Natural Competence in the Hyperthermophilic Archaeon *Pyrococcus furiosus* Facilitates Genetic Manipulation: Construction of Markerless Deletions of Genes Encoding the Two Cytoplasmic Hydrogenases^{∇†}

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In attempts to develop a method of introducing DNA into *Pyrococcus furiosus*, we discovered a variant within the wild-type population that is naturally and efficiently competent for DNA uptake. A *pyrF* gene deletion mutant was constructed in the genome, and the combined transformation and recombination frequencies of this strain allowed marker replacement by direct selection using linear DNA. We have demonstrated the use of this strain, designated COM1, for genetic manipulation. Using genetic selections and counterselections based on uracil biosynthesis, we generated single- and double-deletion mutants of the two gene clusters that encode the two cytoplasmic hydrogenases. The COM1 strain will provide the basis for the development of more sophisticated genetic tools allowing the study and metabolic engineering of this important hyperthermophile.

It would be difficult to overestimate the contribution of genetic manipulation to the study of any biological system, and it is an essential tool for the metabolic engineering of biosynthetic and substrate utilization pathways. This is particularly true for the archaea since, in spite of their environmental and industrial importance, coupled with their unique molecular features, much remains to be learned about their biology (2). The marine hyperthermophilic anaerobe *Pyrococcus furiosus* is of special interest not only for its ability to grow optimally at 100°C and the implications of this trait for its biology but also for industrial applications of its enzymes, as well as its capacity to produce hydrogen efficiently (4, 13, 44). The ability to apply genetic analyses of *P. furiosus* to underpin existing biochemical and molecular studies will contribute greatly to the establishment of *P. furiosus* as a model organism, particularly for biological hydrogen production.

The development of genetic systems in the archaea, in general, presents many unique challenges given the extreme growth requirements of many of these organisms. To date, genetic systems of various levels of sophistication have been developed for representatives of all major groups of archaea, including halophiles, methanogens, thermoacidophiles, and hyperthermophiles (2, 6, 30, 40, 43, 46). A variety of transformation methods are being used, including electroporation, heat shock with or without CaCl₂ treatment, phage-mediated transduction,

spheroplast transformation, liposomes, and, very recently, even conjugation with *Escherichia coli* (2, 12). Transformation via natural competence has been reported in three archaeal species, in comparison to over 60 bacterial species that are known to exhibit this trait (16, 36). Two of them are the methanogens *Methanococcus voltae* PS (7, 27) and *Methanobacterium thermoautotrophicum* Marburg (47); however, transformation frequencies were low, and there have been no follow-up studies regarding natural competence. The other is the hyperthermophile *Thermococcus kodakarensis*, which has an optimal growth temperature of 85°C. Its natural competence has enabled the development of genetic tools for targeted gene deletions, the use of shuttle vectors, and a reporter gene system (32–37). In fact, *T. kodakarensis* was one of the first archaeal hyperthermophiles for which chromosomal manipulations were reported (36), along with *Sulfolobus solfataricus*, for which a transformation system with accompanying shuttle vectors had previously been established (6, 48).

One of the most significant barriers to genetic manipulation of archaea, in general, and hyperthermophiles, in particular, is the lack of selectable markers. Antibiotic selection strategies used in mesophilic bacteria are typically ineffective because the molecular machineries of archaea are not affected by the antibiotic (9, 29) or, in the case of hyperthermophiles, because of the instability of either the drug or the heterologously expressed resistance protein at high temperatures (2, 25). One exception is the drug simvastatin (or mevinolin), first utilized in the haloarchaea (18, 28), which is sufficiently thermostable to inhibit growth of both *T. kodakarensis* (85°C) (23) and *P. furiosus* (45). Simvastatin competitively inhibits 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which converts HMG-CoA to mevalonate, the rate-limiting step in the biosynthesis of isoprenoids, the major component of archaeal membrane lipids. Simvastatin at sufficiently high concentrations leads to cessation of cell growth, while overexpression of HMG-CoA reductase confers resistance (18, 23).

