

ECOLOGY

Life on the thermodynamic edge: Respiratory growth of an acetotrophic methanogen

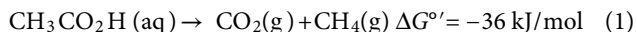
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Although two-thirds of the nearly 1 billion metric tons of methane produced annually in Earth's biosphere derives from acetate, the in situ process has escaped rigorous understanding. The unresolved question concerns the mechanism by which the exceptionally marginal amount of available energy supports acetotrophic growth of methanogenic archaea in the environment. Here, we show that *Methanosarcina acetivorans* conserves energy by Fe(III)-dependent respiratory metabolism of acetate, augmenting production of the greenhouse gas methane. An extensively revised, ecologically relevant, biochemical pathway for acetotrophic growth is presented, in which the conservation of respiratory energy is maximized by electron bifurcation, a previously unknown mechanism of biological energy coupling. The results transform the ecological and biochemical understanding of methanogenesis and the role of iron in the mineralization of organic matter in anaerobic environments.

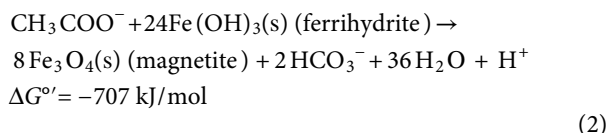
INTRODUCTION

Methanogenic archaea, methanogens, are essential players in the global carbon cycle, producing 1 billion metric tons of methane annually, of which two-thirds derives from the methyl group of acetate by acetotrophic species. They are terminal organisms of microbial food chains that decompose biomass to methane in Earth's diverse anaerobic biospheres. As methane accounts for about 30% of net anthropogenic radiative forcing, acetotrophic methanogens are accountable for a significant share of the global warming that affects climate change (1). Accordingly, it is proposed that the evolution of efficient acetotrophic methanogens produced a methanogenic burst in the end-Permian carbon cycle that contributed to a sharp increase in global warming and Earth's greatest mass extinction (2).

Acetotrophic methanogens are important players in Earth's ecology, yet their role is fragile, considering that they live on the thermodynamic edge (3, 4). On the basis of present understanding, it is not abundantly clear how acetotrophic methanogens conserve enough energy for growth under ideal laboratory conditions, let alone in competitive environments (5). The current acetoclastic pathway relies strictly on a fermentative mode, where the carbonyl group of acetate is oxidized with electrons transferred to the endogenous electron-accepting methyl group that produces methane (4). The net free energy available for growth is exceedingly small considering the requirement for activation of acetate by adenosine triphosphate (ATP) (Eq. 1) (3). The energy available by fermentative



methanogenesis is in stark contrast to acetotrophic nonmethanogenic anaerobes from the domain *Bacteria* that use exogenous electron acceptors such as ferric iron for respiration (Eq. 2) (6).



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The possibility of respiratory-driven acetotrophic growth of *Methanosarcina acetivorans* was advanced by the recent finding that membrane vesicles from acetate-grown cells generate a Na^+ gradient that drives ATP synthesis dependent on the oxidation of ferredoxin (Fdx) and the reduction of Fe(III) citrate (7). Furthermore, the effects of ferrihydrite amendment on the population dynamics of methanogens in rice field soil slurries support the proposal that rice cluster 1 (RC-1) methanogens may conserve energy by reducing Fe(III) (8). Notably, methanogens are of ancient origin, and Fe(III) reduction has been suggested to be of importance in the early evolution of respiration (9). However, Fe(III)-dependent respiratory growth has not been reported for any methanogenic pathway. Here, we show respiratory metabolism of acetate by *M. acetivorans* dependent on the reduction of ferrihydrite that produces a twofold increase in growth and augments production of the greenhouse gas methane. A revised, ecologically relevant, biochemical pathway is proposed, where electron bifurcation (EB) maximizes the conservation of respiratory energy. EB is a recently discovered mechanism of energy conservation in anaerobic microbes, where an electron pair is bifurcated with transfer of one electron to an acceptor with a more negative redox potential driven by transfer of the second electron to an acceptor with a more positive potential (5). The results document respiratory energy conservation previously unrecognized for any methanogenic species, which transforms the ecological and biochemical understanding of methanogenesis and the role of iron in anaerobic environments.

