

Comparison of a New *Thiomicrospira* Strain from the Mid-Atlantic Ridge with Known Hydrothermal Vent Isolates†

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A new autotrophic *Thiomicrospira* strain, MA-3, was isolated from the surface of a polymetal sulfide deposit collected at a Mid-Atlantic Ridge hydrothermal vent site. The DNA homology among three vent isolates, *Thiomicrospira crunogena*, *Thiomicrospira* sp. strain L-12, and *Thiomicrospira* sp. strain MA-3, was 99.3% or higher, grouping them as the same species, *T. crunogena* (type strain, ATCC 35932). The fact that *T. crunogena* and *Thiomicrospira* sp. strain L-12 were isolated from Pacific vent sites demonstrates a cosmopolitan distribution of this species.

Sulfur-oxidizing chemolithotrophs isolated from hydrothermal environments are described as mesophiles and facultative or obligate autotrophs (6, 7, 10, 14, 19) with diverse oxidative capabilities (12, 13, 15). Twelve of ninety-five sulfur-oxidizing isolates from the Galapagos hydrothermal vent site were physiologically described as *Thiomicrospira* strains (19), and one Mid-Atlantic Ridge (MAR) isolate was phylogenetically placed in the same genus (17). Combined molecular and cultivation approaches have greatly furthered the concept of *Thiomicrospira* diversity in marine as well as freshwater habitats (2). Presently fully described sulfur-oxidizing chemolithoautotrophs isolated from deep-sea hydrothermal vents are limited to two species of *Thiomicrospira* (14, 18) and one species of *Thiobacillus* (7), none of which is acidophilic. Furthermore, natural microbial populations as well as certain MAR isolates and the two previously described *Thiomicrospira* isolates from Pacific vent sites (*T. crunogena* and *Thiomicrospira* sp. strain L-12) have been shown to oxidize sulfidic minerals at near-neutral pH (8, 23). The most active of these Atlantic vent isolates (MAR strain MA-3) has been physiologically and phylogenetically characterized and compared to the two different Pacific vent site *Thiomicrospira* isolates.

Strain MA-3 was isolated from enrichments of surface scrapings of a polymetal sulfide rock collected from a depth of 3,620 m at the MAR Trans-Atlantic Geotransverse (TAG) hydrothermal vent site. *T. crunogena* and *Thiomicrospira* sp. strain L-12 were regrown from our culture collection. The medium used for enrichment and laboratory experimentation was artificial seawater (ASW) containing vitamins, trace elements, and phenol red indicator (8). The pH of filter-sterilized (0.2- μ m pore size) medium was set at 7.4 with HCl. The reduced sulfur sources for autotrophic growth were sodium thiosulfate (10 mM [T-ASW]), elemental sulfur (1% [wt/vol]), sodium sulfide (500 μ M) (5), sodium sulfite (1 and 5 mM), and natural and commercial metal sulfides (e.g., pyrite and chalcopyrite) as ground and sterilized slurries (4% [wt/vol]) (8). The organic

