Glucose Metabolism and Acetate Switch in Archaea: the Enzymes in *Haloferax volcanii*

Tom Kuprat,^a Marius Ortjohann,^a Ulrike Johnsen,^a Peter Schönheit^a

^aInstitut für Allgemeine Mikrobiologie, Christian-Albrechts-Universität, Kiel, Germany

Tom Kuprat and Marius Ortjohann contributed equally to this work. Author order was determined both alphabetically and in order of increasing seniority.

ABSTRACT The halophilic archaeon *Haloferax volcanii* has been proposed to degrade glucose via the semiphosphorylative Entner-Doudoroff (spED) pathway. Following our previous studies on key enzymes of this pathway, we now focus on the characterization of enzymes involved in 3-phosphoglycerate conversion to pyruvate, in anaplerosis, and in acetyl coenzyme A (acetyl-CoA) formation from pyruvate. These enzymes include phosphoglycerate mutase, enolase, pyruvate kinase, phosphoenolpyruvate carboxylase, and pyruvate-ferredoxin oxidoreductase. The essential function of these enzymes were shown by transcript analyses and growth experiments with respective deletion mutants. Furthermore, we show that *H. volcanii*—during aerobic growth on glucose—excreted significant amounts of acetate, which was consumed in the stationary phase (acetate switch). The enzyme catalyzing the conversion of acetyl-CoA to acetate as part of the acetate overflow mechanism, an ADP-forming acetyl-CoA synthetase (ACD), was characterized. The functional involvement of ACD in acetate formation and of AMP-forming acetyl-CoA synthetases (ACs) in activation of excreted acetate was proven by using respective deletion mutants. Together, the data provide a comprehensive analysis of enzymes of the spED pathway and of anaplerosis and report the first genetic evidence of the functional involvement of enzymes of the acetate switch in archaea.

IMPORTANCE In this work, we provide a comprehensive analysis of glucose degradation via the semiphosphorylative Entner-Doudoroff pathway in the haloarchaeal model organism *Haloferax volcanii*. The study includes transcriptional analyses, growth experiments with deletion mutants, and characterization of all enzymes involved in the conversion of 3-phosphoglycerate to acetyl coenzyme A (acetyl-CoA) and in anaplerosis. Phylogenetic analyses of several enzymes indicate various lateral gene transfer events from bacteria to haloarchaea. Furthermore, we analyzed the key players involved in the acetate switch, i.e., in the formation (overflow) and subsequent consumption of acetate during aerobic growth on glucose. Together, the data provide novel aspects of glucose degradation, anaplerosis, and acetate switch in *H. volcanii* and thus expand our understanding of the unusual sugar metabolism in archaea.

KEYWORDS *Haloferax volcanii*, glucose degradation, semiphosphorylative Entner-Doudoroff pathway, acetate switch, ADP-forming acetyl-CoA synthetase, pyruvate-ferredoxin oxidoreductase, phosphoenolpyruvate carboxylase

Comparative analyses of the central carbon metabolism in archaea, bacteria, and eukarya revealed a variety of unusual pathways and enzymes in archaea that differ from the canonical pathways of bacteria and eukarya. In particular, sugar metabolism has been studied in detail (1, 2). It was found that anaerobic and microaerophilic hyperthermophilic archaea, including *Pyrococcus* and *Thermococcus* species and *Pyrobaculum aerophilum*, degrade glucose and glucose polymers by modified versions of the Embden-

Citation Kuprat T, Ortjohann M, Johnsen U, Schönheit P. 2021. Glucose metabolism and acetate switch in archaea: the enzymes in *Haloferax volcanii*. J Bacteriol 203:e00690-20. <https://doi.org/10.1128/JB.00690-20>.

Editor William W. Metcalf, University of Illinois at Urbana Champaign

Copyright © 2021 American Society for Microbiology. All Rights Reserved.

Address correspondence to Peter Schönheit, peter.schoenheit@ifam.uni-kiel.de.

Received 17 December 2020

Accepted 28 January 2021

Accepted manuscript posted online 8 February 2021

Published 23 March 2021

Meyerhof pathway, whereas thermoacidophilic archaea, e.g., *Picrophilus torridus* and *Sulfolobus* species, utilize modified Entner-Doudoroff (ED) pathways, namely, the non-phosphorylative ED pathway and the branched ED pathway, respectively (3, 4).

In halophilic archaea, sugar metabolism has been studied in particular in *Haloferax* and *Haloarcula* species (5–10). *Haloferax volcanii*, which represents a model organism of haloarchaea (11), exhibits a high metabolic versatility in utilizing various sugars, hexoses, pentoses and deoxysugars, and other carbon compounds, e.g., acetate, as growth substrates (12). Glucose degradation in *H. volcanii* has been shown to proceed via the semiphosphorylative ED (spED) pathway, and the enzymes of this pathway, involved in the conversion of glucose to 3-phosphoglycerate (3PG), have been characterized (7, 13, 86). They include glucose dehydrogenase, gluconate dehydratase, 2-keto-3-deoxygluconate kinase, and 2-keto-3-deoxy-6-phosphogluconate aldolase. Furthermore, the enzymes involved in the oxidation of glyceraldehyde-3-phosphate (GAP) to 3PG, GAP dehydrogenase, and phosphoglycerate kinase were characterized. However, the enzymes involved in further degradation of 3PG to pyruvate, including phosphoglycerate mutase, enolase, and pyruvate kinase, have not been analyzed. Also, the enzymes that catalyze the conversion of pyruvate to acetyl-CoA, which is further oxidized in the citric acid cycle, and the formation of oxaloacetate as an anaplerotic reaction from glucose have not been characterized in *H. volcanii*.

In previous studies, we have shown that during exponential growth on glucose, the haloarchaea *Haloarcula marismortui* and *H. volcanii* excreted significant amounts of acetate which were consumed in the stationary phase (14, 15). The excretion of acetate from glucose under aerobic conditions (acetate overflow) and subsequent utilization of acetate following complete glucose consumption—together designated acetate switch—have been reported in various bacteria, e.g., *Escherichia coli* and *Bacillus subtilis* (16, 17). It has been proposed that acetate overflow in these bacteria takes place when the rate of glucose degradation exceeds that of subsequent reactions in the citric acid cycle and respiratory chain (18–21). The enzymes involved in the acetate switch in these bacteria have been analyzed; the conversion of acetyl-CoA to acetate is catalyzed by two enzymes, namely, phosphotransacetylase (PTA; $\text{acetyl-CoA} + \text{P}_i \rightarrow \text{acetyl-phosphate} + \text{CoA}$) and acetate kinase (AK; $\text{acetylphosphate} + \text{ADP} \rightarrow \text{acetate} + \text{ATP}$). The respective *pta* and *ackA* genes encoding PTA and AK are transcriptionally upregulated during growth on glucose. The subsequent reutilization of excreted acetate involves an inducible AMP-forming acetyl-CoA synthetase (ACS; $\text{acetate} + \text{CoA} + \text{ATP} \rightarrow \text{acetyl-CoA} + \text{AMP} + \text{PP}_i$). AK and PTA are known as classical enzymes in acetate formation in anaerobic fermentation processes of bacteria, whereas ACS is the predominant enzyme of acetate activation during growth on acetate in bacteria.

Archaea do not use the bacterial enzymes AK and PTA for acetate formation; instead, it has been shown that all acetate-forming archaea, including anaerobic hyperthermophiles and aerobic haloarchaea, utilize an ADP-forming acetyl-CoA synthetase (ACD; $\text{acetyl-CoA} + \text{ADP} + \text{P}_i \rightarrow \text{acetate} + \text{ATP} + \text{CoA}$). ACD is a novel prokaryotic enzyme involved in acetate formation and ATP synthesis replacing the canonical bacterial mechanism involving AK and PTA (22–24). In contrast, the activation of acetate in aerobic archaea is catalyzed by AMP-forming acetyl-CoA synthetase (ACS), and thus, ACS is operative in both archaea and bacteria. Cell extracts of *H. marismortui* and *H. volcanii* were shown to contain ACD activity in exponentially grown cells, whereas stationary-phase cells contain an inducible ACS activity (14, 15). So far, the genes encoding ACD and ACS activities and their functional involvement in the acetate switch have not been demonstrated in haloarchaea.

Here, we report the characterization of the spED pathway enzymes phosphoglycerate mutase, enolase, and pyruvate kinase. Also, the anaplerotic enzyme phosphoenolpyruvate carboxylase and the acetyl-CoA-forming enzyme pyruvate-ferredoxin oxidoreductase (POR) are described. Furthermore, we analyzed the functional involvement of genes associated with the acetate switch from glucose, i.e., ACD and ACS enzymes in *H. volcanii*.

FIG 1 Transcriptional analysis of *gpml* encoding cofactor-independent phosphoglycerate mutase from *H. volcanii*. The gene *gpml* (1,581 nucleotides) is depicted as a gray arrow, with flanking genes as white arrows (A). Northern blotting was performed with RNA from glucose (Glc)- and Casamino Acid (Cas)-grown cells using a probe against *gpml*. 16S rRNA served as a loading control (B).

RESULTS AND DISCUSSION

Haloferax volcanii has been reported to degrade glucose to pyruvate via the semi-phosphorylative Entner-Doudoroff (spED) pathway. In our previous analyses, we have characterized the spED enzymes that catalyze the conversion of glucose to 3-phosphoglycerate (7, 13, 86). Here, we report the characterization of the spED enzymes phosphoglycerate mutase, enolase, and pyruvate kinase. Furthermore, pyruvate-ferredoxin oxidoreductase forming acetyl-CoA from pyruvate was studied. We also analyzed the enzyme phosphoenolpyruvate (PEP) carboxylase involved in anaplerosis to replenish tricarboxylic acid (TCA) cycle intermediates. The functional involvement of the enzymes was studied by transcriptional analyses and by growth experiments with the respective deletion mutants. Furthermore, we show that *H. volcanii* excretes significant amounts of acetate from glucose. The enzyme involved in acetate formation from acetyl-CoA, an ADP-forming acetyl-CoA synthetase, was characterized.

