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Metagenomic Analysis of the Microbial Community at Zodletone Spring (Oklahoma): Insights into the Genome of a Member of the Novel Candidate Division OD1†

Mostafa S. Elshahed,[1]* Fares Z. Najar,[2] Mandy Aycock,[2] Chunmei Qu, Bruce A. Roe,[2] and Lee R. Krumholz[1]

Department of Botany and Microbiology and Institute for Energy and the Environment[1] and Department of Chemistry and Biochemistry and the Advanced Center for Genome Technology,[2] University of Oklahoma, Norman, Oklahoma 73019

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A metagenomic library was constructed from the anaerobic sediments of a mesophilic sulfur spring. Thirty-five bacterial 16S rRNA gene-containing clones were identified in this library. Analysis of a genomic fragment belonging to candidate division OD1 provided useful insights into the physiology and biochemistry of this novel, yet-uncultured candidate division.

16S rRNA gene-based surveys clearly demonstrate that the scope of microbial diversity is much broader than implied by culture-dependent studies (12, 14). One of the most important challenges to microbial ecologists is to elucidate the physiological properties, energy conservation pathways, and ecological significance of recently discovered, yet-uncultured microorganisms. Although creative isolation strategies are bringing some of these "unculturable" microorganisms to pure cultures (3, 9, 18) or stable enrichments (7), the majority of novel bacterial lineages still evade isolation. An interesting alternative to isolation involves cloning and sequencing DNA directly from various ecosystems. This sequence-based analysis (metagenomics) allows an in silico investigation of various metabolic pathways utilized by novel microbial groups (8, 10, 15).

We are currently investigating the microbial diversity in Zodletone Spring, an anaerobic sulfide- and sulfur-rich spring in Oklahoma (4, 5, 11), with the goal of determining physiological features of uncultivated members of the microbial community. In this study, we report on the construction and screening of a metagenomic library from Zodletone Spring sediments and analysis of a 35.7-kb DNA fragment that belongs to candidate division OD1.

The spring location, geochemical characteristics, and microbial diversity have been previously documented (5, 17). DNA extracted from the spring source sediments was separated on 1% low-melting-point agarose using a field inversion gel electrophoresis box (MJ Research Inc, Watertown, MA). The DNA fraction greater than 30 kb was excised from the gel, and the DNA obtained was ligated into a CopyControl cloning vector, pCC1FOS (Epicenter Corp., Madison, WI), and transfected into *Escherichia coli* strain EPI300 according to the manufacturer's instructions.

Library screening for fosmids containing 16S rRNA genes was performed on pooled DNA from 384 clones, which had been treated with Plasmid-Safe ATP-dependent DNase (Epicenter) to minimize *E. coli* chromosomal DNA interference. Each pool was screened using the *Bacteria*-specific primer pairs 8F/805R (5) and 1054-16SF/21-23S R (6). The latter pair amplifies the intergenic spacer region along with approximately the last 500 bp of the 16S rRNA. 16S rRNA gene-containing fosmids within pools were located at the intersection of pooled plate rows and columns using additional sets of primers created to identify hypervariable regions within the target 16S rRNA gene. The detailed procedures for cloning, shotgun library construction, fluorescent-based DNA sequencing, and subsequent analysis were as described previously (1, 2, 13, 16).

Screening of a total of 19,200 clones resulted in the identification of 35 16S rRNA gene-containing fosmids (Table 1). The phylogenetic affiliations of these clones were in accordance with our previous studies regarding the importance of sulfur-transforming and anaerobic fermentative processes in Zodletone Spring source sediments (5). Fosmids with 16S rRNA gene sequences monophyletic with members of the chemolithotrophic sulfide-oxidizing genus *Acidithiobacillus* and the sulfur-respiring genus *Sulfurospirillum* were detected, as well as fosmids belonging to the order *Chromatiales* and to the δ-*Proteobacteria* that could putatively be involved in anoxygenic photosynthesis and sulfur (or sulfate) reduction, respectively. Clones belonging to anaerobic fermentative groups included members of the genus *Syntrophus*, class *Bacteroides*, and order *Clostridiales.* Finally, several fosmids were closely related to 16S rRNA sequences previously encountered in Zodletone Spring (5), including ZFos45e05, related to the Zodletone 16S rRNA clone ZB17, both of which are members of candidate division OD1 (Fig. 1).

