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.
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* DH5α F′ (Invitrogen) was used as the recipient strain for all plasmid constructions, 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 was used to create strains PAO1 containing chromosomal cupA1 lacZ reporter (Arne Riettsch, Case Western Reserve University).

<table>
<thead>
<tr>
<th>Strains or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td>PAO1 <em>cupA</em> lacZ</td>
<td>PAO1 containing chromosomal <em>cupA1</em> lacZ reporter</td>
<td>38</td>
</tr>
<tr>
<td>PAO1 <em>ΔmvaT</em> <em>cupA</em> lacZ</td>
<td>PAO1 <em>cupA</em> lacZ containing deletion of <em>mvaT</em></td>
<td>38</td>
</tr>
<tr>
<td>PAO1 <em>ΔPA2127</em> <em>cupA</em> lacZ</td>
<td>PAO1 <em>cupA</em> lacZ containing deletion of <em>PA2127</em> gene</td>
<td>This study</td>
</tr>
<tr>
<td>PAO1 <em>ΔmvaT ΔPA2127</em> <em>cupA</em> lacZ</td>
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<td>This study</td>
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<tr>
<td>PAO1 <em>ΔmvaT ΔPA2126</em> <em>cupA</em> lacZ</td>
<td>PAO1 <em>ΔmvaT</em> <em>cupA</em> lacZ containing deletion of <em>PA2126</em> gene</td>
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<tr>
<td>PAO1 <em>ΔmvaT</em> <em>PA2127</em> <em>cupA</em> lacZ</td>
<td>PAO1 <em>ΔmvaT</em> <em>cupA</em> lacZ containing partial deletion of <em>PA2127</em> gene</td>
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<td>PAO1 <em>ΔmvaT</em> <em>PA2126.1</em> <em>cupA</em> lacZ</td>
<td>PAO1 <em>ΔmvaT</em> <em>cupA</em> lacZ containing partial deletion of <em>PA2126.1</em> gene</td>
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<tr>
<td>PAO1 <em>ΔmvaT</em> <em>PA2126</em> <em>cupA</em> lacZ</td>
<td>PAO1 <em>ΔmvaT</em> <em>cupA</em> lacZ containing partial deletion of <em>PA2126</em> gene</td>
<td>This study</td>
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<tr>
<td>PAO1 PA2126 <em>lacZ</em></td>
<td>PAO1 containing chromosomal <em>PA2126</em> g-lacZ reporter</td>
<td>This study</td>
</tr>
<tr>
<td>PAO1 <em>ΔmvaT PA2126</em> <em>lacZ</em></td>
<td>PAO1 PA2126 <em>lacZ</em> containing deletion of <em>mvaT</em></td>
<td>This study</td>
</tr>
<tr>
<td>PAO1 <em>ΔmvaT</em> attB::<em>pPA2127-lacZ</em></td>
<td>PAO1 containing chromosomal <em>pPA2127-lacZ</em> reporter integrated at <em>mvaT</em></td>
<td>This study</td>
</tr>
<tr>
<td>PAO1 <em>ΔmvaT</em> attB::<em>pPA2127-lacZ</em></td>
<td>PAO1 <em>ΔmvaT</em> attB::<em>pPA2127-lacZ</em> containing deletion of <em>mvaT</em></td>
<td>This study</td>
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<tr>
<td>PAO1 <em>Δanr</em> <em>cupA</em> lacZ</td>
<td>PAO1 <em>cupA</em> lacZ containing deletion of <em>anr</em></td>
<td>This study</td>
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<tr>
<td>PAO1 <em>Δanr ΔmvaT</em> <em>cupA</em> lacZ</td>
<td>PAO1 <em>ΔmvaT</em> <em>cupA</em> lacZ containing deletion of <em>anr</em></td>
<td>This study</td>
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<tr>
<th>Plasmids</th>
<th>Description</th>
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<tr>
<td>pPSV35</td>
<td>Shuttle vector with gentamicin resistance gene (<em>aacC1</em>), <em>PA</em> origin, <em>lacI</em>, and lacUV5 promoter</td>
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</tr>
<tr>
<td>pPSV35-1</td>
<td>pPSV35 with genomic DNA fragment from <em>P. aeruginosa</em> strain PAK containing part of PA2125 gene through part of PA2129 gene</td>
<td>This study</td>
</tr>
<tr>
<td>pPSV35-2</td>
<td>Derivative of pPSV35-1 containing part of PA2127 gene- <em>cupA</em></td>
<td>This study</td>
</tr>
<tr>
<td>pPSV35-3</td>
<td>Derivative of pPSV35-1 containing part of PA2125 gene through part of PA2127 gene- <em>cupA</em></td>
<td>This study</td>
</tr>
<tr>
<td>pPSV35-4</td>
<td>pPSV35 with genomic DNA fragment from <em>P. aeruginosa</em> strain PAK containing <em>apt</em> and <em>anr</em></td>
<td>This study</td>
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<tr>
<td>p2127</td>
<td>PA2127 gene from PAO1 cloned into pPSV35</td>
<td>This study</td>
</tr>
<tr>
<td>p2126.1</td>
<td>PA2126.1 gene from PAO1 cloned into pPSV35</td>
<td>This study</td>
</tr>
<tr>
<td>p2126</td>
<td>PA2126 gene from PAO1 cloned into pPSV35</td>
<td>This study</td>
</tr>
<tr>
<td>p2127-2126.1</td>
<td>PA2127 and PA2126.1 genes from PAO1 cloned into pPSV35</td>
<td>This study</td>
</tr>
<tr>
<td>p2126-2126.1</td>
<td>PA2126.1 and PA2126 genes from PAO1 cloned into pPSV35</td>
<td>This study</td>
</tr>
<tr>
<td>pM</td>
<td>pMBB67EH vector with carbenicillin resistance gene (<em>bla</em>), <em>lacI</em>, and lacUV5 promoter</td>
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</tr>
<tr>
<td>pM-MvaT</td>
<td>mvaT from PAO1 cloned into pMBB67EH</td>
<td>38</td>
</tr>
<tr>
<td>pAnr</td>
<td>anr from PAO1 cloned into pPSV35</td>
<td>This study</td>
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</table>

