Genetic, Genomic, and Transcriptomic Studies of Pyruvate Metabolism in *Methanosarcina barkeri* Fusaro

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**ABSTRACT**

Pyruvate, a central intermediate in the carbon fixation pathway of methanogenic archaea, is rarely used as an energy source by these organisms. The sole exception to this rule is a genetically uncharacterized *Methanosarcina barkeri* mutant capable of using pyruvate as a sole energy and carbon source (the Pyr \(^+\) phenotype). Here, we provide evidence that suggests that the Pyr \(^+\) mutant is able to metabolize pyruvate by overexpressing pyruvate ferredoxin oxidoreductase (por) and mutating genes involved in central carbon metabolism. Genomic analysis showed that the Pyr \(^+\) strain has two mutations localized to Mbar_A1588, the biotin protein ligase subunit of the pyruvate carboxylase (pyc) operon, and Mbar_A2165, a putative transcriptional regulator. Mutants expressing the Mbar_A1588 mutation showed no growth defect compared to the wild type (WT), yet the strains lacked pyc activity. Recreation of the Mbar_A2165 mutation resulted in a 2-fold increase of Por activity and gene expression, suggesting a role in por transcriptional regulation. Further transcriptomic analysis revealed that Pyr \(^+\) strains also overexpress the gene encoding phosphoenolpyruvate carboxylase, indicating the presence of a previously uncharacterized route for synthesizing oxaloacetate in *M. barkeri* and explaining the unimpaired growth in the absence of Pyc. Surprisingly, stringent repression of the por operon was lethal, even when the media were supplemented with pyruvate and/or Casamino Acids, suggesting that por plays an unidentified essential function in *M. barkeri*.

**IMPORTANCE**

The work presented here reveals a complex interaction between anabolic and catabolic pathways involving pyruvate metabolism in *Methanosarcina barkeri* Fusaro. Among the unexpected findings were an essential role for the enzyme pyruvate-ferredoxin oxidoreductase and an alternate pathway for synthesis of oxaloacetate. These results clarify the mechanism of methanogenic catabolism of pyruvate and expand our understanding of carbon assimilation in methanogens.

A diverse group of strictly anaerobic archaea are responsible for virtually all biologically produced methane, making them key players in the global carbon cycle (1). These unusual microorganisms grow using a very limited number of substrates, obtaining all of their energy from the methanogenic process. While most methanogens use only H\(_2\)-CO\(_2\) or formate as growth substrates, members of the *Methanosarcinales* show some metabolic diversity, with many species using H\(_2\)-CO\(_2\), various one-carbon (C\(_1\)) compounds (e.g., methanol, methylamines, and methyl-sulfides), and acetate as growth substrates. A few methanogenic species can use longer-chain alcohols as energy sources, but these molecules are not assimilated. Instead, they are oxidized to provide the reducing equivalents needed for reduction of CO\(_2\) to methane and then excreted (2). Larger organic compounds, such as sugars and fatty acids, are almost never substrates for methanogenic archaea, although they are cometabolized via syntrophic microbial communities (3). The sole exception to this rule is *Methanosarcina barkeri*, which has been reported to grow on pyruvic acid.

Two reports of methanogenic growth on pyruvate appeared in the early 1990s. In the first, a pyruvate-utilizing (Pyr \(^+\)) mutant of *M. barkeri* Fusaro was obtained after prolonged incubation (8 to 12 weeks) of methanol-grown cell aggregates inoculated into a medium containing pyruvate as the sole substrate (4). Although initial growth was rather slow, repeated passages in pyruvate medium gave rise to a strain with a doubling time of ca. 24 h. The conclusion that growth on pyruvate was enabled by mutation, rather than by adaptation, was based on the observation that the long lag needed for switching from methanol to pyruvate was lost in this strain. Characterization of the mutant suggested that growth involved a pathway in which pyruvate was oxidized to acetyl coenzyme A (acetyl-CoA) and CO\(_2\) using the enzyme pyruvate-ferredoxin oxidoreductase (Por), with subsequent conversion of acetyl-CoA to CO\(_2\) and CH\(_4\) by the standard aceticlastic pathway for methanogenesis (4, 5). The reducing equivalents produced by pyruvate oxidation were also used to reduce CO\(_2\) to CH\(_4\) probably using H\(_2\) as an intermediate. Interestingly, the mutant displayed limited nonmethanogenic growth when methanogenesis was inhibited by addition of bromoethanesulfonic acid (BES); however, growth ceased after ca. 8 generations (5). Thus, the notion that all methanogens are obligate methanogens remains intact. Rajagopal and LeGall also reported growth of *M. barkeri* on pyruvate (6), but using different strains (227, MS, and...
from DSMZ in May 2011. Growth on media solidified with 1.5% agar was as described previously (14). All plating manipulations were carried out under strictly anaerobic conditions in an anaerobic incubator as described previously (15). Puromycin (CalBiochem, San Diego, CA) was added from sterile anaerobic stocks at a final concentration of 2 μg/ml for selection of Methanosarcina strains carrying the puromycin transacetylase gene (pac). The purine analog 8-aza-2,6-diaminopurine (Sigma, St. Louis, MO) was added from sterile anaerobic stocks at a final concentration of 20 μg/ml for selection against the hpt gene. Tetracycline was added to a final concentration of 100 μg/ml to induce expression of genes driven by the pMcrB(tetO1) promoter. Unless otherwise stated, 10% inoculum was used as a starting culture for pyruvate-utilizing strains.

