Extracellular electron transport mediated Fe(III) reduction by a community of alkaliphilic bacteria that use flavins as electron shuttles.

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Keywords: COPR, flavin, alkaliphile, Cr(VI), iron, Fe(III), Fe(II), bioremediation

Running title: Extracellular electron transport by Alkaliphiles

ABSTRACT

The biochemical and molecular mechanisms used by alkaliphilic bacterial communities to reduce metals in the environment are currently unknown. We demonstrate that an alkaliphilic (pH > 9) consortium dominated by *Tissierella, Clostridium* and *Alkaliphilus* sp. are capable of using iron (Fe^{3+}) as a final electron acceptor under anaerobic conditions. Iron reduction is associated with the production of a freely diffusible species that upon rudimentary purification and subsequent spectroscopic, HPLC and electrochemical analysis has been identified as a flavin species displaying properties indistinguishable from riboflavin. Due to the link between iron reduction and the onset of flavin production, it is likely that riboflavin has an import role in extracellular metal reduction by this alkaliphilic community.

Introduction

Iron is the most abundant redox-active metal in soils (1). Iron has two oxidation states that are stable under the geochemical conditions found in soils: Fe(III) under relatively oxic conditions and Fe(II) under reducing conditions (2). Fe-reducing microorganisms can couple the oxidation of a wide variety of organic compounds to the reduction of Fe(III) to Fe(II) during dissimilative metabolism (3). Due to the ubiquity of iron in the subsurface the oxidation of a significant portion of all organic matter in submerged soils and aquatic sediments is coupled to reduction of Fe(III) (3). Numerous Fe-reducing microorganisms from a range of microbial taxa have been isolated from a broad range of environments (4-6).

During anaerobic respiration, bacteria transfer electrons from organic carbon to an electron acceptor that originates outside the cell and use the energy released from these coupled reactions to translocate protons from the cytoplasm to the periplasm (7). This results in an electrochemical gradient (or electromotive force), composed of a membrane potential, $\Delta\Psi$, and a proton concentration gradient across the cytoplasmic membrane, which is used to drive bioenergetic processes such as solute transport and ATP synthesis via oxidative phosphorylation (8). Some alkaliphilic bacteria can exploit the transmembrane electrochemical gradient that arises from a sodium concentration gradient to drive bioenergetic processes in conditions where it is challenging to maintain a proton gradient (9). In aerobic conditions the electron acceptor is oxygen, however in anaerobic conditions, such as found in saturated soils, bacteria can use other electron acceptors, commonly fumarate, nitrate, arsenate, DMSO, Fe(III), Mn(IV), Cr(IV) V(V) oxides and various forms of other carbonaceous and sulfur-based compounds (10-17).

Bacteria often respire with electron acceptors that are passively transported into the periplasmic space. Such respiration involves a lipophilic proton/electron carrier commonly referred to as the quinone/quinol pool located in the cytoplasmic membrane, which transfers electrons to an inner-membrane bound, periplasm facing multi-heme *c*-type cytochrome (18, 19). A number of different terminal reductases can then complete the membrane associated electron transport system (19-23). In pH neutral and acidic environments, bacteria have also been shown to facilitate the transfer of electrons to various compounds that are outside the cell. During extracellular electron transport the inner-membrane bound *c*-type cytochrome is thought to transfer electrons to a series of other multi-heme cytochromes, and by that mechanism, across the periplasm and through the outer membrane (24-27). It has been proposed multi-heme cytochromes then have a central role in electron transfer to metal oxides outside the cell and can be achieved by two mechanisms. The first is by direct attachment of the cell to the electron acceptor, such as metal oxides (3), and has been elegantly demonstrated in the case of the Mtr complex where direct electron transfer was shown by Mtr contact with minerals (28). The second is by the production of soluble extracellular electron shuttles, such as flavins, which are released into the immediate environment around the cell (29-32).

Electron-shuttling compounds are usually organic molecules external to the bacterial cells that can be reversibly oxidized and reduced. These compounds can thus carry electron carriers between bacterial cells and insoluble electron acceptors, enabling long-distance electron transfer (33). As the oxidation and reduction of electron-shuttling compounds are reversible, small catalytic amounts can undergo multiple reduction-oxidation cycles (34). Humic substances that contain quinone moieties were the first electron-shuttling compounds reported to stimulate Fe(III)

oxide reduction (35). To date it has been shown that *Shewanella* sp. and several methanotrophic bacteria can release flavins (i.e. flavin mononucleotide and riboflavin (30, 36)) as electron shuttles. As yet it is uncertain whether bacteria can also release quinone-like compounds as electron shuttles in response to a metabolic requirement (37), or whether this is an opportunistic use of substances found in the environment. Quinone groups in humic acids can act as electron shuttling compounds during the reductive dechlorination of chlorinated solvents, but the reduction rate is pH sensitive in the range 7.2 – 8.0 (38). This was attributed to the varying ease of deprotonation of the redox active groups in the electron shuttling compounds. Further, humic substances contain several different functional groups, which can act as electron shuttling compounds in the range 6.6 - 8.0, and the pH value at which a particular type of functional group is active dependent on substituents neighbouring the redox centre (39).

Several species of bacteria have been shown to reduce Fe(III) in alkaline growth media over the pH range $9 \leq pH \leq 11$ (e.g. *Geoalkalibacter ferrihydriticus* (6); *Alkaliphilus metalliredigens* (40); *Tindallia magadii* (41); *Clostridium beirjerinckii* (42); *Anoxynatronum sibiricum* (43); *Anaerobranca californiensis* (44)). However, as yet, there is little detailed information on the mechanisms of how anaerobic bacteria growing at high pH use iron as a final electron acceptor. Utilising iron is particularly challenging as most Fe(III) phases are relatively insoluble in this pH range (2). Indeed the amount of iron in aqueous solution is estimated to be approximately 10^{-23} M at pH 10 (45). Thus it is speculated that the iron reduction mechanisms of alkaliphilic bacteria must be extremely efficient. Recently it has been shown that adding riboflavin to a community of alkaliphilic soil bacteria grown in-vitro at pH 10 increased the rate at which Fe(III) was reduced suggesting that members of the

community might be able to use riboflavin as an electron shuttle in alkaline conditions (46). However, as electron shuttle catalysed reactions are very pH sensitive (38, 39), it may not be appropriate to extrapolate what is known about the process from near neutral studies to high pH environments.

This study investigates the growth characteristics of a community of bacteria recovered from beneath a waste tip where highly alkaline chromium ore processing residue (COPR) has been dumped. It characterises the bacterial consortium that has become established after repeated growth in an alkaline Fe(III)-containing growth media. Growth of the bacterial consortium by iron reduction is linked to the production of a soluble species that was detected in the growth media. This species was isolated and characterised by spectroscopic and electrochemical analyses.

Methods

Alkaline Fe(III)-Containing (AFC) Media

The AFC media contained $NaH_2PO_4.H_2O$ (0.356 g/l), KCl (0.1 g/l) and 10 ml/l each of standard vitamin and mineral mixtures (47). Fe(III) citrate (2 g/l) and yeast extract (2 g/l) were added as the sole sources of electron acceptors and donors. The pH value of the media was buffered to 9.2 with the addition of Na_2CO_3. The media was boiled for 30 min then purged with nitrogen for 30 min to exclude oxygen. It was placed in 100 ml glass serum bottles, and the headspaces were filled with N_2. The bottles were sealed with butyl rubber stoppers with aluminium crimps, and heat sterilised at 120°C for 20 min. Fe in the AFC media remained soluble as a red coloured Fe(III)-citrate complex but also contained a small amount of a hydrous ferric oxyhydroxide precipitate which formed when the pH was adjusted to pH 9.2. Riboflavin spiked media was made by adding 3.76 $x10^{-2}$ g/l riboflavin to AFC media.

