The domestication of wild sulfate reducers: establishing a continuous culture for maintaining biofilm phenotype.

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Abstract
A major challenge in the field of microbiology is culturing bacterial isolates in a laboratory setting. Careful and elegant methods have been employed to grow a diverse array of bacteria which need highly specific conditions or metabolic requirements in order to replicate. These conditions are usually designed to reflect those of the environment from which the bacteria is isolated from or to specifically enrich for bacteria with certain metabolic or phenotypic traits. Both strategies are likely to select for bacteria that can are adaptable to pressures posed by different environments to that of their environmental ecological niche. The “domestication” of a “wild” bacterial isolate could therefore shape its genome or phenotype is either a permanent or transition manner that is restored by switching conditions. This mini-project addresses what effect growing an early isolate of a sulfate reducing bacterium in a continuous culture on the adaptation and selection for cells able to maintain a specific phenotype of biofilm formation.

Introduction
Sulfate reducing bacteria are ubiquitous in nature but usually inhabit a highly specialised niche where they use sulfate as an electron acceptor in anoxic environments \(^1\). SRBs are able to utilise a range of organic molecules as carbon sources coupled to the reduction of sulfate as well as \(H_2\) and although energy kinetics for these reactions can be low, are key members in sulphur and carbon cycles across systems.

SRBs are present in multiple and diverse habitats including freshwater and marine sediments \(^2,3\). A site sampled extensively in the 2015 Microbial Diversity course, Trunk River, has an interesting stratification of freshwater, brackish and seawater layers throughout the tidal pond system, as established during the course by several MD staff and students. From this site, an inoculum was used from a sample termed “lemonade” which is opaque yellow that plumes in depressions in the shallow regions of the pond. The emergence of a matrix of microbial biofilms occurs, also termed “spaghetti mat” (Sebastian Kopf, 2015) over time from this lemonade plume. An enrichment culture for SRBs was performed using an inoculum from each a “lemonade” sample and sediment sample away from a
“lemonade” plume. In the enrichment culture from the lemonade sample, a biofilm was observed which formed on the glass vessel. Biofilm forming SRB are of particular interest as pose a problem to industrial settings, where the production of H₂S is both toxic and corrosive, such as in the petrochemical industry.

From an ecological perspective, the SRB play an important role in elemental sulfur and carbon cycles. They are key players in shaping microbial community composition both by facilitating growth of sulphur oxidising bacteria and in competition to methanogens and acetogens. Although there is a long history of the study of SRBs, it has been acknowledged that further characterisation of their function and activities within environment is needed, in addition to the isolation of novel types. Part of the challenge in culturing novel bacteria is the phenomenon of strain adaptability through loss of function. One example in a SRB isolate is for Desulfovibrio vulgaris Hildenborough ATCC 29579, of which a mutant which lost a 200 kbp megaplasmid during laboratory culture. In this project, the isolation and genetic characterisation of a novel SRB species has been performed, as well as the application of a less traditional approach for culturing SRB while maintaining biofilm phenotype. The hypothesis being tested with this system is whether the domestication of an early isolate influences the resultant isolate obtained by specific culture method.

Materials and Methods

Bacterial isolation and strain characterisation

An enrichment for sulfate reducing bacteria (SRB) was performed using samples from Trunk River (Duck Island) and Trunk River (Lemonade) inoculum in SRB media in septum vials under anoxic conditions at 30°C in the dark. Laboratory culture of the isolates were in the same media and same conditions. The SRB media contains 1x fresh water base (100x stock with 17.1 mM NaCl, 1.97 mM MgCl₂.6H₂O, 0.68 mM CaCl₂.2H₂O and 6.71 mM KCl), 5 mM NH₄Cl, 1 μM K₂HPO₄, 5 mM MOPS, 1% Resazurin, 20 mM NaSO₄, 1 mM Sodium Acetate, 1 mM Sodium Sulfide, 10 mM Sodium Bicarbonate, 1x Trace Metals (1000x stock contains 20 μM HCl, 7.5 μM FeSO₄.7H₂O, 0.48 μM H₂BO₃, 0.5 μM MnCl₂.4H₂O, 6.8 μM CoCl₂.6H₂O, 1.0 μM NiCl₂.6H₂O, 12 nM CuCl₂.2H₂O, 0.5 μM ZnSO₄.7H₂O, 0.15 μM Na₂MoO₄.2H₂O, 25 nM NaVO₃, 9 nM Na₂WO₄.2H₂O, 23 nM Na₂SeO₃.5H₂O) and 1x multivitamin stock (1000x contains 10 μM MOPS, pH 7.2, 0.1 μg/ml Riboflavin, 0.03 μg/ml Biotin, 0.1/μl Thiamine HCl, 0.1 μg/ml L-Ascorbic acid, 0.1 μg/ml d-Ca-pantothenate, 0.1 μg/ml Folic acid, 0.1 μg/ml Nicotinic acid, 0.1 μg/ml 4-aminobenzoic acid, 0.1 μg/ml pyridoxine HCl, 0.1 μg/ml Lipoic acid, 0.1 μg/ml NAD, 0.1 μg/ml Thiamine pyrophosphate and 0.01 μg/ml Cyanocobalamin). Following autoclaving, the vitamin stock, Sodium sulphide and Sodium bicarbonate were added when media was cool and was then dispensed as needed under anaerobic conditions whilst flushed with N₂.
A SRB isolate was obtained following its passage in dilution to extinction liquid cultures. Cell morphology and biofilms were visualised on a Zeiss Axio Imager 2 and a Zeiss SteREO Discovery V.12 imaged using Zen software.

