Evidence for Autotrophy via the Reverse Tricarboxylic Acid Cycle in the Marine Magnetotactic Coccus Strain MC-1

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Strain MC-1 is a marine, microaerophilic, magnetite-producing, magnetotactic coccus phylogenetically affiliated with the α-Proteobacteria. Strain MC-1 grew chemolithotrophically with sulfide and thiosulfate as electron donors with HCO3-/CO2 as the sole carbon source. Experiments with cells grown microaerobically in liquid with thiosulfate and H2 as electron donors showed that all cell carbon was derived from H14CO3-/12CO2, and therefore that MC-1 is capable of chemolithoautotrophy. Cell extracts did not exhibit ribulose-1,5-bisphosphate carboxylase-oxygenase (RubisCO) activity, nor were RubisCO genes found in the draft genome of MC-1. Thus, unlike other chemolithoautotrophic, magnetotactic bacteria, strain MC-1 does not appear to utilize the Calvin-Benson-Bassham cycle for autotrophy. Cell extracts did not exhibit carbon monoxide dehydrogenase activity, indicating that the acetyl-coenzyme A pathway also does not function in strain MC-1. The 13C content of whole cells of MC-1 relative to the 13C content of the inorganic carbon source (Δ13C) was −11.4 ‰. Cellular fatty acids showed enrichment of 13C relative to whole cells. Strain MC-1 cell extracts showed activities for several key enzymes of the reverse (reductive) tricarboxylic acid (rTCA) cycle including fumarate reductase, pyruvate:acceptor oxidoreductase and 2-oxoglutarate:acceptor oxidoreductase. Although ATP citrate lyase (another key enzyme of the rTCA cycle) activity was not detected in strain MC-1 using commonly used assays, cell extracts did cleave citrate, and the reaction was dependent upon the presence of ATP and coenzyme A. Thus, we infer the presence of an ATP-dependent citrate-cleaving mechanism. These results are consistent with the operation of the rTCA cycle in MC-1. Strain MC-1 appears to be the first known representative of the α-Proteobacteria to use the rTCA cycle for autotrophy.

Magnetotactic bacteria are a morphologically, metabolically, and phylogenetically diverse assemblage of prokaryotes that synthesize intracellular, membrane-bound, single-magnetic-domain crystals of the minerals magnetite (Fe3O4) and greigite (Fe3S4) (4). These unique structures, called magnetosomes (3), are generally organized in chains within the cell (5) and cause the cell to passively align along magnetic field lines (21). Magnetotaxis results from this magnetic alignment combined with active cellular motility (21). The discovery of magnetotaxis 30 years ago by Blakemore (9) was based on the observation of large populations of extremely motile magnetotactic cocci all swimming in the same direction. Since then, the magnetotactic cocci have been found to be ubiquitous in marine, brackish, and freshwater habitats (20, 21, 49). The magnetotactic cocci are characterized structurally by being bilophotrichous (possessing two bundles of flagella on one side of the cell) (21, 38, 39). Cells also often possess pili, and some contain intracellular sulfur-rich globules (13, 21, 38). Magnetotactic cocci display a polar preference in their swimming direction and thus exhibit polar magneto-aerotaxis (21); this distinguishes these magnetotactic bacteria from Magnetospirillum spp., which display axial magneto-aerotaxis (21). Phylogenetically, based on 16S rRNA sequences, all known magnetotactic coccus cluster at the base of the α subdivision of the Proteobacteria, the division that contains almost all of the known Fe3O4-producing magnetotactic bacteria (15, 20, 49). Despite the ubiquity of the magnetotactic cocci and the ease of collecting cells in large numbers (39), only one strain of a magnetotactic coccus, presently called strain MC-1, has been isolated and cultivated in axenic culture (21). Strain MC-1 was isolated from a chemically stratified estuary in the Petaquamscut River (R.I.) and appears to be an obligate microaerophile.

