Identification of Genes in the σ^{22} Regulon of *Pseudomonas aeruginosa* Required for Cell Envelope Homeostasis in Either the Planktonic or the Sessile Mode of Growth

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ABSTRACT **The *Pseudomonas aeruginosa* extracytoplasmic functioning (ECF) sigma factor σ^{22} is encoded by *algT/algU* and is inhibited by anti-sigma factor MucA. σ^{22} was originally discovered for its essential role in the expression of the exopolysaccharide alginate by mucoid strains associated with chronic pulmonary infection. However, σ^{22} is now known to also have a large regulon associated with the response to cell wall stress. Our recent transcriptome analysis identified 293 open reading frames (ORFs) in the σ^{22} stress stimulon that include genes for outer envelope biogenesis and remodeling, although most of the genes have undefined functions. To better understand the σ^{22}-dependent stress response, mutants affected in 27 genes of the σ^{22} stimulon were examined and expression was studied with *lacZ* fusions. Mutants constructed in the 27 genes showed no major change in response to cell wall-acting antibiotics or growth at elevated temperatures nor in alginate production. The mutants were examined for their effects on the expression of the σ^{22}-dependent promoter of the alginate biosynthetic operon (P*algD*) as a measure of σ^{22} derepression from MucA. By testing P*algD* expression under both planktonic and sessile growth conditions, 11 genes were found to play a role in the stress response that activates σ^{22}. Some mutations caused an increase or a decrease in the response to cell wall stress. Interestingly, mutations in 7 of the 11 genes caused constitutive P*algD* expression under nonstressed conditions and thus showed that these genes are involved in maintaining envelope homeostasis. Mutations in PA0062 and PA1324 showed constitutive P*algD* expression during both the planktonic and the sessile modes of growth. However, the PA5178 mutation caused constitutive P*algD* expression only during planktonic growth. In contrast, mutations in PA2717, PA0567, PA3040, and PA0920 caused constitutive P*algD* expression only in the sessile/biofilm mode of growth. This provides evidence that the σ^{22} stimulon for cell envelope homeostasis overlaps with biofilm control mechanisms.**

IMPORTANCE **During chronic lung infections, such as in cystic fibrosis patients, *Pseudomonas aeruginosa* produces the exopolysaccharide alginate and forms biofilms that shield the organisms from the immune response and increase resistance to antibiotics. Activation of alginate genes is under the control of an extracytoplasmic stress response system that releases an alternative sigma factor (σ^{22}) in response to cell wall stress and then activates expression of a large regulon. In this study, a mutant analysis of 27 members of the regulon showed that 11 play a role in envelope homeostasis and affect the stress response system itself. Interestingly, some genes demonstrate effects only in either the planktonic (free-swimming) or the sessile (biofilm) mode of growth, which leads to persistence and antibiotic tolerance. The studies presented here provide an important initial step in dissecting the mechanisms that regulate a critical signal transduction pathway that impacts *P. aeruginosa* pathogenesis.**

Received 29 March 2012 **Accepted** 4 April 2012 **Published** 15 May 2012

Citation Wood LF, Ohman DE. 2012. Identification of genes in the σ^{22} regulon of *Pseudomonas aeruginosa* required for cell envelope homeostasis in either the planktonic or the sessile mode of growth. mBio 3(3):e00094-12. doi:10.1128/mBio.00094-12.

Editor Gerald Pier, Harvard Medical School

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Pseudomonas aeruginosa is a Gram-negative bacterium capable of thriving in diverse environmental niches. It is also an important opportunistic human pathogen and major contributor to chronic lung infections in patients with cystic fibrosis (CF). The ability of this organism to successfully colonize these different environments is often multifactorial. One survival strategy used by *P. aeruginosa* in the CF lung is to overproduce the exopolysaccharide alginate, which gives colonies a distinctive mucoid phenotype and is also a harbinger of increased morbidity and mortality for these patients (1). The alginate barrier in mucoid *P. aeruginosa* has been shown to provide protection against many common antibiotics (2) and to confer increased resistance to phagocytic killing and antibody-dependent bactericidal mechanisms (3–5). The ability of *P. aeruginosa* to form biofilms is another important factor in establishing chronic infections in the lungs of CF patients (6, 7). Mucoid *P. aeruginosa* in a biofilm is more resistant to killing by human leukocytes in the presence of gamma interferon than is its isogenic nonmucoid form, suggesting that alginate plays an important role in protecting mucoid *P. aeruginosa* biofilm bacteria from the human immune system (3).

The master regulator for alginate production in mucoid *P. aeruginosa* is the extracytoplasmic functioning (ECF) sigma fac-

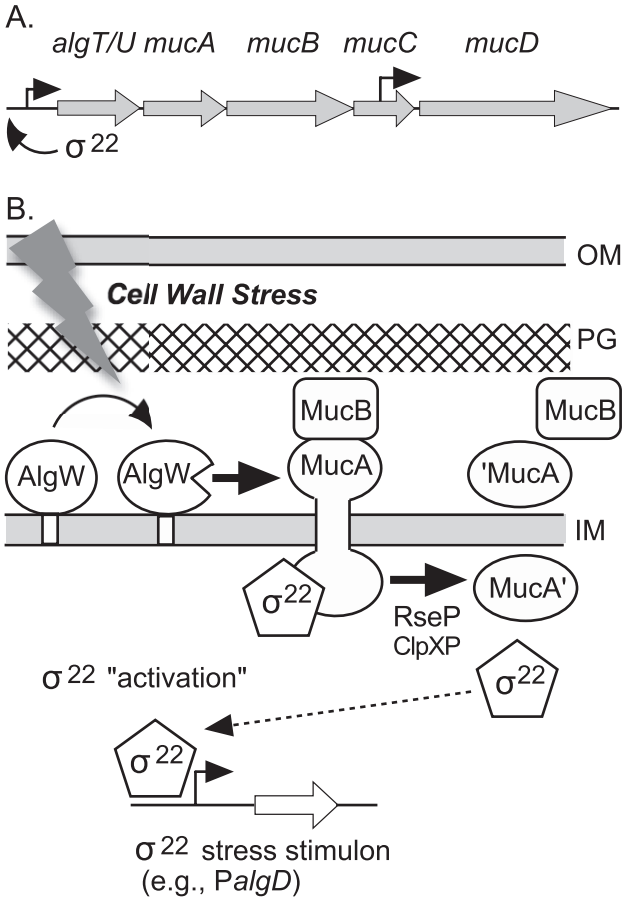

FIG 1 (A) Map of the *algT/U* operon. Arrows indicate promoters. (B) Model for the "activation" of σ^{22} activity by regulated intramembrane proteolytic (RIP) degradation of the anti-sigma factor MucA. Under nonstress conditions, σ^{22} activity is low due to its sequestration at the inner membrane by the MucA-MucB complex. In response to cell wall stress (e.g., D-cycloserine), AlgW cleaves MucA in the C terminus and then RseP (YaeL) and ClpXP cleave the MucA N-terminal region, releasing σ^{22} from posttranslational repression. This allows σ^{22} to complex with core RNA polymerase, thus increasing transcription of target promoters in the σ^{22} stimulon, which includes P*algD* of the alginate biosynthetic operon. Abbreviations: OM, outer membrane; PG, peptidoglycan; IM, inner membrane.

tor, σ^{22} (8, 9). This 22-kDa alternative sigma factor, encoded by *algT* (also known as *algU*), is essential for expression of the *algD* promoter (P*algD*), which controls the expression of the 12-gene alginate biosynthetic operon (*algD-alg8-alg44-algKEGXLIJFA*) (10–12). Three additional positive regulatory proteins under σ^{22} control are required for P*algD* expression: the two-component response regulators AlgB and AlgR (13–15) and a small ribbon-helix-helix family DNA-binding protein, AmrZ (16).

