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Sulfidogenesis in low pH (3.8–4.2) media by a mixed population of acidophilic bacteria

Sakurako Kimura, Kevin B. Hallberg & D. Barrie Johnson*
*School of Biological Sciences, University of Wales, Bangor, LL57 2UW, U.K. (*author for correspondence: e-mail: d.b.johnson@bangor.ac.uk)*

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Abstract

A defined mixed bacterial culture was established which catalyzed dissimilatory sulfate reduction, using glycerol as electron donor, at pH 3.8–4.2. The bacterial consortium comprised a endospore-forming sulfate reducing bacterium (isolate M1) that had been isolated from acidic sediment in a geothermal area of Montserrat (West Indies) and which had 94% sequence identity (of its 16S rRNA gene) to the Gram-positive neutrophile *Desulfosporosinus orientis*, and a Gram-negative (non sulfate-reducing) acidophile (isolate PFBC) that shared 99% gene identity with *"Acidocella aromatica"*. Whilst M1 was an obligate anaerobe, isolate PFBC, as other *Acidocella* spp., only grew in pure culture in aerobic media. Analysis of microbial communities, using a combination of total bacterial counts and fluorescent *in situ* hybridization, confirmed that concurrent growth of both bacteria occurred during sulfidogenesis under strictly anoxic conditions in a pH-controlled fermenter. In pure culture, M1 oxidized glycerol incompletely, producing stoichiometric amounts of acetic acid. In mixed culture with PFBC, however, acetic acid was present only in small concentrations and its occurrence was transient. Since M1 did not oxidize acetic acid, it was inferred that this metabolite was catabolized by *Acidocella* PFBC which, unlike glycerol, was shown to support the growth of this acidophile under aerobic conditions. In fermenter cultures maintained at pH 3.8–4.2, sulfidogenesis resulted in the removal of soluble zinc (as solid phase ZnS) whilst ferrous iron remained in solution. Potential syntrophic interactions, involving hydrogen transfer between M1 and PFBC, are discussed, as is the potential of sulfidogenesis in acidic liquors for the selective recovery of heavy metals from wastewaters.

Abbreviations: AMD – acid mine drainage; EDAX – energy dispersive analysis of X-rays; FISH – fluorescent *in situ* hybridization; OFN – oxygen-free nitrogen; SRB – sulfate reducing bacteria.

Introduction

Acidic environments in which sulfur or sulfide minerals are subjected to (biologically)-accelerated oxidative dissolution characteristically contain large concentrations of soluble sulfate (up to *ca.* 8 M; Johnson 2003). Dissimilatory sulfate reduction might therefore be anticipated to occur within anaerobic zones in these environments, providing that suitable electron donors (such as small molecular weight organic compounds, or hydrogen) are also present. Whilst there have been reports of sulfidogenesis in low pH ecosystems, such as sediments of acid mine drainage (AMD)-impacted streams and lakes (Tuttle et al. 1969; Gyure et al. 1990), efforts directed at isolating and cultivating truly acidophilic or acid-tolerant sulfate reducing bacteria (SRB) have been mostly

unsuccessful. Most of the SRB that have been isolated from acidic mine streams and lakes are neutrophilic and are not active below pH 5.0 (Küsel et al. 2001; Tuttle et al. 1969), suggesting that many, if not all, sulfidogenic bacteria inhabit circum-neutral pH microsites within these sediments.

One of the reasons for the failure to isolate acidophilic SRB has been the use of inappropriate enrichment media. For example, small molecular weight organic acids, such as lactate, are commonly used in growth media for SRB (Postgate 1979). However, in acidic liquors (with pH values below the p*K*a's of aliphatic acids) the acids occur predominantly in their undissociated, lipophilic forms and, as such can transverse bacterial membranes, where they dissociate in the circum-neutral internal cell cytoplasm, causing in dis-equilibrium and the influx of further undissociated acid, and acidification of the cytosol (Norris & Ingledew 1992). Concentrations of organic acids at even <1 mM are lethal to many acidophilic microorganisms (Johnson & Roberto 1997).

