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

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Running Title: Selection of Targeted Deletions in Pyrococcus

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 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 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 (*ΔtrpAB 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 *ΔpyrF*) 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 Δ*pyrF* 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),P_{gdh}-*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.

160 This strategy has several important advantages over conventional deletion
161 construction. It does not require cloning, and only six primers are needed to provide
162 specificity for the target gene (Table S1). Primers of 60 bases are significantly less
163 expensive and eliminate the need for primer purification making pop-out construction of
164 deletions amenable to a high-throughput system. Since both the integration and
165 excision of the P_{gdh}-*pyrF* cassette are selected, this method may be used to target any
166 non-essential gene. The pop-out constructs, as described here, leave a 40 bp 'scar'
167 sequence that remains in the genome after pop-out of the P_{gdh}-*pyrF* marker cassette. If
168 a scarless deletion is desired, this strategy could be modified so that only one 40 bp
169 pop-out sequence is included in the construct, which would recombine with the native
170 sequence on the other side of the P_{gdh}-*pyrF* marker cassette to generate a scarless
171 deletion of the target gene. Alternatively, the 40bp scar sequence provides flexibility for
172 modifying genomic targets by introducing specific sequences such as signal peptides or
173 affinity tags for protein purification. The utility of such tags has been demonstrated (7,
174 16).
175 For the *trpAB* deletion mutant, two rounds of purification, selecting uracil
176 prototrophy, were required to resolve merodiploids generated by the initial marker
177 replacement event. If the deletion is viable, and produces a small or no growth defect,
178 segregation and allelic fixation should be random. In the case of *trpAB*, which we
179 expected to have a mild phenotype with tryptophan added to the medium, two rounds of
180 colony purification were sufficient. The subsequent pop-out strain should have a neutral
181 phenotype with both uracil and tryptophan added to the growth medium, and we found,

182 in fact, that no additional purification was necessary after one round of selection on 5-
183 FOA.
184
185 **The Δ*trpAB* strain is a tight tryptophan auxotroph but not resistant to 5FAA.**
186 As with uracil biosynthesis, tryptophan biosynthesis allows for selection for prototrophy
187 as well as auxotrophy because the wild type allele is counter-selectable. First
188 demonstrated in *S. cerevisiae*, deletion of various genes in the biosynthetic pathway
189 results in a tryptophan auxotroph that is resistant to 5-fluoroanthranilic acid (5-FAA)
190 (36). This anthranilic acid analog is converted to 5-fluorotryptophan by the tryptophan
191 biosynthetic pathway, and incorporation of 5-fluorotryptophan into proteins is toxic. In
192 addition, 5-fluorotryptophan inhibits anthranilate synthase, thereby reducing the
193 synthesis of tryptophan and increasing 5-fluorotryptophan toxicity (23). We found that *P.*
194 *furiosus* is sensitive to 5-FAA on defined medium at a concentration of approximately 2
195 g/L. In *P. furiosus* the tryptophan biosynthetic pathway is predicted to be in an operon
196 consisting of seven genes (*trpA*-*G*) (35). Our first targets for deletion mutagenesis were
197 *trpF* (PF1707), *trpE* (PF1709) and *trpD* (PF1710). Deletions of *trpE* were not obtained
198 most likely for technical reasons and deletions of *trpF* (PF1707) and *trpD* (PF1710)
199 were leaky auxotrophs not resistant to 5-FAA. Deletion of the *trpAB* locus (PF1706-
200 1705, Figure 2) resulted in a tight tryptophan auxotroph, but also did not confer
201 resistance to 5-FAA, suggesting that there are other mechanisms of 5-FAA toxicity in *P.*
202 *furiosus*.
203

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 *ΔpyrF ΔtrpAB* 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 (~10^4 transformants per μg DNA) which were similar to each other and comparable to that previously determined for pJFW018 (~10^4 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 *ΔpyrF)*, 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 μg/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

