

Biofilm Formation in *Pseudomonas aeruginosa*: Fimbrial *cup* Gene Clusters Are Controlled by the Transcriptional Regulator MvaT†

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Pseudomonas aeruginosa is an opportunistic bacterial pathogen which poses a major threat to long-term-hospitalized patients and individuals with cystic fibrosis. The capacity of *P. aeruginosa* to form biofilms is an important requirement for chronic colonization of human tissues and for persistence in implanted medical devices. Various stages of biofilm formation by this organism are mediated by extracellular appendages, such as type IV pili and flagella. Recently, we identified three *P. aeruginosa* gene clusters that were termed *cup* (chaperone-usher pathway) based on their sequence relatedness to the chaperone-usher fimbrial assembly pathway in other bacteria. The *cupA* gene cluster, but not the *cupB* or *cupC* cluster, is required for biofilm formation on abiotic surfaces. In this study, we identified a gene (*mvaT*) encoding a negative regulator of *cupA* expression. Such regulatory control was confirmed by several approaches, including *lacZ* transcriptional fusions, Northern blotting, and transcriptional profiling using DNA microarrays. MvaT also represses the expression of the *cupB* and *cupC* genes, although the extent of the regulatory effect is not as pronounced as with *cupA*. Consistent with this finding, *mvaT* mutants exhibit enhanced biofilm formation. Although the *P. aeruginosa* genome contains a highly homologous gene, *mvaU*, the repression of *cupA* genes is MvaT specific. Thus, MvaT appears to be an important regulatory component within a complex network that controls biofilm formation and maturation in *P. aeruginosa*.

In their natural environment, bacteria grow predominantly in organized communities termed biofilms. The ability to attach to solid surfaces and the subsequent formation of an organized bacterial biofilm community are also important steps in the establishment of chronic bacterial infections and persistence in host tissues (4). It was recently demonstrated that *Pseudomonas aeruginosa* grows as a biofilm in the lungs of cystic fibrosis patients (13, 31). These microbial communities then develop unique characteristics and properties that protect them from external influences, most significant of which is the enhanced resistance of bacteria within the biofilm to antibiotics and host defenses (18). In *P. aeruginosa*, the adherence phenotype is mostly driven by extracellular appendages, such as flagella and type IV pili (22). Flagellar motility appears to be required for approaching surfaces and counteracting repulsive forces. Twitching motility, a mechanism that is mediated by type IV pili, is required for subsequent spreading over the surface. Following formation of microcolonies, the biofilm structure undergoes maturation, where the bacterial community assumes a characteristic architecture. The production of the extracellular polysaccharide alginate and the rhamnolipid surfactant allow the maintenance of biofilm architecture and provide the biofilm with a degree of physical resistance (6, 11,

21). The maturation process is accompanied by the engulfment of microcolonies within a glycocalyx matrix containing aqueous channels allowing the flow of nutrients and waste products but also the transport of oxygen and various signaling molecules. Biofilm maturation in *Pseudomonas* (7), *Burkholderia* (14), and *Aeromonas* (17) has been reported to depend on *N*-acylhomoserine lactone-dependent quorum sensing, although not under all conditions (12). Although the role of quorum sensing as an important mechanism for regulating biofilm maturation cannot be doubted, the overall process is much more complex and involves the integration of many diverse signals from the environment (26), including the availability of nutrients. It is very likely that successful cellular adaptations within the community necessitate the coordination of activities of many genes through complex regulatory networks (16).

Recently, we identified three *P. aeruginosa* gene clusters, termed *cup* for chaperone-usher pathways, which may be involved in the assembly of novel *P. aeruginosa* fimbrial structures that are radically different from the type IV pili (5, 38). Such structures and the pathways involved in their assembly in *P. aeruginosa* have not previously been described. The chaperone-usher pathway takes its name from the components required for assembling the pilin subunits into pili (fimbriae) on the surface of many gram-negative bacteria. The assembly pathway has been most extensively studied in *Escherichia coli*. A so-called chaperone protein binds fimbrial subunits that are secreted into the periplasm through the general export pathway (25, 36). The chaperone-pilin complex is subsequently delivered to the usher protein, which forms a pore in the outer membrane. This usher protein allows the pilin subunits to be

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

TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics | Source or reference |
|-----------------------|--|-----------------------|
| Strains | | |
| <i>E. coli</i> | | |
| TG1 | <i>supE</i> $\Delta(lac-proAB)$ <i>thi hsdR</i> Δ 5 (F' <i>traD36 proA</i> ⁺ <i>B</i> ⁺ <i>lacI</i> ^q Δ M15) | Laboratory collection |
| TOP10F' | F' [<i>lacI</i> ^q Tn10(Tet ^r)] <i>mrcA</i> $\Delta(mrr-hsdRMS-mcrBC)\phi$ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 recA1 araD139 $\Delta(ara-leu)$7697 <i>galU galK rpsL</i>(Str^r) <i>endA1 nupG</i></i> | Invitrogen |
| S17 λ pir | <i>thi pro hsdR hsdM</i> ⁺ <i>recA</i> RP4-2-Tc::Mu-Km::Tn7 λ pir | 30 |
| <i>P. aeruginosa</i> | | |
| PAO1 | Wild type | Laboratory collection |
| PAO1 <i>lecA::lux</i> | <i>lecA::luxABCDE</i> genomic reporter fusion in PAO1 | 42 |
| PAO-P10 | Tn5-B21 <i>mvaT</i> mutant derived from PAO1 <i>lecA::lux</i> | 8 |
| PAO-P48 | <i>mvaU</i> chromosomal deletion mutant derived from PAO1 <i>lecA::lux</i> | This work |
| PAO-P47 | <i>mvaT</i> chromosomal deletion mutant derived from PAO1 | 8 |
| PAO-P46 | <i>mvaU</i> chromosomal deletion mutant derived from PAO1 | This work |
| PAO-MC | <i>cupA3</i> mutation in PAO-P47 | This work |
| Tn5G library | PAK Δ <i>pilA::Tn5G</i> collection | 38 |
| Plasmids | | |
| pCR2.1 | Ap ^r ColE1 f1 <i>ori</i> | Invitrogen |
| pDM4 | Cm ^r , suicide vector carrying the <i>sacBR</i> genes for sucrose selection | 19 |
| pMP220 | Tc ^r IncP, vector for <i>lacZ</i> transcriptional fusions | 33 |
| pMMB190 | Ap ^r , pMMB66EH, <i>tac</i> promoter, <i>lacZ</i> α | 20 |
| pRK2013 | Km ^r ColE1 with Tra ⁺ Mob ⁺ | 9 |
| pUC18 | Ap ^r ColE1 <i>lacI</i> ϕ 80 <i>lacZ</i> α | Boehringer |
| pUCmvaU | 1.4-kb BamHI-PstI PAO1 chromosomal DNA fragment containing <i>mvaU</i> in pUC18 | This work |
| pUC Δ mvaU | 300-bp <i>mvaU</i> deletion in pUCmvaU | This work |
| pDM4 Δ mvaU | pUC Δ mvaU DNA insert cloned in pDM4 | This work |
| pMPFCAS | <i>cupA1S-lacZ</i> transcriptional fusion in pMP220 | This work |
| pMPFCAL | <i>cupA1L-lacZ</i> transcriptional fusion in pMP220 | This work |
| pMMBmvaT | 513-bp PAO1 chromosomal DNA fragment containing <i>mvaT</i> in pMMB190 | This work |
| pUCP18 | Same as pUC18, 1.8-kb stabilizing fragment for maintenance in <i>Pseudomonas</i> spp. | 29 |
| pUCPmvaT | 1.6-kb PAO1 DNA fragment containing <i>mvaT</i> cloned in pUCP18 | 8 |

finally released from the chaperone and assembled into fibrils while crossing the outer membrane (36). Among the genes encoding putative chaperones and usher proteins, those in the *cupA* gene cluster were found to be required for adhesion to inert surfaces, and thus their products are likely to be important in the initiation of biofilm formation (38). Such a requirement was shown to be independent of type IV pili under the conditions tested. The two additional clusters that were identified, *cupB* and *cupC*, had no obvious influence on surface attachment in these conditions (38).

