**Insights into the Assembly of the Alginate Biosynthesis Machinery in *Pseudomonas aeruginosa***

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*Pseudomonas aeruginosa* is an opportunistic pathogenic agent of particular significance to cystic fibrosis patients. This bacterium produces the exopolysaccharide alginate, which is an indicator of poor prognosis for these patients. The proteins required for alginate polymerization and secretion are encoded by genes organized in a single operon; however, the existence of internal promoters has been reported. It has been proposed that these proteins form a multiprotein complex which extends from the inner to outer membrane. Here, experimental evidence supporting such a multiprotein complex was obtained via mutual stability analysis, pull-down assays, and coimmunoprecipitation. The impact of the absence of single proteins or subunits on this multiprotein complex, i.e., on the stability of potentially interacting proteins, as well as on alginate production was investigated. Deletion of *algK* in an alginate-overproducing strain, PDO300, interfered with the polymerization of alginate, suggesting that in the absence of AlgK, the polymerase and copolymerase subunits, Alg8 and Alg44, are destabilized. Based on mutual stability analysis, interactions between AlgE (outer membrane), AlgK (periplasm), AlgX (periplasm), Alg44 (inner membrane), Alg8 (inner membrane), and AlgG (periplasm) were proposed. Coimmunoprecipitation using a FLAG-tagged variant of AlgE further demonstrated its interaction with AlgK. Pull-down assays using histidine-tagged AlgK showed that AlgK interacts with AlgX, which in turn was also copurified with histidine-tagged Alg44. Detection of AlgG and AlgE in PAO1 supported the existence of internal promoters controlling expression of the respective genes. Overall experimental evidence was provided for the existence of a multiprotein complex required for alginate polymerization and secretion.

*Pseudomonas aeruginosa* is an important opportunistic pathogen responsible for many nosocomial infections. Upon infection of the lungs of cystic fibrosis (CF) patients, it converts to a mucoid phenotype, characterized by the overproduction of the exopolysaccharide alginate, which is a polymer of β-1,4-linked β-D-mannuronic acid and its C-5 epimer α-L-guluronic acid (1, 2). Alginate not only protects the pathogen from antibiotics and host immune responses but also can clog the patient’s lungs. These infections are notoriously difficult to eradicate and often lead to the death of the patient (3, 4).

Twelve proteins required for the biosynthesis of alginate are encoded by the *algD* operon (*algD alg8 alg44 algK algE algG algX algl algf algE algA*), while another protein, AlgC, is encoded elsewhere (5, 6). The cytoplasmic proteins AlgA, AlgC, and AlgD convert D-fructose-6-phosphate through a series of reactions into GDP-mannuronic acid, the activated precursor of alginate biosynthesis (7–9). GDP-mannuronic acid is polymerized into a polynmannuronate chain by the inner membrane glycosyltransferase, Alg8, with some involvement of Alg44 (10–12). Alg8 has multiple transmembrane domains and a large cytoplasmic glycosyltransferase domain (10), while Alg44 has a single transmembrane domain separating a cytoplasmic c-di-GMP-binding PilZ domain and a large C-terminal periplasmic domain (11). Upon polymerization, the nascent polynmannuronate chain is proposed to be translocated across the periplasm by a periplasmic scaffold composed of AlgG, AlgL, AlgK, AlgX, and AlgE (13–17). In the periplasm, mannuronic acid residues of the nascent alginate chain are selectively O-acetylated at O2’ and/or O3’ positions by the action of AlgI, AlgJ, and AlgF (18, 19). A component of the proposed periplasmic scaffold, AlgG, is an epimerase which converts D-mannuronic acid into α-L-guluronic acids at the polymer level (17, 20). AlgL, a bifunctional periplasmic protein, serves as an alginate-degrading enzyme while also contributing to the integrity of the periplasmic scaffold (13, 21). AlgK is located in the periplasm, attached to the outer membrane with a lipid moiety, and it contains multiple copies of tetratricopeptide repeat-like motifs, a widespread domain involved in protein-protein interactions (22). The protein AlgX is also secreted into the periplasm and interacts with AlgK and MucD, a serine protease which is involved in the posttranslational regulation of alginate biosynthesis (15, 23, 24). The exact functions of AlgK and AlgX are not clear; however, they are essential for alginate secretion (14, 15, 25). The nascent alginate is secreted out of the cell through AlgE, which forms an electropositive β-barrel pore in the outer membrane (16, 26, 27).

