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Development and Dynamics of *Pseudomonas* sp. Biofilms

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***Pseudomonas* sp. strain B13 and *Pseudomonas putida* OUS82 were genetically tagged with the green fluorescent protein and the *Discosoma* sp. red fluorescent protein, and the development and dynamics occurring in flow chamber-grown two-colored monospecies or mixed-species biofilms were investigated by the use of confocal scanning laser microscopy. Separate red or green fluorescent microcolonies were formed initially, suggesting that the initial small microcolonies were formed simply by growth of substratum attached cells and not by cell aggregation. Red fluorescent microcolonies containing a few green fluorescent cells and green fluorescent microcolonies containing a few red fluorescent cells were frequently observed in both monospecies and two-species biofilms, suggesting that the bacteria moved between the microcolonies. Rapid movement of *P. putida* OUS82 bacteria inside microcolonies was observed before a transition from compact microcolonies to loose irregularly shaped protruding structures occurred. Experiments involving a nonflagellated *P. putida* OUS82 mutant suggested that the movements between and inside microcolonies were flagellum driven. The results are discussed in relation to the prevailing hypothesis that biofilm bacteria are in a physiological state different from planktonic bacteria.**

Surface adhesion of bacteria and subsequent cell binary fission and exopolymer production lead to the formation of bacterial biofilms (see reference 6 for a review). Application of confocal scanning laser microscopy (CSLM) has led to the suggestion that microcolonies are the basic structural units in sessile communities (4). It was found that biofilms are highly hydrated open structures containing a high fraction of exopolymers and large void spaces between the microcolonies (18). Mushroom-shaped microcolonies, separated by channels and voids, were observed in *Pseudomonas fluorescens* biofilms (15, 16). Investigations of multispecies biofilm communities on granular activated carbon in fluidized-bed reactors revealed that growth occurred as discrete microcolony structures separated by channel boundaries (19). Microbial biofilms in river water were shown to have a ridged structure, with microcolonies forming ridges parallel to the direction of flow (23).

Evidence is now emerging that motility may play a role in structure formation in biofilms. After the initial attachment to the substratum, *Pseudomonas aeruginosa* evidently moves on the substratum by means of twitching motility, and it has been suggested that the initial microcolonies are formed by aggregation of bacteria (25). Furthermore, it has been suggested that the initial microcolonies in *Vibrio cholerae* El Tor biofilms are formed via flagellar motility of the cells along the substratum (32). In addition, Wolfaardt et al. (34) and Nielsen et al. (24) observed structural changes in biofilms in response to changing environments, suggesting that established biofilms in some cases may display dynamic behavior. Since it appears that structures in both young and mature biofilms in some cases are formed by movement of bacteria we found it of interest to study the dynamics occurring in developing biofilms. For this purpose two model organisms, *Pseudomonas* sp. strain B13 and *Pseudomonas putida* OUS82, were tagged with the green fluorescent protein (Gfp) and the *Discosoma* sp. red fluorescent protein (DsRed), and the dynamics occurring in monospecies or two-species biofilms initiated with mixtures of green and red fluorescent cells were investigated by the use of CSLM.

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MATERIALS AND METHODS

Strains and media. *P. putida* OUS82 (13), *Pseudomonas* sp. strain B13 (11), and derivatives thereof (see below) were used in the experiments. Phylogenetic analysis using the sequence align editor in the ARB program (kindly provided by Oliver Strunk and Wolfgang Ludwig, Technical University of Munich, Munich, Germany) and 16S rRNA sequences retrieved from GenBank (National Center for Biotechnology Information, Bethesda, Md.) showed that *Pseudomonas* sp. strain B13 is closely related to *P. aeruginosa*. AB10 medium [1.51 mM $(NH_4)_2SO_4$, 3.37 mM Na_2HPO_4, 2.20 mM KH_2PO_4, 179 mM NaCl, 10 μM $CaCl_2$, 0.1 mM $MgCl_2$, 1 μM $FeCl_3$] supplemented with citrate (1 mM) as the carbon source and trace minerals (final concentration [per liter]: 0.2 mg of $CaSO_4$, 0.2 mg of $FeSO_4 \cdot 7H_2O$, 20 μg of $MnSO_4 \cdot H_2O$, 20 μg of $CuSO_4$, 20 μg of $ZnSO_4 \cdot 7H_2O$, 10 μg of $CoSO_4 \cdot 7H_2O$, 10 μg of $NaMoO_4 \cdot H_2O$, 5 μg of H_3BO_3) was used as the biofilm medium (at room temperature). AB medium (3) supplemented with citrate (10 mM) and trace minerals was used for overnight batch cultivation (at 30°C).

Construction of a plasmid containing a $P_{A1/04/03}$-RBSII-*dsRed*-T0-T1 cassette. Plasmid pDsRed (Clontech Laboratories, Inc., Palo Alto, Calif.) encoding DsRed (originally designated drFP583 by Matz et al. [20]) was used as the template for PCR amplification with primers DsRedUp (5′ATATAGCATGCG GTCTTCCAAGAATGTTATCAA3′) and DsRedDown (5′CTCTCAAGCTTC CCGGGTTAAAGGAACAGATGGTGGCG3′). This resulted in a 702-bp fragment containing the *dsRed* gene with a silent point mutation in the second codon, resulting in an *Sph*I site, and with a downstream *Hin*dIII site. A $P_{A1/04/03}$-RBSII-*dsRed*-T0-T1 cassette-containing plasmid was constructed by cutting the 702-bp PCR fragment with *Sph*I and *Hin*dIII and cloning the two fragments (the *dsRed* gene contains an internal *Hin*dIII site) in *Sph*I/*Hin*dIII-cut pJBA46 (1) as described previously (1).

