JOURNAL OF BACTERIOLOGY, Feb. 2010, p. 674–678 Vol. 192, No. 3
0021-9193/10/$12.00 doi:10.1128/JB.01307-09

Function of Ech Hydrogenase in Ferredoxin-Dependent, Membrane-Bound Electron Transport in *Methanosarcina mazei*▽

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Received 1 October 2009/Accepted 13 November 2009

Reduced ferredoxin is an intermediate in the methylotrophic and aceticlastic pathway of methanogenesis and donates electrons to membrane-integral proteins, which transfer electrons to the heterodisulfide reductase. A ferredoxin interaction has been observed previously for the Ech hydrogenase. Here we present a detailed analysis of a *Methanosarcina mazei* Δ*ech* mutant which shows decreased ferredoxin-dependent membrane-bound electron transport activity, a lower growth rate, and faster substrate consumption. Evidence is presented that a second protein whose identity is unknown oxidizes reduced ferredoxin, indicating an involvement in methanogenesis from methylated C_1 compounds.

The aceticlastic pathway of methanogenesis creates approximately 70% (10) of the biologically produced methane and is of great ecological importance, as methane is a potent greenhouse gas. Organisms using this pathway to convert acetate to methane belong exclusively to the genera *Methanosarcina* and *Methanosaeta*. The two carbon atoms of acetate have different fates in the pathway. The methyl moiety is converted to methane, whereas the carbonyl moiety is further oxidized to CO_2 and the electrons derived from this oxidation step are used to reduce ferredoxin (Fd) (6). During methanogenesis from methylated C_1 compounds (methanol and methylamines), one-quarter of the methyl groups are oxidized to obtain electrons for the reduction of heterodisulfide (27). A key enzyme in the oxidative part of methylotrophic methanogenesis is the formylmethanofuran dehydrogenase, which oxidizes the intermediate formylmethanofuran to CO_2 (7). The electrons are transferred to Fd. It has been suggested that reduced ferredoxin (Fd_{red}) donates electrons to the respiratory chain with the heterodisulfide (coenzyme M [CoM]-S-S-CoB) as the terminal electron acceptor and that the reaction is catalyzed by the Fd_{red}:CoM-S-S-CoB oxidoreductase system (7, 24). The direct membrane-bound electron acceptor for Fd_{red} is still a matter of debate; for the Ech hydrogenase, a reduced ferredoxin-accepting, H_2-evolving activity has been observed for *Methanosarcina barkeri* (20), which implies that the H_2:CoM-S-S-CoB oxidoreductase system is involved in electron transport (13). Direct electron flow from the Ech hydrogenase to the heterodisulfide reductase has not been shown to date (20, 21). In contrast to *M. barkeri*, *Methanosarcina acetivorans* lacks the Ech hydrogenase (11). It can nevertheless grow on acetate, which is why another complex present in this organism, the Rnf complex, is thought to be involved in the aceticlastic pathway of methanogenesis as an acceptor for Fd_{red} (8, 10, 17). The *Methanosarcina mazei* genome, however, contains genes coding for the Ech hydrogenase, but this species lacks the Rnf complex (5).

To investigate whether the Ech hydrogenase is the only means by which *M. mazei* channels electrons from Fd_{red} into the respiratory chain, a mutant lacking the Ech hydrogenase (*M. mazei* Δ*ech* mutant) was constructed. Electron transport experiments using Fd_{red} as the electron donor and CoM-S-S-CoB as the electron acceptor were conducted with wild-type and mutant membranes to gain deeper insight into the actual membrane-bound protein complexes that accept electrons from Fd_{red}. Furthermore, an in-depth characterization of the growth and trimethylamine (TMA) consumption of the Δ*ech* mutant was performed, which provided insight into the *in vivo* role of Ech hydrogenase.

