

Differential Regulation of Twitching Motility and Elastase Production by Vfr in *Pseudomonas aeruginosa*

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Vfr, a homolog of *Escherichia coli* cyclic AMP (cAMP) receptor protein, has been shown to regulate quorum sensing, exotoxin A production, and *regA* transcription in *Pseudomonas aeruginosa*. We identified a twitching motility-defective mutant that carries a transposon insertion in *vfr* and confirmed that *vfr* is required for twitching motility by construction of an independent allelic deletion-replacement mutant of *vfr* that exhibited the same phenotype, as well as by the restoration of normal twitching motility by complementation of these mutants with wild-type *vfr*. Vfr-null mutants exhibited severely reduced twitching motility with barely detectable levels of type IV pili, as well as loss of elastase production and altered pyocyanin production. We also identified reduced-twitching variants of quorum-sensing mutants (PAK *lasI*::Tc) with a spontaneous deletion in *vfr* (S. A. Beatson, C. B. Whitchurch, A. B. T. Semmler, and J. S. Mattick, *J. Bacteriol.*, 184:3598–3604, 2002), the net result of which was the loss of five residues (EQERS) from the putative cAMP-binding pocket of Vfr. This allele (VfrΔEQERS) was capable of restoring elastase and pyocyanin production to wild-type levels in *vfr*-null mutants but not their defects in twitching motility. Furthermore, structural analysis of Vfr and VfrΔEQERS in relation to *E. coli* CRP suggests that Vfr is capable of binding both cAMP and cyclic GMP whereas VfrΔEQERS is only capable of responding to cAMP. We suggest that Vfr controls twitching motility and quorum sensing via independent pathways in response to these different signals, bound by the same cyclic nucleotide monophosphate-binding pocket.

Pseudomonas aeruginosa is an opportunistic pathogen of animals and humans that infects immunocompromised hosts (7, 42). Pathogenesis due to this bacterium involves the production of a number of extracellular virulence determinants, including lipases and phospholipases, proteases, exopolysaccharides, alkaline phosphatases, and type IV pili. Type IV pili are flexible surface filaments about 6 nm in diameter that are produced at the poles of the bacterial cell. Type IV pili mediate attachment to the host epithelial tissues and a form of surface translocation termed twitching motility. The mechanism of twitching motility is pilus retraction and extension (9, 30). Twitching motility also appears to be an important virulence factor, as mutants that have nonfunctional type IV pili have reduced infectivity (11, 20). Twitching motility has also been shown to be involved in biofilm formation (34), which may be important during infection (12, 37).

The biogenesis and function of type IV pili in *P. aeruginosa* is dependent on at least 35 genes that are located in several clusters on the chromosome. These include genes encoding the main structural subunit of the pili (PilA or pilin), a leader peptidase, ancillary proteins with prepilin like leader se-

quences, inner and outer membrane proteins, nucleotide-binding proteins, other proteins whose functions are not clear, the RpoN sigma factor, two two-component sensor-regulator pairs, and a complex chemosensory signal transduction system (for a review, see reference 2).

The *P. aeruginosa* virulence factor regulator Vfr is a homolog of the *Escherichia coli* catabolite repressor protein (CRP; reviewed in references 8 and 25) and has been shown to regulate exotoxin A production, *regA* transcription, and quorum sensing via control of *lasR* transcription (1, 55, 56). CRP is known to be important in the control of virulence factor production in a number of eubacteria, including type IVB pili in *Vibrio cholerae* (47) and flagella in *Salmonella* sp. (26). The three-dimensional structure of *E. coli* CRP has been determined (54) and was recently refined to 2.1 Å (35). Structural data, coupled with site-directed mutagenesis studies, have shown the importance of residues conserved among the cyclic nucleotide-binding domains of CRP and its homologs from a range of organisms.

