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Characterization of the *Pseudomonas aeruginosa* Alginate Lyase Gene (*algL*): Cloning, Sequencing, and Expression in *Escherichia coli*

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Mucoid strains of *Pseudomonas aeruginosa* produce a viscous exopolysaccharide called alginate and also express alginate lyase activity which can degrade this polymer. By transposon mutagenesis and gene replacement techniques, the *algL* gene encoding a *P. aeruginosa* alginate lyase enzyme was found to reside between *algG* and *algA* within the alginate biosynthetic gene cluster at 35 min on the *P. aeruginosa* chromosome. DNA sequencing data for *algL* predicted a protein product of ca. 41 kDa, including a 27-amino-acid signal sequence, which would be consistent with its possible localization in the periplasmic space. Expression of the *algL* gene in *Escherichia coli* cells resulted in the expression of alginate lyase activity and the appearance of a new protein of ca. 39 kDa detected on sodium dodecyl sulfate-polyacrylamide gels. In mucoid *P. aeruginosa* strains, expression of *algL* was regulated by AlgB, which also controls expression of other genes within the alginate gene cluster. Since alginate lyase activity is associated with the ability to produce and secrete alginate polymers, alginate lyase may play a role in alginate production.

Pseudomonas aeruginosa is an opportunistic pathogen causing septicemia and severe, often lethal, infections of the respiratory tract, urinary tract, burn wounds, and blood (11). In patients with cystic fibrosis (CF), *P. aeruginosa* causes chronic pulmonary infections which remain intractable to antibiotic therapy, making this pathogen the major cause of morbidity and mortality in these patients (57).

The chronic nature and persistence of these bacteria in the CF lung depend in large part on the elaboration of a viscous mucoid exopolysaccharide coat, called alginate (40). Although mucoid strains of *P. aeruginosa* are rarely found in other clinical situations, their presence in CF patients is highly predictable, suggesting that the CF-affected lung provides a unique environment for inducing alginate production. Secretion of alginate also appears to provide survival advantages to the bacteria, such as by decreasing the uptake and early bactericidal effect of aminoglycosides (3) and inhibiting nonopsonic phagocytosis by monocytes and neutrophils both in vitro (3, 21) and in vivo (2). Once mucoid *P. aeruginosa* cells colonize the CF lung, all efforts to eradicate the bacteria, including aggressive antibiotic therapy and physiotherapy, have proven unsuccessful (57).

P. aeruginosa alginate is composed of a linear polymer of β-1-4-linked D-mannuronic and L-guluronic acids which is variably modified with *O*-acetyl groups on the mannuronic acid residues (27). Much of the pathway of alginate biosynthesis in *P. aeruginosa* has been defined, on the basis of fructose-6-phosphate as the initial precursor molecule (see references 40 and 45 for reviews). Many of the enzymes involved in alginate biosynthesis are clustered on about 18 kb of DNA near *argF* at 35 min on the *P. aeruginosa* chromosomal map (13) (Fig. 1) and appear to form an operon (9). These include *algA*, encoding the bifunctional enzyme phosphomannose isomerase-GDP-mannose pyrophosphorylase (52), *algG*, whose product is required for incorporation of L-guluronate residues into alginate (8), *algD*, which encodes GDP-mannose dehydrogenase (16), and *algE*, located just upstream of *algG*, which is believed to be involved in the later stages of alginate biosynthesis (10). However, the mechanisms of alginate polymer formation and export have not yet been defined.

Regulation of the alginate biosynthetic gene cluster depends on transcriptional regulation of *algD*, the first gene in the cluster (Fig. 1). Genes which have been identified as regulating *algD* include *algT*, *algB*, *algR* (*algR1*), *algQ* (*algR2*), and *algP* (*algR3*) (see reference 40 for review). The *algT* locus at 68 min is involved in the spontaneous conversion between the mucoid and nonmucoid phenotypes (46). In contrast, the *algB* gene at 13 min and the *algRPQ* cluster at 9 min are both responsible for high-level alginate production (17, 29). The products of *algR* (15) and *algB* (63) show similarity to the family of two-component response regulators which control gene expression in response to environmental factors (42). Environmental conditions such as high osmolarity increase the rate of *algD* transcription (4).

Alginates are enzymatically depolymerized by alginate lyases (EC 4.2.2.3), which cleave the 1-4 glycosidic linkage by β elimination, resulting in an unsaturated nonreducing terminus (26). Alginate lyases (also called alginases) have a preference for either L-guluronic or D-mannuronic acid residues and have been identified in a variety of bacteria including marine organisms (14, 19, 54, 55), *Bacillus circulans* (30), *Klebsiella* species (6, 37), and *Pseudomonas* species (55, 61), including *P. aeruginosa* (1, 20, 38, 43).

We have previously described the presence of alginate lyase activity in mucoid *P. aeruginosa* strains (43), and in this paper, we describe the cloning and sequencing of the *P. aeruginosa* alginate lyase gene (*algL*) and its expression in *Escherichia coli*.

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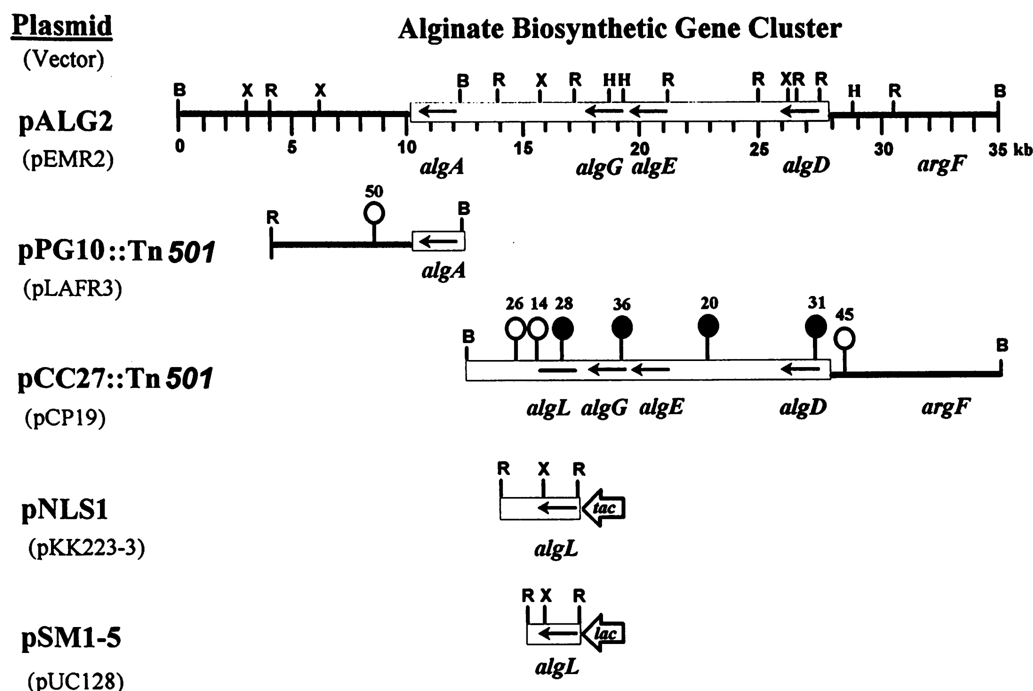

FIG. 1. Restriction and gene maps of *P. aeruginosa* DNA clones used in this study. Open bars represent DNA of the alginate biosynthetic gene cluster. Circles represent Tn*501* insertions which either block (solid circles) or do not affect (open circles) alginate lyase expression. The putative location of *algL* is identified. Approximate fragment sizes: pPG10, 8 kb; pCC27, 23 kb; pNLS1, 3.4 kb; pSM1-5, 1.9 kb. Restriction sites: B, *Bam*HI; X, *Xho*I; R, *Eco*RI; H, *Hin*dIII.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains, phages, and plasmids used in this study are shown in Table 1. *P. aeruginosa* FRD strains 1120, 1128, 1131, 1136, and 1145 containing characterized Tn*501* insertions in the alginate biosynthetic gene cluster were described by Chitnis and Ohman (9). Broad-host-range plasmids with defined Tn*501* insertions within the alginate gene cluster (pCC27::Tn*501*-14 and pCC27::Tn*501*-26) have been described previously (8) and were mobilized into *P. aeruginosa* FRD1 as described below.

