RESEARCH LETTER

Nonmucoid conversion of mucoid *Pseudomonas aeruginosa* induced by sulfate-stimulated growth

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Abstract

Alginate-overproducing mucoid *Pseudomonas aeruginosa*, responsible for chronic airway infections in cystic fibrosis (CF) patients, is resistant to antibiotic treatments and host immune clearance. In this study, we performed a phenotype microarray screen and identified sulfate ion as a molecule that can suppress alginate production. When a mucoid *P. aeruginosa* strain CM21 and additional mucoid isolates were grown with 5% sodium sulfate, significantly decreased levels of alginate were produced. Suppression of alginate production was also induced by other sulfate salts. Expression of a reporter gene fused to the *algD* promoter was considerably decreased when grown with sulfate. Furthermore, bacterial cell shape was abnormally altered in CM21, but not in PAO1, a prototype nonmucoid strain, suggesting that sulfate-stimulated cell shape change is associated with transcriptional suppression of the alginate operon. Finally, a CM21 *lpxC* mutant defective in lipid A biosynthesis continued to produce alginate and maintained the correct cell shape when grown with sulfate. These results suggest a potential involvement of lipoplysaccharide biosynthesis in the sulfate-induced reversion to nonmucoid phenotype. This study proposes a novel strategy that can be potentially applied to treat persistent infection by recalcitrant mucoid *P. aeruginosa*.

Introduction

The Gram-negative bacterium *Pseudomonas aeruginosa* is a major opportunistic pathogen in human cystic fibrosis (CF). In chronic CF airways, *P. aeruginosa* strains that have acquired selected phenotypes are recovered and such phenotypic changes are induced for survival in the harsh environment of the human lung (Burns *et al.*, 2001; Hogardt *et al.*, 2007; Mena *et al.*, 2008). One of these changes is an overproduction of a capsule-like polysaccharide called alginate (Evans & Linker, 1973). Mucoid *P. aeruginosa* strains isolated from sputum of CF patients have a mutation in the *mucA* gene, encoding MucA, a negative regulator of AlgT that activates transcription of the alginate biosynthesis operon (Martin *et al.*, 1993). A truncated version of MucA, encoded by a mutated *mucA* gene, no longer binds to AlgT (Xie *et al.*, 1996), and expression of the alginate biosynthesis operon is continuously activated (Govan & Deretic, 1996; Xie *et al.*, 1996; Boucher *et al.*, 1997; Bragonzi *et al.*, 2009). Besides this major pathway to mucoid conversion, a stringent starvation response or sigma factor competition were also reported to regulate alginate production in *P. aeruginosa* (May *et al.*, 1991; Yin *et al.*, 2013).

In mucoid *P. aeruginosa*, expression of virulence factors is more down-regulated than acute infection, and this is in response to the need to thrive in infected tissue or organs (Hogardt & Heesemann, 2010). However, mucoid *P. aeruginosa* exhibit several unique virulence-associated characteristics compared with nonmucoid counterparts. First, mucoid variants are more resistant to antibiotic treatment (Govan & Fyfe, 1978) and phagocytic immune activity (Cabral *et al.*, 1987). Such a resistant nature is due to the presence of an alginate capsule around the cell. Secondly, mucoid strains are less potent in activating the type III secretion system, an important virulence determinant (Wu *et al.*, 2004). These phenotypes are considered to contribute to the persistent survival in the patient airway (Yu *et al.*, 1998). For efficient treatment, it would

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therefore be beneficial to switch mucoid *P. aeruginosa* back to a nonmucoid form.

When a mucoid *P. aeruginosa* strain was grown statically with reduced oxygen potential, nonmucoid revertants were recovered that acquired spontaneous mutations in the *algT* gene (Wyckoff *et al.*, 2002). In addition, overexpression of the *rpoD* gene resulted in suppressed production of alginate AlgT/U (Yin *et al.*, 2013). These previous studies suggest that reversion to a nonmucoid form can be achieved by genetic alterations. To our knowledge, however, conversion to a nonmucoid form by treatment with a defined chemical compound has not previously been elucidated. Here, we performed a phenotype microarray analysis with a clinically isolated mucoid *P. aeruginosa* strain and revealed that alginate production was suppressed by sulfate ions. This highlights a previously undescribed means to weaken mucoid *P. aeruginosa*, a major threat to human healthcare.

