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Molecular characterization and diversity of thermophilic iron-reducing enrichment cultures from deep subsurface environments

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Aims: The objectives of this work were to explore the diversity in Fe(III)-reducing enrichment cultures from the deep subsurface and to identify strains involved in metal reduction.

Methods and Results: Analyses of 16S ribosomal RNA (rRNA) of enrichments, supplemented with hydrogen, acetate or pyruvate as an electron donor, identified three dominant operational taxonomic units (OTUs). All cultures exhibited considerable diversity (36–24 OTUs), even after being transferred at least nine times. Two OTUs were present in all three cultures, constituting about 65% of the total clones examined.

Conclusions: Dominant OTUs appeared to be most closely related to *Thermoanaerobacter ethanolicus* or *T. kivui*. One OTU, which is potentially responsible for autotrophic Fe(III) reduction, was only about 95% similar to *T. ethanolicus* and may represent a new species.

Significance and Impact of the Study: An unexpectedly high diversity was found in these enrichments and this diversity may be a feature that can be exploited.

INTRODUCTION

Dissimilatory Fe(III) reduction not only influences the biogeochemical cycling of carbon and many metals but also has important implications for the evolution of microbial life (Lovley 1991; Nealson and Saffarini 1994). Micro-organisms capable of oxidizing organic compounds with Fe(III) reduction are phylogenetically diverse. They include members of the gamma and delta Proteobacteria, Gram-positive bacteria and Flexistipes bacteria (Lovley 1991; Boone *et al.* 1994; Nealson and Saffarini 1994; Lonergan *et al.* 1996; Greene *et al.* 1997; Slobodkin *et al.* 1997).

Based on geological evidence, thermophily should be a characteristic associated with Fe(III) reduction. The primitive Earth is hypothesized to have been hotter than at present (Kasting and Ackerman 1986; Ohmoto and Felder 1987), and evidence suggested that dissimilatory Fe(III) reduction could be an early form of microbial respiration (Lovley 1991). Bacterial reduction of Fe(III) under mesophilic conditions is well documented (Lovley 1991, 1993; Nealson and Saffarini 1994). However, little is known about the microbial Fe(III) reduction under psychotrophic (e.g. Zhang *et al.* 1999) or thermophilic conditions. Only recently have several phylogenetically distinctly different thermophilic iron-reducing bacteria been isolated and characterized (Boone *et al.* 1995; Greene *et al.* 1997; Liu *et al.* 1997; Slobodkin *et al.* 1997).

The purpose of this study was to investigate the composition and structure of three enrichment cultures capable of reducing Fe(III)-oxyhydroxide to magnetic iron oxides. These cultures were enriched from geologically- and hydrologically-isolated Cretaceous- and Triassic-age sedimentary basins in the deep (860–2090 m below surface) terrestrial subsurface (Liu *et al.* 1997; Tseng *et al.* 1999). The results presented here are based on 16S rRNA gene-based molecular analysis. The analyses indicated that these iron-reducing communities are diverse but are dominated by a few low G + C Gram-positive bacteria.

There is mounting evidence of very high microbial diversity in environmental samples that may offer a largely untapped store of organisms of potential use in biotechnology. However, assaying microbial diversity in nature is complicated by many factors, and the loss of diversity upon enrichment

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and isolation has become increasingly evident with the use of molecular techniques. The enrichment of microbial cultures in order to isolate specific strains of organisms is a required step in most isolation procedures. However, enrichment and cultivation constrain the microbial community, and many species present in the original environment may be lost (e.g. Dunbar *et al*. 1999). The microbial diversity present in enrichment cultures has been the subject of some investigation (e.g. Jensen *et al*. 1998) but is largely unknown, as are the factors that control the diversity. In some studies, substrates (e.g. Holoman *et al*. 1998) and degree of dilution (Jackson *et al*. 1998) appeared to influence the diversity of enrichment cultures. As part of this study, the influence of substrate on the diversity of iron-reducing enrichments originating from the same parent material was investigated.

MATERIALS AND METHODS

Enrichments and DNA extraction

Thermophilic Fe(III)-reducing enrichment cultures were obtained from the Piceance Basin in Colorado at a depth greater than 860 m below the land surface as previously described (Liu *et al*. 1997). The original enrichment cultures were incubated, in pressure tubes under a H_2–CO_2 (80 : 20) headspace (2 atm pressure) with 70 mmol l^{-1} of amorphous ferric oxyhydroxide in a phosphate-buffered basal medium (PBBM) supplemented with 0·05% yeast extract. The PBBM medium contained (g l^{-1} distilled water): $NaH_2PO_4{\cdot}H_2O$, 0·28; NaCl, 0·9; $MgCl_2{\cdot}6H_2O$, 0·2; $CaCl_2{\cdot}2H_2O$, 0·1; NH_4Cl, 1·0; morpho-linopropanesulphonic acid (MOPS), 0·1; resazurin, 0·001; nitrilotriacetic acid, 0·015; $FeCl_2{\cdot}4H_2O$, 0·002; $MaCl_2{\cdot}6H_2O$, 0·001; $Na_2W_2O_3$, 0·0002; $MnCl_2{\cdot}4H_2O$, 0·001; $CoCl_2{\cdot}6H_2O$, 0·001; $CaCl_2{\cdot}2H_2O$, 0·01; $ZnCl_2$, 0·005; $CuCl_2{\cdot}2H_2O$, 0·00002; H_3BO_3, 0·00005; $Na_2MoO_4{\cdot}2H_2O$, 0·001; NaCl, 0·01; Na_2SeO_3, 0·00017; $NiCl_2{\cdot}6H_2O$, 0·00024; biotin, 0·00002; folic acid, 0·00002; pyridoxine hydrochloride, 0·0001; thiamine hydrochloride, 0·00005; riboflavin, 0·00005; nicotinic acid, 0·00005; DL-calcium pantothenate, 0·00005; vitamin B_{12}, 0·000001; *p*-aminobenzoic acid, 0·00005; and lipoic acid, 0·00005. The pH of the PBBM medium was adjusted to 7·5–7·8. Strict anaerobic techniques were used during medium preparation, bacterial culture and end-product recovery. Initial inoculations were made with 1 g crushed sedimentary rocks or 1 ml drilling fluid 10 ml^{-1} medium, and transfers were made by inoculating 1–5% (v/v) of positive cultures.