DNA manipulations. Most routine genetic manipulations and plasmid extractions were done as described by Sambrook et al. (49). Plasmids used and constructed are shown in Table 1. Plasmids were mutagenized by Tn*501* insertions with plasmid RSF1010::Tn*501* as the transposon donor with protocols previously described (47). The sites of Tn*501* insertions were mapped by restriction fragment analysis. *P. aeruginosa* FRD1150 was constructed by gene replacement as previously described (47). Triparental matings were used to mobilize recombinant plasmids from *E. coli* to *P. aeruginosa* PAO1 with the conjugative helper plasmid pRK2013 by methods detailed elsewhere (28) and transferred to strain FRD1 with phage F116L transduction and gene recombination methods (47). A chloramphenicol acetyltransferase (CAT) polylinker cassette was constructed to permit ready insertion into several different unique restriction enzyme sites within genes of interest. The ca. 2.9-kb *Bam*HI-*Hin*dIII fragment from pRU686 containing the CAT gene (58) was blunted with Klenow fragment and recloned into the Klenow-blunted *Hin*dIII site of pMTL24 (7) to form pMCm. This plasmid contains the CAT coding region flanked on both sides with several identical restriction enzyme sites (see Fig. 4). An oligonucleotide site-directed mutation in *algL* was constructed with the polymerase chain reaction (PCR) method of Mikaelian and Sergeant (41) (see text for details).

DNA sequencing. A series of overlapping deletions of the ca. 1.9-kb insert fragment of clone pSM1-5 (Table 1) were prepared with the Erase-a-Base System (Promega Corporation, Madison, Wis.). Clone pSM1-5 with the gene in the same orientation as the vector promoter was employed for sequential exonuclease III deletions progressing from the left side of the insert as shown in Fig. 2. To sequence the other DNA strand, the 1.9-kb *Eco*RI insert fragment in pSM1-5 was recloned into the same site of pUC128 but in the opposite orientation. The orientation in which the *algL* gene was anti to the vector promoter (pSM2-14) was selected for construction of exonuclease III deletions that were then used for sequencing. Some of these deletions were also recloned into pUC129 as *Hin*dIII-*Eco*RI fragments and tested for their alginate lyase activity in *E. coli* cells on YC alginate plates (see below). In some cases, internal restriction fragments were subcloned in pUC129 and were end-sequenced. Double-stranded DNA sequencing was performed by the dideoxy chain-termination method with Sequenase (United States Biochemicals, Cleveland, Ohio) and deoxyadenosine 5'-[α-^{35}S]thiotriphosphate (>1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.), and both DNA strands were completely sequenced. Resolution of DNA regions containing GC compressions, common to pseudomonad double-stranded DNA sequencing, was accomplished by various strategies, such as replacing dGTP with either 7-deaza-dGTP or dITP (United States Biochemicals), as well as by using terminal deoxynucleotidyl transferase as suggested by Fawcett and Bartlett (22).

TABLE 1. Bacterial strains, phages, and plasmids

Strain, phage, or plasmid	Genotype, phenotype, or description[a]	Source or reference
E. coli		
HB101	*proA2 leuB6 thi-1 lacY1 hsdR hsdM recA13 supE44 rpsL20*	49
DH5α	F^- *lacZ*Δ M15 *endA1 hsdR17 supE44 thi-1 gyrA96 relA1 recA1*	49
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ (*lac-proAB*) F′ [*traD36* $proAB^+$ $lacI^q$ *lacZ*Δ*M15*]	49
JA-221	*hsdR* Δ*trpE5 leuB6 lacY recA thi* F′ ($lacI^q$ $lacZ^+$ $lacY^+$ $lacA^+$ $proA^+$ $proB^+$)	39
MC1061	*hsdR mcrB araD 139* Δ(*araABC-leu*)*7679* Δ*lacX74 galU galK rpsL thi*	49
P. aeruginosa		
PAO1	Prototrophic, Alg^-	32
FRD1	Prototrophic, Alg^+ CF isolate	44
FRD444	*algB2*::Tn*501*	29
FRD1145	Alg^+ Tn*501*-45 (upstream of *algD*) Hg^r	9
FRD1131	Alg^- *algD*::Tn*501*-31, Hg^r	9
FRD1120	Alg^- *alg*::Tn*501*-20 (between *algD* and *algG*) Hg^r	9
FRD1136	Alg^- *algG*::Tn*501*-36 Hg^r	9
FRD1128	Alg^- alg::Tn*501*-28 (between *algG* and *algA*) Hg^r	9
FRD1114	Alg^- *alg*::Tn*501*-14 (between *algG* and *algA*) Hg^r	This study
FRD1126	Alg^- *alg*::Tn*501*-26 (between *algG* and *algA*) Hg^r	This study
FRD1150	Alg^+ *alg*::Tn*501*-50 (downstream of *algA*) Hg^r	This study
Phage, F116L	*P. aeruginosa*-transducing phage	36
Plasmids		
pKK223-3	*E. coli* expression vector	Pharmacia LKB Biotechnology (Piscataway, N.J.)
pUC128/129	*E. coli* cloning vector	35
pBluescript II KS^+	*E. coli* cloning plasmid	Stratagene Cloning Systems (La Jolla, Calif.)
pLAFR3	Cosmid vector derived from pLAFR1 (25), Tc^r	53
pRK415	Broad-host-range cloning vector	35
pRK2013	ColE1-$Tra(RK2)^+$ Km^r	23
RSF1010::Tn*501*	IncQ Hg^r	47
pEMR2	pBR322 *cos oriT* Ap^r/Cb^r Km^r	24
pALG2	pEMR2 with 35-kb DNA from FRD1 containing $argF^+$ $algDGA^+$	8
pCP19	IncP1 *cos oriT* Tc^r	24
pCC27	pCP19 with 23 kb of pALG2 $argF^+$ $algDG^+$	8
pCC27::Tn*501*-14	*alg*::Tn*501*-14 (between *algG* and *algA*)	8
pCC27::Tn*501*-26	*alg*::Tn*501*-26 (between *algG* and *algA*)	8
pPG10	ca. 9-kb *Eco*RI-*Bam*HI fragment from pALG2 cloned into pLAFR3 containing *algA*	This laboratory
pNLS1	ca. 3.4-kb *Eco*RI fragment from pCC27 cloned into the same site of pKK223-3 with the insert oriented in the same direction as the vector *tac* promoter; strongly alginate lyase positive	This study
pSM1-5	ca. 1.9-kb *Eco*RI fragment from pCC27::Tn*501*-14 cloned into the same site in pUC128 with the insert oriented in the same direction as the vector *lac* promoter; strongly alginate lyase positive	This study
pSM2-14	ca. 1.9-kb *Eco*RI fragment from pSM1-5 was recloned into the same site of pUC128 with the insert oriented in the direction opposite to the vector *lac* promoter; weakly alginate lyase positive	This study
pNLS9	ca. 1.9-kb *Eco*RI fragment from pSM1-5 was blunted with S1 nuclease and recloned into the *Eco*RV site of pUC128 with the insert oriented in the direction opposite to the vector *lac* promoter; weakly alginate lyase positive	This study
pNLS10	ca. 1.9-kb *Eco*RI insert from pSM1-5 was cloned into the same site of pBluescript II KS^+ such that the orientation of the *algL* gene was inverse from the vector promoter; weakly alginate lyase positive	This study
pNLS11	ca. 1.9-kb *Eco*RI insert from pSM1-5 was cloned into the same site of pBluescript II KS^+ such that the *algL* gene was in the same orientation as the vector promoter; strongly alginate lyase positive	This study
pNLS12	pSM1-5 DNA was restricted with *Xho*I, and the large fragment remaining was religated; this deleted the 3′ terminus of the predicted *algL* gene; alginate lyase negative	This study
pNLS13	ca. 6.8-kb *Bam*HI-*Hin*dIII fragment from pCC27 recloned into the same sites of pRK415; alginate lyase positive	This study
pMCm	ca. 2.9-kb *Bam*HI-*Hin*dIII fragment from pRU686 (58) encoding chloramphenicol resistance was blunted with Klenow fragment and recloned into the Klenow blunted *Hin*dIII site of pMTL24 (7)	This study
pNLS14	pMCm DNA was restricted with *Kpn*I, and the ca. 2.9-kb CAT fragment was ligated with pNLS13 DNA that had been partially restricted with *Kpn*I; alginate lyase negative	This study
pNLS15	same as pSM1-5, except that a mutationally introduced *Eco*RI site ca. 90 bp upstream of the *Kpn*I site in *algL* destroyed the ATG start codon; very weak alginate lyase activity	This study
pNLS16	ca. 1.5-kb *Eco*RI fragment from pNLS15 was cloned into the same site of pUC129; strongly alginate lyase positive	This study
pNLS17	Δ2.7 deletion fragment of pNLS9 cloned into pUC129; strongly alginate lyase positive	This study

[a] Abbreviations: Alg^+, mucoid due to alginate production; Alg^-, nonmucoid; Hg^r, mercury resistance; Tra^+, transfer by conjugation; Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Cb^r, carbenicillin resistance; Tc^r, tetracycline resistance.