MATERIALS AND METHODS

Purification of proteins. Ferredoxin was purified from *Clostridium pasteurianum* DSM 525^T essentially as described by Mortenson (22). The last steps (dialysis and crystallization) were replaced by ultrafiltration. The ferredoxin content was determined using the bicinchoninic acid method (26). CO dehydrogenase/acetyl-CoA synthase (CODH/ACS) was purified from *Moorella thermoacetica* ATCC 39073 as described by Ragsdale et al. (25), except for the following modifications. The extract was not heat treated or fractionated with ammonium sulfate. The first DEAE-cellulose column was eluted with a step gradient consisting of 0.1 to 0.5 M NaCl. CODH/ACS was then purified further using Q-Sepharose anion-exchange and phenyl-Sepharose hydrophobic interaction columns. Active fractions were concentrated and buffer exchanged into 50 mM Tris-HCl (pH 7.6) using Amicon ultracentrifuge concentrators in an anaerobic chamber (Vacuum Atmospheres). Immediately after purification, the enzyme had a CO oxidation-methyl viologen reduction specific activity of 273 U/mg at 37°C.

Membrane preparations. *M. mazei* DSM 7222 and derivatives of this strain were grown anaerobically at 37°C in *Methanosarcina* medium (DSM medium 120) with TMA as the substrate (12). Subsequent steps were performed anaerobically in an anaerobic chamber (Coy Laboratory Products, United States) under a 97% N_2-3% H_2 atmosphere. In the late exponential phase the cells were harvested and lysed by resuspending them in phosphate buffer (40 mM KH_2PO_4/K_2HPO_4 [pH 7.0], 5 mM dithioerythritol [DTE], 1 μg ml^{-1} resazurin) and incubated with DNase I for 30 to 60 min at 4°C. The cell lysate was ultracentri-

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▽ Published ahead of print on 30 November 2009.

fuged (1 h, 45,000 rpm), the supernatant was discarded, and the membrane pellet was homogenized in 20 mM phosphate buffer (KH_2PO_4/K_2HPO_4, pH 7.0) containing 20 mM $MgSO_4$, 500 mM sucrose, 5 mM DTE, and 1 μg ml^{-1} resazurin. The ultracentrifugation step was repeated, the supernatant was discarded, and the membrane pellet was homogenized in the buffer described above. The protein content was determined using the Bradford method (3).

Enzyme assays. The photometric tests were performed anaerobically in 1.5-ml glass cuvettes with rubber stoppers using a V-550 UV/visible spectrophotometer (Jasco, Germany). Benzylviologen-dependent heterodisulfide reductase activity was determined by the decrease in absorption at 575 nm using 600 μl phosphate buffer [40 mM KH_2PO_4/K_2HPO_4 [pH 7.0] reduced with Ti(III) citrate] containing 625 nmol benzylviologen (ε_{575} = 8.9 mM^{-1} cm^{-1}), 300 nmol $Na_2S_2O_4$, 50 μg *M. mazei* membrane, and 50 nmol CoM-S-S-CoB.

$F_{420}H_2$ dehydrogenase activity was determined by determining the increase in absorption at 420 nm using 600 μl phosphate buffer (40 mM KH_2PO_4/K_2HPO_4 [pH 7.0], 5 mM DTE) containing 15 nmol $F_{420}H_2$ (ε_{420} = 40 mM^{-1} cm^{-1}), 50 μg *M. mazei* membrane, 300 nmol metronidazole, and 180 nmol methylviologen (18). F_{420} was isolated and reduced as described by Abken et al. (1). One unit of activity was defined as 1 μmol electrons transported per min. CoM-S-S-CoB (synthesized as described previously [9, 23]) was quantified in anaerobic glass vials with rubber stoppers containing 250 μl phosphate buffer [40 mM phosphate and 1 μg ml^{-1} resazurin, reduced with Ti(III) citrate] flushed with N_2-CO (5% CO [purity, 1.8] and 95% N_2 [purity, 5.0]) for 1 min. For analysis of CoM-S-S-CoB reduction, 100 nmol CoM-S-S-CoB, 8.9 μg *C. pasteurianum* ferredoxin, 150 μg *M. mazei* membrane, and 75 μg *M. thermoacetica* CO dehydrogenase/acetyl-CoA synthase were added. For CoM-SH/CoB-SH quantification (28) 20-μl samples were taken every 10 min for 1 h and directly used in a modified Ellman's assay; 950 μl Tris buffer (150 mM, pH 8.1) was mixed with the sample and 100 μl Ellman's reagent [5 mM 5,5′-dithiobis(2-nitrobenzoic acid (ε_{412} = 13.6 mM^{-1} cm^{-1}] in 50 mM sodium acetate buffer, pH 5.0], and the absorption at 412 nm was immediately measured.