Subcultures were established in PBBM amended with 0·05% yeast extract with three different electron donors. Electron donors for these three enrichments were pyruvate (10 mmol l^{-1}), acetate (10 mmol l^{-1}) or hydrogen (80% in headspace of 2 atm pressure). The subcultures all contained 70 mmol l^{-1} amorphous ferric oxyhydroxide (prepared by neutralizing a solution of $FeCl_3$ and collecting the washed precipitates) as an electron acceptor. For enrichment, 25 ml pressure tubes containing 10 ml liquid medium were sealed under a N_2–CO_2 (80 : 20 at 101 kN m^{-2} gas pressure) atmosphere with a butyl rubber stopper and aluminium seal. The medium sealed in incubation tubes was sterilized at 121°C for 20 min. The Fe(III) compound and the major electron donor were added to the sealed tubes from anaerobic sterile stock solutions. Bromoethanesulphonate (BESA, final concentration 5 mmol l^{-1}) was added to the medium before inoculation. All incubations were kept in the dark at 60°C unless otherwise specified. The subcultures were transferred more than eight times before molecular analysis. Dilution for each transfer was 1 or 0·3 ml into 10 ml pressure tubes for a 10- or 30-fold dilution at each transfer. The thermophilic Fe(III)-reducing enrichment cultures were placed under autotrophic and fermentative conditions with hydrogen, acetate or pyruvate as the electron donor.

The enrichment cultures were tested for the production of magnetic iron oxides using a magnet outside the tube as a screening tool. The magnetic material was confirmed to be magnetite in later tests (Liu *et al*. 1997). Positive enrichments were used in the molecular analysis.

16S rRNA gene amplification and cloning

Due to the resistance of some Gram-positive bacteria to chemical lysis, a combination of physical and chemical lysis methods was used to isolate the total community DNA (Zhou *et al*. 1996). Cultures (10–30 ml) were centrifuged at 12 000 *g* for DNA extraction. The samples were ground with a mortar and pestle in the presence of sterile sand and liquid nitrogen before addition of the extraction buffer (Zhou *et al*. 1996). The mortar and pestle were washed with extraction buffer; the preparations were then frozen at −70°C and thawed by microwave heating until the preparation was briefly boiling a total of three times. The genomic DNA was then extracted using an SDS-based lysis method (Zhou *et al*. 1995).

The oligonucleotides used for amplifying eubacterial 16S rRNA genes were the primer set of fD1 and rP1, as described by Weisburg *et al*. (1991), with modifications to the linker sequences (Zhou *et al*. 1995). All stocks for PCR amplification were made and procedures performed following the precautions suggested by Kwok and Higuchi (1989). Purified DNA (1 μl) was used as a template in a 20 μl reaction. The conditions for amplifying 16S rRNA genes were described previously (Zhou *et al*. 1995).

The PCR-amplified 16S rRNA gene products were quantified by comparing band intensity on agarose gels with known concentrations of lambda phage DNA. The amplified PCR products were directly ligated to the pCR™ II vector from Invitrogen (San Diego, CA, USA). Ligation and transformation were carried out according to the

manufacturer's instructions. The ratio of inserts to vectors in ligation was 0·5–1. A 2 μl aliquot of the ligation reaction mixture was transformed by heat pulse into *Escherichia coli* INVαF′-competent cells (Invitrogen).

A set of PCR amplification primers specific to the polylinker of the vector pCR^{TM} II was designed to screen the 16S rDNA inserts (Zhou *et al.* 1997). The PCR amplification conditions for this set of primers were previously described (Zhou *et al.* 1997). Initially, 160 white colonies (indicating transformation) of about 300, from each of the three enrichment cultures, were screened for 16S rDNA. However, all (290) of the white colonies from the hydrogen-grown culture were eventually screened due to a high proportion of false-positive clones.

16S rDNA restriction fragment length polymorphism (RFLP) analysis

RFLP analysis was performed in order to differentiate 16S rDNA clones. The PCR amplified products, one-fifth of the 20 μl, were digested using 0·1 U of each of two tetrameric endonuclease pairs (*Msp* I plus *Rsa* I, and *Hha* I plus *Hae* III; Gibco BRL Life Technologies, Gaithersburg, MD, USA). The mixture was digested overnight at 37°C and the resulting RFLP products were separated by gel electrophoresis. Electrophoresis was accomplished in 3·5% Metaphor agarose (FMC Bioproducts, Rockland, ME, USA) prepared according to the manufacturer's instructions with 1 × TBE at 4°C and 7 volts cm^{-1} for 4 h. The gel was stained with 0·5 mg ethidium bromide ml^{-1} and visualized under UV excitation. The RFLP patterns were compared using Molecular Analyst Fingerprinting Plus Software (BIO-RAD, Hercules, CA, USA). As in other studies (Moyer *et al.* 1994, 1995), each unique RFLP pattern was designated as an operational taxonomic unit (OTU).

