able to fully restore twitching motility to *fimL* mutants of both PA103 and PAO1 (Fig. 6A, data not shown). Furthermore, pUCP*vfr*F but not pUCP*vfr*A significantly reduced the autolysis phenotype of PA103Δ*fimL* (data not shown). Thus, it appears that *fimL* mutant phenotypes can be complemented by *vfr* provided *in trans*. Furthermore, our observations suggest that either a high level of *vfr* expression is required for complementation of *fimL* mutants and/or that expression of *vfr* from the endogenous *vfr* promoter in pUCP*vfr*A is poor in *fimL* mutants.

### Vfr *expression and production is reduced in* fimL *mutants when cultured on agar*

As the twitching motility defect in *fimL* mutants could be complemented by extrachromosomal copies of *vfr*, we examined *vfr* transcription in *fimL* mutants to determine whether FimL might regulate *vfr* expression. A *Pvfr–lacZ* transcriptional fusion reporter was constructed using a mini-CTX-*lacZ* promoter reporter system (Becher and Schweizer, 2000; Hoang *et al*., 2000). The mini-CTX system is based on the lysogenic phage ΦCTX and facilitates the integration of genetic elements in single copy at a defined location (phage attachment site *attB*) on the *P. aeruginosa* chromosome. The mini-CTX-*Pvfr–lacZ* reporter was introduced into PA103, PAO1, PA103Δ*fimL* and PAO1Δ*fimL*. A promoterless mini-CTX-*lacZ* was also introduced into the wild-type strains as a negative control. β-Galactosidase activity was assayed from 8 h and 16 h LB plate cultures. PAO1Δ*fimL* shows a reproducible and significant reduction in *Pvfr* activity relative to wild type

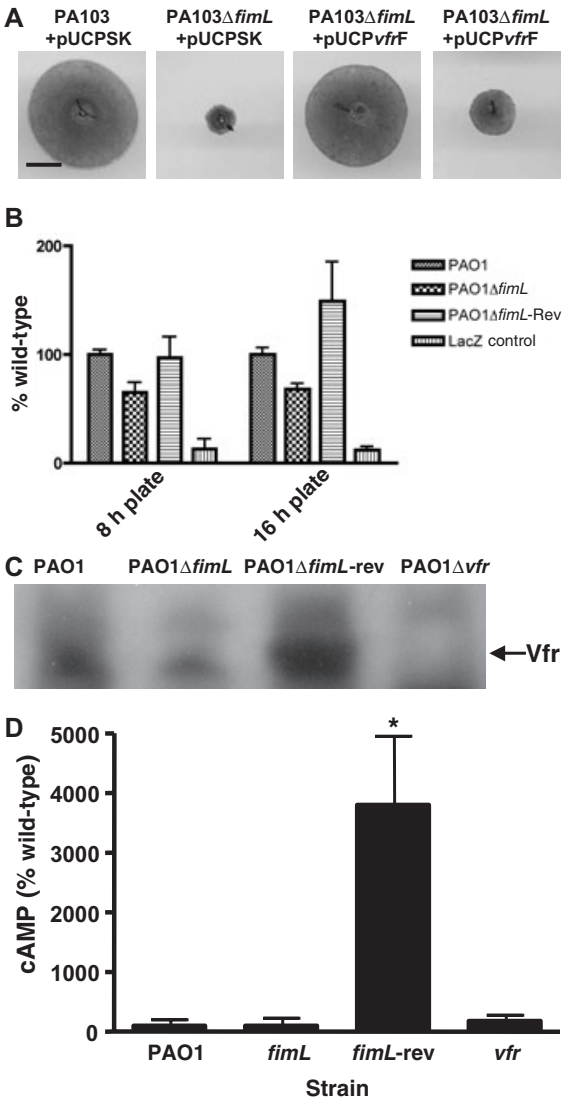


**Fig. 6.** FimL regulates Vfr.
A. Vfr restores twitching motility to PA103Δ*fimL*. Subsurface twitching motility at the agar/plastic interface of tissue culture-treated polystyrene dishes after 16 h incubation at 37°C by PA103 + pUCPSK (vector control); PA103Δ*fimL* + pUCPSK; PA103Δ*fimL* + pUCP*vfr*F (*vfr* with *Plac*); and PA103Δ*fimL* + pUCP*vfr*A (*vfr* against *Plac*). pUCP*vfr*F restores twitching motility in PA103Δ*fimL* to wild-type levels. Partial restoration of twitching motility in PA103Δ*fimL* is seen with pUCP*vfr*A. Similar results were obtained with PAO1Δ*fimL*. All panels are at the same magnification. Bar represents 1 cm.
B. *vfr* promoter activity in plate-grown PAO1 strains. The indicated strains harbouring the *Pvfr–lacZ* fusion were grown on LB agar plates for 8 h or 16 h. The β-galactosidase activities of samples were measured, specific activities (relative to $OD_{600}$) calculated and results normalized to PAO1. Multiple samples were assayed ($n = 9$) and data are presented as means ± SD.
C. Vfr levels. The indicated strains were grown on LB plates for 16 h. Bacteria were enumerated by serial dilution and lysates from equal numbers of bacteria were separated by SDS-PAGE and immunoblotted with a Vfr antibody. Vfr protein levels are decreased in PAO1Δ*fimL* and increased in PAO1Δ*fimL* revertent.
D. cAMP levels. The indicated strains were grown on LB plates for 16 h and cAMP levels measured using an ELISA-based assay. cAMP levels are increased in the PAO1Δ*fimL* revertent (*$P < 0.05$, Student's two-tailed *t*-test compared with wild type).

(66–72% of wild type; $n = 9$–12; $P < 0.05$; Fig. 6B). These findings were confirmed by measuring steady-state Vfr protein levels of plate-grown PAO1 by Western blot analysis. At both 8 h (data not shown) and 16 h (Fig. 6C), we observed decreased levels of Vfr protein in PAO1Δ*fimL*.

While our results thus far suggest that FimL regulates *vfr* transcription, we considered the possibility that FimL could also be modulating Vfr activity by affecting the levels of cAMP, a cofactor necessary for at least some functions regulated by Vfr (West *et al*., 1994). As shown in Fig. 6D, cAMP levels were not significantly different from wild type in PAO1Δ*fimL* and PAO1Δ*vfr*.

Together, our data show that FimL modulates multiple virulence factors, including tfp biogenesis and function, TTS, and autolysis, at least in part, via modulation of *vfr* expression and production. Along with the fact that *fimL* mutants can be complemented with extrachromosomal copies of *vfr*, these results indicate that the reduction in *vfr* production in plate-grown *fimL* mutants in plate culture is contributing to the observed *fimL*-related phenotypes. Thus, it seems that FimL is required for normal *vfr* transcription under conditions in which functional tfp are required for twitching motility, such as for growth on agar surfaces.