Materials and methods

Phenotype microarray, bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are shown in Table 1. Phenotype microarray (Biolog Inc., Hayward, CA) experiments were performed as described elsewhere (Johnson *et al.*, 2008). The mucoid CM21 strain was grown in each well of phenotype microarray plates for 24 h. Bacterial cells were then inoculated into 150 × 20-mm Luria–Bertani (LB) agar plates using a 96-well pin replicator. After 24 h growth at 37 °C, morphology of the CM21 colonies was examined and growth conditions that induced nonmucoid reversion were identified. CF strain *P. aeruginosa* PAO1 and *Escherichia coli* were grown at 37 °C on LB plates or in LB broth. Gentamicin (Sigma-Aldrich, St. Louis, MO) at 50 μg mL^{-1} was added for selecting transformed bacteria. Sodium sulfate, ammonium sulfate, magnesium sulfate and sodium chloride were all purchased from Sigma-Aldridge. The reporter plasmid, pMEXR*algD*, was a kind gift from Dr Toyofuku (University of Tsukuba, Japan) and it has been modified from pMEX9 (Toyofuku *et al.*, 2007) by replacing the *xylE* reporter gene with a gene encoding Ds-Red.

Alginate assay

Pseudomonas aeruginosa strains were grown at 37 °C in LB broth and LB broth containing sodium sulfate (LB+SS), ammonium sulfate (LB+AS), magnesium sulfate (LB+MS) and sodium chloride (LB+SC) for 24 h with vigorous shaking. Bacterial cells were grown in 10 mL LB overnight and a 1-mL aliquot of each bacterial culture was pelleted by centrifugation at 16000 × ***g*** for 5 min. Twenty microliters of culture supernatant was mixed with 80 μL distilled water and assayed for quantification of alginate as described previously (Damron *et al.*, 2009). OD_{600} values of bacterial suspensions were used for normalization. The amount of uronic acid was analysed with a standard curve constructed with the use of alginic acid from brown algae (Sigma-Aldrich).

Transposon random mutagenesis

For random transposon insertion mutagenesis, *E. coli* SM10/λpir harboring pBTK30 (Kim *et al.*, 2012) and CM21 were used as donor and recipient strains, respectively. Gentamicin-resistant trans-conjugants were grown in LB agar plates containing 50 μg mL^{-1} gentamicin, 50 μg mL^{-1} Irgasan (Sigma) and 5% sodium sulfate. Mucoid mutants were selected and the transposon insertion site was determined by arbitrary PCR as described by Kim *et al.* (2012).

Complementation of *lpxC* mutant

A low copy plasmid pJN105 was used for complementation of the *lpxC* mutant. The gentamicin resistance gene of pJN105 was replaced with a carbenicillin resistance marker and named pJN105c (Table 1). The *lpxC* gene of the parental strain CM21 was amplified and cloned into pJN105c. Primers to amplify the *lpxC* ORF and its endogenous promoter region were *lpxC*_EcoRI_F: 5′-CC ATTGAATTCTGGAGAAACCGGTGAAGGTCG-3′ and *lpxC*_ EcoRI_R: 5′-ATGTGGAATTCGAAAACCTTCGCGAAT CCGCC-3′ (EcoRI restriction recognition sequence underlined in each primer sequence). The CM21 *lpxC*::Tn mutant was then transformed with pJN105c::*lpxC* or pJN105c by electroporation. Carbenicillin at 50 or 75 μg mL^{-1} was used for selective growth in broth or on agar plates, respectively.

Growth curve measurement

Pseudomonas aeruginosa strains PAO1 and CM21 were grown in LB or in LB supplemented with 5% sodium sulfate for 20 h at 37 °C with shaking at 200 r.p.m. Initial culture volume was 10 mL and bacterial growth was monitored by measuring OD_{600} values.

DsRed fluorescence mesurement

CM21 transformed with pMEXR*algD* plasmid was incubated in 352 mM sodium sulfate, ammonium sulfate,

Table 1. Strains and plasmids used in the study

Strain or plasmid	Relevant characteristic(s)	Reference or source
P. aeruginosa		
PAO1	Nonmucoid prototype strain; Alg^-	Yoon *et al.* (2002)
FRD1	CF isolate; Alg^+	Yoon *et al.* (2006)
PAO1 *mucA*::Tn	Transposon insertion mutant of *mucA*; Alg^+	This study
CM2	CF isolate; Alg^-	This study
CM4	CF isolate; Alg^+	This study
CM6	CF isolate; Alg^-	This study
CM8	CF isolate; Alg^+	This study
CM9	CF isolate; Alg^+	This study
CM12	CF isolate; Alg^+	This study
CM13	CF isolate; Alg^+	This study
CM15	CF isolate; Alg^+	This study
CM19	CF isolate; Alg^+	This study
CM21	CF isolate; Alg^+	This study
CM22	CF isolate; Alg^-	This study
CM23	CF isolate; Alg^+	This study
CM24	CF isolate; Alg^-	This study
CM25	CF isolate; Alg^-	This study
CM28	CF isolate; Alg^-	This study
CM46	CF isolate; Alg^+	This study
CM51	CF isolate; Alg^+	This study
CM52	CF isolate; Alg^+	This study
CM21 *lpxC*::Tn	Transposon insertion mutant of *lpxC*	This study
CM21 *lpxC*::Tn/pJN105c::*lpxC*	*lpxC*::Tn complementation strain	This study
E. coli		
SM10/λpir	Donor strain, Km^r, *thi-1, thr, leu, tonA, lacY, supE, recA::RP4-2-Tc::Mu, λpir*	Laboratory strain
Plasmids		
pBTK30	Transposon vector for construction of a random mutant library, Gm^r	Kim *et al.* (2012)
pMEXR*algD*	Transcriptional fusion of *algD* promoter with a gene encoding DsRed, Gm^r	This study
pJN105c	pJN105 with carbenicillin resistance marker in replace of gentamicin resistance marker	This study
pJN105c::*lpxC*	pJN105c with wild-type copy of *lpxC* gene with its endogenous promoter	This study

