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

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ABSTRACT

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 *ΔpyrF*) 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 deletions of virtually any non-essential gene.
INTRODUCTION

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 (5, 32). 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 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 (9, 13, 20, 21, 29, 30), 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 is required for homologous recombination (29), but in *S. acidocaldarius*, which is transformed via electroporation, 10-30 bp of homology is sufficient (20). Several methods that rely on homologous recombination have also been developed to construct mutants in mesophilic archaea, including in a *Halobacterium* sp. (24, 37), *Haloferax volcanii* (3, 4) and *Methanosarcina acetivorans* C2A (26).

The isolation of a mutant of *Pyrococcus furiosus*, previously designated COM1 (GLW101: COM1 ΔpyrF), that is efficiently competent for DNA uptake was recently reported (21). This strain is transformed by exogenous DNA without any chemical or physical treatment, as compared 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.
(10), as well as the production of strains that lack some key metabolic enzymes (6) and that overproduce affinity-tagged versions of the native (7) and a subcomplex form (16) of the cytoplasmic hydrogenase.

In this study, we show that 1000 bp efficiently directs 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 constructed deletions by PCR amplification (without cloning) to select marker replacement events with subsequent 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 (∆*trpAB ΔpyrF*) that allows simultaneous nutritional selection of both markers, demonstrated using a recently constructed replicating plasmid for *P. furiosus* (10). 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 of the eukaryotic recombinase *rad51* in *P. furiosus*. Surprisingly, a deletion of *radB* had no apparent effect on either recombination or DNA transformation in the GLW101 (COM1 Δ*pyrF*) strain. Mutants 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 transformation, but the exact nature of this mutation remains unknown.
RESULTS AND DISCUSSION

Fewer than 40 base pairs of homologous DNA allows selection of marker replacements in *P. furiosus*. To investigate the minimum homology required for recombination in *P. furiosus* GLW101 (COM1 ΔpyrF), PCR products containing the *pyrF* gene with flanking DNA regions ranging in length from 0 to 1000 bp were used to restore the ΔpyrF locus in GLW101 to wild type, selecting transformants for uracil prototrophy (Figure 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 DNA containing 1 kb flanking regions and a few transformants detectable for DNA with flanking regions as short as 20 nucleotides (Figure 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 (21). This may contribute little for larger fragments of homology but may well impact 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 (2-10μg/mL) of transforming DNA (21). 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 (10), and a linear wild type *pyrF*-containing fragment with 1 kb flanking homology. For both DNA types,
Transformants were detectable with DNA concentrations as low as 1 ng/mL. When transforming DNA concentration is high (10 μg/mL), the transformation frequency of linear DNA is approximately 1 out of 100-500 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 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 likely 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 (29) 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 Figure 2. The transforming DNA fragment containing the deletion cassette contains pyrF under the control of the promoter for the gene encoding glutamate dehydrogenase (PF1602), Pgdh-pyrF (21), flanked by an additional 40 bp direct repeat sequence with minimal homology to the P. furiosus chromosome and constructed using Splicing
Overlap Extension (SOE) PCR (17). 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 PCR reactions were used to amplify the upstream and downstream flanking regions of \( trpAB \) as well as the \( pyrF \) expression cassette (Figure 2), 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 few as 40 base pairs 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 (Figure S1), 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 (1mL 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 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 (Table S1). 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<sub>gdh</sub>-pyrF cassette are selected, this method may be used to target any non-essential 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<sub>gdh</sub>-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<sub>gdh</sub>-pyrF marker cassette to generate a scarless deletion of the target gene. Alternatively, the 40bp 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 (7, 16).

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 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 \( \Delta trpAB \) strain is a tight tryptophan auxotroph but not resistant to 5FAA.