The use of alternative (non-acid) substrates for enriching acidophilic/acid-tolerant SRB has been more successful. Hard et al. (1997) isolated an SRB from a Norwegian copper mine on solid media that contained methanol as carbon and energy source. The isolate had a pH range for growth of 4.0–9.0. These authors also noted that a characterized SRB, *Desulfovibrio salexigens*, could also grow on methanol in media poised at pH 4.5 and above. Sen & Johnson (1999) used glycerol as electron donor to enrich for sulfidogenic bacteria from sediments from geothermal sites on Montserrat (West Indies) and an abandoned copper mine (Mynydd Parys) in Wales. Sulfidogenesis (and production of alkalinity) was detected in fermenter cultures adjusted to pH 2.0 in the case of one of the enrichments. It was later confirmed that the sulfidogenic cultures were consortia of SRB and other, undefined, bacteria (Sen 2001).

One of the restrictions in using microbial sulfidogenesis to remediate acidic mine waters and recover base metals present within them is that the SRB used in current operations are all neutrophilic, and are highly sensitive to even mildly acidic waters (Postgate 1979). To circumvent this problem, sulfidogenic fermenters used in both full-scale (e.g. at the Budelco zinc refinery in the Netherlands; Boonstra et al. 1999) and bench-scale (e.g. Tabak & Govind 2003; Tabak et al. 2003; Pott & Mattiasson 2004) systems are all "off line", to avoid direct contact with acidic liquors. This involves growing SRB in an isolated neutral pH fermenter where hydrogen sulfide is generated, and from there delivered to a second tank (or tanks) where metal sulfides are precipitated. Acid-tolerant (or acidophilic) SRB would have the potential advantage that a single reactor tank could be used, within which sulfidogenesis and metal removal from inflowing acidic wastewaters would occur simultaneously.

This paper describes sulfidogenesis in low pH (3.8–4.2) media by a defined mixed population of an acetogenic SRB and an acetate-degrading *Acidocella*-like organism. Evidence is presented for a tightly-coupled syntrophic relationship between the two acidophiles, which appears to be of major significance in facilitating biological sulfate reduction at low pH.

Materials and methods

Microorganisms and growth media

A mixed culture of acidophilic bacteria was established in a liquid medium containing 5 mM K_2SO_4, trace elements and basal salts (Johnson 1995). The medium was de-oxygenated with oxygen-free nitrogen (OFN) for 30 min and then sterilized (120 °C, 30 min). A vitamin mixture (Widdel & Pfennig 1981), 5 mM glycerol, 5 mM $ZnSO_4$ and 0.1 mM $FeSO_4$ (heat-sterilized, or filter-sterilized through 0.2 μm membranes, as appropriate) were added to the liquid medium after autoclaving.

The bacteria used were coded "M1" and "PFBC". M1 is a spore-forming SRB that had been isolated by Sen (2001) from an enrichment culture of acidic sediment from Montserrat, by plating onto acidic glycerol-containing overlay solid medium, incubated anaerobically (Sen & Johnson 1999). Sequence analysis of the 16S rRNA gene of this bacterium (partial product, 466 bp) showed that it was most closely related to *Desulfosporosinus orientis* (94% sequence identity). Strain PFBC is an acidophilic heterotrophic bacterium that had been isolated as a contaminant from another supposedly pure culture of *Desulfosporosinus* isolate PFB, and which was not capable of dissimilatory sulfate reduction.

Sequence analysis of the 16S rRNA gene of PFBC (partial product, 656 bp) showed that it was most closely related to "*Acidocella aromatica*" (99% sequence identity; S. Kimura, unpublished data), an apparently obligately aerobic acidophile that was able to catabolize a variety of aromatic compounds, and which was also particularly adept at oxidizing small molecular weight organic acids, such as acetate (Hallberg et al. 1999; Gemmell & Knowles 2000). Isolate PFBC was routinely maintained on a solid medium containing 5 mM fructose (pH 3) incubated aerobically.

Growth of pure cultures of M1 and PFBC

Pure cultures of M1 and PFBC were obtained from repeated single colony isolation, using the solid media referred to above. They were then tested for growth in liquid media (pH 4.0) containing either 5 mM glycerol (+5 mM zinc sulfate for M1), or 2 mM acetic acid, incubated both aerobically and anaerobically at 30 °C. Growth was monitored by enumeration of bacterial cells (using a Thoma counting chamber and phase contrast microscopy) and depletion of substrate. Concentrations of acetic acid were also measured in cultures of M1 grown on glycerol. Sulfidogenesis was indicated by decreasing concentrations of soluble zinc (due to the formation of insoluble ZnS) during culture incubation. Cultures were set up either in shake flasks (100 ml of media in 150 ml flasks, for aerobic cultures) or in 25 ml universal bottles, incubated anaerobically using the Anaero*Gen*^{TM} AN25 system (Oxoid Ltd.).