To advance our understanding of the sequential involvement of these new fimbriae during the establishment of a *P. aeruginosa* biofilm, we sought to determine how the *cup* gene clusters are regulated. In addition, we would like to understand how their regulation is coordinated with the expression of other adhesins and with the general transcriptional response during biofilm formation. Indeed, the identification of a substantial number of genes that are under the control of common biofilm regulators could lead to the development of new therapeutic strategies directed towards the prevention of biofilm formation during infections. Here we describe the identification of a negative regulator of *cupA* gene expression, MvaT. Interestingly, an *mvaT* mutant also exhibits enhanced expression of the *cupB* and *cupC* gene clusters and displays increased surface coverage and greater biofilm depth.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used are listed in Table 1. Strains were grown at 30 or 37°C in L broth (LB), in

M63 minimal medium, or on LB agar plates. *E. coli* TG1 and TOP10F' were used to propagate plasmids. Plasmids were introduced into *P. aeruginosa* strains by electroporation (32) or triparental mating by using plasmid pRK2013 as a helper (9). The transformants were selected on *Pseudomonas* isolation agar containing antibiotics. Plasmids were maintained by adding ampicillin and tetracycline (50 and 15 μ g/ml, respectively) for *E. coli* and carbenicillin, tetracycline, and gentamicin (500, 200, and 50 μ g/ml, respectively) for *P. aeruginosa*.

Plasmid constructions and DNA manipulation. The *cupA1S-* and *cupA1L-lacZ* transcriptional fusions were constructed by PCR amplification of upstream DNA regions from the *cupA1* gene (370 and 1,017 bp, respectively) by using the oligonucleotide couples OFN65-OFN2 and OFN1-OFN2, respectively. PCR amplification products were directly cloned into the pCR2.1 vector by using the TA cloning kit (Invitrogen), nucleotide sequences were verified (Genome Express, Grenoble, France), and EcoRI DNA fragments were subcloned into the low-copy-number vector pMP220 (33), yielding pMPFCAS and pMPFCAL, respectively. The 513-bp fragment containing the *mvaT* gene was PCR amplified using the oligonucleotide pair M1/M2, cloned into pCR2.1, and subsequently subcloned into the pMMB190 vector (20), yielding pMMBmvaT. The *mvaT* gene was also cloned in pUCP18 (29), yielding pUCPmvaT, as described previously (8). Chromosomal DNA was prepared by using the Nucleospin C+T kit (Macherey-Nagel), and PCR amplifications were carried out using HiFi polymerase (Roche). All the oligonucleotides used in this study are listed in Table 2.

Screening of the *P. aeruginosa* Tn5G library. Construction of the Tn5G library was described previously (38). The plasmids carrying *lacZ* transcriptional fusions were introduced into the Tn5 library by using triparental mating. Mutants containing the plasmid were selected on LB plates supplemented with 200 μ g of tetracycline/ml and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) (125 μ g/ml). After overnight growth, blue colonies were identified and isolated on the same medium.

Inverse PCR. Inverse PCR was performed as described previously (38). Briefly, chromosomal DNA from each mutant was prepared by using the Nucleospin C+T kit. DNA was digested with AluI and religated. The circular DNA was used as the matrix for PCR amplification using oligonucleotides OTn1 and OTn2. The DNA fragments thus amplified were cloned into the pCR2.1 vector

TABLE 2. Oligonucleotide sequences

| Oligonucleotide | Nucleotide sequence | Gene or DNA region |
|-----------------|---------------------------------------|--------------------------------|
| OFN1 | 5'-GGAATACCAGTCCGAGGCGGGAT-3' | <i>cupAS-lacZ</i> |
| OFN65 | 5'-CCATAGTCGGAAATACAAGCGTTG-3' | <i>cupAL-lacZ</i> |
| OFN2 | 5'-GCTGAATGTGATAGTGTGGCCG-3' | <i>cupAS</i> and <i>L-lacZ</i> |
| M1 | 5'-CCACCGCCACTCAGCACAGACAAGG-3' | <i>mvaT</i> |
| M2 | 5'-CGCTATTCGCTGGAGACTGGAGGGC-3' | <i>mvaT</i> |
| OTn1 | 5'-GCGCGGATCCTGGAAAACGGGAAAAG-3' | Tn5G |
| OTn2 | 5'-CCATCTCATCAGAGGGTAGT-3' | Tn5G |
| MA101 | 5'-GGCAAACACTATCACATTCAGCCG-3' | <i>cupA1</i> (A1 probe) |
| MA102 | 5'-GGCGGCGGTGTTCCACAGGTTCCC-3' | <i>cupA1</i> (A1 probe) |
| NB101 | 5'-TCGTCAACTTCTCGGGCAACATCAC-3' | <i>cupB1</i> (B1 probe) |
| NB102 | 5'-CAGCGGGATCTTCGTGGTGTGGTC-3' | <i>cupB1</i> (B1 probe) |
| NC101 | 5'-CAGACGGCACCATCAATTTCAAAGG-3' | <i>cupC1</i> (C1 probe) |
| NC102 | 5'-ATAGATGCCAATACCGACGGCCAGT-3' | <i>cupC1</i> (C1 probe) |
| NA201 | 5'-CGGGCAAGCGTGGTGGTGACCG-3' | <i>cupA2</i> (A2 probe) |
| NA202 | 5'-TTGTCCACCGCACCGCCCAGC-3' | <i>cupA2</i> (A2 probe) |
| NA301 | 5'-TCCAACACTACACCTATTCGGCTAC-3' | <i>cupA3</i> (A3 probe) |
| NA302 | 5'-CCGTCTGAGAAATCGTGGAGGAG-3' | <i>cupA3</i> (A3 probe) |
| NA401 | 5'-GGGACGCCGTCTACGAGATGTTTTTC-3' | <i>cupA4</i> (A4 probe) |
| NA402 | 5'-TGTAGGAATAGGGCTGVGAGAGGAT-3' | <i>cupA4</i> (A4 probe) |
| NA501 | 5'-CTGCCGCCCGTATTCGCGCTGG-3' | <i>cupA5</i> (A5 probe) |
| NA502 | 5'-AGTACGGCGTCGGGTGTCCAC-3' | <i>cupA5</i> (A5 probe) |
| NB301 | 5'-CCTGTCTGCTGGCACTGTGTTTC-3' | <i>cupB3</i> (B3 probe) |
| NB302 | 5'-AATAGCTGGGCACCGAGACATA-3' | <i>cupB3</i> (B3 probe) |
| NC301 | 5'-AGGTGTCCGTCTATTCAGGT-3' | <i>cupC3</i> (C3 probe) |
| NC302 | 5'-GGTACGGTTGCTACTGAATTG-3' | <i>cupC3</i> (C3 probe) |
| 464801 | 5'-GCAGCCATTCCGACCTACCAACG-3' | PA4648 probe |
| 464802 | 5'-CGTCGGCGTGGGTGTGCTTTGTCC-3' | PA4648 probe |
| 465101 | 5'-ACCTACCTCTCCGCCAGCCGCT-3' | PA4651 probe |
| 465102 | 5'-CCTGTTCTCGACCGCCAGG-3' | PA4651 probe |
| Pil1 | 5'-ACCCGCTGAAGACCAGCTGTTGAAGA-3' | <i>pilA</i> probe |
| Pil2 | 5'-ATTTACAAGCCCAGACCCATCCGC-3' | <i>pilA</i> probe |
| mvaUUF | 5'-CGGCTGGATCCGCGACTTCGCGGAAAT-3' | Upstream <i>mvaU</i> |
| mvaUDR | 5'-GACGCCTGCAGTTCGGTCCCGGCC-3' | Downstream <i>mvaU</i> |
| mvaUUR | 5'-TGCCTCGCGGAACTGTGCACGTTGGACAT-3' | Upstream <i>mvaU</i> |
| mvaUDF | 5'-ACCGTCGAATCCTGGGTGCACCGCTAAGCCG-3' | Downstream <i>mvaU</i> |

by using the TA cloning kit and transformed into *E. coli* TOP10F' (Invitrogen). DNA sequencing was performed by Genome Express.

Computer analysis. The DNA sequences obtained following inverse PCR were analyzed with the BLASTN program and compared with the PAO1 genome at <http://www.pseudomonas.com>.

β -Galactosidase assay. Overnight cultures, grown in LB, were diluted in LB to an optical density at 600 nm (OD₆₀₀) of 0.1. Growth and β -galactosidase activity were monitored by harvesting samples at different time intervals. β -Galactosidase activity was measured according to the Miller method, based on *o*-nitrophenyl- β -D-galactopyranoside hydrolysis (28). β -Galactosidase activities were expressed in Miller units. β -Galactosidase activity was also observed by looking at blue colonies on plates containing 125 μ g of X-Gal per ml of LB agar.

