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Extracellular Electron Transport-Mediated Fe(III) Reduction by a Community of Alkaliphilic Bacteria That Use Flavins as Electron Shuttles

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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* spp. is 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, high-performance liquid chromatography, and electrochemical analysis, has been identified as a flavin species displaying properties indistinguishable from those of 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.

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 the 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 under conditions where it is challenging to maintain a proton gradient (9). Under aerobic conditions, the electron acceptor is oxygen; however, under anaerobic conditions, such as those found in saturated soils, bacteria can use other electron acceptors, commonly, fumarate, nitrate, arsenate, dimethyl sulfoxide, Fe(III), Mn(IV), Cr(IV), and 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 multiheme cytochrome *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 cytochrome *c*-type cytochrome is thought to transfer electrons to a series of other multiheme cytochromes and, by that mechanism, across the periplasm and through the outer membrane (24–27). It has been proposed that multiheme cytochromes then have a central role in electron transfer to metal oxides outside the cell, which 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* spp. and several methanotrophic bacteria can release flavins (i.e.,

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flavin mononucleotide [FMN] 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 over the range from pH 7.2 to 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 over the range from pH 6.6 to 8.0, and the pH value at which a particular type of functional group is active is dependent on the substituents neighboring the redox center (39).

Several species of bacteria have been shown to reduce Fe(III) in alkaline growth media over the pH range $9 \leq \text{pH} \leq 11$ (e.g., *Geoalkalibacter ferrihydriticus* [6], *Alkaliphilus metalliredigens* [40], *Tindallia magadii* [41], *Clostridium beijerinckii* [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. Utilization of iron is particularly challenging, as most Fe(III) phases are relatively insoluble over 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 under alkaline conditions (46). However, as electron shuttle-catalyzed reactions are very pH sensitive (38, 39), it may not be appropriate to extrapolate what is known about the process from studies performed at nearly neutral pH 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 characterizes the bacterial consortium that has become established after repeated growth in an alkaline Fe(III)-containing (AFC) growth medium. Growth of the bacterial consortium by iron reduction was linked to the production of a soluble species that was detected in the growth medium. This species was isolated and characterized by spectroscopic and electrochemical analyses.

MATERIALS AND METHODS

AFC medium. The AFC medium contained $NaH_2PO_4 \cdot H_2O$ (0.356 g/liter), KCl (0.1 g/liter), and 10 ml/liter each of standard vitamin and mineral mixtures (47). Fe(III) citrate (2 g/liter) and yeast extract (2 g/liter) were added as the sole sources of electron acceptors and donors. The pH value of the medium was buffered to 9.2 with the addition of Na_2CO_3. The medium was boiled for 30 min and 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 aluminum crimps and heat sterilized at 120°C for 20 min. The Fe in the AFC medium remained soluble as a red Fe(III)-citrate complex, but the AFC medium also contained a small amount of a hydrous ferric oxyhydroxide precipitate which formed when the pH was adjusted to 9.2. Riboflavin-spiked medium was made by adding 3.76×10^{-2} g/liter riboflavin to AFC medium.

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 medium used in this study (see references 48 and 49 for details). This community was grown several times in AFC medium, with subsequent bottles inoculated with a 1% (vol/vol) cell suspension from a culture in the upper exponential phase of growth. Upper-exponential-phase growth was determined by a change in the color of the precipitate in the medium from red to black.

Growth characterization. Bottles containing AFC medium 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, syringes, and aseptic technique (50). The pH was measured using an HQ40d pH meter (Hach). Total Fe(II) was measured by dissolving 0.5 ml sample in 2 ml of 0.5 N HCl for 1 h before reacting with ferrozine solution. The color was allowed to develop for 10 min, and then the absorption at 562 nm was measured using a Thermo Scientific BioMate 3 UV/visible spectrophotometer (51). The total amount of ATP was determined by luciferin luciferase assay using a Molecular Probes ATP determination kit (Life Technologies). Cell counting was performed using an improved Neubauer hemocytometer on an Olympus BH-2 microscope.

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

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

DNA extraction and sequencing of the 16S rRNA gene. DNA was extracted from the bacterial community growing in the AFC medium containing yeast extract as the only source of electron donors using a FastDNA spin kit for soils (MP Biomedicals). A 1.5-kb fragment of the 16S rRNA gene was amplified by PCR using broad-specificity primers. The PCR product was ligated into a standard cloning vector and transformed into *Escherichia coli* competent cells to isolate plasmids containing the insert, which were sent for sequencing (see the supplemental material 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 the 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) naive Bayesian classifier (56) (see the supplemental material for details of the sequence analysis).