### *FimL is required for expression of TTSS components*

FimL appears to influence *vfr* transcription under conditions in which *fimL* mutants present *vfr*-related phenotypes such as reduced tfp assembly and twitching motility and increased autolysis on agar plates. PA103 *vfr* and *fimL* mutants are also both defective in killing epithelial cells *in vitro* (Fig. 5). Given that Vfr has been recently reported to control expression of the TTSS apparatus and effectors (Wolfgang *et al*., 2003), we wished to determine whether *fimL* might also be affecting *vfr* transcription under the conditions encountered in the cytotoxicity assays. We assayed β-galactosidase activity from the PA103 mini-CTX-*Pvfr–lacZ* reporter strains after incubation with epithelial cells. These assays showed that *vfr* promoter activity was reduced to about 53% of wild type in PA103Δ*fimL* mutants under these conditions ($n = 6$; $P < 0.05$; Fig. 5D). We also tested the ability of pUCP*vfr*F to complement the cytotoxicity defect of PA103 *fimL* mutants. Expression of Vfr *in trans* from pUCP*vfr*F restored cytotoxicity to PA103Δ*fimL* (Fig. 5B).

We next determined whether the defect in *vfr* transcription observed in *fimL* mutants translates into defects in secretion and/or production of TTSS system components. The proteins ExoU and ExoT are TTSS effectors which are translocated by PA103 into epithelial cells and mediate cytotoxicity and cell-rounding respectively. ExoT production and secretion was assayed from the same samples used for assaying *Pvfr* activity in the presence of HeLa

cells. Western analyses showed that PA103Δ*fimL* was defective in the secretion of ExoT into the media (Fig. 5E). Loss of secretion could result from a block in function of the TTSS apparatus and/or from loss of production. Western analysis of protein production in the bacterial cell showed that PA103Δ*fimL* produced little to no detectable ExoT under these conditions (Fig. 5E), indicating that FimL is required for normal production of ExoT in the presence of epithelial cells. This finding is consistent with a role for FimL in controlling gene expression via Vfr. Taken together, our observations suggest that FimL affects tfp function, cytotoxicity and TTS, at least partly, through modulation of *vfr* expression.

### fimL *mutants spontaneously revert to wild-type phenotypes*

During this study we were surprised to observe that *fimL* mutants of both PA103 and PAO1 often reverted to an apparent wild-type twitching phenotype. In such cases, reversion was characterized by flares of twitching cells erupting from the normally smooth margins of the *fimL* mutant colonies when grown on the agar surface (Fig. 7). Reversion was also evident with stab assays in 1% agar where flares of twitching motility caused by reversion were evident at the agar/plastic interface (Fig. 7). Cultures taken from flares of twitching cells surrounding previously non-twitching colonies invariably yielded a variant with a stable wild-type twitching motility phenotype. Given these observations, it is important to note that throughout all of our assays with *fimL* mutants we were careful to take special precautions to ensure that we were not inadvertently characterizing revertant strains, particularly when *fimL* mutants showed little to no change from wild-type phenotypes. For instance, we routinely confirmed by microscopic examination that the *fimL* colonies used for initiating assays, as well as colonies obtained after completion of the assay, demonstrated no evidence of reversion.

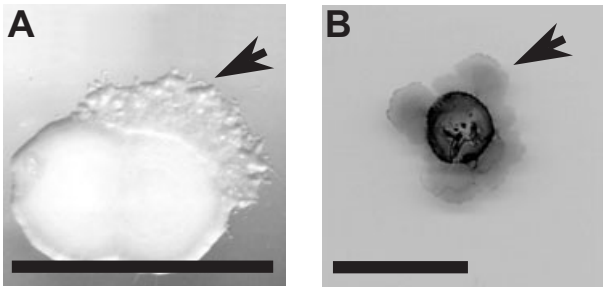


**Fig. 7.** Reversion of *fimL* mutants.
A. Arrow indicates flares of twitching cells (revertants) erupting from the smooth margins of a PA103Δ*fimL* colony grown on LB agar (1.6%). Bar represents 1 cm.
B. Arrow indicates zone of twitching motility caused by reversion observed at the agar/plastic interface during a stab assay of PAO1Δ*fimL* using a standard polystyrene Petri dish. Bar represents 1 cm.

To determine whether *fimL* mutants or *fimL* revertants were hypermutators, we compared their mutation frequency with wild-type strains by measuring the frequency of spontaneous streptomycin or rifampicin resistance development after 24–48 h (data not shown). We found no difference in the general mutation rate between wild type and *fimL* mutants or revertants.

We selected specific revertants for further examination in various phenotypic assays. Twitching motility stab assays and slide microscopy show that PAO1 and PA103 *fimL* revertants (PAO1Δ*fimL*-Rev and PA103Δ*fimL*-Rev) demonstrate wild-type twitching motility under the conditions of these assays (Fig. 2A and B). ELISA and Western assays with anti-PilA anti-serum show that PA103Δ*fimL*-Rev is hyperfimbriate but has normal levels of cell- associated pilin (Fig. 2C and D). PA103Δ*fimL*-Rev shows decreased autolysis and PQS levels when grown on agar plates (Fig. 4A and B), suggesting that high levels of Vfr may repress PQS production. Cytotoxicity and adhesion assays show that these phenotypes are also restored in PA103Δ*fimL*-Rev (Fig. 5A and C). Secretion of ExoT into the media is fully restored in the PA103Δ*fimL*-Rev (Fig. 5E). Production of ExoT within the bacterial cell is also restored in PA103Δ*fimL*-Rev, although this is reduced relative to wild-type ExoT production (Fig. 5E). β-Galactosidase assays with *fimL* revertants isolated from the PAO1 and PA103 mini-CTX-*Pvfr–lacZ* reporter strains show that *fimL* revertants have *Pvfr* activity restored to wild-type levels when assayed from 8 h plate cultures (Fig. 6B for the PAO1 data; data not shown for PA103) or when PA103Δ*fimL*-Rev was cultured in the presence of epithelial cells (Fig. 5D). Interestingly, PAO1Δ*fimL*-Rev, but not PA103 Δ*fimL*-Rev, shows a significant increase in *Pvfr* activity relative to wild type when assayed from plates after 16 h incubation at 37°C ($n = 9$; $P < 0.05$; Fig. 5D for the PAO1 data; data not shown for PA103). The increase in *vfr* transcription observed with plate-grown PAO1Δ*fimL*-Rev correlated well with increased Vfr protein levels (Fig. 6C). Strikingly, cAMP levels were also greatly increased in PAO1Δ*fimL*-Rev, approximately 4- to 10-fold at 8 h (data not shown) and 10- to 40-fold at 16 h (Fig. 6D). Our observations suggest that the suppressor mutation(s) in the *fimL* revertants causes increased levels of *vfr* transcription and production, increased levels of cAMP and increased PQS. These findings are consistent with the ability of pUCP*vfr*F to complement *fimL* mutants (see above).

As *Pvfr* activity is increased in *fimL* revertants, we tested whether the *vfr* promoter or the gene itself might be a site of the extragenic supressor mutation in the *fimL* revertants. We sequenced a 1.3 kb region that encompassed the *vfr* coding region, as well as 110 bp downstream and 559 bp upstream of *vfr* from wild-type PAO1 and PA103, the isogenic *fimL* mutants of each and five independently isolated revertants of each of PAO1Δ*fimL*

and PA103Δ*fimL*. Neither *vfr* nor its promoter contains the site of suppressor mutation in the revertant *fimL* strains (data not shown). As cAMP levels were also elevated in PAO1Δ*fimL*-Rev, we sequenced the coding regions of the two adenylate cyclase genes, CyaA or CyaB, as well as the upstream intergenic regions of 149 and 217 bp, respectively, which should include their respective promoters. We found no mutations in PAO1Δ*fimL*-Rev compared with the wild-type sequence in the database. Given the similarity of FimL to the N-terminal domain of ChpA, it is possible that FimL functions to modulate ChpA activity through interaction with ChpA or its interacting partners. We considered the possibility therefore that the FimL-like domain of ChpA might be a candidate for acquiring the suppressor mutation and sequenced this domain from wild-type and *fimL* revertant strains. Sequence analysis shows that this domain of ChpA is not mutated in the revertants (data not shown). It is likely that the site of secondary suppressor mutation in the revertants is occurring in a gene that encodes an as yet unidentified regulator of *vfr* and adenylate cyclase transcription.