Alkaliphilic Fe(III)-Reducing Bacterial Community

A community of alkaliphilic anaerobic bacteria capable of Fe(III) reduction was cultured from soil taken from beneath a 19th Century COPR waste tip using the AFC media used in this study (see (48, 49) for details). This community was grown on several times in AFC media, with subsequent bottles inoculated with 1% (v/v) of cell suspension from a culture in the upper exponential phase of growth. Upper exponential growth was determined by colour change of the precipitate in the media from red to black.

Growth Characterisation

Bottles containing AFC media were inoculated with the alkaliphilic Fe(III) reducing bacterial community. The bottles were kept at a temperature of 21 ± 1 °C. Periodically they were sampled using needles and syringes and aseptic technique (50). The pH was measured using a HQ40d pH meter (Hach). Total Fe(II) was measured by dissolving 0.5 ml sample in 2 ml of 0.5N HCl for 1 hour before reacting with ferrozine solution. The colour was allowed to develop for 10 min and then absorption at 562 nm was measured using a Thermo Scientific BioMate 3 UV/VIS Spectrophotometer (51). The total amount of Adenosine Triphosphate (ATP) was determined by luciferin luciferase assay using a Molecular Probes ATP Determination Kit (Life Technologies, USA). Cell counting was performed using an improved Neubauer haemocytometer on an Olympus BH-2 microscope.

Growth of the Community with Alternative Electron Donors

Media was prepared as above except the yeast extract concentration was reduced to 0.2 g/l. An alternative electron donor (acetate, lactate, ethanol, methanol or sucrose) was added at a concentration of 20 mM. The alternative growth media was inoculated with 1 % (v/v) of cell suspension from a bacterial community grown on AFC media that was in the upper exponential phase of growth. The bottles were incubated for one week, and 1 % (v/v) was transferred into fresh media and grown on for a second week. Colour change of the media from red to black was taken to indicate iron reduction. Those that showed colour change were grown on into media containing no yeast extract and assessed for iron reduction after a further week.

Bacteria growth on Plates

AFC media was prepared with the addition of 20 g/l agar. After heat sterilisation at 120°C for 20 min, plates were poured keeping the agar media <1.5mm thick. A cell suspension of the community in the upper exponential growth phase was diluted 10x using autoclaved AFC media and 100 µl spread onto the plates. The plates were stored in a sealed box, with an Anaerogen sachet (Oxoid Ltd, UK) to eliminate oxygen, at a temperature of 37˚C. After 2 weeks, single colonies were picked-off and re-streaked on new plates which were then kept under the same conditions. Iron reduction was identified by areas of agar discolouring from red to clear.

DNA Extraction and Sequencing of the 16S rRNA Gene

DNA was extracted from the bacterial community growing in the AFC media containing yeast extract as the only source of electron donors using a FastDNA spin kit for soils (MP Biomedicals, USA). A 1.5 kb fragment of the 16s rRNA gene was

amplified by Polymerase Chain Reaction (PCR) using broad specificity primers. The PCR product was ligated into a standard cloning vector, and transformed into E. coli competent cells to isolate plasmids containing the insert, which were sent for sequencing (see Supplementary Information for details). DNA was also extracted from cell colonies isolated on agar plates, and a portion of the 16S rRNA gene was amplified by PCR and sent for direct sequencing.

The quality of gene sequences was evaluated (52), and putative chimeras were excluded from subsequent analyses. Sequences were grouped into operational taxonomic units (OTUs) (53), and phylogenetic trees were constructed for representative sequences (54, 55). Sequences were classified using the Ribosomal Database Project (RDP) naïve Bayesian Classifier (56) (see S.I. for details of the sequence analysis). Sequences were submitted to the GenBank database (Genbank Numbers: KF362050-KF362117) (56).

Scanning Electron Microscopy (SEM)

A 2 ml sample was taken from a bottle of AFC media in the upper exponential phase of growth and centrifuged at 13,300 × *g* for 5 min to collect the cells and precipitate. The pellet was then re-suspended in deionised H_2O in order to remove soluble phases such as Na_2CO_3, centrifuged again for 5 min. The pellet was transferred to a copper crucible and SEM analysis was performed using a FEI Quanta 650 FEG-ESEM. Energy Dispersive X-ray spectra were collected with an Oxford X-max 80 SDD (liquid nitrogen free) EDS detector and images were collected in secondary electron imaging mode.

Isolation and Quantification of Soluble Electron-Shuttling Compounds

100 ml of culture was centrifuged at 9,000 x *g* for 15 min to separate cells from the growth medium. Culture supernatant was neutralized with HPLC-grade HCl to pH 7, and extracted with 100 ml of ethyl acetate. The bottom aqueous layer was discarded. The pooled organic phase was transferred into an acid-cleaned high-density polyethylene (HDPE) bottle and residual water was removed by drying over sodium sulphate (5 g) at 4°C overnight. The organic phase was then filtered through 0.45 µm polytetrafluoroethylene (PTFE) syringe filter (Sartorius) and dessicated using a rotary evaporator. The resulting residue was dissolved with MilliQ H_2O in an ultrasonic bath (Elma, Elmasonic S30).

A 10 ml column containing 8 g XAD-16 resin (Sigma) was pre-cleaned with 100 % methanol and rinsed thoroughly with deionised H_2O. The ethyl acetate soluble fraction extract was slowly transferred onto the column (XAD-16 is a non-ionic macroreticular resin is designed to adsorb small to medium MW organic substances from aqueous systems and polar solvents by hydrophobic and polar interactions). Compounds that bound to the resin were eluted sequentially with four bed volumes of 10%, 50% and 100% methanol (HPLC grade Merck). The 50% and 100% elutions were pooled and reduced to ~10 ml using a rotary evaporator at <30°C (previous work (45) has shown that Flavins are retained in this fraction). This solution was then transferred to a 15 ml test tube and desiccated by speedvac (Savant SC210A). The resulting dark orange residue was resuspended in either 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 30 mM Na_2SO_4 pH 7.4 or deionised H_2O for further spectroscopy, electrochemical assays and quantification. Unused AFC media was subjected to the same extraction and used as a control.

Flavin quantification was performed by scanning wavelengths from 300-700 nm using a UV-2 UV/Vis spectrophotometer (Unicam). A standard curve was generated by observing known concentrations (0.05 µM, 0.125 µM, 0.25 µM, 0.5 µM, 1 µM) of riboflavin. An extinction coefficient at 455 nm (ε = 12,500 $cm^{-1}M^{-1}$) was used to quantify concentration (57).

Fluorescence Spectroscopy

Fluorescence spectra of purified culture supernatant were measured on a Quanta Master 30 (PTI/Photomed) fluorescence spectrometer using a 1 cm path length. Slit widths of 0.5 and 1.5 mm were used for excitation and emission wavelengths, respectively.

