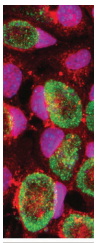

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SHORT COMMUNICATION

Identification and characterization of a novel inhibitor of alginate overproduction in *Pseudomonas aeruginosa*

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In this work the authors identify a novel inhibitor of alginate overproduction in *Pseudomonas aeruginosa* which is an interesting finding that will be of importance to the field of bacterial pathogenesis.

Keywords
Pseudomonas aeruginosa; alginate; biofilms; inhibitor; PA1494; *muiA*.

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Abstract

In this study, we performed whole-genome complementation using a PAO1-derived cosmid library, coupled with *in vitro* transposon mutagenesis, to identify gene locus PA1494 as a novel inhibitor of alginate overproduction in *P. aeruginosa* strains possessing a wild-type *mucA*.

Alginate overproduction is a key mechanism for the development of a chronic lung infection by *P. aeruginosa* in individuals with cystic fibrosis (Govan & Deretic, 1996). Alginate overproduction is achieved through increased transcription of the alginate biosynthetic operon at the *algD* promoter (Deretic *et al.*, 1987). Regulation of alginate overproduction primarily involves the alternative sigma factor AlgU (AlgT, σ^{22}) and its cognate antisigma factor, MucA (Martin *et al.*, 1993a, b). Typically, in low-alginate-producing *P. aeruginosa* strains, AlgU is sequestered by MucA to the inner membrane (Mathee *et al.*, 1997; Rowen & Deretic, 2000). However, with a loss of MucA through mutations, AlgU is free to activate transcription at the *algD* promoter (Martin *et al.*, 1993a, b; Wozniak & Ohman, 1994). Previous reports have also determined an alternative mechanism for alginate overproduction, which requires AlgW, a DegS-like serine protease (Wood *et al.*, 2006; Qiu *et al.*, 2007; Cezairliyan & Sauer, 2009). AlgW is the first intramembrane protease that has been shown to degrade the periplasmic portion of MucA (Cezairliyan & Sauer, 2009). However, there are other proteases, MucP and ClpXP that are also required for alginate overproduction via the degradation of MucA (Qiu *et al.*, 2007, 2008a). All of these proteases regulate alginate production by degrading MucA. Additionally, it has been suggested that preventing the overproduction of alginate (Ramsey & Wozniak, 2005), and more specifically inhibiting the regulated proteolytic degradation of MucA (Damron & Goldberg, 2012), is a potential strategy to prevent the establishment of chronic *P. aeruginosa* infections. In response to these suggestions, we employed whole-genome complementation coupled with *in vitro* transposon mutagenesis to identify a genetic locus that can inhibit alginate overproduction in *P. aeruginosa* strains with a wild-type *mucA*.

Recently, we determined that alginate overproduction in *P. aeruginosa* strain PAO579 (*muc-23*), a derivative of PAO1, is caused by the release of AlgU from MucA via regulated intramembrane proteolysis (Withers *et al.*, 2012, 2013). This proteolytic cascade is initiated by the activation of AlgW through a truncated type-IV pilus (PilA108; Withers *et al.*, 2013). To identify novel inhibitors of alginate overproduction in *P. aeruginosa* strains with a wild-type

mucA, a PAO1-derived, minimal tiling path (MTP) genomic cosmid library (Huang *et al.*, 2000) was conjugated into PAO579 (Govan & Fyfe, 1978). As a result, we identified that cosmid MTP87 could completely suppress alginate overproduction in PAO579 (data not shown). MTP87 covers a region of 22,757 bp from the genome of PAO1 (start: 1,618,021; and end: 1,640,777). To identify the exact gene within this cosmid responsible for the multicopy suppression of alginate overproduction, MTP87 underwent random transposon-mediated *in vitro* mutagenesis, and the mutated cosmids were then conjugated en masse into PAO579 and screened for alginate overproduction (Fig. 1a). We observed the presence of alginate-overproducing clones, indicating a transposon-mediated inactivation of a specific inhibitory gene within cosmid MTP87 (Fig. 1a). PCR and sequence analysis of the mutagenized MTP87 confirmed a single transposon insertion in open reading frame PA1494. Previous transcriptome analyses have shown that PA1494 is up-regulated when *P. aeruginosa* is exposed to azithromycin (Nalca *et al.*, 2006; Kai *et al.*, 2009) and hydrogen peroxide (Chang *et al.*, 2005). However, because PA1494 belongs to a class of unclassified/hypothetical genes and its