The enzymes of the spED pathway and pyruvate-ferredoxin oxidoreductase. Phosphoglycerate mutase. In the genome of *H. volcanii*, the gene *gpml* (HVO_2516) is annotated to encode a putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (iPGM). iPGMs differ from cofactor (2,3-bisphosphoglycerate)-dependent PGMs (dPGMs) that require 2,3-bisphosphoglycerate as effectors. The iPGM from *H. volcanii* has a calculated molecular mass of 57.2 kDa. Transcription of *gpml* analyzed by Northern blotting experiments with cells grown on glucose or Casamino Acids revealed a specific signal of about 1,620 nucleotides each, which corresponds to the length of *gpml* of 1,581 nucleotides indicating expression under both glycolytic and gluconeogenic conditions (Fig. 1). The gene *gpml* was overexpressed in *H. volcanii* H1209, and the recombinant enzyme was purified by Ni-nitrilotriacetic acid (NTA) affinity and size exclusion chromatography. The protein showed on SDS-PAGE a single band of 70 kDa (see Fig. S1 in the supplemental material); by gel filtration, a molecular mass of 62 kDa was determined and characterized iPGM as a monomeric protein. iPGM catalyzed the conversion of 3-phosphoglycerate (3PG) to 2-phosphoglycerate (2PG) with a $V_{\max}$ of 85.4 U/mg and a K_m for 3PG of 2.5 mM. The enzyme also catalyzed the reverse reaction, the conversion of 2PG to 3PG, with a $V_{\max}$ of 9.1 U/mg and a K_m for 2PG of 0.6 mM (see Fig. S2 in the supplemental material).

iPGM from *H. volcanii* showed high sequence identity (63% to 66%) to putative iPGMs from other haloarchaea, e.g., *Haloarcula marismortui*, *Halobacterium salinarum*, and *Haloquadratum walsbyi*, and to characterized iPGMs from the bacteria *E. coli* (41%) and *Geobacillus stearothermophilus* (44%) (25, 26). Less sequence identity (11 to 13%) was found with characterized iPGM from the archaea *Archaeoglobus fulgidus*, *Methanocaldococcus jannaschii*, *Sulfolobus solfataricus*, and *Pyrococcus furiosus* (27–30).

Phylogenetic analysis of iPGMs indicates that the homologs from bacteria, archaea, and eukarya cluster in three distinct, domain-specific clades (Fig. 2). The iPGMs of *H. volcanii* and haloarchaeal homologs are members of the bacterial iPGM cluster, which is distantly related to iPGMs of the archaeal cluster. The close phylogenetic relationship of haloarchaeal iPGMs to bacteria suggest a lateral gene transfer event from bacteria

FIG 2 Phylogenetic relationship of cofactor-independent phosphoglycerate mutase from *H. volcanii* and haloarchaeal homologs, from other archaea, and from bacteria and eukarya. The tree is based upon a multiple amino acid sequence alignment that was generated with ClustalX (83). Numbers at the nodes are bootstrapping values according to neighbor joining. Organism names and UniProt accession numbers are as follows: *Haloferax volcanii*, [D4GTU8](#); *Halobacterium salinarum*, [Q9HNY7](#); *Haloquadratum walsbyi*, [Q18GK9](#); *Haloarcula marismortui*, [Q5UXB9](#); *Archaeoglobus fulgidus*, [O28523](#); *Pyrococcus furiosus*, [P58814](#); *Methanocaldococcus jannaschii*, [Q59007](#); *Sulfolobus solfataricus*, [Q980A0](#); *Thermoplasma acidophilum*, [Q9HL27](#); *Methanothermobacter thermotrophicus*, [O27628](#); *Pyrobaculum aerophilum*, [Q8ZVE4](#); *Geobacillus stearothermophilus*, [Q9X519](#); *Bacillus subtilis*, [P39773](#); *E. coli*, [P37689](#); *Staphylococcus aureus*, PDB entry: [4MY4](#); *Arabidopsis thaliana*, Pgm1, [O04499](#); *A. thaliana*, Pgm2, [Q9M9K1](#); *Zea mays*, [P30792](#); *Trypanosoma brucei*, PDB entry: [3NVL](#).

to haloarchaea. More detailed phylogenetic analyses of iPGMs showing a similar tree topology have been reported previously (27, 29). It should be noted that iPGMs are phylogenetically and structurally unrelated to cofactor (2,3-bisphosphoglycerate)-dependent PGMS (dPGMs). An archaeal dPGM has been characterized from *Thermoplasma acidophilum* (27).

Enolase. In the *H. volcanii* genome, the gene HVO_2774 is annotated as *eno* encoding a putative enolase of 42 kDa. The gene *eno* was overexpressed in *H. volcanii* H1209, and the recombinant protein was purified. The His-tagged enzyme showed on SDS-PAGE a molecular mass of 45 kDa (Fig. S1) and—as analyzed by gel filtration—a molecular mass of 358 kDa characterizing the enolase as a homooctameric protein. The enzyme catalyzed the conversion of 2PG to PEP with a $V_{\max}$ of 121 U/mg and a K_m for

2PG of 0.63 mM. The specific activity in the reverse direction, the conversion of PEP to 2PG, was 41.17 U/mg with a K_m for PEP of 0.92 mM (Fig. S2). Activity was dependent on $MgCl_2$ with a K_m of 2.7 mM.

Enolases are widely distributed in archaea, bacteria, and eukarya and are highly conserved enzymes composed of about 45-kDa subunits. *H. volcanii* enolase showed a sequence identity of 32% to 42% to archaeal homologs from *Picrophilus torridus*, *P. furiosus*, and *S. solfataricus* and of 32 to 43% to the bacteria *E. coli* and *Bacillus subtilis* and to human. The high sequence identity of the enolases from the three domains of life is in accordance with a conserved function in metabolism. However, the oligomeric composition of enolases differs; in eukarya, dimeric enzymes were reported, whereas the enolases from several bacteria, e.g., *Staphylococcus aureus* and *Bacillus subtilis*, are homooctameric proteins (31, 32). In archaea, enolases have been purified from the hyperthermophiles *P. furiosus* and *Methanocaldococcus jannaschii* (33, 34). The enolase from *M. jannaschii* is a homooctamer as shown here for the enolase from *H. volcanii*. Enolases belong to the enolase superfamily which includes, e.g., various sugar acid dehydratase families that all form distinct clusters in accordance with their specific catalytic function. A phylogenetic analysis of members of the enolase family, which includes the *H. volcanii* enolase sequence, and of sugar acid dehydratase families within the enolase superfamily has recently been reported (10).

Pyruvate kinase. Pyruvate kinase from *H. volcanii*, encoded by *pykA* (HVO_0806), has recently been characterized as a homotetrameric protein of 200 kDa. The enzyme showed sigmoidal saturation kinetics with the substrates PEP and ADP and exhibited a moderate allosteric activation by AMP. Detailed phylogenetic analyses of pyruvate kinases from archaea, bacteria, and eukarya indicate that pyruvate kinases from haloarchaea originated from bacterial pyruvate kinases that are activated by AMP (35).

Here, we report the transcriptional regulation of *pykA* and analyzed its functional involvement in glycolysis by growth studies with a deletion mutant. Transcription of *pykA* was analyzed by Northern blotting experiments in cells grown on glucose or pyruvate. A specific signal at 2,000 nucleotides was detected in glucose-grown cells which corresponds to the length of *pykA* of 1,758 nucleotides (Fig. 3). No signal was detected in pyruvate-grown cells. A *pykA* deletion mutant was generated that showed an impaired growth on glucose; wild-type growth was restored by in *trans* complementation with *pykA*. Growth of the mutant on pyruvate was not affected (Fig. 3). These results indicate a specific function of pyruvate kinase from *H. volcanii* in glycolysis. The observed residual growth of the *pykA* mutant suggests that alternative enzyme(s) might functionally replace *pykA* in PEP conversion to pyruvate. A potential candidate might be PEP synthetase for which in the thermophilic archaeon *Thermococcus kodakarensis* a glycolytic function of PEP conversion to pyruvate has been proposed (36). However, in *H. volcanii*, a significant contribution of PEP synthetase in glucose degradation seems unlikely since a PEP synthetase deletion mutant was previously shown to grow on glucose as the wild type (12).

Pyruvate-ferredoxin oxidoreductase. The oxidative decarboxylation of pyruvate to acetyl-CoA in haloarchaea has been reported to be catalyzed by pyruvate-ferredoxin oxidoreductase (POR) (pyruvate + CoA + 2 ferredoxin_{ox} → acetyl-CoA + 2 ferredoxin_{red} + CO₂). The enzyme from *Halobacterium halobium* has been characterized (37, 38). Here, we report the characterization of POR from *H. volcanii*. The functional involvement of POR in glucose degradation, converting pyruvate to acetyl-CoA and CO₂, was analyzed by transcriptional analyses and growth studies with a conditional lethal mutant. In *H. volcanii*, the genes *porA* (HVO_1305; 1,896 nucleotides) and *porB* (HVO_1304, 939 nucleotides) are annotated to encode the α - and β -subunit of POR (Fig. 4). Transcription of *porA* and *porB* was analyzed by Northern blotting with cells grown on glucose or acetate. Using specific probes either against *porA* or *porB*, a transcript signal each of about 2,650 nucleotides was detected in glucose-grown cells (Fig. 4), which corresponds to the sum of the lengths of *porA* and *porB* indicating that both genes are cotranscribed as an operon. No signal was detected in acetate-grown cells, demonstrating a glucose-specific upregulation of POR.

The *porAB* operon was cloned into the vector pTA963 and was homologously

FIG 3 Transcriptional and deletion mutant analyses of *pykA* encoding pyruvate kinase from *H. volcanii*. The gene *pykA* (1,758 nucleotides) is depicted as a gray arrow, with flanking genes as white arrows (A). Northern blotting was performed with RNA from glucose (Glc)- and pyruvate (Pyr)-grown cells using a probe against HVO_0806. 16S rRNA served as loading control (B). Growth of the *pykA* deletion mutant from *H. volcanii* on 25 mM glucose (C) and on 40 mM pyruvate (D). Square, wild type; circle, $\Delta pykA$ mutant; triangle, deletion mutant complemented with *pykA*.

overexpressed. The recombinant enzyme was purified and showed on SDS-PAGE one band at 82 kDa (α -subunit) and a second at 44 kDa (β -subunit) (Fig. S1). The molecular masses on SDS-PAGE were higher than the calculated molecular masses of the α -subunit of 68.4 kDa and β -subunit of 34.5 kDa. Higher apparent molecular masses on SDS-PAGE have been reported for several halophilic proteins due to their high content of acidic amino acids (39, 40). By gel filtration, a molecular mass of about 190 kDa for POR was determined indicating a heterotetrameric $\alpha_2\beta_2$ structure. POR activity was measured under anaerobic conditions by following the pyruvate- and CoA-dependent reduction of methyl viologen as an artificial electron acceptor. Recombinant POR showed an apparent V_{max} of 2.07 U/mg and apparent K_m values for pyruvate and CoA of 4.79 mM and 0.06 mM, respectively (Fig. S2).

