

## Alginate Synthesis in *Pseudomonas aeruginosa*: the Role of AlgL (Alginate Lyase) and AlgX

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Previous studies localized an alginate lyase gene (*algL*) within the alginate biosynthetic gene cluster at 34 min on the *Pseudomonas aeruginosa* chromosome. Insertion of a Tn501 polar transposon in a gene (*algX*) directly upstream of *algL* in mucoid *P. aeruginosa* FRD1 inactivated expression of *algX*, *algL*, and other downstream genes, including *algA*. This strain is phenotypically nonmucoid; however, alginate production could be restored by complementation in *trans* with a plasmid carrying all of the genes inactivated by the insertion, including *algL* and *algX*. Alginate production was also recovered when a merodiploid that generated a complete alginate gene cluster on the chromosome was constructed. However, alginate production by merodiploids formed in the *algX::Tn501* mutant using an alginate cluster with an *algL* deletion was not restored to wild-type levels unless *algL* was provided on a plasmid in *trans*. In addition, complementation studies of Tn501 mutants using plasmids containing specific deletions in either *algL* or *algX* revealed that both genes were required to restore the mucoid phenotype. *Escherichia coli* strains which expressed *algX* produced a unique protein of ~53 kDa, consistent with the gene product predicted from the DNA sequencing data. These studies demonstrate that AlgX, whose biochemical function remains to be defined, and AlgL, which has alginate lyase activity, are both involved in alginate production by *P. aeruginosa*.

*Pseudomonas aeruginosa* is one of the most important opportunistic human pathogens, causing septicemia and severe, often lethal infections of the respiratory tract, urinary tract, burn wounds, eyes, and intestines, as well as other sites (11). *P. aeruginosa* is ubiquitous and exhibits innate resistance to a wide range of antimicrobial agents, making infections with this pathogen both common and difficult to treat. Patients with cystic fibrosis (CF), the most common lethal genetic metabolic disease among Caucasians, have a multisystem disease due to a biochemical defect in the regulation of epithelial chloride transport (52). This defect leads to the accumulation of thick mucus in the lungs causing respiratory congestion and increased susceptibility to bronchopulmonary disease (27, 59). Despite an aggressive host immune response (57), patients with CF usually have chronic pulmonary infections with *P. aeruginosa* which remain intractable to antibiotic treatment (45, 56). The incidence of *P. aeruginosa* colonization in CF patients is very high (60 to 90%) and reaches almost 100% in some clinical studies (18, 33, 59). As a consequence, *P. aeruginosa* lung infections are the predominant cause of morbidity and mortality in CF patients (27, 33, 56, 59).

The persistence of *P. aeruginosa* in the lungs of CF patients, as well as the bacterial resistance to antibiotic action and host-mediated clearance mechanisms, has been attributed to the production of an exopolysaccharide called alginate (see reference 40 for a review). Alginate production decreases the uptake and early bactericidal effect of aminoglycosides (4) and inhibits nonopsonic phagocytosis by monocytes and neutrophils both in vitro (4, 20) and in vivo (3). Baltimore et al (1, 2) demonstrated that the mucoid coating also inhibits opsonic phagocytosis by concealing opsonic immunodeterminants on the bacterial surface. Moreover, the alginate coat increases bacterial adherence to the respiratory epithelia (19, 46), there-

by increasing the rate of colonization within the respiratory tract.

*P. aeruginosa* alginate is a linear, acetylated polymer consisting of  $\beta$ (1-4)-linked D-mannuronate and L-gulonate residues (26). Alginate synthesis is regulated by a complex process involving at least three distinct regions of the bacterial chromosome. A small cluster of genes including *algR* (*algR1*), *algQ* (*algR2*), and *algP* (*algR3*) at 9 min (see reference 15 for a review) and *algB* located at 13 min (30) are required for high-level alginate production. These genetic loci express proteins which are thought to act primarily through DNA binding and bending (14, 15, 60), similar to other proteins known to be members of bacterial two-component signal transduction systems. Located at 68 min is another gene cluster containing *algU* (*algT*), *mucA* (*algS*), and *mucB* (*algN*), which collectively interact to form a molecular switch which is ultimately responsible for the nonmucoid-to-mucoid conversion via the expression of an alternative RNA polymerase sigma factor from *algU* (see reference 16 for a review). Mutational analysis of *algT*, which expresses a protein found to have sequence homology with an *E. coli* global response sigma factor ( $\sigma^E$ ) (17, 35, 38), has revealed that *algT* is involved in regulating expression of the signal transduction receivers *algR* and *algB*, which in turn are required for optimal activation of the alginate biosynthetic gene cluster at 34 min (61). AlgT (AlGU) appears to be inhibited by the gene products of *mucA* and *mucB* (*algN*), which have been proposed to act in a manner analogous to that of anti- $\sigma$  factors (37). Inactivation of *mucA*, either experimentally or as seen in CF-associated isolates (37, 50), or experimental inactivation of *mucB* (36) or *algN* (28), derepresses AlgT (AlGU) activity.