RESULTS

An investigation of respiratory-driven acetotrophic growth of *M. acetivorans* was prompted by the previous finding that everted membrane vesicles from acetate-grown cells generate a Na^+ gradient dependent on the reduction of Fe(III) (7). Acetotrophic growth, gaged by protein production (Fig. 1A), was enhanced by supplementing the medium with 20 mM ferrihydrite. Growth with ferrihydrite was twofold greater when the medium was further supplemented with 0.2 mM anthraquinone-2,6-disulfonate (AQDS), in which the trend was reflected in cell counts (Fig. 2). The low concentration of added AQDS was intended only as a mediator of electron transport to focus on the more ecologically relevant ferrihydrite. This concentration of AQDS had no individual effect on the growth parameters (Fig. 1). Results obtained for cultures amended with ferrihydrite

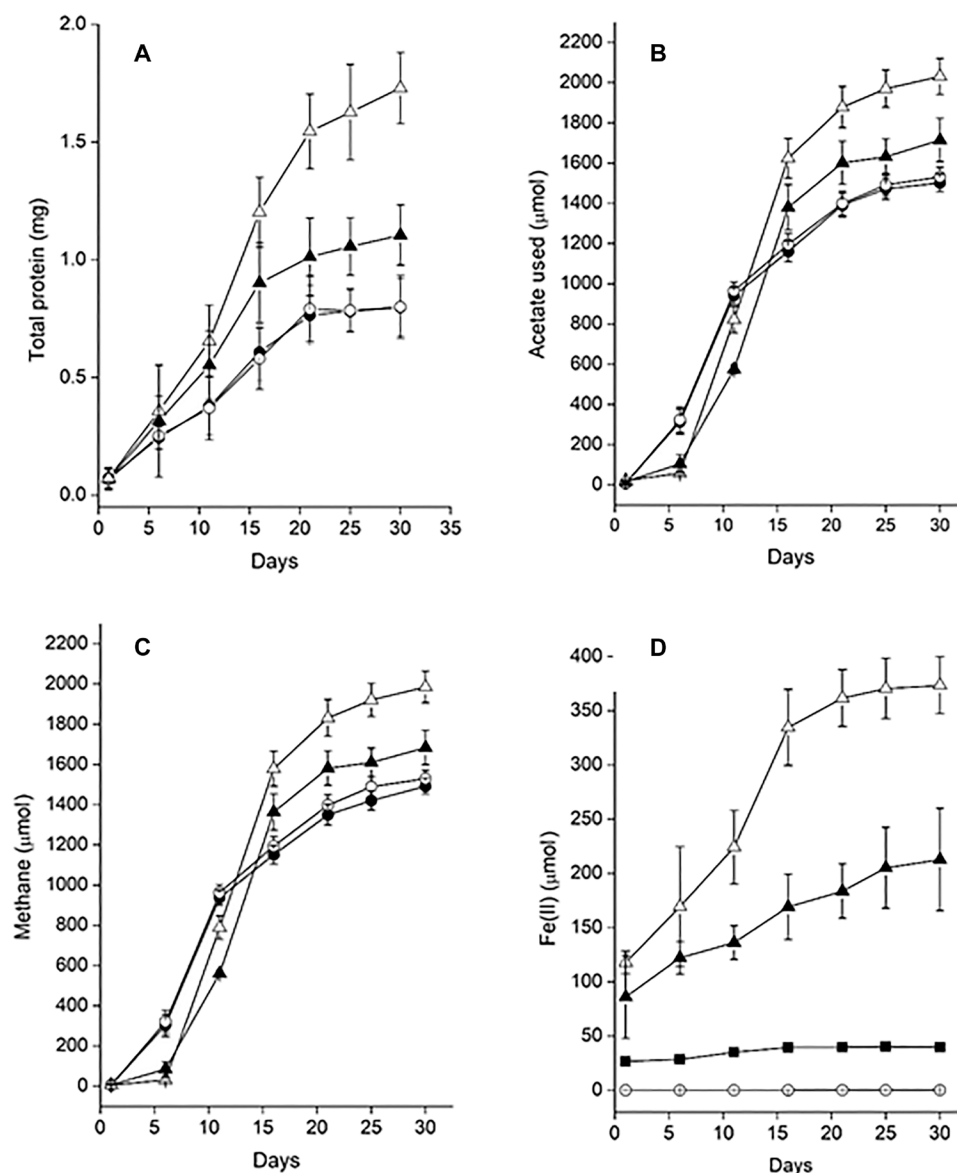


Fig. 1. Time course of growth parameters for acetate-grown cultures of *M. acetivorans* with and without supplements. (A) Protein. (B) Acetate. (C) Methane. (D) Fe(II). ●, without supplements; ○, with 0.2 mM anthraquinone-2,6-disulfonate (AQDS); ▲, with 20 mM ferrihydrite; △, with 0.2 mM AQDS and 20 mM ferrihydrite; ■, uninoculated media with 20 mM ferrihydrite. Values are the mean of three biological replicates. Error bars represent the SD.

and AQDS (Fig. 1, A to D) were replicated in subcultures (fig. S1). Molar growth yields ($Y_{ac} = \text{g protein/mole acetate}$) were 0.6 and 0.9 for cultures amended with ferrihydrite and ferrihydrite plus AQDS, respectively, compared to 0.5 for unamended cultures (Fig. 1 and table S1). Yields increased with increasing amounts of Fe(III) reduced to Fe(II) (Fig. 3).

Supplementation with ferrihydrite also increased the consumption of acetate and production of methane (Fig. 1, B and C). Production of Fe(II) was concurrent with growth in all cultures containing ferrihydrite (Fig. 1D), in which the reddish brown color was replaced with a black precipitate identified as magnetite (fig. S2) characteristic of Fe(III)-respiring anaerobes from the domain *Bacteria* (10). Magnetite was also shown to be a product of ferrihydrite reduction by cultures of *Methanosarcina thermophila*, an acetotroph biochemically related to *M. acetivorans*, although respiratory growth was not