As with uracil biosynthesis, tryptophan biosynthesis allows for selection for prototrophy as well as auxotrophy because the wild type allele is counter-selectable. First demonstrated in \( S. \ ceravisiae \), deletion of various genes in the biosynthetic pathway results in a tryptophan auxotroph that is resistant to 5-fluoroanthranilic acid (5-FAA) (36). 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 (23). We found that \( P. \ furiosus \) is sensitive to 5-FAA on defined medium at a concentration of approximately 2 g/L. In \( P. \ furiosus \) the tryptophan biosynthetic pathway is predicted to be in an operon consisting of seven genes (\( trpA-G \)) (35). Our first targets for deletion mutagenesis were \( trpF \) (PF1707), \( trpE \) (PF1709) and \( trpD \) (PF1710). Deletions of \( trpE \) were not obtained most likely for technical reasons and deletions of \( trpF \) (PF1707) and \( trpD \) (PF1710) were leaky auxotrophs not resistant to 5-FAA. Deletion of the \( trpAB \) locus (PF1706-1705, Figure 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 (10) to generate pJFW070 (Figure 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 transformation efficiencies of the two markers. The efficiencies (~10⁴ transformants per µg DNA) which were similar to each other and comparable to that previously determined for pJFW018 (~10⁴ transformants per µg DNA (10)). 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 (16).

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 (27). This protein has been implicated in recombination and repair in *P. furiosus* by its DNA-binding affinity and interaction with other known recombination proteins (19). Its role as a recombination mediator, rather than a true recombinase is supported by its weak ATPase and strand exchange activities in *P. furiosus* (19) and
recombination and growth defects in deletion mutants of *Haloferax volcanii* (14, 15). 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 resulting colonies identified four that contained 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 non-replicating integrating plasmid (pGLW021) (21), integrating PCR products (*trpAB*::*pyrF* pop-out construction, and amplified wild type *pyrF* with 1 kb flanking regions), as well as 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 3-4 $\mu$g/ml, with plating efficiencies of approximately 1%).

Sensitivity to UV light was used to test recombination related to DNA repair. Survival of the *radB* mutant after exposure to UV doses in the range of 0-10 millijoules on a plate surface was indistinguishable from wild type and GLW101 at all intensities tested (Figure 5A). There was also no difference in the growth rates of the two strains in
defined media (Figure 5B) or under conditions of oxidative shock (Figure 5C) as measured by sensitivity to hydrogen peroxide (34).

The apparent wild type phenotype of JFW04 is somewhat surprising given the severe phenotype of \textit{radB} mutants in the Euryarchaeon \textit{Halofex volcanii} (2, 14, 15). On the other hand, \textit{radB} is not present in any known member of the Crenarchaeota, including \textit{Sulfolobus} species. The apparent wild type phenotype of JFW04 may result from differences in \textit{radB} functional 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, \textit{Pyrococcus furiosus} DSM3638, has proven to be genetically intractable in our hands. Future work with \textit{radB} in the closely related \textit{T. kodakaraensis} could address this issue.

Attempts to construct a deletion of \textit{radA} were unsuccessful. We also attempted to construct a deletion of \textit{radA} (PF1926), another \textit{rad51} homologue (28). In contrast to \textit{RadB}, \textit{RadA} is a true recombinase, with DNA binding, ATPase, and strand exchange activities in \textit{P. furiosus} (19). Deletion of \textit{radA} in \textit{Halofex volcanii} results in recombination and growth defects (38). Transformation of a \textit{radA} pop-out construction into GLW101 also produced hundreds of uracil prototrophic colonies. Eight were screened for the marker replacement event at the \textit{radA} locus, and all contained both \textit{radA} and the \textit{P_{gan}-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. In either liquid
media, or on plates, these strains took at least twice as long to grow to comparable cell density or colony size, as compared to the GLW101 parent. 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., cells 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 HMG-CoA gene for simvastatin resistance selection (Figure S2). We found that GLW101 was readily transformed by this plasmid on defined media containing uracil and simvastatin. We performed the same experiment with a 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 *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 free 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 (18, 25), reported for their commercial use. In all conditions tested, no restriction activity was detected for either the wild type or GLW101. In addition, there are no annotated restriction endonucleases 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* is competent if appropriate conditions were used.

