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

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*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*, *algH*, *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 F5 produces low levels of alginate in vitro and appears nonmucoid (16); however, F5 exhibits a mucoid colony morphology following the introduction of the 200-kb plasmid pPSR12 (16). Mutagenesis of F5(pPSR12) resulted in the isolation of several alginate-defective mutants, including F5.31, which contains a Tn5 insertion in *algL* (21).

Cloning of *algL*. *Escherichia coli* strain (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 pLP5.5, 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 EcoRI/NorI 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 using the thiorbituric 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-pyrurate/min. The protein concentration was determined by measuring the absorbance at 280 nm 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’ EcoRI 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’ EcoRI 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 (ALVPKP 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...
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 *Halo monas 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’-AATCAACAACGGCTCGTACTG GGCTGC-3’), which contained an *Afl*III site (boldface). W207 was replaced with alanine using the primer set 2 (5’-AACCA CTGTTACGCGGCTGCCTGGTCG-3’). 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 (His3Ala) and pLPW207A (Trp3Ala). 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 sodium acetate buffer (pH 5.0 to 8.0) with 50 mM sodium chloride. The pH optimum for AlgL was determined using 15 mM sodium citrate and 30 mM sodium acetate buffer (pH 5.0 to 8.0) with 50 mM sodium chloride. The pH optimum for AlgL was determined using 15 mM sodium citrate and 30 mM sodium acetate buffer (pH 5.0 to 8.0) with 50 mM sodium chloride.

![Diagram](https://example.com/diagram.png)

**TABLE 1.** Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli BL21(DE3)</strong></td>
<td>F'ompT hsdSb (rBmB) gal dcm</td>
<td>Novagen</td>
</tr>
<tr>
<td><strong>P. syringae pv. syringae FF5</strong></td>
<td>Cu'r; contains pPSR12; stably mucoid</td>
<td>16</td>
</tr>
<tr>
<td><strong>P. aeruginosa FRD462</strong></td>
<td>algG4 (polyM-derivative of FRD1)</td>
<td>8</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBluescript SK(+)</td>
<td>Ap'; ColE1 origin</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pET21a</td>
<td>Ap'; ColE1 origin</td>
<td>Novagen</td>
</tr>
<tr>
<td>pET21b</td>
<td>Ap'; ColE1 origin</td>
<td>Novagen</td>
</tr>
<tr>
<td>pAPE6.2</td>
<td>Ap'; a 6.2-kb <em>Eco</em>RI fragment in pBluescript SK+; contains algL from <em>P. syringae</em> FF5</td>
<td>21</td>
</tr>
<tr>
<td>plP3.5</td>
<td>Ap'; a 3.5-kb <em>Eco</em>RI/<em>Mlu</em>I fragment from pAPE6.2 in pBluescript SK+</td>
<td>This study</td>
</tr>
<tr>
<td>plPX1.7</td>
<td>Ap'; a 1.7-kb <em>Xmn</em>I fragment from pAPE6.2 in pBluescript SK+</td>
<td>This study</td>
</tr>
<tr>
<td>plPA1.4</td>
<td>Ap'; a 1.4-kb <em>Afl</em>III fragment from pAPE6.2 in pBluescript SK+</td>
<td>This study</td>
</tr>
<tr>
<td>plP6.8</td>
<td>Ap'; a 1.4-kb <em>Eco</em>RI/<em>Not</em>I fragment from pPA1.4 in pET21a</td>
<td>This study</td>
</tr>
<tr>
<td>plPH204A</td>
<td>Ap'; a 1.1-kb <em>Eco</em>RI/<em>Xho</em>I fragment with mutated algL (His→Ala) in pET21b</td>
<td>This study</td>
</tr>
<tr>
<td>plPW207A</td>
<td>Ap'; a 1.1-kb <em>Eco</em>RI/<em>Xho</em>I fragment with mutated algL (Trp→Ala) in pET21b</td>
<td>This study</td>
</tr>
</tbody>
</table>

a Abbreviations: Ap', ampicillin resistance; Cu', copper resistance.
of alginate or dissemination of the bacteria when they are exposed to conditions unsuitable for survival and growth (3).

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. AF22020. This work was supported by grants AI 36325 (N.L.S.) and AI 43311 (C.L.B.) from the National Institutes of Health. We thank Rick Hatcher and Sally Scott for technical assistance and Alejandro Peñaloza-Vázquez and Lisa Keith for advice and criticism.

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