Schone, Christian; Poehlein, Anja; Jehmlich, Nico; Adlung, Norman; Daniel, Rolf; von Bergen, Martin; Scheller, Silvan; Rother, Michael

Deconstructing Methanosarcina acetivorans into an acetogenic archaeon

Published in:
Proceedings of the National Academy of Sciences of the United States of America

DOI:
10.1073/pnas.2113853119

Published: 11/01/2022

Document Version
Publisher's PDF, also known as Version of record

Published under the following license:
CC BY-NC-ND

Please cite the original version:
Schone, C., Poehlein, A., Jehmlich, N., Adlung, N., Daniel, R., von Bergen, M., Scheller, S., & Rother, M. (2022). Deconstructing Methanosarcina acetivorans into an acetogenic archaeon. Proceedings of the National Academy of Sciences of the United States of America, 119(2), Article e2113853119. https://doi.org/10.1073/pnas.2113853119

This material is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of the repository collections is not permitted, except that material may be duplicated by you for your research use or educational purposes in electronic or print form. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone who is not an authorised user.
Deconstructing *Methanosarcina acetivorans* into an acetogenic archaeon

Christian Schöne*, Anja Poehelein*, Nico Jehmlich*, Norman Adlung*, Rolf Daniel*, Martin von Bergen*, Silvan Scheller*, and Michael Rother**

*Institute of Microbiology, Technische Universität Dresden, 01062 Dresden, Germany; †Institute of Microbiology and Genetics, Georg-August-Universität Göttingen, 37077 Göttingen, Germany; ‡Department of Molecular Systems Biology, Helmholtz Centre for Environmental Research, 04318 Leipzig, Germany; and §Department of Bioproducts and Biosystems, Aalto University, 02150 Espoo, Finland

Edited by Mary Lidstrom, Office of Research, University of Washington, Seattle, WA; received July 27, 2021; accepted November 16, 2021

The reductive acetyl-coenzyme A (acetyl-CoA) pathway, whereby carbon dioxide is sequentially reduced to acetyl-CoA via coenzyme-bound C1 intermediates, is the only autotrophic pathway that can at the same time be the means for energy conservation. A conceptually similar metabolism and a key process in the global carbon cycle is methanogenesis, the biogenic formation of methane. All known methanogenic archaea depend on methanogenesis to sustain growth and use the reductive acetyl-CoA pathway for autotrophic carbon fixation. Here, we converted a methanogen into an acetogen and show that *Methanosarcina acetivorans* can dispense with methanogenesis for energy conservation completely. By targeted disruption of the methanogenic pathway, followed by adaptive evolution, a strain was created that sustained growth via carbon monoxide-dependent acetogenesis. A minute flux (less than 0.2% of the carbon monoxide consumed) through the methane-liberating reaction remained essential, indicating that currently living methanogens utilize metabolites of this reaction also for anabolic purposes. These results suggest that the metabolic flexibility of methanogenic archaea might be much greater than currently known. Also, our ability to deconstruct a methanogen into an acetogen by merely removing cellular functions provides experimental support for the notion that methanogenesis could have evolved from the reductive acetyl-coenzyme A pathway.

*Methanosarcina* | methanogenic | acetogenic | acetyl-CoA pathway

The reduction of two carbon dioxide (CO₂) to an activated acetyl group using inorganic electron donors, the reductive acetyl-coenzyme A (acetyl-CoA) pathway, is considered to be (among) the oldest metabolism(s), its reaction principles possibly predating the genes for the enzymes involved (1–3). It is the only metabolic pathway that can be involved both in carbon fixation and energy conservation. In the bacterial domain, the reductive acetyl-CoA pathway involves tetrahydrofolate (H₄F) as the major one-carbon transferring cofactor (4) and hydrolysis of ATP for the activation of CO₂ to formyl-H₄F (5). Energy conservation through this cytoplasmic pathway can be achieved via membrane-bound oxidoreductases generating an ion motive force (6). In contrast, members of the archaeal domain employ a variant of the pathway that involves tetrahydromethanopterin (H₄MPT) (7) and either flavin-based electron bifurcation or reverse electron transfer for the activation of CO₂ to formyl-H₄MPT (via formyl-methanothiolanate, SI Appendix, Fig. S1) (8).

The archeal acetyl-CoA pathway is mostly employed for carbon assimilation (9, 10). Only *Archaeoglobus fulgidus* and *Methanosarcina acetivorans* were shown to use this pathway for energy conservation by acetogenic carbon monoxide utilization (11, 12). As of yet uncultivated Archaea are suspected to grow acetylogenically based on their genetic inventory (13–15).