## Discussion

Tfp assembly and function, as well as the production and secretion of virulence factors, are controlled by complex regulatory circuits in *P. aeruginosa* (Mattick, 2002). These include sensor/regulator modules that regulate tfp, including AlgR and FimS, PilR and PilS, the Chp system, transcriptional activators such as Vfr and its cofactor cAMP, and three interrelated quorum-sensing systems. We now identify a novel gene product, FimL, that has homology to the N-proximal domain of ChpA and which affects tfp, TTS and autolysis. Furthermore, we show that FimL regulates these diverse processes at least in part through modulation of Vfr production. Finally, we have discovered an unusual property of this gene, unique among genes in the pilus biogenesis pathway. Upon inactivation, the *fimL* mutants acquire secondary suppressor mutations at a significant rate. Our studies indicate that Vfr and FimL are components of a common pathway that co-ordinately controls virulence factors that are critical to the pathogenesis of human infections.

Several lines of evidence suggest that FimL is involved in the regulation of tfp function and twitching motility in *P. aeruginosa*. *fimL* mutants showed decreased twitching motility, and this phenotype was restored by introduction of the cloned *fimL* gene. Surface-assembled tfp are decreased although intracellular levels of PilA are normal in *fimL* mutants. As twitching motility has been shown to involve concerted extension and retraction of multiple tfp (Merz *et al*., 2000; Skerker and Berg, 2001), our findings suggest that FimL affects the regulation of tfp assembly and function rather than production.

Under certain conditions, tfp are required for mature biofilm development. Interestingly, PAO1Δ*fimL* forms mature biofilms under the conditions used in this study. Time-lapse video microscopy shows PAO1Δ*fimL* migrates across the glass surface similarly to wild-type PAO1. However, the mature biofilms appear somewhat irregular and resemble those formed in a ChpA mutant (M. Klausen, C. B. Whitchurch, T. Tolker-Nielsen and J. Engel, unpublished studies), consistent with our hypothesis that FimL may intersect with the Chp signalling system. We were careful to ensure that the biofilm assays were not initiated with *fimL* revertants, and we also determined that reversion had not occurred during the course of the assay.

We present multiple lines of evidence that FimL modulates *vfr* expression and that the defects in tfp biogenesis, twitching motility, TTS and cytotoxicity observed in *fimL* mutants may be accounted for (at least in part) by a decrease in *vfr* expression. First, similar to FimL, *vfr* regulates twitching motility and expression of the TTSS apparatus and effectors (Beatson *et al*., 2002a; Wolfgang *et al*., 2003). Second, provision of *vfr in trans* complements *fimL* mutant phenotypes. Third, *vfr* transcription is depressed in *fimL* mutants when grown on plates or in the presence of epithelial cells. Finally, *vfr* expression is upregulated in a FimL revertant, and Vfr-regulated phenotypes, including twitching motililty and TTS, are restored.

These observations also suggest that FimL functions to differentially control *vfr* expression under specific conditions in which tfp are functional, as is the case for twitching motility or for TTS-dependent killing and disruption of host signalling pathways in epithelial cells. The reduction in *vfr* promoter activity in *fimL* mutants under these conditions is relatively mild (only 50–70% of wild type) but is accompanied by a decrease in Vfr protein levels. Interestingly, *vfr* expression in *fimL* mutants is unaffected in broth-grown cultures (C.B. Whitchurch and J.N. Engel, unpubl. obs.) and we are currently pursuing the significance of this observation.

We also discovered that *fimL* and *vfr* mutants of PA103 show increased autolysis when grown for prolonged time periods on LB plates, while Δ*fimL* revertants exhibit wild-type autolysis and an exaggerated increase in *vfr* and cAMP levels. The mechanism of autolysis is unknown, but has been shown to correlate with PQS production (D'Argenio *et al*., 2002). PQS biosynthesis is a complex process that is regulated by MvfR (also known as PqsR) (Diggle *et al*., 2003; Deziel *et al*., 2004). Recently, 4-hydroxy-2-heptylquinoline (HHQ) has been identified as the immediate precursor to PQS and to have intercellular signalling properties of its own (Deziel *et al*., 2004). Conversion of HHQ to PQS is regulated by LasR but not by Mvfr. However, PQS production is still synthesized, although with delayed kinetics, in a *lasR* mutant (Diggle *et al*., 2003). Interestingly, a *lasR* mutant of PA14 shows

increased autolysis, which correlates with increased HHQ levels. This observation may explain the high baseline autolysis observed in PA103, a strain that is naturally deficient in LasR. Our experiments suggest that PQS is not increased in PA103 *fimL* or *vfr* mutants. We propose, instead, that the observed increase in autolysis in the *fimL* and *vfr* mutants may arise from increased HHQ (or HHQ precursor) levels, which are exaggerated by the absence of LasR in PA103. Autolysis and PQS levels are decreased in the FimL revertent, possibly because of upregulation of an enzyme that degrades HHQ and PQS or that shunts one or more of its precursors into another pathway.

The interrelationship of the PQS/HHQ cell communication systems is intricate and incompletely understood, but is probably important in the pathogenesis of *P. aeruginosa* infections (Collier *et al*., 2002; Guina *et al*., 2003). We could only detect PQS production in PA103 after prolonged growth on plates, at which time it has probably entered stationary-phase growth. Similarly, PQS production in PAO1 and PA14 has been shown to be induced during late logarithmic and stationary phase (Diggle *et al*., 2002; Deziel *et al*., 2004). Together with published data of others demonstrating that Vfr regulates the Las and Rhl quorum-sensing systems (Albus *et al*., 1997), our results suggest that Vfr may regulate all three known quorum-sensing systems of *P. aeruginosa*. Our data are consistent with a model in which FimL regulates Vfr, which functions under the conditions of our assays to negatively regulate PQS production and autolysis. Our findings also support the notion that cAMP, presumably by binding to and modulating Vfr activity, may also regulate PQS production and autolysis.

These observations also suggest that FimL regulates *vfr* expression under specific conditions in which tfp are functional, such as twitching motility or TTS-dependent killing and disruption of host signalling pathways in epithelial cells. The reduction in *vfr* promoter activity in *fimL* mutants is relatively mild (only 50–70% of wild type). Our findings suggest that even a minor reduction in *vfr* transcript levels within the cell has a significant impact on Vfr-dependent phenotypes and/or that FimL is also functioning via other effectors. Indeed, while both *fimL* and *vfr* mutants show reduced levels of surface-assembled tfp compared with wild type, we found that the defect is more severe in *fimL* mutants, an observation which supports the notion that FimL might also be controlling additional gene products necessary for functional tfp. This prediction is supported by initial microarray analysis that reveals that FimL and Vfr transcriptional targets include both genes in common and non-overlapping sets of genes (J. Sargent, J. West and J. Engel, unpubl. obs.).

