Development of a Cre/*lox*-based multiple markerless gene disruption method for the extreme thermophile *Thermus thermophilus*

防衛大学校理工学研究科後期課程

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Introduction

Cre/*loxP* site-specific recombination, which was first reported in 1981 (Sternberg and Hamilton 1981), has become a significant technology to manipulate DNA *in vivo* (Nagy 2000). This recombination is performed between two *loxP* sites using a 38-kDa Cre recombinase. *loxP* is a 34-bp consensus DNA sequence that has an 8-bp core spacer region, defining its orientation, flanked by two 13-bp palindromic sequences, which are the binding sites of Cre (Hoess and Abremski 1985). Cre-mediated recombination results in the excision of the DNA sequence between two *loxP* sites in the same direction, whereas it catalyzes inversion when the two *loxP* sites are in the opposite direction. The efficacy of the Cre/*lox* system in a broad spectrum of biological species and wide variety of applications has made this technology indispensable for *in vivo* genetic manipulation. This system enables various types of recombination, such as conditional recombination, intermolecular recombination, and time-and-space-specific recombination (Nagy 2000). An important application of this system is the removal of selectable markers after gene disruption, which enables the marker to be reused. Marker recycling is a crucial issue for researchers studying organisms that have a limited number of selectable markers, of which extreme thermophiles are a typical example. A shortage of selectable markers has been reported, even for the most studied extreme thermophile, *Thermus thermophilus* (*T. thermophilus*).

T. thermophilus HB27 is a Gram-negative bacterium isolated from a Japanese hot spring, which grows optimally at high temperatures ranging between 65 and 72 °C (Oshima and Imahori 1974). Its natural competence (Koyama et al. 1986), relatively small genome size, consisting of a 1.89-Mb chromosome and 0.23-Mb plasmid pTT27, and available genome sequence (Henne et al. 2004) have made it suitable as a model organism to study thermophilic prokaryotes. The production of a yellow pigment (Oshima and Imahori 1974) and polyploidy,

harboring four to five copies of chromosomes in a cell (Ohtani et al. 2010), are also unique characteristics of *T. thermophilus* HB27. Due to the stability of its proteins and ease of purification, this extreme thermophile was also the focus of a structural genomics project (Yokoyama et al. 2000). Despite its ideal properties as a model organism, a genetic analysis similar to that for mesophiles has been difficult to perform due to the lack of selectable markers for *T. thermophilus*.

Since most of the mesophilic proteins used as selectable markers are sensitive to thermal denaturation at the optimal growth temperature for *T. thermophilus*, their thermostabilities have been improved by random mutagenesis. To date, three examples of antibiotic-resistant genes for kanamycin (Km) (Hoseki et al. 1999), hygromycin (Hm) (Nakamura et al. 2005), and bleomycin (Brouns et al. 2005) are available for *T. thermophilus*; however, it is still a potential bottleneck in the genetic analysis of genes involved in functionally redundant pathways. Therefore, markerless gene disruption is an attractive strategy for research on *T. thermophilus*, and several methods that rely on counter-selection have been reported. Among them, three methods require specific mutant backgrounds responsible for each counter-selectable marker as the parental strains, the Δ*pyrE* (Tamakoshi et al. 1999), Δ*bgl* (Angelov et al. 2013), or Δ*crtB* (Fujita et al. 2015) strain; however, recent studies have demonstrated that counter-selection strategies, in which the *rpsL1* allele (Blas-Galindo et al. 2007), *pheS* allele (Carr et al. 2015), or *codA* gene of *Thermaerobacter marianensis* DSM 12885 (Wang et al. 2016) were used as counter-selectable markers, were applicable to the wild-type strain. When markerless gene disruptants were isolated as colonies resistant to each counter-selection agent, they contained spontaneous selection-resistant mutants depending on the respective background mutant frequencies, for example, 2.5×10^{-7} (Carr et al. 2015) and 10^{-6} to 10^{-7} (Blas-Galindo et al. 2007), and thus additional gene analyses are needed for these

methods, except for Δ*crtB*-based color-selection. These disadvantages are also a potential obstacle to the construction of multiple markerless gene disruptants because they may accumulate spontaneous drug-resistant mutations during repetitive counter-selection. Another strategy that does not require any selection process has recently been reported. In this method, markerless gene disruptants were identified by colony PCR on an enormous scale using the manual screening of colonies grown without selection after a conventional process of transformation, and, hence, it requires a large amount of work (Leis et al. 2014).

The objective of this study was to develop a novel markerless gene disruption method for *T. thermophilus* to overcome the limitations in the counter-selection strategies, namely, the requirement of specific mutant and/or the production of spontaneous selection-resistant mutants. In this respect, I applied a Cre/*lox* system for removal a selectable marker after gene disruption. A Cre/*lox* system has been used in a broad spectrum of biological species for genetic manipulations (Nagy 2000), however, it has not yet been examined in *T. thermophilus*. The activity of Cre recombinase has been experimentally confirmed up to 46 °C (Buchholz et al. 1996), which is slightly less than 50 °C, the minimum growth temperature of *T. thermophilus* HB27 (Ohtani et al. 2010). However, a CD spectral analysis showed that protein denaturation starts at approximately 54 °C (Buchholz et al. 1998). Assuming that Cre is still active at 50 °C, I examined the application of the Cre/*lox* system to markerless gene disruption in *T. thermophilus*. In order to achieve this, two genetic tools, a *loxP–htk–loxP* cassette and the *cre*-expressing plasmid, pSH-Cre, were created. I found that the Cre/*lox* system was compatible with the proliferation of the *T. thermophilus* HB27 strain at the lowest growth temperature (50 °C), and confirmed that the Cre-mediated removal of the selectable marker, *htk* (Hoseki et al. 1999), from the polyploid genome was achieved by the introduction of pSH-Cre into a gene disruptant strain that was constructed by the insertion of the *loxP–htk–loxP* cassette into the TTC1535

gene. Moreover, I succeeded in establishing a triple gene disruptant strain without leaving behind a selectable marker. This is the first example of the disruption of three genes distantly located on the chromosome of *T. thermophilus* in the wild-type background. On the other hand, the Cre-mediated deletion and inversion of the chromosomal region between multiple *loxP* sites occurred in the process of the sequential disruption of multiple genes. In order to avoid these undesired chromosomal rearrangements, I created a *lox66–htk–lox71* cassette that contained mutant *lox* sites (Albert et al. 1995), which allowed for the construction of a double gene disruptant without inducing the undesired deletion. My results showing chromosomal deletions (range, 0.7–122.9 kbp) and inversions (range, 34.5–88.4 kbp) also suggested that this system is applicable to the deletion or inversion of the targeted chromosomal region, which may enable the bioengineering of *T. thermophilus* on an unprecedented scale.

Materials and methods

Basic experiments were performed according to standard protocols described in Molecular cloning: a laboratory manual (Sambrook and Russell 2001).

Bacterial strains and growth conditions

All the *T. thermophilus* strains used in the present study are listed in Table 1, and were grown in PY medium (Ohta et al. 2006) at 70 °C unless otherwise stated. A total of 50 μg/ml of Km and/or 40 μg/ml of Hm was added to the medium when needed. *Escherichia coli* (*E. coli*) DH5α strain, which was used for genetic constructions, was grown at 37 °C in LB medium (Sambrook and Russell 2001). A total of 100 μg/ml ampicillin (Ap), 50 μg/ml Km, or 200 μg/ml Hm was added to the medium when required. In order to make agar plates, 1.5% (w/v) agar (Difco) was added to PY and LB media.

