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 H₂-CO₂ or formate as growth substrates, members of the *Methanosarcinales* show some metabolic diversity, with many species using H₂-CO₂, various one-carbon (C₁) 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 CO₂ 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 CO₂ using the enzyme pyruvate-ferredoxin oxidoreductase (Por), with subsequent conversion of acetyl-CoA to CO₂ and CH₄ by the standard acetoclastic pathway for methanogenesis (4, 5). The reducing equivalents produced by pyruvate oxidation were also used to reduce CO₂ to CH₄, probably using H₂ 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

Received 8 July 2015 Accepted 28 August 2015

Accepted manuscript posted online 8 September 2015

Citation López Muñoz MM, Schönheit P, Metcalf WW. 2015. Genetic, genomic, and transcriptomic studies of pyruvate metabolism in *Methanosarcina barkeri* Fusaro. J Bacteriol 197:3592–3600. doi:10.1128/JB.00551-15.

Editor: G. A. O'Toole

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JB.00551-15>.

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TABLE 1 *Methanosarcina* strains used in this study

<i>M. barkeri</i> strain	Genotype	Source or reference
DSM804	Wild type	DSMZ ^a
Pyr-1	Pyr ⁺ (Table 2)	Laboratory stock
Pyr-2	Pyr ⁺ (Table 2)	4
WWM85	$\Delta hpt::\phi C31int-attP$	12
WWM818	$\Delta hpt::\phi C31int-attP$ pycC1	This study
WWM940	$\Delta hpt::\phi C31int-attP$ pycC1 Mbar_A2165(G59R)	This study
WWM941	$\Delta hpt::\phi C31int-attP$ pMcrB(tetO1)::porCDAB	This study

^a Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures.

UBS). The phenotype of these strains when grown on pyruvate was substantially different than the one observed in the Pyr⁺ mutant of *M. barkeri* Fusaro. *M. barkeri* 227, MS, and UBS grew substantially more slowly (doubling time, ~150 h versus 25 h), with lower levels of methane production (~0.7 mol CH₄/mol pyruvate versus ~1.25 mol CH₄/mol pyruvate) and significantly lower growth yields (~1.7 g/mol CH₄ versus ~14 g/mol CH₄), than the *M. barkeri* Fusaro mutant. Whether growth of these strains on pyruvate required mutation or adaptation was not addressed.

In addition to serving as an energy source, pyruvate plays a critical role as a biosynthetic intermediate for numerous anabolic pathways in methanogens (reviewed in reference 7). Based on biochemical data, *M. barkeri* synthesizes pyruvate by reductive carboxylation of acetyl-CoA mediated by Por, which is a freely reversible enzyme (8, 9). Pyruvate then enters the gluconeogenic pathway via phosphoenolpyruvate (PEP) and the branched tricarboxylic cycle via oxaloacetate (OAA). The former is produced via the enzyme PEP synthase (10), whereas the latter is produced via the biotin-dependent enzyme pyruvate carboxylase (Pyc [11]).

The genetic basis for the Pyr⁺ phenotype of the *M. barkeri* Fusaro mutant has yet to be established, and genetic studies of anabolic metabolism have not been attempted in the organism. In this study, we explored the molecular, genetic, and biochemical bases for growth of the *M. barkeri* Fusaro Pyr⁺ mutant on pyruvate. Our data suggest that growth on pyruvate requires overexpression of Por and loss of Pyc activity. In addition, analysis of the genes encoding enzymes that produce and consume pyruvate revealed a surprisingly complex interaction between the catabolic and anabolic metabolism of this key biosynthetic intermediate.

