The Geoglobus acetivorans Genome: Fe(III) Reduction, Acetate Utilization, Autotrophic Growth, and Degradation of Aromatic Compounds in a Hyperthermophilic Archaeon

Andrey V. Mardanov, a Galina B. Slododkina, b Alexander I. Slobodkin, b Alexey V. Beletsky, a Sergey N. Gavrilov, b Ilya V. Kublanov, b Elizaveta A. Bonch-Osmolovskaya, b Konstantin G. Skryabin, a Nikolai V. Ravin a

Bioengineering Centre, Russian Academy of Sciences, Moscow, Russia; Winogradsky Institute of Microbiology, Russian Academy of Sciences, Moscow, Russia

Geoglobus acetivorans is a hyperthermophilic anaerobic euryarchaeon of the order Archaeoglobales isolated from deep-sea hydrothermal vents. A unique physiological feature of the members of the genus Geoglobus is their obligate dependence on Fe(III) reduction, which plays an important role in the geochemistry of hydrothermal systems. The features of this organism and its complete 1,860,815-bp genome sequence are described in this report. Genome analysis revealed pathways enabling oxidation of molecular hydrogen, proteinaceous substrates, fatty acids, aromatic compounds, n-alkanes, and organic acids, including acetate, through anaerobic respiration linked to Fe(III) reduction. Consistent with the inability of G. acetivorans to grow on carbohydrates, the modified Embden-Meyerhof pathway encoded by the genome is incomplete. Autotrophic CO₂ fixation is enabled by the Wood-Ljungdahl pathway. Reduction of insoluble poorly crystalline Fe(III) oxide depends on the transfer of electrons from the quinone pool to multiheme c-type cytochromes exposed on the cell surface. Direct contact of the cells and Fe(III) oxide particles could be facilitated by pilus-like appendages. Genome analysis indicated the presence of metabolic pathways for anaerobic degradation of aromatic compounds and n-alkanes, although an ability of G. acetivorans to grow on these substrates was not observed in laboratory experiments. Overall, our results suggest that Geoglobus species could play an important role in microbial communities of deep-sea hydrothermal vents as lithoautotrophic producers. An additional role as decomposers would close the biogeochemical cycle of carbon through complete mineralization of various organic compounds via Fe(III) respiration.

Received 20 August 2014 Accepted 19 November 2014 Accepted manuscript posted online 21 November 2014


Editor: R. M. Kelly
Address correspondence to Nikolai V. Ravin, nravin@mail.ru.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.02705-14.

Copyright © 2015, American Society for Microbiology. All Rights Reserved. doi:10.1128/AEM.02705-14.
diplasma aelicultum, “Acidianus manzaensis,” and “Candidatus Aciduliprofundum boonei,” which reduce soluble Fe(III). Among the neutrophilic hyperthermophiles, species of the genera Pyrococulurn, Thermococcus, Ferroglobus, and Geoglobus reduce soluble as well as insoluble Fe(III) compounds (2, 16–21). The physiology and biochemistry of Fe(III) reduction in hyperthermophilic archaea were studied in some detail in representatives of the genus Pyrobcicum. The majority of iron reductase activity in these organisms appeared to be localized in the cytoplasm, which has low cytochrome content; only circumstantial evidence for the participation of the membrane-bound cytochrome-bc1 complex in iron reduction has been provided (22–24). Comparative studies of the genomes of five Pyrobcicum species (P. aerophilum, P. arsenaticum, P. calidifontis, P. islandicum, and “Thermoproteus neutrophilus” [Pyrobcicum neutrophilum comb. nov.]) did not reveal specific genes for iron respiration (25). The complete genomes of Ferroglobus placidus and “Ca. Aciduliprofundum boonei” are also available; however, there are no published data on the genomic determinants of Fe(III) reduction in these organisms (26). It was recently shown that the obligate Fe(III)-reducing hyperthermophilic archaean Geoglobus ahangari uses a direct-contact mechanism for the reduction of Fe(III) oxides to magnetite (27), but genomic determinates of these processes are unknown.

Another very interesting physiological feature of G. aceticovans is the ability to utilize acetate, one of the major products of the anaerobic degradation of organic matter. This ability is rare among thermophilic archaea and so far has been described in crenarchaea of the genus Pyrobcicum (28) and in the Fe(III)-reducing archaean Ferroglobus placidus and Geoglobus ahangari (18).

Here, the complete genome sequence of G. aceticovans strain SBH67 was reported. A whole-genome analysis and analysis of metabolic pathways provide insight into the lifestyle of G. aceticovans, revealing its potential for iron reduction and autotrophic growth. This analysis also reveals genetic determinants potentially enabling degradation of aromatic compounds and n-alkanes.