In *Escherichia coli*, it was found that proteins involved in the biosynthesis of group 1 capsular polysaccharides (serotype K30) form a transenvelope multiprotein complex (28). Based on these results, it was proposed that biosynthesis and translocation of polysaccharides are temporally and spatially coupled by multiprotein complexes. This would also overcome the problems associated with the transport of high-molecular-weight polysaccharides across the inner and outer membranes (29). Recent studies have suggested that proteins involved in the biosynthesis of alginate also form a multiprotein complex which spans the entire bacterial envelope fraction (30, 31). To explore this possibility further, in this study, interactions between various subunits of alginate bio-

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synthesis complex were examined. Furthermore, the role of individual proteins (AlgG, Alg44, AlgE, AlgX, AlgK, and MucD) on the stability of other proposed subunits and the whole alginate biosynthesis complex was investigated. A better understanding of the organization of multiprotein protein complex for alginate biosynthesis could assist toward the identification of new drugs against P. aeruginosa infections. This knowledge would also be helpful for reconstitution of the alginate biosynthesis machinery in vitro systems and to engineer strains for the production of tailor-made alginites.

MATERIALS AND METHODS

Construction of isogenic knockout mutants. PDO300ΔalgK was generated as follows. Fragments from the N terminus (1 to 460 bp) and C terminus (1045 to 1428 bp) of the coding region of algK gene were amplified using Platinum Taq Hi Fi (Invitrogen) polymerase and the primer pairs algKNcEcoF-/algKNBamR and algKCBamF-/algKCCEcoR, respectively. The PCR products were hydrolyzed using BamH1 and ligated together into the pGEM-T Easy vector (Promega, Madison, WI), yielding pGEM-T Easy:algKNC. A 1,100-bp fragment, FRT-aacC1-FRT, encoding a gentamicin resistance gene (aacC1) flanked by FLP recombinase recognition sites (FRT), was obtained by digesting the plasmid pSp5856 with BamH1 (32). The resulting fragment, FRT-aacC1-FRT, was inserted into pGEM-T easy:algKNC, yielding the construct pGEM-T Easy::algKNC6Gm. The plasmid pGEM-T Easy::algKNC6Gm was used as a template for amplifying the algKNC6Gm cassette with Pfx high-fidelity polymerase using the primers algKNC6EcoF and algKCEcoR. The subsequent algK-gentamicin resistance cassette was ligated into the Smal site of the suicide vector pEX100T, yielding pEX100T:algKNC6Gm. This plasmid was transferred into P. aeruginosa PDO300 via conjugation utilizing the E. coli S17-1 (33, 34). Transconjugants were selected for on mineral salt medium (MSM) supplemented with 100 μg/ml of gentamicin and 5% (wt/vol) sucrose (35). The double-crossover event, generating the strain PDO300ΔalgKNC6Gm, was confirmed by gentamicin resistance (300 μg/ml) and PCR by using the primers algK(upXout) and algK(downXout). E. coli SM10 was used as the donor strain to transfer the FLP recombinase-encoding vector pFLP2 (32) into PDO300ΔalgKNC6Gm to remove the gentamicin resistance cassette (FRT-aacC1-FRT). Successful transconjugants were selected on Pseudomonas isolation agar (PIA) containing carbenicillin (300 μg/ml) and subsequently transferred to PIA plates containing 5% sucrose to remove the pFLP2 plasmid. Successful loss of the FRT-aacC1-FRT cassette was confirmed by PCR and sensitivity to gentamicin and carbenicillin. Plasmids pEX100T:ΔalgKNC6Gm and pEX100T:ΔalgKNC6Gm were used to delete algE in PAO1 (generating PAO1ΔalgE) and algX in PDO300ΔalgK (generating PDO300ΔalgKΔalgX) using the same strategy as described above. The bacterial strains and sequences of primers used for PCR are given in Tables S1 and S2 in the supplemental material.

cis-complementation by chromosomal integration of respective genes. To generate mini-CTX:PmucD, the promoter region at —901 bp relative to the algU open reading frame was amplified using primers palgU/HindIII and palgUXbalR (37) and hydrolyzed with HindIII and Xbal; the mucD region was hydrolyzed form plasmid pBBR1MCS-5::mucD (24) using Xbal and SacI, purified, and ligated together with algU promoter in mini-CTX-lacZ (38), which was hydrolyzed with HindIII and SacI, generating mini-CTX:PmucD. To generate plasmids mini-CTX:Palg44, mini-CTX:Palg8, mini-CTX:PalgK, mini-CTX:PalgX, and mini-CTX:PalgE, the algD promoter fragment was hydrolyzed from pGEM-TEasy:PalgD with PstI and HindIII and ligated together with HindIII- and BamHI-hydrolyzed alg44, alg8, algK, and algX into mini-CTX-lacZ hydrolyzed with PstI and BamHI.