We recently demonstrated that the marine magnetotactic bacterial strains MV-1 and MV-2 grow chemolithooautotrophically (6). Cell extracts of these strains show ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) activity, and both strains possess intact form II RubisCO genes (6) and thus rely on the Calvin-Benson-Bassham (CBB) cycle for CO2 fixation and autotrophy. Cells of strain MC-1 also appeared to grow chemolithooautotrophically using sulfide and thiosulfate as electron donors (21, 37). Because strain MC-1 grows microaerophilically (21), it seemed probable that it would also use the CBB cycle for autotrophy and RubisCO for CO2 fixation as in other aerobic chemolithooautotrophic members of the domain Bacteria. However, cell extracts of strain MC-1 did not show RubisCO activity, and typical genes of the various forms of RubisCO are not present in the draft genome sequence of strain MC-1 (sequence available at the Joint Genome Institute website, http://genome.jgi-psf.org/draft_microbes/magm1/magm1.home.html) (6). Recently, we discovered open reading frames (ORFs) in the draft MC-1 genome that have
high sequence identity to pyruvate:ferredoxin oxidoreductase and 2-oxoglutarate:ferredoxin oxidoreductase from autotrophic members of the *Aquificales* that utilize the reverse or reductive tricarboxylic acid (rTCA) cycle for CO2 fixation and autotrophy. This suggested to us that strain MC-1 might also use the rTCA cycle for CO2 fixation. The aim of this study was to further investigate this possibility.

**MATERIALS AND METHODS**

**Bacteria and growth conditions.** The marine magnetotactic coccus strain MC-1 was isolated from water collected from the onic-anoxic interface of the Pettaquamscutt River Estuary, R.I. (15). This strain has now been characterized and will be named shortly (D. A. Bazylinski, unpublished data). Strain MC-1 is an obligate microaerophile and may be an obligate autotroph (D. A. Bazylinski, unpublished results) although it appeared to grow with acetate heterotrophically when it was first isolated (37).

For experiments to determine the contribution of CO2 to cell carbon, cells of strain MC-1 grown microaerobically in semisolid medium at 28°C from small inocula in O2 gradients, with thiosulfate (S2O32-) as an electron donor and radiolabeled [14C]bicarbonate (HCO3-; American Radiolabeled Chemicals, St. Louis, Mo.) as the sole carbon source, as previously described (6).

For enzyme assays, lipid extraction, and isotopic composition measurements, strain MC-1 was grown in liquid medium. Cells were grown in 850 mL of medium in 2-liter glass bottles. The medium consisted of an artificial seawater (6) base to which were added 20% (per liter) inorganic anions, 5 mM of modified Wolfe's mineral elixir (21), 0.25 g of NaHCO3 and 100 µL of 0.2% (wt/vol) aqueous resazurin. The pH of the medium was adjusted to 7.0, 1.26 g of NaHCO3 was added per liter, and the vessel was sealed. The medium was then bubbled with 7.5% CO2 in N2 (flow rate about 100 mL min-1) passed over heated copper wire to remove O2 for 45 min. The medium was sealed and autoclaved. After the autoclaving and cooling steps, the following solutions were injected (per liter): 2 mM ferrous citrate, 1 mg of Na2MoO4, 0.6 g of KH2PO4, 0.9 g of K2HPO4, 0.25 g of fumarate, 100 µM NiCl2; pH 7.0 (34) and then transferred to anaerobic RRNCO medium buffered with 10 mM Tris · HCl, pH 7.0. Cells of *C. tepidum* and *R. rubrum* in 100 mM Tris · HCl, pH 7.0; 10 cm. The reaction was initiated by injection of anaerobic cell extract. Two molecules of CO2 are reduced per each molecule of 2-oxoacid oxidized.

**Analysis and carbon isotope composition of lipids.** (i) Lipid extraction. Cells of strain MC-1 grown autotrophically were freeze-dried and extracted for lipids (55, 61, 62). Lipids were separated on a silicic acid column into fractions containing neutral lipids, glycolipids, and polar lipids (24). The polar lipids were treated using a mild alkaline methanolysis to produce fatty acid methyl esters (FAMES). FAMES were identified using an Agilent 6890 series gas chromatograph interfaced to an Agilent 5973 mass selective detector (61). Mass spectra were determined by electron impact at 70 eV. Methyl heptanoate was used as the internal standard. FAMES were expressed as equivalent peaks against the internal standard.

(ii) Stable carbon isotopes. Carbon isotope compositions of the FAMES were determined using an HP 6890 gas chromatograph connected to a Finnigan MAT Delta Plus-XL mass spectrometer (Scientific Instrument Services, Ringoes, N.J) (61, 62). Carbon isotope compositions of whole cells were also determined. The 13C/12C ratios of whole cells and headspace CO2 were determined using a Delta Plus isotope ratio mass spectrometer (Scientific Instrument Services) with a precision of ±0.2‰.