Alg^+, mucoid phenotype; Alg^-; nonmucoid phenotype.

magnesium sulfate and sodium chloride, and 50 μg mL^{-1} gentamicin added to LB broth, consisting of 1% tryptone (w/v) and 0.5% yeast extract (w/v), with vigorous shaking for 24 h at 37 °C. Each 200-μL aliquot of bacterial culture was transferred to a 96-well plate. Fluorescence was measured using a fluorometer. Excitation and emission wavelength were 555 and 600 nm, respectively. For normalization based on OD_{600} value, each 1-mL aliquots of bacterial culture was pelleted by centrifugation at 16000 $\times$ ***g*** for 5 min and resuspended in phosphate-buffered saline.

Confocal microscopy

Bacterial shape and red fluorescent signal were observed using FV-1000 confocal microscope (Olympus Optical, Tokyo, Japan) following procedures described previously (Yoon *et al.*, 2011; Lee *et al.*, 2012). Excitation and emission wavelength were 555 and 600 nm, respectively, for observation of DsRed fluorescence.

Statistical analysis

Data are expressed as means ± SD. An unpaired Student's *t*-test was used to analyse the average difference between the nonsulfate and sulfate-treated groups, and a P value of < 0.05 was considered statistically significant. All experiments were repeated to verify the reproducibility.

Results

Alginate production was decreased during growth with 5% sodium sulfate

To determine the growth condition that suppresses alginate production, a mucoid CF isolate strain, CM21, was grown in plates of the phenotype microarray (PM). After overnight growth, strain CM21 was reinoculated into LB agar plates using a 96-well pin replicator and we screened for CM21 colonies that showed a nonmucoid phenotype. Among 1919 wells, reduced alginate production was only

observed in D08 well of the Microplate No. 9 that contained 5% sodium sulfate. Quantitative alginate assay further confirmed suppressed alginate production by sodium sulfate. As shown in Fig. 1a, the level of alginate produced by CM21 during growth with sodium sulfate was significantly decreased when compared with that of LB-grown CM21. The suppressed level of alginate produced by CM21 was similar to that produced by PAO1, a nonmucoid prototype *P. aeruginosa* strain (Fig. 1b), suggesting that alginate production was almost completely inhibited by sodium sulfate. A well-known mucoid strain FRD1 (Lee *et al.*, 2013) also responded to the presence of sodium sulfate and decreased production of alginate was observed (Fig. 1a). Moreover, PAO1-derived *mucA* mutants, which were determined to be mucoid (Xie *et al.*, 1996), also responded to the presence of sodium sulfate and exhibited nonmucoid phenotype (Supporting Information, Fig. S1). We then examined whether alginate production is affected by sodium sulfate in a dose-dependent manner. A gradual decrease in alginate production was observed in response to increasing concentrations of sodium sulfate (Fig. 1b). Together, our results demonstrated that sodium sulfate can suppress alginate production in mucoid *P. aeruginosa* strains.

Environmental factors that influence bacterial growth, such as temperature (Leitao *et al.*, 1992) and oxygen potential (Krieg *et al.*, 1986), were reported to play roles in alginate production. We therefore explored whether suppressed alginate production is associated with altered bacterial growth by sodium sulfate. Based on our growth curve experiments shown in Fig. 1c and d, bacterial growth was only marginally affected by sodium sulfate. Although a 2-h delay was observed during the exponential phase of growth, final OD_{600} values were comparable between growth in LB and LB supplemented with 5% sodium sulfate (Fig. 1c and d). Of note is that such a delay was invariably observed, irrespective of whether the tested strain was nonmucoid or mucoid. These results suggest that sulfate-stimulated suppression of alginate production is probably not due to the growth inhibition of the CM21 mucoid strain by sodium sulfate.

Sulfate-stimulated suppression of alginate production in other mucoid *P. aeruginosa* CF isolates

Next, we sought to elucidate the effect of sodium sulfate on bacterial ability to produce alginate in 17 other *P. aeruginosa* CF isolates. Among these strains, CM02, CM06, CM22, CM24, CM25 and CM28 were nonmucoid, while the other 11 strains were mucoid. To verify sulfate-induced conversion to a nonmucoid phenotype in a quantitative manner, we used an alginate assay with bacterial culture supernatants. As shown in Fig. 2, all the mucoid strains showed decreased levels of alginate when grown with 5% sodium sulfate, although bacterial response to sodium sulfate varied between strains. CM4, CM12 and CM51 exhibited the most dramatic decrease in alginate production in response to the treatment, while strain CM52 showed only minimal decrease in the assay (Fig. 2). Again, nonmucoid strains grown in LB media produced low levels of alginate, further verifying the specificity of our alginate assay.