Construction and verification of mutant strains. M.arkeri strains WWM818 and WWM940 were constructed by markerless genetic exchange via liposome-mediated transformation (13, 16). WWM818 was made from WWM85 using pML09. WWM940 was made from WWM818 using pML34. WWM941 was made by gene replacement (17) after transformation of WWM85 to puromycin resistance with pML44. The plasmids used for creating mutants are described in Table S1 in the supplemental material. All plasmids were confirmed by DNA sequencing at the W. M. Keck Center for Comparative and Functional Genomics, University of Illinois. All mutations were verified by PCR and DNA hybridization experiments (data not shown). DNA hybridizations were performed using the DIG System (Roche, Mannheim, Germany) as recommended by the manufacturer using MagnaGraph Nylon transfer membranes (Micron Separations Inc., Westborough, MA). Standard methods were used to isolate and manipulate plasmid DNA from Escherichia coli strains (18). For plasmid descriptions and strain genotypes, refer to Table S1 in the supplemental material and Table 1, respectively.

Genome sequencing and mapping analysis. Genomic DNA from M.arkeri was isolated as described previously (19) and sequenced on the Illumina HiSeq2000 platform at the W. M. Keck Center for Comparative and Functional Genomics, University of Illinois. DNA libraries were prepared with an Illumina TruSeq DNA Sample Prep kit (Illumina, San Diego, CA), quantified by quantitative PCR (qPCR), and sequenced for 100 cycles using a TruSeq SBS sequencing kit (Illumina, San Diego, CA). Reads were trimmed (quality, 0.001; maximum, 100; minimum, 99) and mapped to the M.arkeri reference genome (NC_007333 and NC_007349) using CLC Genomics Workbench 7.0.3 (CLCbio, Aarhus, Denmark). The parameters for mapping were as follows: mismatch, 2; insertion, 3; deletion, 3; length, 0.8; similarity, 0.95; mapped randomly. The same program was used to identify point mutations, insertions, and deletions. The parameters for single nucleotide polymorphism (SNP) detection were as follows: maximum coverage, 10; variant count, 2; variant probability, 0.0. The parameters for insertions-deletions were as follows: P value, 0.0001; mismatch, 3; minimum number of reads, 2. A variant ratio of 0.9 to 1 and a frequency of 90 to 100% were used as selection criteria for the mutations. All mutations identified by this method were confirmed by Sanger sequencing of PCR products amplified from the appropriate strains.

Transcriptome analyses (RNA-seq). Strains were adapted to specific growth substrates for at least 30 generations prior to RNA isolation. RNA was extracted from triplicate cultures grown to mid-exponential phase using a modification of the ZR Fungal/Bacterial RNA MiniPrep kit (Zymo Research, Irvine, CA). Briefly, 10-ml cultures were quickly chilled in a dry-ice/ethanol bath to stop RNA synthesis. The cultures were pelleted by centrifugation for 10 min at 4°C and then resuspended in 800 μl TRIzol reagent (ThermoFisher, Grand Island, NY). Total RNA was extracted following the manufacturer’s instructions. rRNA was depleted using subtractive hybridization employing biotinylated rRNA probes as previously described (20), except that the probes were generated using M.arkeri rRNA genes as templates.

RNA sequencing was performed at the W. M. Keck Center for Comparative and Functional Genomics, University of Illinois, using strand-specific libraries prepared with Illumina ScriptSeq version 2 (Epicentre
Biotechnologies, Madison, WI). Bar-coded libraries were pooled and sequenced for 101 cycles on a HiSeq2000 using the TrueSeq SBS sequencing kit version 3 (Illumina, San Diego, CA). The reads were analyzed using the CLC genomics platform (version 7.5); reads were trimmed based on quality (quality, 0.001; ambiguous, 2; discard minimum, 30, maximum, 100). To remove bias caused by the remaining stable RNAs, the trimmed sequences were mapped to *M. barkeri* rRNA and tRNA sequences using the following parameters: similarity, 0.9; length, 0.85; mismatch, 2; insertion, 3; deletion, 3. The remaining reads were used for the RNA-seq analysis (similarity, 0.9; length, 0.85; mis; 2; ind; 3; del; 3; global alignment, maximum number of reads per read, 10). Expression values were calculated as reads per kilobase per million (RPKM). The reads were normalized, and statistical analysis was performed using the empirical analysis of digital gene expression (21). To determine differential gene expression, reads from each mutant strain were compared against reads of the wild-type (WT) strain when grown in the same substrate. Genes showing expression changes greater than 2-fold or less than −2-fold with *P* values of ≤0.05 were considered to be differentially expressed.

