Disruption of *Thermus thermophilus* genes by homologous recombination using a thermostable kanamycin-resistant marker

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Abstract Genes of an extremely thermophilic bacterium, *Thermus thermophilus*, were disrupted by homologous recombination using a recently developed, thermostable kanamycin-resistant marker. First, the *trpE* gene was disrupted with various constructions of DNA. The transformation efficiency was exponentially increased as the length of the homologous regions flanking the marker gene increased above the minimum length (200–300 bp). We then disrupted five genes of the nucleotide excision repair system and examined their phenotypes. The convenience and high reliability of this method should prompt its application to the high-throughput systematic disruption of the genes of this thermophilic bacterium.

Key words: Thermophile; Selection marker; Functional genomics; UV sensitivity

1. Introduction

Thermus thermophilus HB8 [1] has all the essential genes for the growth in a minimal medium despite its small genome size of 1.74 Mb [2] and can be genetically manipulated [3]. Together with the high stability of its proteins, *T. thermophilus* is very attractive as a model organism to describe the entire system of a basic life form. Structural and functional genomics studies on *T. thermophilus* are now in progress [4]. During the course of this project, the construction and analysis of disruptant strains would not only be informative but also essential especially for analyzing genes of unknown function. However, it has never been reported that genes of thermophiles including *T. thermophilus* are systematically 'knocked out'. One of the major reasons for this is that there has been no positive selection marker available at the optimum growth temperature of the thermophiles.

The *slpA* gene of *T. thermophilus* was previously inactivated by insertional mutagenesis using a moderately thermostable kanamycin-resistant gene [5]. However, it took 48 h of incubation to obtain transformants probably due to the low stability of the marker at 70°C. We recently developed a kanamycin-resistant gene named *HTK*, which is functional beyond the maximum growth temperature of *T. thermophilus* [6]. In this report, we examined the conditions to make *T. thermophilus* disruptants using this marker and then disrupted a series of genes of the nucleotide excision repair system.

2. Materials and methods

2.1. Culture conditions

T. thermophilus HB8 was grown at 70°C in TR medium: 0.4% tryptone (Difco Laboratories, Detroit, MI, USA), 0.2% yeast extract (Oriental Yeast, Tokyo, Japan), and 0.1% NaCl, pH 7.5 (adjusted with NaOH). The TR medium was supplemented with 0.4 mM $CaCl_2$ and 0.4 mM $MgCl_2$ (TT medium) when used for the transformation experiments [3]. To make plates, 1.5% gellan gum (Wako, Osaka, Japan) [7], 1.5 mM $CaCl_2$, and 1.5 mM $MgCl_2$ were added to the TR medium (metals are necessary to solidify the gellan gum). To study the tryptophan auxotrophy, minimal plates were supplemented with 11 μg/ml each of lysine, glycine, serine, methionine, and proline to assist cell growth. The minimal plates contained the following components (per l): 5 g sucrose, 0.75 g K_2HPO_4, 0.25 g KH_2PO_4, 2 g NaCl, 2.5 g $(NH_4)_2SO_4$, 0.1 mg biotin, 1 mg thiamine, 125 mg $MgCl_2{\cdot}6H_2O$, 25 mg $CaCl_2{\cdot}2H_2O$, and 10 g gellan gum, pH 7.2 (adjusted with NaOH).

2.2. Constructions of plasmids and DNA fragments

A 3.4-kb genomic DNA fragment of *T. thermophilus* HB8 that contains the 1.4-kb *trpE* gene [8] was amplified by PCR from a cosmid E019 (unpublished) and subcloned into the plasmid pUC18. The resultant plasmid, pUC18-trpE, was digested with *Bst*EII at two sites in the *trpE* gene, and the larger fragment was ligated with the *HTK* gene, which also contains a promoter sequence, to construct pUC18-ΔtrpE::HTK. In this plasmid, 55% of the coding region of the *trpE* gene is removed, and the *HTK* gene is inserted therein. DNA fragments, which have 100–1000 bp of the *T. thermophilus* genomic DNA regions flanking the *HTK* gene, were amplified by PCR from pUC18-ΔtrpE::HTK. These linear DNA fragments were gel-purified to remove the template plasmid and used for transformation. The transformation was also done with circular DNAs after these fragments were subcloned into pUC18. All the plasmid constructions were done with *Escherichia coli* JM109.

2.3. Transformation of T. thermophilus

The transformation of *T. thermophilus* was carried out by the following procedures [3]: An overnight culture was diluted 1:60 with TT medium and shaken at 70°C for 2 h ($\sim 1\times10^8$ cells/ml). This culture (400 μl) was mixed with a 50-μl DNA solution. The mixture was shaken at 70°C for 2 h and then spread on plates containing 500 μg/ml of kanamycin. The plates were incubated at 70°C for 15 h.

