Biogenesis and Function of *c*-type Cytochromes in the Methanogenic Archaeon, *Methanosarcina acetivorans*

Dinesh Gupta¹, Katie E. Shalvarjian², Dipti D. Nayak^{1*}

Department of Molecular and Cell Biology, University of California, Berkeley, CA, USA¹;

Department of Plant and Microbial Biology, University of California, Berkeley, CA, USA²

Running Head: Assembly and role of *c*-type cytochromes in methanogens

*Address correspondence to:

Dipti D. Nayak (dnayak@berkeley.edu)

Department of Molecular and Cell Biology, 1 Barker Hall #3204, University of California,
Berkeley, CA 94720-3204

Tel: 510-664-5267

Abstract

C-type cytochromes (cyt *c*) are proteins that covalently bind heme and are integral to electron transport chains. A growing body of evidence suggests that cyt *c* play a vital role in both intra- and extra-cellular electron transfer processes in Archaea, especially in members that metabolize methane and other short chain alkanes. Elaborate mechanisms for the biogenesis of cyt *c* are known in Bacteria and Eukarya but this process remains largely uncharacterized in Archaea. Here, we have used the model methanogenic archaeon *Methanosarcina acetivorans* to characterize a distinct form of the system I cyt *c* maturation machinery (referred to as the Ccm machinery henceforth) that is broadly distributed in members of the Archaea. Phenotypic analyses of *M. acetivorans* mutants deficient in essential components of the Ccm machinery reveal that cyt *c* are broadly important for growth and methanogenesis, but the magnitude of their impact can vary substantially depending on the growth substrate. Heterologous expression of a synthetic operon with the Ccm machinery (CcmABCEF) from *M. acetivorans* is both necessary and sufficient for cyt *c* biogenesis in a non-native host (*M. barkeri* Fusaro) that is incapable of cyt *c* biogenesis. Even though components of the Ccm machinery are universally conserved across the Archaea, our evolutionary analyses indicate that different clades of Archaea acquired this pathway through multiple independent horizontal gene transfer events from different groups of Bacteria. Overall, we have demonstrated the convergent evolution of a novel Archaea-specific Ccm machinery for cyt *c* biogenesis and its role in methane metabolism.

Significance Statement

Microorganisms belonging to the domain Archaea play an especially important role in regulating atmospheric methane levels. Specifically, methanogens are the primary source of biogenic methane and anaerobic methanotrophic archaea (ANME) consume a substantial proportion of methane released in marine sediments. Genomic studies have implicated a class of electron-transfer proteins called *c*-type cytochromes as being crucial in mediating archaeal methane metabolism in the environment. However, neither the biogenesis nor the role of *c*-type cytochromes in methane metabolism has ever been investigated. Here, we have used a model methanogen, *Methanosarcina acetivorans*, to characterize a distinct pathway for maturation of *c*-type cytochromes that seems to be uniformly conserved across the Archaea and have also identified substrate-specific functional roles for *c*-type cytochromes during methanogenesis.

Introduction

C-type cytochromes [Cyt(s) *c*] are found across the tree of Life, and are critical to electron transfer processes ranging from aerobic respiration in the mitochondrion of Eukaryotes to intra-/extra-cellular electron transport in Bacteria and Archaea [1–4]. The production of a holo-cyt *c*, that is capable of electron transfer, requires complex post-translational processing to covalently attach iron-protoporphyrin IX (heme *b*) to an unfolded apo-cyt *c* protein [5, 6]. Heme attachment occurs on the extracellular face of the cytoplasmic membrane, and, thus also requires the translocation of the apoprotein and heme across the membrane. The apoprotein is translocated via the Sec pathway [7–9] and a set of proteins collectively known as the cyt *c* maturation system are involved in energy-dependent heme transport, apoprotein handling, and covalent cofactor attachment [5, 6, 10]. Three distinct systems for cyt *c* maturation have been identified thus far, and have been studied extensively in bacterial and eukaryotic model systems [5, 6]. However, despite growing evidence that cyt *c* play a vital role in electron transport processes across members of the Archaea - particularly in microorganisms that metabolize methane and other alkanes- the biogenesis of cyt *c* has never been functionally characterized in an archaeon.

Based on genomic surveys [11–13] of sequenced isolates and metagenomics assembled genomes (MAGs), the vast majority of cyt *c* containing Archaea encode homologs of the System I cyt *c* maturation pathway (Ccm pathway) that is widespread in members of the Bacteria and is also present in the mitochondria of some Plants and Protozoa [5, 14]. The Ccm pathway is best characterized in Gram-negative bacteria, such as *E. coli*, where nine to ten proteins, encoded by *ccmABCDEFGH(I)* and *ccdA* (or *dsbD*), are involved in cyt *c* biogenesis (Supplementary Figure 1) [5, 6]. Typically, the *ccm* genes are encoded in an operon on the chromosome and the *ccmH* open reading frame in *E. coli* is often found as two different genes, annotated as *ccmH* (or *ccl2*)

and *ccmI* (or *cycH*), in bacteria like *Rhodobacter capsulatus* [5]. Genetic and biochemical studies of the Ccm pathway have revealed that the membrane-associated CcmABCD complex translocates heme across the cytoplasmic membrane to form holo-CcmE (Supplementary Figure 1). Heme from holo-CcmE is ultimately transferred to the apo-cyt *c* by the CcmF/H complex (cytochrome synthetase) to form holo-cyt *c*. CcmG and CcdA (or DsbD) reduce the disulfide bond between cysteine residues of heme-binding motifs (CXXCH) in the apo-cyt *c* prior to heme attachment (Supplementary Figure 1)[15–17]. Curiously, most, if not all, archaeal genomes sequenced thus far lack several *ccm* genes (namely *ccmD*, *ccmH*, *ccmI*) that have been shown to be essential for cyt *c* biogenesis in Bacteria like *E. coli*, *Rhodobacter capsulatus*, *Paracoccus denitrificans*, *Bradyrhizobium japonicum*, *Shewanella oneidensis*, and *Desulfovibrio desulfuricans* [10, 18–20]. In bacteria, CcmD facilitates the release of the heme-bound holo-CcmE from the CcmABCD complex [21], while CcmH and CcmI are a part of the protein complex involved in covalent attachment of heme to the apo-cyt *c* (Supplementary Figure 1)[5, 6]. As such, based on evidence from studies with bacteria, the streamlined Ccm machinery found in archaeal genomes, comprised only of CcmABCEFG and CcdA, would be insufficient for the biogenesis of cyt *c*. However, the mature cyt *c* proteins have been identified in many archaeal strains, such as *Haloferax volcanii*, *Ignicoccus hospitalis*, *Pyrobaculum islandicum*, *Ferroplasma acidophilum*, and *Methanosarcina* spp [12, 22–26]. Whether Archaea have replaced CcmD, CcmH, and CcmI with non-orthologous proteins or have reconfigured the Ccm machinery such that *ccmDHI* are no longer essential remains unclear.

In this study, we used the genetically tractable methanogenic archaeon, *Methanosarcina acetivorans*, as a model system to functionally characterize the pathway for biogenesis of cyt *c* in archaea. Most archaea that encode cyt *c* proteins are either recalcitrant to laboratory cultivation

techniques, genetically intractable, or only encode only one cyt *c* that might be essential for growth [11–13, 27]. In contrast, *M. acetivorans* encodes multiple cyt *c* proteins [28], can be easily cultivated in a laboratory [29], and has state-of-the-art genetic tools [30, 31], including inducible gene expression, tests for gene essentiality, and CRISPR-Cas9 based genome editing for complex genetic manipulation [32]. *M. acetivorans* is a methanogen that can only grow by coupling energy conservation to the production of methane gas using acetate or methylated compounds, like methanol, as a growth substrate. At least 5 different cyt *c* proteins are encoded in the *M. acetivorans* genome and these proteins contain between 1 and 7 heme-binding motifs [28]. Recent studies have even proposed distinct roles for some of these cyt *c* proteins in intracellular electron transport during methanogenesis [26, 33–35], extracellular electron transfer (EET) [28] as well as direct interdomain electron transfer (DIET) between *M. acetivorans* and bacterial strains like *Geobacter metallireducens* [36]. Thus, *M. acetivorans* is not only an ideal candidate to dissect the biogenesis of cyt *c* in Archaea but also to functionally characterize the role of cyt *c* in various electron transfer processes that have ramifications on global carbon cycling as well as on the formation of microbial communities in anoxic environments.

Here, we have used a combination of genetic, molecular, and biochemical analyses, to show that *M. acetivorans* and other archaea use a streamlined version of the Ccm machinery that only requires *ccmABCEF* for cyt *c* biogenesis. To this end, we have shown that the *ccmABCEF* from *M. acetivorans* is sufficient to produce holo-cyt *c* in a heterologous host, *Methanosarcina barkeri* Fusaro, that otherwise is incapable of cyt *c* biogenesis. Our physiological analyses also reveal substrate-specific phenotypes for the cyt *c* biogenesis pathway and cyt(s) *c* during growth and methanogenesis in *M. acetivorans*. A closer inspection of the distribution and synteny of the cyt *c* biogenesis genes in methane-metabolizing archaea related to *Methanosarcina* (i.e. belonging

to the Order *Methanosarcinales*) suggests that the cyt *c* biogenesis genes were likely acquired in the last common ancestor of the *Methanosarcinales* and have been lost in many extant clades that also do not encode any cyt *c* genes. Although the streamlined Ccm machinery is conserved across Archaea, our evolutionary analyses suggest that the acquisition of cyt *c* biogenesis in Archaea has occurred through multiple horizontal gene transfer (HGT) events with different members of the Bacteria. Overall, we have used the model methanogenic archaeon, *M. acetivorans*, as a model system to characterize a streamlined form of the Ccm machinery used for the biogenesis of cyt *c* in members of Archaea.

Results

The Ccm machinery is essential for *c*-type cytochrome biogenesis in *M. acetivorans*.

We used a sequence-based approach to identify homologs of eight different genes of the Ccm machinery for cyt *c* maturation at four different chromosomal loci in *M. acetivorans*. The *ccmABC* genes (MA1428-MA1430) are located at one chromosomal locus and the *ccmE* gene (MA4149) is in a putative operon with a geranyl farnesyl diphosphate synthase at another locus (Supplementary Figure 1). Two *ccmF* genes (MA3305 and MA3304 that have been renamed *ccmF1* and *ccmF2* respectively), which likely represent the C- and N- terminal segments of the bacterial *ccmF* locus respectively, are encoded in a putative operon. Finally, *ccmG* and *ccdA* are found adjacent to each other on the chromosome (MA4254 and MA4255) (Supplementary Figure 1). We used our recently developed Cas9-based genome editing technology to generate markerless in-frame deletion mutants of the *ccmABC* locus, *ccmE*, *ccmF1*, *ccmF2*, *ccmG*, *ccdA* as well as the $\Delta ccmF1\Delta ccmF2$ and $\Delta ccmG\Delta ccdA$ double mutants. We picked the $\Delta ccmABC$ mutant for whole-genome sequencing to screen for off-target CRISPR-Cas9 activity. Apart from the deleted locus,

we did not observe any other mutations in the $\Delta ccmABC$ mutant relative to the parent strain (WWM60) (Supplementary Table 1). Consistent with our previous observations, these data suggest that off-target activity is negligible during Cas9-mediated genome editing in *M. acetivorans* [32, 37, 38].

We selected a representative cyt *c* encoded by the *mmcA* gene (locus tag: MA0658) in the *M. acetivorans* genome to assay for cyt *c* maturation in the *ccm* mutants. MmcA is a heptaheme cyt *c* that contains a N-terminal signal peptide and is likely located in the pseudo-periplasm (Supplementary Figure 2). MmcA is a highly expressed cyt *c* that is conserved across methane-metabolizing archaea [28, 39, 40] and holo-MmcA is easy to detect as it contains seven heme-binding motifs. Taken together, these functional and technical features make MmcA an ideal candidate to assay cyt *c* biogenesis. To develop a rapid assay for quantification of holo-cyt *c* in the *ccm* mutants, we built a plasmid-based overexpression system by adding a C-terminal TAP (tandem affinity purification) tag comprised of a 3X FLAG sequence and a twin-Strep sequence to the *mmcA* coding sequence placed under the control of a tetracycline-inducible promoter (Figure 1a). As a control, we transformed the $\Delta mmcA$ mutant with an untagged or TAP-tagged cyt *c* overexpression vector to test if the presence of the tag in the MmcA CDS interferes with protein production or heme-attachment. To identify protein production, we performed an immunoblot with a commercial anti-Flag antibody. Using this technique, we were able to successfully detect the TAP-tagged MmcA in the cell lysate (Figure 1b). To assay for heme attachment (formation of holo-cyt *c*), we used a peroxidase-based heme stain, which detects covalently bound (*c*-type) heme in cyt *c* [41]. Using this technique, we were able to verify that the TAP-tagged MmcA is capable of undergoing covalent modification to generate the corresponding holo cyt *c* (Figure 1b). Taken

together, these controls demonstrate that our overexpression vectors can be used to reliably and rapidly assay the production and covalent modification of MmcA in *M. acetivorans*.

Next, we introduced the plasmid overexpression system with a TAP-tagged MmcA in each of the *ccm* deletion mutants and assayed for the production of tagged MmcA protein and holo-MmcA in each of the resulting strains (Figures 2a-2c). We were unable to detect the tagged protein or holo-MmcA in the $\Delta ccmABC$, $\Delta ccmE$, $\Delta ccmF2$, $\Delta ccmF1ccmF2$ mutants, which suggests that these genes are essential for cyt *c* biogenesis in *M. acetivorans* (Figure 2c). Consistent with previous studies, these results also indicate that the apo-MmcA is immediately targeted for degradation in the absence of a functional Ccm machinery [42–44]. Curiously, we were able to detect faint band corresponding to holo-MmcA in the $\Delta ccmF1$ mutant (Figure 2c). These data suggest that the gene product of *ccmF1* is important but not vital for cyt *c* biogenesis possibly because the catalytically important residues from CcmF in *E. coli* are all present in CcmF2 from *M. acetivorans* (Supplementary Figure 3). Finally, we observed that $\Delta ccmG$ and $\Delta ccdA$ single mutants as well as the $\Delta ccmG\Delta ccdA$ double mutant produced roughly the same amount of holo-MmcA as the parent strain (Figure 2c), which indicates that these genes are not essential for disulfide bond reduction in the apo cyt *c* under highly reducing laboratory growth conditions. Overall, using MmcA as a diagnostic cyt *c*, we have shown that the proteins encoded by *ccmABCEFI2* in *M. acetivorans* constitute a functional, streamlined version of the Ccm machinery for cyt *c* maturation.

Archaeal CcmABC is involved in the formation of a holo-CcmE heme chaperone

In the first steps of cyt *c* biogenesis, heme *b* is transported across the cytoplasmic membrane to generate holo-CcmE: a heme chaperone that covalently binds heme and transports it to the cytochrome *c* synthetase complex [45, 46]. In Gram-negative bacteria, like *E. coli*, heme

transport and holo-CcmE formation is mediated by the CcmABCD complex (Figure 3a). In the CcmABCD complex, CcmD plays an essential role in facilitating the release of holo-CcmE from the CcmABCE adduct [5, 21]. Since *M. acetivorans* and all other sequenced archaeal strains lack CcmD, we investigated the role of the CcmABC complex in the formation of holo-CcmE (Figure 3b). To this end, we developed a plasmid-based overexpression system for CcmE with a C-terminal 1XStrep-1XFlag tag placed under the control of a tetracycline-inducible promoter (Figure 3c). We introduced the C-tagged CcmE overexpression plasmid in the parent strain (WWM60) as well as the $\Delta ccmABC$ and $\Delta ccmE$ mutants and were able to detect tagged- CcmE in protein enriched from the membrane fraction (using an anti-Strep affinity column) by immunoblotting with an anti-FLAG antibody in all plasmid complemented strains (Figure 3d). We were only able to observe the heme-bound holo-CcmE by heme staining the enriched membrane fraction of the plasmid complemented parent strain (WWM60) and $\Delta ccmE$ mutant (Figure 3d). Furthermore, we were unable to detect holo-cyt *c*, including the MmcA protein, by heme staining the total cell lysate of the $\Delta ccmABC$ strain complemented with the CcmE overexpression plasmid, which indicates that overexpression of CcmE does not rescue the *ccmABC* lesion in the cytochrome maturation machinery (Supplementary Figure 4). These data support the hypothesis that the CcmABC complex has evolved to transport heme and produce holo-CcmE independent of CcmD in *M. acetivorans* and other archaea.

A CXXXY motif in archaeal CcmE is required for heme-attachment and protein stability

In bacteria with a Ccm machinery, like *E. coli*, a conserved Histidine residue (H130) present in the HXXXY motif of CcmE covalently binds heme [47]. Notably, all sequenced archaea (and a few bacteria like *Desulfovibrio desulfuricans*) have replaced this Histidine with a Cysteine (Figure 4a) [11, 19]. To test if the Cysteine residue (C120) in the conserved motif (CXXXY) of

CcmE in *M. acetivorans* has a functional role similar to the Histidine residue in *E. coli* or the Cysteine residue in *D. desulfuricans*, we built a plasmid-based overexpression system for mutant alleles of *ccmE* with either a C120A or a C120H substitution and a C-terminal 1XStrep-1XFlag tag under the control of an inducible promoter (Figures 4a and 4b). We introduced these plasmids in the $\Delta ccmE$ mutant and successfully detected the wild-type and C120A CcmE protein in the Strep-enriched membrane fraction by immunoblotting with anti-FLAG antibodies (Figure 4c). Despite repeated attempts, we were unable to detect the C120H CcmE mutant in the membrane or soluble fraction (Figure 4c and Supplementary Figure 5). This outcome is consistent with the hypothesis that the C120H substitution considerably destabilizes CcmE, leading to proteolysis of the gene product. Next, we were able to observe the wild-type CcmE by heme staining the Strep-enriched membrane fraction of the corresponding strain however, no heme stained band corresponding to CcmE was detected for the C120A mutant (Figure 4c). Furthermore, we were unable to detect holo-MmcA or any other holo-cyt *c* by heme staining the total cell lysate of the $\Delta ccmE$ strain complemented with either the C120A or C120H CcmE (Supplementary Figure 6). These results provide strong evidence in support of the hypothesis that the C120 residue of the CXXXY motif is important for heme attachment as well as protein stability in the archaeal CcmE.

A streamlined Ccm machinery comprised of CcmABCEF is necessary and sufficient for *c*-type cytochrome biogenesis in *Methanosarcina* spp.

While our genetic studies with *M. acetivorans* clearly show that the proteins encoded by *ccmABC*, *ccmE*, *ccmF1* and *ccmF2* are essential for cyt *c* biogenesis, they do not inform us of any non-orthologous proteins that might also be involved in this process. To test if the cyt *c* maturation pathway encoded by the *ccm* genes in *M. acetivorans* is both necessary and sufficient for cyt *c* biogenesis, we expressed *mmcA* and a synthetic operon comprised of *ccmABCEF1F2* in a

heterologous host. We chose *Methanosarcina barkeri* Fusaro as a heterologous host for this study as it has the same codon usage pattern as *M. acetivorans*, contains an intact pathway for heme *b* synthesis, and is known to produce *b*-type cytochromes, but does not encode any of the *ccm* genes or cyt *c* in its genome. To confirm that *M. barkeri* Fusaro does not produce cyt *c* we assayed the cell lysate with the peroxidase-based heme stain for cyt *c* described earlier. Using this technique, did not detect any signal of cyt *c* in *M. barkeri* Fusaro (Supplementary Figure 7). We built a plasmid-based expression system containing some or all of the following components: i) *ccmABCEFI2* genes under the control of a tetracycline inducible promoter and ii) *mmcA* (a diagnostic cyt *c*) with a C-terminal TAP tag under the control of a constitutive promoter (Figure 5a). Each of these plasmids was integrated on the *M. barkeri* Fusaro chromosome at a neutral locus using a Φ C31 integrase system described previously [30]. We were able to detect a band corresponding to the TAP-tagged MmcA by immunoblotting with anti-FLAG antibodies and heme staining in *M. barkeri* strains expressing both *mmcA* and *ccm* genes (Figure 5b). These results clearly indicate that the *ccmABCEFI2* genes in *M. acetivorans*, encode a complete, functional and streamlined version of the System I cytochrome *c* maturation machinery previously characterized in Bacteria.