Table 1 *T. thermophilus* strains used in this study

Strain	Genotype	Reference
HB27	Wild type	Oshima and Imahori (1974)
ST1	ΔTTC1535*Kpn*I::*loxP–htk–loxP*	This study
ST1Δ*htk*	ΔTTC1535*Kpn*I::*loxP*	This study
ST2	ΔTTC1535*Kpn*I::*loxP*, ΔTTC1576::*loxP–htk–loxP*	This study
ST2Δ*htk*	ΔTTC1535*Kpn*I::*loxP*, ΔTTC1576::*loxP*	This study
ST2Δ*htk*IN	ΔTTC1535*Kpn*I::*loxP*, ΔTTC1576::*loxP* , IN(TTC1535–1576)	This study
ST3	ΔTTC1454::*loxP–htk–loxP*, ΔTTC1535*Kpn*I::*loxP*, ΔTTC1576::*loxP*	This study
ST3Δ*htk*	ΔTTC1454::*loxP*, ΔTTC1535*Kpn*I::*loxP*, ΔTTC1576::*loxP*	This study
ST4	ΔTTC1535::*lox66–htk–lox71*	This study
ST4Δ*htk*	ΔTTC1535::*lox72*	This study
ST5	ΔTTC1535::*lox72*, ΔTTC1537::*loxP–htk–loxP*	This study
ST5Δ*htk*	ΔTTC1535::*lox72*, ΔTTC1537::*loxP*	This study
ST6	ΔTTC1535::*lox72*, ΔTTC1537::*lox66–htk–lox71*	This study
ST6Δ*htk*	ΔTTC1535::*lox72*, ΔTTC1537::*lox72*	This study
ST7	ΔTTC1535::*loxP–htk–loxP*	This study
ST7Δ*htk*	ΔTTC1535::*loxP*	This study
ST8	ΔTTC1535::*loxP*, ΔTTC1537::*loxP–htk–loxP*	This study

Manipulation of DNA

The plasmids used in the present study were prepared from *E. coli* DH5α using the Wizard Plus SV Minipreps DNA purification system (Promega). DNA fragments were purified from agarose gels or the PCR reaction mixture using the Wizard SV Gel and PCR Clean-Up System (Promega). Three different DNA polymerases were used for PCR depending on the purpose of the experiment. GoTaq Green Master Mix (Promega) was used for the amplification of DNA fragments for TA cloning, a PCR analysis of *T. thermophilus* genomic DNA, and the amplification of DNA fragments for gene disruption. iProof High-Fidelity Master Mix (Bio Rad) was used to clone the genomic DNA fragments of *T. thermophilus*. Pyrobest DNA Polymerase (Takara) was used for inverse PCR during the construction of gene disruption plasmids. PCR was performed according to the manufacturer's instructions. When the purified genomic DNA of *T. thermophilus* was used as a PCR template, the annealing temperature was set to either 60 °C or 65 °C depending on the Tm of the primers. In addition, Ribonuclease (DNase free) (Wako) and Hi-Di Formamide (Thermo Fisher Scientific) were added to the PCR mixture (final concentrations of 0.1 μg/μl and 2.0% (v/v), respectively) in order to improve the amplification of GC-rich DNA. PCR products were separated by 0.8% (w/v) agarose gel electrophoresis and stained with ethidium bromide.

Preparation of *T. thermophilus* genomic DNA

Genomic DNA was prepared from 2 ml of the overnight culture. The culture was centrifuged to pellet the cells. The pellet was resuspended in 1 ml of Pi buffer (33 mM Na_2HPO_4 and 33 mM KH_2PO_4) for washing and cells were re-pelleted. The pellet was resuspended in 300 μl of lysozyme solution (5 mg/ml lysozyme, 0.9% (w/v) glucose, 10 mM EDTA, and 25 mM Tris–HCl pH8.0) and kept on ice for 10 min. Fifteen microliters of 10% (w/v) SDS was added, mixed

gently, and kept on ice for an additional 30 min. The chilled solution was then mixed thoroughly with an equal volume of phenol–chloroform. The mixture was centrifuged (5 min, 16,100 rcf, room temperature) and the supernatant was subjected to ethanol precipitation. Pelleted DNA was rinsed with 70% (v/v) ethanol, dried, and dissolved in 100 μl of TE buffer.

Plasmid construction

The plasmids and synthetic oligonucleotides used in this study are listed in Tables 2 and 3, respectively. Details on plasmid construction are described below.

Table 2 Plasmids used in this study

Plasmid	Relevant characteristics	Reference(s)
pUC18	Cloning vector	Yanisch-Perron et al. (1985)
pBluescript SK(+)	Cloning vector	Short et al. (1988)
pCR2.1-TOPO	Cloning vector	Thermo Fisher Scientific
pGEM-T Easy	Cloning vector	Promega
pTAP60	*htk*, *bla*, *repA* of pTT8, replication origin of pUC18	Ohta et al. (2006)
pBSSK–loxP–RfA–loxP	*loxP*–RfA–*loxP*	Unpublished data, this study
pTN30	*bla*, *supF*, replication origin of pBR322	Hiratsu et al. (2013)
pT8H5-Pslp	*hph5*, *PslpA*, replication origin of pUC19	Takayama et al. (2004); Nakamura et al. (2005)
pSH-Cre	*hph5*, *PslpA-cre*, *repA* of pTT8, replication origin of pBR322	This study
pUC18–loxP–htk–loxP	*Bam*HI-flanked *loxP–htk–loxP*	This study
pUC18-lox66–htk–lox71	*Bam*HI-flanked *lox66–htk–lox71*	This study
pTTC1454	pGEM-T Easy derivative, TTC1454 cloned by TA cloning	This study
pDELloxTTC1454	pTTC1454 derivative, ΔTTC1454::*loxP–htk–loxP*	This study
pTTC1535	pUC18 derivative, TTC1535 cloned at the *Eco*RI/*Hin*dIII site	This study
pDELloxTTC1535K	pTTC1535 derivative, ΔTTC1535*Kpn*I::*loxP–htk–loxP*	This study
pDELloxTTC1535	pTTC1535 derivative, ΔTTC1535::*loxP–htk–loxP*	This study
pDELmloxTTC1535	pTTC1535 derivative, ΔTTC1535::*lox66–htk–lox71*	This study
pTTC1537	pUC18 derivative, TTC1537 cloned at the *Eco*RI/*Xba*I site	This study
pDELloxTTC1537	pTTC1537 derivative, ΔTTC1537::*loxP–htk–loxP*	This study
pDELmloxTTC1537	pTTC1537 derivative, ΔTTC1537::*lox66–htk–lox71*	This study
pTTC1576	pUC18 derivative, TTC1576 cloned at the *Sma*I site	This study
pDELloxTTC1576	pTTC1576 derivative, ΔTTC1576::*loxP–htk–loxP*	This study

Table 3 Synthetic oligonucleotides used in this study

Oligonucleotides	Sequence (5' to 3')[a]	Restriction site
M13-M4	GTTTTCCCAGTCACGAC	-
M13-RV	CAGGAAACAGCTATGAC	-
104	AAAGGTACCTCTTGAGATCCTTTTTTTC	*Kpn*I
140	CTTCTATTCCTTTGCCCTCGGACGAGTG	-
141	CAGATTCGGCCCAAGGTTTACAAAATCC	-
146	ATGCGCCGGGAGATCCTGGTGGCGGCG	-
149	GAAGATCTTGCGGCGGTCCTCCACCTG	*Bgl*II
150	GAAGATCTTGAACCCCAGGGACCCCG	*Bgl*II
161	TGGAGTTCAAGGTGCCCATCCGCAC	-
162	GGCCTCCTGCCCCTCATGGTGAGCC	-
165	CTCCAAGGTGGTCTTTGAGGTGCGG	-
166	GAAGAGGTCCTCCAGGATTAGCTGC	-
180	GGAATTCGGGAGCTTTTAGGGGTAGAGGTG	*Eco*RI
181	CCAAGTACCTCCTGGAAGGGCTTAG	-
182	GAAGATCTGCTGCAGGAGGACCTCGGAGAC	*Bgl*II
183	GAAGATCTTCGCCCGGGAAAGCCCCAAGGAG	*Bgl*II
193	GGGGTACCTCCCACCTCCCCCCGGG	*Kpn*I
194	TGCAGCTCGAGATGTCCAATTTACTGACCGTACACC	*Xho*I
195	GCTCGCTCGAGCTAATCGCCATCTTCCAGCAGGCGC	*Xho*I
196	GGGGTACCGCGGTGGCGGCCGCTCTAG	*Kpn*I
201	CATGGTGGAGCACAAGCGCCTCTTC	-
206	TCGACTAGAATTCATGAAAGGACC	*Eco*RI
207	CGGAATTCAAAATGGTATGCGTTTTGACAC	*Eco*RI
211[b]	CGGGATCC**taccg**TTCGTATAGCATACATTATAC	*Bam*HI
212[b]	CGGGATCC**taccg**TTCGTATAATGTATGCTATAC	*Bam*HI
213	TAGAAGCTTTGTGAGCAAAAGGCCAGC	*Hin*dIII
215	CGGGATCCTCGAGGTCGACGGTATCG	*Bam*HI
229	CGGAATTCTGCCAGGCCTCCACGGCTAG	*Eco*RI
230	TGCTCTAGAAGCGCCCAAAGCCCAGGAG	*Xba*I
231	GAAGATCTTGAAGCAGGCGCATCACGGCCAAG	*Bgl*II
232	GAAGATCTTCGGGAGCTTTTAGGGGTAGAGGTG	*Bgl*II
233	CCTTTACCCCCTTCTCCTCCTCCTC	-
247	ACCGGGAGATGGCGAGGCTCAC	-
lox1[c]	AGCTTATAACTTCGTATAGCATACATTATACGAAGTTAT	*Hin*dIII compatible

