Short Communication

Genetic Evidence for Bacterial Chemolithoautotrophy Based on the Reductive Tricarboxylic Acid Cycle in Groundwater Systems

ALBIN ALFREIDER* and CARSTEN VOGT

1Institute of Ecology, University of Innsbruck, Technikerstr. 25, 6020 Innsbruck, Austria; and 2Department of Isotope Biogeochemistry, Helmholtz Centre for Environmental Research–UFZ, Permoserstraße 15, 04318 Leipzig, Germany

(Received August 11, 2011—Accepted November 29, 2011—Published online February 22, 2012)

Geologically and chemically distinct aquifers were screened for the presence of two genes coding for key enzymes of the reverse tricarboxylic acid (rTCA) cycle in autotrophic bacteria, 2-oxoglutarate : ferredoxin oxidoreductase (aclB) and the beta subunit of ATP citrate lyase enzymes (oorA). From 42 samples investigated, aclB genes were detected in two and oorA genes in six samples retrieved from polluted and sulfidic aquifers. aclB genes were represented by a single phytype of almost identical sequences closely affiliated with chemolithoautotrophic Sulfurimonas species. In contrast, sequences analysis of oorA genes revealed diverse phytypes mainly related to sequences from cultivation-independent studies.

Key words: Groundwater, chemautotroph, CO₂ fixation, reverse tricarboxylic acid cycle

To date, six mechanisms are known by which autotrophic organisms fix carbon (7). A frequent CO₂ fixation strategy in chemolithoautotrophic bacteria occurs via the Calvin-Benson-Bassham cycle (6). The enzyme responsible for the actual fixation of CO₂ is ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) and genes coding for the large subunit of RubisCO are often used to analyse chemolithoautotrophs in cultivation-independent environmental studies. Several investigations have been performed in groundwater systems, which revealed the widespread occurrence of diverse forms of RubisCO and hence autotrophic bacteria assimilating CO₂ via the Calvin cycle pathway in the subsurface (2, 4).

The reductive tricarboxylic acid cycle (rTCA) is, in addition to the Calvin cycle, a carbon fixation pathway which is frequently found in prokaryotes: The rTCA cycle appears to operate in phylogenetically diverse autotrophic bacteria (Aquificae, Nitrosospira, Chlorobi, Proteobacteria), including genera of anoxic phototrophic bacteria, sulfate-reducing bacteria and hyperthermophilic hydrogen-oxidizing bacteria (7, 17). Although special biochemical adaptations to oxic conditions are known, this pathway involves enzymes that are sensitive to oxygen. As a result, rTCA-based CO₂ fixation is mostly found in anaerobes or microaerophiles (7). Cultivation-independent investigation based on the detection of genes coding for key enzymes in the rTCA pathway were mainly performed in hydrothermal vent systems and thermal springs (e.g. 10, 14, 18, 27). The results of these studies suggest that the rTCA cycle is a major autotrophic pathway in thermal and sulfidic environments and molecular evidence also suggests that the Calvin cycle is often less prevalent in these ecosystems. On the other hand, autotrophy based on the rTCA cycle is not restricted to hot ecosystems. Several isolates from bacterial enrichment studies and environmental investigations performed with samples from terrestrial ecosystems, activated sludge, saline lakes, sulfidic caves, nonvent marine systems and an estuary revealed the presence of taxonomically diverse bacteria potentially using the rTCA cycle for CO₂ fixation (e.g. 8, 11, 21, 23, 24, 29, 31, 37 and references therein); however, their ecological significance in these habitats is often not well understood.

To our knowledge, the distribution and diversity of autotrophic bacteria assimilating CO₂ via the rTCA pathway has not been analyzed in groundwater systems. In the present study, 42 groundwater samples from a variety of aquifers were screened for the presence of two genes coding for key enzymes of the rTCA cycle: the alpha subunit of 2-oxoglutarate : ferredoxin oxidoreductase enzymes (oorA genes) and the beta subunit of ATP citrate lyase enzymes (aclB genes). Amplification products, which were successfully retrieved from six samples, were used to construct clone libraries and selected positive clones were sequenced in order to evaluate their phylogenetic relationships.