Nutritional markers are especially useful for genetic selec-

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TABLE 1. *P. furiosus* strains used and constructed in this study

Strain designation/phenotype	Genotype	Parent strain/phenotype	Genome region(s) deleted from parent strain ^a
DSM 3638	Wild type		
COM1	$\Delta pyrF$	DSM 3638	PF1114 (nt 1062504–1063123)
ΔSHI	$\Delta pyrF$ $\Delta PF0891$ – $\Delta PF0894$	COM1	PF0891 to PF0894 (nt 863754–867807)
$\Delta SHII$	$\Delta pyrF$ $\Delta PF1329$ – $\Delta PF1332$	COM1	PF1329 to PF1332 (nt 1250021–1255193)
$\Delta SHI \Delta SHII$	$\Delta pyrF$ $\Delta PF0891$ – $\Delta PF0894$ $\Delta PF1329$ – $\Delta PF1332$	$\Delta SHII$	PF0891 to PF0894 (nt 863754–867807)

^a Locus tags of deleted genes are listed, followed by nucleotides (nt) of the deleted region in parentheses (*P. furiosus* DSM 3638 genome; GenBank accession no. AE009950.1).

tion if an organism is able to grow on a defined medium, and a number of such nutritional selections have been utilized in archaea, including auxotrophies for amino acids (e.g., leucine and tryptophan) (3, 36), thymidine (3, 26), and agmatine (34). A counterselectable marker based on loss of the uracil biosynthetic enzyme orotidine-5'-monophosphate (OMP) decarboxylase, first described in yeast (*Saccharomyces cerevisiae*) (8), has been used successfully in archaeal organisms, including *T. kodakarensis* (20, 28, 35, 36). OMP decarboxylase (*pyrF* in archaea and bacteria) converts the pyrimidine analog 5-fluoroorotic acid (5-FOA) to fluorodeoxyuridine, a toxic product that kills growing cells (8). Mutations in *pyrF* result in uracil auxotrophs that are resistant to 5-FOA.

P. furiosus is a hyperthermophilic anaerobe in the same family as *T. kodakarensis*, but it has a much higher optimal growth temperature (100 versus 85°C) (14). Recently, the *T. kodakarensis* transformation method was used successfully to transform *P. furiosus* with a replicating shuttle vector, using selection for simvastatin resistance (45). However, there have been no reports of genetic manipulation of the *P. furiosus* chromosome. Here we used simvastatin and 5-FOA selections to generate a $\Delta pyrF$ strain, designated COM1. Remarkably, the COM1 strain was naturally competent for DNA uptake at transformation frequencies orders of magnitude higher than those of the wild-type strain. In fact, cells can be transformed directly on selective plates by spotting the lawn with DNA containing a wild-type copy of the *pyrF* gene. The combined transformation and recombination frequencies allow marker replacement by direct selection of linear DNA. This strain has been used to create single- and double-deletion mutants of the two predicted operons encoding the two heterotetrameric cytoplasmic hydrogenases of *P. furiosus*.

MATERIALS AND METHODS

Strains and growth conditions. *E. coli* strain ET12567 (*dam dcm* mutant) containing the nontransmissible helper plasmid pUZ8002 was used for initial attempts at conjugation. *E. coli* strain DH5 α was used for plasmid DNA preparation and as a control for conjugation experiments. General techniques for *E. coli* were performed as described previously (31).

The *P. furiosus* strains used and constructed in this study are listed in Table 1. The medium for *P. furiosus* growth was composed of 1 $\times$ base salts, 1 $\times$ trace minerals, 10 μ M sodium tungstate, and 0.25 mg/ml resazurin, with added cysteine at 0.5 g/liter, sodium sulfide at 0.5 g/liter, sodium bicarbonate at 1 g/liter, and 1 mM sodium phosphate buffer (pH 6.8), and for complex medium, containing combinations of 0.05% (wt/vol) yeast extract, 0.35% (wt/vol) cellobiose, 0.5% (wt/vol) maltose, and 0.5% (wt/vol) casein hydrolysate (enzymatic), or, for defined medium, containing 1 $\times$ vitamin solution, 2 $\times$ 19-amino-acid solution, and 0.35% (wt/vol) cellobiose. The complex medium variations used are as follows: yeast extract, cellobiose, and peptides (YECp); yeast extract and peptides with sulfur (YEP-S⁰); and yeast extract and maltose (YEM). Stock solutions

