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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 archaeon *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 (Δ*pyrF*), 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 (Δ*pyrF* Δ*trpE*::*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 (Δ*pyrF* Δ*trpE*), 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 (Δ*pyrF* Δ*trpE* Δ*hisD*), could also be constructed. The transformation systems developed here now provide the means for extensive genetic studies in this hyperthermophilic archaeon.

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 archaeon 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 archaeon *Sulfolobus solfataricus* using a *lacS*

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
E. coli		
DH5α	*supE44* Δ*lacU169* (Φ80 *lacZ* Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relAI*	Stratagene (La Jolla, CA)
T. kodakaraensis		
KOD1	Wild type	5, 16
KU216	Δ*pyrF*	This study
KW128	KU216 Δ*trpE*::*pyrF*	This study
KH3	KW128 Δ*hisD*::*trpE*	This study
KuW1	KU216 Δ*trpE*::3′ region of *trpE-pyrF*	This study
KUW1	Δ*pyrF* Δ*trpE*	This study
KuWH1	KUW1 Δ*hisD*::3′ region of *hisD-pyrF*	This study
KUWH1	Δ*pyrF* Δ*trpE* Δ*hisD*	This study
KuWc1	KU216 Δ*trpE*::2μ′=*pyrF*-2μ′	This study
KUWc1	Δ*pyrF* Δ*trpE*::2μ′	This study
KuWcHc1	KUWc1 Δ*hisD*::2μ′-*pyrF*-2μ′	This study
KUWcHc1	Δ*pyrF* Δ*trpE*::2μ′ Δ*hisD*::2μ′	This study
Plasmids		
pUC118	Amp^r general cloning vector	Takara Bio (Ohtsu, Japan)
pUD2	pUC118 derivative, *pyrF* marker cassette (P_{pyrF}::*pyrF*)	This study
pUMT2	pUC118 derivative; *trpE* marker cassette (P_{pyrF}::*trpE*)	22
pUCMP	pUC118 derivative; 2μ′-*pyrF*-2μ′	This study
pUDPyrF	pUC118 derivative; Δ*pyrF*	This study
pUDTrpE	pUC118 derivative; Δ*trpE*::*pyrF*	This study
pUDHisD	pUC118 derivative; Δ*hisD*::*trpE*	This study
pUDLysV	pUC118 derivative; Δ*lysV*::*trpE*	This study
pUDTPOP	pUC118 derivative; Δ*trpE*::3′ region of *trpE-pyrF*	This study
pUDHPOP	pUC118 derivative; Δ*hisD*::3′ region of *hisD-pyrF*	This study
pYES2	General expression vector for *Saccharomyces cerevisiae*	Invitrogen (Carlsbad, CA)
pUDTPOPC	pUC118 derivative; Δ*trpE*::2μ′-*pyrF*-2μ′	This study
pUDHPOPC	pUC118 derivative; Δ*hisD*::2μ′-*pyrF*-2μ′	This study

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 (ASW-YT) or a synthetic medium (ASW-AA) (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 by Sambrook and Russell (19). Genomic DNA of *T. kodakaraensis* was isolated as described previously (21). PCR was carried out using KOD -Plus- (Toyobo, Osaka, Japan) as a DNA polymerase, and sequences of 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 fusion in the plasmid pUD (21) with primers PJPRO-R2/PJPYR-F2, each containing a PvuII restriction site, followed by insertion into pUC118 at the HincII site. From the resulting plasmid, pUD2, the PvuII 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 PvuII sites (1,423 bp), has been described previously (22).

Four vectors for disruption of *pyrF*, *trpE*, *hisD*, and *lysV* genes in *T. kodakaraensis* through double-crossover homologous recombination (pUDPyrF, pUDTrpE, pUDHisD, and pUDLysV, respectively) 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* KOD1 genomic DNA using the primer sets PPYR-R/PPYR-F, PTRP-R/PTRP-F, PHISD-R/PHISD-F, and PLYSV-R/PLYSV-F for pUDPyrF, pUDTrpE, pUDHisD, and pUDLysV, respectively. Each amplified DNA fragment was subcloned into pUC118 at the HincII site. The flanking regions of the target gene and the plasmid backbone, excluding the target gene, were then amplified from the respective plasmids using primers PDPYR-R/PDPYR-F, PDTRP-R/PDTRP-F, PDHISD-R/PDHISD-F, and PDLYSV-R/PDLYSV-F, respectively, and the resulting DNA fragments were designated L-PyrF, L-TrpE, L-HisD, and L-LysV, respectively. pUDPyrF (5,010 bp, markerless) (Fig. 1) was obtained by self-ligation of L-PyrF after 5′ phosphorylation of both ends. pUDTrpE (5,925 bp, *pyrF* marker) (Fig. 1), pUDHisD (6,585 bp, *trpE* marker) (Fig. 1), and pUDLysV (6,585 bp, *trpE* marker) were constructed by ligation of the corresponding marker cassette with the fragments L-TrpE, L-HisD, and L-LysV, respectively, with the markers oriented in the same direction as those of the target genes. All plasmids were designed so that upstream and downstream genes, which in some cases overlapped the target gene, were left intact. We also took care not to remove the ribosome-binding sites of downstream genes in order to avoid disturbing their translation. Therefore, in the present study, 5′ regions of *trpE* (19 bp) and *hisD*

