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Microarray Analysis of Global Gene Expression in Mucoid *Pseudomonas aeruginosa*

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***Pseudomonas aeruginosa* is the dominant pathogen causing chronic respiratory infections in cystic fibrosis (CF). After an initial phase characterized by intermittent infections, a chronic colonization is established in CF upon the conversion of *P. aeruginosa* to the mucoid, exopolysaccharide alginate-overproducing phenotype. The emergence of mucoid *P. aeruginosa* in CF is associated with respiratory decline and poor prognosis. The switch to mucoidy in most CF isolates is caused by mutations in the *mucA* gene encoding an anti-sigma factor. The mutations in *mucA* result in the activation of the alternative sigma factor AlgU, the *P. aeruginosa* ortholog of *Escherichia coli* extreme stress sigma factor σ^E. Because of the global nature of the regulators of mucoidy, we have hypothesized that other genes, in addition to those specific for alginate production, must be induced upon conversion to mucoidy, and their production may contribute to the pathogenesis in CF. Here we applied microarray analysis to identify on the whole-genome scale those genes that are coinduced with the AlgU sigmulon upon conversion to mucoidy. Gene expression profiles of AlgU-dependent conversion to mucoidy revealed coinduction of a specific subset of known virulence determinants (the major protease elastase gene, alkaline metalloproteinase gene *aprA*, and the protease secretion factor genes *aprE* and *aprF*) or toxic factors (cyanide synthase) that may have implications for disease in CF. Analysis of promoter regions of the most highly induced genes (>40-fold, $P \leq 10^{-4}$) revealed a previously unrecognized, putative AlgU promoter upstream of the osmotically inducible gene *osmE*. This newly identified AlgU-dependent promoter of *osmE* was confirmed by mapping the mRNA 5′ end by primer extension. The recognition of genes induced in mucoid *P. aeruginosa*, other than those associated with alginate biosynthesis, reported here revealed the identity of previously unappreciated factors potentially contributing to the morbidity and mortality caused by mucoid *P. aeruginosa* in CF.**

Cystic fibrosis (CF) is the most common lethal genetic disorder among Caucasians, with a carrier frequency of 1 in 25 individuals (62). The principal causative agent of morbidity and mortality in CF is the opportunistic pathogen *Pseudomonas aeruginosa* (26). *Pseudomonas* infections in CF follow a characteristic course. The initial intermittent colonization of the CF lung (12) is believed to occur by infection with bacteria acquired from the environment. A more-or-less permanent chronic colonization is established upon conversion of *P. aeruginosa* to the mucoid phenotype (35, 48). As a result, mucoid *P. aeruginosa* is isolated from >90% of all CF patients (21). Infections with mucoid *P. aeruginosa* are associated with heightened inflammation, tissue destruction, and declining pulmonary function (7, 36). It has been recognized that the establishment of chronic mucoid *P. aeruginosa* colonization correlates with a poor prognosis for the CF patient (26, 35, 48).

The most overt characteristic of the mucoid phenotype is the production of a thick mucopolysaccharide layer consisting of the exopolysaccharide alginate (26). Conversion to mucoidy results from mutations that free the stress response sigma factor AlgU (42, 43), the *P. aeruginosa* ortholog of *E. coli* σ^E (66)—also known as AlgT (18)—from negative regulation by the anti-sigma factor *mucA* (10). AlgU induces the production of the exopolysaccharide alginate via activation of the promoter for the alginate biosynthetic operon headed at its 5′ end by the *algD* gene (16, 17, 22). The alternative sigma factor AlgU has been shown to have additional roles other than activation of the alginate biosynthesis pathway (20, 54). For example, AlgU directs transcription of the gene encoding the main heat shock sigma factor RpoH (54), as well as a number of products situated around the genome, including factors that may protect the bacterium from stress, such as *osmC* (20). Among genes previously shown to belong to the AlgU sigmulon (20) are a number of products with proinflammatory potential, including lipoproteins that provoke the host inflammatory response via Toll-like receptor signaling (1) and that induce production of interleukin-8 (IL-8) by human macrophages (20).

Previous studies have relied on classical transcript mapping (56) or a bioinformatics approach, involving identification of recognizable AlgU consensus promoter sequences in the *P. aeruginosa* genome followed by transcriptional mapping of individual promoters (20). In order not only to identify the immediate genes controlled by AlgU (collectively termed the "AlgU sigmulon") (20), but also to uncover all of the genes in *P. aeruginosa* that are induced upon conversion to mucoidy or otherwise affected by AlgU activation, we carried out a whole-genome microarray analysis comparing global gene expression

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profiles in isogenic mucoid and nonmucoid *P. aeruginosa* strains. We found that a specific subset of *P. aeruginosa* virulence factors is induced upon conversion to mucoidy. These and additional observations may have implications for understanding the worsening of disease in CF patients upon conversion to mucoidy in *P. aeruginosa*. Our studies also further expand the AlgU sigmulon, increasing the number of known mapped AlgU (σ^E) promoters.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. aeruginosa* PAO381 (PAO1 leucine auxotroph) and its mucoid derivatives PAO578I (*mucA22*) and PAO578II (*mucA22 sup-2*) have been described previously (8, 23). The nonmucoid strain PAO6865 (*algU*::Tc^r) is derived from PAO578II (9). For RNA isolation, strains were cultured shaking at 37°C overnight in Luria broth (LB). One milliliter of the overnight culture was used to inoculate 100 ml of LB with 0.3 M NaCl (PAO578II and PAO6865) or without salt supplement (PAO381 and PAO578I) for a starting density equal to that of a saturated culture diluted 1:100 and grown for 4 h at 37°C to a mid-log optical density at 600 nm (OD_{600}) of 0.5. Growth curves were determined up to the 4-h harvest and were identical for mucoid and nonmucoid strains.

Genomic searches. PAO1 genomic sequence was obtained from the *Pseudomonas* Genome Project (www.pseudomonas.com) (60). Data were imported for analysis by MacVector sequence analysis software (version 7.0; Eastman Kodak Co.). A subsequence search corresponding to the AlgU consensus (GAACTT-$N_{16/17}$-TCCAA) was carried out, allowing for up to four substitutions to determine potential AlgU promoter sites in the genome. Additional information was obtained from the PseudoCAP annotation project (www.pseudomonas.com).

Primer design and DNA methods. The 16-mer primers GAACTGCACGAC GAGC and CCTCCGTAAGGAAGCG were generated approximately 400 bp upstream and downstream of the *osmE* promoter. These primers were used in a PCR to generate a 0.8-kb fragment from total genomic PAO1 DNA to serve as a sequencing template. A 22-mer primer, GGGTTGCCATCACGAACAGTGC, was designed 60 bp downstream of the suspected AlgU promoter site and oriented to extend back towards the putative promoter to generate a transcript by using reverse transcriptase in primer extension analyses as well as to sequence the promoter region by using a ^{33}P sequencing kit (Amersham, Piscataway, N.J.). Controls (*algD* and *oprF*) for these studies have been described previously (20).