**Enzyme assays.** The Rubisco activity of cell extracts was determined as described by Beulecker et al. (8), except that the dithionite concentration was changed to 5 mM and the pH was adjusted to 7.0. Pyruvate:ferredoxin oxidoreductase and 2-oxoglutarate:acceptor oxidoreductase activities were determined by measuring the pyruvate- or α-ketoglutarate-dependent reduction, respectively, of methyl viologen (MV) at 578 nm (εMV = 9.8 × 10^4 M^-1 cm^-1) (59) spectrophotometrically. The reactions were carried out anaerobically in serum-stoppered cuvettes under N2 at 25°C. The standard assay mixture (final volume, 2 ml) contained 50 mM KHPO4 buffer, pH 7.0; 0.25 mM EDTA; 1 mM MgCl2, 5 mM MV, 1 mM dithiothreitol, and 10 mM α-ketoglutarate as the substrate. Anaerobic cell extract was injected to initiate each reaction. Two molecules of MV are reduced per each molecule of 2-oxoacid oxidized.

Carbon monoxide dehydrogenase activity was also determined spectrophotometrically by measuring the CO-dependent reduction of MV at 578 nm. The reaction was carried out exactly as for the 2-oxoacid assays above except that CO was the substrate. Reaction mixtures were rendered anaerobic using N2 gas, after which the N2 was replaced by CO. Two molecules of MV are reduced per each molecule of CO oxidized.

Fumarate reductive activity was determined spectrophotometrically based on a procedure described for an assay for nitrous oxide reductase (48). This assay uses photochemically reduced (rather than chemically reduced) benzyl viologen (BV; εBV = 20,000 M^-1 cm^-1) (36) as the electron donor. The reaction mixture (1 ml) contained 50 mM KHPO4 buffer, pH 7.0; 1 mM triethanolamine, 10 mM BV, and 2 mM sodium fumarate. The pH of the final reaction mix was 7.0, and reactions were carried out at 25°C. BV was photochemically reduced to the blue cation radical by exposure to a blue fluorescent bulb (Philips F15T8/B; Atlanta Light Bulbs Inc., Tucker, Ga.) at a distance of about 10 cm. The reaction was initiated by injection of anaerobic cell extract. Two molecules of BV are oxidized per each molecule of fumarate reduced.

Two spectrophotometric assays were used to assay ATP-citrate lyase activity. In the first, acetyl-CoA formed from the ATP-dependent cleavage of citrate reacts with hydroxylamine to form acetyl-hydroxylamine, which then forms a colored complex with Fe3+ that can be measured spectrophotometrically (1, 29). The alternative method is a coupled assay: malate formed by the ATP-dependent cleavage of citrate is determined by the oxidation of NADH in the presence of malate dehydrogenase (MDH) (30). The MDH-coupled assay used here was based on the method of Wahlund and Tabita (53). The reaction mixture (1 ml) contained 10 mM MgCl2, 0.1 mM sodium citrate, 2 mM NADH, 3 mM ATP, 0.9 mM CoA, 100 nkat (6.0 µU) MDH, in 100 mM Tris · HCl, pH 7.0. The reaction was initiated by adding NADH. The oxidation of NADH was determined spectrophotometrically at 340 nm (ε = 6.22 M^-1 cm^-1) at 25°C.

Thin-layer chromatography (TLC) in combination with autoradiography was used to analyze the CO-dependent cleavage of citrate reactants. The first thin-layer chromatography (TLC) run was performed on silica gel 60 F254 plates (Merck, Darmstadt, Germany) with a solvent system that can be measured spectrophotometrically (1, 29). The alternative method is a coupled assay: malate formed by the ATP-dependent cleavage of citrate is determined by the oxidation of NADH in the presence of malate dehydrogenase (MDH) (30). The MDH-coupled assay used here was based on the method of Wahlund and Tabita (53). The reaction mixture (1 ml) contained 10 mM MgCl2, 0.1 mM sodium citrate, 2 mM NADH, 3 mM ATP, 0.9 mM CoA, 100 nkat (6.0 µU) MDH, in 100 mM Tris · HCl, pH 7.0. The reaction was initiated by adding NADH. The oxidation of NADH was determined spectrophotometrically at 340 nm (ε = 6.22 M^-1 cm^-1) at 25°C.