of individual components were prepared as described previously (1). The 200 $\times$ vitamin stock solution contained (per liter) 10 mg each of niacin, pantothenate, lipoic acid, *p*-aminobenzoic acid, thiamine (B₁), riboflavin (B₂), pyridoxine (B₆), and cobalamin (B₁₂) and 4 mg each of biotin and folic acid. The 25 $\times$ 19-amino-acid stock solution contained (per liter) 3.125 g each of arginine and proline; 1.25 g each of aspartic acid, glutamine, and valine; 5.0 g each of glutamic acid and glycine; 2.5 g each of asparagine, histidine, isoleucine, leucine, lysine, and threonine; 1.875 g each of alanine, methionine, phenylalanine, serine, and tryptophan; and 0.3 g tyrosine. An additional 0.5 g/liter cysteine was added to the defined medium since the amino acid stock solution lacked cysteine. When used, elemental sulfur was added to liquid cultures at a concentration of 2 g/liter. Liquid cultures were inoculated with a 1 to 2% inoculum or with a single colony and then incubated at 90°C in anaerobic culture bottles or Hungate tubes degassed with three cycles of vacuum and argon.

A solid medium was prepared by mixing an equal volume of liquid medium at a 2 $\times$ concentration with 1% (wt/vol) Phytagel (Sigma) previously autoclaved to solubilize, and both solutions were maintained at 95°C just prior to mixing. The medium was poured into glass petri dishes immediately after mixing. For solid media with added sulfur, 2 mM polysulfide was added immediately after mixing the 2 $\times$ medium and solidifying agent (from a 1 M stock polysulfide solution made by reacting yellow elemental sulfur and sodium sulfide anaerobically in a 4:1-mol ratio). After inoculation, plates were inverted and placed into modified paint tanks (or "anaerobic jars"), which were degassed with three cycles of vacuum and argon and incubated at 90°C for 48 to 64 h.

Vector construction and DNA manipulation. The *P_{gdh}-hmg* cassette (provided by H. Atomi) (23) was cloned into a modified pSET vector (pJHW006, with a modified multiple cloning site and different replication origin, provided by J. Huddleston) to generate pGLW027. A fragment containing 1-kb flanking regions to the portion of *pyrF* (PF1114) which did not overlap with adjacent genes was constructed by overlap PCR and cloned into pGLW027 to make the *pyrF* knock-out vector, pGLW028 (see Fig. S1 in the supplemental material for vector diagrams). The *P_{gdh}-pyrF* cassette was constructed by overlap PCR. A 283-bp portion of the intergenic region upstream of *gdh* (PF1602) was joined with the *pyrF* gene with the addition of a 12-bp sequence containing the T1 terminator from the histone gene *hpyA1* (PF1722) (41). The cassette was cloned into pJHW006 to make pGLW015. The SHI and SHII operon knockout vectors pGLW021 and pKSW001 contained adjacent ~1-kb flanking regions of the SHI operon (PF0891 to PF0894) and the SHII operon (PF1329 to PF1332), respectively, constructed by overlap PCR and cloned into pGLW015 (see Fig. S1 in the supplemental material for vector diagrams). The PCR product of the wild-type *pyrF* genome region used to transform the COM1 background strain was amplified from wild-type genomic DNA with primers amplifying from approximately 1 kb up and downstream from *pyrF*. The primers used in this study are listed in Table S1 in the supplemental material.

Transformation and 5-FOA selection. For the attempted conjugation experiments, two 50-ml cultures of *E. coli* ET12567 harboring the pUZ8002 helper plasmid with and without the transforming plasmid pGLW028 were grown to an optical density of 0.4 to 0.8 (660 nm). Cells were harvested and washed twice in LB medium. Two 50-ml cultures of wild-type *P. furiosus* were grown in YECp complex medium to a density of approximately 2 $\times$ 10⁸ cells/ml (as determined by counting in a Petroff-Hausser counting chamber). The cells were harvested aerobically by centrifugation, washed twice in 1 $\times$ base salts, and suspended in 3 ml 1 $\times$ base salts. This suspension was divided and used to suspend each *E. coli* pellet. The mixtures of *P. furiosus* and *E. coli* cells were incubated at 37°C for 1 h aerobically with gentle shaking and then spread onto plates of YECp complex medium containing 22 μ M simvastatin (Sigma). Plates were incubated aerobically at 37°C for 1 h and then transferred to anaerobic jars and incubated for 48