RNA isolation and microarray analysis. For primer extension analysis RNA was isolated over a CsCl cushion as described previously (20). For microarray analysis and primer extension, RNA was isolated with TRIzol (Invitrogen Life Technologies, Carlsbad, Calif.) and Qiagen RNeasy (Qiagen, Valencia, Calif.) according to the method described by Lory et al. (http://cfgenomics.unc.edu/protocols_rna_prep.htm). Accordingly, two 35-ml aliquots were taken from the 100-ml culture with an OD_{600} of 0.5 and processed in parallel. Cultures were pelleted and resuspended in TRIzol reagent by vortexing. Samples were lysed by sonication for 10 s and incubated at room temperature for 5 min. Following chloroform extraction, RNA was precipitated with isopropanol. RNA was pelleted, washed with 70% ethanol, and dried in a vacuum chamber. RNA was resuspended in water and treated with DNase (RQ1; Promega, Madison, Wis.) at 37°C for 1 h. Protein and nucleotides were removed by phenol-chloroform extraction, and RNA was precipitated in 1 volume of isopropanol. Pelleted RNA was resuspended in water, and like samples were combined. The quantity and purity of RNA were determined by $OD_{260/280}$ spectrometry. tRNA was removed by following the RNeasy (Qiagen) RNA extraction protocol. Samples were eluted with 30 μl of water, and quantity was determined by spectrometry.

Labeled cDNA was generated according to the protocol for the Affymetrix (Santa Clara, Calif.) *Pseudomonas* microarray chip. cDNA was synthesized by annealing random primers (Invitrogen) to purified RNA and extended with SuperScript II (Invitrogen). Transcripts corresponding to *Bacillus subtilis* genes *dap*, *thr*, *phe*, *lys*, and *trp* were spiked into the cDNA synthesis reaction mixture as a control to monitor cDNA synthesis, labeling, hybridization, and staining efficiency (courtesy of Steve Lory, Harvard Medical School). RNA was removed by addition of 1 N NaOH and incubation at 65°C for 30 min. The reaction was neutralized with 1 N HCl, and cDNA was purified with a QIAquick PCR purification kit (Qiagen).

Yields were quantified and cDNA was fragmented with 0.6 U of DNase I (Amersham Pharmacia Biotech) per μg of cDNA for 10 min at 37°C, followed by heat inactivation. Verification of cDNA fragmentation between 50 and 200 bases was confirmed by running samples on RNA 6000 Nano LabChip (Agilent Technologies, Palo Alto, Calif.) analyzed with an Agilent 2100 bioanalyzer. Fragmented cDNA was terminally labeled with biotin-ddUTP (Enzo BioArray terminal labeling kit; Affymetrix) for 30 min at 37°C.

GeneChip *P. aeruginosa* genome array chips (Affymetrix) were hybridized overnight at 50°C in the presence of nonspecific DNA and control B2 oligonucleotide (Affymetrix). The chips were washed, stained, and scanned the following day according to the steps of the Affymetrix Microarray Suite software, version 5.0, for the *Pseudomonas* chip on an Affymetrix GeneChip fluidics station. Chips were first stained with ImmunoPure streptavidin (Pierce Chemical, Rockford, Ill.), followed by biotinylated antistreptavidin goat antibody (Vector Laboratories, Burlingame, Calif.), and finally by R-phycoerythrin-streptavidin (Molecular Probes, Eugene, Oreg.). Chips were scanned on an Affymeterix probe array scanner. The results from three independent identical experiments were merged for each strain. The merged data were used for subsequent comparisons and assessed with Genomax (InforMax, Bethesda, Md.) and Microsoft Excel with Student's *t* test according to the method of Arfin et al. (3). The combined fourfold cutoff value and *P* value of ≤0.001 for inclusion in Tables 2 and 3 have been set arbitrarily high to minimize reporting of false positives. We point out that additional statistical tests (4, 40) can be applied to these data sets to glean additional information as necessary. The original raw data files have been deposited in the Cystic Fibrosis Foundation Therapeutics, Inc.-Genomax shared workspace (http://cfgenomics.unc.edu/supplemental_data/geno_share.htm). Pertinent information on raw data containing hybridization results for specific oligonucleotide sets and confidence intervals for gene expression is available in that database.

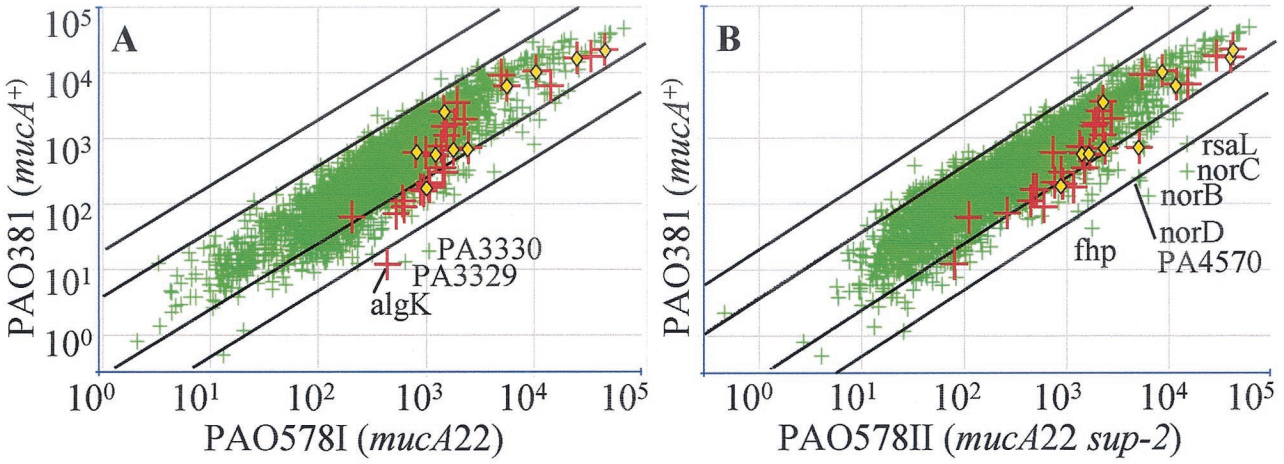
Primer extension analysis. Reverse transcription mapping was carried out as described previously (20). Primers were end labeled by polynucleotide kinase with [γ-^{32}P]ATP (Perkin-Elmer Life Sciences, Boston, Mass.) for 1 h at 37°C. The reaction was stopped with 0.5 M EDTA, diluted with Tris-EDTA (TE), and heat inactivated by incubation at 65°C for 5 min. Labeled primers were annealed to 15 (CsCl isolation) or 3 (TRIzol isolation) μg of total cellular RNA in hybridization buffer (0.5 M KCl, O.24 M Tris-HCl [pH 8.3]) by dissociation at 95°C for 1 min followed immediately by annealing at 55°C for 2 min and stabilization on ice for 15 min. Primers were extended with Superscript II (Invitrogen) according to manufacturer's instructions and loaded adjacently to a sequencing ladder that utilized the same primer (33).

