Ammonia-oxidizing archaea use the most energy-efficient aerobic pathway for CO_2 fixation

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Archaea of the phylum Thaumarchaeota are among the most abundant prokaryotes on Earth and are widely distributed in marine, terrestrial, and geothermal environments. All studied Thaumarchaeota couple the oxidation of ammonia at extremely low concentrations with carbon fixation. As the predominant nitrifiers in the ocean and in various soils, ammonia-oxidizing archaea contribute significantly to the global nitrogen and carbon cycles. Here we provide biochemical evidence that thaumarchaeal ammonia oxidizers assimilate inorganic carbon via a modified version of the autotrophic hydroxypropionate/hydroxybutyrate cycle of Crenarchaeota that is far more energy efficient than any other aerobic autotrophic pathway. The identified genes of this cycle were found in the genomes of all sequenced representatives of the phylum Thaumarchaeota, indicating the environmental significance of this efficient CO_2-fixation pathway. Comparative phylogenetic analysis of proteins of this pathway suggests that the hydroxypropionate/hydroxybutyrate cycle emerged independently in Crenarchaeota and Thaumarchaeota, thus supporting the hypothesis of an early evolutionary separation of both archaeal phyla. We conclude that high efficiency of anabolism exemplified by this autotrophic cycle perfectly suits the lifestyle of ammonia-oxidizing archaea, which thrive at a constantly low energy supply, thus offering a biochemical explanation for their ecological success in nutrient-limited environments.

Nitrosopumilus maritimus | autotrophy

All characterized representatives of the recently proposed phylum Thaumarchaeota (1) are chemolithotrophs that oxidize ammonia aerobically to nitrite via a yet unknown pathway (2). Initial experiments on the first cultured representative of this phylum, the marine archaeon *Nitrosopumilus maritimus* (3) showed many cellular, genomic, and physiological features that reflect an oligophilic lifestyle, being adapted to very low energy supply (3, 4). Indeed, kinetic studies on *N. maritimus* established its extremely high affinity for ammonia/ammonium (hereafter referred to simply as "ammonia") (5). This affinity enables archaeal nitrifiers to grow at extremely low ammonia concentrations (in the low nanomolar range) and thus outcompete their bacterial counterparts in oligotrophic environments such as the open ocean (6–8). Consequently, ammonia-oxidizing archaea (AOA) almost certainly exert primary control over nitrification as well as contributing significantly to primary production in the ocean and in soils (9–13).

Autotrophic CO_2 fixation is the central anabolic process in thaumarchaeal ammonia oxidizers. However, the energy available from the oxidation of the nanomolar concentrations of ammonia typical of the marine environment is very low. Thus, we hypothesized that highly efficient anabolic pathways would provide the thaumarchaea an ecological advantage relative to the ammonia-oxidizing bacteria (AOB) that are more competitive at higher ammonia concentrations. Among the six autotrophic CO_2-fixation pathways described to date, three use oxygen-tolerant enzymes and therefore are regarded as aerobic, i.e., the widespread Calvin–Benson cycle, the 3-hydroxypropionate bicycle in Chloroflexi, and the hydroxypropionate/ hydroxybutyrate (HP/HB) cycle in aerobic Crenarchaeota (14-18). All these cycles have significant energy demands and vary only slightly in the amount of ATP required for the synthesis of cellular precursor metabolites from CO_2, suggesting that they are distinguished by other features (14, 15, 18).

Genomic studies of the Thaumarchaeota suggested a functioning HP/HB cycle (4, 13, 19–22), which was first identified in the thermophilic crenarchaeon *Metallosphaera sedula* (19) and was extended to other aerobic Crenarchaeota (14, 23). Briefly, one acetyl-CoA and two bicarbonate molecules are converted via 3-hydroxypropionate to succinyl-CoA. Succinyl-CoA, in turn, is converted via 4-hydroxybutyrate to two molecules of acetyl-CoA, one of which serves as carbon precursor (Fig. 1). Assignment of a functional HP/HB cycle in Thaumarchaeota has been based solely on the detection of genes coding for characteristic enzymes of this cycle (acetyl-CoA/propionyl-CoA carboxylase, methylmalonyl-CoA mutase, 4-hydroxybutyryl-CoA dehydratase) in the absence of genes diagnostic for the other autotrophic pathways (4, 19, 20). However, certain genes encoding homologs for various enzymes specific to the cycle could not be identified in the genomes of Thaumarchaeota, and experimental evidence for its operation was missing. In addition, *M. sedula* and *N. maritimus* showed differences in stable carbon isotope fractionation relative to the inorganic carbon source. Although *N. maritimus* exhibited a fractionation of *ca.* −20‰ (24), the *M. sedula* biomass showed only a minor depletion of approximately −2‰ (25). Although the different discrimination effects may be attributed to the growth conditions of the two organisms (28 °C at pH 7.5 and 65 °C at