SEM. A 2-ml sample was taken from a bottle of AFC medium with bacteria 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 resuspended in deionized H_2O in order to remove soluble phases, such as Na_2CO_3, and centrifuged again for 5 min. The pellet was transferred to a copper crucible, and scanning electron microscopy (SEM) analysis was performed using an FEI Quanta 650 FEG-ESEM scanning electron microscope. Energy-dispersive X-ray spectra were collected with an Oxford X-max 80 SDD (liquid nitrogen-free) energy-dispersive X-ray spectroscopy (EDS) detector, and images were collected in secondary electron imaging mode.

Isolation and quantification of soluble electron-shuttling compounds. One hundred milliliters of culture was centrifuged at 9,000 × *g* for 15 min to separate cells from the growth medium. Culture supernatant was neutralized with high-performance liquid chromatography (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 sulfate (5 g) at 4°C overnight. The organic phase was then filtered through a 0.45-μm-pore-size polytetrafluoroethylene (PTFE) syringe filter (Sartorius) and desiccated using a rotary evaporator. The resulting residue was dissolved with Milli-Q H_2O in an ultrasonic bath (Elmasonic S30; Elma).

A 10-ml column containing 8 g XAD-16 resin (Sigma) was precleaned with 100% methanol and rinsed thoroughly with deionized H_2O. The ethyl acetate-soluble fraction extract was slowly transferred onto the column (XAD-16 is a nonionic macroreticular resin designed to adsorb low- to medium-molecular-weight organic substances from aqueous systems and polar solvents by hydrophobic and polar interactions). Compounds that bound to the resin were sequentially eluted with 4 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 use of a SpeedVac apparatus (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 deionized H_2O for further spectroscopy, electrochemical assays, and quantification. Unused AFC medium was subjected to the same extraction and used as a control.

Flavin quantification was performed by scanning wavelengths from 300 to 700 nm using a UV-2 UV/visible 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 ($\varepsilon = 12{,}500\ cm^{-1}\ M^{-1}$) was used to quantify the concentration (57).

Fluorescence spectroscopy. The 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. Ultraflat template-stripped gold (TSG) electrodes (surface area [*A*] = 0.2 cm^2) were prepared and cleaned (see reference 58 for details). Self-assembled monolayers (SAMs) were formed on electrodes by incubating them with 1 mM 8-mercaptooctanol in propanol for 16 h. 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 (Ag/AgCl) electrode 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 glove box (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 glove box for at least 24 h before use. Electrochemical measurements were recorded at 21°C using an Autolab electrochemical analyzer with a PGSTAT30 potentiostat, a Scangen module, and an FRA2 frequency analyzer (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 s before cycling at a scan rate (*u*) of 10 mV/s in the potential window from +200 mV to −600 mV (versus Ag/AgCl). Comparison of the CVs for SAM and flavin-modified electrodes indicated 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 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 molecule.

HPLC. For rapid discrimination of flavins, an 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. Ten-microliter samples (equivalent to 100 ng flavin) were injected into an HPLC system consisting of an online degasser (DG-2080-53), a gradient former (LG-1580-02), a PU-980 pump, an AS-1555 autosampler, and a UV-975 UV detector set at 420 nm (all from Jasco, Gross-Umstadt, Germany), as well as an RF-551 fluorescence detector set at 450/520 nm (excitation/emission; Shimadzu, Duisburg, Germany). Separations were performed at a flow rate of 1 ml/min on a LiChrospher 100 RP-18e column (5 μm; 250 by 4 mm; Merck, Darmstadt, Germany) at 25°C. The solvent system consisted of water–0.1% trifluoroacetic acid (phase A) and acetonitrile (phase B) nominally applied as follows: 15% phase B for 5 min, 15% phase B to 50% phase B in 2 min, 50% phase B for 1 min, 50% phase B to 15% phase B in 1 min, and 15% phase B for 4 min. The retention times (means ± standard deviations, *n* = 3) of flavins in this solvent system were 3.76 ± 0.01 min (riboflavin-5′-diphosphate [FAD], which was present as a 6% impurity in the FMN used), 4.64 ± 0.07 min (FMN), and 5.91 ± 0.03 min (riboflavin).

