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Abundance of Reverse Tricarboxylic Acid Cycle Genes in Free-Living Microorganisms at Deep-Sea Hydrothermal Vents

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Since the discovery of hydrothermal vents more than 25 years ago, the Calvin-Bassham-Benson (Calvin) cycle has been considered the principal carbon fixation pathway in this microbe-based ecosystem. However, on the basis of recent molecular data of cultured free-living and noncultured episymbiotic members of the epsilon subdivision of *Proteobacteria* and earlier carbon isotope data of primary consumers, an alternative autotrophic pathway may predominate. Here, genetic and culture-based approaches demonstrated the abundance of reverse tricarboxylic acid cycle genes compared to the abundance of Calvin cycle genes in microbial communities from two geographically distinct deep-sea hydrothermal vents. PCR with degenerate primers for three key genes in the reverse tricarboxylic acid cycle and form I and form II of ribulose 1,5-bisphosphate carboxylase/oxygenase (Calvin cycle marker gene) were utilized to demonstrate the abundance of the reverse tricarboxylic acid cycle genes in diverse vent samples. These genes were also expressed in at least one chimney sample. Diversity, similarity matrix, and phylogenetic analyses of cloned samples and amplified gene products from autotrophic enrichment cultures suggest that the majority of autotrophs that utilize the reverse tricarboxylic acid cycle are members of the epsilon subdivision of *Proteobacteria*. These results parallel the results of previously published molecular surveys of 16S rRNA genes, demonstrating the dominance of members of the epsilon subdivision of *Proteobacteria* in free-living hydrothermal vent communities. Members of the epsilon subdivision of *Proteobacteria* are also ubiquitous in many other microaerophilic to anaerobic sulfidic environments, such as the deep subsurface. Therefore, the reverse tricarboxylic acid cycle may be a major autotrophic pathway in these environments and significantly contribute to global autotrophic processes.

The discovery of deep-sea hydrothermal vents 27 years ago opened a window into a largely unknown and unexplored biosphere that changed our fundamental views about the extent of habitable microbial environments on earth and the possible origins of life itself. One of the most startling discoveries of these early studies was that these vast communities appeared to be supported solely on carbon originating from microbe-based chemoautotrophy and not phototrophy (28). Perhaps the best studied chemoautotrophic system is that used by gamma proteobacterial endosymbionts of the giant tubeworm *Riftia pachyptila*. These endosymbionts function via the Calvin cycle to fix carbon and transfer it to their host, deriving energy from the oxidation of hydrogen sulfide (9, 16). Other deep-sea invertebrate symbioses in associated vent environments and free-living aerobic sulfur oxidizers utilizing the Calvin cycle have since been characterized (8, 29, 36, 41, 55).

Subsequent studies, however, have provided evidence that suggests the significant contribution of other autotrophic pathways at hydrothermal vents. Stable carbon isotopic analyses of primary consumers from a number of hydrothermal vent sites have revealed values significantly heavier than would be predicted from microbe-based chemoautotrophy via the Calvin cycle (49, 50). The reverse tricarboxylic acid (rTCA) cycle is an alternative CO_2 fixation pathway that generates a stable isotopic signature more closely resembling these vent primary consumers (37, 44). To date, the rTCA cycle has been found in only a few microorganisms, including *Chlorobium* species, a few members of the delta subdivision of *Proteobacteria* (i.e., *Desulfobacter hydrogenophilus*), and some members of the thermophilic *Aquificales* order and archaeal *Thermoproteaceae* family (5, 13, 15, 19, 27, 42, 43). A reversal of the entire TCA cycle for carbon dioxide fixation uses four or five ATP molecules and generates one molecule of oxaloacetate from four molecules of CO_2 (18). Key enzymes of the rTCA cycle include ATP citrate lyase (encoded by the *aclBA* gene) and two of the four carbon dioxide-fixing enzymes, 2-oxoglutarate:ferredoxin oxidoreductase (encoded by the *oorDABC* gene), and pyruvate:ferredoxin oxidoreductase (encoded by the *porCDAB* or *nifJ* gene). These enzymes catalyze the reversal of the TCA cycle by making the energetically unfavorable reverse reactions possible. For example, ATP citrate lyase catalyzes the cleavage of citrate into acetyl coenzyme A (acetyl-CoA) and oxaloacetate in a CoA- and ATP-dependent matter (2). Reversal of the TCA cycle requires the combined use of all three enzymes for reductive carboxylation to occur.

Recent molecular and isotopic data indicate that the epsilon proteobacterial community associated with *Alvinella pompejana*, which thrives on the sides of the hotter black smoker chimneys at deep-sea hydrothermal vents, may utilize the rTCA cycle for autotrophic growth. Two of the key genes were present and expressed in the episymbiont community, which is dominated by members of the epsilon subdivision of *Proteobacteria* (7). In addition, there is genetic evidence of the rTCA cycle in at least two cultured autotrophic members of the epsilon subdivision of *Proteobacteria* from hydrothermal vents and one autotrophic member of the epsilon subdivision of *Proteobacteria* isolated from marine sediments (6, 56). Inter-

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estingly, molecular studies indicate that members of the epsilon subdivision of *Proteobacteria* dominate all free-living deep-sea hydrothermal vent microbial environments studied so far (10, 23, 35, 39). Enrichment of a diverse array of chemoautotrophic members of the epsilon subdivision of *Proteobacteria* from hydrothermal vents further supports the results of these molecular studies (1, 6, 33, 46). These chemoautotrophs utilized H_2 or reduced sulfur compounds as electron donors and O_2, nitrate,or elemental sulfur as electron acceptors.