Northern blot hybridization. Total RNA was isolated from 50-ml cultures of strains PAO1, PAO-P46, and PAO-P47 grown in LB. Following centrifugation, bacterial cells pellets were resuspended in 30 ml of Trizol reagent (Gibco BRL). After chloroform extraction and isopropanol precipitation, samples were treated with 20 U of RQ1 DNase (Promega) and cleaned using the RNeasy kit (Qiagen). One percent agarose gels for size fractionating RNA were cast and run using NorthernMax-Gly Gel prep/running buffer (Ambion). Glyoxal Load Dye (Ambion) was used for denaturation of sample RNA, with Millenium Marker (Ambion) included as a molecular weight marker. Twenty micrograms of total RNA was used for analysis.

Transfer of RNA to nylon membranes (Hybond N⁺ membranes; Amersham Pharmacia Biotech) was performed using transfer buffer (Ambion). Every step was performed following the manufacturer's instructions. Digoxigenin (DIG)-labeled DNA probes were generated using the PCR DIG Probe synthesis kit (Roche). The oligonucleotide pairs MA101/MA102, NB101/NB102, and NC101/NC102 were used to generate probes for *cupA1* (A1, 260 bp), *cupB1* (B1, 330 bp), and *cupC1* (C1, 339 bp), respectively. DNA probes for *cupA2* to *cupA5*, *cupB3*, and *cupC3* were also generated by using the oligonucleotide pairs NA201/NA202, NA301/NA302, NA401/NA402, NA501/NA502, NB301/NB302,

and NC301/NC302, respectively. Finally, the DNA probes for PA4648 and PA4651 were generated by using the oligonucleotide pairs 464801/464802 and 465101/465102, respectively. Hybridization was performed overnight at 50°C using at least 5 ng of labeled probe per ml of DIG Easy Hyb buffer (Roche). Washing and blocking reagents came from the DIG Wash and Block buffer set. Blots were developed using Fab fragments from an anti DIG-alkaline phosphatase conjugate (Roche), with detection with CDP-star (Roche). Autoradiography was performed with X-Omat films (Kodak). Every step was performed according to the manufacturer's instructions.

Microarray analysis. Five-hundred-microliter portions of bacterial cultures were treated with 100 μ l of lysozyme (1 mg/ml), the samples were left for 15 min at room temperature, and total RNA was isolated by using the Qiagen RNeasy kit. RQ1 DNase (Promega) was used for DNase treatment, and RNAs were checked on 1% agarose gels (RNase free). Targets for microarrays were subsequently prepared according to the Affymetrix Expression Analysis Protocol Guide with few modifications, and RNA spike controls provided by the Cystic Fibrosis Foundation were included. Briefly, 12 μ g of RNA was used and treated with 1 U of RNase inhibitor (Ambion) per μ l during cDNA synthesis using Superscript II (Invitrogen). cDNA was purified by using a PCR purification kit (Qiagen), and cDNA fragmentation was performed by using 0.06 U of DNase I (Roche) per microgram of cDNA at 37°C for 10 min followed by 10 min at 100°C. The extent of cDNA fragmentation was verified on a 2% agarose gel, and then cDNA was end labeled with biotin ddUTP by using the Enzo BioArray Terminal Labeling kit. Target hybridization to GeneChip *P. aeruginosa* genome arrays (Affymetrix), washing, and scanning were performed using a hybridization oven, a Fluidics station, and a scanner obtained from Affymetrix. Hybridization intensity data were extracted from the scanned array images, and intrachip normalizations were performed using Affymetrix Microarray Suite 5.0 software. The data were subsequently analyzed and experimental comparisons were made using GeneSpring version 4.2.1 (Silicon Genetics).

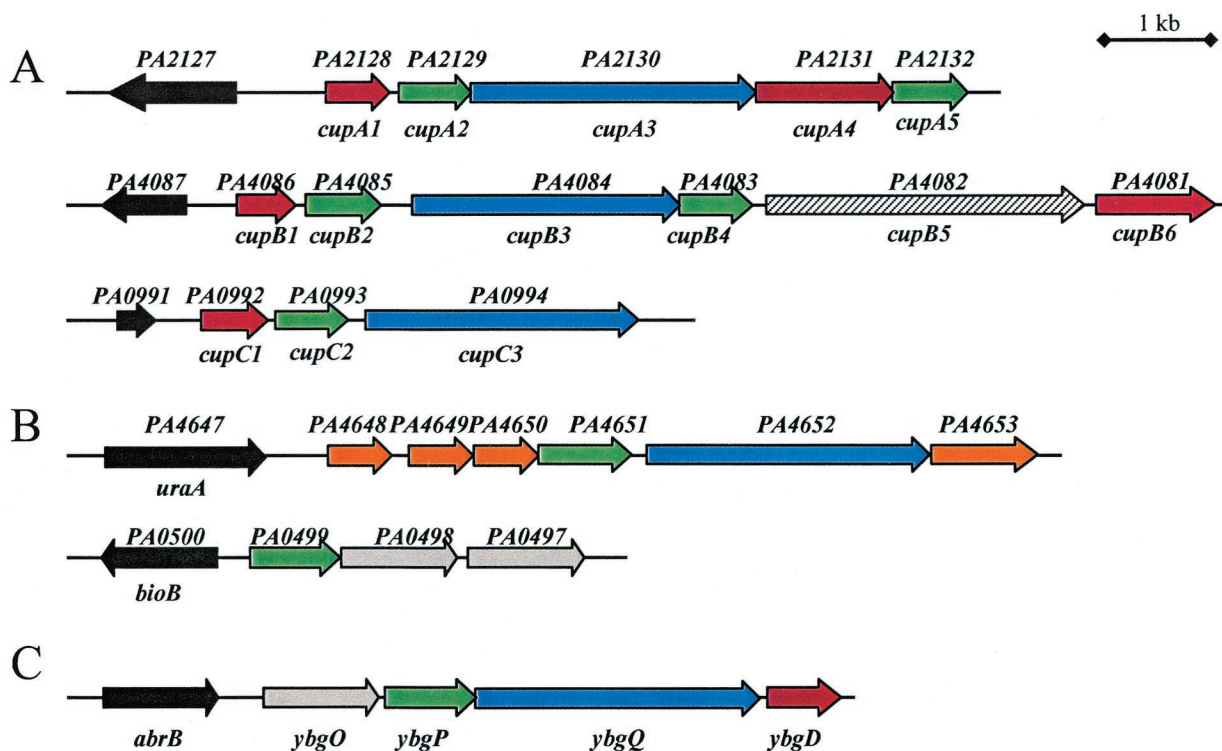


FIG. 1. Organization of fimbrial gene clusters. Genes encoding putative fimbrial subunits are indicated in red, chaperones are indicated in green, and ushers are indicated in blue. Genes located upstream of the putative promoter region of the gene clusters are represented in black. (A) *cupA*, *cupB*, and *cupC* gene clusters from *P. aeruginosa*. The hatched *cupB5* gene encodes a protein homologous to filamentous hemagglutinin. (B) Additional *P. aeruginosa* gene clusters containing fimbrial assembly genes. Genes represented in orange encode proteins containing the COG5430 domain. In grey are *ybgO* homologues that are found in an *E. coli* K-12 fimbrial gene cluster. (C) Fimbrial *ybg* gene cluster from *E. coli* K-12.

Construction of *P. aeruginosa* mutants. The *cupA3* mutation was engineered in the *mvaT* mutant (PAO-P47) as described previously (38), yielding a *mvaT cupA3* mutant termed PAO-MC. An *mvaU* chromosomal deletion mutant of PAO1 lacking 300 internal nucleotides was constructed as follows. Using PAO1 DNA as a template, the intact *mvaU* gene (PA2667) (354 bp) plus 528 bp of upstream flanking DNA and 526 bp of downstream flanking DNA was amplified by using the primer pair *mvaUUF*, containing a BamHI restriction site, and *mvaUDR*, containing a PstI restriction site. The resulting PCR product was digested with BamHI and PstI and cloned into similarly digested pUC18, resulting in plasmid pUC*mvaU*. To introduce a deletion of the recombinant *mvaU* gene, the primer pair *mvaUUR* and *mvaUDF* were used in conjunction with inverse PCR, using pUC*mvaU* DNA as a template. The resulting blunt-ended PCR product containing a 300-bp deletion in *mvaU* was self-ligated, resulting in plasmid pUCΔ*mvaU*. The PCR product was excised from the vector by using BamHI and SphI and cloned into the vector pDM4 (19), digested with BglII and SphI, resulting in plasmid pDM4Δ*mvaU*. Allelic exchange using pDM4Δ*mvaU* contained in *E. coli* S17-1 λpir with PAO1 resulted in a *P. aeruginosa* strain (PAO-P46) containing an in-frame deletion of the *mvaU* gene. This deletion was confirmed by both PCR and Southern blot analysis (data not shown). The *mvaU* deletion mutant of the PAO1 *lecA::lux* strain was constructed by allelic exchange using pDM4Δ*mvaU* contained in *E. coli* S17-1 λpir with PAO1 *lecA::lux*, resulting in strain PAO-P48. The deletion was confirmed by PCR (data not shown).

