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The *Pseudomonas aeruginosa* Vfr Regulator Controls Global Virulence Factor Expression through Cyclic AMP-Dependent and -Independent Mechanisms[▽]†

Erin L. Fuchs,[1]‡ Evan D. Brutinel,[2]‡ Adriana K. Jones,[3]§ Nanette B. Fulcher,[1] Mark L. Urbanowski,[2] Timothy L. Yahr,[2] and Matthew C. Wolfgang[1,3]*

Cystic Fibrosis/Pulmonary Research and Treatment Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599[1]; Department of Microbiology, University of Iowa, Iowa City, Iowa 52242[2]; and Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599[3]

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Vfr is a global regulator of virulence factor expression in the human pathogen *Pseudomonas aeruginosa*. Although indirect evidence suggests that Vfr activity is controlled by cyclic AMP (cAMP), it has been hypothesized that the putative cAMP binding pocket of Vfr may accommodate additional cyclic nucleotides. In this study, we used two different approaches to generate apo-Vfr and examined its ability to bind a representative set of virulence gene promoters in the absence and presence of different allosteric effectors. Of the cyclic nucleotides tested, only cAMP was able to restore DNA binding activity to apo-Vfr. In contrast, cGMP was capable of inhibiting cAMP-Vfr DNA binding. Further, we demonstrate that *vfr* expression is autoregulated and cAMP dependent and involves Vfr binding to a previously unidentified site within the *vfr* promoter region. Using a combination of *in vitro* and *in vivo* approaches, we show that cAMP is required for Vfr-dependent regulation of a specific subset of virulence genes. In contrast, we discovered that Vfr controls expression of the *lasR* promoter in a cAMP-independent manner. In summary, our data support a model in which Vfr controls virulence gene expression by distinct (cAMP-dependent and -independent) mechanisms, which may allow *P. aeruginosa* to fine-tune its virulence program in response to specific host cues or environments.

Pseudomonas aeruginosa is an opportunistic pathogen responsible for a variety of life-threatening infections in immunocompromised individuals and those receiving critical care (12). *P. aeruginosa* is the primary cause of morbidity and mortality in individuals with cystic fibrosis, in whom it causes chronic lung infection (6). Furthermore, indwelling medical devices, severe wounds, burns, surgery, and corneal abrasion predispose otherwise-healthy individuals to infection by this organism (12). The ability of *P. aeruginosa* to cause infection depends on the expression of an array of surface-exposed and secreted virulence factors (40). Many of these factors are directly or indirectly controlled by the transcriptional regulator protein Vfr (*v*irulence *f*actor *r*egulator). Vfr positively regulates production of exotoxin A (ETA or ToxA), type IV pili (Tfp), a type III secretion system (T3SS), and the *las* quorum-sensing system which, in turn, controls the expression of hundreds of additional genes, including multiple virulence factors (2, 4, 44, 54). In addition, Vfr negatively regulates flagellar gene expression (10). A consensus Vfr binding sequence has been proposed (24), and direct binding of Vfr to target promoters has been demonstrated for several genes, including those encoding ToxA (*toxA*), LasR (the *las* quorum-sensing regulator), FleQ (the master regulator of flagellar biogenesis), RegA and PtxR (regulators of *toxA* expression), and CpdA (a cyclic AMP [cAMP] phosphodiesterase) (2, 10, 14, 16, 24). While the global role of Vfr in regulating virulence gene expression has been established, the molecular mechanisms that control Vfr activity and expression are not well understood.

Vfr is a member of the 3′,5′-cAMP receptor protein (CRP) family of transcriptional regulators (55). The best-studied member of this family is *Escherichia coli* CRP, which primarily regulates genes involved in carbon metabolism (20). CRP functions as a homodimer, and its activity is directly controlled by the allosteric regulator cAMP; CRP undergoes a conformational change upon cAMP binding that enables the protein to interact with target promoters in a DNA sequence-specific manner (5, 26, 34–36, 43). CRP is also capable of binding 3′,5′-cGMP (3, 13, 49); however, structural studies indicate that cGMP does not induce the necessary conformational change required for CRP DNA binding (36). Although previous studies have demonstrated that Vfr and CRP have similar affinities for cAMP *in vitro* (48), the roles of cAMP and other cyclic nucleotides in Vfr function have not been directly examined.

P. aeruginosa encodes two intracellular adenylate cyclases (CyaA and CyaB) responsible for cAMP synthesis (58). Mutants lacking both *cyaA* and *cyaB* exhibit reduced virulence factor expression and are severely attenuated in an adult mouse model of acute pneumonia (47, 58). In addition, whole-genome expression profiling revealed that the transcriptomes of *P. aeruginosa* mutants defective in cAMP synthesis or lacking *vfr* are nearly identical, suggesting that Vfr activity is de-

* Corresponding author. Mailing address: Cystic Fibrosis/Pulmonary Research and Treatment Center, University of North Carolina, CB 7248, Chapel Hill, NC 27599. Phone: (919) 966-8679. Fax: (919) 966-5178. E-mail: matthew_wolfgang@med.unc.edu.

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‡ E.L.F. and E.D.B. contributed equally to this work.

§ Present address: Infectious Diseases, Novartis Institutes for BioMedical Research, Cambridge, MA 02139.

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pendent on cAMP availability (58). In support of this notion, a previous study revealed that Vfr is capable of restoring cAMP-dependent gene expression in an *E. coli crp* mutant. However, CRP cannot complement a *P. aeruginosa vfr* mutant, suggesting that Vfr and CRP are not functionally interchangeable (48, 55).

The putative cAMP binding pocket of Vfr contains three additional amino acids relative to that of *E. coli* CRP (4, 55). While most of the residues involved in cAMP binding are conserved, Vfr has a threonine residue at a position equivalent to serine 128 (S128) of *E. coli* CRP (4). Mutational studies have demonstrated that threonine substitution of CRP S128 results in CRP activation by both cAMP and cGMP (28). Furthermore, a spontaneous Vfr mutant ($Vfr_{\Delta EQERS}$) lacking 5 amino acids (EQERS) in the cyclic nucleotide binding domain, including two critical cAMP binding residues conserved among other CRP homologs, retained the ability to regulate expression of a subset of virulence factors (4). Based on these observations, it has been proposed that Vfr may respond to cAMP, cGMP and/or other allosteric regulators (4, 55). While there is currently no evidence that *P. aeruginosa* has the ability to synthesize cGMP (16), it does produce cyclic diguanosine monophosphate (c-di-GMP) and possibly cyclic diadenosine monophosphate (c-di-AMP) (22, 25, 56). Recent findings indicate that some members of the CRP family bind c-di-GMP, which acts as a negative allosteric regulator (27, 51).

While the mechanism of allosteric regulation of Vfr activity is unresolved, there is evidence suggesting that *vfr* expression is controlled at the level of transcription. A previous study identified two putative Vfr binding sites upstream of the *vfr* gene (centered at bp −67.5 and bp −39.5 relative to the transcription start site) (24). Furthermore, it was demonstrated through electrophoretic mobility shift assays (EMSAs) that Vfr could specifically bind to a *vfr* promoter probe containing both putative sites (24). Although the role of these sites in *vfr* promoter activity has not been tested, direct binding of Vfr to this region suggests that *vfr* expression is autoregulated, as is the case for *E. coli crp* (1, 8, 21).

In this study, we directly assessed the cyclic nucleotide requirement for Vfr activity both *in vitro* and *in vivo*. We generated apo-Vfr by two independent methods and demonstrate that Vfr binding to most target promoters specifically requires cAMP. Other cyclic nucleotides did not support Vfr activity. Furthermore, we show that high concentrations of cGMP inhibit the formation of Vfr-DNA complexes. Using DNase I footprinting, we identified a novel Vfr binding site within the *vfr* promoter distinct from the previously proposed binding sites. *In vitro* transcription assays were employed to demonstrate that both cAMP and Vfr are required for positive *vfr* autoregulation. Finally, we provide evidence that the *lasR* promoter is an exception to the cAMP requirement paradigm, since cAMP was not required for Vfr binding to the *lasR* promoter *in vitro* or for activation of the *lasR* promoter activity *in vivo*. Taken together, our findings provide new mechanistic insights into the complex cAMP/Vfr signaling pathway that controls *P. aeruginosa* virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The strains and plasmids used in this study are listed in Table 1. For routine passage, strains were grown at 37°C in LB (Difco). For complementation experiments, pPa-*vfr* was maintained in *P. aeruginosa* with 30 μg/ml carbenicillin (Cb). Bacterial growth in broth culture was assessed based on the optical density at 600 nm (OD_{600}).

P. aeruginosa strains PAK *cyaA cyaB vfr* and PAK *lasR vfr* were constructed by introducing a deletion allele for *vfr* (encoded by the pEXGmΔ*vfr* plasmid) onto the chromosome of PAK *cyaA cyaB* and PAK *lasR*, respectively, using a previously described method (58). PAK *lasR cyaA cyaB* was constructed by sequentially introducing deletion alleles for *cyaA* and *cyaB* (encoded by pEXGmΔ*cyaA* and pEXGmΔ*cyaB*, respectively) onto the chromosome of PAK *lasR*.

