JOURNAL OF BACTERIOLOGY, Dec. 2007, p. 8667–8676 Vol. 189, No. 23
0021-9193/07/$08.00+0 doi:10.1128/JB.01344-07

Local and Global Regulators Linking Anaerobiosis to *cupA* Fimbrial Gene Expression in *Pseudomonas aeruginosa*▽†

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Received 17 August 2007/Accepted 10 September 2007

The *cupA* gene cluster of *Pseudomonas aeruginosa* encodes components and assembly factors of a putative fimbrial structure that enable this opportunistic pathogen to form biofilms on abiotic surfaces. In *P. aeruginosa* the control of *cupA* gene expression is complex, with the H-NS-like MvaT protein functioning to repress phase-variable (on/off) expression of the operon. Here we identify four positive regulators of *cupA* gene expression, including three unusual regulators encoded by the *cgrABC* genes and Anr, a global regulator of anaerobic gene expression. We show that the *cupA* genes are expressed in a phase-variable manner under anaerobic conditions and that the *cgr* genes are essential for this expression. We show further that *cgr* gene expression is negatively controlled by MvaT and positively controlled by Anr and anaerobiosis. Expression of the *cupA* genes therefore appears to involve a regulatory cascade in which anaerobiosis, signaled through Anr, stimulates expression of the *cgr* genes, resulting in a concomitant increase in *cupA* gene expression. Our findings thus provide mechanistic insight into the regulation of *cupA* gene expression and identify anaerobiosis as an inducer of phase-variable *cupA* gene expression, raising the possibility that phase-variable expression of fimbrial genes important for biofilm formation may occur in *P. aeruginosa* persisting in the largely anaerobic environment of the cystic fibrosis host lung.

The gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic pathogen of humans that is notorious for being the principal cause of morbidity and mortality in cystic fibrosis (CF) patients; chronic colonization of the CF lung by *P. aeruginosa* typically leads to progressive lung damage and eventually respiratory failure and death (13). In the CF lung the organism is thought to persist as a biofilm, forming clusters of cells encased in a polymeric matrix (32). In this biofilm mode of growth *P. aeruginosa* exhibits increased resistance to antibiotics and is better able to evade the host immune response (6). Recent evidence suggests that the microbial environment in the CF lung is largely anaerobic (43) and that cells of *P. aeruginosa* persist in the CF lung in anaerobic biofilms (47). Indeed, the biofilm formed by cells of *P. aeruginosa* under anaerobic conditions is especially robust (47).

The *cupA* gene cluster of *P. aeruginosa* encodes components of a putative fimbrial structure that enables this organism to form biofilms on abiotic surfaces (36). Under standard laboratory growth conditions, expression of the *cupA* gene cluster is tightly repressed by MvaT (37), a putative transcription regulator that is thought to functionally resemble members of the H-NS family of nucleoid-associated proteins (34). MvaT from *P. aeruginosa* was originally identified as a global regulator of virulence gene expression (7), and recent microarray analyses have revealed that MvaT controls the expression of at least 150 or so genes in *P. aeruginosa*, with the *cupA* genes being the most tightly repressed (37). Several of the genes within the MvaT regulon are implicated in virulence, and a preponderance of MvaT-controlled genes encode components of putative adhesive structures or surface proteins such as fimbriae (37).

Recent findings indicate that the control of *cupA* gene expression in *P. aeruginosa* is complex. In particular, we have found that in the absence of MvaT, expression of the *cupA* fimbrial gene cluster is phase variable (i.e., the gene cluster exhibits reversible on/off expression) (38). The diversity in the bacterial population that results from phase-variable expression of the *cupA* fimbrial genes might impart a fitness advantage. Although we previously observed phase-variable expression of the *cupA* genes in an *mvaT* mutant background, we did not know whether phase-variable expression of the *cupA* genes could occur in wild-type cells.

Here we present evidence that the *cupA* genes are expressed in a phase-variable manner when wild-type cells of *P. aeruginosa* are grown under anaerobic conditions. Moreover, we identified components of the regulatory network that positively regulates *cupA* gene expression under these conditions. In particular, using a genetic screen, we identified four positive regulators of *cupA* gene expression. These include Anr, a global regulator of anaerobic gene expression, and three regulators whose effects on gene expression are localized primarily to the *cupA* genes. The three local regulators are encoded by the *cgrABC* genes that reside in a putative operon situated immediately upstream of the *cupA* gene cluster. The products of the *cgr* genes do not resemble any classical positive regulator of gene expression; *cgrA* encodes a hypothetical protein of unknown function, whereas *cgrB* encodes a putative acetylase and *cgrC* encodes a protein with homology to the ParB family of DNA-binding proteins that are typically involved in DNA segregation. We show that all three of the *cgr* genes are required for phase-variable expression of the *cupA* genes, either in the context of an *mvaT* mutant background or when wild-type cells are grown anaerobically. We show further that *cgr* gene expression is subject to control by MvaT, Anr, and anaer-

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† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 21 September 2007.

