Alginate Lyase (AlgL) Activity Is Required for Alginate Biosynthesis in *Pseudomonas aeruginosa*

Mark T. Albrecht and Neal L. Schiller*

University of California, Riverside, Riverside, California 92521

Received 12 February 2005/Accepted 1 March 2005

To determine whether AlgL's lyase activity is required for alginate production in *Pseudomonas aeruginosa*, an *algL*Δ::Gm^r mutant (FRD-MA7) was created. *algL* complementation of FRD-MA7 restored alginate production, but *algL* constructs containing mutations inactivating lyase activity did not, demonstrating that the enzymatic activity of AlgL is required for alginate production.

The ability of *Pseudomonas aeruginosa* to produce chronic infection in cystic fibrosis (CF) patients' lungs is based in part on its biosynthesis of alginate, an exopolysaccharide copolymer composed of (1-4)-linked β-D-mannuronic acid blocks interspersed with its C5 epimer α-L-guluronic acid (10, 15). Acquisition of mucoid (alginate-producing) *P. aeruginosa* strains is associated with a decline in pulmonary function and a reduced survival rate among CF patients (2, 17). Alginate can restrict the diffusion of certain antibiotics into the cell and can also inhibit several of the host's major antibacterial defense mechanisms, making it very difficult for CF patients to clear mucoid *P. aeruginosa* from their lungs (see reference 15 for a review).

Most of the alginate biosynthetic genes, including *algD*, *alg8*, *alg44*, *algK*, *algE*, *algG*, *algX*, *algL*, *algI*, *algJ*, *algF*, and *algA*, are found in a chromosomal gene cluster which functions as an operon controlled by the *algD* promoter (1, 24). *algC*, which is also involved in lipopolysaccharide biosynthesis (9), is located outside of this cluster and is expressed from its own promoter (27). Alginate biosynthesis begins in the cytoplasm with fructose-6-phosphate, which is converted to GDP-mannuronic acid via a series of steps involving AlgA, AlgC, and AlgD (see reference 24 for review) and then transported across the periplasmic membrane, possibly by Alg8 and Alg44 (24). Alg8, due to its resemblance to β-glycosyltransferases (21), is considered to be a good candidate for the polymerization of GDP-mannuronic acid residues into a poly(M) chain, but AlgK might also be involved (12). Some of the mannuronic acid residues are acetylated through the action of AlgF, AlgI, and AlgJ (5–7), while others are epimerized into guluronic acid by AlgG (4). It has been hypothesized that these later stages of alginate synthesis occur via a protein complex or scaffold composed of alginate proteins AlgG, AlgK, and AlgX (8, 11, 12, 20). This scaffold is believed to assist in polymer formation by bringing the enzymes and mannuronic acid residues together in one location, facilitating the modification of these residues and guiding the movement of the developing polymer to the outer membrane secretin, AlgE, a protein that appears to form poly-uronic-specific channels for translocation out of the cell

(19). During the later stages in the periplasm, AlgG (8, 11) and AlgX (20) protect the developing polymer from AlgL, a periplasmic alginate lyase that degrades alginate via β-elimination (16, 22). Interestingly, the AlgG protein, but not its mannuronan C5-epimerase, is required for alginate polymer formation (8, 11).

Previous work in our lab using transposon mutagenesis indicated that the AlgL protein is needed for alginate production in *P. aeruginosa* strain FRD1 (16). This study tested our working hypothesis that AlgL's enzymatic activity as an alginate lyase is required for the production of alginate in *P. aeruginosa*.

