INVESTIGATION OF THE ANABOLIC PYRUVATE OXIDOREDUCTASE AND THE FUNCTION OF POREF IN *METHANOCOCCUS MARIPALUDIS*

by

WINSTON CHI-KUANG LIN

(under the direction of WILLIAM B. WHITMAN)

ABSTRACT

The pyruvate oxidoreductase (POR) catalyzes the reversible oxidation of pyruvate. For hydrogenotrophic methanogens, the anabolic POR catalyses the energetically unfavorable reductive carboxylation of acetyl-CoA to form pyruvate, a precursor of organic carbon synthesized in methanogens.

Previously, the methanoccocal POR was purified to homogeneity. It contained five polypeptides, four of which were found to be similar to the four subunit (□, □, □, and □) POR. For further characterization, the *por* gene cluster was isolated and sequenced. Six open reading frames were found, *porABCDEF*. The gene *porE* encoded the fifth subunit that copurified with the POR. Also identified was *porF,* which was similar to *porE*. Both *porE* and *porF* contained high cysteinyl residue content and two Fe/S binding motifs, suggesting that *porE* and *porF* may serve as electron carriers for the methanococcal POR.

To further elucidate the role of PorE and PorF, a deletional mutant strain JJ150 was constructed which lacked *porEF*. Growth of the mutant in minimal McN medium was slower than the growth of the wild type strain JJ1. POR, hydrogenase, and carbon monoxide dehydrogenase activities in JJ150 were similar to the wild type. In contrast, pyruvate-dependent methanogenesis was inhibited in JJ150. Complementation of the mutant with *porEF* restored growth and pyruvate-dependent methanogenesis to wild type levels. Partial complementation of *porE* or *porF* yielded different results. Partial complementation with *porE* restored methanogenesis but not growth. Complementation with *porF* did not restore methanogenesis but did partially restore growth. These results suggested that *porEF* was important for the anabolic POR. Separately, *porE* and *porF* appear to play different roles.

Deletional mutagenesis of the entire *por* gene cluster and homologous recombination into a specific region in the *por* gene cluster, were performed to determine whether the POR was essential to *Methanococcus.* Further investigation will be needed to conclusively determine essentiality, but results suggested that the POR was essential for *Methanococcus*. The inactivation of *porC* was found to have a greater impact on the growth than the inactivation of *porE*. To further investigate the function of PorC, *porC* was deleted to form deletional mutant JJ156. However from these results, the function of porC was inconclusive.

INDEX WORDS: *Methanococcus marpaludis*, Pyruvate oxidoreductase, PorE, PorF Methanogenesis, Carbon monoxide dehydrogenase, Ferredoxin.

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DEDICATION

To my wife, Winlynn for her love, inspiration and patience.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. METHANOGENS

Methanogens are a major physiological group of Archaea. From rRNA trees it has been determined that Archaea are phylogenetically distinct from Bacteria and Eukarya. There are at least two kingdoms within the archaeal domain, the Crenarchaeota (hyperthermophiles and thermoacidophiles like *Thermoproteus* and *Sulfolobus*) and the Euryarchaeota (hyperthermophiles such as *Pyrococcus*, methanogens, and halophiles; Woese et al.,1990). Some hallmarks of Archaea include: membrane lipids contain isoprenoid chains and are ether-linked to glycerol while Bacteria and Eucarya possess straight fatty acyl chains with ester links, unusual modifications of tRNA, and a very distinctive range of antibiotic sensitivities. About 20 to 25 bp upstream of the transcriptional initiation site, many archaeal promoter regions have a conserved TATA-like box binding sites (Box A) that are absent in bacterial cells but reminiscent of eukaryotic promoter regions to which the eukaryal RNA polymerase binds (Brown et al., 1989; Reiter et al., 1990; Hain et al., 1992; Creti et al., 1993). For example, the consensus sequence for the methanogen Box A site is AAANNTTTATATA (Brown et al., 1989). However, despite the similarity of the transcriptional process to eukaryotes, the archaeal genes are organized into bacteria-like operons (Creti, et al., 1993; Langer and Zillig, 1993; Brown and Doolittle, 1997).

Methanogens are organisms of catabolic specialization that obtain energy for growth through methanogenesis. Methanogens are represented by five orders within Archaea, including, *Methanobacteriales, Methanomicrobiales, Methanosarcinales*, *Methanopyrales*, and *Methanococcales*. For a review, see Boone et al. (1993). Although methanogens exhibit a diverse phylogeny, all seem to be able to utilize only simple

compounds, predominantly one-carbon compounds as substrates for methanogenesis. Major substrates utilized during methanogenesis include H_2/CO_2, formate, methyl alcohol, methylamines, methyl sulfide, and acetate. Additionally, secondary alcohols such as 2-propanol, 2-butanol, and to a limited extent, 2-pentanol are partially oxidized by some species to reduce CO_2 to CH_4.

Three main pathways exist for the production of methane. The first pathway is the CO_2 reducing or hydrogenotrophic pathway where CO_2 undergoes four, two electron reductions to form methane (Boone et al., 1993; Rouviere and Wolfe, 1987). H_2 or formate serves as the electron source. A second pathway is the methylotrophic pathway where the methyl group of a C-1 compound is transferred to a methyl carrier (HS-CoM) and reduced to CH_4 (Boone et al.,1993). Methyl groups also serve as electron donors upon oxidation to CO_2. However, H_2 may also be an electron donor (Boone et al, 1993). The final pathway is the aceticlastic pathway where acetate is split to form CO_2 and CH_4 (Boone et al., 1993). As evident by these pathways, methanogens are not able to utilize complex organic substrates with the exception of acetate and several secondary alcohols (Boone et al., 1993; Widdel, 1986).

The genus *Methanococcus* is within the order of *Methanoccales*. Its members have an irregular coccoid shape with a regularly-structured cell wall comprised of protein. (Koval and Jarrell, 1987). All methanogens have been found in marine environments, and they lyse easily in distilled water or in diluted detergents. Most methanococci are motile via flagella. Methanococci typically have low mol. % G+C for genomic DNA in the range of 29-34%. Metabolically, most *Methanococcus* are only able to utilize H_2/CO_2 or formate as energy sources thus making it one of the most limited

methanogens in terms of substrate range. Within *Methanococcus*, the autotrophs utilize a modified Ljundahl-Wood pathway for the CO_2 fixation while the heterotrophs can also assimilate acetate (Whitman, 1985).

2. AUTOTROPHY AND C-1 METABOLISM.

Autotrophy is the process by which carbon dioxide is utilized as the sole or major source of carbon for the formation of organic material for growth. The pathways by which autotrophy can occur are categorized as autotrophic CO_2 fixation. Energy required for autotrophy is obtained through either absorption of light or by oxidation of inorganic compounds. Autotrophs that utilize light or inorganic compounds are called photoautotrophs or chemoautotrophs, respectively. Autotrophic carbon dioxide fixation may occur by four different pathways, the Calvin cycle (also called the reductive pentose phosphate pathway; Calvin, 1962), the reductive tricarboxylic acid (TCA) pathway (Evans et al., 1966, the 3-hydroxypropionate pathway (Holo, 1989), and the reductive acetyl-CoA pathway (Ljungdahl and Wood, 1965),

2a. The Calvin cycle. The Calvin cycle is the most utilized carbon fixation pathway in nature (Tabita, 1995). Originally discovered in green plants, photolithotrophs such as cyanobacteria and facultative autotrophs (such as purple sulfur and purple nonsulfur bacteria) also utilize the Calvin cycle to fix CO_2 into 3-phosphoglyceric acid (3-PGA; for a review see Tabita, 1995). The key enzyme in the cycle is ribulose-1, 5-bisphosphate carboxylase (RubisCO) which is the initial enzyme for CO_2 fixation. Another enzyme, phosphoribulokinase is required to convert ribulose-5-phosphate to ribulose 1,5-bisphosphate (RuBP). For every CO_2 fixed, the cycle requires 3 ATP.

With oxygenic phototrophic growth, H_2O is utilized as an electron donor while CO_2 is utilized as a carbon source. However, with anaerobic phototrophic metabolism, other substrates may be utilized instead of CO_2. One substrate is formate where the formate dehydrogenase is utilized to oxidize formate to CO_2 (Tabita, 1995). From here, the Calvin cycle is utilized for the assimilation of CO_2. *Rhodospirillum rubrum* can also oxidize CO to CO_2 via a membrane-bound carbon monoxide dehydrogenase (CODH/ACS; Bonam et al., 1984). The CO_2 would then be assimilated via the Calvin cycle.

2b. The reductive tricarboxylic acid pathway. First proposed by Evans et al. (1966) and primarily found in the green bacterium *Chlorobium*, the reductive tricarboxylic acid pathway is an alternative pathway to the Calvin cycle. From two CO_2, the reductive TCA cycle forms one molecule of acetyl-CoA, which can be converted to carbohydrates. Although not a widely utilized pathway, *Hydrogenobacter thermophilus*, was found to utilize the reductive TCA cycle (Yoon et al., 1997). Also two archaea, *Thermoproteus neutrophilus* (Schafer et al, 1989) and *Sulfolobus brierleyi* (Kandler and Stetter, 1981) are both thought to possess this cycle

Originally thought to be a secondary pathway to the Calvin cycle for the synthesis of precursors of cellular carbon, it was later determined to be the sole pathway of CO_2 fixation in *Chlorobium*. Evidence arguing against the Calvin cycle is as follows; Although, RuBisCo activity has been reported in *Chlorobium* (Smillie et al.,1962; Tabita et al., 1974), Buchanan et al. could not detect RuBisCo activity in cell free extracts. Additionally, Shively et al. (1986) later determined that RuBisCo gene probes constructed from *Rhodospirillum rubrum* did not hybridize with *Chlorobium* DNA. One

of the key enzymes in the Calvin cycle, phosphoribulokinase, which catalyzes the ATP-dependent phosphorylation of ribulose-5-phosphate to ribulose bisphosphate, was also not found in *Chlorobium thiosulfatophilum* (Sirovag (1975).

It was discovered that the following two ferredoxin-dependent reactions existed in these phototrophic anaerobes:

(1) acetyl-CoA +CO_2 +Fd_{red} -→ pyruvate + HS-CoA +Fd_{ox}

(2) succinyl-CoA +CO_2 +Fd_{red}→ □-oxoglutarate + HS-CoA +Fd_{ox},

They are catalyzed by pyruvate synthase and □-oxoglutarate synthase respectively. These enzymes make it possible to run the TCA cycle in reverse for biosynthetic purposes. However, there were doubts about this pathway because a key enzyme needed for the reductive TCA cycle, citrate lyase, could not be detected. Later Ivanovsky et al. (1980) discovered high levels of ATP-dependent citrate lyase.

2c. The 3-hydroxypropionate pathway. The 3-hydroxypropionate pathway (reviewed by Sirevág, 1995) was discovered in *Chloroflexus aurantiacus*, a member of the green bacteria. Initially thought to utilize the reductive tricarboxylic acid pathway like *Chlorobium*, it was discovered that the pyruvate synthase was the only enzyme present for that pathway in *C. aurantiacus* (Holo and Sirevág, 1986). Later, Holo (1989) found that under photoautotrophic conditions, *C. aurantiacus* excreted 3-hydroxypropionate and suggested that this compound may be an intermediate in a CO_2 fixation pathway. In this cycle, acetyl-CoA is converted to malonyl-CoA via a carboxylation reaction. Through a series of reductions, propionyl-CoA is formed. From another carboxylation reaction, succinyl-CoA is then formed. Malonyl-CoA is then formed from succinyl-CoA and is then cleaved by malonyl-CoA lyase to form acetyl-

CoA and the net fixation product glyoxylate (Sirevág, 1995). From here, acetyl-CoA reenters the cycle while glyoxylate has been hypothesized to be converted to 3-phosphoglyceric acid (Ivanovsky et al., 1993)

2d. The reductive acetyl-CoA pathway and acetogenic autotrophy. The non-cyclical reductive acetyl-CoA pathway (also known as the Ljundahl-Wood pathway) is utilized by acetogenic bacteria, methanogens and some sulfate- reducing bacteria. Initially discovered by Ljungdahl and Wood (Ljungdahl et al., 1966; Ljungdahl and Wood, 1965) in acetogenic bacteria, CO_2 is reduced with a series of reducing equivalents from dehydrogenated substrates to generate ATP from electron transport phosphorylation (Fuchs, 1986). Essentially, the reductive acetyl-CoA pathway in acetogens can be broken down into four steps (Fuchs, 1986; Figure 1.1A). The first step involves the formation of formate from H_2 and CO_2 by formate dehydrogenase. Formate is activated to methyl-tetrahydrofolate (CH_3-THF) by a series of reactions involving formyltetrahydrofolate synthase, methenyl-tetrahydrofolate cyclohydrolase, and methylene tetrahydrofolate reductase. In the second step, the methyl group from CH_3-THF is transferred to a key enzyme in the pathway, the carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS), by a combination of a methytransferase and a corrinoid protein. Third, CODH/ACS concurrently reduces a second CO_2 to CO and condenses the bound methyl group and CO to form a bound acetyl group. Finally, from a reaction with the acetyl group and a bound CoASH, acetyl-CoA is formed.

As mentioned above, the CODH/ACS is the key enzyme in the reductive acetyl-CoA pathway. The CODH/ACS is a bifunctional enzyme which catalyses not only the

reversible reduction of CO_2 to bound CO (Fuchs,1986) but also the catalysis of the final steps of acetyl-CoA synthesis (Ragsdale and Kumar, 1996; Lindahl, 2002).

The use of the enzymes in this pathway by anaerobic organisms is multifaceted. The pathway is utilized in; (1) energy metabolism of acetogenic bacteria for acetate synthesis; (2) energy metabolism in some methanogens for disproportionation of acetate; (3) CO_2 fixation in autotrophic methanogens and acetogens for the biosynthesis of cellular carbon, or for the assimilation of CO_2 (Fuchs, 1986). For example, acetogenic bacteria such as the clostridial species utilize this pathway for the energy metabolism from CO_2 and H_2 resulting in acetate synthesis. Sulfate-reducing bacteria utilize the pathway for acetyl-CoA oxidation to CO_2 for energy metabolism. Aceticlastic methanogens like *Methanosarcina* use a modification of the pathway where acetate is disproportionated into CO_2 and CH_4 for energy (Eikmanns and Thauer, 1984). Autotrophic methanogens such as *Methanococcus* utilize a modified pathway for autotrophic CO_2 fixation for the production of acetyl-CoA for entry into anabolic processes.

3. ONE CARBON METABOLISM IN METHANOGENS

3a. Aceticlastic methanogens. Aceticlastic methanogens includes members of the genera *Methanosarcina* and *Methanosaeta* (formerly *Methanothrix)*. Many species of *Methanosarcina* are able to grow with H_2/CO_2, methanol, methylamines and acetate as substrates. Thus, *Methanosarcina* is metabolically the most versatile methanogen (Ferry, 1993). Growth on H_2/CO_2, however, is slow and can take a period of acclimation (Sowers et al, 1984; Mukhpadhyay et al., 1991). Acetate is the preferred substrate of *Methanosarcina*. However, *Methanosaeta*, is limited to acetate as a substrate for growth.

In acetate metabolism, acetate is disproportionated into CO_2 and CH_4. A key intermediate, acetyl-CoA, is synthesized from acetyl-CoA from acetate kinase and phosphotransacetylase or acetyl-CoA synthetase (in *Methanothrix*) Acetyl-CoA is then either utilized for cell carbon or disproportionated by the CODH/ACS, which catalyzes the cleavage of acetyl-CoA. When acetyl-CoA is cleaved, a methyl group, a carbonyl group and CoA is formed. From here on, the reverse (oxidative) direction of the acetyl-CoA pathway elucidated from the acetogenic bacteria is utilized. While the carbonyl group is oxidized to CO_2, the methyl group formed is then transferred to a corrinoid protein and finally to CoM (HS-CoM) via methyl-H_4 sarcinopterin (CH_3-H_4SPT). CH_3-H_4SPT is then reductively demethylated to methane and all the cofactors are oxidized through the reduction. This form of methanogenesis from acetate is considered to be a fermentation since the electrons from the oxidation of the carbonyl group to CO_2 are used to reduce the methyl group from acetyl-CoA (Ferry,1993; Jablonski et al. 1993).

3b. Facultative autotrophic methanogens. Autotrophic methanogens such as *Methanothermobacter* (formerly known as *Methanobacterium*) and *Methanococcus* spp. assimilate cell carbon primarily by a variation of the reductive acetyl-CoA pathway (modified Ljundahl-Wood pathway) that had been established in acetogenic bacteria (Figure 1.1B). A key difference from acetogenic bacteria is that autotrophic methanogens obtain the methyl group necessary for the synthesis of acetyl-CoA from methyl-tetrahydromethanopterin (CH_3-H_4MPT). CH_3-H_4MPT is an intermediate in the methane biosynthetic pathway from H_2/CO_2. However, the carbonyl group is generated in a similar manner to the clostridial systems (Simpson and Whitman, 1993).

Much of the early work on elucidating the pathway in *Methanothermobacter* relied upon the data obtained from the acetogenic pathway (Whitman, 1994). Initial attempts to determine a pathway for autotrophy via the Calvin cycle failed when neither ribulose-1,5 –bisphosphate carboxylase nor 3-phosphoglycerate was detected in *M. thermautotrophicus* (Zeikus et al., 1977). Also, while most of the enzymes needed for the reductive tricarboxylic acid cycle have been detected, citrate lyase and isocitrate dehydrogenase were absent thus arguing against a completed cycle (Zeikus et al., 1977). In support of the modified acetyl-CoA pathway, Daniels et al. (1977) determined that *M. thermautotrophicus* did contain a functional CODH/ACS where, as the sole energy source, four moles of CO were disproportionated into three moles of CO_2 and one mole of CH_4. While the physiological purpose of the reported CODH/ACS was unknown at the time, this gave an early clue that there may be an analogous acetyl-CoA pathway in methanogens. Additionally, labeling reaction using ^{14}CO showed that CO was incorporated into the carboxyl group of acetyl-CoA (Stupperich et al, 1993). Further evidence for the acetyl-CoA pathway in another methanogen, *Methanococcus* is as follows: DeMoll et al. (1987) discovered a CODH/ACS in *Methanococcus vannielii* with similar properties to the *M. barkeri* enzyme. However it did not contain the acetyl-CoA synthase activity. However, Shieh and Whitman (1988) determined from the related species *Methanococcus maripaludis* that acetyl-CoA synthase activity was present. Acetyl-CoA synthase activity was determined using an acetyl-CoA trap assay. In this assay the pyruvate formed from acetyl-CoA by the pyruvate oxidoreductase (POR) was trapped as lactate via exogenously added LDH and NADH. The lactate produced was then measured spectrophotometrically. Additionally, using $^{14}CO_2$, radiolabel was

incorporated into lactate where the C-1, C-2 and C-3 positions were at 73, 33, and 11% respectively (Shieh and Whitman, 1988). Radiolabel in the C-1 position of lactate would be expected from the formation of pyruvate from acetyl-CoA and CO_2 via the POR reaction (Shieh and Whitman, 1988). The C-2 and C-3 of lactate were thought to be derived from the carbonyl and methyl groups of acetyl-CoA (Shieh and Whitman, 1988; Whitman, 1994). Further evidence of the Ljungdahl-Wood pathway in methanococci was provided when six acetate auxotrophs that were isolated following ethyl methanesulfonate mutagenesis (Ladapo and Whitman, 1990). These auxotrophs were shown to have greatly reduced CODH/ACS activity. Some auxotrophs also had reduced POR (synthase) activity as well. Furthermore, spontaneous revertants of three of the mutants regained both activities thus suggesting a possible link between the POR and the CODH/ACS activities.

4. BIOSYNTHESIS OF CELLULAR CARBON IN *METHANOCOCCUS*

Pyruvate is a central intermediate in the biosynthetic pathways of *Methanococcus* and other autotrophic methanogens (Figure 1.2). Pyruvate is synthesized from acetyl-CoA via the pyruvate oxidoreductase (POR), another site of CO_2 fixation. Enzymes involved in pyruvate metabolism will be discussed in a later section. As mentioned above, most of the acetyl-CoA produced for biosynthesis is from the modified Ljungdahl-Wood pathway. While acetyl-CoA is a precursor of lipids (mevalonate pathway), most of the major biosynthetic pathways require pyruvate as the initial precursor. **(**Table 1.1). Pyruvate and acetyl-CoA requirements for the methanogen are estimated to be 10.7 and 14.5 mmol/g cells dry wt., respectively (Table 1.1). This section discusses the major biosynthetic pathways that require pyruvate in *Methanococcus*.

4a. The reductive incomplete tricarboxylic acid cycle in *Methanococcus*.

Acetyl-CoA enters the major biosynthetic pathways through pyruvate. After pyruvate, the incomplete tricarboxylic acid cycle is utilized to synthesis major cellular components such as amino acids. In methanogens, there exists two types of incomplete tricarboxylic acid cycles: the reductive incomplete TCA cycle (RI-TCA) and the oxidative incomplete TCA cycle (OI-TCA). *Methanococcus* and other autotrophic methanogens like *Methanothermobacter* utilize the reductive incomplete TCA cycle (RI-TCA; Figure 1.2). From early research that attempted to establish whether or not the reductive TCA cycle was the pathway of CO_2 fixation in methanogens , it was initially determined that *Methanothermobacter* contained the early enzymes in the cycle. However, because key enzymes were missing in the cycle, the RI-TCA cycle could not be completed past □-ketoglutarate (Zeikus et al., 1977). Through ^{13}C labeling studies, the RI-TCA cycle was confirmed in *Methanospirillum* (Ekiel et al 1985; Sprott, 1993). Shieh and Whitman (1987) also established through enzymatic assays that the enzymes of the RI-TCA cycle were present in *Methanococcus*. Also established in that study was that *Methanococcus* did not enter the cycle via phosphoenolpyruvate as found in *Methanothermobacter*, but rather, it utilized pyruvate via the pyruvate carboxylase. From the lack of citrate synthase and isocitrate dehydrogenase, it was also confirmed that the IO-TCA cycle did not exist in *Methanococccus* (Shieh and Whitman, 1987). Aceticlastic methanogens such as *Methanosarcina* and *Methanosaeta* utilize the oxidative incomplete TCA cycle (OI-TCA) which requires two acetyl-CoAs and a series of oxidations to form 2-ketoglutarate (Simpson and Whitman, 1983).

Simpson and Whitman (1993) have hypothesized why two incomplete TCA cycles exist in methanogens. They base their explanation on the energetics required for 2-ketoglutarate synthesis, the final product in both incomplete TCA cycles. The RI-TCA cycle (autotrophic methanogens), which requires several reductions to form succinate and a reductive carboxylation to finally form 2-ketoglutarate, may be used when electron donors like H_2 are utilized such as in hydrogenotrophic, autotrophic methanogens. The IO-TCA cycle requires an additional acetyl-CoA for the synthesis of 2-ketoglutarate. Acetotrophic methanogens that are able to grow with exogenous acetate would be able to synthesize enough acetyl-CoA to complete the reactions.

4b. Amino acid biosynthesis. Much of methanogen amino acid biosynthesis is similar to pathways elucidated from bacteria and eukaryotes (Fuchs et al., 1978; Ekiel et al.,1983; Ekiel, et al., 1985; for a review see Simpson and Whitman, 1993). However, several pathways of amino acid biosynthesis do differ in methanogens.

The branched chain amino acids (BCAA) were determined to be synthesized by the acetohydroxy acid pathway. A key difference, is that the precursor for isoleucine, □-ketobutyrate, which is frequently synthesized through threonine, is instead synthesized from pyruvate and acetyl-CoA via the citramalate pathway (Ekiel et al, 1983, Ekiel et al.,1984). Xing and Whitman (1987,1991 and 1992) confirmed that the other enzymes of branched chain amino acid biosynthesis were similar to those found in bacteria.

The synthesis of the aromatic amino acids, phenylalanine and tyrosine were largely determined to be from the shikimate and the chorismate pathways. However based upon ^{13}C labelling patterns of pentoses, erythrose 4-phosphate is probably not the precursor of aromatic amino acids in *M. maripaludis* (Tumbula et al., 1997). In support

of this hypothesis, ORFs encoding the first two genes of this pathway are absent from the genomes of the methanogens and related euryarchaeota.

4c. Carbohydrate, purine and pyrimidine biosynthesis. Carbohydrates are precursors for pentose biosynthesis, pseudomurein, and the common reserve polysaccharide, glycogen (Simpson and Whitman, 1993). Although methanogens only utilize limited substrates for growth and cannot assimilate sugars, glycogen is present in many methanogens (Konig et al., 1985; Pellerin et al., 1987; Ratner et al., 1987). Also, under special conditions, glycogen can be utilized as a substrate for methanogenesis (Konig et al., 1985). Previous research has established that from pyruvate, methanogens utilize the classical pathway of gluconeogenesis to form glycogen (Figure 1.3). The enzymes of gluconeogenesis were confirmed by Yu et al. (1994) in *M. maripaludis* and by ^{13}C labeling studies in *M. jannaschii* (Sprott et al., 1993).

Pentose biosynthesis in *Methanococcus* was determined to proceed through the conventional non-oxidative pentose phosphate pathway with intermediates from the gluconeogenesis pathway, fructose bisphosphate and glyceraldehydes-3-phosphate (Yu et al., 1994; Figure 1.3). Pentoses are utilized for nucleic acid and histamine biosynthesis.

Purine and pyrimidine synthesis were examined by Ekiel et al. (1983) in *M. hungatei* through ^{13} C-labelling studies. Pyrimidines are synthesized from aspartate by the common pathway. The C-5 and C-6 of purines are obtained from glycine while the C-2 and C-8 were derived from the C-2 of acetate instead of the tetrahydrofolate pathway used by eubacteria (Simpson and Whitman, 1993).

5. ENZYMES INVOLVED IN PYRUVATE METABOLISM

In many organisms, key reactions in intermediate metabolism include the oxidative decarboxylation of pyruvate to acetyl-CoA, the biosynthesis of pyruvate from acetyl-CoA, and non-oxidative decarboxylation of pyruvate. Pyruvate is a key intermediate in the breakdown of sugars and in many biosynthetic pathways where it is a precursor for amino acid synthesis, gluconeogenesis, and fermentation products.

