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Production of Mucoid Exopolysaccharide during Development of *Pseudomonas aeruginosa* Biofilms

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Production of mucoid exopolysaccharide by planktonic, chemostat-derived, and adherent *Pseudomonas aeruginosa* 579 bacteria was separately monitored for 7 days by using a *lacZ-algD* promoter-reporter gene and assays of total carbohydrate and metabolic activity. Mucoid exopolysaccharide production was transiently elevated following adherence but declined to planktonic levels by day 7.

Formation of *Pseudomonas aeruginosa* biofilms is expedited by the elaboration of a mucoid exopolysaccharide (MEP) (6) similar to the alginate of marine algae and *Azotobacter vinelandii* (25). Within the biofilm, *P. aeruginosa* is shielded from antibacterial compounds compared with planktonic (floating) *P. aeruginosa* (1, 2, 4, 13, 23). An intact biofilm, with its cocooning MEP, must be essential for the resistance, since *P. aeruginosa* bacteria which have been dispersed from the MEP are as susceptible to aminoglycoside and β-lactam antibiotics as their planktonic counterparts (12, 13). MEP production before and following adherence has not been reported. Such a study is relevant in light of the reported increased temporal resistance of adherent *P. aeruginosa* to tobramycin and piperacillin (1, 2).

We have investigated the production of MEP by planktonic and adherent *P. aeruginosa* bacteria. The influences of planktonic growth rate, adherence to silicone rubber, and NaCl concentration (which is both physiologically relevant in cystic fibrosis and which has been shown to repress MEP production in agar-grown *P. aeruginosa*) on MEP production were explored. The study was designed so that planktonic bacteria growing in a chemostat at defined relatively low or high dilution rates (*D*s) were used to seed the silicone rubber surfaces positioned in a flowthrough device. The adherent bacteria were subsequently exposed to only the growth medium permitting an unambiguous assessment of the development of the biofilms with no inference from incoming planktonic bacteria. Other studies in which colonized surfaces were positioned inside the chemostat (1, 2) or which have maintained the connection between the chemostat and the flowthrough device (16, 27) have not been able to distinguish the planktonic and adherent populations during biofilm development.

P. aeruginosa 579 (*leu38 muc23* $FP2^+$ [10, 11]) containing an *algD* promoter-*lacZ* fusion (see below) was grown at ambient temperature (22 to 25°C) in a defined liquid medium (pH 7.2) containing NaCl (0 or 0.3 M), Na_2HPO_4 (10.0 mM), $(NH_4)_2SO_4$ (7.6 mM), KH_2PO_4 (4.8 mM), monosodium L-glutamate (2.0 mM), $MgSO_4 \cdot 7H_2O$ (1.7 mM), L-leucine (1.0 mM), $FeSO_4 \cdot 7H_2O$ (6.6 μM), and gentamicin sulfate (Sigma Chemical Co., St. Louis, Mo.) (50 $\mu g \cdot ml^{-1}$). By monitoring batch culture growth at 540 nm and using spread plate viable counts (23), 2.0 mM L-glutamate was found to be growth limiting. Whether glutamate was carbon or nitrogen limiting was not settled.

Five hundred milliliters of the medium in a chemostat (Pegasus Industrial Specialties Ltd., Agincourt, Ontario, Canada), aerated by the flow (200 $ml \cdot min^{-1}$) of sterile air, was aseptically inoculated with 10 ml of a 12- to 15-h *P. aeruginosa* 579 batch culture. After 24 h, dilution of the culture by sterile medium was begun. Flow rates of 32 or 122 $ml \cdot h^{-1}$, established by using a Masterflex model 7524-00 microprocessor pump (Cole-Parmer Instruments, Chicago, Ill.), produced a *D* of 0.07 or 0.24 h^{-1}. Cultures were maintained for 3 days in order to allow the populations to achieve stable optical (540 nm) and viable cell densities. After 3 and 4 days, a modified Robbins device (MRD) (23) was connected directly to the chemostat outflow for 60 min. Planktonic bacteria exiting the chemostat flowed past 25 0.5-cm^2 discs of silicone rubber (Silastic; Dow Corning Corp., Midland, Mich.) positioned in series flush with the upper wall of the MRD lumen. After 60 min, the MRD was disconnected from the chemostat and was connected to a reservoir of identically composed medium (day 0). Flow of medium through the MRD and the chemostat was continued from day 0 to day 7. Two 1-ml aliquots of the planktonic suspension and two colonized surfaces were obtained on day 1 (24 to 26 h), day 4 (95 to 98 h), and day 7 (168 to 170 h) for each assay to be described except for the total carbohydrate assay, for which four planktonic and adherent populations were examined at each time point. All experiments were done twice.

