Improved and Versatile Transformation System Allowing Multiple Genetic Manipulations of the Hyperthermophilic Archaeon *Thermococcus kodakaraensis*

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We have recently developed a gene disruption system for the hyperthermophilic archaean *Thermococcus kodakaraensis* by utilizing a pyrF-deficient mutant, KU25, as a host strain and the pyrF gene as a selectable marker. To achieve multiple genetic manipulations for more advanced functional analyses of genes in vivo, it is necessary to establish multiple host-marker systems or to develop a system in which repeated utilization of one marker gene is possible. In this study, we first constructed a new host strain, KU216 (ΔpyrF), by specific and almost complete deletion of endogenous pyrF through homologous recombination. In this refined host, there is no need to consider unknown mutations caused by random mutagenesis, and unlike in the previous host, KU25, there is little, if any, possibility that unintended recombination between the marker gene and the chromosomal allele occurs. Furthermore, a new host-marker combination of a trpE deletant, KW128 (ΔpyrF ΔtrpE::pyrF), and the trpE gene was developed. This system made it possible to isolate transformants through a more simple selection procedure as well as to deduce the transformation efficiency, overcoming practical disadvantages of the first system. The effects of the transformation conditions were also investigated using this system. Finally, we have also established a system in which repeated utilization of the counterselectable pyrF marker is possible through its excision by pop-out recombination. Both endogenous and exogenous sequences could be applied as tandem repeats flanking the marker pyrF for pop-out recombination. A double deletion mutant, KUW1 (ΔpyrF ΔtrpE), constructed with the pop-out strategy, was demonstrated to be a useful host for the dual markers pyrF and trpE. Likewise, a triple deletion mutant, KUWH1 (ΔpyrF ΔtrpE ΔhisD), could also be constructed. The transformation systems developed here now provide the means for extensive genetic studies in this hyperthermophilic archaean.

Recent phylogenetic analysis of living organisms based on rRNA sequences has indicated that hyperthermophiles occupy the deepest and shortest branches in the phylogenetic tree, postulating that the origin and evolution of biological systems may have derived from hyperthermophiles (25). Studies on the unique properties of hyperthermophiles are expected to provide valuable perspectives on the mechanisms that enable them to survive and grow in extreme environments (26). They are also important as potential resources for highly thermostable enzymes (2, 28). Many members of hyperthermophiles belong to the third domain of life, *Archaea*, along with halophiles and methanogens. *Archaea* exhibit a mosaic of features from the other two domains, *Bacteria* and *Eucarya*; intriguingly, their components for information processing are more closely related to those in eucaryotes than those in bacteria. From these interests, genome projects of various types of hyperthermophilic archaea have been performed, and complete genome sequences of more than 10 species have been determined. The genomes of hyperthermophilic archaea are rather small, and consequently, they are predicted to possess simplified versions of various biological machineries and metabolisms composed of small sets of genes. This is a great advantage for elucidating the functions of genes identified by genome analyses, and accumulation of this information may ultimately help to understand the basic principles of life.

The progress of research on hyperthermophilic archaea had been constantly hampered by the limitation of available tools for genetic manipulation. In contrast to several genetic methodologies for mesophilic archaea, halophiles (18, 23), and methanogens (12, 23), which are comparable to those for bacteria, manipulative strategies for hyperthermophilic archaea are still at an early stage. In particular, the development of targeted gene disruption in hyperthermophiles had not been achieved, despite the establishment of shuttle vector systems for several strains in the genera *Sulfolobus* and *Pyrococcus* (1, 4, 9, 24). As gene disruption is a powerful and direct tool for investigation of in vivo gene functions, development of a gene targeting system would certainly be a major breakthrough in the research on hyperthermophilic archaea.

From this viewpoint, we have recently developed a gene targeting system for *Thermococcus kodakaraensis* (21), a sulfur-reducing hyperthermophilic archaean belonging to *Thermococcales* in *Euryarchaeota* (5, 16). We successfully disrupted the trpE gene in *T. kodakaraensis* by homologous recombination utilizing a pyrF-deficient mutant and the pyrF gene as a host strain and a selectable marker, respectively, the first example in hyperthermophiles (21). Gene disruption in the thermoacidophilic archaean *Sulfolobus solfataricus* using a lacS

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marker has also been developed (29). However, in both methods, only one selectable marker gene was available.

In this report, we describe the improvement of the transformation system using the pyrF marker along with the establishment of a new host-marker combination consisting of a trpE deletant and the trpE gene. Moreover, repeated utilization of the pyrF marker was made possible by pop-out excision of the counterselectable marker to achieve multiple genetic manipulations for more advanced functional analyses of genes in vivo.

