The Genome of *M. acetivorans* Reveals Extensive Metabolic and Physiological Diversity

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Methanogenesis, the biological production of methane, plays a pivotal role in the global carbon cycle and contributes significantly to global warming. The majority of methane in nature is derived from acetate. Here we report the complete genome sequence of an acetate-utilizing methanogen, *Methanosarcina acetivorans* C2A. *Methanosarcinae* are the most metabolically diverse methanogens, thrive in a broad range of environments, and are unique among the Archaea in forming complex multicellular structures. This diversity is reflected in the genome of *M. acetivorans*. At 5,751,492 base pairs it is by far the largest known archaeal genome. The 4524 open reading frames code for a strikingly wide and unanticipated variety of metabolic and cellular capabilities. The
The Archaea remain the most poorly understood domain of life despite their importance to the biosphere. Methanogenesis, which plays a pivotal role in the global carbon cycle, is unique to the Archaea. Each year, an estimated 900 million metric tons of methane are biologically produced, representing the major global source for this greenhouse gas and contributing significantly to global warming (Schlesinger 1997). Methanogenesis is critical to the waste-treatment industry and biologically produced methane also represents an important alternative fuel source. At least two-thirds of the methane in nature is derived from acetate, although only two genera of methanogens are known to be capable of utilizing this substrate. We report here the first complete genome sequence of an acetate-utilizing (acetoclastic) methanogen, *Methanosarcina acetivorans* C2A.

The *Methanosarcinae* are metabolically and physiologically the most versatile methanogens. Only *Methanosarcina* species possess all three known pathways for methanogenesis (Fig. 1) and are capable of utilizing no less than nine methanogenic substrates, including acetate. In contrast, all other orders of methanogens possess a single pathway for methanogenesis, and many utilize no more than two substrates. Among methanogens, the *Methanosarcinae* also display extensive environmental diversity. Individual species of *Methanosarcina* have been found in freshwater and marine sediments, decaying leaves and garden soils, oil wells, sewage and animal waste digesters and lagoons, thermophilic digesters, feces of herbivorous animals, and the rumens of ungulates (Zinder 1993).

The *Methanosarcinae* are unique among the Archaea in forming complex multicellular structures during different phases of growth and in response to environmental change (Fig. 2). Within the *Methanosarcinae*, a number of distinct morphological forms have been characterized, including single cells with and without a cell envelope, as well as multicellular packets and lamina (Macario and Conway de Macario 2001). Packets and lamina display internal morphological heterogeneity, suggesting the possibility of cellular differentiation. Moreover, it has been suggested that cells within lamina may display differential production of extracellular material, a potential form of cellular specialization (Macario and Conway de Macario 2001). The formation of multicellular structures has been proposed to act as an adaptation to stress and likely plays a role in the ability of *Methanosarcina* species to colonize diverse environments.

Significantly, powerful methods for genetic analysis exist for *Methanosarcina* species. These tools include plasmid shuttle vectors (Metcalf et al. 1997), very high efficiency transformation (Metcalf et al. 1997), random in vivo transposon mutagenesis (Zhang et al. 2000), directed mutagenesis of specific genes (Zhang et al. 2000), multiple selectable markers (Boccazzi et al. 2000), reporter gene fusions (M. Pritchett and W. Metcalf, unpubl.), integration vectors (Conway de Macario et al. 1996), and anaerobic incubators for large-scale growth of methanogens on solid media (Metcalf et al. 1998). Furthermore, and in contrast to other known methanogens, genetic analysis can be used to study the process of methanogenesis: Because *Methanosarcina* species are able to utilize each of the three known methanogenic pathways, mutants in a single pathway are viable (M. Pritchett and W. Metcalf, unpubl.). The availability of genetic methods allowing immediate exploitation of genomic sequence, coupled with the genetic, physiological, and environmental diversity of *M. acetivorans* make this species an outstanding model organism for the study of archaeal biology. For these reasons, we set out to study the genome of *M. acetivorans*.
RESULTS

Genome Structure and Content
We sequenced \textit{M. acetivorans} using a combination of whole-genome shotgun and directed finishing (see Methods). The genome consists of a single circular chromosome of 5,751,492 base pairs with 42.7\% G + C content (Table 1). \textit{M. acetivorans} has thus the largest genome known for an archaeon and the fourth largest among sequenced prokaryotes. The \textit{M. acetivorans} genome is twice as large as those of its phylogenetically nearest sequenced relatives, \textit{Archaeoglobus fulgidus} and \textit{Halobacterium}, and over three times as large as the two previously sequenced methanogens, \textit{Methanobacterium thermoautotrophicum} and \textit{Methanococcus jannaschii} (Fig. 3).

Origin of Replication
A putative origin of replication (selected as base pair 1) was identified using the method of cumulative skew (Grigoriev 1998), as well as colocalization with features identified in archaeal replication origins (Kelman 2000).

