Non-mucoid Reversion of Mucoid *Pseudomonas aeruginosa* Induced by Sulfate-stimulated Growth

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Non-mucoid Reversion of Mucoid *Pseudomonas aeruginosa* Induced by Sulfate-stimulated Growth

(Directed by Professor Sang Sun Yoon)

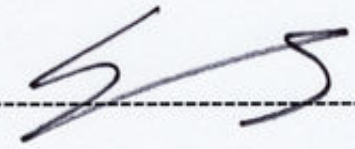
The Master's Thesis
submitted to the Department of Medical Science,
The Graduate School of Yonsei University
in partial fulfillment of the requirements for the
degree ofMaster of Medical Science

Kyung Bae Min

June 2014

This certifies that the Master's Thesis

of Kyung Bae Min is approved.



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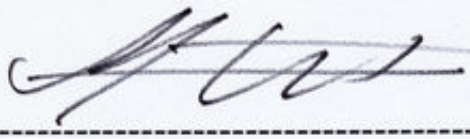

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ACKNOWLEDMENTS

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2014년 6월

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ABSTRACT

Non-mucoid reversion of mucoid *Pseudomonas aeruginosa* induced by sulfate-stimulated growth

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(Directed by Professor Sang Sun Yoon)

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 *algD* promoter was considerably decreased when grown with sulfate. Furthermore, bacterial cell shape was abnormally altered in CM21, but not in PAO1, a prototype non-mucoid 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 LPS biosynthesis in the sulfate-induced reversion to non-mucoid phenotype. Together, this study proposes a novel strategy that can be potentially applied to treat persistent infection by recalcitrant mucoid *P. aeruginosa*.

Key words: *pseudomonas aeruginosa*, alginate production, cystic fibrosis

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I. INTRODUCTION

The Gram-negative bacterium *Pseudomonas aeruginosa* is a major opportunistic pathogen in human respiratory disease, Cystic Fibrosis (CF). In chronic CF, *P. aeruginosa* has been changed to get several selected phenotypes for surviving in harsh human lung environment[1-3]. One of these changes is an overproduction of capsule-like polysaccharide called alginate[4]. Overproduction of alginate leads to morphological change which is seen to gelatinous, mucoid. Mucoid CF strain isolated from sputum of CF patient has mutated *mucA* gene. The MucA is a negative regulator of AlgT/U which is a stress related alternative sigma factor that drives *algD* gene expression[5]. Because of sequestered AlgT/U by MucA[6], malfunctioning MucA continuously leads to express alginate operon that consists of 13 genes, and

that is the primary reason to be a mucoid strain[6-9]. There are several mechanisms that are reported to induce the overproduction of alginate involved in MucA malfunction. In another study, stringent starvation response or sigma factor competition can regulate mucoidy[10,11].

In persistence infection, expression of virulence factor is more down regulated than acute infection, and this is undergone for thriving in infected tissue or organ[12]. Pathological roles of alginate are a barrier of phagocytic killing[13,14] and one of factors to be clinical difficulties by increased antibiotic resistance and evasion of the host immune response[14].

Previous research reported some cases of reversion from mucoid to non-mucoid. This reversion can be selectively arisen from low oxygen tension growth condition[15], caused by spontaneous *algT/U* mutation[16]. In addition, sigma factor RpoD can suppress the alginate production through competition with AlgT/U[10]. Those great studies suggest a regulation process of alginate production, but are not shown to induce suppression of alginate production without genetic techniques. In this work, we performed a Phenotype Microarray with clinically isolated *P. aeruginosa* strain and revealed that alginate production was suppressed by high concentration of sulfate ion. Addition 5% sodium sulfate to media shows that alginate production is reduced and related with LpxC which engages in committed step in lipid A synthesis. Transposon random mutagenesis for selecting a mucoid strain on 5% sulfate added media shows that regulation of lipid A levels is involved in alginate production. This report uncovers a previously undescribed means to enfeeble mucoid *P. aeruginosa*, a formidable adversary to human healthcare.

II. MATERIALS AND METHODS

1. Bacterial strains and growth condition

Bacterial strains and plasmid used in this study are shown in Table 1. CF isolate strains, *P. aeruginosa* PAO1 and *E. coli* are grown at 37˚C in Luria Bertani (LB) agar plate or LB broth. Gentamycin (Sigma-Aldrich Co. St. Louis, MO) and Irgasan (Sigma-Aldrich Co.) were added to media for selecting transformed or conjugated bacteria. Concentration of gentamycin and Irgasan added to LB plate or LB broth were 50μg/ml and 25μg/ml for selecting transformed bacteria. For selecting conjugated CF isolate CM21, 100μg/ml of gentamycin and 25μg/ml of Irgasan were added in LB agar plate. Filtered sodium sulfate, ammonium sulfate, magnesium sulfate and sodium chloride were added in LB plate and LB broth and concentration of each of sulfate salts was equally 352mM. Sodium sulfate, ammonium sulfate, magnesium sulfate and sodium chloride were all purchased from Sigma-Aldridge Co.