Electrochemical assays

Ultra-flat template-stripped gold (TSG) electrodes (surface area, A = 0.2 cm^2) were prepared and cleaned (see (58) for details). Self-assembled monolayers (SAMs) were formed on electrodes by incubating them with 1 mM 8-mercaptooctanol in propanol for 16 hours. After rinsing with propanol and methanol, the electrodes were dried under a nitrogen gas flow and assembled in a bespoke glass electrochemical cell (58). Voltammetry was conducted with a standard 3-electrode setup. A TSG working electrode was embedded in a PTFE holder with a rubber *O*-ring seal; a platinum wire counter electrode and a saturated silver/silver chloride electrode (Ag/AgCl) completed the circuit in the buffer volume (20 mM MOPS, 30 mM Na_2SO_4, pH 7.4) (17). The electrochemical cell was surrounded by a steel mesh Faraday cage and operated inside an N_2 filled glovebox (MBraun MB 150 B-G) where the O_2 levels were <1 ppm. All solutions were purged with N_2 for 1 h and stored in the

glovebox for at least 24 h before use. Electrochemical measurements were recorded at 21°C using an Autolab electrochemical analyser with a PGSTAT30 potentiostat, SCANGEN module and FRA2 frequency analyser (Ecochemie). Electrochemical impedance spectra were recorded for each SAM electrode prior to modification with flavin to control SAM quality. The electrodes were then incubated with approximately 0.1 μM flavin in 20 mM MOPS, 30 mM Na_2SO_4 pH 7.4 for 30 min. The flavin-modified electrode was then washed 3 times with buffer solution to remove non surface-associated flavins.

Analogue cyclic voltammograms (CVs) were recorded by holding the potential at 0.2 V for 5 seconds before cycling at a scan rate (u) of 10 mV/s in the potential window from +200 mV to –600 mV (vs Ag/AgCl). Comparison of the CVs for SAM and flavin-modified electrodes indicate that a thin flavin layer remains bound to the electrode surface. The electroactive coverage of the flavin, Γ, was determined from the integration of the peak areas of the baseline-subtracted signals using SOAS software, available from Dr. C. Léger (59). The coverage is calculated from:

$$Q = nFA\Gamma \quad (1)$$

where Q is the total charge required for oxidation of the bound absorbate, F is the Faraday constant, and n is the number of electrons per flavin.

High performance liquid chromatography

For rapid discrimination of flavins a high performance liquid chromatography (HPLC) separation was used. The purified flavin, commercially available riboflavin (Sigma) and FMN (riboflavin-5′-phosphate; FLUKA, Buchs, Switzerland) were dissolved in water at a concentration of 10 μg/ml. 10 μl samples (equivalent to 100 ng flavin) were injected into a HPLC system consisting of an online degasser DG-2080-53, a

277 gradient former LG-1580-02, a PU-980 pump, an AS-1555 autosampler, a UV-975
278 UV-detector set at 420 nm (all from Jasco, Gross-Umstadt, Germany), and a RF-551
279 fluorescence-detector set at 450/520 nm (excitation/emission) (Shimadzu, Duisburg,
280 Germany). Separations were performed at a flow-rate of 1 ml/min on a LiChrospher
281 100 RP-18e column (5 µm; 250 x 4 mm; Merck, Darmstadt, Germany) at 25°C. The
282 solvent system consisted of water / 0.1 % trifluoroacetic acid (phase A) and
283 acetonitrile (phase B) nominally applied as follows: 15 % B for 5 min, 15 % B to 50 %
284 B in 2 min, 50 % B for 1 min, 50 % B to 15 % B in 1 min, and 15 % B for 4 min.
285 Retention times (means ± SD, n = 3) of flavins in this solvent system were: 3.76 ±
286 0.01 min (FAD, riboflavin-5'-diphosphate; which was present as a 6 % impurity in the
287 FMN used), 4.64 ± 0.07 min (FMN), and 5.91 ± 0.03 min (riboflavin).
288
289 **Results**
290 **Bacteria growth characteristics**
291 Growth of the community of alkaliphilic Fe(III)-reducing bacteria in alkaline
292 Fe(III) containing (AFC) media was characterised by enumeration of cell numbers,
293 ATP and total Fe(II) concentration in the media. Cell numbers, ATP and total Fe(II)
294 showed the same trend. After initial inoculation, there was a lag phase where cells/l
295 stayed roughly constant for 72 hours, after which cell numbers exponentially
296 increased to a peak of ~200 x 10^9 cells/l at 168 hours (Figure 1*A*). Cell numbers
297 stayed at similar levels until 500 hours when they started to slowly decrease.
298 Negligible Fe(II) was recorded until 96 hours had elapsed then the concentration
299 increased to a maximum of ~3500 µM at 216 hours (Figure 1*C*) and subsequently
300 stayed relatively constant until 500 hours. After this time Fe(II) levels started to
301 decrease (data after 600 hours not shown). Trace amounts of ATP were observed

until 96 hours at which point the concentration rapidly increased to the maximum of 1-2 nM after 192 hours (Figure 1*D*). The pH value was consistently 9.1 until 72 hours had elapsed when it started to decrease and reached a final value of 8.5 by ~360 hours (Figure 1*B*).

Growth with Alternative Electron Donors

Growth was observed in the majority of media containing an alternative electron donor after one week (Table 1). When inocula from these bottles were transferred into fresh media, only bottles where either sucrose or ethanol were the primary electron donor exhibited colour change after a further week of incubation. Transfer of inocula from the growth positive bottles to media containing either sucrose or ethanol (as appropriate) as the sole electron donor resulted in no colour change.

Agar Plates and isolate Analysis

Growth of the AFC media culture on agar plates resulted in small colourless colonies on the surface of the plate after 2 weeks. A lessening in the colour density of media/agar plates and the formation of very small dark particles in the agar was associated with colony growth (Supplementary Information Figure S1). The colour change is due to reduction of aqueous Fe(III) in the AFC media and precipitation of Fe(II). SEM analysis of the spent AFC media (see below) suggests that the particles in the agar-AFC media were Vivianite crystals (hydrated iron(II) phosphate). The reduction in colour density extended across wide areas of the plate, so individual colonies were picked off the plates with sterile toothpicks and streaked onto new plates. For about 25% of these streaks there was a reduction in the colour density of media/agar in the immediate vicinity of the streak, which extended about 2mm

beyond the boundary of the cell colonies. Colonies were randomly selected from these plates for rRNA gene sequence analysis.

Community analysis and Streak analysis

The 16s rRNA gene sequences extracted from the AFC media show that all the bacteria within the consortium were from the order Clostridiales within the phylum Firmicutes. Analysis of the 59 sequences using the RDP Classifier (60) indicated that there were three genera represented; 48% of the sequences were *Tissierella* sp. 44% were *Clostridium* sp. and 8% were *Alkaliphilus* sp.). MOTHUR analysis further classified the sequences into 5 OTUs. The *Tissierella* genus contained three OTUs, from which representative sequences were selected and analysed again using the RDP classifier. This showed two of the OTUs to be *Tissierella* sp. (from now on called *Tissierella* strain A and B) with a confidence threshold of 100% and the other, with a threshold of 87% (*Tissierella* strain C). The Clostridia and Alkaliphillus genera both contained one OTU with a confidence threshold of 100% (Figure 2). Representative sequences were selected from each OTU and a taxonomic tree showing their relationship with closely related type strains was constructed (Figure 3).

Direct PCR sequencing of bacteria grown on agar plates showed that the bacteria associated with a reduction in the colour density of media/agar (5 sequences) were all from the genus *Tissierella*. Comparative MOTHUR analysis of these sequences and those from the AFC media showed them to be all from the *Tissierella* strain C. The bacteria from the streaks where there was no change in the colour density of media/agar were much harder to sequence. Four sequences were characterised using the RDP classifier, one from the genus Ochrobactrum, and the other three were unclassified Actinomycetaceae.

