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Proteobacteria, extremophiles and unassigned species dominate in a tape-like showerhead biofilm

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ABSTRACT

The development of showerhead biofilms exposes the user to repeated contact with potentially pathogenic microbes, yet we know relatively little about the content of these aggregates. The aim of the present study was to examine the microbial content of tape-like films found protruding from a domestic showerhead. Culturing showed that the films were dominated by aerobic α- and β-proteobacteria. Three isolates made up almost the entire plate count. These were a *Brevundimonas* species, a metalophilic *Cupriavidus* species and a thermophile, *Geobacillus* species. Furthermore, it was shown that the *Cupriavidus* isolate alone had a high capacity for biofilm formation and thus might be the initiator of biofilm production. A clone library revealed the same general composition. However, half of the 70 clones analyzed could not be assigned to a particular bacterial phylum and of these 29 differed from one another by only 1–2 base pairs, indicating a single species. Thus both the culture dependent and culture independent characterizations suggest a simple yet novel composition. The work is important as the biofilm is fundamentally different in form (tape-like) and content from that of all previously reported ones, where variously *Mycobacterium*, *Methylobacterium* and *Xanthomonas* species have dominated, and extremophiles were not reported.

Introduction

Biofilms are microbial communities that grow attached to surfaces and/or interfaces; they are embedded in a frequently self-produced matrix of extracellular polymeric substances.[1] The development of biofilms in bathroom environments brings the microbes present into close contact with humans. Biofilms have been reported in washstands,[2] on shower curtains[3] and in showerheads.[4,5] They can constitute potential reservoirs for pathogens, particularly for immune-compromised individuals. Showerhead biofilms have, for example, been shown to include and enrich pathogenic (e.g., *Legionella pneumophilia, Mycobacterium avium*) and opportunistic pathogens such as non-tuberculous mycobacteria.[4] Microbes which dislodge from shower biofilms come into

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contact with the skin and also the pulmonary system by way of aerosolization. It has been hypothesized that the recent rise in pulmonary infection with non-tuberculous mycobacteria is linked to the increased use of showers rather than baths.[6] Showerheads can be considered as extreme environments. For example, microbes present in mature biofilms must survive at least short periods of high temperatures, and bathroom biofilms might then enrich for thermophilic species. Most studies have focused on the specific presence of *Legionella pneumophilia* and *Mycobacterium avium*, and little is known of the prevalence and identities of other microorganisms that may be delivered during shower usage. In fact, to the best of our knowledge there exist only two comprehensive investigations of showerhead biofilms.[4,5] A related study of shower curtain biofilms has also been published.[3] In one study,[4] it was reported that the showerhead environment strongly enriched for microbes able to form biofilms in water systems, including *Methylobacterium* spp. and *Sphingomonas* spp. and, most strikingly, non-tuberculous mycobacteria which accounted for more than about one third of the clone library. As the study was non-culture based and did not include metabolic stains, it did not provide information on the metabolically active and culturable fraction of the analyzed biofilms. Strikingly different were the results of pyrosequencing of 16S rRNA genes amplified from biofilms on the outer surface (spray plate) of three showerheads.[5] A preponderance of γ-proteobacteria was demonstrated, with the genus *Xanthomonas* accounting for more than one third of the total sequence information. The corresponding culture-based analysis showed a much more even distribution of a wide-range of gram-negative and gram-positive genera. Given the small number of investigations, the detection of potentially pathogenic bacteria and the wide variation in the results found, there is need for more studies to improve the field. There is great variation in the origins (e.g., surface and groundwaters), temperature and quality of domestic water supplies, as well as in water disinfection practices. We thus hypothesize that as more studies of showerhead biofilms become available, new and strikingly different contents of potential health significance might be revealed. Previous studies have collected biofilms by either swabbing or scraping, and no mention is made of observed variations, if any, in the appearance of biofilms collected. The biofilm analyzed in the present work was unusual in that it presented as free-hanging, tape-like strands which must at least in part be attributable to the properties of the species present in it. The present study uses both culture and non-culture based techniques, to identify the composition of the biofilm. Unlike previous work it also looks at the content of fungi and protozoa and investigates the potential for biofilm formation among individual bacterial isolates. The latter may help to identify the main progenitors of biofilm formation and aid in the development of strategies to control biofilm development.

Materials and methods

Biofilm origin, appearance and collection

The biofilm consisted of strips (>1 cm) of slightly opaque, elastic, tape-like material which had emerged and hung from the vents in a domestic, plastic showerhead. The showerhead was delivered to a local Norwegian water works by a concerned resident who found the material unsightly. The home received UV and hypochlorite treated surface water from a municipal water works. Small pieces of the material were aseptically sectioned with a scalpel and washed carefully with 5 serial changes (10 ml aliquots) of pharmaceutical grade, sterile distilled water prior to analysis.

Live/dead cell staining and epifluorescence microscopy

To determine its general structure, content and metabolic state, washed biofilm sections were stained using the FilmTracer™ LIVE/DEAD® Biofilm viability kit (Invitrogen, CA, USA). In brief, 3 μl SYTO® 9 green fluorescent stain and the same volume of propidium iodide (red fluorescence) was added to 1 ml filter sterilized water (the stain). The stain was pipetted onto biofilm sections which were kept in the dark. After 30 min. the residual stain was removed by two washes in pharmaceutical grade water. The biofilm sections were examined by fluorescence microscopy (Olympus BX40, GmbH) using the antifadent containing immersion oil Citifluor AF87 (Citifluor Ltd, London, UK) and emission filter sets for the green (WIBA-cube, Olympus) and red (WG-cube, Olympus) dyes.

Plate count investigation of the biofilm content (bacteria, fungi, protozoa), and cellular morphologies

Washed biofilm (10 mg) was added to 1 ml of pharmaceutical grade distilled water in an eppendorf tube. Thereafter, 1 g of acid washed, sterile 2 mm glass beads were added to the tube which was then vortexed at maximum speed for 2×5 min with intermittent cooling (2 min) on ice. This approach seemed to completely disrupt the film and free bacteria were visible in the microscope. Aliquots (0.1 ml) were spread on R_2A-agar (Oxoid, Thermo Scientific, UK) to obtain the total mesophilic heterotrophic bacterial plate count and Rose Bengal Chloramphenicol agar (Oxoid) to obtain the total mesophilic heterotrophic fungal plate count. Plates of each agar type were incubated at $22 \pm 2\,^{\circ}C$ (7 days). An additional R_2A plate was incubated at $55 \pm 1\,^{\circ}C$ for 48 h to detect thermophiles. Samples were also spread on sheep blood agar plates (Oxoid) for the detection of rapidly growing strains of potential clinical interest. Plates were examined for colony counts and types after aerobic and anaerobic incubation at $37 \pm 1\,^{\circ}C$ (48 h).

Free-living protozoa were detected as previously described.[7] In brief, washed biofilm pieces (10 mg) were added to non-nutrient agar seeded with heat-killed *Escherichia coli*. Plates were incubated at two different temperatures, $22 \pm 2\,^{\circ}C$ and $37 \pm 1\,^{\circ}C$, and examined for the presence of protozoa and protozoal cysts over a period of 7 days.

DNA-isolation from the biofilm

Extraction of total community DNA from biofilm pieces was performed using the *alternative protocol* in the UltraClean™ Soil DNA Isolation kit (MoBIO Laboratories, CA, USA). In brief, 10 mg washed biofilm was first transferred into the supplied DNA extraction tubes. As an aid to homogenization of the