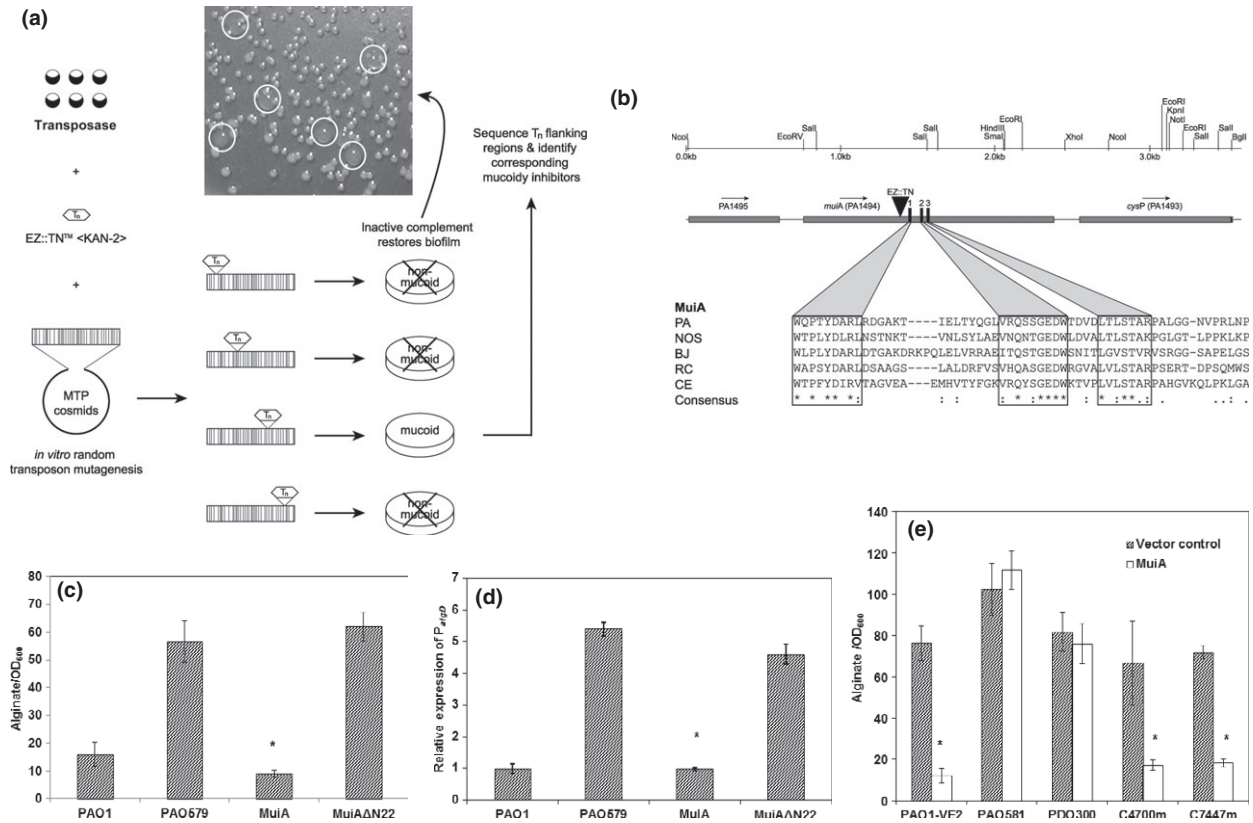

Fig. 1 Identification and characterization of MuiA. (a) MTP87 cosmid was subjected to *in vitro* transposon mutagenesis to generate random gene knockouts. Shown in the inset are PAO579 (*muc-23*) exconjugants carrying cosmid MTP87 randomly mutagenized with an EZ::TN transposon (Epicentre), selected on a PIA plate supplemented with the appropriate antibiotic and incubated at 37 °C for 48 h. (b) Restriction map, gene organization, and Tn insertion in the *muiA* gene. Homology of MuiA with its orthologs. Shown are the most homologous regions (1, 2, and 3): *Rhodobacter capsulatus* (RC; ORF1654; 534aa), *Bradyrhizobium japonicum* (BJ; CAC38742; 560aa), *Nostoc* sp. (NOS; NP_484904; 545aa), and *Caenorhabditis elegans* (CE; NP_500427; 556aa). A single Tn insertion occurred before regions 1, 2, and 3. (c) PAO1 pHERD20T, PAO579 pHERD20T (vector control), PAO579 pHERD20T-*muiA* (wild-type *muiA*), and PAO579 pHERD20T-*muiA*ΔN22 (deletion of N-terminal signal sequence) were grown on PIA plates supplemented with carbenicillin and 0.1% arabinose for 24 h at 37 °C and then for 24 h at room temperature. The alginate was collected and measured using the standard carbazole assay. The values are representative of three independent experiments. Statistical significance was determined using the Student's *t*-test in comparison with PAO579 (*$P < 0.05$). (d) The β-galactosidase activity of the *algD* promoter was measured using PAO1 and PAO579 miniCTX-P_{algD}-lacZ with pHERD20T, pHERD20T-*muiA*, or pHERD20T-*muiA*ΔN22. All strains were incubated at 37 °C on PIA plates supplemented with tetracycline, carbenicillin, and 0.1% arabinose. The values for the mean and standard deviation in Miller units (one Miller unit = $1000 \times (A_{420}/-1.75 \times A_{550}/OD_{600\,nm}\ mL^{-1}\ min^{-1})$) are shown as relative expression as compared to PAO1 and are representative of three independent experiments. Statistical significance was determined using the Student's *t*-test in comparison with PAO579 (*$P < 0.05$). (e) Alginate measurements for various laboratory and clinical strains expressing pHERD20T (vector control) or pHERD20T-*muiA in trans*. Strains were cultured on PIA plates supplemented with 300 μg mL^{-1} of carbenicillin and 0.1% arabinose and incubated for 24 h at 37 °C and then for 24 h at room temperature. The values are representative of three independent experiments. Statistical significance was determined using the Student's *t*-test in comparison with the vector control (*$P < 0.05$).

exact function is unknown, we refer to PA1494 as mucoidy inhibitor gene A or *muiA*.

