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Coordinate Regulation of Bacterial Virulence Genes by a Novel Adenylate Cyclase-Dependent Signaling Pathway

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Summary

Type III secretion systems (TTSSs) are utilized by numerous bacterial pathogens to inject effector proteins directly into host cells. Using a whole-genome microarray, we investigated the conditions and regulatory factors that control the expression of the *Pseudomonas aeruginosa* TTSS. The transcriptional response of known TTSS genes indicates a hierarchical pattern of expression in which a set of secretion apparatus and regulatory genes is constitutively expressed. Further analysis of genes coordinately regulated with those encoding the TTSS led to the identification of a signaling pathway that originates from a membrane-associated adenylate cyclase and controls TTSS gene expression. Transcriptome analysis of mutants lacking the ability to synthesize cAMP or the cAMP binding protein Vfr implicated this pathway in the global regulation of host-directed virulence determinants, including the TTSS.

Introduction

The type III secretion system (TTSS), expressed by a wide range of animal and plant pathogens, represents one of the more specific host-directed bacterial virulence determinants (Hueck, 1998). This protein secretion and delivery system specifically translocates bacterial effectors directly into the host cell cytoplasm. Consequently, the TTSS functions only after intimate contact is established between pathogen and host. After delivery, the activities of these effectors range from simple disruption of intracellular signaling to outright killing (reviewed in Cornelis and Van Gijsegem, 2000; Galan and Collmer, 1999). The regulation of genes encoding effector proteins, their cognate chaperones, and the components of the host cell-targeting secretion apparatus are controlled by a complex regulatory mechanism that assimilates various extracellular and intracellular signals (Francis et al., 2002; Miller, 2002).

Pseudomonas aeruginosa is an opportunistic pathogen, responsible for serious infections of humans with compromised host defense mechanisms (Deretic, 2000). It is now recognized that the relatively large genome of *P. aeruginosa* (~6.3 Mb) carries an extensive repertoire of genes compatible with the various niches that this organism can occupy and includes many potential determinants of virulence (Stover et al., 2000). The armament of *P. aeruginosa* includes a TTSS and four known effectors. These include two ADP-ribosyltransferases (ExoS and ExoT), a protein with host-specific adenylate cyclase activity (ExoY), and an acute cytotoxin (ExoU) (Finck-Barbancon et al., 1997; Yahr et al., 1996a, 1996b, 1998).

Various biochemical and in silico approaches resulted in the identification of approximately 40 *P. aeruginosa* genes associated with TTSS activity (Frank, 1997). The expression of the *P. aeruginosa* TTSS and secretion of effector proteins are induced after contact with mammalian cells and under calcium-limiting growth conditions, analogous to the signals controlling expression of the TTSS in *Yersinia* species (Pettersson et al., 1996; Straley et al., 1993; Vallis et al., 1999). The coordinate expression of TTSS genes in *P. aeruginosa* is in part accomplished by the activity of ExsA, a factor belonging to the AraC family of transcriptional regulators (Frank and Iglewski, 1991). ExsA exerts a positive regulatory effect by binding to a defined site near the promoters of known TTSS genes (Hovey and Frank, 1995; Yahr et al., 1996a). The mechanism by which *P. aeruginosa* virulence factors, including the TTSS, are coordinately regulated in response to external signals is unclear. In this work, we carried out transcriptional profiling utilizing *P. aeruginosa* DNA microarrays to define the range of genes and potential regulatory networks that function under conditions known to regulate expression of the TTSS.

In the course of our studies, we discovered that a subset of known TTSS component genes were constitutively expressed regardless of the secretion-inducing signal or the presence of the positive regulatory factor ExsA, indicating the existence of an expression hierarchy. We further discovered that a number of putative regulatory genes were expressed in a calcium-dependent manner. Systematic inactivation of these genes lead to the identification of regulatory factors that play a role in assimilating environmental signals and regulate the expression of virulence genes, including those that encode the TTSS. One such gene encodes a class III adenylate cyclase (termed CyaB), the second enzyme with the ability to form 3′, 5′-cyclic adenosine monophosphate (cAMP) in *P. aeruginosa*. Under conditions of limiting calcium, both *P. aeruginosa* adenylate cyclases contribute to the total cellular cAMP content and control expression of the TTSS and other calcium-regulated virulence factors via the cAMP binding protein Vfr. The transcriptome of cAMP- and Vfr-deficient mutants indicates that numerous host-directed virulence determinants, including motility systems, attachment organelles, and the type II secretory pathway are coordinately regulated under conditions that control expression of the TTSS. The hierarchical organization of a novel regulatory cascade has thus been established and indicates that the cAMP-signaling pathway in *P. aeruginosa* controls gene expression in the host environment.

Results

Transcriptional Profiling of *P. aeruginosa*

To determine the effect of calcium limitation and the contribution of the ExsA transcription factor on the *P.*

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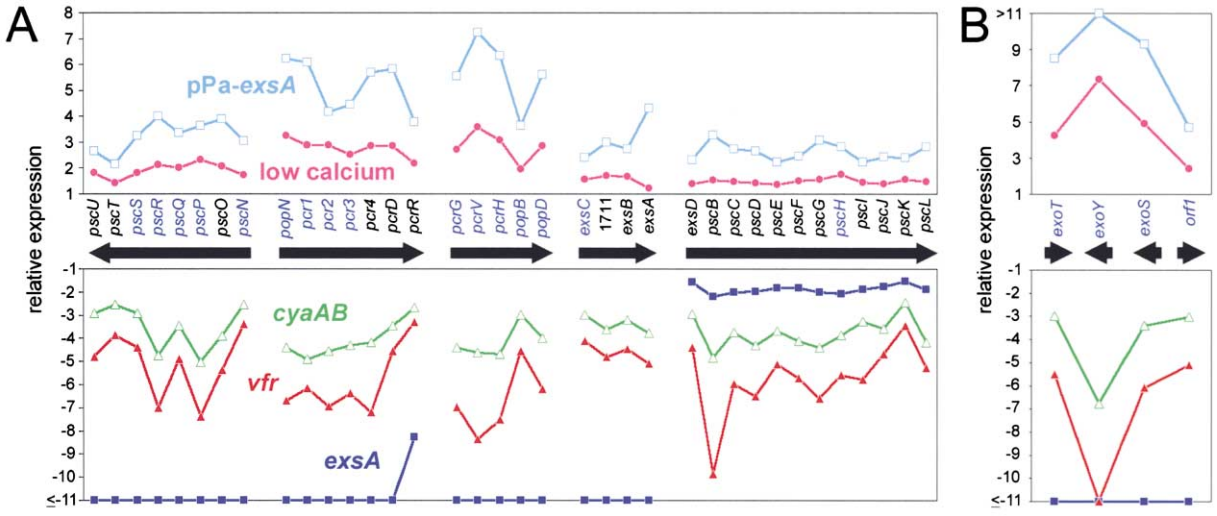

Figure 1. Transcriptional-Profiling Results Indicate Hierarchical Expression of Genes Encoding Components of the Type III Secretion Apparatus and Secreted Effectors

Presented is the average relative expression of known and predicted type III secretion genes; wild-type PAK grown in low-calcium (pink), versus high-calcium, media, overexpression of *exsA* from pPa-*exsA* in wild-type PAK grown under high-calcium conditions relative to the same strain transformed with control plasmid (pPa-*exsA*, light blue), and mutants deleted for *exsA* (blue), *cyaAB* (green), and *vfr* (red) compared with wild-type under calcium-limiting conditions. Genes that showed significant alterations in expression in all profiling experiments are indicated (blue).

