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The *Pseudomonas aeruginosa* Chp Chemosensory System Regulates Intracellular cAMP Levels by Modulating Adenylate Cyclase Activity

Nanette B. Fulcher[1], **Phillip M. Holliday**[2], **Erich Klem**[1], **Martin J. Cann**[2], and **Matthew C. Wolfgang**[1,3,*]

[1]Cystic Fibrosis/Pulmonary Research and Treatment Center, University of North Carolina, Chapel Hill, NC 27599

[2]School of Biological and Biomedical Sciences, Durham University, South Road, Durham DH1 3LE, United Kingdom

[3]Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, NC 27599

Summary

Multiple virulence systems in the opportunistic pathogen *Pseudomonas aeruginosa* are regulated by the second messenger signaling molecule adenosine 3', 5'-cyclic monophosphate (cAMP). Production of cAMP by the putative adenylate cyclase enzyme CyaB represents a critical control point for virulence gene regulation. To identify regulators of CyaB, we screened a transposon insertion library for mutants with reduced intracellular cAMP. The majority of insertions resulting in reduced cAMP mapped to the Chp gene cluster encoding a putative chemotaxis-like chemosensory system. Further genetic analysis of the Chp system revealed that it has both positive and negative effects on intracellular cAMP and that it regulates cAMP levels by modulating CyaB activity. The Chp system was previously implicated in the production and function of type IV pili (TFP). Given that cAMP and the cAMP-dependent transcriptional regulator Vfr control TFP biogenesis gene expression, we explored the relationship between cAMP, the Chp system and TFP regulation. We discovered that the Chp system controls TFP production through modulation of cAMP while control of TFP-dependent twitching motility is cAMP-independent. Overall, our data define a novel function for a chemotaxis-like system in controlling cAMP production and establish a regulatory link between the Chp system, TFP and other cAMP-dependent virulence systems.

Keywords

Pseudomonas aeruginosa; chemosensory system; cAMP; adenylate cyclase; Vfr

Introduction

Pseudomonas aeruginosa is an opportunistic bacterial pathogen responsible for a wide array of human diseases including ulcerative keratitis, soft tissue infection, otitis, urinary tract infection, pneumonia, bacteremia and endocarditis (Driscoll *et al.*, 2007). Infection by *P. aeruginosa* is typically associated with disruption of physical barrier function (burn injury, wounds and medical devices) and defective immune defense mechanisms

*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 matthew_wolfgang@med.unc.edu.

(immunosuppression and acquired immunodeficiency syndrome). Consequently, *P. aeruginosa* is a leading cause of gram-negative hospital-acquired infection (Ruiz *et al.*, 1999; Arancibia *et al.*, 2002). In addition infection with *P. aeruginosa* is the primary cause of morbidity and mortality in individuals with cystic fibrosis where it causes chronic lung infection (Brennan and Geddes, 2002).

Many virulence factors associated with *P. aeruginosa* infection are regulated by the small molecule second messenger adenosine 3', 5'-cyclic monophosphate (cAMP or cyclic AMP) (Wolfgang *et al.*, 2003). In *P. aeruginosa*, cAMP is believed to control gene expression through allosteric regulation of the transcription factor Vfr (Virulence factor regulator), which is a member of the cAMP receptor protein (CRP) family (West *et al.*, 1994). Cyclic AMP and Vfr appear to be the central components controlling a global virulence gene response in *P. aeruginosa* through regulation of multiple virulence systems including type IV pili (TFP) (Beatson *et al.*, 2002; Wolfgang *et al.*, 2003), the type II secretion (T2S) system and secreted toxins (West *et al.*, 1994; Beatson *et al.*, 2002; Wolfgang *et al.*, 2003; Ferrell *et al.*, 2008), type III secretion (T3S) (Wolfgang *et al.*, 2003), quorum sensing (QS) (Albus *et al.*, 1997) and flagellar biogenesis (Dasgupta *et al.*, 2002). Proteome and transcriptome analyses of Vfr-deficient mutants (Suh *et al.*, 2002; Wolfgang *et al.*, 2003) indicate the extent and complexity of the Vfr regulon and also suggest that there may be more systems under Vfr control than are currently recognized.

Cyclic AMP is produced by the enzyme adenylate cyclase (AC) and *P. aeruginosa* encodes two putative intracellular ACs, CyaA and CyaB (Wolfgang *et al.*, 2003). *P. aeruginosa* mutants lacking either *cyaA* or *cyaB* have reduced intracellular cAMP; however, the contribution of CyaB is substantially greater than that of CyaA (Wolfgang *et al.*, 2003). Furthermore, previous studies indicate that CyaB is the primary source of cAMP associated with *P. aeruginosa* virulence (Wolfgang *et al.*, 2003; Smith *et al.*, 2004). CyaB is predicted to be a member of the ubiquitous Class III family of ACs widely distributed in both eukaryotes and prokaryotes (Baker and Kelly, 2004). CyaB consists of an amino (N)-terminal MASE2 domain (membrane associated sensor 2) and a carboxy (C)-terminal AC catalytic domain (Nikolskaya *et al.*, 2003). The MASE2 domain is predicted to function as a membrane anchor and regulator of CyaB catalytic domain activity (Nikolskaya *et al.*, 2003).

Although functional regulation of CyaB has not been demonstrated, we predict that control of cAMP synthesis by CyaB may be an important step in coordinating virulence gene expression. As a first step in our investigation of cAMP regulation, we provide definitive biochemical evidence that *P. aeruginosa* CyaB is capable of synthesizing cAMP *in vitro*. We next performed a genetic screen for regulators of intracellular cAMP and identified genes encoding multiple components of the Chp chemotaxis-like chemosensory system. The Chp system was previously shown to control TFP production and twitching motility (Darzins and Russell, 1997; Whitchurch *et al.*, 2004), a form of surface-associated bacterial movement mediated by the extension and retraction of TFP fibers (Skerker and Berg, 2001). In addition, the Chp system appears to play a critical role in *P. aeruginosa* virulence that cannot be accounted for by its regulation of TFP alone (D'Argenio *et al.*, 2001). Given that cAMP and the cAMP-dependent transcriptional regulator Vfr have been implicated in TFP regulation (Beatson *et al.*, 2002; Wolfgang *et al.*, 2003), we examined the regulatory link between the Chp system, cAMP production, TFP biogenesis and twitching motility. We demonstrate that the Chp system controls intracellular cAMP levels by modulating CyaB activity and that the regulated production of TFP is cAMP-dependent, thereby providing a mechanistic link between the Chp system and TFP biogenesis. In addition, we provide evidence that the Chp system controls TFP function (twitching motility) through a cAMP-independent mechanism. Our finding that the Chp chemosensory system controls cAMP

production suggests that this signaling system also plays a more global regulatory role in *P. aeruginosa* virulence via control of the cAMP/Vfr-dependent virulence regulon.

Results

Biochemical characterization of the AC activity of *P. aeruginosa* CyaB

Inactivation of *cyaB* affects the intracellular cAMP pool in *P. aeruginosa* (Wolfgang *et al.*, 2003), but there is no direct evidence that CyaB is an AC. The predicted catalytic domain of CyaB is located in the C-terminal region of the protein, while the N-terminus encodes a putative membrane-anchoring domain (Nikolskaya *et al.*, 2003). To characterize the AC activity of CyaB, we cloned and expressed the putative catalytic domain (amino acids 217–463) as an N-terminal hexahistidine fusion. The recombinant protein ($CyaB_{217–463}$) was purified (>99% homogeneity) under native conditions (Fig. 1A) and subjected to biochemical analysis. Purified $CyaB_{217–463}$ displayed AC activity in the presence of either Mn^{2+} or Mg^{2+} as the divalent metal co-factor (Fig. 1B). Enzyme parameters were determined in the presence of Mn^{2+}-ATP due to the higher specific activity obtained versus Mg^{2+}-ATP. $CyaB_{217–463}$ displayed pH and temperature optima of 8.5 and 40°C, respectively. The enthalpy of activation (E_A) derived from the linear arm of an Arrhenius plot was 53.5±0.4 kJ mol^{-1}. Kinetic parameters were determined using 250 nM enzyme at pH 7.5 and gave a K_m for ATP of 66.7±4.8 μM and a V_{max} of 230±4.6 nmol cAMP mg^{-1} min^{-1}. A Hill coefficient of 1.01±0.03 indicated non-cooperative binding of ATP at the two predicted active sites (Linder and Schultz, 2003). All values are consistent with previously characterized bacterial and mammalian Class III ACs (Cann *et al.*, 2003; Litvin *et al.*, 2003; Hammer *et al.*, 2006). Analysis of the amino acid sequence of the presumed active site revealed that CyaB most likely belongs to sub-Class IIIb (Linder and Schultz, 2003) and, consistent with this prediction, alanine substitution of substrate specificity-determining residues (K274 and T351) significantly ($p<0.0001$) reduced AC activity (Fig. 1C). These results provide definitive evidence that *P. aeruginosa* CyaB is an AC.