2. Phenotype Microarray

Phenotype Microarray (Biolog Inc., Hayward CA. USA) experiments were performed as described elsewhere[17]. The Phenotype Microarray (PM) was performed as manufacture's protocol with wild type strain *P. aeruginosa* PAO1 and CF isolate mucoid strain CM21. All colonies of PAO1 and CM21 incubated on LB plate 37˚C for 24 hours at were harvested by using sterile cotton swabs. The harvested colonies were suspended in 16ml IF-0 GN base media (Biolog Inc., Catalog#72256) to achieve 42% transmittance (T) in the Biolog Tubidimeter (Biolog Inc., Catalog#3531). 15ml of 42% T cells suspended IF-0 GN base media was added to 90ml of IF-0 media mixed with 0.9ml dye mix A (Biolog Inc., Catalog#74221) to obtain 85% T final cell density for inoculation. 0.6μl of 85% T cell suspension was transferred to 120ml IF-10 media (Biolog Inc., Catalog#72254)

with 1.2 ml dye mix A to inoculate PM Microplate No. 9 to No. 20. 22ml of the 85% T 90ml of IF-0 media inoculum was inoculated to PM Microplate No. 1 and No. 2. Remaining 68ml of 85% T cell suspension was inoculated to PM Microplate No. 3 to No. 8. 680µl of 2M sodium succinate (Sigma-Aldrich Co.) was added to 68ml 85% T cell suspension, and used as sole carbon source of PM Microplate No. 3 to No. 8. All inoculation volume of all wells were 100µl per well. All PM Microplates were incubated at 37˚Cfor 24 hours using OmniLog PM Incubator (Biolog Inc., Catalog#91171).

3. Bacterial transformation and conjugation

Mucoid CF isolate CM21 was electro-transformed as previously described[18]. Transformation from *E.coli* to CF isolate was performed via general cloning protocol to transfer transposon containing plasmid pBTK30.

4. Alginate assay

P. aeruginosa PAO1 and CF isolate 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 hours with vigorous shaking, respectively. Bacterial cells were pelleted by centrifugation at 13,000 rpm for 15 min and resuspended in PBS. OD_{600} values of bacterial suspensions were used for normalization. Bacterial supernatants was diluted to 1:10 ratio with distilled water, and used for carbazole-borate assay to measuring D-mannuronate, as previously described[19]. Distilled water and LB broth were used as blank sample. The amount of uronic acid was analyzed with a standard curve constructed with the use of alginic acid from brown algae (Sigma-Aldrich Co.) which was dissolved in sodium bicarbonate solution. The used concentration of alginic acid from brown algae was 50µg/ml. measurement of bacterial density and amount of alginate was performed in 96-well plate.

5. Growth curve measurement

Wild type strain *P. aeruginosa* PAO1 and CF isolate strain CM21 were incubated in LB and LB+SS condition at 37˚C with full aeration for 16 hours, respectively. Incubation volume of each condition was 20ml in 200ml flask and OD_{600} value of each condition was measured by spectrophotometer by every 2 hours using cuvette.

6. Non-mucoid reverted colony screening

All wells of PM Microplates used for PM experiment were used as sources to screen non-mucoid revertant. 96-well pin replicator was used to inoculate all wells of PM Microplate onto fresh LB agar plates. All inoculates on LB agar plates were incubated at 37˚C for 24 hours. Non-mucoid revertant was screened by comparing to morphological characteristic change between mucoid and non-mucoid .

7. Transposon random mutagenesis and PCR

For a random transposon insertion mutagenesis, *E. coli* SM10λpir harboring pBTK30[20] was used as donor strain. Targeted recipient strain was mucoid CF isolate CM21. Donor and recipient strain were incubated in gentamycin added LB plate and LB plate, respectively. After incubation, all of donor and recipient colonies on the plate were scrapped and mixed on LB plate. The mixture was incubated for 6hours at 37˚C. After 6 hours incubation, the mixture was harvested into 50ml around bottom tube (SPL) and spread onto gentamycin, Irgasan and 5% sodium sulfate added selective agar plate. Gentamycin-resistant trans-conjugants were grown in LB agar plates containing 50μg/ml gentamycin, 25μg/ml Irgasan and 5% sodium sulfate. Mucoid mutants were selected and transposon insertion site was determined by arbitrary PCR reaction as described elsewhere[20].

To confirm transposon insertion, PCR was performed with transposon specific primer and *lpxC* matched primer. *lpxC* matched region primer sequence was 5’

AGTACTTCCTTCGTCAAGGAG 3'. Transposon specific primer sequence was 5' CTTACCAGGCCACGCGTCGACTAGTAC 3'.

8. *mucA* gene sequencing

For sequencing *mucA* gene of CF ioslates, irreversibly reverted CM21 and CF isolate FRD1, CM21, CM2, CM4, CM6, CM8, CM9, CM12, CM13, CM15, CM19, CM22, CM23, CM24, CM25, CM28, CM46, CM51 and CM52 were grown in LB broth at 37˚C for overnight. Genomic DNA of all strains was extracted using G-Spin™ for bacteria Genomic DNA Extraction Kit (iNtRON Biotechnology, Korea) by manufacture's protocol. The genomic DNA was used as template for PCR of *mucA* gene.

Used PCR primers were mucA_Forward 5'-AAGAGAGGTATCGCTATGAGTC-3' and mucA_Backward 5'-AGGTGGTGCGCATGTCTCTC-3' designed to cover upstream 15bp length and downstream 21bp length sequence from *mucA* ORF. PCR products were confirmed using gel electrophoresis with 1.5% agarose gel. *mucA* bands in the gel were extracted using QIAquick® Gel Extraction Kit (QIAGEN, Hilden, Germany) following manufacturer's protocol for sequencing. Sequence information of *mucA* fragment of all strain was obtained from MACROGEN (Korea) and sequence alignment was performed by web based alignment tool CLUSTALW (www.genome.jp).

9. Confocal microscope

Bacterial shape and red fluorescent signal were observed using LSCM (FV-1000; Olympus Optical Co. Ltd., Japan) following procedures described previously[21,22]. Excitation and emission wavelength were 555 and 600 nm for observation of DsRed fluorescence.

