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Spatial Organization of Microbial Biofilm Communities

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Abstract

The application of advanced microscopy and molecular and electrochemical high-resolution methods has provided insights into the structural organization and function of biofilm communities. It appears that cellular properties such as growth differentiation, chemotaxis, and cell-to-cell signaling enable biofilm communities to organize structurally in response to the external conditions and the activities of the different biofilm members. Thereby resource utilization becomes optimized, and processes which require syntrophic relationships or special micro-environments become facilitated.

Introduction

Microbial life in the environment is mostly characterized by multiplicity (many species together), nutrient limitation, changing environments, and a structured distribution of the biomass. It is therefore not too surprising that traditional investigations of bacteria grown in the laboratory as pure cell lines with excess nutrients under constant and controlled conditions in liquid suspensions do not really contribute directly to an understanding of the ecology of microorganisms. The challenge obviously is to address the microbial community scenarios as they appear in the environment with methods and tools that permit detailed studies of relevant features. During the past decade such approaches have been made with increasing success due to the employment of techniques directed toward *in situ* monitoring of the presence and activity of specific bacterial species, or of the biochemical processes taking place, or both. The resolution level of these new methods ranges from single cell dimensions to entire ecosystems.

The introduction of molecular biology to this field of microbial ecology has had a strong impact, because many molecular tools are compatible with the application of light microscopy, e.g., confocal scanning laser microscopy (CSLM). *In situ* identification of individual organisms or groups of organisms using phylogenetic markers (FISH) is essential for a high-resolution description of community structure. Fluorescent reporters are equally essential for detection and localization of specific gene expression. DNA techniques of various kinds have proven valuable for characterization of community composition and reactions to external signals, and new developments of these methods (e.g., DNA array techniques) will further increase the level of information about population profiles and their expression profiles.

What has so far come out of these investigations—among

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other things—is the documentation of a very high degree of organization of microbial communities, which appears to integrate the growth conditions and the species composition in such a way that optimal exploitation of the local resources is achieved. The major purpose of this review is to present some of this documentation and to correlate it with structure/function analysis of simple laboratory-based biofilms; our primary objective is to understand the mechanisms responsible for community organization. We will not claim that just one mechanism will eventually explain how all bacteria get organized—in fact, we claim the opposite, i.e., bacteria will probably organize themselves in many different ways depending on the environmental conditions, the population composition, and of course the influence of various stochastic events. However, it does seem possible—and interesting—to reveal common traits and response patterns, which play important roles in the processes leading to such organization.

In the following, we review and discuss examples from various types of bioreactors, in which naturally occurring microbial biofilm communities have been found to be highly structured and organized. Subsequently, current work with much simpler and carefully controlled biofilm model systems is discussed in the context of searching for explanations for community organization.

Biofilm Communities in Natural Environments or Bioreactor Systems

Biodegrading Biofilms

Investigations of multispecies biofilm communities on granular activated carbon (GAC) in fluidized-bed reactors treating contaminated groundwater showed that growth mainly occurred as discrete microcolony structures separated by channel boundaries, and that these structures were maintained over time [14]. It was also found that open spaces extended from the top of the biofilms to the deep inner regions, forming a channel-like network. The structure of the biofilms on the GAC particles was dependent on the substrate. Ball-shaped microcolonies were found on the GAC particles in fluidized bed reactors treating toluene-containing groundwater, whereas the micro-colonies on the GAC particles in fluidized bed reactors treating BTEX-containing groundwater had a coral-reef appearance.

Sulfate Reducing Bacteria in Biofilms

Ramsing et al. [29] were the first to make integrated use of micro-sensors and fluorescent *in situ* hybridization to study structure-function relationships in biofilms. The vertical distribution of sulfate-reducing bacteria (SRB) was investigated, and gradients of oxygen and hydrogen sulfide were measured in photosynthetic biofilms from the trickling filter of a sewage plant. Hydrogen sulfide was found predominantly in the anoxic zone of the biofilm, and most of the SRB were found in the anoxic layers as dense clusters or assemblies. A similar study, which also included temporal variations, was performed by Santegoeds et al. [30]. By performing fluorescent *in situ* hybridization with a comprehensive panel of specific oligonucleotides, Manz et al. [13] detected clusters of SRB in sludge from both the anaerobic, anoxic, and aerobic zones of an activated sludge tank.