Nucleotide sequence accession numbers. Sequences were submitted to the GenBank database and can be found under accession numbers KF362050 to KF362117.

RESULTS

Bacterial growth characteristics. The growth of the community of alkaliphilic Fe(III)-reducing bacteria in alkaline Fe(III)-containing (AFC) medium was characterized by enumeration of cell numbers, the ATP concentration, and the total Fe(II) concentration in the medium. Cell numbers, the ATP concentration, and the total Fe(II) concentration showed the same trend. After initial inoculation, there was a lag phase where the number of cells/liter stayed roughly constant for 72 h, after which cell numbers exponentially increased to a peak of ~200 × 10^9 cells/liter at 168 h (Fig. 1A). Cell numbers stayed at similar levels until 500 h, when they started to slowly decrease. Negligible Fe(II) was recorded until 96 h had elapsed, and then the concentration increased to a maximum of ~3,500 μM at 216 h (Fig. 1C) and subsequently stayed relatively constant until 500 h. After this time, Fe(II) levels started to decrease (data obtained after 600 h are not shown). Trace amounts of ATP were observed until 96 h, at which point the concentration rapidly increased to the maximum of 1 to 2 nM after 192 h (Fig. 1D). The pH value was consistently 9.1 until 72 h

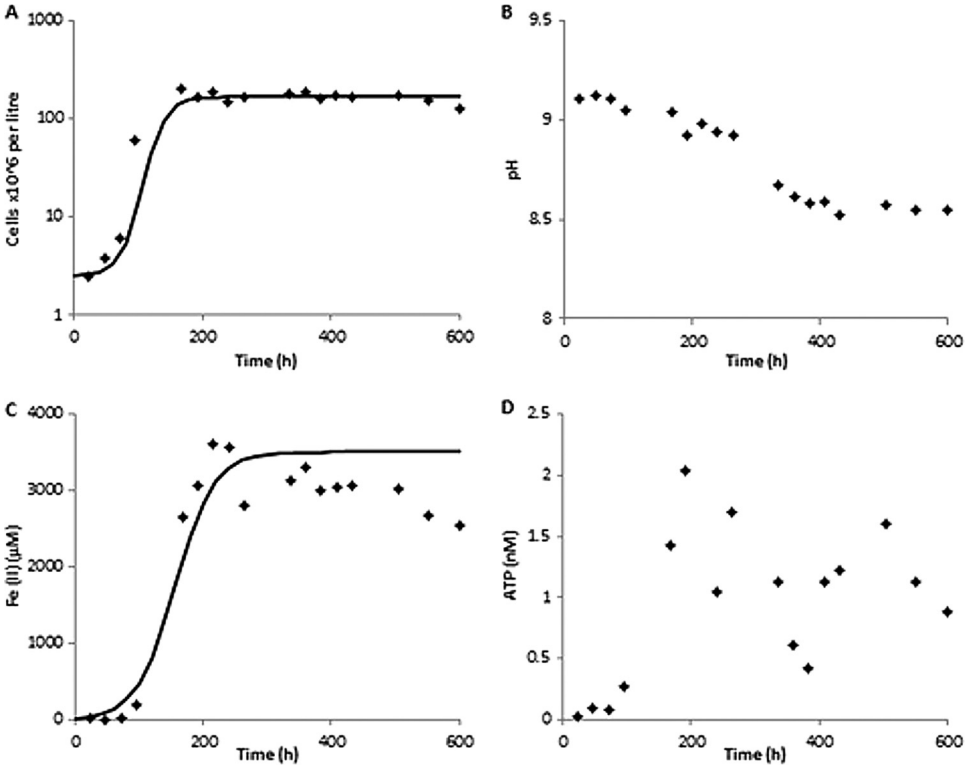

FIG 1 Growth of the iron-reducing consortia in AFC medium: variation of cell numbers (10^6/liter) (A), pH (B), Fe(II) concentration (μmol/liter) (C), and ATP concentration (nmol/liter) (D) with time. Sigmoidal growth curves were fitted to the cell count and Fe(II) data (73).

had elapsed, when it started to decrease and reached a final value of 8.5 by ~360 h (Fig. 1B).