Growth of mixed cultures of M1 and PFBC

Mixed cultures of M1 and PFBC were set up in an Electrolab P350 fermenter (Electrolab, U.K.) incorporating a 2 l growth vessel fitted with pH, temperature and dissolved oxygen monitoring and control. The glycerol- and zinc-containing medium (described above) was sterilized and de-oxygenated by gassing with OFN. Strains M1 and PFBC, pre-grown in pure culture, were introduced, and the culture pH set. The values used were pH 3.8, 4.0 and 4.2. In each case, the pH limits set were 0.1 of a pH unit below and above the set point. The control of pH in the fermenter was maintained by automatic addition of 0.1 M NaOH or 0.1 M H_2SO_4. The amount of sulfuric acid used in pH maintenance was monitored to allow calculations to be made of net sulfate reduction, though this was subject to some degree of error. On each occasion that a batch experiment was carried out, 1 l of spent liquor from the previous run was removed from the fermenter (2 l working volume) and replaced with 1 l of fresh medium as above, but containing 10 mM, rather than 5 mM, of both zinc sulfate and glycerol, so that the initial concentration of these components was ca. 5 mM (though this did vary between experimental runs). The fermenter was maintained at 30 °C, and stirred gently (60 rpm) with a continuous stream of OFN. Samples were withdrawn aseptically at regular (generally daily) intervals to determine the microbial composition of the liquid phase, and to measure the various chemical parameters described below. Replicated or triplicated batch experiments were carried out for each pre-set pH value, and the duration of these experiments was generally 6–10 days.

Determination of relative and total numbers of M1 and PFBC in mixed cultures

Relative numbers of M1 and PFBC in mixed fermenter cultures were determined using fluorescent *in situ* hybridization (FISH). This involved staining the mixed population with a fluorescein-labeled general bacterial probe, and species-specific Cy3-labeled probes (Table 1). Samples (2 ml) from the fermenter culture were centrifuged and re-suspended in 3% (v/v) paraformaldehyde in phosphate buffered saline. Samples were incubated at 4 °C for 2 h in the fixative for fixation. The fixed samples were processed using the method described by (Bond & Banfield 2001). Hybridization (10 μl reaction) was carried out with both 25 ng of a fluorescein-labeled eubacterial probe (EUB338) (Amann et al. 1990) and 25 ng of AcaromCy3 probe (which targeted the *Acidocella*-like PFBC) or aSRBCy3 probe (Frank Roberto, Idaho National Engineering and Environmental Laboratory, U.S.A., unpublished), which targeted the *Desulfosporosinus*-like M1. Unlabelled helper oligonucleotides (Table 1) were also added to the hybridization reaction at the same concentration as the probes (Fuchs et al. 2000). Mounting medium (70% glycerol in 50 mM Tris pH 9.5 containing 23.3 mg/ml 1,4-diazabicyclo[2.2.2]octane (DABCO)) was applied to reduce fading of the

Table 1. 16S rRNA-targeted oligonucleotide probes used in this study

Probe name (ODP nomenclature[a])	Specificity	Sequence (5′–3′)	Helper oligonucleotides (5′–3′)
AcaromCy3 (S-S-Acarm-633-a-A-19)	"*A. aromatica*" isolates (including PFBC)	GTCGCTACGTATCAAAAGC	AGTCCCCAGGTTGAGCCC CAACCCTCTTCCTTACTCTA
aSRBCy3 (S-S-DspM1-446-a-A-19)	*Desulfosporosinus* M1 group	CTGAAGAAACCGTTCTTCC	CTAAAAACAGTACTTTACAATCCG GTCATGTCTCCTCCATATTC

[a] Oligonucleotide Database Project nomenclature (Alm et al. 1996).

probe signal during enumeration. Various concentrations of formamide (0–50%, v/v) were tested to optimize specificity and maximize signal response for each probe using pure cultures of target and other related microorganisms, including *Acidiphilium*-isolate SJH for AcaromCy3 probe and *D. orientis* for aSRBCy3 probe. The optimum formamide concentrations needed for the AcaromCy3 and aSRBCy3 probes were 20 and 30%, respectively. To enumerate *Acidocella* PFBC and *Desulfosporosinus* M1, Cy3-labeled cells were counted relative to those stained with the EUB338 probe.