Successful amplification of aclB and oorA genes was achieved with samples from terrestrial ecosystems, activated sludge, saline lakes, sulfidic caves, nonvent marine systems and an estuary. The results of these studies suggest that the rTCA cycle is a major autotrophic pathway in thermal and sulfidic environments and molecular evidence also suggests that the Calvin cycle is often less prevalent in these ecosystems. On the other hand, autotrophy based on the rTCA cycle is not restricted to hot ecosystems. Several isolates from bacterial enrichment studies and environmental investigations performed with samples from terrestrial ecosystems, activated sludge, saline lakes, sulfidic caves, nonvent marine systems and an estuary revealed the presence of taxonomically diverse bacteria potentially using the rTCA cycle for CO₂ fixation (e.g. 8, 11, 21, 23, 24, 29, 31, 37 and references therein); however, their ecological significance in these habitats is often not well understood.

To our knowledge, the distribution and diversity of autotrophic bacteria assimilating CO₂ via the rTCA pathway has not been analyzed in groundwater systems. In the present study, 42 groundwater samples from a variety of aquifers were screened for the presence of two genes coding for key enzymes of the rTCA cycle: the alpha subunit of 2-oxoglutarate : ferredoxin oxidoreductase enzymes (oorA genes) and the beta subunit of ATP citrate lyase enzymes (aclB genes). Amplification products, which were successfully retrieved from six samples, were used to construct clone libraries and selected positive clones were sequenced in order to evaluate their phylogenetic relationships.

Successful amplification of aclB and oorA genes was achieved with samples from terrestrial ecosystems, activated sludge, saline lakes, sulfidic caves, nonvent marine systems and an estuary. The results of these studies suggest that the rTCA cycle is a major autotrophic pathway in thermal and sulfidic environments and molecular evidence also suggests that the Calvin cycle is often less prevalent in these ecosystems. On the other hand, autotrophy based on the rTCA cycle is not restricted to hot ecosystems. Several isolates from bacterial enrichment studies and environmental investigations performed with samples from terrestrial ecosystems, activated sludge, saline lakes, sulfidic caves, nonvent marine systems and an estuary revealed the presence of taxonomically diverse bacteria potentially using the rTCA cycle for CO₂ fixation (e.g. 8, 11, 21, 23, 24, 29, 31, 37 and references therein); however, their ecological significance in these habitats is often not well understood.

To our knowledge, the distribution and diversity of autotrophic bacteria assimilating CO₂ via the rTCA pathway has not been analyzed in groundwater systems. In the present study, 42 groundwater samples from a variety of aquifers were screened for the presence of two genes coding for key enzymes of the rTCA cycle: the alpha subunit of 2-oxoglutarate : ferredoxin oxidoreductase enzymes (oorA genes) and the beta subunit of ATP citrate lyase enzymes (aclB genes). Amplification products, which were successfully retrieved from six samples, were used to construct clone libraries and selected positive clones were sequenced in order to evaluate their phylogenetic relationships.

Successful amplification of aclB and oorA genes was achieved with samples from terrestrial ecosystems, activated sludge, saline lakes, sulfidic caves, nonvent marine systems and an estuary. The results of these studies suggest that the rTCA cycle is a major autotrophic pathway in thermal and sulfidic environments and molecular evidence also suggests that the Calvin cycle is often less prevalent in these ecosystems. On the other hand, autotrophy based on the rTCA cycle is not restricted to hot ecosystems. Several isolates from bacterial enrichment studies and environmental investigations performed with samples from terrestrial ecosystems, activated sludge, saline lakes, sulfidic caves, nonvent marine systems and an estuary revealed the presence of taxonomically diverse bacteria potentially using the rTCA cycle for CO₂ fixation (e.g. 8, 11, 21, 23, 24, 29, 31, 37 and references therein); however, their ecological significance in these habitats is often not well understood.

To our knowledge, the distribution and diversity of autotrophic bacteria assimilating CO₂ via the rTCA pathway has not been analyzed in groundwater systems. In the present study, 42 groundwater samples from a variety of aquifers were screened for the presence of two genes coding for key enzymes of the rTCA cycle: the alpha subunit of 2-oxoglutarate : ferredoxin oxidoreductase enzymes (oorA genes) and the beta subunit of ATP citrate lyase enzymes (aclB genes). Amplification products, which were successfully retrieved from six samples, were used to construct clone libraries and selected positive clones were sequenced in order to evaluate their phylogenetic relationships.