Insertion of *gfp* and *dsRed* into the chromosome of *P. putida* OUS82 and *Pseudomonas* sp. strain B13. The *gfp* or *dsRed* gene fused to the *Escherichia coli* ribosomal promoter, *rrnB*P1, or the synthetic *lac* promoter, $P_{A1/04/03}$, in the *rrnB*P1-RBSII-*gfp*-T0-T1 (30), $P_{A1/04/03}$-RBSII-*gfp*-T0-T1 (1), and $P_{A1/04/03}$-RBSII-*dsRed*-T0-T1 cassettes was inserted into the chromosome of *P. putida* OUS82 and *Pseudomonas* sp. strain B13 using pUTkan or pUTtc delivery plasmids (10) with the cassettes cloned in the *Not*I site. The delivery plasmids were mobilized from *E. coli* CC118 λpir to the recipients using the helper strain *E. coli* HB101(RK600) as previously described (1). Exconjugants with mini-Tn*5* cassettes inserted in the chromosome were selected on AB plates supplemented with citrate (10 mM) and kanamycin (50 μg/ml) or tetracycline (10 μg/ml).

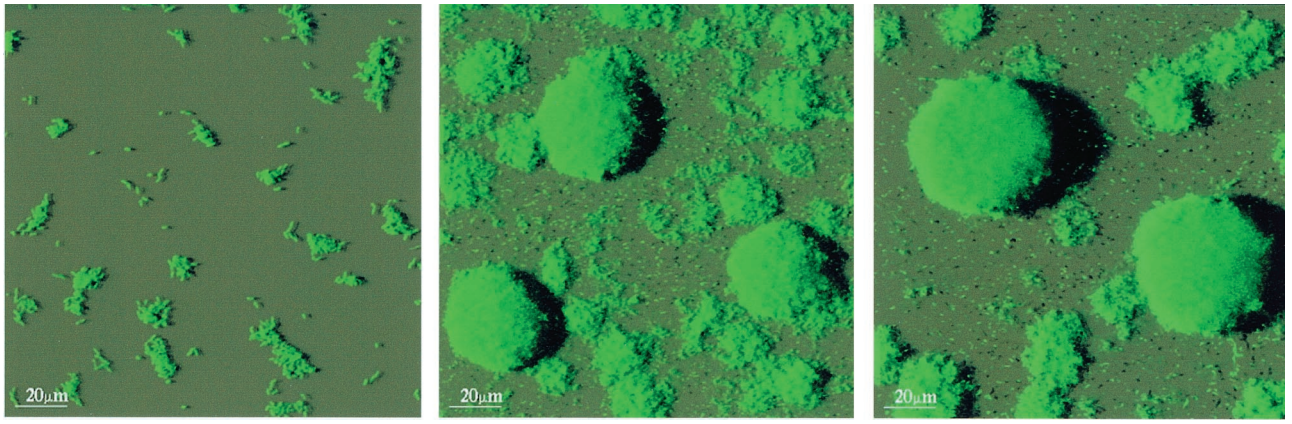

FIG. 1. The spatial structures in a developing B13(GF) biofilm were studied by the use of CSLM. Shadow projection micrographs recorded in a 1 (left)-, 3 (middle)-, and 5 (right)-day-old biofilm are shown.

Fluorescent exconjugants that grew indistinguishably from the parental strains on plates and in biofilms were used in the experiments described here. The green fluorescent derivatives were designated B13(GF) and OUS82(GF), while the red fluorescent derivatives were designated B13(RF) and OUS82(RF).

Construction of nonflagellated *P. putida* OUS82 derivatives. After insertion of the $P_{A1/04/03}$-RBSII-*gfp*-T0-T1 cassette in *P. putida* OUS82, the green fluorescent exconjugants were tested for their ability to swim in plates containing LB medium (2) solidified with 0.35% agar. Five out of 877 exconjugants did not spread in the low-agar plates and did not show swimming motility under microscopic examination. Electron microscopy showed that the nonmotile mutants were nonflagellated (Fla^-) as opposed to the two to four polar flagella carried by wild-type OUS82 cells (data not shown). The regions flanking the mini-Tn*5* cassettes in two of the Fla^- mutants were cloned and sequenced using standard techniques. Subsequent comparative sequence analysis did not reveal high homology of the sequences to any known genes, but it showed that the two Fla^- mutants are independent (data not shown). Since we were unable to genetically define the Fla^- mutants, all experiments in the present report (which involved nonflagellated *P. putida*) were performed in replicate with the two independent Fla^- mutants. Since the two independent Fla^- mutants showed the same biofilm phenotypes, we are confident that their distinct behavior was due to the lack of flagella.

Cultivation of biofilms. Biofilms were cultivated in flow chambers (34) with channel dimensions of 1 by 4 by 40 mm. The flow system was assembled and prepared as described previously (22). Flow chambers were inoculated with overnight cultures of the OUS82 and B13 derivatives diluted 100-fold in 0.9% NaCl. After inoculation the medium flow was stopped for 1 h, and thereafter the medium was pumped through the flow cells at a constant velocity of 0.2 mm/s using a peristaltic pump (model 205S; Watson Marlow, Calmouth, Cornwall, England).

Microscopy and image analysis. Microscopic observation was done using a Carl Zeiss Axioplan 2 epifluorescence microscope equipped with filter set 10 for Gfp detection and filter set 15 for DsRed detection. Analysis of biofilm spatial structures was performed with a confocal scanning laser microscope (model TCS4D; Leica Lasertechnik, GmbH, Heidelberg, Germany), equipped with detectors and filter sets for simultaneous monitoring of Gfp and DsRed fluorescence. Shadow projections and optical sections were generated using the IMARIS software package (Bitplane AG, Zürich, Switzerland) running on a Silicon Graphics Indigo2 workstation (Silicon Graphics, Mountain View, Calif.). The images were processed for display using Photoshop software (Adobe, Mountain View, Calif.).

