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Physiological Versatility of the Extremely Thermoacidophilic Archaeon *Metallosphaera sedula* Supported by Transcriptomic Analysis of Heterotrophic, Autotrophic, and Mixotrophic Growth▽†

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Comparative transcriptomic analysis of autotrophic, heterotrophic, and mixotrophic growth of the archaeon *Metallosphaera sedula* (70°C, pH 2.0) revealed candidates for yet-to-be-confirmed components of the 3-hydroxypropionate/4-hydroxybutyrate pathway and implicated a membrane-bound hydrogenase (Msed_0944-Msed_0946) for growth on H_2. Routes for generation of ATP and reducing equivalents were also identified.

Microbial mixotrophy has been defined as either the capacity to assimilate both organic and inorganic carbon sources simultaneously (18, 20) or the utilization of an inorganic energy source in combination with an organic carbon source (1, 16, 21). Common to either definition is the idea that mixotrophy implicates a relationship between heterotrophy and autotrophy. *Metallosphaera sedula* is an extremely thermoacidophilic archaeon that stands out from related extremophiles by its physiological versatility (Table 1). *M. sedula* grows heterotrophically on peptides, autotrophically by fixing CO_2 by using H_2 as a reductant, and mixotrophically on Casamino Acids and $FeSO_4$ or metal sulfides (1, 2, 8, 10, 12, 17). Recent studies of *M. sedula*'s 3-hydroxypropionate/4-hydroxybutyrate cycle (4, 13, 19), in conjunction with the availability of genome sequence information (3), provided the basis here for examining mixotrophic growth in *M. sedula* more closely by using transcriptomic analysis.

M. sedula (DSMZ 5348) was grown aerobically at 70°C in an orbital shaking oil bath at 70 rpm on DSMZ 88 medium (pH 2). Cells were grown (each with biological repeats) heterotrophically (0.1% tryptone supplement; "H"), autotrophically (headspace composition of 7% CO_2, 14% O_2, 28% H_2, balance N_2; "A"), and mixotrophically (0.1% tryptone supplement plus headspace composition of 7% CO_2, 14% O_2, 28% H_2, balance N_2; "M"). At the fourth pass under each growth condition, cells were inoculated (~10^7 cells/ml) into two 1-liter bottles for each growth mode, containing 300 ml of medium. Cell densities were measured using epifluorescence microscopy. Culture harvesting, microarray construction methods, RNA preparation, slide scanning, and data analysis were as described previously (2, 3), with the exception that Trizol (Invitrogen) was used as the RNA extraction reagent and a Packard BioChip ScanArray 4000 scanner was used for slide scanning. Differential transcription, or "response," was defined as relative changes of ≥2 (where a $\log_2$ value of ±1 means a 2-fold change) having P values of ≥5.4 (Bonferroni correction equivalent to a P value of 4.0×10^{-6} for this microarray). With biological repeats for each condition tested, the four possible combinations (i.e., for autotrophic growth versus heterotrophic growth, four comparisons, A_1-H_1, A_1-H_2, A_2-H_1, and A_2-H_2) were determined and then averaged (A-H). Microarray data are available through the NCBI Gene Expression Omnibus (GEO) database under accession number GSE14978.

Growth physiology of *M. sedula*. Heterotrophically grown *M. sedula* reached stationary phase (9×10^8 cells/ml) at 48 h (t_d = 5.0), while autotrophically grown cells (t_d = 11 to 13 h) were still in exponential phase at 70 h (maximum density, 3×10^8 cells/ml) (Fig. 1). Mixotrophically grown *M. sedula* reached stationary phase (6×10^8 cells/ml) at 18 h (t_d = 3.7 h), suggesting beneficial contributions from organic and inorganic carbon and energy sources.

