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Cultivation of Aerobic Chemoorganotrophic Proteobacteria and Gram-Positive Bacteria from a Hot Spring Microbial Mat

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The diversity of aerobic chemoorganotrophic bacteria inhabiting the Octopus Spring cyanobacterial mat community (Yellowstone National Park) was examined by using serial-dilution enrichment culture and a variety of enrichment conditions to cultivate the numerically significant microbial populations. The most abundant bacterial populations cultivated from dilutions to extinction were obtained from enrichment flasks which contained 9.0×10^2 primary producer (*Synechococcus* spp.) cells in the inoculum. Two isolates exhibited 16S rRNA nucleotide sequences typical of β-proteobacteria. One of these isolates contained a 16S rRNA sequence identical to a sequence type previously observed in the mat by molecular retrieval techniques. Both are distantly related to a new sequence directly retrieved from the mat and contributed by a β-proteobacterial community member. Phenotypically diverse gram-positive isolates genetically similar to *Bacillus flavothermus* were obtained from a variety of dilutions and enrichment types. These isolates exhibited identical 16S rRNA nucleotide sequences through a variable region of the molecule. Of the three unique sequences observed, only one had been previously retrieved from the mat, illustrating both the inability of the cultivation methods to describe the composition of a microbial community and the limitations of the ability of molecular retrieval techniques to describe populations which may be less abundant in microbial communities.

Recent studies investigating microbial species diversity in the Octopus Spring cyanobacterial mat community have revealed a marked disparity between the native 16S rRNA sequence types observed in the mat by molecular retrieval techniques and the 16S rRNA sequences of aerobic chemoorganotrophic bacteria cultivated from this and other geothermal habitats (34, 35, 38). Sequences that were retrieved from the Octopus Spring mat and that may belong to organisms exhibiting aerobic chemoorganotrophic metabolic capabilities include planctomycete, proteobacterial, and gram-positive bacterial representatives, as well as those of relatives of green sulfur and green nonsulfur bacteria (35). However, characterizing the metabolic capabilities of the bacteria which contain retrieved 16S rRNA sequence types is difficult without first cultivating these organisms.

Previous efforts to cultivate aerobic chemoorganotrophic bacteria from alkaline silicious hot spring cyanobacterial mats including Octopus Spring mat have yielded *Bacillus* (5), *Thermomicrobium* (19), *Thermus* (6), and *Chloroflexus* (4) isolates. These studies used similar cultivation techniques characterized by relatively high organic substrate concentrations (usually 0.1% [wt/vol] tryptone and yeast extract in liquid medium). An exception was the cultivation of the oligotrophic bacterium *Isosphaera pallida* in a culture medium devoid of organic substrates (15). Enrichments were often conducted at high incubation temperatures (70°C) regardless of the temperature of the collection site and the existence of temperature-adapted strains (23). Isolates were obtained by directly streaking mat material onto solidified media or by directly adding undiluted inoculum to enrichment flasks. Both of these methods preclude attempts to measure the relative abundance of the organisms cultivated. Since the development of enrichment culture techniques, microbiologists have suspected that these methods may select for the organisms which are best adapted to the enrichment culture environment but which may not be the dominant organisms in nature (33, 41). Our investigations resulted from our suspicion that the selectivity of the enrichment culture environment may explain the discrepancy between cultivated and naturally occurring populations detected by molecular retrieval techniques.

In this study, we attempted to cultivate more numerically abundant aerobic chemoorganotrophic bacteria from the 50 to 55°C region of Octopus Spring cyanobacterial mat community located in Yellowstone National Park. We used serial-dilution enrichment culture (8, 31) to provide a relative measure of the abundance of the isolates obtained and to avoid culture overgrowth by numerically insignificant species (13). We also performed enrichments under seemingly more natural conditions (e.g., incubating at the temperature of sample collection, using more relevant substrates known to be present in the habitat such as mat material and glycolic acid [2], and using lower substrate concentrations). Previously reported results from this study (22) revealed a diversity of *Thermus* isolates cultivated from the Octopus Spring mat whose distribution may be controlled by specialization to different temperatures which occur within the habitat. Here we report the cultivation and 16S rRNA sequence characterization of phenotypically and phylogenetically distinct proteobacterial and gram-positive aerobic chemoorganotrophic bacteria from the Octopus Spring mat.

