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ORIGINAL PAPER

Winston Lin · William B. Whitman

The importance of *porE* and *porF* in the anabolic pyruvate oxidoreductase of *Methanococcus maripaludis*

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Abstract The operon of the anabolic pyruvate oxidoreductase (POR) of *Methanococcus maripaludis* encodes two genes (*porEF*) whose functions are unknown. Because these genes possess sequence similarity to polyferredoxins, they may be electron carriers to the POR. To elucidate whether the methanococcal POR requires PorEF for activity, a deletion mutant, strain JJ150, lacking *porEF* was constructed. Compared to the wild-type strain JJ1, the mutant grew more slowly in minimal medium and minimal plus acetate medium, and pyruvate-dependent methanogenesis was inhibited. In contrast, the methyl-viologen-dependent pyruvate-oxidation activity of POR, carbon monoxide dehydrogenase, and hydrogenase activities of the mutant were similar to those of the wild-type. Upon genetic complementation of the mutant with *porEF* in the methanococcal shuttle vector pMEV2+*porEF*, growth in minimal medium and pyruvate-dependent methanogenesis were restored to wild-type levels. Complementation with *porE* alone restored methanogenesis from pyruvate but not growth in minimal medium. Complementation with *porF* alone 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 in vivo even though they are not required for the dye-dependent activity.

Keywords Pyruvate oxidoreductase · Methanococcus · Archaea · Methanogenesis · Ferredoxin

W. Lin · W. B. Whitman (✉)
Department of Microbiology, University of Georgia,
Athens, GA 30602-2605, USA
Tel.: +1-706-5424219, Fax: +1-706-5422674,
e-mail: whitman@uga.edu

Present address:
W. Lin
Department of Microbiology, University of Massachusetts,
Amherst, Massachusetts, USA

Abbreviations *CODH/ACS* Carbon monoxide dehydrogenase/acetyl-CoA synthase · *POR* Pyruvate oxidoreductase

Introduction

The anabolic pyruvate oxidoreductase (POR) in *Methanococcus maripaludis* catalyzes the energetically unfavorable reductive carboxylation of acetyl-CoA to form pyruvate. Pyruvate, a key precursor for amino acid and sugar biosynthesis in *Methanococcus*, then enters the incomplete reductive tricarboxylic acid and gluconeogenic pathways (Shieh and Whitman 1987; Yu et al. 1994). Upon purification, the methanococcal POR contains five polypeptides, four of which are similar to the α, β, γ, and δ subunits of PORs initially characterized in hyperthermophiles (Blamey and Adams 1993; Adams and Kletsin 1996; Lin et al. 2003). The fifth polypeptide, PorE, appears to be present only in hydrogenotrophic methanogens like *M. maripaludis, Methanocaldococcus jannaschii,* and *Methanothermobacter thermautotrophicus*. Additionally, the *M. maripaludis por* operon encodes ORFs (*porABCDEF*) corresponding to the N-terminal sequences of all five polypeptides found in the purified enzyme and an additional ORF (*porF*) with sequence similarity to *porE* (Lin et al. 2003). The predicted amino acid sequences of *porE* and *porF* contain iron–sulfur motifs, thus implying a possible ferredoxin-like electron transfer function. A homolog to *porE* is also found next to the carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) gene cluster in these hydrogenotrophic methanogens. Like POR, the reduction catalyzed by CODH/ACS is very endothermic when H_2 is the electron donor. Thus, it was proposed that PorE and its homologs are specialized, low-potential electron carriers for the methanococcal POR and other enzymes catalyzing endothermic anabolic reactions (Lin et al. 2003). Evidence supporting this hypothesis comes from work on *Moorella [Clostridium] thermoacetica, Chlorobium tepidum*, and *Hydrogenobacter thermophilus* PORs, which require low-potential ferredoxins for pyruvate synthesis

Table 1 Bacterial strains and plasmids utilized in this study. *POR* Pyruvate oxidoreductase

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 *Methanococcus maripaludis* POR operon	Lin et al. (2003)
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 in which nonessential regions were deleted	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

(Furdui and Ragsdale 2000; Yoon et al. 1996, 1997, 2001). The studies presented here provide additional evidence that *porEF* are involved in pyruvate biosynthesis.

Materials and methods

Bacterial strains, plasmids, media and culture conditions

The sources of the microbial strains and plasmids are listed in Table 1.