Using group-specific hybridization probes and sequence analysis of a gene encoding dissimilatory sulfite reductase (DSR) common to all SRB, Minz et al. [16, 17] found that different SRB groups (clades or lineages) predominated at distinct depths of a photosynthetic microbial mat. The dominating groups of SRB were preferentially localized within the region defined by the oxygen chemocline, but some SRB lineages and clades were found only in the permanently anoxic zone of the mat. This distribution of phylogenetic groups, which to some extent was nonoverlapping, indicated that the different groups serve specific functions in the microbial mat. Available data suggested a close association between SRB and sulfide-oxidizing bacteria in the oxic zone of the microbial mat [16].

In addition to the vertical stratification of SRB in the microbial mat, diurnal changes in the structure probably also occur. The oxygen concentration in the photosynthetic mat changes from day to night, and it has been shown that some SRB move chemotactically away from the highest oxygen concentrations [40] or toward the oxic/anoxic interface [11]. It appears that a full understanding of the processes in microbial mats, which evidently involve syntrophic and microhabitat localized functions as well as diurnal changes, can only be achieved after detailed investigations of the spatial organization of the relevant organisms.

Nitrifying Biofilms

Application of fluorescent *in situ* hybridization and microelectrode technology has also provided information about the spatial organization of nitrifying bacterial populations and their activities in biofilms. The process of nitrification (oxidation of ammonium to nitrate via nitrite) is carried out by two phylogenetically unrelated groups of bacteria, the ammonium oxidizers and the nitrite oxidizers. Although the

structure of nitrifying biofilms is heterogeneous with biomass separated by interstitial voids (e.g., [23]), it has been possible to obtain micro-profiles of oxygen, ammonium, nitrite, and nitrate concentrations, as well as ammonium and nitrite oxidation rates, by averaging measurements from different locations [23, 33–35]. A dense layer of ball-shaped clusters of *Nitrosomonas* cells (ammonium oxidizers) in close physical association with surrounding smaller clusters of *Nitrobacter* cells (nitrite oxidizers) was found in the aerobic zone of a nitrifying biofilm from the trickling filter of an aquaculture [33]. In aggregates from nitrifying fluidized bed reactors the dominant populations of *Nitrosospira* spp. (ammonium oxidizers) and *Nitrospira* spp. (nitrite oxidizers) formed dense clusters that were in contact with each other [34]. The nitrifyng zone (measured with microelectrodes) was restricted to the outer 100 to 150 μm of the aggregates, and the nitrifiers were found in this zone as a dense layer of clusters. The central part of the aggregates was found to be inactive, and significantly fewer nitrifiers were found there [35]. *Nitrosomonas* and *Nitrospira* spp. were found to be the dominant nitrifying populations in domestic wastewater biofilms [23]. The nitrite oxidizing cells formed irregularly shaped aggregates consisting of small microcolonies, which clustered around the microcolonies of ammonia oxidizers.

Methanogenic Biofilms (Granules)

By the use of fluorescent *in situ* hybridization, Harmsen et al. [5, 6] documented how the structure of granular sludge relates to the function of the microorganisms. The functions of the different microorganisms were not monitored directly, but were inferred from the following *a priori* knowledge:(i) Anaerobic degradation of organic matter leads to intermediate formation of alcohols and fatty acids. (ii) These intermediate compounds (e.g., propionate and butyrate) can be converted by acetogens to acetate, hydrogen/formate, and carbon dioxide, provided that the hydrogen partial pressure and formate concentration are low. (iii) Removal of hydrogen and formate is accomplished by methanogens that convert these compounds (and carbon dioxide) to methane. (iv) If sulfate is present, sulfate-reducing bacteria, such as *Desulfobulbus* spp., can convert propionate and sulfate to acetate and hydrogen sulfide independent of the concentrations of hydrogen and formate. (v) The acetate, which is produced with or without sulfate present, can be utilized by acetotrophic methanogens.