examined (11). The ATP/adenosine diphosphate (ADP) ratio in mid-growth of *M. acetivorans* was twofold greater in cultures amended with ferrihydrite and AQDS, reflecting an enhanced energetic state dependent on electron transport (Fig. 4). Cultures supplemented with ferrihydrite and AQDS produced 333.7 μmol of Fe(II) and consumed 45.7 μmol of acetate in excess of methane produced (table S1), which indicates the Fe(III)-dependent oxidation of acetate according to Eq. 2. These results establish Fe(III)-dependent respiratory acetotrophic growth of *M. acetivorans*.

Ferrihydrite supplementation augmented methane production, in which the contribution to growth was examined. Cultures minus ferrihydrite produced 0.53 μg of protein per micromole of methane at the end of growth. Thus, the 1985.4 μmol methane produced at the end of growth accounted for 1052.2 μg of the 1730 μg total protein in cultures amended with ferrihydrite and AQDS (table S1). The

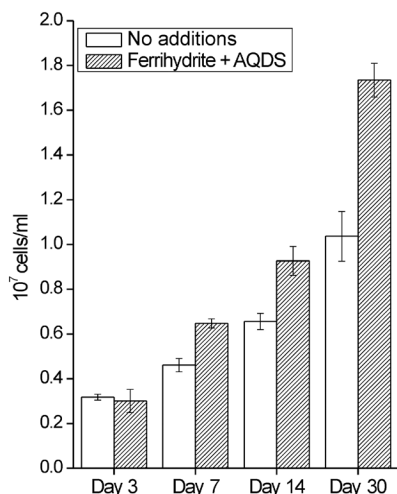


Fig. 2. Cell counts with time for unamended cultures and cultures of *M. acetivorans* amended with ferrihydrite and AQDS. Bars are the mean of three biological replicates, in which the SD is shown by error bars.

balance of 677.7 μg of protein is attributed to Fe(III)-dependent respiratory growth. Parallel calculations showed 892.7 μg versus 207.2 μg of protein attributed to methanogenic versus respiratory growth of cultures containing ferrihydrite minus AQDS (table S1). Although methanogenesis greatly exceeded respiration, 18 and 39% of growth are attributed to respiration in cultures containing ferrihydrite and ferrihydrite plus AQDS explained by the larger free energy available by respiration (Eq. 2) versus methanogenesis (Eq. 1) (3). Although the presence of ferrihydrite produced an initial lag in methanogenesis and acetate utilization (Fig. 1), no lag was observed in growth or reduction of Fe(III), which is attributed to the predominance of respiration.