Other sulfate salts also suppressed alginate production in the mucoid CM21 strain

Our results demonstrated that sodium sulfate can suppress alginate production in all the tested mucoid strains. Next, we examined whether the inhibitory alginate production can also be achieved by other sulfate salts. When strain CM21 was grown in LB containing equal concentrations (352 mM) of ammonium sulfate or magnesium sulfate, we observed a similar degree of suppression of alginate production (Fig. 3). To rule out the possibility that the suppressed alginate production is caused by osmotic stress due to the presence of high concentrations of sulfate salts, we tested the effect of NaCl on alginate production. Of note, CM21 strain produced higher levels of alginate during growth in LB supplemented with NaCl. Together, these results suggest that suppressed production of alginate by sodium sulfate is induced by the sulfate ion, and not by sodium ions or by any secondary effect due to the presence of high osmotic stress.

Sulfate-induced suppression of alginate production is regulated at the transcriptional level

Next, we sought to assess the effect of sulfate on expression of the *algD* gene, the first gene of the alginate biosynthesis operon that encodes GDP-mannose 6-dehydrogenase. To address this, we used a transcriptional fusion construct, pMEXR*algD*, in which the DsRed-coding gene was cloned downstream of the *algD* promoter (Toyofuku *et al.*, 2007, 2014). PAO1 transformed with pMEXR*algD* failed to exhibit a red fluorescent signal, while a robust red fluorescent signal was detected in CM21 harboring the same plasmid (Fig. 4a and f). This result further confirmed that the alginate biosynthesis operon is actively transcribed in the mucoid CM21 strain. Note that the red fluorescent signal was significantly decreased in the CM21/pMEXR*algD* strain grown in LB amended with sodium sulfate, ammonium sulfate or magnesium sulfate (Fig. 4g–i). Consistent with this finding, alginate production was suppressed under these growth conditions (Fig. 3). When grown with NaCl, a culture condition that permitted

