GENETIC STUDIES OF CARBON DIOXIDE FIXATION IN THE METHANE-PRODUCING ARCHAEON *METHANOCOCCUS MARIPALUDIS*

by

WONDUCK KIM

(under the direction of WILLIAM B. WHITMAN)

ABSTRACT

Methanococcus maripaludis is a facultative autotrophic methanogen that utilizes $H_2 + CO_2$ for carbon fixation and energy production. In CO_2 fixation, acetyl-CoA is the precursor of cell carbon. To study autotrophic CO_2 fixation pathways, acetate auxotrophs of *M. maripaludis* were generated by random mutagenesis. The mutagenesis was performed by disruptions of chromosomal genes with a plasmid library in the vector, pMEB.2, and screening by replica plating. JJ117 was one of the nine conditional acetate auxotrophs found and was auxotrophic for both acetate and cobalamin. However, the disrupted gene (*ppm*: phosphopentomutase) in JJ117 did not seem likely to be involved in the cobamide biosynthetic pathway. Cloning and sequencing of the downstream sequence of *ppm* found *cbiJ*, which is convergently transcribed. Thus, the cobamide auxotrophy may have been caused by an effect on *cbiJ* expression. The *cbiJ* was mutagenized by gene replacement, and the resulting mutant was an acetate and cobalamin auxotroph. To understand how acetate supports the growth of JJ117, the cobamide in wild type and JJ117 was quantified by a bioassay. The assay showed that JJ117 produced a small amount of cobamide that might be sufficient for cobamide – dependent activities that are essential for growth. To explain the acetate auxotrophy, acetate is hypothesized to spare the requirement of cobamide by its conversion to acetyl-CoA without cobamide.

Previous research indicated the possible involvement of the *act* (pyruvate-formate lyase activating enzyme) or *ehb* (energy-conserving hydrogenase B) in the pathway of autotrophic CO_2 fixation. These genes were mutagenized by gene replacement and the mutants were characterized. Growth of mutant strains, JJ310 and JJ320, which have mutations in *act,* was stimulated by acetate. However, more research is required to clarify the roles of the *act*. Mutagenesis of *ehb* in *M. maripaludis* generated an acetate auxotroph, S40, suggesting that the gene is involved in autotrophic CO_2 fixation. In addition, methanogenesis from pyruvate was also inhibited in S40, indicating that the hydrogenase encoded by *ehb* may be linked to the activity of pyruvate oxidoreductase (POR). These data are consistent with previous reports that show that this hydrogenase is coupled to endergonic reactions by energy conservation.

INDEX WORDS**:** *Methanococcus maripaludis*, Acetate auxotrophs, Cobamide synthesis, Formate-lyase activating enzyme, Ehb hydrogenase

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DEDICATION

To my parents and brothers

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEWS

I. GENERAL CHARACTERISTICS OF METHANOGENS

Methanogens are prokaryotes that produce methane gas as an essential component of their energy metabolism. Although they are responsible for copious methane production by the gastrointestinal tract of humans and many domestic animals, none are known to be responsible for food borne disease. For the food industry, the primary interest has been in their importance in anaerobic bioreactors for wastewater treatment and animal nutrition.

Methanogens are obligate anaerobes and are common in many environments with a low redox potential, such as the gastrointestinal tracts of animals and termites, sediments of freshwater lakes, rice paddies, sewage, landfills and anaerobic digestors. In these habitats, methanogens catalyze the terminal step in an anaerobic food chain that converts organic matter into methane and CO_2. Anaerobic bacteria and anaerobic eukaryotes perform the initial transformation of the biopolymers in organic matter to substrates for methanogenesis. Thus, consortia of microorganisms are required to produce methane in most habitats, a striking example of which is the symbiotic methanogens of many anaerobic protozoa.

Methanogens are the major source of atmospheric methane, an important greenhouse gas that has been increasing in concentration for the last two hundred years. Given that about half of the methane produced is oxidized by methane-oxidizing bacteria, about 1-2 % of all the organic matter produced by plants each year passes through the methanogenic food chain. Thus, this process is a significant component of the carbon cycle.

The substrate range of the methanogens themselves is quite limited and only three types are utilized (Fig. 1). In the first type, CO_2 is reduced to methane by fairly narrow range of electron donors. Some methanogens can also slowly oxidize a few alcohols, especially ethanol, isopropanol, isobutanol and cyclopentanol. The alcohols are incompletely oxidized to form ketones or carboxylic acids. In the second type of methanogenesis, acetate is fermented to methane and CO_2. Even though this aceticlastic reaction accounts for most of the methane generated in many habitats, it is catalyzed by only a few genera of methanogens. The last type of methanogenesis is the reduction of the methyl group of C1 compounds to methane. C1 compounds utilized include methanol, monomethylanine, dimethylamine, trimethylamine, methanethiol and dimethylsulfide. Usually, a portion of the C1 compound is oxidized to provide electrons for the reduction. Thus, about one quarter of the methyl groups are oxidized to CO_2 and three quarters are reduced to methane. Some methylotrophic methanogens cannot oxidize methyl groups, and H_2 is the electron donor.

1. Diversity and taxonomy

All known methanogens are Archaea, a diverse phylogenetic group that also includes many thermophilic and halophilic prokaryotes. Although first proposed by Carl Woese and his collaborators in 1977 on the basis of their unusual 16 S rRNA sequences, other features also characterize the archaea, including unique structures of their lipids, novel cell walls, abundance in extreme habitats, and eukaryotic type of DNA-dependent RNA polymerase and promoter structure. Recent genomic sequencing has confirmed many of the profound differences between archaea and the more familiar eubacteria.

Lipids are important chemotaxonomic markers in methanogens and other archaea. There are four major differences from the lipids of eubacteria and eukaryotes. First, the hydrocarbon side chains are linked to glycerol with ether instead of ester bonds. Second, the hydrocarbon side chains are based on the C5 isoprenoid unit instead of the C2 acetyl moiety. Third, archeal lipids contain sn-2,3-glycerol, the opposite stereoisomer of the glycerol in eubacterial and eukaryotic lipids. Fourth, archaeal lipids are frequently tetraethers that span the membrane and are formed by a condensation of the isoprenoid side chains. Although ether-linked and branched lipids are found in some eubacteria, the combination of unusual features in archeal lipids argues strongly for entirely different biosynthetic pathway. Within the methanogens, the major core lipids are archaeol (2,3-di-O-phytanyl *sn* –glycerol diether), caldarchaeol (ditetraterpenediyl glycerol tetraether), *sn*-2-hydroxyarchaeol, and sn-3-hydroxyarchaeol. Glycolipids also contain glucose, galactose, N-acetylglucosamine and mannose. Phospholipids also contain inositol, ethanolamine, serine, aminopentanetetrols and glycerol.

Several different types of cell envelopes are present in methanogens. In the simplest type, the cell envelope is composed solely of a protein surface layer or S-layer. The S-layer contains hexagonally arranged protein subunits, which vary in molecular weight and antigenicity between species. Frequently, the S-layer protein is glycosylated. In other methanogens, the wall contains additional polymers, such as methanochondroitin. This compound is similar in structure to chondroitin, which is found in the connective tissue of animals, and plays a vital role in cellular aggregation in some genera. In other methanogens, the cell envelope also contains a protein sheath that is strongly resistant to detergent and proteases. Lastly, methanogens that stain Gram-positive contain pseudo-

murein, a peptidoglycan that superficially resembles the common murein of eubacteria. In pseudomurein, the sugar backbone is composed of L-N-acetyl talosaminuronic acid and D-N-acetylglucosamine. The interpeptide bridge contains only L-amino acids.

Among the archaea, methanogens are unusual in that they are common in moderate as well as extreme habitats. Thus, growth temperatures of methanogens span 100°C, from psychrotrophic to hyperthermophilic environments. Optimal salinities for growth vary from freshwater to saturated brine. In contrast, most methanogens prefer neutral to moderately alkaline pH values and few acidophiles have been described.

Recent taxonomic proposals place the methanogens in 25 genera, representing 12 families and five orders (Table 1). This taxonomy is consistent with the high degree of phenotypic and genotypic diversity found within group. The wide diversity within the group suggests that methanogenesis is an ancient life style. Because it is likely that methanogens are monophyletic, it is also likely that methanogenesis evolved only once and that all modern methanogens share a single ancestor.

2. Methanogenic bioreactors

In a typical anaerobic bioreactor, oxidation of organic matter rapidly exhausts good electron acceptors such as O_2, nitrate, and sulfate. When CO_2 is the only abundant electron acceptor remaining, the conditions become favorable for methanogenesis. Because CO_2 is such a poor electron acceptor, only about 5% of the total energy of combustion in the organic matter is available to support microbial growth, and the formation of microbial biomass or sludge is correspondingly less than that found in a typical aerobic bioreactor. In addition, the fuel requirements and reactor volumes are much smaller for a methanogenic bioreactor, and the process may be significantly less

expensive than for typical aerobic waste treatment. For instance, a 223,000 gallon (843,000 L) anaerobic fluidized bed reactor removing 9,700 lbs (4,400 kg) biochemical oxygen demand (BOD) per day would produce 72,000 ft^3 (2,040,000 L^3) of CH_4 per day and 180 tons of sludge per year. A comparable aerobic treatment would require a 1,700,000 gallon (6,400,000 L) reactor and produce 1800 tons of sludge per year.

During anaerobic digestion, the initial fermentation of organic matter generates H_2, formate, and a wide variety of organic acids such as acetate, propionate, butyrate and lactate as well as alcohols (Fig. 2). Consumption of the H_2 and formate by the methanogens enables syntrophic bacteria to oxidize the organic acids and alcohols to additional H_2 and acetate. This interaction between the syntrophic bacteria and the methanogens, called interspecies H_2 transfer, is necessary because the fermentation of propionate and butyrate to acetate and H_2 is only feasible when the H_2 concentration is extremely low or below 10^{-4} atm. Similarly, fermentation of lactate and ethanol is also greatly stimulated when the H_2 concentration is low. Thus, the anaerobic food chain of fermentative and syntrophic bacteria converts the organic matter into the major substrates for methanogenesis: CO_2, H_2, formate and acetate.

For the fermentation of sugars, typically about two-thirds of the methane is derived from acetate, whereas the remainder is formed by the CO_2 reduction. These ratios are in close agreement with that expected based on the biochemistry of glycolysis. In this pathway, one molecule of hexose is oxidized to two molecules of pyruvate with the reduction of two more molecules of NAD^+. Pyruvate is then oxidized to acetate and CO_2 by the reduction of two more molecules of NAD^+ or NAD^+ equivalents. The four molecules of NADH (or NADH equivalents) are used to reduce one molecule of

CO_2 to methane. The two molecules of acetate are utilized to form two additional molecules of methane and CO_2. Thus, one molecule of hexose is converted into three molecules of methane and CO_2. Even though aceticlastic methanogenesis is more abundant, CO_2 reduction to methane is essential to generate most of the acetate, and both process are interrelated.

The generation times of the aceticlastic methanogens and the syntrophic bacteria frequently exceed 24 h, which limits the turnover time of the anaerobic bioreactor. This problem is overcome by recycling or trapping the anaerobic microbes within the bioreactor, and high volumetric loading rates can be achieved. Nevertheless, the slow growth of these microbes may partially explain the long start-up times for anaerobic bioreactors.

Numerous species of CO_2-reducing methanogens have been isolated from bioreactors, including species of *Methanobacterium*, *Methanothermobacter*, *Methanobrevibacter*, *Methanogenium*, *Methanocorpusculum* and *Methanospirillium*. For the aceticlastic methanogens, species of both *Methanosarcina and Methanosaeta* are usually present. Although *Methanosarcina* grows more rapidly, it is unable to utilize the low concentrations of acetate taken up by *Methanosaeta*.

3. Methanogenesis in the gastrointestinal tract of animals

Methanogenesis in the rumen of cattle and other ruminants is major source of atmospheric methane as well as of considerable importance in the nutrition of these animals. The anaerobic food chain is similar to that found in bioreactors except that CO_2 reduction is the major source of methanogenesis (Fig. 2). Because the residence time of

the rumen contents is less than one day, aceticlastic methanogens and synthrophic bacteria are washed out, preventing the metabolism of acetate and other organic acids. The organic acids can then accumulate to concentrations that can be absorbed by the animal. Although species of *Methanobrevibacter* are commonly isolated from the rumen, species of *Methanomicrobium* and methylotrophic *Methanosarcina* are also present.

The bovine rumen produces 200-400 L of methane per day, which represents a significant loss in energy for the animal. Two common feed additives, monensin (or rumensin) and lasalocid, derive their effectives from their ability to inhibit H_2 production by Gram–positive bacteria in the rumen. The lower availability of H_2 limits methanogenesis, and propionate production is stimulated. The net result is more efficient utilization of low fiber feeds by the animal.

In humans, a similar process occurs in the colon. However, the methanogenic fermentation of only about 10% of people on a Western diet is significant and produces more than a liter of methane per day. At least superficially, this fermentation resembles that found in the rumen, where organic acids are absorbed by the intestines and CO_2 reduction is the source of most of the methane. People who do not produce large amounts of methane probably contain homoacetogenic bacteria instead. These eubacteria are strict anaerobes that oxidize H_2 to reduce CO_2 to acetate. Because this fermentation is slightly less favorable energetically than methanogenesis, CO_2 reduction to acetate is not a major process in many methanogenic habitats, and the factors which enable acetogenesis to dominate in the colon are not understood. In the colon, the most

abundant methanogen is *Methanobrevibacter smithii*. However, low numbers of the methylotroph *Methanosphaera* have also been detected.

II. CARBON ASSIMILATION IN METHANOGENS

1. The reductive acetyl-CoA pathway in methanogens

A modified acetyl-CoA pathway (or Wood/Ljungdahl pathway), which is utilized for carbon fixation and energy production in acetogens and some sulfate-reducing bacteria is found in methanogens. In the Wood/Ljungdahl pathway, acetyl-CoA is synthesized from two CO_2 molecules that are converted into a methyl group and a carbonyl group by two independent pathways. The acetyl-CoA is then the precursor of cell carbon in the bacteria. For energy production, ATP is synthesized during the conversion of acetyl-CoA into acetate, which is excreted (Drake, 1994). This pathway is thought to play an important role in carbon cycling in natural habitats (Drake *et al*., 1997). In sulfate-reducing bacteria, the acetyl-CoA pathway proceeds in both directions, depending on the organisms (Thauer *et al*., 1989). For some autotrophs, it is a pathway of CO_2 fixation. For some heterotrophs, it is utilized for acetate oxidation.

Early studies of *Methanothermobacter thermautotrophicus* suggested that the autotrophic methanogens incorporate CO_2 by a reductive acetyl-CoA pathway. Enzymes involved in a reductive pentose phosphate pathway or a reductive tricarboxylic acid pathway that fixed CO_2 in other bacteria autotrophic bacteria were not found (Zeikus *et al*., 1977).

As in acetogens and the sulfate-reducing bacteria, acetyl-CoA is a precursor of the cellular carbon in methanogens. The study of incorporation of [^{14}C]-labeling acetate to *M. thermautotrophicus* showed that high concentrations of acetate provide up to 65 % of the cell carbon in the methanogen (Fuchs *et al*.,1978). Another incorporation study of [^{14}C]-labeled acetate and pyruvate into *M. thermautotrophicus* suggested acetyl-CoA is a precursor of pyruvate and amino acids (Fuchs and Stupperich, 1980). Research suggested two molecules of CO_2 are required for the synthesis of one molecule of acetyl-CoA (Stupperich and Fuchs, 1983).

The acetyl-CoA pathway in methanogens is different from that of other bacteria in some respects. In methanogens, synthesis of acetyl-CoA is linked to the methanogenesis pathway. Moreover, there is no formation of ATP in the methanogenic acetyl-CoA pathway. The nature of the linkage between acetyl-CoA synthesis and methane production was suggested by the inhibition of both pathways by inhibitors that inactivate corrinoid-dependent reactions (Stupperich and Fuchs, 1984). In addition, it was found *in vitro* that two intermediates of methanogenesis, N^5, N^{10}-methylenetetrahydromethanopterin and N^5-methyltetrahydromethanopterin, stimulated the synthesis of acetyl-CoA in *M. thermautotrophicus*. It was theorized that donation of the methyl group from N^5-methyltetrahydromethanopterin to the acetyl-CoA pathway was responsible for this stimulation (Länge and Fuchs, 1987).

2. CODH/acetyl-CoA decarbonylase synthase

CODH (carbon monoxide dehydrogenase) is a central enzyme for autotrophic CO_2 fixation. The enzyme catalyzes synthesis of acetyl-CoA by combining a carbonyl group

and a methyl group. In methanogens, CODH is found as a component of the CODH/ACDS (CODH/acetyl-CoA decarbonylase synthase) complex.

The presence of CODH activities in autotrophic methanogen was confirmed in *M. maripaludis* by assaying cell extracts grown on $H_2 + CO_2$. The activities were quantified indirectly by the formation of lactate from pyruvate and acetyl-CoA. This assay showed that the activities of CODH were increased by CO, CH_3I and HCHO, and reduced by KCN, an inhibitor of the enzyme (Shieh and Whitman, 1987).

Since acetate can be converted into acetyl-CoA by acetyl-CoA synthetase in the methanogen, the acetate auxotrophs generated by chemical mutagenesis were utilized to investigate the acetyl-CoA pathway. The auxotrophs contain low levels of CODH activity, indicating that acetyl-CoA synthesis by CODH is essential for this pathway. Some of the auxotrophs contained low activities of CODH and POR, suggesting the activities of both enzymes are coupled by common factors (Ladapo and Whitman, 1990).

In aceticlastic methanogens, the CODH/ACDS complex is involved in synthesis and disproportionation of acetyl-CoA. The involvement of the complex in autotrophic biosynthesis is indicated by the formation of acetyl-CoA from CoA, CO and CH_3I by the CODH complex from *M. thermophila* (Abbanat and Ferry, 1991). In a disproportion role, the complex catalyzes the cleavage of acetyl-CoA into a CH_3 moiety, CO and coenzyme A. The CH_3 moiety is transferred to a corrinoid protein then tetrahydrosarcinapterin. From tetrahydrosarcinapterin, the CH_3 moiety is transferred to coenzyme M and reduced into CH_4 in the methanogenesis pathway (Jetten *et al*., 1992).

Either the entire CODH/ACDS complex or one component of the complex has been purified from *Methanosarcina barkeri*, *Methanosarcina thermophila*, *Methanosarcina*

frisia and *Methanococcus vannielii* (Krzycki and Zeikus, 1984: Terlesky *et al*., 1986: Eggen *et al*., 1996: DeMoll *et al*., 1987). The complexes purified from aceticlastic methanogens, *M. barkeri* and *M. thermophila*, are composed of two components with a total of five subunits (α, β, γ, δ, and ε). Investigation of the purified *M. thermophila* complex showed that the two components contain a Ni/Fe-S center (αε) and a Co/Fe-S center (γδ) (Abbanat and Ferry, 1991). The Ni/Fe-S component and the Co/Fe-S component in aceticlastic methanogens were proposed to be involved in reversible conversion of CO and CO_2 and the disproportion of cleaved CH_3 group from acetyl-CoA, respectively (Abbanat and Ferry, 1991: Jablonski *et al*., 1993). Recent research (Murakami and Ragsdale, 2000: Gencic and Grahame, 2003) suggests that the β subunit of the complex also contains the Ni/Fe-S center that is involved in cleavage and assembly of C-C and C-S bonds of acetyl-CoA.

The analysis of genes encoding the CODH/ACDS complex showed the subunits, α, ε, β, δ and γ are encoded by *cdhA*, *cdhB*, *cdhC*, *cdhD* and *cdhE* genes, respectively. Comparison of amino acid sequences of the *cdh* genes indicated that a ferredoxin is present in the complex. Structures of the iron-sulfur clusters are possibly unique since cloning and expression of *cdhA* and *cdhB* of *Methanothrix soehngenii* in *Escherichia coli* and *Desulfovibrio vulgaris* failed (Eggen *et al*.,1991). On the genome of *M. thermophila*, the genes encoding subunits of CODH/ACDS complex (*cdhABCDE*) are clustered and cotranscribed. Northern blotting and primer extension of the gene cluster indicated the presence of multiple transcripts from different promoters in the operon (Maupin-Furlow and Ferry, 1996).

Recently, the DNA sequences encoding the CODH/ACDS complexes in methanogens were identified from complete genomic sequences. In the genome of *Methanocaldococcus jannaschii*, *cdh* homologs are found in three different DNA regions (Bult *et al*., 1996). Alternatively, most of the *cdh* homologs are found in one cluster in *M. maripaludis*. The clusters also include homologs to genes found in formate dehydrogenase and CODH accessory protein. The gene structure of *cdh* homologs in *M. thermautotrophicus* is similar to that of *M. maripaludis* (Smith *et al*., 1997). In *Methanosarcina acetivorans*, gene duplication of the *cdhABCDE* operon was identified (Galagan *et al*., 2002).

3. Activation of acetate into acetyl-CoA

In methanogens, two different activation systems for converting acetate into acetyl-CoA are present. In *Methanosarcina* spp., the activation occurs by the sequential reactions of acetate kinase (acetate + ATP → acetylphosphate + ADP) and phophotransacetylase (acetylphosphate + CoA → acetyl-CoA + Pi). In *Methanosarcina thermophila*, the two genes (*pta* and *ack*) that encoding the enzymes are adjacent on the genome and are coregulated at the transcription level (Singh-Wissman and Ferry, 1995). Acetate kinase and phosphotransacetylase were purified and characterized from this methanogen. The molecular weight of the kinase, which is a homodimer (α_2), was 94,000, and divalent metal ions were essential for activity. The expression of acetate kinase was increased when acetate was present (Aceti and Ferry, 1988). The acetate kinase was classified as a member of the sugar kinase/Hsc70/actin superfamily by a putative binding motif in the nucleotide sequence (Miles *et al*., 2001). Phosphotransacetylase is a monomer (Mr=42,000-52,000) that requires more than 10 mM of potassium and ammonium for

maximum activity *in vitro*. Western blotting indicated that the expression level of the acetate kinase was increased by presence of acetate (Lundie and Ferry, 1989). The detailed catalytic mechanism of the enzymes was revealed by site directed mutagenesis. The study showed that the binding motif sequence of acetate kinase is essential since the mutagenesis of the sequence severely lowered the activities of the enzyme (Miles *et al*., 2001). Another series of site directed mutagenesis experiments was performed for the phosphotransacetylase, which showed the important roles of specific arginine (R310) and cysteine (C159) residues during catalysis (Rasche *et al*., 1997, Iyer and Ferry, 2001).

In other methanogens, including *Methanothrix* spp., *Methanococcus* spp., and *Methanobacterium* spp., the activation of acetyl-CoA is performed by acetyl-CoA synthetase alone. The acetyl-CoA synthetase catalyzes the activation by consuming ATP (acetate + ATP → acetyl-CoA + AMP + PPi). The molecular mass of the synthetase purified from *M. soehngenni* is 146 kDa. It is a α_2 homodimer (Jetten *et al*., 1989). The *acs* gene encoding the enzyme was cloned and expressed in *E. coli* (Eggen *et al*., 1991).

Recent analysis of the genome of *Methanocaldococcus jannaschii* discovered a gene homologous to ADP- forming acetyl-CoA synthetase, which is usually identified in hyperthermophile archaea (Musfeldt and Schonheit, 2002). This enzyme catalyzes reversible conversion of acetyl-CoA into acetate (acetyl-CoA + ADP + Pi ↔ acetate + ATP +CoA). In the hyperthermophile archaea, this formation of acetate is a final step of sugar fermentation, and ATP is produced by substrate level phosphorylation (Schafer *et al*., 1993: Schönheit and Schäfer, 1995).

4. Roles of pyruvate and pyruvate oxidoreductase (POR)

Pyruvate is an essential intermediate for CO_2 fixation in methanogens since it serves as a precursor of amino acids and carbohydrates. In methanogens, pyruvate is formed from reductive carboxylation of acetyl-CoA, catalyzed by pyruvate oxidoreducatse (POR). The enzyme also catalyzes the oxidative decarboxylation of pyruvate into acetyl-CoA. The study of methanogenic POR suggested that specialized ferredoxins functions as the electron carrier. The POR contains three [4Fe-4S] centers and contains thiamine diphosphate as a cofactor. The POR of *M. thermautotrophicus*, *M. jannaschii* and hyperthermophiles is composed of four subunits: α, β, γ, and δ (Bock *et al*., 1996: Bock *et al*.,1997: Tersteegen *et al*.,1997). In contrast, the POR of *M. maripaludis* contains an additional subunit with similarity to a polyferredoxin (Lin, 2002).

In *Methanococcus s*pp., the anabolic (reductive) activities of POR appear to be predominant. When *M. maripaludis* was grown under $H_2 + CO_2$, significant amounts of acetate from the oxidation of pyruvate were not generated. However, when H_2 was absent, the oxidation of pyruvate occurred, which was confirmed by stimulation of methanogenesis from pyruvate in resting cells. This is predicted, as oxidation of pyruvate provides reducing equivalents for the methanogenesis pathway. However, the oxidation is not physiologically significant, as only 4 % of the rate of methanogenesis from $H_2 + CO_2$ was supported by pyruvate. The growth experiments of *Methanococcus voltae* and acetate auxotrophs of *M. maripaludis* confirmed that pyruvate does not function as a source of carbon and energy in the presence of H_2. The low catabolic activities of POR are efficient in the metabolism of the methanogens since it prevents futile cycling (Yang *et al*., 1992).

M. maripaludis grown with alanine and $H_2 + CO_2$ showed carbon flux thorough pyruvate. In this experiment, alanine was the sole nitrogen source, and it was converted into pyruvate and the pyruvate-derived amino acids (leucine, isoleucine and valine) by the activities of alanine dehydrogenase and alanine aminotransferase. Thus, the intracellular flux of pyruvate carbons could be studied by NMR analysis of labeled valine and leucine following growth on radiolabeld alanine. This study showed even in the presence of abundant source of reduced carbon, a large fraction of the acetyl-CoA and pyruvate was formed by autotrophic CO_2 fixation (Yang *et al*., 2002).

In mutant of the aceticlastic methanogen, *Methanosarcina barkeri* (strain Fusaro), pyruvate functions as carbon source and energy source. The acetyl-CoA formed from pyruvate oxidation was a precursor of cell carbon, and the methyl group from the compound is used in methanogenesis (Bock *et al*., 1994: Rajagopal and LeGall, 1994).

5. Amino acid biosynthesis in methanogens

Investigations suggest that the biosynthetic pathways of amino acids in methanogens are analogous to those of bacteria and eukaryotes. Despite the similarities, the methanogenic amino acid synthesis pathways are somewhat modified. As a C-1 acceptor for the purified methanogenic serine transhydroxylmethylase, which catalyzes the conversion of serine into glycine, methanogens utilize tetrahydromethanopterin instead of tetrahydrofolate (Hoyt *et al*.,1986). In methanogens, the branched-chain amino acids, isoleucine, valine and leucine, are synthesized by the acetohydroxy acid pathway as in eubacteria (Xing and Whitman, 1991). However, the precursor of 2-ketobutyrate and isoleucine in the pathway is citramalate, which is synthesized from

acetyl-CoA and CO_2, rather than threonine in the eubacterial pathway (Ekiel *et al*.,1984: Eikmanns *et al*.,1983).

The synthesis of aromatic amino acids in methanogens utilizes the shikimate pathway, which uses erythrose 4-phosphate as a precursor (Ekiel *et al*.,1983). However, it is uncertain if those amino acids in methanogens are derived from the erythrose 4-phosphate, a product of the pentose phosphate pathway. In a ^{13}C-labelling study, carbon labeling of the aromatic amino acids was not consistent with that found in other organisms. Thus, it was considered that methanogens might possess a modified pentose phosphate pathway. Those researchers, however, suggested that there was no modification in the pentose phosphate pathway but that erythrose 4-phosphate was not a precursor of aromatic amino acids (Tumbula *et al*., 1997). Moreover, the activity of 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase was not detected (Fischer *et al*.,1993). In addition, genes homologous to the 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase 3-dehydroquinate synthase were not identified in the genomes of methanogens. Therefore, some unusual biosynthetic pathways of the aromatic amino acids are expected in methanogens.