A well characterized enzyme involved in non-oxidative decarboxylation of pyruvate is the pyruvate decarboxylase (PDC). There are three well-characterized enzyme systems in which pyruvate is oxidatively decarboxylated to acetyl-CoA. They are the pyruvate formate lyase (PFL), the pyruvate dehydrogenase (PDH) and the POR. An additional system, involving the pyruvate oxidase enzyme decarboxylates pyruvate directly to acetate. Both the PDH and pyruvate oxidase are found in aerobic organisms and can also be found in mitochondria as well. The PFL is found in enteric and lactic acid bacteria where pyruvate is metabolized to acetyl-CoA and formate. The POR catalyses a similar reaction to the PDH enzyme but is found mostly in anaerobic organisms. However, unlike the PDH, the POR utilizes low potential ferredoxins rather than NAD^+, thus enabling it to catalyze the reverse reaction where pyruvate is synthesized from acetyl-CoA. Many autotrophic organisms utilizing the reductive acetyl-CoA pathway use the POR enzyme system.

5a. Pyruvate decarboxylase. First detected in yeast, the PDC has been found in fungi, plants, and sparingly in prokaryotes (Candy and Duggleby, 1998). Various fungi and plants that have PDC are, *Saccharomyces cerevisiae*, *Neurospora crassa*, *Aspergillis* species, maize, rice, and soybean (Kellermann et al., 1986; Candy and Duggleby, 1998).

Prokaryotes that contain the PDC include, *Zymomonas mobilis*, *Clostridium botulinum*, *Acetobacter* species, and some *Erwinia* (Bringer-Meyer et al., 1986; König, 1998). The *Zymomonas* PDC is the best characterized enzyme in the prokaryotes. PDC is a key enzyme in homoethanol fermentations and catalyses the nonoxidative decarboxylation of pyruvate to acetaldehyde and CO_2 (Candy and Duggleby, 1998). From here, acetaldehyde is reduced to ethanol by the alcohol dehydrogenase. The *Zymomonas* PDC was found to be a homotetramer with a molecular weigh of 59-65 kDa and is thiamine diphosphate (TPP) dependant (Candy and Duggleby, 1998; König, 1998).

5b. Pyruvate oxidase. The pyruvate oxidase is a membrane-bound or lipid-associated enzyme found in *E. coli* and certain *Lactobacillus* species. The enzyme catalyses the oxidative decarboxylation of pyruvate to form acetate and CO_2 (O'Brien et al, 1977). The reaction is: pyruvate + E(enzyme)-FAD/TPP → acetate + CO_2 + E-$FADH_2$/TPP (Gennis and Hager, 1976). Purified from *E. coli*, it was found to be a 265,000 Da protein with four identical subunits. From SDS gels, each subunit appears to be about 60,000 Da. Two cofactors, FAD and TPP are necessary for catalytic activity (O'Brien, 1977; Reeny and Hager, 1982). FAD appears to be tightly bound to each subunit and must be removed by extremely vigorous methods. The TPP cofactor however, is lost upon purification and must be supplied exogenously for the enzyme to function. The physiological electron acceptor is a membrane bound electron transport system containing cytochrome b_1 and ubiquinones where oxygen is the terminal electron acceptor (Reeny and Hager, 1982; Cunningham and Hager, 1971).

5c. Pyruvate formate-lyase. This anaerobic enzyme catalyzes a key step in glucose fermenting pathways utilized by enterobacteria, *Streptococcus,* several species of

Clostridum, and some lactic acid bacteria (Weidner, 1996). The enzyme is a homodimer consisting of 85-kDa subunits. The molecular weight of the enzyme is 170,000 Da. Induced under anaerobic conditions, the pyruvate formate-lyase catalyses the reversible catalysis of pyruvate + CoASH to form acetyl-CoA + formate (Knappe et al., 1984; Knappe et al., 1974) through two half reactions involving an acetyl-enzyme intermediate with a ping-pong reaction pattern. The reaction is:

(1) E (enzyme) + pyruvate$\leftarrow\rightarrow$ acetyl-E + formate

(2) acetyl-E + CoA$\leftarrow\rightarrow$ E + acetyl-CoA

5d. Pyruvate dehydrogenase. PDH is a large three enzyme complex with a M_r of 4.5 x 10^6. It consists of pyruvate decarboxylase (E1), dihydrolipoate transacetylase (E2), and the flavoprotein dihydrolipoate dehydrogenase (E3) that work in conjunction to catalyze the irreversible oxidation of pyruvate + CoA + NAD^+ $\rightarrow$ acetyl-CoA + NADH + CO_2 (Weiland, 1983; Figure 1.4). Found in *Escherichia coli, Azotobacter vinelandii, Pseudomona aeruginosa* and *Zymomonas mobilis*, this enzyme complex functions in highly regulated aerobic, catabolic pathways. TPP, FAD, and lipoic acid are utilized as cofactors while the electron acceptor is NAD^+ (de Kok, et al., 1998; Wieland, 1983). Regulation is accomplished through an allosteric mechanism and product inhibition (de Kok et al., 1998).

The 100 kDa pyruvate dehydrogenase component (E1) with the TPP cofactor catalyses the initial decarboxylation of pyruvate, which forms a thiamine diphosphate-bound enamine (de Kok et al., 1998). The overall reaction rate of the complex is very similar to the rate of the pyruvate dehydrogenase component, indicating that this is the rate-limiting step. The acetyltransferase component (E2) is a multi-lipoyl domain

structure that initially works in conjunction with E1-P to form acetyl lipoic acid. Additionally, E2 catalyses the transfer of the acetyl group from the acetyl lipoic acid to CoA thus forming acetyl-CoA. From here the dihydrolipoamide dehydrogenase component (E3) reoxidizes the dihydrolipoamide left over from the synthesis of acetyl-CoA with NAD^+.

5e. Pyruvate oxidoreductase. With anaerobic Bacteria and Archaea, a similar reaction is catalyzed by the POR. Aside from the TPP requirement, POR is different from PDH. While PDH is a large multienzyme complex, POR is a small single multisubunit enzyme. Most PORs have been found to be soluble except for the membrane-bound or associated PORs found in the hydrogenosomes in the eukaryote *Trichomonas vaginalis* (Williams et al., 1987) and cell membrane of *Giardia duodenalis* and *Entamoeba histolytica* (Brown et al. 1998; Townson et al, 1996; Reeves et al. 1977). Instead of the lipoic acid and FAD in PDH, Fe-S clusters are found. Except for the halophilic archaeon and *Desulfovibrio africanus*, most PORs are extremely sensitive to oxygen. The *D. africanus* enzyme, while similar to other bacterial PORs, has been hypothesized to contain a disulfide bridge that may protect the [4Fe-4S] clusters (Chabriere et al., 1999). Although several different catalytic mechanisms have been described (see below), low potential electron carriers such as ferredoxins and flavodoxins are utilized rather than $NADP^+$ (with the exception of *Euglena gracilis*; Inui et al., 1987). Importantly, this reaction is reversible, allowing for autotrophic CO_2 fixation and the biosynthesis of pyruvate from acetyl-CoA.

6. CLASSES OF PORS

Currently, PORs have been purified and characterized from several Archaea, Bacteria and some Eukarya (Table 1.2). Based on subunit composition three major forms of POR exist (Figure 1.5), The four subunit $\alpha\beta\gamma\delta$ type (Figure 1.5a), the aerobic archaeal two subunit $\alpha\beta$ type (Figure 1.5b), and the mesophilic bacterial homodimeric α_2-type (Figure 1.5c; Adams and Kletzin, 1996; Zhang et al., 1996).

6a. Four subunit, $\alpha\beta\gamma\delta$ type POR. PORs from hyperthermophiles, including the anaerobic Archaea (*Pyrococcus furiosus*, *Archaeoglobus fulgidus* and *Thermococcus litoralis*) and anaerobic Bacteria (*Thermotoga maritima*) possess a four subunit enzyme (α, β, γ, and δ) with molecular masses of approximately 45, 30, 25, and 15 kDa, respectively (Figure 1.5a). The holoenzyme has a M_r range of about 107 to 220 kDa (Yang et al., submitted; Kunow et al., 1995; Adams and Kletzin, 1996; Hughes et al., 1995). *P. furiosus*, however, has a variation where the γ subunit is shared between the 2-ketoisovalerate oxidoreductase (VOR) and the POR (Kletzin and Adams, 1996). It was reported by Adams and Kletzin (1996) that POR's from *T. litoralis*, and *T. maritima* are octomeric. Despite the similarity in subunit construction to other hyperthermophilic archaea and bacteria, the sulfate-reducing archaeon, *Archaeaoglobus fulgidus* appears to have a heterotetrameric enzyme (Kunow et al., 1995; Adams and Kletzin, 1996). All of these PORs utilize ferredoxin as the electron acceptor. All of these hyperthermophiles oxidize pyruvate with the POR for fermentation.

The gastric pathogen *H. pylori* is the first mesophilic bacterium found to contain a $\alpha\beta\gamma\delta$-type POR with similarity to the hyperthermophilic enzymes (Hughes et al., 1995). While ferredoxin is utilized for electron transfer in the hyperthermophilic organism, *H.*

pylori is reported to utilize a flavodoxin as the electron acceptor (Hughes et al., 1995). Also, recently a 135 kDa □□□□type POR was purified from the aerobic, thermophilic, obligately chemolithoautotrophic hydrogen-oxidizing bacterium, *Hydrogenobacter thermophilus* TK-6 (Yoon et al., 1997). Interestingly, the POR from *H. thermophilus* is the first □□□□type POR purified from an organism utilizing the reductive TCA cycle.

6b. Aerobic archaeal , □□ type POR. The 2-oxoacid:ferredoxin oxidoreductases (OFOR) from the aerobic Archaea, *Sulfolobus* and *Halobacterium,* belong in the same superfamily as the PORs from other organisms **(**Figure 1.5b). The OFORs exhibit broad substrate specificity, which includes 2-oxoglutarate, 2-oxobutyrate, and pyruvate (Zhang et al.,1996; Fukuda and Wakagi, 2002). The OFOR is comprised of two nonidentical subunits at 71 kDa (subunit □) and 36 kDa (subunit □; Zhang et al, 1996; Kerscher and Oesterhelt, 1981a). It was predicted that the OFOR from *Halobacterium salinarium* (*H. halobium*) and *Sulfolobus* sp. Strain 7 contains two TPP and two [4Fe-4S] clusters per $\square_2\square_2$ proteins structures and ferredoxin serves as the electron acceptor (Zhang et al., 1996; Plaga et al., 1991; Kersher and Oesterhelt, 1981a). It is postulated that the □ subunit is the result of a fusion of the □ and □subunits and the deletion of the □subunit from the four subunit POR (Adams and Kletzin, 1996; Zhang et al., 1996). Zhang et al. (1996) also state that the loss of the two [4Fe-4S] cluster binding motifs of the □subunit may be an adaption for protection towards an aerobic environment.

6c. Bacterial and Eukaryal 2□ type POR. PORs from anaerobic mesophilic bacteria appear to have a similar overall size (240 kDa) to the hyperthermophilic PORs (Shah et al., 1983; Meinecke et al., 1989; Brostedt and Norlund, 1991; Plaga et al., 1992; Kletzin and Adams, 1995). However, the PORs are generally found in a homodimer ($\square_2$)

as seen in Figure 1.5c. The bacterial POR is utilized in various functions. For example, *Rhodobacter capsulatis*, *Rhodospirillum rubrum* and *Klebsiella pneumoniae* enzymes couple the oxidation of pyruvate to provide electrons for nitrogen fixation (Yakunin and Hallenbeck, 1998; Brostedt and Nordlund, 1991; Shah et al. 1983). Other organisms such as *Clostridium [Moorella]* spp. utilize the POR during autotrophic growth on H_2+CO. Recently Furdui and Ragsdale (2000) found that the POR from *Clostridium thermoaceticum* could function as a pyruvate synthase with a low potential ferredoxin.

The first crystal structure of a POR complexed with pyruvate was determined from the sulfate-reducer *Desulfovibrio africanus* (Chabriere, 1999a). The elucidation of the structure has enabled a determination of spatial location of the three [4Fe-4S]clusters, the TPP binding motifs, and their coordination within enzyme. Chabriere et al. (1999) have also demonstrated that the [4Fe-4S] clusters and the TPP binding motifs in all three types of POR are structurally analogous, thus reinforcing the hypothesis of Adams and Kletzin (1996) that the four subunit POR may be the ancestral POR.

Eukaryotic PORs are generally found in ancient eukaryotic lineages that do not have mitochondria. Thus they share many of the metabolic features found in bacteria. Eukaryotic organisms that contain POR include *Giardia duodenalis*, *Euglena gracilis*, *Trichomonas vaginalis*, and *Entamoeba histolytica* (Rotte et al., 2001; Inui et al, 1987; Hrdy and Muller,1995; and Reeves et al, 1977). The subunit construction is very similar to that of the mesophilic anaerobic bacteria. The eukaryotic POR appears to be a homodimeric enzyme with an overall Mr of 240,000 to 309,000 and composed of subunits with a M_r of 120,000 to 166,000. The electron acceptor usually is a ferredoxin, although, the *Euglena* POR utilizes $NADP^+$ (Inui et al., 1987). A distinct difference from

other PORs is that most of the eukaryotic PORs are associated with a membrane, although the location of the eukaryotic POR can vary. For example, the POR from *G. duodenalis* and *E. histolytica* are loosely associated with the cell membrane while the POR from *T. vaginalis* is bound to the inside of the hydrogenosomes. Hydrogenosomes are specialized organelles that process pyruvate using the POR and evolve hydrogen gas; Kurland and Andersson, 2000). Hydrogenosomes may have evolved from a symbiosis with an archaeon, possibly an autotrophic methanogen, and a H_2-evolving bacterium (Martin and Mueller, 1998).

7. CATALYTIC MECHANISM OF POR

PORs from various organisms have been identified by their ability to oxidize pyruvate by a catalytic mechanism different from the PDH enzyme. However, there appear to be two different catalytic mechanisms by which PORs from various organisms can oxidize pyruvate.

Two archaeal PORs, from *Halobacterium halobium* and *Pyrococcus furiosus*, have been found to utilize a unique free radical species that has not been found in other PORs. Kerscher and Oesterheld (1981b; Figure 1.6A) found that with *H. halobium* POR, pyruvate is bound to TPP. Decarboxylation of pyruvate follows to form HETPP (hydroxyethyl-TPP). HETPP then donates one electron to a [4Fe-4S] cluster to form a HETPP radical intermediate. CoASH then reacts with the HETPP radical intermediate, causing the transfer of the unpaired electron to the [4Fe-4S] cluster and formation of acetyl CoA. Finally, the [4Fe-4S] clusters are then reoxidized by a ferredoxin. Similarly, a TPP radical-based mechanism has been elucidated for the *P. furiosus* POR. However, the *P. furiosus* POR contains an unusual copper atom, that is believed to replace the

divalent cations such as Mg^{2+} and Mn^{2+} that are usually associated with TPP (Smith et al., 1994; Figure 1.6B). Smith et al. (1994) proposed that a Cu^{2+}/TPP radical binds to pyruvate, thus forming a HETPP radical intermediate. CoASH then is oxidized by Cu^{2+} to form CoAS-Cu^{+}. The HETPP radical intermediate then undergoes a nucleophilic attack by CoAS-Cu^{+} to form acetyl-CoA (Smith et al., 1994).

Evidence has suggested that the bacterial POR does not utilize a radical species in catalysis. For example the POR from *Clostridium pasteurianum* was determined to decarboxylate pyruvate initially to form hydroxyethyl-TPP (Mortenson, 1963). Uyeda and Rabinowitz (1971a, b) confirmed the formation of hydroxyethyl-TPP (HETPP) and further expanded the catalytic mechanism. They found that from the HETPP intermediate, two electrons are passed to [4Fe-4S] clusters. CoASH then accepts the acyl group from HETTP to form acetyl-CoA. Finally, the [4Fe-4S] clusters are reoxidized by an electron acceptor such as a ferredoxin. Additionally, Smith et al. (1994) has found from electron spin resonance (EPR) studies that *T. maritima* POR has a similar reaction and couples the oxidation of CoASH to the reduction of an Fe-S center (Figure 1.7). However, recent evidence from Menon and Ragsdale (1997) suggests that the bacterial POR does indeed have the unique HETPP radical and that others have not detected it because of its rapid decay. Menon and Ragsdale (1997) further contend that the HETPP radical should be detected in all POR when utilizing their detection methods (rapid mixing methods).

8. METHANOCOCCAL POR

Studies by Yang (1994) determined that the methanococcal POR had a similar subunit construction as the hyperthermophilic POR with one exception, an additional

subunit was discovered (referred to as PorE) that eluted with the POR. The molecular weight of *M. maripaludis* POR was found to be about 200,000 with 5 polypeptides (α=47,000, β=33,000, γ=25,000, δ=13,000 and CR=21,500) (Yang, 1994). The α,β,γ,δ subunits from *Methanococcus* POR have high similarity to the subunits of the hyperthermophilic PORs (Yang, 1994). Although a fifth polypeptide was not purified from the hydrogenotrophic *M. thermautotrophicus*, a POR was purified with similar M_rs to the methanococcal POR (Tersteegen et al., 1997). The specific oxidative activity of the *M. thermautotrophicus* POR was found to be similar to *P. furiosus* (Tersteegen et al., 1997). From the aceticlastic methanogen, *Methanosarcina barkeri* (Fusario), the POR was determined to contain three [4Fe-4S] clusters and one TPP binding site (Bock et al., 1997).

With non-methanogenic heterotrophic organisms as described above, the POR can function, *in vitro*, in both the oxidative and the biosynthetic direction. However, under normal physiological conditions these PORs oxidatively decarboxylate pyruvate to acetyl-CoA. Shieh and Whitman (1988) showed that in *Methanococcus*, POR and pyruvate synthase activities were also present in cell extracts. With acetate auxotrophs, it was observed that both POR and pyruvate synthase activities were reduced in comparison to the wild type activities (Ladapo and Whitman, 1990). Further, spontaneous revertants regained wild type levels of both pyruvate synthase and POR activity thus suggesting that both activities are associated with the same enzyme. In all cases, however, the pyruvate synthase activity was much lower than the pyruvate oxidoreductase activity.

Despite the reversibility of the POR reaction *in vitro*, evidence suggests that the methanogenic POR only functions in the biosynthetic direction for carbon assimilation. If pyruvate oxidation were possible in methanococci, expected products would be acetyl-CoA and then acetate. Using an acetate auxotroph, if the POR were able to function catabolically *in vivo,* substitution of pyruvate should be able to replace the requirement for acetate. However, with *M. voltae* and *M. maripaludis* JJ6 and JJ8 (acetate auxotrophs), pyruvate could not support growth. Additionally, Yang et al. (1992) showed that under non-physiological conditions (N_2+CO_2), resting cells of *M. voltae* and *M. maripaludis* could oxidize pyruvate for methanogenesis at rates of 3.4 and 2.8 nmol $min^{-1}mg^{-1}$ respectively. However, the reaction was only 4% of the rates of methanogenesis with H_2 and did not support growth (Yang et al. 1992). Additionally, when H_2 was present, pyruvate oxidation stopped, presumably to prevent a futile cycle. These results suggest that the methanococcal POR functions unidirectionally towards pyruvate biosynthesis under physiological conditions. However, the regulation of pyruvate biosynthesis in methanogens is unknown.

9. HYPOTHESIS AND PURPOSE OF STUDY

In methanogenic habitats, the midpoint of the H^+/H_2 couple is about –296 mV (at 10^{-4} atm) and the acetyl CoA+CO_2/pyruvate couple is about –500 mV. From the chemical equation of pyruvate synthesis,

$$\text{acetyl-CoA} + CO_2 + H^+ + 2e^- \leftrightarrows \text{pyruvate} + \text{CoASH}$$

the $\Delta G°$ can be estimated at +46.6 kJ/mol. This would favor pyruvate oxidation rather than biosynthesis. For pyruvate synthesis to work, the POR must be coupled to an energy source. An obvious energy source for pyruvate synthesis might be the hydrolysis of

ATP. However, Yang (1994) found that with purified *M. maripaludis* POR, ATP did not affect the activity of POR. Moreover, an ATP-binding motif has not been detected in the sequences of homologs in the *M. jannaschii* and *M. thermautotrophicus* total genomic sequences. Thus, it seems likely that ATP is not the energy source for pyruvate synthesis. An alternative may be that the POR is coupled to the proton motive force (PMF) directly or indirectly.

In Model 1 (Figure 1.8a), the POR is coupled to a hydrogenase. The hydrogenase drives the reaction through the translocation of sodium or hydrogen ions. This catalyzes the transfer of electrons from hydrogen gas to an oxidized electron carrier X to form a reduced electron carrier X. Importantly, with the acetyl-CoA/pyruvate couple at –490 mV, electron carrier X must be sufficiently electronegative to be an efficient electron donor for the methanococcal POR. This reduced electron carrier can then donate electrons to the synthesis of pyruvate from acetyl-CoA. In this manner, the POR is indirectly coupled to the PMF.

While cofactor F_{420} has been suggested as an electron carrier for the methanogenic PORs (Zeikus et al, 1977), it is considered to be too electropositive to be an electron donor for POR as depicted in Model 1. Ferredoxins have been found to be the electron carrier for other PORs. Ferredoxins are a particularly attractive possibility because they have a reduction potential range from –200 to –650 mV (Binert, 2000). The low potential ferredoxins may be sufficiently electronegative to be an electron donor for methanogen POR. This assumption is reasonable for *M. maripaludis* because up to seven different ferredoxins and four polyferredoxins have been predicted from the *M. jannaschii* complete genome sequence (Bult et al. 1996). In addition, Menon and

Ragsdale (1997) performed in vitro studies on clostridial POR and clostridial ferredoxin showing that ferredoxins can be utilized as electron donors for pyruvate synthesis.

An alternative model for pyruvate biosynthesis (Model 2, Figure 1.8b) depicts a membrane bound POR. The PMF is directly coupled to the POR for the synthesis of pyruvate. A reduced electron carrier Y donates electrons to the reaction. Finally, this electron carrier can then be reduced by a hydrogenase either directly as depicted or in a coupled fashion. With the $2H^+/H_2$ couple (10^{-3} atm) at -325 mV, electron carrier Y does not need to be extremely electronegative as the electron carrier X in Model 1. While this model is not easily disproven, most PORs purified (with the exception of the eukaryal PORs) were found in the soluble fraction thus suggesting that Model 1 may be more likely.

Recently, complete genome sequences have been completed from *M. jannaschii* and *M. thermautotrophicus* (Bult et al., 1996; Smith et al.,1997). This has enabled a detailed comparison of the various sequences of the POR from methanogens and other organisms. Putative POR subunits from *M. jannaschii* (Mj 0266= □, Mj 0267= □, Mj 0268 = □, Mj 0269 = □) demonstrated high similarity between the □□□ type POR nucleotide sequence of *P. furiosus* POR (this work). Although *M. thermautotrophicus* has a three subunit POR, it appears to possess a fusion of the ORFs encoding for the □ and □ subunit (Smith et al., 1997; This work). Further investigation of the four subunits of the POR from *M. jannaschii* and the three subunits of the POR from *M. thermautotrophicus* corroborated with sequence data presented by Kletzin and Adams (1996). In particular, four conserved cysteine and a TPP-binding domain were found in the □ subunit for both methanogens POR. In addition, two [4Fe-4S] cluster motifs were

found in the □ subunit (subunit □□□ for *M. thermautotrophicus*). Yang (1994) also confirmed these results in *Methanococcus maripaludis* with N-terminal sequences of the purified POR.

Aside from the four orthologous subunits, both *M. jannaschii* and *M. thermautotrophicus* contain two additional ORFs that appear to be associated with POR (Bult et al., 1996; Smith et al.,1997). Using DNA sequences data of these ORFs from *M. jannaschii* (Mj 0264 and Mj0265) and *M. thermautotrophicus* (MTH 1736 and MTH 1737), preliminary data were obtained. With the Genetics Computer Group inc. (GCG) package, it was determined that Mj 0265 (*porE*) , Mj 0264 (*porF*) and the orthologous genes in *M. thermautotrophicus* were very similar (this work). The putative ORFs from *M. jannaschii* and *M. thermautotrophicus* contained many cysteine residues and a FeS motif from the translated polypeptide sequence, suggesting a ferredoxin-like electron carrier and supporting POR model 1 (Figure 1.8a; this work). This evidence supported Yang's (1994) finding of the additional PorE polypeptide (formerly called □) from the *M. maripaludis* POR. From an amino acid sequence comparison of a 20 amino acid N-terminal region of the *M. maripaludis* PorE polypeptide, there was similarity to ORF Mj0265 in *M. jannaschii* (unpublished analysis). Further, investigation of a gene sequence from another enzyme involved in autotrophy, the carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS), showed that with both *M. jannaschii* (Mj0156 = CODH/ACS □ subunit) and *M. thermautotrophicus* (MTh 1713 = CODH/ACS □subunit) contained CODH/ACS/ACS-associated cysteine-rich subunits (Mj 0155 and MTh1714). Additionally, a comparison with various other PORs from other Archaea and Bacteria showed that the additional ORFs (Mj0265 and Mj0264) were

not present with PORs and CODH/ACS sequence clusters outside of the hydrogenotrophic methanogens.

The data presented here leads to the hypothesis that PorE homologs are a necessary component of key autotrophic enzymes such as the POR in some methanogens. To further this hypothesis, the nucleotide sequence of the POR from *Methanococcus* was sequenced, analyzed, and the genes encoding PorE and PorF were mutated. The results of this work show that Por E/F homologs are an important component for the methanogenic POR and may be a novel adaption by hydrogenotrophic methanogens for use in pyruvate synthesis.

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Table 1.1. Hypothetical composition and pyruvate and acetyl-CoA needed in *Methanococcus*[a].