TaqMan real-time PCR. *P. aeruginosa* mucoid strain PAO578II and its nonmucoid (*algU*::Tc^r) derivative strain, PAO6865, were grown as described above. RNA was isolated with RNeasy (Qiagen), treated with RQ1 DNase (Promega) for 1 h at 37°C, and repurified with RNeasy. cDNA was synthesized as previously described. As a control, the reaction was also carried out without reverse transcriptase. Total cDNA was quantitated by spectrophotometry, and exactly 50 ng was used for reverse transcription (RT)-PCR.

RT-PCR was carried out on an ABI Prism 5700 with 500 nM PCR primers and 200 nM probe on 50 ng of isolated cDNA in triplicate. Additionally, RT-PCR of an equivalent volume of the cDNA synthesis reaction mixture that contained no reverse transcriptase indicated there was no contaminating genomic DNA, and a no-template control reaction indicated there was no cross-contamination. Primers and probes were designed for *osmC* and *fusA1* by using Primer Express software (Applied Biosystems, Foster City, Calif.). The PCR primers (Integrated DNA Technologies, Inc., Coralville, Iowa) for *osmC* were CAACCCCTATGG CTTCAATACC and TCAGCTCTTCCGGGTTGGT, and those for *fusA1* were GGCCCGTACTACACCCATCA and CGTCAACGTGGGCACAGATA. The TaqMan probes (Applied Biosystems) were 6-FAM-CGAGGGCGCACC-TA MRA for *osmC* and 6-FAM-CGCTACCGTAATATC-TAMRA for *fusA1*.

RESULTS

Comparative global analysis of gene expression profiles in mucoid and nonmucoid *P. aeruginosa*. We investigated global gene expression in mucoid *P. aeruginosa* by using the recently available *P. aeruginosa* microarray gene chip. A comparison was carried out (Fig. 1A) between the wild-type nonmucoid strain PAO381 and its isogenic *mucA22* mucoid mutant, PAO578I, which constitutively produces alginate. The *mucA*22 mutation truncates the anti-sigma factor MucA and renders AlgU fully active by relieving AlgU from inhibition by MucA (10, 43, 51). The transcripts of genes related to the regulation or production of alginate (Fig. 1A and Table 1) were shifted towards increased expression in the mucoid strain, with an



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ID	Description	Fold activation
PAO578I/PAO381		
PA3329	unknown	38
PA3330	prob. short chain dehydrogenase	64
PA3543	*algK*	40
PAO578II/PAO381		
PA0523	*norC*	52
PA0524	*norB*	54
PA0525	*norD*	20
PA1431	*rsaL*	23
PA2664	*fhp*	61
PA4570	unknown	23

FIG. 1. Global gene expression analysis in mucoid *P. aeruginosa* by transcriptional profiling with GeneChip microarrays. Each plot represents the merged expression data from three independent cultures run on three separate chips for each strain. Diagonal lines delimit regions with ≥4-fold or ≥20-fold induction. Large red crosses indicate alginate biosynthetic and regulatory genes (Table 1), and yellow diamonds indicate genes previously demonstrated to have AlgU-dependent promoters (Table 3). (A) Wild-type nonmucoid strain PAO381 (*mucA*$^+$) compared to its isogenic mucoid mutant, PAO578I, carrying a typical *mucA* mutation (*mucA22*). (B) Nonmucoid strain PAO381 compared to the mucoid derivative, PAO578II (PAO578I *sup-2*), carrying both the *mucA22* mutation responsible for conversion to mucoidy and the *sup-2* mutation (55), which causes a slight attenuation of the mucoid phenotype often observed with CF isolates. (C) Identification (ID) and induction levels of genes designated on the figure The *algK* gene codes for periplasmic alginate secretion protein, *norCBD* codes for NO reductase, *rsaL* codes for the repressor of autoinducer synthase, and *fhp* codes for flavohemoglobin.

observed induction of between twofold and ninefold for the majority of the genes. The *algK* gene, encoding a periplasmic lipoprotein implicated in the polymerization of alginate (32), showed an exceptionally high, 40-fold induction in the mucoid strain PAO578I versus its nonmucoid parent, PAO381. This appears to be due to uncharacteristically low background levels in PAO381 for the *algK* oligonucleotide set tiled on the microarray chip (data not shown), rather than being based on a dramatically elevated expression of *algK* in mucoid PAO578I relative to other alginate genes.

A number of genes not implicated in alginate production were significantly induced in mucoid *P. aeruginosa* (Table 2) and often exceeded the fold induction values observed with the alginate-specific genes. The finding that the alginate biosynthetic genes are not the most highly induced genes (compare Tables 1 and 2), supports our hypothesis that a large number of additional genes are activated either directly or indirectly upon conversion to mucoidy via *mucA* mutations. Thus, the role of AlgU and the effects of its activation extend further than the control of alginate production.

TABLE 1. Microarray expression analysis of mucoid versus nonmucoid *P. aeruginosa* genes or operons related to alginate production[a]