**Phenotypic characterization.** Growth rates were determined by monitoring the optical densities of three or more independent cultures at 600 nm (OD_{600}) using a Bausch and Lomb Spectronic 21. Gene essentiality tests were conducted using a modified procedure described previously (22). Briefly, 10-ml cultures were grown to saturation in HS-methanol broth with tetracycline (100 μg/ml), centrifuged, washed three times with plain HS medium, and then resuspended in 5 ml HS medium. Aliquots of 10 μl of a 10-fold dilution series were then spotted onto agar-solidified HS-methanol media containing some or all of the following: tetracycline (100 μg/ml), pyruvate (100 mM), acetate (100 mM), 0.01% Casamino Acid, and 0.01% yeast extract.

**Determination of enzymatic activities.** Pyruvate carboxylase was assayed from cell extracts by coupling the formation of oxaloacetate to malate dehydrogenase (11). NADH oxidation by malate dehydrogenase was measured spectrophotometrically (ε_{340} = 6.22 mM^{-1} cm^{-1}) at 37°C. Crude cell extracts were prepared from mid-exponential-phase cultures (OD_{600} = 0.5 to 0.6) by centrifuging 10-ml cultures and resuspending them in 500 μl of lysis buffer (3 M KCl, 50 mM Tris-HCl [pH 8], 5% inositol, 5 mM MgCl₂, 2 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride). The reaction mixture consisted of 100 mM Tris-HCl (pH 8), 4 mM MgCl₂, 250 mM KCl, 20 mM KHCO₃, 4 mM ATP, 0.2 mM NADH, 2 U malic dehydrogenase (Sigma-Aldrich, St. Louis, MO), 20 mM sodium pyruvate, and 20 μl crude cell extracts. Reactions were started by addition of 20 μl of sodium pyruvate to 1 ml of prewarmed assay mixture. Preparation of cell extracts and enzyme assays was performed under strictly anaerobic conditions. Phosphoenolpyruvate carboxylase activity was also measured by coupling its reaction to malate dehydrogenase. Crude cell extracts were prepared as described for pyruvate carboxylase activity. The reaction mixture contained 100 mM Tris-HCl (pH 8), 4 mM MgCl₂, 250 mM KCl, 20 mM KHCO₃, 4 mM ATP, 0.2 mM NADH, 2 U malic dehydrogenase (Sigma-Aldrich, St. Louis, MO), 20 mM sodium pyruvate, and 20 μl crude cell extracts. Reactions were started by addition of phosphoenolpyruvate to 1 ml of prewarmed assay mixture. Preparation of cell extracts and assay mixtures was performed under strictly anaerobic conditions.

Pyruvate ferredoxin oxidoreductase was assayed by monitoring the CoA- and pyruvate-dependent reduction of benzyl viologen (ε_{578} = 8.65 mM^{-1} cm^{-1}) (23). Crude cell extracts were prepared as previously described using 50 mM MOPS (morpholinepropanesulfonic acid) (2 mM DTT, pH 7) as the lysis buffer. The assay was performed under strictly anaerobic conditions. The reaction mixture consisted of 2 mM benzyl viologen, 0.1 mM CoA, 10 mM sodium pyruvate, and 10 μl crude cell extracts in a total volume of 1 ml. Reactions were started by addition of CoA to a prewarmed assay mixture.

**Protein concentrations for all assays were determined by the Bradford method** (Coomassie protein reagent, Sigma-Aldrich, St. Louis, MO), using bovine serum albumin (BSA) as a standard.

**Accession numbers.** Genome sequencing data were deposited in the Sequence Read Archive (SRA) in the National Center for Biotechnology Information (NCBI) under accession no. SRP059541. The raw and processed RNA-seq data have been deposited in the Gene Expression Omnibus (GEO) under accession number GSE70370.

**RESULTS**

**Identification of mutations associated with the Pyr⁺ phenotype.** To identify the mutations responsible for the pyruvate-utilizing phenotype of the *M. barkeri* Fusaro mutant, we sequenced the genomes of two cell lines (Pyr-1 and Pyr-2) that had been independently maintained by periodic serial transfer over the course of a decade. We also sequenced the parental strain (DSM804) from the DSMZ stock collection, which represents the closest available relative of the Pyr− mutants.

Eighteen differences, including 16 SNPs and 2 relatively large deletions, between the three newly sequenced strains and the published genome sequence were observed (Table 2). It should be noted that the published *M. barkeri* genome sequence was obtained from a cell line that had also been independently maintained by serial passage in a high-salt medium to promote growth in the single-cell morphology (24). Thus, we anticipated numerous differences between the four strains. Comparison of shared sequence differences allowed reconstruction of the most likely mutagenic history of the four strains (Fig. 1). Thirteen SNPs and both of the deletions are shared between Pyr-1, Pyr-2, and DSM804. These mutations clearly occurred after separation of the cell lines, leading to the genome-sequenced strain and the remaining three strains. Because DSM804 is Pyr−, these mutations do not by themselves confer the ability to utilize pyruvate. A similar argument can be made for a mutation that is found only in Pyr-1, because Pyr-1 and Pyr-2 grow equally well on pyruvate. The two remaining mutations must have arisen in the ancestor of the Pyr− lines, suggesting that one or both are responsible for the ability to use pyruvate. These putative Pyr− mutations include a frameshift in the pycC gene (designated the pycC1 allele) and a glycine-to-arginine mutation (G59R) in the winged-helix-turn-helix motif of a putative transcriptional regulator encoded by the Mbar_A2165 locus (Fig. 2).