Chromosomal transcriptional reporters were constructed by PCR amplifying the *vfr* promoter region (bp −164 to +206 relative to the *vfr* transcriptional start site [39]) and the *lasR* promoter region (bp −264 to +238 relative to the predominant *lasR* T_1 transcriptional start site [2]) from *P. aeruginosa* strain PAK chromosomal DNA using oligonucleotides (see Table S1 in the supplemental material; EcoRI and BamHI restriction sites are underlined) tailed with *att*B1 or *att*B2 sequences for Gateway cloning into pDONR201 (Invitrogen). Both promoter fragments encompassed known or predicted promoter elements and the translational start sites of the corresponding genes. Promoter fragments were removed from pDONR201 by digestion with EcoRI and BamHI and ligated into the corresponding restriction sites of mini-CTX-*lacZ* (23). The resulting plasmids were used to integrate the promoter-*lacZ* fusions onto the chromosome at a vacant ϕCTX phage attachment site of wild-type and mutant *P. aeruginosa* strains as described previously (23).

A *toxA* transcriptional reporter plasmid was constructed by PCR amplifying bp −500 to +100 relative to the *toxA* translational start codon from PAK chromosomal DNA with *toxA* rep 5′ and *toxA* rep 3′ oligonucleotides (see Table S1 in the supplemental material; HindIII and BamHI restriction sites are underlined), digesting with HindIII and BamHI, and ligating into the corresponding restriction sites of the plasmid pR-*lacZ* to create pR*toxA-lacZ*. pR-*lacZ* is a low-copy-number *lacZ* transcriptional reporter plasmid (gift of Arne Rietsch [Case Western Reserve University]). The pR*toxA-lacZ* plasmid was transferred to appropriate strains by conjugation (18) followed by selection on LB agar plates containing 75 μg/ml gentamicin (Gm) and 25 μg/ml irgasan (Irg).

To create a *P. aeruginosa vfr* expression plasmid (pPa-*vfr*), the open reading frame of *vfr* was PCR amplified from strain PAK genomic DNA using primers 5′ *vfr* and 3′ *vfr* (see Table S1 in the supplemental material) and cloned into pMMBV1GW (16) using Gateway cloning (Invitrogen) by a previously described method (58).

Plasmid templates used in the *in vitro* transcription assays were created by cloning the *vfr* promoter region (bp −164 to +206 relative to the *vfr* transcriptional start site [39]) and the *lasR* promoter region (bp −264 to +30 or −264 to +238 relative to the *lasR* T_1 transcriptional start site [2]) upstream of the *rpoC* transcriptional terminator in plasmid pOM90 (38) to create pOM90-*vfr*, pOM90-$lasR_{(-264\ to\ +30)}$, and pOM90-$lasR_{(-264\ to\ +238)}$. The promoter fragments were PCR amplified from strain PAK chromosomal DNA using oligonucleotides tailed with BamHI and EcoRI restriction sites (see Table S1 in the supplemental material), digested with BamHI and EcoRI, and ligated into the corresponding restriction sites of pOM90. For the *in vitro* transcription assay, the predicted size of the *vfr* transcript is 281 nucleotides; the predicted sizes of the *lasR* transcripts are 102 and 132 nucleotides for the pOM90-$lasR_{(-264\ to\ +30)}$ template and 310 and 340 nucleotides for the pOM90-$lasR_{(-264\ to\ +238)}$ template.

Protein purification and generation of apo-Vfr. Purification of cAMP-Vfr, CpdA, a mutant derivative of CpdA (CpdA-N93A), and *P. aeruginosa* RNA polymerase holoenzyme (RNAP) was carried out as described previously (16, 52) (see Fig. S1 in the supplemental material). Apo-Vfr was generated by two independent methods. In the first approach, cAMP-Vfr was incubated with a 5-fold molar excess of purified CpdA or CpdA-N93A for 18 h at 23°C. In some experiments, CpdA was inactivated by heating for 5 min at 95°C prior to incubation with Vfr. In the second approach, bound cAMP was removed from Vfr by denaturing Vfr protein (0.5 ml at 55 μg/ml) by dialysis for 2 h at 4°C against buffer (50 mM Tris-HCl [pH 7.0], 100 mM KCl, 50 mM NaCl, 1 mM dithiothreitol [DTT], 1 mM EDTA, 10% glycerol, 0.5% Tween 20) containing 6 M urea. Vfr was refolded by sequentially reducing the urea concentration to 3 M, 2 M, 1 M, 0.5 M, and 0 M under the dialysis conditions described above in the absence or presence of 50 μM cAMP.

EMSAs. DNA promoter probes were generated by PCR using the indicated oligonucleotides (see Table S1 in the supplemental material) and end labeled using 10 μCi of [γ-^{32}P]ATP (GE Healthcare) and 10 U of T4 polynucleotide kinase (New England Biolabs). EMSAs were performed as previously described (7). Briefly, probes (0.25 nM each) were incubated in binding buffer (10 mM Tris [pH 7.5], 50 mM KCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, and 100 μg/ml bovine serum albumin) containing 5 μg/ml poly(2′-deoxyinosinic-2′-deoxycytidylic acid) [poly(dI-dC); Sigma] for 5 min at 25°C. As noted below and in the

TABLE 1. *P. aeruginosa* strains and plasmids used in this work

Construct	Description[a]	Reference or source
P. aeruginosa strains		
PAK	Wild-type strain	50
PAK *cyaA*	Nonpolar *cyaA* deletion	58
PAK *cyaB*	Nonpolar *cyaB* deletion	58
PAK *cyaA cyaB*	Nonpolar *cyaA cyaB* deletions	58
PAK *vfr*	Nonpolar *vfr* deletion	58
PAK *cyaA cyaB vfr*	Nonpolar *cyaA cyaB vfr* deletions	This study
PAK *lasR*	Nonpolar *lasR* deletion	57
PAK *lasR vfr*	Nonpolar *lasR vfr* deletions	This study
PAK *lasR cyaA cyaB*	Nonpolar *lasR cyaA cyaB* deletions	This study
Plasmids		
pMMBV1GW	Empty *P. aeruginosa* expression vector; Ap^r	16
pPa-*vfr*	*vfr* encoded by pMMBV1GW	This study
pDONOR201	Gateway cloning vector; Kn^r	Invitrogen
pR-*lacZ*	Low-copy-number plasmid for creating transcriptional fusions with *lacZ*; Gm^r	This study
pR*toxA-lacZ*	*toxA* promoter region (bp −500 to +100 relative to *toxA* translational start codon) in HindIII and BamHI sites of pR-*lacZ*	This study
mini-CTX-*lacZ*	Plasmid for chromosomal integration of promoter-*lacZ* transcriptional fusions; Tc^r	23
mini-CTX-P*vfr-lacZ*	*vfr* promoter region (bp −164 to +206 relative to *vfr* transcriptional start site) in EcoRI and BamHI sites of mini-CTX-*lacZ*	16
mini-CTX-P*lasR-lacZ*	*lasR* promoter region (bp −264 to +238, relative to *lasR* T1 transcriptional start site) in EcoRI and BamHI sites of mini-CTX-*lacZ*	This study
pEXGmGW	Gateway-adapted suicide vector; Gm^r	58
pEXGmΔ*vfr*	pEXGmGW carrying *vfr* deletion allele	58
pEXGmΔ*cyaA*	pEXGmGW carrying *cyaA* deletion allele	58
pEXGmΔ*cyaB*	pEXGmGW carrying *cyaB* deletion allele	58
pOM90	Plasmid template for *in vitro* transcription assays; Kn^r	38
pOM90-*vfr*	*vfr* promoter region (bp −164 to +206 relative to *vfr* transcriptional start site) in HindIII and BamHI sites of pOM90	This study
pOM90-$lasR_{(-264\ to\ +30)}$	*lasR* promoter region (bp −264 to +30 relative to *lasR* T1 transcriptional start site) in HindIII and BamHI sites of pOM90	This study
pOM90-$lasR_{(-264\ to\ +238)}$	*lasR* promoter region (bp −264 to +238 relative to *lasR* T1 transcriptional start site) in HindIII and BamHI sites of pOM90	This study

[a] Ap^r, ampicillin resistance marker; Kn^r, kanamycin resistance marker; Gm^r, gentamicin resistance marker; Tc^r, tetracycline resistance marker.

figure legends, cAMP, cGMP, c-di-GMP, or c-di-AMP was also present. cAMP-Vfr or apo-Vfr protein was then added at concentrations (indicated in figure legends) for a final reaction volume of 20 μl and incubated for an additional 15 min at 25°C. Samples were subjected to electrophoresis on a 5% polyacrylamide glycine gel (10 mM Tris [pH 7.5], 380 mM glycine, 1 mM EDTA) at 4°C. Imaging and data analyses were performed using an FLA-7000 PhosphorImager (Fujifilm) and MultiGauge v3.0 software (Fujifilm). EMSAs were repeated a minimum of two times, and representative gels are shown.

DNase I footprinting assays. A single end-labeled γ-^{32}P-labeled double-stranded DNA probe was generated by PCR in which one of the oligonucleotide primers was modified (5 Amino-MC6; Integrated DNA Technologies) at the 5′ end to prevent phosphorylation (see Table S1 in the supplemental material). Probes were subsequently labeled as described above. Footprinting reaction mixtures consisted of a single end-labeled probe (10 fmol) with 5 μg/ml poly(dI-dC) (Sigma) in DNase I reaction buffer (10 mM Tris [pH 8.0], 50 mM KCl, 2 mM $MgCl_2$, 0.5 mM DTT, 100 μg/ml bovine serum albumin, 10% glycerol). cAMP-Vfr was added for a final reaction volume of 25 μl, and the mixture was incubated for 15 min at 25°C. DNase I footprinting and DNA sequencing reactions were performed as previously described (31, 41, 45).