(A) Relative expression of genes encoding structural and regulatory components of the secretion apparatus. Operons are indicated by solid arrows and connected data points.

(B) Relative expression of genes encoding effector proteins and the ExoS-specific chaperone (*orf1*). Arrows indicate the direction of transcription relative to the *P. aeruginosa* chromosome.

aeruginosa strain PAK transcriptome, we conducted three profiling experiments using GeneChip *P. aeruginosa* Genome Arrays (Affymetrix). First, gene expression under calcium replete and deplete (secretion-inducing) growth conditions was determined for PAK. Next, *exsA*-dependent gene expression was determined by profiling a nonpolar *exsA* deletion mutant under secretion-inducing conditions (low calcium). In the third experiment, *exsA* was overexpressed from a plasmid (pPa-*exsA*) in wild-type PAK grown in high-calcium media, and the transcriptome was compared with that derived from the same strain carrying the plasmid vector alone.

Average microarray hybridization intensity data for the 5,678 PAK-specific probe sets (Experimental Procedures) is available as Supplemental Data (see Supplemental Table S1 at http://www.developmentalcell.com/cgi/content/full/4/2/253/DC1). Statistical analysis of the derived transcriptomes indicates that 318 genes exhibited calcium-dependent expression, 31 genes showed altered expression in a nonpolar *exsA* deletion mutant compared with wild-type under low-calcium conditions, and 126 genes showed significant expression differences when *exsA* was overexpressed under noninducing high-calcium conditions (see Supplemental Tables S2–S4 at http://www.developmentalcell.com/cgi/content/full/4/2/253/DC1).

The transcriptional response of each of the known TTSS genes was examined under the three profiling conditions (Figure 1). All of the known TTSS genes showed low-calcium induction, with the three known strain PAK effectors (*exoT*, *exoY*, and *exoS*) being the most responsive (Figure 1B). Deletion of *exsA* resulted in an average reduction in expression of greater than 50-fold for the three effector-encoding genes and four additional operons encoding structural and regulatory components of the secretion apparatus (*pscN-pscU*, *popN-pcrR*, *pcrG-popD*, and *exsC-exsA*). In addition, all of the TTSS genes were induced after overexpression of *exsA* in a calcium-independent manner (Figure 1).

Several notable exceptions in the expression pattern of the TTSS genes were discovered. *exsA* only showed a 20% increase in mRNA level when low- and high-calcium growth conditions were compared, making it the least responsive of all TTSS genes. In addition, the *exsD-pscL* operon of structural genes showed a modest induction under low calcium and a less than 2-fold decrease, on average, in the absence of *exsA*.

Identification of Regulators of TTSS-Mediated Host Cell Cytotoxicity

Twenty-four potential upstream regulators of the TTSS were identified by searching the expression profile-generated list of calcium-dependent genes (see Supplemental Table S2) for those that encoded putative transcription factors or proteins with a potential function in signal transduction (Table 1). To establish a more concise list of candidate TTSS regulators, we examined the influence of calcium concentration on gene expression in a second growth medium under conditions known to induce secretion (Experimental Procedures). Of the original 24 candidate regulator-encoding genes, 11 showed a response to alterations in calcium concentration regardless of growth condition (Table 1). The two-component sensor encoding genes *phoQ* and PA4777

Table 1. Low-Calcium-Dependent Genes Encoding Potential Transcriptional Regulator/Signal Transduction Proteins

P.a. ID[a]	Name	Relative Expression[b] TSB OD_{600} 0.5[c]	Relative Expression[b] LB OD_{600} 3.0[d]	Family/Function
PA0048[e]		**−1.62 ± 0.04**	**−1.50 ± 0.09**	**HTH/transcriptional repressor**
PA0149[e]		**−1.70 ± 0.08**	**−1.87 ± 0.09**	**ECF/Sigma-70 factor**
PA0367		2.31 ± 0.02	1.22 ± 0.01	*tetR*/transcriptional regulator
PA1179[e]	***phoP***	**15.65 ± 0.68**	**14.37 ± 0.87**	**two-component response regulator**
PA1180[e]	*phoQ*	14.94 ± 1.58	11.20 ± 1.75	two-component sensor
PA1229[e]		**−1.58 ± 0.05**	**−2.10 ± 0.07**	***ara*C/transcriptional regulator**
PA1336		2.53 ± 0.15	1.35 ± 0.40	two-component sensor
PA1561	*aer*	2.17 ± 0.05	1.04 ± 0.15	MCP/methyl-accepting chemotaxis protein
PA2657		−1.96 ± 0.07	−1.44 ± 0.04	two-component response regulator
PA2692		−1.52 ± 0.01	−1.31 ± 0.16	*gntR*/transcriptional regulator
PA3192[e]	***gltR***	**1.67 ± 0.02**	**1.54 ± 0.16**	**two-component response regulator**
PA3217[e]		**1.68 ± 0.15**	**2.54 ± 0.84**	**probable adenylate cyclase**
PA3599		1.62 ± 0.15	1.14 ± 0.49	*luxR*/transcriptional regulator
PA4197		−2.23 ± 0.12	1.46 ± 0.07	two-component sensor
PA4290		−2.02 ± 0.02	−1.33 ± 0.27	MCP/methyl-accepting chemotaxis protein
PA4493		2.28 ± 0.02	1.03 ± 0.04	two-component response regulator
PA4494		2.18 ± 0.24	1.36 ± 0.07	two-component sensor
PA4776[e]		**18.15 ± 0.27**	**3.21 ± 0.49**	**two-component response regulator**
PA4777[e]		7.04 ± 0.41	1.59 ± 0.24	two-component sensor
PA4853	*fis*	−1.62 ± 0.03	1.02 ± 0.06	HTH/DNA topology
PA4983		1.62 ± 0.10	1.19 ± 0.10	two-component response regulator
PA5116[e]		2.04 ± 0.01	6.85 ± 3.95	*MerR*/transcriptional regulator
PA5262[e]	***algZ***	**2.01 ± 0.16**	**2.81 ± 1.00**	**two-component sensor**
PA5499	*np20*	1.52 ± 0.07	1.06 ± 0.12	*GntR*/transcriptional regulator

[a] P.a. ID is the unique identifier assigned to each *P. aeruginosa* strain PAO1 ORF during annotation of the genome (http://www.pseudomonas.com).
[b] Expression data is presented as fold change under low-calcium growth relative to high-calcium growth. Negative numbers indicate decreased expression in low calcium. The standard error of duplicate experiments is given.
[c] Bacteria were grown in TSB to an OD_{600} of 0.5. For the high-calcium condition, media were supplemented with 5 mM $CaCl_2$. Low-calcium concentrations were achieved by chelating the growth media with a final concentration of 5 mM EGTA.
[d] Bacteria were grown in LB to an OD_{600} of 3.0. For the high-calcium condition, media were treated with 10 mM Nitrilotriacetic acid (NTA) and 20 mM $CaCl_2$ was added. To achieve low-calcium concentrations, we chelated media with 10 mM NTA.
[e] Genes that showed calcium-dependent changes in expression in both growth conditions. Genes in bold were subjected to disruption mutagenesis.

were eliminated from further study, as the list already contained genes for their cognate response regulators (*phoP* and PA4776).