Significance

CO_2 fixation is the most important biosynthesis process on Earth, enabling autotrophic organisms to synthesize their entire biomass from inorganic carbon at the expense of energy generated by photo- or chemotrophic processes. In the present study we demonstrate an autotrophy pathway that represents the most energy-efficient mechanism for fixing inorganic carbon in the presence of oxygen. This novel variant of the hydroxypropionate/hydroxybutyrate cycle appears to be common in a ubiquitous and abundant group of microorganisms that can thrive in nutrient-limited environments. This discovery offers a biochemical explanation for the remarkable ecological success of the ammonia-oxidizing archaea in extremely nutrient-limited environments typical of most of the open ocean.

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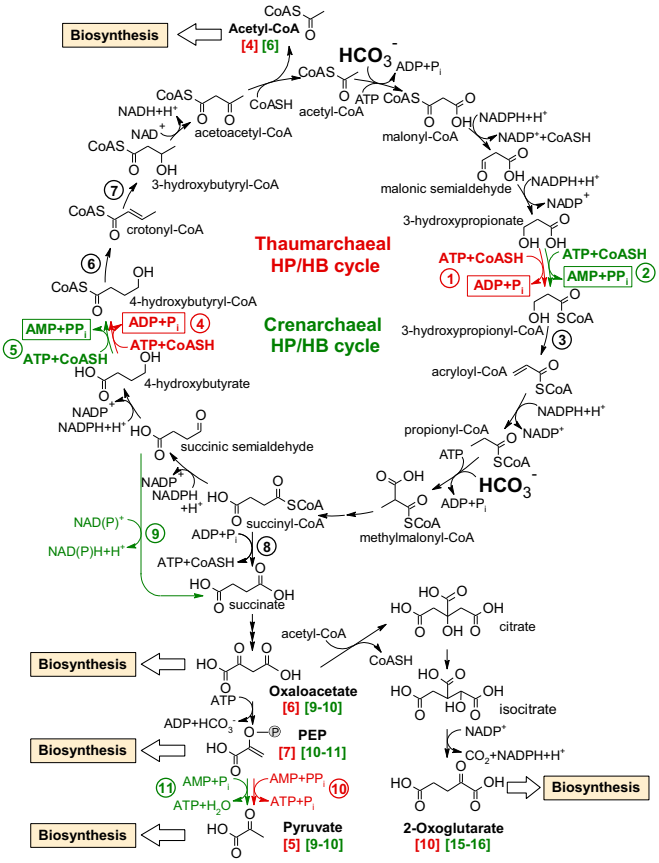

Fig. 1. Reactions of the crenarchaeal and thaumarchaeal variants of the HP/HB cycle (modified from ref. 19). The reactions determining energy efficiency of the crenarchaeal (*M. sedula*) cycle are shown in green, and those for the *N. maritimus* variant are shown in red. Reactions common to both are shown in black. Note that although the two pathways have similar reactions and intermediates, they are significantly different in energy efficiency (Table 4) and evolved independently in Crenarchaeota and Thaumarchaeota (see text). The numbers in square brackets represent moles of high-energy anhydride bonds of ATP required to form 1 mol of the corresponding central precursor metabolites (see also Table 4). Enzymes as numbered in circles are 1, 3-hydroxypropionyl-CoA synthetase (ADP-forming); 2, 3-hydroxypropionyl-CoA synthetase (AMP-forming); 3, 3-hydroxypropionyl-CoA dehydratase; 4, 4-hydroxybutyryl-CoA synthetase (ADP-forming); 5, 4-hydroxybutyryl-CoA synthetase (AMP-forming); 6, 4-hydroxybutyryl-CoA dehydratase; 7, crotonyl-CoA hydratase; 8, succinyl-CoA synthetase (ADP-forming); 9, succinic semialdehyde dehydrogenase; 10, pyruvate-phosphate dikinase; and 11, pyruvate-water dikinase.

pH 2, respectively), differences in carbon-fixing carboxylases cannot be excluded, thus underscoring the need for a biochemical characterization of the autotrophic CO_2 fixation pathway in *N. maritimus*.

Here, using *N. maritimus* as a model organism, we demonstrate that Thaumarchaeota possess a modified version of the HP/HB cycle that is distinct from the cycle operating in Crenarchaeota (Fig. 1). Hence, our study shows that the HP/HB cycle apparently evolved twice in Archaea. Importantly, the identified mechanism for CO_2 fixation is far more energy efficient than any other aerobic autotrophic pathway. Thus, this study provides a plausible biochemical explanation for the ability of AOA to thrive under extremely low-nutrient concentrations.