These plasmids (mini-CTX:Palg44, mini-CTX:Palg8, mini-CTX:PalgK, mini-CTX:PalgX, and mini-CTX:PalgK) were electroporated into PDO300Δalg44, PDO300Δalg8, PDO300ΔalgK, PDO300ΔalgX, and PA01ΔmucD, which were described in previous studies (12, 16, 23, 24, 31), as well as PDO300ΔalgK. Transformants were selected for on PIA supplemented with 150 μg/ml tetracycline. The chromosomal integration of the plasmids was confirmed through PCR using primers PseRUP and PseRDWN. The mini-CTX-lacZ backbone was removed by introducing the flippase-encoding plasmid pFLP2. pFLP2 was cured by cultivating the cells for 24 h on PIA containing 5% (wt/vol) sucrose. Cells sensitive to tetracycline and carbenicillin were analyzed through PCR for removal of the mini-CTX-lacZ backbone. The same strategy was used to integrate the empty vector mini-CTX-lacZ into the chromosome of mutant strains as well as PDO300 and PA01. The oligonucleotide sequence can be found in Table S2 in the supplemental material.

Isolation of envelope fractions. Cells of P. aeruginosa were grown overnight in LB medium in planktonic mode or for 48 h on solid PIA. Cells were harvested by centrifugation or scraping from the plates and washed twice in 100 ml of 10 mM HEPES (pH 7.4) buffer. Cells were resuspended in 10 ml of 10 mM HEPES buffer and lysed by sonication on ice for 12 cycles with 15 s of sonication followed by 15 s of cooldown. After lysis, cells were centrifuged at 8,000 × g for 45 min at 4°C to remove the unbroken cells and cellular debris. The soluble fraction containing the whole-cell lysate was centrifuged at 100,000 × g for 1 h at 4°C to isolate the envelope fraction (inner membrane, outer membrane, and associated proteins). Envelope fractions were used immediately or stored at −80°C for future analysis.

Coimmunoprecipitation of AlgE and pulldown assays. To solubilize the envelope fractions, they were suspended in buffer A (100 mM NaH2PO4 · H2O, 10 mM Tris-HCl [pH 7.5], 1% Triton X-100, 150 mM KCl, and 1% [w/v] glycoproteins) by gently rocking at 4°C for 2 h and insoluble material removed by centrifugation at 50,000 × g for 30 min.

For FLAG-tagged AlgE (AlgEL6F), 1 ml of supernatant was incubated with 40 μl of anti-FLAG IP resin slurry (GenScript, Piscataway, NJ) overnight at 4°C. The mixture was centrifuged at 5,000 × g for 30 s, and supernatant was removed. Resin was washed three times with equilibration buffer (50 mM Tris, 150 mM NaCl [pH 7.4]). A 20-μl volume of 1X SDS-PAGE loading buffer (62.5 mM Tris-HCl [pH 6.8 at 25°C], 2% [wt/vol] SDS, 10% [vol/vol] glycerol, 0.01% [wt/vol] bromophenol blue) was added to the resin and heated at 100°C for 5 min. Resin was again centrifuged at 8,000 × g for 30 s, and the supernatant was subjected to SDS-PAGE and analyzed by Western blotting.

For hexahistidine-tagged AlgK (AlgK-His) pull-down, a His-Spin protein miniprep kit (Zymo Research) was used according to the manufacturer’s instructions, with a few modifications. The solubilized envelope fraction (in buffer A) was incubated with the resin. The affinity gel was washed twice, once with each wash buffer (supplemented with imidazole at 25 and 50 mM, respectively). Proteins of interest were eluted from the affinity gel by incubation for 1 min with 100 μl elution buffer (containing 500 mM imidazole) and centrifugation. The eluted frac-
tion was either stored at −80°C or analyzed by SDS-PAGE and immunoblotting.

**In vivo chemical cross-linking.** Chemical cross-linking was performed using disuccinimidyl glutarate (DSS; Pierce Biotechnology, Rockford, IL) prepared in dimethyl sulfoxide (DMSO). Briefly, 400-ml overnight cultures of *P. aeruginosa* in LB were harvested and washed twice with saline solution and once with phosphate-buffered saline (PBS) (pH 7.5). Cells were resuspended in 5 ml of PBS and incubated with DSS (1.5 mM) for 30 min at 37°C or 2 h on ice. Reactions were terminated by adding Tris-HCl (pH 7.5) at a final concentration of 20 mM for 15 min. Cells were pelleted and resuspended in 5 ml Tris-buffered saline (TBS) (pH 7.8) and then treated with 0.5 ml of lysis buffer (150 mM NaCl, 100 mM Tris-HCl, 0.2% Triton X-100 (pH 8.0)) containing 1 mg/ml lysozyme, 1 mg/ml DNase, and protease inhibitor cocktail set III, EDTA free (Calbiochem Co.). Samples were incubated on ice for 20 min with shaking and subsequently lysed by sonication. Cell debris was removed by centrifugation at 15,800 × g for 30 min at 4°C, and supernatants were subjected to centrifugation at 50,000 × g for 1 h. The pellet constituting the envelope fraction was solubilized in solubilizing buffer (8 M urea, 100 mM Tris-HCl, pH 8.0, 1 M NaCl, 1% Triton X-100, 0.2% N-lauroyl sarcosine, 10 mM imidazole). Histidine-tagged Alg44 (Alg44-His) was purified using a His-Spin protein miniprep kit (Zymo Research) as described above. Purified proteins were separated by SDS-PAGE on a 4 to 15% Mini-Protean TGX precast gel (Bio-Rad) and identified by immunoblotting.