MATERIALS AND METHODS

Cultivation of G. aceticovans. G. aceticovans (strain SBH67) was isolated from a hydrothermal sample collected on the Mid-Atlantic Ridge (1) and was kept in the culture collection of the Laboratory of Hyperthermophilic Microbial Communities, Winogradsky Institute of Microbiology, Russian Academy of Sciences. Cells were grown on anaerobically prepared, bicarbonate-buffered, sterile (135°C, 1 h) liquid medium with acetate (18 mM) as an electron donor and poorly crystalline Fe(III) oxide (ferrihydrite) (90 mM Fe(III)) as an electron acceptor. To assess the necessity of direct cell-to-mineral contact for G. aceticovans growth, ferrihydrite in the culture medium was immobilized onto microporous glass beads (29), was added to reoxidize the medium composition and biochemistry of Fe(III) reduction in hyperthermophilic Geoglobus were studied in some detail in representatives of the genus Pyrobcicum. The majority of iron reductase activity in these organisms appeared to be localized in the cytoplasm, which has low cytochrome content; only circumstantial evidence for the participation of the membrane-bound cytochrome-bc1 complex in iron reduction has been provided (22–24). Comparative studies of the genomes of five Pyrobcicum species (P. aerophilum, P. arsenaticum, P. calidifontis, P. islandicum, and “Thermoproteus neutrophilus” [Pyrobcicum neutrophilum comb. nov.]) did not reveal specific genes for iron respiration (25). The complete genomes of Ferroglobus placidus and “Ca. Aciduliprofundum boonei” are also available; however, there are no published data on the genomic determinants of Fe(III) reduction in these organisms (26). It was recently shown that the obligate Fe(III)-reducing hyperthermophilic archaean Geoglobus ahangari uses a direct-contact mechanism for the reduction of Fe(III) oxides to magnetite (27), but genomic determinates of these processes are unknown.

Another very interesting physiological feature of G. aceticovans is the ability to utilize acetate, one of the major products of the anaerobic degradation of organic matter. This ability is rare among thermophilic archaea and so far has been described in crenarchaea of the genus Pyrobcicum (28) and in the Fe(III)-reducing archaean Ferroglobus placidus and Geoglobus ahangari (18).

Here, the complete genome sequence of G. aceticovans strain SBH67 is reported. A whole-genome analysis and analysis of metabolic pathways provide insight into the lifestyle of G. aceticovans, revealing its potential for iron reduction and autotrophic growth. This analysis also reveals genetic determinants potentially enabling degradation of aromatic compounds and n-alkanes.

MATERIALS AND METHODS

Cultivation of G. aceticovans. G. aceticovans (strain SBH67) was isolated from a hydrothermal sample collected on the Mid-Atlantic Ridge (1) and was kept in the culture collection of the Laboratory of Hyperthermophilic Microbial Communities, Winogradsky Institute of Microbiology, Russian Academy of Sciences. Cells were grown on anaerobically prepared, bicarbonate-buffered, sterile (135°C, 1 h) liquid medium with acetate (18 mM) as an electron donor and poorly crystalline Fe(III) oxide (ferrihydrite) (90 mM Fe(III)) as an electron acceptor. To assess the necessity of direct cell-to-mineral contact for G. aceticovans growth, ferrihydrite in the culture medium was immobilized onto microporous glass beads according to a method described in reference 29. The medium composition and preparation techniques were described previously (1); the medium was additionally supplemented with NaCl (18 g liter⁻¹) and MgCl₂ (4 g liter⁻¹) to increase the salinity. The pH of the medium ranged between 6.5 and 6.8, and the incubation temperature was 80°C.

Genome sequencing. The G. aceticovans genome was sequenced on a Roche GS FLX genome sequencer by the standard protocol for a shotgun genome library. The GS FLX run resulted in the generation of approximately 57 Mb of sequences with an average read length of 221 bp. The GS FLX reads were assembled into 30 large contigs by the use of GS De Novo Assembler 2.0 (454 Life Sciences, Branford, CT). The contigs were oriented into a single scaffold by sequencing 48,070 paired-end reads containing 1.3-kb insertions, and the complete genome sequence was obtained upon the generation and sequencing of appropriate PCR fragments. In total, genomic DNA of G. aceticovans was sequenced with an average of 30-fold coverage.

Genome annotation and analysis. tRNA genes were located with tRNAscan-SE (30). Protein-coding genes were identified with the GLIMMER gene finder (31). Whole-genome annotation and analysis were performed with the AutoFACT annotation tool (32), followed by a round of manual curation. Clustered regularly interspaced short palindromic repeat (CRISPR) loci were identified with CRISPR Finder (33); putative transposon-related proteins were found by searching against the IS database (http://www.is.biotoul.fr). Signal peptides were predicted with SignalP v.3.0 (http://www.cbs.dtu.dk/services/SignalP/) by using the HMM algorithm. Pilins were predicted with the FlaFind 1.2 on-line service (http://signalfind.org/flafind.html).

Preparation of membrane fractions of G. aceticovans. An aliquot of biomass harvested for DNA extraction (see above) was resuspended in 15 ml of 20 mM MES (4-morpholinoethanesulfonic acid) buffer (pH 6.8) with the salinity adjusted to that of the culture medium (1). Ferrihydrite was separated by intensive shaking of the suspension and centrifugation at 1,000 × g for 5 min. The supernatant was further centrifuged at 10,000 × g for 30 min. The pellet of the cells was washed with desalted 20 mM MES buffer and resuspended in it to a final cell density of ca. 10¹⁰ cells per ml. The cells were disrupted with four subsequent cycles of freeze-thaw treatment at −80°C and +30°C, respectively, followed by ultrasonication for 1 min at 23 kHz on a Soniprep 150 Plus device (MSE Ltd., United Kingdom). The cell extract, microscopically checked for the absence of whole cells, was centrifuged for 5 min at 2,000 × g to discard coarse cell debris and mineral particles and for a further 30 min at 20,000 × g, yielding a reddish-to-colorless pellet. The pellet, containing mainly cell membranes, was resuspended in 20 mM desalted MES buffer (pH 6.8) to a final protein content of approximately 600 mg liter⁻¹.

