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Vanadate and triclosan synergistically induce alginate production by *Pseudomonas aeruginosa* strain PAO1

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Summary

Alginate overproduction by *P. aeruginosa* strains, also known as mucoidy, is associated with chronic lung infections in cystic fibrosis (CF). It is not clear how alginate induction occurs in the wild type (wt) *mucA* strains. When grown on *Pseudomonas* isolation agar (PIA), *P. aeruginosa* strains PAO1 and PA14 are nonmucoid producing minimal amounts of alginate. Here we report the addition of ammonium metavanadate (AMV), a phosphatase inhibitor, to PIA (PIA-AMV) induced mucoidy in both these laboratory strains and early lung colonizing nonmucoid isolates with a wt *mucA*. This phenotypic switch was reversible depending on the availability of vanadate salts and triclosan, a component of PIA. Alginate induction in PAO1 on PIA-AMV was correlated with increased proteolytic degradation of MucA, and required envelope proteases AlgW or MucP, and a two-component phosphate regulator, PhoP. Other changes included the addition of palmitate to lipid A, a phenotype also observed in chronic CF isolates. Proteomic analysis revealed the upregulation of stress chaperones, which was confirmed by increased expression of the chaperone/protease MucD. Altogether, these findings suggest a model of alginate induction and the PIA-AMV medium may be suitable for examining early lung colonization phenotypes in CF before the selection of the *mucA* mutants.

Keywords

Alginate induction; Vanadate; AlgU/T; AlgW; LPS; lipid A

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Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium capable of thriving in various niches in the environment. *P. aeruginosa* is also an opportunistic pathogen that can colonize the airways of patients with cystic fibrosis (CF) (Lyczak *et al.*, 2002). Once in the lung, *P. aeruginosa* is subjected to selective pressures that result in the emergence of strains which produce copious amounts of an exopolysaccharide known as alginate (Govan & Deretic, 1996). Alginate overproduction is advantageous for *P. aeruginosa* as it promotes the formation of an extracellular matrix that protects the bacterium from host defenses (Govan & Deretic, 1996, Lyczak *et al.*, 2002).

Alginate overproducing strains of *P. aeruginosa* generally harbor mutations in a gene known as *mucA*. The *mucA* gene is located within the *algUmucABCD* cluster in the genome of *P. aeruginosa*. For the prototypic strain PAO1, these genes correspond to the loci of PA0762-PA0766 within the genome (www.pseudomonas.com). AlgU (also known as AlgT, σ^{22} , or σ^E) (DeVries & Ohman, 1994, Martin *et al.*, 1993a) is an alternative stress-related sigma factor, which belongs to the extracytoplasmic function (ECF) sigma factor superfamily. One of the major functions controlled by AlgU is to promote alginate production. Transcriptome analysis has revealed that AlgU can activate a vast number of genes (Firoved *et al.*, 2002, Firoved & Deretic, 2003, Firoved *et al.*, 2004a, Firoved *et al.*, 2004b, Tart *et al.*, 2005, Wood *et al.*, 2006, Wood & Ohman, 2009b), some of which are beyond alginate regulation. AlgU is required for the oxidative stress and heat shock responses (Yu *et al.*, 1996). Regulation of AlgU occurs at transcriptional and post-translational levels. At the level of transcription, the *algU* operon has five promoters, and two of them are dependent upon AlgU (DeVries & Ohman, 1994, Schurr *et al.*, 1995). At post-translational level, AlgU is negatively regulated by MucA, the cognate anti-sigma factor. MucA is a transmembrane protein whose N-terminus sequesters AlgU to the cytoplasmic membrane. Release of AlgU from MucA causes increased AlgU activity, and the subsequent expression of the alginate biosynthetic operon via the *algD* promoter. The alginate biosynthetic operon (PA3540-PA3551) encodes most all of the genes required to synthesize and secrete alginate. However, the gene encoding the AlgC enzyme, which is involved both lipopolysaccharide (LPS) (Coyne *et al.*, 1994) and alginate synthesis (Zielinski *et al.*, 1991), is located outside of the biosynthetic operon (PA5322). AlgU also activates expression of transcription factors, encoded by *algB* (Wozniak & Ohman, 1991), *algR* (Mohr *et al.*, 1991), and *amrZ* (Baynham *et al.*, 2006), which participate in transcription from the *algD* promoter.

The emergence of mucoid isolates in the CF lung is thought to be a selective process. Environmental isolates of *P. aeruginosa* are generally nonmucoid. The CF patients probably acquire the initial colonizing strains from the environment. Emergence of the *mucA* mutants signifies the onset of chronic infection because overproduction of alginate facilitates the formation of a mucoid biofilm (Govan & Deretic, 1996). But before these mutants become dominant in the CF lung, it is possible that there is a time point that *P. aeruginosa* displays a transitional mucoid phenotype without *mucA* mutations. This probably occurs during the initial colonization to a time before the establishment of chronic infection. But this transitional mucoid phenotype is not well studied due to lack of an *in vitro* growth condition that mimics the induction. Analysis of this phenotype is important because it will help to understand the early colonizing phenotypes and the associated stress conducive in the conversion to the mucoid phenotype. Previous studies have shown the phosphate availability (Terry *et al.*, 1991) and hydrogen peroxide (Mathee *et al.*, 1999) can cause conversion to the stable mucoid phenotype. Furthermore, anaerobiosis can induce alginate expression *in vivo* (Bragonzi *et al.*, 2005). Recently, two studies have shown that cell wall stress agents can activate proteolysis of MucA and alginate gene expression *in vitro* (Wood *et al.*, 2006, Wood & Ohman, 2009b). However, such activation is not enough to result in mucoid

conversion (Wood *et al.*, 2006, Wood & Ohman, 2009b). To achieve alginate overproduction *in vitro* by wt *mucA* strains, genetic manipulations are required. For example, overexpression of the envelope protein MucE (Qiu *et al.*, 2007), or inactivation of a sensor kinase KinB (Damron *et al.*, 2009b) can both cause AlgW-dependent proteolysis of MucA and alginate overproduction (Fig. S1). Within the *algUmucABCD* cluster, inactivation of *mucB* (Martin *et al.*, 1993b) or *mucD* also results in alginate overproduction in the wt *mucA* strains (Boucher *et al.*, 1996). MucB protects the C-terminus of MucA (Cezairliyan & Sauer, 2009); without MucB, increased degradation of MucA likely occurs. When the chaperone protease MucD is absent, MucA degradation is dependent upon the MucP protease (Damron & Yu, 2011). However, each of these models lacks description of the events that occur upstream of regulated proteolysis of MucA by AlgW and MucP. Conditions that cause the envelope proteins to accumulate and/or misfold thereby activating AlgW and the resulting mucoidy have not been characterized.

P. aeruginosa also expresses other CF specific phenotypes upon adaptation to the CF airway. These phenotypes include increased antibiotic tolerance, biofilm formation, loss of flagellar motility, decreased secretion of virulence factors, and changes to the O-antigen and lipid A components of LPS. LPS is the major component of the outer leaflet of the Gram-negative outer membrane and is comprised of three distinct regions: O-antigen, core, and a hydrophobic anchor known as lipid A. O-antigen and core are made up of polysaccharide chains. CF isolates commonly display a rough phenotype due to the loss of the O antigen (Hancock *et al.*, 1983). Lipid A consists of β -(1',6)-linked diglucosamine backbone with phosphates at the 1 and 4' positions, amide-linked fatty acids at the 2 and 2' positions, and ester-linked fatty acids at the 3 and 3' positions. The chain lengths of the fatty acids linked to lipid A are C12 or C10. In *P. aeruginosa*, penta-acylated lipid A is the most common form, however, hexa- and hepta- forms are also found (Moskowitz & Ernst, 2010). Addition of a C16 fatty acid, or palmitate, results in the synthesis of a hexa-acylated lipid A. In a study, 100% of CF isolates have palmitate on lipid A (n=86) (Ernst *et al.*, 2007). It has been suggested that acylation alters the recognition of lipid A by the host and leads to chronic colonization of the CF airway (Moskowitz & Ernst, 2010). These lipid A modifications also change the bacterial cell surface and outer leaflet integrity.

In *Escherichia coli*, modifications to lipid A structure can induce σ^E activity (σ^E is the *E. coli* homologue of AlgU) (Tam & Missiakas, 2005). When palmitate is added to lipid A by the palmitoyltransferase PagP, increased σ^E activity is observed (Tam & Missiakas, 2005). Palmitoylation of lipid A and increased σ^E expression has relevance to *P. aeruginosa* and CF because palmitoylation of lipid A is observed in *P. aeruginosa* strains isolated from the CF airway (Ernst *et al.*, 1999). In *E. coli*, addition of palmitate is regulated by specific growth conditions, such as low divalent cation concentration or by the addition of ammonium metavanadate (NH_4VO_3 , AMV) to the growth medium (Tam & Missiakas, 2005). AMV is a general phosphatase inhibitor that causes increased σ^E activity without a major impact on the growth of *E. coli* (Tam & Missiakas, 2005). We wondered if AMV would induce a similar effect on the activation of σ^E homolog AlgU in *P. aeruginosa*.