Alternatively, H_2:heterodisulfide oxidoreductase activity was determined by replacing CO, CO dehydrogenase/acetyl-CoA synthase, and ferredoxin with H_2. In this analysis, only 50 μg membrane preparation was used, and 1 U of activity was defined as the reduction of 1 μmol CoM-S-S-CoB per min.

Generation of the *M. mazei* Δ*ech* mutant. The *M. mazei* Δ*ech* mutant was generated by homologous recombination using the techniques described by Metcalf et al. (19). Up- and downstream regions of the *ech* gene region were cloned into the two multiple cloning sites of pJK3 (19), linearized with ApaI, and transformed into *M. mazei*. Instead of the gene, a puromycin resistance (*pac*) cassette was inserted. Puromycin-resistant colonies were picked and screened for gene knockout. Gene knockout was confirmed by sequencing and Southern blotting. Homologous recombination led to deletion of *echA* to *echE* (*mm2320* to *mm2324*) and part of *echF* (*mm2325*).

For construction of the *M. mazei* Δ*ech* mutant, primers 5′-CCTACTCGAGGA GTGAATCAGCGAATAGAG-3′ and 5′-GACCGAATTCACAACGTATCCTC CGACCTA-3′ (upstream region of *mm2320*) with XhoI and EcoRI restriction sites and primers 5′-CCAGGAGCTCGGTATGCTAAACCTTGTATT-3′ and 5′-CAA GGGATCCCACTACAAATGTTGTCTCC-3′ (*mm2325* and downstream region) with SacI and BamHI restriction sites were used.

Growth analysis and substrate quantification. The *M. mazei* wild type and the Δ*ech* mutant were grown as described above in 50-ml cultures with TMA as the growth substrate. Growth of the Δ*ech* mutant was also observed with methanol, TMA plus H_2, or TMA plus H_2 and CO_2, but not with acetate as the substrate. The optical density at 600 nm (OD_{600}) was measured with a Helios Epsilon Vis photometer (Thermo Scientific) in the presence of sodium dithionite. At different time points 700-μl samples were taken from TMA-grown cultures. Cells and medium were separated by silicone oil centrifugation (100 μl silicone oil, 5 min, 14,000 rpm) to thoroughly separate cells from the medium.

TMA, dimethylamine (DMA), and monomethylamine (MMA) were quantified as described by Krätzer et al. (15). The concentrations of these compounds were measured in triplicate, and the results were compared to standard curves.

RESULTS AND DISCUSSION

To investigate ferredoxin-mediated membrane-bound electron transport in *M. mazei*, an *in vitro* assay with washed membrane preparations of the *M. mazei* wild-type and Δ*ech* mutant strains grown on trimethylamine was performed. In this assay, electrons were transferred from the initial substrate CO to *M. thermoacetica* CO dehydrogenase/acetyl-CoA synthase (CODH/ACS), which reduces *C. pasteurianum* ferredoxin (Fd). Fd donates electrons to different membrane-bound complexes (described in detail below), which are predicted to reduce methanophenazine (1). Methanophenazine is the electron donor for the cytochrome *b* subunit of the heterodisulfide reductase (Fig. 1), which reduces the terminal electron acceptor CoM-S-S-CoB (4). The activity of the Fd_{red}:heterodisulfide oxidoreductase system, described by the following equation, was about 24 mU/mg membrane protein and was confirmed with three independent membrane preparations obtained from different *M. mazei* cultures (Fig. 2): $2Fd_{red}$ + CoM-S-S-CoM + $2H^+ \rightarrow 2Fd_{ox}$ + HS-CoM + HS-CoB. This value was similar to the values obtained by Peer (24), who obtained an activity of 23 mU/mg for the *Methanosarcina thermophila* CO:CoM-S-S-CoB oxidoreductase system. The apparent requirement for Fd in the *M. mazei* system makes it a suitable tool to investigate Fd-dependent reduction of heterodisulfide. CoM-S-S-CoB reduction was clearly dependent on the presence of CO, CODH/ACS, Fd, and membranes. If one component was omitted, heterodisulfide-reducing activity could not be measured (Fig. 2). Chemical degradation of heterodisulfide could not be observed in the reaction mixtures.

