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Stratified Growth in *Pseudomonas aeruginosa* Biofilms

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In this study, stratified patterns of protein synthesis and growth were demonstrated in *Pseudomonas aeruginosa* biofilms. Spatial patterns of protein synthetic activity inside biofilms were characterized by the use of two green fluorescent protein (GFP) reporter gene constructs. One construct carried an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *gfpmut2* gene encoding a stable GFP. The second construct carried a GFP derivative, *gfp*-AGA, encoding an unstable GFP under the control of the growth-rate-dependent $rrnBp_1$ promoter. Both GFP reporters indicated that active protein synthesis was restricted to a narrow band in the part of the biofilm adjacent to the source of oxygen. The zone of active GFP expression was approximately 60 μm wide in colony biofilms and 30 μm wide in flow cell biofilms. The region of the biofilm in which cells were capable of elongation was mapped by treating colony biofilms with carbenicillin, which blocks cell division, and then measuring individual cell lengths by transmission electron microscopy. Cell elongation was localized at the air interface of the biofilm. The heterogeneous anabolic patterns measured inside these biofilms were likely a result of oxygen limitation in the biofilm. Oxygen microelectrode measurements showed that oxygen only penetrated approximately 50 μm into the biofilm. *P. aeruginosa* was incapable of anaerobic growth in the medium used for this investigation. These results show that while mature *P. aeruginosa* biofilms contain active, growing cells, they can also harbor large numbers of cells that are inactive and not growing.

Biofilms are communities of microorganisms that are associated with surfaces. Cell clusters in biofilms are known to be characterized by gradients in the concentrations of oxygen, nutrients, and metabolic waste products. It is widely recognized that such chemical heterogeneity in microbial biofilms can lead to microorganisms in the biofilm exhibiting different rates of growth and metabolic activity. Even pure cultures of microorganisms growing in biofilms experience these gradients and may exist in a range of metabolic states. The variety of growth states that can be represented in a biofilm, even for a single species, surely contributes to the special ecology and antimicrobial tolerance manifested by biofilms. Given the fundamental significance of growth status, it is surprising that there have been few investigations in which the growth patterns in biofilms have been visualized.

Several studies have investigated spatial patterns of cellular activity inside biofilms, using approaches such as staining with nucleic acid dyes that differentially indicate DNA and RNA (17), hybridization to 16S rRNA with fluorescently tagged oligonucleotide probes (9), the induction of alkaline phosphatase followed by staining with a fluorogenic phosphatase substrate (8, 19), and green fluorescent protein (GFP) expression from a growth-rate-dependent promoter (11). These previous investigations have revealed gradients in metabolic activity in biofilms.

The purpose of the work reported here was to evaluate and apply fluorescent protein-based approaches for mapping spatial patterns of protein synthetic activity in *Pseudomonas aeruginosa* biofilms. We tested three different fluorescent protein-based approaches for visualizing patterns of activity in *P. aeruginosa* biofilms. The first used the *gfpmut2* gene controlled by the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible P_{trc} promoter. This led to the production of a stable GFP when the gene was induced. The second used a protease-sensitive GFP encoded by a *gfp* derivative under the control of a growth-rate-dependent promoter, $rrnBp_1$. The third used a fluorescent timer protein that turns from green to red as the protein matures. The activities reflected by these fluorescent protein constructs were determined by confocal scanning laser microscopy of two different biofilm systems.

MATERIALS AND METHODS

Bacterial strains and media. *P. aeruginosa* PAO1 and its derivatives were used for these studies. *P. aeruginosa* strain AH298 contains a chromosomally encoded gene for GFP under the control of the $rrnBp_1$ promoter. This strain was constructed as described elsewhere (10, 11), except that the kanamycin resistance marker was replaced with tellurite resistance (150 μg/ml) for the selection of transconjugants of *P. aeruginosa*. Because expression from the $rrnBp_1$ promoter is growth rate dependent (11) and because the expressed GFP is an unstable variant (*gfp*-AGA), this reporter has the effect of yielding bright green fluorescence in rapidly growing cells and dim or no fluorescence in slowly growing bacteria or bacteria that are not growing (11). Plasmid pAB1 carries *gfpmut2* under the control of the IPTG-inducible P_{trc} promoter (16). This GFP has enhanced fluorescence, as described by Cormack et al. (4). *P. aeruginosa* expressing *gfpmut2* has been shown to be brightly fluorescent (11). Plasmid pMF335 contains the gene for the timer fluorescent protein (14), a derivative of the *Discosoma* red fluorescent protein that changes from green to red fluorescence as the protein matures. This plasmid was constructed by ligating the pRO1614

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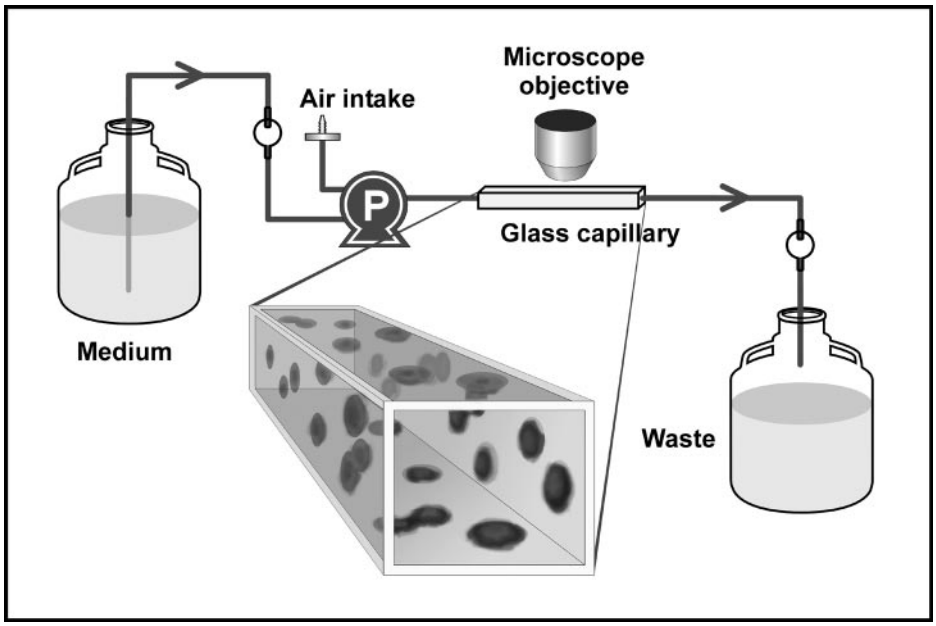

