Microscopic Analysis of Sulfate Reducing Bacterial Consortia

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Introduction.

Liquid enrichments are a very useful tool in microbiology. Using different conditions for enrichment, physiologically diverse organisms can be isolated. In general, liquid enrichments are expected to yield planktonic cultures.

The origin of my project is based on the unusual observation of 'colonies' forming on the glass walls within liquid sulfate reducing bacteria (SRB) enrichments. Microscopic analyses were performed to characterize the morphological diversity and community structure of these interesting aggregates.

Material and Methods.

Cultivation. SRB enrichments were set up using salt water SRB basal medium (containing per L Na_2SO_4 4.0 g, KH_2PO_4 0.2 g, NH_4Cl 0.25 g, NaCl 20.0 g, $MgCl_2 \cdot 6H_20$ 3.0 g, KCl 0.5 g, and $CaCl_2 \cdot 2H_20$ 0.15 g). The basal medium was supplemented after autoclaving with 1.0 ml/l 1000X EDTA-chelated Trace elements solution (dH_20 987 ml, EDTA pH 6.0 5.2 g, $FeSO_4 \cdot 7H_20$ 2.1 g, H_3BO_3 30.0 mg, $MnCl_2 \cdot 4H_20$ 0.1 g, $CoCl_2 \cdot 6H_20$ 0.19 g, $NiCl_2 \cdot 6H_20$ 24.0 mg, $CuCl_2 \cdot 2H_20$ 2.0 mg, $ZnSO_4 \cdot 7H_20$ 144.0 mg, $Na_2MoO_4 \cdot 2H_20$ 36.0 mg, Sodium Vanadate 25.0 mg, $Na_2SeO_3 \cdot 5H_20$ 6.0 mg, and $Na_2WO_4 \cdot 2H_20$ 8.0 mg filter sterilized), 1.0 ml/l 1000X 12-vitamin solution (10 mM Phosphate buffer pH 7.2, Riboflavin 10.0 mg, Thiamine·HCl 100.0 mg, L-Ascorbic acid 100.0 mg, D-Ca-pantothenate 100.0 mg, Folic acid 100.0 mg, Niacinamide 100.0 mg, Nicotinic acid 100.0 mg, 4-aminobenzoic acid 100.0 mg, Pyridoxine·HCl 100.0 mg, Lipoic acid 100.0 mg, NAD 100.0 mg, and Thiamine pyrophosphate 100.0 mg titrated to dissolve with NaOH and filter sterilize. Store frozen in 10.0 ml aliquots), 1.0 ml/l 1000X vitamin B_{12} solution (1 mg/ml, titrate until dissolved with HCl, filter sterilize and store frozen), 30.0 ml/l Bicarbonate solution (84 g/l $NaHCO_3$), and 1.5 ml/l Sulfide solution (240 g/l $Na_2S \cdot 9H_20$). Carbon sources were added at 5 mM (250 µl 1M stock solution) to 50 ml aliquots of medium in Pfennig bottles.

Agar shakes were also attempted to isolate members of the consortia. Briefly, 1% SRB agar containing the appropriate carbon source was used to serially dilute culture supernatant and individual colonies (with and without disruption) using standard techniques.

Microscopy. Phase contrast micrographs were obtained using a Zeiss Axioplan 2 microscope (Zeiss, Germany). Images were captured using the imaging software Metamorph version 4.6 (Universal Imaging Corp.,

Downington, PA). Confocal micrographs were done using a single-photon Zeiss LSM510 confocal microscope. Projections of the confocal data were constructed using the microscope software package. For confocal microscopy aggregates were stained using SYBR-green (Molecular Dynamics). The protocol used for stained was modified from the manufacturers recommendations. Briefly, the dye was diluted 1:10 to make a working stock. This stock was mixed 1:5 with the sample and incubated one hour at room temperature in the dark. The stain was then removed by washing with anti-fade solution [50% glycerol, 50% PBS (120 mM NaCl, 10 mM NaH_2PO_4 pH 7.5), 0.1% p-phenylenediamine (made fresh using 1 ml glycerol/PBS plus 10 µl frozen stock of 10% p-phenylenediamine)] at least three times. All manipulations were carried out using a microscopy chamber constructed by gluing a small washer to a microscope slide. Each step was monitored using the stereomicroscope to track aggregates.

