In situ examination of microbial populations in a model drinking water distribution system

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Abstract A flow cell set-up was used as a model drinking water distribution system to analyze the *in situ* microbial population. Biofilm growth was followed by transmission light microscopy for 81 days and showed a biofilm consisting of microcolonies separated by a monolayer of cells. Protozoans (ciliates and flagellates) were often seen attached to the microcolonies. The biofilm was hybridized with oligonucleotide probes specific for all bacteria and the α- and β-subclass of *Proteobacteria* and visualized with a scanning confocal laser microscope. Hybridization showed that the microcolonies primarily consisted of a mixed population of α- and β-*Proteobacteria*. 65 strains from the inlet water and 20 from the biofilm were isolated on R_2A agar plates and sorted into groups with amplified rDNA restriction analysis. The 16S rDNA gene was sequenced for representatives of the abundant groups. A phylogenetic analysis revealed that the majority of the isolated strains from the bulk water and biofilm were affiliated to the family of *Comamonadaceae* in the β-lineage of *Proteobacteria*. The majority of the strains from the α-lineage were affiliated to the family of *Sphingomonadaceae*. We were unable to detect any strains from the *Pseudomonas* genus and found a low abundance of bacteria affiliated to the γ-subclass of *Proteobacteria* where *Pseudomonas* and *E. coli* are positioned. The analysis revealed a high bacterial diversity in the water phase as well as the biofilm, but no strains were found in both environments.

Keywords Biofilm; *Comamonadaceae*; drinking water; FISH; phylogeny; *Sphingomonadaceae*

Introduction

The composition of microbial populations in drinking water distribution systems is important in understanding bacteriological processes, including the fate of pathogens. In low nutrient environments it may be assumed that bacteria adapted to oligotrophic conditions are the first colonizers of the system whereas potential pathogens like *E. coli* or eukaryotic organisms are considered to be secondary colonizers feeding on remnants and excretions. Therefore, to perform a risk analysis for the pathogens in water supply systems, it is necessary to understand the ecology of the indigenous population including their interactions with the surroundings (Szewzyk *et al*., 2000). At present, the composition of microbial populations in potable water is mostly treated as "a black box" and at best addressed with heterotrophic plate count or total count enumeration.

Another aspect of the microbial ecology in water supply systems is the fact that several microorganisms are used as indicators for various parameters of the hygienic quality. For example, a *P. fluorescens* P17 is used in the assimilable organic carbon test assay (AOC) as an indicator of the accessibility and amount of organic nutrients in water (van der Kooij, 1992). To evaluate if the metabolic potential of *P. fluorescens* is representative for the indigenous microflora, a deeper insight into the composition and physiology of the autochthonous bacterial population is needed.

Using a standard flow cell set-up supplied with drinking water one can visualize the microbial distribution as well as their structural composition using fluorescent *in situ* hybridization combined with microscopical techniques. With this approach we wanted to

analyze which microorganisms that colonized the surface and their spatial distribution and compare it with the free-flowing community.

Materials and methods

Flow cell set-up

The biofilm was grown in small flow cells made of Plexiglas, with a microscope coverslip attached on top with silicone-glue (Wolfaardt *et al.*, 1994). This construction made it possible to follow the biofilm build-up and microbial diversity by transmission light microscopy without destruction of the sample. The system was operated with laminar flow ($v = 15.5$ mm/s $\Rightarrow$ $N_{Re} = 15.5$) at 23°C. For a more detailed description of the set-up, see Christensen *et al.* (1999). Water from a household tap at the Technical University of Denmark was continually fed to the system and the indigenous microbial population served as inoculum for the flow cells.

Biofilm analysis

The formation of biofilm was followed for 81 days with transmission light microscopy. The biofilm was also examined with fluorescent *in situ* hybridization (FISH) followed by scanning confocal laser microscopy (SCLM) (for a detailed description, see Christensen *et al.*, 1999). Prior to hybridization, the biofilm was fixed with cold 4% formaldehyde and embedded in 20% polyacrylamide to preserve the hydrated structure (Møller *et al.*, 1998). Hybridizations were done at 46°C and 30% formamide (final concentration after addition of probe) with the Eub338 (Amann *et al.*, 1990), ALF1b, and BET42a probes (Manz *et al.*, 1992). The hybridized biofilm was analyzed with scanning confocal laser microcopy and multichannel simulated fluorescence projection (SFP) images were generated using IMARIS software (Bitplane AG, Zürich, Switzerland).

