Method for isolation of auxotrophs in the methanogenic archaeabacteria: Role of the acetyl-CoA pathway of autotrophic CO2 fixation in *Methanococcus maripaludis*

(Methanogens/pyruvate synthase/CO dehydrogenase)

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**ABSTRACT** A procedure was developed for the enrichment of auxotrophs in the antibiotic-insensitive archaeabacterium *Methanococcus*. After mutagenesis with ethyl methanesulfonate, growing cells were selectively killed upon exposure to the base analogs 6-azauracil and 8-azahypoxanthine for 48 hr. Using this method, eight independent acetate auxotrophs of *Methanococcus maripaludis* were isolated. Six of the auxotrophs had an absolute growth requirement for aceate and contained 1–16% of the wild-type levels of CO dehydrogenase. Three of these six also contained 14–29% of the wild-type levels of pyruvate oxidoreductase and 12–30% of the wild-type levels of pyruvate synthase. Two spontaneous revertants of these latter auxotrophs regained the ability to grow normally in the absence of acetate and wild-type levels of CO dehydrogenase, acetyl-CoA synthase, pyruvate oxidoreductase, and pyruvate synthase. Likewise, a spontaneous revertant of an auxotroph with reduced levels of CO dehydrogenase and wild-type levels of pyruvate oxidoreductase regained the ability to grow normally in the absence of acetate and wild-type levels of CO dehydrogenase and acetyl-CoA synthase. Two additional auxotrophs grew poorly in the absence of acetate but contained wild-type levels of CO dehydrogenase and pyruvate oxidoreductase. These results provide direct genetic evidence for the *Ljungdahl–Wood pathway* [Ljungdahl, L. G. (1986) Annu. Rev. Microbiol. 40, 415–450; Wood, H. G., Ragsdale, S. W. & Pezacka, E. (1986) Trends Biochem. Sci. 11, 14–18] of autotrophic acetyl-CoA biosynthesis in the methanogenic archaeabacteria. Moreover, it suggests that the acetyl-CoA and pyruvate synthases may share a common protein or coenzyme component, be linked genetically, or be regulated by a common system.

Autotrophy is very ancient. On the basis of the isotopic fractionation of ancient carbonates and organic matter, autotrophy may have existed on earth 3.5 billion years ago (1). In the absence of more direct information about early life, comparative physiology offers one approach to gain insight in the nature of Precambrian organisms. Thus, if an ancient pathway of CO2 fixation exists in modern organisms, it may be distributed phylogenetically among very diverse organisms. Three pathways of autotrophic CO2 fixation have been reported for the archaeabacteria and the eubacteria, which represent the extremes of bacterial evolution (2). These include the *Ljungdahl–Wood* pathway of autotrophic acetyl-CoA synthesis (3–5), the Calvin cycle (6, 7), and the reductive citric acid cycle (8, 9). Therefore, these three pathways are likely candidates for a pathway from a common ancestor to modern bacteria. Although there is substantial evidence for the *Ljungdahl–Wood* pathway in methanogenic archaeabacteria, much of it is circumstantial. For instance, CO dehydrogenase (CODH), which is a key enzyme of this pathway, has been isolated from a number of methanogens (10–12). In contrast to the eubacterial enzyme, the biosynthetic activity of the purified archaeabacterial enzyme has not been demonstrated. Although biosynthetic activity can be demonstrated in crude extracts, it is only a small fraction of the amount necessary to support autotrophic growth (13, 14). In addition, *Methanococcus voltae* is capable of substantial autotrophic CO2 fixation, yet it contains very low levels of CODH (15). Moreover, in the aceticlastic methanogens, the physiological function of the CODH is the cleavage of acetyl-CoA and not autotrophy (16, 17). Therefore, the role of this enzyme system in autotrophic methanogens is ambiguous.

