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Characterization of bacterial diversity associated with deep sea ferromanganese nodules from the South China Sea[§]

De-Chao Zhang, Yan-Xia Liu, and Xin-Zheng Li*

Institute of Oceanology, Chinese Academy of Sciences, Qingdao, 266071, P. R. China

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Deep sea ferromanganese (FeMn) nodules contain metallic mineral resources and have great economic potential. In this study, a combination of culture-dependent and culture-independent (16S rRNA genes clone library and pyrosequencing) methods was used to investigate the bacterial diversity in FeMn nodules from Jiaolong Seamount, the South China Sea. Eleven bacterial strains including some moderate thermophiles were isolated. The majority of strains belonged to the phylum *Proteobacteria*; one isolate belonged to the phylum *Firmicutes*. A total of 259 near full-length bacterial 16S rRNA gene sequences in a clone library and 67,079 valid reads obtained using pyrosequencing indicated that members of the *Gammaproteobacteria* dominated, with the most abundant bacterial genera being *Pseudomonas* and *Alteromonas*. Sequence analysis indicated the presence of many organisms whose closest relatives are known manganese oxidizers, iron reducers, hydrogen-oxidizing bacteria and methylotrophs. This is the first reported investigation of bacterial diversity associated with deep sea FeMn nodules from the South China Sea.

Keywords: deep sea, ferromanganese, bacterial diversity

Introduction

Manganese and iron occur in seawater at extremely low concentrations (approximately 0.0004 ppm), but can account for > 30% of the total mass in polymetallic nodules (Wang and Müller, 2009). Ferromanganese (FeMn) nodules are a common type of authigenic deposit in oxygenated aquatic environments. They grow very slowly (a few mm/million years) by the accretion of very fine-grained and colloidal oxyhydroxides onto solid substrates, and often form dense pavements over large areas of the seafloor (Calvert, 1978; Jauhari and Pattan, 2000). Nodules of FeMn have been found on the seafloor in all oceans and seas, with the exception of the Mediterranean and Red seas, at depths of at least 100 m down to 6,000 m (Wang *et al*., 2009).

In addition to mineralization, polymetallic nodules are also formed by biologically driven processes involving microorganisms (biomineralization). Free-living and biofilm-forming bacteria within the nodules precipitate inorganic oxides on their surfaces, in reactions including the oxidation of soluble Mn (II) to insoluble Mn (III, IV) (Nealson *et al*., 1988; Francis *et al*., 2001a). In recent years many studies of microbial diversity in ocean nodule provinces and FeMn micronodules have been performed using culture-independent methods (Stein *et al*., 2001; Xu *et al*., 2005; Li *et al*., 2011; Tully and Heidelberg, 2013; Wu *et al*., 2013). However, knowledge of the microbial diversity associated with oceanic FeMn nodules is very limited.

The deep sea FeMn nodules in this study were collected from the base of a dormant hydrothermal vent at the Jiaolong seamount. Here, we report on culturable bacterial strains and the bacterial community associated with the nodules, based on a combination of culture-dependent and culture-independent analyses. This is the first reported investigation of the microbial diversity in deep sea FeMn nodules from the South China Sea.

Materials and Methods

Sample collection

Deep sea FeMn nodules were collected from a water depth of 3573 m at a dormant hydrothermal vent at the Jiaolong seamount (South China Sea) using the manned submersible 'Jiaolong'. The submersible was launched from the scientific exploration vessel 'Xiangyanghong 09', during an experimental application cruise on 3 July 2013 (Supplementary data Figs. S1 and S2). The temperature at the sampling station was approximately 3–4°C. The nodules were collected undamaged, and stored at -20°C until laboratory analysis. Samples taken from the nodule surface (0–3 mm) were dried at 105°C for 12 h, weighed, ground to a homogeneous powder, and compressed into powder pellets for elemental determination. The concentrations of MnO and Fe_2O_3 in the nodule samples were determined using inductively coupled plasma mass spectrometry (ICP-MS) (Wu *et al*., 2013).

Isolation, characterization and phylogenetic analysis of culturable bacteria

Samples of entire FeMn nodules were shaken for 15 min at 120 rpm in 90 ml of sterile 1% sodium pyrophosphate. Appropriate dilutions, prepared in sterile saline solution (0.9% NaCl), were plated onto Marine agar 2216 (Difco)

*For correspondence. E-mail: lixzh@qdio.ac.cn
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(0.5% peptone, 0.1% yeast extract, 0.01% Ferric citrate, 1.945% NaCl, 0.59% $MgCl_2$, 0.324% $MgSO_4$, 0.18% $CaCl_2$, 0.055% KCl, 0.016% Na_2CO_3, 0.008% KBr, 0.034% $SrCl_2$, 0.0022% H_3BO_3, 0.0004% Na_2SiO_3, 0.00024% NaF, 0.00016% NH_4NO_3, 0.0008% Na_2HPO_4, 1,000 ml distilled water, pH 7.6), Mn agar (0.2% peptone, 0.01% yeast extract, 0.01% KNO_3, 0.02% $MnCl_2{\cdot}4H_2O$, 1.5% agar, 1,000 ml aged seawater; pH 7) and iron bacteria medium [0.5% peptone, 0.02% $MgSO_4{\cdot}7H_2O$, 0.02% $(NH_4)_3Fe$ $(C_6H_5O_7)_2$, 0.005% $CaCl_2$, 0.001% $FeCl_3{\cdot}6H_2O$, 0.005% $MnSO_4{\cdot}H_2O$, 1.5% agar, 1,000 ml aged seawater, pH 7] and incubated at 25°C for one week. For the isolates obtained, growth under anaerobic conditions, the growth temperature range, enzyme production and susceptibility to antibiotics were determined as described previously (Zhang *et al*., 2013), except that R2A agar was replaced by Marine agar 2216. Salt tolerance was assessed at 25°C on plates containing 0.5% peptone, 0.1% yeast extract and 1.5% agar. Resistance to the heavy metal Mn^{2+} was determined in a medium containing 0.5% peptone, 0.1% yeast extract, 2% NaCl, 1.5% agar and Mn^{2+} (10–300 mM, supplied as $MnCl_2{\cdot}4H_2O$). The metal manganese was provided in a soluble and bioavailable form. Isolates were subjected to 16S rRNA gene sequence analysis, as described previously (Zhang *et al*., 2012).