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

Agar plates and isolate analysis. Growth of the AFC medium culture on agar plates resulted in small colorless colonies on the surface of the plate after 2 weeks. A lessening in the color density of the medium/agar in the plates and the formation of very small dark particles in the agar was associated with colony growth (see Fig. S1 in the supplemental material). The color change is due to the reduction of aqueous Fe(III) in the AFC medium and the precipitation of Fe(II). SEM analysis of the spent AFC medium (see below) suggests that the particles in the agar-AFC medium were vivianite crystals [hydrated iron(II) phosphate]. The reduction in color 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 color density of the medium/agar in the immediate vicinity of the streak, which extended about 2 mm beyond the boundary of the cell colonies. Colonies were randomly selected from these plates for rRNA gene sequence analysis.

TABLE 1 Iron reduction by the alkaliphilic bacterial community when grown on different electron donors

Electron donor	Outcome at wk[a]:		
	1	2	3
Acetate	++−	−−−	
Lactate	++−	−−−	
Ethanol	+++	+++	−−−
Methanol	++−	−−−	
Sucrose	+++	+++	−−−

[a] The three symbols represent positive (+) and negative (−) outcomes in each of three replicates.

Community analysis and streak analysis. The 16S rRNA gene sequences extracted from the AFC medium showed 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* spp., 44% were *Clostridium* spp., and 8% were *Alkaliphilus* spp. Analysis with the mothur program further classified the sequences into 5 OTUs. The *Tissierella* genus contained three OTUs, from which representative sequences were selected and analyzed again using the RDP classifier. This showed two of the OTUs to be *Tissierella* spp. (from here on, *Tissierella* strains A and B) with a confidence threshold of 100% and the other OTU to be a *Tissierella* sp. with a threshold of 87% (*Tissierella* strain C). The *Clostridium* and *Alkaliphilus* genera each contained one OTU with a confidence threshold of 100% (Fig. 2). Representative sequences were selected from each OTU,

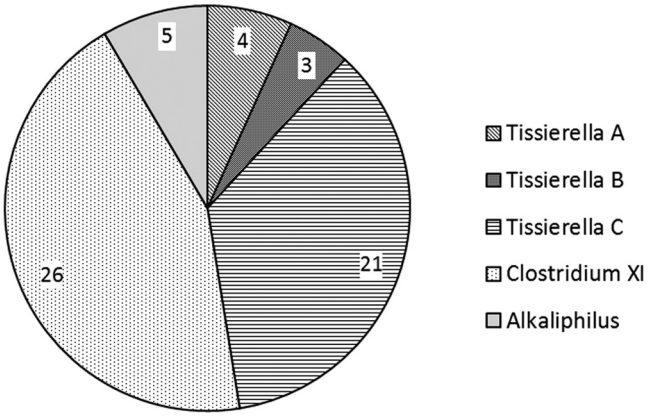

FIG 2 Microbial community grown on alkaline Fe(III)-containing medium; sequence allocation to OTUs was determined by the mothur program.

and a taxonomic tree showing their relationship with closely related type strains was constructed (Fig. 3).

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

Analysis for soluble electron-shuttling compounds. To investigate whether a soluble electron-shuttling compound was involved in Fe(III) reduction by the consortium, the spectral properties of spent medium were studied at four stages of growth. The culture supernatants were examined at times of 24, 72, 168, and 336 h (1, 3, 7, and 14 days, respectively) for optical signatures indicative of quinones or flavins (unused AFC medium was used as the control). Scanning of the culture supernatants over a wavelength range from 200 to 700 nm revealed spectral features that increased in amplitude with the age of the culture and that were compatible with the accumulation of flavins in the medium (Fig. 4A). The extracts from XAD column purification exhibited spectral features (Fig. 4B) indistinguishable from those exhibited by commercially available riboflavin (61) (the extract from the unused medium produced no detectable peaks). Upon excitation at 441 nm, the XAD column extract exhibited a broad emission peak in its fluorescence spectrum at between 475 and 650 nm, with a maximum at 517 nm (Fig. 4D). This feature, exhibited by commercially available riboflavin (also shown in Fig. 4D), is diagnostic for the isoalloxazine ring structure in flavin species (62). To corroborate these findings with the Fe(II)-dependent growth of the culture, the amount of flavin produced at each stage of growth was compared to the level of Fe(II) accumulation in the culture medium. Interestingly, there was a direct correlation between the appearance of flavin and the generation of Fe(II) during the growth phase of the bacterial consortium (Fig. 4C).