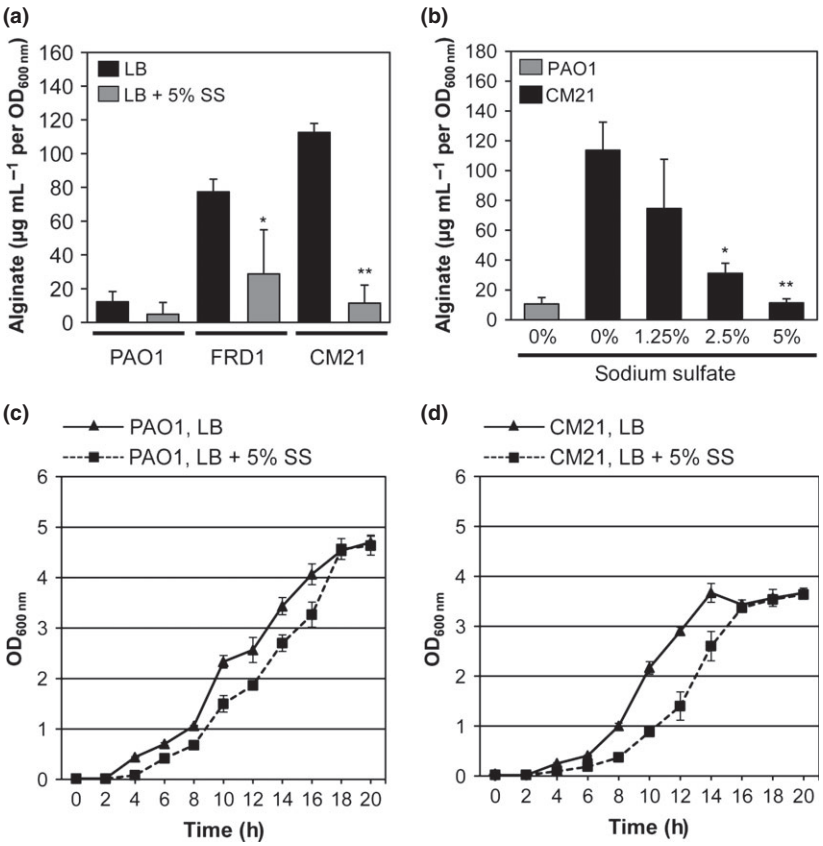

Fig. 1. Identification of sodium sulfate as a molecule that can suppress alginate production. (a) Levels of alginate produced by PAO1 (nonmucoid), CF isolate FRD1 (mucoid) and CM21 (mucoid) strains in LB (black bars) and in LB containing 5% sodium sulfate (SS, gray bars). The number of replicates was three and mean values ± SD are displayed in each bar. *$P < 0.05$ vs. alginate levels from LB-grown FRD1, **$P < 0.005$ vs. alginate levels from LB-grown CM21. (b) Dose-dependent effect of sodium sulfate on alginate production. Mucoid CM21 strain was grown in LB with increasing concentrations of sodium sulfate. *$P < 0.05$ vs. alginate levels from CM21 grown in LB, **$P < 0.005$ vs. alginate levels from LB-grown CM21. The number of replicates was six and mean values ± SD are displayed in each bar. (c, d) Effect of 5% sodium sulfate on bacterial growth. (c) Nonmucoid wild-type PAO1 and (d) mucoid CF isolate CM21 were grown in LB (solid line with black triangles) and in LB containing 5% sulfate (dotted line with black squares) for 20 h. Bacterial growth was monitored by measuring the optical density (OD) at 600 nm every 2 h.

robust alginate production, the CM21/pMEXR*algD* strain exhibited a comparable level of red fluorescent signal (Fig. 4j). Consistent with the image analysis, red fluorescent intensities of CM21/pMEXR*algD* cells were significantly decreased when grown with sodium sulfate, ammonium sulfate and magnesium sulfate (Fig. S2). Intriguingly, we observed significant cell shape changes of the mucoid CM21 strain in response to growth with sodium sulfate, ammonium sulfate or magnesium sulfate. A round cell shape was observed when grown with sodium sulfate (Fig. 4g) or ammonium sulfate (Fig. 4h), whereas CM21 strain became highly elongated upon growth with magnesium sulfate (Fig. 4i). Cell shape changes were not detected in the nonmucoid PAO1 strain under these growth conditions (Fig. 4b–d). Together, our results suggest that sulfate-induced suppression of alginate production is probably accompanied by bacterial cell shape change.

Isolation of CM21-derived mutants that remained mucoid during growth with sodium sulfate

To provide insight into the nonmucoid conversion in response to sodium sulfate, we screened a library of CM21 transposon insertion mutants looking for a mutant that remained mucoid during sulfate-stimulated growth. Among *c.* 32 000 mutants tested, three independent mutants were found to be mucoid in LB agar plates containing 5% sodium sulfate. Importantly, all of the three mutants were determined to have transposon insertion in the *lpxC* (PA4406) gene. Transposon insertion occurred at different sites, all of which were located near the 3′ end of the gene (data not shown).The *lpxC* gene encodes LpxC enzyme, UDP-3-*N*-acetylglucosamine deacetylase, a key enzyme involved in lipid A biosynthesis, the first committed step for lipopolysaccharide (LPS) production (Hyland *et al.*, 1997). The *lpxC*::Tn mutant maintained a mucoid phenotype in the presence of 5% sodium sulfate, while its parental CM21 strain again showed nonmucoid colonies in the same growth media, and a quantitative alginate assay further verified that the *lpxC*::Tn mutant continuously produced alginate in LB media containing sodium sulfate (Fig. 5a and b). When the mutant was complemented with its wild-type copy of the *lpxC* gene, sulfate responsiveness was recovered and thus alginate production was markedly decreased (Fig. 5c and d). Importantly, the normal rod-shape morphology was not changed after growth with sulfate salts (Fig. S3). These