Construction of a quantitative cAMP reporter system

The activity of AC enzymes is typically regulated by cellular factors in response to intracellular and extracellular signals. However, there is currently no information regarding the signals or mechanisms that control activity of the major *P. aeruginosa* AC CyaB. To facilitate high-throughput screens for identifying candidate regulators of cAMP synthesis, we constructed a transcriptional reporter system for monitoring intracellular cAMP levels based on the *E. coli lac*P1 promoter. In *E. coli*, CRP activates transcription of *lacZ* in a cAMP-dependent manner by binding a CRP consensus sequence in the P1 promoter region of the *lac* operon (Ebright *et al.*, 1984). *P. aeruginosa* Vfr was shown to interact with conserved nucleotides in the *lac*P1 promoter and restores cAMP-dependent *lacZ* transcription when expressed in an *E. coli crp* mutant (West *et al.*, 1994; Suh *et al.*, 2002; Kanack *et al.*, 2006). Therefore we predicted that activity of the *lac*P1 promoter would be cAMP- and Vfr-dependent in *P. aeruginosa*. We created a transcriptional reporter by fusing the *E. coli lac*P1 promoter to the *lacZ* gene and then introduced the construct into the vacant ϕCTX phage attachment site on the chromosome of *P. aeruginosa* strain PAK (Fig. 2A). To test whether *lac*P1-*lacZ* transcription was sensitive to changes in intracellular cAMP, we assessed reporter activity in a set of isogenic mutant strains with an inactivated copy of *cyaA*, *cyaB* or both AC genes (*cyaAB*). The relative pattern of β-galactosidase activity in the AC mutants accurately reflected their respective intracellular cAMP levels as determined by direct measurement (Fig. 2B). We next compared intracellular cAMP and reporter activity in a *vfr* mutant. Reporter activity was eliminated in the *vfr* mutant, indicating that the *lac*P1 promoter is Vfr-dependent in *P. aeruginosa*. In contrast, the *vfr* mutant showed an approximately 50% reduction in intracellular cAMP compared to the wild type strain. This

reduction suggests that Vfr may regulate expression of one or both AC genes or other factors that affect AC activity. The role of Vfr in regulating intracellular cAMP will be explored elsewhere. The correlation between *lac*P1-*lacZ* reporter activity and cAMP levels indicates that transcription from the *E. coli lac*P1 promoter can be used as a surrogate readout for intracellular cAMP production in *P. aeruginosa*.

Identification of *P. aeruginosa* genes encoding candidate CyaB regulators

To identify potential regulators of CyaB function we performed a transposon (Tn) mutagenesis screen in strain PAK carrying the cAMP-dependent transcriptional reporter system described above. The screen was carried out in a *cyaA* mutant strain such that CyaB was the only source of intracellular cAMP. We generated a library of approximately 60,000 random Tn insertion mutants and identified 125 candidates with reduced reporter activity by plating on LB agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Because the genome sequence of strain PAK is not available, the site of Tn insertion was determined for 121 of the mutants by comparing DNA sequence derived from each of the Tn-chromosomal junctions to the genome sequence of strain PAO1 (Stover *et al.*, 2000; Winsor *et al.*, 2009) (Table 1 and Fig. S1). Multiple Tn insertions occurred in *cyaB* and *vfr*, thus demonstrating that the screen produced mutants with the desired phenotype. The majority of the novel target genes identified in the Tn screen were previously implicated in the regulation of TFP biogenesis and/or twitching motility (Table 1). Both TFP production and twitching motility were previously shown to be Vfr-dependent (Beatson *et al.*, 2002; Wolfgang *et al.*, 2003). Multiple Tn insertions occurred in the *pilGHIJKchpABC* (Chp) gene cluster (Fig. S1), which encodes a chemotaxis-like chemosensory signal transduction system (Fig. S2)(Darzins and Russell, 1997; Whitchurch *et al.*, 2004) predicted to regulate TFP. There were Tn insertions in the *pilA* and *pilB* genes, which encode pilin, the major structural subunit of TFP, and PilB, an ATPase required for TFP assembly, respectively (Strom and Lory, 1986; Turner *et al.*, 1993). We also recovered mutants with Tn insertions in *fimL* and *fimV*, the gene products of which are required for wild type levels of twitching motility and TFP production (Semmler *et al.*, 2000; Whitchurch *et al.*, 2005). FimL is homologous to the N-terminus of the Chp system protein ChpA while FimV has no known homolog. The only targets identified in the screen not associated with TFP production or function were PA1821, *grpE* and *mexF*. PA1821 is immediately upstream of *fimL* (Fig. S1) and is predicted to encode an enoyl-CoA hydratase/isomerase. The *grpE* and *mexF* genes encode a cytoplasmic heat shock protein and an inner membrane component of a multidrug efflux pump, respectively.

To validate the Tn screen, we performed β-galactosidase assays to quantify reporter activity in a representative Tn insertion mutant for each candidate regulator (Fig. 3). All of the Tn insertion mutants displayed reduced reporter activity, with the majority having activity equivalent to that of the *cyaAB* mutant. As a control, we showed that a randomly selected Tn mutant (*pirA*::Tn) that formed a blue colony on the primary selection plates had activity equivalent to that of the parent (*cyaA*) strain.

Because the *lac*P1-*lacZ* reporter is Vfr-dependent, the regulatory effect of cAMP and Vfr cannot be distinguished. However, we discovered that the addition of cAMP to the growth media was sufficient to rescue reporter activity in an AC mutant (*cyaAB*) but not in a *vfr* mutant (Fig. 2C). This result indicates that *P. aeruginosa*, like other gram-negative bacteria, can transport extracellular cAMP (Saier *et al.*, 1975), albeit by an unknown mechanism. To determine if reduced reporter activity in the Tn mutants was due to reduced intracellular cAMP, we tested whether the phenotype could be complemented by exogenous cAMP. With the exception of the Tn insertions in *vfr*, reporter activity was restored in all of the Tn mutants in the presence of exogenous cAMP (data not shown), indicating that the defect in each of the mutants specifically affects intracellular cAMP.

Multiple Chp chemosensing system and TFP regulatory genes affect intracellular cAMP

Given the established connection between the cAMP-dependent transcriptional regulator Vfr and TFP (Beatson *et al.*, 2002; Wolfgang *et al.*, 2003), we focused on the genes previously implicated in regulating TFP production and/or twitching motility for their role in cAMP regulation. To confirm that the targets identified in our screen affect intracellular cAMP, we constructed individual non-polar deletion mutants for the genes encoding PilA, PilB, FimL, FimV, PilG (CheY), PilI (CheW), PilJ (MCP) and ChpA (CheA). For the Chp chemosensory system components (Fig. S2) the *E. coli* Che system homolog is indicated in parentheses. In addition, we individually deleted the remaining Chp system genes encoding PilH (CheY), PilK (CheR), ChpB (CheB) and ChpC (CheW) to assess their contribution to intracellular cAMP levels. To facilitate complementation of the deletion mutants using a LacI-regulated plasmid-based gene expression system, it was necessary to modify the *lac*P1-*lacZ* cAMP reporter, which is subject to LacI repression. We eliminated the *lac*I binding sites within the *lac*P1 promoter and demonstrated that the resulting construct (*lac*P1Δ*lacI-lacZ*) is not responsive to LacI but is still cAMP-dependent (Fig. S3). Each of the non-polar deletion mutations was introduced into a *cyaA* mutant strain carrying a chromosomal copy of the *lac*P1Δ*lacI-lacZ* reporter. We determined the level of reporter activity and the level of intracellular cAMP in each mutant strain (Fig. 4A). In general, reporter activity mirrored direct cAMP measurements, with the exception of the *pilH* mutant, where reporter activity is likely to be at its maximum. A subset of mutants (*pilG*, *pilI*, *pilJ*, *chpA*, *fimL* and *fimV*) had cAMP levels equivalent to that of the adenylate cyclase (*cyaAB*) double mutant while the *pilH* and *pilK* mutants had higher cAMP levels than the parent (*cyaA*) strain. Intracellular cAMP levels in the *pilA*, *chpB* and *chpC* mutants were not statistically different relative to the parent strain, but their values for the reporter assay were. The values for the *pilB* mutant were not different than that of the parent strain in either assay. Together, these results indicate that *fimL*, *fimV*, *pilA* and the members of the Chp gene cluster (*pilGHIJKchpABC*) all affect intracellular cAMP and suggest that the individual components exert either a positive (*pilG*, *pilI*, *pilJ*, *chpA*, *chpC*, *fimL* and *fimV*) or negative (*pilH*, *pilK* and *chpB*) regulatory effect on intracellular cAMP.

To confirm the role of the identified TFP/Chp genes in regulating cAMP, we determined whether the cAMP phenotype of the mutants could be complemented *in trans*. For the mutants with altered intracellular cAMP, we created expression plasmids by cloning the coding region of each gene into multiple derivatives of the *P. aeruginosa* expression vector pMMB67EH containing different versions of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *tac* promoter (see experimental procedures). We transferred the plasmids to their respective mutant strains in the PAK*cyaA*::*lac*P1Δ*lacI-lacZ* background and determined the optimal induction conditions for each gene by assessing β-galactosidase activity. For each of the mutants, we established an expression level at which the corresponding gene either restored cAMP reporter activity (*pilA*, *fimL*, *fimV*, *pilG*, *pilI*, *pilJ*, *chpA* and *chpC*) or reduced elevated cAMP reporter activity to near wild type levels (*pilH* and *chpB*) (Fig. 4B). We were unable to complement the *pilK* mutant at any of the expression levels tested (data not shown). To investigate the possibility that deletion of *pilK* coding region affected distal gene expression, we made an alternative version of the *pilK* mutant by ablating the predicted translational start site. Both versions of the *pilK* mutant displayed equivalent levels of reporter activity, but plasmid expression of *pilK* failed to complement either of the mutants. Therefore we could not confirm the cAMP phenotype of the *pilK* mutant. Although the role of *pilK* in regulating cAMP remains inconclusive, the fact that mutants lacking the other TFP regulatory genes identified in our screen had altered cAMP and could be complemented *in trans* supports the conclusion that these genes contribute to the regulation of intracellular cAMP in *P. aeruginosa*.