Novel candidate division OD1 members are globally distributed in marine and terrestrial habitats and appear to be mainly present in anoxic environments (Fig. 1; see also Table S1 in the supplemental materials). Fosmid ZFos45e05, belonging to candidate division OD1, was sequenced and open reading frames characterized (Fig. 2 and Table 2; see also Table S2). The rRNA operon organization within ZFos45e05 was differ-

* Corresponding author. Mailing address: University of Oklahoma, Department of Botany and Microbiology 770 Van Vleet Oval, Norman, OK 73019. Phone: (405) 325-5255. Fax: (405) 325-7619. E-mail: Mostafa@ou.edu.

† Supplemental material for this article may be found at http://aem.asm.org/.

TABLE 1. Fosmids containing 16S rRNA genes identified in the library

Phylum	Fosmid name	Closest match (accession no.)	% Similarity	Affiliation
Proteobacteria				
α-*Proteobacteria*	ZFos25g11	*Azospirillum* sp. strain 5C (AF413109)	99	Genus *Azospirillum*
γ-*Proteobacteria*	ZFos3b04	*Acidithiobacillus albertensis* (AJ459804)	97	Genus *Acidithiobacillus*
	ZFos29d02	Heavy metal impacted soil clone KCM-B-83 (AJ581596)	94	Order *Chromatiales*
	ZFos32e11	*Acinetobacter* sp. strain ATCC 31012 (AF542963)	95	Genus *Acinetobacter*
	ZFos33d04	*Stenotrophomonas maltophilia* (DQ41193)	96	Genus *Stenotrophomonas*
	ZFos44D	*Psychrobacter frigidicola* (AJ609556)	92	Genus *Psychrobacter*
	ZFos39g11	*Acinetobacter* sp. strain Wuba16 (AF336348)	97	Genus *Acinetobacter*
δ-*Proteobacteria*	ZFos33i05	*Desulfobacterium cetonicum* (AJ237603)	87	δ-*Proteobacteria*
	ZFos14e13	*Syntrophus gentianae* (X85132)	95	Genus *Syntrophus*
	ZFos52C	*Syntrophus gentianae* (X85132)	97	Genus *Syntrophus*
ε-*Proteobacteria*	ZFos2f11	*Sulfurospirillum* sp. strain EK7 (AJ535704)	96	Genus *Sulfurospirillum*
	ZFos9d02	*Sulfurospirillum* sp. strain EK7 (AJ535704)	99	Genus *Sulfurospirillum*
Chloroflexi	ZFos9b11	Coastal marine sediment clone TIHP368-10 (AB031632)	93	Group V sediment *Chloroflexi*
	ZFos48f09	Coastal marine sediment clone TIHP368-10 (AB031632)	91	Group V sediment *Chloroflexi*
	ZFos40d07	Uranium waste pile clone Sh765B-TzT-6 (AJ519642)	91	Class *Dehalococcoidetes*
Bacteroidetes	ZFos25h14	Protein-degrading consortium clone BSA1B-13	97	Class *Bacteroides*
	ZFos44a23	Clinical isolate strain 47077 (AF227830)	94	Order *Sphingobacteriales*
	ZFos45e01	Bovine rumen clone p2b04ct-1 (AY578446)	99	Genus *Prevotella*
	ZFos45c09	Bovine rumen clone p1g07ct-1 (AY578417)	91	Class *Bacteroides*
Planctomyces	ZFos39a01	Salt marsh clone SIMO-1913 (AY711279)	89	Class *Planctomycetaceae*
Candidate division OD1	ZFos45e05	Japan trench clone BD7-4 (AB015580)	82	Candidate division OD1
Actinobacteria	ZFos12D	Dichloropropane dechloronating clone SHA-34 (AJ306762)	96	Family *Streptomycetaceae*
	ZFos29a01	*Arthrobacter gandensis* (AJ491108)	97	Genus *Arthrobacter*
	ZFos33B	Oil storage cavity clone KB20 (AB074931)	98	Family *Coriobacteriaceae*
	ZFos35D	Oil storage cavity clone KB20 (AB074931)	98	Family *Coriobacteriaceae*
*Firmicute*s	ZFos1A	Trichlorobenzene consortium clone SJA-143 (AJ009494)	92	Family *Acidaminococcaceae*
	ZFos19h03	*Anaerospora hongkongensis* (AY372051)	97	Family *Acidaminococcaceae*
	ZFos28B	*Sporomusa aerivorans* (AJ506192)	97	Genus *Sporomusa*
	ZFos31b07	Artesian basin clone R82 (AF407695)	94	Family *Clostridiaceae*
	ZFos39g02	Oil reservoir clone PL-35B11 (AY570624)	96	Family *Clostridiaceae*
	ZFos37b08	Uranium-contaminated sediment clone ph5Lac302-37 (AY527741)	94	Family *Acidaminococcaceae*
	ZFos40d08	*Sporomusa aerivorans* (AJ506192)	96	Genus *Sporomusa*
	ZFos44a01	*Butyrivibrio fibrisolvens* (X89980)	93	Family *Lachnospiraceae*
	ZFos46b02	*Anaerospora hongkongensis* (AY372051)	97	Family *Acidaminococcaceae*
	ZFos50a03	Bovine rumen clone p3a03ct-1 (AY578513)	93	Family *Lachnospiraceae*