Molecular analyses. Single aggregates were removed from liquid culture and incubated in growth medium for 10 minutes at 95°C to lyse cells. The resulting fluid was used as template for PCR using a delta-proteobacteria specific primer (delta96F, 5'-AGTARAGYGGCGCAC) with a universal reverse primer (1391R). All aggregates were PCR positive using delta-proteobacteria specific primers (data not shown).

Results and Discussion.

Imaging of Whole Colonies. Colonies forming within the liquid enrichment were fairly standard in form and size within individual enrichments. Typical sizes were approximately 200 nm in width by 300 nm long. Based on confocal imaging the colonies were approximately 100 nm in depth. The colony appearance was influenced by the particular enrichments in which they were observed. Nominally, the only difference between each bottle was the carbon source and inoculum (although each group shared a single inoculum). Each of these primary enrichments were established and maintained by an individual student, which may have influenced the structural differences observed in colony morphology. Figure 1 shows a typical colony formed using formate (5 mM

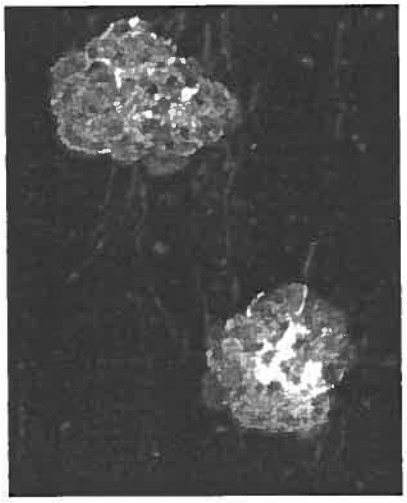

Figure 1. Colonies formed in SRB liquid enrichments grown using formate as carbon source. Note filaments adhering to glass wall.

plus 100 μM acetate). These colonies appeared to be composed of elliptical units that were similar in size and arranged in a 'grape cluster' configuration. There were also apparent filaments that exhibited gliding motility based on observations of the filaments within the enrichment. In comparison, the bottle using 5mM ethanol for carbon source (Figure 2) developed structures that look like micro-colonies, and did not contain as many filamentous organisms.

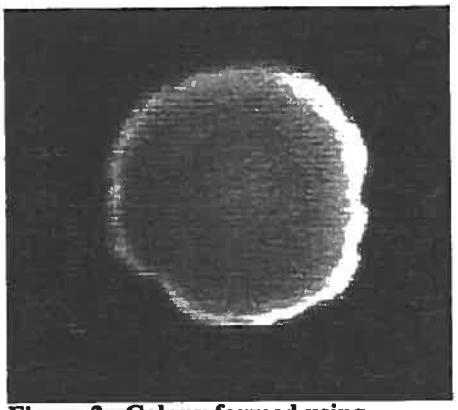

Figure 2. Colony formed using ethanol as carbon source.

Phase Microscopy analysis. The appearance of single aggregates, removed from the bottles, was investigated by dropping cover slips on to individual aggregates placed on microscope slides. This greatly compacted the samples and led to some misleading impressions about the density of cells within individual aggregates from the formate enrichments. Based on these observations a large number of motile and non-motile cells of various morphologies were also observed around the perimeter of the compressed aggregate. These initial observations also skewed the interpretation of the importance of the filaments for aggregate construction in the formate enrichment. The number of filaments was relatively low compared to the apparent bulk of biomass present in the compressed aggregate (Figure 3). One intriguing observation was the consistent size of the units of the 'grape-cluster' colony morphology formed in the formate enrichment. The best explanation for this phenomenon is the tendency of gliding bacteria to