Spectrophotometric detection of c-type cytochromes of G. aceticovans. This assay was based on the rapid scanning of the UV light-visible light absorbance spectrum of a sample, allowing determination of the redox state of cytochromes by specific absorbance peaks, as described previously (14). In the current study, the assay was modified to decrease the influence of excess dithionite on the results of cytochrome reoxidation. Soluble [Fe(III)-EDTA] or insoluble (ferrihydrite) forms of Fe(III) were added to the samples until the complete disappearance of a broad absorbance peak at 352 to 363 nm (corresponding to reduced dithionite). Subsequently, 1 mM Fe(III)-EDTA or 3 mM Fe(III) as ferrihydrite, immobilized onto microporous glass beads (29), was added to reoxidize the cytochromes. The incubation temperature corresponded to the growth optimum (80°C) of the organism.

Nucleotide sequence accession number. The annotated genome sequence of G. aceticovans has been deposited in the GenBank database under accession no. CP009552.

RESULTS AND DISCUSSION

General features of the genome. G. aceticovans has a single circular chromosome of 1,860,815 bp with an average G+C content of 46.8% without extrachromosomal elements (Table 1). There is a single copy of the 16S-23S rRNA operon and a single distantly located 55 rRNA gene. A total of 47 tRNA genes coding for all 20 amino acids are scattered throughout the genome; 2 of them contain introns.

The chromosome displays a single major local minimum of cumulative GC skew profile data that likely corresponds to the DNA replication origin (see Fig. S1 in the supplemental material). A single origin of replication was previously found in Archaeoglobus fulgidus, whereas multiple replication origins in some methanogens and halophiles have been suggested previously (34).

Using a combination of coding potential prediction and similarity searches, 2,230 potential protein-encoding genes, with an average length of 758 bp, covering 90.8% of the genome, were
identified. Through similarity and domain searches for the predicted protein products, the function of 1,357 (60.9%) protein-encoding genes was predicted with different degrees of confidence and generalization. The function of the remaining 873 genes could not be predicted from the deduced amino acid sequences; among them, 345 genes were unique to *G. acetivorans*, with no significant similarity to any known sequences.

Consistent with its affiliation to the order *Archaeoglobales*, *G. acetivorans* shares more than half of its proteome with those of *Ferrolobus placidus* (1,486 proteins) and *A. fulgidus* (1,302 proteins), while 1,061 proteins of *G. acetivorans* are also present in *Archaeoglobus profundus*. Pairwise comparison by all-versus-all BLAST analysis of the genomes of *G. acetivorans* and *F. placidus* revealed only short regions of gene order conservation (see Fig. S2 in the supplemental material).

The search for putative mobile elements revealed 16 transposases, 13 of which belong to the IS481 family. The almost identical ~1-kb sequences of all 13 copies of this IS element suggest that it invaded the *G. acetivorans* genome recently on the evolutionary time scale and may remain functional. Other transposases, Gace_1139 of the IS240 family, Gace_1546 of the IS605 family, and Gace_2130 of the IS4 family, are present in single copies in the genome. The genome of *G. acetivorans* contains 5 CRISPR (clustered regularly interspaced short palindromic repeat) loci, containing 2, 3, 14, 12, and 4 spacer-repeat units. The *G. acetivorans* CRISPR system belongs to type I as determined by the presence of the characteristic *cas3* gene (35).

**Flagella and pili.** The ability of microorganisms to sense environmental conditions by the activity of the chemotaxis system and actively move toward more-favorable locations by the activity of the flagellum is a widespread phenomenon. Flagellar motility could be especially advantageous to *G. acetivorans*, as it provides a mechanism to access insoluble Fe(III) oxide particles. However, in the original description, it was reported to lack flagella (1), in contrast to most *Archaeoglobi*, including *A. fulgidus* (36) and *F. placidus* (37). However, the genome sequence of *G. acetivorans* revealed the presence of a fla gene cluster (Gace_0653 to Gace_0660) and a distantly located preflagellin peptidase flaK gene (Gace_1897). The gene order in the fla cluster of *G. acetivorans* is identical to that in *A. fulgidus*, which belongs to fla2-type clusters according to the results presented in reference 38. As well as the *A. fulgidus* genome, the genome of *G. acetivorans* lacks a fla1 cluster, supporting the hypothesis of its loss after horizontal gene transfer (HGT) of the fla2 cluster from *Crenarchaeota to Archaeoglobi* via *Methanomicrobia* (38). Interestingly, *F. placidus* has a fla1-type cluster, indicating that HGT of fla2 to the common ancestor of *Geoglobus* and *Archaeoglobus* had already occurred after its divergence from *Ferroglobus*. The genetic components for a chemotaxis system (Gace_0661 to Gace_0672) are encoded near the fla gene cluster, and they were also transferred from *Methanomicrobiu*.

In addition to fla genes, several pilins have been predicted with the FlaFind 1.2 online service (39). The FlaFind search retrieved 19 sequences in the genome of *G. acetivorans*, two of which encode the FlaB structural flagellin (Gace_0653, 0654). A local BLAST search against a set of all archaeal sequences assigned as “pilins” in the Uniprot database on 4 July 2014 revealed that the three FlaFind-positive proteins of *G. acetivorans* are related to archaeal pilins. One protein is Gace_2093, which shares homology with the PilA major structural pilin of a haloarchaeon (*Natronomomas pharaonis*) and possesses a characteristic N-terminal hydrophobic motif belonging to the Duf1628 domain, recently identified in *Haloferax volcanii* (39). Another putative component of the *G. acetivorans* pilus structure is encoded by Gace_2093, which shares weak similarity with proteins involved in flagellin/pilin biosynthesis (40). No other genes involved in assembly of pili have been identified in the genomic context of these putative pilins; however, homologs of the PilT family ATPase required for archaeal pilus and flagellum assembly have been identified (Gace_1614, etc.).