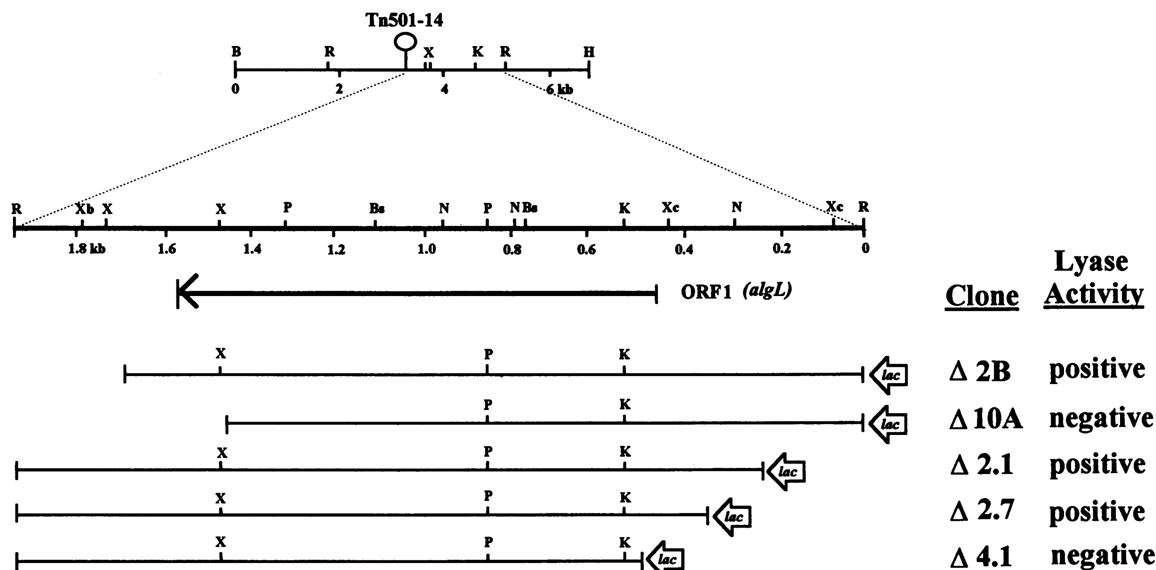

FIG. 2. Identification of the ca. 1.9-kb *Eco*RI DNA fragment (pSM1-5) encoding alginate lyase which was sequenced, as well as various deletion fragments which were used to localize the *algL* gene. The transcriptionally active orientation of ORF1 is shown by the arrow. Restriction sites: B, *Bam*HI; X, *Xho*I; R, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Xb, *Xba*I; P, *Pst*I; N, *Not*I; Bs, *Bst*EII; Xc, *Xcm*I.

Culture media. *E. coli* and *P. aeruginosa* strains were routinely cultured in L broth (10 g of tryptone, 5 g of yeast extract, and 5 g of sodium chloride per liter of distilled water [pH 7.5]) at 37°C, except for cultures grown to measure alginate lyase activity (see below). Antibiotics were used for selection at the following concentrations (per milliliter): ampicillin, 100 μg; carbenicillin, 300 μg; chloramphenicol, 34 μg; kanamycin, 25 μg; mercuric chloride, 18 μg; and tetracycline, 15 μg for *E. coli* or 100 μg for *P. aeruginosa*. The minimal medium used for selection of *P. aeruginosa* after triparental matings was that of Vogel and Bonner (59).

Alginate lyase plate assay. YC alginate medium was modified from the assay for pectate lyases (34) to permit assay of alginate lyase. It contained 17.5 g of NaCl, 2 g of ammonium sulfate, 0.2 g of $MgSO_4 \cdot 7H_2O$, 3 g of Casamino Acids, and 2 g of yeast extract per liter of water. Sodium alginate from *Macrocystis pyrifera* (Sigma Chemical Co., St. Louis, Mo.) was added to the medium at 0.5% (wt/vol) while stirred vigorously in a Waring blender, the pH was adjusted to ca. 8.0 with 10 N NaOH, and agar was added to 0.6%. After autoclaving, the medium (with antibiotics added as needed) was poured into petri plates and allowed to dry thoroughly before use. Selected colonies of *P. aeruginosa* or *E. coli* carrying various alginate lyase constructs were transferred onto the plates with toothpicks and were incubated at 37°C. Strongly positive reactions were visualized as depressions ≥1 cm in the medium surrounding colonies that appeared within 24 h.

Measurement of alginate lyase activity. Extracts for alginate lyase activity were prepared from overnight cultures grown in modified Luria broth which contained 0.3 M NaCl. Bacteria were collected by centrifugation, washed once with saline, and resuspended in 1/10 the original volume of 0.03 M Tris-HCl buffer (pH 7.5) containing 0.2 M $MgCl_2$. Alginate lyase release from the bacterial cells was done with a modification of the temperature shock method of Hoshino and Kageyama (33). Briefly, the cell suspension was subjected to 4 cycles of 37°C incubation for 10 min followed by incubation for 15 min at 0°C, and then it was centrifuged at 8,000 × *g* for 15 min. The supernatants were either tested immediately for alginate lyase activity or stored at −70°C.

The typical reaction mixture (250 μl) included the bacterial extract, buffer (0.03 M Tris-HCl [pH 8.5] containing 9 mM $MgCl_2$ and 0.5 M NaCl), and sodium alginate substrate (2 mg/ml) mixed in a 1:1:0.5 ratio, respectively. The reaction mixtures were incubated at 37°C for 20 min, and enzyme activity was measured by the thiobarbituric acid assay of Weissbach and Hurwitz (62). The results were expressed in enzyme units (EU), where 1 EU was defined as the amount of enzyme required to produce 1 nmol of β-formyl-pyruvate per min per ml at 37°C (43).

Polyacrylamide gel electrophoresis. *E. coli* JA-221 cells containing various *algL* constructs were grown for ca. 3 h in 15 ml of Luria medium supplemented with ampicillin at 28°C until the cell density was ca. 0.5 at 600 nm. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to 1 mM, and the cells were grown for an additional 6 h at 28°C. Cells were collected by centrifugation for 5 min at 5,000 × *g* and were resuspended in 2 ml of water. An equal volume of the cell suspension and 2.5× Laemmli sample solution were mixed and heated for 5 min in a boiling water bath. The samples were then run on a 1-mm-thick Laemmli 10% polyacrylamide-sodium dodecyl sulfate (SDS) gel and were stained with Coomassie blue R250 as previously described (12).

Nucleotide sequence accession number. The nucleotide sequence of *algL* shown in Fig. 3 has been deposited in the DDBJ, EMBL, and GenBank DNA data bases under accession no. L09724.

RESULTS

Location of the alginate lyase gene. Previous studies demonstrated that whereas crude enzyme extracts from mucoid *P. aeruginosa* strains typically express alginate lyase activity, extracts from nonmucoid variants of these strains have little if any detectable enzyme activity (50). Furthermore, alginate lyase production in some mucoid strains (including FRD1) is stimulated by the presence of high salt concentra-

EcoRI 10 20 30 40 50 XcmI 60
GAATTCGCCA CCCACTACGA CATGGCGCAG AAGAGCTTCT ACCGCCAGGC CATGCCGCTG

70 80 90 100 110 120
GTGGACAACG GCTGCTCCGG CCGGAAGACC GTACTCAGCC GCAAGGTCAA GCTGCGCCAG

130 140 150 160 170 180
GGCCGCAACG AGGTGCTGCT GAACAGCGCC GCGCTACCGA TCCGTAGCGG CAGCTACGTC

190 200 210 220 230 240
GCCGACGTGA CCTACAGCGA CCCTTCGGTA CACGAGTTGA AGAACACCAT CTGGTACATG

Δ2.1(+)
250 260 270 280 290 NotI 300
AACGGCCGCC GCGAGCAGTT GAAGATCGAG CAGTCGAAAG CCGTCGATAC CGGCGGCCGC

310 320 330 340 350 360
TACGTCTTCC AACTGCGCAA CGACAGCGAC TGGGCCGACC AGCAGTTCCT TTCCCTGGAA

Δ2.7(+)
370 380 390 400 410 420
ATCGAAGCGC CGGAGGACAT GCCCCAGGGC CTGGAGGTCC AGGCCAGCAT CTGCCAGGCG

430 440 XcmI 450 460 470
GCGCCCGCCA AGGCCAGCCA GTCGGTGGCC GGGAGGTAAA CG ATG AAA ACG TCC CAC
Met Lys Thr Ser His

480 490 500 510 520 530
CTG ATC CGT ATC GCC CTG CCC GGT GCC CTC GCC GCG GCA TTG CTC GCC AGC CAG
Leu Ile Arg Ile Ala Leu Pro Gly Ala Leu Ala Ala Ala Leu Leu Ala Ser Gln