M. maripaludis was grown in 28-ml stoppered tubes with 275 kPa H_2/CO_2 gas (80:20, v/v) at 37 °C in McN mineral 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%(v/v) vitamin mixture (Whitman et al.1986) and 3 mM 2-mercaptoethanesulfonic acid), modified McCA+alanine in which the sole nitrogen source, ammonium chloride, was replaced with 2.5 mM alanine, and McCAP (McCA plus 10 mM pyruvate). In some cases, either puromycin or neomycin was added to final concentrations of 2.5 μg ml^{-1} or 500 μg ml^{-1}, respectively.

Large-scale cultures for preparation of cell-free extracts were grown in an 11-l fermentor as described by Shieh and Whitman (1988) except that McCA was used instead of McC. The fermentor was prepared as follows: McCA (11 l) with the sodium bicarbonate reduced to 2 g l^{-1} was prepared and autoclaved under H_2/CO_2 gas for 20 min. Following the autoclave cycle, the fermentor was cooled to 37 °C with H_2/CO_2 at 100 kPa and a flow rate of 100–250 ml/min. Additionally, 5 ml of a 20% (w/v) 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, the cells were examined for the mutant phenotype by monitoring growth in McCA and McN media in 28-ml stoppered tubes and methane evolution from pyruvate as described later. The experiments were carried out 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 containing 1 mM dithiolthreitol and 1 mM cysteine-HCl, pH 6.8, with 0.5 mg DNase I per 10 g wet weight of cells, as described by Shieh and Whitman (1987). The resuspended cells were aliquoted into 2-ml volumes and stored at –20°C 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, Calif., USA). For liquid and solid media, kanamycin (50 μg ml^{-1}) or ampicillin (50 μg ml^{-1}) were added to cultures containing the plasmids 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, New York, USA).

Transformation and plasmid purification

M. maripaludis was transformed by the polyethylene glycol method (Tumbula et al. 1994). *E. coli* Top 10 transformations were done using a Gene Pulser electroporator (BioRad, Richmond, Calif., USA) at 200 W, 2.5 kV, and 25 mF 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

porEF deletions in the *M. maripaludis* genome were accomplished using pIJA03+CR (Fig. 1). The plasmid pIJA03 is an integration vector that lacks an origin of replication for methanococci and contains a *pac* cassette (Statholopolous et al. 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 (Gernhardt et al. 1990; Statholopolos et al. 2001). To construct pIJA03+CR, a 1049 bp region within *porD* was amplified by PCR using the primers 5′Crinact-MCS1 (5′-CCCCCCGCAGATCTGT-TGTAGGTCTTGGTGGAAGG-3′) and 3′Crinact-MCS1 (5′-CCC-CCCGCTCTAGAAGACCGTATTCGTCGCATTTC Y-3′). These primers introduced *Bgl*II and *Xba*I sites (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,