Consistent with the molecular analyses, additional electron microscopic studies have revealed the presence of two types of filamentous structures in *G. acetivorans*—flagella and tiny pilus-like appendages (Fig. 1). The presence of flagella implies motility of the organism, while pili, connecting the cells with Fe(III) minerals (Fig. 18), are more likely to serve for adhesion than for twitching motility. The production of two distinct types of filaments, flagella and curled pilus-like thin appendages, was also reported for *G. ahangari* (27). Interestingly, cells simultaneously producing both flagellum- and pilus-like structures have not been identified in *G. acetivorans* cultures, suggesting that these processes are strictly regulated in the organism.

**Central metabolism.** *G. acetivorans* was reported to be unable to grow on carbohydrates (1). Consistently, we found no glycoside hydrolase-coding genes in the genome. Moreover, the set of enzymes of the modified Embden-Meyerhof pathway (41) encoded

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Value</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genome size (bp)</td>
<td>1,860,815</td>
<td>100</td>
</tr>
<tr>
<td>No. of predicted origins of replication</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>G+C content (bp)</td>
<td>871,618</td>
<td>46.8</td>
</tr>
<tr>
<td>Coding regions (bp)</td>
<td>1,689,252</td>
<td>90.8</td>
</tr>
<tr>
<td>No. of RNA operons (165-235)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>No. of SS RNA genes</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>No. of RNA genes</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>No. of predicted protein-coding genes</td>
<td>2,230</td>
<td>100</td>
</tr>
<tr>
<td>No. of protein-coding genes with assigned functions</td>
<td>1,357</td>
<td>60.9</td>
</tr>
<tr>
<td>No. of predicted genes unique to <em>G. acetivorans</em></td>
<td>345</td>
<td>15.5</td>
</tr>
<tr>
<td>No. of genes assigned to COGs</td>
<td>1399</td>
<td>62.7</td>
</tr>
<tr>
<td>No. of genes encoding transposases</td>
<td>16</td>
<td>0.7</td>
</tr>
<tr>
<td>No. of CRISPR arrays</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

*COGs, clusters of orthologous groups.*

**FIG 1** Electron micrographs of *G. acetivorans* cells. (A) Flagellum-like structures. (B) Pili-like structures connecting two cells with an Fe(III) mineral particle. Average dimensions of pili: diameter, 7 nm; length, 300 nm. Note the different sizes of the scale bars in panels A and B and the different thicknesses of the filaments. The fine structure was studied using a JEM-100 electron microscope. Negative staining of whole cells was done with 2% phosphotungstic acid.
by the genome of *G. acetivorans* is present but incomplete. The first step, glucose phosphorylation, could be accomplished by the Rho-associated protein kinase (ROK) family, encoded by Gace_2137. Additional steps can be catalyzed by glucose-6-phosphate isomerase (Gace_0363), ATP-dependent phosphofructokinase (Gace_0807 and Gace_1608), fructose 1,6-bisphosphate aldolase (Gace_1230 and Gace_1261), triosephosphate isomerase (Gace_2159), NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (Gace_0477), phosphoglycerate kinase (Gace_1063), phosphoglycerate mutase (Gace_1916 and Gace_0373), and enolase (Gace_1602). However, genes encoding pyruvate kinase were not identified. The genome of *G. acetivorans* also lacks genes encoding the key enzymes of the modified Entner-Doudoroff pathway of glucose oxidation, such as glucose dehydrogenase, gluconate dehydratase, 2-keto-3-deoxy-gluconate kinase, and 2-keto-3-deoxy-(6-phospho) gluconate aldolase. Genes encoding enzymes of the oxidative pentose phosphate pathway, glucose-6-phosphate dehydrogenase, ribulose-5-phosphate epimerase, transketolase, and transaldolase, are also lacking. Therefore, consistent with the inability of *G. acetivorans* to grow on carbohydrates, we found no complete pathway of carbohydrate catabolism in its genome.

Glucconeogenesis in *G. acetivorans* seems to be operative because all genes encoding required enzymes of the Embden-Meyerhof pathway, including enzymes functioning irreversibly in the anabolic direction—phosphoenolpyruvate synthetase (Gace_e_0215) and fructose-1,6-bisphosphatase (Gace_2256)—are present. Thus, the *G. acetivorans* genome encodes a complete set of enzymes sufficient for hexose phosphate synthesis, while synthesis of pentoses from hexoses may occur via the ribulose monophosphate pathway, encoded in the genome.

In contrast to carbohydrates, proteinaceous substrates can support the growth of *G. acetivorans* (1). Their hydrolysis may be performed by about 20 different peptidases, some of which were predicted to carry signal peptides, which could indicate their export from the cell. Amino acid catabolism occurs in the cells of *G. acetivorans*, possibly using pathways similar to those studied in *Thermococcales* (42–44) and assumed to function also in other archaea. Various 2-oxoacids produced during amino acid fermentation are further oxidized to the corresponding coenzyme A (CoA) derivatives, with the formation of reduced ferredoxin due to the activity of 2-oxoacid:ferredoxin oxidoreductases of a different specificity. In particular, there are two copies of pyruvate ferredoxin oxidoreductase (POR; Gace_0695 to Gace_0698 and Gace_1414 to Gace_1417) which generate acetyl-CoA and CO2 from pyruvate, as occurs in *F. placidus* (26). Acetyl-CoA is converted to acetate with the production of ATP due to the catalytic activity of acetyl-CoA synthetases. In the presence of external electron acceptors, acetyl-CoA may be completely oxidized via the tricarboxylic acid (TCA) cycle encoded by the *G. acetivorans* genome.