10. Statistical analysis

Data are expressed as means ± SD. Unpaired Student's t-test was used to analyze the data and a p-value of <0.05 was considered statistically significant. All the experiments were repeated to verify the reproducibility.

Table 1. Strains and plasmids used in the study

Strain or plasmid	Relevant characteristic(s)	Reference or source
P. aeruginosa		
PAO1	Wild type strain; Alg^-	Laboratory strain
FRD1	Wild type strain; Alg^+	Laboratory strain
CM21	CF isolate strain; Alg^+	This study
CM2	CF isolate strain; Alg^+	This study
CM4	CF isolate strain; Alg^+	This study
CM6	CF isolate strain; Alg^-	This study
CM8	CF isolate strain; Alg^+	This study
CM9	CF isolate strain; Alg^+	This study
CM12	CF isolate strain; Alg^+	This study
CM13	CF isolate strain; Alg^+	This study
CM15	CF isolate strain; Alg^+	This study
CM19	CF isolate strain; Alg^+	This study
CM22	CF isolate strain; Alg^-	This study
CM23	CF isolate strain; Alg^+	This study
CM24	CF isolate strain; Alg^-	This study
CM25	CF isolate strain; Alg^-	This study
CM28	CF isolate strain; Alg^-	This study
CM46	CF isolate strain; Alg^+	This study
CM51	CF isolate strain; Alg^+	This study
CM52	CF isolate strain; Alg^+	This study
CM21Δ*lpxC*	Transposon insertion mutant of *lpxC* (PA4406)	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	GM^r, transposon cassette inserted plasmid for transposon random mutagenesis	Kim S, et al., 2012
pMEXR*algD*	Transcriptional DsRed-P*algD* fusion, DsRed version of pMEXRGFP, GM^r	Toyofuku M, *et al.*, 2007

Alg^- represents non-mucoid phenotype.

Alg^+ represents mucoid phenotype.

III.RESULTS

1. Alginate production was decreased during growth with 5% sodium sulfate

To unravel a growth condition that suppresses alginate production, a mucoid CF isolate strain, CM21 was grown in plates of the Phenotype Microarray (PM). After overnight growth, CM21 strain was re inoculated into LB agar plates using a 96-well pin replicator and we screened for CM21 colonies that showed non-mucoid phenotype. Because operative principle of the PM detects bacterial respirational growth phenotype affected by single molecule contained in each well, subject mucoid CF isolate strain should have similar growth rate to wild type strain PAO1 which is used as reference strain. Therefore, CF isolate CM21 was chosen as subject strain since CM21 showed robust growth rate in LB condition similar to PAO1 (Fig. 3). Experimental scheme was depicted in Fig. 1. Result of the screening, reduced alginate production was only observed in D08 well of the Microplate No. 9 that contained 5% of sodium sulfate. This reverted inoculate from the well containing 5% sodium sulfate was reproducibly generated and continuously showed non-mucoid phenotype. Quantitative alginate assay further confirmed suppressed alginate production by sodium sulfate. As shown in Fig. 2a, the level of alginate produced by CM21 during growth with sodium sulfate was less than 10% of that produced by LB-grown CM21. The suppressed level of alginate produced by CM21 was similar to that produced by PAO1, a non-mucoid prototype *P. aeruginosa* strain (Fig. 2b) suggesting that alginate production was almost completely inhibited by sodium sulfate. A well-characterized mucoid strain FRD1[23] also responded to the presence of sodium sulfate and decreased production of alginate was observed (Fig. 2a). 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 the increasing concentrations of sodium sulfate (Fig. 2b). Together, our results demonstrated a successful identification of sodium sulfate as a molecule that

can suppress alginate production in mucoid *P. aeruginosa* strains.

