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Exopolysaccharide Production in Biofilms: Substratum Activation of Alginate Gene Expression by *Pseudomonas aeruginosa*

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Reporter gene technology was employed to detect the activity of an alginate promoter of *Pseudomonas aeruginosa* when the organism was grown as a biofilm on a Teflon mesh substratum and as planktonic cells in liquid medium. Alginate biosynthetic activity was determined with a mucoid cell line derived from a cystic fibrosis isolate and containing an alginate *algC* promoter fused to a *lacZ* reporter gene. Reporter activity was demonstrated with chromogenic and fluorogenic substrates for β-galactosidase. Expression of *algC* was shown to be upregulated in biofilm cells compared with planktonic cells in liquid medium. Gene up-expression correlated with alginate biosynthesis as measured by Fourier transform infrared spectroscopy, uronic acid accumulation, and alginate-specific enzyme-linked immunosorbent assay. The *algC* promoter was shown to have maximum activity in planktonic cultures during the late lag and early log phases of the cell growth cycle. During a time course experiment, biofilm *algC* activity exceeded planktonic activity except during the period immediately following inoculation into fresh medium. In continuous-culture experiments, conversion of *lacZ* substrate was demonstrated microscopically in individual cells by epifluorescence microscopy.

In 1966, Linker and Jones first reported the production of alginate in *Pseudomonas aeruginosa* (22). This organism has been shown to preferentially assume an attached state, embedding itself in exopolymer composed of alginate (8). Alginate is a linear copolymer of β-1,4-linked D-mannuronic acid and its C-5 epimer L-guluronic acid. Alginate as a biofilm matrix polymer plays an important role in diseases such as cystic fibrosis (CF) (5, 16) and urinary tract infections (26), in the fouling of man-made materials (6), and at surfaces in the natural environment (15). The pathway of alginate biosynthesis by *P. aeruginosa* is summarized in Fig. 1.

The activation of a critical alginate promoter, *algD*, by *P. aeruginosa* has been shown to take place during nitrogen limitation, during membrane perturbation induced by ethanol, and when cells were exposed to media of high osmolarity (12, 13). Similar to the *algD* promoter, the *algC* promoter has been shown to be activated by environmental signals such as high osmolarity, and this activation is dependent on the presence of a response regulator protein, AlgR1 (34). The term activation is used in this paper to refer to an increase in promoter activity observed in response to an environmental signal which results in up-expression of the gene under that promoter's control.

The activities of the alginate biosynthetic enzymes are extremely low even in mucoid strains of *P. aeruginosa* and are either greatly reduced or absent in nonmucoid strains (27, 29, 32). Because of the low activity of these enzymes, reporter genes have been used to detect the activity of the alginate promoters, rather than the direct measurement of the enzymes. The gene *algC*, encoding phosphomannomutase, was examined in the present study because it is a key regulation point in the alginate biosynthetic pathway. The promoter sequence for *algC* along with 1.0 kb of upstream DNA was fused to a promoterless *lacZ* (β-galactosidase) reporter gene to form the plasmid designated pNZ63 (33, 34). The plasmid was introduced into the stable mucoid *P. aeruginosa* strain 8830.

Strain 8830 was selected for the present study because of the large amount of alginate it produces and its very low rate of reversion to the nonmucoid form (10). Strain 8830 was isolated as a stable mucoid variant following ethyl methanesulfonate mutagenesis of strain 8822, a spontaneous nonmucoid revertant of a CF isolate, strain 8821 (10). The exopolysaccharide produced on solid media by strain 8830 has been shown by Darzins and Chakrabarty (10) to contain alginate, as determined by the method of Knutson and Jeanes (21).

In view of the preference of *P. aeruginosa* to form biofilms composed of cells and associated exopolymer, it was of interest to determine what effect attachment of cells to a substratum would have on alginate production. In the current effort, substratum activation of the *algC* promoter is demonstrated in *P. aeruginosa* 8830 and related to alginate synthesis.

MATERIALS AND METHODS

Strains and media. The bacterium used in this study was *P. aeruginosa* 8830 containing the *algC-lacZ* transcriptional fusion plasmid pNZ63. This reporter plasmid and its characteristics have been described previously (33, 34).