MATERIALS AND METHODS

Cultivation of isolates. Sample collection and enrichment culture conditions were as described by Nold and Ward (22). Briefly, cyanobacterial mat samples were collected in September and October 1992 and November 1993 from the shoulder region of Octopus Spring (50 to 55°C, pH 8.5). The samples were kept between 46 and 50°C for 3 h in transit to the laboratory; once in the laboratory, they were homogenized with a Dounce tissue homogenizer and serially diluted (1:10) in sterile medium D (9) before inoculation. Carbon sources included glycolic acid (0.1% [wt/vol] glycolic acid in Castenholz medium D amended with 33% [vol/vol] Octopus Spring water [GLD]), casein (4% [wt/vol] casein in Castenholz medium D amended with 33% [vol/vol] Octopus Spring water [CND]), solidified autoclaved mat homogenate (10% [vol/vol] cyanobacterial mat homog-

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enate with 3% [wt/vol] agar in Castenholz medium D amended with 33% [vol/vol] Octopus Spring water [MTD]), and a standard substrate used to cultivate *Thermus* species (6), i.e., 0.1% (wt/vol) tryptone and 0.1% (wt/vol) yeast extract in Castenholz medium D (TYD) (see Table 1). The sample for TYD enrichments was collected on 30 September 1992, and the sample for MTD, CND, and GLD enrichments was collected on 29 October 1992. For samples collected on a later sampling date (9 November 1993), 0.1, 0.01, and 0.001% (wt/vol) tryptone and yeast extract were used as carbon sources. Liquid enrichments (50 ml of GLD, TYD, or CND in 300-ml shake flasks) were inoculated with 5 ml of serially diluted mat homogenate, incubated at 50°C with shaking (150 rpm) until turbid, and then transferred to solidified medium containing 3% agar for isolation. Solidified mat homogenate was spread inoculated with 100 μl of serially diluted inoculum and placed in a static incubator at 50°C. Individual colonies which exhibited unique and stable phenotypic properties (colony color, cell morphology, spore formation, motility) were restreaked for purification and perpetuated from each medium type. Cyanobacterial (*Synechococcus* spp.) cells were enumerated by direct microscopic count with appropriate dilutions of mat homogenate, using a Petroff-Hausser counting chamber. Total direct counts of cyanobacterial cells in undiluted homogenized mat inocula were 5.3×10^8 unicells ml^{-1} (TYD) and 1.8×10^9 unicells ml^{-1} (GLD, CND, and MTD). The abundance of each isolate is reported relative to the number of *Synechococcus* cells present in the diluted inoculum source used to inoculate the flask in which the isolate was observed.

Characterization of 16S rRNA sequences. 16S rRNA sequence data were generated for each phenotypically unique isolate which grew to the highest dilution in each enrichment type. Harvested cells were lysed by following an established enzymatic protocol (36). Nucleic acids were extracted, the 16S rRNA gene was amplified by PCR and cloned, and full-length (*Escherichia coli* positions 28 to 1483) (7) 16S rRNA sequence data were generated for isolates ac-15, ac-16, and ac-18 as described by Kopczynski et al. (20). Partial 16S rRNA sequence data were generated for the remaining isolates by directly sequencing PCR products with the Sequenase PCR product-sequencing kit (United States Biochemical, Cleveland, Ohio) as specified by the manufacturer. The 16S rRNA sequence we report here, which was retrieved directly from the mat was obtained from Octopus Spring library V-L by previously described methods (39). This sequence was previously reported as clone OS-V-L-28 (35) but was not characterized. Sequences were aligned and similarity values were calculated with the SeqEdit program version 3.0.4 provided by the Ribosomal Database Project (RDP) at the University of Illinois (21). All available sequence data, including ambiguous bases and alignment gaps, were included in similarity calculations. Sequences were analyzed for potential chimeric structures with the Check_Chimera program, available through the RDP. Phylogenetic trees were constructed with the programs DNADIST, SEQBOOT, FITCH, and CONSENSE from the Phylogenetic Inference Package (PHYLIP) version 3.57c (11), with representative sequences derived from the RDP.