249 defined media (Figure 5B) or under conditions of oxidative shock (Figure 5C) as
250 measured by sensitivity to hydrogen peroxide (34).
251 The apparent wild type phenotype of JFW04 is somewhat surprising given the
252 severe phenotype of *radB* mutants in the Euryarchaeon *Haloferax volcanii* (2, 14, 15).
253 On the other hand, *radB* is not present in any known member of the Crenarchaeota,
254 including *Sulfolobus* species. The apparent wild type phenotype of JFW04 may result
255 from differences in *radB* functional divergence within the Euryarchaeota. It could also be
256 the result of the GLW101 genetic background, but it is not possible to address this, as
257 the wild type strain, *Pyrococcus furiosus* DSM3638, has proven to be genetically
258 intractable in our hands. Future work with *radB* in the closely related *T. kodakaraensis*
259 could address this issue.
260
261 **Attempts to construct a deletion of *radA* were unsuccessful.** We also attempted to
262 construct a deletion of *radA* (PF1926), another *rad51* homologue (28). In contrast to
263 RadB, RadA is a true recombinase, with DNA binding, ATPase, and strand exchange
264 activities in *P. furiosus* (19). Deletion of *radA* in *Haloferax volcanii* results in
265 recombination and growth defects (38). Transformation of a *radA* pop-out construction
266 into GLW101 also produced hundreds of uracil prototrophic colonies. Eight were
267 screened for the marker replacement event at the *radA* locus, and all contained both
268 *radA* and the P_{gdh}-*pyrF* marker replacement. Six rounds of colony purification, which
269 were sufficient to purify other deletion mutants, failed to resolve these merodiploids, and
270 repeated attempts to isolate a clean marker replacement strain were unsuccessful. In
271 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 Δ*pyrF*) (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 20μM uracil from a 1% inoculum (~7 generations) and then plating onto defined media containing 20μM 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 10μL 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 (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 (33) was used to generate a random 40bp sequence (5' aagtgagcgtgttacgccgagacccggtttcgtctctcat 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_{gdh}-*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±4ºC. 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 ± 2ºC. 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 58ºC, and a 70 second extension at 72ºC. Products were purified using a DNA Clean & Concentrator™-25 column (Zymo Research).Three fragments were put together by two

364 rounds of (SOE) PCR (17). The *trpAB* upstream flanking region was joined to the P_{gdh}-
365 *pyrF* marker cassette, and in a separate reaction, the P_{gdh}-*pyrF* marker cassette was
366 also joined to the *trpAB* 3' flanking region. (SOE) PCR was performed using ~50ng of
367 each template DNA in a 50μL reaction. Prior to thermal cycling, template was denatured
368 without primers, allowed to anneal at 58ºC, and extended for 10 minutes at 72ºC. 30
369 cycles of amplification were performed as before, with the same end primers used to
370 generate the template products with the extension time increased to 120 seconds.
371 These two products were purified and used as template for another (SOE) PCR. In the
372 second (SOE) PCR, the first annealing step was omitted and the two fragments were
373 allowed to anneal and extend at 72ºC for 10 minutes. 30 cycles of amplification were
374 performed as before with JF392 and JF395 and extension time increased to 180
375 seconds. The 3 kb PCR product was then purified and transformed into the GLW101
376 strain. Eight uracil prototrophs were picked into liquid defined medium without uracil and
377 grown overnight at 90ºC. Putative transformants were screened for the marker
378 replacement by PCR with JF392 and JF395, using conditions described to generate the
379 pop-out PCR product (Figure 2). The final deletion mutant was confirmed by sequencing
380 of the *trpAB* region, which contained the sequence as designed.
381
382 **Construction of plasmids.** To construct pJFW051 a 4.4 kb fragment was amplified by
383 PCR from pJFW018 using primers JF264 and JF269. The P_{gdh}-*hmg* cassette (22) was
384 amplified from pGLW28 (21) using primers GL021 and GL022, treated with T4
385 polynucleotide kinase and ligated into the 4.4 kb fragment. A cassette, containing the
386 wild type *trpAB* alleles under the transcriptional control of the phosphoenolpyruvate

synthase (PF0043) promoter (P_{pep}-*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_{pep}-*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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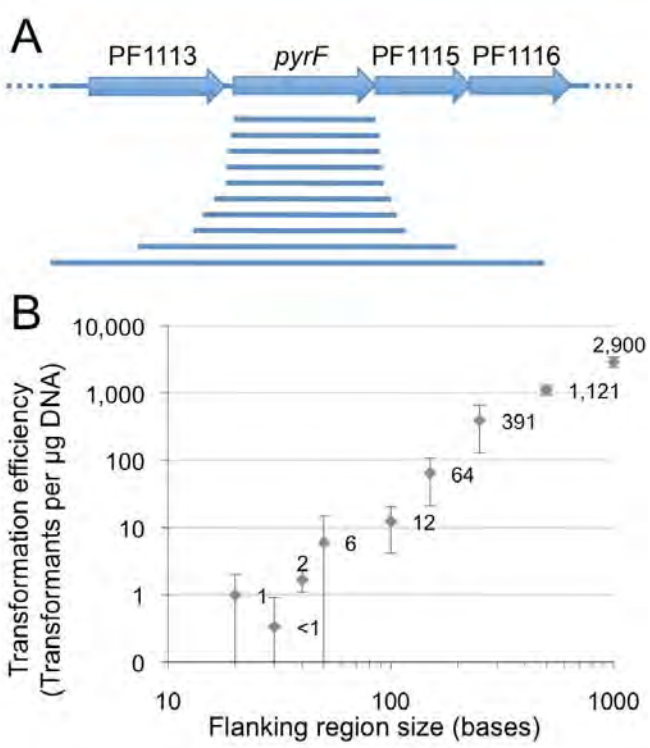
429 **Figure 1.**