Isolation and phylogenetic analysis of culturable strains

Bacterial strains were isolated on R2A medium (10 d, 23°C). Selected colonies were re-streaked twice and harvested on plates since many strains did not grow in liquid culture. DNA from the harvested cell-cultures was extracted according to Grimberg *et al.* (1989). All strains were hybridized with Eub338, ALF1b, BET42a and Gam42a to get a broad phylogenetic affiliation. The 16S rDNA gene was PCR amplified using 9F (5′-GAG TTT GAT CCT GGC TCAG-3′) and 1492R (5′-GGC/T TAC CTT GTT ACG ACTT-3′) (Lane, 1991) primer set and an annealing temperature of 52°C. The isolated strains were analyzed by amplified rDNA restriction analysis (ARDRA) using the restriction enzymes *RsaI* and *MspI*. The obtained bandpatterns were separated on a 1.7% agarose gel and analyzed using GeneTools (Syngene, Cambridge, UK). The 16S rDNA of selected representatives of OTUs (operational taxonomical units) were sequenced using the same primer set as PCR and aligned with the ARB software package (Strunk *et al.*, 2001). A phylogenetic analysis was performed using both the neighbor-joining method (w. Jukes–Cantor distance correction) and maximum parsimony and the result of the two algorithms was compared.

Results and discussion

Biofilm structure

The biofilm formation in the flow cells was followed for 81 days with transmission light microscopy to give a first impression of the biological system without interrupting it. Figures 1A–1D show the microbial colonization and biofilm development in a flow chamber. Many single cells were immobilized at the surface after 4 days, and a few microcolonies had been formed. The bacteria were mostly cocci or rod shaped. A few bigger microcolonies had developed after 12 days, and protozoans were grazing on these larger microcolonies. By day 17, the microcolonies had developed further, however, there

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were still large areas of a monolayer of cells surrounding the microbial stacks. At day 24, larger microcolonies with protozoans attached was observed. This biofilm structure was commonly observed during the rest of the study.

Several models predicting the biofilm structure have been proposed by different researchers (Wimpenny and Colasanti, 1997). The heterogeneous mosaic biofilm model proposes that biofilms consist of microcolonies attached to substratum at the base, but well separated from their neighbors (Walker *et al.*, 1995). Between the microcolonies individual cells attach to the surface forming a very thin film layer. The main difference to the water-channel model proposed by Costerton *et al.* (1994) is sparseness of the observed microcolonies, which stand as unconnected towers surrounded by the water phase. Using a cellular automaton model, Wimpenny and Colasanti (1997) showed that a structure closely related to the heterogeneous mosaic biofilm model could be formed at low substrate concentration. The observations in this study are consistent with this model, indicating that the low carbon concentration in the inlet water results in a biofilm consisting of microcolonies scattered around the surface separated by large areas colonized only by single cells.

Biofilm composition

A high number of protozoans were observed in connection to the biofilm. Several morphologically distinct types such as flagellates and ciliates (round and oval) were present. Most types were observed repeatedly during the study and were present in the biofilm in high numbers from an early stage. Figure 1E shows an example of protozoan abundance in the flow chamber after only 12 days. The eukaryotes were more or less evenly distributed on the substratum and phenomena like grazing fronts as previously reported (Kalmbach *et al.*, 1997a) were not observed. The number of eukaryotes did not appear to be influenced by the amount of bacterial biomass, since a significant number of protozoans were seen even at periods with low bacterial numbers. A protist community seems to be linked to biofilms formed in drinking water distribution systems (Foissner, 1996; Kalmbach *et al.*, 1997a; Sibille *et al.*, 1998). This can have potential health implications as various pathogenic bacterial species proliferate in association with protozoans, e.g. *Legionella* (Atlas, 1999) and *Mycobacterium* (Steinert *et al.*, 1998).

The bacterial biofilm was hybridized with both α- and β-*Proteobacteria* and eubacterial probes (Figure 1F) and visualized using a scanning confocal laser microscope. The bacteria formed small, loosely spaced microcolonies on the substratum, in accordance to the observations from transmission light microscopy. The microcolonies were approximately 10 μm high. All *Proteobacteria* should fluoresce with the eubacterial probe and one could see that there were a high number of orange colored cells (EUB338 + BET42a) and only a few red cells (BET42a), indicating a low degree of unspecific staining. The photomicrograph shows that the bacteria affiliated to β-*Proteobacteria* are dominant together with a significant number belonging to the α-*Proteobacteria*. Bacteria affiliated to the β-subclass of *Proteobacteria* seem to constitute an important part of the *in situ* biofilm population in drinking water distribution systems (Manz *et al.*, 1993; Kalmbach *et al.*, 1997b). Moreover, the microcolonies consisted of mixed populations of α- and β-*Proteobacteria*. This could indicate interactions between various species in the biofilm.