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

Strain or plasmid	Description	Source or reference
Strains		
PAO1	Wild type	Arne Rietsch (Case Western Reserve University)
PAO1 *cupA lacZ*	PAO1 containing chromosomal *cupA1 lacZ* reporter	38
PAO1 Δ*mvaT cupA lacZ*	PAO1 *cupA lacZ* containing deletion of *mvaT*	38
PAO1 ΔPA2127 *cupA lacZ*	PAO1 *cupA lacZ* containing deletion of PA2127 gene	This study
PAO1 ΔPA2126 *cupA lacZ*	PAO1 *cupA lacZ* containing deletion of PA2126 gene	This study
PAO1 Δ*mvaT* ΔPA2127 *cupA lacZ*	PAO1 Δ*mvaT cupA lacZ* containing deletion of PA2127 gene	This study
PAO1 Δ*mvaT* ΔPA2126 *cupA lacZ*	PAO1 Δ*mvaT cupA lacZ* containing deletion of PA2126 gene	This study
PAO1 Δ*mvaT* PA2127 *cupA lacZ*	PAO1 Δ*mvaT cupA lacZ* containing partial deletion of PA2127 gene	This study
PAO1 Δ*mvaT* PA2126.1 *cupA lacZ*	PAO1 Δ*mvaT cupA lacZ* containing partial deletion of PA2126.1 gene	This study
PAO1 Δ*mvaT* PA2126 *cupA lacZ*	PAO1 Δ*mvaT cupA lacZ* containing partial deletion of PA2126 gene	This study
PAO1 PA2126 *lacZ*	PAO1 containing chromosomal PA2126 gene-*lacZ* reporter	This study
PAO1 Δ*mvaT* PA2126 *lacZ*	PAO1 PA2126 *lacZ* containing deletion of *mvaT*	This study
PAO1 *attB*::pPA2127-*lacZ*	PAO1 containing chromosomal pPA2127-*lacZ* reporter integrated at ϕCTX attachment site	This study
PAO1 Δ*mvaT attB*::pPA2127-*lacZ*	PAO1 *attB*::pPA2127-*lacZ* containing deletion of *mvaT*	This study
PAO1 Δ*anr cupA lacZ*	PAO1 *cupA lacZ* containing deletion of *anr*	This study
PAO1 Δ*anr* Δ*mvaT cupA lacZ*	PAO1 Δ*mvaT cupA lacZ* containing deletion of *anr*	This study
Plasmids		
pPSV35	Shuttle vector with gentamicin resistance gene (*aacC1*), PA origin, *lacI*^{q}, and *lacUV5* promoter	24
pPSV35-1	pPSV35 with genomic DNA fragment from *P. aeruginosa* strain PAK containing part of PA2125 gene through part of PA2129 gene	This study
pPSV35-2	Derivative of pPSV35-1 containing part of PA2127 gene-*cupA1* intergenic region through part of PA2129 gene	This study
pPSV35-3	Derivative of pPSV35-1 containing part of PA2125 gene through part of PA2127 gene-*cupA1* intergenic region	This study
pPSV35-4	pPSV35 with genomic DNA fragment from *P. aeruginosa* strain PAK containing *apt* and *anr*	This study
p2127	PA2127 gene from PAO1 cloned into pPSV35	This study
p2126.1	PA2126.1 gene from PAO1 cloned into pPSV35	This study
p2126	PA2126 gene from PAO1 cloned into pPSV35	This study
p2127-2126.1	PA2127 and PA2126.1 genes from PAO1 cloned into pPSV35	This study
p2126.1-2126	PA2126.1 and PA2126 genes from PAO1 cloned into pPSV35	This study
p2126-2127	PA2126 and PA2127 genes from PAO1 cloned into pPSV35	This study
pM	pMMB67EH vector with carbenicillin resistance gene (*bla*), *lacI*^{q}, and *tac* promoter	12
pM-MvaT	*mvaT* from PAO1 cloned into pMMB67EH	38
pAnr	*anr* from PAO1 cloned into pPSV35	This study

obiosis. Our finding that anaerobiosis induces phase-variable *cupA* gene expression raises the possibility that phase-variable expression of fimbrial genes important for biofilm formation may occur in *P. aeruginosa* persisting in the CF host lung, where the microbial environment is thought to be largely anaerobic.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. All *P. aeruginosa* strains used in this study are listed in Table 1. *Escherichia coli* DH5αF′IQ (Invitrogen) was used as the recipient strain for all plasmid constructs, and *E. coli* strain SM10 was used to mate plasmids into *P. aeruginosa*. *P. aeruginosa* was grown in LB for all experiments except those whose results are shown in Fig. 5; in the latter experiments LB with 2.5 g/liter NaCl and 1% KNO_3 (LBN) was used as the growth medium. When *E. coli* was grown, antibiotics were used when necessary at the following concentrations: gentamicin, 15 μg/ml; carbenicillin, 100 μg/ml; and tetracycline, 5 μg/ml. When *P. aeruginosa* was grown, antibiotics were used when necessary at the following concentrations: gentamicin, 25 μg/ml for liquid cultures and 60 μg/ml for solid media; carbenicillin, 300 μg/ml; and tetracycline, 200 μg/ml. Phase-on and phase-off colonies were visualized following growth on LB agar or LBN agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (75 μg/ml). Anaerobic growth conditions were achieved using an anaerobic growth chamber together with a gas pack (AnaeroPack system; PML Microbiologicals). To confirm that anaerobic conditions were attained, in each experiment a Δ*anr* strain (PAO1 Δ*anr cupA lacZ*; see below) was incubated alongside the experimental strain(s). As reported previously, *anr* mutants do not grow under anaerobic conditions in which nitrate is used as the terminal electron acceptor (46, 48).

Construction of strains and plasmids. Deletion constructs for the PA2127 and PA2126 genes were generated by amplifying flanking regions by PCR and then splicing the flanking regions together by overlap extension PCR. The deletions were in frame and contained the following linker sequences: 5′-GAATTC-3′ and 5′-AAGCTTTGGGAG-3′, respectively. The resulting PCR products were then cloned into plasmids pEX18Gm (17) and pEXG2 (24), yielding plasmids pEX-ΔPA2127 and pEX-ΔPA2126. These plasmids were then used to create strains PAO1 ΔPA2127 *cupA lacZ*, PAO1 ΔPA2126 *cupA lacZ*, PAO1 Δ*mvaT* ΔPA2127 *cupA lacZ*, and PAO1 Δ*mvaT* ΔPA2126 *cupA lacZ* containing in-frame deletions of the PA2127 and PA2126 genes by allelic exchange. Deletions were confirmed by PCR. Strains PAO1 *cupA lacZ* and PAO1 Δ*mvaT cupA lacZ* have been described previously (38).

Partial deletion constructs for the PA2127, PA2126.1, and PA2126 genes were generated using the same principle, and this yielded plasmids pEX-PΔ2127, pEX-PΔ2126.1, and pEX-PΔ2126, which allowed deletion of all but the last codon of the PA2127 gene, replacement of the intergenic region between the PA2127 and PA2126 genes by the linker 5′-AAGCTT-3′, and deletion of all but the 27 first codons of the PA2126 gene, respectively. These plasmids were then used to create strains PAO1 Δ*mvaT* PA2127 *cupA lacZ*, PAO1 Δ*mvaT* PA2126.1 *cupA lacZ*, and PAO1 Δ*mvaT* PA2126 *cupA lacZ* containing partial deletions of

the PA2127, PA2126.1, and PA2126 genes, respectively, by allelic exchange. Deletions were confirmed by PCR.

The PA2126 *lacZ* reporter strain contained the *lacZ* gene integrated immediately downstream of the PA2126 gene on the PAO1 chromosome and was made by allelic exchange. Flanking PCR products were amplified and spliced together in order to add KpnI, NcoI, and SphI sites one base after the PA2126 gene stop codon. The resulting PCR product was cloned on a SacI/PacI fragment into pEXG2 (24), yielding plasmid pEXG2-PA2126. The *lacZ* gene was subsequently cloned into this construct on a KpnI/SphI fragment derived from plasmid pP18-*lacZ* (Arne Rietsch, unpublished data), generating plasmid pEXG2-PA2126-*lacZ*. This plasmid was then used to create reporter strains PAO1 PA2126 *lacZ* and PAO1 Δ*mvaT* PA2126 *lacZ* by allelic exchange.