**Pyruvate carboxylase activity is abolished by the pycC1 mutation.** In *M. barkeri*, OAA is synthesized by pyruvate carboxylase (Pyc), a two-subunit, biotin-dependent enzyme encoded by the pycBAC-slp operon. The α and β subunits of Pyc are encoded by *pycA* and *pycB*, while *pycC* encodes a bifunctional protein that covalently attaches the essential biotin cofactor to PycB while also serving as a transcriptional repressor for biotin-biosynthetic genes (11). The frameshift mutation that we identified occurs early in the Pyr− lines, suggesting that one or both are responsible for the inability to use pyruvate. These putative Pyr− mutations include a frameshift in the pycC gene (designated the pycC1 allele) and a glycine-to-arginine mutation (G59R) in the winged-helix-turn-helix motif of a putative transcriptional regulator encoded by the Mbar_A2165 locus (Fig. 2).

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It is highly unlikely that the pycC1 strain obtains oxaloacetate from the medium, because oxaloacetate undergoes rapid β-decarboxylation to pyruvate under physiological conditions (25); therefore, the viability of the pycC1 mutant suggests that *M. barkeri* has an alternate pathway for OAA biosynthesis. Many organisms, including some methanogenic archaea, use the enzyme
TABLE 2 DNA sequence polymorphisms in M. barkeri Fusaro genome sequencesa

<table>
<thead>
<tr>
<th>Locus tag (region)b</th>
<th>Annotation of altered gene</th>
<th>Observed polymorphism</th>
<th>Amino acid change</th>
<th>Presence or absence of:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mbar_A0055 (bp 67649)</td>
<td>CAAX protease self-immunity</td>
<td>T→C</td>
<td>Leu→Ser</td>
<td>+ + +</td>
</tr>
<tr>
<td>Mbar_A0540 (bp 642250)</td>
<td>Oligosaccharide amylase</td>
<td>A→G</td>
<td>Arg→Gly</td>
<td>+ + +</td>
</tr>
<tr>
<td>Mbar_A1119 (bp 1377047)</td>
<td>UDP-glucose 6-dehydrogenase</td>
<td>T→C</td>
<td>NA</td>
<td>+ + +</td>
</tr>
<tr>
<td>Mbar_A1236 (bp 1533125)</td>
<td>Hypothetical protein</td>
<td>ΔT</td>
<td>Ile→Phe</td>
<td>+ + +</td>
</tr>
<tr>
<td>Mbar_A1407 (bp 1736118)</td>
<td>Transposase</td>
<td>T→C</td>
<td>Thr→Ala</td>
<td>+ + +</td>
</tr>
<tr>
<td>Mbar_A1588 (bp 1958354)</td>
<td>Biotin-(acetyl-CoA carboxylase) ligase</td>
<td>ΔA</td>
<td>stop codon</td>
<td>+ + +</td>
</tr>
<tr>
<td>Mbar_A1758 (bp 237232)</td>
<td>Predicted 5-layer protein</td>
<td>G→A</td>
<td>Arg→Gln</td>
<td>+ + +</td>
</tr>
<tr>
<td>Mbar_A2011 (bp 2739641)</td>
<td>Predicted transcriptional regulator</td>
<td>ΔA</td>
<td>NA</td>
<td>+ + +</td>
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<tr>
<td>Mbar_A2632 (bp 4695769)</td>
<td>Flagellar assembly protein H</td>
<td>ΔA</td>
<td>Frameshift</td>
<td>+ + +</td>
</tr>
<tr>
<td>Mbar_A3644 (bp 4733572)</td>
<td>DNA-directed RNA polymerase subunit A</td>
<td>G→A</td>
<td>Arg→Cys</td>
<td>+ + +</td>
</tr>
<tr>
<td>Mbar_A3692 (bp 1514571–1514834)</td>
<td>Winged helix-turn-helix transcription repressor DNA binding</td>
<td>Δ264 bp</td>
<td>NA</td>
<td>+ + +</td>
</tr>
<tr>
<td>Mbar_A3656 (bp 1705 bp)</td>
<td>Transposase</td>
<td>Δ1,705 bp</td>
<td>NA</td>
<td>+ + +</td>
</tr>
</tbody>
</table>

a Putative Pyr+ mutations are shown in boldface.

b Numbering and sequence differences are relative to GenBank accession number CP000099.1.

c Presence (+) or absence (−) of the indicated polymorphism is shown.

d NA, not applicable (the change is not within a coding sequence).

phosphoenolpyruvate carboxylase (Ppc) for this purpose (26). Although previous efforts failed to detect Ppc activity in M. barkeri cell extracts (10), a homolog of the archaeal ppc gene is found in the M. barkeri genome sequence (locus tag Mbar_A2632). Based on the idea that Ppc activity might be expressed in Pyr+, a putative Ppc enzyme mentioned above, and because pyruvate is thought to be metabolized via acetate, after growth on methanol with pyruvate and acetate, methanol with acetate, and acetate alone. Pairwise comparisons of mRNA abundance were then made between each pair of the three strains for each of the growth conditions. The full data set, including parsed tables showing only significant differences and those shared in common between the two mutant cell lines, is presented in Tables S2 to S6 in the supplemental material.