The large cluster of alginate structural genes appears to function as an operon (8). Transcriptional activation of the promoter of *algD*, the first gene in this cluster, is associated with conversion to the alginate-producing (Alg<sup>+</sup>) phenotype (13, 51). Contained within this operon are *algA*, which encodes a bifunctional enzyme acting as both a phosphomannose isom-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Phenotype, genotype, or description	Source or reference
<i>P. aeruginosa</i> FRD1	Prototrophic, Alg <sup>+</sup> CF isolate	44
<i>P. aeruginosa</i> FRD1114	Alg <sup>-</sup> (Tn501 inserted immediately downstream of <i>algL</i> ) Hg <sup>r</sup>	48
<i>P. aeruginosa</i> FRD1128	Alg <sup>-</sup> <i>algX</i> ::Tn501-28 Hg <sup>r</sup>	48
<i>E. coli</i> JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB) F'</i> [ <i>traD36 proAB<sup>+</sup> lacI<sup>Δ</sup> lacZΔM15</i> ]	47
<i>E. coli</i> HMS174(DE3)	F <sup>-</sup> <i>recA r<sub>k12</sub><sup>-</sup> m<sub>k12</sub><sup>-</sup> Rif<sup>r</sup>(ΔDE3)</i>	55
pEMR2	pBR322 <i>cos oriT</i> Ap <sup>r</sup> /Cb <sup>r</sup> Km <sup>r</sup>	22
pALG2	pEMR2 with 35-kb <i>Bam</i> HI fragment from FRD1 containing <i>argF<sup>+</sup> algDGLFA<sup>+</sup></i>	7
pSM4	pALG2 <i>ΔalgL</i>	This study
pSM5	pRSET5A with <i>algX</i> inserted into vector <i>Nde</i> I- <i>Pst</i> I restriction sites	This study
pUCP21	Broad-host-range expression vector with <i>lac</i> promoter; Ap <sup>r</sup> /Cb <sup>r</sup> mob <sup>-</sup>	58
pRK415	Broad-host-range expression vector with <i>lac</i> promoter; Tc <sup>r</sup> mob <sup>+</sup>	31
pNLS18	ca. 1.6-kb <i>algL</i> DNA fragment ligated into <i>Hind</i> III- <i>Eco</i> RI sites of pRK415 for AlgL expression	This study
pNLS42	ca. 8.8-kb <i>Hind</i> III- <i>Sst</i> I ( <i>algXLFA<sup>+</sup></i> ) DNA fragment ligated into pUCP21; mob <sup>-</sup> Ap <sup>r</sup> /Cb <sup>r</sup>	This study
pNLS43	ca. 8.4-kb <i>Hind</i> III- <i>Sst</i> I ( <i>algLFA<sup>+</sup> ΔalgX</i> ) DNA fragment ligated into pUCP21; mob <sup>-</sup> Ap <sup>r</sup> /Cb <sup>r</sup>	This study
pNLS44	ca. 8.35-kb <i>Hind</i> III- <i>Sst</i> I ( <i>algXFA<sup>+</sup> ΔalgL</i> ) DNA fragment ligated into pUCP21; mob <sup>-</sup> Ap <sup>r</sup> /Cb <sup>r</sup>	This study
pRK2013	ColE1- <i>tra</i> (RK2) <sup>+</sup> Km <sup>r</sup>	21
pMF36	Broad-host-range vector containing a <i>tac</i> promoter; mob <sup>+</sup> Ap <sup>r</sup> /Cb <sup>r</sup>	24
pMF39	pMF36 vector containing <i>algG</i> , <i>algX</i> , and <i>algL</i> on a ca. 4.5-kb <i>Nco</i> I- <i>Xba</i> I fragment properly oriented for expression from the vector <i>tac</i> promoter; Ap <sup>r</sup> /Cb <sup>r</sup> mob <sup>+</sup>	M. Franklin
pNLS30	pUC129 containing <i>algX</i> and <i>algL</i> on a ca. 3.5-kb <i>Hind</i> III- <i>Xba</i> I fragment from pMF39	This study
pUC129	pUC119-derivative cloning vector; Ap <sup>r</sup>	31
pRSET5A	pBluescript backbone containing bacteriophage T7 expression elements of pET3; Ap <sup>r</sup>	49
pLysS	pACYC184 with bacteriophage T7 lysozyme gene inserted into vector <i>Bam</i> HI site; gene is inserted with start site away from vector Tc <sup>r</sup> promoter; Cm <sup>r</sup>	55

<sup>a</sup> Abbreviations: Alg<sup>+</sup>, mucoid because of alginate production; Alg<sup>-</sup>, nonmucoid; Tc<sup>r</sup>, tetracycline resistance; Rif<sup>r</sup>, rifampin resistance; Hg<sup>r</sup>, mercury resistance; Km<sup>r</sup>, kanamycin resistance; Ap<sup>r</sup>, ampicillin resistance; Cb<sup>r</sup>, carbenicillin resistance; Cm<sup>r</sup>, chloramphenicol resistance; Tra<sup>+</sup>, transfer by conjugation.

erase and a GDP-mannose pyrophosphorylase (53); *algD*, which expresses a GDP-mannose dehydrogenase (13); *algF*, a gene involved in alginate acetylation (24, 54); and *algG*, which encodes a C-5 epimerase (7, 23). Several other open reading frames (ORFs) found within the operon, including *algE* (9), *alg-44* (34), and *alg-8* (34), have also been described; however, the roles of the proteins encoded by these genes in alginate biosynthesis are unknown.

Alginates are enzymatically depolymerized by alginate lyases (EC 4.2.2.3), which cleave the 1-4 glycosidic linkage by  $\beta$ -elimination, resulting in an unsaturated, nonreducing terminus (25). We previously showed that *algL*, the *P. aeruginosa* gene for alginate lyase (48), is located within the alginate biosynthetic gene cluster, between *algG* and *algF*. The location of this gene within the biosynthetic gene cluster and its coregulation with genes involved in alginate production suggested that AlgL may be required for synthesis of the alginate polymer by *P. aeruginosa*. This paper presents the results obtained from two complementary molecular strategies, utilizing transposon-mutagenized, nonmucoid variants of the CF isolate FRD1, which conclusively demonstrate the involvement of both *algL* and *algX*, the gene immediately upstream of *algL*, in alginate production by *P. aeruginosa*. Expression of *algX* in *Escherichia coli* produced a unique protein of ~53 kDa consistent with the gene product predicted from its DNA sequence.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** Bacterial strains and plasmids used in this study are described in Table 1. Strains were routinely cultured in L broth (1.0% Bacto-tryptone, 0.5% yeast extract, 0.5% NaCl) or on L agar plates (L broth with 1.5% agar) with antibiotics as needed. For some studies, a 1:1 mixture of L agar and Pseudomonas Isolation Agar (Difco Laboratories, Detroit, Mich.) (P/L agar plates) was used. Unless otherwise indicated, antibiotics were used at the following concentrations (in micrograms per milliliter): ampicillin, 100 for *E. coli*; carbenicillin, 300 for *P. aeruginosa*; kanamycin, 50 for *E. coli*; chloramphenicol,

50 for *E. coli*; mercuric chloride, 18 for *P. aeruginosa*; tetracycline, 25 for *E. coli* and 100 for *P. aeruginosa*.

**Recombinant-plasmid construction.** General procedures for plasmid preparation (including restriction digests, ligations, and transformations) were performed as described by Sambrook et al. (47) with *E. coli* JM109. When needed, plasmids were isolated from *E. coli* by using the Wizard mini plasmid preparation kit (Promega Corporation, Madison, Wis.).

**Construction of plasmid vectors pNLS42, pNLS43, and pNLS44.** Each of the plasmid vectors pNLS42, pNLS43, and pNLS44 (see Fig. 1) was derived from pNLS37, a pRK415 vector containing an 8.8-kb *Hind*III-*Sst*I fragment from pALG2 expressing the biosynthetic gene cluster from *algX* through *algA*. pNLS42 was constructed by isolating the entire 8.8-kb *Hind*III-*Sst*I fragment from pNLS37 and ligating it into pUCP21, a vector chosen for enhanced expression of these genes in *P. aeruginosa* (58). pNLS44 is comparable to pNLS42 except that during its construction, the 465-bp *Pst*I fragment internal to *algL* was removed. pNLS43 is comparable to pNLS42 except that during its construction, the 384-bp *Xcm*I fragment located at the 3' terminus of the *algX* region was removed (this fragment encompasses the site into which the Tn501 transposon had inserted into the FRD1128 chromosome).

