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Molecular analysis of a sulphate-reducing consortium used to treat metal-containing effluents

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Abstract

A sulphate-reducing consortium used in a bioprocess to remove toxic metals from solution as insoluble sulphides, was characterised using molecular (PCR-based) and traditional culturing techniques. After prolonged cultivation under anoxic biofilm-forming conditions, the mixed culture contained a low diversity of sulphate-reducing bacteria, dominated by one strain closely related to *Desulfomicrobium norvegicum*, identified by three independent PCR-based analyses. The genetic targets used were the 16S rRNA gene, the 16S-23S rRNA gene intergenic spacer region and the disulfite reductase (*dsr*) gene, which is conserved amongst all known sulphate-reducing bacteria. This organism was also isolated by conventional anaerobic techniques, confirming its presence in the mixed culture. A surprising diversity of other non-sulphate-reducing facultative and obligate anaerobes were detected, supporting a model of the symbiotic/commensal nature of carbon and energy fluxes in such a mixed culture while suggesting the physiological capacity for a wide range of biotransformations by this stable microbial consortium.

Introduction

Sulphate often occurs as a counter-ion to metals in contaminated water, and when reduced to sulphide by sulphate-reducing bacteria (SRB), can effectively remove a broad range of metals as highly insoluble sulphides (White *et al.* 1998, 2003). An added advantage of this approach to metal bioremediation, particularly pertinent to the treatment of acidic metal contaminated waste (White & Gadd 1998), is the subsequent neutralization of the effluent via mechanisms that include the direct utilization of protons during sulphate reduction and the conversion of a strong acid (H_2SO_4) to a weak acid (HS^-) (White & Gadd 1998). For these reasons, sulphate-reducing bacteria have been used to treat metal contaminated waters in a range of configurations including artificial wetlands, fixed bed anaerobic filters and sludge reactors (White *et al.* 1998, Lloyd *et al.* 2004). The best studied example used a sludge-blanket reactor to treat water contaminated with sulphate and metals (principally zinc) from the smelting works at Budel-Dorplein in the Netherlands. Metal concentrations were reduced to the sub-ppb range (Barnes *et al.* 1994) and the process was subsequently expanded to commercial scale. A range of bioreactor configurations based on this system are now marketed under the registered trademark "Thiopaq®" by Paques BV of the Netherlands (http://www.paques.nl).

Another study aimed to further develop this anaerobic process and integrate it into a programme to treat metal-contaminated soils (White *et al*. 1998). Metals were leached from contaminated soil using sulphur-oxidising bacteria, yielding a metal-laden acidic liquor which was then treated using sulphate-reducing bacteria, resulting in neutralization of the effluent and precipitation of the metals as insoluble sulphides. Advantages associated with the use of a mixed culture in the sulphide precipitation step included a lower susceptibility to contamination by other organisms, improved adaptation to perturbations during reactor operation (e.g. changes in redox potential, temperature, pH and nutrient concentrations) and a more complete use of substrates by the microbial consortium (White *et al*. 1998). Although stable mixed cultures have been used in established sulphidogenic metal treatment processes (Barnes *et al*. 1994, Drury 1999), the microbial populations used remain ill-defined. The aim of this study was, therefore, to elucidate the microbiological components of the mixed culture utilized in a sulphidogenic metal treatment bioreactor (White *et al*. 1998, 2003). Such information is important so that changes in reactor performance can be correlated with microbial community structure. In addition, the range of other contaminants that can be utilized may be predicted from a better knowledge of the bacterial species within the community. To address this problem, relatively non-biased polymerase chain reaction (PCR)-based techniques were selected as the method of choice in combination with traditional culturing techniques.

Materials and Methods

Mixed culture

The mixed sulphate-reducing culture was obtained from a brackish estuarine sediment of the river Tay (White & Gadd 1996, White *et al*. 1998). Enrichments were carried out using a defined medium modified from that of Widdel and Pfennig (1981) with 2.24 gl^{-1} Na lactate as the sole carbon/energy source and 4.00 gl^{-1} Na_2 SO_4 as the terminal electron acceptor. Cultures were selected for biofilm growth by repeatedly batch-culturing to stationary phase in the presence of a polystyrene coupon (to which cells preferentially attached) and using segments of the coupon to inoculate further subcultures. This resulted in a mixed culture with a strong propensity for attached growth. Biofilm-adapted cultures were further selected for metal and acid tolerance, as described by White and Gadd (1996).