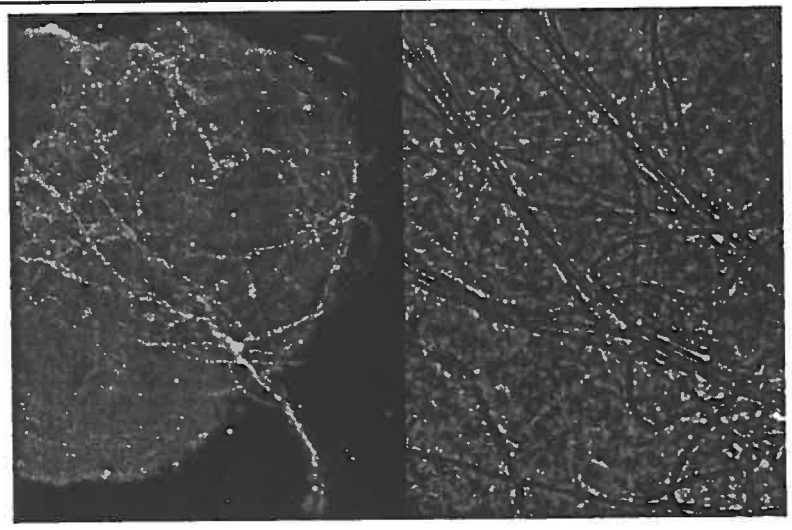

Figure 3. Dark-field image of aggregate at 10X (left) and 40X phase contrast micrograph of selected filaments (right). Note the apparent lack of filaments in some regions of the aggregate and general appearance of large amounts of biomass not specifically associated with the filaments.

'double-back' during gliding and form elliptical tracks. Other bacteria would then colonize these tracks/filaments forming the basis for the observed structure. As these biofilms were formed it created more surfaces for the gliding organisms to colonize (probably the limiting 'substrate' in this system) resulting in the three dimensional accumulation of biomass in regularly shaped units.

The large numbers of filaments present on the enrichment vessel walls independent of aggregate formation supports the idea that the aggregation was a means to increase surface area to support more growth of the filamentous SRBs. The presence of the filamentous SRBs is noteworthy due to the unusual method for enrichment (liquid culture with no growth supports for gliding). Future attempts at obtaining axenic cultures of these organisms would be worth pursuing. The isolation of these types of SRB by shakes is noted as being difficult due to "commensals and competitors" that suggest the propensity to aggregate is a common property. This was also observed in my attempts to isolate organisms associated with the aggregates by shakes. Primarily agar degraders were produced in the shakes and subsequently destroying the shakes preventing any isolated cultures. The specific property of agar degradation was very common in most of our salt-water enrichments containing agar, which makes the presence of the phenotypic property difficult to assess in the context of the aggregates. However, the property of degrading complex polysaccharides seems to be common in salt-water environments. Based on published images, filaments that closely resemble *Desulfonema magnum* and *Desulfonema limicola* (Figure 4) were obtained (Chapter 183, The Prokaryotes online).

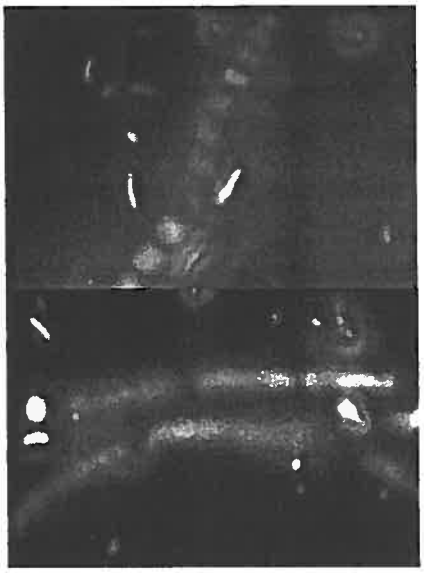

Figure 4. Images of filaments stained with SYBR-green at 100X. The individual cells within the filaments along with phase contrast observations (not shown) allow for the identification of a *Desulfonema limicola*-like organism (top) and a *Desulfonema magnum*-like organism (bottom).