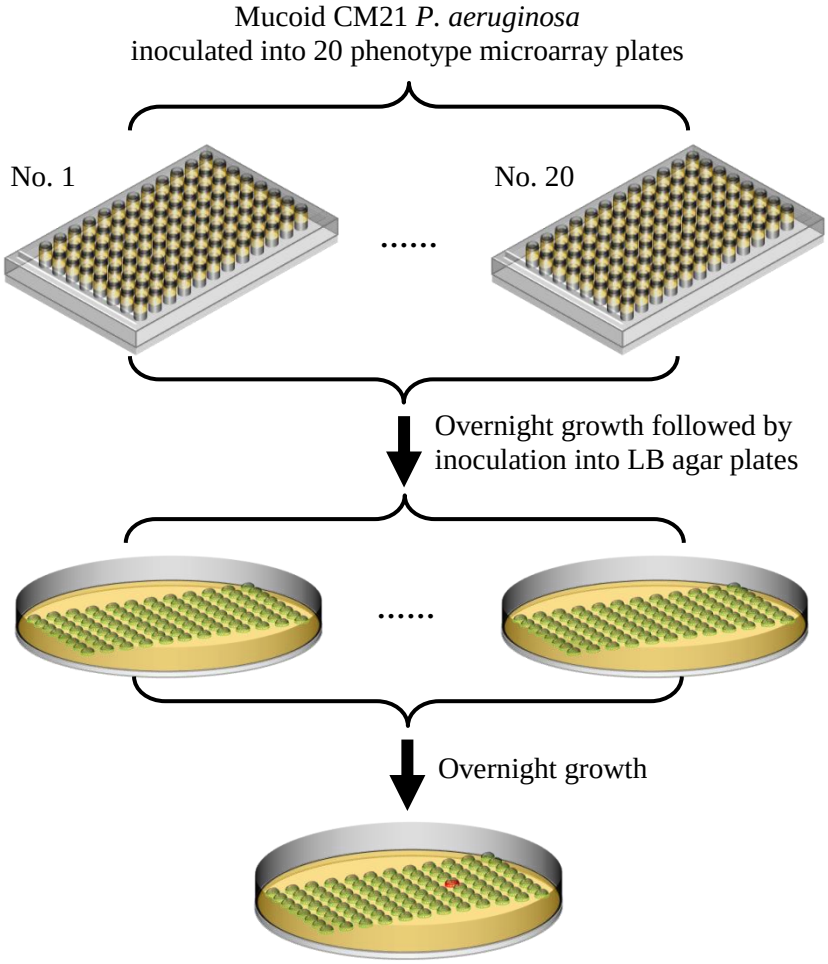

Figure. 1. Simplified scheme of the screening procedures. A mucoid CM21 strain was grown in each well of twenty different Phenotype Microarray plates. After overnight growth, bacterial cells were re inoculated into LB agar plates and bacterial colonies were screened for non-mucoid revertants.

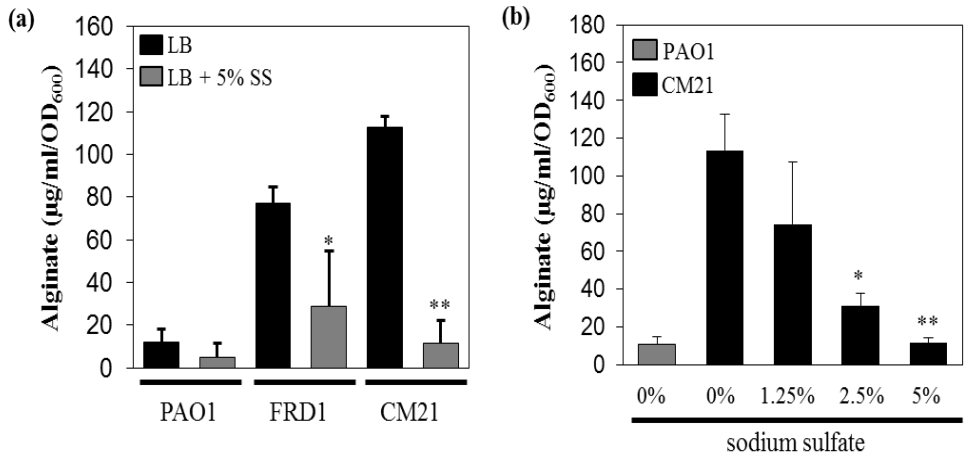

Figure. 2. Identification of sodium sulfate as a molecule that can suppress alginate production. (a) Levels of alginate produced by PAO1 (non-mucoid), CF isolate FRD1 (mucoid) and CM21 (mucoid) strains in LB (black bars) and in LB containing 5% sodium sulfate (SS, gray bars). Values of means ± SD are displayed. *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.

2. 5% sodium sulfate stimulated growth feature was not involved in alginate suppression

Environmental factors that influence bacterial growth, such as temperature[24] and oxygen potential[25] 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. 3, bacterial growth was only marginally affected by sodium sulfate. Although a 2-hour delay was observed during exponential phase, final OD_{600} values were comparable between growth in LB and LB supplemented with sodium sulfate (Fig. 3). Of note is that such a delay was invariably observed, irrespective of whether the tested strain was non-mucoid or mucoid. These results suggest that sulfate-stimulated suppression of alginate production is not likely due to the growth inhibition of the CM21 mucoid strain by sodium sulfate.

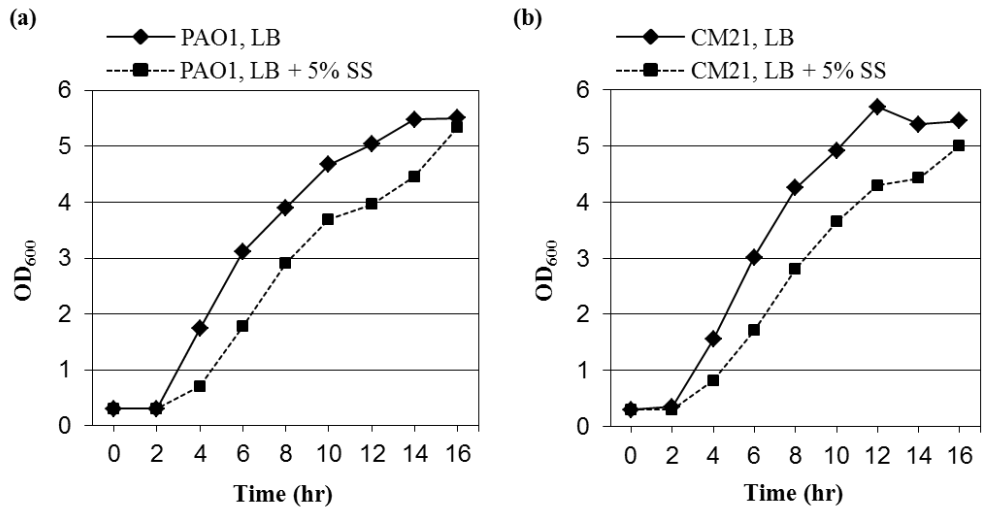

Figure. 3. Growth curve comparison between LB and 5% sodium sulfate added LB. (a, b) Effect of 5 % sodium sulfate on bacterial growth. (a) Non-mucoid wild type PAO1 and (b) mucoid CF isolate CM21 were grown in LB (solid line with black triangles) and in LB containing 5% sulfate (dotted line with black squares) for 16 hours. Bacterial growth was monitored by measuring optical density (OD) at 600 nm every 2 hours.