Δ4.1 (-)
540 550 KpnI 560 570 580
GTC AGC CAG GCC GCC GAC CTG GTA CCC CCG CCC GGC TAC TAC GCG GCG GTC GGC
Val Ser Gln Ala Ala Asp Leu Val Pro Pro Pro Gly Tyr Tyr Ala Ala Val Gly
↑

590 600 610 620 630
GAG CGC AAG GGC AGC GCC GGC AGC TGC CCC GCG GTG CCG CCG CCG TAT ACC GGC
Glu Arg Lys Gly Ser Ala Gly Ser Cys Pro Ala Val Pro Pro Pro Tyr Thr Gly

640 650 660 670 680 690
AGC CTG GTC TTC ACC AGC AAA TAC GAA GGC TCC GAT TCG GCG CGG GCG ACC CTC
Ser Leu Val Phe Thr Ser Lys Tyr Glu Gly Ser Asp Ser Ala Arg Ala Thr Leu

700 710 720 730 740
AAC GTC AAG GCG GAG AAG ACC TTC CGC TCG CAG ATC AAG GAC ATC ACC GAC ATG
Asn Val Lys Ala Glu Lys Thr Phe Arg Ser Gln Ile Lys Asp Ile Thr Asp Met

750 760 770 BstEII 780 790 NotI 800
GAG CGC GGC GCT ACC AAG CTG GTC ACC CAG TAC ATG CGC AGC GGC CGC GAC GGC
Glu Arg Gly Ala Thr Lys Leu Val Thr Gln Tyr Met Arg Ser Gly Arg Asp Gly

810 820 830 840 850
GAC CTG GCC TGC GCA CTG AAC TGG ATG AGC GCC TGG GCC CGC GCC GGC GCC CTG
Asp Leu Ala Cys Ala Leu Asn Trp Met Ser Ala Trp Ala Arg Ala Gly Ala Leu

PstI 860 870 880 890 900
CAG AGC GAC GAC TTC AAC CAC ACC GGC AAG TCC ATG CGC AAA TGG GCG CTG GGC
Gln Ser Asp Asp Phe Asn His Thr Gly Lys Ser Met Arg Lys Trp Ala Leu Gly

910 920 930 940 950 NotI 960
AGC CTC TCC GGC GCC TAC ATG CGC CTG AAG TTC TCC AGC TCG CGG CCG CTC GCG
Ser Leu Ser Gly Ala Tyr Met Arg Leu Lys Phe Ser Ser Ser Arg Pro Leu Ala

970 980 990 1000 1010
GCC CAC GCC GAG CAG AGC CGG GAA ATC GAG GAC TGG TTC GCC CGG CTC GGC ACC
Ala His Ala Glu Gln Ser Arg Glu Ile Glu Asp Trp Phe Ala Arg Leu Gly Thr

1020 1030 1040 1050 1060 1070
CAG GTA GTC CGC GAC TGG AGC GGC CTG CCG CTG AAG AAG ATC AAC AAC CAT TCC
Gln Val Val Arg Asp Trp Ser Gly Leu Pro Leu Lys Lys Ile Asn Asn His Ser

1080 1090 1100 1110 BstEII 1120
TAC TGG GCG GCC TGG TCG GTG ATG TCC ACC GCG GTG GTG ACC AAC CGC CGC GAC
Tyr Trp Ala Ala Trp Ser Val Met Ser Thr Ala Val Val Thr Asn Arg Arg Asp

1130 1140 1150 1160 1170
CTC TTC GAC TGG GCG GTG AGC GAG TTC AAG GTC GCC GCC AAC CAG GTC GAC GAG
Leu Phe Asp Trp Ala Val Ser Glu Phe Lys Val Ala Ala Asn Gln Val Asp Glu

1180 1190 1200 1210 1220 1230
CAG GGC TTC CTG CCC AAC GAA CTC AAG CGC CGC CAG CGC GCC CTC GCC TAC CAC
Gln Gly Phe Leu Pro Asn Glu Leu Lys Arg Arg Gln Arg Ala Leu Ala Tyr His

1240 1250 1260 1270 1280
AAC TAT GCG CTG CCA CCG CTG GCG ATG ATC GCC GCG TTC GCC CAG GTC AAC GGC
Asn Tyr Ala Leu Pro Pro Leu Ala Met Ile Ala Ala Phe Ala Gln Val Asn Gly

1290 1300 1310 1320 PstI 1330 1340
GTC GAC CTG CGC CAG GAG AAC CAC GGC GCC CTG CAG CGC CTG GCC GAG CGG GTG
Val Asp Leu Arg Gln Glu Asn His Gly Ala Leu Gln Arg Leu Ala Glu Arg Val

1350 1360 1370 1380 1390
ATG AAG GGA GTC GAC GAC GAG GAA ACC TTC GAG GAG AAG ACC GGC GAG GAC CAG
Met Lys Gly Val Asp Asp Glu Glu Thr Phe Glu Glu Lys Thr Gly Glu Asp Gln

1400 1410 1420 1430 1440
GAC ATG ACC GAC CTC AAG GTC GAC AAC AAG TAC GCC TGG CTG GAG CCC TAC TGC
Asp Met Thr Asp Leu Lys Val Asp Asn Lys Tyr Ala Trp Leu Glu Pro Tyr Cys

Δ10A (-)
1450 1460 1470 ↓ 1480 XhoI 1490 1500
GCC CTC TAC CGC TGC GAG CCG AAG ATG CTC GAG GCG AAG AAG GAC CGC GAG CCG
Ala Leu Tyr Arg Cys Glu Pro Lys Met Leu Glu Ala Lys Lys Asp Arg Glu Pro

1510 1520 1530 1540 1550
TTC AAC AGT TTC CGC CTC GGC GGC GAA GTG ACG CGG GTG TTC AGC CGC GAA GGG
Phe Asn Ser Phe Arg Leu Gly Gly Glu Val Thr Arg Val Phe Ser Arg Glu Gly

1560 1570 1580 1590 1600 1610
GGA AGT TGA GGG C GTGGCGGGAA GCGGGCGCAC AGGCCGGGGG CCACGCGCCC
Gly Ser

1620 1630 1640 1650 1660 1670
CTCTACCCCG CTCGCCGACC AGGCGCCACA CCCCGACCCA TGCGCGACAG ACGCGGGCGG

Δ2B (+)
1680 1690 1700 1710 1720 1730
GCGCAACACC TTAGGCAACA CCACGGGGAG GAAAACGGCG GGTCGTCCCG CCTTTCCTCG

XhoI 1740 1750 1760 1770 1780 1790
AGCGCTCCAG GGCGCGGAAA GGCAGGGCCG ACCTGAATGG TTGACGCTCT AGAGAGAGAG

1800 1810 1820 1830 1840 1850
ACGGCATGGT CTTTTCTTCA AACGTGTTCC TGTTCCTGTT CCTGCCGGTC TTTCTCGGCC

1860 1870 1880 1890 1900 1910
TGTACTACCT GAGCGGCGAA CGTTACCGGA ACCTGCTGCT GCTGATCGCC AGCTACGTGT

1920 1930
TCTAGGGGGG CTCGCAGAAT TC

FIG. 3. Nucleotide sequence of the 1.9-kb *Eco*RI fragment (strand equivalent to the mRNA strand is shown). The amino acid sequence for the translated *algL* gene is shown. Locations of selected restriction sites are denoted above the sequence. The termination points of various exonuclease deletion fragments are identified by down arrows, while an up arrow shows the location of the cleavage site of the signal peptide (between Ala-27 and Ala-28) determined by N-terminal amino acid sequencing of mature AlgL. The end of the *Pseudomonas* DNA sequence and the beginning of the DNA sequence of the transposon Tn*501* occur at nucleotide position 1915 (Tn*501* DNA sequence is underlined).

tions (0.2 to 0.5 M NaCl) in the growth media (50), a condition which has also been found to stimulate the *algD* promoter and alginate biosynthesis (4). These findings suggested that alginate production and alginate lyase expression are coregulated. To further test this hypothesis, strains FRD1 and FRD444 (*algB2*::Tn*501*) were grown in 5× peptone broth as previously described (29). Crude enzyme preparations were prepared from bacterial pellets by heat shock treatment and were examined for alginate lyase activity with the thiobarbituric acid assay. Goldberg and Ohman (29) had previously shown that *algB* mutants (such as FRD444) produced much less alginate than the FRD1 parental strain. We confirmed this observation and also determined that strain FRD444 expressed 25× less alginate lyase activity than strain FRD1 (data not shown). This demonstrated that both alginate biosynthesis and alginate lyase expression are controlled by AlgB. It was recently shown that AlgB is a positive transcriptional regulator of *algD*, the first gene of the alginate biosynthetic cluster (63).