The goals of this work were to develop an *in vitro* growth medium for the analysis of alginate induction in *P. aeruginosa* and to characterize the nature of environmental sensing which activates alginate overproduction. Here, we report *Pseudomonas* isolation agar (PIA) supplemented with AMV (PIA-AMV) can induce mucoidy in strains of *P. aeruginosa* with a wt *mucA*. We found that AMV and triclosan can synergistically activate alginate overproduction. To our knowledge, this is the first example that mucoidy can be artificially induced in *P. aeruginosa in vitro*, independent of mutations. We also show that alginate induction in PAO1 is due to the increased MucA degradation leading to activation of AlgU. Furthermore, growth on PIA-AMV induces synthesis of palmitoylated lipid A. It also seems

that growth of PAO1 on PIA-AMV may cause protein misfolding in the envelope because there is an increase in the expression of general stress chaperones including the chaperone protease MucD. We propose that the PIA-AMV medium simulates environmental conditions which can activate alginate overproduction in *P. aeruginosa*.

Results

P. aeruginosa PAO1 cultivated on PIA-AMV is mucoid

In *E. coli*, modifications to lipid A structure have been shown to induce a σ^E -dependent response (Tam & Missiakas, 2005). To determine if AMV could induce activation of the *P. aeruginosa* σ^E homologue, AlgU, the wt nonmucoid strain PAO1 was cultivated on the PIA-AMV medium. Interestingly, mucoid colony morphology of PAO1 was observed (Fig. 1A). PIA is a complex growth medium for isolation and selection of pseudomonads. PAO1 on PIA is always nonmucoid. The AMV concentration was then optimized for this phenotype. We determined PIA supplemented with 0.27 mM AMV resulted in the highest visible mucoid phenotype. Other salts of metavanadate, such as cesium, sodium and potassium could also induce this phenotype similar to AMV (data not shown), suggesting that the vanadate component of the salt is responsible for this phenotype. Carbazole analysis for uronic acid content was performed on cell free suspensions. The uronic acid content of PAO1 cultured on PIA-AMV was 26 ± 7 $\mu\text{g}/\text{mL}/\text{OD}_{600}$, but PAO1 on PIA was not detectable as determined by the method of collection and carbazole analysis as described in this study. For comparison, *mucA25* mucoid strain PAO581 (Qiu *et al.*, 2008b) on PIA produced the same amount of alginate upon 24 hr growth. To determine if the exopolysaccharide was alginate, standard nuclear magnetic resonance (NMR) analysis of precipitated polysaccharide was performed. As expected, the precipitated material of PAO1 on PIA-AMV was alginate and composed of 33% guluronic acid and 67% mannuronic acid (Fig. 1A). The composition of PAO581 alginate (Qiu *et al.*, 2008b) was 31% guluronic acid and 69% mannuronic acid.

To establish that the AMV-mediated mucoidy in PAO1 was not due to the mutation of *mucA*, the colonies on PIA-AMV were then transferred to PIA lacking AMV. This returned the bacterium to wt nonmucoid morphology (Fig. 1B). So AMV on PIA has an alginate induction effect. We wondered whether AMV was solely responsible for this phenotype or components of PIA were also required. When PAO1 was cultivated on LB supplemented with 0.27 mM AMV, the inducible mucoid phenotype was not evident (data not shown). This suggests PIA has some ingredient(s) that facilitate the induction. PIA contains triclosan (also known as irgasan), a cell wall stress agent that has been shown to activate *algD* expression in *P. aeruginosa* (Wood *et al.*, 2006). When triclosan was added to LB containing 0.27 mM AMV, the inducible mucoid phenotype was observed (data not shown). To determine if AMV or triclosan alone could activate alginate overproduction, AMV or triclosan from 0-10 mM and 0-2000 $\mu\text{g ml}^{-1}$ respectively, in L agar was used to grow PAO1. PAO1 showed a reduced growth on high concentrations of AMV, but was nonmucoid (data not shown). PAO1 is capable of growing on high amounts of triclosan (Chuanchuen *et al.*, 2003, Zhu *et al.*, 2010). On LB agar, PAO1 was nonmucoid for all concentration of triclosan tested (data not shown). PIA also contains 15 mM magnesium chloride to stimulate pyocyanin production for identification of *P. aeruginosa*. When the magnesium chloride concentration was lowered to 1.5 mM in PIA-AMV, alginate overproduction ceased (data not shown), suggesting that a high divalent cation concentration is needed for alginate overproduction by PAO1 on PIA-AMV. Collectively, these data indicate the combination of triclosan, AMV and high magnesium are required for strain PAO1 to overproduce alginate.

PIA-AMV induces mucoid conversion in strains with wt alleles of *algU mucA*

Since PIA-AMV activated alginate production in strain PAO1 with a wt *mucA*, we wondered if this inducible phenotype can be reproduced in other laboratory strains. Commonly used *P. aeruginosa* strains PA14 and PAK were both mucoid on PIA-AMV (Table 1). However, strain PA103 did not overproduce alginate, suggesting that PA103 may have a mutation that impairs alginate synthesis. We hypothesized that the most likely place for a mutation in PA103, which blocks alginate overproduction, would be *algU*. So we sequenced *algU* from PA103 but found no mutations in PA103 in *algU* (Table 1). Therefore, it seems PA103 may have an unknown mutation(s) compared to PAO1. We next tested CF *P. aeruginosa* isolates for alginate induction (Table 1). Early colonizing CF isolates with a wt *mucA* (C01232, C3715, C7406) were nonmucoid on PIA, but they all became mucoid on PIA-AMV. Therefore, AMV has an alginate induction effect in strains with a wt *mucA*. We then examined whether AMV could also have an alginate inhibitory effect in strains that were already mucoid such as the *mucA* mutants. Mucoid CF isolate, FRD1 (*algT⁺mucA22*) was tested on PIA-AMV and remained mucoid, indicating the absence of such an effect. However, nonmucoid CF strain FRD2 and CF149 were not mucoid on PIA-AMV. FRD2 and CF149 have suppressor mutations in *algT/U* (Damron *et al.*, 2009a, DeVries & Ohman, 1994, Ohman & Chakrabarty, 1981), which would block alginate production. These results suggest that PIA-AMV will induce mucoid conversion in nonmucoid laboratory and CF strains with the genotype of *algU⁺mucA⁺*. The strains with *algU* suppressor mutations will not respond to induction on PIA-AMV.

Alginate induction in PAO1 requires two sigma factors, AlgU and RpoN

We tested a group of sigma factor mutants for alginate induction. As expected, we observed the *algU* and *rpoN* genes in PAO1 were both required for alginate induction on PIA-AMV medium (Table 1). Heat shock sigma factor RpoH and stationary phase growth sigma factor RpoS have also been previously associated with alginate production. AlgU can drive expression of RpoH (Schurr & Deretic, 1997). Here we observed *rpoH* was not required for mucoidy on PIA-AMV (Table 1). This is somewhat expected because *rpoH* expression is downstream of AlgU (Schurr and Deretic, 1997). Previous work has shown that inactivation of *rpoS* causes decreased alginate production in FRD1 (Suh *et al.*, 1999). But *rpoS* was not required for alginate induction in PAO1 on PIA-AMV.

Alginate induction in alginate regulator and biosynthetic gene mutants

Mutation of the negative regulators of AlgU such as *mucA* (Martin *et al.*, 1993c), *mucB* (Goldberg *et al.*, 1993, Martin *et al.*, 1993b), *mucD* (Boucher *et al.*, 1996), *kinB* (Damron *et al.*, 2009b) cause alginate overproduction. Mutants in the negative regulators of AlgU all remained mucoid on PIA-AMV (Table 1). The gene *mucC* has been associated with alginate overproduction, but has not been fully characterized (Boucher *et al.*, 1997). Here, we observed that alginate overproduction occurred in the *mucC* mutant (Table 1). We next tested the requirement of alginate biosynthetic genes using this system. As anticipated, alginate biosynthetic genes *algD* and *algE*, encoding GDP-mannose dehydrogenase and the alginate porin pump, respectively, were both required for mucoidy on PIA-AMV (Table 1). Several two-component regulatory systems are involved in regulation of alginate production. Alginate overproduction requires *algR*, which encodes a transcription factor involved in *algD* expression (Deretic *et al.*, 1989). PAO1*algR* was not mucoid on PIA-AMV (Table 1). However, the cognate histidine kinase of *algR*, encoded by *algZ*, was not required for alginate overproduction on PIA-AMV. The NtrC-family transcription factor, AlgB, has been shown to be required for *algD* expression (Leech *et al.*, 2008) as well as mucoidy in a wt *mucA* strains (Damron *et al.*, 2009b). As predicted, PAO1*algB* remained nonmucoid on PIA-AMV (Table 1).

