Metabolic Commensalism and Competition in a Two-Species Microbial Consortium

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We analyzed metabolic interactions and the importance of specific structural relationships in a benzyl alcohol-degrading microbial consortium comprising two species, Pseudomonas putida strain R1 and Acinetobacter strain C6, both of which are able to utilize benzyl alcohol as their sole carbon and energy source. The organisms were grown either as surface-attached organisms (biofilms) in flow chambers or as suspended cultures in chemostats. The numbers of CFU of P. putida R1 and Acinetobacter strain C6 were determined in chemostats and from the effluents of the flow chambers. When the two species were grown together in chemostats with limiting concentrations of benzyl alcohol, Acinetobacter strain C6 outnumbered P. putida R1 (500:1), whereas under similar growth conditions in biofilms, P. putida R1 was present in higher numbers than Acinetobacter strain C6 (5:1). In order to explain this difference, investigations of microbial activities and structural relationships were carried out in the biofilms. Insertion into P. putida R1 of a fusion between the growth rate-regulated rRNA promoter rrnB1 and a gfp gene encoding an unstable variant of the green fluorescent protein made it possible to monitor the physiological activity of P. putida R1 cells at different positions in the biofilms. Combining this with fluorescent in situ hybridization and scanning confocal laser microscopy showed that the two organisms compete or display commensal interactions depending on their relative physical positioning in the biofilm. In the initial phase of biofilm development, the growth activity of P. putida R1 was shown to be higher near microcolonies of Acinetobacter strain C6. High-pressure liquid chromatography analysis showed that in the effluent of the Acinetobacter strain C6 monoculture biofilm the metabolic intermediate benzoate accumulated, whereas in the biculture biofilms this was not the case, suggesting that in these biofilms the excess benzoate produced by Acinetobacter strain C6 leaks into the surrounding environment, from where it is metabolized by P. putida R1. After a few days, Acinetobacter strain C6 colonies were overgrown by P. putida R1 cells and new structures developed, in which microcolonies of Acinetobacter strain C6 cells were established in the upper layer of the biofilm. In this way the two organisms developed structural relationships allowing Acinetobacter strain C6 to be close to the bulk liquid with high concentrations of benzyl alcohol and allowing P. putida R1 to benefit from the benzoate leaking from Acinetobacter strain C6. We conclude that in chemostats, where the organisms cannot establish fixed positions, the two strains will compete for the primary carbon source, benzyl alcohol, which apparently gives Acinetobacter strain C6 a growth advantage, probably because it converts benzyl alcohol to benzoate with a higher yield per time unit than P. putida R1. In biofilms, however, the organisms establish structured, surface-attached consortia, in which heterogeneous ecological niches develop, and under these conditions competition for the primary carbon source is not the only determinant of biomass and population structure.

Bacteria often live in consortia bound to surfaces, such as in biofilms, flocs, or granules (5). Under these conditions the bacteria are positioned in a heterogeneous environment with gradients of nutrients and waste products as a consequence of diffusion and mass transport processes, and it is therefore to be expected that this heterogeneity is reflected in the physiology of the individual cells. In agreement with this, consortia like biofilms often appear as rather complex and heterogeneous assemblies consisting of clusters of bacteria embedded in polymeric substances, which are separated by void regions (cell-free channels) (12, 13, 27, 28). The development of the cell-free regions may support transport of nutrients and waste products to and from the deeper layers of the biofilms (7). For example, DeBeer et al. (8) showed by using microelectrodes that at the same depths in a biofilm, oxygen concentrations in the void regions were much higher than those in adjacent clusters of biomass.

The development of what seem to be structurally organized communities may argue for the presence of overall regulatory elements, which control the formation of the community structures (6). However, changes in structural organization have been shown to be significantly affected by the nutrients supplied to the community (13, 27). Furthermore, mathematical modeling of bacterial growth in biofilms has indicated that simple rules based on nutrient gradients, diffusion rates, and biomass production may determine basic features of biofilm structures (18, 26). Thus, even though there may be regulatory factors that are actively involved in control of biofilm formation, parameters like mass transport, substrate concentrations, diffusion gradients, detachment-attachment mechanisms, and flow rates probably all have significant influence on biofilm structures.

Syntrophic relationships between different organisms have
been demonstrated in several microbial ecosystems, such as the interspecies electron transfer from H₂ or formate in anaerobic digesters (1, 25) and the relationship between ammonia-oxidizing and nitrite-oxidizing species in nitrifying communities (22). Communities involving xenobiotic degradation (for a review, see reference 21) and oral communities (2) are other examples of tight metabolic associations between community species.