FIG. 1. Capillary reactor for biofilm growth. The growth medium was pumped continuously through a 1-mm-square glass capillary that was inoculated with *P. aeruginosa*.

origin of replication from plasmid pMF17 (6) to the unique EcoRI site of pTIMER (BD Biosciences Clontech, Palo Alto, Calif.). The pMF335 construct allowed the stable replication of pTIMER in *P. aeruginosa* and the expression of the fluorescent timer protein under the control of the P_{lac} promoter. Plasmids pMF335 and pAB1 were introduced into *P. aeruginosa* by triparental mating with the conjugation helper plasmid pRK2013 (5). Transconjugants were selected on *Pseudomonas* isolation agar (Difco) containing carbenicillin (300 μg/ml). Bacteria were grown in tryptic soy broth (TSB) or on tryptic soy agar (TSA) plates at 37°C. One-tenth strength TSB was used for flowthrough biofilm experiments.

GFP induction in planktonic cells. Overnight planktonic cultures of *P. aeruginosa* PAO1(pAB1) were grown with shaking in TSB supplemented with 150 μg of carbenicillin/ml. These cultures were diluted to an optical density (at 600 nm, with a 1-cm path length) of 0.040 in 150 ml of fresh TSB. The cultures were incubated at 37°C and sampled every 20 min throughout the duration of the experiment. At each time point, 3.6 ml of culture was sampled from the flask and pipetted in 200-μl aliquots into a black-sided clear-bottomed 96-well plate (Costar; Corning Incorporated, Corning, N.Y.). Fluorescence intensities were measured with a Bio-Tek FL600 microplate fluorescence reader, with excitation at 485 nm and emission at 530 nm. The fluorescence intensity values were averaged for 16 samples for each time point. After 2 h, the culture was divided into two equal volumes. The bacteria in one flask were induced with 1 mM IPTG, while those in the other flask served as a control without induction.

For tests of GFP induction in an anaerobic environment, nitrogen gas was bubbled through TSB in two anaerobic vials. The vials were sealed and autoclaved. Bacteria from aerated overnight cultures of *P. aeruginosa* PAO1(pAB1) in TSB with 150 μg of carbenicillin/ml were injected into the anaerobic TSB to achieve a final optical density of 0.040. The beginning culture volume in each vial was approximately 80 ml. The vials were sampled every 30 min for 2 h with sterile needles and syringes that were purged with nitrogen. GFP fluorescence intensities were determined for these samples as described above. After 2 h, IPTG was added to one vial to a final concentration of 1 mM. The same amount of sterile buffer was added to the control. Sampling continued as described above every 30 min for 4 h.

Colony biofilm preparation. Colony biofilms were grown on polycarbonate membranes resting on agar plates (1, 16). Planktonic cultures of *P. aeruginosa* were grown overnight with shaking in TSB and diluted to an optical density (at 600 nm, with a 1-cm path length) of 0.050 in TSB. Carbenicillin (150 μg/ml) was added to the broth used to grow the inoculum for the preparation of PAO1 with fluorescent timer protein, PAO1(pMF335), or PAO1(pAB1). The antibiotic was not included in the agar medium used to grow colony biofilms. One 5-μl drop of diluted planktonic culture was used to inoculate individual sterile, black, polycarbonate membrane filters (25-mm diameter, 0.2-μm pore size; Poretics Corp., Livermore, Calif.) resting on TSA. The membranes were sterilized by UV exposure (15 min per side) prior to inoculation. The plates were inverted and incubated at 37°C for 48 h, and the membrane-supported biofilms were transferred to fresh culture medium once after 24 h. For the strain with the IPTG-inducible GFP, the biofilms were transferred to agar medium containing 1 mM IPTG for an additional 4 h. To confirm the presence of bacteria in the uninduced region of the PAO1(pAB1) colony biofilms, we used a rhodamine counterstain. Rhodamine B (5 μg/ml) was added to the TSA plates used for the final 4 h of incubation to counterstain bacteria that did not express GFP.

For anaerobic control experiments, TSA plates were incubated in anaerobic bags with sealing bars (BBL GasPak system; Becton Dickinson Microbiology, Franklin Lakes, N.J.) for 24 h prior to membrane inoculation. Colony biofilms were prepared as described above except that immediately after membrane inoculation, the plates were again placed inside anaerobic bags for incubation at 37°C. At the time of sampling, only the plate being sampled was removed from its anaerobic bag.

Freeze sectioning and microscopy. Colony biofilms were embedded in a histological cryoembedding medium (Tissue-TEK O.C.T. compound; VWR Scientific Products, Willard, Ohio), and dry ice was used to solidify the embedding medium. Membrane colony biofilms were placed on a flat piece of flexible metal, and the colonies were covered with embedding medium. The metal was placed on dry ice and the embedding medium was allowed to freeze. Membranes, colonies, and the embedding medium were detached from the metal sheet, and the edges of the membranes were trimmed. The colonies were then placed directly on dry ice, with the embedded side down and the exposed membrane side up. Embedding medium was used to cover the exposed membranes, which were then frozen.

Embedded colonies were sectioned into 5-μm-thick cross sections by use of a Leica CM1800 refrigerated microtome. The sections were placed on glass slides and then examined by confocal scanning laser microscopy.