Sludge from a sucrose-fed up-flow anaerobic sludge blanket (UASB) reactor was shown to consist of three layers: An exterior layer containing mainly bacteria; a second layer composed of microcolonies of acetotrophic methanogens and mixed microcolonies containing acetogens and hydrogen/formate-utilizing methanogens; and a central part, with large cavities, inorganic materials, and some methanogenic microcolonies (see Fig. 1). As inferred from *a priori* knowledge, the bacteria in the first layer converted sucrose to propionate, butyrate, and acetate, whereas in the second layer, the location of acetogens and hydrogen/formate-utilizing methanogens synthrophically juxtapositioned in microcolonies constituted the basis for conversion of propionate and butyrate to methane and acetate, and the acetotrophic methanogens present in the neighboring microcolonies converted acetate to methane. The structure of sludge from a propionate/butyrate/acetate-fed UASB reactor was different from the structure of the sucrose-grown sludge (both reactors were initially inoculated with sludge from a sugar beet wastewater UASB plant). The exterior layer of bacteria was absent, the methanogens (especially acetotrophic) were located throughout, and the syntrophic, mixed microcolonies were located more toward the center of the granules.

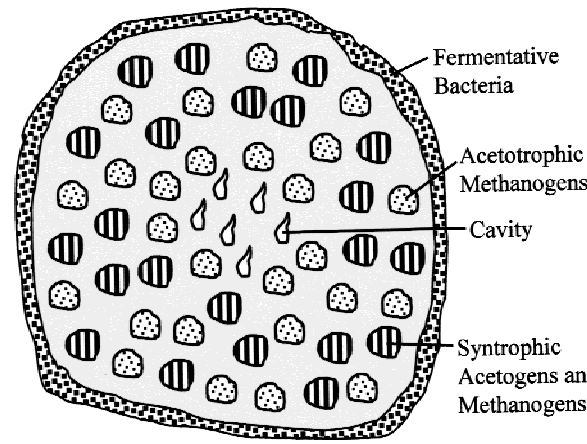

Fig. 1. Schematic drawing showing the spatial structure of granular sludge from a sucrose-fed UASB reactor as described in [5] and [6]. Individual groups of bacteria were identified by the use of fluorescent *in situ* hybridization.

Sludge from a potato-processing UASB plant showed the same structure as described for the sucrose-fed sludge, except that a low number of *Desulfobulbus* spp. were located in the outer layer of bacteria. This sludge was used to inoculate a UASB reactor fed with propionate-containing medium and another UASB reactor fed with propiate/sulfate-containing medium. The propionate-grown sludge lacked the outer bacterial layer and consisted of a mixture of acetotrophic

methanogen microcolonies and syntrophic acetogen–methanogen-containing microcolonies. The propionate/sulfate-grown sludge consisted of an outer layer containing mainly *Desulfobulbus* spp., and below this layer methanogenic microcolonies were present, but syntrophic acetogen–methanogen-containing microcolonies were not found. Apparently, the unconditional conversion of propionate to acetate and hydrogen sulfide by the *Desulfobulbus* spp. located in the outer layer prevented the formation of the syntrophic mixed microcolonies.

Microelectrode analysis of methane and hydrogen sulfide profiles and determinations of the spatial organization of the organisms in methanogenic, methanogenic–sulfidogenic, and sulfidogenic sludge was recently performed [31]. The activity of sulfate reducing bacteria was limited to the outer 50 to 100 μm of methanogenic–sulfidogenic sludge, and to the outer 200 to 300 μm of sulfidogenic sludge. The activity of the methanogenic bacteria in the methanogenic and methanogenic–sulfidogenic sludge was located inwards, starting at ca. 100 μm from the aggregate surface.