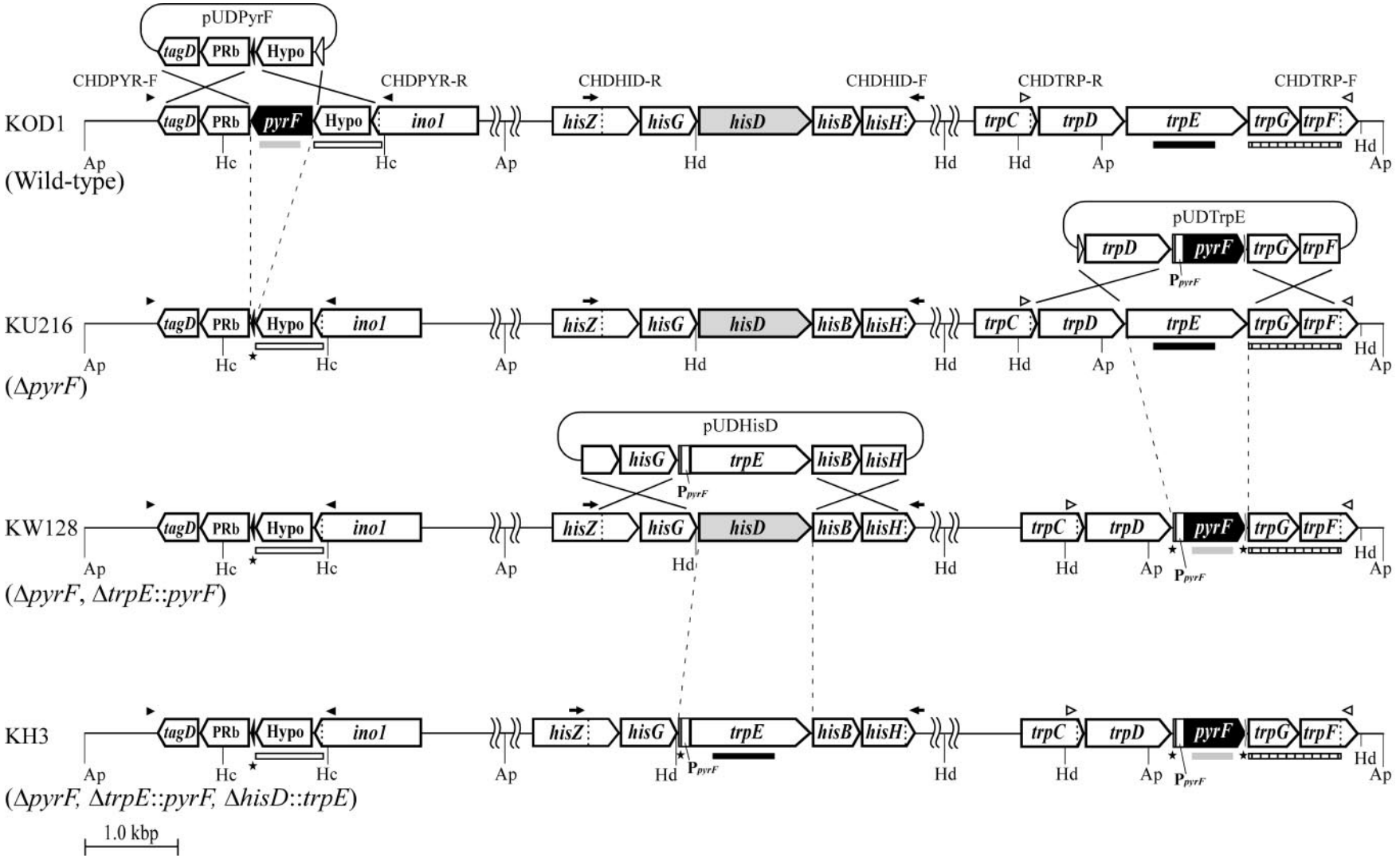


FIG. 1. Schematic diagram of targeted disruption of *pyrF*, *trpE*, and *hisD* in *T. kodakaraensis* KOD1, KU216, and KW128 using pUDPyrF, pUDTrpE, and pUDHisD, respectively. Relevant regions of the chromosome are illustrated for (from the top) strains KOD1, KU216, KW128, and KH3. The positions of primer 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. P_{pyrF} 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.

(14 bp) and 3′ regions of *pyrF* (21 bp) and *trpE* (8 bp) were preserved after recombination.

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 disruption 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 0.8 kbp 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-TrpE and L-HisD, respectively. The plasmids harboring the duplicated 3′ regions of *trpE* and *hisD* in the same direction were selected to obtain pUDTPOP (see Fig. 4A) and pUDHPOP, respectively.

In type II vectors, we applied a nucleotide sequence derived from exogenous DNA, a portion of the 2μ region (designated as 2μ′) in the yeast-*E. coli* shuttle vector pYES2, as the tandem repeat region. Two DNA fragments, 0.35 kbp of nucleotide sequences in the 2μ region in pYES2, were amplified using primer sets POPCR-R/POPCR-F, containing XbaI and EcoRI-SmaI sites, respectively, and POPCL-R/POPCL-F, containing SphI-SmaI and PstI sites, respectively. The former and latter DNA fragments were digested with XbaI/EcoRI and Sph1/PstI, respectively, and then inserted into the corresponding sites in pUD2. The resulting plasmid harboring the *pyrF* marker flanked by tandem repeats of 2μ′ regions was named pUCMP. The 2μ′-*pyrF*-2μ′ region can be excised from pUCMP by SmaI as a universal marker cassette for pop-out recombination of the *pyrF* marker and was used to construct pUDTPOPC for *trpE* disruption (see Fig. 4B) and pUDHPOPC for *hisD* disruption by ligation