The PA2127 *lacZ* reporter strain contained the promoter region of the PA2127 gene (pPA2127) cloned upstream of *lacZ* and inserted at the ϕCTX attachment site on the PAO1 chromosome (18). The 637 bp of DNA upstream of the PA2127 gene start codon was amplified by PCR and cloned upstream of *lacZ* in the Mini CTX *lacZ* vector (18), yielding plasmid Mini CTX PA2127-*lacZ*. This plasmid was then used essentially as described previously (18) to introduce the pPA2127-*lacZ* fusion at the ϕCTX attachment site on the chromosomes of PAO1 and PAO1 Δ*mvaT*, creating reporter strains PAO1 *attB*::pPA2127-*lacZ* and PAO1 Δ*mvaT attB*::pPA2127-*lacZ*, respectively.

A deletion construct for *anr* was generated by amplifying flanking regions by PCR and then splicing the flanking regions together by overlap extension PCR. The deletion was in frame and contained the linker sequence 5′-GAATTC-3′. The resulting PCR product was then cloned into plasmid pEX18Gm (17), yielding plasmid pEX-Δ*anr*. This plasmid was then used to create strains PAO1 Δ*anr cupA lacZ* and PAO1 Δ*mvaT* Δ*anr cupA lacZ*, each containing an in-frame deletion of *anr*, by allelic exchange. The *anr* deletion in each strain was confirmed by PCR.

The *P. aeruginosa* genomic DNA library was a gift from Arne Rietsch (Case Western Reserve University). The library was made from genomic DNA of *P. aeruginosa* strain PAK that had been partially digested with Sau3AI and size fractionated. DNA in the 2- to 5-kb size range was cloned into BamHI-digested pPSV35 (24) to make the library. Plasmids pPSV35-1 (see Fig. 1) and pPSV35-4 (see Fig. 4) were isolated from the library. To make pPSV35-2, a HindIII fragment (containing the PA2126, PA2126.1, and PA2127 genes) was excised from pPSV35-1, and the backbone was recircularized. The same HindIII fragment was subcloned into pPSV35, generating plasmid pPSV35-3.

Plasmids p2127, p2126.1, p2126, and pAnr are derivatives of pPSV35 and direct the synthesis of the PA2127, PA2126.1, PA2126, and Anr proteins, respectively, under control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *lacUV5* promoter. The plasmids were made by cloning PCR-amplified DNA fragments containing each of the PA2127, PA2126.1, PA2126, and *anr* genes from *P. aeruginosa* strain PAO1 into pPSV35.

Plasmids p2127-2126.1 and p2126.1-2126 were made by cloning PCR-amplified DNA fragments containing either the PA2127 and PA2126.1 genes or the PA2126.1 and PA2126 genes from *P. aeruginosa* strain PAO1 into pPSV35. Plasmid p2126-2127 was made by cloning a PCR-amplified DNA fragment containing the PA2126, PA2126.1, and PA2127 genes from *P. aeruginosa* strain PAO1 into pPSV35. Plasmid pM-MvaT directs the synthesis of *P. aeruginosa* MvaT under control of the IPTG-inducible *tac* promoter and has been described previously (38). Plasmid pMMB67EH, from which pM-MvaT was derived, has been described previously (12).

β-Galactosidase assays. Cells were grown at 37°C either in LB with aeration or in LBN under anaerobic conditions. Media were supplemented as needed with gentamicin (25 μg/ml) or carbenicillin (300 μg/ml) and IPTG at the concentration indicated. Cells were permeabilized with sodium dodecyl sulfate and $CHCl_3$ and assayed for β-galactosidase activity as described previously (8). Assays were performed at least three times in triplicate on separate occasions. Representative data sets are shown below. The values are averages based on one experiment.

Microarray experiments. Cells of PAO1 containing plasmid pPSV35-3 or pPSV35 were grown with aeration at 37°C in LB containing gentamicin (25 μg/ml). Duplicate cultures of each strain were inoculated at a starting optical density at 600 nm of 0.01 and grown to an optical density at 600 nm of ~0.5 (corresponding to the mid-logarithmic phase of growth). RNA isolation, cDNA synthesis, and cDNA fragmentation and labeling were performed essentially as described previously (42). Labeled samples were hybridized to Affymetrix GeneChip *P. aeruginosa* genome arrays (Affymetrix). Data were analyzed for statistically significant changes in gene expression using GeneSpring GX. The genes whose expression changed fivefold or more, with a P value of ≤0.01, are listed in Table 2.

TABLE 2. Microarray analysis of genes controlled by overexpression of the PA2126 and PA2127 genes during the mid-logarithmic phase of growth

Gene	Designation	Fold change	Description
PA2129	*cupA2*	144.7	CupA2, chaperone
PA2130	*cupA3*	48.1	CupA3, usher
PA2132	*cupA5*	15.5	CupA5, chaperone
PA3384	*phnC*	6.2	PhnC, component of ABC transporter
PA0718		6.0	Hypothetical from bacteriophage Pf1
PA0238		5.3	Hypothetical
PA4603		−5.6	Hypothetical
PA0679		−6.7	Hypothetical
PA1328		−7.5	Transcription regulator, LysR family
PA4186		−7.8	Hypothetical
PA5256	*dsbH*	−8.1	DsbH, disulfide bond formation protein
PA4799		−15.2	Hypothetical

RESULTS

Genetic screen for positive regulators of *cupA* gene expression. When cells of *P. aeruginosa* strain PAO1 are grown at 37°C in LB, expression of the *cupA* fimbrial genes is tightly repressed by MvaT (37, 38). In an attempt to identify positive regulators of *cupA* gene expression, we screened a plasmid library of *P. aeruginosa* genomic DNA for plasmids that stimulated expression of a chromosomal *cupA lacZ* reporter. Specifically, we introduced into reporter strain PAO1 *cupA lacZ* (38) a library of plasmids containing different portions of *P. aeruginosa* genomic DNA and isolated the transformants that gave rise to blue colonies on LB agar plates containing X-Gal. Plasmids isolated from these transformants were retransformed into reporter strain PAO1 *cupA lacZ* to confirm the plasmid linkage of the observed phenotype. Restriction analysis of the isolated plasmids and subsequent sequence analysis of the corresponding genomic DNA inserts revealed that we had isolated two different plasmids that stimulated *cupA* gene expression. One of these plasmids, referred to here as pPSV35-1, contained DNA spanning part of the PA2125 gene through the middle of the *cupA2* (PA2129) gene (referred to as insert 1) (Fig. 1A). We have previously shown that the *cupA* genes are expressed in a phase-variable manner in the absence of MvaT (38). We found that the *cupA* genes are also expressed in a phase-variable manner in cells containing plasmid pPSV35-1 (Fig. 1B and data not shown). In particular, reporter strain cells carrying plasmid pPSV35-1 gave rise to both dark and pale blue colonies on LB agar plates containing X-Gal; when cells were restreaked onto LB agar plates containing X-Gal, dark blue colonies gave rise to both dark and pale blue colonies, and pale blue colonies gave rise to both pale and dark blue colonies. Quantification of *cupA* gene expression revealed that there is at least an ~320-fold difference in *cupA* gene expression between phase-on cells (cells derived from phase-on, dark blue colonies) carrying pPSV35-1 and wild-type cells containing the pPSV35 parent plasmid (Fig. 1B).

