

# The Genome Sequence of the Crenarchaeon *Acidilobus saccharovorans* Supports a New Order, *Acidilobales*, and Suggests an Important Ecological Role in Terrestrial Acidic Hot Springs<sup>∇‡</sup>

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***Acidilobus saccharovorans* is an anaerobic, organotrophic, thermoacidophilic crenarchaeon isolated from a terrestrial hot spring. We report the complete genome sequence of *A. saccharovorans*, which has permitted the prediction of genes for Embden-Meyerhof and Entner-Doudoroff pathways and genes associated with the oxidative tricarboxylic acid cycle. The electron transfer chain is branched with two sites of proton translocation and is linked to the reduction of elemental sulfur and thiosulfate. The genomic data suggest an important role of the order *Acidilobales* in thermoacidophilic ecosystems whereby its members can perform a complete oxidation of organic substrates, closing the anaerobic carbon cycle.**

Acidophilic microorganisms are widely dispersed in natural acidic environments, including volcanic hot springs, and are, in the majority, aerobes (14). However, such anoxic, high-temperature, acidic environments are inhabited by metabolically versatile anaerobic thermoacidophiles of the archaeal phylum *Crenarchaeota*. Lithoautotrophic thermoacidophiles oxidize molecular hydrogen in the course of elemental sulfur (S<sup>0</sup>) respiration. Organotrophs couple the oxidation of organic substrates to the reduction of S<sup>0</sup> or thiosulfate. They all belong to the genus *Acidilobus* in the family *Acidilobaceae* and to the genus *Caldisphaera* in the family *Caldisphaeraceae* (4, 13, 22, 24). *Acidilobaceae* and *Caldisphaeraceae* form the crenarchaeal order *Acidilobales* (24). *Acidilobus saccharovorans* was isolated from an acidic hot spring of Uzon Caldera, Kamchatka, Russia (24). It is an obligately anaerobic acidophile with a range of growth from pH 2.5 to 5.8 (optimum at pH 3.5 to 4) and a temperature range from 60 to 90°C (optimum at 80 to 85°C). It utilizes a wide range of proteinaceous and carbohydrate substrates and cannot grow lithoautotrophically on H<sub>2</sub> and CO<sub>2</sub> (24). S<sup>0</sup> and thiosulfate stimulate growth and are reduced to H<sub>2</sub>S. Protons cannot serve as electron acceptors, since no H<sub>2</sub> is produced during growth in the absence of S<sup>0</sup> (24). Genomic sequences of aerobic, thermoacidophilic euryarchaea *Thermoplasma acidophilum* (26) and *Picrophilus torridus* (8) give an insight into the thermoacidophilic survival strategy. However, no genomes of obligately anaerobic, thermoacidophilic archaea were available until now. Here we present the genome of

*A. saccharovorans* and show that it encodes numerous hydrolytic enzymes and metabolic pathways necessary for the utilization and complete mineralization of organic substrates in its natural habitat, acidic hot springs.

**Genome sequencing and annotation.** *A. saccharovorans* 345-15<sup>T</sup> (DSM 16705) was obtained from the culture collection of the Laboratory of Hyperthermophilic Microbial Communities, the Winogradsky Institute of Microbiology of the Russian Academy of Sciences, and grown as described previously (24). The *A. saccharovorans* genome was sequenced on a Roche GS FLX genome sequencer by the standard protocol for a shotgun genome library. The GS FLX run (1/4 plate) resulted in the generation of about 22 Mb of sequences with an average read length of 230 bp. The GS FLX reads were assembled into 11 contigs by a GS De Novo Assembler (Roche). The contigs were oriented into scaffolds, and the complete genome sequence was obtained upon the generation and sequencing of appropriate PCR fragments. The rRNA genes were identified with RNAmmer (17). tRNA genes were located with tRNAscan-SE (19). Protein-encoding genes were identified with the GLIMMER gene finder (5), followed by a round of manual curation. Genome annotation was performed with the AutoFACT tool (16). Protein similarity was analyzed by BLASTP search (1). Signal peptides were predicted with SignalP version 3.0 (3).