to 64 h at 90°C anaerobically. For natural transformation, aliquots of *P. furiosus* culture typically grown to mid-log phase ($\sim 2 \times 10^8$ cells/ml) in defined liquid medium were mixed with DNA at a concentration of 2 to 10 ng DNA per μ l of culture, spread in 30- μ l aliquots onto defined solid medium lacking uracil (for COM1 transformations) or containing 16 μ M simvastatin (for COM1 and wild-type transformation comparison), and plates were placed inverted in anaerobic jars and incubated at 90°C for ~ 64 h. Serial dilutions of culture were made in order to calculate plating efficiencies. The transformation frequencies reported herein take into account the number of cells plated as determined by culture cell counts (this does not take into account the plating efficiency), and, where indicated, the total amount of DNA added (i.e., the number of transformants per microgram of DNA per 10^8 cells). Colonies were picked into 4 to 6 ml of liquid medium in Hungate tubes—either complex medium (YECF) with simvastatin or defined medium without uracil—and incubated anaerobically overnight at 90°C. For 5-FOA selection, 30 μ l of culture was plated directly onto complex medium plates containing 8 mM 5-FOA and 20 μ M uracil with 2 mM polysulfide added just prior to plate pouring. Colonies resistant to 5-FOA were cultured similarly in nonselective complex medium for genomic DNA isolation and screening. After PCR confirmation of a deletion, the resulting strains were passaged twice on solid medium for colony purification.

Genomic DNA isolation. For genomic DNA isolation, cells from 1 ml of overnight *P. furiosus* culture were harvested and suspended in 100 μ l buffer A (25% sucrose, 50 mM Tris-HCl, 40 mM EDTA, pH 7.4) followed by addition of 250 μ l 6 M guanidinium HCl–20 mM Tris, pH 8.5, with incubation at 70°C for 5 min. Genomic DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1; buffered at pH 8), ethanol precipitated, and suspended in 50 μ l 10 mM Tris buffer, pH 8.0.

Screening, purification, and sequence verification of mutants. Transformant colonies were inoculated into liquid medium for genomic DNA extraction and subsequent PCR screening of the target region. Primers were designed to amplify up and downstream from the homologous regions used to construct the deletion (see Table S1 in the supplemental material). For PCRs, the extension time utilized was sufficient to allow for amplification of the wild-type allele, if it was still present. After initial screening, transformants containing the expected deletion were further purified by two additional passages under selection on solid medium and screened a second time by PCR to check for segregation of the deleted allele. The deletions were then verified in the purified isolates by sequence analysis. A PCR product was generated from genomic DNA by using primers outside the homologous regions used to construct the deletion, and internal primers were used to sequence the PCR product.

RNA extraction and RT-qPCR analyses. Total RNA was extracted from cell extracts of *P. furiosus* with acid-phenol (39) and stored at -80°C until needed. RNA was treated with Turbo DNase (Ambion) for 30 min at 37°C and further purified using the Absolutely RNA clean up kit (Agilent Technologies). cDNA was then prepared using the AffinityScript quantitative PCR (qPCR) cDNA synthesis kit (Agilent Technologies). All quantitative reverse transcription-PCR (RT-qPCR) experiments were carried out with an Mx3000P instrument (Stratagene) with the Brilliant SYBR green qPCR master mix (Agilent Technologies). The gene encoding the pyruvate ferredoxin oxidoreductase gamma subunit (PF0971) was used as an internal control for RNA quality. Table S1 in the supplemental material lists the primers used in RT-qPCR experiments.

RESULTS

Construction and characterization of a *P. furiosus* Δ *pyrF* strain. To generate a deletion of *pyrF* (PF1114) in the *P. furiosus* chromosome, a deletion of the gene was constructed on a plasmid containing the *P. furiosus* HMG-CoA reductase gene (*hmg*; PF1848) under the control of the *P. furiosus* glutamate dehydrogenase (*gdh*; PF1602) promoter for selection with simvastatin. We found that the sensitivity of *P. furiosus* to simvastatin on solid medium varies depending on medium type (defined versus complex), density of plated cells, and length of incubation time, with the MIC ranging from approximately 10 μ M to over 24 μ M. We had limited success in obtaining a clean background for selection with simvastatin, presumably due to a high rate of spontaneous resistance to simvastatin from mutations causing native gene amplification, as noted elsewhere (2,