Genes
A total of 4524 open reading frames (ORFs) longer than 200 bp were identified as putative protein-coding genes (see Methods). A summary of the characteristics of these genes is shown in Table 1. The average protein-coding region is 936 bp. Predicted coding sequences cover 74\% of the genome. This gene density is low compared to other sequenced archaea (for ex-

<table>
<thead>
<tr>
<th>Table 1. \textit{Methanosarcina acetivorans} Genome Features</th>
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<tr>
<td><strong>General</strong></td>
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<tr>
<td>Size</td>
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<td>% G + C</td>
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<td>Protein-coding genes</td>
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<td>RNA genes</td>
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<td>Pseudogenes</td>
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<td><strong>Predicted protein-coding sequences</strong></td>
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<td>Identified by similarity to known sequences</td>
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<td>Conserved hypothetical proteins</td>
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<td>Predicted proteins (no similarity to known sequences)</td>
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<td><strong>Multigene families</strong></td>
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<td>Number of genes in multigene families</td>
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<td><strong>Domain comparison</strong></td>
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<td>Genes with top BLASTX hit to domain</td>
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<td>Archaea</td>
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<td>Bacteria</td>
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<td>\textit{Methanococcus jannaschii}</td>
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<td>\textit{Halobacterium sp NRC-1}</td>
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<td>Genes with BBH hits</td>
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<td>\textit{Archaeoglobus fulgidus}</td>
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<td>\textit{Methanococcus thermoautotrophicum}</td>
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<td><strong>Conserved clusters</strong></td>
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<td>\textit{Methanococcus thermoautotrophicum}</td>
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<td>\textit{Pyrococcus horikoshii}</td>
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<td>\textit{Halobacterium sp NRC-1}</td>
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*Best bidirectional hit: Defined between two species as a pair of proteins, each of which has its top best \textit{BLASTP} hit to the other when all proteins from both species are compared to one another.

*A cluster in one species was defined as a set of genes with each member in the set no further than 10 genes from at least 1 other gene in the set. A conserved cluster was defined as a cluster in \textit{M. acetivorans} with each member of the cluster having a best bidirectional hit (BBH) with a member of a single cluster in a second species. Thus, a conserved cluster consists of a set of genes “close” in the \textit{M. acetivorans} genome whose BBH genes are also “close” in the genome of the second species. The number of genes indicates the number of genes in conserved clusters.

Figure 2. Different morphological forms of \textit{Methanosarcina acetivorans}. Thin-section electron micrographs showing \textit{M. acetivorans} growing as both single cells (center of micrograph) and within multicellular aggregates (top left, bottom right). Cells were harvested during late-exponential growth in medium containing sodium acetate and prepared for electron microscopy as described previously (Sowers and Ferry 1983). Electron micrographs were taken with a JOEL JEM 100B transmission electron microscope.
multigene families containing 315 predicted proteins that
apted genes belong to one of 539 multigene families (Table 2),
accounted for by multigene families. Nearly half of all anno-
A. fulgidus
are 1.3–1.4 times larger on average than related families
archaeal genomes (see Methods) indicate likely gene family
average length of intergenic regions is 341 bp, nearly three times
sequenced prokaryotic genomes (data not shown). The aver-
negative correlation between gene density and genome size in
aerobic halophilic
Pyrococcus abysii
jannaschii
lineage giving rise to the anaerobic sulfate-reducing
Methanosarcineae
are described below.

Multigene Families
A considerable portion of the M. acetivorans genome can be
accounted for by multigene families. Nearly half of all anno-
tated genes belong to one of 539 multigene families (Table 2),
with 98 families containing 315 predicted proteins that ap-
pear to be unique to M. acetivorans. Comparisons with related
archaeal genomes (see Methods) indicate likely gene family
expansion in M. acetivorans. Multigene families in M. acet-
ivors are 1.3–1.4 times larger on average than related families
in A. fulgidus, Halobacterium spp., M. thermoautotrophicum, M.
jannaschii, Pyrococcus abyssi, and Pyrococcus horikoshii. Of
course, family contraction in these related species cannot be
ruled out with the available data. In comparison, the number
of proteins in M. acetivorans without homology to other
known proteins was two to three times larger than in these
species. Thus both the differences in gene family size and the
greater number of apparently unique genes appear to contribute
to the larger genome of M. acetivorans relative to these related
archaean species.

The largest multigene families include several transport-related
families. In all, at least 243 genes were members of transport-related
families and ~160 membrane transport systems were identified. A
small number of large families ac-
count for a significant proportion
of the membrane transporters. For
example, 47 ABC transporter genes
putatively involved in iron chelate
uptake were identified including 17
different binding proteins, more
than twice the number of iron ABC
transporter genes than in any other
sequenced prokaryote (http://www-
biology.ucsd.edu/~ipaulsen/transport/). Although originally
isolated in an iron-rich environ-
ment, these predicted genes suggest
the ability of M. acetivorans to adapt
to iron-poor environments, as well
as to outcompete other organisms for iron uptake. Both pos-
sibilities underscore the adaptability of M. acetivorans.