The Chp chemotaxis-like system regulates intracellular cAMP levels by modulating CyaB activity

The candidate CyaB regulators affect intracellular cAMP, but the mechanism of regulation is not known. We hypothesized that these regulators could affect the intracellular cAMP pool via cAMP synthesis at the level of *cyaB* expression or CyaB activity or through altered cAMP degradation. *P. aeruginosa* possesses cAMP phosphodiesterase activity, which results in cAMP hydrolysis (Siegel *et al.*, 1977), but this activity has not been genetically defined. To address cAMP regulation by the Chp system, we first evaluated the amount of CyaB protein produced in each of the deletion mutants by immunoblotting with CyaB-specific antibody (Fig. 5). Despite the fact that the mutants had altered intracellular cAMP (Fig. 4A), the level of CyaB produced was equivalent to that of the parent strain (*cyaA*). This result indicates that the amount of CyaB enzyme does not account for altered levels of cAMP in the TFP/Chp system mutants.

We further examined the regulatory effect of the Chp system on cAMP by evaluating the effect of PilG and PilH on cAMP pools. PilG and PilH, which are both CheY homologs (Fig. S2)(Darzins, 1993;Darzins, 1994), have opposite effects on intracellular cAMP accumulation (Fig. 4A). We tested the ability of different cAMP sources to restore wild type levels of cAMP reporter activity in strains lacking AC activity in addition to either *pilG* or *pilH* (Fig. 6A). Because of the potential regulatory effect of PilG and PilH on CyaB, we used plasmid-based overexpression of *P. aeruginosa cyaA* (pMMBV2-*cyaA*) and addition of exogenous cAMP as alternative means to restore intracellular cAMP. Both alternative sources of cAMP restored reporter activity in the *cyaAB*, *cyaABpilG* and *cyaABpilH* mutants to equivalent levels. The fact that the level of *cyaA* expression or exogenous cAMP that rescued the *cyaAB* mutant phenotype also complemented the triple mutants indicates that PilG and PilH do not affect intracellular cAMP levels via cAMP degradation. Plasmid-based *cyaB* expression restored reporter activity in the *cyaAB* mutant, but failed to restore reporter activity in the *cyaABpilG* mutant and resulted in higher reporter activity in the *cyaABpilH* mutant, suggesting that the differential effects of PilG and PilH on intracellular cAMP are specific for CyaB. There were equivalent levels of CyaB protein in each of the *cyaB*-expressing strains (Fig. 6B), eliminating the possibility that different amounts of the enzyme accounted for different levels of reporter activity. Taken together, these data support a model in which PilG and PilH affect CyaB activity but have specific and opposite effects.

The Chp chemotaxis-like system controls TFP production but not twitching motility via regulation of intracellular cAMP

Based on the previous findings that Vfr affects TFP production and twitching motility, and that cAMP and Vfr are required for the expression of multiple TFP biogenesis genes (Beatson *et al.*, 2002; Wolfgang *et al.*, 2003), we hypothesized that the TFP regulators identified by our screen may affect TFP production and twitching motility through their control of cAMP. To test this hypothesis, we first established whether each of the mutants has a TFP production/function defect corresponding to its respective level of intracellular cAMP. The available information regarding the TFP phenotypes of the *fimL*, *fimV* and Chp locus mutants, however, comes from multiple studies using different *P. aerguinosa* strains and different methodologies (Darzins and Russell, 1997; Semmler *et al.*, 2000; Whitchurch *et al.*, 2004; Whitchurch *et al.*, 2005). Therefore, we systematically evaluated the TFP phenotypes of the non-polar isogenic mutants in strain PAK. As an indicator of TFP production, we examined the amount of pilin, the major subunit of TFP, that could be recovered from the bacterial surface (Fig. 7A). To determine the sensitivity of our assay and to estimate relative TFP levels, we compared serial dilutions of the pilin-containing surface fractions recovered from the mutant and parental (*cyaA*) strains (Fig. S4). The level of surface pilin recovered from the *pilH* and *chpB* mutants was approximately 4-fold and 2-fold

greater than that of the parent strain, respectively. The level of surface pilin recovered from the *pilK* mutant was elevated but less than 2-fold greater than that of the parent strain. In contrast, the parent strain produced approximately 2-fold more surface pilin than the *chpC* mutant and 50-fold more surface pilin than the *cyaAB* and *fimL* mutants. For the remainder of the mutants (*fimV*, *pilG*, *pilI*, *pilJ* and *chpA*) surface pilin levels were below the limit of detection of our assay (<1% of the parental strain), a phenotype similar to that of the *pilA* mutant, which lacks pilin and serves as a negative control. With the exception of the *pilA* mutant, all of the mutants produced intracellular pilin as assessed by immunoblot of whole bacterial cell lysates (Fig. 7B). This result indicates that altered pilin synthesis does not account for the differences in TFP production in the affected mutants and suggests that the corresponding defects are at the level of TFP assembly. In addition, altered TFP production in the *pilA*, *pilG*, *pilH*, *pilI*, *pilJ*, *chpA*, *chpB*, *chpC*, *fimL* and *fimV* mutants could be complemented by plasmid expression of the corresponding gene (data not shown). Although slightly more surface pilin was recovered from the *cyaAB* and *fimL* mutants than any of the Chp mutants lacking cAMP (*pilG*, *pilI*, *pilJ* and *chpA*), the overall trend was that mutants with reduced cAMP had a corresponding reduction in surface TFP production while mutants with increased cAMP (*pilH*, *pilK* and *chpB*) had greater levels of surface pilin (Fig. 4A and 7A).

We also tested TFP-dependent twitching motility as an indicator of pilus function by measuring the extent of subsurface colony expansion (Fig. 7C). The *cyaAB*, *pilK*, *chpB*, *chpC* and *fimL* mutants produced twitching motility zones similar to that of the parent strain. However, in contrast to the *pilK*, *chpB* and *chpC* mutants, the *cyaAB* and *fimL* mutants had reduced cAMP and TFP (Fig. 4A and 7A). The *pilG*, *pilH*, *pilI*, *pilJ*, *chpA* and *fimV* mutants had a severe twitching motility defect; however, only the *pilG*, *pilI*, *pilJ*, *chpA* and *fimV* mutants had a corresponding defect in cAMP production and surface TFP, whereas the *pilH* mutant produced elevated levels of cAMP and TFP. For the mutants with altered twitching motility, we were able to complement the defect by plasmid-based expression of the corresponding gene (data not shown). The fact that most of the cAMP-lacking TFP/Chp mutants (*pilG*, *pilI*, *pilJ*, *chpA* and *fimV*) had a more severe twitching motility defect than the double AC (*cyaAB*) mutant and that the *pilH* mutant was defective for twitching motility despite having elevated cAMP (Fig. 4A and 7C) indicates that cAMP levels do not correlate with twitching motility. Overall, these results suggest that the TFP/Chp system may control TFP function in a cAMP-independent manner.

To further explore the hypothesis that the Chp system controls TFP biogenesis but not twitching motility via cAMP, we determined whether restoration of wild type levels of intracellular cAMP can complement the altered TFP phenotypes associated with the *pilG* and *pilH* Chp system mutants. As a first step, we determined that inclusion of 20 mM cAMP in LB agar restored TFP production (Fig. 8A), twitching motility (Fig. 8C) and cAMP-dependent reporter activity (data not shown) to wild type levels in the *cyaAB* mutant. We then assessed TFP production and twitching motility in the *cyaABpilG* and *cyaABpilH* triple mutant strains in the absence or presence of 20 mM cAMP (Fig. 8). In the absence of exogenous cAMP, the *cyaABpilG* mutant was similar to its *cyaApilG* parent strain in that it lacked TFP and was defective for twitching motility. Complementation of the *cyaABpilG* mutant with exogenous cAMP restored TFP production but not twitching motility. The *cyaABpilH* mutant (lacking endogenous cAMP) produced less TFP than its parental strain (*cyaApilH*), which synthesizes excess cAMP (Fig. 4A); however, there was no difference in twitching motility between these strains. Complementation of the *cyaABpilH* mutant with exogenous cAMP resulted in an increase in TFP production but not twitching motility. Immunoblot analysis of whole cell lysates showed that each of the mutant strains was capable of producing pilin (Fig. 8B), indicating that altered TFP production was due to a defect in assembly and not subunit availability. In summary, complementation of the

cyaABpilG and *cyaABpilH* triple mutants with exogenous cAMP resulted in increased TFP levels (Fig. 8A), suggesting that the Chp system controls TFP production via cAMP. In contrast, adjusting intracellular cAMP to wild type levels failed to alleviate the twitching motility defects of either the *cyaABpilG* or *cyaABpilH* triple mutants (Fig. 8C), indicating that components of the Chp system regulate TFP function independent of cAMP. Taken together, these findings support a model in which cAMP is the primary mediator for Chp system control of TFP biogenesis, but not TFP function.

Discussion

The results of this study provide evidence for new regulatory connections in the complex pathway controlling *P. aeruginosa* virulence factor production and suggest that regulation of intracellular cAMP by the Chp chemosensory system represents a mechanism by which environmental sensing is coupled to virulence gene expression. The regulation of intracellular cAMP by the Chp system represents an output not previously recognized for related bacterial chemosensory systems (Wadhams and Armitage, 2004; Berleman and Bauer, 2005; Kato *et al.*, 2008; Mignot and Kirby, 2008; Porter *et al.*, 2008). We hypothesize that the Chp system regulates TFP and other cAMP-dependent virulence systems in response to as yet unidentified signals in the host environment via the following steps: i) signal reception, ii) signal transduction, iii) modulation of AC activity and iv) cAMP/Vfr-dependent virulence gene regulation (Fig. 9).