**Protein analysis.** A total of 20 μg of protein was loaded and separated by SDS-PAGE using an 8% polyacrylamide gel. The resulting gels were either stained with Coomassie blue or used for immunoblotting using an iBlot dry-blotting system (Invitrogen). Nitrocellulose membranes were blocked using skim milk (5%, wt/vol) in Tris-buffered saline containing Tween 20 (0.05% vol/vol) (TBST) for 1 h at room temperature or overnight at 4°C. After the membrane had been washed three times with TBST, it was treated with the primary antibody, raised in rabbit, of interest in TBST containing skim milk (2%, wt/vol) for 1 h at room temperature: anti-Alg44 (1:10,000), anti-AlgK (1:10,000), anti-AlgG (1:1,000), anti-AlgX (1:7,000), or anti-AlgE (1:5,000). Membranes were then washed three times and incubated with the commercial secondary antibody (Abcam, Cambridge, United Kingdom), anti-IgG anti-rabbit antibodies labeled with horseradish peroxidase (HRP) (1:10,000) in TBST containing skim milk (2%, wt/vol) for 1 h at room temperature. After three washes, membranes were resolved with SuperSignal West Pico chemiluminescent substrate (Thermoscientific, Rockford, IL) and developed on X-ray film (Kodak, Rochester, NY). For the detection of hexahistidine-tagged proteins, a HisProbe-HRP kit (Thermoscientific, Rockford, IL) was used according to the manufacturer’s instructions.

**Alginate quantification.** Alginate was isolated and uronic acid content quantified using *Pseudomonas* isolation media (agar for alginate, liquid medium for free uronic acids) as described previously (16). To assess the free uronic acids, 2 ml of overnight culture was centrifuged, and supernatant was filtered through a Vivaspin-500 (GE Healthcare) filter device with a molecular mass cutoff of 10 kDa. The uronic acids in the flowthrough, which consist of free uronic acids and short alginate degradation products, were measured as described below.

**Solid surface attachment assay.** Attachment of cells to solid surface was measured as has been described (39). Briefly, relevant strains of *P. aeruginosa* were grown overnight, and the optical density at 600 nm (OD600) was measured. Using an appropriate amount of culture, a final dilution of 1:100 in LB medium was made. Eight wells of three sterile microplates (Greiner Bio-one) were inoculated with 100 μl of diluted culture and incubated at 37°C for 24 h. After incubation, nonadherent bacteria were removed by gentle washing as has been described (39).

**RESULTS**

**Effect of algK deletion on alginate biosynthesis.** A marker-free isogenic deletion mutant of algK was generated in *P. aeruginosa* PDO300 to investigate its role in alginate biosynthesis. Immunoblot analysis of envelope fractions isolated from PDO300 deleted algK showed that AlgK was absent (Fig. 1A, lane 2). Similar to what has been described previously (14), this strain did not produce detectable levels of alginate (Fig. 1B). Recent studies have shown that deletion of AlgG, AlgX, and AlgE resulted in the secretion of free uronic acids, which are considered degradation products of alginate caused by the action of alginate lyase, AlgL (15–17). To assess whether the loss of mucoidy of the algK mutants is caused by the inability to polymerize alginate or because of its degradation in the periplasm, uronic acid quantification was performed. Contrary to what has been described previously for algK mutants, no free uronic acids were detected for algK deletion mutants in this case (Fig. 2). Similar to the results for algK mutants, no free uronic acids were produced by alg44 and algx mutants of PDO300 (Fig. 2B). These results suggested that deletion of algK and algX has an effect on the stability of Alg44 and/or Alg8, leading to a defect in the polymerization of alginate.

**Complementation of the algK knockout mutant.** In order to rule out the possibility of polar effects resulting from the deletion of algK, PDO300 deleted algK was complemented with algK either in trans (pBBR1MCS-5:algK) or in cis (mini-CTX::algK). The presence of AlgK was restored in the complemented strains (Fig. 1A;
also, see Fig. S1, lane 14, in the supplemental material). Interestingly, in the complemented strains AlgK was detected in the envelope fractions only when cells were harvested from solid medium but was absent when envelopes were obtained from cells grown in liquid medium (Fig. 3, lane 12; also, see Fig. S1, lane 14, in the supplemental material). The amount of alginate produced by PDO300/algK/H11001 pBBR1MCS-5 was 4-fold less than that produced by PDO300/H11001 pBBR1MCS-5 (Fig. 1B), and the amount of alginate produced by PDO300/algK/H11001 mini-CTX::algK was 4.4-fold less than that produced by PDO300/mini-CTX (Fig. 3B).