Biofilm formation assay. Biofilm formation on stainless steel was assayed as follows. Prior to inoculation, 10 ml of LB was seeded with fresh bacterial colonies and incubated overnight at 37°C. The turbidity of the cultures (600 nm) was adjusted to 1.0 (±0.1), and 100-μl aliquots were used to inoculate petri dishes containing three stainless steel coupons in 10 ml of 0.1× LB. Plates were incubated at 37°C on a rotary shaker (60 rpm) for 72 h.

Visualization and analysis of biofilm structure. Stainless steel coupons were rinsed twice in sterile phosphate-buffered saline and air dried. They were then heat fixed, stained in 0.1% acridine orange for 2.5 min, and rinsed in phosphate-buffered saline. The coupons were then examined for bacterial attachment by using an inverted fluorescence microscope. Surface coverage was estimated as described previously (17) using the LUCIA image analysis program (version 4.11).

Time- and cell-density-dependent measurement of bioluminescence. Bioluminescence was determined as a function of cell density using a combined automated luminometer-spectrometer (Anthos Labtech LUCYI) as described previously (42). Overnight cultures of *P. aeruginosa* were diluted 1:1,000 in fresh LB medium, and 0.2 ml was inoculated into microtiter plates. Luminescence and turbidity of the cultures at 495 nm (OD₄₉₅) were automatically determined every 30 min (as relative light units per unit of OD₄₉₅).

RESULTS

Expression of the *cupA* gene fusion in *P. aeruginosa* PAO1.

To investigate the activity of the *cupA* promoter region, two transcriptional fusions were constructed using various lengths of DNA located upstream from the *cupA1* start codon. The *cupA1* gene is the first gene of a cluster encoding components involved in fimbrial biogenesis via the chaperone-usher pathway (*cupA1* to *cupA5*) (Fig. 1A). An open reading frame (ORF) (PA2127 in the PAO1 genome annotation) encodes a protein which shares 40% identity with the hypothetical *E. coli ybdN* gene product. It is located 850 bp upstream of *cupA1* and transcribed in the reverse orientation. Fragments of 370 and 1,017 bp, the last one containing the whole PA2127-*cupA1* intergenic region, were PCR amplified using the oligonucleotide pairs OFN65/OFN2 and OFN1/OFN2, respectively. The DNA fragments were cloned into the pMP220 vector, yielding the transcriptional fusions *cupA1S-lacZ* (S for short) and *cupA1L-lacZ* (L for large) carried on plasmids pMPFCAS and pMPFCAL, respectively. The fidelity of each cloned fragment was verified by DNA sequencing.

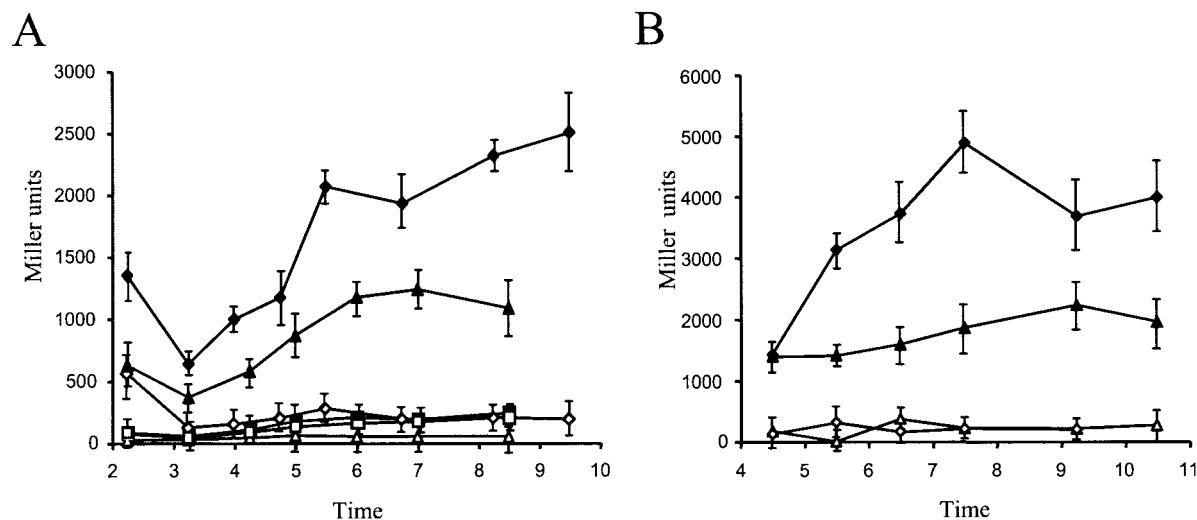


FIG. 2. Expression of the *cupA* gene cluster. (A) The expression level of the *cupA_{IS}-lacZ* (pMPFCAS) (diamonds) and *cupA_{IL}-lacZ* (pMPFCAL) (triangles) transcriptional fusions from the wild-type PAO1 strain (open symbols) or the isogenic *mvaT* mutant (filled symbols) were compared to those obtained with the pMP220 cloning vector control (squares). (B) Expression of the *cupA_{IS}-lacZ* (pMPFCAS) (diamonds) and *cupA_{IL}-lacZ* (pMPFCAL) (triangles) transcriptional fusions from the *mvaT* mutant containing a plasmid encoding the *mvaT* gene (pMMBmvaT) (open symbols) or the cloning vector (pMMB190) (filled symbols). Each experiment was independently repeated three times, and error bars indicate standard deviations.

The recombinant plasmids were introduced into the *P. aeruginosa* wild-type strain PAO1. The recombinant strains were subsequently grown in LB medium, and β -galactosidase activity was measured at different time points over the growth period (Fig. 2A). Both transcriptional fusions exhibited similar β -galactosidase activity levels compared with that observed for the pMP220 control. The same comparative results were obtained when these strains were grown in minimal medium (M63) supplemented with glucose and Casamino Acids at different temperatures (30 or 37°C) in flask cultures or in microtiter plate wells (data not shown).

Isolation of *P. aeruginosa* mutants exhibiting detectable *cupA* gene expression. Because of the low β -galactosidase activity originating from the *cupA₁* promoter-*lacZ* constructs in *P. aeruginosa*, we reasoned that *cupA* gene expression in the *P. aeruginosa* wild-type strain PAO1 was repressed in the conditions of our assay. The pMPFCAL plasmid, carrying the *cupA_{IL}-lacZ* fusion, was thus introduced into a *P. aeruginosa* mutant collection generated by random insertion of a Tn5 modified transposon, Tn5G (38). The recombinant clones were plated on selective media containing X-Gal. Three clones that exhibited an intense blue color, revealing an increased level of β -galactosidase activity, were selected and further analyzed. The genomic DNA from these clones was isolated and subjected to inverse PCR using divergent oligonucleotides that match the 3' end of the transposon, as previously described (38). Determination of the nucleotide sequence of the PCR products obtained revealed that, in all three clones, the transposon had inserted into a gene that was annotated as PA4315 on the *P. aeruginosa* genome website (www.pseudomonas.com) (34). This ORF encodes a protein that is 82% similar to the P16 subunit of the *Pseudomonas mevalonii* heteromeric transcriptional regulator MvaT (27). This gene has also recently been found to encode a novel regulator of virulence gene expression in *P. aeruginosa*, which is termed MvaT (8).

Examination of the *P. aeruginosa* PAO1 genome database revealed the presence of a second MvaT P16 subunit homologue (PA2667). It is 64% similar to that of *P. mevalonii* (it is also 51% identical and 68% similar to PA4315). We have therefore designated PA4315 as *mvaT* and PA2667 as *mvaU*. Further analysis of the genome databases indicates that MvaT P16 subunit homologues, with sequence conservation spread evenly throughout the proteins, are highly conserved in the *Pseudomonas* genus genomes, including *P. putida*, *P. syringae*, *P. fluorescens*, *P. aeruginosa*, *P. mevalonii*, and the closely related *Azotobacter vinelandii*. Recently, these MvaT proteins have been described as a novel class of H-NS-like proteins. Indeed, even if they show no significant homology with any known H-NS protein at the sequence level, MvaT proteins are predicted to share the same three-dimensional structure as the *E. coli* H-NS and seem to be functionally related (35).