Isolation of SRB was achieved using a shake-tube protocol, using the above defined medium, with the addition of a further 39.5 mg l^{-1} $FeSO_4$ and 1%(w/v) high-purity agar. Biofilm cultures were disrupted by vortexing and a 10%(v/v) inoculum was added to shake tubes held at 40 °C, under nitrogen, with gentle mixing. Tenfold serial dilutions were sealed under a N_2 headspace and incubated in the dark at 30 °C for four weeks. SRB colonies were visible by the presence of black iron sulphide, and were extracted from the lowest dilution tubes containing isolated colonies, using a sterile capillary tube. The extracted colonies were re-inoculated into further shake-tube series and the above was repeated twice, before colonies were inoculated into liquid medium and incubated as described above, selecting for biofilm growth. Colony purity was checked by plating under aerobic and anaerobic conditions into medium containing lactate or glucose as the sole carbon/energy source, prior to molecular characterisation.

A number of isolates were obtained and a single morphotype (designated I1) (see below) was dominant amongst these. Further metabolic tests showed that this SRB isolate was able to grow by the incomplete oxidation of lactate to acetate and CO_2, coupled to the reduction of sulphate. Ethanol could also be used as a carbon/energy source, but not glucose, and molecular hydrogen could be utilised as a source of electrons for sulphate reduction. Lactate oxidation also took place with sulphite and thiosulphate as electron acceptors, but nitrate could not be utilised.

Amplification of 16S rRNA gene sequences

DNA was extracted from growth media using a Fast DNA spin kit (UltraClean, Soil DNA Isolation Kit, MO BIO Laboratories INC, Solana Beach, CA, USA). A fragment of the 16S rRNA gene, approximately 520 b.p., was amplified by PCR from samples using the broad-specificity primers 8F and 519r following published protocols (Islam *et al*. 2004). In addition, a 1542 bp product was amplified from DNA purified from an axenic

culture of Strain I1 using primers pA and pH' (Edwards *et al*. 1989) using a similar PCR protocol. Purity of the amplified products was determined by electrophoresis in Tris-borate-EDTA (TBE) gel. DNA was stained with ethidium bromide and viewed under short-wave UV light using a BioRad Geldoc 2000 system (BioRad, Hemel Hempstead, Herts, UK).

Amplification of disulfite reductase (DSR) gene sequences

A fragment of the DSR gene, approximately 1.9 kb, in length was amplified from samples using the SRB specific primers dsr1F and dsr4R (Wagner *et al*. 1998). The PCR amplification protocol used with the dsr1F and dsr4R primers was: initial denaturation at 94 °C for 4 min, melting at 94 °C for 30 s, annealing at 54 °C for 1 min, elongation at 72 °C for 2 min; 30 cycles, followed by a final extension step at 72 °C for 5 min. Purity of the amplified product was determined as described previously.

Restriction Fragment Length Polymorphism (RFLP) analysis

PCR products were purified using a QIAquick PCR purification kit (Qiagen, Crawley, W. Sussex, UK) and ligated directly into the cloning vector pCR 2.1 (Invitrogen, Paisley, UK) prior to transformation into *E. coli* TOP 10 competent cells. White transformants that grew on LB agar containing ampicillin and X-Gal were screened for an insert using PCR. Primers were complementary to the flanking regions of the PCR insertion site of the pCR 2.1 cloning vector. The PCR method was: an initial denaturation at 94 °C for 4 min, melting at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min; 35 cycles, followed by a final extension step at 72 °C for 5 min. The resulting PCR products were purified using a QIAquick kit and treated with restriction endonucleases *Sau3A* and *Msp I*. The restriction enzyme digests were separated using an agarose TBE gel.