To determine total numbers of bacteria, fixed samples (50 μl) were suspended in 10 ml filtered distilled water and adsorbed onto 25 mm black polycarbonate filters (0.2 μm pore-size; Millipore, Billerica, U.S.A.) using a 1225 sampling manifold (Millipore). The fixed samples were washed twice by drawing filtered distilled water through the membrane before staining with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 10 min. The sample fixed on the membrane was washed twice with filtered distilled water then placed onto a drop of CITIFLUOR^{TM} AF87 (Citifluor Ltd., U.K.) on a slide glass. In order to prevent fading of the dye, mounting medium (90% glycerol in 50 mM Tris pH 9.5 containing 23.3 mg/ml 1,4-diazabicyclo[2.2.2]octane (DABCO)) was applied directly onto the membrane. Enumeration of fluorescein/Cy3- and DAPI-stained bacteria was carried out using a Nikon ECLIPSE E66 microscope (Nikon Corporation, Japan).

Analytical techniques

Acetate, sulfate and glycerol were all determined using a Dionex DX-320 ion chromatograph (Dionex, Sunnyvale, U.S.A.). Acetate and sulfate were determined together by suppressed ion chromatography, using an Ion Pac® AS11 column and an ASRS ULTRA 4 mm Suppressor and an IC 25 ion chromatograph, and glycerol using a Carbo Pac^{TM} Pa10 column and an ED 40 amperometric detector. In both cases, KOH was used as eluent (15 mM for glycerol and a gradient from 15 to 45 mM in the case of acetate and sulfate). Data were analyzed using the Chromeleon® software package (Dionex, U.S.A.).

Concentrations of soluble zinc in fermenter cultures were determined using atomic absorption

spectrometry (SP9-10, Pye Unicam, England). Soluble ferrous iron was determined using the FerroZine reagent (Lovley & Phillips 1987).

Precipitates formed within the fermenter were analyzed using energy dispersive analysis of X-rays (EDAX). Samples were dehydrated by successive incubation for 3 min in 50, 80 and 95% (v/v) ethanol. Following air-drying, the samples were placed onto aluminum stubs and viewed using a Hitachi S-520 scanning electron microscope (Hitachi, Japan). The elemental composition of the solid phase materials was determined using an Oxford Instruments Link Isis III EDAX System (Oxford Instrument, Witney, Oxon, England).

Results

Growth of isolates M1 and PFBC in pure culture

The sulfate-reducer, *Desulfosporosinus* M1, was confirmed to be an obligate anaerobe. Of the four culture permutations tested, successful growth of pure cultures of M1 only occurred in 5 mM glycerol medium under anoxic conditions. This SRB was shown to be acetogenic, producing equimolar amounts of acetate from the oxidation of glycerol (data not shown). In contrast, although it had been isolated from an anoxic sulfidogenic mixed culture, *Acidocella* PFBC could not grow in pure culture in the absence of oxygen, either using glycerol or acetic acid as carbon and energy sources. This isolate grew readily in the presence of oxygen in 3 mM acetic acid (pH 3.0) medium, but could not metabolize glycerol aerobically. The latter trait has also been noted for closely related strains of "*A. aromatica*" (Hallberg & Johnson 2001).

Mixed cultures of acidophiles grown in fermenter cultures

Changes in concentrations of glycerol, acetic acid, sulfate and soluble zinc in the mixed fermenter cultures are shown in Figures 1–3. Continuous monitoring of dissolved oxygen confirmed that the cultures were anoxic in all of the experimental runs. At pH 4.0 (Figure 1), the oxidation of glycerol was tightly coupled to changes in the concentration of soluble zinc. Production of sulfide resulted in the precipitation of ZnS, so that concentrations of soluble zinc would have reflected

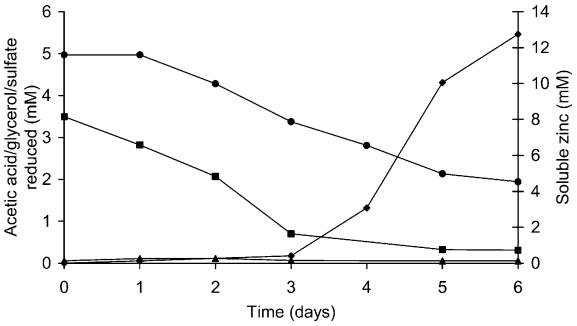

Figure 1. Relationships between substrate utilization, sulfate reduction and precipitation of zinc in a mixed culture fermenter maintained at pH 4.0. Key: (▲), acetic acid; (■), glycerol; (◆), sulfate reduced; (●), soluble zinc.