AlgK with a hexahistidine tag (AlgK-His) also partially restored alginate production and presence of the AlgK protein in the envelope fraction (Fig. 1A, lane 4, and B).

**Mutual stability analysis.** Experimental evidence from recent studies suggested the existence of a multiprotein complex involved in the polymerization and secretion of alginate (31). Here the stability of the complex was assessed by removing individual subunits of the proposed envelope-spanning multiprotein complex. The impact of proposed subunits on the stability of potentially interacting subunits was investigated. Mutants of PDO300 lacking algE, algX, algK, alg8, and alg44 and PAO1 lacking mucD were used. These mutants were complemented in cis to address stoichiometric issues; here, cis-complementation introduces a single copy of a gene under the control of algD promoter into the chromosomal DNA of the respective strain. Strains PDO300 and PAO1 were used as positive and negative controls, respectively. The proteins AlgE, AlgX, AlgK, AlgG, and Alg44 were detected in the envelope fraction of PDO300 (Fig. 3, lane 1). As expected, AlgE, AlgK, AlgX and Alg44 were absent in the envelope fractions of PAO1 which does not produce detectable amount of alginate as shown by uronic acid assay (Fig. 3A, lane 2, and B). Interestingly, a distinct band for AlgG was detected in PAO1 (Fig. 3A, lane 2). Deletion of alg8 resulted in the absence of AlgK, AlgX, and Alg44 from the PDO300/alg8 envelope fraction (Fig. 3A, lane 3). Expression of alg8 in cis could not restore these proteins to the detectable levels (Fig. 3A, lane 4). The presence and absence of AlgG did not appear to affect the stability of AlgE (Fig. 3A, lanes 3 and 4).
AlgK interacts with AlgE and AlgX in vivo. Our mutual stability analysis suggested an interaction between AlgE and AlgK.

Although this interaction has been previously been proposed based on structural and domain homologies to other proteins, no experimental evidence had been so far provided AlgK contains tetratricopeptide-repeats (TRP) which are involved in protein-protein interaction. Furthermore, it has been proposed that, BcsC and PgaA proteins involved in the secretion of cellulose and N-acetyl-d-glucosamine, respectively, have a domain architecture which represents fusion of AlgK and AlgE (41, 42). Despite these precedencies, no direct evidence has been provided for this long proposed interaction between AlgE and AlgK (22, 27). To investigate this further, a coimmunoprecipitation (co-IP) assay was performed by expressing in P. aeruginosa PDO300ΔalgK, a variant of AlgE with the FLAG epitope inserted into the extracellular loop 6 (pBBR1MCS-5:algEL6F). This variant was selected because it can fully restore alginate production and is present in the OM when produced in P. aeruginosa PDO300ΔalgK in trans (16). Wild-type AlgE with no FLAG epitope was used as a negative control. Anti-FLAG IP resin was used to isolate AlgEL6F from solubilized envelope fractions of cells harvested from solid medium. A distinct band of AlgE and AlgK was detected in the elution fraction. As expected, no band for AlgE or AlgK was detected in the negative control (Fig. 4A). The

FIG 4 Co-IP and pulldown assays show that AlgK interacts with AlgE and AlgX. (A) Solubilized envelope fractions of P. aeruginosa PDO300ΔalgE (pBBR1MCS-5:algE) grown on solid medium (lane 1) or harboring plasmid pBBR1MCS-5:algEL6F (lane 2) were incubated with anti-FLAG co-IP resin. The elution fraction was probed with specific antibodies as described in Materials and Methods. Relevant lanes from the same immunoblot were combined, and only the relevant part of the same immunoblot was shown. (B) Pulldown assay using AlgK-His. P. aeruginosa PDO300ΔalgK harboring either pBBR1MCS-5:algK (lane 1) or pBBR1MCS-5:algK-6his (lane 2) was grown on solid medium, and the envelope fraction was isolated. Solubilized envelope fractions were subjected to metal ion affinity chromatography and probed with the indicated antibodies. Co-IP and pulldown assays were performed twice.

4). AlgG was detected in both the Δalg8 mutant and its complemented strain (PDO300Δalg8 + mini-CTX:alg8) at a level higher than that of the wild-type PDO300 (Fig. 3, lanes 3 and 4), though AlgG was present in slightly larger amounts in the complemented mutant.

Deletion of alg44 destabilized AlgK and AlgX but not AlgE and AlgG (Fig. 3A, lane 5). Complementation with alg44 restored AlgK and AlgX (Fig. 3A, lane 6). These findings indicate that Alg44 potentially interacts with either AlgX or AlgK or both.