The distribution of all confirmed 16S rDNA clones among these OTUs for each enrichment culture was determined. Different OTUs were numbered in the order of their abundance. To determine whether the *in situ* bacterial diversity was well represented by the 16S rDNA clones examined, refraction analysis (Tipper 1979) was performed. The cumulative number of OTUs was plotted as a function of the clone number, which was randomly assigned to each clone on the order of initial detection. A non-linear regression analysis was performed using the Sigma Plot Software (SPSS Inc., Chicago, IL, USA) to predict the level at which no additional unique clones would have been found (the saturation level).

rDNA sequencing

To determine the phylogenetic identity of the iron-reducing bacteria, 21 clones from different representative OTUs having more than two clones were selected for sequencing. The DNA sequences of the 16S rRNA genes were determined directly using the PCR-amplified DNA as the sequencing template. The amplified PCR products were purified using the Wizard^{TM} PCR Preps DNA purification system (Promega, Madison, WI, USA) according to the manufacturer's instructions. DNA sequences were determined with automated fluorescent *Taq* cycle sequencing using the ABI Catalyst 800 and ABI 373 A Sequencer (Applied Biosystems, Forester City, CA, USA) at Michigan State University. Approximately 100 ng of the purified DNA were used for each automated fluorescent sequencing reaction. The primer used for sequencing the 5′ end of the 16S rDNA (R529) spanned the *E. coli* 16S rRNA gene positions of 529–512. Clone P-3 was further sequenced using nine additional primers because phylogenetic studies based on the 5′ end partial sequences showed that the OTU represented by the clone P-3 was distinctly different from other bacteria (Liu *et al.* 1997). The following additional primers were used to obtain full 16S rDNA sequences: FD1 (8–27), F270 (246–261), F519 (519–533), F788 (787–802), F1099 (1099–1144), R350 (357–342), R925 (920–906), R1240 (1242–1227) and RP1 (1511–1492).

Phylogenetic analysis

Sequences were assembled using the assembling programs in the Genetic Computer Group (GCG) software package (Devereaux *et al.* 1984). The sequences were checked with the programs Check_Chimera from the Ribosomal Database Project (RDP) (Larsen *et al.* 1993; Maidak *et al.* 1997) and mglobalCHI (Komatsoulis and Waterman 1997). Preliminary analysis was done by searching the current databases (GenBank release 101·0 and EMBL release 50·0) using the program FASTA. Sequences were then aligned manually to the 16S rDNA sequences of the species that showed the highest similarity scores in the outputs of FASTA in the previously aligned 16S rDNA sequence database, RDP. using the GDE multiple sequence editor program from RDP. Initial phylogenetic screening was constructed using the DNA distance program, Neighbour-Joining, in the PHYLIP package (Felsenstein 1989). All 16S rDNA sequences of Gram-positive bacteria in RDP, plus the closely-related 16S rDNA sequences which were absent in RDP, were used in the initial screening. Appropriate subsets of 16S rDNA sequences were selected, based on the initial phylogenetic results, and subjected to final phylogenetic analysis through the maximum likelihood method with the program fastDNAml from the RDP. Final phylogenetic trees were constructed with a transition/transversion ratio of 2·0 using jumbled orders of 10 for the addition of taxa.

The nucleic acid sequence accession numbers in GenBank are:

Table 1 16S rDNA clone diversity for the three thermophilic iron-reducing enrichment cultures

Enrichments	White colonies screened	Total 16S rDNA clones	Unique 16S rDNA clones	Numbers of RFLP patterns detected by			
				Msp I + Rsa I	Hha I + Hae III	Diversity (H)	Richness
Hydrogen	296	107	36	24 (66·7%)	10 (33·3%)	2·58	7·49
Acetate	160	97	30	17 (56·7%)	13 (43·3%)	2·38	6·35
Pyruvate	160	140	24	15 (62·5%)	9 (37·5%)	2·31	4·85

P-3, AF325224; A-35, AF325225; P-2, AF325226; P-26, AF325227; A-55, AF325228; A-100, AF325229; P-132, AF325230; P-36, AF325231; P-1, AF325232; A-15, AF325233; P-4, AF325234; P-47, AF325235; P-5, AF325236; P-6, AF325237; H-12, AF325238; H-22, AF325239; A-40, AF325240; H-92, AF325241; H-52, AF325242; A-112, AF325243; H-112, AF325244.

RESULTS

16S rDNA RFLP analysis

A total of 36 (of 107 16S rDNA clones), 30 (of 97) and 24 (of 140) RFLP patterns were identified from the 16S rDNA clones for hydrogen-, acetate- and pyruvate-grown cultures (Table 1), respectively. While many clones, the false positives, contained no detectable inserts, a sufficient number of the clones (36–88%) had a 16S rDNA insertion detectable by PCR amplification with the vector-specific primers (Table 1). A majority of the unique 16S rDNA clones among these enrichments was detected with the restriction enzymes *Msp* I plus *Rsa* I. However, an additional 33–43% of the unique patterns were obtained by the secondary enzyme digestion (Table 1) with the restriction enzymes *Hha* I plus *Hae* II.