β-Galactosidase (β-gal) activity, total carbohydrate, and metabolic activity (see below for each) were expressed per 10^6 viable bacteria, since only living bacteria would be capable of producing MEP. CFU per milliliter or square centimeter varied by less than 1 logarithm unit for each combination of *D* and NaCl concentration over the 7-day experimental period. Variation would not be expected for the planktonic (chemostat) populations. Our observations of the plateau in the viable counts of *P. aeruginosa* 579 on silicone rubber by 24 to 26 h (Fig. 1) are different from reports of a gradual increase (over a few days) in the adherent viable counts (1, 2, 16). *P. aeruginosa* 579 populations progress from an adherent monolayer to multicell-thick populations immobilized in exopolymer (20). As the biofilm thickens, the bacteria adjacent to the substratum may be nonviable while the cells at the periphery of the biofilm may be viable and capable of growth (6). Thus, it is conceivable

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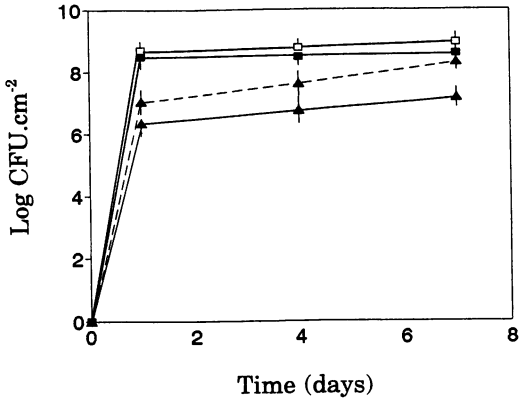

FIG. 1. Enumeration of viable bacteria in adherent *P. aeruginosa* 579 populations (log CFU per square centimeter) following the adhesion of planktonic bacteria grown at a *D* of 0.07 or 0.24 h^{-1}. CFU of two ultrasonically dispersed adherent populations were determined at each time point in two experiments by spread plating 100-μl aliquots of phosphate-buffered saline serial dilutions onto brain heart infusion agar. The data are means ± 2 standard errors (n = 4). Populations which developed following the adherence of planktonic bacteria cultured in the absence of NaCl at a *D* of 0.07 (-▲-) or 0.24 (-■-) h^{-1} or in the presence of 0.3 M NaCl at a *D* of 0.07 (-▲-) or 0.24 (-□-) h^{-1} were enumerated on days 0, 1, 4, and 7. The NaCl concentrations of the particular planktonic population were maintained for the adherent population.