***In vitro* transcription assays.** Plasmid templates (2 nM) were incubated in the absence or presence of refolded apo-Vfr (100 nM) and/or cAMP (100 nM) in 1× transcription buffer (40 mM Tris-HCl [pH 7.5], 150 mM KCl, 10 mM $MgCl_2$, 0.01% Tween 20, and 1 mM DTT) containing 0.75 mM rATP, rGTP, and rCTP for 10 min at 25°C. Purified *P. aeruginosa* RNA polymerase holoenzyme, known to be largely σ^{70} saturated (52), was then added (10 nM) and the mixture was incubated for 5 min at 25°C. Finally, 0.75 mM rUTP and 5 μCi [α-^{32}P]CTP in 1× transcription buffer containing heparin (50 μg/ml) were added for a final reaction volume of 20 μl, and transcription was allowed to proceed for 10 min at 30°C. Reactions were terminated by adding 20 μl of stop buffer (98% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), heated to 95°C for 5 min, and electrophoresed on 5% denaturing urea polyacrylamide gels. Phosphorimaging and densitometry were performed using a FLA-7000 PhosphorImager (Fujifilm) and MultiGage v3.0 software (Fujifilm), respectively.

β-Galactosidase assays. Overnight cultures of strains containing *vfr* or *lasR* promoter-*lacZ* fusions were diluted 1:100 into LB and grown to an OD_{600} of 1 or 5, respectively. Cb (30 μg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG; 40 μM) were added to the medium as indicated in the figure legends. Strains harboring both pR*toxA-lacZ* and pPa-*vfr* were grown overnight in LB containing 30 μg/ml Cb and 15 μg/ml Gm, washed twice with deferrated (33) Bacto tryptic soy broth (DTSB; Becton Dickinson), and inoculated into fresh DTSB containing 30 μg/ml Cb, 15 μg/ml Gm, and 40 μM IPTG. Starting with a culture OD_{600} of 0.025, bacteria were grown for 8 h, and 1 ml of culture supernatant was collected. For all samples, β-galactosidase activity measurements were carried out as described previously (32), except that optical density determinations were made using a 96-well plate reader (Bio-Tek). Each assay was repeated at least three times.

Western blot assays. Bacteria were grown as described for β-galactosidase assays. Whole-cell lysates and culture supernatants were prepared for detection of Vfr and secreted ToxA, respectively, as previously described (16). All Western blot assays were repeated a minimum of three times with independently derived protein samples, and representative blots are shown.

Statistical analysis. The two-tailed unpaired *t* test was used for data comparison where appropriate using Prism v5.0b (GraphPad Software).

RESULTS

Vfr binds target promoters with different affinities *in vitro*. The nucleotide sequence and position of Vfr binding sites within target promoters vary substantially (2, 10, 14, 16, 24). To determine the affinity of Vfr for a representative set of target

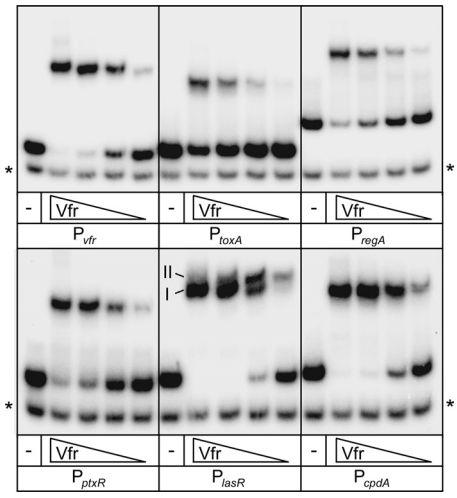

FIG. 1. EMSAs showing the specific DNA binding activities of cAMP-Vfr to P_{vfr}, P_{toxA}, P_{regA}, P_{ptxR}, P_{lasR}, and P_{cpdA} promoter probes. Specific and nonspecific probes (0.25 nM each; note that the specific activity of the nonspecific probe is lower than that of the specific probes) were incubated in the absence (−) or presence of various concentrations of cAMP-Vfr (113, 38, 13, and 4 nM) for 15 min followed by electrophoresis and phosphorimaging. Mobilities of the promoter probes, encompassing known (P_{toxA}, P_{regA}, P_{ptxR}, P_{lasR}, and P_{cpdA}) or predicted (P_{vfr}) Vfr binding sites, were retarded in a Vfr concentration-dependent manner, indicating the formation of specific protein-DNA complexes. The nonspecific probe (~160 bp) is indicated by an asterisk. cAMP-Vfr-dependent P_{lasR} shift complexes I and II are indicated by Roman numerals.

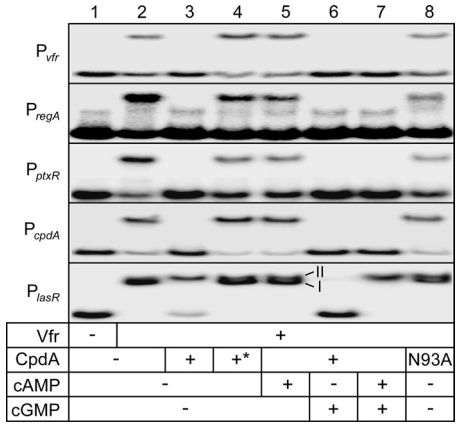

FIG. 2. Cyclic AMP and cyclic GMP differentially affect Vfr-DNA binding activity as assessed in EMSAs. Lanes 1 and 2 represent promoter probes (0.25 nM each) that were incubated in the absence or presence of cAMP-Vfr, respectively, for 15 min followed by electrophoresis and phosphorimaging. Lanes 3, 5, 6, and 7 represent samples in which the cAMP-Vfr complex was incubated with the cAMP phosphodiesterase CpdA prior to the addition of specific promoter probes. As controls, cAMP-Vfr was also incubated with heat-inactivated CpdA (*; lane 4) or a catalytically inactive CpdA mutant (N93A; lane 8). Binding reaction mixtures contained no exogenously added nucleotides (lanes 1 to 4 and 8), 50 μM cAMP (lane 5), 1 mM cGMP (lane 6), or both nucleotides (lane 7). P_{lasR} shift complexes I and II are indicated by Roman numerals. The final concentration of Vfr protein in the EMSAs was 13 nM (P_{vfr} and P_{cpdA}) or 38 nM (P_{regA}, P_{ptxR}, and P_{lasR}).

promoters, we used quantitative EMSAs. Vfr protein was expressed in *P. aeruginosa* and isolated from cellular lysates using a cAMP-agarose affinity purification column (see Fig. S1 in the supplemental material). Specific promoter probes (P_{vfr}, P_{toxA}, P_{regA}, P_{ptxR}, P_{lasR}, and P_{cpdA}) encompassing known or predicted Vfr binding sites (2, 14, 16, 24) were generated by PCR. For each binding assay, cAMP-Vfr was incubated with the specific promoter probe (~200 bp), a nonspecific control probe (~160 bp), and poly(dI-dC) and analyzed by native polyacrylamide gel electrophoresis and phosphorimaging. The addition of cAMP-Vfr had no effect on mobility of the nonspecific DNA probe (Fig. 1). In contrast, mobilities of the P_{vfr}, P_{toxA}, P_{regA}, P_{ptxR}, P_{lasR}, and P_{cpdA} promoter probes were retarded in a Vfr concentration-dependent fashion, indicating the formation of specific protein-DNA complexes (Fig. 1). The fraction of probe that shifted as a function of Vfr concentration was used to calculate the apparent equilibrium constant (K_{eq}) for cAMP-Vfr binding. The apparent K_{eq} for the promoter probes varied over an 18-fold range (Table 2), with cAMP-Vfr having the highest affinity for P_{lasR} and P_{cpdA} and the lowest affinity for P_{toxA}. Interestingly, our results showed two distinct P_{lasR} shift products that were not previously reported (2). The higher-mobility product (complex I) was the predominant form at the highest cAMP-Vfr concentration tested (113 nM), and the lower-mobility shift product (complex II) was observed as the cAMP-Vfr concentration was reduced. At the lowest cAMP-Vfr concentration examined (4 nM), complex II was the dominant shift product detected. The reason for the two distinct P_{lasR} shift products is unclear; however, the subtle change in mobility likely reflects a difference in the conformation of the protein-DNA complex rather than variation in the number of Vfr molecules bound per probe.

TABLE 2. Apparent K_{eq} for cAMP-Vfr binding to promoter DNA

Promoter	K_{eq}	SEM	n^a
vfr	17.0	4.5	3
toxA	95.0	10.3	2
regA	23.0	1.4	2
ptxR	15.4	4.8	2
lasR	5.3	0.4	2
cpdA	7.5	1.9	2

[a] Number of replicates for each quantitative EMSA.