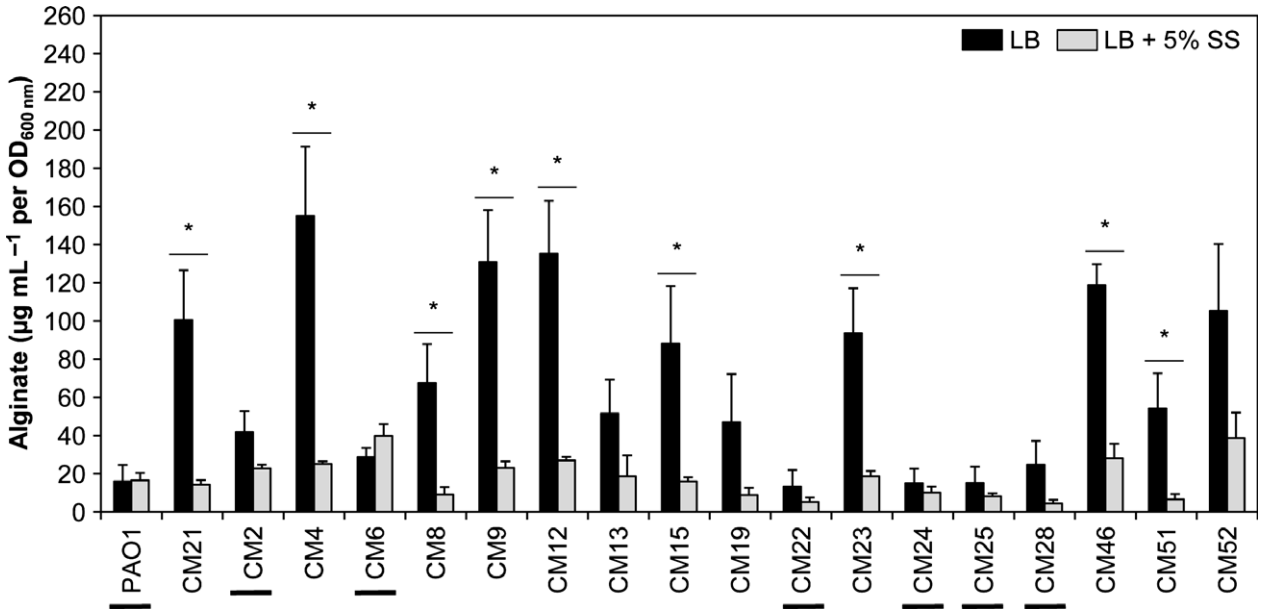

Fig. 2. Effect of 5% sodium sulfate on alginate production by various CF isolates. Seventeen additional CF isolates (CM2–CM52) were grown in LB (black bars) and in LB + 5% sodium sulfate (gray bars) and alginate levels were measured. Six strains (underlined) were nonmucoid. The experiment was performed in triplicate and mean values ± SD are displayed in each bar. *$P < 0.05$ vs. alginate levels from LB-grown cells.

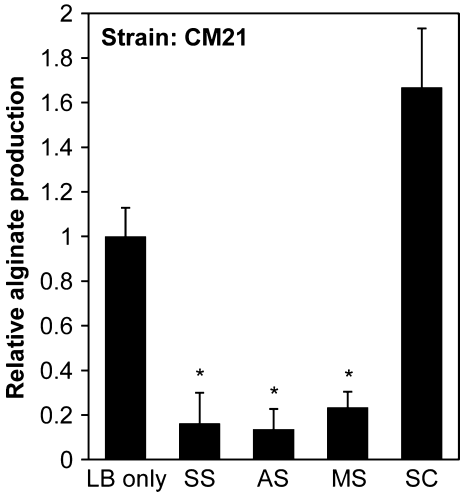

Fig. 3. Effects of diverse sulfate ions on alginate production. Strain CM21 was grown in LB containing 5% sodium sulfate (SS), ammonium sulfate (AS), magnesium sulfate (MS) or sodium chloride (SC). The alginate level produced by CM21 grown in plain LB was used as a control and normalized to 1.0. The number of replicates was six and mean values ± SD are displayed in each bar. *$P < 0.05$ vs. alginate levels from LB-grown CM21.

results suggest that changes in cell shape are associated with nonmucoid conversion and a cellular mechanism for LPS biosynthesis plays a role in responding to sulfate salts.

Discussion

Most *P. aeruginosa* isolates from chronic CF patients have the capacity to overproduce alginate, a capsular polysaccharide, to protect themselves from the stressful environment of the lung (Yoon *et al.*, 2006). As the alginate acts as a physical barrier to antimicrobial agents and host immune cells (May *et al.*, 1991), suppression of alginate production would be beneficial in treating CF patients. In this study, we identified the sulfate ion as a molecule that can specifically suppress alginate production in mucoid *P. aeruginosa* strains. Furthermore, we presented genetic evidence that strongly suggests a potential involvement of LPS biosynthesis in this process.

PM has been used to assess the effects of diverse chemical compounds on bacterial growth (Zhang & Biswas, 2009; Bochner *et al.*, 2010; Peleg *et al.*, 2012; Chen *et al.*, 2013) and therefore provide a better understanding of the phenotype-to-genotype relationship (Bochner, 2003). PM is also used to analyse bacterial resistance to a variety of stress conditions, such as osmotic shock, pH changes and antimicrobial agents (Bochner *et al.*, 2008). In the aforementioned studies, bacterial responses were monitored by measuring the rate of the organism's respiration, which reflects bacterial growth (Vaas *et al.*, 2012). In this study, PM was applied to identify a chemical that can alter bacterial colony morphotype. This was made possible by simply examining the colony phenotype of a mucoid *P. aeruginosa* strain that had been grown in each well of the PM.