On the basis of the information given above, a likely location for the alginate lyase gene is within the alginate biosynthetic gene cluster at 35 min on the *P. aeruginosa* chromosome (45). To test this hypothesis, the effect of Tn*501* (8.2-kb) insertions placed within or adjacent to the alginate gene cluster on alginate lyase activity was examined. Insertion of the Tn*501* transposon within the biosynthetic gene cluster would affect transcription of downstream genes because the gene cluster appears to function as an operon (9). Restriction mapping studies were used to identify the location of these insertions (Fig. 1). As shown in Table 2, Tn*501* insertions located within the cluster blocked alginate formation, whereas insertions Tn*501*-45 and Tn*501*-50 (which are located outside the cluster) did not. While most of the insertions which blocked alginate biosynthesis also blocked alginate lyase activity, Tn*501* insertions 14 and 26 blocked alginate production but not alginate lyase activity. The finding that Tn*501*-28 but not Tn*501*-14 blocked expression of alginate lyase suggested that the alginate lyase gene

TABLE 2. Effect of transposon mutagenesis on alginate biosynthesis and alginate lyase production

Strain	Colony type[a]	Alginate lyase (avg EU/ml [no. of expts])[b]
FRD1 (wild type)	Mucoid	22.3 (4)
FRD1145 (Tn*501*-45)	Mucoid[c]	2.1 (3)
FRD1131 (Tn*501*-31)	Nonmucoid	0.3 (3)
FRD1120 (Tn*501*-20)	Nonmucoid	0.4 (4)
FRD1136 (Tn*501*-36)	Nonmucoid	1.2 (3)
FRD1128 (Tn*501*-28)	Nonmucoid	0.3 (9)
FRD1114 (Tn*501*-14)	Nonmucoid	50.4 (5)
FRD1126 (Tn*501*-26)	Nonmucoid	52.1 (2)
FRD1150 (Tn*501*-50)	Mucoid	55.2 (6)

[a] Qualitative determination on the basis of colony morphology.
[b] Determined with the thiobarbituric acid assay.
[c] Although mucoid, the amount of mucoid exopolysaccharide produced by FRD1145 was visibly less than that observed with FRD1.

(which we designated *algL*) was located at or near the Tn*501*-28 insertion site (Fig. 1).

To ensure that the alginate lyase-negative phenotype of the *alg*::Tn*501* mutants resulted from the Tn*501* insertions and was not due to spontaneous nonmucoid conversion at *algT* (46) or to mutations at other regulatory loci (e.g., *algB* or *algRPQ*; see reference 40), the complete alginate gene cluster was reconstructed in the chromosome of the *alg*::Tn*501* mutants. pALG2 (Fig. 1), which contains the entire alginate gene cluster, was conjugally transferred to *P. aeruginosa* by triparental mating and selection for carbenicillin resistance. The integration of pALG2 into the chromosome of the FRD1::Tn*501* derivatives occurred at a single cross-over by homologous recombination because this plasmid has a narrow host range (9). This resulted in a complete functional alginate gene cluster, and colonies displayed the mucoid phenotype. Complementation of the Tn*501*-mutagenized genes with pALG2 confirmed that the Tn*501* insertions within the designated areas shown in Fig. 1 were responsible for blocking mucoidy and alginate lyase expression.

Expression of the *algL* gene in *E. coli*. Genetic manipulations of the *algL* region of the alginate gene cluster were performed to determine the location of *algL* and whether it encodes alginate lyase. A 3.4-kb *Eco*RI fragment from pCC27, which should include the *algL* region (Fig. 1), was cloned into the *Eco*RI site of expression vector pKK223-3 to generate pNLS1. Ampicillin-resistant transformants of *E. coli* JM109 were screened for the presence of the 3.4-kb *Eco*RI fragment in both orientations behind the resident *tac* promoter in the vector. These strains, as well as the vector-transformed control, were grown to early log phase, and the *tac* promoter was induced with 1 mM IPTG. Growth was allowed to continue for an additional 4 h at 37°C. The bacteria were harvested, crude enzyme extracts were prepared, and alginate lyase activity was measured and compared with that in FRD1. As shown in Table 3, there was no alginate lyase activity in *E. coli* JM109 (pKK223-3), but activity was demonstrated in JM109 (pNLS1), which carried the 3.4-kb *Eco*RI fragment in the orientation shown in Fig. 1. Constructs which carried this same insert in the opposite orientation did not express enzymatic activity (extracts were examined with the thiobarbituric acid assay). These results showed that the *algL* gene encodes an alginate lyase and that it is oriented in the same direction as other alginate biosynthetic genes in the cluster (Fig. 1).

TABLE 3. Alginate lyase expression in *E. coli*

Strain	EU/ml[a]	Protein (mg/ml)	Sp act (EU/mg of protein)
P. aeruginosa FRD1	18.70	0.321	58.26
E. coli JM109 (pKK223-3)	0.0	0.436	0.0
E. coli JM109 (pNLS1)	66.79	0.458	145.83

[a] EU per milliliter were determined with the thiobarbituric acid assay. Data represent the average of triplicate determinations.

The location of Tn*501*-14 just beyond the apparent alginate lyase coding region provided a convenient transposon *Eco*RI site just downstream of the two *Xho*I sites (see the top of Fig. 2), which was exploited for further subcloning. The resultant ca. 1.9-kb *Eco*RI DNA fragment from pCC27::Tn*501*-14 was therefore isolated and cloned into pUC128 in both orientations. Strong alginate lyase activity, as determined by depressions on YC alginate plates, was observed when this fragment was oriented with the *Xho*I sites positioned furthest downstream of the *lac* promoter in pUC128 (pSM1-5), but weak enzymatic activity was also detected when the insert was in the opposite orientation (pSM2-14). This 1.9-kb *Eco*RI insert was also cloned in both orientations in pBluescript II KS$^+$. When the transcribing orientation was downstream of the *lac* promoter (pNLS11; Table 1), high levels of alginate lyase activity in *E. coli* cells were observed on YC alginate plates, while the opposite orientation (pNLS10) expressed low levels of activity.

Sequencing the *algL* gene. The 1.9-kb *Eco*RI fragment encoding the alginate lyase gene was sequenced. Since only one orientation of this fragment relative to the *lac* promoter in pUC plasmids resulted in high levels of alginate lyase production in *E. coli* cells, it was assumed that the reading frame occurred on the DNA strand shown in Fig. 3. The DNA sequence data (Fig. 3) showed the occurrence on this DNA strand of two overlapping, long open reading frames (ORFs). On the basis of the information described below, the smaller one of these, ORF1, appeared to encode the alginate lyase activity, and is therefore termed *algL*. ORF1 initiated with the ATG at base 463 and terminated with a TGA codon at base 1564. Location of the termination codon at this position was supported by exonuclease III deletion Δ2B at base 1679, which was fully active in the YC alginate lyase plate test, and by deletion Δ10A at position 1471, which was devoid of detectable activity (Fig. 2). A plasmid construct (pNLS12) in which the ca. 300-bp *Xho*I fragment (Fig. 3) was deleted was also alginate lyase negative, as predicted from placement of the translational stop codon. The translational start codon was located between deletion Δ2.7 at base 368, which was fully alginate lyase positive, and deletion Δ4.1 at base 539, which was devoid of activity (Fig. 2). The only ATG start codon between these deletions which was correctly located relative to a Shine-Dalgarno box occurred at base 463. An ATG codon in the same reading frame at base 379 was followed by a translational termination codon at base 457. A contiguous reading frame extends from position 1 of the sequenced region to the TAA codon at position 457. This could represent the 3′ end of another ORF which occurs immediately upstream of *algL*, but data concerning this possibility are not yet available.