The *Ljungdahl–Wood* pathway catalyzes the complete synthesis of acetyl-CoA from two molecules of CO2. In methanogens the methyl carbon of acetyl-CoA is believed to be obtained from the methyltetrahydromethanopterin, an intermediate in the reduction of CO2 to methane (5). In extracts, methyltetrahydromethanopterin is also formed by a chemical reaction between formaldehyde and tetrahydromethanopterin and the enzymatic reduction of the product methyltetrahydromethanopterin (18). By analogy with the clostridial system, the methyl carbon is probably transferred to a corrinoid protein and then to the acetyl-CoA synthase (3, 4). Methyl iodide may react directly with the corrinoid protein or the acetyl-CoA synthase to provide an alternative source of the methyl carbon (19). The carboxyl carbon of acetyl-CoA is obtained from the reduction of CO2 to carbon monoxide, the CODH activity. Alternatively, the acetyl-CoA synthase can bind CO directly for acetyl-CoA synthesis.

To obtain direct evidence for the role of the *Ljungdahl–Wood* pathway in autotrophic methanogens, acetate auxotrophs of the facultative autotroph *Methanococcus maripaludis* were isolated by an enrichment procedure developed for this purpose. These auxotrophs were examined for CODH and acetyl-CoA synthase activity to establish the physiological function of these enzymes. Because of the general nature of this selection against growing cells, this method should also be useful for the selection of auxotrophic mutants in other antibiotic-insensitive archaeabacteria.

**MATERIALS AND METHODS**

*Media and Growth of Bacteria.* *M. maripaludis* strain II was grown anaerobically with H2 and CO2 as substrates for methanogenesis (14, 20). The media used were minimal medium (McN), which contained CO2 as the sole carbon source.

Abbreviations: CODH, CO dehydrogenase; POR, pyruvate oxidoreductase; zUra, 6-azauracil; zHyp, 8-azahypoxanthine; LDH, lactate dehydrogenase.

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source (21); McC, which contained 10 mM sodium acetate in the minimal medium; McC, which contained 10 g of vitamin-free Casamino acids (Difco) per liter of minimal medium; and McAC, which contained acetate and Casamino acids in the minimal medium. Traces of acetate in the vitamin-free Casamino acids were removed by steam distillation of a 20% solution for 1 hr. After steam distillation, the acetate concentration in McC medium was found to be <1.30 mM by the acetate kinase assay and <0.029 mM by bioassay with M. voltae (22, 23). All media also contained 2.8 mM cysteine as a reductant. Solid medium was prepared by the addition of 1% bacteriological agar (Fishere). Stock cultures of the wild-type and derived mutants were maintained in 20% glycerol and McAC medium at −70°C (21).

Mutagenesis and Isolation of Mutants. To remove oxygen from a stock solution of ethyl methanesulfonate, a 0.2 M solution was taken into the anaerobic chamber and incubated in a loosely stoppered vial for 24 hr. Wild-type cells of M. maripaludis JJ1 were mutagenized by adding 0.1 ml of the ethyl methanesulfonate solution to a 5-ml culture during the early logarithmic growth phase in McN medium (24). After pressurizing with H2/CO2, 80:20 (vol/vol), to 200 kPa, the culture was incubated at 37°C for 1 hr. Cells were centrifuged in the culture tube at 2500 rpm for 20 min in a Dannon/IEC centrifuge (model CRU 5000) and washed three times with McN medium. After resuspending in 5 ml of McN medium, 1.0 ml was inoculated into 5 ml of McA medium. When the turbidity at 660 nm increased to about 0.4 cm−1, 0.2 ml was transferred to McC medium containing 1 mg of 6-azauracil (zUra) and 8-azaahypoxanthine (zHyp) per ml. The cultures were then pressurized with H2/CO2 to 200 kPa and incubated for 48 hr at 37°C. The surviving cells were recovered by centrifugation, washed twice in McN medium, and grown to the midlogarithmic phase in McA medium. This enrichment with base analogues was repeated twice. Cultures were then plated on McA agar medium and incubated for 10–14 days (25). Colonies were replica-plated with sterile toothpicks onto McN, McC, McA, and McAC agar and incubated for 10–14 days. Colonies that formed on plates of McA and McAC media, but not on McN or McC agar media, were picked with a sterile syringe into 5 ml of McA broth for further characterization. After growth, the McA broth culture was washed twice with McN medium and resuspended in 5 ml of McN medium. Portions of this culture (0.1 ml) were then inoculated into McN, McA, McC, and McAC media. Frozen stock cultures were prepared from the cultures that showed no growth in McN or McC medium. Independent isolates were obtained from separate experiments, and different pools of wild-type cells were used for mutagenesis and mutant isolation of each independent auxotroph.