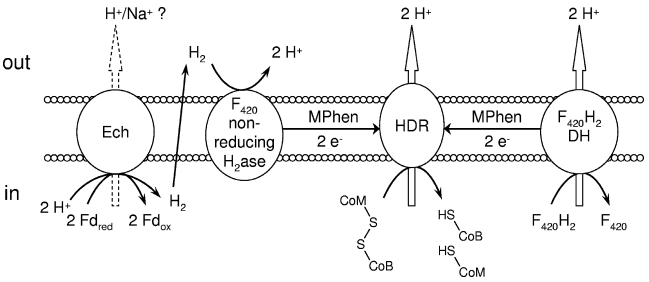

FIG. 1. Proposed model of membrane-bound electron transfer in *M. mazei*. Proton translocation has been shown for the heterodisulfide reductase (14) and $F_{420}H_2$ dehydrogenase (2). Proton translocation for the Ech hydrogenase is assumed. H_2ase, hydrogenase; DH, dehydrogenase; HDR, heterodisulfide reductase; MPhen, methanophenazine.

According to the current model (Fig. 1), Fd_{red} produced in the aceticlastic pathway, as well as in the methylotrophic pathway, is oxidized by the Ech hydrogenase, which releases molecular hydrogen (20). The Ech hydrogenase is membrane bound and consists of six subunits (EchA to EchF). Hydrogen produced in the first reaction is reoxidized by the F_{420} nonreducing hydrogenase (Fig. 1), and electrons are subsequently shuttled to the heterodisulfide reductase, which reduces CoM-S-S-CoB (14). In *M. barkeri*, Fd_{red}-oxidizing and H_2-evolving activities have been observed for the purified Ech hydrogenase, which supports the hypothesis described above (20). The coupling of Ech hydrogenase activity and CoM-S-S-CoB reduction, however, has not been shown to date. To analyze this process in more detail, we constructed an *M. mazei* Δ*ech* mutant and used the membrane fraction in the CoM-S-S-CoB reduction assays. The activity of the *M. mazei* Δ*ech* mutant membrane fraction was about 50% of the activity of the wild-type membrane fraction (Fig. 2). The decrease in activity confirms that in *M. mazei* the Ech hydrogenase also accepts electrons from Fd_{red}. However, the Fd_{red}-oxidizing activity was not completely absent in the *M. mazei* Δ*ech* mutant and was one-

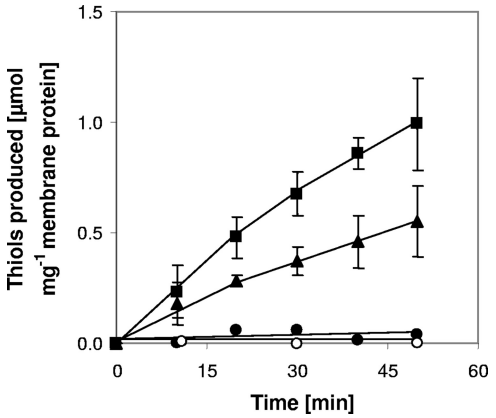

FIG. 2. Fd-dependent heterodisulfide reduction with wild-type and Δ*ech* mutant cytoplasmic membranes. The assay mixtures (total volume, 250 μl) contained 100 nmol CoM-S-S-CoB, 8.9 μg Fd, 150 μg membrane, and 75 μg CODH under an atmosphere consisting of N_2 (95%) and CO (5%). ■, wild-type membranes; ▲, membranes from Δ*ech* mutant; ●, control without Fd; ○, control without CO (100% N_2). Other control experiments (omission of CO dehydrogenase, membranes, or heterodisulfide) were performed, and it was found that heterodisulfide reduction was abolished.