The production of *c*-type cytochromes is important for growth of *M. acetivorans*

Multiple cyt *c* are encoded in the genome of *M. acetivorans* and some of them, like MmcA, are associated with the electron transport chain; yet the cyt *c* maturation machinery does not seem to be essential under standard growth conditions (i.e. in medium with trimethylamine hydrochloride as the sole carbon and energy source). To determine the physiological role of the Ccm machinery as well as the cyt(s) *c* produced by this process, we assayed the growth characteristics of the $\Delta mmcA$, $\Delta ccmE$ and $\Delta ccmABC$ mutants on growth substrates that represent

a variety of methanogenesis pathways and thermodynamic regimes for *M. acetivorans* (Figure 6 and Supplementary Figure 8). In these analyses, the $\Delta ccmE$ and $\Delta ccmABC$ mutants represent lesions in the Ccm pathway that would prevent maturation of all expressed cyt *c*, whereas the $\Delta mmcA$ mutant represents an in-frame deletion in a specific cyt *c* found in the membrane-associated Rnf (**R**hodobacter **n**itrogen **f**ixation) complex that catalyzes the transfer of electrons between ferredoxin and methanophenazine (a membrane-bound electron carrier) coupled to Na⁺ translocation [26, 34] (Supplementary Figure 2b). We assayed growth on substrates that represent the two modes of methanogenesis in *M. acetivorans*: the methylotrophic pathway and the acetoclastic pathway. During methylotrophic growth, on compounds like trimethylamine hydrochloride (TMA), methanol or dimethyl sulfide (DMS), cells disproportionate the methylated compound to produce carbon dioxide and methane in a 3:1 ratio (Supplementary Figures 8a, 8b and 8d). In contrast, during growth on acetate, via the acetoclastic pathway, the substrate undergoes dismutation to produce carbon dioxide and methane in a 1:1 ratio (Supplementary Figure 8c). Furthermore, the free energy yields ($\Delta G^{\circ'}$) of these substrates, from -164.5 kJ/mol TMA to -36.0 kJ/mol acetate, also captures the entire bioenergetic landscape of methanogenesis.

We observed substantially slower growth than the parent strain for the *ccm*-deletion mutants ($\Delta ccmABC$ and $\Delta ccmE$) as well as the cyt *c* deletion mutant ($\Delta mmcA$) under all conditions tested; however, the magnitude of the growth defect varied dramatically (Figure 6; Supplementary Table 2). In growth medium with acetate as the sole substrate, we did not detect any growth for the *ccm*-deletion mutants or the $\Delta mmcA$ mutant (over the course of 7 weeks), which indicate that holo-MmcA and, possibly, other cyt *c* produced by the Ccm pathway are essential for acetoclastic methanogenesis (Figure 6c; Supplementary Table 2). In contrast, cyt *c* are not essential but important during growth on methylated compounds however, the growth characteristics of each

mutant varied substantially (Figures 6a, 6b, and 6d; Supplementary Table 2). Notably, despite a shorter lag, the *ccm*-deletion mutants grew slower than the parent strain on DMS (Figure 6d). On TMA and DMS, the *ccm*-deletion mutants and $\Delta mmcA$ mutant had a slower growth rate (by a factor of 0.60 and 0.64, respectively) compared to the parent (WWM60) (Figures 6a and 6d; Supplementary Table 2). But, the growth of the *ccm*-deletion mutants and the $\Delta mmcA$ mutant was indistinguishable from each other (Supplementary Table 2). Taken together, these data indicate that although MmcA is dispensable, it is the sole physiologically relevant cyt *c* during growth on TMA and DMS. In contrast, on methanol, the *ccm*-deletion mutants grew significantly slower than the parent (WWM60) and the $\Delta mmcA$ mutant as well (by a factor of 0.34 and 0.92, respectively) (Figure 6b; Supplementary Table 2). These data suggest that MmcA is the predominant cyt *c* synthesized on methanol, and is vital for growth, but other cyt *c* encoded in the genome also play a minor but physiologically relevant role. Our growth analyses underscore the importance of cyt *c* on the physiology of *M. acetivorans* and highlight that the role of individual cyt(s) *c* can vary in a substrate-specific manner.

Evolutionary Analysis of *c*-type Cytochrome biogenesis in Archaea

Even though cyt *c* are important for optimal growth and methanogenesis in *M. acetivorans*, the Ccm machinery and cyt *c* proteins are absent in the close relative *M. barkeri* Fusaro. This uneven distribution of cyt *c* in strains within the Genus *Methanosarcina* could be due to gene loss in strains like *M. barkeri* Fusaro or gene gain by horizontal gene transfer (HGT) of the Ccm machinery and cyt *c* genes in strains like *M. acetivorans*. To distinguish between the two evolutionary hypotheses, we mapped the distribution of the Ccm machinery on the species tree of strains belonging to the genus *Methanosarcina* that are present in the Genome Taxonomy Database (GTDB) [48]. The species tree is based on core genome alignment of the corresponding strains

and was obtained from Annotree [49] (Figure 7a). Either all genes of the Ccm machinery are present in a strain or the whole machinery is completely absent (Figure 7a). The strains that encode the Ccm machinery are not monophyletic i.e. the Ccm machinery is not just present in one single clade of *Methanosarcina* strains. This observation strongly supports the hypothesis that the Ccm machinery was present in the last common ancestor of extant *Methanosarcina* spp. and strains like *M. barkeri* Fusaro have lost these genes (Figure 7a). This hypothesis is corroborated by the synteny of the chromosomal locus surrounding the *ccmABC*, *ccmE*, and *ccmF1ccmF2* genes across all *Methanosarcina* spp. (Figure 7b). Notably, strains like *M. barkeri* MS and *M. vacuolata* contain a truncated ORF (192 bp and 195 bp, respectively) that has sequence homology to the C-terminus of *ccmE* likely as a scar of a gene loss event (Figures 7a and 7b). To test whether the Ccm machinery was acquired by HGT in the last common ancestor of extant strains within the Genus *Methanosarcina* or is present in other Genera within the Family *Methanosarcinaceae*, we mapped the distribution of the Ccm machinery (i.e. the presence of the *ccmABCEFIIF2* genes) across the *Methanosarcinaceae* (Supplementary Figure 9). The Ccm machinery is broadly distributed in methanogens and anaerobic methane oxidizing archaea (ANME) across the *Methanosarcinaceae* suggesting an important role for cyt *c* in methane metabolism across this clade (Supplementary Figure 9). Whilst the Ccm machinery is universally conserved in some Genera, like *Methanococcoides*, *Methanosalsum*, and *Methanohalobium*, this whole machinery seems to have been lost in several members of other Genera, like *Methanosarcina*, *Methanolobus*, *Methanoperedens* and *Methanomethylovorans* (Supplementary Figure 9). Thus, it is possible that loss of the Ccm machinery and cyt *c* proteins occurs frequently as an adaptation to certain environmental conditions.

To test if the Ccm machinery in members of the *Methanosarcinaceae* was vertically inherited from an archaeal ancestor or acquired through inter-domain HGT from bacterial clades, we constructed maximum-likelihood phylogenetic trees of each *ccm* gene from *M. acetivorans* (Figure 8; Supplementary Figures 10-15). For each of the *ccm* genes, one or more clades comprising of strains belonging to the Order *Methanosarcinales* is nested within clades derived from different bacterial groups (Figure 8; Supplementary Figures 10-15). Based on these trees, it is evident that Ccm machinery in the *Methanosarcinales* was acquired by HGT from bacteria. It is also likely that different *ccm* genes were acquired from different bacteria, which is further corroborated by the observation that these genes are not present in an operon contrary their chromosomal arrangement in bacterial strains (Supplementary Figure 1, 10-15). Taken together, the streamlined Ccm machinery, which lacks CcmDHI, is not ancestral to the Archaea, rather it has been acquired through multiple inter-domain HGT events between Archaea and Bacteria. While it is not possible to rule out the role of historical contingency in the notable absence of CcmDHI, it is tempting to speculate that unique features of the archaeal cell render CcmHDIG and CcdA dispensable such that a streamlined Ccm machinery comprising of CcmABCEF is sufficient for cyt *c* maturation in Archaea.

Discussion

Cyt *c* are widely found in members of the Archaea where they catalyze numerous electron transfer processes that facilitate microbial interactions and fuel biogeochemical cycles. The biogenesis of cyt *c* is complex and is mediated by a dedicated pathway that can vary substantially across the tree of Life. In the vast majority of eukaryotes, a single protein called cyt *c* heme lyase (CCHL) is involved in the biogenesis of cyt *c* [5]. In contrast, bacteria contain more complex pathways that are fine tuned to work with lower concentrations of heme [5]. The most complex of

these pathways, called the Ccm machinery, requires at least nine to ten proteins (CcmABCDEFGH(I) and CcdA/DsbD) that work in concert for the biogenesis of cyt *c*. The biochemical details of the Ccm machinery in bacteria has been studied in great detail using model systems over the past few decades and the role of each *ccm* gene is well established at this point [5, 6]. Previous research has shown that archaea encode some components of the bacterial s Ccm machinery [11, 12] however, orthologs of at least three proteins (CcmD, CcmH and CcmI) that are absolutely critical for cyt *c* maturation in bacteria are universally absent in archaeal genomes. Thus, it was unclear how the Ccm machinery is being repurposed (if at all) for cyt *c* biogenesis in archaea. In this study, we use the methanogen *Methanosarcina acetivorans* to characterize a streamlined form of the Ccm system comprised of only five proteins (CcmABCEF) that likely serves as the predominant route for cyt *c* biogenesis in members of the Archaea (Figure 2 and Figure 5).

Based on our findings, there are three key differences between the Ccm machinery in Bacteria and Archaea. While *ccmD* seems to be uniformly absent in archaeal genomes [11] it is universally present in bacterial genomes and is essential for cyt *c* biogenesis in a number of strains ranging from *E. coli* and *R. capsultus* to *D. desulfuricans* [5, 19, 21]. In bacteria, CcmD is a small (69 aa long) transmembrane protein that associates with CcmABC and facilitates the release of holo-CcmE in the periplasm after heme transport and attachment [21, 46]. In *M. acetivorans*, the CcmABC complex is capable of forming holo-CcmE and releasing it into the pseudo-periplasm in a CcmD independent manner (Figure 3). It is unlikely that the *ccmD* gene has been fused to *ccmA*, *ccmB*, or *ccmC* based on the sequence length and alignment of the archaeal genes with their bacterial counterparts (Supplementary Figures 10-12). It is also unlikely that the CcmD independent mechanism for the release of holo-CcmE from CcmABC might be linked to the CXXXY heme-binding motif in CcmE because some bacteria, like *D. desulfuricans*, that encode

ccmD and require this gene product for cyt *c* biogenesis also contain a CXXXY heme-binding motif in CcmE (Figure 4) [19]. One plausible hypothesis for the absence of CcmD might be the unusual architecture of archaeal membranes. The archaeal cytoplasmic membrane is composed of isoprenoid lipids (phytanyl units), compared to phospholipids in bacterial cytoplasmic membranes. This lipid composition might alter how CcmABC and CcmE are anchored into the membrane and render CcmD inconsequential during the formation and release of holo-CcmE.

Next, the archaeal CcmE universally contains a CXXXY heme-binding motif whereas bacterial CcmE can either have a HXXXY motif or a CXXXY motif depending on the strain [5, 19]. In *E. coli*, a H130C allele of CcmE can still bind heme but cannot transfer the heme to the CcmFH complex [19, 50, 51]. In contrast, a C120H mutation in *M. acetivorans* seems to destabilize the protein to such an extent that it can no longer be detected (Figure 4 and Supplementary Figure 5). Similarly, no heme-bound CcmE was detected for the C127H allele of CcmE derived from the bacterium *D. desulfuricans* [19]. Taken together, these data suggest that the Cys and His residues involved in heme-binding CcmE are not interchangeable. In fact, many other as yet uncharacterized features of CcmE, possibly unique to these two groups of CcmE proteins, are likely to be equally important for the formation of holo-CcmE and the transfer of heme to the apo cyt *c* via interactions between CcmE and CcmF as well other proteins. Whether the absence of a HXXXY motif containing CcmE in archaea is just an artifact of historical contingency or due to functional/evolutionary constraints remains unclear.

Finally, the cyt *c* synthetase complex in bacteria contains two (CcmFH or CcmFI) or three (CcmFHI) proteins depending on the strain whereas the archaeal counterpart only contains CcmF. In *M. acetivorans*, the CcmF gene is split into two coding sequences (*ccmF1* and *ccmF2*), each of which might be derived from different bacterial groups (Figure 8 and Supplementary Figures 14,

15). Functionally important domains of the cyt *c* synthetase complex including the two histidine residues that coordinate the pseudo-periplasmic heme *b* as well as the highly conserved WWD domain are all present in CcmF2 (Supplementary Figure 3). CcmF1 probably plays a secondary role in cyt *c* biogenesis, which might explain why a small amount of holo cyt *c* can still be detected in the Δ *ccmF1* mutant of *M. acetivorans* (Figure 2). In bacteria and *Arabidopsis*, CcmH/I have been shown to interact with the apo cyt *c* and deliver it to CcmF [5, 16, 52]. It is unlikely that *M. acetivorans* and other archaea have replaced CcmH/I with a non-orthologous protein as heterologous expression of *ccmABCEF1F2* in the non-native host, *M. barkeri* Fusaro, seems to be sufficient for cyt *c* biogenesis (Figure 5). However, at present, we cannot distinguish between the possibility that *M. barkeri* Fusaro also encodes this non-orthologous protein or that the standalone CcmF in *M. acetivorans* performs the function of CcmFH in *E. coli*.

Within the Archaea, cyt *c* are especially enriched in the Family *Methanosarcinaceae* wherein these proteins have likely fostered tremendous metabolic innovation [53]. Our work and other studies [28, 35, 36, 39] show that cyt *c* like MmcA are intricately coupled to growth under a variety of conditions in the marine methanogen *M. acetivorans* (Figure 6). Furthermore, membrane-spanning multi-heme cyt *c* have been shown to facilitate anaerobic methane oxidation by microbial consortia comprised of anaerobic methanotrophic archaea (ANME) and sulfate reducing bacteria (SRB) [54–57] and recent studies also suggest an important role for cyt *c* in facilitating anaerobic alkane metabolism in several members of the *Methanosarcinaceae* [40]. Since ANME and alkane oxidizing archaea are yet to be isolated in pure culture, our ability to manipulate the biogenesis of cyt *c* also renders *M. acetivorans* as an ideal platform to perform functional analyses of diverse cyt *c* across the *Methanosarcinaceae*. Curiously, even though cyt *c* are widely present in members of the *Methanosarcinaceae*, there are many extant strains, such as *M. barkeri* Fusaro, that lack the

Ccm machinery altogether and do not encode any cyt *c* proteins in their genome. Our evolutionary analyses provide strong evidence that the uneven distribution of the Ccm machinery and cyt *c* in the *Methanosarcinaceae* is due to gene loss events rather than independent gene gain events (Figure 7; Supplementary Figure 9). It is also worth noting that many of the extant lineages that lack the Ccm machinery and cyt *c* are found in freshwater or anthropogenic sources (like waste digestors); reciprocally, the Ccm machinery and diverse cyt *c* are especially conserved in lineages derived from marine environments (Figure 7; Supplementary Figure 9). This pattern is indicative of an environment-specific selective pressure to retain or discard cyt *c* and the associated biogenesis machinery. However, the specific biotic or abiotic environmental cues that underlie this pattern remain unclear at the moment.

Previous studies [11, 12] have used some of the unique features that unify the archaeal Ccm machinery, notably the absence of CcmDHI and a conserved CXXXY heme binding domain in CcmE, to support an ancestral origin of the Ccm machinery in the Archaea. Our phylogenetic analyses (Figure 8 and Supplementary Figures 10-15) contradict this hypothesis and provide strong evidence supporting the hypothesis that there have been many independent HGT events that have cross-pollinated the Ccm machinery between the Archaea and Bacteria. In fact, even the Ccm machinery in one single archaeon, like *M. acetivorans*, may not have been derived from a single source (Figure 8 and Supplementary Figures 10-15). Taken together, the streamlined Ccm machinery in the Archaea is functionally uniform despite independent evolutionary origins.

Materials and Methods

In silico design of single guide RNAs (sgRNAs) for Cas9-mediated genome editing. Twenty bp target sequences for Cas9-mediated genome editing in this study are listed in Supplementary Table 3. All target sequences were chosen using the CRISPR site finder tool in Geneious Prime

version 11.0 with the following parameters: *i*) the PAM site was set to NGG at the 3' end, *ii*) 0 mismatches were allowed against off-targets, and *iii*) 0 mismatches were allowed to be indels. Activity scores were predicted using the methods described previously [58]. The *M. acetivorans* chromosome and the plasmid pC2A were used to score off-target binding sites.

Plasmid construction. All plasmids used in this study are listed in Supplementary Table 4 and the primers used to generate the plasmids are listed in Supplementary Table 5. Plasmids for Cas9 mediated genome editing were designed using pDN201 as the backbone described previously [32]. Briefly, PCR fragments with the *P_{mtaCBI}* promoter from *M. acetivorans*, sgRNA(s) targeting gene(s) of interest, and the *mtaCBI* terminator from *M. acetivorans* were fused to *AscI* linearized pDN201 using the Gibson assembly method as described previously [32]. Subsequently, PCR fragments with the repair template were fused to the sgRNA containing vector linearized with *PmeI* using the Gibson assembly method as described previously [32]. All plasmid-based overexpression constructs were constructed using pJK029A[30] as the backbone. Fragments containing the gene of interest and promoter, terminators or tandem affinity purification tags as indicated in Supplementary Table 4 were amplified and fused to the *P_{mcrB(tetO4)}* promoter in pJK029A [30] linearized with *NdeI* and *HindIII* using the Gibson assembly method as described previously [32]. A cointegrate of the pDN201-derived plasmid or pJK029A-derived plasmid and pAMG40, containing the pC2A origin of replication, was obtained using the Gateway BP Clonase II Enzyme Mix (ThermoFisher Scientific, Waltham, MA, USA) prior to transformation in *Methanosarcina spp.* Standard techniques were used for the isolation and manipulation of plasmid DNA. WM4489, a DH10 β derivative engineered to control copy-number of oriV-based plasmids [59], was used as the host strain for all plasmids generated in this study (Supplementary Table 4). WM4489 was transformed by electroporation at 1.8 kV using an *E. coli* Gene Pulser (Bio-Rad,

Hercules, CA). All pDN201-derived plasmids were verified by Sanger sequencing at the UC Berkeley DNA Sequencing Facility and all pAMG40 cointegrates were verified by restriction endonuclease analysis.