with the fragments L-TrpE 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 $CaCl_2$ method was applied as described previously (21). The $pyrF^+$ strains with uracil prototrophy were selected by cultivation twice in ASW-AA liquid medium prior to the plate cultivation due to the lack of strictness in uracil auxotrophy of the *pyrF* host on plate medium.

With *trpE* as a selectable marker, transformation was performed as follows. After cultivation of the host strain in ASW-YT liquid medium, approximately 4×10^8 cells at the late exponential phase were harvested (17,000 × *g*, 5 min), resuspended in 200 μl of 0.8× ASW medium, and kept on ice for 30 min. Three micrograms of DNA was added into the suspension, and the cells were incubated on ice for 1 h, followed by a heat shock at 85°C for 45 s and further incubation on ice for 10 min. A modified ASW-YT liquid medium (1.3 ml containing 2.0 ml/liter of polysulfide solution [21] instead of elemental sulfur) was added to the transformed cells, and the suspension was incubated at 85°C for 2 h for outgrowth. The cells were then harvested (17,000 × *g*, 5 min), resuspended in 200 μl of 0.8× ASW, and directly spread onto a selective synthetic ASW-AA plate not containing tryptophan (ASW-AAW$^-$). After cultivation for 5 to 8 days at 85°C, the transformants grown on the plate medium, tryptophan prototrophs, were isolated. Transformation efficiencies were determined by counting colony numbers of tryptophan prototrophs, and all values mentioned in the text are averages of the results of three independent experiments.

Genotypes of transformants obtained in this study were analyzed by PCR using primer sets that anneal outside of the homologous regions, by sequencing of the targeted regions, and/or by Southern hybridization (see below). When necessary, colonies of candidates grown on the selective plate medium were analyzed by colony PCR.