352

353 **Analysis for of Soluble Electron-Shuttling Compounds**

354 To investigate whether a soluble electron shuttling compound was involved in
355 Fe(III) by the consortium, the spectral properties of spent media were studied at four
356 stages of growth. Time points at 24, 72, 168 and 336 h (1, 3, 7 and 14 days) were
357 examined for optical signatures indicative of quinones or flavins (unused AFC media
358 was used as the control). Scanning the culture supernatants over a wavelength
359 range 200-700 nm revealed spectral features that increased in amplitude with the
360 age of the culture that is compatible with accumulation of flavinoids in the media
361 (Figure 4*A*). The extracts from XAD-column purification exhibited spectral features
362 (Figure 4*B*) indistinguishable from those exhibited by commercially available
363 riboflavin (61) (the extract from the unused media produced no detectable peaks).
364 Upon excitation at 441 nm, the XAD-column extract exhibited a broad emission peak
365 between 475 and 650 nm in its fluorescence spectra with a maximum at 517 nm
366 (Figure 4*D*). This feature, exhibited by commercially available riboflavin (also shown
367 in Figure 4*D*), is diagnostic for the isoalloxazine ring structure in flavin species (62).
368 To corroborate these findings with the Fe(II)-dependent growth of the culture, the
369 amount of flavin produced at each stage of growth was compared to Fe(II)
370 accumulation in the culture medium. Interestingly, there is a direct correlation
371 between the appearance of flavin and generation of Fe(II) during the growth phrase
372 of the bacterial consortium (Figure 4*C*).

373 Cyclic voltammetry (Figure 5*A*) revealed that the surface immobilized XAD-
374 column extract is capable of transferring electrons to and from a metal species, with
375 oxidation and reduction peak potentials of -0.18 mV and -0.25 mV vs SHE
376 respectively. Furthermore, the electrochemical profile of the column extract is very

similar to that obtained from commercially available pure riboflavin. Once the peaks were baseline corrected to remove any slope bias from the scans (Figure 5*B*), it was revealed that the electrochemical coverage and peak potentials of the column extract were almost identical to those of commercially available riboflavin (Figure 5*A* and 5*B*). Thus both the surface adsorption and packing characteristics of the column extract are indistinguishable from riboflavin.

However, the spectral, fluorescence and electrochemical properties investigated here are common to FAD, FMN and riboflavin, so to further discern the identity of the flavin species HPLC spectroscopy was performed. HPLC analysis of the surface immobilized XAD-column extract revealed a single peak which, when compared to commercially available riboflavin, FMN and FAD eluted at the same retention volume as riboflavin (Figure 6).

Growth in Media Spike with Riboflavin

To further corroborate the role of riboflavin in Fe(III) reduction, growth media was spiked with riboflavin. Bacteria grown in AFC media supplemented with riboflavin resulted in the production of Fe(II) after 48 hours, half the time of the bacteria in the base AFC media (Figure 7). The exponential phase of growth for the bacteria in riboflavin amended media was complete after 144 hours.

SEM

The precipitate recovered from the microcosms containing AFC media after cell growth appeared to be black in colour and crystalline in nature. Under SEM analysis the primary features seen were flattened prismatic crystals, roughly 30 x 5 x 5 µm in size (Figure 8). Between the crystals was an amorphous gel which cracked as the

sample was dried. EDS spot analysis of crystals (insert in Figure 8) gave similar spectra with distinct peaks for O, P, and Fe, and a small S peak (there were also Cu peaks associated with the copper crucible which contained the sample). The flattened prismatic crystals have the morphology of Vivianite ($Fe_3(PO_4)_2.8H_2O$) (63) (the sulphur peak in the EDS spectra is probably associated with the amorphous background phase). Vivianite is a common phase when Fe(III) is bio-reduced in the media containing high concentrations of soluble phosphate (64).

Discussion

The identity of alkaliphilic community

After repeated growth on AFC media (50+ growth cycles since isolation from the soil), sequencing data shows that there are still several genera of bacteria in the iron reducing community. This suggests that either all the bacteria present are able to respire independently using the AFC media or a symbiotic relationship has developed between the differing types of bacteria whereby one requires the respiration products of another for growth. The AFC media contained yeast extract which is a complex mixture of organic compounds, including amino acids and polysaccharides (65). Yeast extract can support a wide range of metabolic processes, and this may explain the range of species in the consortium. None of the alternative electron donors supported long-term growth of the consortium. In media containing sucrose or ethanol with a low concentration of yeast extract, bacterial growth was recorded however no growth was observed without it. Thus it is clear that yeast extract contains something that is vital for iron reduction that is not supplied by the base media. Several other alkaliphilic organisms are reported to

grow poorly on single organic compounds and require the presence of complex electron donor species (66, 67).

Nearly half (48%) of the sequences characterised from the AFC media were from the genus *Tissierella* with Mothur analysis showing they could be further separated into three OTUs, *Tissierella* A, B and C. *Tissierella sp.* are obligate anaerobic, gram negative, non-sporeforming rods (68). All OTUs were most closely related to the type strain Tissierella Preacuta (seqmatch scores are A = 75%, B = 80% and C = 86%). 44% of the sequences characterised were from a single OTU in the genus Clostridium XI and were up to 100% similar to type strain *Clostridium mangenoti*. Found in many soils around the world (69), *Clostridium mangenoti* is an extremely hardy anaerobe whose spores are able to resist low temperature, vacuums and high levels of radiation (70). Therefore it is no surprise that it can exist in the harsh geochemical environment in the original soil with high pH and in the presence of chromate. 8% of the bacteria sequenced were from a single OTU in the genus the Alkaliphilus most closely related to the type strain *Alkaliphilus oremlandii* (seqmatch score 83%) (71). Bacteria from the Alkaliphilus genus are obligate alkaliphilic anaerobes that have been found in deep subsurface alkaline environments (72). Members of this genus have been shown to reduce numerous Fe(III) phases (4, 40), as well as groundwater contaminants such as arsenic (71).

The isolation of bacterial colonies in streaks on agar plates identified species that can reduce iron remote from the cell location. The streaks that visibly cleared the media only contained bacteria of the genus *Tissierella*, which MOTHER analysis showed to be part of the OTU C. This fact, together with the observation that *Tissierella* forms a significant part of the AFC media consortium, suggests that *Tissierella* may be the principle bacteria producing the electron-shuttling compound.

Extensive efforts to reintroduce these Tissierella C streaks into AFC media for further investigation were unsuccessful. It should be noted that these data do not preclude the possibility that other bacteria species in the consortium are also producing a soluble electron-shuttling compound. Transferring the bacteria from aqueous to agar media will exert a strain on members of the consortium, which some bacteria may not be able to tolerate. Similarly the relatively small sample size could mean that other bacteria capable of flavin production were not seen by chance.

The sequences obtained from the streaks which didn't clear were identified as bacteria not seen in the initial population from the AFC media. This is not a surprise as environmental samples usually contain many different bacteria strains which can tolerate the media in which they are cultured, but never reach the exponential stage of growth. When growth conditions and competitive pressures are changed initially minor constituents of a bacterial population can become more significant.

The alkaliphilic community secrete flavins to transfer electrons extracellularly

When the bacterial community is grown on AFC media at pH 9.2, cell growth occurs slightly before the increase in Fe(II) (both have been modelled in Figure 1 by a logistic sigmoidal growth function (73); see S.I. for details). During the period of cell growth and Fe(III) reduction a water soluble organic compound was released into solution. The concentration of this extracellular compound increased during the exponential growth phase, but decreased slightly in late stationary phase (see Figure 4*A* and 4*B*) suggesting its release is not associated with cell lysis.