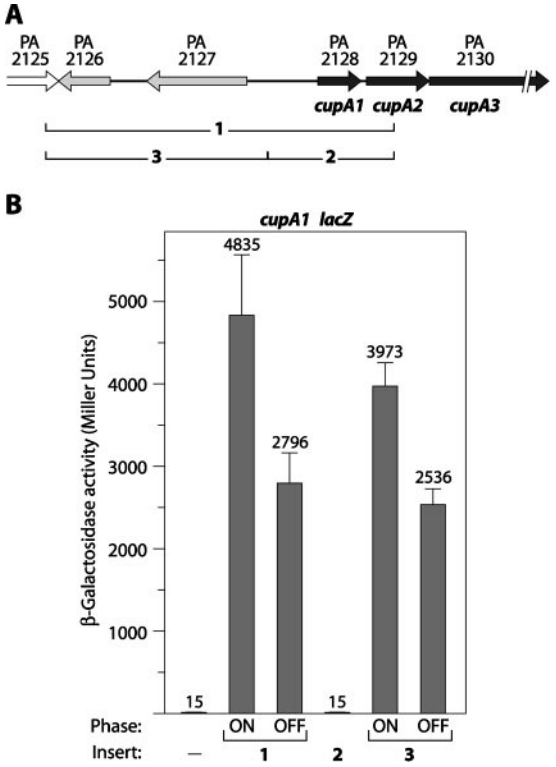

FIG. 1. Plasmids containing the PA2126 and PA2127 genes promote phase-variable expression of the *cupA* genes in *P. aeruginosa*. (A) Organization of genes within and upstream of the *cupA* gene cluster. The DNAs corresponding to inserts 1, 2, and 3 are indicated at the bottom. (B) Quantification of *cupA lacZ* expression in cells of the wild-type reporter strain harboring plasmids with the indicated DNA inserts. Cells were grown in the presence of 1 mM IPTG and assayed for β-galactosidase activity. Where indicated, cultures were obtained from phase-on or phase-off colonies. Phase-variable expression of the *cupA lacZ* reporter was not observed in cells containing the parent plasmid pPSV35 or in cells containing the plasmid carrying insert 2 (pPSV35-2).

To better define which portion of insert 1 was responsible for the stimulatory effect on *cupA* gene expression, we subcloned two different portions of insert 1 (corresponding to inserts 2 and 3 shown in Fig. 1A) into the pPSV35 parent vector. The plasmid containing insert 2 failed to stimulate *cupA* gene expression, whereas the plasmid containing insert 3 stimulated *cupA* gene expression to levels approaching those seen in cells with the plasmid containing insert 1 (Fig. 1B). As we had seen for cells with the plasmid containing insert 1, cells with the plasmid containing insert 3 expressed the *cupA* genes in a phase-variable manner (Fig. 1B and data not shown). Although the genomic DNA in plasmid pPSV35-1 and its derivatives originates from *P. aeruginosa* strain PAK, cells with a plasmid (p2126-2127) containing the PA2126 and PA2127 genes from *P. aeruginosa* strain PAO1 also expressed the *cupA* genes in a phase-variable manner (data not shown). These findings suggest that plasmids containing the PA2126 and PA2127 genes can promote phase-variable expression of the *cupA* genes in *P. aeruginosa*.

Genes that map immediately upstream of the *cupA* operon positively control *cupA* gene expression. The PA2126 and PA2127 genes encode a ParB-like protein and a conserved hypothetical protein of unknown function, respectively (Fig. 2A). Strikingly, homologs of the PA2126 and PA2127 genes have previously been implicated in the control of genes encoding immunoglobulin-binding proteins in *E. coli* (27). We reasoned that if the PA2126 and PA2127 genes were important for *cupA* gene expression, then deletion of either one of these genes should reduce *cupA* gene expression in an *mvaT* mutant background. To test these predictions, we introduced in-frame deletions of either the PA2126 gene (ΔPA2126) or the PA2127 gene (ΔPA2127) into an *mvaT* mutant derivative of our *cupA* reporter strain (38). Deletion of either the PA2126 or PA2127 gene dramatically reduced *cupA* gene expression in the Δ*mvaT* mutant background (Fig. 2B). Surprisingly, phase-variable expression of the *cupA* genes could not be complemented in either the Δ*mvaT* ΔPA2126 mutant background or the Δ*mvaT* ΔPA2127 mutant background with either the PA2126 gene or the PA2127 gene in *trans* (Fig. 2B). We discovered that our failure to complement either the ΔPA2126 or ΔPA2127 in-frame deletion mutant was due to the fact that there is a third gene, referred to here as the PA2126.1 gene (see the supplemental material), not previously annotated on the PAO1 genome (http://www.pseudomonas.com) and overlapping both the PA2126 and PA2127 genes (Fig. 2A), that is also required for *cupA* gene expression. Therefore, both our ΔPA2126 and ΔPA2127 in-frame deletion mutants also contained mutations in the PA2126.1 gene. In keeping with this idea, the ΔPA2126 mutant could be complemented by a plasmid expressing both the PA2126.1 and PA2126 genes, and the ΔPA2127 mutant could be complemented by a plasmid expressing both the PA2127 and PA2126.1 genes (Fig. 2B). The PA2126.1 gene appears to encode a protein with homology to members of the Gcn5-related *N*-acetyltransferase (GNAT) superfamily (40).

To assess the individual contributions of the PA2126, PA2126.1, and PA2127 genes to *cupA* gene expression, we constructed three additional mutant strains. Specifically, we introduced mutations affecting the PA2126, PA2126.1, or PA2127 gene into our Δ*mvaT cupA lacZ* reporter strain. Figure 2C shows that mutation of the PA2126, PA2126.1, or PA2127 gene abolished *cupA* gene expression in the Δ*mvaT* mutant background. Furthermore, the effects of the PA2126, PA2126.1, and PA2127 gene mutations on *cupA* gene expression could be complemented with the corresponding wild-type gene in *trans* (Fig. 2C). Note that although the plasmid expressing the PA2127 gene alone only weakly complemented the PA2127 mutant (Fig. 2C), the importance of the PA2127 gene in *cupA* gene expression was further demonstrated by the fact that a plasmid expressing both the PA2127 and PA2126.1 genes could complement the in-frame PA2127 deletion mutant (ΔPA2127) (Fig. 2B), whereas a plasmid expressing the PA2126.1 gene alone could not do this (data not shown). Our findings suggest that the PA2127, PA2126.1, and PA2126 genes are important for expression of the *cupA* genes in an *mvaT* mutant background. We therefore designated the PA2127, PA2126.1, and PA2126 genes *c*upA *g*ene *r*egulator *A* (*cgrA*), *cgrB*, and *cgrC*, respectively.