For growth of the auxotrophic or wild-type strain in a 10-liter fermentor, McA medium was used under an atmosphere of H2/CO2 (100 kPa). At the end of growth, cultures of the auxotrophs were screened for the presence of spontaneous revertants. A portion of the culture was removed aseptically, washed in 5 ml of McN medium, and serially diluted to about 5 × 105 cells/ml in McN and McA media. These tubes were monitored for turbidity for 5 days. Cell pastes were only used if no growth was observed in McN medium.

Spontaneous zUra-resistant mutants were isolated after inoculating 5 × 108 cells into McNA medium containing 1 mg of zUra per ml. When an optical density of 0.4 cm−1 was obtained, 0.1 ml of this culture was plated on McA agar containing 1 mg of zUra per ml. After growth, colonies were initially picked into McA medium and maintained thereafter in the same medium containing zUra. Spontaneous revertants of the auxotrophs were obtained by washing 5 × 108 cells of the auxotrophs in McN medium and resuspending them in the same medium. When growth was observed, usually after 2–3 days, the cultures were plated on McN agar medium, and isolated colonies of the revertants were picked.

Preparation of Cell Extracts and Enzymatic Assays. After growth in a 10-liter fermentor, cells were harvested in the early stationary phase in a Sharples continuous flow centrifuge (26). After harvesting, all procedures were performed using strictly anaerobic techniques (14). The cells were resuspended in 10 ml of 25 mM K-Pipes buffer containing 0.5 mg of DNase per 10 g of wet weight of cells. Cell lysis was completed by freezing for 1 hr at −20°C. After thawing, the cell paste was centrifuged at 30,000 × g for 30 min at 4°C. Protein was measured by the Lowry method following digestion in NaOH (27).

The enzymatic assay were performed in a Gilford spectrophotometer at 37°C. CODH and pyruvate oxidoreductase (POR) were assayed by following the CO-dependent and pyruvate-dependent reduction of methyl viologen (14). One unit of enzyme activity was equal to the reduction of 1 nmol of methyl viologen per min. Under the conditions employed, 1 unit/mg of protein for both enzymes was significantly different from the background. Hydrogenase activity was determined by the H2-dependent reduction of methyl viologen (28). One unit of hydrogenase was equal to the reduction of 1 μmol of methyl viologen per min.

Acetyl-CoA synthesis was measured in a coupled assay with endogenous pyruvate synthase and added lactate dehydrogenase (LDH) (14). Extracts were precultivated in a 37°C water bath for 1 hr to remove endogenous substrates. The basic assay contained LDH, 10 μl containing 25 units; bromoethanesulfonate, 1 mM; and NADH, 0.4 mM, under an atmosphere of H2/CO2. Some assays contained these additional components: formaldehyde, 1 mM; methyl iodide, 1 mM; and CO2, 100 kPa. Undialyzed cell extract (0.2 ml) was added to each vial in the anaerobic glove box. The vials were flushed with H2/CO2 and incubated at 37°C for 40 min prior to terminating the reaction with 60% perchloric acid and measuring the lactate formed (29). The acetyl-CoA synthase activity was then taken as the nmol of lactate formed per min after subtracting the lactate synthesized in a control assay without CO2. The pyruvate synthase assay contained acetyl phosphate, 0.2 mM, and phosphotransacetylase, 10 μl containing 20 units, in the basic assay.