3. Results and discussion

3.1. DNAs used for transformation

We first examined the relationship between the lengths of

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Abbreviations: *HTK*, a thermostable kanamycin-resistant marker gene; UV, ultraviolet light; *trcf*, the gene for a transcription-repair coupling factor of *Thermus thermophilus* HB8

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the homologous regions and the efficiencies of the transformation. The *trpE* gene was targeted in this experiment, which encodes a large subunit of anthranilate synthase of the tryptophan biosynthesis pathway [8]. When transformation was done with linear DNA fragments, it was found necessary to attach the homologous regions of >300 bp at both ends of the *HTK* gene, and the transformation efficiencies increased exponentially above the minimum length (Fig. 1A, open circles). Relatively high efficiencies (150–5000 transformants/μg DNA) were obtained with the lengths of 500–1000 bp. On the other hand, when the DNA fragments were subcloned into a plasmid and the circular DNAs were used, the transformation efficiency with the 300-bp homologous regions at both ends of the *HTK* gene (2×300-bp homologous regions) increased 100-fold, and the minimum length could be reduced to 200 bp (Fig. 1A, closed triangles). There are three possible factors that cause this difference: (i) the form of the DNA, linear or circular, (ii) base modifications, such as methylation, of the plasmid DNA prepared from *E. coli*, and (iii) the non-homologous region derived from the plasmid.

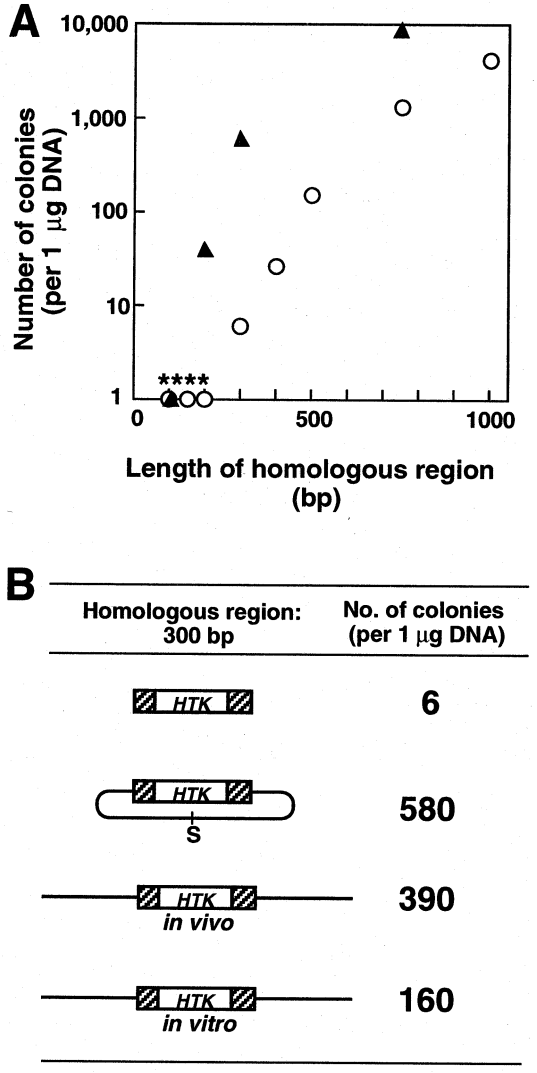

Fig. 1. Transformation efficiencies with various DNAs. A: Transformation experiments were done with DNAs that have homologous regions of indicated lengths at both ends of the *HTK* gene. Open circles indicate results with linear DNA fragments amplified by PCR. The DNA fragments were cloned into pUC18, and the circular plasmid DNAs were prepared from *E. coli* and used to transform *T. thermophilus* (closed triangles). Asterisks indicate that no transformant was obtained with up to 2 μg DNA. Colony numbers varied ±30% between experiments. B: Transformation efficiencies of four different constructions with homologous regions of the same length: (i) a linear fragment with 2×300-bp homologous regions; (ii) (i) was inserted into the *Nde*I and *Xba*I sites of pUC18, and the resultant plasmid was prepared from *E. coli* (an *Spe*I site (S) was incorporated in the middle of the vector); (iii) (ii) was linearized with *Spe*I; (iv) the same fragment as (iii) was amplified by PCR using (ii) as the template. (iii) and (iv) have two 1.2-kb non-homologous regions derived from pUC18.