MvaT specifically down-regulates the *cupA₁* promoter. The plasmid pMPFCAL was introduced into a PAO1 *mvaT* deletion mutant (PAO-P47), and the β -galactosidase activity was determined to confirm the influence of MvaT on gene expression from the *cupA₁* promoter. In these conditions, the level of β -galactosidase was increased up to sixfold when compared with the expression level observed in the wild-type strain PAO1 (Fig. 2A). Furthermore, there is a reproducible difference in the activation of gene expression when the two *cupA* transcriptional fusions are considered. That containing the shorter region (*cupA_{IS}-lacZ*) is twofold up-regulated when compared with the larger fusion (*cupA_{IL}-lacZ*), which encompasses an additional 550-bp DNA region (Fig. 2A). This suggests that an additional, as yet uncharacterized, *cis*-controlling element might exist within the upstream DNA fragment of the larger fusion. To verify that the increase in β -galactosidase activity from the *cupA-lacZ* transcriptional fusion was a direct consequence of the absence of MvaT, the *mvaT* gene was cloned into the broad-host-range pMMB190, yielding pMMBmvaT. This

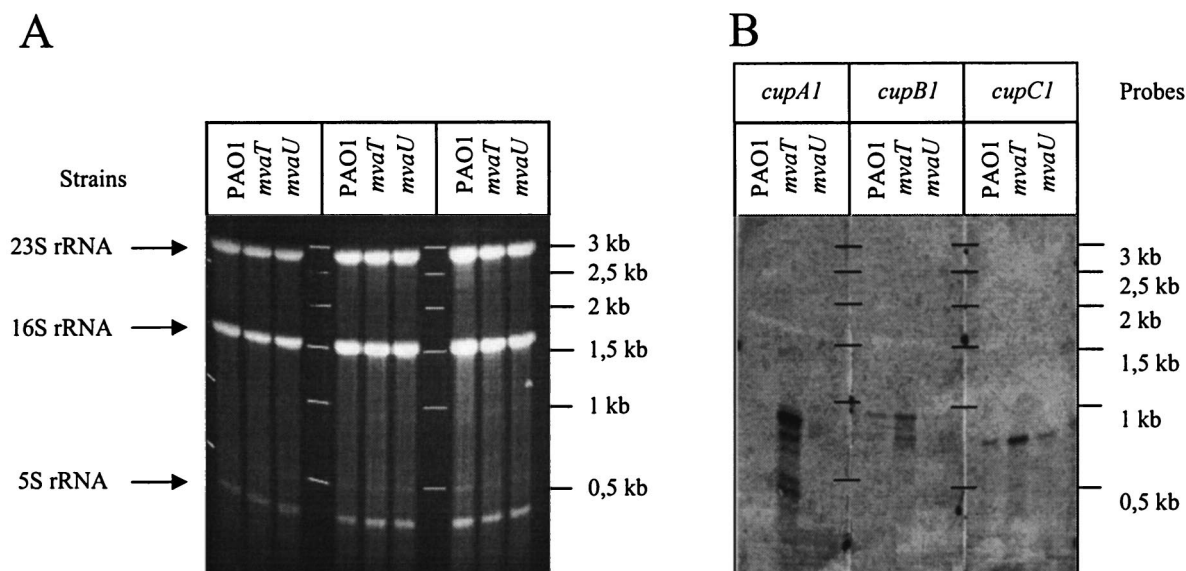


FIG. 3. Northern blot analysis. (A) Total RNA was extracted from various *P. aeruginosa* strains, including PAO1 and the *mvaT* (PAO-P47) and *mvaU* (PAO-P46) isogenic mutants, and loaded on a 1% agarose gel. The positions of the major bands corresponding to the 23S, 16S, and 5S rRNA are indicated on the left, whereas the molecular size standards are indicated on the right. (B) After electrophoresis the RNA was transferred onto nitrocellulose and hybridized with one of various probes, including *cupA1*, *cupB1*, and *cupC1* probes. Each experiment was independently repeated up to three times with no significant variation observed.

recombinant plasmid was introduced into PAO-P47 (*mvaT*) containing pMPFCAL or pMPFCAS to complement the *mvaT* mutation. Interestingly, the level of β -galactosidase was strongly reduced in the *mvaT* mutant carrying pMMB*mvaT* whereas it remained high when the plasmid control pMMB190 was used (Fig. 2B). This observation confirmed that expression from the *cupA1* promoter is repressed when MvaT is present.

The influence of MvaT on *cupA* gene cluster expression was also analyzed by Northern blot hybridization in order to evaluate which of the *cupA* genes could be transcribed from the *cupA1* promoter. RNAs were extracted from PAO1 and PAO-P47 (*mvaT*) as described in Materials and Methods. RNA samples were run on a 1% agarose gel and transferred onto a nylon membrane. DNA probes, which match with each of the *cupA* genes (Fig. 1A and Table 2), were engineered using the PCR DIG Probe synthesis kit (Roche) and used for hybridizing the membrane. The position of the transcripts was visualized by using anti-DIG antibodies and autoradiography (Fig. 3). Using the *cupA1* probe, we clearly detected a transcript of approximately 780 nucleotides, slightly longer than the full-length *cupA1* gene (551 bp), in the *mvaT* mutant but not in the wild-type strain. This observation confirmed that transcription from the *cupA1* promoter could not clearly be detected in the wild-type background but that a deletion of the gene encoding the MvaT transcriptional regulator considerably enhanced transcription of the *cupA1* gene. Moreover, the size of the mRNA indicates that the *cupA1* gene is monocistronic or that its transcript is rapidly processed from a larger precursor. Transcripts corresponding to the *cupA2* to *cupA5* cluster region could not be detected (data not shown). This might be due to a much lower level of transcription of these genes or mRNA instability compared to *cupA1*. Finally, using a *pilA* control probe, we could detect no variation in the transcript level of

this gene between the PAO1 wild-type strain and the *mvaT* mutant (data not shown).

***mvaT* mutants exhibit enhanced biofilm formation.** Since the *cupA* gene cluster is required for biofilm formation on abiotic surfaces and as MvaT negatively regulates *cupA* gene cluster expression, we examined the biofilm phenotype of the *mvaT* mutant on stainless steel surfaces. The surface coverage of the biofilms formed by each *P. aeruginosa* strain on stainless steel coupons in static biofilm experiments after 72 h at 37°C was analyzed (see Materials and Methods). The data were obtained from 60 independent measurements of biofilms stained with 0.1% (wt/vol) acridine orange and revealed a mean twofold increase in surface coverage for the *mvaT* mutant PAO-47 compared with the wild type (data not shown). Figure 4 illustrates the enhanced biofilm depth and surface coverage of the *mvaT* mutant as compared with the wild-type strain. We also used another technique to quantify bacterial attachment to polystyrene surfaces, a crystal violet-based assay (23), and similarly PAO-P47 showed enhanced biofilm formation (data not shown). These data are consistent with a role for *mvaT* as a negative regulator of *cupA* gene expression since these genes have previously been shown to be involved in initial attachment (38). Moreover, on stainless steel, we observed a significant increase in biofilm depth for PAO-P47 as compared with the wild-type strain PAO1. This increase is revealed by the large augmentation of microcolony size, as shown in Fig. 4C.

To supplement these data, we tested the influence of *mvaT* with respect to surface coverage on stainless steel in different genetic backgrounds. For example, overexpression of *mvaT* from pUCP*mvaT* in a wild-type strain of *P. aeruginosa* resulted in reduced surface coverage (Fig. 5A), which could be a result of down-regulation of a gene such as *cupA1* or *lecA* (8) (Fig. 2).