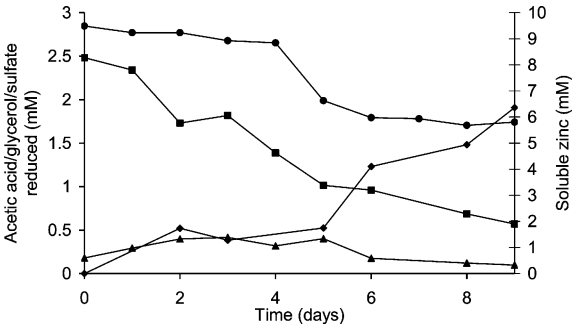

Figure 2. Relationships between substrate utilization, sulfate reduction and precipitation of zinc in a mixed culture fermenter maintained at pH 3.8. Key: (▲), acetic acid; (■), glycerol; (◆), sulfate reduced; (●), soluble zinc.

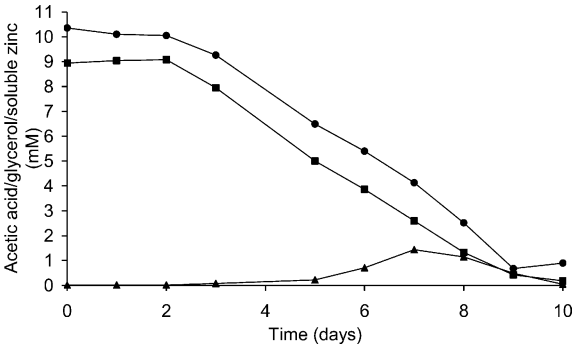

Figure 3. Relationship between substrate utilization and sulfate reduction, determined as precipitation of zinc, in a mixed culture fermenter maintained at pH 4.2. Key: (▲), acetic acid; (■), glycerol; (●), soluble zinc.

sulfate reduction. Although acetic acid was detectable throughout the incubation period, concentrations never rose above 0.12 mM. With

this fermenter run, 3.7 mmol/l of glycerol were oxidized, 5.5 mmol/l sulfate reduced and 7.1 mmol/l soluble zinc were removed. The net change in acetic acid concentration was < 0.01 mM. In contrast to zinc, concentrations of soluble ferrous iron did not change during the culture run.

Corresponding data from a fermenter culture run at pH 3.8 are shown in Figure 2. Glycerol oxidation appeared to be less tightly coupled to sulfate reduction during the first phase (days 1–5) but paralleled each other more closely after that time. Removal of soluble zinc corresponded to sulfate reduction throughout. More acetic acid (0.4 mM at day 3) was detected in this culture than at pH 4.0, but again this was transient, and the final concentration of acetic acid was actually less at the end of this fermenter run than at day 0 (that present at the start was due to carry over from the previous experimental run). In this batch experiment, 1.9 mmol/l glycerol was oxidized and 3.6 mmol/l zinc was precipitated (as ZnS).

Data from a mixed culture fermenter run at pH 4.2 are shown in Figure 3. In this case, the initial concentrations of glycerol and soluble zinc were approximately twice those of the pH 4.0 run. Again, glycerol oxidation was tightly coupled to zinc precipitation, presumably due to sulfidogenesis (no data for sulfate reduction were obtained in this particular run). A total of 8.8 mmol of glycerol were oxidized, and 9.7 mmol zinc precipitated (both/l) in this experiment. The concentration of acetic acid in this culture remained at <0.1 mM until day 4, but then increased, reaching a peak of 1.4 mM (the greatest concentration detected in any of the mixed cultures) before declining to 0.02 mM by the end of the experiment.

Solid phase products formed within the pH 4.0 fermenter culture were analyzed by EDAX. The results, shown in Figure 4, confirmed that ZnS was formed as a solid phase product. The absence of peaks at 6.4 and 7.1 keV indicate that no iron sulfides were produced (Figure 4).