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

Next, we sought to elucidate the effect of sodium sulfate on bacterial capability to produce alginate in 17 more *P. aeruginosa* CF isolates. Among these strains, CM02, CM06, CM22, CM24, CM25 and CM28 were non-mucoid, while the other 12 strains were mucoid. To verify sulfate-induced reversion to non-mucoid phenotype in a quantitative manner, we performed alginate assay using bacterial culture supernatants. As shown in Fig. 4, all of mucoid strains produced decreased levels of alginate when grown with sodium sulfate, although bacterial response to sodium sulfate varied strain to strain. CM04, CM46 and CM 52 showed the highest sensitivity to the sulfate. CM4, CM8 and CM51 strains exhibited the most dramatic decrease in alginate production in response to the treatment, while CM51 strain showed only minimal decrease in the assay (Fig. 4). This result implied that almost CF isolates showed to have tendency to suppress the alginate. Although every CF isolates was not genetically identified, mucoid CF isolates were commonly expressing alginate synthesis operon to overproduce the alginate using a same alginate synthesis protein complex spanned inner and outer membrane. When this complex is failed, alginate would not be synthesized or could be degraded. Besides, mutation in *mucA* gene of all CF isolates was confirmed. Each CF isolate had different mutated region in *mucA* gene, which supported that CF isolates had widespread mutation in the genome by malfunctioned mismatch repair system. Based on these facts, incubation with appropriate concentration of sodium sulfate can reduce the amount of alginate. Again, non-mucoid strains grown in LB media produced low levels of alginate further verifying the specificity of our alginate assay. Therefore, sulfate induced alginate reduction effect is common among mucoid CF isolates and sulfate is effective only to mucoid strain.

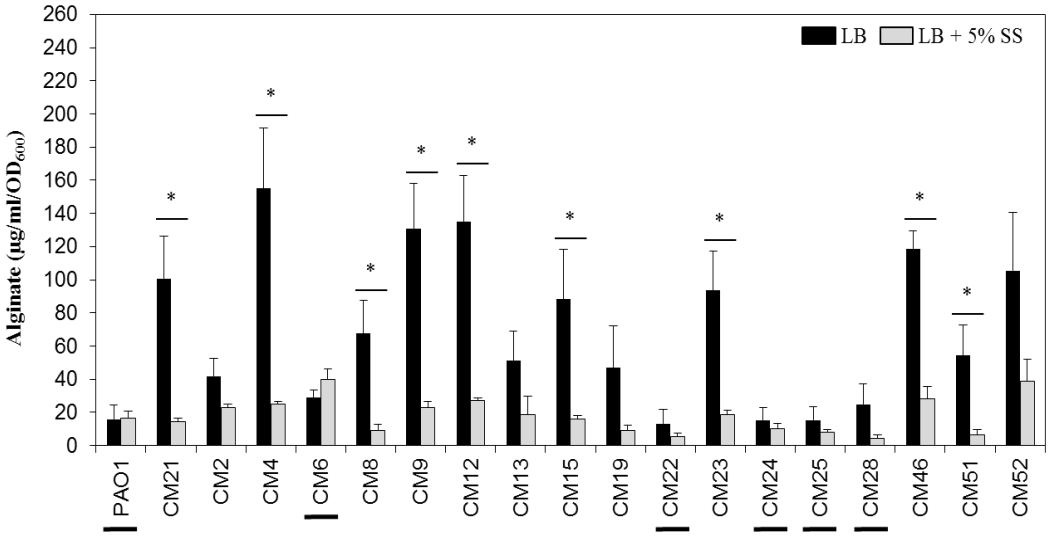

Figure. 4. Effect of 5 % sodium sulfate on alginate production by various CF isolates. Seventeen more CF isolates (CM2 ~ CM52) were grown in LB (black bars) and in LB + 5% sodium sulfate (gray bars) and alginate levels were measured. Six strains (underlined) were non-mucoid. *p<0.05 vs. alginate levels from LB-grown cells.

4. 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 CM21 strain was grown in LB containing equal concentrations of ammonium sulfate or magnesium sulfate, we observed similar degree of suppression of alginate production (Fig. 5). 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 sulfate ion, neither by sodium ion nor by any secondary effect due to the presence of high osmotic stress.

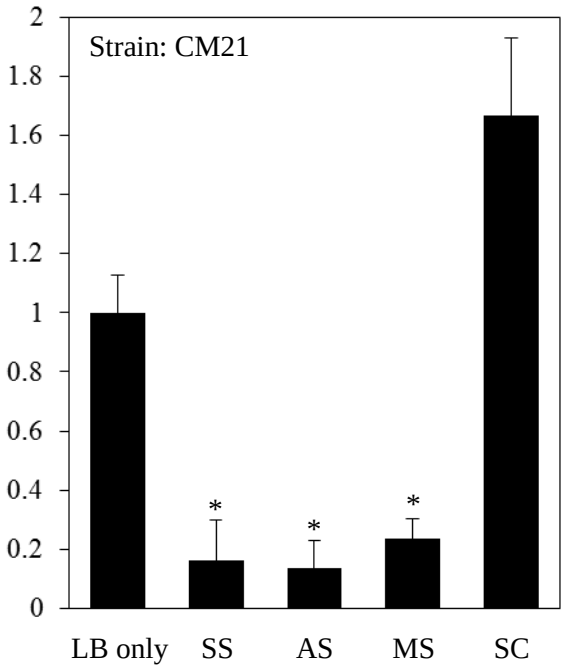

Figure. 5. Effects of diverse sulfate ions on alginate production. CM21 strain was grown in LB containing 5% sodium sulfate (SS), ammonium sulfate (AS), magnesium sulfate (MS) or sodium chloride (SC). Alginate level produced by CM21 grown in plain LB was used as a control and normalized to 1.0. *p<0.05 vs. alginate levels from LB-grown CM21.