Amplification of the 16S rRNA gene was performed using 5 µl of culture in a colony PCR, in a reaction as follows: 25 µl Promega GoTaq® G2 Hot Start Green Master Mix, 4 µl of each oligonucleotide 8F and 1391R (universal bacterial 16S rRNA gene primers) and 13.0 µl nuclease-free water. PCR cycle conditions were a denaturation step at 95°C for 2 min, and 25 cycles of a denaturation step at 95°C for 30 sec, annealing step at 55°C for 30 sec and extension step at 72°C for 1.5 min with a final extension step at 72°C for 10 min. The PCR product was sequenced using Sanger sequencing and the sequence was searched, aligned and a phylogenetic tree generated using the ribosomal database project (RDP: accessed online at https://rdp.cme.msu.edu/). The tree file was imported to MEGA v6 ⁶. A blastn search of the 16S rRNA sequence was performed against the NCBI nt/nr database, accessed online at https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch, 18/08/2015).

Bacterial genomic DNA was extracted using three methods, due to low biomass of the culture. In the first extraction, 15 ml of a turbid culture was filtered onto 0.2 µM Isopore™ Membrane filters which were then used in a modified protocol using PowerFeacal® DNA isolation kit (MoBio, USA). Cells were removed from the filters by bead beating for 10 min at maximum speed setting, and the gDNA isolation performed following the manufacturer’s guidelines for Gram negative bacteria. A second extraction was performed following a series of ten successive centrifugation steps, where 1.5 ml of culture was centrifuged for 15,000 xg for 30 min, the supernatant was aspirated and 1.5 ml of new media was added and this step repeated for maximal recovery of a pellet. Genomic DNA was extracted from this using the Wizard® Genomic DNA purification kit (Promega, USA) following the manufacturer’s guidelines for Gram negative bacteria. A third extraction was performed using 6 ml of culture which was filtered onto a Isopore™ filter and resuspended in TRIzol® (Life Technologies, USA) with bead beating and extraction with Chloroform purification by Srijak Bhatnagar. In brief, the filter was added to 1 ml TRIzol and bead beated for 10 min. The supernatant had then 5 mg Achromopeptidase added and incubated for 20 min at 37°C. To this, 1 ml of 1% SDS and 5 mg of Proteinase K was added, and further incubated for 1.5 hrs at 55°C. Afterwards, 3.6 ml of 5M NaCl and 15 ml of Chloroform was added and the sample mixed for 20 min at 6 rpm. The sample was then centrifuged at 5000 xg for 20 min. The supernatant was transferred to a clean Eppendorf and 30 ml of Isopropanol added. The precipitated DNA was removed and rinsed with 1 ml of 70% EtOH, and the pellet was air dried before resuspension in MilliQ H₂O. DNA quantity and quality was assessed using a Nanodrop and Quantas Fluorometer with a QuantiFlour® dsDNA kit according to the manufacturer’s
guidelines. Illumina libraries were prepared by Srijak Bhatnagar for shotgun whole genome sequencing using a MiSeq System.

**Biofilm characterisation**

*Desulfovibrio sp. 15KH1* cultures with visible biofilm and aggregated growth were used to determine the biofilm matrix by staining with a final concentration of 20 µM Propidium iodide for DNA and 20 µg/ml WGA Alexa Fluor 488 for extracellular polysaccharide. The samples were stained directly without washing steps as the medium does not contain sugars for unspecific binding. The dyes were selected for complimentary emission peaks of 617 nm and 488 nm, respectively.