Construction of *algL*Δ::Gm^r using FRD1::pJLS3. A ~6.9-kb HindIII-BamHI fragment from pNLS42 (22) containing wild-type *algL* from *P. aeruginosa* strain FRD1 was cloned into pUC19 (New England Biolabs) to create pMA1. This vector was digested with BstZ171 and XhoI to delete most of *algL* and 162 bp downstream of *algL*. Plasmid pSJ12 (12) encodes a gentamicin resistance (Gm^r) cassette with its promoter sequence intact but lacking its transcriptional stop sequence. This ~0.7-kb Gm^r cassette was amplified via PCR with primers provided with restriction sites for BstZ171 and XhoI, at 5' and 3', respectively, so that the antibiotic resistance cassette could be cloned into pMA1 and the antibiotic resistance marker expressed from its own promoter in the appropriate orientation as the alginate biosynthesis genes, generating pMA2. The resulting ~6.6-kb HindIII-BamHI sequence containing *algL*Δ::Gm^r from pMA2 was excised, blunt ended with Klenow polymerase, and cloned into the SmaI site of pEX100T, a gene replacement vector that does not replicate in *P. aeruginosa* and confers sucrose sensitivity from *sacB* (23); this generated pMA3. The ~0.24-kb XhoI fragment encompassing the *algL* terminus and 162 bp downstream of *algL* that was lost when deleting *algL* was isolated from pMA1 and cloned back into pMA3 in the proper orientation, determined via sequencing, giving rise to the *algL*Δ::Gm^r allelic exchange vector pMA4. Triparental mating was used to mobilize pMA4 from GeneHog cells (Invitrogen, Carlsbad, CA) with the aid of helper plasmid pRK2013 (3) to *P. aeruginosa* strain FRD1::pJLS3 (Cb^r Gm^s), which has the alginate biosynthetic cluster under *Ptac* control, allowing alginate production to be induced in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Merodiploids resulting from a single-crossover homologous recombination event were selected for on LA-PIA (a 1:1 mixture of LB

* Corresponding author. Mailing address: Division of Biomedical Sciences, University of California, Riverside, Riverside, CA 92521-0121. Phone: (951) 827-4569. Fax: (951) 827-5504. E-mail: neal.schiller@ucr.edu.

FIG. 1. FRD-MA7 (*algL* Δ ::Gm^r) is nonmucoid but is restored to the mucoid phenotype after complementation with a functional *algL* provided in *trans* by pNLS18. Each strain was grown on LA-PIA with the appropriate antibiotics and with or without IPTG as indicated for 48 h at 37°C.

agar and *Pseudomonas* isolation agar [both from Difco Laboratories]) plates with 250 μ g/ml of gentamicin (20). Mucoid merodiploids were then grown in Luria-Bertani broth, Lennox (Difco) for 18 h and plated onto LA-PIA with 250 μ g/ml of gentamicin containing 7.5% sucrose to select for colonies that had undergone double crossovers leading to gene replacement.

The *algL*-to-*algL* Δ ::Gm^r chromosomal exchange was confirmed via PCR and DNA sequencing in all of the nonmucoid recombinants tested, and one of these, designated FRD-MA7, was selected for further study. When induced with IPTG, FRD-MA7 displayed the same nonmucoid phenotype as its uninduced parent (Fig. 1). Complementation of FRD-MA7 with pNLS18 (22) and IPTG induction revealed that the mucoid phenotype could be restored in FRD-MA7 by *algL* provided in *trans* on pNLS18 (Fig. 1), demonstrating that the *algL* Δ ::Gm^r mutation was responsible for the observed nonmucoid phenotype.

FRD-MA7 lacks alginate lyase activity and does not produce uronic acid. FRD1::pJLS3, FRD-MA7, and FRD-MA7/pNLS18 were grown overnight at 37°C in 64 ml of modified alginate-producing (MAP) medium (4) supplemented with or without IPTG and the appropriate antibiotics. Cells were collected from 50 ml of the culture by centrifugation, washed twice, and resuspended in 30 mM Tris-HCl (pH 7.5) containing 0.2 M MgCl₂. The periplasmic fraction was isolated from these cells by temperature shock (22), and the number of alginate lyase enzyme units per mg of protein present was determined as described previously (16, 22). As expected, FRD-MA7 did not have any detectable alginate lyase activity, whereas complementation of FRD-MA7 with pNLS18 restored lyase activity (165.4 $\pm$ 15.6 U/mg for FRD-MA7/pNLS18 versus 156.1 $\pm$ 40.0 U/mg for FRD1::pJLS3) (data reflect means $\pm$ standard errors of the means from IPTG-induced cultures).