ent from that observed within most bacterial genomes. The 16S rRNA gene appears to be separate from both the 23S and the 5S rRNA genes. Thirteen tRNA genes were identified around the 16S rRNA gene. Fosmid ZFos45e05 has an overall low G+C content (34.9%). The consistently low G+C content in all genes argues that this value is a true reflection of the G+C content of the entire genome of the microorganism. Out of 33 protein-coding open reading frames (ORFs), 13 had no orthologs in the database (Table 2). Apparent phylogenetic affiliation of the remaining 20 ORFs indicated that their most closely related orthologs were dispersed among the bacterial, archaeal, and eukaryotic phyla. Phylogenetic analysis of three putative housekeeping genes in ZFos45e05 [DNA polymerase 1 (ORF 1), tRNA (guanine-N1)-methyltransferase (ORF 10),

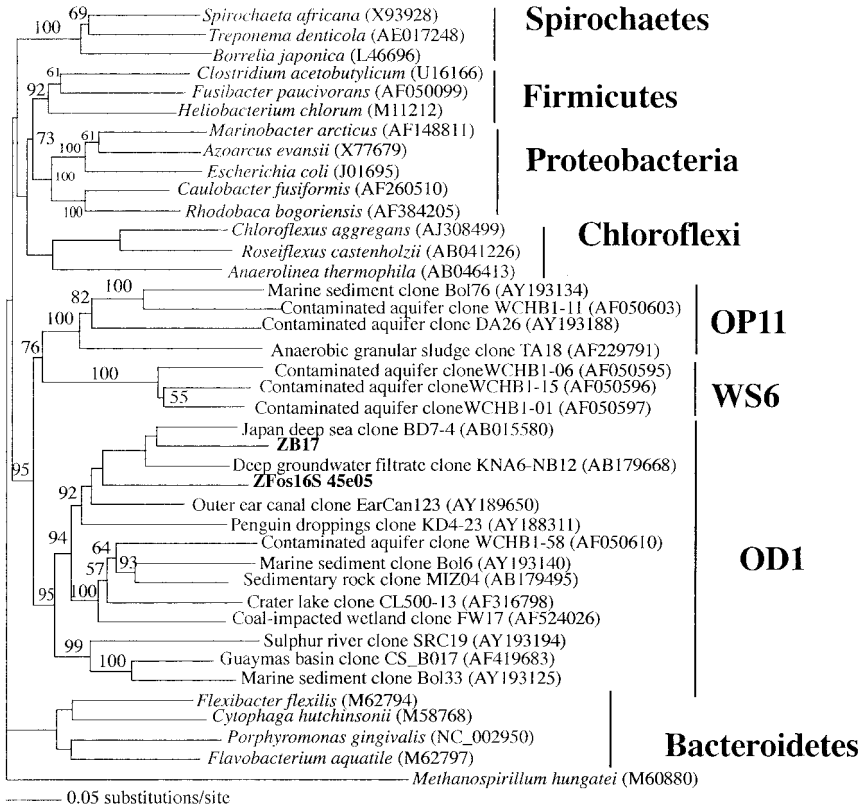

FIG. 1. Distance dendrogram representing the phylogenetic affiliation of selected members of the novel candidate division OD1, including the 16S rRNA gene from fosmid ZFos45e05 and 16S rRNA clone ZB17, previously encountered during a 16S rRNA gene survey of Zodletone Spring (5). The tree was constructed as previously described (4). A more detailed phylogenetic analysis, as well as a list of partial and complete OD1 16S rRNA clones identified in the GenBank database, is available in the supplemental materials (Fig. S1; Table S1).

and large ribosomal subunit protein L19 (ORF 32)] (Fig. 3) supported the hypothesis that candidate division OD1 is not closely related to any bacterial division with genome-sequenced representatives. The large sequence divergence which resulted in deep branching points to the potential genomic novelty of this yet-uncultured phylum of *Bacteria*.

