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Characterization of Alginate Lyase from *Pseudomonas syringae* pv. syringae

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The gene encoding alginate lyase (*algL*) in *Pseudomonas syringae* pv. syringae was cloned, sequenced, and overexpressed in *Escherichia coli*. Alginate lyase activity was optimal when the pH was 7.0 and when assays were conducted at 42°C in the presence of 0.2 M NaCl. In substrate specificity studies, AlgL from *P. syringae* showed a preference for deacetylated polymannuronic acid. Sequence alignment with other alginate lyases revealed conserved regions within AlgL likely to be important for the structure and/or function of the enzyme. Site-directed mutagenesis of histidine and tryptophan residues at positions 204 and 207, respectively, indicated that these amino acids are critical for lyase activity.

Pseudomonas syringae pv. syringae causes disease in many plant species and produces the exopolysaccharide alginate, a linear polymer of O-acetylated β-1,4-linked D-mannuronic and L-guluronic residues (7, 11). Alginate functions as a virulence factor in *P. syringae* and also enhances epiphytic fitness, resistance to desiccation, and tolerance to toxic molecules (22, 29).

Pseudomonas aeruginosa is a leading cause of mortality in cystic fibrosis patients (24). Alginate contributes to the virulence of *P. aeruginosa* and protects the organism from antibiotics (13) and phagocytosis (1). The alginate biosynthetic and regulatory genes are located in several discrete regions of the *P. aeruginosa* chromosome (9). The alginate biosynthetic operon in *P. aeruginosa* is located at 34 min (4), and it is arranged similarly in *P. syringae* (21). Several genes in *P. syringae* have been identified that have homologs in *P. aeruginosa*, including *algA*, *algD*, *algF*, *algG*, *alg44*, *alg8*, *algL*, and *algT* (14, 21). Of particular interest to us was *algL*, which encodes alginate lyase.

Alginate lyases depolymerize alginate by cleaving the β-1,4 glycosidic bond, resulting in a molecule containing an unsaturated uronic acid residue at the nonreducing end (10, 15, 27). They prefer D-mannuronic or L-guluronic acid residues and may be affected by acetylation (10, 27). Alginate lyases from bacteria, algae, invertebrates, fungi, and bacteriophages have been characterized (27).

P. syringae pv. syringae FF5 produces low levels of alginate in vitro and appears nonmucoid (16); however, FF5 exhibits a mucoid colony morphology following the introduction of the 200-kb plasmid pPSR12 (16). Mutagenesis of FF5(pPSR12) resulted in the isolation of several alginate-defective mutants, including FF5.31, which contains a Tn*5* insertion in *algL* (21).

Cloning of *algL*. *Escherichia coli* strains (Table 1) were maintained on L medium (Difco Laboratories, Detroit, Mich.) at 37°C, and ampicillin was added at a concentration of 100 μg/ml. *Pseudomonas* spp. were grown on King's medium B (17) and cultured at 28°C (*P. syringae*) or 37°C (*P. aeruginosa*). pAPE6.2, which contains *algL*, *algF*, and *algA* (21), was used to construct pLP3.5, pLPX1.7, and pLPA1.4 (Fig. 1; Table 1). All genes were oriented to facilitate transcription from the T7 promoter of pBluescript SK+. To optimize the expression of *algL*, a 1.4-kb *Eco*RI/*Not*I fragment from pLPA1.4 was subcloned in pET21a in the same orientation as the T7 promoter and named pLP6.8 (Fig. 1).

Overproduction of AlgL and measurement of alginate lyase activity. *E. coli* BL21(DE3) cells containing various constructs were grown at 37°C until the optical density at 600 nm was ~0.6. Isopropyl-β-D-thiogalactopyranoside was added to a concentration of 1.0 mM, and cells were incubated an additional 3 h at 37°C. Whole-cell protein extracts were prepared and separated on sodium dodecyl sulfate-polyacrylamide gels (23). For the isolation of periplasmic alginate lyase, cells were grown at 27°C to an optical density at 600 nm of 0.6, induced with isopropyl-β-D-thiogalactopyranoside (1.0 mM), and incubated an additional 6 h. Cells were collected by centrifugation (8,000 × *g* for 15 min), and the periplasmic fraction was isolated by temperature shock (5). Alginate lyase activity was measured by the thiobarbituric acid assay (26) and recorded as enzyme units (EU), with 1 EU equal to the amount of AlgL needed to produce 1 μmol of β-formyl-pyruvate/min. The protein concentration was determined by measuring the A_{280} where an absorbance of 1.0 = 1 mg of protein/ml.

