Genome of Methanoregula boonei 6A8 reveals adaptations to oligotrophic peatland environments

Suzanna Bräuer,1 Hinsby Cadillo-Quiroz,2 Nikos Kyrpides,3 Tanja Woyke,3 Lynne Goodwin,4 Chris Detter,4 Sheila Podell,5 Joseph B. Yavitt,6 and Stephen H. Zinder7

Correspondence
Suzanna Bräuer
brauersl@appstate.edu

1Department of Biology, Appalachian State University, Boone, NC 28608, USA
2Swette Center for Environmental Biotechnology at the Biodesign Institute, Arizona State University, Tempe, AZ 85287-4501, USA
3Department of Energy, Joint Genome Institute, Walnut Creek, CA 94598, USA
4Los Alamos National Laboratory, Los Alamos, NM 87545, USA
5Scripps Institution of Oceanography, La Jolla, CA 92039, USA
6Department of Natural Resources, Cornell University, Ithaca, NY 14853, USA
7Department of Microbiology, Cornell University, Ithaca, NY 14853, USA

Analysis of the genome sequence of Methanoregula boonei strain 6A8, an acidophilic methanogen isolated from an ombrotrophic (rain-fed) peat bog, has revealed unique features that likely allow it to survive in acidic, nutrient-poor conditions. First, M. boonei is predicted to generate ATP using protons that are abundant in peat, rather than sodium ions that are scarce, and the sequence of a membrane-bound methyltransferase, believed to pump Na+ in all methanogens, shows differences in key amino acid residues. Further, perhaps reflecting the hypokalemic status of many peat bogs, M. boonei demonstrates redundancy in the predicted potassium uptake genes trk, kdp and kup, some of which may have been horizontally transferred to methanogens from bacteria, possibly Geobacter spp. Overall, the putative functions of the potassium uptake, ATPase and methyltransferase genes may, at least in part, explain the cosmopolitan success of group E1/E2 and related methanogenic archaea in acidic peat bogs.

INTRODUCTION

Methanoregula boonei is an acidophilic methanogen isolated from an ombrotrophic peat bog (McLean Bog) in New York State, USA (Bräuer et al., 2006a). A member of the Euryarchaeal order Methanomicrobiales, this archaeon demonstrates physiological evidence of adaptation to nutrient-poor low ionic strength environments, such as ability to grow at 0.4 mM Na+ and sensitivity to > 50 mM sodium (Bräuer et al., 2011) in contrast to methanogens described elsewhere (Jarrell & Kalmokoff, 1988). As M. boonei has been described previously (Bräuer et al., 2011), this paper will focus on a summary of genomic evidence revealing the presence of putative genes specific for proton-rich and sodium- and potassium-poor environments.

M. boonei is the type strain (DSMZ = 21154T, JCM = 14090T) within the type genus of the family Methanoregulaceae (Sakai et al., 2012). Cultures are dimorphic, containing thin rods (0.2–0.3 μm in diameter and 0.8–3.0 μm long) and irregular cocci (0.2–0.8 μm in diameter). In PM1 medium, M. boonei appears to be an obligate hydrogenotroph and is unable to utilize formate, acetate, methanol, ethanol, 2-propanol, butanol or trimethylamine (Bräuer et al., 2011). Optimal growth conditions are near 35–37 °C and pH 5.1, with growth occurring at pH values as low as 3.8.