**METHODS**

**P. furiosus** strains, media, and growth conditions. *P. furiosus* DSM 3638 (11) wild type, GLW101 (COM1 ΔpyrF) (21) and other strains were grown anaerobically in a defined medium with cellobiose as carbon source (21) at 90°C for 16-18 hours in 100 mL serum bottles containing 50 mL of liquid medium or on media solidified with phytagel (1% w/v) for 64 hours. For growth of GLW101 and other uracil auxotrophic strains, defined media contained 20 μM uracil. Transformation of GLW101 was performed as described (21). 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 20μM uracil and lacking tryptophan. Transformation efficiency reported here was calculated as the number of transformant colonies per μg of DNA added and does not take into account plating.
efficiency which is typically 1-5%. Transformation frequency was calculated as the proportion of transformant colonies to total cells and does 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 media. 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 media containing 20 μM uracil from a 1% inoculum (~7 generations) and then plating onto defined media containing 20 μM uracil and 3mM 5-FOA.

Restriction endonuclease assays. Cell free extracts (CFE) were prepared from 1 L cultures as described previously (8). Endonuclease assays were performed in 10 μL reaction volumes using 0.5-1 μg of pJFW051 DNA. Varying amounts of CFE (from 0 to 20 μg total protein) were added, in separate reactions, incubated in a NEBuffer 4 (20mM Tris–acetate pH 7.9, 50 mM potassium acetate, 10 mM magnesium acetate, and 1 mM dithiothreitol) (New England Biolabs). Reactions were prepared on ice and incubated at 90°C for 15 minutes. Similar assays were performed to detect the type I or type III restriction endonucleases. These assays were performed as before, except with the inclusion of 1mM ATP, 80 μM 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...
1000 to 0 bp was performed using the following primer sets: GL055-GL058;
pyrF500bpF-pyrF500bpR; pyrF250bpF-pyrF250bpR; pyrF150bpF-pyrF150bpR;
pyrF100bpF-pyrF100bpR; pyrF50bpF-pyrF50bpR; pyrF40bpF-pyrF40bpR; pyrF30bpF-
pyrF30bpR; pyrF20bpF-pyrF20bpR; pyrF10bpF-pyrF10bpR; pyrF0bpF-pyrF0bpR (Table
S1). These products were purified using a DNA Clean & Concentrator™-25 column
and transformed into the GLW101 strain. Three biological replicates
were performed for each PCR product.

**trpAB pop-out marker replacement strategy.** Sequence Manipulation Suite (33) was
used to generate a random 40bp sequence (5'
aagtgacgtgtacgcgagacccggtttcgtctctcat 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, JF394-JF395, Table S1) were used to amplify trpAB 1kb
flanking regions. The \( P_{gdr-pyrF} \) marker cassette was amplified from pJFW017 (10)
plasmid DNA using primers JF355.3 and JF356.3. The specific annealing regions of
these primers were designed for melting temperatures at 55±4°C. The overlap tails were
30-35 bases in length and designed so that the overlapping regions between PCR
products would be 20-25 bases in length, melting at 62 ± 2°C. PCR was performed
using *PfuTurbo* polymerase in a 50 μL reaction volume according to manufacturer’s
instructions (Stratagene). Thermal cycling included 30 cycles with annealing at 58°C,
and a 70 second 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 (SOE) PCR (17). 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 ~50ng of
each template DNA in a 50μL reaction. Prior to thermal cycling, template was denatured
without primers, allowed to anneal at 58ºC, and extended for 10 minutes at 72ºC. 30
cycles of amplification were performed as before, with the same end primers used to
generate the template products with the extension time increased to 120 seconds.
These two products were purified and used as 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 72ºC for 10 minutes. 30 cycles of amplification were
performed as before with JF392 and JF395 and extension time increased to 180
seconds. 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 90ºC. Putative transformants were screened for the marker
replacement by PCR with JF392 and JF395, using conditions described to generate the
pop-out PCR product (Figure 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 \( P_{gdh\text{-}hmg} \) cassette (22) was
amplified from pGLW28 (21) 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
synthase (PF0043) promoter (P<sub>pep</sub>-trpAB) was constructed by (SOE) PCR. A 126bp portion of the intergenic region upstream of PEP synthase was amplified from wild type gDNA using primers GL158 and WN008. The trpAB genes (PF1705, PF1706) were amplified using primers WN009 and WN010 and joined to the fragment containing the PEP regulatory region and a 12bp terminator from the hpyA1 gene (PF1722) (31). The P<sub>pep</sub>-trpAB cassette was treated with T4 polynucleotide kinase and ligated into the pJFW018 plasmid (10) that had been digested with EcoRV and treated with shrimp alkaline phosphatase. E. coli strain DH5α 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.).

