

Recombinogenic Properties of *Pyrococcus furiosus* Strain COM1 Enable Rapid Selection of Targeted Mutants

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We recently reported the isolation of a mutant of *Pyrococcus furiosus*, COM1, that is naturally and efficiently competent for DNA uptake. While we do not know the exact nature of this mutation, the combined transformation and recombination frequencies of this strain allow marker replacement by direct selection using linear DNA. In testing the limits of its recombination efficiency, we discovered that marker replacement was possible with as few as 40 nucleotides of flanking homology to the target region. We utilized this ability to design a strategy for selection of constructed deletions using PCR products with subsequent excision, or “pop-out,” of the selected marker. We used this method to construct a “markerless” deletion of the *trpAB* locus in the GLW101 (COM1 Δ *pyrF*) background to generate a strain (JFW02) that is a tight tryptophan auxotroph, providing a genetic background with two auxotrophic markers for further strain construction. The utility of *trpAB* as a selectable marker was demonstrated using prototrophic selection of plasmids and genomic DNA containing the wild-type *trpAB* alleles. A deletion of *radB* was also constructed that, surprisingly, had no obvious effect on either recombination or transformation, suggesting that its gene product is not involved in the COM1 phenotype. Attempts to construct a *radA* deletion mutation were unsuccessful, suggesting that this may be an essential gene. The ease and speed of this procedure will facilitate the construction of strains with multiple genetic changes and allow the construction of mutants with deletions of virtually any nonessential gene.

Hyperthermophilic archaea are of special interest because of their evolutionary history and unique physiology, as well as several important biotechnology applications associated with their thermostable enzymes (4, 31). The development of genetic methods for this diverse group has presented many challenges, in part because of their extreme growth requirements. Recent progress has been made, however, in the ability to transform a variety of species by taking advantage of the fact that some are highly recombinogenic and/or able to take up DNA via natural competence. *Sulfolobus* sp., *Thermococcus kodakaraensis*, and *Pyrococcus furiosus* can all be transformed by linear DNA fragments (8, 12, 19, 20, 28, 29), but the length of the homologous flanking region needed for marker replacement varies. In *T. kodakaraensis*, which is naturally competent, more than 100 bp of homologous flanking region are required for homologous recombination (28), but in *Sulfolobus acidocaldarius*, which is transformed via electroporation, 10 to 30 bp of homology is sufficient (19). Several methods that rely on homologous recombination have also been developed to construct mutants in mesophilic archaea, including in *Halobacterium* sp. (23, 36), *Haloferax volcanii* (2, 3), and *Methanosarcina acetivorans* C2A (25).

The isolation of a mutant of *Pyrococcus furiosus*, previously designated COM1 (strain GLW101 [Δ *pyrF*]), that is efficiently competent for DNA uptake was recently reported (20). This strain is transformed by exogenous DNA without any chemical or physical treatment, as opposed to the wild-type *P. furiosus*, in which transformants were not obtained under the same conditions. Subsequently, this strain has enabled the construction of replicating shuttle vectors based on the chromosomal replication origin (9), as well as the production of strains that lack some key metabolic enzymes (5) and that overproduce affinity-tagged versions of the native (6) and a subcomplex form (15) of the cytoplasmic hydrogenase.

In this study, we show that 1,000 bp efficiently direct integration into the chromosome and as few as 40 bp allow efficient homologous recombination in the *P. furiosus* GLW101 chromosome using linear DNA fragments. Using this ability of GLW101 to recombine short segments of homologous DNA, a strategy was developed for generating deletion mutants by PCR amplification (without cloning) to select marker replacement events with subsequent excision, or “pop-out,” of the selected marker. An important feature of this method is that it allows direct selection of targeted mutants. We used this approach to generate a markerless deletion of *trpAB*, generating a strain (Δ *trpAB* Δ *pyrF*) that allows simultaneous nutritional selection of both markers, as demonstrated using a recently constructed replicating plasmid for *P. furiosus* (9). To examine the role of genes predicted to be important for recombination, we used this method to attempt to delete *radA* and *radB*, both homologues in *P. furiosus* of the eukaryotic recombinase *rad51*. Surprisingly, deletion of *radB* had no apparent effect on either recombination or DNA transformation in the GLW101 (COM1 Δ *pyrF*) background. Mutants with a deletion of *radA* were not obtained, suggesting that deletion of this gene may be lethal. Further analysis of the GLW101 strain suggests that the natural competence phenotype does not result from uracil starvation or the loss of a restriction enzyme as a barrier to DNA trans-

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

Strain	Genotype	Parent strain	Reference or source
DSM3638	Wild type		11
GLW101	COM1 Δ <i>pyrF</i>	DSM 3638	21
GLW102	COM1 Δ <i>pyrF trpF::P_{gdh}-pyrF</i>	GLW101	This work
GLW103	COM1 Δ <i>pyrF trpD::P_{gdh}-pyrF</i>	GLW101	This work
GLW104	COM1 Δ <i>pyrF::pyrF</i>	GLW101	This work
JFW01	COM1 Δ <i>pyrF trpAB::P_{gdh}-pyrF</i>	GLW101	This work
JFW02	COM1 Δ <i>pyrF</i> Δ <i>trpAB</i>	JFW01	This work
JFW03	COM1 Δ <i>pyrF radB::P_{gdh}-pyrF</i>	GLW101	This work
JFW04	COM1 Δ <i>pyrF</i> Δ <i>radB</i>	JFW03	This work

formation, but the exact nature of this mutation remains unknown.