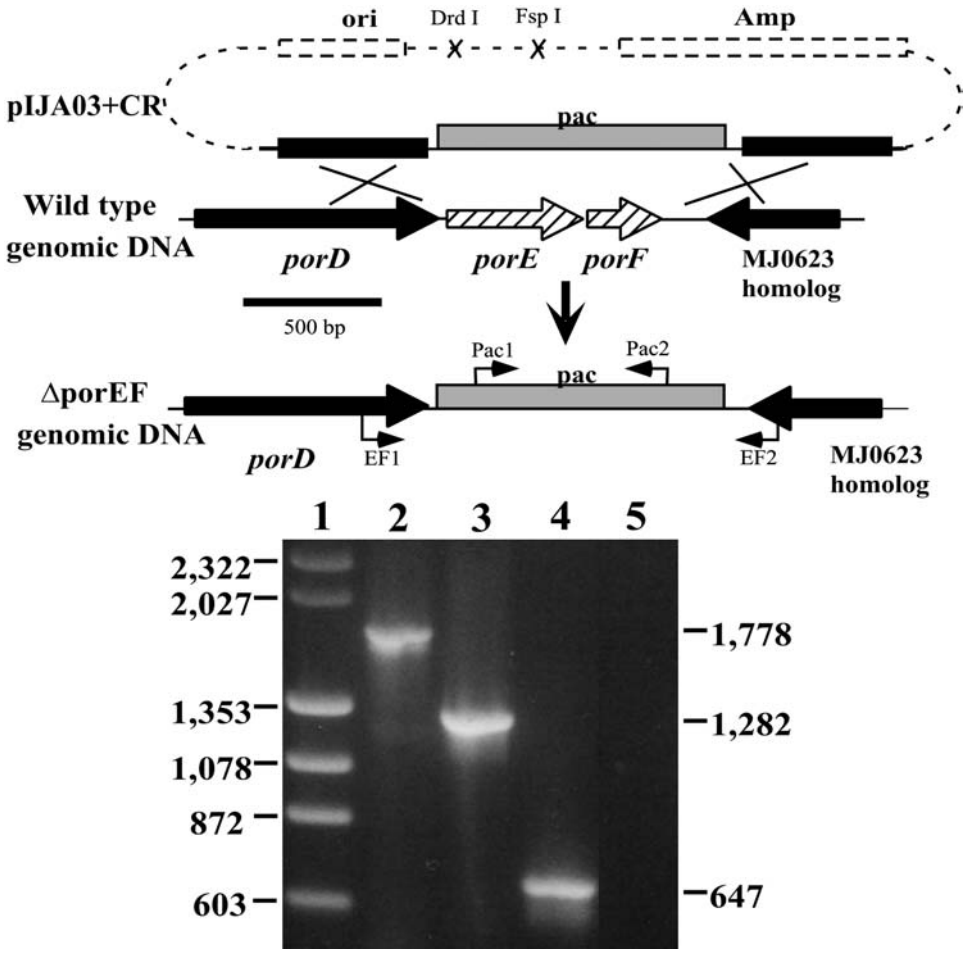

Fig. 1a, b Replacement of *Methanococcus maripaludis porEF* genes with the *pac* cassette. **a** Directed replacement and inactivation of *porE* and *porF* with the *pac* cassette was accomplished through double homologous recombination using pIJA03+CR. Prior to transformation, pIJA03+CR was digested with *Drd*I and *Fsp*I to produce the linear plasmid. **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 mutant strain JJ 150 with EF primers, *lane 3* amplification of strain JJ1 (wild-type) using EF primers, *lane 4* amplification of strain JJ150 with Pac primers, *lane 5*, amplification of strain JJ1 using Pac primers

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 carried out for 30 cycles at 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min and 30 s. The final extension time was 5 min at 72 °C. The orientation of each insert was confirmed by restriction mapping.

Prior to transformation, pIJA03+CR was linearized by digestion with *Drd*I and *Fsp*I. 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, McCAP, and modified McCA+alanine plates 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 McCA 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 stored 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 (Tumbula et al. 1995). The cells were aliquoted into 0.2-ml fractions, sealed into airtight cyrogenic tubes (Corning, New York, USA) 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 for each condition and was repeated at least once.

Methanogenesis from pyruvate

Cells were grown in McCA or McN under H_2/CO_2 to early stationary phase or an absorbance at 600 nm of 0.8–0.85. The cultures were centrifuged at 2,500×*g* (Beckman centrifuge) for 15 min at room temperature. Cell pellets were washed once and resuspended in 5 ml Mc buffer (Yang et al. 1992). Mc buffer was similar in composition to McN except that the bicarbonate concentration was reduced from 5 g/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 min, 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 sodium pyruvate at 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, Tex., USA) and a PorapakQ 80/100 column (Alltech, Houston, Tex., USA). The temperatures of the detector, injector, and column were 250, 180 and 120 °C, 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 done under strictly anoxic 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×*g* for 30 min at 4 °C. The supernatant was used in the subsequent assays. The protein concentration of the supernatant was measured using the BCA method (Pierce Chemical, Ohio, USA).

POR activity was measured anoxically as pyruvate and HS-CoA-dependent methyl-viologen (MV) reduction (Shieh and Whitman

1988). Activities of the carbon monoxide dehydrogenase/ acetyl-CoA synthase (CODH/ACS; Shieh and Whitman1988) and hydrogenase (Ragsdale and Ljungdahl 1984) were measured similarly as CO- or H_2-dependent reduction of methyl viologen, respectively.

Construction of the methanococcal expression vector pMEV-2

The plasmid pMEV-2 developed in this study was derived from pMEV1 and the cosmid pBK-CMV (Stratagene, Calif., USA), 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 were deleted (Gardner 2000). 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). To replace the *pac* cassette in pMEV1, the *neo*/*kan* cassette from pBK-CMV was amplified using the primers MEV/Neo-Bam (5′-CCCCCCGGATCCGAGGCC TAGGCTTTTGCAAA-3′) 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. PCR amplification was carried out as described above, and the amplicon was digested with *Bam*HI and *Eco*0109 for directed ligation into pMEV1 to create pMEV2. The region chosen for the ligation of the *neo*/*kan* amplicon placed it under the control of the P*mcr* promoter. The structure of pMEV2 was verified through restriction mapping.