Three OTUs dominated the three enrichment cultures (Fig.1). Together, these three OTUs accounted for 64%, 65% and 64% of the 16S rDNA clones from the hydrogen-, acetate- and pyruvate-grown cultures, respectively. The remainder of the OTUs remained at much lower levels than the three dominants and accounted for only about 36% of the 16S rDNA clones. The most prevalent member of this group (P-4) accounted for less than 10% of the clones in the pyruvate enrichment, less than 1% in the hydrogen enrichment (H-205), and was not detected in the acetate enrichment (Table 2).

Table 2 The OTUs common in pyruvate-, acetate- and hydrogen-grown communities with a representative clone for each OUT is listed

Pyruvate	Acetate	Hydrogen
P-9 (24·3)*	A-3 (37·1)	H-2 (30·8)
P-3 (19·3)	A-10 (17·5)	H-3 (19·0)
P-1 (20·7)	–	H-5 (14·3)
P-4 (9·3)	–	H-205 (0·9)
P-26 (5·0)	–	H-273 (0·9)
–	A-35 (4·1)	H-58 (1·9)
P-52 (0·7)	–	H-112 (1·9)
P-110 (0·7)	A-130 (1·0)	–
P-124 (0·7)	–	H-48 (0·9)

* The number in parenthesis indicates the percentage of this OTU in total 16S rDNA clones examined.

The 16S rDNA clone diversity appeared to be highest in the hydrogen-grown culture and lowest in the pyruvate culture. For example, after screening 60 16S rDNA clones (accounting for 56, 62 and 43% of the total clones of the hydrogen-, acetate- and pyruvate-grown cultures), only 61% (20 out of 36) of total OTUs were encountered for the hydrogen enrichment. In contrast at this point, 76% (23 out of 30) and 75% (18 out of 24) of the OTUs had been encountered for the acetate- and pyruvate-grown cultures, respectively. In addition, non-linear regression analysis indicated that the actual saturation level of OTU detection was at 178, 46 and 29 clones for the hydrogen-, acetate- and pyruvate-grown culture, respectively (Fig. 2).

Overall, the results suggest that, except for the lower diversity, the hydrogen-grown community was more similar to the pyruvate-grown community than to the acetate-grown community. More OTUs were shared between the hydrogen- and pyruvate-grown cultures than between either and the acetate-grown culture (Table 2). One of the dominant OTUs (represented by clones P-1 and H-5) was not observed in acetate-grown culture. However, the other two dominant OTUs (represented by clones P-3 and P-9, A-3 and A-10, and H-2 and H-3) were shared by all three of the enrichment cultures (Table 2).

Sequence data and phylogenetic analyses

The 21 clones sequenced grouped rather tightly together. An 82·1–99·8% sequence similarity was observed among the 5′ end of the 16S rDNA sequences (373–469 bp) (Table 3). No obvious evidence of chimeric artifacts for these partial sequences was detected using the programs Check_Chimera and mglobalCHI.

Phylogenetic analysis, established by a maximum likelihood method for all of the clones sequenced, revealed the presence of three primary groups that all belong to the *Acetogenium*