Historically, studies of chemoautotrophs from deep-sea hydrothermal vents relied on laboratory-based ^{14}C labeling and detection of key Calvin cycle enzymes in vent samples as well as culture-based experiments (30, 36, 48, 55). Although these approaches detect autotrophic processes, they are limited to the particular autotrophy assay or enrichment conditions used. More recently, PCR with primers specific for either ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) genes or two key rTCA cycle genes has successfully detected the presence and diversity of microorganisms that have the potential to utilize the Calvin or rTCA cycle for autotrophy (7, 14). For this study, these molecular approaches were combined and refined to detect and phylogenetically characterize three key rTCA cycle genes and two forms of the RubisCO gene from a variety of geographically distinct vent samples. Expression of these genes was also assayed via a gene expression analysis based on reverse transcription-PCR (RT-PCR). While PCR has its own inherent biases (54), these studies enabled us to determine which autotrophic pathways are being utilized and identify likely contributors and their significance to the fixed carbon pool at deep-sea hydrothermal vents.

MATERIALS AND METHODS

Sample collection, nucleic acid extraction, and reverse transcription. Chimney samples were collected on three cruises of the deep-sea vessel DSV *Alvin*. The cruises were from the Atlantis Rise to the East Pacific Rise in November 1999 (9°N, M vent, Io vent), January 2000 (Guaymas Basin samples [G534, G585, G609, and G855]), and November 2001 (9°N, P vent). Chimney samples were placed into a 4°C sterile seawater-filled container and brought to the surface. Samples were immediately subsectioned and frozen at −80°C until nucleic acid extraction could be performed. DNA was extracted as described previously (6). RNA was extracted from sample G534 (Rebeccas Roost flange) by a modified acid-phenol extraction protocol (7), to which a bead beating step (40 s in a Fast Prep machine with 0.1- and 2.5-mm-diameter zirconia and silica beads) was added during the cell lysis step to ensure complete lysis of the cells. Nucleic acids were quantified by spectrophotometry and stored at −80°C. Approximately 250 ng of RNA from Rebeccas Roost was reverse transcribed into cDNA by a previously described technique (7).

Enrichment and DNA extraction of sulfur-oxidizing autotrophs. Sulfur-oxidizing autotrophs were enriched by incubating chimney- or *Alvinella pompejana*-associated samples (tubes or scrapings) into sulfide-oxygen or sulfide-nitrate gradient tubes on board ship. Gradients were set up by making a bottom agar layer of 20 mM sulfide, adding a 4-ml 0.3% Noble agar layer (in sterile seawater) on top mixed with the inoculum (approximately 0.5 ml of sample) and 2 mM NO_3^-, where indicated. The headspaces of the tubes were exchanged to H_2:CO_2 (80:20), with the addition of air (1 to 2% O_2) in some cases for microaerophilic growth. Tubes were incubated either at room temperature or 42 or 55°C until evidence of growth occurred (1 to 3 days). Subcultures were made at least two times from these enrichments. DNA was extracted from the cells, and denaturing gradient gel electrophoresis of 16S rRNA genes was performed and clone libraries were constructed by previously described methods (6).

PCR amplifications and cloning. PCR detection of the ATP citrate lyase beta subunit (*aclB*) was based on previously designed primers (7). For this study, we designed degenerate primers for both forms of RubisCO, form I (*cbbL*) and form II (*cbbM*), both forms of the pyruvate:ferredoxin oxidoreductase gene (*por* and *nifJ*), and a reverse primer combined with a previously designed primer that amplifies a larger region of the 2-oxoglutarate:ferredoxin oxidoreductase gene (*oorA*) than that described previously (7). New primers designed for this study include *oorA* 345R (5′-CTTGCAGCCTGTNGGMAKNCCNGT), *porA* 900F (5′-GATCAGGTCCTTCAGNCCNTTCCC), *porA* 1101R (5′-RTCICTTYCIC CIARACC), *nifJ* 2459F (5′-CIGGITGYGGIGAAACICC), *nifJ* 2933R (5′-CCI ATRTCRTAIGCCCAICCRTC), *cbbL* F (5′-GACTTCACCAAAGACGAC GA), *cbbL* R (5′-TCGAACTTGATTTCTTTCCA), *cbbM* F (5′-TTCTGGCTG GGBGGHGAYTTYATYAARAAYGACGA), and *cbbM* R (5′-CCGTGRCC RGCVCGRTGGTARTG). Primer design was based on amino acid alignments for at least six phylogenetically distinct bacteria and/or archaea, as appropriate (Table 1). Expected PCR sizes for these amplifications are as follows: for *aclB*, 312 bp; for *oorA*, 110 bp with old primers and 278 bp with new primers; for *porA*, 201 bp; for *nifJ*, 474 bp; for *cbbL*, 711 bp; and for *cbbM*, 328 bp.