substrates were glucose, lactate, galactose, peptone, yeast extract, and Casamino Acids, tested individually at 0.1% in ASW medium. Agar (1.5%) medium made with T-ASW was used for the initial isolation, counting of CFU, and stock culture maintenance. Growth was determined by acridine orange epifluorescent cell counts (11), pH indicator change, elemental sulfur deposition, and incorporation of radiolabeled ¹⁴CO₂. The latter was done in T-ASW or metal sulfide-amended ASW medium with radiolabeled NaH¹⁴CO₃ (8, 21, 23). In testing for the possible excretion of acid-stable products during CO₂ incorporation in growing cultures at 24°C, the procedure of Ruby and Jannasch (18) was modified by acidifying the filtrate to pH 3.0 and then sparging with air to remove residual radiolabeled bicarbonate. Maximum growth rates were determined at 24°C in 100 and 10% air-saturated media by direct cell counts and by ¹⁴CO₂ incorporation. The growth temperature range was determined with liquid cultures (1°C increments above 38°C). The pH range for growth was tested in T-ASW set at initial pH values in the range of 4.5 to 9.0 at 0.5-pH intervals. Iron oxidation supporting CO₂ incorporation was studied with ASW medium with 10 mM ferrous sulfate at pH 7.0 with a gaseous headspace of 20, 2, or 1% oxygen (balanced by N₂). T-ASW with 1% oxygen in the gas phase served as the positive control. Iron toxicity was tested in oxygen-free (N₂ gassed) ASW medium containing from 0 to 90 mM ferrous sulfate at near-neutral pH and inoculated with pregrown, centrifuged, and washed cells. Viability, as CFU, was assessed by plating of subsamples at various time points on T-ASW agar medium. Nitrate respiration was tested by counting cell number increase in anoxic T-ASW medium supplemented with 2 mM KNO₃. The requirement for sodium ions and the possible substitution of potassium were tested in NaCl-free T-ASW medium (18). Effects of ambient versus in situ pressure (1 × 10⁵ Pa and 350 × 10⁵ Pa) on growth were measured by CO₂ incorporation at 24°C (14, 18). The 16S rRNA-encoding gene from isolate MA-3 was amplified by using primers and PCR conditions as described by Muyzer et al. (17). The 16S rRNA sequence of strain MA-3 was aligned manually, by using the sequence editor SEQAPP (9), to sequences of other bacteria obtained from the Ribosomal Database Project (RDP [16]), and from GenBank (1). The DNA base composition for G+C, the ubiquinone analysis, and the DNA-DNA hybridizations were all carried out as described in detail by Brinkhoff et al. (3).

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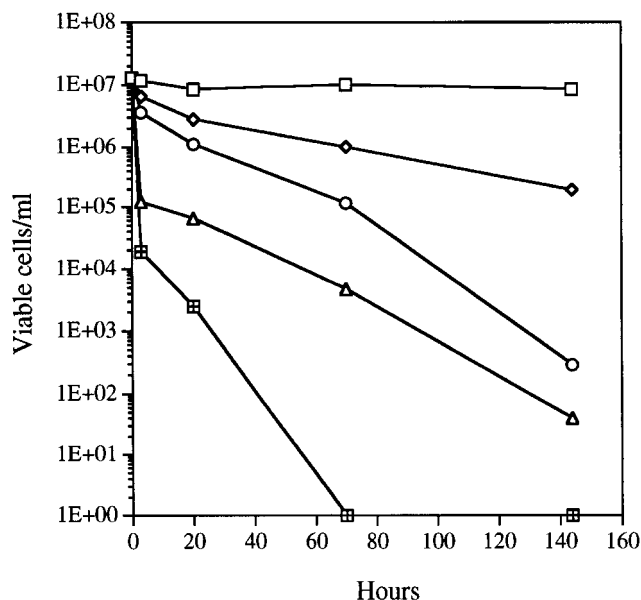


FIG. 1. Survival (CFU) of pregrown cells of strain MA-3 exposed to varied concentrations of Fe^{2+} at near-neutral pH under anoxic conditions at 23°C. Symbols represent concentrations of iron: □, none; ◇, 10 mM; ○, 30 mM; △, 60 mM; ⊠, 90 mM.

Strain MA-3 is an autotrophic, obligately aerobic, gram-negative, motile (by phase microscopy) and slightly vibrioid bacterium measuring 0.5 to 0.7 by 1.3 to 2.0 μm . The colonies on T-ASW agar are white, smooth, and entire and produce elemental sulfur and sulfate (i.e., sulfuric acid). The strain grows aerobically on reduced sulfur compounds (H_2S , sulfur, and thiosulfate) and sulfidic minerals, all of which may be available in its natural vent habitat, but not on sulfite. It did not demonstrate nitrate respiration. The growth rate on thiosulfate at 24°C is 0.8 h^{-1} (doubling time of 51 min) in the presence of either 100 or 10% air-saturated medium. It did not grow heterotrophically on any of the organic compounds tested. Strain MA-3 of the species *T. crunogena* has been deposited as ATCC 700270 in the American Type Culture Collection.