**MATERIALS AND METHODS**

**Strains and growth conditions.** The strains and plasmids used in this study are listed in Table 1. *T. kodakaraensis* KOD1 and its derivatives were cultivated under strictly anaerobic conditions at 85°C in a rich growth medium (AW-YT) or a synthetic medium (AW-CAA) (22). The preparation of plate medium and cultivation of the cells on it were performed as described previously (21). Further modifications of the medium for investigation of auxotrophy of mutant strains and selection of transformants are described in the text.

*Escherichia coli* strain DH5α, used for general DNA manipulation, was routinely cultivated at 37°C in Luria-Bertani (LB) medium (19) and supplemented with 50 μg/ml ampicillin when needed.

**General DNA manipulation.** General DNA manipulation was performed as described previously (19). Genetic DNA of *T. kodakaraensis* was isolated as described previously (21). PCR was carried out using KOD-Plus (Toyobo, Osaka, Japan) or TaKaRa EX Taq DNA polymerase, and sequences of the primers used for PCR in this study are available upon request. When necessary, DNA fragments amplified by PCR were phosphorylated by T4 kinase (Toyobo). Restriction enzymes and modifying enzymes were purchased from TaKaRa Bio (Ohtsu, Japan) or Toyobo. DNA fragments after agarose gel electrophoresis were recovered and purified with GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom). Plasmid DNA was isolated using QIAGEN (Hilden, Germany) plasmid kits. DNA sequencing was performed using a BigDye Terminator Cycle Sequencing kit, version 3.0, and a model 3100 capillary sequencer (Applied BioSystems, Foster City, CA).

**Construction of disruption vectors.** Construction of marker cassettes and several disruption vectors was carried out as described below. A pyrF marker cassette was constructed by amplification of the putative pyrF promoter-pyrF gene fragment in the plasmid pUD (21) with primers PJPRO-R2/PJPRYR-F2, each containing a Pvull restriction site, followed by insertion into pUC118 at the HincII site. From the resulting plasmid, pUD2, the Pvull restriction fragment (763 bp) for use as the marker cassette. Construction of the trpE marker cassette, the putative pyrF promoter-trpE gene fusion flanked by PavII sites (1,423 bp), has been described previously (22).

Four DNA fragments containing the respective target gene together with its flanking regions (about 1,000 bp, except for 732 bp for the 5′-flanking region of pyrF) were amplified from *T. kodakaraensis* KOD1 genomic DNA using the primer sets PPyR-R/PPYR-F, PTPR-R/PTPR-F, PHISD-R/PHISD-F, and PLYSV-R/PLYSV-F for pUDPyrF, pUDPtpE, pUDPHisD, and pUDPlysV, respectively. Each amplified DNA fragment was subcloned into pUC118 at the PvuII restriction sites. Cloned fragments were sequenced to confirm that the respective target gene was not altered.

Four DNA fragments containing the respective target gene together with its flanking regions (about 1,000 bp, except for 732 bp for the 5′-flanking region of pyrF) were amplified from *T. kodakaraensis* genomic DNA using the primer sets PPyR-R/PPYR-F, PTPR-R/PTPR-F, PHISD-R/PHISD-F, and PLYSV-R/Plysv-F, respectively, and the resulting DNA fragments were designated L-PyrF, L-TrpE, L-HisD, and L-LysV, respectively. L-PyrF (5,010 bp, markerless) (Fig. 1) was obtained by self-ligation of L-PyrF after 5′ purification and digestion with BglII and HincII (Fig. 1). L-TrpE (5,295 bp, pyrF marker) (Fig. 1), L-HisD (6,585 bp, trpE marker) (Fig. 1), and L-LysV (6,585 bp, trpE marker) were obtained by self-ligation of the corresponding marker cassette with the fragments L-TrpE, L-HisD, and L-LysV, respectively. Each amplified DNA fragment was subcloned into pUC118 at the HincII site. From the resulting plasmid, pUD2, the Pvull restriction fragment (763 bp) for use as the marker cassette. Construction of the trpE marker cassette, the putative pyrF promoter-trpE gene fusion flanked by PavII sites (1,423 bp), has been described previously (22).

Three vectors for disruption of pyrF, trpE, hisD, and lysV genes in *T. kodakaraensis* were constructed as follows. Four DNA fragments containing the respective target gene together with its flanking regions (about 1,000 bp, except for 732 bp for the 5′-flanking region of pyrF) were amplified from *T. kodakaraensis* genomic DNA using the primer sets PPyR-R/PPYR-F, PTPR-R/PTPR-F, PHISD-R/PHISD-F, and PLYSV-R/Plysv-F, respectively, and the resulting DNA fragments were designated L-PyrF, L-TrpE, L-HisD, and L-LysV, respectively. L-PyrF (5,010 bp, markerless) (Fig. 1) was obtained by self-ligation of L-PyrF after 5′ purification and digestion with BglII and HincII (Fig. 1). L-TrpE (5,295 bp, pyrF marker) (Fig. 1), L-HisD (6,585 bp, trpE marker) (Fig. 1), and L-LysV (6,585 bp, trpE marker) were obtained by self-ligation of the corresponding marker cassette with the fragments L-TrpE, L-HisD, and L-LysV, respectively. Each amplified DNA fragment was subcloned into pUC118 at the HincII site. From the resulting plasmid, pUD2, the Pvull restriction fragment (763 bp) for use as the marker cassette. Construction of the trpE marker cassette, the putative pyrF promoter-trpE gene fusion flanked by PavII sites (1,423 bp), has been described previously (22).