430

431

432 **Figure 1.** A: The wild type *pyrF* region and PCR amplified fragments (indicated as

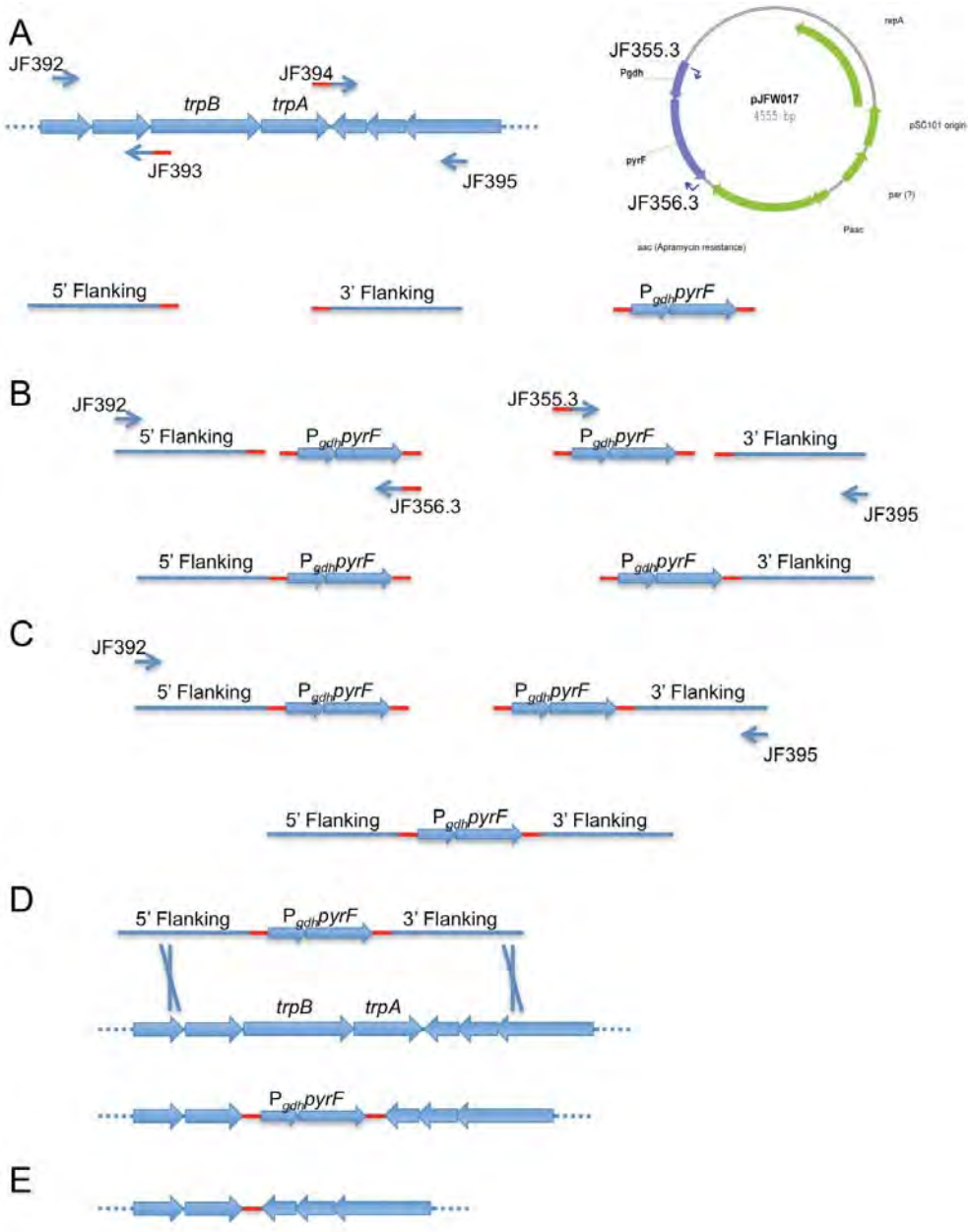
433 lines below the chromosomal region) with varying lengths of flanking sequence

434 used to transform GLW101 selecting uracil prototrophy. B: Transformation

435 efficiencies using PCR products. 1µg of DNA was used to transform ~10^7cells.