lox2[c]	AGCTATAACTTCGTATAATGTATGCTATACGAAGTTATA	*Hin*dIII compatible
lox3[c]	AATTATAACTTCGTATAGCATACATTATACGAAGTTATG	*Eco*RI compatible
lox4[c]	AATTCATAACTTCGTATAATGTATGCTATACGAAGTTAT	*Eco*RI compatible
n1	AGATCTTCTATTCCTTTGCCCTCGG	*Bgl*II
n2	AGATCTCGGCCCAAGGTTTACAAAA	*Bgl*II
n3	GTTAATCATGTTGGTTACGCTG	-
n4	AAGCTTTTCCCCGAAAAGTGCCA	*Hin*dIII
n5	TGGAGTTCAAGGTGCCCATCCG	-
n6	AGCTTTGGTCCGAGAGGGTG	-
n7	GAGCTCGGATCCCGTTGACG	*Bam*HI
n8	TGCAGCGGATCCAACATGATTAAC	*Bam*HI
n9	GACTTCGCCCTCAACATGGA	-
n10	TTCTCCGCCTTGGTCTTGAG	-

[a]Restriction sites used for cloning are *underlined*.

[b]Regarding the primers 211 and 212, the bases shown in *bold lower-case letters* indicate the regions specific to the mutant *lox* sites, *lox71* and *lox66*, respectively.

[c]Regarding the primers lox1-4, the regions compatible with the restriction sites are *underlined*.

Construction of a *loxP–htk–loxP* cassette

pUC18–loxP–htk–loxP harboring a *loxP–htk–loxP* cassette (Fig.1a) was constructed as summarized in Fig. 2. The *loxP* sequences were obtained from pBSSK–loxP–RfA–loxP (unpublished work) consisting of a Reading Frame Cassette A (RfA) fragment (Invitrogen) sandwiched by two *loxP* sites oriented directly. In brief, the synthetic DNAs, lox1/lox2 for LoxPH and lox3/lox4 for LoxPRI, listed in Table 3 were annealed and ligated, respectively, into the *Hin*dIII site and *Eco*RI site of a pBluescript II SK (+) (pBSSK) (Short et al. 1988) derivative containing RfA at the *Eco*RV site of the vector to construct pBSSK–loxP–RfA–loxP. In the present study, RfA in the plasmid was replaced with the *htk* fragment. A 1.1-kbp DNA region, including *htk*, of pTAP60 (Ohta et al. 2006) was amplified by PCR using the primers n7/n8 and subcloned into pCR2.1-TOPO by TA cloning to generate pCR2.1-htk. The RfA fragment of pBSSK–loxP–RfA–loxP was removed by *Eco*RV digestion and the 1.1-kbp *htk* fragment treated by T4 DNA polymerase (Takara) after the *Bam*HI digestion of pCR2.1-htk was inserted therein to generate pBSSK–loxP–htk–loxP. The *loxP–htk–loxP* cassette of pBSSK–loxP–htk–loxP was amplified by PCR using the primers M13-RV/215 to generate *Bam*HI sites at both ends. The resultant 1.3-kbp PCR product was digested by *Bam*HI and ligated to the corresponding site in pUC18 (Yanisch-Perron et al. 1985) to generate pUC18–loxP–htk–loxP. The *Bam*HI-flanked *loxP–htk–loxP* cassette of the plasmid was used to construct the series of plasmids for gene disruption.

Construction of a *lox66–htk–lox71* cassette

pUC18–lox66–htk–lox71 harboring a *lox66–htk–lox71* cassette (Fig. 1a) was constructed as summarized in Fig. 2. pBSSK–loxP–htk–loxP was used as the template for PCR by the primers 206/211 and 207/212 in order to replace its *loxP* sites with *lox71* and *lox66*, respectively. The

resultant PCR products, *lox71–'htk* and *htk–lox66*, were digested by *Eco*RI and *Bam*HI and ligated to the corresponding sites of pUC18 and pBSSK, respectively. Both of the resultant plasmids were digested by *Hin*dIII and *Eco*RI, and a 0.4-kbp fragment from pUC18-(207/212) was ligated to the corresponding site of pBSSK-(206/211) in order to generate pBSSK–lox66–htk–lox71 containing the *lox66–htk–lox71* cassette. Since the transformation of *E. coli* DH5α with the ligation product was unsuccessful, it was used as a template for PCR by the primers M13-M4/M13-RV in order to amplify the *lox66–htk–lox71* cassette. The 1.4-kbp PCR product was digested by *Bam*HI and the resultant 1.2-kbp fragment was ligated to the corresponding site of pUC18 in order to construct pUC18–lox66–htk–lox71. A *lox66–htk–lox71* cassette was prepared from this plasmid, and was also used to construct gene disruption plasmids.

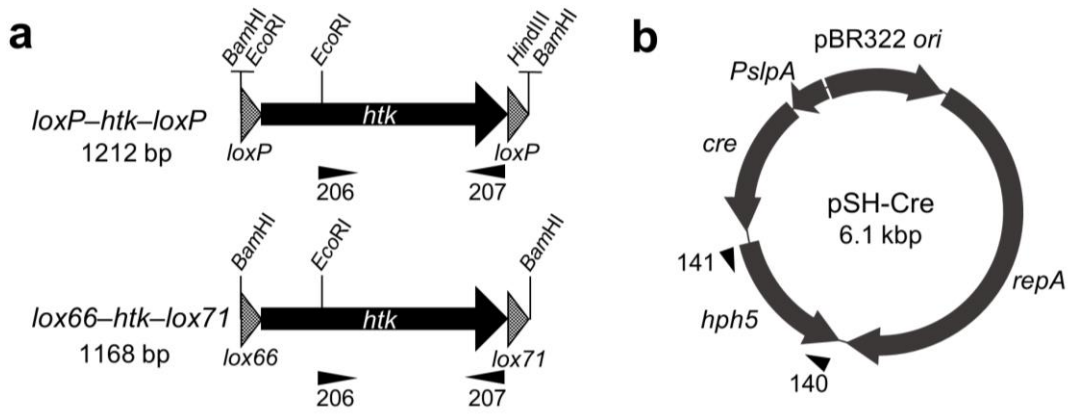

Fig. 1 Genetic tools for the application of the Cre/*lox* system to the markerless gene disruption in *T. thermophilus* HB27. **a** Structural features of the *loxP–htk–loxP* and *lox66–htk–lox71* cassettes. *Shaded triangles* depict the orientation of the *lox* sites, as described by Albert et al. (1995). The *htk* gene is a thermostable Km-resistant gene. **b** Structural features of the *cre*-expressing vector pSH-Cre. The *cre* gene is under the control of the strong *PslpA* promoter. The *hph5* gene is a thermostable Hm-resistant gene. The *repA* region is the replication origin in *T. thermophilus*. The positions of the primers used for PCR analyses are indicated by *filled triangles*.