**Construction of pSM4.** The wild-type *algL* gene of pALG2 was exchanged for the *algL::cat* gene of pNLS14 (previously described in reference 48) by an interplasmid exchange technique (41). Rare double crossover, homologous recombination events occurred in RecA<sup>+</sup> *E. coli* C600. Following overnight incubation, the cosmid vector of the double transformants was packaged by making a  $\lambda$ CL857 lysate which was used to transduce *E. coli* JM109. Bacteria containing double-crossover recombinants were identified as ampicillin-resistant, chloramphenicol-resistant, and tetracycline-sensitive colonies. Restriction digests of the vectors isolated from these bacteria confirmed the proper construction, designated pSM3, which was next digested with *Xba*I to remove the *algL::cat* region and all but 87 bp of the chromosomal *algL* gene. After religation, constructs recovered from transformed bacteria were digested with *Xba*I to ensure that the *algL* region of pSM3 had been deleted. This plasmid was designated pSM4.

**Triparental matings.** Triparental matings were used to mobilize recombinant plasmids from *E. coli* to *P. aeruginosa* with the conjugative helper plasmid pRK2013 by methods detailed elsewhere (29), with the following minor modification. After a 6-h incubation at 37°C, the filter containing the mating mixtures was placed into a sterile tube containing 5 ml of 0.85% NaCl and vortexed to dislodge the bacteria. P/L agar plates containing carbenicillin were inoculated with 100  $\mu$ l of a 1:10 dilution of the bacterial suspension and incubated at 37°C until colonies appeared. Individual transconjugants were scored for reversion to the mucoid phenotype.

**Electroporation.** Electroporation-competent *P. aeruginosa* FRD1114 and FRD1128 cells were prepared by harvesting 100-ml overnight cultures by cen-

trifugation and washing them three successive times with 15 ml of ice-cold 10% glycerol. After the final wash, the bacteria were resuspended in 5 ml of 10% glycerol, aliquoted, and frozen at  $-80^{\circ}\text{C}$ . Electroporation was performed by the *Pseudomonas putida* protocol as described by the manufacturer of the electroporator, BTX Electronic Genetics (San Diego, Calif.). A 100- $\mu\text{l}$  aliquot of thawed competent bacteria was mixed with 1  $\mu\text{g}$  of plasmid DNA and loaded into prechilled 2-mm cuvettes. The bacteria were electroporated at 2.5 kV/cm, 129 W, and 0 capacitance for  $\sim 4.6$  ms. Immediately following electroporation, 500  $\mu\text{l}$  of KMB medium (2% proteose peptone, 1% glycerol, 6 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 6.5 mM  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ , pH 7.0) was added to each cuvette. Suspensions were transferred from the cuvettes to microcentrifuge tubes, where bacteria recovered during a 3-h incubation at  $37^{\circ}\text{C}$  with gentle inversion. P/L agar plates containing carbenicillin were inoculated with 100  $\mu\text{l}$  of these bacteria and incubated at  $37^{\circ}\text{C}$  until colonies appeared. Individual transformants were transferred to P/L agar plates containing carbenicillin, previously coated with 80  $\mu\text{l}$  of 100 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), incubated at  $37^{\circ}\text{C}$ , and scored for alginate production.

**Alginate assay.** To measure alginate production, strains were grown on L agar plates with appropriate antibiotics for  $\sim 28$  h at  $37^{\circ}\text{C}$ . The bacteria were then swabbed into 10 ml of 0.85% NaCl, and tubes containing the mixture were vortexed vigorously and centrifuged to remove the bacteria for subsequent weighing. The alginate remaining in the supernatant was precipitated by the addition of 25 ml of 95% ethanol. The alginate precipitates were collected by centrifugation and resuspended in 2 ml of 0.85% NaCl. The uronic acid concentration was determined by the colorimetric assay described by Knutson and Jeanes (32).

**DNA sequencing.** Double-stranded DNA sequencing was done by the dideoxynucleotide chain termination method with Sequenase (Amersham Corp., Arlington Heights, Ill.) and deoxyadenosine 5' [ $\alpha$ - $^{35}\text{S}$ ]thiotriphosphate (specific activity,  $>1,000$  Ci/mmol; Amersham Corp.) to completely sequence both strands of the DNA between *algG* and *algL*. Sequence reactions were initially performed with dGTP nucleotide and termination mixes. Resolution of GC compression artifacts, a complication commonly observed when pseudomonad DNA is sequenced, was achieved by replacing the dGTP nucleotides used in the sequencing reactions with either dITP or 7-deaza-dGTP nucleotide analogs. Custom oligonucleotide primers were obtained from Gibco BRL, Grand Island, N.Y.

**PCR.** PCR employing the plasmid template pNLS30 with primers designed to introduce the *NdeI* (CATATG) and *PstI* (CTGCAG) restriction sites encompassing *algX* was performed in a final volume of 50  $\mu\text{l}$  which contained 2 mM  $\text{Mg}^{2+}$ , 400  $\mu\text{M}$  deoxynucleoside triphosphate, 0.4  $\mu\text{M}$  each primer, 750 ng of plasmid template, 2.5 U of Vent DNA polymerase (New England Biolabs), and 10% formamide. Following an initial incubation of  $95^{\circ}\text{C}$  for 5 min, insert amplification was performed in 30 cycles of  $95^{\circ}\text{C}$  for 1.5 min,  $56^{\circ}\text{C}$  for 2 min, and  $75^{\circ}\text{C}$  for 3 min. The reaction was concluded with a final incubation of  $72^{\circ}\text{C}$  for 15 min. PCR products were electrophoresed on a 0.8% Tris-acetate-EDTA agarose gel and visualized with ethidium bromide.

**Expression of AlgX and polyacrylamide gel electrophoresis.** *E. coli* HMS174 (DE3) was sequentially transformed with both pLysS and pSM5. A single ampicillin- and chloramphenicol-resistant colony was used to inoculate 5 ml of L broth containing ampicillin (200  $\mu\text{g}/\text{ml}$ ) and chloramphenicol (50  $\mu\text{g}/\text{ml}$ ). After incubation at  $37^{\circ}\text{C}$  for 2 h, the suspension was centrifuged, the supernatant was replaced with 5 ml of L broth containing the two antibiotics at identical concentrations, and the culture was incubated at  $37^{\circ}\text{C}$ . This medium exchange process was repeated at 1.5-h intervals until the bacterial culture reached an  $A_{600}$  of  $\sim 0.7$ . At this time, expression of the endogenous T7 polymerase carried on the DE3 bacteriophage was induced by adding IPTG to 1 mM and incubating for another 2 h at  $37^{\circ}\text{C}$ . After centrifugation, the pellet was resuspended in 200  $\mu\text{l}$  of doubly distilled  $\text{H}_2\text{O}$  and frozen at  $-20^{\circ}\text{C}$ . A negative-control sample was prepared by the same procedure with the HMS174(DE3) lysogen carrying both pLysS and pRSET5A. Equal volumes of the cell suspension and  $2.5\times$  Laemmli sample solution were mixed and heated to  $95^{\circ}\text{C}$  for 5 min. The samples were then run on a 9% polyacrylamide-sodium dodecyl sulfate (SDS) gel, 1.5 mm thick, and stained with Coomassie blue R250 as previously described (12).