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

Next, we sought to assess the effect of sulfate on the expression of *algD* gene, the first gene of the alginate biosynthesis operon that encodes GDP-mannose 6-dehydrogenase. To address this issue, we used a transcriptional fusion construct, pMEXR*algD*, in which DsRed-coding gene was cloned downstream of *algD* promoter[26]. PAO1 transformed with pMEXR*algD* failed to exhibit red fluorescent signal, while robust red fluorescent signal was detected in CM21 harboring the same plasmid (Fig. 6a and f). This result further confirmed that alginate biosynthesis operon is actively transcribed in the mucoid CM21 strain. It is of note that red fluorescent signal was significantly decreased in CM21/pMEXR*algD* strain grown in LB amended with sodium sulfate, ammonium sulfate or magnesium sulfate (Fig. 6g~i). Consistent with this finding, alginate production was suppressed under these growth conditions (Fig. 5). When grown with NaCl, a culture condition that permitted robust alginate production, the CM21/pMEXR*algD* strain exhibited comparable level of red fluorescent signal (Fig. 6j). Intriguingly, we observed significant cell shape changes of the mucoid CM21 strain in response to the growth with sodium sulfate, ammonium sulfate or magnesium sulfate. Round cell shape was observed, when grown with sodium sulfate (Fig. 6g) or ammonium sulfate (Fig. 6h), whereas CM21 strain became highly elongated upon growth with magnesium sulfate (Fig. 6i). Cell shape changes were not detected in non-mucoid PAO1 strain under these growth conditions (Fig. 6b~d). Together, our results suggest that sulfate-induced suppression of alginate production is likely accompanied with bacterial cell shape change.

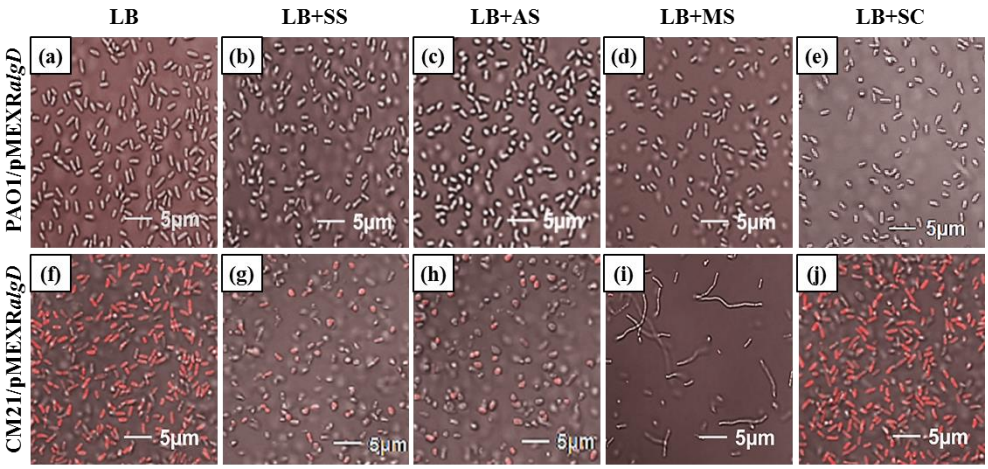

Figure. 6. Confocal microscopic analysis of *algD* promoter reporter strains. Non-mucoid wild type strain PAO1 and mucoid CF isolate strain CM21 were transformed with the plasmid pMEXR*algD*, in which *algD* promoter was fused with the coding sequence of DsRed red fluorescent protein. Reporter strains 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 the identical magnification

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

To provide an insight into the non-mucoid reversion 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. By conjugation, transposon vector, pBTK30 was introduced into mucoid CF isolate CM21. Among ~32,000 mutants tested, 4 mutants were found to be mucoid in LB agar plates containing 5% sodium sulfate. Importantly, all of the 4 mutants were determined to have transposon insertion in *lpxC* (PA4406) gene. By arbitrary PCR and sequencing, we confirmed that transposon insertion occurred at different sites, all of which were located near the 3' end of the gene. Shown in Fig. 7a is an *lpxC* mutant, where transposon was inserted the most downstream of the coding sequence. Transposon insertion was ensured by PCR with transposon specific primer and primer of sequencing read region in *lpxC* gene from arbitrary PCR to observe a 500bp-sized PCR product band by 1.5% agarose gel electrophoresis (Fig. 7b).In this PCR, genomic DNA of CM21 and 4 *lpxC* mutants were used as template. Therefore, there was no band in parental CM21which has no transposon insertion. However, we discarded M2 mutant since CM21Δ*lpxC* M2 mutant was confirmed to have multiple transposon insertion. The *lpxC* gene encodes LpxC enzyme, UDP-3-N-acetylglucosamine deacetylase, a key enzyme involved in lipid A biosynthesis, the first committed step for LPS production[27]. Δ*lpxC* mutant maintained mucoid phenotype in the presence of 5% sodium sulfate, while its parental CM21 strain again showed non-mucoid colony type in the same growth media (Fig. 8a). Quantitative alginate assay also showed that the mutant produced comparable levels of alginate during growth with sulfate (Fig. 8b). Likewise, the mutant did not respond to ammonium sulfate or magnesium sulfate and therefore remained mucoid

(data not shown). Importantly, normal rod-shape morphology was not changed after the growth with sulfate salts (Fig. 8c). These results suggest that changes in cell shape are associated with non-mucoid reversion and a cellular mechanism for LPS biosynthesis plays a role in responding to sulfate salts.