Transcriptomic analysis revealed that certain open reading frames (ORFs) responded to heterotrophic growth conditions (see Table S1 in the supplemental material). For example, succinyl coenzyme A (CoA) synthetase (Msed_1581-Msed_1582), a tricarboxylic acid (TCA) cycle intermediate, was upregulated under heterotrophic growth versus autotrophic growth conditions (see Table S2 in the supplemental material). As expected, the autotrophic transcriptome was characterized by the upregulation of several amino acid biosynthesis gene clusters (see Table S3 in the supplemental material). Riboflavin and thiamine biosynthesis genes (Msed_0046-Msed_0048, Msed_0906, and Msed_2221) were also observed to be upregulated under autotrophic growth conditions compared to under heterotrophic and mixotrophic growth conditions. Very few ORFs responded uniquely to mixotrophic conditions (see Table S4 in the supplemental material). However, several ORFs in the 3-hydroxypropionate/4-hydroxybutyrate cycle were transcribed at higher levels during mixotrophic growth versus autotrophic growth (Msed_709, 2-fold; Msed_1426, 2-fold) (see Table S5 in the supplemental material). Thus, while a strong shift away from inorganic carbon fixation was observed, CO_2 present in the headspace air may still be assimilated during mixotrophic growth.

Inorganic carbon fixation. It is not yet known how, or in what form, inorganic carbon is assimilated by *M. sedula*. CO_2 could diffuse through the cell membrane, or CO_2 (or bicarbon-

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TABLE 1. Selected genes in extreme thermoacidophile genomes implicated in carbon and energy source utilization

Component	Presence in genome of[a]:												
	Ss	St	Sa	Pt	Ms	Cm	Si_L	Si_M	Si_U	Si_{YGN}	Ab	Aa	Sm
Genes encoding terminal oxidase:													
SoxABCDD′L	X	X	X		X		X	X	X	X		?	?
SoxEFGHIM	X	X	X	X	X	X	X	X	X	X		?	?
DoxBCE	X	X	X	X	X	X	X	X	X	X		X	?
Iron oxidation genes													
FoxABCD gene		X			X							?	X
Rusticyanin 1 gene					X	X	X	X	X	X		?	?
Rusticyanin 2 gene					X				X	X		?	?
Sulfur oxidation genes													
Sor gene		X		X								X	X
TetH gene		X		X	X	X	X	X	X	X		?	?
DoxDA/TQO gene	X	X		X	X		X	X	X	X		X	X
CO_2 fixation													
3-Hydroxypropionate/4-hydroxybutyrate genes	X	X	X		X		X	X	X	X		?	?
H_2 utilization													
Hydrogenase (NiFe) gene					X		?		X		X	X	?

[a] Ss, *Sulfolobus solfataricus*; St, *Sulfolobus tokodaii*; Sa, *Sulfolobus acidocaldarius*; Pt, *Picrophilus torridus*; Ms, *Metallosphaera sedula*; Cm, *Caldivirga maquilingensis*; Si, *Sulfolobus islandicus* strains L, M, U, YG, and YN; Ab, *Aciduliprofundum boonei*; Aa, *Acidianus ambivalens*; Sm, *Sulfolobus metallicus*; X, present; ?, unknown.

ate) could enter the cell via transporters. Msed_1539 is annotated as an Rh family ammonium transporter (IPR001905); note that Rh proteins in the green alga *Chlamydomonas reinhardtii* most likely function as CO_2 gas channels (14). Msed_1539 was upregulated (4-fold) under autotrophic growth compared to under either heterotrophic or mixotrophic growth conditions and possibly plays a role in CO_2 fixation.

Carbonic anhydrase catalyzes the interconversion of CO_2 into bicarbonate. Msed_0390 (β-class) and Msed_1618 (γ-class) appear to encode carbonic anhydrase homologs, although only the Msed_0390 transcript was upregulated under autotrophic conditions compared to heterotrophic conditions (see Table S6 in the supplemental material). A similar protein in *Acidianus ambivalens* (CAC83596; 36% amino acid [aa] identity) was previously characterized as a soluble cytochrome *b*; however, no activity was detected when the protein was tested for carbonic anhydrase function (6).

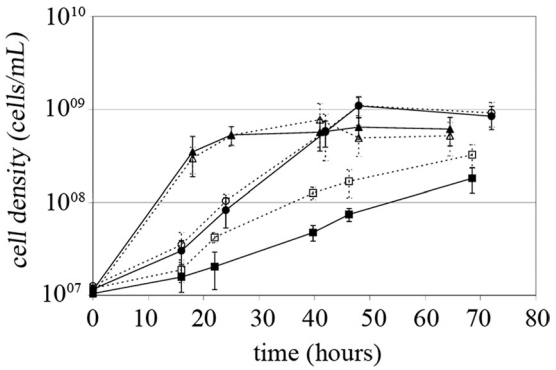

FIG. 1. Growth curves for the third passage of heterotrophic (●), autotrophic (■), and mixotrophic (▲) growth cultures of *M. sedula*. Biological repeats are represented by open symbols and broken lines. Error bars represent σ.