A globally important archael energy metabolism is methanogenesis. It shares reactions with the reductive acetyl-CoA pathway but lacks the carbonyl and extends the methyl branch (Fig. 1). This extension encompasses 1) the exergonic transfer of the H₄MPT-bound methyl group to 2-mercaptoethanesulfonate (coenzyme M, HS-CoM) by the membrane-integral, sodium-dependent (i.e., energy-converting) N₇-methyl-H₄MPT:HS-CoM methyltransferase (Mtr) (16), which generates a sodium motive force; 2) the reduction of methyl-S-CoM to methane, catalyzed by methyl-S-CoM reductase (Mcr) (17) using 7-mercaptoheptanoylthreonine phosphate (coenzyme B, HS-CoB) as a reductant; and 3) the reduction of the resulting heterodisulfide, CoM-S-S-CoB, by heterodisulfide reductase (Hdr) (Fig. 1). The last archaeal common ancestor was proposed to have been a methanogen (18–20), which seems counternintuitive considering that all autotrophic methanogens use the reductive acetyl-CoA pathway for carbon fixation and that the methanogenic "extension" of the methyl branch involves additional enzymes (the Mtr-Mcr-Hdr “module” Fig. 1) and sophisticated catalysis (the Mcr reaction has currently no precedence in laboratory chemistry) as well as an unusual principle of energetic coupling (generation of a sodium motive force by methyl transfer) (21). Despite the acetogenic lifestyle being not well documented for extant Archaea, evolving the methanogenic trait from an acetogenic ancestor seems plausible. In this communication, we deconstructed (i.e., simplified) *Methanosarcina acetivorans*, a member of the methanogenic Euryarchaeota, into an acetogen. Previous analyses showed that growth of *M. acetivorans* on carbon monoxide (CO) coincides with an approximate eightfold reduction (as compared to Methanol as substrate) in abundance of energy-converting Mtr

Author contributions: C.S., S.S., and M.R. designed research; C.S., A.P., N.J., and N.A. performed research; C.S., A.P., N.J., N.A., R.D., M.v.B., S.S., and M.R. analyzed data; and C.S., A.P., N.J., N.A., R.D., M.v.B., S.S., and M.R. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

This article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

1To whom correspondence may be addressed. Email: michael.rother@tu-dresden.de.

Published January 6, 2022.
Removing Mtr Leads to "Methyl Auxotrophy" in *M. acetivorans*. In order to create physiological reference data, the carboxidotrophic characteristics of *M. acetivorans* strain M42, used as wild type in this study, were assessed prior to mutagenesis (Fig. 2A). M42 converts more than 75% of the CO supplied to acetate (4 CO consumed per acetate produced) and less than 10% to methane (4 CO consumed per methane produced) during growth (Fig. 2A), confirming that acetogenesis rather than methanogenesis is the major CO-dependent catabolic pathway under this condition (*SI Appendix*, Fig. S1) (11). To investigate whether the organism's catabolism can be shifted completely toward acetogenesis, the operon encoding Mtr (N^2^-methyl-H4MPT:HS-CoM methyltransferase), Mcr (methyl-S-CoM reductase), and Hdr (heterodisulfide reductase); for simplicity, thiol-containing cofactors coenzyme A, B, and M are abbreviated, and the input of electrons is not shown.

(22). After the removal of the Mtr-encoding genes (i.e., disruption of the methyl branch toward methane, Fig. 1), an *M. acetivorans* strain was selected, which grows in a sustained fashion acetogenically on CO. Despite this dramatic change of physiology, Mcr remained essential in the strain, most likely because of the requirement of the CoM-S-S-CoB heterodisulfide in at least one unknown essential anabolic reaction. The requirement of methanogenesis can, thus, be independent from energy conservation for *M. acetivorans*, which could represent a transition state in the evolution of an acetogenic into a methanogenic archaeon.

**Results**

**Removing Mtr Leads to "Methyl Auxotrophy" in *M. acetivorans*.** In order to create physiological reference data, the carboxidotrophic characteristics of *M. acetivorans* strain M42, used as wild type in this study, were assessed prior to mutagenesis (Fig. 2). M42 converts more than 75% of the CO supplied to acetate (4 CO consumed per acetate produced) and less than 10% to methane (4 CO consumed per methane produced) during growth (Fig. 2), confirming that acetogenesis rather than methanogenesis is the major CO-dependent catabolic pathway under this condition (*SI Appendix*, Fig. S1) (11). To investigate whether the organism’s catabolism can be shifted completely toward acetogenesis, the operon encoding Mtr, *mtrEDC-BAFGH* (MA0269-MA0276), was deleted from the chromosome (*SI Appendix*, Fig. S1) using a combination of CO and MeOH as energy substrates. The resulting mutant strain MKOmtr3 (*SI Appendix*, Table S1) was unable to grow on either MeOH or CO alone during 12 mo of incubation (*SI Appendix*, Table S2). While the requirement of Mtr during methylotrophic growth was expected (*SI Appendix*, Fig. S1), it was surprising that carboxidotrophic growth of MKOmtr3 was not possible considering its acetogenic potential (Fig. 2).

MKOmtr3 did grow on a combination of acetate and methanol (Fig. 3A). The methanol concentrations required (Fig. 3A, Inset) were substantial, which suggests that MKOmtr3 grew via methyl reduction like the corresponding *Methanosarcina barkeri* Δ*mtr* mutant (23). Under this condition, acetate serves as electron donor and methanol as electron acceptor (*SI Appendix*, Fig. S1). In contrast, growth on CO and methanol (the condition under which the mutant was isolated) required less than 2 mM MeOH for maximal yield (Fig. 3B), which rules out energy conservation of MKOmtr3 via methyl reduction, that is, methanogenesis. If exogenous methyl groups are essential for the CO-dependent growth of MKOmtr3 but not catabolic methanogenic substrates, most likely they were required for anabolic purposes. As MKOmtr3 quantitatively converted the methanol supplied to methane during CO-dependent growth (*SI Appendix*, Fig. S2), methyl-S-CoM, through which exogenous methyl groups are funneled toward central metabolism (24), could be ruled out as the required anabolic metabolite. The free thiols HS-CoM and HS-CoB are also unlikely candidates, as they would not get oxidized, that is, be present, in the absence of exogenous methyl groups. Instead, the CoM-S-CoB heterodisulfide appeared as the metabolite, most likely causing "methyl auxotrophy" in MKOmtr3.