Examination of the multigene families also highlights
the importance of surface structures to M. acetivorans. The
second largest multigene family consists of 62 annotated sur-
face proteins. The majority of these genes were predicted not
to contain transmembrane domains, suggesting that these
proteins are secreted and play a role in generating the cell
envelope (or S-layer), as well as an extracellular matrix during
the formation of multicellular structures (see below).

Finally, widespread genetic diversity was also observed at
the level of cellular systems. For example, M. acetivorans is
distinct among all prokaryotes in possessing all of the four
major molecular chaperoning systems, including the GroES/L
system, which has not been described in any other archaeon.
Additional examples of the genetic diversity of M. acetivorans
are described below.

Methanogenesis
The only known energy-yielding process available to metha-
nogenic organisms is methanogenesis (Fig. 1) and the impor-
tance of this metabolic process is reflected in the genome of
M. acetivorans. Nearly 200 genes related to methanogenesis
were identified. These include genes required for known steps
in methanogenesis from methylated compounds such as
methanol and methylated amines (the methylotrophic path-
way; Fig. 4A) and those required for methanogenesis from
acetate (the acetoclastic pathway; Fig. 4B). Many of these
genes are present in multiple copies in the M. acetivorans ge-
nome. These include two or three copies each of genes en-
coding components of the methyl transferase systems for
methanol, trimethylamine, dimethylamine and monometh-
ylamine, and two copies of a six-gene operon encoding the
acetyl-CoA decarboxylase/synthase (ACDS) complex essential
for the acetoclastic pathway. The duplication of the ACDS
complex in M. acetivorans is particularly striking; the two op-
erons are 95% identical over a 6.6-kbp stretch, including a subsection of nearly 4 kbp with only 6 nucleotide changes.

**Methanogenic Specialization**

The biochemical functions of the multiple methanogenic genes provide strong evidence that *M. acetivorans* specializes in the utilization of acetate and a variety of one-carbon compounds as growth substrates. It is possible that these multiple gene copies provide added flexibility in the utilization of these substrates. The individual copies of the duplicated genes could display differential regulation or encode enzymes with altered kinetic properties, allowing the organism to rapidly change between substrates and to cope with variable substrate concentrations. The emphasis on the utilization of multiple substrates is consistent with the hypothesis that *Methanosarcina* species succeed in nature by following a generalist strategy (Zinder 1993). This is in contrast to the previously sequenced genomes of *M. thermotrotophicum* and *M. jan-naschii* that specialize in the utilization of a single substrate, molecular hydrogen (H₂).

**Hydrogen Utilization**

*M. acetivorans* differs from many other *Methanosarcina* species in being incapable of growing methanogenically using H₂ to reduce CO₂ (the hydrogenotrophic pathway; Sowers et al. 1984). The hydrogenotrophic pathway shares many steps in common with the oxidative branch of the methylo trophic pathway and utilizes the same enzymes for these steps, although the reactions are run in the reductive rather than the oxidative direction (Fig. 1). Electrons required only for the reduction reactions are provided by the oxidation of molecular hydrogen by enzymes known as hydrogenases. These hydrogenases are characteristic enzymes of the hydrogenotrophic pathway and would not be expected to play a role in the oxidative reactions.

Therefore, it was surprising that most of the hydrogenases identified in hydrogenotrophic *Methanosarcina* species were also present in the *M. acetivorans* genome. The genes include a homolog of the *Methanosarcina barkeri* *frh* operon encoding an F₄₂₀-reducing hydrogenase (Vaupel and Thauer 1998) and homologs of two *Methanosarcina mazei* operons encoding F₄₂₀-nonreducing hydrogenases (in which F₄₂₀ is a redox coenzyme unique to methanogenesis). The identification of these hydrogenases in *M. acetivorans* strongly implies that it is capable of metabolizing hydrogen for purposes other than CO₂ reduction. Thus, a reassessment of the role of hydrogen metabolism in so-called nonhydrogenotrophic organisms is warranted.

The inability of *M. acetivorans* to grow methanogenically using H₂/CO₂ may be explained by the absence of two critical hydrogenases. Neither the F₄₂₀-reducing hydrogenase encoded by the *fre* operon (Vaupel and Thauer 1998) nor the energy-conserving ferredoxin-dependent hydrogenase encoded by the *ech* operon (Kunkel et al. 1998; Meuer et al. 1999) are present in *M. acetivorans*, whereas both are present in *M. barkeri*, which is capable of methanogenesis using H₂/CO₂. The presence of these two genes in hydrogen-utilizing methanogens, and their absence in *M. acetivorans*, provides compelling evidence for their roles in hydrogenotrophic methanogenesis.