Successful amplification of aclB and oorA genes was achieved with samples from terrestrial ecosystems, activated sludge, saline lakes, sulfidic caves, nonvent marine systems and an estuary. The results of these studies suggest that the rTCA cycle is a major autotrophic pathway in thermal and sulfidic environments and molecular evidence also suggests that the Calvin cycle is often less prevalent in these ecosystems. On the other hand, autotrophy based on the rTCA cycle is not restricted to hot ecosystems. Several isolates from bacterial enrichment studies and environmental investigations performed with samples from terrestrial ecosystems, activated sludge, saline lakes, sulfidic caves, nonvent marine systems and an estuary revealed the presence of taxonomically diverse bacteria potentially using the rTCA cycle for CO₂ fixation (e.g. 8, 11, 21, 23, 24, 29, 31, 37 and references therein); however, their ecological significance in these habitats is often not well understood.
important electron-accepting process at the site. Although oxygen concentrations were not measured in situ, the presence of sulfide in the investigated sampling sites indicates anoxic, sulfidic conditions in the aquifer (Table 1).

Groundwater systems, where rTCA cycle genes were not detected, included sampling campaigns performed in non-polluted and shallow groundwater systems (Salzburg, Austria), a methyl tert-butyl ether (MTBE), BTEX and ammonia-polluted groundwater remediation test site near Leuna (Germany) and from deep subsurface samples retrieved by exploratory drilling along the projected Brenner Base Tunnel (Tyrol, Austria) (Table 1); these sampling sites are described in more detail elsewhere (4).

For DNA extraction, volumes between 300 to 500 mL groundwater were concentrated on filters (pore size 0.22 μm; Durapore, Millipore, Bedford, MA, USA) and immediately frozen until analysis. Aquifer sediments from the underground columns in Zeitz and Bitterfeld were sampled with sample lances, immediately transferred to an anoxic jar (AnaeroCult A; Merck, Germany), transported at 4°C and stored at −20°C before further processing. DNA extraction of groundwater and sediment samples was performed with the FastDNA Spin Kit for soil (Qbiogene, Carlsbad, CA, USA) according to the manufacturer’s instructions and as described by Alfreider et al. (4). Two sets of oligonucleotide primers were used for PCR amplification of aciB and oorA genes as described in Campbell et al. (9) and Campbell and Cary (10). PCR products of appropriate length were cloned into a pDrive Cloning Vector (Qiagen, Valencia, CA, USA) according to the protocols provided by the manufacturer and as described in Alfreider et al. (4). Plasmid DNA was isolated (Qiagen plasmid kit; Qiagen), and clones were screened for the presence of inserts by PCR using vector-specific primers. Full PCR fragments were sequenced with capillary genetic analyzers (ABI 3730 or ABI3700; Applied Biosystems, Foster City, CA, USA) by an external sequencing facility (Macrogen, Seoul, Korea). Closest relatives to oorA and aciB nucleotide sequences and deduced amino acid sequences were obtained using NCBI’s sequence similarity search tools BLASTN and BLASTP (5), the IMG/M data management and analysis system for metagenomes (26) and CAMERA metagenomic resources (30). Deduced amino acids were aligned using Clustal W, as provided by MEGA 4 software and analysis system for metagenomes (26) and CAMERA.