Cyclic AMP is required for Vfr binding to *vfr*, *regA*, *ptxR*, and *cpdA* promoter DNA. To determine the role of cAMP in Vfr function, we used two different approaches to generate cAMP-free Vfr (apo-Vfr). As a first approach, we incubated the cAMP-Vfr complex overnight with a 5-fold molar excess of purified *P. aeruginosa* CpdA (see Fig. S1 in the supplemental material), a cAMP phosphodiesterase that degrades cAMP (16). To determine whether CpdA treatment affected the ability of Vfr to form complexes with its target promoters, the Vfr and CpdA mixture was used in EMSA experiments in the absence or presence of exogenous cAMP. CpdA treatment eliminated the formation of Vfr shift complexes with the P_{vfr}, P_{regA}, P_{ptxR}, and P_{cpdA} promoter probes (Fig. 2, lane 3). Interestingly, CpdA-treated Vfr was unable to form complex I with P_{lasR} but retained the ability to form complex II (discussed below). When cAMP-Vfr was incubated overnight in the absence of CpdA (lane 2), with heat-inactivated CpdA (lane 4), or with a catalytically inactive CpdA mutant (CpdA-N93A;

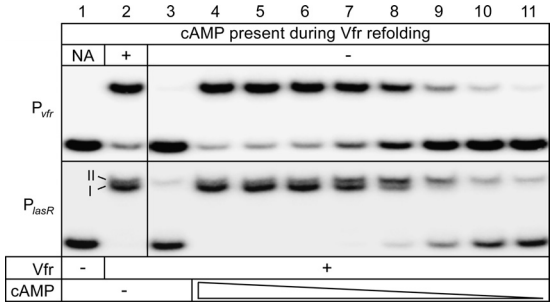

FIG. 3. Cyclic AMP is required for the binding of apo-Vfr to P_{vfr} and the formation of P_{lasR} complex I but not P_{lasR} complex II. EMSA results show DNA binding activity of denatured Vfr (38 nM) that was refolded in the presence (lane 2) or absence (lanes 3 to 11) of 50 μM cAMP. For lanes 4 to 11, DNA binding reaction mixtures (15-min incubation) contained various concentrations of exogenous cAMP (left to right, 800, 400, 200, 100, 50, 25, 12, and 6 nM). P_{lasR} shift complexes I and II are indicated by Roman numerals.

lane 8) (16), Vfr retained DNA binding activity. The latter results indicate that the loss of Vfr-dependent DNA binding was specifically associated with CpdA activity. The ability of CpdA-treated Vfr to shift the P_{vfr}, P_{regA}, P_{ptxR}, and P_{cpdA} probes was rescued by the addition of cAMP (50 μM) to the DNA binding reaction mixtures (lane 5). These results indicate that cAMP is necessary for Vfr binding to P_{vfr}, P_{regA}, P_{ptxR}, and P_{cpdA}.

To further assess the contribution of cAMP to Vfr function, we generated apo-Vfr by a second, nonenzymatic approach. Specifically, cAMP-Vfr was denatured in the presence of 6 M urea and cAMP was removed by dialysis. Vfr was then refolded by sequentially reducing the urea concentration in the absence or presence of 50 μM cAMP, and the DNA binding activity of recovered Vfr was examined by EMSA. Whereas Vfr refolded in the presence of cAMP retained the ability to bind the P_{vfr} probe (Fig. 3, lane 2), Vfr refolded in the absence of cAMP was unable to shift P_{vfr} (lane 3). These results are consistent with those obtained with CpdA-treated Vfr (Fig. 2) and suggest that refolding of Vfr in the absence of cAMP generates apo-Vfr. Cyclic AMP restored the DNA binding activity of refolded apo-Vfr for P_{vfr} in a concentration-dependent manner (Fig. 3, lanes 4 to 11). A 50% shift of the P_{vfr} probe occurred at ~50 nM cAMP (lane 8), and the maximal shift was achieved at 200 nM cAMP (lane 6). Taken together, these results demonstrate that for a representative set of Vfr-dependent promoters, cAMP is required for the DNA binding activity of Vfr.

Cyclic AMP-independent binding of Vfr to the *lasR* promoter. In contrast to the P_{vfr}, P_{regA}, P_{ptxR}, and P_{cpdA} probes, our EMSA studies suggest that both cAMP-Vfr and apo-Vfr bind to the P_{lasR} probe. As mentioned above (Fig. 2, lane 3), incubation of cAMP-Vfr with CpdA eliminated formation of the higher-mobility Vfr-P_{lasR} complex (complex I); however, CpdA treatment had no effect on the formation of the lower-mobility complex (complex II). The addition of excess cAMP (50 μM) specifically restored formation of complex I (Fig. 2, lane 3 versus 5). Similar results were observed with refolded apo-Vfr; in the absence of exogenous cAMP only complex II was detected (Fig. 3, lane 3). The addition of exogenous cAMP to refolded apo-Vfr resulted in the formation of complex I in a concentration-dependent manner. For refolded apo-Vfr, ~25 nM cAMP was sufficient to shift 50% of the P_{lasR} probe (lane 9), and the maximal shift was achieved between 50 and 100 nM cAMP (Fig. 3). These results indicate that Vfr is capable of forming distinct complexes with P_{lasR} in a cAMP concentration-dependent manner and suggest that cAMP-Vfr and apo-Vfr may be responsible for the different P_{lasR} shift products (complex I and II, respectively). As such, it appears that P_{lasR} is a unique promoter in that Vfr can bind the P_{lasR} probe *in vitro* without being fully saturated with cAMP. Although the two Vfr-P_{lasR} shift products were also detected in EMSAs using Vfr presumed to be cAMP saturated (Fig. 1), it is possible that Vfr was not fully occupied with cAMP at the lower protein concentrations as a consequence of dilution. To test the possibility that cAMP dissociates from the cAMP-Vfr complex upon dilution, we examined the affinity of cAMP-Vfr for P_{lasR} and P_{cpdA} at various dilutions by EMSA in the presence of excess cAMP (50 μM). The K_{eq} for cAMP-Vfr binding to P_{lasR} and P_{cpdA} was unaffected by the addition of excess cAMP (see Fig. S2 in the supplemental material), and both conditions gave results identical to those in Fig. 1, indicating that the purified cAMP-Vfr complex is fully saturated with cAMP even when diluted to low nanomolar concentrations. Taken together, these results indicate that Vfr forms distinct complexes with P_{lasR} in both a Vfr and cAMP concentration-dependent manner.

Cyclic GMP inhibits the formation of Vfr-DNA complexes. To address whether cGMP can regulate Vfr function, CpdA-generated apo-Vfr was incubated with an excess of cGMP (1 mM). Whereas cAMP restores DNA binding activity of apo-Vfr, cGMP was unable to restore Vfr binding to the P_{vfr}, P_{regA}, P_{ptxR}, or P_{cpdA} probes (Fig. 2, lane 6). Furthermore, cGMP did not support the formation of Vfr-P_{lasR} complex I and appeared to reduce formation of complex II, suggesting that cGMP inhibits Vfr DNA binding activity. To further examine this possibility, we conducted competition experiments by simultaneously adding both cAMP and cGMP to CpdA-generated apo-Vfr protein. The presence of 50 μM cAMP and excess cGMP (1 mM) prevented Vfr from binding to the P_{vfr}, P_{regA}, P_{ptxR}, and P_{cpdA} probes and inhibited formation of P_{lasR} complex I (Fig. 2, lane 5 versus 7). While addition of cGMP alone inhibited formation of Vfr-P_{lasR} complex II, it appears that complex II was produced when both cGMP and cAMP were present. Currently, we are unable to explain this discrepancy. We next examined the effect of cGMP on Vfr binding to P_{vfr}, P_{toxA}, and P_{lasR} promoter probes using refolded apo-Vfr. Similar to the results with CpdA-generated apo-Vfr, the addition of cGMP alone was not sufficient to restore binding of apo-Vfr to P_{vfr} and P_{toxA}, and it inhibited formation of Vfr-P_{lasR} complex II (Fig. 4, lane 4). Again, consistent with the above results, the presence of both cAMP (50 μM) and excess cGMP (1 mM) prevented Vfr from binding to the P_{vfr} and P_{toxA} probes and formation of Vfr-P_{lasR} complex I (Fig. 4, lane 7).

To determine the amount of cGMP required for inhibition of Vfr DNA binding activity, we conducted a titration experiment using the P_{vfr} probe. Refolded apo-Vfr shifted 90% of P_{vfr} in the presence of 100 nM cAMP (Fig. 5, lane 3), and 50% inhibition of the shift complex required an ~2,500-fold molar excess of cGMP (250 μM; lane 6). Vfr binding activity was unaffected in the presence of a 300-fold molar excess of cGMP

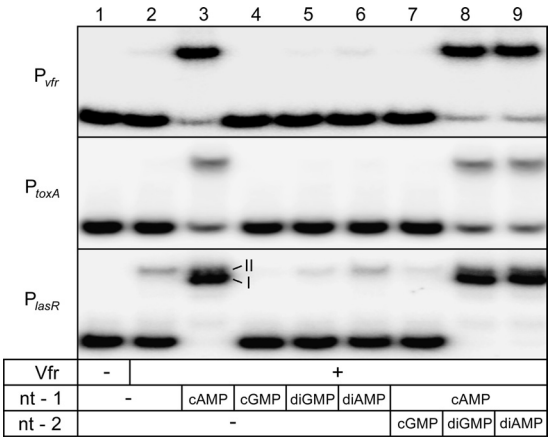

FIG. 4. Analysis of the nucleotide requirements for Vfr-DNA binding activity. Denatured Vfr that was refolded in the absence of cAMP was used in EMSAs to examine the ability of apo-Vfr to bind P_{vfr}, P_{toxA}, and P_{lasR} probes in the presence of different nucleotides. EMSAs were performed with no exogenous nucleotide added (lane 2) or in the presence of 50 μM cAMP (lane 3), 1 mM cGMP (lane 4), 1 mM c-di-GMP (lane 5), or 1 mM c-di-AMP (lane 6). Lanes 7 to 9 represent samples in which cAMP (50 μM) was added in addition to 1 mM cGMP, c-di-GMP, or c-di-AMP, respectively. The final concentration of apo-Vfr in EMSAs was 38 nM for P_{vfr} and P_{lasR} and 113 nM for P_{toxA}. P_{lasR} shift complexes I and II are indicated in lane 3.