The *muiA* gene is predicted to encode a polypeptide of 551 amino acids with a predicted molecular mass of 61 kDa and an isoelectric point (pI) of 5.5. Located immediately downstream is the *E. coli* periplasmic sulfate-binding ortholog (*cysP*: PA1493). The *muiA* gene is predicted to use GTG as a start codon with a typical type-I signal sequence encoding 22 amino acids (NH_2-MNRLAASPLLFAGLFASAPLLA-COOH; Lewenza *et al.*, 2005), and previous proteomic analysis detected MuiA in the periplasm of PAO1. Based on MALDI-TOF/TOF survey on the proteome of the PAO1 cell envelope, the relative abundance of MuiA was in the same range as the alginate negative regulator MucB, but less than the major porin protein OprF (Imperi *et al.*, 2009). Additionally, we confirmed the results presented in the previous studies by detecting the presence of a hemagglutinin-tagged MuiA in the periplasm using cell fractionation and Western blot analysis (data not shown). Through BLAST analysis, we determined that MuiA is highly conserved among other *P. aeruginosa* strains; however, no orthologs were identified in *E. coli* species or other Pseudomonads. MuiA orthologs were found in other organisms including *Rhodobacter capsulatus*, *Bradyrhizobium japonicum*, *Nostoc* sp., and *Caenorhabditis elegans* (Fig. 1b). These orthologs are all of similar size ranging from 530 to 560 amino acids in length and are classified as conserved hypothetical proteins. An internal region of MuiA (232–274aa) displayed three highly conserved regions. In addition, the transposon insertion in MTP87 was located 15 bps in front of these conserved domains (Fig. 1b).

To confirm whether *muiA* is responsible for suppressing alginate overproduction, we used standard molecular techniques (Russell, 2001) to clone *muiA* into the shuttle vector pHERD20T, which contains the P_{BAD} arabinose-inducible promoter (Qiu *et al.*, 2008b). PAO1 pHERD20T, PAO579 pHERD20T (vector control), and PAO579 pHERD20T-*muiA* were cultured on PIA supplemented with carbenicillin and 0.1% arabinose, and the amount of alginate was measured using the standard carbazole assay (Knutson & Jeanes, 1968). When compared to PAO1 and the vector control, there was a decrease in alginate overproduction when *muiA* was expressed *in trans* (Fig. 1c). Additionally, we observed that pHERD20T-*muiA* can suppress mucoidy even in the absence of arabinose on PIA, suggesting that the basal expression from pHERD20T-*muiA* was sufficient for suppression (data not shown). We also observe that the removal of the N-terminal signal sequence (pHERD20T-*muiA*ΔN22) abrogated MuiA's ability to suppress alginate overproduction in PAO579 (Fig. 1c). Also, we observed that the in-frame deletion of *muiA* in strain PAO1 did not result in alginate overproduction, suggesting that MuiA does not play a central role in alginate regulation (data not shown). These results suggest that MuiA suppresses alginate overproduction after localization to the periplasm, and can act as a multicopy suppressor for alginate overproduction in PAO579.