Conclusions from the Analysis of Biofilm Communities in Natural Environments or Bioreactor Systems

The described examples confirm classical descriptions from the early days of microbiology and microbial ecology of microbial ecosystems, in which various types of bacteria, displaying their broad repertoire of physiology, are organized in layers or domains in response to a combination of the external environmental conditions and the activities of their neighbors. The application of the molecular and electrochemical high-resolution methods have changed the scale of investigation from the macrosystem analysis of the past to the detailed microscopic resolution levels of the present. But the conclusions are basically the same: We can explain the community organization from knowledge about the composition of the environment and the microorganisms present in the ecosystem. Bacteria adapt to the environment—external as well as local—in such a way that resource utilization becomes optimized, by responding to signals and nutrients according to their genotypic potential. In addition, local selective forces will of course result in population changes according to the specific interactions between the composition of the environment and the organisms present. Future investigations will reveal whether there are general rules governing community development, and interesting approaches include studies over time of community developmental features, analysis of transcription patterns in key organisms, and introduction of specific mutants affected in "community traits" or global regulatory functions. However, at this point we should like to argue that the search for consensus explanations for community establishment and development may be best described as a "wild goose chase."

Biofilm Model Communities in Laboratory-Based Systems

Studies from the natural environment or from man-made bioreactors do not reveal how the microorganisms respond to the environmental signals, and how signal transduction eventually results in organized structures in complex communities. In order to develop a basis for this understanding, it is essential to create research scenarios that allow us to monitor closely these events under conditions that may be controlled and precisely determined. The following examples of microbial community analysis are based on much simpler consortia of bacteria kept in laboratory systems (flow chambers and in a few cases microtitration plates). The major advantages of these systems are (1) the controllable nutrient conditions, (2) the possibility of determining organism composition (including genetically varying the strains), and (3) the ability to follow developmental phases of a microbial community from the initial colonization steps to terminal events leading to removal of the biomass.

Initiation of Biofilm Development

Knowledge about the molecular mechanisms operating in biofilm development is now emerging. It was recently shown that major changes in the pattern of gene expression occur during biofilm formation: 38% of the genes in *Escherichia coli* were found to be differentially expressed in cells attaching to the wells of microtitration plates in comparison to planktonic cells [28]. Genes induced by high osmolarity, oxygen limitation, and high cell density were among the differentially expressed. Flagella or flagellar driven motility was found to have a role in the initial cell-to-surface interactions for both *E. coli* and *Pseudomonas aeruginosa* [24, 27]. Evidence indicating that for *E. coli* type I pili and curli are required for the initial surface attachment was provided [27, 42]. For *P. aeruginosa* the polar localized type IV pili evidently have a role in microcolony formation as they enable this organism to move on the substratum, and because a mutant deficient in type IV pili synthesis as opposed to the wild type did not form microcolonies [24]. In addition, it

has been proposed that microcolony formation in *Vibrio cholerae* EL Tor biofilms involves flagellar driven aggregation of surface-associated bacteria [43].

Extracellular Polymeric Substances in Biofilms

The microcolonies in most biofilms are surrounded by large amounts of extracellular polymeric substances (EPS) [12]. By monitoring expression from an *algC–lacZ* fusion in *P. aeruginosa,* using a fluorescent LacZ substrate, it was shown that genes encoding synthesis of alginate become induced after the initial attachment process [2]. Likewise, it was shown that genes encoding synthesis of colanic acid become induced in *E. coli* during biofilm formation [28]. Although it has been reported that EPS stabilize the spatial structure in *V. cholerae* EL Tor biofilms [43], it is not yet known whether EPS have a role in shaping the spatial structures in biofilms. Evidence has been provided that EPS can facilitate storage of nutrients in biofilms for subsequent intake during periods of carbon limitation [47, 48].