Eight of the nine remaining candidate TTSS regulators were inactivated by a rapid gene disruption technique (Experimental Procedures). Only PA5116 could not be disrupted after repeated attempts. To determine whether any of the inactivated genes were important in regulating the TTSS in response to host cell contact, we assessed each of the corresponding mutants for its cytotoxic effect on cultured CHO cells, as measured by the release of lactate dehydrogenase (LDH). This assay has been previously utilized to identify novel TTSS regulators and to show that mammalian cell killing specifically requires delivery of effectors by the TTSS (Dacheux et al., 2002; Kang et al., 1997). Three mutants showed a dramatic reduction (>85%) in their ability to cause LDH release compared with wild-type (Figure 2A). The most attenuated mutants included those with disrupted genes encoding members of two different two-component regulator systems (*gltR* and *algZ*) and a gene (PA3217) whose product was annotated as a probable adenylate cyclase.

A Candidate Regulator of the TTSS Is Predicted to Encode a Unique Membrane-Associated Adenylate Cyclase

We chose PA3217 for further analysis because of its potential involvement in signaling through the second messenger cAMP. Moreover, the potential role of this probable adenylate cyclase in regulation of the TTSS was intriguing, considering that the genome of *P. aeruginosa* contains a gene for another adenylate cyclase (*cyaA*), sharing a high level of similarity with the class I adenylate cyclases present in the genomes of all bacteria sequenced to date. A more comprehensive sequence analysis of the putative PA3217 gene product indicated the presence of two conserved domains (see Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/4/2/253/DC1). The carboxy-terminal portion of the PA3217 gene product shares defining features and a high degree of homology with the catalytic domain of a growing family of protozoan and eubacterial class III adenylate cyclases (see Supplemental Figure S1) (Danchin, 1993; Liu et al., 1997). The amino terminus of the PA3217-encoded protein is predicted to possess five transmembrane helices and shows homology to a small family of receptor-like domains of unknown function (see Supplemental Figure S1).

Production of cAMP in *P. aeruginosa* by Two Adenylate Cyclases

To determine whether the *P. aeruginosa* gene products of PA3217 and *cyaA* are capable of generating cAMP, we cloned and expressed each gene in an *E. coli* strain defective for cAMP synthesis, owing to a disruption of

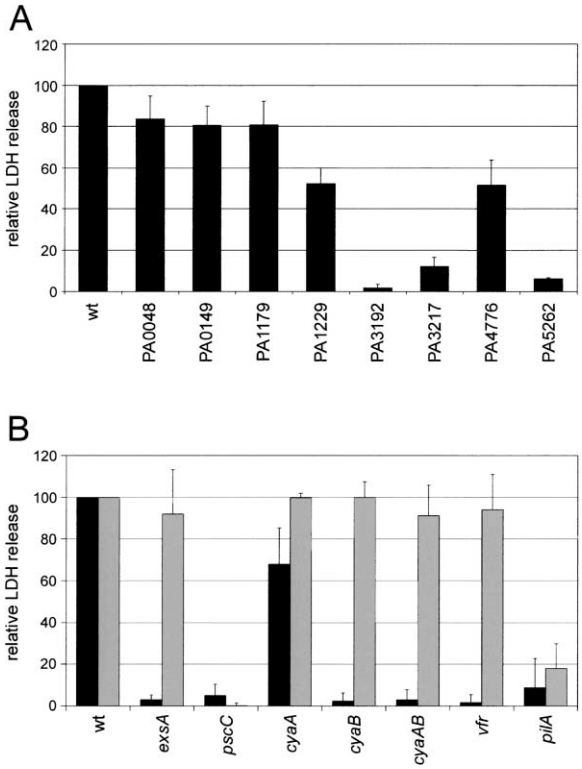

Figure 2. Release of Lactate Dehydrogenase (LDH) from *P. aeruginosa*-Infected CHO Cells

LDH release was quantified with a commercially available assay kit (Roche) after 5 hr of exposure to wild-type and mutant *P. aeruginosa* strains at an infection multiplicity of 10:1. Bars represent average LDH release induced by mutants relative to wild-type.

(A) LDH release from CHO cells infected with *P. aeruginosa* mutants disrupted for calcium-dependent genes encoding putative regulator/signal transduction proteins. Disrupted genes are indicated.

(B) Deletion mutants defective for components of the cAMP-signaling pathway and known TTSS genes show reduced host cell cytotoxicity. Deleted genes are indicated. Black bars represent *P. aeruginosa* strains carrying a control vector, pMMB67EH. Gray bars indicate strains overexpressing *exsA* (pPa-*exsA*). Error bars represent the standard deviation for triplicate experiments.

its sole endogenous adenylate cyclase gene, *cyaA*. Both *P. aeruginosa* genes and a clone of the wild-type *E. coli cyaA* gene restored cAMP-dependent utilization of lactose and maltose (Karimova et al., 1998) in the mutant *E. coli* strain (see Supplemental Figure S2 at http://www.developmentalcell.com/cgi/content/full/4/2/253/DC1).On the basis of this finding, PA3217 was renamed *cyaB*.

To examine the contribution of *cyaB* and *cyaA* to the total cellular cAMP content in *P. aeruginosa*, we constructed nonpolar deletion mutants and measured cAMP levels in wild-type and mutant strains (Figure 3). The steady-state level of cAMP in wild-type PAK increased by approximately 2-fold when grown in low-calcium media compared with high-calcium media. The *cyaA* mutant showed a slight reduction in cAMP levels, while the *cyaB* mutant was severely impaired for cAMP production under both high- and low-calcium growth. Increased levels of cAMP were detected under low-calcium propagation in both mutant backgrounds. On the basis of these results, it appears that CyaB generates the majority of cAMP in *P. aeruginosa*; however, both adenylate cyclases respond to calcium limitation and play a role in cAMP production. A strain deleted for both adenylate cyclase genes (*cyaAB)* showed background levels of cAMP. Complementation of the double mutant with expression plasmids carrying either *cyaB* or *cyaA* (pPa-*cyaB* and pPa-*cyaA*, respectively) resulted in high levels of cAMP (Figure 3).