Cyclic voltammetry (Fig. 5A) revealed that the surface-immobilized XAD column extract was capable of transferring electrons to and from a metal species with oxidation and reduction peak

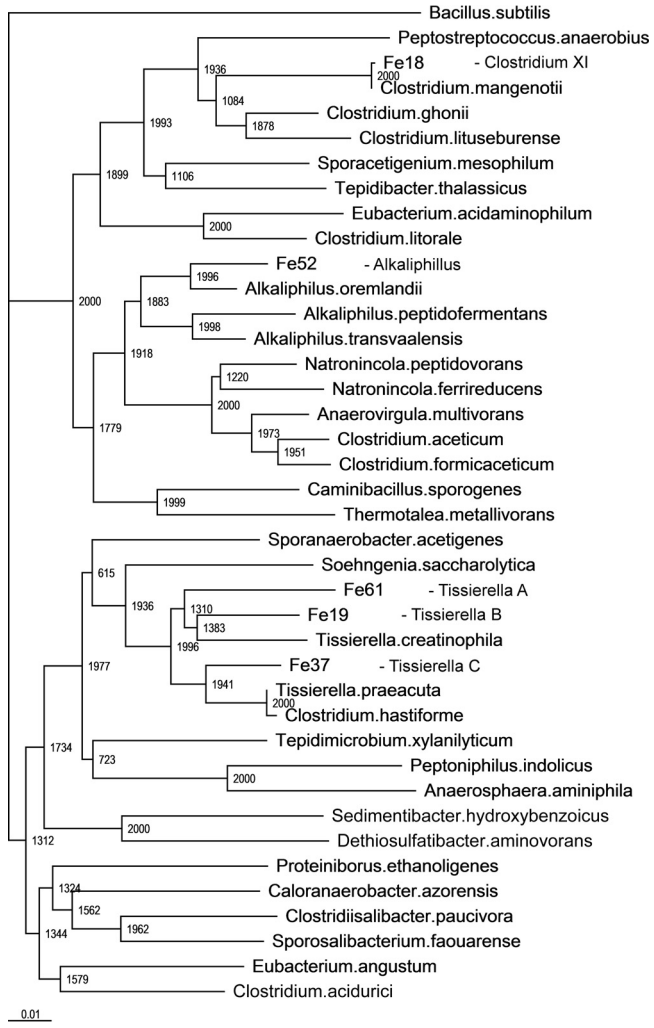

FIG 3 Taxonomic tree showing the relationships between representative sequences from each OTU and closely related type strains. The scale bar corresponds to 0.01 nucleotide substitutions per site, and bootstrap values from 2,000 replications are shown at branch points.

potentials of −0.18 mV and −0.25 mV versus standard hydrogen electrode (SHE), respectively. Furthermore, the electrochemical profile of the column extract was very similar to that obtained from commercially available pure riboflavin. Once the peaks were baseline corrected to remove any slope bias from the scans (Fig. 5B), it was revealed that the electrochemical coverage and peak potentials of the column extract were almost identical to those of commercially available riboflavin (Fig. 5A and B). Thus, both the surface adsorption and packing characteristics of the column extract were indistinguishable from those of riboflavin.

However, the spectral, fluorescence, and electrochemical properties investigated here were 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. Comparison with chromatographs for commercially available riboflavin, FMN, and FAD showed that this peak was eluted at the same retention time as riboflavin (Fig. 6).