Sample DNA extraction

Extraction of DNA from the nodules was achieved using a modified phenol–chloroform extraction method (Zhou *et al*., 1996). Briefly, 1.5 g nodule powder was suspended in 150 µl EDTA (0.5 M, pH 8.0) and 500 µl lysis buffer (500 µl 1 M Tris-HCl, 200 µl 0.5 M EDTA, 1 ml 5 M NaCl, 5 ml 10% SDS, diluted to 50 ml to total volume and autoclaved), and 35 µl proteinase K solution (20 mg/ml) was added. The mixture was incubated at 55°C for 2 h in a shaking water bath to effect complete lysis, then 200 µl 5 M NaCl was added and the mixture was stored on ice for 5 min. The mixture was purified by extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and centrifugation (13,000 rpm for 10 min), then extraction with chloroform:isoamyl (24:1) and recentrifugation.

Nucleic acids were precipitated from the solution by adding 1 volume of isopropanol, incubating at -20°C for 20 min, then centrifuging for 10 min at 13,000 rpm. After washing with 75% ethanol the precipitated DNA was purified by incubation at 37°C for 30 min in 100 µl TE solution to which 5 µl RNase A was added. The DNA extracts were run on 1% agarose gels and the DNA concentration was determined using a Qubit 2.0 Fluorometer.

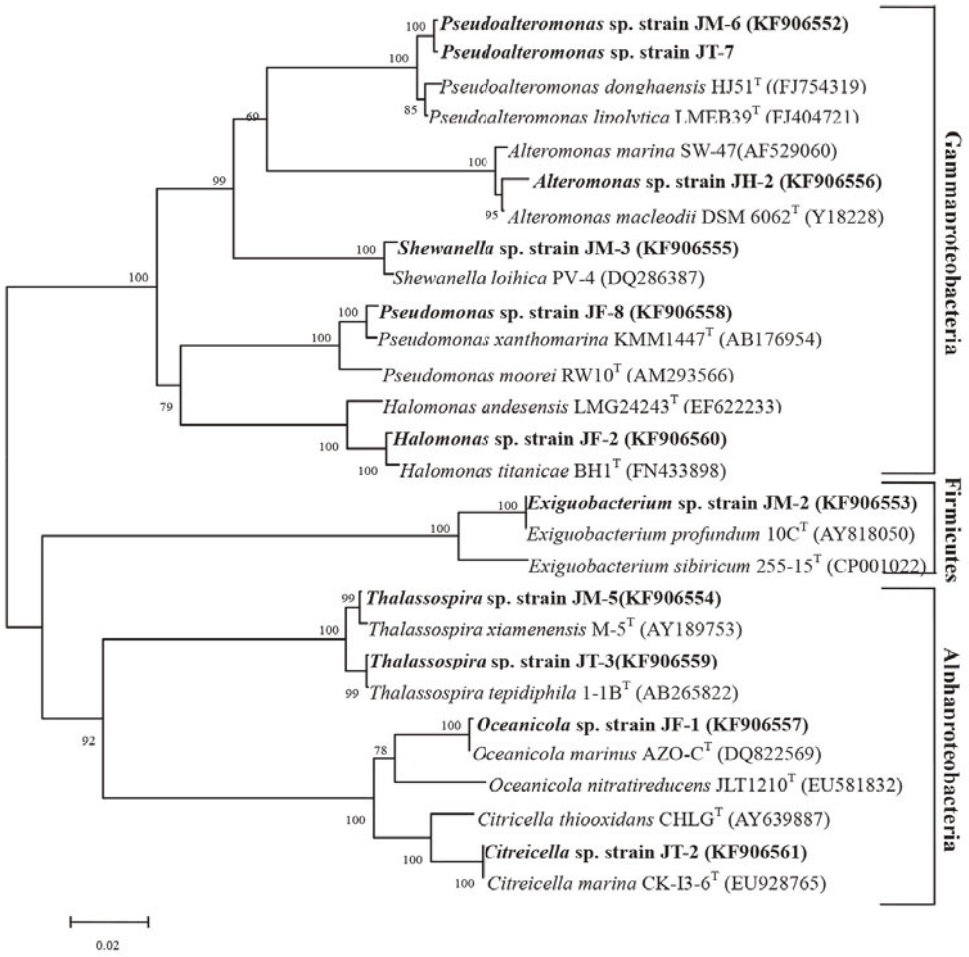

Fig. 1. Phylogenetic relationships of 11 culturable bacterial strains isolated in this study and related to the *Proteobacteria* and *Firmicutes*. The tree was inferred by neighbor-joining analysis. Bootstrap values (%) are based on 1,000 replicates and are shown for branches with more than 50% support. GenBank accession numbers of 16S rRNA sequences are given inparentheses. Bar, 0.02% sequence divergence. Strains isolated in this study are indicated in bold.

16S rRNA gene clone library and phylogenetic analysis

Bacterial 16S rRNA genes were amplified by PCR using two universal primers (Zhang *et al.*, 2006). The PCR reagents and their final concentrations in a 50 µl reaction mixture were: 1.5 U of Taq polymerase, 1× PCR buffer, 0.2 mM of each deoxynucleoside triphosphate, 0.3 µl bovine serum albumin (10 mg/ml), 10 µM of each primer, and 5 µl of DNA extract. The PCR conditions, construction of the 16S rRNA gene clone library, and the restriction fragment length polymorphism (RFLP) analysis were performed as described previously (Zhang *et al.*, 2012). Sequencing reactions were carried out at Chinese Sangon Biotech (China).

Sequences were checked for chimeras using the CHECK_CHIMERA software of the Ribosomal Database Project II, and cross-checked using the Pintail program (Maidak *et al.*, 2001). Sequences having > 98% similarity and matching the same GenBank sequence were assigned to the same phylotype. Sequences that demonstrated strong homology were then aligned to reference sequences using Clustal X1.8, and phylogenetic analysis was performed as described previously (Margesin and Zhang, 2013).