PCR conditions were as described previously for *aclB* (7) with the following changes in annealing temperatures for genes: 54°C for *aclB*, 49°C for *porA*, 55°C for *nifJ*, 52°C for *oorA*, 53°C for *cbbL*, and 58°C for *cbbM*. All amplifications were performed on a Robocycler 96 (Stratagene) as described previously (7). Cycling conditions were optimized for each primer set with DNA from the following organisms: *aclB* (*Alvinella pompejana* symbiont fosmid 7G3), *oorA* (*Helicobacter pylori* and *A. pompejana* symbiont community), *porA* (*H. pylori*), *nifJ* (*Escherichia coli* and *Chlorobium tepidum*), *cbbL* (*Synechococcus* sp. and Delaware Inland Bays water), and *cbbM* (*Riftia pachyptila* trophosome symbiont). Between 1 and 10 ng of chimney DNA was used for amplifications. Between 2 and 5 μl of the reverse-transcribed sample was used for RT-PCR amplifications of the Guaymas Basin RNA sample. Amplicons were cloned into a TA-TOPO vector (Invitrogen, Carlsbad, Calif.), and approximately 48 correct-sized clones from each library were restricted and grouped according to their restriction fragment length polymorphism (RFLP) patterns (Table 1). Unique RFLP groups were sequenced as described previously (7). All clones were sequenced from the *porA* clone libraries due to the small size of the insert (~201 bp). The total numbers of sequences from each library from the 9°N P vent are as follows (the gene and number of clones are shown): *aclB*, 36; *porA*, 26; *oorA*, 44; *cbbL*, 30; *cbbM*, 37; and *nifJ*, 14. The total numbers of sequences from each library from Guaymas Basin samples are as follows (the gene and number of clones are shown): *aclB*, 31; *porA*, 27; *oorA*, 23; and *nifJ*, 7. In addition, *oorA* and *porA* clones were sequenced from *Persephonella marina*, "*Candidatus* Arcobacter sulfidicus," and the epsilon proteobacterial strain, Am-H, isolated from hydrothermal vents (6, 38, 56).

Phylogenetic analysis. Nucleic acid sequences obtained from the clone libraries were translated and aligned to similar sequences obtained from the GenBank database using the DNASTAR sequence analysis program and Clustal W (Table 1). Neighbor-joining distance trees and bootstrapped protein parsimony trees (based on 100 replicates) were generated with the PHYLIP package in the Genetic Data Environment (GDE) package as described previously (7). Similarity matrices were generated with PHYLIP using the amino acid similarity matrix algorithm. Trees were generally based on alignments of somewhat less than the full-length amplified region (Table 1). Insertions and deletions were not included in the calculations. Trees were viewed with Treeview X version 0.4.1 (http://darwin.zoology.gla.ac.uk/~rpage/treeviewx/).

Nucleotide sequence accession numbers. All nucleotide sequences have been deposited in GenBank and assigned the following accession numbers: AY430817 to AY431025 and AY553054 to AY553061. Phylogenetic trees of all sequence alignments (*aclB*, *oorA*, *porA*, *nifJ*, and *cbbL*/*cbbM*) have been submitted to TreeBASE (M1789-M1993).

RESULTS

Twelve free-living communities from two geographically, physically, and chemically distinct hydrothermal vent sites along the East Pacific Rise (EPR) were examined. The Axial Summit Caldera (9°N50/104°W17; depth of 2,500 m) is a basalt-based system, characterized by relatively large chimney structures and high-temperature effluent (21). The Guaymas Basin ridge axis (27°N/111°W24; depth of 2,000 m) is covered in a thick layer of sediment (~400 m), with small diffuse flow chimneys and flanges with thick bacterial mats (12, 47). While the chemistry of these vent sites may differ dramatically, they both contain compounds necessary for chemoautotrophic processes (12).

We used six sets of degenerate primers to amplify key Calvin

TABLE 1. Genes, restriction enzymes used, and alignment information

Gene[a]	Expected size (bp)	Restriction enzyme(s)	Alignment sequence	Accession no.	Alignment[b]	No. of aa[c] in alignment
aclB	312	*D*deI/*M*seI	7G3 (*A. pompejana* symbiont fosmid clone)	AY312991	MVAGG. . .GGP	96
			6C6 (*A. pompejana* symbiont fosmid clone)	AY312990		
			Lupinus albus	CAC86996		
			Arabidopsis thaliana	AAL25637		
			Caenorhabditis elegans	NP_508280		
			Mus musculus	AF332051.1		
			Homo sapiens	XP036462		
oorA	278(110)	*H*aeIII/*M*seI	*Helicobacter pylori*	AF021094	FFGG. . . .GLPT (FFGG. . .QMEDE)	91 (36)
			Hydrogenobacter thermophilus	AB046578		
			Thermotoga maritima	H72323		
			Thermoanaerobacter tengcongenis	AE013094		
			Geobacter metallireducens	NZ_AAAS01000001		
			Desulfovibrio desulfuricans G20	NZ_AABI01000006		
			Campylobacter jejuni	NC_002163		
porA	201	None	*Chlorobium tepidum* TLS	NC_002932	PFP. . .GE/GS/R	51
			Helicobacter pylori	NP_223754.1		
			Clostridium tetani E88	NP_783049		
			Thermotoga maritima	O05651		
			Archaeoglobus fulgidus DSM 4304	NP_070527		
			Pyrococcus furiosus	NP_578695		
			Methanococcus jannaschii	NP_247239		
nifJ	474	*A*luI	*Fusobacterium nucleatum* subsp. *vincentii*	EAA24989	GCGE. . .WAYDI	137
			Nostoc sp. strain *PCC 7120*	NP_485951		
			Desulfitobacterium hafniense	ZP_00098862		
			Desulfovibrio desulfuricans G20	ZP_00128897		
			Geobacter metallireducens	ZP_00080327		
			Thermoanaerobacter tengcongenis	NP_622125		
			Clostridium pasteurianum	AAC18834		
			Chlorobium tepidum TLS	AAM72853		
			Salmonella enterica serotype Typhimurium LT2	NP_460610		
			Escherichia coli	AAC74460		
			Campylobacter jejuni	NP_282614		
cbbL	711	*A*luI/*R*saI	*Chlamydomonas reinhardtii*	J01399	QPFM. . . .HVHR	76
			Anacystis nidulans	X03220		
			Synechococcus sp. strain *WH7803*	U46156		
			Hydrogenovibrio marinus	D43622		
			Thiobacillus ferrooxidans	X70355		
			Thiobacillus sp.	M34536		
			Thiobacillus denitrificans	L42940		
			Hydrogenophilus thermoluteolus	D30764		
cbbM	328	*H*aeIII	*Rhodospirillum rubrum*	X00286	QVFC. . . .TARR	76
			Rhodobacter capsulatus	U23145		
			Thiobacillus denitrificans	L37437		
			Riftia pachyptila endosymbiont	AF047688		
rbcL			*Methanococcus jannaschii*	U67564		