MvaT controls expression of the *cgr* genes. MvaT has previously been shown to repress *cupA* gene expression (37, 38). However, it is not known whether MvaT exerts its effect on *cupA* gene expression directly (for example, by binding to the

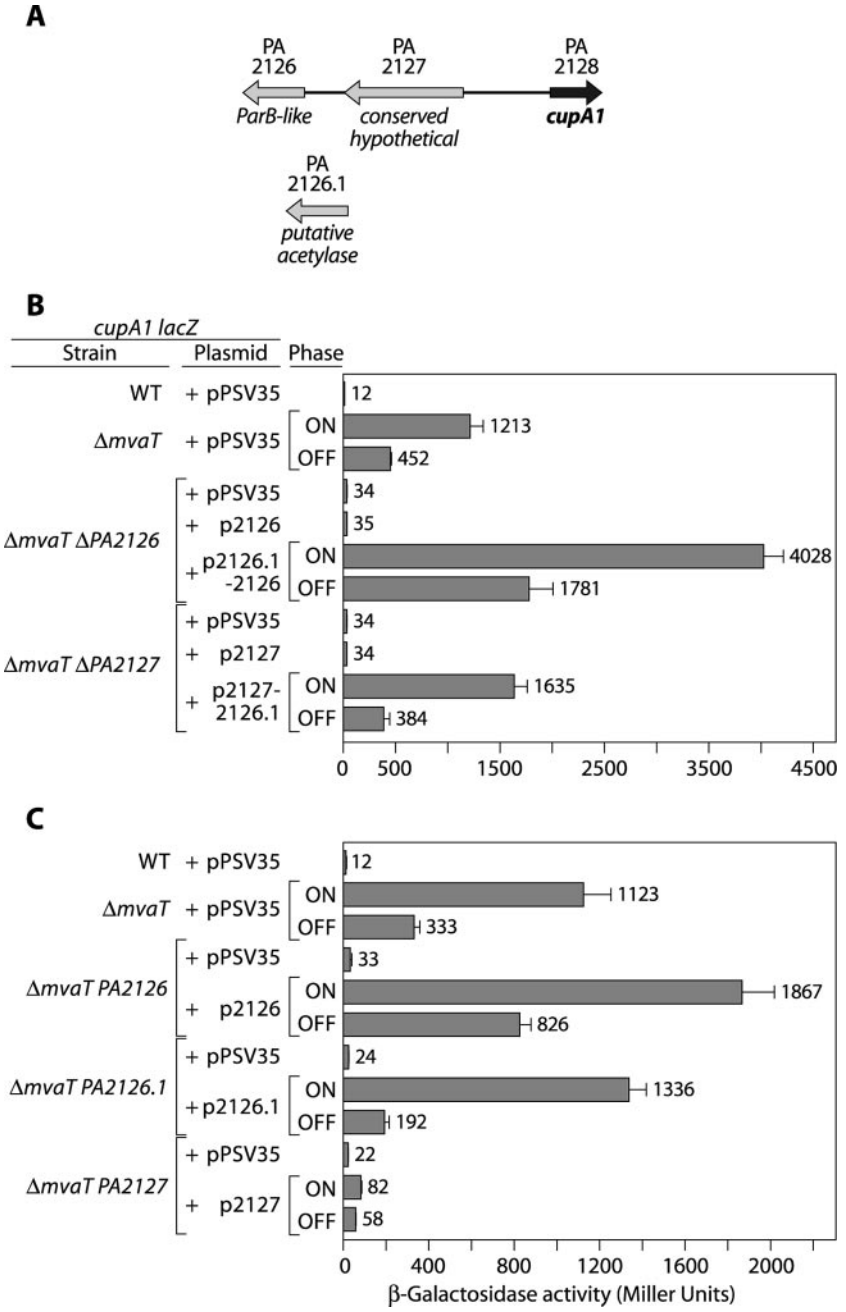

FIG. 2. Genes that map immediately upstream of the *cupA* operon are required for *cupA* gene expression in an *mvaT* mutant background. (A) Organization of genes that map immediately upstream of the *cupA* gene cluster. The PA2126 gene encodes a ParB-like protein; the PA2126.1 gene, which was discovered in this study, encodes a putative acetylase; and the PA2127 gene encodes a hypothetical protein of unknown function. (B) Quantification of *cupA lacZ* expression in cells of the wild-type, Δ*mvaT*, Δ*mvaT* ΔPA2126, and Δ*mvaT* ΔPA2127 reporter strains containing the indicated plasmids. (C) Quantification of *cupA lacZ* expression in cells of the wild-type, Δ*mvaT*, Δ*mvaT* PA2126, Δ*mvaT* PA2126.1, and Δ*mvaT* PA2127 reporter strains containing the indicated plasmids. For panels B and C cells were grown in the presence of 1 mM IPTG and assayed for β-galactosidase activity. Plasmids p2126, p2126.1, p2127, p2126.1-2126, and p2127-2126.1 direct the synthesis of the PA2126, PA2126.1, PA2127, PA2126.1 plus PA2126, and PA2127 plus PA2126.1 proteins, respectively, and were derived from the parent vector pPSV35. Where indicated, cultures were obtained from phase-on or phase-off colonies. Note that overexpression of the PA2126, PA2126.1 (data not shown), or PA2127 gene does not suffice to promote phase-variable expression of the *cupA* genes. WT, wild type.

cupA promoter region) or indirectly through effects on other regulators that control *cupA* gene expression or both. In order to test whether MvaT controls *cupA* gene expression, at least in part, through an effect on expression of the *cgr* genes, we constructed two additional reporter strains. In one of these strains *lacZ* was placed downstream of the PA2126 (*cgrC*) gene on the PAO1 chromosome. In the other strain, the *cupA1*-PA2127 intergenic region was placed upstream of *lacZ* such