Nucleotide sequence accession numbers. New 16S rRNA sequences are reported herein for isolates ac-15, ac-16, and ac-18 (GenBank accession numbers U46749, U46748, and U46747, respectively) and for one 16S rRNA sequence retrieved directly from the Octopus Spring mat (Octopus Spring type R; GenBank accession number U46750).

RESULTS

The outcome of enrichment culture experiments conducted on samples collected in September and October 1992 is summarized in Table 1. While phenotypically diverse bacterial isolates were obtained from TYD, MTD, and GLD enrichments, the enrichment containing CND as the carbon source yielded only one isolate (ac-12). Growth occurred to different dilutions depending on the medium type. Enrichments containing GLD as a carbon source yielded an isolate (ac-21) which was cultivated from a 10^8-fold dilution, which initially contained 9.0×10^2 *Synechococcus* cells. TYD, CND, and MTD enrichments all yielded less abundant isolates cultivated from 10^3- to 10^5-fold dilutions, initially containing 2.7×10^7 to 9.0×10^5 *Synechococcus* cells, respectively. The extent to which the inoculum was diluted before incubation affected the type of growth observed in liquid enrichment cultures. Less abundant populations (10^2- to 10^5-fold dilutions) exhibited turbid growth, while more abundant populations growing in GLD enrichments (10^6- to 10^8-fold dilutions) were characterized by filamentous organisms growing as faint orange pellicles detected at the interface between air and water in the shaken enrichment flask. These sheathed filamentous organisms (isolates ac-19, ac-20, and ac-21) were isolated by streaking pellicle samples onto solidified medium D preinoculated with *Synechococcus* isolate C1 (13). Subsequent transfer of colonies to liquid enrichments failed, thereby precluding 16S rRNA sequence characterization of these isolates.

TABLE 1. Bacterial isolates cultivated from the 50 to 55°C Octopus Spring cyanobacterial mat community sampled during October and November 1992

Isolate	Medium[a]	Highest dilution	Distinguishing characteristics	16S rRNA sequence type identical to:
ac-3	TYD	10^{-2}	Motile coccobacillus	ac-15
ac-4	TYD	10^{-3}	Brown coccobacillus	ac-18
ac-5	TYD	10^{-3}	Yellow sporulating rod	ac-18
ac-8	MTD	10^{-4}	Motile coccobacillus	ac-15
ac-9	MTD	10^{-4}	White sporulating rod	ac-18
ac-10	MTD	10^{-5}	Motile rod	ac-16
ac-11	MTD	10^{-5}	Sheathed orange rod, 0.5 by 5 μm	ND[b]
ac-12	CND	10^{-5}	Motile rod	ac-16
ac-13	GLD	10^{-4}	Yellow sporulating rod	ac-18
ac-15[c]	GLD	10^{-6}	Motile coccobacillus	ac-15
ac-16[c]	GLD	10^{-6}	Motile rod	ac-16
ac-18[c]	GLD	10^{-7}	Colorless coccobacillus	ac-18
ac-19	GLD	10^{-7}	Sheathed orange trichome, 0.5 by 200 μm	ND
ac-20	GLD	10^{-7}	Sheathed orange trichome, 1 by 10–50 μm	ND
ac-21	GLD	10^{-8}	Sheathed orange trichome, 0.6 by 10–50 μm	ND

[a] The pH of all media was adjusted to 8.2 before autoclaving.
[b] ND, no data.
[c] Isolate from which full-length 16S rDNA sequence data are available.

In enrichment culture experiments conducted on samples collected in November 1993, the carbon source concentration did not influence the extent of growth (all TYD concentrations exhibited growth to a 10^8-fold dilution), but evidence of growth was observed sooner in the more dilute enrichments (0.001 and 0.01% TYD) (6 days) than in 0.1% TYD (44 days). Growth at the highest dilutions resembled the orange filamentous pellicle described above, and characterization attempts similarly failed.