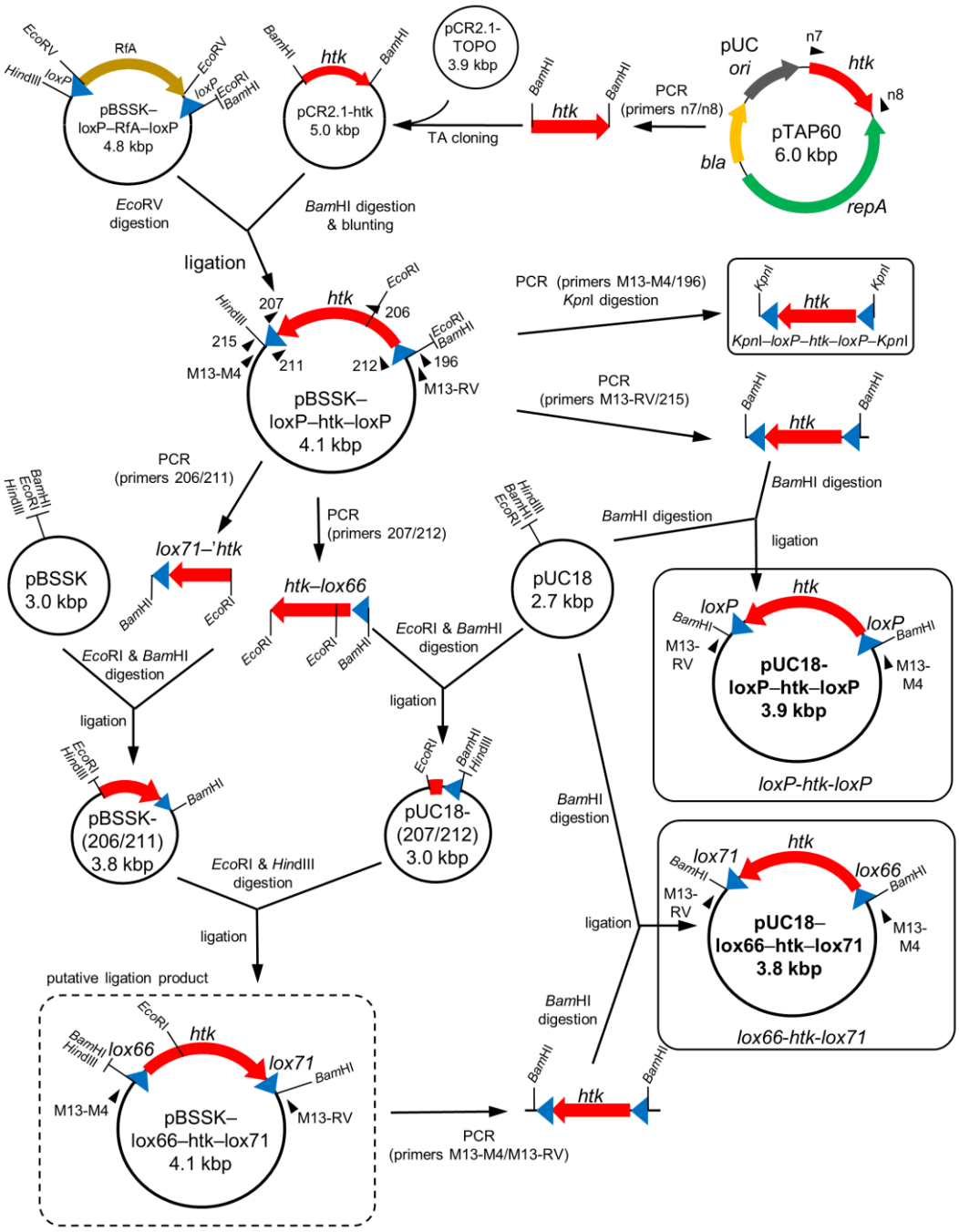

Fig. 2 Construction of plasmids containing a *loxP–htk–loxP* cassette or *lox66–htk–lox71* cassette. pUC18–loxP–htk–loxP contained a *loxP–htk–loxP* cassette, in which a Km-selectable marker, *htk*, is flanked by two directly oriented *loxP* sites. pUC18–lox66–htk–lox71 contained a *lox66–htk–lox71* cassette. *lox66* and *lox71* are mutant *lox* sites. The positions of the primers used for PCR are indicated by *filled triangles*.

Construction of plasmids for gene disruption

The plasmids for gene disruption were constructed as indicated in Fig. 3. In brief, genomic DNA fragments, which contain disruption target genes along with their up- and downstream flanking regions, were subcloned into the cloning vectors listed in Table 2. The disruption cassette, either *loxP–htk–loxP* or *lox66–htk–lox71*, was then inserted into the coding region of the target gene in order to construct gene disruption plasmids. The gene disruption constructs in the resultant plasmids were amplified by PCR using the primers M13-M4/M13-RV, which were purified from agarose gels and used to produce the gene disruptants of *T. thermophilus*.

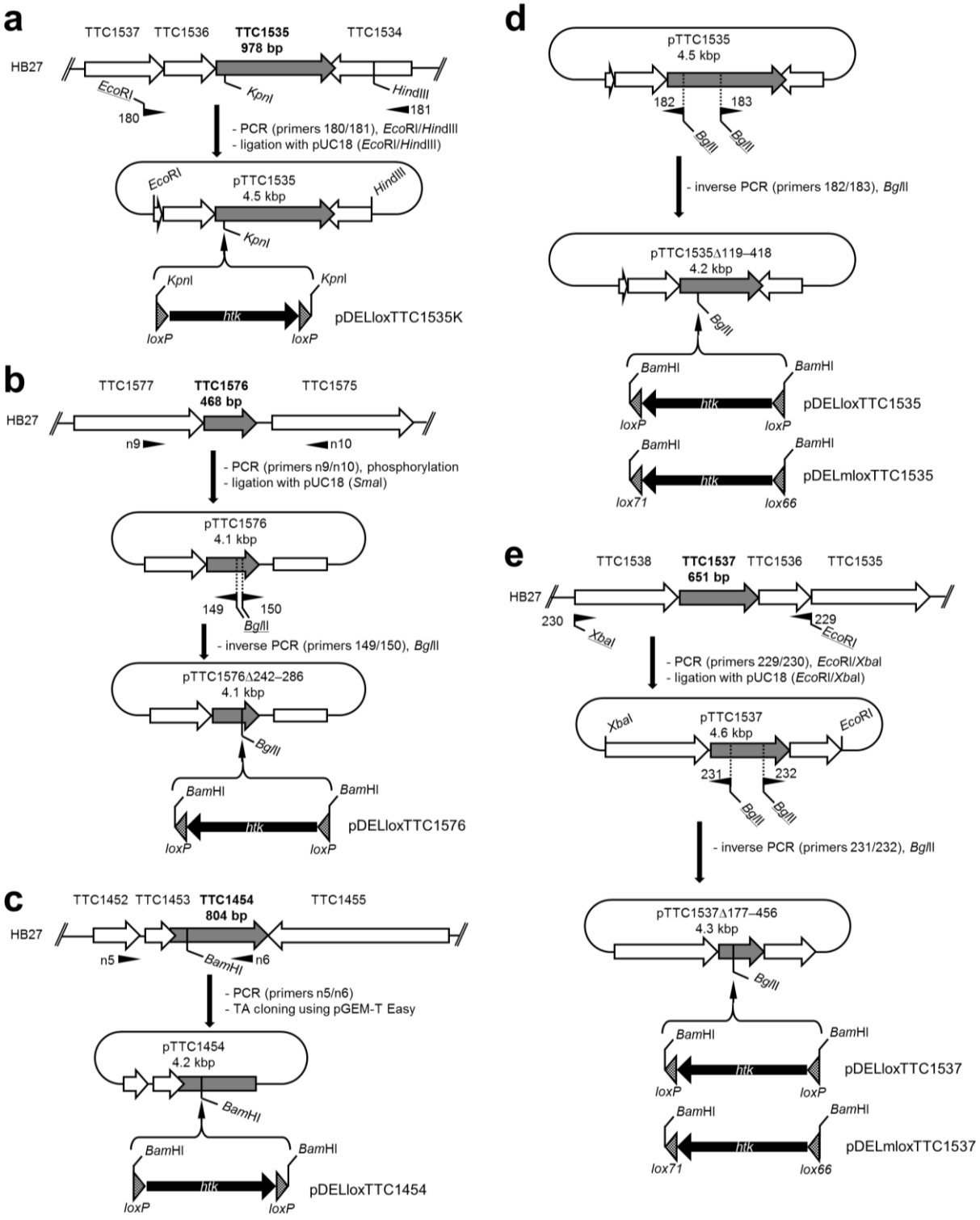
a
TTC1537
TTC1536
TTC1535
978 bp
TTC1534
HB27
EcoRI
KpnI
HindIII
180
181
- PCR (primers 180/181), EcoRI/HindIII
- ligation with pUC18 (EcoRI/HindIII)
pTTC1535
4.5 kbp
KpnI
hlk
loxP
pDELloxTTC1535K
b
TTC1577
TTC1576
468 bp
TTC1575
n9
n10
- PCR (primers n9/n10), phosphorylation
- ligation with pUC18 (SmaI)
pTTC1576
4.1 kbp
149
150
BglII
- inverse PCR (primers 149/150), BglII
pTTC1576Δ242–286
4.1 kbp
BamHI
pDELloxTTC1576
c
TTC1452
TTC1453
TTC1454
804 bp
TTC1455
n5
n6
- PCR (primers n5/n6)
- TA cloning using pGEM-T Easy
pTTC1454
4.2 kbp
pDELloxTTC1454
d
pTTC1535
4.5 kbp
182
183
- inverse PCR (primers 182/183), BglII
pTTC1535Δ119–418
4.2 kbp
pDELloxTTC1535
lox71
lox66
pDELmloxTTC1535
e
TTC1538
TTC1537
651 bp
TTC1536
TTC1535
230
229
XbaI
- PCR (primers 229/230), EcoRI/XbaI
- ligation with pUC18 (EcoRI/XbaI)
pTTC1537
4.6 kbp
231
232
- inverse PCR (primers 231/232), BglII
pTTC1537Δ177–456
4.3 kbp
pDELloxTTC1537
pDELmloxTTC1537