Complement of Δ*porEF* mutants

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 et al.1994). Transformants were screened on McCA plates containing neomycin.

Results and discussion

Mutagenesis of *porEF* and complementation

To determine whether *porEF* is required for POR activity, deletion mutants were constructed by transformation of the *M. maripaludis* wild-type strain JJ1 with pIJA03+CR. This plasmid contained the *pac* cassette, encoding puromycin resistance in methanococci, between the genes flanking *porEF* on the *M. maripaludis* genome. Upon transformation with the linearized plasmid, the wild-type was expected to acquire puromycin resistance through replacement of *porEF* with the *pac* cassette (Fig. 1). Because pyruvate is 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 of wild-type cells is obtained from alanine (Yang et al. 2002). In all those media, the transformation efficiency was about 3×10^4 transformants per μg of DNA, comparable to that of the positive control pBD1 in the same experiments. Transformation with pBD1 deletes a portion of the gene encoding cysteinyl-tRNA synthetase, which is cryptic in *M. maripaludis* (Statholopolous et al. 2001). The high transformation efficiency insured that the mutants were not derived from rare genetic events or multiple mutations and that the transformants were capable of growth on McCA medium without pyruvate or alanine.

The genotype of the mutants was confirmed by PCR amplification with flanking primers (EF1 and EF2), which resulted in a 1.8-kb product for the Δ*porEF::pac* mutant. This increase from the 1,282-bp amplicon of the wild-type resulted from replacement of the 790-bp *porEF* region with the 1.3-kb *pac* cassette (Fig. 1). Additionally, primers internal to the *pac* cassette produced a PCR product of the expected size from the deletion mutant but not from JJ1 genomic DNA, which did not possess the *pac* cassette. All three of the deletion mutants that were isolated, including strain JJ150, contained the correct genotype (Fig. 1 and data not shown).

Growth of the Δ*porEF::pac* mutant

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 mutant strain JJ150 were monitored with rich (McCA), minimal (McN), and minimal+acetate (McNA) medium. The effect of pyruvate on growth was not determined because a degradation product of pyruvate forms spontaneously in methanococcal medium and inhibits growth (data not shown). Presumably, this product is the dimer parapyruvate, and acetate is not detected following incubations of uninoculated medium with pyruvate (Korff 1969, Yang et al. 1992). The growth of strain JJ150 was comparable to that of the wild-type in rich medium, where many of the monomers required for growth were provided (Fig. 2A). However, during the exponential phase strain JJ150 grew more slowly than the wild-type in minimal medium, where higher levels of pyruvate biosynthesis would be required. Because cultures enter into a linear growth phase at high cell density due to H_2 limitation, exponential growth is only observed at low cell densities, below optical densities of 0.4 in Fig. 2A. Nevertheless, this difference was consistently observed in six independent experiments. The doubling times (means±standard deviations of four cultures) of the wild-type in McN mineral and McCA complex media were nearly the same, 3.5±0.9 h and 2.8±0.5 h, respectively. In

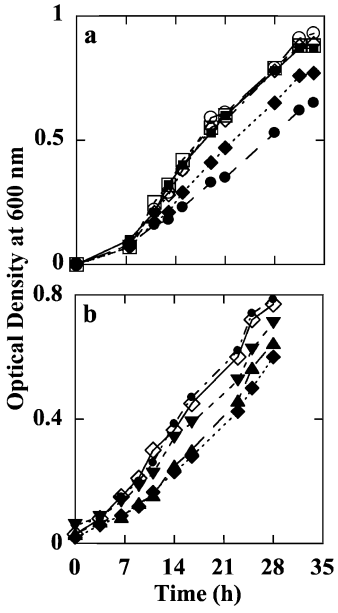

Fig 2a, b Growth of Δ*porEF::pac* mutant strain JJ150 and complementation by pMEV2+*porEF*. **a** The effect of Δ*porEF::pac* deletion on growth at 37 °C on H_2/CO_2. Growth of strain JJ1 (wild-type) in McCA (□), McN (◊), and McNA(○). Growth of *porEF* mutant strain JJ150 in McCA (■), McN (◆), and McNA (●). **b** Complementation of *porEF*. All strains were grown in McN at 37 °C under H_2/CO_2: strain JJ1 (wild-type, ◊), strain JJ150 (*porEF,* ◆), strain JJ154 (*porEF+porE,* ▲), strain JJ155 (*porEF+porF,* ▼), strain JJ153 (*porEF+porEF*, ●)

contrast, the doubling times of mutant strain JJ150 were 5.1±2.1 h and 3.6±0.6 h, respectively, and the addition of acetate caused even poorer growth (Fig. 2A). 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.