### Table 1. Description of samples and detection of aciB and oorA genes based on PCR amplification

| Sampling site | Depths (mbs) | Oxygen mg L⁻¹ | Sulfide mg L⁻¹ | Sulfate mg L⁻¹ | Ammonium mg L⁻¹ | Nitrate mg L⁻¹ | Nitrite mg L⁻¹ | Main pollutant | aciB | oorA |
|---------------|--------------|---------------|---------------|---------------|----------------|---------------|---------------|---------------|------|------|
| Municipal groundwater Salzburg (5 samples) | | | | | | | | | | |
| Groundwater samples (S1, S2, S3, S5, S6) | 2.9–10.1 | 3.6–8.5 | b.d. | 7.8–31.3 | b.d. | 2.5–39 | b.d. | Non-polluted | – | – |
| Reactor facility Bitterfeld (5 samples) | | | | | | | | | | |
| Aquifer sediment samples (B1, B2, B3, B4) | column b.d. | n.d. | 681–871 | 5–5.4 | b.d. | b.d. | Chlorobenzene | – | – |
| Groundwater inflow from horizontal well (B5) | 23 | b.d. | b.d.–1 | 714 | 5.3 | b.d. | b.d. | Chlorobenzene | + | + |
| Pilot Plant Leuna (6 samples) | | | | | | | | | | |
| Groundwater samples (L1–L6) | channels | 0.4–1.6 | b.d.–0.04 | 473–601 | 45.6–61.4 | b.d.–3.49 | b.d.–1.7 | MTBE | – | – |
| Test field Zeitz (10 samples) | | | | | | | | | | |
| Pumice sample (Z1) | column | n.d. | 23.5 | 332 | 2.5 | n.d. | n.d. | Benzene | – | – |
| Coarse sand sample (Z2) | column | n.d. | 12.2 | 391 | 1.9 | n.d. | n.d. | Benzene | – | – |
| Groundwater sample—column inflow (Z3) | n.d. | 11.1 | 391 | 2.2 | n.d. | n.d. | Benzene | – | – |
| Groundwater sample (Z4) | 13.9 | n.d. | 0.6 | n.d. | 1.1 | n.d. | n.d. | Benzene | – | – |
| Groundwater sample (Z5) | 19.1 | n.d. | 8.1 | n.d. | 5.5 | n.d. | n.d. | Benzene | + | + |
| Groundwater sample (Z6) | 42 | n.d. | 4.6 | n.d. | 3.6 | n.d. | n.d. | Benzene | – | + |
| Groundwater sample (Z7) | 47 | n.d. | 4 | n.d. | 3.7 | n.d. | n.d. | Benzene | – | – |
| Groundwater sample (Z8) | 53 | n.d. | 0.7 | n.d. | 3.3 | n.d. | n.d. | Benzene | – | + |
| Groundwater sample (Z9) | 20 | n.d. | 0.7 | n.d. | 7.7 | n.d. | n.d. | Benzene | – | – |
| Groundwater sample (Z11) | 11.5 | n.d. | 0.3 | n.d. | b.d. | n.d. | n.d. | Benzene | – | + |
| Deep subsurface—Brenner base tunnel (16 samples) | | | | | | | | | | |
| Groundwater samples (V1–V5; P1, P2; N; A1–A3; E1–E3; M1, M2) | 48–780 | n.d. | b.d. | 14–1296 | b.d.–0.24 | b.d.–2.56 | b.d.–0.04 | Non-polluted | – | – |

b.d.: below detection limit
n.d.: not determined
+ PCR amplification product (samples underlined)
– no PCR amplification product
products were cloned and positive clones with the proper insert length were selected for sequence analysis. ATP citrate lyase genes were detected in sampling station Bitterfeld B5 with seven identical sequences (based on deduced amino acid sequences) and one sequence was retrieved from the groundwater well Zeitz Z5 (Fig. 1). Phylogenetic analysis revealed that these sequences formed a unique cluster and showed 90 to 91% amino acid sequence similarities with ε-Proteobacterial aclB sequences. These closest relatives include deep-sea hydrothermal Sulfurimonas isolates (32), deep-sea hydrothermal chemolithoautotrophic isolates of ε-Proteobacteria, epibiotic bacteria associated with the hydrothermal vent polychaete Alvinella pompejana (9) and free-living microorganisms obtained from deep-sea hydrothermal vents (10). However, the sequence with the highest similarity to aclB genes obtained from samples B5 and Z5 was found in the non-vent bacterium Sulfurimonas denitrificans, a mesophilic sulfur oxidizer isolated from coastal marine sediments (31, 34).