TTSS-Mediated Cytotoxicity Requires Both cAMP and the cAMP Binding Transcription Factor Vfr

cAMP can act as a transcriptional signaling molecule by binding to, and activating, transcription factors of the CRP family (*c*AMP *r*eceptor *p*roteins). Vfr, a *P. aeruginosa* member of this family, has been implicated in the regulation of twitching motility, quorum sensing, pyocyanin production, repression of flagellar biosynthesis, and the expression of numerous proteins secreted by the type II pathway (Albus et al., 1997; Beatson et al., 2002; Dasgupta et al., 2002; West et al., 1994). In order to determine whether cAMP generated by CyaB and CyaA acts through Vfr, we assessed deletion mutants defective for each for their ability to cause cytotoxicity in CHO cells (Figure 2B). The *cyaA* deletion led to a 30% reduction in host cell LDH release. In contrast, cytotoxicity was substantially more attenuated (>95%) in the *cyaB* deletion mutant as well as in the *exsA* and *pscC* mutants, which are known to be defective in type III secretion. This reduction was more dramatic than that seen in the PA3217 (*cyaB*) disruption mutant, which may have had residual activity because of incomplete inactivation (Figure 2A). The simultaneous deletion of both adenylate cyclase genes (*cyaAB*) did not result in a significant decrease in cytotoxicity compared with the *cyaB* mutant, indicating that CyaB is the major mediator of camp-dependent host contact signaling. The *vfr* mutant showed levels of cytotoxicity similar to those seen in the *cyaB* mutant backgrounds and in the TTSS-defective mutants lacking ExsA and PscC.

The Lack of TTSS-Mediated Host Cell Cytotoxicity in cAMP-Signaling Mutants Is Not Due to a Defect in Host Cell Interaction

Studies have shown that TTSS-mediated host cell cytotoxicity is preceded by interaction of *P. aeruginosa* with host cells via their type IV pili (Tfp) (Comolli et al., 1999; Kang et al., 1997). Mutants in *vfr* show decreased expression of Tfp and Tfp-associated twitching motility (Table 2) (Beatson et al., 2002). To determine whether this defect influences pilus-mediated adherence to host cells, we performed binding assays. A slight reduction in adherence was detected in the *vfr* and double-adenylate cyclase mutants; however, the differences were not statistically significant when compared with wild-type (Table 2). The *cyaB* and *cyaA* mutants showed wild-type levels of adherence, indicating that the TTSS-mediated cytotoxicity defect in these backgrounds is not due to a defect in host cell binding. Strains lacking the TTSS regulator ExsA and the secretion apparatus component PscC appeared to have a slight increase in adherence levels. The nonpiliated *pilA* mutant showed a greater

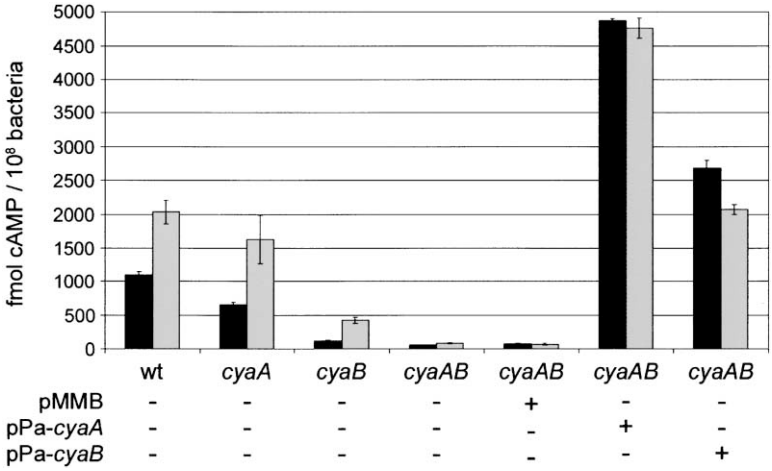

Figure 3. Intracellular cAMP Levels Are Dependent on Two Adenylate Cyclases and Extracellular Calcium Concentration

Intracellular cAMP was measured with an enzyme immunoassay detection kit (Amersham). Wild-type and mutant strains were grown to an OD_{600} of 1.0 in TBS supplemented with 5 mM $CaCl_2$ (black bars) or 5 mM EGTA (gray bars). Bacteria from 1.0 ml of culture were collected by centrifugation, washed twice in phosphate-buffered saline, and sonicated in 500 μl lysis buffer 1B (provided with assay kit). Lysates were assayed according to the manufacturer's protocol. The double-adenylate cyclase mutant was complemented with expression plasmids encoding *cyaA*, *cyaB*, or a vector control (pPa-cyaA, pPa-cyaB, and pMMB, respectively). Values represent the average of three assays. Error bars indicate the standard deviation.

than 10-fold reduction in adherence. This mutant is capable of expressing the TTSS and secreting effectors into culture supernatants (data not shown), but does not induce a cytotoxic response in cultured mammalian cells (Figure 2B). Reduced growth rate in the mutants could also account for reduced cytotoxicity; however, in all cases, the deletion mutants showed rates of growth identical to that of the wild-type parental strain regardless of growth media.

To further rule out possible defects at the level of host cell interaction, we overexpressed *exsA* in each of the mutant backgrounds and assessed their ability to induce a cytotoxic response. When *exsA* was overexpressed from the plasmid pPa-*exsA*, wild-type levels of LDH release were restored in mutants defective for both adenylate cyclase genes and *vfr* (Figure 2B). pPa-*exsA* was also able to complement the *exsA* deletion strain. Cytotoxicity, as assessed by LDH release, was not restored in the *pscC* or *pilA* deletion backgrounds, owing to their inherent secretion and adherence defects, respectively. The restoration of cytotoxicity by overexpression of *exsA* demonstrates that a precursory host cell interaction is established in the *cya* and *vfr* mutants. Moreover, the cAMP signal most likely acts upstream, or at the level, of ExsA.

Role of cAMP in Translation and Secretion of Effectors

To test for possible defects in protein synthesis and secretion, we grew wild-type and mutant strains under high- and low-calcium conditions and examined expression of the TTSS effector ExoS by immunoblotting (Figure 4). Wild-type PAK showed readily detectable ExoS antigen in both supernatant (secreted) and pellet (cell-associated) fractions after growth in low-calcium media. Both single adenylate cyclase mutants showed some accumulation of ExoS in the low calcium fractions, but at reduced levels compared with wild-type. Expression of ExoS in the *cyaB* mutant was slightly more reduced than in a *cyaA* background. The double-adenylate cyclase mutant (*cyaAB*) had no detectable ExoS antigen in the supernatant fractions. A faint ExoS signal was detectable in the bacterial pellet fraction for this mutant, indicating that low levels of ExoS still accumulate, even in the absence of detectable levels of cAMP (Figure 4B). ExoS antigen was not detected in the *vfr* mutant or in the TTSS mutants *exsA* and *pscC*. Complementation of these mutants with *exsA* (pPa-*exsA*) restored ExoS synthesis and calcium-dependent secretion in the *exsA*, *cyaAB*, and *vfr* mutants. This is consistent with the ability of ectopically expressed *exsA* to restore cytotoxicity in