DISCUSSION

Revised, ecologically relevant, biochemical pathway

Figure 5 shows the pathway proposed for acetotrophic growth of *M. acetivorans* in the presence of ferrihydrite. The extensively revised pathway is based on results presented here and on previous biochemical understanding of *M. acetivorans* (4). Hydrolysis of ATP drives the activation of acetate to acetyl-coenzyme A (CoA) that is decarbonylated with the methyl group transferred to tetrahydrosarcinapterin (H_4SPT). The carbonyl group is oxidized to CO_2 with electrons transferred to Fdx. A fraction of the methyl groups from $\text{CH}_3\text{-H}_4\text{SPT}$ are transferred to coenzyme M (HSCoM) catalyzed by the membrane-bound Mtr ($\text{CH}_3\text{-H}_4\text{SPT:HSCoM}$ methyltransferase) complex coupled to translocation of $2\text{Na}^+/\text{CH}_3\text{-H}_4\text{SPT}$. The methyl group of $\text{CH}_3\text{-SCoM}$ is reduced to CH_4 by methyl-coenzyme M reductase (Mcr), with electrons donated by coenzyme B (HSCoB) and accompanied by the formation of CoMS-SCoB. The heterodisulfide is reduced with Fdx^{2-} to regenerate HSCoM and HSCoB involving a membrane-bound electron transport chain composed of the *Rhodobacter* nitrogen fixation (Rnf) complex, cytochrome c (Cyt c), a quinone-like electron carrier methanophenazine (MP), and the heterodisulfide reductase (HdrED), as previously described (12). The electron transfer is coupled to generation of Na^+ and H^+ gradients driving ATP synthesis by the ATP synthase dependent on both Na^+ and H^+ , in which the optimal ratio is accomplished with the Na^+/H^+

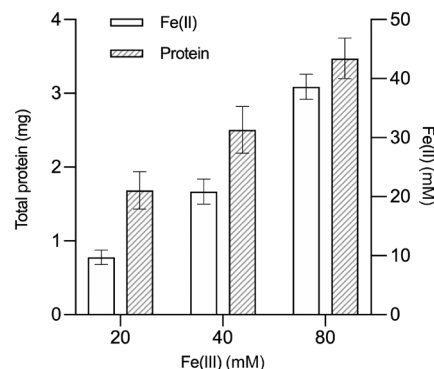


Fig. 3. Production of protein and Fe(II) at the end of growth correlated with increasing concentrations of ferrihydrite in the media that contained 0.2 mM AQDS. Bars are the mean of three biological replicates, in which the SD is shown by error bars.

antiporter Mrp (13). Oxidation of the carbonyl group with transfer of electrons to an endogenous electron acceptor fits the definition of fermentation.

The balance of methyl groups from $\text{CH}_3\text{-H}_4\text{SPT}$ is oxidized to CO_2 in accordance with the oxidative branch of the pathway for growth of *M. acetivorans* with methylotrophic substrates (i.e., methanol), where coenzyme F_{420} (F_{420}) accepts electrons (Fig. 5) (14). At this juncture, electron transport deviates from fermentation, as exogenous Fe(III) is the final electron acceptor, which fits the definition of respiration. Growth with methylotrophic substrates such as methanol involves reoxidation of F_{420}H_2 by F_{420}H_2 dehydrogenase (Fpo) that donates electrons to HdrED, mediated by MP, thereby generating a H^+ gradient driving ATP synthesis (15). However, the involvement of Fpo coupled with AQDS-mediated electron transport from HdrED to ferrihydrite is an unlikely alternative for acetotrophic growth (fig. S3), as Fpo is down-regulated in acetate-grown versus methanol-grown cells (16). Thus, it is proposed that F_{420}H_2 is reoxidized by the electron bifurcating (EB) HdrED heterodisulfide reductase ($\text{HdrA}_2\text{B}_2\text{C}_2$), which is up-regulated in acetate-grown versus methanol-grown cells (17). Electrons from F_{420}H_2 are bifurcated with reduction of Fdx and CoMS-SCoB, thereby sparing Fdx^{2-} otherwise required for methanogenesis.