**Fig. 1.** The acetyl CoA pathway and methanogenesis. Whether H4Fo r acetogenic into a methanogenic archaeon.

**Fig. 2.** Converting a methanogen into an acetogen. Biomass production (blue, OD578), CO consumption (black, values have to be multiplied by 5 to get the actual amounts), and formation of acetate (green), formate (purple), and methane (red) in M42 (A, wild type) and MKOmtrSF (B, suppressor of mtr deletion, *SI Appendix*, Table S3) during carboxidotrophic growth.

**Fig. S1.** Methane formation in a differently scaled y-axis; shown are mean values and their SDs (error bars) of 6 (M42) or 5 (MKOmtrSF) independent cultures (serum bottles), respectively; the result was reproduced twice.
the mtr phenotype (no growth on CO alone) and which had apparently also eliminated the requirement for the respective compound (SI Appendix, Table S2), had occurred and moved through the population. Clonal isolates of the potential suppressor mutants were obtained with CO as the sole energy source by picking single colonies from streaks on agar plates. One suppressor MKOmtrSF, the balancing of metabolites produced from acetate (40 mM) and MeOH (150 kPa) and MeOH (25 mM) (SI Appendix, Table S3), was out of 3% of its genome. From analyzing the sequences of the lesion’s boundaries (SI Appendix, Fig. S3), no obvious mechanism for the deletion could be deduced. Whether any of the 159 open reading frames (SI Appendix, Table S5) deleted, an aberrant regulatory effect causing altered gene expression (five putative transcriptional regulators are encoded in the region missing, SI Appendix, Fig. S5), or the codon deletion in MA1819 (SI Appendix, Table S6) caused the phenotype of MKOmtrSF could not be unraveled, if only for the sheer number of genes affected.

To learn how the genomic changes of MKOmtrSF affected its protein inventory, it was compared to that of the wild type and to that of the parental strain MKOmtr3 through quantitative proteomic analysis. Of the predicted 4,660 proteins of M. acetivorans C2A (Reference Sequence Database NC_003552), 50 were differentially abundant (using a threshold of ±2.5-fold) in MKOmtrSF compared to the wild type and 45 compared to its parental strain MKOmtr3 (SI Appendix, Table S6). Generally, removing Mtr in M. acetivorans led to increased abundance of proteins involved in methylotrophic metabolism (e.g., MtmC1, MtbA, RamA, MtpA, and MtsF; SI Appendix, Table S6), which may serve the purpose of making various sources of methyl groups accessible for the strain’s “methyl auxotrophy” (SI Appendix, Fig. S2). A remarkable change in the proteome of the mtr suppressor MKOmtrSF compared to its parental strain was the decrease of proteins involved in methylotrophic metabolism (e.g., RamA, MtpCAP, MtsF, Table S4), or the codon deletion in MA1819 (SI Appendix, Table S6), lending support to the idea that a feature of mtr suppression is to reduce the capacity for methylotrophic metabolism.

The most striking proteomic feature of MKOmtrSF was that HdrD was absent (SI Appendix, Table S6). Together with HdrE, HdrD constitutes the terminal oxidoreductase of the respiratory chain of Methanosarcina species (8) (SI Appendix, Fig. S1). Consequently, MKOmtrSF essentially lacked membrane-associated Hdr activity (i.e., HdrED) (Fig. A). Thus, MKOmtrSF eliminates catalytic, that is, respiratory CoM-S-S-CoB reduction in order to grow acetogenically on CO. To confirm this conclusion, the HdrED-encoding genes (MA0687-MA0688) were deleted in MKOmtrSF (SI Appendix, Fig. S4). The resulting hdrED deletion mutant, designated MKOmtrhdr (SI Appendix, Table S1), displayed phenotypic characteristics very similar to those of its parental strain (Table 1, Fig. 2B, and SI Appendix, Fig. S4). Thus, the terminal oxidoreductase of the methanogenic respiratory chain, otherwise essential (20) for energy conservation (as a chemiosmotic coupling site) and for reduction of the CoM-S-S-CoB heterodisulfide, is, like Mtr, dispensable for CO-dependent, aceticogenic M. acetivorans.

Mcr Remains Essential. Despite the fact that MKOmtrSF produced only minute amounts of methane (Fig. 2B), neither Mcr activity in vitro (Fig. 4B) nor the abundance of its subunits (SI Appendix, Table S6) were altered much compared to its parental strain. The presence of either BES or 3-NOP, both potent

Shutting Off Methanogenic Respiration. To identify the mutation(s) that suppressed the mtr phenotype in MKOmtrSF and, thus, to unravel the basis for the dramatic change in physiology, its genome was (re)sequenced and compared to that of the wild type and of the parental strain MKOmtr3. All but one of the single nucleotide polymorphisms were present in all three strains (SI Appendix, Table S4) and are therefore not responsible for MKOmtrSF’s phenotype. Unique to MKOmtrSF is a deletion of a threonine codon in MA1819, which encodes a homolog of AspB (25). Strikingly, MKOmtrSF also carries a 177,114-base pair deletion (compared to the reference strains), which corresponds to approximately 3% of its genome. From analyzing the sequences of the lesion’s boundaries (SI Appendix, Fig. S3), no obvious mechanism for the deletion could be deduced. Whether any of the 159 open reading frames (SI Appendix, Table S5) deleted, a regulatory effect causing altered gene expression (five putative transcriptional regulators are encoded in the region missing, SI Appendix, Fig. S5), or the codon deletion in MA1819 (SI Appendix, Table S6) caused the phenotype of MKOmtrSF could not be unraveled, if only for the sheer number of genes affected.