Signal reception

Currently the nature of the signal(s) received by the Chp system is not known. Previously, low calcium and high osmolarity *in vitro* culture conditions were shown to affect intracellular cAMP (Wolfgang *et al.*, 2003; Rietsch and Mekalanos, 2006), but it is not known if these signals are relevant *in vivo* or if they act through the Chp system. Other possible signals include phosphatidylethanolamine and phosphatidylcholine, which are chemoattractants for TFP-dependent twitching motility in *P. aeruginosa* (Kearns *et al.*, 2001; Barker *et al.*, 2004; Miller *et al.*, 2008). An intriguing possibility is that TFP fibers themselves relay signaling information for regulating cAMP, similar to the mechanism in *Myxococcus xanthus* where TFP are proposed to serve as sensors for the Dif chemosensory system that controls TFP-dependent social motility (Black *et al.*, 2006).

Signal transduction

Our analysis of the individual Chp system components suggests that cAMP levels are responsive to a stimulatory signal transduced by the Chp system as well as a Chp-dependent signal attenuation/feedback mechanism. Using a combination of forward and reverse genetics we showed that *pilG*, *pilI*, *pilJ*, *chpA* and *chpC* had a positive effect on cAMP, while *pilH* and *chpB* had a negative effect. While preliminary, these results support a model in which Chp system regulation of cAMP involves both activation and adaptation pathways, which are conserved features among bacterial chemosensing systems (Fig. S2). Our finding that *pilG*, *pilI*, *pilJ*, *chpA* and *chpC* all had a positive effect on cAMP is consistent with these components acting sequentially in the signal relay. The fact that a strain lacking *chpB*, which encodes a putative methylesterase, has elevated cAMP suggests that the Chp system is subject to regulation by a traditional adaptation pathway involving MCP methylation/demethylation. However, further confirmation of the adaptation pathway is necessary given our inability to confirm the phenotype of the *pilK* (methlytransferase) mutant.

An interesting feature of the Chp system is that the CheY-like proteins PilG and PilH have different effects on cAMP production by CyaB. CheY-like proteins are typically activated by CheA-dependent phosphorylation in response to signal detection and, in many γ-

proteobacteria, signal termination via dephosphorylation is mediated by CheZ (Wadhams and Armitage, 2004). PilG and PilH both possess conserved phosphorylation sites; however, no CheZ-like phosphatase is associated with the *P. aeruginosa* Chp system. We hypothesize that signal termination for the Chp system may occur by a mechanism similar to that originally described in the *Sinorhizobium meliloti* chemotaxis system in which one CheY-like protein serves as a phosphate sink by accepting phosphoryl groups from a second CheY-like effector (Sourjik and Schmitt, 1998). Based on this model, we predict that phosphorylated PilG is a positive effector of CyaB activity and that PilH is not an effector but instead a phosphate sink, which is consistent with its negative effect on CyaB activity. An alternative hypothesis is that PilG and PilH are both true effectors, but each has a different rate of phosphorylation and/or spontaneous dephosphorylation, allowing them to act sequentially to first activate (PilG) and then inhibit (PilH) cAMP production.

Modulation of AC activity

This study provides evidence that the *P. aeruginosa* Chp system regulates cAMP through alteration of CyaB activity. Based on the structural similarities between *P. aeruginosa* CyaB and other Class III ACs, the enzyme is predicted to have at least two potential regulatory mechanisms. First, the adjacent membrane-spanning MASE2 domain, like other regulatory modules present in Class III ACs, is likely to be involved in the detection of intracellular or extracellular signals that are relayed to the catalytic domain (Nikolskaya *et al.*, 2003; Linder, 2006). Second, CyaB belongs to the subset of Class III ACs (Class IIIb) in which the catalytic domain is subject to direct regulation by the bicarbonate ion (Cann *et al.*, 2003; Linder, 2006). To our knowledge, regulation of *P. aeruginosa* CyaB by the Chp system is the first example of a bacterial Class III AC controlled by specific signaling proteins. However, it remains to be determined if Chp system regulation of CyaB is direct and whether signal detection involves interaction with the MASE2 and/or catalytic domains.

cAMP/Vfr-dependent virulence gene regulation

Although a direct role for cAMP in Vfr activation has not been demonstrated, we predict cAMP is required for Vfr activity in regulating Vfr-dependent virulence systems such as T3S, QS and the TFP biogenesis genes. Of the cAMP regulators identified in our screen, only FimL has been previously linked to Vfr regulation (Whitchurch *et al.*, 2005). FimL was shown to control multiple virulence factors in strain PAO1, including TFP biogenesis and function, T3S, and biofilm formation, via its regulation of Vfr. The authors ruled out cAMP's involvement because *fimL* had no effect on intracellular cAMP levels but the evidence that *fimL* twitching revertants had elevated cAMP makes the interpretation less clear. In contrast, we showed a definitive role for *fimL* in cAMP regulation in strain PAK. Although FimL is homologous to the N-terminus of ChpA, it remains to be determined if there is a functional relationship between FimL and the Chp chemosensing system.

The fact that multiple *P. aeruginosa* virulence factors are regulated by cAMP implicates FimL, FimV and the Chp system components in general virulence system regulation. In fact, other groups have reported significantly reduced virulence phenotypes associated with mutations in these genes. Tn insertion mutants in *fimV* and the *chp* gene cluster were attenuated in a *Drosophila* infection model, an effect that could not be accounted for by the loss of TFP function alone (D'Argenio *et al.*, 2001). In cell culture assays, inactivation of *fimV*, *fimL* or *pilJ* had a greater effect on cytotoxicity than adherence (Kang *et al.*, 1997; Ahn *et al.*, 2004; Whitchurch *et al.*, 2005), suggesting these mutants have defects in multiple virulence systems. The adherence and cytotoxic phenotypes of these mutants are similar to those of mutants lacking AC activity or Vfr (Wolfgang *et al.*, 2003), which is consistent with the involvement of these genes in cAMP regulation. In addition, the fact that *fimL* and *pilJ* mutants were shown to have impaired T3SS activation (Whitchurch *et al.*, 2005;

Zolfaghar *et al.*, 2005) provides direct evidence for regulation of cAMP-dependent virulence factors by the TFP/Chp components.

Our analysis of TFP function also revealed that although twitching motility is ultimately dependent on TFP biogenesis, the Chp system exerts cAMP-independent regulatory control over TFP function. Currently the mechanism by which the Chp system regulates twitching motility is not known but one possibility is via regulation of TFP extension and retraction (Winther-Larsen and Koomey, 2002), which is mediated by the ATPases PilB and PilT, respectively (Whitchurch *et al.*, 1991; Turner *et al.*, 1993; Chiang *et al.*, 2008).

The majority of the Tn insertions in our screen for cAMP regulators mapped to genes previously implicated in TFP regulation, including those encoding TFP structural components (*pilA*) or regulators of TFP biogenesis and/or function (*fimL*, *fimV*, *pilB*, *pilG*, *pilI*, *pilJ* and *chpA*). We recovered multiple Tn insertions in the PA1821 locus, which is immediately upstream of *fimL* and encodes a probable enoyl-CoA hydratase/isomerase (Whitchurch *et al.*, 2005). We believe the phenotype of the PA1821 Tn mutants is due to a polar effect on *fimL* but we did not confirm this hypothesis. The non-polar *pilB* mutant did not recapitulate the phenotype of the *pilB* Tn mutant, so it was not pursued further. However, this outcome did serve to demonstrate that not all TFP biogenesis factors affect cAMP, which is consistent with the fact that we recovered only a subset of the over 40 known TFP-related genes (Mattick, 2002; Huang *et al.*, 2003; Whitchurch *et al.*, 2005) in our screen. In contrast to the *pilB* mutant, which does not assemble TFP, the *pilA* mutant showed a modest reduction in cAMP. At this time it is unclear whether pilin (encoded by *pilA*) affects cAMP through the Chp system or through an independent mechanism; the same is true for FimV, another cAMP regulator with no known chemotaxis protein homolog. The *fimV* gene is required for TFP production and twitching motility in *P. aeruginosa* and is proposed to be involved in cell wall reorganization (Semmler *et al.*, 2000; Ahn *et al.*, 2004), a function that may directly or indirectly affect Chp system output.

In conclusion, this study has revealed a number of new regulators of the *P. aeruginosa* cAMP signaling pathway, including the members of the Chp chemosensory system. We also identified the specific regulatory effect of the Chp system in controlling AC activity and future studies will determine whether the relationship between the Chp system and CyaB is direct or indirect. Ultimately, defining the signal(s) that initiate the *P. aeruginosa* virulence programme via the Chp system will facilitate our understanding of the machinery for host environmental sensing in this pathogenic bacterium.

Experimental procedures

Bacterial strains and growth conditions

All mutant *P. aeruginosa* strains used in this study were derived from the prototypic laboratory strain PAK and are referenced in Table S1. For routine passage, *E. coli* and *P. aeruginosa* were grown at 37°C in Luria Bertani (LB) medium. pMMB-based expression plasmids were maintained in *P. aeruginosa* with 150 μg ml^{-1} carbenicillin (Cb), except where noted. Bacterial growth in broth culture was assessed by optical density at 600 nm (OD_{600}).

Native purification of the CyaB catalytic domain

To create an expression construct for the C-terminal CyaB catalytic domain, we amplified the portion of the *cyaB* coding region corresponding to amino acid positions 217 to 463 by PCR using the 5' cyaB K217 and 3' cyaB T223 oligonucleotides (Table S2) and genomic DNA from *P. aeruginosa* strain PAK as a template. The resulting fragment contained engineered BamHI and PstI restriction endonuclease site at the 5' end and at the 3' end,

respectively. The *cyaB*$_{217–463}$ fragment was cut and inserted into the pQE30 vector (Qiagen) between the BamHI and PstI sites, creating pQE30*cyaB*$_{217–463}$. The plasmid was transferred to *E. coli* M15[pREP4] for expression of 6×His-tagged CyaB$_{217–463}$. Bacteria were grown in LB broth at 30°C until OD_{600} = 0.6, followed by induction with 20 μM IPTG for an additional 20 hours at 22°C. Purification of CyaB$_{217–463}$ using Ni-NTA affinity resin (Qiagen) was carried out using the methods described for purification of the *Anabaena* CyaB1 catalytic domain (Kanacher *et al.*, 2002).