**Preparation of cell extracts.** Cell extracts of MC-1, *C. tepidum*, and *R. rubrum* were prepared by harvesting cells grown in liquid culture by centrifugation at 6,000 × g for 15 min at 4°C. MC-1 cells were washed once in artificial seawater buffered with 10 mM Tris · HCl, pH 7.0. Cells of *C. tepidum* were washed once in 100 mM Tris · HCl, pH 7.0; *R. rubrum* cells were washed once in 50 mM KHPO4 buffer, pH 7.0. Cells were then resuspended in 50 mM KHPO4, pH 7.0, and lyzed by two to three passages through a French pressure cell at 124 MPa. The lysate was kept anaerobic by releasing it into a N2-filled tube connected to the exit of the French pressure cell and by carrying out all transfers of the lysate in an anaerobic chamber (Coy Laboratory Products, Grass Lake, Mich.). Cell lysates were centrifuged anaerobically at 10,000 × g for 30 min at 4°C to remove nonlysed cells, cell debris, and magnetosomes when present. Protein in cell extracts was measured using Coomassie brilliant blue (10) with a Bio-Rad protein assay kit (Bio-Rad Laboratorys, Hercules, Calif.). Lyophilized bovine serum albumin was used as a standard (Sigma-Aldrich, St. Louis, Mo.).
Cellulose F; EMD Chemicals Inc., Gibbstown, N.J.) were equilibrated in a sealed glass chamber containing 1-pantonalformic acid:water (48:8:48:8:2.4). A total of 10 μl of reaction mixture was spotted onto the plate at timed intervals. TLC proceeded until the solvent front reached the top of the plate. 

RESULTS

Growth experiments. Cells of strain MC-1 can now be grown routinely in liquid culture. Initiation of growth, however, is dependent on the formation of an [O2] gradient in the medium. This, in turn, was dependent on the amount of O2 that was introduced into anaerobic medium and the size of the inoculum (number of cells). Typically, the amount of O2 that allowed growth to proceed from an initial inoculum of 1% was about 0.5% O2 in the headspace. Higher amounts resulted in much longer lag periods or no growth. Growth of strain MC-1 always initiated at the oxic-oxic interface (based on the color of the resazurin in the medium), and eventually cells formed a macroscopic biofilm on the glass at the surface of the medium.

The growth medium contained S2O3 as the electron donor and radiolabeled H14CO3 as the sole carbon source. Growth in the form of a thick layer at the surface. At this point, additional O2 could be introduced to the culture, which resulted in increased growth cannot be measured without disrupting the gradient, thereby interfering with cell growth if not completely inhibiting it. Using cell numbers before and after growth, we estimate an average doubling time of ~30 h for strain MC-1 in these gradient cultures.

The growth medium contained S2O3− as the electron donor and no organic carbon other than a small amount of vitamins and cysteine. Cells of strain MC-1 did not grow, however, when S2O3− was omitted. The major source of carbon in the medium was CO2/HCO3−. Cells produced internal S-rich globules when grown on S2O3− (D. A. Bazylinski and B. L. Cox, unpublished data). Elemental S also precipitated external to the cell in the growth medium (data not shown).

Bicarbonate/O2 as source of cell carbon. Cells of strain MC-1 were grown microaerobically from small inocula in [O2] gradients with S2O3− as an electron donor and radiolabeled H14CO3− as the sole carbon source. For each experiment, the amount of 14C from H14CO3− incorporated into protein was compared with the calculated amount of protein carbon determined by protein measurements and an average carbon content of protein of 54% (16, 32) (Table 1). The results show that the average percentage (triplicate analysis) of protein carbon derived from H14CO3− in cells of strain MC-1 was 99.9 ± 2.5%.

Profiles and carbon isotopic compositions of whole cells and fatty acids of strain MC-1. The fatty acid profile of strain MC-1 was dominated by 16:1, 16:0, and 18:1 (Table 2) fatty acids. The isotopic compositions of these fatty acids were −21.8 to −24.5‰, which were 3.7 to 6.5‰ higher (more positive) than that of whole cells (−28 ‰) (Table 2).