Table 2. Genotype and Phenotype of *P. aeruginosa* Strains Used in This Study

Strain	Defective Alleles (Amino Acids Deleted)	Twitching Motility (mm)[a]	CHO Cell Adherence (Bacteria/Cell)[b]	Reference
PAK	wild-type	29.7 ± 1.3	2.2 ± 1.4	D. Bradley
PAKΔ*pilA*	*pilA* (101–126)	0.0 ± 0.0	0.2 ± 0.1	Kagami et al., 1998
PAKΔ*exsA*	*exsA* (7–279)	30.4 ± 1.1	3.7 ± 2.1	this study
PAKΔ*pscC*	*pscC* (10–567)	29.4 ± 0.9	4.5 ± 1.3	this study
PAKΔ*cyaA*	*cyaA* (12–937)	29.4 ± 1.6	2.7 ± 0.6	this study
PAKΔ*cyaB*	*cyaB* (18–453)	25.9 ± 1.3	3.1 ± 1.7	this study
PAKΔ*cyaAB*	*cyaA* (12–937), *cyaB* (18–453)	20.6 ± 1.8	1.6 ± 0.6	this study
PAKΔ*vfr*	*vfr* (17–214)	12.3 ± 1.8	1.4 ± 0.8	this study

[a] Twitching motility was quantified with a previously published technique (Darzins, 1993). The zone of growth (motility zone) between the agar and plate was measured (millimeters). Measurements represent the average from five assays.

[b] Bacterial adherence to cultured CHO cells was quantified by a modified version of the described technique (Cervin et al., 1994). CHO cells grown to 80–90% confluency ($\sim 2.5 \times 10^5$ cells) were washed, and bacteria derived from exponentially growing cultures were added at a multiplicity of 50 in 1.0 ml of fresh F12 media (Gibco BRL) without serum. After 1 hr incubation at 37°C in the presence of 5% CO_2, nonadherent bacteria were removed by washing twice with 1× phosphate-buffered saline. Coverslips were fixed and stained, and adherent bacteria were quantified microscopically as described (Cervin et al., 1994).

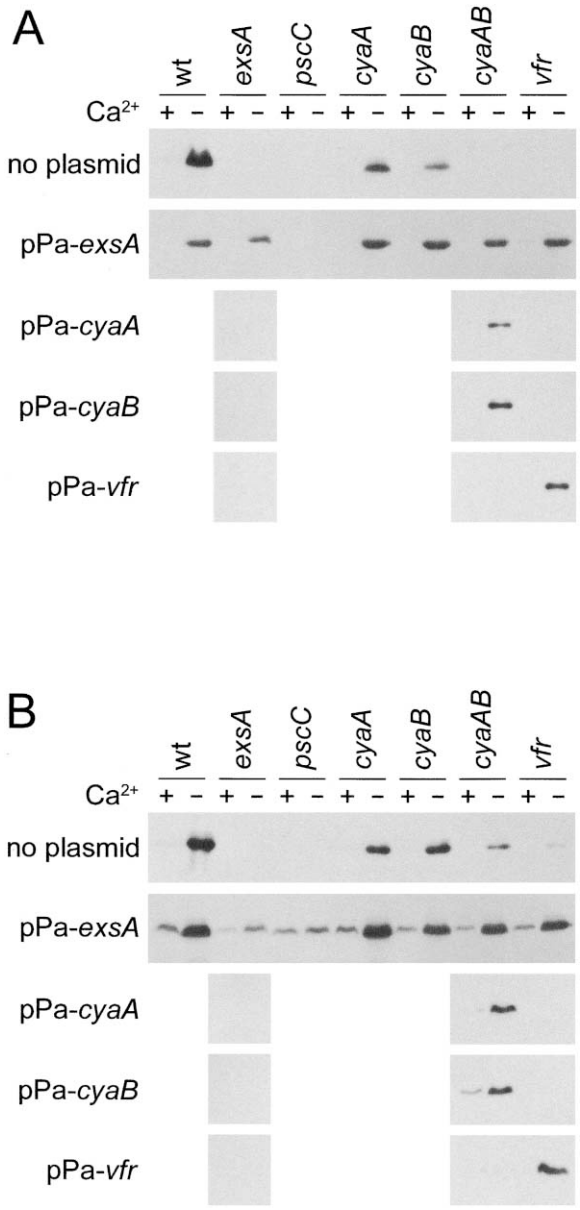

Figure 4. Components of the cAMP-Signaling Pathway and ExsA Are Required for Low-Calcium-Induced Translation and Secretion of ExoS

Wild-type and mutant *P. aeruginosa* strains were grown under high-calcium (+) or low-calcium (−) conditions. Supernatant (A) and pellet (B) fractions derived from an equivalent number of bacteria were analyzed by SDS-PAGE and immunoblotted with ExoS-specific antisera. Deleted genes are indicated. Mutant strains were complemented with expression plasmids encoding *exsA*, *cyaA*, *cyaB*, and *vfr*, as indicated.

each of these mutants (Figure 2B). As expected, overexpression of *exsA* restored ExoS synthesis, but not secretion, in the *pscC* mutant, which lacks an essential component of the secretion machinery (Figure 4). Complementation with plasmid-encoded copies of *cyaA*, *cyaB*, and *vfr* (pPa-*cyaA*, pPa-*cyaB*, and pPa-*vfr*, respectively) restored ExoS expression and secretion only in the corresponding mutants.

Transcriptional Regulation of TTSS Genes by *P. aeruginosa* Adenylate Cyclases and Vfr

In order to determine whether alterations in transcript level can account for the patterns of ExoS synthesis seen above, we constructed a transcriptional reporter in which the *exoS* promoter controlled the expression of the β-galactosidase-encoding gene *lacZ*. When wild-type PAK was grown in low-calcium media, the *exoS* promoter showed a 9-fold increase in activity compared with expression in high-calcium media (Figure 5). Mutants defective for *exsA* were not able to activate *exoS* in response to low calcium. Both of these results are consistent with transcriptional-profiling data (Figure 1) and the expression patterns seen for the ExoS effector protein (Figure 4). The *pscC* mutant was also unaffected by low-calcium stimulation. Both *cya* mutants showed a greater than 60% reduction in low-calcium-dependent activation of the *exoS* promoter compared with wild-type, consistent with the reduction in ExoS protein levels seen by immunoblot analysis (Figure 4). Under low-calcium growth, the double-adenylate cyclase and *vfr* mutants showed a 5- to 6-fold reduction in *exoS* promoter activity compared with wild-type. However, in these mutants, the *exoS* promoter was still slightly responsive to calcium limitation, indicating a possible secondary effect of this extracellular signal on transcription. Complementation of the mutants with pPa-*exsA* resulted in hyperactivity of the *exoS* promoter in the wild-type and in the *cya* and *vfr* mutants (Figure 5). The same plasmid only restored *exoS* promoter activity to wild-type levels in the *exsA* mutant. The absence of hyperactivation in this case is most likely due to the absence of basal *exsA* expression, which is present in the other mutants. Lower activation in the *exsA*-complemented *pscC* mutant is consistent with previous findings that showed negative-feedback regulation in this secretion mutant (Yahr et al., 1996b). Complementation with *cyaA*, *cyaB*, and *vfr* only restored low-calcium-dependent activity in the corresponding mutant backgrounds (Figure 5). This establishes a hierarchy in which the adenylate cyclases and Vfr act upstream of ExsA.