FIG. 1. Strategy for obtaining a *pyrF* deletion and PCR analysis of the *pyrF* deletion in the COM1 strain. (A) A diagram of the *pyrF* genome region is shown with the *pyrF* deletion plasmid having 1-kb regions from up- and downstream of *pyrF* for homologous recombination and also containing the *P_{gdh}-hmg* cassette for selection of simvastatin resistance. Homologous recombination can occur at the upstream or downstream *pyrF* flanking regions, integrating the plasmid into the genome and generating a strain that is simvastatin resistant. Selection on 5-FOA selects for loss of the plasmid along with deletion of the *pyrF* gene. Bent arrows depict primers used for verification of *pyrF* deletion. (B) Gel depicting PCR products of the *pyrF* genome region in the COM1 strain compared to the wild type, amplified by primers outside the up- and downstream regions used for homologous recombination (see bent arrows in panel A).

34). The strategy for construction of a *pyrF* deletion strain is depicted in Fig. 1A.

We tried a number of methods to introduce plasmid DNA into *P. furiosus*, including the heat shock method used successfully in *T. kodakarensis* (35, 36), but did not obtain transformants by simvastatin selection. However, an attempt at conjugation of *P. furiosus* (DSM 3638) with *E. coli* yielded two transformants (from 10^9 *P. furiosus* cells), which were verified by PCR analysis to contain the *P_{gdh}-hmg* cassette. Subsequent experiments showed that *E. coli* was not required for transformation and that *P. furiosus* is naturally competent for DNA uptake. The initial selection of these transformants likely resulted from uptake of DNA released from lysed *E. coli* cells. In other experiments, transformants were also obtained with the nonconjugative DH5 α strain of *E. coli*, confirming that DNA

FIG. 2. COM1 is a uracil auxotroph. Growth curves of the COM1 strain (triangles) compared to the wild type (circles) in the presence (open symbols) and absence (closed symbols) of uracil. Culture growth was monitored by optical density at 660 nm. The slight increase in optical density for the COM1 strain cultured without uracil reflects a slight darkening of the medium due to incubation at 98°C. The lack of growth in the COM1 strain was verified by assaying protein concentrations for each time point. Each point represents an average of samples from two independent cultures, with error bars showing standard deviation.

was being taken up by *P. furiosus* through a mechanism other than conjugation (data not shown).

The two simvastatin-resistant transformants contained plasmids integrated into the *P. furiosus* chromosome at three different locations, as determined by PCR analyses: at both the upstream and downstream flanking regions of the *pyrF* gene and at the *hmg* locus, which has 1.2 kb of homology with the plasmid (see Fig. S2 in the supplemental material). The isolates were subcultured once on simvastatin-containing plates for colony purification and then cultured in medium without simvastatin but containing uracil to allow a second crossover event to eliminate the plasmid. The MIC of 5-FOA on YECF complex medium was determined to be 8 mM. Hundreds of colonies were observed on complex medium containing 5-FOA, and of 28 screened by PCR, all contained a deletion of *pyrF*. Further purification of the isolates by an additional passage on solid medium resulted in loss of the plasmid at the *hmg* locus. PCR and sequence analysis of the *pyrF* locus for the isolate designated COM1 verified the deletion and that the plasmid was lost from the *hmg* locus, generating a wild-type gene. No transcript from the *pyrF* locus was detected by RT-qPCR (see Fig. S3 in the supplemental material). The COM1 strain was used for all further genetic manipulation.

A comparison of the growth rates of the *P. furiosus* wild-type and *P. furiosus* COM1 strains in defined medium in the presence and absence of uracil is shown in Fig. 2. The COM1 strain did not grow in the absence of uracil but did grow in medium supplemented with uracil and had a growth rate comparable to that of the wild type. As *pyrF* is potentially part of a four-gene operon (Fig. 1A), RT-qPCR was used to confirm that expression of the surrounding genes (PF1113, PF1115, and PF1116) was not affected significantly as a result of deletion of *pyrF* (data not shown).