MATERIALS AND METHODS

***P. furiosus* strains, media, and growth conditions.** *P. furiosus* DSM3638 (10) wild type, GLW101 (COM1 Δ *pyrF*) (20), and other strains (Table 1) were grown anaerobically in a defined medium with cellobiose as the carbon source (20) at 90°C for 16 to 18 h in 100-ml serum bottles containing 50 ml of liquid medium or on medium solidified with Phytigel (1% wt/vol) for 64 h. For growth of GLW101 and other uracil auxotrophic strains, the defined medium contained 20 μ M uracil. Transformation of GLW101 was performed as described previously (20). The GLW104 strain was generated by transforming GLW101 with a PCR product containing the wild-type *pyrF* allele and selecting for uracil prototrophy. Transformation of the JFW02 strain was performed similarly, but tryptophan prototrophic selection was performed on a medium with or without 20 μ M uracil and lacking tryptophan. The transformation efficiencies reported here were calculated as the number of transformant colonies per μ g of DNA added and do not take into account plating efficiencies, which are typically 1 to 5%. Transformation frequencies were calculated as the proportions of transformant colonies to total cells and do take into account cell viability.

Purification of intermediate strains was performed by plating 10^{-3} dilutions of transformant cultures onto selective plate medium (without uracil) and picking isolated colonies into selective liquid medium. JFW01 and JFW03 were both purified to homogeneity in two rounds of colony purification. We were never able to obtain a pure marker replacement mutant for *radA*. Pop-out recombination was accomplished by growing strains with the marker replacement cassette inserted into the chromosome in defined cellobiose medium containing 20 μ M uracil from a 1% inoculum (~7 generations) and then plating onto defined medium containing 20 μ M uracil and 3 mM 5-fluoroorotic acid (5-FOA).

Restriction endonuclease assays. Cell-free extracts (CFE) were prepared from 1-liter cultures as described previously (7). Endonuclease assays were performed in 10- μ l reaction mixture volumes using 0.5 to 1 μ g of pJFW051 DNA. Various amounts of CFE (from 0 to 20 μ g total protein) were added to separate reaction mixtures that were incubated in NEBuffer 4 (20 mM Tris-acetate, pH 7.9, 50 mM potassium acetate, 10 mM magnesium acetate, and 1 mM dithiothreitol; New England Biolabs). Reaction mixtures were prepared on ice and incubated at 90°C for 15 min. Similar assays were performed to detect the type I or type III restriction endonucleases. These assays were performed as described above except with the inclusion of 1 mM ATP, 80 μ M S-adenosylmethionine (SAM), or both.

PCR amplification and transformation of the wild-type *pyrF* gene. PCR amplification of the wild-type *pyrF* gene with flanking regions ranging in length from 1,000 to 0 bp (Fig. 1A) was performed using the following primer sets: GL055-GL058, *pyrF*500bpF-*pyrF*500bpR, *pyrF*250bpF-*pyrF*250bpR, *pyrF*150bpF-*pyrF*150bpR, *pyrF*100bpF-*pyrF*100bpR, *pyrF*50bpF-*pyrF*50bpR, *pyrF*40bpF-*pyrF*40bpR, *pyrF*30bpF-*pyrF*30bpR, *pyrF*20bpF-

*pyrF*20bpR, *pyrF*10bpF-*pyrF*10bpR, and *pyrF*0bpF-*pyrF*0bpR (see Table S1 in the supplemental material). These products were purified using a DNA Clean & Concentrator-25 column (Zymo Research) and transformed into the GLW101 strain. Three biological replicates were performed for each PCR product.

***trpAB* pop-out marker replacement strategy.** Sequence Manipulation Suite (32) was used to generate a random 40-bp sequence (5' AAGT GAGCGTGTACGCCGAGACCCGGTTTCGTCTCTCAT 3') that was altered slightly at the 3' end to prevent hairpin or self-annealing structures that could be problematic in PCR. This sequence was introduced into the pop-out PCR product using 5' primer tails. Two primer sets (JF392-JF393 and JF394-JF395; see Table S1 in the supplemental material) were used to amplify 1-kb regions flanking *trpAB*. The *P_{gdh}-pyrF* marker cassette was amplified from pJFW017 (9) plasmid DNA using primers JF355.3 and JF356.3. The specific annealing regions of these primers were designed for melting temperatures at $55 \pm 4^\circ\text{C}$ (mean \pm standard deviation). The overlap tails were 30 to 35 bases in length and designed so that the overlapping regions between PCR products would be 20 to 25 bases in length, melting at $62 \pm 2^\circ\text{C}$. PCR was performed using *PfuTurbo* polymerase in a 50- μ l reaction mixture volume according to the manufacturer's instructions (Stratagene). Thermal cycling included 30 cycles with annealing at 58°C and a 70-s extension at 72°C. Products were purified using a DNA Clean & Concentrator-25 column (Zymo Research). Three fragments were put together by two rounds of splicing by overlap extension (SOE) PCR (16). The *trpAB* upstream flanking region was joined to the *P_{gdh}-pyrF* marker cassette, and in a separate reaction, the *P_{gdh}-pyrF* marker cassette was also joined to the *trpAB* 3' flanking region. SOE PCR was performed using ~50 ng of each template DNA in a 50- μ l reaction mixture. Prior to thermal cycling, the template was denatured without primers, allowed to anneal at 58°C, and extended for 10 min at 72°C. Thirty cycles of amplification were performed as described above, with the same end primers used to generate the template products but with the extension time increased to 120 s. These two products were purified and used as the template for another SOE PCR. In the second SOE PCR, the first annealing step was omitted and the two fragments were allowed to anneal and extend at 72°C for 10 min. Thirty cycles of amplification were performed as described above, with primers JF392 and JF395 and extension time increased to 180 s. The 3-kb PCR product was then purified and transformed into the GLW101 strain. Eight uracil prototrophs were picked into liquid defined medium without uracil and grown overnight at 90°C. Putative transformants were screened for the marker replacement by PCR with JF392 and JF395, using the conditions described to generate the pop-out PCR product (see Fig. 2). The final deletion mutant was confirmed by sequencing of the *trpAB* region, which contained the sequence as designed.