The *algL* ORF shown in Fig. 3 encoded a protein of 367 amino acids with a computer-determined molecular weight of 40,826. The N terminus of the protein product contained amino acids that closely fit those predicted to function as a

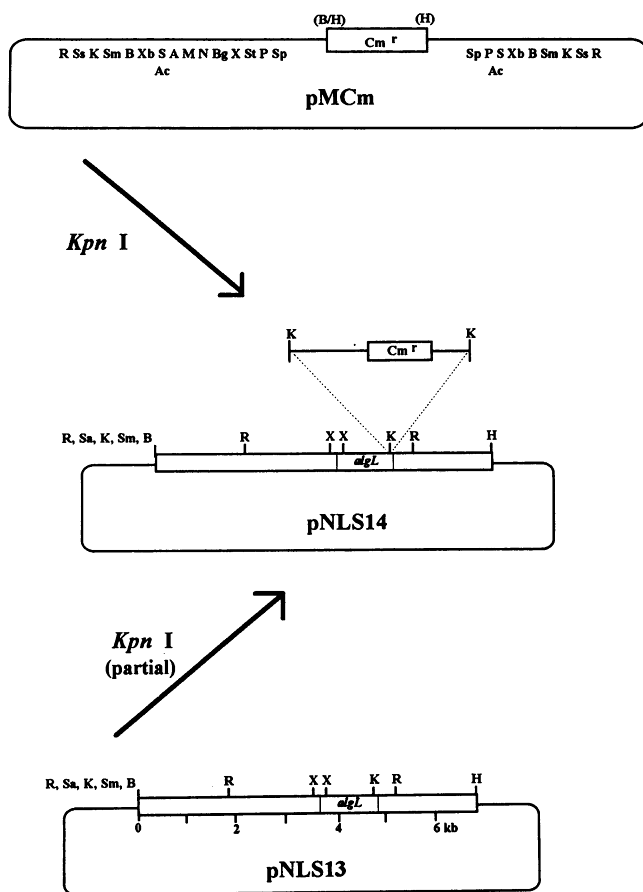

FIG. 4. Construction of a CAT-containing insertion mutant in the *algL* gene of pNLS13. Restriction sites: A, *Aat*I; Ac, *Acc*I; B, *Bam*HI; Bg, *Bgl*II; R, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; M, *Mlu*I; N, *Nco*I; P, *Pst*I; Sa, *Sac*I; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; Ss, *Sst*I; St, *Stu*I; X, *Xho*I; Xb, *Xba*I.

leader peptide (60). Thus, several basic and polar amino acids occurred in the first eight amino acids, followed by a string of 13 hydrophobic residues and then six C-region amino acids, several of which are polar. The putative signal peptide sequence was predicted to cleave between two alanine residues, amino acids 27 and 28 (Fig. 3). This was confirmed by N-terminal amino acid sequencing data, which are discussed in more detail later. The resulting mature secreted protein thus contained 340 amino acids with a predicted molecular weight of 38,112. The ORF encompassing the pre-protein was 68% G+C overall and 92% G+C in the third coding position. Three *Not*I restriction sites occurred in the sequenced region as well as several sites for other G+C-rich restriction enzymes. Such a high G+C content is similar to those of other *P. aeruginosa* genes, including another member of the *alg* operon, *algE* (10). The *algL* protein did not show significant homology to any other protein in the SwisProt (release 24) data base when the programs of Devereaux et al. (18) were used. Direct comparison of the *algL* sequence with *pel* genes encoding pectate lyase enzymes (31, 56) also did not disclose significant homology.

Insertion and oligo site-directed mutagenesis of *algL*. pMCm DNA was restricted with *Kpn*I, and the 2.9-kb CAT fragment was ligated with pNLS13 DNA (Fig. 4) that had been partially restricted with *Kpn*I and transformed into *E. coli*. The resulting *E. coli* transformants were screened to select a plasmid (pNLS14) in which the CAT cartridge was inserted into the unique *Kpn*I site of the *algL* gene (Fig. 4). As predicted, this construct was devoid of alginate lyase activity in *E. coli* cells, further supporting the identity of ORF1 as *algL*, the alginate lyase gene.

A mutation was constructed in which the putative start (ATG) and second (AAA) codons of *algL* were altered by mutation in order to confirm their role in the coding sequence. This was done by the PCR method of Mikaelian and Sergeant (41) in which the ca. 600-bp *Kpn*I fragment of pSM1-5 was amplified in the presence of the oligonucleotide 5′GGGAGGTAAACGAATTCAACGTCCCAC3′ (where altered codons 1 and 2 are underlined). The PCR-amplified product from the ORF *Kpn*I site to the ATG removed at position 463 was sequenced and confirmed to be correct. The PCR products were cloned into pUC128, transformed into *E. coli* DH5α, and the resultant plasmids were screened for an *Eco*RI site (GAATTC) introduced by the mutagenesis at position 462 (Fig. 3). One such plasmid was retained, and the DNA sequence of the mutated *algL* gene was confirmed by sequence analysis. The ca. 600-bp *Kpn*I fragment was then recloned in the correct orientation into the wild-type *algL* gene of pNLS11. This construct, called pNLS15, was transformed into *E. coli* cells.

When expressed in *E. coli* JM109 or JA-221, pNLS15 demonstrated little or no alginate lyase activity on YC alginate plates, further supporting the prediction that the methionine residue at position 463 is the translational initiation site of *algL*. This was further confirmed when the ca. 1.5-kb *Eco*RI insert fragment from pNLS15 was cloned into pUC129 to generate pNLS16. This should result in a translational fusion of *algL* with the *lacZ*α fragment of pUC129. When transformed into *E. coli* JA-221, a high level of alginate lyase activity was observed on YC alginate plates. These data confirm the occurrence of the ATG at base 463 as the translational start of *algL*.

Expression of alginate lyase activity. Several *E. coli* strains were evaluated for their ability to maintain *algL* plasmid constructs and express alginate lyase activity. Strain DH5α did not maintain several of the high-expression constructs well, exhibiting growth inhibition on Luria-ampicillin medium and alteration of the plasmids associated with loss of alginate lyase activity. Strain JM109 suitably maintained most of the plasmid constructs when grown on Luria-ampicillin plates but did not produce as high levels of activity of alginate lyase as strains MC1061 or JA-221. Plasmids leading to high levels of alginate lyase activity were also not maintained well in strain MC1061 but were retained through several single colony transfers in strain JA-221 on Luria-ampicillin plates supplemented with 0.3% glucose. Despite the fact that strain JA-221 has an episomal *lac* I^q gene which should aid in repressing vector *lac* promoters in the absence of IPTG induction, the addition of glucose was necessary to further repress the vector promoter. Strain JA-221 therefore appeared to be the best expression host of those screened for the *algL* gene.

SDS gel electrophoresis of whole *E. coli* JA-221 cells showed the presence of a ca. 39-kDa band that was unique to *algL* plasmid constructs exhibiting strong alginate lyase activity on YC alginate plates. As shown in Fig. 5, the 1.9-kb *Eco*RI fragment containing *algL* cloned in the down-promoter orientation in pBluescript II KS$^+$ (pNLS11, lane 4) or pUC128 (pSM1-5, lane 7) led to the appearance of the ca. 39-kDa band. Cells carrying the cloning plasmid only

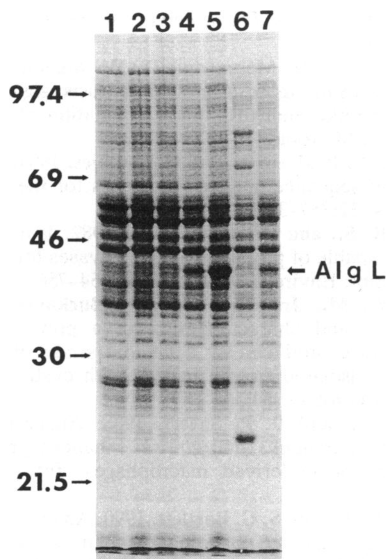

FIG. 5. SDS-polyacrylamide gel electrophoresis of Coomasie blue-stained proteins from whole *E. coli* JA-221 cells containing various constructs of *algL*. Lanes: 1, pUC129 only; 2, pNLS12; 3, pNLS10; 4, pNLS11; 5, pNLS17; 6, pNLS15; 7, pSM1-5. The numbers to the left of the figure mark the position of the molecular weight markers (in thousands).

(pUC129, lane 1), a deletion clone in which the sequence 3′ to the *Xho*I site at base 1477 was removed (pNLS12, lane 2), or the anti-promoter orientation in pBluescript II KS$^+$ (pNLS10, lane 3) did not produce a band that could be discerned from normal *E. coli* proteins. A clone in which the ATG start codon was removed by oligo-mutagenesis (pNLS15) also did not exhibit a detectable band at 39 kDa (lane 6). A very strong 39-kDa band was shown by cells containing the Δ2.7 deletion clone in pUC129 (pNLS17, lane 5). Since the 39-kDa protein matched closely in size that predicted for *algL* by DNA sequence data and was produced only from *E. coli* cells carrying alginate lyase-positive clones, the 39-kDa protein is most likely the *algL* gene product.