To analyze the functional involvement of POR in pyruvate conversion to acetyl-CoA, a *porAB* conditional lethal mutant was generated. In this mutant, the *porAB* operon was under the control of a tryptophan inducible promoter showing expression of *porAB* only in the presence of tryptophan. The *porAB* mutant did not grow on glucose (Fig. 4) or on pyruvate (not shown) in the absence of added tryptophan, whereas the mutant grew on both substrates upon addition of tryptophan. In contrast, growth of the *porAB* mutant on acetate was not affected, excluding a possible role in acetyl-CoA conversion to pyruvate in the anabolism (Fig. 4). This finding is in accordance with that of previous studies showing that in *H. volcanii* pyruvate is formed from acetyl-CoA involving the glyoxylic acid cycle and two malic enzymes (12, 41).

The finding that the *porAB* mutant did not grow on glucose or pyruvate indicates that POR in *H. volcanii* cannot be replaced by alternative enzymes, such as a pyruvate dehydrogenase (PDH) complex, the typical enzyme of pyruvate conversion to acetyl-

FIG 4 Transcriptional and conditional lethal mutant analyses of the *porAB* operon from *H. volcanii*. Genomic organization of *porB* (HVO_1304; 939 nucleotides) and *porA* (HVO_1305; 1,896 nucleotides) encoding the α - and β -subunit of the pyruvate-ferredoxin oxidoreductase are given as gray arrows; flanking genes HVO_1306 (1,158 nucleotides) and HVO_1303 (429 nucleotides) are given as white arrows. The intergenic regions upstream of *porA* and downstream of *porB* are 199 and 117 nucleotides, respectively; three nucleotides are between *porA* and *porB*. (A). Northern blotting of the *porAB* operon was performed with RNA from glucose (Glc)- and acetate (Ac)-grown cells using probes against *porA* (left) and *porB* (right). 16S rRNA served as loading control (B). Growth of the conditional lethal mutant of *porAB* on 15 mM glucose (C) and 40 mM acetate (D). Square, wild type; circle, *porAB* mutant without tryptophan; triangle, the mutant after addition of tryptophan.

CoA in aerobic bacteria and eukarya (pyruvate + CoA + NAD⁺ → acetyl-CoA + CO₂ + NADH). In *H. volcanii*, genes encoding three 2-oxoacid dehydrogenase complexes have been identified; however, catalytic activities of these complexes, including pyruvate dehydrogenase, have not been shown (42–44). The reason why in haloarchaea and other aerobic archaea PORs rather than PDH complexes catalyze the pyruvate oxidation to acetyl-CoA is still a matter of debate (45).

POR of *H. volcanii* shows high sequence identity (34% for α -subunit; 38% for β -subunit) to the well-characterized POR from *Halobacterium halobium* (37, 38). As the *H. volcanii* POR, the *Halobacterium* enzyme is a 200-kDa $\alpha_2\beta_2$ heterotetramer. In contrast, characterized PORs from the aerobic archaea *S. solfataricus* and *Aeropyrum pernix* constitute 100-kDa $\alpha\beta$ dimeric enzymes showing sequence homology to the α - and β -subunits of POR from *H. volcanii*. POR from anaerobic archaea, e.g., *P. furiosus* and *Methanosarcina barkeri*, and from the bacterium *Thermotoga maritima* constitute heterotetrameric proteins composed of four different subunits with molecular masses of 47, 30, 24, and 15 kDa (46–48). However, PORs from many anaerobic bacteria, including

FIG 5 Transcriptional analysis of *ppc* encoding phosphoenolpyruvate carboxylase from *H. volcanii*. The gene *ppc* (2,694 nucleotides) is depicted as a gray arrow, with flanking genes as white arrows (A). Northern blotting was performed with RNA from glucose (Glc)- and acetate (Ac)-grown cells using a probe against *ppc*. 16S rRNA served as loading control (B).

Klebsiella pneumonia and *Clostridium acetobutylicum*, are 240-kDa homodimeric enzymes composed of 120-kDa subunits (49, 50).

PEP carboxylase—the anaplerotic enzyme during growth on glucose. Acetyl-CoA formed by POR is further oxidized to two molecules of CO₂ via reactions of the citric acid cycle (51). Anaplerotic reactions, i.e., PEP carboxylase or pyruvate carboxylase, during growth on glucose to replenish the cycle have not been characterized in *H. volcanii*. In the genome of *H. volcanii*, the gene HVO_2621 is annotated as *ppc* to encode a putative PEP carboxylase (PPC) with a calculated molecular mass of 102.3 kDa. Transcription of *ppc* was analyzed by Northern blotting with cells grown on glucose or acetate. A specific transcript of about 2,650 nucleotides which corresponds to the size of the *ppc* gene (2,694 nucleotides) was detected in glucose-grown cells. This signal was absent in acetate-grown cells indicating glucose-specific induction of *ppc* expression (Fig. 5).

The gene *ppc* was overexpressed, and the recombinant enzyme was purified by Ni-NTA affinity and size exclusion chromatography. PPC showed on SDS-PAGE a single band of 115 kDa (Fig. S1); by gel filtration, a molecular mass of 140 kDa was determined, indicating PPC to be a monomeric protein. The recombinant enzyme catalyzed the bicarbonate-dependent conversion of PEP to oxaloacetate and phosphate. The activity requires the presence of acetyl-CoA, which is known to be an allosteric activator of PPC from bacteria. The apparent K_m for acetyl-CoA was 0.195 mM (Fig. 6). PPC activity in dependence of PEP concentration, in the presence of 1 mM acetyl-CoA, followed Michaelis-Menten kinetics with an apparent V_{max} of 2.4 U/mg and a K_m for PEP of 0.5 mM. At 0.025 mM acetyl-CoA, significantly lower PPC activity was detected (Fig. 6). The addition of fructose-1,6-bisphosphate and glucose-6-phosphate (up to 50 mM), which have been shown to act as allosteric effectors of PPC from *E. coli* and maize, did not affect PPC activity of *H. volcanii*. PPC was strictly dependent on divalent cations, with Mn²⁺ being most effective (2.2 U/mg at 5 mM MnCl₂). The activity with MgCl₂ (5 mM) was less than 5% compared with MnCl₂. PPC activity was inhibited by aspartate, an allosteric inhibitor of PPC from bacteria, archaea, and plants. Inhibition was dependent on the concentrations of acetyl-CoA and PEP. e.g., at 0.1 mM acetyl-CoA and 1 mM PEP the addition of 1 mM aspartate caused an inhibition of activity by 85%.

To demonstrate an essential anaplerotic role of PPC, we generated a deletion mutant of *ppc*. The mutant did not grow on glucose; wild-type growth was recovered by complementation with *ppc*. Growth of the *ppc* mutant on acetate was not affected (Fig. 7). These data indicate that PPC is the essential anaplerotic reaction during growth on glucose but not on acetate. It has been shown previously that during growth on acetate, *H. volcanii* uses the glyoxylic acid cycle as anaplerotic sequence (12). Furthermore, the *ppc* mutant did not grow on pyruvate (Fig. 7), indicating that PPC is the essential anaplerotic reaction also on pyruvate as growth substrate, which is converted to PEP, the substrate of PPC, via PEP synthetase (12).

FIG 6 Dependence of phosphoenolpyruvate carboxylase activity from *H. volcanii* on acetyl-CoA concentration in the presence of 2.5 mM PEP (A) and on PEP concentration (B) in the presence of 1 mM acetyl-CoA (circles) or 0.025 mM acetyl-CoA (squares). Data are fitted to the Michaelis-Menten equation.

PEP carboxylases have been characterized from various bacteria, eukarya, and from a few archaea. Bacterial and eukaryal PPCs are usually homotetramers of about 100-kDa subunits and are allosterically activated in bacteria by acetyl-CoA (52, 53). The PPC from *H. volcanii* is composed of a 100-kDa subunit and was activated by acetyl-CoA,

FIG 7 Growth of deletion mutant of *ppc* encoding phosphoenolpyruvate carboxylase from *H. volcanii*. Growth was performed on 15 mM glucose (A), on 40 mM pyruvate (B), and on 40 mM acetate (C). Square, wild type; circle, deletion mutant; triangle, deletion mutant complemented with *ppc*.

i.e., showed features typical for bacterial PPCs, but it differs from bacterial PPCs, being a monomeric protein. In the archaeal domain PPCs have been characterized from *S. solfataricus*, *Sulfolobus acidocaldarius*, *Methanothermobacter thermautotrophicus*, and *Methanothermus sociabilis*. These archaeal PPCs are homotetramers composed of small subunits (55 to 60 kDa) and are not activated by acetyl-CoA as an allosteric effector. However, these enzymes showed allosteric inhibition by aspartate (54–57).

PPC from *H. volcanii* showed the highest sequence identity (64% to 71%) to putative homologs from the haloarchaea *Haloarcula marismortui*, *Halorubrum lacusprofundi*, and *Halorhabdus utahensis*. All these putative haloarchaeal PPC homologs are composed of large, 100-kDa subunits. High sequence identity was also found with characterized bacterial PPCs, e.g., from *E. coli* (30%) and *Rhodopseudomonas palustris* (32%), and PPCs from eukarya, e.g., *Zea mays* (26%) (58–60). A significantly lower sequence identity (<10%) of *H. volcanii* PPC was found with characterized and putative PPCs from archaea. A sequence identity of less than 10% was also found with PPCs from a few Gram-positive bacteria, e.g., *Clostridium perfringens* (61), and two putative PPCs from haloarchaea, i.e., *Halobacterium salinarum* and *Haloarcula vallismortis*.