Barcoded 454 pyrosequencing of the 16S rRNA gene, and data analysis

Sequencing using an Illumina MiSeq platform was performed as described by Caporaso *et al.* (2012). PCR amplifications were conducted using the 515f/806r primer set, which amplifies the V4 region of 16S rRNA gene. This primer set was selected as it exhibited few biases and was expected to yield accurate phylogenetic and taxonomic information. The DNA was amplified following the protocol described by Caporaso *et al.* (2011). The full paired-end sequence reads were 245 nt in length, following removal of primers and barcodes.

Pairs of reads from the original DNA fragments were merged using FLASH (Magoč and Salzberg, 2011). Sequences were analyzed using the QIIME software package (Caporaso *et al.*, 2010). The reads were first filtered using QIIME quality filters, then a workflow (pick_de_novo_otus.py) was used to pick operational taxonomic units (OTUs). Sequences were assigned to OTUs at 97% similarity. Sequences representative of each OTU were selected, and the RDP classifier (Wang *et al.*, 2007) was used to assign taxonomic data to each representative sequence.

Nucleotide sequence accession numbers

All sequences generated for the bacterial isolates in this study were deposited in the GenBank database (accession numbers KF906552–KF906562). Accession numbers for the bacteria-related clones in the library were KF906563–KF906601. Complete datasets for the barcoded pyrosequencing of the 16S rRNA gene were submitted to the NCBI Short Read Archive under accession number SRR1279009.

Results

Characterization of culturable bacterial diversity

The majority of the strains (10/11; 91%) belonged to the

Table 1. Properties of 11 bacterial strains isolated from the deep-sea FeMn nodules. +, positive; -, negative; w, weak.

Strain properties	*Pseudoalteromonas* sp. JM-6	*Pseudoalteromonas* sp. JT-7	*Alteromonas* sp. JH-2	*Shewanella* sp. JM-3	*Pseudomonas* sp. JF-8	*Halomonas* sp. JF-2	*Exiguobacterium* sp. JM-2	*Thalassospira* sp. JM-5	*Thalassospira* sp. JT-3	*Oceanicola* sp. JF-1	*Citreicella* sp. JT-2
Growth temperature range (°C)	4–50	4–50	4–45	4–45	4–55	4–50	4–53	4–55	4–55	4–50	4–50
Resistance to antibiotics (30 µg/ml):											
Rifampicin	-	-	-	-	+	+	-	+	-	-	-
Ampicillin	-	-	-	+	+	+	-	+	-	+	-
Kanamycin	+	+	-	-	-	-	+	+	-	-	-
Tetracycline	+	+	-	-	+	+	-	+	+	+	-
Chloramphenicol	-	-	-	-	+	-	-	+	-	-	-
Resistance to Mn^{2+}:											
100 mM	+	+	+	+	+	+	+	+	+	+	+
150 mM	+	+	-	w	+	+	+	w	+	w	+
200 mM	w	w	-	-	+	+	+	-	-	-	+
Enzymatic activity:											
Protease	+	+	-	+	-	-	-	-	-	-	-
Amylase	-	-	+	-	+	-	+	-	-	+	-

phylum *Proteobacteria*, with a predominance of the classes *Alphaproteobacteria* and *Gammaproteobacteria*. Only one representative of the *Firmicutes* (1/11) was present (Fig. 1). Based on phenotypic characteristics and 16S rRNA gene sequencing, the 11 strains were assigned to nine genera: *Alteromonas*, *Pseudoalteromonas*, *Shewanella*, *Pseudomonas*, *Halomonas*, *Thalassospira*, *Oceanicola*, *Citreicella*, and *Exiguobacterium*. The nearest phylogenetic neighbors of three strains (JM-2, JM-3, and JF-2) had been isolated from hydrothermal habitats including a deep sea hydrothermal vent on the East Pacific Rise (Crapart *et al*., 2007), a hydrothermal vent of the Loihi seamount in the Pacific Ocean (Gao *et al*., 2006), and hydrothermal plumes in north and south Pacific Ocean vent fields (Kaye *et al*., 2004).

The properties of the 11 bacterial isolates are shown in Table 1. All could grow on Marine agar 2216 at 4–45°C, and some were moderately thermophilic; the upper temperature limit for growth was 55°C. All isolates had high Mn^{2+} tolerance (100 mM), with *E. profundum* JM-2 exhibiting the highest tolerance (220 mM).