**Carbon Monoxide Metabolism**

Certain *Methanosarcina* species have been shown to grow using carbon monoxide (CO) as a methanogenic substrate. It has been hypothesized that oxidation of CO is mediated by the CO dehydrogenase (CODH), now known to be part of the multisubunit ACDS complex (O’Brien et al. 1984), during methanogenic growth on this substrate. Therefore, it was unexpected that *M. acetivorans* was found to contain two homologs of the single-subunit enzyme utilized by *Rhodospirillum rubrum* for growth on CO (Fig. 4B). The presence of this single-subunit enzyme indicates a possible alternative mechanism for methanogenic growth on CO. Furthermore, the fact that this enzyme is used by *R. rubrum* for nonmethanogenic growth on CO raises the surprising possibility that *M. acetivorans* may grow nonmethanogenically, using CO as a substrate. This would require a major reassessment of the metabolism of methanogens, which heretofore have been thought to rely solely on methanogenesis for energy production.

**Undiscovered Growth Substrates**

A number of genes were identified indicating the possibility that other, as yet unidentified, growth substrates may be utilized by *M. acetivorans*. As shown in Figure 4A, methylo trophic methanogenesis begins with the transfer of the methyl group from a variety of substrates to coenzyme M, via two methyl-transferases, designated MT1 and MT2, and a substrate-specific methylo trophic corrinoid protein (containing a modified B₁₂ derivative). Fourteen MT2 and 15 corrinoid protein-encoding genes were identified in the *M. acetivorans* genome. These include three proteins with both MT2 and corrinoid-binding domains. Two of the MT2 homologs and 10 of

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**Table 2. Largest 30 Multigene Families**

<table>
<thead>
<tr>
<th>Name</th>
<th>Size</th>
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<tbody>
<tr>
<td>ABC transporter ATP-binding protein</td>
<td>85</td>
</tr>
<tr>
<td>Surface protein</td>
<td>62</td>
</tr>
<tr>
<td>Sensory transduction histidine kinase</td>
<td>49</td>
</tr>
<tr>
<td>Methyltransferase</td>
<td>46</td>
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<tr>
<td>Predicted protein</td>
<td>29</td>
</tr>
<tr>
<td>Oligopeptide ABC transporter (permease)</td>
<td>28</td>
</tr>
<tr>
<td>Conserved hypothetical protein</td>
<td>25</td>
</tr>
<tr>
<td>Transposase</td>
<td>21</td>
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<tr>
<td>Iron ABC transporter, solute-binding protein</td>
<td>21</td>
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<tr>
<td>Transposase</td>
<td>20</td>
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<tr>
<td>Oligopeptide ABC transporter, solute-binding protein</td>
<td>19</td>
</tr>
<tr>
<td>Iron-sulphur flavoprotein</td>
<td>18</td>
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<tr>
<td>Transposase</td>
<td>18</td>
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<tr>
<td>lsoB-like ATP-binding protein</td>
<td>18</td>
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<tr>
<td>Conserved hypothetical protein</td>
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<tr>
<td>MFS transporter family protein</td>
<td>14</td>
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<tr>
<td>Polysaccharide biosynthesis protein/LPS biosynthesis protein/sugar transferase</td>
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</tr>
<tr>
<td>Predicted protein</td>
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</tr>
<tr>
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<tr>
<td>Fe (II) ABC transporter, permease protein</td>
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<td>Nitrogenase, subunit</td>
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<tr>
<td>Transposase</td>
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<td>Methylamine corrinoid protein</td>
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<tr>
<td>Metallo cofactor biosynthesis protein family</td>
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<td>O-linked GlcNAc transferase</td>
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<tr>
<td>Response regulator</td>
<td>11</td>
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<tr>
<td>Sulfate/molybdate/phosphate ABC transporter, permease protein</td>
<td>11</td>
</tr>
<tr>
<td>Transcription repressor; MarR family protein</td>
<td>10</td>
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the corrinoid proteins are known to be involved in the utilization of either methanol or methylated amines (see Fig. 4A). The remaining homologs have not been attributed to known substrates. Some of these are likely to be involved in the use of methyl-sulfides, substrates known to be used by *M. acetivorans*. However, no close homologs of the known *M. barkeri* methyl-sulfide methyltransferase genes (Paul and Krzycki 1996) were identified in *M. acetivorans*. It is also possible that additional MT1 homologs exist but were not identified, as these proteins do not constitute a homologous family that would allow identification by sequence comparison. Therefore, 12 unaccounted MT2 homologs and five unaccounted corrinoid proteins appear to be encoded by *M. acetivorans* genome (Fig. 4A), suggesting that additional one-carbon compounds may act as energy-yielding substrates for this organism.

Central Metabolism

**Oxygen Metabolism**

Methanogenesis is a form of anaerobic respiration using compounds such as CO\(_2\) or methanol as terminal electron acceptors (Fig. 1). This is analogous to the use of oxygen as the terminal electron acceptor by mitochondria and many bacteria, or the use of nitrate, sulfate, or iron by various microbes. Despite decades of search, no methanogen has ever been observed to utilize nonmethanogenic electron acceptors to support growth. Moreover, methanogens are exquisitely sensitive to oxygen under laboratory conditions and are rapidly killed by even very low concentrations of O\(_2\).