Isolates clustered into three phylogenetically distinct groups: proteobacteria, gram-positive bacteria, and *Thermus* species (previously reported in reference 22) (Fig. 1). Isolate ac-15 contains a 16S rRNA nucleotide sequence which is identical to the previously retrieved β-proteobacterial Octopus Spring type N sequence (34) over the 277 nucleotides available for comparison (Table 2). Isolate ac-16 and the retrieved but previously uncharacterized Octopus Spring type R sequence also displayed similarity to proteobacterial sequences (Table 2). Isolates ac-15 and ac-16 and the Octopus Spring type R sequence are compared with other mat proteobacterial sequence types and representatives of the major proteobacterial lines of descent in Fig. 2. Analysis of diagnostic secondary structure (*E. coli* positions 140 to 223) and diagnostic nucleotide signatures (*E. coli* positions 50, 108, 124, 640, 690, 722, 760, 812, 871, 929, 947, and 1234) (42), sequence similarity (Table 2) results, and tree (Fig. 2) results all support the inference that isolates ac-15, ac-16, and the retrieved Octopus Spring type R sequence belong to the β subdivision of the proteobacteria. Isolate ac-18 exhibited a 16S rRNA nucleotide sequence nearly identical to the sequence from *Bacillus flavothermus*, a group 5 *Bacillus* species in the low-G+C-content subdivision of the gram-positive bacteria (25) cultivated from a hot spring habitat (16) (Fig. 1; Table 2). We found no evidence of chimera formation in the 16S rRNA sequences of any of the isolates by using Check_Chimera (low maximum improvement scores and lack

of peakedness) and secondary-structure analyses; however, detecting chimeration can be problematic (20, 28).

Stable phenotypic differences were displayed by isolates ac-4, ac-5 (which was phenotypically similar to ac-13), ac-9, and ac-18 (Table 1), yet all exhibited gram positive 16S rRNA nucleotide sequences identical to those of isolate ac-18 through the region 1086 to 1295, which includes the V9 variable region of the molecule (nucleotides 1110 to 1276). Only two of these isolates (isolates ac-5 and ac-13) exhibited phenotypic similarity to *B. flavothermus* (i.e., rod-shaped morphology, spore formation, and yellow colony color) (16). Other isolates displayed similar morphology and identical β proteobacterial 16S rRNA sequence types. Motile rods (isolates ac-10, ac-12, and ac-16) and motile coccobacilli (isolates ac-3, ac-8, and ac-15) were isolated from different dilutions and substrate types and displayed identical 16S rRNA nucleotide sequences through nucleotides 1087 to 1289 and 1085 to 1280, respectively.

DISCUSSION

Here we present the results of cultivation and full-length 16S rRNA sequence characterization of an organism whose sequence was previously observed in the Octopus Spring mat by molecular retrieval techniques (34). We detect no unambiguous 16S rRNA nucleotide differences between isolate ac-15 and the previously retrieved sequence fragment Octopus Spring type N (Table 2). It appears that the type N population corresponds to an aerobic chemoorganotrophic motile coccobacillus of β-proteobacterial descent, which is able to utilize glycolic acid, tryptone and yeast extract, and mat homogenate as carbon sources. Other examples of bacterial populations detected by both cultivation and 16S rRNA sequence retrieval are rare; Ferris et al. (13) successfully cultivated a relevant thermophilic cyanobacterium from this habitat, and Huber et al. (17) obtained an archaeal isolate whose 16S rRNA was previously detected.

Isolates ac-15 (i.e., Octopus Spring type N) and ac-16 are the first proteobacterial isolates to be cultivated from the Octopus Spring mat community (35). These isolates are phylogenetically related to members of the β subdivision of the proteobacteria, a physiologically diverse clustering of organisms which display nitrogen fixation (18, 26, 40), aerobic and anaerobic respiration (32), and photoautotrophic and photoheterotrophic capabilities (40). While isolates ac-15 and ac-16 clearly exhibit aerobic chemoorganotrophic metabolic capabilities, they may, like other β-proteobacteria, display physiological versatility. Thus, their metabolic functions exhibited in the Octopus Spring mat community are currently unknown. Four of the five known proteobacterial sequences detected in the Octopus Spring mat are most similar to members of the β subdivision. We have observed a similar pattern of multiple representatives within one phylogenetic type in the cyanobacterial, green sulfur, green nonsulfur, and *Thermus*/*Deinococcus* lines of descent (12, 22, 30). One possible explanation for this recurring pattern could be that progenitor bacteria within a phylogenetic group became specialized to conditions which vary in the habitat, resulting in subsequent evolutionary radiation and the observed diversity of modern 16S rRNA types (37, 43).