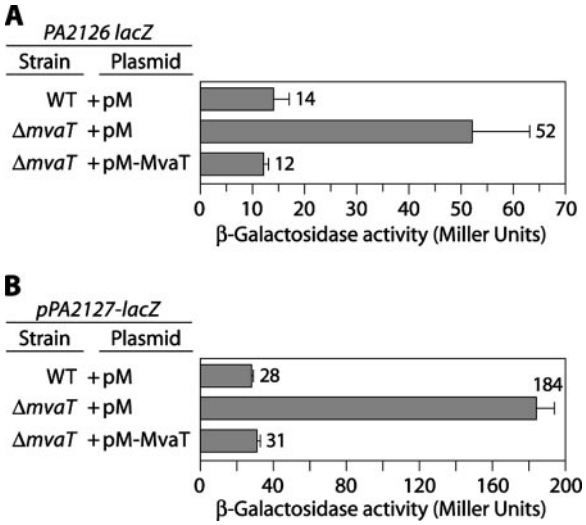

FIG. 3. MvaT represses expression of the *cgr* genes. (A) Quantification of PA2126-*lacZ* expression in cells of the wild-type reporter strain and the Δ*mvaT* mutant derivative harboring the indicated plasmids. Plasmid pM-MvaT directs the synthesis of MvaT and was derived from plasmid pMMB67EH, which is designated pM here. The PA2126-*lacZ* reporter strain contains the *lacZ* gene placed downstream of the PA2126 gene on the PAO1 chromosome. Cells were grown in the presence of 1 mM IPTG and assayed for β-galactosidase activity. (B) Quantification of PA2127-*lacZ* expression in cells of the wild-type reporter strain (PAO1 *attB*::pPA2127-*lacZ*) and the Δ*mvaT* mutant derivative harboring the indicated plasmids. The plasmids and experimental conditions are the same as those described above for panel A. The PA2127-*lacZ* reporter strains contain the PA2127 promoter(s) driving expression of a linked *lacZ* reporter gene integrated as a single copy into the ϕCTX attachment site on the PAO1 chromosome. WT, wild type.

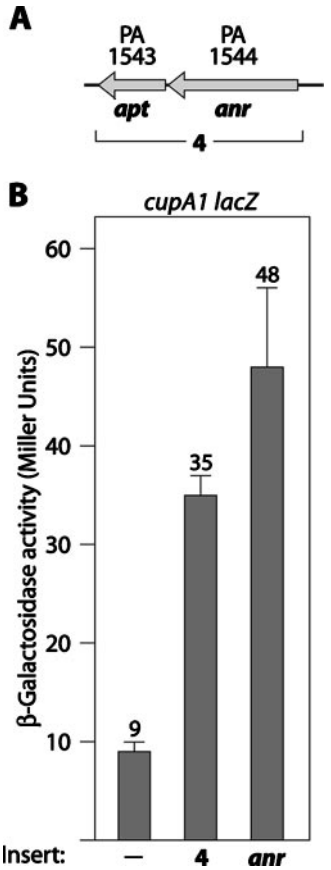

FIG. 4. Plasmids containing the *anr* gene stimulate *cupA* gene expression in *P. aeruginosa*. (A) Organization of the *apt* and *anr* genes. DNA corresponding to the DNA contained on insert 4 is indicated. (B) Quantification of *cupA lacZ* expression in cells of the wild-type reporter strain harboring plasmids with the indicated DNA inserts. Plasmid pAnr, a derivative of pPSV35, was the source of the *anr* gene. Cells were grown in the presence of 1 mM IPTG and assayed for β-galactosidase activity.

that the putative PA2127 promoter(s) was driving expression of *lacZ* and a single copy of the resulting PA2127 gene-*lacZ* reporter fusion was inserted into the ϕCTX attachment site in the PAO1 chromosome (18). Subsequently, we introduced an in-frame deletion of the *mvaT* gene into each of these reporter strains. Figure 3A shows that deletion of *mvaT* resulted in an ~4-fold increase in expression of the PA2126 (*cgrC*) gene and presumably the entire *cgrABC* operon. This effect of the *mvaT* deletion could be complemented with the *mvaT* gene supplied in *trans* (Fig. 3A). Consistent with these findings, deletion of *mvaT* resulted in an ~6-fold increase in expression of the PA2127-*lacZ* reporter (Fig. 3B). These findings suggest that expression of the *cgr* genes is repressed, either directly or indirectly, by MvaT. We infer from this that MvaT represses *cupA* gene expression, at least in part, by repressing expression of the *cgr* genes, which positively regulate *cupA* gene expression. Note that although the *cupA* genes are expressed in a phase-variable manner in an *mvaT* mutant background (38), we did not see phase-variable expression of either the PA2126-*lacZ* reporter or the PA2127-*lacZ* reporter in the absence of MvaT. Phase-variable expression of the *cgr* genes is therefore unlikely to account for the phase-variable expression of the *cupA* genes that occurs in an *mvaT* mutant background.

Microarray analyses of genes regulated by the *cgr* gene cluster. Expression of the *cgr* genes from a multicopy plasmid results in an ~320-fold increase in *cupA* gene expression (Fig. 1B). To determine whether the expression of other genes in addition to those of the *cupA* operon is controlled by the *cgr* genes, we used DNA microarrays. In particular, we compared the global gene expression profiles of cells carrying a multicopy plasmid encoding the *cgr* locus and cells carrying a control plasmid using the *P. aeruginosa* GeneChip microarrays from Affymetrix. Table 2 shows that the expression of only 12 genes changed fivefold or more when the *cgr* genes were overexpressed, and of these, the *cupA* genes were most strongly affected. These findings suggest that the products of the *cgr* genes function primarily as local regulators and do not control the expression of many of the other MvaT-controlled genes in *P. aeruginosa* (37).

Anr can influence *cupA* gene expression. Our genetic screen for positive regulators of *cupA* gene expression identified a second plasmid that stimulated *cupA* gene expression. This plasmid had a relatively modest effect on *cupA* gene expression and contained DNA encompassing the *apt* and *anr* genes encoding adenine phosphoribosyltransferase and the Fnr-like transcription activator Anr, respectively (Fig. 4). Moreover, a plasmid expressing *anr* alone had an equal stimulatory effect

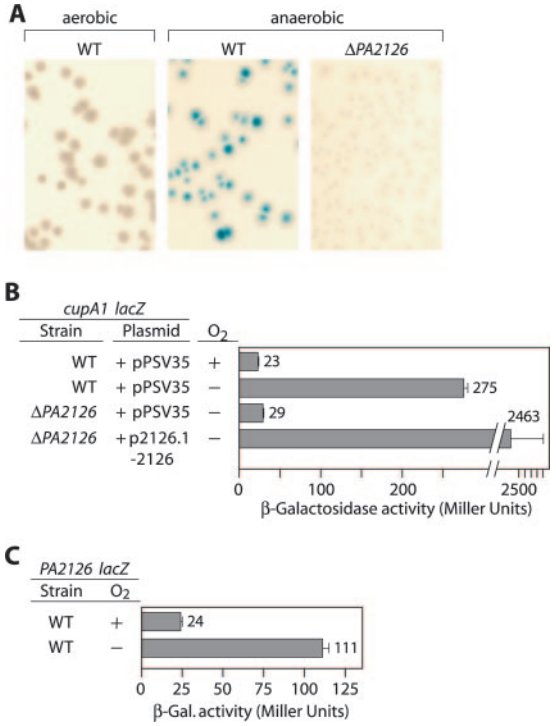

FIG. 5. Anaerobiosis promotes phase-variable expression of the *cupA* genes and stimulates expression of the *cgr* genes. (A) Phenotypes of the wild-type *cupA lacZ* reporter strain grown under aerobic or anaerobic conditions and the ΔPA2126 *cupA lacZ* reporter strain grown under anaerobic conditions. Strains were grown at 37°C on LBN agar containing X-Gal. Only the wild-type reporter strain gave rise to blue (phase-on) and pale blue (phase-off) colonies when cells were grown under anaerobic conditions. (B) Quantification of *cupA lacZ* expression in cells of the wild-type and ΔPA2126 reporter strains containing the indicated plasmids. Cells were grown in the presence or absence of O_2 as indicated. Plasmid p2126.1-2126 directs the synthesis of PA2126.1 plus PA2126 and is derived from the parent vector pPSV35. (C) Quantification of PA2126-*lacZ* expression in cells of the wild-type reporter strain grown in the presence or absence of O_2. WT, wild type; β-Gal., β-galactosidase.