BL21(DE3) cells containing pLP3.5 and pLPA1.4 had AlgL activity, whereas cells containing pLPX1.7 did not (Table 2). These results localized *algL* between nucleotides 400 and 1800 with respect to the 5′ *Eco*RI site in pLP3.5, a hypothesis which was confirmed by sequence analysis. AlgL was overproduced in *E. coli* BL21(DE3) containing pLP6.8, and high levels of lyase activity correlated with the induction of a ~40-kDa band, which was found to be related to AlgL from *P. aeruginosa* when analyzed by immunoblotting.

Sequence analysis. The translational start for *algL* was located at bp 477 with respect to the 5′ *Eco*RI site in pAPE6.2 (Fig. 1), and the sequence extended to a stop codon at bp 1611. A potential ribosome-binding site was present 7 bp upstream from the start codon. The deduced protein product of *algL* contained 378 amino acids with a predicted N-terminal signal peptide. The N terminus of partially purified AlgL was sequenced, and the first 10 residues (A L V P P K G Y D A) confirmed that the protein was cleaved between 2 alanine residues (A28 and A29). AlgL was found to have a mass of

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TABLE 1. Bacterial strains and plasmids used in this study[a]

Strain or plasmid	Relevant characteristics	Source or reference
E. coli BL21(DE3)	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm*	Novagen
P. syringae pv. syringae FF5	Cu^r; contains pPSR12; stably mucoid	16
P. aeruginosa FRD462	*algG4* (polyM-producing derivative of FRD1)	8
Plasmids		
pBluescript SK(+)	Ap^r; ColE1 origin	Stratagene
pET21a	Ap^r; ColE1 origin	Novagen
pET21b	Ap^r; ColE1 origin	Novagen
pAPE6.2	Ap^r; a 6.2-kb *Eco*RI fragment in pBluescript SK+; contains *algL* from *P. syringae* FF5	21
pLP3.5	Ap^r; a 3.5-kb *Eco*RI/*Mlu*I(*Sma*I) fragment from pAPE6.2 in pBluescript SK+	This study
pLPX1.7	Ap^r; a 1.7-kb *Xmn*I fragment from pAPE6.2 in pBluescript SK+	This study
pLPA1.4	Ap^r; a 1.4-kb *Afl*III fragment from pAPE6.2 in pBluescript SK+	This study
pLP6.8	Ap^r; a 1.4-kb *Eco*RI/*Not*I fragment from pLPA1.4 in pET21a	This study
pLPH204A	Ap^r; a 1.1-kb *Eco*RI/*Xho*I fragment with mutated *algL* (His→Ala) in pET21b	This study
pLPW207A	Ap^r; a 1.1-kb *Eco*RI/*Xho*I fragment with mutated *algL* (Trp→Ala) in pET21b	This study

[a] Abbreviations: Ap^r, ampicillin resistance; Cu^r, copper resistance.

42,541 Da and an isoelectric point of 8.19 when analyzed using PeptideSort (version 10.0; University of Wisconsin Genetics Computer Group).

Multiple sequence alignments of AlgL and site-directed mutagenesis. AlgL from *P. syringae* was related to AlgL from *Halomonas marina* (76%), *P. aeruginosa* (63%), *Azotobacter chroococcum* (61%), and *Azotobacter vinelandii* (59%). CLUSTALX (25) was used to construct a multiple sequence alignment of alginate lyases. The region containing NNHSYW (residues 202 to 207 in *P. syringae* AlgL) was conserved among bacterial alginate lyases and included the active site identified in the crystal structure of alginate lyase A1-III from *Sphingomonas* (28). The importance of these residues in the activity of AlgL from *P. syringae* was investigated by replacing the histidine (H204) and tryptophan (W207) residues with alanine.