Alginate induction in PAO1 requires MucA proteases AlgW and MucP

Since PAO1 has a wt *mucA* gene, alginate induction would relieve suppression on the proteolytic degradation of MucA. Two proteases, AlgW and MucP, are critical for the degradation of wt MucA. Therefore, we tested PAO1 strains lacking each of the two characterized protease genes, *algW* and *mucP*. Both *algW* and *mucP* were required for mucoidy on PIA-AMV (Table 1). This suggested that alginate induction on PIA-AMV was occurring due to the activation of these two proteases. Prc protease is a periplasmic protease needed for mucoidy in *mucA* mutants (Reiling *et al.*, 2005). However, *prc* is dispensible for mucoidy under conditions in which AlgW is activated (Qiu *et al.*, 2007). Without *prc*, mucoidy occurred on PIA-AMV, which indicated Prc had a minimal role in regulated proteolysis of wt MucA in PAO1 (Table 1). MucE is the only identified envelope protein that activates regulated proteolysis of MucA. However, many *P. aeruginosa* outer membrane proteins have C-termini peptide sequences that could activate AlgW (Qiu *et al.*, 2007). With *mucE* inactivated in PAO1, alginate induction still occurred. This suggests that *mucE* is not involved in alginate induction in PAO1 on PIA-AMV.

AMV increases *PalgU* and *PalgD* promoter activity in PAO1

As mentioned, without the proteases AlgW or MucP, alginate induction was not observed (Table 1). This suggests MucA degradation may be occurring in PAO1 on PIA-AMV. The proteolysis of MucA results in increased promoter activity of *algU* (Damron *et al.*, 2009b, Qiu *et al.*, 2007). This effect is due to the fact that two of the *algU* promoters are dependent upon AlgU (DeVries & Ohman, 1994, Schurr *et al.*, 1995). To observe the effect of PIA-AMV on the alginate-related promoters *PalgU* and *PalgD*, a single copy of the *algU* or *algD* promoters fused with *lacZ* (Damron *et al.*, 2009b), were integrated onto the chromosomes of PAO1, PAO1 Δ *algW*, PAO1 Δ *rpoN*, PAO1 Δ *algB*. The β -galactosidase activities were measured on PIA and PIA-AMV (Fig. 2AB). Compared to the activities on PIA, PAO1 on PIA-AMV had high *PalgU* and *PalgD* activities, which were dependent upon *algW* (Fig. 2AB). This result suggests that AlgW-dependent regulated proteolysis is the mechanism for alginate induction on PIA-AMV. In the absence of *rpoN*, high *PalgU* but low *PalgD* activities were observed (Fig. 2AB). This is consistent with a model that RpoN activates transcription at *PalgD* (Boucher *et al.*, 2000). In the absence of *algB*, there was decreased *PalgU* expression associated with minimal *PalgD* expression. Previously we have noted in a *kinB* mutant that *PalgU* activity can be dependent on *algB* (Damron *et al.*, 2009b).

PIA-AMV induced mucoidy of PAO1 causes decreased stability of MucA and an increase in MucD expression

Since *algW* was required for alginate induction on PIA-AMV, we hypothesized that MucA was being degraded under this condition. Previously, we have reported degradation of HA-epitope tagged MucA expressed from a multicopy plasmid in *kinB* mutant (Damron *et al.*, 2009b), *mucD* mutant (Damron & Yu, 2011), and *mucE* overexpressing strains (Qiu *et al.*, 2007). To improve the resolution for measuring HA-MucA degradation *in vivo*, we constructed a single copy of HA-*mucA* (Fig. S2) on the chromosome shuttled via miniCTX1 (Hoang *et al.*, 2000). Western blot analysis revealed that when PAO1 was grown on PIA-AMV, there was less HA-MucA detected (Fig. 3A lanes 1 and 2). Since the *PalgU* promoters were used to drive expression of HA-*mucA*, it should be noted that PIA-AMV caused 7-fold increase in *PalgU* activity (Fig. 2A). Therefore, PIA-AMV caused high expression of HA-MucA, but HA-MucA in PAO1 on PIA-AMV was detected in a low quantity (Fig. 3A). Our interpretation of this result is that the growth of PAO1 on PIA-AMV caused increased MucA degradation which leads to AlgU activation and alginate overproduction.

When the chaperone protease *mucD* is inactivated, alginate overproduction occurs (Boucher *et al.*, 1996). Inactivation of *mucD* causes instability of MucA (Damron & Yu, 2011, Wood & Ohman, 2009a). Since MucD appears to control proteins that activate regulated proteolysis of MucA, we used MucD expression as an indicator of protein quality in the periplasm. Western blot analysis with cell lysates of PAO1 on PIA and PIA-AMV revealed that MucD expression increased on PIA-AMV (Fig. 3B). This suggests a possibility that the protein quality of the periplasm was affected by PAO1 growth on PIA-AMV compared to PIA. However, the MucD upregulation on PIA-AMV was not sufficient to suppress mucoidy.

PhoP, but not PmrAB, is required for induced mucoidy in PAO1

Previously Zhou *et al.* (Zhou *et al.*, 1999) showed that one effect of cultivating *E. coli* in the presence of AMV was synthesis of alternative forms of lipid A. The transcription factor PhoP is required for expression of the genes that encode the enzymes which modify lipid A (Ernst *et al.*, 1999). We reasoned that lipid A modifications may induce alginate overproduction on PIA-AMV. PAO1 growing on PIA-AMV required *phoP* for alginate overproduction (Table 1), which suggests a link between modified lipid A and activation of alginate production. In *E. coli*, PagP is the enzyme that adds palmitate to lipid A; however a homologue in *P. aeruginosa* has not yet been identified (Bishop *et al.*, 2005). In *E. coli*, it has been shown that overexpression of the PagP enzyme caused increased σ^E activity (Tam & Missiakas, 2005). Zhou *et al.* (Zhou *et al.*, 1999) showed that the addition of aminoarabinose to lipid A occurs due to AMV in the culture media. In light of this, we thought it logical to test *pmrA* and *pmrB* mutants that control aminoarabinose modification of lipid A in *P. aeruginosa* (McPhee *et al.*, 2003). Neither *pmrA* nor *pmrB* were required for mucoidy on PIA-AMV (Table 1). Together, this suggests the composition of lipid A in the cell may influence the conserved σ^E system in *P. aeruginosa*.

PAO1 lipid A is modified with palmitate when cultivated on PIA-AMV

To determine if vanadate played a role in altering the structure of lipid A, mass spectrometry analysis was performed on lipid A extracted after growth on PIA-AMV (Fig. 4ABC). Consistent with previous reports (Ernst *et al.*, 2006, Ernst *et al.*, 2007), lipid A structures of PAO1 were a blend of penta- and hexa-acylated molecules, identified on spectra by mass to charge ratios (m/z) of 1447 and 1463 (penta) and 1617 and 1633 (hexa) (Fig. 4C). The difference of 16 mass units in Fig. 4 spectra represents the presence of an additional hydroxyl residue in the lipid A structure. Hexa-acylated lipid A (m/z 1617) was the major type of lipid A from PAO1 on PIA though penta-acylated lipid A (m/z 1447) was also observed. Interestingly, when PAO1 was cultured on PIA-AMV, a third species of lipid A was observed that represents a second hexa-acylated lipid A (m/z 1685). This ion species results from the addition of palmitate (C16) to the penta-acylated species (m/z 1447) (Fig. 4B and C). Interestingly, and different to previous results using *E. coli*, the addition of aminoarabinose was not detected (Fig. 4ABC).

Since PhoP was required for mucoidy on PIA-AMV, it was imperative to test an *oprH* mutant because the genes *phoPQ* and *oprH* are genetically linked and are autoregulated via *phoP* (Macfarlane *et al.*, 1999). Here, we observed that *oprH* was not required for mucoidy of PAO1 on PIA-AMV. Recently it has been shown that the absence of *phoQ* upregulates alginate genes (Gooderham *et al.*, 2009). Collectively, these data implicate a role of *phoP* in the induction of the alginate pathway, but *oprH* is not essential for alginate induction (Table 1).