Growth in medium spiked with riboflavin. To further corrob-

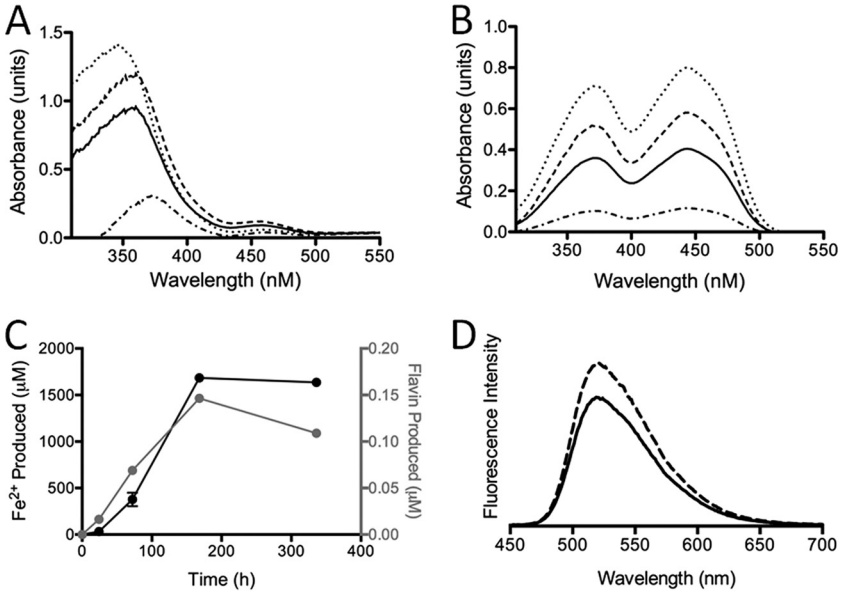

FIG 4 Spectroscopy of culture supernatants. (A and B) UV-visible spectra of culture medium supernatants at various stages of alkaliphilic consortium growth (A) or the extracellular compounds isolated (B). Data for samples taken at day 1 (dash-dot lines), day 3 (solid lines), day 7 (dotted lines), and day 14 (dashed lines) are shown. (C) Comparison of the flavin produced with Fe(III) conversion to Fe(II) using the quantification information from panel B. Black symbols indicate Fe^{2+} produced and gray symbols indicate flavin produced. (D) Fluorescence spectra of extracellular compounds isolated from culture medium supernatant (dashed line) compared to those of commercial pure riboflavin (solid line). Upon excitation at 441 nm, the emission spectra were monitored at between 450 and 700 nm. The results shown are representative of those from two biological replicates.

orate the role of riboflavin in Fe(III) reduction, growth medium was spiked with riboflavin. Bacteria grown in AFC medium supplemented with riboflavin produced Fe(II) after 48 h, half the time of the bacteria in the base AFC medium (Fig. 7). The exponential phase of growth for the bacteria in riboflavin-amended medium was complete after 144 h.

SEM. The precipitate recovered from the microcosms containing AFC medium after cell growth appeared black and crystalline in nature. Under SEM analysis, the primary features seen were flattened prismatic crystals roughly 30 by 5 by 5 μm in size (Fig. 8). Between the crystals was an amorphous gel which cracked as the sample was dried. EDS spot analysis of crystals (Fig. 8, inset) gave similar spectra with distinct peaks for O, P, and Fe and a small S peak (there were also Cu peaks, which were associated with the copper crucible which contained the sample). The flattened prismatic crystals have the morphology of vivianite [$Fe_3(PO_4)_2 \cdot 8H_2O$] (63) (the sulfur peak in the EDS spectrum is probably associated with the amorphous background phase).

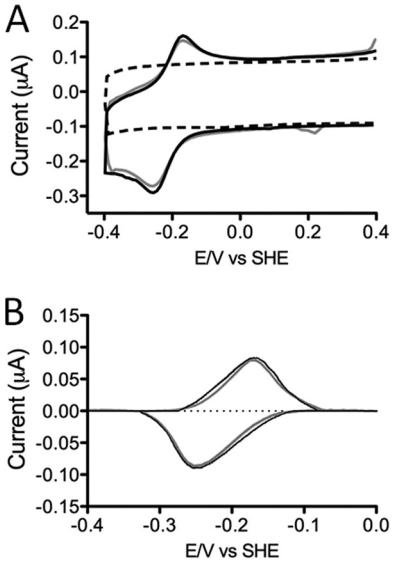

FIG 5 Cyclic voltammetry (CV) of 8-OH-modified TSG electrode before (blank) and after formation of a flavin film. All CVs were recorded in 20 mM MOPS, 30 mM Na_2SO_4 buffer (pH 7.4) at a 10-mV/s scan rate. (A) CVs showing the redox chemistry of immobilized purified flavin extract (gray lines) compared to the redox chemistries of 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 panel A. The results shown are representative of those from three replicate experiments. E/V, electrode potential (volts); SHE, standard hydrogen electrode.