An amino acid sequence alignment of PPC from *H. volcanii* and from *E. coli* is shown in Fig. 8. The sequences contain the two consensus motifs typical for PPCs. The predicted secondary structure of PPC from *H. volcanii* matches well to the secondary elements of the PPC structure from *E. coli* (Fig. 8) (52). Also, the amino acids shown to be involved in the binding of Mn^{2+} , PEP (62), and of the allosteric inhibitor aspartate (52) are completely conserved in the PPC from *H. volcanii*.

Phylogenetic analysis of characterized and putative PPC sequences from archaea, bacteria, and eukarya indicates that PPCs form two distinct clusters (Fig. 9), a bacterial type and an archaeal type, as has been previously reported (54, 55). One cluster comprises PPCs from bacteria and eukarya and also contains the PPCs from *H. volcanii* and related homologs, which are all composed of about 100-kDa subunits. The second cluster comprises small subunit PPCs from archaea and also includes three PPCs from Gram-positive bacteria and the two PPCs from haloarchaea, i.e., *H. salinarum* and *H. vallismortis*. The attribution of haloarchaeal PPCs to both the bacterial/eukaryal and the archaeal cluster suggests a different phylogenetic evolutionary history of haloarchaeal PPCs. The close relationship of *H. volcanii* PPC and homologs with bacterial PPCs indicates a lateral gene transfer event from bacteria to these haloarchaea. Multiple horizontal gene transfers in evolution within the PPC family have previously been reported (63).

ACD—the key enzyme of acetate overflow from glucose in *H. volcanii*. During growth on 25 mM glucose and 0.5% yeast extract, *H. volcanii* excretes—in the exponential-growth phase—about 6 mM acetate which is completely consumed in the stationary phase (Fig. 10). The enzyme functionally involved in acetate formation from acetyl-CoA was characterized as ADP-forming acetyl-CoA synthetase (ACD), encoded by the gene *acd* (HVO_1000). Transcriptional analyses, growth studies with a deletion mutant, and biochemical characterization of the recombinant ACD are reported.

The gene *ptaN* (HVO_0999) is located adjacent to the *acd* gene and encodes a putative N-terminal domain of phosphotransacetylase. Northern blot analysis with a probe against the *acd* gene revealed a transcript signal at about 3,200 nucleotides in glucose-grown cells, which corresponds to the sum of the lengths of *acd* and *ptaN*, indicating cotranscription of both genes (Fig. 11). In addition to the signal at 3,200 nucleotides, a signal at 1,200 nucleotides was detected with a probe against *ptaN*, suggesting that *ptaN* is part of the operon and is also transcribed as a single transcript (Fig. 11). The additional transcription of *ptaN* as a single transcript is in accordance with the identification of potential basal promoter elements, i.e., a TATA box that centered at –27 and a “WW” motif at –10/–11 (W stands for A or T) (64), in the intergenic region (of 26 nucleotides) of both genes. Significantly weaker transcript signals were detected with

RNA from cells grown on acetate, indicating glucose-specific upregulation of these two genes.

ib.asm.org 11

April 2021 Volume 203 Issue 8 e00690-20 jb.asm.org.org 12

FIG 10 Acetate switch during growth on glucose in the wild type, *acd* mutant, and quadruple *acs* mutant from *H. volcanii*. (A) Growth of the wild type on 25 mM glucose (gray circles), consumption of glucose (open circles), and acetate formation (black circles); acetate formation in the Δacd mutant (triangles) and in the Δacd mutant that was complemented with *acd* in trans (squares). (B) Growth of the quadruple *acs* mutant on 25 mM glucose (gray triangles); acetate formed was not consumed in this *acs* mutant (black triangles) compared with acetate formed that was consumed in the stationary phase in the wild type (circles).

of the aryl acid phenyl acetate (59%). Indolacetate and succinate were used at rates less than 1.5%.

To analyze the functional involvement of ACD in acetate overflow from glucose in *H. volcanii*, an *acd* deletion mutant was generated. Growth of the Δacd mutant on glucose was not affected (not shown); however, the amount of acetate formed in the Δacd mutant in the exponential-growth phase was reduced by about 70% compared with the wild type (Fig. 10). Wild-type levels of acetate formation were recovered by complementation of the mutant with *acd*. These results indicate that ACD is functionally involved in acetate formation during overflow metabolism of *H. volcanii* on glucose.

Molecular and kinetic properties of *H. volcanii* ACD compared with those of ACDI isoenzymes. The ACD from *H. volcanii* showed high sequence identity (66%) to the characterized ACD from *H. marismortui* (65). Both haloarchaeal ACDs are homodimeric proteins of 75 kDa subunits which show high sequence similarity to ACDs from hyperthermophilic archaea, e.g., *Pyrococcus furiosus* and *Pyrobaculum aerophilum*, that are 140-kDa heterotetrameric, $\alpha_2\beta_2$ enzymes (65–67). Thus, the haloarchaeal ACDs are fusions of the homologous α -subunits (47 kDa) and β -subunits (25 kDa) of hyperthermophilic archaea. The hyperthermophilic archaeon *Archaeoglobus fulgidus* contains both, ACD homologs composed of $\alpha_2\beta_2$ subunits or dimers of fused α - and β -subunits (68). Furthermore, in contrast to the haloarchaeal ACDs, which each contain one ACD

FIG 11 Transcriptional analyses of the *acd* operon from *H. volcanii*. Genomic organization of *acd* (HVO_1000; 2,094 nucleotides) and *ptaN* (HVO_0999; 1,137 nucleotides) encoding ADP-forming acetyl-CoA synthetase and a putative N-terminal domain of phosphotransacetylase, respectively. The genes *acd* and *ptaN* are given as gray arrows; the flanking genes HVO_1001 (261 nucleotides) and HVO_0998 (189 nucleotides) are given as white arrows. (A). Northern blotting of the operon was performed with RNA from glucose (Glc)- and acetate (Ac)-grown cells using probes against *acd* (B) and *ptaN* (C). 16S rRNA served as loading control.

enzyme, the hyperthermophilic archaea *P. furiosus*, *Thermococcus kodakarensis*, and *A. fulgidus* contain several ACD isoenzymes of different metabolic function, e.g., in the degradation of sugars and amino acids (68–70). The ACD isoenzymes I and II of *P. furiosus* differ with respect to their substrate specificity as follows: the ACDI isoenzyme preferentially utilizes acetyl-CoA over aryl-CoA esters, such as phenylacetyl-CoA, and is primarily involved in sugar fermentation. In contrast, the ACDII isoenzyme preferentially catalyzes the conversion of aryl-CoA esters and is primarily implicated in the degradation of aromatic amino acids (66, 67).

The ACDs of *H. volcanii* and of *H. marismortui* predominantly use acetyl-CoA, which classifies the haloarchaeal ACDs as ACDI isoenzymes involved in acetate formation from glucose. The *H. volcanii* ACD—in contrast to *H. marismortui* ACD—showed significant activity with phenylacetyl-CoA, suggesting an additional role in aromatic amino acid degradation.

TABLE 1 Molecular and catalytic properties of ADP-forming acetyl-CoA synthetase from *H. volcanii*

Parameter	Substrate	Data
Apparent molecular mass of enzyme (kDa)		
Native		163
Subunit		79
Calculated		74.5
Oligomeric structure		α_2
Apparent $V_{\max}$ value (U/mg) (direction of acetate formation)		59.1
Apparent K_m value (mM)	Acetyl-CoA	0.051
	ADP	0.12
	P _i	2.0
Apparent $V_{\max}$ value (U/mg) (direction of acetyl-CoA formation)		44.9
Apparent K_m value (mM)	Acetate	2.0
	ATP	0.77
	CoA	0.3
pH optimum		7.5
KCl optimum (M)		0.5
MgCl ₂ optimum (mM)		5

ACS enzymes are involved in activation of excreted acetate. In a previous publication, we studied the growth of *H. volcanii* on acetate and identified four paralogous AMP-forming acetyl-CoA synthetases (ACSs) that are involved in the activation of acetate to acetyl-CoA. This was concluded by showing that growth of a quadruple mutant of ACS1, ACS2, ACS7, and ACS9 on acetate was completely prevented (12). Here, we tested the growth of this quadruple ACS mutant on glucose to analyze its effect on the consumption of the excreted acetate in the stationary phase. This mutant did not affect the growth kinetics and the formation of acetate in the exponential-growth phase; however, the consumption of acetate in the stationary-growth phase was completely prevented (Fig. 10).

Together, the data show that the excretion of acetate is catalyzed by ACD, whereas the reutilization involves ACS enzymes. These data are in accordance with enzyme measurements reported for *H. marismortui* (15).

Conclusions. In the present communication, we report a comprehensive description of enzymes of the semiphosphorylative Entner-Doudoroff (spED) pathway in the model haloarchaeon *Haloferax volcanii*. Following previous analyses, we analyzed here the enzymes of the lower part of the spED pathway, including phosphoglycerate mutase (PGM), enolase, and pyruvate kinase. Furthermore, PEP carboxylase (PPC) as an anaplerotic enzyme and pyruvate-ferredoxin oxidoreductase catalyzing the acetyl-CoA formation from pyruvate were characterized (Fig. 12). Phylogenetic analyses of several enzymes of the spED pathway, including iPGM and PPC, and as shown in our previous reports, 2-keto-3-deoxy-6-phosphogluconate aldolase, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate kinase, indicate that *H. volcanii* acquired these enzymes via lateral gene transfer events from bacteria (7, 13, 35). These findings are in accordance with a previous bioinformatic study by Martin and coworkers, proposing that the evolution of the heterotrophic lifestyle of haloarchaea, including sugar degradation pathways, was the result of massive lateral transfer of bacterial genes (71). Furthermore, we present genetic and enzymatic evidence of the key players of acetate switch during aerobic growth on glucose in archaea. The functional involvement of the enzymes in acetate formation and consumption was demonstrated by analyses with knockout mutants (Fig. 12). The enzyme catalyzing acetate overflow in *H. volcanii* was characterized as an ADP-forming acetyl-CoA synthetase, an enzyme reported so far to be specific for acetate formation in the course of fermentation of sugars in anaerobic archaea. By demonstrating a function of ACD in acetate formation in aerobic glucose metabolism of haloarchaea, we extend the metabolic role of ACD in archaeal metabolism. Furthermore, acetate consumption as part of the acetate switch mechanism involves AMP-forming acetyl-CoA synthetases. Together, the data expand our understanding of glucose degradation and of the acetate switch in the domain of archaea.