Adenylate cyclase assay

AC assays were performed in a final volume of 100 μl and typically contained 50 mM buffer (pH 7.5, MOPS; pH 8.5, Tris/HCl), 2 mM [2,8-^{3}H]cAMP (150 Bq), and [α-^{32}P] (25 kBq) as substrate (Salomon *et al.*, 1974). Other assay conditions are as indicated in the figure legend. Protein concentrations were adjusted to maintain substrate utilization at less than 10%. Kinetic constants were determined over a substrate concentration range of 1 to 1000 μM.

P. aeruginosa strain construction

To create a cAMP-dependent transcriptional reporter for use in *P. aeruginosa*, we used a region of the *E. coli lac*P1 promoter extending from 175 bp upstream of the transcriptional start site to 104 bp downstream of the translational start site of *lacZ*; this region is predicted to contain elements necessary for *lacZ* transcription, including a CRP binding site (Busby and Ebright, 1999). A fragment containing the *lac*P1 promoter region was PCR amplified from *E. coli* strain HB101 genomic DNA with EclacP1 rep 5' and EclacP1 rep 3' oligonucleotides (Table S2) and digested with EcoRI and BamHI and cloned between the EcoRI and BamHI sites of the mini-CTX-*lacZ* plasmid, creating mini-CTX-P*lac*P1-*lacZ*. The resulting plasmid was integrated into the vacant ϕCTX phage chromosomal attachment site in strain PAK using previously described methods (Hoang *et al.*, 2000). To construct isogenic AC and *vfr* mutants in the *lac*P1-*lacZ* reporter background, PAK::*lac*P1-*lacZ* was subjected to allelic exchange with individual suicide vectors (pEXGmΔ*cyaA*, pEXGmΔ*cyaB*, or pEXGmΔ*vfr*) using previously described methods (Wolfgang *et al.*, 2003). To create the double AC (*cyaAB*) mutant, the PAK*cyaA*::*lac*P1-*lacZ* strain was used in a subsequent round of allelic exchange with pEXGmΔ*cyaB*. Construction of a version of the *lac*P1-*lacZ* reporter lacking LacI binding sites (named *lac*P1Δ*lacI*-*lacZ*) is described in the supplemental text.

Deletion alleles for *pilA*, *pilB*, *pilG*, *pilH*, *pilI*, *pilJ*, *pilK*, *chpA*, *chpB*, *chpC*, *fimL* and *fimV* were constructed using SOE-PCR as described (Wolfgang *et al.*, 2003) using PAK genomic DNA as a template and the appropriate SOE oligonucleotide pairs (Table S2). Deletion alleles were cloned into pEXGmGW, pEXG2GW or pDONRX (plasmid construction described in supplemental text) by Gateway cloning and the resulting plasmids were used to introduce the various mutations into PAK*cyaA*::*lac*P1Δ*lacI*-*lacZ* by allelic exchange as described (Wolfgang *et al.*, 2003).

To create gene expression plasmids, the open reading frames (ORFs) of *P. aeruginosa cyaA*, *cyaB*, *pilG*, *pilH*, *pilI*, *pilJ*, *pilK*, *chpA*, *chpB*, *chpC*, *fimL* and *fimV* were amplified from PAK genomic DNA using the appropriate oligonucleotide pairs (Table S2; (Wolfgang *et al.*, 2003) and cloned into Gateway vectors as described previously (Wolfgang *et al.*, 2003). Briefly, PCR fragments were tailed with *att*B1 and *att*B2 sequences, cloned into the pDONR201 entry vector and then transferred to different versions of the pMMBGW destination vector. pMMBV1 and pMMBV2 have altered −35 and −10 regions resulting in reduced transcriptional expression relative to that of pMMB (S. Lory, Harvard Medical School, unpublished). The pMMBV3, pMMBV4 and pMMBV5 plasmids are versions of

pMMB, pMMBV1, and pMMBV2, respectively, with an additional *lac* operator; this site was added by changing the sequence between the −35 and −10 sites from ATTAATCATCGGCTCG to TTGTGAGCGGATAACAA. These versions of the plasmids allow lower expression levels compared to the original series such that the maximal expression level of pMMB>pMMBV1>pMMBV2 and pMMBV3>pMMBV4>pMMBV5. All pMMB-based expression plasmids were transferred to the appropriate PAK strain by conjugation (Furste *et al.*, 1986) followed by selection on LB agar plates containing 150 μg ml^{-1} Cb and 25 μg ml^{-1} irgasan (Irg). For complementation of cAMP reporter activity in the TFP/Chp mutants, the target genes were expressed in the following plasmids: pMMB (*pilA*, *pilG* and *pilH*), pMMBV1 (*pilI*, *pilK*, and *fimV*), pMMBV2 (*chpC* and *fimL*), pBMMBV4 (*chpA*), and pMMBV5 (*chpB* and *pilJ*).

β-galactosidase assay

Assays for β-galactosidase activity were conducted as described (Wolfgang *et al.*, 2003) using bacteria grown to mid-logarithmic growth phase in LB broth or LB broth containing 30 μg ml^{-1} Cb and the indicated amount of IPTG, or using bacteria grown on LB agar plates with or without 20 mM cAMP as indicated.

cAMP assay

For determination of intracellular cAMP concentration, bacteria were grown in LB broth at 37°C to OD_{600} = 1.0. Bacteria were collected by centrifugation at 13, 000 × *g* for 2 minutes at 4°C and washed twice with 1 ml cold 0.9 M NaCl. Pellets were suspended in 100 μl 0.1N HCl and incubated on ice for 10 minutes with occasional vortexing to lyse bacteria. Lysates were centrifuged at 13, 000 × *g* for 5 minutes at 4°C to remove cellular material and the supernatant was assayed for cAMP using an enzyme-linked immunosorbant assay (Cayman Chemical) following the manufacturer's protocol for sample acetylation. Duplicate bacterial pellets for protein determination were suspended in 100 μl phosphate buffered saline (PBS) and were lysed by three freeze/thaw cycles followed by centrifugation at 13,000 × *g* for 5 minutes to pellet unbroken cells. The protein concentration of the supernatant was determined by BCA protein assay (Pierce). Assay values for cAMP were converted to intracellular concentrations (μM) based on the estimated cellular volume per mg of protein (D'Souza-Ault *et al.*, 1993).

Tn insertion library construction and screening

To create a *P. aeruginosa* Tn insertion mutant library, we performed Tn mutagenesis of PAK*cyaA*::*lac*P1-*lacZ* using the plasmid pBTK30, which contains a Gm resistance-marked transposable element and a *mariner C9* transposase (Goodman *et al.*, 2004). Suspensions of *E. coli* strain SM10 harboring pBTK30 and the *P. aeruginosa* recipient (OD_{600} = 40–50) were mixed at a ratio of 2:1 and pipetted onto LB plates and incubated for 2 hours at 37°C. Bacteria were then suspended in LB and dilutions were plated on LB plates containing 75 μg ml^{-1} Gm, 25 μg ml^{-1} Irg and 40 μg ml^{-1} X-gal. Plates were incubated at 37°C for 24 hours. The *cyaA* mutant forms blue colonies on X-gal; colonies that appeared less blue (presumably due to reduced reporter activity) were selected from plates containing approximately 1000–2000 colonies per plate. Candidate clones were patched onto LB X-gal plates and grown at 22°C to confirm the color phenotype. The site of Tn insertion for each clone was determined by PCR with semi-random and Tn-specific oligonucleotides followed by sequencing of the Tn-chromosome junctions as described (Goodman *et al.*, 2004). The Tn insertion location was identified by comparison to the genome sequence of *P. aeruginosa* strain PAO1 (Stover *et al.*, 2000; Winsor *et al.*, 2009). To test cAMP complementation of the Tn mutant phenotype, bacteria were patched on LB X-gal plates with either 50 mM cAMP or no cAMP and incubated at 22°C for 24 hours. Complementation by cAMP was scored by comparing the color of individual Tn mutants on plates with and without

exogenous cAMP. The *lac*P1-*lacZ* reporter strains harboring the *cyaA*, *cyaAB* or *vfr* mutations were patched on each plate as controls to ensure consistent color development between plates with and without cAMP.

Pilus preparation

TFP sheared from the bacterial surface were isolated from *P. aeruginosa* grown on LB agar plates or, where indicated, LB agar plates containing 20 mM cAMP. For complementation assays with plasmid-harboring strains, bacteria were grown on LB plates containing 30 μg ml^{-1} Cb and IPTG ranging from 0 to 500 μM. Bacteria were suspended in 1 ml of 0.15 M NaCl and 0.2% formaldehyde and vortexed for 1 minute. Cells were removed by centrifugation at 12,000 × *g* for 5 minutes. The supernatant was transferred to a microfuge tube, adjusted to 0.1 M $MgCl_2$, and incubated at 4°C for 3 hours. Following centrifugation at 12,000 × *g* for 5 minutes, the resulting pellet was suspended in SDS-PAGE sample buffer, resolved on an 18% polyacrylamide gel and visualized by GelCode Blue Stain (Pierce). The relative amount of pilin monomer demonstrated by SDS-PAGE was used as an indicator of the abundance of TFP on the bacterial surface.