Capillary biofilm system. Biofilms were grown in glass capillary tubes under continuous flow conditions (Fig. 1). The glass tubes had square cross sections, allowing direct microscopic observation of the biofilms growing on the inside of the tubes through the flat tube walls. The capillaries were mounted in a flow cell holder to reduce breakage. The capillaries had a nominal inside dimension of 900 μm and a wall thickness of 170 ± 10 μm (Friedrich & Dimmock, Millville, N.J.). The flow cell apparatus consisted of a vented medium feed carboy (4-liter capacity), a flow break, a filtered air entry, a peristaltic pump, the capillary and flow cell holder, an inoculation port, and a waste carboy. These components were connected by silicone rubber tubing. We have found that the development of biofilm cell clusters of *P. aeruginosa* is enhanced by pumping air through the capillary along with the liquid medium. Therefore, the system also contained a T

connector just upstream of the glass capillary to allow mixing of the air and medium flows. Medium and system components were sterilized separately by autoclaving and then connected after cooling in a biological hood.

The capillary flow system was inoculated with 2 ml of an overnight culture of *P. aeruginosa* with an optical density at 600 nm of 0.001 to 0.005. For inoculation, the flow was stopped and the tubing was clamped downstream of the inoculation port. The inoculum was injected via the port to fill the glass capillary. The tubing upstream of the glass tube was clamped, and the system was allowed to stand without flow for 24 h. After this time, the flow of medium (1/10-strength TSB) was initiated at a flow rate of 20 ml h^{-1}. Air was pumped through the capillary at the same flow rate as the medium by use of a parallel tube in the same peristaltic pump. This resulted in slug flow of the medium and air bubbles through the capillary tube. Biofilms were counterstained by injecting a solution of rhodamine B at 50 mg $liter^{-1}$ into the capillary. Biofilms were observed by scanning confocal laser microscopy after 24 h of continuous flow at 37°C.

Microscopy and image analysis. Confocal scanning laser microscopy was performed with a Leica TCS NT confocal scanning laser microscope, with excitation at 488 and 568 nm and with emission collected at 500 to 530 nm (green channel) and 585 to 615 nm (red channel). Microscope images were analyzed by use of the line-scan function of MetaMorph image analysis software (Universal Imaging Co., Downingtown, Pa.). For colony biofilm experiments, the relative GFP intensities were measured for at least three independently grown biofilms and for three independent transects across each biofilm. Depth profiles of GFP intensity were measured perpendicular to the membrane supporting the colony biofilm. The interface between the biofilm and the membrane was set at zero on this spatial axis. Intensity profiles were used to measure the biofilm thickness and the dimensions of the green fluorescent band of active growth produced by GFP from strains PAO1(pAB1) and AH298. The biofilm thickness was measured as the distance from the membrane to the biofilm-air interface.

Oxygen penetration. Oxygen concentration profiles in colony biofilms were measured with a dissolved oxygen microelectrode. The oxygen microelectrode was based on the principle of the common amperometric Clark oxygen electrode. It consisted of an outer casing sealed at the sensor tip with an oxygen-permeable silicone membrane. The casing was fabricated from a Pasteur pipette that was tapered down to an active sensor tip of 15 μm. A buffered electrolyte solution consisting of 0.3 M K_2CO_3, 0.2 M $KHCO_3$, and 1 M KCl filled the internal cavity. Three electrodes occupied the internal cavity as well: they included a gold-tipped, glass-encased platinum cathode at which oxygen diffusing in through the silicone membrane was reduced; a silver-silver chloride counterelectrode, which served as the current return; and a guard electrode, which reduced the amount of unwanted oxygen entering from the back of the electrode. A potential of −0.8 volts of direct current was applied between the cathode and the counterelectrode. The current from the cathode, which is proportional to the concentration of oxygen in the bulk external solution, was measured with a picoammeter in the range of 0 to 3 nA. The same potential was applied between the guard electrode and the counterelectrode to reduce the background signal while measuring low concentrations of oxygen. The electrode was calibrated in air, and a zero level was obtained by placing the electrode tip in a 0.5% agar gel containing a suspension (0.2%) of ferrous sulfide (3). Oxygen concentration profiles were obtained by lowering the oxygen microelectrode into the biofilm by use of a computer-controlled stepping motor (16).

Transmission electron microscopy. Colony biofilms were grown for 48 h on TSA and then exposed to 150 μg of carbenicillin/ml for 12 h by the transfer of biofilms to TSA plates supplemented with the antibiotic. An untreated control was grown for 60 h on TSA. These specimens were fixed overnight by moving the colony biofilm to a filter pad soaked in 5% glutaraldehyde. The biofilms were postfixed in 2% osmium tetroxide, dehydrated in an ethanol series, and treated with propylene oxide. Colonies were embedded in Spurr's epoxy resin. Ultrathin sections were cut, placed on copper grids, and stained with uranyl acetate and Reynold's lead citrate. The sections were examined on an LEO 912 AB transmission electron microscope. Cell lengths were measured and displayed as a function of their relative positions in the biofilm. Cell lengths, measured along the major axis of the cell, were measured manually from enlarged prints.

RESULTS

Stratified pattern of GFP fluorescence in *P. aeruginosa* colony biofilms. *P. aeruginosa* formed colony biofilms that were approximately 150 to 300 μm thick after 48 h of development (Table 1). Cell numbers in colony biofilms were similar for strains PAO1, PAO1(pAB1), and AH298, with a mean viable areal cell density of approximately 2×10^9 CFU cm^{-2}. Strain PAO1(pAB1) carries a plasmid containing a *gfpmut2* gene, which encodes a stable GFP that is induced in the presence of IPTG. The addition of IPTG to an aerobic exponential-phase planktonic culture resulted in the development of green fluorescence in the culture, as determined by fluorimetry (Fig. 2A). No increase in fluorescence was observed in the absence of IPTG or in cells cultivated under anaerobic conditions (Fig. 2B). Optical density measurements conducted in parallel showed that *P. aeruginosa* grew under aerobic conditions, but not under anaerobic conditions, in TSB (Fig. 3). When *P. aeruginosa* PAO1(pAB1) was cultivated in colony biofilms under uninduced conditions, no green fluorescence was observed. Figure 4A shows an uninduced PAO1(pAB1) colony biofilm counterstained with rhodamine B. In contrast, bright green fluorescence located all along the air interface of the colony biofilm was visible in PAO1(pAB1) colony biofilms that were exposed to IPTG for 4 h (Fig. 4B).

