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ClpXP proteases positively regulate alginate overexpression and mucoid conversion in *Pseudomonas aeruginosa*

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Abstract

Overproduction of the exopolysaccharide alginate and conversion to a mucoid phenotype in *Pseudomonas aeruginosa* are markers for the onset of chronic lung infection in cystic fibrosis (CF). Alginate production is regulated by the extracytoplasmic function (ECF) σ factor AlgU/T and the cognate anti-σ factor MucA. Many clinical mucoid isolates carry loss-of-function mutations in *mucA*. These mutations, including the most common *mucA22* allele, cause C-terminal truncations in MucA, indicating that an inability to regulate AlgU activity by MucA is associated with conversion to the mucoid phenotype. Here we report that a mutation in a stable mucoid strain derived from the parental strain PAO1, designated PAO581, that does not contain the *mucA22* allele, was due to a single-base deletion in *mucA* (ΔT180), generating another type of C-terminal truncation. A global mariner transposon screen in PAO581 for non-mucoid isolates led to the identification of three regulators of alginate production, *clpP* (PA1801), *clpX* (PA1802), and a *clpP* paralogue (PA3326, designated *clpP2*). The PAO581 null mutants of *clpP*, *clpX* and *clpP2* showed decreased AlgU transcriptional activity and an accumulation of haemagglutinin (HA)-tagged N-terminal MucA protein with an apparent molecular mass of 15 kDa. The *clpP* and *clpX* mutants of a CF mucoid isolate revert to the non-mucoid phenotype. The ClpXP and ClpP2 proteins appear to be part of a proteolytic network that degrades the cytoplasmic portion of truncated MucA proteins to release the sequestered AlgU, which drives alginate biosynthesis.

INTRODUCTION

Acquisition of chronic pulmonary infection with *Pseudomonas aeruginosa* is a major cause of morbidity and mortality in patients with cystic fibrosis (CF). The emergence of clinical

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The GenBank/EMBL/DDBJ accession number for the *algUmucABCD* sequence of *Pseudomonas aeruginosa* PAO581 is EF635219.

A supplementary figure showing genomic profiles of PAO1 and the isogenic strain PAO581 (PAO1 *mucA25*), and a supplementary table listing mariner transposon insertions leading to the non-mucoid phenotype in the stable mucoid strain, are available with the online version of this paper.

mucoid isolates of *P. aeruginosa* in a CF patient, due to overproduction of a capsular polysaccharide, alginate, is an indicator of decreased life expectancy (Govan & Deretic, 1996; Henry *et al.*, 1992). The accumulation of mucus plugs in the airways, composed in part of bacterial alginate, reduces airway conductance, accelerating the rate of decline in pulmonary function. Copious amounts of alginate also enable the pathogen to grow as compact microcolonies (Worlitzsch *et al.*, 2002), referred to as biofilms by some investigators, which increases the organism's resistance to host phagocytosis and antibiotics. Extensive research efforts over the past three decades have led to the basic understanding of the biosynthesis and regulation of alginate production in *P. aeruginosa*. Increased transcription of genes that encode alginate biosynthetic enzymes involves multiple regulatory and signalling molecules (Govan & Deretic, 1996; Lyczak *et al.*, 2002; Ramsey & Wozniak, 2005). The key to mucoid conversion is the expression of the *algD* operon, whose transcription is increased when the extracytoplasmic function (ECF) σ factor AlgU (also called AlgT) binds to the promoter of this operon. Additional regulation is engendered by the two-component signal transduction systems encompassing both FimS–AlgR and KinB–AlgB, and the transcriptional factor AlgZ (Baynham & Wozniak, 1996), which has recently been renamed AmrZ (Baynham *et al.*, 2006).

The activity of AlgU is regulated by the *algUmucABCD* cluster, which encodes a cognate anti-σ factor, MucA, as well as the negative regulators MucB and MucD. Disruption of the production of these three proteins leads to mucoid conversion. However, disruption of *mucC* does not result in mucoid conversion. Coincidently there is no *mucC* orthologue in the genome of a related alginate-producing organism, *Pseudomonas syringae* (Keith & Bender, 2001). The regulation of AlgU activity follows a paradigm similar to that of *Escherichia coli* σ^E (Qiu *et al.*, 2007). According to the model, the N terminus of RseA (the MucA orthologue) is bound by ClpP (Flynn *et al.*, 2003), and is degraded by the ClpXP protease complex (Chaba *et al.*, 2007). The degradation of RseA is initiated by the inner membrane-associated proteases DegS and YaeL (RseP) (Alba *et al.*, 2002; Alba & Gross, 2004; Grigorova *et al.*, 2004; Kanehara *et al.*, 2003; Walsh *et al.*, 2003), followed by ClpXP-mediated degradation of the cytoplasmic N terminus of RseA to release the sequestered σ^E into the cytoplasm (Chaba *et al.*, 2007). This model also suggests that MucB binds to the periplasmic domain of MucA stabilizing this anti-σ factor. MucD is an orthologue of *E. coli* DegP, an HtrA family serine protease involved in the degradation of misfolded proteins, and is crucial for protein quality control in the periplasm. MucD negatively regulates mucoidy (Boucher *et al.*, 1996; Yorgey *et al.*, 2001). We have previously provided evidence that MucD suppresses alginate overproduction by targeting misfolded and unfolded polypeptides that otherwise lead to activation of AlgW, which subsequently initiates the proteolysis of MucA and release of active AlgU (Qiu *et al.*, 2007). Of note, AlgW production was initially thought to suppress alginate overproduction (Boucher *et al.*, 1996). However, the homologue of AlgW in *E. coli*, DegS, is a protease that acts on the periplasmic domain of a MucA homologue in *E. coli*, RseA, to activate σ^E (Alba & Gross, 2004). Using mariner-based transposition mutagenesis in two non-mucoid strains of *P. aeruginosa*, PAO1 and PA14, we have previously identified a small envelope protein, MucE, whose production activates AlgW, leading to increased alginate production (Qiu *et al.*, 2007). Furthermore, through screening of inducers of alginate overproduction, cycloserine has been identified as an

inducer of AlgW, leading to alginate overproduction (Wood *et al.*, 2006). The regulatory protease AlgW most likely degrades the portion of MucA found in the periplasm; however, MucA has an extensive cytoplasmic domain which is likely where the protein binds and sequesters the AlgU transcriptional activator. To date, no cytoplasmic proteases have been identified that affect the steady-state levels of the cytoplasmic domain of MucA.

Among different mucoid *P. aeruginosa* isolates from CF patients as well as in mucoid laboratory isolates, missense and nonsense mutations have been identified throughout the *mucA* gene (Anthony *et al.*, 2002; Boucher *et al.*, 1997). For instance, both CF patient isolate FRD1 and the laboratory-induced mucoid variant of PAO1, PAO578, harbour the same mutation in *mucA* (designated the *mucA22* allele), and these strains have been utilized extensively to study the regulation of alginate synthesis (Govan & Deretic, 1996; Ramsey & Wozniak, 2005). The *mucA* mutant alleles have been identified in about 44 % of *P. aeruginosa* isolates from an Australian adult CF centre (Anthony *et al.*, 2002), but in as many as 84% of mucoid *P. aeruginosa* isolates from other populations of CF patients (Boucher *et al.*, 1997). Additional sequencing studies of the *mucA* gene from CF sputum isolates have identified some early colonizing strains that are free of the *mucA* mutations (Qiu *et al.*, 2007). Thus, while *mucA* allelic variants such as *mucA22* can commonly lead to the mucoid phenotype, other variants or controlling factors are also involved in producing this phenotype.