Proteomic comparison of PAO1 grown on PIA vs. PIA-AMV

Growth of PAO1 mutants on PIA-AMV was used to directly test the importance of alginate related genes in the overproduction of alginate (Table 1). However, we expected other effects might have occurred that were overlooked by our direct analysis. To determine effects of PIA-AMV on the proteome of PAO1, a shotgun proteomics approach was utilized. Total protein extracts were submitted to Multidimensional Protein Identification Technology (MudPIT). MudPIT analysis involves two liquid-column chromatographic separations followed by MALDI-TOF MS (Matrix-assisted laser desorption-time of flight mass spectrometry) peptide fingerprinting to identify peptides with a complex sample. PAO1 was cultured on PIA and PIA-AMV. Proteins were extracted by lysis using an acid-labile surfactant and MS was performed. 1845 peptides were identified with a cutoff level of 99 % confidence corresponding to 69 unique proteins in the two samples. Fig. 5 shows the various classes of proteins identified and Table 2 shows selected proteins identified in the two samples. Table S2 provides all of the peptides identified in the two samples. Of interest many proteins associated with metabolism were identified in the PIA-AMV sample (Fig. 5). Several potential explanations exist for this observation. It is likely that since PAO1 is mucoid on PIA-AMV, then increased metabolism is necessary for alginate production. Interestingly, a two-component histidine kinase referred to as AruS (PA4982), which has been shown to be associated with the arginine transaminase pathway (Yang & Lu, 2007), has been identified with a high number of peptides in PAO1 cultured on PIA-AMV (Table 2 and Table S2). Observation of this high number of peptides corresponding to AruS, suggests that AMV does alter histidine kinases. AruS has been previously identified as important in early infection of CF airway in a phage display library (Beckmann *et al.*, 2005). It is possible AruS may be a direct target of AMV, but we cannot exclude the possibility that AruS was upregulated as part of the metabolic scheme for increased alginate production.

Since PAO1 growing on PIA-AMV produces alginate, we would predict that AlgD would be identified in the proteome (Table 2). Furthermore, because PAO1 has high AlgU activity on PIA-AMV (Fig. 2A), we would expect flagella would be down regulated since AlgU represses flagella synthesis (Garrett *et al.*, 1999). FliC or flagelin B was observed with less peptides in PAO1 on PIA-AMV than PIA (Table 2).

There were more proteins corresponding to secreted factors observed after growth on PIA than on PIA-AMV (Fig. 5 and Table 2). Interestingly, PstS which is an extracellular phosphate binding protein that is secreted out of the cell as finger like appendages (Zaborina *et al.*, 2008) was only observed in PAO1 grown on PIA and not on PIA-AMV (Table 2 and Table S2). Inversely, phospholipase C was observed only in the PIA-AMV sample and not in the PIA sample (Table 2). Catalase, which is involved in adaptation and protection, was observed on both PIA and PIA-AMV. However, adaptation and protection proteins such as superoxide dismutase and thiol peroxidase were only detected in PAO1 grown on PIA-AMV. SodB or superoxide dismutase has been previously shown to be responsible for resistance to vanadium (Baysse *et al.*, 2000). Growth on PIA-AMV likely caused increased SodB to deal with the antibacterial properties of vanadium.

It is known in *E. coli* that the DegS protease is activated by misfolded proteins (Ades *et al.*, 1999). Previously when *E. coli* was cultured in AMV, there was no apparent change in the profiles of outer membrane proteins (OMPs) (Tam & Missiakas, 2005). Our MudPIT proteome data also suggested little change in the total amount of OMPs identified since major outer membrane porin OprF has an equal percent of peptides identified on both PIA and PIA-AMV (Table 2). However, we have previously shown that overexpression of OprF alone does not activate alginate production (Qiu *et al.*, 2008a). Also, OprO was identified in the PIA-AMV sample (Table 2). OprO has a C-terminal sequence (YVF) which can activate alginate production (Qiu *et al.*, 2007). It is possible that increased OprO could activate

AlgW resulting in MucA degradation. If PIA-AMV causes general protein misfolding, identification of chaperone proteases would occur in MudPIT MS analysis. A greater amount of the chaperones GroEL, DnaK and GrpE were identified in the PIA-AMV sample than the PIA sample (Table 2). This suggests PIA-AMV may cause protein misfolding which would cause upregulation of chaperones to maintain the cellular integrity. Previously it has been observed that chronically infected CF patients have a high antibody titre against GroEL (Ulanova *et al.*, 1997). Since PIA-AMV seems to upregulate GroEL (Table 2), this further suggests PIA-AMV mimics the CF lung condition. In terms of the other chaperones, DnaK has been shown to have a role in regulated accumulation of heat shock proteins in *Pseudomonas putida* (Kobayashi *et al.*, 2010). It has not been demonstrated if inactivation of any of these chaperones would activate alginate overproduction. MudPIT analysis revealed that PIA-AMV affects the proteome mainly in the classes of metabolism and chaperones.

Discussion

Alginate overproduction by *P. aeruginosa* has been extensively examined because this phenotype marks the onset of chronic infection that will ultimately lead to the decline in health of a CF patient. Further complicating this phenotype is the fact that the CF lung environment selects for mutation(s) in *P. aeruginosa* that alter antibiotic resistance, motility, virulence factors, and many other phenotypes. Little information is known about the early infecting *P. aeruginosa* strains other than they are nonmucoid and similar to environmental strains. Previously, we suggested that the biodiversity of CF isolates may be a cause for the variation of morbidity and mortality in CF (Head & Yu, 2004). Since most agree the CF lung drives evolution of the initial infecting strains, it could be speculated the best chance for therapeutic intervention is before the emergence of resistant mutants. Therefore, the study of environmental strains and their response to CF conditions is necessary.

It has been shown that alginate production genes are induced before any mutations in *mucA* have occurred (Bragonzi *et al.*, 2005). This suggests that alginate production may be protective to the colonizing strains in the CF lung. Furthermore, the alginate biosynthetic enzyme gene *algD* has been shown to be necessary for the development of chronic infections using initially nonmucoid strains (Coleman *et al.*, 2003). Our recent work, as well as the work of others (Cezairliyan & Sauer, 2009, Damron *et al.*, 2009b, Damron & Yu, 2011, Qiu *et al.*, 2008b, Qiu *et al.*, 2007, Wood *et al.*, 2006, Wood & Ohman, 2009a), has detailed that proteolytic cleavage of MucA by AlgW and MucP as a mechanism for alginate production independent of mutation in *mucA*. Interestingly, in a study which utilized signature tagged mutagenesis, *algU* and *algW* were required for chronic infection in a rat model (Potvin *et al.*, 2003). These findings preceded the characterization of AlgW as the regulated protease of MucA and now further support the notion that AlgW and the proteolysis of MucA are important in pathogenesis. Collectively, these studies suggest alginate production may contribute to early infection.

In this study, we have defined a medium (PIA-AMV) which induces mucoidy in PAO1. Cell wall synthesis inhibitors such as triclosan in PIA have been shown to induce expression of alginate genes, but not to a level high enough to cause mucoidy (Wood *et al.*, 2006). Here, we show that PIA-AMV causes alginate overproduction through the AlgW-dependent pathway and utilized most all of the previously described alginate genes as well as other genes not previously associated in alginate overproduction. The PIA-AMV model suggests that modifications to the cellular envelope can activate the AlgW-dependent pathway for alginate production (Fig. 6). However, is PIA-AMV a relevant condition or an artificial lab condition? PIA-AMV medium caused palmitoylation of lipid A (Fig. 4) and hexa-acylated lipid A, which we observed here in this study, has also been previously observed in chronic

P. aeruginosa isolates from the airways of CF patients (Ernst *et al.*, 1999). It has been suggested that palmitoylation of lipid A could create a situation where β -barrel OMPs are not inserted into the outer leaflet (Bishop, 2008, Tam & Missiakas, 2005), resulting in the activation of the σ^E system. It has been proposed that the *E. coli* σ^E system functions as the 'canary in a coal mine' (Alba & Gross, 2004). It is possible that the AlgU system functions the same way downstream as σ^E , but responds to different environmental cues by overproducing alginate to remedy the situation and maintain homeostasis. It is also possible that this response is for protection from stress agents (Fig. 6). Since AlgU is upregulated and proteolysis of MucA is activated, periplasmic housekeeping chaperones should be increased in expression on PIA-AMV. If OMPs are not being properly inserted or folded, then we would expect chaperones would be upregulated to compensate. MudPIT MS analysis of the proteome of PAO1 cultured on PIA-AMV suggests that chaperones are upregulated on PIA-AMV (Table 2). From these data, we may also consider the increased expression of chaperone/protease MucD indicates that the periplasmic protein quality may be affected by growth on PIA-AMV. Our data suggest a model where outer leaflet changes can activate the AlgW-dependent pathway (Fig. 6). As mentioned above, we observed lipid A modification when PAO1 is cultured on PIA-AMV (Fig. 4). In spite of this data, we do not know if palmitoylation of lipid A is directly responsible for the activation of alginate production. Regulation of the lipid A modification transcription factor *phoP* is required for mucoid phenotype of PIA-AMV (Table 1). Interestingly, the same lipid A modifications observed on PIA-AMV also occur in CF isolates (Ernst *et al.*, 1999). Here we also looked at O-antigen expression and did not observe changes in A- or B-band O antigen due to PIA-AMV (Fig. S3)