**TABLE 1. Strains and plasmids used in this study**

<table>
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<th>Strain or plasmid</th>
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<th>Source or reference</th>
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<td></td>
</tr>
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<td>Stratagene (La Jolla, CA)</td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
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<td>ΔhisD::3′ region of hisD-pyrF</td>
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</tr>
<tr>
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<td>ΔpyrF ΔtrpE::3′</td>
<td>This study</td>
</tr>
<tr>
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<td>ΔpyrF ΔtrpE::3′</td>
<td>This study</td>
</tr>
<tr>
<td>T. kodakaraensis</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>Amp′ general cloning vector</td>
<td>Takara Bio (Ohtsu, Japan)</td>
</tr>
<tr>
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</tr>
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<td>pUC118 derivative; ΔtrpE::pyrF</td>
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<td>pUC118 derivative; ΔhisD::trpE</td>
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<td>pUDPlysV</td>
<td>pUC118 derivative; ΔlysV::trpE</td>
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<td>This study</td>
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<td>pYES2</td>
<td>General expression vector for Saccharomyces cerevisiae</td>
<td>Invitrogen (Carlsbad, CA)</td>
</tr>
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<td>pUDPHisDPOP</td>
<td>pUC118 derivative; ΔhisD::3′-pyrF-2′-µ′</td>
<td>This study</td>
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FIG. 1. Schematic diagram of targeted disruption of pyrF, trpE, and hisD in T. kodakaraensis KOD1, KU216, and KW128 using pUDPyrF, pUDTrpE, and pUDHisD, respectively. Relevant regions of the chromosome are illustrated for (from the top) strains KOD1, KU216, KW128, and KH3. The positions of primer sets used for analyses of targeted disruption of pyrF (CHDPYR-F/CHDPYR-R, closed arrowheads), trpE (CHDTRP-R/CHDTRP-F, open arrowheads), and hisD (CHDHID-R/CHDHID-F, closed arrows) are indicated. The gray, closed, open, and striped boldface bars indicate each region spanned by the pyrF, trpE, pyrF upstream, and trpE downstream probes used in Southern blot analyses, respectively. The gray, closed arrow indicates the putative promoter region of the operon containing pyrF. Black stars indicate regions of target genes that were left intact in order to avoid disturbing nearby genes as described in Materials and Methods.

Gene name abbreviations: Hypo, hypothetical gene; ino1, myo-inositol-1-phosphate synthase; PRb, predicted RNA-binding protein; tagD, cytidylyltransferase. Restriction site abbreviations: Ap, ApaI; Hc, HincII; Hd, HindIII.
To examine the effect of the lengths of the homologous regions on recombination efficiency at the hisD locus, three linear DNA fragments, DH-L1, DH-L2, and DH-L3, with 1,000 bp, 500 bp, and 100 bp of homologous regions, were amplified from pUDHisD using the primer sets HD-1000R/HD-1000F, HD-500R/HD-500F, and HD-100R/HD-100F, respectively. The fragments DH-L2 and DH-L3 were inserted into pUC118 at the HincII site to obtain the circular disursion plasmids pUDHisD2 and pUDHisD3 harboring 500 bp and 100 bp of homologous regions, respectively.

**Construction of pop-out vectors for repeated utilization of pyrF marker.** Two types of disruption vectors with tandem repeat regions flanking the pyrF gene were constructed for gene disruption and subsequent excision of the pyrF marker by pop-out recombination. Type I vectors harbor an additional copy of the 3'-flanking region of the respective target genes positioned upstream of the pyrF marker. After the first double-crossover homologous recombination, this additional region leads to a structure in which the marker gene is flanked by two tandem homologous regions. Approximately 0.85 kb of the 3'-flanking regions of trpE, and hisD genes was amplified from T. kodakaraensis genomic DNA using primers DPOPT-R/DPOPT-F and DPOPH-R/DPOPH-F, respectively. The DPOPT-R and DPOPH-R primers and the DPOPT-F and DPOPH-F primers contain SphI and PstI sites, respectively, outside the annealing sequences. Each amplified DNA fragment was digested with SphI and PstI and inserted into pUD2 at the corresponding sites. From each resulting plasmid, the pyrF marker cassette adjacent to the 3' region of the target gene was excised by HindIII and XbaI digestion, blunted by Blunting High (Toyobo), and then ligated with L-Trp and L-HisD, respectively. The plasmids harboring the duplicated 3' regions of the pyrF marker adjacent to the L-Trp and L-HisD regions were selected to obtain pUDTPOP (see Fig. 4A) and pUDHPPOP, respectively.