Laser Scanning Confocal Microscopy (LSCM). This was the most useful method for observing the aggregates formed. The three dimensional aspect coupled to the use of SYBR-green allowed the discrimination of cells from other types of biomass and the general spatial relationships between cells. In general the aggregates in the formate enrichment contained very

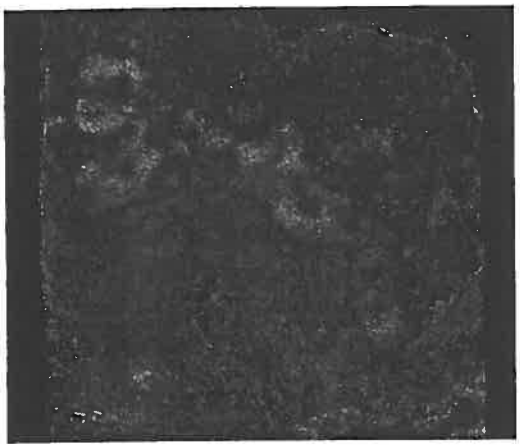

Figure 5. Glass proximal surface projection of the SRB aggregate from the LSCM data gathered at 63X. Note the high levels of staining along the edges of the aggregate and within certain regions of the aggregate.

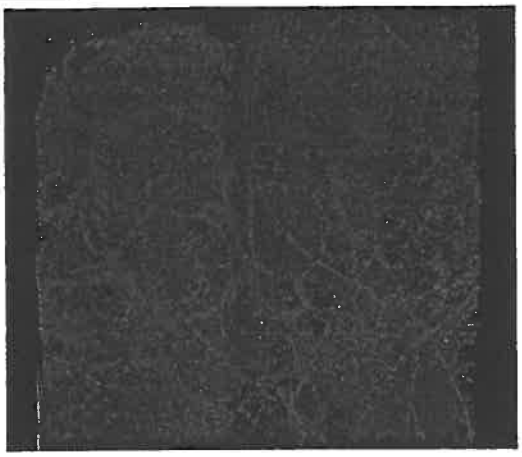

Figure 6. Liquid proximal surface projection of the SRB aggregate from the LSCM data gathered at 63X. Note the distribution of filaments throughout the aggregate and relatively light staining near filaments. There are also channels and holes present within the aggregate.

few cells not specifically associated with the filaments. This is apparent from the density of staining associated with the filaments and lack of staining in other regions of the aggregate. A thin band of intense staining at the edge of the aggregates also suggests that another community was establishing along the margins of the aggregates (Figure 5 and 6). The compacted samples observed under phase microscopy contained motile cells, including spirochetes, which are presumably fermentative. My current hypothesis is these organisms were degrading the materials, such as EPS, produced by the aggregate organisms. Subsequent observations made by compressing the samples used for LSCM showed the areas of intense stain contained filaments that were highly colonized by other bacteria.

Concluding Remarks. The phenomenon observed during the enrichment and transfer of the aggregates formed in liquid culture is interesting from several standpoints. First, the isolation of surface attached organisms and the absence of planktonic organisms during liquid enrichment was completely unexpected. This is not the typical result of liquid enrichments. Secondly, the idea of isolating 'community' was very intriguing. The ability to transfer the phenotype of aggregate formation in subsequent liquid enrichment will allow for the analysis of the community that is forming to ask questions about community structure and stability. A future project directed at isolating the *Desulfonema*-like organisms from Sippewissett Swamp would also be useful due to the limited number of

cultures (three species based on NCBI taxonomy browser http://www.ncbi.nlm.nih.gov/Taxonomy/) for this genera.