σ^{22} is encoded by the *algT/U-mucABCD* operon along with posttranslational regulatory proteins (Fig. 1A). MucA is an anti-sigma factor and primary inhibitor of σ^{22} activity; it acts by sequestering the sigma factor at the cytoplasmic membrane. Mutations in *mucA* are the most common cause of the constitutive expression of alginate observed in CF isolates of mucoid *P. aeruginosa* (17). MucA spans the inner membrane with the N-terminal cytoplasmic domain binding σ^{22} and the C-terminal periplasmic domain binding MucB (18, 19) (Fig. 1B). The formation of this macromolecular membrane complex results in diminished expression of σ^{22}-regulated genes. The *mucD* gene encodes an HtrA/DegP-like periplasmic protease that apparently degrades peptide signals that lead to σ^{22} release and thus helps maintain the integrity of the σ^{22}-MucAB complex. The *mucD* gene is also under the control of an internal promoter within *mucC*, a gene of unknown function (20).

Liberation of σ^{22} from the inner membrane complex and the resultant increase in σ^{22}-mediated transcription occur by regulated intramembrane proteolytic (RIP) destruction of MucA (Fig. 1B). Cell wall stress triggers the activation of this process, such as by exposure to cell wall-active antibiotics (e.g., D-cycloserine) or compounds that disrupt biological membranes (e.g., Tween and triclosan) or by overproduction of certain outer membrane proteins (e.g., MucE) (19, 21, 22). AlgW is an inner membrane endoserine protease that senses envelope stress conditions by binding sequence-specific polypeptide signal molecules at its PDZ domain, which relieves steric inhibition of the catalytic domain and allows AlgW to cleave MucA in the periplasmic domain (18). The initiating AlgW-dependent cleavage of MucA is followed by further degradation of the truncated anti-sigma polypeptide through the actions of RseP (YaeL) protease and several cytoplasmic ClpXP proteases, resulting in the ultimate release of σ^{22} so that it can complex with core RNA polymerase and direct transcription of its target genes (18, 21–23).

We have analyzed the transcriptional profiles of genes upregulated by σ^{22} activation in response to cell wall stress (19). Exposing *P. aeruginosa* PAO1 to D-cycloserine, which directly targets peptidoglycan synthesis, results in the increased expression of 293 genes that are dependent on σ^{22} deregulation. Among those upregulated are the genes for alginate biosynthesis and its regulation. Other members of the σ^{22} stimulon include genes involved in peptidoglycan biosynthesis (*mdoH*, *mrcB*, and *mpl*), lipopolysaccharide (LPS) biosynthesis (*wzz*, *rmlD*, *wbpH*, and *wbpD*), and genes encoding proteins with known adaptive or protective functions, such as bacitracin resistance protein (BacA), chloroperoxidase (Cpo), and two mechanosensitive channels encoded by PA4394 and PA4614 (19). A number of lipoproteins that are σ^{22} dependent have predicted roles in outer membrane repair and/or maintenance. However, the large majority of genes in the σ^{22} stimulon encode hypothetical or conserved hypothetical proteins for which limited biological information is known. It has also been shown that a mutation in *algT/algU*, which disrupts the whole σ^{22} stimulon of 293 genes, also results in less robust (i.e., less shear-resistant) biofilms (24). However, it is unknown which genes of the stimulon are involved with biofilm formation.

In this study, we sought to better understand the σ^{22}-mediated stress response by characterizing mutants with defects in specific σ^{22}-dependent genes for potential roles in maintaining cell envelope integrity. This analysis revealed that several gene products play a role in envelope homeostasis and thus affect the stress response system itself. Further study revealed that some of these genes demonstrate this effect only in either the planktonic (free-swimming) or the sessile (biofilm) mode of growth.

RESULTS

Construction of σ^{22} stress stimulon mutants. There are 293 known genes in the σ^{22} stress stimulon of strain PAO1 as determined by a transcriptome analysis of planktonically grown bacteria subjected to cell wall stress with D-cycloserine (19). In this study, we selected a set of undercharacterized genes within the σ^{22}

TABLE 1 Verification that selected genes were σ^{22} dependent by using a *lacZ* fusion analysis of each promoter in *algT* (σ^{22}-deficient) and *mucA* (anti-sigma-factor-deficient) backgrounds

Gene	Gene product description[a]	Stress induction (15/60 min)[b]	P-*lacZ* WT:*algT*:*mucA* activity[c]
PA0059	OsmC, redox protein, osmotically induced	9.9/8.9	156:15:450
PA0062	Hypothetical, predicted type II lipoprotein	11.4/10.5	ND
PA0460	Hypothetical, predicted periplasmic	18.9/7.7	1,569:116:5,161
PA0567[d]	Proteolipid homolog, membrane integrity	6.0/6.2	220:142:976
PA0854	FumC2, fumarate hydrase	4.7/10.7	ND
PA0919	Hypothetical, predicted secretion usher	3.0/<2	Operon
PA0920[d]	Aminoacyl-phosphatidylglycerol synthase	3.0/<2	3,264:2,825:5,239
PA1243	Predicted transcriptional regulator, PAS domain	2.0/<2	87:30:307
PA1323	Hypothetical, DUF883 family	19.8/15.9	269:13:1,278
PA1324	Predicted to bind/transport polysaccharides	19.1/14.3	Operon
PA2167	Hypothetical, unclassified	<2/20.7	72:21:470
PA2176	Hypothetical, unclassified	<2/7.0	76:19:296
PA2177[d]	Predicted sensor response hybrid, PAS domain	2.2/<2	99:101:244
PA2717	Cpo, chloroperoxidase	<2/14.4	ND
PA3040[d]	Hypothetical, DUF883 family	7.1/6.2	1,352:1,073:4,148
PA3459[d]	Predicted glutamine amidotransferase	4.4/<2	402:415:839
PA3691	Hypothetical, predicted lipoprotein	15.7/17.8	608:13:2,216
PA3795[d]	Predicted oxidoreductase	5.2/8.8	707:605:1,593
PA3819	SlyB homolog, outer membrane protein	10.3/8.2	1,221:568:3,351
PA4311	Predicted glycosyltransferase	4.0/6.9	ND
PA4394	Predicted mechanosensitive channel, McsS	3.0/4.0	130:15:797
PA4717[d]	Predicted periplasmic metalloprotease	5.3/6.3	1,404:1,101:3,215
PA5107	Lipocalin Blc, outer membrane protein	8.1/4.4	Operon
PA5108	Hypothetical, predicted lipoprotein	7.5/3.0	714:387:3,732
PA5178	Hypothetical, LysM domain	9.6/7.1	452:284:2,532
PA5212	Hypothetical, unclassified	15.9/7.8	1,555:44:5,758
PA5424[d]	Hypothetical, predicted inner membrane protein	8.3/7.4	807:660:3,904