METHODS

Preparation of DNA and genome sequencing. M. boonei was cultured as described previously (Bräuer et al., 2006b). An exponentially growing culture (1L) was harvested by cold centrifugation and DNA was extracted using a GNOME DNA isolation kit (MP Biomedicals), following the manufacturer’s protocols except that a final concentration of 0.1% SDS was added in addition to the cell lysis/denaturating solution to increase cell lysis. Genomic DNA was then evaluated for quality and concentration prior to sequencing.
The genome of *M. boonei* 6A8 was sequenced at the Joint Genome Institute (JGI) using a combination of 3, 8 and 40 kb (fosmid) DNA libraries. All general aspects of library construction and sequencing performed at the JGI can be found at http://jgi.doe.gov/. Draft assemblies were based on 37,430 total reads. All three libraries provided 13 × coverage of the genome. The Phred/Phrap/Consed software package (www.phrap.com) was used for sequence assembly and quality assessment (Ewing & Green, 1998; Ewing et al., 1998; Gordon et al., 1998). After the shotgun stage, reads were assembled with parallel Phrap (High Performance Software). Possible misassemblies were corrected with Dupfinisher (Han & Chain, 2006). Gaps between contigs were closed by editing in Consed or custom primer walk. A total of 921 additional reactions were necessary to close gaps and to raise the quality of the finished sequence. The completed genome sequences of *M. boonei* 6A8 contains 37,526 reads, achieving a mean of 13-fold sequence coverage per base with an error rate of less than 1 in 100,000.

Additional gene functional annotation and comparative analyses were performed within the Integrated Microbial Genomes (IMG/ER) platform (Markowitz et al., 2006). Alignments of functional genes were conducted in BioEdit using CLUSTAL W (Larkin et al., 2007). Phylogenetic trees were reconstructed using the PHYLIP software.
package (Felsenstein, 2004) by conducting both neighbour-joining and maximum-likelihood analysis.

**Nucleotide accession number.** The complete genome sequence of *M. boonei* strain 6A8 is available in the National Center for Biotechnology Information database (Wheeler et al., 2007) under GenBank/EMBL/DDBJ accession number NC_009712. Additionally, the genome is available in the IMG system (Markowitz et al., 2006) and the JGI genome portal (Grigoriev et al., 2012).

### RESULTS AND DISCUSSION

#### Genome sequencing and annotation information

*M. boonei* was selected for sequencing due to its potential energy production (methane), biogeochemical importance in global carbon cycling and occurrence in habitats that are unique for cultured methanogens, i.e. proton rich and nutrient element poor. Sequencing, assembly and annotation were conducted by the Department of Energy JGI. A summary of the genome sequencing information can be found in Table 1.

#### Genome properties

The genome consists of one single circular chromosome of approximately 2.5 million bp with 2518 genes identified, including one rRNA gene operon (Table 1, Fig. 1). Highlighting our dearth of knowledge of methanogenic archaea, only 36% of the genes were associated with one of the well-defined cluster of orthologous groups (COG) categories (Tatusov et al., 2000), with the remaining 64% either not associated with a COG (39%), or associated only by general (14%) or unknown (12%) function (Table 2). The majority of the COG genes in *M. boonei* were predicted to be involved in energy production and conversion (9%), translation (8%), and transport and metabolism of amino-acids (8%), coenzymes (6%) and ions (6%).

#### Adaptation to high proton concentrations

In McLean Bog, the pH is near 4 and the H\(^+\) concentration is approximately 10\(^{-4}\) M (100 \(\mu\)M), three orders of magnitude greater than at pH 7. Moreover, sphagnum moss impedes the flow of mineral-rich groundwater into the bog so that the only water source is rain, essentially distilled water. Consequently, Na\(^+\) concentrations are typically low. For example, Na\(^+\) concentrations were measured as only 2 \(\mu\)M in the McLean Bog porewater (Bräuer et al., 2004). These extremely low external Na\(^+\) concentrations make developing a sodium motive force challenging; however, sodium motive force is considered essential to energy conservation by methanogens (Schlegel & Müller, 2013).