The homology between FimL and the N-terminus of ChpA, a complex CheA-like histidine kinase that also regulates tfp biogenesis and twitching motility (Whitchurch *et al*., 2004), is intriguing and prompts the hypothesis that FimL modulates this signal transduction system. However, the relationship between FimL, Vfr and the Chp chemosensory regulon may be complex. While our data places FimL upstream of Vfr, it has been reported that the Chp system components, along with most of the known tfp genes, are themselves targets of Vfr (Wolfgang *et al*., 2003). Overexpression of Chp system components did not restore twitching motility to *fimL* mutants, suggesting that FimL may regulate additional factors required for twitching motility, as suggested by preliminary microarray studies. Nonetheless, FimL may still have a modulatory role on the activity of ChpA or its partners in phosphotransfer relays.

Our studies have uncovered a role for FimL in regulating TTS. *fimL* mutants show loss of adherence to epithelial cells, presumably caused by reduced levels of functional surface-assembled tfp. The decrease in ExoU-mediated cytotoxicity can be explained in part by the reduction in adhesion. Remarkably, we found that *fimL* mutants are also defective in the production, secretion and translocation of TTSS effectors. This finding can be explained by decreased production of Vfr, which has recently been shown to regulate transcription of the TTS apparatus and effectors (Wolfgang *et al*., 2003), as well as by the loss of surface pili, which have recently been shown to be required for production and translocation of TTS effectors (T. Jakobsen and J. Engel, unpubl. data).

One of the most intriguing aspects of this study has been the observation that the *fimL* mutant phenotype is readily overcome presumably by an extragenic spontaneous suppressor mutation. Importantly, *fimL* mutants and revertants are not hypermutators. The revertents appear to overproduce Vfr and cAMP, which may synergistically act to increase transcription of many downstream gene targets. The high selective pressure for developing twitching motility has been amply demonstrated by the reversion of *P. aeruginosa* PAK *lasI* and *rhlI* null mutants. After having undergone mutations in *vfr* and *algR* (respectively) to achieve a defective twitching phenotype, these mutants develop further compensatory mutations at high frequency when stabbed in solid media (Beatson *et al*., 2002b). As the site of the compensatory mutation in the FimL revertants is not in Vfr, nor in the genes encoding adenylate cyclase (*cyaA* or *cyaB*), or the N-terminal domain of *ChpA*, we predict that it is occurring in a gene that encodes a regulator of *vfr* and adenylate cyclase transcription. Given the lack of homology with transcriptional regulators, it is highly likely that FimL controls *vfr* transcription indirectly via one or more intermediary proteins. Identifying the site(s) of suppressor mutation in *fimL* revertants is an important avenue of future research to expand our understanding of how FimL modulates *vfr* expression in *P. aeruginosa*. Further analysis of FimL, Vfr

and ChpA will help elucidate the mechanism via which these proteins intersect to control tfp biogenesis and function, TTS, PQS and HHQ production, and other aspects of *P. aeruginosa* virulence.

## Experimental procedures

### *Bacterial strains, plasmids and media*

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* were routinely cultured in LB broth or on LB agar containing the appropriate antibiotic at 37°C. *P. aeruginosa* was routinely cultured in LB or on LB agar (1–1.6%) or Vogel-Bonner (VBM) agar (Vogel and Bonner, 1956) plus the appropriate antibiotic at 37°C unless otherwise indicated. For light microscopy, the cells were grown on media that contained 4 g $l^{-1}$ tryptone, 2 g $l^{-1}$ yeast extract, 2 g $l^{-1}$ NaCl, 1 g $l^{-1}$ $MgSO_4 \cdot 7H_2O$ and 8 g $l^{-1}$ GelGro (ICN) as a solidifying agent. The following antibiotic concentrations were used for the selection of *E. coli*: tetracycline 5 μg $ml^{-1}$ for plasmid selection and 40 μg $ml^{-1}$ for cosmid selection; ampicillin 100 μg $ml^{-1}$, chloramphenicol 25 μg $ml^{-1}$ and kanamycin 50 μg $ml^{-1}$. The concentration of antibiotics for the selection