DNA sequencing and phylogenetic analysis

Nucleotide sequences were determined by the dideoxynucleotide method (Islam *et al*. 2004). An ABI Prism BigDye Terminator Cycle Sequencing Kit was used in combination with an ABI Prism 877 Integrated Thermal Cycler and ABI Prism 377 DNA Sequencer (Perkin Elmer Applied Biosystems, Warrington, UK). Sequences (typically 500 base pairs in length) were analysed against the NCBI (USA) database using BLAST program packages and matched to known 16S rRNA gene sequences. Gene sequences were aligned using ClustalX software package and corrected manually. The TREECON package was used for distance analysis using the Jukes and Cantor correction. A phylogenetic tree was constructed from the distance matrix via neighbour joining (Islam *et al*. 2004).

Intergenic Spacer Analysis Fingerprinting

The 16S-23S intergenic spacer region from the bacterial RNA operon was amplified from DNA purified from sediment samples using PCR, as described previously, using primers S-D-Bact-1522-b-S-20 (eubacterial 16S rRNA small subunit), and L-D-Bact-132-a-A-18 (eubacterial 23S rRNA large subunit (Ranjard *et al*. 2001)), the former labelled at the 5′ end with HEX (6-carboxyhexafluorescein) fluorochrome. The HEX-labelled PCR product contained the intergenic gene sequence and approximately 20 b.p. of the S-D-Bact-1522-b-S-20 primer, and 130 b.p. of the 23S rRNA gene. The intergenic spacer fragments were resolved on a 5% polyacrylamide gel and run under denaturing conditions for 4.5 hours at 3000 V on an ABI Prism 377 DNA Sequencer (Perkin Elmer Applied Biosystems, Warrington, UK). The data were analysed using GeneScan 3.1 software (Perkin Elmer) which converted fluorescence data into electrophoregrams; peaks represented fragments of different sizes, with peak heights proportional to the quantity of fragment in the mixed product.

Results

Molecular analysis of the sulphate-reducing bacterial consortium

Initial molecular analyses focused on a characterization of the 16S rRNA genes in a clone library constructed from a PCR product that had been

amplified from the microbial consortium using broad specificity bacterial primers. Amplifications using primers specific for Archaea did not yield a PCR product, suggesting that these prokaryotes were not numerically significant in the microbial consortium. Approximately 20% of the 57 clones that were screened from the clone library corresponded to 16S rRNA genes affiliated to known sulphate-reducing bacteria (Table 1, Figure 1). All were members of the δ-Proteobacteria. The predominant sulphate-reducing bacterium detected in the mixed culture was most closely related (99% homology over 500 base pairs) to the sulphate-reducing bacterium *Desulfomicrobium norvegicum* (Figure 2). The partial sequence was also identical to that of a 1542 base pair product that corresponded to the almost complete 16S rRNA gene sequence that was amplified from an axenic culture (strain I1) isolated from the consortium, suggesting that this isolate corresponded to the *Desulfomicrobium* detected in the clone library. Although there is undoubtedly some bias imposed by the PCR-based technique used in this study, it is fair to conclude that the sulphate-reducing bacterium isolated from the mixed culture is numerically significant in the mixed culture and may be the dominant sulphate-reducing bacterium present. The hypothesis that this organism is a significant component of the mixed culture is further supported by the retrieval of the corresponding disulfite reductase (DSR) gene from the mixed culture by PCR (Figure 3). DSR genes are useful functional gene markers for sulphate-reducing bacteria, as they are present in all sulphate-reducing prokaryotes (Wagner *et al.* 1998). The DSR genes from the mixed culture were amplified, cloned and yielded only 2 RFLP types from 15 clones, 67 % of the clone library corresponded to the DSR gene most closely related to that of *Desulfobacterium macestii* (96% match over 744 bp), and was identical to that amplified from the pure culture identified as a *Desulfomicrobium* sp. (see above). It should be noted that *Desulfobacterium macestii* has recently been reclassified as a *Desulfomicrobium* strain (Hippe *et al.* 2003) in keeping with our phylogenetic analysis of the pure culture reported here. The other 33 % corresponded to a distinct DSR gene most closely related to that of *Desulfomicrobium baculatum* (96% match over 762bp). Additional evidence that the *Desulfomicrobium* strain isolated and also detected by 16S rRNA and DSR gene analysis in the mixed culture, was a numerically significant member of this community was the clear presence of two major peaks corresponding to the 16S-23S rRNA gene intergenic spacer regions amplified from this organism, in the mixed microbial community ARISA profile (Figure. 5). More than one peak from a single organism is not unexpected given the multiple copies of the rRNA genes present in many bacteria (Nazaret, pers. communication).