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 ΔpyrF) background, we have found that deletion of radB has no detectable phenotype, but 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* (12). The increased transformation frequency associated with the
GLW101 strain makes it particularly useful for the study of natural competence,
CRISPR 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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of Georgia.
**Figure 1.**

A: The wild type pyrF region and PCR amplified fragments (indicated as lines below the chromosomal region) with varying lengths of flanking sequence used to transform GLW101 selecting uracil prototrophy. B: Transformation efficiencies using PCR products. 1μg of DNA was used to transform ~10^7 cells.
Figure 2.
Figure 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: 1kb flanking regions are amplified from gDNA 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.
Figure 3. Construction of the *trpAB* pop-out markerless deletion. Panel A. Lanes: 1) 1kb 5' flanking amplicon, 2) 1kb 3' flanking amplicon, 3) P<sub>gdh</sub>-pyrF marker cassette, 4) overlapped 5' flanking to P<sub>gdh</sub>-pyrF marker cassette, 5) overlapped 3' flanking to P<sub>gdh</sub>-pyrF marker cassette, 6) *trpAB* pop-out marker replacement cassette. Panel 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: 1Kb DNA ladder, *trpAB* locus in 1: GLW101, 2: JFW01, and 3: JFW02.
Figure 4. Construction of pJFW070. Panel A: GL158 and WN008 were used to amplify the PEP synthase promoter ($P_{\text{pep}}$). WN009 and WN010 were used to amplify $\text{trpAB}$ from wild type genomic DNA. B: These fragments were joined by (SOE) PCR to produce the $P_{\text{pep}}$-$\text{trpAB}$ marker cassette, which was treated with T4 PNK and ligated into the pJFW018 fragment produced by EcoRV digestion and SAP treatment, producing pJFW070.
Figure 5

A

Relative Survival

UV dose (mJ)

B

Optical density (660 nm)

Time (h)

C

Optical density (660 nm)

Time (h)
Figure 5. Characterization of JFW04. Wild type (blue), GLW101 (red) and JFW04 (green) were exposed to UV radiation on a plate surface with doses ranging from 0-10mJ. Relative survival was calculated as the proportion of colonies compared to the unexposed control. Panel B. Growth curve of the JFW04 strain (circles) compared to GLW101 (squares) and wild type (X). Culture growth was monitored by optical density at 660nm. Panel C. Oxidative stress response of the JFW04 strain (X) compared to GLW101 (diamonds) and wild type (triangles). Time of H₂O₂ addition is indicated by arrows. Each point represents an average of samples from two or more independent cultures, with error bars showing standard deviation.
Table 1. *P. furiosus* strains used and constructed in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Parent strain</th>
<th>Reference</th>
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<tbody>
<tr>
<td>DSM3638</td>
<td>Wild type</td>
<td>-</td>
<td>(11)</td>
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<tr>
<td>GLW101</td>
<td>COM1 ΔpyrF</td>
<td>DSM 3638</td>
<td>(21)</td>
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<td>GLW101</td>
<td>This work</td>
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<tr>
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<td>COM1 ΔpyrF trpAB::Pgdh-pyrF</td>
<td>GLW101</td>
<td>This work.</td>
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<tr>
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<tr>
<td>JFW04</td>
<td>COM1 ΔpyrF ΔradB</td>
<td>JFW03</td>
<td>This work.</td>
</tr>
</tbody>
</table>


the *Escherichia coli* Red and *Sulfolobus acidocaldarius* recombination systems. Molecular Microbiology 69:1255-1265.


and RadB Are Involved in Homologous Recombination in *Pyrococcus furiosus*. The Journal of Biological Chemistry **275**:33782-33790.