Enzyme assays. RubisCO activity was not detected in cell extracts of strain MC-1. Cell extracts of chemolithoautotrophically grown strain MV-1 were used as a positive control (6) and

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<th>Parameter or component</th>
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<td>Whole cells-CO2</td>
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* Values were determined in duplicate MC-1 samples designated MC-1A and MC-1B.
* Values were determined for MC-1A plus MC-1B.
* Dominated by 16:1ω7c.
* Weighed average of 16:1ω7c and 16:1a of an unknown double-bond position.
* Dominated by 18:1ω7c.
* Isotopic composition for 18:1ω7c only.

δ13C, %C incorporated into protein fraction as a percentage of total CO2 fixed.
showed a RubisCO-specific activity of 10.2 nmol of CO₂ fixed min⁻¹ mg⁻¹ of cell protein. Strain MV-1 is known to utilize the CBB cycle (6).

MC-1 cell extracts showed both pyruvate:acceptor (MV) and 2-oxoglutarate:acceptor (MV) oxidoreductase activity: 3.9 ± 0.9 nmol min⁻¹ mg⁻¹ of cell protein⁻¹ and 9.1 ± 5.5 nmol min⁻¹ mg⁻¹ of cell protein⁻¹, respectively (values given as mean ± standard deviation of triplicate analyses). In both cases, the reduction of MV was dependent upon the presence of the substrate, CoA, or cell extract. Activity was not detected when the cell extract was heated to 100°C for 5 min prior to its introduction in the assay mixture. Fumarate reductase activity in the cell extract was heated to 100°C for 5 min prior to its introduction in the assay mixture. Fumarate reductase activity was determined to be 21.4 nmol min⁻¹ mg⁻¹ of cell protein. Strain MV-1 is known to use the TCA cycle (6).

Cells of strain MC-1 grow well microaerobically in semisolid liquid growth medium containing an [O₂] gradient and S. O₂⁻ as an electron donor. The main source of carbon is CO₂/HCO₃⁻, and the medium contained only traces of organic carbon as low amounts of vitamins and cysteine, which they

**DISCUSSION**

Cells of strain MC-1 grow well microaerobically in semisolid liquid growth medium containing an [O₂] gradient and S. O₂⁻ as an electron donor. The main source of carbon is CO₂/HCO₃⁻, and the medium contained only traces of organic carbon as low amounts of vitamins and cysteine, which they
cannot grow on alone. It thus seems likely that strain MC-1 grows as a chemolithoautotroph in this medium. To demonstrate autotrophy in strain MC-1, we determined both the amount of $^{13}$C from H$^{14}$CO$_3^-$ incorporated into protein and the total amount of protein in separate experimental cultures. We then calculated the amount of protein carbon from protein determinations using an average carbon content of protein of 54% (16, 32). The amount of $^{13}$C from H$^{14}$CO$_3^-$ incorporated into protein was compared to the calculated amount of protein carbon. The percentage of calculated protein carbon derived from H$^{14}$CO$_3^-$ was 99.9% for strain MC-1. We infer from this result that all cell carbon in strain MC-1 grown with S$_2$O$_3^{2-}$ comes from H$^{14}$CO$_3^-$/$^{12}$CO$_2$, and we conclude that strain MC-1 grows chemolithoautotrophically.

Because strain MC-1 is an obligate microaerophile (4, 20) and because other magnetotactic bacteria have been shown to use the CBB cycle for autotrophy, we suspected that it would also use the CBB cycle and RubisCO for CO$_2$ fixation as do most other aerobic chemolithoautotrophic bacteria. Cell extracts of strain MC-1 did not exhibit RubisCO activity, however, and the various forms of RubisCO genes could not be found in the draft genome sequence of strain MC-1 (6). We have also ruled out the possibility that strain MC-1 uses the acetyl-CoA pathway because we did not detect CO dehydrogenase activity, nor could we identify genes for this enzyme in the draft genome. We cannot unequivocally rule out operation of the 3-hydroxypropionate pathway in addition to the rTCA cycle, although we regard this as unlikely, given that we did not find genes for key enzymes of this pathway (malonyl-CoA synthetase and propionyl-CoA synthase) in the draft genome of MC-1.