6. Carbohydrate biosynthesis in methanogens

In methanogens, gluconeogenesis and the pentose phosphate pathway synthesize carbohydrates. Like other archaea, methanogens utilize the enzymes of the classical Embden-Meyerhoff-Parnas (EMP) pathway for gluconeogenesis. ^{14}C-labeling studies indicated the presence of the EMP pathway in *M. thermautotrophicus* (Jansen *et al*., 1982). Moreover, the activities of enzymes involved in the EMP pathway were detected

in *M. maripaludis* (Yu *et al*., 1994). It was reported that *Methanothermobacter* spp. utilizes the EMP pathway only for anabolic gluconeogenesis (Fuchs *et al*., 1983), whereas in *M. maripaludis* the pathway is for both anabolic and catabolic reactions (Yu *et al*., 1994).

Information from labeling and ^{13}C-NMR of pathway intermediates indicate that two different pentose phosphate pathways are present in methanogens. In the oxidative pentose phosphate pathway, which is found in *M. thermautotrophicus, M. barkeri, Methanbacterium bryantii, Methanospirilium hungatei,* and *Methanosphaera stadtmanae* (Choquet *et al*.,1994), pentoses are synthesized by oxidative decarboxylation from 6-phosphogluconate. In contrast, *Methanococcus* spp., possess the non-oxidative pentose phosphate pathway, where elevated levels of transketolase and transaldolase activity are necessary. The nonoxidation pathway conserves CO_2, which is released during the oxidative pathway. In addition, NADPH is not produced during the nonoxidative biosynthesis (Yu *et al*., 1994).

7. Incomplete TCA cycles in methanogens

The incomplete TCA (tricarboxylic acid) cycles of methanogens are unusual. As in the TCA cycle in other organisms, intermediates of the incomplete cycles are converted into cellular compounds such as amino acids. Two different incomplete TCA cycles are found in methanogens; an incomplete reductive TCA cycle and an incomplete oxidative TCA cycle. Probably, the presence of these cycles is dependent on the physiologies of the methanogens, since the reductive cycle is found in autotrophic methanogens while aceticlastic methanogens possess the oxidative cycle (Ekiel *et al*., 1985: Sprott *et*

al.,1993). The oxidative cycle loses one molecule of CO_2 for the synthesis of α–ketoglutarate. This pathway appears not to influence the growth of aceticlastic methanogens much because the organism could use acetate as carbon and energy sources. In the reductive cycle, α–ketoglutarate is biosynthesized from oxaloacetate by reductive carboxylation. This system is advantageous to autotrophic methanogens that can supply a source of the reduction from $H_2 + CO_2$ (Simpson and Whitman, 1993).

III. COBAMIDES

1. Structure and roles of cobamide

Cobamides are a class of the complex cofactors synthesized in bacteria and archaea. They are composed of a central tetrapyrrole ring (corrin ring) with inserted cobalt, an upper ligand, a lower ligand, and an aminopropanol moiety that links the ring and lower ligands (Fig. 3). Tetrapyrrole-derived structures are also found in hemes, sirohemes, chrolophylls, and coenzyme F_{430}. Various moieties are found in the lower ligand positions of cobamides, including 5, 6-dimethylbenzimidazole (Cameron *et al*., 1989: Johnson and Escalante-Semerena, 1992: Stupperich *et al*., 1988), 5-methylbenzimidazole (Kräutler *et al*., 1988), 5-hydroxybenzimidazole, adenine (Stupperich and Kräutler, 1988), 5-methoxybenzimidazole (Wurm *et al*., 1975), 5-methoxy-6-methylbenzimidazole, p-cresol (Stupperich *et al*., 1988) and phenol (Stupperich *et al*., 1989). Possible upper ligands include methyl, hydroxyl, cyano (Glusker, 1995) or glutathonyl moieties (Glusker, 1995, Pezacka *et al*., 1990).

Cobamides are cofactors of enzymes that catalyze either methyl transfer or intramolecular rearrangement (Rondon *et al*., 1997). Enzymatic reactions that involve methyl transfer are found in methionine synthesis (methionine synthase), acetate production in acetogenic bacteria, and methanogenesis in methanogens.

Cobamides containing 5'-deoxyadenosyl as an upper ligand are involved in intramolecular rearrangement. During rearrangement reactions, a Co-C bond of the cofactor is cleaved to form a radical that captures a hydrogen atom from substrate molecules. Adenosyl-cobamide has been described as a cofactor in mutases (methylmalonyl-CoA, methyleneglutarate, glutamate, L- α-lysine, D-β -lysine, ornithine), dehydrases (diol, glycerol), lyases (ethanolamine, ammonia lyase) and a ribonucleotide reductase that catalyzes the synthesis reaction of deoxynucleotides (Halpern, 1985).

2. Biosynthesis of cobamides

The biosynthesis of cobamide is complicated and usually requires at least 20 enzymes (Fig. 4) (Cameron *et al*., 1989). The pathway can be divided into three parts: synthesis of cobinamide, synthesis of lower ligands, and assembly (Roth *et al*., 1993). The study of cobamide synthesis has been predominantly concerned with the synthesis of cobalamin (Coα–[α-(5, 6-dimethylbenzimidazole)]-cobamide) in bacteria.

The early steps of cobamide synthesis are common to those of compounds containing tetrapyrrole rings. The first step of cobamide synthesis is formation of 5-aminolevulinic acid. Two different pathways exist for the synthesis of 5-aminolevulinic acid in cobamide synthesizing organisms. In the C-4 (Shemin) pathway found in animals, yeasts, and some bacteria, succinyl-CoA and glycine are converted into 5-aminolevulinic

acid (Jordan and Shemin, 1972). Alternatively, in the C-5 pathway found in cyanobacteia, anaerobic bacteria, and higher plants, 2-oxoglutarate and glutamate are converted into 5-aminolevulinic acid (Beale *et al*., 1975: Klein *et al*., 1980: Hollriegl *et al*.,1982). Two molecules of 5-aminolevulinic acids are then converted into porphobilinogen (PBG) that is the precursor of uroporphyrinogen III.

Numerous enzymes are involved in the conversion of uroporphyrinogen III into cobamide. In the conversion pathway, precorrins (corrin ring structures without cobalt) synthesized from uroporphyrinogen III are modified by methylation, amidation, reduction, decarboxylation, methyl migration, and insertion and reduction of cobalt. The modifications result in the synthesis of a cobyric acid derivative that contains eight methyl groups and cobalt. The methylases in this pathway utilize S-adenosylmethionine (AdoMet) as source of the methyl groups. In aerobic bacteria, the cobalt is inserted into hydrogenobyrinic acid *a*, *c* diamide, which is formed late in the pathway from uroporphyrinogen III. In anaerobic bacteria such as *Propionibacterium* spp. and *Salmonella* spp., the insertion occurs in precorrin-2, which is an early step in the pathway (Roth *et al*., 1993, Roth *et al*., 1996, Cameron *et al*., 1989).

Aerobic bacteria and anaerobic bacteria also possess different biosynthetic pathways for the lower ligands and the aminopropanol moiety of the cobamide. While aerobic bacteria utilize only riboflavin (FMN) for synthesis of the 5, 6-dimethylbenzimidazole (Horig and Renz, 1980), anaerobic bacteria require several precursors such as erythrose, methionine and glycine (Höllriegl *et al*., 1982: Vogt *et al*., 1988: Vogt and Renz, 1988) for this synthesis. This alternative pathway is indispensable for the anaerobic bacteria since oxygen is required for the synthesis of 5, 6-dimethylbenzimidazole from FMN.

The aminopropanol moiety was thought be derived from decarboxylation of threonine in bacteria; however, current research proposes that threonine is not a precursor (Roth *et al*., 1996). The synthesis of cobamide is completed by linkage of upper ligand, lower ligand and an aminopropanol moiety into a complete cobamide.

3. Molecular studies of cobamide synthesis

Genetic research of cobamide synthesis has been performed extensively in *Salmonella enterica* serovar Typhimurium and *Pseudomonas denitrificans*. Genes that are responsible for synthesis and transport of cobalamin have been identified by the construction and analysis of mutants. With this approach, different organizations of cobamide biosynthetic genes were identified among bacteria. In the genome of *S. enterica* serovar Typhimurium, most of genes (25 genes) are clustered (Roth *et al*., 1993), while in *P. denitrificans* the genes are scattered into four regions (Cameron *et al*., 1989). Moreover, it was found that the sequences of two genes from *P. dentrificans* and five genes from *S. enterica* serovar Typhimurium that encode proteins for cobamide synthesis do not possess similarity to any genes in other organisms. Diverse gene organization patterns are also found in other cobamide-synthesizing organisms, suggesting the gene organization is not conserved due to the variety of the biosynthetic pathways (Roth *et al*.,1996).

The cobalamin synthesis is regulated at the transcription level in *S. enterica* serovar Typhimurium. In the regulation, the PocR protein, which is activated by propanediol, induces the transcription of two adjacent operons: the *cob* operon for cobalamin synthesis and the *pub* operon for degradation of propanediol. The propanediol degradation

provides carbon or energy sources to the bacteria, but requires cobalamin for synthesis of the enzymes (Rondon and Escalante-Semerena, 1992; Bobik *et al*.,1992). In contrast to the induction by propanediol, cobalamin also functions as a repressor of the *cob* operon (Richter-Dahlfors and Andersson**,** 1992). It was suggested that the global regulation systems, Crp/Cya and ArcA/ArcB, also affect the synthesis by inducing the transcription of genes encoding the regulatory proteins, PocR and PduF, which are required for catabolism of propanediol (Ailion *et al*., 1993, Chen *et al*., 1995).

4. Cobamides of methanogens

The isolation and identification of cobamides from thirteen methanogens identified two different types: factor III (Coα-[α–5-hydroxybenzimidazole)]-cobamide) and pseudo vitamin B_{12} (Co α–[α-(7-adenyl)]-cobamide). Moreover, cobalamin, which is frequently observed in other bacteria, was not isolated from the methanogens. Factor III was identified from all of the *Methanobacterium* spp. and most species of *Methanomicrobiales* tested. Pseudo vitamin B_{12} was identified from *Methanococcus* spp. and *Methanoplanus limicola* (Stupperich and Kräutler, 1988).

The amounts of total cobamides are relatively high in the methanogens. Cobamides isolated from thirteen methanogen species by high-performance liquid chromatography (HPLC) were presented in concentrations of 100-1,400 nmol/g dry cell weight (Stupperich and Kräutler, 1988). Subsequent studies found a larger range of the cobamide content (10-9,480 nmol/g protein) (Gorris and van der Drifit, 1994). These values are enormous compared to cobalamin concentration of *E. coli* (< 0 .01 nmol/g dry cell weight) (Stupperich and Kräutler, 1988).

The high concentration of cobamides suggests the coenzyme is involved in fundamental reactions in methanogens. The increasing production of methane by addition of corrinoids indicates an enzyme of methanogenesis contains a cobamide cofactor. Isolation of the methyl transferring enzymes from methanogens showed cobamides are cofactors of the acetyl-CoA decarbonylase/synthase complex (ACDS) (Abbanat and Ferry, 1991), N^5-methyltetrahydromethanopterin: CoM methyltransferase (Thauer *et al*., 1993: Kengen *et al*., 1992) and methanol:HS-CoM mehtyltransferase (Van der Meijden *et al*., 1983a, 1983b).

The methanol: HS-CoM methyltransferase complex is required for the use of methanol as an energy substrate in methylotrophic methanogens. The complex purified from *Methanosarcina barkeri* is composed of two enzymes: MT1 and MT2. MT1 of the complex contain a cobamide for the transfer of the methyl group from methanol to coenzyme M (Van der Meijden *et al*., 1983a, 1983b).

5. Biosynthesis of factor III

Thus far, the biosynthetic pathways of cobamide have been investigated only for factor III in methanogens. The biosynthetic pathway of pseudo vitamin B_{12} has never been elucidated. The analysis of a ^{14}C-radiolabeling incorporation pattern in *M. thermautotrophicus* indicated that the precursor of the tetrapyrrole in factor III, 5-aminolevulinic acid, is synthesized by the C-5 pathway (Gilles *et al*.,1983). The biosynthesis of factor III was also studied in *M. barkeri* by ^{14}C- labeling incorporation. The presence of the C-5 biosynthetic pathway was also confirmed in this methanogen by analyzing incorporation patterns of glutamate into the tetrapyrrole ring. In addition, it

was found, as in anaerobic bacteria, that 5-hydroxybenzimidazole is derived from glycine (Scherer *et al*., 1984).

The biosynthetic pathway of cobamide in *M. thermautotrophicus* might not be only used in the generation of factor III. The synthesis pathway could also be used for synthesis of other cobamides such as cobalamin. In addition, the biosynthesis of cobalamin in *M. thermautotrophicus* did not affect its growth, indicating that the cobamide- requiring enzymes are capable of using other types of cobamides. This unique metabolism is possible since methanogens possess transport proteins for various types of cobamides and bases (Stupperrich *et al*., 1987). For methanogens, this physiology might be a survival strategy in natural habitats where nutrient sources are not abundant.

More detailed information on pathways of factor III synthesis in *Methanothermobacter* spp. was obtained by ^{13}C-NMR analysis (Eisenreich and Bacher, 1991). This study showed that the biosynthetic pathways from porphobilinogen to the pyrrole ring and methylations of the ring were identical to those used in prokaryotic organisms. However, the methanogenic pathway utilizes pyruvate or lactate as a precursor for the synthesis of aminopropanol. Moreover, the study showed that riboflavin (FMN) (as in aerobic bacteria) or erythrose phosphate (as in anaerobic bacteria) does not serve as a precursor of the benzene ring of the 5-hydroxybenzimidazole. The presence of a modified pentose phosphate pathway in the methanogen was inferred from the labeling patterns of the aromatic amino acids and erythrose 4-phosphate. However, the study of Tumbula *et al*. (1997) suggested that a modified pathway of aromatic amino acid biosynthesis is responsible for the unexpected labeling.

A recent study showed that a modified pathway of cobamide synthesis found in *M. thermautotrophicus* ΔH, was also present in *Halobacterium* spp.(Woodson *et al*., 2003). A comparison of genome sequences of archaea and bacteria indicated that orthologues of *cobU*, which encodes nucleotide triphosphate (NTP):5'-deoxyadenosyl cobinamide kinase/GTP: adenosylcobinamide-phosphate guanylyltransferase in bacteria (Maggio-Hall and Escalante-Semerena, 1999) are absent from archaeal genomes. However, the gene product of *cobY* may substitute for the activity of CobU. CobU is a bifunctional enzyme that has nucleotidyltransferase and kinase activities. However, CobY is only involved in the nucleotidyltransferase reaction. Furthermore, *cobY* complemented a *cobU* mutation in *S. enterica* serovar Typhimurium, suggesting the kinase activity of CobU might not be essential for the *de novo* biosynthesis of cobamides for archaea and bacteria (Thomas and Escalante-Semerena, 2000).

IV. HYDROGENASES

1. The classification of hydrogenases

Hydrogenases are enzymes that are involved in the reactions of reversible hydrogen splitting. The enzymes are widely distributed among organisms that include bacteria, archaea, and eukaryotes. The activities of hydrogenases are observed in energy conservation and hydrogen production. Most hydrogenases contain metals, which are components of [Fe-S] clusters, [Ni-Fe] sites, and [Fe] sites. Hydrogenases can be divided into several groups depending on their properties and structures. Recently, Vignais *et al.*(2001) classified the enzymes into three groups depending on the metal

types in the active sites: the [NiFe]-hydrogenases, the [Fe]-hydrogenases and the metal - free hydrogenases.

2. Function of hydrogenases

[NiFe]-hydrogenases are composed of at least two subunits (heteromultimers): the large (α) and small (β) subunits. Due to functional diversities, [NiFe]-hydrogenases are classified into four groups (Wu and Mandrand, 1993). The first group of the [Ni-Fe] hydrogenases catalyzes the oxidation of H_2 in the membrane, indicating that the enzymes are involved in energy conservation. These hydrogenases are found in the anaerobic bacteria and archaea that do not use O_2 as terminal electron acceptors in ATP production. In nitrogen-fixing bacteria, the hydrogenase removes H_2 that otherwise inhibits the activity of nitrogenases and also recycles the H_2 to the energy production pathway (Adams *et al*., 1981).

The second group of [Ni-Fe] hydrogenases is located in the cytoplasm. This group includes a hydrogenase that oxidizes H_2 produced from nitrogenase in N_2-fixing cyanobacteria and a H_2-sensing hydrogenase involved in bacterial signal transduction with histidine kinase and a response regulator (Vignais *et al*., 2001).

The hydrogenases in the third group of [Ni-Fe] hydrogenases are able to bind cofactors in the cytoplasm. This group includes the F_{420} -reducing hydrogenases in methanogens, bifunctional hydrogenases in hyperthermophiles, methyl viologen-reducing hydrogenases, and bidirectional NAD-linked hydrogenases. The role of the F_{420} -reducing hydrogenases is described in more detail in below. The bifunctional hydrogenases are found in hyperthermophiles such as *Pyrococcus furiosus* (Adams,

1990). In the archaea, the hydrogenases encoded by *hydA* and *hydP/shyA* are involved in reduction of sulfur to dispose extra reducing equivalents from sugar fermentation and formation of hydrogen (Ma *et al*., 2000: Silva *et al*., 2000). The bidirectional hydrogenases catalyze the reversible reactions of hydrogen. One example is a hydrogenase from cyanobacteria which controls the rate of electron transport during the light reactions of photosynthesis (Appel *et al*., 2000).

The fourth group includes membrane bound H_2-evolving hydrogenases: hydrogenase 3 in *E. coli*, CO-induced hydrogenase in *Rhodospirillium rubrum* and Ech hydrogenase in archaea. These hydrogenases contain more than six subunits and possess high similarity of their amino acid sequences to NADH: quinone oxidoreductase (Tersteegen and Hedderich, 1999). The subunits include large subunits and small subunits containing a [Ni-Fe] binding motif and ferredoxin, respectively. The other subunits are transmembrane proteins, ferredoxins and hydrophilic proteins. These hydrogenases are located in the membrane, are involved in energy conservation, and generate a proton motive force by proton translocation (Fox *et al*., 1996: Meuer *et al*., 1999).

In contrast to the [NiFe]-hydrogenases, most of the [Fe]-hydrogenases are monomers. Most of the enzymes catalyze the reactions of hydrogen production, and are capable of interacting with various types of acceptors and donors. The roles of [Fe]-hydrogenases are found in recycling of H_2 and disposing of the extra reductants during anaerobic energy production. These hydrogenases contain an H cluster that is the active site of the enzyme (Adams, 1990). The metal-free hydrogenase in the methanogens is described below.

3. Hydrogenases in methanogens

Four different hydrogenases have been found in methanogens: F_{420} -reducing hydrogenase, F_{420}–non reducing hydrogenase, metal- free hydrogenase, and Ech hydrogenase.

a. F_{420} -reducing hydrogenase

The F_{420} -reducing hydrogenase catalyzes the reduction of coenzyme F_{420} that is an essential electron carrier in methanogens. The reduced-coenzyme F_{420} provides reducing equivalents for formation of methylene-H_4MPT and methyl- H_4MPT during methanogenesis (Fig. 5) (Deppenmeier *et al*., 1996). The enzyme in *M. thermautotrophicus* is composed of three subunits (α, β, γ), and the largest subunit (the α subunit) contains the [Ni-Fe] active site. [Fe-S] clusters and flavins are also present in this hydrogenase (Fox *et al*., 1987: Alex *et al*., 1990).

In *M. thermautotrophicus,* the hydrogenase is encoded by a gene cluster of four genes (*frh*ADGB). Polyferredoxin was identified as a component of the enzyme. In *Methanococcus voltae,* two operons, *frc* and *fru*, encode F_{420} -reducing hydrogenases. Frc and Fru hydrogenases have high sequence similarity of the genes although the Fru hydrogenase contains selenocysteins in one of the subunits (Sorgenfrei, 1997). The genome of *M. barkeri* also contains operons encoding two isozymes of F_{420} -reducing hydrogenases. The two operons were expressed during growth with H_2+ CO_2, methanol, and trimethylamine, whereas the addition of acetate did not induce expression (Vaupel and Thauer, 1998).

b. F_{420}-nonreducing hydrogenase

The role of F_{420}-nonreducing hydrogenase is well studied in aceticlastic *Methanosarcina* strains. In these methanogens, two isozymes of the hydrogenase, Vho and Vht are present. Vho is involved in the reduction of CoM-S-S-CoB during methanogenesis. During the reduction of CoM-S-S-CoB, H_2 in the cytoplasm is oxidized by the VhoA subunit of the hydrogenase. Then, the electrons from the oxidation are transferred to an electron carrier, methanophenazine, via the subunits, VhoG and VhoC. The VhoC contains cytochrome *b* (Deppenmeier *et al*., 1995). The oxidation of the carrier provides electrons for the reduction of heterodisulfide reductase. The protons are also translocated into the periplasm by the heterodisulfide reductase during the reduction of CoM-S-S-CoB (Deppenmeier, 1996: Deppenmeier, 1999).

Vht catalyzes the oxidation of H_2 during synthesis of formylmethanofuran from CO_2, H_2 and methanofuran. The reducing equivalent formed by the oxidation is transferred into the VhtC subunit of the hydrogenase, the electron carrier polyferredoxin, and finally onto formyl-MF dehydrogenase. Linkage to the energy conservation system is expected since these reactions are endergonic (Deppenmeier *et al*., 1996: Deppenmeier, 1999).

The F_{420}-nonreducing hydrogenases in autotrophic methanogens differ from those of aceticalstic methanogens by the absence of the cytochrome subunit (Cytb1). In *M. thermautotrophicus*, two isozymes of the F_{420}-nonreducing hydrogenases are encoded (Woo *et al.*, 1993) by operons that were sequenced (*mvh*DGAB). The genome of *Methanococcus voltae* also contains two operons, *vhuDGAUB* and *vhuDGAB*, which encode the F_{420}-nonreducing hydrogenases. However, Vhu and Vhc are different since Vhu contains selenium in an additional subunit (VhuU) (Sorgenfrei, 1997).

c. H_2-forming methylenetetrahydromethanopterin dehydrogenase (Hmd)

The metal-free hydrogenase, H_2-forming methylenetetrahydromethanopterin dehydrogenase (Hmd) catalyzes the reversible reduction of methenyl-H_4MPT into N^5, N^{10}-methylene-H_4MPT during methanogenesis. The purified Hmd from *M. thermautotrophicus* demonstrated that the hydrogenase is composed of 43 kDa monomer (Zirngibl *et al*., 1990). Methylene-H_4MPT is also synthesized by the combined reactions of F_{420}-reducing hydrogenase and F_{420}-dependent methylenetetrahydromethanopterin dehydrogenase (Mtd). In addition, Hmd and Mtd reduce F_{420} in the presence of methenyl-H_4MPT.

Methanogens do not absolutely require the activities of Hmd since Mtd and F_{420}-reducing hydrogenase could catalyze the synthesis of N^5, N^{10}-methylene-H_4MPT. Under nickel limitation, the activities of Hmd and Mtd increased, but, the F_{420}-reducing hydrogenase was inactivated. The reduction of F_{420} was entirely performed by the Hmd and Mtd due to absence of active F_{420} reducing hydrogenase (Afting *et al*., 1998).

d. Ech hydrogenase

The Ech (energy conserving hydrogenase or energy converting hydrogenase) in methanogens was first purified from *Methanosarcina barkeri* as a complex of Ech hydrogenase and heterodisulfide reductase. The purified hydrogenase showed high hydrogenase activity, and the amino acid sequences of the enzyme did not possess high similarity to other methanogenic hydrogenases. The purified Ech hydrogenase was composed of six subunits, which is consistent with the six genes (*echABCDEF*) in the operon encoding the hydrogenase (Künkel *et al*., 1998).

The hydrogenase was purified again with efficient purification techniques and was assayed to investigate its biochemical properties. The assays showed the enzyme oxidizes and reduces ferredoxin, and converts CO into CO_2 and H_2 (Meuer *et al*.,1999).

In the genomes of *Methanothermobacter* spp. and *Methanococcus* spp, two operons encoding Ech hydrogenases, *eha* and *ehb*, are found. A transcription study of the two *ech* operons in *M. thermautotrophicus* suggested different transcription levels of Eha and Ehb. Reverse transcription (RT)-PCR analysis showed that the amount of *ehb* transcript was larger than *eha* transcript when cells had saturating levels of H_2. These data might indicate the Eha and Ehb are involved in anabolic and catabolic pathways, respectively. Additionally, gene compositions of the two operons are not identical: the *eha* operon contains a larger number of genes (Tersteegen and Hedderich, 1999).

The Ech hydrogenases in methanogens are probably involved in energy conservation and reverse electron flow. The growth and biochemical studies of a mutant of *M. barkeri* in which *ech* was deleted suggested that the hydrogenase is coupled to endergonic reactions in the methanogen. The reactions include synthesis of acetyl-CoA from H_2+CO_2 or pyruvate, synthesis of formyl-MF, and reduction of CoM-S-S-CoB (Fig. 5). It was proposed that the hydrogenase is coupled to diverse enzymes including the CODH/ACDS complex, POR and formyl-MF dehydrogenase, and is utilized to overcome the energetically unfavorable barriers. During the reverse electron flow, the hydrogenase may function as an electron pump to utilize the proton motive force to generate strong reductants from H_2 gas (Meuer *et al*., 2002).

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TABLE 1-1. Genera of methanogenic archaea

Genera	Morphology	Substrates	Optimal temperature (C°)	Habitats
Methanobacterium	Rod	H2, (for, iP, iB)	37-45	Sewage, bioreactor, marshy soil, alkaline sediment, oil reservoir waters
Methanobrevibacter	Coccobacillus	H2, for	37-40	Rumen, sludge, human and animal feces, wet wood of trees
Methanosphaera	Coccus	H2 + m	37	Human feces, rabbit colon
Methanothermobacter	Rod	H2, (for)	55-65	Sewage, river sediment
Methanothermus	Rod	H2	80-88	Solfataric water and mud
Methanococcus	Coccus	H2, for	35-40	Marine sediments, salt marsh
Methanothermococcus	Coccus	H2, for	60-65	Heated marine sediments
Methanocaldococcus	Coccus	H2	80-85	Marine hydrothermal vents
Methanotorris	Irregular coccus	H2	88	Marine hydrothermal vents
Methanomicrobium	Short rod	H2, for	40	Rumen
Methanoculleus	Irregular coccus	H2, for, (iP, iB)	20-45	Marine and river sediment, bioreactor
Methanofollis	Irregular coccus	H2, for (iP, IB,cP)	37-40	Solfataric pool mud
Methanogenium	Irregular coccus	H2, for, (iP, iB, E, P, B, cPe, iPe)	15-57	Marine and fresh sediment, bioreactors
Methanolacinia	Irregular rod	H2,iP,iB,cPe	40	Marine sediment
Methanoplanus	Irregular disk, plates	H2, for, (iP)	32-40	Swamp, marine sediment, oil reservoir waters
Methanocorpusculum	Irregular coccus	H2, for, (iP, iB)	30-40	Bioreactor, lake sediment
Methanospirillum	Spirillum	H2, for, (iP,iB)	30-37	Bioreactor, freshwater sediment
Methanocalculus	Irregular cocus	H2, for	38	
Methanosarcina	Aggregates, coccus, macrocyst	m, MeN, ac, (H2)	30-40, 50-55	Freshwater and marine sediments, bioreactor, rumen, soil, sewage
Methanococcoides	Irregular coccus	m, MeN	23-35	Marine water and sediment
Methanohalobium	Flat polygon	MeN	40-55	Salt lagoons
Methanohalophilus	Irregular coccus	m, MeN	26-36	Saline lake sediment, stromatolite associated mat
Methanolobus	Irregular coccus	m. MeN, (MeS)	37	Marine sediment
Methanosalsum	Irregular coccus	m. MeN, MeS	35-45	Saline lake sediment
Methanosaeta	Sheathed rod	Ac	35-40 55-60	Sewage, bioreactor, landfill

ac: acetate, B: butanol, cP: cyclopentanol, E: ethanol, for: formate, iB: isobutanol, iP: isopropanol, iPe: isopentanol, m: methanol, MeN: methylamines, MeS; dimethylsulfide or methanethiol, P: I-propanol

Parenthesis indicate that the substrate is utilized by only some species or strains

Figure 1-1.

Overview of the three types of methanogenesis.

Type I: CO_2 is reduced to methane via the C1 carrier methanofuran (not shown) and tetrahydromethanopterin (H_4MPT), a folate analog that is virtually unique to methanogens. The electrons for the reduction of CO_2 to methane are obtained form H_2, formate, or a few secondary and primary alcohols.