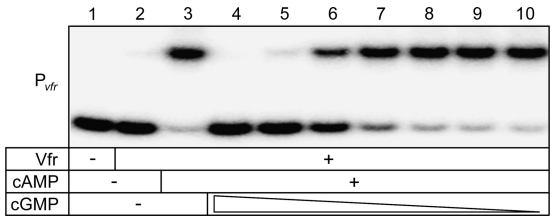

FIG. 5. Kinetics of cGMP inhibition of Vfr binding to P_{vfr} as determined by EMSA. Samples contained P_{vfr} (0.25 nM) in the absence (lane 1) or presence of refolded apo-Vfr (38 nM) with no exogenous nucleotide added (lane 2), 100 nM cAMP (lane 3), or both 100 nM cAMP and various concentrations of cGMP (lanes 4 to 10; 1,000, 500, 250, 125, 60, 30, and 15 μM).

(30 μM; lane 9). While these results indicate that cGMP can act as an inhibitor of cAMP-dependent Vfr DNA binding activity *in vitro*, the high concentration of cGMP required is unlikely to be achieved *in vivo* (see Discussion). Furthermore, it remains to be determined whether cGMP acts as a competitive or allosteric inhibitor.

The inhibitory effect of cGMP raised the question as to whether other cyclic nucleotides affect the DNA binding activity of Vfr. When added alone (1 mM) to refolded apo-Vfr, neither c-di-GMP nor c-di-AMP supported the formation of Vfr-DNA complexes with the P_{vfr}, P_{toxA}, or P_{lasR} promoter probes (Fig. 4, lanes 5 and 6). In addition, neither signaling molecule inhibited the formation of Vfr-DNA complexes in the presence of cAMP (lanes 8 and 9). Thus, our data indicate that these nucleotides are unlikely to play a direct role in regulating Vfr function.

Vfr binds to a single site within the *vfr* promoter *in vitro*. The *vfr* promoter region is predicted to encompass two putative Vfr binding sites, centered at bp −67.5 and −39.5 relative to the transcriptional start site (24) (Fig. 6A). However, binding to either site has not been experimentally proven. Although both sites were present in the P_{vfr} probe examined by EMSA (Fig. 1 and 6A), our data and those of a previous study (24) demonstrate that only a single Vfr-dependent shift product is formed. While not definitive, these results suggest that Vfr may bind to a single site within P_{vfr}. To address this issue, DNase I footprinting of P_{vfr} was performed. In the presence of cAMP-Vfr, a 32-bp sequence (bp −73 to −42 relative to the *vfr* transcriptional start site) was protected from DNase I cleavage (Fig. 6B). The protected region partially overlapped the two previously predicted binding sites and the intervening sequence (Fig. 6A). Within the protected region, enhanced cleavage sites were detected at bp −61 and bp −50/−51 (Fig. 6B). The size of the footprint and spacing of the DNase I hypersensitivity sites are consistent with Vfr footprints of the *toxA*, *regA*, *ptxR*, *fleQ*, *lasR*, and *cpdA* promoters (2, 10, 14, 16, 24) and suggest that Vfr also binds to a single site within P_{vfr}. We identified a putative Vfr binding site (5′-GGATCACAGTC:CTGATAGC TGC) within the protected region by aligning the position of the DNase I hypersensitivity sites with those found in other published Vfr DNase I footprints (Fig. 6B; see also Fig. S3 in the supplemental material). Enhanced DNase I cleavage is associated with distortion of the helical DNA structure and occurs at positions 5 and 6 within the conserved half-sites of CRP and Vfr binding sequences (24) (see Fig. S3). CRP has been shown to induce ~40° bends at equivalent positions in its target promoters (42), a distortion that likely accounts for increased DNase I sensitivity.

The putative Vfr binding site (5′-GGATCACAGTC:CTGA TAGCTGC; underlined sequence portions are the conserved half-sites to which Vfr is predicted to bind) identified in the *vfr* promoter shares limited conservation with the proposed Vfr consensus binding sequence (5′-ANWWTGNGAWNY:AGW TCACAT, where dimeric Vfr is predicted to bind two half-sites) (24); however, the upstream half-site (TCACA) within the *vfr* promoter is identical to the consensus downstream half-site, suggesting that Vfr may tolerate a high degree of variability in the organization of its target binding sites. While further analysis is needed to confirm the exact Vfr binding site, our data support the notion that Vfr binds to its own promoter at a single site.

To determine whether Vfr binding is sufficient for activation of P_{vfr}, *in vitro* transcription assays were performed in the absence and presence of cAMP and using refolded apo-Vfr, RNA polymerase isolated from *P. aeruginosa* that is largely σ^{70} saturated (see Fig. S1 in the supplemental material), and a plasmid template carrying the *vfr* promoter region (bp −164 to +206 relative to the transcriptional start) (Fig. 6A). A σ^{70}-dependent transcript (~320 nucleotides [nt]), produced from the plasmid backbone, was detected under all conditions and served as a positive transcription control (Fig. 6C). Whereas a faint band, corresponding in size to the predicted plasmid-encoded *vfr* transcript (~281 nt), was detected in the absence of cAMP or Vfr, the presence of both refolded apo-Vfr and cAMP (100 nM) increased the amount of the *vfr* transcript ~11-fold as determined by densitometry. In addition, we also observed a cAMP-Vfr-dependent transcript (~115 nt) generated from the plasmid backbone that fortuitously served as an internal control for cAMP-Vfr-dependent transcription. These