[a] PAO1 gene names and descriptions were obtained from the *Pseudomonas* Genome Database (33) and are listed numerically. Sequence-defined transposon insertions in each gene were purchased from the University of Washington Genome Center. All were transduced into the PAO1/PDO1 reference isolate and verified by PCR analysis.
[b] Stress induction shows data previously described (19) for the fold increase of the genes' transcriptional activity expressed from the PAO1 chromosome when treated with D-cycloserine (400 μg/ml) for 15 or 60 min as determined by microarray analysis.
[c] PAO1 (wild type [WT]), PDO-LS586 (*algT*), and PDO351 (*mucA*) strains containing each *lacZ* fusion plasmid were grown in L broth at 37°C with aeration, collected during logarithmic growth, and assayed for β-galactosidase activity. Data show each gene's promoter activity (Miller units) when fused to *lacZ* (P-*lacZ*) when expressed in the wild type or in an *algT* or *mucA* mutant. "Operon" indicates that the gene is in an operon of a gene already tested. ND, not determined.
[d] The mutant did not show the predicted σ^{22} stimulon phenotype until tested under cell wall stress conditions (see Table 2).

stress stimulon (Table 1) for a mutant analysis to shed light on the output of this complex stress response system. These genes can be under the direct or indirect control of σ^{22}; previous studies show that at least two of them (PA0059 and PA3819) have promoter regions with the σ^{22} consensus sequence (25). Transposon insertion mutants of PAO1 with sequence-defined insertions in genes of the σ^{22} stress stimulon were obtained from the University of Washington Genomics Resource Center (WGRC). To ensure a consistent PAO1 strain background, which is known to show some diversity among laboratory strains (26), each WGRC transposon-mutated allele was transduced into this laboratory's reference PAO1 isolate (also known as PDO1). In all, 27 transduced transposon insertions were constructed for study and are shown in Table 1 along with their stress induction values from the microarray analyses (19). Each was tested by PCR to verify that the transposon was in the correct gene (see Materials and Methods).

Verification of membership in the σ^{22} stress stimulon. To verify that the genes in Table 1 were indeed upregulated by σ^{22} activation, plasmids that contained each gene's upstream promoter region transcriptionally fused to *lacZ* using broad-host-range vector pSS269 were constructed (27). All the reporter plasmids showed β-galactosidase activity in wild-type PAO1 (Table 1), indicating that their promoters had been cloned to form *lacZ* fusions. Compared to PAO1, the promoter activity of σ^{22} regulon members should show reduced expression in an *algT* mutant (PDO-LS586) devoid of σ^{22} and high expression in a *mucA* mutant (PDO351) lacking the anti-sigma factor for σ^{22}. In general, these genes' promoter reporters (P-*lacZ*) showed this predicted expression pattern, indicating that they were directly or indirectly under the control of σ^{22} (Table 1).

Many of the promoters fused to *lacZ* (P-*lacZ*) showed a dramatic reduction in β-galactosidase activity in the *algT* mutant (i.e., devoid of σ^{22}) as expected, but some had only a modest reduction or none in the *algT* mutant (i.e., PA0567, PA0920, PA2177, PA3040, PA3459, PA3795, PA4717, and PA5424) (Table 1). Thus, their P-*lacZ* reporters were examined under conditions of cell wall stress in wild-type PAO1 and in an *algT* (PDO-LS586) mutant (Table 2). The results showed that all but one (PA0567) had a ≥5-fold increase in β-galactosidase activity in PAO1 as a result of exposure to D-cycloserine, which as predicted was not observed in the *algT* mutant. With PA0567-*lacZ*, β-galactosidase activity rose by only 2.3-fold following cell wall stress and then activity actually increased instead of decreasing in the *algT* mutant, suggesting that the regulation of PA0567 is more complex. Nevertheless, we kept this gene in our study because PA0567-*lacZ* transcriptional activity was markedly elevated in the *mucA* mutant (Table 1), indicating that this gene was under σ^{22} control.

TABLE 2 Confirmation of σ^{22}-dependent gene expression during cell wall stress for selected promoters

Promoter-*lacZ* fusion[a]	Fold increase in β-galactosidase activity due to D-cycloserine treatment	
	PAO1	*algT*
PA0567-*lacZ*	2.3	10.9
PA0920-*lacZ*	5.6	2.6
PA2177-*lacZ*	14.2	2.3
PA3040-*lacZ*	10.0	2.2
PA3459-*lacZ*	9.0	2.4
PA3795-*lacZ*	11.4	3.3
PA4717-*lacZ*	8.8	2.8
PA5424-*lacZ*	19.2	1.7

[a] Listed are promoter-*lacZ* fusions from Table 1 that did not yield the predicted phenotypes under unstressed conditions in *algT* and/or *mucA* mutant backgrounds. Here they were compared for β-galactosidase activity under cell wall stress conditions in PAO1 and an *algT* mutant by exposure to D-cycloserine. Bacteria were grown under routine lab conditions (L broth with aeration at 37°C) to an OD_{600} of 0.3 and treated with a sub-MIC level (400 μg/ml) of D-cycloserine for 60 min. The fold increase in β-galactosidase activity (Miller units) shown is a comparison to that of untreated control cultures. In PAO1 containing functional σ^{22}, all 8 promoter fusions above showed an increase in transcriptional activity when exposed to D-cycloserine. None of these promoters, except PA0567-*lacZ*, showed high induction in the σ^{22} knockout, PAO1*algT*, indicating their dependence on σ^{22} for increased expression during cell wall stress.

Phenotypes of σ^{22} stimulon mutants. Because the genes of the σ^{22} stress stimulon were discovered by their upregulation following peptidoglycan damage, we looked at the mutants for changes in sensitivity to cell wall-inhibiting antibiotics (i.e., D-cycloserine, fosfomycin, and carbenicillin [Cb]); however, none showed an altered zone of inhibition compared to parent strain PAO1 in a standard disk diffusion assay (data not shown). Sensitivity to elevated growth temperatures is also a common phenotype of mutants defective in the ability to recover from stress, but the pattern of growth at 43°C in L broth with aeration showed no change from that of wild-type PAO1 (data not shown). However, when the growth temperature was raised to 45°C, the PA0062 and PA1324 mutants did show slightly lower growth rates than did PAO1 (Fig. 2). Because previous studies indicate a link between the σ^{22} stimulon and the regulation of biofilm formation (24), we looked for biofilm defects in the mutants with knockouts in genes of the σ^{22} stimulon. We examined microbial adherence to the walls of polystyrene tubes (24-h incubation at 37°C) and at the ability to form air-to-medium interface flocculation in statically grown culture (over 12 days at 25°C) using methods previously described (28).

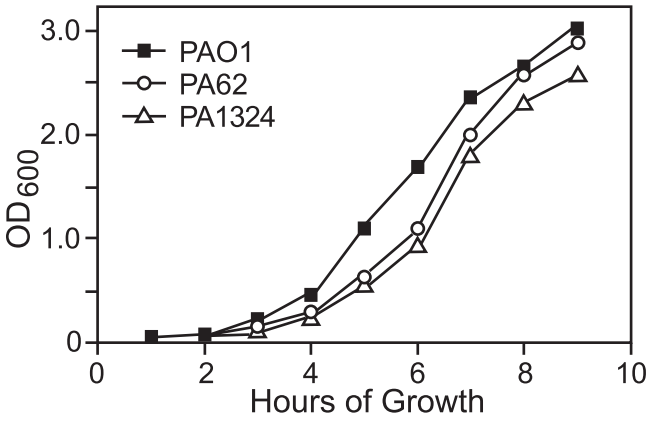

FIG 2 Demonstration of a temperature-sensitive (Ts) growth phenotype. Mutants with Tn*5*-related (Tc^r) insertions in genes of the σ^{22} stimulon were examined for growth defects compared to the parental strain, PAO1, in L broth at 45°C in a shaking water bath incubator. Among the 27 mutants examined, mutations in PA0062 or PA1324 caused a modest temperature-sensitive growth defect at 45°C. Shown is one of three experiments, all of which showed comparable results.