Strains and growth media. All strains used in this study are listed in Supplementary Table 6. All archaeal strains were grown in single-cell morphology [29] at 37°C without shaking in bicarbonate-buffered high-salt (HS) liquid medium with N₂/CO₂ (80/20) in the headspace. For transformations and growth analyses, 10 mL cultures were grown in Balch tubes with N₂/CO₂ (80/20) at 8-10 psi in the headspace. For protein purifications, 250 mL cultures were grown in anaerobic bottles with N₂/CO₂ (80/20) at 3-5 psi in the headspace. All *M. barkeri* Fusaro cells were cultivated in liquid medium supplemented with 125 mM methanol. For mutant generation, *M. acetivorans* and *M. barkeri* Fusaro were plated on agar solidified HS medium (1.6% agar w/v) with 50 mM TMA or 62.5 mM Methanol as the carbon and energy substrate respectively. Solid media plates were incubated in an intra-chamber anaerobic incubator maintained at 37°C with N₂/CO₂/H₂S (79.9/20/0.1) in the headspace, as described previously [60]. Puromycin (RPI, Mount Prospect, IL, USA) to a final concentration of 2 µg/mL and the purine analog 8ADP (CarboSynth, San Diego, CA, USA) to a final concentration of 20 µg/mL were added from sterile, anaerobic stock solutions to select for transformants containing the *pac* (puromycin transacetylase) cassette and to select against the *hpt* (phosphoribosyltransferase) cassette encoded on pC2A-based plasmids, respectively. Anaerobic, sterile stocks of tetracycline hydrochloride in deionized water were prepared fresh shortly before use and added to a final concentration of 100 µg/mL. All mutant strains were verified by Sanger sequencing at the UC Berkeley DNA Sequencing Facility. All *Escherichia coli* strains were grown in Lysogeny broth (LB) or LB-agar at 37 °C with appropriate antibiotics (25 µg/mL kanamycin, and/or 10 µg/mL chloramphenicol) as indicated for different

constructs (Supplementary Table 4). All liquid cultures of *E. coli* were grown shaking at 250 rpm. For plasmid preparation, cultures were supplemented with 10 mM rhamnose to increase the plasmid copy number of the pDN201 and pJK029A derived plasmids.

Transformation of *Methanosarcina* spp. Liposome-mediated transformation was used for *M. acetivorans* and *M. barkeri* Fusaro as described previously [61]. Late-exponential phase culture of *M. acetivorans* (10 mL with TMA) or *M. barkeri* Fusaro (50 mL with MeOH) and 2 µg of plasmid DNA were used for each transformation. Briefly, cells were centrifuged, the supernatant was carefully decanted, and the pellet was resuspended in 1 mL bicarbonate buffered isotonic sucrose (0.85 M) containing 100 µM cysteine. Two µg plasmid DNA and 25 µL DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate) (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) were added to the cell suspension and incubated for 4 hours at room temperature in an anaerobic chamber with CO₂/H₂/N₂ (20/4/balance) in the headspace. The mix of cells, plasmid DNA and DOTAP was inoculated in HS-medium with the appropriate growth substrate growth medium and incubated at 37 °C for 12-16 hours prior to plating. *M. acetivorans* cells were spread on puromycin-containing agar-solidified medium using a spreader whereas a *M. barkeri* Fusaro cells were plated used the top-agar method described previously [29, 62].

Whole genome resequencing of CRISPR-edited *Methanosarcina acetivorans* mutants. A 10 mL culture of DDN029 in HS + 50 mM trimethylamine hydrochloride incubated at 37 °C was harvested at late-exponential phase (OD₆₀₀ ~0.8). Genomic DNA was extracted using the Qiagen blood and tissue kit (Qiagen, Hilden, Germany) and the concentration of genomic DNA was measured using a Nanodrop One Microvolume UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Library preparation and Illumina sequencing was performed at the Microbial Genome Sequencing Center, Pittsburgh, PA, USA. Illumina sequencing reads were

aligned to the *Methanosarcina acetivorans* C2A genome and mutations were identified using Breseq version 0.35.5 [63]. Illumina sequencing reads for DDN029 have been deposited to the Sequencing Reads Archive (SRA) with the following BioProject accession number: PRJNA800036.

Growth assays for *M. acetivorans*. *M. acetivorans* strains were grown in single-cell morphology [29] in bicarbonate-buffered high salt (HS) liquid medium containing 125 mM methanol, 50 mM trimethylamine hydrochloride (TMA), 40 mM sodium acetate, or 20 mM dimethylsulfide (DMS). Most substrates were added to the medium prior to sterilization. DMS was added from an anaerobic stock solution maintained at 4 °C immediately prior to inoculation. For growth analyses, 10 mL cultures were grown in sealed Balch tubes with N₂/CO₂ (80/20) at 8-10 psi in the headspace. Growth measurements were conducted with at least three independent biological replicates derived from colony-purified isolates using optical density readings at 600 nm (OD₆₀₀). Optical density readings were obtained from a UV-Vis Spectrophotometer (Gensys 50, Thermo Fisher Scientific, Waltham, MA, USA) outfitted with an adjustable test tube holder that could directly take OD₆₀₀ readings from cultures in a Balch tube. A Balch tube containing 10mL HS medium with the appropriate growth substrate was used as the ‘Blank’ for OD₆₀₀ measurements. For growth on methanol, TMA, and DMS, cells were acclimated to the growth substrate for a minimum of five generations prior to quantitative growth measurements. Growth measurements on acetate were performed with cells transferred from HS+TMA medium. A 1:20 dilution of mid-late-exponential phase cultures was used as the inoculum for growth analyses. Growth curves were plotted on a log base 10 scale and growth rate measurements were performed individually for each replicate by calculating the slope of the line that fits as many data points on the growth curve for a linear regression coefficient ($R^2 \geq 0.99$). For maximum OD₆₀₀ measurements, cells were diluted 1:10 in

HS-medium containing the appropriate growth substrate. Growth curve plots and statistical analyses were obtained using GraphPad Prism 9.0.0.

Purification of membrane fraction containing Tandem Affinity Purification (TAP) tagged

CcmE. Protein purification was performed with 250 mL of mid-exponential phase culture grown in HS + 50 mM TMA at 36°C without shaking under aerobic conditions. Cells were harvested by centrifugation ($6000 \times g$) for 20 min at 4 °C and was lysed in 10 mL of Wash buffer (50 mM NaH_2PO_4 , 300 mM NaCl, pH = 8.0) on ice. The cell lysate was clarified by centrifugation at $10,000 \times g$ for 10 min at 4°C, followed by separation of soluble and membrane fractions via high-speed ultracentrifugation at $100,000 \times g$ for 45 min at 4 °C. The membrane pellets were solubilized in 4 mL Wash buffer with 1% Triton 100-X (Sigma-Aldrich, Saint Louis, MO, USA). The solubilized membrane fraction was loaded on a column containing 0.5 mL Strep-tactin Superflow plus resin (50% suspension; Qiagen, Hilden, Germany) equilibrated with 4 mL of the Wash buffer. The column was washed twice with 4 mL of Wash buffer by gravity flow and the purified protein was eluted in four fractions with 0.5 mL Elution buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 2.5 mM desthiobiotin, pH = 8.0) per fraction. The protein was concentrated using 10kDa Amicon filter (Merck Millipore, Burlington, MA, USA). The protein concentration in each fraction was estimated using the Bradford reagent (Sigma-Aldrich, Saint Louis, MO, USA) with BSA (bovine serum albumin) as the standard per the manufacturer's instructions.

Heme staining and immunoblotting. Heme staining using heme peroxidase assays were performed as described previously [41, 64]. For heme staining MmcA, total cell lysate of *M. acetivorans* or *M. barkeri* Fusaro was incubated at 65°C for 4 minutes. For heme staining CcmE, cell lysate/enriched-protein samples were not heat treated. Protein samples were resolved by 12% Mini-Protean TGX denaturing SDS-PAGE gel (Bio-Rad, Hercules, CA, USA) and transblotted to

0.2 μ m PVDF membrane (Bio-Rad, Hercules, CA, USA) using Trans-Blot Turbo transfer system (Bio-Rad, Hercules, CA, USA). For heme staining, the SuperSignal West Femto kit (Thermo Scientific, Waltham, MA, USA) was used to detect the heme signal on the transblotted PVDF membrane and imaging was performed with ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA). All the heme stain blots were washed with 50 mL stripping buffer (60 mM Tris pH= 7 containing 2% SDS and 7 μ L/mL β -mercaptoethanol) shaking at 50 rpm for 1 hour at 50 °C and confirmed for the absence of any peroxidase-based signal from heme before they were used for immunoblots. FLAG-tagged proteins were probed with immunoblotting using monoclonal anti-Flag M2-Peroxidase (HRP) antibody (Sigma-Aldrich, Saint Louis, MO, USA) (1/50,000X dilution) and Immobilon Western Chemiluminescent HRP Substrate (Millipore, Burlington, MA, USA) was used for signal detection. Imaging was performed with ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA). Near equal loading of total protein was estimated using the Bradford reagent (Sigma-Aldrich, Saint Louis, MO, USA) with BSA (bovine serum albumin) as the standard per the manufacturer's instructions.

Bioinformatics analyses. Phylogenetic trees of *Methanosarcina* spp. and other strains within the *Methanosarcinales* (as depicted in Figure 7a and Supplementary Figure 9) were obtained from AnnoTree [49]. Annotree was used for functional annotation of *ccm* genes using the following KEGG annotations: *ccmA* (K02913), *ccmB* (K02194), *ccmC* (K02195), *ccmE* (K02197), *ccmF* (02198). For gene trees, 500 closest homologs were extracted from the NCBI non-redundant protein database using the corresponding Ccm gene sequence from *M. acetivorans* as the query in BLAST-P searches. The amino acid sequences of these proteins were aligned using MUSCLE 3.7 on the CIPRES Science Gateway cluster V3.3 [65]. Maximum-likelihood trees were generated using RAxML v8.2.12 with the Jones-Taylor-Thornton (JTT) substitution matrix on the CIPRES

Science Gateway cluster V3.3 [65]. Trees were displayed using Fig Tree v1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>). Gene ortholog neighborhood was obtained using the bidirectional best hits for the corresponding *ccm* gene using the Integrated Microbial Genomes and Microbiomes (IMG) platform containing annotated isolate genome and metagenome datasets sequenced at the Joint Genome Institute (JGI)[66]

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Author contributions

D.G. contributed to conceptualization, data curation, formal analysis, methodology, and writing. K.E.S. contributed to data curation, formal analysis, and methodology. D.D.N contributed to

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602 **Competing Interests**

603 The authors do not declare any competing interests.

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Figure Legends

Figure 1: a) The design of diagnostic constructs to use the native heptaheme *c*-type cytochrome, MmcA, to map the cytochrome *c* biogenesis pathway in *Methanosarcina acetivorans*. *i)* The control construct (pM) contains a *mmcA* coding sequence whereas *ii)* the test construct (pM-C) contains a *mmcA* coding sequence with a C-terminal translational fusion of a tandem affinity purification (TAP) tag comprised of a 3X FLAG sequence and a Twin-Strep sequence. In each construct, gene expression is driven by a tetracycline-inducible medium-strength promoter, *PmcrB(tetO4)*, and a transcriptional terminator of the *mcr* operon from *M. acetivorans* is provided after the last coding sequence. **b)** Assays to measure holo-MmcA formation by covalent heme attachment and detect the tagged MmcA protein in whole cell lysates of *M. acetivorans* mutants. *(Left)* A heme peroxidase-based assay is used to detect the presence of proteins with covalently bound heme in whole cells lysates. Using this assay, both untagged (Lane 3) and tagged (Lane 5) heme-bound holo-MmcA can be detected upon induction of the genes from each plasmid construct described above. The tagged MmcA protein runs at a higher molecular weight due to the presence of a *ca.* 7.3 kDa TAP tag at the C-terminus compared to the native MmcA protein. *(Right)* Immunoblotting with commercial anti-FLAG antibody can be used detect MmcA production in the test construct containing a C-terminal TAP tag fused to the *mmcA* gene (Lanes 4 and 5). (-) indicates that no tetracycline was added to the growth medium and (+) indicates that 100 µg/ml tetracycline was added to the growth medium. An equal amount of whole cell lysate protein (~80 µg) was loaded in each lane.

Figure 2: a) Chromosomal organization of the system I cytochrome *c* maturation machinery (Ccm) genes in *M. acetivorans*. Double dashed lines indicate that the genes are located more than 110 kbp away on the chromosome. Genes located at the same chromosomal locus are shown in

the same color. **b)** A schematic showing the genotype of strains used to assay the role of individual *ccm* genes in the maturation of *c*-type cytochromes. In addition to the corresponding *ccm* deletion on the chromosome, these strains also contain a self-replicating plasmid (pM-C) where the *mmcA* coding sequence with a C-terminal tandem affinity purification (TAP) tag comprised of a 3X FLAG sequence and a Twin-Strep sequence is placed under the control of a tetracycline-inducible medium-strength promoter, *PmcrB(tetO4)*. In these strains, the expression of the C-terminal TAP tagged MmcA can be induced by the addition of tetracycline to the growth medium. **c)** A heme peroxidase-based assay to measure the formation of heme-bound holo-MmcA (top) and Western blots (WB) with anti-FLAG antibody to detect the production of C-terminal TAP tagged MmcA (bottom). Neither holo-MmcA nor tagged protein could be detected in the $\Delta ccmCBA$, $\Delta ccmE$, $\Delta ccmF2$ single mutants and the $\Delta ccmF1\Delta ccmF2$ double mutant suggesting that the apo-MmcA undergoes rapid proteolysis in the absence of a functional Ccm machinery. All assays were conducted with whole cell lysates of cultures grown in medium containing 100 μ g/ml tetracycline to induce the expression of C-terminal TAP tagged MmcA. An equal amount of whole cell lysate protein (~60 μ g) was loaded in each lane. The vector control used for this experiment is pJK029A as described previously in [30].

Figure 3: a) The formation of heme-bound holo-CcmE in bacteria containing the System I Ccm machinery is facilitated by the CcmABCD complex. The ATP-dependent CcmABC complex translocates heme *b* from the cytosol to CcmE and CcmD facilitates the dissociation of holo-CcmE from the CcmABCDE adduct. **b)** Since archaea lack the *ccmD* gene the formation of holo-CcmE is unclear. One hypothesis, shown here, is that the CcmABC complex in archaea mediates the dissociation of holo-ccmE independent of CcmD. **c)** The design of a test construct (pE-C) containing the *ccmE* coding sequence with C-terminal translational fusion of a modified tandem

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refer to two independently colonies of the *M. barkeri* Fusaro cointegrate expressing the Ccm machinery and MmcA from *M. acetivorans*. An equal amount of whole cell lysate (~60 µg) was loaded in each lane. The vector control used for this experiment is pJK029A as described elsewhere [30].

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Figure 7: a) A phylogenetic tree for strains belonging to the genus *Methanosarcina* in the Genome Taxonomy Database (GTDB) was obtained from AnnoTree [49]. The presence or absence of genes functionally annotated as *ccmA*, *ccmB*, *ccmC*, *ccmE*, *ccmF1* and *ccmF2* in the genome of individual strains is shown in dark green (present) or black (absent) respectively. A few strains like *M. barkeri* MS, *M. vacuolata*, and *M. sp.* UBA5 encode a truncated copy of *ccmE* as indicated in light green. **b)** Chromosomal organization of genes surrounding the *ccmABC*, *ccmE*, and *ccmF1ccmF2* locus in *Methanosarcina* strains. Genes of the same color (except light yellow) represent members of the same orthologous group. Synteny of the *ccmABC*, *ccmE*, and *ccmF1ccmF2* chromosomal locus across members of the Genus *Methanosarcina* supports the hypothesis that these genes were present in the last common ancestor and have been lost in several extant lineages.

904 **Figure 8:** Maximum-likelihood phylogenetic trees of 500 **a)** CcmE and **b)** CcmF2 sequences
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a

Constructs for heterologous expression of system-I Ccm and MmcA

b

Methanosarcina barkeri Fusaro

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a

CcmE

b

CcmF2

■ Methane metabolizing archaea
 ■ *Chloroflexi*
■ Actinobacteria
 ■ Nitrosospirinae
 ■ *Desulfovibrio* spp.
 ■ Acidobacteria

■ *Gemmatimonadates*
■ *Bacillus* spp.
 ■ Marinimicrobia
 ■ Other archaea
 ■ Other bacteria

Figure 8: Maximum-likelihood phylogenetic trees of 500 **a)** CcmE and **b)** CcmF2 sequences obtained from the NCBI non-redundant (nr) protein database using the corresponding sequence from *Methanosarcina acetivorans* as the search query. Sequences belonging to certain functional groups or evolutionarily related groups of archaea and bacteria are shown in colors denoted in the legend at the bottom. These gene trees are indicative of multiple Horizontal Gene Transfer (HGT) events between members of the Archaea and Bacteria.

Supplementary Material

Title: Functional Characterization of *c*-type Cytochrome Biogenesis in the Archaeon, *Methanosarcina acetivorans*

Authors: Dinesh Gupta¹, Katie E. Shalvarjian², Dipti D. Nayak^{1*}

Department of Molecular and Cell Biology, University of California, Berkeley, CA, USA¹;

Department of Plant and Microbial Biology, University of California, Berkeley, CA, USA²

Running Head: *c*-type cytochrome biogenesis in archaea

*Address correspondence to:

Dipti D. Nayak (dnayak@berkeley.edu)

Department of Molecular and Cell Biology, 1 Barker Hall #3204, University of California,
Berkeley, CA 94720-3204

Tel: 510-664-5267

Supplementary Figure 1: a) Chromosomal organization of the System I c-type cytochrome maturation (Ccm) machinery genes in a few representative bacteria and the archaeon, *Methanosarcina acetivorans*. In bacteria like **i)** *Escherichia coli* **ii)** *Rhodobacter capsulatus* and **iii)** *Desulfovibrio desulfuricans*, 9-10 genes (*ccm*ABCDEF $\overline{GHI}$ and *dsbD/ccdA*) are involved in the maturation of c-type cytochromes (cyt c), and most of the *ccm* genes are found in an operon. **iv)** The *ccm* genes in the archaeon *M. acetivorans* and other methane metabolizing archaea belonging to the Order *Methanosarcinales* are not found in a single operon and, notably, homologs of three essential genes, *ccmD*, *ccmH*, and *ccmI*, are absent. Double dashed lines indicate a distance >110 kbp on the chromosome. **b)** An overview of the System I Ccm machinery from *E. coli*. The apo cyt-c is transported across the inner cytoplasmic membrane into the periplasmic space using the Sec system. The CcmABCD membrane-bound complex transports heme b to form heme-bound holo-CcmE in an ATP-dependent manner. Holo-CcmE delivers heme b to the membrane-bound cyt c synthetase complex, CcmFH, that catalyzes the formation of holo cyt c. CcmG and DsbD/CcdA facilitate the transfer of electrons to reduce the disulfide bond between the cysteine residues in the CXXCH motif of the apo cyt c prior to the formation of holo cyt c. Figure adapted from Kranz *et al.*, 2009 (1).

a**>MmcA (MA0658) *M. acetivorans***

MVIMNRLNLLVSGVAVLLLLLAAGAYSSLGYSGNDAIASHYMTKGEWSDSV**CGGCH**FGVYENVNNSYHVQ
 VNMSRWSPLTNFDLETSGEEWVKKFGMYHPGGPLAKYGIDID**CMMCHE**KYGLYDFDARAEAIANGDF
 ANANSLAVANFSATAQSDPLHLFVYTANVLTPYPLLIVFHDAVNGAPIS**CAQQRCH**RIDVETSAVMWADE
 EDFEESDAHAANGVE**CTECH**HTEAFIITS DHQIGRGNTSGTPDLPDSHYDDTMR**SCDDAECH**AGISHGP
 FADSHMEFLA**CEACH**TPELPGGDLPGGNVLESFSWQNGEREDVYRSDSQPALAWYNGNFGDVLPSVDT
 RNDTDVKVTPFNNTGTWWDAGTDPEVLANPNTSISTGDPIPVQYVKAADANGDGEVTVTEEMQAYDADG
 DGEADYPNAVLRITVELYYQVAHSIVSSDIGLADPYT**CKDCH**GNEAVIDWAALGYEQDPGGESSAVKSI
 AVTYDKPRPVEVETEPAL*

b

Supplementary Figure 2: a) Amino acid sequence of the c-type cytochrome (cyt c) MmcA from *Methanosarcina acetivorans*. The predicted signal peptide is shown in blue, five canonical heme-binding motifs (CXXCH) are shown in red and two non-canonical heme-binding motifs (CXXXCH and CXXXXCH) are shown in orange. Based on these observations, MmcA is a heptaheme cyt c that is present in the pseudo-periplasm of *M. acetivorans*. **b)** MmcA is associated with the **R**hodobacter **n**itrogen **f**ixation (Rnf) bioenergetic complex in methanogens and other alkane metabolizing archaea. The Rnf complex catalyzes the electron transfer reaction between cytosolic Ferredoxin (Fd) and the membrane-associated electron carrier Methanophenazine (MP) coupled to the translocation of Na⁺ ions across the cytoplasmic membrane. MmcA has been hypothesized to play two roles in the context of the Rnf bioenergetic complex: i) it is involved in the extracellular transfer of electrons from/to Fd or ii) it directly interacts with MP to mediate the final step in the intracellular electron transport reactions from Fd to MP. The reduced MPH₂ generated by the Rnf complex is regenerated by the membrane-bound Heterodisulfide reductase (Hdr) that couples the translocation of protons (H⁺) to the transfer of electrons from MP to the CoM-S-S-CoB disulfide that acts as the terminal electron acceptor in cytochrome-containing methanogens like *Methanosarcina acetivorans*.