All experiments were conducted with a defined culture medium containing the following, in grams per liter: sodium lactate, 0.05; sodium succinate, 0.05; ammonium nitrate, 0.05; KH_2PO_4, 0.19; K_2HPO_4, 0.63; Hutner salts (7), 0.01; glucose, 2.0; and L-histidine, 0.01. Carbenicillin (350 mg/liter for liquid medium and 600 mg/liter for solid medium) was added to maintain plasmids in the cells.

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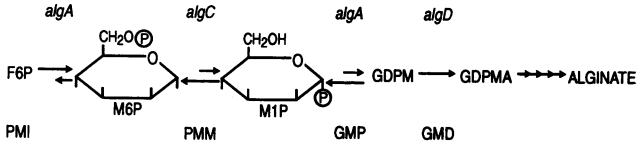

FIG. 1. Alginate biosynthetic pathway in *P. aeruginosa*. Enzymes catalyzing the known reactions in the pathway are as follows: PMI, phosphomannose isomerase; PMM, phosphomannomutase; GMP, GDP-mannose pyrophosphorylase; and GMD, GDP-mannose dehydrogenase. The genes encoding these enzymes are indicated above the respective enzyme name. The known reaction intermediates in the pathway are F6P, fructose 6-phosphate; M6P, mannose 6-phosphate; M1P, mannose 1-phosphate; GDPM, GDP-mannose; and GDPMA, GDP-mannuronic acid. The steps between the formation of GDP-mannuronic acid and that of alginate include polymerization, acetylation, export, and epimerization (shown by arrows) and are not yet well characterized.

Biofilm versus planktonic cell expression of alginate synthesis. Cultures were prepared from stocks maintained at −70°C in skim milk media by inoculating 0.1 ml into Erlenmeyer starter culture flasks (2 by 50 ml) containing 25 ml of culture medium supplemented with carbenicillin. Starter cultures were incubated at room temperature for 48 h or until cultures became turbid. Cell suspensions of 10 ml were inoculated into 1-liter Erlenmeyer flasks containing 500 ml of culture medium supplemented with carbenicillin. Two duplicate flasks containing sterile medium and a Teflon mesh having a surface area of 332.8 cm^2 were used to culture the biofilm population. Two additional duplicate flasks containing no mesh were used to culture the planktonic cell population. Flasks were incubated at 24°C for 96 h on a rotary shaker (175 rpm).

Time course experiment. A time course experiment was run to examine the relationship of *algC* gene expression to the bacterial growth cycle. Starter cultures of *P. aeruginosa* were prepared as described above. Three separate flasks each containing 500 ml of sterile medium supplemented with carbenicillin were inoculated with 10 ml of starter culture in late log phase (as determined by turbidity). These flasks were incubated at 24°C on a rotary shaker (175 rpm). Samples from each flask were taken during lag, log, and stationary phases of the growth cycle and immediately assayed for turbidity, total cell counts, and β-galactosidase activity. Subsamples of 10 ml were transferred to chromic acid-washed scintillation vials and stored at −70°C for subsequent determination of protein and uronic acids.

At the end of the stationary phase, 10 ml of culture from one flask was transferred to 500 ml of sterile culture medium supplemented with carbenicillin to examine the effects of fresh medium on alginate regulation. Cells were analyzed for β-galactosidase activity, and cell densities were determined by optical density at 600 nm. In addition, 10 ml of late-stationary-phase culture was transferred from one of the three original flasks to each of seven flasks containing 250 ml of sterile culture medium supplemented with carbenicillin and containing 3.5 g of Teflon mesh having a surface area of 20.7 cm^2. The cells which were attached to the Teflon mesh were harvested at intervals from different flasks over a period of 48 h. Cells were immediately analyzed for β-galactosidase activity and were enumerated by total cell counts by the method of Hobbie et al. (17).

Cell counts. Cells were enumerated by the spread plate method, using R2A agar (1). A cell suspension of 10 ml was removed from each resuspended washed population. These samples were homogenized with a Tekmar Tissue-Mizer at maximum speed for 30 s at 4°C. A subsample of the homogenized cell suspension was analyzed by phase-contrast microscopy to determine that cells did not display clumping. Cells were enumerated in triplicate and reported as CFU per milliliter.