However, no obvious alteration from the wild-type PAO1 biofilm phenotypes could be observed with any of the mutants (data not shown).

The σ^{22} stress stimulon genes are coregulated with those for alginate production, so we examined the possibility that some of the mutants might be altered in envelope homeostasis that would activate AlgW protease sufficiently to cause alginate biosynthesis. However, none showed a mucoid phenotype on agar plates, nor was production of alginate measurably above background PAO1 levels (data not shown). To determine if any of these mutations might cause a block in alginate production, the gene-specific transposon insertions (Tc^r) were transduced into the chromosome of an isogenic mucoid strain, PDO351 (*mucA*::Gm^r). However, all of these double mutants retained the mucoid phenotype and produced levels of alginate similar to that produced by PDO351 (data not shown), indicating that none of the genes under study was required for high-level alginate production.

Effect of mutations in the σ^{22} stimulon on P*algD* induction in planktonic or sessile culture. To determine whether any of the selected genes in the σ^{22} stress stimulon have a role in envelope homeostasis, we examined the mutants for effects on σ^{22} activation, which responds with high sensitivity to disturbances in the cell wall. Expression of P*algD*, a well-characterized σ^{22}-dependent promoter for an important virulence factor, was examined using a *lacZ* transcriptional fusion (P*algD-lacZ*) as an indicator of the σ^{22} activity level under unstressed and stressed conditions. When wild-type PAO1 carried a P*algD-lacZ* reporter plasmid (pLW149a) and was grown under planktonic conditions (i.e., L broth with aeration), it produced only a low level of β-galactosidase (13 ± 7 Miller units), but when stressed by exposure to D-cycloserine at a sub-MIC for 60 min, expression increased ~100-fold (1,161 ± 231 Miller units). Thus, when normalized to 100%, the planktonic P*algD-lacZ* untreated/treated ratio of expression in PAO1 was typically ~1:100 (Table 3). This 1:100 ratio was then compared to the expression of P*algD-lacZ* in the mutants of the σ^{22} stress stimulon under the same conditions.

We also employed another tool for measuring P*algD* induction, but under sessile conditions using a plate bioassay (22). Here, a low-copy-number reporter plasmid (pLW117, Gm^r) has the *algD* promoter fused to a promoterless *cat* gene (P*algD-cat*) such that growth occurs in the presence of chloramphenicol (Cm) when P*algD* is activated. When a culture of PAO1(pLW117) was

TABLE 3 Comparison of P*algD* induction in mutants of the σ^{22} stimulon when in the planktonic and sessile states of growth

Phenotype	Strain/ mutation	Activity of untreated planktonic P*algD-lacZ*:activity of that treated with D-cycloserine[a]	Sessile P*algD-cat* treated with D-cycloserine[b]	Putative role of gene product
Wild type	PAO1	1:100	Ring	
Alterations in stressed P*algD* expression	PA3459	1:67	Weaker ring	Osmoprotectant
	PA5424	1:52	Weaker ring	Membrane protein
	PA1243	1:153	Ring	Sensor/regulator
	PA5107	1:134	Ring	Lipocalin
Growth-independent alterations in unstressed P*algD* expression	PA0062	6:131 (Ts)	Lawn	Lipoprotein
	PA1324	1.7:151 (Ts)	Lawn	Polysaccharide binding
Planktonic growth-specific alterations in unstressed P*algD* expression	PA5178	7:215	Ring	LysM, BON domains
Sessile growth-specific alterations in unstressed P*algD* expression	PA0567	1:100	Lawn	UPF0057 domain
	PA3040	1:100	Lawn	DUF883 family
	PA2717	1:142	Lawn	Chloroperoxidase
	PA0920	1:55	Lawn	A-PG synthase[c]

[a] P*algD-lacZ* Miller units of β-galactosidase in PAO1 strains were 13 ($\pm$7) if untreated and 1,161 ($\pm$231) if treated with D-cycloserine. To be significant in the fold change, the ratio needed to be $<$0.5 or $>$1.5 for untreated cultures and $<$80 or $>$120 for treated cultures. When maximum activity was normalized to 100%, the untreated/treated ratio of P*algD-lacZ* expression in PAO1 was typically 1:100. Ts indicates a temperature-sensitive growth defect at 45°C.

[b] P*algD-cat* activity in PAO1 strains was estimated on L agar plates containing chloramphenicol to prevent growth unless the *cat* fusion was activated by D-cycloserine placed in the center of the plate, which resulted in a ring of growth. In some mutants, a weaker ring of growth was observed. A lawn of growth indicated constitutive P*algD-lacZ* expression under such sessile conditions.

[c] A-PG, aminoacyl-phosphatidyl glycerol.

spread onto an L agar plate containing Cm (50 μg/ml) and D-cycloserine was spotted in the center of the plate, a ring of growth formed around the antibiotic due to the activation of the P*algD-cat* fusion (Fig. 3, PAO1). When the P*algD-cat* fusion was tested in a *mucA* mutant (PDO351), a lawn of growth on the Cm plate was observed because σ^{22} is constitutively active (Fig. 3,

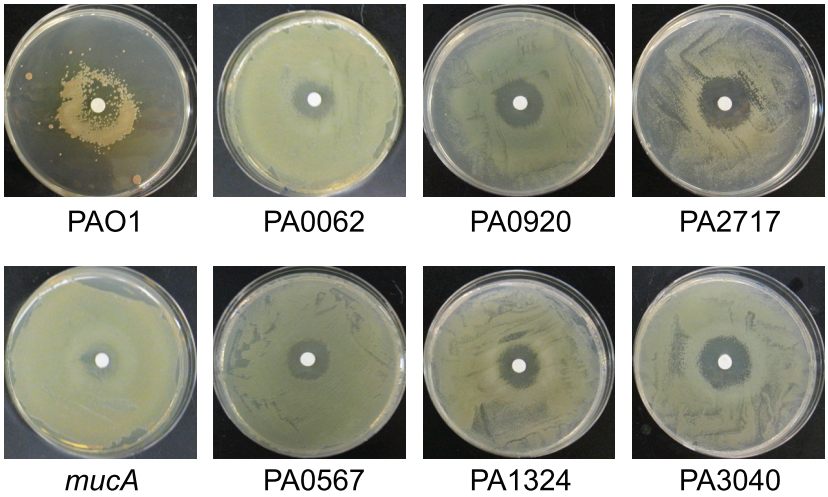

FIG 3 Sessile growth assay for observing the effects of mutations in genes of the σ^{22} stimulon on the activation of the σ^{22}-dependent promoter, P*algD*. Shown are pictures of plates of L agar plus chloramphenicol (Cm) coated with mutant derivatives of PAO1 that hosted pLW117, a plasmid containing P*algD-cat*, which confers Cm^r and thus growth when activated. A 5-mm filter disk impregnated with D-cycloserine was placed in the center of the plate. With PAO1, exposure to D-cycloserine (10 μg) in the center of the plate produced a ring of Cm^r growth after 3 days at 25°C. A *mucA* mutant (devoid of the σ^{22} anti-sigma factor) is shown as an example of σ^{22} derepression. The other plates show a lawn of growth with the mutants indicated, which occurred with or without D-cycloserine and indicated constitutive activation of P*algD-cat* due to the mutation.

mucA); this lawn phenotype did not require stress for induction (data not shown). These P*algD-cat* phenotypes allowed us to test σ^{22} activity under sessile (i.e., biofilm-like) conditions for comparison to the above P*algD-lacZ* phenotypes under planktonic culture conditions.