All cultured members of the groups *Methanobacteriales/Methanococcales* lack cytochromes, and the only known energy-conserving step is Na\(^+\) pumping coupled to methyl group transfer by the membrane-bound enzyme complex methyltetrahydromethanopterin : coenzyme M methyltransferase (Mtr) (Schlegel & Müller, 2013; Thauer et al., 2008). Further, the A\(_1\)A\(_0\) ATPase/synthases studied among the members of the groups *Methanobacteriales/Methanococcales* have clearly been shown to pump Na\(^+\) (McMillan et al., 2011; Mulkidjanian et al., 2008). In contrast, the cytochrome-containing methanogens in the *Methanosarcinales* have Mtr complexes, but also have steps that pump protons (Schlegel & Müller, 2013; Thauer et al., 2008), and evidence has favoured H\(^+\) pumping by the ATPases in these organisms (Müller et al., 1999; Pisa et al., 2007). More recently, it was demonstrated that the A\(_1\)A\(_0\) archaeal ATPase/synthase in *Methanosarcina acetivorans* is ‘promiscuous’, pumping either Na\(^+\) or H\(^+\) (Schlegel et al., 2012) with both ions possible at neutral pH, especially at seawater salinity of 0.4 M Na\(^+\), whereas only protons were pumped at pH 5. *M. boonei* belongs to the *Methanomicrobiales*, which lack cytochromes like the *Methanobacteriales/Methanococcales* cluster, but are more closely related to the *Methanosarcinales*, and it is not clear which patterns of bioenergetics this group follows.

It is the ATP/K subunit of the membrane bound A\(_0\) subunit of the A\(_1\)A\(_0\) ATPase/synthase complex that is responsible for

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**Table 2. Genes associated with COG functional categories**

<table>
<thead>
<tr>
<th>COG category</th>
<th>No. of genes</th>
<th>% of total*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy production and conversion</td>
<td>157</td>
<td>9.35</td>
</tr>
<tr>
<td>Translation, ribosomal structure and biogenesis</td>
<td>134</td>
<td>7.98</td>
</tr>
<tr>
<td>Amino acid transport and metabolism</td>
<td>133</td>
<td>7.92</td>
</tr>
<tr>
<td>Coenzyme transport and metabolism</td>
<td>108</td>
<td>6.43</td>
</tr>
<tr>
<td>Inorganic ion transport and metabolism</td>
<td>96</td>
<td>5.71</td>
</tr>
<tr>
<td>Signal transduction mechanisms</td>
<td>95</td>
<td>5.65</td>
</tr>
<tr>
<td>Transcription</td>
<td>83</td>
<td>4.94</td>
</tr>
<tr>
<td>Post-translational modification, protein turnover, chaperones</td>
<td>69</td>
<td>4.11</td>
</tr>
<tr>
<td>Replication, recombination and repair</td>
<td>69</td>
<td>4.11</td>
</tr>
<tr>
<td>Carbohydrate transport and metabolism</td>
<td>61</td>
<td>3.63</td>
</tr>
<tr>
<td>Cell wall/membrane/envelope biogenesis</td>
<td>55</td>
<td>3.27</td>
</tr>
<tr>
<td>Nucleotide transport and metabolism</td>
<td>53</td>
<td>3.15</td>
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<tr>
<td>Cell motility</td>
<td>28</td>
<td>1.67</td>
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<tr>
<td>Defence mechanisms</td>
<td>27</td>
<td>1.61</td>
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<tr>
<td>Lipid transport and metabolism</td>
<td>24</td>
<td>1.43</td>
</tr>
<tr>
<td>Intracellular trafficking, secretion and vesicular transport</td>
<td>22</td>
<td>1.31</td>
</tr>
<tr>
<td>Cell cycle control, cell division, chromosome partitioning</td>
<td>18</td>
<td>1.07</td>
</tr>
<tr>
<td>Secondary metabolites biosynthesis, transport and catabolism</td>
<td>15</td>
<td>0.89</td>
</tr>
<tr>
<td>Chromatin structure and dynamics</td>
<td>2</td>
<td>0.12</td>
</tr>
<tr>
<td>General function prediction only</td>
<td>227</td>
<td>13.51</td>
</tr>
<tr>
<td>Function unknown</td>
<td>204</td>
<td>12.14</td>
</tr>
<tr>
<td>Not in a COG category</td>
<td>971</td>
<td>38.56</td>
</tr>
</tbody>
</table>

*The total is based on the total number of protein encoding genes in the annotated genome.*
pumping either H\(^+\) or Na\(^+\), with the ion typically binding to a conserved aspartate or a glutamate located within a transmembrane helix. In Table 3, partial sequences of the AtpC/K are aligned, and residues critical to Na\(^+\) binding in the Methanobacteriales/Methanococcales (Grüber et al., 2014; McMillan et al., 2011) are indicated in bold. Also shown in bold are residues in the \textit{M. acetivorans} sequence that are considered crucial to being able to bind either Na\(^+\) or H\(^+\). The \textit{M. boonei} sequence shares these residues as well as many others with \textit{M. acetivorans}, and it is likely that its ATPase pumps protons near pH 4.