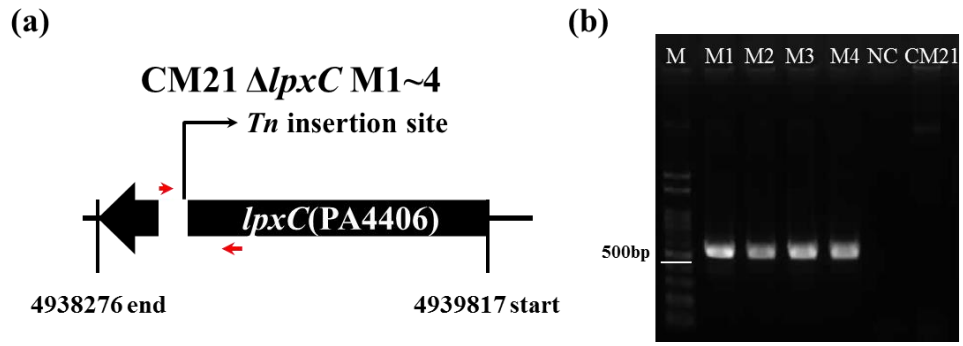

Figure. 7. Transposon insertion site and insertion conformational gel electrophoresis. (a) Arbitrary PCR confirmed transposon (*Tn*) insertion site (white box) was located at 3' end of *lpxC* gene (PA4406). Upper red arrow means *Tn* specific primer annealing site, and under red arrow means *lpxC* matched region primer annealing site. (b) 1.5% agarose gel electrophoresis of *lpxC-Tn* insertion confirmation. M; marker, M1, M2, M3 and M4; selected *lpxC* mutants, NC; negative control (primer only), CM21; CM21 genomic DNA template.

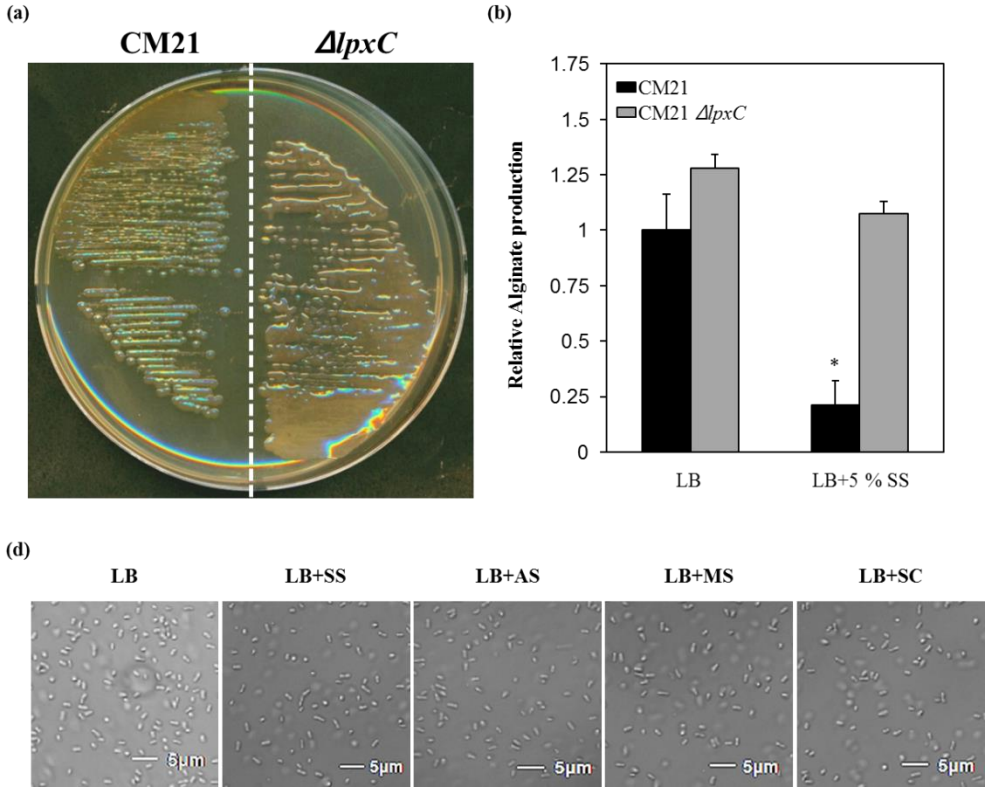

Figure. 8. Effect of *lpxC* gene mutation on the capability of CM21 to produce alginate. (a) Colony morphotype of Δ*lpxC* CM21 mutant in LB agar plate containing 5% sodium sulfate. (b) Levels of alginate produced by CM21 (black bars) and its Δ*lpxC* mutant (gray bars). Alginate level produced by CM21 grown in plain LB was used as a control and normalized to 1.0. (c) Confocal microscope images of CM21Δ*lpxC* strain grown under 5% sodium sulfate (SS), ammonium sulfate (AS), magnesium sulfate (MS) and sodium chloride (SC). CM21Δ*lpxC* showed consistent rod type cell shape under these growth conditions.*p<0.05 vs. alginate levels from LB-grown Δ*lpxC* mutant.

IV. DISCUSSION

Most of *P. aeruginosa* isolates from chronic CF patients have capacity to overproduce alginate, capsular polysaccharide to protect themselves from stressful lung environments[28]. Since the alginate acts as a physical barrier to antimicrobial agents and host immune cells[11], suppression of alginate production would be beneficial for treating CF patients. In this study, we identified 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.