Positive selection of *pyrF*-deleted strains with 5-FOA. A *pyrF* deletion mutant, KU216, and several *pyrF* pop-out recombinants were positively selected on a synthetic plate medium supplemented with 0.75% 5-fluoroorotic acid (5-FOA) (Wako Pure Chemicals, Osaka, Japan) (8) and 10 μg/ml uracil (Kohjin, Tokyo, Japan) (ASW-AA-FU). In construction of the Δ*pyrF* strain KU216, *T. kodakaraensis* KOD1 was cultivated twice in a uracil-free ASW-AA medium to repress growth of spontaneous mutants deficient in *pyrF* and/or *pyrE* genes as well as to avoid carryover of uracil. The wild-type cells at the late exponential phase were harvested and transformed with pUDPyrF by the $CaCl_2$ procedure (21). The further transformed cells were grown in ASW-AA liquid medium supplemented with 10 μg/ml uracil to allow the generation of *pyrF* deletion mutants. Cultures were then spread onto ASW-AA-FU plate medium, and the 5-FOA-resistant mutants were isolated and analyzed.

For repeated utilization of the *pyrF* marker, excision of the counterselectable *pyrF* marker inserted within the target locus on the chromosome was achieved through pop-out recombination between tandem repeats flanking the *pyrF* marker. *T. kodakaraensis* KU216 was transformed with a pop-out vector, pUDTPOP or pUDTPOPC, by the $CaCl_2$ method. A desired $pyrF^+$ strain was isolated, and the cells grown in 20 ml of ASW-YT medium were inoculated onto ASW-AA-FU plate medium with an appropriate cell density. The resulting 5-FOA-resistant mutants were isolated and analyzed. Frequencies for generation of 5-FOA-resistant mutants were determined by counting colony numbers on ASW-AA-FU plate medium and colony numbers on that without 5-FOA, and all values mentioned in the text are averages of the results of three independent experiments. The double mutants with deletion of both *pyrF* and *trpE* were further transformed with pUDHPOP and pUDHPOPC for disruption of the *hisD* gene to demonstrate further repeated utilization of *pyrF* marker, according to the procedures mentioned above.

Hybridization analyses. Southern blot analyses were carried out with 5.0 μg of genomic DNAs digested with appropriate restriction enzymes, and the overall procedures were performed as described previously (22). The primer sets used for preparation of *pyrF* upstream, *pyrF*, and *trpE* downstream probes shown in Fig. 1 were PPYRF-R/PDPYRF-F, PROPYR-R/PROPYR-F, and PDTRP-R/PTRP-F, respectively. The *trpE* probe was prepared as described previously (22).

RESULTS

Construction of the *pyrF* deletion mutant. We previously isolated a uracil-auxotrophic PyrF-deficient mutant, KU25, 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 3A). Moreover, the sole signal detected in the Southern blot analysis of KU216 denied the occurrence of nonhomologous recombination. Sequencing analysis of the target region was also consistent with the Δ*pyrF* genotype formed by double-crossover homologous recombination. We further confirmed that the *pyrE* gene in KU216, whose inactivation is another

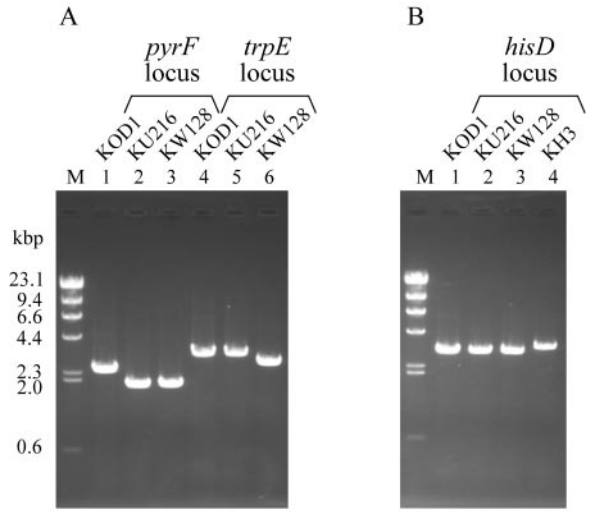


FIG. 2. PCR analyses of *T. kodakaraensis* strains KU216 (Δ*pyrF*), KW128 (Δ*pyrF* Δ*trpE*::*pyrF*), and KH3 (Δ*pyrF* Δ*trpE*::*pyrF* Δ*hisD*::*trpE*). (A) Amplification of *pyrF* and *trpE* loci in strains KOD1, KU216, and KW128 using CHDPYR-R/CHDPYR-F and CHDTRP-R/CHDTRP-F as primer sets, respectively. (B) Amplification of the *hisD* locus in *T. kodakaraensis* KOD1, KU216, KW128, and KH3 using CHDHID-R and CHDHID-F as primers. Primers used for these analyses are displayed in Fig. 1. M represents the DNA size marker, HindIII-digested λ DNA.