Description (gene)	Identification	PAO578I/PAO381[b]		PAO578II/PAO6865[c]	
		Fold activation	*P* value	Fold activation	*P* value
Sigma factor (*algU*)	PA0762	2.0	9.0E-02	49.2	2E-03**
Anti-sigma factor (*mucA*)	PA0763	2.4	4.1E-02*	20.8	3E-04**
Negative regulator of alginate biosynthesis (*mucB*)	PA0764	1.2	3.3E-01	7.0	1E-03**
Negative regulator of alginate biosynthesis (*mucC*)	PA0765	1.1	5.3E-01	4.1	6E-03**
Serine protease (*mucD*)	PA0766	1.1	5.1E-01	2.2	1E-03**
GDP-mannose 6-dehydrogenase (*algD*)	PA3540	4.0	7E-03**	7.0	2.4E-02*
Alginate biosynthesis protein (*alg8*)	PA3541	4.0	2E-03**	2.9	2.8E-01
Alginate biosynthesis protein (*alg44*)	PA3542	5.0	6E-03**	3.4	1.0E-02*
Alginate biosynthetic protein (*algK*)	PA3543	40.2	8E-03**	5.8	5.1E-02*
Outer membrane protein (*algE*)	PA3544	5.0	5.3E-02*	3.2	7.3E-02
Alginate-c5-mannuronan-epimerase (*algG*)	PA3545	8.7	8E-03**	3.5	7.7E-02
Alginate biosynthesis protein (*algX*)	PA3546	5.2	1.9E-02*	4.1	5.0E-02*
Poly (β-D-mannuronate) lyase (*algL*)	PA3547	8.5	3E-03**	6.3	1.3E-01
Alginate *o*-acetyltransferase (*algI*)	PA3548	7.5	9E-03**	4.1	3.8E-02*
Alginate *o*-acetyltransferase (*algJ*)	PA3549	7.7	1E-03**	7.6	1.2E-02*
Alginate *o*-acetyltransferase (*algF*)	PA3550	6.6	7.0E-02	16.0	3.9E-02*
Phosphomannose isomerase (*algA*)	PA3551	4.4	4E-03**	4.3	6.0E-02
Alginate regulatory protein (*algP*)	PA5253	−1.8	2E-03**	−1.2	3.4E-01
Alginate regulatory protein (*algQ*)	PA5255	−1.7	6.1E-02	−1.4	1.3E-01
Alginate two-component regulatory protein (*algR*)	PA5261	1.4	3.5E-01	3.1	7.8E-02
Alginate two-component regulatory protein (*algZ*)	PA5262	1.8	9E-03**	2.2	2E-03**

[a] Values represent higher (positive) or lower (negative) expression in the mucoid strain than in the nonmucoid strain. *, $P \leq 0.05$; **, $P \leq 0.01$.
[b] Mucoid strain PAO578I (*mucA22*) and its nonmucoid wild-type parental strain, PAO381 (*mucA*$^+$).
[c] Mucoid strain PAO578II (*mucA22 sup-2 algU*$^+$) and its nonmucoid *algU* mutant derivative, PAO6865 (*algU*::Tc^r).

We also observed only a small induction in the transcripts of the extracellular sigma factor (ECF) AlgU and the cotranscribed negative regulator MucA in the mucoid strain PAO578I compared to that in its wild-type parent, PAO381 (Table 1). AlgU positively regulates its own promoter, most likely contributing to the small induction observed (Table 1). Because *algU* is also transcribed from a number of AlgU-independent promoters (56), multiple transcripts initiated by different RNA polymerase holoenzymes may mask a contribution of AlgU when analyzed by microarrays. Furthermore, since the majority of the regulation of AlgU occurs at the posttranslational level by MucA (51), a large induction of AlgU transcription may not be a prerequisite to observe dramatic effects on alginate production. The putative alginate regulatory genes *algP* and *algQ* did not show induction in mucoid *P. aeruginosa*, and if anything, *algP* showed slightly reduced transcription. The *algP* gene encodes a *P. aeruginosa* histone-like element (37). The *algQ* gene was previously thought to encode a kinase phosphorylating the response regulator AlgR (52), but later was shown to be the ortholog of an anti-sigma factor affecting the major sigma factor RpoD (σ^{70}) (19, 34). The out-of-sync transcription of *algP* and *algQ* relative to the rest of the *alg* genes suggests a rather indirect participation, if any, of these factors.

Global analysis of AlgU-dependent expression profiles in mucoid *P. aeruginosa*. We next tested how inactivation of AlgU in mucoid (*mucA* mutant) *P. aeruginosa* affected global gene expression. For this purpose, a derivative of PAO578I, PAO578II (PAO578I *sup-2*), which has an additional *sup-2* mutation that renders it responsive to growth conditions for maximal production of alginate, was employed (55). This strain, unlike PAO578I, in which *algU* inactivation cannot be achieved, permits inactivation of *algU*. The strain PAO6865 is an *algU*::Tc^r derivative of PAO578II (9). When the microarray expression data (Fig. 1B) from PAO578II and PAO6865 were compared, genes with a previously demonstrated AlgU-dependent promoter (20) (Fig. 1B and Table 3) showed a wide range of expression ratios. The highest levels of differential AlgU-dependent expression were observed with the following genes: (i) *slyB* (*ycfJ*), encoding a putative porin (41); (ii) *lptA*, encoding a lipoprotein capable of inducing IL-8 production by human monocytes (20); (iii) *osmC* and *osmE*, inducible by elevated osmolarity and potentially playing a role in stress defense (13, 27, 28) (with OsmE also being a lipoprotein thus having a proinflammatory potential); and (iv) *pfpI*, encoding a homolog (64% similar) of the major protease from *Pyrococcus furiosus* (29). In some cases, such as with the *algU-mucABCD* gene cluster, a gradient of induction could be detected along the putative operon (Table 1) consistent with a transcription gradient or mRNA degradation from the 3′ end of an operonic transcript. The alginate biosynthetic cluster, extending from *algD* to *algA*, showed a relatively uniform induction, *algD* yielding the strongest AlgU-dependent signal in the cluster, with the exception of *algF* and *algJ*.

Induction of virulence determinants associated with conversion to mucoidy in *P. aeruginosa*. In addition to alginate production, several factors that may play a role in *P. aeruginosa* pathogenesis are induced (fold induction of ≥4, $P \leq 0.001$) upon conversion to mucoidy (Table 2). (i) *lasB*, the gene that encodes the virulence factor elastase (5), was induced in mucoid *P. aeruginosa,* consistent with an association previously described in the sputum of CF patients (59). (ii) Also consistent with the previously reported in vivo data (59) was a smaller induction of *lasA* (not included in Table 2; induction of 3.3-fold, $P = 0.004$), encoding a protease that is able to degrade host elastin and collagen (61). (iii) A weak induction, if any, of exotoxin A (*toxA* gene [not shown in Table 2]) was also

TABLE 2. Microarray analysis of global gene expression in mucoid *P. aeruginosa* strain PAO578I (*mucA22*) compared to its nonmucoid parent, PAO381 (*mucA*$^+$)[a]