EB conserves energy inherent in F_{420}H_2 that maximizes the thermodynamic efficiency. No growth or methane was detected in cultures amended with the Mcr inhibitor 2-bromoethanesulfonic acid. This result supports respiratory acetotrophic growth dependent on production of methane and CoMS-SCoB by Mcr, which is essential for reoxidation of F_{420}H_2 by EB in the proposed pathway (Fig. 5). Respiration reliant on methanogenesis indicates no alternative mechanisms for Fe(III)-dependent reoxidation independent of methanogenesis (fig. S3). Thus, the upper limit of methyl group oxidation is 50% of the acetate metabolized.

In respiratory electron transport of the proposed pathway (Fig. 5), Rnf oxidizes Fdx^{2-} with transfer of electrons to Cyt c that reduces ferrihydrite. The previously determined translocation of $2\text{Na}^+/\text{Fe(III)}$ reduced by the Rnf/Cyt c complex contrasts with the reported translocation of $0.04\text{Na}^+/\text{CoMS-SCoB}$ reduced by fermentative electron transport, results indicating greater efficiency of the former versus the latter (7, 18). Oxidation of Fdx^{2-} by the more efficient $\text{Fdx}^{2-}:\text{Fe(III)}$ couple is consistent with greater Y_{ac} for ferrihydrite-amended versus unamended cultures. Further amendment with AQDS increased the production of Fe(II) by twofold, in which the

pathway predicts a corresponding increase in acetate conversion to 2CO_2 , consistent with the observed twofold increase in Y_{ac} compared to cultures amended with ferrihydrite alone. Amendment with AQDS also increased methane production, although the contribution to the final yield was marginal compared to respiration. It is probable that AQDS stimulated the $\text{Fdx}^{2-}:\text{Fe(III)}$ couple by mediating electron transport between Cyt c and Fe(III), thereby enabling greater acetate oxidation to CO_2 . It has been proposed that AQDS is replaced with humic acids in nature, although it is debated and beyond the scope of this investigation (19). The pathway is supported by the previously reported in vitro translocation of Na^+ coupled to membrane-bound electron transport to Fe(III) and unpublished results showing respiratory growth of *M. acetivorans* with methanol dependent on the reduction of AQDS and ferrihydrite (7).

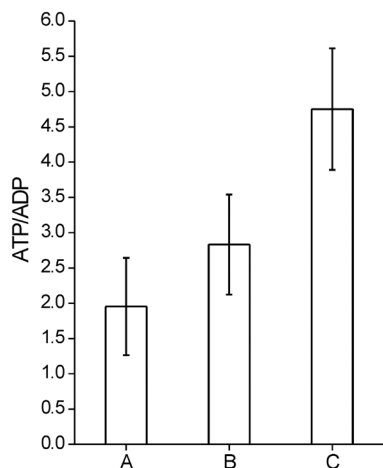
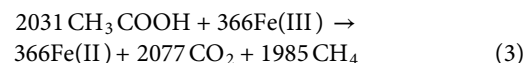


Fig. 4. The ATP/ADP ratio of acetate-grown *M. acetivorans* sampled at midgrowth. (A) No additions. (B) Ferrihydrite. (C) Ferrihydrite and AQDS. Bars are the mean of three biological replicates, in which the SD is shown by error bars.