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 (Δ*pyrF*) 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 $CaCl_2$ 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 (Δ*pyrF* Δ*trpE*::*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 (P_{pyrF}::*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$^-$ plate medium. As a result, we could obtain tryptophan prototrophs with a transformation efficiency of approx-

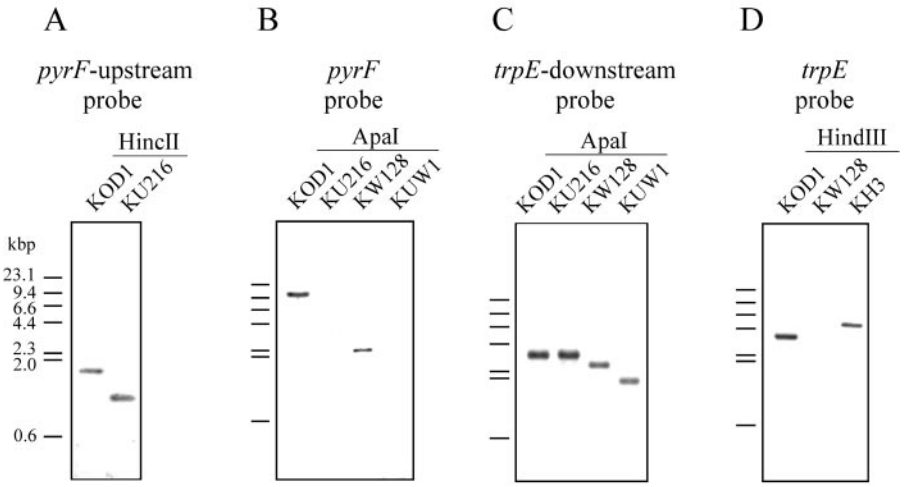
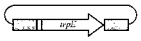
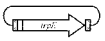
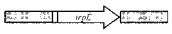
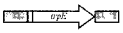


FIG. 3. Southern blot analyses of *T. kodakaraensis* strains KU216 (Δ*pyrF*), KW128 (Δ*pyrF* Δ*trpE*::*pyrF*), and KH3 (Δ*pyrF* Δ*trpE*::*pyrF* Δ*hisD*::*trpE*). (A) The *pyrF* upstream probe was used against genomic DNAs of KOD1 and KU216 digested with HincII. (B) The *pyrF* probe was used against genomic DNAs of KOD1, KU216, KW128, and KUW1 digested with ApaI. (C) The *trpE* downstream probe was used against genomic DNAs of KOD1, KU216, KW128, and KUW1 digested with ApaI. (D) The *trpE* probe was used against genomic DNAs of KOD1, KW128, and KH3 digested with HindIII. The bars on the left side of each panel indicate the mobility of fragments in the DNA size marker, HindIII-digested λ DNA. Regions spanned by probes used for these analyses are displayed in Fig. 1.

TABLE 2. Transformation of *T. kodakaraensis* using various DNAs[a]

DNA added to the cells	DNA form	Length of homologous regions (bp)	DNA structure	Transformation efficiency (Trp^+ prototroph/μg DNA/4 × 10^8 cells)
pUDHisD	Circular	1,000		10^2
pUDHisD2	Circular	500		10^1
pUDHisD3	Circular	100		None
DH-L1	Linear	1,000		10^2
DH-L2	Linear	500		10^1
DH-L3	Linear	100		None

[a] Transformation was performed without $CaCl_2$, 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 × 10^2/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 (Δ*pyrF* Δ*trpE*::*pyrF* Δ*hisD*::*trpE*) 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 $CaCl_2$ treatment on the transformation. In the course of the *hisD* disruption, cells of KW128 were resuspended in transformation buffer containing 80 mM $CaCl_2$ or 0.8× ASW, treated with pUDHisD, and directly inoculated onto ASW-AAW^- 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 $CaCl_2$ treatment, transformation efficiencies were within a similar level of approximately 2 × 10^2/μg DNA, indicating that the $CaCl_2$ 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 allele 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 (10^1/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 (Δ*trpE*::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 3×10^{-4}. One of the several strains isolated was designated KUW1, and its genotype (Δ*pyrF* Δ*trpE*) 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 (Δ*pyrF* Δ*trpE* Δ*hisD*). 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, KuWc1, 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 KUWc1 (Δ*pyrF* Δ*trpE*::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 8×10^{-4}. Further transformation with a similar type II vector, pUDHPOPC, for *hisD* disruption followed by the pop-out event gave the strain KUWcHc1 (Δ*pyrF* Δ*trpE*::2μ′ Δ*hisD*::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 (Δ*pyrF* Δ*trpE*), and confirmed that the *pyrF* marker was applicable for transformation of KUW1 in the course of constructing strain KuWH1 (Δ*pyrF* Δ*trpE* Δ*hisD*::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 (Δ*pyrF*), KW128 (Δ*pyrF* Δ*trpE*::*pyrF*), and KUW1 (Δ*pyrF* Δ*trpE*) 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 rgy_{Tk} (6), imp_{Tk}, and fbp_{Tk} 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 Δ*trpE* 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 10^2/μg DNA. In addition to our previous finding that transformation of *T. kodakaraensis* could