Component	% total wt[b,c]	μmol/g of cells in *E. coli*	corrected μmol/cells in *M. maripaludis*[b,c]	Estimated pyruvates consumed in biosynthesis[e]	μmol pyr/g of cells	Additional acetyl-CoA not used for pyruvate biosynthesis (μmol/g of cells)
Protein[a]	**63%**					
Alanine		488	559	1	559	
arginine		281	322	1	322	
asparagine		229	262	1	262	
Aspartate		229	262	1	262	
Cysteine		87	100	1	100	
Glutamate		250	286	1	286	
Glutamine		250	286	1	286	
Glycine		582	667	1	667	
Histidine		90	103	4.34	447	
Isoleucine		276	316	2	632	316
Leucine		428	490	2	981	490
Lysine		326	373	2	747	
Methionine		146	167	1	167	
Phenylalanine		176	202	3	605	
Proline		210	241	1	241	
Serine		205	235	1	235	
Threonine		241	276	1	276	
Tryptophan		54	62	4.67	289	
Tyrosine		131	150	3	450	
Valine		402	460	2	921	

Component	% total wt[b,c]	μmol/g of cells in *E. coli*	corrected μmol/cells in *M. maripaludi* [b,c]	Pyruvates consumed in pathway[e]	μmol pyr/g of cells	additional acetyl-CoA not used for pyruvate biosynthesis (μmol/g of cells)
Nucleic Acids[a]	**24%**					
AMP		165	165	2.67	441	
GMP		203	203	2.67	542	
CMP		126	126	2.67	336	
UMP		136	136	2.67	363	
dAMP		24.6	25	2.67	66	
dGMP		25.4	25	2.67	68	
dCMP		25.4	25	2.67	68	
dTMP		24.6	25	2.67	66	
Lipids[a]	**10%**					
glycerol		161	161	1	161	
Serine		97	97	1	97	
C20 isoprenoid		258	258	0		3096
Glycogen[d] (glucose eq.)	**0.5%**	154	31	2	62	
Total pyruvate or acetyl-CoA (μmol/g)					**10746**	**3902**

a. Estimated and calculated from Neidhart and Umbarger,1996; Whitman et al., 1982
b. Neidhart and Umbarger, 1996 *E.coli* composition estimates were: protein, 55%; nucleic acids, 23.6%; lipids, 9.1%; glycogen, 2.5% and small molecules, 3.5%.
c. Correction for *M. maripaludis* total cell composition was calculated from Whitman et al., 1982
d. Estimated from Yu et al., 1994
e. Common pathways as outlined in Figures 2 and 3 were used except that isoleucine was biosynthesized via the citramalate pathway.

Table 1.2. Overview of pyruvate oxidoreductases in organisms. (Modified and recompiled from Yang, 1994)

			ELECTRON ACCEPTORS*(next page)								
Microbes	**Type of PO R**	**Role/ Function**	**FAD**	**FMN**	**FD**	**RD**	**FLD**	**NAD^+**	**$NADP^+$**	**F_{420}**	**Reference**
Methanococcus maripaludis	+PorE/F	synthesis/CO_2 incorporation	+	+		+		-	-	-	Yang et al. submitted Yang, 1994
Methanothermobacter thermautotrophicus		synthesis/CO_2 incorporation						-		+/?	Zeikus et al., 1977 Tersteegen et al, 1997
Methanosarcina barkeri		synthesis/CO_2 incorporation			+					-	Bock, 1994; Hatchickian, 1982
Halobacterium salinarium		oxidation/TCA cycle			+						Kersher and Oesterhelt, 1981 Schafer et al., 1993
Sulfolobus acidocaldarius		oxidation/ acetate fermentation			+						Kersher et al 1982
Sulfolobus sp. strain 7		oxidation/ acetate fermentation			+		+				Zhang et al., 1996
Pyrococcus furiosus		oxidation/ acetate fermentation			+						Blamey et al., 1994
Archaeoglobus fulgidus		oxidation/ acetate fermentation			+						Kunow et al., 1995
Thermotoga maritime		oxidation/ acetate fermentation			+						Blamey and Adams, 1993
Hydrogenobacter thermophilus TK-6		reductive TCA cycle	+	+	+			-	-		Yoon et al, 1996
Helicobacter pylori		oxidation/ acetate fermentation			+		+				Hughes et al., 1995
Desulfovibrio africanus	2	oxidation/ acetate fermentation			+		+				Pieulle et al., 1995
Klebsiella pneumonia	2	oxidation/N_2-fixation			-		+				Shah et al.,1983
Rhodobacter capsulatus	2	oxidation/N_2-fixation			+		+				Yakunin et al., 1998

Microbes	Type of POR	Role/ Function	FAD	FMN	FD	RD	FLD	NAD^+	$NADP^+$	F_{420}	Reference
Bacteroides succinogenes	2	oxidation/ acetate fermentation	+	+				-	-		Miller, 1978
Clostridum acidi-urici	2	oxidation/ acetate fermentation	+	+	+	+		-	-		Rabinowitz, 1971
Chlorobium tepedum	2	reductive TCA cycle	+	+							Yoon et al.,1995
Anaeroplasma spp.	2	oxidation/ acetate fermentation	+								Petzel and Hartman, 1990
Anabaena cylindrical	2	oxidation/N_2-fixation			+						Bothe and Oesterhelt, 1974
Anabaena variabilis	2	oxidation/N_2-fixation			+						Leach and Carr, 1971
Rhodospirillum rubrum	2	oxidation/N_2-fixation			+						Brostedt and Nordlund, 1991
Euglena gracilis	2	?	+	+	-		-	+			Inui et al., 1987
Trichomonas vaginalis	2	oxidation/ SLP-ATP production	+	+	+		-	-			Lindmark et al., 1975 Williams et al., 1987
Entamoeba histolytica	2	oxidation/ acetate fermentation		+	+	+		-	-		Reeves et al., 1977
Giardia duodenalis	2	?			+						Brown et al., 1997

* FAD= flavin adenine dinuclotide FMN=flavin mononucleotide
FD=ferredoxin FLD=flavodoxin RD=rubredoxin
FLD= flavodoxin NAD^+= nicotinamide adenine dinucleotide
$NADP^+$=NAD phosphate F_{420}=coenzyme F_{420}

Figure 1.1. Comparison of the acetogenic (clostridial) Ljundahl-Wood Pathway (A) to the methanogenic modified Ljundahl-Wood Pathway (B). (Modified from Jones et al., 1987; Simpson and Whitman 1993.)

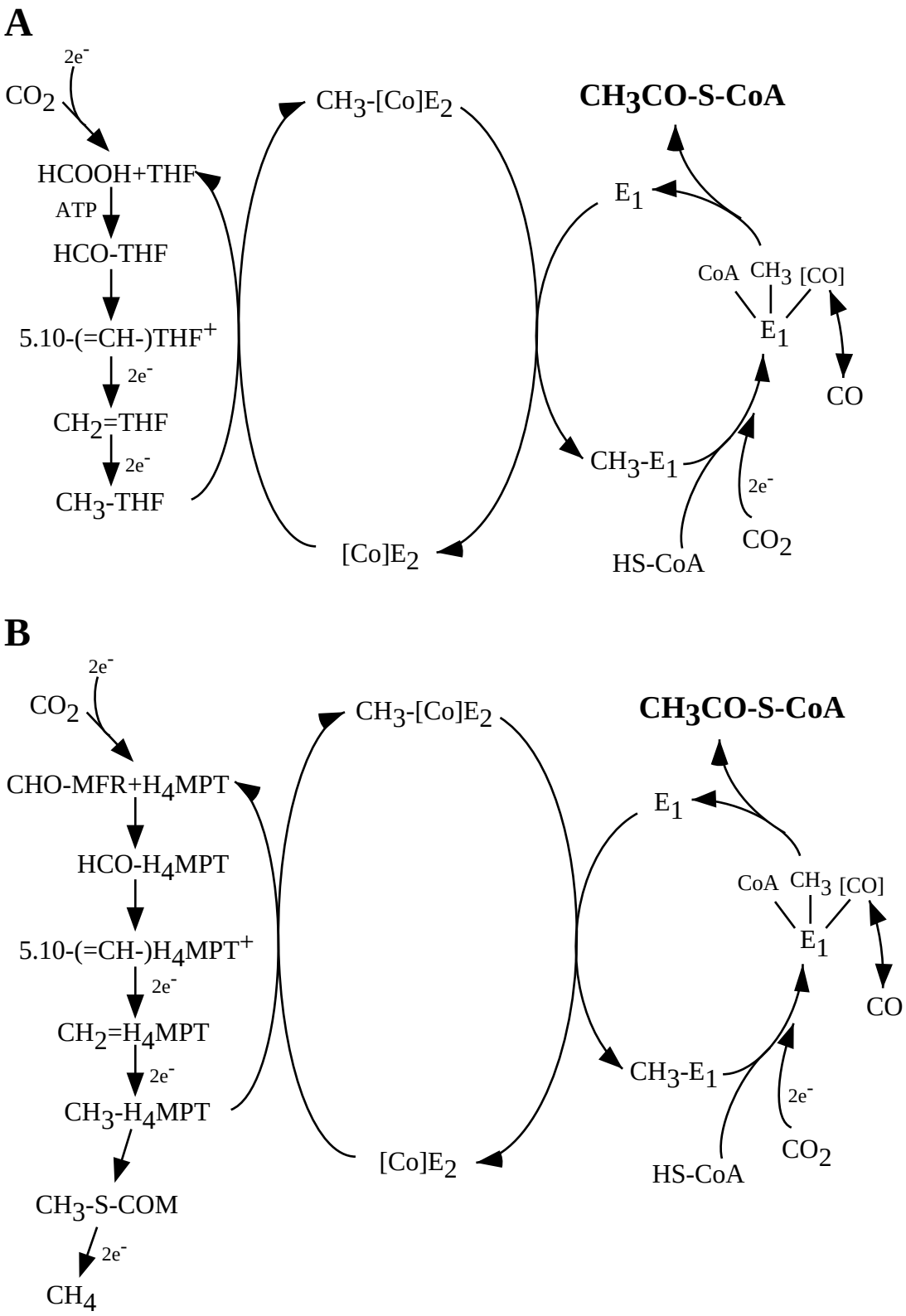

A
2e-
CO2
HCOOH+THF
ATP
HCO-THF
5.10-(=CH-)THF+
2e-
CH2=THF
2e-
CH3-THF
CH3-[Co]E2
[Co]E2
CH3CO-S-CoA
E1
CoA CH3 [CO]
E1
CO
CH3-E1
2e-
CO2
HS-CoA
B
2e-
CO2
CHO-MFR+H4MPT
HCO-H4MPT
5.10-(=CH-)H4MPT+
2e-
CH2=H4MPT
2e-
CH3-H4MPT
CH3-S-COM
2e-
CH4
CH3-[Co]E2
[Co]E2
CH3CO-S-CoA
E1
CoA CH3 [CO]
E1
CO
CH3-E1
2e-
CO2
HS-CoA

Figure 1.2. Major biosynthetic pathways found in Methanococcus. Includes the reductive incomplete TCA cycle (RI-TCA) and the gluconeogenesis pathway for glycogen storage. (Modified from Shieh and Whitman, 1987)

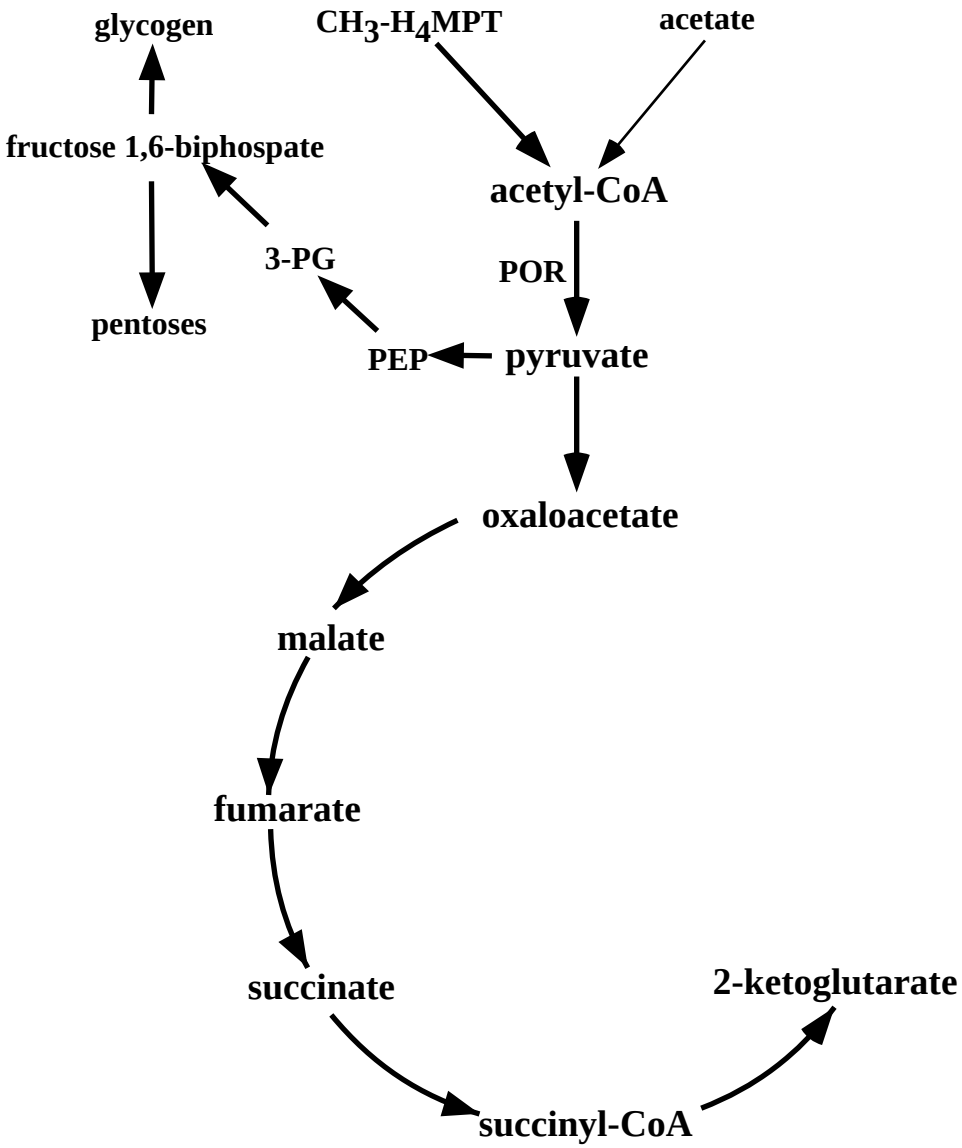

glycogen
CH3-H4MPT
acetate
fructose 1,6-biphospate
acetyl-CoA
3-PG
POR
pentoses
PEP
pyruvate
oxaloacetate
malate
fumarate
succinate
2-ketoglutarate
succinyl-CoA

Figure 1.3. Proposed pathway of carbohydrate, glycogen and ribose synthesis in *Methanococcus*. Modified from Yu et al., 1994.

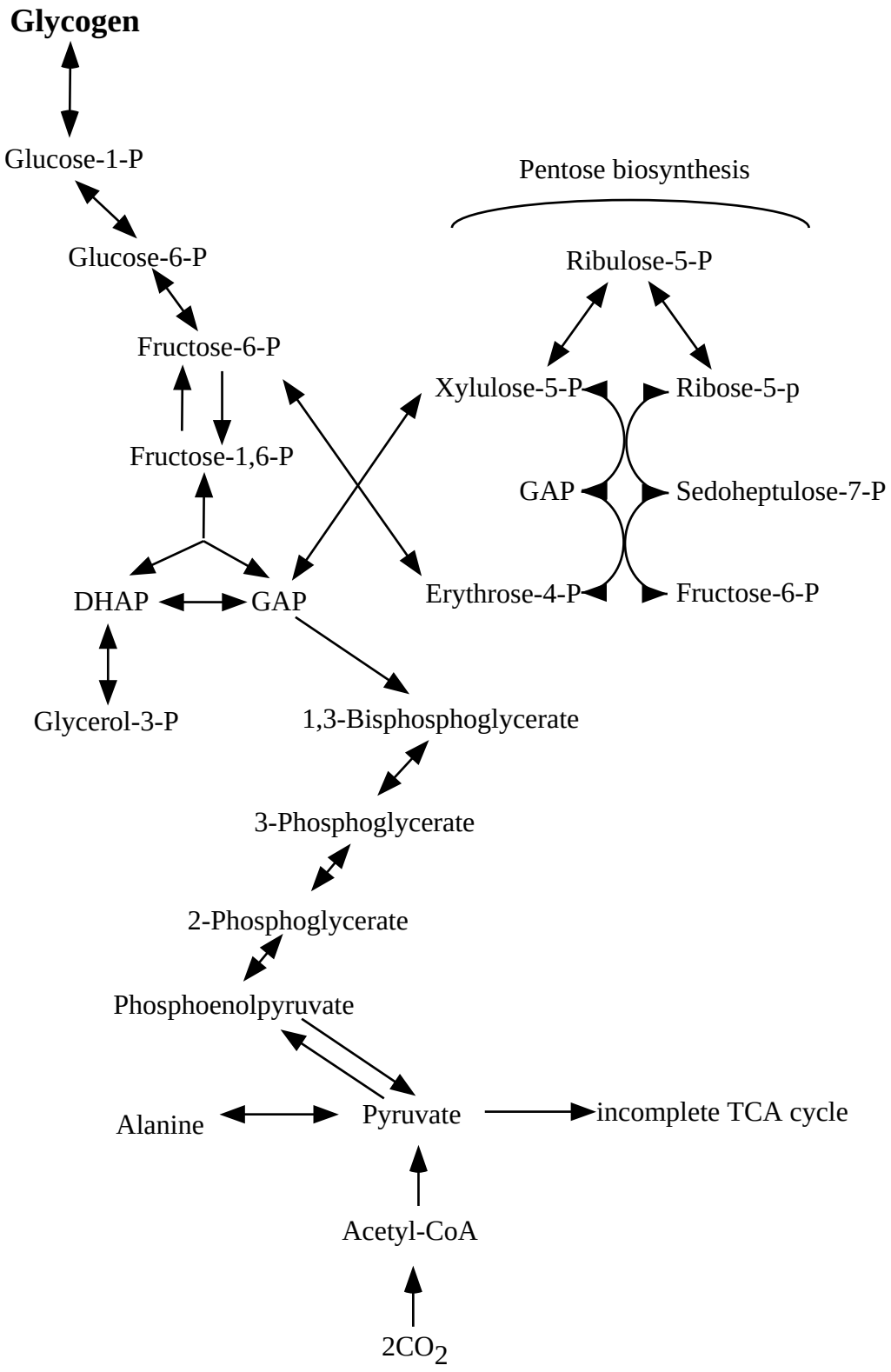

Glycogen
Glucose-1-P
Glucose-6-P
Fructose-6-P
Fructose-1,6-P
DHAP
GAP
Glycerol-3-P
1,3-Bisphosphoglycerate
3-Phosphoglycerate
2-Phosphoglycerate
Phosphoenolpyruvate
Alanine
Pyruvate
incomplete TCA cycle
Acetyl-CoA
2CO2
Pentose biosynthesis
Ribulose-5-P
Xylulose-5-P
Ribose-5-p
GAP
Sedoheptulose-7-P
Erythrose-4-P
Fructose-6-P

Figure 1.4. Sequence of reaction in the enzyme complex pyruvate dehydrogenase. E1 is the pyruvate dehydrogenase/decarboxylase. E2 is the acetyltransferase component. E3 is the dihydrolipoamide dehydrogenase component. E1 oxidatively decarboxylates pyruvate in conjunction with TPP to form acetyl lipoic acid which is attached to E2. E2 also catalyzes the transfer of the acetyl group to HS-CoA to form acetyl-CoA. E3 utilizes NAD^+ to reoxidize the dihydrolipoamide group from E2 (Modified from de Kok et al, 1998)

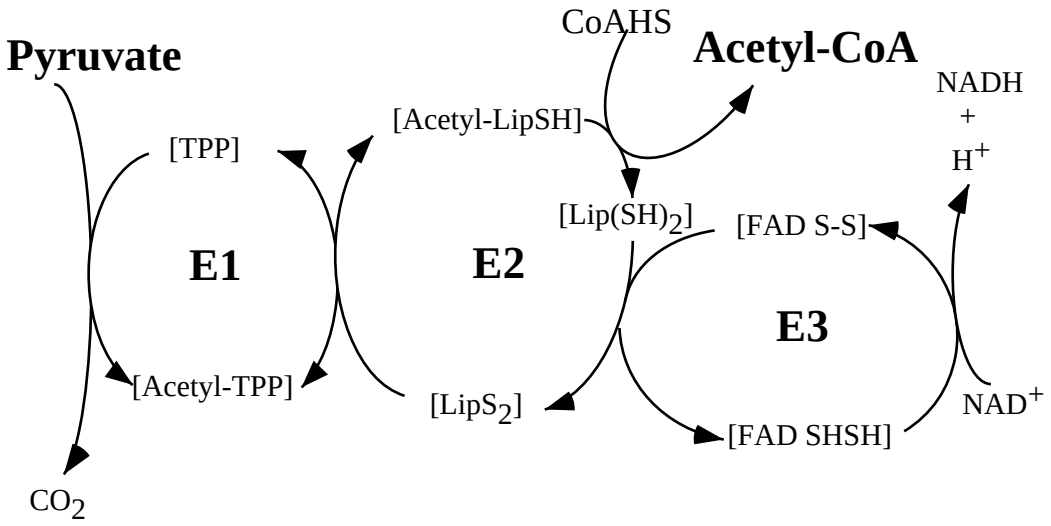
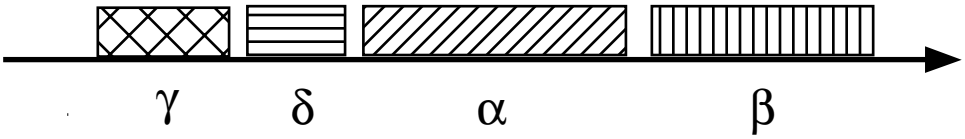

Pyruvate
CoAHS
Acetyl-CoA
NADH
+
H+
[TPP]
[Acetyl-LipSH]
[Lip(SH)2]
[FAD S-S]
E1
E2
E3
[Acetyl-TPP]
[LipS2]
[FAD SHSH]
NAD+
CO2

Figure 1.5. Types of PORs. A. □□□□type POR commonly found in hyperthermophiles. B. □□ type POR commonly found in aerobic Archaea. C. 2□ type PORs commonly found in mesophilic bacteria and eukaryotes. Similar hatch/shading marks denote regions of homology between subunits or within subunits.

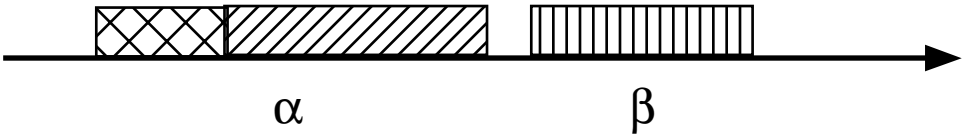
A.

αβδγ **type POR**



B.

αβ/α2β2 **type POR**



C.

2α **type POR**

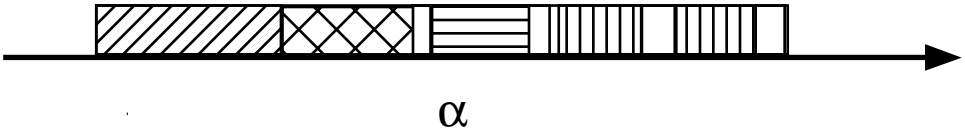


Figure 1.6. Proposed POR catalytic pathways for the archaea *Halobacterium salinarium* (A) and *Pyrococcus furiosus* (B). From Kersher and Oesterhelt , 1982 (diagram A); and Smith et al., 1994 (diagram B).

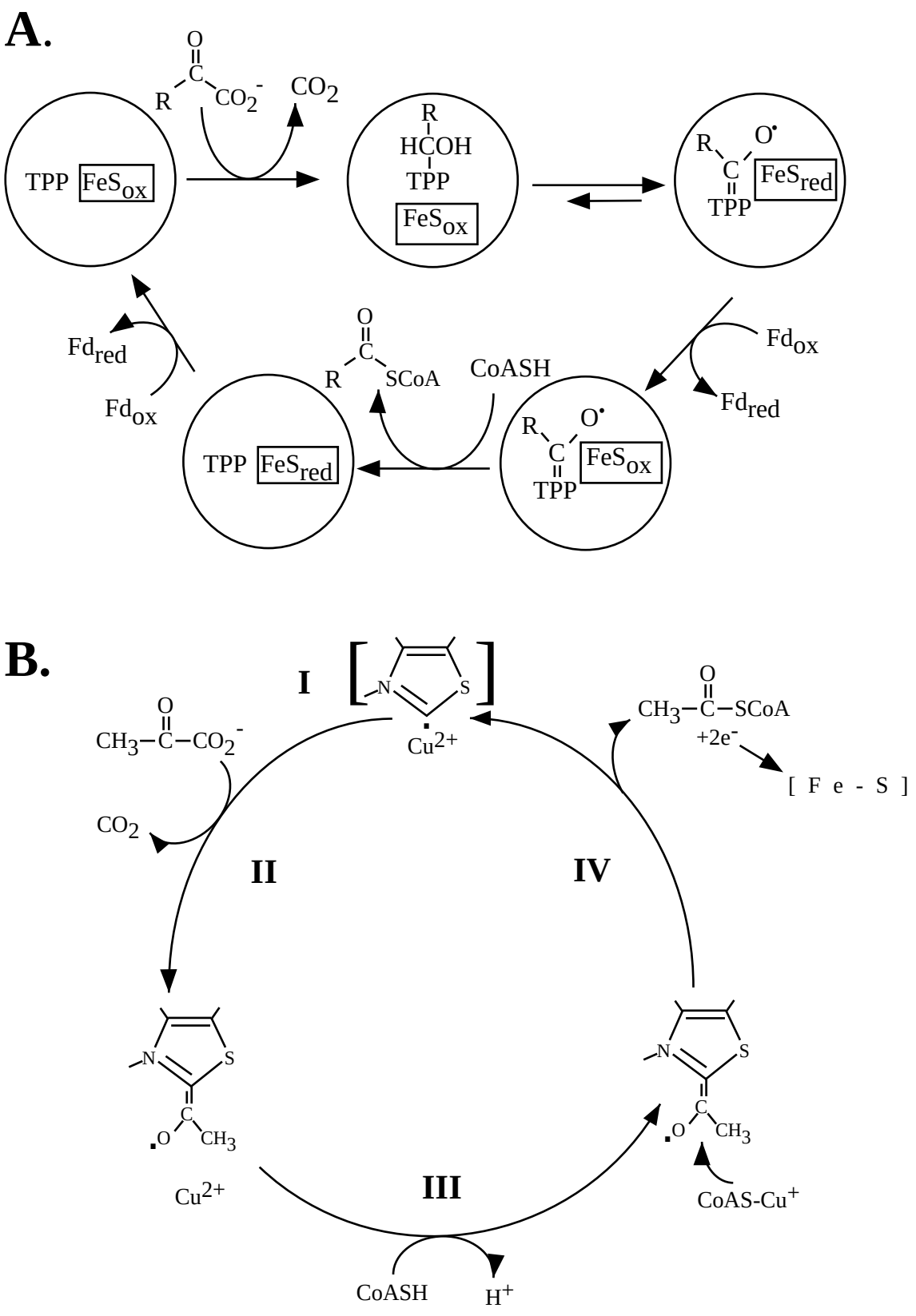

A.
TPP FeSox
CO2
R
HCOH
TPP
FeSox
R
O•
C
TPP
FeSred
Fdox
Fdred
CoASH
R
SCoA
TPP FeSred
R
O•
C
TPP
FeSox
Fdred
Fdox
B.
I
Cu2+
CH3–C–CO2-
CO2
II
N
S
C
O
CH3
Cu2+
III
CoASH
H+
IV
CH3–C–SCoA
+2e-
[F e - S]
CoAS-Cu+

Figure 1.7. Proposed catalytic pathway for the POR from the bacterium *Thermotoga maritime*. (From Smith et al., 1994).