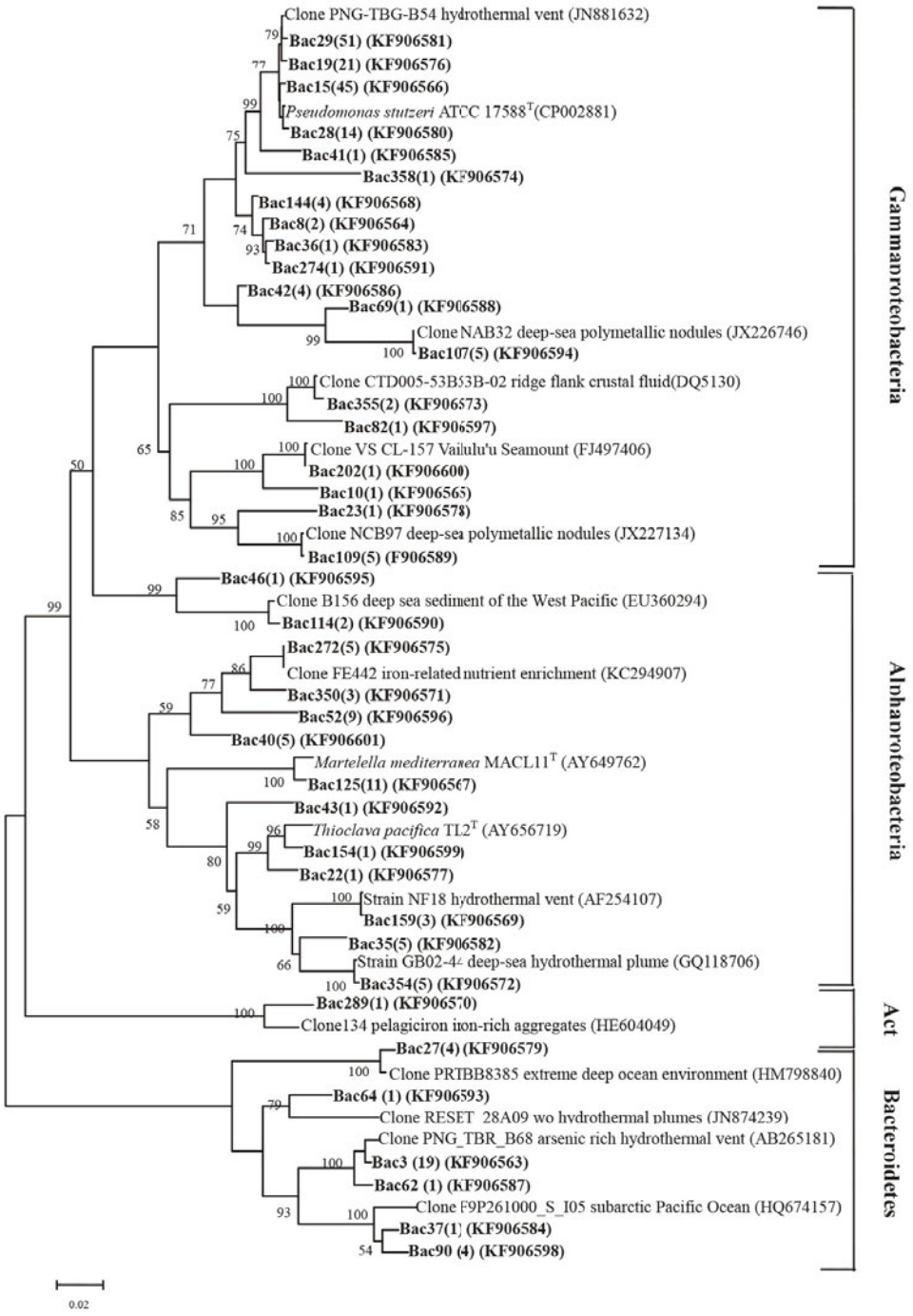

Fig. 2. Phylogenetic relationships of bacterial clone sequences and environmental 16S rRNA sequences related to the *Gammaproteobacteria*, *Alphaproteobacteria*, *Actinobacteria* (Act), and *Bacteroidetes*. The tree was inferred by neighbor-joining analysis. Bootstrap values (%) are based on 1,000 replicates and are shown for branches with more than 50% support. GenBank accession numbers of 16S rRNA sequences are given in parentheses. Bar, 0.02% sequence divergence. Clone sequences with their prevalence (in brackets) in the clone library are indicated in bold.

Tests to determine bacterial enzyme production showed protease production by isolates JM-6, JT-7, and JM-3, which were affiliated to *Pseudoalteromonas* and *Shewanella*. Isolates JH-2, JF-8, JM-2, and JF-1 produced amylase.

Diversity of bacterial 16S rRNA gene sequences in the clone library

A total of 259 bacterial 16S rRNA gene sequences were retrieved and grouped into 39 phylotypes (Fig. 2). The bacterial clone library represented an estimated coverage of 95.7%. The 39 bacterial phylotypes could be divided into three phyla: *Proteobacteria* (225 clones; 87%), *Bacteroidetes* (30 clones; 12%), and *Actinobacteria* (4 clones; 1%). *Proteobacteria* dominated the bacterial clone library, and comprised 32 phylotypes representing the classes *Alphaproteobacteria* and *Gammaproteobacteria*. *Gammaproteobacteria* showed the greatest diversity among the *Proteobacteria* (19 phylotypes). The dominance of *Gammaproteobacteria* (168 clones; 65%) was largely because of the high proportions of phylotypes Bac29 (51 clones; 20%), Bac15 (45 clones; 17%), Bac19 (21 clones; 8%), and Bac28 (14 clones; 5%). *Bacteroidetes* was the second largest non-*Proteobacteria* group detected, comprising 30 clones that accounted for 12% of the bacterial community.

Pyrosequencing and sequence analysis

A total of 67,079 valid reads and 6912 operational taxonomic units (OTUs) were obtained using the QIIME software package. The sequences were classified to 13 phyla or groups using the UCLUST program. *Proteobacteria* (74%, relative abundance) and *Bacteroidetes* (23%) were the two most dominant phyla. *Crenarchaeota* (0.01%) was the only *Archaea* phylum detected in the FeMn nodules. *Gammaproteobacteria* comprised the highest percentage in the phylum *Proteobacteria*, most of which belonged to the genera *Pseudomonas* (39% of the total reads) and *Alteromonas* (32%).

Discussion

The percentages of MnO and Fe_2O_3 in the FeMn nodules in the present study were 40.0% and 9.8%, respectively. Nodules are typically composed of the transition metals Mn (20–30%) and Fe (5–25%) (Wang and Müller, 2009). The percentage of MnO found (40%) is higher than usually reported for FeMn nodules and the sediments in deep sea areas where nodules occur (Li *et al.*, 2011; Wu *et al.*, 2013). Prior to this study there had been no reports of microbial diversity associated with FeMn nodules in the South China Sea.

The culturable fraction of the microbial community provided the basis for further studies considering the biomineralization process, in which a living organism governs the precipitation of elements and/or ions from the deep sea chemical environment. The 11 deep sea isolates cultured in this study belonged to nine genera. They were found to have high Mn^{2+} tolerance (100 mM), with *Exiguobacterium* sp. strain JM-2 having the greatest resistance to Mn^{2+} (220 mM). Microorganisms resistant to Mn^{2+} may be involved in the oxidation of soluble Mn^{2+} during the formation FeMn nodules. The resistance to Mn^{2+} is possibly associated with genes encoding multi-copper oxidases required for Mn (II) oxidation (Francis and Tebo, 2001), which were found in the genome sequence of *Exiguobacterium* sp. strain JM-2 with 2998 open reading frames (ORFs; unpublished data). In addition, a novel plasmid (15.57 kb, 19 ORFs) was isolated from this strain. Interesting, the moderately thermophilic strains JM-2, JF-8, JT-3, and JM-5 could also grow at 4–5°C, which was the temperature at the sampling station.