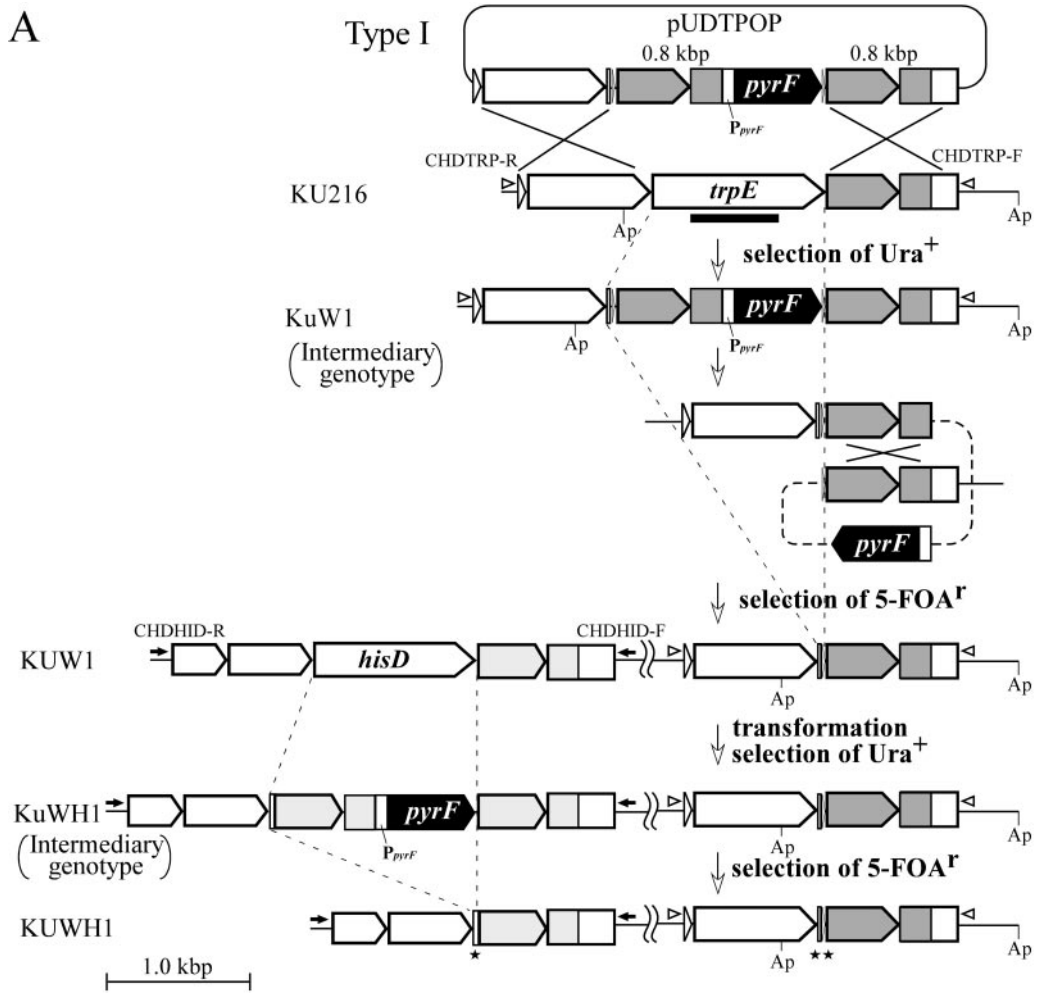


FIG. 4. Schematic diagram of sequential disruption of *trpE* and *hisD* through excision of the *pyrF* marker by pop-out recombination. (A) Construction of strains KUW1 (Δ*pyrF* Δ*trpE*) and KUWH1 (Δ*pyrF* Δ*trpE* Δ*hisD*) using type I pop-out vectors harboring tandem repeats of the endogenous 3′ region of the target gene flanking *pyrF* on both sides. (B) Construction of strains KUWc1 and KUWcHc1 using type II pop-out vectors harboring tandem repeats of the exogenous 2μ′ region flanking *pyrF* on both sides. The regions shaded in gray indicate the tandem repeat regions in each strategy. Open arrowheads and closed arrows indicate primer sets CHDTRP-R/CHDTRP-F and CHDHID-R/CHDHID-F for analyses of targeted disruption of *trpE* and *hisD*, respectively. Restriction site abbreviation: Ap, ApaI. All genes adjacent to the target genes are the same as those mentioned in the legend of Fig. 1.