Fig. 3 Construction of gene disruption plasmids. Primers are shown in *filled triangles*. **a** pDELloxTTC1535K. A 1995-bp region of *T. thermophilus* HB27 genomic DNA containing the 978-bp TTC1535 gene was amplified by PCR using the primers 180/181. The *Eco*RI/*Hin*dIII digest of the PCR product (1849 bp) was subcloned into the corresponding site of pUC18. The resultant plasmid, pTTC1535, was cleaved at the unique *Kpn*I site on the coding region of TTC1535, at which a *Kpn*I-flanked *loxP–htk–loxP* cassette was inserted to generate pDELloxTTC1535K. The *Kpn*I-flanked *loxP–htk–loxP* cassette was constructed by amplifying the *loxP–htk–loxP* fragment from pBSSK–loxP–htk–loxP (Fig. 2) using the primers M13-M4/196. The PCR product was digested by *Kpn*I and the resultant 1254-bp fragment was used for the construction of the plasmid. **b** pDELloxTTC1576. A 1448-bp region of *T. thermophilus* HB27 genomic DNA containing the 468-bp TTC1576 gene was amplified by PCR using the primers n9/n10. The PCR product was phosphorylated by T4 polynucleotide kinase (Toyobo) and ligated with *Sma*I-digested pUC18. The resultant plasmid, pTTC1576, was used as the template for inverse PCR with the primers 149/150, which have a *Bgl*II restriction site at their overhang. The resultant PCR product that lacks nucleotides (nt) 242 to 286 of the TTC1576 gene was digested by *Bgl*II (pTTC1576Δ242–286) and ligated with the *loxP–htk–loxP* cassette from *Bam*HI-digested pUC18–loxP–htk–loxP to generate pDELloxTTC1576. **c** pDELloxTTC1454. A 1151-bp region of *T. thermophilus* HB27 genomic DNA containing the first 643 bp of the TTC1454 gene was amplified by PCR using the primers n5/n6. The PCR product was cloned into the pGEM-T Easy vector. The resultant plasmid, pTTC1454, was cleaved at the unique *Bam*HI site on the coding region of TTC1454 and ligated with the *loxP–htk–loxP* cassette to generate pDELlox1454. **d** pDELloxTTC1535 and pDELmloxTTC1535. pTTC1535 was used as the template for inverse PCR using the primers 182/183, which have a *Bgl*II restriction site at their overhang. The resultant PCR product that lacks nt 119 to 418 of the TTC1535 gene was digested by *Bgl*II (pTTC1535Δ119–418) and ligated with the *loxP–htk–loxP* cassette and *lox66–htk–lox71* cassette to generate pDELloxTTC1535 and pDELmloxTTC1535, respectively. **e** pDELloxTTC1537 and pDELmloxTTC1537. A 1947-bp region of *T. thermophilus* HB27 genomic DNA containing the 651-bp TTC1537 gene was amplified by PCR using the primers 229/230. The *Xba*I/*Eco*RI digest of the PCR product was subcloned into the corresponding site of pUC18. The resultant plasmid, pTTC1537 was used as a template for inverse PCR using the primers 231/232, which have a *Bgl*II restriction site at their overhang. The resultant PCR product that lacks nt 177 to 456 of the TTC1537 gene was digested by *Bgl*II (pTTC1537Δ177–456) and ligated with the *loxP–htk–loxP* cassette and *lox66–htk–lox71* cassette to generate pDELloxTTC1537 and pDELmloxTTC1537, respectively. *Shaded triangles* show the orientation of the *lox* sites. Restriction sites with *underlines* are introduced by PCR using primers with the restriction site at the overhang.

Construction of a *cre*-expressing plasmid

The *cre*-expressing vector pSH-Cre (Fig. 1b) was constructed as shown in Fig. 4. A 1.2-kbp DNA region, including the thermostable Hm-resistant gene, *hph5* (Nakamura et al. 2005), of pT8H5-Pslp (Takayama et al. 2004; Nakamura et al. 2005) was amplified by PCR using the primers n1/n2 and subcloned into pCR2.1-TOPO by TA cloning to generate pCR2.1-hph5. The resultant plasmid was then digested by *Bgl*II and the *hph5* fragment was ligated with *Bam*HI-digested pTN30 (Hiratsu et al. 2013) to create pTN30-hph5. The *repA* region for the replication origin in *T. thermophilus* (Aoki and Itoh 2007) was amplified from pTAP60 by PCR using the primers n3/n4, and the resultant 2.8-kbp DNA fragment was subcloned into pCR2.1-TOPO by TA cloning. The resultant pCR2.1-repA was digested with *Hin*dIII and *Pst*I, and the *repA*-containing 2.8-kbp fragment was ligated to the corresponding sites of pTN30-hph5. The resultant plasmid, pKAT925 is an *E. coli*-*T. thermophilus* shuttle vector. Since *hph5* is useful for plasmid maintenance in *E. coli* and *T. thermophilus*, the *bla* gene was removed from pKAT925. A 1.0-kbp region of pTN30 including pBR322 *ori* was initially amplified by PCR using the primers M13-M4/104. The PCR product was digested with *Kpn*I and *Hin*dIII, which was then ligated with a 4.1-kbp fragment of *Kpn*I/*Hin*dIII-digested pKAT925 to construct pKAT925s.