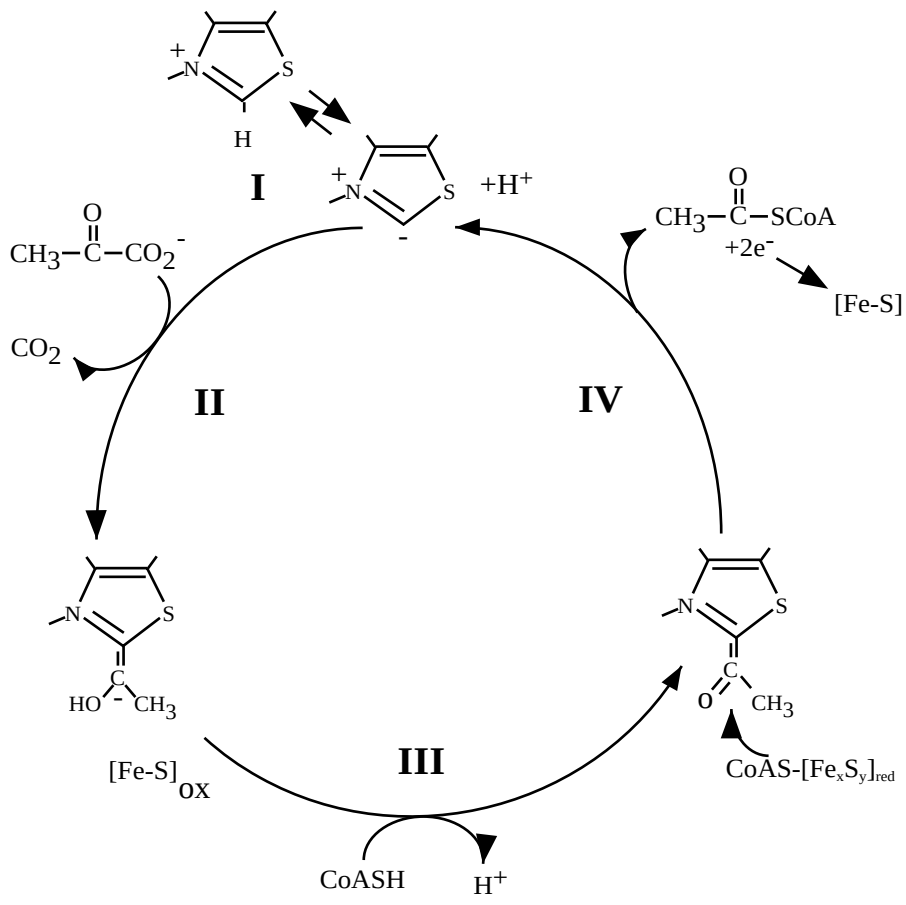
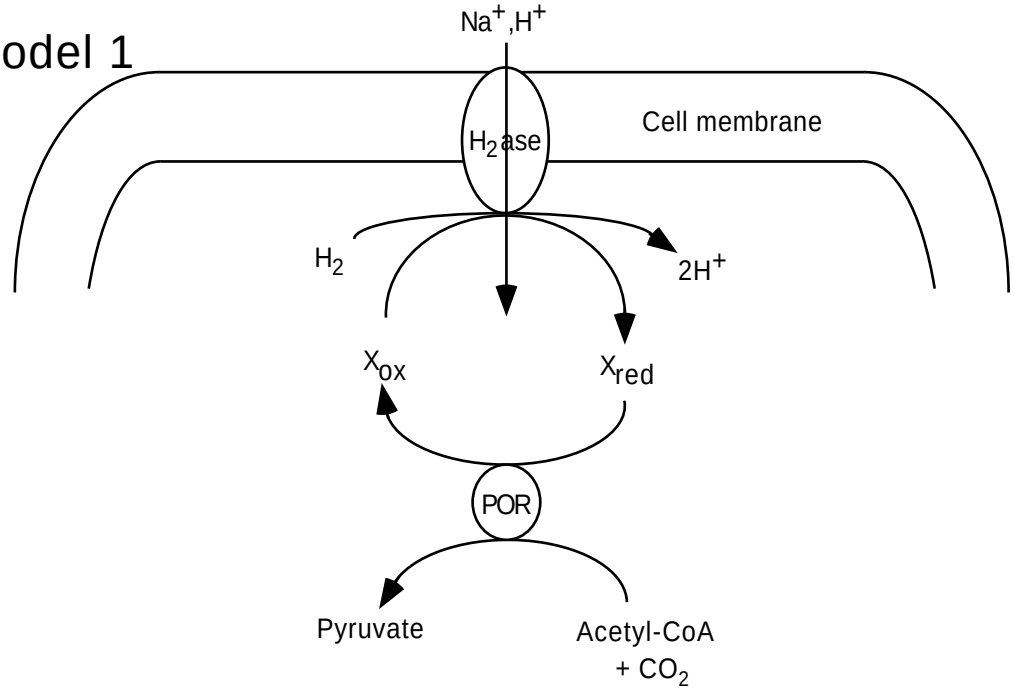
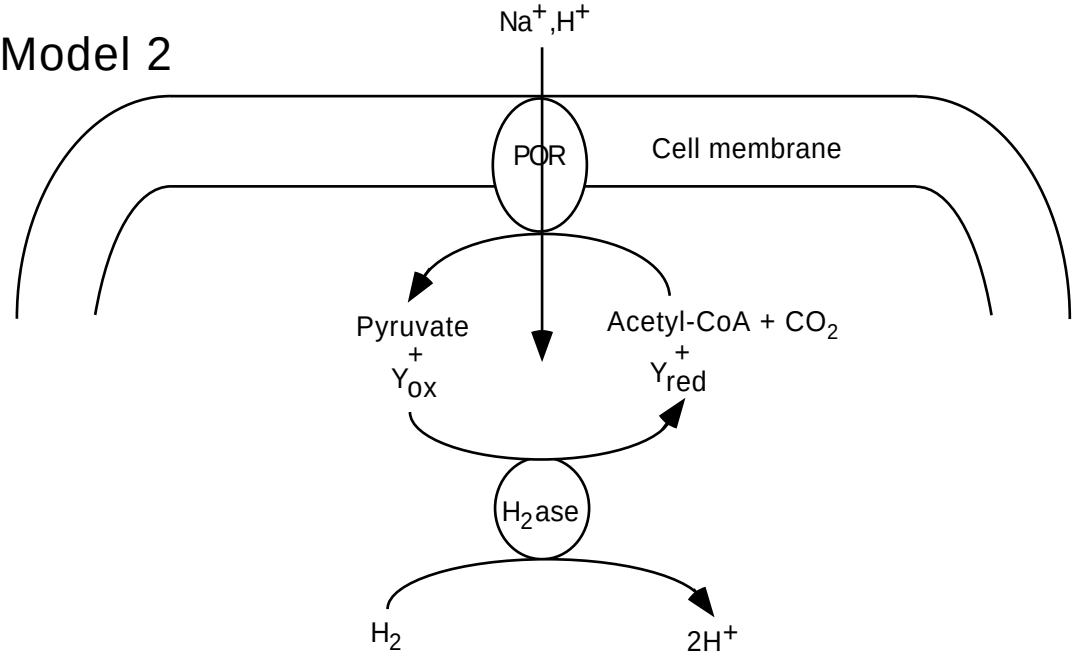

I
II
III
IV
+H+
CH3–C(=O)–CO2-
CO2
CH3–C(=O)–SCoA
+2e-
[Fe-S]
[Fe-S]ox
CoASH
H+
CoAS-[FexSy]red

Figure 1.8. Models for pyruvate synthesis by POR in hydrogenotrophic methanogens. Model 1. POR is linked to PMF through an extremely electronegative electron donor and hydrogenase. Model 2. POR is membrane bound and utilizes the energy in the PMF to biosynthesize pyruvate from an electropositive electron donor.

Model 1



Model 2



CHAPTER 2

THE GENES OF THE ANABOLIC PYRUVATE OXIDOREDUCTASE FROM *Methanococcus maripaludis* STRAIN JJ[1]

[1] Lin W.C. and W.B. Whitman. To be submitted to *Archives of Microbiology*.

ABSTRACT

In autotrophic methanogens, pyruvate oxidoreductase (POR) plays a key role in the assimilation of CO_2 and biosynthesis of organic carbon. Previously, this enzyme was purified to homogeneity and contained five polypeptides with M_rs of 47,000, 33,000, 25,000, 21,500, and 13,000. From the previously determined N-terminal sequence, four of the polypeptides were determined to be similar to subunits commonly associated with this enzyme from other Archaea. However, the polypeptide with a M_r of 21,500 had not been previously observed in PORs. From this work, nucleotide sequencing of the gene cluster encoding the POR revealed six open reading frames (*porABCDEF*). The genes *porABCD* corresponded to the subunits previously identified in PORs. It was determined that *porE* was the gene that encoded the 21,500 M_r polypeptide from the N-terminal sequence. Additionally, the translated polypeptide PorE, contained a high cysteinyl residue content and a motif indicative of a Fe/S cluster. The *porF* gene also possessed a high sequence similarity to *porE*, a high cysteinyl residue content, and two Fe/S cluster motifs. Homologs to both *porE* and *porF* were also present in the genomic sequences of the autotrophic methanogens, *Methanococcus jannaschii* and *Methanothermobacter thermautotrophicus*. Based upon these results, it is proposed that PorE and PorF are components of a specialized system required to transfer low potential electrons for pyruvate biosynthesis.

INTRODUCTION

Methanogenic bacteria are a major physiological group of Archaea that obtain energy for growth by producing methane. Many typical hydrogenotrophic methanogens, such as *Methanococcus maripaludis*, are unable to oxidize complex organic substrates

and the major methanogenic substrates are H_2+CO_2 or formate (Whitman, 2001). The major carbon sources are either CO_2 or acetate. During autotrophic CO_2 fixation, acetyl-CoA is formed by the Ljungdahl-Wood pathway (Shieh and Whitman, 1988; Ladapo and Whitman, 1990). When acetate is present, acetyl-CoA is formed by acetate thiokinase (Shieh and Whitman, 1987). From acetyl-CoA, carbon enters the major biosynthetic pathways by reductive carboxylation catalyzed by pyruvate oxidoreductase (POR; Shieh and Whitman, 1987). Therefore this reaction is important for both autotrophic growth and heterotrophic growth on acetate.

The activity of POR has been detected in a wide range of microorganisms, including the Archaea, Bacteria, anaerobic protozoa, and fungi. POR catalyzes a reversible reaction that has different physiological roles in microorganisms (for reviews, see Adams and Kletzin, 1996; and Chabriere et al, 1999). For many heterotrophic bacteria, pyruvate oxidation by POR provides the electrons for nitrogen fixation and anaerobic respiration (Bogusz et al., 1981). Pyruvate is also oxidized during the acetate fermentation of sugars in homoacetogenic bacteria (Furdui and Ragsdale, 2000). In autotrophic bacteria or bacteria that utilize acetate as a carbon source, POR biosynthesizes pyruvate. Because the biosynthetic reaction requires a strong reductant, it has been more difficult to demonstrate. The biosynthetic activity of the enzyme from the hydrogenotrophic bacterium *Hydrogenobacter thermophilus* was demonstrated with dithionite-reduced ferredoxin (Yoon et al., 1997). Similarly, in the presence of ferredoxin, carbon monoxide dehydrogenase (CODH) and CO, the POR from *Moorella [Clostridia] thermoaceticum* readily biosynthesizes pyruvate (Furdui and Ragsdale, 2000). Lastly, low potential ferredoxins reduced by the light reaction of spinach

chloroplasts or *Chlorobium* reaction centers drive pyruvate biosynthesis by the *Chlorobium tepidum* POR (Yoon et al, 2001; Yoon et al, 1999). However, with the exception of the light-driven reductions, these reactions are nonphysiological, and the mechanisms by which hydrogen-consuming lithotrophs generate low potential electron donors for POR are not clearly understood.

In our previous studies on whole cells of methanococci, the oxidative activity of POR could not be demonstrated *in vivo* under the normal growth conditions with H_2 (Yang et al, 1992). For instance, in an acetate auxotroph of *M. maripaludis*, the nutritional requirement for acetate cannot be replaced by pyruvate (Yang et al., 1992). Similarly, the close relative *Methanococcus voltae* requires acetate for growth and pyruvate will not substitute (Whitman et al., 1982). However, in the absence of the physiological electron donors H_2 and formate, methanogenesis from pyruvate was detected in the resting cells of both *M. maripaludis* and *M. voltae* (Yang et al. 1992). Therefore, at least for some conditions, pyruvate is taken up and oxidized. Pyruvate was also used as the sole energy and carbon source by mutants of *Methanosarcina barkeri* (Bock et al., 1994; Rajagopal and LeGall, 1994). These results suggested that the direction of catalysis by POR is strictly regulated in methanogenesis. In methanococci, the oxidative activity of POR is readily demonstrated in cell-free extracts, but the biosynthetic activity is extremely labile. For instance, incubation of extracts under N_2 gas for one hour reduces the activity by 60% (Shieh and Whitman, 1988). In addition, the physiological electron carrier is not certain. A ferredoxin purified from the closely related *Methanothermococcus thermolithotrophicus* was not reduced by POR (Hatchikian et al, 1989). However, the genomic sequence of *M. maripaludis* contains 18 ORFs

annotated as ferredoxin or ferredoxin-related proteins (J. Leigh, personal communication), so it is possible that another ferredoxin may be coupled to this enzyme. Furthermore, the POR from *M. maripaludis* was purified to homogeneity (Yang et al., submitted; Yang et al 1994). From this purification, the methanococcal POR was determined to be similar to the four subunit hyperthermophilic POR from *Pyrococcus furiosus*. However, an additional polypeptide co-purified with the POR. Therefore, in order to characterize this enzyme further in methanococci, the nucleotide sequence of its genes were determined.

MATERIALS AND METHODS

Bacterial Strains. *M. maripaludis* strain JJ was obtained from W.J. Jones (Jones et. al., 1983). *Escherichia coli* strain Top 10 was obtained from Invitrogen (Carlsbad, Ca).

Media, culture conditions, and preparation of cell extracts. *M. maripaludis* was grown with 275 kPa of H_2:CO_2 (80:20) at 37°C in McN (mineral medium) or McC (complex medium minus vitamin solution) as described by Whitman et al. (1986). Broth cultures, 5 ml, were grown in 28 ml stoppered tubes (Bellco. Elrudo, NJ) using strictly anaerobic procedures (Balch et al. 1979). Cultures were repressurized several times each day.

E. coli strain Top 10 was grown at 37°C on low salt Luria-Bertaini medium as described by Invitrogen (Carlsbad, CA). For liquid and solid medium, kanamycin (50 □g ml^{-1}) and ampicillin (50 □g mg^{-1}) were used depending on the plasmid (pZeRO-2 or pIJA03/02). Plasmids were transformed into *E. coli* strain Top 10 with a Gene Pulser (BioRad, Richmond, Ca) at 200 □, 2.5 kV, and 25 □F with 0.2 mm gap cuvettes.

Growth of both *M. maripaludis* and *E. coli* were monitored at 600 nm with a spectrophotometer (Spectronic 20, Baush and Lomb).

Probe construction, Southern hybridization and screening of clones. A probe, MJPOR, containing a portion of the *Methanococcus jannaschii* genes for POR was constructed from plasmid AMJHM83 (American Type Culture Collection, MD). The cloned DNA was excised from the plasmid using *Sac*I and *Xba*I. The resulting 1.7 kb fragment was gel-purified using the Wizard plus Miniprep DNA Purification System (Promega, Madison, WI) and radiolabelled using a modified random nucleotide-primed synthesis method (Lehman, 1989). In this method, the fragment was first denatured in a boiling water bathe for 10 minutes and incubated on ice. The denatured fragment was labelled following an incubation with a mixture of nucleotides (dTTP, dCTP, dGTP, 2 nM each), hexanucleotide mix (Boehringer Mannheim, Germany), Klenow fragment (5U), and 20 nM [a^{32} P] dATP (3000Ci/mMol) at 37 C for 4 hrs. It was then desalted using a NucTrap Probe Purification column (Stratagene, La Jolla, Ca).

M. maripaludis genomic DNA was isolated by a modification of the procedure of Saito and Miura (1963) as described previously (Gardner and Whitman, 1999). Prior to Southern hybridization, the *M. maripaludis* genomic DNA was digested with *Hin*dIII, *Eco*RI or both enzymes at the same time. For Southern Hybridization, the digested DNA fragments (15 mg) were electrophoresed and transferred to a Magnacharge nylon membrane (MSI, Ma). Prehybridization and hybridization using the probe MJPOR was performed at 28°C for 4 and 10 hrs, respectively, in 2XSSC. Membrane washing was performed twice for 15 min at 38°C in 0.5 X SSC. Following a 10 hr exposure to the phosphor screen, the hybridization was visualized with Phosphorimager:SI (Molecular

Dynamics, Sunnyvale, CA) and quantified using the ImageQuant v. 1.0 software (Molecular Dynamics, Sunnyvale, CA). The hybridization revealed a 1.4 kb *Hind*III fragment, a 6.1 kb *Eco*RI fragment and a 1.4 kb fragment from the double digest.

Because of the low stringency of the hybridization of the MJPOR probe to the *M. maripaludis* DNA, the *Hin*dIII fragment was isolated first and used as a probe for the 6.1 kb *Eco*R1 fragment. First, the genomic DNA was digested with *Hin*dIII and electrophoresed, and the 1.4 kb region of the gel was excised. The DNA was purified with the Wizard Plus Miniprep system (Promega, WI) and ligated into *Hin*dIII-digested pZErO cloning vector (Invitrogen, Ca) The ligation mixture was then transformed into *E.coli* Top10 cells (Invitrogen, Ca). Plasmid DNA purified from kanamycin resistant isolates were screened by Southern hybridization with the probe MJPOR using the same condition as described above. Those clones hybridizing to MJPOR were grown in 5 ml cultures of LB broth plus kanamycin and purified by Qiagen spin minipreps. The plasmid DNA (pZErO+POR *Hin*dIII) from the clones was sequences and shown to contain sequences homologous to published POR sequences. The radiolabelled probe MmPOR was then constructed from the plasmid via PCR using primers PorMj83-5' (5'-AGTCATCCTCTGCCTTTTCA-3') and PorMj83-3' (5'-GATTACAAGACCTT CAGGG-3'). The amplification solution consisted of 50 pg DNA template, 25 pmol of each primer, 200 mM dNTP (dTTP, dCTP, dGTP), 20 nM of [α-^{32}P] dATP(3000 Ci/nmol), and 1.5 U of Taq polymerase (Promega, Madison, Wi) along with the reaction buffer supplied with the enzyme. After the denaturation of the DNA at 95°C for 5 minutes, the PCR consisted of 25 cycles of 94°C, 1 min; 50 °C, 1 min; and 72 °C, 1 min. The final PCR amplicon was separated from unincorporated nucleotides with a NucTrap

Probe purifiacatin column. The 6.1 kb *Eco*RI fragment was then isolated from a partial library of *Eco*RI-digested genomic DNA in the same manner as the *Hin*dIII fragment except that the MmPOR□□ probe was used.

Sequencing and analysis. Plasmids from *E. coli* Top 10 isolates hybridizing to the probes were obtained from a 5 ml culture of LB broth plus kanamycin and purified (Quiagen spin miniprep, Ca). The genomic inserts were sequenced with M13 reverse primer 5'-CAGGAAACAGCTATGAC-3' and the M13 forward primer 5'-GTAAAACGACGGCCAG-3', which were complementary to flanking regions of pZErO-2 (Invitrogen, Ca). Through primer walking, the DNA was sequenced on both strands. Sequencing was performed on an Applied Biosystems automated sequencer (PE Applied Biosystems, Ca) at the Molecular Genetics Facility at the University of Georgia. The sequences were assembled using the program Sequencher (Gene Codes Corp., MI).

Sequence analysis of *Pyrococcus furiosus, Methanothermobacter thermautotrophicus, Methanococcus jannaschii* and *M. maripaludis*, and *Helicobacter pylori* POR was accomplished through GCG (genetic Computer Group, Madison, WI) program MOTIFS (identification of potential Fe-S sites). The TPP binding sites were identified through the manual input of the consensus sequence, GDG{X}{7}E{X}{4}A{X}{13}N (Hawkins et al., 1989), using the MOTIFS program as well. Identification of hydrophobic regions in the POR sequences was accomplished using the web based programs TMpred (www.ch.embnet.org) and Protscale (ExPasy .com).

For the phylogenetic analysis, nucleic acid sequences were obtained from the GenBank-EMBL database and translated into polypeptide sequences using the FRAMES

programs from GCG. Similarity searches were performed using BLAST and FASTA programs from GCG (Genetics Computer Group, Madison, WI) and the TIGR website. Sequences of homologs from other euryarchaeotes of about the same size were chosen for further analysis. Unaltered sequences, subsets of sequences, and conserved regions within the sequences were used for multiple alignments using PILEUP and BOXSHADE programs of GCG. Phylogenetic analyses were performed using PHYLIP v. 3.57 (Felsenstein, 1989). Evolutionary distances were determined with PROTDIST, and the Neighbor-joining and Fitch-Margoliash dendrograms were generated with NEIGHBOR and FITCH, respectively. The SEQBOOT program was used to calculate bootstrap values for both algorithms based upon 100 replicate trees. Parsimony analyses were performed with PROTPARS. The Genbank accession number for the *M. maripaludis* 6.1 kbp *Eco*RI fragment containing the POR genes is AF230199.

RESULTS

Identification of the *M. maripaludis por* cluster. The *M. jannaschii* genomic sequence possessed two clusters of ORFs annotated as ferredoxin-dependent oxidoreductases (Bult et al., 1996). One of these was identified as the POR based upon similarity to the N-terminal sequences from the *M. maripaludis* polypeptides. A probe for the *M. maripaludis* genes, MJPOR (Figure 2.1), was then constructed from the plasmid AMJHM83. This plasmid contained a 1.7 kb insert that included *porC* in its entirety and portions of *porB* and *porD* from *M. jannaschii*. Southern hybridization identified 6.1 kb and 1.4 kb fragments from *Eco*R1- and *Hin*dIII- digested *M. maripaludis* genomic DNA, respectively. Double digestion of genomic DNA confirmed that the *Hin*dIII fragment was internal to the *Eco*RI fragment. A partial library was

created using *Hin*dIII- digested *M. maripaludis* genomic DNA and screened by hybridization to MJPOR. Upon sequencing, the clone containing the 1.4 kb *Hin*dIII insert DNA was found to include sequences homologous to a region within the *porC* from the *M. jannaschii por* gene cluster. The *Hin*dIII fragment from *M. maripaludis* was then used as a probe to isolate a clone possessing the 6.1 kb *Eco*RI fragment.

Analysis of the nucleotide sequence of the 6.1 kb *Eco*RI fragment revealed nine complete and one partial ORFs, four of which (*porABCD*) had homology to POR genes identified from *P. furiosus* and *M. jannaschii* (Figure 2.1; Adams and Kletzin, 1996; Bult et al., 1996). The homologous genes from *M. thermautotrophicus* appeared to contain a fusion of the *porA* and *porB* genes from these other organism. The percent similarity of the amino acid sequences of the genes in the *M. maripaludis por* gene cluster to *P. furiosus, M. jannaschii,* and *M. thermautotrophicus* genes was in the range of 48-79% (Figure 2.1). The genes *porA*, *porB*, *porC*, *porD* were 527, 254, 1160, and 890 bp, respectively, and encoded polypeptides of approximately 19.6, 9.4, 42.1, and 33.2 kDa, respectively. These estimated molecular weights corresponded favorably to the M_r of the polypeptides from the purified enzyme (Yang el al., submitted; Yang, 1994). Additionally, the deduced N-terminal sequences of *porABCD* corresponded to the N-terminal sequences of the *M. maripaludis* POR □, □, □, and □ subunits. Further analysis of the *porABCD* sequences revealed potential ribosomal binding sites for all four ORFs, motifs characteristic of conserved [4Fe-4S] cluster and TPP-binding site in *porD* (Figure 2.2). These motifs were very similar to those found in the *P. furiosus* and *M. jannaschii* genes (Figure 2.2) and fully consistent with the proposed crystal structure of the *D. africanus* POR (Chabriere et al., 1999). Additionally, the hydropathy plots were

generated for the identification of possible transmembrane regions in *M. maripaludis*. The α subunits contained one potential transmembrane region while the β subunit did not have any predicted transmembrane regions. The γ and δ subunits both contained two to four potential transmembrane regions. The translated PorE and PorF did not contain any predicted transmembrane regions. However the similarity of the putative transmembrane regions in the *M. maripaludis* subunits were very similar to the soluble PORs found in *M. thermautotrophicus* and *P. furiosus* (Figure 2.2) suggesting that the *M. maripaludis* POR is not membrane bound.