The presence of acetyl-CoA synthetase and the TCA cycle may explain the ability of *G. acetivorans* to oxidize acetate to CO2 in the presence of an external electron acceptor (1). *G. acetivorans* could also utilize the acetyl-CoA pathway for acetate oxidation to CO2 (see below), as also described in acetate-oxidizing sulfate-reducing bacteria (45) and acetylastic methanogens (46).

*G. acetivorans* is able to oxidize propionate (1), which is consistent with the presence of a propionate metabolic pathway similar to that found in the sulfate-reducing firmicute Desulfotomaculum kueneniai (47). The pathway includes glutonate CoA-transferase (Gace_1311 and -1312), propionyl-CoA carboxylase (Gace_0750), methylmalonyl-CoA epimerase (Gace_0751), and methylmalonyl-CoA mutase (Gace_0747 and -0752 and Gace_2171 and -2172). Succinyl-CoA produced in these reactions may be oxidized in the TCA cycle.

*G. acetivorans* is able to grow on stearate and palmitate (1). The utilization of fatty acids may be enabled by a complete β-oxidation pathway encoded in the genome. This trait rarely occurs among archaea and was found in *A. fulgidus* (48), *Acidibacillus saccharovorans* (49), and *Vulcanisaeta moutonovskii* (50). We did not find any signal peptide-containing esterases or lipases in the genome of *G. acetivorans*, and growth on triacylglycerols or lipids was not observed.

**Autotrophic pathways.** Similarly to other described Archaeoglobaceae species (except *A. profundus* and *A. infectus*), *G. acetivorans* is capable of autotrophic growth using CO2 as a carbon source. Several different pathways for CO2 fixation have been reported for hyperthermophilic archaea (51). The key enzymes of the reverse TCA, 3-hydroxypropionate, and Calvin-Benson pathways were not identified. Similarly to *F. placidus*, the genome of *G. acetivorans* contains the acetyl-CoA reductive (Wood-Ljungdahl) pathway. This pathway consists of two branches, each reducing CO2 into methyl and carboxyl moieties, respectively, which are then joined to form acetyl-CoA (52). A methyl branch reduces CO2 into a methyl group by a sequence of reactions proceeding in a manner reverse to that of methanogenesis, and a carboxyl branch converts a second CO2 molecule into a carboxyl group using the CO2 dehydrogenase/acetyl-CoA synthase complex. All genes in this pathway were identified (see Table S1 in the supplemental material) except for that encoding formate dehydrogenase, the first enzyme of a methyl branch.

An alternative pathway of formate production could be the activity of pyruvate formate lyase (PLF), which catalyzes the conversion of pyruvate and coenzyme A to formate and acetyl-CoA. This enzyme may be encoded by the genes Gace_0240 (catalytic enzyme) and Gace_0241 (activating enzyme); however, detailed analyses suggest that these enzymes are probably alkylsuccinate synthase and its activator (see below). Therefore, the source of formate entering the Wood-Ljungdahl pathway is unclear.

According to the original description (1), *G. acetivorans* is capable of growth using H2 as the sole electron donor. No organic carbon source was required for growth on hydrogen. At least three hydrogenases, as well as a set of hydrogenase maturation proteins, are encoded by the *G. acetivorans* genome. The first is the cytoplasmic three-subunit methyl viologen-reducing hydrogenase (Gace_0709, Gace_0710, and Gace_0711 coding for MvhD, MvhG, and MvhA subunits, respectively), a part of the H2:heterodisulfide oxidoreductase complex that catalyzes the reduction of the coenzyme M-coenzyme B heterodisulfide and is typically involved in cytoplasmic redox balance. Two other hydrogenases, assigned to group 1 (53), are membrane-bound respiratory complexes that could perform respiratory hydrogen oxidation linked to quinone reduction. Such NiFe hydrogenases link the oxidation of H2 to the reduction of the terminal electron acceptor [Fe(III) oxide in case of *G. acetivorans*], with the recovery of energy in the form of a proton motive force. The first complex encoded in the proximity of genes of methyl viologen-reducing hydrogenase and heterodisulfide reductase subunits consists of the NiFe hydrogenase large subunit (Gace_0714), a small subunit (Gace_0715) with
a twin-arginine translocation (Tat) signal peptide, and a membrane-bound cytochrome β subunit (Gace_0716) which connects the hydrogenase to the quinone pool of the respiratory chain in the membrane. The second distantly located cluster (Gace_1723 to -1725) encodes the same set of subunits.

The transmembrane proton gradient generated by membrane-bound hydrogenases and the F_{432}H_{3}quione oxidoreductase complex (Gace_0005 to -0015) may be used for ATP synthesis by V-type ATPase encoded by genes Gace_1729 to 1737.