436

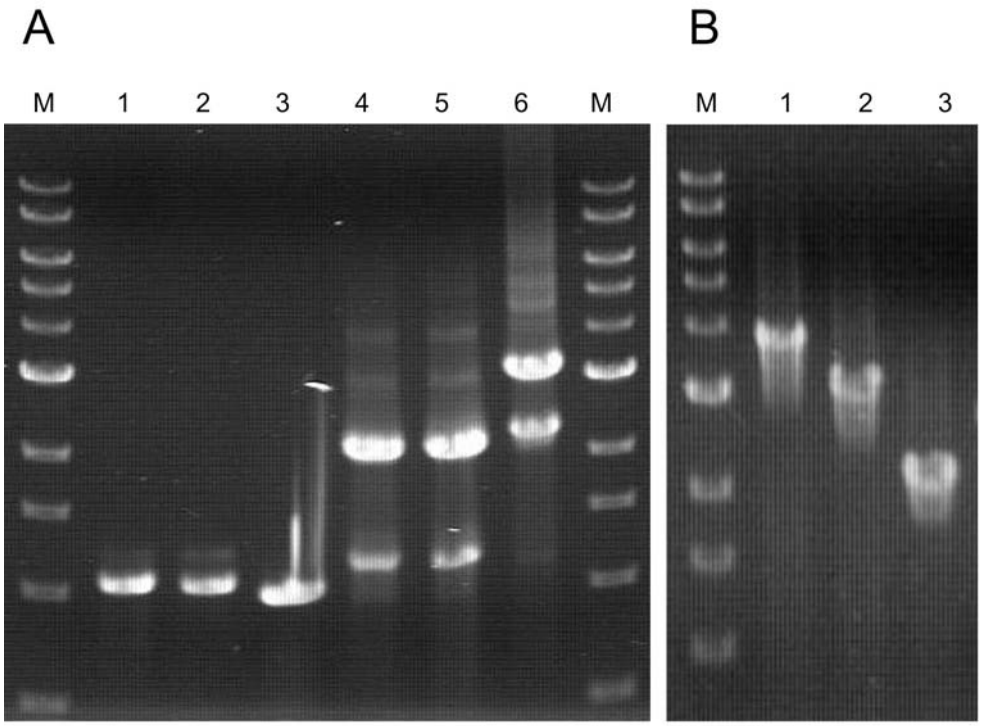
436 **Figure 2.**



437

438 **Figure 2.** Pop-out marker replacement strategy. Six primers are used to construct a
439 pop-out PCR product that is used to direct marker replacement and subsequent
440 excision of the selected marker. A: 1kb flanking regions are amplified from gDNA and
441 P_{gdh}-*pyrF* is amplified from pJFW017. Overlap tails for (SOE) PCR introduce the 'pop-
442 out scar' sequence and are indicated in red. B: (SOE) PCR generates two overlap
443 products. C: A second (SOE) PCR generates the final pop-out construct. D:
444 Transformation into *P. furiosus* allows for selection of the marker replacement event. E:
445 5-FOA selection of the pop-out cassette generates a markerless deletion.
446