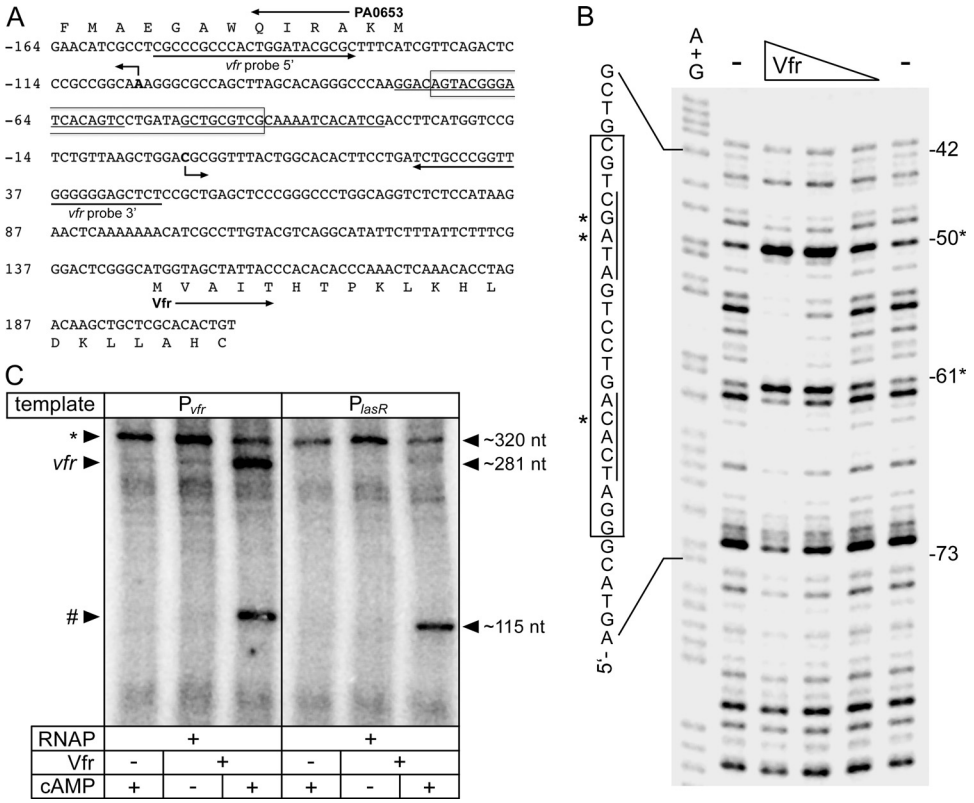

FIG. 6. Binding of cAMP-Vfr to a single site within the *vfr* promoter is sufficient for activation of *vfr* transcription *in vitro*. (A) Diagram of the *vfr* promoter region used as template for *in vitro* transcription reactions and *in vivo* promoter reporter (P_{vfr}-*lacZ*) assays. Numbering (in bp) is relative to the previously identified (39) *vfr* transcription start site (indicated in bold type and marked by the arrow, which shows the direction of transcription). Also indicated is the transcription start site for the divergently transcribed gene PA0653. Amino acid residues for partial coding sequences of Vfr and the PA0653 gene product are shown. The previously predicted Vfr binding sites (24) (centered at bp −39.5 and −67.5) are underlined, and the sequence that was protected from DNase I cleavage by cAMP-Vfr (shownn in panel B) is boxed. The locations of oligonucleotide sequences (*vfr* probe 5′ and *vfr* probe 3′) used to generate P_{vfr} probes for EMSAs and DNase I footprinting are indicated by arrows. (B) DNase I footprinting of the *vfr* promoter region by cAMP-Vfr. Samples contained a DNA fragment (0.4 nM) corresponding to bp −153 to 48 relative to the *vfr* transcription start site; the top strand of the DNA probe was radiolabeled on a single end. Prior to treatment with DNase I, the DNA probe was incubated in the absence (−) or presence of cAMP-Vfr (114, 13, or 1 nM). DNase I-generated fragments were separated by electrophoresis, and Maxam-Gilbert (A+G) sequencing ladders were made using the same DNA. The sequence of DNase I cleavage altered by cAMP-Vfr is indicated, the putative Vfr binding site is boxed, and the predicted half-sites are shown by vertical lines to the right of the sequence. Numbers indicate the distance (in bp) relative to the transcription start site, and asterisks indicate positions of enhanced DNase I cleavage. (C) *In vitro* transcription from P_{vfr} and P_{lasR}. Assay mixtures contained 10 nM *P. aeruginosa* σ^{70} RNAP and 2 nM plasmid DNA template containing bp −164 to +206 of P_{vfr} (relative to its transcription start site) or bp −264 to +30 of P_{lasR} (relative to the *lasR* T_1 transcription start site). Where indicated, 100 nM refolded apo-Vfr and/or 100 nM cAMP was present. The lengths in nucleotides of transcripts from the P_{vfr} promoter (~281 nt), the σ^{70}-dependent control transcript produced from the plasmid backbone (*; ~320 nt), and a cAMP-Vfr-dependent transcript produced from the plasmid backbone (#; ~115 nt) are indicated.

data, along with the EMSA and DNase I footprinting results, suggest that binding of cAMP-Vfr to a single site in the *vfr* promoter is sufficient to activate P_{vfr} *in vitro*.

***vfr* expression is autoregulated and cAMP dependent.** The finding that Vfr activates *vfr* transcription *in vitro* suggests that Vfr is subject to autoregulation *in vivo*. To test this hypothesis, β-galactosidase activity was measured in wild-type and *vfr* mutant strains from a *lacZ* transcriptional reporter carrying the same *vfr* promoter fragment used for the *in vitro* transcription assays (Fig. 6A). The reporter was introduced at the *P. aeruginosa* chromosomal φCTX phage attachment site (23). The wild-type strain displayed ~6-fold more reporter activity than the *vfr* mutant ($P < 0.0001$), indicating that Vfr positively regulates its own expression (Fig. 7A).

To address the *in vivo* role of cAMP in Vfr regulation, *vfr* promoter activity was measured in strains lacking one or both of the endogenous adenylate cyclases (CyaA and CyaB) (Fig. 7A). While β-galactosidase activity in the *cyaA* mutant was unaffected, activity in the *cyaB* mutant was reduced by more than 50% compared to the wild type ($P < 0.0001$). Furthermore, activity in the *cyaA cyaB* double mutant was significantly reduced ($P < 0.0001$) to a level similar to that observed for the *vfr* mutant. Reduction in *vfr* promoter activity corresponded to reduced Vfr protein levels, as determined by Western blot analysis (Fig. 7A, bottom panel). Thus, *vfr* expression is autoregulated and dependent on cAMP levels *in vivo*. Based on our EMSA studies, the simplest interpretation is that cAMP is required for Vfr DNA binding activity *in vivo*. Alternatively, cAMP binding may stabilize Vfr and protect it from degradation.

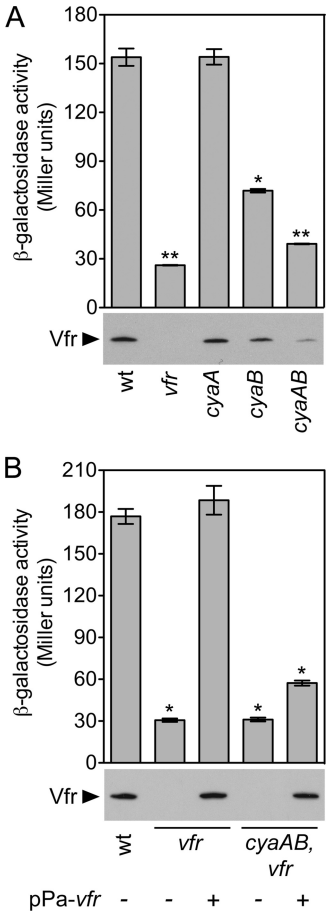

FIG. 7. *In vivo vfr* expression is autoregulated and cAMP dependent. (A) Activity of the *vfr* promoter as measured in a β-galactosidase assay in wild-type (wt), *vfr*, *cyaA*, *cyaB*, and *cyaA cyaB (cyaAB)* mutant strains containing the chromosomal P_{vfr}-*lacZ* reporter ($n = 4$; standard errors of the means are shown). Strains were grown in LB to an OD_{600} of 1. β-Galactosidase activity (in Miller units) for the *cyaB* mutant (*) and for the *cyaA cyaB* and *vfr* mutants (**) was significantly different ($P < 0.001$) compared pairwise to the activity for all other strains shown. (B) β-Galactosidase activity of P_{vfr}-*lacZ* in wt, the *vfr* mutant, and the *cyaA cyaB vfr* (*cyaAB vfr*) triple mutant strains containing empty vector or the same vector encoding Vfr (pPa-*vfr*) ($n \geq 4$; standard errrors of the means are shown). Cultures were grown in LB containing Cb (30 μg/ml) and IPTG (40 μM) to an OD_{600} of 1. β-Galactosidase activity for the indicated strains (*) was significantly different ($P < 0.0001$) than the activity for the wild-type strain and the complemented *vfr* mutant. The bottom portions of panels A and B show Western blot results with whole-cell lysates from the indicated strains probed with Vfr-specific antiserum. Lysates were normalized based on total cellular protein.

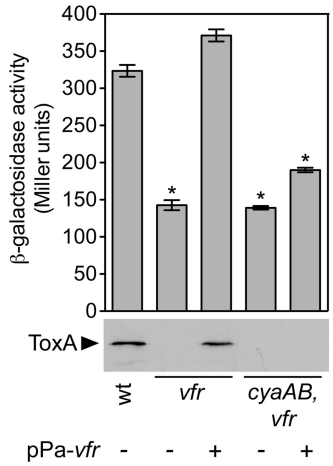

FIG. 8. Cyclic AMP is required for Vfr-dependent expression of *toxA* and secretion of its gene product ToxA. (Top) Activity of the *toxA* promoter as measured by a β-galactosidase assay in wild-type (wt), *vfr* mutant, and *cyaA cyaB vfr* triple mutant (*cyaAB vfr*) strains harboring the pR*toxA-lacZ* transcriptional reporter plasmid ($n = 6$; standard errors of the means are indicated). Strains also contained empty vector or the Vfr expression plasmid (pPa-*vfr*). Samples were taken from late-stationary-phase cultures grown in DTSB (low iron) containing 30 μg/ml Cb, 15 μg/ml Gm, and 40 μM IPTG. β-Galactosidase activities for the indicated strains (*) were significantly different ($P < 0.0001$) than the activity for the wild-type strain and the complemented *vfr* mutant. (Bottom) Trichloroacetic acid-precipitated supernatants from cultures grown as described above were resolved by SDS-PAGE and probed with ToxA-specific antiserum.

To distinguish whether cAMP affects Vfr transcriptional activity or Vfr protein stability, we compared *vfr* promoter activity when Vfr was ectopically expressed from an IPTG-inducible *tac* promoter to equivalent levels in the *vfr* mutant and in a triple mutant strain lacking both adenylate cyclases and chromosomal *vfr* (*cyaAB vfr*) (Fig. 7B). When expressed to levels similar to those observed in a wild-type strain (Fig. 7B, bottom panel), plasmid-encoded Vfr was sufficient to fully restore *vfr* promoter activity in the *vfr* mutant. In contrast, plasmid-encoded Vfr did not substantially increase *vfr* promoter activity in the *cyaAB vfr* triple mutant, demonstrating that intracellular cAMP is required for Vfr activity *in vivo*. Taken together, our results indicate that cAMP modulates the cellular levels of Vfr protein via Vfr autoregulation.