Four genes in ZFos45e05 potentially encode enzymes involved in metabolic processes. These include two phosphoenolpyruvate synthase gene paralogs (ORFs 13 and 15), both of which have an archaeal affiliation based on BLASTp analysis. The other metabolism-related genes were pyruvate formate lyase-activating enzyme (ORF 23) and oxygen-sensitive ribonucleoside triphosphate reductase (ORF 24), both of which are present only in anaerobic or facultative anaerobic microorganisms (Table S2). The genomic organization in which the latter two genes are adjacent has also been observed in *Archaea* (e.g., fosmids belonging to anaerobic methane oxidizing microorganisms [8]), *Pyrococcus abyssi*, and *Pyrolobus fumarii*. This similar phylogeny and genomic organization might be indicative of ancient evolutionary traits or the occurrence of horizontal gene transfer between the aforementioned groups.

The small size of the sequenced OD1 fosmid coupled with our inability to detect more OD1 fosmids within the library renders information regarding this unique group of bacteria limited. However, this study provided several interesting ob-

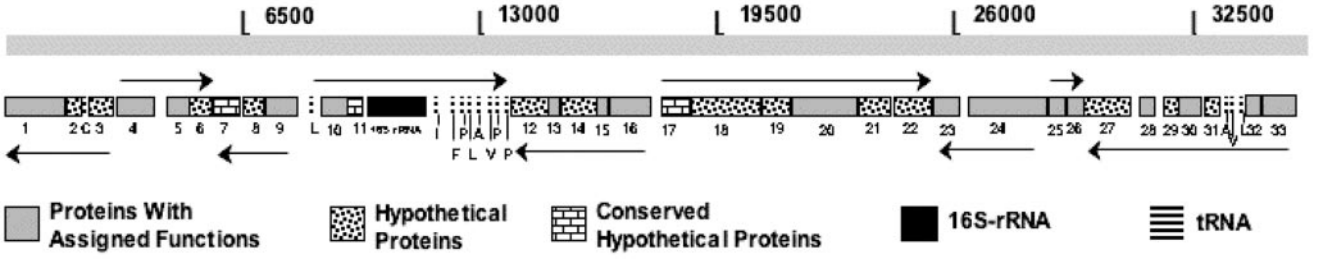

FIG. 2. Genomic map of OD1 fosmid ZFos45e05. ORFs are shaded according to their putative functions. More information regarding putative functions of ZFos45e05 ORFs is available in the supplemental materials (Table S2).

TABLE 2. General characteristics of ZFos45e05 OD1 fosmid

Characteristic	ZFos45e05
Length (bp)	35,743
G+C%	34.9
G+C% (coding regions)	40.4
rRNA operon	16S
No. of ORFs	33
No. of tRNA	13
Hypothetical proteins (unique)	13
Conserved hypothetical proteins (no. of hits to other hypothetical proteins)	3
Phylogenetic affiliations of most similar orthologs[a]	*Proteobacteria* (5), *Firmicutes* (3), *Aquifex* (1), *Fusobacteria* (1), *Actinobacteria* (1), *Chloroflexi* (1), *Archaea* (5), eukaryotes (3)

[a] Numbers in parentheses are numbers of ORFs within ZFos45e05 most closely similar to each lineage.