To learn how the genomic changes of MKOmtrSF affected its protein inventory, it was compared to that of the wild type and to that of the parental strain MKOmtr3 through quantitative proteomic analysis. Of the predicted 4,660 proteins of M. acetivorans C2A (Reference Sequence Database NC_003552), 50 were differentially abundant (using a threshold of ±2.5-fold) in MKOmtrSF compared to the wild type and 45 compared to its parental strain MKOmtr3 (SI Appendix, Table S6). Generally, removing Mtr in M. acetivorans led to increased abundance of proteins involved in methylotrophic metabolism (e.g., MtmC1, MtbA, RamA, MtpA, and MtsF; SI Appendix, Table S6), which may serve the purpose of making various sources of methyl groups accessible for the strain’s “methyl auxotrophy” (SI Appendix, Fig. S2). A remarkable change in the proteome of the mtr suppressor MKOmtrSF compared to its parental strain was the decrease of proteins involved in methylotrophic metabolism (e.g., RamA, MtpCAP, MtsF, Table S4), or the codon deletion in MA1819 (SI Appendix, Table S6), lending support to the idea that a feature of mtr suppression is to reduce the capacity for methylotrophic metabolism.

The most striking proteomic feature of MKOmtrSF was that HdrD was absent (SI Appendix, Table S6). Together with HdrE, HdrD constitutes the terminal oxidoreductase of the respiratory chain of Methanosarcina species (8) (SI Appendix, Fig. S1). Consequently, MKOmtrSF essentially lacked membrane-associated Hdr activity (i.e., HdrED) (Fig. A). Thus, MKOmtrSF eliminates catalytic, that is, respiratory CoM-S-S-CoB reduction in order to grow acetogenically on CO. To confirm this conclusion, the HdrED-encoding genes (MA0687-MA0688) were deleted in MKOmtrSF (SI Appendix, Fig. S4). The resulting hdrED deletion mutant, designated MKOmtrhdr (SI Appendix, Table S1), displayed phenotypic characteristics very similar to those of its parental strain (Table 1, Fig. 2B, and SI Appendix, Fig. S4). Thus, the terminal oxidoreductase of the methanogenic respiratory chain, otherwise essential (20) for energy conservation (as a chemiosmotic coupling site) and for reduction of the CoM-S-S-CoB heterodisulfide, is, like Mtr, dispensable for CO-dependent, aceticogenic M. acetivorans.

Mcr Remains Essential. Despite the fact that MKOmtrSF produced only minute amounts of methane (Fig. 2B), neither Mcr activity in vitro (Fig. 4B) nor the abundance of its subunits (SI Appendix, Table S6) were altered much compared to its parental strain. The presence of either BES or 3-NOP, both potent

Schone et al.
Deconstructing Methanosarcina acetivorans into an acetogenic archaeon
https://doi.org/10.1073/pnas.2113853119
methanogenic microorganisms have been considered obligate methane producers (31), obviously because methane is the end product of their energy metabolism. Here, we demonstrate that a cytochrome-containing methanogen, *Methanosarcina acetivorans*, is able to conserve energy and grow—in a sustained fashion—without relying on methanogenesis. That the methane-releasing reaction catalyzed by Mcr remains essential in MKOmtrSF—despite the fact that its energy metabolism is now acetogenic.

**Table 1. Growth parameters of *M. acetivorans* on CO**

| Strain                  | Growth rate [h⁻¹] | Doubling time [h] | Final OD₅₇₈  | Yield [g/mol CO] | Yield [g/mol acetate] |
|-------------------------|-------------------|------------------|-------------|-----------------|-----------------------|
| M42                     | 0.043 ± 0.002     | 16.0 ± 0.6       | 1.81 ± 0.05 | 2.7 ± 0.7       | 20.5 ± 3.6            |
| MKOmtr3 (+2 mM MeOH)    | 0.040 ± 0.001     | 17.4 ± 0.6       | 1.71 ± 0.22 | n.d.            | n.d.                  |
| MKOmtrSF                | 0.040 ± 0.001     | 17.5 ± 0.5       | 1.27 ± 0.03 | 2.0 ± 0.6       | 11.2 ± 2.2            |
| MKOmrthdr               | 0.032 ± 0.001     | 21.6 ± 0.4       | 1.37 ± 0.07 | 2.3 ± 1.0       | 14.8 ± 4.1            |

n.d., not determined.

inhibitors of methanogenesis in vivo (27, 28) via blocking the activity of Mcr (28–30), completely abrogated growth of MKOmtrSF (*SI Appendix*, Fig. S5). Furthermore, all our efforts to delete (all or some of) the Mcr-encoding genes, mcrBDCGA, by using three different genetic constructs failed (*SI Appendix*, Table S7). Taken together, Mcr remains essential in MKOmtrSF—despite the fact that its energy metabolism is now acetogenic.