The mutant was also grown on ammonia-free medium with alanine as the nitrogen source. Under these conditions, alanine is a major source of intracellular pyruvate (Yang et al. 2002). Both the mutant and the wild-type grew extremely slowly under these conditions in mineral+acetate as well as in complex medium. In addition, upon repeated transfers of strain JJ150 in minimal medium (with ammonia as the N-source), the growth properties became indistinguishable from those of 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, strain JJ153 was constructed in which 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 strain JJ153 (Fig. 2B). Additionally, strains JJ154 and JJ155, which contained either *porE* or *porF* cloned into pMEV2, respectively, provided partial complementation. By itself, *porE* had only a small effect, while growth with *porF* alone was nearly sufficient to restore a wild-type pattern of growth (Fig. 2B). Nevertheless, the requirement for both *porE* and *porF* for full complementation suggests that the two genes play important yet somewhat different roles.

Table 2 Methyl-viologen-dependent oxidation activities of POR, carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS), and hydrogenase in extracts of *M. maripaludis* strains JJ1 and JJ150. Means and standard deviations are based upon four assays of extracts from two independent cultures grown in complex medium. Activities between the two strains were not significantly different at P=0.05

Strain	Specific activity (μmol min^{-1} mg $protein^{-1}$) of:		
	POR	CODH/ACS	Hydrogenase
JJ1 (wild-type)	0.59±0.12	0.20±0.06	420±340
JJ150	0.83±0.25	0.10±0.05	380±170

POR activity in the Δ*porEF::pac* mutant

The enzymatic activity of the POR in cell extracts of mutant strain JJ150 and the wild-type JJ1 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 strain JJ150 and wild-type JJ1 grown in complex medium were similar for all three enzymes (Table 2). Likewise, the specific activities for these three enzymes in the wild-type and strain JJ150 cells grown in miminal medium were also similar to each other (data not shown). These results indicate that the deletion did not have a large affect on the levels of POR and its dye-dependent activity.

Pyruvate-dependent methanogenesis of the Δ*porEF::pac* mutant

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 *porEF::pac* mutant, where the activity was comparable to that found with endogenous substrates (Table 3). In contrast, methanogenesis with H_2 as the electron donor was not affected. Pyruvate-dependent methanogenesis was fully restored in strains JJ153 and JJ154, which were the deletion mutants complemented with *porEF* and *porE*, respectively (Table 3). In contrast, the phenotype of strain JJ155, in which the *porEF::pac* deletion was complemented by only *porF*, was indistinguishable from that of the original mutant. These results provided further evidence that *porE* and *porF* possess different functions.

Table 3 Methanogenesis from pyruvate by resting cells of the Δ*porEF::pac* mutant of *M. maripaludis*.Cells were incubated under N_2/CO_2 with 50 mM pyruvate or H_2/CO_2. Activity was determined after 9 h, except for H_2, which was determined after 4 h. *ND* Not determined. Means and standard deviations are based upon four determinations. Activities with H_2 were based upon two determinations, thus standard deviations are not given

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

Summary

Lin et al. (2003) previously demonstrated that *porE* and *porF* contained motifs indicative of Fe–S clusters and proposed that PorE and PorF were electron carriers to the anabolic POR. While we have not elucidated the specific functions of PorE and PorF, we have shown here that *porE* and *porF* are important components of the anabolic POR in hydrogenotrophic methanogens. The deletion of *porE* and *porF* affected growth and oxidation of pyruvate. Complementation of *porEF* restored both of these functions. Furthermore, PorE and PorF complemented these POR functions differently, indicating that they play different roles. Implicit in these results, PorE, which copurified with the POR enzyme (Lin et al.2003), may be tightly 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 affect pyruvate-dependent methanogenesis. However, PorF did partially restore the growth phenotype. Based upon these results, PorF may function as an electron donor to the POR. While more research is necessary to elucidate the specific function of PorE and PorF, these proteins may represent a novel adaptation of the POR by the hydrogenotrophic methanogens for pyruvate biosynthesis.

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