CcmF (<i>E. coli</i>)	MMPEIGNGLLCLALGIALLLSVYPLWGVARGDARMMASSRLFAWLLFMSVAGAFVLVNA	60
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	--MNTGMGLIWIAGASGLLAFLTSLLYFFRQDRKFLALSEKLELI--AGVGLVFAIFLLA	56
	: * ** : * . . ** : * . * * : : * . : : * . . * : : *	
CcmF (<i>E. coli</i>)	--FVVNDFTVTYVASNSNTQLPVWYRVAATWGAHEGSLLLWVLLMSGWTFAVAIFSQRIP	118
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	FYLLGVDLTQYSYVFQHSSTDLAWHYRFSALWAGQEGSFLIWTGFIIVML-AITRFTGTGK	115
	: : * ** : : * . * : : * : : * : : * : : * : : * : : *	
CcmF (<i>E. coli</i>)	LDIVARVLAIMGV---SVGFLLFILFTSNPFSRT-----LPNFPIEG	158
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	VLRETEIFSIMRSVSLFVASVFLLLLALKNPFSMYYTGTGVPDVTNWNLFAPFPVVSYG	175
	: : : : * * * . . . : : * * * . . . * . *	
CcmF (<i>E. coli</i>)	RDLNPLLQDPGLIFPPLLYMGYGVGSVAFAFAIASLLSGRLDSTYARFTRPWTLAAWIF	218
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	QGMNPLLNRNPWMAIHPPLLFLGYAAFTLPFSAIGGLVL--KDSRWSELATGWMRVSWLF	233
	: : * : : * : : * : : * : : * : : * : : * : : * : : * : : *	
	176 WWD Domain 261	
CcmF (<i>E. coli</i>)	LTIGIVLGSAWYAYELGWGGW-WFWDPVENASFMPWLVTALMHS LAVTEQRASFKAATL	277
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	LTLGIGFGAFWAYEVLGWGAWYWTWDPVETSSLIPLWLTATAYLHAKLRAR-QGEYGFLLP	292
	***** : * : * * * * * * * * * * * * * * * : : * : : :	
CcmF (<i>E. coli</i>)	LLAISAFSLCLLGTFLVRSGVLVSVHAFASDPARGMFILAFMVLVIGSGSLLLFAARGHKV	337
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	MLALVSFILVIFSTFVTRSGLVSVHWSQDFTMEGMIIALFLVVLIGSSAVLLVRKYFSE	352
	: * : : * * : : * : * : * : * : * : * : * : * : * : * : * : *	
CcmF (<i>E. coli</i>)	RS-----RVNNALWSRESLLLANNVLLVAAMLVLLGTLPLVHKQL	379
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	ELYLFMKFYFLTFFSRFFMKKLSVDLTVRSTIFATVSV-LVLLAALITMGLLTPFILRV	411
	. : : : * * : : : : * * : : : * * : : : * * : : : *	
CcmF (<i>E. coli</i>)	GLGSISIGEPFFNTMTFTWLMVPFALLGVGPLVRWGRDRPRKIRNLLIIA--FISTLVLS	437
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	TGEEILLDSAYFNLRAALPTLALVMLLTCLLL--GSAGKK--EALLALGLGVAGSVLSV	467
	. * . . : * * : : : : * * : * : * : . * * . . . : : :	
CcmF (<i>E. coli</i>)	LLLPLWLFESKVVAMTVLGLAMACWIAVLAIAEAAALRISRGTKTTFSYWGMVA AHLGLAVT	497
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	VFSPFSSLPVNIISFSL--AVALFAVMYRLLS---SKEKTLKGLT LRRAGSHI IHLGVVLL	522
	: : * : : : : * * : : : : . : * * : * * : : * : : *	
CcmF (<i>E. coli</i>)	IVGIAFSQNSYVERDVRMKSGSDVDIHEYRFTFRDVKEVT---G---PNWRGGVAT----	547
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	LVGILFSTNMKLEDSAAVPVGEVGTFSQMGYSIHVTNITSIGIEGAPYGN YQGSAYVSTVY	582
	: * * * * . * . . : * : : : : : : : * * : * : * . .	
CcmF (<i>E. coli</i>)	IGVTRDGKPETVLVYAEKRYNT--AGSMTEAAIDGGITRDLYAALGE-ELENGAWAVRL	604
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	FDVYRWGQPFDS--GQVYISDFKWEQSYTETIYHRLLEELFIAPKSVDTKTQTVELYV	640
	: . * * * : . : * * . . * : * . * : : * : * . : : : : :	
CcmF (<i>E. coli</i>)	YYKPFVRWIWAGGLMMALGGLLCLFDPRYRKRVPNPQKTAPEAA-----	647
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	RKVPFMTSLWGGFYLMVLGILMLFISDSLAGE---KGSPGNGLSGTLD SEREQVNGIK	696
	** : : * . * : * . * * : : . . * * *	
CcmF (<i>E. coli</i>)	-----	647
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	NDSKKKRVSKKNV	709

Supplementary Figure 3: Alignment of the CcmF amino acid sequence from *Escherichia coli* with the concatenated CcmF2-CcmF1 amino acid sequences from *Methanosarcina acetivorans*. Based on this alignment, the CcmF2 sequence (in blue) from *M. acetivorans* is homologous to N-terminus of CcmF from bacteria and the CcmF1 (in purple) sequence of *M. acetivorans* is homologous to the C-terminus of CcmF from bacteria. The highly conserved WWD domain, and three conserved histidine residues that serve as axial ligands for heme *b* in the membrane (His261 in *E. coli*) and the periplasm (His176 and His 303 in *E. coli*) are all conserved in the CcmF2 sequence of *M. acetivorans*.

Supplementary Figure 4: Expression of CcmE with a C-terminal 1XFLAG-1X Strep tag from a plasmid (pE) in the parent strain of *Methanosarcina acetivorans* (WWM60) and the $\Delta ccmE$ background leads to the production of heme-bound holo-MmcA as detected by a heme peroxidase assay of whole cell lysates (Lanes 1, 3 of heme-stained gel). Thus, the C-tagged CcmE is functional and facilitates the maturation of native c-type cytochromes (cyt c) like MmcA. In contrast, expression of C-tagged CcmE in the $\Delta ccmABC$ background does not produce any holo-MmcA (Lane 2 of heme stained gel). In the absence of CcmABC, holo-CcmE does not form, which, in turn, prevents the maturation of native cyt c like MmcA. All cultures were grown in medium containing 100 $\mu\text{g/ml}$ tetracycline to induce the expression of C-tagged CcmE from the plasmid pE. An equal amount of protein ($\sim 20 \mu\text{g}$) was loaded in each lane.

$\Delta ccmE$ *M. acetivorans*

Supplementary Figure 5: Expression of the C120H mutant of CcmE from *Methanosarcina acetivorans* with a C-terminal 1XFLAG-1X Strep tag in the $\Delta ccmE$ background does not lead to the production of any CcmE protein that can be detected by Heme peroxidase assays (top gel) or Western Blot using an anti-Flag antibody (bottom gel) in the Strep-enriched membrane fraction (Lane 1) or the soluble fraction (Lane 2). In contrast, expression of the WT sequence CcmE from *M. acetivorans* with a C-terminal 1XFLAG-1X Strep tag in the $\Delta ccmE$ background leads to the production of protein that can be detected by Heme peroxidase assays (top gel) and Western Blot using an anti-Flag antibody (bottom gel) in the Strep-enriched soluble fraction (Lane 3) and the membrane fraction (see Figure 4). Strep-enriched membrane or soluble fraction represents protein eluted after passing the membrane or the soluble fraction through a Strep-affinity column. The C120H mutant was sequenced verified prior to protein purification. Based on these data, it is highly likely that the C120H substitution destabilizes the CcmE protein. All cultures were grown in medium containing 100 $\mu\text{g/ml}$ tetracycline to induce the expression of the tagged CcmE protein. An equal amount of protein ($\sim 1 \mu\text{g}$) was loaded in each lane.

Supplementary Figure 6: Expression of the wildtype (WT) sequence of CcmE from *Methanosarcina acetivorans* with a C-terminal 1XFLAG-1X Strep tag (C-tagged CcmE) in the $\Delta ccmE$ background leads to the production of heme-bound holo-MmcA as detected by a heme peroxidase assay of whole cell lysates (Lane 1 of heme-stained gel). Thus, the C-tagged WT sequence of CcmE is functional and facilitates the maturation of native c-type cytochromes (cyt c) like MmcA. In contrast, expression of C-tagged C120H or C120A mutants of CcmE in the $\Delta ccmE$ background does not produce any holo-MmcA (Lanes 2,3 of heme stained gel). Thus, the C120H and C120A mutants disrupt the function of CcmE, which, in turn, prevents the maturation of native cyt c like MmcA. All cultures were grown in medium containing 100 $\mu\text{g/ml}$ tetracycline to induce the expression of C-tagged CcmE. An equal amount of protein ($\sim 20 \mu\text{g}$) was loaded in each lane.

Supplementary Figure 7: (Left) Heme-peroxidase assays to detect proteins that covalent bind heme (including c-type cytochromes) in whole cell lysates of *Methanosarcina acetivorans* (Lanes 1, 2, 3) and *Methanosarcina barkeri* (Lanes 4, 5, 6) grown in high-salt minimal medium with 125 mM methanol as the sole source of carbon and energy. Three different concentrations (~30, 60, 90 µg) of whole cell lysate were used per strain for these assays as shown in the Coomassie gel control (right).

Supplementary Figure 8: **a)** Methanogenic metabolism of trimethylamine (TMA) in *Methanosarcina acetivorans*. Green arrows represent TMA, dimethylamine (DMA), monomethylamine (MMA) specific methyltransferase reactions that catalyze the transfer of each of the methyl groups in TMA to ultimately form methyl-CoM (CH₃-CoM). CH₃-CoM undergoes a disproportionation reaction to generate CO₂ and CH₄ in a 3:1 ratio. **b)** Methanogenic metabolism of methanol in *Methanosarcina acetivorans*. The purple arrow represents the methanol specific reaction that catalyzes the transfer of the methyl group to ultimately form methyl-CoM (CH₃-CoM). CH₃-CoM undergoes a disproportionation reaction to generate CO₂ and CH₄ in a 3:1 ratio. **c)** Methanogenic metabolism of acetate in *Methanosarcina acetivorans*. The yellow arrow represents the reaction catalyzed by the acetyl-CoA decarbonylase/synthase (ACDS) enzyme complex that splits acetate into an enzyme bound carbonyl group and a methyl group that is transferred to the C₁ carrier tetrahydrosarcinopterin (H₄SPT). Oxidation of the carbonyl group to CO₂ produces electrons that can be used to reduce the methyl group to CH₄. During acetoclastic methanogenesis, CO₂ and CH₄ are produced in a 1:1 ratio. **d)** Methanogenic metabolism of dimethyl sulfide (DMS) in *Methanosarcina acetivorans*. The orange arrows represent DMA and methanethiol (MeSH) specific reactions that catalyze the transfer of the methyl groups from DMS to ultimately form methyl-CoM (CH₃-CoM). CH₃-CoM undergoes a disproportionation reaction to generate CO₂ and CH₄ in a 3:1 ratio.

Supplementary Figure 9: A phylogenomic reconstruction of the relationship between various genera (depicted by different colors along the circumference of the radial tree) within the Family *Methanosarcinaceae* from Annotree (Mendler *et al.*, 2019). Purple lines depict strains that encode homologs of *ccmABCEF1F2* in their genome whereas black lines depict strains that lack homologs of *ccmABCEF1F2* in their genome. Based on the pattern of distribution of the *ccmABCEF1F2* genes it is likely that these genes were present in the last common ancestor of the *Methanosarcinaceae* and have been lost in members of various Genera including *Methanosarcina* spp., *Methanohalophilus* spp., *Methanomethylovorans* spp., *Methanolobus* spp. and *Methanoperedens* spp.

Phylogenetic tree of CcmA

- Methane metabolizing archaea
- Chloroflexi*
- Nitrosospinae*
- Other bacteria

Supplementary Figure 10: An unrooted maximum-likelihood tree of 500 CcmA sequences retrieved from the NCBI non-redundant protein sequence database using the corresponding sequence from *Methanosarcina acetivorans* as the search query. Branch labels indicate support values.

Phylogenetic tree of CcmB

Methane metabolizing archaea

Chloroflexi

Nitrosospinae

Other bacteria

Supplementary Figure 11: An unrooted maximum-likelihood tree of 500 CcmB sequences retrieved from the NCBI non-redundant protein sequence database using the corresponding sequence from *Methanosarcina acetivorans* as the search query. Branch labels indicate support values.

Phylogenetic tree of CcmC

Supplementary Figure 12: An unrooted maximum-likelihood tree of 500 CcmC sequences retrieved from the NCBI non-redundant protein sequence database using the corresponding sequence from *Methanosarcina acetivorans* as the search query. Branch labels indicate support values.

Phylogenetic tree of CcmE

- Methane metabolizing archaea
- Chloroflexi*
- Actinobacteria
- Nitrosospinae*
- Desulfovibrio spp.*
- Acidobacteria
- Bacillus spp.*
- Other archaea
- Other bacteria

Supplementary Figure 13: An unrooted maximum-likelihood tree of 500 CcmE sequences retrieved from the NCBI non-redundant protein sequence database using the corresponding sequence from *Methanosarcina acetivorans* as the search query. Branch labels indicate support values.

Phylogenetic tree of CcmF1

Methane metabolizing archaea

Chloroflexi

Psychrobacter spp.

Sulfate reducing bacteria

Acidobacteria

Other bacteria

Supplementary Figure 14: An unrooted maximum-likelihood tree of 500 CcmF1 sequences retrieved from the NCBI non-redundant protein sequence database using the corresponding sequence from *Methanosarcina acetivorans* as the search query. Branch labels indicate support values.

Phylogenetic tree of CcmF2

Supplementary Figure 15: An unrooted maximum-likelihood tree of 500 CcmF2 sequences retrieved from the NCBI non-redundant protein sequence database using the corresponding sequence from *Methanosarcina acetivorans* as the search query. Branch labels indicate support values.

Supplementary Table 1: List of mutations in CRISPR-edited mutant strain DDN029 containing a $\Delta ccmABC$ in-frame deletion mutation

Position	Mutation	Notes
487691	C added	Present in Parent (WWM60)
836182-836771	$\Delta hpt::PmcrB-tetR$	Present in Parent (WWM60)
941168	A5->6	Present in Parent (WWM60)
1314120	$\Delta 1bp$	Present in Parent (WWM60)
1698001	$\Delta 2684 bp$	$\Delta ccmABC$ (only present in DDN029)
2086881-82	TC->CT	Present in Parent (WWM60)
2086886	G->T	Present in Parent (WWM60)
2534543	C added	Present in Parent (WWM60)
2836646	A->G (F64L)	Present in Parent (WWM60)
2867059	T->G (H313Q)	Present in Parent (WWM60)
3433201	$\Delta 1bp$	Present in Parent (WWM60)
4295452	$\Delta 1bp$	Present in Parent (WWM60)
4874567	$\Delta 1bp$	Present in Parent (WWM60)
4945345	C added	Present in Parent (WWM60)
5078585	G->A (M1M)	Present in Parent (WWM60)

Supplementary Table 2: Growth data of *M. acetivorans* strains shown in Figure 6

<i>M. acetivorans</i> strains	Doubling time (Td) (hrs)	Max.OD ₆₀₀ (OD ^M)	P value for WT vs mutants (Td/OD ^M)	P value for $\Delta mmcA$ vs Δccn mutants (Td/OD ^M)	P value for $\Delta ccnABC$ vs $\Delta ccnE$ mutants (Td/OD ^M)
Trimethylamine (TMA) as a growth substrate					
WWM60 (WT)	12.13 ± 0.04	1.29 ± 0.01			
$\Delta mmcA$	19.89 ± 0.44	1.14 ± 0.02	3.73E-05 (***)/ 0.0013 (**)		
$\Delta ccnABC$	20.08 ± 0.37	1.15 ± 0.02	2.70E-05 (***)/ 0.0011 (**)	0.4661 (NS)/ 0.1832 (NS)	
$\Delta ccnE$	20.19 ± 0.15	1.15 ± 0.01	2.21E-06 (***)/ 0.0002 (**)	0.2523 (NS)/ 0.5187 (NS)	0.3856 (NS)/ 0.8431 (NS)
Methanol (MeOH) as a growth substrate					
WWM60 (WT)	10.11 ± 0.23	1.13 ± 0.00			
$\Delta mmcA$	27.23 ± 0.28	0.92 ± 0.00	6.66E-07 (***)/ 8.30E-07 (***)		
$\Delta ccnABC$	29.40 ± 0.58	0.90 ± 0.01	1.89E-05 (***)/ 2.28E-05 (***)	0.0131(*)/ 0.0477(*)	
$\Delta ccnE$	29.52 ± 0.28	0.90 ± 0.01	5.85E-08 (***)/ 4.71E-06 (***)	6.06E-05 (***)/ 0.0036(**)	0.7980 (NS)/ 0.0749(NS)
Acetate as a growth substrate					
WWM60 (WT)	77.93 ± 4.32	0.22 ± 0.01			
$\Delta mmcA$	No growth observed [#]	-	-		
$\Delta ccnABC$	No growth observed [#]	-	-	-	
$\Delta ccnE$	No growth observed [#]	-	-	-	-
Dimethyl sulfide (DMS) as a growth substrate					
WWM60 (WT)	32.6 ± 0.55	0.35 ± 0.01			
$\Delta mmcA$	53.7 ± 5.66	0.26 ± 0.02	0.0247 (*)/ 0.0053(**)		
$\Delta ccnABC$	51.1 ± 2.84	0.33 ± 0.00	0.0093 (***)/ 0.0474 (*)	0.2515 (NS)/ 0.0248 (*)	
$\Delta ccnE$	41.6 ± 1.68	0.36 ± 0.00	0.0055 (***)/ 0.4266 (NS)	0.0876 (NS)/ 0.0215 (*)	0.0568 (NS)/ 0.0148 (*)

