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Regulation of the Alginate Biosynthesis Gene *algC* in *Pseudomonas aeruginosa* during Biofilm Development in Continuous Culture

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Reporter gene technology was used to observe the regulation of the alginate biosynthesis gene, *algC* in a mucoid strain of *Pseudomonas aeruginosa* in developing and mature biofilms in continuous culture on Teflon and glass substrata. The plasmid pNZ63, carrying an *algC-lacZ* transcriptional fusion, was shown to not be diluted in continuous culture over a period of 25 days in the absence of selection pressure. Biofilm cells under bulk phase steady-state conditions demonstrated fluctuations in *algC* expression over a 16-day period, but no trend of increased or decreased expression over the time interval was indicated. In vivo detection of *algC* up-expression in developing biofilms was performed with a fluorogenic substrate for the plasmid-borne *lacZ* gene product (β-galactosidase) by using microscopy coupled with image analysis. By this technique, cells were tracked over time and analyzed for *algC* activity. During the initial stages of biofilm development, cells already attached to a glass surface for at least 15 min exhibited up-expression of *algC*, detectable as the development of whole-cell fluorescence. However, initial cell attachment to the substratum appeared to be independent of *algC* promoter activity. Furthermore, cells not exhibiting *algC* up-expression were shown to be less capable of remaining at a glass surface under flowing conditions than were cells in which *algC* up-expression was detected.

Pseudomonas aeruginosa has been shown to preferentially assume a biofilm mode of growth (9), embedding itself in a gelatinous organic polymer matrix composed primarily of alginate (10). Biofilms produced by these organisms have been shown to be resistant to antibiotic therapy (1), resulting in chronic infections of the urinary tract (22, 24) and lung epithelia of cystic fibrosis patients (5, 19). *P. aeruginosa* biofilms have also been shown to be important as fouling agents in industrial process systems (6) and to form at surfaces in the natural environment (17). Control of these biofilms continues to be problematic, in part as a result of a lack of understanding of the in situ regulation of biofilm matrix polymer formation.

Previous work has shown alginate production to be up-regulated in *P. aeruginosa* during nitrogen limitation (14), during membrane perturbation induced by ethanol, when cells were exposed to media of high osmolarity (15), and under conditions of high oxygen tension (3). Recently, we have reported that a specific gene (*algC*) involved in alginate biosynthesis by this organism is upregulated as the result of attachment to a surface (13). Little is known, however, of the regulation of alginate by this organism in biofilms in continuous culture.

Investigations into the regulation of alginate in continuous culture have been largely performed on total extracellular polymeric substance (EPS) or on exopolysaccharides, which in *P. aeruginosa* are believed to include alginate. Bakke et al. (2) examined the production of EPS by *P. aeruginosa* biofilms, showing that EPS biosynthesis was not directly proportional to substrate flux. This work did not, however, examine the regulation of EPS production. Robinson et al. (27) found that EPS formation was inversely related to the growth rate of *P. aeruginosa* in suspension in a chemostat; however, these results were not duplicated in biofilms. Vandevivere and Kirchman (28) found higher rates of exopolysaccharide synthesis in a subsurface bacterium attached to sand under conditions of continuous flow when compared with the rates in unattached bacteria.

In the present work, the behavior of a principal gene in the alginate biosynthesis pathway of *P. aeruginosa* in axenic biofilms was examined at the population level as well as at the single-cell level. The gene *algC*, encoding the enzyme phosphomannomutase, was studied because it is an essential enzyme for biosynthesis of alginate and a key point of regulation in the alginate pathway. The gene product of *algC* acts as a bifunctional enzyme, having phosphomannomutase activity (converting mannose 6-phosphate to mannose 1-phosphate), as well as phosphoglucomutase activity (interconverting glucose 6-phosphate and glucose 1-phosphate) (29). In its role as phosphomannomutase, the *algC* gene product is responsible for the formation of alginate, and in its role as phosphoglucomutase, the same gene product is responsible for the production of lipopolysaccharide (LPS) (11). The promoter sequence for *algC*, along with 1.0 kb of upstream DNA, was fused to a promoterless *lacZ* reporter gene to form the plasmid pNZ63 (30). The plasmid was introduced into the stable mucoid *P. aeruginosa* 8830. Strain 8830 was used in a previous study to demonstrate the up-regulation of *algC* by attachment to glass and Teflon substrata.

MATERIALS AND METHODS

Bacteria 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 by Zielinski et al. (30, 31).

All batch and continuous culture experiments were conducted with a defined culture medium containing sodium lactate (0.05 g/liter), sodium succinate (0.05 g/liter), ammonium nitrate (0.381 g/liter), KH_2PO_4 (0.19 g/liter), K_2HPO_4 (0.63 g/liter), Hutner salts (7) (0.01 g/liter), glucose (1.0 g/liter), and L-histidine (0.01 g/liter). Solid YTG medium was used for the enumeration of bacteria recovered from batch and continuous-culture experiments. When grown on this medium, *P. aeruginosa* 8830 produces mucoid colonies which show up-expressed *algC* activity. YTG medium contained the following components in grams per liter: yeast extract, 5.0; tryptone, 10.0; glucose, 2.0; and Bacto Agar, 1.5. YTG medium amended with carbenicillin was prepared with 350 mg of the filter-sterilized

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antibiotic per liter added to the medium following autoclave sterilization. YTG medium amended with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was prepared by adding 0.04 g of the β-galactoside per liter in *N,N*-dimethyl formamide. Phosphate buffer was prepared by adding 7.0 g of K_2HPO_4 and 3.0 g of KH_2PO_4 to 1 liter of ultrapure water (pH 7.0).