on *cupA* gene expression (Fig. 4B), whereas a plasmid expressing *apt* alone had no effect on *cupA* gene expression (data not shown). These findings suggest that Anr can influence *cupA* gene expression, either directly or indirectly. Although Anr typically controls the expression of genes under anaerobic conditions (29, 48), Anr can also influence gene expression under aerobic conditions (26). To determine whether Anr is essential for *cupA* gene expression under aerobic conditions, we created a derivative of our PAO1 Δ*mvaT cupA lacZ* reporter strain carrying an in-frame deletion of the *anr* gene. Quantification of *cupA* gene expression in this Δ*anr* mutant revealed that deletion of *anr* resulted in a modest decrease (less than twofold) in *cupA* gene expression (data not shown), suggesting that unlike the products of the *cgr* genes, Anr is not essential for *cupA* gene expression in the absence of MvaT. We were unable to accurately assess the contribution of Anr to *cupA* gene expression under anaerobic conditions because *anr* is essential under the conditions used in our experiments, in which nitrate is used as the terminal electron acceptor to support anaerobic respiration (46, 48).

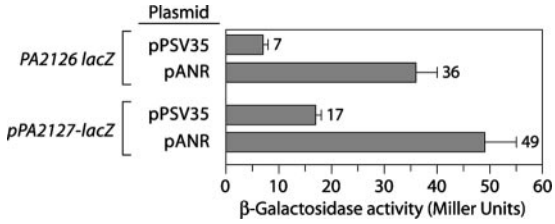

FIG. 6. Plasmid containing the *anr* gene stimulates *cgr* gene expression in *P. aeruginosa*: quantification of PA2126-*lacZ* expression and quantification of PA2127-*lacZ* expression in cells of wild-type reporter strains harboring the indicated plasmids. Cells were grown in the presence of 1 mM IPTG and assayed for β-galactosidase activity. Plasmid pAnr, a derivative of pPSV35, was the source of the *anr* gene.

Anaerobiosis promotes phase-variable expression of the *cupA* genes. We have previously shown that the *cupA* genes are expressed in a phase-variable manner in the absence of MvaT (38). However, we have been unable to detect phase-variable expression of the *cupA* genes in the presence of MvaT (i.e., in wild-type cells). Because Anr can influence *cupA* gene expression (Fig. 4) and because Anr typically controls the expression of genes under anaerobic conditions, we next asked whether the *cupA* genes were expressed in a phase-variable manner under anaerobic conditions. Cells of the PAO1 *cupA lacZ* reporter strain gave rise to both blue and pale blue colonies on LB agar plates containing nitrate and X-Gal when the cells were grown anaerobically but not when the cells were grown aerobically (Fig. 5A). When cells were restreaked on LB agar plates containing X-Gal and nitrate, following incubation under anaerobic conditions, blue colonies gave rise to both blue and pale blue colonies, and pale blue colonies gave rise to both blue and pale blue colonies (data not shown). These findings are consistent with the idea that the *cupA* genes are expressed in a phase-variable manner when cells are grown anaerobically. Moreover, expression of the *cupA* genes under anaerobic conditions is dependent on the *cgr* genes since phase-variable expression of the *cupA* genes could not be detected under anaerobic conditions in the ΔPA2126 mutant (containing mutations in both the PA2126/*cgrC* and PA2126.1/*cgrB* genes) (Fig. 5A and B) or in any other *cgr* mutant strain (data not shown).

Anaerobiosis stimulates expression of the *cgr* genes. Because the *cgr* genes are important for *cupA* gene expression under anaerobic conditions, we next asked whether expression of the *cgr* genes was controlled by anaerobiosis. Figure 5C shows that expression of the PA2126-*lacZ* reporter was ~4-fold higher in cells grown under anaerobic conditions than in cells grown aerobically. We inferred from this that expression of the PA2126 (*cgrC*) gene and presumably the rest of the *cgr* genes is upregulated in response to growth under anaerobic conditions.

Anr stimulates expression of the *cgr* genes. Expression of *anr* from a multicopy plasmid stimulates *cupA* gene expression (Fig. 4B). To begin to address whether Anr mediates its stimulatory effect through an effect on the *cgr* genes, we introduced a plasmid containing *anr* together with a control plasmid into the PA2126-*lacZ* and PA2127-*lacZ* reporter strains and quantified reporter gene expression by measuring β-galactosidase activity. Figure 6 shows that expression of *anr* from a multicopy plasmid resulted in an ~6-fold increase in expression of the PA2126 gene and an ~3-fold increase in expression of the PA2127-*lacZ* reporter.

These findings suggest that Anr can influence expression of the *cgr* genes. Taken together, our findings suggest that anaerobiosis promotes phase-variable expression of the *cupA* genes, at least in part, through an effect on *cgr* gene expression. Moreover, they suggest that the effect of anaerobiosis on *cgr* gene expression is mediated, either directly or indirectly, by Anr.

DISCUSSION

Using a genetic screen, we identified four previously undescribed positive regulators of *cupA* gene expression in *P. aeruginosa*. These regulators include three local regulators encoded by the *cgrABC* genes and Anr, a global regulator of anaerobic gene expression. Moreover, through the identification of Anr, we were able to identify anaerobiosis as one environmental condition under which the *cupA* genes are expressed in a phase-variable manner; previously, we had observed phase-variable expression of the *cupA* genes only in the absence of MvaT (i.e., in an *mvaT* mutant background) (38). All three of the *cgr* genes appear to be required for phase-variable expression of the *cupA* genes. Although we suspect that the *cgr* genes are required for *cupA* gene expression, it is also possible that they simply serve to switch on phase-variable expression of the *cupA* operon, or both.