The P*algD-lacZ* and P*algD-cat* reporter plasmids (pLW149a and pLW117, respectively) were transferred to each of the mutant strains for comparison of the effects of planktonic and sessile conditions. When grown under the same planktonic conditions, most of them produced β-galactosidase at the same ~1:100 untreated/treated ratios, similar to the wild type. Likewise, in the sessile state of growth, most showed the same ring of growth as did the wild type upon exposure to D-cycloserine. However, there were 11 mutants that showed distinguishing phenotypes compared to the wild type and are presented in Table 3. Four mutants showed wild-type levels of P*algD-lacZ* activity under nonstressed conditions but showed alterations in the response to cell wall stress. Two (PA3459 and PA5424) showed reduced responsiveness to stress in the planktonic assay; in the sessile assay, they also displayed a consistently weaker ring of growth (data not shown). Two other mutants (PA1243 and PA5107) showed an increased response to stress when in planktonic culture, although a normal ring of growth was seen in the plate assay rather than an enlarged ring, which might have been predicted.

Three mutants (PA0062, PA1324, and PA5178) had activity higher than that of the wild-type P*algD-lacZ* under planktonic and unstressed conditions (Table 3), indicating that loss of this gene product caused σ^{22} activation even without stress. This suggests that the loss of these gene products affected envelope homeostasis such that AlgW-mediated degradation of MucA is increased. Two of these (PA0062 and PA1324) showed the same results under sessile-biofilm conditions when carrying P*algD-cat* and produced a lawn (Fig. 3), which was consistent with elevated σ^{22} activation in the absence of stress. Both of these mutants also showed a temperature-sensitive phenotype at 45°C (Fig. 2). Interestingly, the PA5178 mutant produced a wild-type ring of growth when P*algD-cat* was induced by D-cycloserine, rather than a lawn, indicating that its role in envelope homeostasis was observable only under planktonic conditions.

There were four other mutants (PA0567, PA3040, PA2717, and PA0920) with elevated σ^{22} activity that grew as a lawn when expressing *algD-cat* (Fig. 3), indicating constitutive P*algD-cat* expression under unstressed sessile conditions. However, all showed normal P*algD-lacZ* activity under unstressed planktonic conditions (Table 3). Thus, the roles of these σ^{22} stimulon gene products in maintaining envelope homeostasis were dependent on the sessile-biofilm state of growth.

DISCUSSION

The σ^{22} stimulon is a large stress response system in *P. aeruginosa* that includes the genes for the production of alginate, an important virulence factor; however, most of the gene products in the stimulon have undefined functions (19). Here we sought to better understand the σ^{22} stress response system by examining the expression of 27 undercharacterized genes of this stimulon and the phenotypes resulting from their mutations. Interestingly, none of the mutations of this stress response stimulon had a major effect on typical stress-related phenotypes like growth rate, temperature sensitivity, or alginate production.

We considered the recent observation that a mutation in *algT/U*, which disrupts the whole σ^{22} stimulon, results in less robust (i.e., less shear-resistant) biofilms (24). Biofilm growth of *P. aeruginosa* occurs on natural surfaces in the environment and during human infections, such as in the lungs of CF patients, causing chronic bronchopneumonia (6, 7). The development of a biofilm is initiated by planktonic (freely moving) bacteria that attach to a surface (become sessile) and form microcolonies that produce a polymeric matrix and become increasingly tolerant to antibiotics (29). When we compared the 293 genes in the σ^{22} stimulon (19) to those activated during biofilm development (30), we found that 59% of the σ^{22} stimulon genes were in common. Among the genes selected in our study, all but one (PA0919) are upregulated in PAO1 biofilms as determined by cluster analysis of whole-genome expression profiles of PAO1 transcriptomes derived from planktonic cultures that developed into mature biofilms (30). In addition, a recent screen of a strain PA14 transposon insertion library for biofilm defects revealed that mutants in 3 of the genes studied here (PA0854, PA1243, and PA4394) exhibit reduced biofilm formation as observed by microscopic analysis (31). Given this link between the σ^{22} stimulon and biofilm formation, we examined our σ^{22} stimulon mutants for biofilm defects. However, no obvious alteration from the wild-type biofilm phenotypes was observed using simple biofilm models. Instead, our studies focused on potential effects that the mutations might have on envelope homeostasis, as measured by σ^{22} activity on the alginate operon promoter, P*algD*, during planktonic and sessile growth conditions.

We expected that many of the genes in the σ^{22} stimulon would contribute to cell envelope homeostasis because their expression was upregulated during extracytoplasmic stress induced by exposure to D-cycloserine. This is an antibiotic that directly causes disruption of the peptidoglycan cell wall. Disruption of envelope homeostasis results in the activation of σ^{22}, which can be measured by the expression of a σ^{22} promoter. One such σ^{22} promoter is P*algD*, the promoter of the alginate operon. Like other characterized ECF sigma factors, such as σ^{E} in *Escherichia coli* (32), σ^{22} is known to be activated by stress conditions via signal RIP degradation of the anti-sigma factor that normally sequesters the sigma factor, thus releasing or "activating" sigma factor activity (Fig. 1). We have previously shown that in *P. aeruginosa* PAO1, σ^{22} activation is highly responsive to cell wall stress (19, 22). Indeed, our mutant analysis of 27 genes of the σ^{22} stimulon revealed that over one-third of them (i.e., 11 of 27) had effects on σ^{22} activation under conditions of stress or nonstress.

Four mutants that altered the ability of *P. aeruginosa* to respond to cell wall stress with σ^{22} activation were found. Mutations in PA3459 and PA5424 reduced P*algD* responsiveness to cell wall stress under planktonic and sessile conditions. This suggests that their gene products are required for full activation of the stress response system involved in σ^{22} control. PA3459 is the first gene of a three-gene operon (33) that putatively encodes functions involved in the synthesis of a cytoplasmic osmoprotectant, *N*-acetylglutaminyl-glutamine amide (NAGGN) and is upregulated in response to osmotic stress (34). Thus, loss of this osmoprotectant resulted in a reduced ability to respond to cell wall stress in both planktonic and sessile states of growth. PA5424 encodes a small (81-amino-acid) conserved hypothetical protein predicted to contain 3 transmembrane helices and to localize to the inner membrane. Its closest homolog is the hypothetical protein YeaQ in *E. coli*, sharing 61% similarity, but for which little else is known.

