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## Role of Alginate Lyase in Cell Detachment of *Pseudomonas aeruginosa*

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**The exopolysaccharide alginate of *Pseudomonas aeruginosa* was shown to be important in determining the degree of cell detachment from an agar surface. Nonmucoid strain 8822 gave rise to 50-fold more sloughed cells than mucoid strains 8821 and 8830. Alginate anchors the bacteria to the agar surface, thereby influencing the extent of detachment. The role of the *P. aeruginosa* alginate lyase in the process of cell sloughing was investigated. Increased expression of the alginate lyase in mucoid strain 8830 led to alginate degradation and increased cell detachment. Similar effects were seen both when the alginate lyase was induced at the initial stage of cell inoculation and when it was induced at a later stage of growth. It appears that high-molecular-weight alginate polymers are required to efficiently retain the bacteria within the growth film. When expressed from a regulated promoter, the alginate lyase can induce enhanced sloughing of cells because of degradation of the alginate. This suggests a possible role for the lyase in the development of bacterial growth films.**

*Pseudomonas aeruginosa* is known to produce an exopolysaccharide alginate under specific conditions. Alginate is a linear random polymer of  $\beta$ -1-4-linked D-mannuronic acid and L-guluronic acid (17). The mannuronate residues are modified to various degrees by O-acetyl groups (14). Alginate is important in the development of chronic bronchopulmonary infections in cystic fibrosis patients. It has been shown to play a role in colonization by increasing the adherence of the bacteria to solid surfaces (4, 25, 26, 30). Alginate is involved in the persistence of the pseudomonal infection by protecting against antibiotic-mediated and polymorphonuclear leukocyte-directed phagocytosis and killing (4, 5, 21). The formation of biofilms in the lung provides added protection for the bacteria (2, 20, 21), and extracellular polysaccharides, such as alginate, appear to be involved in such biofilm development. It has been shown that the production of an extracellular polysaccharide is important for the microcolony formation of two freshwater bacterial isolates (1a). Biofilms are important in medical pathogenesis as they provide a means to evade the immune system, keep the cells hydrated, trap nutrients, and immobilize the cells (12). Adhesion of microbes to solid surfaces has been shown to influence a wide range of bacterial activities, including the expression of alginate biosynthetic genes (12, 15, 20, 34).

Most of the alginate biosynthetic genes of *P. aeruginosa* are clustered at 34 min on the chromosome (Fig. 1A) (28). The *algL* gene, which codes for alginate lyase, is also located within this cluster (6, 31). Alginate lyase enzymes cleave the 4-O-linked glycosidic bonds between uronate residues by an eliminative mechanism to produce unsaturated sugar derivatives (18). The alginate lyase (AlgL) of *P. aeruginosa* has optimal activity against nonacetylated polymannuronic acid (16, 24, 29). The role of the *P. aeruginosa* alginate lyase in alginate production is intriguing. Several other microbes, including *Bacillus circulans* and two marine *Pseudomonas* species, that possess such an enzyme can utilize alginate as a carbon source

(19, 22, 38). However, it appears that *P. aeruginosa* 8821 and 8830 are unable to do so (unpublished results). The *algL* gene of *P. aeruginosa* is dispensable for alginate production. Disruption of the *algL* gene results in a nonmucoid phenotype that can be changed to a mucoid phenotype solely by the presence in *trans* of the downstream gene *algA* (6). AlgL could be involved in alginate modification as it could be important for determining the molecular size of the alginate polymer produced. A decrease in polymer length could affect the properties of the alginate, including its ability to enhance attachment of the bacteria to solid surfaces. We set out to see if increased expression of the alginate lyase in *P. aeruginosa* would alter the size of the alginate synthesized and if this would in turn affect the adherence properties of the bacteria.

### MATERIALS AND METHODS

**Organisms and plasmids.** The bacterial strains, vectors, and recombinant plasmids used in this study are shown in Table 1.

**Sloughing analysis.** The *P. aeruginosa* strains were grown overnight in Luria broth (Difco Laboratories) with carbenicillin added to 450  $\mu$ g/ml when appropriate. An aliquot of 200  $\mu$ l was spread on each Pseudomonas Isolation Agar (PIA; Difco Laboratories) plate, with 1 mM IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) supplemented when appropriate. The surface area of each plate was 57 cm<sup>2</sup>. The plates were incubated at 37°C for 72 h. A 5-ml portion of 0.9% NaCl was gently layered on top of each plate, and the plates were allowed to incubate at room temperature for 5 min. The total numbers of sloughed cells (CFU) were determined by serial dilution of the NaCl washes and by plate counts.