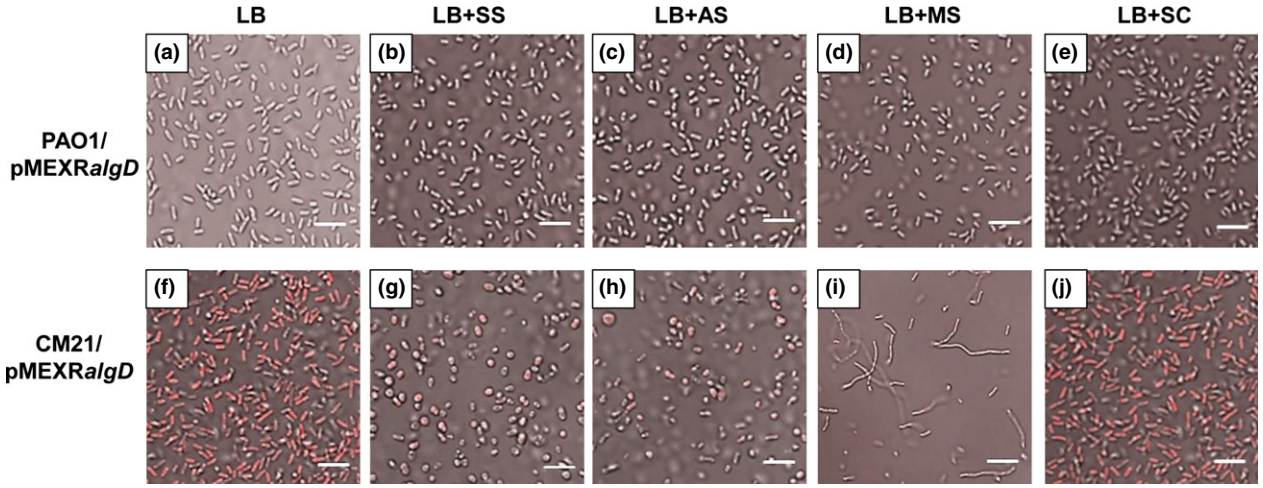

Fig. 4. Confocal micrographs of *algD* promoter reporter strains. Nonmucoid wild-type strain PAO1 and mucoid CF isolate strain CM21 were transformed with the plasmid pMEXR*algD*, in which the *algD* promoter was fused with the coding sequence of DsRed red fluorescent protein. Reporter strains were grown in LB (a and f), LB+SS (b and g), LB+AS (c and h), LB+MS (d and i) or LB+SC (e and j) and analyzed for red fluorescence. All images were acquired at ×1000 magnification. Scale bars = 5 μm.