Relative effects of these factors were analyzed using four DNA constructions (Fig. 1B). Circularization increased the transformation efficiency only 1.5-fold (580/390). The effect of the DNA modifications in *E. coli* was about 2-fold (390/160). Thus, most of the 100-fold difference (580/6) would be attributed to the attachment of the non-homologous region. Transformation by homologous recombination using a given DNA would be affected by the efficiency with which host cells take up the DNA, the stability of the DNA in the host cells, and the efficiency of the recombination event. The non-homologous region might prevent DNA from being degraded by exonucleases, or *T. thermophilus* cells might more efficiently take up larger DNA fragments.

3.2. Verification of the genotype and phenotype

The results of Fig. 1 were obtained using only the resistance to kanamycin as the marker of successful recombination. To confirm that the *trpE* gene on the chromosome was recombined as intended, we analyzed the genotype of three independent kanamycin-resistant clones by Southern hybridization (Fig. 2A). These clones were those obtained using a circular DNA with 2×200-bp homologous regions. As shown in Fig. 2A, single bands of predicted sizes were detected in all clones for both the *Nco*I and *Bam*HI digestions. It has been reported that both novel DNA joints should be independently checked to verify the correct recombination [9,10]. Thus, we also confirmed the upstream joint by PCR using a pair of primers; one anneals at the 5′ end of the *HTK* gene and the other at 1000 bp upstream. In all three kanamycin-resistant clones bands of 1000 bp were detected, whereas no bands were detected in the wild-type strain (data not shown).

The *trpE* strain is expected to show auxotrophy for tryptophan. The wild-type and kanamycin-resistant strains were cultured in the presence or absence of tryptophan (Fig. 2B). The kanamycin-resistant strain grew only in the presence of tryptophan, while the wild-type strain grew on both plates.

These results indicate that the ratio of 'true' disruptants to all kanamycin-resistant clones is very high, that is, this screening system is highly reliable. We confirmed this notion by disrupting other genes using the same method.

3.3. Disruption of the nucleotide excision repair genes

Five genes of the nucleotide excision repair system were disrupted: *uvrA* [11], *uvrB* [12], *uvrC* (unpublished), *uvrD* [13], and the gene encoding a transcription-repair coupling factor (*trcf*) (unpublished). Circular DNAs with 2×200-bp homologous regions were constructed as in the case of the *trpE* gene and used for the transformation. The transformation efficiencies were 1–8 colonies/μg DNA.

The genotypes of the disruptants (two independent clones for each strain) were verified by PCR using primer pairs sim-