**Discussion**

Since their discovery, methanogenic microorganisms have been considered obligate methane producers (31), obviously because...
MK Omtr3, respectively) (SI Appendix, Table S3). Unaccounted for carbon and electrons indicate that beside the known catabolic products acetate, CO2, and formate (methanethiol and dimethylsulfide (41) were not produced), MK OmtrSF (and MK Omtr3, respectively) generate at least one additional metabolite from H2—and more reduced than—CO. It is, therefore, conceivable that reoxidation of MPH2 might be coupled to the formation of (an) unknown metabolite(s). Other questions raised by the data presented here are how the strain generates methyl-S-CoM from CO in the absence of Mtr and which of the many factors lost in the strain are responsible for its dramatic phenotypic shift (SI Appendix, Fig. S1 and Supplementary Information Text).

Since acetogenic M. acetivorans MK OmtrSF was obtained solely by removing genes (and one codon of a gene, SI Appendix, Table S4) combined with adaptive evolution (i.e., selection-driven adjustment of the metabolic repertoire present), it is conceivable that an H2MPT-containing acetogenic ancestor of methanogens, lacking cytochromes, Mtr, Mcr, andHdr (and the corresponding biosynthesis and maturation machinery) (42), might have evolved methanogenesis (SI Appendix, Supplementary Information Text). Such a scenario would be compatible with the substantial differences in the methyl branch of the bacterial and the archaeal variant of the reductive acetyl-CoA pathway (43) and with the idea of the reductive acetyl-CoA pathway being the first metabolism (3).

It is possible that other groups of chemolithotrophic microorganisms (e.g., acetogenic and sulfate-reducing bacteria) (44, 45), methanogenic archaea are metabolically very restricted, both regarding the range of electron donors they use, which include only H2, C1 compounds, acetate, and a few secondary alcohols (31), and electron acceptors (CO2 and methyl groups), which all lead to the formation of methane and of the CoM-S-CoB heterodisulfide (8). The number of experimentally validated electron donors for methanogenesis is increasing (46–48), and analyses of metagenomes even suggest sugar- and amino acid–dependent facultative methanogenic lifestyles so far not captured through cultivation (13, 49). However, alternative electron acceptors were, thus far, not reported to confer to methanogens the ability to grow non-methanogenically in a sustained fashion (i.e., for more than a few generations). Since such ability is routinely tested by using inhibitors of Mcr, most commonly BES (50–52), the anabolic requirement of the CoM-S-CoB heterodisulfide (the product of the Mcr reaction) reported here may shroud the capacity of methanogens for sustained nonmethanogenic energy conservation, which might be much more extensive than currently known.

Materials and Methods

Microbiological and Molecular Methods. Standard conditions were used for plasmid constructions, growth, and transformation of Escherichia coli strains (53). Plasmids and strains are presented in SI Appendix, Table S1. M. acetivorans strains were grown in High-Salt (HS) medium (54, 55) or in Pipes-HS (P-HS) medium, where piperazine-bis(2-ethanesulfonic acid) [60 mM, pH 7.4] was added to media from anaerobic sterile stock solutions to 5 mM and 2-bromoethanesulfonate (BES, Sigma-Aldrich) and 3-nitrooxypropanol [3-nitrooxypropanol (NOP, synthesized at the Department of Chemistry (TU Dresden) as described (56)) were added to media from anaerobic sterile stock solutions to 5 mM and 50 μM, respectively. M. acetivorans was genetically manipulated as described (41, 57, 58). Growth of M. acetivorans in liquid cultures was monitored via its optical density (OD) at 578 nm (OD 578) using a spectrophotometer (Thermo Scientific) for Balch tubes or using an Ultraspec 2000 spectrophotometer (Pharmacia Biotech) for measurements of diluted culture samples. For the determination of cell yields, three replicate cultures were grown on CO in P-HS medium and passed through predried (30 min, 80 °C), weighed cellulose nitrate filters (0.45 μm pore size) (Sartorius), which were washed with 25 mL P-HS, dried at 80 °C, and weighted. CO2, acetate, and CH4 were quantified at the beginning and at the end of the experiment to derive the amounts of CO consumed and of acetate and methane formed.

Metabolite Analyses. Methane, methanethiol, and dimethylsulfide in the gas phase were quantified using a GC-2010 Plus gas chromatograph (Shimadzu GmbH) equipped with an Optima-WAXPlus column (length, 30 m; diameter, 0.32 μm; film thickness, 0.5 μm) (Macherey-Nagel) developed with N2 or He as the carrier gas at a column flow rate of 3.39 (N2) or 2.22 (He) ml·min−1. The temperature of the flame ionization detector was set to 250 °C, that of the column oven to 40 °C, and that of the split (2:1) injector to 200 °C. CO and CO2 in the gas phase (after acidification of the sample with 0.06 vol concentrated HCl) were quantified with the same chromatograph but using a thermal conductivity detector, which was set with a positive polarity at 80 °C, 45 mA, makeup flow 8 mL·min−1 for N2 and 120 °C, 95 mA, makeup flow 7.5 mL·min−1 for He. The column used for this purpose was Carboxen 1010 PLOT (length, 30 m; diameter, 0.32 mm; film thickness, 15 μm) (Sigma-Aldrich), set to 80 °C, developed with N2 or He as the carrier gas at a column flow rate of 3.8 or 3 mL·min−1, respectively. The temperature of the split (5:1 for He, 2:1 for N2) injector was set to 200 °C. Gas phase samples (50 μL) were injected with a gas-tight sample lock syringe (Hamilton). Acetate and formate in cleared supernatant samples were determined enzymatically by using an acetic or formic acid kit,respectively (R-Biopharm), and following the manufacturer’s instructions.