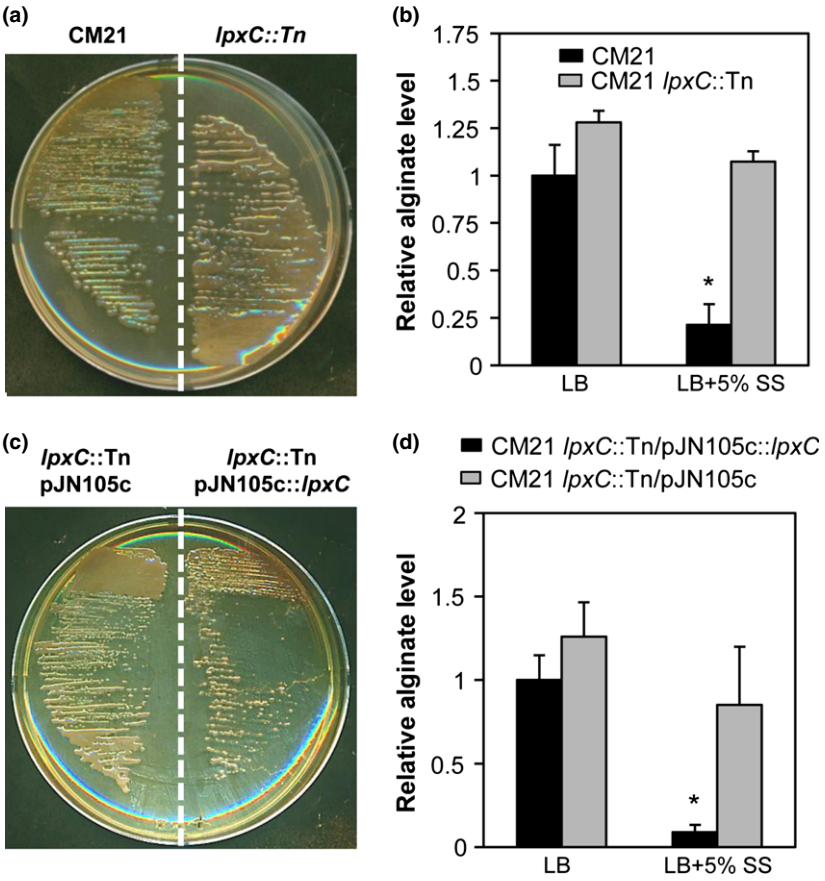

Fig. 5. Effect of the *lpxC* gene mutation on the ability of CM21 to produce alginate. (a) Colony morphotype of CM21 and CM21 *lpxC*::Tn mutant in an LB agar plate containing 5% sodium sulfate. (b) Levels of alginate produced by CM21 (black bars) and its *lpxC* mutant (gray bars). The alginate level produced by CM21 grown in plain LB was used as a control and normalized to 1.0. The experiment was performed in triplicate and mean values ± SD are displayed in each bar. **P* < 0.05 vs. alginate levels from LB-grown CM21. (c) Colony morphotype of the control strain, CM21 *lpxC*::Tn/pJN105c, and complemented strain, CM21 *lpxC*::Tn/pJN105c::*lpxC*. (d) Levels of alginate produced by the *lpxC* complemented strain, CM21 *lpxC*::Tn/pJN105c::*lpxC* (black bars), and the control strain, CM21 *lpxC*::Tn/pJN105c (gray bars). The alginate level produced by the complemented strain grown in plain LB was used as a control and normalized to 1.0. The experiment was performed in triplicate and mean values ± SD are displayed in each bar. **P* < 0.05 vs. alginate levels from LB-grown CM21 *lpxC*::Tn/pJN105c::*lpxC*. The experiment was performed in triplicate and means ± SD are displayed in each bar. **P* < 0.05 vs. alginate levels from LB-grown *lpxC*::Tn mutant.

Our results clearly suggested that sulfate-induced suppression of alginate production occurred in association with cell shape change. Because such a morphological change was not observed in nonmucoid PAO1, the sensitivity of mucoid strains to sulfate ions is probably due to the distinct differences in cell surface properties between

mucoid and nonmucoid cells. To produce and secrete alginate, a polymeric substance with high molecular weight, mucoid *P. aeruginosa* requires a multiprotein complex spanning the outer membrane, the periplasm and the inner membrane (Remminghorst & Rehm, 2006). Our results also showed that the transcriptional activity of the *algD* promoter was down-regulated in the presence of the sulfate ion. It will be important to investigate how sulfate-induced changes in cell surface properties lead to the signal that eventually suppressed the transcription of the alginate operon.

Changes in bacterial cell morphology are triggered by various stimuli. During anaerobic NO_3^- respiration, cell elongation was observed in nonmucoid *P. aeruginosa* (Yoon *et al.*, 2011). Of note is that such a unique cell shape change was not detected in mucoid *P. aeruginosa*. Together, these results suggest that bacterial response to a given stimulation may differ in nonmucoid vs. mucoid *P. aeruginosa*. Recently, it was reported that nonmucoid strains of *P. aeruginosa* rapidly converted to a spherical shape in response to treatment with a class of β-lactam antibiotics (Monahan *et al.*, 2014). Again, it will be of interest to examine if the similar change can be induced in mucoid strains.

Transposon mutants disrupted in the *lpxC* gene were not responsive to sulfate ions and remained mucoid in the presence of 5% sulfate. The recovery of *lpxC* mutants was counterintuitive, because the *lpxC* gene was considered to be an essential gene (Barb & Zhou, 2008). For the regulation of lipid A level, *E. coli* utilizes FtsH protease that recognizes a conserved degradation motif located in the C-terminal region of LpxC (Ogura *et al.*, 1999). As the level of lipid A increases, FtsH protease degrades LpxC to maintain balanced lipid A levels for membrane stability and viability (Katz & Ron, 2008). Unlike *E. coli* LpxC, *P. aeruginosa* LpxC was reported to be highly stable under conditions in which FtsH is active (Langklotz *et al.*, 2011). Moreover, chemical inhibitors for *E. coli* LpxC failed to inhibit *P. aeruginosa* LpxC (Mdluli *et al.*, 2006). These findings suggest that regulation of LPS content in *P. aeruginosa* may not be mediated by a conventional process that involves proteolytic degradation of LpxC. In the current study, three independent *lpxC* mutants were recovered with robust ability to produce alginate in sulfate media. Importantly, the mutant cells maintained their normal morphology (Fig. S3). Although more experiments are necessary to precisely determine the mechanism involved, uninterrupted production of lipid A is required for the sulfate-induced conversion to a nonmucoid phenotype in mucoid *P. aeruginosa* strains. Of note is that lipid A extracted from mucoid *P. aeruginosa* isolates was found to be modified with palmitate or aminoarabinose and these modifications were not detected in environmental isolates (Ernst *et al.*, 2007). Therefore, it is likely that an altered lipid A moiety is responsible for the "mucoid-specific" response to excess sulfate.

In conclusion, we have identified a novel mechanism by which the mucoid phenotype of clinical *P. aeruginosa* isolates can be shut off. Development of an effective strategy to treat mucoid *P. aeruginosa* infection is necessary and will be facilitated by establishing a means of reverting mucoid isolates to the nonmucoid phenotype. We anticipate that our current results will stimulate further investigations, with the ultimate goal of eradicating this clinically important opportunistic pathogen.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Colony morphology of PAO1 *mucA*::Tn strain grown in LB agar plate containing either 0% or 5% sodium sulfate.

Fig. S2. Relative fluorescence level of CM21 transformed with pMEXR*algD* plasmid.

Fig. S3. Confocal microscope images of CM21 *lpxC*::Tn strain grown under 5% sodium sulfate (SS), ammonium sulfate (AS), magnesium sulfate (MS) and sodium chloride (SC).