**Table 1.** Bacterial strains and plasmids used in this study.

| Strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| Strains | | |
| *E. coli* | | |
| DH5α | *endA1 hsdR17* ($r_K^- m_K^+$) *glnV44 thi-1 recA1 gyrA relA1 Δ(lacIZYA-argF)U169 deoR [ϕ80dlacΔ(lacZ)M15]* | Laboratory collection |
| S17-1 | *thi pro hsdR recA chr::RP4-2* | Simon *et al.* (1983) |
| BL21(DE3) | $F^-$ *ompT hsdS* ($r^-_B m^-_B$)*gal dcm* (DE3) | Laboratory collection |
| *P. aeruginosa* | | |
| PAO1 | Wild-type *P. aeruginosa* strain ATCC 15692 | Holloway and Morga (1986) |
| PA103 | Wild-type cytotoxic *P. aeruginosa* strain | Liu (1966) |
| PAO1293 | $Cm^R$ derivative of PAO1 | de Lorenzo *et al.* (1990) |
| PAO1293–31E6 | PAO1293 with mTn*5*-Tc in *fimL* | Beatson *et al.* (2002a) |
| PA103 mutant I | PA103 with Tn*5*-$Gm^R$ insertion in *fimL* | Kang *et al.* (1997) |
| PA103 mut N | PA103 with Tn*5*-$Gm^R$ insertion in *pscJ* | Kang *et al.* (1997) |
| PAO1Δ*pilA* | PAO1 (ATCC 15692) with *pilA* inactivated by allelic displacement; $Tel^R$ | This study |
| PA103*pilA*::$Gm^R$ | PA103 with $Gm^R$ cassette in *pilA* | This study |
| PAO1*nudC*::$Tc^R$ | PAO1 with EZ::TN<TET-1> insertion in *nudC* | This study |
| PA103*nudC*::$Tc^R$ | PA103 with EZ::TN<TET-1> insertion in *nudC* | This study |
| PA103Δ*fimL* | PA103 with in-frame deletion of *fimL* | This study |
| PA103Δ*fimL*-Rev | Twitching revertant isolated from PA103Δ*fimL* | This study |
| PAO1 Δ*fimL* | PAO1 with in-frame deletion of *fimL* | This study |
| PAO1Δ*fimL*-Rev | Twitching revertant isolated from PAO1Δ*fimL* | This study |
| PA103 Δ*vfr* | PA103 with in-frame deletion of *vfr* | This study |
| PAO1 Δ*vfr* | PAO1 with in-frame deletion of *vfr* | This study |
| $PAO1_{GFP}$ | PAO1 tagged with eGFP in mini-Tn*7*, $Gm^R$ | This study |
| $PAO1_{CFP}$ | PAO1 tagged with eCFP in mini-Tn*7*, $Gm^R$ | This study |
| $PAO1_{YFP}$ | PAO1 tagged with eYFP in mini-Tn*7*, $Gm^R$ | This study |
| PAO1 $\Delta fimL_{GFP}$ | PAO1Δ*fimL* tagged with eGFP in mini-Tn*7*, $Gm^R$ | This study |
| PAO1 $\Delta fimL_{CFP}$ | PAO1Δ*fimL* tagged with eCFP in mini-Tn*7*, $Gm^R$ | This study |
| PAO1 $\Delta fimL_{YFP}$ | PAO1Δ*fimL* tagged with eYFP in mini-Tn*7*, $Gm^R$ | This study |
| PACW108 | PA103 with mini-CTX-*Pvfr–lacZ* | This study |
| PACW109 | PA103Δ*fimL* with mini-CTX-*Pvfr–lacZ* | This study |
| PACW123 | Twitching revertant isolated from PACW109 | This study |
| PACW118 | PA103 with mini-CTX-*lacZ* | This study |
| PACW114 | PAO1 with mini-CTX-*Pvfr–lacZ* | This study |
| PACW115 | PAO1Δ*fimL* with mini-CTX-*Pvfr–lacZ* | This study |
| PACW122 | Twitching revertant isolated from PACW115 | This study |
| PACW120 | PAO1 with mini-CTX-*lacZ* | This study |
| Plasmids | | |
| pOK12, pUCK21 | *E. coli* cloning vectors, $Km^R$ | Vieira and Messing (1991) |
| pUCPSK | *P. aeruginosa–E. coli* shuttle vector, $Ap^R$ | Watson *et al.* (1996) |
| pBluescript II SK | *E. coli* cloning vector, $Ap^R$ | Stratagene |
| pGem-T | *E. coli* cloning vector, $Ap^R$ | Promega |
| pGem-7 | *E. coli* T7 expression vector, $Ap^R$ | Promega |
| pMMB208 | Broad host range vector with inducible *Ptac* promoter, $Cm^R$ | Morales *et al.* (1991) |
| pEX100T | *P. aeruginosa* suicide vector, $Ap^R$ | Schweizer and Hoang (1995) |
| pX1918GT | Source of $Gm^R$ cassette, $Ap^R$ | Schweizer and Hoang (1995) |
| pRIC380 | *P. aeruginosa* suicide vector, $Ap^R$ | Alm and Mattick (1996) |
| pJEN34 | pRIC380 carrying EZ::TN<TET-1> in $Ap^R$ gene, $Tc^R$ | Whitchurch *et al.* (2004) |
| mini-CTX-*lacZ* | Contains promoterless *lacZ* for constructing transcriptional fusions, $Tc^R$ | Becher and Schweizer (2000) |
| pFLP2 | Encodes Flp recombinase | Hoang *et al.* (1998) |
| pLAFR5SK1 | $Tc^R$ cosmid vector | Hauser and Engel (1999) |
| pUCP*vfrF* | 1.3 kb *Xho*I fragment containing *vfr* in pUCPSK with *Plac* | Beatson *et al.* (2002a) |
| pUCP*vfrA* | 1.3 kb *Xho*I fragment containing *vfr* in pUCPSK against *Plac* | Beatson *et al.* (2002a) |

**Table 1.** *cont.*

| Strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| pMO01539 | pLA2917 containing partial *Sau*3A PAO1 chromosomal DNA fragment spanning the *pilG-K*, *chpA-E* cluster | Whitchurch *et al.* (2004) |
| pUCPChpA | 8 kb *Sac*I/*Cla*I fragment from pMO01539 containing *chpA* in pUCPSK | Whitchurch *et al.* (2004) |
| pMMBChpA in pMMB206 | 8 kb *Sac*I/*Cla*I fragment from pMO01539 containing *chpA* | Whitchurch *et al.* (2004) |
| pCos5-9B | pLAFR5SK1 cosmid of PA103 genomic DNA containing PA1822 (*fimL*) | This study |
| pJC207+ | 2 kb *Kpn*I/*Eco*47III fragment containing only *fimL* from pCos5-9B in pGem-7, *fimL* in the direction of T7 promoter | This study |
| pJC207– | 2 kb *Kpn*I/*Eco*47III fragment containing only *fimL* from pCos5-9B in pGem-7, *fimL* against the T7 promoter | This study |
| pSB62.4 | Marker rescue clone of PAO1293-31E6 genomic DNA in pBluescript II SK, contains site of transposon insertion, $Ap^R$, $Tc^R$ | This study |
| pCW70 | mini-CTX-*lacZ* containing *vfr* promoter region | This study |
| pCW32 | 8 kb *Bam*HI fragment containing *fimL* from pCos5-9B in pUCPSK | This study |
| pCW32-EZ::TN-7 | pCW32 with EZ::TN<TET-1> insertion in *fimL* | This study |
| pCW32-EZ::TN-8 | pCW32 with EZ::TN<TET-1> insertion in *nudC* | This study |
| pAKO-gent | pEX100T with *Hin*dIII fragment containing 3.2 kb PA103*pilA*::$Gm^R$; *pilA* deleted from 1.2 kb *Hin*dIII PA103 genomic fragment and replaced with $Gm^R$ | This study |
| pSB415.1A | 5 kb *Not*I fragment containing *nudC*::EZ::TN<TET-1> from pCW32-EZ::TN-8 in pUK21 | This study |
| pSB415.2A | 5 kb *Spe*I fragment from pSB415.1 A in pRIC380 | This study |
| pJEN24 | pGEM-T containing 1 kb region 5′ of *fimL* | This study |
| pJEN25 | pGEM-T containing 1 kb region 3′ of *fimL* | This study |
| pJEN26 | pOK12 containing 1 kb 5′ *fimL* PCR fragment from pJEN24 | This study |
| pJEN27 | pJEN26 containing 1 kb 3′ *fimL* PCR fragment from pJEN25 | This study |
| pJEN36 | pJEN34 carrying Δ*fimL* from pJEN27 on *Spe*I fragment | This study |
| pJEN39 | pGEM-T containing 1 kb region 5′ of *vfr* | This study |
| pJEN42 | pGEM-T containing 1 kb region 3′ of *vfr* | This study |
| pJEN55 | *Eco*RI/*Hin*dIII fragment from pJEN39 and *Hin*dIII/*Bam*HI fragment from pJEN42 concatamerized and cloned into *Eco*RI/*Bam*HI sites of pOK12 | This study |
| pJEN51 | pJEN34 carrying Δ*vfr* from pJEN55 on *Spe*I fragment | This study |
| pJEN53a | 6.4 kb *Eco*RI fragment containing *pilG-K* from pMO01539 in pUCPSK with *Plac* | This study |
| pJEN54 | 1.7 kb *Eco*RI/*Sac*I fragment from pJEN53a in pUCPSK, contains 'FimL-like' region of ChpA with *Plac* | This study |
| pJB23-1 | 3.7 kb *Eco*RI fragment from pCW32-EZ::TN-8 in pMMB208, contains *fimL* with *Ptac* | This study |
| pJB23-2 | 3.7 kb *Eco*RI fragment from pCW32-EZ::TN-8 in pMMB208, contains *fimL* against *Ptac* | This study |
| pJB24 | 1.7 kb *Eco*RI/*Sac*I fragment from pJEN54 in pMMB208, contains 'FimL-like' region of ChpA with *Ptac* | This study |

Cm, chloramphenicol; Tc, tetracycline; Gm, gentamicin; Km, kanamycin; Ap ampicillin; Tel, tellurite.

of *P. aeruginosa* were carbenicillin 250 μg $ml^{-1}$, chloramphenicol 250 μg $ml^{-1}$, rifampicin 50 μg $ml^{-1}$, streptomycin 500 μg $ml^{-1}$, tellurite 150 μg $ml^{-1}$ and tetracycline 200 μg $ml^{-1}$.