Results

Activities of HP/HB Cycle Enzymes in Cell Extracts of *N. maritimus*. Direct evidence for the operation of the HP/HB cycle in Thaumarchaeota was provided by biochemical analysis of cell extracts generated from 12–15 L batch cultures of *N. maritimus*. Cells were grown autotrophically to a density of 3×10^7 cells/mL (~0.6 mg/L dry mass) with a generation time of 35 h, which requires a specific carbon-fixation rate of 27 nmol·min^{-1}·mg^{-1} protein. If two molecules of CO_2 are fixed in one turn of the autotrophic CO_2 fixation cycle, the minimal in vivo specific activity of this enzyme system is 13.5 nmol·min^{-1}·mg^{-1} protein.

In accordance with results of genomic studies, the activities of the key enzyme of the Calvin–Benson cycle, ribulose 1,5-bisphosphate carboxylase/oxygenase, and of the 3-hydroxypropionate bicycle, mesaconyl-CoA C1–C4 CoA-transferase, could not be detected in cell extracts (*SI Appendix*, Table S1). In contrast, the key carboxylase of the HP/HB cycle, a biotin-containing acetyl-CoA/propionyl-CoA carboxylase, was active (Table 1). The activity was dependent on the addition of ATP and was completely inhibited by avidin, a specific inhibitor of biotin-dependent enzymes. Moreover, *N. maritimus* expressed a biotinylated protein, as shown by biotin staining of cell extracts with a size matching the predicted mass of the biotin carboxyl carrier protein of this carboxylase (Nmar_0274, 18.5 kDa) (*SI Appendix*, Fig. S1). The observed low activity probably is caused by the low stability of the enzyme complex. For example, in vitro *Escherichia coli* acetyl-CoA carboxylase is one of the least stable of the characterized enzymes (26), and its activity can hardly be detected in cell extracts.

N. maritimus cell extracts also catalyzed the postulated conversion of 3-hydroxypropionate into propionyl-CoA as well as the conversion of 4-hydroxybutyrate into two molecules of acetyl-CoA (*SI Appendix*, Figs. S2 and S3). The other enzyme activities necessary for the functioning of the HP/HB cycle were detected also (Table 1). The conversion of 4-hydroxybutyrate into two molecules of acetyl-CoA could be measured under oxic conditions (ambient air), showing that the key enzyme required for this conversion, 4-hydroxybutyryl-CoA dehydratase, is oxygen tolerant.

Acetyl-CoA is the product of the proposed HP/HB cycle and is used for biosynthesis (Fig. 1). However, in *N. maritimus*, acetyl-CoA cannot be reductively carboxylated to pyruvate as it is in anaerobes (*SI Appendix*, Table S1), so another assimilation mechanism is required. A similar situation occurs in *M. sedula*, where, by an additional half turn of the cycle, acetyl-CoA plus another two bicarbonate molecules are converted into succinyl-CoA. Succinyl-CoA then is converted to malate or oxaloacetate, which can be decarboxylated to pyruvate or phosphoenolpyruvate (27). Hence the precursor metabolites, primarily pyruvate, phosphoenolpyruvate, oxaloacetate, 2-oxoglutarate, and a few others, are generated from succinyl-CoA (Fig. 1). Accordingly, *N. maritimus* possesses an ADP-forming succinyl-CoA synthetase (Fig. 1 and *SI Appendix*, Table S1) operating in the reverse direction to drain carbon precursor metabolites off the HP/HB cycle. Phosphoenolpyruvate carboxykinase (Nmar_0392) is the only enzyme encoded in the genome of *N. maritimus* capable of catalyzing oxaloacetate conversion to phosphoenolpyruvate and thus connecting pools of C_4 and C_3 compounds.

Characterization of Thaumarchaeal 4-hydroxybutyryl-CoA Dehydratase. The apparent oxygen insensitivity of 4-hydroxybutyryl-CoA dehydratase in cell extracts suggested that the key enzyme of the HP/HB cycle might be adapted to the aerobic operation of the cycle in *N. maritimus*. This possibility raised our interest, because closely related clostridial 4-hydroxybutyryl-CoA dehydratases had been reported to be extremely oxygen-sensitive (28, 29). The presence of the oxygen-tolerant 4-hydroxybutyryl-CoA dehydratase in Thaumarchaeota is a biochemical requirement for the operation of the HP/HB cycle under aerobic conditions. The gene encoding 4-hydroxybutyryl-CoA dehydratase (Nmar_0207) was cloned and heterologously expressed in *E. coli*. The corresponding protein was purified anaerobically, and its properties were compared with the properties of heterologously produced 4-hydroxybutyryl-CoA dehydratase from *Clostridium aminobutyricum* that participates in 4-aminobutyrate fermentation (28). The two enzymes are highly similar, sharing 58% amino acid sequence identity and 72% sequence similarity. Correspondingly, their K_m and V_{max} values for 4-hydroxybutyryl-CoA were almost identical