MATERIALS AND METHODS

Strains, media, and growth conditions. The *Methanosarcina* strains used in the study are shown in Table 1. All *Methanosarcina* strains were grown under strictly anaerobic conditions. Derivatives of *M. barkeri* WWM85 (12) were grown in single-cell morphology at 37°C in high-salt (HS) medium (13). *M. barkeri* DSM804, Pyr-1, and Pyr-2 were grown in aggregated-cell morphology at 37°C in imidazole medium as previously described (4). The growth substrate provided was 125 mM methanol, 120 mM acetate, 100 mM sodium pyruvate, or a mixture of methanol (125 mM) and acetate (120 mM), methanol (125 mM) and pyruvate (100 mM), methanol (125 mM) and acetate (120 mM) plus pyruvate (100 mM), or methanol (125 mM) with H₂-CO₂ (80:20) at 200 kPa over ambient pressure. The substrates were sterilized by filtration using a 0.2-μm filter. *M. barkeri* Pyr-1 was independently maintained after transfer to the Metcalf laboratory in January 2004; *M. barkeri* Pyr-2 has been maintained in the Schönheit laboratory since its isolation (4). *M. barkeri* DSM804 was received

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. barkeri* 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 (Micon 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. barkeri* 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 DNaseq 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. barkeri* 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, 90. 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. barkeri* 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 TruSeq 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 μ g/ml), centrifuged, washed three times with plain HS medium, and then resuspended in 5 ml HS medium. Aliquots of 10 μ l of a 10-fold dilution series were then spotted onto agar-solidified HS-methanol media containing some or all of the following: tetracycline (100 μ g/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 ($\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) 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 μ l of lysis buffer (3 M KCl, 50 mM Tris-HCl [pH 8], 5% inositol, 5 mM MgCl_2 , 2 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride). The reaction mixture consisted of 100 mM Tris-HCl (pH 8), 4 mM MgCl_2 , 250 mM KCl, 20 mM KHCO_3 , 4 mM ATP, 0.2 mM NADH, 2 U malic dehydrogenase (Sigma-Aldrich, St. Louis, MO), 20 mM sodium pyruvate, and 20 μ l crude cell extracts. Reactions were started by addition of 20 μ l 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 MgCl_2 , 250 mM KCl, 20 mM KHCO_3 , 4 mM ATP, 0.2 mM NADH, 2 U malic dehydrogenase, 2 mM phosphoenolpyruvic acid monosodium salt, and 20 μ l 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 ($\epsilon_{578} = 8.65 \text{ mM}^{-1} \text{ cm}^{-1}$) (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 μ l 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 biotin protein ligase domain (Fig. 2). Thus, it is likely that the mutation results in a nonfunctional *Pyc*. To validate this idea, we constructed and characterized a *pycC1* mutant in the strain background derived from the genome-sequenced strain (WWM85). As expected, the resulting strain (WWM818) lacked *Pyc* activity; however, growth of the *pycC1* mutant in HS-methanol medium was similar to that of its isogenic parent (Fig. 3).

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 sequences^a

Locus tag (region) ^b	Annotation of altered gene	Observed polymorphism	Amino acid change	Presence or absence of:		
				Pyr-1	Pyr-2	DSM804
Mbar_A0055 (bp 67649)	CAAX protease self-immunity	T→C	Leu→Ser	+	+	+
Mbar_A0540 (bp 642250)	Oligosaccharide amylase	A→G	Arg→Gly	+	+	+
Intergenic (bp 1366984)	NA ^d	T→C	NA	+	+	+
Mbar_A1119 (bp 1377047)	UDP-glucose 6-dehydrogenase	ΔT	Ile→Phe	+	+	+
Mbar_A1236 (bp 1533125)	Hypothetical protein	T→C	Thr→Ala	+	+	+
Mbar_A1407 (bp 1736118)	Transposase	T→C	Val→Ala	+	+	+
Intergenic (bp 1857490)	NA	Ins:AT	NA	+	+	+
Mbar_A1588 (bp 1958354)	Biotin-(acetyl-CoA carboxylase) ligase	ΔA	stop codon	+	+	—
Mbar_A1758 (bp 257232)	Predicted S-layer protein	G→A	Arg→Gln	+	+	+
Mbar_A2165 (bp 2739641)	Predicted transcriptional regulator	G→A	Gly→Arg	+	+	—
Intergenic (bp 3911323)	NA	ΔA	NA	+	—	—
Mbar_A3249 (bp 4169592)	Histidine kinase-like ATPases	ΔA	Frameshift	+	+	+
Intergenic (bp 4445293)	NA	A→G	NA	+	+	+
Intergenic (bp 4462290)	NA	C→T	NA	+	+	+
Mbar_A3644 (bp 4695769)	Flagellar assembly protein H	G→A	Arg→Cys	+	+	+
Mbar_A3692 (bp 4753372)	DNA-directed RNA polymerase subunit A	T→C	Ile→Met	+	+	+
Mbar_A1226 (bp 1514571–1514834)	Winged helix-turn-helix transcription repressor DNA binding	Δ264 bp	NA	+	+	+
Mbar_A3656 (bp 4713304–4715008)	Transposase	Δ1,705 bp	NA	+	+	+