Although the *algL* Δ ::Gm^r mutation in FRD-MA7 results in a nonmucoid phenotype, this does not exclude the possibility that some form of poly-uronic acid is being synthesized. For these studies, the remaining 14 ml of cultures from the experiment above was split into two 7-ml aliquots, and the cells were collected by centrifugation, dried, and weighed while the culture supernatants were placed in Spectra/Por 6 dialysis membranes (Fisher Scientific, Pittsburgh, PA). To examine the ex-

tent of polymerization, we used 1-kDa dialysis membranes, which would retain polymers of ≥ 5 subunits, and 10-kDa dialysis membranes, which would retain polymers with ≥ 50 subunits. The culture supernatants were dialyzed to equilibrium overnight against 7 ml of 10 mM Tris-HCl (pH 7.6), and then the two fractions corresponding to the dialyzed (inside the tubing) and dialysate (outside the tubing) were collected for analysis. The remaining supernatant was extensively dialyzed for an additional 48 h prior to collecting the dialyzed fraction. Concentrations of uronic acid in these samples were determined using the carbazole assay (14) and a standard curve based on *Macrocystis pyrifera* alginate (Sigma), and they are reported as mg of uronic acid/g (dry weight) of cells (Fig. 2).

In the absence of IPTG, FRD1::pJLS3 produces 23.5 $\pm$ 3.8 mg/g of uronic acid, whereas in the presence of IPTG it produces 546.2 $\pm$ 35.1 mg/g. The difference between these values, 522.8 $\pm$ 33.4 mg/g, represents the amount of IPTG-inducible uronic acid made by FRD1::pJLS3, and this was high-molecular-weight, nondialyzable uronic acid (compare the values for inside versus outside the dialysis bag in Fig. 2). In contrast, FRD-MA7 produces 12.3 $\pm$ 2.0 mg/g of uronic acid in the absence of IPTG and only 15.1 $\pm$ 2.2 mg/g in the presence of IPTG. These values are not significantly different ($P > 0.05$), indicating that alginate biosynthesis in FRD-MA7 is not induced in the presence of IPTG, due to the deletion of *algL*. Polymeric uronic acid production by FRD-MA7 was restored to $\sim 70\%$ of parental levels when complemented with pNLS18. The data obtained in similar experiments using 10-kDa dialysis membranes were essentially the same (data not shown). These findings demonstrate that FRD-MA7 does not produce extracellular poly-uronic acid.

To explore the possibility that FRD-MA7 produces uronic acid trapped within the cell, we measured undialyzed extracellular and intracellular uronic acid levels in FRD1::pJLS3 and FRD-MA7. After growth in MAP (with or without IPTG) for 20 h at 37°C, the cells were pelleted by centrifugation and the supernatants were collected. The cell pellets were then washed, weighed, resuspended in Tris-HCl (pH 7.6), and passed three times through a French pressure cell press (American Inst. Co. Inc., Silver Spring, MD); the cellular debris was removed via centrifugation. Analysis of samples from inside and outside the cell demonstrated that FRD-MA7 induced with IPTG produces only 3.0 $\pm$ 0.9 mg/g of extracellular uronic acid and 0.4 $\pm$ 0.2 mg/g of intracellular uronic acid above background levels (i.e., uronic acid levels found in uninduced samples), while FRD1::pJLS3 induced with IPTG produces 240.7 $\pm$ 7.0 mg/g of uronic acid above background levels, and this uronic acid was found exclusively outside the cell (data not shown). These data further suggest that the *algL* Δ ::Gm^r mutation blocks alginate biosynthesis from the IPTG-inducible alginate operon.