Functions of Biofilm Channels

Application of CLSM showed that biofilms are highly hydrated open structures containing a high fraction of EPS and large void spaces between microcolonies [12]. Mushroom-shaped secondary structures, separated by channels and voids, were observed in *Pseudomonas fluorescens* biofilms [9, 10]. Following up on the microscopic discoveries of the heterogeneous distribution of biomass in biofilms, and the existence of channels and void spaces, many attempts have been made to assign functionality to these structural elements. Evidence has been provided that the cell-free channels and pores present in biofilms increase the influx of substrate and nutrients to the inner parts of the biofilm and facilitate efflux of wastes. Liquid flow was shown to occur in the channels and pores of a trispecies model biofilm by tracking fluorescent beads using CSLM, and it was shown that the path and velocity of the flow was directed by the shape and thickness of the channels and pores [39]. Using a combination of microelectrodes and CSLM, it was shown that oxygen transport from the pores into the cell clusters was of the same magnitude as that directly from the bulk liquid into the cell clusters [4]. Using CSLM and *in situ* hybridization with a fluorescent probe targeting *Pseudomonas putida* and another probe targeting all eubacteria, the three-dimensional structure of a toluene-fed multispecies biofilm and the spatial distribution and activity of a toluene degrading *P. putida* biofilm member were investigated [20]. Microcolonies of the *P. putida* strain were found throughout the heterogeneous channel-containing biofilm, and judged from the ribosomal RNA content of the hybridized cells (see below), they were equally active independent of their location, indicating that substrate was supplied via the channels to the inner regions of the biofilm.

Heterogeneous Growth Activity in Biofilms

Knowledge of the distribution of bacterial growth activity in biofilms is fundamental for the understanding of the structural development and performance of biofilm, and may shed light on biofilm properties such as resistance toward antibiotics and biocides. In order to monitor growth activity at the level of single cells and local subpopulations, the well-established correlation between growth rate and ribosomal content [32] has been exploited. Quantification of the fluorescent light emitted from *in situ* rRNA hybridized cells indicated that sulfate-reducing bacteria in a young multispecies biofilm were more active than the sulfate-reducing bacteria in an established biofilm [26]. In another study, attached *P. putida* cells in a chemostat were shown to grow with a constant growth rate independent of the dilution rate, in contrast to the planktonic part of the population [19]. However, since both slow-growing and nongrowing bacteria have low ribosomal contents, and because cellular ribosomal contents only slowly adjust to new growth rates, quantitative *in situ* hybridization may not be suitable for measuring growth activity in heterogeneous and dynamic biofilms.

In order to obtain a better measure of *in situ* growth activity, a fusion between the *E. coli* ribosomal *rrnB*P1 promoter and the *gfp*(AAV) gene, encoding an unstable Gfp protein, was constructed [37]. Insertion of this fusion into the chromosomes of a range of proteobacteria (e.g., *E. coli, P. putida, P. aeruginosa, P. fluorescens,* and *Pseudomonas* sp. B13) produced growth activity reporter strains that emit green fluorescence with an intensity proportional to the growth activity [22, 37, and unpublished results]. When microcolonies of a *P. putida*::P*rrnB*P1-*gfp*(AAV) strain growing in a biofilm reached a critical size, the light emitted by the cells decreased in the center of the microcolonies and eventually throughout the microcolonies, indicating that the cells displayed different levels of growth activity correlating with their location in the biofilm and with the biomass of the biofilm [37].

The heterogeneous distribution of growth activity in biofilms has also been observed through the use of other ap-

proaches. A *Klebsiella pneumoniae* biofilm embedded, sectioned, and stained with acridine orange showed green in the interior with an orange band running along the biofilm–bulk fluid interface [44]. Since acridine orange stains RNA red and DNA green, this result indicated that the cells closest to the bulk liquid had the highest rRNA content. Observations of increased growth activity of the cells closest to the bulk liquid and channels have also been made from approaches that are independent of ribosomal contents. Gyrase inhibiting compounds (such as nalidixic acid and fleroxacin) can be used to estimate cell growth potential on the basis of the amount of cell elongation that occurs after exposure, and fleroxacin treatment of a *P. fluorescens* biofilm caused a gradient of cell elongation, with the greatest amount of cell elongation occurring near the biofilm–liquid interface [10]. Induction of alkaline phosphatase (APase) occurs after phosphate deprivation preferentially in actively growing cells, and when *P. aeruginosa* biofilms were shifted to low-phosphate medium, APase activity was detected (using a fluorogenic substrate) in a well-defined band immediately adjacent to the biofilm–bulk fluid interface [8]. Evidence was presented that growth in the interior of the *P. aeruginosa* biofilms was oxygen-limited [49].