Plasmid retention studies. The activity of the constitutive marker for carbenicillin resistance on the pNZ63 fusion plasmid was examined to determine whether the presence of antibiotic pressure or cell attachment would influence the plasmid copy number. The study organism was examined under three separate growth conditions: planktonic cells grown in batch in defined culture medium, planktonic cells grown in batch in defined culture medium amended with 350 mg of carbenicillin per liter, and biofilm cells attached to Teflon mesh during growth in batch in defined culture medium amended with 350 mg of carbenicillin per liter. Cell cultures of *P. aeruginosa* were recovered from frozen skim milk stock and grown until turbid in preculture flasks of defined culture medium supplemented with 350 mg of carbenicillin per liter. An inoculum of 0.1 ml of preculture was added to each of three sets of quintuplicate 250-ml flasks containing 100 ml of defined culture medium. Each set corresponded to one of the three populations listed above. Cells were grown at 25°C on a rotary shaker at 175 rpm until cultures reached the stationary phase. Cells were centrifuged at 16,274 × *g* for 30 min, washed three times in PO_4 buffer, and frozen at −40°C. Cells attached to Teflon mesh were removed by scraping with a sterile brush, resuspended in defined culture medium, centrifuged, and washed as above. All samples were resuspended in 2 ml of PO_4 buffer (pH 7.0). The washing procedure was intended both to concentrate the sample and to remove carbenicillin from the preparation.

β-Lactamase assay. β-Lactamase activity was determined by a procedure based on that described by Cohenford et al. (8). Whole-cell samples were thawed and lysed at 0°C by cavitation for two 30-s bursts at high setting with a Bronwill Biosonik IV ultrasonic cell disrupter. Aliquots (0.5 ml) of lysed cell suspensions were mixed with 1.0 ml of β-lactam substrate (100 μg of carbenicillin per liter), vortexed, and allowed to stand for 10 min at 25°C. The reaction was terminated by the addition of 1.5 ml of neocuproine-copper reagent. Samples were vortexed and incubated at 25°C for an additional 30 min to allow color development. The β-lactam acid generated by the enzyme when reacted with the neocuproine-copper solution yielded an orange color with an absorbance maximum at 454.5 nm. All samples were analyzed at the above wavelength in a Milton Roy Spectronic 601 spectrophotometer.

Continuous culture studies. *P. aeruginosa* was grown in continuous culture to determine the ability of biofilm bacteria to retain the pNZ63 plasmid in the absence of antibiotic pressure and to evaluate *algC* expression in biofilm cells. A continuous-culture vessel having a liquid volume of 375 ml and containing defined culture medium was used without supplemented carbenicillin to grow biofilms for up to 22 days. The culture vessel was inoculated with 10 ml of late-log-phase starter culture in defined culture medium containing 350 mg of carbenicillin per liter. Thus, the carbenicillin concentration in the vessel was 0.93 mg/liter prior to the initiation of flowing conditions. The bacteria were allowed to colonize the surfaces within the culture vessel over a period of 24 h in batch mode. Fluid samples were collected from the culture vessel at the end of the batch mode period during two experimental runs to represent the planktonic cell population. Following the initial batch period, flow was turned on and adjusted to achieve washout conditions, resulting in a dilution rate in the culture vessel (D) of 3.53 h^{-1}. Previous experiments in our laboratory have shown *P. aeruginosa* 8830 to have a growth rate (μ) of 0.69 h^{-1} when grown in defined culture medium. Therefore, cells collected in the effluent after the onset of flowing conditions would have been shed from the growing biofilm on the culture vessel surfaces, where they were immune from washout.

Three separate continuous-culture experiments were run with Teflon mesh having a surface area of 137.2 cm^2 as a substratum for biofilm growth within the continuous-culture vessel. Teflon was used in this study because it has previously been shown to promote biofilm growth and *algC* up-expression in *P. aeruginosa* 8830 in batch culture (13). Bacteria shed from the biofilm under flowing conditions were collected each day from the effluent of the continuous-culture vessel to determine whether these cells would maintain their plasmid conferred antibiotic resistance in the absence of antibiotic pressure. These bacteria were enumerated on solid YTG medium containing 350 mg of carbenicillin per liter to recover CFU containing a functional β-lactamase marker and on solid YTG medium to determine total bacterial numbers. The efficiency of plasmid retention was determined as the fraction of carbenicillin-resistant CFU recovered relative to the total CFU recovered. Following growth on YTG, colonies were replica plated onto YTG amended with 350 mg of carbenicillin per liter to determine whether injury was responsible for decreased resistance to carbenicillin. For a discussion of injury in bacteria, see references 21 and 22. The number of CFU recovered on carbenicillin-containing plates was compared with the number of CFU recovered on YTG plates containing X-Gal, to ensure that the retention of antibiotic resistance was associated with retention of the β-galactosidase gene.

In the effort to evaluate *algC* expression during biofilm development in continuous culture, biofilms were grown in the culture vessel until the point at which bacteria shed into the effluent were shown to maintain a constant density as determined by standard plate count techniques with YTG medium. After several days of maintenance of relatively constant effluent cell densities, Teflon mesh substrata containing biofilms were recovered from two continuous-culture experiments. The biofilm bacteria were aseptically removed from the mesh by scraping with a sterile brush. These were suspended in sterile culture medium and frozen for subsequent analysis. These samples were analyzed for uronic acid accumulation, protein content, β-lactamase specific activity, and β-galactosidase specific activity. Scraped biofilm samples were subjected to the same analyses as planktonic samples recovered after the 24-h batch culture.