How do the *cgr* genes positively regulate *cupA* gene expression? Our microarray experiments suggested that the effects of the Cgr regulators are largely limited to the *cupA* genes. The Cgr regulators themselves are unusual in that they do not resemble any classical positive regulator of gene expression. CgrC (PA2126) belongs to the ParB family of DNA-binding proteins, which often contain a helix-turn-helix DNA-binding motif and are typically involved in DNA partitioning (15, 19, 31). However, ParB family members are also known to control gene expression. For example, the ParB protein from the *E. coli* P1 plasmid can repress expression of genes flanking the P1 centromere (25). Although most ParB-like proteins that influence gene expression tend to function as repressors or silencers (5, 21, 23, 25, 45), there are several examples where ParB-like proteins positively regulate gene expression. Indeed, VirB from *Shigella flexneri* is a ParB-like protein that functions exclusively as a transcription regulator and mediates its positive effects on virulence gene expression by displacing the negative regulator H-NS (4, 35). Of particular relevance to the Cgr system, the ParB-like IbrB protein has been implicated in the positive control of gene expression in *E. coli* strain ECOR-9 (27). In particular, it is thought that IbrB functions together with IbrA, a homolog of CgrA, to coregulate expression of the prophage-associated *eib* genes, which encode immunoglobulin-binding proteins. However, the mechanism of action of IbrA and IbrB is unknown.

Sequence analysis and structural prediction algorithms suggest that CgrA is a member of the adenine nucleotide α-hydrolase superfamily. This family includes the phosphoadenosine phosphosulfate/adenosine phosphosulfate reductases, ATP sulfurylases, and N-type ATP pyrophosphatases (28). Like IbrA (27), CgrA contains a putative phosphoadenosine phosphosulfate/adenosine phosphosulfate-binding domain, and it will be interesting to determine whether this domain within CgrA is important for its activity.

CgrB is a putative member of the GNAT family of acetyl transferases (40). Although acetylases belonging to the GNAT family play important roles in regulating gene expression in eukaryotes (22, 33), there are few examples in which acetylases are known to influence gene expression in prokaryotes (10, 41). Perhaps CgrB acetylates either CgrA or CgrC to promote its activity. It is also possible that CgrB targets a small molecule, the acetylation of which is required for either CgrA or CgrC to function. Nevertheless, whether CgrB is truly an acetylase and how CgrB functions together with CgrA and CgrC to positively control *cupA* gene expression remain to be determined.

Because CgrC is predicted to be a DNA-binding protein, we speculate that CgrC may regulate *cupA* gene expression by binding directly to the *cupA1* promoter region and, together with CgrA and CgrB, function either to remove a repressor from the *cupA* promoter DNA or to activate transcription from the *cupA1* promoter(s). If the Cgr proteins do function to remove a repressor from the *cupA1* promoter region, that repressor is unlikely to be MvaT, since we have shown that the *cgr* genes are required for *cupA* gene expression in the absence of MvaT.

It is important to note that we do not yet know the mechanism governing phase-variable expression of the *cupA* genes. It is possible that the *cgr* genes themselves somehow mediate phase-variable expression of the *cupA* genes or that some yet-to-be-identified factor(s) is responsible.

Mechanism by which MvaT represses *cupA* gene expression. We and others have previously shown that MvaT represses expression of the *cupA* genes (37, 38). Here we found that MvaT represses *cgr* gene expression, suggesting that MvaT represses *cupA* gene expression, at least in part, by repressing the expression of genes encoding positive regulators of *cupA* gene expression. Our findings are consistent with those of a previous study in which the *cgrA* (PA2127) transcript was found by DNA microarray to be ~2-fold more abundant in cells of an *mvaT* mutant strain than in cells of the wild-type strain (37).

Phase-variable expression of the *cupA* genes under anaerobic conditions. We have presented evidence that the *cupA* genes are expressed in a phase-variable manner when cells are grown anaerobically. Consistent with these findings, previous microarray analyses found that *cupA* gene expression was induced during anaerobic growth (1, 9) and during growth under microaerophilic conditions (1). We have also shown that the presence of *anr* on a multicopy plasmid can stimulate expression of both the *cupA* genes and the *cgr* genes under aerobic conditions and that *cgr* gene expression is upregulated during anaerobic growth. In support of the latter finding, a recent proteomic analysis of *P. aeruginosa* revealed that CgrA (PA2127) was more abundant in cells grown anaerobically than in cells grown aerobically (44). Our findings suggest that under anaerobic conditions, expression of the *cgr* genes is induced in an Anr-dependent fashion, resulting in a concomitant increase in *cupA* gene expression. Although we do not yet know whether Anr regulates expression of the *cgr* or *cupA* genes (or both) directly, we have discovered a putative Anr-binding site within the *cgrA-cupA1* intergenic region (Sandra Castang and S. L. Dove, unpublished). Because *P. aeruginosa* encodes a second Anr ortholog, called Dnr (2), which recognizes similar binding sites and whose expression is dependent upon Anr (3,

14, 30), it is important to determine whether any role that this site might play is a direct result of Anr binding.

Several lines of evidence suggest that the microbial environment in the chronically infected CF lung is largely anaerobic (43, 47). Our finding that phase-variable expression of the *cupA* genes occurs under anaerobic conditions raises the possibility that phase-variable expression of the *cupA* genes may occur in cells of *P. aeruginosa* growing in the CF host lung. Because the *cupA* genes can influence biofilm formation in vitro (11, 20, 36), it is important to determine whether the *cupA* genes are expressed in vivo and, if they are, what role they play in the host lung environment. Perhaps, under the anaerobic conditions of the CF host lung, expression of the *cupA* genes (in phase-on cells) facilitates the initial formation of the biofilm. Subsequent switching to the phase-off expression state might better enable cells to persist either because it could facilitate escape from the biofilm and subsequent colonization of other sites or because it could facilitate immune evasion by cells that no longer produce immunogenic CupA fimbriae. Indeed, in many instances the phase-variable expression of bacterial surface structures is thought to provide a mechanism for immune evasion (39). Any heterogeneity in the cell population that would result from phase-variable expression of the *cupA* genes may therefore contribute to the long-term survival of *P. aeruginosa* in the CF host lung.

We have shown that anaerobic growth is an important environmental cue for phase-variable expression of the *cupA* genes. Although several other phase variation systems are responsive to environmental conditions (16, 39), the *cupA* system appears to be an example of a system in which phase variation occurs only in response to a specific environmental stimulus. Precisely how the components of the regulatory cascade that we have described are integrated into the regulatory mechanism controlling phase-variable expression of the *cupA* genes remains to be determined.

ACKNOWLEDGMENTS

We thank Arne Rietsch for the *P. aeruginosa* PAK genomic library, Anja Brencic for help with microarray experiments, and Renate Hellmiss for artwork. We thank Ann Hochschild, Stephen Lory, Joseph Mougous, and Arne Rietsch for discussions and comments on the manuscript.

This work was supported by National Institutes of Health grant AI069007 (to S.L.D.). J.S.S. was supported by a postdoctoral fellowship from the Cystic Fibrosis Foundation.

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