Although numerous differences in mRNA abundance were observed, we reasoned that differential expression would need to be seen in both Pyr-1 and Pyr-2 in order to be relevant to the Pyr+ phenotype. Relatively few genes met this criterion (Table 3). Most notable among them are the genes of the por operon, which were expressed at significantly higher levels in the Pyr+ mutants. This phenotype was especially pronounced when the cells were grown in the presence of pyruvate or acetate. This substrate-specific phenotype can be explained by the observation that DSM804 downregulates expression of por when pyruvate or acetate is present, whereas Pyr-1 and Pyr-2 do not (see Table S7 in the supplemental material compared to Table 3 and Tables S2 to S6). This regulatory phenotype exaggerates the differences seen between the mutants and their parent in media with added pyruvate or acetate and minimizes the differences seen in media without these additions. Two other genes displayed similar behavior: Mbar_A2632, which encodes the putative Ppc enzyme mentioned above, and Mbar_A2011, which encodes an S-layer protein.

Genes involved in nitrogen metabolism also showed substantially different expression in the Pyr+ mutants, although no clear pattern could be observed (Table 3). Thus, some nitrogen assimilation genes were upregulated and others downregulated. Moreover, the expression pattern was reversed for many of these genes on transfer from methanol medium containing either pyruvate or acetate to medium with either methanol or acetate alone. We do not currently have a satisfactory explanation for this observation. Surprisingly, the expression of genes predicted to be regulated by PycC (27), including the pyc and bioY operons, is not significantly

FIG 1 Reconstruction of the most likely genetic history of the four sequenced M. barkeri Fusaro genomes based on shared sequence differences.
FIG 2 Graphic depiction of the putative Pyr+ mutations. A frameshift mutation, designated *pycC1*, in the *Mbar_A1588* locus introduces a stop codon in the biotin protein ligase domain of the PycC protein. The mutated allele retains the potential to produce a truncated protein encompassing the DNA-binding domain of PycC. The second mutation results in a Gly-to-Arg change in the helix-turn-helix domain of the putative DNA-binding transcriptional regulator encoded by the *Mbar_A2165* locus. The locus tags for the genes are shown above the arrows. *Mbar_A2164* encodes a putative GTP binding protein, *Mbar_A2166* encodes a putative phosphoribosyl-ATP-pyrophosphatase, and *Mbar_A2167* encodes a putative 3-isopropylmalate dehydratase. Mutation positions are shown by asterisks in the diagram and by bold letters in the amino acid and nucleotide sequences below.

FIG 3 Characterization of the *pycC1* mutant. (Top) Pyc activity was measured in a coupled reaction using malate dehydrogenase (Mdh) to reduce enzymatically produced oxaloacetate to malate with concomitant oxidation of NADH. Activity for the complete reaction using extracts from the parental strain (WWM85) and the *pycC1* mutant (WWM818) are shown, as are control reactions using *pycC1* cell extract but lacking the pyruvate substrate (+/- Pyr) and coupling enzyme (-/- Mdh). The error bars represent the standard deviations of three biological replicates. (Bottom) Growth of the parental strain (WWM85) and the *pycC1* mutant (WWM818) on HS-methanol medium. The error bars represent the standard deviations of three biological replicates.

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changed in the *pycC1* mutants under most of the conditions examined.

**Mbar_A2165** is involved in regulation of Por. To examine the role of the *Mbar_A2165* (G59R) allele in pyruvate metabolism, we attempted to recreate the mutation in the WWM85 strain background, yet despite numerous attempts, we were unable to obtain the mutant. We were, however, able construct a double mutant (WWM940) containing both the *pycC1* mutation and the G59R change in *Mbar_A2165*. Taken together, these data suggest that the *Mbar_A2165*(G59R) mutation is lethal in the presence of a wild-type *pycC* allele. To assess the regulatory phenotype of this mutation, we conducted transcriptional profiling of the *pycC1* single mutant, the *pycC1 Mbar_A2165*(G59R) double mutant, and their isogenic parent (see Table S8 in the supplemental material). Comparison of the *pycC1* mutant with the parent revealed only 14 differentially expressed genes, none of which have obvious roles in pyruvate metabolism. In contrast, comparison of the double mutant to the parent revealed 34 differentially expressed genes, including *porA* (+2.3-fold), *porB* (+2.2-fold), and *ppc* (*Mbar_A2632*; +2.5-fold). Comparison of the single mutant to the double mutant showed 78 regulated genes, including the *por* operon (*porA*, +2.9-fold; *porB*, +3.1-fold; *porC*, +3.1-fold; *porD*, +2.75-fold) but not *ppc*. Consistent with the transcriptional data, Por activity was more than 2-fold higher when the *Mbar_A2165*(G59R) mutation was present (specific activities: WWM85, 0.59 ± 0.06; WWM818, 0.50 ± 0.04; WWM940, 1.28 ± 0.22).