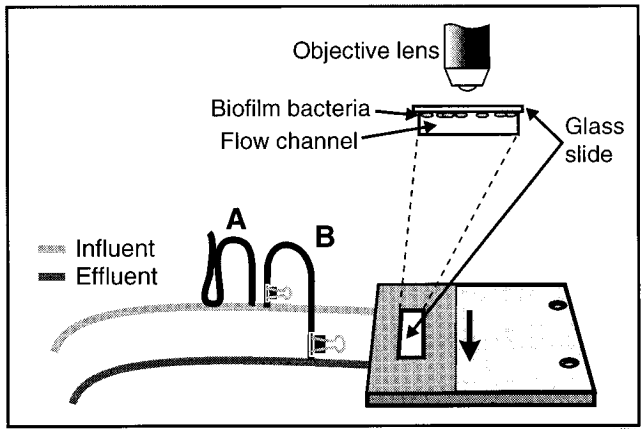

FIG. 1. Schematic representation of continuous-culture flow cell used to monitor specific reporter gene activity in individual bacteria. The flow cell is inoculated with bacteria, which are allowed to attach to the inner surface of the glass slide, where they may be viewed under UV or white-light illumination. The arrow represents the direction of flow. A, inoculation loop; B, loop used to divert air bubbles from flow channel.

During the period of relatively stable effluent cell densities, samples were collected daily from the reactor effluent and enumerated on YTG medium. Subsamples were frozen at −40°C and subsequently analyzed for β-galactosidase specific activity and protein concentration to evaluate *algC* expression.

β-Galactosidase assay. The β-galactosidase assay was performed on thawed samples with a Milton Roy Spectronic 601 spectrophotometer by the procedure of Miller (23) as described previously (13). β-Galactosidase specific activity is reported as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) converted per minute per milligram of protein at 25°C and pH 7.0.

Lowry protein assay. The Lowry protein assay was performed on thawed samples as described previously (13) and analyzed with a Milton Roy Spectronic 601 spectrophotometer.

Uronic acid assay. Total uronic acids were measured in thawed samples of scraped biofilm and whole culture by the method of Kintner and Van Buren (20) with a Milton Roy Spectronic 601 spectrophotometer.

Microscopic analysis of *algC* activity in continuous culture. In a previous paper, we reported the use of a fluorogenic galactoside which, when hydrolyzed by the pNZ63 reporter gene product (β-galactosidase), can be detected by epifluorescence microscopy within individual cells of *P. aeruginosa* (13). This fluorogenic compound was used in the present study as the substrate for reporter enzyme activity to evaluate *algC* expression in individual bacteria in continuous culture. To determine the effect of the presence of the methylumbelliferyl-β-D-galactopyranoside on the viability of the study bacteria, a test was performed in which the bacteria were grown for 52 h in batch culture in the presence and absence of 0.01 g of the fluorogenic substrate per liter in defined culture medium. Bacteria were sampled throughout the incubation period, and their ability to be cultured on solid YTG medium was evaluated.

A continuous-culture apparatus was developed to monitor in situ reporter gene expression of individual bacteria during initial stages of biofilm development (Fig. 1). The apparatus was configured as a once-through flow cell system. The influent defined culture medium supplemented with 350 mg of carbenicillin per liter was retained in a 4-liter glass reservoir. Methylumbelliferyl-β-D-galactopyranoside dissolved in 5.0 ml of *N,N*-dimethyl formamide was added to the defined culture medium to a final concentration of 0.01 g/liter. Medium from the influent reservoir was pumped through silicone tubing via a Masterflex pump to an aeration flask sparged with filtered air. The aerated medium was pumped to a flat plate flow cell by using a Masterflex 8 roller-head peristaltic pump at a flow rate of 0.13 ml min^{-1}. The flow cell was constructed of polycarbonate and was 0.1 mm deep, 1.4 cm wide, and 4.0 cm long; the upper face was capped with a glass coverslip. The glass coverslip was used as a substratum for bacterial attachment and biofilm development because it is a relatively inert material, is transparent, and has been shown to result in *algC* activity similar to that observed for Teflon mesh (unpublished data). Flow through the cell was laminar, having a Reynolds number of 0.17 and a fluid residence time of 0.43 min. The flow cell was sealed to prevent contamination and affixed to the stage of an Olympus BH2 micro-

TABLE 1. *P. aeruginosa* 8830 plasmid-borne β-lactamase specific activity

Population	Antibiotic presence[a]	β-Lactamase sp act[b] (mean ± SD)
Batch[c]		
Biofilm	+	55.1 ± 7.1
Planktonic	+	37.9 ± 7.6
Planktonic	−	38.5 ± 6.4
Continuous culture[d]		
Biofilm	−	51.6 ± 1.93

[a] Carbenicillin, 350 mg/liter.
[b] Nanomoles of carbenicillin converted per milligram of protein per minute.
[c] Mean of five separate experiments.
[d] Mean of two separate experiments.

scope containing a BH2-RFL fluorescent attachment. Medium leaving the flow cell was pumped to an effluent reservoir via silicone tubing. The entire system was closed to the outside environment but maintained in equilibrium with atmospheric pressure by a 0.2-μm-pore-size gas permeable filter fitted to each flask.

Log phase *P. aeruginosa* cells were inoculated through a septum approximately 1 cm upstream from the flow cell while flow was maintained. Bacteria were allowed to attach to and grow on the surfaces of the system downstream from the site of inoculation over a period of 24 h. Flow through the system was then increased to remove any bacteria attached to the inside surface of the coverslip (as determined by microscopy). Bacteria shed from biofilm upstream from the flow cell were then allowed to recolonize the surfaces of the flow cell under conditions of normal flow. After 2 h, cells attached to the inner surface of the glass coverslip were viewed by transmitted light with phase contrast at a magnification of ×1,025 to detect total cells. The same field was then observed under UV excitation with a UG1 blue Olympus filter block to detect the conversion of the methylumbelliferyl substrate. Fluorescent and phase-contrast images were recorded with an American Innovisions image analysis system. Each image was taken by using an integration of 50 scans to increase the amount of detectable fluorescence and to collect data on only those bacteria which were attached to the coverslip. Planktonic bacteria flowing beneath the coverslip were not detectable during the integration period, because of their motion. Information on the number and location of attached bacteria within a single field was collected at approximately 15-min intervals for a period of 165 min. All images were stored as separate files for subsequent retrieval and analysis.