A Novel Regulatory Cascade Coordinates Host-Directed Virulence

To determine the range of genes affected by the cAMP-signaling pathway, we again employed microarrays. Transcriptional profiles for the double-adenylate cyclase and *vfr* mutants were determined under calcium-limiting conditions (see Supplemental Table S1). A total of 206 genes were significantly altered in the *vfr* deletion mutant compared with wild-type, and 181 genes were altered in the double-adenylate cyclase mutant (see Supplemetnal Tables S5 and S6 at http://www.developmentalcell.com/cgi/content/full/4/2/253/DC1). The overlap of responsive genes in both conditions was extensive (162 genes). This observation is in direct support of a model in which the cAMP product generated by the adenylate cyclases acts through Vfr. Interestingly, the magnitude of the expression defect for these genes is greater in the *vfr* mutant than in the double-adenylate cyclase mutant (Figure 1; see Supplemental Tables S5 and S6). This is either due to low levels of cAMP present in the cell or residual affinity of Vfr for promoters in the absence of the cAMP cofactor.

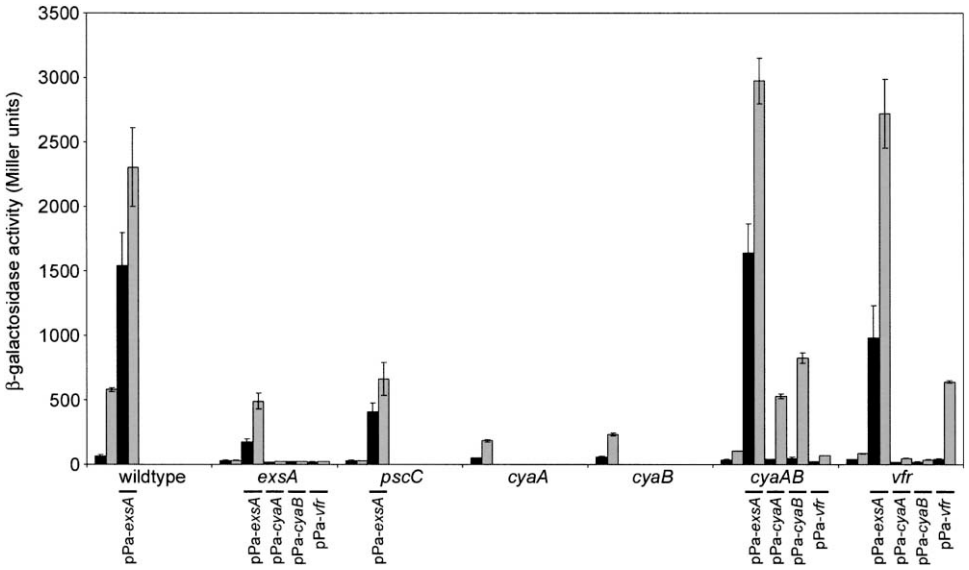

Figure 5. The *P. aeruginosa* cAMP-Signaling Pathway Is Required for *exoS* Transcription

A transcriptional reporter was engineered by cloning a 973 bp DNA fragment carrying the *exoS* promoter upstream of the *lacZ* gene encoding β-galactosidase in plasmid mini-CTX-*lacZ* (Hoang et al., 2000). The resulting construct was moved onto the chromosome of strain PAK and its derivatives as described (Hoang et al., 2000). The effect of *exsA*, *pscC*, and cAMP-signaling pathway deletion mutations on reporter activity was measured (Miller, 1972). Reporter strains were grown in high-calcium (black bars) or low-calcium (gray bars) media (TSB) to an OD_{600} of 1.0–2.0 prior to assays. Mutants were complemented with expression plasmids encoding *exsA*, *cyaA*, *cyaB*, and *vfr*, as indicated. Average values and standard deviations are presented from triplicate assays.

Transcription of all of the known TTSS genes was dependent on adenylate cyclase activity and Vfr (Figure 1). These results are consistent with a model in which the cAMP pathway acts upstream of ExsA. However, 67 of the Vfr-dependent genes showed altered regulation when *exsA* was overexpressed, including 34 genes that are not predicted to be involved in the TTSS. In fact, the remaining Vfr-dependent genes (see Supplemental Tables S1 and S5) showed a similar trend, but did not meet the statistical cutoff. These results indicate that overexpression of *exsA* can activate the cAMP signaling pathway in the absence of the low-calcium signal. An additional outcome of this analysis was the finding that the *exsD-pscL* operon is much more responsive in the *vfr* deletion mutant than in a mutant lacking ExsA.

Examination of the genes showing altered expression in the *vfr* deletion mutant indicated that the components of four well-studied virulence pathways were affected (see Supplemental Figure S3 at http://www.developmentalcell.com/cgi/content/full/4/2/253/DC1). They include the genes involved in biogenesis of Tfp and flagella and in the formation of the TTSS and type II secretion system. In addition, genes that regulate or are predicted to regulate bacterial chemotaxis were also affected, a likely consequence of alterations in the two motility systems.

Discussion

We took advantage of a known TTSS regulatory signal, calcium limitation, to derive a transcriptional profile for *P. aeruginosa*. We also determined the contribution of ExsA, a known regulator of TTSS gene expression, by assessing the transcriptional impact of its depletion and overexpression. Analysis of the transcriptomes generated in these studies provided interesting insights into the pattern of assimilation of regulatory signals during expression of the multicomponent TTSS and its effectors.

While most secretion apparatus component and effector genes were regulated by calcium limitation and ExsA, expression of *exsA* itself was relatively unaffected by calcium limitation. Given these results, we conclude that the low-calcium response does not significantly stimulate *exsA* transcription and that the activating properties of ExsA must be regulated at some other level. In addition, the *exsD-pscL* operon, which encodes components of the secretion apparatus, was expressed regardless of ExsA and calcium limitation. The presence of the *exsD-pscL* transcript under conditions that are not conducive to secretion suggests that the assembly of this complex protein delivery machinery may proceed in two stages, similar to the ordered assembly of the highly related flagellar structure. A structure consisting of the products of the *exsD-pscL* operon could be constitutively present in the cell envelope or could be assembled rapidly under secretion-inducing conditions. Either scenario may facilitate the subsequent assembly and localization of additional apparatus components. This is consistent with published reports and our observation that the absence of PscC, the outer membrane secretin component of the secretion apparatus encoded within the *exsD-pscL* gene cluster, results in a complete block in expression of all genes specifying TTSS components (data not shown; Yahr et al., 1996b) and effectors (Figures 4 and 5). It has been recently shown that ExsD, encoded by the first gene of the *exsD-pscL* operon, acts as a repressor of TTSS genes and binds to ExsA (McCaw

et al., 2002). This is consistent with a model in which the products of the *exsD-pscL* operon and *exsA* are constitutively expressed in the absence of a secretion-inducing signal. Interaction between ExsA and ExsD under these conditions represses the expression of additional TTSS genes. Under secretion-inducing conditions repression is relieved by an unknown mechanism, allowing the expression of additional secretion components, apparatus assembly, and secretion.