In type II vectors, we applied a nucleotide sequence derived from exogenous DNA, a portion of the 2a region (designated as 2a') in the yeast-E. coli shuttle primer pyES2, as the tandem repeat region. Two DNA fragments, 0.35 kb of nucleotide sequences in the 2a region in pyES2, were amplified using primer sets POPCR-R/POPCR-F, containing XbaI and EcoRI-SmaI sites, respectively, and HD-500R/HD-500F, containing SphI and PstI sites, respectively. Each fragment contained SphI and PstI sites, respectively, outside the annealing sequences. The resulting plasmid harboring the pyrF marker flanked by tandem repeats of 2a' regions named pUCMP. The 2a'-pyrF-2a' region can be excised from pUCMP by SmaI as an universal marker cassette for pop-out recombination of the pyrF marker and was used to construct pUTDPOP for trpE disruption (see Fig. 4B) and pUDHPPOP for hisD disruption by ligation with the fragments L-Trp and L-HisD, respectively.

**Transformation of T. kodakaraensis.** All steps involved in the genetic manipulation of T. kodakaraensis were performed under anaerobic conditions with the exception of centrifugation for cell harvesting. In the case of transformation utilizing the pyrF marker, the CaCl2 method was applied as described previously (21). For repeated utilization of the pyrF marker for disruption (see Fig. 1) were failed to obtain a desired counterselectable marker. The transformants were selected on a plate medium containing 5-FOA, tryptophan, and pyrF gene. We previously isolated a uracil-auxotrophic PyrF-deficient mutant, KU216, of T. kodakaraensis by UV mutagenesis and then constructed a gene targeting system utilizing this strain and the pyrF gene as a host and a selectable marker, respectively (21). However, the possibility remained that various mutations induced by UV irradiation were present in other genes on the KU25 chromosome. In addition, since the pyrF mutation in KU25 was only a 1-bp deletion, unintended recombination between the marker pyrF gene and the mutated allele on the chromosome could always occur, becoming problematic when the homologous regions of the target gene were shortened to 500 bp (21). To solve these problems, we constructed a new host strain whose endogenous pyrF gene was specifically and almost completely deleted by homologous recombination, as illustrated in Fig. 1. T. kodakaraensis KOD1 was transformed with pUDPyrF as described in Materials and Methods, and several candidates for pyrF deletion were positively selected with 5-FOA. One of the isolates was designated KU216 and was confirmed to require uracil for growth in ASW-AA liquid medium. The genotype of KU216 was determined by PCR using the primer pair CHDPYR-R/CHDPYR-F and Southern blot analyses using a probe of the pyrF upstream region. Both analyses demonstrated that the target region in KU216 was shorter than the native locus in wild-type KOD1 as expected (Fig. 2A and C)]. Moreover, the sole signal detected in the Southern blot analysis of KU216 denoted the occurrence of nonhomologous recombination. Sequencing analysis of the target region was also consistent with the ΔpyrF genotype formed by double-crossover homologous recombination. We further confirmed that the pyrE gene in KU216, whose inactivation is another

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**RESULTS**

**Construction of the pyrF deletion mutant.** We previously isolated a uracil-auxotrophic PyrF-deficient mutant, KU216, of T. kodakaraensis by UV mutagenesis and then constructed a gene targeting system utilizing this strain and the pyrF gene as a host and a selectable marker, respectively (21). However, the possibility remained that various mutations induced by UV irradiation were present in other genes on the KU25 chromosome. In addition, since the pyrF mutation in KU25 was only a 1-bp deletion, unintended recombination between the marker pyrF gene and the mutated allele on the chromosome could always occur, becoming problematic when the homologous regions of the target gene were shortened to 500 bp (21). To solve these problems, we constructed a new host strain whose endogenous pyrF gene was specifically and almost completely deleted by homologous recombination, as illustrated in Fig. 1. T. kodakaraensis KOD1 was transformed with pUDPyrF as described in Materials and Methods, and several candidates for pyrF deletion were positively selected with 5-FOA. One of the isolates was designated KU216 and was confirmed to require uracil for growth in ASW-AA liquid medium. The genotype of KU216 was determined by PCR using the primer pair CHDPYR-R/CHDPYR-F and Southern blot analyses using a probe of the pyrF upstream region. Both analyses demonstrated that the target region in KU216 was shorter than the native locus in wild-type KOD1 as expected (Fig. 2A and C). Moreover, the sole signal detected in the Southern blot analysis of KU216 denoted the occurrence of nonhomologous recombination. Sequencing analysis of the target region was also consistent with the ΔpyrF genotype formed by double-crossover homologous recombination. We further confirmed that the pyrE gene in KU216, whose inactivation is another
factor responsible for uracil auxotrophy and 5-FOA resistance, remained intact. These results indicated that the uracil auxotrophy of KU216 was caused solely by the deficiency of pyrF.