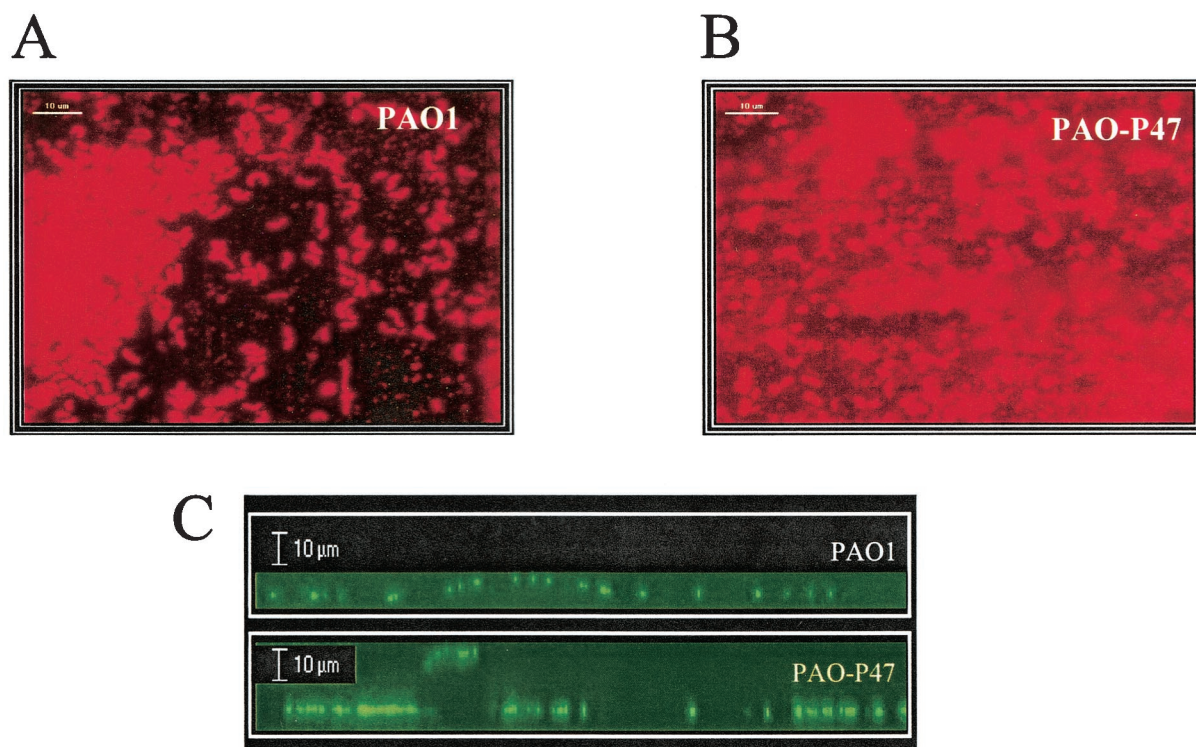


FIG. 4. Biofilm formation by *P. aeruginosa* PAO1 and PAO-P47 (*mvaT* mutant) on stainless steel. (A and B) Characteristics of the biofilms formed by PAO1 and PAO-P47, respectively. (C) Depth of biofilms formed by PAO1 and PAO-P47 in a static model as determined using confocal laser scanning microscopy.

However, surface coverage of a *mvaT lecA* double mutant, PAO-P10 (8), was increased as compared to the *lecA* single mutant (PAO1 *lecA::lux*) (Fig. 5B). This indicates that adhesive structures encoded by genes different from *lecA*, but also overexpressed in the *mvaT* background, may be responsible for the increased biofilm phenotype. Finally, surface coverage of a *mvaT cupA3* double mutant (PAO-MC) was decreased as compared to the *mvaT* single mutant (PAO-P47) (Fig. 5C), suggesting that in the *mvaT* background, the CupA system strongly contributes to the enhanced biofilm phenotype.

Influence of MvaT on *cupB* and *cupC* gene expression. Two additional gene clusters encoding components of the chaperone-usher pathway have previously been identified in *P. aeruginosa* (5, 38). These gene clusters, termed *cupB1* to *cupB6* (PA4086 to PA4081) and *cupC1* to *cupC3* (PA0992 to PA0994) (Fig. 1A), were examined with respect to their expression in a *P. aeruginosa* wild-type or *mvaT*-negative genetic background.

We analyzed the transcription of *cupB* and *cupC* gene clusters using Northern blot hybridization. DNA probes were designed for two of the *cupB* and *cupC* genes (*cupB1* and -3 and *cupC1* and -3), encoding, respectively, the predicted major pilin subunit and the usher for each system (Fig. 1). In contrast to what we observed with the *cupA* gene cluster, transcripts could be detected in the wild-type PAO1 strain with B1 and C1 probes (Fig. 3). Interestingly, the RNA levels appeared to be increased by two- to threefold in both cases in PAO-P47 (*mvaT*) (Fig. 3). These results suggest that *cupB1* and *cupC1* gene expression might also be controlled in an MvaT-dependent manner. The sizes of the transcripts detected with B1 and

C1 are approximately 885 and 730 nucleotides, respectively (Fig. 3). For information, the length of the *cupB1* and *cupC1* genes is 569 and 617 bp, respectively. Similarly as with the *cupA* gene cluster, it seems that only transcripts corresponding to the first gene of the cluster could be detected, since no RNA could be detected using either B3 or C3 probes in an *mvaT* or wild-type genetic background (data not shown).

Analysis of *cup* gene expression in an *mvaT* genetic background using DNA microarrays. We examined the range of genes controlled by MvaT, because there was a reasonable probability that in addition to a set of gene clusters (*cup* genes) it may exert a global influence on other genes involved in bacterial attachment and biofilm formation. The microarray used (Affymetrix GeneChip *P. aeruginosa* genome array), contains probes representing the whole PAO1 genome. We were therefore able to compare global gene expression of the parental PAO1 strain and its isogenic *mvaT* mutant. Bacterial strains were grown at 37°C to OD₆₀₀ of 3 in LB medium. We chose these conditions for the analysis since they were those in which we observed a large change in *cupA1* gene expression with *lacZ* transcriptional fusions as well as Northern blot studies. Two independent microarray experiments were performed, and the data obtained were merged and quantified by using the Microarray Suite (Affymetrix) and GeneSpring (Silicon Genetics) softwares. Strikingly, *cupA1* gene (PA2128) expression was increased 15-fold in the *mvaT* mutant, which is the most drastic change observed in gene expression in this analysis, whereas other *cupA* genes presented a 4.12-fold (*cupA2*) or 2.96-fold (*cupA3*) up-regulation (Table 3). These values are in agree-

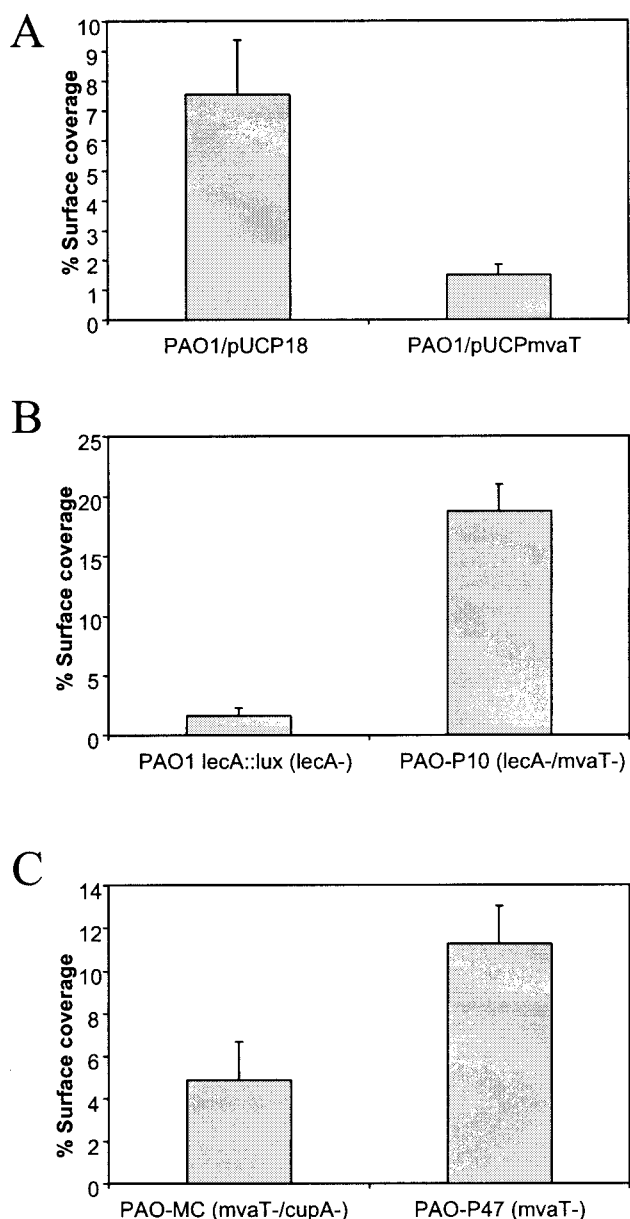


FIG. 5. Quantitation of biofilm formation by *P. aeruginosa* on stainless steel. (A) Influence of overexpression of *mvaT* from pUCPmvaT in the wild-type PAO1 strain. (B) Influence of the *mvaT* mutation within a *lecA* genetic background (PAO1-P10). PAO *lecA::lux* is the parental *lecA* mutant. (C) Influence of the *cupA3* mutation within a *mvaT*-negative background (PAO-MC). PAO-P47 is the parental *mvaT* mutant.