The pyruvate ferredoxin oxidoreductase gene is an essential gene in *M.arkeri* Fusaro. To probe the role of Por activity in pyruvate metabolism, the native *porCDAB* promoter was replaced by a tetracycline-regulated promoter (generating strain WWM941). Consistent with Por being required for pyruvate biosynthesis (10), the strain grew well on methanol medium in the presence of the inducer tetracycline but failed to grow in its absence (Fig. 4A and B). Similar results were obtained in liquid medium when the strain was grown on acetate, methanol with H2-CO2, and a methanol-acetate mixture (data not shown). However, unlike a previously characterized *M.arkeri* ech mutant, which is a pyruvate auxotroph (28), this strain failed to grow in the absence of inducer in media supplemented with various combinations of pyruvate, acetate, Casamino Acids, and yeast extract (Fig. 4C through H). The failure of supplementation to restore growth of the por-depleted strain indicates that Por, or one of its subunits, plays an additional essential role(s) beyond pyruvate biosynthesis.

Attempts to recreate the Pyr+ phenotype. Based on the results presented above, we expected that either the *pycC1* or *Mbar_A2165*(G59R) mutation, or both together, would be required for growth on pyruvate. Unfortunately, a direct test of this
TABLE 3 Selected mRNA abundance changes in Pyr-1 and Pyr-2 relative to DSM804

<table>
<thead>
<tr>
<th>Locus tag</th>
<th>Annotation</th>
<th>Fold change</th>
<th>MeOH/Pyr</th>
<th>MeOH/Pyr/Ac</th>
<th>MeOH/Ac</th>
<th>MeOH</th>
<th>Acetate</th>
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<tbody>
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<td>Mbar_A0999</td>
<td>por operon</td>
<td>6.8</td>
<td>4.9</td>
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<td>5.7</td>
<td>4.2</td>
<td>1.2</td>
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<td>por operon</td>
<td>7.7</td>
<td>4.3</td>
<td>2.5</td>
<td>5.8</td>
<td>4.4</td>
<td>1.7</td>
</tr>
<tr>
<td>Mbar_A1001</td>
<td>por operon</td>
<td>11.1</td>
<td>7.1</td>
<td>3.8</td>
<td>9.1</td>
<td>4.9</td>
<td>2.1</td>
</tr>
<tr>
<td>Mbar_A1002</td>
<td>por operon</td>
<td>15.3</td>
<td>7.9</td>
<td>4.5</td>
<td>9.0</td>
<td>3.9</td>
<td>1.2</td>
</tr>
<tr>
<td>Mbar_A2011</td>
<td>S-layer protein</td>
<td>34.2</td>
<td>19.7</td>
<td>8.6</td>
<td>82.7</td>
<td>39.5</td>
<td>-1.1</td>
</tr>
<tr>
<td>Mbar_A2632</td>
<td>Pep carboxylase</td>
<td>4.4</td>
<td>3.7</td>
<td>2.9</td>
<td>4.37</td>
<td>2.9</td>
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Putative PycC-regulated genes

<table>
<thead>
<tr>
<th>Locus tag</th>
<th>Annotation</th>
<th>Fold change</th>
<th>MeOH/Pyr</th>
<th>MeOH/Pyr/Ac</th>
<th>MeOH/Ac</th>
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<th>Acetate</th>
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<tbody>
<tr>
<td>Mbar_A0583</td>
<td>bioY operon</td>
<td>1.1</td>
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Nitrogen fixation genes

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<th>MeOH/Pyr</th>
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a The full data sets for each condition, including parsed tables showing only significant differences, are presented in Tables S1 to S5 in the supplemental material.

b Fold change of expression values of mutant strains compared to WT M. barkeri grown in the same substrate. Statistically significant values (P < 0.05) are shown in boldface. Positive changes represent upregulation of the gene of interest. Similarly, a negative change indicates downregulation of the gene of interest.

hypothesis is experimentally difficult due to the morphological properties of Methanosarcina cells and the unusual physiology of the Pyr-1 and Pyr-2 cell lines. Most Methanosarcina species grow in large cellular aggregates that are encased in a thick polysaccharide cell wall. This cell wall presents a significant barrier to uptake of foreign DNA, and despite numerous attempts, to our knowledge, no one has yet succeeded in transforming aggregated Methanosarcina cells. However, prolonged cultivation of aggregated cells in media of high osmotic strength with high concentrations of divalent cations results in conversion to a highly transformable

FIG 4 The por operon is essential for viability in M. barkeri. (A and B) The parental strain, which expresses por from its normal promoter (por+) (WWM85), and a derivative that expresses por from a tetracycline-dependent promoter (por+tet) (WWM941) were pregrown in HS-methanol broth with tetracycline and then washed, serially diluted, and plated on HS-methanol agar in the presence (A) or absence (B) of tetracycline. (C to H) Cultures were also plated on HS-methanol agar without tetracycline, supplemented with 100 mM pyruvate (C); 10 mM pyruvate and 100 mM acetate (D); 0.1% Casamino Acids (E); 0.1% yeast extract (F); 100 mM pyruvate, 0.01% Casamino Acids, 0.01% yeast extract (G); or 100 mM pyruvate, 0.1% Casamino Acids, 0.1% yeast extract (H).
single-cell morphology that lacks the polysaccharide cell wall (13, 24). Thus, the WWM85 cell line used in this study grows as single cells and is genetically tractable, while the DSM804, Pyr-1, and Pyr-2 strains grow as aggregates and are not transformable.