At near-neutral pH, strain MA-3 will utilize commercial pyrite or natural metal sulfides (primarily chalcopyrite) collected from MAR vent sites. Growth on these minerals is optimal at pH 6.5 to 7.0, with doubling times averaging from 10 to 50 h, depending on the mineral, and ceases when the pH drops below 6.0 to 5.5. On thiosulfate, strain MA-3 grows optimally at pH 7.5 and ceases growth at comparably low pHs.

Strain MA-3 did not grow autotrophically on ferrous iron as a substrate. In fact, ferrous iron was significantly toxic at increasing concentrations (Fig. 1). In the iron-free control, cells remained almost completely viable (as CFU) over the experimental time period. Growth of this isolate at a hydrostatic pressure of its normal habitat in the deep sea (ca. $350 \times 10^5 \text{ Pa}$) did not show a barophilic adaptation; however, the organism is quite barotolerant, demonstrating a rate of CO_2 fixation at $350 \times 10^5 \text{ Pa}$ approximately 83% of the rate measured at $1 \times 10^5 \text{ Pa}$. The reduction in growth rate is similar to that reported for *T. crunogena* and *Thiomicrospira* sp. strain L-12 at their in situ pressure of $250 \times 10^5 \text{ Pa}$ (14, 18). Strain MA-3 grows over a temperature range of 4 to 41°C (optimum of 28 to 32°C). This maximum growth temperature of 41°C is the highest reported for the three species. Cells did not grow at 42°C, but the organism survived for 4 days at this temperature and then grew at a lower temperature (24°C), while cells incubated at 44°C for an equivalent time period did not grow upon transfer. Sodium is required for growth of strain MA-3, because no growth occurred at concentrations of less than 50 mM NaCl over 1 week. Optimum growth occurred at 200 to 400 mM NaCl. Potassium, as KCl, was unable to substitute for sodium. In T-ASW medium, strain MA-3 excreted approximately 4 to 5% of its fixed carbon as acid-stable organic compounds, of unknown composition, during exponential growth and a maximum of 12% in the stationary phase.

Most physiological characteristics of strain MA-3 are similar to those of the two *Thiomicrospira* isolates described earlier, *T. crunogena* and *Thiomicrospira* sp. strain L-12 (e.g., motility and utilization of the same reduced sulfur sources), including the presence of ubiquinone Q-8 with traces of Q-7. Differences among the three isolates are listed in Table 1. In its maximum rate of growth on thiosulfate and its growth temperature range, strain MA-3 resembles *T. crunogena* more than *Thiomicrospira* sp. strain L-12. Similarly, slight differences hold for the excretion of fixed carbon, pH optima, minimum salinities, and G+C contents. The 16S rRNA-encoding gene of strain MA-3 was nearly completely sequenced to determine its phylogenetic affiliation. By parsimony analysis of the 16S rRNA sequences, the new isolate was grouped with other *Thiomicrospira* species isolated from hydrothermal vent communities, i.e., *T. crunogena* and *Thiomicrospira* sp. strain L-12. The closest relative is *T. crunogena* (14), to which it has a sequence similarity value higher than 99%. Only 2 bp differences could be found between the sequences of *T. crunogena* and strain MA-3: at *Escherichia coli* positions (4) 978 and 1015, there was a G in the sequence of *T. crunogena* versus an A in the sequence of strain MA-3. The DNA-DNA homologies among the three strains are presented in Table 2. Strain MA-3 has a 99.3% homology

TABLE 1. Growth characteristics of three *Thiomicrospira* isolates from deep-sea hydrothermal vents