Deletion of algE resulted in AlgK and AlgX not being detected, while levels of Alg44 were reduced (Fig. 3A, lane 7). Expression of algE in cis restored the presence of AlgK and AlgX and restored the levels of Alg44 (Fig. 3A, lane 8). These results are consistent with the proposed interaction between AlgE and AlgK, which in turn interacts with AlgX (22, 24).

Interestingly, deletion of algX (PDO300ΔalgX) destabilized all of the components (AlgE, AlgK, and Alg44) except AlgG (Fig. 3A, lane 9). All of the components were restored when AlgX was expressed in cis (Fig. 3A, lane 10). AlgX has been shown to interact with AlgK (24). The destabilization of AlgK in the algX mutant further supports this interaction. The destabilization of AlgE observed in the algX mutant might be an indirect effect of the destabilization of AlgK. However, deletion of AlgK alone did not completely destabilize AlgE. These results indicated that AlgX plays a role in localization of AlgE to the outer membrane (OM). In strain PDO300ΔalgK, AlgX and Alg44 were completely destabilized, while levels of AlgE were reduced (Fig. 3A, lane 11). Interestingly, the presence of these components could not be restored in the algK-complemented mutants during planktonic growth (Fig. 3A, lane 12). The strains with algK complemented in cis did not produce AlgK at detectable levels. Intriguingly, however, when cells were harvested from solid medium (as opposed to liquid culture), AlgK was restored to normal levels (see Fig. S1, lane 14, in the supplemental material).

Unexpectedly, a mutant of PAO1 lacking mucD, a negative regulator of alginate biosynthesis, did not show detectable levels of Alg44, AlgK, and AlgX when cells were grown in planktonic mode (Fig. 3A, lane 13); however, in PAO1ΔmucD, AlgE, AlgK, AlgX, and Alg44 were detected when cells were grown on solid medium and alginate was produced (Fig. 3B; also, see Fig. S1, lane 11, in the supplemental material).

Deletion of alg8, algE, algK, algX, and alg44 in PDO300 caused a complete loss of production of alginate. Production of alginate was restored, although the amount of alginate was less than in the wild-type strain PDO300, when these components were expressed in cis in their respective knockout strains (Fig. 3B). Among all the strains, the algK-, algX-, and algE-complemented strains produced the smallest amount of alginate. This suggests a strict stoichiometric requirement of these proteins for the formation of functional alginate biosynthesis complexes.

Complementation of PDO300Δalg8 with alg8 or PDO300ΔalgK with algK restored the presence of proposed subunits of the alginate biosynthesis complex when cells were grown on solid medium, which ruled out any polar effects caused by these deletions (see Fig. S1, lanes 4 and 14, in the supplemental material). Alginate is overproduced during growth in biofilm mode (40), which suggested that on solid medium, the transcription from algD operon increases and/or the alginate biosynthesis multiprotein complex is more active/abundant.
elution fraction was also probed with anti-AlgX and anti-Alg44 antibodies but no band for AlgX or Alg44 was detected.

To investigate the proposed AlgK-AlgE interaction from the perspective of AlgK, a pulldown assay using AlgK-His (AlgK with a C-terminal hexahistidine tag) were done. To this end, AlgK-His and wild-type AlgK (negative control) were independently expressed in PDO300 ΔalgK in trans. Cells were either grown in liquid medium and treated with cross-linkers or harvested directly from solid medium without cross-linking. AlgK-His was purified from solubilized envelope fractions of these cells and analyzed by immunoblotting. However, under these experimental conditions AlgE was not copurified with AlgK-His. To determine whether AlgK interacts with other components, immunoblotting with AlgX and Alg44 antibodies was carried out. We found that only AlgX was copurified with AlgK-His (Fig. 4B). As expected, AlgK and AlgX were not detected in the negative control (Fig. 4B).

**Interaction between Alg44 and AlgX.** Results from mutual stability experiments suggested an interaction between Alg44 and AlgK/AlgX. To investigate this further, we carried out a pulldown assay using PDO300Δalg44 (12) carrying plasmids for expression of either wild-type Alg44 or Alg44-His (Alg44 with hexahistidine tag). Wild-type Alg44 was used as a control. A noncleavable chemical cross-linker, DSG, with spacer arm length of 7.7 Å for stabilization of protein-protein interactions was used. As expected, these pulldown experiments using immobilized metal ion affinity chromatography, and the control strain PDO300Δalg44+pBBR1MCS-5:alg44 did not enable purification of Alg44 or AlgX (Fig. 5). However, with cell lysates containing Alg44-His, an additional protein with an apparent molecular mass of ~90 kDa which bound both anti-Alg44 and anti-AlgX antibodies was detected (Fig. 5A and B). The molecular mass of this protein is approximately equal to the combined molecular mass of Alg44 and AlgX (91.57 kDa). In the absence of cross-linker or when Alg44-His was purified from a mutant deficient in alg44 and algX (PDO300Δalg44ΔalgX), this ~90-kDa protein was no longer detectable (Fig. 5A and B, lanes 1 and 2). These results suggested that in vivo, Alg44 interacts with AlgX. Under the experimental conditions used here, neither AlgK nor AlgE was copurified with Alg44.