Cell harvesting. Teflon mesh was aseptically removed from culture flasks and gently rinsed with sterile medium to remove loosely adherent cells. The remaining attached cells (biofilm population) were aseptically recovered by scraping the cells from the mesh with a sterile brush into 250 ml of chilled sterile medium. Both the biofilm population and the planktonic population were washed by centrifugation (250 ml in sterile acid-washed centrifuge tubes) at 16,274 × *g* for 20 min at 4°C and resuspended in sterile chilled culture medium. Resuspended cultures were assayed for viable cell numbers on R2A agar immediately following centrifugation. Subsamples were frozen at −70°C for subsequent protein analysis, β-galactosidase activity, uronic acids, and Fourier transform infrared spectroscopy.

Portions of scraped and unscraped mesh were immersed in 2% formaldehyde, stained with acridine orange, and examined by fluorescence microscopy to check culture purity and cell removal. No cells were observed on the surface of the scraped mesh samples.

β-Galactosidase assay. Aliquots of 0.6 ml of thawed cell suspension were mixed with 0.4 ml of Z-buffer (24) and lysed with 1 drop of chloroform and 2 drops of 0.1% sodium dodecyl sulfate per ml of cell suspension. Lysed cell suspensions were incubated for 5 h with 0.2 mg of *o*-nitrophenyl-β-D-galactoside (ONPG) and analyzed with a Varian DMS 90 spectrophotometer by the procedure of Miller (24). β-Galactosidase specific activity is reported as micromoles of ONP produced per minute per milligram of protein or per cell at 25°C, pH 7.0.

Lowry protein assay. The Lowry protein assay was performed on thawed samples with a Varian DMS 90 spectrophotometer following the procedure as modified by Peterson (28). Protein is reported in milligrams per milliliter of resuspended cell culture.

Collection of infrared spectra. Total carbohydrate associated with washed, resuspended biofilm cells recovered from the Teflon mesh and planktonic cells were determined by attenuated total-reflectance Fourier transform infrared spectroscopy. Cell suspensions were transferred to an open boat Micro Circle cell (Spectra Tech, Stamford, Conn.) containing a germanium internal reflection element. Samples were dried on the internal reflection element and then positioned in the front and back beams on the optical bench of a Perkin-Elmer model 1800 spectrometer equipped with a KBr beam splitter and a liquid N_2-cooled, medium-range, mercury-cadmium-telluride detector (5,000 to 580 cm^{-1}). Spectra were collected from each circle cell by using the single-beam mode over the range of 2,000 to 700 cm^{-1} and ratios to spectra collected from each cell prior to introduction of the cell suspensions were determined. Spectra of cell suspensions dried on the internal reflection element were compared with a spectrum of sodium alginate (Sigma Chemical Co., St. Louis, Mo.) to verify the peak assignment. The amounts of total carbohydrate and protein associated with the dried cells were obtained from the areas of the absorbance peaks centered at 1,050 cm^{-1} for complex sugar rings and 1,534 cm^{-1} for amide II, respectively (25).

Uronic acid assay. Total uronic acids were measured in thawed samples of washed cell suspensions by the method of

TABLE 1. *P. aeruginosa* 8830 response to growth on Teflon mesh

Population	β-Galactosidase sp act (μmol of ONPG converted min^{-1} mg of $protein^{-1}$)[a]		FTIR[b]	Uronic acids (ng produced μg of $protein^{-1}$)[c]		ELISA
	Expt 1	Expt 2		Expt 1	Expt 2	
Biofilm	10.0 ± 1.6	10.0 ± 1.2	25.83	18.1 ± 3.3	25.9 ± 6.5	+
Suspended cells	2.6 ± 0.85	2.1 ± 1.8	12.05	7.6 ± 1.7	8.7 ± 1.6	+

[a] ± 95% confidence interval of three replicates.
[b] Ratio of infrared absorbance peak areas contributed by total carbohydrates (1,050 cm^{-1}) and amide II protein (1,534 cm^{-1}).
[c] ± Standard deviation of three replicates.

Kintner and Van Buren (19), using a Varian DMS 90 spectrophotometer.

