Bubble biofilm: Bacterial colonization of air-air interface

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ABSTRACT

Microbial mats or biofilms are known to colonize a wide range of substrates in aquatic environments. These dense benthic communities efficiently recycle nutrients and often exhibit high tolerance to environmental stressors, characteristics that enable them to inhabit harsh ecological niches. In some special cases, floating biofilms form at the air-water interface residing on top of a hydrophobic microlayer. Here, we describe biofilms that reside at the air-air interface by forming gas bubbles (bubble biofilms) in the former Ytterby mine, Sweden. The bubbles are built by micrometer thick membrane-like biofilm that holds enough water to sustain microbial activity. Molecular identification shows that the biofilm communities are dominated by the neuston bacterium Nevskia. Gas bubbles contain mostly air with a slightly elevated concentration of carbon dioxide. Biofilm formation and development was monitored in situ using a time-lapse camera over one year, taking one image every second hour. The bubbles were stable over long periods of time (weeks, even months) and gas build-up occurred in pulses as if the bedrock suddenly exhaled. The result was however not a passive inflation of a dying biofilm becoming more fragile with time (as a result of overstretching of the organic material). To the contrary, microbial growth lead to a more robust, hydrophobic bubble biofilm that kept the bubbles inflated for extended periods (several weeks, and in some cases even months).

Introduction

Benthic microbial communities typically organize themselves into biofilms or microbial mats, attached to a solid substrate. This ecological model of organization has been highly successful throughout Earth’s history with evidence of sedimentary microbial mats dating back to the Archaean time (e.g., Ref. [1]). Microbial mat communities are densely packed together in ecosystems where nutrients, electron donors, and acceptors are tightly and efficiently recycled (e.g., Ref. [2]). These communities are embedded in extracellular polymeric substances (EPS), acting as diffusion barriers that allow for a wide range of metabolic activities to coexist. The protective effect of EPS combined with highly flexible metabolisms strongly improves the tolerance of those communities to environmental stressors, explaining that we find microbial mats and biofilms in extreme environments.

In some instances, the hydrophobic nature of certain microbial EPS allows communities to colonize the air-water interface by forming floating biofilms [3,4]. Although such strategy is not fully understood, these aerobic communities seem to benefit from access to gaseous phases on one side and nutrients from the water on the other side [3]. Here, we document for the first time, bacterial communities that colonize the air-air interface by forming a peculiar ‘bubble biofilm’ attached to walls in tunnels leading to the main shaft of the former Ytterby mine [5,6]. Although it is unclear if these bubble biofilms represent a local curiosity or a larger ecological strategy, it provides another striking evidence of the extraordinary ability of the microbial world to adjust to any environmental challenge.

Materials and methods

The Ytterby mine area - site description

The former quartz and feldspar mine, also known for the discovery of tantalum and seven of the rare earth elements, is located on the shores of the Baltic Sea in the Stockholm archipelago, Sweden (59° 42’ 84” N, 18° 35’ 38” E). After closing in 1933, it reopened during the cold war era in

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Ethyne, Propane, Propene, Propyne were also measured but only present in collected from fracture water and bubble bio precipitates associated with water bearing fractures in the mine tunnel. Analyses of gas trapped by bubble bio precipitates on the rock wall inside both bubbles.

Table 1
Analyses of gas trapped by biofilm (Bubbles 1 and 2, see Fig. 1A and B); gas trapped by biofilm on Mn deposit (Bubble 3, see Fig. 1C), immature biofilm bubble, and (Bubble 4, see Fig. 1C) mature biofilm bubble. (N₂, Ethane, Ethene, Ethyne, Propane, Propene, Propyne were also measured but only present in traces).

| Gas (ppm) | Tunnel air | Bubble 1 | Bubble 2 | Bubble 3 | Bubble 4 |
|-----------|------------|----------|----------|----------|----------|
| H₂        | <3.00      | <3.00    | <3.00    | <3.00    | <3.00    |
| O₂        | 173000     | 168000   | 176000   | 175000   | 174000   |
| O₂ + Ar*  | 183000     | 177000   | 183000   | 189000   | 185000   |
| N₂        | 783000     | 788000   | 791000   | 781000   | 792000   |
| CO        | <20.00     | <20.00   | <20.00   | <20.00   | <20.00   |
| CO₂       | 398        | 563      | 435      | 574      | 496      |
| CH₄       | 13.6       | 10.8     | 12.0     | 13.0     | 19.9     |

* High oxygen levels make it difficult to separate oxygen from argon. Therefore oxygen is also reported as argon + oxygen as a combined peak. The remaining gas, to receive 100% analysed gas, could be helium that was not analysed.