Prior work has identified two mucoid derivatives of strain PAO1, PAO579 and PAO581, harbouring uncharacterized genetic changes, designated *muc-23* (PAO579) and *muc-25* (PAO581) variants (Fyfe & Govan, 1983). We chose the latter strain to search for additional components that regulate alginate production in *P. aeruginosa*, and to identify the genetic change of the *mucA25* variant in PAO581. We were able to determine via physical mapping of the chromosome of PAO581 that no large-scale DNA rearrangements or deletions had occurred in comparison with the sequenced parental strain PAO1 (Stover *et al.*, 2000). The wild-type MucA protein is a trans-inner-membrane protein with 194 aa (Mathee *et al.*, 1997). According to the NCBI Conserved Domain Database (www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml), MucA, like *E. coli* RseA, has two functional domains, one located in the cytoplasm ($MucA_{7–57}$, equivalent to the N-terminal region of RseA) and the other in the periplasm ($MucA_{113–170}$, equivalent to the C-terminal region of RseA). In addition, the $MucA_{84–104}$ region has been shown elsewhere to contain a transmembrane domain (Mathee *et al.*, 1997; Rowen & Deretic, 2000). In this study, we found that the mucoid phenotype of PAO581 was due to a frameshift mutation resulting in the production of a truncated MucA protein, MucA25, which only retained the predicted cytoplasmic domain. Additional analysis of non-mucoid variants generated in PAO581 using a mariner-based saturation transposon mutagenesis approach identified proteases encoded within the *clpP–clpX* cluster (PA1801 and PA1802) and the *clpP2* (PA3326) gene as new regulators of alginate biosynthesis. We demonstrate, for the MucA25 variant, that stabilization of the mucoid phenotype is due to the susceptibility of these truncated forms of MucA to proteolysis by the ClpXP and ClpP2 proteases, leading to the degradation of N-terminal MucA and release of the sequestered AlgU to activate alginate biosynthesis.

METHODS

Strains and plasmids

E. coli DH5α was used as the host for molecular cloning. *P. aeruginosa* strains PAO1 and PAO581 were grown at 37 °C (unless specified) in Luria broth (LB), or on Luria (L) agar or *Pseudomonas* Isolation Agar (PIA; Difco) plates, supplemented with gentamicin or carbenicillin (300 μg ml^{-1}) when required. The strains of *P. aeruginosa* used for conjugative transfer of plasmids were grown at 42 °C in LB. The *E. coli* strains were grown in LB, or on L agar supplemented with carbenicillin (100 μg ml^{-1}), gentamicin (13 μg ml^{-1}) or kanamycin (40 μg ml^{-1}), when required. Other strains as well as plasmids used are listed in Table 1.

Transformation and conjugation

Either electroporation or a chemical transformation method was used for the transformation of *E. coli* (Qiu *et al.*, 2007). Triparental conjugations, using pRK2013 as the helper plasmid (Figurski & Helinski, 1979), were used for genetic manipulation of *P. aeruginosa* PAO581 and PAO1 strains.

Transposon mutagenesis and complementation analysis

Biparental conjugations were carried out for transposon mutagenesis, using *E. coli* SM10 λpir carrying plasmid pFAC as the donor strain (Wong & Mekalanos, 2000) and PAO581 as the recipient strain. After 4–6 h of mating of *P. aeruginosa* and *E. coli* cells on L agar plates at 37 °C, bacteria were streaked onto PIA plates supplemented with 300 μg gentamicin ml^{-1}. Non-mucoid colonies were identified and subjected to further genetic analyses. The chromosomal DNA of non-mucoid mutants was isolated using the QIAamp genomic DNA kit (Qiagen). About 2 μg DNA was digested with *Sal*I overnight at 37 °C followed by purification and self-ligation using Fast-Link DNA ligase (Epicentre). The circular closed DNA was used for inverse PCR using GM3OUT and GM5OUT primers (Table 1), which were designed based on the sequence of the Gm^r gene, and the PCR products were purified and sequenced.

PFGE and Southern hybridization

PFGE coupled with Southern hybridization analyses were carried out as described previously (Head & Yu, 2004) for comparison of the genome organization between *P. aeruginosa* strains PAO581 and PAO1. Southern blot hybridization was also used to monitor the copy number of transposon insertions using the Gm^r gene as the probe template.

Western blot analyses

Bacterial cells were harvested from LB (supplemented with 150 μg carbenicillin ml^{-1}). OD_{600} was measured and equal amounts of bacterial cells for each sample to be used were pelleted. The bacterial cells were washed with cold PBS buffer (pH 7.4) once and then pelleted for protein extraction. The total cellular protein extracts were prepared using the ReadyPreps kit (Epicentre). Protein samples were heated at 95 °C for 5 min, electrophoresed on an SDS-PAGE gel (15% acrylamide), and transferred onto a nitrocellulose membrane

(pore size 0.1 μm, Schleicher & Schuell). Immunoblots were developed by using rat anti-haemagglutinin (HA) monoclonal antibody (Roche) or rabbit anti-RNAP (RNA polymerase α-subunit) polyclonal antibody (courtesy of M. J. Chamberlin, University of California, Berkeley) as the primary antibody, and horseradish peroxidase-labelled goat anti-rat (Roche) or anti-rabbit IgG (Kirkegaard & Perry laboratories) as the secondary antibody. Enhanced chemiluminescence (ECL; Amersham Biosciences) was used for detection.

β-Galactosidase activity assay

The *P. aeruginosa* strains carrying the $P1_{algU}$ promoter–*lacZ* fusion were grown on three different PIA plates at 37 °C, and cells were harvested and resuspended in cold PBS buffer. OD_{600} was recorded, and β-galactosidase activity was measured after SDS/chloroform permeabilization for each sample and the Miller units were recorded. One Miller unit is equivalent to $1000 \times (A_{420}/–1.75 \times A_{550}/OD_{600}\ ml^{-1}\ min^{-1})$. The reported values represent the average in triplicate of at least three experiments.

Alginate assay

The alginate assay was based on a method published previously (Knutson & Jeanes, 1968) with the following modifications. *P. aeruginosa* and mutants were grown at 37 °C on 50 ml PIA plates in triplicate for 24 h. Bacterial growth was removed from plates and suspended in 40 ml PBS. The OD_{600} was recorded. The alginate standard curve was made using D-mannuronic acid lactone (Sigma) in the range 0–100 μg ml^{-1}.

Statistical analysis

Analysis of β-galactosidase was done with one-way ANOVA followed by a post-hoc analysis of all relevant pair-wise comparisons. Analysis of alginate assays was done using the *t* test. All analyses were performed using SigmaStat software (v. 3.5, Systat Software).