Another question of interest is whether the Mtr complex in \textit{M. boonei} pumps Na\(^+\). In \textit{Methanosarcina mazei} and \textit{Methanothermobacter marburgensis}, Na\(^+\) pumping was demonstrated for the Mtr complex, thereby leading to a sodium motive force. From these results, it was extrapolated that all Mtr complexes pump Na\(^+\) (Schlegel & Müller, 2013). The pumping is attributed to the membrane-bound MtrE subunit, and a specific aspartate (indicated by the bold D) predicted to be within a transmembrane helix, part of the motif 168-1WGGTAAGGG167D168 that is conserved between the two organisms (Gottschalk & Thauer, 2001). \textit{M. boonei} and other members of the \textit{Methanomicrobiales} have an asparagine instead of aspartate at that position (position 190 in Fig. S1, available in the online Supplementary Material) in their MtrE sequences, which renders that residue unable to pump cations. There is a glutamate at position 253 in Fig. S1 within a region (predicted to be a transmembrane alpha helix by the IMG website) that is conserved amongst \textit{Methanomicrobiales} as well as some other methanogens, but is not present in the \textit{M. marburgensis} or \textit{Methanosarcina Barkeri} sequences. This residue may play a role in pumping but, as of now, it is

Table 3. Amino acid alignment of AtpCK demonstrating the two conserved glutamine (Q) and tyrosine (Y) residues (shown in bold) (identified by: McMillan et al., 2011; Mulkidjian et al., 2008; Sakai et al., 2011) that appear to be unique for methanogens predicted to have sodium-driven ATPases versus those predicted to have proton-driven or sodium-proton driven ATPases

\begin{center}
\begin{tabular}{|l|l|}
\hline
Organism & Partial amino acid alignment \\
\hline
\textit{Methanosarcina mazei} strain G01 & KAVGAGLVG KALGVSGLQG VMIAGAAAGVA VAENKDMFGL ALFLTVIPET IVFLGVLLL VY \\
\textit{Methanospirillum hungatii} JF1 & VPAGAAFAV GAGAGAIGAQS KIAGAGAG KVRPSAGT VJVLLEIPET L VLGFVLLL MI \\
\textit{Methanosaeta thermophila} & KALGAGAAI VTLGASLQIA KDGTGAAIGA MAENQFGK GLILTVIPET IVFLGVLLL VL \\
\textit{Methanobrevibacter ruminantium} & KAIGAGALAT VTLASLQAE KIAGTAAGA MAENQFGLK GLILTVIPET IVFLGVLLL LL \\
\textit{Methanobrevibacter ruminantium} C2A & IAGASIAA TLGASAIAE KIAGTAAGA MAENQFGLK GLILTVIPET IVFLGVLLL LI \\
\textit{Methanosarcina aberdeenensis} & SAVAGAAGL TLIQGAGLQ AAGAAATGAM QAENKDMFGL ALFLTVIPET IVFLGVLLL VL \\
\textit{Methanobrevibacter \textit{M. boonei}} & KAIGGGLAGL TLAGAGLGQ AAAGAAATGAM QAENKDMFGL ALFLTVIPET IVFLGVLLL VL \\
\textit{Methanobrevibacter \textit{M. boonei}} & IAGGAGAAGL TAQGAGLGQ AAAGAAATGAM QAENKDMFGL ALFLTVIPET IVFLGVLLL VL \\
\textit{Methanobrevibacter \textit{M. boonei}} & IAGGAGAAGL TAQGAGLGQ AAAGAAATGAM QAENKDMFGL ALFLTVIPET IVFLGVLLL VL \\
\textit{Methanobrevibacter \textit{M. boonei}} & IAGGAGAAGL TAQGAGLGQ AAAGAAATGAM QAENKDMFGL ALFLTVIPET IVFLGVLLL VL \\
\hline
\end{tabular}
\end{center}
unclear whether the Mtr complex in *M. boonei* or other *Methanomicrobiales* pumps Na\(^+\) or H\(^+\), or perhaps is not a pump at all. Because of these fundamental differences in the MtrE sequences between *Methanomicrobiales* and other methanogens, the role of Mtr in their bioenergetics warrants examination, especially since it is considered the only site for energy conservation in these organisms.