[a] *aclB* encodes ATP citrate lyase, beta subunit; *oorA* encodes 2-oxoglutarate:ferredoxin oxidoreductase, alpha subunit; *porA* encodes pyruvate:ferredoxin oxidoreductase, alpha subunit; *nifJ* encodes pyruvate flavodoxin/ferrodoxin oxidoreductase; *cbbL* encodes ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit; *cbbM* encodes form II ribulose 1,5-bisphosphate carboxylase/oxygenase; and *rbcL* encodes form III ribulose-bisphosphate carboxylase.

[b] Alignment of sequence from the indicated amino acids to the indicated amino acids.

[c] aa, amino acids.

and rTCA cycle genes from vent community samples. While both RubisCO and ribulose 5-phosphate kinase are required for the operation of the Calvin cycle, we amplified only portions of genes encoding form I (*cbbL*) and form II (*cbbM*) RubisCO as Calvin cycle markers (45). The form III, or archaeal, RubisCO was not included in this analysis, as a functional Calvin cycle has not been demonstrated in members of the domain *Archaea* that actively express this enzyme (17). Three key genes of the rTCA cycle were amplified: ATP citrate lyase (*aclAB*); pyruvate:ferredoxin oxidoreductase (or ferredoxin-dependent pyruvate synthase), which has two independent genes (heterodimeric pyruvate:ferredoxin oxidoreductase [*porCDAB*] and homodimeric ferredoxin-dependent pyruvate synthase [*nifJ*]); and 2-oxoglutarate:ferredoxin oxidoreductase (or α-ketoglutarate:acceptor oxidoreductase) (*oorDABC*) (18, 45).

We found that all 12 chimney samples contained the required rTCA genes *aclB* and *nifJ* and 10 of 12 were positive for the rTCA genes *porA* and *oorA*. Of these 12 samples, only seven and three contained genes for forms I (*cbbL*) and II (*cbbM*) of RubisCO, respectively. RT-PCR expression analysis of a Guaymas Basin chimney sample (G534 or G538) determined the presence of mRNA arising from both Calvin and rTCA cycle genes. Expression was demonstrated for all genes except *cbbM* (form II of RubisCO), identical to the results obtained when DNA from the same sample was amplified (Fig. 1).

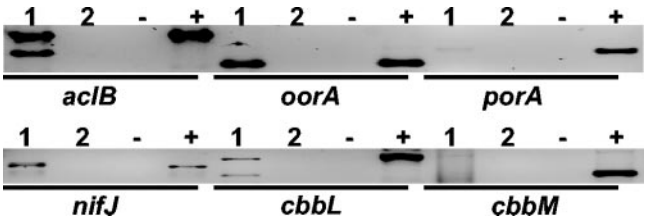

FIG. 1. Expression analysis of key autotrophic genes in a Guaymas Basin chimney sample (G538). Lanes 1, RT-PCR samples; lanes 2, the same RT-PCR samples with no added reverse transcriptase; lanes − and +, negative and positive controls for each gene, respectively. *aclB* encodes ATP citrate lyase, beta subunit; *oorA* encodes 2-oxoglutarate: ferredoxin oxidoreductase, alpha subunit; *porA* encodes pyruvate: ferredoxin oxidoreductase, alpha subunit; *nifJ* encodes pyruvate flavodoxin/ferrodoxin oxidoreductase; *cbbL* encodes ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit; and *cbbM* encodes form II ribulose 1,5-bisphosphate carboxylase/oxygenase.

Diversity of autotrophic genes. Genetic diversity in the Calvin and rTCA pathways was analyzed by generating clone libraries of all positive amplifications from two distinct chimney samples from the 9°N P vent (POB, P vent outside bottom) and Guaymas Basin sample G534 or G538. All six genes were amplified from the 9°N P vent, suggesting a diverse chemoautotrophic community. Only the *cbbM* gene failed to amplify from Guaymas Basin samples. Clone libraries were also generated from the *aclB* amplicons from the 9°N Io vent (outside and inside) chimney samples and all amplicons from the RT-PCR experiment and grouped by RFLP analysis. The clones from the *porA* libraries were not restricted due to the small size of the insert. Amplicons with unique RFLP patterns were sequenced and included in the phylogenetic analysis.

The numbers of different sequences, based on their translated amino acids, were generally very similar between most of the libraries. For instance, the number of clones (normalized to 100) containing sequences coding for different amino acids from the *aclB*, *oorA*, *cbbL*, and *cbbM* 9°N P-vent libraries were 27, 14, 33, and 22, respectively. Similar numbers of clones were observed with the Guaymas Basin *aclB* library (29 of 100), while the Guaymas Basin *oorA* library was more diverse (39 of 100). The number of different clones observed from the *porA* libraries was much greater (9°N P vent, 54 of 100; Guaymas Basin, 74 of 100), perhaps due to the non-RFLP grouping of the clones before sequencing and/or the diversity of the amplified region. In all cases, the extent of clone diversity in the libraries indicated that the populations were not fully characterized. However, the diversity observed was enough to demonstrate the range of sequence types within the population, as evidenced by both protein similarities and phylogenetic analyses of the cloned sequences (see below).