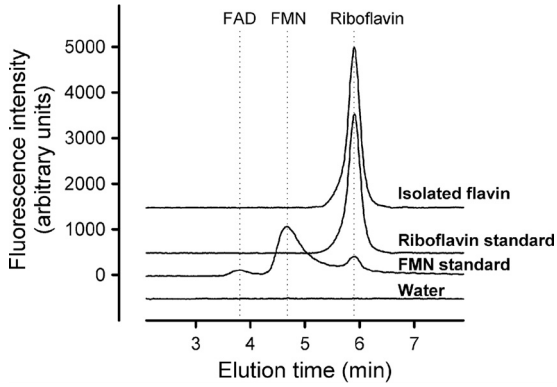

FIG 6 Reversed-phase HPLC of the isolated flavin, riboflavin standard, and an FMN preparation which contained quantifiable amounts of riboflavin and FAD. One hundred nanograms of each sample was analyzed.

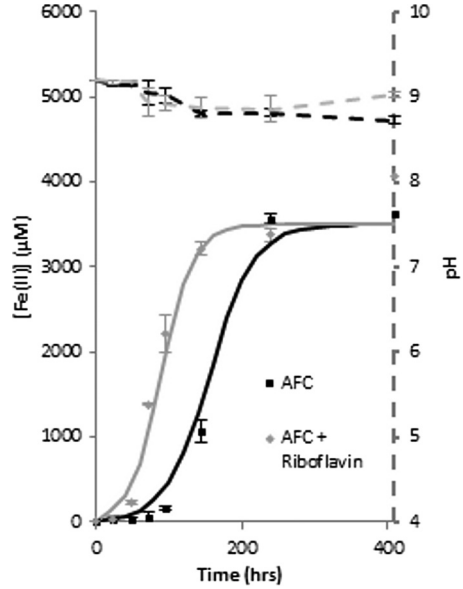

FIG 7 Average Fe(II) production and pH value during the growth of the iron-reducing consortia in AFC medium spiked with riboflavin. Sigmoidal growth curves were fitted to the Fe(II) data (73). Error bars indicate 1 standard deviation from the mean.

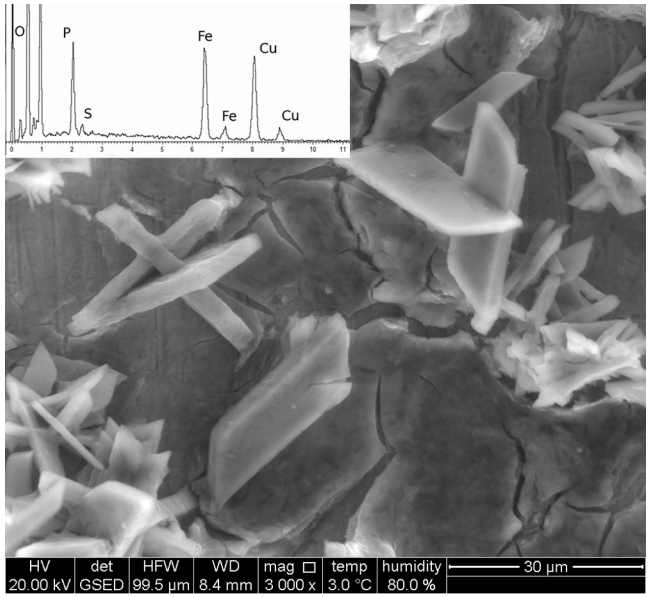

FIG 8 Electron micrograph of the precipitate recovered from the spent AFC medium. HV, high voltage; det GSED, detector, gaseous secondary electron detector; HFW, horizontal field width; WD, working distance; mag, magnification.

Vivianite is a common phase when Fe(III) is bioreduced in medium containing high concentrations of soluble phosphate (64).