Construction of plasmids. To construct pJFW051, a 4.4-kb fragment was amplified by PCR from pJFW018 using primers JF264 and JF269. The *P_{gdh}-hmg* cassette (21) was amplified from pGLW28 (20) using primers GL021 and GL022, treated with T4 polynucleotide kinase, and ligated into the 4.4-kb fragment. A cassette containing the wild-type *trpAB* alleles under the transcriptional control of the phosphoenolpyruvate (PEP) synthase (PF0043) promoter (*P_{pep}-trpAB*) was constructed by SOE PCR. A 126-bp portion of the intergenic region upstream of the PEP synthase gene was amplified from wild-type genomic DNA using primers GL158 and WN008. The *trpAB* genes (PF1705 and PF1706) were amplified using primers WN009 and WN010 and joined to the fragment containing the PEP regulatory region and a 12-bp terminator from the *hpyA1* gene (PF1722) (30). The *P_{pep}-trpAB* cassette was treated with T4 polynucleotide kinase and ligated into the pJFW018 plasmid (9) that had been digested with EcoRV and treated with shrimp alkaline phosphatase. *Escherichia coli* strain DH5 α cells were transformed by electroporation in a 2-mm-gap cuvette at 2.5 V. The plasmid constructions were confirmed by restriction analysis. Plasmid DNA was isolated from liquid cultures by using QIAprep spin miniprep columns (Qiagen, Inc.).

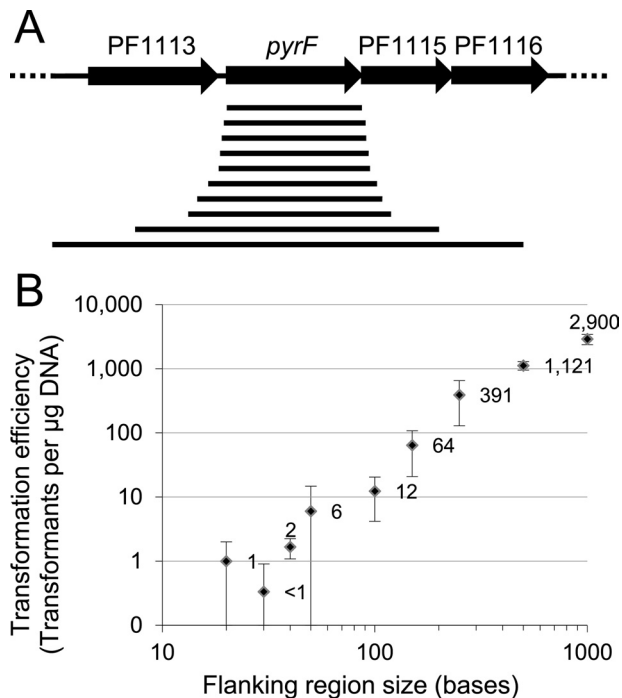


FIG 1 (A) The wild-type *pyrF* region and PCR-amplified fragments (indicated as lines below the chromosomal region) with various lengths of flanking sequence used to transform GLW101, selecting uracil prototrophy. (B) Transformation efficiencies using PCR products. Amounts of 1 μg of DNA were used to transform $\sim 10^7$ cells.

RESULTS AND DISCUSSION

Fewer than 40 bp of homologous DNA allows selection of marker replacements in *P. furiosus*. To investigate the minimum homology required for recombination in *P. furiosus* GLW101 (COM1 $\Delta pyrF$), PCR products containing the *pyrF* gene with flanking DNA regions ranging in length from 0 to 1,000 bp were used to restore the $\Delta pyrF$ locus in GLW101 to the wild type, selecting transformants for uracil prototrophy (Fig. 1A). The transformation efficiency was measured as the number of uracil prototrophic transformants per μg of DNA. The transformation efficiency increased exponentially with the increase in flanking region length, with up to 10^3 transformants per μg of DNA containing 1-kb flanking regions and a few transformants detectable for DNA with flanking regions as short as 20 nucleotides (Fig. 1B). It is important to note that the annotated *pyrF* open reading frame overlaps with the open reading frame of the downstream gene, so the constructed *pyrF* deletion retains the last 16 bases of *pyrF* to include the full-length downstream open reading frame. As a result, the 3' flanking region contained 16 bases of additional homology to the *pyrF* gene (20). This may contribute little for larger fragments of homology but may well affect the interpretation of the data for the very short fragments. However, our results clearly show that recombination can occur between 40-base sequences.