Development of an improved transformation system using a trpE marker. It has been found that pyrF-deficient mutant KU25 (with a point mutation in pyrF) could grow on uracil-free plate medium despite its uracil auxotrophy in the liquid medium, probably due to some pyrimidine-related compounds in the solidifier (21). KU216 (ΔpyrF) also showed the same property. In the use of uracil auxotrophs of this organism as host strains, this property brings about a complicated procedure for isolation of prototrophs after transformation; two rounds of cultivation in uracil-free liquid medium were necessary prior to colony isolation, hampering calculation of transformation efficiency. We therefore attempted to utilize a trpE deletion mutant and the trpE gene as a host strain and a selectable marker, respectively, because the previously constructed trpE deletion mutant, KW4, showed strict tryptophan auxotrophy both in liquid medium and on plate medium (21). As shown in Fig. 1, almost the entire coding region of trpE on the chromosome of KU216 was replaced by the pyrF marker with the CaCl2 method as described for the construction of KW4 (21). Colony PCR analysis after the final plate culture suggested that all of the three uracil prototrophs examined were trpE deletion mutants. One of the isolates was designated KW128, and the expected genotype (ΔpyrF ΔtrpE::pyrF) was confirmed by PCR using CHDTRP-R/CHDTRP-F (Fig. 2A); Southern blot using pyrF, trpE downstream, and trpE probes (Fig. 3B, C, and D, respectively); and sequencing analyses.

We then investigated the capability of strain KW128 and the trpE gene as a host-marker system for transformation of T. kodakaraensis. For this purpose, the hisD gene, encoding histidinol dehydrogenase within a probable histidine biosynthesis operon (his operon) in T. kodakaraensis, was chosen as a target gene to be disrupted. KW128 was transformed with the hisD disruption vector pUDHisD harboring a trpE marker cassette (PpyrF::trpE). After treatment with the plasmid DNA, cells were incubated in rich medium (modified ASW-YT) at 85°C for 2 h, aiming to promote homologous recombination before cultivation on a selective plate medium. The washed cells after outgrowth were directly inoculated onto tryptophan-deficient ASW-AAW/H11002 plate medium. As a result, we could obtain tryptophan prototrophs with a transformation efficiency of approx-
Table 2. Transformation of *T. kodakaraensis* using various DNAs<sup>a</sup>

<table>
<thead>
<tr>
<th>DNA added to the cells</th>
<th>DNA form</th>
<th>Length of homologous regions (bp)</th>
<th>DNA structure</th>
<th>Transformation efficiency (Trp&lt;sup&gt;+&lt;/sup&gt; prototroph/μg DNA/4 × 10&lt;sup&gt;8&lt;/sup&gt; cells)</th>
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<sup>a</sup> Transformation was performed without CaCl<sub>2</sub>, and outgrowth was carried out for 2 h. The order of magnitude of transformation efficiency is the mean obtained from three independent experiments. The circular plasmids were prepared using an E. coli DH5α strain harboring a methylation system, while the linear DNAs were prepared by PCR without methylation.

Imately 1 × 10<sup>7</sup>/μg DNA, while a control experiment without the exogenous DNA gave no tryptophan prototrophs. Colony PCR analysis suggested that 7 out of 10 tryptophan prototrophs examined were hisD deletion mutants. The genotype of one of the isolates, designated KH3, was confirmed to be as expected (ΔpyrF ΔtrpE::ΔhisD ΔtrpE) by PCR using the primer pair CHDHID-R/CHDHID-F (Fig. 2B). Southern blot using the trpE probe (Fig. 3D), and sequencing analyses. Strain KH3 displayed strict histidine auxotrophy with an inability to grow in ASW-AA liquid medium without histidine (data not shown), indicating that the his operon is actually involved in histidine biosynthesis in *T. kodakaraensis*. These results demonstrated that the combination of the new host, KW128, and the trpE gene was applicable to the transformation of *T. kodakaraensis*. This also indicates that the genes downstream of trpE in the trp operon are functioning and that our disruption of trpE did not lead to notable polar effects in this operon. This system enables us to select transformants by a simple procedure without repeated cultivation in liquid medium and to evaluate transformation efficiencies, overcoming the practical disadvantages in the previous system using pyrF as a selectable marker.