**General features of the genome.** *A. saccharovorans* has a single circular chromosome of 1,496,453 bp with no extrachromosomal elements (see Fig. S1 in the supplemental material). There is a single copy of the 16S-23S rRNA operon and a single distantly located 5S rRNA gene. A total of 45 tRNA genes coding for all 20 amino acids are scattered over the genome. The cumulative GC skew profile (9, 18) displays two local minima that likely correspond to the DNA replication origins (see Fig. S1 in the supplemental material). Genome annotation reveals 1,499 potential protein-encoding genes, 972 (65%) of which can be functionally assigned. The functions of the remaining 527 genes cannot be predicted from the deduced

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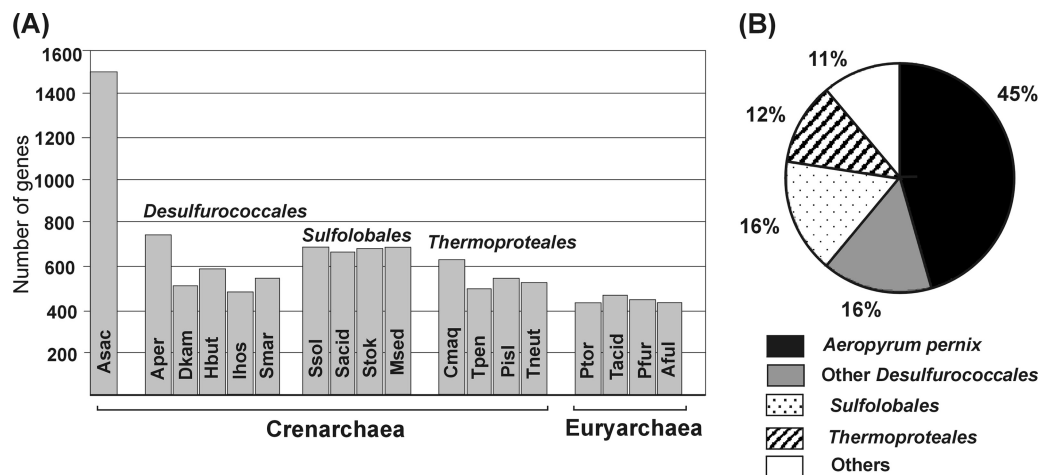


FIG. 1. Comparisons of proteomes of *A. saccharovorans* and other archaea. (A) The numbers of *A. saccharovorans* protein-encoding genes present in the genomes of other archaea. Genes were considered present in a pair of genomes if the region of similarity covered  $>70\%$  of the corresponding protein with an E value of  $<10^{-10}$ . Abbreviations: Asac, *A. saccharovorans*; Aper, *Aeropyrum pernix*; Dkam, *Desulfurococcus kamchatkensis*; Hbut, *Hyperthermus butylicus*; Ihos, *Ignicoccus hospitalis*; Smar, *Staphylothermus marinus*; Ssol, *Sulfolobus solfataricus*; Sacid, *Sulfolobus acidocaldarius*; Stok, *Sulfolobus tokodaii*; Msed, *Metallosphaera sedula*; Cmaq, *Caldivirga maquilensis*; Tpen, *Thermophilum pendens*; Pisl, *Pyrobaculum islandicum*; Tneut, *Thermoproteus neutrophilus*; Ptor, *Picrophilus torridus*; Tacid, *Thermoplasma acidophilum*; Pful, *Pyrococcus furiosus*; Aful, *Archaeoglobus fulgidus*. (B) The best BLASTP hits of *A. saccharovorans* proteins. Significant BLASTP hits were detected for 1,218 of 1,499 proteins found in the *A. saccharovorans* genome. Proteins unique to *A. saccharovorans* were excluded from this analysis.

amino acid sequences; 246 of them are unique to *A. saccharovorans*.