An SDS-polyacrylamide gel of whole *E. coli* JA-221 cells containing pNLS17 was electroblotted onto a polyvinylidene fluoride membrane, the 39-kDa protein band was excised, and its N-terminal amino acid sequence was determined by the University of California, Riverside, Biotechnology Instrumentation Facility. The N-terminal amino acid sequence (16 amino acids) exactly matched that predicted from the nucleotide sequence shown in Fig. 3, starting with the Ala-28 residue, confirming the predicted location of the cleavage of the signal peptide sequence (Fig. 3).

DISCUSSION

These results demonstrate that the alginate lyase structural gene (*algL*) is located within the alginate biosynthetic gene cluster between *algG* and *algA* at 35 min on the *P. aeruginosa* chromosome. This location had been predicted by the observations that alginate lyase production was controlled by AlgB, which also regulates the alginate biosynthetic gene cluster (63), and was stimulated by high salt concentrations (50), which also upregulate alginate biosynthesis (4). The results of our genetic manipulations in *P. aeruginosa* are also consistent with recent studies which show that genes in the alginate cluster may be organized as an operon (9).

Strong alginate lyase activity was detected when the *algL* gene was inserted behind vector promoters in the orientation shown in Fig. 1, consistent with the orientation of known biosynthetic genes in the operon. However, low but clearly detectable levels of activity were observed when the gene was oriented in the opposite direction (noted with YC alginate plates). This suggests the possibility of a weak internal promoter for *algL*. Chu et al. (10) also detected a weak internal promoter for *algE*, a gene which is found upstream of *algL* but downstream of *algD* and within the biosynthetic operon-like structure. The importance of an internal promoter for *algL* in the in vivo regulation of this enzyme needs further study.

The observation that the *P. aeruginosa* FRD1 alginate lyase enzyme produced in *E. coli* was approximately 39 kDa is comparable to the size of the semi-purified *P. aeruginosa* enzyme from strain WcM#2 (50). The inferred amino acid sequence derived from our DNA sequence analysis of *algL* showed a potential signal sequence for AlgL. Periplasmic localization of the lyase is consistent with the procedures required to release the enzyme from both *P. aeruginosa* and *E. coli* strains and is an area currently under investigation.

Expression of the cloned *algL* gene in *E. coli* not only verified the location of the structural gene for alginate lyase but also provided a source of the enzyme which can be used for protein purification and characterization studies. Other investigators have recently cloned alginate lyases from other species, including a guluronidase from *Klebsiella pneumoniae* (6) and a mannuronidase from a marine bacterium (5). Similarities between AlgL and the sequences of these enzymes, once they are reported, may provide information on the structure-function relationships of the *P. aeruginosa* alginate lyase.

The occurrence of *algL* within the biosynthetic operon suggests that the enzyme it encodes might have a role in alginate biosynthesis. Certainly, the production of this alginate lyase does not prohibit alginate polymer formation. However, the mucoid phenotype of a nonpolar mutation in *algL*, which does not block *algA* expression, should reveal whether AlgL is necessary or somewhat inhibitory to maximal polymer formation. Localization of *algL* within the biosynthetic cluster may actually facilitate regulation of the system in vivo, a model proposed by Romeo et al. (48) to explain the presence of catabolic genes within the glycogen biosynthetic cluster in *E. coli*. Alternatively, AlgL, which is functionally a depolymerase in vitro, may function in vivo as a component of an alginate polymerization reaction complex.

Characterization of *P. aeruginosa algL* may also have practical applications. The inability of current therapeutic strategies to clear mucoid *P. aeruginosa* strains from the lungs of CF patients has stimulated interest in developing novel strategies for eradicating the bacteria from patients. One possibility is to use a combination of alginate-degrading enzymes and conventional antibiotic therapy. Previous studies have shown that alginate lyase treatment of mucoid *P. aeruginosa* can render these bacteria more susceptible to nonopsonic phagocytosis and antibiotics both in vitro (3, 21) and in vivo (2). Studies are in progress to determine whether alginate lyase can reduce the viscosity of some CF sputum samples and in this capacity also provide an additive effect to DNase (51) in reducing sputum viscosity. The cloning of this gene and its overexpression in *E. coli* provide us with a source of enzyme which can be purified and characterized

and used in studies to evaluate potential therapeutic applications.

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REFERENCES

1. **Bartell, P. F., T. E. Orr, and G. K. H. Lam.** 1966. Polysaccharide depolymerase associated with bacteriophage infection. J. Bacteriol. **92:**56–62.
2. **Bayer, A. S., S. Park, M. C. Ramos, C. C. Nast, F. Eftekhar, and N. L. Schiller.** 1992. Effects of alginase on the natural history and antibiotic therapy of experimental endocarditis caused by mucoid *Pseudomonas aeruginosa*. Infect. Immun. **60:**3979–3985.
3. **Bayer, A. S., D. P. Speert, S. Park, J. Tu, M. Witt, C. C. Nast, and D. C. Norman.** 1991. Functional role of mucoid exopolysaccharide (alginate) in antibiotic-induced and polymorphonuclear leukocyte-mediated killing of *Pseudomonas aeruginosa*. Infect. Immun. **59:**302–308.
4. **Berry, A., J. D. DeVault, and A. M. Chakrabarty.** 1989. High osmolarity is a signal for enhanced *algD* transcription in mucoid and nonmucoid *Pseudomonas aeruginosa* strains. J. Bacteriol. **171:**2312–2317.
5. **Brown, B. J., J. F. Preston, and L. O. Ingram.** 1991. Cloning of alginate lyase gene (*alxM*) and expression in *Escherichia coli*. Appl. Environ. Microbiol. **57:**1870–1872.
6. **Caswell, R. C., P. Gacesa, K. E. Lutrell, and A. J. Weightman.** 1989. Molecular cloning and heterologous expression of a *Klebsiella pneumoniae* gene encoding alginate lyase. Gene **75:**127–134.
7. **Chambers, S. P., S. E. Prior, D. A. Barstow, and N. P. Minton.** 1988. The pMTL *nic*$^-$ cloning vectors. I. Improved pUC polylinker regions to facilitate the use of sonicated DNA for nucleotide sequencing. Gene **68:**139–149.
8. **Chitnis, C. E., and D. E. Ohman.** 1990. Cloning of *Pseudomonas aeruginosa algG*, which controls alginate structure. J. Bacteriol. **172:**2894–2900.
9. **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.
10. **Chu, L., T. B. May, A. M. Chakrabarty, and T. K. Misra.** 1991. Nucleotide sequence and expression of the *algE* gene involved in alginate biosynthesis by *Pseudomonas aeruginosa*. Gene **107:**1–10.
11. **Cross, A., J. R. Allen, J. Burke, G. Ducel, A. Harris, J. John, D. Johnson, M. Lew, B. MacMillan, P. Meers, R. Skalova, R. Wenzel, and J. Tenney.** 1983. Nosocomial infections due to *Pseudomonas aeruginosa*: review of recent trends. Rev. Infect. Dis. **5**(Suppl.)**:**S837–845.
12. **Dahler, G. S., F. Barras, and N. T. Keen.** 1990. Cloning of genes encoding extracellular metalloproteases from *Erwinia chrysanthemi* EC16. J. Bacteriol. **172:**5803–5815.
13. **Darzins, A., S.-K. Wang, R. I. Vanags, and A. M. Chakrabarty.** 1985. Clustering of mutations affecting alginic acid biosynthesis in mucoid *Pseudomonas aeruginosa*. J. Bacteriol. **164:**516–524.
14. **Davidson, I. W., I. W. Sutherland, and C. J. Lawson.** 1976. Purification and properties of an alginate lyase from a marine bacterium. Biochem. J. **159:**707–713.
15. **Deretic, V., R. Dikshit, W. M. Konyecsni, A. M. Chakrabarty, and T. K. Misra.** 1989. The *algR* gene, which regulates mucoidy in *Pseudomonas aeruginosa*, belongs to a class of environmentally responsive genes. J. Bacteriol. **171:**1278–1283.
16. **Deretic, V., J. F. Gill, and A. M. Chakrabarty.** 1987. Gene *algD* coding for GDP-mannose dehydrogenase is transcriptionally activated in mucoid *Pseudomonas aeruginosa*. J. Bacteriol. **169:**351–358.
17. **Deretic, V., C. D. Mohr, and D. W. Martin.** 1991. Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: signal transduction and histone-like elements in the regulation of bacterial virulence. Mol. Microbiol. **5:**1577–1583.
18. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
19. **Doubet, R. S., and R. S. Quatrano.** 1982. Isolation of marine bacteria capable of producing specific lyases for alginate degradation. Appl. Environ. Microbiol. **44:**754–756.
20. **Dunne, W. M., Jr., and F. L. A. Buckmire.** 1985. Partial purification and characterization of a polymannuronic acid depolymerase produced by a mucoid strain of *Pseudomonas aeruginosa* isolated from a patient with cystic fibrosis. Appl. Environ. Microbiol. **50:**562–567.
21. **Eftekhar, F., and D. P. Speert.** 1988. Alginase treatment of mucoid *Pseudomonas aeruginosa* enhances phagocytosis by human monocyte-derived macrophages. Infect. Immun. **56:**2788–2793.
22. **Fawcett, T. W., and S. G. Bartlett.** 1990. An effective method for eliminating "artifact banding" when sequencing double stranded DNA templates. BioTechniques **9:**46–48.
23. **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.
24. **Flynn, J. L., and D. E. Ohman.** 1988. Use of a gene replacement cosmid vector for cloning alginate conversion genes from mucoid and nonmucoid *Pseudomonas aeruginosa* strains: *algS* controls expression of *algT*. J. Bacteriol. **170:**3228–3236.
25. **Friedman, A. M., S. R. Long, S. E. Brown, W. J. Buikema, and F. M. Ausubel.** 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene **18:**289–296.
26. **Gacesa, P.** 1987. Alginate modifying enzymes: a proposed unified mechanism of action for the lyases and epimerases. FEBS Lett. **212:**199–202.
27. **Gacesa, P., and N. J. Russell.** 1990. The structure and properties of alginate, p. 29–49. *In* P. Gacesa and N. J. Russell (ed.), *Pseudomonas* infection and alginates: biochemistry, genetics and pathology. Chapman and Hall, London.
28. **Goldberg, J. B., and D. E. Ohman.** 1984. Cloning and expression in *Pseudomonas aeruginosa* of a gene involved in the production of alginate. J. Bacteriol. **158:**1115–1121.
29. **Goldberg, J. B., and D. E. Ohman.** 1987. Construction and characterization of *Pseudomonas aeruginosa algB* mutants: role of *algB* in high-level production of alginate. J. Bacteriol. **169:**1593–1602.
30. **Hansen, J. B., R. S. Doubet, and J. Ram.** 1984. Alginase enzyme production by *Bacillus circulans*. Appl. Environ. Microbiol. **47:**704–709.
31. **Hinton, J. C. D., J. M. Sidebotham, D. R. Gill, and G. P. C. Salmond.** 1989. Extracellular and periplasmic isoenzymes of pectate lyase from *Erwinia carotovora* subspecies *carotovora* belong to different gene families. Mol. Microbiol. **3:**1785–1795.
32. **Holloway, B. W., V. Krishnapillai, and A. F. Morgan.** 1979. Chromosomal genetics of *Pseudomonas*. Microbiol. Rev. **43:**73–102.
33. **Hoshino, T., and M. Kageyama.** 1980. Purification and properties of a binding protein for branched-chain amino acids in *Pseudomonas aeruginosa*. J. Bacteriol. **141:**1055–1063.
34. **Keen, N. T., D. Dahlbeck, B. Staskawicz, and W. Belser.** 1984. Molecular cloning of pectate lyase genes from *Erwinia chrysanthemi* and their expression in *Escherichia coli*. J. Bacteriol. **159:**825–831.
35. **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.
36. **Krishnapillai, V.** 1971. A novel transducing phage. Its role in recognition of a possible new host-controlled modification sys-