Two biofilm cultures produced what appeared to be plaques and to investigate the excision and release of phages from biofilm cells, the supernatants of both were examined using transmission electron microscopy. To prepare samples for negative staining, 1.5 ml of supernatant was centrifuged at 10,000 xg for 10 min and filtered using a 0.2 µM filter. A 10 µl volume of each sample was absorbed to glow discharged, formvar and carbon coated copper TEM grids. After 10 min, the sample was removed, washed three times with MilliQ H₂O (Millipore, USA) and stained with 2% uranyl acetate for 30 sec. The grids were viewed using a Joel 200 and imaged using AMT software.

**Chemostat development**

The development of a chemostat was optimised during this project for the continuous culture of the sulfate reducing isolate. Initially six conditions were set up, three of each planktonic and biofilm cultures. All vessels were inoculated with a 1.5 ml of turbid culture from an early passage (P4 - P5) isolate culture and grown at 30°C. All cultures were in 15 ml liquid media and the vessels for biofilm culture were partially filled with 52.5 g of 3 mm diameter glass beads. This equates to 42.405 cm³ additional surface area. The six cultures included a planktonic and biofilm vessel in each of the three conditions; fast flow rate in 1 mM Acetate as a carbon source, and slow flow rate in 1 mM Acetate and 10 mM Acetate media. Flow rates for the in and out flow medium were optimised to be at a slow rate of 21 µl/min with a projected doubling time of 138 min using a 1.14 mm diameter tubing and a fast rate of 29 µl/min with a 100 min doubling time using a 1.30 mm tubing and pump rate of 0.5. The media was added as a drip system to avoid growth of culture into the media reservoir. Outflow of media was taken from bottom of the vessel to ensure mixing of nutrients and vessels were degassed in the headspace with N₂.

The chemostat setup is outside of an anaerobic chamber for ease of access. For this, it was essential to include an outflow to vent H₂S produced by the SRB in addition to an inflow of N₂ to maintain an anoxic environment. Five versions of chemostat vessel and setup were devised in response to the challenges posed by establishing an anoxic, small scale, multiple bioreactor system.
Following the first setup as described above, one amendment to this includes changing needles to those with different gauges for the in and out flow media, and gas lines in order to balance pressure in the vessels. A third setup was generated which used a different pump system with a reduction in conditions to fast and slow flow rates with a 1 mM Acetate added to the media. The flow rates were established at 0.1 and 0.2. A fourth setup reduced the four in/out flows (an active inflow of media and gas, and passive out flow for spent media and H2S) to three lines, with an actively pumped in media line, an inflow of pressurised N2 and passive outflow of H2S and media to a waste reservoir that was then vented. Also an oxygen scrubber was installed to the ultrapure N2 before degassing of the vessels. The final and fifth setup balanced the pressures for maintain the passive outflow, with larger gauge needles and tubing for the outflow, elevation of the vessels and uncoupling and splitting of the N2 supply to the media vessel and the chemostat vessels for separate flow control.

**Growth dynamics and biofilm formation of Desulfovibrio sp. 15KH1 in continuous culture**

Monitoring of the chemostat cultures was performed using phase microscopy for an idea of the health of the culture via cell numbers and motility. To assess culture growth, optical density measurements were obtained over a daily time course at 600 nm using 600 µl of culture, post vortex to disrupt biofilm aggregates. Secondly, as a proxy for culture growth, the depletion of acetate was measured using High-performance liquid chromatography (HPLC). For this 450 µl of culture had 50 µl of 5N H2SO4 added which was then centrifuged at 10,000 xg for 5 min and filtered through a 0.2 µM filter. The samples were analysed in a 20 µl injection with HPLC-Aminex HPX-87H 300 mm x 7.8 mm, 60C, 0.6 mL/min flow, 5 mM H2SO4 (isocratic) with a Shimadzu™ High pressure liquid chromatography system. U.V. detected of acetate peak values were normalised according to the Refractive Index Detection of MOPS in each sample. Samples for analysis using Ion Chromatography (IC) were centrifuged at 10,000 xg for 5 min and filtered using a 0.2 µM filter. For each sample, 200 µl was added to 1.8 ml Mill-Q water in a 1:10 dilution and analysed on a Thermo Scientific™ Dionex™ Ion Chromatogram with an AS-18 column.

**Total cell enumeration in continuous cultures**

To quantify the number of cells in each culture, for the planktonic, 6 ml of culture was collected on 0.2 µM filters and for the biofilm, 3 ml of culture. Filters were stained using 1 µg/ml DAPI solution as part of a mounting fixative containing 4:1 mix of Citifluor and Vectashield on glass slides which were then enumerated under U.V.