Analysis of *porEF*. Immediately downstream of *porD* were two ORFs, named here as *porE* and *porF*. These ORFs were expected to encode polypeptides of 167 and 138 amino acids, 18 and 17 of which were cysteinyl residues, respectively. They were named PorE and PorF to avoid confusion with the previously named subunits of the catabolic PORs. One motif indicative of [4Fe-4S] clusters was present in *porE*, while two motifs were present in *porF*, suggesting a possible function in electron transfer (Figure 2.3). In addition, there were a total of 16 conserved cysteine residues found in the polypeptide. The estimated molecular weight of PorE was 18.5 kDa, which was similar to the determined M_r of the 21,500 MW polypeptide that copurified with the POR. In addition, the N-terminal sequence of this polypeptide was identical to that of the translation product of *porE*.

Homologs with high similarity to PorE and PorF were also present immediately downstream of the *por* gene clusters in the genomic sequences of the autotrophic methanogens *M. jannaschii* (MJ0265 and MJ0264, respectively) and *M. thermautotrophicus* (MTH1737 and MTH1736, respectively) but not in the gene clusters

encoding PORs from other archaea such as *Archaeoglobus fulgidus* and *P. furiosus*. Other homologous cysteine-rich ORFs were also found immediately downstream to the CODH/ACS gene cluster in *M. maripaludis, M. jannaschii*, and *M. thermautotrophicus*. In phylogenetic analyses, the homologs associated with *porF* and CODH/ACS gene clusters grouped together (Figure 2.4). Even though the bootstrap support for these clusters was low, the clusters were robust enough to be found by all three algorithms used and in both an alignment of the nearly complete sequences and an alignment of only the most conserved regions. While the *porE* homologs formed a cluster with low bootstrap support in the Neighbor-joining and the Fitch-Margolaish analysis (Figure 2.4), MTH1737 formed a deep branch associated with the homologs from the CODH/ACS gene cluster in the most parsimonious trees. The somewhat different placement by different algorithms was not surprising given the small sizes of these sequences and the small number of informative positions. Together, the phylogenetic similarities and locations on the genomes suggested that these genes played similar functions in the different hydrogenotrophic methanogens and that they were conserved within these organisms.

Other homologs with high sequence similarity formed separate clades. *M. jannaschii* possessed two additional homologs that were much larger in size. MJ1193 was greater that twice the size of *porE* and a subunit of the methyl viologen-reducing hydrogenase. It was also homologous to the *M. thermautotrophicus* ORF MTH1133. Similarly, the homolog MJ1303 contained greater than 300 amino acid residues more than *porE*. The *M. thermautotrophicus* genome sequence contained one additional homolog, MTH0105, that appeared to represent a fusion to a subunit of glutamate

synthase. *A. fulgidus* possessed four homologs (AF1202, AF0076, AF0950, AF2385) of about the same size as *porE*, but they were not linked to the gene clusters encoding the POR or CODH/ACS and did not group with he methanococcal ORFs in phylogenetic analyses (Figure 2.4). AF1202 was found in the genome near a subunit of formate dehydrogenase and was similar to a homolog in *Pyrococcus abyssii* PAB1390 (Figure 2.4), but there was no evidence for the function of the other ORFs. In addition, *A. fulgidus* contained AF0175, a homolog that was larger than *porE* and near a dimethylsulfoxide reductase subunit in the genome. Lastly, additional homologs were found in the *P. abyssii* and *P. horikoshi* genomic sequences. The ORFs PAB2028 and PH0893 (in *P. horikoshi*) were very similar and appeared next to the subunit of formaldehyde dehydrogenase. The ORFs PAB0213 and PH1750 were also very similar to each other and were near a sarcosine oxidase homolog in the genomic sequence. In addition to these homologs of high similarity, there were a large number of homologs of low similarity from a variety of bacteria. These ORFs were typically annotated as formate hydrogen-lyase subunit 2, homologs of YsaA and HydN, glutamate synthase, or ferredoxin.

DISCUSSION

Autotrophic methanogens such as *Methanococcus* utilize the POR anabolically to carboxylate acetyl-CoA formed via a modified Ljungdahl-Wood pathway into pyruvate. From here, pyruvate enters major biosynthetic pathways for the formation of cellular components. However, POR from non-autotrophic anaerobes such as *Pyrococcus* and *Clostridia* functions primarily to oxidatively decarboxylate pyruvate into acetyl-CoA**.** However, all POR can catalyze the reverse reaction in vitro.

The methanococcal POR appears to contain at least five subunits, each with a M_r of about 47,000, 33,000, 25,000, 21,000 and 13,000 corresponding to genes *porC,D,B,E* and *A* respectively. The 21,000 M_r fifth polypeptide, PorE contains cysteinyl residues in an Fe/S motif. In addition, nucleotide sequencing data demonstrated a potential sixth ORF, *porF* that has homology to *porE*. Other autotrophic methanogens, *M. jannaschii* and *M. thermoautotrophicum*, also show similar POR gene clusters to the *M. maripaludis* POR gene cluster. Interestingly, four of the polypeptides (□, □, □, and □) in the anabolic methanococcal POR are similar to the POR from the thermophilic archaea, *Pyrococcus furiosus*, and the extremely thermophilic eubacterium, *Thermotoga* which utilizes the POR for catabolic purposes (Blamey and Adams, 1994). Therefore, the homology of the methanococcal, methanobacterial and the pyrococcal POR leads to the conclusion that although each catalyzes a different reaction *in vivo*, the catabolic POR from *Pyrococcus* and four subunits of the anabolic methanococcal POR are homologous. Thus, catabolic and anabolic reactions may be catalyzed by closely similar enzymes but under different conditions.

From the natural environmental conditions that methanogens are found, the E' of the H_2/H^+ couple is –296 mV at 10^{-4} atm of H_2 (Zinder, 1993). Considering that the midpoint potential of the acetyl-CoA/pyruvate couple is about –500 mV (calculated from Thauer et al., 1977) and the calculated □G is +39 kJ/mol for the reduction of acetyl-CoA + CO_2, the reaction would be unfavorable in the presence of any physiologically likely concentration of substrates. This implies that additional energy is needed to drive the reaction.

How the hydrogenotrophic methanogen POR catalyses the bioenergetically unfavorable pyruvate synthesis is still unknown. One possibility is the hydrolysis of a high energy substrate such as ATP may be needed to drive pyruvate synthesis. This would assume that the hydrolysis product was necessary for pyruvate oxidation. However, Yang (1992) determined that ADP did not effect the activities of the purified POR and the catalytic properties of the methanococcal POR were similar to other PORs (For a review, see Adams and Kletzin, 1996). In addition, no evidence for other substrates were found from other sources. Additionally, the POR from *M. thermautotrophicus* appears to be a typical POR (Tersteegen et al., 1997)

Another possibility is the direct coupling of pyruvate synthesis to the proton motive force. In this model, the POR would be expected to be membrane associated. Although this possibility cannot be easily excluded, the purified methanococcal POR was found in the soluble fraction thus demonstrating that it is not an intrinsic membrane protein. Additionally, the hydropathy analysis on the methanococcal POR (Figure 2.2) demonstrate that the methanococcal POR is similar to the soluble □□□type POR from *P. furiosus*.

With the observation that most of the microorganisms that have H_2-evolving hydrogenase or nitrogenase activity exhibit physiological pyruvate oxidation activity, and those that have the H_2-consuming hydrogenase activity display pyruvate synthase activity, pyruvate synthesis by the methanogenic POR may be driven by the energy derived from a hydrogenase-linked proton-motive force (PMF). The energy in the PMF would be utilized to drive the unfavorable reduction of a very electronegative electron acceptor by H_2. Two homologs of the membrane-bound Ech hydrogenase found in *M.*

thermautotrophicus and *M. barkeri* are found in *M. maripaludis* (W.B. Whitman, unpublished results, and J. Leigh, personal communications; Kunkel et al, 1998; Meuer et al., 1999). This enzyme system is homologous to components of the mitochondrial complex I and is believed to couple H_2-oxidation to the PMF (Albracht et al. 2000). In addition, Meuer et al. (2002) has reported that Ech hydrogenase mutants of *Methanosarcina* lose the ability to biosynthesize pyruvate. This implies that the hydrogenase may play a role in generating strong reductants for the POR in *Methanosarcina* This leads to the question of what is needed to drive pyruvate synthesis assuming that the reaction is coupled to an H_2-consuming hydrogenase. Since the physiological function of methanococcal POR is catalyzing the synthesis of pyruvate from acetyl-CoA and CO_2 at a redox potential of pyruvate/acetyl-CoA of about –500 mV, it needs a hydrogenase coupled electron carrier that is negative enough to donate electrons to drive pyruvate synthesis. Unfortunately, the physiological electron donor for methanococcal POR is not clear. Previous results show that coenzyme F_{420}, the most common electron carrier in methanococci, is not the electron donor to the methanococcal POR (Yang, submitted; Yang, 1994). Additionally, Yang et al. (submitted) determined that FAD, FMN and rubredoxin are not the physiological electron acceptors as well. The utilization of C-type cytochromes as the electron donor is also not promising since the POR activity obtained with C-type cytochrome was quite low. Additionally, C-type cytochromes have only been detected in methanol-grown *Methanosarcina* but not in H_2/CO_2 grown *Methanosarcina* and *Methanococcus* (Kuhn et al., 1983).

One possible compound that can serve as the electron donor of methanococcal POR is ferredoxin. The redox potential range of ferredoxins is about -200 mV to -650

mV (Binert, 2000) which may be negative enough for pyruvate synthesis. Additionally, ferredoxins have been reported to be used by most of the PORs examined. In fact, Furdui and Ragsdale (2000) report the utilization of ferredoxin from *Moorella [Clostridium] thermoaceticum* as a sufficient electron donor for pyruvate synthesis with only a 10-fold lower K_{cat}/K_m value than that of pyruvate oxidation. A clostridial type ferredoxin, [4Fe-4S]-ferredoxin, also has been detected in *M. thermolithotrophicus* (Hatchikian et. al., 1989). Additionally, 18 ORFs with ferredoxin signatures have been detected in the genome sequence from *M. jannaschii* (J. Leigh, personal communication).

While an electron donor has not been found, this does not preclude the possibility of a specialized electron donor for the methanococcal POR. The genes *porEF* which are seemingly unique to PORs from autotrophic methanogens, appear to contain an abundance of cysteinyl residues in a [4Fe-4S] signature in their translated polypeptides. These clusters would suggest a ferredoxin-like electron carrying role. The co-purification of PorE with the POR indicates that it is able to bind to the POR complex in some manner. While the homologs to PorE and PorF are not well characterized, all play a role in electron carriers. Annotated as formate hydrogenlyase subunit 2, a domain of the large subunit of glutamate synthase, or ferredoxin, they appear to function over a broad range of midpoint potentials more electronegative than quinones (Böck and Sawers, 1996). In addition, analysis has revealed an ORF with homology to *porEF* immediately downstream to the autotrophic methanogen carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH) gene. The CODH is another major enzyme involved with autotrophy in methanogens and links methanogenesis to a modified Ljundahl-Wood pathway for the synthesis of acetyl-CoA. Furthermore, in other CODH/ACS and POR gene clusters from

other organisms, notably from the aceticlastic methanogen *Methanosarcina*, these cysteine-rich genes are not present at neither the POR nor the CODH/ACS. Analysis of the genome sequence from *A. fulgidus*, another autotrophic archaeon, has revealed ORFs homologous to *porEF*. However, these ORFs were not associated with any operon. The association of these gene with autotrophic enzymes in methanogens suggests a connection between these genes and autotropy. While further experiments will be necessary, it is proposed that these genes may encode proteins with extremely low redox potential capable of donating electrons for pyruvate synthesis (POR) or acetyl-CoA synthesis (CODH/ACS).

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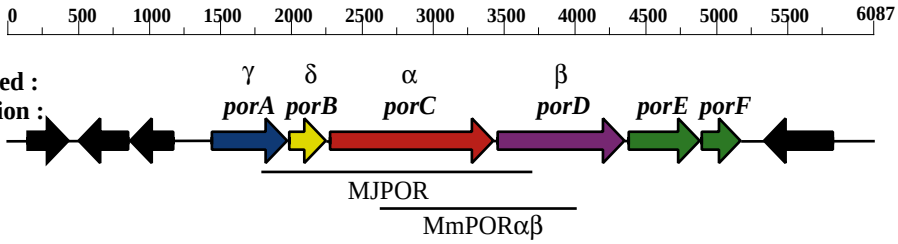
Figure 2.1. Sequence of the 6.1 kb *Eco*RI fragment containing the *por* genes from *M. maripaludis* genomic DNA. MJPOR represents the location of the *M. jannaschii* clone that was used to isolate the original *M. maripaludis Hin*dIII fragment MmPOR□□. Hybridization to the *Hin*dIII fragment was used to isolate this *Eco*RI fragment encoding the entire gene cluster.



***M. jannaschii* homologs**

MJ number:	1160	0068	0273	0269	0268	0267	0266	0265	0264	0623

Amino acid identity (%)

***M. jannaschii* :**	73	77	71	72	77	73	79	63	53	56
***M. thermautotrophicus* :**				67	64	50	62	41	49	
***P. furiosus* :**				59	60	50	48			

Figure 2.2. Comparative analysis and motifs found in POR subunits from several Archaea.. The following symbols are used: (▭) potential Fe/S binding motif, (▭▭▭) potential transmembrane helices, (▭) TPP binding motif, and (ı) conserved cysteinyl residues that bind an Fe-S cluster. Lines interconnecting motifs indicates conserved motifs. *M. maripaludis* strain JJ (MM-JJ), *M. jannaschii* (MJ), *M. thermautotrophicus*(Mth), *P. furiosus*(PF), *A. fulgidus*(AF)

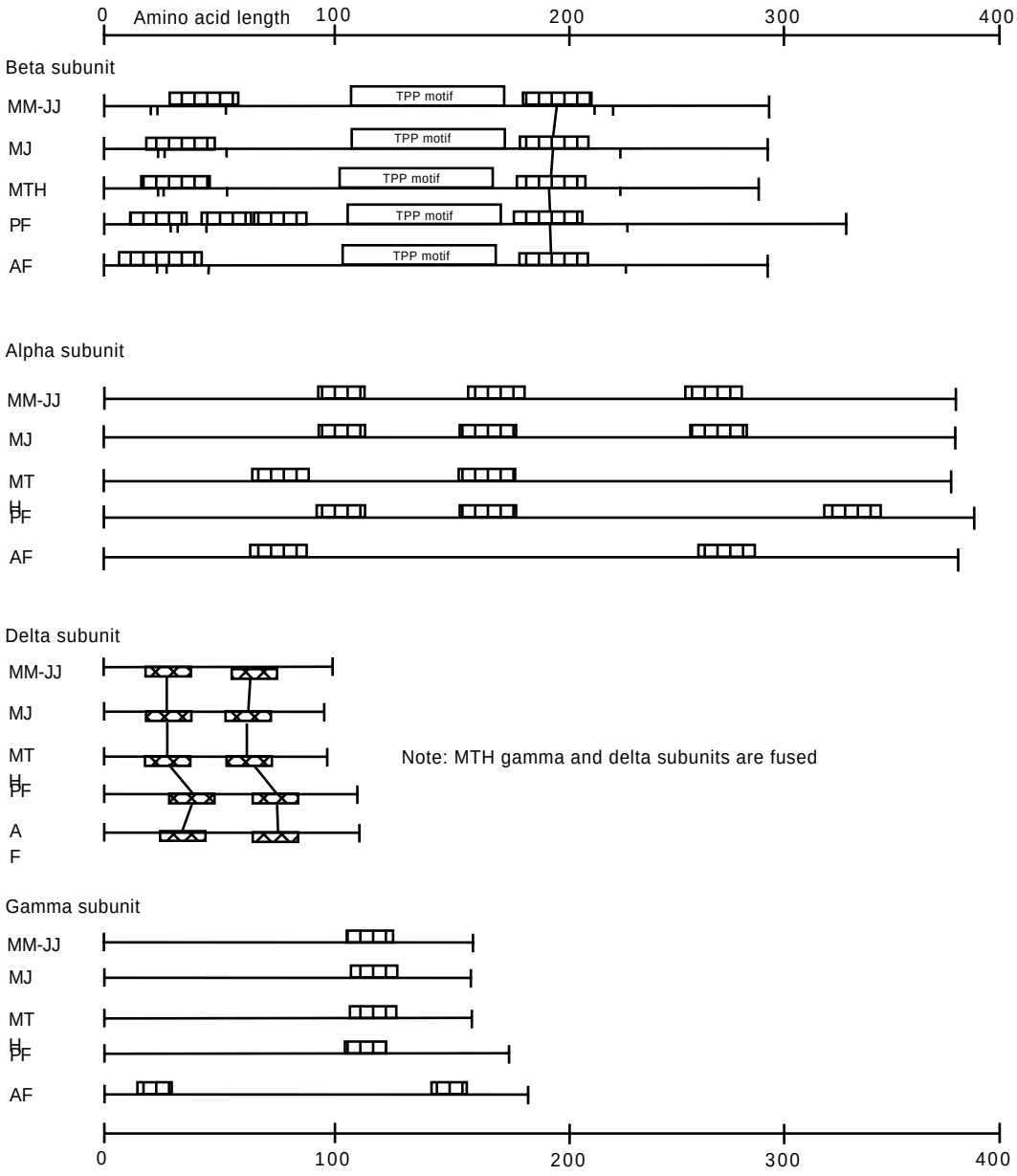
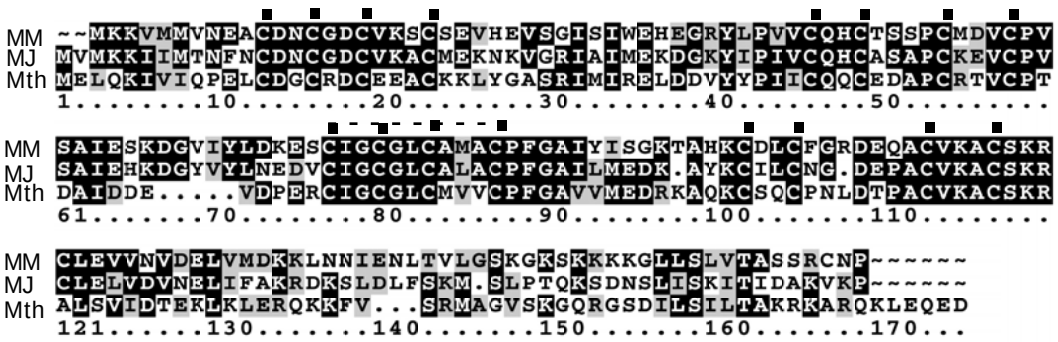

0
Amino acid length
100
200
300
400
Beta subunit
MM-JJ
MJ
MTH
PF
AF
TPP motif
Alpha subunit
MM-JJ
MJ
MT
H
PF
AF
Delta subunit
MM-JJ
MJ
MT
H
PF
A
F
Note: MTH gamma and delta subunits are fused
Gamma subunit
MM-JJ
MJ
MT
H
PF
AF
0
100
200
300
400

Figure 2.3. Comparative analysis and motifs found in PorE and PorF homologs from *M. maripaludis* strain JJ (MM), *M. jannaschii* (MJ). and *M. thermautotrophicus* (Mth). Cysteine residues are denoted by a dot. Dotted lines denotes areas where C-X(2)-C-X(2)-C-X(3)-C-[PEG] consensus sequence for 4Fe-4S binding sequence were found.

PorE homologs



PorF homologs

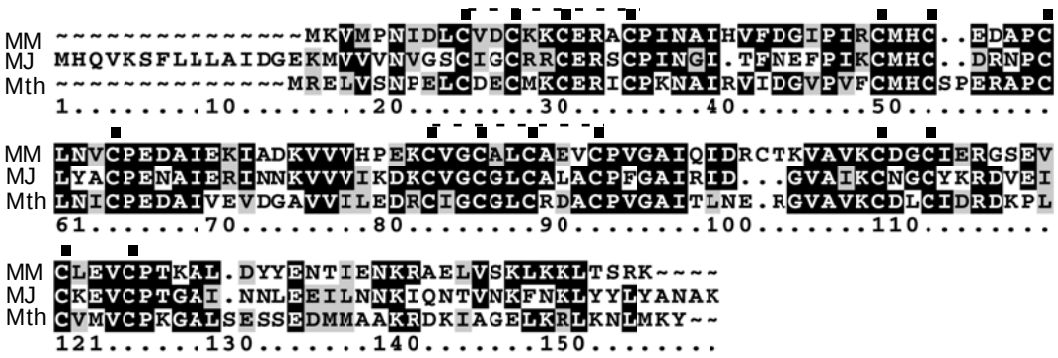
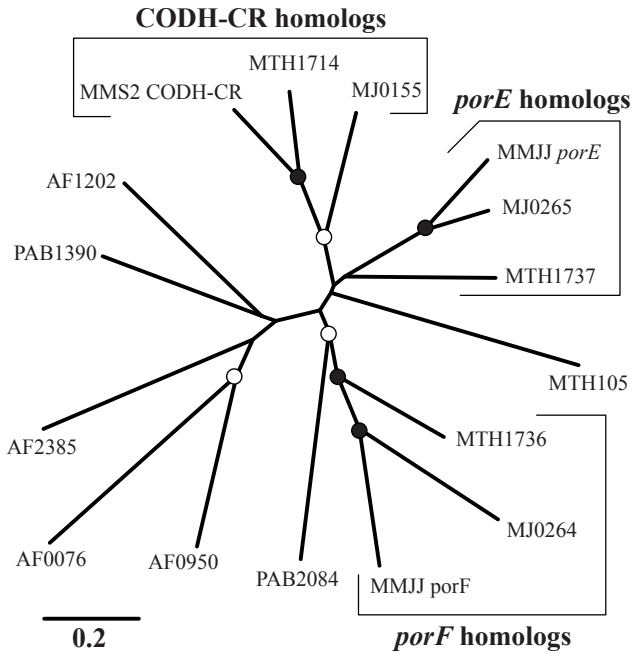


Figure 2.4. Phylogenetic relationship of the PorE homologs associated with the POR and CODH/ACS gene clusters of the hydrogenotrophic methanogens *M. maripaludis* (MMJJ for strain JJ and MMS2 for strain S2), *M thermautotrophicus* (MTH) and *M. jannaschii* (MJ). For comparison, additional homologous ORFs of about the same size and high sequence similarity were included from the euryarchaeotes *Archaeoglobus fulgidus* (AF) and *Pyrococcus abyssii* (PAB). The dendrogram was based upon and alignment of conserved positions and was generated by the Fitch-Margoliash algorithm. It was largely congruent with the dendrogram generated by Neighbor-joining (not shown). Bootstrap values for both dendrograms were nearly identical. Values >90% are indicated by (●), values >70% are indicated by (○). The scale bar represents 0.2 expected amino acid substitutions per site.


CODH-CR homologs
MTH1714
MMS2 CODH-CR
MJ0155
porE homologs
MMJJ porE
AF1202
MJ0265
PAB1390
MTH1737
MTH105
MTH1736
AF2385
MJ0264
AF0076
AF0950
PAB2084
MMJJ porF
0.2
porF homologs

CHAPTER 3

THE ROLE OF *porE* AND *porF* IN THE ANABOLIC POR OF *Methanococcus maripaludis*[1]

1. Lin, W.C. and W.B. Whitman. To be submitted to *Molecular Microbiology.*

ABSTRACT

The anabolic methanococcal pyruvate oxidoreductase (POR) was previously purified, and the genes were sequenced. Four of the genes (*porABCD*) are similar to the PORs from other organisms. The other two genes (*porEF*) appear to encode Fe-S proteins similar to ferredoxins thus suggesting an electron carrying function. To elucidate whether the methanococcal POR required PorEF, a deletion mutant, strain JJ150, lacking *porEF* was constructed. The mutant grew more slowly in minimal medium and minimal plus acetate medium than the wild type strain JJ1. The methyl-viologen dependent POR, carbon monoxide dehydrogenase, and hydrogenase activities of the mutant were similar to the activities in the wild type. Pyruvate-dependent methanogenesis was inhibited in the mutant. Upon genetic complementation of the mutant with *porEF* in the methanococcal shuttle vector pMEV2+*porEF*, growth in minimal medium and pyruvate-dependent methanogenesis was restored to wild-type levels. Complementation with *porE* restored methanogenesis from pyruvate but not growth in minimal medium. Complementation with *porF* partially restored growth but not methanogenesis from pyruvate. Although the specific roles of *porE* and *porF* have not been determined, these results suggest that PorEF play important roles in the anabolic POR.