The pathway (Fig. 5) predicts the stoichiometry shown in Eq. 3



by substituting the amount of acetate consumed for methanogenesis (1985.4 μmol) and the balance by respiration (45.7 μmol) in cultures amended with ferrihydrite and AQDS (table S1). The amount of Fe(II) predicted by the pathway (366 μmol) is in close agreement with that determined experimentally (333.7 μmol) at the end of growth (table S1). The pathway also predicts that 86.5 and 166.9 μmol of ATP were produced at the end of growth by Fe(III)-dependent respiration in the absence and presence of AQDS, considering that 173.0 and 333.7 μmol of Fe(III) were reduced (table S1). Because 30.5 and 45.7 μmol of ATP are required for activation of acetate, 56.0 and 121.2 μmol of ATP were available for growth in the absence and presence of AQDS. Thus, respiratory growth was enhanced 46% by amendment with AQDS, consistent with the 50% increase in Y_{ac} .

Amendment of media with ferrihydrite augmented growth, acetate utilization, and methanogenesis by *M. acetivorans*, in contrast to the reported inhibition in other methanogens attributed to the unproductive diversion of electrons to Fe(III) and the increase in redox potential of the media (11, 19, 20). Although AQDS was present in low concentration (0.2 mM) with no consequence, the higher concentration of 20 mM ferrihydrite increased the redox potential of the standard media for *M. acetivorans*. The indifference to increased redox potential is potentially attributable to the exceptional tolerance of *M. acetivorans* to oxidative stress (21). The augmentation of methanogenesis in the presence of ferrihydrite may be attributable to the increased level of ATP which facilitated transport of acetate by phosphorylation in the cytoplasm and the subsequent conversion of acetyl phosphate to acetyl-CoA, thereby activating the acetyl group essential for C—C bond cleavage, producing the methyl and carbonyl groups for methanogenesis.

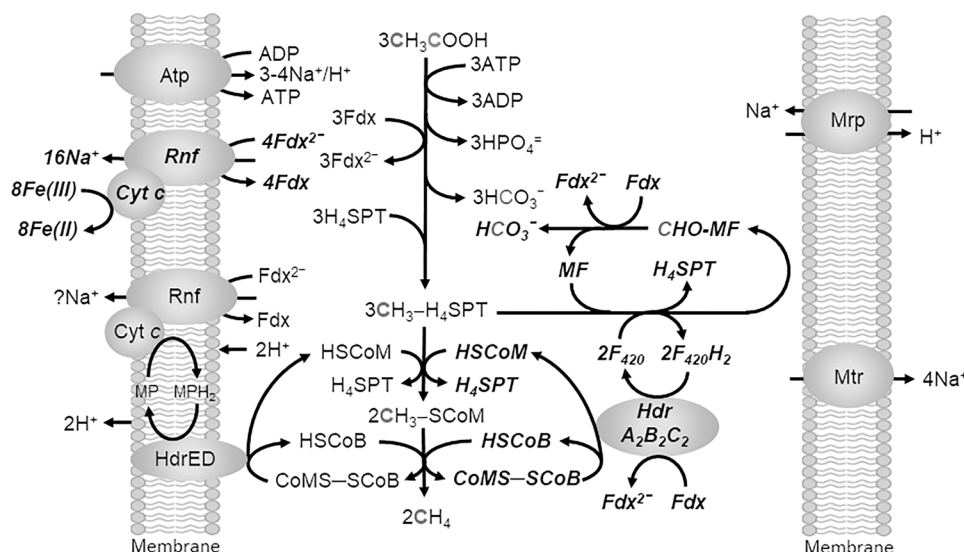


Fig. 5. The aceticlastic pathway proposed for growth of *M. acetivorans* in the presence of ferrihydrite. The stoichiometry shown is arbitrary. The respiratory metabolism of acetate is shown in bolded and italicized font. Atp, ATP synthase; Rnf, homolog of the *Rhodobacter* nitrogen fixation complex; Fdx, ferredoxin; Cyt c, multiheme cytochrome c; MP, methanophenazine; HdrED, membrane-bound heterodisulfide reductase; H4SPT, tetrahydrosarcinapterin; HSCoM, coenzyme M; HSCoB, coenzyme B; MF, methanofuran; HdrA2B2C2, cytoplasmic heterodisulfide reductase; F420, coenzyme F420; Mtr, $\text{CH}_3\text{-H}_4\text{SPT:HSCoM}$ methyltransferase; Mrp, multisubunit sodium/proton antiporter.