Protein similarities. Protein similarities based on translated sequences of PCR products were calculated for five of six genes from the libraries described above (Fig. 2). *nifJ* sequence data were not included, based on the low number of phylogenetic similarities to either the *aclB* or *oorA* tree (data not shown). The numbers of clones from *aclB*, *oorA*, and *porA* sequences (rTCA cycle genes) that formed a clade within the presumed epsilon *Proteobacteria* group were similar between the 9°N P vent and Guaymas Basin samples. As an example, within the rTCA-specific gene clone libraries, there were three and six (*aclB*), four and five (*oorA*), and seven and seven (*porA*) sequence groups that formed a clade with known epsilon proteobacterial sequences for the 9°N P vent and Guaymas Basin samples, respectively. Within the 9°N P vent and Guaymas Basin *porA* libraries, there were three and seven groups, respectively, of translated sequences with less than 60% similarity to either the known epsilon proteobacterial or *Persephonella marina (Aquificales*-type) sequences. The most abundant group of these occurred in the Guaymas Basin sample and was not more than 59% similar to any other known *porA* sequence over a stretch of 51 amino acids. A number of the less frequent clones were most similar to archaeal *porA* sequences (data not shown).

Clone libraries of the *cbbL* and *cbbM* gene amplicons were generated from the 9°N P-vent sample only. Both libraries contained two clusters of sequences. Within the *cbbL* library, the dominant RFLP group of *cbbL* sequences was most similar to the sequence of an endosymbiont of a *Pogonophora* found in the Japan Trench, while the two less abundant groups were most similar to the sequences of two gamma proteobacterium-related RubisCOs (Fig. 2 and 3). The two most abundant groups of *cbbM* library clones were most similar to the *cbbM* of *Thiobacillus denitrificans* (Fig. 2 and 3) (22). The two less prevalent groups of clones were more distantly related, and their closest relatives were from *T. denitrificans* and the *Riftia pachyptila* endosymbiont (40).

Phylogenetic relatedness of key rTCA genes. To estimate the evolutionary relatedness of the key genes of the rTCA cycle, phylogenetic analyses were performed based on translations of *aclB* and *oorA* clone sequences. Since only sequences from the *aclB* and *oorA* genes from two previous cultures of members of the epsilon subdivision of *Proteobacteria* were characterized, we also included sequences obtained from other sulfur-oxidizing enrichments from the 9°N vent site. As measured by denaturing gradient gel electrophoresis analysis of the 16S rRNA genes (data not shown), as well as a limited 16S rRNA gene clone diversity analysis, the autotrophic enrichments generally contained one to five microorganisms, with the dominant microorganism being a member of the epsilon subdivision of *Proteobacteria* (Table 2). 16S rRNA gene sequencing of the bacteria from two enrichment cultures (3833-42 and 3838-RT [Table 2]) was performed to further characterize the dominant autotrophs in those cultures. The cultured bacteria most closely related (>92%) to the 3833-42 and 3838-RT enrichments were the autotrophic epsilon *Proteobacteria Caminibacter hydrogeniphilus* and *Sulfurimonas autotrophica*, respectively (1, 26).

Clone libraries were generated with the *aclB* and *oorA* amplicons from the 9°N P-vent sample as well as the Guaymas Basin sample (both DNA and RNA). The *aclB* and *oorA* trees showed very similar phylogenies, with two exceptions (Fig. 4). Both trees indicated a predominance of clones that form a clade in the known epsilon proteobacterial group. There were, however, a large number of clones from the 9°N-P vent *aclB* library that formed a cluster with the known *Aquificales*-type representative (*P. marina*) and did not have any corresponding *oorA* similarity. Likewise, there was a cluster of *oorA* clones from the Guaymas Basin sample that formed a distinct deeply branching clade that lacked any corresponding *aclB* counterpart. These results were verified with a second *aclB* library