**Biofilm quantification**

At the end time point, the biomass which was adhered to the glass beads as a presumed biofilm was quantified using crystal violet following a modified protocol which is described here. Five
beads per sample were used including a control of only beads that were not used in the cultures, in triplicate with 200 µl of 1% CV added. This was incubated at room temperature for 30 min. The beads were washed three times with an excess of PBS until clear and the crystal violet was eluted using 200 µl of 30% acetic acid. The absorption for each was measured at 595 nm.

**Results and Discussion**

**Isolate characterisation**

The SRB isolate has a cell morphology which is consistent with *Desulfovibrio* species (Fig. 1). Plaque like holes were observed in the mature biofilm from its initial enrichment culture and also in a subsequent passage culture which was exposed to air, and became oxic as indicated by a colour change of the redox indicator resazurin. Temperate phages which can excise from *Desulfovibrio* sp. have been documented previously, however no phage like particles were observed under transmission electron microscopy although material which appeared to be abiotic, possibly sulfur deposits covered the grids, suggesting optimisation of the absorption and staining method was needed.

Sequencing of *Desulfovibrio sp 15KH1* 16S rRNA gene resulted in a 857 bp product which phylogenetic analysis confirmed its identification as a member of this genus (Fig. 2). A blastn search against the NCBI nt/nr database revealed that this sequence shares a 93% identity to an uncultured bacterium with a query coverage of 99% and an E value of 0.0. The next best hit is to a cultured isolate, *Desulfovibrio psychrotolerans*, with an identity of 93% which had been isolated from a salt water lake in the Himalayas. A taxa name of *Desulfovibrio sp. 15KH1* has been assigned for reference purposes. The most commonly described SRB belong to the Deltaproteobacteria which includes the *Desulfovibrio* genus. It is interesting that this isolate matches closest to a freshwater isolate and that the closest alignments do not appear to overlap with those present in a marine biofilm containing SRBs.

**Fig. 1. Isolate characterisation and biofilm formation** Left: Biofilm aggregate visualised using phase contrast microscopy. Top right: Biofilm with plaque like holes on the wall of the glass septum vessel. Bottom right: Bacterial aggregate clumping in culture.
Biofilm characterisation

To characterise the biofilm from a batch culture of Desulfovibrio sp. 15KH1, two fluorescent stains were used in conjugation; Propidium iodide which stains extracellular DNA (eDNA) and DNA within cells with compromised membranes, and WGA Alexa Fluor 488 which stains exopolysaccharide (EPS). The resulting fluorescence of the biofilms show that both eDNA and EPS are components of the isolate biofilm (Fig. 3.). This result contrasts with a previous report that biofilms formed by D. vulgaris Hildenborough ATCC 29579 are held together by protein filaments or flagella, and may indicate that the culture used in this study is mixed. It also shows there is a mixture of membrane intact and compromised cells in the biofilm.
Chemostat development and optimisation

The development and troubleshooting of the chemostat design is summarised in Fig. 4. The final working setup consisted of a two conditions of fast and slow rate with a planktonic and biofilm vessel for each (Figs. 5 and 6). The cultures were kept at a volume of 15 ml which was initially inoculated with 1.5 ml culture. The slow rate is approximately 14.4 ml/min, with a projected doubling rate of 30 min and fast rate is 29.4 ml/min and doubling rate of 63 min. The biofilm vessel with 52.5 g beads (1500 3 mm beads provided 42.405 cm³ surface area). The inflow was actively pumped into the vessels and pressurised N₂ to maintain both an anoxic culture and to create pressure for the removal of media and H₂S from the continuous cultures. The media was added as a drip method, the outflow needle was placed at the height of the volume for effluent and the N₂ was bubbled into the bottom of the vessels for maximum degassing and mixing of nutrients.
Fig. 4. Development of chemostat vessels for the continuous culture of *Desulfovibrio sp. 15KH1*. Multiple set ups illustrated with notes on troubleshooting and modifications to the setup.
Growth dynamics of *Desulfovibrio sp. 15KH1* in continuous culture.

The working chemostat was running for 3 days and to three measurements to assess growth during this time were used; OD, acetate and sulfate. Colony Forming Units (CFU) counts were not obtained as an earlier attempt at growing *Desulfovibrio sp. 15KH1* on agar plates was unsuccessful. Batch cultures which had been growing for 6, 10 and 12 days were used as positive controls. For the OD measurement, all chemostat samples, absorbance were under 0.06, but for the 10 day culture, absorbance was 0.14 (Fig. 6). The acetate and sulfate depletion was observed only in the batch cultures (Fig. 6).