446 **Figure 3**



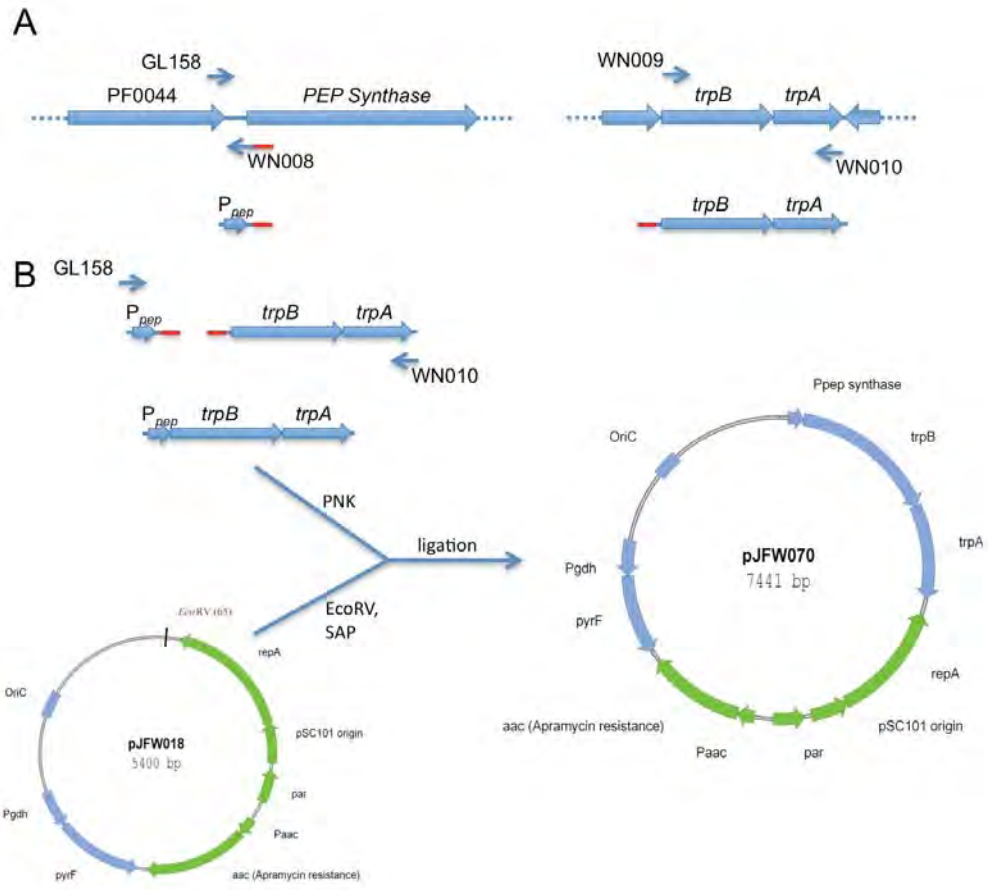
447

448

449 **Figure 3.** Construction of the *trpAB* pop-out markerless deletion. Panel A. Lanes: 1) 1kb
450 5' flanking amplicon, 2) 1kb 3' flanking amplicon, 3) P_{gdh}-*pyrF* marker cassette, 4)
451 overlapped 5' flanking to P_{gdh}-*pyrF* marker cassette, 5) overlapped 3' flanking to P_{gdh}-
452 *pyrF* marker cassette, 6) *trpAB* pop-out marker replacement cassette. Panel B. PCR
453 amplification of the genomic regions surrounding the *trpAB* loci showing the marker
454 replacement, and subsequent pop-out (confirmed by DNA sequencing of the PCR
455 products). Lanes M: 1Kb DNA ladder*, trpAB* locus in 1: GLW101, 2: JFW01, and
456 3:JFW02.