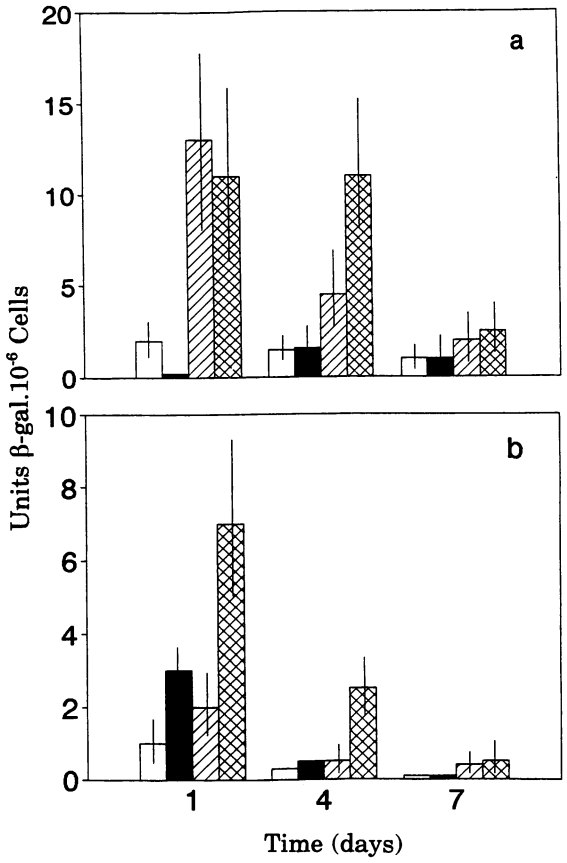

FIG. 2. Units of β-gal activity per 10^6 viable *P. aeruginosa* 579 cells at *D*s of 0.07 (a) and 0.24 (b) h^{-1}. The data are means ± 2 standard errors of four planktonic or adherent populations. Open bars, planktonic bacteria with 0.3 M NaCl; solid bars, planktonic bacteria without NaCl; hatched bars, adherent bacteria with 0.3 M NaCl; crosshatched bars, adherent bacteria without NaCl.

that while the spatial distribution of the living cells within the biofilm changed over time their number could remain constant.

MEP transcription was quantified (21) on days 1, 4, and 7 by using a fusion between *lacZ* (encoding β-gal) and the *algD* promoter. *algD* encodes GDP-mannose dehydrogenase (24), which catalyzes the irreversible conversion of GDP-mannose to GDP-mannuronate (24) in the MEP biosynthetic pathway. Transcription from the *algD* promoter, indicating the bacterial commitment towards MEP manufacture, would produce β-gal. To construct the fusion, the 9.2-kb *Hin*dIII fragment from the cosmid clone pCC27 (19) was cloned into pBluescript II SK in both orientations to give pSDF1 and pSDF4. The promoter region of *algD* was cloned by deleting the *Xho*I and *Eco*RI fragments from the pSDF4 construct, resulting in pSDF7. The *algD* promoter region was cloned from pSDF7 into the broad-host-range vectors pML6 and pML7 (19) as *Kpn*I-*Xba*I fragments to produce pSDF13 and pSDF15, which conferred gentamicin resistance to *P. aeruginosa* 579. Prior to transformation, the bacteria required a gentamicin MIC (17) of 1.25 μg · ml^{-1} (n = 6). pML6 and pML7 were chosen for the construction of the *algD* transcription fusion because it has been shown that the copy numbers of these plasmids are constant and stable in *Pseudomonas* species (19).

On days 1 and 4, the β-gal activity of 10^6 viable planktonic *P. aeruginosa* 579 bacteria cultured at a *D* of 0.07 h^{-1} with or without 0.3 M NaCl was significantly less (analysis of variance, $P < 0.05$) than the activity of 10^6 viable adherent bacteria from the corresponding populations developed in the medium with or without 0.3 M NaCl (Fig. 2a). By day 7, the planktonic and adherent bacterial β-gal activities were similar (Fig. 2a). Use of the lower *D* produced higher planktonic and adherent bacterial β-gal activities than those obtained when the planktonic bacteria were cultured at a *D* of 0.24 h^{-1} (Fig. 2b). Following adherence of the faster-growing planktonic bacteria, the NaCl-free adherent populations also displayed significantly elevated β-gal activities ($P < 0.05$) on days 1 and 4 compared with the corresponding planktonic populations (Fig. 2b). By day 7, the planktonic and adherent bacterial β-gal activities were similar (Fig. 2b). β-gal activity was absent in the negative controls (uninoculated medium with or without 0.3 M NaCl and 10^8 viable *Proteus mirabilis* ATCC 29906 bacteria · ml^{-1}). The influence of 0.3 M NaCl on β-gal (*algD*) activity was equivocal. When bacteria cultured at a D of 0.07 or 0.24 h^{-1} adhered to silicone rubber, the NaCl-free adherent populations displayed significantly raised ($P < 0.05$) β-gal activities on day 4 or on both day 1 and day 4, respectively, compared with those adherent populations which had been exposed to 0.3 M NaCl (Fig. 2). The presence of NaCl appeared to depress but not eliminate transcription from the *algD* promoter in the adherent populations. A previous study utilizing agar-grown *P. aeruginosa* 579 showed that *algD* activity was repressed by 0.3 M NaCl (8). The disparate observations concerning the influence of NaCl on *algD* activity may reflect an influence of the substratum on bacterial gene expression (7).