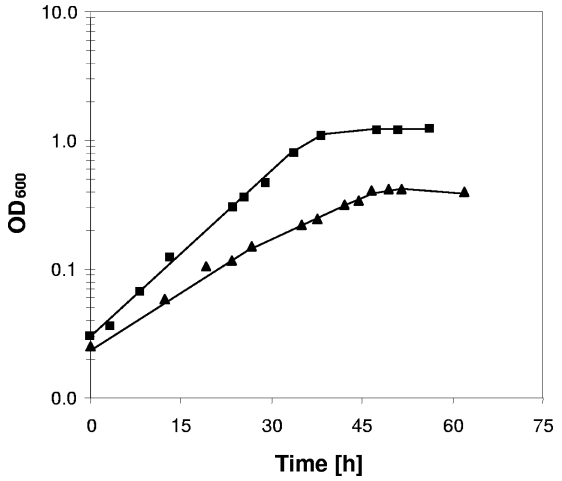

FIG. 3. Growth curves for the wild type (■) and Δ*ech* mutant (▲) during growth on TMA.

half of the wild-type activity. Hence, it is tempting to speculate that there is at least one membrane-bound protein that is different from the Ech hydrogenase and accepts electrons from Fd_{red}, allowing the cell to obtain energy by electron transport phosphorylation during aceticlastic growth as well as methylotrophic growth. In *M. acetivorans*, genes for the Ech hydrogenase are not present, but the organism can nevertheless grow on acetate, in which Fd_{red} oxidation by a membrane-bound protein complex is crucial. Proteome analysis suggested that another membrane-bound complex, referred to as Rnf, is involved in acetate metabolism and hence in the respiratory chain and the oxidation of Fd_{red} (16, 17). In contrast, *M. mazei* possesses an Ech hydrogenase, but the genomic data indicate that the Rnf complex is not present.

To ensure correct functioning of the Fd-independent parts of the respiratory chain (Fig. 1), the H_2:heterodisulfide reductase activity assay, benzylviologen-dependent heterodisulfide reductase assay, and $F_{420}H_2$ dehydrogenase activity assay were performed with the wild-type strain and deletion mutant membrane preparations. The H_2:heterodisulfide reductase exhibited an activity of approximately 150 mU/mg protein in the wild type and in the *ech* mutant, indicating that there was a fivefold increase compared to the CO system. The higher activity of the H_2:CoM-S-S-CoB oxidoreductase system probably resulted from limited interaction of the nonnative reaction components with the Ech hydrogenase in our test system. Also, the benzylviologen-dependent heterodisulfide reductase and $F_{420}H_2$ dehydrogenase activities were similar for all *M. mazei* membranes tested. Hence, deletion of the Ech hydrogenase had no influence on the H_2- and $F_{420}H_2$-dependent electron transport systems in *M. mazei* (Fig. 1).

The *M. mazei* Δ*ech* mutant did not grow on acetate, as observed previously by Meuer et al. (21) for an *M. barkeri* Δ*ech* mutant. When trimethylamine (TMA) was used as a growth substrate, distinct differences in growth behavior were observed. TMA is demethylated stepwise to dimethylamine (DMA) and monomethylamine (MMA), portions of which are excreted into the growth medium. DMA occurs only as an intermediate product at very low concentrations in the culture supernatant, whereas MMA accumulates and is used only when TMA and DMA are completely consumed (15). The respective methyl groups are transferred to CoM via substrate-specific methyl transferases, yielding methyl-CoM (15). Three-quarters of the methyl moieties are reduced to CH_4, leading to the formation of heterodisulfide (CoM-S-S-CoB) (Fig. 1). One-quarter of the methyl-CoM is oxidized to CO_2, yielding reducing equivalents or electrons that are transferred to F_{420} and ferredoxin, respectively (27) (Fig. 1). $F_{420}H_2$ is oxidized by the membrane-bound $F_{420}H_2$ dehydrogenase (Fig. 1), whereas Fd_{red} is recycled by the Fd_{red}:heterodisulfide oxidoreductase system, which is Ech independent in the Δ*ech* mutant.