As described above, we were able to recreate a pycC1 single mutant or a pycC1 Mbar_A2165(G59R) double mutant in the WWM85 strain background. Because the single-celled strains lyse in the low-salt medium used for cultivation of DSM804, Pyr-1, and Pyr-2, we characterized the growth phenotype of these strains in HS-pyruvate medium. Surprisingly, the aggregated Pyr-1 and Pyr-2 strains failed to grow on pyruvate in HS medium. Pyr-1 and Pyr-2 grow well in HS media with other substrates, suggesting that the medium lacks some component of the low-salt medium that is required for growth on pyruvate or that growth on pyruvate is blocked by some component of the high-salt medium. To examine this, we tested numerous variants of HS medium that included the components of low-salt medium at a range of concentrations. We also tried variants with decreasing concentrations of the components of the high-salt medium; however, we did not change the concentrations of NaCl (400 mM) or MgCl₂ (50 mM), as they are required to prevent lysis. Although some of these media allowed limited pyruvate-dependent growth of Pyr-1 and Pyr-2, none would support growth upon serial transfer. We also attempted to develop a high-osmotic-strength, low-salt medium using sucrose and MgCl₂, but obtained similar results. Consequently, we are currently unable to assess whether the pycC1 and/or Mbar_A2165(G59R) allele is necessary and sufficient for growth.

**DISCUSSION**

Previous studies showed that wild-type strains of *M. barkeri* Fusaro are incapable of growth on pyruvate but that it is possible to select mutants with the ability to do so (4). Our analysis of these mutants suggests that the Pyr⁺ phenotype depends on the pycC1 and/or the Mbar_A2165(G59R) allele, because these mutations represent the only shared sequence differences between the Pyr⁺ strains and their isogenic Pyr⁻ parent. Unfortunately, we were unable to rigorously establish whether one or both of the alleles are required due to the physiological and morphological constraints of our current experimental system. Thus, we cannot exclude the possibility that additional polymorphisms between the DSM804 and WWM85 cell lines contribute to the Pyr⁺ phenotype. Nevertheless, our data strongly suggest that both mutations are required for the phenotype because it appears that the Mbar_A2165(G59R) mutation is lethal in the absence of pycC1.

Our data suggest that the ability to grow on pyruvate requires two substantial changes in central metabolism (Fig. 5). First, instead of using the Pyc-catalyzed carboxylation of pyruvate to form OAA, the strains use a two-step pathway involving conversion of pyruvate to PEP followed by conversion of PEP to OAA via the anabolic pathway for production of biomass. Subsequent conversion of the biosynthetic enzyme to take on a catabolic role, oxidizing pyruvate to acetyl-CoA and CO₂. Subsequent conversion of acetyl-CoA to acetate, a regulatory phenotype seen in DSM804 but not in Pyr-1 and Pyr-2. The nonconservative glycine-to-arginine change in the winged helix-turn-helix motif of the Mbar_A2165-encoded protein could easily prevent DNA binding to cognate promoters, thereby abrogating expression. These physiological differences suggest a model for growth of the Pyr⁺ mutant. The key feature of this model is the inability of the mutant to downregulate Por in response to pyruvate. High Por activity in the presence of exogenous pyruvate would thus allow the biosynthetic enzyme to take on a catabolic role, oxidizing pyruvate to acetyl-CoA and CO₂. Subsequent conversion of acetyl-CoA to methane could then occur via the standard aceticlastic pathway, as previously suggested. The requirement for the pycC1 mutation has two possible explanations in this model. In the first, pycC1 is required, because the Mbar_A2165(G59R) mutation that allows Por overexpression is lethal in the presence of a functional Pyc. We note that this mutation simultaneously increases expression of Ppc and speculate that the loss of Pyc is required to prevent futile cycling between OAA and pyruvate when both enzymes are present, as seen in other organisms (29). Alternatively, the loss of Pyc may direct pyruvate into the oxida-