It is a widely accepted fact that the majority of microbes in the environment remain uncultivated, and direct recovery of bacterial 16S rDNA theoretically represents the entire microbial community. Although 16S rDNA gene-based microbial community analysis can survey higher capacity than culture-based survey, it is also well known that even deep sequencing often fail to sample entire community due to potentially dominating "rare biosphere" (Lynch and Neufeld, 2015). Molecular cloning libraries can provide detailed information on single clone sequences, because near full-length bacterial 16S rRNA gene sequence can be obtained, but 454 pyrosequencing data provides more comprehensive information. Thus, combining a clone library and pyrosequencing analysis in this study provided the most detailed analysis of the microbial community composition associated with nodules.

Bacterial phylotypes in the library tended to cluster with uncultured deep sea and hydrothermal vent clones. Phylotypes in the *Proteobacteria* were most abundant, and many (Bac29, Bac107, Bac109, Bac114, Bac159, and Bac354) were related to uncultivated environmental clones recovered from a variety of sources including an arsenic rich and shallow-sea hydrothermal vent (Meyer-Dombard *et al.*, 2013), the Galapagos Rift hydrothermal vent (Peña *et al.*, 2013), a cobalt-rich crust deposit region in the Pacific Ocean (Li *et al.*, 2011), deep sea sediment of the west Pacific Ocean (Wang *et al.*, 2008), a Galapagos hydrothermal vent (Teske *et al.*, 2000) and the Guaymas Basin hydrothermal plume (Dick and Tebo, 2010). Phylotypes in the *Bacteroidetes* were the second largest group in the library. Phylotype Bac27 was 98.9% identical to clone PRTBB8385 from an extreme deep ocean environment (Eloe *et al.*, 2011); phylotype Bac64 had a sequence that was similar to that obtained from hydrothermal plumes 115 m southwest of Ty/Iovents (East Pacific Rise; Sylvan *et al.*, 2012); and phylotype Bac289 was related to clone 134, from northern basin redoxcline iron-rich aggregates ('iron snow') associated with acidic lignite mine lake 77 (Liu *et al.*, 2013).

Manganese-oxidizing bacteria are abundant and widely distributed in the environment including in ferromanganese deposits, stratified soils and the Indian ridge system basalt (Sujith *et al.*, 2014). Possible evidence for manganese oxidation in FeMn nodules in this study came from phylotypes in the clone library and OTUs derived from pyrosequencing, which were related to manganese-oxidizing bacteria. The majority of taxa identified by pyrosequencing belonged to the genus *Pseudomonas* (39%), and most were related to *P. stutzeri* and *P. alcaligenes*. *Pseudomonas* strains readily form biofilms and have the ability to oxidize Mn (II), especially the well-studied strains *P. putida* GB-1 and *P. putida* MnB1. These have long been used as model organisms for the study of Mn (II) oxidation, with studies involving *P. putida* MnB1

dating to the 1970s (Geszvain *et al*., 2013). In this study, four bacterial phylotypes (Bac29, Bac19, Bac15, and Bac28) comprising 131 clones in the clone library had 96.7–97.5% sequence similarity to *P. putida*. Other potential manganese-oxidizing bacteria detected at lower frequencies using pyrosequencing included *Hyphomicrobium* spp. (0.07% of reads), *Exiguobacterium* spp. (0.05%), *Alcanivorax* spp. (0.04%), *Cupriavidus* spp. (0.01%), and *Deinococcus* spp. (0.01%). *Hyphomicrobium* has been reported to be involved in the oxidation and deposition of manganese, and has been found to be abundant in manganese deposits from numerous locations worldwide (Tyler, 1970). Clones belonging to *Pseudomonas* and *Hyphomicrobium* have been detected in the Clarion-Clipperton zone in the eastern Pacific Ocean (Wang *et al*., 2010), freshwater ferromanganous micronodules and sediments (Stein *et al*., 2001), and the cobalt-rich crust deposit region in the Pacific Ocean (Li *et al*., 2011). Sujith *et al*. (2014) reported that Mn-oxidizing bacteria *Exiguobacterium* spp. and *Alcanivorax* spp. isolated from Indian ridge system basalt having Mn-oxide coatings were involved in the mobilization of Mn. *Deinococcus radiodurans* has evolved a very efficient manganese regulation mechanism that involves a high intracellular Mn/Fe ratio, and confers resistance to extreme conditions (Sun *et al*., 2012).

Iron-reducing bacteria exert significant control on iron migration and biomineralization, and biogeochemical cycling. Iron-reducing bacteria broadly inhabit deep sea environments and often co-occur with Mn (II)-oxidizing bacteria in communities associated with ferromanganous micronodules (Stein *et al*., 2001). The second most abundant group by pyrosequencing was *Alteromonas* (32%). *Alteromonas putrefaciens* was the first organism reported to grow with hydrogen as an electron donor and Mn (IV) as an electron acceptor, and the first organism reported to couple the oxidation of formate to the reduction of Fe (III) or Mn (IV) (Lovley *et al*., 1989). Many sequences detected by pyrosequencing were related to the iron-reducing bacteria *Acidovorax* spp. (3.1% of reads), *Stenotrophomonas* spp. (0.1%), and *Shewanella* spp. (0.2%). The iron-reducing activity of *Stenotrophomonas maltophilia* has been applied to phosphate removal from the returned liquor of a municipal wastewater treatment plant, and *S. maltophilia* BK was shown to be able to reduce Fe (III) to Fe (II) under anaerobic conditions using xenobiotics as sole sources of carbon (Ivanov *et al*., 2005). Close relatives of *Stenotrophomonas* were detected in the cobalt-rich crust deposit region in the Pacific Ocean (Li *et al*., 2011). *Shewanella piezotolerans* WP3^{T} was reported to be able to reduce ferrihydrite at pressures up to 50 MPa, and may be extensively involved in iron biogeochemical cycling in deep sea environments (Xiao *et al*., 2007).