Isolate ac-18 contains the first thermophilic *Bacillus*-like 16S rRNA sequence observed in the Octopus Spring mat (35). Isolates ac-4, ac-5 (and ac-13), ac-9, and ac-18 are phenotypically distinct (Table 1) but share this 16S rRNA nucleotide sequence at least through the V9 variable region (nucleotides 1239 to 1298). This could be due to either the highly conserved nature of the 16S rRNA molecule (42) or undetected differences in other regions of the molecule. Since only limited data were obtained from isolates which exhibited identical 16S rRNA sequences (207 to 222 nucleotides), we cannot reject the latter possibility. Sequence similarity within phenotypically diverse thermophilic gram-positive bacteria has been previously observed (3). Caution should be applied when interpreting bacterial diversity detected by a conservative genetic marker, since populations exhibiting identical 16S rRNA nucleotide sequences may contain highly related yet phenotypically distinct members.

By observing the extent of growth from a serially diluted inoculum, an estimate of the relative abundance of cultivated strains can be obtained (8). The highest dilution at which we observed isolate ac-15 (Octopus Spring type N) (10^6-fold) originally contained almost 5 orders of magnitude more *Synechococcus* cells in the inoculum (9.0×10^4), indicating the relative numerical insignificance of this cultivated species in native mat material. If we can assume that the frequency at which a se-