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Fig. 5. **Final chemostat setup.** Left: Diagram of final setup of chemostat for two conditions, fast and slow flow rates for planktonic and biofilm cultures containing glass beads with inflow of media in drip system, inflow of N₂ and gas and media effluent outflow from culture maximal volume level. Right: Chemostat setup of vessels in water bath with inflow media (black tubing), N₂ inflow (yellow tubing) and effluent outflow (white tubing).
Fig. 6. Top: Optical density at 600 nm of chemostat and batch culture. Middle: Acetate concentration in chemostat and batch cultures. Bottom: Sulfate concentrations in chemostat and batch cultures. Some analyses repeatedly failed and the data is not plotted here. Abbreviations as follows; SB = slow biofilm, SP = slow planktonic, FB = fast biofilm, FP = fast planktonic. Number indicates day of chemostat.
Total cell enumeration in continuous cultures

At the end time point, liquid culture from both biofilm and planktonic vessels was used to enumerate total cell numbers according to staining with DAPI (Fig. 7.). For counting, the samples were filtered onto 0.2 μM pore sized membranes and stains prior to counting. For this, 6 ml of planktonic culture was used and 3 ml of biofilm culture was used for each count. This resulted in total cell numbers of approximately $1.8 \times 10^4$ cells ml$^{-1}$ in the fast biofilm culture, $2.2 \times 10^3$ cells ml$^{-1}$ in the slow biofilm culture, $2.18 \times 10^2$ cells ml$^{-1}$ in the fast planktonic and 63 cells ml$^{-1}$ in the slow planktonic culture.

Biofilm quantification

To assess the amount of biofilm grown in the bead vessels, at the end of the experiment the amount of total biomass adhering to five beads per assay was quantified using crystal violet staining. There was heterogeneity observed between beads (Fig. 8). The control beads did bind crystal violet but to a lesser degree of beads from either slow or fast biofilm, and there is a trend that the fast
biofilm culture had greater amount of crystal violet binding which indicates the greatest amount of biofilm (Fig. 9.).

**Fig. 8. Crystal violet stain on glass beads from continuous culture vessels.** On the left hand side is the assay for fast biofilm and on the right hand side for slow biofilm. Each tube contains five beads, which have stained heterogeneously.

**Fig. 9. Crystal violet stain as a measurement of biomass of biofilms formed on glass beads.** Assays for beads from the slow biofilm (SB) and fast biofilm (FB) and control beads (C) which did not have exposure to bacterial culture. The control beads did bind an amount of crystal violet however this is less than the biofilm beads. The greatest amount of biofilm is on the beads from the fast biofilm culture.

**Conclusions and summary.**

The isolation of a sulfate reducing bacterium in the first weeks of the course presented an interesting opportunity to test whether a phenotype from an early passage that was subsequently lost during a traditional culture methods could be maintained. To do so, a chemosotat system for the
continuous culture was established as assessed by final cell counts from the end time point and biofilm formation on glass beads. These counts demonstrated differential growth between the conditions used and biomass as a biofilm.

This bacterium requires anoxic culture and also produces H\textsubscript{2}S gas. This presents challenges in growing cultures and manipulating in even traditional laboratory culture. Considerable development and troubleshooting was required for the chemostat design and implementation meant that the continuous culture was performed for four days. This is not as long as originally planned to then characterise the resulting bacterial populations following continuous culture over two weeks.

The methods used to monitor the growth during continuous culture were not successful in providing data to show growth of the culture but this was likely due to the low biomass at such an early stage of culture. Future work would include further modifications to the chemostat design, for example separation of the biofilm and planktonic culture pump system to remove problems with pressurisation of the vessels to ensure efficient removal of effluent. If additional time was available for longer continuous culture, the cultures would be assessed for growth dynamics over time and the ability to form biofilms from the planktonic cultures at the end time points.

Despite the challenges involved in its setup and short running time, the chemostat system functioned well enough to say that the greater cell numbers and biofilm formed in the fast biofilm continuous culture could selectively maintain this phenotype over the other conditions, although these may be because they are suboptimal for the isolate’s growth.

Future work includes analysis of the whole genome sequencing data which can also be used to identify whether the isolate has prophage/s which may have been induced and caused the plaques observed in the initial and oxic exposed cultures.

To conclude, the results of this project has characterised a putative novel species of sulfate reducing bacteria from Trunk River, a site that has been little studied in the long history of the Microbial Diversity Course at the Marine Biological Laboratory. The biofilm of this isolate was shown to contain both EPS and eDNA in the matrix. A chemostat for the continuous culture of this microorganism was established and the end results suggest that this approach may be used to select for different phenotypes in the early cultivation of wild microbes which could be applied to other systems and species.
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References.