MATERIALS AND METHODS

Growth experiments. *H. volcanii* H26 and deletion mutants grew aerobically at 42°C in 100-ml Erlenmeyer flasks containing 20 ml of synthetic medium (7, 72) supplemented with glucose (15 or 25 mM), acetate (40 mM), or pyruvate (40 mM). In growth experiments analyzing the acetate switch, the medium contained 25 mM glucose and 0.5% yeast extract. Due to the chromosomal deletion of the orotate phosphoribosyltransferase gene (*pyrE2*) in strain H26, the medium was supplemented with uracil (50 µg/ml). For complementation experiments, deletion mutants were transformed with the *pyrE2*-containing plasmid pTA963 that carried the respective target gene, and growth was performed without the addition of uracil. Expression of target genes was induced by tryptophan (80 to 200 µM). Growth was followed by measuring the optical density at 578 or 600 nm. Each growth experiment was reproduced independently. Glucose and acetate were determined enzymatically as described (73, 74).

Generation of deletion mutants and of a conditional lethal mutant of *porAB*. Markerless gene deletion mutants of HVO_0806, HVO_2621, and HVO_1000 were constructed using the pop-in/pop-out strategy (8, 75). In each case, the up- and downstream regions of the target gene (each of about 500 nucleotides) were amplified by two PCRs (see Table S4 in the supplemental material). Then, the two fragments were fused by PCR, generating a deletion product that was ligated into the vector pTA131. These plasmids carrying the *pyrE2* gene for selection in *H. volcanii* H26 were each multiplied in *Escherichia coli* XL1 Blue MRF' followed by transformation of *H. volcanii* H26. Cells that have integrated the respective plasmid into the genome via homologous recombination (pop-in) were selected and cultivated in uracil-free medium with 1% Casamino Acids. Pop-out clones were generated by plating pop-in cells on medium containing both uracil (30 µg/ml) and 5-fluoroorotic acid (50 µg/ml), followed by passaging the

FIG 12 Glucose degradation and acetate switch in *H. volcanii*. Enzymes shown to be functionally involved in glucose degradation via the semiphosphorylative Entner-Doudoroff pathway, anaplerosis, acetyl-CoA formation from pyruvate, and in acetate formation and consumption are highlighted by gray background. Enzymes that were characterized in previous studies are indicated by a frame (7, 13, 39). Locus tags are given in parentheses. KDG, 2-keto-3-deoxygluconate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; GAP, glyceraldehyde-3-phosphate; 1,3BPg, 1,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; GDH, glucose dehydrogenase (HVO_1083); GAD, gluconate dehydratase (HVO_1488); KDGK, KDG kinase (HVO_0549); KDPGA, KDPG aldolase (HVO_0950); GAPDH, GAP dehydrogenase (HVO_0481); PGK, phosphoglycerate kinase (HVO_0480); iPGM, cofactor-independent phosphoglycerate mutase (HVO_2516); ENO, enolase (HVO_2774); PK, pyruvate kinase (HVO_0806); POR, pyruvate-ferredoxin oxidoreductase (HVO_1305 and HVO_1304); ACS, AMP-forming acetyl-CoA synthetase (ACS1, HVO_0894; ACS2, HVO_0896; ACS7, HVO_A0156; ACS9, HVO_A0551); ACD, ADP-forming acetyl-CoA synthetase (HVO_1000); PPC, PEP carboxylase (HVO_2621); TCA, tricarboxylic acid cycle.

pop-out cells in liquid medium. 5-Fluoroorotic acid is toxic for cells containing the *pyrE2* gene, and thus, only cells that had experienced a second homologous recombination event (pop-out) were able to grow. Pop-out clones that were either wild type or the deletion variant of the indicated gene were screened for successful deletion by PCR followed by Southern blotting.

For complementation experiments of the mutants Δ HVO_0806, Δ HVO_2621, and Δ HVO_1000, the strains were transformed with the plasmids also used for overexpression (see below) containing the respective wild-type genes under the control of a tryptophanase (*ptnA*) promoter. Expression of target genes was induced by the addition of tryptophan up to 200 μ M.

A conditional lethal mutant of the *porAB* operon was generated. In this mutant, chromosomal expression of the *porAB* operon was controlled by the inducible *ptnA* promoter, showing *porAB* expression only in the presence of the inductor tryptophan (76, 77). Therefore, the *ptnA* promoter was fused to the first 195 nucleotides of *porA* by PCR (Table S4), followed by ligating this fusion product into the suicide vector pTA131 that carries the *pyrE2* gene for selection in *H. volcanii* H26. *H. volcanii* H26 was transformed with the plasmid that integrated in the genome via homologous recombination at the *porA* site (homologous region at the first 195 nucleotides), resulting in a strain in which *ptnA* controls the genome-encoded *porAB* operon. After confirmation of the conditional lethal mutation by PCR and Southern hybridization, growth experiments were performed in the presence (80 μ M) and absence of tryptophan.

Homologous overexpression and purification of His-tagged enzymes. Plasmids for homologous overexpression of target genes were constructed as described (78). Genes were amplified from genomic DNA of *H. volcanii* and were ligated into the plasmid pTA963. The *porAB* operon was amplified and as

one fragment ligated into pTA963 (see Table S2 in the supplemental material). By this cloning strategy, the genes were fused to 6× CAC at the 5′ end encoding 6 histidine residues. Plasmids were multiplied in *E. coli* XL1-Blue MRF′ and used for transformation of *H. volcanii* H1209. For overexpression of His-tagged fusion proteins, the transformed H1209 cells grew in complex medium (8, 78) up to an optical density at 600 nm of about 0.5, and expression was induced by the addition of 2 mM tryptophan. After 10 to 15 h of further growth, cells were harvested by centrifugation. For purification of protein, cell pellets were suspended in 50 mM Tris-HCl (pH 8.2) containing 1.5 M KCl and 5 mM imidazole and disrupted using a French pressure cell, followed by centrifugation. The supernatant was applied onto a Ni-NTA affinity column, and specific elution of the protein was performed with 100 to 250 mM imidazole. This protein solution was applied onto a Superdex 200 HiLoad 16/60 column (GE Healthcare), and elution was performed by an isocratic flow in 50 mM Tris-HCl (pH 7.5) containing 1.5 M KCl. Purification of PEP carboxylase was performed by the same procedure in the presence of 5 mM dithiothreitol (DTT).

Characterization of recombinant, His-tagged enzymes. Enzyme activities were measured at 42°C. Coupling enzymes were purchased from Sigma-Aldrich, Roche, or Megazyme. It was ensured that the coupling enzymes were not rate limiting. Kinetic data were fitted to the Michaelis-Menten equation with Origin2015 software.

Phosphoglycerate mutase (PGM) activity (3-phosphoglycerate $\rightleftharpoons$ 2-phosphoglycerate) was measured in both directions. (i) In the direction of 2-phosphoglycerate (2PG) formation, the assay mixture (1 ml) contained 50 mM Tris-HCl (pH 7.5), 1.5 M KCl, 2 mM ADP, 5 mM MgCl₂, 0.3 mM NADH, 8 mM 3-phosphoglycerate (3PG), 0.5 U enolase, 2 U pyruvate kinase, 5.5 U lactate dehydrogenase, and up to 1.9 μg protein. (ii) In the direction of 3PG formation, the assay mixture (1 ml) contained 50 mM Tris-HCl (pH 7.5), 1.5 M KCl, 5 mM MgCl₂, 2.5 mM ATP, 2.5 mM PEP, 0.3 mM NADH, 5 mM 2PG, 3.9 U phosphoglycerate kinase, 2 U pyruvate kinase, 5.5 U lactate dehydrogenase, and up to 1.9 μg protein.

Enolase activity (2PG $\rightleftharpoons$ phosphoenolpyruvate) was measured in both directions as either phosphoenolpyruvate (PEP) formation or consumption at 240 nm using a molar extinction coefficient for PEP of 0.96 mM⁻¹ cm⁻¹. The molar extinction coefficient was calculated from a standard curve with PEP up to a concentration of 1.75 mM. The assay mixtures (1 ml) contained 50 mM Tris-HCl (pH 7.5), 1.5 M KCl, 5 mM MgCl₂, 5 mM 2PG or 1.6 mM PEP, and up to 3.2 μg protein. The *K_m* value for MgCl₂ was determined in the direction of PEP formation with 2.5 mM 2PG.

Pyruvate-ferredoxin oxidoreductase activity (pyruvate + CoA + 2 MV_{ox} $\rightarrow$ acetyl-CoA + 2 MV_{red} + CO₂) was measured under anaerobic conditions in stoppered glass cuvettes (volume 1 ml) under a N₂ gasphase by following the pyruvate- and CoA-dependent reduction of methyl viologen (MV) using a molar extinction coefficient for MV at 578 nm of 9.8 mM⁻¹ cm⁻¹ (79). The assay mixture contained 0.1 M Tris-HCl (pH 7.0), 2 M KCl, 5 mM MV, 10 mM pyruvate, 0.3 mM CoA, and 4.6 μg protein. To slightly reduce the assay mixture, 1 to 2 μl of a 100 mM sodium-dithionite solution was added.

PEP carboxylase activity (PEP + HCO₃⁻ $\rightarrow$ oxaloacetate + P_i) was measured in the direction of oxaloacetate formation by coupling the reaction to the oxidation of NADH with malate dehydrogenase. The assay mixture (0.3 ml) contained 100 mM Tris-HCl (pH 7.5), 1.5 M KCl, 5 mM MnCl₂, 10 mM NaHCO₃, 0.3 mM NADH and 2.5 mM PEP, 4 U malate dehydrogenase, 0 to 1 mM acetyl-CoA, and up to 12.3 μg protein. Glucose-6-phosphate and fructose-1,6-bisphosphate as allosteric activators were tested at concentrations up to 50 mM. Aspartate as an allosteric inhibitor was tested at 1 mM in the presence of 0.1 mM acetyl-CoA and 1 mM PEP. The reaction was started by the addition of acetyl-CoA.