We performed further PCR analyses to evaluate the genotype of one of the three Trp prototrophs that were not hisD deletion mutants. PCR analyses indicated the occurrence of single-crossover recombination within the homologous region downstream of hisD, leading to Trp prototrophy. Interestingly, PCR analyses also implied the presence of the original plasmid, pUDHisD, suggesting a spontaneous popping out of the plasmid from the chromosome in these cells.

**Effects of transformation conditions and length of homologous regions on transformation efficiency.** With the new system using the trpE marker, we investigated the effects of CaCl<sub>2</sub> treatment on the transformation. In the course of the hisD disruption, cells of KW128 were resuspended in transformation buffer containing 80 mM CaCl<sub>2</sub> or 0.8 × ASW, treated with pUDHisD, and directly inoculated onto ASW-AAW<sup>−</sup> plate medium. In these experiments, outgrowth of the transformed cells was omitted to avoid precipitation that was probably formed between calcium cations in the transformation buffer and phosphate groups in the outgrowth medium. The numbers of colonies with tryptophan prototrophy grown on the selective plate medium were then counted. Regardless of the presence or absence of CaCl<sub>2</sub> treatment, transformation efficiencies were within a similar level of approximately 2 × 10<sup>2</sup>/μg DNA, indicating that the CaCl<sub>2</sub> treatment did not have an apparent effect on transformation efficiency. The results also imply that the 2-h outgrowth procedure does not significantly enhance the transformation efficiency.

Next, we investigated the effects of the length of homologous regions that flank the target gene. In the previous gene disruption system using KU25 as the host strain, the use of a linear DNA harboring homologous regions of 500 bp resulted in predominant homologous recombination between the pyrF marker in the exogenous DNA and the mutated allele on the host chromosome instead of the intended recombination (21). In contrast, the new host, KW128, allowed us to evaluate more precisely the effects of length of homologous regions without recombination at the marker (trpE) locus, as the allelic on the host chromosome had been almost entirely removed. KW128 was transformed with circular and linear DNAs with 1,000, 500, and 100 bp of homologous regions for the disruption of hisD. As shown in Table 2, DNAs with 1,000-bp homologous regions led to successful homologous recombination regardless of the DNA form. Homologous regions of 500 bp were also sufficient to bring about homologous recombination, although the efficiencies became lower (10<sup>1</sup>/μg DNA). Both circular and linear DNAs with 100-bp homologous regions gave no tryptophan prototrophs, suggesting that this length was too short to promote effective homologous recombination in this organism under the conditions examined.

**Repeated utilization of pyrF marker through pop-out recombination.** The use of a single selectable marker often limits genetic modification of the host chromosome to one trial, as the marker remains in the recipient cells, and thus, it can no longer be used for subsequent transformation. Therefore, in addition to developing useful selectable markers, we set out to develop a more versatile system enabling multiple cycles of transformation. This would expand our capabilities in elucidating gene function, enabling us to perform multiple gene dis-
ruptions or one gene disruption followed by complementation with an exogenous gene. Based on a strategy described previously for yeast (3), we chose the counterselectable marker pyrF. In this procedure, the pyrF marker that is inserted into the chromosome after the first transformation would undergo excision through pop-out recombination that occurs between tandem repeat regions located on both sides of the marker gene. The resulting markerless transformant can be isolated by positive selection using 5-FOA.

Here, two kinds of vector constructs were adopted for promotion of the pop-out event. In the type I vector, a 3’-flanking region of the target gene was applied as the region repeated in tandem. For gene disruption of trpE and subsequent excision of the pyrF marker, a vector, pUDTPOP, was constructed by inserting a fusion of the trpE 3’ region and the pyrF marker cassette between the homologous regions designed for trpE disruption, as shown in Fig. 4A. In this plasmid, the pyrF marker is directly sandwiched by endogenous 0.8-kbp sequences of the trpE 3’ region. KU216 was transformed with pUDTPOP, and one pyrF+ strain with an intermediary genotype (ΔtrpE::3’-trpE-pyrF), KuW1, could be isolated. PCR analysis of the trpE locus of KuW1 was consistent with replacement of trpE by pyrF along with the additional trpE 3’-flanking region located upstream of the marker (Fig. 5A, main band in lane 2). Subsequent cultivation of KuW1 on the plate medium containing 5-FOA resulted in the generation of 5-FOA-resistant colonies with a frequency of $3 \times 10^{-4}$. One of the several strains isolated was designated KUW1, and its genotype (ΔpyrF ΔtrpE) was analyzed. First, PCR analysis of the trpE locus led to the amplification of a shorter DNA fragment (Fig. 5A, lane 3). Furthermore, a signal corresponding to a shorter fragment with the trpE downstream probe was detected (Fig. 3C), along with the disappearance of a signal with the pyrF probe (Fig. 3B), in Southern blot analyses. These results clearly indicated pop-out recombination between the tandem repeats. Sequencing analysis also confirmed the intended excision of the pyrF marker. Interestingly, minor amplification of a fragment corresponding to the chromosome structure formed after the pop-out event was also detected with total DNA isolated from KuW1 cells after a few cultivations under nonselective conditions (absence of 5-FOA) (Fig. 5A, lane 2). Although we could not quantify the efficiency of the pop-out event, the results suggest that the molecular construct used here allows the pop-out recombination to occur at efficiencies that can be detected even under nonselective conditions. Furthermore, KuW1 was transformed with pUDHPOP, a vector designed for disruption of hisD and subsequent pop-out excision of pyrF using the same strategy. As a result of PCR analyses of the pyrF, trpE, and hisD loci (Fig. 5B, lanes 3, 6, and 9), we confirmed that the final isolate, KUWH1, was a triple mutant with the expected genotype (ΔpyrF ΔtrpE ΔhisD). These results demonstrated that by using type I pop-out vectors, we can repeatedly utilize the pyrF marker for multiple gene disruptions.