**Genomic determinants of Fe(III) reduction.** Fe(III) appears to be the only electron acceptor supporting the growth of _G. acetivorans_. Consistently, we found no nitrate or sulfate reduction pathways previously identified in _ Archaeoglobales_ able to use these electron acceptors (26, 48, 54). During the growth of _G. acetivorans_, approximately 30% of the initial amount of Fe(III) [supplied as insoluble poorly crystalline Fe(III) oxide] was converted to extracellular magnetite particles.

It is generally recognized that the biochemical machinery driving dissipatory Fe(III) reduction is built up of three major groups of multiheme c-type cytochromes: cytochromes associated with the cytoplasmic membrane that accept electrons from the quinone pool of the electron transfer chain; electron-shuttling periplasmic cytochromes; and a group of cytochromes associated with the outer membrane which accept electrons from the periplasmic shuttles and transfer them to the insoluble acceptor [Fe(III) mineral] contacting the cell surface (55, 56). These assertions were deduced on the basis of extensive investigations of Fe(III)-reduction mechanisms in model mesophilic Gram-negative bacteria of the genera _Geobacter_ and _Shewanella_. The mechanisms have been recently suggested for Fe(III)-reducing _Archaea_ because multiheme c-type cytochromes have been identified in _Pyrobaculum calidifontis_ (24) and _Pyrobaculum_ sp. 1860 (57), and specific ferrihydrite-reducing heme c-containing proteins, required for the reduction of insoluble Fe(III) oxide, have been detected in the fraction sheared from the cell’s outer surface of _G. ahangari_ (27).

We have screened the _G. acetivorans_ genome for the presence of multiheme c-type cytochrome genes. The analysis revealed 16 open reading frames (ORFs) encoding putative multiheme cytochromes, possessing from 4 to 32 heme c-binding motifs within one or several conserved domains of the multiheme cytochrome family (CL0517 of _Pfam_ 26.0). Among these, 13 proteins (Gace_0099, -0304, -0430, -1340, -1360, -1361, -1826, -1843 to -1847) were predicted to be localized in the cell envelope, possessing transmembrane helices and/or signal peptides. The retrieved protein sequences were screened for homology with previously described multiheme cytochromes, driving the reduction of insoluble Fe(III) in _Shewanella oneidensis_ and _Geobacter sulfurreducens_. Seven proteins organized in two putative operons were predicted to be the determinants of ferrihydride reduction according to the applied procedure. The most probable operon of Fe(III) reduction in _G. acetivorans_ consists of the genes Gace_1843 to -1847, encoding five multiheme c-type cytochromes with predicted membrane localizations that are necessary to contact the insoluble electron acceptor (ferrihydrite). Among these genes, Gace_1847 encodes a putative protein that includes several c-type multiheme domains, an outer surface membrane anchor region, and two hematite-binding motifs, described previously for a putative terminal Fe(III)-oxide reductase of _Shewanella oneidensis_ (58). One of these motifs in Gace_1847 is adjacent to a heme c-binding motif, supporting the idea of the involvement of putative hematite-binding residues in electron transfer processes performed by this protein. Similar hematite-binding motifs have been revealed in Gace_1843 to -1845, indicating the presence of a complex system for attachment to the Fe(III) oxide surface. It should be noted that homologs with predicted functions have not been retrieved from the Uniprot database (http://www.uniprot.org) for any of the genes comprising the operon. However, comparisons with genomes of closely related organisms of the genera _Ferroglobus_ and _Archaeoglobus_ revealed genes homologous to Gace_1843 to -1847 only in Fe(III)-reducing _F. placidus_, with an average sequence identity of ca. 40%.

An additional Fe(III)-reducing complex of membrane-bound multihemes could be represented by Gace_1360 to -1361, as each of the proteins encoded by these genes possesses five c-type hemes and one to five putative hematite-binding motifs. However, the absence of homologs with predicted functions encoded by Gace_1360 to -1361 does not allow a reliable prediction of function for these cytochromes.

Alternative Fe(III) reductases could be represented in _G. acetivorans_ by two membrane-bound oxidoreductases encoded by genes Gace_0099 to -0102 and genes Gace_1341 to -1344. Each complex consists of four subunits: a c-type cytochrome of the NapC family containing 12 heme motifs (Gace_0099 and Gace_1341), an NrfD-like transmembrane protein (Gace_0100 and Gace_1342), an electron transfer 4Fe-4S ferredoxin iron-sulfur protein (Gace_0101 and Gace_1343), and a multiheme c-type cytochrome (Gace_0102 and Gace_1344). The amino acid sequence identity between the corresponding subunits in the two complexes is in the range of 30% to 50%. In both complexes, the iron-sulfur subunits and one (Gace_1344) or both (Gace_0099 and -0102) c-type cytochromes contain N-terminal signal peptides and are probably targeted to the outer surface of the cytoplasmic membrane. Therefore, these oxidoreductases could accept electrons from the quinone pool and perform extracellular reduction of Fe(III). Note that similar complexes are present in the genome of the Fe(III)-reducing archaeon _F. placidus_ but are absent in _A. fulgidus_ and _A. profundus_, which are unable to reduce Fe(III).