TABLE 1. Biofilm thicknesses, active zone thicknesses, and oxygen penetration depths in *P. aeruginosa* colony biofilms[d]

Strain	Biofilm thickness[a] (μm)	Active zone thickness[b] (μm)	Oxygen penetration depth[c] (μm)
PAO1	209 ± 37	Not visible	50.6 ± 1.2
AH298	154 ± 23	63 ± 16	46.0 ± 5.3
PAO1(pMF335)	ND	ND	48.6 ± 7.3
PAO1(pAB1)	298 ± 60	60 ± 12	ND

[a] Thickness was determined from GFP-expressing frozen cross sections as the perpendicular distance from the membrane interface to the outermost fluorescent material.
[b] The active zone dimension was measured as the width at the peak half-height of the GFP fluorescence intensity in a frozen cross section.
[c] Oxygen penetration depth was determined as the distance into the biofilm at which the first derivative of the oxygen concentration reached 5% of its maximum value.
[d] Data are means ± standard deviations. ND, not determined.

Similar results were observed with *P. aeruginosa* AH298, which carries a *gfp* derivative encoding an unstable GFP under control of the growth-rate-dependent $rrnBp_1$ promoter. For this strain, rapidly growing cells are bright green, while slowly growing cells are dim or dark. In colony biofilms formed by strain AH298, green fluorescence was localized in a band adjacent to the air interface of the colony (Fig. 4C). No such distinct band could be discerned in biofilms formed by wild-type PAO1 (not shown).

We also attempted to visualize spatial patterns of activity by using a fluorescent timer protein construct. This strain carries a plasmid coding for a fluorescent protein that has been shown to change from green to red fluorescence in an oxygen-dependent maturation process as the protein ages. One possibility was that this construct would identify areas of new growth in green and areas of aged cells in red. Both green and red fluorescence was observed in colony biofilms, but the two colors were distributed uniformly throughout the depth of the biofilm (not shown).

The bands of GFP expression for strain PAO1(pAB1) and AH298 occupied about one-third to one-fourth of the colony thickness. The dimension of the fluorescent region averaged 60 ± 12 μm for PAO1(pAB1) and 63 ± 16 μm for AH298 (Table 1). The zone of active protein synthesis revealed by these

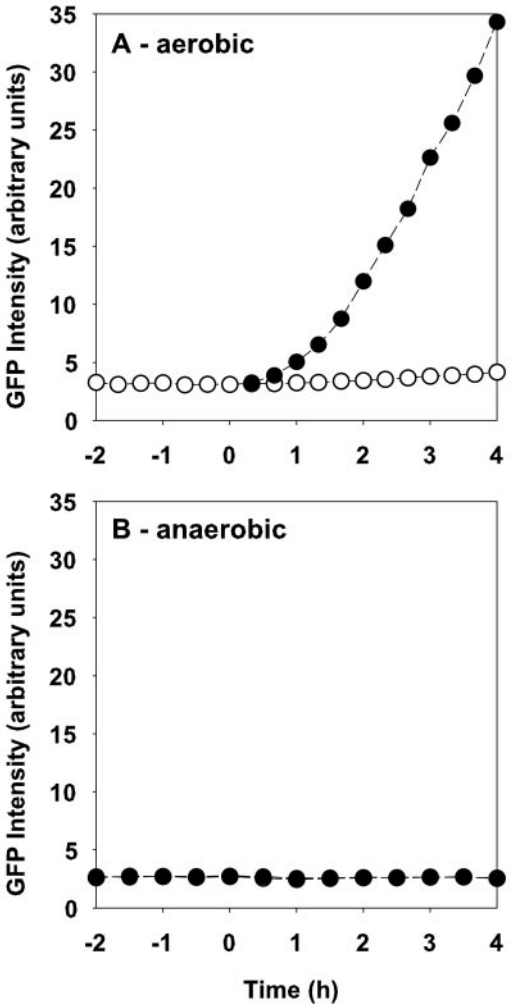

FIG. 2. Induction of GFP in planktonic *P. aeruginosa* cultures under aerobic (A) or anaerobic (B) conditions. Time zero on the *x* axis corresponds to the addition of 1 mM IPTG to the culture. ●, IPTG added; ○, negative control.

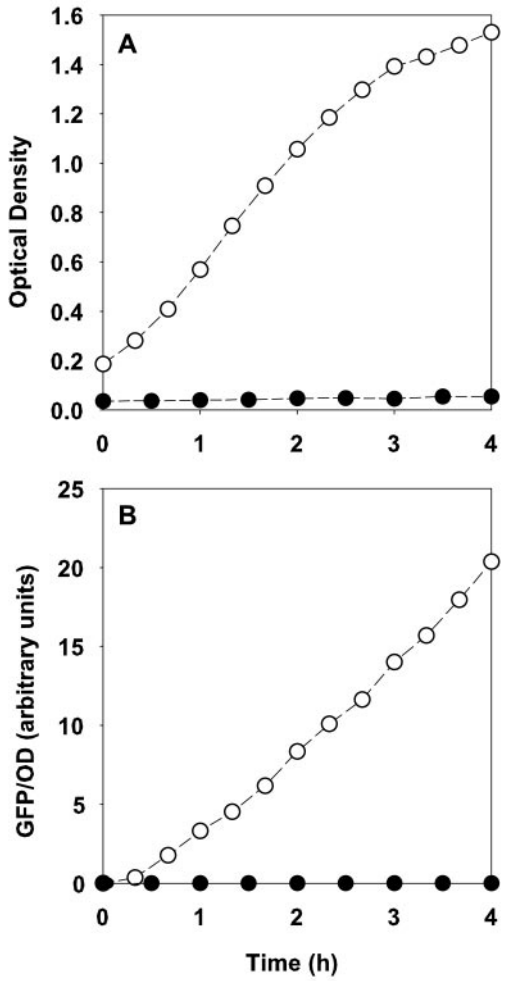

FIG. 3. Optical density (A) and specific GFP fluorescence (B) in planktonic *P. aeruginosa* cultures induced with IPTG under aerobic (○) and anaerobic (●) conditions. Time zero on the *x* axis corresponds to the addition of 1 mM IPTG to the culture. Specific GFP fluorescence was calculated as follows: (GFP fluorescence − initial fluorescence)/optical density (OD) at 600 nm.

constructs coincided with the aerobic layer of the biofilm, as determined by the use of oxygen microelectrodes (Fig. 5). Oxygen penetrated about 50 μm into the colony biofilms formed by any of the *P. aeruginosa* strains (Table 1). The measured depth of oxygen penetration was not statistically significantly different for PAO1, AH298, or PAO1(pMF335) colony biofilms ($P > 0.35$ by a two-tailed *t* test).