The microarray analysis also resulted in the identification of a number of genes encoding putative regulatory elements that could account for TTSS expression in response to environmental signals. To verify that such genes may be part of a virulence regulon or, more specifically, involved in the host cell contact-dependent regulation of the TTSS, we engineered strains defective for each and assessed their ability to induce a cytotoxic effect in cultured CHO cells. Mutations in three genes (*algZ*, *gltR*, and PA3217) resulted in significant attenuation of cytotoxicity. We did not find a correlation between the magnitude of change in expression level and effect on cytotoxicity for this set of regulators. In fact, the three candidate regulators that showed the greatest attenuation of cytotoxicity had only modest, but reproducible, expression changes, which may reflect transcriptional fine-tuning. The protein product of these candidate genes may be regulated primarily at the functional level. We conclude that reproducible changes in expression may be as important as magnitude when predicting involvement of genes in a particular pathway.

Loss of cytotoxicity due to insertional inactivation of *algZ* is very likely due to the lack of expression of Tfp (Whitchurch et al., 1996), which are an absolute requirement for the establishment of an intimate contact between *P. aeruginosa* and its target cell. In an adhesion assay, this mutant was unable to attach to CHO cells (data not shown). GltR is the response regulator of a two-component system previously shown to be involved in glucose transport in *P. aeruginosa* (Sage et al., 1996). The involvement of GltR in cytotoxicity is unclear. Unlike the *algZ* mutant the *gltR* mutant showed normal adhesion to CHO cells (data not shown).

The third calcium-responsive gene (PA3217) was annotated as a putative adenylate cyclase. We demonstrate that it does, in fact, encode a protein with adenylate cyclase activity, both in *E. coli* and in *P. aeruginosa,* and have named it *cyaB*. We also show that a second unrelated *P. aeruginosa* adenylate cyclase, CyaA, has activity in *E.coli* and *P. aeruginosa*. This raised the possibility that cAMP may regulate a subset of genes that are coordinated with the TTSS in *P. aeruginosa*. This is supported by recent studies that have shown that cAMP is involved in the regulation of the *Yersinia* TTSS as well as in the regulation of host-specific virulence determinants in *Vibrio cholerae* (Li et al., 2002; Petersen and Young, 2002; Skorupski and Taylor, 1997). Using a series of defined mutants, we analyzed the relative contributions of the two adenylate cyclases to the generation of cAMP. Under the experimental conditions examined, the total cAMP pool in *P. aeruginosa* required the expression of both enzymes (Figure 3), and only a double mutant (*cyaAB*) was devoid of detectable levels of cAMP. Interestingly, calcium-limiting conditions lead to an increase in the levels of cAMP in both *cyaA* and *cyaB* backgrounds; we were unable, however, to detect any effects of calcium limitation on the expression of *cyaA* using microarrays. This suggests that calcium may regulate the activity of CyaA and control both the expression of *cyaB* and the activity of its protein product.

We used several additional assays to evaluate the role of cAMP and the cAMP binding protein Vfr on the expression of the TTSS. Levels of secreted ExoS protein and transcription from the *exoS* promoter showed similar dependence on CyaA and CyaB, with only a double *cyaA* and *cyaB* mutation or deletion of *vfr* resulting in a complete lack of expression of ExoS under calcium-limiting conditions. When utilizing a cell cytotoxicity assay as a measure of TTSS function, we observed a more severe defect in a *cyaB* mutant than in a strain lacking *cyaA*. This difference may reflect the transmission of a second signal through the cAMP pathway, namely, attachment of the bacteria to host cells. In fact, work by Wolf-Watz and coworkers showed that adherence of *Y. pseudotuberculosis* to host cells leads to upregulation of TTSS genes, even in calcium-replete medium (Pettersson et al., 1996). This indicates that two unrelated signals (limiting calcium and host cell contact) could independently activate TTSS gene expression. We conclude that, in *P. aeruginosa*, these two signals both act through the cAMP pathway. The observed differences between the phenotypes of *cyaA* and *cyaB* mutants can be explained in terms of differential expression and activity of each enzyme in response to these specific environmental cues. It is noteworthy that CyaB and all of the class III adenylate cyclases possess several putative transmembrane segments, which suggests membrane localization. Consequently, CyaB may act as a membrane sensor that detects contact with host cells.

Several observations indicate that activation of the cAMP-signaling pathway alone does not fully account for the low-calcium response. First, overexpression of either adenylate cyclase gene, which results in high levels of intracellular cAMP (Figure 3), does not activate transcription, translation, or secretion of *exoS* and its product in high-calcium media (Figures 4 and 5). Second, calcium limitation is required for in vitro secretion of ExoS, regardless of transcription and translation (Figure 4). These results demonstrate that transcriptional activation of the TTSS components and secretion of effectors are genetically separable events. In addition, these findings suggest that the low-calcium signal is either sensed by two separate pathways or that these two pathways are linked by a common upstream sensing mechanism. Interestingly, overexpression of *exsA* from a plasmid under high-calcium (nonsecretion) conditions results in the upregulation of non-TTSS genes that are dependent on Vfr and cAMP. This result suggests that overexpression of *exsA* can activate the cAMP signaling pathway in the absence of the low-calcium signal. The same genes were not affected by deletion of *exsA* or *pscC* (data not shown).

Defects in the cAMP-signaling pathway were complemented by overexpression of *exsA* with regard to *exoS* transcription, expression, and host cell cytotoxicity (Figures 2, 4 and 5). In addition, overexpression of *cyaA*, *cyaB*, and *vfr* were not sufficient to complement an *exsA* mutant (Figures 4 and 5). This establishes a genetic hierarchy in which cAMP and Vfr act upstream, or at the

level, of ExsA. It remains to be determined whether Vfr directly influences *exsA* transcription or the transcription of other TTSS genes. No sequence similar to either of the two published Vfr binding sites could be identified in the vicinity of known TTSS genes, including *exsA*. While it is not known what, if anything, activates ExsA, it can be envisioned that Vfr affects ExsA activity directly by acting as a coactivator. As such, Vfr could increase the affinity of ExsA for TTSS gene promoters. Overexpression of exsA could bypass the need for Vfr, which would be consistent with the above results.