**Biochemical analysis of Fe(III) reduction.** In our study, growth of _G. acetivorans_ appeared to depend strictly on direct access to the surface of an insoluble electron acceptor (ferrihydrite); no growth was observed upon ferrihydrite sequestration from cells, even in the case of porous glass beads leaving a minor surface layer of the mineral for direct contact with cells. A fraction of the cell membranes of _G. acetivorans_, grown with freely accessible ferrihydrite, revealed a characteristic absorbance peak of oxidized c-type cytochromes at 407 nm (Fig. 2). Reduction of this fraction with dithionite returned peaks at 424, 522, and 553 nm in a redox difference absorption spectrum, which is characteristic of reduced c-type cytochromes. The fraction was susceptible to further complete reoxidation by soluble Fe(III)-EDTA and partial reoxidation by ferrihydrite, indicating the presence of Fe(III)-reducing cytochromes in the cell membranes (Fig. 2). Incomplete reoxidation with ferrihydrite was revealed by retention of an absorbance peak at 424 nm and decreased absorbance ratios at 424 nm and 407 nm, corresponding to γ bands of reduced and oxidized cytochromes c, respectively, and the complete disappearance of the α band of reduced cytochrome c at 553 nm. Such incomplete reoxidation indicates that only a restricted fraction of...
membrane-associated c-type cytochromes in *G. aceticivorans* are able to interact with an extracellular electron acceptor. Interestingly, in the case of reoxidation with ferricydrite, but not with soluble Fe(III)-EDTA, the γ extrema of the reduced and oxidized cytochrome forms showed a slight red-shift (Fig. 2B); this has been reported to indicate heme c conformational destabilization upon redox transformation and interaction with hematein (59).

The complete reoxidation of cytochromes with soluble Fe(III)-EDTA correlates with the previously reported ability of *G. aceticivorans* to grow at a low rate with Fe(III) citrate as an electron acceptor (1); this implies that the membrane fraction of *G. aceticivorans* cells contains other iron reductases along with terminal oxidoreductases that are able to interact with an insoluble Fe(III) form. We propose that these Fe(III)-reducing cytochromes form a chain to transfer electrons from the quinone pool to extracellular Fe(III) via terminal iron reductases, directly interacting with insoluble Fe(III) on the cell surface. Such a chain was previously proposed in the Fe(III)-reducing bacteria *Carboxydothermus ferrireducens*, *Thermococcus温泉*, and *Methylothermus roseus* (14, 60, 61). The same chain may also function in *G. ahangari*, in which cell surface-associated c-type cytochromes, specifically required for the reduction of insoluble Fe(III) oxides, have been been detected together with other heme-containing proteins providing cell growth with soluble Fe(III) citrate (27).

It is now clear from the genome analysis that key determinants of insoluble Fe(III) reduction in *Geoglobus* are the same as in the case of model mesophilic iron reducers (i.e., multiheme c-type cytochromes). *G. aceticivorans* contains the largest number of c-type multiheme cytochrome genes among Fe(III)-reducing thermophilic *Archaea* reported so far; this correlates with the strict dependence of *Geoglobus* species on insoluble Fe(III) forms as electron acceptors supporting cell growth.

**Degradation of aromatic compounds and n-alkanes.** Until now, *F. placidus* was the only hyperthermophilic archaeon known to be capable of anaerobically oxidizing aromatic compounds. It can grow by oxidizing benzoate and phenol to carbon dioxide, with Fe(III) serving as the sole electron acceptor (62). The benzene degradation pathway identified in *F. placidus* (63), including benzoate-CoA ligase (Gace_0785), class I bdz-type benzyol-CoA reductase BdzOPON (Gace_0796 to -0799), cyclohex-1-ene-1-carboxyl-CoA hydratase BdhK (Gace_0765), 2-hydroxycyclohexane-1-carboxyl-CoA dehydrogenase BdhH (Gace_077), 2-ketocyclohexane-carboxyl-CoA hydrolase Bdh (Gace_0789), and didehydro-pimeloyl-CoA hydratase Dph (Gace_0804), is conserved in *G. aceticivorans* (see Table S1 in the supplemental material). The final product, 3-hydroxypimeloyl-CoA, could be converted into acetyl-CoA by beta-oxidation and finally be fully oxidized to CO2 by the tricarboxylic acid cycle.

In the *F. placidus* genome, most of genes relevant to the degradation of aromatic compounds are located in three clusters (63). Cluster 3 (Ferp_0074 to -0101), comprising genes involved in phenol metabolism, is absent in *G. aceticivorans*. Genes of clusters 1 and 2 (Ferp_1026 to -1049 and Ferp_1169 to -1195), involved in benzoate metabolism, in the *G. aceticivorans* genome are located in one genome region (Fig. 3). This benzoate metabolism cluster...
(Gace_0763 to -0804; Fig. 3) also includes homologs of *F. plcadus* genes located outside main clusters but probably involved in benzoate degradation such as *badH* (Ferp_1233), *badI* (Ferp_1040), and Gace_0771 to -0775 (Ferp_1227 to -1232), presumably associated with aromatic metabolism and induced in *F. plcadus* cells grown on benzoate (63). The benzoate metabolism cluster in the *G. activorans* genome seems to be present in a most compact ancestral form; this cluster became split and rearranged in the *F. plcadus* genome.

In spite of the presence of the benzoate degradation genes, the capability of *G. activorans* to utilize benzoate was not observed during the initial characterization of this organism (1), and iterated experiments did not reveal growth on benzoate in a range of different concentrations. It is possible that other aromatic substrates may be metabolized by the same pathway or that the benzoate degradation abilities might be exhibited only under specific unknown environmental conditions, which are yet to be reproduced in laboratory experiments.