Nucleotide sequence accession numbers. The nucleotide sequences of the sites of mini-Tn*5* insertion in the two *P. putida* Fla^- mutants have been deposited in GenBank under accession numbers AF295532 and AF295533.

RESULTS

In order to study the spatial localization of the model organisms, *P. putida* OUS82 and *Pseudomonas* sp. strain B13, in flow chamber-grown biofilms by the use of CSLM, we tagged the bacteria with the Gfp or the DsRed by chromosomal insertion of mini-Tn*5* cassettes containing the *gfp* or *dsRed* genes fused to strong promoters (see Materials and Methods for details). The resulting strains, OUS82(GF), OUS82(RF), B13(GF), and B13(RF), were green or red fluorescent and grew indistinguishably from the respective wild-type strains on agar plates and in biofilms (data not shown). The relative viability of B13(RF) and OUS82(RF) in overnight cultures was slightly higher (by a factor of approximately 1.4) than the relative viability of B13(GF) and OUS82(GF). Insertion of the *dsRed* cassette in the chromosome of OUS82 resulted in only weakly red fluorescent clones. The red fluorescence of the DsRed-containing bacteria was in general inversely correlated with their growth rate, and stationary-phase bacteria were highly red fluorescent (data not shown), suggesting that maturation of the DsRed protein into the fluorescent form occurs slowly in bacteria. Furthermore, the swimming *dsRed*-containing bacteria in the flow chambers were only weakly red fluorescent.

Two kinds of CSLM micrographs are presented in the present study. The shadow projections show a top view with illumination from the side so that the shadow indicates the three-dimensional shape of the microcolonies or structures and their distance to the substratum. The optical sections show a single horizontal layer inside the biofilms, and they are used to visualize cells inside the microcolonies or structures.

Development and dynamics in *Pseudomonas* sp. strain B13 biofilms. Flow chambers irrigated with citrate minimal medium were inoculated with a small number of B13(GF) bacteria from an overnight culture, and the biofilm formation was monitored by CLSM. As shown in Fig. 1, *Pseudomonas* sp. strain B13 initially formed flat irregularly shaped microcolonies that eventually became dense ball-shaped microcolonies. Except for the earliest phase, a large number of swimming bacteria were present in all phases of biofilm formation. Because these bacteria moved during the recording of the CLSM micrographs they appear mostly as dots in Fig. 1. Since the laminar bulk liquid flow rate (200 μm/s) in the flow chamber was higher than the maximal swimming velocity reported for *Pseudomonas* spp. (85 μm/s [14]), a population of swimming bacteria could not exist in the flow chamber macroenvironment. However, a laminar bulk liquid flow rate of 200 μm/s corresponds to a surface boundary layer flow rate of less than 10 μm/s (17), so the bacteria could swim in all directions (including upstream) in the microenvironment surrounding the biofilm.

In order to investigate the dynamics in developing B13 biofilms, flow chambers were inoculated with a small number of bacteria from 1:1 mixtures of B13(GF) and B13(RF) overnight cultures, and the spatial distribution of the green and red fluorescent bacteria was recorded as the biofilm developed.

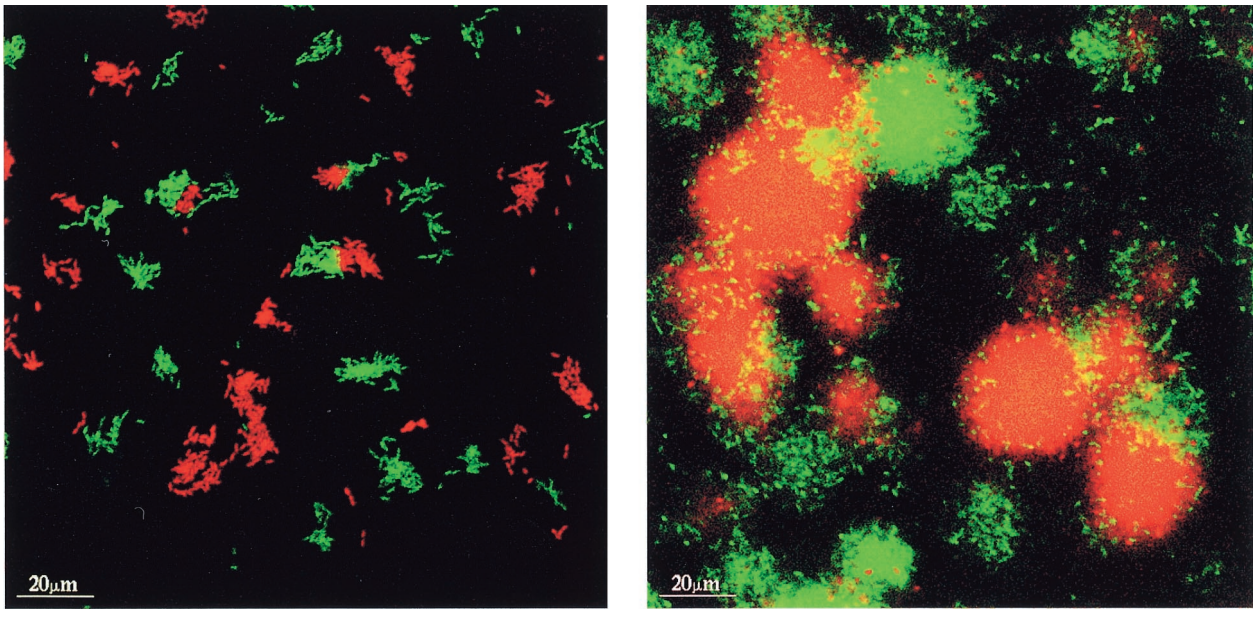

FIG. 2. The location of green and red fluorescent bacteria within horizontal sections of a developing B13(GF)-B13(RF) biofilm was studied by the use of CSLM. Optical section micrographs recorded in a 1 (left)- and 5 (right)-day-old biofilm are shown.