RESULTS

Identification of the *muc-25* mutation as a single-base deletion, ΔT180, in *mucA*

The stable mucoid variant, PAO581 was isolated *in vitro* following the incubation of the non-mucoid *P. aeruginosa* PAO with phage E79 (Fyfe & Govan, 1980). Since this strain carried undefined *muc* mutation(s) (designated the *muc-25* variant; Fyfe & Govan, 1983), we first tested whether there were large genomic alterations in this strain. Using macrorestriction digestions of the genome coupled with separation by PFGE, no large-scale recombination, inversion, deletion, or amplification was noted in PAO581 as compared with PAO1 (see Supplementary Fig. S1). This is consistent with earlier data (Fyfe & Govan, 1983) which indicate that the mucoid phenotypes associated with *muc* mutation(s) designated *muc-22*, *muc-23* and *muc-25* are not associated with major changes in DNA organization.

Previously, the *muc-25* locus of PAO581 had been roughly mapped to the region between *pruAB* (67.5 min) and *hisI* (69 min), very close to the *algUmucABCD* cluster (68 min) (Fyfe & Govan, 1983). Therefore, we sequenced the *algUmucABCD* genes of PAO581. A single base deletion at T180 of *mucA* was identified, leading to creation of a premature stop codon

(TGA) at position 285. The resulting frameshift encoded a truncated polypeptide of 94 aa containing the N-terminal 59 aa of MucA. This protein was probably located in the cytoplasm due to the absence of the transmembrane ($MucA_{84–104}$) and periplasmic ($MucA_{113–170}$) domains. No mutation was found in the *algU*, *mucB*, *mucC* or *mucD* genes of PAO581. To determine whether this mutation could be identified in naturally occurring mucoid *P. aeruginosa* isolates, we sequenced the *algU–mucA* genes of a series of variant mucoid isolates obtained from the lungs of transgenic CF mice that emerged after 6–12 months of chronic lung infection (Coleman *et al.*, 2003). Interestingly, the same *mucA* mutation as that of PAO581 was identified in one of the mucoid isolates (Strain 1003, Table 1). We next replaced the wild-type *mucA* gene in PAO1 with the *mucA*ΔT180 allele from PAO581, and the resultant strain PAO1DR1 became mucoid, as expected (data not shown). The mucoid phenotype of both PAO581 and PAO1DR1 could be suppressed by the introduction of wild-type *mucA in trans* on a plasmid (pUCP20T-*mucA*) (*lac* promoter, data not shown). Thus, the previously uncharacterized *muc-25* mutation was due to deletion of a single base at T180 in the *mucA* gene, and has been designated the *mucA25* allele.

Transposon insertions in *tig*, *clpP*, *clpX* and *clpP2* render the *muc-25* mucoid PAO581 non-mucoid

To determine whether other factors might be important for the expression of the mucoid phenotype in PAO581, we used mariner transposon mutagenesis, which gives rise to high-density insertion of a TA-flanked gentamicin-resistance marker into the chromosome of target bacteria, including *P. aeruginosa* (Qiu *et al.*, 2007; Rubin *et al.*, 1999; Wong & Mekalanos, 2000). In total, 86 non-mucoid mutants were isolated out of about 100 000 Gm^R mutants of PAO581 screened, and 60 clones were found with single insertions (see Supplementary Table S1). Insertion sites were identified by inverse PCR and sequencing. The majority of the non-mucoid mutants harboured a single insertion, as confirmed by Southern hybridization using the Gm^r cassette as a probe (data not shown). As expected, most insertions were mapped to the known positive regulatory genes, including *algU*, *algR* (Deretic *et al.*, 1989), *algB* (Goldberg & Dahnke, 1992) and *amrZ* (*algZ*) (Baynham & Wozniak, 1996; Tart *et al.*, 2006). These results, while confirming the previous findings, also validated the reliability and efficiency of the mariner-based transposon mutagenesis. There were about two to three independent transposon insertions in the first seven genes of the 18 kb alginate biosynthetic operon, *algD* (PA3540), *alg8* (PA3541), *alg44* (PA3542), *algK* (PA3543), *algE* (PA3544), *algG* (PA3545) and *algX* (PA3546), and in the unlinked *algC* (PA5322) gene. However, no insertions were found in the other five genes, *algL* (PA3547), *algI* (PA3548), *algJ* (PA3549), *algF* (PA3450) and *algA* (PA3551). These results were consistent with previous findings, since transposon inactivation of *algL* is only possible if the transcription of the entire *algD* operon is suppressed (Jain & Ohman, 2005), and inactivation of *algI*, *algJ* and *algF* would not lead to a complete loss of mucoidy, as these genes encode proteins involved in alginate acetylation, but not biosynthesis (Franklin *et al.*, 2004).

Several other metabolic genes were also disrupted by transposon insertions, including *carA* (PA4758, encoding the small subunit of carbamoylphosphate synthase), *carB* (PA4756, encoding the large subunit of carbamoylphosphate synthetase), *purF* (PA5426, encoding the

glutamine phosphoribosylpyrophosphate amidotransferase), *purM* (PA0945, encoding the phosphoribosylformyglycinamidine cycloligase), and *pyrD* (PA3050, encoding dihydroorotate dehydrogenase). However, the role of these metabolic genes in the mucoid phenotype was not investigated further.

Of greater interest were the transposon insertions in the non-mucoid mutants mapped to the *tig-clpP-clpX* (PA1800–1802) polycistronic loci and a monocistronic locus, PA3326, a gene paralogous to *clpP* (designated *clpP2*) (Fig. 1, Table 1). The *clpP2* gene encodes a polypeptide of 201 aa, homologous to ClpP (PA1801, 213 aa). Eleven out of the 51 transposon insertions into coding sequences (21.6%) were mapped to the *tig* (two insertions), *clpP* (three insertions), *clpX* (four insertions) and *clpP2* (two insertions) loci. Southern blot analyses confirmed that only a single copy of the transposon was inserted into the chromosome in these null mutants (data not shown). The relevance of these genes to mucoid conversion was confirmed by complementation tests. The mucoid phenotype and alginate synthesis could be restored to the *tig* (PAO581DR23), *clpP* (PAO581DR58) and *clpX* (PAO581DR45) null mutants by the pUCP20T-borne *tig-clpP-clpX* (pUCP20T-*tig-clpXP*) genes *in trans* (Table 2, Fig. 2). In addition, introduction of pUCP20-*clpP2* could restore mucoidy to the *clpP2* null mutants of PAO581DR3 (Table 2) and PAO581DR9 (not shown), suggesting that the ClpP2 protease is also required for the mucoid phenotype. However, the non-mucoid phenotype of the *tig* null mutants, PAO581DR23 and PAO581DR6, seemed to result from polar effects of the transposon insertion on the downstream *clpP* and *clpX*, since pUCP20T-*tig* did not restore alginate synthesis (Table 2, Fig. 2, PAO581DR23). Both pUCP20T-*tig-clpXP* and pUCP20T-*clpXP* induced a high level of alginate production in the *tig* mutant PAO581DR23 (Table 2). To confirm the function of ClpXP and to verify that the transposon insertion did not cause other unknown mutations, we cloned the Gm^R interrupted allele of PAO581DR58 (*clpP*::Gm^R) into the suicide vector pEX100T and used it to disrupt the *clpP* gene of PAO581. The resultant *clpP* mutant of PAO581 was non-mucoid, and the mucoid phenotype could be complemented by plasmid-borne *clpP in trans*.