Figure 2 shows the proposed 3-hydroxypropionate/4-hydroxybutyrate cycle in *M. sedula* (4), along with the normalized transcription levels for each candidate ORF for each growth mode (see also Table S5 in the supplemental material). Several of the known inorganic carbon fixation cycle enzyme transcripts were upregulated under autotrophic conditions compared to under heterotrophic conditions, most notably malonyl-CoA reductase (Msed_0709; 25-fold). While 3-hydroxypropionyl-CoA dehydratase (Msed_2001) and methylmalonyl epimerase (Msed_0639) were not responsive to growth mode, these ORFs were constitutively transcribed at relatively high levels. For the putative 4-hydroxybutyryl-CoA dehydratase (step 11, Msed_1220 or Msed_1321), only Msed_1321 was triggered by autotrophic versus heterotrophic conditions, supporting its proposed functional role as the 4-hydroxybutyryl-CoA dehydratase gene (4, 9). Several gene candidates were proposed as putative crotonyl-CoA hydratases (step 12); however, none were higher under autotrophic versus heterotrophic conditions (autotrophy versus heterotrophy). Interestingly, the 3-hydroxypropionyl-CoA dehydratase (step 5, Msed_2001) was recently shown to also be active on (*S*)-hydroxbutyryl-CoA, forming crotonyl-CoA (19). Of the candidates proposed for the putative acetoacetyl-CoA β-ketothiolase, Msed_0656 was the only ORF that exhibited upregulation under autotrophic conditions. Three additional ORFs meriting further autotrophy-related investigation include Msed_2087, annotated as encoding an acyl-CoA dehydrogenase domain-containing protein (upregulated 8-fold during both autotrophy versus heterotrophy and autotrophy versus mixotrophy), Msed_1994 (COG 3435; upregulated 4-fold during autotrophy versus heterotrophy), located on the strand opposite the re-