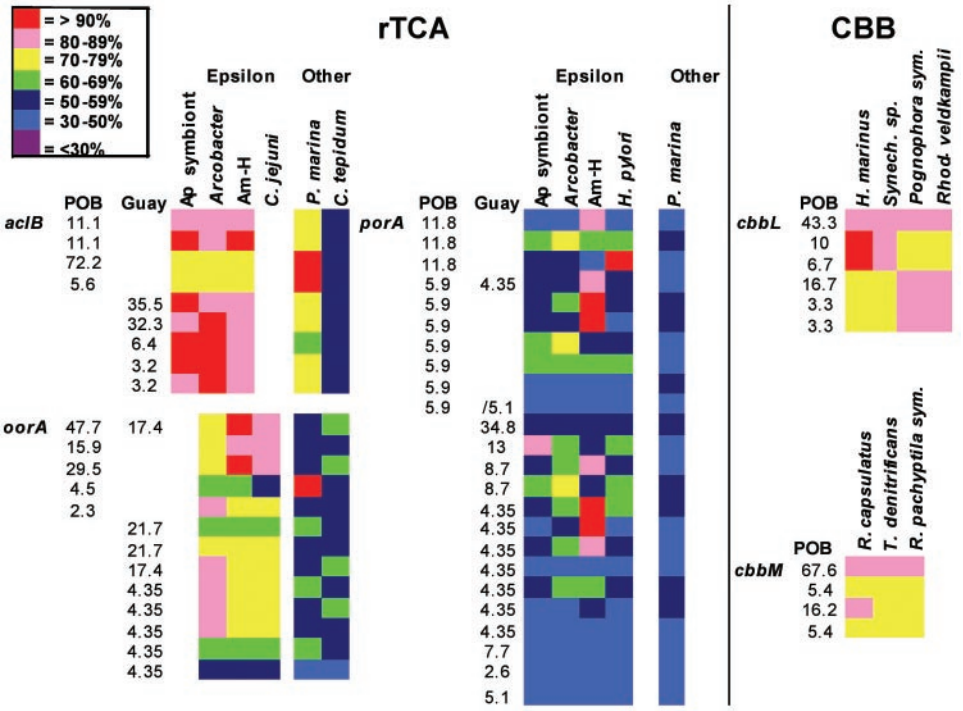

FIG. 2. Protein similarities of key rTCA and Calvin cycle autotrophic genes. The genes were generated from clone libraries obtained from the 9°N P vent (POB) and Guaymas Basin bacterial samples (Guay) (G534 or G538). Nucleic acid sequences were translated and grouped together if they shared the same RFLP pattern or were greater than 95% identical at the amino acid level. They were then aligned to and similarity matrices generated against known sequences in the GenBank database (e.g., members of the epsilon subdivision of the *Proteobacteria* and other bacteria). *aclB* encodes ATP citrate lyase, beta subunit; *oorA* encodes 2-oxoglutarate:ferredoxin oxidoreductase, alpha subunit; *porA* encodes pyruvate: ferredoxin oxidoreductase, alpha subunit; *nifJ* encodes pyruvate flavodoxin/ferrodoxin oxidoreductase; *cbbL* encodes ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit; *cbbM* encodes form II ribulose 1,5-bisphosphate carboxylase/oxygenase. Ap symbiont, *Alvinella pompejana* symbiont; *Synech. sp.*, *Synechococcus* sp.; *Pognophora sym.*, *Pognophora* symbiont; *Rhod. veldkampii*, *Rhodobacter veldkampii*; *R. capsulatus*, *Rhodobacter capsulatus*; *R. pachyptila sym.*, *Riftia pachyptila* symbiont.

generated with the 9°N P-vent sample and sequence analysis of libraries generated with the Guaymas Basin RT-PCR amplification products.

Phylogenetic analysis of the shorter *porA* amplicon gave almost identical distance tree topologies as the *oorA* tree, including the large cluster of clones not related to either the epsilon proteobacterial or *P. marina* sequences (data not shown). It had, however, several unreliable bootstrap values, probably due to a combination of *porA* divergence and short sequence. The *nifJ* phylogenetic data showed no clear phylogenetic groups that were similar to either the *aclB* or *oorA* gene sequence (data not shown). Although most of the *nifJ* clones grouped in the same clade as the *Campylobacter jejuni nifJ* sequence, no clones were more than 50% similar at the amino acid level.

DISCUSSION

Chemoautotrophy at deep-sea hydrothermal vents occurs via at least the rTCA and Calvin cycles, with the possibility that other pathways are being utilized, as evidenced by the presence of key autotrophic genes shown in this study in combination with stable isotope analysis of primary vent consumers (49, 50). Expression analysis, as well as the overwhelming presence and phylogenetic diversity of rTCA genes, indicate that the principal pathway utilized in hydrothermal vent free-living microbial communities is the rTCA cycle. Phylogenetic analysis of the key rTCA genes indicates that the majority group with known epsilon proteobacterial sequences.

It is not surprising that autotrophic members of the epsilon subdivision of *Proteobacteria* use the rTCA cycle. They are known for their metabolic and thermal versatility, utilizing a variety of electron donors and acceptors, generally under microaerophilic to anaerobic conditions while growing between 20 and 60°C (46). Autotrophic members of the order *Aquificales*, which grow at higher temperatures, utilize similar metabolic strategies and the rTCA cycle (7, 38). Hydrothermal vent diffuse flow sites are areas of intense mixing between two types of fluids: the high temperature end-member fluids that are strongly reducing and ambient seawater (2°C) that is fully oxygenated. In situ measurements of sulfide-rich high-temperature (>30°C) diffuse flow vents surrounding alvinellid communities detect little to no oxygen, providing an ideal habitat for members of the epsilon subdivision of *Proteobacteria* and the order *Aquificales* that thrive in these conditions (32, 38).

These experiments did not unequivocally show the presence and expression of key rTCA cycle genes within a single organism, except within the enriched autotrophs. However, on the basis of the phylogenetic positions of the clones within the distance trees and their relatedness to cultured representatives, we surmise that the *aclB*, *oorA*, and *porA* genes that form