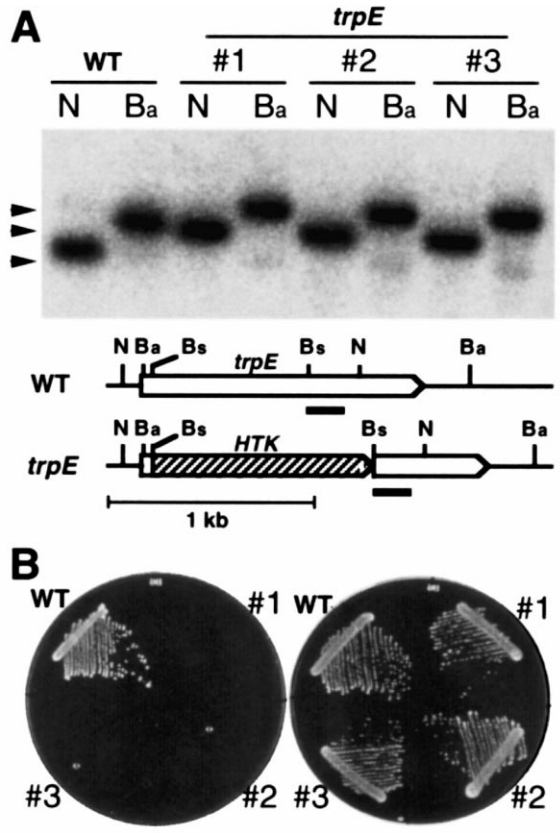

Fig. 2. Verification of the genotype and phenotype of the *trpE* strain constructed using a circular DNA with 2×200-bp homologous regions. A: Genomic DNAs (0.3 μg) of the wild-type strain and three independent clones of the *trpE* strain were digested with *Nco*I (N) and *Bam*HI (Ba) and analyzed by Southern hybridization. Schematic representations of the genomic regions surrounding the *trpE* gene are also shown. In the *trpE* strain, the *HTK* gene is inserted at the *Bst*EII sites (Bs). One of the 200-bp homologous regions was amplified by PCR (underlined), radiolabeled using 50 μCi [α-^{32}P]dCTP (3000 Ci/mmol) and '*Bca*BEST Labeling Kit' (Takara Biomedicals, Kyoto, Japan), and used as the probe (10 ng of the labeled DNA). Arrowheads indicate the positions of the molecular weight markers: 1.88, 1.49, and 0.93 kb. Predicted band sizes for the wild-type strain are 1.20 and 1.60 kb for the *Nco*I and *Bam*HI digestions, respectively. Those for the *trpE* strain are 1.52 and 1.92 kb for the *Nco*I and *Bam*HI digestions, respectively. B: Each strain was grown in TR medium to ∼1×10^8 cells/ml. The cells were harvested, washed with sterile water, and streaked on plates in the presence (right) or absence (left) of tryptophan. The plates were incubated at 70°C for 15 h.

ilar to that used for the *trpE* strain. As a result, a band of expected size was detected in each kanamycin-resistant clone, whereas no band was detected in the wild-type strain (data not shown). This result indicates that the *HTK* gene is inserted in the target genes.

Next, the ultraviolet light (UV) sensitivity of these disruptants was examined. The *uvrA*, *uvrB*, and *uvrC* genes encode subunit A, B, and C, respectively, of the UvrABC excinuclease [14]. The *uvrD* gene encodes DNA helicase II [14], and *trcf* encodes a factor that functions in a phenomenon known as strand-specific repair, in which nucleotide excision repair preferentially occurs in the transcribed strand of an actively transcribed DNA region [15]. As shown in Fig. 3, the *uvrA*, *uvrB*, and *uvrC* strains show increased UV sensitivity, and the similar sensitivity of these strains is consistent with the fact that these gene products work intimately in the initial steps of the repair process [14]. The UV sensitivity of the *trcf* strain is slightly increased. There is no significant difference in the UV sensitivity between the *uvrD* and wild-type strains. This is interesting because the *uvrD* strains of *E. coli* and *Salmonella typhimurium* show increased UV sensitivity [16–18]. Some other helicase(s) might be able to compensate for UvrD in *T. thermophilus*.

3.4. Gene disruption using two-step PCR

All the above experiments were done for the already cloned genes. To clone genes is time-consuming and not suitable for processing many samples in parallel, and adequate restriction sites are not available for many genes. Thus, the following model experiment targeting the *trpE* gene was done to explore a more general application. Instead of amplifying a linear DNA fragment from pUC18-ΔtrpE::HTK, the fragment was obtained as follows [19,20]: First, two 500-bp DNA fragments were directly amplified from the genomic DNA of *T. thermophilus*. One of the two primers for each PCR was designed so that the product has a 20-bp extension homologous to one end of the *HTK* gene. Then, the two fragments and *HTK* gene were assembled into one fragment by the second PCR with the 20-bp overlapping sequences. The resultant DNA fragment was used for the transformation. The efficiency was about 70 colonies/μg DNA, which is similar to that for the linear fragment with 2×500-bp homologous regions in Fig. 1A. Six clones were analyzed, and all of them were auxotrophic for tryptophan (data not shown).

3.5. Conclusions

There was no significant difference in the transformation efficiency among the six genes disrupted in this paper. So far, more than 30 kanamycin-resistant clones were examined on their genotypes and/or phenotypes, and in all of them the target genes were adequately disrupted. Together with the successful disruption with a DNA fragment constructed by the two-step PCR, the low false-positive rate of the present system would greatly facilitate the future high-throughput disruption of *T. thermophilus* genes.

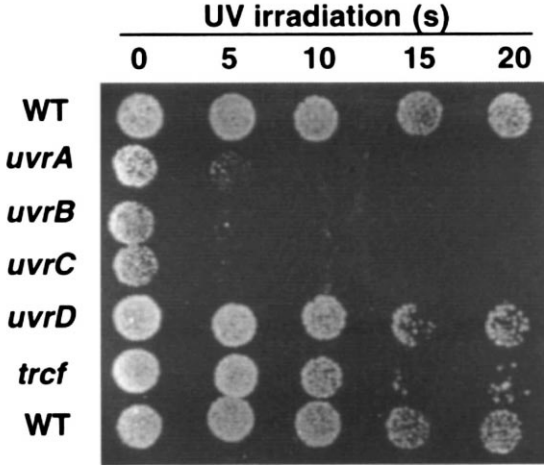

Fig. 3. UV sensitivity. Each strain was grown in TR medium to ∼1×10^8 cells/ml, diluted to 1×10^5 cells/ml with the medium, and spotted on a plate (500 cells/spot). After UV irradiation for 0–20 s using a UV lamp (254 nm, 2 W/m^2) at a distance of 5 cm, the plate was incubated at 70°C for 15 h.

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