All data represent the mean ± standard deviation of at least 3 biological replicates at 37 °C

[#] No growth observed at 37 °C for 50 days

P > 0.05 (non-significant, NS); P ≤ 0.05 (*); P ≤ 0.01 (**); P ≤ 0.0001 (***) using a two-sided Student's t-test

Supplementary Table 3: List of target sequences used in this study

Gene (locus tag)	Target sequence (+ PAM)	Location on <i>M. acetivorans</i> chromosome
<i>mmcA</i> (MA0658)	AGTTACAGCAATACTCTTCA <u>CGG</u> TGAATACCCAAGAGAAGAAT <u>AGG</u>	776339 – 776358 (- strand) 774977 – 774996 (- strand)
<i>ccmABC</i> (MA1428-1430)	AAAAGAGGGTCAAAAAGAGC <u>AGG</u> AGGTCAAGGGCTTTCAGGAC <u>AGG</u>	1698029 – 1698048 (+ strand) 1700643 – 1700662 (+ strand)
<i>ccmF₂</i> (MA3304)	CAGGCAGGACAGGAAATTTT <u>TGG</u> GTCCCTGATTCCCTGGCTTA <u>CGG</u>	4078616 – 4078635 (- strand) 4077905 – 4077924 (- strand)
<i>ccmF₁</i> (MA3305)	CACAGAGACTGTAGCAAATA <u>TGG</u> TCTCCCGGAAACGGCCTTTC <u>CGG</u>	4079700 – 4079719 (+ strand) 4078,828 – 4078847 (- strand)
<i>ccmE</i> (MA4149)	TGGTGTGACATCATTTGAAC <u>TGG</u>	5066266 - 5066285 (+ strand)
<i>ccmG</i> (MA4254)	GCATCTGTAATCTTTACGGC <u>AGG</u>	5177988 – 5178007 (+ strand)
<i>ccdA</i> (MA4255)	TCTCCTTGTAGGGCTCTGGA <u>TGG</u>	5179184 – 5179203 (+ strand)

Supplementary Table 4: List of plasmids used in this study

Plasmid	Features	Source
pAMG40	Vector for fosmid retrofitting that contains pC2A and λ attB	(Guss et al. 2008)
pJK027A	Vector with <i>PmcB(tetO1)</i> promoter fusion to <i>uidA</i> that contains ϕ C31-attB and λ attP	(Guss et al. 2008)
pJK029A	Vector with <i>PmcB(tetO4)</i> promoter fusion to <i>uidA</i> that contains ϕ C31-attB and λ attP	(Guss et al. 2008)
pDN201	pJK027A-derived plasmid with <i>PmcB(tetO1)</i> promoter fusion to Spy <i>cas9</i>	(Nayak and Metcalf, 2017)
pDN401	pDN201-derived plasmid with synthetic fragments containing <i>PmtaCB1</i> promoter fusion to sgRNAs in tandem targeting the <i>mmcA</i> (MA0658) coding sequence (CDS)	This study
pDN402	pDN401-derived plasmid containing a repair template to generate an in-frame deletion of the <i>mmcA</i> (MA0658) CDS	This study
pDN403	Cointegrate of pDN401 and pAMG40	This study
pDN404	Cointegrate of pDN402 and pAMG40	This study
pDN406	pJK029A-derived plasmid with <i>PmcB(tetO4)</i> promoter fusion to <i>mmcA</i> (MA0658) CDS from <i>M. acetivorans</i> with a C-terminal tandem affinity purification tag (containing a 3X FLAG tag and a Twin-Strep tag)	This study
pDN409	Cointegrate of pDN406 and pAMG40	This study
pDN411	pDN201-derived plasmid with synthetic fragments containing <i>PmtaCB1</i> promoter fusion to sgRNAs in tandem targeting the <i>ccmE</i> (MA4149) coding sequence (CDS)	This study
pDN412	pDN411-derived plasmid containing a repair template to generate an in-frame deletion of the <i>ccmE</i> (MA4149) CDS	This study
pDN413	Cointegrate of pDN411 and pAMG40	This study
pDN414	Cointegrate of pDN412 and pAMG40	This study
pDN421	pDN201-derived plasmid with synthetic fragments containing <i>PmtaCB1</i> promoter fusion to sgRNAs in tandem targeting the <i>ccmF1</i> (MA3305) coding sequence (CDS)	This study
pDN422	pDN201-derived plasmid with synthetic fragments containing <i>PmtaCB1</i> promoter fusion to sgRNAs in tandem targeting the <i>ccmF2</i> (MA3304) coding sequence (CDS)	This study
pDN423	pDN201-derived plasmid with synthetic fragments containing <i>PmtaCB1</i> promoter fusion to sgRNAs in tandem targeting the <i>ccmF1F2</i> (MA3305-3304) coding sequences (CDS)	This study
pDN424	pDN421-derived plasmid containing a repair template to generate an in-frame deletion of the <i>ccmF1</i> (MA3305) CDS	This study
pDN425	pDN422-derived plasmid containing a repair template to generate an in-frame deletion of the <i>ccmF2</i> (MA3304) CDS	This study
pDN426	pDN423-derived plasmid containing a repair template to generate an in-frame deletion of the <i>ccmF1F2</i> (MA3305-3304) CDS	This study
pDN427	Cointegrate of pDN421 and pAMG40	This study
pDN428	Cointegrate of pDN422 and pAMG40	This study
pDN429	Cointegrate of pDN423 and pAMG40	This study
pDN430	Cointegrate of pDN424 and pAMG40	This study
pDN431	Cointegrate of pDN425 and pAMG40	This study
pDN432	Cointegrate of pDN426 and pAMG40	This study
pDN443	pDN201-derived plasmid with synthetic fragments containing <i>PmtaCB1</i> promoter fusion to sgRNAs in tandem targeting the <i>ccmABC</i> (MA1428-1430) coding sequences (CDS)	This study
pDN444	pDN443-derived plasmid containing a repair template to generate an in-frame deletion of the <i>ccmABC</i> (MA1428-1430) CDS	This study
pDN445	Cointegrate of pDN443 and pAMG40	This study
pDN446	Cointegrate of pDN444 and pAMG40	This study
pDPG001	pDN201-derived plasmid with synthetic fragments containing <i>PmtaCB1</i> promoter fusion to sgRNAs in tandem targeting the <i>ccmG</i> (MA4254) coding sequence (CDS)	This study
pDPG002	pDN201-derived plasmid with synthetic fragments containing <i>PmtaCB1</i> promoter fusion to sgRNAs in tandem targeting the <i>ccdA</i> (MA4255) coding sequence (CDS)	This study
pDPG003	pDN201-derived plasmid with synthetic fragments containing <i>PmtaCB1</i> promoter fusion to sgRNAs in tandem targeting the <i>ccmG-ccdA</i> (MA4254-4255) coding sequences (CDS)	This study
pDPG004	pDPG001-derived plasmid containing a repair template to generate an in-frame deletion of the <i>ccmG</i> (MA4254) CDS	This study

pDPG005	pDPG002-derived plasmid containing a repair template to generate an in-frame deletion of the <i>ccdA</i> (MA4255) CDS	This study
pDPG006	pDPG003-derived plasmid containing a repair template to generate an in-frame deletion of the <i>ccmG-ccdA</i> (MA4254-4254) CDS	This study
pDPG007	Cointegrate of pDPG004 and pAMG40	This study
pDPG008	Cointegrate of pDPG005 and pAMG40	This study
pDPG009	Cointegrate of pDPG006 and pAMG40	This study
pDPG010	Cointegrate of pJK029A and pAMG40	This study
pDPG014	pJK029A-derived plasmid with <i>PmcB(tetO4)</i> promoter fusion to <i>ccmABC</i> (MA1428-1430)- <i>ccmE</i> (MA4149)- <i>ccmF1F2</i> (MA3305-3304) CDS from <i>M. acetivorans</i>	This study
pDPG015	pJK029A-derived plasmid with <i>M. barkeri</i> <i>PserC</i> (constitutive) promoter fusion to <i>mmcA</i> (MA0658) CDS from <i>M. acetivorans</i> with a C-terminal tandem affinity purification tag (containing a 3X FLAG tag and a Twin-Strep sequence)	This study
pDPG017	pJK029A-derived plasmid with <i>PmcB(tetO4)</i> promoter fusion to <i>ccmABC</i> (MA1428-1430)- <i>ccmE</i> (MA4149)- <i>ccmF1F2</i> (MA3305-3304) CDS from <i>M. acetivorans</i> and <i>M. barkeri</i> <i>PserC</i> (constitutive) promoter fusion to <i>mmcA</i> (MA0658) CDS from <i>M. acetivorans</i> with a C-terminal tandem affinity purification tag (containing a 3X FLAG tag and a Twin-Strep sequence)	This study
pKES30	pJK029A-derived plasmid with <i>PmcB(tetO4)</i> promoter fusion to <i>ccmE</i> (MA4149) CDS from <i>M. acetivorans</i> with a C-terminal tandem affinity purification tag (containing a 1X Strep and a 1X FLAG sequence)	This study
pKES031	Cointegrate of pKES30 and pAMG40	This study
pKES038	pJK029A-derived plasmid with <i>PmcB(tetO4)</i> promoter fusion to <i>ccmE</i> (MA4149) CDS with C120H substitution with a C-terminal tandem affinity purification tag (containing a 1X Strep and a 1X FLAG sequence)	This study
pKES039	Cointegrate of pKES38 and pAMG40	This study
pKES040	pJK029A-derived plasmid with <i>PmcB(tetO4)</i> promoter fusion to <i>ccmE</i> (MA4149) CDS with C120A substitution with a C-terminal tandem affinity purification tag (containing a 1X Strep and a 1X FLAG sequence)	This study
pKES041	Cointegrate of pKES40 and pAMG40	This study

Note: pDN201/pJK029A derived plasmids contain the chloramphenicol resistance marker, pAMG40 contains kanamycin resistance marker, and the co-integrates of pDN201/pJK029A derived plasmids and pAMG40 contain both contain chloramphenicol and kanamycin resistance markers.

Supplementary Table 5: List of primers used in this study

Primer Name	Primer Sequence (Overhangs for Gibson assembly are shown in red; sequences to introduce C120A or C120H mutations in <i>ccmE</i> are shown in purple)
<i>mtaBCI</i> -f for pDPG001, 002, 003	<u>TCGAAGTTTAAACCTGCAGG</u> GAATTACACGATCACTAATTTT
<i>mtaBCI</i> -sgRNA-r for pDPG001	<u>GCCGTAAAGATTACAGATGC</u> ACATGTGCTTACAGCATAAT
sgRNA-scaffold-f for pDPG001	<u>GCATCTGTAATCTTTACGGC</u> GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
sgRNA-scaffold-r for pDPG001	<u>TAAGCGGCCGCGATCGCCGG</u> TACATGAGGGCTGAAAAGCCG
<i>mtaBCI</i> -sgRN-r for pDPG002	<u>TCCAGAGCCCTACAAGGAGA</u> ACATGTGCTTACAGCATA
sgRNA-scaffold-f for pDPG002	<u>TCTCCTTG TAGGGCTCTGGA</u> GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
sgRNA-scaffold-r for pDPG002	<u>TCCAGAGCCCTACAAGGAGA</u> TTCGTTTTCTCTTTTAGGTGACTG
pDPG004-us-f	<u>GCCTTTTTTTTTTCGAAGTTT</u> CAGAAAAGAGTTTTTGAAAGTGCAGC
pDPG004-us-r1	<u>AGTAAAGAACGGCTCTTTC</u> CAGGATTAGTGGTATAATTAGTTTATTC
pDPG004-ds-f	<u>TAATTATACCACTAATCTCTG</u> GAAAGAGCCGTTCTTTACTATGAG
pDPG004-ds-r	<u>TGTAATTCCTGCAGGTTT</u> TACAGTAAGAACTATGAGTCAGGAG
pDPG005-us-f	<u>GCCTTTTTTTTTTCGAAGTTT</u> CCCTGAATGCAGAGCTGAAA
pDPG005-us-r	<u>TGAGGTGGTTCTGATAGGC</u> AACCACGGGTGAAATAGCTTC
pDPG005-ds-f1	<u>AAGCTATTTACCCGTGGTT</u> GCCTATCAGAACCACCTCAG
pDPG005-ds-r	<u>TGTAATTCCTGCAGGTTT</u> CTTTCATGTTTAGGGGACCTGC
pDPG006-us-r2	<u>TGAGGTGGTTCTGATAGGC</u> CAGGATTAGTGGTATAATTAGTTTATTCATG
pDPG006-ds-f2	<u>TAATTATACCACTAATCTCTG</u> GCCTATCAGAACCACCTCAGC
pDPG004_seq-f	AATTCCTACCTTCAGGCTCAAG
pDPG004_seq-r	TGACGCAGGATTTGAAGTTCAG
pDPG004, 005, 006_seq-r	ACAATGTCATTATTCAGATCAAGCGG
pDPG005_seq-f	ACTTCAAATCCTGCGTCAGTTGG
pDPG005_seq-r	TGTGTCAAATGTATATTGTTTGGTACGC
US_1kb-us- <i>ccmG</i> _seq-f	ACGGGAGAACGTCAGGTGAG
DS_1kb-ds- <i>ccmG</i> _seq-r	TGCCGGTGAAACTTCCTGTATTTG
US_1kb-us- <i>ccdA</i> -seq-f	TCCATAGCAAGGGCTCTGTCAC
DS_1kb-ds- <i>ccdA</i> -seq-r	TCGTAGCAATCGCAAGGGAAAGTG
<i>ccmABC</i> -f for pDPG014	<u>AATAAATTAAGGAGGAAATTCA</u> ATGGAACGCGGTCTCCATAAG
<i>ccmABC</i> -r for pDPG014	<u>TTTTAAAACCCCGTATGGCTCG</u> TTATGTAACACAAATTGAAAAACACAC
<i>ccmE</i> -f for pDPG014	<u>TTTTTCAATTTGTGTTACATAA</u> CGAGCCATACGGGGTTTTAAATG
<i>ccmE</i> -r for pDPG014	<u>ATTAAATGGGGAATAATAGTTT</u> TTACTCCGTATACTTTGACGGGC
<i>ccmF</i> ₁ <i>F</i> ₂ -f for pDPG014	<u>CCGTCAAAGTATACGGAGTAA</u> AAACTATTATTCCCCATTTAATTG
<i>ccmF</i> ₁ <i>F</i> ₂ -r for pDPG014	<u>TACATTATACGAAGTTATCAAGA</u> TCATTCTCGCTAAAATACTTC
<i>PserC</i> -f for pDPG015	<u>AGCCTTTTTTTTTTCGAAGTTT</u> GATCTCAACCACCTTTTTTC
<i>PserC</i> -r for pDPG015	<u>TAACCTATTTCATAATGACCAC</u> ATCTTTTCCTTTTTTGGTACTG
<i>mmcA</i> -TAP-f for pDPG015	<u>AGTACCAAAAAAGGAAAAAGAT</u> GTGGTCATTATGAATAGGTT

<i>mmcA</i> -TAP-r for pDPG015	<u>TAAAGTGTGTTGATTATTGAGT</u> TTAACCTGATTTTTCAAACTG
<i>Tmcr</i> (Ma)-f for pDPG015	<u>CCTCAGTTTGAAAAATCAGGTTAA</u> ACTCAATAAATCAAACACTTTAAAC
<i>Tmcr</i> (Ma)-r for pDPG015	<u>GCCGGCGCGCCTGCAGGTTT</u> GATTTCCGGAAAAACAGTAAAG
pDPG014_seq-r1	TCCTTCGGCAAGATTAGAAG
pDPG014_seq-f1	CTTCTAATCTTGCCGAAGG
pDPG014_seq-r2	TGCTTACATAAGCCGAACC
pDPG014_seq-f2	TCGGCTTATGTAAGCACCG
pDPG014_seq-r3	TGAAAAGATCTCGGTCTCC
pDPG014_seq-f3	TCAATTATGAGGTCGGTTTCG
pDPG014_seq-r4	AGTCCTCAAAACATGAGAAATC
pDPG014_seq-f4	TCATGTTTTGAGGACTTCAGG
pDPG014_seq-r5	ATCTCAGGCAGCAAATTTCC
pDPG014_seq-f5	TAGGAAATTTGCTGCCTGAG
pDPG014_seq-r6	ATCCTTTGCCCAGATCGAAC
pDPG014_seq-f6	TCGATCTGGGCAAAGGATG
<i>ccmE</i> -f for pKES30, 38, 40	<u>TGATTTTAATAAAATTAAGGAGGAAATTCAT</u> ATGAATAAAAAAAGAAATCACTGC
<i>ccmE</i> -r1 for pKES30	<u>CAAACCTGAGGATGTGACCACCCGCCACCCT</u> CCGTATACTTTGACGGGCA
<i>ccmE</i> -r2 for pKES30	<u>GTCGTCGTCCTTGAGTCCCCGCCACCT</u> TTTCAAACCTGAGGATGTGACC
<i>ccmE</i> (C120A)-r1 for pKES40	<u>TTACTCCGTATACTTTGACGGTGC</u> TCCGGTAATGATCTTATTTGCC
<i>ccmE</i> (C120H)-r1 for pKES38	<u>TTACTCCGTATACTTTGACGGGTG</u> TCCGGTAATGATCTTATTTGCC
<i>ccmE</i> (C120A)-r2 for pKES40	<u>CAAACCTGAGGATGTGACCACCCGCCACCCTC</u> CGTATACTTTGACGGTGC
<i>ccmE</i> (C120H)-r2 for pKES38	<u>CAAACCTGAGGATGTGACCACCCGCCACCCTC</u> CGTATACTTTGACGGGTG
<i>ccmE</i> -r3 for pKES30, 38, 40	<u>GCATACATTATACGAAGTTATCAAGATTAGAT</u> GTCGTCGTCCTTGTAGTC
pDN402_us_f	<u>TTTGAGCCTTTTTTTTCGAAGTTTAAAC</u> CGG TTG ACT ATG CAG GTA CG
pDN402_us_r	CAG TAG AAG CAG CAC AGC GA
pDN402_ds_f	<u>GTGTCGGGAGTCGCTGTGCTGCTTCTACTG</u> GTA ACT TAC GAT AAG CCA AGA CC
pDN402_ds_r	<u>GTGTAATTCGGCGCGCCTGCAGGTTTAAAC</u> GAC GGT ATT CTT AAC CAC AGG
DDN009_seq_f	CCT GTT TGG TCC AAT ACA TC
DDN009_seq_r	ACT TCA GCT TCT GTC TGG AC
<i>mmcA</i> _f for pDN409	<u>TGATTTTAATAAAATTAAGGAGGAAATTCAT</u> GTG GTC ATT ATG AAT AGG TT
<i>mmcA</i> _r for pDN409	GAG TGC AGG CTC CGT TTC A
C_TAPtag_f for pDN409	<u>CCTGTAGAAGTTGAAACGGAGCCTGCACTC</u> GAC GAT GAC GAC AAG GGT GG
C_TAPtag_r for pDN409	<u>AGCATACATTATACGAAGTTATCAAGATTA</u> ACCTGATTTTCAAACCTGAGG
pDN409_seq_f	TGA GAT TTC CCT ATC AGT GAT
pDN409_seq_r	CTC ACT AAA GGG ATA ACT TCG
pDN412_us_f	<u>TTTGAGCCTTTTTTTTCGAAGTTTAAAC</u> CAC CGG TAA GAA AAG AAT CTG G
pDN412_us_r	<u>GGTAATGATCTTATTTGCCTCAAACGTAGA</u> AGC AAC TGC AAG CAG TGA TT
pDN412_ds_f	TCT ACG TTT GAG GCA AAT AAG
pDN412_ds_r	<u>GTGTAATTCGGCGCGCCTGCAGGTTTAAAC</u> CTA AAT GGT ATC ATT TGT GGC