***P. aeruginosa* does not grow on TSA under anaerobic conditions.** GFP requires oxygen for the activation of fluorescence (15). In a biofilm that may contain anoxic zones, a lack of fluorescence could be due to an absence of protein synthetic activity, but it is also possible that the protein was made and simply did not mature to a fluorescent state because of the local absence of oxygen. To help discern these possibilities, we tested the growth of *P. aeruginosa* under anaerobic conditions without the addition of an alternative electron acceptor. No growth was detected in anaerobically incubated batch planktonic cultures (Fig. 3A). When *P. aeruginosa* was grown aerobically on TSA, the viable cell numbers in a colony biofilm increased during 48 h of development, from 8×10^4 to 5×10^9 CFU. When the strains were incubated anaerobically on TSA, the viable cell numbers increased very little, from 8×10^4 CFU at the time of inoculation to 1.3×10^5 CFU. No growth was visible when *P. aeruginosa* was streaked onto a TSA plate and incubated anaerobically, even after several days. These observations confirm that *P. aeruginosa* is not capable of anaerobic growth on the medium that we used.

Stratified pattern of cell elongation in antibiotic-treated colony biofilms. When *P. aeruginosa* is exposed to the antibiotic carbenicillin, cell division is blocked and growing cells form filaments. We used this behavior as another means to localize regions of growth in colony biofilms. Transmission electron microscopy of colony biofilms left untreated for 48 h showed short rods, which are characteristic of *P. aeruginosa* (Fig. 6, top row). The untreated biofilms also harbored significant numbers of lysed cells. Lysed cells predominated in the colony interior, with fewer lysed cells near the air interface. When 48-h colony biofilms were exposed to 150 μg of carbenicillin

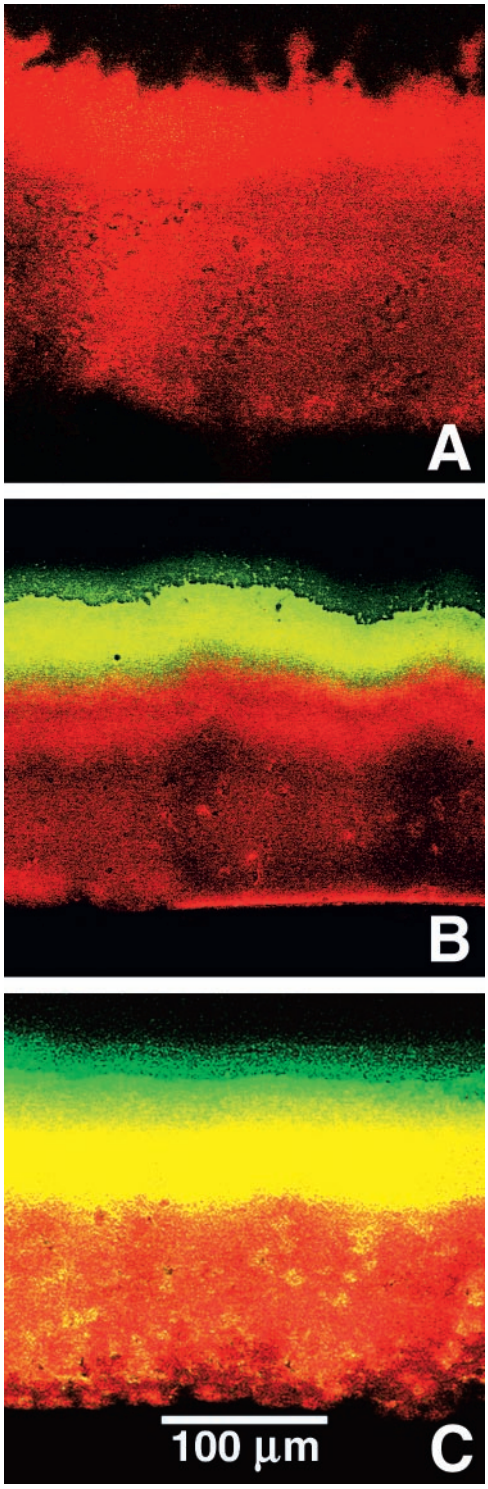

FIG. 4. Stratified patterns of GFP expression in frozen sections of *P. aeruginosa* colony biofilms. Green areas are due to GFP and red areas are due to the rhodamine B counterstain. Panel A shows a negative control in which a colony biofilm formed by strain PAO1(pAB1) was not induced. Panel B shows a biofilm of the same strain after 4 h of induction with IPTG. Panel C shows a colony biofilm formed by the reporter strain AH298.