Immunoblotting

Whole cell lysates for detection of pilin and CyaB were prepared from bacteria grown in LB broth until OD_{600} = 1. Lysates were normalized based on total protein. Strains harboring plasmids were grown in the presence of 30 μg ml^{-1} Cb and the indicated amount of IPTG. Pilin samples were collected by centrifugation and suspended in 100 μl of SDS-PAGE sample buffer and incubated at 95°C for 10 minutes. The lysate was diluted 1:10 and 2.5μl was resolved on 18% SDS-polyacrylamide gels and transferred to nitrocellulose. For CyaB samples, bacterial pellets were resuspended in 100 μl H_2O and treated with 1 μl DNase (10 mg ml^{-1}) for 10 minutes at 37°C. SDS-PAGE sample buffer (100 μl) was then added followed by solubilization at 42°C for 45 minutes. CyaB samples were run on 12% SDS-polyacrylamide gels and transferred to nitrocellulose. For detection of pilin, membranes were probed with PKL1 anti-pilin monoclonal antibody (Yu *et al.*, 1994) (gift of Randall Irvin, University of Alberta; 1:30,000 dilution) followed by an HRP-conjugated goat anti-mouse Ig antibody (Promega; 1:100,000 dilution). For detection of CyaB, membranes were probed with affinity purified anti-CyaB rabbit serum directed against a peptide determinant (DKERVIQALQQAAERLRDKVIL) in the C-terminal region of CyaB (Proteintech Group, Inc.; 1:5,000 dilution) followed by a horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ig (Rockland; 1:25,000 dilution). Blots were developed with enhanced chemiluminescense reagents (Millipore) and visualized by autoradiography.

Twitching motility assay

Strains were grown overnight at 37°C on LB agar plates and bacteria were stab-inoculated to the bottom of 100 mm tissue culture-treated dishes (Corning) containing 5 ml LB/1% agar or LB/1% agar plus 20 mM cAMP. For complementation assays with plasmid-harboring strains, bacteria were grown in the presence of 30 μg ml^{-1} Cb and IPTG ranging from 0 to 500 μM. Plates were incubated for 40 hours at 37°C in a humidified chamber and the zone of subsurface bacterial spread was measured.

Statistical analysis

Calculations to assess statistical significance for values obtained in all assays were made using an unpaired *t* test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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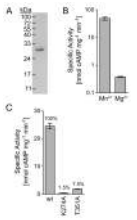

FIG. 1. Biochemical characterization of *P. aeruginosa* CyaB

A) Coomassie Blue-stained SDS-polyacrylamide gel of purified recombinant $CyaB_{217–463}$. A 1.5 μg portion of protein was applied and molecular-mass standards (in kDa) are indicated. B) $CyaB_{217–463}$ specific activity (n=4; ± standard error of the mean (SEM)) in the presence of 200 μM Mn^{2+}-ATP (68 nM protein, 40°C, pH 8.5) or 1 mM Mg^{2+}-ATP (13 μM protein, 40°C, pH 8.5). **C)** Wild type and mutant (K274A or T351A) $CyaB_{217–463}$ specific activities (n=8; ± SEM) assayed with 250 μM Mn^{2+}-ATP (0.7 μM protein, 40°C, pH 7.5). Percentage indicates the portion of wild type activity. All values were significantly different ($p<0.0001$) compared to the value for wild type CyaB.

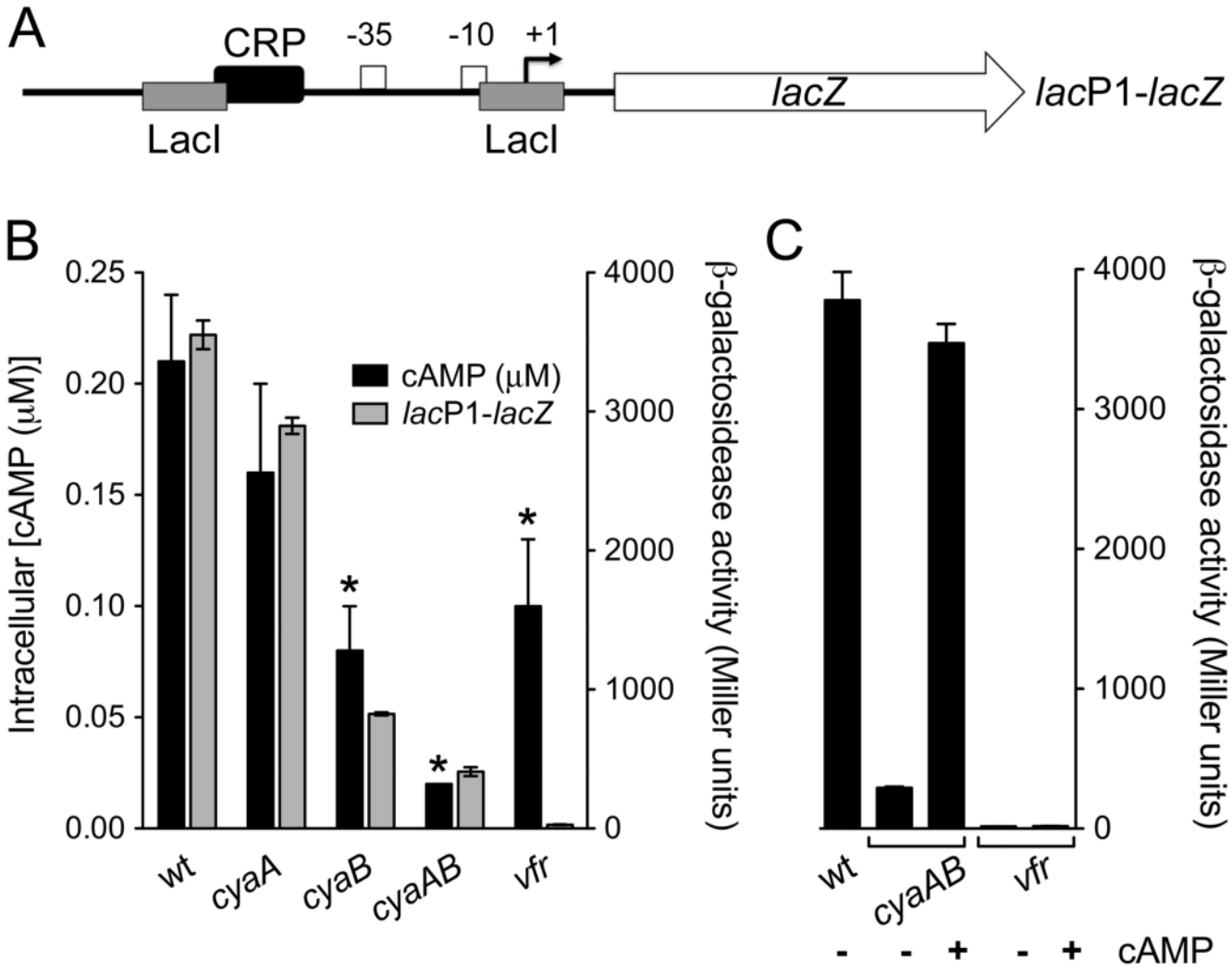

FIG. 2. Activity of the *lac*P1-*lacZ* transcriptional reporter is cAMP-dependent and reflects the level of intracellular cAMP

A) Schematic diagram of the *lac*P1-*lacZ* promoter reporter. The *lac*P1 promoter region of the *E. coli lacZ* gene contains two LacI binding sites (gray rectangles) and a CRP binding site (black rectangle). The transcriptional start site (+1) and −35 and −10 regions are indicated. The region of the *lac*P1 promoter extending 177 bp upstream of the transcriptional start site was fused to the *lacZ* gene. B) Direct measurement of cAMP by enzyme immunoassay (left axis, black bars) and β-galactosidase activity (right axis, gray bars) in the wild type strain (wt), the AC mutants (*cyaA*, *cyaB* and *cyaAB*), or the *vfr* mutant (*vfr*); all strains contained the *lac*P1-*lacZ* reporter. For the β-galactosidase assay, the data represent three independent experiments with four replicates each and values are reported as the mean ± SEM; values for all strains are significantly different ($p<0.0001$) when compared pair wise to the values for all other strains shown. For the cAMP assay, the data represent three independent experiments, each with three replicates and values are reported as the mean ± SEM. The asterisk (*) indicates that the values for the indicated strains are significantly different ($p\leq0.019$) compared to the value for the wild type strain. C) A defect in cAMP synthesis can be restored by exogenous cAMP. β-galactosidase activity in the wild type strain (wt), AC double mutant (*cyaAB*) or *vfr* mutant (*vfr*) grown in the presence (+) or

absence (−) of 50 mM cAMP. All strains contained the *lac*P1-*lacZ* reporter. The data represent two independent experiments with three replicates each and values are reported as the mean ± SEM.

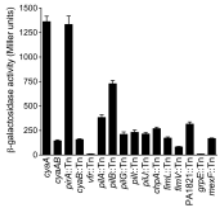

FIG. 3. Quantitation of cAMP reporter activity in Tn insertion mutants

β-galactosidase activity of the *cyaA* mutant (parent), the AC double mutant (*cyaAB*) and representative Tn insertion mutants in the PAK*cyaA*::*lac*P1-*lacZ* background grown in broth culture to mid-logarithmic growth phase. The gene disrupted by Tn insertion is indicated for each strain. The *pirA* mutant is a clone from the Tn insertion library screen that had a blue colony phenotype on X-gal indicator plates. The data represent three independent experiments with four replicates each and values are reported as the mean ± SEM. All values (except *pirA*::Tn) were significantly different ($p<0.0001$) compared to the value for the parent strain.