The silicon tubes used to connect the flow cells are believed to contain biologically usable compounds such as solvents or plasticizers. This could change the structure and composition of the biofilm due to selection of organisms that are able to degrade these compounds or simply a general elevation of the carbon and energy source. The presence of protozoans in the biofilm could also be determined by the low flow rate under which the system was operated. Thus, the specific model set-up used may influence the resulting community structure.
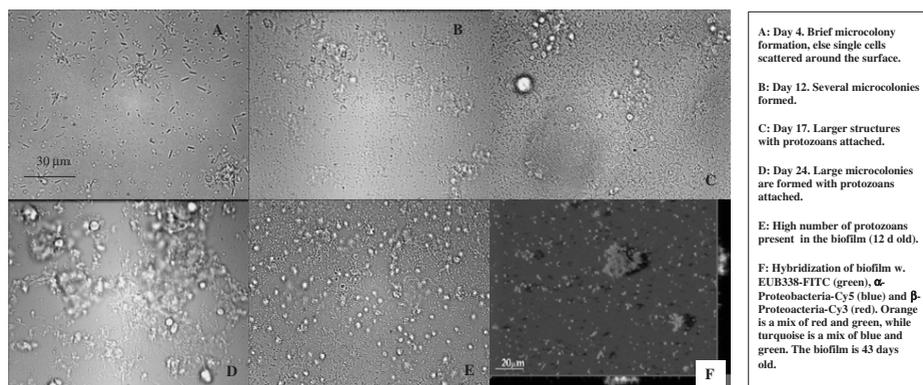
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A: Day 4. Brief microcolony formation, else single cells scattered around the surface.

B: Day 12. Several microcolonies formed.

C: Day 17. Larger structures with protozoans attached.

D: Day 24. Large microcolonies are formed with protozoans attached.

E: High number of protozoans present in the biofilm (12 d old).

F: Hybridization of biofilm w. EUB338-FITC (green), **α**-Proteobacteria-Cy5 (blue) and **β**-Proteoacteria-Cy3 (red). Orange is a mix of red and green, while turquoise is a mix of blue and green. The biofilm is 43 days old.

Figure 1 Development of the biofilm and microbial colonization (see legend for details of each image)

Table 1 List of OTU's generated from RFLP analysis of strain from the bulk water phase and biofilm. α, β and γ refers to the subclass of *Proteobacteria*. Note: x = partial sequencing (approximately 800 bp). xx = full sequencing (1,500 bp)

OTU	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Inlet water	1	–	1	2	4	1	1	1	1	–	–	–	–	–	–	–	35	2	1	4	1	1	1	1	5	1	1
Biofilm	–	4	–	–	–	–	–	–		5	6	1	1	1	1	1	–	–	–	–	–	–	–	–	–	–	–
Phylogeny	β	α	β	?	β	α	α	?	?	α	β	β	α	α	α	α	β	β	?	γ	β	?	α	?	α	α	β
Sequenced	x	xx	–	–	x	–	–	–	–	xx	xx	–	–	–	–	–	xx	–	–	x	xx	–	–	–	x	–	x

Phylogenetic analysis of isolates

65 bacterial strains from the inlet water and 20 from the biofilm were isolated on R$_2$A agar plates. Using restriction fragment length polymorphism on PCR amplified 16S rDNA (ARDRA), these were grouped into 27 operational taxonomical units (OTU) and a representative from each of the abundant OTUs was sequenced. *MspI* and *RsaI* were used for the restriction analysis, since they have a high resolution in separating strains affiliated to the *Proteobacteria* phylum (Moyer *et al.*, 1996). FISH analysis of the strains showed that all OTUs were phylogenetic homogeneous, supporting the results from ARDRA. Table 1 lists the results from the restriction analysis, the phylogenetic affiliation and strains that were sequenced.