INTRODUCTION

The anabolic pyruvate oxidoreductase (POR) in *Methanococcus maripaludis* catalyses the energetically unfavorable reductive carboxylation of acetyl-CoA to form pyruvate. Pyruvate, a key precursor for cellular components in *Methanococcus*, then enters the incomplete reductive tricarboxylic acid pathway, a major biosynthetic pathway. Reported previously, the methanococcal POR was purified and its nucleotide sequence

determined. The purified POR contained five polypeptides, four of which were similar to the subunits of PORs (□,□,□, and □) initially characterized in hyperthermophiles (Blamey and Adams, 1993, Adams and Kletsin, 1996). The fifth polypeptide, PorE appeared to be found only in hydrogenotrophic methanogens like *M. maripaludis, Methanocaldococcus jannaschii,* and *Methanothermobacter thermautotrophicus*. Additionally, the *M. maripaludis por* operon encoded open reading frames (*porABCDEF*) corresponding to the N-terminal sequences of all five polypeptides found in the purified enzyme and an additional ORF (*porF*) with homology to *porE*. The predicted polypeptide sequences of *porE* and *porF* contained iron-sulfur motifs, thus implying a possible ferredoxin-like electron transfer function that may be specific for the methanococcal POR. A homolog to *porE* was also found next to the carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH) gene cluster in these hydrogenotrophic methanogens. The reduction of CO_2 to CO catalyzed by this enzyme is also very endothermic when H_2 is the electron donor. Ferredoxins have a reduction potential ranging from –200 to –650 mV (Beinert, 2000). Thus, it was proposed that ferredoxins or low potential ferredoxin-like electron carriers could be electron donors to the methanococcal POR (Yang et al., submitted). Evidence supporting this hypothesis comes from work on the *Moorella [Clostridium] thermoacetica, Chlorobium tepidum*, and *Hydrogenobacter thermophilus* PORs, which were shown to synthesize pyruvate when coupled to low potential ferredoxins (Furdui and Ragsdale, 2000; Yoon et al., 2001; Yoon et al., 1997, Yoon et al., 1996). The studies presented here provide additional evidence that *porEF* are involved in pyruvate biosynthesis as well as insight into the potential function of a *porE* homolog associated with the CODH/ACS gene cluster.

MATERIALS AND METHODS.

Plasmids and Bacterial strains. Refer to Table 3.1.

Media and culture condition. *M. maripaludis* was grown in 28 ml stoppered tubes with 275 kPa H_2/CO_2 gas (80:20 [vol/vol]) at 37 ˚C in McN medium as described previously (Jones et al., 1983; Whitman et al., 1986). Variations of McN medium used were: McNA (mineral medium plus 10 mM sodium acetate) and McCA (McNA plus 2% Casamino acids, 1% (vol/vol) ml of vitamin mixture [Whitman et al., 1986] and 3 mM 2-mercaptoethanesulfonic acid), modified McCA in which the sole nitrogen source, ammonium chloride, was replaced with 2.5 mM alanine, and McCAP (McCA + 10 mM pyruvate. In some cases, either puromycin or neomycin was added to final concentrations of 2.5 mg ml^{-1} or 500 mg ml^{-1}, respectively.

For growth at low H_2 concentrations, *M. maripaludis* was grown in McN medium in modified 160 ml stoppered serum bottles. These bottles possessed a 20 ml side arm fused to the side so that absorbance measurements could be taken in a Spectronic 20 (Bauch and Lomb, NY). With CO_2 at a constant partial pressure of 55 kPa, the following partial pressures of H_2:N_2 gas were used, 0:220, 55:165, 82.5:137.5, 165:55, and 220:0. Final pressure within the bottles was 275 kPa. Growth was monitored for approximately thirty hours.

Large scale cultures for preparation of cell free extracts were grown in an 11 liter fermentor as described by Shieh and Whitman (1988) except that McCA was used instead of McC. The fermentor was prepared by the following method. Eleven liters of McCA with the sodium bicarbonate reduced to 2 g $liter^{-1}$ was prepared and autoclaved under H_2/CO_2 gas for 20 minutes. Following the autoclave cycle, the fermentor was

cooled to 37ºC with H_2/CO_2 at 100 kPa at a flow rate of 100 to 250 ml/min. Additionally, 5 ml of a 20% (wt/vol) solution of $Na_2S{\cdot}9H_2O$ was added twice a day during growth. The inoculum was 200 ml of culture grown on the same medium in bottles. Prior to harvesting of cells, the cells were examined for mutant phenotype through the monitoring of growth in McCA and McN media in 28 ml stoppered tubes and by the monitoring of the methane evolution from pyruvate as described later. The experiments were performed to insure that reversion by mutations at a second site had not occurred. Cells were harvested in the early stationary phase with a Sharples continuous-flow centrifuge. The harvested cells were resuspended with 25 mM PIPES-KOH buffer, plus 1 mM dithiolthreitol and 1 mM cysteine-HCL, pH 6.8 with 0.5 mg of DNase I per 10g wet weight of cells as described by Shieh and Whitman (1987). The resuspended cells were aliquoted into 2 ml volumes and stored at –20ºC under H_2 gas until further use.

E. coli strain Top 10 was grown at 37°C on low salt Luria-Bertaini medium as described by Invitrogen (Carlsbad, CA). For liquid and solid medium, kanamycin (50 □g ml^{-1}) and ampicillin (50 □g ml^{-1}) were used depending on the plasmid (pZErO-2 or pIJA03, respectively).

Growth of both *M. maripaludis* and *E. coli* were monitored at 600 nm with a Spectronic 20 spectrophotometer (Baush and Lomb, NY).

Transformation, and plasmid purification. *M. maripaludis* transformations were performed by the polyethylene glycol method (Tumbula et al., 1994). *E. coli* Top 10 transformations were performed using a Gene Pulser electroporator (BioRad, Richmond, Ca) at 200 □, 2.5 kV, and 25 □F with 0.2 mm gap cuvettes. Plasmids were

purified using the QIAgen spin miniprep kit (Qiagen, Germany) according to the manufacturer's instructions.

Construction of Δ*porEF::pac* mutants. The *porEF* gene deletions in the *M. maripaludis* genome were accomplished using pIJA03+CR (Figure 3.1). The vector pIJA03 is an integration vector that lacks an origin of replication for methanococci and contains a *pac* cassette (Statholopolous, 2001). The *pac* cassette, which encodes puromycin resistance, is flanked by two multiple cloning sites (MCS) to allow either single or double recombination into the *M. maripaludis* genome for directed gene deletions (Statholopolos, 2001; Gernhardt et al., 1990). To construct pIJA03 + CR, a 1049 bp region within *porB* was amplified by PCR using the primers, 5'Crinact-MCS1 (5'-CCCCCCGCAGATCTGTTGTAGGTCTTGGTGGAAGG-3'), and 3'Crinact-MCS1 (5'-CCCCCCGCTCTAGAAGACCGTATTCGTCGCATTTC Y-3'). These primers introduced *Bgl*II and *Xba*I sites as underlined into the PCR amplicon. The PCR amplicon was digested with *Bgl*II and *Xba*I and ligated into the MCS1 upstream of the *pac* cassette. Similarly, an 800 bp region immediately downstream of *porF* was amplified using primers 5'CRinactMCS2 (5'-CCCCCCGCACTAGTCGAGGCAGTGAAGTATGTCT-3') and 3'CRinactMCS2 (5'-CCCCCCCGGTACCCGAGGCAGTGAAGTATGTCT-3'), which introduced *Spe*I and *Kpn*I sites. This PCR amplicon was digested, gel purified and ligated into the MCS2 downstream of the *pac* cassette in pIJA03. Both PCR reactions were performed for 30 cycles at 94 ºC for 1 min, 50 ºC for 1 min, 72 ºC for 2 min and 30 sec. The final extension time was 5 min at 72 ºC. The orientation of each insert was confirmed by restriction mapping.

Prior to the transformation, pIJA03+CR was linearized by digestion with *Drd*I and *Fsp*I, resulting in a fragment of 4378 bp that contained the two inserts necessary for replacement of *porEF* with the *pac* cassette. This fragment was gel purified and transformed into wild type *M. maripaludis* strain JJ1 cells grown to early stationary phase. After transformation, the cells were screened on McCA, McCA + 10 mM pyruvate, and a modified McCA$^-$+alanine (the sole nitrogen source, ammonium chloride was replaced by 2.5 mM alanine) plates with that contained puromycin.

From the transformation, random puromycin-resistant isolates were restreaked on the same medium and transferred to stoppered culture tubes containing 5 ml of McCA liquid medium plus puromycin. The culture tubes containing the isolates were pressurized to 275 kPa and incubated at 37° C. After growth, 2 ml were used for determination of genotype and phenotype while the remaining 3 ml of the culture were prepared as frozen stocks. To prepare the frozen stocks, the culture tube was transferred to an anaerobic glove box where the remaining culture was centrifuged in a microfuge. The supernatant was discarded and replaced with 1 ml of 30% glycerol + McCA medium. The cells were aliquoted into 0.2 ml fractions, sealed into airtight cyrogenic tubes (Corning, NY) and stored at –80°C. In order to avoid potential second site revertants, subsequent growth experiments were conducted after the first transfer from the frozen stocks. Each experiment included duplicates from each condition and was repeated at least once.

Methanogenesis from pyruvate . Cells were grown in McCA or McN under H_2/CO_2 to late exponential phase or an absorbance at 600 nm of 0.8 - 0.85. The cultures were centrifuged at 2500 X g (Beckman centrifuge) for 15 min at room temperature.

The cell pellets were washed once and resuspended with 5 ml of Mc buffer (Yang et al, 1992). Mc buffer was similar in composition to McN except that the bicarbonate concentration was reduced from 5g/L to 2 g/L and the gas phase was 183 kPa of N_2+CO_2 (80:20 v/v). After resuspension, the cells were flushed with N_2/CO_2 for 15 minutes, and 1 ml of the cell suspension was transferred to a 2.6 ml stoppered serum vial containing an atmosphere of N_2/CO_2 and sufficient sodium pyruvate for a final concentration of 50 mM. Methane from the headspace of the serum vial was measured with a gas chromatograph equipped with a flame ionization detector (FID; Varian, gas chromatograph model 3700, Sugar Land, TX) and a PorapakQ 80/100 column (Alltech, Houston TX). The temperatures of the detector, injector, and column were 250, 180 and 120 ºC, respectively. A standard curve was constructed with 1% methane in N_2 gas.

Preparation of cell-free extracts and enzymatic assays. All cell preparations and assays were performed under strictly anaerobic conditions. An aliquoted cell suspension, (2 ml) was thawed under a stream of H_2. The cells lysed upon thawing, and the suspension was centrifuged at 30,000 X g for 30 min at 4ºC. The supernatant was used in the subsequent assays. The protein concentration of the supernatant was measured using the BCA method (Pierce Chemical, OH).

Activity of POR was measured anaerobically as pyruvate and HS-CoA-dependent methyl-viologen (MV) reduction (Shieh and Whitman, 1988). The assay buffer was 100 mM Tricine-KOH, pH 8.6, 10 mM methyl viologen (MV), 5 mM $MgCl_2$ and 1 mM thiamine pyrophosphate (TPP). After the addition of 20 mM pyruvate and 100 mM HS-CoA in 1 ml of assay buffer, the assay was performed at 37 ºC under a N_2 atmosphere. The increase of absorbance was measured at 578 nm. The extinction coefficient of MV

was 9.7$mM^{-1}cm^{-1}$. One unit was defined as 1 mmol of MV reduced per min. Activity of POR was measured at least twice per extract and on at least two extracts from separately prepared cultures.

Activities of the carbon monoxide dehydrogenase/ acetyl-CoA synthase (CODH/ACS; Shieh and Whitman,1988) and hydrogenase (Ragsdale and Ljungdahl, 1984) were measured similarly as CO- or H_2-dependant reduction of MV, respectively. The buffer was 50 mM potassium phosphate, pH 7.5, with 20 mM MV. After addition of the cell extracts to 1 ml of assay buffer, the cuvette was flushed with either CO or H_2, and the increase of absorbance at 578 nm was monitored at 37 ºC. To produce O_2-free CO, the gas was bubbled through 25 mM MV solution that was reduced with 10 mM sodium dithionite.

Pyruvate synthesis was measured through the *in vitro* lactate trap as described by Shieh and Whitman (1988) with the following modifications; the cell extract was not preincubated for 1 h at 37 C; for determination of CO effect on pyruvate synthesis, H_2/CO_2 gas was added to atmospheric pressure and CO was overpressurized to 183 kPa; for determination of CO_2 dependency for pyruvate synthesis, H_2 gas replaced H_2/CO_2.

Construction of pMEV-2. The methanococcal expression vector, pMEV-2 (Figure 3.2) developed in this study was derived from pMEV1 and the cosmid pBK-CMV (Stratagene,Ca), which contained a *neo*/*kan* cassette for neomycin resistance. The vector pMEV1 was based upon pWLG30+lacZ (Gardner and Whitman, 1999) except that regions near the ORFLESS 1 and 2 have been deleted (Gardner and Whitman, unpublished). The region downstream of the *pac* promoter (P*mcr*) containing the puromycin gene and the terminator (T*mcr*) from pMEV1 were replaced with the *neo*/*kan*

cassette from pBK-CMV (Stratagene, Ca). To replace the *pac* cassette in pMEV1, the *neo*/*kan* cassette from pBK-CMV was amplified using the primers MEV/Neo-Bam (5'-CCCCCCGGATCCGAGGCC TAGGCTTTTGCAAA3') and MEV/Neo-Eco (5'-CCCCCCAGGGCCTAGTAACCT GAGGCTATGGCA-3') which contained *Bam*HI and *Eco*0109 sites, respectively, as underlined. The amplified *neo/kan* cassette contained the HSV-TK polyA terminator but not the SV-40 promoter. The PCR amplification was performed as described above and the amplicon was digested with *Bam*HI and *Eco*0109 for directed ligation into pMEV1 to create pMEV2 (Figure 3.2). The region chosen for the ligation of the *neo/kan* amplicon would place the insert under the control of the P*mcr* promoter. The structure of, pMEV2, was verified through restriction mapping.

Complement of Δ*porEF* mutants. The genes *porE*, *porF* and *porEF* were amplified from the *M. maripaludis* genomic DNA. For amplification of *porEF*, the primers used were 5'POR-E (5'- CCCCCCATGCATGAAAAAAGTAATGATGGT-3') and 3'-POR-EF (5'- CCCCCCTCTAGAAGAAAGAAAAATTGATG-3'). For amplification of *porE*, the primers used were 5'POR-E and 3'POR-E (5'-CCCCCCTCTAGAACTT CACCAGATAATTTTA-3'). For amplication of *porF*, the primers used were 5'POR-F (5'- CCCCCCATGCATGAAGGTAATGCCAAATAT-3') and 3'POR-EF. The 5' primers and the 3' primers contained *Nsi*I and *Xba*I restriction sites, respectively. After amplification, pMEV2, and *porE*, *porF*, and *porEF* gene fragments were digested with *Nsi*I and *Xba*I, and ligated to form pMEV2+*porE*, pMEV2+ *porF*, or pMEV2+*porEF*. The plasmids were transformed into *E. coli* Top 10 for storage. These plasmids were transformed into *M. maripaludis* strain JJ150 by the

polyethylene-glycol method (Tumbula, 1994). Transformants were screened on McCA plates containing neomycin.

RESULTS AND DISCUSSION

Mutagenesis of *porEF* and complementation. To determine if *porEF* was required for POR activity, deletion mutants were constructed by transformation of the wild type strain JJ1 with pIJA03+CR, a plasmid containing portions of the *M. maripaludis por* gene insertions flanking the *pac* cassette as shown in Figure 3.1. Upon transformation with the linearized plasmid, the wild type was expected to acquire puromycin resistance through replacement of the *porEF* genes with the *pac* cassette. Because pyruvate was an essential precursor for monomer biosynthesis in methanococci, the transformants were plated in a variety of media designed to minimize the pyruvate requirement. McCA provided amino acids, to spare the pyruvate required for amino acid biosynthesis. McCA + 10 mM pyruvate was also used, but methanococci take up pyruvate poorly and higher concentrations are inhibitory (Yang et al. 1992). Lastly, medium with alanine as the sole nitrogen source was also used. In this medium 70% of the cellular pyruvate demand is obtained from alanine (Yang et al, in press). In all those media, the transformation efficiency was about 3 x 10^4 per μg of DNA and comparable to that of pBD1in the same experiments. Transformation with pBD1 deletes a portion of the gene encoding the cysteinyl-tRNA synthetase, which is cryptic in *M. maripaludis* (Statholopolous, 2001). The high transformation efficiency insured that the mutants were not derived from rare genetic events or multiple mutations.

The genotype of the mutants were confirmed by PCR amplification with flanking primers (EF1and EF2), which resulted in a 1.8 kb product for the Δ*porEF::pac* mutant.

This increase from the 1282 bp amplicon of the wild-type resulted from replacement of the 790 bp *porEF* region with the 1.3 kb *pac* cassette (Figure 3.1). Additionally, primers internal to the *pac* cassette produced a PCR product of the expected size from deletion mutant but not JJ1 genomic DNA. Three deletion mutants including JJ150 were isolated that contained the correct genotype.

Deletion of *porEF* affects growth. Pyruvate is an essential precursor for monomer biosynthesis in methanogens. Therefore, the Δ*porEF::pac* mutant was expected to grow poorly. To test this hypothesis, the H_2/CO_2-dependent growth characteristics of the JJ150 were monitored with rich (McCA), minimal (McN), and minimal + acetate (McNA) medium. The affect of pyruvate on growth was not determined because the degradatation products of pyruvate that formed spontaneously in the medium inhibited the growth of the wild type and the mutant. Also, initial experiments included alanine in ammonia-free McCA and McN media. Under these conditions, alanine is deaminated into pyruvate (Yang et al. in press). However, alanine was not used in later experiments due to the extremely slow growth of both the mutant and the wild type under these conditions. The growth of JJ150 were comparable to the wild type in rich medium, where many of the monomers required for growth were provided (Figure 3.3A). However, the growth was slower than the wild type in minimal medium, where higher levels of pyruvate biosynthesis would be required. This difference was consistently observed in six independent experiments. Although not consistently observed in independent experiments, the addition of acetate caused even poorer growth of the mutant (Figure 3.3). This phenotype is understandable if growth in the Δ*porEF::pac* mutant was dependent on the *porE* homolog associated with the

CODH/ACS operon. In this case, exogenous acetate would be expected to lower expression of this gene and inhibit pyruvate biosynthesis. However, due to inconsistent results, further characterization of the acetate effect on mutant growth will be required. The observed doubling times for the mutant in complex, minimal, and minimal + acetate were 3.6 ± 0.59, 5.1 ± 2.1, and 5.1 ± 1.0 hours respectively. The observed doubling times for the wild type in complex, minimal, and minimal + acetate were 2.8 ± 0.53, 3.4 ± 0.90, and 3.4 ± 0.78 hours respectively. However, upon repeated transfers of JJ150 in minimal medium, the growth properties became indistinguishable from wild type, indicating that selection occurred for second site mutations that compensated for the original mutation.

To confirm that the observed phenotype was a result of the Δ*porEF::pac* mutation, JJ155 was constructed where the deletion was complemented with *porEF* cloned into the methanococcal expression vector pMEV2. The growth in minimal medium was restored to wild type levels in JJ155 (Figure 3.3B). Additionally, JJ153 and JJ154, which contained either *porE* or *porF,* respectively, provided partial complementation. By itself, *porE* had only a small effect, while growth with *porF* alone was nearly sufficient to restore wild-type growth. Nevertheless, the requirement for both *porE* and *porF* for full complementation suggests that both genes play important yet somewhat different roles.

To differentiate the mutant from the wild type further, the H_2 concentration in the headspace was varied. The rationale for this experiment was that if PorE and/or PorF were the physiological electron carriers to the POR, the mutant might be more sensitive to the H_2 concentration than the wild type. However, at the lowest concentrations of H_2 tested, growth was very slow and the difference in growth rate between JJ150 and

JJ1(WT) diminished (data not shown). The results suggested that anabolism was not growth limiting under those conditions.

Determination of enzymatic activity. From cell extracts of the JJ150 and the wild-type JJ1, the enzymatic activity of the POR was determined. Due to the oxygen-labile nature of POR, the activity of two other oxygen-labile enzymes, the hydrogenase and the CODH/ACS were determined as controls. With methyl viologen as the electron acceptor, activities in JJ150 and JJ1grown in complex medium were similar for all three enzymes (Table 3.2). Likewise, the specific activities for these three enzymes in JJ1 and JJ150 cells grown in miminal medium were also similar to each other. To examine the pyruvate synthase activity, a pyruvate-trap coupled assay using lactate dehydrogenase was employed (Shieh and Whitman, 1988). This assay system trapped pyruvate formed in cell extracts into lactate, which was not further metabolized in those extracts. Results obtained from McCA grown cells indicated that while there was pyruvate synthase activity in the extracts, it was not CO_2-dependent (Table 3.3). This indicated that the observed activity may have arisen from endogenous substrates. Additionally, the addition of CO gas did not further stimulate pyruvate synthase activity.

Deletion of *porEF* has an effect on pyruvate-dependent methanogenesis. Pyruvate serves as an electron donor for methanogenesis in resting cells of methanococci in the absence of the physiological electron donors H_2 and formate (Yang et al., 1992). If PorE and/or PorF are involved in electron transfer to the POR, the deletion of *porEF* would be expected to inhibit the in vivo oxidation of pyruvate and pyruvate–dependent methanogenesis. In fact, pyruvate–dependent methanogenesis was severely inhibited in the Δ*porEF::pac* mutant, where the activity was comparable to that found with

endogenous substrates (Table 3.4). In contrast, methanogenesis with H_2 as the electron donor was not affected. Pyruvate-dependent methanogenesis was fully restored in JJ153 and JJ155, which were the deletion mutants complemented with *porE* and *porEF*, respectively (Table 3.4). In contrast, the phenotype of JJ154, in which the Δ*porEF::pac* deletion was complemented by only *porF*, was indistinguishable from the original mutant. These results provided further evidence that *porE* and *porF* possess different functions. Additionally, cultures of JJ150 that had been serially transferred on minimal medium and lost the distinctive growth phenotype remained deficient in pyruvate-dependent methanogenesis.

Yang et al. (submitted) previously demonstrated that the *porE* and *porF* genes contained motifs indicative of Fe-S clusters and proposed that PorE and PorF were electron carriers to the anabolic POR. While we have not fully elucidated the specific function of PorE and PorF, we show here that *porE* and *porF* are important components of the anabolic POR in hydrogenotrophic methanogens. The deletion of *porE* and *porF* affected both growth and oxidation of pyruvate. Complementation of *porEF* restored both of these functions. Furthermore, the PorE and PorF complemented these POR functions differently, indicating that they play different roles. While more research is necessary to elucidate the specific function of PorE and PorF, these proteins may represent a novel adaptation of the □□□□-type POR by the hydrogenotrophic methanogens for pyruvate biosynthesis.

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Table 3.1. Bacterial strains and plasmids utilized in this study

Bacterial strain or plasmid	Genotype or description	Source or reference
M. maripaludis strains		
JJ1	Wild type	Jones et al., 1983
JJ150	Δ*porEF*::*pac*	This work
JJ153	Δ*porEF*::*pac*/pMEV2+*porEF*	This work
JJ154	Δ*porEF*::*pac*/pMEV2+*porE*	This work
JJ155	Δ*porEF*::*pac*/pMEV2+*porF*	This work
E. coli Top10'	General cloning strain	Invitrogen
Plasmids		
pZero +POR	Kan^r cloning vector plus *M. maripaludis* POR operon	This work
pIJA03	Pur^r methanogen integration vector	Stathopoulos et al., 2001
pIJA03 + CR	Contains inserts to allow *porEF* deletion in *M. maripaludis* genome	This work
pMEV1	pur^r shuttle vector; derived from pWLG30+lac where nonessential regions were deleted	W. Gardner, 2000
pBK-CMV	Cosmid that contains Neo^r gene	Stratagene
pMEV2	derived from pMEV1, neo^r shuttle vector	This work
pMEV2+*porE*	pMEV2 with *porE*	This work
pMEV2+*porF*	pMEV2 with *porF*	This work
pMEV2+*porEF*	pMEV2 with *porEF*	This work

Table 3.2. Activities of POR, CODH/ACS, and hydrogenase in extracts of *M. maripaludis* JJ1 and JJ150 grown in McNA medium

	Specific activity of		
Strain	POR	CODH/ACS	Hydrogenase
JJ1 (WT)	0.593 ± 0.123	0.199 ± 0.063	416 ± 341
JJ150	0.826 ± 0.251	0.095 ± 0.047	384 ± 169

Values are based upon four assays of extracts from two independent cultures grown in complex medium.

Table 3.3. LDH trap for pyruvate synthase activity with cells grown in minimal medium

Additions,[a,b]	Strain	lactate formed (nmol)
NADH(2.5 μmol)	JJ1	0
	JJ150	0
LDH (25 U)	JJ1	0
	JJ150	0
NADH + LDH	JJ1	128±48
	JJ150	96±50
NADH +LDH +CO	JJ1	116±31
	JJ150	116±63
NADH + LDH +H_2	JJ1	99±42
	JJ150	124±84

a. The assay contained 8 to 10 mg of protein.
b. The assay was conducted in an atmosphere of H_2/CO_2 except for NADH + LDH + H_2.

Table 3.4. Methanogenesis from pyruvate by resting cells of the Δ*porEF::pac* mutant of *Methanococcus maripaludis.*

			Activity [nmol CH_4 $(min)^{-1}$(mg cells dry wt)$^{-1}$] with substrate added		
Strain	Genotype	Plasmid	Pyruvate	None	H_2
JJ1	WT	-	1.13 ± 0.35	0.18 ± 0.02	360
JJ150	Δ*porEF*::*pac*	-	0.27 ± 0.09	0.21 ± 0.03	355
JJ153	Δ*porEF*::*pac*	pMEV2+*porE*	1.32 ± 0.50	0.18 ± 0.03	ND
JJ154	Δ*porEF*::*pac*	pMEV2+*porF*	0.21 ± 0.04	0.21 ± 0.01	ND
JJ155	Δ*porEF*::*pac*	pMEV2+*porEF*	1.58 ± 0.21	0.20 ± 0.01	ND

Cells were incubated under N_2/CO_2 or H_2/CO_2 with 50 mM pyruvate. Activity was determined after 9 h except for H_2, which was determined after 4 h. ND = Not determined. Data are based upon 4 determinations.

Figure 3.1. Replacement of *M. maripaludis porEF* genes with the *pac* cassette. A. Directed replacement and inactivation of *porE* and *porF* genes with the *pac* cassette was accomplished through double homologous recombination using pIJA03+CR. Prior to transformation, pIJA03+CR was linearized with *Drd*I and *Fsp*I. B. Verification of the genotype of the mutant was determined through PCR. Two sets of primers were used: EF1 and EF2, Pac1 and Pac2. Lane 1, DNA size standards. Lane 2, Amplification of JJ150 mutant with EF primers. Lane 3, Amplification of JJ1 (WT) using EF primers. Lane 4, Amplification of JJ150 with Pac primers. Lane 5, Amplification of JJ1 (WT) using Pac primers.