Enzymes. All manipulations were carried out under strictly anaerobic conditions using gas-tight vials or inside an anaerobic glove box (Coy) containing N2:H2 (96:4 [vol/vol]). Lysates of M. acetivorans were prepared from cultures at exponential growth phase (OD 1.4 to 1.0) by lysing cells (sedimenting MPhH2) in the respective enzyme activity buffer for 30 min on ice. After repeated sedimentation, the supernatant represented the cleared lysate. To separate cytoplasmic and membrane fraction, cleared lysate was subjected to ultracentrifugation at 150,000 × g for 2 h. The resulting supernatant represented the cytoplasmic fraction. The pellet was resuspended in buffer and subjected to ultracentrifugation again. The resulting pellet represented the membrane fraction. The protein in cell fractions was quantified with the method of Bradford (59) using bovine serum albumin as standard. Reduced viologen:CoM-S-CoB oxidoreductase (heterodisulfide reductase) activity was quantified in cytoplasmic and membrane fractions of M. acetivorans as described (60), except that the assay buffer consisted of 50 mM Tris HCl (pH 7.6) and 2 mM dithiothreitol. CoM-S-CoB–dependent oxidation of benzylovioligen (BVI) (ABCR GmbH & Co. KG) was followed at 604 nm (ε = 10.7 × 104 M−1 cm−1) and spectrophotometrically reduced with 0.1 M N2:H2 (96:4, respectively). The temperature of the split (5:1 for He, 2:1 for N2) injector was set to 200 °C. Gas phase samples (50 μL) were injected with a gas-tight sample lock syringe (Hamilton). Acetate and formate in cleared supernatant samples were determined enzymatically by using an acetic or formic acid kit, respectively (R-Biopharm), and following the manufacturer’s instructions.

Genome Analysis. For genome sequencing, DNA was isolated via the Wizard Genomic DNA Purification Kit (Promega). Illumina shotgun paired-end sequencing libraries were prepared using the Nextera XT Library Preparation Kit (Illumina) according to manufacturer’s instructions and sequenced on a MiSeq instrument and the MiSeq reagent kit version 3 (2 × 300 bp) as recommended by the manufacturer (Illumina). Trimmomatic v0.39 (63) was used for quality filtering of the raw reads and Bowtie2 (64) for the mapping on the reference genome M. acetivorans C2A genome sequence (65) (accession no. NC_003552). The results were analyzed with Geneious Prime 2019. The raw reads of the analyzed strains have been deposited in the Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra) under accession numbers SRR13272466 (M42) (66), SRR13272467 (Mkomtr3) (67), SRR13272468 (MkomtrSF.12) (68), and SRR13272469 (MkomtrSF.1) (69), respectively.

Proteome Analysis. A total of 1 mL exponential growing cultures were centrifuged for 10 min at 12,000 × g and 4 °C. After protein extraction and proteolytic cleavage, the peptide lysates were injected into nano high-performance liquid chromatography (UltiMate 3000 RSLCnano, Dionex, thermo Fisher Scientific). Peptide separation was performed on a C18 reverse-phase trapping column (C18 PepMap100, 300 μm × 5 mm, particle size 5 μm, nano viper, thermo Fisher Scientific) followed by a C18 reverse-phase analytical column (Acclai

Schoene et al. Deconstructing Methanosarcina acetivorans into an acetogenic archaean
PepMap 100, 75 μm × 25 cm, particle size 3 μm, nanoViper, Thermo Fisher Scientific. A mass spectrometric analysis of peptides was performed on a Q Exactive HF mass spectrometer (Thermo Fisher Scientific) coupled with a TriVersa NanoMate (Advion, Ltd.) source in liquid chromatography (LC) chip coupling mode. LC gradient, ionization mode, and mass spectrometry mode have been used as described (70). Raw data were processed with Proteome Discoverer (v2.2, Thermo Fisher Scientific). Search settings for the Sequest HT search engine were set to trypsin (Full), max. missed cleavage 2, precursor mass tolerance ± 10 ppm, fragment mass tolerance ± 0.2 Da. The raw files were searched against the protein-coding sequences of M. acetivorans. Peptides representing proteins eliminated through targeted (Hpt in all strains, MtrECCABFG in MK0mtr3 and MK0mtrSF) or spontaneous mutation(s) (MA2190-MA2335 in MK0mtrSF) were discarded, as they are not present in the respective strain. The false discovery rates (FDR) were determined with the node Percolator (71).

1. C. Huber, G. Wächtlershauer, Activated acetic acid by carbon fixation on (Fe,Ni)S under primordial conditions. Science 276, 245–247 (1997).