Description (gene)[b]	Identification	Fold activation	*P* value
Toxins/toxin secretion			
Alkaline metalloproteinase (*aprA*)	PA1249	9	8E-04
3-Oxoacyl-[acyl carrier protein] synthase III FabH2	PA3333	9	2E-04
Elastase (*lasB*)	PA3724	8	3E-05
Alkaline protease secretion protein (*aprF*)	PA1248	8	3E-06
Hydrogen cyanide synthase (*hcnC*)	PA2195	6	1E-03
Hydrogen cyanide synthase (*hcnA*)	PA2193	6	2E-04
Phospholipase accessory protein (*plcR*)	PA0843	4	1E-03
Alkaline protease secretion protein (*aprE*)	PA1247	4	4E-05
Membrane proteins/transport			
Probable binding protein component of ABC transporter	PA0203	11	4E-04
Hypothetical membrane protein	PA0702	8	9E-04
Probable outer membrane efflux protein	PA3521	7	2E-04
Probable outer membrane protein	PA2760	−5	1E-05
Enzymes and unknown			
Probable short chain dehydrogenase	PA3330	64	4E-07
Unknown	PA3329	38	8E-05
Probable nonribosomal peptide synthetase	PA2302	10	3E-04
Probable aminopeptidase	PA2939	8	7E-04
Probable FAD-dependent monooxygenase	PA2587	6	3E-04
Probable FAD-dependent monooxygenase	PA3328	6	9E-04
Probable nonribosomal peptide synthetase	PA3327	5	4E-04
Probable nonribosomal peptide synthetase	PA2305	4	1E-03
Motility and attachment			
Type 4 fimbrial biogenesis protein (*fimT*)	PA4549	9	1E-04
Type 4 fimbrial biogenesis protein (*pilM*)	PA5044	−4	5E-04
Transcriptional regulation			
Probable transcriptional regulator	PA2681	5	2E-05
Metabolism			
Cytochrome P450	PA3331	9	4E-04
Probable cytochrome *c*	PA1555	−4	2E-04
Probable cytochrome *c* oxidase subunit	PA1556	−4	9E-06
Cytochrome c_{551} peroxidase (*ccpR*)	PA4587	−4	3E-04

[a] Values represent higher (positive) or lower (negative) expression in the mucoid strain than in the nonmucoid strain. For a fold difference of ≥4-fold, $P \leq 0.001$.
[b] Only genes with known or homology-based proposed function (annotated in the *P. aeruginosa* genome) are included. PA3329 (unknown) is included to highlight its induction along with that of the adjacent genes suggestive of a putative operonic structure.

observed (1.7-fold, $P = 0.01$). (iv) The *aprA* gene, encoding the alkaline metalloproteinase implicated in obtaining iron at the site of infection through the degradation of transferrin (57), and genes for its secretion, *aprE* and *aprF*, were among the most highly induced genes. Since alkaline protease is activated by elastase (LasB) (46) and is important for the activation of the LasA protease (65), it appears that conversion to mucoidy simultaneously induces genes for production of the major proteases and proteolytic activation cascades in *P. aeruginosa*. (v) The *plcR* gene is important for the function of the hemolytic phospholipase C (PlcH), and when knocked out results in 10-times-less hemolytic activity by phospholipase C (14). Thus, conversion to mucoidy may play a role in facilitating phospholipase action.

Additionally, genes producing secreted factors that may be intoxicating to the host were coinduced with conversion to mucoidy (Table 2). The enzymes encoded by the *hcnABC* cluster, *hcnA* (sixfold induction, $P = 2E-04$), *hcnC* (sixfold induction, $P = 1E-03$ [shown in Table 1]), and *hcnB* (fourfold induction, $P = 3E-03$) generate the secreted, extremely toxic secondary metabolite hydrogen cyanide (HCN). HCN has been shown to contribute to the pathogenesis of *P. aeruginosa* (24). Cyanide production inhibits cytochrome *c* oxidase, resulting in a block in the terminal component in mitochondrial respiration (6). There was also an induction of genes encoding putative nonribosomal peptide synthetases in mucoid *P. aeruginosa* (Table 2). These types of enzymes utilize unconventional amino acids and modifications to generate short peptides with a variety of biological activities, including disabling defense systems in the host, such as the immunosuppressant cyclosporine (53).

Activation of defense mechanisms in mucoid *P. aeruginosa* with attenuated alginate expression due to a *sup-2* suppressor mutation. Many mucoid *P. aeruginosa* CF isolates typically resemble the PAO578II strain in terms of their somewhat attenuated alginate production, attributed to a suppressor mutation termed "*sup-2*" (18, 55). Since it is typical to coisolate type I (with constitutive alginate expression as in PAO578I) and type II (as in PAO578II, which requires additional specific environmental conditions to display a mucoid phenotype)

TABLE 3. Microarray analysis of ECF sigma factor AlgU (*P. aeruginosa* σ^E)-dependent gene expression in *P. aeruginosa*[a]