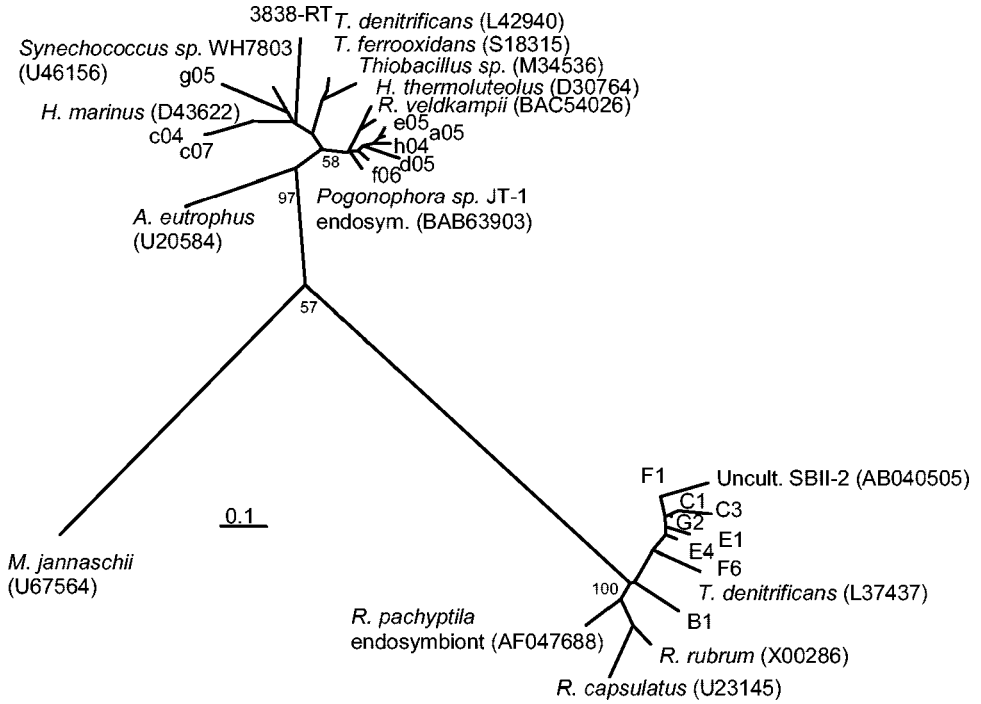

FIG. 3. Phylogenetic analysis of translated *cbbL* (ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit) and *cbbM* (form II ribulose 1,5-bisphosphate carboxylase/oxygenase) clone libraries from the 9°N P vent (POB) sample. The neighbor-joining distance tree was generated on the basis of translated amino acids (minus insertions and deletions) from the *cbbL* and *cbbM* genes. Bootstrap values from 100 resamplings are indicated prior to the branch points of the tree. The scale bar represents the calculated number of changes per amino acid position. Clones identical at the amino acid level were not included in the trees. Accession numbers are shown in parentheses. *A. eutrophus*, *Alcaligenes eutrophus*; *T. ferrooxidans*, *Thiobacillus ferrooxidans*; *H. thermoluteolus*, *Hydrogenophilus thermoluteolus*; *R. veldkampii*, *Rhodobacter veldkampii*; endosym., endosymbiont; Uncult., uncultured; *R. rubrum*, *Rhodospirillum rubrum*; *R. capsulatus*, *Rhodobacter capsulatus*; *M. jannaschii*, *Methanococcus jannaschii*.

a clade within the known members of the epsilon subdivision of *Proteobacteria* most likely occur within the same groups and possibly in the same organisms. The evolutionary relatedness of the *aclB*, *oorA*, and *porA* genes within the epsilon proteobacterial and *Aquificales* groups, as well as the presence of rTCA cycle genes in *Chlorobium* species and in some members of the domain *Archaea*, also suggests that the rTCA cycle has been present from an early evolutionary point (7, 18, 25).

The phylogenetic results from the *aclB* and *oorA* trees that demonstrate nonevolutionarily similar (nonparallel) clones are not surprising for two reasons. First, we used degenerate primers that may not have amplified all possible genes, given their nucleic acid heterogeneities. Recently, Aoshima et al. described an alternative enzyme system in *Hydrogenobacter thermophilus* TK-6 that has ATP citrate lyase activity but uses a citryl-CoA intermediate (3, 4). The genes involved (citryl-CoA lyase [*ccl*] and citryl-CoA synthase [*ccs*]) are related to the ATP citrate lyase gene. There are several areas of homology between the *ccs* and *aclB* genes, but the primers used in this study most likely would not amplify *ccs* from *H. thermophilus* TK-6. Second, the rTCA cycle genes are also expressed in other metabolic pathways, or the cycle can be reversed for heterotrophic oxidative processes (24, 34). For instance, pyruvate:ferredoxin oxidoreductase is used in the second step of the autotrophic acetyl-CoA pathway (18). The presence and expression of *porA* genes that are not related to the known epsilon proteobacterial sequences and that group with known archaeal sequences in some cases suggests the potential use of the acetyl-CoA pathway by free-living microorganisms at deep-sea hydrothermal vents. Additional culture-based studies along with molecular studies involving the use of primers specific for the key gene of the acetyl-CoA pathway (acetyl-CoA synthase/CO dehydrogenase) would be needed for this determination (18).

TABLE 2. Molecular characterization of sulfur-oxidizing enrichments

Dive/enrichment	Temp (°C)	Electron acceptor	Dominant DGGE band[a]	No. of other bands	rTCA genes	Calvin cycle genes *cbbL*/*cbbM*
3833/Sub2[b]	42	O_2	1	0	+	−/−
3833/Sub2	42	NO_3^-	1	1	+	Weak +/−
3836/Sub1	42	O_2	1	4	+	−/−
3837/Sub1	42 or 55	O_2	1	5	+	−/−
3838[b]	RT[c]	O_2	1	4	+	Weak +/−
3838	42	O_2	2	4	+	−/−

[a] All sequenced dominant band(s) grouped with members of the epsilon subdivision of *Proteobacteria.* DGGE, denaturing gradient gel electrophoresis.
[b] rTCA and *cbbL* gene fragments (Fig. 2 and 3) and dominant 16S rRNA genes sequenced (corresponding to bp 1 to 519 of *E. coli* 16S rRNA genes.
[c] RT, room temperature.