Mutant *algL* genes were constructed by a two-step PCR (2) using mutagenic oligonucleotides and primers located at the 5′ and 3′ ends of *algL*. H204 was replaced with alanine (GCG) using the primer set 1 (5′ AATCAACA**ACGCGT**CGTACTG GGCTGC), which contained an *Afl*III site (boldface). W207 was replaced with alanine using the primer set 2 (5′ AACCA CTCGTACGCGGCTGCCTGGTCG). The products of the first PCR were ~700 or 500 bp when the mutagenic oligonucleotides were used with the 5′- or 3′-end primers, respectively. The products of the first PCR were combined and used as a template in a second PCR with the 5′- and 3′-end primers. The resulting 1.1-kb PCR products were subcloned as *Eco*RI-*Xho*I fragments into pET21b, resulting in pLPH204A (His→Ala) and pLPW207A (Trp→Ala). When these constructs were overproduced in *E. coli* BL21(DE3), neither mutant protein had lyase activity, suggesting a role for these residues in substrate binding or enzyme catalysis (28).

Biochemical properties of AlgL. The pH optimum for AlgL was investigated using 15 mM sodium citrate and 30 mM

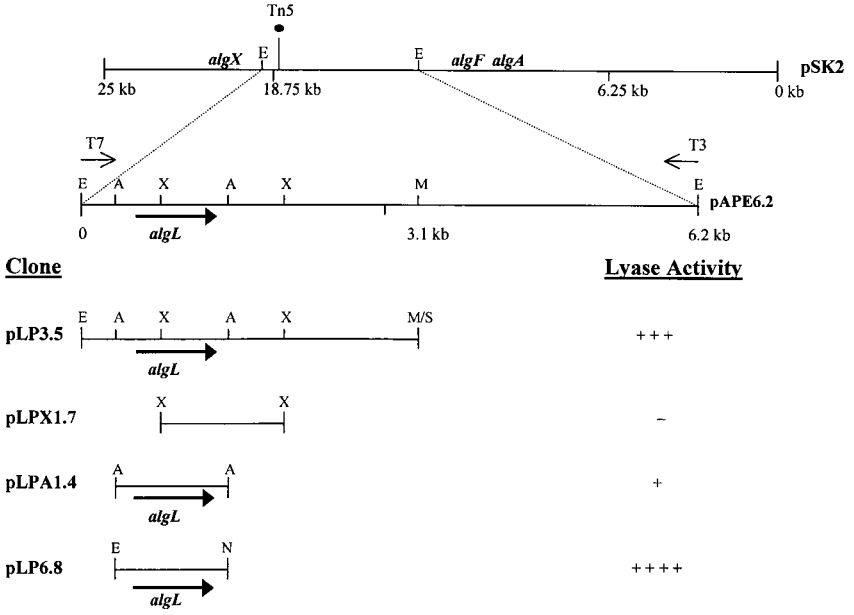

FIG. 1. Constructs used to localize, sequence, and overexpress *algL* from *P. syringae* pv. syringae. pSK2 contains the alginate structural gene cluster from *P. syringae* pv. syringae FF5 (21). The location of the Tn*5* insertion in the *algL* mutant FF5.31 is indicated (●), and the transcriptional orientation for *algL* is shown (→). Restriction site abbreviations: A, *Afl*III; E, *Eco*RI; M, *Mlu*I; N, *Not*I; S, *Sal*I; and X, *Xmn*I. Symbols for lyase activity: –, no activity; +, 50 to 100; +++, 500 to 750; ++++, 750 to 1,000.

TABLE 2. Alginate lyase activity in *E. coli* BL21(DE3) containing various constructs

Construct	AlgL activity (EU/ml)[a]	Protein concentration (mg/ml)	Specific activity (EU/mg)
pBluescript SK+	0	3.15	0
pLP3.5	523	4.42	118
pLPX1.7	0	2.86	0
pLPA1.4	82	2.27	36
pET21a	0	3.10	0
pLP6.8	806	3.65	221

[a] Numbers of EU/ml were determined with the thiobarbituric acid assay. See the text for an explanation.

$NaPO_4$ (pHs 5 and 6), 30 mM $NaPO_4$ (pH 7), and 30 mM Tris (pHs 8 and 9). The optimum pH for AlgL was 7.0, and only 50% activity was obtained at pH 5.0. AlgL activity was not reduced by 1 mM EDTA, indicating that the enzyme does not require divalent cations. Therefore, all subsequent assays were conducted at pH 7.0 without divalent cations. AlgL did not require NaCl for activity; however, the addition of 0.2 M NaCl enhanced lyase activity by ~70%. The optimum temperature for AlgL was 42°C, which is similar to that for other intracellular lyases (12, 19). The kinetics of AlgL were measured using different concentrations of sodium alginate from *Macrocytis pyrifera* (Sigma Chemical Co., St. Louis, Mo.) as described previously (6). The apparent K_m for AlgL was 3.4×10^{-4} M (sugar residues) when the data were analyzed using HYPER.EXE, version 1.1s (http://www.liv.ac.uk/~jse/software.html), and the maximal catalytic rate was 2.2×10^4 per s.