Table 1. Specific activities of the enzymes of the HP/HB cycle in cell extracts of *N. maritimus*

Enzyme	Specific activity, nmol·min^{-1}·mg^{-1} protein	Genes in *N. maritimus*
Acetyl-CoA carboxylase	1.2 ± 0.1	Nmar_0272/0273/0274
Malonyl-CoA reductase (NADPH)*	62 ± 6	?
Malonic semialdehyde reductase (NADPH)	290 ± 40	?
3-Hydroxypropionyl-CoA synthetase (ADP-forming)	25 ± 5	Nmar_1309
3-Hydroxypropionyl-CoA dehydratase	480 ± 140	Nmar_1308
Acryloyl-CoA reductase	≥1.6[†]	?
Propionyl-CoA carboxylase	0.5 ± 0.1	Nmar_0272/0273/0274
Methylmalonyl-CoA epimerase	ND	Nmar_0953
Methylmalonyl-CoA mutase	ND	Nmar_0954/0958
Succinyl-CoA reductase (NADPH)*	18 ± 2	?
Succinic semialdehyde reductase (NADPH)*	400 ± 30	?
4-Hydroxybutyryl-CoA synthetase (ADP-forming)	20 ± 5	Nmar_0206
4-Hydroxybutyryl-CoA dehydratase	≥1.5[†]	Nmar_0207
Crotonyl-CoA hydratase [(*S*)-3-hydroxybutyryl-CoA forming]	16,500 ± 1,400	Nmar_1308
(*S*)-3-Hydroxybutyryl-CoA dehydrogenase (NAD$^+$)[‡]	160 ± 20	Nmar_1028
Acetoacetyl-CoA β-ketothiolase	≥1.5[†]	Nmar_0841 and/or Nmar_1631

ND, not determined; ?, gene cannot be identified unambiguously based on bioinformatic analysis. The data are mean values and deviations of several independent enzyme assays.

*No activity with NADH was detected.

[†]Estimated from the rate of 3-hydroxypropionate conversion to propionyl-CoA (for acryloyl-CoA reductase) or from the rate of 4-hydroxybutyrate conversion to acetyl-CoA (for 4-hydroxybutyryl-CoA dehydratase and acetoacetyl-CoA β-ketothiolase).

[‡]No activity with NADP$^+$ was detected.

(*N. maritimus*: V_{max} 25 ± 2 μmol·min^{-1}·mg^{-1} protein, K_m 0.06 ± 0.02 mM; *C. aminobutyricum*: V_{max} 19 ± 1 μmol·min^{-1}·mg^{-1} protein, K_m 0.056 ± 0.03 mM).

However, the two enzymes were strikingly different in their oxygen tolerance. The clostridial enzyme was oxygen sensitive and had a half-life of 14 min under aerobic conditions, whereas the 4-hydroxybutyryl-CoA dehydratase of *N. maritimus* was almost two orders of magnitude more stable in the presence of oxygen, retaining 50% of activity even after 46 h of incubation in the presence of oxygen (*SI Appendix*, Fig. S4). These data suggest that the thaumarchaeal 4-hydroxybutyryl-CoA dehydratase is well suited to function in aerobic organisms.

Characterization of Previously Unidentified Enzymes Involved in the HP/HB Cycle of *N. maritimus*. Cell extract measurements revealed that the two CoA ligases acting on 3-hydroxypropionate and 4-hydroxybutyrate were ADP-forming (Fig. 1 and Table 1), whereas aerobic Crenarchaeota possess AMP-forming ligases (19, 30, 31). Because this issue is important for evaluating the energy efficiency of the cycle, we studied these enzymes in greater detail. The genome of *N. maritimus* contains two genes that are homologous to the ADP-forming acetyl-CoA synthetase from *Archaeoglobus fulgidus* (32), Nmar_1309 (38% identity to AF1938) and Nmar_0206 (41% identity to AF1211) and that may encode the required enzymatic activities. These genes were synthesized, cloned, and heterologously expressed in *E. coli*. The purified proteins were active and catalyzed the ATP-dependent conversion of 3-hydroxypropionate (Nmar_1309) and 4-hydroxybutyrate (Nmar_0206) with ATP and CoA into 3-hydroxypropionyl-CoA and 4-hydroxybutyryl-CoA, respectively, with ADP and P_i being formed. Although these proteins activated some other organic acids, the catalytic efficiency for these substrates was poor, thus identifying them as 3-hydroxypropionyl-CoA synthetase (Nmar_1309) and 4-hydroxybutyryl-CoA synthetase (Nmar_0206) (Tables 2 and 3). As discussed below, the presence of these two ADP-forming acyl-CoA synthetases is an important feature in making this cycle the most cost-effective autotrophic pathway in aerobes.