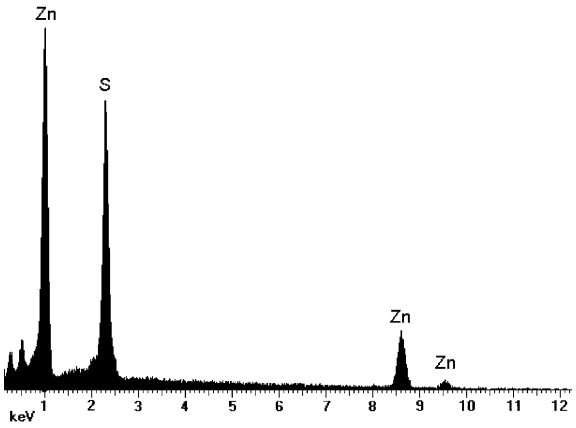

Figure 4. Composition of solid-phase products of the sulfidogenic mixed culture determined by energy dispersive analysis of X-rays (EDAX). The Kα1 (8.6 keV), La1 (9.5 keV) and La2 (1.0 keV) peaks of zinc along with the Kα1 peak (2.4 keV) of sulfur have been labeled.

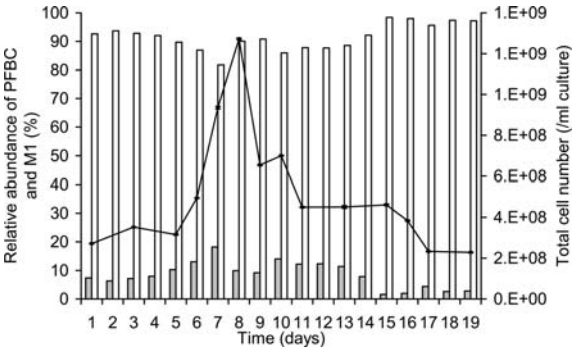

Figure 5. Microbial population dynamics in a pH 4.0 fermenter culture. Relative abundance of "*A. aromatica*" PFBC (unshaded bars) and *Desulfosporosinus* M1 (shaded bars) as determined by FISH analysis; line graph, total bacterial numbers, determined by DAPI staining.

Microbial community dynamics

Changes in total and relative bacterial numbers in a replicate pH 4.0 fermenter culture run are illustrated in Figure 5. The relative abundance of M1 increased between day 4 and 7 while glycerol was oxidized (data not shown), and decreased on days 8 and 9 at the same time that the concentration of acetic acid peaked (data not shown). *Desulfosporosinus* M1 appeared to recover from day 10 before its abundance, relative to PFBC, decreased again from day 14. On numerical basis, *Acidocella* PFBC accounted for more than 80% of the fermenter population throughout the incubation time though, since M1 cells are significantly larger (biovolume approximately 10 times) than those of PFBC, both microbes were equivalent on a biomass basis in the fermenter cultures.

Discussion

Previous work by Sen (2001) and Sen & Johnson (1999) had resulted in the enrichment of acidophilic (or acid-tolerant) SRB from acid sediments in Montserrat and the Parys copper mine, in north Wales. Pure cultures of the *Desulfosporosinus*-like isolate (M1) obtained from the Montserrat enrichment oxidized glycerol, producing acetic acid in equimolar amounts, as in Equation 1:

$$4C_3H_8O_3 + 3SO_4^{2-} + 6H^+ \rightarrow 4CH_3COOH + 4CO_2 + 3H_2S + 8H_2O \quad (1)$$

Incomplete substrate oxidation, producing acetic acid as an end product, is characteristic of many related SRB (Castro et al. 2000). Partial oxidation of glycerol results in the amount of sulfate reduced/mole of substrate oxidized being significantly less than if the substrate is completely oxidized to CO_2. In low pH liquors, acetic acid, occurring predominantly as the undissociated acid, also tends to be toxic to microbes at relatively low concentrations.