Applications of scanning confocal laser microscopy (SCLM), fluorescence in situ hybridization (FISH), and microelectrodes have led to a rapidly increasing understanding of structure-function relationships in microbial communities. FISH and the use of microelectrodes have shown that the nitrite-oxidizing bacteria are clustered around ammonia-oxidizing bacteria in nitrite-oxidizing zones (inner part of the biofilm) in a nitrifying wastewater treatment biofilm (17). In addition, Ramsing et al. (20) demonstrated a negative correlation between sulfate-reducing bacteria and the oxygen profile in a photosynthetic biofilm, and in anaerobic granular sludge digesters the structural relationship between different species was shown to be highly organized (9, 11, 23).

To obtain a better understanding of the function of channel structures and of structural relationships between species in relation to the overall functionality of the microbial communities, we also need to be able to determine the physiological state of the individual cells in the microbial consortium. Recently, a reporter system based on a fusion between the rRNA promoter from Escherichia coli and a reporter gene encoding an unstable derivative of the green fluorescent protein (GFP) was developed (24). This reporter system has been used to monitor growth activity in the present study of species relationships in the native isolation of Pseudomonas putida strain C6 and Acinetobacter sp. strain C6 and Pseudomonas putida strain R1.

### MATERIALS AND METHODS

**Strain and growth conditions.** Throughout this investigation, derivatives of *P. putida* R1 and *Acinetobacter* strain C6 described in Table 1 were used. *P. putida* R1 (SM1700) with the mini Tn5-Km-<sup>rrnB</sup>-<sup>gfp</sup>[AGA]-<sup>T₆r</sup>-<sup>T₆</sup> cassette inserted into the chromosome; Na<sup>+</sup><sup>+</sup> subclass of class *Proteobacteria*<sup>a</sup> This work

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<td>Acinetobacter sp. strain C6 (CKL01)</td>
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<sup>a</sup> P. putida R1 (JB156) from reference 3.
<sup>b</sup> From reference 14.
<sup>c</sup> From reference 15.

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From reference 15.

From reference 14.

From reference 15.
Embedding and 16S rRNA hybridization of hydrated biofilm samples. In order to avoid further degradation of the unstable GFP, the embedding and hybridization procedure, as previously described (3, 4, 15), was slightly modified. The biofilms were fixed by carefully inoculating 500 μl of ice-cold 4% paraformaldehyde solution directly into the flow channel, which was then kept on ice for at least 1 h to ensure complete fixation of all cells in the biofilm. The fixed biofilms were washed with 1× phosphate-buffered saline by pumping the solution through the channels for 20 min (flow rate, 0.25 mm s⁻¹), and finally the biofilm was embedded in 1 ml of 20% acrylamide solution containing 200:1 acrylamide-bisacrylamide (Sequagel; National Diagnostics, Atlanta, Ga.), 8 μl of N,N,N',N'-tetramethylethylenediamine (Kodak International Biotechnologies Inc., New Haven, Conn.), and 20 μl of 1% ammonium persulfate (International Biotechnologies Inc.). After the polyacrylamide was allowed to solidify for at least 1 h, the glass coverslip was carefully loosened from the flow cell, and the biofilm containing the polyacrylamide block was lifted out of the flow channel. The block was subsequently cut into slices of approximately 5 mm, placed on a six-well hybridization slide (Novakemi ab, Enskede, Sweden), and prehybridized at 37°C in 45 μl of the hybridization buffer (washing solution I [0.9 M NaCl, 100 mM Tris, pH 7.2] containing 30% formamide at 37°C). After 30 min, the prehybridization buffer was removed and 30 μl of the hybridization buffer containing 75 ng of each probe was added to the hybridization well. The slide was incubated at 37°C for at least 3 h in a moisturized chamber. The polyacrylamide blocks were washed in 45 μl of washing solution I for 30 min at 37°C, followed by washing in 45 μl of washing solution II (0.9 M NaCl, 100 mM Tris, pH 7.2) for another 30 min at 37°C. Finally, the acrylamide blocks were rinsed in 45 μl of Milli-Q water and then immediately mounted on an object glass with a drop of SlowFade phosphate-buffered saline-based antifade solution (Molecular Probes) and a coverslip on top.