Here, we report that Vfr controls twitching motility in *P. aeruginosa*. The *lasI* and *rhlI* quorum-sensing genes had been previously reported to be required for twitching motility (17), and therefore it appeared possible that Vfr might control twitching motility via *lasR* [which is dependent on the LasI biosynthetic product, *N*-(3-oxododecanoyl)-L-homoserine lactone, for function]. However, mutations in the quorum-sensing genes *lasI*, *lasR*, *rhlI*, and *rhlR* have no obvious effect on twitching motility (6) although, interestingly, three independent twitching-defective mutants that spontaneously arose in the *lasI* mutant background had all suffered a specific 15-nucleo-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) ^a
Strains	
<i>E. coli</i>	
DH5 α	<i>endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>glnV44 thi-1 recA1 gyrA relA1</i> Δ (<i>lacIZYA-argF</i>)U169 [ϕ 80 <i>dlaC</i> Δ (<i>lacZ</i>)M15]
S17-1.....	<i>thi pro hsdR recA chr::RP4-2</i> (45)
<i>P. aeruginosa</i>	
PAK.....	Wild-type <i>P. aeruginosa</i> strain K ^b
PAO1.....	Wild-type <i>P. aeruginosa</i> strain ATCC 15692
PAO1293.....	Cm ^r derivative of <i>P. aeruginosa</i> PAO1
PAK <i>pilA::Tc</i>	Previously referred to as AWK (53)
118C8.....	PAO1293 mTn5-Tc <i>vfr</i> mutant identified during transposon library screen
PAK <i>vfr::mTn 5-Tc</i>	PAK with same mTn5-Tc insertion as 118C8
PAO1 <i>vfr::Tc</i>	PAO1 with Tc cartridge inserted as blunt fragment into unique <i>BclI</i> site of <i>vfr</i>
R306.....	PAK Tn5-B21 <i>pilV</i> mutant (21)
Plasmids	
pSM-TET.....	Source of Tc ^r cassette (31)
pRIC380.....	<i>P. aeruginosa</i> suicide vector (4)
pOK12.....	Km ^r cloning vector (50)
pUCPSK.....	<i>P. aeruginosa-E. coli</i> shuttle vector (52)
pBluescript II SK.....	<i>E. coli</i> cloning vector with Ap selection (Stratagene)
pMO011925.....	pLA2917 cosmid from Holloway library found to contain <i>vfr</i> (56)
pUCP <i>vfrA</i>	1.3-kb <i>XhoI</i> fragment containing <i>vfr</i> cloned from pMO011925 into pUCPSK
pUCP <i>vfrF</i>	1.3-kb <i>XhoI</i> fragment containing <i>vfr</i> cloned from pMO011925 into pUCPSK
pSB77.12.....	Marker rescue clone from <i>PstI</i> digest of 118C8 (carries <i>vfr::mTn 5-Tc</i> insert)
pSB172.11.....	6.2-kb <i>PstI</i> insert (including 2.2-kb mTn 5-Tc) from pSB77.12 cloned into pOK12
pSB172.2.....	6.2-kb <i>SpeI</i> insert from pSB172.11 cloned into pRIC380
pSB201.13A.....	pUCP <i>vfrA</i> with Tc ^r cassette from pSM-TET inserted within blunted <i>BclI</i> site
pSB206.1A.....	4.3-kb <i>NotI-KpnI</i> insert (including 3-kb Tc ^r cassette) from pSB201.13A cloned into pOK12
pSB216.1A.....	4.3-kb <i>SpeI</i> insert from pSB206.1A cloned into pRIC380
pSB299.15A.....	<i>vfr</i> amplified from wild-type PAO1 and cloned into pUCPSK against <i>lac</i>
pSB299.17A.....	<i>vfr</i> amplified from PAK <i>lasI</i> -RT and cloned into pUCPSK against <i>lac</i>

^a Antibiotic resistance abbreviations: Tc, tetracycline; Cm, chloramphenicol; Km, kanamycin; Ap, ampicillin.

^b Source: David Bradley, Memorial University of Newfoundland, St. John's, Newfoundland, Canada.

tide (nt) deletion within the cyclic AMP (cAMP)-binding pocket of Vfr (Vfr Δ EQERS). Analysis of this allele indicates that Vfr controls twitching motility and the production of quorum-sensing-related virulence factors by two distinct and independent pathways.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *E. coli* strain DH5 α was used in all genetic manipulations and in the preparation of DNA sequencing templates, and *E. coli* S17-1 was used as the donor strain in bacterial conjugation (45). Details of bacterial strains, plasmid construction, and the primers used in this study are described in Table 1. *P. aeruginosa* competent cells and transformations were prepared by MgCl₂ treatment as described previously (29). *E. coli* and *P. aeruginosa* liquid cultures were maintained in Luria-Bertani (LB) broth (41), and solid medium (LBA) was prepared by adding 1 to 1.5% Select agar (Gibco-BRL). *Pseudomonas* Isolation Agar (PIA; Difco) was used for pyocyanin quantitation from plate cultures. Light microscopy was performed with nutrient medium (4 g of Tryptone liter⁻¹, 2 g of yeast extract liter⁻¹, 2 g of NaCl liter⁻¹) solidified with 8 g of GelGro (ICN) liter⁻¹ for greater optical clarity. The following antibiotic concentrations were used for selection of *E. coli*: 12.5 μ g of tetracycline ml⁻¹ for plasmid selection, 40 μ g of tetracycline ml⁻¹ for cosmid selection, 100 μ g of ampicillin ml⁻¹, and 50 μ g of kanamycin ml⁻¹. The concentrations of antibiotics used for selection of *P. aeruginosa* were 500 μ g of carbenicillin ml⁻¹, 250 μ g of chloramphenicol ml⁻¹, 20 μ g of rifampin ml⁻¹, and 200 μ g of tetracycline ml⁻¹.

DNA manipulations. DNA manipulations, plasmid DNA extractions, Southern hybridization, and radiolabeling of probe DNA were performed by standard procedures (41). The enzymes for DNA manipulation were supplied by Roche and New England Biolabs.

Construction and screening of PAO1293 transposon mutant library. *P. aeruginosa* strain PAO1293, a chloramphenicol-resistant derivative of completely se-

quenced strain PAO1, was mutagenized with the 2.2-kb mini-Tn 5-*tet* (hereafter abbreviated mTn 5-Tc) transposable element. A library containing 92,260 tetracycline-resistant clones was characterized by the following four criteria: (i) estimation of the frequency of exconjugant mutants, (ii) genomic profiling of 100 Tc^r clones selected via PCR amplification of an internal 398-nt Tc gene product, (iii) Southern hybridization, and (iv) sequencing of mTn 5-Tc insertion endpoints for 30 clones. On the basis of these criteria and biostatistical analysis as a binomial probability, the library was estimated to cover the 6.3-Mb chromosome at 14.6 genome equivalents.

In order to facilitate mass screening for mutants deficient in twitching motility, aliquots of the original library were diluted and plated on LBA containing tetracycline and ~12,000 individual colonies were randomly picked into 96-well microtiter plates that were stored at -70°C after incubation. A 96-prong twitching stab tool was constructed with stainless steel prongs in a Perspex base to allow rapid screening of the entire gridded library for loss of twitching motility. After overnight incubation, mutants with absent or reduced twitching motility were selected and confirmatory twitching stabs were performed manually (see below).