Alginate lyase's activity is required for alginate polymer formation. To determine whether AlgL's enzymatic activity or physical presence is required for alginate biosynthesis, we created two mutant AlgL proteins using site-directed mutagenesis. These proteins were designated AlgL-H202Q and AlgL-Y256F, based on their respective mutations located within the active cleft. The 1.5-kb EcoRI-HindIII *algL* DNA fragment from pNLS18 was cloned into pUC19 to obtain pMA5. The basic amino acid histidine 202 (CAT), located in the NN HSYW conserved region of alginate lyase, and the hydropho-

FIG. 2. Poly-uronic acid is not produced in the absence of AlgL. Supernatants from cultures of FRD1::pJLS3, FRD-MA7, FRD-MA7/pNLS18, FRD-MA7/pMAH202Q, and FRD-MA7/pMAY256F were obtained after 20 h of incubation, with or without IPTG induction, and dialyzed in membranes with a 1-kDa molecular mass cutoff. Samples for uronic acid analysis were collected from inside and outside the dialysis bag after equilibrium dialysis (23 h), represented as “inside” and “outside” on the abscissa, and again inside the dialysis bag after extensive dialysis (48 h), represented as “final” on the abscissa. Data are reported as mg of uronic acid present per g (dry weight) of cells and represent the mean $\pm$ standard error of the mean from three replicates obtained in seven (FRD1::pJLS3), four (FRD-MA7 and FRD-MA7/pNLS18), or three (FRD-MA7/pMAH202Q and FRD-MA7/pMAY256F) separate experiments. Data shown represent the amount of uronic acid produced in IPTG-induced cultures after background uronic acid levels from the uninduced (-IPTG) strains had been subtracted. The asterisk designates a mean of 0.0 ± 0.0 . *P* values were determined using a one-way analysis of variance followed by Tukey’s posttest, where *P* < 0.05 was considered significant.

bic amino acid tyrosine 256 (TAC), were targeted for mutagenesis due to their potential roles in the enzymatic cleavage of alginate (25, 26). Using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA), H202Q (*CAG*) and Y256F (*TTC*) mutations were generated to obtain pMA6 and pMA7, respectively. These replacement residues were chosen since they are not ionizable and therefore should not interact with the alginate polymer. Furthermore, we would expect that these single point mutations would have no significant effect on the tertiary structure of AlgL. The mutagenized *algL* genes were cloned into pRK415 (13), sequenced to confirm the mutations, and designated pMAH202Q and pMAY256F, respectively. These vectors were electroporated into the *algL* Δ mutant, and transformants were selected for on LA-PIA plates with 100 μ g/ml of tetracycline supplemented with IPTG and confirmed via PCR analysis and sequencing. Expression of the mutant lyase proteins within the periplasm was confirmed via Western blotting (Fig. 3) as previously described (20) with minor modifications, i.e., using periplasmic fractions from cells grown in 50 ml of MAP medium (with or without IPTG), anti-AlgL rabbit antiserum (18), and donkey anti-rabbit immunoglobulin G horseradish peroxidase-linked antibody (Amersham Biosciences, Piscataway, NJ).

FRD-MA7 complemented with AlgL-H202Q and AlgL-Y256F lacked lyase activity, was nonmucoid, and was phenotypically identical to FRD-MA7 (data not shown). Dialysis of culture supernatants revealed that the uronic acid concentrations obtained both inside and outside the 1-kDa dialysis bag with FRD-MA7/pMAH202Q and FRD-MA7/pMAY256F

were not significantly above uninduced background levels (*P* > 0.05) (Fig. 2). Analysis of undialyzed extracellular and intracellular fractions from FRD-MA7/pMAH202Q and FRD-MA7/pMAY256F revealed that uronic acid levels in these strains were also not significantly above uninduced background levels (*P* > 0.05) (data not shown). These results suggest that in the absence of a lyase-active AlgL protein, *P. aeruginosa* produces only background levels of uronic acid, levels which are significantly reduced (*P* $\leq$ 0.01) relative to IPTG-induced FRD1::pJLS3 and FRD-MA7/pNLS18.