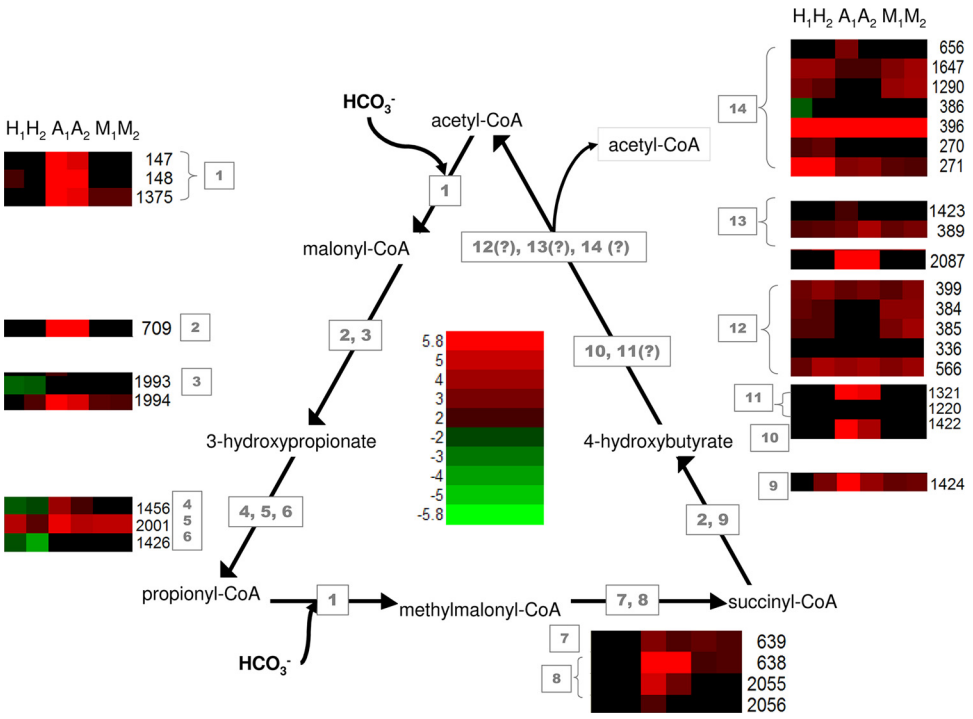

FIG. 2. Transcriptional levels of *M. sedula* ORFs implicated in the 3-hydroxypropionate/4-hydroxbutyrate cycle (based on the *M. sedula* model proposed in reference 4) during heterotrophic (H_1 and H_2), autotrophic (A_1 and A_2), and mixotrophic (M_1 and M_2) growth. Heat plots were constructed using AFM 4.0 (5). Red indicates high transcription, and green indicates low transcription; corresponding numbers represent least-squares means of normalized $\log_2$-transformed transcription levels relative to the overall average transcription level of 0. Enzymes: 1 = acetyl-CoA/propionyl-CoA carboxylase, 2 = malonyl-CoA/succinyl-CoA reductase, 3 = malonic semialdehyde reductase, 4 = 3-hydroxypropionyl-CoA synthetase, 5 = 3-hydroxypropionyl-CoA dehydratase, 6 = acryloyl-CoA reductase, 7 = methylmalonyl-CoA epimerase, 8 = methylmalonyl-CoA mutase, 9 = succinate semialdehyde reductase, 10 = 4-hydroxybutyryl-CoA synthetase, 11 = 4-hydroxybutyryl-CoA dehydratase, 12 = crotonyl-CoA hydratase, 13 = (*S*)-3-hydroxybutyryl-CoA dehydrogenase, 14 = acetoacetyl-CoA β-ketothiolase.

cently characterized malonic semialdehyde reductase gene (13), and Msed_2056 (ArgK-like), whose overlapping position with the ORF encoding the second subunit of the methylmalonyl-CoA mutase (Msed_2055) indicates the potential for cotranscription.

Hydrogenases. Two different loci in the *M. sedula* genome appear to encode NiFe hydrogenases. Msed_0913 to Msed_0950 represent loci encoding two hydrogenases (three putative α subunits and two β subunits), multiple accessory proteins, and multiple hypothetical proteins for which no GenBank database matches can be identified. Msed_0944 and Msed_0945 encode the small and large subunits (HynSL; 33% and 28% aa identity, respectively) of a NiFe hydrogenase, while Msed_0946 and Msed_0947 transcripts are similar to the Isp1 cytochrome *b* membrane anchor (22% aa identity) and Isp2 Fe-S oxidoreductase-like protein (24% aa identity) of the thermoacidophilic group I NiFe hydrogenase, previously described for *Acidianus ambivalens* (15). The only other archaeal gene with similarities to group I NiFe hydrogenases comes from the recently sequenced genome of *Sulfolobus islandicus* U.3.28 (contig56_2390). Results of a phylogenetic analysis of β subunits (similar to those in references 15 and 23) suggest that both thermoacidophilic hydrogenases should be classified as group I (data not shown).

Msed_0923 and Msed_0924 transcripts are homologous to the β and α subunits of NiFe hydrogenases. Neither signal peptides nor transmembrane helices are predicted for the β subunit, suggesting that the hydrogenase encoded by Msed_0923 and Msed_0924 is cytoplasmic versus membrane associated.

Only one hydrogenase responded when *M. sedula* was grown aerobically in the presence of hydrogen (Fig. 3; see also Table S7 in the supplemental material). The membrane-bound hydrogenase was significantly upregulated under autotrophic growth compared to under heterotrophic growth (Msed_0944 to Msed_0946, 7.4- to 12.6-fold). Multiple Hyp-encoding ORFs, an FeS oxidoreductase-like protein, and several hypothetical proteins in the same region also had similar response patterns. The lack of response from the ORFs encoding the soluble hydrogenase suggests that this enzyme may not be activated by the presence of H_2 in an aerobic environment and, therefore, not function as an uptake hydrogenase. However, the normalized transcript levels detected for these ORFs were lower than the overall average transcript levels, which is consistent with the low synthesis levels reported for the group II, H_2-sensing, regulatory hydrogenase (HupUV) in *Rhodobacter capsulatus* (24).