Only two other sulphate-reducing bacteria were detected in the clone library, with phylogenetic affiliations based on distinct 16S rRNA

Table 1. Sequence analysis of 16S rRNA partial gene clone library prepared from a sulphate-reducing consortium.

RFLP Type	Closest Matching Bacterium (Accession number)	%Match (Identities)	% Present	Phylogenetic Division
DUN-M4	*Rhodobacter capsulatus* (M34129)	96% (435/451)	19.25%	Alpha Proteobacteria
DUN-M9	*Delftia acidovorans strain* LE/66 (AJ516044)	92% (382/411)	1.75%	Beta Proteobacteria
DUN-A17	*Azoarcus communis* (AF011343)	95% (452/472)	8.75%	Beta Proteobacteria
DUN-A23	*Desulfomicrobium norvegicum* (AJ277897)	99% (498/500)	15.75%	Delta Proteobacteria
DUN-M2	*Desulfovibrio dechloracetivorans* (AF230530)	95% (418/437)	1.75%	Delta Proteobacteria
DUN-M10	*Desulfobacterium macestii* (AJ237604)	96% (412/427)	3.50%	Delta Proteobacteria
DUN-A12	*Escherichia coli* K-12 MG1655 (U00096)	84% (254/300)	5.25%	Gamma Proteobacteria
DUN-A13	*Photorhabdus luminescens* (AY444555)	97% (489/501)	1.75%	Gamma Proteobacteria
DUN-A7	*Acholeplasma polakii* (AF031479)	94% (400/425)	1.75%	Acholeplasmatales
DUN-A2	*Dysgonomonas wimpennyi* (AY643492)	91% (455/500)	12.25%	Bacteroides
DUN-A15	*Clostridium lactatifermentans* (AY033434)	92% (367/397)	12.25%	Clostridiales
DUN-A9	*Acetobacterium carbinolicum* (X96956)	97% (473/484)	1.75%	Clostridiales
DUN-M1	*Sphingobacterium mizutae* (X67853)	92% (252/273)	7.00%	Flavobacteria
DUN-A1	*Spirochaeta bajacaliforniensis* (AJ698859)	90% (165/182)	5.25%	Spirochaetales
DUN-A5	*Sphingobacterium comitans* (X91814)	91% (213/232)	1.75%	Sphingobacteria