The sites of mTn 5-Tc insertion from mutants with absent or reduced twitching motility were determined by generating and sequencing marker rescue clones. Briefly, *P. aeruginosa* mTn 5-Tc mutant chromosomal DNA was digested with *PstI* or *XhoI* and then shotgun cloned into pUCPSK and used to transform *E. coli* DH5 α cells that were subsequently plated on LBA containing ampicillin and tetracycline. Plasmid DNA was extracted from ampicillin- and tetracycline-resistant colonies, and the site of transposon insertion was identified by sequence analysis as described below. Mutant 118C8 was found to contain an mTn 5-Tc insertion within *vfr*.

Sequencing and sequence analysis. Automated DNA sequencing was performed by the Australian Genome Research Facility (University of Queensland, Brisbane, Queensland, Australia) by using big-dye terminator chemistries and *Taq* cycle sequencing kits from Perkin-Elmer Applied Biosystems and analysis on an ABI PRISM 377 DNA sequencer (Perkin-Elmer Applied Biosystems). A

primer (Tn5.I; 5'-GCGGCCAGATCTGATCAAGAG-3') complementary to a region near the I terminus of the mini-Tn5 cassette was used to obtain the sequence adjacent to the point of transposon insertion in marker rescue clones. Primer Tn5.I was manufactured by Pacific Oligos, Lismore, New South Wales, Australia. BLAST (5) searches of the *P. aeruginosa* PAO1 unfinished genome sequence at the National Center for Biotechnology Information (Bethesda, Md.) identified the position of transposon insertion, and the DNA sequence from that region was obtained via the Institute for Molecular Bioscience *P. aeruginosa* interactive database (<http://www.bit.uq.edu.au/pseudomonas>) (13). Further BLAST searches were performed by using the nonredundant nucleotide and protein databases at the National Center for Biotechnology Information. Where quoted, protein identities were calculated from a CLUSTALW pairwise alignment with an open gap penalty of 10 and a gap extension penalty of 0.1 with a blosum 30 similarity matrix.

Construction of isogenic mutants. *vfr* allelic-exchange mutants were constructed by using the sucrose selection system described previously (4, 43). To construct PAK *vfr*::mTn 5-Tc, the *vfr*::mTn 5-Tc allele was subcloned from the marker rescue clone of mutant 118C8 into the vector pOK12. To construct PAO1 *vfr*::Tc, the 1.3-kb *XhoI* fragment from pUCP *vfrA* was subcloned into pOK12 and the tetracycline cassette from pSM-TET was inserted as a blunt fragment into the *BclI* site (5' extensions were removed with mung bean nuclease) within *vfr*. The resulting clones were then digested with *SpeI*, and the disrupted genes were inserted into the suicide vector pRIC380 (Table 1). This vector carries the genes *sacBR*, which promote sensitivity to sucrose, and *oriT*, which enables conjugal transfer. The constructs were then used to transform *E. coli* donor strain S17-1 in preparation for mating with *P. aeruginosa*. Following conjugation, the transconjugants were plated on 5% sucrose medium containing tetracycline to select for colonies in which the plasmid had been excised while leaving the homologously recombined mutated gene in the chromosome. Mutants were confirmed by Southern blot analysis and examined with the subsurface twitching assay.

Twitching motility assay. Twitching motility was assayed as described previously (3). Briefly, the *P. aeruginosa* strain to be tested was stab inoculated through a 1% agar plate and, after overnight growth at 37°C, the zone of twitching motility between the agar and petri dish interface was visualized by staining with Coomassie brilliant blue R250.

Western blotting. Whole cells from LBA plates were resuspended to an optical density at 600 nm of 1.0 in 50 mM sodium carbonate buffer, pH 9.6. One-milliliter samples were centrifuged, and the cell pellet was resuspended in 100 μ l of sample buffer (60 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate, 10% glycerol, 5% β -mercaptoethanol, 0.001% bromophenol blue). To reduce viscosity, the samples were sheared by 20 passages through a 27½-gauge needle and heated to 100°C for 5 min. Samples were then displayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 15% polyacrylamide gel and a 5% stacking gel (27) and transferred electrophoretically to Hybond-C nitrocellulose (Amersham) with a Tris-glycine buffer system (49). Proteins were detected with anti-PilA serum (1:5,000), followed by goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (1:5,000; Boehringer Mannheim).

ELISAs. Enzyme-linked immunosorbent assays (ELISAs) (15) to determine the amount of surface pili were performed as follows. Cells were resuspended in 50 mM sodium carbonate buffer, pH 9.6, at an optical density at 600 nm of 1.0, and 200 μ l was loaded into wells of a 96-well ELISA plate. After overnight incubation at 4°C, the wells were washed with phosphate-buffered saline containing 0.1% Tween 20, blocked with 3% bovine serum albumin for 1 h, and then exposed to an anti-PilA antibody at a starting dilution of 1:500 for 2 h at 37°C. After removal of antisera, the wells were again washed with phosphate-buffered saline containing 0.1% Tween 20, and then goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase was added (1:5,000) and the mixture was incubated for 2 h at 37°C. Detection was done with 20 mg of *p*-nitrophenyl phosphate (Sigma) ml⁻¹ in 1 M Tris buffer (pH 8.0), and the tray was read at 405 nm with an ELISA reader (Bio-Rad).

Light microscopy. Light microscopy was performed as described previously (44). Briefly, sterile microscope slides were submerged in molten GelGro medium to obtain a thin layer of medium coating the slide. The slides were allowed to set in a horizontal position and air dried briefly prior to use. The slides were then inoculated with a small loopful of bacteria taken from an overnight plate culture. A sterile glass coverslip was placed over the point of inoculation, and the slide was transferred to a large petri dish containing a moist tissue and sealed with Nescofilm (Bando Chemical Industries) to maintain humid conditions. Incubation times ranged from 2 to 6 h at 37°C. Slide cultures were examined with a Zeiss Axioskop 50 microscope with Nomarski facilities at magnifications of $\times 200$ to $\times 400$.