In another strategy, using type II vectors, an exogenous DNA sequence was applied for the tandem repeats, as in a strategy described previously (3) (Fig. 4B). We adopted a 0.35-kbp sequence derived from the 2μ region in the yeast plasmid pYES2 as the exogenous sequence, designated as 2μ′, and constructed a new cassette consisting of the pyrF marker flanked on both sides by the 2μ′ regions. The 2μ′-pyrF-2μ′ fusion was then inserted between the 5′- and 3′-flanking regions of trpE for homologous recombination, and KU216 was transformed with the resulting vector, pUDTPOPC. As seen in the case of KuW1, PCR analysis of a pyrF+ intermediate strain, KuWE1, exhibited a major band corresponding to the intended replacement of trpE by the 2μ′-pyrF-2μ′ cassette, along with a faint band indicating subsequent pop-out recombination without 5-FOA (Fig. 5A, lane 4). By positive selection with 5-FOA, we could obtain KUW1 (ΔpyrF ΔtrpE::2μ′ ΔhisD::2μ′′) harboring one copy of the 2μ′ region in the place of trpE. In the case of this experiment, the frequency of the generation of 5-FOA-resistant colonies was $8 \times 10^{-7}$. Further transformation with a similar type II vector, pUDHPOPC, for hisD disruption followed by the pop-out event gave the strain KUWHe1 (ΔpyrF ΔtrpE::2μ′ ΔhisD::2μ′′). Genotypes of these strains were confirmed by PCR analyses of the pyrF, trpE, and hisD loci (Fig. 5C, lanes 2 and 3, 5 and 6, and 8 and 9). In conclusion, the exogenous sequence derived from yeast plasmid could also be applied as tandem repeats for repeated utilization of the pyrF marker.

Utilization of two genetic markers, pyrF and trpE, in a double deletion mutant, KUW1. As described above, we created the double deletion mutant KUW1, in which pyrF and trpE genes were almost entirely removed (ΔpyrF ΔtrpE), and confirmed that the pyrF marker was applicable for transformation of KUW1 in the course of constructing strain KUWH1 (ΔpyrF ΔtrpE ΔhisD::3′-hisD-pyrF). Using the trpE marker, we further constructed the plasmid pUDLysV for disruption of the lysV gene in a predicted lysine biosynthesis operon and performed transformation experiments on KUW1 and KuWH1. The lysV deletion mutants were successfully obtained from these host strains by using trpE as a selectable marker (data not shown), indicating the usefulness of KUW1 as a host strain in which both independent and sequential utilization of these two markers are possible.

DISCUSSION

This study reports the development of improved transformation systems for the hyperthermophilic archaeon T. kodakaraensis. Strains KU216 (ΔpyrF), KW128 (ΔpyrF ΔtrpE::pyrF), and KUW1 (ΔpyrF ΔtrpE) were constructed as hosts by directed gene deletion through homologous recombination. Therefore, there is no longer a need to consider unknown mutations caused by random mutagenesis. Furthermore, undesirable recombination between marker genes and the chromosome alleles will no longer be problematic. KU216 was used as a basic strain for the construction of further mutants of T. kodakaraensis. The trpE deletant KW128 and the trpE gene have already been applied as a host and a selectable marker for disruption of tyrG (6), impG, and fbpG genes (22), demonstrating the usefulness of this system for various gene disruptions.