Although respiratory metabolism is a decided thermodynamic advantage over fermentative methanogenesis, only 20% of the acetate was metabolized by respiration. This paradox is unexplained except for the possibility that the culture conditions chosen were suboptimal, such as the concentrations of AQDS and ferrihydrite. AQDS is an artificial electron carrier intended to replace physiological electron carriers that may be more effective. Furthermore, *M. acetivorans* strain C2A has been maintained in the laboratory for decades since isolation, which may have evolved a diminished capacity for electron transport to ferrihydrite.

Ecological implications

Methanogenesis and Fe(III) reduction are the most important processes for mineralization of organic matter in freshwater and sulfate-limited anaerobic environments (22). Yet, the link between Fe(III) reduction and methanogenesis is complex and obscure. A consensus has emerged that only acetotrophic nonmethanogens reduce Fe(III) in environments where they coexist with methanogens. For example, an investigation of rice paddy soil concluded that *Geobacter* oxidizes acetate and reduces ferrihydrite to magnetite that is the conductive mineral facilitating electron transfer to *Methanosarcina* species that reduce CO₂ to CH₄ (23). An investigation of rice paddy soils concluded that Fe(III)-reducing nonmethanogens outcompete methanogens for acetate (24). However, an investigation of the rice paddy root environment showed an increase of acetotrophic *Methanosarcina* when high acetate concentrations become available (25). Furthermore, it is reported that Fe(III) reduction and acetotrophic methanogenesis occur simultaneously in diverse environments (23, 26).

M. acetivorans was isolated from a biomass-rich coastal marine sediment, environments where sulfate is limiting and Fe(III) is an electron acceptor (27, 28). The previously unrecognized advantages of respiratory acetotrophic growth and metabolism of acetate by *M. acetivorans* suggest that metabolically similar methanogens are capable of competing with nonmethanogenic Fe(III)-reducing acetotrophic microbes, which challenges the current dogma. Furthermore, both respiration and methanogenesis provides *M. acetivorans* with the ability to outcompete nonmethanogenic acetotrophic microbes when Fe(III) becomes limiting in the environment. However, this model may not apply to competition with acetotrophic *Methanosaeta* species that metabolize acetate to 1 mM, which is below the 10 mM threshold reported here for *M. acetivorans* (8, 25). Last, it is reported that methylotrophic methanogens oxidize substrates to CO₂ with transfer of electrons to sulfate-reducing species, presenting the possibility of reducing exogenous electron acceptors coupled to the respiratory conservation of energy (29, 30).

MATERIALS AND METHODS

Anaerobic methods

Standard anaerobic techniques were as previously described (31, 32). Gases were passed through a column of hot reduced copper filings to remove traces of oxygen. All transfers and samplings of cultures were performed anaerobically in an atmosphere of 100% N₂.

Media composition and growth

M. acetivorans strain C2A was cultured in 55-ml serum bottles, as described previously (33). The standard high-salt medium (25 ml) containing 90 mM acetate was supplemented with 0.2 mM AQDS and 20 mM ferrihydrite unless indicated otherwise. Although sup-

plementation with AQDS had no significant effect on the redox potential of standard medium, further addition of ferrihydrite changed the potential from −144 to −107 mV (standard hydrogen electrode). The final pH of all media was adjusted to 7.2. All experiments used freshly prepared ferrihydrite that was synthesized as described previously with the following modification (34). Briefly, 40 ml of a 0.4 M FeCl₃ solution (pH 0.98) was adjusted to pH 7.0 by adding 1 M NaOH over a period of 3 hours with vigorous stirring. The preparation was washed three times with fivefold volumes of distilled water to obtain a 0.4 M stock that was stored in the dark under an atmosphere of 100% N₂.

Analytical

Methane and acetate were determined by gas chromatography (model A890BGC, Agilent Technologies) using a Stabilwax column (34 m by 530 μm by 0.2 μm) and flame ionization detector. For the quantification of acetate, a culture sample volume of 0.5 ml was filtered through a polyether sulfone membrane (pore size, 0.22 μm), and the filtrate was acidified with formic acid before injection.