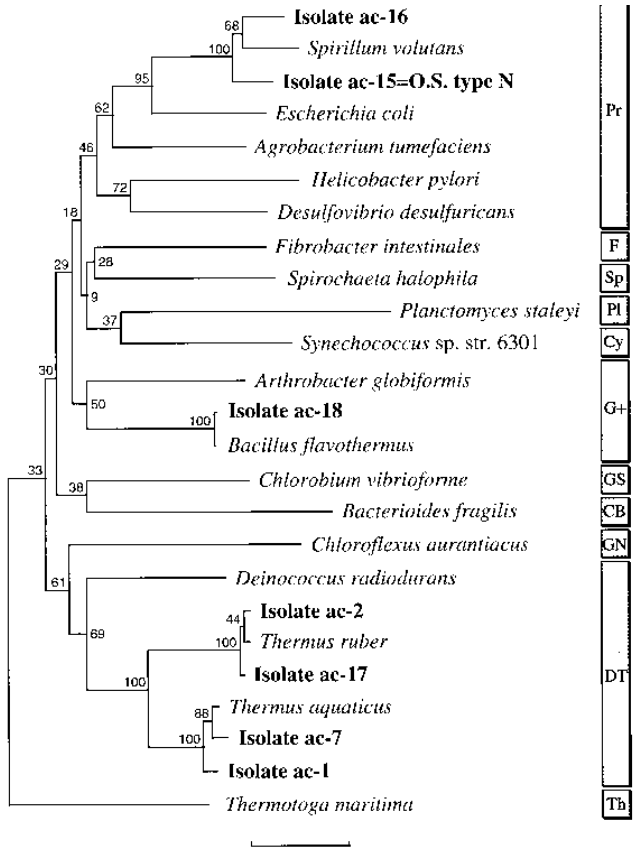

FIG. 1. Distance matrix phylogenetic tree showing the placement of 16S rRNA sequences of aerobic chemoorganotrophic isolates cultivated from the Octopus Spring (O.S.) cyanobacterial mat community relative to those of representatives of the major bacterial lines of descent. Representatives were chosen from the following lines: Pr, proteobacteria; F, *Fibrobacter*; Sp, spirochetes and relatives; Pl, *Planctomyces* and relatives; Cy, cyanobacteria; G+, gram-positive bacteria; GS, green sulfur bacteria; CB, *Flexibacter-Cytophaga-Bacteroides*; GN, green nonsulfur bacteria and relatives; DT, *Deinococcus-Thermus* subdivision of the green nonsulfur bacteria; Th, *Thermotogales. Thermus* sequences reported by Nold and Ward (22) are included to exhibit the full phylogenetic range of the aerobic chemoorganotrophic bacteria cultivated during these studies (isolates denoted by boldface type). The consensus values at the nodes indicate the number of times the group consisting of the species to the right of the node occurred among 100 trees inferred from the bootstrapped data set sampled by analysis of restricted nucleotide positions (39) which were common to all sequences (at least 898 nucleotides). This tree was rooted by using the 16S rRNA sequence of *Methanobacterium thermoautotrophicum*. The scale bar represents 0.05 fixed point mutation per sequence position.

TABLE 2. 16S rRNA sequence similarities of Octopus Spring isolates and selected proteobacterial and gram-positive sequence types

Sequence[a]	% Similarity to and no. of unambiguous differences with sequence[b]:									
	1	2	3	4	5	6	7	8	9	10
Proteobacteria										
1. Isolate ac-15 (1,392)		**0**	172	142	66	152	177	365	363	385
2. O.S. type N (277)	**99.8**		29	22	27	28	44	65	65	79
3. Isolate ac-16 (1,456)	87.7	89.7		161	60	**87**	185	357	358	391
4. O.S. type R (1,346)	88.9	91.9	88.0		56	148	181	333	334	349
5. O.S. type G (588)	88.8	90.2	89.8	90.6		70	93	120	120	145
6. *Azoarcus denitrificans* (1,458)	89.1	90.0	**93.9**	89.0	88.2		193	367	365	402
7. O.S. type O (728)	76.0	77.6	75.0	75.0	79.8	73.8		170	169	211
Gram-positive bacteria										
8. Isolate ac-18 (1,478)	74.6	76.8	76.1	75.9	79.9	75.5	77.1		**6**	341
9. *Bacillus flavothermus* (1,477)	74.7	76.8	76.0	75.8	79.9	75.6	77.2	**99.6**		340
10. *Thermotoga maritima* (1,481)	73.4	72.6	74.0	74.9	75.9	73.3	71.7	77.2	77.2	

[a] O.S., Octopus Spring. Number of nucleotides available for comparison in parentheses.
[b] Values in the lower left are percent sequence similarities based on all available sequence data, and values in the upper right are the absolute number of unambiguous nucleotide substitutions. The boldface values indicate the closest relatives of isolates ac-15, ac-16, and ac-18.

quence is observed in cloning libraries reflects the abundance of that sequence in nature, the low abundance of isolate ac-15 in mat homogenate may explain why the Octopus Spring type N sequence was detected only once by molecular retrieval techniques (34). Isolates ac-16 and ac-18 were cultivated from similar dilutions (10^6- and 10^7-fold, respectively), but these populations have not been previously observed in cloning libraries constructed from Octopus Spring mat nucleic acid.

Isolates ac-19, ac-20, and ac-21, which exhibited growth in the highest dilutions (10^7- and 10^8-fold), could not be grown to sufficient quantity for sequence analysis. These organisms grew as sheathed trichomes similar to the *Chloroflexus* species described by Pierson and Castenholz (24) and also obtained from aerobic chemoorganotrophic enrichments by Brock (4). Glycolate has previously been identified as a substrate for aerobic chemoorganotrophic metabolism in the Octopus Spring mat. Under illuminated conditions, glycolate is excreted by photosynthetically active *Synechococcus* cells and is readily incorporated by filamentous *Chloroflexus*-like organisms (2). This result confirms the importance of glycolate as a carbon source for aerobic chemoorganotrophy in the Octopus Spring mat and illustrates the importance of using ecologically relevant carbon substrates to cultivate the more numerically abundant bacterial species. Combining cultivation and molecular retrieval approaches has allowed us to confirm the *Chloroflexus*-like nature of the sheathed trichomes which grow to the highest dilutions in GLD medium (30).