Microscopy and image analysis. All microscopic observations and image acquisitions were performed with a TCD4D SCLM (Leica Laserotechnik GmbH, Heidelberg, Germany) equipped with an argon-krypton laser and three detectors and filter sets for simultaneous monitoring of fluorescein isothiocyanate-GFP and the indocarbocyanine dyes CY3 and CY5. The x-y images were presented as extended-focus images, which are produced by taking the confocal images from the different depths of the biofilm and projecting them into a single image. The extended-focus images and vertical cross sections through the biofilm were generated by using the IMARIS software package (Bitplane AG, Zurich, Switzerland) running on an Indigo2 workstation (Silicon Graphics, Mountain View, Calif.). Images were further processed for display by using Photoshop software (Adobe, Mountain View, Calif.).

HPLC analysis. Samples subjected to high-performance liquid chromatography (HPLC) analysis were taken from the effluent of the flow channels. The samples were filtered through a 0.2-μm-pore-size filter, and the contents of benzyol alcohol and benzoate were measured with a Shimadzu (Tokyo, Japan) HPLC equipped with a Supelcosil LC-18 reverse-phase column (Supelco Park, Bellefonte, Pa.) and a UV-visible detector set at 206 nm. The mobile phase was a solution of 40% acetonitrile (HPLC grade; Sigma Aldrich) and 60% NaH₂PO₄ (50 mM, pH 3.0) supplied at a flow rate of 1 ml min⁻¹.

Analysis of biofilm thickness. The thicknesses of the biofilms were measured using a specific function on the digitally controlled microscope (DMRXA microscope; Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany) which makes it possible to measure the distance between two focused planes.

RESULTS

Quantitative analysis of mixed-species and monospecies consortia of P. putida R1 and Acinetobacter strain C6 in chemostats and in flow chambers. P. putida R1 and Acinetobacter strain C6 were grown as suspended cultures in chemostats, in which heterogeneous structural relationships between the organisms are not established. Acinetobacter strain C6 and P. putida R1 were established either as monocultures (Fig. 1A) or together in a mixed culture (Fig. 1B). As shown in Fig. 1, constant cell densities were reached in the monoculture and mixed-culture chemostats after approximately 3 and 5 days, respectively. With respect to Acinetobacter strain C6, the cell density remained constant in both chemostats over the course of the next 5 days until the experiment was terminated, whereas a slight increase in P. putida R1 cell numbers was observed during the last 2 days. In both the mixed and monospecies cultures Acinetobacter strain C6 reached the same cell density of approximately 10⁹ CFU/ml. In contrast, the cell densities of P. putida R1 were lower than the corresponding levels of Acinetobacter strain C6 in both the mixed-species and the monospecies chemostats. When growing alone in the chemostat, the cell density of P. putida R1 was between 10 and 100 times lower than that of Acinetobacter strain C6, whereas in the chemostats in which Acinetobacter strain C6 and P. putida R1 were mixed, the density of P. putida R1 was reduced to a 500- to 1,000-fold-lower level relative to that of Acinetobacter strain C6. These results show that Acinetobacter strain C6 produces more biomass than P. putida R1.

To investigate how the observed differences in the metabolic efficiency affected the composition in surface-associated cultures over time, nondestructive in situ methods were employed. Two parameters were measured: (i) biofilm thickness and (ii) CFU of cells collected from the flow chamber effluents per milliliter. Although the ratios between biomass, voids, and

FIG. 1. Time course analysis of the numbers of P. putida R1 (■) and Acinetobacter strain C6 (●) cells collected from chemostats where the strains were established either as monospecies cultures (A) or as mixed cultures (B). CFU were enumerated on LB plates containing the appropriate antibiotics for selection of P. putida R1 and Acinetobacter strain C6, respectively. Error bars indicate standard deviations.
channels may vary in internal parts of the biofilm (which was not taken into account when measuring biofilm thickness), the thickness of the biofilm may, as shown for other biofilm consortia (10), be used as a relative measure of the total biomass accumulated in the flow channels. As shown in Fig. 2, both Acinetobacter strain C6 and P. putida R1 monocultures reached a thickness of about 7 to 10 \( \mu \)m, whereas the binary biofilm reached a thickness of approximately 12 to 14 \( \mu \)m.