Inspection of the *N. maritimus* genome sequence revealed that certain genes coding for proteins of the HP/HB cycle occur in distinct clusters. For instance, the gene for the 4-hydroxybutyryl-CoA synthetase is colocalized with the gene for the 4-hydroxybutyryl-CoA dehydratase (Nmar_0207), the latter catalyzing the following reaction of the cycle. The 3-hydroxypropionyl-CoA synthetase gene is colocalized with the gene for an enoyl-CoA hydratase of the crotonase family (Nmar_1308) that may represent a 3-hydroxypropionyl-CoA dehydratase, the next enzyme in the reaction sequence. Indeed, heterologously produced Nmar_1308 catalyzed 3-hydroxypropionyl-CoA dehydration to acryloyl-CoA (V_{max} 74 ± 9 μmol·min^{-1}·mg^{-1} protein, K_m 0.18 ± 0.06 mM) as well as crotonyl-CoA hydration to 3-hydroxybutyryl-CoA (V_{max} 3,110 ± 230 μmol·min^{-1}·mg^{-1} protein, K_m 0.16 ± 0.03 mM). Both activities are essential for the proposed cycle. The ratio of 3-hydroxypropionyl-CoA dehydratase and crotonyl-CoA hydratase activities for the heterologously produced enzyme was similar to the ratio of these activities in cell extracts (Table 1). This similarity suggests that Nmar_1308 is responsible for both conversions in vivo and functions as promiscuous 3-hydroxypropionyl-CoA dehydratase/crotonyl-CoA hydratase (Fig. 1).

Phylogenetic Analysis Indicates Convergent Evolution of the HP/HB Cycle. In addition to verifying experimentally the presence of three previously unaccounted genes of the HP/HB cycle in *N. maritimus*, we were able to identify most other genes for the enzymes of this pathway bioinformatically (Table 1). Using this information, we performed a comparative phylogenetic analysis to trace the evolution of the HP/HB cycle (Fig. 2 and see *SI Appendix*, Figs. S6–S13 and *SI Appendix*, *SI Text* for details). The identified genes of the HP/HB cycle were found in the genomes of all sequenced representatives of the phylum Thaumarchaeota, suggesting a common ability to grow autotrophically via this variant of the HP/HB cycle. Additionally, the corresponding proteins formed separate "thaumarchaeal clusters," which were not specifically associated with crenarchaeal sequences. Even for the key enzyme of the cycle, 4-hydroxybutyryl-CoA dehydratase, thaumarchaeal proteins cluster together with bacterial representatives rather than with crenarchaeal ones (Fig. 2*D*). This clustering suggests a convergent evolution of autotrophic CO_2 fixation cycles in Cren- and Thaumarchaeota.

Comparison of the Growth Yield of AOA and AOB. AOB use the costly Calvin–Benson cycle, whereas AOA use the identified cost-effective pathway, features that should severely affect the growth yields of these two groups. To compare the growth yield of AOA and their bacterial counterparts, we grew *N. maritimus* strain SCM1 and the AOB *Nitrosococcus oceani* in unbuffered

Table 2. General properties of recombinant 3-hydroxypropionyl-CoA synthetase and recombinant 4-hydroxybutyryl-CoA synthetase from *N. maritimus*

Property	3-Hydroxypropionyl-CoA synthetase	4-Hydroxybutyryl-CoA synthetase
Reaction catalyzed	3-hydroxypropionate + ATP + CoA → 3-hydroxypropionyl-CoA + ADP + P_i	4-hydroxybutyrate + ATP + CoA → 4-hydroxybutyryl-CoA + ADP + P_i
Gene	Nmar_1309	Nmar_0206
Molecular mass, kDa	76	76
Predicted molecular mass, kDa*	76.1	75.6
Divalent cations required for the activity	Mg^{2+} or Mn^{2+} (no activity with Ni^{2+}, Co^{2+}, and Ca^{2+})	Mg^{2+} or Mn^{2+} (no activity with Ni^{2+}, Co^{2+}, and Ca^{2+})

*Predicted for the native protein without polyhistidine tags.

synthetic medium containing 0.5 mM NH_4Cl (*SI Appendix*, Fig. S5). The doubling time of *N. oceani* was significantly shorter than the doubling time of *N. maritimus* (15 h versus 28 h). However, the specific growth yield of *N. maritimus* was significantly greater, 1.3 g dry mass formed per mole of ammonia oxidized in contrast to only 0.8 g/mol for *N. oceani.* Therefore, *N. maritimus* couples ammonia oxidation with biomass formation in a more efficient way than *N. oceani*.