The numbers of predicted protein-encoding genes of *A. saccharovorans* that have homologs in other archaeal genomes are shown in Fig. 1A. The closest relative of *A. saccharovorans* is the aerobic, neutrophilic crenarchaeon *Aeropyrum pernix*; they share about half of their proteomes ( $\sim 740$  proteins). Fewer *A. saccharovorans* proteins have homologs in the genomes of other members of the crenarchaeal orders *Desulfurococcales* and *Thermoproteales* (Fig. 1B). Notably, *A. saccharovorans* shares higher numbers of proteins (661 to 687, 16% of best BLASTP hits) with the phylogenetically distant aerobic thermoacidophiles of the order *Sulfolobales* (24). Predicted functions of most of the 199 proteins of *A. saccharovorans* showing the best BLASTP hits in *Sulfolobales* proteomes are related to transport (54 proteins) or are unknown (86 proteins). *A. saccharovorans* and *Sulfolobales* often coexist in acidic hot springs, and lateral gene transfer may be responsible for the relatively high similarity between their proteomes. Extensive lateral gene transfer was suggested to explain the high fraction of genes shared by *Sulfolobales* and aerobic euryarchaeal thermoacidophiles *P. torridus* and *T. acidophilum* (8, 26). However, it is unlikely that extensive gene transfer occurred between *A. saccharovorans* and euryarchaeal thermoacidophiles, because the numbers of proteins shared with *P. torridus* (428) and *T. acidophilum* (459), as well as with nonacidophilic euryarchaea *Pyrococcus furiosus* (440) and *Archaeoglobus fulgidus* (427), are similar.

**Metabolism of proteins, carbohydrates, and lipids.** Growth of *A. saccharovorans* on proteins and peptides may be enabled by the function of several extracellular proteolytic enzymes. The imported peptides can be fermented with concomitant substrate-level phosphorylation to generate ATP (Fig. 2; see Table S1 in the supplemental material). The presence of genes

for extra- and intracellular glycoside hydrolases and sugar transporters (Fig. 2 and Table S1) correlates with the growth of *A. saccharovorans* on carbohydrates (24). Notably, the *A. saccharovorans* genome encodes at least two primary ABC-type sugar transport systems (Table S1) and more than 20 secondary transporters. A similar high ratio of secondary transport systems to ATP-consuming primary transport systems was found in the thermoacidophile *P. torridus* (8), suggesting that the high external proton concentration is extensively used by *A. saccharovorans* for transport processes.

Glucose in archaea is metabolized to pyruvate via either the Embden-Meyerhof (EM) pathway or the Entner-Doudoroff (ED) pathway. Both modified EM and ED pathways are encoded by *A. saccharovorans* genome (Fig. 2), suggesting their operation in parallel as found in *Thermoproteus tenax* (32). The ED pathway can be beneficial at the upper temperature range of growth, since it avoids the most heat-labile intermediates (31). The EM pathway may account for the synthesis of fructose-6-phosphate used in the ribulose monophosphate pathway (Fig. 2).

The genome contains 14 genes encoding esterases and genes encoding a complete  $\beta$ -oxidation pathway, suggesting the ability of *A. saccharovorans* to utilize triacylglycerides and long-chain fatty acids available from the environment (Fig. 2 and Table S1). To our knowledge, besides *A. saccharovorans*, *A. fulgidus* is the only archaeon to encode the enzymes of  $\beta$ -oxidation (15).

**Conservation of energy.** *A. saccharovorans* may conserve energy by substrate-level phosphorylation as well as by oxidative phosphorylation. During growth in the presence of  $S^0$  as a terminal electron acceptor,  $H_2S$  is produced and acetate is formed (24). It is likely that in the presence of  $S^0$ , acetyl coenzyme A (acetyl-CoA) generated from the oxidation of substrates is concomitantly converted to acetate by fermenta-