DDN010_seq_f	CAA CTG AAA ATG ACT TTT CG
DDN010_seq_r	ATA CAC CTG ATA AAG TTA GTG
pDN424_us_f	TTG TGA AAT TGC TCA CGA AC
pDN424_us_r	GAG AAC ATC GCT TAG CTT TTT C
pDN424_ds_f	TTTTTTATGAAAAAGCTAAGCGATGTTCTC GCT CAG GTA AAC GGA ATC AA
pDN424_ds_r	GTGTAATTCGGCGCGCCTGCAGGTTTAAAC AAA GGT CGA GAA GAT GAC C
pDN425_us_f	TTTGGAGCCTTTTTTTTTTCGAAGTTTAAAC CAG CAC CAT ATT TGC TAC AG
pDN425_us_r	TAT CCA GAT GAG TCC CAT TC
pDN425_ds_f	ATGAATACTGGAATGGGACTCATCTGGATA GTT CTC CTT GTC AGG AAG TA
pDN425_ds_r	GTGTAATTCGGCGCGCCTGCAGGTTTAAAC GTG AAG CCA ACA ACA ACT TCC
pDN426_ds_f	GTTTTTTATGAAAAAGCTAAGCGATGTTCTC GTT CTC CTT GTC AGG AAG TA
DDN011_012_013_seq_f	TTT TCG TCC AGC ACT TTT AC
DDN011_012_013_seq_r	TAC CAG GGT TCT TTC TTG AC
pDN444_us_f	TTTGGAGCCTTTTTTTTTTCGAAGTTTAAAC GAG CAG GAA GAA AAG AGC TTG
pDN444_us_r	TCCGGCAGATCTGCGTGCCTCAAGTTCCTC TGA TAG TTC CCT TAT GGA GAC C
pDN444_ds_f	GAG GAA CTT GAC GCA CGC AG
pDN444_ds_r	GTGTAATTCGGCGCGCCTGCAGGTTTAAAC CGG CAT AAT CCG CTC TGA CTG
DDN029_seq_f	GGA GTT CCA GGT TAC AGA CTG
DDN029_seq_r	GTC TGG TGC TGA TTA TGG AC

Supplementary Table 6: List of *Methanosarcina* strains used in this study

Strains	Genotype	Construction details	Source
<i>M. acetivorans</i> strains			
WWM60	$\Delta hpt::PmcrB-tetR$	-	(Guss et al., 2008)
DDN009	$\Delta hpt::PmcrB-tetR, \Delta mmcA$	WWM60 was transformed to Pur ^R with pDN404; plasmid-cured strain was isolated by plating on medium with 8ADP	This study
DDN010	$\Delta hpt::PmcrB-tetR, \Delta ccmE$	WWM60 was transformed to Pur ^R with pDN414; plasmid-cured strain was isolated by plating on medium with 8ADP	This study
DDN011	$\Delta hpt::PmcrB-tetR, \Delta ccmF_1$	WWM60 was transformed to Pur ^R with pDN430; plasmid-cured strain was isolated by plating on medium with 8ADP	This study
DDN012	$\Delta hpt::PmcrB-tetR, \Delta ccmF_2$	WWM60 was transformed to Pur ^R with pDN431; plasmid-cured strain was isolated by plating on medium with 8ADP	This study
DDN013	$\Delta hpt::PmcrB-tetR, \Delta ccmF_1 \Delta ccmF_2$	WWM60 was transformed to Pur ^R with pDN432; plasmid-cured strain was isolated by plating on medium with 8ADP	This study
DDN016	DDN009/ pDN409 [<i>PmcrB(tetO4)-mmcA-C-TAP tag</i>]	DDN009 was transformed to Pur ^R with pDN409; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN029	$\Delta hpt::PmcrB-tetR, \Delta ccmABC$	WWM60 was transformed to Pur ^R with pDN446; plasmid-cured strain was isolated by plating on medium with 8ADP	This study
DDN033	$\Delta hpt::PmcrB-tetR, \Delta ccmG$	WWM60 was transformed to Pur ^R with pDPG007; plasmid-cured strain was isolated by plating on medium with 8ADP	This study
DDN034	$\Delta hpt::PmcrB-tetR, \Delta ccdA$	WWM60 was transformed to Pur ^R with pDPG008; plasmid-cured strain was isolated by plating on medium with 8ADP	This study
DDN035	$\Delta hpt::PmcrB-tetR, \Delta ccmG \Delta ccdA$	WWM60 was transformed to Pur ^R with pDPG009; plasmid-cured strain was isolated by plating on medium with 8ADP	This study
DDN037	DDN009/pDPG010 [<i>PmcrB(tetO4)-uidA</i>]	DDN009 was transformed to Pur ^R with pDPG010; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN038	WWM60/pDPG010 [<i>PmcrB(tetO4)-uidA</i>]	WWM60 was transformed to Pur ^R with pDPG010; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN039	WWM60/pDN409 [<i>PmcrB(tetO4)-mmcA-C-TAP tag</i>]	WWM60 was transformed to Pur ^R with pDN409; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN040	DDN010/pDN409 [<i>PmcrB(tetO4)-mmcA-C-TAP tag</i>]	DDN010 was transformed to Pur ^R with pDN409; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN041	DDN029/pDN409 [<i>PmcrB(tetO4)-mmcA-C-TAP tag</i>]	DDN029 was transformed to Pur ^R with pDN409; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN047	DDN013/pDN409 [<i>PmcrB(tetO4)-mmcA-C-TAP tag</i>]	DDN013 was transformed to Pur ^R with pDN409; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN052	DDN012/pDN409 [<i>PmcrB(tetO4)-mmcA-C-TAP tag</i>]	DDN012 was transformed to Pur ^R with pDN409; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN054	DDN011/pDN409 [<i>PmcrB(tetO4)-mmcA-C-TAP tag</i>]	DDN011 was transformed to Pur ^R with pDN409; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN060	DDN033/pDN409 [<i>PmcrB(tetO4)-mmcA-C-TAP tag</i>]	DDN033 was transformed to Pur ^R with pDN409; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN062	DDN034/pDN409 [<i>PmcrB(tetO4)-mmcA-C-TAP tag</i>]	DDN034 was transformed to Pur ^R with pDN409; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN064	DDN035/pDN409 [<i>PmcrB(tetO4)-mmcA-C-TAP tag</i>]	DDN035 was transformed to Pur ^R with pDN409; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN078	DDN010/pKES31 [<i>PmcrB(tetO4)-ccmE-C-TAP^m tag</i>]	DDN010 was transformed to Pur ^R with pKES031; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN091	DDN029/pKES31 [<i>PmcrB(tetO4)-ccmE-C-TAP^m tag</i>]	DDN029 was transformed to Pur ^R with pKES031; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study

DDN092	DDN010/pKES39 [PmcrB(tetO4)-ccmE(C120H)-C-TAP ^m tag]	DDN010 was transformed to Pur ^R with pKES039; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN093	DDN010/pKES41 [PmcrB(tetO4)-ccmE(C120A)-C-TAP ^m tag]	DDN010 was transformed to Pur ^R with pKES041; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN099	WWM60/ pKES31 [PmcrB(tetO4)-ccmE-C-TAP ^m tag]	WWM60 was transformed to Pur ^R with pKES031; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
<i>M. barkeri</i> strains			
WWM155	$\Delta hpt:: PmcrB-tetR-phiC3Int-attP$	-	(Guss et al., 2008)
DDN085	WWM155/pJK029A [PmcrB(tetO4)-uidA]	WWM155 was transformed to Pur ^R with pJK029; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN086	WWM155/pDPG014 [PmcrB(tetO4) -(ccmABCEF ₁ F ₂) _{Ma}]	WWM155 was transformed to Pur ^R with pDPG014; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN087	WWM155/pDPG015 [PserC-(mmcA) _{Ma} -C-TAP tag]	WWM155 was transformed to Pur ^R with pDPG015; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN088	WWM155/pDPG017 [PserC-(mmcA) _{Ma} -C-TAP tag, PmcrB(tetO4)-(ccmABCEF ₁ F ₂) _{Ma}]	WWM155 was transformed to Pur ^R with pDPG017; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study

Supplementary References

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Supplementary Material

Title: Functional Characterization of *c*-type Cytochrome Biogenesis in the Archaeon, *Methanosarcina acetivorans*

Authors: Dinesh Gupta¹, Katie E. Shalvarjian², Dipti D. Nayak^{1*}

Department of Molecular and Cell Biology, University of California, Berkeley, CA, USA¹;

Department of Plant and Microbial Biology, University of California, Berkeley, CA, USA²

Running Head: *c*-type cytochrome biogenesis in archaea

*Address correspondence to:

Dipti D. Nayak (dnayak@berkeley.edu)

Department of Molecular and Cell Biology, 1 Barker Hall #3204, University of California,
Berkeley, CA 94720-3204

Tel: 510-664-5267

Supplementary Figure 1: a) Chromosomal organization of the System I c-type cytochrome maturation (Ccm) machinery genes in a few representative bacteria and the archaeon, *Methanosarcina acetivorans*. In bacteria like **i)** *Escherichia coli* **ii)** *Rhodobacter capsulatus* and **iii)** *Desulfovibrio desulfuricans*, 9-10 genes (*ccmABCDEF GHI* and *dsbD/ccdA*) are involved in the maturation of c-type cytochromes (cyt c), and most of the *ccm* genes are found in an operon. **iv)** The *ccm* genes in the archaeon *M. acetivorans* and other methane metabolizing archaea belonging to the Order *Methanosarcinales* are not found in a single operon and, notably, homologs of three essential genes, *ccmD*, *ccmH*, and *ccmI*, are absent. Double dashed lines indicate a distance >110 kbp on the chromosome. **b)** An overview of the System I Ccm machinery from *E. coli*. The apo cyt-c is transported across the inner cytoplasmic membrane into the periplasmic space using the Sec system. The CcmABCD membrane-bound complex transports heme b to form heme-bound holo-CcmE in an ATP-dependent manner. Holo-CcmE delivers heme b to the membrane-bound cyt c synthetase complex, CcmFHCcmF, that catalyzes the formation of holo cyt c. CcmG and DsbD/CcdA facilitate the transfer of electrons to reduce the disulfide bond between the cysteine residues in the CXXCH motif of the apo cyt c prior to the formation of holo cyt c. Figure adapted from Kranz *et al.*, 2009 (1).

a**>MmcA (MA0658) *M. acetivorans***

MVIMNRLNLLVSGVAVLLLLLAAGAYSSLGYSGNDAIASHYMTKGEWSDSV**CGGCH**FGVYENVNNSYHVQ
 VNMSRWSPLTNFDLETSGEEWVKKFGMYHPGGPLAKYGIDID**CMMCHE**KYGLYDFDARAEAIANGDF
 ANANSLAVANFSATAQSDPLHLFVYTANVLTPYPLLIVFHDVANGAPIS**CAQQRCH**RIDVETSAVMWADE
 EDFEESDAHAANGVE**CTECH**HTEAFIITS DHQIGRGNTSGTPDLPDSHYDDTMR**SCDDAECH**AGISHGP
 FADSHMEFLA**CEACH**TPELPGGDLPGGNVLESFSWQNGEREDVYRSDSQPALAWYNGNFGDVLPSVDT
 RNDTDVKVTPFNNTGTWWDAGTDPEVLANPNTSISTGDPPIPVQYVKAADANGDGEVTVTEEMQAYDADG
 DGEADYPNAVLRITVELYYQVAHSIVSSDIGLADPYT**CKDCH**GNEAVIDWAALGYEQDPGGESSAVKSI
 AVTYDKPRPVEVETEPAL*

b

Supplementary Figure 2: a) Amino acid sequence of the c-type cytochrome (cyt c) MmcA from *Methanosarcina acetivorans*. The predicted signal peptide is shown in blue, five canonical heme-binding motifs (CXXCH) are shown in red and two non-canonical heme-binding motifs (CXXXCH and CXXXXCH) are shown in orange. Based on these observations, MmcA is a heptaheme cyt c that is present in the pseudo-periplasm of *M. acetivorans*. **b)** MmcA is associated with the **R**hodobacter **n**itrogen **f**ixation (Rnf) bioenergetic complex in methanogens and other alkane metabolizing archaea. The Rnf complex catalyzes the electron transfer reaction between cytosolic Ferredoxin (Fd) and the membrane-associated electron carrier Methanophenazine (MP) coupled to the translocation of Na⁺ ions across the cytoplasmic membrane. MmcA has been hypothesized to play two roles in the context of the Rnf bioenergetic complex: i) it is involved in the extracellular transfer of electrons from/to Fd or ii) it directly interacts with MP to mediate the final step in the intracellular electron transport reactions from Fd to MP. The reduced MPH₂ generated by the Rnf complex is regenerated by the membrane-bound Heterodisulfide reductase (Hdr) that couples the translocation of protons (H⁺) to the transfer of electrons from MP to the CoM-S-S-CoB disulfide that acts as the terminal electron acceptor in cytochrome-containing methanogens like *Methanosarcina acetivorans*.

CcmF (<i>E. coli</i>)	MMPEIGNGLLCLALGIALLLSVYPLWGVARGDARMMASSRLFAWLLFMSVAGAFVLVNA	60
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	--MNTGMGLIWIAGASGLLAFLTSLLYFFRQDRKFLALSEKLELI--AGVGLVFAIFLLA	56
	: * ** : * . . ** : * . * * : : * . : : . * . * : : *	
CcmF (<i>E. coli</i>)	--FVVNDFTVTYVASNSNTQLPVWYRVAATWGAHEGSLLLWVLLMSGWTFAVAIFSQRIP	118
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	FYLLGVDLTQYSYVFQHSSTDLAWHYRFSALWAGQEGSFLIWTGFIIVML-AITRFTGTGK	115
	: : * ** : : * . * : : * : : * : : * : : * : : * : : *	
CcmF (<i>E. coli</i>)	LDIVARVLAIMGV---SVGFLLFILFTSNPFSRT-----LPNFPIEG	158
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	VLRETEIFSIMRSVSLFVASVFLLLLALKNPFSMYYTGTGVPDVTNWNLFAPFPVVSYG	175
	: : : : * * * . . . : : * * * . . . * . *	
CcmF (<i>E. coli</i>)	RDLNPLLQDPGLIFPPLLYMGYVGFSAFAFAIASLLSGRLDSTYARFTRPWTLAAWIF	218
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	QGMNPLLNRNPWMAIHPPLLFLGYAAFTLPFSAAGGLVL--KDSRWSELATGWMRVSWLF	233
	: : * : : * : : * : : * : : * : : * : : * : : * : : * : : *	
	176 WWD Domain 261	
CcmF (<i>E. coli</i>)	LTLGIVLGSAAWYYELGWGGW-WFWDPVENASFMPWLVTALMHS LAVTEQRASFKAATL	277
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	LTLGIGFGAFWAYEVLGWGAWYWTWDPVETSSLIPLWTATAYLHAKLRAR-QGEYGFLLP	292
	***** : * : * * * * * * * * * * * * : : * : : *	
CcmF (<i>E. coli</i>)	LLAISAFSLCLLGTFLVRSGVLVSVFAFASDPARGMFILAFMVLVIGSGSLLLFAARGHKV	337
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	MLALVSFILVIFSTFVTRSGLVSVHWSQDFTMEGMIIALFLVVLIGSSAVLLVRKYFSE	352
	: * : : * * : : * : * : * : * : : . . * : * * : : * : * : : . .	
CcmF (<i>E. coli</i>)	RS-----RVNNALWSRESLLLANNVLLVAAMLVLLGTLPLVHKQL	379
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	ELYLFMKFYFLFTFSRFFMKKLSVDLTVRSTIFATVSV-LVLLAALITMGLLTPFILRV	411
	. : : : * * : : : : * * : : : * * * : : * * * : : *	
CcmF (<i>E. coli</i>)	GLGSISIGEPFFNTMFTWLMVPFALLGVGPLVRWGRDRPRKIRNLLIIA--FISTLVLS	437
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	TGEEILLDSAYFNLRAALPTLALVMLLTCLLL--GSAGKK--EALLALGLGVAGSVLSV	467
	. * . . : * * : : : : * * : * : * : . * * . . : : :	
CcmF (<i>E. coli</i>)	LLLPLWLFESKVVAMTVLGLAMACWIAVLAIAEAAALRISRGTKTTSFYWGMVA AHLGLAVT	497
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	VFSPFSSLPVNIISFSL--AVALFAVMYRLLS---SKEKTLKGLTRRAGSHIHLGVVLL	522
	: : * : : : : * * : : : : . . : : * * : * * : : *	
CcmF (<i>E. coli</i>)	IVGIAFSQNSYVERDVRMKSGSDVDIHEYRFTFRDVKEVT---G---PNWRGGVAT----	547
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	LVGILFSTNMKLEDSAAVPVGEVGTFSQMGYSIHVTNITSIGIEGAPYGN YQGSAYVSTVY	582
	: * * * * . * . . : * : : : : : : : * * : * : * . .	
CcmF (<i>E. coli</i>)	IGVTRDGKPETVLVYAEKRYNT--AGSMTEAAIDGGITRDLYAALGE-ELENGAWAVRL	604
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	FDVYRWGQPFDS--GQVYISDFKWEQSYTETIYHRLLEELFIAPKSVDTKTQTVELYV	640
	: . * * * * . : * * . . * : * . * : : * : * . : : : : :	
CcmF (<i>E. coli</i>)	YYKPFVRWIWAGGLMMALGGLLCLFDPRYRKRVPNPQKTAPEAA-----	647
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	RKVPFMTSLWGGFYLMVLGILMLFISDSLAGE---KGSPGNGLSGTLD SEREQVNGIK	696
	* : : * : * : * : * : * : : . . * : * .	
CcmF (<i>E. coli</i>)	-----	647
CcmF ₂ F ₁ (<i>M. acetivorans</i>)	NDSKKKRVSKKNV	709

Supplementary Figure 3: Alignment of the CcmF amino acid sequence from *Escherichia coli* with the concatenated CcmF2-CcmF1 amino acid sequences from *Methanosarcina acetivorans*. Based on this alignment, the CcmF2 sequence (in blue) from *M. acetivorans* is homologous to N-terminus of CcmF from bacteria and the CcmF1 (in purple) sequence of *M. acetivorans* is homologous to the C-terminus of CcmF from bacteria. The highly conserved WWD domain, and three conserved histidine residues that serve as axial ligands for heme *b* in the membrane (His261 in *E. coli*) and the periplasm (His176 and His 303 in *E. coli*) are all conserved in the CcmF2 sequence of *M. acetivorans*.

Supplementary Figure 4: Expression of CcmE with a C-terminal 1XFLAG-1X Strep tag from a plasmid (pE) in the parent strain of *Methanosarcina acetivorans* (WWM60) and the $\Delta ccmE$ background leads to the production of heme-bound holo-MmcA as detected by a heme peroxidase assay of whole cell lysates (Lanes 1, 3 of heme-stained gel). Thus, the C-tagged CcmE is functional and facilitates the maturation of native c-type cytochromes (cyt c) like MmcA. In contrast, expression of C-tagged CcmE in the $\Delta ccmABC$ background does not produce any holo-MmcA (Lane 2 of heme stained gel). In the absence of CcmABC, holo-CcmE does not form, which, in turn, prevents the maturation of native cyt c like MmcA. All cultures were grown in medium containing 100 $\mu\text{g/ml}$ tetracycline to induce the expression of C-tagged CcmE from the plasmid pE. An equal amount of protein ($\sim 20 \mu\text{g}$) was loaded in each lane.