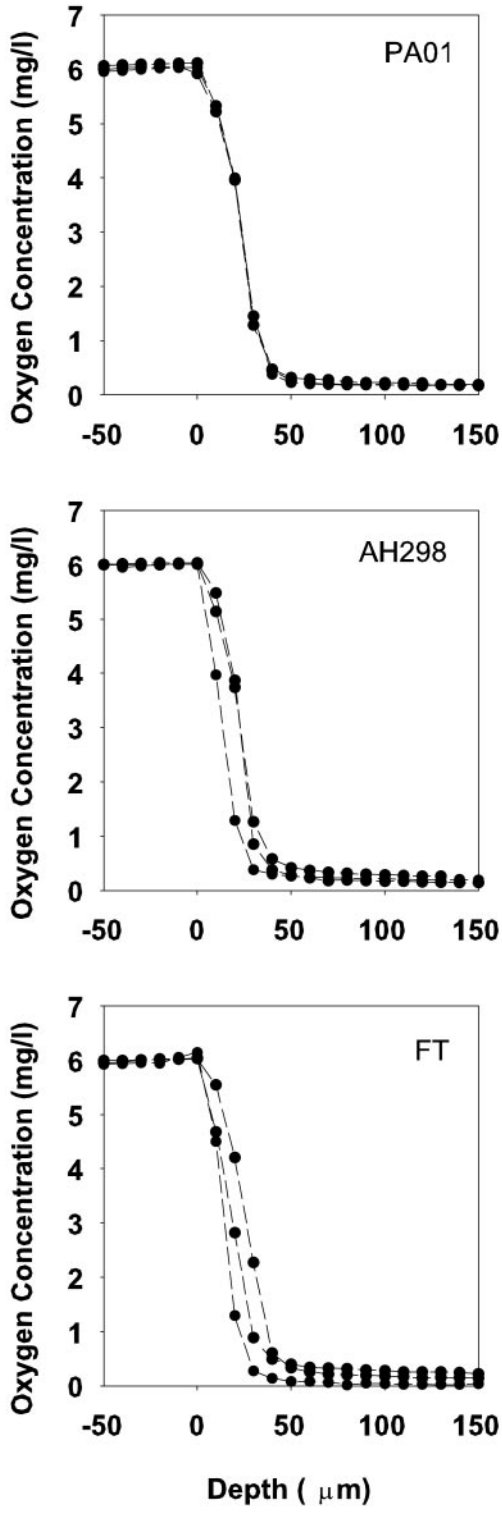

FIG. 5. Oxygen concentration profiles for *P. aeruginosa* colony biofilms. Triplicate data sets are shown for each strain. Depth zero on the *x* axis corresponds to the air-colony interface.

ml^{-1} for an additional 12 h, filamentous cells were evident, but only near the colony-air interface (Fig. 6, bottom left panel). Cell elongation was not observed for cells in the interior of the carbenicillin-treated biofilms or near the membrane (Fig. 6,

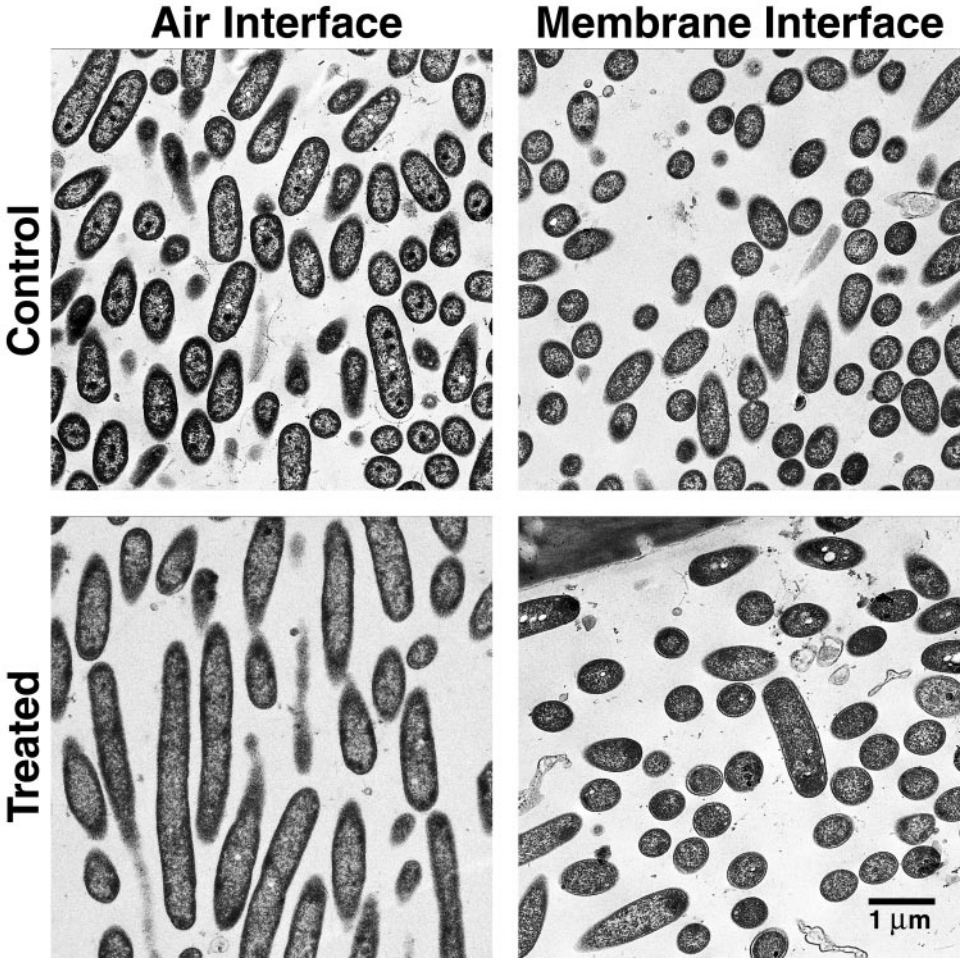

FIG. 6. Transmission electron microscopy of *P. aeruginosa* PAO1 in colony biofilms. Treated specimens were exposed to 150 μg of carbenicillin ml^{-1} in TSA for 12 h. The control remained on TSA for the same 12-h period.

bottom right panel). Filamentation was observed on the opposite interface of the colony from where the antibiotic was delivered, showing that carbenicillin penetrated the biofilm but only affected cells at the air interface. The distribution of elongated cells in the antibiotic-treated specimen and in the untreated control is shown in Fig. 7. Since cell elongation depends on growth, this result demonstrates that growth occurs in a layer of the biofilm near the air interface and suggests that there is no growth in strata of the biofilm that are more distant from the oxygen source.