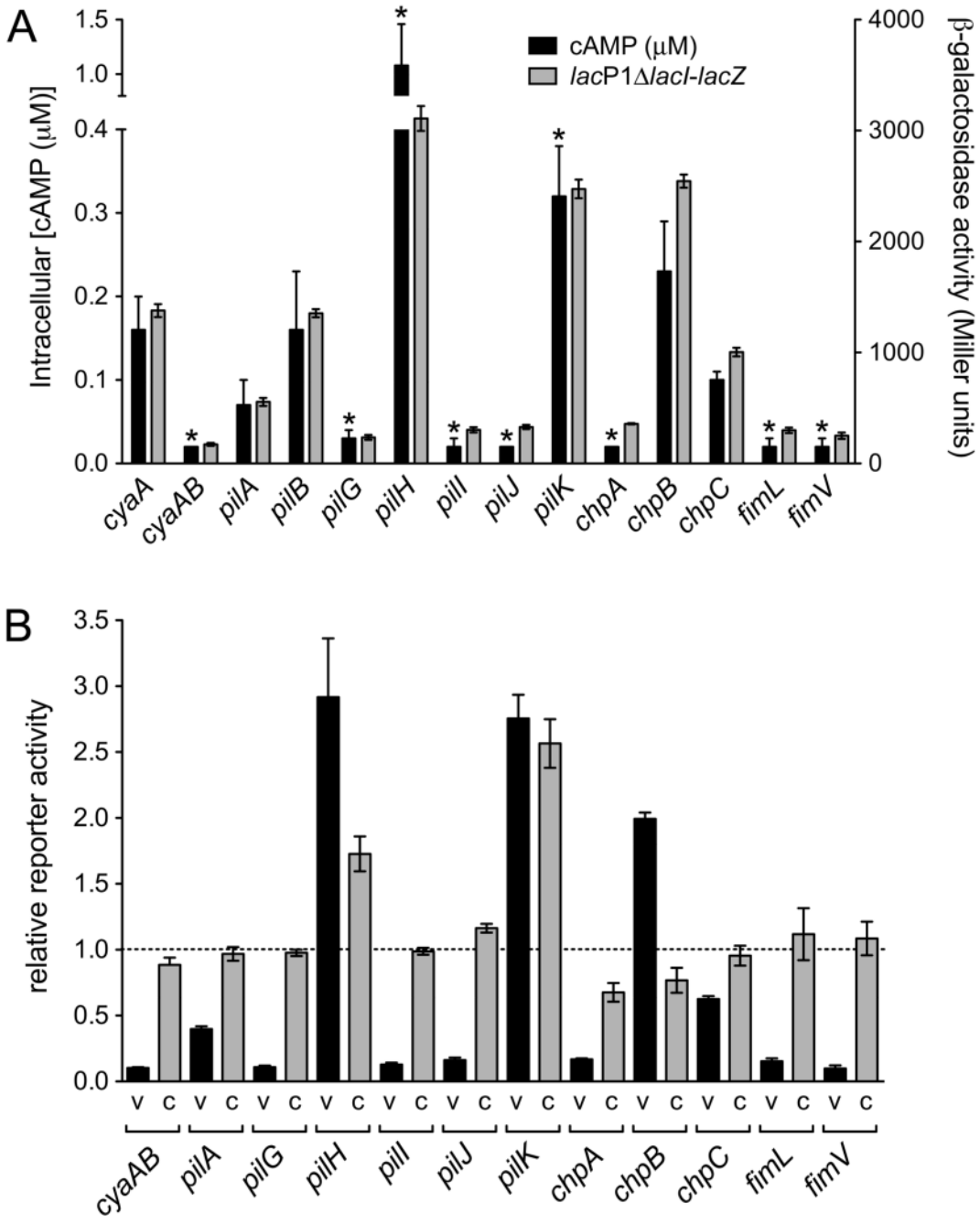

FIG. 4. Inactivation of TFP/Chp genes affects intracellular cAMP

A) Direct measurement of cAMP by enzyme immunoassay (left axis, black bars) and β-galactosidase activity (right axis, gray bars) in the *cyaA* mutant (parent), the double AC mutant (*cyaAB*), or the indicated non-polar deletion mutant in the PAK*cyaA*::*lac*P1Δ*lacI-lacZ* background. For the β-galactosidase assay, the data represent three independent experiments with two replicates each and values are reported as the mean ± SEM; all values (except *pilB*) were significantly different ($p \leq 0.0003$) compared to the value for the parent strain. For the cAMP assay, the data represent three independent experiments with three replicates each and values are reported as the mean ± SEM; the asterisk (*) indicates that the values for the indicated strains are significantly different ($p \leq 0.04$) compared to the value for

the parent strain. B) Complementation of the TFP/Chp mutants by expression of the corresponding gene *in trans*. Activity of the *lac*P1Δ*lacI-lacZ* reporter in the non-polar deletion mutant strains harboring either the empty pMMB67EH vector (V, black bars) or the pMMB-based plasmid (C, gray bars) encoding the corresponding complementing gene. The TFP/Chp genes were each expressed in the pMMB plasmid backbone listed in the experimental procedures. The *cyaAB* mutant (*cyaAB*) harboring either pMMB67EH (V) or pMMBV2-*cyaA* (C) serve as controls for complementation of *lac*P1Δ*lacI-lacZ* reporter activity. Strains were grown in broth culture containing IPTG at the concentration determined to be optimal for complementation of the mutant phenotype (see text) and assayed at mid-logarithmic growth phase. Relative reporter activity is reported as the β-galactosidase activity of an individual mutant strain divided by that of the *cyaA* mutant parent strain harboring pMMB67EH. The data represent a minimum of three independent experiments with two replicates each and values are reported at the mean ± SEM.

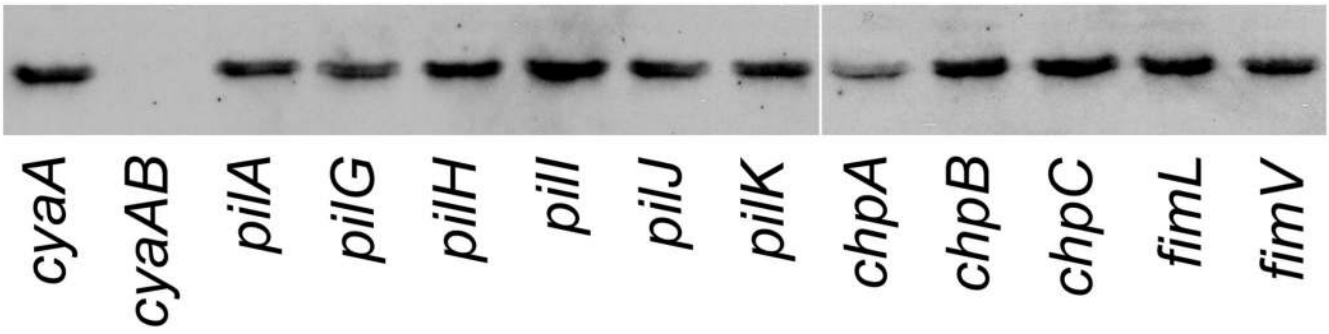

FIG. 5. CyaB protein levels do not account for altered intracellular cAMP in the TFP/Chp mutants

CyaB immunoblot of whole cell lysates from the *cyaA* parent strain (*cyaA*), the double AC mutant (*cyaAB*) and the indicated mutants in the PAK*cyaA*::*lac*P1Δ*lacI-lacZ* background. Samples were normalized based on total protein and blots were probed with affinity-purified CyaB-specific rabbit antiserum.

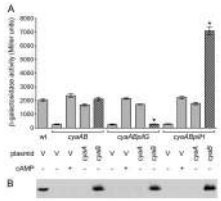

FIG. 6. The Chp system regulates cAMP at the level of CyaB activity

β-galactosidase activity of the wild type strain (wt), the double AC mutant (*cyaAB*), and the *cyaAB*, *pilG* (*cyaABpilG*) and *cyaAB*, *pilH* (*cyaABpilH*) triple mutants. All strains contained the *lac*P1Δ*lacI-lacZ* reporter and carried either the pMMB67EH empty vector or pMMBV2-*cyaA* (*cyaA*) or pMMBV2-*cyaB* (*cyaB*). Bacteria were grown in broth culture in the presence (+) or absence (−) of 50 mM cAMP. The data represent two independent experiments with four replicates each and values are reported as the mean ± SEM. The asterisk (*) indicates that the values for the indicated strains are significantly different ($p<0.0001$) when compared pair wise to the value for the *cyaAB* mutant carrying pMMBV2-*cyaB*. B) CyaB immunoblot of whole-cell lysates from the above strains. Samples were normalized based on bacterial number and blots were probed with affinity-purified CyaB-specific rabbit antiserum.