2. M. Preiner et al., A hydrogen-dependent geochemical analogue of primordial carbon and energy metabolism. Nature 474, 534–542 (2020).

3. W. F. Martin, Older than genes: The acyl CoA pathway and origins. Front. Microbiol. 11, 817 (2020).

4. L. G. Jungdahl, The autotrophic pathway of acetyl synthesis in acetogenic bacteria. Annu. Rev. Microbiol. 46, 415–450 (1992).

5. A. Y. Sun, L. Jungdahl, G. W. Total, Synthesis of acetate from CO₂ in CO₂ reduction via the acetyl-CoA carbon-carbon cleavage reaction in cell extracts. J. Bacteriol. 193, 215–219 (1981).

6. K. Schuchmann, V. Müller, Autoytrophy at the thermodynamic limit of life: A model for energy conservation in acetogenic bacteria. Nat. Rev. Microbiol. 12, 809–821 (2014).

7. J. C. Escalante-Semerena, K. L. Rinehart Jr., R. S. Wolfe, Tetrahydromethanopterin, a carbon carrier in methanogenesis. J. Biol. Chem. 259, 9447–9455 (1984).

8. R. K. Thauer, A. K. Kaster, H. Seedorf, W. Buckel, R. Hedderich, Methanogenic archaea: Ecologically relevant differences in energy conservation. Nat. Rev. Microbiol. 6, 579–591 (2008).

9. D. A. Miller, M. R. M. Hind, K. R. Thauer, Anaerobic lactate oxidation to CO₂ by Archaeoglobus fulgidus via the carbon monoxide dehydrogenase pathway: Demonstration of the acetyl-CoA-carbon-carbon cleavage reaction in cell extracts. Arch. Microbiol. 153, 215–218 (1989).

10. P. G. Simpson, W. B. Whitman, “Anabolic pathways in methanogens” in Methanogenesis, G. J. Ferry, Ed. (Springer, New York, 1993), pp. 445–472.

11. M. Rother, W. W. Metcalf, Anaerobic growth of Methanococcus maripaludis on formate, carbon monoxide: An unusual way of life for a methanogenic archaeon. Proc. Natl. Acad. Sci. U.S.A. 102, 16664–16669 (2005).

12. J. T. Keltjens, G. D. Vogels, Conversion of methanol and methylamines to methane and carbon dioxide’’ in Methanogenesis, G. J. Ferry, Ed. (Springer, New York, 1993), pp. 253–293.

13. D. R. Laake, C. Schwerdler, J. E. Bailey, U. Sauer, Acetate-specific stress response in acetate-resistant bacteria: An analysis of protein patterns. Biotechnol. Prog. 13, 552–562 (1997).

14. N. R. Buan, W. W. Metcalf, Methanogenesis by Methanosarcina acetivorans involves two structurally and functionally distinct classes of heterodisulfide reductase. Mol. Microbiol. 75, 843–853 (2010).

15. W. E. Baich, R. S. Wolfe, Specificity and biological distribution of coenzyme M (2-mercaptoethanesulfonic acid) J. Bacteriol. 137, 256–263 (1999).

16. E. C. Duin et al., Mode of action elucidated for the specific reduction of methane emissions from ruminants by the small molecule 3-nitrooxypropanol. Proc. Natl. Acad. Sci. U.S.A. 113, 6172–6177 (2016).

17. U. Ermler, W. Grabarse, S. Shima, M. Goubeaud, R. K. Thauer, Crystal structure of Methanobacterium thermautotrophicus operon in (Fe,Ni)S. Biophys. Acta 1381–1384 (2001).

18. K. Raymann, C. Brochier-Armanet, S. Gribaldo, The two-domain tree of life is non-energy driven when CO is the electron donor. Annu. Rev. Microbiol. 69, 81–103 (2015).

19. K. Decker, K. Jungermann, R. K. Thauer, Energy production in anaerobic organisms. Angew. Chem. Int. Ed. Engl. 9, 138–158 (1970).

20. B. Becher, V. Müller, G. Gottschalk, Conversion of methanol to methane and carbon dioxide in Methanobacterium thermoautotrophicus. Phil. Trans. R. Soc. London B, 339, 702–733 (1992).

21. B. Becher, V. Müller, G. Gottschalk, Methyl- and tetrahydromethylmethyl-enzyme: Coenzyme M methyltransferase of Methanosarcina strain GO1 is a N(2)-translocating membrane protein. J. Bacteriol. 174, 7656–7660 (1992).

22. U. Ermir, W. Grabarse, S. Shima, M. Goubeaud, R. K. Thauer, Crystal structure of methyl-coenzyme M reductase: The key enzyme of biological methane formation. Science 278, 1457–1462 (1997).

23. K. Raymann, C. Brochier-Armanet, S. Gribaldo, The two-domain tree of life is linked to a new root for the Archaea. Proc. Natl. Acad. Sci. U.S.A. 112, 6670–6675 (2015).

24. G. Gorrell, P. S. Adam, S. Gribaldo, Methanogenesis and the Wood-Ljungdahl pathway: An ancient, versatile, and fragile association. Genome Biol. 8, 1706–1711 (2007).

25. R. A. Bergheis et al., Hydrogenotrophic methanogenesis in archaeal phylum Pyroscarcinaeota reveals the shared ancestry of all methanogens. Proc. Natl. Acad. Sci. U.S.A. 116, 5037–5040 (2019).