Decreased expression of β-gal by the adherent bacteria may have been due to the decreased production of MEP. If so, the total detectable carbohydrate would plateau. To assess this theory, an experiment in which 1-ml aliquots of

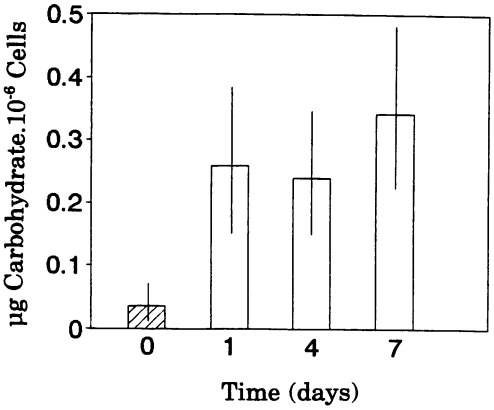

FIG. 3. Total *P. aeruginosa* 579 carbohydrate (in micrograms per 10^6 viable cells) of planktonic bacteria grown at a *D* of 0.07 h^{-1} without NaCl on days 1, 4, and 7 days after adherence. One-milliliter volumes of the planktonic suspension were collected from the chemostat immediately following the disconnection of the MRD from the chemostat (day 0). Adherent populations were recovered on days 1, 4, and 7. The data are means ± 2 standard errors (n = 8).

planktonic bacteria (D = 0.07 h^{-1}, no NaCl) were collected on day 0 was performed twice. Colonized surfaces were recovered on days 1, 4, and 7. Each sample was assayed for total carbohydrate (9) by using D-glucose (Sigma Chemical Co.; 0.55 to 551.88 mM) as a standard. Detectable carbohydrate was expressed in micrograms per 10^6 viable bacteria. One day following adhesion, the level of total carbohydrate of the adherent bacteria was significantly higher ($P < 0.05$) than the level displayed by the planktonic bacteria recovered on day 0 (Fig. 3). Thereafter, the total carbohydrate in the adherent populations plateaued (Fig. 3).

Concurrently with the β-gal assays, planktonic and adherent populations were assayed for the electron transport system (ETS)-mediated reduction of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) to INT-formazan, in order to quantify biomass production by the respiring bacteria (15). Planktonic bacteria were deposited by filtration onto a 12-mm-diameter, 0.22-µm-pore-size GF filter (Millipore Corp.). Nonadherent bacteria were removed by rinsing each surface with 3 1-ml volumes of 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.0) (Sigma Chemical Co.). The membranes and colonized surfaces recovered from the MRD were individually analyzed as described elsewhere (3), with the amount of INT-formazan formed in micrograms per 10^6 viable cells per hour determined against duplicate standard curves of INT-formazan (Sigma Chemical Co.; 0.212 to 21.218 µM). As negative controls, planktonic bacteria, intact biofilms, and suspensions of bacteria which had been ultrasonically dispersed (23) from biofilms (n = 3 for each) were exposed to 2.0% (vol/vol) formaldehyde for 30 min prior to the application of INT. To verify that the reduction of INT observed by using intact biofilms was maximal and was not restricted by diffusion limitations, intact and dispersed viable biofilms (n = 2 for each) were assayed.

ETS activity of adherent *P. aeruginosa* 579 populations (Fig. 4) was reminiscent of the temporal pattern of β-gal (*algD* promoter) activity. At a *D* of 0.07 h^{-1}, the absence of NaCl produced a rate of formazan production by the adherent bacteria which was significantly higher ($P < 0.05$) on days 1 and 4 than the rate in the presence of NaCl (Fig. 4a). These differences were not apparent when the planktonic