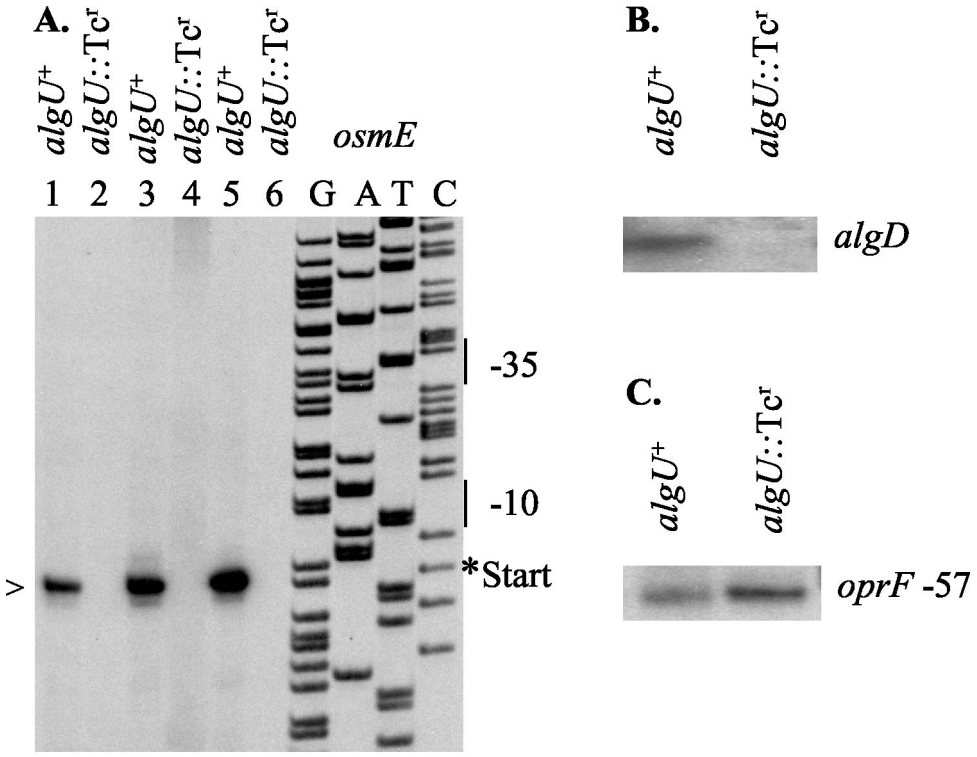
Description (gene)	Identification	Fold activation[b]	*P* value
Previously identified AlgU-dependent genes			
Hypothetical membrane protein (*slyB* [*ycfJ*])	PA3819	56	2E-04
Hypothetical lipoprotein (*lptA*)	PA1592	35	5E-06
Osmotically inducible protein (*osmC*)	PA0059	24 (91)	4E-04
GDP-mannose 6-dehydrogenase (*algD*)	PA3540	7	2.4E-02
Hypothetical protein (*asmD3*)	PA3902	4	1E-03
Hypothetical protein (*asmD2*)	PA3952	3	6.5E-02
Hypothetical protein (*asmB2*)	PA0856	2	1.0E-02
Sigma factor (*rpoH*)	PAO376	1	5.3E-01
Outer membrane protein (*oprF*)	PA1777	1	7.1E-01
Peptidyl-prolyl *cis-trans* isomerase (*lptB*)	PA3262	1	2.3E-01
Expression change ≥4-fold ($P \leq 0.001$)			
Membrane proteins			
Osmotically inducible lipoprotein (*osmE*)	PA4876	49	1E-04
Hypothetical membrane protein	PA5182	18	7E-04
Probable permease of ABC transporter	PA3890	11	1E-04
Hypothetical membrane protein	PA2777	11	7E-04
Hypothetical membrane protein	PA3041	6	1E-04
Hypothetical membrane protein	PA2148	4	1E-04
Type III export protein *pscE*	PA1718	−4	1E-03
Probable lipoprotein localization protein (*olB*)	PA4668	−4	1E-03
Transcription/translation			
Possible protease (*pfpI*)	PA0355	33	1E-03
tRNA (guanine-N1)-methyltransferase (*trmD*)	PA3743	−4	3E-05
16S rRNA processing protein (*rimM*)	PA3744	−4	1E-03
30S ribosomal protein S8 (*rpsH*)	PA4249	−4	5E-04
50S ribosomal protein L15 (*rplO*)	PA4244	−4	1E-03
Polyribonucleotide nucleotidyltransferase (*pnp*)	PA4740	−4	4E-04
50S ribosomal protein L23 (*rplW*)	PA4261	−7	1E-03
Elongation factor G (*fusA1*)	PA4266	−10 (−30)	3E-04
Metabolism			
UDP-glucose 4-epimerase (*galE*)	PA1384	7	2E-04
Probable c-type cytochrome (*nirN*)	PA0509	6	2E-05
Probable glutamine amidotransferase	PA3459	5	2E-04
Maleylacetoacetate isomerase	PA2007	5	5E-05
Heme d1 biosynthesis protein (*nirJ*)	PA0511	5	1E-03
Branched-chain alpha-keto acid dehydrogenase (*bkdB*)	PA2249	5	1E-03
Homocysteine synthase (*metY*)	PA5025	−4	1E-03
Aspartate carbamoyltransferase (*pyrB*)	PA0402	−5	4E-05
Phosphoribosylamine-glycine ligase (*purD*)	PA4855	−4	8E-04
Transcriptional regulation			
Transcriptional regulator (*pyrR*)	PA0403	−4	1E-03
Chemotaxis			
Probable chemotaxis transducer	PA2573	4	3E-04
Probable chemotaxis transducer	PA2867	−5	2E-04

[a] The following strains were compared: mucoid strain PAO578II (*mucA22 sup-2 algU*$^+$) and its nonmucoid derivative, PAO6865 (*algU*::Tc^r).
[b] Numbers in parentheses show results of RT-PCR analyses of the corresponding genes as described in Materials and Methods. The displayed fold induction levels are calculated as $2^{\Delta CT}$, where ΔCT represents the difference in CT of the two samples being compared, with CT representing the PCR cycle that crosses the preset logarithmic phase threshold.

strains of mucoid *P. aeruginosa* from CF lungs, we also used microarray analysis to compare PAO578II (PAO381 *mucA22 sup-2*) with the parental strain, PAO381 (*mucA*$^+$ *sup-2*^{0}) (Fig. 1B and C).

Among the most highly induced genes in the comparison of PAO578II with PAO381 (Fig. 1) were *norB* and *norC*, encoding the b and c subunits, respectively, of the *P. aeruginosa* nitric oxide (NO) reductase (2), which could play a role in bacterial defense against host innate immune clearance mechanisms, such as reactive nitrogen intermediates (31). Another member of this operon, *norD*, the activity of which is required for NO reductase activity, also showed elevated transcription (15). Also potentially important in NO detoxification is the induced *fhp* gene encoding a *P. aeruginosa* flavohemoglobin homolog. Fhp has been shown in other organisms to detoxify NO under aerobic conditions (25).

Confirmation of the predicted *osmE* AlgU (σ^E)-dependent promoter by RT mapping. The *osmE* gene showed one of the highest AlgU-dependent activation levels in mucoid *P. aeruginosa* (49-fold; Table 3) and was chosen for further potential



D. *osmE* promoter

-35 -10 *

GAACTCTGCGCGAGGGCCTGCGT**TCCAA**TGTTCGC

Consensus sequence

GAACTT----------16/17 bp----------TCCAA--5/6 bp +1

FIG. 2. Mapping of the AlgU-dependent *osmE* promoter. (A) Primer extension mapping of the *osmE* mRNA 5′ end corresponding to the AlgU consensus promoter. Total RNA was isolated from the mucoid strain PAO578II (*mucA22 sup-2*) (lanes 1, 3, and 5) or its *algU* knockout nonmucoid derivative, PAO6865 (PAO578II *algU*::Tc^r) (lanes 2, 4, and 6). Lanes 1 and 2 contain 3 μg of RNA from a batch used in microarray analysis, and lanes 3 to 6 contain 15 μg of RNA obtained by a CsCl isolation procedure (20). Primer extension products were run adjacently to a sequencing ladder generated with the same primer. Bars denote the AlgU promoter consensus sequence. >, AlgU-dependent mRNA start site. (B) A previously identified AlgU-dependent promoter, *algD*, used as a positive control for AlgU-dependent transcription. (C) A promoter 57 bp upstream of the *oprF* gene, which is independent of AlgU, used to demonstrate equivalent loading of RNA (a negative control for AlgU-dependent transcription). (D) The *osmE* AlgU-dependent promoter sequence with the −35 and −10 regions indicated by boldface letters. A T→C substitution relative to the strict AlgU consensus −35 region is underlined. (Note that this is the first AlgU [σ^E] promoter with a variant nucleotide in this position.) An asterisk marks the transcriptional start site.