Discussion

Method-related challenges of this study were associated with the extremely small cell dimensions of *N. maritimus* and its oligotrophic character, resulting in growth with a generation time of more than a day and maximum cell densities of only 5×10^7 cells/mL. In addition to direct biochemical analysis using cell extracts generated from 12–15 L batch cultures, we further validated enzyme function and gene annotation by expressing four thaumarchaeal enzymes in *E. coli*. Together, the detailed biochemical characterization of carbon fixation in *N. maritimus* provided compelling evidence for the operation of an energy-efficient variant of the HP/HB cycle in Thaumarchaeota, both confirming and extending a mechanism of CO_2 fixation only suggested by genomic analysis.

What makes the thaumarchaeal HP/HB cycle so special? This autotrophic cycle is more energy efficient than any other aerobic carbon-fixation pathway. The efficiency can be explained by three specific adaptions. First, the activation of 3-hydroxypropionate and 4-hydroxybutyrate proceeds via ADP- rather than AMP-producing enzymes; hence, one turn of the cycle requires two high-energy bonds less than one turn of the crenarchaeal HP/HB pathway (Fig. 1). Second, the participation of promiscuous enzymes catalyzing multiple reactions in the thaumarchaeal HP/HB cycle, such as acetyl-CoA/propionyl-CoA carboxylase and 3-hydroxypropionyl-CoA dehydratase/crotonyl-CoA hydratase, reduces the cost of protein biosynthesis. Third, the oxygen stability of 4-hydroxybutyryl-CoA dehydratase that until now has been assumed to require anoxic conditions because of its radical mechanism (33) is a prerequisite for the aerobic operation of the thaumarchaeal HP/HB cycle, lowering enzyme maintenance and turnover costs further. Interestingly, 4-hydroxybutyryl-CoA dehydratase from *M. sedula* also is robust in the presence of oxygen, as reported during revision of this work (34).

Comparative phylogenetic analyses pointed to convergent evolution of the HP/HB cycle, almost certainly having been invented independently in the crenarchaeal and thaumarchaeal lineages. The thaumarchaeal and crenarchaeal variants of the HP/HB cycle involve analogous reactions to fix CO_2, but they use a divergent set of mostly unrelated enzymes. Thus, the advantage in energy conferred by this pathway explains its thrice-repeated independent emergence during evolution, namely in the thaumarchaeal and crenarchaeal versions of the HP/HB cycle and in the 3-hydroxypropionate bicycle in Chloroflexi (14, 19, 35). Independent emergence of the HP/HB cycle provides additional support for the hypothesis of an early evolutionary divergence of the Thaumarchaeota and its status as a separate phylum (1). Still, only few full-genome sequences of the representatives of Thaumarchaeota are available, and further phylogenetic analyses that include new members of the Thaumarchaeota are required to confirm these conclusions.

The development of this economical cycle no doubt has contributed to the ecological success of AOA. Accordingly, CO_2 fixation through the thaumarchaeal HP/HB cycle requires one-third less energy than via the Calvin–Benson cycle functioning in AOB (36) (Table 4). Furthermore, CO_2 fixation via the HP/HB cycle is not accompanied by losses caused by the oxygenase side-reaction of ribulose-1,5-bisphosphate carboxylase/oxygenase, which leads to an additional loss of about 20% of fixed carbon in organisms using the Calvin–Benson cycle (15, 37). In fact, comparison between the specific growth yields obtained from cultures of *N. maritimus* and the marine AOB *N. oceani* grown under exactly the same conditions indicates that *N. maritimus* uses the energy gained by ammonia oxidation for biomass formation in a much more efficient way than its bacterial counterpart (*SI Appendix*, Fig. S5). The specific growth yield of *N. maritimus* was ~1.5 higher than the value determined for

Table 3. Catalytic properties of recombinant 3-hydroxypropionyl-CoA synthetase and recombinant 4-hydroxybutyryl-CoA synthetase from *N. maritimus*

	3-Hydroxypropionyl-CoA synthetase			4-Hydroxybutyryl-CoA synthetase		
Substrate	V_{max}, μmol·min $^{-1}$·mg $^{-1}$ protein	K_m, mM	k_{cat}/K_m	V_{max}, μmol·min $^{-1}$·mg $^{-1}$ protein	K_m, mM	k_{cat}/K_m
3-Hydroxypropionate	**0.59 ± 0.03**	**1.2 ± 0.2**	**0.64**	–	–	–
4-Hydroxybutyrate	0.48 ± 0.04	5.6 ± 1.0	0.11	**1.4 ± 0.01**	**0.37 ± 0.06**	**4.88**
Acetate	–	–	–	0.22 ± 0.01	200 ± 5	0.001
Propionate	0.50 ± 0.10	17.0 ± 6.0	0.04	0.10 ± 0.01	88 ± 7	0.002
Butyrate	0.54 ± 0.01	12.4 ± 0.3	0.06	0.61 ± 0.05	5 ± 1	0.16
ATP	0.59 ± 0.03	0.6 ± 0.1	1.28	1.7 ± 0.2	0.22 ± 0.07	9.96
CoA	0.60 ± 0.10	0.16 ± 0.09	4.85	1.4 ± 0.1	0.16 ± 0.05	11.28

Neither synthetase was active with the following substrates: crotonate, acetoacetate, (*S*)-3-hydroxybutyrate, (*R*)-3-hydroxybutyrate, succinate, (*RS*)-malate, itaconate, (*S*)-citramalate. –, no activity detected.