In this study, we present several strong lines of evidence that MC-1 utilizes the rTCA cycle for CO$_2$ fixation and autotrophy when grown chemolithoautotrophically. First, all chemolithoautrophs produce biomass characterized by discrimination against $^{13}$C during CO$_2$ fixation. Of the four known autotrophic pathways in Bacteria and Archaea, the rTCA cycle and 3-hydroxypropionate cycle typically discriminate less strongly against $^{13}$C than the CBB cycle and reductive acetyl-CoA pathway. Isotope fractionation between biomass and CO$_2$ ($-11.6%e$) (Table 2) for MC-1 falls within the range of values previously obtained for the rTCA cycle (22, 41). This range is much lower than that of the CBB cycle ($-20$ to $-26%e$) and the reductive acetyl-CoA pathway ($-34$ to $-40%e$) and is lower than the 3-hydroxypropionate cycle ($-13.7%e$) (26, 51). However, fractionation values around $-13%e$ were obtained for one mesophilic ε-proteobacterial species ("Candidatus Archo bacter sulfidicus") that was recently demonstrated to use the rTCA cycle (28, 56).

Isotopic compositions of cellular fatty acids provide additional proof of the operation of the rTCA cycle in strain MC-1. In the rTCA cycle, straight-chain fatty acids are hypothesized to be synthesized from the pool of acetyl-CoA that is generated by the first step (citrate cleavage) of the cycle (47). Thus, straight-chain lipids are enriched in $^{13}$C relative to whole cells; that is, the $\delta^{13}$C of straight chain lipids should be greater than that of whole cells. Strain MC-1 exhibits fractionations of 3.7 to 6.5%e between fatty acids and whole cells (Table 2). Green and purple sulfur bacteria that use the rTCA cycle for carbon fixation produce lipids that are 2 to 16% enriched in $^{13}$C relative to biomass (50). The values for MC-1 are less than the 10 to 16%e enrichment observed in the green sulfur phototroph Chlorobium liniicola but higher than the range of 2 to 4%e seen in Thiocapsa roseopersicina (50), Thermococcus ruber (31), and Persephonella marina (60, 63), all of which potentially use the rTCA cycle (or a modification of this pathway) for carbon assimilation (31, 50, 63).

Finally, the presence of specific enzyme activities key to the rTCA cycle, as well as the presence of the genes for most of these enzymes in the genome of strain MC-1, indicates that this organism relies on the rTCA cycle for autotrophy. The activities of 2-oxoglutarate:acceptor oxidoreductase, pyruvate:acceptor oxidoreductase, and fumarate reductase were demonstrated in cell extracts of strain MC-1. Pyruvate:acceptor oxidoreductase and 2-oxoglutarate:acceptor oxidoreductase catalyze the reductive carboxylation of acetyl-CoA and succinyl-CoA to pyruvate and 2-oxoglutarate, respectively, in the rTCA cycle (17). In C. limicola, the electron donor for these reactions is ferredoxin (17). Fumarate reductase, a key enzyme of the rTCA cycle (28), catalyzes the reduction of fumarate to succinate, thereby replacing succinate dehydrogenase of the oxidative TCA cycle (17). The archaeon Thermoproteus neutrophilus, which appears to use the rTCA cycle, turns off fumarate reductase during heterotrophic growth by repression and/or inactivation of this enzyme (7, 43).

We identified putative genes in the draft genome of MC-1 for 2-oxoglutarate:acceptor oxidoreductase, pyruvate:acceptor oxidoreductase, and succinyl-CoA synthetase, a reversible enzyme also involved in the rTCA cycle. These genes are found in the same region of the genome (Fig. 2). The two putative genes that code for the α and β subunits of 2-oxoglutarate:acceptor oxidoreductase indicate that this enzyme belongs to the $\alpha$β-type enzyme family found in Hydrogenobacter thermophilus (but not Aquifex aeolicus) and some archaeal species (57, 58). The arrangement of these putative genes in strain MC-1 is similar to that in H. thermophilus in that the α and β subunit genes are immediately followed by an ORF of unknown but presumably identical function based on sequence identity.