RESULTS

Isolation of Auxotrophs. Like other archaebacteria, the methanococci are insensitive to many common antibiotics, and methods for the selective enrichment of auxotrophs have been unavailable. Bacitracin, which has been employed successfully for this purpose in Methanobacterium spp. and certain halobacteria (24, 30), is ineffective in the methanococci (T. L. Bowen and W. B. W., unpublished results). The identification of several bacteriocidal nucleobase analogs in M. voltae suggested an alternative for the enrichment of auxotrophic mutants (25). To develop this method, the facultative autotroph M. maripaludis was treated with 4 mM of the mutagen ethyl methanesulfonate for 1 hr, conditions that reduced the viability of the culture by about 75%. Enrichment for acetate auxotrophs was then performed as described in the experimental procedures using zHyp and zUra. Eight independent acetate auxotrophs were obtained by this method (Table 1).

To determine the general utility of the enrichment procedure, the sensitivity of one of the auxotrophs to the nucleobase analogues under growing and nongrowing conditions was examined (Fig. 1). Under the growth condition with acetate, the viability of the auxotroph was reduced by nearly three orders of magnitude in the presence of the analogues. In the absence of acetate, a condition in which the auxotroph did not grow, the decline in viability was much smaller, and
Table 1. Strains of M. maripaludis

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth without acetate*</th>
<th>Phenotype†</th>
<th>Parental strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>JJ1</td>
<td>+</td>
<td>Aut* (wild type)</td>
<td>—</td>
</tr>
<tr>
<td>JJ4</td>
<td>(+)</td>
<td>Aut-1</td>
<td>JJ1</td>
</tr>
<tr>
<td>JJ6</td>
<td>—</td>
<td>Aut-2</td>
<td>JJ1</td>
</tr>
<tr>
<td>JJ7</td>
<td>—</td>
<td>Aut-3</td>
<td>JJ1</td>
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<tr>
<td>JJ8</td>
<td>—</td>
<td>Aut-4</td>
<td>JJ1</td>
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<tr>
<td>JJ9</td>
<td>—</td>
<td>Aut-5</td>
<td>JJ1</td>
</tr>
<tr>
<td>JJ10</td>
<td>(+)</td>
<td>Aut-6</td>
<td>JJ1</td>
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<tr>
<td>JJ11</td>
<td>—</td>
<td>Aut-7</td>
<td>JJ1</td>
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<tr>
<td>JJ12</td>
<td>—</td>
<td>Aut-8</td>
<td>JJ1</td>
</tr>
<tr>
<td>JJ13</td>
<td>+</td>
<td>Aut-2, Rut-1</td>
<td>JJ6</td>
</tr>
<tr>
<td>JJ14</td>
<td>—</td>
<td>Aut-5, Zur-1</td>
<td>JJ9</td>
</tr>
<tr>
<td>JJ15</td>
<td>+</td>
<td>Aut-5, Zur-1, Rut-2</td>
<td>JJ14</td>
</tr>
<tr>
<td>JJ16</td>
<td>+</td>
<td>Aut-7, Rut-3</td>
<td>JJ11</td>
</tr>
</tbody>
</table>

*+, The growth rates without acetate in McN and McC medium were >70% of the wild-type rate; (+), the growth rates in McN and McC media were 40–70% of the wild-type rate; —, no growth in McN or McC medium within 5 days.

†Aut*, capable of autotrophic growth; Aut-, requires acetate for growth; Rut, revertant of Aut− that is capable of autotrophic growth; Zur, resistant to zUr.

the analogues had no effect. Therefore, the final enrichment for cells that were incapable of growth under this condition was about 1000-fold. In addition, enrichment with either of the analogues singly was unsuccessful, presumably because of the rapid selection for analogue-resistant mutants (data not shown; ref. 25). Therefore, the combination of bacteriocidal nucleobase analogues was necessary to selectively kill growing cells under these conditions.