Cyclic AMP is necessary for Vfr-dependent expression of *toxA*. To examine the role of cAMP in regulating Vfr-dependent virulence factor expression, we evaluated the transcriptional activity of the *toxA* promoter. The regulation of *toxA* expression is complex and involves numerous regulators, including the products of the Vfr-dependent *regA* and *ptxR* genes (11, 14). In addition, our EMSA experiments and previous studies suggest that *toxA* expression is also directly regulated by Vfr (11, 15, 24, 55); however, the role of cAMP in *toxA* expression *in vivo* has not been tested. β-Galactosidase assays were used to measure *toxA* promoter activity in strains harboring a plasmid-borne *toxA* transcriptional reporter (pR*toxA-lacZ*). Consistent with published data (11), *toxA* promoter activity was significantly reduced ($P < 0.0001$) in the *vfr* mutant, and ectopic expression of Vfr in this strain background was sufficient to restore transcriptional activity to that of the wild-type strain (Fig. 8). Expression from the *toxA* promoter was also significantly reduced ($P < 0.0001$) in the *cyaAB vfr* triple mutant; however, complementation with plasmid-encoded Vfr was not sufficient to restore wild-type promoter activity. To determine whether *toxA* expression correlated with secretion of the *toxA* gene product, ToxA, we assessed protein levels in culture supernatants by Western blot analysis (Fig. 8, bottom panel). Consistent with previously published data (11, 16, 55), the *vfr* mutant did not secrete detectable levels of ToxA, and plasmid-encoded Vfr complemented the *vfr* mutant phenotype (Fig. 8, bottom panel). In contrast, ectopic expression of Vfr in

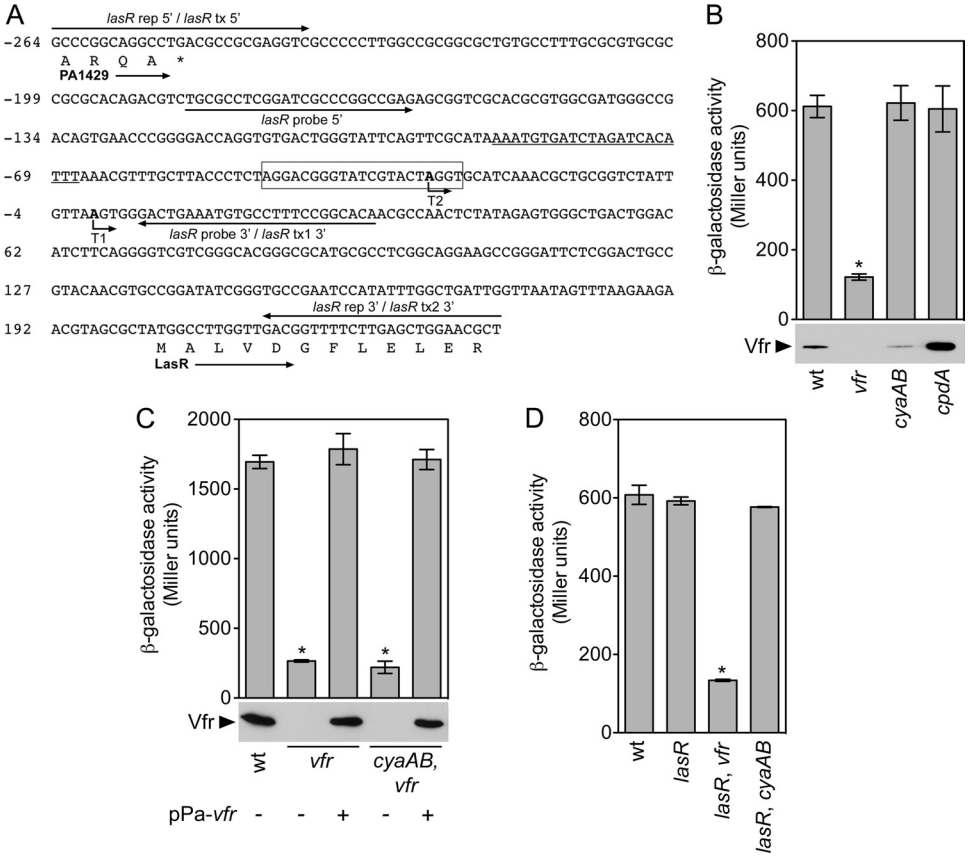

FIG. 9. *In vivo* expression of *lasR* is dependent on Vfr but not cAMP. (A) Diagram of the *lasR* promoter region used to generate the *lasR* promoter reporter (P_{lasR}-*lacZ*). Numbering (in bp) is relative to the *lasR* T_1 transcriptional start site (2). T_1 and T_2 transcriptional start sites are indicated in bold type and marked by the arrow, which shows the direction of transcription. Amino acid residues for the partial coding sequences of LasR and the upstream gene product PA1429 are shown. The Vfr binding site is underlined, and a putative *lux* box, associated with quorum-dependent gene regulation, is boxed (2). The locations of oligonucleotide sequences used to generate the P_{lasR} EMSA probe (*lasR* probe 5′/*lasR* probe 3′), the *in vivo* transcriptional reporter (*lasR* rep 5′/*lasR* rep 3′), and two different *in vitro* transcription templates (*lasR* tx 5′/*lasR* tx1 3′ and *lasR* tx 5′/*lasR* tx2 3′) are indicated by arrows. (B, top panel) β-Galactosidase activity (in Miller units) measured from the chromosomal P_{lasR}-*lacZ* reporter in wild-type (wt) and *vfr*, *cyaA cyaB* (*cyaAB*), and *cpdA* mutant strains (means ± standard error of the means; $n \geq 3$). Strains were grown in LB broth to an OD_{600} of 5. The asterisk signifies that β-galactosidase activity of the *vfr* mutant is significantly different ($P < 0.0001$) compared pairwise to activity for the other strains shown. (Bottom panel) Western blot of whole-cell lysates normalized for total protein and probed with Vfr-specific antiserum. (C, top panel) β-Galactosidase activity from the P_{lasR}-*lacZ* reporter in wt, *vfr* mutant, and *cyaA cyaB vfr* (*cyaAB vfr*) triple mutant strains containing empty vector or the same vector encoding Vfr (pPa-*vfr*). Cultures were grown in LB containing Cb (30 μg/ml) and IPTG (40 μM) to an OD_{600} of 5. Results are presented as means ± standard errors of the means ($n \geq 3$). The asterisks signify that the β-galactosidase activities of *vfr* and *cyaA cyaB vfr* mutant strains containing empty vector were significantly different ($P < 0.0001$) compared pairwise to activity for all other strains. (Bottom panel) Western blot of whole-cell lysates normalized for total protein and probed with Vfr-specific antiserum. (D) β-Galactosidase activity from the P_{lasR}-*lacZ* reporter in the wt strain and *lasR*, *lasR vfr* (*lasR vfr*), and *lasR cyaA cyaB* (*lasR cyaAB*) mutants (means ± standard errors of the means; $n \geq 3$). Strains were grown in LB to an OD_{600} of 5. The asterisk signifies that the β-galactosidase activity of the *lasR vfr* (*lasR vfr*) mutant is significantly different ($P < 0.0001$) compared pairwise to activities of other strains.

the *cyaAB vfr* triple mutant was not sufficient to restore ToxA secretion. Thus, like the *vfr* promoter, cAMP directly affects Vfr-dependent transcription of *toxA*.

Cyclic AMP is not required for Vfr-dependent expression of *lasR in vivo*. In contrast to the P_{vfr}, P_{toxA}, P_{ptxR}, P_{regA}, and P_{cpdA} probes, our EMSA studies suggest that both apo-Vfr and cAMP-Vfr can bind the P_{lasR} probe *in vitro*. To evaluate the role of cAMP in Vfr-dependent activation of *lasR in vivo*, we constructed a chromosomal transcriptional reporter in which the *lasR* promoter (bp −264 to +238 relative to the *lasR* T_1 transcriptional start site) (Fig. 9A) was fused to *lacZ*. β-Galactosidase activity from the *lasR* promoter reporter was then compared for the wild-type strain, the *vfr* mutant, and the double adenylate cyclase (*cyaAB*) mutant. As previously reported (2), there was a significant reduction ($P < 0.0001$) in reporter activity in the *vfr* mutant compared to the wild-type strain (Fig. 9B). In contrast, *lasR* promoter activity was unaffected in the *cyaAB* double mutant. These data indicate that *in vivo* expression of *lasR* does not require cAMP synthesis. We previously showed that deletion of the *P. aeruginosa* cAMP phosphodiesterase gene *cpdA* results in a 30-fold increase in intracellular cAMP and a 12-fold increase in *vfr* expression (16). To evaluate the effects of increased cAMP and Vfr protein levels on *lasR* expression, we measured *lasR* promoter reporter activity in an isogenic *cpdA* mutant. Like the *cyaAB* mutant, the *cpdA* mutant retained wild-type levels of reporter

activity (Fig. 9B). Thus, the results of our *in vitro* studies correlate with our *in vivo* finding that cAMP synthesis is not required for Vfr to bind the *lasR* promoter. Furthermore, our results indicate that while Vfr is required for *lasR* expression, promoter activity is unaffected over a wide range of Vfr protein levels (Fig. 9B, bottom panel) and cAMP concentrations. To further confirm these results, we demonstrated that plasmid-encoded Vfr is sufficient to restore *lasR* promoter activity in both a *vfr* mutant and a *cyaAB vfr* triple mutant (Fig. 9C).

Vfr is not sufficient to activate σ^{70}-dependent transcription from P_{lasR} *in vitro*. To determine whether binding of apo-Vfr or cAMP-Vfr is sufficient for activation of P_{lasR}, we conducted *in vitro* transcription assays in the absence and presence of refolded apo-Vfr and/or cAMP and using *P. aeruginosa* RNA polymerase, which was largely σ^{70} saturated, and a plasmid template carrying the *lasR* promoter region (bp −264 to +30 relative to the *lasR* T_1 transcriptional start site) (Fig. 9A). No *lasR*-specific transcripts (expected sizes of 102 and 132 nt) were detected under any of the reaction conditions used (Fig. 6C). However, both cAMP-Vfr-dependent and -independent control transcripts were produced from the vector backbone, indicating that the transcription reactions worked as expected. To rule out the possibility that *lasR* transcription is initiated at a site downstream of the T_1 and T_2 sites identified by S1 nuclease protection assays (2), we created a second *in vitro* transcription template carrying the same full-length promoter fragment (bp −264 to +238 relative to the T_1 transcriptional start site) as used in the *in vivo* promoter reporter assays (Fig. 9A). Again, both control transcripts were produced from the vector backbone, but *lasR*-specific transcripts were not detected (data not shown). Taken together, these results suggest that an additional factor(s) is required for Vfr-dependent activation of *lasR* or that an alternative σ factor (other than σ^{70}) is necessary for the recruitment of RNA polymerase to the *lasR* promoter.