Substrate specificity of AlgL. Alginate was isolated from *P. syringae* FF5(pPSR12) and *P. aeruginosa* FRD462 using established methods (20) and deacetylated as described previously (8). The substrate specificity of AlgL from *P. syringae* was evaluated using sodium alginate from *M. pyrifera*, polymannuronate (polyM) alginate from *P. aeruginosa* FRD462 before and after deacetylation, and alginate from *P. syringae* FF5(pPSR12). AlgL degraded deacetylated polyM alginate more efficiently than the other substrates, indicating a preference for polyM and suggesting that acetylation interferes with lyase activity (Fig. 2). Furthermore, AlgL from *P. syringae* degraded its own alginate, which may indicate a role for AlgL in the biosynthesis of alginate or dissemination of the bacteria when they are exposed to conditions unsuitable for survival and growth (3).

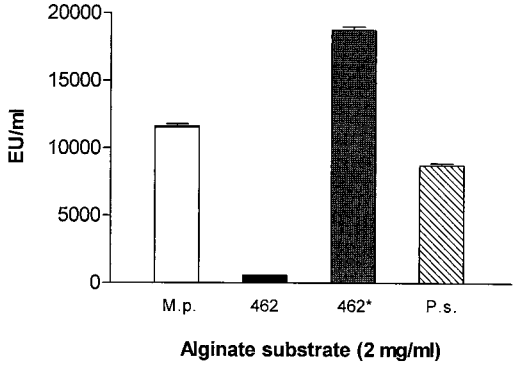

FIG. 2. Substrate specificity of alginate lyase. The lyase activity of extracts obtained after temperature shock was evaluated in enzyme reaction buffer (30 mM sodium phosphate, pH 7.0) at 37°C for 2 min using the following substrates at 2 mg/ml: sodium alginate from *M. pyrifera* (M.p.); polymannuronic acid (polyM) from *P. aeruginosa* FRD462 (462); deacetylated polyM (462*); and alginate isolated from *P. syringae* pv. syringae FF5(pPSR12) (P.s.). The data represent the mean ± the standard deviation ($n = 4$).

Alginate plays an important role in the virulence of both *P. syringae* and *P. aeruginosa*, and *algL* mutants of both species produce less alginate than wild-type strains (18, 21). The lyases from both pseudomonads degrade their own alginate, which is consistent with a role in cleaving preformed alginate and/or in determining the length of the alginate polymer. Elucidating the role of AlgL will provide a better understanding of alginate biosynthesis in both organisms and the diseases they cause in plant and animal hosts.

Nucleotide sequence accession number. The nucleotide sequence for *algL* from *P. syringae* was deposited in GenBank under accession no. AF222020.