AlgU promoter sequence investigations. A candidate promoter, not previously recognized by a bioinformatics approach using a stringent AlgU (*P. aeruginosa* σ^E) promoter consensus sequence (20), was found 37 bp upstream of the lipoprotein *osmE* translational start site. The putative *osmE* promoter sequence showed one T→C substitution (underlined) in the −35 consensus region (GAACTC) and a perfect −10 *P. aeruginosa* consensus region (TCCAA) (Fig. 2D). We next mapped the *osmE* mRNA 5′ end (Fig. 2A) by primer extension (RT) analysis. When run adjacently to a sequencing ladder, an *osmE* transcript was observed initiating 6 bp downstream of the predicted AlgU −10 consensus sequence (Fig. 2A). The transcript was present in the mucoid strain PAO578II (AlgU$^+$ *mucA22*) with a functional AlgU sigma factor, but the corresponding band was absent in the *algU*-knockout strain PAO6865 (*algU*::Tc^r *mucA22*) derived from PAO578II. These data show *osmE* promoter dependence on AlgU. Furthermore, the mRNA 5′ end was positioned correctly relative to the predicted AlgU (σ^E) −35 and −10 promoter regions. As a control, the promoter of *algD* was included, and as expected, it showed AlgU dependence (Fig. 2B). An AlgU-independent promoter 57 bp upstream of *oprF* was also included as an RNA quality and loading control (Fig. 2C). The mapping of the *osmE* promoter validated the microarray results for the *osmE*

gene. In addition to mapping the *osmE* promoter and showing its dependence on AlgU, we also validated the microarray data by examining two genes at opposite ends of the expression spectrum (*osmC* and *fusA1*; Table 3) using real-time PCR. The results showed for *osmC* a 91-fold increase and for *fusA1* a 30-fold reduction in *algU*$^+$ cells relative to *algU* mutant cells. This is in keeping with the 24-fold increase in *osmC* expression and 10-fold decrease in *fusA1* levels by microarrays.

DISCUSSION

In this study, we utilized microarrays to probe global gene expression associated with conversion to mucoidy in *P. aeruginosa*. The observed increased expression in mucoid strains of a number of previously described AlgU-dependent promoters (20) validated our microarray analysis. In conjunction with bioinformatics tools, a new AlgU-dependent promoter has been identified upstream of the highly induced *osmE* gene, encoding an osmotically responsive lipoprotein, OsmE, underscoring the validity of the microarray findings. Our data show that AlgU is a global stress response sigma factor that induces a number of systems in *P. aeruginosa*, not only the alginate system. Furthermore, we report the identity of gene subsets encoding virulence factors specifically induced with conversion to mucoidy, which include proteases and toxic products such as HCN.

Genes with previously demonstrated AlgU-dependent promoters (20) show a wide range of induction levels (Table 3). The *slyB*, *lptA*, *osmC*, and *algD* genes represent a category that has a strong if not complete dependence on AlgU for induction. Other genes that have an AlgU-dependent promoter, but which do not show a strong induction in mucoid strains (*asmD3*, *asmD2*, *asmB2*, *rpoH*, *oprF*, and *lptB*) may also be under the control of additional regulators or promoters, such as in the case of *oprF*, encoding the major outer membrane protein porin F of *P. aeruginosa*. The *oprF* gene has two additional promoters, one of which is dependent on SigX (11), and thus the contributions of AlgU may be masked. The *lptB* gene was previously shown to have two overlapping promoters—one dependent on AlgU and a second one independent of AlgU, which compensates for the absence of AlgU-dependent transcripts in the AlgU mutant strain PAO6865 through its increased activity (20).

Transcription profiles in the CF-specific, mucoid phenotype suggest that conversion to mucoidy may have specific deleterious effects in the CF patient, in addition to the known roles of mucoidy in antiphagocytosis (38, 47), hypochlorite and reactive oxygen intermediate scavenging (39, 58), and specialized, aggressive biofilm formation distinct from that of environmental biofilms (30). Regarding the issues associated with biofilm formation, it is not clear at this point whether the biofilms studied by using nonmucoid *P. aeruginosa* grown on environmental substrates have a direct relationship (if any) to mucoid *P. aeruginosa* in CF. A comparison of our microarray data with those reported for *P. aeruginosa* isolates grown on granite rock (Table 4) shows little correlation, if any, in gene expression trends. This comparison does not take into account the significant differences in experimental designs, and potential overlaps cannot be ruled out at this point.

In our studies, induction of genes encoding several potent virulence factors (elastase, LasA protease, alkaline proteinase, and PlcR) and dangerous toxic products (HCN) was observed. Although the microarray analysis of mucoid *P. aeruginosa* showed a general induction of the alginate biosynthetic pathway, somewhat surprisingly, the alginate genes (with the notable exception of *algK*) did not receive the highest scores. We also did not observe induction of some of the putative regulators of alginate production, *algP* and *algQ*. However, even the well-documented regulators of alginate production, the *algU-mucABCD* cluster and *algZR* did not show signs of a high expression differential between mucoid strain PAO578I and its nonmucoid parental strain, PAO381.

Because alginate production is energetically taxing to the bacterium, there may exist a selective pressure in *P. aeruginosa* strains carrying *mucA* mutations to down-regulate this system. Good evidence for the existence of such pressure is the accumulation of second site suppressor mutations, such as *sup-2*, both in laboratory strains and in CF isolates (49, 55). This burden is further demonstrated by the highly unstable mucoid phenotype (18, 55). Intriguingly, the suppressor mutations not only down-regulate alginate production to metabolically sustainable levels, but simultaneously activate additional defense systems (*norB*, *norC*, and *fhp*) (Fig. 1B and C) that may protect the bacteria from NO and the downstream highly bactericidal combinatorial products of NO and reactive oxygen intermediates. Incidentally, alginate itself is a scavenger of reactive oxygen intermediates, and it seems that by slight attenuation of its production to energetically more favorable levels via *sup-2* mutations, *P. aeruginosa* isolates from CF simultaneously gain resistance to another source of bactericidal radicals, viz. NO and reactive nitrogen intermediates.

Additional products coinduced with the conversion to a mucoid phenotype are known virulence traits of *P. aeruginosa*. Secretion of a freely diffusible toxic secondary metabolite, HCN, by *P. aeruginosa* is known to inhibit cytochrome *c* oxidase of the mitochondria (64). Recently, HCN has been demonstrated to be a potent *P. aeruginosa* virulence factor according to a *Caenorhabditis elegans* infection model (24). HCN has also been implicated in the inhibition of several other metalloenzymes, such as catalase, peroxidase, superoxide dismutase, nitrate reductase, and nitrogenase (6), and as a result may play an important role in bacterial defense against reactive oxygen and nitrogen intermediates produced by the host.