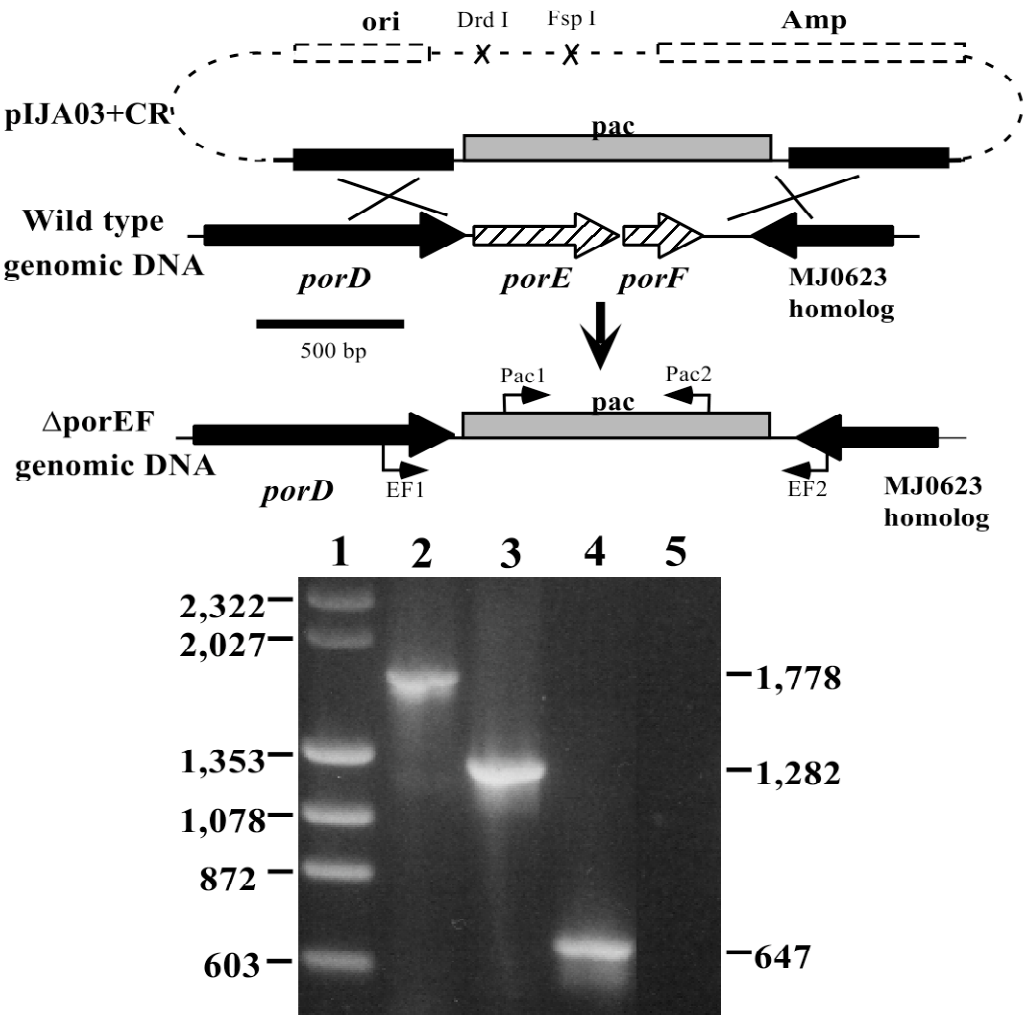

ori
Drd I
Fsp I
Amp
pIJA03+CR
pac
Wild type
genomic DNA
porD
porE
porF
MJ0623
homolog
500 bp
Pac1
Pac2
pac
ΔporEF
genomic DNA
porD
EF1
EF2
MJ0623
homolog
1
2
3
4
5
2,322
2,027
1,353
1,078
872
603
1,778
1,282
647

Figure 3.2. Construction of methanococcal expression vector pMEV2 encoding neomycin resistance. pMEV2 was constructed from pMEV1 and pBK-CMV (Stratagene). Through PCR with the primers, MEV/Neo-Bam and MEV/Neo-Eco, the *neo/kan* cassette was amplified from pBK-CMV. MEV/Neo-Bam and MEV/Neo-Eco contained *BamH*I and *Eco*0109 sites engineered into the sequence. The resulting *neo/kan* amplicon and pMEV1 were digested with *BamH*I and *Eco*0109 and ligated to form pMEV2. Ligation of an gene into the *Nsi*I site would allow the insert to be expressed under the control of P*hmv*A in methanococci. amp=ampicillin resistance gene, neo=neomycin resistance cassette, pac=puromycin resistance cassette, ORFless= regions of DNA without start sites.

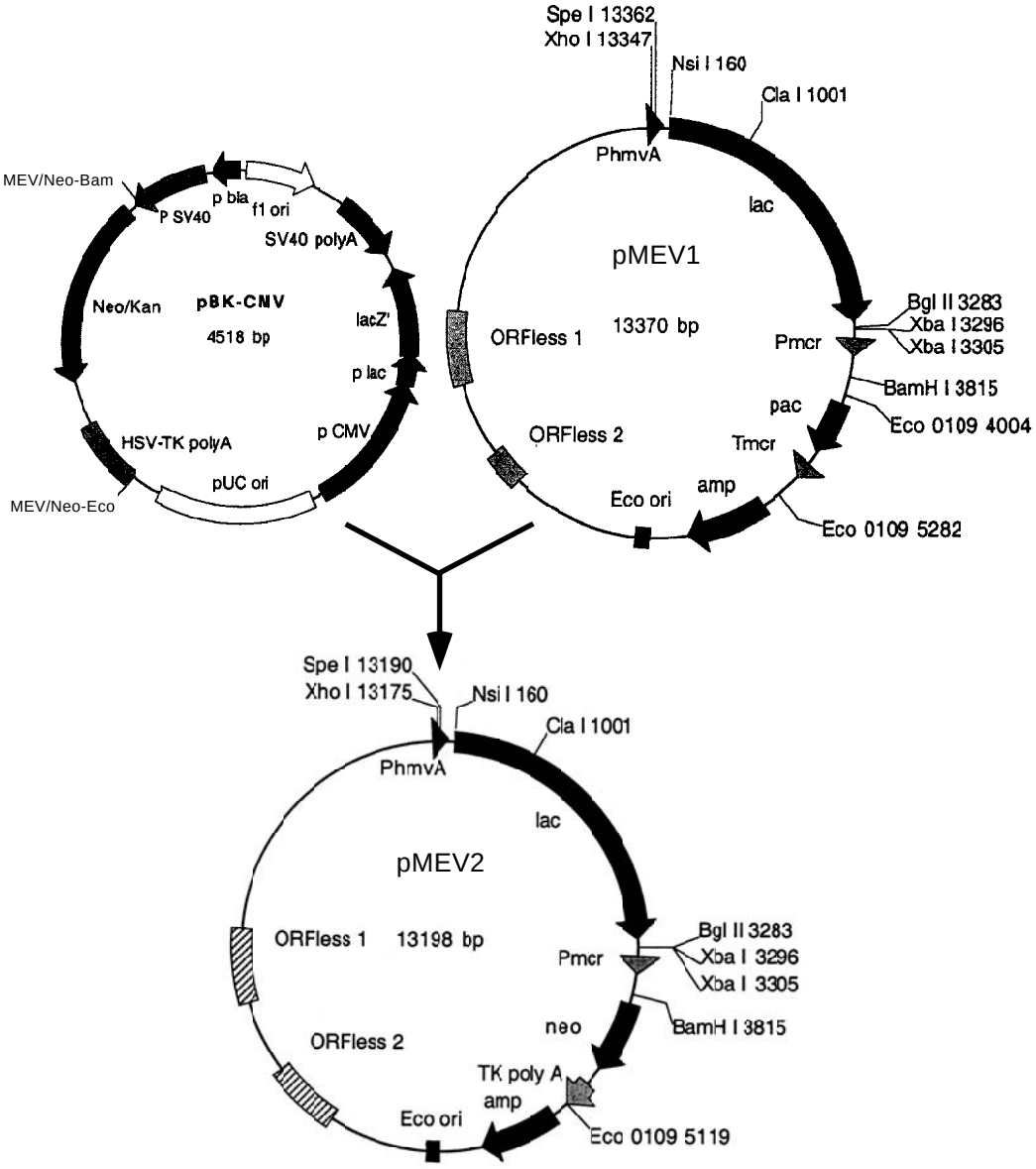
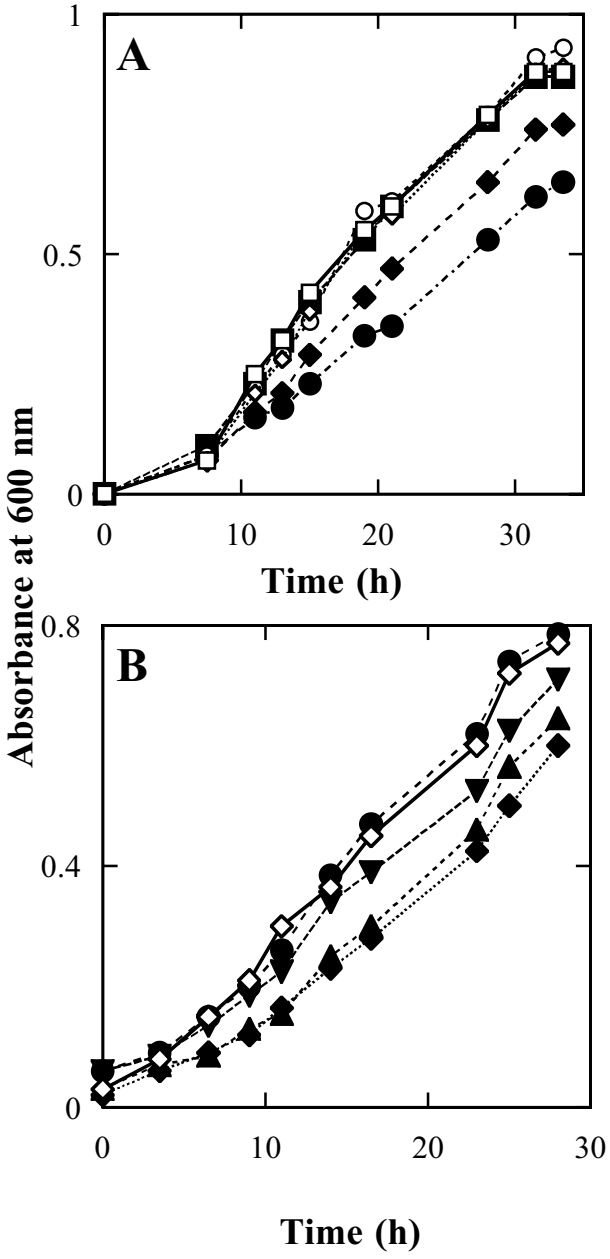

MEV/Neo-Bam
P SV40
p bla
f1 ori
SV40 polyA
Neo/Kan
pBK-CMV
4518 bp
lacZ'
p lac
p CMV
HSV-TK polyA
pUC ori
MEV/Neo-Eco
Spe I 13362
Xho I 13347
Nsi I 160
Cla I 1001
PhmvA
lac
pMEV1
ORFless 1
13370 bp
Pmcr
Bgl II 3283
Xba I 3296
Xba I 3305
BamH I 3815
pac
Eco 0109 4004
ORFless 2
Tmcr
amp
Eco ori
Eco 0109 5282
Spe I 13190
Xho I 13175
Nsi I 160
Cla I 1001
PhmvA
lac
pMEV2
ORFless 1
13198 bp
Pmcr
Bgl II 3283
Xba I 3296
Xba I 3305
neo
BamH I 3815
ORFless 2
TK poly A
amp
Eco ori
Eco 0109 5119

Figure 3.3. Growth of the Δ*porEF* mutant JJ150 and complementation by pMEV2+*porEF*. A. The effect of Δ*porEF* deletion on *M. maripaludis*. Growth was performed at 37°C under H_2/CO_2. Growth of JJ1 (WT) in McCA (□), McN (◇), and McNA(△). Growth of the Δ*porEF* mutant JJ150 in McCA (■), McN ( ), and McNA (●). B. Complementation of Δ*porEF*. All strains were grown in McN at 37°C under H_2/CO_2: JJ1 (WT ◇), JJ150 (Δ*porEF*), JJ153 (Δ*porEF*+*porE* ▲), JJ154 (Δ*porEF* + *porF* ▼), JJ155 (Δ*porEF* + *porEF* ●).


A
B
Absorbance at 600 nm
Time (h)
Time (h)
0
0.5
1
0
10
20
30
0.4
0.8

CHAPTER 4

PRELIMINARY STUDIES ON THE ESSENTIALITY OF POR AND THE EFFECTS OF DELETIONAL MUTAGENESIS OF THE GENES, *porC* AND *porD* FROM *Methanococcus maripaludis*

SUMMARY

The methanogen *Methanococcus maripaludis* utilizes an unusual anabolic POR five polypeptides for the biosynthesis of cellular carbon. Nucleotide sequencing determined that four of the subunits of the POR (□,□,□, and □) are similar to the four subunit POR from hyperthermophiles, while the fifth subunit encoded by *porE* and an additional subunit *porF* potentially encoded for Fe-S proteins. While *porE* and *porF* were shown to be important to the anabolic POR in previous research, *porABCD* have not been investigated. Results obtained from the deletion of the POR gene cluster and directed inactivation of *porC* and *porD*, which encode the □ and □ subunits respectively, suggested that the POR was essential for *M. maripaludis*. Although, the genotype was not confirmed, preliminary evidence from directed deletion of the *porC* and *porD* demonstrated that *M. maripaludis* mutants had an extended lag period in growth. However results were inconclusive.

INTRODUCTION

The pyruvate oxidoreductase (POR) catalyzes the reversible oxidation of pyruvate and has different physiological roles in microorganism (Adams and Kletzin, 1996). Pyruvate is oxidized by POR in heterotrophic bacteria for nitrogen fixation and anaerobic respiration or for acetate fermentation of sugars in homoacetogenic bacteria (Adams and Kletzin, 1996; Chabriere et al., 1999; Bogutz et al., 1981). In the reverse direction, pyruvate, a central intermediate in biosynthetic pathways in *Methanococcus*, is synthesized from acetyl-CoA and CO_2. However, the physiological electron acceptor has not been determined at this time. Other organisms such as *Clostridium thermoaceticum* and *Chlorobium* have been shown to be able to utilize the POR with low potential

electron carriers for the biosynthesis of pyruvate as well (Furdui and Ragsdale, 2000; Yoon et al., 2001; Yoon et al., 1999).

It was previously demonstrated that there were primarily three types of PORs; the four subunit, □□□□ hyperthermophilic type, the two subunit □□ aerobic archaeal type, and the homodimer $□_2$ bacterial type (Adams and Kletzin, 1996). However, previous research has shown that the purified methanococcal POR contains five polypeptides: □,□,□,□and PorE. Interestingly, four of the subunits are similar to those of the four subunit hyperthermophilic POR (□, □, □, and □subunits; Yang et al., submitted). Further investigation of the *por* gene cluster of *Methanococcus* confirmed the presence of genes encoding the five subunits as well as an additional gene, *porF*, found in the *por* gene cluster. From deletion experiments, PorE and PorF were shown to be important in POR catalysis in both the oxidative and the reductive reaction in *Methanococcus*. Additionally, PorE and PorF may be the physiological electron carriers for the POR and may be a novel adaptation for anabolism. Since pyruvate is a central intermediate in the biosynthetic pathways of *Methanococcus*, it is hypothesized that the POR is essential. In this research, we performed preliminary investigations to determine whether the POR is essential for *Methanococcus* and attempt to delete the genes encoding the □ and □ subunits of the methanococcal POR.

MATERIALS AND METHODS

Plasmids and Bacterial strains. Refer to Table 4.1.

Media and culture condition. *M. maripaludis* was grown in 28 ml stoppered tubes with 275 kPa H_2/CO_2 gas (80:20 [vol/vol]) at 37 ˚C in McN medium as described previously (Jones et al., 1983; Whitman et al., 1986). Variations of McN medium used

were: McNA (mineral medium plus 10 mM sodium acetate), McCA (McN plus 2% Casamino acids, 1 ml/100 ml of medium of vitamin mixture [Whitman et al., 1986] and 3 mM 2-mercaptoethanesulfonic acid), modified McCA in which the sole nitrogen source, ammonium chloride, was replaced with 2.5 mM alanine, and McCA + 10 mM pyruvate. In some cases, puromycin was added to a final concentration of 2.5 g ml^{-1}.

E. coli strain Top 10 was grown at 37°C on low salt Luria-Bertaini medium as described by Invitrogen (Carlsbad, CA). For liquid and solid medium, kanamycin (50 g ml^{-1}) and ampicillin (50 g ml^{-1}) were used depending on the plasmid (pZErO-2 or pIJA03, respectively).

Growth of both *M. maripaludis* and *E. coli* were monitored at 600 nm with a Spectronic 20 spectrophotometer (Baush and Lomb, NY).

Transformation, and plasmid purification. *M. maripaludis* transformations were performed by the polyethylene glycol method (Tumbula et al., 1994). *E. coli* Top 10 transformations were performed using a Gene Pulser electroporator (BioRad, Richmond, Ca) at 200 , 2.5 kV, and 25 F with 0.2 mm gap cuvettes. Plasmids were purified using the QIAgen spin miniprep kit (Qiagen, Germany) according to the manufacturer's instructions.

PCR conditions. All PCR reactions were performed for 30 cycles at 94 ºC for 1 min, 50 ºC for 1 min, 72 ºC for 2 min and 30 sec. The final extension time was 5 min at 72 ºC.

Construction of Δ*porABCDEF::pac* and Δ*porCD::pac* mutants. The plasmids pIJA03+ *porABCDEF* and pIJA03 + *porCD* were constructed in an attempt to produce

por gene cluster or *porCD* gene deletions, respectively, in the *M. maripaludis* genome (Figure 4.1). The vector pIJA03 is an integration vector that lacks an origin of replication for methanococci and contains the *pac* cassette (Statholopolous, 2001). The *pac* cassette, which encodes for puromycin resistance, is flanked by two multiple cloning sites (MCS) to allow either single or double recombination into the *M. maripaludis* genome for directed gene deletions (Statholopolos, 2001; Gernhardt et al., 1990).

To construct pIJA03 + *porABCDEF*, a 971 bp region immediately upstream of the start of *porA* (Figure 4.2) was PCR amplified using the primers, 5'porABCDEF-MCS1 (5'-CCCCCCGCAGATCTGACATCCACGTCTTCAACGG -3') and 3'porABCDEF-MCS1 (5'CCCCCCGCTCTAGACCCTCTTCCGTGGAATCTAA -3'). These primers introduced *Bgl*II and *Xba*I sites (as underlined) into the PCR amplicon. The PCR amplicon was digested with *Bgl*II and *Xba*I and ligated into MCS1 upstream of the *pac* cassette. Similarly, an 811 bp region immediately downstream of *porF* (Figure 4.2) was amplified using primers 5'porABCDEF-MCS2(5'CCCCCCGCACTAGTCGAGGCAGT GAAGTATGTCT-3') and 3'porABCDEF-MCS2 (5'-CCCCCCCGGTACCCCTCCC TTATCTTTCGAACCC-3'), which introduced *Spe*I and *Kpn*I sites. This PCR amplicon was digested, gel purified and ligated into MCS2 downstream of the *pac* cassette in pIJA03.

To construct pIJA03 + *porCD*, a 1140 bp region that included the region upstream of *porA*, *porA* in it's entirety, and most of *porB* (Figure 4.2) was PCR amplified using the primers 5'porCD-MCS1 (5'-CCCCCCGCAGATCTGAGCCTTGCTCCAGCATCAAT -3') and 3'porCD-MCS1 (5'CCCCCCGCTCTAGACAGCCTTCAGGACAGAAGAT -3'). These primers introduced *Bgl*II and *Xba*I sites (as underlined) into the PCR

amplicon and the PCR amplicon was digested with *Bgl*II and *Xba*I and ligated into MCS1 upstream of the *pac* cassette. Similarly, a 994 bp region that included the latter portion of *porD* and all of *porE* (Figure2) was amplified using primers 5'porCD-MCS2 (5'CCCCCCGCACTAGTAAGCCTTATCCGTGGGGAAA-3') and 3'porCD-MCS2 (5'-CCCCCCCGGCTAGCTGCGTTTATAGGACATGCCC-3'), which introduced *Spe*I and *Nhe*I sites. This PCR amplicon was digested, gel purified and ligated into MCS2 downstream of the *pac* cassette in pIJA03.

Linearized pIJA03+*porABCDEF* or pIJA03+*porCD* were transformed into *M. maripaludis* in attempts to make Δ*porABCDEF::pac* and Δ*porCD::pac* mutants (Table 4.1). Linearization of pIJA03+*porABCDEF* and pIJA03+*porCD* was accomplished by digestion with *Drd*I and *Fsp*I. The gel purified fragment was transformed into wild type *M. maripaludis* strain JJ1 cells grown to early stationary phase. After transformation, the cells were screened on McCA, McCA +pyruvate, and modified McCA +alanine plates that contained puromycin.

From the transformation, random puromycin-resistant isolates were chosen and transferred to stoppered culture tubes containing 5 ml of McCA liquid medium plus puromycin. The culture tubes containing the isolates were pressurized to 275 kPa and incubated with H_2+CO_2 at 37° C. After growth, 2 ml were used for determination of genotype and phenotype while the remaining 3 ml of the culture was prepared as frozen stocks. The presence of the puromycin cassette was checked with the primers, CheckPur 5' (5'-AGATGTTAGAAGCGGGATCAAAGATGGCGG-3') and CheckPur 3' (3'-GGCCGTCCCTATAGGACGACTATTTTGTT-3'). These primers were located immediately inside the puromycin cassette. The growth characteristics of the isolates

were determined with McN or McCA liquid medium. The growth experiments set contained duplicate experiments per medium.

To prepare the frozen stocks, the culture tube was transferred to an anaerobic glove box where 3 mls of culture was centrifuged. The supernatant was discarded and replaced with 1 ml of 30% glycerol + McCA media. The cells were aliquoted into 0.2 ml fractions, sealed into airtight cyrogenic tubes (Corning, NY) and stored at –80°C.

Construction of single insertion mutants. To attempt the insertional inactivation of the *porC* and *porE* genes, and single gene fragments were cloned into the MCS1 of pIJA03 (Figures 3). However, initially the *por* nucleotide sequence region necessary for the cloning was PCR amplified and blunt ended ligated into *EcoR*V restricted pZErO-2 vector (Invitrogen, Ca). The primers used for *porC* (subunit) inactivation were SPOR BglII-5' (5'-GCAGATCTGTATCCAATTACCCCCCAAAC-3') and SPOR XbaI-3' (5'-GCTCTAGAAGTAGTTTGGATCACCAAGC-3') for a 552 bp product (Figure 4.2). The primers used for *porE* inactivation were SPOR BglII-5' (5'-GCAGATCTTACAGCATCAATAAGTTACCC-3') and SPORCRXbaI-3' (5'-GCTCTAGATACATCCATACAAGGTGATC-3') for a 507 bp product (Figure 4.2). Incorporated into the primers were *Bgl*II and *Xba*I restriction sites as underlined in the sequence. Once ligated and transformed into *E. coli* Top 10, the purified pZErO-2 + *porC* or pZErO-2 +*porE* was restricted with *Bgl*II and *Xba*I and ligated into pIJA03. The resulting vectors, pIJA03+ *porC* and pIJA03+*porE* were then separately transformed into *M. maripaludis* JJ1 via the polyethylene-glycol method (Tumbula et al., 1994).

Localization of insertion of pIJA03+*porC*. Determination of the region of homologous recombination was performed on transformants from the pIJA03+*porC*

(Figure 4.4). Using genomic DNA purified from the transformants, the DNA was digested with *Cla*I. One *Cla*I restriction site is known to be contained in the pIJA03 multiple cloning site, MCS2. The second site would then be within adjacent regions of the *M. maripaludis* genomic DNA. The digested DNA was circularized with ligase and transformed into *E. coli* Top10. The cell containing the circularized DNA that included the pIJA03 vector was selected on ampicillin-containing plates. The resulting plasmid was isolated, purified and sequenced using the IJA#1 primer (5'-AAAGTGCCACCT GACCTCT-3') or IJA#2 (5'-AAGAGCGCCCAATACGCAA-3'). Sequencing was performed on an Applied Biosystems automated sequencer (PE Applied Biosystems, CA) at the Molecular Genetics Facility at the University of Georgia. The sequences were assembled and viewed using the program Sequencher (Gene Codes Corp., MI).

RESULTS AND DISCUSSION

Determination of POR essentiality in *Methanococcus*. Pyruvate, a key biosynthetic intermediate, is formed from acetyl-CoA and CO_2 via the pyruvate oxidoreductase (POR). In fact, a majority of cellular carbon is formed from pyruvate, which leads to the hypothesis that the POR is essential for *M. maripaludis*. Two methods were utilized to investigate the essentiality of the POR: (1) deletion mutagenesis of the entire *por* gene cluster *porABCDEF* and (2) single homologous recombination into specific regions in the *por* gene cluster.

To determine if the *por* gene cluster were required for POR activity, deletion mutants were constructed by transformation of the wild type strain JJ1 with a derivative of pIJA03 with portions of the *M. maripaludis* genome near the *por* genes flanking the *pac* cassette as shown in Figure 4.1. Upon transformation with the linearized plasmid,

the wild type would acquire puromycin resistance through two homologous transformation events, where the *porABCDEF* genes would be replaced with the *pac* cassette. However, after three attempts, no transformants were observed. In contrast, the control plasmid pBD1 yielded 6 x10^3 transformants per μg of DNA. These results, while not conclusive, implied that the POR was essential to *M. maripaludis* and that deletion of the *por* gene cluster would be lethal to the cell.