457

458 **Figure 4.**

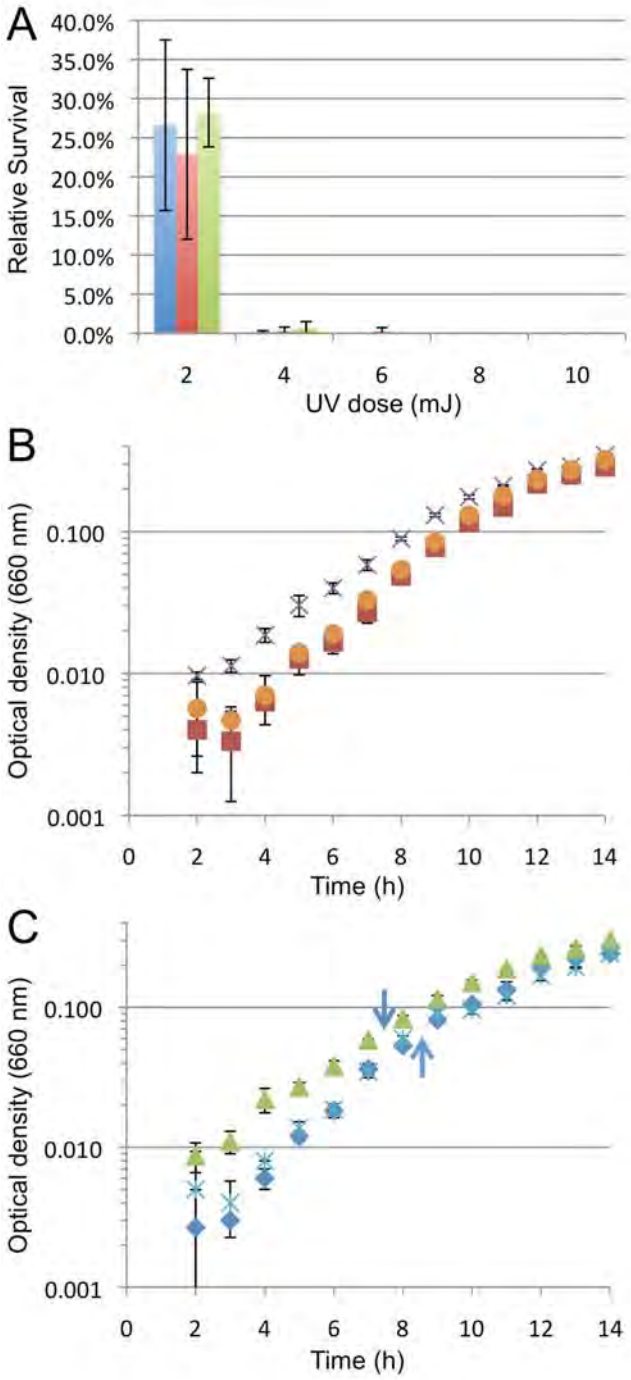


459

460

461 **Figure 4.** Construction of pJFW070. Panel A: GL158 and WN008 were used to amplify
462 the *PEP synthase* promoter (P_{pep}). WN009 and WN010 were used to amplify *trpAB* from
463 wild type genomic DNA. B: These fragments were joined by (SOE) PCR to produce the
464 P_{pep}-*trpAB* marker cassette, which was treated with T4 PNK and ligated into the
465 pJFW018 fragment produced by EcoRV digestion and SAP treatment, producing
466 pJFW070.

467 **Figure 5**



468

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 H_2O_2 addition is indicated by arrows. Each point represents an average of samples from two or more independent cultures, with error bars showing standard deviation.

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

Strain	Genotype	Parent strain	Reference
DSM3638	Wild type	-	(11)
GLW101	COM1 Δ*pyrF*	DSM 3638	(21)
GLW102	COM1 Δ*pyrF trpF::*P_{gdh}*-pyrF*	GLW101	This work.
GLW103	COM1 Δ*pyrF trpD::*P_{gdh}*-pyrF*	GLW101	This work.
GLW104	COM1 Δ*pyrF*::*pyrF*	GLW101	This work
JFW01	COM1 Δ*pyrF trpAB::*P_{gdh}*-pyrF*	GLW101	This work.
JFW02	COM1 Δ*pyrF* Δ*trpAB*	JFW01	This work.
JFW03	COM1 Δ*pyrF radB::*P_{gdh}*-pyrF*	GLW101	This work.
JFW04	COM1 Δ*pyrF* Δ*radB*	JFW03	This work.

479

480

REFRENCES

1. **Alani, E., L. Cao, and N. Kleckner.** 1987. A method for gene disruption that allows repeated use of URA3 selection in the construction of multiply disrupted yeast strains. Genetics **116:**541-5.
2. **Allers, T.** 2011. Personal Communication.
3. **Allers, T., H. P. Ngo, M. Mevarech, and R. G. Lloyd.** 2004. Development of additional selectable markers for the halophilic archaeon *Haloferax volcanii* based on the *leuB* and *trpA* genes. Appl Environ Microbiol **70:**943-53.
4. **Bitan-Banin, G., R. Ortenberg, and M. Mevarech.** 2003. Development of a gene knockout system for the halophilic archaeon *Haloferax volcanii* by use of the *pyrE* gene. J Bacteriol **185:**772-8.
5. **Blumer-Schuette, S. E., I. Kataeva, J. Westpheling, M. W. W. Adams, and R. M. Kelly.** 2008. Extremely thermophilic microorganisms for biomass conversion: status and prospects. Current Opinion in Biotechnology **19:**210-217.
6. **Bridger, S. L., S. M. Clarkson, K. Stirrett, M. B. Debarry, G. L. Lipscomb, G. J. Schut, J. Westpheling, R. A. Scott, and M. W. Adams.** 2011. Deletion Strains Reveal Metabolic Roles for Key Elemental Sulfur-Responsive Proteins in *Pyrococcus furiosus*. J Bacteriol **193:**6498-504.
7. **Chandrayan, S. K., P. M. McTernan, R. C. Hopkins, J. Sun, F. E. Jenney, Jr., and M. W. Adams.** 2012. Engineering hyperthermophilic archaeon

Pyrococcus furiosus to overproduce its cytoplasmic [NiFe]-hydrogenase. J Biol Chem **287:**3257-64.