Type II: In aceticlastic methanogenesis, the methyl group of acetate is transferred to H_4MPT. The electrons for the reduction of methyl group to methane are obtained from the oxidation of the carboxyl group of acetate.

Type III: In methylotrophic methanogenesis, the methyl group is transferred to coenzyme M (CoM, 2-mercaptoethanesulfonic acid), the terminal C1 carrier in the pathway of methanogenesis from acetate and CO_2.

Methyl groups are also oxidized by a reversal of the pathway of CO_2 reduction. The last step in the pathway, the reduction of methyl group to methane is common to all types of methanogenesis and requires two additional unique coenzymes (not shown). The first is coenzyme F_{430}, a nickel tetrapyrrole that is tightly bound to the methylreductase enzyme. The second is 7-mercaptoheptanoylthreonine phosphate, which serves as the proximal electron donor to the methylreductase.

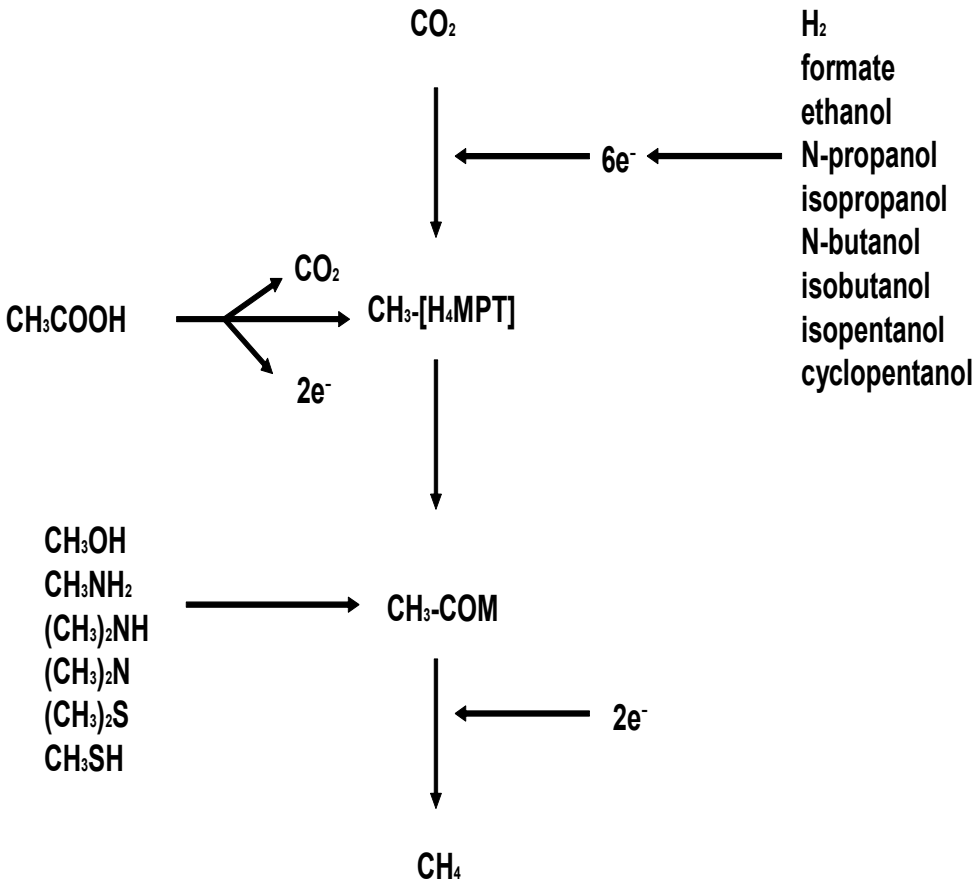
CO2
H2
formate
ethanol
N-propanol
isopropanol
N-butanol
isobutanol
isopentanol
cyclopentanol
6e-
CO2
CH3COOH
CH3-[H4MPT]
2e-
CH3OH
CH3NH2
(CH3)2NH
(CH3)2N
(CH3)2S
CH3SH
CH3-COM
2e-
CH4

Figure 1-2.

Methanogenic food chain in a bioreactor and rumen. Organic polymers are degraded to monomers and then organic acids and alcohols, H_2 and CO_2 by fermentative organisms. Syntrophic organisms convert the organic acids and alcohols into acetate, H_2, formate and CO_2, which are the substrates for the methanogenic archaea. Broken lines indicate reactions that do not occur in the rumen and colon habitats.

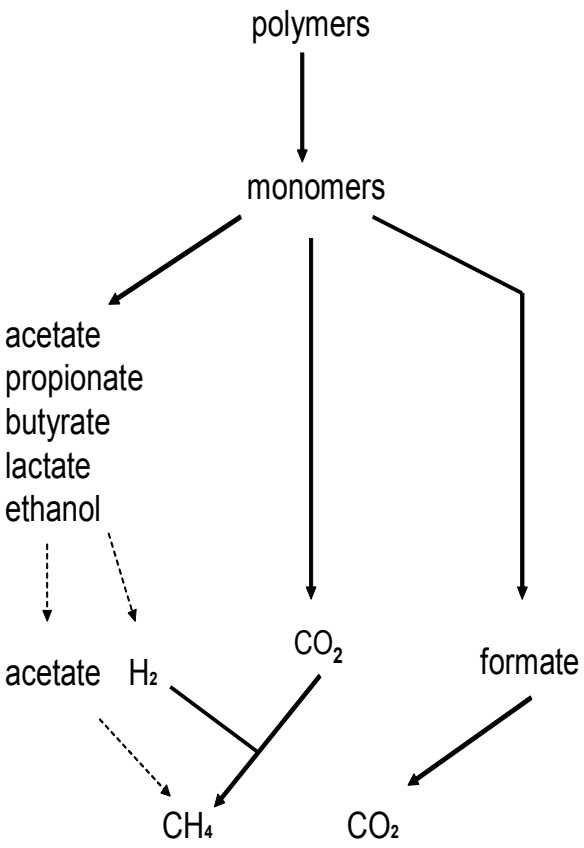
polymers
monomers
acetate
propionate
butyrate
lactate
ethanol
acetate
H2
CO2
formate
CH4
CO2

Figure 1-3.

Structures of cobamides (adapted from http://www.ce.udel.edu/~pei/B12.html).

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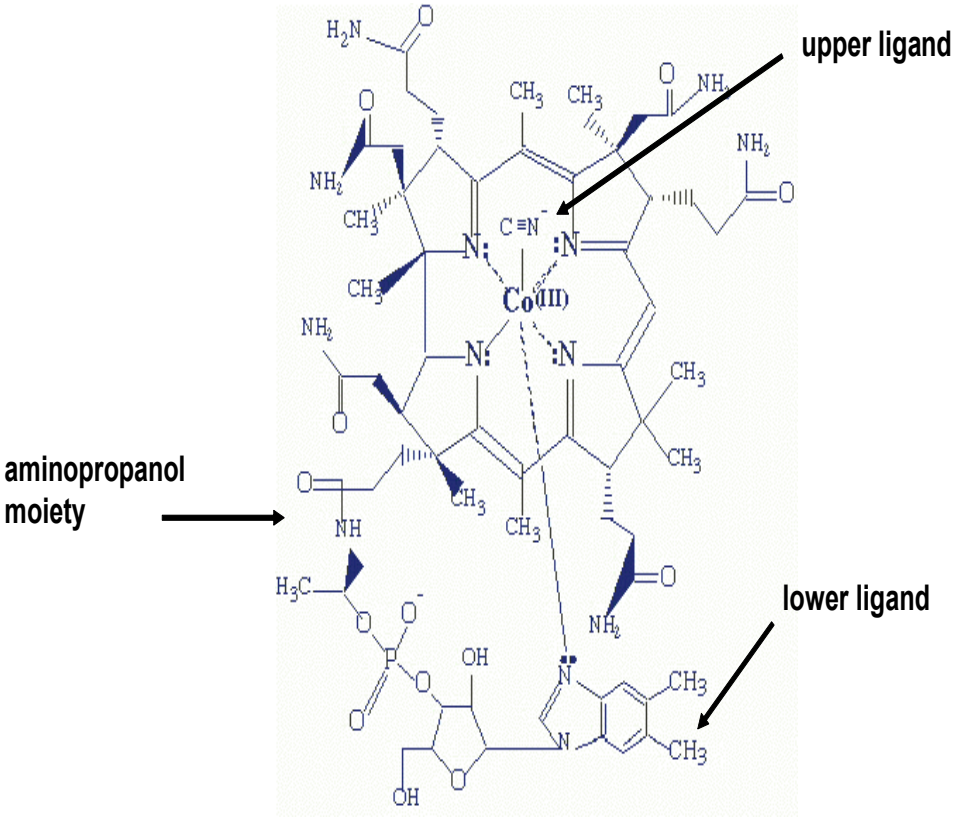

upper ligand
aminopropanol moiety
lower ligand

Figure 1-4.

The pathway of cobalamin synthesis in bacteria (adapted from Roth *et al*., 1993).

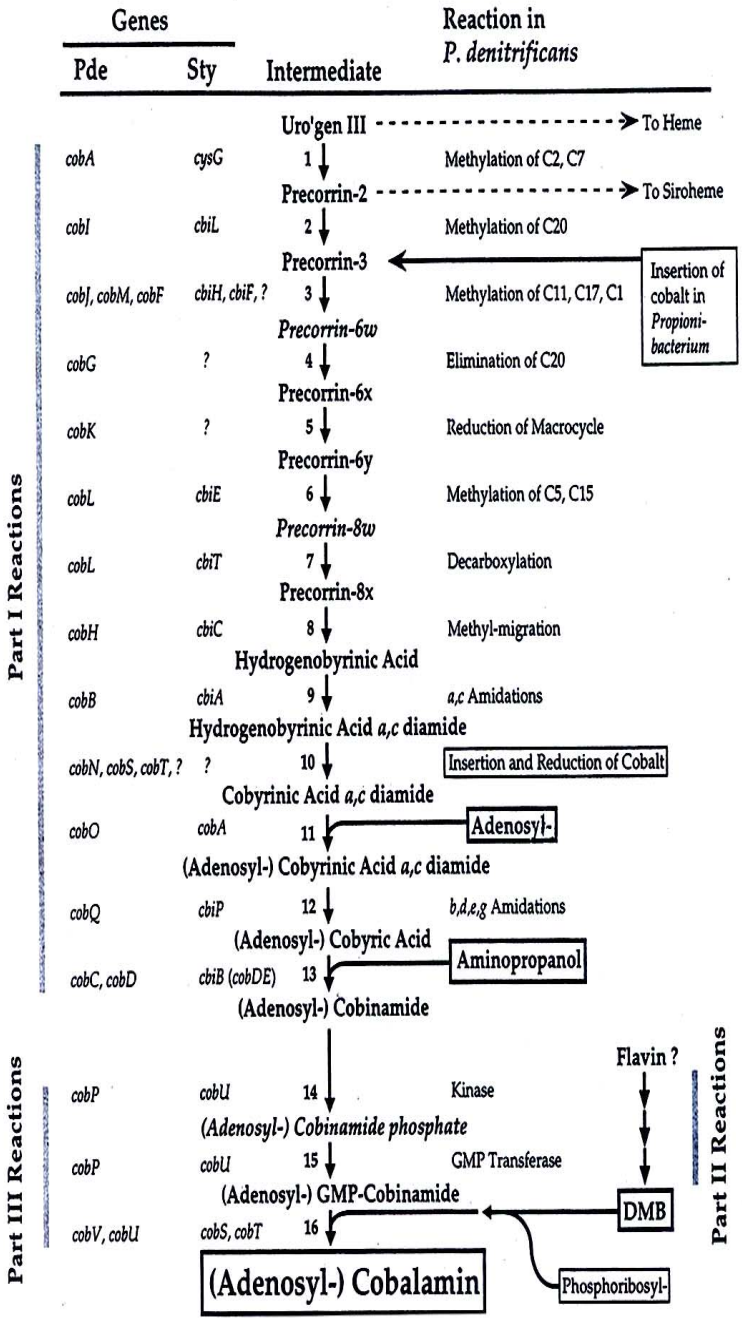
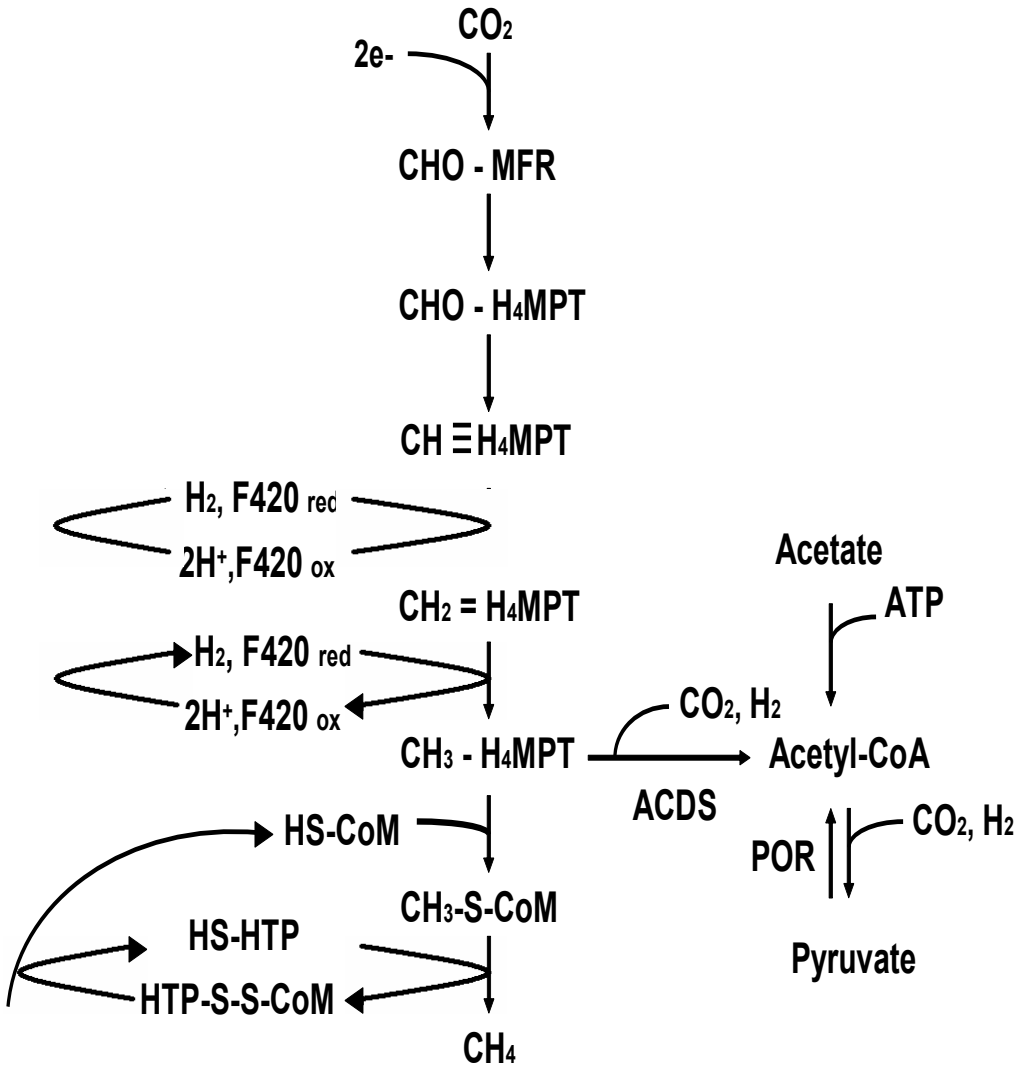

Genes
Pde
Sty
Intermediate
Reaction in
P. denitrificans
Uro'gen III
To Heme
cobA
cysG
1
Methylation of C2, C7
Precorrin-2
To Siroheme
cobI
cbiL
2
Methylation of C20
Precorrin-3
Insertion of cobalt in Propioni-bacterium
cobJ, cobM, cobF
cbiH, cbiF, ?
3
Methylation of C11, C17, C1
Precorrin-6w
cobG
?
4
Elimination of C20
Precorrin-6x
cobK
?
5
Reduction of Macrocycle
Precorrin-6y
cobL
cbiE
6
Methylation of C5, C15
Precorrin-8w
cobL
cbiT
7
Decarboxylation
Precorrin-8x
cobH
cbiC
8
Methyl-migration
Hydrogenobyrinic Acid
cobB
cbiA
9
a,c Amidations
Hydrogenobyrinic Acid a,c diamide
cobN, cobS, cobT, ?
?
10
Insertion and Reduction of Cobalt
Cobyrinic Acid a,c diamide
cobO
cobA
11
Adenosyl-
(Adenosyl-) Cobyrinic Acid a,c diamide
cobQ
cbiP
12
b,d,e,g Amidations
(Adenosyl-) Cobyric Acid
cobC, cobD
cbiB (cobDE)
13
Aminopropanol
(Adenosyl-) Cobinamide
Flavin ?
cobP
cobU
14
Kinase
(Adenosyl-) Cobinamide phosphate
cobP
cobU
15
GMP Transferase
(Adenosyl-) GMP-Cobinamide
DMB
cobV, cobU
cobS, cobT
16
(Adenosyl-) Cobalamin
Phosphoribosyl-
Part I Reactions
Part II Reactions
Part III Reactions

Figure1-5.

Pathway of methanogenesis and synthesis of acetyl-CoA in methanogens. MFR: methanofuran, H_4MPT: tetrahydromethanopterin, F_{420}: deazafalvin, CoM: 2-mercaptoethanesulfonate, HS-HTP: 7-mercaptoheptanoyl-threonine phosphate.


CO2
2e-
CHO - MFR
CHO - H4MPT
CH ≡ H4MPT
H2, F420 red
2H+,F420 ox
CH2 = H4MPT
H2, F420 red
2H+,F420 ox
CH3 - H4MPT
CO2, H2
Acetate
ATP
Acetyl-CoA
ACDS
POR
CO2, H2
Pyruvate
HS-CoM
CH3-S-CoM
HS-HTP
HTP-S-S-CoM
CH4

CHAPTER II

ISOLATION OF ACETATE AUXOTROPHS OF THE METHANE-PRODUCING ARCHAEON *METHANOCOCCUS MARIPALUDIS* BY RANDOM MUTAGENESIS[1]

[1] Kim, W. and W. B. Whitman. 1999. Genetics. **152:**1429-1437.

ABSTRACT

In order to learn more about autotrophic growth of methanococci, nine conditional mutants of *Methanococcus maripaludis* were isolated after transformation of the wild type with a random library in pMEB.2, a suicide plasmid bearing the puromycin resistance cassette *pac*. These mutants grew poorly in mineral medium and required acetate or complex organic supplements such as yeast extract for normal growth. One mutant, JJ104, was a leaky acetate auxotroph. A plasmid, pWDK104, was recovered from this mutant by electroporation of a plasmid preparation into *Escherichia coli*. Transformation of wild-type *M. maripaludis* with pWDK104 produced JJ104-1, a mutant with the same phenotype as JJ104, thus establishing that insertion of pWDK104 into the genome was responsible for the phenotype. pWDK104 contained portions of the methanococcal genes encoding an ABC transporter closely related to MJ1367-MJ1368 of *Methanococcus jannaschii*. Because high levels of molybdate, tungstate and selenite restored growth to wild-type levels, this transporter may be specific for these oxyanions. A second acetate auxotroph, JJ117, had an absolute growth requirement for either acetate or cobalamin, and wild-type growth was only observed in the presence of both. Cobinamide, 5',6'-dimethylbenzimidazole, and 2-aminopropanol did not replace cobalamin. This phenotype was correlated with tandem insertions in the genome but not single insertions and appeared to have resulted from an indirect effect on cobamide metabolism. Plasmids rescued from other mutants contained portions of ORFs denoted in *M. jannaschii* as endoglucanase (MJ0555), transketolase (MJ0681), thiamine biosynthetic protein *thiI* (MJ0931), and several hypothetical proteins (MJ1031, MJ0835 and MJ0835.1).

INTRODUCTION

Methanococcus maripaludis is a marine, methanogenic archaeon that utilizes H_2+CO_2 or formate as sole carbon and energy sources (14). During autotrophic growth, CO_2 is assimilated by a modified Ljungdahl-Wood pathway of acetyl-CoA biosynthesis (35). In this pathway, methyltetrahydromethanopterin produced during methanogenesis donates a methyl group to the corrinoid Fe/S protein of the acetyl-CoA decarbonylase/synthase complex (ACDS, also called CO dehydrogenase). This complex reduces CO_2 to the oxidation level of CO and combines it with HS-CoA and the methyl group to form acetyl-CoA. In *M. maripaludis*, the biosynthetic activity as well as a number of diagnostic partial activities of the ACDS complex have been detected in cell free extracts (26). In addition, acetate auxotrophs isolated following ethyl methanesulfonate mutagenesis had low levels of ACDS activity, and spontaneous revertants recovered wild-type levels of ACDS activity (16). While this evidence is indicative of a role for the ACDS complex, the complex has never been isolated from an autotrophic methanogen, and its role in autotrophy is largely inferred from the properties of the enzyme in the homoacetogenic clostridia and aceticlastic methanogens.

To identify additional factors important for autotrophy in *M. maripaludis*, random insertional mutagenesis was performed to isolate mutants unable to grow autotrophically. In this approach, cells were transformed with a genomic library in pMEB.2, a pUC derivative bearing the puromycin-resistance cassette *pac* (10). This plasmid is unable to replicate in methanococci, and puromycin resistance can only be acquired by homologous recombination into the genome at the site of the cloned genomic DNA (24). If the cloned fragment is internal to a gene or operon, integration will disrupt expression of the gene

and/or downstream genes. While similar techniques have been utilized to inactivate specific genes in the methanococci (3, 13); this is the first report of its application to random mutagenesis.

MATERIAL AND METHODS

Strains. The wild type strain of *Methanococcus maripaludis*, strain JJ, was obtained from W. J. Jones (14). *Escherichia coli* SURE was obtained from Stratagene (La Jolla, CA).

Media and culture conditions. *M. maripaludis* was grown in mineral medium (McN) at 37°C with $H_2 + CO_2$ as the carbon and energy source as described previously (34). Unless specified otherwise, 5 ml cultures were grown in 28 ml stoppered tubes using strictly anaerobic procedures (2). The initial gas pressure was 240 kPa, and tubes were repressurized two or three times a day. McA was composed of McN plus 10 mM sodium acetate. McC was composed of McN plus 0.2 % (wt/vol) yeast extract. McAC was composed of McA plus 0.2 % yeast extract. $McAC^+$ was composed of McAC plus 0.2 % (wt/vol) Casamino acids, 1 % (vol/vol) vitamin solution (37), 0.15 % (wt/vol) each of valine, isoleucine, and leucine, and 0.01 mM pantoyllactone. Growth on agar plates was performed as described previously (31). Solid medium had the same composition as broth except that the $NaHCO_3$ concentration was reduced to 2 g L^{-1} and 1 % (wt/vol) Noble agar (Difco) was added. Replica plating was performed with sterile toothpicks that had been incubated in the anaerobic chamber for 1 day. Growth of 200 ml cultures was performed in one liter bottles and McAC medium as described previously (3). For cleaning, culture tubes, stoppers and flasks were autoclaved in 0.2 N NaOH for 20 min.

After cooling, the glassware and stoppers were rinsed in tap water followed by deionized water.

Construction of library. A 200 ml culture of *M. maripaludis* was grown overnight and harvested by centrifugation for 15 min at 7,300 g at 4°C. The cell pellet was washed in 30 ml of 0.4 M NaCl and resuspended in 3 ml of 0.15 M NaCl + 0.1 M sodium EDTA (pH 8.0). To lyse the cells, the cell suspension was placed in a -70°C freezer. The suspension was then thawed in a water bath at room temperature, and 5 ml of phenol-chloroform-isoamyl alcohol extraction solution were added (22). After gently mixing, the solution was centrifuged for 10 min at 8,200 g and 4°C. The aqueous phase was then recentrifuged for 20 min at 8,200 g and 4°C to remove precipitated sulfide and protein. Finally, the DNA was collected by ethanol precipitation and washed with 70 % ethanol (23). After drying, the pellet was resuspended in a minimum volume of TE buffer (10 mM Tris chloride, pH 8.0, and 1 mM sodium EDTA, pH 8.0) at 4°C.

To remove RNA, the DNA was treated with 1 µl of DNase-free RNase (23) for 1 day at 4°C. To prepare DNA fragments, 10 µl of DNA (3 µg μl^{-1}) was diluted into 90 µl of 0.1 SSC, where SSC was 0.15 M NaCl and 15 mM sodium citrate, pH 7.0. The solution was then sonicated at 0°C for 12 min in intervals of 0.5 min. For partial repair, 11 µl of the DNA solution was treated with 10 U of Klenow fragment (10 U μl^{-1}, Promega, Madison WI) and 8 µl of 0.5 mM NTPs. After 15 min at 30°C, 3 µl of 0.5 M sodium EDTA (pH 8.0) was added to terminate the reaction. The vector pMEB.2 (10) was digested with *Eco*RV and dephosphorylated with calf intestinal alkaline phosphatase (23). For ligation, 75 ng of the dephosphorylated vector and 23 ng of the partially repaired genomic DNA were combined with 200 U of T4 DNA ligase (Promega) and buffer in a total volume of

20 µl and incubated overnight at room temperature. After ligation, the DNA solution was diluted with 4 parts of water, and 1 µl was electroporated into *E. coli* SURE. The electroporation was repeated 10 times. The transformants were pooled, diluted to 50 ml in LB broth plus ampicillin (120 µg ml^{-1}), and incubated for 8 h at 37°C. After growth, the cell suspension was diluted with 50 ml of LB broth + 30 % (vol/vol) glycerol, and 1 ml portions were stored at -70°C. The total number of transformants obtained was 3 x 10^5, 75 % of which contained cloned DNA.

To transform *M. maripaludis*, 0.1 ml of the frozen cell suspension was inoculated into 10 ml of LB broth + ampicillin (50 µg ml^{-1}). After growth overnight at 37°C, the plasmids were purified from 1.5 ml of culture with Wizard Plus Miniprep systems (Promega). Purified plasmid, 3 µg in 40 µl of deionized water, was diluted with 60 µl of TE buffer in a microfuge tube. The plasmid preparation was then transferred to the anaerobic chamber and incubated for 1 day to allow O_2 to diffuse from the solution. Upon transformation of a 5 ml culture of *M. maripaludis*, the transformants were inoculated into 20 ml of $McAC^+$ containing 2.5 µg puromycin ml^{-1} in a 160 ml serum bottle and incubated for 2 days under H_2+CO_2 at 37°C to allow for growth. The culture was then dispensed into 4 sterile culture tubes and centrifuged at 2,000 g for 20 min at room temperature, and the cells were resuspended in a one-fifth volume of McC + 25 % glycerol (32). One ml portions of the glycerol-containing cell suspensions were stored at -70°C.

Transformation, plasmid purification, electrophoresis. *M. maripaludis* was transformed by the polyethylene glycol method of Tumbula *et al*. (33). *E. coli* was transformed by electroporation with a BioRad (Hercules, CA) Gene Pulser set at 25 µF

and 2.46 kV (23). Plasmids were purified from *E. coli* and *M. maripaludis* using the Wizard Plus Miniprep DNA purification system (Promega) following the manufacturers instructions. DNA was electrophoresed on 0.8 % agarose gels as described by SAMBROOK *et al*. (23).

Base analog selection and isolation of mutants. Auxotrophic mutants of *M. maripaludis* were enriched by a modification of the nucleobase selection of Ladapo and Whitman (16). Enrichment medium was prepared by adding 50 mg of 8-azahypoxanthine and 10 mg of 6-azauracil to sterile culture tubes. The culture tubes were incubated for 1 day in the anaerobic chamber before adding 5 ml of sterile McN medium. After stoppering, the gas atmosphere was exchanged with $H_2 + CO_2$. The frozen stock culture of the transformants, 0.2 ml, was inoculated into 5 ml of $McAC^+$ medium and incubated overnight at 37°C. When the A_{600nm} was 0.7, 0.2 ml was inoculated into the enrichment medium. After incubation for 2 days at 37°C, the enrichment culture was centrifuged at 2,000 g for 20 min at room temperature, and the cell pellet was resuspended in 5 ml of McN medium. The cells were washed one additional time and resuspended in $McAC^+$ medium. The culture was then incubated at 37°C until the A_{600} was >0.5, and 0.2 ml was inoculated into enrichment medium. After incubation for 2 days, the cells were washed in McN medium and resuspended in McC^+ medium as described above. Culture, 0.2 ml, was then plated on McC^+ plus 2.5 μg ml^{-1} of puromycin. Plates were incubated for 7 days, and isolated colonies were replica plated on McN, McA, McC, and $McAC^+$ media.