Figure 2 shows optical sections of the two-colored monospecies biofilm. The green and red fluorescent B13 derivatives initially formed separate small microcolonies, but in later phases of biofilm formation the red fluorescent microcolonies often contained a few green fluorescent bacteria while the green fluorescent microcolonies often contained a few red fluorescent bacteria. This suggested that the initial microcolonies were formed by the growth of substratum-attached bacteria (as opposed to microcolony formation by aggregation of bacteria) and that swimming bacteria reentered the microcolonies.

Development and dynamics in *P. putida* OUS82 biofilms. Flow chambers irrigated with citrate minimal medium were inoculated with a small number of OUS82(GF) bacteria from an overnight culture, and the biofilm formation was monitored by CLSM. As shown in Fig. 3, OUS82(GF) initially formed small irregularly shaped microcolonies, but after about 3 days of growth, loose irregularly shaped protruding structures were formed. Epifluorescence microscopic inspection of the biofilm showed that the transition from compact microcolonies into the loose structures was preceded by rapid circular movement of the bacteria inside the microcolonies, which eventually leads to dissolution of the microcolonies. This phenomenon is illustrated in Fig. 4A with optical sections of the same location recorded at 20-s intervals. (A movie showing the phenomenon better can be viewed at http://ulla-brinch.homepage.dk.) In order to investigate if the observed rapid movement of the OUS82(GF) cells was caused by swimming motility, we included a nonflagellated *P. putida* OUS82 derivative, OUS82 (Fla$^-$), in the investigation (see Materials and Methods for details). The OUS82(Fla$^-$) cells did not move inside the microcolonies (Fig. 4B), and the compact microcolonies were not dissolved in OUS82(Fla$^-$) biofilms (Fig. 5), suggesting that the rapid movement of the bacteria inside the microcolonies and the transition from compact microcolonies to the loose structures involved flagellum-driven motility. Optical sectioning of a

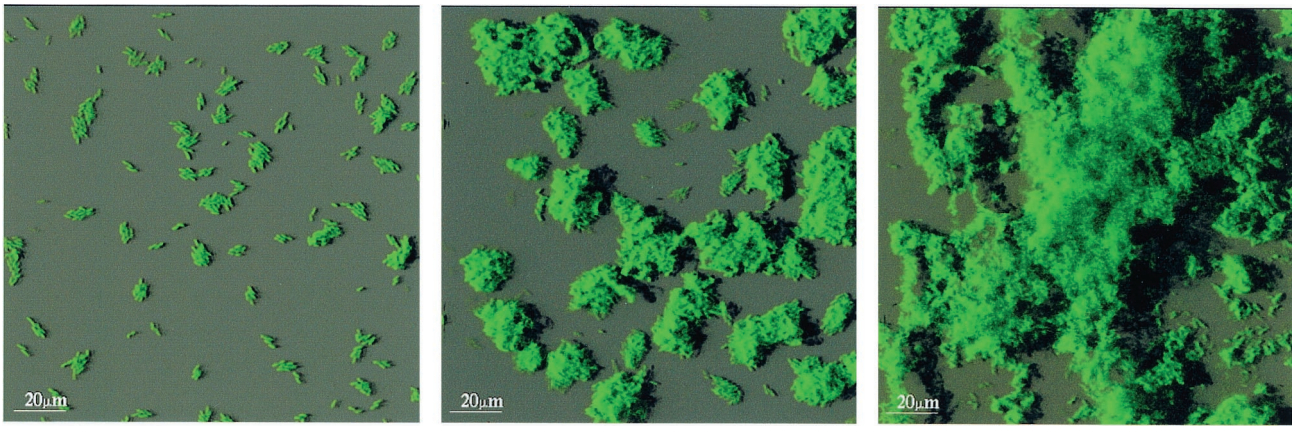

FIG. 3. The spatial structures in a developing OUS82(GF) biofilm were studied by the use of CSLM. Shadow projection micrographs recorded in a 1 (left)-, 3 (middle)-, and 5 (right)-day-old biofilm are shown.