$\Delta ccmE$ *M. acetivorans*

Supplementary Figure 5: Expression of the C120H mutant of CcmE from *Methanosarcina acetivorans* with a C-terminal 1XFLAG-1X Strep tag in the $\Delta ccmE$ background does not lead to the production of any CcmE protein that can be detected by Heme peroxidase assays (top gel) or Western Blot using an anti-Flag antibody (bottom gel) in the Strep-enriched membrane fraction (Lane 1) or the soluble fraction (Lane 2). In contrast, expression of the WT sequence CcmE from *M. acetivorans* with a C-terminal 1XFLAG-1X Strep tag in the $\Delta ccmE$ background leads to the production of protein that can be detected by Heme peroxidase assays (top gel) and Western Blot using an anti-Flag antibody (bottom gel) in the Strep-enriched soluble fraction (Lane 3) and the membrane fraction (see Figure 4). Strep-enriched membrane or soluble fraction represents protein eluted after passing the membrane or the soluble fraction through a Strep-affinity column. The C120H mutant was sequenced verified prior to protein purification. Based on these data, it is highly likely that the C120H substitution destabilizes the CcmE protein. All cultures were grown in medium containing 100 μ g/ml tetracycline to induce the expression of the tagged CcmE protein. An equal amount of protein (~ 1 μ g) was loaded in each lane.

Supplementary Figure 6: Expression of the wildtype (WT) sequence of CcmE from *Methanosarcina acetivorans* with a C-terminal 1XFLAG-1X Strep tag (C-tagged CcmE) in the $\Delta ccmE$ background leads to the production of heme-bound holo-MmcA as detected by a heme peroxidase assay of whole cell lysates (Lane 1 of heme-stained gel). Thus, the C-tagged WT sequence of CcmE is functional and facilitates the maturation of native c-type cytochromes (cyt c) like MmcA. In contrast, expression of C-tagged C120H or C120A mutants of CcmE in the $\Delta ccmE$ background does not produce any holo-MmcA (Lanes 2,3 of heme stained gel). Thus, the C120H and C120A mutants disrupt the function of CcmE, which, in turn, prevents the maturation of native cyt c like MmcA. All cultures were grown in medium containing 100 $\mu\text{g/ml}$ tetracycline to induce the expression of C-tagged CcmE. An equal amount of protein ($\sim 20 \mu\text{g}$) was loaded in each lane.

Supplementary Figure 7: (Left) Heme-peroxidase assays to detect proteins that covalent bind heme (including c-type cytochromes) in whole cell lysates of *Methanosarcina acetivorans* (Lanes 1, 2, 3) and *Methanosarcina barkeri* (Lanes 4, 5, 6) grown in high-salt minimal medium with 125 mM methanol as the sole source of carbon and energy. Three different concentrations (~30, 60, 90 µg) of whole cell lysate were used per strain for these assays as shown in the Coomassie gel control (right).

Supplementary Figure 8: **a)** Methanogenic metabolism of trimethylamine (TMA) in *Methanosarcina acetivorans*. Green arrows represent TMA, dimethylamine (DMA), monomethylamine (MMA) specific methyltransferase reactions that catalyze the transfer of each of the methyl groups in TMA to ultimately form methyl-CoM (CH₃-CoM). CH₃-CoM undergoes a disproportionation reaction to generate CO₂ and CH₄ in a 3:1 ratio. **b)** Methanogenic metabolism of methanol in *Methanosarcina acetivorans*. The purple arrow represents the methanol specific reaction that catalyzes the transfer of the methyl group to ultimately form methyl-CoM (CH₃-CoM). CH₃-CoM undergoes a disproportionation reaction to generate CO₂ and CH₄ in a 3:1 ratio. **c)** Methanogenic metabolism of acetate in *Methanosarcina acetivorans*. The yellow arrow represents the reaction catalyzed by the acetyl-CoA decarbonylase/synthase (ACDS) enzyme complex that splits acetate into an enzyme bound carbonyl group and a methyl group that is transferred to the C₁ carrier tetrahydrosarcinopterin (H₄SPT). Oxidation of the carbonyl group to CO₂ produces electrons that can be used to reduce the methyl group to CH₄. During acetoclastic methanogenesis, CO₂ and CH₄ are produced in a 1:1 ratio. **d)** Methanogenic metabolism of dimethyl sulfide (DMS) in *Methanosarcina acetivorans*. The orange arrows represent DMA and methanethiol (MeSH) specific reactions that catalyze the transfer of the methyl groups from DMS to ultimately form methyl-CoM (CH₃-CoM). CH₃-CoM undergoes a disproportionation reaction to generate CO₂ and CH₄ in a 3:1 ratio.

Supplementary Figure 9: A phylogenomic reconstruction of the relationship between various genera (depicted by different colors along the circumference of the radial tree) within the Family *Methanosarcinaceae* from Annotree (Mendler *et al.*, 2019). Purple lines depict strains that encode homologs of *ccmABCEF1F2* in their genome whereas black lines depict strains that lack homologs of *ccmABCEF1F2* in their genome. Based on the pattern of distribution of the *ccmABCEF1F2* genes it is likely that these genes were present in the last common ancestor of the *Methanosarcinaceae* and have been lost in members of various Genera including *Methanosarcina* spp., *Methanohalophilus* spp., *Methanomethylovorans* spp., *Methanolobus* spp. and *Methanoperedens* spp.

Phylogenetic tree of CcmA

Supplementary Figure 10: An unrooted maximum-likelihood tree of 500 CcmA sequences retrieved from the NCBI non-redundant protein sequence database using the corresponding sequence from *Methanosarcina acetivorans* as the search query. Branch labels indicate support values.

Phylogenetic tree of CcmB

■ Methane metabolizing archaea

Chloroflexi

Nitrosospinae

■ Other bacteria

Supplementary Figure 11: An unrooted maximum-likelihood tree of 500 CcmB sequences retrieved from the NCBI non-redundant protein sequence database using the corresponding sequence from *Methanosarcina acetivorans* as the search query. Branch labels indicate support values.

Phylogenetic tree of CcmC

Supplementary Figure 12: An unrooted maximum-likelihood tree of 500 CcmC sequences retrieved from the NCBI non-redundant protein sequence database using the corresponding sequence from *Methanosarcina acetivorans* as the search query. Branch labels indicate support values.

Phylogenetic tree of CcmE

- Methane metabolizing archaea
- Chloroflexi*
- Actinobacteria
- Nitrosospinae*
- Desulfovibrio spp.*
- Acidobacteria
- Bacillus spp.*
- Other archaea
- Other bacteria

Supplementary Figure 13: An unrooted maximum-likelihood tree of 500 CcmE sequences retrieved from the NCBI non-redundant protein sequence database using the corresponding sequence from *Methanosarcina acetivorans* as the search query. Branch labels indicate support values.

Phylogenetic tree of CcmF1

Methane metabolizing archaea

Chloroflexi

Psychrobacter spp.

Sulfate reducing bacteria

Acidobacteria

Other bacteria

Supplementary Figure 14: An unrooted maximum-likelihood tree of 500 CcmF1 sequences retrieved from the NCBI non-redundant protein sequence database using the corresponding sequence from *Methanosarcina acetivorans* as the search query. Branch labels indicate support values.

Phylogenetic tree of CcmF2

Supplementary Figure 15: An unrooted maximum-likelihood tree of 500 CcmF2 sequences retrieved from the NCBI non-redundant protein sequence database using the corresponding sequence from *Methanosarcina acetivorans* as the search query. Branch labels indicate support values.

Supplementary Table 1: List of mutations in CRISPR-edited mutant strain DDN029 containing a $\Delta ccmABC$ in-frame deletion mutation

Position	Mutation	Notes
487691	C added	Present in Parent (WWM60)
836182-836771	$\Delta hpt::PmcrB-tetR$	Present in Parent (WWM60)
941168	A5->6	Present in Parent (WWM60)
1314120	$\Delta 1bp$	Present in Parent (WWM60)
1698001	$\Delta 2684 bp$	$\Delta ccmABC$ (only present in DDN029)
2086881-82	TC->CT	Present in Parent (WWM60)
2086886	G->T	Present in Parent (WWM60)
2534543	C added	Present in Parent (WWM60)
2836646	A->G (F64L)	Present in Parent (WWM60)
2867059	T->G (H313Q)	Present in Parent (WWM60)
3433201	$\Delta 1bp$	Present in Parent (WWM60)
4295452	$\Delta 1bp$	Present in Parent (WWM60)
4874567	$\Delta 1bp$	Present in Parent (WWM60)
4945345	C added	Present in Parent (WWM60)
5078585	G->A (M1M)	Present in Parent (WWM60)

Supplementary Table 2: Growth data of *M. acetivorans* strains shown in Figure 6

<i>M. acetivorans</i> strains	Doubling time (Td) (hrs)	Max.OD ₆₀₀ (OD ^M)	P value for WT vs mutants (Td/OD ^M)	P value for $\Delta mmcA$ vs Δccn mutants (Td/OD ^M)	P value for $\Delta ccnABC$ vs $\Delta ccnE$ mutants (Td/OD ^M)
Trimethylamine (TMA) as a growth substrate					
WWM60 (WT)	12.13 ± 0.04	1.29 ± 0.01			
$\Delta mmcA$	19.89 ± 0.44	1.14 ± 0.02	3.73E-05 (***)/ 0.0013 (**)		
$\Delta ccnABC$	20.08 ± 0.37	1.15 ± 0.02	2.70E-05 (***)/ 0.0011 (**)	0.4661 (NS)/ 0.1832 (NS)	
$\Delta ccnE$	20.19 ± 0.15	1.15 ± 0.01	2.21E-06 (***)/ 0.0002 (**)	0.2523 (NS)/ 0.5187 (NS)	0.3856 (NS)/ 0.8431 (NS)
Methanol (MeOH) as a growth substrate					
WWM60 (WT)	10.11 ± 0.23	1.13 ± 0.00			
$\Delta mmcA$	27.23 ± 0.28	0.92 ± 0.00	6.66E-07 (***)/ 8.30E-07 (***)		
$\Delta ccnABC$	29.40 ± 0.58	0.90 ± 0.01	1.89E-05 (***)/ 2.28E-05 (***)	0.0131(*)/ 0.0477(*)	
$\Delta ccnE$	29.52 ± 0.28	0.90 ± 0.01	5.85E-08 (***)/ 4.71E-06 (***)	6.06E-05 (***)/ 0.0036(**)	0.7980 (NS)/ 0.0749(NS)
Acetate as a growth substrate					
WWM60 (WT)	77.93 ± 4.32	0.22 ± 0.01			
$\Delta mmcA$	No growth observed [#]	-	-		
$\Delta ccnABC$	No growth observed [#]	-	-	-	
$\Delta ccnE$	No growth observed [#]	-	-	-	-
Dimethyl sulfide (DMS) as a growth substrate					
WWM60 (WT)	32.6 ± 0.55	0.35 ± 0.01			
$\Delta mmcA$	53.7 ± 5.66	0.26 ± 0.02	0.0247 (*)/ 0.0053(**)		
$\Delta ccnABC$	51.1 ± 2.84	0.33 ± 0.00	0.0093 (***)/ 0.0474 (*)	0.2515 (NS)/ 0.0248 (*)	
$\Delta ccnE$	41.6 ± 1.68	0.36 ± 0.00	0.0055 (***)/ 0.4266 (NS)	0.0876 (NS)/ 0.0215 (*)	0.0568 (NS)/ 0.0148 (*)

All data represent the mean ± standard deviation of at least 3 biological replicates at 37 °C

No growth observed at 37 °C for 50 days

P > 0.05 (non-significant, NS); P ≤ 0.05 (*); P ≤ 0.01 (**); P ≤ 0.0001 (***) using a two-sided Student's t-test

Supplementary Table 3: List of target sequences used in this study

Gene (locus tag)	Target sequence (+ PAM)	Location on <i>M. acetivorans</i> chromosome
<i>mmcA</i> (MA0658)	AGTTACAGCAATACTCTTCA <u>CGG</u> TGAATACCCAAGAGAAGAAT <u>AGG</u>	776339 – 776358 (- strand) 774977 – 774996 (- strand)
<i>ccmABC</i> (MA1428-1430)	AAAAGAGGGTCAAAAAGAGC <u>AGG</u> AGGTCAAGGGCTTTCAGGAC <u>AGG</u>	1698029 – 1698048 (+ strand) 1700643 – 1700662 (+ strand)
<i>ccmF₂</i> (MA3304)	CAGGCAGGACAGGAAATTTT <u>TGG</u> GTCCCTGATTCCCTGGCTTA <u>CGG</u>	4078616 – 4078635 (- strand) 4077905 – 4077924 (- strand)
<i>ccmF₁</i> (MA3305)	CACAGAGACTGTAGCAAATA <u>TGG</u> TCTCCCGGAAACGGCCTTTC <u>CGG</u>	4079700 – 4079719 (+ strand) 4078,828 – 4078847 (- strand)
<i>ccmE</i> (MA4149)	TGGTGTGACATCATTTGAAC <u>TGG</u>	5066266 - 5066285 (+ strand)
<i>ccmG</i> (MA4254)	GCATCTGTAATCTTTACGGC <u>AGG</u>	5177988 – 5178007 (+ strand)
<i>ccdA</i> (MA4255)	TCTCCTTGTAGGGCTCTGGA <u>TGG</u>	5179184 – 5179203 (+ strand)

Supplementary Table 4: List of plasmids used in this study

Plasmid	Features	Source
pAMG40	Vector for fosmid retrofitting that contains pC2A and λ attB	(Guss et al. 2008)
pJK027A	Vector with <i>PmcB(tetO1)</i> promoter fusion to <i>uidA</i> that contains ϕ C31-attB and λ attP	(Guss et al. 2008)
pJK029A	Vector with <i>PmcB(tetO4)</i> promoter fusion to <i>uidA</i> that contains ϕ C31-attB and λ attP	(Guss et al. 2008)
pDN201	pJK027A-derived plasmid with <i>PmcB(tetO1)</i> promoter fusion to Spy <i>cas9</i>	(Nayak and Metcalf, 2017)
pDN401	pDN201-derived plasmid with synthetic fragments containing <i>PmtaCB1</i> promoter fusion to sgRNAs in tandem targeting the <i>mmcA</i> (MA0658) coding sequence (CDS)	This study
pDN402	pDN401-derived plasmid containing a repair template to generate an in-frame deletion of the <i>mmcA</i> (MA0658) CDS	This study
pDN403	Cointegrate of pDN401 and pAMG40	This study
pDN404	Cointegrate of pDN402 and pAMG40	This study
pDN406	pJK029A-derived plasmid with <i>PmcB(tetO4)</i> promoter fusion to <i>mmcA</i> (MA0658) CDS from <i>M. acetivorans</i> with a C-terminal tandem affinity purification tag (containing a 3X FLAG tag and a Twin-Strep tag)	This study
pDN409	Cointegrate of pDN406 and pAMG40	This study
pDN411	pDN201-derived plasmid with synthetic fragments containing <i>PmtaCB1</i> promoter fusion to sgRNAs in tandem targeting the <i>ccmE</i> (MA4149) coding sequence (CDS)	This study
pDN412	pDN411-derived plasmid containing a repair template to generate an in-frame deletion of the <i>ccmE</i> (MA4149) CDS	This study
pDN413	Cointegrate of pDN411 and pAMG40	This study
pDN414	Cointegrate of pDN412 and pAMG40	This study
pDN421	pDN201-derived plasmid with synthetic fragments containing <i>PmtaCB1</i> promoter fusion to sgRNAs in tandem targeting the <i>ccmF1</i> (MA3305) coding sequence (CDS)	This study
pDN422	pDN201-derived plasmid with synthetic fragments containing <i>PmtaCB1</i> promoter fusion to sgRNAs in tandem targeting the <i>ccmF2</i> (MA3304) coding sequence (CDS)	This study
pDN423	pDN201-derived plasmid with synthetic fragments containing <i>PmtaCB1</i> promoter fusion to sgRNAs in tandem targeting the <i>ccmF1F2</i> (MA3305-3304) coding sequences (CDS)	This study
pDN424	pDN421-derived plasmid containing a repair template to generate an in-frame deletion of the <i>ccmF1</i> (MA3305) CDS	This study
pDN425	pDN422-derived plasmid containing a repair template to generate an in-frame deletion of the <i>ccmF2</i> (MA3304) CDS	This study
pDN426	pDN423-derived plasmid containing a repair template to generate an in-frame deletion of the <i>ccmF1F2</i> (MA3305-3304) CDS	This study
pDN427	Cointegrate of pDN421 and pAMG40	This study
pDN428	Cointegrate of pDN422 and pAMG40	This study
pDN429	Cointegrate of pDN423 and pAMG40	This study
pDN430	Cointegrate of pDN424 and pAMG40	This study
pDN431	Cointegrate of pDN425 and pAMG40	This study
pDN432	Cointegrate of pDN426 and pAMG40	This study
pDN443	pDN201-derived plasmid with synthetic fragments containing <i>PmtaCB1</i> promoter fusion to sgRNAs in tandem targeting the <i>ccmABC</i> (MA1428-1430) coding sequences (CDS)	This study
pDN444	pDN443-derived plasmid containing a repair template to generate an in-frame deletion of the <i>ccmABC</i> (MA1428-1430) CDS	This study
pDN445	Cointegrate of pDN443 and pAMG40	This study
pDN446	Cointegrate of pDN444 and pAMG40	This study
pDPG001	pDN201-derived plasmid with synthetic fragments containing <i>PmtaCB1</i> promoter fusion to sgRNAs in tandem targeting the <i>ccmG</i> (MA4254) coding sequence (CDS)	This study
pDPG002	pDN201-derived plasmid with synthetic fragments containing <i>PmtaCB1</i> promoter fusion to sgRNAs in tandem targeting the <i>ccdA</i> (MA4255) coding sequence (CDS)	This study
pDPG003	pDN201-derived plasmid with synthetic fragments containing <i>PmtaCB1</i> promoter fusion to sgRNAs in tandem targeting the <i>ccmG-ccdA</i> (MA4254-4255) coding sequences (CDS)	This study
pDPG004	pDPG001-derived plasmid containing a repair template to generate an in-frame deletion of the <i>ccmG</i> (MA4254) CDS	This study

pDPG005	pDPG002-derived plasmid containing a repair template to generate an in-frame deletion of the <i>ccdA</i> (MA4255) CDS	This study
pDPG006	pDPG003-derived plasmid containing a repair template to generate an in-frame deletion of the <i>ccmG-ccdA</i> (MA4254-4254) CDS	This study
pDPG007	Cointegrate of pDPG004 and pAMG40	This study
pDPG008	Cointegrate of pDPG005 and pAMG40	This study
pDPG009	Cointegrate of pDPG006 and pAMG40	This study
pDPG010	Cointegrate of pJK029A and pAMG40	This study
pDPG014	pJK029A-derived plasmid with <i>PmcB(tetO4)</i> promoter fusion to <i>ccmABC</i> (MA1428-1430)- <i>ccmE</i> (MA4149)- <i>ccmF1F2</i> (MA3305-3304) CDS from <i>M. acetivorans</i>	This study
pDPG015	pJK029A-derived plasmid with <i>M. barkeri</i> <i>PserC</i> (constitutive) promoter fusion to <i>mmcA</i> (MA0658) CDS from <i>M. acetivorans</i> with a C-terminal tandem affinity purification tag (containing a 3X FLAG tag and a Twin-Strep sequence)	This study
pDPG017	pJK029A-derived plasmid with <i>PmcB(tetO4)</i> promoter fusion to <i>ccmABC</i> (MA1428-1430)- <i>ccmE</i> (MA4149)- <i>ccmF1F2</i> (MA3305-3304) CDS from <i>M. acetivorans</i> and <i>M. barkeri</i> <i>PserC</i> (constitutive) promoter fusion to <i>mmcA</i> (MA0658) CDS from <i>M. acetivorans</i> with a C-terminal tandem affinity purification tag (containing a 3X FLAG tag and a Twin-Strep sequence)	This study
pKES30	pJK029A-derived plasmid with <i>PmcB(tetO4)</i> promoter fusion to <i>ccmE</i> (MA4149) CDS from <i>M. acetivorans</i> with a C-terminal tandem affinity purification tag (containing a 1X Strep and a 1X FLAG sequence)	This study
pKES031	Cointegrate of pKES30 and pAMG40	This study
pKES038	pJK029A-derived plasmid with <i>PmcB(tetO4)</i> promoter fusion to <i>ccmE</i> (MA4149) CDS with C120H substitution with a C-terminal tandem affinity purification tag (containing a 1X Strep and a 1X FLAG sequence)	This study
pKES039	Cointegrate of pKES38 and pAMG40	This study
pKES040	pJK029A-derived plasmid with <i>PmcB(tetO4)</i> promoter fusion to <i>ccmE</i> (MA4149) CDS with C120A substitution with a C-terminal tandem affinity purification tag (containing a 1X Strep and a 1X FLAG sequence)	This study
pKES041	Cointegrate of pKES40 and pAMG40	This study

Note: pDN201/pJK029A derived plasmids contain the chloramphenicol resistance marker, pAMG40 contains kanamycin resistance marker, and the co-integrates of pDN201/pJK029A derived plasmids and pAMG40 contain both contain chloramphenicol and kanamycin resistance markers.