Expression from the *lasR* promoter was previously reported to be cell density dependent, and a putative *lux* box associated with quorum-sensing-dependent gene expression was identified within the *lasR* promoter (2). Given the possibility that *lasR* may be autoregulated, we examined Vfr-dependent expression of the *lasR* promoter reporter in a *lasR* mutant (Fig. 9D). β-Galactosidase activity in the *lasR* mutant was unaffected compared to the wild-type strain. Furthermore, reporter activity in a *lasR vfr* double mutant was indistinguishable from the *vfr* mutant (Fig. 9A and D). To determine if LasR facilitates Vfr-dependent expression from the *lasR* promoter *in vivo* in the absence of cAMP, we assessed promoter reporter activity in a *lasR cyaA cyaB* triple mutant. Again, *lasR* deletion did not affect promoter reporter activity, indicating that a factor other than LasR is likely to contribute to Vfr-dependent transcription from the *lasR* promoter.

DISCUSSION

In this study, we demonstrate that cAMP acts as a positive regulator of Vfr by promoting Vfr DNA binding to multiple virulence gene promoters. Although we demonstrated that the cyclic nucleotide specificity of Vfr is similar to that of *E. coli* CRP, it is not clear whether the changes in protein structure that occur in CRP in response to cAMP binding also occur in Vfr. In CRP, binding of cAMP causes a series of transitions in the protein structure that ultimately contribute to a repositioning of the DNA binding domain to an orientation compatible for specific interactions with DNA (34–36, 43). Although Vfr and CRP are highly homologous (67% identical and 91% similar) (55), it is possible that particular residue differences, such as those within the Vfr nucleotide binding pocket (4, 55), induce conformational effects that are different than those occurring in CRP, resulting in unique interactions between Vfr and RNAP and/or target promoter DNA. Ultimately, structural studies comparing cAMP-Vfr and apo-Vfr are needed to determine the nature of the allosteric change induced by cAMP and may provide insight as to why CRP cannot functionally substitute for Vfr (48, 55). Further, we cannot formally rule out the possibility that Vfr is controlled by an effector that does not regulate CRP and is different from the cyclic nucleotides tested in this study.

We observed that Vfr-DNA binding activity was inhibited by cGMP, which is a property that is also exhibited by CRP (3, 13, 49). However, the relevance of Vfr regulation by cGMP *in vivo* is questionable, since inhibition of Vfr-DNA binding activity required a 2,500-fold excess of cGMP when cAMP levels were within a biologically relevant range (16, 17). Overall, there have been few reports of the presence of cGMP in bacteria, and thus a physiological role of this nucleotide in prokaryotes has yet to be defined (29). The *P. aeruginosa* genome encodes a single enzyme (CyaB) with homology to guanylate cyclase, but we have shown that CyaB has adenylate cyclase activity and possesses critical substrate-determining residues consistent with ATP being the preferred substrate (17, 30). Although the *P. aeruginosa* cyclic nucleotide phosphodiesterase CpdA was shown to exhibit cGMP phosphodiesterase activity *in vitro*, intracellular cGMP levels remained below the limit of detection (≤0.01 μM) in mutants lacking *cpdA* (16). Taken together, these findings suggest that cGMP is unlikely to play a biological role in Vfr control.

A potential control point in regulating the activity of the cAMP/Vfr signaling pathway is at the level of *vfr* expression. We have provided both *in vivo* and *in vitro* evidence that Vfr regulates transcription from its own promoter and that *vfr* expression is cAMP dependent. Our data demonstrate that Vfr is a positive regulatory factor with respect to its own transcription. In contrast, the *E. coli crp* promoter is negatively autoregulated by CRP, except at high concentrations of cAMP, where positive autoregulation has been reported (1, 8, 21). The different modes of autoregulation displayed by the *crp* and *vfr* promoters are presumably due to their different promoter architectures and reflect their specific regulatory roles in *E. coli* and *P. aeruginosa* (catabolite repression versus virulence factor expression, respectively). Our DNase I footprinting result indicates that cAMP-Vfr binds to a single unique site centered approximately 58 bp upstream of the *vfr* transcriptional start site. This spacing is similar to that of the secondary CRP binding site (CRPII) responsible for positive *crp* autoregulation (21), raising the possibility that transcriptional activation by Vfr and CRP at these sites occurs by a similar mechanism. In addition, the fact that Vfr binds a single unique region within the *vfr* promoter *in vitro* was surprising, given how closely the two predicted Vfr binding sites matched the consensus sequence (24); this result suggests that some Vfr bind-

ing sites may be difficult to predict based on bioinformatic data alone.

Our observation that cAMP was not required for Vfr binding to the *lasR* promoter *in vitro* or for *lasR* promoter activity *in vivo* provides an exception to our overall finding that expression of multiple virulence genes requires cAMP for Vfr activation. While we cannot formally rule out the possibility that *P. aeruginosa* produces low levels of cAMP by some other mechanism, intracellular levels of cAMP are greatly reduced in a *cyaAB* mutant compared to a wild-type strain (16, 17, 58). Neither apo-Vfr nor cAMP-Vfr was sufficient to activate transcription from the *lasR* promoter *in vitro*, suggesting that an additional factor(s) is required for Vfr-dependent expression of *lasR*, such as an additional transcriptional regulator or an alternative sigma factor (different from σ^{70}). We ruled out the possibility that LasR itself regulates *lasR* and/or promotes Vfr-dependent activation, as we observed no effect on *lasR* reporter activity in strains lacking *lasR*. An alternative candidate is GacA, the transcriptional regulator of the GacS/GacA signaling cascade involved in extracellular polysaccharide synthesis and biofilm formation (19, 53). GacA was shown to regulate *lasR* expression *in vivo* by an unknown mechanism (37), and further experiments are needed to establish whether this regulator and/or other downstream factors are critical for regulation of *lasR* by apo-Vfr.

Although the mechanism for apo-Vfr activation of *lasR* remains to be determined, our findings may account for the previously reported phenotypes of a Vfr mutant in which 5 amino acid residues from the putative cAMP binding domain were deleted ($Vfr_{\Delta EQERS}$) (4). Beatson et al. showed that plasmid-based expression of $Vfr_{\Delta EQERS}$ in a *vfr* mutant restored quorum-sensing-dependent production of elastase but not Tfp-dependent twitching motility. To explain this phenomenon, those authors proposed that $Vfr_{\Delta EQERS}$ might be responding to cGMP or another effector to differentially regulate gene expression. Taking into account the findings presented in our study, it is more likely that $Vfr_{\Delta EQERS}$, like apo-Vfr, activates *lasR* expression, which in turn would support the subsequent expression of downstream quorum-sensing factors like elastase. Nevertheless, the results from both studies still raise the question as to why the *las* quorum-sensing system is dependent on Vfr but not cAMP. It is possible that the simultaneous activation of the cAMP-dependent and quorum-sensing-dependent regulons is counterproductive under conditions that alter intracellular cAMP levels.

Since Vfr activity is ultimately dependent upon the cellular concentration of cAMP (except in the case of *lasR* regulation), understanding the mechanisms by which cAMP levels are controlled is critical to expanding our knowledge of virulence factor regulation in *P. aeruginosa*. Currently, the environmental signals that trigger *P. aeruginosa* to upregulate or downregulate cAMP/Vfr-dependent virulence factor production are not known. Given that multiple cAMP-dependent virulence factors are required for acute *P. aeruginosa* infection and that acute virulence factor inhibition is associated with chronic *P. aeruginosa* infection, we hypothesize that bacterial cAMP synthesis is stimulated upon encountering the host environment but then is subject to downregulation upon onset of the chronic state. We predict that intracellular cAMP fluctuations occur in response to spatio-temporal host signals that are unique to the environments encountered during acute and chronic infection. Further, cAMP downregulation may occur prior to (or in addition to) the well-documented genetic inactivation of virulence factors and their regulators (such as Vfr) that occurs among chronic infection isolates (46). In terms of cAMP activation, we have shown that the Chp chemotaxis-like chemosensory signal transduction system controls cAMP levels via modulation of CyaB adenylate cyclase activity (17). Once cAMP is synthesized, the results of the present study suggest that Vfr regulates virulence promoters in either a cAMP-dependent (*ptxR*, *regA*, and *toxA*) or cAMP-independent (*lasR*) manner. The cAMP-dependent branch of the pathway, in combination with *vfr* autoregulation, may serve as a signal amplification loop, whereby subtle changes in intracellular cAMP can have large effects on gene expression. The cAMP-independent branch may allow differential regulation of Vfr-dependent (but not cAMP-dependent) systems, such as quorum sensing. Ultimately, the cAMP signaling cascade is predicted to be reset following CyaB activation by the cAMP phosphodiesterase CpdA. Further investigation is needed to define environmental cues responsible for activating cAMP/Vfr signaling and the mechanisms by which these signals are integrated into the pathway in an effort to understand the specific impacts on virulence regulation in different phases of *P. aeruginosa* infection.

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