### *Recombinant DNA techniques and sequence analysis*

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). The preparation of *E. coli* competent cells and transformation protocols were followed according to Sambrook *et al.* (1989). Preparation of *P. aeruginosa* competent cells and transformations were performed as described previously (Mattick *et al.*, 1987). pCW32 was subjected to *in vitro* transposon mutagenesis using the EZ::TN<TET-1> kit (Epicenter Technologies). Double-stranded DNA (dsDNA) was prepared for sequencing either using a modified alkaline lysis method involving PEG precipitation (Applied Biosystems) or with QIAprep Spin MiniPrep Kits (Qiagen). Sequencing was performed at off-site facilities. The 3303 bp sequence of the *fimL* gene and the surrounding region was entered into GenBank under the Accession No. AF083252. Nucleotide and predicted protein sequences were analysed using MACVECTOR (Oxford Molecular Group), BLAST (http://www.ncbi.nlm.nih.gov/BLAST; Altschul *et al.*, 1997), PFAM (http://www.sanger.ac.uk/Software/Pfam/ Bateman *et al.*, 2002) and SMART (http://smart.embl-heidelberg.de/; Schultz *et al.*, 1998; 2000; Letunic *et al.*, 2002).

### *Identification of PA103 mutant I transposon insertion site*

A library of PA103 genomic fragments cloned into cosmid pLAFR5SK1 (Hauser *et al.*, 1998) was screened by mating *E. coli* strain S17-1 containing the PA103 cosmid library to PA103 mutant I. These strains were mated on LB agar over-

night at 37°C and exconjugants were selected on VBM agar containing tetracycline. Pools of eight cosmid clones were initially screened, followed by screening for individual cosmids from pools that restored twitching motility to PA103 mutant I.

### *Identification of the PAO1293 fimL mutant*

*Pseudomonas aeruginosa* strain PAO1293, a chloramphenicol-resistant derivative of the completely sequenced PAO1 strain, was mutagenized with a 2.2 kb mTn*5*-Tc transposable element (de Lorenzo *et al*., 1990) and a library of over 12 000 mutants were screened by twitching stab assay for defects in twitching motility (Beatson *et al*., 2002a). One such twitching-defective mutant PAO1293-31E6 was selected for further study. To identify the point of transposon insertion marker rescue cloning of the chromosomal fragment containing the transposon (and associated tetracycline resistance marker) was performed by ligating a *Pst*I digest of chromosomal DNA from 31E6 into pBluescript II SK (to produce pSB62.4). The DNA sequence flanking the point of insertion was determined by automated sequencing using a primer designed to match one end of the transposon (primer Tn*5*.I: 5′-GCGGCCAG ATCTGATCAAGAG-3′).

### *Bacterial protein expression studies*

T7 expression was accomplished following the procedure of Studier and Moffatt (1986) using *E. coli* strain BL21(DE3). *fimL* was cloned as a 3.3 kb *Kpn*I fragment from pCos5-9B into pGEM-7 either in the same direction as the T7 promoter (pJC207+) or in the opposite direction of the T7 promoter (pJC207–). One millilitre of a bacterial culture at an $A_{600}$ of 0.1 was incubated in M9 minimal medium supplemented with 0.5% methionine assay medium (Difco, Detroit, MI) for 60 min at 37°C and then induced with 1 mM IPTG for another 60 min at 37°C. Cultures were subsequently incubated with 100 μg $ml^{-1}$ rifampicin for 60 min at 37°C before labelling with 10 μCi $\mu l^{-1}$ Tran$^{35}$S-Label Metabolic Labeling Reagent (ICN Pharmaceuticals, Costa Mesa, CA). Samples were centrifuged, resuspended in 50 μl of SDS-PAGE sample buffer (Laemmli, 1970), boiled for 5 min and then loaded onto 10% SDS-PAGE gels (Laemmli, 1970) followed by autoradiography.

### *Construction of isogenic mutants*

In-frame deletions of *fimL* were constructed as follows. An approximately 1 kb region 5′ of *fimL* and including the first 96 bp of *fimL* was amplified with primers fimL1 (5′-GCGG GATCCGTTTAGCGCAATCATCGAAG-3′) and fimL2 (5′-GCGAAGCTTGGCAATGAACTGCTCAAGAC-3′). An approximately 1 kb region 3′ of *fimL* and encompassing the final 326 bp of *fimL* was amplified with primers fimL3 (5′-GCGAAGCTTATCACCGCGTACCTGGAATC-3′) and fimL4 (5′-GCGGGATCCTCCTCGATCTCGTCTTCCTG). The PCR products were cloned into pGEM-T and sequence was confirmed. Construction of the *fimL* deletion suicide clone for allelic exchange is described in Table 1.

In-frame deletions of *vfr* was constructed as follows. An approximately 1 kb region 5′ of *vfr* and including the first 32 bp of *vfr* was amplified with primers vfr1 (5′-GAATTCGTC GATGTACTGCACGTAGG-3′) and vfr2 (5′-AAGCTTTTTGAG TTTGGGTGTGTGGG-3′). An approximately 1 kb region 3′ of *vfr* and encompassing the final 11 bp of *vfr* was amplified with primers vfr5 (5′-AAGCTTGGCACCCGCTGAACAGCACC-3′) and vfr6 (5′-GGATCCATTCAACTGGCCCACGATGC). The PCR products were cloned into pGEM-T and sequence was confirmed. The cloned PCR products were excised and the fragments concatamerized and cloned into pOK12. The *vfr* in-frame deletion construct was then shuttled into the suicide vector pJEN34.

Other suicide clones for allelic exchange used in this study were constructed as described in Table 1. Allelic exchange mutants were constructed using the sucrose selection system described previously (Schweizer, 1992; Alm and Mattick, 1996). All suicide clones contain the genes *sacB*/*sacR* which promote sensitivity to sucrose, and *oriT* which enables conjugal transfer. The constructs were then transformed into the *E. coli* donor strain S17-1 in preparation for mating into *P. aeruginosa*. After conjugation, transconjugates were selected on 5% sucrose media. This allows selection of colonies in which the plasmid sequences have been excised while leaving the homologously recombined mutated gene in the chromosome. All mutants were genotypically confirmed by Southern blotting.

### *Motility assays*

In all assays the strains to be tested were inoculated from fresh cultures grown overnight on LB agar (1.6%). Twitching motility stab assays in LB solidified with 1% agar (BBL) were performed as described previously (Alm and Mattick, 1995) except that tissue culture-treated polystyrene dishes (Corning) were used. Microscopic analysis of twitching motility on GelGro (ICN) slides was performed as described previously (Semmler *et al*., 1999).

Swarming motility was assayed on plates composed of 0.8% nutrient broth (Oxoid) supplemented with 0.5% glucose and solidified with 0.5% agar (BBL) (Rashid and Kornberg, 2000). Plates were dried overnight at room temperature and the strains to be tested were point inoculated to the surface of the agar and incubated at 37°C for 6 h.