ELISA test for presence of alginate. Suspensions of washed, biofilm, and planktonic cells were assayed for alginate, using an enzyme-linked immunosorbent assay (ELISA) following the procedure of Irvin and Ceri (18). Authentic *P. aeruginosa* alginate was prepared by the method of Darzins and Chakrabarty (10) and was used as a positive control. Sterile culture medium supplemented with carbenicillin (350 mg/liter) was used as a negative control. Wells in a polystyrene microtiter plate were coated with sample for 24 h at 4°C. Bovine serum albumin (0.1% in deionized water) was added to each well and allowed to coat the wells for 2 h. Mouse anti-alginate antiserum (1:1,000 dilution) was added and allowed to react for 2 h. An aliquot of 0.4 mg of anti-mouse immunoglobulin M peroxidase conjugate per ml was added to each well, and wells were incubated for 2 h. *O*-phenylenediamine dihydrochloride was prepared in phosphate-citrate buffer to 0.4 mg/ml and added to each well to react with horseradish peroxidase. Reactions were terminated with 3 M H_2SO_4. All reactions were carried out at room temperature. Wells were inspected visually for the indicated color change.

***algC* expression by biofilm cells in continuous culture.** A continuous-culture apparatus was used to monitor in situ reporter gene activity of biofilm cells by epifluorescence microscopy. A glass capillary having a rectangular cross section with internal dimensions of 0.3-mm depth, 6.0-mm width, and 2.0-cm length was used as a substratum for growth of biofilm cells. The capillary was fitted to a customized holder which could be mounted atop a microscope stage. Medium was pumped from a sterile reservoir via surgical tubing through the glass capillary to a waste reservoir. An inoculation port was positioned upstream from the capillary. The continuous-culture apparatus was closed to the outside environment but maintained in equilibrium with atmospheric pressure by use of gas-permeable filters. Defined culture medium used in these experiments was amended with methylumbelliferyl β-D-galactoside. Two continuous-culture systems were operated in parallel. The first was inoculated with *P. aeruginosa* 8830 harboring the pNZ63 plasmid. The second system was used as a control and was inoculated with a *P. aeruginosa* strain 8830 not carrying the *lacZ* gene. This was done to ensure that any observed fluorescence was due to the presence of converted substrate and not to some other fluorescence phenomenon. The strain used in the control line is not carbenicillin resistant; therefore, the medium for culture of that organism contained no antibiotic. Both systems were run at a flow rate of 0.19 ml min^{-1}, producing laminar flow within the capillary with a Reynolds number of 1.0. Residence time in the capillary was 18.9 min, less than the doubling time of the organism in suspension, allowing only biofilm organisms to be retained within the capillary.

Confocal laser microscopy. Imaging of in vivo expression of β-galactosidase gene expression was performed with an Olympus BH-2 microscope connected to a Bio-Rad MRC 600 Confocal Laser Scanning attachment. Biofilm cells attached to the walls of a glass capillary were observed with the microscope, using reflected light to illuminate all cells. The same field was then scanned with laser light at an excitation wavelength of 488 nm and an emission wavelength of 510 nm to observe fluorescence due to β-galactosidase conversion of the methylumbelliferyl substrate. Images were recorded for bacteria containing the pNZ63 plasmid with *lacZ* under the control of the *algC* promoter and for bacteria not containing the *lacZ* gene.

RESULTS

Biofilm versus planktonic cell expression of alginate synthesis. β-Galactosidase was used as a reporter of *algC* promoter activation to determine whether attachment to a Teflon surface influences the regulation of alginate biosynthesis in *P. aeruginosa*. When the activity of biofilm cells grown on Teflon mesh was compared with that of planktonic cells grown in suspension, the results demonstrated a greater than threefold increase in reporter gene activity per milligram of protein for the biofilm population (Table 1).

Fourier transform infrared spectroscopy of biofilm and planktonic cell preparations revealed that total carbohydrate, when normalized to amide II protein, was 2.14-fold higher in the biofilm population (Table 1). Similarly, when normalized to protein, biofilm cells exhibited a greater than twofold-higher uronic acid content than planktonic cells after 96 h of incubation (Table 1).