Regarding the expression of *cre*, the *PslpA-cre* region, in which *cre* is under the control of the strong *T. thermophilus* promoter *PslpA* (Faraldo et al. 1992), was cloned to pKAT925s. The 1.0-kbp ORF of the *cre* gene in the 705-Cre plasmid (GeneBridges, Germany) was amplified by PCR using the primers 194/195. The PCR product was digested by *Xho*I and cloned into the corresponding site of pBSSK to generate pBSSK-Cre. pT8H5-Pslp was digested with *Xba*I and *Sal*I, and the 0.3-kbp fragment containing the promoter was ligated to the corresponding site of pBSSK-Cre to generate pBSSK-PslpA-Cre. *PslpA-cre* was amplified from

pBSSK-PslpA-Cre using the primers M13-M4/196. The resultant 1.4-kbp PCR product was digested by *Kpn*I and ligated to the corresponding site of pKAT925s in order to construct pKAT925s-Cre. Since the *supF* gene was irrelevant to the purpose of this study, it was removed from pKAT925s-Cre. A 2.0-kbp region including *cre* and pBR322 *ori* in pKAT925s-Cre was PCR-amplified using the primers 195/213. The PCR product was digested with *Eco*RV and *Hin*dIII, and the resultant 1.5-kbp fragment was ligated with *Eco*RV/*Hin*dIII-digested pKAT925s-Cre to construct pSH-Cre.

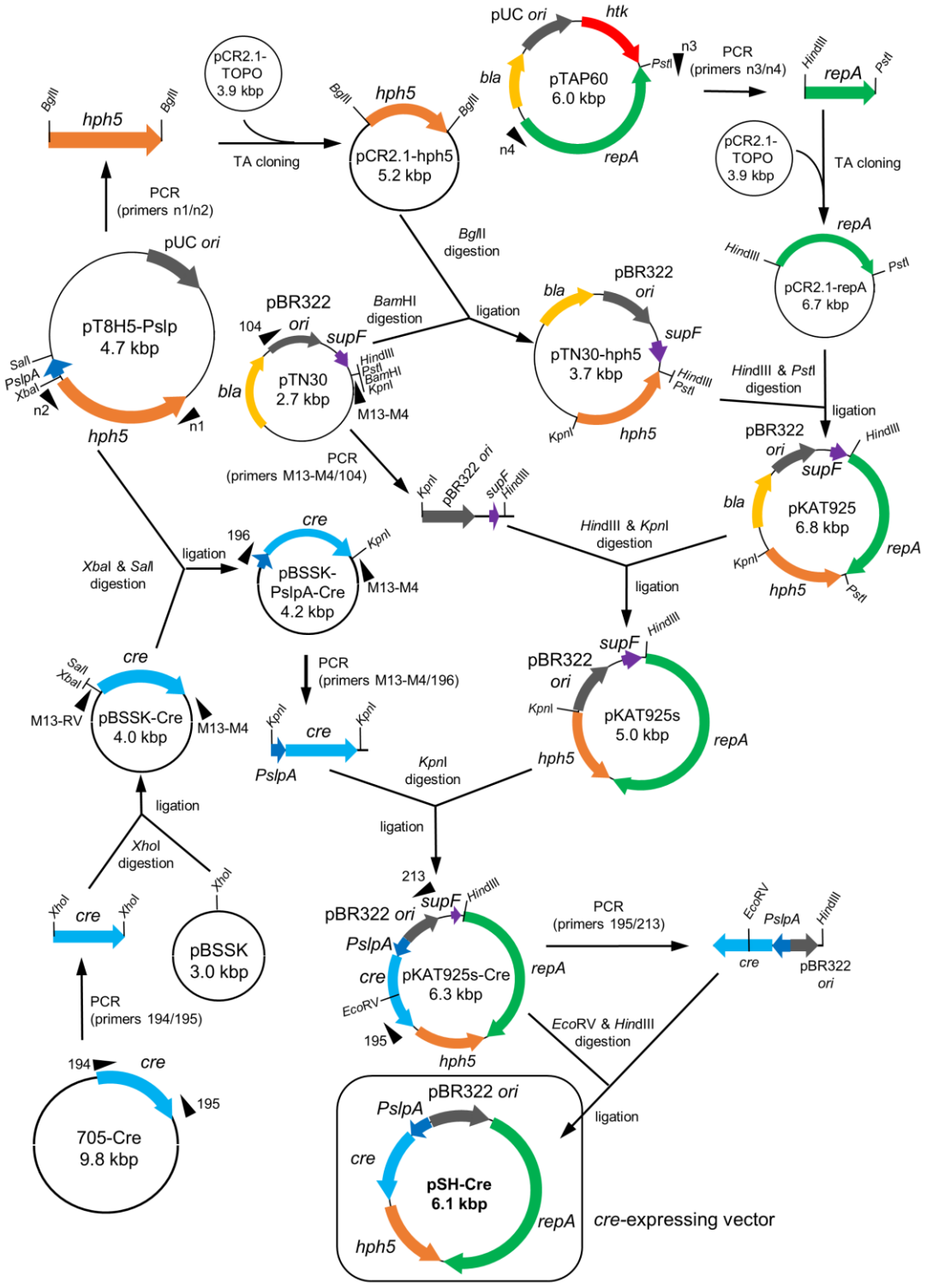

Fig. 4 Construction of the *cre*-expressing vector pSH-Cre. pSH-Cre is a *E. coli*-*T. thermophilus* shuttle vector containing the Hm-selectable marker, *hph5*. The *cre* gene is under the control of the strong *T. thermophilus* promoter *PslpA*. The positions of the primers used for PCR are indicated by *filled triangles*.

Transformation of *T. thermophilus*

The transformation of *T. thermophilus* was performed based on the protocol described by Hoseki et al. (1999). Transformants were selected on PY agar plates with appropriate antibiotics for an overnight incubation at 70 °C. In order to confirm transformation, selected colonies were streaked on the same plates and incubated overnight. Single-colony isolates were subjected to a genomic analysis to confirm transformation.

Gene disruption in *T. thermophilus* using the *loxP–htk–loxP* cassette

In the first step of markerless gene disruption, target genes were disrupted using the PCR product amplified from the gene disruption plasmids constructed for each disruption target gene (Fig. 3). Transformation was performed as described above. Transformants were selected by Km resistance, which was conferred by the *htk* gene in the *loxP–htk–loxP* cassette. In order to confirm gene disruption, the complete replacement of the target gene on multiple chromosomes with the disruption construct was identified by a PCR analysis using the primers specific to each disruption target gene.

Removal of the *htk*-selectable marker by Cre-mediated recombination

In order to remove *htk* from the chromosome, 50–150 ng of the Hm-selectable *cre*-expressing plasmid pSH-Cre was transformed into *loxP–htk–loxP* or *lox66–htk–lox71* gene disruptant mutants. Transformation was performed as described above and the transformants were selected on PY/Km+Hm plates. In order to induce Cre/*lox* site-specific recombination for the removal of *htk* between two *lox* sites, single-colony isolates were re-streaked on PY/Hm plates and incubated at 50 °C for 5–7 days. The Hm-resistant colonies that grew were replica plated on PY and PY/Km plates and incubated at 70 °C overnight in order to select Km-sensitive candidates.

Km-sensitive clones were selected as candidates for markerless gene disruptants, and the corresponding colonies grown on PY plates were cultured in 3 ml of PY medium without antibiotics. Genomic DNA was prepared from the culture and the removal of *htk* was confirmed by a series of PCR analyses.

Curing pSH-Cre from *T. thermophilus*

pSH-Cre was removed from gene disruptants after the removal of *htk* had been confirmed by PCR. Gene disruptants harboring pSH-Cre were cultured at 70 °C overnight in 3 ml of PY medium without Hm. The culture was then diluted to 1:100 in 3 ml of fresh PY medium and grown overnight. The medium was preheated to 70 °C before the dilution to keep Cre inactive and prevent undesired recombination throughout the process. Subculturing was repeated twice, giving three overnight cultures, and 100 μl of the 10^{-6} dilution culture was spread on PY and PY/Hm agar plates and then incubated at 70 °C overnight. Four to 12 of the colonies grown on the PY plate were cultured at 70 °C overnight in 3 ml of PY medium. In order to confirm the absence of pSH-Cre, genomic DNA prepared from the culture was subjected to a PCR analysis using the primers 140/141 for the amplification of a 1.2-kbp DNA fragment for *hph5* on the plasmid (Fig. 1b).