![FIG 5 Proposed anabolic and catabolic metabolism of pyruvate in *M. barkeri* Fusaro. The native aceticlastic pathway, which is used for catabolism of pyruvate, is shown in blue, and the anabolic pathway for production of biomass is shown in red. The enzymes proposed to catalyze specific steps are shown in green: Mcr, methyl-CoM reductase; Mtr, CoM-H₄SPT methytransferase; Cdh, carbon monoxide dehydrogenase; Por, pyruvate-ferredoxin oxidoreductase; Ppc, pyruvate carboxylase; Pps, PEP carboxylase; and Pps, PEP synthase. Overexpression of Por and Ppc in the Pyr⁺ mutants, coupled with loss of Pyc, is sufficient to allow growth on pyruvate as the sole methanogenic substrate. Abbreviations: CoM-H₄SPT, coenzyme M-tetrahydrosarcinapterin; CoM-S-S-CoB, heterodisulfide of coenzyme M and coenzyme B; Fd₆ox and Fd₆red, oxidized and reduced ferredoxin, respectively.](http://jb.asm.org/)

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itive pathway by blocking its direct conversion to OAA. In this scenario, the kinetic parameters of the two-step pathway would favor channeling of pyruvate into the oxidative pathway, rather than the biosynthetic pathway.

Our inability to recreate the Pyr\(^+\) phenotype in the single-cell morphology suggests the possibility that the aggregated-cell morphology is necessary to observe the phenotype. During growth on pyruvate Pyr\(^+\) strains convert pyruvate to acetyl-CoA, CO\(_2\), and reduced ferredoxin by oxidative decarboxylation; then, CO\(_2\) is reduced by ferredoxin to produce CH\(_4\) (Fig. 5) (4). It is possible that the population of Pyr\(^+\) cells in the aggregates is not biochemically homogeneous, with one subset of the population performing hydrotrophic methanogenesis and the second aceticlastic methanogenesis. It has been reported that these two reactions can be coordinated between two methanogenic strains (30). Since H\(_2\) inhibits aceticlastic growth, in the cell aggregates, hydro-

enotrophic methanogens would lower the H\(_2\) partial pressure to a level that would allow aceticlastic methanogenesis to occur and therefore allow the organism to metabolize pyruvate. If this sy-

trophic association in cell aggregates is necessary for the organism to metabolize pyruvate, the phenotype would not be observed in the single-cell morphology strains used in our experiments.

Perhaps the most surprising result was the observation that porCDAB expression is essential in M. barkeri, even when media are supplemented with pyruvate. In many anaerobic microorgan-

isms, Por plays a catabolic role similar to that observed in the M. barkeri mutants. Por-deficient mutants have been made in several of these organisms, and although the mutants lose the ability to utilize specific substrates, they are viable and generally healthy (31, 32). In contrast, autotrophic methanogens typically use Por as a biosynthetic enzyme to produce pyruvate by reductive carboxyla-

tion of acetyl-CoA (7). Based on the fact that the mutants grow on pyruvate and that pyruvate supplementation can rescue an M. barkeri ech mutant (28), this phenotype is not caused by an inability to take up the substrate. Thus, Por clearly has an additional role beyond synthesis of pyruvate. We suspect that this additional function is also biosynthetic, consistent with the nature of the encoding gene and because supplementation did allow slight growth of the Por-depleted cells (Fig 4C through H). Biochemical characterization of M. barkeri Por showed that the enzyme has a rather narrow substrate range, using only pyruvate and 2-oxo-

butyrate (in the oxidative direction) (23). Thus, it is conceivable that the enzyme is required for production of either propionyl-CoA or 2-oxo-butyrate, according to the following equation: propionyl-CoA + CO\(_2\) + reduced ferredoxin\(\leftrightarrow\)2-oxo-butyrate + oxidized ferredoxin.

Por enzymes from other sources have additional activities, including hydrogenase and pyruvate decarboxylase (33,34), so it is possible that one of these activities is required for viability. It is also possible that one of the proteins in the porCDAB operon acts as a subunit for another required enzyme complex. Candidates include the small ferredoxin encoded by porD or one of the cata-

lytic subunits, which have been shown to be interchangeable in other 2-oxo acid-ferredoxin oxidoreductases (31, 35). If, as sus-
pected, a biosynthetic block causes the lethal phenotype, then the required intermediate either is not present in yeast extract or Casamino Acids, or the cell does not take it up.

Finally, our data shed some light on the role of pycC in biotin-

dependent gene regulation. In E. coli, mutations in the biotin-

protein ligase domain of the homologous protein affect the regu-

lation of biotin-biosynthetic genes (36). M. barkeri does not carry genes for biotin biosynthesis; however, in silico analysis has re-

vealed the presence of two PycC binding sites in the genome, one presumably regulating the biotin transporter bioY and a second one upstream of the pyc operon (27). Our transcriptomic analysis did not reveal consistent and statistically significant changes in the expression of these genes across substrates. Thus, M. barkeri PycC does not regulate the expression of genes related to biotin metab-

olism, at least at levels that can be detected by the RNA-seq meth-

odology used here.

Taken together, our results suggest that pyruvate utilization in M. barkeri is an intricate process that balances the needs of ana-

bolic and catabolic pathways. Moreover, the finding of novel anaerotic reactions for the synthesis of phosphoenolpyruvate (Pps, pyruvate-phosphate dikinase, and pyruvate-water dikinase) and oxaloacetate (Ppc) and the essentiality of Por suggest that further analyses of central metabolism in Methanosarcina are war-

rated.

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