Since the *tig-clpP-clpX* gene cluster has been identified to be involved in the regulation of alginate synthesis, the *tig-clpP-clpX* cluster of PAO581 was subjected to sequence analyses. Compared with the *tig-clpP-clpX* cluster of the parental nonmucoid strain PAO1, some missense mutations were found, suggesting that these polymorphisms could be required for the mucoid phenotype of PAO581. Therefore, the *tig-clpXP* and *clpXP* genes of PAO581 were cloned into pUCP20T to test for mucoid induction in PAO1. However, overexpression of these PAO581-derived genes, *tig-clpXP* (PAO581), *clpXP* (PAO581) or the *clpP2* gene from PAO581 (pUCP20T-*clpP2*), was not sufficient to cause mucoid conversion in PAO1 (Table 2). Furthermore, plasmid-borne alleles from PAO581 were able to restore the mucoid phenotype of the non-mucoid mutants PAO581DR58 (*clpP*::Gm^R), PA581DR23 (*tig*::Gm^R), PAO581DR45 (*clpX*::Gm^R) and PAO581DR3 (*clpP2*::Gm^R) (Table 2). The pUCP20T-*tig-clpXP* genes from PAO1 could also complement *in trans* the null mutations of *tig*, *clpX* and *clpP* in PAO581 (results were identical to those recorded for PAO581-derived alleles in Table 2). These results indicate that the polymorphisms of the *tig-clpXP* loci were not responsible for the mucoid conversion in PAO581, and represented functional allelic variants

that likely arose when PAO1 was mutagenized to produce the mucoid variant of PAO581 or during the laboratory passages of PAO581.

AlgU activity is significantly decreased in the *clpP*, *clpX* and *clpP*2 null mutants

AlgU activity was assayed in various strains, including PAO1 and PAO581, and the isogenic null mutants of *tig*, *clpX*, *clpP* and *clpP2*, as well as two other non-mucoid strains with mutations in *algU* (PAO581DR7) or *algR* (PAO581DR51) (Table 1). The P1 promoter of *algU* has been reported to be an AlgU-dependent autoregulatory promoter (Firoved & Deretic, 2003; Schurr *et al.*, 1995). The $P1_{algU}$ promoter–*lacZ* fusion was integrated into the CTX phage attachment site (*attB*) on the bacterial chromosome of PAO581 and the isogenic mutants (Hoang *et al.*, 2000). AlgU expression was 2.2-fold higher in PAO581 than that in the wild-type non-mucoid strain PAO1 ($P<0.001$ ANOVA, $P<0.05$ Dunnett's test) thus validating that the mucoid phenotype of PAO581 was caused by the increased level of functional AlgU (Fig. 3). Furthermore, AlgU activities of the isogenic, non-mucoid *tig*, *clpX*, *clpP* and *clpP*2 null mutants of PAO581 were comparable to that of PAO1, and were two to threefold lower than that of PAO581 ($P<0.001$ ANOVA, $P<0.05$ Dunnett's test for pair-wise comparisons). A very low level of β-galactosidase activity was detected in the *algU* null mutant PAO581DR7 (negative control), which was 2.8-and sixfold lower than that of PAO1 and PAO581, respectively (Fig. 3). The AlgU activity was decreased slightly in the *algR* null mutant PAO581DR51 (positive control), but was 1.8-fold higher than that of PAO1 (Fig. 3, $P<0.001$ ANOVA, $P<0.05$ Dunnett's test). AlgR is an AlgU-dependent downstream regulatory gene for alginate synthesis (Deretic *et al.*, 1989), and its disruption could not affect the activity of the upstream regulator AlgU. These results suggest that ClpX, ClpP and ClpP2 are involved in the increase in AlgU activity, which is associated with increased alginate synthesis and the subsequent development of the mucoid phenotype.

Overexpression of *algU* restores the mucoid phenotype to the *tig*, *clpP*, *clpX* and *clpP*2 null mutants of PAO581

The above results suggest that the *tig* and *clp* mutants could not degrade the anti-σ factor MucA protein to release AlgU, thus producing the non-mucoid phenotype in *P. aeruginosa*. If this is the case, overexpression of AlgU in these mutants should bypass the requirement for *tig*, *clpP*, *clpX* and *clpP*2. Therefore, the pUCP20T-P_{BAD}-*algU* construct was introduced into these null mutants. All of these conjugants converted to the mucoid phenotype when inoculated onto PIA plates supplemented with 0.5–2.5% arabinose, whereas in the absence of arabinose the strains remained nonmucoid on PIA plates (data not shown). These results suggest that the endogenous AlgU is sequestered by the truncated MucA25 protein in the *tig*, *clpX*, *clpP* and *clpP*2 null mutants of PAO581, as these cofactors are apparently needed for release of AlgU from the anti-σ factor MucA.

Both truncated MucA proteins and intact *tig-clpP-clpX* and *clpP*2 are required for the mucoid phenotype in *P. aeruginosa*

To determine whether the conversion of *P. aeruginosa* to the mucoid phenotype is dependent on both a truncated MucA protein and the Clp proteases, which could degrade the cytoplasmic portion of this sequestering anti-σ factor, we created the HA-tagged *mucA25* and HA-tagged wild-type *mucA* fusions in the pBAD/pUCP20T vector, and transferred

these constructs into PAO1, PAO581, PAO581DR23 (*tig*::Gm^R), PAO581DR45 (*clpX*::Gm^R), PAO51DR58 (*clpP*::Gm^R) and PAO581DR3 (*clpP2*::Gm^R) (Table 1). As expected, there was no phenotypic change in wild-type PAO1 expressing HA-tagged *mucA25* (Fig. 4). However, overexpression of the *mucA25* allele suppressed the mucoid phenotype in PAO581, while the vector control had no effects. Similarly, all PAO581 strains carrying the wild-type *mucA* allele *in trans* remained mucoid when arabinose was absent, but converted to non-mucoid when the level of arabinose was >0.01% (data not shown). Similarly, the IPTG-inducible pVDtac24-based construct pDR206, carrying the HA tag–*mucA* fusion, and pDR207, carrying an HA tag–*mucA22* fusion, could also suppress the mucoid phenotype of PAO581 under IPTG induction (data not shown). However, higher levels of inducers (>0.05% arabinose or 5 mM IPTG) were required for the *mucA25*- or *mucA22*-carrying constructs to suppress the mucoid phenotype of PAO581 than for mucoid suppression by the wild-type *mucA*-carrying constructs. These results indicate that the mucoid phenotype of PAO581 is due to the truncation of anti-σ factor MucA as a consequence of *mucA25* mutation, and that ClpXP might be responsible for degrading the N terminus of MucA.