Results

Gene disruption using the *loxP–htk–loxP* cassette

In many biological species, a Cre/*lox* system has been applied for the removal of selectable markers after gene disruption; however, it has not yet been examined in extreme thermophiles. In order to clarify whether this system is applicable to markerless gene disruption in *T. thermophilus*, the most studied extreme thermophile, I constructed two genetic tools, a *loxP–htk–loxP* cassette and pSH-Cre, respectively (Fig. 1a, b). The *loxP–htk–loxP* cassette was used to disrupt a target gene, which consisted of the thermostable Km-resistant gene, *htk*, flanked by two *loxP* sites in the same direction. Therefore, a gene disruptant was selected by Km resistance and subsequently transformed with pSH-Cre to remove *htk* via Cre-mediated recombination. The *cre* gene in pSH-Cre is under the control of the strong *PslpA* promoter in *T. thermophilus*.

To examine the efficacy of the two genetic tools, a markerless gene disruption experiment targeting TTC1535, a chromosomal gene of *T. thermophilus* HB27, was performed. In order to disrupt TTC1535 using the *loxP–htk–loxP* cassette, a gene disruption plasmid was constructed as summarized in Fig. 3a. The gene disruption plasmid, pDELloxTTC1535K, was used as a template for PCR to amplify a gene disruption construct, the 3143-bp DNA fragment containing ΔTTC1535*Kpn*I::*loxP–htk–loxP* (Fig. 5a). The gene disruption of TTC1535 was performed by transforming the wild-type *T. thermophilus* HB27 strain with the resultant PCR product, as described in the Materials and methods. To confirm the disruption of TTC1535, genomic DNA was prepared from four of the Km-resistant clones, and subjected to a genomic PCR analysis using the primers 180 and 181. As shown in Fig. 6a, the TTC1535 region in multiple copies of chromosomal DNA was completely replaced by ΔTTC1535*Kpn*I::*loxP–htk–loxP*; a 2.0-kbp fragment (Fig. 5a) was amplified from wild-type genomic DNA (lane 1), whereas a 3.2-kbp fragment corresponding to the size of ΔTTC1535*Kpn*I::*loxP–htk–loxP* (Fig.

5b) was amplified from the genomic DNA of all four transformants (lanes 2–5). The resultant TTC1535 disruptant, ΔTTC1535*Kpn*I::*loxP–htk–loxP*, was designated as the ST1 strain.

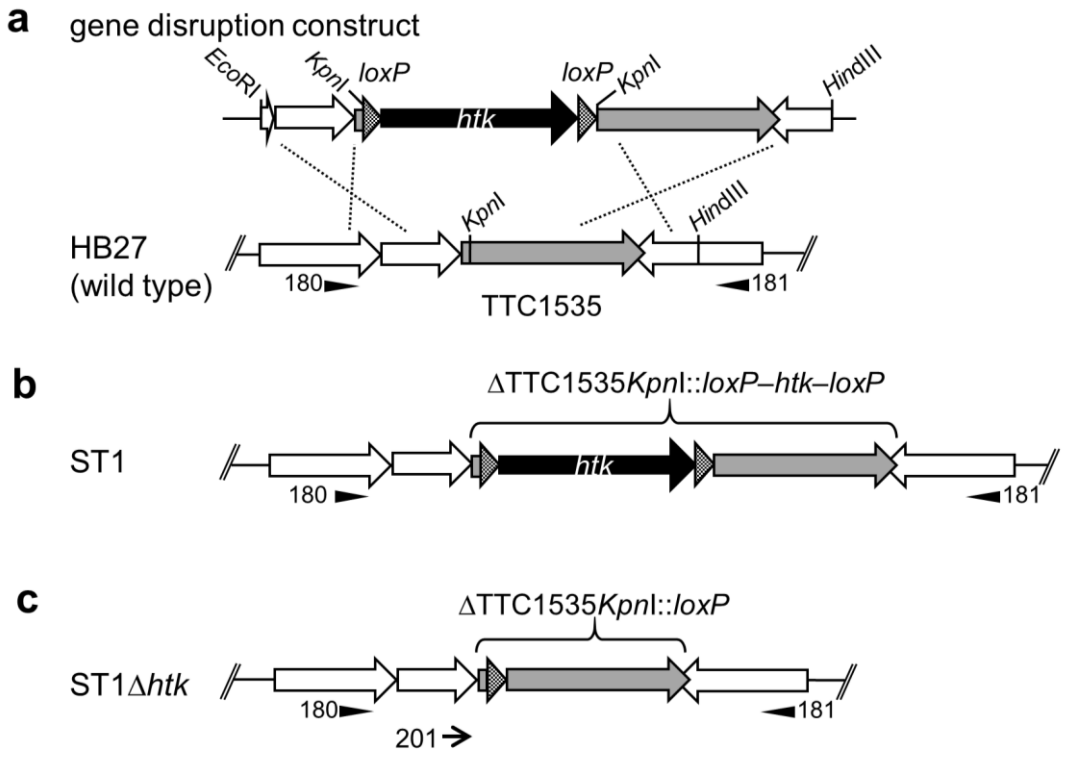

Fig. 5 Description of the markerless gene disruption of TTC1535. **a** Disruption of TTC1535 by homologous recombination. The *lower* and *upper* parts represent the genomic TTC1535 region of the wild-type strain and the gene disruption construct for TTC1535, respectively. **b** and **c** represent the genomic TTC1535 region in ST1 and ST1Δ*htk*, respectively. Primer 201 (*thin arrow*) was used for DNA sequencing. The positions of the primers used for PCR analyses are indicated by *filled triangles*.

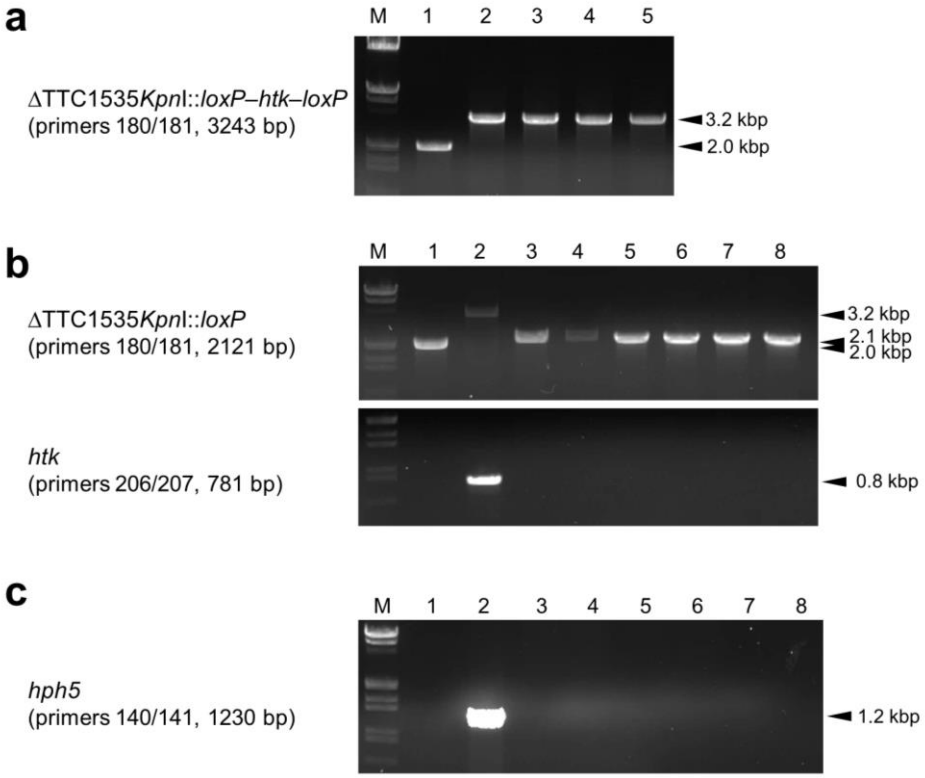

Fig. 6 PCR analyses of the TTC1535 disruptants. The genomic regions analyzed by PCR are indicated on the *left*. Each primer set and the expected lengths (bp) of the objective PCR products are also indicated in *parentheses*. The sizes (kbp) of the PCR products are indicated on the *right*. M indicates the lambda DNA/*Eco*RI+*Hin*dIII size marker. **a** Analysis of the gene disruption of TTC1535 using a *loxP–htk–loxP* cassette. The expected length of the PCR product for HB27 is 1995 bp. *Lane 1* HB27, *lanes 2–5* Km-resistant clones. **b** Analyses of the removal of *htk* from ST1. (*Top*) PCR analysis using the TTC1535-specific primers. The expected lengths of the PCR products for HB27 and ST1 are 1995 bp and 3243 bp, respectively. (*Bottom*) PCR analysis using the *htk*-specific primers. *Lane 1* HB27; *lane 2* ST1; *lanes 3–8* Km-sensitive ST1 clones. **c** Analysis for the absence of pSH-Cre in ST1Δ*htk*/pSH-Cre clones after three passages. The absence of pSH-Cre was examined by the amplification of *hph5* on the plasmid. *Lane 1* HB27; *lane 2* ST1Δ*htk*/pSH-Cre; *lanes 3–8* ST1Δ*htk* candidate clones.