Three-dimensional protein homology modeling. With the SWISS-MODEL server (<http://www.expasy.ch/swissmod/SWISS-MODEL.html>) and the Swiss Protein Data Bank (PDB) viewer (<http://www.expasy.ch/spdbv/>) (19), three-dimensional homology models of Vfr and Vfr Δ EQERS were automatically constructed with the cAMP-CRP structure as a template (PDB entry PDB 1D 1G6N) (35). In the Vfr model, residues forming the cyclic nucleotide monophosphate (cNMP)-binding pocket all have main-chain dihedral angles that are in allowed regions of the Ramachandran plot. However, the additional residues in Vfr between β strands 6 and 7 make it impossible to predict the exact conformation of this nonconserved loop with the CRP structure alone. Therefore, the sequence between β strands 6 and 7 in Vfr (LFEKEGSE) was modeled with the loop-building function of the Swiss PDB viewer and the nonconserved loop database. Multiple loop conformations with favorable torsion (main-chain dihedral angles with the allowed regions of the Ramachandran plot) and energetics were identified when β strands 6 and 7 were constrained at L77 and E86, respectively. Therefore, the conformation predicted for Vfr β strands 6 and 7 is theoretically possible despite the difference in the intervening loop. The same procedure was used to optimize the model of Vfr Δ EQERS.

Elastase assay. Assay of the elastolytic activity of *P. aeruginosa* spent culture supernatant was done essentially as previously described (33). Briefly, bacterial cultures were incubated with shaking at 37°C for 16 h in LB broth. After centrifugation of the culture, 500 μ l of supernatant (diluted 1:10) was added to 10 mg of Elastin Congo Red (Sigma) in glass tubes and the mixture was incubated at 37°C for 16 h. After centrifugation of the slurry, the supernatant was diluted 1:10 and the absorbance at 495 nm was measured with a spectrophotometer zeroed on a control Elastin Congo Red sample incubated with LB broth alone. The relative elastolytic activity of each strain is represented by the absorbance at 495 nm of triplicate assays after adjustment for dilution.

Assay for pyocyanin production. For plate assays, LBA or PIA plates were seeded with 100 μ l of overnight broth culture and spread plated to achieve confluent growth prior to incubation for 16 h at 37°C. Plate cultures were shaken gently after the addition of 5 ml of H₂O to the plate surface. After 30 min, the slurry was removed by pipette and the cells were removed by centrifugation. Pyocyanin was extracted from culture supernatants and measured essentially as described by Essar et al. (16). Briefly, 500 liters of culture supernatant was extracted three times with 0.333 liter of diethyl ether and the pyocyanin was extracted from the pooled ether fractions into 0.2 M HCl as a red pigment that was subsequently measured spectrophotometrically at 520 nm.

RESULTS

Vfr is required for twitching motility. During a screen of a *P. aeruginosa* PAO1293 mTn 5-Tc transposon library, we identified a mutant (118C8) that exhibited a large (~70%) reduction in twitching motility levels, as measured by the standard subsurface agar assay. The growth rate of 118C8 was not significantly less than that of the PAO1293 parent (data not shown), indicating that the loss of twitching motility was not simply due to a growth defect. In order to characterize the site of transposon insertion in 118C8, we rescued a *PstI* chromosomal fragment containing the mTn 5-Tc marker. Sequencing outward from the transposon with primer Tn5.I indicated that *P. aeruginosa* 118C8 contained a mTn 5-Tc insertion within *vfr* (Fig. 1A).

A cosmid encompassing the *vfr* gene was isolated from the Holloway *P. aeruginosa* PAO1 cosmid library (22, 39), and a 4-kb *PstI* fragment containing *vfr* was subcloned into pUCPSK. From this intermediary plasmid, a 1.3-kb *XhoI* fragment containing *vfr* was subcloned into pUCPSK in both orientations relative to the *lac* promoter (Fig. 1B). The defective twitching motility of 118C8 was restored to wild-type levels by complementation with wild-type *vfr* in either orientation (data not shown), indicating that the mTn 5-Tc transposon insertion in *vfr*, and not a secondary-site mutation, was the cause of the twitching defect.

To allow comparison with the other twitching-defective mutant strains identified previously in this laboratory (21), the