Our data suggest the PIA-AMV medium induces clinically relevant phenotypes, but is there vanadate in the CF lung environment? More specifically, is there enough vanadate and stress to activate alginate overproduction? Vanadium is commonly found in minerals such as sandstone, coal, shale, crude oil, and tar. It is therefore conceivable that all humans come in contact with this element. Vanadium is the least abundant element in the human body and in a healthy 70 kg person there is about 0.11 mg (Emsley, 1988). However, vanadium accumulates in the human lung with age and can reach a level of $6.5 \mu\text{g g}^{-1}$ in elderly people due to decreased alveolar and mucociliary clearance (Waters, 1977). To our knowledge, it is not known how much vanadium is in the CF lung. However, it is known that the CF lung has decreased mucociliary clearance. Assuming the CF lung has the same amount of vanadium as an elderly person, how does that compare to the amount of vanadium in PIA-AMV? Fortunately, PIA-AMV medium contains $6.1 \mu\text{g g}^{-1}$ vanadium, which is nearly equivalent to the amount found in elderly humans. However, it is not clear if compounds such as triclosan exist in the lung that would inhibit fatty acid synthesis, but there is little to argue that the CF lung condition applies stress to *P. aeruginosa*. Of additional interest, Ascidians, otherwise known as sea squirts, are creatures that accumulate massive amounts of vanadium in vanadocytes (vanadium-containing blood cells). Recently, a chloride channel has been identified that participates in transport of vanadium from seawater into vanadocytes. The chloride channel of vanadocytes is similar to the mammalian CIC3/4/5-type chloride channels (Ueki *et al.*, 2003). This is interesting because the defect in CF is the cystic fibrosis transmembrane conductance regulator which is also a chloride ion channel. Collectively these data from the literature, combined with our data presented here, suggest that PIA-AMV may mimic a clinically relevant phenotype of *P. aeruginosa*.

CF patients are typically colonized with nonmucoid *P. aeruginosa* (Govan & Deretic, 1996) that would theoretically resemble the laboratory strain PAO1, producing minimal amounts of alginate. However, the pathophysiological significance associated with the phenotypic changes from initial colonizing nonmucoid, to hypothetical temporary mucoid, to the constitutively mucoid is not fully understood. Because PIA-AMV induces alginate

production in nonmucoid strains with *algU*⁺ *mucA*⁺, this medium will allow examination of the dynamics and distribution of the infecting population of *P. aeruginosa* in CF. It is not clear at what stage of the infection the *algU* mutant emerges, and whether the emergence and/or dominance of this mutant correlates with an acute exacerbation in CF. Since a wt *algU*, encoding a functional AlgU is required for alginate induction on PIA-AMV (Table 1), it may be possible to quickly screen isolates for mutations in *algU*. This is of importance because mutation of *algU* results in increased virulence (Yu *et al.*, 1996). Surveillance of CF patient sputa for the emergence of *algU* mutants via the cultivation on PIA-AMV may provide vital information about the *P. aeruginosa* infecting population to the clinician. The PIA-AMV medium creates a condition that causes alginate overproduction and lipid A modification that have been observed in CF isolates. We are currently testing *P. aeruginosa* non-redundant mutant libraries on PIA-AMV to determine all of the genes required for alginate production. It will also be interesting to see the effect of PIA-AMV on the transcriptome of *P. aeruginosa*. In conclusion, we suggest PIA-AMV medium can serve as an *in vitro* model to address questions regarding the initial interaction of *P. aeruginosa* and the CF airway.

Experimental procedures

Bacterial strains, media, growth conditions, and oligonucleotides—Bacterial strains and plasmids used in this study are indicated in Table S1. To clone miniCTX1-*PalgU*-*algU*-*HAmucA* as shown in Fig. S2, splicing by overlap PCR (SOE-PCR) was used to generate the DNA fragment that was ligated into miniCTX1 to be shuttled to the PAO1 chromosome. A meridiplod strain was generated by integrating the HA-tagged *mucA* into the *attB* attachment site of the PAO1 chromosome. PCR and sequencing were used to show that the HA-*mucA* construct was properly integrated into the *attB* site (data not shown). Since the HA-*mucA* was expressed from a copy of the *algU* promoters, HA-MucA expression is physiologically similar to wt MucA of that strain. This system allows for better detection of changes in MucA concentration since HA-MucA is not expressed from a multicopy plasmid. *P. aeruginosa* strains were grown at 37° C in lysogeny broth Lennox formulation (10g tryptone, 5 g yeast extract, 5 g NaCl) (LB), on LB agar or *Pseudomonas* isolation agar plates (PIA; Difco) or on PIA-AMV. PIA plates were prepared containing 20 mL of glycerol per liter as recommended by the manufacturer. PIA-AMV plates were prepared as follows. One liter of PIA medium with 20 mL of glycerol was autoclaved and cooled. 20 mL of distilled water was boiled with a stir bar to which 50 mg of ammonium metavanadate (Sigma-Aldrich) was added. After several minutes the AMV dissolved and tinted the liquid yellow. 12.5 mL of the 2.5 mg mL⁻¹ AMV solution was then added to the molten PIA to make PIA-AMV with AMV at a concentration of 0.27 mM. For contrast in digital images shown in Fig. 1A, Congo Red (Sigma-Aldrich) was added at 20 mg L⁻¹. Oligonucleotides used in this study are indicated in Table S1.

Carbozole determination of uronic acid content—*P. aeruginosa* strains were grown at 37°C on PIA or PIA-AMV for 24hr. Bacteria and alginate were removed from plates with PBS and suspended in 100 mL of PBS per plate. The optical density at OD₆₀₀ of bacterial suspension in PBS was measured and adjusted. Cells were then cleared from suspension by centrifugation. The supernatants containing bacterial alginates combined with 3 volumes of cold ethanol (99%), mixed thoroughly to precipitate the alginate fibers that were removed through filtration by 10 µM titanium dutch weave filters (Dorstener Wire Tech). The collected alginate was used for carbozole assay for the amounts of the uronic acid using a standard curve made with D-mannuronic acid lactone (Sigma-Aldrich) in the range of 0-100 µg mL⁻¹ as described (Knutson & Jeanes, 1968).

Analysis of bacterial alginate via nuclear magnetic resonance (NMR)—Cells and alginate were harvested by scraping the colonies off PIA-AMV plates and re-suspended in phosphate buffered saline (PBS). Cell pellets were removed by centrifugation, and the supernatants were combined with 3 volumes of cold ethanol (99%), mixed thoroughly to precipitate the alginate fibers that were removed through filtration by 10 μ M titanium Dutch weave filters (Dorstener Wire Tech). The alginate fibers were then dried under a vacuum. The ^1H -NMR analysis of the seaweed and bacterial alginates were performed according to the protocol F2259-03 of the American Society for Testing and Materials (ASTM) International. Alginic acid, from brown seaweed, (Sigma-Aldrich Catalog A7003) was used as a standard. To determine the composition and sequence of alginates by ^1H -NMR, the alginate samples (~10 mg) were dissolved in 10 mL distilled water with pH adjusted to 5.6 with 1 M HCl and incubated at 100°C for 1 h. Next the pH was reduced to 3.8 followed by incubation at 80°C for 30 min. After the two-step acid hydrolysis, the pH was elevated to 7.5 with 1 M NaOH before the samples were subject to freeze-drying. The dried samples were re-dissolved in 1 mL 99.9 % D_2O , and underwent 3 additional cycles of freeze-drying were performed to remove the excess exchangeable protons. The samples were solubilized in 0.5 mL 99.96% D_2O , transferred to a NMR tube and analyzed by high-field ^1H -NMR spectroscopy at 80°C using a JEOL JNM-ECP600 (600-MHz) spectrometer at 378K. Acetone- d_6 (2.04 ppm) was used as an internal standard in the samples. The composition, expressed as percent of monomers G (F_G) and M (F_M) was determined from the integration of the relevant ^1H -NMR spectra. The area of each peak, which is proportional to the amount of residues giving rise to the signal, was used to calculate the above parameters.

β -galactosidase activity assay—The promoter fusion constructs miniCTX- P_{algU} -*lacZ* and miniCTX- P_{algD} -*lacZ* were integrated onto the *P. aeruginosa* chromosome at the CTX phage *att* site (Hoang *et al.*, 2000). β -galactosidase activity assay was based on the method as originally described by Miller (Miller, 1972) with the following modifications. The cells were grown on PIA and PIA-AMV plates in triplicate for 24hr at 37° C and harvested in PBS. The β -galactosidase activity was assayed after toluene permeabilization of the cells. The reported values represent the average in triplicate of three independent experiments. The values displayed are normalized to PAO1 cultured on PIA medium.