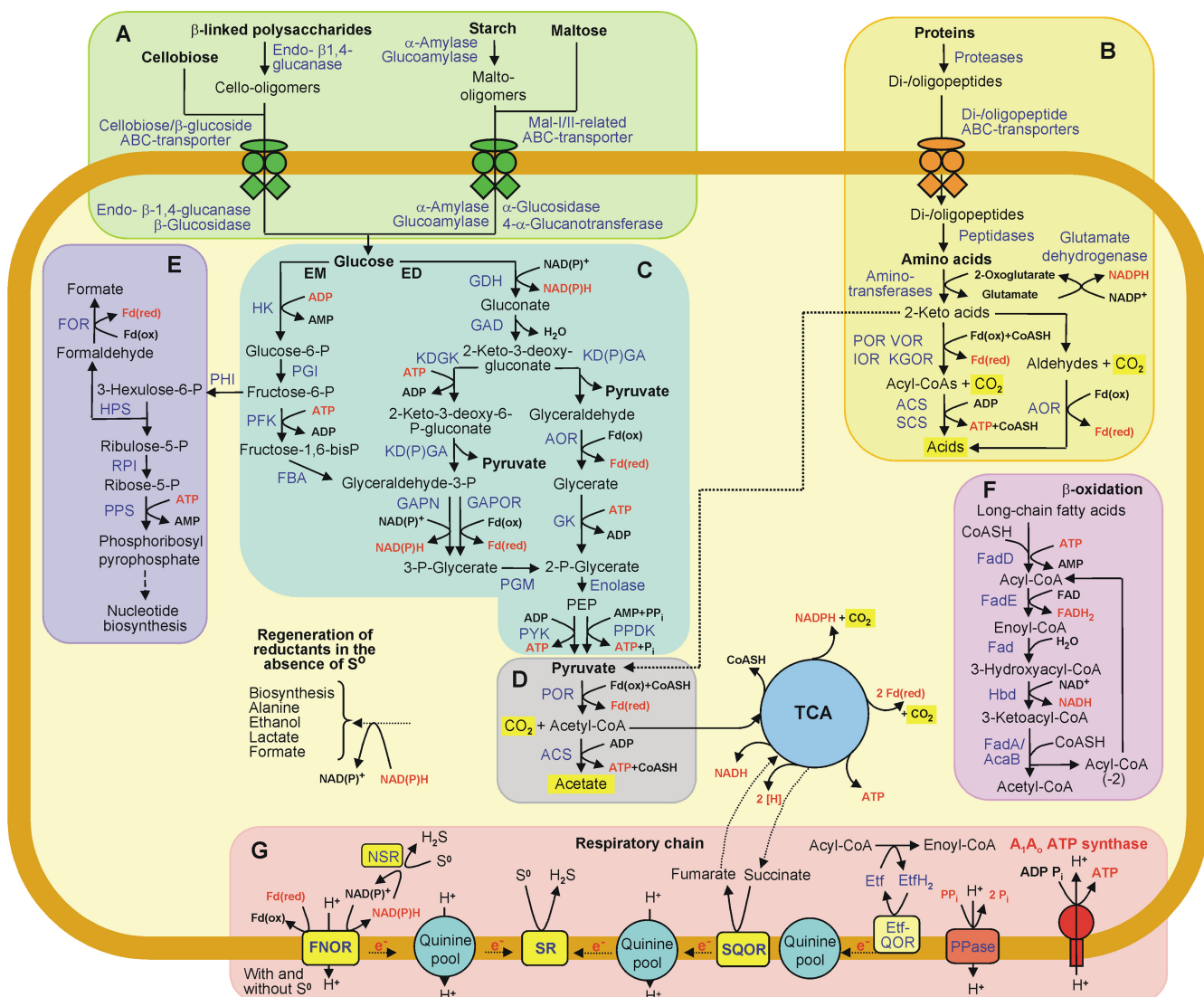


FIG. 2. Overview of catabolic pathways encoded by the *A. saccharovorans* genome. Substrates utilized are in boldface, enzymes and proteins encoded by genes identified on the genome are in blue, and energy-rich intermediate compounds are in red. Panels: A, utilization of carbohydrates; B, utilization of proteins; C, glycolysis (EM and ED pathways); D, pyruvate degradation; E, pentose phosphate synthesis; F,  $\beta$ -oxidation of long-chain fatty acids; G, formation of proton motive force coupled with ATP generation. TCA, oxidative tricarboxylic acid cycle. Abbreviations: POR, pyruvate:ferredoxin oxidoreductase; VOR, 2-ketoisovalerate:ferredoxin oxidoreductase; IOR, indolepyruvate:ferredoxin oxidoreductase; KGOR, 2-ketoglutarate:ferredoxin oxidoreductase; ACS, acetyl-CoA synthetase; SCS, succinyl-CoA synthetase; AOR, aldehyde:ferredoxin oxidoreductase; HK, ADP-dependent hexokinase; PGI, phosphoglucose isomerase; PFK, ADP-dependent phosphofructokinase; FBA, fructose-1,6-bisphosphate aldolase; GAPOR, glyceraldehyde-3-phosphate:ferredoxin oxidoreductase; GAPN, nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase; PGM, phosphoglycerate mutase; PYK, pyruvate kinase; PPK, pyruvate phosphate dikinase; PEP, phosphoenolpyruvate; GDH, glucose dehydrogenase; GAD, gluconate dehydratase; KDGK, 2-keto-3-deoxy-gluconate kinase; KD(P)GA, 2-keto-3-deoxy-(6-phospho)gluconate