In contrast to the aclB genes, the analysis of sequences coding for 2-oxoglutarate:ferredoxin oxidoreductase enzymes showed a high degree of diversity. The amino acid sequences deduced from oorA genes sequences retrieved from horizontal well B5 (sampling station Bitterfeld) revealed three phylogenotypes (Fig. 2). Sequence Bitterfeld B5-A5 was affiliated with several sequences obtained from sampling stations Z5, Z6 and Z11 of the Zeitz aquifer. Sequence Bitterfeld B5-2 and the closely related sequence Zeitz Z6-B21 are affiliated with Syntrophobacter fumaroxidans str. MPOB (89% sequence similarity). Two Bitterfeld B5 clones (B5-A9 and B5-A6) were clearly separated from all other oorA genotypes retrieved in this study (Fig. 2). Both sequences clustered with oorA genes derived from deep-sea hydrothermal chemolithoautotrophic isolates and cultivation-independent studies. Sulfurimonas denitrificans was the closest relative with 94% amino acid similarity. As already mentioned above, Sulfurimonas denitrificans and other Sulfurimonas species were also the closest relatives of all aclB sequences observed at sampling station Bitterfeld B5. S. denitrificans, an obligate chemolithoautotrophic ε-Proteobacterium, is a metabolically versatile bacterium with the potential to use a variety of compounds as electron donors, e.g. reduced sulfur compounds, hydrogen or formate (31). S. denitrificans, which was originally isolated from coastal marine sediments (34), is also suggested to be a key player in the nitrogen cycle in the Baltic Sea, responsible for chemolithoautotrophic denitrification (8, 13). In addition to nitrate, S. denitrificans can use inorganic sulfur compounds or oxygen as electron acceptors (31).

The oorA sequences derived from the sampling stations Zeitz were widespread along the phylogenetic tree. Most sequences were not closely affiliated with known oorA genotypes deposited in public databases and therefore these genotypes were also distantly related to cultivated representatives. Consequently, it would be too speculative to deduce ecophysiological characteristics from these sequences. Comparison with deduced amino acid sequences retrieved from sequences of public databases showed closest similarities ranging between 76% and 92%. For example one cluster of oorA sequences from groundwater well Zeitz Z11 (clones 31, B46, B47, B50) was affiliated (84%–88% deduced amino acid identity) with an oorA gene obtained from a metagenomic study of a hypersaline mat from Guerrero Negro, Mexico, which is characterized by steep physicochemical gradients on the millimeter scale (22). Several oorA genes retrieved in our study were affiliated with sequences of different metagenomic investigations, including a thermophilic terephthalate-degrading microbial community growing at around 55°C that was developed in a lab-scale bioreactor (IMG/M database, not published), oorA genes from a highly stable reductive dechlorinating bioreactor (IMG/M database, not published), microbial communities from Yellowstone...
hot springs and hot spring pools (IMG/M database, not published) and symbionts from the marine oligochaete Olavius algarvensis (38). Summarizing the phylogenetic analysis based on genes coding for 2-oxoglutarate:ferredoxin oxidoreductase enzymes revealed that the majority of sequences were affiliated with **oorA** sequences derived from cultivation independent studies, generally thermal ecosystems and/or environments that are characterized by distinct concentration gradients and different redox states of biogeochemically important elements supporting reverse tricarboxylic acid cycle as the autotrophic pathway.

The presence of phylogenetically distant **oorA** sequences, however, suggests the occurrence of **oorA** phylotypes coding for 2-oxoglutarate:ferredoxin oxidoreductase enzymes involved in other (non rTCA-related) biochemical processes. A reaction that could specifically be of interest at study site Zeitz is the potential role of 2-oxoglutarate:ferredoxin oxidoreductase for aromatic ring reduction (12), since field site Zeitz is contaminated with BTEX compounds with benzene as the main pollutant. Degradation of benzene and toluene under sulfate-reducing conditions has been demonstrated at the site (36), and some of the **oorA** sequences are affiliated with sequences detected in sulfate-reducing or putative syntrophic bacteria (Fig. 2) that are potentially involved in anaerobic BTEX degradation.

To our knowledge, this is the first comprehensive study demonstrating the existence of bacteria with genetic potential using a pathway other than the Calvin-Benson-Bassham cycle involved in other (non rTCA-related) biochemical processes. Fig. 2. Neighbor-joining phylogenetic tree calculated from deduced amino acids of **oorA** sequences obtained in this study (shown in bold) and public databases. Accession numbers of sequences are given in parentheses. Reference sequences from metagenomic libraries were obtained from the Joint Genome Institute database (IMG) and CAMERA database (JCVI_PEP). Bootstrap values are shown as percentages of 1,000 replicates and values over 50% are indicated on nodes.
for CO₂ fixation in groundwater ecosystems. From earlier investigations based on RubiscoCO analysis we have learned that in samples retrieved from the same groundwater sampling sites, the Calvin cycle is widely distributed (in 85% of 48 samples) and used by diverse obligate and facultative chemolithoautotrophic Proteobacteria for CO₂ fixation (4).