Aerobic hydrogen-oxidizing bacteria, which can grow autotrophically using hydrogen as an electron donor and oxygen as the terminal electron acceptor, are widely distributed in marine environments, geothermal regions and oligotrophic environments (Yoon *et al*., 2008). *Hydrogenophaga* strains in the family *Comamonadaceae* are neutrophilic and well-known hydrogen oxidizers. OTUs closely related to hydrogen-oxidizing *Hydrogenophaga* were detected at low abundance (0.14%) by pyrosequencing, indicating that the nodule microbial communities could partially be driven by hydrogen.

Methane is a major C1 compound whose metabolism forms an important part of the global carbon cycle. Methylotrophic bacteria play an important role in maintaining the balance of C_1 compounds in ocean environments (Chistoserdova, 2011). The FeMn nodules in this study had relatively low abundances of *Methylotenera* (0.6%), in the family *Methylophilaceae*, and *Methyloversatilis* (0.3%) in the family *Rhodocyclaceae*. The recently reported genome sequence of *Methyloversatilis universalis* strain FAM5, the first cultivated methylotrophic member of the order *Rhodocyclales*, provides a framework for analysis of the evolution of the methylotrophic ability (Kittichotirat *et al*., 2011).

Members of the *Archaea* are a minor component of the microbial community in thin FeMn oxides (Santelli *et al*., 2008). The OTU recovered by pyrosequencing was related to the genus *Nitrosopumilus* (0.01%); this genus belongs to the *Thaumarchaeota*, which is a new phylum of the Archaea proposed by Brochier-Armanet *et al*. (2008). Chemolithoautotrophic ammonia-oxidizing *Thaumarchaeota* able to grow using ammonium as the energy source have been detected in Mn oxides coating old seamounts in the western Pacific Ocean (Könneke *et al*., 2005; Nitahara *et al*., 2011). *Thaumarchaeota* have also been found in FeMn nodules and adjacent sediments in the central South Pacific Gyre (SPG), where FeMn nodules can comprise up to 70% of the exposed surface sediment. The *Thaumarchaea* are among the most abundant groups of organisms on the planet, and all members are believed to be capable of the first step of nitrification (ammonia oxidation) and carbon fixation (Hatzenpichler, 2012; Tully *et al*., 2012; Tully and Heidelberg, 2013). The presence of *Thaumarchaea* in energy-limited SPG environments suggests the possibility of a food web supported by ammonia oxidation and carbon fixation (Tully and Heidelberg, 2013).

Conclusions

This study provided novel information regarding the bacterial diversity associated with FeMn nodules from the South China Sea, and supports our hypotheses that FeMn nodules represent unique microhabitats, and that bacterial communities may contribute to metal cycling and the production of FeMn deposits.

The strains isolated from the FeMn nodules were found to be able to grow at 4–45°C and the upper temperature limit for growth was 55°C. All isolates were resistant to a very high Mn^{2+} concentration (100 mM), and *Exiguobacterium* sp. strain JM-2, which contained a novel plasmid (15.57 kb), had the greatest resistance to Mn^{2+} (220 mM). Interesting, some of the moderately thermophilic isolates could also grow at 4–5°C, which was the temperature at the sampling station. The results show that the microorganisms associated with deep sea FeMn nodules may have evolved mechanisms for adaptation to diverse thermal regimes and environments characterized by heavy metal stress.

Most of the DNA sequences recovered from the FeMn nodules tended to cluster with uncultured deep sea and hydrothermal vent clones, rather than isolates from established groups in other environments. These results are consistent

with the fact that the FeMn nodules were collected from the bottom of a dormant hydrothermal vent at the Jiaolong seamount. We found that *Gammaproteobacteria* were dominant, and the most abundant bacterial groups were *Pseudomonas* and *Alteromonas*. Similar results were reported in previous studies of microbial communities in deep sea polymetallic nodules, and in the sediments in deep sea nodule areas (Xu *et al*., 2005; Tully and Heidelberg, 2013; Wu *et al*., 2013).

Some bacteria were potentially related to metal (e.g. Mn and Fe) cycling, which is an important process in the formation and growth of polymetallic nodules. Sequence analysis showed the presence of many organisms whose closest relatives are known manganese oxidizers (*Pseudomonas*, *Exiguobacterium*, *Alcanivorax*, *Cupriavidus*, *Hyphomicrobium*, and *Deinococcus*), iron reducers (*Alteromonas*, *Stenotrophomonas*, *Acidovorax*, and *Shewanella*), hydrogen oxidizers (*Hydrogenophaga*), methylotrophs (*Methylotenera*, *Methyloversatilis*, and *Methylotenera*) and *Thaumarchaea* (*Nitrosopumilus*). Our results suggest that manganese-oxidizing bacteria may occur together with iron-reducing bacteria in FeMn nodules. Bacteria possibly involved in the cycling of hydrogen and C1 compounds were also detected. The ammonia-oxidizing *Archaea* detected may play a role as primary producers in the microbial ecosystem of hydrogenetic FeMn crusts. The discovery of some moderately thermophilic bacteria having high Mn^{2+} tolerance provides a starting point for further research into the role these microbes play in FeMn nodule formation.

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References

Brochier-Armanet, C., Boussau, B., Gribaldo, S., and Forterre, P. 2008. Mesophilic *crenarchaeota*: Proposal for a third archaeal phylum, the *Thaumarchaeota*. *Nat. Rev. Microbiol.* **6**, 245–252.

Calvert, S.E. 1978. Iron-manganese nodules, Sedimentology, Encyclopedia of Earth Science, Hutchinson & Ross, Inc., Dowden, PA, USA.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., *et al.* 2010. QIIME allows analysis of high-throughput community sequencing data. *Nat. Meth.* **7**, 335–336.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., *et al.* 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* **6**, 1621–1624.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N., and Knight, R. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. USA* **108**, 4516–4522.

Chistoserdova, L. 2011 Methylotrophy in a lake: from metagenomics to single-organism physiology. *Appl. Environ. Microbiol.* **77**, 4705–4711.