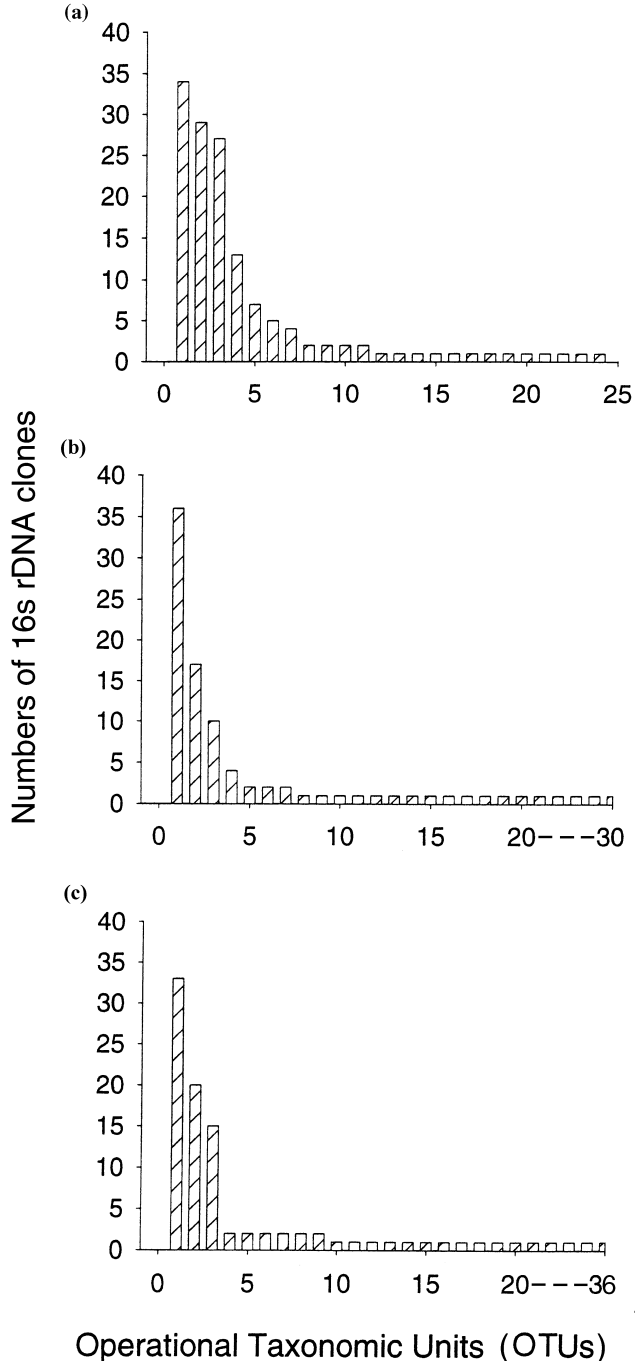

Fig. 1 Distribution of 16S rDNA clones from the thermophilic iron-reducing communities among different OTUs, each defined as a unique RFLP pattern. The OTUs are shown in order of clone abundance. (a) Pyruvate; (b) acetate; (c) hydrogen

subgroup within the *Syntrophomonas* group of the *Clostridia* subphylum of Gram-positive bacteria (Fig. 3). These three groups represented 50·2% of the clones. The most frequently encountered group of clones was the P-9 (OTU 1) cluster. This cluster was composed of clones P-1 (OTU 3), P-5 (OTU 10), P-47 (OTU 9), A-15 (OTU 3), A-40 (OTU 5), A-112 (OTU 6), H-12 (OTU 4), H-22 (OTU 5), H-52 (OTU 6), H-92 (OTU 7) and H-112 (OTU 8). The similarities of the sequenced regions ranged from 89·7 to 99·8% among these clones (Table 1). This cluster is most closely related to *T. ethanolicus* with a similarity of 92·4–98·4% (Fig. 3, Table 3), and accounted for 26·7% of the clones.

The second most frequently encountered group of clones was the P-3 (OTU 2) cluster, which was composed of P-2 (OTU 7), P-26 (OTU 5), A-35 (OTU 4), A-55 (OTU 7) and A-100 (OTU 8). There were 94·3–99·5% similarities among these clones, and 81·3–89·6% similarities between these clones and the other clones in the P-9 cluster (Table 3). This cluster was most closely related to *T. ethanolicus* with a similarity of 83·9–95·3% (Fig. 3, Table 3), and accounted for 17·7% of the clones. Sequence comparison of the full sequence, after sequencing with the nine additional primers, showed that the clone P-3 from this cluster had 95·8% similarity to the strain *T. ethanolicus* ATCC 3322. The signature sequences of this clone most closely matched the signature sequences of the members of low G + C Gram-positive bacteria (Table 4). However, the majority of the sequence variation was located at the 5′ end region of the 16S rDNA gene. There was only 81·3% similarity of the first 300 bp to *T. ethanolicus* ATCC 3322, whereas the rest of the gene was 99·6% similar to this strain. This phenomenon was also observed when the sequence for P3 was compared with the next most similar isolate, *Thermoanaerobacter kivui*, with 98·2% similarity at the 3′ end and only 80·4% similarity at the 5′ end.

The third related phylogroup was the P-4 (OTU 4) cluster, which was composed of P-36 (OTU 6) and P-132 (OTU 11). There were 99·5–99·8% similarities among these three clones, 82·9–94·4% similarities to the other P-9 cluster and 85·4–87·1% to the P-3 cluster. This cluster was also closely related to *T. ethanolicus*, with a similarity of 92·9–93·4% (Fig. 3, Table 3), and accounted for 5·8% of the clones.

DISCUSSION

Despite at least nine transfers and dilutions, the thermophilic Fe(III) reducing enrichment cultures retained a surprisingly high degree of diversity. Considering that the original enrichment was with hydrogen, it is reasonable that the final diversity was highest in the hydrogen enrichments. There was considerably less diversity in the acetate and pyruvate cultures.

In these million-years-old deep subsurface basin samples, H_2 oxidation-coupled Fe(III) reduction was observed under autotrophic conditions. However, it was not possible to isolate such an autotroph in pure culture. This is a dilemma frequently facing environmental microbiologists: how to