aldolase; GK, glycerate kinase; PHI, 6-phospho-3-hexuloisomerase; HPS, 3-hexulose-6-phosphate synthase; RPI, ribose-5-phosphate isomerase; PPS, phosphoribosyl pyrophosphate synthase; FOR, formaldehyde:ferredoxin oxidoreductase; Fd(ox), oxidized ferredoxin; Fd(red), reduced ferredoxin; FNOR, ferredoxin:NAD(P)<sup>+</sup> oxidoreductase complex; NSR, NAD(P)H:elemental sulfur oxidoreductase; PPase, H<sup>+</sup>-translocating pyrophosphatase; SQOR, succinate:quinone oxidoreductase complex; SR, sulfur reductase complex; Etf, electron transfer flavoprotein; Etf-QOR, electron transfer protein-quinone oxidoreductase; CoASH, coenzyme A; FAD, flavin adenine dinucleotide; FADH<sub>2</sub>, reduced FAD.

tion pathway as well as oxidized to CO<sub>2</sub> by anaerobic respiration (Fig. 2). The *A. saccharovorans* genome encodes all eight enzymes of the oxidative tricarboxylic acid (TCA) cycle (Fig. 2 and Table S1). This cycle operates in *T. tenax* and *Pyrobaculum islandicum* and was proposed to function in both oxidative and reductive directions, enabling the complete oxidation of or-

ganic substrates to CO<sub>2</sub> and H<sub>2</sub>S, as well as CO<sub>2</sub> fixation during autotrophic growth (12, 30, 31, 32). In *A. saccharovorans*, which cannot grow autotrophically (24), the TCA cycle appears to operate only in the oxidative direction. Indeed, citrate lyase, the key enzyme of the reductive TCA cycle, is not encoded.