Western blot analysis of proteins—Total cell lysates were prepared with Protea Preps (Protea Biosciences) by manufacturer's protocol. Protein lysates were quantified by D_C assay (BIO-RAD). 50 μ g of protein was boiled in SDS-loading buffer. The samples were electrophoresed on 15% ProteaGel (Protea Biosciences) SDS-PAGE polyacrylamide and then electro blotted (Trans-Blott Cell, BIO-RAD) on to 0.45 μ m nitrocellulose. The membrane was blocked with 3% non-fat dry milk in PBS (pH 7.4). Primary antibodies were diluted 1:5000 in 3% non-fat dry milk in PBS. The membranes were probed with mouse monoclonal antibodies against alpha RNA subunit polymerase subunit (Neoclone) and rat monoclonal antibody against HA (Roche) or anti-MucD (Wood & Ohman, 2006) overnight at 4°C with shaking. HRP-labeled goat anti-mouse IgG or HRP-labeled anti-rabbit IgG were diluted 1:10000 in 3% non-fat dry milk in PBS and used as the secondary antibodies. Advanced ECL or ECL chemiluminescence (Amersham Biosciences) was used for detecting HRP-labeled goat anti-mouse IgG or anti-rabbit IgG (Roche) by the manufacturer's protocol. The signals were detected with an EC3 Imaging System (UVP) by capturing with a BioChemi HR camera (UVP) or by exposure to Amersham Hyperfilm ECL (Amersham Biosciences). For re-probing, membranes were stripped with 62.5 mM Tris-HCl pH 6.8, 2% SDS, 100 mM β -mercaptoethanol for 15 min at 50°C and then washed in PBS.

Isolation of LPS—LPS was isolated using a rapid small-scale isolation method (Yi & Hackett, 2000). In brief, 1.0 mL of Tri-reagent (Molecular Research Center) was added to

cell culture pellets resuspended in 10 mL LB and incubated at room temperature for 15 minutes. 200 μ L of chloroform was added, vortexed, and incubated for an additional 15 minutes. Samples were centrifuged for 10 min at $13,500 \times g$ and the aqueous layer was removed. Five hundred μ L of water was added to the lower organic layer and vortexed. After 15 to 30 minutes, samples were centrifuged again and the aqueous layers were combined. This process was repeated two more times. The combined aqueous layers were frozen and lyophilized overnight.

In order to hydrolyze the lipid A from the LPS, 500 μ L of 1% (wt/vol) SDS in 10 mM Na-acetate at pH 4.5 was added to lyophilized sample. Samples were incubated at 100°C for 1 hour, frozen, and lyophilized. The samples were then washed with 100 μ L water and 1 mL of acidified ethanol (100 μ L 4M HCl in 20 mL 95% ethanol). Samples were centrifuged at $2,350 \times g$ for 5 minutes and ethanol removed. The pellet was washed three times in 1 mL of 95% ethanol and the entire series of washes repeated twice. Samples were resuspended in 500 μ L of water, frozen and lyophilized.

Mass spectrometry procedures for lipid A—Negative-ion matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS) experiments were performed as previously described with the following modifications (Ernst *et al.*, 1999, Guo *et al.*, 1998). Lyophilized lipid A was dissolved in 10 μ L 5-chloro-2-mercaptobenzothiazole (CMBT) MALDI matrix in chloroform/methanol 1:1 (v/v), and then 1 μ L applied onto the sample plate. All MALDI-TOF experiments were performed using a Bruker Autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics Inc.). Each spectrum was an average of 300 shots. ES tuning mix (Agilent) was used to calibrate the MALDI-TOF MS.

Total protein preparation for MS—Total protein preparations of PAO1 cultured on PIA and PIA-AMV were obtained by processing protein lysates with ProteaPrep MS Cell Lysis kit (Protea Biosciences), per manufacturer's instructions. Protea MS Preps use Progenta anionic acid surfactant II to disrupt membranes. The surfactant is then inactivated by acidification with trifluoroacetic acid as instructed by the manufacturer. The lack of SDS or other detergents increases the sensitivity of downstream mass spectrometry analysis. Protein concentrations were determined using Bio-Rad D_C Protein Assay. SDS-PAGE (14% polyacrylamide) with Coomassie staining was performed to determine the complexity of the samples and to insure protease degradation had not occurred to the samples.

Multidimensional Protein Identification Technology (MudPIT) mass spectrometric analysis—To identify the proteins present in *P. aeruginosa* total cell lysates, MudPIT with tandem MALDI TOF-TOF mass spectrometry was employed. Total protein samples (50 μ g) were desalted using C_4 ProteaTip SpinTips (Protea Biosciences) as per manufacturer's protocol. The collected samples were lyophilized and dissolved in 100 μ L of 50mM ammonium bicarbonate in 20% acetonitrile for tryptic digestion. The samples were then reduced and alkylated with 10 μ L of 250mM DTT (60min/ 55°C), and 10 μ L of 625mM iodoacetamide (60min/room temperature/in the dark). Proteolytic digestion was performed in 50mM ammonium bicarbonate buffer using a trypsin to protein ratio of 1:100. The digestion was carried out overnight at 37°C . The digests were cleaned by repeated lyophilizing and reconstituting in a 0.1M acetic acid solution. After final lyophilization, the digests were reconstituted in a strong cation exchange loading buffer (5mM ammonium formate in 20% acetonitrile, pH 3.0) to be fractionated with ProteaTip spin tips as per manufacturer's protocol. The SpinTip was transferred to a new centrifuge tube to collect the sample during elution with 200 μ L of elution solution. Eight different elution solutions were used to fractionate the peptides (20, 60, 100, 150, 200, 250, 400, 500 mM ammonium formate in 20% acetonitrile) in a step-wise manner. The collected fractions were cleaned by repeated

lyophilization and reconstitution in a 0.1M acetic acid solution. After the final lyophilization, the digests were reconstituted in LC run buffer. The fractions were then submitted to LC MALDI spotting and MALDI TOF/TOF spectral analysis by Protea Biosciences as detailed previously (Damron *et al.*, 2009a) to survey the proteome of the *P. aeruginosa* samples. Peptides corresponding to proteins were counted and reported as the relative percent of peptides identified in the sample. The classifications of each proteins indicated were derived from the Pseudomonas Genome Database (Winsor *et al.*, 2009).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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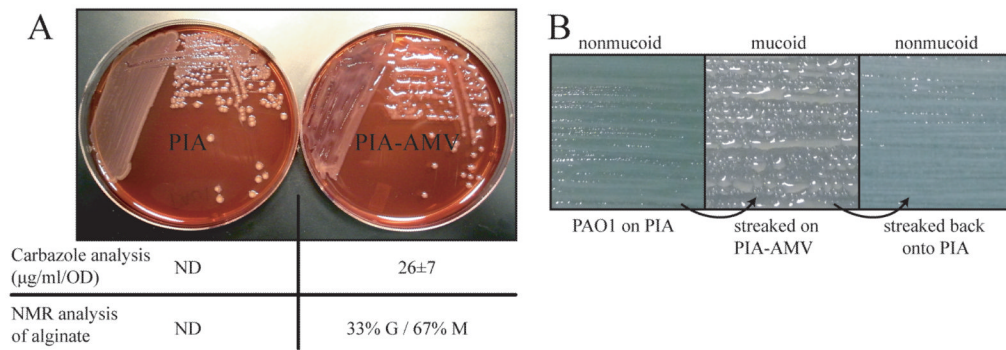


Figure 1. PIA-AMV induces alginate production in strain PAO1

A. Shown is strain PAO1 cultured on PIA and PIA supplemented with 0.27 mM AMV for 24 hr at 37°. Both PIA and PIA-AMV plates in the picture contained Congo Red for imaging contrast. The average uronic acid content and the ratio of guluronate to mannuronate residues of the alginate produced (G/M) are indicated. NMR analysis was used to determine the percentage of G and M residues in the alginate produced by PAO1 on PIA-AMV. ND indicates the alginate concentration was below the detection limit. B. PAO1 was cultured on PIA and then streaked on PIA-AMV where alginate overproduction occurs. When mucoid PAO1 was transferred back to PIA, alginate production ceased and the strain was nonmucoid. These passages were performed daily for two weeks during which PAO1 was mucoid on PIA-AMV and nonmucoid on PIA.

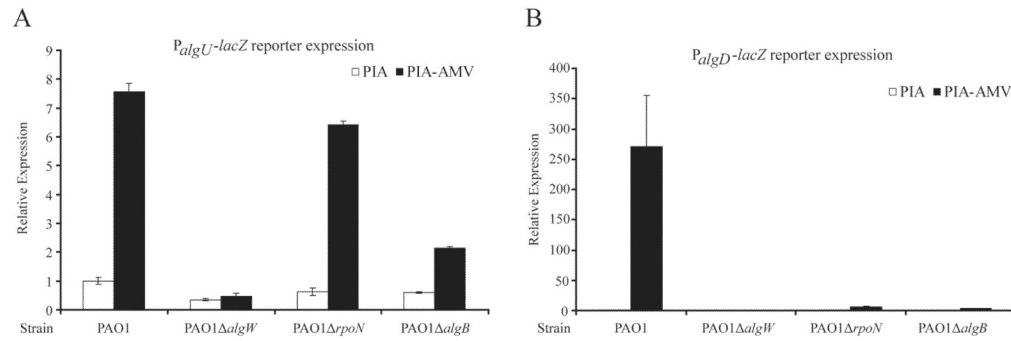


Figure 2. *PalgU* and *PalgD* expression on PIA-AMV

PalgU-lacZ and *PalgD-lacZ* reporter constructs were integrated into the chromosome of the strains as indicated via the miniCTX1 chromosomal shuttle vector (Hoang *et al.*, 2000). β -galactosidase activities were determined after 24 hr growth on PIA or PIA-AMV. Values were normalized to PAO1 on PIA and indicated as mean \pm SD from three independent experiments. A. The *PalgU* activity of *P. aeruginosa* strains on PIA and PIA-AMV. B. The *PalgD* activity of *P. aeruginosa* strains on PIA and PIA-AMV. No *PalgD* activity was detected for strain PAO1 on PIA or strain PAO1 Δ *algW* on PIA or PIA-AMV.