Previously, Storey et al. found a correlation between *algD* transcripts and *lasB* transcripts in the sputa of CF patients (59). They also noted a weaker but significant correlation of *algD* with *lasA* transcripts. These findings closely mirror the results we obtained with microarray analysis of mucoid *P. aeruginosa* strains carrying *mucA* but not *sup-2* mutations. The *lasB* gene was strongly induced in mucoid *P. aeruginosa*, as was *lasA*, albeit to a lesser extent. The correlation of transcripts observed by microarray analysis in this study with those observed in vivo suggests that at least some aspects of the microarray analysis of mucoid *P. aeruginosa* in vitro may accurately reflect the situation in the CF lung.

Consistent with the role of AlgU as the founding member (42, 44) of the extracytoplasmic function (ECF) class of sigma factors (45, 50), we found a number of AlgU-inducible genes, encoding products predicted to be associated with membranes (Tables 2 and 3). While at least some of these genes may be

TABLE 4. Comparison of mucoid *P. aeruginosa* and reported environmental biofilm gene expression

Description (gene)	Identification	Biofilm[a,b] fold activation	Mucoid/nonmucoid[c]	
			Fold activation	*P* value
Bacteriophage genes				
Hypothetical protein of bacteriophage Pf1	PA0718	22.6	1.6	2E-01
Helix-destabilizing protein of bacteriophage Pf1	PA0720	35.2	1.4	9E-02
Hypothetical protein of bacteriophage Pf1	PA0721	26.6	1.4	6E-01
Hypothetical protein of bacteriophage Pf1	PA0722	64.2	−1.0	9E-01
Coat protein B of bacteriophage Pf1 (*coaB*)	PA0723	83.5	−1.5	2E-01
Probable coat protein A of bacteriophage Pf1	PA0724	10.1	1.7	6E-02
Hypothetical protein of bacteriophage Pf1	PA0725	9.9	1.4	7E-02
Hypothetical protein of bacteriophage Pf1	PA0726	8.9*	2.3	4E-02
Hypothetical protein from bacteriophage Pf1	PA0727	14.6	1.6	9E-02
Motility and attachment				
Probable fimbrial protein	PA2128	−16.5*	−4.0	3E-02
Probable pilus assembly chaperone	PA2129	−2.4	1.2	6E-01
Type 4 fimbrial precursor PilA	PA4525	−6.6*	−2.5	2E-02
Flagellar hook protein FlgE	PA1080	−2*	−1.7	3E-02
Flagellin type B, FliC	PA1092	−2.3*	−2.9	1E-02
Flagellar capping protein FliD	PA1094	−2.1*	−1.9	7E-03
Probable pilus assembly chaperone	PA2129	−2.4	1.2	6E-01
Translation				
50S ribosomal protein L28 (*rpmB*)	PA5316	4.4	−2.4	1E-01
50S ribosomal protein L19 (*rplS*)	PA3742	2.7	−3.0	9E-02
50S ribosomal protein L23 (*rplW*)	PA4261	2.3	−2.6	6E-03
50S ribosomal protein L4	PA4262	2.4	−2.3	5E-02
30S ribosomal protein S7 (*rpsG*)	PA4267	2.2	−1.5	2E-02
50S ribosomal protein L18 (*rplR*)	PA4247	2.3	−1.9	7E-03
Translation initiation factor IF-2 (*infB*)	PA4744	2.1	−1.4	1E-02
Ribosome modulation factor (*rmf*)	PA3049	−5.3	−1.3	3E-01
ATP-binding protease component ClpA	PA2620	−2.1*	−1.3	2E-02
Metabolism				
Cytochrome *c* oxidase, subunit II (*coxB*)	PA0105	−2.9	1.7	1E-03
Cytochrome *c* oxidase, subunit I (*coxA*)	PA0106	−2.7	2.0	1E-01
Cytochrome *c* oxidase, subunit III (*coIII*)	PA0108	−2.9	2.4	1E-03
Urease beta subunit (*ureB*)	PA4867	63.1	−1.2	2E-01
Lipoate-protein ligase B (*lipB*)	PA3997	2.8	−1.1	6E-01
Leucine dehydrogenase (*glpD*)	PA3584	−4.1	1.1	7E-01
Leucine dehydrogenase (*ldh*)	PA3418	−2.5	1.0	8E-01
Membrane proteins or secretion				
TolA protein (*tolA*)	PA0971	3.9*	3.1	2E-02
Translocation protein TatA	PA5068	2.4	1.3	1E-01
Translocation protein TatB	PA5069	6.9	1.8	6E-02
Outer membrane lipoprotein OmlA	PA4765	2.4	−1.7	2E-03
Probable porin	PA3038	−3.5	−1.2	6E-01
Exoenzyme S synthesis protein C (*exsC*)	PA1710	−2.5*	−3.0	3E-02
Probable sodium-solute symporter	PA3234	−2.3	1.2	3E-01
Regulation				
Probable transcriptional regulator	PA2547	3.1	1.5	2E-01
Sigma factor RpoH	PA0376	2.3	1.1	6E-01
Sigma factor RpoS	PA3622	−2.3	1.1	4E-01
Probable two-component response regulator	PA4296	−2.2	1.0	9E-01
Other				
Rod shape-determining protein MreC	PA4480	3.1	1.2	9E-02
Probable DNA-binding protein	PA5348	−4.6*	−1.5	1E-02
Probable glycosyl hydrolase	PA2160	−2.3	2.1	3E-02
Methylated DNA-protein-cysteine methyltransferase (*ogt*)	PA0995	−2.1	−1.5	2E-01

[a] Environmental biofilm data as presented in Table 1 of the article by Whiteley et al. (63), reporting differential gene expression in *P. aeruginosa* PAO1 grown as biofilm relative to planktonic growth.

[b] Asterisks indicate correlation of gene expression trends in mucoid and biofilm phenotypes.

[c] Mucoid PAO578I and nonmucoid PAO381. The genes included are those reported by Whiteley et al. (63) to display differential expression in the biofilm mode of growth.

involved in the production of alginate, others seem to be involved in bacterial adhesion (Table 2). Our data also indicate a potential link between conversion to mucoidy and drug resistance mechanisms, such as through drug efflux (e.g., PA3521 is induced sevenfold in the mucoid strain) (Table 2). Identification via microarray analysis of *P. aeruginosa* membrane proteins associated with the medically relevant mucoid phenotype may offer new vaccine candidates to prevent or combat chronic *P. aeruginosa* infections. The action of proteases or metabolic enzymes induced in mucoid *P. aeruginosa* could also be considered as future drug targets for intervention with critical bacterial activities coinduced with mucoidy during colonization in CF. The studies presented here define additional genes associated with conversion to a mucoid phenotype in *P. aeruginosa* that will be of importance to fully understand pathology in the CF host.

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