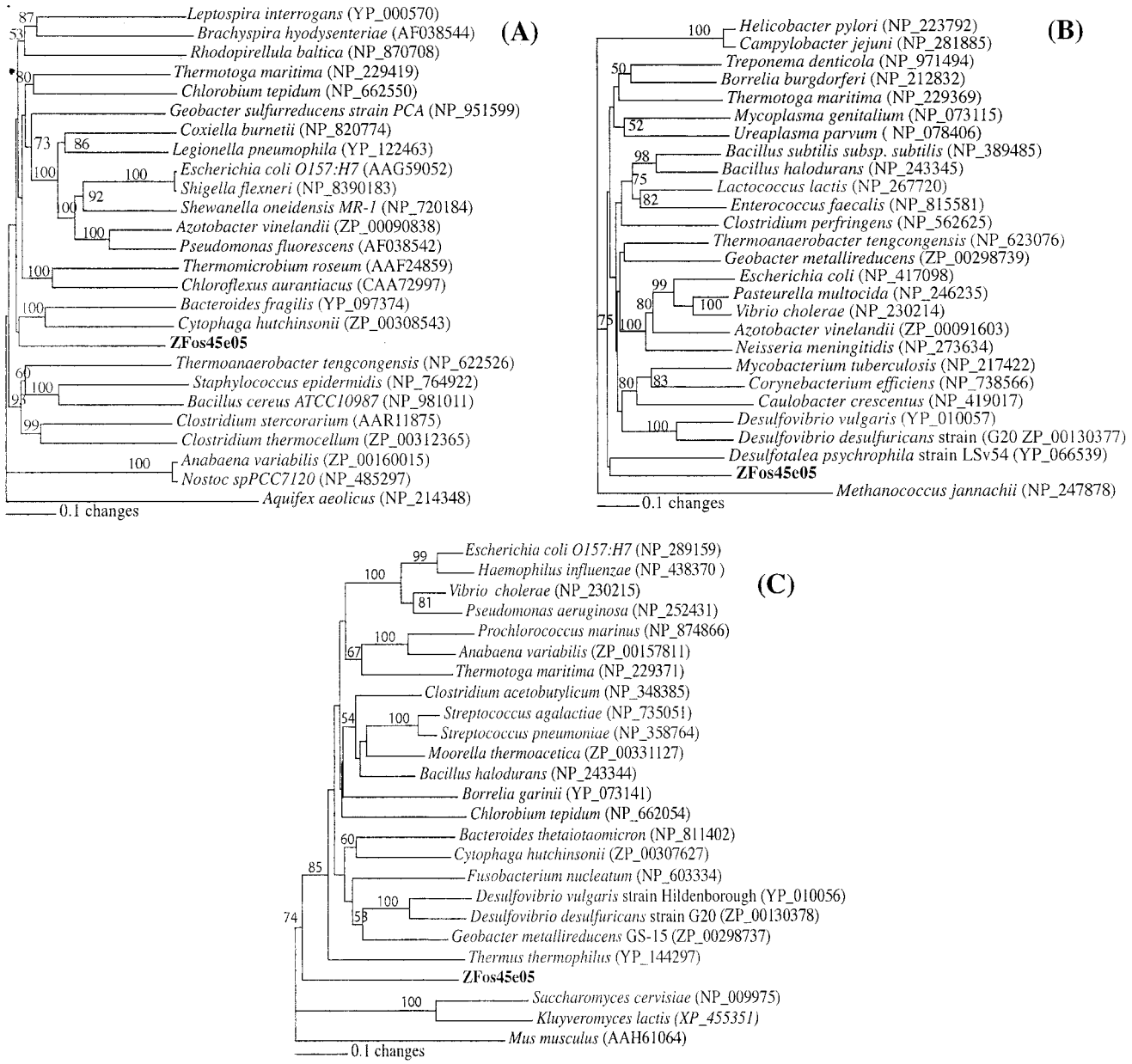

FIG. 3. Distance dendrogram evaluating the phylogenetic position of three protein-encoding ORFs from candidate division OD1 fosmid ZFos45e05 compared to their orthologs from previously sequenced bacterial genomes: DNA polymerase 1 (ORF 1) (A), tRNA (guanine-N1)-methyltransferase (ORF 10) (B), and large ribosomal subunit protein L19 (ORF 32) (C).

servations, including an unusual rRNA operon organization, low G+C content, low sequence similarity of OD1 putative gene products to their orthologs, the presence of genes encoding oxygen-sensitive enzymes, and an apparent archaeal affiliation and archaeal genomic organization of OD1 genes involved in metabolic processes. We are currently evaluating different strategies to locate more OD1 fosmids based on the information generated by sequencing ZFos45e05. This could aid in elucidating some of the metabolic pathways utilized by this bacterial division as well as in understanding its ecological significance in anaerobic ecosystems.

Nucleotide sequence accession numbers. Sequences obtained in this study have been deposited in GenBank under accession numbers DQ227583 to DQ227617 and AC160099.