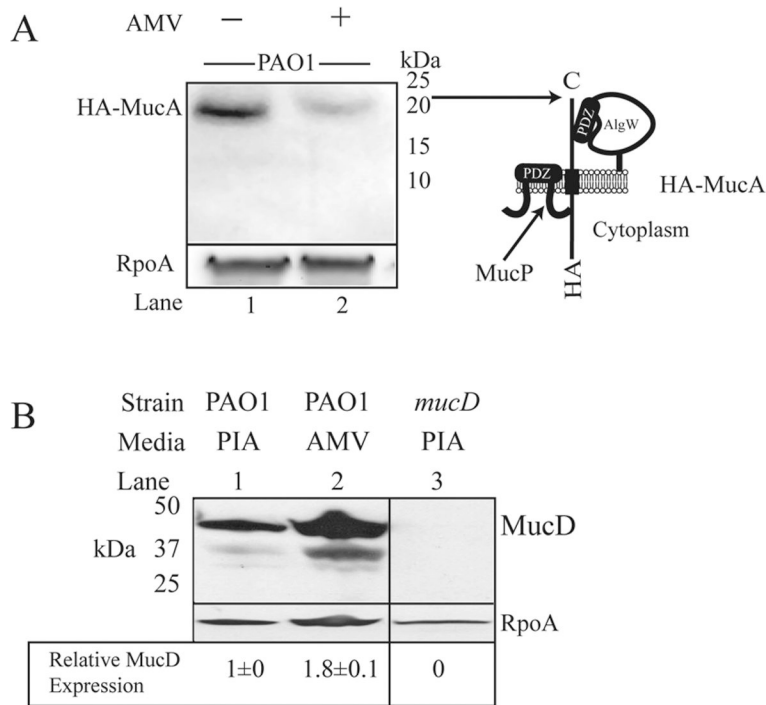


Figure 3. Strain PAO1 shows a decreased level of HA-MucA and increased MucD expression on PIA-AMV

A. Western blot analysis was performed as previously described (Damron *et al.*, 2009b). Cell lysates were prepared from cells after 24 hr growth on PIA and PIA-AMV. The membranes were probed with anti-HA (Roche) and anti-alpha subunit of RNAP antibodies (Neoclone) as a loading control. Shown are representative panels of blots from three independent experiments with 50µg of total lysate. Positions of apparent molecular masses are indicated aligned with a schematic of HA-tagged MucA C. Total protein lysates of PAO1 cultured on PIA and PIA-AMV as well as a *mucD* mutant strain, were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed sequentially with anti-MucD (Wood & Ohman, 2006) and anti-alpha RNA polymerase (Neoclone). MucD signal was normalized to 1 for PAO1 on PIA for comparison to PAO1 on PIA-AMV. Shown is a representative of three independent experiments with standard deviation indicated for biological replicates.

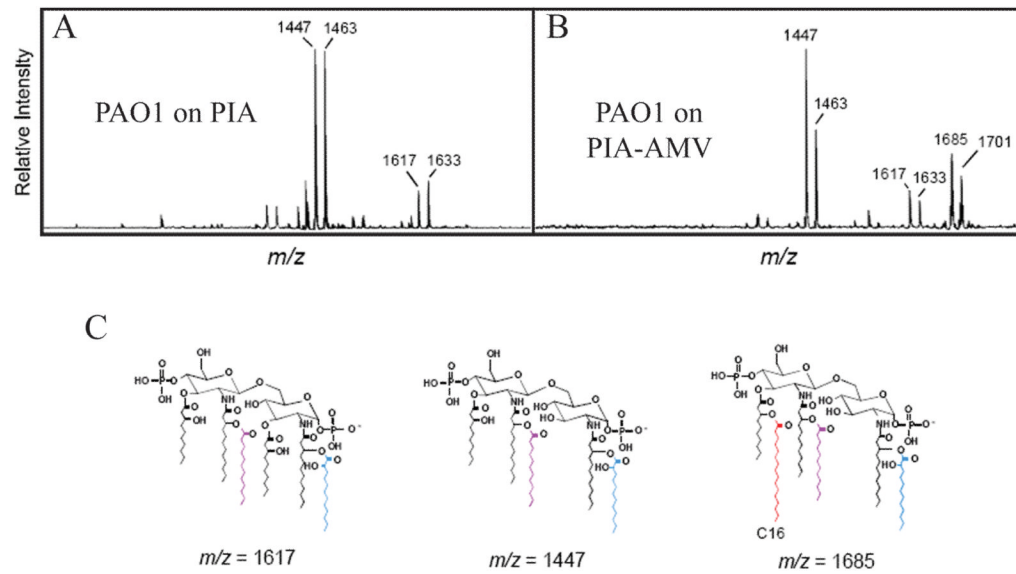


Figure 4. PIA-AMV regulation of *P. aeruginosa* lipid A modification

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS) in the negative-reflection ion mode of lipid A isolated from *P. aeruginosa* PAO1 grown in the absence or presence of vanadate. (A) PAO1 grown on PIA. (B) PAO1 grown on PIA-AMV. (C) Indicates the three structures of the lipid A molecules identified.

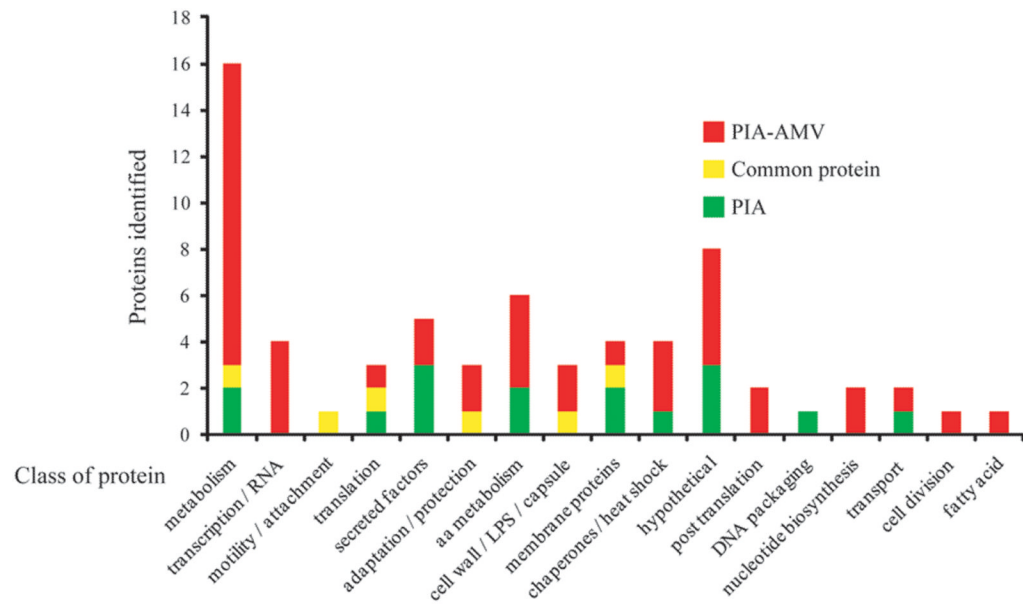


Figure 5. Distribution of proteins as determined by MudPIT mass spectrometry of PAO1 on PIA versus PIA-AMV

Proteins were grouped into classes (Winsor *et al.*, 2009) based on the proteins identified by MudPIT MS analysis. Proteins found on PIA and PIA-AMV are indicated in green and red, respectively. Proteins that were common for both samples are indicated in yellow.