**DISCUSSION**

Here the role of individual subunits of the alginate polymerization/secretion machinery in assembly and stability of the proposed multiprotein complex was investigated (30). One of the components, AlgK, is a periplasmic lipoprotein anchored to the inner leaflet of the outer membrane by its lipid moiety (22). This protein has multiple copies of the tetratricopeptide repeat motif commonly found in proteins involved in the assembly of multiprotein complexes (22). To elucidate the role of AlgK in alginate biosynthesis, an isogenic algK deletion mutant in the constitutive alginate overproducer *P. aeruginosa* PDO300 was generated. This mutant, PDO300ΔalgK, failed to produce high-molecular-weight alginate (Fig. 1B), a phenotype consistent with a previous study using the algK mutant of strain FRD1, FRD1100 (14). Interestingly, PDO300ΔalgK produced almost no free uronic acid oligomers, while FRD1100 produced significantly more (Fig. 2) (14). The lack of free uronic acids, which are thought to be the product of alginate degradation in the periplasm by alginate lyase, indicated that in the absence of AlgK, alginate polymerization might not occur. This would suggest that AlgK may play a role in the polymerization of alginate or more likely play a role in the stability of the polymerases Alg8 and/or Alg44 in the inner membrane.

This difference between our observations and those in FRD1100 could be due to a difference in parent strain and method of mutant generation. In FRD1100, the algK open reading frame was replaced with a gentamicin resistance cassette containing a promoter which would elevate the transcription of downstream genes such as algA, which is involved in alginate precursor (uronic acid) synthesis. In contrast, PDO300ΔalgK has no extra promoter or marker present. The yield of free uronic acids produced by PDO300ΔalgK was comparable to that of PDO300ΔalgX and PDO300Δalg44, suggesting that AlgK (and AlgX) have roles in alginate polymerization, possibly through stabilizing Alg8 and Alg44, the polymerase and copolymerase of alginate synthesis, re-
respectively (Fig. 2). This was supported by our mutual stability assays of the various mutants (PDO300ΔalgK, PDO300Δalg44, and PDO300ΔalgX), which demonstrated that AlgK, Alg44, and AlgX were interdependent for stability, suggesting that these proteins might interact (Fig. 3A).

Mutual stability experiments were performed to identify potential protein-protein interactions between subunits of the alginate biosynthesis machinery. The principle of mutual stability is that the deletion of a subunit would destabilize its interacting partner. To provide further experimental support beyond mutual stability analysis, pull-down and co-IP assays were used to demonstrate the interactions. The results of mutual stability analysis with PDO300ΔalgE (Fig. 3A, lane 7) and the previous observation that AlgK was required for the proper localization of AlgE to the outer membrane (22) provided a strong basis for further exploring the proposed AlgK-AlgE interaction. A co-IP assay was employed, under native conditions, using FLAG-tagged AlgE, which provided strong evidence for an interaction between AlgK and AlgE (Fig. 4A). Similar to this, functional homologues of AlgE and AlgK (HmsH and HmsF, respectively) from the poly-β-1,6-N-acetyl-d-glucosamine (PGA) secretion system of Yersinia pestis have been found to interact (43). AlgK appears to be a weakly produced protein, as suggested by our inability to detect AlgK in cells grown in planktonic growth mode (Fig. 3A, lane 12). Hence, to increase the likelihood of identifying potential interaction partners (AlgX, Alg44, and AlgE) of AlgK, pulldown assays from cells grown on solid medium were performed; this biofilm mode of growth is characterized by increased alginate production and possibly by an increase in the number of alginate biosynthesis complexes. Pulldown experiments using AlgK-His demonstrated that AlgK interacts with AlgX, a finding consistent with previous work (24) (Fig. 4B).

Mutual stability analyses using Δalg44 and ΔalgX mutants suggested an interaction between these components (Fig. 3, lanes 5, 6, 9, and 10). Furthermore, the periplasmic C-terminal domain of Alg44 is similar to membrane fusion proteins of multidrug efflux pumps and is thought to interact with the periplasmic and/or outer membrane components of the alginate biosynthesis machinery (10–12). To investigate this possibility, pulldown assays using Alg44-His were performed. Under native conditions, in the absence of cross-linker DSG, no interaction partner was found, suggesting that interactions of Alg44 are weak and/or transient or dependent on an intact membrane. However, in the presence of DSG, a protein with an apparent molecular mass of ~90 kDa corresponding to the combined molecular masses of AlgX (49.9 kDa) and Alg44 (41.2 kDa) was detected using anti-Alg44 or anti-AlgX antibodies (Fig. 5A and B). These data suggested an interaction between Alg44 and AlgX (Fig. 6). The interaction between Alg44-AlgX-AlgK explains why reduced amounts of free uronic acid were detected for the algK and algX mutant, respectively, because in both mutants the copolymerase Alg44 was destabilized, which in turn impaired alginate polymerization (Fig. 3A, lanes 9 and 11). Hence, no alginate was synthesized in the periplasm, and thus there was no degradation by the alginate lyase to lead to free uronic acid formation.