The *P. furiosus* COM1 strain is naturally competent for uptake of both circular DNA and linear DNA. The strategy for using the COM1 strain as a background for genetic manipulation rests on the ability to complement the deletion with the wild-type allele, allowing uracil prototrophic selection, as depicted in Fig. 3A. A cassette was constructed for expression of

FIG. 3. Strategy for obtaining an SHI operon deletion and PCR analyses of the cytoplasmic hydrogenase operon deletions. (A) The SHI operon genome region is shown with the SHI operon deletion plasmid with 1-kb regions from up- and downstream of the operon for homologous recombination and also containing the P_{gdh} -*pyrF* cassette for selection of uracil prototrophy. Homologous recombination can occur at either the upstream or downstream SHI operon flanking regions, integrating the plasmid into the genome and generating a strain that is a uracil prototroph. PF0891, PF0892, PF0893, and PF0894 represent the genes coding for the SHI beta, gamma, delta, and alpha subunits, respectively. (B) Gel depicting PCR products of the SHI and SHII operon genome regions in the Δ SHI, Δ SHII, and Δ SHI Δ SHII strains compared to the COM1 strain, amplified by primers with at least one primer outside the homologous recombination regions.

the wild-type *pyrF* gene under the transcriptional control of the *gdh* promoter with a short terminator sequence (T1) from the *hpyA1* gene (41) at the 3' end of the *pyrF* gene. Using a plasmid containing this cassette along with 1-kb flanking regions to a genomic target (pGLW021), we obtained on the order of 10^4 transformants per μ g DNA per 10^8 cells, suggesting that *P. furiosus* is not only naturally competent but that the COM1 strain is highly efficient for DNA uptake. Hundreds of transformants were obtained by simply mixing plasmid DNA containing a wild-type copy of the *pyrF* gene with a small volume of culture (2 ng DNA per μ l culture) under aerobic conditions and plating this mixture onto defined medium without uracil for selection of transformants under anaerobic growth conditions at 90 to 98°C. No colonies were observed in the absence of added DNA, even when plating over 100-fold more cells than were used for transformation. There was also no significant change in the number of observed transformants as a result of varying the carbon source in the defined medium (cellobiose, maltose, or malto-oligosaccharides). Early, mid- or late-log-phase cells were capable of undergoing transformation, including aliquots of cells from cultures that had been

stored at room temperature (anaerobically) for up to 2 weeks. Since plating was carried out aerobically for convenience, we observed variable plating efficiencies (up to 80%), with the average being approximately 10%.

To test transformation with linear DNA, PCR products containing the wild-type *pyrF* gene were generated with 1-kb flanking regions to *pyrF* and used to transform the COM1 strain. Transformants were obtained at frequencies in the range of 10^5 per 10^8 cells, and even the addition of intact wild-type genomic DNA repaired the *pyrF* locus (see Fig. S4 in the supplemental material). The COM1 strain was also transformed by spotting DNA onto a lawn of cells on plates. We noted that transformant colonies that were screened (directly after subculturing the colonies once in liquid medium) often contained a mixture of the wild-type and mutant alleles (see Fig. S4 and S5 in the supplemental material), and this can be attributed to the existence of multiple chromosomal copies in *P. furiosus* (24). It was therefore necessary to allow genome segregation of deletion mutants with subsequent colony purification. The combination of natural competence and recombination in the COM1 strain results in efficient marker replacement using linear as well as circular DNA.

Given the high frequencies of natural transformation of the COM1 strain, we sought to compare it to the wild type. The *pyrF* deletion plasmid containing the P_{gdh} -*hmg* cassette was used to transform both the COM1 and wild-type strains, selecting for simvastatin resistance. For experiments in which we observed no transformants for the wild type (in 10^8 cells), we observed on the order of 10^5 transformants per 10^8 cells for the COM1 strain. This dramatic difference in transformation frequencies between the two strains may reflect changes in DNA uptake, homologous recombination, or both; however, the underlying mechanism(s) involved is as yet unknown.