The use of the trpE marker made it possible not only to isolate transformants by a simple selection procedure based on the strict tryptophan auxotrophy of ΔtrpE strains but also to score the transformation efficiency. The disruption of the hisD gene in KW128 was performed using the trpE marker with an efficiency of approximately $10^{8}$/μg DNA. In addition to our previous finding that transformation of T. kodakaraensis could
occur without CaCl$_2$ treatment of the recipient cells (21), it was further clarified here that the CaCl$_2$ treatment did not have an apparent effect on the transformation efficiency. This natural competency of *T. kodakaraensis* was a property quite distinct from that of the closely related archaeon *Pyrococcus abyssi*, of which transformation with a shuttle vector required the use of a polyethylene glycol (PEG)-mediated spheroplast method for uptake of extracellular DNA ($10^2$ to $10^3$ pg DNA) (14). Gene disruption by double-crossover homologous recombination has recently been reported in *S. solfataricus* using electroporation; however, the efficiencies and the effects of transformation conditions were not documented (29). When compared to the efficiencies of natural transformation of mesophilic archaea through single-crossover homologous recombination, the efficiency for *T. kodakaraensis* was larger than the $10^3$ pg DNA reported for *Methanococcus voltae* PS (17) and similar to the levels of $10^5$ to $10^6$ pg DNA observed for *Methanococcus maripaludis* (20, 27). However, it was lower than those for *M. voltae* by an electroporation-mediated procedure ($10^5$ pg DNA) (17) and for *M. maripaludis* by a PEG-mediated procedure ($10^5$ pg DNA) (27). In most cases in the transformation of mesophilic archaea with autonomously replicating plasmids, much higher efficiencies ($10^5$ to $10^8$ pg DNA) have been reported by using PEG- or liposome-mediated methods (15, 23). In general, transformation through homologous recombination is supposed to be affected by DNA uptake efficiency, intracellular stability of the exogenous DNA, and recombination efficiency in host cells. Although it has not been clarified which factor is mainly responsible for the transformation efficiency in *T. kodakaraensis*, adoption of PEG, liposome, or electroporation methodology in the transformation procedure may further enhance the efficiency. Indeed, in the transformation of *M. voltae*...
and *M. maripaludis* with integration vectors, dramatic improvements of efficiencies over those of natural transformation were achieved by electroporation- and PEG-mediated transformation, respectively.

In *T. kodakaraensis*, efficient recombination at the target locus was possible with homologous regions of 1,000 bp. Unlike the case of *M. voltae* (17), the type of added DNA, circular or linear, did not seriously affect the transformation efficiency. This result may reflect the different DNA uptake and restriction machineries between these organisms. Reducing the length of the homologous regions by half (500 bp) still led to recombination but with lower efficiencies, whereas 100-bp homologous regions seem to be too short. Under our conditions reported here, homologous regions longer than at least 100 bp appear to be necessary for effective double-crossover recombination in *T. kodakaraensis*. This fact hampers our use of the far-easier PCR-based molecular construction reported in several yeast strains, where constructs with homologous regions of only about 50 bp are applicable (7, 11, 13). However, there is the possibility that higher intracellular concentration of DNAs effective for formation of recombination complexes, which can be achieved by enhancement of uptake efficiency and/or stability of exogenous DNAs, will allow recombination with extremely shortened homologous regions.

In addition to the new host-marker systems, we demonstrated the repeated utilization of the single *pyrF* marker through pop-out recombination between tandem repeats flanking the marker genes, followed by positive selection of the *pyrF*-excised strains with 5-FOA. Both an endogenous 3′ region of the target (type I vectors) and an exogenous 2µ region from a yeast plasmid (type II vectors) could be applied, and the pop-out recombination was found to occur in *T. kodakaraensis* even in the absence of 5-FOA. The type II vectors, using the 2µ′-*pyrF*-2µ′ fusion, can be applied as a universal cassette for any gene disruption and subsequent reuse of the marker, saving us several steps to tailor tandem repeat regions for each target gene. In this strategy, one copy of the exogenous sequence will consequently remain on the host chromosome with each gene disruption. Multiple copies of the sequence after repeated utilization may lead to instability of the chromosome caused by removal of regions between the exogenous sequences, especially when they are introduced at nearby position from each other in the same orientation on the chromosome. However, the facile occurrence of pop-out recombi-
nation in *T. kodakaraensis* might be applicable to promote artificial large-scale rearrangement of the genome, for example, for creation of a minimum genome for hyperthermophilic life. Using the pop-out strategy, we have constructed a double deletion mutant, KUW1 (ΔpyrF ΔtrpE), and a triple deletion mutant, KUWH1 (ΔpyrF ΔtrpE ΔhisD), using type I vectors. The strain KUW1 is, as demonstrated above, useful for further multiple genetic manipulations of *T. kodakaraensis* with two kinds of markers, trpE and the repeatedly utilizable pyrF. Along with the recent complete genome analysis of *T. kodakaraensis* (10), the multiple-transformation system developed in this study is expected to expand the versatility of using this archaeon as a model organism for research on hyperthermophilic archaea.

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