Verification that the carbohydrate detected by Fourier transform infrared spectroscopy and uronic acids detected by the colorimetric assay were contributed by alginate was obtained by an ELISA specific for L-guluronic acid. Both biofilm and planktonic cells produced a positive reaction which was not observed in controls not containing alginate. No effort was made to use this assay to detect quantitative differences in alginate content of the two cell populations.

Time course characterization of alginate synthesis. Activation of the *algC* promoter was evaluated at different stages of the cell growth cycle. Planktonic cell cultures of *P. aeruginosa* were sampled over the course of the growth curve (69 h) and assayed for reporter gene (*lacZ*) specific activity. The β-galactosidase enzyme did not accumulate over the course of the cell growth cycle. Maximum *algC* promoter activity was observed during the early log phase of growth, with a decrease in activity detected during mid- and late log phases. A subsequent increase in activity was noted as cells entered

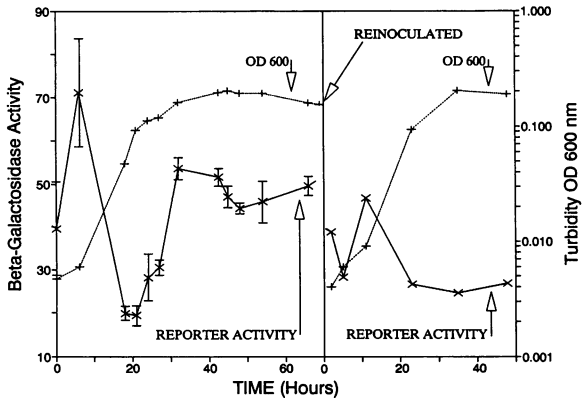

FIG. 2. Relationship of β-galactosidase specific activity (micromoles of ONPG converted milliliter^{-1} minute^{-1} optical density at 600 nm $[OD_{600}]^{-1}$) to cell growth cycle (OD_{600}) of planktonic population in batch culture. Dashed line represents average OD_{600} measurements for three replicates; solid line depicts average activity measurements for three replicates. Vertical bars represent ±1 standard deviation.

stationary phase, when they became limited in nitrogen, and again as they entered the death phase (Fig. 2). When cells from one of the replicate cultures were reinoculated into fresh medium, *algC* activity decreased initially and then increased during early log phase as observed previously, displaying minimum activity at stationary phase (Fig. 2). The activity of the *algC* promoter after reinoculation into fresh medium did not attain the maximum value observed during the previous growth cycle. Since replicate experiments were not performed as part of the reinoculation study, error bars are not displayed with the data.

In a separate experiment, *algC* activity of the biofilm cell population was measured over the same time course as the planktonic cell culture (Fig. 3). Maximum activation of the *algC* promoter was observed at approximately 24 h for biofilm cells, possibly the result of attaining a starvation state. With the exception of the initial 12-h period following inoculation, the biofilm cell population showed higher reporter gene activity when normalized to cell number than did the planktonic cell population. The maximum activity of the biofilm cells was more than five times higher than the maximum activity of the planktonic cells.

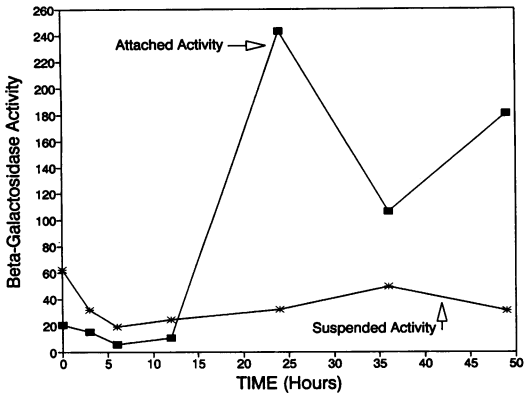

FIG. 3. Comparison of β-galactosidase specific activity (nanomoles of ONPG converted milliliter^{-1} minute^{-1} cell number^{-1}) in biofilm (attached) and planktonic (suspended) cell populations in batch culture.