### Adaptation to low potassium concentrations

Similar to the case for Na\(^+\), the K\(^+\) concentrations in McLean Bog porewater are extremely low, less than 25 \(\mu\)M (Bräuer et al., 2004), and cells typically accumulate K\(^+\) (Epstein, 2003) as well as expel Na\(^+\). *M. boonei* is predicted to carry genes for three different K\(^+\) uptake mechanisms including the low-affinity *trk* genes that many methanogens carry, in addition to the medium-affinity *kup* genes and the ATP-driven high-affinity *kdp* genes, both of which are more rarely found among methanogenic archaea (Table S1). Only one other methanogen (sequenced to date) carries all three predicted K\(^+\) uptake systems, *Methanosphaerula palustris* E1-9c, and it was also isolated from a peatland ecosystem (Cadillo-Quiroz et al., 2009; Cadillo-Quiroz et al., 2008), a fen in which the pH was neutral but the K\(^+\) porewater concentrations were only 3–8 \(\mu\)M (Dettling et al., 2007).

In *Escherichia coli*, the *kdp* uptake system shows both high specificity and high affinity for potassium, and is required for growth during extreme potassium limitation (Altendorf & Epstein, 1996; Epstein, 2003; Epstein et al., 1990). *E. coli* cultures with a mutation in the *kdp* genes have shown growth deficiencies at K\(^+\) concentrations below 300 \(\mu\)M (Rhoads et al., 1976). Highlighting its importance in *M. boonei*, the *kdp* operon has been duplicated and can be identified in two locations in the genome (Fig. 2).

Compared to *E. coli*, both predicted KdpA proteins in *M. boonei* (Mboo 0443 and 0894) have all four regions (I, 112-NTNWQ-116; II, 230-TNGGG-234; III, 343-SCGAV-347; IV, 468-NNGSA-472; *E. coli* numbering) demonstrated experimentally (Bertrand et al., 2004; Buurman et al., 1995; Dorus et al., 2001; Schrader et al., 2000; van der Laan et al., 2002) and in 3D structural models (Greie, 2011; Hu et al., 2008) to be responsible for K\(^+\) binding. Originally identified by the HGT-detection program, DarkHorse (Podell & Gaasterland, 2007), the predicted KdpA proteins in *M. boonei* and in other methanogens phylogenetically within the Proteobacteria, perhaps most closely resembling those of *Geobacter* spp. (Fig. 3). Since there are apparently three closely related clades of methanogen KdpA protein sequences, it is unclear how many transfer events have occurred. Moreover, the KdpC protein is predicted to be fused to the N-terminal of KdpA (Fig. 2) in both sets of genes, an arrangement shared with *Methanomassiliicoccus luminyensis*, a methanogen isolated from human faeces (Dridi et al., 2012) belonging to a new phylum related to *Thermoplasma* and only able to use H\(_2\) and methanol for methanogenesis. All other methanoarchaea, including the closely related *Methanosphaerula*, have the canonical *kdpABC* gene order. Thus, the arrangement and close phylogenetic relationship of their *kdpCA* genes relative to that of other organisms suggests that a gene transfer event occurred between ancestors of *M. boonei* and *M. luminyensis*.