Analysis of microscopic images. Stored digital images of bacteria were retrieved and displayed on a computer screen. Clear-plastic overlays placed on the screen were used to mark the time and position of cells attached to the inner surface of the glass coverslip. Three distinct subpopulations of bacteria were identified: (i) bacteria that were already attached to the coverslip in the previous observation period, (ii) bacteria that had attached to the coverslip since the previous observation period, and (iii) bacteria that had detached from the coverslip since the previous observation period. Bacteria within each of the three subpopulations were recorded as being fluorescent or nonfluorescent, indicating whether they manifested *algC* up-expression.

RESULTS

Plasmid retention studies. The transcriptional fusion plasmid pNZ63 contains an inducible β-galactosidase gene controlled by the promoter region of the *algC* gene of *P. aeruginosa*. Studies of reporter gene activity with such a plasmid are valid only when the copy number variation from cell to cell does not influence the interpretation of reporter gene activity measurements. To evaluate the stability of the pNZ63 plasmid in *P. aeruginosa* 8830, we decided to examine the activity of the β-lactamase marker which is constitutively expressed and not under *algC* promoter control. When β-lactamase specific activity was compared between planktonic cells in the presence and absence of β-lactam antibiotic pressure in batch culture, no significant difference was observed (Table 1). This indicates that the presence of antibiotic does not significantly influence the average copy number of plasmid within the population. When β-lactamase specific activity of biofilm bacteria was compared with that of planktonic cells when both were grown in the presence of antibiotic in batch culture, the average specific activity of the biofilm population was approximately 1.5 times of that of the planktonic population (Table 1). This result indicates that plasmid copy number may be 50% higher in the biofilm population than in the planktonic population.

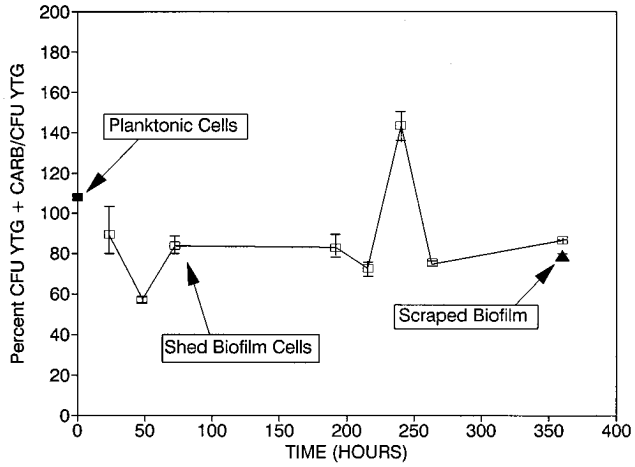

FIG. 2. Cells grown in continuous culture that display carbenicillin resistance, expressed as a percentage of total cells. Symbols: ■, planktonic cells collected from reactor before onset of continuous-flow operation; □, cells shed from the biofilm into the effluent; ▲, total biofilm cells scraped from Teflon mesh. Error bars represent ±1 standard deviation for triplicate samples.

The biofilm bacterial population which developed in the absence of antibiotic in continuous culture over a period of 18 and 22 days showed no significant difference in β-lactamase specific activity when compared with the population of bacteria growing as a biofilm in batch culture in the presence of antibiotic (Table 1). These results indicated that carbenicillin resistance was not lost by the vast majority of bacterial cells within the biofilm population during growth in continuous culture in the absence of antibiotic pressure over a period of at least 18 days.

To determine whether subpopulations of *P. aeruginosa* 8830 containing pNZ63 lose carbenicillin resistance after removal of antibiotic pressure, individual cells that had detached from growing biofilm were recovered from the effluent of the continuous-culture vessel operated under washout conditions and screened for their ability to grow in the presence of antibiotic. Samples were removed from the continuous-culture vessel and plated on selective media on a daily basis to examine the resistance of individual CFU to antibiotic. Microscopic analysis showed that CFU were composed of single cells. Results from a representative experiment with Teflon mesh as a substratum for biofilm growth in the absence of antibiotic are shown in Fig. 2. Over a period of 360 h, no overall decrease in carbenicillin resistance was observed for cells shed from the biofilm into the effluent. These results indicated that the population of bacteria shed from the biofilm showed no net loss of plasmid-borne β-lactamase activity during the time course of the experiment.

The datum point labeled "Scraped Biofilm" in Fig. 2 indicates the population of total biofilm cells scraped from the mesh at the end of the experiment which were culturable in the presence and absence of antibiotic. The percentage of scraped biofilm cells resistant to carbenicillin was not significantly different from that of shed cells, indicating that the shed cells did not represent a subpopulation of the total biofilm population with respect to plasmid copy number. No overall loss of carbenicillin resistance with time was demonstrated during any of the continuous-culture experiments.

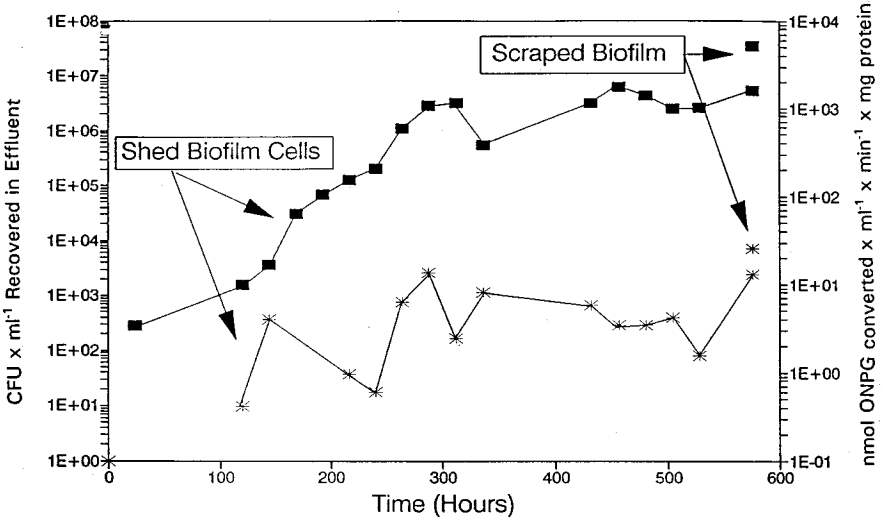

FIG. 3. Specific activity of *algC* reporter gene (∗) and viable cell numbers (■) for *P. aeruginosa* 8830 from continuous-culture vessel effluent. Cells shed from the biofilm into the effluent and total biofilm cells scraped from Teflon mesh are represented.