The cells collected from the effluent are those cells that detach from the flow chamber biofilm. Only in cases where the biofilm cell density has reached a steady state for each species will the effluent cells become an exact representation of the biofilm population. On the other hand, previous studies have shown (3) that in control experiments in which Acinetobacter strain C6 and P. putida R1 were the only species or the predominant species, the effluent data do represent reliable estimates of the entire biofilm population. Determinations of the biofilm effluent cell numbers showed that a ratio of approximately 1:5 of Acinetobacter strain C6 to P. putida R1 in the mixed biofilm was reached after a few days (Fig. 3B). Varying the ratio of Acinetobacter strain C6 and P. putida R1 in the inoculum did not change this ratio significantly (data not shown). After 2 to 3 days the effluent cell numbers of Acinetobacter strain C6 in the monoculture (Fig. 3A) and mixed-culture (Fig. 3B) biofilms had reached a level which remained nearly constant throughout the rest of the experiment. In the monoculture biofilm of P. putida R1, the cell number increased at a lower rate but reached higher numbers than Acinetobacter strain C6 (Fig. 3A), whereas a constant high level of P. putida R1 in the mixed biofilm was reached within a few days (Fig. 3B). Thus, the presence of Acinetobacter strain C6 apparently results in a faster establishment of P. putida R1 in the mixed consortium.

It was previously observed that in mixed-species biofilms growing with benzyl alcohol as the sole carbon and energy source, the expression of the benzoate-inducible promoter, Pm, from the TOL pathway inserted into P. putida R1 was induced in regions near microcolonies of Acinetobacter strain C6 (15), and it was speculated that benzoate leaking from Acinetobacter strain C6 caused the induction of Pm in P. putida R1. In order to investigate this explanation, we performed HPLC analysis of benzyl alcohol and benzoate in flow channel effluents, and at the same time this analysis was used to further assess the metabolic efficiencies of the two organisms.

Samples were taken at different time points after initial colonization of mono- and mixed-culture flow chamber biofilms of P. putida R1 and Acinetobacter strain C6. In the P. putida R1 monoculture biofilm (Fig. 4A), the amount of degraded benzyl alcohol increased slowly over the entire sampling period, reaching 75% conversion after 10 days. In the Acinetobacter strain C6 monoculture biofilm, more than 90% of the benzyl alcohol was catabolized 4 days after the initial colonization, but in contrast to the case for the P. putida R1 biofilm, a large amount of benzoate was accumulated (Fig. 4B). Thus, Acinetobacter strain C6 does in fact leak benzoate from cells when grown as a biofilm. The effluent from a mixed...
culture of the two species showed removal of benzyl alcohol as well as efficient degradation of benzoate (Fig. 4).

**In situ analysis of the mixed biofilm consortium.** To further analyze the metabolic interactions between *Acinetobacter* strain C6 and *P. putida* R1 cells and their consequences for the structural development of the consortium, mixed-species biofilms were fixed and embedded 1, 2, 3, and 6 days after the initial colonization. For specific identification of the two organisms, we used FISH with the fluorescence-labeled probes PP986 and ACN449, targeting *P. putida* R1 and *Acinetobacter* strain C6, respectively. By employing SCLM in combination with FISH, the exact positions of *Acinetobacter* strain C6 and *P. putida* R1 cells could be visualized. The changes and distributions of microbial growth activity of *P. putida* R1 cells were analyzed by insertion of a transposon with the growth phase-regulated promoter *rrnB P1* fused to a *gfp* gene encoding an unstable variant of GFP. Applications of this type of monitor system inserted into *P. putida* R1 for determinations of growth activity in biofilms have previously been demonstrated (24). This reporter system can be used to distinguish between fast-growing (bright green) and slow-growing or nongrowing (weak or no signal) cells.

The images presented in Fig. 5 represent examples of the most frequently occurring structural relationships between the two species observed at days 1, 2, 3, and 6 after the initial colonization. In order to ensure that the changing structures observed were a consequence of a systematic process and not just some random process occurring in some cases and not in others, the experiments was run in five independent rounds each time with three independent flow channels running in parallel. In all experiments the same developmental pattern was observed, indicating that the dynamic changes in the structural relationships between the two species are caused by non-random processes.

One day after inoculation of the strains, the *P. putida* R1 cells with the highest apparent growth activity (highest GFP signal) were observed in the regions near small microcolonies of *Acinetobacter* strain C6 cells (Fig. 5A). As the *Acinetobacter* strain C6 microcolonies grew in size, the *P. putida* R1 cells with highest growth activity were located in the center of the colonies (Fig. 5B). At day 3, two distinct types of associations had developed. In one of these, *P. putida* R1 was excluded from the center of the rather large *Acinetobacter* strain C6 colonies (Fig. 5C), and the cells with the highest growth activity were now observed at the periphery of the colonies. After 6 days, many of these *Acinetobacter* strain C6 microcolonies were overgrown by *P. putida* R1 cells and the fluorescence intensity of the *P. putida* R1 cells near the *Acinetobacter* strain C6 colonies was reduced to low levels (Fig. 5D). In the other type of association, small *Acinetobacter* strain C6 microcolonies started to build up in the upper layers of the *P. putida* R1 biofilm (Fig. 5E). The result was production of large structures (at least 100 to 200 μm thick) in which *Acinetobacter* strain C6 microcolonies became integrated in the *P. putida* R1 cell structures (Fig. 5F).