Swimming motility was assayed by stab inoculation of the strain to be tested into swim agar plates (LB solidified with 0.3% agar) followed by incubation for 6 h at 37°C. Motility was assessed qualitatively by examining the circular turbid zone formed by the bacterial cells migrating away from the point of inoculation.

### *Phage sensitivity assays*

Phage sensitivity was assayed using the tfp-specific *P. aeruginosa* bacteriophage PO4. The phage stock was titred by adding serial dilutions of the phage to 100 μl of PA103 grown to stationary phase, and the mixture was combined with 0.7% LB top agarose and then poured onto LB agar. Phage sensitivity was assayed in strains PA103, PA103 mutant I (*fimL*) and PA103*pilA*::Gm$^R$ by spotting 5 μl of serially diluted phage

onto top agarose seeded with 100 μl of bacteria which has been prepared by diluting a stationary-phase culture to an $A_{600}$ of 0.6 in LB broth.

### *Autolysis assays*

Overnight LB cultures of the strains to be tested were diluted 1:100 into fresh LB, grown with vigorous aeration at 37°C to mid-log phase and 100 μl plated onto LB agar plates. After incubation at 37°C for 16–24 h, the plates were examined for the characteristic 'plaque-like' clearing associated with autolysis.

### *PQS assays*

Overnight cultures were diluted 1:100 in 5 ml LB and grown with vigorous aeration at 37°C to mid-log phase. 100 μl of each culture was plated on LB agar and grown at 37°C in a humidified bag for 8 or 16 h. The entire bacterial lawn was resuspended in 5 ml of phosphate-buffered saline (PBS) and PQS was extracted as previously described (Gallagher *et al*., 2002). Briefly, 300 μl of culture resuspension was extracted twice with 900 μl of acidified ethyl acetate by vigorously vortexing for at least 30 s, followed by centrifugation at 16 000 *g* for 5 min. An aliquot (800 μl) of the upper organic phase was transferred to a microcentrifuge tube and allowed to dry overnight at room temperature. The dried extracts were resuspended in 20 μl of a 1:1 acetonitrile:methanol mixture by vortexing and pulse spinning multiple times. The entire volume of each extract was loaded onto a Silca Gel 60 F254 plate (20 × 20 cm; EM Science) which had been activated by soaking in 5% $KH_2PO_4$ and baking at 100°C for 1 h. Thin layer chromatography (TLC) was performed with a solvent mixture containing 17:2:1 methylene chloride:acetonitrile:1,4-dioxane. When the solvent front neared the top of the plate, the plates were removed, air dried and photographed under long-wave UV illumination using a GelDoc photoimager (Bio-Rad). Quantification was performed using the Quantity One software (Bio-Rad).

### *Biofilm assays*

PAO1 (ATCC 15692) and the isogenic PAO1Δ*fimL* were fluorescently tagged at an intergenic neutral chromosomal locus with *gfp*, *cfp* or *yfp* in mini-Tn*7* constructs as described previously (Klausen *et al*., 2003b). Biofilm cultivation, image acquisition and analyses were performed as described previously (Klausen *et al*., 2003a,b). In these studies modified FAB medium (Heydorn *et al*., 2000) was supplemented with 30 mM glucose for batch overnight cultures and with 0.3 mM glucose for biofilm cultivation.

### *cAMP assays*

Three single colonies for each strain were used to inoculate 5 ml of starter cultures that were grown in LB overnight shaking at 37°C, diluted 1:100 into 5 ml of LB and were grown with vigorous aeration at 37°C until mid-log growth. One hundred microlitres were plated onto a 1.6% LB plate and incubated at 37°C in a humidified tray for 8 or 16 h. PBS (5 ml) was added to the plate to remove the bacteria and vortexed vigorously. The $A_{600}$ was measured and 10–20 μl were used to measure cAMP levels according to manufacturer's instructions (cAMP Biotrak EIA System, Amersham Biosciences). The cells were diluted into PBS (500 ml), centrifuged at 8000 r.p.m. (eppendorf microfuge) and resuspended in 500 μl of Lysis Buffer 1B. The pellet was frozen at –80°C, and thawed at room temperature, sonicated (Branson sonifier 45) three times for 10 s on ice and 100 μl was used to measure cAMP using an ELISA non-acetylation assay.

### *PilA immunoblotting and ELISA*

Rabbit polyclonal anti-sera to PA103 PilA was generated using the C-terminal 18 amino acids of PilA (TCTSTQEEMF IPKGCNEP) (Johnson *et al*., 1986) conjugated to keyhole limpet haemocyanin (Animal Pharm Services). Detection of PilA in whole cell and in sheared surface-assembled tfp samples were performed essentially as described elsewhere (Whitchurch *et al*., 2004) except for the following differences. In this study, overnight LB cultures were subcultured 1:100, incubated to mid-log phase and 100 μl plated onto LB agar plates. After incubation at 37°C for 8 h the bacteria were resuspended in PBS and ELISA and preparation of whole cell and surface tfp samples performed as described previously (Whitchurch *et al*., 2004). ELISAs were performed in Immulon 4HBX Microtiter® Immunoassay plates (DYNEX Technologies) and $A_{405}$ of wells was measured using a microplate spectrophotometer (SPECTRAmax® 340PC384, Molecular Devices) Samples for PilA immunoblots were displayed with NuPAGE 12% Bis-Tris Gel using 1× NuPAGE MES SDS Running Buffer (Invitrogen) and electroblotted onto Immobilon-P transfer membrane (Millipore) in the Tris-glycine system described by Towbin *et al*. (1979). Membranes were blocked with 5% milk, probed with a 1:40 000 dilution of primary anti-PilA antibody in 1% skim milk powder in PBS. Membranes were then incubated with a 1:10 000 dilution of goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (HRP) (Jackson Immunoresearch) in 1% skim milk powder in PBS followed by detection by enhanced chemiluminescence (ECL) using the ECL Western Blotting Detection Reagents from Amersham Biosciences.

### *Vfr immunoblotting*

Plate-grown bacteria (8 and 16 h, prepared as described for the cAMP assays) were resuspended in PBS ($A_{600} \approx 1$), pelleted and resuspended in 50 μl of Laemmli buffer, passed three times through a 27 g needle to shear bacterial DNA, and 10 μl were electrophoresed through one 10% Nu-PAGE gel (Invitrogen). Immunoblotting was performed as described above, using a rabbit polyclonal antibody to Vfr (a kind gift of Dr Susan West, University of Wisconsin; 1:5000 dilution) followed by incubation with a goat anti-rabbit HRP (1:10 000) dilution.

### *Adhesion assays*

Bacteria were grown at 37°C for 18 h with shaking. The

culture was diluted to an $A_{600}$ of 0.08 and grown shaking to an $A_{600}$ of ≈0.5. The exact inoculum was enumerated by serial dilution onto LB plates. HeLa cells ($10^5$ per well) were grown for 16–18 h on 12-well transwell tissue culture plates (COSTAR Corning, 12 mm diameter, 0.4 μm pore size), washed twice with PBS, and the medium was replaced with 1 ml of MEM-Lite (MEM; Sigma Chemical), 20 mM Hepes buffer (pH 8.0), 3.5% sodium bicarbonate in the upper compartment and 1.5 ml of MEM-Lite in the lower compartment. Cells were infected with a multiplicity of infection (moi) of 250 ($OD_{600}$ 1 = $10^9$ bacteria per millilitre) for 1 h at 37°C, the filters were excised with a scalpel and washed four times in PBS. Cells were lysed by incubation for 30 min at 25°C as previously described (Comolli *et al*., 1999b). Bacterial counts were enumerated by serial dilution onto LB plates and normalized to the input inoculum.