The extracellular compound exhibited UV/vis spectral features indistinguishable from those of commercially available riboflavin. Further it has surface adsorption characteristics and surface packing on TSG electrodes, and oxidises and reduces

476 with essentially the same redox potentials, as riboflavin. Lastly, HPLC analysis
477 showed this to be a single compound a chromatogram matching the retention time of
478 commercially available riboflavin. Thus, taking into account the overwhelming
479 agreement in the data, it is deduced that the extracellular compound is riboflavin.
480 When riboflavin was spiked into AFC media containing the bacterial community
481 Fe(III) reduction started sooner and was quicker than in unspiked media, strongly
482 suggesting that the riboflavin is involved in the mechanism of Fe(III) reduction. When
483 isolates from the community were grown on AFC-agar plates the media cleared at
484 mm scale distances from the “streaks” demonstrating that iron reduction was
485 occurring remote from the cell location.

486 There is a wide body of evidence that flavins can act as an electron shuttling
487 compound during extracellular electron transport to iron in circum-neutral pH
488 environments. For example *Shewanella* species release flavins and this increases
489 the ability of cells to reduce Fe(III) oxides into Fe(II) in cellular respiration (29-32).
490 Thus it seems extremely likely that the extracellular, riboflavin-like compound
491 released to solution by the alkaliphilic iron reducing community during growth is
492 acting as an electron-shuttling compound, and has a role in Fe(III) reduction; the first
493 time that this has been shown to occur at alkaline pH. Given that even mesophilic
494 bacteria can adopt a wide variety of mechanisms to perform similar roles
495 physiological functions when interacting with their environment (74), and the stress
496 of a challenging environment has led extremophilic bacteria to evolve distinctly
497 different mechanisms in many cases (45, 75), it is striking that the electron shuttling
498 compound found in this study of alkaliphiles is indistinguishable from that used by
499 mesophiles. Interestingly flavins have also been found in the culture supernatants of
500 several methanotrophic species (36), indicating that this method of extracellular

electron transfer may be more widespread among anaerobic communities living on the brink of life than first thought.

Bioremediative potential

The bacterial consortium investigated in this study was recovered from beneath a waste tip where alkaline, Cr(VI) containing COPR leachate has been migrating into the underlying soil layer for over 100 years (76). Chromium has accumulated in this soil within a mixed Cr(III)–Fe(III) oxy-hydroxide phase. The most likely mechanism of chromium retention is abiotic reduction by microbially produced soil associated Fe(II) (48). Hence, microbially Fe(III) reduction at high pH can have important consequences for the mobility of redox sensitive contaminants at alkaline contaminated sites, and promoting microbial Fe(III) reduction could form the basis of a treatment strategy for such sites in the future.

An issue at some industrially contaminated sites is that the waste can have very high pH. Common industrial processes, such as iron and steel making, aluminium and chromium extraction, and lime and cement manufacture, produce a waste form with a pH > 12 (76-79). Many of these wastes contain elevated concentration of redox-sensitive, potentially mobile, toxic metals (e.g. As, V, Cr). Thus the near-waste environment is particularly harsh, so soil bacteria will tend to favour micro-habitats where they are protected from the bulk chemical flux by buffering reactions occurring with the soil minerals and respiration products (80, 81). The production of a soluble electron-shuttling compound enhances the potential success of any bioremediation scheme, as the electron shuttling compounds can diffuse out from these niche environments where the bacteria respire, and produce reduced iron even where the soil is highly leachate affected. There is some evidence

of this at the sampling site, where 45→75% of the microbially available iron is Fe(II) despite an average soil pH value of 11→12.5, and this may account for why the soil has accumulated 0.3%→0.5% (w/w) Cr(III), despite the soil receiving a continual flux of Cr(VI) containing leachate from the waste (48). The use of a soluble electron-shuttling compound will increase the amount of soil Fe(III) available for bioreduction many fold, even where it is present in high pH zones unsuitable for bacterial respiration, thus increasing the overall bioreduction capacity of the soil. Another interesting point to note is that although flavin electron-shuttles are well suited to perform one or two electron transfers (i.e. those interactions involving Fe(III)-minerals and cell cytochromes; (29), flavin electron-shuttles do not specifically target Fe(III) compounds. Flavins will react with the other oxidised compound it encounters with a high enough reductive potential, thus direct reduction of some groundwater contaminants (e.g. U(VI) → U(IV)) by this bacteria community may be possible.

Acknowledgements

SJF would like to acknowledge his funding from the John Henry Garner Scholarship at the University of Leeds. The authors would like to acknowledge the work of Dr Rob Whittleston in the initial isolation of the alkaliphilic iron reducing bacterial community.

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761 **TABLE 1.** Iron reduction by the alkaliphilc bacterial community when grown on
762 different electron donors (+/– indicates a positive and negative outcome in each
763 replicate).

Electron Donor	Week 1	Week 2	Week 3
Acetate	++-	---	
Lactate	++-	---	
Ethanol	+++	+++	---
Methanol	++-	---	
Sucrose	+++	+++	---

764

765

766 **FIGURE 1**

767 Growth of the iron reducing consortia in AFC media: Variation of (A): Cell numbers
768 x10^6/l (B) pH, (C) Fe(II) (µmol/l) and (D) ATP (nmol/l) with time. Sigmoidal growth
769 curves have been fitted to the cell count and Fe(II) data (73).

770

771 **FIGURE 2**

772 Microbial community grown on alkaline Fe(III) containing media; sequence allocation
773 to Operation Taxonomic Units was determined by the MOTHUR program.

774

775 **FIGURE 3**

776 Taxonomic tree showing the relationships between representative sequences from
777 each OTU and closley related type strains (the scale bar corresponds to 0.01

778 nucleotide substitutions per site and bootstrap values from 2000 replications are
779 shown at branch points).
780
781 **FIGURE 4**
782 **Spectroscopy of culture supernatants**
783 UV-visible spectra of (*A*) culture media supernatant at various stages of alkaliphilic
784 consortium growth or (*B*) extracellular compounds isolated. Data is shown from
785 samples taken as day 1 (dash-dot lines), day 3 (solid lines), day 7 (dotted lines) and
786 day 14 (dashed lines). (*C*) Compares the flavin produced with Fe(III) conversion to
787 Fe(II) using the quantification information from (*B*). (*D*) Fluorescence spectra of
788 extracellular compounds isolated from culture media supernatant (dashed line)
789 compared to those from commercial pure riboflavin (solid line). Upon excitation at
790 441 nm, the emission spectra were monitored between 450 and 700 nm. Results
791 shown are representative of two biological replicates.
792
793 **FIGURE 5**
794 **Cyclic voltammetry (CV) of 8-OH-modified TSG electrode before (blank) and**
795 **after formation of a flavin film.**
796 All CVs were recorded in 20mM MOPS, 30mM Na_2SO_4 buffer (pH 7.4) at a 10 mV/s
797 scan rate. (*A*) CVs showing redox chemistry of immobilized purified flavin extract
798 (*grey lines*) compared to commercially pure riboflavin (*black lines*) and a blank SAM
799 (*dashed lines*). (*B*) Baseline correct voltammogram for immobilized purified flavin
800 extract from the CV presented in (*A*). Results shown are representative of three
801 replicate experiments.
802

FIGURE 6

Reversed phase HPLC of the isolated flavin, riboflavin standard, and an FMN preparation, which contains quantifiable amounts of riboflavin and FAD. 100 ng of each sample were analyzed.

FIGURE 7

Average Fe(II) production and pH value during the growth of the iron reducing consortia in AFC media spiked with riboflavin. Sigmoidal growth curves are fitted to the Fe(II) data (73). Error bars indicate one standard deviation from the mean.