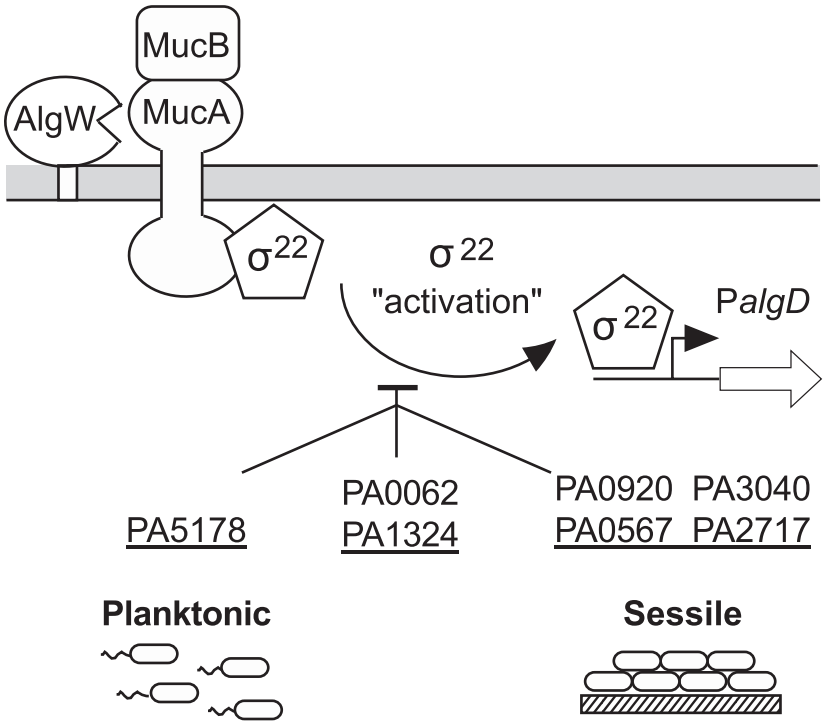

FIG 4 Illustration of the roles of specific gene products in the σ^{22} stimulon on the "activation" of σ^{22} activity (i.e., by release from the MucA-MucB inhibitory complex). This was determined by examining the effect of mutations on envelope homeostasis, which leads to activation of P*algD*. Mutations showed their effects when *P. aeruginosa* grew in the planktonic state of growth (left), the sessile/biofilm mode (right), or both (center).

Mutants with defects in PA1243 and PA5107 showed elevated expression during cell wall stress that could be measured during planktonic growth. PA1243 is predicted to encode a sensor/response regulator hybrid protein, located in the cytoplasmic membrane. It contains both a histidine kinase domain and a sensory domain (with a PAS motif). Thus, PA1243 may directly control a subset of genes in the σ^{22} stimulon under conditions recognized by its sensor domain. Interestingly, it was reported that a transposon insertion in PA1243 of strain PA14 exhibits reduced biofilm formation (31). PA5107 is predicted to encode a bacterial lipocalin (Blc), which is an outer membrane lipoprotein. Blc in *E. coli* is known to be expressed under cell envelope stress conditions caused by high osmolarity (35). Recent reports indicate that *E. coli* Blc is a dimer with a binding preference for lysophospholipids, which suggests a role for this protein in the storage/transport of lipids important for membrane biogenesis and repair (36).

Of particular interest to this study were the 7 mutants that demonstrated elevated σ^{22} activation even in the absence of stress: PA0062, PA1324, PA5178, PA2717, PA0567, PA3040, and PA0920. This suggests that these gene products are important for maintaining envelope homeostasis and/or preventing AlgW from spontaneously activating the σ^{22} stress response (Fig. 4). Mutations in PA0062 and PA1324 caused higher expression of P*algD* with and without stress induction, and this was observed under planktonic and sessile conditions. Interestingly, mutations in PA0062 or PA1324 also caused mild temperature-sensitive growth defects at 45°C. PA0062 encodes a hypothetical uncharacterized protein that is probably a lipoprotein. The PA1324 open reading frame (ORF) is classified in the *Pseudomonas* Genome Database (33) as an unknown hypothetical protein with a predicted type II lipoprotein signal. Recently, a nuclear magnetic resonance (NMR) structure of the PA1324 protein predicted a pre-albumin-like fold, and based on ligand screening studies, it is postulated to be involved in the binding and/or transport of polysaccharides (37).

The PA5178 mutant showed higher constitutive and induced expression of P*algD* only during planktonic growth, while induced and uninduced P*algD* expression appeared normal under sessile conditions. Thus, PA5178 plays a role in envelope homeostasis primarily during the planktonic mode of growth. PA5178 encodes an unclassified hypothetical protein that is predicted to contain one LysM domain, which is a widely distributed peptidoglycan-binding domain (38), suggesting that it acts directly on the cell wall. It also has a BON domain, which is a putative phospholipid-binding region (39). A proteomic analysis of *P. aeruginosa* outer membranes shows that the cellular concentration of this protein is increased with increasing resistance to several antibiotics, including ampicillin (40).

The rest of the mutants (PA0920, PA0567, PA3040, and PA2717) showed defects in unstressed envelope homeostasis that could be observed only in the sessile state of growth (Fig. 4). Mutations in PA0567 and PA3040 had no effect on P*algD* expression and induction during planktonic growth but caused constitutive P*algD* expression under sessile growth. Thus, the effects due to the loss of these gene products were severe enough to trigger the σ^{22} stress response only in adherent cultures. PA0567 encodes a small, 52-amino-acid, hydrophobic peptide with 2 predicted transmembrane helices. It shares 78% similarity with the proteolipid YqaE of *E. coli*, and both are members of the Pfam domain UPF0057. UPF0057 proteolipids have been characterized in yeast and plants as stress-responsive proteins that help maintain membrane integrity under environmental conditions of high salinity and low temperatures (41). PA3040 encodes an ortholog of the DUF883 family of membrane proteins. It is the first gene of a three-gene operon expressing hypothetical proteins. Interestingly, another member of the DUF883 protein family is PA1323, which was also included in this study of σ^{22} stimulon members, although its inactivation did not lead to any apparent alteration in σ^{22} activity.

PA2717 is predicted to encode a cytoplasmic chloroperoxidase (Cpo). The PA2717 mutant was normal for unstressed P*algD* activity under planktonic conditions, but elevated P*algD* expression was seen under planktonic stressed conditions. It formed a lawn in the plate assay, indicating constitutive P*algD* activity under sessile conditions, suggesting that it normally attenuates σ^{22} activation in the absence of stress.

The mutation in PA0920 was especially interesting in that under planktonic conditions P*algD* expression was normal if unstressed but showed lower responsiveness (i.e., ~50% P*algD-lacZ* activity) upon cell wall stress. However, the PA0920 mutation had the opposite effect under sessile growth and caused constitutive

P*algD* expression. PA0920 has recently been shown to encode an integral inner membrane enzyme required for the production of 2′-alanyl-phosphatidylglycerol in *P. aeruginosa* membranes (42). A PA0920 mutant analysis showed that alanyl-phosphatidylglycerol in *P. aeruginosa* PAO1 membranes confers increased resistance to the toxic effects of the heavy metal Cr^{3+}, the osmolyte sodium lactate, the cationic peptide protamine sulfate, and cefsulodin, a β-lactam that impedes cross-linking of peptidoglycan. PA0920 is induced under acidic growth conditions, and here we show that it is also induced 5-fold by D-cycloserine as part of the σ^{22} stimulon. Changing the amount of alanyl-phosphatidylglycerol in *P. aeruginosa* membranes is a mechanism to control the fluidity and permeability of the cellular membranes, which are essential to maintaining envelope homeostasis (42). Here we also observed that loss of alanyl-phosphatidylglycerol in PAO1 membranes resulted in a disruption of envelope homeostasis leading to σ^{22} activation, but only under sessile/biofilm conditions.