Small subunit rRNA gene amplification, sequencing and phylogenetic analysis

Amplification of the targeted small subunit rRNA gene was conducted following a two-step PCR protocol using the universal primers’ combination: 519 forward and 1391 reverse as described in Spang et al. [7]; using HotStarTaq (Qiagen). Samples were sequenced on a MiSeq Illumina platform using Reagent kit v3, (600-cycle) at the SciLifeLab sequencing facility at Uppsala University, Sweden. Sequence features (here described as representative operational taxonomic unit, OTUs) were clustered at the 97% sequence identity level using QIME2 (vsearch cluster-features-de-novo option). Taxonomic assignment of the OTUs was conducted using a naïve Bayesian classifier in QIME2 (feature-classifier classify-sklearn) using a confidence score of 0.7 (μ-confidence) against the SILVA v132 database. To construct the phylogenetic tree, OTUs corresponding to the Solimonadaceae family were used as query sequences against the Genbank nucleotide database using BLASTN to retrieve the top 20 sequences from each OTU. The resulting sequences were aligned using MAFFT-Q-INS-I [8] and the Ytterby OTUs were added using the “-add” option. The alignment was trimmed to only include the amplicon region for both reference and Ytterby sequences. Maximum likelihood phylogenetic inference was conducted on the unmasked alignment with IQ-TREE v 2.0 [9] under the best scoring model of evolution selected with ModelFinder (TIM3+F+I1 + I4 + G4) with 1000 ultrafast bootstraps. Sequences obtained in this study were deposited in the NCBI Sequence Read Archive (SRA) as part of project number PRJN544894. Sample accession numbers for the four biofilm samples are SAMN11898234, SAMN11898235, SAMN11898230 and SAMN11898229.

Gas analyses

Gas trapped by bubble biofilm was sampled from four different locations: two samples of gas trapped by biofilm associated with a manganese oxide deposit (Fig. 1A, B), and two samples of gas trapped by biofilm of different maturity covering initial manganese oxide precipitates located in the same tunnel (Fig. 1C). Analyses of gases were conducted by Microbial Analytics Sweden AB. Three gas chromatograph systems equipped with three different detectors were used. Methane (CH₄) > 20 ppm, nitrogen (N₂) and oxygen (O₂) were partly analysed on a Varian CP-3800 gas chromatograph (Agilent Technologies Inc., CA, USA), equipped with a thermal conductivity detector (TCD) and high resolution capillary column (25 m*0.53 mm *20 μm) CP7430 (Bruker, select permanent gases/CO₂ HR) and partly on DANI Master GC, equipped with a TCD and column MXT-Molsieve 5A Plot (30 m*0.53 mm*50 μm). Carbon monoxide (CO) was also analysed using the latter system. Helium (He) was used as a carrier gas in both systems. CH₄ and hydrocarbon gases (C1–C3) < 20 ppm were partly analysed on Varian CP-3800 gas chromatograph equipped with a flame ionization detector (FID) and carboxen column (2 m*1.8 in. *2.1 μm) Ultimatel CP99969, with N₂ as carrier gas and partly on Bruker 450 gas chromatograph (Bruker Daltonics, Scandinavia AB, Solna, Sweden) equipped with an PDHID detector (Valco Instruments Company, Inc, Houston, USA) and column Porabond Q (50 m*0.53 mm, ID) CP7355, with He as carrier gas. Hydrogen (H₂), oxygen and dinitrogen monoxide (N₂O) were also analysed on a Bruker 450 gas chromatograph equipped with a PDHID detector and column MOLSIECW 5A PLOT (25 m* 0.32 mm, ID) CP7536. He was used as carrier gas. All chromatographs were calibrated using certified gas mixes (Air Liquide, Specialty gases, Krefeld, Germany).
**Results**

**Bubble biofilm formation**

Bubble biofilm formation and development were monitored *in situ* using a Brinno BCC200 professional time-lapse camera over one year, taking one image every second hour. Time-lapse movies show that gas bubbles were stable over long periods of time (weeks, even months) and that gas build-up occurred in pulses as if the bedrock suddenly exhaled.
Over time, the bubbles matured and formed a robust hydrophobic biofilm that kept the bubbles inflated for extended periods (SI 2). No visible deflation was observed in the monitored bubbles, but occasional growth pulses. With time, the bubble biofilm migrated down the rock wall creating long chains or clusters of bubbles that occasionally shrank during the process due to mechanical strain (SI 3). Otherwise, only strong physical disturbances altered their evolution. For example, at times of high water supply, bubbles were washed down the bedrock and did not have time to settle (SI 4). Gas analyses show that bubbles contained mostly air with slightly elevated concentrations of carbon dioxide (Table 1). The average amount of carbon dioxide measured in the four bubbles, 517 ppm, was significantly higher ($p < 0.025$) than concentrations in the ambient tunnel air, 398 ppm.