In previous work, we routinely used high concentrations (2 to 10 $\mu\text{g}/\text{ml}$) of transforming DNA (20). To determine the relationship between transforming DNA concentration and transformation frequency, we transformed GLW101 with a range of DNA concentrations, using both a replicating shuttle vector, pJFW018 (9), and a linear wild-type *pyrF*-containing fragment with 1 kb of

flanking homology. For both DNA types, transformants were detectable with DNA concentrations as low as 1 ng/ml. When the transforming DNA concentration is high (10 $\mu\text{g}/\text{ml}$), the transformation frequency of linear DNA is approximately 1 out of 100 to 500 viable cells. Transformation with linear DNA fragments requires both DNA uptake and integration into the genome. Given the long regions of homology and the overabundance of DNA, it is likely that DNA uptake is the more (though not necessarily the only) limiting factor. These results are consistent with a model of natural competence in which only a small subset of cells are competent but they are very efficiently transformed. Taken together, these data suggest that transformation is probably very efficient with ample regions of homology, even at very low DNA concentrations. Transformation with very short regions of homology may be possible, but the efficiency may be prohibitively low for practical applications.

Sequence homology within the PCR products used for selection of marker replacement allows pop-out of the selected marker. Selection of marker replacements using the wild-type copy of *pyrF* results in strains that are uracil prototrophs not useful for further mutant construction using *pyrF* as a selectable marker. To overcome this, we adapted a strategy that had been used successfully in yeast (1) and *T. kodakaraensis* (28) for pop-out of the wild-type *pyrF* allele. An example of this strategy, targeting two genes involved in tryptophan biosynthesis, *trpAB*, is shown in Fig. 2. The transforming DNA fragment containing the deletion cassette contains *pyrF* under the control of the promoter for the gene encoding glutamate dehydrogenase (PF1602), P_{gdh} -*pyrF* (20), flanked by an additional 40-bp direct-repeat sequence with minimal homology to the *P. furiosus* chromosome and constructed using splicing by overlap extension (SOE) PCR (16). A direct-repeat sequence of 40 bp was sufficient to allow for pop-out of the P_{gdh} -*pyrF* marker, and these regions were introduced into the transformation construct using PCR primers shorter than 60 bases. Three separate PCRs were used to amplify the upstream and downstream flanking regions of *trpAB*, as well as the *pyrF* expression cassette (Fig. 2 and 3), and these products were then joined by two successive rounds of SOE PCR, using a total of six primers for the construction. A proofreading polymerase was used to minimize the potential for introducing changes during polymerization. It is interesting to note that, while larger regions of homology are necessary for efficient marker replacement, as little as 40 bp allows efficient pop-out of the selected marker, suggesting that recombination, apart from transformation, is also very efficient.

Transformation of the *trpAB* pop-out construction into the GLW101 strain resulted in hundreds of uracil prototrophic colonies, suggesting that marker replacement at this locus was also very efficient. Eight of these colonies were picked for PCR screening (see Fig. S1 in the supplemental material), and one was purified to homogeneity. This intermediate strain, JFW01, was a uracil prototroph and a tryptophan auxotroph. To select the pop-out event at the *trpAB* locus, JFW01 was grown in liquid medium containing uracil and then (1 ml of culture) was grown on solid medium containing both uracil and 5-fluoroorotic acid (5-FOA). All 5-FOA-resistant colonies (10 total) were screened for pop-out of the *pyrF* cassette, and the frequency of pop-out was approximately 10^{-7} . One of these strains was designated JFW02. JFW02 is a tight tryptophan auxotroph, and excision of the P_{gdh} -*pyrF* marker restored 5-FOA resistance and uracil auxotrophy. JFW02