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REFERENCES

1. **Bodenteich, A., S. L. Chissoe, Y. F. Wang, and B. A. Roe.** 1993. Shotgun cloning as the strategy of choice to generate templates for high-throughput dideoxynucleotide sequencing, p. 42–50. *In* J. C. Venter (ed.), Automated DNA sequencing and analysis techniques. Academic Press, London, United Kingdom.
2. **Chissoe, S. L., A. Bodenteich, Y. F. Wang, Y. P. Wang, S. W. Burian, C. Dennis, J. Crabtree, A. Freeman, K. Iyer, L. Jian, Y. Ma, H. J. McLaury, H. Q. Pan, O. Sharan, S. Toth, Z. Wong, G. Zhang, N. Heisterkamp, J. Groffen, and B. A. Roe.** 1995. Sequence and analysis of the human ABL gene, the BCR gene, and regions involved in the Philadelphia chromosomal translocation. Genomics **27:**67–82.
3. **Connon, S. A., and S. J. Giovannoni.** 2002. High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. Appl. Environ. Microbiol. **68:**3878–3885.
4. **Elshahed, M. S., F. Z. Najar, B. A. Roe, A. Oren, T. A. Dewers, and L. R. Krumholz.** 2004. Survey of archaeal diversity reveals an abundance of halophilic *Archaea* in a low-salt, sulfide- and sulfur-rich spring. Appl. Environ. Microbiol. **70:**2230–2239.
5. **Elshahed, M. S., J. M. Senko, F. Z. Najar, S. M. Kenton, B. A. Roe, T. A. Dewers, J. R. Spear, and L. R. Krumholz.** 2003. Bacterial diversity and sulfur cycling in a mesophilic sulfide-rich spring. Appl. Environ. Microbiol. **69:** 5609–5621.
6. **Garcia-Martinez, J., S. G. Acinas, A. I. Anton, and F. Rodriguez-Valera.** 1999. Use of the 16S–23S ribosomal genes spacer region in studies of prokaryotic diversity. J. Microbiol. Methods **36:**55–64.
7. **Girguis, P. R., V. J. Orphan, S. J. Hallam, and E. F. DeLong.** 2003. Growth and methane oxidation rates of anaerobic methanotrophic archaea in a continuous-flow bioreactor. Appl. Environ. Microbiol. **69:**5472–5482.
8. **Hallam, S. J., N. Putnam, C. M. Preston, J. C. Detter, D. Rokhsar, P. M. Richardson, and E. F. DeLong.** 2004. Reverse methanogenesis: testing the hypothesis with environmental genomics. Science **305:**1457–1462.
9. **Kaeberlein, T., K. Lewis, and S. S. Epstein.** 2002. Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. Science **269:**1127–1129.
10. **Liles, M. R., B. F. Manske, S. B. Bintrim, J. Handelsman, and R. M. Goodman.** 2003. A census of rRNA genes and linked genomic sequences within a soil metagenomic library. Appl. Environ. Microbiol. **69:**2684–2691.
11. **Luo, Q., L. R. Krumholz, F. Z. Najar, B. A. Roe, A. D. Peacock, D. C. White, and M. S. Elshahed.** 2005. Diversity of the microeukaryotic community in sulfide-rich Zodletone Spring. Appl. Environ. Microbiol. **71:**6175–6184.
12. **Pace, N. R.** 1997. A molecular view of microbial diversity and the biosphere. Science **276:**734–740.
13. **Pan, H. Q., Y. P. Wang, S. L. Chissoe, A. Bodenteich, Z. Wang, K. Iyer, S. W. Clifton, J. S. Crabtree, and B. A. Roe.** 1994. The complete nucleotide sequence of the SacBII domain of the P1 pAD10-SacBII cloning vector and three cosmid cloning vectors: pTCF, svPHEP, and LAWRIST16. Genet. Anal. Tech. Appl. **11:**181–186.
14. **Rappe, M. S., and S. J. Giovannoni.** 2003. The uncultured microbial majority. Annu. Rev. Microbiol. **57:**369–394.
15. **Riesenfeld, C. S., P. D. Schloss, and J. Handelsman.** 2004. Metagenomics: genomic analysis of microbial communities. Annu. Rev. Genetics **38:**525–552.
16. **Roe, B. A.** 2004. Shotgun library construction for DNA sequencing. Methods Mol. Biol. **255:**171–187.
17. **Senko, J. M., B. S. Campbell, J. R. Henricksen, M. S. Elshahed, T. A. Dewers, and L. R. Krumholz.** 2004. Barite deposition mediated by phototrophic sulfide-oxidizing bacteria. Geochim. Cosmochim. Acta **68:**773–780.
18. **Zengler, K., G. Toledo, M. Rappe, J. Elkins, E. J. Mathur, J. M. Short, and M. Keller.** 2002. Cultivating the uncultured. Proc. Natl. Acad. Sci. USA **99:**15681–15686.