Recovery of resistant cells was, on average, lower than 100% of the total recoverable cells in all experiments. To check the discrepancy between total and resistant CFU recovered, replica plating was performed by transferring cells from each colony of planktonic shed biofilm and biofilm bacteria recovered on nonselective medium to carbenicillin-containing medium. Of the colonies transferred, 100% were shown to be carbenicillin resistant. No statistical difference was observed between CFU having antibiotic resistance and CFU having β-galactosidase activity (data not shown). These results indicated that the bacteria which were not recovered on antibiotic-containing medium when plated directly from the continuous culture effluent were a population of injured cells. Injured bacteria are those which are not recoverable on selective medium unless they are resuscitated first on a nonselective medium (21, 22).

Continuous-culture mature-biofilm experiment. Once it had been established that pNZ63 did not dilute out of the population of biofilm cells in the absence of antibiotic pressure, it became feasible to run experiments to examine the regulation of the *algC* gene over time in a mature biofilm with a plasmid-borne reporter. Three independent chemostat experiments were run with Teflon mesh as a substratum for biofilm growth: two for 432 h, and the other for 576 h. After establishing an attached population of cells by operating the reactor in batch mode for 24 h, flow of culture medium was initiated and adjusted to achieve washout conditions. After several days, the density of viable cells shed from the biofilm in the reactor effluent stabilized to a level fluctuating around a mean of 5.08×10^6 CFU/ml (standard deviation, $\pm 2.37 \times 10^6$) for the three separate experiments. Figure 3 shows results from the chemostat experiment which was run for 576 h. With the exception of a low cell density obtained at 336 h, shed-cell densities appeared to plateau between 2×10^6 and 6×10^6 CFU/ml after 288 h (Fig. 3). This range of shed-cell densities was typical of that exhibited by cells shed from biofilms in previous experiments. Uncontrollable episodic detachment of bacteria from the biofilm may have accounted for observed variations in effluent cell densities.

Alginate accumulation based on uronic acid concentrations and expression of *algC* based on reporter gene product specific activity were determined for the planktonic cell population at the end of the 24-h batch culture period and for the scraped biofilm population at the end of the continuous culture period for two separate chemostat experiments (Table 2). Expression of *algC* was nearly 20-fold higher and alginate levels were over 2-fold higher in the biofilm population than in the suspended-cell population, respectively (Table 2).

Expression of *algC* was also evaluated in the population of bacteria shed from the biofilm which were recovered from the chemostat effluent of the experiment run for 576 h. Fluctuations in specific activity of reporter gene product were observed as the biofilm developed throughout the course of the experiment (Fig. 3). The average β-galactosidase specific activity for the shed cells collected in the effluent from 288 through 576 h is shown in Table 2. This averaged activity had a value which fell between those derived for scraped biofilm cells and planktonic cells, indicating an intermediate stage in *algC* activity (Table 2).

Evaluation of *algC* expression in individual cells in early stages of biofilm development. A novel microscopic technique was used for observing single-cell *algC* expression during the initial phases of biofilm development. By using a once-through flow cell reactor system, gene expression was monitored in bacteria which had detached from an upstream biofilm and subsequently reattached to a glass coverslip, where they could be observed by fluorescence and transmitted-white-light microscopy. To determine that the fluorogenic galactoside used

TABLE 2. *P. aeruginosa* 8830 continuous-culture biofilm activity versus planktonic cell activity

Population	Uronic acids (ng produced/μg of protein)	β-Galactosidase sp act (nmol/min/mg of protein)
Biofilm[a]	80.0 ± 0.14	19.35 ± 8.8
Planktonic[a]	36.5 ± 0.70	1.06 ± 0.05
Shed cell[b]	ND[c]	6.21 ± 4.3

[a] Mean of two separate experiments ± 1 standard deviation.
[b] Mean of nine samples from one chemostat experiment.
[c] ND, not done.