**DISCUSSION**

Competition for substrate is considered to be one of the major evolutionary driving forces in the bacterial world, and numerous experimental data obtained in the laboratory under well-controlled conditions show how different organisms, or variants of one organism, may effectively outcompete others because of better utilization of a given energy source. Such competition experiments may be performed very convincingly in chemostats, where nutrient limitation allows for metabolic competition among the cells present in the reactor. One important factor for optimal performance of a chemostat is the homogeneous distribution of the suspended cells, and it is considered important to prevent surface attachment of the cells to the reactor walls. Under such optimal conditions, in which only one nutrient is limiting for growth, it may be expected that an organism will totally outcompete all others if it has an improved efficiency of substrate utilization relative to the others. The concentration of the particular nutrient will always be below the threshold level for the less efficient cells, and the rate of washout is therefore higher than the rate of cell proliferation for these cells.

In the present study the two organisms were grown as monospecies and as mixed-species cultures with benzyl alcohol as the
FIG. 5. SCLM micrographs showing the structural relationships between *Acinetobacter* strain C6 and *P. putida* R1 cells with low and high activity, respectively, in a mixed biofilm consortium which was supplied with 0.5 mM benzyl alcohol as the sole carbon source. At days 1 (A), 2 (B), 3 (C and E), and 6 (D and F) after inoculation, biofilms were embedded and hybridized. *P. putida* R1 and *Acinetobacter* strain C6 were hybridized with PP986 labeled with CY5 (blue) and ACN449 labeled with CY3 (red), respectively. The active *P. putida* R1 cells were monitored as cells emitting green fluorescence due to the *rrnB1-gfp* [AGA] fusion inserted in the chromosome of *P. putida* R1. These cells appear as cyan due to
the combination of green (GFP) and blue (hybridization). For each panel similar images were collected from at least two organisms reached nearly stable cell densities, in which the cell number ratio was approximately 1 to 500 in favor of Acinetobacter strain C6. This again showed the competitive advantage of Acinetobacter strain C6, but it also showed an unexpected stable presence of \textit{P. putida} R1 at a low but significant level. In the flow chamber-based biofilms, effluent cell counts showed that within a few days the two organisms reached nearly stable cell densities, in which \textit{P. putida} R1 was present in higher numbers than Acinetobacter strain C6 (approximately 5 to 1). Furthermore, measurements of the biofilm thickness suggested that the simultaneous presence of both strains resulted in a significant increase of the overall biomass in the biofilm. Thus, despite the better utilization of benzyl alcohol by \textit{Acinetobacter} strain C6, \textit{P. putida} R1 was maintained in both growth systems. This finding may be explained by the previous suggestion that growing with benzyl alcohol as the carbon source makes \textit{Acinetobacter} strain C6 cells excrete benzoate, a carbon source readily utilized by \textit{P. putida} R1, into the reactor environment (15). This hypothesis has been confirmed in the present study. The HPLC analysis of the effluent from flow chambers shows that benzoate accumulates in \textit{Acinetobacter} strain C6 monospecies biofilms, whereas in the presence of \textit{P. putida} R1 almost all the excreted benzoate is degraded. In the \textit{P. putida} R1 monospecies biofilm, degradation of benzyl alcohol was rather inefficient, and only a low concentration of benzoate was detected in the effluent. This suggests that \textit{Acinetobacter} strain C6 relatively easily converts benzyl alcohol to benzoate, whereas the degradation of benzoate is slow, resulting in a metabolic bottleneck that leads to accumulation of benzoate. In contrast, \textit{P. putida} R1 encodes a better pathway for degradation of benzoate, which has the capacity to mineralize both the benzoate derived from the conversion of benzyl alcohol to benzoate via its own degradation pathway and the external supply from \textit{Acinetobacter} strain C6.