C-terminal-truncated MucA proteins are stabilized in the *tig*, *clpX*, *clpP* and *clpP2* null mutants

The *in vivo* stability of MucA protein was monitored by the Western blot analysis of the HA-tagged wild-type MucA protein and HA-tagged MucA25 proteins in PAO1 and PAO581. Similar levels of wild-type HA–MucA fusion protein (~25 kDa) could be detected in PAO1, PAO581 and the *tig-clp* mutants of PAO581 (data not shown), suggesting that the full-length HA-tagged MucA protein is stable in all of these genetic backgrounds. However, the levels of the truncated MucA25 (~15 kDa) proteins were 4.3-fold lower in PAO1 than in PAO581 after arabinose induction (Fig. 4). The levels of HA-tagged MucA25 proteins were also increased in the *clp*– mutants of PAO581 compared with PAO1 (Fig. 4). The level of HA-tagged MucA25 was over threefold higher in PAO581DR3 (*clpP2*::Gm^R) and PAO581DR58 (*clpP*::Gm^R) than in PAO581 (Fig. 4). There was an accumulation of the MucA25 variant protein under the condition of arabinose induction in the null mutants of *tig*, *clpX*, *clpP* and *clpP2* (data not shown). Therefore, in the absence of the ClpXP protease, MucA25 was not degraded, suggesting that this protease complex may be responsible for the degradation of the N terminus of MucA25 to release the sequestered AlgU into the cytoplasm for activating the transcription of alginate biosynthetic genes.

AlgW and MucP are not required for the mucoid phenotype of PAO581

In *E. coli*, the mature form of DegS is a periplasmic protease that regulates the activity of σ^E, as does the *P. aeruginosa* AlgW protein, the orthologue of DegS, which initiates MucA proteolysis. AlgW-mediated proteolysis can also be activated by the accumulation of misfolded periplasmic proteins (Qiu *et al.*, 2007). To determine whether AlgW is needed for the mucoid phenotype in *mucA25* mutant PAO581, we inactivated *algW* (PA4446) by inserting a Tet^R marker (*algW*::Tet^R) into the chromosomal copy of *algW* and also created an in-frame deletion of *algW* in PAO581. Both mutants still retained the mucoid phenotype, indicating that *algW* is not required for mucoidy in PAO581. We next disrupted the *mucP* (PA3649) gene of PAO581, which encodes another inner membrane-bound protease

orthologous to *E. coli* YaeL (RseP). MucP is also involved in the cleavage of MucA. The *mucP* (*mucP*::*Tet*^{R}) mutant remained highly mucoid, indicating that this protease was not required for MucA cleavage and the release of AlgU in PAO581. It appears as if the truncation of MucA bypasses requirements for AlgW- and MucP-mediated cleavage of the periplasmic portion of MucA, because the truncated MucA proteins are susceptible to degradation by the active ClpXP complexes acting on the cytoplasmic portion.

ClpXP is required for the mucoid phenotype in wild-type PAO1 and a stable mucoid CF isolate

As described previously, the *mucE* gene (PA4033) encodes a small periplasmic protein whose overexpression causes mucoid conversion in PAO1 and other isolates of *P. aeruginosa* with the wild-type *mucA* gene. Therefore, overexpression of *mucE* provides a strong inducing signal that leads to AlgU activation and alginate synthesis (Qiu *et al.*, 2007), which provides a convenient way to test for the requirement of a specific gene in mucoid conversion. To determine whether ClpP and ClpX are required for mucoid conversion in wild-type PAO1, or ClpXP is a specific case for PAO581 alone, we replaced the *clpP* and *clpX* genes of PAO1 with the *Gm*^{R}-disrupted alleles of PAO581DR58 (*clpP*::*Gm*^{R}) and PAO581DR45 (*clpX*::*Gm*^{R}), respectively. These mutants were introduced with the *mucE*-overexpressing plasmid. The *clpP* and *clpX* mutants of PAO1 were unable to convert to the mucoid phenotype when *mucE* was induced. This suggests that ClpP is required for mucoid conversion in PAO1. To determine whether ClpXP is required for the maintenance of the mucoid phenotype in clinical isolates, we disrupted those genes in CF24, a stable mucoid strain isolated from a CF patient (Boucher *et al.*, 1997). The resultant *clpP* and *clpX* mutants became non-mucoid and the mucoid phenotype could be restored by the plasmid-borne *clpXP*, suggesting that *clpP* and *clpX* are also necessary for the mucoid phenotype that arises in clinical isolates (data not shown).

DISCUSSION

We have presented evidence that ClpXP and ClpP2 proteins are needed for the degradation of the N-terminal portion of MucA resident in the cytoplasm of *P. aeruginosa*, and that the resultant increase in AlgU activity leads to the increased biosynthesis of alginate. By analogy to *E. coli*, it appears that a similar process occurs in *P. aeruginosa* and underlies the emergence of the mucoid phenotype associated with progression of lung disease in CF patients, as described in our proposed model for the role of Clp proteases in the MucA25-mediated mucoid phenotype (Fig. 5). However, no periplasmic protease genes were targeted in any of the non-mucoid transposon insertion mutants of PAO581. The truncation of MucA resulting from the single-base deletion leads to the loss of the periplasmic C terminus. Therefore, cleavage of truncated MucA by the *E. coli* orthologous proteases AlgW (DegS) and MucP (RseP/YaeL) is not necessary for the liberation of AlgU and production of the mucoid phenotype in PAO581 with the *mucA25* allele.

ClpXP is an ATP-dependent serine protease complex responsible for the regulated degradation of proteins and SsrA-mediated protein quality control (Gottesman *et al.*, 1997; Gottesman, 1999). The ring hexamers of the ClpX ATPase bind, denature and then

translocate protein substrates into the degradation chamber of the double-heptameric-ring peptidase ClpP (Joshi *et al.*, 2004). In *Bacillus subtilis*, ClpXP is involved in a series of cellular processes, including stationary-phase gene expression, competence development and sporulation (Msadek *et al.*, 1998), and it is involved in virulence in some pathogenic bacteria (Butler *et al.*, 2006). In addition, the ClpXP protease complexes regulate flagellum synthesis via the master regulator FlhD/FlhC in *Salmonella enterica* serovar Typhimurium (Tomoyasu *et al.*, 2002). ClpXP also controls the expression of type III protein secretion systems through regulation of RpoS and GlrR levels in *E. coli* (Iyoda & Watanabe, 2005). The results presented here show that in *P. aeruginosa* ClpXP regulates alginate synthesis via degradation of the cytoplasmic portion of the truncated anti-σ factor MucA25. Whether or not *P. aeruginosa* ClpXP regulates additional cellular processes in *P. aeruginosa*, as it does in *E. coli* and *Salmonella*, is not yet known.

Notably, the overexpression of the *tig-clpXP* cluster did not induce the mucoid phenotype in wild-type PAO1, indicating that ClpXP activity alone cannot degrade the full-length MucA. The periplasmic domain of the full-length protein likely must first be cleaved by the periplasmic proteases AlgW and MucP, as has been described in the orthologous system in *E. coli* (Alba *et al.*, 2002; Alba & Gross, 2004). It is also notable that unlike most eubacteria, such as *E. coli*, that harbour only a single copy of the *clpP* gene (Porankiewicz *et al.*, 1999), *P. aeruginosa* harbours three copies of *clpP* genes, including *clpP*, encoding the orthologue of the *E. coli* ClpP protein, and the paralogous *clpP2* (PA3326) and *clpP3* (PA2189) genes, encoding hypothetical ClpP proteins. In this study we showed that both *clpP* and *clpP2* are involved in the increase in alginate synthesis in strains with truncated MucA proteins. The *clpP2* gene in the *P. aeruginosa* chromosome lacks the adjacent ORF encoding an ATPase that is found in other ClpP homologues. We also found some sequence heterogeneity in the N termini of the *clpP* and *clpP2* genes, although the significance of this is unknown.