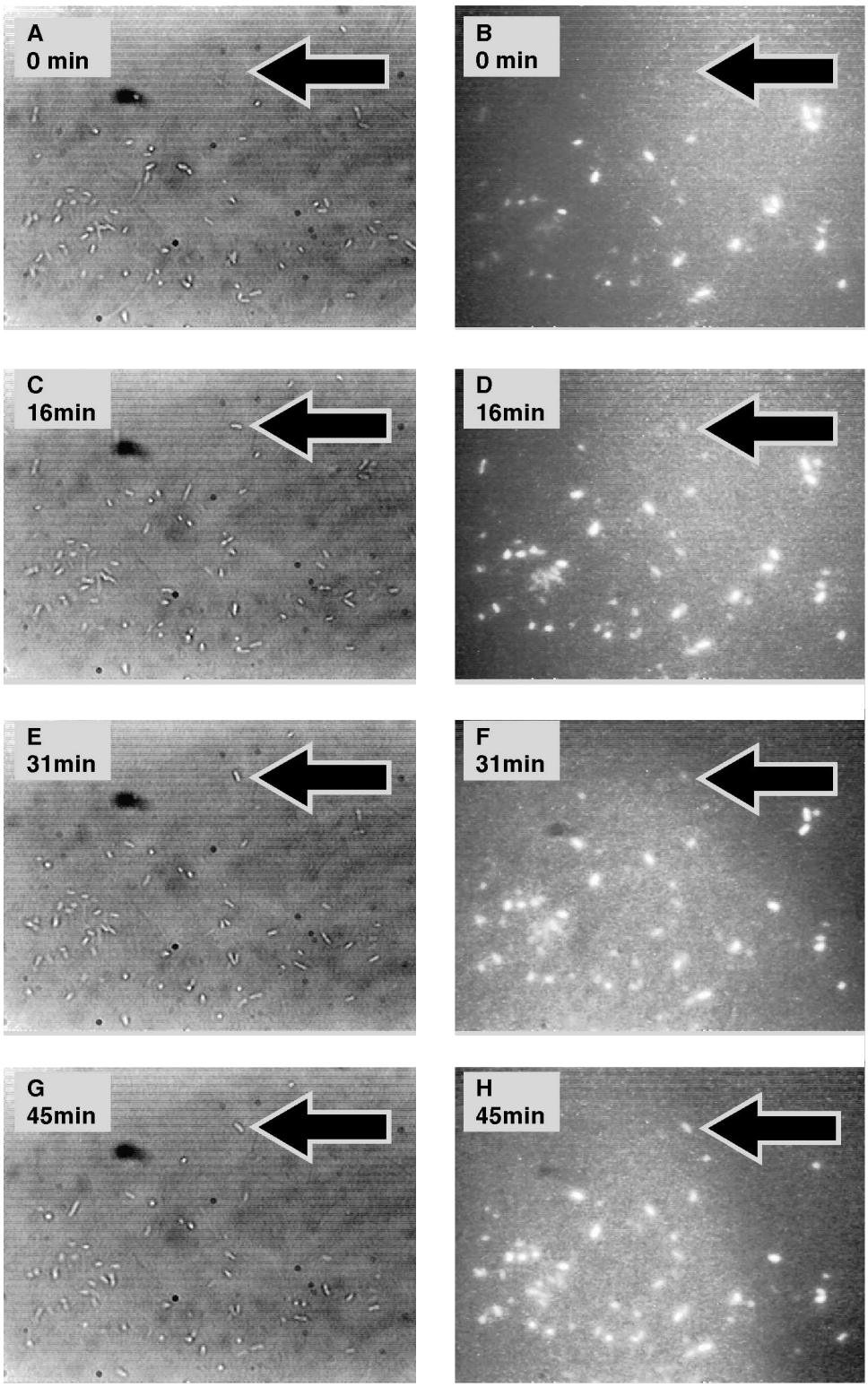

FIG. 4. Photomicrograph of *P. aeruginosa* 8830 cells attached to a glass substratum. Magnification, ×1,025. Phase-contrast image of total cells (left) and fluorescent cells displaying β-galactosidase activity (right). The arrow denotes a cell attaching to the surface, with subsequent *algC* reporter gene up-expression (see text).

in these experiments was not toxic to the cells, an experiment was run to evaluate the influence of methylumbelliferyl-β-D-galactoside on cell growth. Results from the experiment showed no significant difference between the growth curves of cells recovered from flasks containing the fluorogenic compound and those recovered from flasks without the substrate (data not shown).

Figure 4 is a series of photomicrographs taken via image

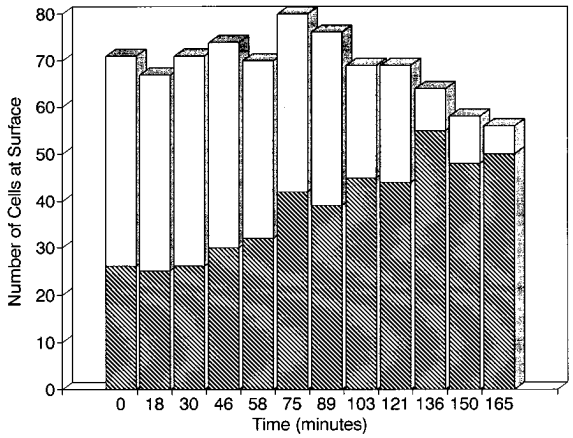

FIG. 5. Bacteria attached to the inner surface of the glass coverslip in a flow cell culture chamber. Hatched bars represent fluorescent cells; open bars represent nonfluorescent cells.

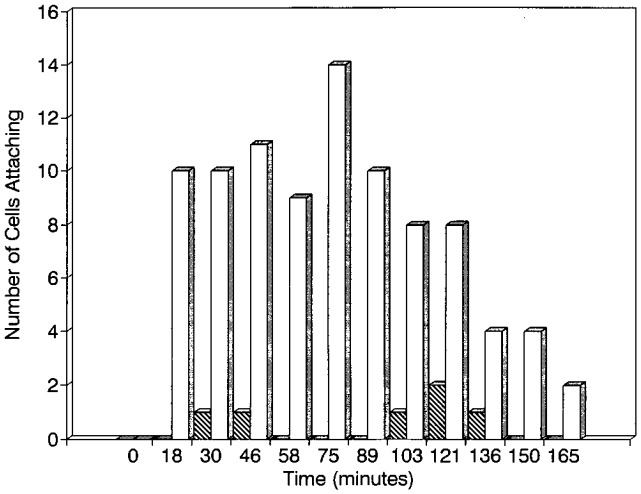

FIG. 6. Attachment of bacteria to the inner surface of the glass coverslip in a flow cell culture chamber. Each time point on the abscissa represents bacteria which have attached to the substratum since the previous time point. Hatched bars represent fluorescent cells; open bars represent nonfluorescent cells.

analysis during the continuous-culture microscopy experiment which tracks the attachment and subsequent up-expression of *algC* in a bacterial cell. At $t = 0$, some bacteria have already attached to the surface (Fig. 4A), and a fraction of these demonstrate *algC* up-expression as indicated by whole-cell fluorescence (Fig. 4B). After 16 min, a new bacterium has attached to a previously uncolonized area of the substratum (Fig. 4C, arrow). This bacterium does not demonstrate fluorescence at $t = 16$ min (Fig. 4D, arrow) or at $t = 31$ min (Fig. 4E and F, arrow). At $t = 45$ min, the bacterium is still present at its attachment site (Fig. 4G, arrow), and has begun to fluoresce (Fig. 4H, arrow).