Microbiologically). To confirm that anaerobic conditions were attained, in each experiment a *Δanr* strain (PAO1 *Δanr* *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′-GAATTCC-3′ and 5′-AAGCTTGGGAGGAG-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 ΔmvaT ΔPA2127 *cupA* lacZ, and PAO1 ΔmvaT 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 ΔmvaT *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-PA2127, pEX-PA2126.1, and pEX-PA2126, 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 ΔmvaT PA2127 *cupA* lacZ, PAO1 ΔmvaT PA2126.1 *cupA* lacZ, and PAO1 ΔmvaT 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 Km, Neo, and SpH sites one base after the PA2126 gene stop codon. The resulting PCR product was cloned on a SacI/FacI fragment into pEX2G (24), yielding plasmid pEX2G-PA2126. The lacZ gene was subsequently cloned into this construct on a KpnI/SphI fragment derived from plasmid pPh18- lacZ (Arne Rietsch, unpublished data), generating plasmid pEX2G-PA2126-lacZ. This plasmid was then used to create reporter strains PAO1 PA2126 lacZ and PAO1 ΔmutA T 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 6CTX 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 anr. This plasmid was then used essentially as described previously (18) to introduce the pPA2127-lacZ fusion into the λCTX attachment site on the chromosomes of PAO1 and PAO1 ΔmutA T, creating reporter strains PAO1 attB::pPA2127-lacZ and PAO1 ΔmutA T 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-Δanr. This plasmid was then used to create strains PAO1 Δanr cupA lacZ and PAO1 ΔmutA T Δanr 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 Sau3A1 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 p2126.1-Δanr and p2126-Δanr 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 lac 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 CHCl₃, 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.

**RESULTS**

**Gene Designation** Fold change Description

<table>
<thead>
<tr>
<th>Gene</th>
<th>Designation</th>
<th>Fold change</th>
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<tr>
<td>PA2129</td>
<td>cupA2</td>
<td>144.7</td>
<td>CupA2, chaperone</td>
</tr>
<tr>
<td>PA2130</td>
<td>cupA3</td>
<td>48.1</td>
<td>CupA3, usher</td>
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<tr>
<td>PA2132</td>
<td>cupA5</td>
<td>15.5</td>
<td>CupA5, chaperone</td>
</tr>
<tr>
<td>PA3384</td>
<td>phnC</td>
<td>6.2</td>
<td>PhnC, component of ABC transporter</td>
</tr>
<tr>
<td>PA0718</td>
<td></td>
<td>6.0</td>
<td>Hypothetical</td>
</tr>
<tr>
<td>PA0238</td>
<td></td>
<td>5.3</td>
<td>Hypothetical</td>
</tr>
<tr>
<td>PA4603</td>
<td></td>
<td>−5.6</td>
<td>Hypothetical</td>
</tr>
<tr>
<td>PA0679</td>
<td></td>
<td>−6.7</td>
<td>Hypothetical</td>
</tr>
<tr>
<td>PA1328</td>
<td></td>
<td>−7.5</td>
<td>Transcription regulator, LysR family</td>
</tr>
<tr>
<td>PA4186</td>
<td></td>
<td>−7.8</td>
<td>Hypothetical</td>
</tr>
<tr>
<td>PA5256</td>
<td>dsbH</td>
<td>−8.1</td>
<td>DsbH, disulfide bond formation protein</td>
</tr>
<tr>
<td>PA4799</td>
<td></td>
<td>−15.2</td>
<td>Hypothetical</td>
</tr>
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**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 of 0.5 (corresponding to the mid-logarithmic phase of growth). RNA isolation, cDNA synthesis, and cDNA fragmentaion 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**

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</table>
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 PA2127 and PA2126.1 genes (Fig. 2B). The PA2126.1 gene appears to encode a protein with homology to members of the Gen5-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 in 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 *cupA* gene regulator 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
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
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 δCTX 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 strain (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 cgr 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. 4). 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...
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 (29, 48), typically controls the expression of genes under anaerobic conditions, either directly or indirectly. Although Anr can influence cupA gene expression (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.

**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 cupA genes in the presence of MvaT (i.e., in wild-type cells). Because Anr can influence cupA gene expression (Fig. 5A) or in any other cupr 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 a ~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 acetyltransferases (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 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 *cgrABC-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,
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 site might play is a direct result of Anr binding.

Several lines of evidence suggest that the microbial environment in the host lung is bacterially colonized. This work was supported by National Institutes of Health grant AI089007 (to S.L.D.). J.S.S. was supported by postdoctoral fellowship from the Cystic Fibrosis Foundation.

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