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 polyethyleneglycol (PEG)-mediated spheroplast method for uptake of extracellular DNA (10^2 to 10^3/μg 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 10^1/μg DNA reported for *Methanococcus voltae* PS (17) and similar to the levels of 10^0~10^3/μg DNA observed for *Methanococcus maripaludis* (20, 27). However, it was lower than those for *M. voltae* by an electroporation-mediated procedure (10^3/μg DNA) (17) and for *M. maripaludis* by a PEG-mediated procedure (10^5/μg DNA) (27). In most cases in the transformation of mesophilic archaea with autonomously replicating plasmids, much higher efficiencies (10^5~10^8/μg 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*

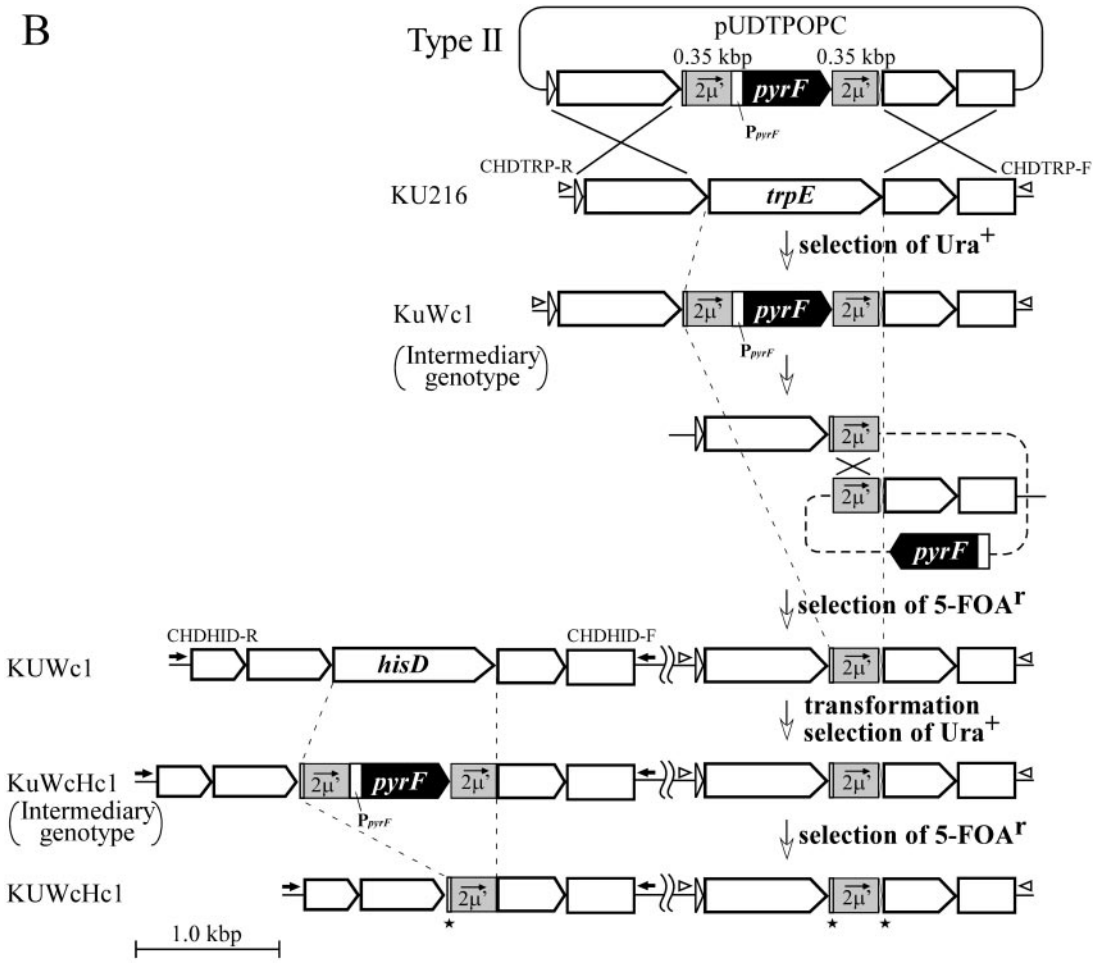


FIG. 4—*Continued*.