8. **Chung, D., J. R. Huddleston, J. Farkas, and J. Westpheling.** 2011. Identification and characterization of CbeI, a novel thermostable restriction enzyme from *Caldicellulosiruptor bescii* DSM 6725 and a member of a new subfamily of HaeIII-like enzymes Journal of Industrial Microbiology & Biotechnology.

9. **Deng, L., H. Zhu, Z. Chen, Y. X. Liang, and Q. She.** 2009. Unmarked gene deletion and host-vector system for the hyperthermophilic crenarchaeon *Sulfolobus islandicus*. Extremophiles **13:**735-46.

10. **Farkas, J., D. Chung, M. Debarry, M. W. Adams, and J. Westpheling.** 2011. Defining Components of the Chromosomal Origin of Replication of the Hyperthermophilic Archaeon, *Pyrococcus furiosus*, Needed for Construction of a Stable Replicating Shuttle Vector. Appl Environ Microbiol **77:**6343-6349.

11. **Fiala, G., and K. O. Stetter.** 1986. *Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100°C. Archives of Microbiology **145:**56-61.

12. **Fukui, T., H. Atomi, T. Kanai, R. Matsumi, S. Fujiwara, and T. Imanaka.** 2005. Complete genome sequence of the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 and comparison with *Pyrococcus* genomes. Genome Res **15:**352-63.

13. **Grogan, D. W., and K. R. Stengel.** 2008. Recombination of synthetic oligonucleotides with prokaryotic chromosomes: substrate requirements of

525 the *Escherichia coli*/λRed and *Sulfolobus acidocaldarius* recombination
526 systems. Molecular Microbiology **69:**1255-1265.
527 14. **Haldenby, S.** 2007. Genetic Analysis of RadB, a Paralogue of the Archaeal
528 Rad51/RecA Homologue, RadA. University of Nottingham.
529 15. **Haldenby, S., M. F. White, and T. Allers.** 2009. RecA family proteins in
530 archaea: RadA and its cousins. Biochemical Society Transactions **37:**102-
531 107.
532 16. **Hopkins, R. C., J. Sun, F. E. Jenney, Jr., S. K. Chandrayan, P. M.**
533 **McTernan, and M. W. Adams.** 2011. Homologous Expression of a
534 Subcomplex of *Pyrococcus furiosus* Hydrogenase that Interacts with
535 Pyruvate Ferredoxin Oxidoreductase. PLoS ONE **6:**e26569.
536 17. **Horton, R. M., Z. L. Cai, S. N. Ho, and L. R. Pease.** 1990. Gene splicing by
537 overlap extension: tailor-made genes using the polymerase chain reaction.
538 Biotechniques **8:**528-535.
539 18. **Ishikawa, K., M. Watanabe, T. Kuroita, I. Uchiyama, J. M. Bujnicki, B.**
540 **Kawakami, M. Tanokura, and I. Kobayashi.** 2005. Discovery of a novel
541 restriction endonuclease by genome comparison and application of a wheat-
542 germ-based cell-free translation assay: *Pab*I (5'-GTA/C) from the
543 hyperthermophilic archaeon *Pyrococcus abyssi*. Nucleic Acids Research
544 **33:**e112.
545 19. **Komori, K., T. Miyata, J. DiRuggiero, R. Holley-Shanks, I. Hayashi, I. K.**
546 **O. Cann, K. Mayanagi, H. Shinagawa, and Y. Ishino.** 2000. Both RadA

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

20. **Kurosawa, N., and D. W. Grogan.** 2005. Homologous recombination of exogenous DNA with the *Sulfolobus acidocaldarius* genome: properties and uses. FEMS Microbiol Lett **253:**141-9.

21. **Lipscomb, G. L., K. Stirrett, G. J. Schut, F. Yang, F. E. Jenney, Jr., R. A. Scott, M. W. Adams, and J. Westpheling.** 2011. Natural competence in the hyperthermophilic archaeon *Pyrococcus furiosus* facilitates genetic manipulation: construction of markerless deletions of genes encoding the two cytoplasmic hydrogenases. Appl Environ Microbiol **77:**2232-8.