Construction of markerless deletion mutants of the *P. furiosus* cytoplasmic hydrogenases. *P. furiosus* contains two cytoplasmic (soluble) hydrogenases (SHI and SHII) each encoded by four genes predicted to be in an operon, PF0891 to PF0894 (coding for the SHI beta, gamma, delta, and alpha subunits, respectively) and PF1329 to PF1332 (coding for the SHII beta, gamma, delta, and alpha subunits, respectively) (21, 22). Deletions of each were constructed on plasmids containing the P_{gdh} -*pyrF* cassette. The deletion strategy is depicted in Fig. 3A. Plasmid DNA containing a deletion of one of the operons was transformed into the COM1 strain. After selection for uracil prototrophy, the resulting transformants were screened by PCR to confirm integration of the plasmid at the targeted genome regions. Transformants were cultured in defined medium without uracil and then spread onto YEP-S⁰ complex medium plates containing 5-FOA and uracil to select double-crossover events removing the integrated plasmid containing the wild-type *pyrF* gene. Previous microarray analyses showed that expression of both hydrogenases was significantly downregulated in the presence of elemental sulfur (1, 38), so sulfur in the form of polysulfide was added to the 5-FOA selection plates to reduce any possible detrimental effects of the deletions. We initially passaged the cells in nonselective liquid medium with added uracil prior to selection on plates containing 5-FOA to relieve selective pressure for the *pyrF* marker, but found that this step was not necessary. Recombi-

nation appears to be sufficiently efficient in *P. furiosus* to allow loss of the selected marker on 5-FOA by plating directly from cultures grown in medium lacking uracil.

PCR amplification of the SHI and SHII operon genome regions was used to screen 5-FOA-resistant isolates. Of 12 isolates screened for deletion of the SHI operon, 5 contained clean deletions and 4 contained products indicating a mixture of the wild type and the deletion mutant (see Fig. S5 in the supplemental material). Of 14 isolates screened for deletion of the SHII operon, seven contained clean deletions and seven were mixtures of the wild type and the deletion mutant (Fig. S5). Selected isolates containing clean deletions were further purified on solid medium, and the deletion was confirmed by PCR analyses (Fig. 3B). The Δ *pyrF* ΔPF0891–PF0894 and Δ *pyrF* ΔPF1329–PF1332 strains (designated ΔSHI and ΔSHII, respectively) were verified by sequencing PCR products amplified from outside the homologous regions used to generate the deletions. The absence of the operon transcripts was confirmed by RT-qPCR with primer pairs targeting each gene within the operons.

The hydrogenase mutants were cultured in YEM complex medium in the absence of S⁰ to examine the growth phenotype. Surprisingly, no differences were observed in the growth of either the ΔSHI or ΔSHII mutant compared to the COM1 parent strain (see Fig. S6 in the supplemental material).

Construction of a ΔSHI ΔSHII mutant. An important feature of this strategy for genetic manipulation allows for the iterative selection and counterselection of marker replacements to make multiple deletions in the same strain. To generate a mutant lacking both cytoplasmic hydrogenases, the ΔSHII strain was transformed with the SHI operon deletion plasmid. Transformants were selected for uracil prototrophy and counterselected for loss of the *pyrF* allele on YEP-S⁰ complex medium containing 5-FOA and uracil. Of 12 isolates screened by PCR, 4 contained a clean deletion of the SHI operon, while 3 contained mixtures of the wild type and the deletion mutant (see Fig. S4 in the supplemental material). The mutants were further purified on solid medium and analyzed by PCR (Fig. 3B). The ΔSHI ΔSHII strain (Δ *pyrF* ΔPF0891–PF0894 ΔPF1329–PF1332) was verified by sequencing of PCR products generated from the corresponding genome region, and RT-qPCR using primer pairs targeting each gene within each operon confirmed the absence of transcripts from both operons.

The ΔSHI ΔSHII mutant was cultured in YEM medium (without S⁰), and this mutant also displayed no differences in growth compared to COM1 under the conditions tested (see Fig. S6 in the supplemental material).

DISCUSSION

Here we report the construction of a deletion of the *pyrF* locus in the *P. furiosus* chromosome and the discovery that this strain, designated COM1, is remarkably efficient and naturally competent for uptake of both circular DNA and linear DNA. The combination of DNA uptake and recombination is sufficiently efficient to allow the generation of marker replacement by direct selection using linear DNA. The ability to use PCR products to generate deletions with selection is a major step forward in terms of ease of manipulation and an important

genetic tool for the study of *P. furiosus*. The ability to use this methodology to generate single and multiple mutations in the same strain will facilitate the analysis of the physiology and metabolism of this important hyperthermophilic archaeon, as well as allowing its metabolic engineering.