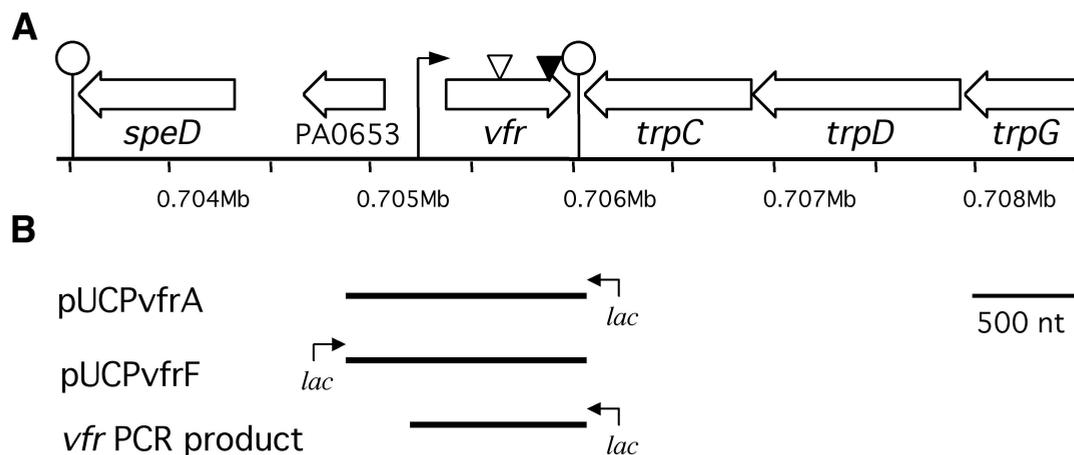


FIG. 1. Genetic organization of the *vfr* locus. (A) ORFs, putative *rho*-independent terminators, and the *vfr* transcriptional start site are represented by hollow arrows, lollipops, and a small filled arrow, respectively. The locations of transposon insertions are indicated by triangles. The filled triangle is the site of mTn 5-Tc insertion in 118C8. Subsequently, this insertion was transferred to *P. aeruginosa* PAK to create PAK *vfr*::mTn 5-Tc. The hollow triangle is the site of insertion of the tetracycline resistance cassette in PAO1 *vfr*::Tc. Numbers below the solid line correspond to the *P. aeruginosa* PAO1 genome sequence. (B) Bold lines denote the region cloned from pMO011925 to create pUCP *vfrA* and pUCP *vfrF* and the region amplified by PCR from wild-type and PAK *lasI*-RT chromosomal DNAs and subsequently cloned to create pSB299.15A and pSB299.17A, respectively.

majority of which were constructed with a *P. aeruginosa* strain K (PAK) parent, we also reconstructed the *vfr* knockout mutation in PAK by transferring the mTn 5-Tc insertion by allelic exchange. As observed with 118C8, the PAK *vfr*::mTn 5-Tc mutant has a severe defect in twitching motility characterized by a small, misshaped twitching zone by stab assay (Fig. 2). Although the twitching defect in PAK *vfr*::mTn 5-Tc is not as severe as that observed in PAK *pilA*::Tc (which lacks type IV pili and is devoid of any twitching motility), the strain is clearly distinguishable from the wild type. Light microscopy revealed that the twitching edge of the PAK *vfr*::mTn 5-Tc mutant was not undifferentiated, like that of PAK *pilA*::Tc, but showed large rafts of cells moving outward, albeit slowly, from the colony (data not shown). However, these rafts were unable to break away from the colony edge and there was no subsequent formation of the intricate lattice-like network of cells characteristic of wild-type twitching motility (44).

The mTn 5-Tc insertion was very close to the *vfr* stop codon (17 nt upstream), raising the possibility that the partial-twitching phenotype may be due to a truncation of Vfr rather than a true null mutation. To address this concern, we created an independent isogenic knockout mutant by inserting a tetracycline resistance cassette centrally within *vfr* in *P. aeruginosa* PAO1 to create PAO1 *vfr*::Tc (Fig. 1A). PAO1 *vfr*::Tc similarly exhibited a severe defect in twitching motility that could be

complemented by pUCP*vfrA* and pUCP*vfrF* (data not shown), confirming that loss of *vfr* causes the observed twitching defect. On the other hand, the *vfr* mutation in strain PAK *vfr*::mTn 5-Tc could only be complemented by pUCP *vfrA* (*vfr* cloned in the opposite orientation with respect to the *lac* promoter), whereas twitching motility was actually inhibited by pUCP *vfrF* in wild-type PAK (presumably due to overexpression of *vfr* in this clone), indicating that there are differences between strains PAK and PAO in the balance of the regulatory circuitry connecting Vfr and twitching motility.

***vfr*-null mutants are defective in type IV pilus assembly.** To further characterize the defect in *vfr*-null mutants, we undertook ELISA and Western analyses. ELISA of whole cells with antisera against the major structural subunit PilA indicated that *vfr* mutants have very low levels of surface pili (Fig. 3A). Western blots of sheared surface pili with anti-PilA sera similarly demonstrated that the *vfr* mutant has a small but significant amount of surface piliation, which may explain its ability to twitch slowly (Fig. 3B). Western blots of whole-cell lysates showed that PAK *vfr*::mTn 5-Tc produces wild-type levels of cellular PilA (Fig. 3B), indicating that the *vfr* mutation does not cause a defect in *pilA* transcription. Therefore, the twitching defect in *vfr*-null mutants is most likely caused by an inability to properly coregulate the assembly or function of surface pili, rather than by a defect in the production of *pilA*. Given the high homology of Vfr to CRP and its previously described function as a transcriptional regulator, it is most likely that Vfr acts as a transcriptional regulator of a gene(s) encoding functional elements of type IV pili or signal transduction pathways that control twitching motility. However, the target(s) that is affected by Vfr in the control of twitching motility has yet to be determined.

Vfr appears to be capable of activation by both cAMP and cGMP. A sequence comparison of Vfr and *E. coli* CRP (Fig. 4) shows that Vfr shares with CRP five of the six residues in the cAMP-binding domain involved in the strongest noncovalent

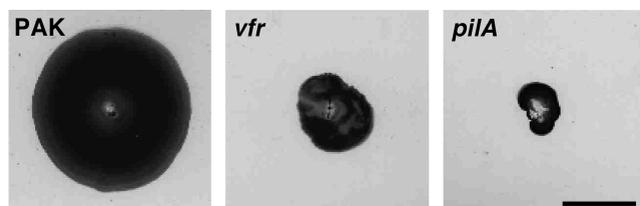


FIG. 2. Subsurface twitching motility assay of wild-type *P. aeruginosa* PAK and *pilA* and *vfr* mutants. Bar, 1 cm.