Combining the approach described above with image analysis, three distinct populations of bacteria were monitored over a period of 165 min. These were (i) bacteria that were already attached to the coverslip in the previous observation period, (ii) bacteria that had attached to the coverslip since the previous observation period, and (iii) bacteria that had detached from the coverslip since the previous observation period. Figure 5 shows the total bacteria attached to the surface of the glass coverslip recorded at roughly 15-min intervals. At the beginning of observation, 63% of cells already attached to the surface were nonfluorescent. As the experiment progressed, the number of nonfluorescent cells at the substratum decreased from 45 to 6 while the number of fluorescent bacteria at the substratum increased from 26 to 50. At the end of the observation period, 89% of the cells at the surface were shown to be fluorescent.

Figure 6 depicts numbers of bacteria from the bulk aqueous phase that had attached to the coverslip at roughly 15-min intervals over the 165-min observation period. Each time point indicates the number of fluorescent or nonfluorescent bacteria that have attached to some location within the field of view since the previous observation period. As can be seen, most cells (over 93%) were not fluorescent at the time of attachment to the substratum. Of the cells that had attached to the substratum, many eventually detached and entered the bulk phase. Of the total that detached from the substratum, over 70% were nonfluorescent, indicating that they were not actively expressing the *algC* gene.

Figure 7 compares the fluorescent and nonfluorescent bacteria which had detached from the substratum since the previous observation period as a percentage, respectively, of the total fluorescent or nonfluorescent population present at the surface. Bacteria that were not up-expressed for *algC* transcription (the nonfluorescent population) showed a higher propensity to detach from the surface than did the bacteria which were up-expressed for *algC* transcription (the fluorescent population).

Finally, Fig. 8 shows bacteria attached to the substratum which had switched from nonfluorescent to fluorescent and vice versa, demonstrating that these cells had changed regulation of *algC* transcription while attached to the substratum. The majority of attached cells switched from nonfluorescent to fluorescent, with only a small fraction losing fluorescence during the time they were attached to the substratum.

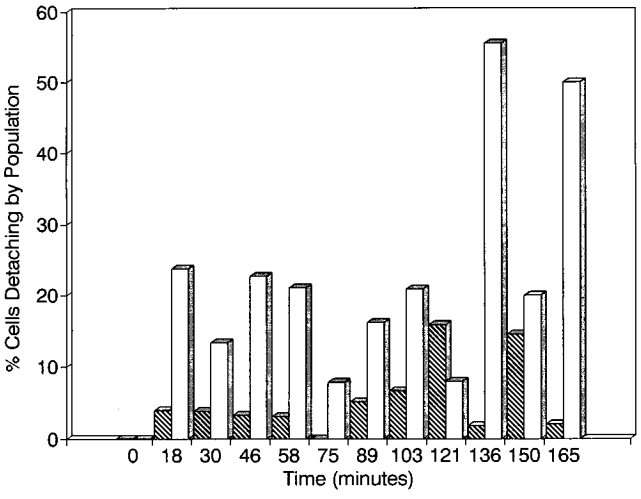

FIG. 7. Bacteria detaching from the inner surface of the glass coverslip in a flow cell culture chamber. Each time point on the abscissa represents the percentage of attached fluorescent or nonfluorescent bacteria that have detached from the substratum and entered the bulk liquid since the previous time point. Hatched bars represent fluorescent cells; open bars represent nonfluorescent cells.

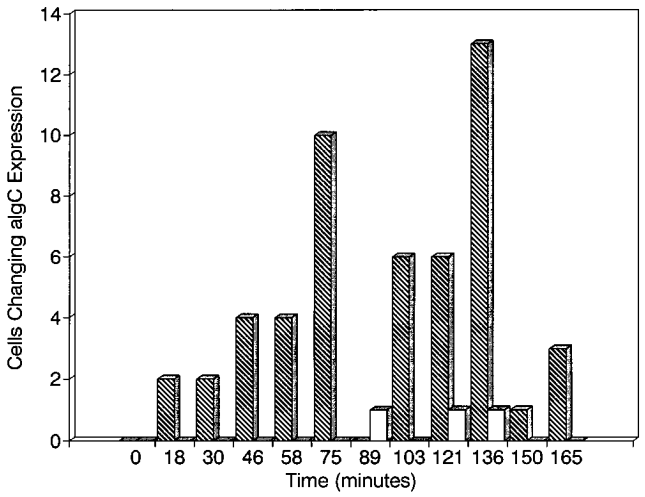

FIG. 8. Change in regulation of *algC* activity by attached bacteria. Hatched bars represent attached nonfluorescent cells that have become fluorescent; open bars represent attached fluorescent cells that have become nonfluorescent.

DISCUSSION

In the current study, we have found that the transcriptional fusion plasmid pNZ63, which carries the reporter construct for *algC* promoter activity, was not lost from *P. aeruginosa* 8830 during growth in batch and continuous culture in the absence of antibiotic pressure. This result was important, because it provided assurance that differences in expression of the plasmid-borne reporter would not be due to the influence of changing copy number in the biofilm population. Differences in antibiotic resistance which were observed between bacteria recovered on antibiotic-containing medium and nonselective media (Fig. 2) could all be accounted for by injury detected by replica plating. It may be argued that all or part of the plasmid had integrated into the host chromosome during the course of our experiments. If this were the case, the integration would have to occur throughout the population and in four separate experiments. We believe this to be an unlikely possibility. Our studies have shown, however, that if integration had occurred, both the antibiotic resistance marker and the *algC-lacZ* transcriptional fusion remained intact and functional. Differences in β-lactamase activity were observed between the biofilm and planktonic cell populations. The physiological basis of these differences is not known.