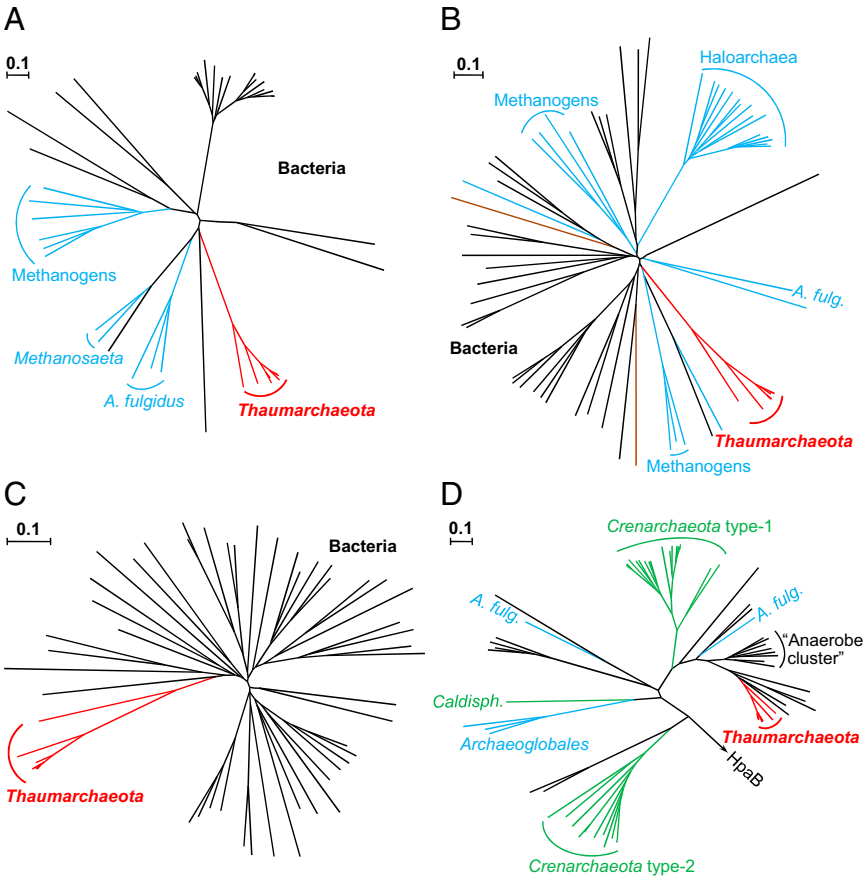

Fig. 2. Phylogenetic trees of *N. maritimus* HP/HB cycle enzymes identified in this study and of the key enzyme of the cycle, 4-hydroxybutyryl-CoA dehydratase. (*A*) Homologs of 3-hydroxypropionyl-CoA synthetase from Nmar_1309. (*B*) Homologs of the 4-hydroxybutyryl-CoA synthetase (Nmar_0206). (*C*) Homologs of 3-hydroxypropionyl-CoA dehydratase/crotonyl-CoA hydratase (Nmar_1308). (*D*) Homologs of 4-hydroxybutyryl-CoA dehydratase (Nmar_0207). Thaumarchaeal sequences are shown in red, crenarchaeal in green, euryarchaeal in blue, eukaryotic in brown, and bacterial in black. The tree is based on amino acid sequence analysis. Tree topology and evolutionary distances are given by the neighbor-joining method with Poisson correction. (Scale bars: a difference of 0.1 substitutions per site.) For details of the tree construction, see *SI Appendix*, Figs. S6 and S10–S12. The accession numbers of the sequences used for the construction of the tree are listed *in SI Appendix*, Table S4. *A. fulg., A. fulgidus; Caldisph., Caldisphaera*.

N. oceani. (Note, however, that the growth rate of *N. oceani* was significantly higher than the growth rate of *N. maritimus*.) Therefore, the growth yields of AOA and AOB distinctly mirror the different energy requirements of the corresponding autotrophic pathways. Consequently, these data offer a biochemical explanation for the dominance of AOA, relative to AOB, in environments with low ammonia availability such as open ocean water (7, 10), where they often account for more than 20% of all planktonic microorganisms (6).