Southern hybridizations. Genomic DNA was purified by the miniprep procedure of Wilson (1). Genomic DNA, 14 μg, was treated with 72 U of *Eco*RI for 48 h. After

electrophoresis of 6 μg, the DNA was transferred to positively charged nylon membranes (Boehringer Mannheim) following the manufacturer's protocol. The probe, pWDK117, was labeled with Dig-dUTP according to the protocol of the Genius labeling kit (Boehringer Mannheim). Prehybridization and hybridization were performed at 65°C for 12 and 24 h, respectively. Hybridization was visualized with CSPD Ready-to-Use Chemiluminescent Substrate (Boehringer Mannheim).

Isolation and sequencing of plasmids from transformants. To obtain the plasmids from the *M. maripaludis* mutants, a 5 ml culture in McC^{+} plus 2.5 μg ml^{-1} puromycin was harvested by centrifugation at 2,000 g for 20 min at room temperature. The cells were resuspended in 200 μl of a solution containing 50 mM Tris chloride, pH 7.5, 10 mM sodium EDTA, and 100 μg ml^{-1} DNase-free RNase A. The suspension was then treated using the Wizard Plus Miniprep DNA Purification System (Promega) according to the manufacturer's instructions. A portion of the purified DNA, 15 μl in deionized water, was dialyzed for 2 h at room temperature. Dialysis was performed by placing a drop of the suspension on the surface of a 0.025 μm filter disk (2.5 cm diameter, Type VS, Millipore, Bedford, MA), and the filter disk was floated on the surface of 20 ml of deionized water in a petri dish. After dialysis, *E. coli* SURE cells were electroporated with 1-5 μl of the DNA suspension.

The plasmids were then isolated from the *E. coli* transformants as described above. The genomic inserts were then sequenced using the primers 5'-AGGCACCCCAGGCTTTACAC and 5'-GCGTTTTTTATTACCTACTA, which were complementary to the flanking regions of pMEB.2. Sequencing was performed on an Applied Biosystems model 373 automated sequencer at the Molecular Genetics

Instrumentation facility at the University of Georgia. Sequences were analyzed with the FASTA and BLAST programs available through GCG (University of Wisconsin) and at the TIGR website (http://www.tigr.org).

Construction of pWDK117-11 and pWDK117-P. An internal fragment of pWDK117 was subcloned. For pWDK117-11, pWDK117 was digested with *Bam*HI and *Bgl*II, and the 682 internal fragment was gel purified and ligated into the *Bgl*II site of pWGL11 (9). In the resulting plasmid, the direction of transcription of the gene fragment was in the opposite direction of the *pac* cassette. For pWDK117-P, pWDK117 was digested with *Bgl*II, and the 316 bp product containing the 5-end of the ORF was gel purified and ligated into the *Bgl*II site of pIJA02. This plasmid was similar to pWLG14 and contained the *M. voltae* histone promoter P*hmvA* immediately upstream of the *Bgl*II site (9).

Accession numbers. The GenBank accession numbers for the *M. maripaludis* DNA cloned in pWDK101, pWDK103, pWDK104, pWDK106, pWDK107, pWDK117 were AF147208, AF146562, AF146563, AF146564, AF146565, and AF146566, respectively.

RESULTS

Library construction. A plasmid library for random mutagenesis of *M. maripaludis* was constructed by blunt end ligation of sonicated genomic DNA into the *Eco*RV site of pMEB.2. The initial library was electroporated into *E. coli*, where 3×10^4 transformants were found. However, of 28 transformants screened, only 21 possessed cloned genomic DNA. Thus, the number of clones in the library was estimated as 2.3×10^4. The size distribution of 20 of these clones was: <0.5 kb, 4; 0.5-1.0 kb, 4; 1.0-1.5 kb, 9; 1.5-2.0 kb,

2; >2.0 kb, 1. Upon transformation of *M. maripaludis*, 3.3 x 10^4 transformants were found. Assuming a genome size of 2,000 kb and average clone size of 1 kb, a library of 9,200 transformants would have a 99% probability of containing any gene of interest. By this criterion, the library was large enough to provide insertions throughout the genome. However, replica plating of 700 transformants of *M. maripaludis* failed to identify conditional mutants that grew poorly in minimal medium (McN). The failure to identify large numbers of conditional mutants could have resulted from a number of affects. If the cloned DNA was too large, it would not be internal to an operon and integration of the plasmid would not disrupt function. Although the average size of operons in methanococci is not known, in *M. jannaschii* about 50% of the ORFs are larger than 600 bp. If the average transcriptional unit is only two ORFs, only a small percentage of the clones in the library would be internal. This problem may have been exacerbated by amplification in *M. maripaludis,* which would enrich for transformants from large clones that have a higher recombination frequency. In addition, auxotrophs which grew poorly in the complex medium may have been lost during the amplification in *M. maripaludis*. Lastly, the amplification of the library in *E. coli* could have further reduced its variability. In any case, the reason for the low number of conditional mutants in the library was not explored further.

Isolation and characterization of JJ104 and other auxotrophs. Because of the low frequency of conditional mutants, auxotrophs were enriched by base analog selection. In the first experiment, eight potential auxotrophs were identified among 100 transformants (Table 1). These auxotrophs, JJ101-JJ108, all grew poorly in mineral medium. For one of them, JJ104, good growth was restored by the addition of acetate. JJ108 required yeast

extract for good growth. The five of the remaining six auxotrophs required McAC$^+$, a highly enriched medium containing acetate as well as amino acids and vitamins, for good growth. Lastly, JJ107 grew poorly even in rich medium.

If the phenotypes were caused by integration of plasmids by homologous recombination, then revertants should occur at a low frequency due to recombination of the plasmids out of the genome. Because the plasmids lack a methanococcal origin of replication, they would normally be lost from the culture. Even though their abundance was expected to be low, it was possible to isolate these plasmids by electroporation of plasmid preparations from the *M. maripaludis* auxotrophs into *E. coli*. For seven of the eight mutants, plasmids were isolated in this fashion (Table 1). In all cases examined by restriction endonuclease digestions, the plasmids isolated from one mutant either contained genomic inserts of the same size or a genomic insert was absent and the digestion pattern was the same as the parent pMEB.2. These latter plasmids were discarded. Sequencing of the ends of the inserts indicated that pWDK101, pWDK102, and pWDK105 were identical. Presumably, the strains JJ101, JJ102, and JJ105 which yielded these plasmids represented multiple isolations of the same mutant during the enrichment and were not formed by independent mutations. Nevertheless, this result demonstrated that the same plasmid could be recovered from multiple clones of a mutant. Even after several attempts, it was not possible to isolate a plasmid from JJ108.

Sequencing of the genomic inserts in the plasmids identified open reading frames homologous to ORFs in the genomic sequences of *Methanococcus jannaschii* and *Methanobacterium thermoautotrophicum* (Fig. 1; 6, 27). pWDK104 encoded components homologous to ABC transporters, including the 3'-end of an integral

membrane protein and the 5'-end of an ATP-binding subunit. The partial sequence of the integral membrane protein component had high similarity to the *M. jannaschii* ORF annotated as a sulfate/thiosulfate transporter and contained the conserved EAA loop region. The second partial ORF had high similarity to the *M. jannaschii* ORF annotated as a sulfate permease and contained the Walker motif A (5). pWDK101 contained three small ORFs of 75-110 amino acid residues. None of these ORFs displayed significant sequence similarity to ORFs in *M. jannaschii* or *M. thermoautotrophicum*. Because of their small size and the absence of homologs, it was not clear that these ORFs were in fact expressed. pWDK103 contained an ORF homologous to the 3'-end of a hypothetical membrane-spanning protein common to *M. jannaschii* and *M. thermoautotrophicum*, an ORF with no known homologs, as well as an ORF homologous to the thiamine biosynthetic protein *thiI*. However, when compared to the *M. jannaschii thiI*, the *M. maripaludis* ORF was missing 101 amino acid residues from the N-terminal. Examination of other reading frames did not produce evidence for a frame shift in the sequence. Therefore, this gene appeared to be truncated or possibly split in *M. maripaludis.* pWDK106 contained a pair of ORFs with homology to the hypothetical proteins in *M. jannaschii* MJ0835 and MJ0835.1. This plasmid and pWDK104 were the only cases where the gene order in *M. jannaschii* was conserved in *M. maripaludis*. Lastly, pWDK107 contained two divergently translated ORFs. The large ORF had homology to an ORF annotated as an endo-1,4-β-glucanase in both *M. jannaschii* and *M. thermoautotrophicum*. The small ORF was homologous to the transketolase of *M. jannaschii*. *M. thermoautotrophicum* does not possess this enzyme, and no homolog was observed (27).

Because the methanococcal genome is AT rich, it is difficult to recognize transcriptional start sites based upon sequence information alone. Nevertheless, based upon the organization of the ORFs, pWDK103, pWDK104, pWDK106 may well have encoded regions internal to an operon, as expected from the method of mutagenesis. However pWDK107, which encoded two divergently translated ORFs, was likely to overlap the transcriptional units, and a simple insertion of the vector would not have been expected to inactivate these genes. In this case, the phenotype could have resulted from more complex insertional events.

Southern hybridizations of the genomic DNA of these mutants with pMEB.2 as the probe indicated that these plasmids had integrated as tandem repeats (data not shown). However, it was not determined if the repeats represented the initial product of the recombination or were subsequently formed during the enrichment and subculturing of the mutants. In other experiments (see below), tandem repeats appeared to represent about 10 % of the products formed by recombination of similar plasmids into the genome. Presumably, the high proportion of tandem repeats obtained in these experiments could have resulted from the initial selection of the methanococcal library in puromycin-containing medium.

Phenotype of JJ104. In the initial screening by replica plating, growth of JJ104 was stimulated by acetate. Similarly, growth was also stimulated by yeast extract, which contains acetate in addition to amino acids and other potential nutrients. To confirm that the phenotype was due to insertion of pWDK104, the wild-type strain JJ1 was transformed with pWDK104 to form JJ104-1. Like the original mutant, growth of JJ104-1 was stimulated by acetate (Fig. 2), confirming that the insertion of pWDK104 was

sufficient to produce the original mutation. In addition, growth of JJ104-1 was stimulated by 0.2 % (wt/vol) Casamino acids (data not shown). The ability of Casamino acids to substitute for acetate suggested that the growth stimulation was due to a general sparing of stress by organic carbon sources and not a specific requirement for acetate (16).

Sulfide is abundant in the habitats of methanococci and can serve as a sole sulfur source, and most mesophilic methanococci do not have a nutritional requirement for sulfate and other sulfur oxyanions (34). Thus, even though some methanococci can assimilate sulfur oxyanions like thiosulfate (8), a mutation in a sulfur oxyanion transporter would not be expected to have a phenotype in sulfide-containing medium. Therefore, in spite of its assignment, the ABC transporter homolog encoded by pWDK104 was probably involved in uptake of some nutrient other than sulfur oxyanions. In support of this hypothesis, growth of JJ104-1 was stimulated by the addition of a mixture of molybdate, tungstate, and selenite (Fig. 2). For the wild-type JJ1, a nutritional requirement for these oxyanions was only apparent after the second transfer in medium without added oxyanions (data not shown). Presumably, oxyanions contaminating the medium supported the nutritional requirements of the wild type during the first transfer. Thus, both the wild type and JJ104-1 required these oxyanions for growth, but JJ104-1 appeared to require higher concentrations. The addition of selenite (10 μM) by itself was also stimulatory, although it was not as effective as the mixture of three oxyanions (data not shown). The addition of tungstate (10 μM) by itself was inhibitory. This result would be expected if uptake of oxyanions in the mutant was performed by a nonspecific transporter and high levels of tungstate inhibited uptake of the other essential oxyanions.

Isolation of JJ117. In a second round of enrichment with base analogs, four additional auxotrophs were identified. One of these, JJ117, had an absolute requirement of acetate for good growth and was characterized further (Table 1). Upon electroporation of a plasmid preparation from JJ117 into *E. coli*, two transformants were found. Upon *Eco*RI digestion, the plasmids in both transformants appeared identical. The cloned genomic DNA of one, pWDK117, was sequenced, and a large ORF was identified (Fig. 1). This ORF was homologous to two *M. jannaschii* ORFs, MJ0010 and MJ1612 (6). The *M. maripaludis* ORF also appeared to be truncated and missing the 27 and 6 C-terminal amino acids of MJ0010 and MJ1612, respectively. Moreover, the amino acid similarity was only 40% and 27% with MJ0010 and MJ1612, respectively, or much lower than observed for likely orthologs between *M. jannaschii* and *M. maripaludis*. Although MJ0010 and MJ1612 were both homologs of a *Streptomyces* gene (6), the functional assignment of this gene has been called into question. It was originally believed to encode phosphonopyruvate decarboxylase (21). This enzyme catalyzes the formation of phosphonoacetaldehyde from phosphonopyruvate and is a required early step in antibiotic biosynthesis. However, subsequent authors pointed out that the gene lacks a thiamine pyrophosphate binding motif and is unlikely to be a decarboxylase (25). The methanococcal ORFs also do not possess thiamin pyrophosphate binding motifs, and their functions are not known.

Phenotype of JJ117. In the initial screening, JJ117 failed to grow on plates in the absence of acetate. This phenotype was confirmed in broth cultures. Washed cells or very high dilutions of cultures failed to grow in mineral medium even after 10 days (Fig. 3 and data not shown). However, in the presence of acetate, growth was restored.

Moreover, the addition of Casamino acids or a mixture of branched-chain amino acids failed to support growth, suggesting that the nutritional requirement for acetate was specific and not due to a general sparing of energy metabolism by organic carbon sources. Surprisingly, a mixture of water soluble vitamins also supported growth (Fig. 3). Of the vitamins, only cobalamin (1 μg L^{-1}) supported growth, and thiamine (50 μg L^{-1}), biotin (20 μg L^{-1}), para-aminobenzoic acid (50 μg L^{-1}), folic acid (20 μg L^{-1}), riboflavin (50 μg L^{-1}), nicotinic acid (50 μg L^{-1}), pyridoxine HCl (100 μg L^{-1}), and DL-calcium pantothenate (50 μg L^{-1}) had no affect (data not shown). Good growth was also obtained with 80 μg L^{-1} or 0.06 μM cobalamin, and no further stimulation was observed with concentrations as high as 7.5 μM (data not shown). Structural components of cobamides including cobinamide (0.8 μM), 5',6'-dimethylbenzimidazole (34 μM), 2'-hydroxybenzimidazole (34 μM), 5'-methylbenzimidazole (34 μM), adenine (37 μM), guanine (37 μM) and 2-aminopropanol (1% vol/vol) did not support growth of JJ117 and did not inhibit growth of the wild-type JJ1 (data not shown). In methanococci, the natural cobamide contains 5'-hydroxybenzimidazole and not 5',6'-dimethylbenzimidazole as found in cobalamin (11). Because 5'-hydroxybenzimidazole was not commercially available, a wide range of nucleosides and potential axial ligands were tested on the assumption that at least some of them could be assimilated. The failure of the components of cobalamin to replace the nutritional requirement in JJ117 suggested that the mutation in JJ117 did not affect biosynthesis of the cobinamide, benzimidazole, or the aminopropanol linker. Thus, the phenotype could result from an inability to assemble these components or by an indirect affect on cobamide metabolism.

Genotype of JJ117. To determine if the phenotype of JJ117 was caused by insertion of pWDK117, the wild type JJ1 was transformed with the plasmid. While two out of 17 transformants were unable to grow in mineral medium, the remainder grew normally. One of the auxotrophs, JJ118, was examined by Southern hybridization (Fig. 4). In this experiment, the chromosomal DNA was digested with *Eco*RI. Hybridization with pWDK117 to itself produced two bands, representing the 2.6 and 3.0 kb *Eco*RI fragments. This plasmid hybridized weakly to a 9 kb fragment in the genomic DNA of the wild type. Presumably, this band contained the wild type gene that had been cloned on the insert. The genomic DNA of JJ117 and JJ118 contained 2.6, 3.0, 4.4, and 7.0 kb fragments that hybridized to pWDK117. In addition, a 6 kb fragment was observed in JJ117, which may have represented a incomplete digestion product. This pattern would be expected if pWDK117 integrated into the genome as tandem copies. In contrast, a transformant with normal growth properties, JJ119, contained only the 2.6, 4.4, and 7.0 kb *Eco*RI fragments. This pattern was expected if only a single copy of the plasmid had integrated. Thus, the auxotrophic phenotype appeared to be associated with tandem insertions of the plasmid.

Because single insertions of the pWDK117 did not produce the auxotrophic phenotype, it seemed unlikely that the phenotype was due to disruption of the large ORF. This hypothesis was supported by three additional observations. First, pWDK117 probably contained most of the ORF, and based upon its alignment with MJ0010 it was only missing 20-80 bp of the 3'-end. After homologous recombination, transformants with a single insertion such as JJ119 would be expected to express a nearly full length ORF, which might retain activity and support normal growth. Second, a portion of the large

ORF representing amino acid residues 81-309 was subcloned to produce a plasmid pWDK117-11. Transformation with pWDK117-11 should further truncate the large ORF from 403 to 309 amino acids. Of 47 transformants tested, none were auxotrophic. Third, it was also possible that the large ORF was essential for growth even in the presence of acetate. In this model, the truncated ORF would have had partial activity that only allowed for growth in the presence of acetate. Insertions which completely inactivated the gene would be lethal unless other more complication genetic events also occurred, such as tandem insertions of the plasmid. If this were the case, the transformation efficiency of pWDK117-11 would be expected to be lower than that observed for pWDK104, which contains a genomic DNA fragment of nearly the same size as pWDK117-11. However, the transformation efficiencies of the two plasmids were nearly the same, indicating that the large ORF was not essential (data not shown). For these reasons, the phenotype of JJ117 did not appear to result from disruption of the large ORF.

It was also possible that the phenotype of JJ117 could be due to overexpression of the large ORF. In this scenario, the large ORF might be a cobamide-binding protein whose overexpression would titrate cobamides out of enzymes in methanogenesis and other essential functions. Acetate might spare this defect if biosynthesis of the cobamide Fe/S protein in the acetyl-CoA synthase complex represented a major pool of intracellular cobamide. To test this hypothesis, the wild-type strain was transformed with an integration expression vector, pWDK117-P. This plasmid contained the moderately strong histone promoter from *M. voltae*, P_{hmvA}, and 316 bp of the 5'-end of the large ORF. Upon integration, it was expected to place the genomic copy of the large ORF under control of P_{hmvA} and to cause overexpression of the gene product (9). Of the five

transformants examined, all grew well in minimal medium, and none were auxotrophic for acetate or cobalamin. In addition, examination of the sequence of the large ORF failed to identify a cobalamin-binding motif (7, 20). Thus, these experiments failed to provide evidence in support of this model.

DISCUSSION

Transposon mutagenesis is a valuable technique that has only be applied sporadically to the archaea (30). For instance, pMudpur, which contained the Mu transposon and the *pac* cassette for puromycin selection in methanococci, was created to mutagenize cloned methanococcal *nif* DNA (4). Upon transformation of the cloned DNA into *M. maripaludis*, transposon insertion was achieved by selecting for puromycin resistance.

An alternative to transposon mutagenesis is to disrupt genes by transformation with a nonreplicative plasmid containing randomly cloned fragments of genomic DNA (17,18,19). Campbell-like integration events insert the plasmid via homologous recombination at sites throughout the genome. The plasmid is inserted between two identical copies of the original cloned DNA. For this general strategy to be applied to the methanococci, it is necessary to obtain large numbers of random insertions throughout the genome, to recover the plasmid after isolation of specific mutants, and to confirm the phenotype after transforming the wild type with the plasmids isolated from specific mutants. For *M. maripaludis*, it has been possible to perform each of these steps during the isolation of acetate auxotrophs. Therefore, this method may have general utility in methanococcal genetics.

The first mutant, JJ104, was a leaky acetate auxotroph whose growth was also stimulated by amino acids and the oxyanions molybdate, tungstate and selenite. Southern hybridization indicated that it contained tandem insertions of the vector into a homolog of an ABC transporter. This family of transporters has been characterized in the archaea by genomic sequencing (6,15), and a maltose transporter from *Thermococcus litoralis* has been studied biochemically (12,39). JJ104 is the first mutant isolated in archaea that disrupted their function in vivo. Although the transport function was not measured directly, the phenotype is consistent with a role in molybdate, tungstate, and selenite uptake. These oxyanions are required for biosynthesis of the active forms of the pterin coenzymes of the formylmethanofuran and formate dehydrogenases, the selenium hydrogenases, selenium-containing tRNAs, and probably other undiscovered macromolecules (28, 29, 38). Both amino acids and acetate can be major carbon sources for the facultative autotroph *M. maripaludis* (37) and might be expected to stimulate when growth is limited for these essential oxyanions.

The second acetate auxotroph, JJ117, had an absolute requirement for either acetate or cobalamin. However, this phenotype probably did not result from simple inactivation of a gene in *M. maripaludis*. First, the original mutant contained tandem repeats of the vector and the cloned gene. Tandem repeats of insertion vectors are relatively common and have been observed before in methanococci (10, 24). However, transformants of pWDK117 containing a single insert were not auxotrophic. When transformed with a plasmid pWDK117-11 containing smaller, internal fragments of the gene, auxotrophs were again not found. These experiments argue strongly against insertional inactivation causing the phenotype. Even though it was not possible to establish a causal link

between the genotype and phenotype in JJ117, these experiments describe a new genetic locus important in acetate and cobamide metabolism in *M. maripaludis*.

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Table 2-1. Mutants of *M. maripaludis* isolated by random insertional mutagenesis

Mutant	McN	McA	McC	McAC$^+$	plasmid isolated [b]
JJ101	±	±	±	+	pWDK101
JJ102	±	±	±	+	pWDK102
JJ103	±	±	±	+	pWDK103
JJ104	±	+	+	+	pWDK104
JJ105	±	±	±	+	pWDK105
JJ106	±	±	±	+	pWDK106
JJ107	±	±	±	±	pWDK107
JJ108	±	±	+	+	None [c]
JJ117	-	+	+	+	pWDK117

[a]Growth of mutants on replica plates containing mineral (autotrophic) medium (McN), mineral medium + acetate (McA), mineral medium + yeast extract (McC), and rich, complex medium (McAC$^+$). -, no growth; ±, poor growth, +, good growth.

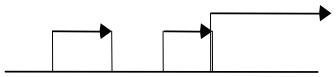
[b]Plasmid isolated from the mutant by electroporation into *E. coli*.

[c]No plasmid was isolated.

Figure 2-1. Sequences of *M. maripaludis* genomic DNA cloned on plasmids isolated from the transformants. The cloned DNA was inserted at the *Eco*RV site adjacent to the *pac* cassette on pMEB.2. In the orientation shown, the *pac* cassette would be on the right. The direction of translation of the ORFs is indicated by arrows. Homologs in the genomic sequences of *M. jannaschii* (MJ) and *Methanobacterium thermoautotrophicum* (MT) are indicated immediately above the ORFs, and the % sequence identity is indicated in parenthesis. The 5'- and 3'-end of the putative genes are indicated by vertical lines from the arrow. The alignment to the *M. jannaschii* homolog was used to choose between alternative start codons suggested by the sequence analysis. When no homolog was found, the ends of the putative genes were taken to be the putative translational start (ATG, GTG, or TTG codons) and termination sites.

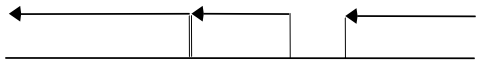
pWDK101

MJ no homologs **MTH** no homologs



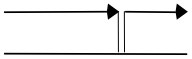
pWDK103

MJ 0931 thil (47%) **MJ** no homolog **MJ 0584** hypothetical protein (28 %)
MTH 1685 thil (36%) **MTH** no homolog **MTH 0894** hypothetical protein (23 %)



pWDK104

MJ 1368 ABC transporter, permease (71%) **MJ 1367** ABC transporter, ATP binding protein (68%)
MTH 0921 ABC transporter, permease (33%) **MTH 0920** ABC transporter, ATP binding protein (35%)

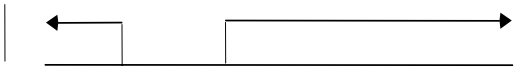


pWDK106

MJ 835.1 hypothetical protein (37%) **MJ 0835** hypothetical protein (43%)
MTH no homolog **MTH** no homolog

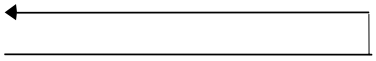
pWDK107

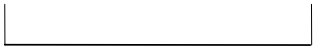
MJ 0681 transketolase (60%) **MJ 0555** endoglucanase (72%)
MTH no homolog **MTH 0437** endoglucanase (57%)



pWDK117

MJ 0010 phosphonopyruvate decarboxylase (41%)
MTH 1591 phosphonopyruvate decarboxylase (26%)





1 kb

Figure 2-2. Stimulation of growth of the acetate auxotroph JJ104-1 by acetate and the oxyanions molybdate, tungstate, and selenite. Growth of JJ104-1 in McN medium (❍), in McN plus 10 mM acetate (●), and in McN plus 10 μM each of sodium molybdate, sodium tungstate, and sodium selenite (Δ). Growth of the wild type JJ1 in McN medium without additions (□). Growth of the wild type in the presence of acetate or the oxyanions was identical to growth in McN medium without additions (data not shown). The inoculum was 8 x 10^6 cells or 4 % (vol/vol) that had been grown in $McAC^+$ and washed twice in McN medium.

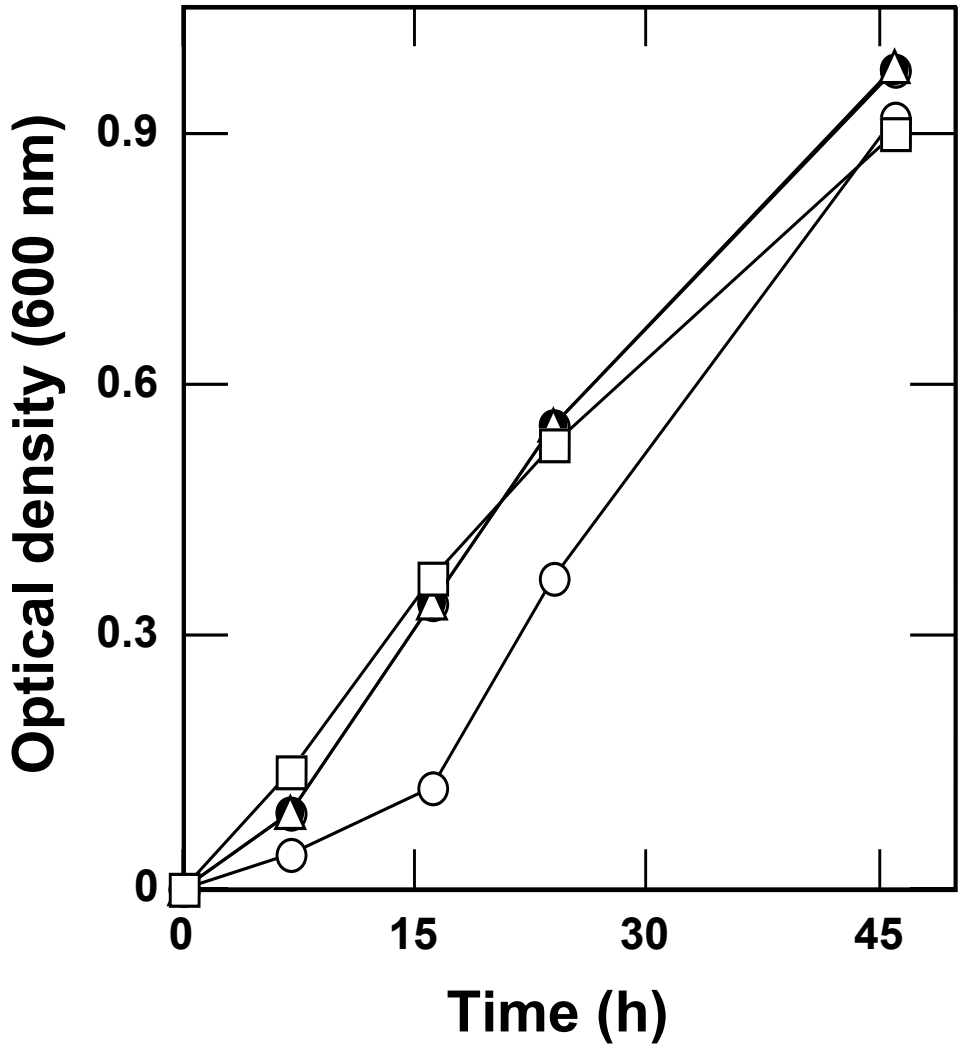

Optical density (600 nm)
0.9
0.6
0.3
0
0
15
30
45
Time (h)

Figure 2-3. Stimulation of growth of the acetate auxotroph JJ117 by acetate and vitamins. Growth of JJ117 in McN medium (❍), in McN plus 10 mM acetate (●), in McN plus a mixture of water soluble vitamins (Δ), and in McN plus both acetate and vitamins (▲). Growth of the wild type in McN medium (□). The inoculum was 6 x 10^3 cells that had been grown in $McAC^+$ medium.