**Nucleotide sequence accession number.** The nucleotide sequence for *algX* (see Fig. 6) has been deposited in the DDBJ, EMBL, and GenBank DNA databases, and the entire nucleotide sequence of *algG*-*algX*-*algL* has been given the accession no. U27829.

## RESULTS

**Cloning and sequencing of *algX*.** In our previous study (48), transposon mutagenesis was used to inactivate chromosomal *algL* in mucoid ( $\text{Alg}^+$ ) *P. aeruginosa* FRD1. Restriction mapping studies identified the relative locations of these insertions, which were later confirmed by DNA sequence analysis. Transposon insertion Tn501-28 was located in a putative gene (*algX*)  $\sim 150$  nucleotides upstream of the ATG codon initiating the *algL* sequence (Fig. 1). In order to sequence the *algX* locus, a

3.5-kb *HindIII*-*XbaI* fragment, containing the 3' region of *algG*, the *algX* locus, and *algL*, was isolated from pMF39, ligated into a similarly digested pUC129 cloning vector, and used to transform *E. coli* JM109. Plasmids were isolated from ampicillin-resistant transformants and analyzed for proper construction by *HindIII*-*XbaI* restriction digests. Vectors found to contain the proper insert were subsequently designated pNLS30.

The parameters of the *algX* gene were established by sequencing the DNA located between *algG* and *algL* (GenBank accession numbers U06720 and L09724, respectively). Double-stranded-DNA sequencing of pNLS30 was initiated by designing oligonucleotide primers that annealed to the 3' end of *algG* (nucleotides 1680 to 1697) and the 5' end of *algL* (nucleotides 37 to 21) which, when used in a sequencing reaction, would extend sequence information from *algG* and *algL* into the region containing the putative *algX* ORF. New primers were synthesized on the basis of the acquired sequence, allowing both strands of the region to be completely sequenced in a "walking" fashion. Analysis of strand complementarity confirmed the sequence data obtained (Fig. 2).

The *algX* ORF, initiating at the ATG start codon at nucleotide 40 and extending to a TAA stop codon at nucleotide 1462, encodes a polypeptide of 474 amino acids with a computer-determined molecular weight of 52,553 and a predicted pI of 7.52. The ORF is 66.7% G+C overall and  $\sim 94\%$  G+C in the third codon position. Such a high G+C content is similar to that of other *P. aeruginosa* genes, including *algL* (48). A nonredundant database search of the Brookhaven Protein Data Bank, the Swiss-Prot (release 31.0, March 1995) database, and the GenBank database was done at the National Center for Biotechnology Information by using the Blast Network service. The search revealed that the AlgX polypeptide does not share any significant sequence homology with any of the proteins currently contained within these databases.

**Role of *algL* in alginate production.** Because of the operonic nature of the biosynthetic gene cluster (8), Tn501-28 (*algX*::Tn501) is a polar mutation which inactivates *algX*, *algL*, and other downstream biosynthetic genes (such as *algF* and *algA*), thus rendering this FRD1 mutant (designated FRD1128) phenotypically nonmucoid ( $\text{Alg}^-$ ). This phenotype was confirmed quantitatively by measuring the uronic acid content of each strain; whereas the  $\text{Alg}^+$  parental strain FRD1 typically produces  $>50$   $\mu\text{g}$  of uronic acid per mg (wet weight) of bacteria, FRD1128 produced  $1.15 \pm 1.99$   $\mu\text{g}$  of uronic acid per mg (wet weight) of bacteria (Table 2). Another transposon insertion, Tn501-14, located  $\sim 350$  nucleotides below the 3' end of the *algL* coding region (Fig. 1), did not affect *algL* expression but still rendered this FRD1 mutant (designated FRD1114)  $\text{Alg}^-$  because of its polar effect on *algA*, an essential gene encoding the enzyme which catalyzes the first reaction of alginate biosynthesis (53). Confirmation that the nonmucoid phenotypes of FRD1128 and FRD1114 were due to the indicated transposon insertions, and not spontaneous mutations of *algT* (*algU*) (17, 37) or *algR* (61), was achieved by mating the plasmid pALG2, a ColE1-based replicon carrying the entire wild-type alginate biosynthetic operon, into the mutant strains. This vector, which is unable to autonomously replicate within *P. aeruginosa*, integrates into the host chromosome by homologous recombination, generating a merodiploid. Following conjugation and selection on carbenicillin-containing P/L agar plates,  $>95\%$  of the pALG2 merodiploids were  $\text{Alg}^+$  within 24 h (Table 2), indicating that the  $\text{Alg}^-$  phenotypes of FRD1128 and FRD1114 are due to the mutational effect of the transposon insertions within the biosynthetic operon. Moreover, these studies demonstrated that these strains can be converted to the