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REFERENCES

1. **Baltimore, R. S., and D. G. Shedd.** 1983. The role of complement in the opsonization of mucoid and non-mucoid strains of *Pseudomonas aeruginosa*. Pediatr. Res. **17:**952–958.
2. **Barettino, D., M. Feigenbutz, R. Valcárcel, and H. G. Stunnenberg.** 1994. Improved method for PCR-mediated site-directed mutagenesis. Nucleic Acids Res. **22:**541–542.
3. **Boyd, A., and A. M. Chakrabarty.** 1994. Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **60:**2355–2359.
4. **Chitnis, C. E., and D. E. Ohman.** 1993. Genetic analysis of the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* shows evidence for an operonic structure. Mol. Microbiol. **8:**583–590.
5. **Eftekhar, F., and N. L. Schiller.** 1994. Partial purification and characterization of a mannuronan-specific alginate lyase from *Pseudomonas aeruginosa*. Curr. Microbiol. **29:**37–42.
6. **Ertesvåg, H., F. Erlien, G. Skjåk-Bræk, B. H. A. Rehm, and S. Valla.** 1998. Biochemical properties and substrate specificities of a recombinantly produced *Azotobacter vinelandii* alginate lyase. J. Bacteriol. **180:**3779–3784.
7. **Fett, W. F., S. F. Osman, M. L. Fishman, and T. S. Siebles III.** 1986. Alginate production by plant-pathogenic pseudomonads. J. Bacteriol. **52:**466–473.
8. **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.
9. **Gacesa, P.** 1998. Bacterial alginate biosynthesis—recent progress and future prospects. Microbiology **144:**1133–1143.
10. **Gacesa, P.** 1992. Enzymic degradation of alginates. Int. J. Biochem. **24:**545–552.
11. **Gross, M., and K. Rudolf.** 1987. Demonstration of levan and alginate in bean plants (*Phaseolus vulgaris*) infected by *Pseudomonas syringae* pv. phaseolicola. J. Phytopathol. **120:**9–19.
12. **Haraguchi, K., and T. Kodama.** 1996. Purification and properties of poly(β-D-mannuronate)lyase from *Azotobacter chroococcum*. Appl. Microbiol. Biotechnol. **44:**576–581.
13. **Hatch, R. A., and N. L. Schiller.** 1998. Alginate lyase promotes diffusion of aminoglycosides through the extracellular polysaccharide of mucoid *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **42:**974–977.
14. **Keith, L. M. W., and C. L. Bender.** 1999. AlgT (σ^{22}) controls alginate production and tolerance to environmental stress in *Pseudomonas syringae*. J. Bacteriol. **181:**7167–7184.
15. **Kennedy, L., K. McDowell, and I. W. Sutherland.** 1992. Alginases from *Azotobacter* species. J. Gen. Microbiol. **138:**2465–2471.
16. **Kidambi, S. P., G. W. Sundin, D. A. Palmer, A. M. Chakrabarty, and C. L. Bender.** 1995. Copper as a signal for alginate synthesis in *Pseudomonas syringae* pv. syringae. Appl. Environ. Microbiol. **61:**2172–2179.
17. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. **44:**301–307.
18. **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.
19. **Nakagawa, A., T. Ozaki, K. Chubachi, T. Hosoyama, T. Okubo, S. Iyobe, and T. Suzuki.** 1998. An effective method for isolating alginate lyase-producing *Bacillus* sp. ATB-1015 strain and purification and characterization of the lyase. J. Appl. Microbiol. **84:**328–335.
20. **Ohman, D. E., and A. M. Chakrabarty.** 1981. Genetic mapping of chromosomal determinants for the production of the exopolysaccharide alginate in a *Pseudomonas aeruginosa* cystic fibrosis isolate. Infect. Immun. **33:**142–148.
21. **Peñaloza-Vázquez, A., S. P. Kidambi, A. M. Chakrabarty, and C. L. Bender.**

1997. Characterization of the alginate biosynthetic gene cluster in *Pseudomonas syringae* pv. syringae. J. Bacteriol. **179:**4464–4472.
22. **Rudolph, K. W. E., M. Gross, F. Ebrahim-Nesbat, M. Nollenburg, A. Zomorodian, K. Wydra, M. Neugebauer, U. Hettwer, W. El-Shouny, B. Sonnenberg, and Z. Klement.** 1994. The role of extracellular polysaccharide as virulence factors for phytopathogenic pseudomonads and xanthomonads, p. 357–448. *In* C. I. Kado and J. H. Crosa (ed.), Molecular mechanisms of bacterial virulence. Kluwer Academic Publishers, Dordrecht, The Netherlands.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Thomassen, M. J., C. A. Demko, and C. F. Doershuk.** 1987. Cystic fibrosis: a review of pulmonary infections and interventions. Pediatr. Pulmonol. **3:** 334–351.
25. **Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins.** 1997. The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25:**4876–4882.
26. **Weissbach, A., and J. Hurwitz.** 1959. The formation of 2-keto-3-deoxyheptonic acid in extracts of *Escherichia coli*. J. Biol. Chem. **234:**705–709.
27. **Wong, T. Y., L. A. Preston, and N. L. Schiller.** 2000. Alginate lyase—review of major sources and enzyme characteristics, structure-function analysis, biological roles and applications. Annu. Rev. Microbiol. **54:**289–340.
28. **Yoon, H. J., B. Mikami, W. Hashimoto, and K. Murata.** 1999. Crystal structure of alginate lyase A1-III from *Sphingomonas* species A1 at 1.78Å resolution. J. Mol. Biol. **290:**505–514.
29. **Yu, J., A. Peñaloza-Vázquez, A. M. Chakrabarty, and C. L. Bender.** 1999. Involvement of the exopolysaccharide alginate in the virulence and epiphytic fitness of *Pseudomonas syringae* pv. syringae. Mol. Microbiol. **33:**712–720.