In *E. coli* and many other bacteria, KdpD is a membrane-bound osmosensitive K\(^+\)-sensing histidine kinase component, and KdpE is the response regulator of a two-component transcriptional regulatory system that induces *kdp* genes when K\(^+\) is low (Nakashima et al., 1992; Poolman & Glaser, 1998) (Fig. 2). A number of methanoarchaea with *kdp* genes possess a *kdpD* gene (Table S1). In *M. boonei*, the *kdpD* gene is predicted to encode a truncated protein lacking the histidine kinase domain (Table S2) and to also lack *kdpE* compared to bacteria. Thus, it is unlikely that KdpD is a transcriptional regulator and it may play some other role in regulating activity of the Kdp or other proteins, since it still maintains membrane-bound sensing domains. Some Bacteria, including *Cyanobacteria* and *Deinococcus radiodurans*,

**Fig. 2.** Diagram of high affinity, ATP-driven potassium uptake (*kdp*) gene arrangement in *Geobacter* spp., which shows the canonical arrangement, compared to the two *kdp* operons [Mboo 0894-6 (top) and Mboo 0443-4 (bottom)] in *M. boonei* 6A8. This figure was modified from an image on the IMG website (https://img.jgi.doe.gov/) (Markowitz et al., 2006). Genes encoding for KdpA, KdpB, KdpC, KdpD or KdpE subunits are indicated by an A, B, C, D or E, respectively. The fused *kdpC/A* gene is indicated by a CA and the predicted pseudogene *kpdD* is indicated by a ‘D’.
also contain a truncated kdpD and lack kdpE (Ballal et al., 2007). Although kdpF is also absent, this gene is non-essential for potassium transport in vivo, according to studies in E. coli (Gaßel et al., 1999).

Similarly, the kup genes may have also been horizontally transferred between Geobacteraceae and Methanosarcina spp. and several members of the Methanomicrobiales (Fig. 4). However, the data are less robust since the Geobacter spp. KupA protein sequences do not cluster with other Proteobacteria, so the direction of transfer is unclear. Still, it’s most likely that the methanoarchaea KupA sequences were derived from Bacteria, according to the phylogenetic clustering (Fig. 4). Essentially all of the methanoarchaea possess low-affinity potassium uptake genes (trk; Table S1); thus, these genes will not be discussed.

Fig. 3. Neighbour-joining dendrogram of KdpA. The tree was rooted using deep-branching members of the Thermoplasmales. Archaeal sequences are indicated with an A. Accession numbers or IMG gene numbers follow the colon. Bootstrap values greater than 60 are shown for nodes that were supported by maximum-likelihood analysis. M. boonei is shown in bold. The scale bar indicates the number of protein changes per site.
CONCLUSION

Organisms related to M. boonei in the E1/E2 cluster, R10, or fen cluster are widespread throughout acidic to moderately acidic peatlands in Germany (Hamberger et al., 2008; Wüst et al., 2009), England (Edwards et al., 1998; Hales et al., 1996), Russia (Kotsyurbenko et al., 2007), Scandinavia (Galand et al., 2005; Høj et al., 2005), the United States (Basiliko et al., 2003; Cadillo-Quiroz et al., 2006; Hawkins et al., 2014) and Canada (Godin et al., 2012; Yavitt et al., 2012).
Further, this group tends to dominate in ombrotrophic bogs and is often outcompeted in minerotrophic fens, where the methanogenic community becomes more diverse (Galand 

et al., 2005; Kotsyurbenko, 2010; Kotsyurbenko et al., 2007). For example, microbial diversity was shown to increase along a gradient from pH 4.2 in an ombrotrophic bog to 5.1 in a mesotrophic fen in Finland (Juottonen et al., 2005). Similarly, a fen in Minnesota was found to have higher diversity than that of a nearby bog (Lin et al., 2012). The genome of M. boonei harbours evidence of adaptation to a proton-rich, sodium-poor and potassium-poor environment, which may, in part, explain the cosmopolitan success of this and related organisms in acidic peat bogs.

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