Metabolic Interactions in Biofilms

As described above, the use of microelectrodes is a powerful technique for monitoring metabolic activities in relation to the structure of microbial communities. Metabolic activity may alternatively be recorded at the level of gene expression in the cells. RT-PCR-based methods for monitoring gene expression at the single cell level have been developed [7, 41]. These methods work well for suspended bacteria or monolayers of bacteria, but monitoring gene expression in individual cells in a biofilm is still a challenge. However, monitoring of gene expression at the single-cell level in complex microbial communities is possible in model systems that include organisms that carry reporter genes. By the use of this approach, interspecies metabolic cross-talk in a toluene-degrading biofilm was demonstrated [21]. The *P. putida* strain in the biofilm was substituted with a genetically modified strain containing the *xylS* gene and a fusion between the Pm promoter (from the Tol degradation pathway) and the *gfp* gene. XylS in combination with benzoates activates transcription from the Pm promoter, and the reporter bacteria therefore become green fluorescent in the presence of benzoates. By the use of fluorescent *in situ* hybridization and CSLM it was shown that the reporter *P. putida* strain became green fluorescent only when it was close to *Acinetobacter* microcolonies in the biofilm. Additional experiments showed that the *Acinetobacter* strain leaks benzoate when it grows with benzyl alcohol as the carbon source, and that the *P. putida* strain has a high affinity for benzoate (unpublished results).

The physical association of the *P. putida* and *Acinetobacter* species was not very tight. In another biofilm model system, a much closer coupling between two metabolically interacting species was observed [22]. In this model system *Burkholderia* sp. LB400 was capable of degrading 3-chlorobiphenyl to 3-chlorobenzoate, whereas *Pseudomonas* sp. B13 (FR1) could mineralize 3-chlorobenzoate, but was unable to degrade 3-chlorobiphenyl. When the consortium was grown on 3-chlorobiphenyl medium, it consisted predominantly of mixed microcolonies, but when it was grown on citrate medium (metabolizable by both organisms), it consisted predominantly of separate microcolonies of the two species (see Fig. 2). When a citrate-grown consortium was fed 3-chlorobiphenyl medium, the structure changed toward mixed microcolonies within 2 days after the substrate shift. Additional experiments indicated that *Pseudomonas* sp. B13(FR1) is chemotactic to 3-chlorobenzoate (unpublished results), suggesting that the substrate-induced structure formation may be a consequence of chemotactic motility. The *P. putida* strain from the toluene-degrading biofilm described above was shown not to be chemotactic to benzoate (unpublished results). However, close association between the *Acinetobacter* species and a benzoate-chemotactic *P. putida* strain was subsequently observed in a benzyl alcohol–grown biofilm (unpublished results).

Structure changes induced by a substrate shift were also observed by Wolfaardt et al. [46]. They studied a biofilm consortium capable of degrading the herbicide diclofop. When this consortium was grown on diclofop medium, a highly differentiated biofilm with specific patterns of intra- and intergeneric cellular coaggregation was formed. However, when the consortium was grown on tryptic soy broth, a biofilm showing no variation in thickness, structure, and spatial orientation of the cells was formed. After a shift in carbon source from tryptic soy broth to diclofop medium, the biofilm acquired the typical structure of diclofop-grown biofilms in only 2 days. The organisms and the nature of the interactions in the study of Wolfaardt et al. [46] were unknown.