The analysis revealed a high bacterial diversity in the water phase as well as the biofilm, but no OTUs were found in both environments. This result indicates a low interaction between the two niches, but more strains need to be analyzed to be conclusive. The majority of the strains in the planktonic phase were β-*Proteobacteria* (69%) whereas only a few strains from the α- and γ-subclass were found. Few strains could not be affiliated with the *Proteobacteria*. The distribution was somewhat different among the sessile population. The majority of the strains were affiliated with the α-subclass (65%), whereas the β-subclass only constituted a minority (35%). In contrast FISH showed that the β-lineage dominated. This difference may be due to the selection of bacteria by cultivation on plates. In most cases less than 1% of the total microbial population is cultivable (Amann *et al.*, 1995). The bias of cultivation was also demonstrated in a recent study of a German drinking water distribution system; the *in situ* dominant genus (*Aquabacterium*) in a young biofilm was only found in low abundance on plates (Kalmbach *et al.*, 1997b).

A phylogenetic reconstruction based on sequences of 16S rRNA with both the neighbor-joining and maximum parsimony methods positioned the abundant culturable species similarly. An analysis based on phylogenetic reconstruction gives a better identification than a basic GenBank search because it takes character data into account, whereas a GenBank search is based on a similarity index.

Table 2 Phylogenetic inference analyzed with neighbor-joining and maximum parsimony method and named using *Bergey's Manual*. The resolution is defined as the possible level of identification from the phylogenetic reconstruction (tree)

OTU	Phylogeny of sequenced strains	16S rDNA resolution
1	*Oxalobacteraceae*	Family
2	*Sphingomonaceae/Blastomonas* spp.	Family/genus
5	*Rhodocyclaceae*	Family
10	*Sphingomonas* yanoikuyae	Species
11	*Comamonas* spp.	Genus
17	*Comamonadaceae*	Family
20	*Xanthomonadaceae*	Family
21	*Comamonadaceae*	Family
25	*Sphingomonas* spp.	Genus
27	*Comamonadaceae*	Family

A conservative view on the level of identification (e.g. genus vs. species) has been applied in positioning the isolated strains. It has been shown that the resolution power of 16S rRNA analysis is limited, so it is dubious whether 16S rRNA analysis can be used for identification at the species level. DNA–DNA reassociation values are currently the golden standard for assigning new isolates at species level. However, studies have proven that there is not a direct correlation between 16S rRNA homology and DNA–DNA reassociation-level (Stackebrandt and Goebel, 1994). Lateral gene transfer may be one reason for this discrepancy (Doolittle, 1999). Thus, various phenotypic characteristics such as substrate utilization profile, serology, etc. are often necessary to assign new strains at species level (Vandamme *et al.*, 1996).

Strains from OTU 11, 17, 21, and 27 (>50% of total isolates) belong to the family of *Comamonadaceae*. Bacteria from this family are known to proliferate in oligotrophic environments and have previously been isolated from a drinking water supply system (Norton and LeChevallier, 2000). In a study of various distribution networks in northern Europe, a group of strains from *Comamonadaceae* belonging to the genus *Aquabacterium* dominated the biofilm (Kalmbach *et al.*, 1999). Strains from OTU 2, 10, and 25 (>66% of α-*Proteobacteria*) are affiliated with the *Sphingomonaceae* family and seemed to be highly abundant, in particular in the biofilm. Strains from this family have also previously been isolated from different water supply systems (Norton and LeChevallier, 2000; Koskinen *et al.*, 2000) and are shown to be adapted to oligotrophic environments (Fegatella and Cavicchioli, 2000). Studies have stated that pseudomonads dominate and reflect the metabolic potential of the microbial population in drinking water distribution systems, but here the strains were isolated on a richer media (Ribas *et al.*, 2000). In this study we were unable to detect any strains from this genus and found a low abundance of bacteria affiliated to the γ-subclass of *Proteobacteria* where *Pseudomonas* and *E. coli* are positioned.

Conclusion

This study confirms previous studies that species from the α- and β-subclass of *Proteobacteria* are highly abundant in drinking water distribution systems. Isolated species were affiliated to the *Sphingomonadaceae* from the α-lineage and to the *Comamonadaceae* from the β-lineage of *Proteobacteria*. Strains from these phylogenetic groups have previously been found in oligotrophic environments including water distribution networks and are in some cases highly abundant. These findings suggest that pseudomonads might not reflect the composition and metabolic potential of the indigenous microbial population in oligotrophic environments like a water supply system. The *in situ* analysis shows that the biofilm consists of a mixed population of various bacterial strains as well as protozoans.

The degree of association (e.g. mixed microcolonies and attached protozoans) indicates that the different organisms interact.

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