Characterization of Auxotrophs. Six of the eight auxotrophs had an absolute growth requirement for acetate in defined or complex medium (Table 1). In the presence of acetate, their growth rates were >60% of the wild-type in both media. CODH is a component of the acetyl-CoA synthase, a key enzyme in the Ljungdahl–Wood pathway (3, 4). The CODH activity was greatly reduced in extracts of all six of these auxotrophs to 1–16% of the wild-type level (Table 2). Because the CODH is an exceptionally oxygen-labile enzyme, the activities of two other oxygen-labile enzymes were examined as controls. Extracts of three of the auxotrophs contained wild-type levels of hydrogenase (Table 2). The levels of hydrogenase in extracts of JJ8 and JJ11 were higher than the wild-type level, and the level in JJ9 was only 70% of the wild-type level. Likewise, extracts of three of the auxotrophs, JJ8, JJ11, and JJ12, contained 68–100% of the wild-type level of POR. Therefore, the reduced CODH activity in extracts of the auxotrophs was not due to exposure to air during growth or preparation of the extracts. These results provided direct evidence for the role of CODH in acetate synthesis in the methanococci.

Surprisingly, extracts of three of the auxotrophs, JJ6, JJ7, and JJ9, contained only 14–29% of the wild-type level of OR, an activity associated with pyruvate synthase (Table 2). Pyruvate synthase is a major anabolic enzyme during heterotrophic and autotrophic growth of the methanococci (14, 26). To confirm these results, a spontaneous zUra-resistant derivative of JJ9 was isolated. This auxotroph, JJ14, contained the same levels of CODH, POR, and hydrogenase as the parent strain (Table 2). Therefore, the reduced CODH and POR activities were properties of a single cell type and not a result of a mixture of the original auxotroph and spontaneous revertants or contaminants acquired during growth in the fermentor.

In contrast, two of the eight auxotrophs isolated grew slowly in the absence of acetate. These auxotrophs, JJ4 and JJ10, grew at 40% and 55% of the wild-type rate, respectively, in defined and complex media without acetate. For JJ4, the cell yield was also reduced by 50% in the absence of acetate. In media with acetate, these auxotrophs grew as well as the wild type. JJ4 and JJ10 contained nearly wild-type levels of CODH, POR, and hydrogenase (Table 2). Although JJ10 contained slightly reduced levels of CODH, 78% of the wild-type level, it is unlikely that this factor was sufficient to explain the slow autotrophic growth rate. Possibly, the mutation or mutations in these auxotrophs inactivated other enzyme systems necessary for efficient autotrophic growth but not essential for autotrophy.

To examine the physiological activities of the acetyl-CoA and pyruvate synthases, a coupled assay with LDH was employed (14). In this assay system, autotrophic acetyl-CoA was carboxylated by endogenous pyruvate synthase prior to being reduced by added LDH. The lactate formed was not metabolized further in these extracts. To examine pyruvate synthase activity, an acetyl-CoA generating system was added to extracts. The levels of acetyl-CoA and pyruvate synthase in extracts of the wild-type cells were typical for heterotrophically grown cells and about one-third of that found in autotrophically grown cells (14). In extracts of JJ8, the levels of pyruvate synthase were close to those found in the wild type (Table 3). This result was consistent with the
A major goal of this work was to obtain genetic evidence for the role of CODH and acetyl-CoA synthase in autotrophy in the methanococci. Two types of evidence were obtained. (i) Six independent auxotrophs that were incapable of autotrophic growth were isolated. All six contained reduced levels of CODH. In at least two cases where the physiological activity of acetyl-CoA synthase could be assayed unambiguously, this activity was also greatly reduced. (ii) Revertants of three of these auxotrophs regained the ability to grow autotrophically and had wild-type levels of CODH and acetyl-CoA synthase. These results provide strong support for a direct role of CODH and acetyl-CoA synthase in autotrophy in the methanococci. In *Methanobacterium ivanovii*, three acetyl auxotrophs were characterized that contained wild-type levels of CODH (24). Presumably, these auxotrophs contain mutations in other components of the acetyl-CoA synthase or other pathways required for autotrophic growth.