ment with the previous results obtained by Northern blot analysis indicating that only *cupA1* transcripts are clearly detectable in an *mvaT* background and thus could be considered as good internal controls to validate the variation in expression of other genes. The up-regulation of *cupB* and *cupC* gene expression was also confirmed by the microarray analysis. Specifically, *cupB1* and *cupC1* transcripts had a 2.43- and 2.9-fold increase, respectively, in the *mvaT* mutant (Table 3). Based on the microarray analysis, 156 genes showed at least a twofold change in their mRNA levels in the *mvaT* mutant compared to

the wild-type (see Tables S4 and S5 in the supplemental material). Among these, 104 genes showed a twofold or greater increase in mRNA levels in the *mvaT* mutant (see Table S4 in supplemental material). Interestingly, one of the genes (annotated PA4651) that shows the highest level of differential expression (5.1-fold) encodes a probable pilus assembly chaperone (Table 3). This gene belongs to a larger cluster in which a gene encoding an usher homologue could be found (PA4652) but no genes encoding fimbrial subunits were identified (Fig. 1B). However, most of the genes in the cluster (PA4648, -4649, -4650, and -4653) are at least threefold up-regulated in the *mvaT* mutant (Table 3). Using Northern blot analysis and DNA probes directed against PA4648 and PA4651, we observed higher transcript levels corresponding to these genes in the *mvaT* mutant than in the wild-type strain (data not shown), thus confirming the microarray data. Four of the genes (PA4648, PA4649, PA4650, and PA4653) encode proteins with similarities to protein U, which is found at the surface of the *Myxococcus xanthus* spore (10). Moreover, another gene, PA0498, was identified with a 3.13-fold up-regulation (Table 3). This ORF is found clustered with PA0499 and PA0497, with PA0499 encoding a probable pilus assembly chaperone (Fig. 1B). PA0497 and PA0498 encode highly homologous proteins that are 38% similar to YbgO from *E. coli* (Fig. 1C). Strikingly, the *ybgO* gene belongs to a cluster containing genes coding for a chaperone (YbgP), an usher (YbgQ), and a fimbrial subunit (YbgD) (3). The fold change in expression of all *cup*-related genes in the *mvaT* mutant is reported in Table 3. Another interesting genetic locus up-regulated in *mvaT* (see Table S4 in the supplemental material) contains the genes PA4306 to PA4297, which encode components reported in *Actinobacillus actinomycetemcomitans* as being important for tight adherence (Tad) (15). Finally, the *lecA* gene, encoding the PA-IL lectin, has previously been described as an MvaT target (8). We found a 4.02-fold up-regulation of *lecA* in the

TABLE 3. *cup*-related genes that are up-regulated in the *mvaT* mutant^a

| PA No. | Gene | Gene product characteristic | Fold change |
|---------------|--|---------------------------------|---------------|
| PA2128 | <i>cupA1</i> | Fimbrial subunit | 15.920 |
| PA2129 | <i>cupA2</i> | Pilus assembly chaperone | 4.120 |
| PA2130 | <i>cupA3</i> | Usher protein | 2.960 |
| PA2131 | <i>cupA4</i> | Putative adhesin subunit | 1.830 |
| PA2132 | <i>cupA5</i> | Pilus assembly chaperone | 1.485 |
| PA4086 | <i>cupB1</i> | Fimbrial subunit | 2.430 |
| PA4085 | <i>cupB2</i> | Pilus assembly chaperone | 2.400 |
| PA4084 | <i>cupB3</i> | Usher protein | 0.739 |
| PA4083 | <i>cupB4</i> | Pilus assembly chaperone | 1.519 |
| PA4081 | <i>cupB6</i> | Putative adhesin subunit | 1.728 |
| PA0992 | <i>cupC1</i> | Fimbrial subunit | 2.900 |
| PA0993 | <i>cupC2</i> | Pilus assembly chaperone | 1.950 |
| PA0994 | <i>cupC3</i> | Usher protein | 1.595 |
| PA4648 | Hypothetical protein (COG5430) | 5.360 | |
| PA4649 | Hypothetical protein (COG5430) | 3.700 | |
| PA4650 | Hypothetical protein (COG5430) | 6.580 | |
| PA4651 | Pilus assembly chaperone | 5.100 | |
| PA4652 | Usher protein | 2.560 | |
| PA4653 | Hypothetical protein (COG5430) | 3.480 | |
| PA0498 | Hypothetical protein-YbgO homolog | 3.130 | |

^a Genes with a change above twofold are indicated in bold.

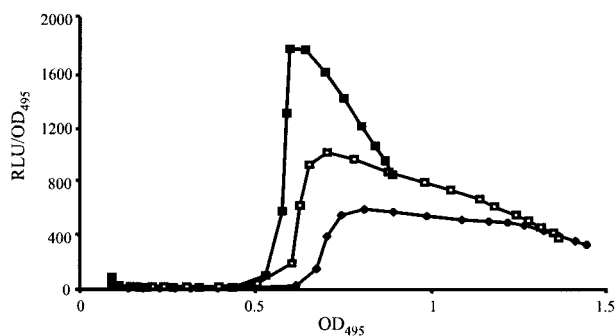


FIG. 6. Mutation in *mvaU* derepresses *lecA*. Influence of *mvaT* and *mvaU* on the expression of a *P. aeruginosa lecA::lux* chromosomal fusion (PAO1 *lecA::lux*). Lux-related bioluminescence in PAO1 *lecA::lux* (closed diamonds), a PAO1 *lecA::lux*-containing *mvaT* mutant (PAO-P10) (closed squares), and a PAO1 *lecA::lux*-containing *mvaU* mutant (PAO-P48) (open squares) was measured as a function of light output per cell. Relative light units and OD₄₉₅ were determined as described in the Materials and Methods.

mvaT mutant (see Table S4 in the supplemental material), confirming our previous results (8) and again validating the overall data obtained with the microarray.

MvaU does not influence *cup* gene expression or biofilm surface coverage. The gene annotated PA2667 encodes a second *P. aeruginosa* regulator belonging to the MvaT family (MvaU). To determine whether *mvaU* is also involved in the regulation of *cupA* gene expression and biofilm formation, a *P. aeruginosa mvaU* deletion mutant was constructed (PAO-P46). Interestingly, when the *cupAL-lacZ* transcriptional fusion was introduced into this strain, no increase in β -galactosidase activity was observed (data not shown). Similar results were obtained with the *cupAS-lacZ* transcriptional fusion (data not shown). In addition, Northern blot analysis revealed that no *cupA1* transcript could be detected in the *mvaU* mutant, in contrast to that observed for the *mvaT* mutant (Fig. 3). The two- to threefold increase in *cupB1* and *cupC1* mRNA levels observed in the *mvaT* mutant in Northern blot analysis was also absent in the *mvaU* mutant. These results indicate that the repression exerted on the *cupA1* promoter, and to a lesser extent on the *cupB1* and *cupC1* promoters, is MvaT dependent and does not appear to involve any cross-talk with the MvaU protein. Furthermore, analysis of surface coverage on stainless steel coupons indicates that there is no significant difference between the *mvaU* mutant (PAO-P46) biofilm and that of the PAO1 wild type (data not shown). However, MvaT and MvaU may share a number of targets, as is the case for *lecA*, the gene encoding the PA-IL lectin. Indeed, we showed that in both the *mvaT* and *mvaU* mutants, the expression of *lecA* is enhanced (Fig. 6).

DISCUSSION

Biofilm formation in a variety of model systems has been used to understand many intriguing aspects of microbial life in communities within a particular niche such as the environment or an infected host. In recent years, genetic approaches have facilitated characterization of the molecular mechanisms involved in this process (21). We have also used genetic approaches to isolate mutants of *P. aeruginosa* affected in biofilm

formation, and this led to the identification of *cup* genes, encoding components of a so-called chaperone-usher pathway, that play an important role at certain stages of biofilm formation (38). In this study, we (i) investigated the expression of the *P. aeruginosa cup* gene clusters; (ii) uncovered the role of MvaT, a transcriptional regulator belonging to a family of proteins which so far have been found only in the pseudomonads, but which could be functionally related to the H-NS proteins (8, 35); and (iii) established the contribution of MvaT to biofilm formation.