Stratified pattern of GFP fluorescence in *P. aeruginosa* capillary tube biofilms. To determine whether the inducible GFP reporter described above could be applied in a noninvasive manner, we induced *P. aeruginosa* PAO1(pAB1) biofilms developed in glass capillary tubes with IPTG for 4 h and then examined them for fluorescence by using confocal scanning laser microscopy. These experiments differed from those with colony biofilms in that there was a continuous flow of medium and the biofilms did not have to be frozen and sectioned prior to examination. The same medium and temperature were used to grow biofilms in capillary tubes as were used for colony biofilms.

After 24 h of growth, large clusters of bacterial microcolo-

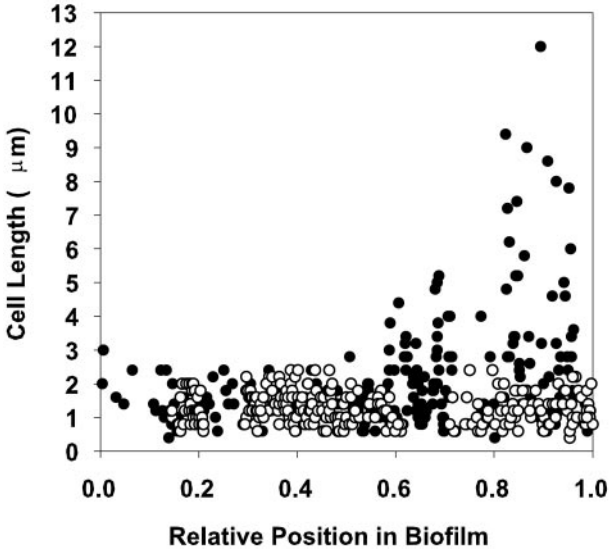

FIG. 7. Distribution of cell lengths within *P. aeruginosa* colony biofilms treated with carbenicillin (●) and in an untreated control (○). The distance scale on the *x* axis is the perpendicular distance of a cell from the membrane divided by the total thickness of the biofilm.

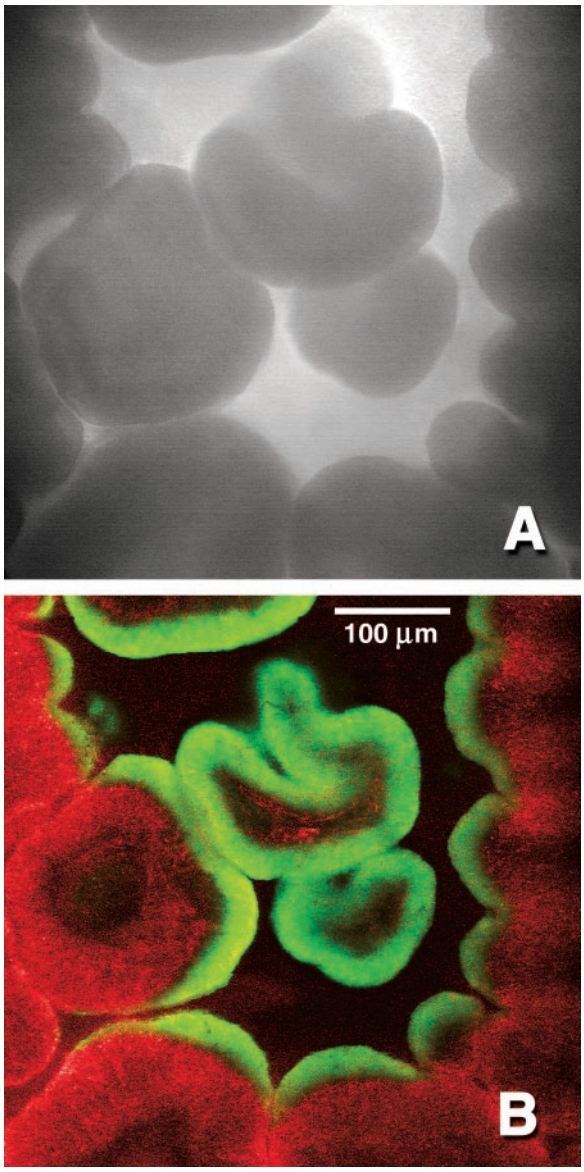

FIG. 8. Stratified pattern of GFP expression in *P. aeruginosa* biofilms grown in glass capillary tubes under continuous flow conditions. Strain PAO1(pAB1) was grown for 24 h and then induced with IPTG for 4 h. Panel A shows a laser transmission view, and panel B shows a fluorescence image of the same spot. Green areas are due to GFP and red areas are due to the rhodamine B counterstain.

nies were evident on the walls of the capillaries (Fig. 8A). The expression of GFP was limited to the surfaces of these clusters and was primarily evident in the vicinity of interstices (Fig. 8B). The dimension of the zone of GFP expression in this system was approximately 25 to 30 μm.

Attempts to map spatial patterns of growth in the presence of nitrate were not fruitful. We attempted to measure the patterns of GFP expression and cell elongation in *P. aeruginosa* colony biofilms growing on TSA supplemented with nitrate. One would anticipate that the addition of nitrate would lead to growth in anaerobic layers of the biofilm that were incapable of growth in the absence of nitrate in earlier experiments. But because GFP activation is oxygen dependent, we could also predict that this growth would not be visible, i.e., anaerobic synthesis of GFP would occur, but the GFP would not become fluorescent. When PAO1(pAB1) was grown on TSA supplemented with 1% potassium nitrate and induced with IPTG, GFP induction along the air interface of the colony was observed as usual. No additional expression of GFP was visible elsewhere in the colony. There was no indication of GFP expression along the agar interface of the colony, which was adjacent to the source of nitrate. We also attempted to localize growth in colony biofilms growing on TSA supplemented with nitrate by examining specimens of cell elongation after exposure to carbenicillin. No filamentous cells were seen in such samples, not even at the air interface of the biofilm. Since we know that viable cell numbers increase in colony biofilms grown on media containing nitrate, we concluded that nitrate interferes with the filamentation that is normally induced by carbenicillin treatment. In summary, attempts to map spatial patterns of growth in biofilms growing on a medium containing nitrate were not fruitful. Neither the inducible GFP nor the cell elongation techniques were able to provide information about the pattern of growth in *P. aeruginosa* colony biofilms grown in the presence of nitrate that could be unambiguously interpreted.