FIGURE 8

Electron micrograph of the precipitate recovered from the spent AFC media.

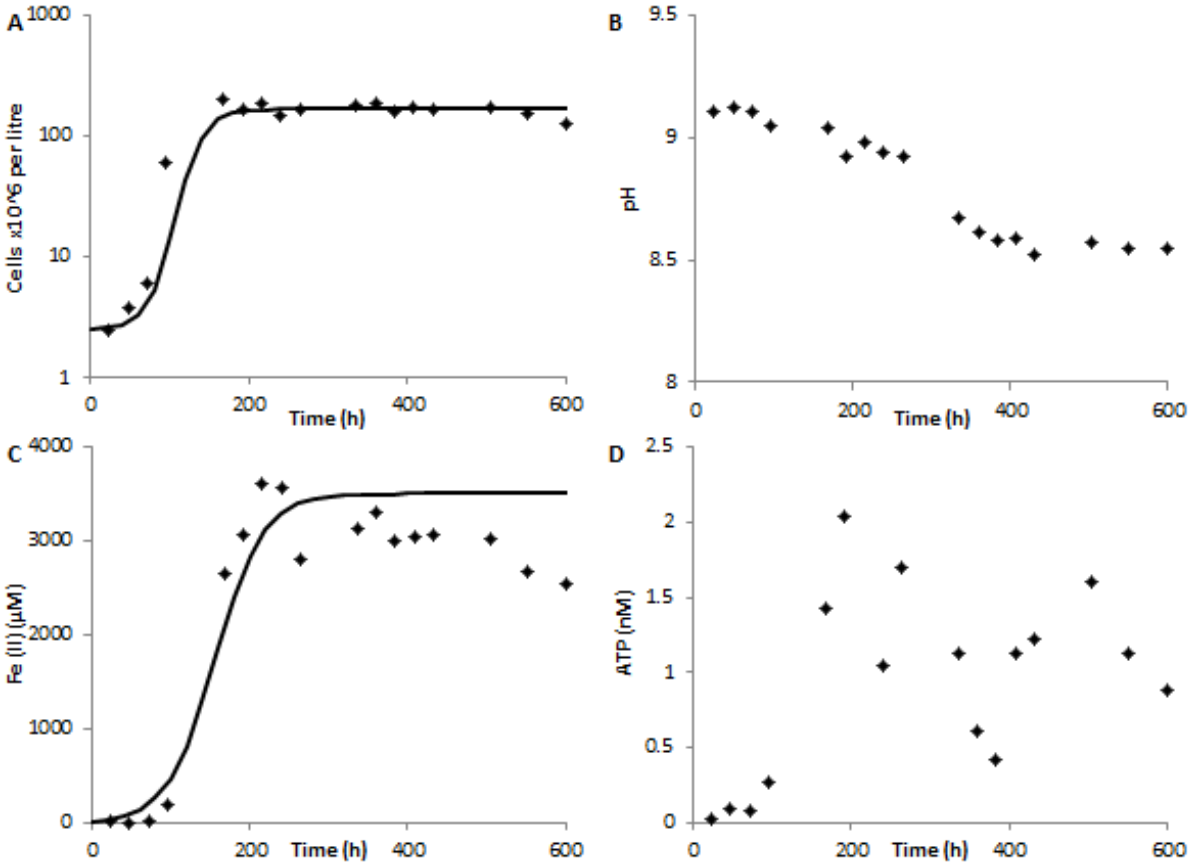


FIGURE 1. Growth of the iron reducing consortia in AFC media: Variation of (A): Cell numbers x10^6/l (B) pH, (C) Fe(II) (µmol/l) and (D) ATP (nmol/l) with time. Sigmoidal growth curves are fitted to the cell count and Fe(II) data (73).

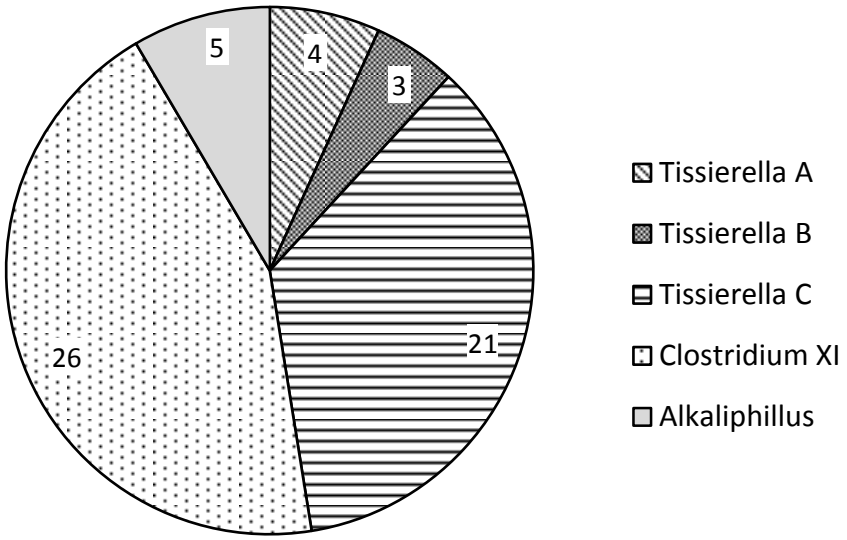


FIGURE 2. Microbial community grown on alkaline Fe(III) containing media; sequence allocation to Operation Taxonomic Units was determined by the MOTHUR program.