ADP-forming acetyl-CoA synthetase (ACD) activity (acetyl-CoA + ADP + P_i $\rightleftharpoons$ acetate + ATP + CoA) was measured in both directions as described elsewhere (80) using two assay systems in 200 μl. (i) The P_i- and ADP-dependent CoA release from acetyl-CoA was monitored with Ellman's thiolreagent (5,5′-dithiobis-2-nitrobenzoic acid; DTNB) by measuring the formation of thiophenolate anion at 412 nm (ϵ = 13.6 mM⁻¹ cm⁻¹) (81). The assay mixture contained 100 mM HEPES-KOH (pH 7.5), 0.5 M KCl, 5 mM MgCl₂, 0.1 mM DTNB, 0.1 mM acetyl-CoA, 1 mM ADP, 10 mM KH₂PO₄, and up to 0.17 μg protein. The pH dependence was measured between pH 5.5 to 8.0 using piperazine (pH 5.5 to 6.0), MES (pH 6.0 to 7.0), and HEPES-KOH (pH 7.0 to 8.0), at 100 mM each. The salt dependence was measured up to 3 M KCl and 25 mM MgCl₂. The substrate specificity of ACD for CoA esters was measured by replacing acetyl-CoA with 0.1 mM phenylacetyl-CoA or isobutyryl-CoA. (ii) The CoA- and acetate-dependent ADP formation from ATP was measured by coupling the reaction with the oxidation of NADH (ϵ = 6.2 mM⁻¹ cm⁻¹) at 340 nm via pyruvate kinase and lactate dehydrogenase. The assay mixture contained 100 mM HEPES-KOH (pH 7.5), 0.5 M KCl, 5 mM MgCl₂, 10 mM acetate, 2 mM ATP, 1 mM CoA, 2.5 mM PEP, 0.3 mM NADH, 0.8 U lactate dehydrogenase, 1.2 U pyruvate kinase, and up to 0.17 μg protein. The specificity of ACD for acids was measured by replacing acetate with 10 mM each of propionate, butyrate, isobutyrate, isovalerate, phenylacetate, indole-3-acetate, or succinate.

Analytical assays. Determination of the molecular masses of native enzymes was performed on a Superdex 200 HighLoad column (1.6 by 60 cm) with a flow rate of 1 ml/min using 50 mM Tris-HCl (pH 7.4), containing 1.5 mM KCl. The column was calibrated with the high-molecular-weight (HMW) and low-molecular-weight (LMW) kits (GE Healthcare) as specified by the manufacturer. The purity of proteins was analyzed by SDS-PAGE. Protein concentration was determined by the method of Bradford with bovine serum albumin V as the standard.

Transcriptional analyses. RNA from exponentially grown cells of *H. volcanii* (at optical densities of about 1.3) was isolated as described previously (82). Northern blot analyses were performed with 4 to 15 μg of RNA (6). Probes were amplified using the PCR DIG probe synthesis kit (Roche Diagnostics, Mannheim, Germany) (for primer sequences, see Table S3 in the supplemental material). Transcript sizes were determined with the RiboRuler high-range RNA ladder (Thermo Fisher Scientific, Schwerte, Germany).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

REFERENCES

1. Siebers B, Schönheit P. 2005. Unusual pathways and enzymes of central carbohydrate metabolism in Archaea. *Curr Opin Microbiol* 8:695–705. <https://doi.org/10.1016/j.mib.2005.10.014>.
2. Bräsen C, Esser D, Rauch B, Siebers B. 2014. Carbohydrate metabolism in Archaea: current insights into unusual enzymes and pathways and their regulation. *Microbiol Mol Biol Rev* 78:89–175. <https://doi.org/10.1128/MMBR.00041-13>.
3. Reher M, Fuhrer T, Bott M, Schönheit P. 2010. The nonphosphorylative Entner-Doudoroff pathway in the thermoacidophilic euryarchaeon *Picrophilus torridus* involves a novel 2-keto-3-deoxygluconate-specific aldolase. *J Bacteriol* 192:964–974. <https://doi.org/10.1128/JB.01281-09>.
4. Zaitsev V, Johnsen U, Reher M, Ortjohann M, Taylor GL, Danson MJ, Schönheit P, Crennell S. 2018. Insights into substrate specificity of archaeal Entner-Doudoroff aldolases: the structures of *Picrophilus torridus* 2-keto-3-deoxygluconate aldolase, and *Sulfolobus solfataricus* 2-keto-3-deoxy(6-phospho)-gluconate aldolase in complex with 2-keto-3-deoxy-6-phosphogluconate. *Biochemistry* 57:3797–3806. <https://doi.org/10.1021/acs.biochem.8b00535>.
5. Johnsen U, Dambeck M, Zaiss H, Fuhrer T, Soppa J, Sauer U, Schönheit P. 2009. D-Xylose degradation pathway in the halophilic archaeon *Haloferax volcanii*. *J Biol Chem* 284:27290–27303. <https://doi.org/10.1074/jbc.M109.003814>.
6. Pickl A, Johnsen U, Schönheit P. 2012. Fructose degradation in the haloarchaeon *Haloferax volcanii* involves a bacterial type phosphoenolpyruvate-dependent phosphotransferase system, fructose-1-phosphate kinase, and class II fructose-1,6-bisphosphate aldolase. *J Bacteriol* 194:3088–3097. <https://doi.org/10.1128/JB.00200-12>.
7. Sutter JM, Tästensen JB, Johnsen U, Soppa J, Schönheit P. 2016. Key enzymes of the semiphosphorylative Entner-Doudoroff pathway in the haloarchaeon *Haloferax volcanii*: characterization of glucose dehydrogenase, gluconate dehydratase, and 2-keto-3-deoxy-6-phosphogluconate aldolase. *J Bacteriol* 198:2251–2262. <https://doi.org/10.1128/JB.00286-16>.
8. Reinhardt A, Johnsen U, Schönheit P. 2019. L-Rhamnose catabolism in archaea. *Mol Microbiol* 111:1093–1108. <https://doi.org/10.1111/mmi.14213>.
9. Tästensen JB, Johnsen U, Reinhardt A, Ortjohann M, Schönheit P. 2020. D-Galactose catabolism in archaea: operation of the DeLey-Doudoroff pathway in *Haloferax volcanii*. *FEMS Microbiol Lett* 367:fnaa29. <https://doi.org/10.1093/femsle/fnaa029>.
10. Johnsen U, Sutter JM, Reinhardt A, Pickl A, Wang R, Xiang H, Schönheit P. 2019. D-Ribose catabolism in archaea: discovery of a novel oxidative pathway in *Haloarcula* species. *J Bacteriol* 202:e00608-19. <https://doi.org/10.1128/JB.00608-19>.
11. Pohlschröder M, Schulze S. 2019. *Haloferax volcanii*. *Trends Microbiol* 27:86–87. <https://doi.org/10.1016/j.tim.2018.10.004>.
12. Kuprat T, Johnsen U, Ortjohann M, Schönheit P. 2020. Acetate metabolism in Archaea: characterization of an acetate transporter and of enzymes involved in acetate activation and gluconeogenesis in *Haloferax volcanii*. *Front Microbiol* 11:3102. <https://doi.org/10.3389/fmicb.2020.604926>.
13. Tästensen JB, Schönheit P. 2018. Two distinct glyceraldehyde-3-phosphate dehydrogenases in glycolysis and gluconeogenesis in the archaeon *Haloferax volcanii*. *FEBS Lett* 592:1524–1534. <https://doi.org/10.1002/1873-3468.13037>.
14. Bräsen C, Schönheit P. 2001. Mechanisms of acetate formation and acetate activation in halophilic archaea. *Arch Microbiol* 175:360–368. <https://doi.org/10.1007/s002030100273>.
15. Bräsen C, Schönheit P. 2004. Regulation of acetate and acetyl-CoA converting enzymes during growth on acetate and/or glucose in the halophilic archaeon *Haloarcula marismortui*. *FEMS Microbiol Lett* 241:21–26. <https://doi.org/10.1016/j.femsle.2004.09.033>.
16. Tobisch S, Zuhlke D, Bernhardt J, Stulke J, Hecker M. 1999. Role of CcpA in regulation of the central pathways of carbon catabolism in *Bacillus subtilis*. *J Bacteriol* 181:6996–7004. <https://doi.org/10.1128/JB.181.22.6996-7004.1999>.
17. Oh MK, Rohlin L, Kao KC, Liao JC. 2002. Global expression profiling of acetate-grown *Escherichia coli*. *J Biol Chem* 277:13175–13183. <https://doi.org/10.1074/jbc.M110809200>.
18. Grundy FJ, Waters DA, Allen SH, Henkin TM. 1993. Regulation of the *Bacillus subtilis* acetate kinase gene by CcpA. *J Bacteriol* 175:7348–7355. <https://doi.org/10.1128/jb.175.22.7348-7355.1993>.
19. Wolfe AJ. 2005. The acetate switch. *Microbiol Mol Biol Rev* 69:12–50. <https://doi.org/10.1128/MMBR.69.1.12-50.2005>.
20. Presecan-Siedel E, Galinier A, Longin R, Deutscher J, Danchin A, Glaser P, Martin-Verstraete I. 1999. Catabolite regulation of the pta gene as part of carbon flow pathways in *Bacillus subtilis*. *J Bacteriol* 181:6889–6897. <https://doi.org/10.1128/JB.181.22.6889-6897.1999>.
21. el-Mansi EM, Holms WH. 1989. Control of carbon flux to acetate excretion during growth of *Escherichia coli* in batch and continuous cultures. *J Gen Microbiol* 135:2875–2883. <https://doi.org/10.1099/00221287-135-11-2875>.
22. Schäfer T, Selig M, Schönheit P. 1993. Acetyl-CoA synthetase (ADP-forming) in archaea, a novel enzyme involved in acetate formation and ATP synthesis. *Arch Microbiol* 159:72–83. <https://doi.org/10.1007/BF00244267>.
23. Bräsen C, Schmidt M, Grötzinger J, Schönheit P. 2008. Reaction mechanism and structural model of ADP-forming Acetyl-CoA synthetase from the hyperthermophilic archaeon *Pyrococcus furiosus*: evidence for a second active site histidine residue. *J Biol Chem* 283:15409–15418. <https://doi.org/10.1074/jbc.M710218200>.
24. Weiße RH, Faust A, Schmidt M, Schönheit P, Scheidig AJ. 2016. Structure of NDP-forming acetyl-CoA synthetase ACD1 reveals a large rearrangement for phosphoryl transfer. *Proc Natl Acad Sci U S A* 113:E519–E528. <https://doi.org/10.1073/pnas.1518614113>.
25. Fraser HI, Kvaratskhelia M, White MF. 1999. The two analogous phosphoglycerate mutases of *Escherichia coli*. *FEBS Lett* 455:344–348. [https://doi.org/10.1016/S0014-5793\(99\)00910-2](https://doi.org/10.1016/S0014-5793(99)00910-2).
26. Jedrzejewski MJ, Chander M, Setlow P, Krishnasamy G. 2000. Mechanism of catalysis of the cofactor-independent phosphoglycerate mutase from *Bacillus stearothermophilus*. Crystal structure of the complex with 2-phosphoglycerate. *J Biol Chem* 275:23146–23153. <https://doi.org/10.1074/jbc.M002544200>.
27. Johnsen U, Schönheit P. 2007. Characterization of cofactor-dependent and cofactor-independent phosphoglycerate mutases from Archaea. *Extremophiles* 11:647. <https://doi.org/10.1007/s00792-007-0094-x>.
28. Potters MB, Solow BT, Bischoff KM, Graham DE, Lower BH, Helm R, Kennelly PJ. 2003. Phosphoprotein with phosphoglycerate mutase activity from the archaeon *Sulfolobus solfataricus*. *J Bacteriol* 185:2112–2121. <https://doi.org/10.1128/jb.185.7.2112-2121.2003>.
29. Van der Oost J, Huynen MA, Verhees CH. 2002. Molecular characterization of phosphoglycerate mutase in archaea. *FEMS Microbiol Lett* 212:111–120. <https://doi.org/10.1111/j.1574-6968.2002.tb11253.x>.
30. Graham DE, Xu H, White RH. 2002. A divergent archaeal member of the alkaline phosphatase binuclear metalloenzyme superfamily has phosphoglycerate mutase activity. *FEBS Lett* 517:190–194. [https://doi.org/10.1016/S0014-5793\(02\)02619-4](https://doi.org/10.1016/S0014-5793(02)02619-4).
31. Wu Y, Wang C, Lin S, Wu M, Han L, Tian C, Zhang X, Zang J. 2015. Octameric structure of *Staphylococcus aureus* enolase in complex with phosphoenolpyruvate. *Acta Crystallogr D Biol Crystallogr* 71:2457–2470. <https://doi.org/10.1107/S1399004715018830>.
32. Newman JA, Hewitt L, Rodrigues C, Solovyova AS, Harwood CR, Lewis RJ. 2012. Dissection of the network of interactions that links RNA processing with glycolysis in the *Bacillus subtilis* degradosome. *J Mol Biol* 416:121–136. <https://doi.org/10.1016/j.jmb.2011.12.024>.
33. Peak MJ, Peak JG, Stevens FJ, Blamey J, Mai X, Zhou ZH, Adams MWW. 1994. The hyperthermophilic glycolytic enzyme enolase in the archaeon, *Pyrococcus furiosus*: comparison with mesophilic enolases. *Arch Biochem Biophys* 313:280–286. <https://doi.org/10.1006/abbi.1994.1389>.
34. Yamamoto H, Kunishima N. 2008. Purification, crystallization and preliminary crystallographic study of the putative enolase MJ0232 from the hyperthermophilic archaeon *Methanococcus jannaschii*. *Acta Crystallogr Sect F Struct Biol Cryst Commun* 64:1087–1090. <https://doi.org/10.1107/S1744309108034180>.