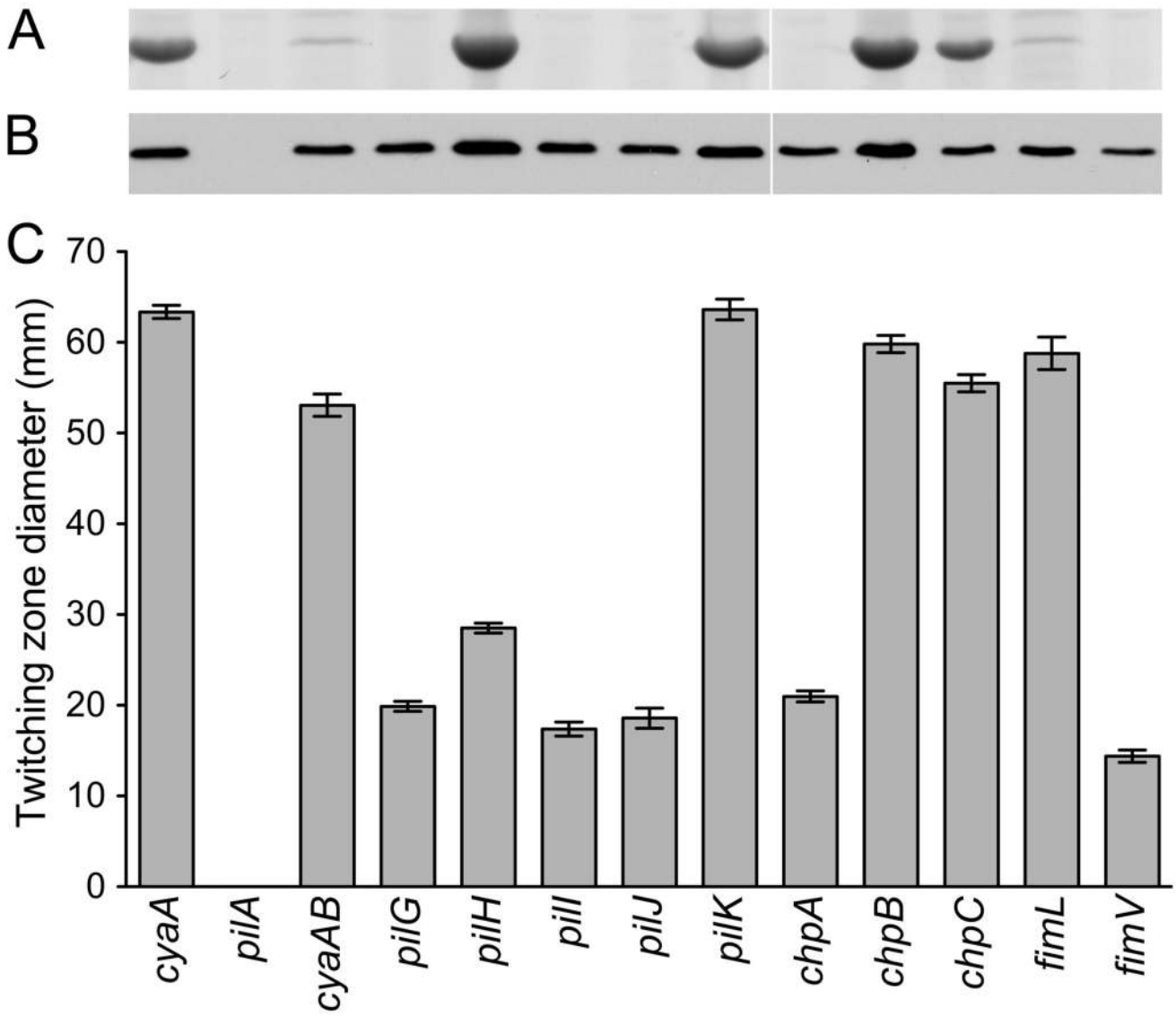

FIG. 7. The role of the TFP/Chp genes in TFP production and twitching motility

A) Coomassie Blue-stained SDS-polyacrylamide gel showing the amount of pilin in purified surface pilus fractions from the *cyaA* mutant (*cyaA*), a non-piliated *pilA* mutant (*pilA*), the double AC mutant (*cyaAB*) and the indicated mutants in the PAK*cyaA*::*lac*P1Δ*lacI-lacZ* background. Samples for pilus preparations were normalized based on bacterial number. Pilus preps were performed three times and a representative gel is shown. B) Immunoblot of whole-cell lysates from the above strains probed with a pilin-specific monoclonal antibody. C) Twitching motility zones for the indicated strains. The data represent at least three independent experiments with a minimum of three replicates each and values are reported as the mean ± SEM.

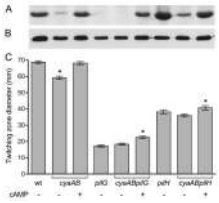

FIG. 8. The Chp system regulates TFP production but not twitching motility via control of intracellular cAMP

A) Coomassie Blue-stained SDS-polyacrylamide gel showing the amount of pilin in purified pilus fractions from the wild type (wt) strain, the double AC mutant (*cyaAB*), the *pilG* and *pilH* mutants in a *cyaA* background (*pilG* and *pilH*, respectively) or the *cyaAB*, *pilG* (*cyaABpilG*) and *cyaAB*, *pilH* (*cyaABpilH*) triple mutants. All strains contained the *lac*P1Δ*lacI-lacZ* reporter. Bacteria were grown on LB agar plates in the presence (+) or absence (−) of 20 mM cAMP and were normalized based on bacterial number. B) Immunoblot of whole-cell lysates from the above strains probed with a pilin-specific monoclonal antibody. C) Twitching motility zones for the indicated strains grown in the presence (+) or absence (−) of 20 mM cAMP. The data represent two independent experiments with three replicates each and values are reported as the mean ± SEM. The asterisk (*) indicates that the values for the indicated strains are significantly different ($p<0.001$) compared to the value for the *cyaAB* mutant grown in the presence of cAMP.

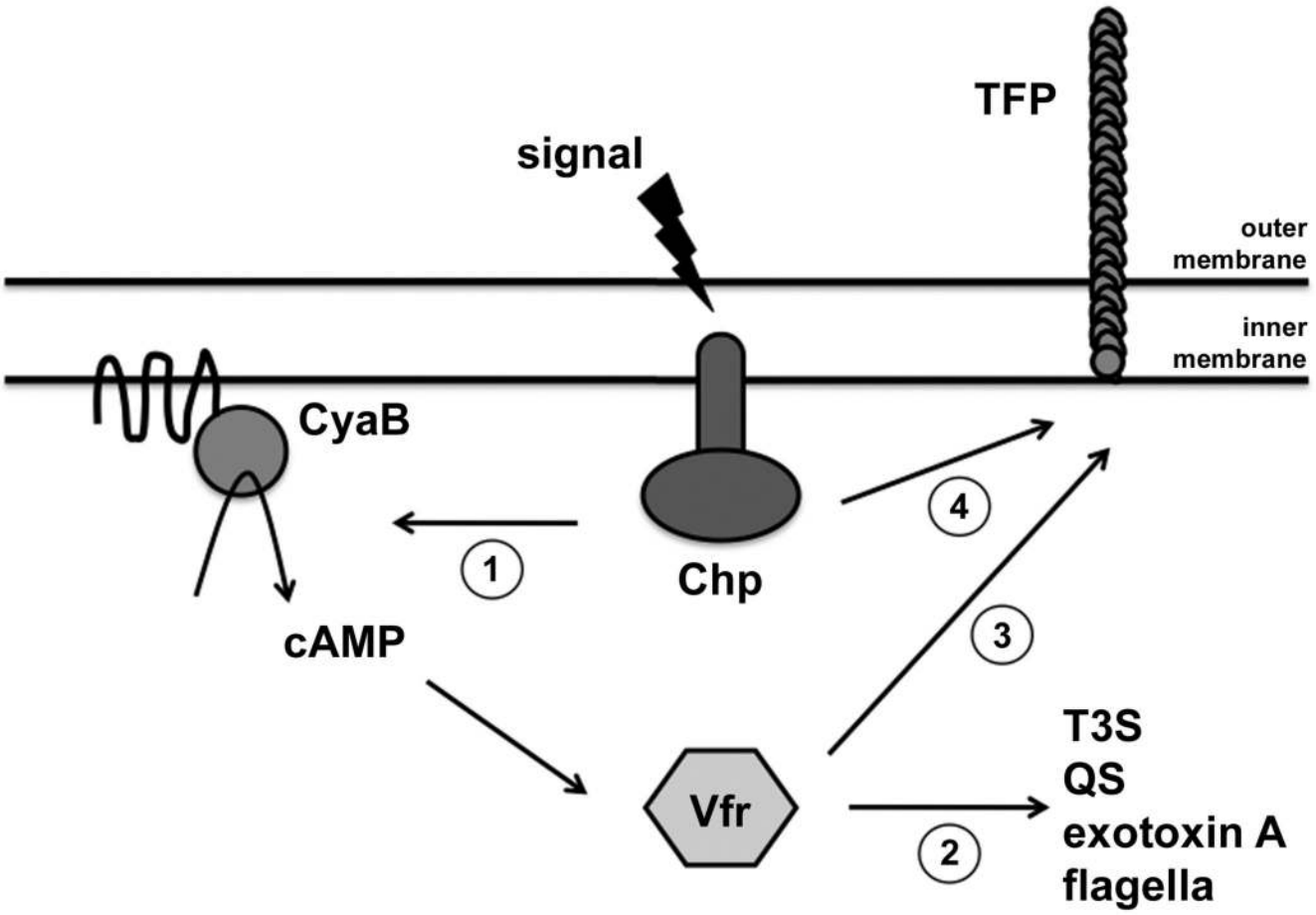

FIG. 9. Model for coupling environmental sensing to virulence factor expression via the *P. aeruginosa* Chp chemosensory system

1) In response to unknown signal(s), the Chp system controls cAMP synthesis by modulating activity of the AC enzyme CyaB, which is predicted to be integral inner membrane protein. The cAMP-dependent transcription factor Vfr mediates transcriptional activation of multiple virulence systems, including 2) T3S, QS, flagellar biosynthesis, multiple toxins and degradative enzymes and 3) the structural and regulatory factors responsible for TFP biogenesis. 4) The Chp system also exerts a regulatory effect on TFP function (twitching motility) that is cAMP-independent.

Table 1

Transposon insertion mutants with altered cAMP reporter activity

number	locus*	function / pathway
39	*cyaB*	adenylate cyclase, cAMP synthesis
27	*vfr*	cAMP-dependent transcriptional regulator
13	*fimV*	TFP / biogenesis (?)
10	*pilJ*	TFP / biogenesis, chemosensing
9	*chpA*	TFP / biogenesis, chemosensing
8	*fimL*	TFP / biogenesis, chemosensing (?)
5	*pilG*	TFP / biogenesis, chemosensing
3	*pilI*	TFP / biogenesis, chemosensing
3	PA1821	probable enoyl-CoA hydratase/isomerase
1	*pilA*	TFP / biogenesis
1	*pilB*	TFP / biogenesis
1	*grpE*	chaperone, heatshock protein
1	*mexF*	multi-drug efflux

*site of transposon insertion lies within the coding sequence or putative promoter region of the indicated gene.