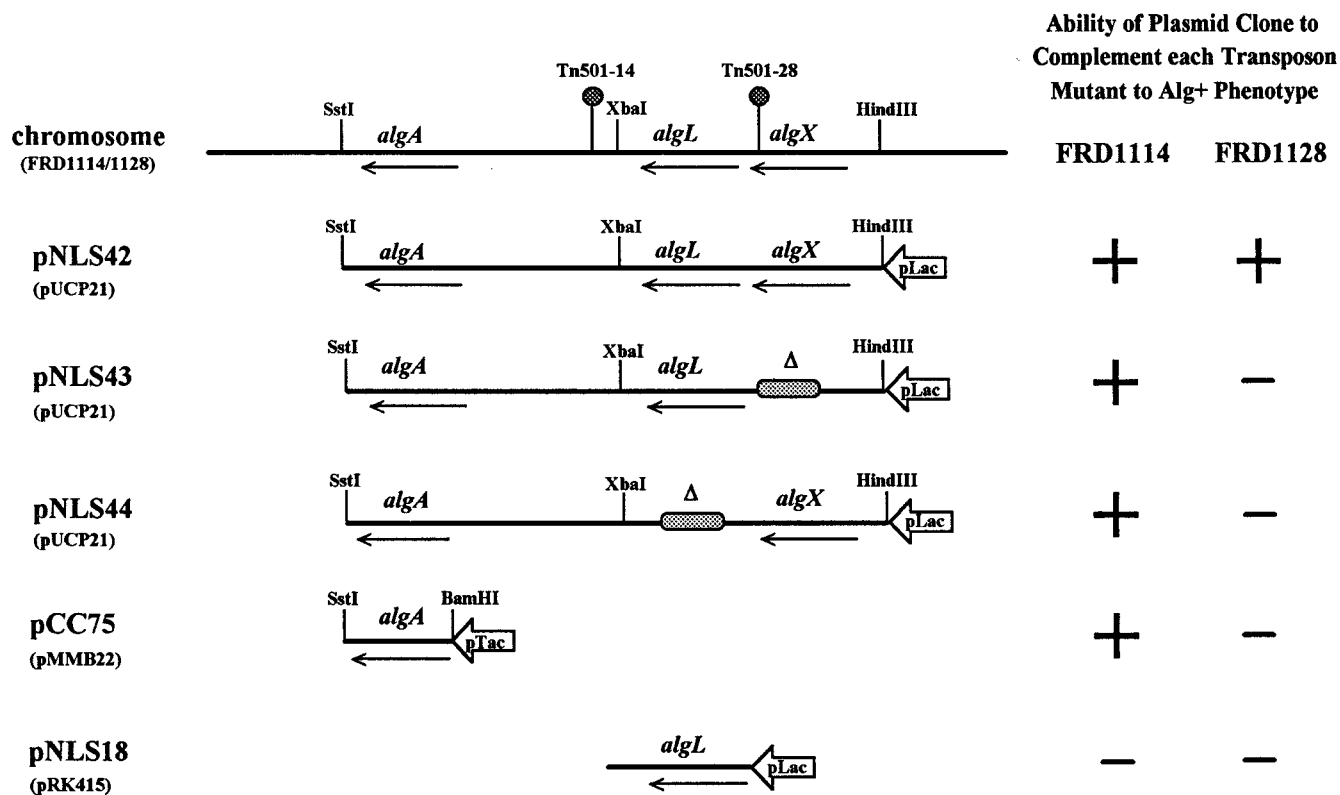


FIG. 1. Plasmid constructs used for FRD1114 and FRD1128 complementation analysis. The locations of the transposons in FRD1114 and FRD1128 are shown at the top. The alginate biosynthetic genes contained within each construct and their orientations relative to the various vector promoters are also depicted (vectors used for preparation of specific constructs are identified in parentheses beneath the construct designations). The shaded segments denote regions which were deleted from the plasmid constructs.

Alg<sup>+</sup> phenotype by genes contained on pALG2. Presumably, those colonies not converted to the mucoid state by this merodiploid strategy had undergone spontaneous mutations in either the *algT* (*algU*) or *algR* regions, rendering them nonmucoid.

To determine whether AlgL functions in alginate production, we used the same merodiploid strategy with the plasmid pSM4, an *algL* deletion variant of pALG2. (The construction of pSM4 is described in Materials and Methods.) That the biosynthetic operon in pSM4 was otherwise intact was confirmed by demonstrating that >95% of the FRD1114::pSM4 recombinants were Alg<sup>+</sup> within 24 h of incubation at 37°C (Table 2), similar to the FRD1114::pALG2 recombinants.

In contrast to the results observed with FRD1114::pSM4, >95% of the FRD1128::pSM4 recombinants remained Alg<sup>-</sup> (Table 2), suggesting that AlgL is involved in biosynthesis. To confirm that the nonmucoid phenotype of these FRD1128::pSM4 merodiploids was due to the absence of AlgL, pNLS18, an autonomously replicating, broad-host-range vector that contains *algL* below its *lac* promoter (Fig. 1), was mated into FRD1128::pSM4. Transconjugants, which appeared phenotypically identical to the FRD1128::pALG2 merodiploids, rapidly produced alginate (data not shown). The ability of pNLS18, which supplies only *algL* in *trans*, to restore wild-type levels of alginate synthesis in FRD1128::pSM4 implicates AlgL as a necessary component of normal alginate synthesis.

**Both *algL* and *algX* participate in alginate production.** Complementation studies using FRD1114 and FRD1128 were performed to determine whether *algL*, *algX*, or both are needed to restore alginate synthesis in the nonmucoid transposon mutants FRD1114 and FRD1128.

Transformation of FRD1114 with pCC75 (Fig. 1), which expresses *algA* under control of a *tac* promoter, converted this strain to Alg<sup>+</sup>. The restoration of alginate synthesis in FRD1114, whose transposon is inserted immediately downstream of *algL*, therefore requires only *algA* (*algF*, involved in alginate acetylation, is not needed to build the polymer). In contrast, FRD1128, with Tn501-28 inserted in *algX*, upstream of *algL*, was not restored to the mucoid phenotype by transformation with pCC75 (Fig. 1). This observation suggested that either *algL* and/or *algX* is required for alginate synthesis, as these are the only genes blocked by the transposon in FRD1128 which are unaffected in FRD1114.

Transformation of FRD1128 with pNLS42, a plasmid vector which expresses all genes affected by the Tn501-28 insertion, restored alginate production (Fig. 1 and Table 2), demonstrating that (i) *in-trans* complementation of the FRD1128 mutant to the mucoid state is achievable and (ii) either AlgL, AlgX, or both are required for the synthesis of alginate. To specifically determine whether *algL* and/or *algX* is required for alginate production, plasmid variants of pNLS42 individually containing *algL* or *algX* deletions were prepared and used to transform FRD1128 or FRD1114. (Plasmid constructions are described in Materials and Methods, and plasmid maps are depicted in Fig. 1). The ability of these transformants to synthesize alginate was then determined.

pNLS44 is similar to pNLS42 except that a 465-bp *Pst*I fragment internal to *algL* was deleted. Transformation of FRD1114 with pNLS44 converted this strain to the mucoid phenotype (Table 2), demonstrating the expression of *algA*. In contrast, colonies of FRD1128 transformed with pNLS44 remained phenotypically nonmucoid, with alginate concentra-