In conclusion, we have found σ^{22}-dependent genes whose loss resulted in altered stress responsiveness or constitutive activation of the σ^{22} sensory system that monitors cell envelope homeostasis. The effects were often dependent upon whether *P. aeruginosa* was in a planktonic or a sessile state of growth, suggesting an overlap with biofilm control mechanisms. Continued studies on the role of σ^{22} stimulon genes may reveal other links to the biofilm mode of growth and potential solutions for disrupting biofilms that often lead to antibiotic tolerance and persistence during infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains of *P. aeruginosa* used in this study are shown in Table 4. The PAO1 parent strain used in this study, also known as PDO1 (22), is a spontaneous Cm^s isolate of the original Cm^r PAO1 strain obtained from B. W. Holloway (43). *E. coli* DH5α was used for routine plasmid manipulations. Bacteria were routinely cultured in L broth (10 g tryptone, 5 g yeast extract, and 5 g NaCl each per liter) or on L agar. LPIA plates were a 1:1 mix of L agar and *Pseudomonas* isolation agar (Difco) and were used to counterselect against *E. coli* following conjugal plasmid transfers to *P. aeruginosa*. Antibiotics (Sigma) were used at the concentrations indicated: gentamicin (Gm), 20 μg/ml and 100 μg/ml for *E. coli* and *P. aeruginosa*, respectively; tetracycline (Tc), 20 μg/ml and 60 μg/ml for *E. coli* and *P. aeruginosa*, respectively; kanamycin (Km), 30 μg/ml, and ampicillin (Ap), 100 μg/ml, both for *E. coli*; and carbenicillin (Cb), 100 μg/ml for *P. aeruginosa*.

Mutant strain construction. Mutants of *P. aeruginosa* PAO1 with sequence-defined transposon (Tn*5*-derived, Tc^r) insertions of Tn*5-lacZ* or Tn*5-phoA* were purchased from the University of Washington Genomics Resource Center (WGRC, http://www.gs.washington.edu/labs/manoil/libraryindex.htm). Such Tn*5*-marked alleles were transduced into this laboratory's PAO1 reference isolate (PDO1) with selection for tetracycline resistance (Tc^r). Plate lysates with the generalized transducing phage F116L (43) were made with each WGRC mutant by incubating 10 ml F116L lysate at various 1:10 dilutions with 0.1 ml of an overnight L broth culture containing 10 mM $MgSO_4$ and 5 mM $CaCl_2$, which was then incubated at room temperature for 10 min to permit adsorption. Molten top agar at 50°C (3 ml L broth with 0.7% agar) was added to each tube and immediately poured onto the surface of an L agar plate. After 24 h at 37°C, 3 ml of L broth was added to a plate showing confluent plaques, the top agar was extracted with a sterile spreader, and the mix was centrifuged to remove the agar and bacterial cells. The supernatant was passed through a 0.45-μm filter to remove any residual bacteria. For transductions, 0.1 ml of a transducing lysate was mixed with 0.2 ml of the *P. aeruginosa* host strain grown overnight in L broth, which was incubated at room temperature for 15 min and was then shaken at 225 rpm at 37°C for 1 h; the culture was then plated onto L agar containing selective antibiotics and incubated at 37°C until colonies were observed. All transduced transposon insertions were examined by PCR to verify that each insertion was in the correct gene. For this, primers specific for Tn*5-lacZ* (LacZ148, GGGTAACGCCAGGGTTTTCC) or Tn*5-phoA* (PhoA138, CGGGTGCAGTAATATCGCCCT) and primers specific for the 5′ and 3′ ends of the gene of interest using sequences obtained from the *Pseudomonas* Genome Database (33) were used in standard PCRs with *Taq* polymerase; the products were observed using agarose gel electrophoresis for size analysis. To examine the effect of such mutations on the mucoid phenotype, Tn*5* (Tc^r)-mutated alleles were transduced into PDO351 (i.e., *mucA*::Gm). There were some F116L lysates of WGRC Tn*5* mutants (i.e., PA0062, PA567, PA920, PA1323, PA1324, PA2717, PA3040, PA3819, PA4717, PA5107, and PA5424) where transductants were rare, in which case the *mucA*::*aac*CI (Gm^r) allele from PDO351 was transduced into verified PAO1/PDO1::Tn*5* (Tc^r) mutants instead. Information on domains in proteins of interest was found in the *Pseudomonas* Genome Database (33) found at http://www.pseudomonas.com.

Disk diffusion assay. The relative level of resistance to antibiotics by mutants was compared to that of the wild type by a standard disk diffusion assay. A sample (0.1 ml) of culture in the early logarithmic phase was spread onto an L agar plate, and then a 5-mm paper disk impregnated with the antibiotic was placed in the center to permit radial diffusion. After 24 h of incubation at 37°C, the diameter of the ring of growth inhibition was measured.

Assay for alginate. *P. aeruginosa* strains were grown on L agar plates at 37°C for 48 h, and then the growth was resuspended in saline. Bacterial cells were removed by centrifugation, and then the supernatant was tested for alginate (which is composed of two uronic acids) using the carbazole-spectrophotometric method for the assay of uronic acids described by Knutson and Jeanes (44). Alginic acid from *Macrocystis pyrifera* (Sigma) was used as the standard.

Promoter-*lacZ* reporter plasmids. High-fidelity *Pfu* Turbo (Stratagene) was used to amplify DNA by PCR with all primers custom synthesized by Eurofins MWG Operon using sequences available in the *Pseudomonas* Genome Database (33). Promoter regions obtained by PCR amplification included DNA ~0.5 kb upstream of each selected ORF, which was cloned into the broad-host-range transcriptional *lacZ* reporter pSS269 (27). Reporter plasmids were transferred from *E. coli* to *P. aeruginosa* strains by triparental mating as described elsewhere (27) using helper plasmid pRK2013 to mobilize the *oriT*-containing plasmids to *P. aeruginosa* with selection on LPIA plates containing an appropriate selective antibiotic. It was observed that reporter plasmids in some Tn*5* mutants (i.e., PA0062, PA0920, PA1243, PA1324, PA2177, and PA3819) were somewhat unstable, and so fresh conjugations of reporter plasmids into these mutants were followed immediately by β-galactosidase assays in order to obtain consistent results. Transcriptional activity of reporter constructs was determined by measuring β-galactosidase activity, reported in Miller units, from promoter-*lacZ* fusions. For assays verifying σ^{22} dependence, strains were grown in L broth to an optical density at 600 nm (OD_{600}) of 1.0, at which time 0.1-ml aliquots were assayed. To determine promoter activation in response to cell wall stress induced by D-cycloserine, strains were grown in 25 ml L broth at 37°C to an OD_{600} of 0.3; cultures were split in half, D-cycloserine was added to one half (400 μg/ml), and then both were incubated for an additional 60 min, at which time 0.1-ml samples of untreated and treated cultures were assayed for β-galactosidase activity.