Crapart, S., Fardeau, M.L., Cayol, J.L., Thomas, P., Sery, C., Ollivier, B., and Combet-Blanc, Y. 2007. *Exiguobacterium profundum* sp. nov., a moderately thermophilic, lactic acid-producing bacterium isolated from a deep sea hydrothermal vent. *Int. J. Syst. Evol. Microbiol.* **57**, 287–292.

Dick, G.J. and Tebo, B.M. 2010. Microbial diversity and biogeochemistry of the Guaymas Basin deep sea hydrothermal plume. *Environ. Microbiol.* **12**, 1334–1347.

Eloe, E.A., Shulse, C.N., Fadrosh, D.W., Williamson, S.J., Allen, E.E., and Bartlett, D.H. 2011. Compositional differences in particle-associated and free-living microbial assemblages from an extreme deep-ocean environment. *Environ. Microbiol.* **3**, 449–458.

Francis, C.A., Co, E.M., and Tebo, B.M. 2001a. Enzymatic manganese (II) oxidation by a marine alpha-proteobacterium. *Appl. Environ. Microbiol.* **67**, 4024–4029.

Francis, C.A. and Tebo, B.M. 2001b. Multicopper oxidase genes from diverse Mn(II)-oxidizing and non-Mn(II)-oxidizing *Pseudomonas* strains. *Appl. Environ. Microbiol.* **67**, 4272–4278.

Gao, H., Obraztova, A., Stewart, N., Popa, R., Fredrickson, J.K., Tiedje, J.M., Nealson, K.H., and Zhou, J. 2006. *Shewanella loihica* sp. nov., isolated from iron-rich microbial mats in the Pacific Ocean. *Int. J. Syst. Evol. Microbiol.* **56**, 1911–1916.

Geszvain, K., McCarthy, J.K., and Tebo, B.M. 2013. Elimination of manganese (II, III) oxidation in *Pseudomonas putida* GB-1 by a double knockout of two putative multicopper oxidase genes. *Appl. Environ. Microbiol.* **79**, 357–366.

Hatzenpichler, R. 2012. Diversity, physiology and niche differentiation of ammonia-oxidizing archaea. *Appl. Environ. Microbiol.* **78**, 7501–7510.

Ivanov, V., Stabnikov, V., Zhuang, W.Q., Tay, J.H., and Tay, S.T.L. 2005. Phosphate removal from the returned liquor of municipal wastewater treatment plant using iron-reducing bacteria. *J. Appl. Microbiol.* **98**, 1152–1161.

Jauhari, P. and Pattan, J.N. 2000. Ferromanganese nodules from the central Indian Ocean basin, pp. 171–195. *In* Cronan, D.S. (ed.) Handbook of marine mineral deposits. CRC, Boca Raton, FL, USA.

Kaye, J.Z., Márquez, M.C., Ventosa, A., and Baross, J.A. 2004. *Halomonas neptunia* sp. nov., *Halomonas sulfidaeris* sp. nov., *Halomonas axialensis* sp. nov. and *Halomonas hydrothermalis* sp. nov.: halophilic bacteria isolated from deep sea hydrothermal-vent environments. *Int. J. Syst. Evol. Microbiol.* **54**, 499–511.

Kittichotirat, W., Good, N.M., Hall, R., Bringe, F., Lajus, A., Médigue, C., Smalley, N.E., Beck, D., Bumgarner, R., Vuilleumier, S., *et al.* 2011.Genome sequence of *Methyloversatilis universalis* FAM5^{T}, a methylotrophic representative of the order rhodocyclales. *J. Bacteriol.* **193**, 4541–4542.

Könneke, M., Bernhard, A.E., de la Torre, J.R., Walker, C.B., Waterbury, J.B., and Stahl, D.A. 2005. Isolation of an autotrophic ammoniaoxidizing marine archaeon. *Nature* **437**, 543–546.

Li, L., Xu, X.W., Jiang, X.W., Wang, C.S., Zhang, D.S., Ni, J.Y., and Wu, M. 2011. Microbial diversity in deep sea sediment from the cobalt-rich crust deposit region in the Pacific Ocean. *FEMS Microbiol. Ecol.* **78**, 565–685.

Liu, S., Chourey, K., Reiche, M., Nietzsche, S., Shah, M.B., Neu, T.R., Hettich, R.L., and Kusel, K. 2013. Insights into the structure and metabolic function of microbes that shape pelagic iron-rich aggregates ('iron snow'). *Appl. Environ. Microbiol.* **79**, 4272–4281.

Lovley, D.R., Phillips, E.J., and Lonergan, D.J. 1989. Hydrogen and formate oxidation coupled to dissimilatory reduction of iron or manganese by *Alteromonas putrefaciens*. *Appl. Environ. Microbiol.* **55**, 700–706.

Lynch, M.D. and Neufeld, J.D. 2015. Ecology and exploration of the rare biosphere. *Nat. Rev. Microbiol.* **13**, 217–229.

Magoč, T. and Salzberg, S.L. 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* **27**, 2957–2963.

Maidak, B.L., Cole, J.R., Lilburn, T.G., Parker, C.T., Saxman, P.R., Farris, R.J., Garrity, G.M., Olsen, G.J., Schmidt, T.M., and Tiedje, J.M. 2001. The RDP-II (Ribosomal Database Project). *Nucleic Acids Res.* **29**, 173–174.

Margesin, R. and Zhang, D.C. 2013. *Humitalea rosea* gen. nov., sp. nov., an aerobic bacteriochlorophyll-containing bacterium of the family Acetobacteraceae isolated from soil. *Int. J. Syst. Evol. Microbiol.* **63**, 1411–1461.

Meyer-Dombard, D.R., Amend, J.P., and Osburn, M.R. 2013. Microbial diversity and potential for arsenic and iron biogeochemical cycling at an arsenic rich, shallow-sea hydrothermal vent (Tutum Bay, Papua New Guinea). *Chem. Geol.* **348**, 37–47.

Nealson, K.H., Tebo, B.M., and Rosson, R.A. 1988. Occurrence and mechanisms of microbial oxidation of manganese. *Adv. Appl. Microbiol.* **33**, 279–318.

Nitahara, S., Kato, S., Urabe, T., Usui, A., and Yamagishi, A. 2011. Molecular characterization of the microbial community in hydrogenetic ferromanganese crusts of the Takuyo-Daigo Seamount, northwest Pacific. *FEMS Microbiol.* **321**, 121–129.

