Monitoring of cellular activities in multispecies bacterial surface communities

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ABSTRACT

We have developed a number of molecular methods and tools useful in the field of microbial physiology and ecology - especially in the analysis of microbial community performance. These methods allow detailed investigations at the single-cell level of bacterial physiological activities, specific gene expression, gene transfer and cell-to-cell communication. Community features such as surface colonization, metabolic interactions, utilization of carbon and energy sources, bacterial motility, microcolony structure and activity, have been analysed in various types of surface bound bacterial communities of different complexities. The major and highly significant conclusion from these studies is that bacteria living attached to surfaces - the dominant form of bacterial life in nature - organize themselves in heterogenous consortia, in which organisms, activities and structural features are distributed in compartments. It is possible that part of this complexity is controlled by chemical communication between the community members.

Introduction

Bacterial activity in natural settings is nearly always connected with surface bound microbial communities. The life of bacteria in such biofilms is in many respects quite different from the planktonic life of bacteria in suspension - the traditional laboratory scenario. These differences imply: 1) Surface communities develop internal heterogeneities; 2) structure/function relationships in surface communities are important targets for investigations of biological activities; 3) it is important to define both cellular and community responses to changing environmental conditions for surface communities. Our research activities have aimed at meeting the experimental challenges connected with investigations of complex adaptive communities at two different levels: Creation of a theoretical framework for investigation strategies, and development of a number of experimental tools and approaches applicable to detailed studies of mixed surface communities.

We have chosen to develop simple laboratory model systems for these investigations of surface bound microbial communities, and central in our studies is development of moelcular tools allowing us to analyse structure/function relationships at the single-cell level. In particular, we have focused on microbial activities at the level of physiological activity and specific gene expression activity.

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Materials and Methods

Biological Systems.

The majority of our in-house investigations are performed in *flow-chambers*, in which surface communities are established as biofilms on glass slides, and nutrients are supplied as continuous flows through channels [3, 14]. The advantages of this system are: reproducibility, ease of operation, direct mounting of chamber on microscope, controllable populations and nutritional conditions, and possibilities of investigator controlled perturbations. We have worked out routine protocols for analysis of the biofilms under conditions where important features such as three-dimensional structures are unaffected by the analysis (e.g. embedding of biofilms in polyacrylamide prior to fixation and subsequent manipulations [7]).

Bacterial Identification.

Identification of bacterial strains and species is based on the use of rRNA sequence information and design of fluorescent probes targeting the ribosomal RNA [1]. We have applied and optimized this method of detection to all the biosystems mentioned above. Introduction of new and/or unknown organisms leads to rRNA sequence determination from which phylogenetic allocation is determined and probes may be designed [15].

Microscopy and Image Analysis.

The fluorescence microscope is central in all our community investigations. We use microscopic set-ups for standard microscopy and image capturing: Zeiss Axioplan Epifluorescence microscopes equipped with CCD cameras. From these we obtain high-resolution micrographs of two-dimensional objects, and in addition we also obtain quantitative information in connection with image analysis using the Cellstat software, which we developed for measurements of fluorescence intensities, cell dimensions, patterns of cell organization etc. [14]. For structural analysis of biofilms we use a scanning confocal laser microscope (Leica) equipped with an extra UV laser. This microscope yields high-resolution three-dimensional images of our bacterial communities [4, 15], and due to the confocal principle it allows visualization of bacteria even in connection with high backgrounds of autofluorescence (e.g. plant root zones).

Detection of mRNA in single cells.

The method of *in situ* rRNA hybridization rests on the large number of rRNA target molecules in the individual cells. The numbers of the most abundant mRNA molecules in bacteria are orders of magnitude lower and therefore not detectable by the same approach of hybridization. Based on the method developed by Hodson et al. [11], we have developed an alternative method for single-cell detection of specific mRNA molecules based on a semi-nested *in situ* RT-PCR amplification (biotin-labelled primers) of the target sequence. Through an extra amplification step using a coupled enzyme reaction (biotin/streptavidin coupled with HRP), it is possible to detect even a single mRNA molecule in a bacterial cell [21].

Reporter Gene Technology.

In our investigations of microbial communities we most often have the possibility of employing genetically modified strains, since the population composition at least to some

extent is defined by the investigator. It has therefore been extremely useful to construct indicator strains, in which specific combinations of an indigenous gene in the organism and a reporter gene compatible with detection in the light microscope have been fused. We have made use of the *lacZ* reporter gene for which we developed a single-cell fluorescent assay based on X-gal [17]. The major advantage of this reporter is that it does not require oxygen for its activity, but it is a draw-back that substrate (X-gal) must be added to the cells to allow detection. The *luxA,B* genes have been very useful for detection of very low bioluminescence signals [18, 19]. Bioluminescence, however, depends on oxygen and reduced nucleotides (energy metabolism), which limits its application in certain contexts. Moreover, it is not compatible with scanning confocal laser microscopy. The *gfp* gene expressing the green fluorescent protein, Gfp, is so far the most useful reporter due to its clear fluorescence detectable in single cells and the lack of substrate [5]. We have constructed a large library of tools based on Gfp to be used as reporters in a number of applications [6, 10, 16]. Through genetic manipulation of the C-terminal end of the protein, we furthermore designed a series of Gfp reporter proteins with severely reduced stability; these unstable reporter proteins allow studies of changing expression levels in the cell [2]. The potential problem with Gfp as a reporter is the requirement for oxygen in the formation of the fluorophore in the protein, but we have recently shown that the fluorophore is formed effectively even under severe oxygen limitations [20].