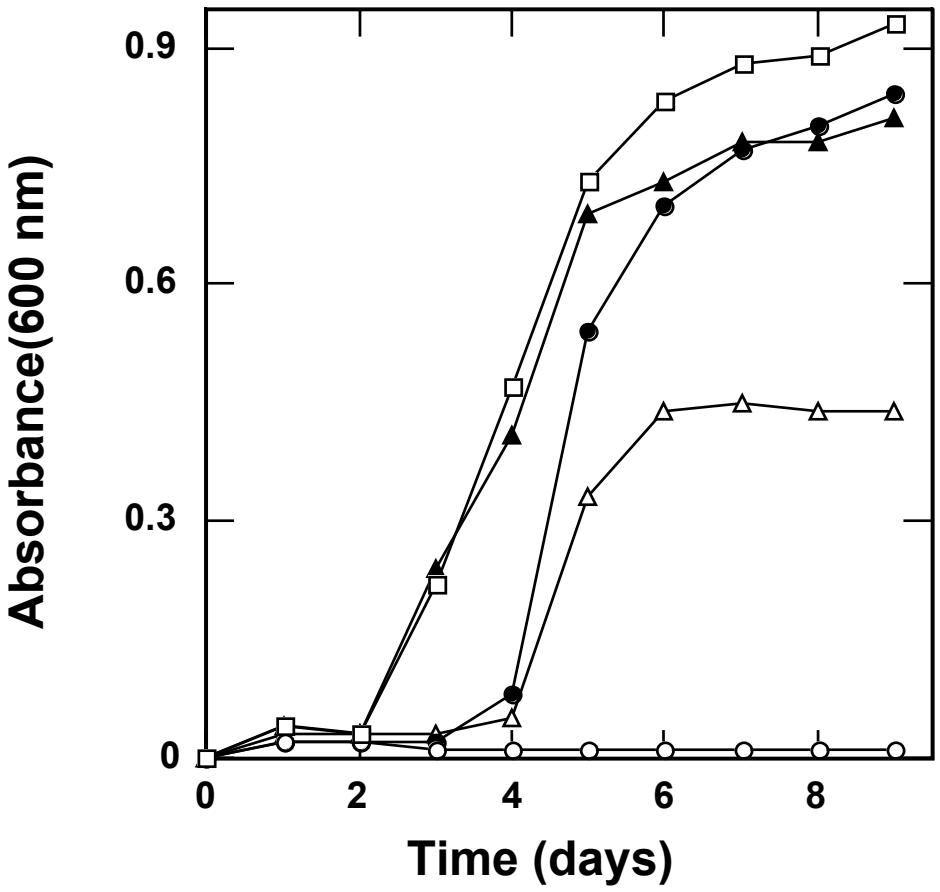
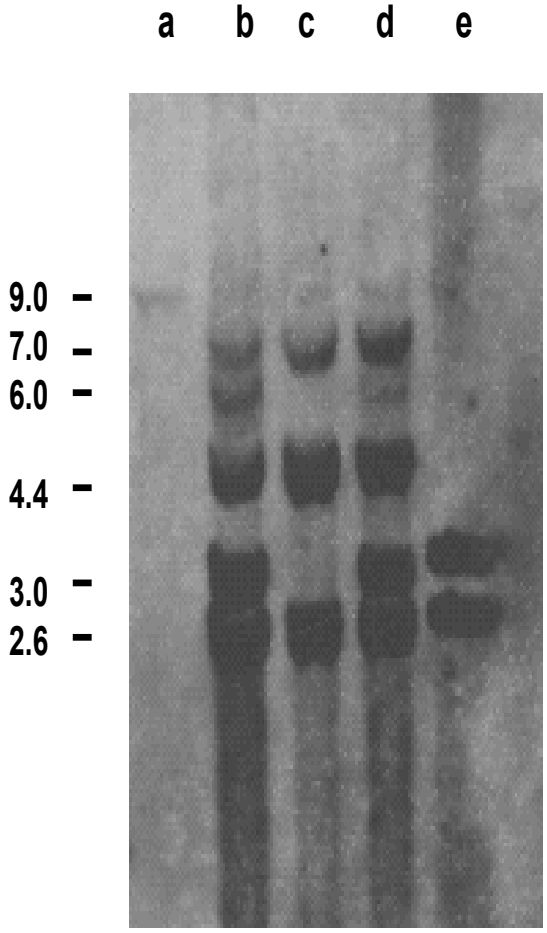

Absorbance(600 nm)
0.9
0.6
0.3
0
0
2
4
6
8
Time (days)

Figure 2-4. Southern hybridization of pWDK117 to genomic DNA from *M. maripaludis* strains JJ1, JJ117, JJ118, and JJ119. DNA was treated with *Eco*RI prior to electrophoresis. The DNA was from a) the wild-type strain JJ1, b) the original auxotroph JJ117, c) the transformant JJ119 with normal growth properties, d) the transformant JJ118 that was auxotrophic, and e) the plasmid pWDK117. The molecular weights of the major bands are indicated in the left margin.


a b c d e
9.0
7.0
6.0
4.4
3.0
2.6

CHAPTER III

MUTAGENESIS OF *CBIJ* OF *METHANOCOCCUS MARIPALUDIS*

INTRODUCTION

Methanococcus maripaludis is a facultative, autotrophic methanogen that produces methane from $H_2 + CO_2$ (12). In methanogens, cobamide is a cofactor of the N^5-methyltetrahydromethanopterin: CoM methyltransferase that catalyzes an essential step during methane production (14). The coenzyme is also a component of the acetyl-CoA decarbonylase/synthase complex (ACDS) that is required for autotrophic acetyl-CoA biosynthesis by the modified Ljungdahl-Wood pathway (1). While many methanogens contain the cobamide factor III (Coα-[(5- hydroxybenzimidazoyl)]-cobamide), methanococci contain pseudo vitamin B_{12} (Coα-[α-(7-adenyl)]-cobamide) (22). While the biosynthesis of factor III has been studied in *Methanobacterium* spp. and *Methanosarcina* spp. (5, 19, 23), the pathway of pseudo vitamin B_{12} biosynthesis has not been extensively examined. In previous research, a mutant JJ117 of *M. maripaludis* was isolated by random mutagenesis and enrichment for an acetate auxotroph (15). Growth of this mutant was dependent on either acetate or cobalamin, and best growth was obtained with both compounds. The mutation was due to tandem insertion of a plasmid into a homolog of phosphopentose mutase (*ppm*), a gene which was not known to play a role in either acetate or cobamide biosynthesis (15). Thus, it was possible that the auxotrophic phenotype of the mutant JJ117 was due to an effect on expression of a neighboring gene. In this work, experiments were performed to discover and characterize the gene directly responsible for the auxotrophic phenotype of JJ117.

MATERIALS AND METHODS

Strains, media and growth conditions. *M. maripaludis* JJ was obtained from W. J. Jones (11). *Escherichia coli* strains of SURE, XL-1 Blue MRF' were from Stratagene (La Jolla, CA), and TOP10 was from Invitrogen (Carlsbad, CA). Methanococci were grown at 37°C with 275 kPa of H_2: CO_2 (80:20) gas in the minimal medium McN with other additions (12). Other media used were: McNA, minimal medium plus 10 mM acetate; McYA, McNA medium plus 0.2 g/L of yeast extract; McNV, minimal media plus 1 μM of cobalamin; McAV, McNA plus 1 μM of cobalamin; McYAV, McYA medium plus 1 μM of cobalamin. The inocula for growth experiments were grown to an absorbance (at 600 nm) of 0.2 to 0.5 in McYAV medium. For growth of methanococci with puromycin, a concentration of 2.5 μg/ml was used. Prior to inoculation, cultures were diluted in McN medium. Glassware for growth experiments were cleaned in 0.1 M HCl solution for at least 12 h at room temperature and autoclaved for 20 min. Stoppers for 28 ml culture tubes were autoclaved with 0.2 N NaOH for 20 min. The glassware and stoppers were rinsed in tap and deionized water after the autoclaving. Solid medium for *M. maripaludis* was prepared in the anaerobic chamber as described (25).

Molecular biology techniques. Ligation was performed at 4°C or 16°C with 1-3 units (Promega, Madison, WI) or 400 units (New England Biolabs, Beverly, MA) of T4 DNA ligase, respectively, as described by the manufacturers. Plasmids from *E.coli* were purified with Wizard plus mini prep kits (Promega) or QIAprep spin miniprep kits (Qiagen, Hilden, Germany). QIAquick gel extraction kit (Qiagen) was used for gel purification of DNA. Restriction enzymes, alkaline phosphatase, Klenow fragments were purchased from New England Biolabs or Promega. DNA was transformed into

E.coli with a Gene Pulser Electroporator (Bio-Rad, Hercules, CA) set at 25 F and 2.50 kV or with the One Shot kit (Invitrogen, Carlsbad, CA). *E. coli* was screened on LB agar plates or LB broth medium with 50 μg/ml of ampicillin. Total genomic DNA of *M. maripaludis* was purified by a mini-prep method described previously (2). Plasmid DNA, 5 ng, or 50 ng of genomic DNA was used as template for PCR amplifications. For PCR, 0.1 μM or 0.2 μM of primers were added. Unless specified differently, the PCR reactions were performed with the Ready-To-Go Kit (Amersham Pharmacia Biotech) with initial denaturation of 94°C for 4 min followed by 30 cycles of denaturation at 94 C° for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. Sequencing was carried out on an Applied Biosystems (Foster city, CA) model 373 automated sequencer in the Molecular Genetics Instrumentation Facility at the University of Georgia. Sequences were analyzed with the programs of GCG (University of Wisconsin), TIGR (http://www/tigr.org) and ERGO (http://wit.integratedgenomics.com/ERGO/).

Transformation for *M. maripaludis*. Polyethyleneglycol transformation procedures for *M. maripaludis* have been described previously (24). Transformations for gene replacement were performed with linear forms of the plasmids which were constructed by digestion with the specified restriction enzymes and subsequent gel purification.

Plasmid construction and identification of flanking sequences. Construction of pWDK117-11 was described previously (Table 1). JJ117-11, which was isolated from the transformation of the wild type strain by pWDK117-11, was cultured in McYAV plus puromycin (Table 2). Genomic DNA was isolated and treated with 20 units of *Eco*RI overnight at 37°C. The digested DNA was purified with a Wizard DNA clean-up kit

(Promega), and 10 µl of the sample containing 0.14 µg of DNA was religated with T4 DNA ligase. The ligation mixture was electroporated into *E.coli* SURE after drop dialysis against distilled water on a VSWP filter paper (pore size 0.025 µm, Millipore). Plasmids that were purified from the *E.coli* transformants were screened by digestion with *Eco*RI and *Eco*RV. The presence of the flanking genomic DNA on the plasmid was confirmed by the observation of a 2.0 kb digestion product, which was larger than the equivalent fragment of pWDK117-11. The plasmid containing the flanking sequence was named pWDK117-12. pWDK120 was constructed by ligating a 0.35 kb PCR amplification product from the new sequence (see below) into the *Nsi*I site of pIJA03. For the PCR, 2.5 units of *pfu* polymerase (Stratagene) and the primers, 5'-TAATTTTAACCACCACAACA (positions +89-103 in Fig.1) and 5'-GGAAGTATCCTCACAATCAG (positions +454-469 in Fig.1) were employed. For the PCR, the standard PCR conditions were used except that of the annealing temperature was 51.5°C. The PCR product was gel purified and ligated into the *Eco*RV site of p-ZErO. After isolation, the plasmid bearing the clone was digested with *Nsi*I, and the resulting fragment was ligated into pIJA03, completing construction of pWDK120. To identify sequences further upstream, pWDK120 was transformed into the wild type to form JJ120. Total DNA from JJ120, 1 µg, was treated with 30 units of *Hin*dIII overnight at 37°C. The digested DNA was religated and electroporated as described above. The resulting plasmids were screened by restriction with *Xba*I and *Xho*I. An increase in size of the 0.07 kb fragment of the vector to 1.0 kb indicated the presence of the upstream sequences. This plasmid was named pWDK117-13. To clone the region downstream of the *ppm* homolog in the mutant JJ117, a strategy similar to that of Hildebrant and Nellen

(10) was used. Genomic DNA, 1 μg was digested with 5 units of *Afl*III and 10 units of *Nru*I. These two sites were present in the vector portion of the tandem repeats immediately upstream of the cloned DNA. Therefore, restriction with two enzymes prevented ligation of the vector from tandem repeats. The only product expected would be formed by ligation between the *Afl*III sites in the vector and upstream in the genome. The resulting plasmid, pWDK117-14, contained an additional 0.55 kb of genomic DNA.

pWDK121 was constructed in pIJA03 to make a gene replacement of *cbiJ*. The upstream fragment (0.9 kb) was obtained by digestion of pWDK117-13 with 20 units of *Nde*I, gel purification, and ligation into the *Nde*I site of pIJA03. The resulting plasmids were screened to identify pWDK121a, which possessed the insert in the same transcriptional orientation as the puromycin transacetylase gene on the *pac* cassette. The second fragment was obtained by PCR amplification of the *ppm* homolog in pWDK117-12. The primers used were: 5'-CCCCCCGGTACCATCGTGTTCTAATCTCAATGG (positions +866-886 in Fig. 1) and 5'-CCCCCCGCTAGCTTAGATTTTGACGGACTTTTG (positions +2000-2020 in Fig.1), where the underlined positions represent *Kpn*I and *Nhe*I restriction sites, respectively. The PCR conditions were the same as used for construction of pWDK120 except that 25 cycles were performed. The product was then digested with 10 units of *Kpn*I and *Nhe*I for 2 h and gel purified. The development of pWDK121 was completed by insertion of the purified product into the *Kpn*I and *Nhe*I sites of pWDK121a.

pWDK200 was constructed by ligating the strong S-layer promoter (P_{sla}) into pIJA03. The promoter was obtained from pSla-*vhcI* (13) by 25 cycles of PCR amplification with two primers: 5'-TAAAGTGACTAGTCAATTTCGAAAGTAATAAAAATAAT and 5'-

ATTTGCATGCATATGCACCTTTTGTTTTTATTTT. The PCR conditions were denaturation at 94°C for 1 min, annealing at 48.8°C for 5 min, and extension at 65.0°C for 2 min. The 0.25 kb product was then gel purified and inserted into the *Eco*RV site of pZErO-2. To obtain the fragment containing the promoter sequence, the plasmid was digested with *Bam*HI-*Nsi*I. The digested fragment was then inserted into the *BamH*I - *Nsi*I sites of pIJA03. pWDK201 was developed to insert P*sla* prior to the genomic copy of the *ppm* homolog after transformation. For the construction, the 5' end of *ppm* was cloned next to P*sla* in pWDK200. First *ppm* was PCR amplified with two primers: 5'-GCGGTGGCATGCATGAAAACAATCGT (underline indicates *Nsi*I site, positions +2021-2045 in Fig.1) and 5'-ATTACGGCGGCCTTTTCCACAG (positions +1080-1101 in Fig.1) from genomic DNA. The product was treated with *Nsi*I and *Bam*HI and cloned into the *Nsi*I-*Bgl*II sites of PIJA200. This treatment was successful because the PCR product possessed an internal *BamH*I site at positions +1101-1105. pWDK202 was constructed to insert P*sla* prior to the genomic copy of *cbiJ* following transformation. Two primers 5'-CCGTAATAGAAGAAATGGATG (positions +1066-1086 in Fig.1) and 5'-TTGCATGCATAACATATGGATTCGTGGCGGA (underline indicates *Nsi*I site, positions +4-24 in Fig.1) were used to PCR amplify a 1.0 kb portion of *cbiJ-ppm*. Genomic DNA of the wild type was used as the template. The standard conditions were used for PCR amplification except that 25 cycles were performed, the annealing temperature was 59.5°C, and extension was for 2 min and 30 sec. The PCR product was digested with *Nsi*I and ligated with into the *Nsi*I site of pIJA200. In addition to the *Nsi*I site in the primer, the PCR product possessed another *Nsi*I site in the amplified DNA

(positions +1063-1068 in Fig.1). The orientations of the inserted fragments were determined by restriction of the plasmids with *Hin*dIII plus *Bam*HI.

pWDK240 and pWDK241 were derivatives of the expression shuttle vector pWLG40 + *LacZ* and were used for overexpression of the *ppm* and *cbiJ* antisense mRNA. Because *ppm* contained an internal *Nsi*I site (at position +1065-1068 in Fig.1), pWDK220 was first constructed by ligating the 0.97 kb *ppm* fragment from the *Nsi*I-*Xba*I digestion of pWDK201 into the *Nsi*I -*Xba*I sites of pWLG40 + *Lac*. This vector was then digested with *Bgl*II and *Xba*I. Because *ppm* contained an internal *Bgl*II site at positions +1785-1790, the product could then be used to clone the 3'-end of *ppm* without using *Nsi*I. The remaining portions of *ppm* were obtained by PCR amplification. For pWDK240, the primers were AAGTTTAGTAGATAGGGCGG (positions +2042-2061) and 5'-CTAGTCTAGATAGCAAATACGACTTCGGGGA (underline indicates *Xba*I site, positions -93-73 in Fig.1). The PCR was performed by the standard conditions except that the annealing was performed at 60°C and the extension was performed for 3 min and 30 sec. For pWDK241, the primers were: 5'- TTAGATTTTGACGGACTTTTG (positions +2000-2019 in Fig.1) and 5'-CTAGTCTAGAGCTTTATCCATGTAATTTAATATTAA (underline indicates *Xba*I site, positions +828-853 in Fig.1). Standard conditions were used for the PCR except that only 25 cycles were performed and extension was for 2 min and 30 sec. Both PCR products were digested with *Bgl*II and *Xba*I, ligated into the *Bgl*II-*Xba*I digested pWDK220. To make pWDK242 and pWDK243, these PCR products were digested with *Nsi*I and *Xba*I to yield 0.96 kb and 0.2 kb gene fragments, respectively, and ligated into pWLG40 + *LacZ* and 0.2 kb and 0.96 kb gene fragments were obtained, respectively.

Because of the internal *Nsi*I site at positions +1063-1068 in *ppm*, these constructions contained only the 3' portion of *ppm*.

PCR confirmation of Δ*cbiJ* mutation. To confirm the replacement of the genomic *cbiJ* with the *pac* cassette, primer sequences for the PCR were selected from 100 bp upstream and 200 bp downstream of the *cbiJ*: P1(5'-TAGCAAATACGACTTCGGGGA, positions -93-73 in Fig. 1) and P4 (5'-GATGGGCGCGTTTGTAAATTC, positions +975-995). Other primers were complementary to the *pac* cassette: P3 (5'-TGCAAGAACTCTTCCTCACG) and P4 (5'-GTCTCTTTCACCAGCAGCTT). The reactions contained 50 ng of template DNA in 1 µl, 1 µl of each primer, 2.5 µl of DMSO (dimethylsulfoxide), and 19.5 µl of distilled water in the Ready-To-Go PCR tubes (Amersham Pharmacia Biotech). The standard PCR conditions were used except that the extension time was for 3 min.

Southern hybridization. Genomic DNA, 2 µg, was digested by *Acc*I (15 units) for 29 h or *Bam*HI (20 units) for 44 h. The restricted DNA was separated on a 1.0 % (wt/vol) agarose gel and transferred to a positively charged nylon membrane (Micro separations Inc.) by the downward capillary method (17). Prehybridization and hybridization were performed at 65°C overnight in a solution of 2 x SSC, 0.01% (w/v) SDS, 0.1% (w/v) N-lauroylsarcosine and 1% blocking reagent (nonfat milk). Membranes were washed twice in solutions of 2 x SSC plus 0.1 % SDS for 5 minutes at room temperature and 2 x SSC plus 1% SDS for 15 minutes at 65°C. The probe was radiolabeled with [α-P^{32}] dATP (ICN, Aurora, OH) according to a random priming method (17). The template for the random priming reaction was the 0.96 kb *Nde*I fragment (Positions-951-+6 in Fig.1) from pWDK121. Approximately 2.5 x 10^7 dpm of

labeled probe was added into the hybridization. Prior to visualization of the membranes with ImageQuant 1.1 software, the membranes were exposed in a Phosphoimager cassette for 36 h. The molecular weight markers were constructed by digestion of the PCR amplificand of the wild type genomic DNA and primers P1-P4 with *Acc*I, *Bam*HI and *Acc*I plus *Pvu*II.

Measurement of cobamide concentration. Cobamides were extracted from cells as described by Gorris *et al*. (8). In this experiment, 100 ml of mid-exponential phase cultures (A600= 0.45-0.55) were harvested by centrifugation at 4°C. After the extraction, the total volume was 25 ml. Of this, 4 ml was concentrated to dryness in a by High Speed Vac (Uniequip, Martinsried, Germany), and resuspended in 50 µl of distilled water. The cobamide concentration was estimated as described by Maggio-Hall and Escalante-Semerena (16) and Thomas and Escalante-Semerena (23). For this experiment, Vogel-Bonner medium overlaid by 3 ml of medium containing *Salmonella enterica* serovar Typhimurium TR6583 [*metE205 ara-9*] or JE1299 [*metE*205 *ara*-9 *btuB7*::*MudJ*] were spotted with 2.5 µl or 5 µl of various dilutions of the methanococcal extracts. The standards were spots of cobalamin solutions containing 0.0025 to 20 pmol of cobalamin in 2.5 µl or 5 µl. The quantity of the cobamide in the methanogens was determined by comparing the diameters of the confluent growth between the standard and samples. For the control JE1299, 2.5 µl or 5 µl of methionine (0.4 to 3 µmol) solutions were also spotted.

Accession number. The GenBank accession number for the *M. maripaludis cbiJ* and surrounding sequence is AF402610.

RESULTS

Identification of flanking sequence of *ppm* homolog. In previous studies, the insertion of tandem repeats of the plasmid pWDK117 into the *M. maripaludis* homolog of the *Methanocaldococcus jannaschii* gene MJ0010 yielded an acetate and cobamide auxotroph (15). However, inactivation of the MJ0010 homolog did not appear to be responsible for the observed phenotype for the following reasons. First, the insertion was only expected to produce a small truncation of the ORF, and it seemed unlikely that this would be sufficient to inactivate the gene product. Second, mutants bearing a single insertion, which would also produce the truncated ORF, were not auxotrophic, and their growth properties were indistinguishable from wild type. Therefore, the phenotype depended on the presence of the tandem repeats. Third, the MJ0010 homolog possessed sequence similarity to *ppm* (phosphopentose mutase), and it was difficult to rationalize a role for this gene product in cobamide or acetate biosynthesis (15). Thus, it was hypothesized that inactivation of a downstream gene might be responsible for the auxotrophic phenotype.

The downstream genes were cloned by transforming *M. maripaludis* with pWDK117-11 to yield JJ117-11 (Table1). The plasmid pWDK117-11 contained a 0.68 kb gene fragment internal to the *ppm* homolog and was expected to integrate within this gene by homologous recombination. The genomic DNA of JJ117-11 was digested with *Eco*RI, ligated and transformed into *E.coli*. In this fashion, pWDK117-12 was isolated (Fig. 1). Sequencing of pWDK117-12 revealed that it contained genomic DNA, including 0.3 kb of the *ppm* homolog and 1.0 kb of a downstream ORF. To obtain additional flanking genes, a portion of this downstream ORF was cloned into the insertion plasmid pIJA03 to

form pWDK120. After transformation of the wild type strain, pWDK117-13 was isolated following *Hin*dIII digestion and transformation into *E. coli*. The sequencing of this plasmid revealed additional downstream genes (Fig. 1).

Analysis of the sequences of these clones identified the 3' region of the *ppm* homolog, which had been missing in the previous clones (15). Analysis of the ORF immediately downstream of the *ppm* homolog showed that it possessed 52% sequence similarity to the *cbiJ* homolog of *Methanocaldococcus jannaschii* (Fig. 1). Downstream of the *cbiJ* homolog were a hypothetical ORF that did not possess similarity to any known gene and the 3'- end of an ORF that was only 180 bp and too short to identify.

Although the original mutant JJ117 with an insertion of tandem repeats possessed a severe auxotrophic phenotype, other transformants formed by integration of single copies of other vectors at the same site were not auxotrophs (15). For this reason, it seemed possible that the cobamide auxotrophy of JJ117 may have been due to a second mutation in *cbiJ*, possibly caused by the formation of the tandem repeats. To test this hypothesis, the 3'-end of *cbiJ* was cloned and sequenced from JJ117 (positions +394-+945 in Fig. 1), but no differences were observed between the sequence of the gene from the wild type and that of the mutant. Thus, the phenotype of JJ117 did not appear to be due to deletion or other mutation in the 3' portion of *cbiJ*.

Mutagenesis of *cbiJ* in *M. maripaludis*. If the original phenotype of JJ117 were due to an effect on expression of *cbiJ*, deletion of *cbiJ* would be expected to yield a mutant with the same phenotype. Therefore, the mutants JJ121 and JJ122 were constructed in which the *cbiJ* was replaced with the *pac* cassette (Fig. 2). The genotypes of these mutants were confirmed by PCR amplification and Southern hybridization (Fig. 2 and

data not shown). First, PCR amplification of the genomic DNA was performed to verify the gene replacement of *cbiJ* in JJ121 and JJ122. Although the primer pair p1-p4 failed to yield a product with JJ121 (data not shown), the expected 1.1 kb product was found with the wild type (Fig. 2 and data not shown). However, amplification of JJ121 DNA with primer pair p2-p3 confirmed the placement of the *pac* cassette in the genome, and amplification with the primer pair p2-p4 confirmed the replacement downstream of *ppm* (Fig. 2). Because primer pair p1-p3 also failed to produce an amplicand in either JJ121 genomic DNA or the plasmid control, it seemed likely that the high GC content of the puromycin transacetylase gene prevented amplification across the 5' boundary of the cassette (9). Thus, it was not possible to demonstrate the 5'- end of the gene replacement and the absence of tandem insertions by this method. Southern hybridization was then used to confirm the gene replacement of *cbiJ* in the mutants. In the first hybridization, *Acc*I-digested genomic DNA produced a 2.2 kb band for wild type and 1.6 kb band for the mutants JJ121 and JJ122 (Fig. 3). These were the sizes expected for a replacement of *cbiJ* with the *pac* cassette (Fig. 2). Similarly, hybridization of *Bam*HI digested genomic DNA produced a 1.8 kb band for wild type and a 2.4 kb band for the mutants (Fig. 3). These results confirmed the gene replacement of *cbiJ* by a single copy of the *pac* cassette.

Phenotype of the Δ*cbiJ* mutants. The original mutant JJ117 was an auxotroph that required either acetate or cobamide for growth (15). In the presence of both compounds, growth was comparable to that of the wild type. For the *ΔcbiJ* mutant JJ121, a similar phenotype was observed. Addition of acetate, cobamide or acetate plus cobamide allowed growth in minimal media (Fig. 4 and data not shown). To determine if JJ117

and JJ121 required similar concentrations of cobamide for growth, the cultures were incubated in minimal medium plus 0.05, 0.3, and 1.0 μM of cobamide. Based upon a cobamide content of methanococci of 485 nmol per gram of protein (7), the concentration necessary to support full growth in minimal medium was expected to be 0.3 μM. Both JJ117 and JJ121 grew in medium containing 0.3 μM and 1.0 μM of cobalamin. However, no growth was observed with 0.05 μM of cobalamin. These results confirmed the similarity of their phenotypes.

In some experiments when high inocula were used, JJ121 grew in minimal medium without acetate or cobalamin after a long lag. Upon subsequent transfers to minimal medium, a lag was no longer observed. Therefore, the occasional growth observed in minimal medium appeared to be due to selection for revertants. Because the revertants remained puromycin resistant, the mutation presumably occurred at a second site.