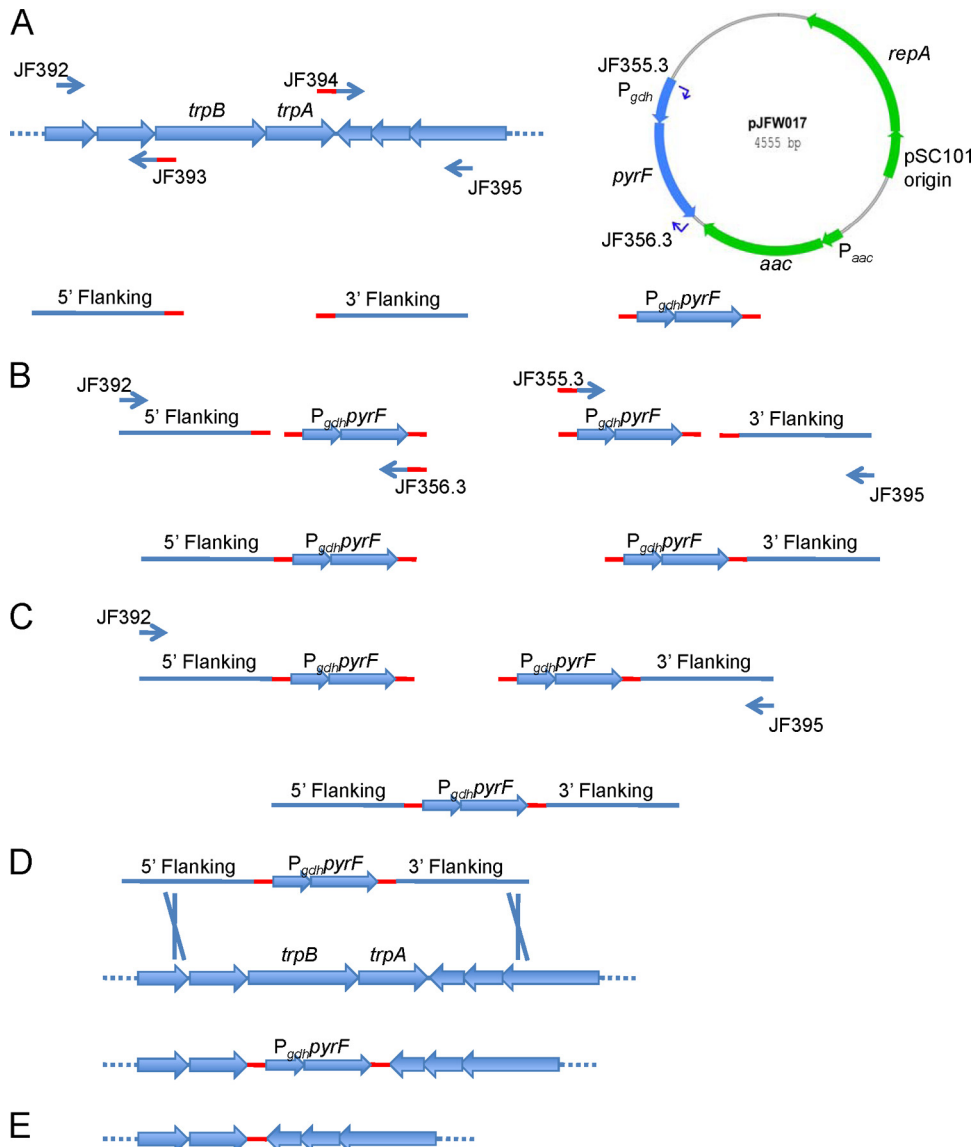


FIG 2 Pop-out marker replacement strategy. Six primers are used to construct a pop-out PCR product that is used to direct marker replacement and subsequent excision of the selected marker. (A) One-kilobase flanking regions are amplified from genomic DNA, and P_{gdh} -*pyrF* is amplified from pJFW017. Overlap tails for SOE PCR introduce the “pop-out scar” sequence and are indicated in red. (B) SOE PCR generates two overlap products. (C) A second SOE PCR generates the final pop-out construct. (D) Transformation into *P. furiosus* allows for selection of the marker replacement event. (E) 5-FOA selection of the pop-out cassette generates a markerless deletion.

is therefore a double auxotroph and suitable for further genetic manipulation.

This strategy has several important advantages over conventional deletion construction. It does not require cloning, and only six primers are needed to provide specificity for the target gene (see Table S1 in the supplemental material). Primers of 60 bases are significantly less expensive and eliminate the need for primer purification, making pop-out construction of deletions amenable to a high-throughput system. Since both the integration and excision of the P_{gdh} -*pyrF* cassette are selected, this method may be used to target any nonessential gene. The pop-out constructs, as described here, leave a 40-bp “scar” sequence that remains in the genome after pop-out of the P_{gdh} -*pyrF* marker cassette. If a scarless deletion is desired, this strategy could be modified so that only one

40-bp pop-out sequence is included in the construct, which would recombine with the native sequence on the other side of the P_{gdh} -*pyrF* marker cassette to generate a scarless deletion of the target gene. Alternatively, the 40-bp scar sequence provides flexibility for modifying genomic targets by introducing specific sequences, such as signal peptides or affinity tags for protein purification. The utility of such tags has been demonstrated (6, 15).

For the *trpAB* deletion mutant, two rounds of purification, selecting uracil prototrophy, were required to resolve merodiploids generated by the initial marker replacement event. If the deletion is viable and produces a small or no growth defect, segregation and allelic fixation should be random. In the case of *trpAB*, which we expected to have a mild phenotype with tryptophan added to the medium, two rounds of colony purification

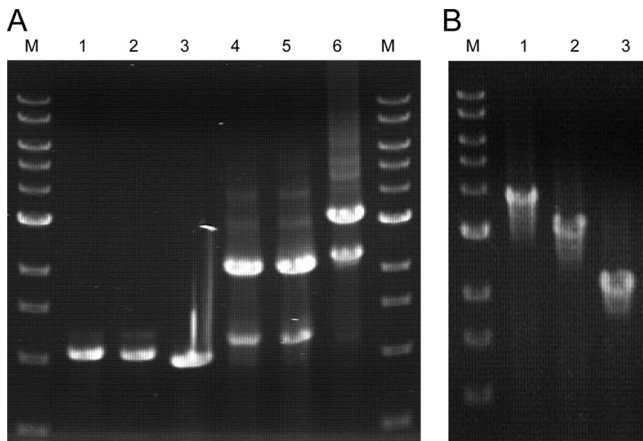


FIG 3 Construction of the *trpAB* pop-out markerless deletion. (A) Lanes: M, 1-kb DNA ladder; 1, 1-kb 5' flanking amplicon; 2, 1-kb 3' flanking amplicon; 3, P_{gdh} -*pyrF* marker cassette; 4, overlapped 5' flanking region to P_{gdh} -*pyrF* marker cassette; 5, overlapped 3' flanking region to P_{gdh} -*pyrF* marker cassette; 6, *trpAB* pop-out marker replacement cassette. (B) PCR amplification of the genomic regions surrounding the *trpAB* loci showing the marker replacement and subsequent pop-out (confirmed by DNA sequencing of the PCR products). Lanes: M, 1-kb DNA ladder; 1, *trpAB* locus in GLW101; 2, *trpAB* locus in JFW01; 3, *trpAB* locus in JFW02.

were sufficient. The subsequent pop-out strain should have a neutral phenotype with both uracil and tryptophan added to the growth medium, and we found, in fact, that no additional purification was necessary after one round of selection on 5-FOA.