### *Cytotoxicity assay*

HeLa cells ($2 \times 10^5$) were seeded in 24-well tissue culture plate(s) 16, 17, 18 h before infection and grown overnight at 37°C in the presence of 5% $CO_2$. Cells were washed three times with PBS and overlayed with 1 ml of MEM-Lite. Bacteria were grown as described for the adhesion assay and HeLa cells were infected with an moi of 50. After 5 h of cocultivation, the amount of lactate dehydrogenase released into the medium (150 μl aliquot) was determined according to the manufacturer's instructions (Promega CytoTox 96 Non Radioactive cytotoxicity assay G1780).

### *ExoT Immunoblotting*

HeLa cells ($2 \times 10^6$) were seeded in 10 cm tissue culture plate(s) 16, 17, 18 h before infection and grown overnight at 37°C in the presence of 5% $CO_2$. Cells were washed three times with PBS and overlayed with 8 ml of MEM-Lite. Bacteria were grown as described for the adhesion assay and HeLa cells were infected with an moi of 150 and incubated for 1.5 h. To quantify ExoT production and secretion, 6 ml of culture medium was subjected to centrifugation (7000 *g* for 20 min at 4°C) to pellet bacteria. Before centrifugation, viable counts were enumerated by serial dilution onto LB plates.Supernatant (5 ml) was filtered through low protein binding PES filters (Pall) and proteins were precipitated with 55% ammonium sulphate. After incubation on ice for 18 h, precipitated proteins were concentrated by centrifugation at 13 000 *g* at 4°C for 20 min. Pellets were resuspended in 250 μl of PBS. Equal amounts of sample were loaded on a 10% NuPAGE Bis-Tris gel (Invitrogen) using 1× NuPAGE MOPS buffer. To determine bacterial production of ExoT, the bacterial pellet was resuspended in 100 μl of BugBuster protein extraction reagent (Novagen) and 1 μl of Benzonase (Novagen) was added. After 30 min incubation at room temperature, extracts were centrifuged at 20 000 *g* for 15 min at 4°C. Supernatants were collected and protein concentrations were determined with the BCA protein assay kit (Pierce) according to the manufacturer's instructions. Equal amounts of total proteins were electrophoresed on a 10% NuPAGE Bis-Tris gel (Invitrogen) using 1× NuPAGE MOPS buffer. Proteins were visualized by Western blot analysis essentially as described for PilA immunoblotting except that membranes were blocked with 5% BSA, and a 1:6000 dilution of anti-ExoT anti-sera (Hauser *et al*., 1998) and a 1:2500 dilution of the HRP-conjugated goat anti-rabbit antisera were used.

### *β-Galactosidase reporter assays*

The promoter region of *vfr* was amplified by PCR using the primers *Pvfr*-forward (5′-AAGCTTAGGAAGGCTTCGC AGCTCTC-3′) and *Pvfr*-reverse (5′-GGATCCGTCTAGGTG TTTGAGTTTGG-3′) and cloned into the mini-CTX-*lacZ* integration vector (Becher and Schweizer, 2000; Hoang *et al*., 2000). This clone (pCW70) encompasses the region −487 to +46 relative to the first nucleotide of the start codon of *vfr* cloned upstream of the promoterless *lacZ* gene. pCW70 and the promoterless mini-CTX-*lacZ* integration vector were transformed into *E. coli* S17-1 and integrated into the *P. aeruginosa* chromosome as previously described (Hoang *et al*., 2000). Unwanted plasmid sequences were removed by utilizing the Flp-FRT recombination procedure (Hoang *et al*., 1998).

Assays of *Pvfr* promoter activity from LB broth and plate cultures were performed as follows. *P. aeruginosa* strains containing mini-CTX-*Pvfr–lacZ* or the promoterless mini-CTX-*lacZ* were grown overnight from single colonies in LB broth. Overnight cultures were diluted 1:100 into LB broth and incubated with vigorous aeration at 37°C for 8 h or 16 h. To assay β-galactosidase activity from plate grown cultures, overnight LB broth cultures were diluted 1:100, grown with vigorous aeration at 37°C to mid-log phase and 100 μl plated onto LB agar plates. After incubation at 37°C for 8 h, the bacteria were resuspended in 5 ml of PBS by vortexing. *Pvfr* activity in the presence of HeLa cells was performed as follows. PA103 strains containing mini-CTX-*Pvfr–lacZ* or the promoterless mini-CTX-*lacZ* were co-cultivated with HeLa cells for 1.5 h as described in the procedure for immonoblotting of ExoT secretion and production in the presence of HeLa cells. Bacteria were centrifuged and the bacterial pellet was resuspended in 250 μl of PBS and 200 μl used to assay for β-galactosidase activity.

β-Galactosidase assays were performed as previously described (Miller, 1972) with modifications to adapt it to 96-well plate. Aliquots (200 μl) of each sample were transferred to the first row of an 8 × 12-well microtitre plate. Samples were serially diluted in PBS twofold down the eight rows of the plate, the $OD_{600}$ of each well was recorded using a microplate spectrophotometer (SPECTRAmax® 340PC384, Molecular Devices) and the plate was stored at −80°C. The plate was thawed at room temperature, 100 μl of B-PER lysis buffer (Promega) added to each well and the plate incubated at 37°C for 1 h. Fifty microlitres of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) solution at 4 mg $ml^{-1}$ in PBS were added to each well and the $OD_{420}$ was measured for 30 min at 1 min intervals using a microplate spectrophotometer (SPECTRAmax® 340PC384, Molecular Devices) and analysed using SoftMaxPro® 4.3.1 (Molecular Devices). Maximum rates of β-galactosidase activity in undiluted culture and $OD_{600}$ of undiluted culture were calculated from suitable dilutions. Specific activity was calculated by dividing the maximum rate with the calculated $OD_{600}$ of undiluted cultures.

*Mutation rate assays*

Minimal inhibitory concentrations of rifampicin and streptomycin were determined for both PAO1 and PA103 as described elsewhere (Andrews, 2001). Mutation rate assays were performed as described previously (Oliver *et al*., 2000) except for the following modification. Single colonies were inoculated into 5 ml of LB broth and incubated for 20 h at 37°C. Overnight cultures were diluted 1:100 into 25 ml of LB broth and incubated 37°C overnight. Selective LB agar plates contained either rifampicin (50 μg $ml^{-1}$) or streptomycin (500 μg $ml^{-1}$).

*Statistical analyses*

Results are presented as means ± SD. Student's two-tailed *t*-test, one-way ANOVA and Student–Newman–Keuls tests were used to determine differences between means. Values of $P < 0.05$ were considered to be significant.

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## Note added in proof

While this paper was in revision, Shan *et al.* (2004) reported that PA1822 is involved in twitching motility (*Microbiology* **150:** 2653–2661).

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