Microbial community composition

DNA analyses indicated that all bubble biofilm samples were dominated by members of the Gammaproteobacteria class (between 79% and 93% of the total prokaryotic community), in which sequences belonging to the Solimonadaceae family and in particular the Nevskia genus were predominant (Fig. 2). The relative abundance of Nevskia in the fracture water (feeding the system) was very low: 0.9% 16S rRNA gene reads compared to an average of 65.9% in the biofilm samples. This group of Nevskia bacteria clustered into two OTUs: one OTU is 97.76% similar to Nevskia ramosa strain Soe1 DSM 11499 (NR_025269 [10], and the second OTU is 98.9% similar to Nevskia ramosa strain MAFF 211643 (AB518684, Kawai, NCBI GenBank 2019). Within the same family there was also a high relative abundance of sequences belonging to the Panacagrimonas genus. A phylogenetic tree was constructed to show the position of Solimonadaceae sequences obtained in this work (Fig. 3).

In general there was little variation among the four biofilm samples with the exception of the Bacteroidetes and Alphaproteobacteria. In the Bacteroidetes group there were substantial differences in terms of relative abundance (ranging from 0.2% to 12% of the 16S rRNA gene reads). Sequences mainly clustered within the Microscillaceae family which is a group of chemoorganotrophic, strictly aerobic bacteria that are capable of gliding motility [11]. Within the Alphaproteobacteria there were also differences in relative abundance between the samples (ranging from 3.2% to 11.7% of the 16S rRNA gene reads), but here the groups that contributed most to these differences remained undetermined.

Discussion

When growing undisturbed, Nevskia form microcolonies at the air-freshwater interface that display a rosette- or bush-like morphology (SI 1). Over time, the bubbles matured and formed a robust hydrophobic biofilm that kept the bubbles inflated for extended periods (SI 2). No visible deflation was observed in the monitored bubbles, but occasional growth pulses. With time, the bubble biofilm migrated down the rock wall creating long chains or clusters of bubbles that occasionally shrank during the process due to mechanical strain (SI 3). Otherwise, only strong physical disturbances altered their evolution. For example, at times of high water supply, bubbles were washed down the bedrock and did not have time to settle (SI 4). Gas analyses show that bubbles contained mostly air with slightly elevated concentrations of carbon dioxide (Table 1). The average amount of carbon dioxide measured in the four bubbles, 517 ppm, was significantly higher ($p < 0.025$) than concentrations in the ambient tunnel air, 398 ppm.
These strictly chemoorganotrophic aerobes are mainly found in shallow aquatic environments such as swamps, ponds, pools, and so forth (see references in Ref. [12]). They are defined as epineuston, i.e., organisms that float on the water surface. Communities are mainly located outside the water phase where they reside on top of a hydrophobic microlayer that develops on the water surface [10,12,14]. The result is an opaque pellicle (floating biofilm), with a hydrophobic nature similar to that of paraffin [15].

In contrast, the Nevskia dominating biofilm in Ytterby does not reside entirely at the boundary between water and air but rather at the bubble gas-air interface Fig. 4. It creates a micrometer size membrane-like biofilm in contact in air on both sides, but still holding sufficient water to sustain microbial activity. Indeed, the result is not a passive inflation of a dying biofilm becoming more fragile with time (as a result of overstretching of the organic material). It instead represents a continuous microbial growth that produces a thicker, more robust, and less transparent bubble biofilm, indicating a more mature stage (Fig. 1C). Although the trapped gas mainly reflects the ambient tunnel air, pressure has to be slightly higher inside the bubble to keep it inflated.

The stable nature of these bubbles can either be explained by (1) a constant influx of gas compensating the possible diffusion losses through the biofilm, or (2) a biofilm that is gas-tight and does not allow for much diffusion over time. In option one, the gas would either be produced by microbial activity or by groundwater CO2 degassing (reequilibration of the recharge fracture water with the tunnel air following Henry’s law). In those cases, we would expect an enrichment in a specific gas within the bubble atmosphere (e.g., CO2, CH4, H2). In our measurements we only record a minor elevation in CO2. The enrichment could theoretically also be prevented by the equilibration with the tunnel air through a gas bubble atmosphere (e.g., CO2, CH4, H2). In our measurements we only be prevented by the equilibration with the tunnel air through a gas bubble atmosphere (e.g., CO2, CH4, H2). In our measurements we only.

Data curation, Funding acquisition, Writing - review & editing. Bert Allard: Writing - review & editing. Rolf Hallberg: Methodology, Writing - review & editing. Felix Homa: Data curation. Tom Martin: Supervision, Writing - review & editing. Thijjs G.J. Ettema: Funding acquisition, Supervision, Writing - review & editing. Christophe Dupraz: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Writing - original draft, Writing - review & editing.

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Appendix A. Supplementary data

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