The present study supports the hypothesis that AlgL’s lyase activity is critical to alginate biosynthesis. Although its exact

FIG. 3. Transformation and expression of *algL* Δ H202Q and *algL* Δ Y256F in FRD-MA7 cultures were verified using Western blot analysis. Periplasmic fractions from FRD-MA7/pNLS18, FRD-MA7/pMAH202Q, and FRD-MA7/pMAY256F cultures were resolved on a 14% sodium dodecyl sulfate-polyacrylamide gel, transferred to a nitrocellulose membrane, probed with AlgL antiserum, and visualized on ECL hyperfilm. Lanes 1 and 2, FRD-MA7/pNLS18; lanes 3 and 4, FRD-MA7/pMAH202Q; lanes 5 and 6, FRD-MA7/pMAY256F. Samples in lanes 1, 3, and 5 were from cultures induced with IPTG, while samples in lanes 2, 4, and 6 were not. This photo is representative of blots from three separate experiments.

role in the biosynthesis of alginate remains to be determined, we propose that AlgL functions as part of the scaffold complex with AlgG, AlgX, and AlgK.

We thank Dennis Ohman for providing *P. aeruginosa* strain FRD1::pJLS3 and plasmid pSJ12.

REFERENCES

- Chitnis, C. E., and D. E. Ohman. 1993. Genetic analysis of the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* shows evidence of an operonic structure. *Mol. Microbiol.* **8**:583–590.
- Demko, C. A., P. J. Byard, and P. B. Davis. 1995. Gender differences in cystic fibrosis: *Pseudomonas aeruginosa* infection. *J. Clin. Epidemiol.* **48**:1041–1049.
- Figurski, D., and D. R. Helinski. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc. Natl. Acad. Sci. USA* **76**:1648–1652.
- Franklin, M. J., C. E. Chitnis, P. Gacesa, A. Sonesson, D. C. White, and D. E. Ohman. 1994. *Pseudomonas aeruginosa* AlgG is a polymer level alginate C5-mannuronan epimerase. *J. Bacteriol.* **176**:1821–1830.
- Franklin, M. J., and D. E. Ohman. 1993. Identification of *algF* in the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* which is required for alginate acetylation. *J. Bacteriol.* **175**:5057–5065.
- Franklin, M. J., and D. E. Ohman. 1996. Identification of *algI* and *algJ* in the *Pseudomonas aeruginosa* alginate biosynthetic gene cluster which are required for alginate O acetylation. *J. Bacteriol.* **178**:2186–2195.
- Franklin, M. J., and D. E. Ohman. 2002. Mutant analysis and cellular localization of the AlgI, AlgJ, and AlgF proteins required for O acetylation of alginate in *Pseudomonas aeruginosa*. *J. Bacteriol.* **184**:3000–3007.
- Gimmestad, M., H. Sletta, H. Ertesvag, K. Bakkevig, S. Jain, S.-J. Suh, G. Skjak-Braek, T. E. Ellingsen, D. E. Ohman, and S. Valla. 2003. The *Pseudomonas fluorescens* AlgG protein, but not its mannuronan C-5-epimerase activity, is needed for alginate polymer formation. *J. Bacteriol.* **185**:3515–3523.
- Goldberg, J. B., K. Hatano, and G. B. Pier. 1993. Synthesis of lipopolysaccharide O side chains by *Pseudomonas aeruginosa* PAO1 requires the enzyme phosphomannomutase. *J. Bacteriol.* **175**:1605–1611.
- Hutchison, M. L., and J. R. W. Govan. 1999. Pathogenicity of microbes associated with cystic fibrosis. *Microbes Infect.* **1**:1005–1014.