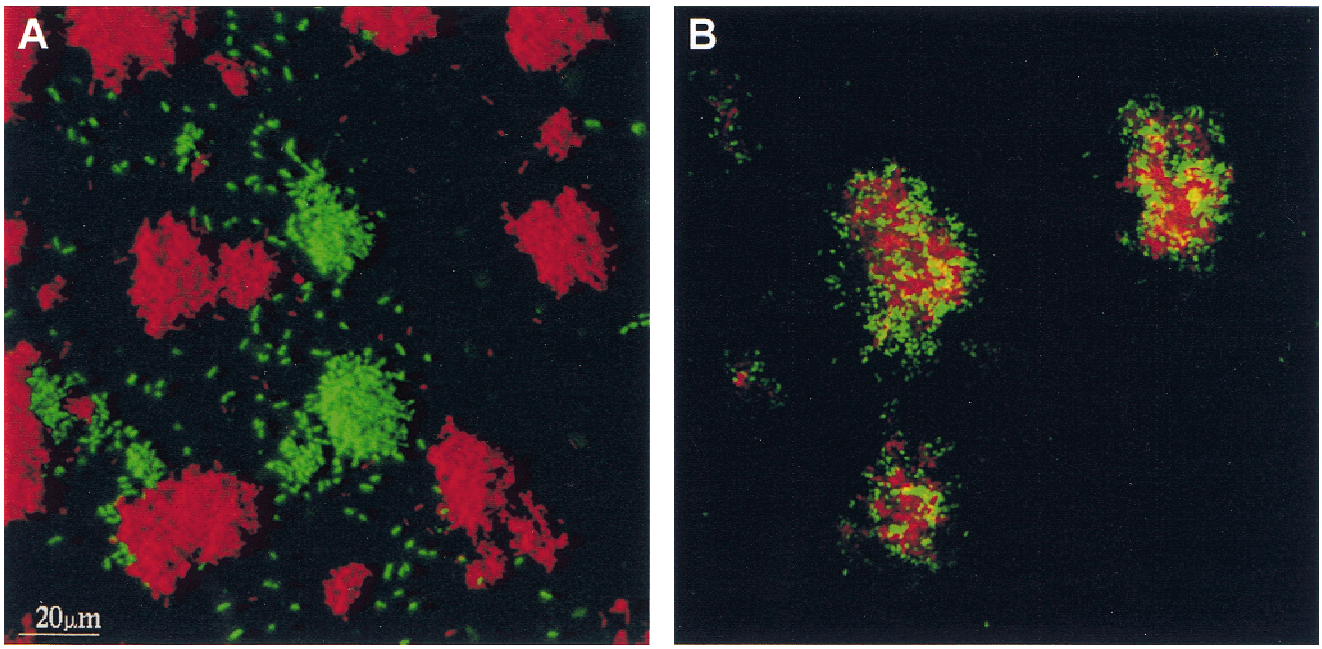

Fig. 2. CSLM micrographs of biofilms consisting of *Burkholderia* sp. LB 400 and *Pseudomonas* sp. B13(FR1). Fluorescent *in situ* hybridization with species-specific oligonucleotide probes stained *Burkholderia* sp. LB 400 red and *Pseudomonas* sp. B13(FR1) green. The biofilms were grown in flow cells irrigated with citrate minimal medium (**A**) or 3-chlorobiphenyl minimal medium (**B**). Reprinted from [22] with permission from Blackwell Science.

Conclusions from the Analysis of Biofilm Model Communities in Laboratory-Based Systems

The great resolution power of the combination of molecular biology and CSLM has been used by many to get a deeper insight into structure/function relationships in microbial communities. When employed in the context of flow-chamber biofilms (or equivalent systems) it has been possible to trace developments of specific microstructures to the level of individual cellular interactions. One significant take-home lesson from such investigations has been, once more, that many interesting features of microbial community life eventually can be described and understood from the basis of physiological activities. Synthrophies or commensalisms established in many natural and man-made contexts because of the diversity of bacterial metabolism have now been documented at all levels, from the macroscopic/global scale to close associations between single cells in microscopic clusters in biofilms. It is particularly interesting to see that even weak selective forces in favor of close interactions and couplings between different bacteria take place in microbial communities, resulting in specific organizational features in relation to the population composition and the environmental conditions. The rapid increase in numbers of genomic sequences in combination with the development of molecular *in situ* techniques will in the future result in much more focused investigations of specific relations between organism genotypes and developmental strategies for microbial community development. The interesting question is whether the traditional objective of molecular biology—the search for cause/effect relationships—is at all relevant and compatible with community complexity. In other words, does it make sense to attempt to understand the development of a multispecies community on basis of an analysis of specific gene expression in individual cells?