We did not detect ATP-citrate lyase activity in MC-1 with commonly used assays and could not find evidence for genes for any ATP-dependent citrate-cleaving enzymes (ATP-citrate lyase, citryl-CoA synthetase, and citryl-CoA lyase) in the draft genome. Nevertheless, cell extracts showed ATP-dependent citrate cleavage activity. In the oxidative TCA cycle, citrate synthase catalyzes the condensation of acetyl-CoA and oxaloacetate to form citrate; for bacteria that employ the rTCA cycle for CO$_2$ fixation and autotrophy, this reaction is carried out in reverse: citrate is cleaved to acetyl-CoA and oxaloacetate (17). Our results for MC-1 show malate as a major product, with no detectable fumarate, and an extremely faint succinate spot (Fig. 1); these data were similar to those obtained for the archaeon T. neutrophilus, although the latter shows no visible spot representing acetyl-CoA (7). The results obtained when NADH was withheld from the reaction are problematic, given that acetyl-CoA might be expected to be a product of citrate cleavage independent of the presence of NADH. This, combined with the absence of known citrate-cleaving enzyme genes in the draft genome of strain MC-1, suggests that the citrate...
cleavage mechanism in MC-1 may be different from that of other bacterial species that use the rTCA cycle. Many such bacteria (e.g., representatives of Chlorobiales and e-Proteobacteria) possess a two-subunit ATP-citrate lyase that catalyzes the cleavage of citrate in a single reaction (28, 33). However, *H. thermophilus* and *A. acicola* cleave citrate using two separate enzymes: citryl-CoA synthetase activates citrate with CoA to form citryl-CoA, which citryl-CoA lyase cleaves to produce oxaloacetate and acetyl-CoA (1, 2). In other prokaryotes demonstrated or inferred to use the rTCA cycle for carbon fixation, the mechanism for citrate cleavage is unknown. Moreover, demonstration of ATP-dependent citrate cleavage activity has not always correlated with the identification of the genes or protein(s) responsible. For example, several representatives of the Thermoproteaceae are thought to use the rTCA cycle for autotrophic CO$_2$ fixation based on enzyme activities (e.g., *T. neutrophilus* [7] or *Pyrobaculum islandicum* [27]) or on the identification of putative genes for enzymes of the oxidative or reductive TCA cycles in the complete genomes (e.g., *Pyrolobus aerophilum* [19] or *Thermoproteus tenax* [46]). For *T. tenax*, the genes responsible for the ATP-dependent cleavage of citrate could not be identified (19, 46). Siebers et al. [46] proposed an alternative mechanism for citrate cleavage in *T. tenax* that has yet to be verified experimentally. Thus, it seems that disparate enzymatic mechanisms for ATP-dependent citrate cleavage are represented among prokaryotes. In light of this, and given that ATP-citrate lyase is also expressed in some δ-Proteobacteria during organotrophic growth (40, 44), we concur with Hügler et al. [28] in advising caution with regard to the use of acl genes as functional markers for the presence of the rTCA cycle (11, 12).

Within the domain Bacteria, the rTCA cycle has been demonstrated for representatives of the Aquificales (1, 2, 7, 45), Chlorobiales (17, 23, 33), δ-Proteobacteria (44), and e-Proteobacteria (28). This study represents the first evidence of the operation of the rTCA cycle in a member of the δ-Proteobacteria group. The magnetotactic cocci show no close affinities to any other α-Proteobacteria and appear to constitute a unique lineage that diverged early from the main branch of the α-Proteobacteria (15, 18) and are only distantly related to other magnetotactic α-proteobacteria (e.g., Magnetospirillum spp., strain MV-1). Representatives of both the Bacteria and Archaea are known to employ the rTCA cycle, and it has been hypothesized by some to be the primordial metabolic cycle for CO$_2$ fixation (42, 52). To date, all known species in the domain Bacteria that utilize the rTCA cycle for CO$_2$ fixation and autotrophy are found in the Aquificales, Chlorobiales, and Proteobacteria (28). In contrast to the Proteobacteria clade, the Aquificales and Chlorobiales are generally regarded as deep-branching lineages within the domain Bacteria. However, the distribution of the rTCA cycle in these groups is in accord with another phylogenetic hypothesis, which holds that these groups are contained within a clade that branched off relatively late, and that the δ- and ε-Proteobacteria were the first subdivisions to emerge after the Proteobacteria split from the Aquificales, followed by the α-Proteobacteria, and finally the β- and γ-Proteobacteria (25). The operation of the rTCA cycle in strain MC-1 is congruent with this hypothesis, given the basal position of MC-1 within the α-Proteobacteria lineage. Alternatively, lateral gene transfer may be the major factor for the distribution of the rTCA cycle in prokaryotes.

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