Anaerobic bacteria with the capacity for *n*-alkane degradation have been isolated relatively recently. The most widespread mechanism of activation of *n*-alkanes is the formation of fumarate at a secondary carbon atom, resulting in a substituted succinate, using a glycol radical as an initiator (64). This reaction is similar to the anaerobic activation of aromatic compounds that leads to benzylsuccinate via a benzyllsuccinate synthase. Genes encoding alkylsuccinate synthase (*assA*) have been identified in *Desulfatibacillum alkenivorans* AK-01 (65). Similar genes have been annotated as *masD* ([1-methylalkyl] succinate synthase (MasI)) in *Azorarcus* sp. HXN1 (66). Recently, genes encoding the catalytic (AssA/BssA) subunit of alkylsuccinate synthase and activating protein (AssD/BsdD) were identified in the genome of the euryarchaean *A. fulgidus* VC-16 (67), which can oxidize saturated hydrocarbons (*n*-alkanes in the range C10 to C21). These genes, AF1449 and AF1450, are similar to those encoding pyruvate formate lyase and pyruvate formate lyase-activating enzyme, respectively. Close homologs of these genes, Gace_0240 (69% identical to AF1449) and Gace_0241 (57% identical to AF1550), are present in the genome of *G. activorans*. The amino acid sequence of Gace_0240 exhibits conserved indels that are specific to AssA and BssA proteins but not to pyruvate formate lyases. Furthermore, enzymes acting as pyruvate formate lyases display two adjacent conserved cysteine residues, whereas at this site, AssA and BssA, like AF1449 and Gace_0240, contain only one conserved cysteine that accepts the radical from the glycol residue and initiates catalysis (67). Likewise, the N-terminal regions of activating enzymes, AF1450 and Gace_0241, contain two cysteine-rich regions that would be involved in [FeS] cluster binding, a feature that is unique to alkyl- and benzylsuccinate synthases and is not found in pyruvate formate lyase-activating enzymes (67). Altogether, these data suggest that the enzymes encoded by Gace_0240 and Gace_0241 are alkyl- or benzylsuccinate synthases and activating enzymes, respectively. Similar enzymes are encoded in the genomes of *A. fulgidus* and *A. sulfatit-lidus* PM70-1 (67) but are absent in *F. plcadus* and the closely related *A. profundus* DSM563. It was proposed that the AssA and AssD genes were acquired through horizontal transfer from *Bacteria* (67). The present results suggest that the transfer occurred to the common ancestor of *Archaeoglobus* (except for *A. profundus*) and *Geoglobus*.

Once formed, (1-methylalkyl)succinate undergoes carbon-skeleton rearrangement and subsequent decarboxylation prior to beta-oxidation. It was suggested previously (68) that the rearrangement and decarboxylation can be catalyzed by methylmalonyl-CoA mutase and methylmalonoyl-CoA decarboxylase, respectively. These enzymes are encoded in the *G. activorans* genome (Gace_0747 to -0752 and Gace_2171 to -2172). The encoded methylmalonyl-CoA pathway is important for regeneration of the fumarate used for the initial activation reaction because propionyl-CoA, derived from the beta-oxidation, can be transformed to succinate and further oxidized to fumarate by succinate dehydrogenase. The ability of *G. activorans* to utilize *n*-alkanes has not been tested and remains to be investigated in laboratory experiments.

Conclusions. The complete genome sequence of *G. activorans* provides a wealth of new information about how this unusual organism exploits its environment. Metabolic pathways and energy generation mechanisms predicted from the genome explain its growth with molecular hydrogen and various organic substrates potentially present in deep-sea hydrothermal vents, proteinaceous compounds, fatty acids, aldehydes, and organic acids, including acetate. In particular, acetate is one of the major metabolic products of the organic matter fermentation exhibited under anaerobic conditions by many hyperthermophiles. Genome data also imply the ability of *G. activorans* to metabolize aromatic compounds and *n*-alkanes, although these traits remain to be experimentally evaluated. Overall, these metabolic features are typical *of Archaeoglobales* growing as scavengers on low-molecular-weight compounds.

The metabolic hallmark of *G. activorans* is its obligate dependence on dissimilatory Fe(III) reduction. Manzella et al. (27) recently found that *G. ahangari* cells attach and transfer electrons to Fe(III) oxides using redox-active proteins exposed on the cell surface. Our genome analysis and spectrophotometric characterization of cell membranes revealed that the key determinants of insoluble Fe(III) reduction in *G. activorans* are the same as in the case of model mesophilic iron reducers (i.e., multiheme c-type cytochromes). As in *G. ahangari*, Fe(III) reduction depends on direct contact of the cells with insoluble Fe(III) particles that could be mediated by pilus-like structures. Overall, our results suggest that *Geoglobus* species play an important role in the microbial communities of deep-sea hydrothermal vents, closing the biogeochemical cycle of carbon through complete mineralization of low-molecular-weight organic substrates via Fe(III) respiration. They could also act as primary producers performing autotrophic CO2 fixation coupled to growth with H2 as the sole electron donor.

Acknowledgments. We thank Vitali A. Svetlitchenyi for valuable insight and effective discussions.

This work was supported by the Russian Foundation for Basic Research (RFB) [complex project: grants 13-04-40206 to A.V.M. and 13-04-40205 to E.A.B.-O.] and by the program “Molecular and Cellular Biology” of the Russian Academy of Science (A.V.M.). The work of S.N.G. on iron reduction was supported by RFB grant 13-04-02157.

References


2. Kashfi K, Tor JM, Holmes DE, Gaw Van Praagh CV, Reysenbach AL, Lovley DR. 2002. *Geoglobus ahangari* gen. nov., sp. nov., a novel hyper-