For experiments in which IPTG was added to cells after inoculation, disposable Nalgene membrane filter units were used. These units consist of two 115-ml chambers separated by a cellulose nitrate membrane. A 15-ml portion of PIA was pipetted onto the membrane at the base of the top chamber. This formed an agar surface area of 16 cm<sup>2</sup>. A 75- $\mu$ l portion of an overnight culture was plated on this surface. The bottom chamber was filled with water, such that the liquid was in contact with the underside of the membrane. IPTG was added to the water at various times of incubation to produce a final

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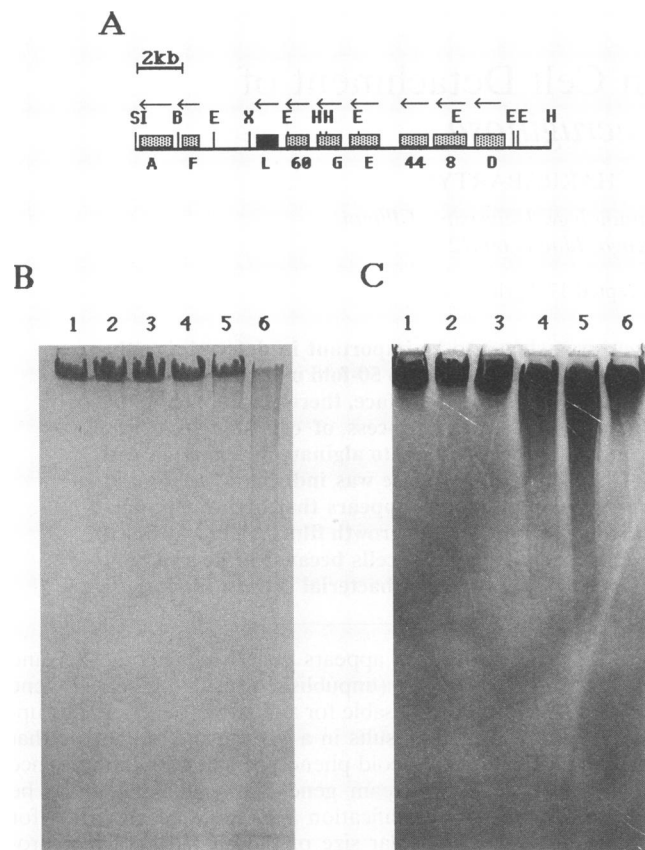


FIG. 1. (A) The alginate biosynthetic gene cluster at 34 min on the *P. aeruginosa* chromosome. The genes are transcribed from right to left (*algD* to *algA*) as shown by the arrows (28). The functions of the gene products are as follows: AlgD, GDP-mannose-dehydrogenase; Alg8 and Alg44, probable membrane proteins involved in alginate polymerization; AlgE, probable outer membrane porin; AlgG, epimerase; Alg60, essential alginate gene product of unknown function; AlgL, alginate lyase; AlgF, alginate acetylase; AlgA, phosphomannose isomerase-GDP-mannose pyrophosphorylase (6, 9, 11, 28). The boxes show the positions of the alginate genes that are known to be in this cluster. The *algL* gene is shown as a black box. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; SI, *Sst*I; X, *Xba*I. (B and C) Effect of induced alginate lyase activity on alginate degradation. (B) A 10- $\mu$ g portion of alginate was analyzed on a 5% polyacrylamide gel. Lanes: 1, 8821; 2, 8830; 3, 8830/pMMB22 without IPTG; 4, 8830/pMMB22 with IPTG; 5, 8830/pSK700 without IPTG; 6, 8830/pSK700 with IPTG. (C) A 35- $\mu$ g portion of alginate was run on a 5% polyacrylamide gel. Lanes: 1, 8830/pMMB22 with IPTG added at 24 h; 2, 8830/pMMB22 without IPTG; 3, 8830/pMMB22 without IPTG; 4, 8830/pSK700 with IPTG added at 24 h; 5, 8830/pSK700 with IPTG added at 0 h; 6, 8830/pSK700 without IPTG.

concentration of 1 mM. All units were incubated at 37°C for a total of 48 h. Subsequent analysis was identical to that described above except that 2 ml of 0.9% NaCl was added to collect the sloughed cells.