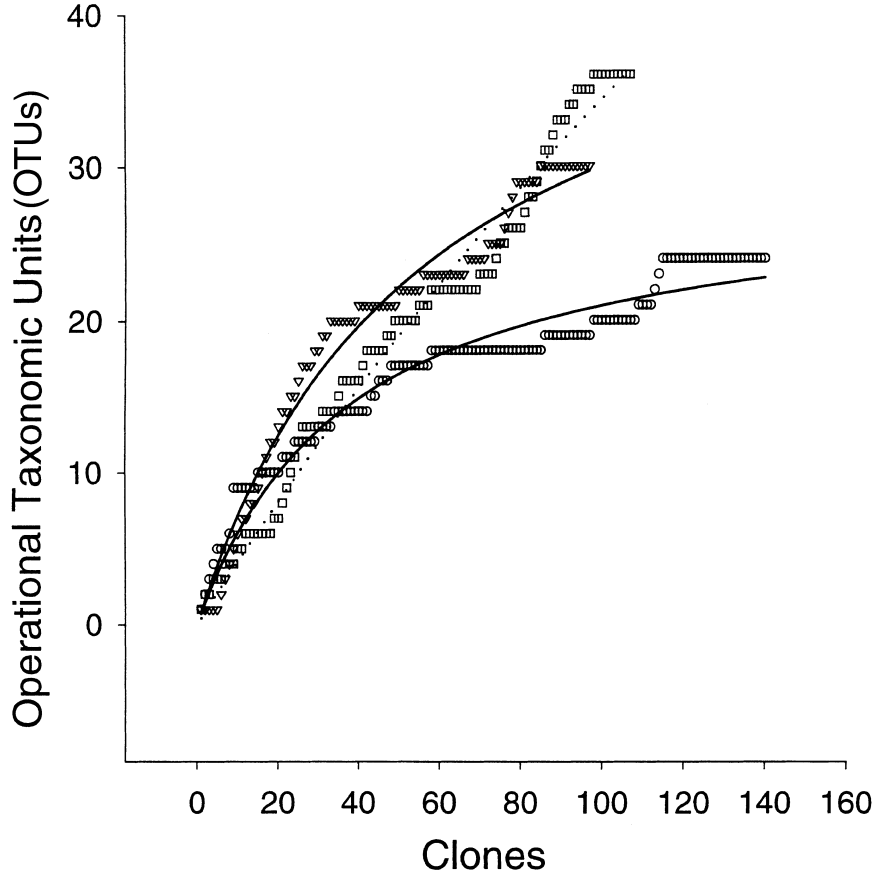

Fig. 2 Evaluation of the representation of the clones obtained from the thermophilic iron-reducing communities by rarefraction analysis (Tipper 1979; Moyer *et al.* 1994). The 16S rDNA clones were numbered based on their order of initial detection, which is assumed to be stochastic relative to the distribution of clones in the clone library. (○), Clones from pyruvate culture; (▽), clones from acetate culture; (□), clones from hydrogen culture; (—), pyruvate regression; (—), acetate regressio; (….), hydrogen regression

reliably assess the microbially-mediated mass and energy flow through an ecosystem when only a small fraction of microbial species can be cultured in the laboratory (Wayne *et al.* 1987). This is a relatively common problem when dealing with environmental isolates and here, was addressed using a differential cultivation scheme.

It was hypothesized that the major (group of) Fe(III)-reducing bacteria should be present in all three types of cultures as all retained the ability to reduce iron. In addition, the autotrophic Fe(III)-reducing bacterium should be present at least in the hydrogen-grown cultures as they are the key to this process. The molecular analyses of the microbial community structure of the three types of enrichment cultures yielded evidence in support of this hypothesis. Despite the difference in the OTU composition, all three types of Fe(III)-reducing enrichment cultures shared two of the three dominant OTUs. These two OTUs, represented by clones P-3 and P-9, are the most likely candidates for H_2 oxidation-coupled Fe(III) reduction.

It is speculated that the dominant OTU represented by the clone P-3 was most likely responsible for H_2 oxidation-coupled Fe(III) reduction and magnetite production. This speculation is based on a test of the capability of autotrophic Fe(III) reduction by a pure culture (TOR-39) isolated from a different thermophilic Fe(III)-reducing enrichment culture. As is the P-9 clone (97·2% sequence similarity), TOR-39 is closely related to *T. ethanolicus* (98·9% sequence similarity), and it was isolated in the present study of another deep subsurface site. The results showed that TOR-39 was incapable of autotrophic growth (data not shown) and, by inference, clone P-9 may also be incapable of autotrophic Fe(III) reduction. Thus, clone P-3 is the likely autotroph. However, further experiments such as *in situ* rRNA hybridization are needed to test this hypothesis.

The sequence data indicate that these thermophilic bacteria represent a novel group capable of reducing Fe(III). Full sequence analysis of clone P-3 revealed a 96% similarity to its closest identified match, *T. ethanolicus*. This degree of divergence in 16S rRNA genes may represent a novel species or genus of bacteria (Boone *et al.* 1996). The 16S rDNA sequence from the clone P-3 was also distinctly different from all other known thermophilic iron-reducing bacteria isolated from different environments. The 16S rDNA sequence from clone P-3 was 83·4% similar to *Thermoterrabacterium ferrireducens* isolated from a hot spring (Slobodkin *et al.* 1997), 81·1% to *Deferribacter thermophilus* isolated from a petroleum reservoir (Greene *et al.* 1997) and 83·9% to *Bacillus infernus* from deep subsurface (Boone *et al.* 1995).

Table 3 Pairwise levels of sequence identity between the clones in these thermophilic iron-reducing communities and some close-related species