The autotrophic lifestyle of marine AOA was confirmed earlier by radiocarbon studies on diagnostic archaeal lipids revealing that more than 80% of the thaumarchaeal biomass in natural seawater originates from inorganic carbon and by incorporation of radiolabeled bicarbonate (11, 38). Estimations suggest that Thaumarchaeota contribute to around 1% to the total marine primary production and contribute significantly to the global carbon cycle (11, 38). Because the relative abundance of Thaumarchaeota increases with depth (up to 40% of all pelagic microorganisms; ref. 6), the thaumarchaeal variant of the HP/HB cycle probably represents the most important CO_2-fixation pathways below the photic zone in the meso- and bathypelagic realm of the ocean.

Apart from providing an explanation for the capacity of the Thaumarchaeota to thrive in extreme oligotrophic environments, there are clear biotechnological applications that could derive from the present study. In recent years, carboxylating enzymes and autotrophic CO_2-fixation pathways have attracted much attention because of their potential future applications for the production of industrial chemicals using synthetic biology approaches (39–41). Autotrophy based on acetyl-CoA/propionyl-CoA

Table 4. Comparison of the energy efficiency of aerobic autotrophic CO_2 fixation cycles

1 mole precursor or 1 g biomass	Calvin–Benson cycle*	3-Hydroxypropionate bicycle	Crenarchaeal HP/HB cycle	Thaumarchaeal HP/HB cycle
Acetyl-CoA	7	7	6	4
Pyruvate	7	7	9–10†	5
Phosphoenolpyruvate	8	9	10–11†	7
Oxaloacetate	8	9	9–10†	6
2-Oxoglutarate	15	16	15–16†	10
Biomass (1 g)	0.12*	0.13	0.13–0.15†	0.09

The numbers represent moles of high-energy anhydride bonds of ATP required to form 1 mole of central precursor metabolite or of the main precursors for the synthesis of 1 g of dry biomass. For the details of calculations, see *SI Appendix, SI Text*. The estimated amount of the central metabolic precursors acetyl-CoA, pyruvate, phosphoenolpyruvate, oxaloacetate, and 2-oxoglutarate necessary for the synthesis of 1 g of dry cells was taken from ref. 42.

*Note that the costs of the oxygenase side reaction of the key enzyme of the Calvin–Benson cycle (ribulose 1,5-bisphosphate carboxylase) are not taken into account in this table. C_3 plants lose about 20% of fixed carbon in photorespiration (15, 37).

†The costs of the synthesis of pyruvate, phosphoenolpyruvate, oxaloacetate, and 2-oxoglutarate in *M. sedula* vary depending on the pathway of succinyl-CoA conversion to succinate (27).

carboxylase rather than on ribulose-1,5-bisphosphate carboxylase/oxygenase would seem to be an attractive model for the development of a synthetic carbon-fixation pathway. The HP/HB cycle already has been suggested as a potential tool for biotechnological applications (40, 41), and we suspect that, apart from the biological importance, our discovery could have significant applied value.

Materials and Methods

N. maritimus strain SCM1 was cultivated autotrophically under aerobic conditions with ammonia as a sole energy source at 28 °C as described previously (3, 5). Cell extracts were prepared using a French pressure cell. 3-Hydroxypropionate conversion to propionyl-CoA and 4-hydroxybutyrate conversion to acetyl-CoA were measured by following the formation of propionyl-CoA and acetyl-CoA, respectively, with HPLC. Ribulose-1,5-bisphosphate carboxylase/oxygenase and acetyl-CoA and propionyl-CoA carboxylases were measured radiochemically by determining substrate-dependent fixation of $^{14}CO_2$. Other enzyme assays were performed using standard biochemical techniques either spectrophotometrically or by following the product formation in an ultra-performance liquid chromatography (UPLC)-based assay. HPLC and UPLC were performed using a reversed-phase C_{18} column, the reaction products were detected by absorbance at 260 nm, and the amount of product(s) was calculated from the relative peak area. The genes for Nmar_1308 and Nmar_0207 were amplified from genomic DNA of *N. maritimus*, and the genes for Nmar_1309 and Nmar_0206 were synthesized by Eurofins MWG Operon. The codon use in the genes was optimized for the expression in *E. coli*. The gene for clostridial 4-hydroxybutyryl-CoA dehydratase was amplified from genomic DNA of *C. aminobutyricum*. The genes from *N. maritimus* were cloned in the expression vector pET16b containing an N-terminal His-tag, and the clostridial gene was cloned in the expression vector pET28b (N-terminal His-tag). All proteins were heterologously overproduced in *E. coli* and purified using Ni-NTA column. Detailed materials and methods can be found in *SI Appendix, SI Materials and Methods*.

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