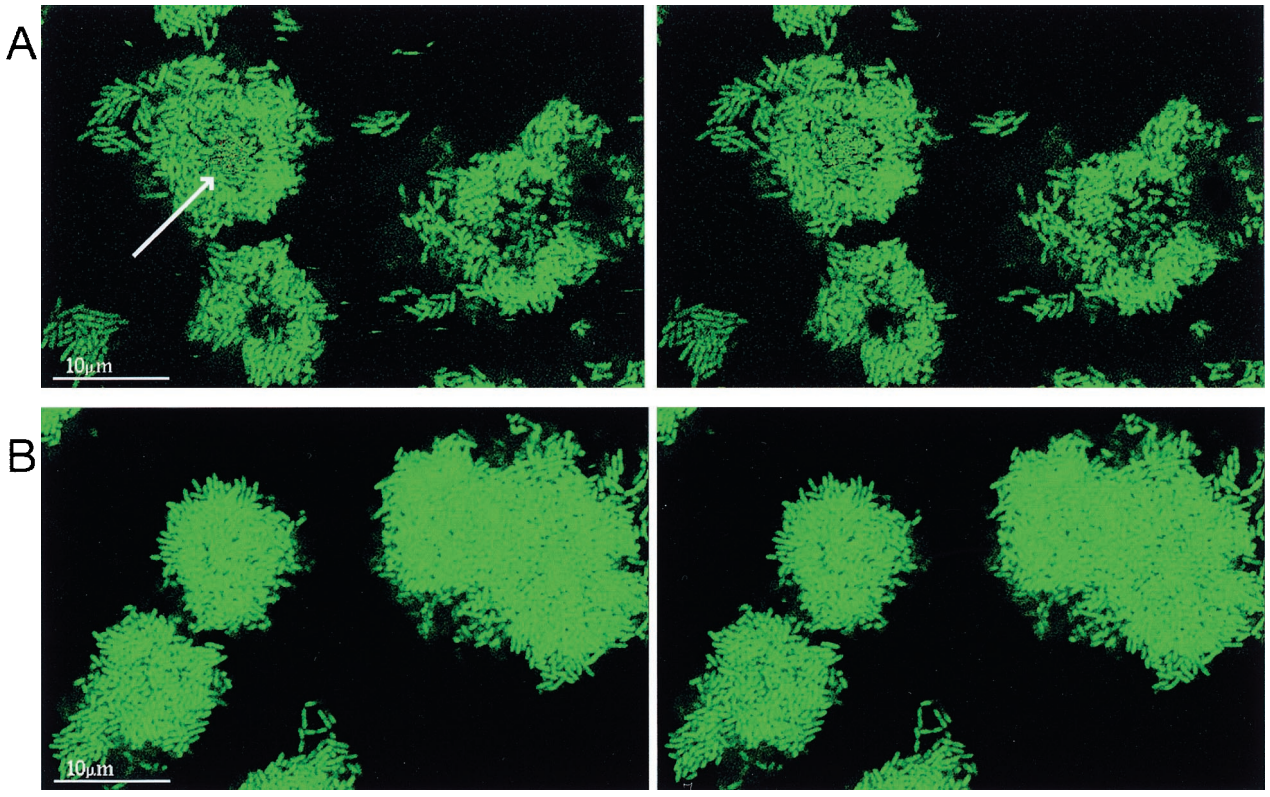

FIG. 4. The location of bacteria within horizontal sections of a 3-day-old OUS82(GF) biofilm (A) and a 3-day-old OUS82(Fla$^-$) biofilm (B) was studied by the use of CSLM. The left optical section micrographs were recorded 20 s before the right optical section micrographs. The mowing cells inside one of the OUS82(GF) microcolonies, which do not appear in the same position on the left and right micrographs, are pointed out.

biofilm initiated with a 1:1 mixture of OUS82(GF) and OUS82 (RF) cells showed that separate green or red fluorescent microcolonies were formed initially and that the loose structures contained both red and green fluorescent cells (Fig. 6). This suggested that the initial microcolonies were formed by the growth of substratum-attached cells (and not by cell aggregation) and that the loose structures contained a mixture of bacteria from different microcolonies.

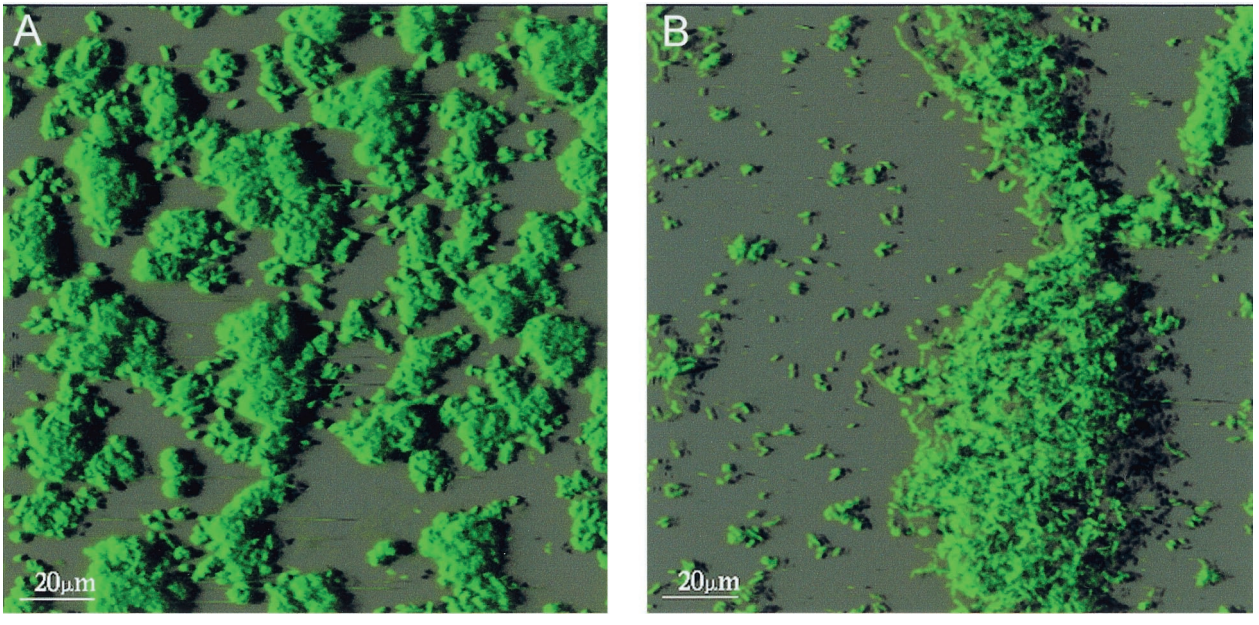

FIG. 5. The spatial structures in OUS82(GF) and OUS82(Fla$^-$) biofilms were studied by the use of CSLM. Shadow projection micrographs of a 7-day-old OUS82 (Fla$^-$) biofilm (A) and a 7-day-old OUS82(GF) biofilm (B) are shown.