The group I uptake hydrogenases have an energy conservation function, contributing to the generation of proton motive force by transferring electrons, via quinones, to cytochromes or heterodisulfide reductases with proton pumping capabilities (11, 22, 23). Both the *M. sedula* SoxABCDD′ quinol/terminal oxidase cluster (Msed_0285-Msed_0292) and components of

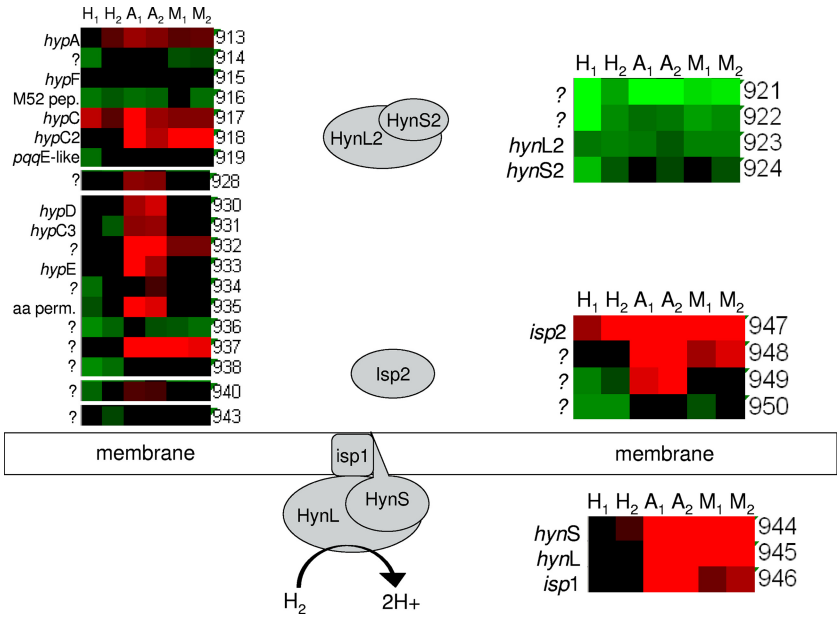

FIG. 3. Transcriptional levels of *M. sedula* hydrogenase-associated ORFs during heterotrophic (H_1 and H_2), autotrophic (A_1 and A_2), and mixotrophic (M_1 and M_2) growth. Heat plots were constructed using AFM 4.0 (5). Red indicates high transcription, green indicates low transcription, and corresponding numbers represent least-squares means of normalized $\log_2$-transformed transcription levels relative to the overall average transcription level of 0.

the heterodisulfide-like cluster (Msed1542-Msed1550), previously reported to be upregulated in the presence of reduced inorganic sulfur compounds (RISCs) (2), were also found to be upregulated here on H_2 (see Table S8 in the supplemental material). In *Aquifex aeolicus*, sulfur reductase (Sre-like subunits) was found in a supercomplex with a group I hydrogenase and bc_1 components during growth on elemental sulfur and hydrogen (7). In *M. sedula*, the *sre*-like complex (Msed_0810-Msed_0818) was found to be upregulated on RISCs (2) but not in the presence of hydrogen (see Table S8 in the supplemental material). The Sre complex studied in *A. ambivalens* was inactive unless it was also coupled with the group I hydrogenase (15). A review of the *M. sedula* group I hydrogenase transcriptional patterns in the presence of elemental sulfur (GEO accession no. 12044) shows that both the α subunit and the cytochrome *b* membrane anchor (Msed_0945 and Msed_0946) were stimulated by elemental sulfur compared to the absence of elemental sulfur, the presence of Fe(II), or the presence of more oxidized forms of sulfur (tetrathionate or sulfate) (see Table S9 in the supplemental material). The cytoplasmic ferredoxin/*isp2*-like transcript (Msed_0947), potentially involved with electron transfer from the hydrogenase, was observed to be downregulated in the presence of elemental sulfur compared to the same conditions.

Transcriptomic analysis of heterotrophic, autotrophic, and mixotrophic growth provided new insights into the growth physiology of *M. sedula*. Confirmed and putative components of the 3-hydroxypropionate/4-hydroxybutyrate pathway could be tracked as a function of growth mode. The membrane-bound hydrogenase responsible for allowing *M. sedula* autotrophic growth on an H_2-based inorganic energy source was identified, and potential routes for contribution to the generation of ATP (via proton motive force) and reducing equivalents could be proposed. The higher *M. sedula* growth rates observed under mixotrophic conditions most likely relate to the synergistic use of available carbon and energy sources.

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