Mucoid strains of *P. aeruginosa* recovered from CF patients usually produce truncated MucA proteins (Anthony *et al.*, 2002; Boucher *et al.*, 1997). It appears as if these truncated proteins are more stable in mucoid strains than in wild-type strains with full-length *mucA* (Figs 4 and 5). We observed that the levels of the truncated MucA25 protein were higher in mucoid PAO581 than in wild-type PAO1 when expression was induced from the P_{BAD} promoter using different concentrations of arabinose (Fig. 4). However, the increased expression of the MucA25 protein converted PAO581 from a mucoid to non-mucoid phenotype, indicating that the increased sequestration of AlgU prevented high-level synthesis of alginate. The stability of the truncated MucA proteins in mucoid strains may be needed to impose some level of regulation on the biosynthesis of alginate when the organism acquires the mucoid phenotype. Thus, additional control over alginate synthesis in mucoid strains might come via the ClpP proteases, which may in turn be influenced by the ClpX ATPase. However, before concluding that this hypothesis is valid, further experimental results are needed.

The involvement of at least two related but unlinked Clp proteases, ClpP and ClpP2, in alginate synthesis in stable mucoid *P. aeruginosa* strains would be consistent with the concept that alginate production must be regulated even when the polysaccharide is produced in copious quantities. In *P. aeruginosa* PAO581, both ClpP and ClpP2 were required for degradation of truncated MucA and production of the mucoid phenotype. Thus,

differential expression of these two proteases could allow a mucoid cell to control the level of alginate synthesized. However, the two Clp proteases differed in their functionality when used to complement chromosomal mutations. Plasmid-borne *tig-clpXP*, *clpXP* and *clpP* were all able to complement the insertionally inactivated *clpP* and *clpP2* genes. This indicates that ClpP can provide the functionality needed for conversion to the mucoid phenotype in the absence of ClpP2, if sufficient levels of ClpP are made. However, the converse was not true, as pUCP20-*clpP2* could not complement the *clpP* null mutant PAO581DR58. This outcome suggests that there are differences in the protease activity of ClpP and ClpP2 on the MucA25/AlgU complex, consistent with the finding that the AlgU activity of PAO581DR3 (*clpP2*::Gm^R) is significantly higher than that of PAO581DR58 (*clpP*::Gm^R) (Fig. 4).

The regulation of alginate synthesis in *P. aeruginosa* is complicated, with direct control from proteins encoded by the *algUmucABCD* operon and the *algR*, *algW*, *mucP* and *amrZ* loci. However, it appears there are additional regulatory circuits, as shown here, indicating that the *clpXP* and *clpP2* genes encode proteins likely to be involved in regulating alginate synthesis via their action on MucA/AlgU cytoplasmic complexes. The AlgsU protein also affects the production of other *P. aeruginosa* virulence factors, including inhibition of flagellum formation by regulating expression of *fleQ* (Tart *et al.*, 2005, 2006), and expression of the RpoH heat shock-response σ factor. Another alginate regulatory factor, AlgR, inhibits the activity of the *P. aeruginosa* type III secretion system (Wu *et al.*, 2004). Overall, the interactions of all of these factors and the use of master control circuits are likely to be critical for maintaining a stable and viable phenotype for the mucoid variants of *P. aeruginosa*.

In summary, by using mariner-based transposition and complementation assays, we have identified the *tig-clpP-clpX* and *clpP2* loci as encoding regulatory factors that control alginate synthesis in mucoid *P. aeruginosa*. We also identified the *muc-25* mutation as a single-base deletion (T180) in *mucA*. These findings add to the already extensive knowledge about regulation of alginate synthesis in *P. aeruginosa*, and identify additional factors that regulate how this organism can convert to the mucoid phenotype needed for maximal pathogenesis in the CF lung, yet also show that our knowledge of regulation of alginate synthesis may still not be complete.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CF	cystic fibrosis
HA	haemagglutinin

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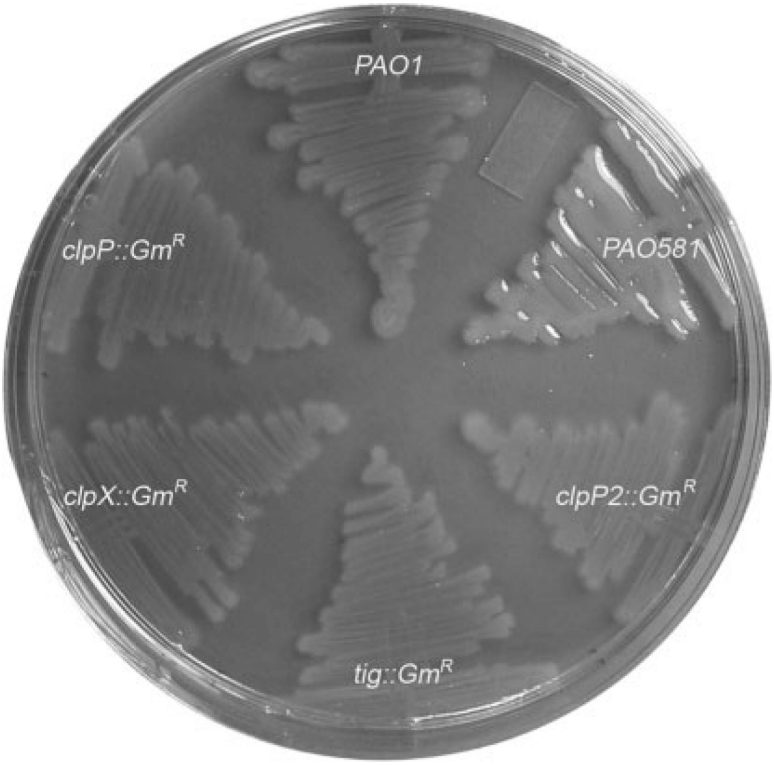

Fig. 1.

Colony morphologies of *P. aeruginosa* PAO1, mucoid variant PAO581 (PAO1 *mucA25*), and the null mutants of *tig* (PAO581DR23), *clpP* (PAO581DR58), *clpX* (PAO581DR45) and *clpP2* (PAO581DR3) on a PIA plate after growth at 37 °C for 24 h.

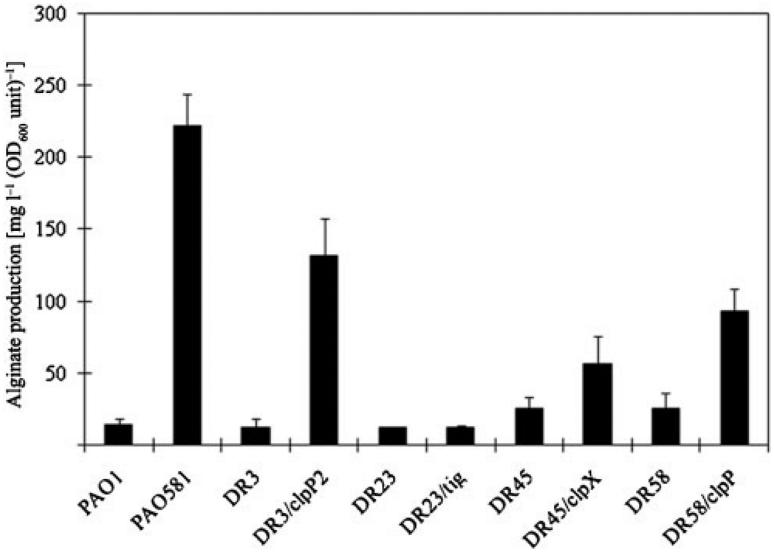

Fig. 2.