Interestingly, M1 grows successfully on acidic "overlay" media (Johnson 1995), which contain acetotrophic acidophiles in the gel underlayer, but not on non-overlay plates (Sen & Johnson 1999). Similarly, it has been difficult to sustain the growth of M1 in acidified liquid media in pure culture, though mixed cultures with acetotrophic acidophiles are far more robust (S. Kimura, data not shown). Strain PFBC is such a "partner acidophile". It was isolated from an enrichment SRB culture from Parys mine sediment and, when inoculated into a pure liquid culture of M1, resulted in a mixed culture that, in contrast to M1 alone, was capable of sustained and reproducible sulfidogenesis in low pH media. Although the lower pH limit for sulfidogenesis by this consortium has not yet been determined, it has been observed at pH 3.7 (the lower limit of the pH 3.8 fermenter run).

According to Equation 1, the stoichiometry between glycerol oxidized and sulfate reduced, assuming incomplete oxidation of the former, is 4:3. One hydrogen sulfide is produced for each sulfate reduced, and this in turn should result in the precipitation (at the pH range used) of equimolar amounts of zinc (Equation 2).

$$Zn^{2+} + H_2S \rightarrow ZnS + 2H^+ \quad (2)$$

The results from the fermenter experiments showed clearly that the mixed culture of M1 and PFBC was behaving differently to that anticipated for the pure culture. For one thing, acetic acid was not accumulating in stoichiometric amounts as glycerol was oxidized. Although acetic acid was found throughout incubation in all experimental runs, concentrations were much lower than predicted by Equation 1, and this metabolite was noted to be transient, increasing and then decreasing in concentration during the fermenter runs. Secondly, in most cases the amounts of sulfate reduced and of zinc precipitated were much greater than predicted by Equation 1.

Data from pure cultures of M1 and PFBC showed that only one of these (M1) could metabolize glycerol and only one (PFBC) could metabolize acetic acid. Given this, in the mixed culture, glycerol would have been oxidized (by M1) to acetic acid, which would be further oxidized (by PFBC) to CO_2. Since M1 is an SRB, its electron acceptor would be sulfate. A theoretical problem arises in the case of PFBC, however, since the only electron acceptor known to be used by this (and related) acidophiles is molecular oxygen. Extensive testing has confirmed that PFBC does not reduce sulfate, and potential alternative electron acceptors (such as nitrate and soluble ferric iron) are not used by "*A. aromatica*" (Hallberg et al. 1999) and, in any case were not present in the liquid medium used.

Confirmation that both M1 and PFBC were growing in mixed fermenter cultures came from combined total and relative bacterial counts using DAPI staining and FISH. The latter showed that relative numbers of the two bacteria fluctuated throughout incubation, with M1 being, in general, more abundant, relatively, in the earlier than in the later phases of the fermenter run at pH 4.0. It is intriguing that an apparently obligately aerobic acidophile (PFBC) was growing concurrently with an obligate anaerobe (M1). The conditions within the fermenter were maintained as strictly anoxic throughout each experiment. One hypothetical explanation for this phenomenon is that it is a novel example of microbial syntrophy. Such interactions are known to be common amongst anaerobes (including SRB) but appear to be less

important with aerobic microorganisms (Madigan et al. 2003). Most syntrophic reactions involve transfer of hydrogen gas from one partner organism to another, and can result in single reactions which are energetically unfavorable (i.e. having positive δG values) proceeding when coupled by a second, microbially-catalyzed reaction (Madigan et al. 2003). The data from the present study show that acetic acid was being consumed, presumably by PFBC (as M1 is an acetogen rather than an acetotroph). In the absence of an extraneous electron acceptor, acetic acid could be metabolized to CO_2 and H_2 by PFBC. Although this is an energetically-unfavorable reaction ($\delta G = +82$ kJ/mol at pH 4), if the H_2 produced is consumed immediately by M1 and coupled to sulfate reduction, then the overall reaction becomes exergonic ($\delta G = -199$ kJ/mol at pH 4). Interestingly, more sulfate was reduced in fermenter mixed cultures than could be accounted for by glycerol to acetate oxidation by M1 alone. Other work (unpublished) has confirmed that the *Desulfosporosinus*-like SRB M1 can utilize hydrogen as an electron donor to fuel sulfate reduction.

The demonstration of sulfidogenesis in controlled laboratory cultures maintained at low pH provides the basis for new applications for remediating metal-rich wastewaters and recovering metals from them. At pH *ca.* 4, zinc and iron were successfully separated; zinc being removed as ZnS precipitates while the ferrous iron remained in solution. At lower pH values (beyond those used in the present study), metals such as copper and zinc could also, in theory, be recovered selectively using sulfidogenic technology.

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