35. Johnsen U, Reinhardt A, Landan G, Tria FDK, Turner JM, Davies C, Schönheit P. 2019. New views on an old enzyme: allosteric regulation and evolution of archaeal pyruvate kinases. *FEBS J* 286:2471–2489. <https://doi.org/10.1111/febs.14837>.
36. Imanaka H, Yamatsu A, Fukui T, Atomi H, Imanaka T. 2006. Phosphoenolpyruvate synthase plays an essential role for glycolysis in the modified Embden-Meyerhof pathway in *Thermococcus kodakarensis*. *Mol Microbiol* 61:898–909. <https://doi.org/10.1111/j.1365-2958.2006.05287.x>.
37. Kerschler L, Oesterhelt D. 1981. Purification and properties of two 2-oxoacid:ferredoxin oxidoreductases from *Halobacterium halobium*. *Eur J Biochem* 116:587–594. <https://doi.org/10.1111/j.1432-1033.1981.tb05376.x>.
38. Plaga W, Lottspeich F, Oesterhelt D. 1992. Improved purification, crystallization and primary structure of pyruvate:ferredoxin oxidoreductase from *Halobacterium halobium*. *Eur J Biochem* 205:391–397. <https://doi.org/10.1111/j.1432-1033.1992.tb16792.x>.
39. Pickl A, Johnsen U, Archer RM, Schönheit P. 2014. Identification and characterization of 2-keto-3-deoxygluconate kinase and 2-keto-3-deoxygalactonate kinase in the haloarchaeon *Haloferax volcanii*. *FEMS Microbiol Lett* 361:76–83. <https://doi.org/10.1111/1574-6968.12617>.
40. Bonete MJ, Pire C, LLorca FI, Camacho ML. 1996. Glucose dehydrogenase from the halophilic Archaeon *Haloferax mediterranei*: enzyme purification, characterisation and N-terminal sequence. *FEBS Lett* 383:227–229. [https://doi.org/10.1016/0014-5793\(96\)00235-9](https://doi.org/10.1016/0014-5793(96)00235-9).
41. Serrano JA, Camacho M, Bonete MJ. 1998. Operation of glyoxylate cycle in halophilic archaea: presence of malate synthase and isocitrate lyase in *Haloferax volcanii*. *FEBS Lett* 434:13–16. [https://doi.org/10.1016/S0014-5793\(98\)00911-9](https://doi.org/10.1016/S0014-5793(98)00911-9).
42. Sisignano M, Morbitzer D, Gätgens J, Oldiges M, Soppe J. 2010. A 2-oxoacid dehydrogenase complex of *Haloferax volcanii* is essential for growth on isoleucine but not on other branched-chain amino acids. *Microbiology (Reading)* 156:521–529. <https://doi.org/10.1099/mic.0.033449-0>.
43. van OJ, Soppe J. 2007. Three 2-oxoacid dehydrogenase operons in *Haloferax volcanii*: expression, deletion mutants and evolution. *Microbiology (Reading)* 153:3303–3313. <https://doi.org/10.1099/mic.0.2007/008508-0>.
44. Al-Mailem DM, Hough DW, Danson MJ. 2008. The 2-oxoacid dehydrogenase multienzyme complex of *Haloferax volcanii*. *Extremophiles* 12:89–96. <https://doi.org/10.1007/s00792-007-0091-0>.
45. Heath C, Posner MG, Aass HC, Upadhyay A, Scott DJ, Hough DW, Danson MJ. 2007. The 2-oxoacid dehydrogenase multi-enzyme complex of the archaeon *Thermoplasma acidophilum*—recombinant expression, assembly and characterization. *FEBS J* 274:5406–5415. <https://doi.org/10.1111/j.1742-4658.2007.06067.x>.
46. Bock A-K, Kunow J, Glasemacher J, Schönheit P. 1996. Catalytic properties, molecular composition and sequence alignments of pyruvate:ferredoxin oxidoreductase from the methanogenic archaeon *Methanosarcina barkeri* (strain Fusaro). *Eur J Biochem* 237:35–44. <https://doi.org/10.1111/j.1432-1033.1996.0035n.x>.
47. Blamey JM, Adams MWW. 1993. Purification and characterization of pyruvate:ferredoxin oxidoreductase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *Biochim Biophys Acta* 1161:19–27. [https://doi.org/10.1016/0167-4838\(93\)90190-3](https://doi.org/10.1016/0167-4838(93)90190-3).
48. Blamey JM, Adams MWW. 1994. Characterization of an ancestral type of pyruvate:ferredoxin oxidoreductase from the hyperthermophilic bacterium, *Thermotoga maritima*. *Biochemistry* 33:1000–1007. <https://doi.org/10.1021/bi00170a019>.
49. Wahl RC, Orme-Johnson WH. 1987. Clostridial pyruvate oxidoreductase and the pyruvate-oxidizing enzyme specific to nitrogen fixation in *Klebsiella pneumoniae* are similar enzymes. *J Biol Chem* 262:10489–10496. [https://doi.org/10.1016/S0021-9258\(18\)60987-1](https://doi.org/10.1016/S0021-9258(18)60987-1).
50. Meinecke B, Bertram J, Gottschalk G. 1989. Purification and characterization of the pyruvate:ferredoxin oxidoreductase from *Clostridium acetobutylicum*. *Arch Microbiol* 152:244–250. <https://doi.org/10.1007/BF00409658>.
51. Falb M, Müller K, Königsmaier L, Oberwinkler T, Horn P, von GS, Gonzalez O, Pfeiffer F, Bornberg-Bauer E, Oesterhelt D. 2008. Metabolism of halophilic archaea. *Extremophiles* 12:177–196. <https://doi.org/10.1007/s00792-008-0138-x>.
52. Kai Y, Matsumura H, Inoue T, Terada K, Nagara Y, Yoshinaga T, Kihara A, Tsumura K, Izui K. 1999. Three-dimensional structure of phosphoenolpyruvate carboxylase: a proposed mechanism for allosteric inhibition. *Proc Natl Acad Sci U S A* 96:823–828. <https://doi.org/10.1073/pnas.96.3.823>.
53. Izui K, Matsumura H, Furumoto T, Kai Y. 2004. Phosphoenolpyruvate carboxylase: a new era of structural biology. *Annu Rev Plant Biol* 55:69–84. <https://doi.org/10.1146/annurev.arplant.55.031903.141619>.
54. Ettema TJ, Makarova KS, Jellema GL, Gierman HJ, Koonin EV, Huynen MA, De Vos WM, van der OJ. 2004. Identification and functional verification of archaeal-type phosphoenolpyruvate carboxylase, a missing link in archaeal central carbohydrate metabolism. *J Bacteriol* 186:7754–7762. <https://doi.org/10.1128/JB.186.22.7754-7762.2004>.
55. Patel HM, Kraszewski JL, Mukhopadhyay B. 2004. The phosphoenolpyruvate carboxylase from *Methanothermobacter thermautotrophicus* has a novel structure. *J Bacteriol* 186:5129–5137. <https://doi.org/10.1128/JB.186.15.5129-5137.2004>.
56. Sako Y, Takai K, Uchida A, Ishida Y. 1996. Purification and characterization of phosphoenolpyruvate carboxylase from the hyperthermophilic archaeon *Methanothermobacter sociabilis*. *FEBS Lett* 392:148–152. [https://doi.org/10.1016/0014-5793\(96\)00805-8](https://doi.org/10.1016/0014-5793(96)00805-8).
57. Sako Y, Takai K, Nishizaka T, Ishida Y. 2006. Biochemical relationship of phosphoenolpyruvate carboxylases (PEPCs) from thermophilic archaea. *FEMS Microbiol Lett* 153:159–165. <https://doi.org/10.1111/j.1574-6968.1997.tb10477.x>.
58. Teraoka H, Izui K, Katsuki H. 1972. Phosphoenolpyruvate carboxylase of *Escherichia coli*: alteration of allosteric properties by photooxidation. *Arch Biochem Biophys* 152:821–827. [https://doi.org/10.1016/0003-9861\(72\)90278-0](https://doi.org/10.1016/0003-9861(72)90278-0).
59. Inui M, Dumay V, Zahn K, Yamagata H, Yukawa H. 1997. Structural and functional analysis of the phosphoenolpyruvate carboxylase gene from the purple nonsulfur bacterium *Rhodospseudomonas palustris* No. 7. *J Bacteriol* 179:4942–4945. <https://doi.org/10.1128/jb.179.15.4942-4945.1997>.
60. Ting IP, Osmond CB. 1973. Multiple forms of plant phosphoenolpyruvate carboxylase associated with different metabolic pathways. *Plant Physiol* 51:448–453. <https://doi.org/10.1104/pp.51.3.448>.
61. Dharmarajan L, Kraszewski JL, Mukhopadhyay B, Dunten PW. 2011. Structure of an archaeal-type phosphoenolpyruvate carboxylase sensitive to inhibition by aspartate. *Proteins* 79:1820–1829. <https://doi.org/10.1002/prot.23006>.
62. Matsumura H, Terada M, Shirakata S, Inoue T, Yoshinaga T, Izui K, Kai Y. 1999. Plausible phosphoenolpyruvate binding site revealed by 2.6 Å structure of Mn²⁺-bound phosphoenolpyruvate carboxylase from *Escherichia coli*. *FEBS Lett* 458:93–96. [https://doi.org/10.1016/S0014-5793\(99\)01103-5](https://doi.org/10.1016/S0014-5793(99)01103-5).
63. Peng Y, Cai J, Wang W, Su B. 2012. Multiple inter-kingdom horizontal gene transfers in the evolution of the phosphoenolpyruvate carboxylase gene family. *PLoS One* 7:e51159. <https://doi.org/10.1371/journal.pone.0051159>.
64. Brenneis M, Hering O, Lange C, Soppe J. 2007. Experimental characterization of *Cis*-acting elements important for translation and transcription in halophilic archaea. *PLoS Genet* 3:e229. <https://doi.org/10.1371/journal.pgen.0030229>.
65. Bräsen C, Schönheit P. 2004. Unusual ADP-forming acetyl-coenzyme A synthetases from the mesophilic halophilic euryarchaeon *Haloarcula marismortui* and from the hyperthermophilic crenarchaeon *Pyrobaculum aerophilum*. *Arch Microbiol* 182:277–287. <https://doi.org/10.1007/s00203-004-0702-4>.
66. Mai X, Adams MWW. 1996. Purification and characterization of two reversible and ADP-dependent acetyl coenzyme A synthetases from the hyperthermophilic archaeon *Pyrococcus furiosus*. *J Bacteriol* 178:5897–5903. <https://doi.org/10.1128/jb.178.20.5897-5903.1996>.
67. Glasemacher J, Bock A-K, Schmid R, Schönheit P. 1997. Purification and properties of acetyl-CoA synthetase (ADP-forming), an archaeal enzyme of acetate formation and ATP synthesis, from the hyperthermophile *Pyrococcus furiosus*. *Eur J Biochem* 244:561–567. <https://doi.org/10.1111/j.1432-1033.1997.00561.x>.
68. Musfeldt M, Schönheit P. 2002. Novel type of ADP-forming acetyl coenzyme A synthetase in hyperthermophilic archaea: heterologous expression and characterization of isoenzymes from the sulfate reducer *Archaeoglobus fulgidus* and the methanogen *Methanococcus jannaschii*. *J Bacteriol* 184:636–644. <https://doi.org/10.1128/jb.184.3.636-644.2002>.
69. Awano T, Wilming A, Tomita H, Yokooji Y, Fukui T, Imanaka T, Atomi H. 2014. Characterization of two members among the five ADP-forming acyl coenzyme A (acyl-CoA) synthetases reveals the presence of a 2-(imidazol-4-yl)acetyl-CoA synthetase in *Thermococcus kodakarensis*. *J Bacteriol* 196:140–147. <https://doi.org/10.1128/JB.00877-13>.
70. Scott JW, Poole FL, Adams MW. 2014. Characterization of ten heterotetrameric NDP-dependent acyl-CoA synthetases of the hyperthermophilic archaeon *Pyrococcus furiosus*. *Archaea* 2014:176863. <https://doi.org/10.1155/2014/176863>.