DISCUSSION

Identity of the alkaliphilic community. After repeated growth on AFC medium (50-plus growth cycles since isolation from the soil), sequencing data showed that there were still several genera of bacteria in the iron-reducing community. This suggests that either all the bacteria present were able to respire independently using the AFC medium or a symbiotic relationship had developed between the different types of bacteria whereby one required the respiration products of another for growth. The AFC medium 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 the long-term growth of the consortium. In medium 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 and that is not supplied by the base medium. 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 characterized from the AFC medium were from the genus *Tissierella*, with mothur analysis showing that they could be further separated into three OTUs, referred to here as *Tissierella* strains A, B, and C. *Tissierella* spp. are obligate anaerobic, Gram-negative, non-spore-forming rods (68). All OTUs were most closely related to the type strain *Tissierella praeacuta* (Seqmatch scores, strain A = 75%, strain B = 80%, and strain C = 86%). Forty-four percent of the sequences characterized were from a single OTU in the genus *Clostridium* XI and were up to 100% similar to the sequence of type strain *Clostridium mangenotii*. Found in many soils around the world (69), *Clostridium mangenotii* 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. Eight percent of the bacteria sequenced were from a single OTU in the genus *Alkaliphilus* and were 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 remotely from the cell location. The streaks that visibly cleared the medium contained only bacteria of the genus *Tissierella*, which mothur analysis showed to be part of OTU *Tissierella* strain C. This fact, together with the observation that *Tissierella* forms a significant part of the AFC medium consortium, suggests that members of the genus *Tissierella* may be the principle bacteria producing the electron-shuttling compound. Extensive efforts to reintroduce these *Tissierella* strain C streaks into AFC medium for further investigation were unsuccessful. It should be noted that these data do not preclude the possibility that other bacterial species in the consortium were also producing a soluble electron-shuttling compound. Transferring the bacteria from aqueous medium to agar medium exerts 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 did not clear were identified as bacteria not seen in the initial population from the AFC medium. This is not a surprise, as environmental samples

usually contain many different bacterial strains which can tolerate the medium 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 secretes flavins to transfer electrons extracellularly. When the bacterial community was grown on AFC medium at pH 9.2, cell growth occurred slightly before the increase in Fe(II) (both are modeled in Fig. 1 by use of a logistic sigmoidal growth function [73]; see the supplemental material 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 (Fig. 4A and B), suggesting that its release was not associated with cell lysis.

The extracellular compound exhibited UV/visible spectral features indistinguishable from those of commercially available riboflavin. Further, it had surface adsorption characteristics and surface packing on TSG electrodes and oxidized and reduced with essentially the same redox potentials as riboflavin. Lastly, HPLC analysis showed this to be a single compound, and the retention time of this compound on the column matched the retention time of commercially available riboflavin. Thus, taking into account the overwhelming agreement in the data, it was deduced that the extracellular compound is riboflavin. When riboflavin was spiked into AFC medium containing the bacterial community, Fe(III) reduction started sooner and was quicker than that in unspiked medium, strongly suggesting that riboflavin is involved in the mechanism of Fe(III) reduction. When isolates from the community were grown on AFC medium agar plates, the medium cleared at mm-scale distances from the streaks, demonstrating that iron reduction was occurring remotely from the cell location.

There is a wide body of evidence that flavins can act as electron-shuttling compounds during extracellular electron transport to iron in circumneutral pH environments. For example, *Shewanella* species release flavins, and this increases the ability of cells to reduce Fe(III) oxides into Fe(II) in cellular respiration (29–32). Thus, it seems extremely likely that the extracellular, riboflavin-like compound released into solution by the alkaliphilic iron-reducing community during growth was acting as an electron-shuttling compound and had a role in Fe(III) reduction; this is the first time that this has been shown to occur at alkaline pH. Given that even mesophilic bacteria can adopt a wide variety of mechanisms to perform similar physiological functions when interacting with their environment (74) and the stress of a challenging environment has led extremophilic bacteria to evolve distinctly different mechanisms in many cases (45, 75), it is striking that the electron-shuttling compound found in this study of alkaliphiles is indistinguishable from that used by mesophiles. Interestingly, flavins have also been found in the culture supernatants of 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) oxyhydroxide phase. The most likely mechanism of chromium retention is abiotic reduction by microbially produced soil-associated Fe(II) (48). Hence, microbial 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 a very high pH. Common industrial processes, such as iron and steel making, aluminum and chromium extraction, and lime and cement manufacture, produce a waste form with a pH of >12 (76–79). Many of these wastes contain elevated concentrations of redox-sensitive, potentially mobile, toxic metals (e.g., As, V, Cr). Thus, the environment near the waste is particularly harsh, so soil bacteria tend to favor microhabitats 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 affected by the leachate. There is some evidence of this at the sampling site, where 45 to 75% of the microbially available iron is Fe(II), despite an average soil pH value of 11 to 12.5, and this may account for why the soil has accumulated 0.3% to 0.5% (wt/wt) Cr(III), even though the soil receives 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 manyfold, 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 oxidized compounds that it encounters with a high enough reductive potential; thus, the direct reduction of some groundwater contaminants [e.g., U(VI) → U(IV)] by this bacterial community may be possible.

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