It is tempting to speculate that if community organization in multispecies communities may be explained on the basis of weak and strong metabolic interactions combined with the bacterial chemotactic properties, then even in the simplest monospecies communities structural differentiation may be explained in a similar way: After the initial colonization phase, during which single adhering cells form the first microscopic colonies, further community growth is preferentially determined by metabolic interactions within and between the microcolonies. Controlled by the local microenvironments created within and around the cell clusters, the motility apparatus of the individual cells respond to the

gradients of these local environments, leading to attraction or repellent reorganization activities. In this way, syntrophic relationships may even develop between isogenic cells in a monospecies community, because of differential phenotypic expressions in different parts of the community.

Most bacteria seem to possess the capacity to adhere to surfaces (important for the initial colonization events), to move in response to the existing gradients of nutritional signals in their immediate vicinity (important for the formation of syntrophic and other relationships), and to rapidly convert available resources to growth activity (important for the structured increase in biomass). An important question is whether other types of microbial interactions are necessary for the development of organized community structures and activities.

Regulation of the Organization of Microbial Communities

The examples presented above strongly suggest that structures of sessile communities are closely related to the interactions and activities that occur in the communities. We now have evidence that the channels and pores in mono- and multispecies biofilms allow influx of substrates and efflux of waste products, and that the spatial organization of the different organisms in multispecies biofilms reflects syntrophic and microhabitat localized processes. It is not fully understood what controls the spatial organization of the microorganisms in biofilms, but at least two hypotheses that are very different, but not mutually exclusive, have been offered.

The first hypothesis is mechanistic and supported by computer simulations of biofilm growth, which explains various structural forms in biofilms as a result of differences in local substrate availability [25, 45]. According to this model, heterogeneous biofilm structures develop when biofilm growth occurs under substrate-transfer-limited conditions. Local consumption of substrate creates substrate gradients, whereby organisms situated on "mounds" have more substrate available than organisms situated in "valley." The mechanistic hypothesis does not take into account cell motility toward substrates (i.e., chemotaxis), but as discussed above, the organization found especially in syntrophic multispecies biofilms suggests an active involvement of chemotactic motility during the development of community structures.

The other hypothesis is a morphogenetic hypothesis, which suggests that bacteria in biofilms differentiate and express genes that directly control the spatial organization of the organisms (e.g., [1]). It has been suggested that cell-to-cell communication plays a role in determining the spatial organization of the bacteria in sessile communities (e.g., [1, 18, 36]). Intercellular communication mediated by acylated homoserine lactones (AHL) has been shown to be involved in regulation of specific gene expression as a function of population density, and could be an example of a community regulator. It has in fact been documented that bacteria in biofilms from widely different ecosystems such as river stones and urethral catheters produce AHLs [15, 38]. Moreover, a *P. aeruginosa* mutant deficient for synthesis of an AHL produced flat and undifferentiated biofilms, but addition of the AHL to the medium resulted in production of a structured biofilm typical of the wild-type strain [3].

We should like to argue that it probably does not make sense to make firm decisions about one or the other explanation as the rule for community development. It is on one hand nearly impossible to eliminate the mechanistic model, since we know that bacteria as individuals must react to nutrient gradients in ways indicated by the model. On the other hand we also know now that bacterial signaling does take place and that intercellular interactions of this type often affect the regulatory activities in the cells resulting in coordinated performances. We should therefore avoid generalized interpretations based on specific views of microbial communities either as aggregates of selfish individuals constantly competing for nutrients, or as multicellular organisms governed by hormone-based communication control. The interesting and challenging view is that microbes in fact develop complex communities under the influence of many activities and events: Stochastic colonization of the surface, creation of nutritional gradients and subsequent growth differentiation, chemotactic movements within the community creating redistribution of biomass, establishment of synthrophic relationships, and excretion of regulatory communication signals leading to construction of new organizational forms. The complexity of these biological systems has until recently been an impossible barrier against detailed investigations; now we have tools that enable us to initiate the analysis and obtain the first answers to the more pertinent questions. We should, however, remember that even though we may now begin to tackle the complexity of microbial community organization, complexity remains an inherent quality of these systems, and we should therefore be prepared for some quite complex answers to our questions. If we focus only on one small aspect of this complexity, we may

be able to jump to quick conclusions, but the validity of these conclusions will be as limited as our focus.

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