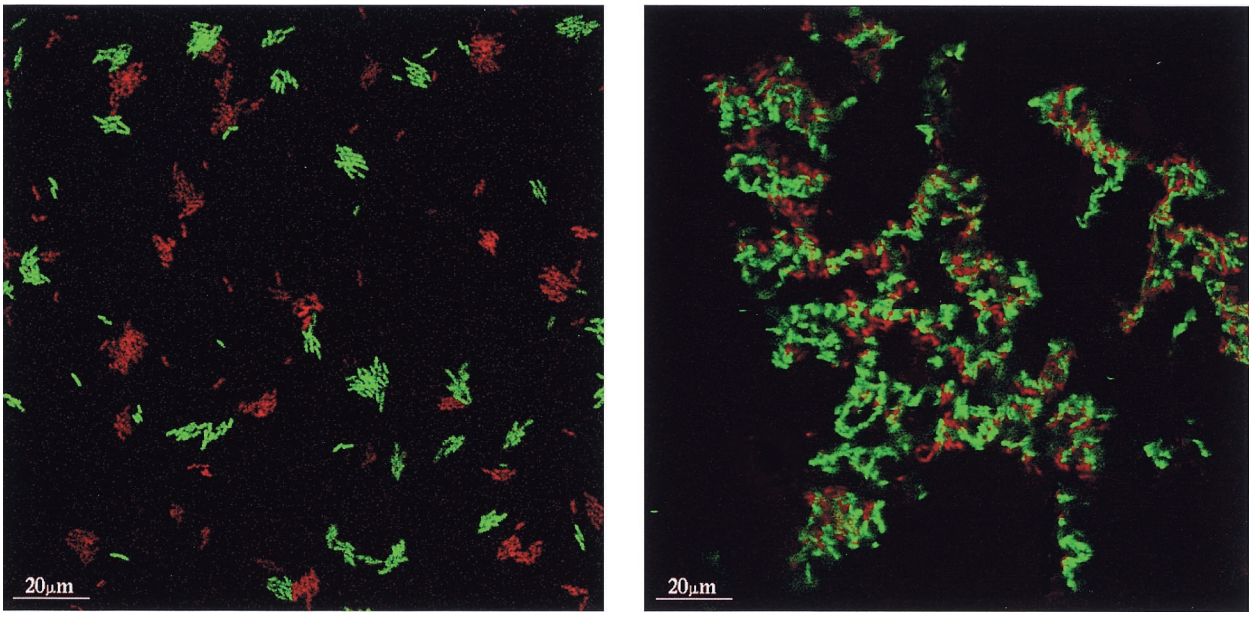

FIG. 6. The location of green and red fluorescent bacteria within horizontal sections of a developing OUS82(GF)-OUS82(RF) biofilm was studied by the use of CSLM. Optical section micrographs recorded in a 1 (left)- and 5 (right)-day-old biofilm are shown.

Development and dynamics in mixed-species biofilms. Biofilm development and dynamics were also investigated in B13/OUS82 mixed-species biofilms. As shown in Fig. 7, B13(RF) and OUS82(GF) formed their distinct structures also in the mixed-species biofilm. Optical sectioning of the biofilm showed that the dense B13(RF) microcolonies often contained a few OUS82(GF) bacteria, while the loose OUS82(GF) structures often contained a few B13(RF) bacteria (Fig. 8). In order to investigate if the observed movement of bacteria between the microcolonies was caused by swimming of the bacteria or could be caused by other kinds of motility or the medium flow, we included OUS82(Fla$^-$) in the investigation. In a 4-day-old B13(RF)-OUS82(GF) biofilm 25 out of 35 randomly chosen B13(RF) microcolonies contained OUS82(GF) cells, whereas in a 4-day-old B13(RF)-OUS82(Fla$^-$) biofilm only 4 out of 35 randomly chosen B13(RF) microcolonies contained OUS82 (Fla$^-$) cells. This indicated that bacterial swimming had a role in the dynamics occurring in the biofilms. The finding that some of the B13(RF) microcolonies did contain OUS82(Fla$^-$) cells could be due to the fact that a large fraction of the nonmotile cells constantly was shed from OUS82(Fla$^-$)-containing biofilms and carried by the medium flow.

The above-described experiments suggested either that the moving bacteria initially stick to the surface of the microcolonies by the use of flagella and become enclosed as the microcolonies grow or that they use flagellar motility to actively enter the microcolonies. In order to distinguish between these possibilities, B13(RF) was grown in flow chambers, and when the distinct ball-shaped microcolonies were formed, OUS82 (GF) or OUS82(Fla$^-$) cells were introduced. The fate of the incoming bacteria was then monitored by the use of CLSM. OUS82(GF) cells were frequently detected inside the red fluorescent B13(RF) microcolonies already at the earliest CSLM recording 1 h after introduction of the green fluorescent cells, while the OUS82(Fla$^-$) cells only rarely were found inside the red fluorescent microcolonies (data not shown). This suggested

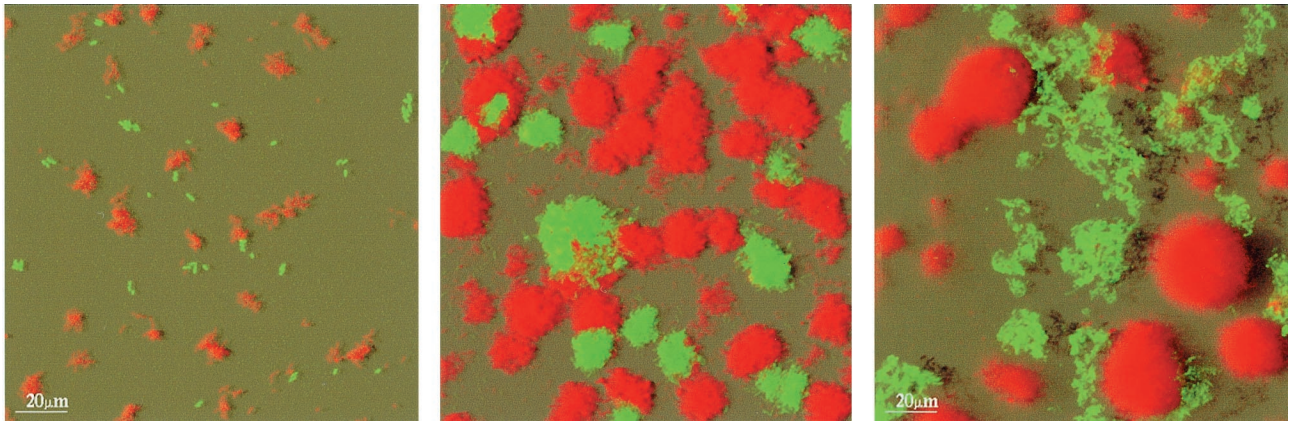

FIG. 7. The spatial structures in a developing B13(RF)-OUS82(GF) biofilm were studied by the use of CSLM. Shadow projection micrographs of a 1 (left)-, 2 (middle)-, and 5 (right)-day-old biofilm are shown.