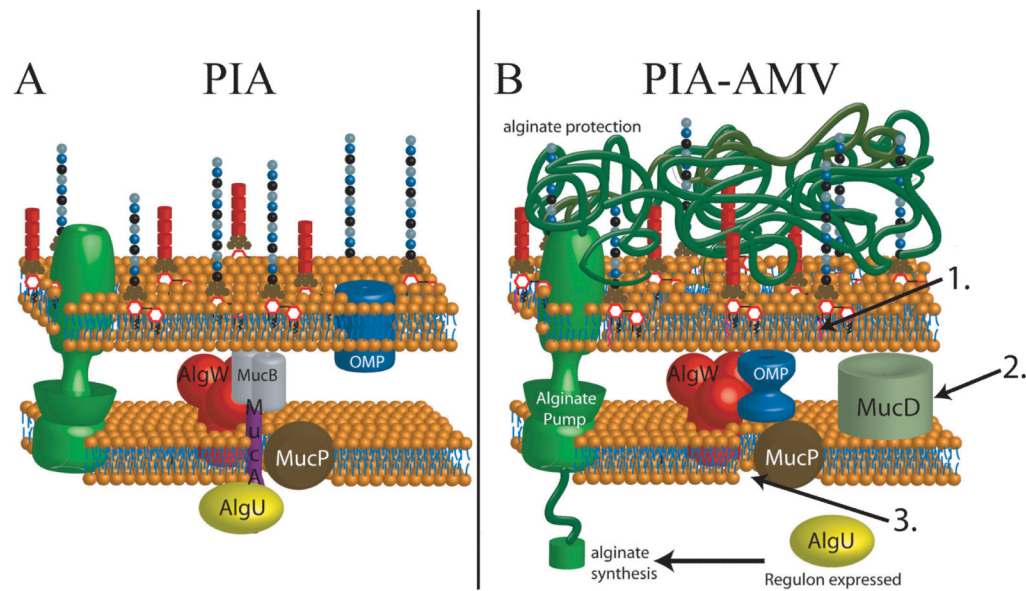


Figure 6. Model for PIA-AMV induction of alginate production in *P. aeruginosa* with wt MucA
 A. On PIA, PAO1 is nonmucooid due to AlgU being sequestered by anti-sigma factor MucA.
 B. PIA-AMV employs ammonium metavanadate, which induces synthesis of pamitoylated lipid A (1.). The combination of AMV and triclosan (a cell wall stress agent) induces alginate overproduction. It may be possible pamitoylated lipid A is a side effect of AMV induction. However, without *phoP*, which controls these modifications, alginate production does not occur (Table 1). This condition induces increased expression of chaperone protease MucD (2.) and AlgW-regulated proteolysis of MucA (3.). Based on the data presented here, we propose that it is likely that MucD is upregulated along with other chaperones because OMPs are misfolded due to the modified lipid A and the cell wall stress caused by triclosan. Regulated proteolysis of MucA leads to activation of AlgU (3.) and alginate production for protection due to the perturbations in the envelope.

Table 1

Alginate induction in *P. aeruginosa* strains on PIA-AMV

Strain or mutants ^a	Genotype of <i>algU mucA</i> , inactivated genes or description of strain	Phenotype ^b	
		PIA	PIA-AMV
<u>Laboratory strains</u>			
PAO1	<i>algU</i> ⁺ <i>mucA</i> ⁺	NM	M
PA14	<i>algU</i> ⁺ <i>mucA</i> ⁺	NM	M
PAK	<i>algU</i> ⁺ <i>mucA</i> ⁺	NM	M
PA103	<i>algU</i> ⁺ <i>mucA</i> ⁺	NM	NM
<u>CF clinical strains</u>			
C0132	Early colonizing CF isolate, <i>algU</i> ⁺ <i>mucA</i> ⁺	NM	M
C3715	Early colonizing CF isolate, <i>algU</i> ⁺ <i>mucA</i> ⁺	NM	M
C7406	Early colonizing CF isolate, <i>algU</i> ⁺ <i>mucA</i> ⁺	NM	M
FRD1	Mucoid CF isolate, <i>algT(U)</i> ⁺ <i>mucA22</i>	M	M
FRD2	Spontaneous nonmucoid CF isolate, <i>mucA22</i> with <i>algT(U)</i> suppressor mutation	NM	NM
CF149	Nonmucoid CF isolate, <i>mucA</i> mutant with <i>algU</i> suppressor mutation	NM	NM
<u>Sigma factors</u>			
PAO1Δ <i>algU</i>	In-frame deletion of alginate master regulator/sigma factor AlgU (σ^{22})	NM	NM
PAO1 <i>rpoH</i>	Knockout of the heat shock sigma factor RpoH (σ^{32})	NM	M
PAO1Δ <i>rpoN</i>	In-frame deletion of the alternative sigma factor RpoN (σ^{54})	NM	NM
PAO1 <i>rpoS::tet</i>	Knockout of the alternative sigma factor RpoS (σ^{38})	NM	M
<u>Alginate regulators</u>			
PAO581	<i>mucA25</i> encoding the N-terminal portion of anti-sigma factor MucA	M	M
PAO1 <i>mucB</i>	Knockout of <i>mucB</i> encoding a protein that the C-terminus of MucA	M	M
PAO1 <i>mucC</i>	Knockout of <i>mucC</i> encoding a protein with unknown function	NM	M
PAO1 <i>mucD</i>	Knockout of <i>mucD</i> encoding a periplasmic chaperone / protease	M	M
PAO1 <i>kinB</i>	Knockout of <i>kinB</i> encoding a two-component histidine kinase	M	M
<u>Alginate biosynthesis</u>			
PAO1 <i>algD</i>	Knockout of <i>algD</i> encoding initial enzyme in alginate biosynthesis	NM	NM
PAO1 <i>algE</i>	Knockout of <i>algE</i> encoding alginate biosynthesis porin pump	NM	NM

Strain or mutants ^a	Genotype of <i>algU mucA</i> , inactivated genes or description of strain	Phenotype ^b	
		PIA	PIA-AMV
<i>Transcription factor</i>			
PAO1Δ <i>algR</i>	In-frame deletion of transcriptional activator AlgR of <i>P_{algD}</i>	NM	NM
PAO1 <i>algZ</i>	Knockout of a two-component histidine kinase	NM	M
PAO1Δ <i>algB</i>	In-frame deletion of transcriptional activator AlgB of <i>P_{algD}</i>	NM	NM
<i>Alginate proteases</i>			
PAO1Δ <i>algW</i>	In-frame deletion of <i>algW</i> encoding an envelope protease for MucA	NM	NM
PAO1 <i>mucP</i>	Knockout of <i>mucP</i> encoding an envelope protease for MucA	NM	NM
PAO1 <i>prc</i>	Knockout of <i>mucP</i> encoding a tail-specific protease for MucA	NM	M
<i>Lipid A enzyme</i>			
PAO1Δ <i>phoP</i>	In-frame deletion of <i>phoP</i> encoding a regulator of palmitoylation of lipid A	NM	NM
PAO1 <i>pmrA</i>	Knockout of <i>pmrA</i> encoding a regulator of aminoarabinose of lipid A	NM	M
PAO1 <i>pmrB</i>	Knockout of <i>pmrB</i> encoding a histidine kinase/regulator of aminoarabinose of lipid A	NM	M
<i>Envelope proteins</i>			
PAO1 <i>oprH</i>	Knockout of <i>oprH</i> encoding PhoP-regulated OMP	NM	M
PAO1 <i>mucE</i>	Knockout of <i>mucE</i> encoding AlgW activating envelope protein	NM	M

^aThe origin of the strains and references are indicated in Supplemental Table 1

^bThe phenotype is indicated as NM or M for nonmucoid or mucoid respectively. Mucoid phenotype was assigned to strains that were similar to PAO1 cultured on PIA-AMV. This phenotype was further characterized by the standard observation of exopolysaccharide strings upon touch of a colony with wooden toothpick.

^cND stands for not determined.

Table 2
Selected proteins identified by MudPIT MS from strain PAO1 cultured on PIA and PIA-AMV

PA loci	Class	Protein	Product Name	PAO1 on PIA		PAO1 on PIA-AMV	
				Peptides	relative % of peptides	Peptides	relative % of peptides
PA3540	cell wall / LPS / capsule	AlgD	GDP-mannose 6-dehydrogenase			24	2.0
PA4982	Metabolism	AruS	two-component sensor kinase			80	6.5
PA2976	transcription / RNA	Rne	ribonuclease E			72	5.9
PA1092	motility / attachment	FlhC	flagellin type B	133	21.5	62	5.1
PA5369	secreted factors	PstS	periplasmic phosphate-binding protein	72	11.6		
PA3319	secreted factors	PlcN	non-hemolytic phospholipase C			51	4.2
PA4236	adaptation / protection	KatA	Catalase	50	8.1	36	2.9
PA4366	adaptation / protection	SodB	superoxide dismutase			11	0.9
PA2532	adaptation / protection	Tpx	thiol peroxidase			10	0.8
PA1777	membrane proteins	OprF	structural outer membrane porin	16	2.6	35	2.9
PA0976	membrane proteins	OprL	peptidoglycan associated lipoprotein	13	2.1		
PA1053	membrane proteins		conserved hypothetical protein	4	0.6		
PA3280	membrane proteins	OptO	pyrophosphate-specific outer membrane porin			6	0.5
PA4385	chaperones / heat shock	GroEL	GroEL protein			29	2.4
PA4761	chaperones / heat shock	DnaK	DnaK protein			19	1.5
PA4762	chaperones / heat shock	GrpE	heat shock protein			17	1.4
PA1793	chaperones / heat shock	PpiB	peptidyl-prolyl cis-trans isomerase B	4	0.6		