22. **Matsumi, R., K. Manabe, T. Fukui, H. Atomi, and T. Imanaka.** 2007. Disruption of a sugar transporter gene cluster in a hyperthermophilic archaeon using a host-marker system based on antibiotic resistance. J Bacteriol **189:**2683-91.

23. **Miozzari, G., P. Niederberger, and R. Hutter.** 1977. Action of tryptophan analogues in *Saccharomyces cerevisiae*. Arch Microbiol **115:**307-16.

24. **Peck, R. F., S. DasSarma, and M. P. Krebs.** 2000. Homologous gene knockout in the archaeon *Halobacterium salinarum* with *ura3* as a counterselectable marker. Mol Microbiol **35:**667-76.

25. **Pingoud, V., C. Conzelmann, S. Kinzebach, A. Sudina, V. Metelev, E. Kubareva, J. M. Bujnicki, R. Lurz, G. Luder, S. Y. Xu, and A. Pingoud.** 2003. PspGI, a type II restriction endonuclease from the extreme thermophile *Pyrococcus* sp. structural and functional studies to investigate

an evolutionary relationship with several mesophilic restriction enzymes. J Mol Biol **329:**913-29.

26. **Pritchett, M. A., J. K. Zhang, and W. W. Metcalf.** 2004. Development of a markerless genetic exchange method for *Methanosarcina acetivorans* C2A and its use in construction of new genetic tools for methanogenic archaea. Appl Environ Microbiol **70:**1425-33.

27. **Sandler, S. J., P. Hugenholtz, C. Schleper, E. F. DeLong, N. R. Pace, and A. J. Clark.** 1999. Diversity of *radA* genes from cultured and uncultured Archaea: Comparative analysis of putative RadA proteins and their use as a phylogenetic marker. Journal of Bacteriology **181:**907-915.

28. **Sandler, S. J., L. H. Satin, H. S. Samra, and A. J. Clark.** 1996. *recA*-like genes from three archaean species with putative protein products similar to Rad51 and Dmc1 proteins of the yeast *Saccharomyces cerevisiae*. Nucleic Acids Res **24:**2125-32.

29. **Sato, T., T. Fukui, H. Atomi, and T. Imanaka.** 2005. Improved and Versatile Transformation System Allowing Multiple Genetic Manipulations of the Hyperthermophilic Archaeon *Thermococcus kodakaraensis*. Applied and Environmental Microbiology **71:**3889-3899.

30. **Sato, T., T. Fukui, H. Atomi, and T. Imanaka.** 2003. Targeted Gene Disruption by Homologous Recombination in the Hyperthermophilic Archaeon *Thermococcus kodakaraensis* KOD1. Journal of Bacteriology **185:**210-220.

31. **Spitalny, P., and M. Thomm.** 2008. A polymerase III-like reinitiation mechanism is operating in regulation of histone expression in archaea. Mol Microbiol **67:**958-70.

32. **Stetter, K. O.** 1996. Hyperthermophilic procaryotes. FEMS Microbiology Reviews **18:**149-158.

33. **Stothard, P.** 2000. The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. Biotechniques **28:**1102, 1104.

34. **Strand, K. R., C. Sun, T. Li, F. E. Jenney, Jr., G. J. Schut, and M. W. Adams.** 2010. Oxidative stress protection and the repair response to hydrogen peroxide in the hyperthermophilic archaeon *Pyrococcus furiosus* and in related species. Arch Microbiol **192:**447-59.

35. **Tang, X., S. Ezaki, S. Fujiwara, M. Takagi, H. Atomi, and T. Imanaka.** 1999. The tryptophan biosynthesis gene cluster trpCDEGFBA from *Pyrococcus kodakaraensis* KOD1 is regulated at the transcriptional level and expressed as a single mRNA. Mol Gen Genet **262:**815-21.

36. **Toyn, J. H., P. L. Gunyuzlu, W. H. White, L. A. Thompson, and G. F. Hollis.** 2000. A counterselection for the tryptophan pathway in yeast: 5-fluoroanthranilic acid resistance. Yeast **16:**553-60.

37. **Wang, G., S. P. Kennedy, S. Fasiludeen, C. Rensing, and S. DasSarma.** 2004. Arsenic resistance in *Halobacterium* sp. strain NRC-1 examined by using an improved gene knockout system. J Bacteriol **186:**3187-94.

614 38. **Woods, W. G., and M. L. Dyall-Smith.** 1997. Construction and analysis of a
615 recombination-deficient (*radA*) mutant of *Haloferax volcanii*. Molecular
616 Microbiology **23:**791-797.
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