During growth of *A. saccharovorans* by anaerobic respiration in the presence of  $S^0$ , reduced ferredoxin, NAD(P)H, and H produced in the TCA cycle can be oxidized by a concerted action of a set of membrane-bound protein complexes and cytoplasmic proteins, resulting in the generation of transmembrane proton gradient. The genome analysis reveals a putative membrane-bound protein complex (ASAC\_0373 to ASAC\_0383), which is partially homologous to bacterial NADH:quinone oxidoreductase (respiratory complex I). From the 14 subunits (NuoA to NuoN) present in *Escherichia coli* complex I (7), this putative *A. saccharovorans* complex contains 11 subunits (NuoA to NuoD and NuoH to NuoN), while three subunits (NuoEFG) known to form the dehydrogenase domain involved in NADH binding and oxidation (28) are missing. Thus, the *A. saccharovorans* complex most likely accepts electrons from a donor other than NADH. ASAC\_0380 is also homologous to the MbxL subunit of the membrane-bound oxidoreductase (MBX) complex from *P. furiosus* (29). It has been shown that in the MBX complex, the oxidation of ferredoxin and the reduction of NAD(P)<sup>+</sup> are coupled to the generation of a proton motive force in the presence of  $S^0$  (29). A similar function of the MBX complex in the crenarchaea *D. kamchatkensis* (25) and *S. marinus* (2), as well as in the euryarchaeon *T. sibiricus* (20), has been proposed. Based on these data, we propose that ASAC\_0373-ASAC\_0383 encodes a proton-translocating ferredoxin:NAD(P)<sup>+</sup> oxidoreductase complex (FNOR) which accepts electrons from reduced ferredoxin and transfers them to NAD(P)<sup>+</sup>, thereby translocating protons and establishing a proton gradient (Fig. 2). NAD(P)H is then oxidized by the encoded NAD(P)H:elemental sulfur oxidoreductase (NSR) (ASAC\_1028), forming H<sub>2</sub>S, or enters other processes linked to NAD(P)H reoxidation (e.g., thiosulfate reduction). In addition, the NuoB (Nqo6) subunit, which transfers electrons to menaquinone in *Thermus thermophilus* complex I (27), is present in the *A. saccharovorans* FNOR complex (ASAC\_0382), suggesting the possibility of electron transfer not only to NAD(P)<sup>+</sup> but also to quinones in the respiratory chain and further to membrane-bound sulfur reductase (SR; ASAC\_1394 to ASAC\_1397) (Fig. 2; see Table S1 in the supplemental material).

The transmembrane proton gradient could also be generated by the function of the predicted integral membrane protein H<sup>+</sup>-translocating pyrophosphatase (PPase; ASAC\_1013) closely related to functionally characterized enzyme from *Pyrobaculum aerophilum* (6). The pyrophosphatase may work in concert with H<sup>+</sup>-ATP synthase to scavenge energy from biosynthetic waste pyrophosphate in order to maintain the proton gradient, especially under energy-limiting conditions.

Besides involving the proposed FNOR complex, the respiratory chain of *A. saccharovorans* presumably involves the succinate:quinone oxidoreductase complex (SQOR; ASAC\_1440 to ASAC\_1443), which is the membrane-bound component of the oxidative TCA cycle. Electrons from the oxidation of succinate in the TCA cycle would be delivered by SQOR to the quinones in the membrane.

The genome contains a gene cluster (corresponding to ASAC\_0264 to ASAC\_0267) encoding two subunits of electron transfer flavoprotein (EtfAB; ASAC\_0264 and ASAC\_0265, respectively), a ferredoxin-like protein (ASAC\_0266), and an electron transfer flavoprotein-quinone oxidoreductase (Etf-

QOR; ASAC\_0267). In analogy to the mitochondrial enzymes, ASAC\_0264 to ASAC\_0267 might be involved in the oxidation of fatty acids and some amino acids (10). These substrates would be oxidized by acyl-CoA dehydrogenases (FadE) with the Etf as an acceptor, which would pass the electrons to Etf-QOR and further to quinone in the respiratory chain of *A. saccharovorans*.

The genome analysis also suggests a second site of  $S^0$  reduction in *A. saccharovorans*. It involves a predicted membrane-bound SR complex, which would function as the terminal oxidase of  $S^0$ -dependent respiration. Based on the sequence analyses, we propose that the SR is composed of the hydrophilic catalytic subunit ASAC\_1397, the electron transfer subunit ASAC\_1396, and the membrane-bound subunits ASAC\_1395 and ASAC\_1394.

Summarizing the data on the electron transfer in *A. saccharovorans*, we propose that its respiratory chain functioning during the growth with  $S^0$  is branched (Fig. 2). First, the electrons from reduced ferredoxin could be utilized by FNOR and NSR. It would be linked to proton translocation and the first site of  $S^0$  reduction. Second, a part of the electrons from FNOR, as well as the electrons from SQOR and Etf-QOR, would be transferred to the quinone pool. Oxidation of quinol by the SR would also be linked to proton translocation and the second site of reduction of  $S^0$ . During growth in the absence of  $S^0$ , the TCA cycle is not functional and the respiratory chain involving the SQOR complex, quinones, and SR seems not to operate. However, a proton gradient still could be established by the oxidation of reduced ferredoxin in the reaction of the proposed FNOR complex linked to reoxidation of NAD(P)H in reactions yielding reduced fermentation products.