To further explain the differences in ratios between \textit{P. putida} R1 and \textit{Acinetobacter} strain C6 in the flow chambers and chemostats, a detailed analysis of the distribution and activity of the \textit{P. putida} R1 cells in the flow chamber biofilms was carried out. In situ rRNA hybridization for identification of the individual organisms was used to analyze the spatial distribution of the two strains in the flow chamber biofilms. After 2 to 3 days of growth, large numbers of \textit{P. putida} R1 cells were found to cluster around large surface-associated microcolonies of \textit{Acinetobacter} strain C6. By employing the \textit{rrnBP1:gfp[AGA]} monitor cassette inserted into \textit{P. putida} R1, it was shown that the \textit{P. putida} R1 cells in these regions had a higher growth activity than those further away from the \textit{Acinetobacter} strain C6 microcolonies. Similar distribution patterns have also been observed in nitrifying biofilms (17) and in a two-species biofilm growing with chlorobiphenyl as the sole carbon and energy source (16). However, in neither of these studies was it possible for the individual strains to be established as monospecies biofilms under the conditions described, and thus, in contrast to the present study, there was no competition for the primary carbon source in the mixed biofilms.

For the \textit{Acinetobacter} strain C6 microcolonies that were initially established at the flow chamber glass surface, a stable structural relationship between \textit{P. putida} R1 and \textit{Acinetobacter} strain C6 cells was not maintained, because \textit{P. putida} R1 started to overgrow the \textit{Acinetobacter} strain C6 microcolonies (Fig. 5C and D). The result was a significant reduction in the growth activity of \textit{P. putida} R1 (reduced GFP signal) in these regions. We suggest that the establishment of \textit{P. putida} R1 cells on the outside of the surface-associated microcolonies reduced the supply of benzyl alcohol to the \textit{Acinetobacter} strain C6 cells and thereby also the production of benzoate from these cells. Instead, small microcolonies of \textit{Acinetobacter} strain C6 cells became established in the upper layer of the \textit{P. putida} R1 biofilm where they were near the medium flow and thereby exposed to a constant supply of benzyl alcohol. This allowed the \textit{Acinetobacter} strain C6 cells to accumulate benzoate in these regions, which was metabolized by the associated \textit{P. putida} R1 cells, which obtained a growth advantage over other, nonassociated \textit{P. putida} R1 cells (Fig. 5E). We suggest that due to the metabolic interactions, these mixed \textit{Acinetobacter} strain C6-\textit{P. putida} R1 structures grew faster than the rest of the biofilm and resulted in the production of large structures of biofilm. Thus, taking all these observations together, the two organisms in the biofilm competed as well as exhibited commensal interactions, depending on the physical positioning of the organism. Similar interacting mechanisms, i.e., different species being able to degrade the primary substrate but having different capacities for degradation of the metabolic intermediates, may be expected to prevail in natural environments. This may explain why some natural communities grown on a single carbon source, such as in the studies presented by Wolfaardt et al. (27) and Møller et al. (13), often develop quite complex biofilm compositions and organizations.

The present investigation of the structure-function relationships in a binary biofilm growing with benzyl alcohol as the only added carbon and energy source thus offers an explanation of the contradictory population data from suspended cultures in chemostats and in flow chamber biofilms, respectively. In the
chemostat the individual cells are all surrounded by the same environmental conditions, and they cannot stay in the reactor if their growth rate is lower than the dilution rate (no adherence). In the biofilm there is a range of conditions surrounding the cells due to the heterogeneity of the consortium structure; i.e., microniches develop in which the supplies of primary and secondary nutrients differ significantly. There will therefore be locations in the biofilm where the conditions favor one or the other or both of the organisms, depending on the local structure. In addition, the cells may adhere to the surface or to each other, thus reducing washout. One important consequence of the biofilm configuration, therefore, is that substrates may be more optimally utilized by the consortium (increased mineralization of metabolic intermediates), resulting in faster degradation of the primary nutrient and a faster buildup of biomass. The reorganization of mixed-species consortia in response to the nutrient conditions in order to achieve optimal conditions for the present organisms may require active motility coupled with chemotaxis, cell-to-cell communication signals for coordinated organizational development, or a liquid flow moving cells around for detachment and reattachment. In the present consortium neither chemotaxis nor cell-to-cell communication is the obvious mechanism behind the presented development of the consortium, because our unpublished investigations have not so far shown evidence of any of these properties in \textit{P. putida} R1. It is therefore concluded that passive transport of cells by the flow through the biofilm may be solely responsible for the continuous structural development taking place in the consortium.

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REFERENCES