It thus is interesting that a putative operon was identified, designated cydAB, encoding an *M. acetivorans* homolog of *Escherichia coli* cytochrome d oxidase. Cytochrome d oxidase is a key step in O\(_2\)-dependent respiration in *E. coli* under low O\(_2\) conditions (Cotter et al. 1997). It transfers electrons from quinones to O\(_2\). In *E. coli*, the reaction contributes to the production of a membrane electrochemical gradient that can be used for ATP synthesis.

The primary role of cytochrome d oxidase in *M. acetivorans* is unclear. Cytochrome d oxidase has been suggested to play a role in protection from oxygen toxicity in numerous organisms, presumably by consuming O\(_2\) and lowering its concentration (Cotter et al. 1997; Duwat et al. 2001; Endley et al. 2001). Such a protective role thus is possible in *M. acetivorans*. In addition to cydAB, two superoxide dismutases, one superoxide reductase, and one catalase were identified. Each of these proteins normally...
functions in protection from reactive oxygen species. Alternatively, cytochrome d oxidase may play a role in energy conservation. In particular, it has been shown that *Lactococcus lactis*, often considered a strict anaerobe, is capable of O₂-dependent respiration using a cytochrome d oxidase homolog (Duwat et al. 2001). The finding of cytochrome d oxidase encoding genes thus suggests the possibility that *M. acetivorans* may be capable of O₂-dependent respiration under appropriate circumstances. This possibility will require careful study to evaluate. In this regard, it is important to note that *Methanosarcina* species are not known to contain quinones, although genes homologous to those known to be involved in ubiquinone and menaquinone biosynthesis were identified.

**Nitrogen Fixation**

Methanogens are the only archaea known to fix nitrogen. Nitrogen fixation is mediated by a nitrogenase complex consisting of two metalloproteins, a dinitrogenase, and a dinitrogenase reductase. Three types of nitrogenases are known: a molybdenum- and iron-containing nitrogenase, a vanadium- and iron-containing nitrogenase, and an iron-only-containing nitrogenase. The latter two nitrogenases are termed alternative nitrogenases and have been hypothesized to serve as mechanisms of nitrogen fixation in molybdenum-limiting environments.

Unique among the archaea, *M. acetivorans* appears to possess all three types of nitrogenases. The molybdenum nitrogenase gene cluster is most similar to that of the gram-positive bacterium *Clostridium pasteurianum*, whereas the vanadium alternative nitrogenase cluster is similar to that of the cyanobacterium *Anabaena variabilis* (Thiel 1993). The iron-only nitrogenase cluster lacks a homolog encoding the dinitrogenase reductase; however, a gene just upstream encodes a protein with 91% identity to the dinitrogenase reductase of the vanadium nitrogenase cluster and may function as the dinitrogenase reductase for the iron-only complex. No archaeon outside the genus *Methanosarcina* is known to contain more than one nitrogenase system. Some bacteria are known to possess three sets of nitrogenase genes, including *Azotobacter vinelandii* (Bishop and Premakumar 1992), an organism considered highly adapted to nitrogen fixation. The presence of all three nitrogenases in *M. acetivorans* suggests that the importance of nitrogen fixation to *M. acetivorans* and again underscores the adaptability of this organism.

**Information Processing**

**Transcription and Gene Regulation**

Transcriptional regulation within archaea in general, and within the methanogens in particular, is not fully understood. The minimal transcriptional apparatus for most archaeal promoters consists of a TATA-box-binding protein (TBP) and an archaeal transcription factor B (TFB), complexed with an archaeal RNA polymerase (RNAP; Bell and Jackson 2001). Interestingly, *M. acetivorans* appears to code for three substantially different TBPs, in addition to a single TFB. Although multiple TBP and TFB have been described in several archaea (Kawarabayasi et al. 1998; Thompson et al. 1999; Ng et al. 2000), *M. acetivorans* (together with *M. barkeri*; E.Conway de Macario, unpubl.) is the only known methanogen with multiple identified TBPs. The presence of multiple TBPs raises the possibility of differential gene expression through the formation of alternative TFB–TFB–RNA polymerase complexes as previously suggested for *Halobacterium* species (Baliga et al. 2000). However, all other archaea with multiple TBPs also contain multiple TFBs. The presence of only one TFB in *M. acetivorans*, along with available genetic methods, offers the capability to investigate the role of multiple TBPs independently of the potential role of multiple TFBs.

Several additional transcription factors were identified, including a single archaeal transcription factor E (TFE), a single archaeal transcription factor S (TFS), and numerous bacterial-like regulators. Comparisons between these archaeal and bacterial regulators indicate a strikingly different profile for bacterial-like regulators in *M. acetivorans*. For example, *M. acetivorans* possesses a family of 15 tetR regulator proteins and a family of 11 marR repressor proteins, whereas no other sequenced archaeon possesses more than 3 members of either family, and *A. fulgidus* contains only one of each. In contrast, only a single *Lip* regulatory protein was identified in *M. acetivorans*, whereas 14 were identified in *A. fulgidus*.