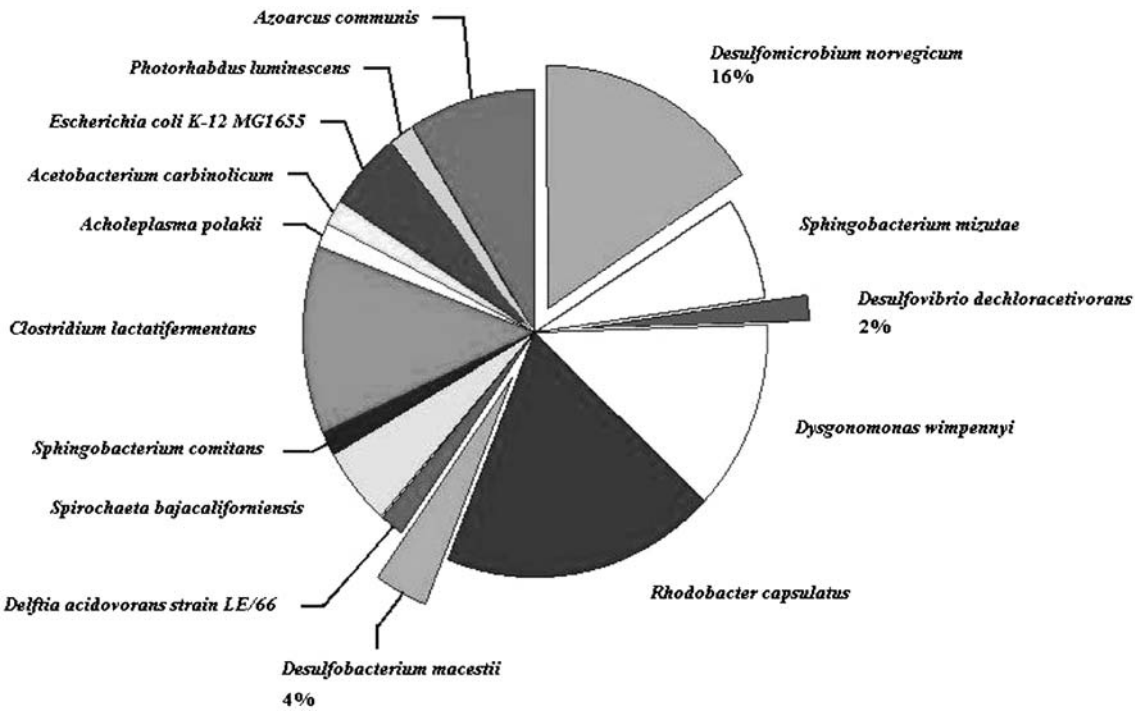

Figure 1. Phylogenetic affiliations (closest know relatives) of bacteria detected in a stable sulphate-reducing consortium using PCR with broad-specificity primers for bacterial 16S rRNA genes.

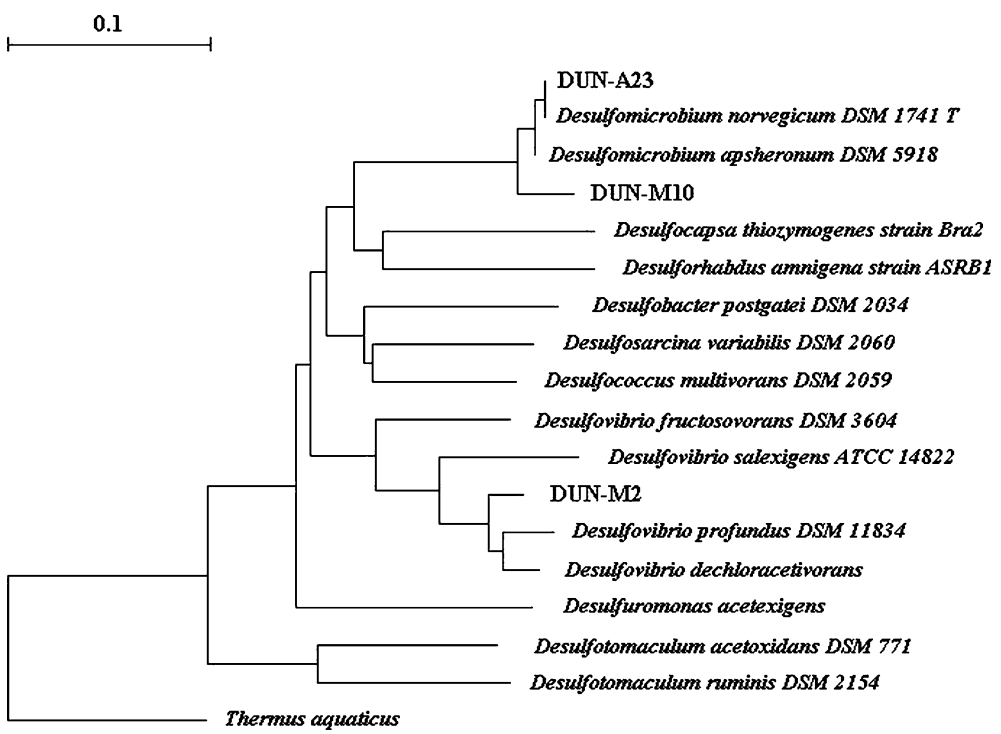

Figure 2. Phylogenetic placement of three sulphate-reducing bacteria detected in the sulphate-reducing consortium. Sequence DUN-A23 corresponded to the sequence of strain I1 isolated from the consortium.