10 20 **SD box** 40 50 60  
**GAAGGACGAG CCGAAGCTTCA GGACTATAGGA CCGAGAGCC ATG AAA ACC GGT ACT TTC CGA**  
 M K T R T S R  
 70 80 90 100 110  
 CTG TTC CGC CTG AGC GCC CTG GCC GGC CTC TGC CTG GCC CAG GCA GCC CTG  
 L P R L L S A L A A G L C L A Q A A L  
 120 130 140 150 160  
 GCC GCC GAC CCC GGC GCG CCG AGC TAC CAG GGC CTG CCG GCC GGC AAC CTG  
 A A D P G A A P S Y Q A L P A G N L  
 170 180 **HNFI** 200 210 220  
 TGC CCG GCC GCC GAC GAC TGC CCG TAC AAC ACC AAG TAC CTG GGC TTC TTC  
 C P A A A D D S R Y N T K Y L G F P  
 230 240 250 260 270  
 ACC GAC CTG GTC CAG GCC CAG GAC GAC TGG CTG TTC TGC ACC ACC TAC GAC CTG  
 T H L V Q A Q D D W L P R T T Y D L  
 280 290 300 310 320 330  
 CGT ACC GAC TTC GGC ACC AGC GAA GGC TGG CGC GAA CTC CGC GCA CTG CGC  
 R T D P G T S A E G W R E L R A L R  
 340 350 360 370 380  
 GAC GAA CTC AAG CGC AAG GGC ATC GAC CTG GTC GTC TAC CAG CCG ACC CGC  
 D E L K R K G I E L V V Y Q P T R  
 390 400 410 420 430  
 GGC CTG GTC AAC CGC GAG AAG CTC AGC CCA GCG GAA AAG GCC GGC TTC GAC TAC  
 G L V N R E K L S P A E K A G P D Y  
 440 450 460 470 480 490  
 GAG CTG GCG AAG AAG AAC TAC CTG GCG ACC ATC GCC CGC TTC CGC CAG GCC GGC  
 E L A K K N Y L A T I A R F R Q A G  
 500 510 520 530 540  
 ATC TGG ACC CCG GAC TTC TCG CCG CTG TTC GAC GAG AAG GAG GAC GGC TAC  
 I W T P D P S P L F D E K E E H A Y  
 550 560 **HNFI** 580 590 600  
 TAC TTC AAG GGC GAC CAC TGG ACT CCC CAT GGC GCC CGG GGC AGC GCG AAG  
 Y P K G D H H W T P H G A R R S A K  
 610 620 630 640 650  
 ATC GTG GCC GAG ACG CTG AAG GTC GTC CCC GGC TTC GAG GAG ATC CCG AAG AAG  
 I V A E T L K Q V P G F E E I P K K  
 660 670 680 690 700  
 CAG TTC GAA AGC AAG CGC GTC GGC CTG TCC AAG CTC GGC ACC TTC CAC AAG  
 Q P E S K R V G L L S K L G T F H K  
 710 720 730 740 750 760  
 GCC GCC GCG CAA CTC TGC GGC AAG ACC TAC ACC CAG TAC GTC GAC CGC TTC  
 A A A Q L C G N S Y A T Q Y V D R K F  
 770 780 790 800 810  
 GAG ACC GAG CCG GTC GGC ACC GAC AGC GGC GAT CTG TTC GGC GAC GGC GGC  
 E T E P V G A S D S G D L F G D G G  
 820 830 840 850 860 870  
 AAC CCG CAG ATC ATC GAG GTC GGC ACC TCC AAC AGC GGC CCG GCC TAC AAC TTC  
 N P Q I A L V G T S N S G P A Y N P  
 880 890 900 910 920  
 GCC GGC TTC CTG GAG GAG TTC AGC GGC GCC GAC ATC CTC AAC AAC GCG GTG TCC  
 A G F L E E F S G A D I L N N A V S  
 930 940 950 960 970 980  
 GGC GGC GGC TTC GAC AGT TCG CTG GCG GGC TAC ATG ACC AGC GAG GAA TTC CAC  
 G G G F P D S S L L A Y M T S E E P H  
 990 **BEFLI** 1010 1020 1030  
 AAG AAC CCG CCG AAG ATC CTC ATC TGG GAA TTC GCC ACC CAC TAC GAC ATG CCG  
 K N P P K I L I W E F A T H Y D M A  
 1040 1050 **Xcml** 1080 1090 1100 1110 1120 1130 1140  
 CAG AAG AGC TTC TAC CGC CAG GCC ATG CCG CTG GTC GAC AAC GGC TTC TCC GGC  
 Q K S P Y R Q A M P L V D N G C S G  
 1150 1160 1170 1180 1190  
 CTG CTG AAC AGC GCC GCG CTA CCG ATC GGT AGC GGC AGC TAC GTC GCC GAC GTC  
 L L N S A A L P I R S G S Y V A D V  
 1200 1210 1220 1230 1240  
 ACC TAC AGC GAC CCT TCG GTA CAC GAG TTG AAG AAC ACC ATC TGG TAC ATG AAC  
 T Y S D P S V H E L K N T I W Y M N  
 1250 1260 1270 1280 1290 1300  
 GGC CGC CGC GAG CAG TTG AAG ATC GAG CAG TCG AAA GCC GTC GAT ACC GGC GGC  
 G R R E Q L K I E Q S K A A V D T G G  
 1310 1320 1330 1340 1350  
 CCG TAC GTC TTC CAA CTG CCG AAC GAC AGC GAC TGG GCC GAC CAG CAG TTC CTT  
 R Y V P Q L R N D S D W A D Q Q P L  
 1360 1370 1380 1390 1400 1410  
 TTC CTG GAA ATC GAA GCG CCG GAG GAC ATG ACC CCC CAG GGC CTG GAG GTC CAG GCC  
 S L E I E A P E D M P Q G L E V Q A  
 1420 1430 1440 1450 1460  
 AGC ATC TGC CAG GCG GCG CCC GCC AAC GCC AGC CAG TCG GTG GCC GAG AGG TAA  
 S I C Q A A P A K A S Q S V A G R .  
 1470 1480 1490 1500 1510 1520 1530  
 ACGATG AAAAGCTCC ACCGTATCCG TAAAGCCCTG CCGGATGCC TGGGCGGGG ATTAGCTCCG  
 1540 1550 **Xcml** **Kcml**  
**AGCCAGATCA GCCAGGCCGC GGACTATAGGA CCGCC**

FIG. 2. Nucleotide sequence of *algX* and amino acid sequence for AlgX. Locations of selected endonuclease restriction sites are denoted above the sequence. The Shine-Dalgarno sequence is shown in boldface type. Underlined sequences depict the nucleotides of the 3' end of *algG* (upstream of *algX*) and the 5' end of *algL*.

TABLE 2. Alginate production by various *P. aeruginosa* strains

Study and strains	$\mu\text{g}$ of uronic acid/ mg (wet wt) of bacteria <sup>a</sup>
<b>Merodiploid</b>	
FRD1114 .....	1.24 $\pm$ 2.15
FRD1114::pALG2 .....	34.88 $\pm$ 2.11
FRD1114::pSM4 .....	33.83 $\pm$ 5.21
FRD1128 .....	1.15 $\pm$ 1.99
FRD1128::pALG2 .....	62.63 $\pm$ 8.33
FRD1128::pSM4 .....	2.15 $\pm$ 3.72
<b>Complementation</b>	
FRD1114 .....	1.24 $\pm$ 2.15
FRD1114(pNLS42) .....	13.22 $\pm$ 7.32
FRD1114(pNLS43) .....	29.28 $\pm$ 0.80
FRD1114(pNLS44) .....	24.82 $\pm$ 4.96
FRD1128 .....	1.15 $\pm$ 1.99
FRD1128(pNLS42) .....	30.11 $\pm$ 11.90
FRD1128(pNLS43) .....	0.03 $\pm$ 0.06
FRD1128(pNLS44) .....	5.46 $\pm$ 2.02

<sup>a</sup> Data represent means  $\pm$  standard deviations based on at least three experiments.

tions only slightly above baseline values (Table 2). Since this construct contains all of the biosynthetic genes of pNLS42 except *algL*, these results further support previous conclusions indicating that AlgL is involved in alginate production.

pNLS43 is similar to pNLS42 except that a 384-bp *XcmI* fragment was removed from the 3' terminus of the *algX* region (the site of the Tn501-28 insertion). While transformation of FRD1114 with pNLS43 rendered this strain mucoid, transformation of FRD1128 by pNLS43 did not (Table 2). Since the only difference between pNLS43 and pNLS42 is the deletion of *algX*, this result strongly argues that AlgX is required for alginate polymer production.