The extent of phylogenetic diversity of form I RubisCO in this study was less than that observed by Elsaied and Naganuma in a previous study analyzing RubisCO molecular diversity of bacteria at deep-sea hydrothermal vents (14). All of

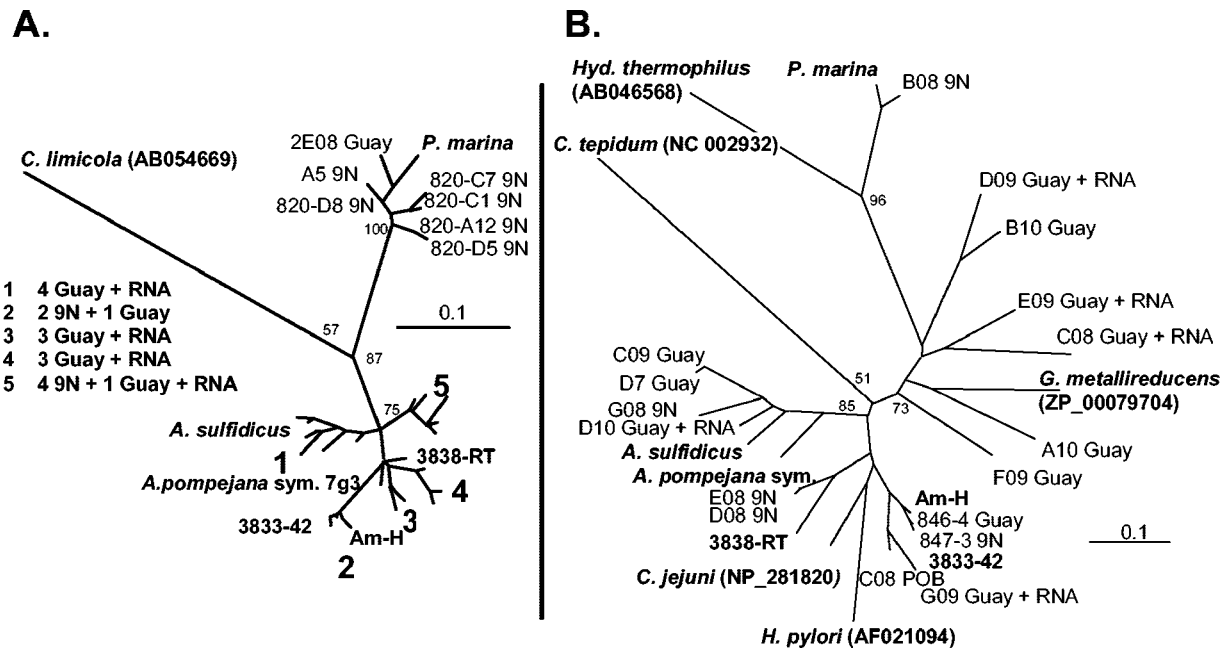

FIG. 4. Phylogenetic analysis of translated *aclB* (ATP citrate lyase, beta subunit) (A) and *oorA* (2-oxoglutarate:ferredoxin oxidoreductase, alpha subunit) (B) clone sequences. Clones labeled with 9N are from the 9°N P-vent sample and those labeled with Guay are from the Guaymas Basin bacterial sample. Guaymas clones originating from RNA (Guay + RNA) and clusters 1 to 5 are indicated. Neighbor-joining distance trees were generated on the basis of alignment of 96 and 91 translated amino acids (minus insertions and deletions) from the *aclB* and *oorA* genes, respectively. The *Chlorobium limicola aclB* sequence (BAB21375) distance was resized to half of its original length for sizing reasons. Bootstrap values from 100 resamplings are indicated prior to the branch points of the tree. The scale bar represents the calculated number of changes per amino acid position. Boldface type refers to known or previously characterized gene sequences. Accession numbers are shown in parentheses. Clones identical at the amino acid level were not included in the trees. *A. pompejana* sym., *Alvinella pompejana* symbiont; *Hyd. thermophilus*, *Hydrogenobacter thermophilus*; *G. metallireducens*, *Geobacter metallireducens*.

the form I (*cbbL*) sequences observed from the P vent (9°N) sample clustered with the type IA *cbbL* sequences, while both type IA and IB sequences were found from deep-sea hydrothermal vent water and chimney samples in the previous study (14). Interestingly, except for the one enrichment sample (3838-RT), we did not find sequences closely related to *Thiobacillus*-like *cbbL* genes, which have previously been isolated from hydrothermal vents (11). Form II RubisCO (*cbbM*) diversity was less than that of form I in the hydrothermal vent we examined and paralleled previously published work (14).

This is the first demonstration of the overwhelming presence and expression of key rTCA cycle genes in at least two groups of free-living microorganisms from deep-sea hydrothermal vents. While members of the epsilon subdivision of *Proteobacteria* and members of the *Aquificales* order are globally ubiquitous in a variety of environmental habitats besides hydrothermal vents (39), the epsilon *Proteobacteria* dominate deep-sea vents (23, 35, 39, 46) and possibly the deep subsurface (31, 52) where the total biomass of prokaryotes has been estimated to exceed that of all other habitats on earth combined (53). Although the Calvin cycle is widely distributed (14, 45), our molecular evidence suggests it is less prevalent in the hydrothermal vent samples we analyzed. This study provides evolutionary insight for the hypothesis that a version of the rTCA cycle was the first autotrophic pathway to have evolved (51) and demonstrates that it is an important carbon fixation pathway in deep-sea hydrothermal vents and potentially in other environments, such as the deep subsurface.

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