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1. P-1	1·000	0·988	0·986	0·998	0·993	0·991	0·897	0·986	0·963	0·970	0·973	0·920	0·878	0·852	0·871	0·855	0·855	0·846	0·939	0·937	0·942	0·975	0·877
2. P-5		1·000	0·979	0·991	0·988	0·986	0·892	0·984	0·958	0·974	0·968	0·914	0·857	0·857	0·859	0·862	0·855	0·850	0·918	0·918	0·918	0·977	0·876
3. P-9			1·000	0·988	0·986	0·984	0·900	0·981	0·966	0·974	0·976	0·922	0·852	0·845	0·847	0·849	0·842	0·838	0·915	0·913	0·915	0·972	0·871
4. P-47				1·000	0·998	0·995	0·900	0·991	0·966	0·981	0·976	0·922	0·868	0·857	0·862	0·860	0·853	0·848	0·930	0·929	0·927	0·984	0·883
5. A-15					1·000	0·992	0·929	0·996	0·992	0·986	0·992	0·949	0·880	0·868	0·873	0·876	0·848	0·866	0·941	0·939	0·944	0·983	0·891
6. A-40						1·000	0·934	0·994	0·990	0·984	0·989	0·952	0·878	0·863	0·871	0·878	0·846	0·855	0·939	0·937	0·942	0·979	0·887
7. A-112							1·000	0·934	0·926	0·913	0·920	0·949	0·821	0·845	0·816	0·853	0·840	0·813	0·832	0·829	0·832	0·924	0·858
8. H-12								1·000	0·992	0·984	0·987	0·946	0·875	0·861	0·869	0·870	0·848	0·849	0·934	0·932	0·937	0·976	0·883
9. H-22									1·000	0·979	0·984	0·944	0·872	0·888	0·867	0·896	0·890	0·864	0·888	0·885	0·888	0·979	0·906
10. H-52										1·000	0·984	0·941	0·861	0·856	0·855	0·861	0·854	0·835	0·913	0·913	0·911	0·972	0·879
11. H-92											1·000	0·936	0·877	0·885	0·872	0·891	0·885	0·864	0·896	0·893	0·896	0·981	0·907
12. H-112												1·000	0·842	0·863	0·837	0·869	0·861	0·828	0·845	0·842	0·845	0·941	0·869
13. P-2													1·000	0·991	0·993	0·988	0·984	0·984	0·871	0·866	0·868	0·875	0·842
14. P-3														1·000	0·995	0·994	0·954	0·976	0·862	0·859	0·862	0·953	0·930
15. P-26															1·000	0·993	0·988	0·984	0·864	0·861	0·862	0·869	0·836
16. A-35																1·000	0·954	0·969	0·864	0·861	0·864	0·880	0·848
17. A-55																	1·000	0·943	0·857	0·854	0·857	0·839	0·809
18. A-100																		1·000	0·857	0·854	0·857	0·868	0·842
19. P-4																			1·000	0·998	0·998	0·932	0·906
20. P-36																				1·000	0·995	0·929	0·904
21. P-132																					1·000	0·934	0·909
22. T.e.																						1·000	0·953
23. T.k.																							1·000

T.e.:*Thermoanaerobacter ethanolicus*; T.k.: *T. kivui.*

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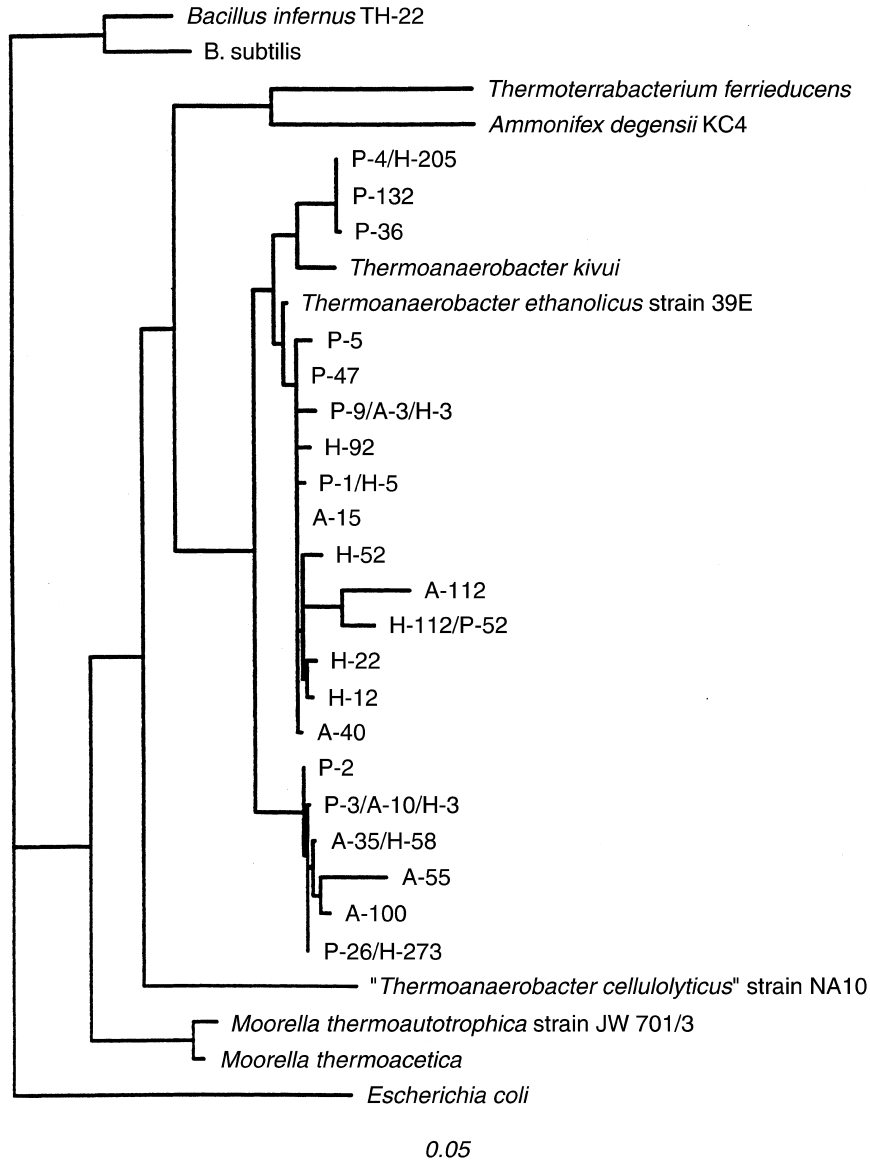

Fig. 3 Phylogenetic relationships of the 21 phylotypes sequenced from the thermophilic iron-reducing communities. The tree was established by maximum likelihood method based on the partial 16S rRNA gene sequences with *Escherichia coli* as the outgroup

These bacteria are distinctly different from all known mesophilic iron-reducing bacteria (Lonergan *et al.* 1996).