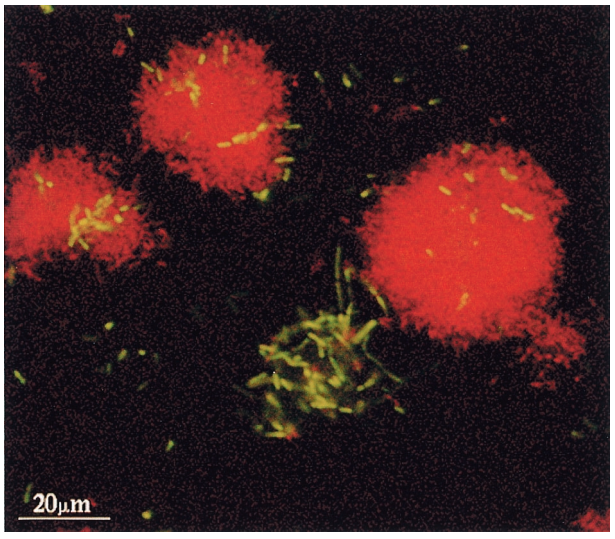

FIG. 8. The location of green and red fluorescent bacteria within horizontal sections of a developing B13(RF)-OUS82(GF) biofilm was studied by the use of CSLM. An optical section micrograph recorded in a 5-day-old biofilm is shown.

that the incoming bacteria were indeed able to penetrate the microcolonies and that this process is flagellum dependent. A CSLM recording performed 3 days after introduction of the green fluorescent cells showed that they were still present as single cells within the red fluorescent microcolonies, not as cell aggregates (Fig. 9). This suggested either that the incoming bacteria were not dividing but remained in the microcolonies or that the cells were rapidly entering and exiting the microcolonies so the average residence time was not sufficient to allow buildup of a population. In support of the latter suggestion, moving OUS82(GF) bacteria could be visualized inside B13(RF) microcolonies by recording optical sections at the same location at 1-min intervals. Figure 9 shows the green fluorescent OUS82(GF) bacteria at different positions inside the red fluorescent B13(RF) microcolonies in two optical sections recorded at the same location with a 1-min time lapse.

DISCUSSION

The model organisms used in the present study represent two different kinds of biofilm formation. Both bacteria form flat microcolonies initially, but in the later phase of biofilm formation *Pseudomonas* sp. strain B13 forms ball-shaped microcolonies whereas *P. putida* OUS82 forms loose protruding structures. Although the reasons for the different behavior of the two pseudomonads are unknown, it was considered advantageous in the present study to use bacteria that display different kinds of biofilm formation.

The use of *gfp* and *dsRed* as reporter genes or genetic tags for distinguishing between different species, identical species, or wild type and mutants is undoubtedly a very useful approach for on-line studies of biofilms. Recently it has been proposed to use dual labeling with a UV-excitable Gfp variant and wild-type Gfp in biofilm studies (7). In our hands, however, UV excitation kills the bacteria, so this approach cannot be used for nondestructive on-line monitoring.

It is widely claimed that bacteria in biofilms are sessile cells in a physiological state different from that of planktonic cells (see references 6 and 12 for reviews), and some reports documenting differential gene expression in sessile (surface-attached) bacteria have appeared (8, 26, 29). Prigent-Combaret et al. (29) reported that expression of 38% of the genes in *E. coli* differ between sessile and planktonic cells. Among these, the *fliC* gene was repressed in sessile bacteria, and flagella were not detected on the sessile bacteria. The present work suggests, however, that bacteria in biofilms display both temporal and spatial variation with respect to differentiation. Some of the bacteria were apparently nonmotile sessile bacteria, but a large fraction of the biofilm bacteria occasionally swam from one microcolony and into another. The *P. putida* OUS82 bacteria were apparently nonmotile and sessile inside the microcolonies in the early phase of biofilm development, but after 3 days of growth, presumably when the microcolonies had reached a critical size, the bacteria started to swim rapidly in circles, the compact microcolonies were dissolved, and loose structures containing bacteria from different microcolonies were formed.

We consequently suggest that biofilms contain both sessile populations and planktonic populations. The ability of a fraction of the bacteria in a biofilm to respond as planktonic cells may allow the biofilm community to respond efficiently to changing environments. Such responses were observed in a two-species model consortium capable of commensal or noncommensal growth (24). When this consortium was grown in flow chambers under commensal conditions it consisted predominantly of mixed microcolonies containing both species, and when the consortium was grown under noncommensal