In the present study, genes for the rTCA cycle were only found in six samples at two sampling locations, with groundwater characterized by anoxic and sulfidic conditions and contaminated with organics. One obvious reason for the limited distribution is the properties of certain enzymes in the rTCA cycle, which are sensitive to oxygen. As a result, the rTCA cycle is only found in anaerobes or microaerophiles. So far, the reductive TCA cycle has been believed to be the main strategy for chemolithoautotrophic CO₂ fixation in hydrothermal vents and other hot ecosystems. The microbial communities in these habitats are usually dominated by autotrophic ε-Proteobacteria and Aquificales, which generally fix CO₂ via the rTCA pathway (10, 17, 27). CO₂ fixation based on the rTCA pathway was also shown for a small number of bacterial representatives phylogenetically related to Chlororibiales, δ-Proteobacteria and magnetotactic cocci affiliated with the class of α-Proteobacteria (37), but their environmental importance has yet to be determined.

Previous studies at sampling sites Zeitz and Bitterfeld, focusing on the bacterial composition based on 16S rDNA analysis, revealed the dominance of β-Proteobacteria and a broad diversity of other phyla (1, 3). Samples from the Bitterfeld site revealed two clone sequences which were phylogenetically classified into the class of ε-Proteobacteria. Interestingly, the 16S rDNA sequence of one of these clones (RA9C8) was affiliated with the facultatively anaerobic, chemolithoautotrophic, sulfur-oxidizing bacterium Sulfuricurvum kujiense (20) and representatives of the genus Sulfurimonas, including S. denitrificans and S. autotrophica (data not shown). A recent study also revealed 16S rDNA sequences related to ε-Proteobacteria at field site Zeitz (19). A phylotype affiliated to the genus Sulfurovum was found to be abundant in a column system operating under close to in situ conditions (19); this phylotype has been shown to be involved in anaerobic benzene degradation (15). Furthermore, a phylotype closely related to S. denitrificans has been recently identified in nitrate-dependent sulfide-oxidizing enrichment cultures prepared from groundwater from field site Zeitz.

The lifestyle of the versatile ε-Proteobacteria in organics-contaminated sulfidic sites is not well understood and should be the focus of future studies. Some members of this taxonomic group are autotrophic, using e.g. reduced sulfur compounds or hydrogen as electron donors, oxygen, nitrate or metals as electron acceptors, and fixing carbon via the rTCA cycle. In contrast, Sulfitrupsirillum or Arcobacter species have been described for fermentative metabolism (25). Recently, phylotypes affiliated mainly to the genus Sulfuricurvum were found to be abundant in toluene-degrading sulfate-reducing enrichment cultures prepared from tar-oil contaminated aquifer sediment samples; their function remained unclear (28). Notably, Hubert et al. (16) observed that similar phylotypes were dominant in oil formation waters from a Canadian oil sand reservoir; the authors speculate that some of these might use also sulfur-containing crude oil compounds as carbon and/or sulfur sources.

Acknowledgements

This study was funded by the Austrian Science Fund to AA (FWF P17649). We thank Jörg Ahlheim and Ralf Trabitzsch (both UFZ) for logistic support, sample collection and technical assistance in the field.