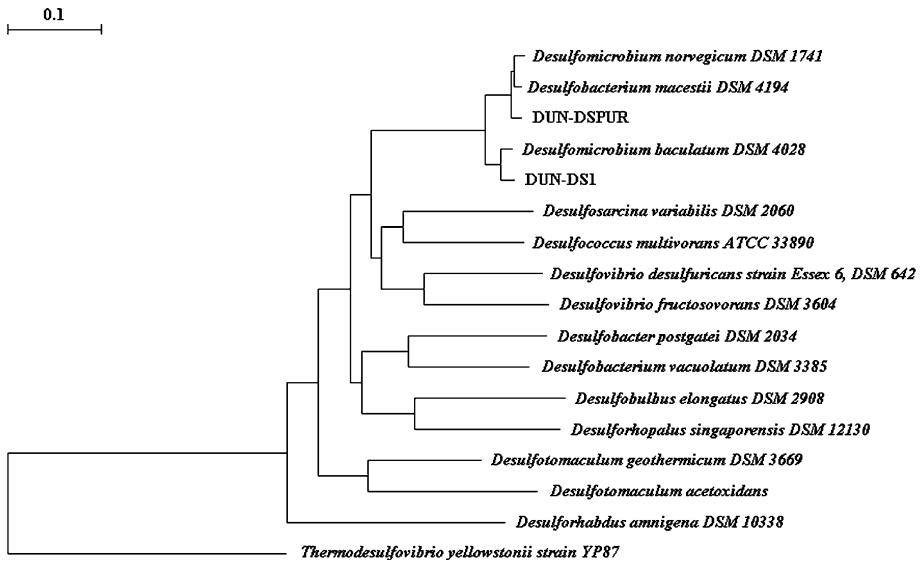

Figure 3. Phylogenetic placement of two distinct DSR sequences detected in a sulphate-reducing consortium. Sequence DUN-DSPUR constituted 67% of the sequences in the close library, and also corresponded to the DSR sequence of strain I1 isolated from the consortium.

gene sequences closest to *Desulfobacterium macestii* (now *Desulfomicrobium macesti*) and *Desulfovibrio dechloracetivorans*. As noted previously, the DSR gene corresponding to the former organism was also detected in the mixed culture. In keeping with the pleomorphic nature of the mixed culture revealed by light microscopy used in combination with Gram-staining, sequences from several other distinct Gram-negative and Gram-positive genera were detected. Throughout the extended cultivation of the mixed sulfidogenic community, spore-forming bacteria were often noted during microscopic investigations, but Gram-positive spore forming sulphate-reducing bacteria, such as *Desulfotomaculum* sp, were not detected or isolated. It is probable that the spore formers revealed by Gram-staining were clostridial species, which contributed 12% of the clone library. The mixed culture also contained symbiotic organisms capable of oxidizing SRB-produced acetate to CO_2 under anaerobic conditions, as well as one or more facultative aerobes, amongst which a filamentous morphotype with gliding motility was abundant. A similar type was also a common secondary coloniser, under anaerobic conditions, of mixed sulphidogenic biofilms dominated by *Desulfomicrobium* morphotypes (see Figure 4).

Discussion

After prolonged cultivation, the mixed culture contained a low diversity of sulphate-reducing bacteria, with one strain closely related to *Desulfomicrobium norvegicum* and this was identified as a numerically-significant member of the population by three independent PCR-based analyses. This organism was also isolated by conventional anaerobic techniques, confirming its presence in the mixed culture. In fact, microscopic observations of the Gram-negative cultures revealed short rods with single polar flagella and vibrioid motility typical of the *Desulfomicrobium* genus (Holt 1994). The relatively low diversity of sulphate-reducing bacteria in the culture probably reflects the selective enrichment process used, including the selection for maximal sulphate reduction and metal and acid tolerance (White & Gadd 1996a 1996b, White *et al*. 1998).