To explain the phenotype of the original mutant JJ117, it seemed possible that overexpression of *ppm* from tandem repeats interfered with the expression of *cbiJ*. For instance, the production of *cbiJ* antisense mRNA might inhibit either the translation of *cbiJ* or the stability of the mRNA. Therefore, integration and expression vectors containing different regions of *ppm* and *cbiJ* were transformed into wild-type *M. maripaludis* to determine if overexpression of *ppm* caused cobamide auxotrophy (Fig. 5). The transformation efficiencies of the expression vectors were comparable to controls to insure that the efficiencies were high. Thus, the transformants were not formed by unusual or rare genetic events. In JJ201, the very strong P*sla* promoter was integrated upstream of the genomic copy of *ppm* (Fig. 5). This construction was expected to result in a merodiploid with very high expression the copy of *ppm* adjacent to *cbiJ* (6). For the

characterization of the transformants, growth experiments were performed in McN, McA and McV broth media after isolating the transformants from McYAV agar plates. In these experiments, significantly slower growth of the transformants was not observed in the McN medium (data not shown). These results indicated that the transformants were not auxotrophic. However, because merodiploids are unstable in *M. maripaludis*, it is possible that JJ201 was overgrown by revertants. Therefore, other constructions were attempted where *ppm* was overexpressed from the shuttle vectors, JJ240-JJ243 (Fig. 5). However, these transformants also failed to show the auxotrophic phenotype. Therefore, another model of gene regulation might be required to explain the effect on *cbiJ* expression.

Quantification of cobamide in the wild type and JJ117. To understand the stimulation of growth of JJ117 with acetate in minimal media, the cobamide content of the strain was measured. The stimulation with acetate was unexpected because acetate is not likely to be specifically involved in the biosynthesis of cobamide. Moreover, cobamides are components of the N^5-methyltetrahydromethanopterin: CoM methyltransferase, an essential enzyme in the pathway of methanogens. Therefore, cobamides were expected to be essential for growth. Thus, it was hypothesized that a small amount of cobamide was synthesized in JJ117, which was sufficient to allow for growth in the presence of acetate. In *M. maripaludis*, acetate can be converted into acetyl–CoA (20). In the absence of acetate, the acetyl-CoA decarbonylase/synthase (ACDS) is required for growth. This enzyme complex also requires cobamide. Therefore, the addition of acetate into minimal medium might reduce the total cobamide demand and allow for growth on reduced levels of cobamide. To verify this hypothesis,

the levels of cobamides in extracts of wild type and JJ117 were determined with the indicator strain *S. enterica* serovar Typhimurium TR6583 (16, 23). Detectible but low amount of cobamides were found in extracts of the mutant JJ117 (Fig. 6A). Because *S. enterica* serovar Typhimurium TR 6583 also responds to methionine, *S. enterica* serovar Typhimurium JE1299 was utilized as an indicator for this amino acid. The levels of methionine in the methanococcal extracts were too low to produce a response in the assay (Fig. 6B). This result indicates that a small amount of the cobamide was synthesized in the Δ*cbiJ* mutant. From other experiments, the cobamide concentrations were estimated from the diameter of confluent growth from strain TR6583. For the wild-type, the levels of cobamide were 800-1100 nmol (g protein)$^{-1}$ following growth in McN and McNA medium. For JJ117, only 15 nmol (g protein)$^{-1}$ was found following growth in McA. Following growth of JJ117 in McV medium, which contained cobalamin, the levels were 1200 nmol (g protein)$^{-1}$ or comparable to wild-type cells. The levels of cobamide determined by this bioassay were similar to the levels of comparable to 145-620 nmol (g protein)$^{-1}$ determined in methanococci by liquid chromatography (22).

DISCUSSION

In this project, we sought to explain the cobamide and acetate auxotrophy of the mutant JJ117, which contained tandem repeats of an insertion vector and portions of *ppm*, a homolog of phosphopentose mutase. The discovery of a homolog of *cbiJ* immediately downstream of *ppm* suggested that inhibition of expression of this gene was the cause of cobamide auxotrophy. The gene *cbiJ* encodes the precorrin 6-X reductase that catalyzes conversion of precorrin 6-X to precorrin 6-Y during an early step in

cobamide synthesis, and *Paracoccus* mutants of *cbiJ* are cobamide auxotrophs (3,18). However, the amino acid sequence of the *cbiJ* homolog in *M. maripaludis* and the other Archaea possesses only 24-33 % identity to the *Paracoccus* and other bacterial genes, so it was necessary to demonstrate its function in methanococci. Thus, the cobamide auxotrophy of the *cbiJ* mutant JJ121 confirmed the requirement of the gene for cobamide synthesis in *M. maripaludis*.

Although good growth of the original mutant JJ117 was only found in the presence of both acetate and cobamide, some growth was also observed in the presence of either acetate or cobamide alone. This phenotype could have resulted from two independent leaky mutants, one each in the pathways of acetate and cobamide biosynthesis. The discovery of acetate auxotrophy in the Δ*cbiJ* mutants eliminated this possibility. Cobamides are required for methyltetrahydromethanopterin: CoM methyltransferase as well as the acetyl–CoA decarbonylase/synthase reactions in methanococci. Because the first reaction is a required step in methanogens, mutations in cobamide biosynthesis were expected to be lethal. The growth of the JJ121 on acetate alone suggested that the mutants contained small amounts of cobamide synthesized by an alternative reaction that bypasses *cbiJ*. In this hypothesis, the amount of the cobamide biosynthesized by the bypass reaction would not be sufficient to maintain normal growth in minimal medium. However, acetate would spare the cobamide requirement because it was no longer required by the acetyl-CoA decarbonylase/synthase and the small amount of remaining cobamide would be sufficient for the methyltransferase. This conclusion was supported by showing that the JJ117 in fact contained 1-2% of the cobamide of the wild-type.

The *cbiJ* and the *ppm* homologs in *M. maripaludis* are convergently transcribed. The *cbiJ* homologs (named *cobK*) are also convergently transcribed with other genes in *Rhodococcus* sp., *Pseudomonas denitrificans* and *Paracoccus denitrificans* (4,18). In *M. maripaludis*, the *cbiJ* and *ppm* homolog were separated by four bases and potential termination structures of stem loop and oligo thymidines overlapped the in 3' regions of the messages. Thus, it seemed possible that mutation in *ppm* could affect the expression of *cbiJ* through interactions of their mRNAs. For instance, overexpression of *ppm* could produce a large quantity of antisense *cbiJ* mRNA that might inhibit translation of *cbiJ*. However, overexpression of the *ppm* homolog in either *cis* or *trans* failed to generate the auxotrophic phenotype in minimal medium. Therefore, the mechanism of the effect of the original mutation in JJ117 on *cbiJ* expression is still not understood.

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TABLE 3-1. Plasmids used in this study

Plasmids	Characteristics	References
pIJA03	Contains modified *pac* cassette (pur^R), developed from pMEB.2	Stathopoulus *et al*.2001 (21)
pWLG40 + *LacZ*	Expression shuttle vector for methanococci, contains *Lac Z*	This work
pWDK200	pIJA03 + S-layer promoter	This work
pWDK117	pMEB.2 + 1.2 kb of *ppm* from *M.maripaludis* JJ	Kim and Whitman, 1999 (15)
pWDK117-11	pMEB.2 + 0.68 kb *Bgl*II-*Bam*HI fragment of *ppm*	Kim and Whitman, 1999 (15)
pWDK117-12	pWDK117-11 + 1.0 kb of DNA downstream of *ppm*	This work
pWDK117-13	pWDK120 + 0.70 kb of DNA upstream of *cbiJ*	This work
pWDK117-14	pMEB.2 + 0.55 kb of DNA downstream of *ppm* in strain JJ117	This work
pWDK120	pIJA03 + 0.38 kb of *cbiJ*	This work
pWDK121a	pIJA03 + 0.90 kb of DNA upstream of *cbiJ*	This work
pWDK121	pWDK121a + 1.10 kb of DNA downstream of *cbiJ*	This work
pWDK201	Inserts P*sla* upstream of *ppm* upon transformation	This work
pWDK202	Inserts P*sla* upstream of *cbiJ* upon transformation	This work
pWDK220	pWDK40 + plus 0.97 kb *ppm*	This work
pWDK240	Inserts 2.10 kb DNA from *ppm* into pWDK40	This work
pWDK241	Inserts 1.20 kb DNA from *ppm* into pWDK40	This work
pWDK242	Inserts 1.10 kb DNA from *ppm-cbiJ* into pWDK40	This work
pWDK243	Inserts 0.20 kb DNA from *ppm* into pWDK40	This work

Table 3-2. Strains used in this study

Strains	Characteristics	References
*M.maripaludis*JJ	Wild-type strain	Jones *et al*. 1983 (11)
JJ117	Transformant of pWDK117, contains tandem insertions, cobalamin and acetate auxotroph	Kim and Whitman, 1999 (15)
JJ117-11	Transformant of pWDK117-11	Kim and Whitman, 1999 (15)
JJ117-12	Transformant of pWDK117-12	This work
JJ117-13	Transformant of pWDK117-13	This work
JJ117-14	Transformant of pWDK117-14	This work
JJ120	Transformant of pWDK120, disruption of *cbiJ*	This work
JJ121 JJ122	Transformant of pWDK121, deletion of *cbiJ*	This work
JJ201	Transformant of pWDK201, *ppm* under control of P*sla*	This work
JJ202	Transformant of pWDK202, *cbiJ* under control of P*sla*	This work
JJ240	Transformant of pWDK240, overexpress *ppm* and potential *cbiJ* antisense mRNA	This work
JJ241	Transformant of pWDK241, overexpress *ppm*	This work
JJ242	Transformant of pWDK242, overexpress 3' portion of *ppm* and potential antisense *cbiJ* mRNA	This work
JJ243	Transformant of pWDK243, overexpress 3' portion of *ppm*	This work

Figure 3-1.

Map of the genes downstream of the *ppm* homolog. Numbering of the DNA sequence begins with the putative transcriptional start codon of *cbiJ*. The nucleotide sequences of the termination regions of *cbiJ* and the *ppm* homolog are shown on the top. The bold characters in the sequences indicate potential termination codons and stem loop structures. H and E represent *Hin*dIII and *Eco*RI sites that were used for identification of flanking sequences of *cbiJ*. A represents the *Afl*III site used in the construction of pWDK117-14. A. The region of genomic DNA cloned in pWDK117-11. B. The region of genomic DNA cloned in pWDK117-12. C. The region of genomic DNA cloned in pWDK117-13. D. The region of genomic DNA from the auxotroph JJ117 cloned in pWDK117-14. E. The region deleted in JJ121 following gene replacement. F. The region used as a probe during the Southern blots of JJ121 and JJ122.

E I V N Y L K N V *

+793

GAAATAGTAAATTATCTAAAAAATGTA**TAA**ATATTTATTTTAAGTGTT**CTATATCCT**C**A**TCA**TT****AGGA**A**TATAG**TCCAA
CTTTATCATTTAATAGATTTTTTACATATTTATA**AAT**AAAATTCACAA**GATATAGGA**G**T**AGT**A**A**TCCT**T**ATATC**AGGTT

+714

* K L H E I D E D N P I Y D L

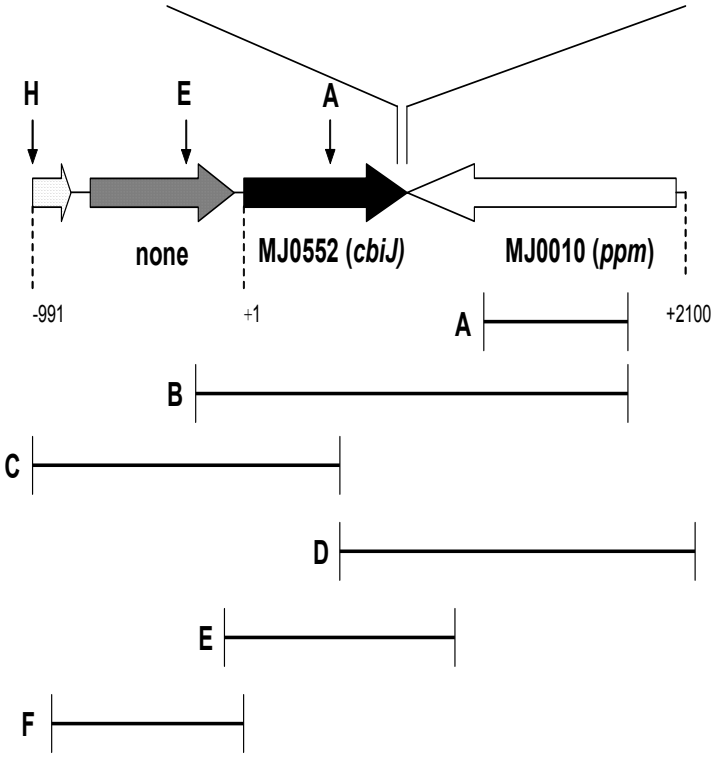

Figure 3-2.

A. The scheme for construction of the *cbiJ* deletion by gene replacement in *M. maripaludis.* A and B indicate locations of the restriction sites for *Acc*I and *Bam*HI, which were used during the Southern hybridization. Also, locations of primers p1- p4 for the PCR are indicated.

B. Identification of the gene replacement in JJ121. PCR with primers to the flanking genes and the *pac* cassette was used to demonstrate insertion of the *pac* cassette into the genome of *M. maripaludis*. A, C, E showed the PCR products of the cloned gene on pWDK121, the bands of B, D, F were produced by PCR amplification of total genomic DNA of JJ121. G represented the product from wild type JJ. A, B: PCR products amplified with p1-p3. C, D: PCR products amplified with p2-p4. E, F: PCR amplified with p2-p3. G:PCR products amplified with p1-p4

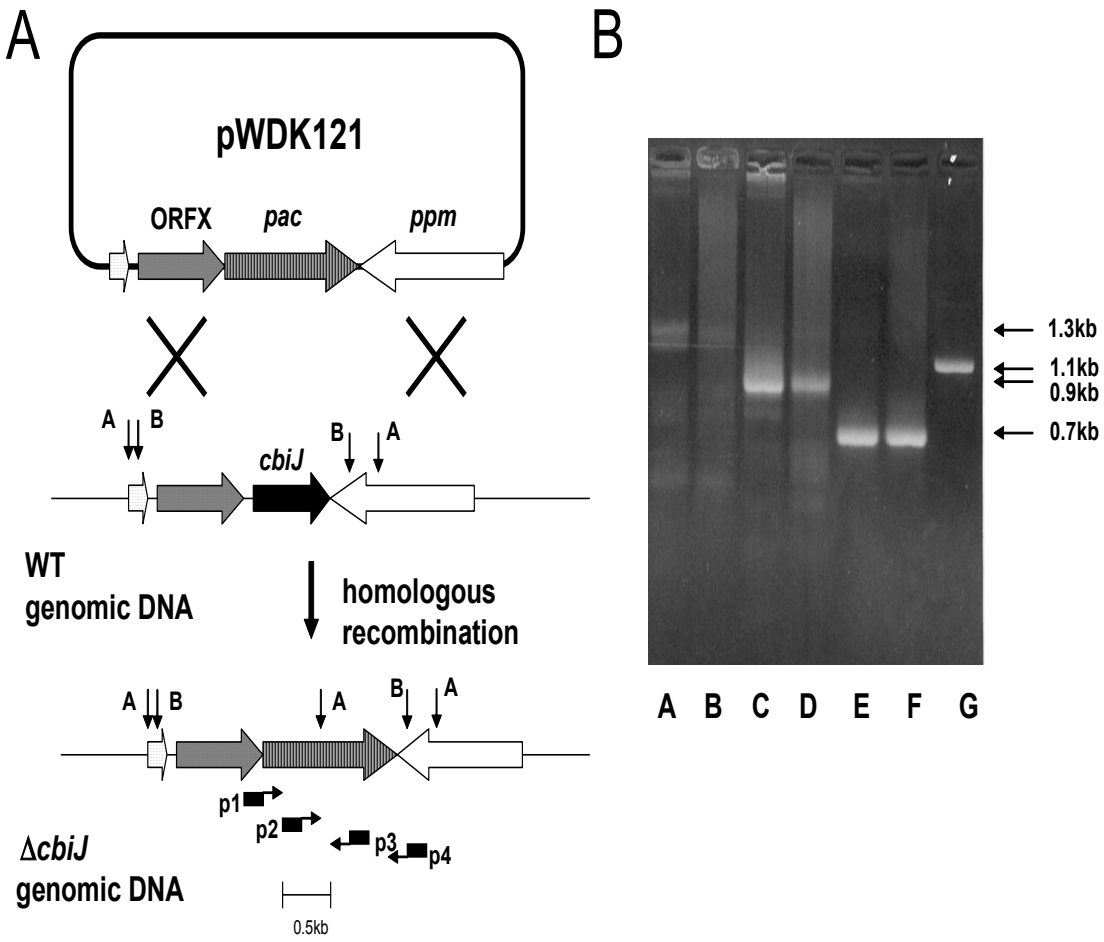
A
pWDK121
ORFX
pac
ppm
A
B
B
A
cbiJ
WT
genomic DNA
homologous
recombination
A
B
A
B
A
p1
p2
p3
p4
ΔcbiJ
genomic DNA
0.5kb
B
1.3kb
1.1kb
0.9kb
0.7kb
A B C D E F G

Figure 3-3.

Southern hybridizations of wild type and the Δ*cbiJ* mutants, JJ121 and JJ122. Genomic DNA was restricted by either *Acc*I or *Bam*HI. The probe was constructed by random priming of a linear portion of pWDK121 corresponding to positions -951-+6 (Figure 1). Controls were PCR amplificands of genomic DNA that had been digested with the restriction enzymes.

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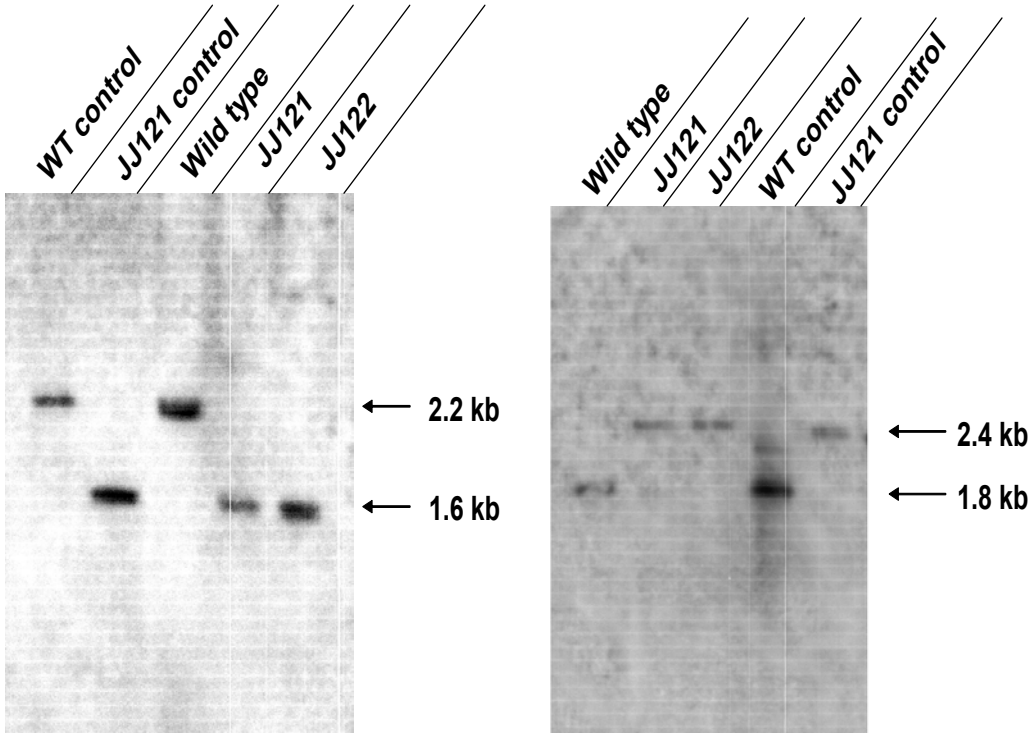

WT control
JJ121 control
Wild type
JJ121
JJ122
2.2 kb
1.6 kb
AccI digestion
Wild type
JJ121
JJ122
WT control
JJ121 control
2.4 kb
1.8 kb
BamHI digestion

Figure 3-4.

Stimulation of growth of the Δ*cbiJ* mutant JJ121 by acetate and cobalamin. Growth of wild type in McN medium (○), McN plus 10mM of acetate (Δ), and McN plus 1 μM of vitamin B_{12} (□). Growth of the mutant JJ121 in McN medium (●), McN plus 10 mM of acetate (▲), McN plus 1 μM of vitamin B_{12} (■). The inoculum was 4.6 X 10^5 cells that had been grown in McYAV medium.

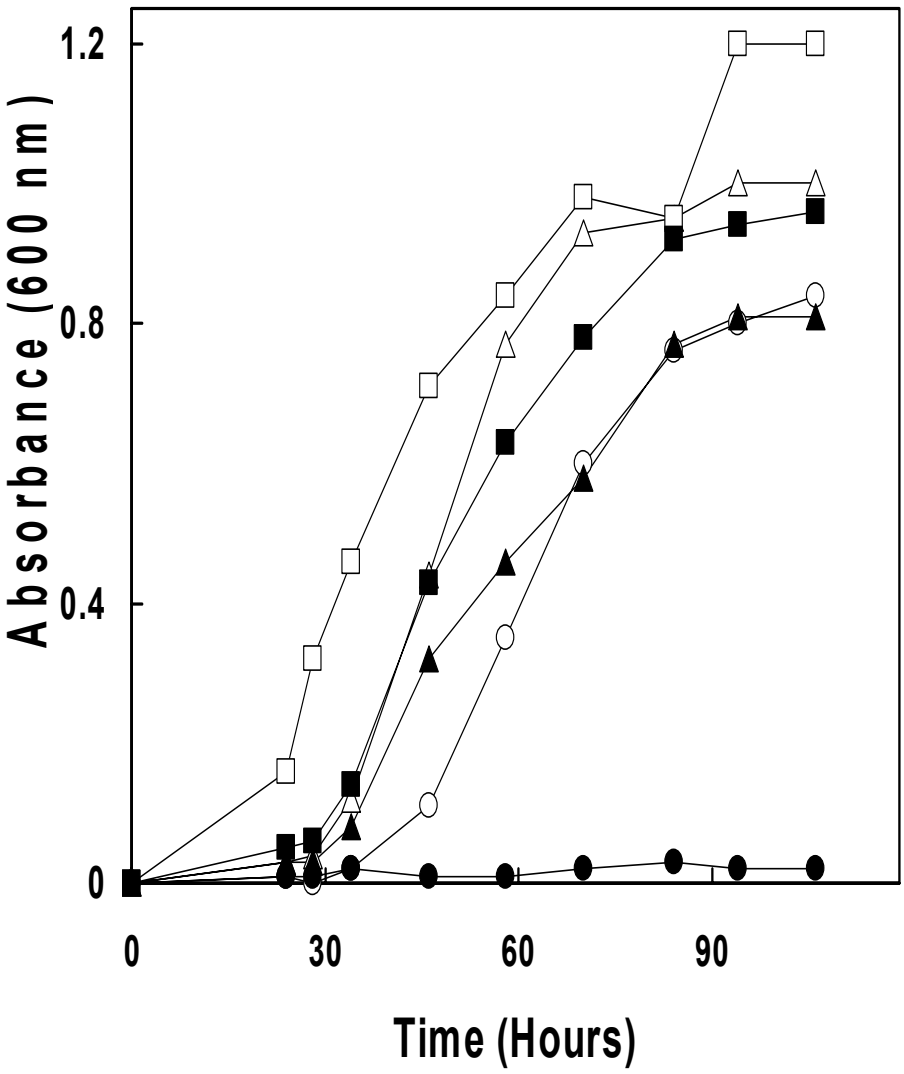

1.2
0.8
0.4
0
Absorbance (600 nm)
0
30
60
90
Time (Hours)

Figure 3-5.

Overexpression of *ppm-cbiJ* in *Methanococcus maripaludis*. The thick lines indicate the DNA fragments cloned into the vectors for overexpression. P*sla* and P*hmvA* represent promoter sequences for the S-layer gene and a histone gene in *Methanococcus voltae*, respectively. Both promoters are believed to be highly expressed in *M. maripaludis*.

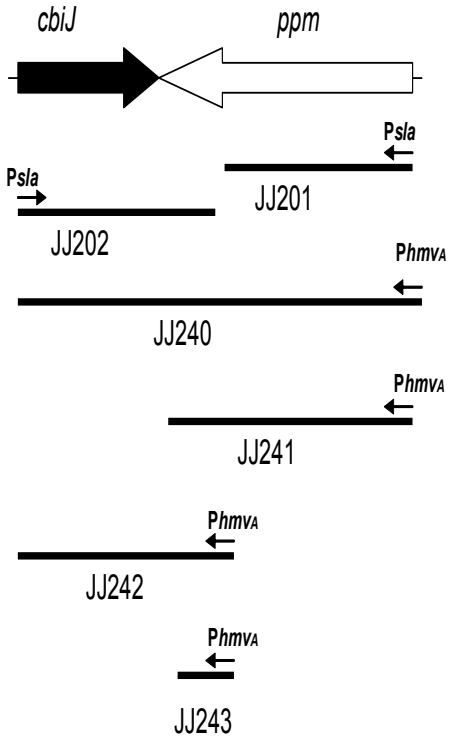
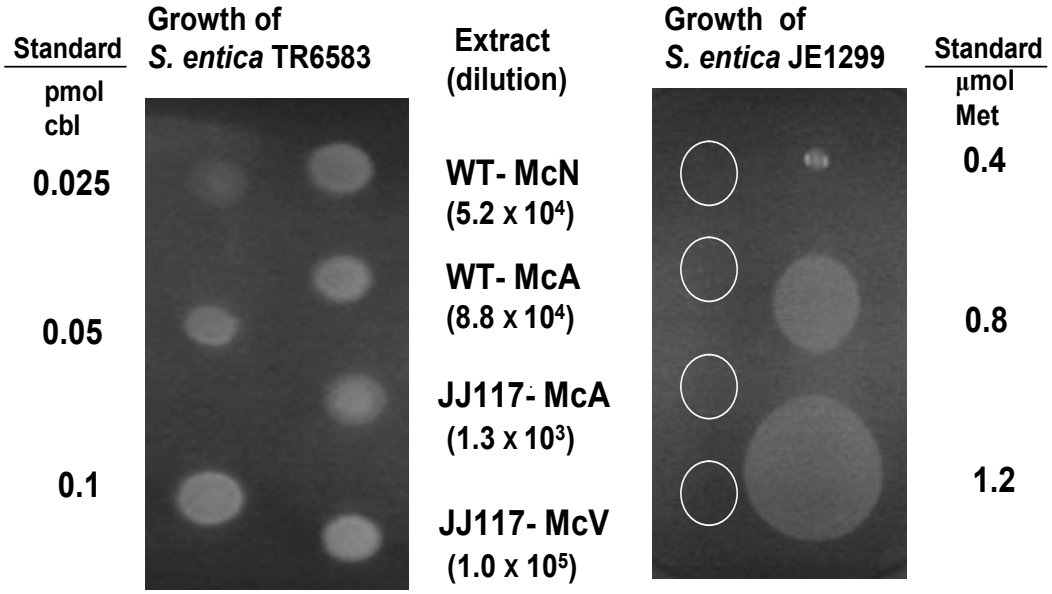

cbiJ
ppm
Psla
Psla
JJ201
JJ202
PhmvA
JJ240
PhmvA
JJ241
PhmvA
JJ242
PhmvA
JJ243

Figure 3-6.

Bioassay for cobamides in cell extracts of the wild type and JJ117.

All spots were 2.5 µl, and extracts were diluted by the indicated amounts to produce zones of confluent growth. *S. enterica* serovar Typhimurium TR6583 is dependent upon either cobamide or methionine. *S. enterica* serovar Typhimurium JE1299 requires methionine for growth and does not respond to cobamides. For this control, undiluted methanococcal extracts were used, and the locations of the spots were indicated by the circles.