71. Nelson-Sathi S, Dagan T, Landan G, Janssen A, Steel M, McInerney JO, Deppenmeier U, Martin WF. 2012. Acquisition of 1,000 eubacterial genes physiologically transformed a methanogen at the origin of Haloarchaea. *Proc Natl Acad Sci U S A* 109:20537–20542. <https://doi.org/10.1073/pnas.1209119109>.
72. Dambeck M, Soppa J. 2008. Characterization of a *Haloferax volcanii* member of the enolase superfamily: deletion mutant construction, expression analysis, and transcriptome comparison. *Arch Microbiol* 190:341–353. <https://doi.org/10.1007/s00203-008-0379-1>.
73. Kunst A, Draeger B, Ziegenhorn J. 1981. Colorimetric methods with glucose oxidase and peroxidase, p 178–185. In Bergmeyer H-U (ed), *Methods of enzymatic analysis*, 3rd ed. Verlag Chemie, Weinheim, Germany.
74. Beutler HO. 1984. Acetate determination with acetyl-CoA synthase, p 639–645. In Bergmeyer H-U (ed), *Methods of enzymatic analysis*, third edition. Verlag Chemie, Weinheim, Germany.
75. Allers T, Mevarech M. 2005. Archaeal genetics—the third way. *Nat Rev Genet* 6:58–73. <https://doi.org/10.1038/nrg1504>.
76. Cerletti M, Martinez MJ, Gimenez MI, Sastre DE, Paggi RA, De Castro RE. 2014. The LonB protease controls membrane lipids composition and is essential for viability in the extremophilic haloarchaeon *Haloferax volcanii*. *Environ Microbiol* 16:1779–1792. <https://doi.org/10.1111/1462-2920.12385>.
77. Large A, Stamme C, Lange C, Duan Z, Allers T, Soppa J, Lund PA. 2007. Characterization of a tightly controlled promoter of the halophilic archaeon *Haloferax volcanii* and its use in the analysis of the essential *cct1* gene. *Mol Microbiol* 66:1092–1106. <https://doi.org/10.1111/j.1365-2958.2007.05980.x>.
78. Allers T, Barak S, Liddell S, Wardell K, Mevarech M. 2010. Improved strains and plasmid vectors for conditional overexpression of His-tagged proteins in *Haloferax volcanii*. *Appl Environ Microbiol* 76:1759–1769. <https://doi.org/10.1128/AEM.02670-09>.
79. Trudinger PA. 1970. On the absorbancy of reduced methyl viologen. *Anal Biochem* 36:222–225. [https://doi.org/10.1016/0003-2697\(70\)90351-9](https://doi.org/10.1016/0003-2697(70)90351-9).
80. Bräsen C, Schönheit P. 2004. Unusual ADP-forming acetyl-coenzyme A synthetases from the mesophilic halophilic euryarchaeon *Haloarcula marismortui* and from the hyperthermophilic crenarchaeon *Pyrobaculum aerophilum*. *Arch Microbiol* 182:277–287. <https://doi.org/10.1007/s00203-004-0702-4>.
81. Ellman GL. 1958. A colorimetric method for determining low concentrations of mercaptans. *Arch Biochem Biophys* 74:443–450. [https://doi.org/10.1016/0003-9861\(58\)90014-6](https://doi.org/10.1016/0003-9861(58)90014-6).
82. Johnsen U, Sutter J-M, Zaiß H, Schönheit P. 2013. L-Arabinose degradation pathway in the haloarchaeon *Haloferax volcanii* involves a novel type of L-arabinose dehydrogenase. *Extremophiles* 17:897–909. <https://doi.org/10.1007/s00792-013-0572-2>.
83. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* 23:2947–2948. <https://doi.org/10.1093/bioinformatics/btm404>.
84. Jones DT. 1999. Protein secondary structure prediction based on position-specific scoring matrices. *J Mol Biol* 292:195–202. <https://doi.org/10.1006/jmbi.1999.3091>.
85. Robert X, Gouet P. 2014. Deciphering key features in protein structures with the new ENDscript server. *Nucleic Acids Res* 42:W320–W324. <https://doi.org/10.1093/nar/gku316>.
86. Pickl A, Johnsen U, Archer RM, Schönheit P. 2014. Identification and characterization of 2-keto-3-deoxygluconate kinase and 2-keto-3-deoxygalactonate kinase in the haloarchaeon *Haloferax volcanii*. *FEMS Microbiol Lett* 361:76–83. <https://doi.org/10.1111/1574-6968.12617>.