**Translation**

*M. acetivorans* was found to contain both a class I and a class II lysyl-tRNA synthetase. The class I lysyl-tRNA synthetase is found in many archaea and some bacteria, and the class II synthetase is found in most bacteria and all eukarya (Soll et al. 2000). *M. acetivorans* (together with *M. barkeri*; G. Sririvasa, G. Paul, and J. Krzycki, unpubl.) represents the first instance of any organism in any domain possessing both classes.

We speculate that the possession of both classes of lysyl-tRNA synthetase may relate to the presence of read-through amber codons (UAG codons not read as a translation stop). It has been noted in methanogens that all known methylamine monooxygenase genes contain a single conserved in-frame amber codon read-through amber codon (Paul et al. 2000), and studies have shown that this codon corresponds to a lysine residue in the product of one of these genes (James et al. 2001). Recent studies have also identified a putative amber decoding tRNA (J. Krzycki, unpubl.). This raises the possibility of conserved amber read-through codons in other gene families. In fact, a preliminary analysis identified a family of four putative transposase genes each containing an in-frame amber codon at an identical position. In addition, a methylcobamide-CoM methylase of unknown function was also identified with an amber codon. Furthermore, use of UAG as a stop codon appears depressed in *M. acetivorans* as compared with related species, supporting the hypothesis of an alternative role for this codon. For example, only 5% of coding regions in *M. acetivorans* end with UAG in comparison to 19% in *A. fulgidus*. Further analysis is required to determine the extent of the read-through-amber-codon phenomenon.

**Multicellular Structures**

*Methanosarcinae* are the only archaea known to undergo major morphological changes involving the formation of distinct multicellular structures (Fig. 2; Macario and Conway de Macario 2001). Thus, *M. acetivorans* provides a model for dissecting the mechanisms regulating multicellularity in this domain of life. *M. acetivorans* synthesizes a cell envelope, termed an S-layer, which consists of protein subunits adjacent to the cell membrane (Sowers et al. 1984a; Kandler and Konig 1993). Under certain conditions, the organism also grows as multiple cells, each with an S-layer, contained within a common sacculus-like structure. Furthermore, *M. acetivorans* is capable of growing as multicellular bodies embedded within a complex extracellular matrix. The importance of the formation of mul-
ticellular structures is underscored by the number and diversity of proteins potentially associated with this process.

**Polysaccharide Biosynthesis**
Over 30 predicted gene products were identified having sequence similarity to polysaccharide biosynthetic enzymes. Many occurred in clusters of adjacent genes. One cluster, characteristic of a typical bacterial capsular biosynthetic operon, includes a dolichyl-phosphate glycosyl transferase, several additional glycosyltransferases, and a gene predicted to belong to a family of polysaccharide extrusion systems. Several other genes have high sequence identity to other hypothetical biosynthetic genes in both S-layer-producing species and pseudo-murein-producing species. This suggests that these genes code for synthesis of extracellular polysaccharides such as lipopolysaccharides, S-layer glycoproteins, and methanochondroitin because pathways for the biosynthesis of all three involve sequential addition of UDP-PP-monomosaccharides to a glycan chain (Hartmann and Konig 1989; Kandler and Konig 1998).

**Surface Proteins**
*M. acetivorans* contains a large number of surface proteins with a diverse array of domains and architectures (Fig. 5). For instance, the second largest multigene family (Table 2) consists of 62 proteins, the majority of which lack transmembrane domains and are homologous to surface antigens in *M. mazie* and thus are likely components of the S-layer (Mayerhofer et al. 1995). In addition, many genes in this family are large multidomain proteins containing domains homologous to metazoan cell adhesion surface receptor protein domains, including polycystic kidney disease (PKD) domains (or C repeats in *M. mazie* [Mayerhofer et al. 1995]) and YVTN β-propeller domains (or AB repeats in *M. mazie* [Mayerhofer et al. 1995]; Fig 5; H. Jing, in prep.). This suggests an additional regulatory or structural role in the formation of *M. acetivorans* multicellular structures. Consistent with the unique ability to form multicellular structures, the fraction of total proteins that are S-layer proteins and the fraction of proteins that contain PKD domains or YVTN β-propeller domains are strikingly larger in *M. acetivorans* than in other sequenced archaea. For example, *A. fulgidus* possesses only two proteins containing PKD domains. Furthermore, the number and diversity of surface proteins provides a mechanism for generating variations in surface structures, consistent with the antigenic variation observed in *M. mazie* (Yao et al. 1992).

**Environmental Sensing and Interaction**

**Signal Transduction**
The genome of *M. acetivorans* indicates a wide repertoire of behavioral responses to external stimuli via two-component regulatory systems. The basic two-component system, as characterized in bacteria, consists of a sensory transduction histidine kinase and a cognate response regulator protein. Typically these two components are paired in a one-to-one ratio. Activation of the sensory transduction histidine kinase causes autophosphorylation and subsequent phosphoryl transfer to the receiver domain of the response regulator. Most bacterial response regulators include an additional effector domain responsible for the final cellular output.