When growth of the *M. mazei* wild-type strain with TMA as the substrate was compared with growth of the Δ*ech* mutant with the same substrate, apparent doubling times of 7.7 h for the wild type and 9.1 h for the Δ*ech* mutant were observed. Furthermore, the wild type grew to an OD_{600} of 1.2, whereas the Δ*ech* mutant stopped growing at an OD_{600} of around 0.5 (Fig. 3). The TMA consumption rate of the Δ*ech* mutant was twofold higher than that of the wild type; TMA was completely consumed at an OD_{600} of 0.48 for the Δ*ech* mutant and at an OD_{600} of 0.80 for the wild type (Fig. 4A). As mentioned above, MMA accumulates in the culture supernatant as a product from TMA and DMA breakdown and is used only when TMA and DMA are completely metabolized. Thus, the MMA concentration in the culture supernatant first increases to a certain level and then decreases. The MMA formation rate was 2.7-fold higher in the Δ*ech* mutant than in the wild type. The MMA degradation rate showed the same tendency; it was 2.5-fold higher in the Δ*ech* mutant than in the wild type (Fig. 4B).

In summary, the electron transport deficiency of the Δ*ech* mutant is reflected in its growth behavior, as it grows slower but uses more substrate to generate less biomass. Taken together, the data indicate that there is a deficient energy coupling site in the Δ*ech* mutant; thus, an H^+- or Na^+-translocat-

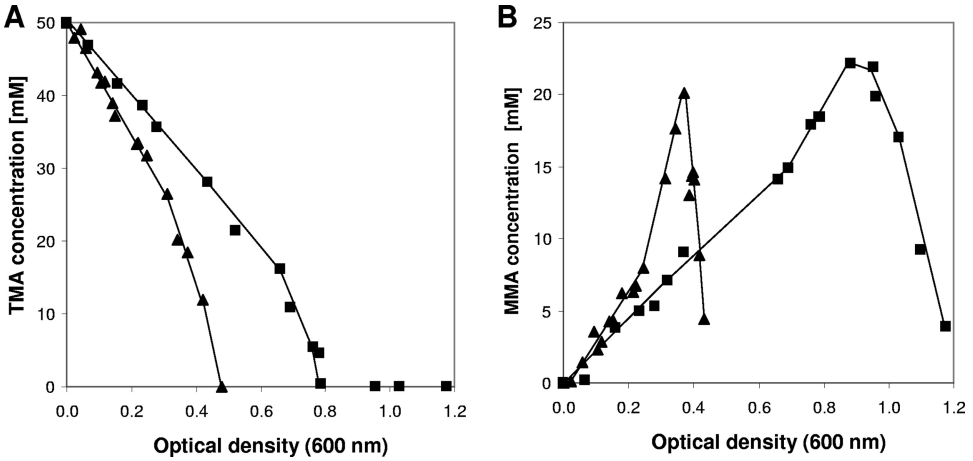

FIG. 4. Substrate utilization by the wild type (■) and the Δ*ech* mutant (▲) during growth with 50 mM TMA as the substrate. (A) TMA consumption. (B) MMA production and consumption.

ing activity of the Ech hydrogenase, as proposed (but not demonstrated) by Meuer et al. (20), is very likely. This hypothesis also explains why the Δ*ech* mutant strain does not grow on acetate. The free energy change associated with methanogenesis from 1 mol acetate allows synthesis of only a little more than 1 mol ATP ($\Delta G^{o\prime} = -36$ kJ/mol CH_4 [6]), but 1 mol ATP is used in acetate activation to form acetyl-CoA. Therefore, this methanogen lives close to the thermodynamic limit, and the loss of the Ech hydrogenase as an ion-translocating enzyme would lead to severe deficiencies in energy conservation. The second yet unidentified $ferredoxin_{red}$-oxidizing protein obviously cannot compensate for this defect.

Overall, the results described here indicate that the ferredoxin-mediated membrane-bound electron transport chain is more complex than previously thought and that the Ech hydrogenase, as well as another unknown membrane-bound protein, is involved in aceticlastic and methylotrophic methanogenesis in *M. mazei*.

ACKNOWLEDGMENTS

We thank Elisabeth Schwab for technical assistance and Paul Schweiger for critical reading of the manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft (grant De488/9-1).

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