It is clear that expression of all TTSS genes in response to environmental signals (both calcium limitation and host cell contact) requires adenylate cyclase activity and the transcriptional regulator Vfr, including the *exsD-pscL* operon (which is only marginally dependent on *exsA*) (Figure 1). Furthermore, transcriptional profiling of *cya* and *vfr* mutants indicates that cAMP-mediated signaling extends beyond the TTSS. A total of 162 genes whose expression was dependent on cAMP and Vfr were identified. This is consistent with recent findings that the expression of more than 60 proteins in *P. aeruginosa* are dependent on Vfr (Suh et al., 2002). The products of the genes identified in our study predominantly fit into four discrete virulence pathways (see Supplemental Figure S3): biogenesis of Tfp and flagella (involved in attachment to host cells and motility) and components of the type II and III secretion systems (required for the delivery of host-specific toxin and effectors). The regulation of genes in these pathways is consistent with a model in which cAMP signaling allows *P. aeruginosa* to sense the human host environment. In this model, change in the concentration of calcium and/or contact with the surface of a host cell is transmitted to an adenylate cyclase, which is very likely the membrane-associated CyaB protein. Production of cAMP, together with Vfr, activates the expression of host cell-specific virulence factors. Upregulation of Tfp (which mediates polar attachment) and concomitant downregulation of flagellar expression (which occupies the same pole as pili) may promote a tighter interaction between *P. aeruginosa* and host cells after initial contact is made. This interaction is essential for the subsequent translocation of TTSS effectors. Coordination with the type II secretion system would also be advantageous, since proteins secreted by this pathway have specific targets within host cells.

We have exploited DNA microarrays to define novel global regulatory networks that control the expression of virulence genes in *P. aeruginosa*. Our discovery that cAMP plays a role in contact-dependent signaling raises interesting questions about the coevolution of signal transduction pathways with specific coregulated virulence genes. The range of cAMP-regulated genes in *P. aeruginosa* appears to consist largely of genes directly or indirectly associated with the pathogenic life stage of this organism. Particularly noteworthy is the role of CyaB, an apparently redundant adenylate cyclase in *P. aeruginosa*, which, due to its membrane localization, may serve as a primary sensor for the assimilation of host-generated signals and further enhance the ability of this common environmental organism to cause a broad range of life-threatening human infections.

Experimental Procedures

P. aeruginosa Strains and Deletion Mutants

All mutant *P. aeruginosa* strains used in this study were derived from the wild-type clinical isolate PAK (D. Bradley). Deletion alleles were assembled by removing an internal fragment of coding sequence from each gene by a modified version of the polymerase chain reaction (PCR)-based technique termed splicing by overlap extension (SOE) (Warrens et al., 1997). Table 2 indicates the mutant alleles constructed for this study and the region deleted, in amino acids, from the relevant gene product. PCR reactions were carried out with the GC-Rich PCR System (Roche). Specific primer sequences are available upon request (M. Wolfgang). Flanking primers were designed to anneal 800–1200 base pairs (bp) upstream and downstream of each deletion/splice junction, respectively. These primers were tailed with *att*B1 and *att*B2 sequences, respectively, as described in the Gateway Cloning Technology Instruction Manual (Invitrogen). The resulting PCR products were cloned with Gateway Technology into pEXGmGW, which was adapted from pEX18Gm (Hoang et al., 1998) with the Gateway Vector Conversion Kit (Invitrogen). Mutant alleles were introduced onto the chromosome of PAK as described (Hoang et al., 1998).

Type III Secretion-Inducing Growth Conditions

For expression-profiling experiments, strain PAK and its isogenic derivatives were grown in tryptic soy broth (TSB; Difco) supplemented with either 5 mM $CaCl_2$ (high calcium) or 5 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA; low calcium) to mid-exponential growth phase (OD_{600} 0.5). For strains carrying expression plasmids, carbenicillin (150 μg/ml) was added to the media as well as IPTG (500 μM). For alternate secretion-inducing conditions, PAK was grown to late-exponential growth phase (OD_{600} 3.0) in Luria-Bertani medium (LB) with high and low calcium. Calcium depletion was achieved by treating LB media with the chelating agent nitrilotriacetic acid (NTA) to a final concentration of 10 mM. To create high-calcium media, we added $CaCl_2$ (20 mM final) to the NTA treated LB.

Rapid Gene Disruption

An internal fragment of each target gene was amplified by PCR. Products, which ranged in size from 250–500 bp, were tailed with *att*B1 and *att*B2 sequences by inclusion in the PCR primers. The products were cloned with Gateway Technology (Invitrogen) in pEXGmGW. Chromosomal integration was selected as described (Hoang et al., 1998) and maintained by propagation in the presence of gentamicin (75 μg/ml).

Expression Clones

Complete open reading frames (ORFs) were PCR-amplified with primers corresponding to the coding sequence for the first eight amino acid residues and the reverse complement of the nucleotide sequence for the last eight amino acid residues of the full-length polypeptides. In all cases the start and stop codons were set to ATG and TGA, respectively, regardless of the actual sequence. The 5′ primer (encompassing the start codon) was tailed with 5′-G**AG GAGG**ATATTC. The underlined sequence introduces a ribosome binding site upstream of each ORF. The 3′ primer (encompassing the stop codon) was tailed with 5′-AGAAAGCTGGGTT. After the first round of PCR (20 cycles), 5 μl of a 50 μl reaction was used as template in a second reaction containing $ATTB1^{start}$ (5′-GGGGACAA GTTTGTACAAAAAAGCAGGCTCGAGGAGGATATTCATG) and $ATT B2^{stop}$ (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTTCA). Products were cloned with Gateway Technology (Invitrogen) into pMMBGW. pMMBGW was adapted from pMMB67EH (Furste et al., 1986) with the Gateway Vector Conversion Kit (Invitrogen). Expression plasmids were moved into *P. aeruginosa* strains by conjugation (Furste et al., 1986) and selection on carbenicillin (150 μg/ml).

Probe Specificity and Microarray Techniques and Analysis

GeneChip *Pseudomonas aeruginosa* Genome Array probe sets specific for strain PAK were determined by hybridization of chromosomal DNA. Details are provided in the Supplemental Experimental Procedures at http://www.developmentalcell.com/cgi/content/full/

4/2/253/DC1. Protocols for RNA preparation, microarray hybridization, and data analysis are included in the Supplemental Experimental Procedures.

Bacterial Fractionation and Immunoblotting

Overnight bacterial cultures were diluted 1:50 into TSB with either 5 mM $CaCl_2$ or 5 mM EGTA and grown for 4 hr at 37°C and 300 rpm. The OD_{600} of the resulting culture was typically between 2.0–3.0. A total of 1.4 ml of the culture was transferred to a microfuge tube, and the bacteria were sedimented by centrifugation at 15,000 × g for 15 min. A total of 1.0 ml of the supernatant was precipitated with trichloroacetic acid (TCA). The bacteria pellet was resuspended in 1.0 ml of phosphate-buffered saline and TCA precipitated. Proteins in the supernatant and pellet fractions were resuspended in 50 or 70 μl of sample buffer (Motley and Lory, 1999), respectively. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, probed with antibody to ExoS, and visualized by ECL as described (Motley and Lory, 1999). ExoS-specific antibodies were the generous gift of D. Frank.

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