Alginate production by *P. aeruginosa* PAO1, PAO581 and the isogenic mutants of PAO581 carrying pUCP20T- or pUCP20T-based constructs. The strains were grown at 37 °C for 24 h on PIA plates supplemented with carbenicillin. DR3/*clpP*2, DR23/*tig*, DR45/*clpX* and DR58/*clpP* represent PAO581DR3 (*clpP*2::*Gm*^{R}), PAO581DR23 (*tig*::*Gm*^{R}), PAO581DR45 (*clpX*::*Gm*^{R}) and PAO581DR58 (*clpP*::*Gm*^{R}) carrying the respective complementing plasmids pUCP20T-*clpP*2, pUCP20T-*tig*, pUCP20T-*clpX* and pUCP20T-*clpP*. The empty vector pUCP20T was used as a control in the remaining strains. Significant differences were noted between PAO1 and PAO581, DR3 and DR3/ClpP2, and DR58 and DR58/*clpP* (P<0.001, *t* test). There was no difference between DR23 and DR23/*tig* (P=0.902) or DR45 and DR45/*clpX* (P=0.068). Bars indicate mean ± SD.

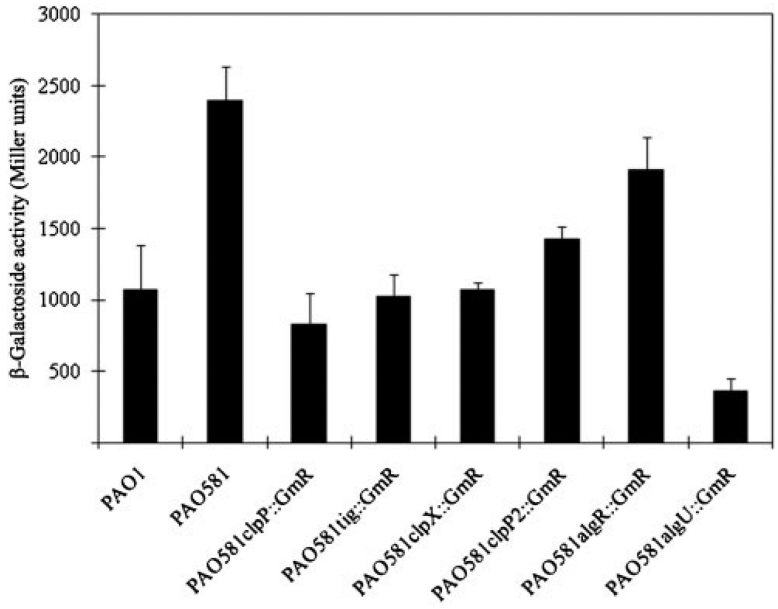

Fig. 3.

β-Galactosidase activity (Miller units) from the *algU*-dependent $P1_{algU}$-*lacZ* reporter on the chromosome of PAO1, PAO581, and the isogenic mutants of *tig* (PAO581DR23), *clpP* (PAO581DR58), *clpX* (PAO581DR45) and *clpP2* (PAO581DR3). PAO581DR7 (*algU*::Gm^R) and PAO581DR51 (*algR*::Gm^R) served as negative and positive controls, respectively. Comparison between PAO581 and its mutants indicates that *β*-galactosidase activity was significantly higher in PAO581 than in the mutants or PAO1 ($P<0.001$, ANOVA; $P<0.05$, Dunnett's test), except for PAO581 versus PAO581DR51 ($P>0.05$). Bars indicate mean ± SD.

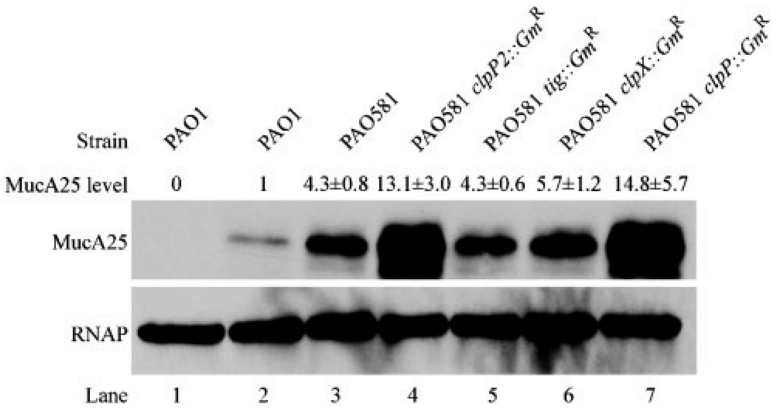

Fig. 4.

Western blot analysis of N-terminal HA-tagged MucA25 protein in *P. aeruginosa*. Lane 1, PAO1/pUCP20T-pBAD (vector control); lanes 2–7, HA-MucA25 expressed from pUCP20T-P_{BAD}-*mucA25* under the induction of 0.05% arabinose. Rabbit polyclonal antibody against the *P. aeruginosa* RNA polymerase α-subunit (RNAP) was used as a loading control.

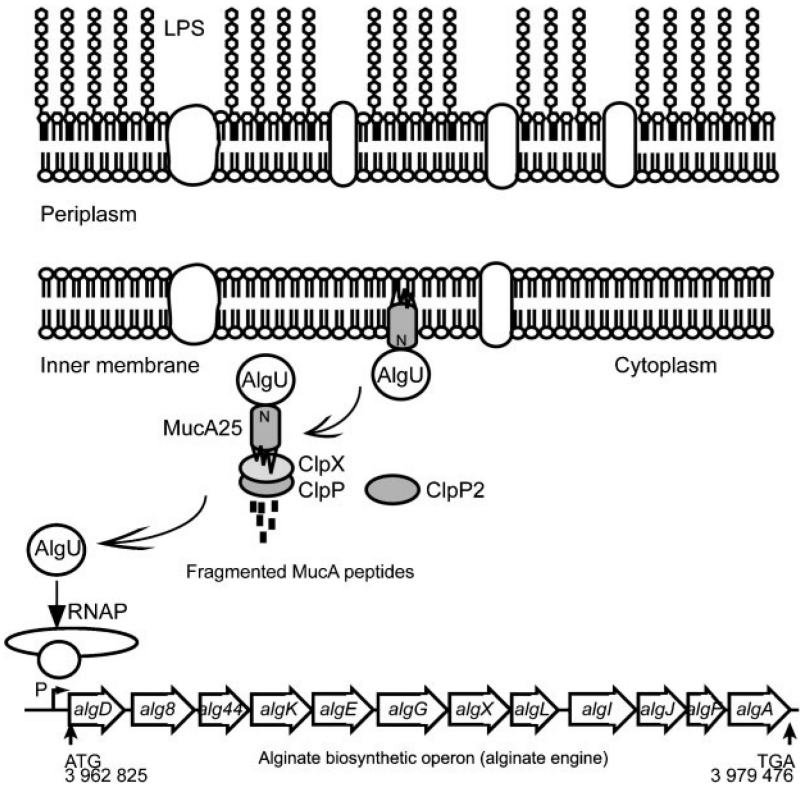

Fig. 5.