The total cell protein of the culture was determined by first pelleting cells from a 0.5 ml culture sample by anaerobic centrifugation (4000g). The pellet was anaerobically washed once with 1 ml of 50 mM tris-HCl buffer (pH 7.5) containing 20 mM MgCl₂ and 1 mM EDTA at 4°C. The pelleted cells were resuspended in 100 μl of lysis buffer (100 mM Pipes, pH 6.8) containing 1 mM EDTA by vigorous stirring and pipetting for 10 min with further incubation at 4°C for 30 min to achieve complete cell lysis. The protein concentration of the lysate was then determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

Cell counts were determined using an epifluorescence microscope (Keyence BZ-9000 Fluorescence Microscope, Germany) with a Bright-Line hemacytometer (Hausser Scientific, Horsham, PA, USA). For all experiments, 10 μl of culture was loaded into the hemacytometer, and images of the four outer squares of all grids (volume of 0.1 mm³ or 10^{−4} ml) were processed by ImageJ software (National Institutes of Health) to determine cell counts. The Fe(II) minerals formed during Fe(III) reduction were dissolved with oxalate as described previously (35). After Fe(II) mineral dissolution, the 100-μl sample was centrifuged (13,000g) and cells from the pellet were resuspended in 90 μl of 50 mM Hepes buffer (pH 7.0) containing 10 mM MgCl₂. The number of cells per milliliter of suspension was calculated by taking the average of the total number of cells per outer square and by multiplying with 10⁴. Samples stained with SYTO 9 were counted using the following settings: filter set GFP (green fluorescent protein) OP-87763; excitation wavelength, 470 ± 40 nm; emission wavelength, 525 ± 50 nm; dichromatic mirror wavelength, 495 nm. Cells were also counted based on coenzyme F₄₂₀ autofluorescence using the following settings: filter set FITC (fluorescein isothiocyanate); excitation wavelength, 467 ± 98 nm; emission wavelength, 513 ± 56 nm; dichromatic mirror wavelength, 506 nm. Autofluorescence derived from coenzyme F₄₂₀ has been reported earlier (11, 36).

Fe(II) was determined by the ferrozine method as described previously (37). Briefly, iron oxide particles contained in 0.1 ml of culture were stabilized by the immediate addition of 0.1 ml of 2 M HCl and incubated in the dark for 24 hours, after which 10 μl was added to 990 μl of 0.1% ferrozine in 0.2 M Hepes buffer (pH 7). After 5-min incubation, the absorbance at 562 nm was determined and compared to Fe(II) standards of ferrous ethylenediammonium sulfate in 1 M HCl.

Samples for the determination of cellular ATP/ADP ratios were prepared as described previously (38). The ratios were determined with a commercial kit (MAK135-1KT, Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Briefly, the assay involves two steps. In the first step, the working reagent lyses cells to release ATP and ADP. In the presence of luciferase, ATP reacts with luciferin to produce light, in which the intensity is a direct measure of the intracellular ATP concentration. In the second step, ADP is enzymatically converted to ATP that reacts with luciferin, producing an intensity of light proportional to the total ADP and ATP in the sample. The light intensity was read with an illuminometer (SpectraMax M3, M4, M5, and M5e Multi-Mode Microplate Readers, Molecular Devices Inc., CA, USA).

An x-ray diffraction (XRD) analysis was performed to identify major Fe species formed from ferrihydrite after cultivation. Freeze-dried samples (100 mg) were pressed into discs at 200 kgf cm⁻² for 30 s. X-ray diffractometry data were collected on a Bruker D8 Advance x-ray diffractometer using Cu K α radiation.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/5/8/eaaw9059/DC1>

Fig. S1. Growth parameters of subcultures amended with both AQDS and ferrihydrite.

Fig. S2. The XRD spectra.

Fig. S3. Alternate pathways of F₄₂₀H₂ reoxidation independent of methanogenesis.

Table S1. Culture parameters shown in Fig. 2.

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