- Jain, S., M. J. Franklin, H. Ertesvag, S. Valla, and D. E. Ohman. 2003. The dual roles of AlgG in C-5-epimerization and secretion of alginate polymers in *Pseudomonas aeruginosa*. *Mol. Microbiol.* **47**:1123–1133.
- Jain, S., and D. E. Ohman. 1998. Deletion of *algK* in mucoid *Pseudomonas aeruginosa* blocks alginate polymer formation and results in uronic acid secretion. *J. Bacteriol.* **180**:634–643.
- Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger. 1988. Improved broad-host range plasmids for DNA cloning in gram-negative bacteria. *Gene* **70**:191–197.
- Knutson, C. A., and A. Jeanes. 1968. A new modification of the carbazole analysis. *Anal. Biochem.* **24**:470–481.
- Lyczak, J. B., C. L. Cannon, and G. B. Pier. 2002. Lung infections associated with cystic fibrosis. *Clin. Microbiol. Rev.* **15**:194–222.
- Monday, S. R., and N. L. Schiller. 1996. Alginate synthesis in *Pseudomonas aeruginosa*: the role of AlgL (alginate lyase) and AlgX. *J. Bacteriol.* **178**:625–632.
- Pedersen, S. S., N. Hoiby, F. Espersen, and C. Koch. 1992. Role of alginate in infection with mucoid *Pseudomonas aeruginosa* in cystic fibrosis. *Thorax* **47**:6–13.
- Preston, L., C. Bender, and N. Schiller. 2001. Analysis and expression of *algL*, which encodes alginate lyase in *Pseudomonas syringae* pv. *syringae*. *DNA Sequence* **12**:455–461.
- Rehm, B. H. A., G. Boheim, J. Tommassen, and U. K. Winkler. 1994. Overexpression of *algE* in *Escherichia coli*: subcellular localization, purification, and ion channel properties. *J. Bacteriol.* **176**:5639–5647.
- Robles-Price, A., T. Y. Wong, H. Sletta, S. Valla, and N. L. Schiller. 2004. AlgX is a periplasmic protein required for alginate biosynthesis in *Pseudomonas aeruginosa*. *J. Bacteriol.* **186**:7369–7377.
- Saxena, I. M., R. M. Brown, Jr., M. Fevre, R. A. Geremia, and B. Henrissat. 1995. Multidomain architecture of beta-glycosyl transferases: implications for mechanism of action. *J. Bacteriol.* **177**:1419–1424.
- Schiller, N. L., S. R. Monday, C. M. Bender, N. T. Keen, and D. E. Ohman. 1993. Characterization of the *Pseudomonas aeruginosa* alginate lyase gene (*algL*): cloning, sequencing, and expression in *Escherichia coli*. *J. Bacteriol.* **175**:4780–4789.
- Schweizer, H. P., and T. T. Hoang. 1995. An improved system for gene replacement and *xylE* fusion analysis in *Pseudomonas aeruginosa*. *Gene* **158**:15–22.
- Shankar, S., R. W. Ye, D. Schlichtman, and A. M. Chakrabarty. 1995. Exopolysaccharide alginate synthesis in *Pseudomonas aeruginosa*: enzymology and regulation of gene expression. *Adv. Enzymol. Relat. Areas Mol. Biol.* **70**:221–255.
- Yoon, H., W. Hashimoto, O. Miyake, K. Murata, and B. Mikami. 2001. Crystal structure of alginate lyase A1-III complexed with trisaccharide product at 2.0 Å resolution. *J. Mol. Biol.* **307**:9–16.
- Yoon, H., B. Mikami, W. Hashimoto, and K. Murata. 1999. Crystal structure of alginate lyase A1-III from *Sphingomonas* species A1 and 1.78 Å resolution. *J. Mol. Biol.* **290**:505–514.
- Zielinski, N. A., A. M. Chakrabarty, and A. Berry. 1991. Characterization and regulation of the *Pseudomonas aeruginosa* *algC* gene encoding phosphomannomutase. *J. Biol. Chem.* **266**:9754–9763.