References

1. Alfreider, A., C. Vogt, and W. Babel. 2002. Microbial diversity in an in situ reactor system treating monochlorobenzene contaminated groundwater as revealed by 16S ribosomal DNA analysis. System. Appl. Microbiol. 25:232–240.
2. Alfreider, A., C. Vogt, D. Hoffmann, and W. Babel. 2003. Diversity of ribulose-1,5-biphosphate carboxylase/oxygenase large-subunit genes from groundwater and aquifer microorganisms. Microb. Ecol. 45:17–328.
3. Alfreider, A., and C. Vogt. 2007. Bacterial diversity and aerobic biodegradation potential in a BTEx-contaminated aquifer. Water Air Soil Pollut. 183:415–426.
4. Alfreider, A., C. Vogt, M. Kaiser, and R. Psenner. 2009. Distribution and diversity of autotrophic bacteria in groundwater systems based on the analysis of RuBisCO genotypes. System. Appl. Microbiol. 32:140–150.
5. Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403–410.
6. Badger, M.R., and E.J. Bek. 2008. Multiple Rubisco forms in proteobacteria: their functional significance in relation to CO₂ acquisition by the CBB cycle. J. Exp. Bot. 59:1525–1541.
7. Berg, I.A. 2011. Ecological aspects of the distribution of different autotrophic CO₂ fixation pathways. Appl. Environ. Microbiol. 77:1925–1936.
8. Bretar, I., M. Labrenz, S. Flavier, J. Botel, H. Kuosa, R. Christen, and M.G. Hoeftle. 2006. Identification of a Thiomicrospira denitrificans-like epsilonproteobacterium as a catalyst for autotrophic denitrification in the central Baltic Sea. Appl. Environ. Microbiol. 72:1364–1372.
9. Campbell, B.J., J.L. Stein, and S.C. Cary. 2003. Evidence of tricarboxylic acid cycle pathways in free-living microorganisms at deep-sea hydrothermal vents. Appl. Environ. Microbiol. 70:6282–6289.
10. Campbell, B.J., A.S. Engel, M.L. Porter, and K. Takai. 2006. The rTCA cycle. In contrast, ε-Proteobacteria represent the major portion of chemoun- totrophic bacteria in sulfidic waters of pelagic redoxclines of the Baltic and Black Seas. Appl. Environ. Microbiol. 74:7546–7551.
11. Hall, J.R., K.R. Mitchell, O. Jackson-Weaver, A.S. Kooser, B.R. Cron, L.I. Crossley, and C.D. Takacs-Vesbach. 2008. Molecular characterization of the diversity and distribution of a thermal spring microbial community using 16S rDNA and metabolic genes. Appl. Environ. Microbiol. 74:4910–4922.
12. Herrmann, S., S. Kleinsteuber, A. Chatzinotas, S. Kuppardt, T. Lueders, H.H. Richnow, and C. Vogt. 2010. Functional characterization of an anaerobic benzene-degrading enrichment culture by DNA stable isotope probing. Environ. Microbiol. 12:401–411.
13. Hubert, C.R.J., T.B.P. Oldenburg, M. Fustie, et al. 2012. Massive dominance of Epsilonproteobacteria in formation waters from a Canadian oil sands reservoir containing severely biodegraded oil. Environ. Microbiol. 14:387–404.
17. Huegler, M., C.O. Wirsen, G. Fuhs, C.D. Taylor, and S.M. Sievert. 2005. Evidence for autotrophic CO$_2$ fixation via the reductive tricarboxylic acid cycle by members of the ε subdivision of proteobacteria. J. Bacteriol. 187:3020–3027.

18. Huegler, M., A. Gaertner, and J.F. Imhoff. 2010. Functional genes as markers for sulfur cycling and CO$_2$ fixation in microbial communities of hydrothermal vents of the Logatchev field. FEMS Microbiol. Ecol. 73:526–537.

19. Kleinsteuber, S., K.M. Schleinitz, J. Breitfeld, H. Harms, H.H. Richnow, and C. Vogt. 2008. Molecular characterization of bacterial communities mineralizing benzene under sulfate-reducing conditions. FEMS Microbiol. Ecol. 66:143–157.

20. Kodama, Y., and K. Watanabe. 2004. *Sulfuricurvum kujiense* gen. nov., sp. nov., a facultatively anaerobic, chemolithoautotrophic, sulfur-oxidizing bacterium isolated from an underground crude-oil storage cavity. Int. J. Syst. Evol. Microbiol. 54:2297–2300.

21. Kovaleva, O.L., T.P. Tourova, G. Muyzer, T.V. Kolganova, and D.Y. Sorokin. 2011. Diversity of RuBisCO and ATP citrate lyase genes in soda lake sediments. FEMS Microbiol. Ecol. 75:37–47.

22. Kunin, V., J. Raes, J.K. Harris, et al. 2008. Millimeter-scale genetic gradients and community-level molecular convergence in a hypersaline microbial mat. Mol. Syst. Biol. 4:198.