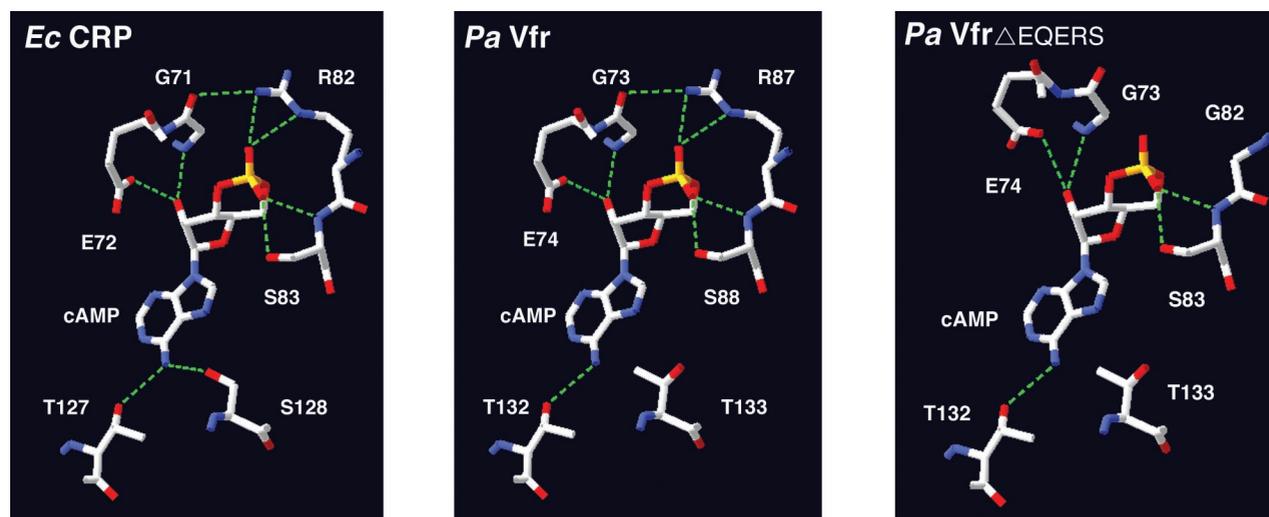


FIG. 5. Homology-based structural models of the cNMP-binding domains of *P. aeruginosa* (*Pa*) Vfr and Vfr Δ EQERS based on *E. coli* (*Ec*) CRP, which served as a template. CRP, Vfr, and Vfr Δ EQERS are shown in stick form and colored in accordance with standard CPK. Only the six residues of CRP (and their corresponding residues in Vfr and Vfr Δ EQERS) that make the strongest contact with cAMP are shown. Hydrogen bonds (as determined from the three-dimensional structure of CRP) are represented by dotted green lines. To permit comparison, the residues of Vfr Δ EQERS are numbered in accordance with the scheme for Vfr (i.e., T132 and T133 of Vfr are actually T127 and T128 of Vfr Δ EQERS).

deletion is dependent on both the longer chain length of Vfr relative to CRP between β strands 6 and 7 and the similarity of the imperfect tandem repeats (i.e., EKEGS/EQERS). However, it should be noted from the model that the substitution of a glycine for the essential arginine residue removes the ability to form some noncovalent bonds with the cyclic phosphate moiety of a cNMP molecule. Therefore, it is likely that Vfr Δ EQERS has less affinity for cNMP molecules, which may affect the allostery imparted by cNMP binding.

Vfr Δ EQERS retains the ability to control elastase and pyocyanin production. In light of the potential for Vfr Δ EQERS to bind cAMP, we were interested in whether this mutant protein retains any functionality. By transforming PAO1 *vfr*::Tc with cloned PCR amplimers of *vfr* from wild-type PAK and PAK *lasI*-RT, we could measure the ability of Vfr and Vfr Δ EQERS, respectively, to rescue the phenotype of the *vfr*-null mutant. As expected, Vfr Δ EQERS could not complement the defective twitching motility phenotype of PAO1 *vfr*::Tc (Table 2). Vfr has also been reported to control the expression of *lasR*, which is required for expression of a range of quorum-sensing-related factors, including elastase (1). Consistent with a lack of *lasR*

expression, PAO1 *vfr*::Tc (Table 2) and PAK *vfr*::mTn 5-Tc (data not shown) are deficient in elastolytic activity. The PAO1 *vfr*::Tc elastase deficiency can be complemented by introducing wild-type *vfr* on a plasmid. Moreover, Vfr Δ EQERS was also able to restore wild-type levels of elastase production in PAO1 *vfr*::Tc (Table 2). Therefore, although the loss of EQERS drastically affected the ability of Vfr to control twitching motility, it had no effect on the ability of Vfr to control elastase production.

We were interested in whether defects in the production of any other Vfr-controlled virulence factors could be similarly complemented by Vfr Δ EQERS. We had observed that when cultured in LB broth, PAO1 *vfr*::Tc produces greatly reduced quantities of pyocyanin compared to those produced by the wild-type strain (data not shown). Interestingly, we found that PAO1 *vfr*::Tc, when it was cultured on PIA plates, produced a greater quantity of pyocyanin than the wild type, whereas the opposite was true when LBA was used (Table 2). As observed with elastase production, wild-type levels of pyocyanin production were restored to PAO1 *vfr*::Tc when it was complemented with either Vfr or Vfr Δ EQERS, on both LBA and PIA (Table 2).