tem in *Pseudomonas aeruginosa*. Mol. Gen. Genet. **114**:134–143.

37. **Lange, B., J. Wingender, and U. K. Winkler.** 1989. Isolation and characterization of an alginate lyase from *Klebsiella aerogenes*. Arch. Microbiol. **152**:302–308.
38. **Linker, A., and L. R. Evans.** 1984. Isolation and characterization of an alginase from mucoid strains of *Pseudomonas aeruginosa*. J. Bacteriol. **159**:958–964.
39. **Masui, Y., J. Coleman, and M. Inouye.** 1983. Multipurpose expression cloning vehicles in *Escherichia coli*, p. 15–32. *In* M. Inouye (ed.), Experimental manipulation of gene expression. Academic Press, Inc., Orlando, Fla.
40. **May, T. B., D. Shinabarger, R. Maharaj, J. Kato, L. Chu, J. D. DeVault, S. Roychoudhury, N. A. Zielinski, A. Berry, R. K. Rothmel, T. K. Misra, and A. M. Chakrabarty.** 1991. Alginate synthesis by *Pseudomonas aeruginosa*: a key pathogenic factor in chronic pulmonary infections of cystic fibrosis patients. Clin. Microbiol. Rev. **4**:191–206.
41. **Mikaelian, I., and A. Sergeant.** 1992. A general and fast method to generate multiple site directed mutations. Nucleic Acids Res. **20**:376.
42. **Miller, J. F., J. J. Mekalanos, and S. Falkow.** 1989. Coordinate regulation and sensory transduction in the control of bacterial virulence. Science **243**:916–922.
43. **Nguyen, L. K., and N. L. Schiller.** 1989. Identification of a slime exopolysaccharide depolymerase in mucoid strains of *Pseudomonas aeruginosa*. Curr. Microbiol. **18**:323–329.
44. **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.
45. **Ohman, D. E., and J. B. Goldberg.** 1990. Genetics of alginate biosynthesis in *Pseudomonas aeruginosa*, p. 206–220. *In* P. Gacesa and N. J. Russell (ed.), *Pseudomonas* infection and alginates: biochemistry, genetics and pathology. Chapman and Hall, London.
46. **Ohman, D. E., J. B. Goldberg, and J. L. Flynn.** 1990. Molecular analysis of the genetic switch activating alginate production, p. 28–35. *In* S. Silver, A. M. Chakrabarty, B. Iglewski, and S. Kaplan (ed.), *Pseudomonas*: biotransformations, pathogenesis and evolving biotechnology. American Society for Microbiology, Washington, D.C.
47. **Ohman, D. E., M. A. West, J. L. Flynn, and J. B. Goldberg.** 1985. Method for gene replacement in *Pseudomonas aeruginosa* used in construction of *recA* mutants: *recA*-independent instability of alginate production. J. Bacteriol. **162**:1068–1074.
48. **Romeo, T., A. Kumar, and J. Preiss.** 1988. Analysis of the *Escherichia coli* glycogen gene cluster suggests that catabolic enzymes are encoded among the biosynthetic genes. Gene **70**:363–376.
49. **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.
50. **Schiller, N. L.** Unpublished studies.
51. **Shak, S., D. J. Capon, R. Hellmiss, S. A. Marsters, and C. L. Baker.** 1990. Recombinant human DNase I reduces the viscosity of cystic fibrosis sputum. Proc. Natl. Acad. Sci. USA **87**:9188–9192.
52. **Shinabarger, D., A. Berry, T. B. May, R. Rothmel, A. Fialho, and A. M. Chakrabarty.** 1991. Purification and characterization of phosphomannose isomerase-guanosine diphospho-D-mannose pyrophosphorylase—a bifunctional enzyme in the alginate biosynthetic pathway of *Pseudomonas aeruginosa*. J. Biol. Chem. **266**:2080–2088.
53. **Staskawicz, B., D. Dahlbeck, N. Keen, and C. Napoli.** 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. **169**:5789–5794.
54. **Stevens, R. A., and R. E. Levin.** 1977. Purification and characteristics of an alginase from *Alginovibrio aquatilis*. Appl. Environ. Microbiol. **33**:1156–1161.
55. **Sutherland, I. W., and G. A. Keen.** 1981. Alginases from *Beneckea pelagia* and *Pseudomonas* spp. J. Appl. Biochem. **3**:48–57.
56. **Tamaki, S. J., S. Gold, M. Robeson, S. Manulis, and N. T. Keen.** 1988. Structure and organization of the *pel* genes from *Erwinia chrysanthemi* EC16. J. Bacteriol. **170**:3468–3478.
57. **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.
58. **Ubben, D., and R. Schmitt.** 1987. A transposable promoter and transposable promoter probes derived from Tn*1721*. Gene **53**:127–134.
59. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218**:97–106.
60. **von Heijne, G.** 1985. Signal sequences. The limits of variation. J. Mol. Biol. **184**:99–105.
61. **von Riesen, V. L.** 1980. Digestion of algin by *Pseudomonas maltophilia* and *Pseudomonas putida*. Appl. Environ. Microbiol. **39**:92–96.
62. **Weissbach, A., and J. Hurwitz.** 1959. The formation of 2-keto-3-deoxyheptonic acid in extracts of *Escherichia coli* B. J. Biol. Chem. **234**:705–709.
63. **Wozniak, D. J., and D. E. Ohman.** 1991. *Pseudomonas aeruginosa* AlgB, a two-component response regulator of the NtrC family, is required for *algD* transcription. J. Bacteriol. **173**:1406–1413.