23. La Cono, V., F. Smidile, G. Bortoluzzi, et al. 2011. Unveiling microbial life in new deep-sea hypersaline Lake Thetis. Part I: Prokaryotes and environmental settings. Environ. Microbiol. 13:2250–2268.

24. Luecker, S., M. Wagner, F. Maixner, et al. 2010. A *Nitrospira* metagenome illuminates the physiology and evolution of globally important nitrite-oxidizing bacteria. Proc. Natl. Acad. Sci. U.S.A. 107:13479–13484.

25. Luijten, M.L.G.C., J. de Weerdt, H. Smidt, H.T.S. Boschker, W.M. de Vos, G. Schraa, and A.J.M. Stams. 2003. Description of *Sulfurospirillum halorespirans* sp. nov., an anaerobic, tetrachlortoethene-respiring bacterium, and transfer of *Dehalospirillum multivorans* to the genus *Sulfurospirillum* as *Sulfurospirillum multivorans* comb. nov. Int. J. Syst. Evol. Microbiol. 53:787–793.

26. Markowitz, V.M., N.N. Ivanova, E. Szeto, et al. 2008. IMG/M: a data management and analysis system for metagenomes. Nucleic Acids Res. 36:D534–538.

27. Perner, M., R. Seifert, S. Weber, A. Koschinsky, K. Schmidt, H. Strauss, M. Peters, K. Haase, and J.F. Imhoff. 2007. Microbial CO$_2$ fixation and sulphur cycling associated with low-temperature emissions at the Lilliput hydrothermal field, southern Mid-Atlantic Ridge (9°S). Environ. Microbiol. 9:1186–1201.

28. Pilloni, G., F. von Netzer, M. Engel, and T. Lueders. 2011. Electron acceptor-dependent identification of key anaerobic toluene degraders at a tar-oil-contaminated aquifer by Pyro-SIP. FEMS Microbiol. Ecol. 78:165–175.

29. Porter, M.L., and A.S. Engel. 2008. Diversity of uncultured epsilonproteobacteria from terrestrial sulfidic caves and springs. Appl. Environ. Microbiol. 74:4973–4977.

30. Seshadri, R., S.A. Kravitz, L. Smarr, P. Gilna, and M. Frazier. 2007. CAMERA: a community resource for metagenomics. PLoS Biol. 5:e75.

31. Sievert, S.M., K.M. Scott, M.G. Klotz, et al. 2008. The genome of the epsilonproteobacterial chemolithooautotrophic *Sulfurimonas denitrificans*. Appl. Environ. Microbiol. 74:1145–1156.

32. Takai, K., B.J. Campbell, S.C. Cary, et al. 2005. Enzymatic and genetic characterization of carbon and energy metabolism by deep-sea hydrothermal chemolithooautotrophic isolates of Epsilonproteobacteria. Appl. Environ. Microbiol. 71:7310–7320.

33. Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Mol. Biol. Evol. 24:1596–1599.

34. Timmer-Ten Hoor, A. 1975. A new type of thiosulphate oxidizing, nitrate reducing microorganism: *Thiomicrospira denitrificans* sp. nov. Neth. J. Sea. Res. 9:344–350.

35. Vogt, C., A. Alfreider, H. Lorbeer, D. Hoffmann, L. Wuensche, and W. Babel. 2004. Bioremediation of chlorobenzene-contaminated ground water in an *in situ* reactor mediated by hydrogen peroxide. J. Contam. Hydrol. 68:121–141.

36. Vogt, C., S. Goedeke, H.C. Treutler, H. Weiss, M. Schirmer, and H.H. Richnow. 2007. Benzene oxidation under sulfate-reducing conditions in columns simulating in situ conditions. Biodegradation 18:625–636.

37. Williams, T.J., C.L. Zhang, J.H. Scott, and D.A. Bazylinski. 2006. Evidence for autotrophy via the reverse tricarboxylic acid cycle for the marine magnetotactic cocccus strain MC-1. Appl. Environ. Microbiol. 72:1322–1329.

38. Woyke, T., H. Teeling, N.N. Ivanova, et al. 2006. Symbiosis insights through metagenomic analysis of a microbial consortium. Nature 443:950–955.