TABLE 2. Phenotypic complementation of *P. aeruginosa* PAO1 *vfr*::Tc

Strain (plasmid)	Vfr allele ^b	Phenotype ^a			
		Twitching motility ^c	Elastase ^d	Pyocyanin ^e	
				LBA	PIA
PAO1(pUCPSK)	None	25.0 \pm 0.8	0.47 \pm 0.13	0.34 \pm 0.09	0.55 \pm 0.11
PAO1 <i>vfr</i> ::Tc(pUCPSK)	None	3.8 \pm 0.4	0.04 \pm 0.03	0.07 \pm 0.03	4.13 \pm 0.29
PAO1 <i>vfr</i> ::Tc(pSB299.15A)	Wild type	24.5 \pm 0.7	0.41 \pm 0.13	0.17 \pm 0.05	0.39 \pm 0.04
PAO1 <i>vfr</i> ::Tc(pSB299.17A)	Δ EQERS	5.3 \pm 0.4	0.33 \pm 0.08	0.24 \pm 0.02	0.76 \pm 0.10

^a Mean results and standard deviations from three independent assays are given.

^b Vfr allele carried on plasmid.

^c Twitching zone diameter (millimeters) after 24 h of incubation.

^d Elastolytic activity (optical density at 495 nm) of 16-h culture supernatant as determined by the Elastin Congo Red assay (33).

^e Pyocyanin production (optical density at 520 nm) was assayed as described in Materials and Methods from 16-h PIA or LBA plate cultures.

These results provide strong support for the idea that Vfr possesses the novel ability to differentially activate (or repress) discrete regulons in response to two different signals (probably cAMP and cGMP) recognized in the same cNMP-binding domain (see Discussion). Such a model suggests that the partial loss of function observed in Vfr Δ EQERS is due to an inability to bind and/or be activated by either cAMP or cGMP while retaining the ability to respond to the other.

DISCUSSION

Vfr has been previously described as a global regulator of virulence factors because of its control of *lasR*, *toxR*, and *regA* transcription (1, 55, 56). Here we show that Vfr also controls twitching motility in *P. aeruginosa*. Vfr is very similar to *E. coli* CRP, with 67% amino acid sequence identity and 91% similarity. The amino acids of CRP that form the structural domains involved in cAMP binding, DNA binding, and interaction with RNA polymerase are identical or highly conserved in Vfr (56). CRP homologs from other pathogenic eubacteria are also involved in the regulation of virulence factors (26, 46), including the assembly and function of *E. coli* Pap pili (18) and *V. cholerae* toxin-coregulated (type IV) pili (47).

Our characterization of the piliation defect in PAK *vfr*::mTn 5-Tc indicates that Vfr is required for the normal function of type IV pili. Importantly, mutants lacking *vfr* are still able to produce detectable amounts of surface pili, although they are greatly reduced compared to those produced by the wild type, and retain the ability to move by twitching motility, albeit slowly and aberrantly. In light of recent advances that confirm that the pilus retraction mechanism is the basis of twitching motility (30), it is possible that *vfr* mutants harbor a defect in pilus assembly or, conversely, exhibit an overactive rate of pilus retraction. Vfr has previously been shown to control the transcription of a number of genes (*lasR*, *toxA*, *regA*, etc.), and it is therefore most likely that Vfr acts as a transcriptional regulator of a gene(s) encoding functional elements of type IV pili or signal transduction pathways that control twitching motility. Of the known Vfr targets, only *lasR* has been previously implicated in twitching motility (via association with *lasI*) (17). However, it is now clear that the *las* and *rhl* quorum-sensing systems are not, in fact, required for twitching motility (6).

Here we show that *vfr* mutants of *P. aeruginosa* are deficient in elastase production, consistent with the reported role of Vfr in the control of *lasR* expression (1). We have also found that Vfr appears to activate and repress pyocyanin production, depending on the culture conditions. Our findings that production of both elastase and pyocyanin is restored to wild-type levels when PAO1 *vfr*::Tc is complemented with either Vfr or Vfr Δ EQERS show that although the mutant form of Vfr is incapable of activating twitching motility, it can still control the production of other virulence factors.

Although Vfr control of pyocyanin production has not been previously reported, a *lasR*-null mutant has been reported to have extracellular pyocyanin levels approximately 45% of those of the wild type (24). More recently, a *lasR* mutant was found to have pyocyanin production that was 8% of that of the wild type after 8 h of incubation but to produce more pyocyanin than the wild type after 14 h of incubation (14). Therefore, our finding that Vfr appears to activate and repress pyo-

cyanin production, depending on the culture conditions, is not as unlikely as it may first appear. Overproduction of pyocyanin by *las* mutants on PIA agar has not been previously reported, but this phenomenon has been observed by others with an independent *lasR* mutant (S. P. Diggle, personal communication). Moreover, when our *lasR*, *lasI*, *rhlR*, and *rhlI* mutants (6) were assayed for pyocyanin production with PIA medium, those containing only a *las* mutation overproduced pyocyanin to the extent observed for *vfr* mutants, whereas any mutant containing an *rhl* mutation produced no pyocyanin (data not shown), consistent with the reported control of pyocyanin production by RhlR/I (10, 24, 32). Therefore, it appears that Vfr controls pyocyanin production (both positively and negatively) via LasR. Although these results strongly suggest that Vfr Δ EQERS provided in *trans* is capable of activating *lasR* transcription in a *vfr* mutant, we cannot entirely rule out the possibilities that Vfr also controls elastase and/or pyocyanin production by other (as yet unrecognized) pathways and that production of these virulence factors is restored by Vfr Δ EQERS in a *lasR*-independent manner.