**Alginate analysis.** The cells remaining on the plates following removal of the sloughed cells were washed and suspended in 0.9% NaCl, and the alginate was isolated as previously described (27). The alginate was quantified by the carbazole method of Knutson and Jensen with manuronic acid as a standard (23).

A 5% nondenaturing polyacrylamide gel was used to analyze

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	References
<i>P. aeruginosa</i>		
8821	<i>his-1</i> Alg <sup>+</sup> (mucoid cystic fibrosis isolate)	13
8822	<i>his-1</i> Alg <sup>-</sup> (spontaneous nonmucoid derivative of 8821)	13
8830	<i>his-1</i> Alg <sup>+</sup> (stable mucoid mutagen-induced derivative of 8822)	13
Plasmids		
pMMB22	IncQ Ap <sup>r</sup> <i>Ptac lacI</i> <sup>a</sup>	3
pSK700	1.8-kb <i>Eco</i> RI- <i>Xba</i> I <i>algL</i> gene fragment in pMMB22	6, 35

the alginate qualitatively (7, 8). The gel consisted of 0.4 M Tris-HCl (pH 8.8) and 5% acrylamide-bisacrylamide. A 25- $\mu$ l portion of alginate was prepared in a solution containing 25  $\mu$ l of glycerol, 25  $\mu$ l of 1.5 M Tris-HCl (pH 8.8), and 5  $\mu$ l of 0.05% bromophenol blue. The gel was run in a buffer of 0.025 M Tris (pH 9.0) and 0.19 M glycine at 15 mA for the first 40 min and then at 25 mA until the dye reached the bottom of the gel. The alginate was visualized by being stained with 0.08% toluidine blue-O in 7% glacial acetic acid for 45 min and then destained with water (7, 8).

**Alginate lyase measurements.** Alginate lyase activity was assayed by using a modification of the method of Boyd et al. (6). The cell pellet from the alginate isolation described above was washed with 0.9% NaCl. The cells were broken by sonication in a solution containing 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, and 10 mM MgCl<sub>2</sub>. This was followed by a 15-min spin at 10,000  $\times$  *g*. Aliquots of the cell-free supernatant fraction were added to an assay solution containing 50 mM PIPES [piperazine *N,N*-bis(2-ethano-sulfonic acid, disodium salt), pH 7.5], 350 mM NaCl, 10 mM MgCl<sub>2</sub>, and 800  $\mu$ g of sodium alginate (Sigma) per ml and incubated for 10 min at 37°C. Thiobarbituric acid assays were performed to measure amounts of unsaturated sugar products (36). One unit of activity is defined as 1 nmol of  $\beta$ -formyl-pyruvate produced min<sup>-1</sup> ml of crude extract<sup>-1</sup>.

## RESULTS

**Correlation of alginate production with decreased cell detachment.** Mucoid *Pseudomonas aeruginosa* strains 8821 and 8830 produced 15 to 22 mg of alginate per plate after 3 days of growth on PIA (Table 2). No alginate could be detected for

TABLE 2. Alginate production, cell detachment, and alginate lyase measurements for *P. aeruginosa* strains 8822, 8821, and 8830<sup>a</sup>

Strain	Phenotype	Amt of alginate (mg/plate)	Detached cells (CFU, 10 <sup>6</sup> )	Amt of alginate lyase sp act (U/mg of protein) <sup>b</sup>
8822	Nonmucoid	ND <sup>c</sup>	287 $\pm$ 164	2.3 $\pm$ 2.2
8821	Mucoid	15.6 $\pm$ 1.5	5.0 $\pm$ 1.3	3.5 $\pm$ 1.2
8830	Mucoid	21.6 $\pm$ 4.0	5.9 $\pm$ 2.7	0.5 $\pm$ 0.9

<sup>a</sup> Data are mean values of three independent experiments  $\pm$  standard deviations.

<sup>b</sup> Both the mucoid strains and the nonmucoid strains grown on PIA plates had very low levels of alginate lyase specific activity, averages of which are shown. These amounts were insufficient to cause significant alginate capsule degradation.

<sup>c</sup> ND, not detectable (<100  $\mu$ g of alginate per plate).