**Expression of AlgX.** AlgX was expressed from the bacteriophage T7 RNA polymerase promoter of the pRSET5A expression vector. To prepare the AlgX expression vector, it was necessary to introduce, by the PCR process, an *NdeI* restriction site (CATATG) at the 5' end of *algX* and a *PstI* restriction site (CTGCAG) immediately downstream of the 3' end of *algX*. The *NdeI* site, which encompasses the *algX* start ATG, allowed introduction of *algX* into the vector *NdeI* restriction site oriented in such a fashion that AlgX expression would proceed from the vector T7 promoter and ribosome binding site as a translational fusion. PCR was performed with the plasmid template pNLS30 and primers designed to introduce the required restriction sites. The amplification products were simultaneously digested with *NdeI* and *PstI*, ethanol precipitated, and ligated into a similarly digested and phosphatase-treated pRSET5A expression plasmid. Proper vector construction was confirmed by restriction digestion and DNA sequencing with both vector T7 promoter- and *algX*-specific primers. The construct was designated pSM5.

As described in Materials and Methods, *E. coli* HMS174 (DE3)(pLysS/pSM5) and HMS174(DE3)(pLysS/pRSET5A) were cultured and IPTG treated to induce expression of the bacteriophage T7 RNA polymerase necessary for transcription of DNA contained below the pRSET5A T7 promoter. Whole-cell lysates of each culture were electrophoresed on a 9% polyacrylamide-SDS slab gel. As shown in Fig. 3, a strongly expressed protein band was visualized at the predicted AlgX molecular mass (~53 kDa) only in the sample which contained pSM5.

## DISCUSSION

During our previous studies of *algL* (48), we were surprised to find that the gene encoding alginate lyase, an alginate-degrading enzyme, was located within the alginate biosynthetic gene cluster and positively coregulated with alginate synthesis. This observation prompted our study to determine what role, if any, AlgL had in alginate production. By using a strain of the mucoid CF patient isolate FRD1 rendered nonmucoid by Tn501 insertion in *algX*, a gene immediately upstream of *algL*, restoration of alginate production by complementation in *trans* was found to require a plasmid carrying all of the genes inactivated by the insertion, including *algL* and *algX*. Alginate production was also recovered when a merodiploid that generated a complete alginate gene cluster on the chromosome was formed. However, alginate production by merodiploids formed in this *algX*::Tn501 mutant using an alginate cluster with an *algL* deletion was not restored to wild-type levels unless *algL* was provided on a plasmid in *trans*. In addition, complementation studies of Tn501 mutants using plasmids containing specific deletions in either *algL* or *algX* revealed that both gene regions were required for restoration of the mucoid phenotype.

On the basis of these studies, AlgL clearly plays a role in alginate production. Although AlgL is required for the normal production of alginate, it may not be essential for polymerizing alginate, consistent with the suggestion of Boyd et al. (6). May and Chakrabarty (39) recently hypothesized that AlgL might cleave the nascent alginate polymer to provide short oligomers for priming the polymerization reaction. Consistent with this model, hyperexpression of *algL* in FRD1 resulted in colonies resembling the parental FRD1 (i.e., Alg<sup>+</sup>) after 24 h of incubation; however, continued incubation led to rapid degradation of the alginate in colonies hyperexpressing AlgL (42). Moreover, the alginate from these hyperexpressing strains displayed a wide range in size in comparison with that of FRD1 (43), confirming that it is being rapidly degraded (10). Boyd

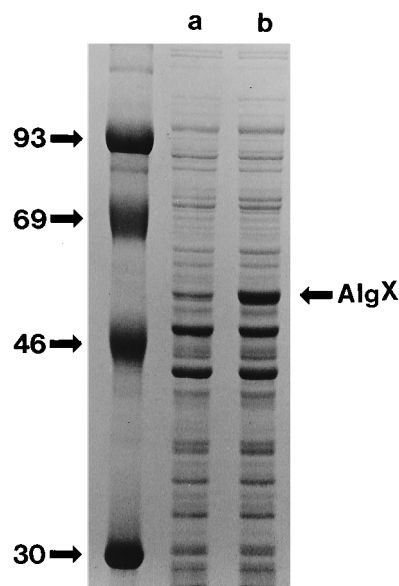


FIG. 3. Coomassie blue-stained SDS-polyacrylamide gel containing proteins from whole-cell lysates of HMS174(DE3)(pLysS) transformed with either pRSET5A or pSM5. Lane a, HMS174(DE3)(pLysS/pRSET5A); lane b, HMS174(DE3)(pLysS/pSM5). The numbers to the left mark the positions of the molecular mass standards (in kilodaltons).

and Chakrabarty (5) have recently reported similar observations.

The experiments described here provide strong evidence that in addition to AlgL, the gene region directly upstream of *algL*, which we have named *algX*, is also important in alginate production. The identification of an additional gene within this chromosomal region important for alginate synthesis was not unexpected, since several key components in alginate synthesis, including the polymerase and proteins involved in alginate transport and export, have not yet been described. The cloning and sequencing of *algX* predicted an ~53-kDa protein product, which was visualized by hyperexpression of AlgX in *E. coli*. Current efforts to purify and characterize both AlgL and AlgX in order to clarify their roles in alginate biosynthesis by *P. aeruginosa* are under way.