In contrast, the genome of *M. acetivorans* indicates a different approach to two-component signal transduction. The sensory transduction histidine kinases comprise the third largest multigene family in *M. acetivorans* with 30 representatives (not including 2 cheA genes involved in chemotaxis signaling). The majority of these contain one or more PAS domains (Ponting and Aravind 1997; Pellequer et al. 1998), predicted to be small-molecule-binding domains, although the exact nature of the small molecules they may bind cannot be determined by similarity alone. In contrast, the genome contains only 18 response regulator receiver domains (excluding 2 cheY and 2 cheB genes). Seven of these occurrences are as domains within histidine kinases themselves. Of the remaining 11 occurrences, 10 are in small single-domain proteins con-
taining only the response regulator receiver domain itself, whereas only a single protein is of sufficient length to contain a bacterial-like effector domain.

The differences in the nature of two-component signaling in *M. acetivorans*, relative to bacteria, led us to examine the genomes of other archaea. A departure from the one-to-one relationship between kinases and regulators has been observed in other archaea, and this appears to be the case for all archaea with annotated two-component systems. In addition, most annotated response regulators in archaea appear to be single-domain proteins. These observations imply a different mode of action for two-component signal-transduction systems in archaea in comparison to well-characterized bacterial species such as *E. coli*. This implication, to our knowledge, has not been previously noted. One possibility is a very high degree of signal integration at the response regulator level with, on average, five histidine kinases per autonomous receiver domain. Alternatively, the kinases may phosphorylate other substrates in addition to recognizable receiver domains. In addition, receiver-domain peptides may interact with as yet uncharacterized effector domains on separate peptides.

**Flagella and Motility**

Although motility has not been observed in any Methanosarcinaceae, genes encoding products for both flagella and chemotaxis are present. *M. acetivorans* contains a single flagellin cluster organized in a manner similar to that in *A. fulgidus*. This cluster includes two flagellin genes followed by three genes with conserved sequences and appearing in the same order as in *A. fulgidus*, followed by flagella accessory genes H, I, and J. The flagellin genes appear more similar to those of *A. fulgidus* than to those of methanococci. From microspacing it appears that all of these genes could be cotranscribed, in keeping with observations in methanococci (Kalmokoff and Jarrell 1991), extreme halophiles (Patenge et al. 2001), and Pyrococcus (Nagahisa et al. 1999).

*M. acetivorans* also possesses two very similar clusters of chemotaxis genes, che-1 and che-2, both of which comprise an apparently complete chemotactic system. The che-1 cluster lies adjacent to the flagellin cluster and consists of homologs of *cheD*, *cheC*, *cheA*, *cheB*, *cheY*, and *cheW*, and a methyl-accepting chemotaxis protein. The che-2 cluster contains homologs of *cheD*, *cheC*, *cheA*, *cheB*, *cheY*, and *cheW*, and contains a methyl-accepting chemotaxis protein, and *cheW*. This cluster also contains a single-domain response regulator.

Despite the presence of flagellin and chemotaxis genes, we were unable to detect processing of preflagellin in a preflagellin peptidase assay using *Methanococcus voltae* preflagellin and membranes obtained from *M. acetivorans* growing as single cells (Correia and Jarrell 2000). Thus, it seems likely that flagella and chemotaxis genes are expressed in specific environmental conditions not previously created in culture (as has been seen for other organisms [Faguy et al. 1993; Mukhopadhyay et al. 2000]), possibly in conjunction with changes in morphology.

**DISCUSSION**

Methanosarcinaceae are unsurpassed among methanogens in terms of metabolic, physiological, and environmental versatility and are unique among archaea in forming complex multicellular structures. These capabilities are reflected in the genome of *M. acetivorans*, the largest known for an archaeon. The genome reveals extensive genetic diversity and redundancy underlying the ability of *M. acetivorans* to adapt to various environmental conditions. In addition, the genome sequence indicates the potential for entirely unexpected metabolic capabilities. These include the possibilities of methanogenesis from as yet undiscovered substrates, non-methanogenic growth-utilizing CO, and perhaps even O2-dependent respiration.

The powerful genetic methods available for *M. acetivorans* allow the immediate exploitation of the complete genome sequence for understanding the organism’s biochemical and environmental diversity and the regulatory mechanisms that underlie its adaptive abilities. In addition, these methods permit functional studies of the over 1300 predicted proteins lacking homology to known proteins. Moreover, the existence of ongoing sequencing efforts for *M. mazei* (Göttingen Genomics Laboratory at http://www.g2l.bio.uni-goettingen.de/), *Methanoscarsina thermophila*, and *Methanosarcina barkeri* (DOE Joint Genome Institute [JGI] at http://www.jgi.doe.gov/JGI_genomic.html/) promises unparalleled opportunity for comparative studies. Such studies offer the potential for elucidating the evolutionary basis of the extraordinary genetic diversity of the Methanosarcinaceae, leading in turn to a greater understanding of the archaeal domain of life.