The apparent diversity of other bacteria in the mixed culture unable to grow using sulphate as an electron acceptor under anaerobic conditions, including some heterotrophs, is surprising but undoubtedly reflects the potential of the culture to treat a wider diversity of effluents including those containing more complex nutrients and other organic contaminants not present in the defined medium used in this study. This finding also

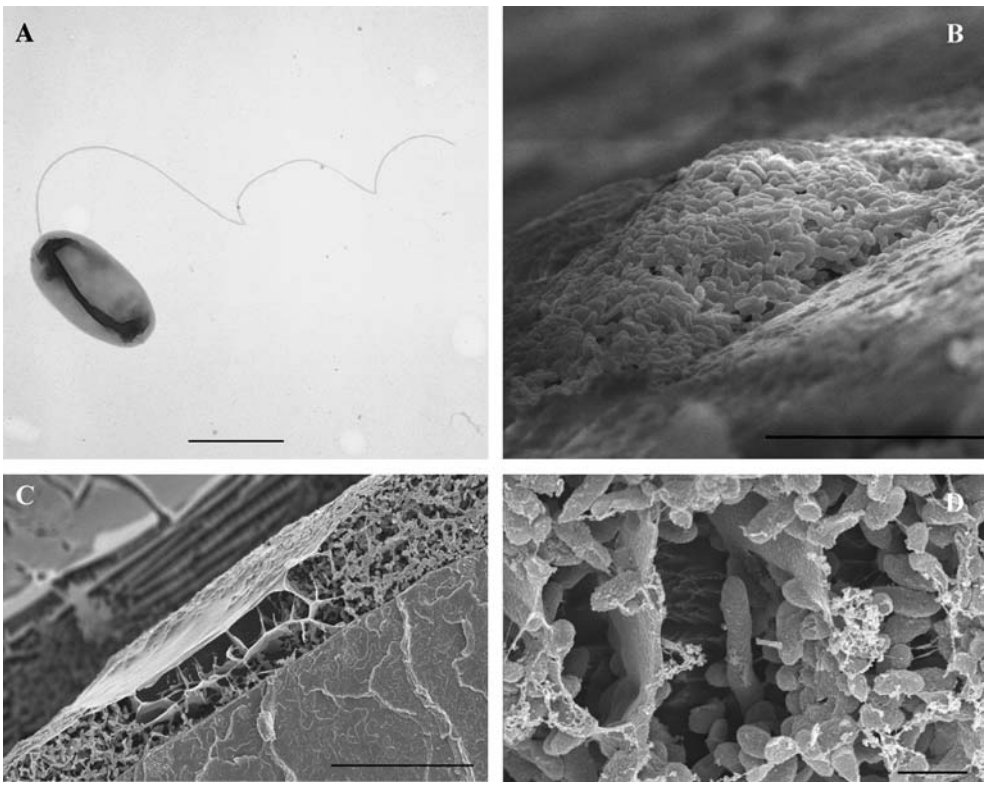

Figure 4. (A) Negatively-stained TEM mount of a single *Desulfomicrobium sp*. (I1) cell harvested from the planktonic phase, showing a typical single polar flagellum.Bar = 1μm. (B) Oblique-angle ESEM image of surface-attached cells, forming part of a typically heterogeneous mixed-species biofilm.Bar = 10μm (C) FESEM cryosection of the same biofilm shows densely-populated cell clusters, interspersed by cell-free regions, within a matrix of extracellular polymers.Bar = 25μm (D) *Desulfomicrobium sp*. morphotypes (short rods) dominated mixed biofilms under laboratory conditions, although other cell types (cocci and filamentous bacteria) were also visible. Fine-grained metal sulphides were precipitated within the biofilm, associated with the extracellular matrix.Bar = 2μm.

supports a model of the symbiotic/commensal nature of carbon and energy fluxes in such a mixed culture. For example, the incomplete oxidation of lactate to acetate by SRB would provide a utilisable carbon/energy source for associated heterotrophs. The abundant EPS matrix in SRB biofilms may also have provided a more complex nutrient resource capable of supporting a diverse bacterial community, as well as contributing to metal capture (White & Gadd 2000). This was reflected in the higher metabolic rate, greater biomass production and more efficient metal removal capacity recorded