Phenotype Microarray (PM) has been used to assess effects of diverse chemical compounds on bacterial growth[29-32] and therefore, provide a better understanding of phenotype-to-genotype relationship[33]. PM is also used to analyze bacterial resistance to a variety of stress conditions, such as osmotic shock, pH changes and antimicrobial agents[34]. In the aforementioned studies, bacterial responses were monitored by measuring the rate of organism's respiration, which reflects bacterial growth[35]. In this study, PM was applied to identify a chemical that can alter bacterial colony morphotype. This finding was made possible by simply examining the colony phenotype of a mucoid *P. aeruginosa* strain that had been grown in each well of PM.

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 non-mucoid PAO1, the sensitivity of mucoid strains to sulfate ion is likely due to the distinct differences in cell surface properties between mucoid and non-mucoid cells. In order to produce and secrete alginate, a polymeric substance with high molecular weight, mucoid *P. aeruginosa* requires a multi-protein complex spanning the outer membrane, the periplasm and

the inner membrane[36]. Our results also showed that the transcriptional activity of the *algD* promoter was down regulated in the presence of sulfate ion. It will be of interest to investigate how sulfate-induced changes in cell surface property lead to the signal that eventually suppressed the transcription of the alginate operon.Furthermore,96-well plate based MIC measurement experiment showed no significant difference in MIC of carbenicillin, streptomycin, gentamycin, erythromycin, chloramphenicol and colistin between irreversibly reverted non-mucoidCM21 and mucoid CM21 (data not shown).These results showed that sulfate stimulation is not associated with other virulence characteristics of mucoid strains. Moreover, almost mucoid CF isolates were known to have various genetic alterations including mutated *mucA* gene resulted from adaption in CF lung environment. Interestingly, as seen in Fig. 4, although each of CF isolates had different altered genetic properties, sulfate induced alginate inhibition was shown in only mucoid strains. And sequencing results of *mucA* gene encoding MucA, a negative regulator of alginate synthesis operon, showed diverse mutations in *mucA* gene of mucoid CF isolates. Again, these findings supported that sulfate ion can associate with alginate synthesis pathway and cell surface properties.

Transposon mutants disrupted in *lpxC* gene were not responsive to sulfate ion and remained mucoid in the presence of 5% sulfate. The recovery of *lpxC* mutants was counterintuitive, because *lpxC* gene was considered to be an essential gene[37]. 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[38]. As the level of lipid A increases, FtsH protease degrades LpxC to maintain balanced lipid A levels for membrane stability and viability[39]. Unlike *E. coli* LpxC, *P. aeruginosa* LpxC was reported to be highly stable under a condition in which FtsH is active[40]. Moreover, chemical inhibitors for *E. coli* LpxC failed to inhibit *P. aeruginosa* LpxC[41]. These findings suggested 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, 3 independent *lpxC* mutants were recovered with robust capability to produce alginate in sulfate media. Importantly, the mutant cells maintained their normal morphology. Although more experiments are necessary to precisely determine the mechanism, uninterrupted production of lipid A is required for the sulfate-induced reversion to non-mucoid phenotype in mucoid *P. aeruginosa* strains. Of note is that lipid A extracted from mucoid *P. aeruginosa* isolates were found to be modified with palmitate or aminoarabinose and these modifications were not detected in environmental isolates[42]. Therefore, it is likely that altered lipid A moiety is responsible for the "mucoid-specific" response to excess amount of sulfate ion.

In conclusion, we identified a novel mechanism by which the mucoid phenotype of clinical *P. aeruginosa* isolates can be shut off. Development of effective strategy to treat mucoid *P. aeruginosa* infection is necessary and will be facilitated by establishing a means of reverting mucoid isolates to non-mucoid 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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<ABSTRACT (IN KOREA)>

황산염 매개 성장에 의한 점액성 녹농균의 비점액성화

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민경배

그람 음성 박테리아인 녹농균은 낭포성 섬유증 환자에서 주로 발견되는 기회감염성병원균이며, 숙주의 면역작용과 항생제에 대한 내성을 가지고 있다. 본 연구에서, Phenotype Microarray 선별과정을 수행하였고 황산염 이온이 점액성 녹농균의 알긴산 생성을 억제하는 물질임을 동정하였다. 점액성 환자분리 녹농균 균주인 CM21과 다른 점액성 녹농균 균주들을 5%의 황산나트륨이 첨가된 배지에서 배양하였을 때, 알긴산의 생성이 상당히 감소하였고 다른 종류의 황산염화합물도 알긴산 생성억제효과를 보였으며, 이 효과는 전사수준에서 일어남을 확인하였다. 또한, 황산염이 첨가된 배지에서 배양한 CM21과 비점액성 균주인 PAO1 중 점액성 균주인 CM21의 세포형태에 상당한 변이가 있음을 관찰하였고, 이를 통하여 세포형태의 변화가 전사수준에서의 알긴산 생합성 오페론의 활성억제와 연계되어있음을 확인하였다. CM21 균주를 대상으로 무작위적인 돌연변이를 유도한 결과, lipid A 생성에 핵심효소를 생성하는 *lpxC*유전자에 돌연변이를 가질 경우, 황산염이온에 의한 알긴산 생성억제 및 세포 형태변이 효과를 받지 않았다. 위의 결과들을 통하여, 황산염이온에 의해 유도된 세포형태변화를 포함한 비점액성화 전환에 LPS 생합성 과정이 잠재적으로 연관되어 있음을 제시하였다. 본 연구의 결과들을 통하여, 어려움이 많았

던 점액성 녹농균 만성감염치료에 잠재적으로 적용 될 수 있는 새로운 치료방법을 개발해 낼 수 있을 것으로 기대된다.

핵심되는말: 녹농균, 알긴산, 낭포성섬유증