**METHODS**

**Growth Conditions**

*Methanosarcina* strains were grown in single-cell morphology (Sowers et al. 1993) at 35°C in HS broth medium containing 125 mM methanol plus 40 mM sodium acetate (HS-MA medium) (Metcalf et al. 1996).

**Sequencing, Assembly, and Finishing**

The genome was sequenced by the whole-genome shotgun method. Genomic DNA was isolated from *M. acetivorans* as described by Boccazzi et al. (2000). m13 (1.5-kb inserts) and plasmid (4-kb inserts) libraries were generated as described at http://www-genome.wi.mit.edu/. Methanosarcina Fosmid (40-kb inserts) library construction is described elsewhere (Zhang et al. 2002).

Sequencing methods are described at http://www-genome.wi.mit.edu/. Plasmid and Fosmid inserts were sequenced from both ends to generate paired-reads. We generated sequence coverage of 7× from plasmids, 1× from M13 and 0.076× from Fosmids and assembled it with Phrap. Initial analysis of the assembly was done with the Mapper software (M.C. Zody, pers. comm.) to select gap-spanning clones for finishing. Two hundred gaps spanned by plasmid clones were closed by transposon-based sequencing using the EZ:TN (KAN-2) kit from Epicentre. Forty-eight gaps spanned only by Fosmids were closed by sequencing Fosmid-derived PCR products. Sequence from 28 unspanned gaps was obtained from fragments generated by combinatorial PCR using genomic DNA as template and pooled primers (Tettelin et al. 1999). One unspanned gap was closed by sequencing a small-insert library (McMurray et al. 1998) produced from an 8.5-kb PCR product. Regions of low sequence quality were resolved by (1) use of ABI dGTP Big Dye Terminator sequencing mix, (2) transposon-primed sequencing of plasmid clones, or (3) sequencing PCR products obtained from plasmid genomic template. Paired-reads within the assembly were visualized with the Mapper software and used in assembly validation. Regions of the assembly spanned by paired-reads occurring with the appropriate orientation and spacing were considered valid. Validation of 99.99% of the genome was performed in this manner, whereas only 6270 bases of the finished assembly were spanned solely by sequenced PCR products. Regions
of the assembly containing nonsensical paired-reads were analyzed further. Eighteen of these regions proved to have been misassembled by Phrap and were resolved manually. The finished genome sequence was manually inspected for quality and edited using the Staden package viewer Gap4. During annotation 480 possible sequence errors (based on breaks in ORFs) were identified. These were manually reviewed and one was shown to be an editing error and was corrected. Five of the possible errors were not unambiguous by quality but after resequencing were shown to have been correct.

Annotation and Analysis

The _M. acetivorans_ genome was annotated using the Calhoun annotation system (J. Galagan, in prep.). ORFs likely to encode proteins were predicted using GLIMMER (Delcher et al. 1999), and all ORFs were searched against two sets of protein family Hidden Markov Models (HMM), Pfam (Bateman et al. 2000) and TIGRFAM (Haft et al. 2001), using the HMNER program (Durbin et al. 1998). The entire genome was also searched against the public protein databases using BLASTX with threshold E < 1e-5. ORFs identified by GLIMMER were refined in length and all ORFs with similarity to a protein family HMM or known proteins were annotated as genes. Genes were assigned identities by a team of seven annotators and three annotation reviewers. For genes with similarities to known proteins, the literature was consulted to identify those proteins experimentally characterized, and this information was captured by the Calhoun system. To supplement and verify the Calhoun automated annotation process, the genome sequence was applied to the annotation pipeline provided with the Comprehensive Microbial Resource (Peterson et al. 2001). Comparisons of results from both systems displayed no substantial inconsistencies.

Multigene families were constructed by searching each annotated gene against all other genes using BLASTP, requiring matches with _E_ ≤ 1e-5 over 60% of the longer gene length, and subsequently clustering genes with matches. An identical analysis was performed on 14 other archaeal and bacterial species. Corresponding families in each of these species were based on using best bidirectional BLASTP hits (E ≤ 1e-5, score > 10, hit length ≥ 60%) between family members. Transfer RNAs were identified using the tRNAscan-SE program (version X). The origin of replication was identified as the point of maximum cumulative AT skew (defined as the cumulative sum of A—T/A+T on one strand).

Additional annotation and extensive genome analysis were performed as part of the _Methanosarcina acetivorans_ Community Annotation Project. Over 30 members of the scientific community participated in the project. Gene annotations were submitted via a web interface and detailed analyses of the genome annotation performed. The project culminated in a two-day Genome Analysis Meeting at the Whitehead Institute Center for Genome Research.

Additional details, analysis results, and the genome sequence are available at http://www-genome.wi.mit.edu/ and at http://www.tigr.org/CMR.

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**M. acetivorans Genome Sequence**

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