Results

We have carried out an analysis of a toluene degrading gas reactor based on a microbial community from a local aquifer. The important outcome of this analysis was that one specific organism – a strain of *P. putida* – despite its fairly low representation in the community (ca. 4%) was responsible for about 65% of the removal of toluene [15]. We created a bacterial community of reduced complexity from this bio-reactor, consisting of 7 isolated strains of which three are able to use toluene as their sole carbon and energy source (*P. putida, Acinetobacter* sp. and *Burkholderia cepacia*), whereas the last four are not. In some experiments, only the three toluene degrading strains have been employed. This community has been central in a range of experimental scenarios, in which we have investigated colonization patterns, growth physiology, structural organization, structure/function relationships, and plasmid transfer.

Biofilms composed of the 3 or 7 species (identified by *in situ* rRNA hybridization) were established in the flow chambers under conditions, where benzyl alcohol was the only available carbon source (requires a toluene degrading pathway for its utilization). The first important observation made was that a small but stable population of the non-degrading strains was maintained in the community. These organisms must live on substrates derived from the degrading organisms (food-chain).

Quantitative *in situ* rRNA hybridization useful for estimating bacterial growth rates [9] showed that cells located near the surface (far from the substrate flow) grow much slower than those located near the biofilm surface (close to the substrate flow) [13]. There is a nutrient gradient down through the biofilm. In a close-up analysis of the distribution of growth activity in the community an alternative reporter of growth was utilized: A fusion between one of the growth rate dependent rRNA promoters from *E. coli* and the gene for unstable Gfp was constructed. This reporter is very useful for identification of actively growing cells, because any stop of growth will result in rapid disappearance of green fluorescence from the cells. When introduced in *P. putida* it showed the expected

phenotype. In the biofilms it could be shown that in micro-colonies there was only a thin surface layer of actively growing cells (green fluorescent), whereas the majority of cells in the inner parts of the colonies seemed to grow very slowly or not at all. This indicates that also in the microenvironments there are very heterogeneous conditions and sub-populations.

Closer examination of two of the strains in the community (*P. putida* and *Acinetobacter*) revealed a metabolic cross-talk between them. When one of the promoters of the TOL operon (induced by an intermediate in the degradation pathway (benzoate)) was fused to the *gfp* reporter gene in *P. putida*, and a biofilm with the other species in the toluene degrading community was established, it was found that the TOL promoter was only induced in *P. putida* cells closely associated with micro-colonies of *Acinetobacter* [16]. This indicates once more that specific micro-environments exist in such surface communities, and that nutrients and other chemical components are heterogeneously distributed.

Discussion

The flow-chamber biofilm has been a very central model system for complex microbial communities in essentially all our work. In the Results section, a sample of observations and information obtained from one community growing on toluene derivative carbon sources has been presented, and the data show that several organisms may be able to coexist stably despite significant differences in growth rates. The dominant feature of the mixed communities is heterogeneity, and there are good indications of reproducibility of pattern formation when the same mixture of organisms grows under similar conditions. It thus seems possible to make consistent conclusions concerning structure/function relationships in such communities, which is a key point in the theoretical work developed in the present program.

The SCIO model was developed as an investigation platform for systematic descriptions of microbial communities [12]. The four system parameters, S (structure), C (coordination), I (interactions) and O (organisms), have been useful as guides for the method development, and essentially all four may now be monitored in any complex community. **Structure** analysis is carried out by the scanning confocal laser microscope, **Coordination** is recorded as ordered community behavior in response to environmental changes and possibly influenced by cell-to-cell communication signals, **Interactions** are documented by specific reporters reacting to factors in the surroundings (including metabolites from other community members), and **Organisms** are identified by specific tagging markers or through *in situ* rRNA hybridization. In the SCIO model the four parameters have been combined to describe degrees of coupling (the S and O parameters) and regulation (the I and C parameters) in the communities, and based on such extrapolations of the parameter analysis we have proposed the possibility of a systemic taxonomy. In our work with different types of flow-chamber communities we have already observed features indicating that such taxonomic descriptions are in fact relevant and informative. The recent communication by Davies et al. [8] suggesting that quorum sensing signals may have a direct or an indirect effect on biofilm structure formation is an interesting example of coordination in microbial communities which deserves a great deal of attention.

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