The other carbon substrates (i.e., CND, TYD, and MTD) yielded growth to only a 10^5-fold dilution. Further growth in these enrichments may have been inhibited by the inappropriateness of the carbon source provided or the relatively high substrate concentrations provided in these enrichments. To test the hypothesis that substrate concentration influences the extent of growth in serial dilution enrichment culture, 0.1, 0.01, and 0.001% tryptone and yeast extract were provided in separate dilution series. The observation that the substrate concentration did not influence the extent of growth (all substrate concentrations yielded growth to a 10^8-fold dilution) was unexpected, since the levels of soluble organic substrates in densely populated cyanobacterial mats may be quite low, favoring organisms adapted to low substrate concentrations. The aerobic mat heterotroph *Isosphaera pallida* was successfully cultivated at oligotrophic substrate concentrations (i.e., unamended mineral salts medium) (15). The relatively high substrate concentrations provided in the enrichments reported in this paper may have selectively recovered only the populations adapted to high organic carbon concentrations.

These results allow the comparison of cloning and cultivation techniques as methods to describe microbial diversity in natural environments. Although attempts were made to cultivate the more numerically abundant aerobic chemoorganotrophic bacteria from the Octopus Spring mat by using serial-dilution enrichment culture and possibly more natural incubation conditions, we still failed to cultivate most organisms whose 16S rRNA sequences were previously retrieved. Since samples for cultivation and molecular cloning experiments were not collected simultaneously, this failure may have been due to seasonal bacterial population variation. However, recent studies of 16S rRNA sequence type variation in the Octopus Spring mat have shown remarkable seasonal stability of bacterial populations (14). Alternatively, our failure to cultivate organisms

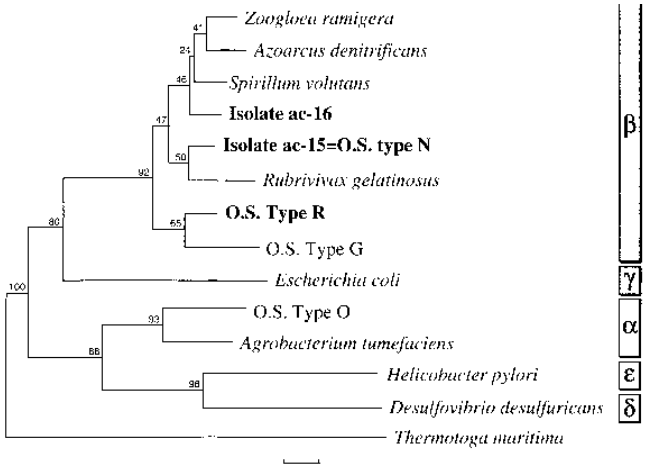

FIG. 2. Distance matrix phylogenetic tree showing the placement of 16S rRNA sequences of cultivated and cloned proteobacterial Octopus Spring (O.S.) mat populations relative to those of representatives of the major proteobacterial lines of descent (α, β, γ, δ, and ε). Boldface type denotes proteobacterial sequences reported in this paper. The consensus values at the nodes indicate the number of times the group consisting of the species to the right of the node occurred among 100 trees inferred from the bootstrapped data set sampled by analysis of nucleotides which align with *E. coli* 16S rRNA positions 802 to 825, 875 to 886, 1046 to 1114, 1157 to 1250, and 1287 to 1392. The scale bar represents 0.01 fixed point mutation per sequence position.

with retrieved 16S rRNA sequences may indicate either that the retrieved sequences do not correspond to an aerobic chemoorganotrophic metabolism or that we did not cultivate the relevant organisms. The inability of enrichment culture to recover predominant populations is well documented (1), and isolation techniques which require growth as a colony on solid media may further limit the retrieval of relevant organisms (29, 37); therefore, the finding of simplified species diversity as a result of using cultivation methods is not unexpected. However, 16S rRNA sequence retrieval methods may also underestimate species diversity by detecting only the species whose nucleic acids are readily cloned or PCR amplified (10, 27). Although we expect the 16S rRNA of numerically abundant organisms to appear in cloning libraries, these techniques may suffer from a lack of sensitivity, resulting in the inability to detect less abundant populations such as those presented in this paper.

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