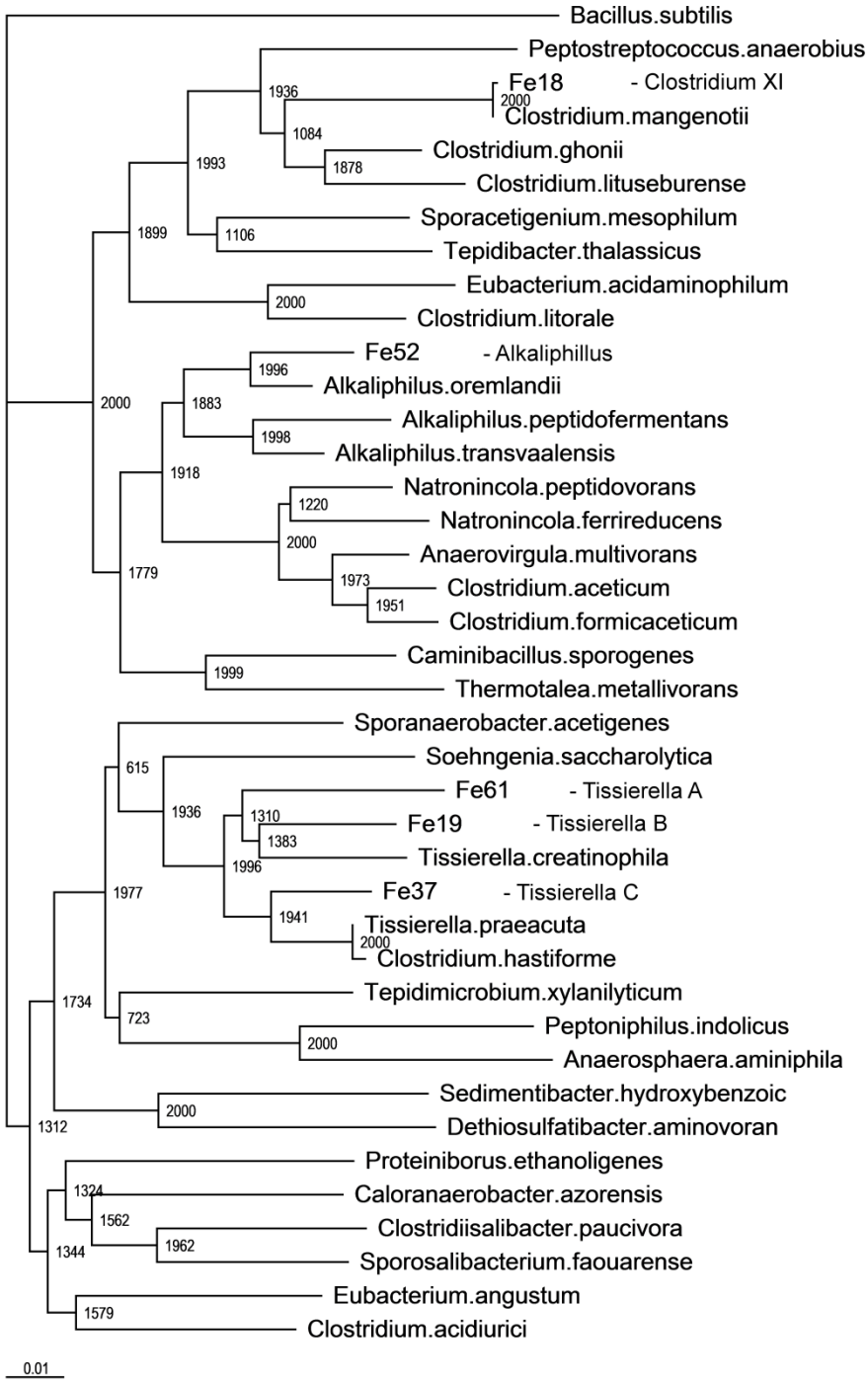
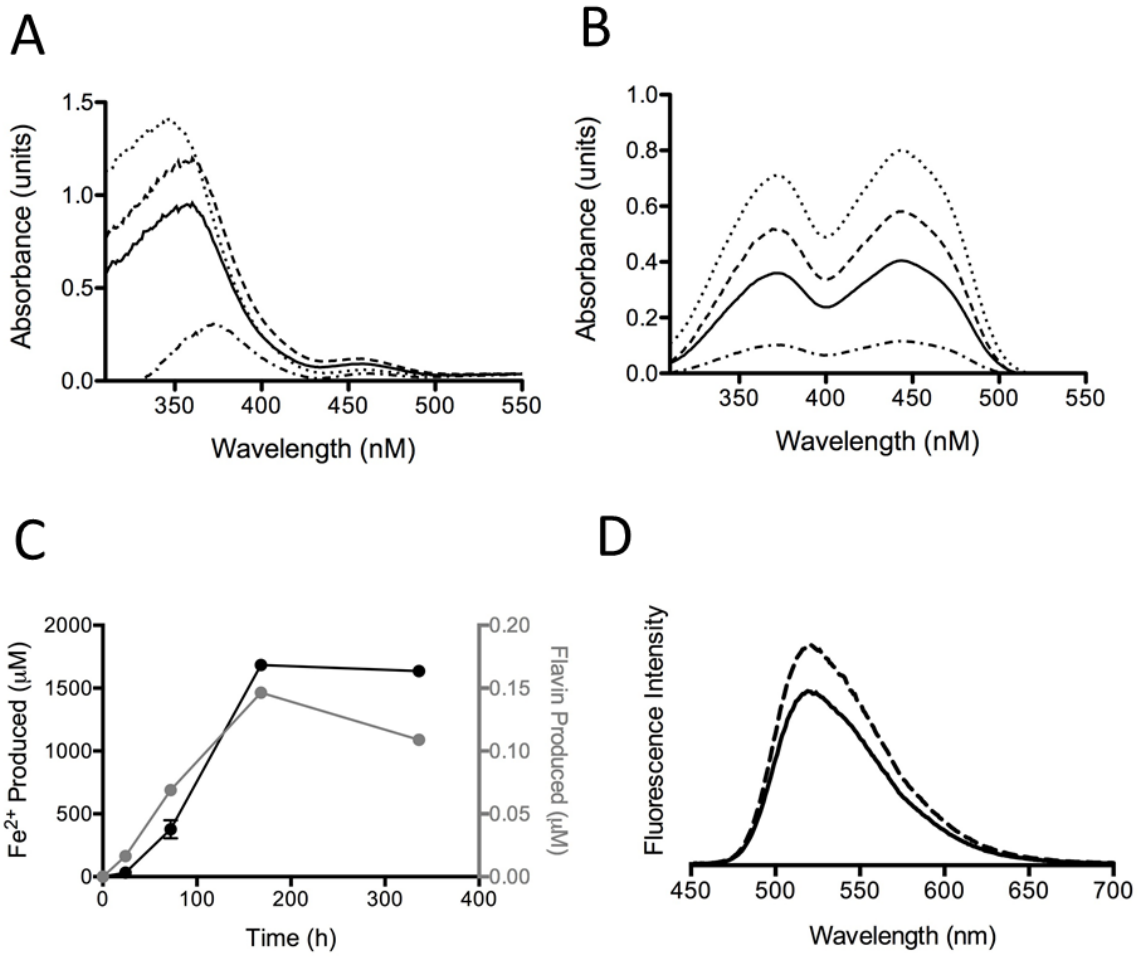


FIGURE 3. Taxonomic tree showing the relationships between representative sequences from each OTU and closley related type strains (the scale bar corresponds to 0.01 nucleotide substitutions per site and bootstrap values from 2000 replications are shown at branch points).



834

835 **FIGURE 4. Spectroscopy of culture supernatants.** UV-visible spectra of (*A*)
836 culture media supernatant at various stages of alkaliphilic consortium growth or (*B*)
837 extracellular compounds isolated. Data is shown from samples taken as day 1 (dash-
838 dot lines), day 3 (solid lines), day 7 (dotted lines) and day 14 (dashed lines). (*C*)
839 Compares the flavin produced with Fe(III) conversion to Fe(II) using the
840 quantification information from (*B*). (*D*) Fluorescence spectra of extracellular
841 compounds isolated from culture media supernatant (dashed line) compared to
842 those from commercial pure riboflavin (solid line). Upon excitation at 441 nm, the
843 emission spectra were monitored between 450 and 700 nm. Results shown are
844 representative of two biological replicates.