Previously, it was reported that alginate overproduction in PAO579 was due to increased transcriptional activity at the P_{algD} promoter site of the alginate biosynthetic operon (Boucher *et al.*, 2000; Withers *et al.*, 2013). To determine the effect that the expression of *muiA* has on P_{algD} activity, we used PAO1 and PAO579 merodiploid strains carrying a chromosomal copy of the *algD* promoter fused with a reporter gene, *lacZ* (generated via miniCTX-PalgD-lacZ). Next, we conjugated pHERD20T (vector control), pHERD20T-*muiA*, and pHERD20T-*muiA*ΔN22 into the PAO1 or PAO579 miniCTX-P_{algD}-*lacZ* and cultured them on PIA plates supplemented with carbenicillin, tetracycline, and 0.1% arabinose. We measured the transcriptional activity of P_{algD} promoter using the Miller assay (Miller, 1972). As expected, the level of transcriptional activity in PAO579 pHERD20T was significantly higher than that in PAO1 (Fig. 1d). The activity at P_{algD} decreased when pHERD20T-*muiA* was expressed *in trans* in PAO579 (Fig. 1d). Additionally, we observed that expression of pHERD20T-*muiA*ΔN22 did not result in a decrease in P_{algD} activity in PAO579 (Fig. 1d). Based on these results, we concluded that expression of *muiA* suppresses transcriptional activity at the alginate biosynthetic operon at the *algD* promoter.

To determine the overall robustness and to elucidate the possible mechanism by which MuiA suppresses alginate overproduction, we conjugated pHERD20T-*muiA* into a variety of laboratory and clinical strains. We observed that expression of *muiA in trans* suppressed alginate overproduction in PAO1-VE2 (Fig. 1e). PAO1-VE2 is a derivative of PAO1 and overproduces alginate due to the activation of AlgW by MucE, a small envelope protein (Qiu *et al.*, 2007; Cezairliyan & Sauer, 2009). Similarly, expression of *muiA in trans* was able to suppress alginate overproduction in clinical strains C7447m and C4700m, both possessing a wild-type MucA (Fig. 1e). The decrease in alginate overproduction observed in PAO1-VE2, C7447m, and C4700m was statistically significant when compared to the vector control ($P < 0.05$). However, expression of *muiA* was unable to suppress alginate overproduction in the PAO1-derived *mucA25* strain, PAO581 (Fig. 1e). PAO581 carries a truncated MucA25 protein, which lacks the transmembrane domain of the wild-type MucA, suggesting that MucA25 is likely localized in the cytoplasm (Qiu *et al.*, 2008a). Additionally, expression of *muiA* did not suppress alginate overproduction in the strain PDO300 (Fig. 1e). PDO300 carries a truncated MucA22 protein. It is important to note that none of the strains examined in this study, aside from PAO579, have three tandem mutations resulting in a truncation of type-IV pili. Therefore, the suppression of alginate overproduction through the expression of MuiA is not specific to those strains possessing a truncation in type-IV pili. Together, these data suggest that MuiA's ability to suppress alginate overproduction is not unique to PAO579. However it is only effective at suppressing alginate overproduction in strains with a wild-type MucA.

In summary, we coupled whole-genome complementation of a PAO1 cosmid library and *in vitro* transposon mutagenesis to identify the genetic locus PA1494 (*muiA*) as a novel inhibitor of alginate overproduction in *P. aeruginosa* strains with a wild-type *mucA*. Additionally, we determined that expression of *muiA in trans* resulted in a decrease in

alginate production, as well as transcriptional activity at the P_{algD} promoter. Lastly, expression of *muiA in trans* was only able to suppress alginate overproduction in various clinical and laboratory strains possessing a wild-type MucA.

Author Contributions

T.R.W. designed and performed experiments, analyzed data, and wrote the manuscript. Y.Y. and H.D.Y. analyzed data and helped in revising the manuscript.

Disclosures

The author Hongwei D. Yu is the Chief Science Officer and Co-founder of Progenesis Technologies, LLC.

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