To investigate further whether the POR were essential for *M. maripaludis*, the single homologous recombination technique was utilized, where the plasmids pIJA03+*porC* and pIJA03+*porE* were transformed into *M. maripaludis* (Figure 4.3). The vectors pIJA03+*porC* and pIJA03+*porE* contained a 500bp region within *porC* or a 550 bp region spanning across the 3' region of *porD* and *porE* in its entirety, respectively (Figure 4.3). Upon transformation with the plasmid, the wild type would acquire puromycin resistance through homologous recombination and insertion of the plasmid into the target region. As controls, two other constructs pIJA03+*cysS* and pIJA03+*hrdA* were utilized (constructed by I. Anderson; Statholopolous, 2001). The vector pIJA03+*cysS* was encoded by the cysteinyl-tRNA synthetase and was considered to not be essential because of a bifunctional prolyl-tRNA synthetase (Statholopolous, 2001). The vector pIJA03+*hrdA* contained a 495 bp portion of the heterodisulfide reductase A gene (*hyd*A) and was believed to be essential (Statholopolos, 2001). Upon transformation, the *porC* plasmid yielded 20% of the number of transformants as the *porE* containing plasmid (Table 4.2). However, the number of transformants was nearly equivalent to those of pIJA+*cysS* (Table 4.2). These results did not support the hypothesis that the POR was essential. In addition, the size of the colonies formed from

the pIJA03+*porC* transformants were 3 to 4 times smaller than the other transformants (Table 4.2). These results suggested that it could be possible to inactivate *porC* but also such mutations would be more deleterious to growth than mutations in *porE*. A possible explanation for the observed results was that pIJA03+*porC* was recombining within the genes of homologous oxidoreductases (OR). In support of this hypothesis, *M. maripaludis* contains three other homologs of *porC*. In order to investigate this possibility, the location of the insertion of the vector was determined as described in (Figure 4.4). While primer IJA#2 did not yield any sequences, the sequence from primer IJA#1, included 50 bp of *porC* near the expected site of insertion that ended at a *cla*I site. The *cla*I site was followed by vector sequence, indicating that no more than 150 bp of flanking DNA had been cloned. Although the region sequenced was very limited, it was sufficient to determine that the vector had integrated into the correct region.

Preliminary studies on the effects of *porCD* deletion on growth. Pyruvate is an essential precursor for monomer biosynthesis in methanogens. The subunit appears to be largely structural, while the subunit is particularly important since it contains an unusual Fe-S binding motif and a TPP-binding site. Therefore, it was expected that deletion of *porC* and *por D,* which encode for the and the subunits respectively, would cause the *M. maripaludis* mutant to grow poorly. To determine if *porCD* were required for POR activity, transformation of the wild type strain JJ1 with pIJA03+*porCD* (Figure 4.1). Upon transformation with the linearized plasmid, the wild type would acquire puromycin resistance through two homologous transformation events where the *porCD* genes would be replaced with the *pac* cassette. In order to determine the genotype of the mutant, PCR primers, CheckPur 5' and CheckPur 3' that flanked the *por*

region were used. However, no products were detected in both the wild type and the mutant (JJ156). Therefore, determination of the genotype of the mutant could not be confirmed through PCR. It is not certain as to why no amplicons were seen in the wild type and the mutant. It is possible that the particular region of DNA may not readily amplify. As an alternative method, Southern blot hybridization could be used to determine the genotype of the mutant. However, it was not attempted for this study. Growth of the mutant JJ156 appeared to be inhibited on minimal medium when compared to growth on McCA. However, since it was not possible to verify the genotype of the mutant, it was not known whether the deletion mutagenesis occurred in the correct genes or if deletion of *porCD* occurred at all. As mentioned above, there are several other oxidoreductases (OR) that exist in methanogens (Tersteegen et al., 1997) and may have homology to the subunits of the POR. It is conceivable that the deletion mutagenesis with the vector occurred in another OR with a homologous sequence.

ACKNOWLEDGEMENTS

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Table 4.1. Bacterial strains and plasmids utilized in this study

Bacterial strain or plasmid	Genotype or description	Source or reference
***M. maripaludis* strains**		
JJ1	Wild type	Jones et al., 1983
JJ156	Δ*porCD*::*pac*	This work
***E. coli* Top10'**	General cloning strain	Invitrogen
Plasmids		
pZErO	General cloning vector	Invitrogen
pZErO +*porC*	Intermediate vector utilized for construction of pIJA03+*porC*	This work
pZErO +*porE*	Intermediate vector utilized for construction of pIJA03+*porE*	This work
pIJA03	Pur^r methanogen integration vector	Stathopoulos et al., 2001
pIJA03 + *porCD*	For construction of Δ*porCD* deletion in *M. maripaludis* genome	This work
pIJA03 +*porABCDEF*	For construction of Δ*porABCDEF* deletion in *M. maripaludis* genome	This work
pIJA03 +*porC*	Contains single insert homologous to region within *porC* to allow insertional inactivation of *porC*	This work
pIJA03 +*porE*	Contains clone of the 3' end of *porD* and a majority of *porE* to allow insertional inactivation of *porE*	This work
pBD1	For construction of Δ*cysS* deletion in *M. maripaludis* genome	Stathopoulos et al., 2001
pIJA03+*hrdA*	Contains clone of the *hydA* to allow insertional inactivation of *hyd*	Stathopoulos et al., 2001
pIJA03+*cysS*	Contains clone of the *cysS* to allow insertional inactivation of *cysS*	Stathopoulos et al., 2001

TABLE 4.2. Transformation of *M. maripaludis* by pIJA03 integration vectors for determination of por gene essentiality.

Plasmid	Size of insert (bp)[1]	Total number of transformants/mg DNA[2]	Size of colony (mm)[3]
pIJA03+*porC*	551	38±8	0.5
pIJA03+*porE*	507	182±19	1.9
pIJA03+*hrdA*	495	9±3	1.4
pIJA03+*cysS*	507	48±8	1.7

[1] Size of *M. maripaludis* genomic DNA inserted into pIJA03

[2] Transformation by PEG method (Tumbula) with 1 g supercoiled plasmid DNA

[3] Average size of five random colonies

Figure 4.1. Strategy for replacement of *M. maripaludis porABCDEF* or *porCD* genes with the *pac* cassette. Directed replacement and inactivation of (A) *porABCDEF* or (B) *porCD* genes was attempted by transformation with the vectors shown. Double homologous recombination of pIJA03 + *porABCDEF* or pIJA03+*porCD* with the genome would cause replacement of the *porABCDEF* or *porCD* genes with the *pac* cassette. Prior to transformation, the plasmids were linearized with *Drd*I and *Fsp*I.

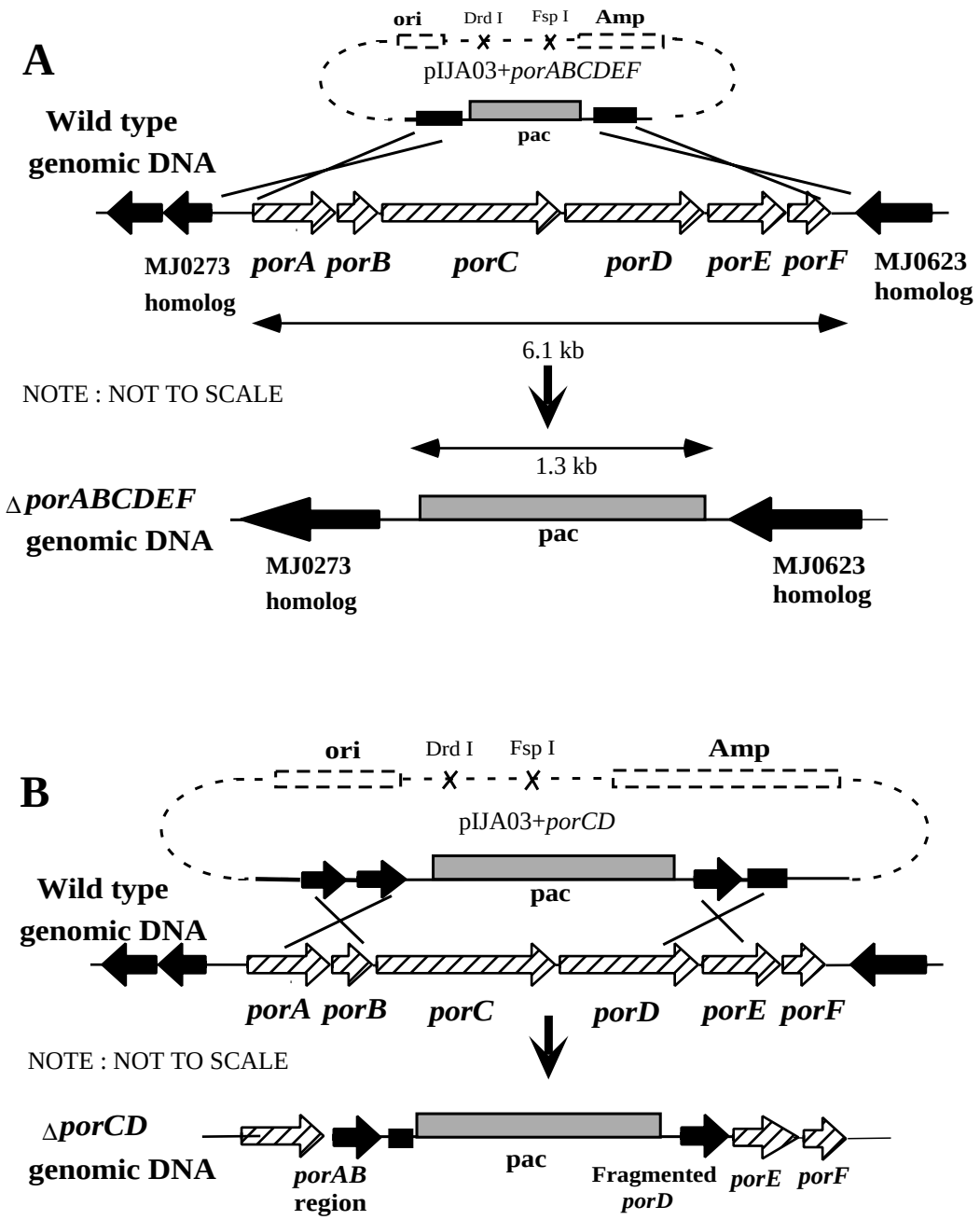

A
ori
Drd I
Fsp I
Amp
pIJA03+porABCDEF
pac
Wild type
genomic DNA
MJ0273
homolog
porA porB
porC
porD
porE porF
MJ0623
homolog
6.1 kb
NOTE : NOT TO SCALE
1.3 kb
ΔporABCDEF
genomic DNA
pac
MJ0273
homolog
MJ0623
homolog
B
ori
Drd I
Fsp I
Amp
pIJA03+porCD
pac
Wild type
genomic DNA
porA porB
porC
porD
porE porF
NOTE : NOT TO SCALE
ΔporCD
genomic DNA
porAB
region
pac
Fragmented
porD
porE
porF

Figure 4.2. Map of PCR amplicands used for construction of gene replacement and insertional inactivation vectors. Dotted line denotes region of deletion for the gene replacement vectors. A. Amplicands and primers for the *porABCDEF* deletion: (1) 5'porABCDEF-MCS1; (2) 3'porABCDEF-MCS1; (3) 5'porABCDEF-MCS2 and (4) 3'porABCDEF-MCS2. B. Primers for *porCD* deletion: (1) 5' porCD-MCS1; (2) 3' porCD-MCS1, (3) 5'porCD-MCS2 and (4) 3'porCD-MCS2. C. Primers for insertional inactivation of *porC*: (1) SPOR□BglII5' and (2) SPOR□XbaI-3'. D. Primers for insertional inactivation of porE: (1) SPOR□BglII5' and (2) SPORCRXbaI-3'.

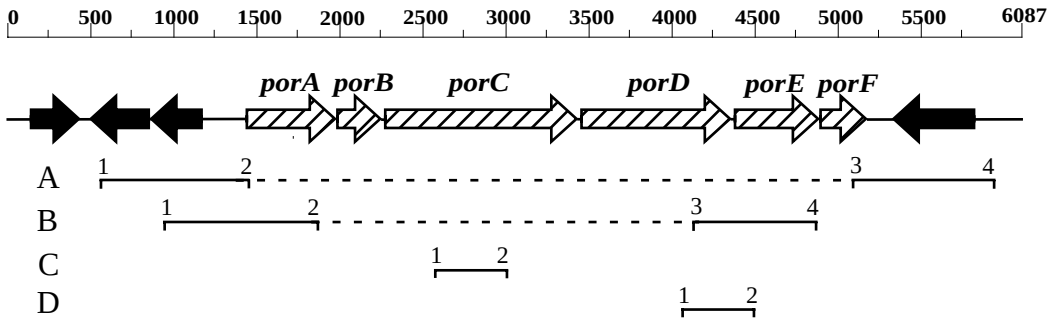
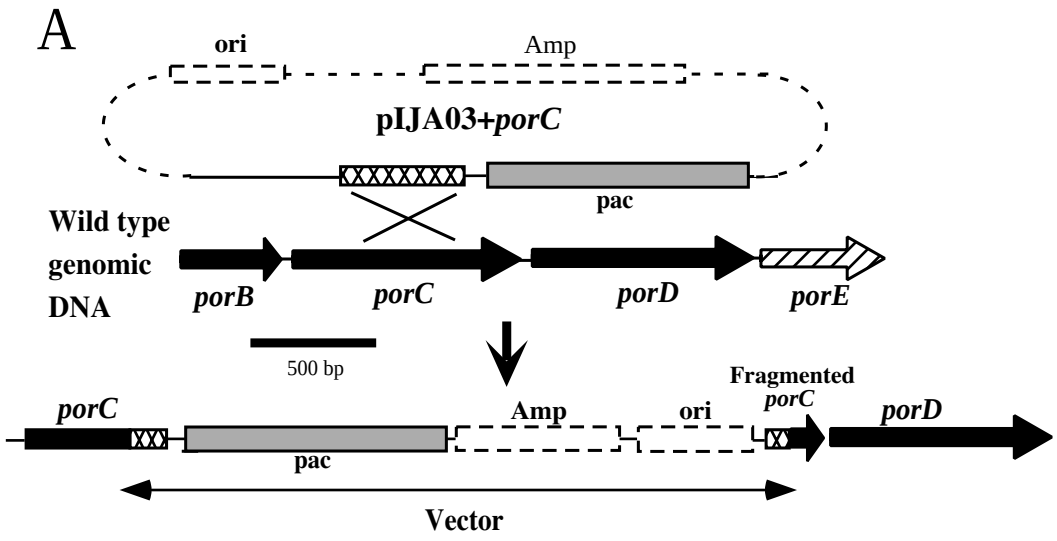

0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
5500
6087
porA
porB
porC
porD
porE
porF
A
B
C
D
1
2
3
4

Figure 4.3. Strategy for insertional inactivation of *porC* or *porE* through single homologous recombination with pIJA03+*porC* or pIJA03+*porE*. This strategy assumes that this gene cluster contains a single promoter 5' of *porA*. A. Inactivation with pIJA03+*porC*. B. Inactivation with pIJA03+*porE*.


A
ori
Amp
pIJA03+porC
pac
Wild type
genomic
DNA
porB
porC
porD
porE
500 bp
porC
Amp
ori
Fragmented
porC
porD
pac
Vector

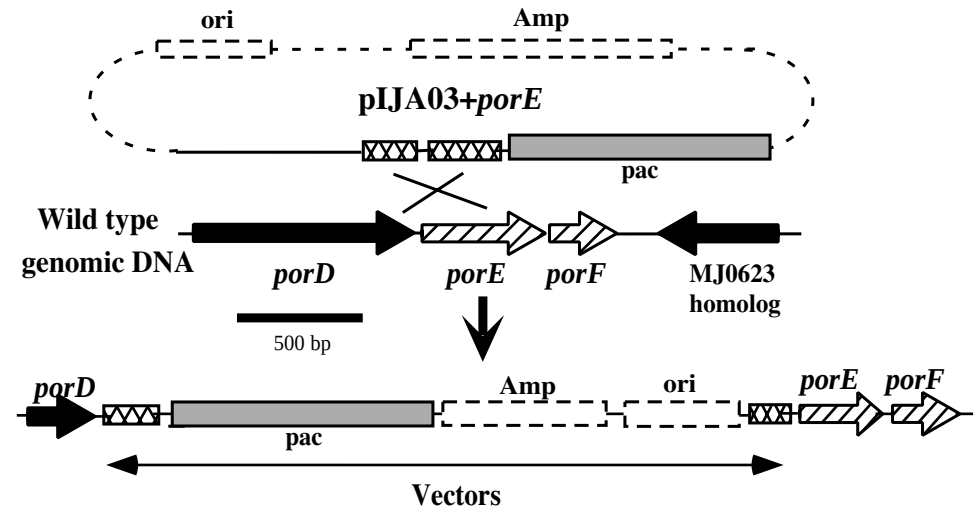
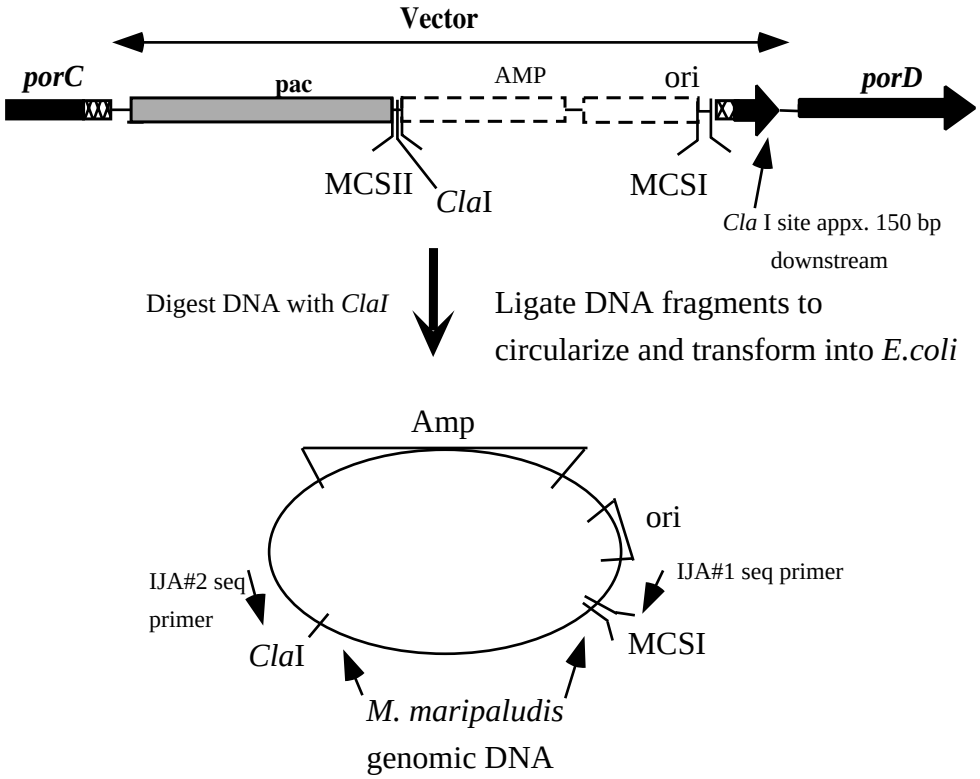
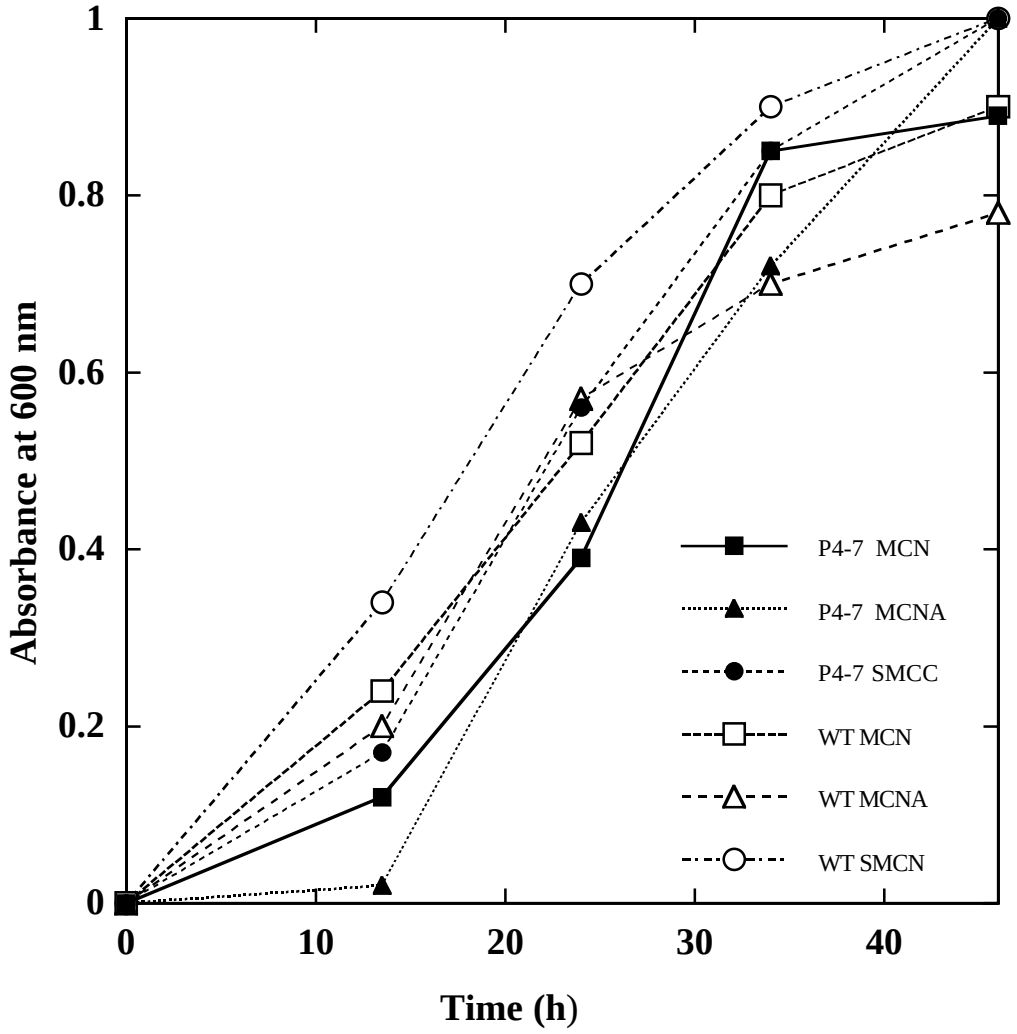

B
ori
Amp
pIJA03+porE
pac
Wild type
genomic DNA
porD
porE
porF
MJ0623
homolog
500 bp
porD
Amp
ori
porE
porF
pac
Vectors

Figure 4.4. Determination of the location of the insert of pIJA03+ *porC*. Genomic DNA of a transformant of pIJA03 +*porC* was digested with *cla*I. A *cla*I site was in the MCS2 of pIJA03 as well as the flanking genomic DNA. The cut DNA was treated with ligase and transformed into *E. coli*. After selection with ampicillin, plasmids containing a portion of the flanking DNA was isolated. Using IJA#2 and IJA#1 sequencing primers, the genomic DNA was then sequenced.



Sequence with primers

Figure 4.5. Growth of the Δ*porCD* mutant JJ156. The effect of *porCD* deletion on *M. maripaludis*. Growth was initiated with a ten-fold diluted culture and was grown at 37°C under H_2/CO_2. Growth of JJ1 (WT) in McCA (○), McNA (△), and McN (□). Growth of JJ156 in McCA (●), McNA (▲) and McN (■).


1
0.8
0.6
0.4
0.2
0
Absorbance at 600 nm
0
10
20
30
40
Time (h)
P4-7 MCN
P4-7 MCNA
P4-7 SMCC
WT MCN
WT MCNA
WT SMCN

CHAPTER 5

CONCLUSIONS

In methanogenic habitats, the midpoint of the H^+/H_2 couple is about –296 mV (at 10^{-4} atm) and the acetyl CoA+CO_2/pyruvate couple is about –500 mV. From the chemical equation of pyruvate synthesis,

$$\text{acetyl-CoA} + CO_2 + H^+ + 2e^- \leftrightarrow \text{pyruvate} + \text{CoASH}$$

the $\Delta G°$ can be estimated at +39 kJ/mol. This would favor pyruvate oxidation rather than biosynthesis. For pyruvate synthesis to work, the POR must be coupled to an energy source. In our model, the POR is coupled to a hydrogenase which can drive the reaction through translocation of hydrogen or sodium ions. A transfer of electrons would then reduce a low potential electron carrier, which can then drive the synthesis of pyruvate from acetyl-CoA In support of this model, low potential ferredoxins have been shown to be utilized as electron donors for pyruvate synthesis in *Chlorobium* and *Moorella*. From this work, the discovery of *porE* and *porF,* both of which contained predicted Fe/S binding motifs, has lead to the hypothesis that these proteins may be ferredoxin-like and serve as the physiological electron donors to the anabolic POR in hydrogenotrophic methanogens such as *Methanococcus*. Interestingly, from sequence analyses a gene similar to *porE* is also associated with the carbon monoxide dehydrogenase (CODH). The CODH is another important anabolic enzyme thought to be coupled to a hydrogenase and may require a low potential electron donor.

Through mutagenesis of *porEF* in *Methanococcus*, it was shown that *porEF* affected the growth and pyruvate-dependent methanogenesis. The complementation of *porEF* restored the growth and pyruvate-dependent methanogenesis to wild-type levels, further confirming that *porEF* played an important role in the anabolic POR of *Methanococcus*. Furthermore, partial complementation of the *porE* and *porF*

demonstrated that individually, each had a different role in the anabolic POR. Implicit from these results, PorE, which copurified with the POR enzyme, may be loosely bound to the POR and may serve to coordinate electrons for the POR in both the oxidative and reductive direction of the reaction. PorF did not seem to be important in the oxidative direction because it failed to restore pyruvate-dependent methanogenesis. However, PorF did partially restore the growth phenotype. Based upon these results and further supposition, PorF may function as the electron donor to the POR. However, specific roles have not been determined for PorE and PorF. What is known conclusively is that porEF is needed for the POR, but individually they have different effects on the POR. Further investigation will be needed to determine their relevance and function to the anabolic POR of *Methanococcus* and other hydrogenotrophic methanogens.