^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⁺ strains, we attempted, unsuccessfully, to assay this activity in crude extracts of the *pycC1* mutant. Thus, the route of OAA synthesis in the mutant could not be biochemically established.

Global transcriptomic profiling of Pyr⁺ mutants. Because both of the putative Pyr⁺ mutations occur in genes encoding transcriptional regulators, it seems likely that altered gene expression plays a role in the ability of the mutants to use pyruvate as a growth substrate. To test this, we performed global transcriptional profiling of the mutant and parental strains via RNA-seq. Because the parental strain is incapable of growth on pyruvate, we isolated

RNA after growth on methanol with and without added pyruvate 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 down-regulates 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 difference 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.

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. barkeri* 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. barkeri 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

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.

TABLE 3 Selected mRNA abundance changes in Pyr-1 and Pyr-2 relative to DSM804^a

		Fold change ^b									
Locus tag	Annotation	MeOH/Pyr		MeOH/Pyr/Ac		MeOH/Ac		MeOH		Acetate	
		Pyr-1	Pyr-2	Pyr-1	Pyr-2	Pyr-1	Pyr-2	Pyr-1	Pyr-2	Pyr-1	Pyr-2
Pyruvate-induced genes											
Mbar_A0999	<i>por</i> operon	6.8	5.1	4.9	2.6	7.8	4.2	2.4	1.2	1.2	−1.3
Mbar_A1000	<i>por</i> operon	7.7	4.3	4.3	2.5	5.8	3.0	4.2	1.2	1.1	−1.6
Mbar_A1001	<i>por</i> operon	11.4	7.1	7.1	3.8	9.1	4.1	4.1	1.1	1.4	−1.4
Mbar_A1002	<i>por</i> operon	15.3	7.9	8.1	4.5	9.0	3.9	3.6	1.1	1.2	−1.5
Mbar_A2011	S-layer protein	34.2	19.7	8.6	4.8	82.7	39.5	−1.1	−3.5	2.0	1.3
Mbar_A2632	Pep carboxylase	4.4	3.7	2.9	1.6	4.37	2.9	4.0	1.9	−1.2	−1.4
Putative PycC-regulated genes											
Mbar_A0583	<i>bioY</i> operon	1.1	1.1	−1.3	−1.3	−1.4	−1.1	1.5	1.8	1.2	1.2
Mbar_A0584	<i>bioY</i> operon	−1.0	−1.1	1.1	1.1	−1.2	−1.4	1.7	1.7	1.1	−1.1
Mbar_A0585	<i>bioY</i> operon	1.6	1.3	1.2	1.1	1.5	1.3	1.8	1.5	−1.1	−1.1
Mbar_A0586	<i>bioY</i> operon	2.3	1.9	1.7	1.1	2.5	1.8	3.6	1.6	1.8	1.4
Mbar_A1586	<i>pyc</i> operon	1.5	1.2	1.0	1.1	1.5	1.0	2.8	1.3	1.7	1.2
Mbar_A1587	<i>pyc</i> operon	3.8	1.9	1.8	1.7	3.3	2.0	2.0	−1.1	1.2	−1.1
Mbar_A1588	<i>pyc</i> operon	1.6	1.3	1.2	1.2	2.4	1.7	1.2	−1.3	1.3	1.1
Mbar_A1589	<i>pyc</i> operon	1.9	1.2	1.2	1.1	1.3	1.0	1.3	−1.5	1.2	−1.0
Nitrogen fixation genes											
Mbar_A0165	Mo-nitrogenase operon	2.4	1.2	2.3	1.7	2.8	2.0	−2.1	−1.8	−1.2	−1.2
Mbar_A0166	Mo-nitrogenase operon	3.9	1.7	4.1	2.8	3.5	2.4	−3.0	−2.4	−1.6	−1.6
Mbar_A0167	Mo-nitrogenase operon	3.5	1.4	5.4	3.6	5.8	3.9	−4.8	−3.6	−3.1	−3.4
Mbar_A0168	Mo-nitrogenase operon	4.2	1.7	6.0	3.9	6.2	4.2	−4.2	−3.3	−3.4	−3.2
Mbar_A0169	Mo-Nitrogenase operon	5.3	1.9	6.4	4.1	5.3	3.5	−3.7	−2.9	−3.5	−2.9
Mbar_A0170	Mo-nitrogenase operon	4.1	1.8	5.9	3.9	8.8	6.0	−7.1	−5.7	−1.9	−1.6
Mbar_A0171	Mo-nitrogenase operon	6.7	2.7	8.9	5.6	−1.2	5.5	−4.9	−4.5	−4.3	−4.5