Supplementary Table 5: List of primers used in this study

Primer Name	Primer Sequence (Overhangs for Gibson assembly are shown in red; sequences to introduce C120A or C120H mutations in <i>ccmE</i> are shown in purple)
<i>mtaBCI</i> -f for pDPG001, 002, 003	<u>TCGAAGTTTAAACCTGCAGG</u> GAATTACACGATCACTAATTTT
<i>mtaBCI</i> -sgRNA-r for pDPG001	<u>GCCGTAAAGATTACAGATGC</u> ACATGTGCTTACAGCATAAT
sgRNA-scaffold-f for pDPG001	<u>GCATCTGTAATCTTTACGGC</u> GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
sgRNA-scaffold-r for pDPG001	<u>TAAGCGGCCGCGATCGCCGG</u> TACATGAGGGCTGAAAAGCCG
<i>mtaBCI</i> -sgRN-r for pDPG002	<u>TCCAGAGCCCTACAAGGAGA</u> ACATGTGCTTACAGCATA
sgRNA-scaffold-f for pDPG002	<u>TCTCCTTG TAGGGCTCTGGA</u> GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
sgRNA-scaffold-r for pDPG002	<u>TCCAGAGCCCTACAAGGAGA</u> TTCGTTTTCTCTTTTAGGTGACTG
pDPG004-us-f	<u>GCCTTTTTTTTTTCGAAGTTT</u> CAGAAAAGAGTTTTTGAAAGTGCAGC
pDPG004-us-r1	<u>AGTAAAGAACGGCTCTTTC</u> CAGGATTAGTGGTATAATTAGTTTATTC
pDPG004-ds-f	<u>TAATTATACCACTAATCTCTG</u> GAAAGAGCCGTTCTTTACTATGAG
pDPG004-ds-r	<u>TGTAATTCCTGCAGGTTT</u> TACAGTAAGAACTATGAGTCAGGAG
pDPG005-us-f	<u>GCCTTTTTTTTTTCGAAGTTT</u> CCCTGAATGCAGAGCTGAAA
pDPG005-us-r	<u>TGAGGTGGTTCTGATAGGC</u> AACCACGGGTGAAATAGCTTC
pDPG005-ds-f1	<u>AAGCTATTTACCCGTGGTT</u> GCCTATCAGAACCACCTCAG
pDPG005-ds-r	<u>TGTAATTCCTGCAGGTTT</u> CTTTCATGTTTAGGGGACCTGC
pDPG006-us-r2	<u>TGAGGTGGTTCTGATAGGC</u> CAGGATTAGTGGTATAATTAGTTTATTCATG
pDPG006-ds-f2	<u>TAATTATACCACTAATCTCTG</u> GCCTATCAGAACCACCTCAGC
pDPG004_seq-f	AATTCCTACCTTCAGGCTCAAG
pDPG004_seq-r	TGACGCAGGATTTGAAGTTCAG
pDPG004, 005, 006_seq-r	ACAATGTCATTATTCAGATCAAGCGG
pDPG005_seq-f	ACTTCAAATCCTGCGTCAGTTGG
pDPG005_seq-r	TGTGTCAAATGTATATTGTTTGGTACGC
US_1kb-us- <i>ccmG</i> _seq-f	ACGGGAGAACGTCAGGTGAG
DS_1kb-ds- <i>ccmG</i> _seq-r	TGCCGGTGAAACTTCCTGTATTTG
US_1kb-us- <i>ccdA</i> -seq-f	TCCATAGCAAGGGCTCTGTCAC
DS_1kb-ds- <i>ccdA</i> -seq-r	TCGTAGCAATCGCAAGGGAAAGTG
<i>ccmABC</i> -f for pDPG014	<u>AATAAATTAAGGAGGAAATTCA</u> ATGAAAAACGCGGTCTCCATAAG
<i>ccmABC</i> -r for pDPG014	<u>TTTTAAAACCCCGTATGGCTCG</u> TTATGTAACACAAATTGAAAAACACAC
<i>ccmE</i> -f for pDPG014	<u>TTTTTCAATTTGTGTTACATAA</u> CGAGCCATACGGGGTTTTAAATG
<i>ccmE</i> -r for pDPG014	<u>ATTAAATGGGGAATAATAGTTT</u> TTACTCCGTATACTTTGACGGGC
<i>ccmF</i> ₁ <i>F</i> ₂ -f for pDPG014	<u>CCGTCAAAGTATACGGAGTAA</u> AAACTATTATTCCCCATTTAATTG
<i>ccmF</i> ₁ <i>F</i> ₂ -r for pDPG014	<u>TACATTATACGAAGTTATCAAGA</u> TCATTCTCGCTAAAATACTTC
<i>PserC</i> -f for pDPG015	<u>AGCCTTTTTTTTTTCGAAGTTT</u> GATCTCAACCACCTTTTTTC
<i>PserC</i> -r for pDPG015	<u>TAACCTATTTCATAATGACCAC</u> ATCTTTTCCTTTTTTGGTACTG
<i>mmcA</i> -TAP-f for pDPG015	<u>AGTACCAAAAAAGGAAAAAGAT</u> GTGGTCATTATGAATAGGTT

<i>mmcA</i> -TAP-r for pDPG015	<u>TAAAGTGTGTTGATTATTGAGT</u> TTAACCTGATTTTTCAAACTG
<i>Tmcr</i> (Ma)-f for pDPG015	<u>CCTCAGTTTGAAAAATCAGGTTAA</u> ACTCAATAAATCAAACACTTTAAAC
<i>Tmcr</i> (Ma)-r for pDPG015	<u>GCCGGCGCGCCTGCAGGTTT</u> GATTTCCGGAAAAACAGTAAAG
pDPG014_seq-r1	TCCTTCGGCAAGATTAGAAAG
pDPG014_seq-f1	CTTCTAATCTTGCCGAAGG
pDPG014_seq-r2	TGCTTACATAAGCCGAACC
pDPG014_seq-f2	TCGGCTTATGTAAGCACCG
pDPG014_seq-r3	TGAAAAGATCTCGGTCTCC
pDPG014_seq-f3	TCAATTATGAGGTCGGTTTCG
pDPG014_seq-r4	AGTCCTCAAAACATGAGAAATC
pDPG014_seq-f4	TCATGTTTTGAGGACTTCAGG
pDPG014_seq-r5	ATCTCAGGCAGCAAATTTCC
pDPG014_seq-f5	TAGGAAATTTGCTGCCTGAG
pDPG014_seq-r6	ATCCTTTGCCCAGATCGAAC
pDPG014_seq-f6	TCGATCTGGGCAAAGGATG
<i>ccmE</i> -f for pKES30, 38, 40	<u>TGATTTTAATAAAATTAAGGAGGAAATTCAT</u> ATGAATAAAAAAAGAAATCACTGC
<i>ccmE</i> -r1 for pKES30	<u>CAAACCTGAGGATGTGACCACCCGCCACCCT</u> CCGTATACTTTGACGGGCA
<i>ccmE</i> -r2 for pKES30	<u>GTCGTCGTCCTTGAGTCCCCGCCACCT</u> TTTCAAACCTGAGGATGTGACC
<i>ccmE</i> (C120A)-r1 for pKES40	<u>TTACTCCGTATACTTTGACGGTGC</u> TCCGGTAATGATCTTATTTGCC
<i>ccmE</i> (C120H)-r1 for pKES38	<u>TTACTCCGTATACTTTGACGGGTG</u> TCCGGTAATGATCTTATTTGCC
<i>ccmE</i> (C120A)-r2 for pKES40	<u>CAAACCTGAGGATGTGACCACCCGCCACCCTC</u> CGTATACTTTGACGGTGC
<i>ccmE</i> (C120H)-r2 for pKES38	<u>CAAACCTGAGGATGTGACCACCCGCCACCCTC</u> CGTATACTTTGACGGGTG
<i>ccmE</i> -r3 for pKES30, 38, 40	<u>GCATACATTATACGAAGTTATCAAGATTAGAT</u> GTCGTCGTCCTTGTAGTC
pDN402_us_f	<u>TTTGAGCCTTTTTTTTCGAAGTTTAAAC</u> CGG TTG ACT ATG CAG GTA CG
pDN402_us_r	CAG TAG AAG CAG CAC AGC GA
pDN402_ds_f	<u>GTGTCGGGAGTCGCTGTGCTGCTTCTACTG</u> GTA ACT TAC GAT AAG CCA AGA CC
pDN402_ds_r	<u>GTGTAATTCGGCGCGCCTGCAGGTTTAAAC</u> GAC GGT ATT CTT AAC CAC AGG
DDN009_seq_f	CCT GTT TGG TCC AAT ACA TC
DDN009_seq_r	ACT TCA GCT TCT GTC TGG AC
<i>mmcA</i> _f for pDN409	<u>TGATTTTAATAAAATTAAGGAGGAAATTCAT</u> GTG GTC ATT ATG AAT AGG TT
<i>mmcA</i> _r for pDN409	GAG TGC AGG CTC CGT TTC A
C_TAPtag_f for pDN409	<u>CCTGTAGAAGTTGAAACGGAGCCTGCACTC</u> GAC GAT GAC GAC AAG GGT GG
C_TAPtag_r for pDN409	<u>AGCATACATTATACGAAGTTATCAAGATTA</u> ACCTGATTTTCAAACCTGAGG
pDN409_seq_f	TGA GAT TTC CCT ATC AGT GAT
pDN409_seq_r	CTC ACT AAA GGG ATA ACT TCG
pDN412_us_f	<u>TTTGAGCCTTTTTTTTCGAAGTTTAAAC</u> CAC CGG TAA GAA AAG AAT CTG G
pDN412_us_r	<u>GGTAATGATCTTATTTGCCTCAAACGTAGA</u> AGC AAC TGC AAG CAG TGA TT
pDN412_ds_f	TCT ACG TTT GAG GCA AAT AAG
pDN412_ds_r	<u>GTGTAATTCGGCGCGCCTGCAGGTTTAAAC</u> CTA AAT GGT ATC ATT TGT GGC

DDN010_seq_f	CAA CTG AAA ATG ACT TTT CG
DDN010_seq_r	ATA CAC CTG ATA AAG TTA GTG
pDN424_us_f	TTG TGA AAT TGC TCA CGA AC
pDN424_us_r	GAG AAC ATC GCT TAG CTT TTT C
pDN424_ds_f	TTTTTTATGAAAAAGCTAAGCGATGTTCTC GCT CAG GTA AAC GGA ATC AA
pDN424_ds_r	GTGTAATTCGGCGCGCCTGCAGGTTTAAAC AAA GGT CGA GAA GAT GAC C
pDN425_us_f	TTTGGAGCCTTTTTTTTTTCGAAGTTTAAAC CAG CAC CAT ATT TGC TAC AG
pDN425_us_r	TAT CCA GAT GAG TCC CAT TC
pDN425_ds_f	ATGAATACTGGAATGGGACTCATCTGGATA GTT CTC CTT GTC AGG AAG TA
pDN425_ds_r	GTGTAATTCGGCGCGCCTGCAGGTTTAAAC GTG AAG CCA ACA ACA ACT TCC
pDN426_ds_f	GTTTTTTATGAAAAAGCTAAGCGATGTTCTC GTT CTC CTT GTC AGG AAG TA
DDN011_012_013_seq_f	TTT TCG TCC AGC ACT TTT AC
DDN011_012_013_seq_r	TAC CAG GGT TCT TTC TTG AC
pDN444_us_f	TTTGGAGCCTTTTTTTTTTCGAAGTTTAAAC GAG CAG GAA GAA AAG AGC TTG
pDN444_us_r	TCCGGCAGATCTGCGTGCCTCAAGTTCCTC TGA TAG TTC CCT TAT GGA GAC C
pDN444_ds_f	GAG GAA CTT GAC GCA CGC AG
pDN444_ds_r	GTGTAATTCGGCGCGCCTGCAGGTTTAAAC CGG CAT AAT CCG CTC TGA CTG
DDN029_seq_f	GGA GTT CCA GGT TAC AGA CTG
DDN029_seq_r	GTC TGG TGC TGA TTA TGG AC

Supplementary Table 6: List of *Methanosarcina* strains used in this study

Strains	Genotype	Construction details	Source
<i>M. acetivorans</i> strains			
WWM60	$\Delta hpt::PmcB-tetR$	-	(Guss et al., 2008)
DDN009	$\Delta hpt::PmcB-tetR, \Delta mmcA$	WWM60 was transformed to Pur ^R with pDN404; plasmid-cured strain was isolated by plating on medium with 8ADP	This study
DDN010	$\Delta hpt::PmcB-tetR, \Delta ccmE$	WWM60 was transformed to Pur ^R with pDN414; plasmid-cured strain was isolated by plating on medium with 8ADP	This study
DDN011	$\Delta hpt::PmcB-tetR, \Delta ccmF_1$	WWM60 was transformed to Pur ^R with pDN430; plasmid-cured strain was isolated by plating on medium with 8ADP	This study
DDN012	$\Delta hpt::PmcB-tetR, \Delta ccmF_2$	WWM60 was transformed to Pur ^R with pDN431; plasmid-cured strain was isolated by plating on medium with 8ADP	This study
DDN013	$\Delta hpt::PmcB-tetR, \Delta ccmF_1 \Delta ccmF_2$	WWM60 was transformed to Pur ^R with pDN432; plasmid-cured strain was isolated by plating on medium with 8ADP	This study
DDN016	DDN009/ pDN409 [<i>PmcB(tetO4)-mmcA-C-TAP tag</i>]	DDN009 was transformed to Pur ^R with pDN409; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN029	$\Delta hpt::PmcB-tetR, \Delta ccmABC$	WWM60 was transformed to Pur ^R with pDN446; plasmid-cured strain was isolated by plating on medium with 8ADP	This study
DDN033	$\Delta hpt::PmcB-tetR, \Delta ccmG$	WWM60 was transformed to Pur ^R with pDPG007; plasmid-cured strain was isolated by plating on medium with 8ADP	This study
DDN034	$\Delta hpt::PmcB-tetR, \Delta ccdA$	WWM60 was transformed to Pur ^R with pDPG008; plasmid-cured strain was isolated by plating on medium with 8ADP	This study
DDN035	$\Delta hpt::PmcB-tetR, \Delta ccmG \Delta ccdA$	WWM60 was transformed to Pur ^R with pDPG009; plasmid-cured strain was isolated by plating on medium with 8ADP	This study
DDN037	DDN009/pDPG010 [<i>PmcB(tetO4)-uidA</i>]	DDN009 was transformed to Pur ^R with pDPG010; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN038	WWM60/pDPG010 [<i>PmcB(tetO4)-uidA</i>]	WWM60 was transformed to Pur ^R with pDPG010; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN039	WWM60/pDN409 [<i>PmcB(tetO4)-mmcA-C-TAP tag</i>]	WWM60 was transformed to Pur ^R with pDN409; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN040	DDN010/pDN409 [<i>PmcB(tetO4)-mmcA-C-TAP tag</i>]	DDN010 was transformed to Pur ^R with pDN409; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN041	DDN029/pDN409 [<i>PmcB(tetO4)-mmcA-C-TAP tag</i>]	DDN029 was transformed to Pur ^R with pDN409; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN047	DDN013/pDN409 [<i>PmcB(tetO4)-mmcA-C-TAP tag</i>]	DDN013 was transformed to Pur ^R with pDN409; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN052	DDN012/pDN409 [<i>PmcB(tetO4)-mmcA-C-TAP tag</i>]	DDN012 was transformed to Pur ^R with pDN409; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN054	DDN011/pDN409 [<i>PmcB(tetO4)-mmcA-C-TAP tag</i>]	DDN011 was transformed to Pur ^R with pDN409; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN060	DDN033/pDN409 [<i>PmcB(tetO4)-mmcA-C-TAP tag</i>]	DDN033 was transformed to Pur ^R with pDN409; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN062	DDN034/pDN409 [<i>PmcB(tetO4)-mmcA-C-TAP tag</i>]	DDN034 was transformed to Pur ^R with pDN409; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN064	DDN035/pDN409 [<i>PmcB(tetO4)-mmcA-C-TAP tag</i>]	DDN035 was transformed to Pur ^R with pDN409; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN078	DDN010/pKES31 [<i>PmcB(tetO4)-ccmE-C-TAP^m tag</i>]	DDN010 was transformed to Pur ^R with pKES031; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN091	DDN029/pKES31 [<i>PmcB(tetO4)-ccmE-C-TAP^m tag</i>]	DDN029 was transformed to Pur ^R with pKES031; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study

DDN092	DDN010/pKES39 [PmcrB(tetO4)-ccmE(C120H)-C-TAP ^m tag]	DDN010 was transformed to Pur ^R with pKES039; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN093	DDN010/pKES41 [PmcrB(tetO4)-ccmE(C120A)-C-TAP ^m tag]	DDN010 was transformed to Pur ^R with pKES041; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN099	WWM60/ pKES31 [PmcrB(tetO4)-ccmE-C-TAP ^m tag]	WWM60 was transformed to Pur ^R with pKES031; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
<i>M. barkeri</i> strains			
WWM155	$\Delta hpt:: PmcrB-tetR-phiC3Int-attP$	-	(Guss et al., 2008)
DDN085	WWM155/pJK029A [PmcrB(tetO4)-uidA]	WWM155 was transformed to Pur ^R with pJK029; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN086	WWM155/pDPG014 [PmcrB(tetO4) -(ccmABCEF ₁ F ₂) _{Ma}]	WWM155 was transformed to Pur ^R with pDPG014; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN087	WWM155/pDPG015 [PserC-(mmcA) _{Ma} -C-TAP tag]	WWM155 was transformed to Pur ^R with pDPG015; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study
DDN088	WWM155/pDPG017 [PserC-(mmcA) _{Ma} -C-TAP tag, PmcrB(tetO4)-(ccmABCEF ₁ F ₂) _{Ma}]	WWM155 was transformed to Pur ^R with pDPG017; isolates were verified by Sanger sequencing and grown in HS medium supplemented with 2 µg/mL Puromycin	This study

Supplementary References

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