DISCUSSION

GFP-based reporter gene constructs in *P. aeruginosa* have revealed a stratified pattern of protein synthesis in biofilms formed by this microorganism. Protein synthesis occurred in a relatively narrow zone, of about 30 to 60 μm wide, at the interface between the biofilm and the source of oxygen. The remainder of the biofilm showed little or no protein synthetic activity. Qualitatively similar stratified activities were apparent in two biofilms with different structures, one of which was grown under static conditions and the other of which was grown in a continuous flow reactor. This lends confidence that the heterogeneous distribution of protein synthetic activity was not an artifact of a particular experimental system.

The zone of GFP fluorescence in induced colony biofilms overlapped the zone of cell filamentation in biofilms exposed to carbenicillin, with both activities being localized to the air interface of the biofilm. The elongation of filaments requires not only protein synthesis, but also the coordinated synthesis of the other macromolecular constituents of the cell. Filamentation can be taken as an indication that cells are actively growing, not just making proteins in the absence of other biomass synthetic activities.

The results obtained in this study are in general agreement with the few other studies in which the metabolically active zone of a biofilm has been measured quantitatively. Xu and coworkers (18, 19) reported zones of de novo protein synthesis in *P. aeruginosa* biofilms grown in drip-flow reactors, as indicated by the induction of alkaline phosphatase, of approximately 20 to 30 μm. Sternberg et al. detected layered growth in *Pseudomonas putida* biofilms (11). Wentland et al. (17) measured growing regions of 11 to 28 μm in *Klebsiella pneumoniae* biofilms by using a technique based on acridine orange staining. Stewart and Robertson (13) measured the zones of active protein synthesis in artificial *Escherichia coli* biofilms by using radioisotope labeling and autoradiography and found them to

be 4 to 30 μm, depending on the glucose concentration in the medium. In all of these examples, the active layer was located at the biofilm-nutrient source interface. The dimension of the zone of anabolic activity in a biofilm can be expected to depend on the microorganism, substrate concentrations in the water bathing the biofilm, and other environmental factors. These measurements collectively suggest that stratified metabolic activity, in which activity is limited to a region with a width measured in tens of micrometers, may be a common feature of bacterial biofilms.

The heterogeneous pattern of protein synthetic activity inside *P. aeruginosa* biofilms is likely a result of oxygen limitation in the biofilms. Dissolved oxygen microelectrode measurements showed that oxygen penetration is limited and that the dimension of the oxic zone is similar to the dimension of the zone of protein synthesis. We know that *P. aeruginosa* is not capable of growth without oxygen on the medium used for this study. The absence of oxygen, therefore, would be sufficient to explain the lack of protein synthetic activity in deeper regions of the biofilm. In the colony biofilm system in particular, the limitation of some other nutrient can clearly be ruled out. The zone of protein synthesis was located at the opposite interface of the biofilm from where nutrients, supplied by the agar medium, originated.

GFP-based approaches may reveal only aerobic growth since the maturation of fluorescence is thought to be oxygen dependent (15). Because *P. aeruginosa* does not grow on TSA in the absence of oxygen, there can be no anaerobic growth in the system used for this work, and therefore the aerobic growth visualized by GFP represents all of the bacterial growth. This interpretation is supported by the planktonic experiment results shown in Fig. 2B and 3B. If some anaerobic expression of GFP had occurred in these experiments, fluorescence would have been expected to develop after sampling of the cultures. The samples were placed into 96-well plates that were exposed to the atmosphere, which would have allowed for the maturation of GFP fluorescence had the protein been present.

Another potential artifact is the possibility that the inducing agent, IPTG, failed to fully penetrate the biofilm. The superficial zone of GFP induction could simply represent the fact that IPTG only accessed the surface layers of the biofilm. This explanation is not tenable in the colony biofilm system because the IPTG was delivered from the agar side of the biofilm but the GFP was expressed at the opposite interface of the biofilm. IPTG is not metabolized, and the time scale for its diffusive penetration can be estimated, by using calculations outlined by Stewart (12), to be approximately 7 min in a 300-μm-thick biofilm. This is much shorter than the 4-h induction period used in the experiments reported here. Experimental data and theoretical considerations both indicate that IPTG penetrated the biofilms adequately.

In some environments, including the lungs, nitrate and nitrite are present and could serve as alternative electron acceptors for the anaerobic growth of *P. aeruginosa* (7, 20). Although the addition of nitrate stimulates growth in the absence of oxygen, Borriello et al. reported that nitrate amendment reduces the efficacy of many antibiotics against *P. aeruginosa* in biofilms (2). The role of nitrates in altering the growth and physiology of *P. aeruginosa* in biofilms is an interesting topic for further research. Unfortunately, neither of the techniques used in the present study to characterize growth patterns in biofilms was able to provide unambiguous data regarding the distribution of protein synthetic activity and growth in biofilms growing in the presence of nitrate.

Growth status is a critical parameter for understanding the ecology and function of microorganisms in biofilms. It is obvious that the growth state of a microorganism is one of the key determinants of its ability to compete and persist. The growth phase is also known to modulate the production of virulence factors. The possibility that biofilms harbor slow-growing and nongrowing cells is one of the leading hypotheses to explain the reduced susceptibility of biofilms to antibiotics and other antimicrobial challenges. A recognition of the variety of growth states represented in a biofilm and the characterization of those states are critical to understanding these and other phenomena of biofilms. The ability to interpret the results of genomic and proteomic analyses applied to biofilm systems is currently hampered by the lack of information about growth patterns in the biofilm. These are just some of the areas of biofilm research that would be facilitated by the development of techniques for characterizing spatial patterns of activity inside biofilms. The fluorescent protein-based techniques described in this article represent one of many possible strategies for mapping microbial activities.

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