Protein-protein interaction was proposed to occur between the putative polymerase (Wzy) and copolymerase (Wzc) of the E. coli type I capsular polysaccharide biosynthesis system; however, experimental evidence for this interaction is still lacking (29). Wzy and Wzc can be considered analogues of Alg8 and Alg44 of the alginate biosynthesis machinery. Interestingly, Alg44 and its interacting partners (AlgX-AlgK) were completely destabilized in PDO300Δalg8 (Fig. 3A, lane 3). Alg44 is a putative copolymerase and deletion of alg44, its cytoplasmic c-di-GMP-binding PilZ domain or its periplasmic MFP domain resulted in loss of alginate production (11, 12). This provided evidence that Alg44 has an additional regulatory role in alginate production. The binding of c-di-GMP may induce conformational changes in Alg44 which may be required to activate Alg8. These findings and mutual stability analysis of the alg8 mutant suggested that Alg44 and Alg8 may interact. In addition, E. coli BcsA protein, which is involved in cellulose polymerization, contains both a glycosyltransferase domain and a PilZ domain, which further suggests that interaction between Alg8 and Alg44 might be required for polymerization to occur (41, 44). However, future research using Alg8 pulldown assays will be required to test this hypothesis.

MucD is a negative regulator of alginate biosynthesis which has been shown previously to interact with AlgX, which in turn interacts with AlgK. Deletion of mucD in PAO1 (PAO1ΔmucD) leads to increased transcription of algD operon and overproduction of alginate (45, 46). Based on this and our results, it is difficult to conclude what structural role MucD might have in alginate production.

Previous studies have suggested that algE may have its own internal promoters (other than the promoter upstream of algD) (47). The presence of a faint band for AlgE and a strong band for AlgG in PAO1 (Fig. 3A, lane 2; also, see Fig. S1 in the supplemental
material) supports independent expression of the two genes. Indeed, a recent publication described the existence of internal promoters for \( \text{algE} \) and \( \text{algG} \), suggesting that independent regulation of \( \text{algG} \) expression controls the degree of alginate epimerization and hence its material properties (48). Although strain PAO1 is nonmucoid, alginate has been demonstrated to be produced by this strain at low levels through the use of highly sensitive immunofluorescence microscopy (49). The elevated \( \text{AlgG} \) presence in PAO1 may result in the production of alginites containing a higher proportion of guluronic acid residues. Alternatively, this suggested that \( \text{AlgG} \) has other functions apart from its function in alginate production.

The OM protein OmpX of \( Y. \) pestis has been described as playing a role in adhesion (50). The role of \( \text{AlgE} \) in attachment to polystyrene microtiter plates was evaluated by comparing PAO1\(\text{AlgE} \) to wild-type PAO1. However, the results suggested that \( \text{AlgE} \) does not play a role in attachment (data not shown).

In the present study, pulldown experiments, co-IP, and mutual stability analysis provided evidence for the existence of a multiprotein complex constituting the alginate polymerization and secretion machinery as well as resolving protein-protein interactions between individual subunits of this complex (Table 1; Fig. 6). It was shown that the OM protein \( \text{AlgE} \) interacts with periplasmic \( \text{AlgK} \). Furthermore, \( \text{AlgK} \) was shown to interact with periplasmic \( \text{AlgX} \), which in turn interacts with the inner membrane protein \( \text{Alg44} \). These results support the existence of transmembrane multiprotein complex facilitating alginate polymerization and secretion. However, it has been proposed that \( \text{AlgI}, \text{AlgJ}, \) and \( \text{AlgF} \) form a complex but apparently are not involved in alginate polymerization or secretion (18). Based on the results obtained by mutual stability and protein-protein interaction assays, we have proposed a model for assembly of the alginate biosynthesis machinery (Fig. 6).

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**TABLE 1 Proposed interaction between various components of the alginate biosynthesis complex**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Proposed interaction(s)</th>
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<tbody>
<tr>
<td>Alg8</td>
<td>Alg44, AlgG</td>
</tr>
<tr>
<td>Alg44</td>
<td>Alg8, AlgX</td>
</tr>
<tr>
<td>AlgE</td>
<td>AlgK</td>
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<tr>
<td>AlgK</td>
<td>Alg44, AlgK</td>
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<tr>
<td>AlgG</td>
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* a Mutual stability data.
* b Pulldown data or communoprecipitation.
* c Based on previously published data (24).

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