Standard
pmol
cbl
0.025
0.05
0.1
Growth of
S. entica TR6583
Extract
(dilution)
WT- McN
(5.2 x 10^4)
WT- McA
(8.8 x 10^4)
JJ117- McA
(1.3 x 10^3)
JJ117- McV
(1.0 x 10^5)
Growth of
S. entica JE1299
Standard
µmol
Met
0.4
0.8
1.2

CHAPTER IV

INVESTIGATION OF ACETATE AUXOTROPHS OF *METHANOCOCCUS MARIPALUDIS*

INTRODUCTION

Methanococcus maripaludis is an autotrophic methanogen that produces methane from $H_2 + CO_2$ to obtain energy for growth. In this organism, acetyl-CoA serves as a precursor of cellular carbon and is a major intermediate in autotrophic CO_2 fixation. The biosynthesis of acetyl-CoA is carried out by the modified Ljungdahl-Wood pathway in which the acetyl-CoA decarbonylase/synthase complex (ACDS) is a key enzyme (Whitman, 1994). Alternatively, the acetyl-CoA is synthesized from free acetate by acetyl-CoA synthetase (Shieh and Whitman, 1987). Thus, exogenous acetate can compensate for mutations in the pathway of autotrophic acetyl-CoA biosynthesis, which allows for genetic approaches to the study of CO_2 fixation.

Previous research isolated and characterized acetate auxotrophs (Kim and Whitman, 1999; Yu, 1997). In these studies, acetate auxotrophs of *M. maripaludis* were obtained by random insertional mutagenesis. JJ21 was one of the acetate auxotrophs isolated following transformation of the wild type with an insertion vector (Yu, 1997). However, the genotype of JJ21 was not obvious due to the chimeric structures of genes cloned on the integration vector. The clone was composed of parts of the genes *act* (encoding the pyruvate formate-lyase activating enzyme), *ehbH* (encoding a subunit of the energy conserving hydrogenase), a hypothetical gene, and a homolog of a drug-resistant gene. *ehbH* was predicted to be a gene responsible for the auxotrophy. To test the hypothesis, this gene segment was cloned into new integration vector, and transformed into wild-type *M. maripaludis* to generate a mutant.

The Ehb hydrogenase is a membrane bound [Ni-Fe] hydrogenase that has recently been identified in Archaea. In *Methanothermobacter* and *Methanococcus*, two forms are

found, Eha and Ehb. Each form is encoded by 15-20 genes, many of which encode membrane proteins with homology to components of the NADH: quinone oxidoreductase (Künkel *et al*., 1998, Sapra *et al*., 2000 and Meuer *et al*., 1999). For these reasons, the enzyme is believed to couple H_2 oxidation or H^+ reduction to the proton motive force. In *Methanothermobacter thermautotrophicus*, the genes of the operons were analyzed, and the transcription levels of the operons were investigated. This study demonstrated that the transcription level of *ehb* is higher than that of *eha* regardless of the H_2 availability, and H_2 limitation increased the levels of both Eha and Ehb (Meuer *et al.*, 1999). The Eha hydrogenase has also been purified from *Methanosarcina barkeri*, which contains only one form (Meuer *et al*., 1999), and was proposed to be involved in coupling proton motive force to energy conservation during the cleavage of acetyl-CoA (Meuer *et al*., 1999). Later, it was suggested that the coupling system is also required to overcome the endergonic barriers for biosynthesis of pyruvate and methane from $H_2 + CO_2$, which are catalyzed by pyruvate oxidoreductase (POR) and formylmethanofuran dehydrogenase (CHO-MF), respectively (Meuer *et al*., 2002).

METHODS AND MATERIALS

Strains, media and growth conditions. Strains and plasmids used in this research are listed in Table 1 and Table 2. Methanogens were incubated under $H_2 + CO_2$ (80:20 [v:v]) gas at 37°C. Preparation of broth and agar media was described previously (Jones *et al*., 1983 and Tumbula *et al*., 1995). The media used were McN (minimal medium), McNA (McN plus 10 mM sodium acetate), McYA (McNA plus 0.2 g/L yeast extract) and McCaA (McNA plus 0.2 g/L Casamino acids). For growth experiments, glassware

was washed in 0.1 M HCl overnight, autoclaved, and rinsed with tap and deionized water. Stoppers for 28 ml aluminum seal tubes were washed with water after autoclaving in 0.2 N NaOH.

Molecular techniques. Ligations were performed as instructed by the manufacturer of the ligase (Promega, Madison, WI). The QIAprep spin miniprep kit (Qiagen, Hilden, Germany) was used for plasmid purification, and the QIAquick gel extraction kit (Qiagen) was used for purification of DNA from gels. For washing of DNA, the Wizard DNA clean-up kit (Promega) was used. PCR was performed with the Ready To Go Kit (Amersham Pharmacia Biotech, Piscataway, NJ). In PCR, 5 ng of plasmid DNA or 50 ng of genomic DNA and 0.1 μM to 0.2 μM of primers were used. Genomic DNA of methanococci was purified by the method described previously (Ausubel *et al*., 1994). Unless specified differently, PCR was performed for 30 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min and extension at 72°C for 2.5 min.

Transformation. An electroporator (Bio-Rad Lab, Melville, N.Y) or the One Shot kit (Invitrogen, Carlsbad, CA) were used for DNA transformation of *E.coli*. The electroporations were performed at 2.50 kV, 200 Ω. Methanococci were transformed by the polyethylene glycol mediated method (Tumbula *et al*., 1994). Linear plasmids for the transformation were constructed by restriction of circular plasmids with proper restriction enzymes. Transformants were plated on McYA medium containing 2.5 μl/ml of puromycin and restreaked on the same medium. The colonies were picked into McYA broth medium plus puromycin, and glycerol stocks were prepared when the cultures reaches early stationary phase (Tumbula *et al*., 1994). For transformation of pWDK310, pWDK320 and pWDK40, McYA medium was necessary to obtain acetate auxotrophs.

Identification of flanking sequences. Identification of flanking genomic sequences in the transformants of pJIA10 and pJP21 was based on the technique described previously (Hildebrant and Nellen, 1991, Kim and Whitman, unpublished). To identify the flanking sequences in JJ10, the transformants of pIJA10, 1 μg of genomic DNA was digested by 30 units of *Bam*HI or *Bgl*II for 48 h. The digested DNA was ligated with T4 DNA ligase, dialyzed with filter paper (pore size 0.025 μm, Millipore) for 2 h, and electroporated into *E.coli* SURE. Plasmids were purified from *E.coli* and then digested with *Xho*I and *Xba*I to confirm the presence of the flanking sequences. The plasmids containing the upstream and downstream sequence were named pIJA10A and pIJA10B, respectively.

To identify the flanking sequences in JJ21, the transformant from pJP21, 1 μg of genomic DNA was treated with 25 units of *Cla*I and *Not*I for 8 h, and the plasmid pJP21A was isolated as described above. The restriction was performed with two enzymes due to the presence of tandem repeats of the inserts on the genome of JJ21. The presence of flanking sequences was confirmed by digestion with *Spe*I and *Cla*I. This method was successful for identification of the upstream DNA. To isolate the downstream DNA, pJP21B was constructed by ligating a 0.8 kb *Eco*47III-*Eco*RV fragment from pJP21A into the *Pvu*II site of pIJA03. This increased the number of restriction sites for subsequent steps. pJP21B was transformed into *M. maripaludis* JJ, the genomic DNA of the resulting strain JJ21B was digested by *Bam*HI and *Xho*I, and the flanking sequence was identified with the same methods described above. The presence of the flanking sequence in the plasmid, named pJP21C, was confirmed by digestion with *Hin*dIII and *Xba*I.

Construction of other plasmids. For gene replacement of portions of *act*, pWDK310 and pWDK320 were constructed. For the construction of pWDK310a, a 1.4 kb fragment from the *Nde*I- *Bam*HI digestion of pJP21B and a 0.8 kb fragment from the *Eco*RV digestion of pJP21C were ligated into the corresponding sites of pIJA03. pWDK320 was constructed by ligation of a 1.6 kb fragment from a *Nsi*I digestion of pJP21B and a 0.96 kb fragment from an *Hpa*I digestion of pJP21C into pIJA03. The *Hpa*I digested fragment was inserted into an *Eco*47III site of pIJA03. Digestion of the plasmid with *Nde*I and *Cla*I was used to confirm that the fragments possessed the same transcription orientation as the *pac* cassette.

pIJA10, an integration vector for gene disruption of *ehbH*, was constructed by inserting a 0.5 kb PCR product into *Eco*RI-*Mlu*I sites of pIJA03. The primers for the PCR were 5'-CCGAATTCCTGAAGAACCGCTATC (underline indicates *Eco*RI restriction site, positions + 5083-5103 in Fig. 4) and 5'-GTACGCGTGGTCTTGGTGGGCATC (underline indicates *Mlu*I restriction sequence, positions + 5562-5577 in Fig. 4). The PCR product was cloned into *Eco*RI-*Mlu*I sites of pZErO-2 and then into pIJA03. The PCR conditions were 30 cycles of denaturation at 94°C for 45 seconds, annealing at 49.5°C for 45 seconds and extension at 72°C for 90 seconds, followed by 10 minute incubation at 72°C. For the PCR, 2.5 units of *pfu* DNA polymerase (Strategene) were used.

pWDK40, pWDK50 and pWDK60 were constructed for gene replacements of different portions of *ehb*. For the constructions, two PCR products amplified from genomic DNA of *M. maripaludis* were inserted into pIJA03. For construction of pWDK40, the first fragment was obtained from PCR with two primers:5'-

CGCGGATCCACCTTTTCTCCATACCGTTTTGTT (underline indicates *Bam*HI restriction site, positions -199- -177 in Fig. 4) and 5'-CTAGTCTAGACCATAGCAAAGCCCAATAATAAGC (underline indicated *Xba*I restriction site, positions +994-+1017 in Fig. 4). The PCR conditions were the same as standard conditions. The gene fragment was then digested with 20 units of *Bam*HI and *Xba*I for 2 h at 37°C, gel purified, and cloned into the *Bam*HI – *Xba*I sites of pIJA03. The other 1.6 kb PCR fragment for the construction was amplified with primers :5'-CGGGGTACCAAACGAAATTGGAAGGGTATGGAC (underline indicated *Kpn*I restriction site, positions +1801-+1824 in Fig. 4) and 5'-CTAGCTAGCACAGGTTCCGCAGGTAATACATGA (underline indicated *Nhe*I restriction site, positions +3331-3354 in Fig. 4). The PCR conditions were the same as standard conditions except that the annealing temperature was 64°C. Following the digestion of the product with *Kpn*I and *Nhe*I and gel purification, the product was ligated into the *Kpn*I – *Nhe*I sites of pIJA03, completing the construction of pWDK40. The first PCR for the construction of pWDK50 was performed with primers: 5'-CGCGGATCCATCTGTAAAAATGTATGTCCTATCG (underline indicates *Bam*HI site, positions +3439-3463 in Fig. 4) and 5'-CTAGTCTAGACAGTATTCGCATTTTTCAGTATTTA (underline indicates *Xba*I site, positions +4550-4574 in Fig. 4). The PCR product was digested by *Bam*HI and *Xba*I after gel purification and inserted into the *Bam*HI and *Xba*I sites of pIJA03 to form pWDK50A. A second PCR was performed with primers: 5'-CGGGGTACCTCATGCCATTACTGAGTTTTATTAT (underline indicates *Kpn*I, positions +5878-5890 in Fig. 4) and 5'-

CTAGCTAGCAACTGATATTGTGAGGGTAAAACTTT (underline indicates *Nhe*I positions + 7138-7162 in Fig. 4). After restriction of the amplicand and pWDK50A with *Kpn*I and *Nhe*I, ligation yielded pWDK50. The primers for the first PCR for the construction of pWDK60 were: 5'-CGCGGATCCCCAAACGATGCAATAGATAAGGGCG (underline indicates *Bam*HI site, positions +4523-4546 in Fig. 2) and 5'-CTAGTCTAGAAGGTCTTGGAGGGCATCCTGGA (underline indicates *Xba*I site, positions +5557-5578 in Fig. 4). The PCR was performed with the conditions of standard PCR expect the annealing temperature was 59°C. The resulting PCR product was gel purified, digested by 15 units of *Bam*HI and *Xba*I. The digested product was ligated into the *Bam*HI and *Xba*I sites of pIJA03, constructing pWDK60A. Another fragment was PCR amplified with two primers: 5'-CGGGTACCGGTTTATCCTGTTTCATTATTTGCA (underline indicates *Kpn*I site, positions +6566-6590 in Fig. 4) and 5'-CTAGCTAGCGAGAATTAATTAGCGCCTACCATGT (underline indicates *Nhe*I site, positions + 7715-7738 in Fig. 4). The fragment was digested with 15 units of *Kpn*I and *Nhe*I, and inserted into the *Kpn*I and *Nhe*I sites of pWDK60A, constructing pWDK60.

pIJA175 and pIJA176 are integration vectors for disruption of *cdhA* and *cdhBC* respectively. To construct pIJA175, PCR was performed with primers: 5'-CGAAGATCTCAGAATGCGGTTGGTG (underline indicates *Bgl*II site), and 5'-GGTCTAGAGCATCAAAAATTCCTTCA (underline indicates *Xba*I site). PCR conditions were 30 cycles of 94°C for 45 s, annealing at 51°C for 45 s and at 72°C for 90 s. A 0.49 kb PCR product from the primers was cloned into *Bgl*II and *Xba*I sites of

pZero-2 and then pIJA03. pIJA176 was constructed by inserting a PCR product into *Bam*HI and *Mlu*I sites of pIJA03. The primers for the PCR were: 5'-GCGGATCCTTTATCGGGGTTACTTACT (underline indicates *Bam*HI site), 5'-CGACGCGTGCATAAATCCCTGAA (underline indicates *Mlu*I site). The PCR was performed by 30 cycles of 94°C for 45 s, annealing at 49.5°C for 45 s and at 72°C for 90 s. A PCR amplicand was inserted into *Bam*HI- *Mlu*I site of pZero-2 and then pIJA03 sequentially.

PCR confirmation of gene replacement. PCR was performed to verify the genotypes of JJ310 and JJ320. The PCR was performed with the standard conditions except that the annealing temperature was 55°C and the extension time was 3 min. For JJ310, the primers were P1: 5'-GGAGGCACGTTATGTCTGAA (positions + 4743-4762 in Fig. 1) and P2: 5'-TACATCGCAGAGGTTTCCAC (positions +6004-6023 in Fig. 1). The primers for JJ320 were P3: 5'-TGAAACAATGCCTATGGTCC (positions + 5547-5566 in Fig. 1) and P4: 5'-CATCCGAACGGCTTTATTGT (positions + 6932-6950 in Fig. 1).

Southern hybridization. Genomic DNA, 1 μg, was treated with 20 units of *Eco*RV and *Bgl*II for 16 h at 37°C. The restricted DNA was transferred to a positively charged nylon membrane (Boehringer Mannheim) after separation on a 1.0 % agarose gel. The probe for the hybridization was constructed by PCR with genomic DNA of *M.maripaludis* S2 as the template. The primers used were 5'-GCAGTAGTTATGGCAGATGACC (positions + 724-746 in Fig. 4 and 5'-GAGTGTCAAAACCTCTCCAATCGAA (positions +2320-2345 in Fig. 4. In the reaction, 25 μCi of [α-P^{32}] dATP (ICN, Aurora, OH) was used. The PCR conditions were 25 cycles of dwelling at 94°C for 4 min, denaturation at 94°C for 1 min, annealing

at 58°C for 1 min and amplification at 72°C for 2 min. Pre-hybridization was performed at 65°C overnight in solution of 2 x SSC, 0.01 % (w/v) SDS, 0.1 % (w/v) N-lauroylsarcosine and 1 % blocking reagent (nonfat milk). For the hybridization, approximately 4 X 10^7 dpm of probe was added into the solution. Washing of the membrane was done in solution of 2 X SSC plus 0.1% SDS for 5 min and 0.2 X SSC plus 0.1 % SDS for 15 min. For subsequent analysis with the ImageQuant 1.1 software program, the membrane was exposed in a Phosphoimager for 3 h.

Methanogenesis. Methane production from 100 mM pyruvate was described previously (Yang *et al*., 1992). In this experiment, 5 ml of mid-exponential phase cultures (A_{600}=0.45-0.75) were grown in McCA. After washing the cultures with the buffer and resuspending to the same cell density, 1 ml aliquots of the cultures were distributed into 2.6 ml sealed anaerobic vials. Pyruvate, 100 mM, was added into some vials to investigate the effect of pyruvate on methanogenesis. The cells in the vials were incubated overnight at 37°C in an anaerobic chamber (Coy laboratories, Ann Arbor, MI). For positive controls, 0.1 ml of the resting cells was incubated for 1 h under 70 kPa of H_2 + CO_2. Methane was measured with a Shimazu GC-8A gas chromatography by flame ionization detection on a DB-624 column run at 60°C (J & W Scientific, Folsom, CA). N_2 was used as the carrier gas, and the injector temperature was 200 °C.

Extraction of membrane fractions. Membranes from *M. maripaludis* were prepared as described by Meuer *et al*. (2002) using anaerobic methods. For this procedure, 200 ml of cultures from mid-exponential phase (A_{600} = 0.4-0.6) in McCaA were harvested by centrifugation at 5,000 X g for 20 min at 4°C. S40 cultures were inoculated at least 12 h earlier than the S2 to obtain similar cell densities at the same

time. The cell pellets were frozen at -20°C for 1 h, which lysed the cells completely. Upon thawing, the lysed cells were resuspended with 11 ml of MOPS/DTT buffer (50 mM 3-(N-Morpholino) propanesulfonic acid, 2 mM dithiothreitol, pH 7.0, prepared under N_2 gas). For determination of protein and hydrogenase activity, 1 ml of the resuspended culture was saved, and 10 ml of the cultures were centrifuged at 10,000 X g for 30 min. After the centrifugation, pellets were resuspended with 2 ml of MOPS/DTT buffer, and 1 ml of the supernatant was saved for subsequent assays. The remaining 9 ml of supernatant was centrifugated at 150,000 X g and 4°C for 2 h with rotor type 70TI / 70.1T1 (Beckman, Fullerton, CA). After the centrifugation, the pellets were resuspended with 3 ml of the MOPS/DTT buffer. Concentrations of total protein were determined by using the BCA protein assay kit (Pierce, Rockford, IL). Prior to protein determination, all of the protein samples were denatured by heating at 90°C for 30 min in 0.1 N NaOH.

Enzyme assay. Hydrogenase activities were determined as described previously (Ladapo and Whitman, 1990). The assay contained MOPS/DTT buffer (50 mM MOPS, 2 mM DTT, pH 7.0) and 2 mM of methylviologen in cuvettes prepared by flushing with 100 % N_2. A small amount of sodium dithionite was added to the cuvettes before initiating the assay. Enzyme activities were measured at 37°C in a spectrophotometer (Beckman DU640D, Fullerton, CA) after adding 1 ml of H_2 (100 %) gas to the cuvettes. The hydrogenase activities of each sample were measured in duplicates within several hours following extraction of the fractions. 1 U of activity was defined as the reduction of 1 μmol of methylviologen per min. In total, five replicate experiments were performed for the determination of activities.

RESULTS

Identification of flanking sequences of a homolog of *act*. Previously, an acetate auxotroph of *M. maripaludis* JJ was isolated following transformation with pJP21, a derivative of pBK-CMV containing cloned *M. maripaludis* DNA. With the vector, selection was for the neomycin resistance marker controlled by the SV40 promoter, which is apparently expressed in methanococci. However, the cloned DNA of pJP21 was chimeric and contained four gene fragments from different parts of the genome that were ligated together during its construction (Yu, 1997). Thus, the location of the mutation in the auxotroph was not known (Yu, 1997). To determine the site of insertion in JJ21, approximately 5.0 kb of the DNA flanking the integrated plasmid was cloned and sequenced. Because the truncated *act* region, which encoded a homolog of pyruvate-formate lyase activating enzyme (PFL-AE), of the plasmid was continuous with the flanking genomic sequence, the *act* gene was the site of insertion in JJ21. The *act* homolog (RMM01257) was located in a cluster of genes that included two methyltransferase homologs and six homologs of genes for iron transport (Fig. 1). In addition to this homolog, two more *act* homologs (RMM01023, RMM01059) were identified from the genomic sequence of *M. maripaludis* S2 (J. Leigh, personal communication).

The roles of the pyruvate formate-lyase activating enzyme have not been studied in methanogens. Interestingly, a homolog of *pfl* (pyruvate-formate lyase), the usual substrate of PFL-AE, was not identified in the *M. maripaludis* genome. Bacterial pyruvate-formate lyase (PFL) catalyzes the conversion of pyruvate into acetyl-CoA and formate during the anaerobic fermentation of sugars (Knappe *et al*., 1974). PFL-AE is

involved in the post-translational activation of PFL by catalyzing the formation of a radical on a glycyl residue of the PFL using S-adenosylmethionine and dihydroflavodoxin as substrates (Knappe *et al*., 1984: Frey *et al*., 1994). The free radical activation of PFL was proposed to be essential during the catalytic cycle of the enzyme (Plaga *et al*., 1988). In *Clostridium*, *E. coli* and *Haemophilus influenza*, *pfl* and *act* are adjacent on the genome, although the genes are not co-regulated at the transcriptional level (Weidner and Sawers, 1996; Sauter and Sawers, 1990; Fleischmann *et al*., 1995). In *Streptococcus bovis*, the two genes are found at separate loci but their transcription is co-regulated (Asanuma and Hino, 2002). PFL-AE was shown to require iron for activity, and a $[3Fe\text{-}4S]^{+}$ cluster was identified in the enzyme (Conradt *et al*., 1984; Broderick *et al*., 2000). The presence of six homologs for genes encoding iron transport in the *M. maripaludis act* gene cluster that contains the *act* homolog suggests the functional coupling of iron uptake and *act*, similar to what has been found in *E. coli* (Knappe *et al*., 1984).

Phenotypes and genotypes of JJ310 and JJ320. The wild-type strain JJ was transformed with pWDK310 and pWDK320 to mutagenize the *act* homolog. Transformation efficiencies were higher than those of a control gene upon plating in McN, McA and McYA media (data not shown). After the transformation, the transformants were picked from McYA plates, transferred into McYA broth plus puromycin. In preliminary experiments, the growths of the original mutant JJ121 as well as that of JJ310 and JJ320 were stimulated by acetate to a greater extent than the wild type (Fig. 2). These results support the hypothesis that *act* is required for good growth under auxotrophic conditions.

The genotypes of JJ310 and JJ320 were verified by PCR (Fig. 3) with primer sequences selected from upstream and downstream of the expected deletion. In PCR amplification with P1-P2, the expected PCR product was 1,260 bp for wild type and 1,920 bp for JJ310. With primers P3-P4, the expected sizes were 1,390 bp for wild type and 1,650 bp for JJ320. Those sizes were observed after agarose gel electrophoresis of amplification products of JJ310 and JJ320 (Fig. 3).

Requirement of *ehb* for growth on acetate. Because a portion of *ehb* was encoded on pJP21, an integration vector was constructed to disrupt the *ehbH* on the genomic DNA (Fig. 4). This vector pIJA10 was expected to integrate by homologous recombination at a single site to produce a merodiploid. Because the vector would be inserted within the *ehb* gene cluster, it was expected to prevent the transcription of the 3'- end of the operon. As controls, plasmids that disrupted *cdhA* and *cdhBC*, which encode subunits of the acetyl-CoA decarbonylase/synthase, were used. Following transformation with these plasmids, no transformants were observed on minimal medium, although good frequencies were observed in medium with acetate or yeast extract (Table 3). This result confirmed the importance of *cdhA* and *cdhBC* for autotrophic acetate biosynthesis. Because no transformants directed to *ehbH* were observed in minimal medium, this gene also appeared to play a role in acetate biosynthesis. However, these mutants appeared to be very unstable because transformants isolated from McNA or McYA media did not possess the acetate auxotrophy (data not shown).

Stable mutagenesis of *ehb*. pWDK40 was transformed into *M.maripaludis* S2 to replace a 1.1 kb portion of *ehbB* with the *pac* cassette encoding puromycin resistance. The mutation was also expected to be polar and to prevent the transcription of

downstream genes (Fig. 4). To confirm the genotype, Southern hybridization was performed for two transformants S40 and S41. For S40, two major bands were observed (Fig. 5). One at 2.5 kb was expected for replacement of a 1.1 kb portion of *ehbB* with the 1.6 kb *pac* cassette. The higher molecular weight band was observed in the wild-type DNA as well and presumably represents the upstream DNA. In the Southern hybridization of S40 evidence for both the wild-type and truncated gene were found (Fig. 5). Because the transformation was performed with the circular plasmid, this mutation was probably produced by a single homologous recombination that inserted both the vector and modified *ehb* gene into the genome.

Phenotype of *M. maripaludis* S40. The auxotrophic phenotype of S40 was investigated by comparing the growth rate of the transformant with that of wild-type *M. maripaludis* S2 in McN, McA and McYA. In the growth experiment, S40 grew slowly in minimal medium (McN) and medium with just acetate (McNA). In contrast, the growth rate S40 was comparable to wild type in the presence of acetate and yeast extract (McYA) (Fig. 6).

In *Methanosarcina*, mutagenesis of the Ech hydrogenase inhibited methane production from H_2 +CO_2, presumably by blocking the formyl-methanofuran dehydrogenase reaction (Meuer *et al*., 2002). However, methanogenesis from H_2 + CO_2 by S40 was not affected by the mutation (Table 4). Therefore, *ehb* is not required for formylmethanofuran biosynthesis in methanococci. In a nonphysiological reaction, high concentrations of pyruvate oxidoreductase (POR) can also serve as an electron donor for methanogenesis in methanococci (Yang *et al*., 1992). The pyruvate oxidoreductase (POR) was proposed to be coupled to the Ehb hydrogenase through two small iron-sulfur proteins PorE and PorF

(Lin *et al*., submitted). In the Δ *ehbB* strain S40, methanogenesis from pyruvate was reduced by about 40% (Table 4). This difference was significant upon an ANOVA analysis of data from three experiments at a confidence level of 99.0 %.

Ech hydrogenases are membrane bound, so the levels of hydrogenase activity in membranes of S40 were determined to examine the mutational effects to the enzymes. In replicate experiments, hydrogenase activities in membrane from S40 were somewhat lower than membranes from wild-type cells (Table 5). The means and standard deviation of the specific activities of the membranes from the high speed centrifugation of wild-type and S40 extracts were 143 ± 25 and 123 ± 33 μmol min^{-1} mg^{-1}, respectively. However, this difference was not significant at the 95% confidence level according to ANOVA analysis.

DISCUSSION

Mutagenesis experiments with JJ310 and JJ320 were performed to investigate if PFL-AE is involved in an autotrophic CO_2 assimilation pathway in *M. maripaludis*. The phenotypes of the transformants indicated that PFL-AE might be involved in CO_2 fixation. However, the auxotrophic phenotypes of the transformants were not as severe as that of JJ21. Possibly, phenotypic reversion might have been occurred due to mixing spontaneous puromycin resistant mutants and either JJ310 or JJ320 during transformation. The presence of low levels of amplicands with the same molecular weight as the wild type during PCR verification of *act* genotypes supported this interpretation. In addition, the possibility of a leaky auxotrophic phenotype of the

mutants cannot be excluded. Currently, to clarify the genotype and phenotype of the *act* mutant more research is required.

PFL-AE homologs in *M. maripaludis* are dissimilar to those of bacteria. *Pfl* is absent from methanococcal genomes suggests that pyruvate oxidoreductase (POR) alone is responsible for the reversible conversion of acetyl-CoA to pyruvate. Thus, the methanococcal PFL-AE must catalyze other reactions than the activation of PFL. The analysis also showed similarities of the PFL-AE between methanogens and bacteria. Motifs indicative of a radical SAM superfamily (CXXXCXXC) in the bacterial PFL-AE were also identified in the *act* homologs in *M. maripaludis*. In addition, the sequence analysis of the operon containing the *act* homolog (RMM 01257) suggests that iron, which is a cofactor of PFL-AE, is also a cofactor of the PFL-AE homolog.

Two putative *ech* gene clusters, which indicate gene duplication, are found in species of *Methanococcus* and *Methanothermobacter*, although only one *ech* gene cluster is found in species of *Methanosarcina*. In some methanogens, gene duplications are also found in the gene clusters that encode F_{420}-reducing hydrogenase and F_{420}-nonreducing hydrogenase (Sorgenfrei *et al*., 1997; Vaupel and Thauer, 1998). The two homologous *ech* operons may originate from a gene duplication event during the evolution of the hydrogenotrophic methanogens *M. maripaludis* (Vignais *et al*., 2001) since the gene compositions of the operons are similar. It is possible that two Echs are functionally redundant, which could be an advantageous adaptation in a changing environment. However, it is also possible that the roles of each hydrogenase are different since some of the genes in the operons are different and relatively low amino acid sequence similarities occur between those proteins that are homologous.