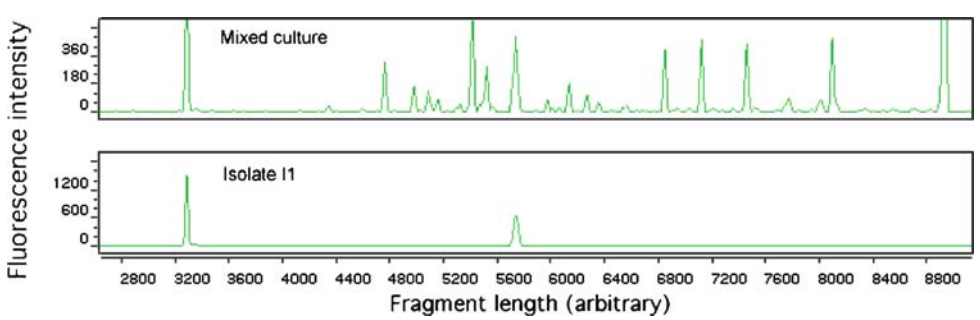

Figure 5. ARISA analysis of the sulphate-reducing consortium and isolate I1.

during biofilm growth of mixed cultures, compared to single-strain cultures (White & Gadd 2000).

The physiology and function of SRB communities is receiving increasing attention not only in relation to important environmental processes occurring under anaerobic conditions and/or at oxic/anoxic interfaces, but also in relation to bioremedial applications for treatment of metal(loid) contaminated effluents (Kolmert & Johnson 2001, Hockin & Gadd 2003). The distribution of sulfate-reducing bacteria (SRB) in contaminated groundwater at a uranium mill tailings disposal site was investigated by sequencing of clone libraries of PCR-amplified dissimilatory sulfite reductase (DSR) gene fragments and phospholipid fatty acid (PLFA) biomarker analysis (Chang *et al*. 2001). A marked diversity among the DSR sequences included those from δ-Proteobacteria, Gram-positive organisms, and the *Nitrospira* division. Among the findings from this approach was that *Desulfotomaculum* possessed remarkable tolerance and adaptation to high levels of uranium and implicated the organism's involvement in natural attenuation of uranium (Chang *et al*. 2001). Our work has similarly highlighted the most important SRB in the mixed community and emphasised its role in metal bioprecipitation within the biofilm.

In situ visualisation and characterisation of metal-containing minerals formed within SRB biofilms from metal-challenged bioreactors can be used in conjunction with analytical chemical techniques and chemical speciation programming to develop quantitative models of metal and metalloid removal by mixed sulphidogenic cultures White *et al*. 2003). As part of this work, it is clearly important to understand the nature of the microbial community and the key organisms involved. Such findings can serve as a basis for future process optimisation and are also relevant to wider applications, such as the treatment of wastes containing organic pollutants. Mixed culture biofilms obviously may possess a wider physiological potential for such a treatment approach, with some of the organisms detected here being capable of organic compound degradation. SRB capable of degrading a wide range of organics including aromatics have been isolated including members of the genera *Desulfobacterium* (Schnell *et al*. 1989) which was a component of the mixed SRB culture examined in this study. In fact, all known sulfate-reducing bacteria which utilize aromatic compounds belong to the Desulfobacteriaceae (Rabus *et al*. 1996). Other bacterial genera identified in the SRB consortium included other bacteria with degradative properties. These include *Delftia acidovorans* that has documented ability to degrade complex organics including 2-(4-sulfophenyl)butyrate (SPB) (Schulz *et al*. 2000) and, in a multi-species consortium, enhanced linuron degradation (Dejonghe *et al*. 2003). *Acetobacterium* spp. are capable of anaerobic degradation of various aromatic compounds (Bache & Pfenning 1981) including some by reductive dechlorination (Terzenbach & Blaut 1994). Further studies on the functionality of mixed SRB consortia together with detailed characterization of the organisms involved would clearly be useful in assessing potential treatment methods for metal and organic-containing wastes.

Acknowledgments

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