TABLE 3. Alginate production, cell detachment, and alginate lyase measurements of 8830/pMMB22 and 8830/pSK700 in the presence and absence of 1 mM IPTG<sup>a</sup>

Strain	Amt of alginate (mg/plate)	Detached cells (CFU, 10 <sup>8</sup> )	Amt of alginate lyase sp act (U/mg of protein)
8830/pMMB22 without IPTG	24.7 ± 5.1	5.9 ± 2.7	1.1 ± 1.9
8830/pMMB22 with IPTG	21.9 ± 6.4	6.8 ± 1.1	1.4 ± 1.4
8830/pSK700 without IPTG	24.2 ± 3.8	6.3 ± 2.1	10.9 ± 3.4
8830/pSK700 with IPTG	15.5 ± 2.7	102 ± 35	160 ± 32

<sup>a</sup> Data are means of three independent experiments ± standard deviations.

strain 8822. This nonmucoid strain gave rise to nearly 50-fold more detached cells than were observed for the mucoid strains 8821 and 8830. The small number of sloughed cells observed with 8821 and 8830 correlated with the large amounts of alginate that were produced by these strains. On the other hand, 8822, which gave rise to many more detached cells, produced no detectable alginate. All strains had low levels of alginate lyase activity.

**Effect of increased expression of alginate lyase on alginate degradation and cell detachment.** The stable mucoid strain 8830 harboring the vector pMMB22 or the *algL* plasmid pSK700 was used for these experiments (Table 1). IPTG induces alginate lyase expression from the *tac* promoter of plasmid pSK700. pMMB22 serves as the vector control. The level of alginate lyase activity of 8830/pMMB22 grown in the presence or absence of IPTG was low (Table 3). 8830/pSK700 grown in the absence of IPTG had a higher level of alginate lyase activity than the vector control because of the leakiness of the *tac* promoter in *P. aeruginosa*. This approximately 10-fold increase in alginate lyase specific activity was not sufficient to alter the amount of alginate produced or to affect cell detachment (Table 3).

8830/pSK700 with IPTG showed a high level of alginate lyase specific activity because of increased expression of the *algL* gene from the *tac* promoter. The amount of cell detachment from a growth film of 8830/pSK700 grown in the presence of IPTG was 17-fold greater than that observed for 8830/pMMB22. The amount of alginate produced by 8830/pSK700 with IPTG was similar to that produced by 8821 (Table 2). Thus, the increase in sloughing observed for 8830/pSK700 with IPTG cannot be explained solely by a decrease in the amount of alginate present. However, the increase in cell detachment does correlate with the degree of depolymerization of the alginate (Fig. 1B). Alginate samples of the above-described strains were found to be quite different from each other qualitatively when they were visualized on a 5% polyacrylamide gel. The alginate of 8830/pSK700 with IPTG was greatly

degraded as seen by its polydispersed pattern. The alginate samples of 8830/pMMB22 with and without IPTG and 8830/pSK700 without IPTG each showed a high-molecular-weight monodispersed band with little or no alginate degradation (Fig. 1B).

**Effect of alginate lyase induction on cell detachment at various stages of growth.** 8830/pMMB22 and 8830/pSK700 were analyzed for cell detachment by using Nalgene filter units. In order to induce the alginate lyase of pSK700, IPTG was added to the bottom chamber either at the time of inoculation (0 h) or 24 h after inoculation. All filter units were incubated at 37°C for 48 h. Addition of IPTG caused a large increase in alginate lyase specific activity (Table 4), and extensive alginate degradation was observed for both IPTG-induced 8830/pSK700 samples (Fig. 1C). Induction of the alginate lyase in 8830/pSK700 at 0 h resulted in a threefold reduction in the amount of alginate produced. The number of detached cells increased 9- to 16-fold over the number produced by the vector control. A less pronounced decrease in alginate formation was seen when IPTG was added at 24 h than when it was added at 0 h. Cell detachment increased fourfold to eightfold over that of the vector control, compared with a 9- to 16-fold increase when the alginate lyase was induced at 0 h.

## DISCUSSION

It has been previously proposed that alginate plays a role in the attachment of *P. aeruginosa* cells to solid surfaces and in biofilm formation (2, 12, 15, 20). Cell attachment and biofilm formation are important factors in the development and persistence of chronic *P. aeruginosa* infections in the lungs of cystic fibrosis patients (2, 4, 5, 20, 21). An important aspect of *P. aeruginosa* biofilm development is the mode of cell detachment once the film is formed. It is known that discrete multicellular aggregates slough off from the biofilm, leading to uneven thickness of the film (33). We developed a simple semiquantitative assay in order to measure the degree of cell detachment. This method involves determination of the number of CFU that are detached in a saline wash from a growth film of bacteria on an agar surface. This system was not designed for precise biofilm analysis, but it did allow a physiological study of the effect of alginate lyase on cell detachment to be performed without specialized equipment. Using this method, we have shown that a nonmucoid strain of *P. aeruginosa* has enhanced ability to slough cells from an agar surface compared with that of mucoid strains. It appears that alginate serves to anchor the cells in a growth film, resulting in reduced cell detachment.