Schematic diagram for the *mucA25* mutation-caused mucoid phenotype in *P. aeruginosa* PAO581 and the cytoplasmic proteolysis involved in the regulation of alginate biosynthesis. The single-base deletion of T180 results in a premature stop codon in *mucA* and C-terminal truncation of MucA to MucA25. This truncation is before the transmembrane domain (residues 84–104) of wild-type MucA, but the location of MucA25 has yet to be proven. The truncated N terminus of MucA25 is subjected to the proteolysis by ClpXP and ClpP2, leading to the release of the sequestered AlgU to drive the transcription of the alginate biosynthesis operon *algD–A*.

Table 1

Strains and plasmids used in this study

Strain, plasmid or primer	Phenotype, genotype or sequence	Source or reference
***P. aeruginosa* strains**		
PAO1	Prototroph	P. Phibbs *
PAO1DR1	Mucoid PAO1 derivative *mucA25*	This study
PAO1DR2	PAO1 *clpP*::Gm^R	This study
PAO1DR3	PAO1 *clpX*::Gm^R	This study
PAO581	Mucoid PAO1 *muc-25* derivative, PAO1 *mucA25*	J. Govan †
PAO581Δ*algIJF*	PAO581 with in-frame deletion of *algI*, *algJ* and *algF*	This study
PAO581Δ*algW*	PAO581 with in-frame deletion of *algW*	This study
PAO581Δ*mucP*	PAO581 *mucP*::Tet^R	This study
PAO581DR3	PAO581 *clpP2*::Gm^R non-mucoid	This study
PAO581DR7	PAO581 *algU*::Gm^R non-mucoid	This study
PAO581DR23	PAO581 *tig*::Gm^R non-mucoid	This study
PAO581DR45	PAO581 *clpX*::Gm^R non-mucoid	This study
PAO581DR51	PAO581 *algR*::Gm^R non-mucoid	This study
PAO581DR58	PAO581 *clpP*::Gm^R non-mucoid	This study
CF24	Isolate from a patient with CF, mucoid	V. Deretic ‡
CF24DR1	CF24 *clpP*::Gm^R non-mucoid	This study
CF24DR2	CF24 *clpX*::Gm^R non-mucoid	This study
Strain 1003	Isolate of *P. aeruginosa* from the lung of a CF mouse, mucoid	This study
***E. coli* strains**		
TOP10	DH5α derivative	Invitrogen
SM10/λpir	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu *λpirR6K* Km^R	Laboratory strain
Plasmids		
pFAC	Mini-*himar*I mariner transposon with a selectable marker Gm^R (*aacC1*) Ap^R	Wong & Mekalanos (2000)
pRK2013	Km^R Tra Mob ColE1	Figurski & Helinski (1979)
pUCP20T	*E. coli–Pseudomonas* shuttle vector, ori1600 P_{lac}, Ap^R	West *et al.* (1994)
P_{algUP1}-*lacZ*	200 bp PCR product including P_{algUP1} with additional 56 bp upstream in mini-ctx-*lacZ*	Qiu *et al.* (2007)
pCR4-TOPO	TA cloning vector; 3.9 kb; Ap^R Km^R	Invitrogen
pUCP20T-*tig-clpPX*	*tig-clpP-clpX* (3.5 kb) from PAO1 in pUCP20 *Bam*HI/*Xba*I	This study
pUCP20T-*tig-clpPX*(581)	*tig-clpP-clpX* (3.5 kb) from PAO581 in pUCP20 *Bam*HI/*Xba*I	This study
pUCP20T-*clpP2*	*clpP2* (PA3326) from PAO1 in pUCP20 *Xba*I/*Hin*dIII	This study
pUCP20T-*tig*	*tig* (1.5 kb) from PAO1 in pUCP20 *Bam*HI/*Kpn*I	This study
pUCP20T-*clpPX*	*clpP-clpX* (2.1 kb) from PAO1 in pUCP20 *Xba*I/*Hin*dIII	This study
pUCP20T-*clpPX*(581)	*clpP-clpX* (2.1 kb) from PAO581 in pUCP20 *Xba*I/*Hin*dIII	This study
pUCP20T-*clpP*	*clpP* (0.7 kb) from PAO1 in pUCP20 *Xba*I/*Hin*dIII	This study
pUCP20T-*clpX*	*clpX* (1.3 kb) from PAO1 in pUCP20 *Xba*I/*Hin*dIII	This study

Strain, plasmid or primer	Phenotype, genotype or sequence	Source or reference
pUCP20T-P_{BAD}-*algU*	*araC*-P_{BAD}-*algU* fusion in pUCP20 *Xba*I/*Hin*dIII	This study
pEX100T	*Pseudomonas* suicide vector, *sacB*, *oriT*, Cb^R	Schweizer & Hoang (1995)
pEX-*mucA25*	*mucA25* allele of PAO581 in pEX100T	This study
pEX100-Δ*algW*	1.4 kb *algW*-flanked fragment with in-frame deletion of *algW* in pEX100T	Qiu *et al.* (2007)
pEX-*clpP*::Gm^R	*clpP*::Gm^R (*aacC1*) from PAO581DR58 in pEX100T	This study
pEX-*clpX*::Gm^R	*clpX*::Gm^R (*aacC1*) from PAO581DR45 in pEX100T	This study
pBAD/pUCP20T	*E. coli–Pseudomonas* shuttle vector, ori1600 P_{BAD}, *araC*, Ap^R	This study
pUCP20T-P_{BAD}-*mucA*	HA tag-*mucA* in pBAD/pUCP20 *Eco*RI/*Hin*dIII	This study
pUCP20T-P_{BAD}-*mucA25*	HA tag-*mucA25* in pBAD/pUCP20 *Eco*RI/*Hin*dIII	This study
Primers		
Gm5OUT	GACTGCCCTGCTGCGTAACA	This study
Gm3OUT	GGGCATACGGGAAGAAGTGA	This study

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Table 2

Complementation analyses on the non-mucoid transposon mutants in *tig* (PA1800), *clpP* (PA1801), *clpX* (PA1802) and *clpP2* (PA3326) loci of *P. aeruginosa* PAO581

***P. aeruginosa* strain**	**Complementing plasmid**							
	pUCP20	**pUCP-*tig-clpXP***	**pUCP-*tig-clpXP581***	**pUCP-*tig***	**pUCP-*clpXP***	**pUCP-*clpP***	**pUCP-*clpX***	**pUCP-*clpP2***
PAO1	NM	NM	NM	NM	NM	NM	NM	NM
PAO581 (*mucA25*)	M	–	–	–	–	–	–	–
PAO581DR3 (*clpP2*::Gm^R)	NM	M	M	–	M	M	–	M
PAO581DR23 (*tig*::Gm^R)	NM	M	M	NM	M	M	–	–
PAO581DR45 (*clpX*::Gm^R)	NM	M	M	–	M	NM	M	–
PAO581DR58 (*clpP*::Gm^R)	NM	M	M	–	M	M	–	NM

NM, non-mucoid; M, mucoid; –, not tested. The strains were grown on PIA plates supplemented with 300 μg carbenicillin ml^{-1} and were scored for phenotype as depicted in Fig. 1.