^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 on pyruvate.

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 enzyme Ppc. The enzyme necessary for conversion of pyruvate to PEP is not clear, because *M. barkeri* carries genes encoding both phosphoenolpyruvate synthase (Pps) and pyruvate phosphate dikinase (Pdk) (Fig. 5). Although we were unable to demonstrate Ppc activity in extracts of the mutants, it is known that the archaeal enzyme can be unstable (26). Given that OAA biosynthesis is essential and that the *ppc* gene is upregulated in the mutants, it is highly likely that Ppc is functionally expressed in the Pyr-1 and Pyr-2 cell lines. The second key change found in the Pyr⁺ strains is a substantially higher level of Por activity. Transcriptomic analy-

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 methyltransferase; Cdh, carbon monoxide dehydrogenase; Por, pyruvate-ferredoxin oxidoreductase; Pyc, pyruvate carboxylase; Ppc, PEP carboxylase; and Pps, PEP synthase. Over-expression 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; Fd_{ox} and Fd_{red}, oxidized and reduced ferredoxin, respectively.

ses of recreated mutants show that the Mbar_A2165(G59R) mutation is responsible for increased expression of both Por and Ppc. Our data do not address whether this regulation is direct or indirect, but the phenotype is probably caused by the inability to repress expression of *por* and *ppc* in response to pyruvate and/or 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 repression.

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-

tive 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 hydrogenotrophic methanogenesis and the second acetateclastic methanogenesis. It has been reported that these two reactions can be coordinated between two methanogenic strains (30). Since H_2 inhibits acetateclastic growth, in the cell aggregates, hydrogenotrophic methanogens would lower the H_2 partial pressure to a level that would allow acetateclastic methanogenesis to occur and therefore allow the organism to metabolize pyruvate. If this syntrophic 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 microorganisms, 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 carboxylation 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-oxobutyrate (in the oxidative direction) (23). Thus, it is conceivable that the enzyme is required for production of either propionyl-CoA or 2-oxobutyrate, according to the following equation: propionyl-CoA + CO_2 + reduced ferredoxin $\leftrightarrow$ 2-oxobutyrate + 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 catalytic subunits, which have been shown to be interchangeable in other 2-oxo acid-ferredoxin oxidoreductases (31, 35). If, as suspected, 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 revealed 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 metabolism, at least at levels that can be detected by the RNA-seq methodology used here.

Taken together, our results suggest that pyruvate utilization in *M. barkeri* is an intricate process that balances the needs of anabolic and catabolic pathways. Moreover, the finding of novel anaplerotic 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 warranted.

ACKNOWLEDGMENTS

We thank Petra Kohler, Alvaro Hernández, and the staff at the W. M. Keck Center for Comparative and Functional Genomics, University of Illinois, for help with genome sequencing and RNA-seq experiments.

This work was supported by a grant from the Energy Biosciences Institute. M.M.L.M. was partly supported by a National Institute of General Medical Sciences (NIGMS), NIH Chemistry, Biology Interface Training Grant (5T32-GM070421).

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