26. B. Becher, V. Müller, G. Gottschalk, The methyl-tetrahydromethylmethyl-enzyme: Coenzyme M methyltransferase of Methanosarcina strain GO1 is a primary sodium pump. FEMS Microbiol. Lett. 65, 239–243 (1990).

27. D. J. Lessner et al., An unconventional pathway for reduction of CO₂ to methane in CO₂-grown Methanosarcina acetivorans revealed by proteomics. Proc. Natl. Acad. Sci. U.S.A. 103, 17921–17926 (2006).
45. A. K. Bock, A. Priegerkraft, P. Schönheit, Pyruvate – A novel substrate for growth and methane formation in Methanosarcina barkeri. Arch. Microbiol. 161, 33–46 (1994).

46. T. Uchiyama, K. Ito, K. Mori, H. Tsurumaru, S. Harayama, Iron-corroding methanogen isolated from a crude-oil storage tank. Appl. Environ. Microbiol. 76, 1783–1788 (2010).

47. S. T. Lohner, J. S. Deutzmann, B. E. Logan, J. Leigh, A. M. Spormann, Hydrogenase-reductase from Methanosarcina acetivorans. Eur. J. Biochem. 217, 587–595 (1993).

48. D. Prakash, S. S. Chauhan, J. G. Ferry, Life on the thermodynamic edge: Respiratory bacteria. Microbiol. Mol. Biol. Rev. 66, 597–681 (2002).

49. I. Vanwonterghem et al., Methanolotrophic methanogenesis discovered in the archaeal phylum Verstraetearchaeota. Nat. Microbiol. 1, 16170 (2016).

50. A. K. Bock, P. Schönheit, Growth of Methanosarcina barkeri (Fusaro) under nonmethano- nogenic conditions by the fermentation of pyruvate to acetate: ATP synthesis via the mechanism of substrate level phosphorylation. J. Bacteriol. 177, 2002–2007 (1995).

51. D. Prakash, S. S. Chauhan, J. G. Ferry, Life on the thermodynamic edge: Respiratory growth as single cells at elevated osmolarity. J. Bacteriol. 182, 2611–2618 (2000).

52. K. R. Sowers, J. E. Boone, R. P. Gunsalus, Disaggregation of Methanosarcina spp. and growth as single cells at elevated osmolarity. Appl. Environ. Microbiol. 59, 3832–3839 (1993).

53. K. R. Sowers, K. M. Noll, “Techniques for anaerobic growth” in Methanogens, K. R. Sowers, H. J. Schreier, Eds. (Cold Spring Harbor Laboratory Press, Plainview, NY, vol 2, 1995), pp. 15–47.

54. M. Biava et al., Novel analgesic/anti-inflammatory agents: Diarylpyrrole acetic esters endowed with nitric oxide releasing properties. J. Med. Chem. 54, 7759–7771 (2011).

55. W. W. Metcalf, J. K. Zhang, E. Apolinario, K. R. Sowers, R. S. Wolfe, A genetic system for Archaea of the genus Methanosarcina: Liposome-mediated transformation and construction of shuttle vectors. Proc. Natl. Acad. Sci. U.S.A. 94, 2626–2631 (1997).

56. P. Bocazzi, J. K. Zhang, W. W. Metcalf, Generation of dominant selectable markers for resistance to pseudomonic acid by cloning and mutagenesis of the ileS gene from the archaean Methanosarcina barkeri fusaro. J. Bacteriol. 182, 2611–2618 (2000).

57. W. W. Metcalf, J. K. Zhang, E. Apolinario, K. R. Sowers, R. S. Wolfe, A genetic system for resistance to pseudomonic acid by cloning and mutagenesis of the ileS gene from the archaean Methanosarcina barkeri fusaro. J. Bacteriol. 182, 2611–2618 (2000).

58. M. M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254 (1976).

59. R. Hedderich, A. Berkessel, R. K. Thauer, Catalytic properties of the heterodisulfide reductase involved in the final step of methanogenesis. FEBS Lett. 255, 67–71 (1989).

60. L. G. Bonacker, S. Baudner, E. Morschel, R. Böcher, R. K. Thauer, Properties of the two isoenzymes of methyl-coenzyme M reductase in Methanobacterium thermoautotro- phicum. Eur. J. Biochem. 177, 587–595 (1993).

61. Scheller, M. Goenrich, R. K. Thauer, B. Jann, Methyl-coenzyme M reductase from methanogenic archaea: Isotope effects on the formation and anaerobic oxidation of methane. J. Am. Chem. Soc. 135, 14975–14984 (2013).

62. A. M. Bolger, M. Lohse, B. Usadel, Trimomatic: A flexible trimmer for illumina sequence data. Bioinformatics 30, 2114–2120 (2014).

63. B. Langmead, S. L. Salzberg, Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357–359 (2012).

64. J. E. Galagan et al., The genome of M. acetivorans reveals extensive metabolic and physiological diversity. Genome Res. 12, 532–542 (2002).

65. J. E. Galagan et al., The PRIDE database and related tools and resources in 2019: Improving support for quantification data. Nucleic Acids Res. 47, D442–D450 (2019).

66. N. Jehmlich, PXD022016, Proteomics Identification Database. https://www.ebi.ac.uk/pride/archive/projects/PXD022016. Deposited 16 October 2020.