Some anaerobic *Crenarchaeota* were reported to use in addition to  $S^0$  other terminal electron acceptors, including sulfate, thiosulfate, and nitrate. The presence of thiosulfate stimulated the growth of *A. saccharovorans* and led to production of H<sub>2</sub>S (24). We have predicted the function of ASAC\_1397 as a component of membrane-bound SR complex (see above). Nevertheless, in analogy with thiosulfate reductase of *Salmonella enterica* involved also in the reduction of  $S^0$  (11), the dual function of ASAC\_1397 in the reduction of  $S^0$  as well as of thiosulfate can be suggested.

The established proton gradient can be used by ATP synthase for the synthesis of ATP. The genome of *A. saccharovorans* encodes a single A<sub>1</sub>A<sub>o</sub> ATP synthase composed of nine subunits (Table S1). The subunit K (c) (ASAC\_0393) of the membrane-embedded motor does not contain conserved residues involved in Na<sup>+</sup> binding in Na<sup>+</sup>-ATP synthases (21), strongly suggesting that H<sup>+</sup> is the coupling ion in the A<sub>1</sub>A<sub>o</sub> ATP synthase. During growth of *A. saccharovorans* in the absence of  $S^0$ , the required proton gradient could be established only partially by the function of the FNOR complex and H<sup>+</sup>-translocating PPase. An additional proton gradient required for maintenance of normal intracellular pH could be generated by the reverse activity of A<sub>1</sub>A<sub>o</sub> ATP synthase.

**Possible ecological role of *Acidilobales*.** Organic substances reaching terrestrial acidic hot springs from surrounding areas as well as produced by lithoautotrophic microorganisms serve as substrates for organotrophic thermoacidophiles. Metabolic activity of these organisms is supported by fast anaerobic oxidation of [<sup>14</sup>C]sucrose to CO<sub>2</sub> in acidic hot springs (23). Cat-

abolic pathways and energy generation mechanisms predicted from the genome of *A. saccharovorans* explain its growth on proteins, carbohydrates, and lipids (Fig. 2). Though phylogenetically *Acidilobales* are most closely related to *Desulfurococcales*, they metabolically resemble *Thermoproteales*. Similarly to *T. tenax*, *A. saccharovorans* possesses both the modified EM and ED pathways of glucose catabolism. Another feature common between *A. saccharovorans* and *Thermoproteales* is the presence of the TCA cycle, which enables a complete oxidation of organic substances in the presence of external anaerobic electron acceptors. Adaptation of *A. saccharovorans* to acidic conditions is reflected by a high ratio of secondary transporters to ATP-dependent primary transporters, the suggested reversibility of H<sup>+</sup>-ATP synthase, the function of the encoded H<sup>+</sup>-translocating PPase, and the proposed two proton translocation sites in the anaerobic respiratory chain.

Physiological functions predicted by *A. saccharovorans* genome analysis (Fig. 2) and the wide distribution of *Acidilobus* spp. in acidic hot springs (23, 24) suggest an important role of *Acidilobales* in microbial communities of such habitats, closing the anaerobic carbon cycle through complete mineralization of organic substrates via S<sup>0</sup> respiration. Contrary to mesophilic microbial communities, in which the process of anaerobic destruction of organic substances consists of several stages performed by different groups of microorganisms (hydrolysis, fermentation, syntrophic reactions, and terminal oxidation), in thermal environments this could be achieved by a single group of organisms, *Thermoproteales* in neutral hot springs and *Acidilobales* in acidic ones.

**Nucleotide sequence accession number.** The annotated genome sequence of *A. saccharovorans* 345-15<sup>T</sup> has been deposited in the GenBank database under accession number CP001742.

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