845

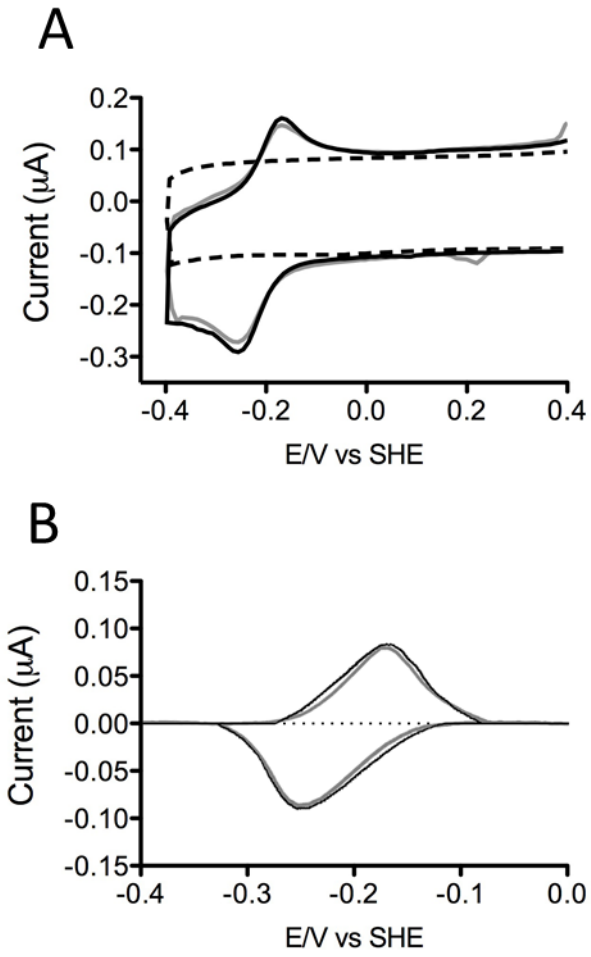


FIGURE 5. Cyclic voltammetry (CV) of 8-OH-modified TSG electrode before (blank) and after formation of a flavin film. All CVs were recorded in 20mM MOPS, 30mM Na_2SO_4 buffer (pH 7.4) at a 10 mV/s scan rate. (*A*) CVs showing redox chemistry of immobilized purified flavin extract (*grey lines*) compared to commercially pure riboflavin (*black lines*) and a blank SAM (*dashed lines*). (*B*) Baseline correct voltammogram for immobilized purified flavin extract from the CV presented in (*A*). Results shown are representative of three replicate experiments.

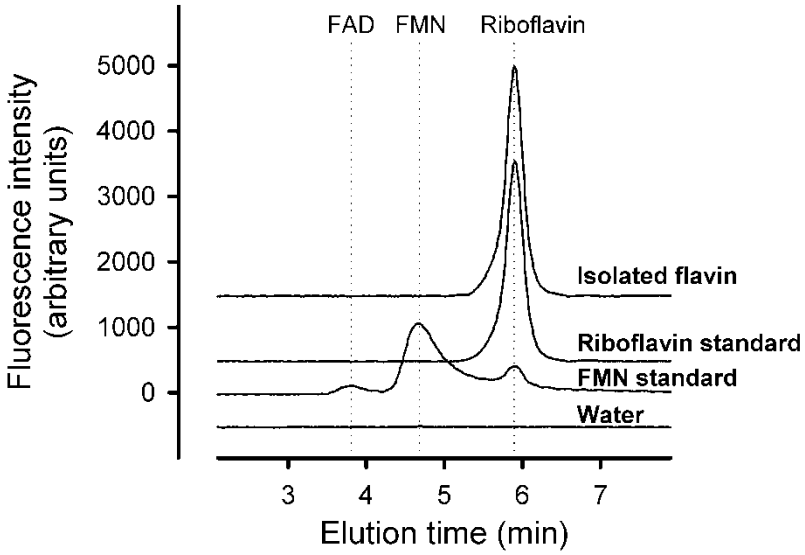
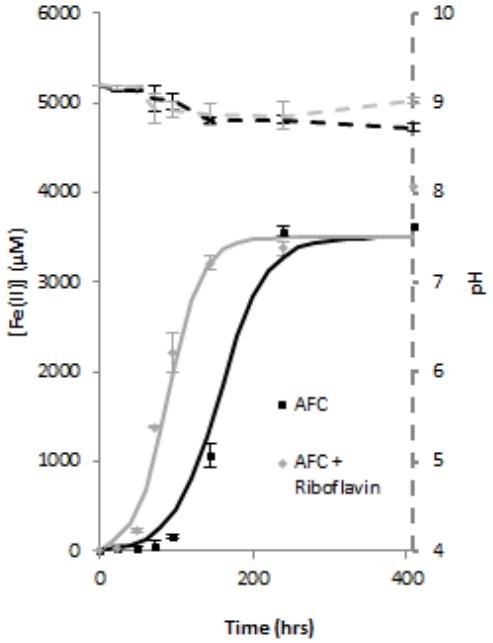


FIGURE 6. Reversed phase HPLC of the isolated flavin, riboflavin standard, and an FMN preparation, which contains quantifiable amounts of riboflavin and FAD. 100 ng of each sample were analyzed.



863

864 **FIGURE 7.** Average Fe(II) production and pH value during the growth of the iron
865 reducing consortia in AFC media spiked with riboflavin. Sigmoidal growth curves are
866 fitted to the Fe(II) data (73). Error bars indicate one standard deviation from the
867 mean.

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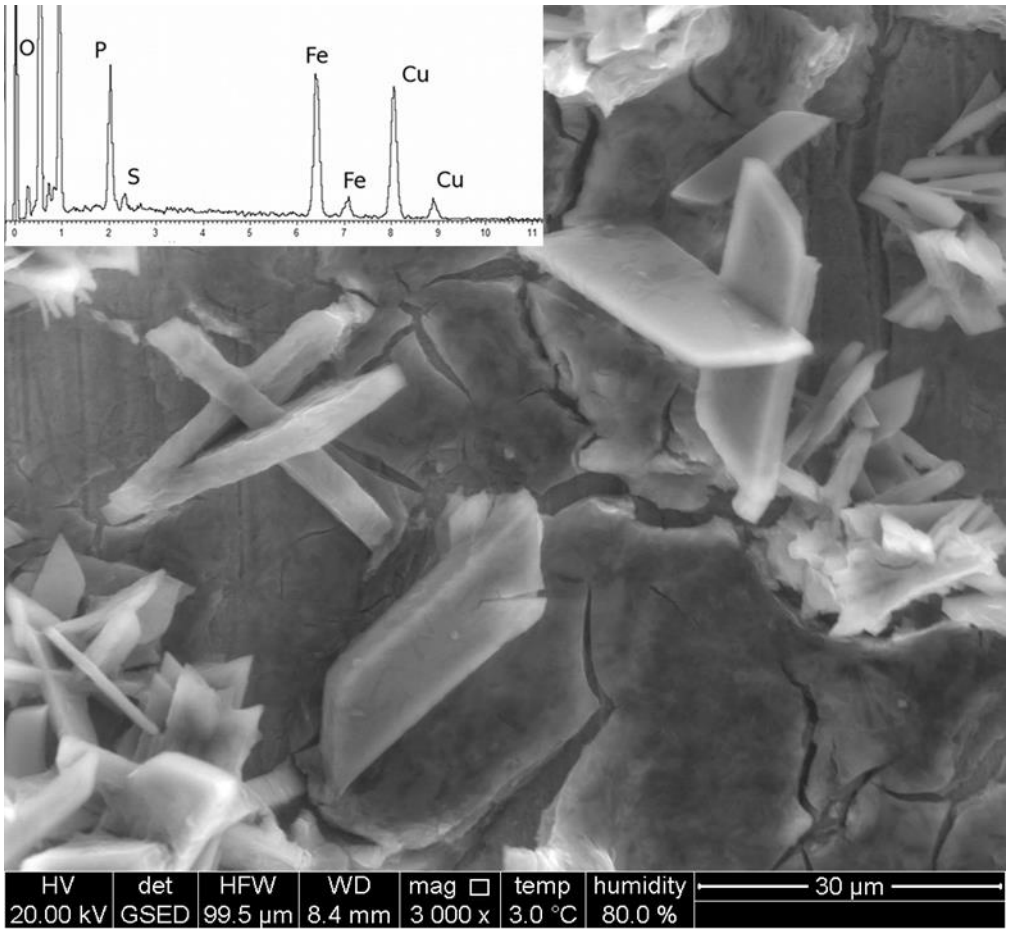

870

871 **FIGURE 8.** Electron micrograph of the precipitate recovered from the spent AFC

872 media.

873