When compared with the parental wild-type strain, a *P. aeruginosa cupA* mutant was unable to attach to the wells of polystyrene microtiter plates when grown at 30 or 37°C in minimal medium supplemented with Casamino Acids (38). This observation suggested that under these growth conditions, in which a distinct CupA-dependent phenotype is apparent, the *cupA* genes must be expressed at a significant level. However, using different *lacZ* transcriptional fusions to the *cupA1* promoter region, we were unable to detect high β -galactosidase activity during the growth of *P. aeruginosa* in microtiter plate wells or in flasks in either minimal or rich medium at 30 or 37°C. One obvious explanation is that the *cupA* genes are only poorly expressed. Nevertheless, it is a possibility that certain adhesins, such as those related to the *cupA* gene cluster, are only transiently expressed, i.e. not before bacterial cells reach, or contact, a surface and not after the biofilm reaches a certain level of maturation. Involvement of regulatory circuits, such as the CpxRA pathway, in biofilm formation following surface contact has already been proposed (24). Moreover, it is noteworthy that the *cupA1* gene (PA2128) is down-regulated once the biofilm established (41). It is thus a possibility that only those cells which reach the wall of the microtiter plate wells will support strong induction of *cup* genes, whereas most of the planktonic bacterial culture, or the cells embedded within the biofilm structure, will keep a low level of *cup* gene expression. To determine whether *cupA* expression is repressed at some stages via a regulatory element, we introduced a *cupA1* promoter fusion into a library of random *P. aeruginosa* Tn5 mutants and screened for strains in which *cupA1* expression was up-regulated. Three distinct mutants were obtained, each of which contained a Tn5 insertion in the same gene, *mvaT*. This gene encodes a homologue of the P16 subunit of the *P. mevalonii* transcriptional regulator MvaT (27).

MvaT was originally identified as a regulator of the *P. mevalonii* operon *mvaAB*, which encodes two enzymes required for mevalonate catabolism, (27). MvaT was shown to bind to a region located at positions -48 to -84 upstream from the *mvaA* translational start codon (39). Analysis of the *cupA1* promoter regions, as well as those of *cupB1*, *cupC1*, and *lecA*, analyzed since they also appeared to be down-regulated by MvaT, failed to identify any significant regions of homology with the *mvaA* cis-acting element. Moreover, no obviously identifiable repeats or conserved DNA domains could be detected when these promoter regions were compared. Finally, and because of the lack of consensus sequences, it is possible that the control exerted by MvaT on its various targets, including *cupA1*, might be direct or indirect.

Interestingly, the *cup* gene clusters are similar to the fimbrial gene clusters of other organisms in that they contain genes encoding an usher, a chaperone, and one or more fimbrial

subunits. Strikingly, Northern blot analysis showed that in all three cases, only a transcript with a size corresponding to that of the first gene of each cluster, *cupA1*, *cupB1*, and *cupC1*, could clearly be identified. It was previously shown that with the *E. coli* gene clusters encoding the Pap or F1845 fimbriae, a polycistronic precursor mRNA is degraded to a relatively stable monocistronic transcript. Such a transcript exclusively contains the gene encoding the major fimbrial subunit, *papA* or *daaE*, respectively (1, 2). These observations strongly suggest that CupA1, CupB1, and CupC1 are major subunits of fimbrial structures assembled by a chaperone-usher pathway. Remarkably, in *E. coli*, the H-NS protein is known to be involved in regulating the expression of genes responsible for the assembly of the Pap pili and the type 1 pili, the best-studied of the chaperone-usher pathways. The assertion by Tendeng and collaborators (35) that MvaT proteins and H-NS proteins are functionally linked is thus reinforced by our observations, which indicate that *P. aeruginosa* MvaT and *E. coli* H-NS are both involved in fimbrial gene expression control.

In *P. aeruginosa*, biofilm formation is strongly dependent on the expression of extracellular appendages such as flagella, type IV pili, or the putative fimbrial structures encoded by the *cupA* genes (22, 38). Given the inability of *cupA* mutants to attach to an abiotic surface and the negative effect exerted through MvaT on *cupA* expression, *mvaT* mutants would be predicted to exhibit enhanced adhesion properties. Consistent with this prediction, we have shown that the biofilm produced on stainless steel coupons by the *P. aeruginosa mvaT* mutant shows greater surface coverage and depth than that produced by the wild type. However, MvaT may control the expression of other yet uncharacterized genes and some may also contribute to the enhanced biofilm-forming capacity of the *mvaT* mutant. Transcriptional profiling of the *mvaT* mutant clearly showed that the absence of this regulatory factor can indeed cause both an increase and a decrease in the levels of mRNA for a substantial number of genes (see Tables S4 and S5 in the supplemental material). In addition to the already known *cup* genes, we identified novel, but incomplete, clusters containing genes encoding a chaperone and an usher protein homologue (Fig. 1B and Table 3), which were also dependent on MvaT. A gene cluster containing PA4651 and PA4652, the chaperone- and usher-encoding genes, respectively, was found up-regulated in the *mvaT* background. Intriguingly, no gene encoding a protein with significant similarities to fimbrial subunits could be identified in this cluster. However, the four other ORFs (PA4648, PA4649, PA4650, and PA4653) encode proteins that contain a characteristic domain found in the protein U precursor of *M. xanthus* (31). This protein is produced as a precursor with a canonical signal peptide and is assembled on the spore surface after crossing the membrane. The protein U domain, called SCPU (pfam05229), is included in a larger conserved domain identified as COG5430. The COG5430 domain could represent a novel type of putative pilin domain, and the PA4648-PA4653 gene cluster could be involved in the assembly of fimbrial structures according to the chaperone-usher model. A similar gene cluster has recently been identified in *Acinetobacter baumannii* and has been shown to be involved in biofilm formation by this organism (37). A second but incomplete fimbrial assembly system was found to be up-regulated in the *mvaT* mutant. In this cluster no usher-encoding gene could be

identified but only an ORF encoding a putative chaperone, PA0499. The two other ORFs of the cluster, PA0497 and PA0498, encode signal peptide-containing proteins similar to the secretory protein YbgO (Fig. 1B). In *E. coli*, the *ybgO* gene is found clustered with genes encoding chaperone-usher components (3) (Fig. 1C). Whether the putative structures assembled by these novel systems are important for adhesion under certain conditions, as is the case for the CupB and CupC systems, remains to be determined.

We also report in this study the existence of a second MvaT-like regulator, MvaU. We could show that in common with MvaT, MvaU is a negative regulator of *lecA* gene expression, encoding the PA-IL lectin, which has been reported to function as an adhesin (40), but in contrast has no influence on *cup* gene expression. This indicates that the MvaT-dependent expression of the *cupA*, *cupB*, and *cupC* genes is strictly dependent on MvaT. Intriguingly, microarray data revealed that *mvaU* is up-regulated in an *mvaT* background (see Table S4 in the supplemental material). Yet, in this context, increased MvaU levels do not seem sufficient to prevent up-regulation of *lecA* in response to the lack of a functional MvaT protein. This observation may confirm that both regulators, even though homologous, are not exchangeable and the relationship, if any, might be complex. To help with understanding these data, it should be noted that the repression effect on *lecA* due to MvaT is nearly twice that due to MvaU (Fig. 6). Finally, because the *mvaU* mutant biofilm exhibited no clear increased surface coverage, we concluded that in the *P. aeruginosa mvaT* mutant, the up-regulation of *lecA* is not sufficient to unmask the enhanced biofilm phenotype of the *mvaT* strain. In addition, the up-regulation of the *cupA* genes in the *mvaT* mutant might thus strongly contribute to the enhancement of biofilm formation in this strain. To support this suggestion, we introduced a *cupA* mutation in the *mvaT* mutant, which resulted in decreased bacterial surface coverage (Fig. 5C). In contrast, introduction of the *mvaT* mutation in a *lecA* mutant still increased bacterial surface coverage (Fig. 5B). In light of these observations, it is possible that the products of the *cupA* genes play an important role in the initial colonization of surfaces, whereas the *lecA*-encoded protein contributes to cell-cell interaction or biofilm maturation at a later stage.

In conclusion, MvaT in *P. aeruginosa* is likely to be an important component of a complex regulatory network involved in biofilm formation. In future work, we will try to determine which conditions affect the repression of the *cup* genes and how this relates to the biofilm developmental cycle. Furthermore, it will also be necessary to define in detail the nature of the MvaT regulon and identify its target DNA binding sites in *P. aeruginosa*. For this purpose, we will purify MvaT in order to perform DNA band shift assays of the putative target genes. Finally, it is challenging to believe that understanding the function of MvaT and its contribution to the lifestyle of *P. aeruginosa* may uncover new opportunities for controlling biofilm-associated infections.

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