The specific nature of the methanococcal auxotrophs is not known. One auxotroph, JJ12, contains only 1% of the wild-type CODH activity and low acetyl-CoA synthase activity but wild-type levels of POR and hydrogenase. Although other explanations are possible, this pattern is consistent with a mutation in the structural gene or genes for acetyl-CoA synthase. Two auxotrophs, J8 and JJ11, contain very low levels of CODH, slightly reduced levels of POR, and slightly elevated levels of hydrogenase. The possible site of a mutation or mutations in these auxotrophs is difficult to predict in the absence of more detailed knowledge of the acetyl-CoA synthase.

Three of the methanococcal auxotrophs, JJ6, JJ7, and JJ9, contain greatly reduced levels of POR and pyruvate synthase as well as CODH. This result was unexpected, and a linkage of acetyl-CoA and pyruvate synthesis has not been previously observed. Because the revertants of these auxotrophs regained all three activities, the simultaneous loss of these characters may have resulted from the inactivation of a component common to both systems. The nature of this component is speculative, but a number of rationales for these auxotrophs are plausible. The acetyl-CoA and pyruvate synthases catalyze reactions that are thermodynamically unfavorable under standard conditions and that require strong reductants. Thus, both synthases could be coupled to membrane proton or ion gradients in vivo, and the inactivation of a common component necessary for this coupling could disrupt synthesis or assembly of the enzyme complexes. In support of this hypothesis, the reduction of CO2 to CO by *Methanosarcina* requires participation of the proton motive force (31). Alternatively, both enzyme systems could utilize the same protein or coenzyme as an electron donor. The synthesis of the electron donor could be defective in the auxotrophs. Lastly, other enzymes could provide a common activator protein. For the methylreductase in methanogens, catalysis by component C requires activation by ATP, H2, and the protein components A2, A3a, and A3b (32). A similar system could be required for activation of other enzyme systems in the methanococci. Likewise, the acetyl-CoA synthase of *Clostridium thermocaceticum* requires activation by CODH disulfide reductase (33). It is possible that a disulfide reductase is also required for activation of the methanococcal acetyl-CoA and pyruvate synthases.

Alternatively, the linkage observed between POR and CODH in these mutants could be due to a number of genetic factors. For instance, if the structural genes for these enzymes constitute an operon, polar or regulatory mutations could reduce the expression of both genes in concert. Likewise, if the structural genes are unlinked but share a trans-acting regulatory component, mutations in the regulatory system could reduce gene expression for both activities. In addition, it is also possible that a mutation in a third enzyme system could lead to an over- or underproduction of a regulatory metabolite and thus reduce the expression of the acetyl-CoA and pyruvate synthases. However, in the absence of more detailed genetic and biochemical information about these enzyme systems, it is not possible to distinguish between these possibilities.

### DISCUSSION

A major goal of this work was to obtain genetic evidence for the role of CODH and acetyl-CoA synthase in autotrophy in the methanococci. Two types of evidence were obtained. (i) Six independent auxotrophs that were incapable of autotrophic growth were isolated. All six contained reduced levels of CODH. In at least two cases where the physiological activity of acetyl-CoA synthase could be assayed unambiguously, this activity was also greatly reduced. (ii) Revertants of three of these auxotrophs regained the ability to grow autotrophically and had wild-type levels of CODH and acetyl-CoA synthase. These results provide strong support for a direct role of CODH and acetyl-CoA synthase in autotrophy in the methanococci. In *Methanobacterium ivanovii*, three acetyl auxotrophs were characterized that contained wild-type levels of CODH (24). Presumably, these auxotrophs contain mutations in other components of the acetyl-CoA synthase or other pathways required for autotrophic growth.

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Lastly, there remains the question of how the auxotrophs with reduced levels of POR and pyruvate synthase grow heterotrophically in the presence of acetate. Because some pyruvate synthase activity remains in these mutants, it is possible that low levels are sufficient to support growth. Alternatively, the cells may contain an alternative mechanism of pyruvate synthesis that is not observed in the presence of high levels of pyruvate synthase.

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