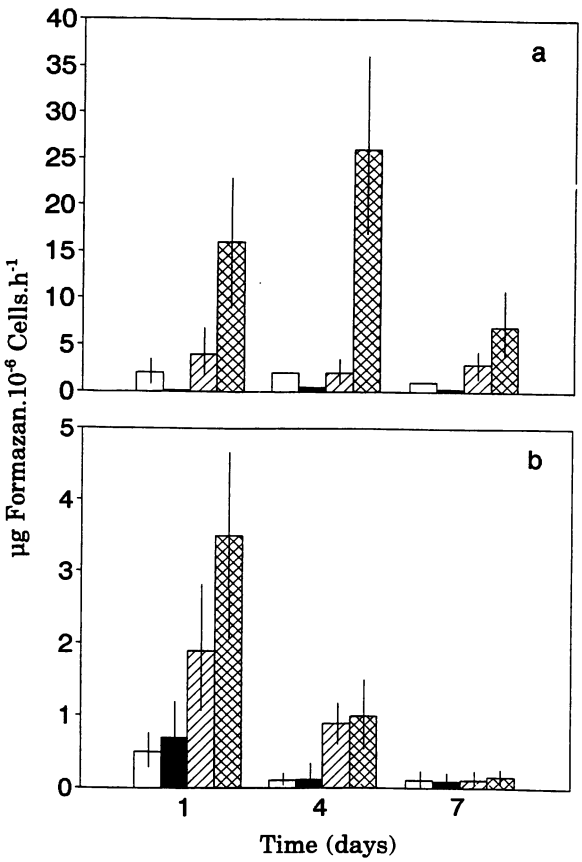

FIG. 4. ETS activities (in micrograms of formazan per 10^6 viable planktonic and adherent *P. aeruginosa* 579 bacteria per hour) at *D*s of 0.07 (a) and 0.24 (b) h^{-1} on days 1, 4, and 7. The assay was performed as described elsewhere (4) on intact biofilms or following the deposition of planktonic bacteria onto membranes. The data are means ± 2 standard errors for four planktonic or adherent bacterial populations. Open bars, planktonic bacteria with 0.3 M NaCl; solid bars, planktonic bacteria without NaCl; hatched bars, adherent bacteria with 0.3 M NaCl; crosshatched bars, adherent bacteria without NaCl.

bacteria which initially adhered to the substrata were grown at a *D* of 0.24 h^{-1} (Fig. 4b). ETS activity required viable bacteria since planktonic and sessile samples exposed to 2% (vol/vol) formaldehyde for 30 min did not reduce INT. The similar rates of INT reduction in two dispersed and two undispersed biofilms recovered on day 7 (data not shown) indicated that the values presented in Fig. 4 were maximal. Sampling within the first 24 h after adhesion will be required to determine whether the elevated sessile metabolic activity was caused by adhesion per se or by the energy demands necessary for the induction of the multienzyme MEP biosynthetic pathway (24) and the subsequent production of MEP. We favor the latter explanation because (i) the elevated ETS activity was prolonged (up to 4 days), inconsistent with a short-term stimulatory response caused by adhesion, and (ii) because the ETS activities of the planktonic bacteria grown at a *D* of 0.07 or 0.24 h^{-1} were similar.

The present observations offer strong evidence for the enhanced but transient production of MEP following the adherence of *P. aeruginosa* 579. Confirmation will be provided by the β-gal and carbohydrate analyses of samples

obtained from the same MRD during the same experiment and analyses using *P. aeruginosa* 579 containing a genomic *lacZ-algD* promoter fusion. The latter experiments are under way (26).

Production of MEP imposes a diffusion barrier on adherent *P. aeruginosa* (12, 14). The resultant altered environmental condition(s) within the biofilm would stimulate the phenotypically plastic (4, 6) adherent *P. aeruginosa* bacteria to adopt physiologies different from that of the bacteria within the defined environment of the chemostat. Adoption of a slower growth rate by the adherent bacteria may be one physiological change (4). As evidenced here, another physiological alteration may be the genotypic and phenotypic curtailing of MEP production. Studies which have concluded that adherent *P. aeruginosa* populations exhibit a temporally increasing resistance to tobramycin and piperacillin (1, 2) failed to separate the planktonic and adherent populations during exposure to the aminoglycoside and β-lactam antibiotics. Our intentional separation of these populations during biofilm development has implicated the biofilm in the temporally observed antibiotic resistance. Experiments in which the antibiotic susceptibility of adherent *P. aeruginosa* 579 populations will be assessed after 1, 4, and 7 days are planned.

Finally, our observations may be germane in light of the coordinate regulation of MEP and elastase production in *P. aeruginosa* cystic fibrosis isolates and strains, including strain 579 (22). In the lungs of cystic fibrosis patients, the MEP-enclosed *P. aeruginosa* populations could harbor bacteria actively producing the exopolymer while other bacteria, shielded from host-related and exogenous antibacterial compounds in an environment repressive for the production of MEP, might instead produce elastase. Establishment of a biofilm or mucoid aggregate could facilitate the persistence of *P. aeruginosa* and provide a protective focus for the action of tissue-destructive virulence factors.

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