The Δ *trpAB* strain is a tight tryptophan auxotroph but not resistant to 5-FAA. As with uracil biosynthesis, tryptophan biosynthesis allows for selection for both prototrophy and auxotrophy because the wild-type allele is counterselectable. First demonstrated in *Saccharomyces cerevisiae*, deletion of various genes in the biosynthetic pathway results in a tryptophan auxotroph that is resistant to 5-fluoroanthranilic acid (5-FAA) (35). This anthranilic acid analog is converted to 5-fluorotryptophan by the tryptophan biosynthetic pathway, and incorporation of 5-fluorotryptophan into proteins is toxic. In addition, 5-fluorotryptophan inhibits anthranilate synthase, thereby reducing the synthesis of tryptophan and increasing 5-fluorotryptophan toxicity (22). We found that *P. furiosus* is sensitive to 5-FAA on defined medium at a concentration of approximately 2 g/liter. In *P. furiosus*, the tryptophan biosynthetic pathway is predicted to be in an operon consisting of seven genes (*trpA* to *-G*) (34). Our first targets for deletion mutagenesis were *trpF* (PF1707), *trpE* (PF1709), and *trpD* (PF1710). Mutants with deletions of *trpE* were not obtained, most likely for technical reasons, and mutants with deletions of *trpF* (PF1707) and *trpD* (PF1710) were leaky auxotrophs not resistant to 5-FAA. Deletion of the *trpAB* locus (PF1705 and PF1706) (Fig. 2) resulted in a tight tryptophan auxotroph but also did not confer resistance to 5-FAA, suggesting that there are other mechanisms of 5-FAA toxicity in *P. furiosus*.

Complementation of the Δ *trpAB* strain by the wild-type *trpAB* alleles restores tryptophan prototrophy. To test the utility of the *trpAB* deletion mutant for prototrophic selection, the wild-type *trpAB* alleles were cloned onto a replicating shuttle vector that also contained the *pyrF* expression cassette (9) to generate pJFW070 (Fig. 4). The COM1 Δ *pyrF* Δ *trpAB* strain (JFW02) was readily transformed by this plasmid, selecting either uracil or tryptophan prototrophy.

Since both markers are contained on the same plasmid, we were able to compare the transformation efficiencies of the two markers. The efficiencies ($\sim 10^4$ transformants per μ g DNA) were similar to each other and comparable to that previously determined for pJFW018 ($\sim 10^4$ transformants per μ g DNA [9]). JFW02 could also be transformed to tryptophan prototrophy using wild-type genomic DNA. This strain will be important for applications that require multiple simultaneous selections in the same strain, such as maintaining a replicating shuttle vector with one marker and using the other marker to perform chromosomal manipulations. Selecting tryptophan prototrophy also provides an additional alternative to uracil prototrophy or a requirement of agmatine for growth (15).

A deletion of *radB* has no obvious effect on recombination in the GLW101 genetic background. To investigate the highly recombinant nature of GLW101 (COM1 Δ *pyrF*), we constructed a markerless deletion of *radB* (PF0021), a homologue of the eukaryotic *rad51* gene (26). This protein has been implicated in recombination and repair in *P. furiosus* by its DNA binding affinity and interaction with other known recombination proteins (18). Its role as a recombination mediator rather than a true recombinase is supported by its weak ATPase and strand exchange activities in *P. furiosus* (18) and recombination and growth defects in deletion mutants of *Haloferax volcanii* (13, 14). The *radB* pop-out PCR product was constructed using the same approach used for *trpAB*. Hundreds of uracil prototrophic colonies were obtained, and of eight colonies screened, one was purified to homogeneity. This intermediate strain, designated JFW03, was grown in liquid medium containing uracil and plated on solid medium containing both uracil and 5-FOA. PCR amplification of the *radB* locus from the resulting colonies identified four that had pop-out of the *pyrF* allele, which was confirmed by DNA sequencing of the PCR products. One of these was designated JFW04. The fact that pop-out was readily selected in this *radB* marker replacement mutant suggests that deletion of *radB* had no significant effect on recombination.

To further investigate recombination in the *radB* deletion mutant, we transformed the JFW04 strain with several different DNA types: a replicating plasmid (pJFW018), a nonreplicating integrating plasmid (pGLW021) (20), integrating PCR products (*trpAB*::*pyrF* pop-out construction and amplified wild-type *pyrF* with 1-kb flanking regions), and wild-type (DSM3638) genomic DNA. All the DNA types transformed JFW04 to uracil prototrophy at equivalent frequencies (on the order of 10^{-2} to 10^{-3} transformants per viable cell count at a DNA concentration of 3 to 4 μ g/ml, with plating efficiencies of approximately 1%).

Sensitivity to UV light was used to test recombination related to DNA repair. The survival of the *radB* mutant after exposure to UV doses in the range of 0 to 10 mJ on a plate surface was indistinguishable from the survival of the wild type and GLW101 at all intensities tested (Fig. 5A). There was also no difference in the growth rates of the two strains in defined medium (Fig. 5B) or under conditions of oxidative shock (Fig. 5C), as measured by sensitivity to hydrogen peroxide (33).