We have also shown that increased expression of the alginate lyase protein in mucoid strains of *P. aeruginosa* led to alginate degradation and accordingly to alginate of lower

TABLE 4. Alginate production, cell detachment, and alginate lyase measurements for 8830/pMMB22 and 8830/pSK700 with IPTG treatments at various times of growth<sup>a</sup>

Strain	Time of IPTG treatment (h)	Amt of alginate (mg/filter unit)	Detached cells (CFU, 10 <sup>8</sup> )	Amt of alginate lyase sp act (U/mg of protein)
8830/pMMB22	No treatment	4.6 ± 0.5	4.3 ± 1.1	0.2 ± 0.4
	0	5.2 ± 2.3	2.9 ± 1.7	1.0 ± 1.2
	24	4.6 ± 2.3	5.2 ± 3.2	1.6 ± 2.1
8830/pSK700	No treatment	5.7 ± 1.8	3.3 ± 1.7	2.5 ± 0.6
	0	1.8 ± 1.6	46.6 ± 14.1	46.5 ± 4.3
	24	3.9 ± 0.5	18.7 ± 6.3	32.2 ± 6.5

<sup>a</sup> Data are the means of three independent experiments ± standard deviations.

molecular weight. This resulted in enhanced sloughing of cells. Shorter-length alginate polymers have decreased viscosity compared with longer polymers. This decrease in viscosity could be responsible for the reduced ability of the shorter alginate polymers to retain bacterial cells on a solid surface. Furthermore, a less polymerized exopolysaccharide could result in a less dense extracellular matrix, thus facilitating detachment of the bacteria. It thus seems that not only is alginate production important for cell immobilization, but the nature of the alginate is also significant. The *P. aeruginosa* AlgL protein is involved in determining the nature of the alginate. The methanogenic archaeobacterium *Methanosarcina mazei* produces a disaggregatase enzyme whose activity shows similarity to that of *P. aeruginosa* alginate lyase (37). The disaggregatase causes cellular aggregates to break apart into separate cells by hydrolytic cleavage of the heteropolysaccharide capsule. Conditions that are generally unfavorable for growth are associated with disaggregatase activity.

It is interesting that the *algL* gene is located in the middle of the alginate biosynthetic gene cluster (6, 31) (Fig. 1A). It has been proposed that this cluster has an operon-like structure and is transcriptionally regulated from the promoter of the upstream gene *algD* (10). This being the case, the degradative *algL* gene would presumably be transcriptionally regulated in the same manner as the biosynthetic genes. However, there is evidence for individual transcriptional control within the cluster itself (11, 32). It is also possible that there is translational control or posttranslational control of gene expression and activity. Thus, the regulation of the gene for alginate lyase activity may not necessarily be the same as that of the other genes in the cluster. Indeed, it would be futile for *P. aeruginosa* to express *algL* coordinately with the biosynthetic genes. It would be interesting to see how the *algL* gene is expressed and to compare this expression with the expression of biosynthetic genes such as *algD* and *algA*. Further studies on the regulation of *algL* gene expression are under way.

It should be noted that *P. aeruginosa* has been reported to produce a distinct low-molecular-weight polysaccharide other than alginate, which enhances the attachment of *P. aeruginosa* to precoated surfaces (1). The enhanced detachment of cells from the growth film as a function of increased alginate lyase expression reinforces the fact that alginate plays an important role in cellular adherence to solid surfaces. The role of *algL* in wild-type *P. aeruginosa* may be to bring about the release of cells from a solid surface or biofilm such that these cells may aid in the spread of the organism to different niches within the cystic fibrosis-infected lung as well as on environmental surfaces. It may be beneficial to the organism to be able to slough off under certain circumstances, particularly during nutritional starvation, anaerobiosis, or other environmental stresses. Future studies of the adhesion and detachment properties of *P. aeruginosa* biofilms with regard to alginate lyase expression would augment these physiological experiments and provide a means to investigate the surface effect of the substratum on alginate lyase gene expression.

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