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#### REFERENCES

- Baltimore, R. S., and M. Mitchell. 1980. Immunologic investigations of mucoid strains of *Pseudomonas aeruginosa*: comparison of susceptibility to opsonic antibody in mucoid and nonmucoid strains. *J. Infect. Dis.* **141**: 238–247.
- 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.
- Bayer, A. S., S. Park, M. C. Ramos, C. C. Nast, F. Eftekhar, and N. L. Schiller. 1992. Effects of alginate on the natural history and antibiotic therapy of experimental endocarditis caused by mucoid *Pseudomonas aeruginosa*. *Infect. Immun.* **60**:3979–3985.
- 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.
- Boyd, A., and A. M. Chakrabarty. 1994. Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* **60**:2355–2359.
- Boyd, A., M. Ghosh, T. B. May, D. Shinabarger, R. Keogh, and A. M. Chakrabarty. 1993. Sequence of the *algL* gene of *Pseudomonas aeruginosa* and purification of its alginate lyase product. *Gene* **131**:1–8.
- Chitnis, C. E., and D. E. Ohman. 1990. Cloning of *Pseudomonas aeruginosa algG*, which controls alginate structure. *J. Bacteriol.* **172**:2894–2900.
- 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.
- 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.
- Coss, D., and N. L. Schiller. 1995. Unpublished data.
- Cross, A., J. R. Allen, J. Burke, G. Duce, A. Harris, J. John, D. Johnson, M. Lew, B. MacMillan, R. Skalova, R. Wenzel, and J. Tenney. 1983. Nosocomial infections due to *Pseudomonas aeruginosa*: review of recent trends. *Rev. Infect. Dis.* **5**(Suppl.):S837–S845.
- 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.
- 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**:249–257.
- 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.
- Deretic, V., M. J. Schurr, J. C. Boucher, and D. W. Martin. 1994. Conversion of *Pseudomonas aeruginosa* to mucoidy in cystic fibrosis: environmental stress and regulation of bacterial virulence by alternative sigma factors. *J. Bacteriol.* **176**:2773–2780.
- Deretic, V., M. J. Schurr, and H. Yu. 1995. *Pseudomonas aeruginosa*, mucoidy and the chronic infection phenotype in cystic fibrosis. *Trends Microbiol.* **3**: 351–356.
- DeVries, C. A., and D. E. Ohman. 1994. Mucoid-to-nonmucoid conversion in alginate-producing *Pseudomonas aeruginosa* often results from spontaneous mutations in *algT*, encoding a putative alternative sigma factor, and shows evidence for autoregulation. *J. Bacteriol.* **176**:6677–6687.
- Doggett, R. G., G. M. Harrison, R. N. Stillwell, and E. S. Wallis. 1966. An atypical *Pseudomonas aeruginosa* associated with cystic fibrosis of the pancreas. *J. Pediatr.* **68**:215–221.
- Doig, P., N. R. Smith, T. Todd, and R. T. Irvin. 1987. Characterization of the binding of *Pseudomonas aeruginosa* alginate to human epithelial cells. *Infect. Immun.* **55**:864–873.
- Eftekhar, F., and D. P. Speert. 1988. Alginate treatment of mucoid *Pseudomonas aeruginosa* enhances phagocytosis by human monocyte-derived macrophages. *Infect. Immun.* **56**:2788–2793.
- 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.
- 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**:3228–3226.
- 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.
- Gacesa, P., R. C. Caswell, and P. Kille. 1989. Bacterial alginases. *Antibiot. Chemother. (Basel)* **42**:67–71.
- 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.
- George, R. H. 1987. *Pseudomonas* infection in cystic fibrosis. *Arch. Dis. Child.* **62**:438–439.
- Goldberg, J. B., W. L. Gorman, J. L. Flynn, and D. E. Ohman. 1993. A mutation in *algN* permits *trans* activation of alginate production by *algT* in *Pseudomonas* species. *J. Bacteriol.* **175**:1303–1308.
- 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.
- 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.
- 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. 1976. A new modification of the carbazole reaction: application to heteropolysaccharides. *Anal. Biochem.* **24**:470–481.
- Kulczycki, L. L., T. M. Murphy, and J. A. Bellanti. 1978. *Pseudomonas* colonization in cystic fibrosis: a study of 160 patients. *JAMA* **240**:30–34.
- Maharaj, R., T. B. May, S.-K. Wang, and A. M. Chakrabarty. 1993. Sequence of the *alg8* and *alg44* genes involved in the synthesis of alginate by *Pseudomonas aeruginosa*. *Gene* **136**:267–269.
- Martin, D. W., B. W. Holloway, and V. Deretic. 1993. Characterization of a locus determining the mucoid status of *Pseudomonas aeruginosa*: AlgU shows sequence similarities with a *Bacillus* sigma factor. *J. Bacteriol.* **175**: 1153–1164.
- Martin, D. W., M. J. Schurr, M. H. Mudd, and V. Deretic. 1993. Differentiation of *Pseudomonas aeruginosa* into the alginate-producing form: inactivation of *mucB* causes conversion to mucoidy. *Mol. Microbiol.* **9**:497–506.
- Martin, D. W., M. J. Schurr, M. H. Mudd, J. R. W. Govan, B. W. Holloway, and V. Deretic. 1993. Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. *Proc. Natl. Acad. Sci. USA* **90**: 8377–8381.
- Martin, D. W., M. J. Schurr, H. Yu, and V. Deretic. 1994. Analysis of promoters controlled by the putative sigma factor AlgU regulating conversion to mucoidy in *Pseudomonas aeruginosa*: relationship to  $\sigma^F$  and stress response. *J. Bacteriol.* **176**:6688–6696.
- May, T. B., and A. M. Chakrabarty. 1994. *Pseudomonas aeruginosa*: genes and enzymes of alginate synthesis. *Trends Microbiol.* **2**:151–157.
- 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.
- Melver, K. S., and D. E. Ohman. Personal communication.
- Munday, S. R., M. J. Franklin, D. E. Ohman, and N. L. Schiller. 1994. Role of AlgL in alginate synthesis in *Pseudomonas aeruginosa*, abstr. D-185, p. 129. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology 1994. American Society for Microbiology, Washington, D.C.

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. **Pier, G. B.** 1985. Pulmonary disease associated with *Pseudomonas aeruginosa* in cystic fibrosis: current status of the host-bacterium interaction. *J. Infect. Dis.* **151**:575–580.
46. **Ramphal, R., C. Guay, and G. B. Pier.** 1987. *Pseudomonas aeruginosa* adhesins for tracheobronchial mucin. *Infect. Immun.* **55**:600–603.
47. **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.
48. **Schiller, N. L., S. R. Monday, C. M. Boyd, 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.
49. **Schoepfer, R.** 1993. The pRSET family of T7 promoter expression vectors for *Escherichia coli*. *Gene* **124**:83–85.
50. **Schurr, M. J., D. W. Martin, M. H. Mudd, and V. Deretic.** 1994. Gene cluster controlling conversion to alginate-overproducing phenotype in *Pseudomonas aeruginosa*: functional analysis in a heterologous host and role in the instability of mucoidy. *J. Bacteriol.* **176**:3375–3382.
51. **Schurr, M. J., D. W. Martin, M. H. Mudd, N. S. Hibler, J. C. Boucher, and V. Deretic.** 1993. The *algD* promoter: regulation of alginate production by *Pseudomonas aeruginosa* in cystic fibrosis. *Cell. Mol. Biol. Res.* **39**:371–376.
52. **Sferra, T. J., and F. S. Collins.** 1993. The molecular biology of cystic fibrosis. *Annu. Rev. Med.* **44**:133–144.
53. **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. *J. Biol. Chem.* **266**:2080–2088.
54. **Shinabarger, D., T. B. May, A. Boyd, M. Ghosh, and A. M. Chakrabarty.** 1993. Nucleotide sequence and expression of the *Pseudomonas aeruginosa algF* gene controlling acetylation of alginate. *Mol. Microbiol.* **9**:1027–1035.
55. **Studier, F., A. Rosenberg, J. Dunn, and J. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185**:60–84.
56. **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.
57. **Warner, J. O.** 1992. Immunology of cystic fibrosis. *Br. Med. Bull.* **48**:893–911.
58. **West, S. E. H., H. P. Schweizer, C. Dall, A. K. Sample, and L. J. Runyen-Janecky.** 1994. Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. *Gene* **128**:81–86.
59. **Wood, R. E., T. F. Boat, and C. F. Doershuk.** 1976. Cystic fibrosis: state of the art. *Am. Rev. Respir. Dis.* **113**:833–878.
60. **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.
61. **Wozniak, D. J., and D. E. Ohman.** 1994. Transcriptional analysis of the *Pseudomonas aeruginosa* genes *algR*, *algB*, and *algD* reveals a hierarchy of alginate gene expression which is modulated by *algT*. *J. Bacteriol.* **176**:6007–6014.