Transcriptional control by Vfr is exerted by binding to consensus CRP-binding sites. For example, *lasR* activation in *P. aeruginosa* can be prevented by replacing nucleotides within the consensus CRP-binding sites (1). In this context, it is of interest that a potential Vfr DNA-binding site has been identified upstream of *fimS* and that Vfr has been shown to bind to this sequence in gel shift assays (S. E. H. West, personal communication). FimS and AlgR are an atypical sensor-regulator pair that has been shown to be required for normal twitching motility (57), and therefore it is possible that Vfr controls twitching motility via transcriptional control of *fimS*. A possible connection between *vfr* and *fimS*/*algR* is particularly intriguing given our finding that spontaneous nontwitching *lasI* and *rhlI* mutants can be caused by mutations in *vfr* and *algR*, respectively (6). In both cases, the twitching phenotype could be restored by compensatory mutations in *vfr* and *algR*, suggesting that modulation of this putative pathway by phase variation represents a key strategy for survival and/or pathogenesis.

There is growing evidence that Vfr activates different sets of genes in response to different signals. When the cAMP-binding domain of CRP is fused to the DNA-binding domain of Vfr and vice versa, both hybrids are able to activate *E. coli lacZ* and *P. aeruginosa lasR*, but neither is able to activate *regA* or *toxA* (West et al., *Pseudomonas* '99). Recently, it has been confirmed that Vfr does bind cAMP and is only able to bind to the *E. coli lac* promoter in the presence of cAMP (48). Here we show that Vfr Δ EQERS, which has an altered cNMP-binding pocket, is still capable of restoring elastase and pyocyanin production to *vfr*-null mutants despite being unable to complement their twitching deficiency. Furthermore, a three-dimensional homology-based structure suggests that despite the loss of five residues from the cNMP-binding pocket, Vfr Δ EQERS appears to be capable of binding cAMP. Taken together, these findings suggest that Vfr is able to regulate elastase and pyocyanin production via activation of *lasR* in response to one cNMP signal, probably cAMP (given that *E. coli* CAP/Vfr fusions can activate *lasR*), but that Vfr is activated to control twitching motility (and probably also *toxA* and *regA*) in response to another. Although we have not identified

cGMP as the latter, it is likely to be the case as cNMP-binding domains are known to be capable of binding both cAMP and cGMP, with activation being nucleotide dependent. *E. coli* CRP is only activated by cAMP (35) but can bind both cAMP and cGMP, whereas CRP with the site-directed mutation S128T is activated by both types of cyclic nucleotides (28), suggesting that Vfr, which also has a threonine in the position corresponding to CRP S128, may be activated by both cAMP and cGMP. As far as we are aware, this is the first report suggesting that the target specificity of a CRP homolog may be modulated by binding more than one type of cNMP.

In *P. aeruginosa*, intracellular levels of cAMP do not correlate with carbon source availability (36) and, unlike CRP, Vfr does not appear to be required for catabolite repression (40, 48). Despite this, Vfr can substitute for CRP in a cAMP-dependent fashion (56), indicating that Vfr can be activated by cAMP. In *P. aeruginosa*, such activation is likely to be in response to environmental signals other than glucose availability. The nature of these signals and intermediary signaling proteins remains unclear. *P. aeruginosa* appears to utilize guanine nucleotides, including ppGpp and cyclic di-GMP, widely as physiological regulators (13, 23). The high G+C content of *P. aeruginosa* also indicates that there may be a high intracellular level of guanine-based nucleotides. Although there is no direct evidence of a link to intracellular cGMP levels, in *P. aeruginosa*, the synthesis of GTP, ppGpp, and inorganic polyphosphate is known to be sequentially promoted by the onset of stationary phase (23) and inorganic polyphosphate has recently been implicated in twitching motility (38). Therefore, the notion that cGMP may be a physiological signal for Vfr is worthy of further exploration.

An alternative hypothesis is that Vfr activates (or represses) various genes in response to binding of cAMP alone. In this model, differential regulation is achieved by cooperating with different regulatory proteins and sigma factors, as is observed in some other CRP homologs (26, 51). In this case, the partial loss of activity observed in Vfr Δ EQERS may be caused by an inability to effect an appropriate allosteric transition. This hypothesis is supported by the observation that the deletion in Vfr Δ EQERS shortens β strands 6 and 7 so as to radically reposition bulky residues corresponding to CRP F76, E77, and E78 and weaken the potential noncovalent bonds with cAMP, whereas in Vfr, despite the additional three residues, overall structural similarity to CRP is maintained. In CRP, β strands 6 and 7 contact the coiled-coil C helices to form part of the dimer interface and are thought to be partly responsible for the allosteric transition effected by cAMP binding (35). Therefore, even if Vfr Δ EQERS could still bind cAMP, the intersubunit interactions might be disrupted, with a concomitant loss of the ability to interact effectively with an associated regulatory protein and target DNA. Such a loss of allostery may negatively affect the control of twitching motility, but not elastase and pyocyanin production, if there were major differences in the consensus binding sequences and/or an additional protein or sigma factor were required for complete activation of one regulon but not the other. Until more is known about the mechanism by which Vfr controls twitching motility, we cannot rule out this hypothesis.

We discovered during the characterization of PAK *lasI*-RT induced-twitching mutants (PAK *lasI*-RT) (Vfr Δ EQERS) that

when twitching stab assays were incubated for several days (presumably providing strong selective pressure to resume twitching motility), these mutants spontaneously developed point mutations in *vfr* that compensated for the EQERS deletion (6). Although the *vfr* alleles from only two such compensatory spontaneous mutants were sequenced in that study, we found that it was relatively simple to generate large numbers of independent compensatory mutations by plating PAK *lasI*-RT on solid medium. Thus, by using the natural mutational ability of *P. aeruginosa* in conjunction with complementation studies and the three-dimensional structure of Vfr, it should be possible to rapidly formulate a comprehensive structure-function map and reach a greater understanding of the mechanism of Vfr function. We anticipate that in the future there will be a greater reliance on studies that exploit the ability of bacteria to select for compensatory mutations under positive selection, particularly where complex regulatory circuits are concerned.

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