The acetate auxotrophy of S40 suggests that the Ehb hydrogenase is part of an autotrophic biosynthetic pathway in *M. maripaludis*. The stimulation of growth by acetate in the Ehb mutant implies that Ehb is linked to synthesis of acetyl-CoA. The synthesis of acetyl-CoA that is catalyzed by the acetyl-CoA decarbonylase/synthase complex (ACDS) is endergonic. Therefore, the reaction requires an input of energy, probably generated by reverse electron transport. Previously, the Ech hydrogenases were proposed to couple the proton motive force to ferredoxin-mediated electron transfer during the synthesis of acetyl-CoA in methanogens (Bott and Thauer, 1987; Meuer *et al*., 1999; Meuer *et al*., 2000).

The biochemical assays indicate that the mutation in the *ehb* operon did not effect the methane production from $H_2 + CO_2$ in *M. maripaludis*. Previous reports show that mutation in the Ech hydrogenase from *M. barkeri* abolished methane production from $H_2 + CO_2$ since the endergonic synthesis of formyl methanofuran (CHO-MF) was dependent on the activities of the hydrogenase (Meuer *et al*., 2002). In case of *M. maripaludis*, it is possible that only the Eha hydrogenase is coupled to the synthesis of CHO-MF; therefore, methane production from $H_2 + CO_2$ would not be affected by a mutation in *ehb*. Another possibility is an increase in the level of *eha* transcription in the absence of Ehb, where both are active in methanogenesis from $H_2 + CO_2$.

The significant reduction in the rate of methanogenesis from pyruvate in S40 suggests that the Ehb hydrogenase couples the oxidation of H_2 to the activities of pyruvate oxidoreducatse (POR). The oxidation of H_2 by a membrane–bound hydrogenase could provide the strong reducing equivalents that are necessary for the conversion of acetyl-CoA into pyruvate and for methanogenesis. This experiment suggested that the mutation

in Ehb significantly decreased the flow of electrons from pyruvate oxidation to the methanogenesis pathway.

Several hypotheses would explain the similar hydrogenase activities of S2 and S40. First, the *eha* operon may be upregulated in response to the decrease in the Ehb activities. To confirm this model, the regulation of these operons should be investigated. Second, the activities of Ehb hydrogenase could have been inaccurately measured due to its sensitivity to oxygen exposure or instability following cell lysis (Tersteegen and Hedderich**,** 1999). Finally, the *ehb* operon might be expressed at a much lower level than *eha* operon and most of the detected activities were due to the Eha hydrogenase.

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TABLE 4-1. Plasmids used in this study

Plasmids	Characteristics	References
pIJA03	Derivative of pMEB.2, contains *pac* cassette	Stathopoulus *et al*., 2001
pIJA10	pIJA03 + 0.5 kb of *ehbH*	This work
pIJA10B	pJIA03 + 1.1 kb DNA upstream of *ehbH*	This work
pIJA10C	pIJA03 + 0.3 kb DNA downstream of *ehbH*	This work
pJP21	pBK-CMV + chimeric clone	Yu, 1997
pJP21A	pIJA03 + 0.7 kb of *pfa*	This work
pJP21B	pJP21 + 2.3 kb DNA upstream of *pfa*	This work
pJP21C	pJP21A + 1.0 kb DNA downstream of *pfa*	This work
pWDK40	pIJA03 + DNA fragments for gene replacement of *ehbB*	This work
pWDK50	pIJA03 + DNA fragments for gene replacement of *ehbG*, *ehbH*, *ehbI*	This work
pWDK60	pIJA03 + DNA fragments for gene replacement of *ehbH, ehbI*	This work
pWDK310	pIJA03 + DNA fragments for gene replacement of *act*	This work
pWDK320	pIJA03 + DNA fragments for gene replacement of *act*	This work

TABLE 4-2. *M. maripaludis* strains used in this study

Strains	Characteristics	References
JJ	Wild type	Jones *et al*., 1983
JJ10	Transformant of pIJA10	This work
JJ21	Transformant of pJP21	Yu, 1997
JJ21A	Transformant of pJP21A	This work
JJ310-313	Transformants of pWDK310	This work
JJ320-324	Transformant of pWDK320	This work
S2	Wild type	Whitman *et al*., 1986
S40	Transformant of pWDK40, gene replacement of *ebhB*	This work
S41	Transformant of pWDK40, gene disruption of *ebhB*	This work
S50	Transformant of pWDK50, gene replacement of *ehbG, ehbH* and *ehbI*	This work
S60	Transformant of pWDK60, gene replacement of *ehbH* and *ehbI*	This work

TABLE 4-3. Transformation efficiencies of selected insertion vectors in *M.maripaludis* JJ[a]

Plasmid	Gene	Gene product	Number of transformants (per µg of DNA)		
			McN	McA	McYA
pIJA175	*cdhA*	ACDS	0	131	200
pIJA176	*cdhBC*	ACDS	0	88	25
pIJA10	*ehbH*	Ehb	0	150	400

a Each plasmid contains an internal portion of the gene that was 488 – 495 bp in length..

TABLE 4-4. Methanogenesis from pyruvate by resting cells of *M.maripaludis* [a]

Strains	Methanogenesis (nmol CH_4 min^{-1} mg cell dry wt^{-1})		
	No addition	+ pyr	+H_2/CO_2
M.maripaludis S2	0.3 ± 0.1	3.0 ± 1.9	161 ± 71
M.maripaludis S40	0.4 ± 0.3	2.0 ± 0.9	164 ± 118

[a] The results shown were average of three experiments. Each value represents mean ± standard deviation. In these experiments, the variation between duplicates from the same experiment was always less than 15 %.

TABLE 4-5. Membrane-associated hydrogenase activities from *M.maripaludis* S2 and S40[a]

Fraction [b]	S2		S40	
	Units	Protein (mg)	Units	Protein (mg)
Cell extract	2400	37.6	1635	27.4
Low speed centrifugation:				
supernatant	1700	17.5	890	12.4
pellet	330	17.0	170	12.0
High speed centrifugation:				
supernatant	630	8.6	520	6.8
pellet	780	5.4	330	2.7

a Data are the averages of duplicate determinations and from one representation experiment. The specific activities from five experiments are given in the text.

b The cells were lysed by freeze-thawing to produce the extract, which was centrifugated at 10,000 X g for 30 min to produce the low speed supernatant and pellet. The supernatant was then centrifuged at 150,000 X g for 120 min to produce the high speed supernatant and pellet.

Figure 4-1.

Map of the operon containing an *act* homolog from the genomic sequence of *M.maripaludis* S2 (Leigh, personal communication). Numbering of the DNA sequence begins with the putative start codon of the first gene in the operon. A. The region of genomic DNA that was sequenced from *M.maripaludis* JJ. B. The region of genomic DNA cloned into pJP21B. C. The region of genomic DNA cloned into pJP21C. D and E. The regions of DNA which were cloned into pWDK310 and pWDK320, respectively. The dotted lines represented the DNA regions deleted by gene replacement in the transformant.

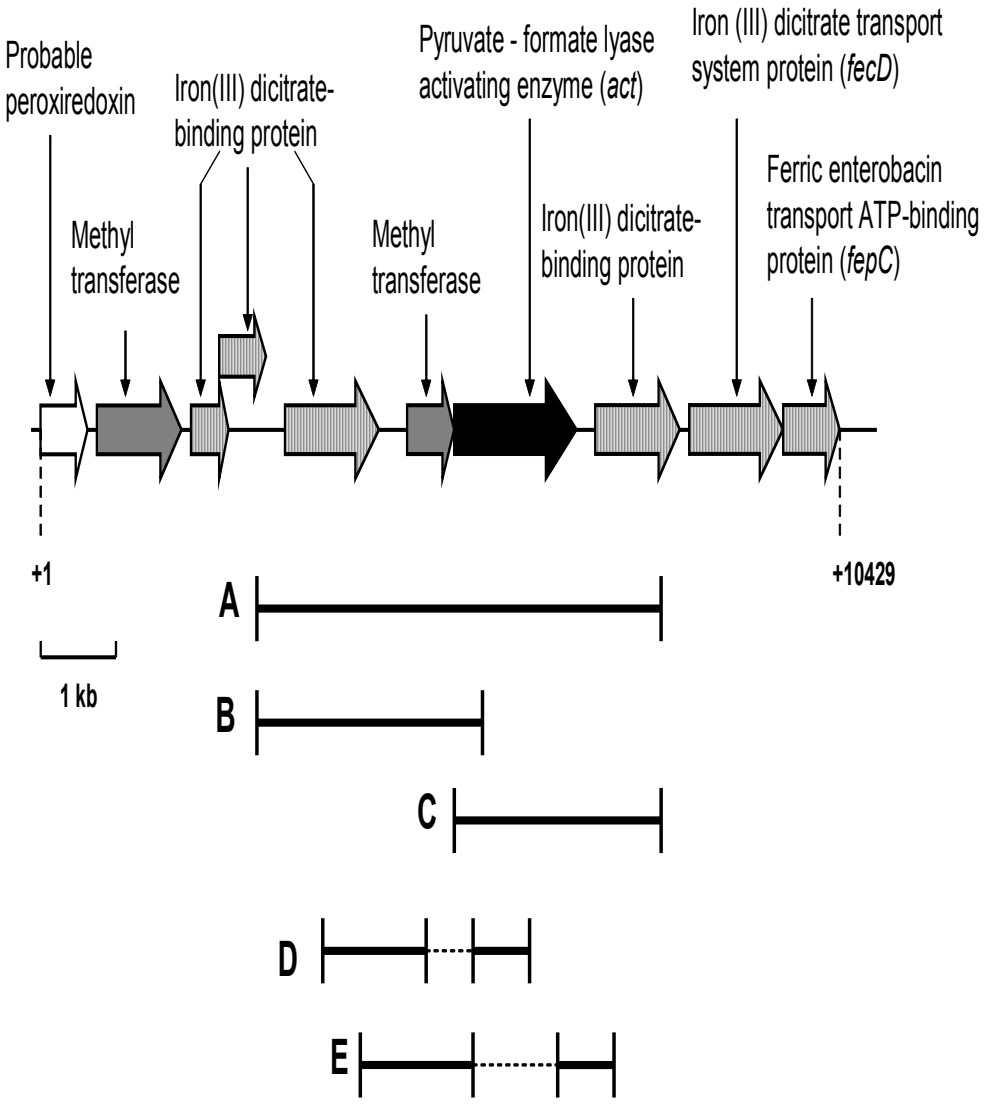

Probable peroxiredoxin
Iron(III) dicitrate-binding protein
Pyruvate - formate lyase activating enzyme (act)
Iron (III) dicitrate transport system protein (fecD)
Methyl transferase
Methyl transferase
Iron(III) dicitrate-binding protein
Ferric enterobacin transport ATP-binding protein (fepC)
+1
+10429
1 kb
A
B
C
D
E

Figure 4-2.

Growth patterns of *M. maripaludis* JJ310, JJ320 and JJ21 in McN and McA.

Growth of wild type in McN medium (○), McA plus acetate (●). Growth of JJ310 in McN (Δ), McN plus acetate (▲). Growth of JJ320 in McN (□), McN plus acetate (■). Growth of JJ21 in McN (◊), McN plus acetate (♦).

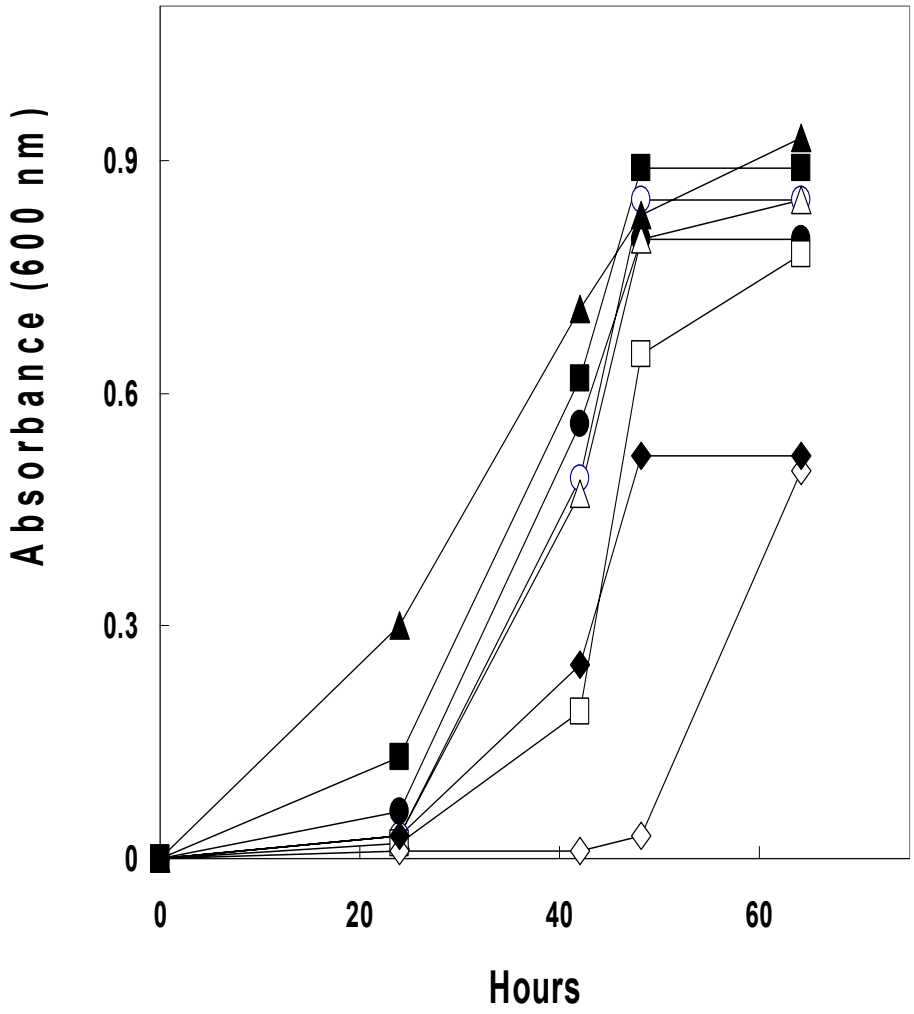

Absorbance (600 nm)
0.9
0.6
0.3
0
0
20
40
60
Hours

Figure 4-3.

Gene replacement of the *act* region by *pac* cassette and confirmation of genotypes of JJ310 and JJ320 by PCR amplification. PCR with primers to the flanking genes was used to demonstrate gene replacement on the genome of *M. maripaludis*. A: gene replacement of the *act* region by *pac* cassette. A: PCR products amplified with p1-p2. The lanes of 1, 2, 3, 4, 5, 6 show λ *Hin*d III marker, the PCR amplicand of *M. maripaludis* JJ, JJ310, JJ311, JJ312 and JJ313, respectively. B: PCR products amplified with p3-p4. The lanes of 1, 2, 3, 4, 5, 6, 7 show λ *Hin*d III marker, the PCR amplicand of *M. maripaludis* JJ, JJ320, JJ321, JJ322, JJ323 and JJ324, respectively.

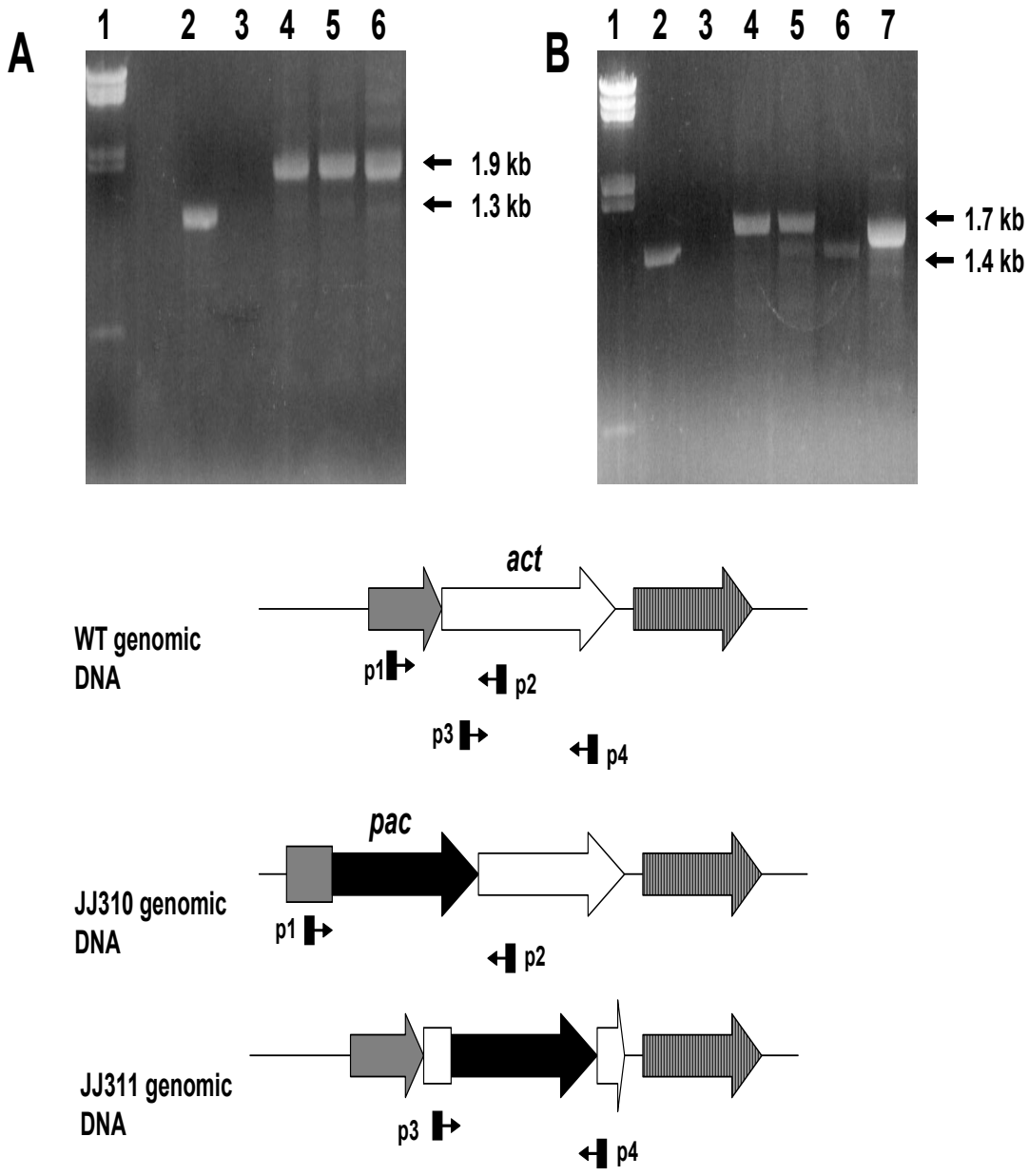

A
1 2 3 4 5 6
1.9 kb
1.3 kb
B
1 2 3 4 5 6 7
1.7 kb
1.4 kb
act
WT genomic DNA
p1
p2
p3
p4
pac
JJ310 genomic DNA
p1
p2
JJ311 genomic DNA
p3
p4

Figure 4-4.

Map of *ehb* operon of *M. maripaludis*. Numbering of the DNA sequence begins with the putative start codon of *ehbA*. B and E represent the sites of *Bgl*II and *Eco*RV used during the Southern hybridization. A. The region of genomic DNA of *M. maripaludis* JJ cloned into pIJA10 B. The region of genomic DNA of *M. maripaludis* JJ cloned in pIJA10B C. The region of genomic DNA of *M. maripaludis* JJ cloned in pIJA10C. D. The thick lines indicate the region of genomic DNA of *M. maripaludis* S2 cloned in pWDK40. The dotted line indicates the DNA deleted during the gene replacement in S40. E. The region used as probe for the Southern blot of *M. maripaludis* S2 and S40. F., G. The thick lines indicate the regions of cloned DNA in pWDK50 and pWDK60 respectively. The dotted lines show the deleted region by the replacement in S50 and S60 respectively.

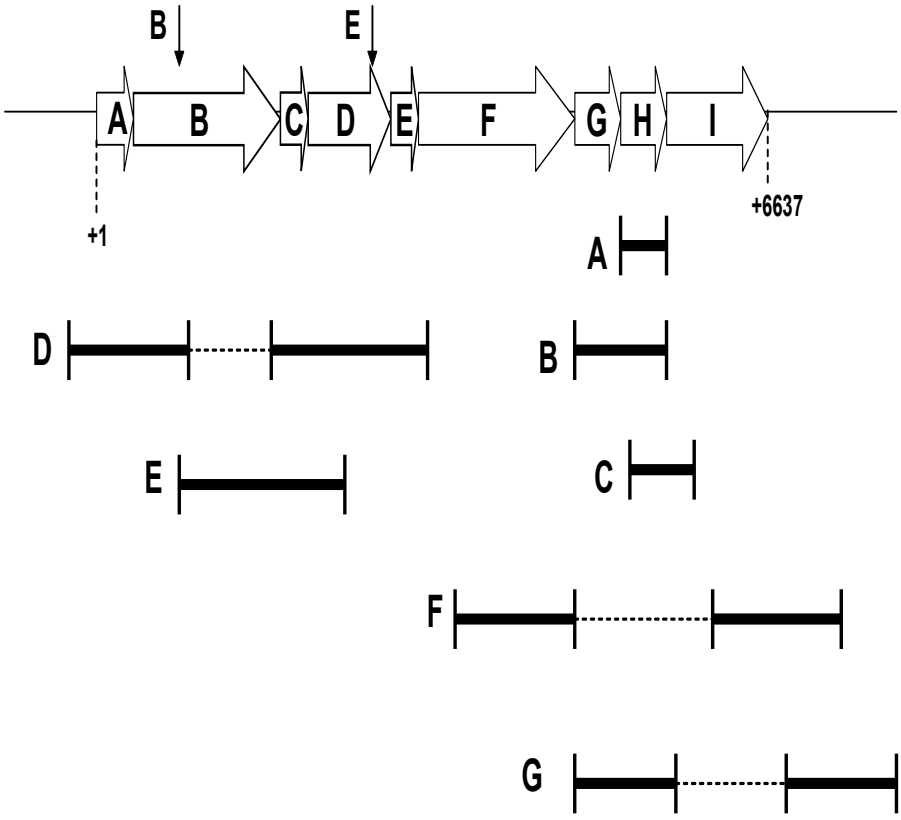

B
E
A
B
C
D
E
F
G
H
I
+1
+6637
A
D
B
E
C
F
G

Figure 4-5. Southern hybridization of genomic DNA of *M. maripaludis* S2, S40 and S41. The genomic DNA was digested with *Bgl*II and *Eco*RV.

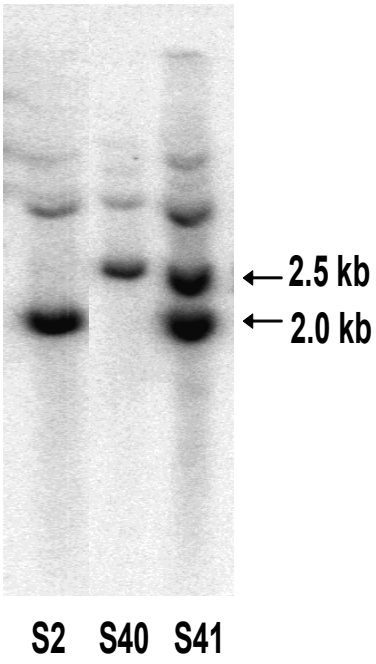
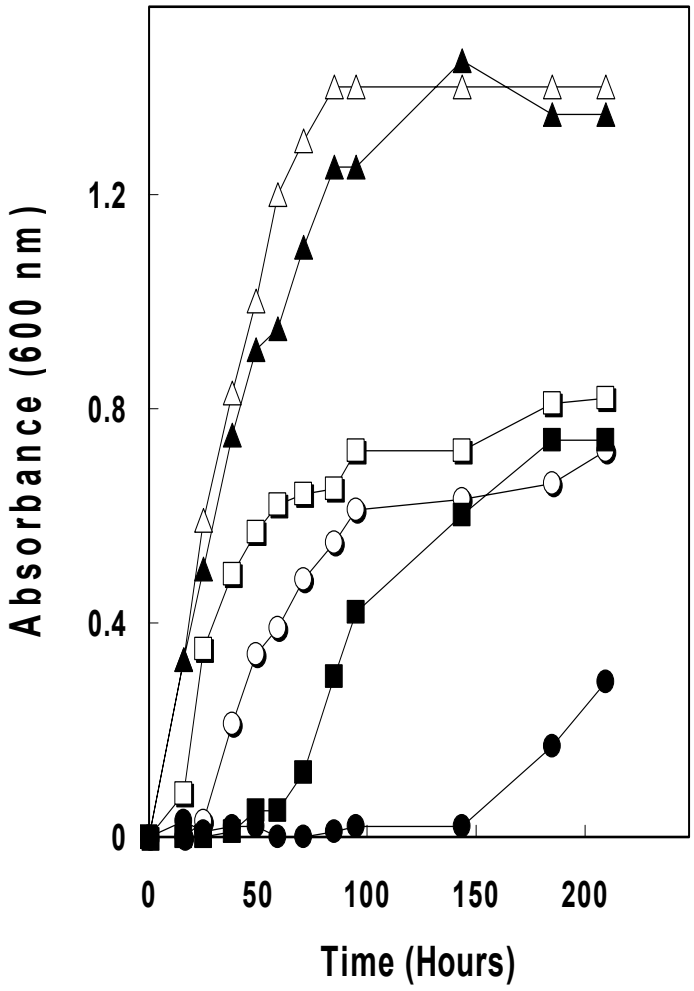

2.5 kb
2.0 kb
S2
S40
S41

Figure 4-6.

Stimulation of growth of *M. maripaludis* S40 by acetate and yeast extract. Growth of wild type in McN medium(○), McN plus acetate(□), and McN plus acetate and yeast extract(Δ). Growth of *M.maripaludis* S40 in McN medium (●), McN plus acetate(■) and McN plus acetate and yeast extract(▲).


Absorbance (600 nm)
1.2
0.8
0.4
0
0
50
100
150
200
Time (Hours)

CHAPTER V

CONCLUSION

CONCLUSION

CO_2 fixation in autotrophic methanogens is complicated by association with numerous pathways. In anabolic metabolism, autotrophic synthesis of acetyl-CoA is linked to methanogenesis and the activity of Ehb hydrogenase. The formation of both acetyl-CoA and methane require cobamide as a cofactor. Studies of JJ117 and JJ121, which are autotrophic for both cobamide and acetate, confirm the crucial role of cobamide in autotrophic CO_2 fixation. These studies suggest that the inhibition of expression of *cbiJ* by tandem repeats of the adjacent *ppm* caused the auxotrophy. Currently, more studies are needed to confirm the inhibition.

Quantification of the total cobamides in wild-type and the mutant cells confirm that JJ117 could grow in the presence of acetate with very low levels of intracellular cobamides. Thus, acetate spared the requirement of cobamide in JJ117 by serving as a source of acetyl-CoA that could be produced in the absence of cobamide. Synthesis of small amount of cobamide in the mutant indicated that alternative biosynthetic pathways can compensate for mutation in *cbiJ*.

Pyruvate-formate lyase activating enzyme (Act) is also possibly involved in autotrophic CO_2 fixation in *M. maripaludis*. Although *act* was identified as a gene required for good growth of JJ21 in medium without acetate, the substrate of the enzyme was not identified in the genome of *M. maripaludis*. The activity of the enzyme appears to require iron, which is also the case in *E. coli*. The role of Act in *M. maripaludis* is expected to be somewhat different from previously studied enzymes.

The acetate auxotrophic phenotype of S40 suggests that Ehb is involved in anabolic pathways in *M. maripaludis*. The auxotrophy of S40 indicates the Ehb provides

exogenous energy for the endergonic synthesis of acetyl-CoA catalyzed by acetyl-CoA decarbonylase/synthase complex (ACDS). The Ehb probably is also coupled to activity of pyruvate oxidoreductase (POR), which was indicated by partial inhibition of methane production from pyruvate in S40. However, reduced activity of Ehb from the mutation was not confirmed, possibly due to lability of the enzyme or background from Eha. While these studies indicate that Ehb and Eha are not functionally equivalent, further studies will be needed to clarify the extent of overlap between these two enzyme complexes.