Using a continuous-culture apparatus, we demonstrated that the specific activity of the *algC* reporter gene product in biofilm bacteria was more than 19 times higher than in planktonic bacteria. This result was consistent with previous studies in our laboratory in which we demonstrated higher levels of *algC* expression in biofilm bacteria grown in batch culture than in planktonic cells grown in batch culture (13). Accumulation of uronic acids was also higher in the continuous-culture biofilm than in planktonic bacteria, although the difference was only slightly more than twofold. The discrepancy between *algC* reporter activity and accumulation of uronic acids may reflect a difference in the end product resulting from phosphomannomutase activity. According to Goldberg et al. (18), *P. aeruginosa* PAO1 requires the enzyme encoded by *algC* for the synthesis of LPS O side chains. The up-expression of *algC* may therefore reflect the production of either LPS or alginate. Since mannose residues within LPS would not be detected by the uronic acids assay performed in this study, LPS production may explain the difference observed between *algC* up-expression and accumulation of uronic acids. Experiments are in progress to determine the fraction of *algC* activity that is dedicated to LPS synthesis during biofilm development. Other factors may affect the relationship between the level of reporter gene expression and accumulation of the end product of *algC* transcription. Accumulation of uronic acids and *lacZ* (reporter) enzyme activity do not necessarily have to vary proportionally to one another. The differences observed between *algC* activity and accumulation of uronic acids may result from differences in rates of formation and activity of enzymes required for processing of mannose 1-phosphate into the end product, alginate. Furthermore, exopolymers such as alginate may be shed into the bulk liquid by the biofilm growing in continuous culture, thus, reducing the net accumulation of uronic acids within the biofilm.

The population of bacteria shed from the biofilm into the effluent of the continuous-culture vessel was shown to have a β-galactosidase specific activity that was intermediate between those of the biofilm population and the planktonic cell population. This result indicates that as bacteria are shed from the biofilm, the *algC* gene is down-regulated. The level of down-regulation is expected to continue until the level observed for planktonic bacteria is achieved. It should be noted that it is also possible that the bacteria shed from the biofilm into the effluent were a subpopulation of biofilm cells which had a lower level of *algC* activity than the average of the total biofilm population. Such a population may be more likely to detach from the biofilm as a result of diminished levels of alginate biosynthesis, as was observed during our microscopic analysis of *algC* activity.

In this study, we have introduced a novel approach to detect and record in situ gene activity within single bacterial cells attached to a surface. With this approach, we are, to the best of our knowledge, the first to report in vivo regulation of a specific bacterial gene at the level of the single cell. Results from these studies indicated that the glass substratum promoted up-expression of *algC* in these bacteria. As biofilms developed, *algC* expression was correlated with the ability of bacteria to remain at the glass substratum. Furthermore, *algC* up-expression was not shown to be a prerequisite to surface attachment, indicating that alginate biosynthesis is not necessary for attachment to the glass surface. However, down-expression of *algC* appears to be associated with subsequent detachment of the bacteria from the surface.

The microscopic analyses of *algC* expression showed that substratum-associated cells can lose fluorescence after a period (usually not less than 30 min), although the β-galactoside substrate is not limiting. Furthermore, some of these cells that had lost fluorescence were shown to regain fluorescence after some period. These observations indicated that loss of fluorescence was not due to irreversible inactivation of the cells. Although a change in permeability to the substrate may account for the above phenomenon, it is more likely to be due to a loss of β-galactosidase activity and hence to reduced expression of the *algC* gene within the cells. Such a conclusion is consistent with the time frame involved in the switch from a fluorescent cell to a nonfluorescent one. Furthermore, we observed that when cells lost their fluorescence, they did so entirely. If the shutdown of a transport mechanism were responsible, some leakiness would be expected, resulting in diminished fluorescence.

The mechanism of the regulation reported above is not known at present. It is possible that activation of *algC* transcription is affected by a member of the homoserine lactone family of autoinducers. The *P. aeruginosa* gene products LasI and LasR are known to be activators of extracellular virulence

factors regulating the production of proteases and exotoxins (16, 25, 26). These compounds are involved in bacterial quorum sensing and are known to cause induction of gene expression only in situations of relatively high population density. Although alginate is an extracellular virulence factor of *P. aeruginosa*, we do not find evidence for quorum sensing in the microscopic analyses we have performed with this organism. Bacteria which were up-expressed for *algC* activity at the surface of the glass coverslip during our experiments were often found juxtaposed to bacteria which showed no detectable *algC* activity. Furthermore, activation of *algC* when observed at the single-cell level was never observed to occur as a group or population phenomenon.

Sensory transduction involving some surface characteristic is possibly responsible for the up-expression of the gene shortly after interaction with the substratum. If this is the case, both glass and Teflon surfaces (or the conditioning films that adhere to those surfaces in liquid medium) promote similar outcomes with respect to regulation. Activation of genes in bacteria following association with different surfaces has been described by a number of authors in recent years. Dagostino et al. (12) reported activation of an unknown gene in *Pseudomonas* sp. strain S9 on polystyrene, Belas et al. (4) demonstrated activation of the *laf* gene in *Vibrio parahaemolyticus* when grown on agar medium, Vandevivere and Kirchman (28) demonstrated surface activation of exopolysaccharide biosynthesis by a subsurface bacterium, and in our laboratory, we have reported the activation of the *algC* gene in *P. aeruginosa* after attachment to Teflon and glass (13). In this paper, we have demonstrated changes in the expression of *algC* in *P. aeruginosa* following attachment to a substratum in continuous culture.

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