The results of this study support the assertion that metal reduction may be a characteristic that is widespread in the domain of bacteria (Lonergan *et al.* 1996). The existence of such phylogenetically-distinct thermophilic iron-reducing bacteria in the geologically-isolated, millions of years-old deep subsurface samples also supports the hypothesis that dissimilatory iron-reducing bacteria are an early form of microbial respiration (Lovley 1991).

The full sequence for the dominant OTU represented by P-3 also showed large variation between the 5′ and 3′ end of the genes when they were compared with the 16S rDNA sequence from *T. ethanolicus* ATCC 3322. The variation suggests that the greater similarity at the 3′ end is either characteristic of the *Thermoanaerobacter*, or that P-3 could be a PCR-generated chimeric artifact. Several lines of evidence indicate that it is unlikely that the P-3 sequence is an artifact. First, when the closest sequences in the database, *T. kivui* and *T. ethanolicus*, are compared with each other there is a comparable discrepancy between the similarity at the 3′ end, 98·3%, and the 5′ end, 91·5%. Thus, this difference may be a characteristic of the *Thermoanaerobacter*. In addition, no obvious evidence for chimeric structure was suggested by the chimeric structure-checking programs, Check_Chimera and mglobalCHI. Second, secondary structure analysis showed that the 16S rDNA sequence from P-3 does form a correct secondary structure. Most of the base changes at the 5′ end make sense in terms of forming secondary structure. Also, no more similar 16S rDNA

Table 4 Results of a signature sequence analysis with clone P-3 and other taxa*

Signature base‡				
	Gram positive bacteria			
Position†	Consensus	OUT P-3	Low G + C	High G + C
168	G	G	G/a	U/G/a
906	G	G	G/a	A
955	U	U	U	A/C
998	N	G	C/g	A/g
1116	U	U	U/c	C/U
1167	A	A	A/c/u	U/c
1224	U	C	U/c	U/a
1410	A	A	A	G

* Signature sequences were obtained from Woese (1987).

† Standard *E. coli* 16S rRNA numbering.

‡ A capital letter indicates a major base that accounts for >90% of assayable cases and a lower case letter indicates a minor base that is found in <15% of the assayable cases.

sequences, except the 16S rDNA sequence from *T. ethanolicus* ATCC 3322, was found when using both 5′ (300 bp) and 3′ (rest of the sequences) end sequences in searching the sequence databases with the FASTA program. Finally, P-3 was predominant in the 16S rDNA clone libraries from all three enrichments. If the PCR-generated chimera is a random process, it is unlikely that a single clone would predominant in all three of the clone libraries.

Although the diversity of the enrichments may not be completely represented by the clone library, the level of analysis was more than sufficient to detect the community's dominant OTUs. A significant decrease in the rate of OTU detection was observed in rarefaction curves in pyruvate- and acetate-grown cultures (Fig. 2). While more than 70% of the OTUs were detected within the first 60 16S rDNA clones, less than 25% of the total OTUs were detected among the remaining 40–60% of the clones. This suggests that most of the diversity in the clone library was detected by RFLP analysis for these two enrichment communities. The rates of OTU detection decreased but much less steeply for the hydrogen-grown culture. Thus, the *in situ* diversity may not be well represented, and could be much higher than the diversity in the 16S clone library. However, the dominant OTUs were detected within the first 15 clones analysed in all three cultures, and only rare members of the enrichment should have been missed.

Molecular techniques are revealing wide diversity in a number of types of enrichment cultures, such as lignin enrichments (Gonzalez *et al.* 1996) and methanogenic archaeal enrichments (Chin *et al.* 1999). High diversity of closely related strains in repeatedly transferred enrichments has been noted using molecular analysis techniques for carbon tetrachloride-degrading enrichments (Zhou *et al.* 1999). In 'natural' enrichments, such as zones of contamination, there have been examples of a variety of apparently closely-related species, e.g. Geobacteraceae (Rooney-Varga *et al.* 1999), being detected in the same sample. Thus, diversity may prove to be an unrecognized feature of enrichment cultures that reflects high diversity of closely-related bacteria in the environment.

In summary, the molecular analysis of the differentially-grown thermophilic Fe(III)-reducing bacterial cultures provided important information on microbial community structure. The sequence information will facilitate the design of appropriate molecular probes in future analysis of dynamic changes in the Fe(III)-reducing community that can lead to a more precise identification of various Fe(III)-reducing bacteria in different environments.

If high diversity is a common feature of enrichment cultures, this phenomenon should be considered in the isolation of bacteria for biotechnology applications. Divergence of 1–5% in 16S rDNA sequence is relatively small from a microbial phylogenetic viewpoint, but these small differences may indicate larger differences in other important physiological aspects. Differences in kinetics, substrates used and products produced could be critical in determining the utility of a strain for biotechnology applications. Thus, it might be wise to attempt to obtain and screen more isolates, even if they are 'closely' related, in order to obtain strains with different physiological properties.

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