Continuous-culture experiment. A flow cell mounted on a microscope stage was used to evaluate *algC* promoter activity in individual cells attached to a glass surface. When methylumbelliferyl β-D-galactoside was present in the medium flowing through the glass capillary which had been inoculated with cells carrying the pNZ63 plasmid (which contained the β-galactosidase gene under the control of the *algC* promoter), 30% of the cells adhering to the flow cell exhibited fluorescence 48 h after inoculation (Fig. 4). Cells not carrying the *lacZ* gene showed no fluorescence in the presence of the methylumbelliferyl substrate. This observation indicated that fluorescence in the pNZ63 plasmid-carrying population was due solely to reporter gene (and, therefore, *algC* gene) activation. These observations demonstrate the variability in expression of alginate genes within a population of cells adhering to a glass surface.

DISCUSSION

While *P. aeruginosa* is known to form biofilms on natural surfaces and CF lung epithelial cells (6, 8), very little is known of the role of adherence to a surface in triggering exopolysaccharide formation. The results described in this paper demonstrate activation of the *algC* promoter in *P. aeruginosa* 8830 which is necessary for the production of the exopolysaccharide alginate. This activation resulted from association of the bacteria with a Teflon substratum. This is, to our knowledge, the first report on the activation of an alginate gene promoter as a consequence of a cell's attachment to a solid surface.

Surface activation of bacterial genes has been reported previously by Dagostino et al. (9) in *Pseudomonas* sp. strain S9 growing on polystyrene microtiter plates. In that study transposon mutagenesis was used to insert promoterless *lacZ* genes into recipient organisms, giving some the ability to display β-galactosidase activity at a surface but not in liquid or on agar media. The authors, however, did not identify the specific target genes controlling *lacZ* activation. In a separate study by Belas et al. (3), the *laf* gene was activated in *Vibrio parahaemolyticus* on agar medium and in a liquid medium of high viscosity, but not in a liquid medium of low viscosity. In that study the authors concluded that gene activation was a consequence of medium viscosity.

In CF lung infections, a succession from nonmucoid to mucoid cells (14) is thought to reflect an adaptation by *P. aeruginosa* to an environment deficient in water and high in K^+, Na^+, HCO_3^-, and Cl^- (20). Laboratory studies have shown that high osmolarity activates *P. aeruginosa* transcription of *algD* (4) and *algC* (34), leading to enhanced alginate production. Ethanol has likewise been shown to enhance alginate synthesis by that organism on solid and in liquid media (13). The effects of ethanol and salt are believed to result in a response leading to bacterial membrane perturbations similar to what is found in the environment of the CF lung. In a separate study, Roberson and Firestone (31) showed that desiccation of *P. aeruginosa* growing in a sand matrix resulted in more exopolymer than when cells were grown at high water potential. Activation of the *algC* promoter in cells grown on a surface, as demonstrated in the present work, may be the result of decreasing water or nutrient levels at the point of contact with the substratum.

Production of alginate has been shown to vary throughout the cell growth cycle. Annison and Couperwhite (2) observed the production of alginate almost exclusively during