The apparent wild-type phenotype of JFW04 is somewhat surprising given the severe phenotype of *radB* mutants in the euryarchaeote *Haloferax volcanii* (13, 14; T. Allers, personal communication). On the other hand, *radB* is not present in any known member of the *Crenarchaeota*, including *Sulfolobus* species. The apparent wild-type phenotype of JFW04 may result from differences in *radB* functional

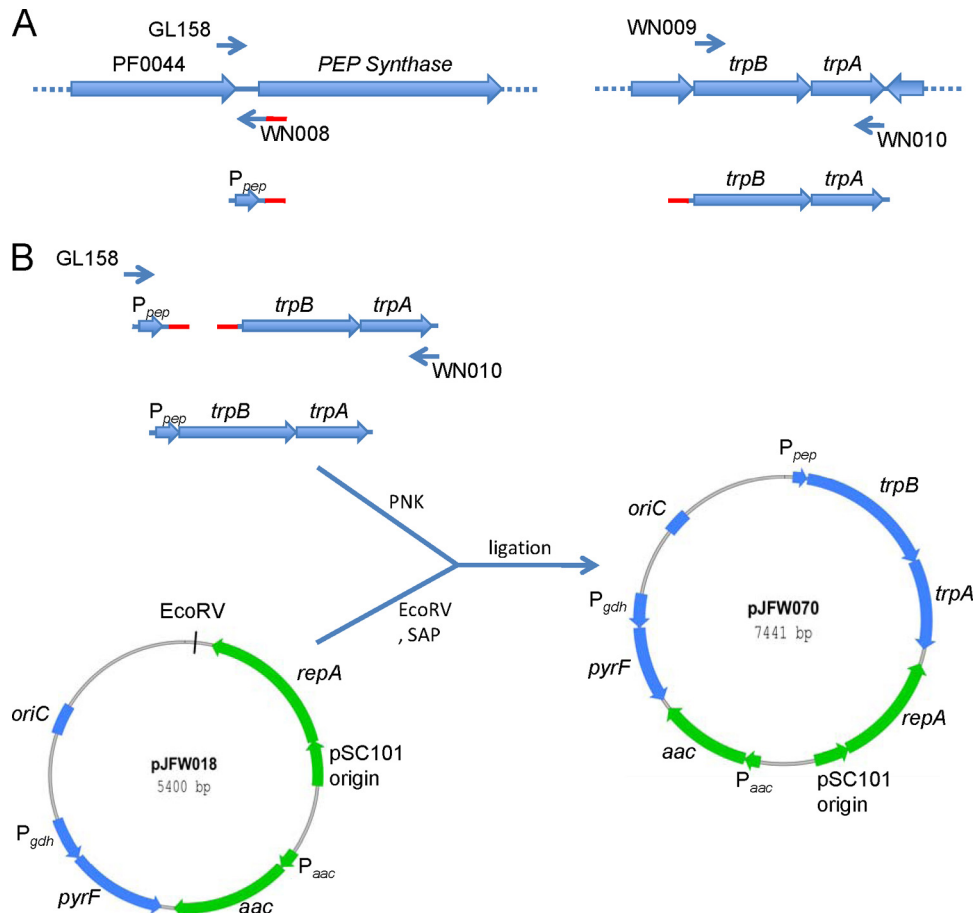


FIG 4 Construction of pJFW070. (A) Primers GL158 and WN008 were used to amplify the PEP synthase promoter (P_{peg}). Primers WN009 and WN010 were used to amplify *trpAB* from wild-type genomic DNA. (B) These fragments were joined by SOE PCR to produce the P_{peg} -*trpAB* marker cassette, which was treated with T4 polynucleotide kinase (PNK) and ligated into the pJFW018 fragment produced by EcoRV digestion and shrimp alkaline phosphatase (SAP) treatment, producing pJFW070.

divergence within the *Euryarchaeota*. It could also be the result of the GLW101 genetic background, but it is not possible to address this, as the wild-type strain, *Pyrococcus furiosus* DSM3638, has proven to be genetically intractable in our hands. Future work with *radB* in the closely related *T. kodakaraensis* could address this issue.

Attempts to construct a mutant with a deletion of *radA* were unsuccessful. We also attempted to construct a mutant with a deletion of *radA* (PF1926), another *rad51* homologue (27). In contrast to RadB, RadA is a true recombinase, with DNA binding, ATPase, and strand exchange activities in *P. furiosus* (18). Deletion of *radA* in *Haloferax volcanii* results in recombination and growth defects (37). Transformation of a *radA* pop-out construction into GLW101 also produced hundreds of uracil prototrophic colonies. Eight were screened for the marker replacement event at the *radA* locus, and all contained both *radA* and the P_{gdh} -*pyrF* marker replacement. Six rounds of colony purification, which were sufficient to purify other deletion mutants, failed to resolve these merodiploids, and repeated attempts to isolate a clean marker replacement strain were unsuccessful. In addition, the unresolved merodiploids showed a severe growth defect. Either in liquid medium or on plates, these strains took at least twice as long as the GLW101 parent to grow to a comparable cell density or colony size. These data suggest that a deletion of the *radA* gene

results in a severe phenotype and that *radA* may, in fact, be essential for viability in *P. furiosus*, although further experiments would be required to prove this conclusively.