Bioassay for P*algD* induction on a solid substrate. Plasmid pKK61 with an *algD-cat* fusion in low-copy-number vector pLAFR1 (Tc^r) was previously described (45). Because the mutants in this study were Tc^r due to the transposon insertions, a Gm^r derivative of pKK61 named pLW117 was constructed to permit selection of the plasmid transferred to the mutant strains. pLW117 was constructed by cloning an *aac*CIΩ (Gm^r) cartridge (46) into the HindIII site of pKK61. Bioassays of PAO1/PDO1::Tn strains (Cm^s) carrying pLW117 were performed as previously described

TABLE 4 *P. aeruginosa* strains and plasmids used in this study[a]

Strain or plasmid	Genotype/phenotype	Source or reference
P. aeruginosa		
PAO1/PDO1	Wild type, Cm^s	This laboratory
PDO-LS586	*algT*::*aac*CI	47
PDO351	*mucA*::*aac*CI (Gm^r) Alg^+	22
PDO358	*algW*::*aac*CIΩ	22
PDO-PA0059	PDO1, PA0059::Tn (Tc^r) (transduced)	This study
PDO-PA0062	PDO1, PA0062::Tn (Tc^r) (transduced)	This study
PDO-PA0460	PDO1, PA0460::Tn (Tc^r) (transduced)	This study
PDO-PA0567	PDO1, PA0567::Tn (Tc^r) (transduced)	This study
PDO-PA0854	PDO1, PA0854::Tn (Tc^r) (transduced)	This study
PDO-PA0919	PDO1, PA0919::Tn (Tc^r) (transduced)	This study
PDO-PA0920	PDO1, PA0920::Tn (Tc^r) (transduced)	This study
PDO-PA1243	PDO1, PA1243::Tn (Tc^r) (transduced)	This study
PDO-PA1323	PDO1, PA1323::Tn (Tc^r) (transduced)	This study
PDO-PA1324	PDO1, PA1324::Tn (Tc^r) (transduced)	This study
PDO-PA2167	PDO1, PA2167::Tn (Tc^r) (transduced)	This study
PDO-PA2176	PDO1, PA2176::Tn (Tc^r) (transduced)	This study
PDO-PA2177	PDO1, PA2177::Tn (Tc^r) (transduced)	This study
PDO-PA2717	PDO1, PA2717::Tn (Tc^r) (transduced)	This study
PDO-PA3040	PDO1, PA3040::Tn (Tc^r) (transduced)	This study
PDO-PA3459	PDO1, PA3459::Tn (Tc^r) (transduced)	This study
PDO-PA3691	PDO1, PA3691::Tn (Tc^r) (transduced)	This study
PDO-PA3795	PDO1, PA3795::Tn (Tc^r) (transduced)	This study
PDO-PA3819	PDO1, PA3819::Tn (Tc^r) (transduced)	This study
PDO-PA4311	PDO1, PA4311::Tn (Tc^r) (transduced)	This study
PDO-PA4394	PDO1, PA4394::Tn (Tc^r) (transduced)	This study
PDO-PA4717	PDO1, PA4717::Tn (Tc^r) (transduced)	This study
PDO-PA5107	PDO1, PA5107::Tn (Tc^r) (transduced)	This study
PDO-PA5108	PDO1, PA5108::Tn (Tc^r) (transduced)	This study
PDO-PA5178	PDO1, PA5178::Tn (Tc^r) (transduced)	This study
PDO-PA5212	PDO1, PA5212::Tn (Tc^r) (transduced)	This study
PDO-PA5424	PDO1, PA5424::Tn (Tc^r) (transduced)	This study
Plasmids		
pKK61	pCP19 ($oriV_{RK2}$, Tc^r) P*algD-cat*	22
pLW117	pKK61 *aacC1*(Gm^r) P*algD-cat*	This study
pLW127	pSS269, PA0059*osmC*(−239/+50)-*lacZ*	19
pLW148	pSS269, PA5178(−501/+50)-*lacZ*	This study
pLW149a	pSS269, PA3540 *algD*(−925/+50)-*lacZ*	19
pLW150	pSS269, PA4394(−255/+60)-*lacZ*	This study
pLW152	pSS269, PA3819(−660/+70)-*lacZ*	This study
pLW155	pSS269, PA3691(−372/+59)-*lacZ*	19
pLW166	pSS269, PA5108(−163/+70)-*lacZ*	19
pLW168	pSS269, PA4717(−1130/+50)-*lacZ*	19
pLW179	pSS269, PA1323(−450/+50)-*lacZ*	This study
pLW182	pSS269, PA2176(−450/+50)-*lacZ*	This study
pLW183	pSS269, PA2177(−450/+50)-*lacZ*	This study
pLW185	pSS269, PA2167(−500/+50)-*lacZ*	This study
pLW186	pSS269, PA0460(−500/+50)-*lacZ*	This study
pLW187	pSS269, PA0567(−500/+50)-*lacZ*	This study
pLW188	pSS269, PA0920(−500/+50)-*lacZ*	This study
pLW189	pSS269, PA3040(−500/+50)-*lacZ*	This study
pLW190	pSS269, PA3459(−500/+50)-*lacZ*	This study
pLW191	pSS269, PA3795(−500/+50)-*lacZ*	This study
pLW194	pSS269, PA5212(−500/+50)-*lacZ*	This study
pLW195	pSS269, PA5424(−500/+50)-*lacZ*	This study
pRK2013	ColE1-Tra(RK2)$^+$ Km^r	48
pSS269	pSS223 *lacZ* SF Ap^r	27

[a] All *P. aeruginosa* strains were derived from PDO1, a spontaneous Cm^r isolate of strain PAO1 previously described (28). Abbreviations: Gm^r, *aac*CI-encoded gentamicin resistance; Ap^r, ampicillin/carbenicillin resistance; Km^r, kanamycin resistance; Alg^+, mucoid due to alginate overproduction; *lacZ*, β-galactosidase reporter in transcriptional fusions; SF, stabilization fragment for replication in *P. aeruginosa*. Numbers in parentheses before *lacZ* indicate the promoter region in base pairs relative to the start of translation (+1) of the open reading frame used to make the transcriptional reporter.

(22). Briefly, expression of *algD-cat* was tested by incubating cultures to an OD_{600} of 1.2, at which time a 1.5-ml sample was centrifuged, and the bacterial cell pellet was resuspended in 10 mM $MgSO_4$ to an OD_{600} of 0.2. A 25-μl sample of this cell suspension was spread onto an L agar plate containing chloramphenicol at 50 μg/ml. A 5-mm filter disk impregnated with 1 mg D-cycloserine (i.e., 10 μl of a 100-mg/ml stock solution) was

placed in the center of the plate. The test plates were then incubated at 25°C for 3 days and examined for a ring of Cm^r growth around the disk, indicating induced expression of *algD-cat*.

Biofilm assays. Static L broth cultures of PAO1 and mutant derivatives were compared for their abilities to adhere to the walls of polystyrene tubes after a 24-h incubation at 37°C as previously described (28). Also, the ability to form an air-to-medium interface flocculation during static-growth culture at 25°C was performed as previously described (28), and the flocculation was examined daily over a 12-day period.

ACKNOWLEDGMENTS

We gratefully acknowledge the VCU Nucleic Acids Core Facility for assistance in the microarray analyses.

This work was supported by Public Health Service grant AI-19146 from the National Institute of Allergy and Infectious Diseases (D.E.O.) and in part by Veterans Administration medical research grant I01BX000477 (D.E.O.).

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