Organism	Source	Maximum growth rate (h^{-1})	°C temp range (optimum)	pH range (optimum)	% Fixed carbon excreted (24 h)	% Growth in situ bar/1 bar	Minimum NaCl requirement (mM)	G+C content
<i>T. crunogena</i> ^a	Vestimentiferan tube worm casing, 21°N EPR	0.8	4–38.5 (28–32)	5.0–8.5 (7.75)	8.5	80	45	44.2 ± 0.2 ^c
<i>Thiomicrospira</i> sp. strain L-12 ^b	Mussel periostracum, Galapagos Rift	0.32	10–35 (25)	5.5–8.5 (8.0)	9.0	75	80	44.4 ± 0.2 ^c
<i>Thiomicrospira</i> sp. strain MA-3	Polymetal sulfide rock, TAG site, MAR	0.8	4–41 (28–32)	5.5–8.5 (7.5)	12	83	50	44.6 ± 0.3

^a Data from reference 14.

^b Data from reference 18.

^c Data from this study.

TABLE 2. Percentages of DNA-DNA homology for three *Thiomicrospira* isolates from deep-sea hydrothermal vents and *T. pelophila*, the type species of the genus

Isolate	% Homology to:		
	<i>T. pelophila</i>	<i>Thiomicrospira</i> sp. strain L-12	<i>T. crunogena</i>
<i>Thiomicrospira</i> sp. strain L-12	27		
<i>T. crunogena</i>	33.5	100	
<i>Thiomicrospira</i> sp. strain MA-3	27	99.3	99.3

to the other two strains. The two Pacific vent strains, *T. crunogena* and *Thiomicrospira* sp. strain L-12, share 100% homology to each other. The type species of the genus *Thiomicrospira*, *T. pelophila*, shows only a 27 to 33.5% homology to all three vent strains. This value, being below the homology threshold of 60 to 70%, defines (20) *T. pelophila* as a separate species.

This study has resulted in two new findings. (i) Despite physiological differences among the three isolates compared, their almost identical 16S rRNA sequences and DNA-DNA hybridization values define them as one species (20, 22), *T. crunogena* (type strain, ATCC 35932). (ii) The identification of these isolates from MAR and East Pacific Rise (EPR) deep-sea hydrothermal vent sites indicates a highly cosmopolitan occurrence of this species. In view of the 16S rRNA sequence similarity of the three isolates compared in this paper, a further strain isolated from the MAR, MA2-6 (17), has a similarity value of about 97% with *T. crunogena*, indicating it is a separate species (20). On the other hand, a new intertidal *Thiomicrospira* isolate, strain JB-B2, shows a 99% similarity to both *T. crunogena* and *Thiomicrospira* sp. strain L-12 (2) and, therefore, may be yet another strain of the species *T. crunogena*.

In view of the greater than 99.3% DNA homology of the three *T. crunogena* strains compared, the observed differences of a physiological nature under equal growth conditions (Table 1) indicate a certain range of phenotypic adaptations to environmentally different vent sites. The metabolic capability of metal sulfide oxidation affords these chemolithotrophs an additional and stable source of energy in environments where massive polymetal sulfide deposits exist, namely at deep-sea hydrothermal vent sites. This versatility may contribute to the fact that the genus *Thiomicrospira* appears to dominate, by molecular analysis (17), the sulfur-oxidizing bacterial communities at deep-sea hydrothermal vents. The present work presents a striking case of wide geographic distribution of a single sulfur-oxidizing bacterial species at Atlantic and Pacific deep-sea vent sites. If this can indeed be extended toward intertidal zones, as suggested by the genetic relatedness of strain JB-B2 (2), a truly worldwide distribution of the species *T. crunogena* would be apparent.

Nucleotide sequence accession number. The 16S rRNA sequence of strain MA-3 is available from GenBank under accession no. AF069959.

The ubiquinones were identified by B. Tindall, and the G+C determination and DNA-DNA hybridizations were done by J. Burghardt, both of the DSMZ identification service (Braunschweig, Germany).

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