The exact nature of the mutation leading to the COM1 phenotype is unknown. The nature of competence in GLW101 is of considerable biological interest. We hypothesized that the initial transformation event, integrating pGLW021, selected using simvastatin resistance, was a rare event, never seen again, but the deletion of *pyrF* in this transformant resulted in a strain that was starved for uracil, and uracil starvation resulted in competence as a mechanism to take up DNA as a source of uracil. Competence would then depend on uracil starvation, i.e., cell growth on plates without uracil, as in prototrophic selection using the wild-type *pyrF* allele. To test this, we constructed a shuttle vector, pJFW051, which is similar to pJFW018 but contains the 3-hydroxy-3-methylglutaryl coenzyme A gene for simvastatin resistance selection (see Fig. S2 in the supplemental material). We found that GLW101 was readily transformed by this plasmid on defined medium containing uracil and simvastatin. We performed the same experiment with GLW104, a GLW101-derived strain with a restored *pyrF* gene, and transformation of this strain was equally efficient. Since uracil starvation is not an issue with the restored

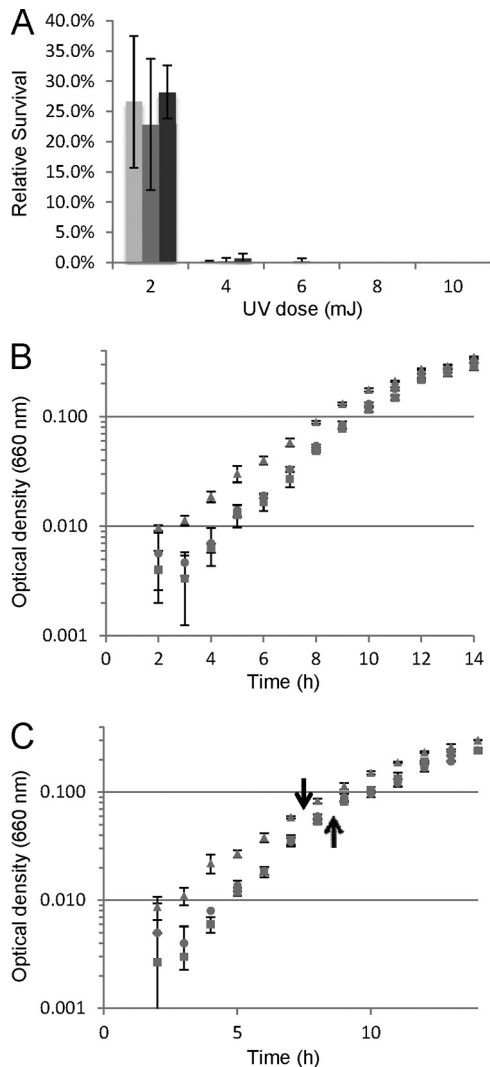


FIG 5 Characterization of JFW04. (A) Wild-type (light grey), GLW101 (grey), and JFW04 (dark grey) strains were exposed to UV radiation on a plate surface with doses ranging from 0 to 10 mJ. Relative survival was calculated as the proportion of colonies compared to the number for the unexposed control. (B) Growth curve of the JFW04 strain (circles) compared to the growth curves of GLW101 (squares) and the wild type (triangles). Culture growth was monitored by optical density at 660 nm. (C) Oxidative stress response of the JFW04 (circles) strain compared to the oxidative stress responses of GLW101 (squares) and the wild type (triangles). H_2O_2 was added at an OD_{660} of ~ 0.07 (7.5 h for wild type, 8.5 h for GLW101 and JFW04). Times of H_2O_2 addition are indicated by arrows. Each point represents the average of samples from two or more independent cultures, and the error bars show standard deviations.

pyrF deletion, this would indicate that competence does not result from uracil starvation.

We also hypothesized that competence might be the result of a mutation in a restriction system, as restriction of heterologous DNA is often a barrier to transformation. To test this, cell extracts were prepared from wild-type *P. furiosus* and GL101 cultures and incubated with pJFW051 plasmid DNA, using conditions suitable for other restriction enzymes from *Pyrococcus* species (17, 24), reported for their commercial use. Under all conditions tested, no restriction activity was detected for either the wild type or GLW101. In addition, there are no annotated restriction endonu-

cleases in the *P. furiosus* genome. Interestingly, there are also no homologues of competence genes. We also emphasize that many “naturally competent” organisms do not exhibit competence under all conditions, and it is possible that wild-type *P. furiosus* will be competent if appropriate conditions are used.

Conclusions. *P. furiosus* is an excellent model system for the study of DNA recombination, repair, and natural competence in the *Archaea*. The methods reported here will facilitate future studies by decreasing the time and expense required to generate marker replacement and deletion mutants. The *trpAB* deletion provides another selectable marker and will enable more sophisticated genetic analyses involving the maintenance of multiple selectable markers. In the GLW101 (COM1 $\Delta pyrF$) background, we have found that the deletion of *radB* has no detectable phenotype but the deletion of *radA* has a severe, possibly lethal phenotype. It is not known if this is true for *P. furiosus* in general or is peculiar to the GLW101 strain, since the nature of competence in GLW101 is not yet understood. This is the subject of ongoing and future investigations, which will be further facilitated by the work presented here.

The application of this method in *P. furiosus* will be especially valuable in elucidating the function of the ~ 600 genes which are unique to *P. furiosus* as compared to *T. kodakaraensis* (11). The increased transformation frequency associated with the GLW101 strain makes it particularly useful for the study of natural competence, CRISPR (clusters of regularly interspaced short palindromic repeats) function, and homologous recombination in general. In addition, the pop-out strategy can be adapted to generate tagged proteins in two steps *in vivo* and will have other uses that make strain construction rapid.

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