High frequencies of transformation were observed with the *P. furiosus* COM1 strain by adding DNA directly to a small volume of cell culture and spreading this mixture on selective plates. The previously reported method for transformation of *T. kodakarensis* relies on a modified CaCl_2 “heat shock” procedure reported for *M. voltae* PS (7), involving an incubation on ice, followed by a short “heat shock” at 85°C, followed by a second incubation on ice. The transformation frequency observed for *P. furiosus* COM1 with uracil prototrophic selection is significantly higher than that reported for *T. kodakarensis* (selection of tryptophan prototrophy in a $\Delta\text{pyrF } \Delta\text{trpF::pyrF}$ mutant). For linear DNA fragments containing ~1 kb of homologous regions, on the order of 10^5 transformants per μg DNA per 10^8 cells were obtained, compared with fewer than 100 transformants per μg DNA per 10^8 cells of *T. kodakarensis* when using linear DNA having the same length of homologous regions (35). These numbers correspond to transformation frequencies (per μg DNA) of 10^{-3} for *P. furiosus* (ΔpyrF) and 10^{-7} for *T. kodakarensis* ($\Delta\text{pyrF } \Delta\text{trpF::pyrF}$). The frequency of natural transformants observed for *P. furiosus* COM1 (up to 10^{-3}) approaches those reported for the highly naturally competent thermophilic bacterium *Thermus thermophilus* (10^{-2}) (17) and fall within the range of frequencies observed for other naturally competent bacteria, including *Acinetobacter baylyi* (7×10^{-3}), *Bacillus subtilis* (3.5×10^{-2}), and *Haemophilus influenzae* (7×10^{-3}) (19).

While we cannot separate the contribution of DNA uptake from that of homologous recombination since we are not using a replicating shuttle vector, the fact that we often observed more than one plasmid integration event in the same transformant suggests that recombination is efficient. This is also supported by previous work showing that *P. furiosus* has the ability to repair its chromosome after complete fragmentation by gamma irradiation (11). Efficient recombination is also suggested by the fact that loss of the *pyrF* gene can be selected for directly on plates containing 5-FOA with no intervening growth in nonselective medium. The factors responsible for the increased transformation frequencies with uptake of both circular DNA and linear DNA in the COM1 strain are not clear, but our data suggest that while wild-type *P. furiosus* has a mechanism for natural competence, there are changes in COM1 that markedly increase this ability. There are no obvious homologs of known DNA translocation machinery in the *P. furiosus* genome, such as those found in *B. subtilis*, *Streptococcus pneumoniae*, or *T. thermophilus* (5, 10); although apparent homologs to some of the internal DNA processing enzymes often associated with natural competence such as DprA (PF1313), Ssb (PF2020), and RecA (PF1926) appear to be present. The increased natural competence of COM1 is likely due to some mutation(s) present in the original wild-type cell from which the COM1 strain was generated, and the nature of such a change is currently under investigation.

One limitation of the strategy utilized herein for targeted deletion of the hydrogenase operons is the lack of a direct selection for deletion of the target gene. This is a problem for

deletions with severe phenotypes as removal of the integrated plasmid containing the deletion can occur without loss of the target gene. For deletion of genes or operons that might result in a growth defect, a marker replacement strategy can be used wherein the $\text{P}_{\text{gdh-pyrF}}$ cassette is placed between target gene flanking regions on a plasmid or linear PCR product. Uracil prototrophic selection can then be used directly for simultaneous transformation and gene deletion.

As an example application of the COM1 strain to make chromosomal manipulations in *P. furiosus*, we deleted the operons encoding either or both cytoplasmic hydrogenases. Surprisingly, no growth phenotype was observed for any of the mutants, and this raises questions about their proposed roles in the primary metabolism in *P. furiosus* (e.g., see reference 15). Studying the pathways of hydrogen production in *P. furiosus* is of great interest because of the increasing concern for production of alternative energy sources, such as hydrogen, to replace fossil fuels. Hyperthermophiles such as *P. furiosus* could potentially have an important role in biological hydrogen production systems (15, 42), and although the cytoplasmic hydrogenases have been characterized *in vitro* (21, 22), as demonstrated herein, their *in vivo* functions have yet to be established. We anticipate that the ability to investigate their functions *in vivo* through genetic manipulation, together with the roles of related enzymes, will provide insight into the mechanisms of hydrogen uptake and production in *P. furiosus* as well as in related hyperthermophiles.

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