Peña, A., Busquets, A., Gomila, M., Mayol, J., Bosch, R., Nogales, B., García-Valdés, E., Bennasar, A., and Lalucat, J. 2013. Draft genome of *Pseudomonas stutzeri* strain NF13, a nitrogen fixer isolated from the galapagos rift hydrothermal vent. *Genome Announc.* **1**, e0011313.

Santelli, C.M., Orcutt, B.N., Banning, E., Bach, W., Moyer, C.L., Sogin, M.L., Staudigel, H., and Edwards, K.J. 2008. Abundance and diversity of microbial life in ocean crust. *Nature* **453**, 653–656.

Stein, L.Y., La Duc, M.T., Grundl, T.J., and Nealson, K.H. 2001. Bacterial and archaeal populations associated with freshwater ferromanganous micronodules and sediments. *Environ. Microbiol.* **3**, 10–18.

Sujith, P.P., Mourya, B.S., Krishnamurthi, S., Meena, R.M., and Loka Bharathi, P.A. 2014. Mobilization of manganese by basalt associated Mn(II)-oxidizing bacteria from the Indian Ridge System. *Chemosphere* **95**, 486–495.

Sun, H., Li, M., Xu, G., Chen, H., Jiao, J., Tian, B., Wang, L., and Hua, Y. 2012. Regulation of MntH by a dual Mn(II)- and Fe(II)-dependent transcriptional repressor (DR2539) in *Deinococcus radiodurans*. *PLoS One* **7**, e35057.

Sylvan, J.B., Pyenson, B.C., Rouxel, O., German, C.R., and Edwards, K.J. 2012. Time-series analysis of two hydrothermal plumes at 9°50′N East Pacific Rise reveals distinct, heterogeneous bacterial populations. *Geobiology* **10**, 178–192.

Teske, A., Brinkhoff, T., Muyzer, G., Moser, D.P., Rethmeier, J., and Jannasch, H.W. 2000. Diversity of thiosulfate-oxidizing bacteria from marine sediments and hydrothermal vents. *Appl. Environ. Microbiol.* **66**, 3125–3133.

Tully, B.J. and Heidelberg, J.F. 2013. Microbial communities associated with ferromanganese nodules and the surrounding sediments. *Front Microbiol.* **4**, 1–10.

Tully, B., Nelson, W., and Heidelberg, J. 2012. Metagenomic analysis of a complex marine planktonic thaumarchaeal community from the Gulfof Maine. *Environ. Microbiol.* **14**, 254–267.

Tyler, P.A. 1970. *Hyphomicrobia* and the oxidation of manganesse in aquatic ecosystems. *Antonie van Leeuwenhoek* **36**, 567–578.

Xiao, X., Wang, P., Zeng, X., Bartlett, D.H., and Wang, F. 2007. *Shewanella psychrophila* sp. nov. and *Shewanella piezotolerans* sp. nov., isolated from west Pacific deep sea sediment. *Int. J. Syst. Evol. Microbiol.* **57**, 60–65.

Xu, M.X., Wang, P., Wang, F.P., and Xiao, X. 2005. Microbial diversity at a deep sea station of the Pacific Nodule Province. *Biodivers. Conserv.* **14**, 3363–3380.

Wang, Q., Garrity, G.M., Tiedje, J.M., and Cole, J.R. 2007 Naive bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* **73**, 5261–5267.

Wang, B., Lai, Q., Cui, Z., Tan, T., and Shao, Z. 2008. A pyrene-degrading consortium from deep sea sediment of the West Pacific and its key member *Cycloclasticus* sp. P1. *Environ. Microbiol.* **10**, 1948–1963.

Wang, C.S., Liao, L., Xu, H.X., Xu, X.W., Wu, M., and Zhu, L.Z. 2010. Bacterial diversity in the sediment from polymetallic nodule fields of the Clarion-Clipperton Fracture Zone. *J. Microbiol.* **48**, 573–585.

Wang, X.H. and Müller, W.E.G. 2009. Marine biominerals: perspectives and challenges for polymetallic nodules and crusts. *Trends Biotechnol.* **27**, 375–383.

Wang, X.H., Schloßmacher, U., Wiens, M., Schröder, H.C., and Müller, W.E.G. 2009. Biogenic origin of polymetallic nodules from the Clarion-Clipperton Zone in the Eastern Pacific Ocean: electron microscopic and EDX evidence. *Mar. Biotechnol.* **11**, 99–108.

Wu, Y.H., Liao, L., Wang, C.S., Ma, W.L., Meng, F.X., Wu, M., and Xu, X.W. 2013. A comparison of microbial communities in deep sea polymetallic nodules and the surrounding sediments in the Pacific Ocean. *Deep Sea Research I.* **79**, 40–49.

Yoon, K.S., Tsukada, N., Sakai, Y., Ishii, M., Igarashi, Y., and Nishihara, H. 2008. Isolation and characterization of a new facultatively autotrophic hydrogen-oxidizing *Betaproteobacterium*, *Hydrogenophaga* sp. AH-24. *FEMS Microbiol. Lett.* **278**, 94–100.

Zhang, D.C., Brouchkov, A., Griva, G., Schinner, F., and Margesin, R. 2013. Isolation and characterization of bacteria from ancient Siberian permafrost sediment. *Biology* **2**, 85–106.

Zhang, D.C., Mörtelmaier, C., and Margesin, R. 2012. Characterization of the bacterial and archaeal diversity in hydrocarbon-contaminated soil. *Sci. Total Environ.* **421-422**, 184–196.

Zhang, D.C., Wang, H.X., Liu, H.C., Dong, X.Z., and Zhou, P.J. 2006. *Flavobacterium glaciei* sp. nov., a novel psychrophilic bacterium isolated from the China No.1 glacier. *Int. J. Syst. Evol. Microbiol.* **56**, 2921–2925.

Zhou, J., Bruns, M.A., and Tiedje, J.M. 1996. DNA recovery from soils of diverse composition. *Appl. Environ. Microbiol.* **62**, 316–322.