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-

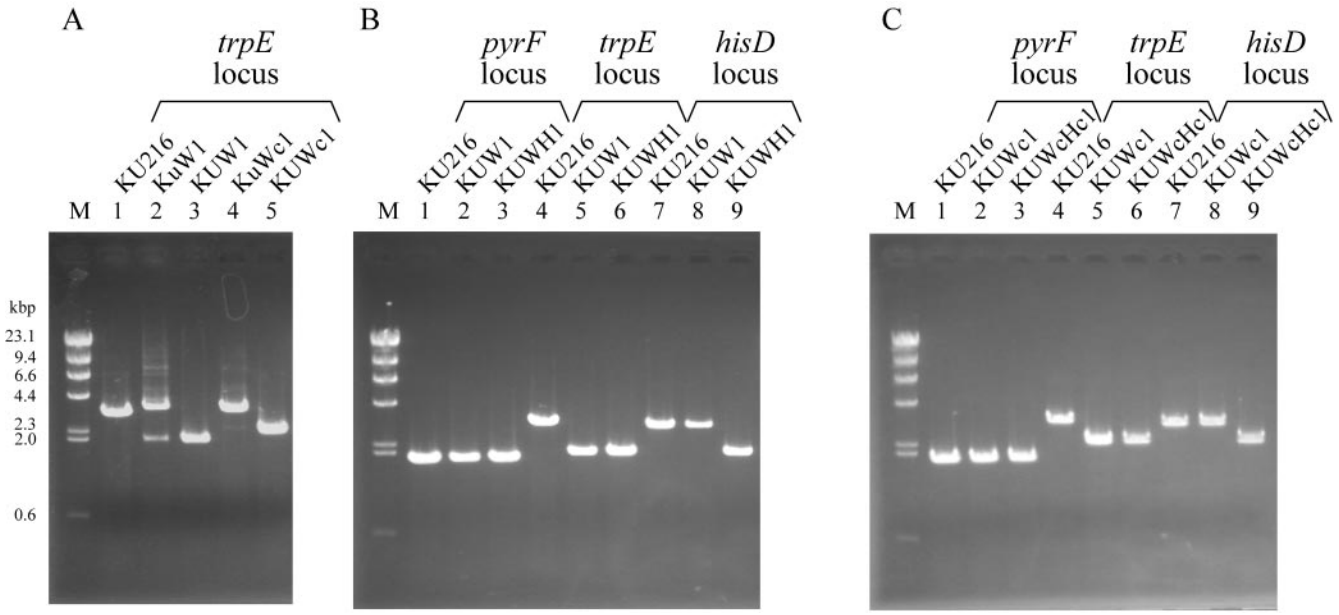


FIG. 5. PCR analyses of *pyrF-trpE* double deletion mutants and *pyrF-trpE-hisD* triple deletion mutants of *T. kodakaraensis* constructed by repeated utilization of the *pyrF* marker using pop-out strategy. (A) Amplification of the *trpE* locus in strains KU216, KuW1, KUW1, KuWc1, and KUWc1 using CHDTRP-R/CHDTRP-F as a primer set. (B) Amplification of *pyrF*, *trpE*, and *hisD* loci in strains KU216, KUW1, and KUWH1 using CHDPYR-R/CHDPYR-F, CHDTRP-R/CHDTRP-F, and CHDHID-R/CHDHID-F as primer sets, respectively. (C) Amplification of *pyrF*, *trpE*, and *hisD* loci in strains KU216, KUWc1, and KUWcHc1 using CHDPYR-R/CHDPYR-F, CHDTRP-R/CHDTRP-F, and CHDHID-R/CHDHID-F as primer sets, respectively. Primer sets used for these analyses were displayed in Fig. 1 and 4. M represents the DNA size marker, HindIII-digested λ DNA.

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 (Δ*pyrF* Δ*trpE*), and a triple deletion mutant, KUWH1 (Δ*pyrF* Δ*trpE* Δ*hisD*), 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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