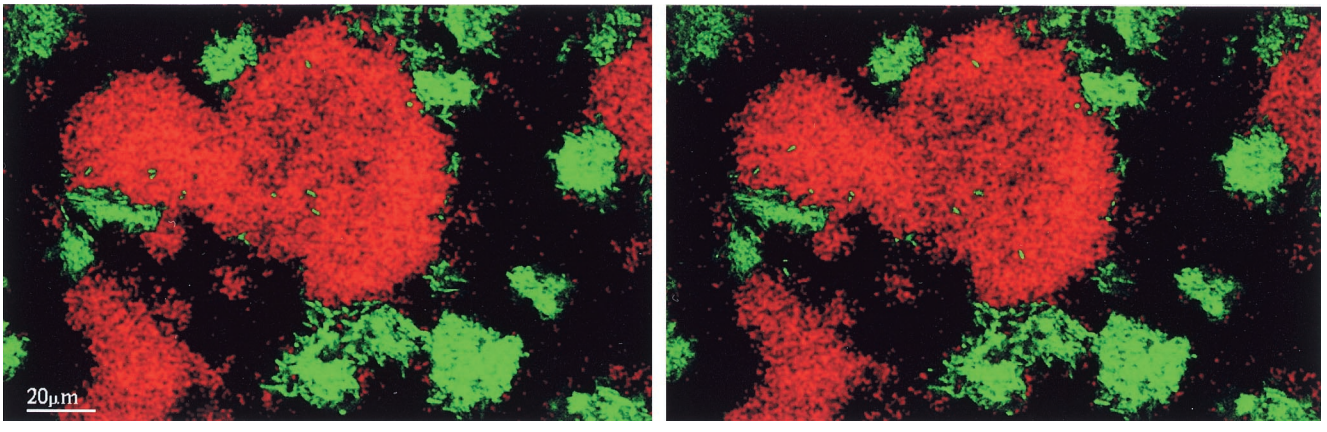

FIG. 9. Movement of green fluorescent OUS82(GF) bacteria inside red fluorescent B13(RF) microcolonies was studied by the use of CSLM. The left optical section micrograph was recorded 1 min before the right optical section micrograph. The appearance of OUS82(GF) bacteria in different positions inside the B13(RF) microcolony on the two micrographs suggests that the OUS82(GF) bacteria moved inside the B13(RF) microcolony.

conditions it consisted predominantly of separate microcolonies of the two species (although dynamics similar to those reported here were observed). However, a shift from noncommensal to commensal conditions resulted in a radical structural change towards mixed microcolonies within 2 days after the substrate shift. Further investigations have revealed that this effective community response may be explained as a consequence of chemotactic motility (unpublished results). As chemotactic motility is characteristic of planktonic cells, the ability of a fraction of the bacteria to respond as planktonic cells may have enabled the rapid structure change.

Evidence has been provided that the initial microcolonies in *P. aeruginosa* biofilms are formed by aggregation of bacteria via twitching motility (25) and that the initial microcolonies in *V. cholerae* El Tor biofilms are formed by aggregation of cells via flagellar motility along the substratum (32). If a biofilm is initiated with a 1:1 mixture of green and red fluorescent bacteria, the formation of microcolonies through aggregation of the bacteria would result in mixed microcolonies containing equal amounts of red and green fluorescent bacteria. The observation in the present work of biofilms consisting initially of separate red or green fluorescent microcolonies suggests that formation of microcolonies through aggregation of bacteria does not play a significant role in *P. putida* OUS82 or *Pseudomonas* sp. strain B13 biofilms under the conditions used in the present study. Instead, the microcolonies seem to be formed by clonal growth from single cells attached to the substratum.

Nonmotile mutants of *E. coli*, *P. fluorescens*, *P. aeruginosa*, and *V. cholerae* were previously shown to be deficient in biofilm formation on the wells of microtiter dishes when they were grown in rich or semidefined media (25, 26, 28, 32). However, the nonmotile *P. fluorescens* mutants did form biofilms on the wells of the microtiter dishes when they were grown in minimal medium supplemented with citrate (26). In the present work the bacteria formed biofilms on glass surfaces in flow chambers irrigated with minimal medium supplemented with citrate, and the nonflagellated *P. putida* OUS82 derivative initially formed biofilms with the same efficiency as the motile *P. putida* OUS82 strain.

At least two previously proposed hypotheses may explain why the many swimming bacteria do not colonize and fill the channels between the microcolonies (or loose structures). Computer simulations, explaining various structural forms in biofilms as a result of differences in local substrate availability (27, 33), suggest that the channels in the biofilms do not become colonized because of substrate limitation. On the other hand, recent studies have suggested that cell-to-cell communication occurs in biofilms (21, 31) and that *P. aeruginosa* cells deficient in synthesis of signal molecules form unstructured biofilms (5, 9). A high concentration of signal molecules in the microcolonies may affect the swimming bacteria so that they do not colonize the channels.

In the context of the present results the interactions between the planktonic, swimming bacteria, and the established microcolonies can be discussed on the basis of the following hypotheses. The first hypothesis is based simply on stochastics; because there is a large fraction of swimming bacteria in the biofilms some of them may accidentally invade the microcolonies. The second hypothesis is based on chemotaxis; a substrate gradient may direct the bacteria away from the microcolonies, but gradients of different metabolites leaking from the microcolonies may direct the swimming bacteria towards these. Between the microcolonies the substrate concentration may be very low (27, 33), whereas the concentration of metabolites leaking from the microcolonies may be higher. Therefore, the swimming bacteria in some locations of the biofilm may be directed towards the microcolonies. The third hypothesis is based on cell-to-cell communication; a high concentration of signal-molecules in the microcolonies of a biofilm may affect the swimming bacteria so that they stay in the biofilm microenvironment and return to the microcolonies as long as the conditions for biofilm life are favorable. We cannot at present with certainty distinguish between these different explanations.

In the present paper we have reported observations of bacterial movement occurring in developing biofilms. From these experiments we have concluded that biofilms are dynamic structures, and we have made suggestions that the bacteria in biofilms may display varying states of differentiation dependent on their temporal and spatial location in the biofilm. At present, however, the specific gene expression in so-called differentiated sessile bacteria is generally not known, so a more comprehensive analysis of states of bacterial differentiation in biofilms, for example by the use of fluorescent reporter genes, must await such knowledge.

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