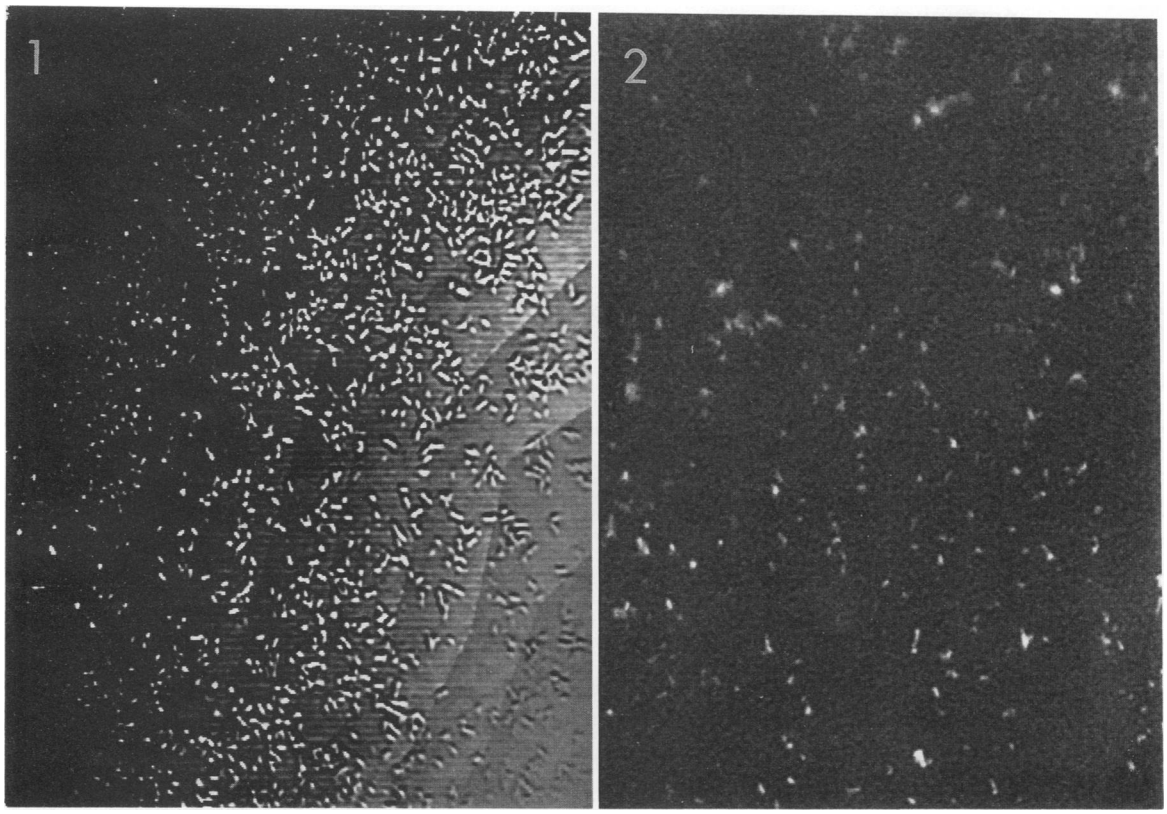

FIG. 4. Scanning confocal laser micrograph of *P. aeruginosa* attached to the inner surface of a glass flow cell. (1) Cells illuminated with reflected white light, showing all bacteria in the field. (2) Fluorescing cells excited by laser light at a wavelength of 488 nm, showing cells which are producing β-galactosidase, indicating *algC* promoter activation.

the exponential phase of growth with *P. aeruginosa*, as did Deavin et al. with *Azotobacter vinelandii* (11). In a separate series of experiments, Piggot et al. (30) found that exopolysaccharide production did not commence in *P. aeruginosa* until late in the exponential phase and continued maximally during the stationary phase of growth. In our studies, we found maximum *algC* reporter activity to occur in the late lag and early exponential phases of the growth cycle for planktonic cells in batch culture. During stationary phase, when the culture became limited in nitrogen, we saw a second increase in exopolymer production, paralleling the data of Piggot et al. (30). Biofilm cells also display variation in *algC* promoter activity. However, overall, activity was greater than with planktonic cells for the duration of the growth cycle.

There is a possibility that the differences in promoter activity observed in the two populations resulted from different average plasmid copy numbers in the respective populations. This is unlikely, however, in view of the fact that the cells of each population were derived from the same stock, precultured under the same conditions, and subsequently exposed to identical media during growth.

Using a fluorescent substrate to evaluate in vivo β-galactosidase activity in continuous culture, we demonstrated an accumulation of fluorescent product within individual cells attached to the surface of a glass capillary. This product was detected microscopically in real time, so that alginate promoter activity in attached cells was evaluated without destructive interference.

Fluorescence by attached bacteria carrying the pNZ63 plasmid demonstrated that the cells were actively expressing the *algC* reporter gene product as had been observed in our batch culture experiments. Interestingly, not all cells were fluorescent at that time even though they were in continuous culture. This implies that differences in the level of exopolysaccharide gene expression exist within the attached cell population. This may be due to the age, nutritional status, or degree or nature of adherence of the cells to the solid surface. Furthermore, this result demonstrates that a continuous detectable level of alginate production is not necessary for cells to remain attached to the substratum.

In this paper, we have reported the activation of a specific gene involved in the production of bacterial exopolysaccharide as the result of attachment to a surface. This activation can be detected and quantified in individual cells attached to a substratum as well as in whole cultures. The mechanism of activation and the range of surfaces upon which such activation occurs are not known at present. Further work is required to examine these unknown aspects of exopolysaccharide production by *P. aeruginosa*.

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