Removal of the *htk* gene by Cre-mediated recombination

In order to remove *htk* from chromosomal ΔTTC1535*Kpn*I::*loxP–htk–loxP*, pSH-Cre was introduced into ST1. A CD spectral analysis previously indicated that the denaturation of Cre starts at approximately 54 °C (Buchholz et al. 1998). Therefore, I assumed that Cre-mediated recombination was accomplished at 50 °C, which is the minimum growth temperature of *T. thermophilus* HB27 (Ohtani et al. 2010). Six colonies of ST1/pSH-Cre transformants were streaked on PY/Hm plates and incubated at 50 °C for five days to form large colonies for selection from each of the six sections. When they were restreaked on PY and PY/Km plates for an overnight incubation at 70 °C, all six clones grew normally on PY plates, but showed no or extremely poor growth on PY/Km plates. The removal of *htk* by Cre-mediated recombination was confirmed by a genomic PCR analysis for the six clones grown on PY plates (Fig. 6b, top); a 2.1-kbp DNA fragment corresponding to the size of ΔTTC1535*Kpn*I::*loxP* was amplified for all clones (lanes 3–8). Furthermore, a sequence analysis of the PCR product using primer 201 (Fig. 5c) identified the junction that was completely identical to the *loxP* sequence produced by Cre-mediated recombination of the *loxP–htk–loxP* cassette (Fig. 7a). These results clearly indicate that the removal of *htk* was successfully performed by Cre/*lox* site-specific recombination *in vivo*. Due to the polyploidy of *T. thermophilus* (Ohtani et al. 2010), the complete removal of *htk* from all copies of the chromosomal DNA of ST1/pSH-Cre was also confirmed by PCR with the *htk*-specific primers, 206/207 (Fig. 1a); an *htk*-derived DNA fragment was not detected for the six clones (Fig. 6b, bottom, lanes 3–8). These results indicate that the Cre/*lox* system worked efficiently in *T. thermophilus* cells at 50 °C, and I successfully performed the markerless gene disruption of TTC1535.

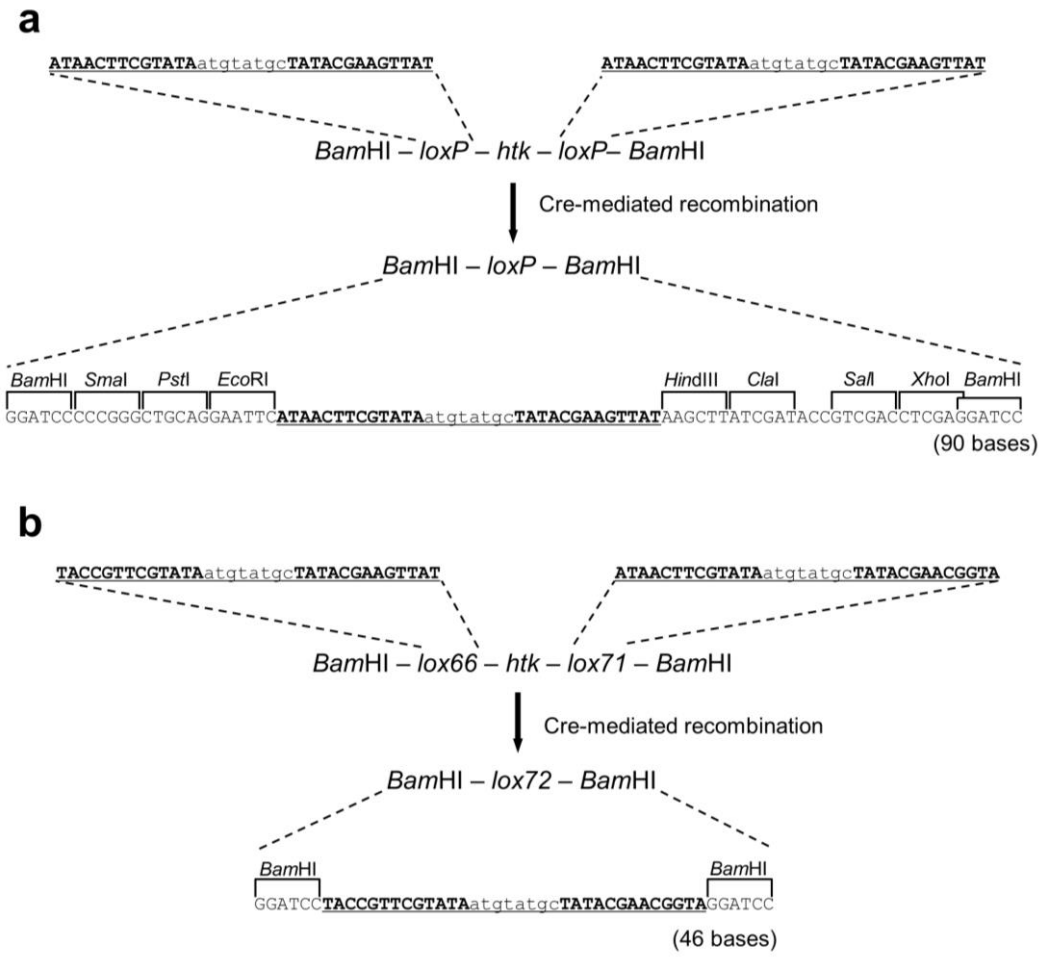

Fig. 7 Nucleotide sequences of *loxP–htk–loxP* and *lox66–htk–lox71* cassettes after Cre-mediated recombination. **a** The *loxP* site produced by the Cre-mediated recombination of the *loxP–htk–loxP* cassette. **b** The *lox72* site produced by the Cre-mediated recombination of the *lox66–htk–lox71* cassette. Recombination between *lox66* and *lox71* generates *lox72*. The 34-bp *lox* sites are *underlined*, and each palindromic sequence for Cre binding is indicated by *bold letters*. The core spacer sequence is shown in *lower case letters*. Restriction sites are indicated on sequences.

pSH-Cre curing from the markerless gene disruptant

pSH-Cre curing from one of the six clones was performed in order to establish the ΔTTC1535*Kpn*I::*loxP* strain, designated as ST1Δ*htk* (Fig. 5c). One of the primary cultures of ST1Δ*htk*/pSH-Cre was initiated in PY liquid medium and incubated at 70 °C without Hm. After three passages, it was plated on PY plates and incubated at 70 °C overnight. Six colonies were selected and their plasmid contents were analyzed by PCR with the *hph5*-specific primers, 140/141 (Fig. 1b); while an *hph5*-derived 1.2-kbp DNA fragment was amplified for ST1/pSH-Cre (Fig. 6c, lane 2), the fragment was not detected for the six clones examined (lanes 3–8). In addition, these clones were unable to grow on PY/Hm plates; therefore, I concluded that pSH-Cre was completely abolished and the ST1Δ*htk* strain was successfully established. These results demonstrate the efficacy of the two genetic tools, the *loxP–htk–loxP* cassette and pSH-Cre, in the disruption of the chromosomal genes of *T. thermophilus* without leaving the selectable marker, *htk*, in the polyploid genome.

Construction of a double markerless gene disruptant

Second gene disruption was performed for ST1Δ*htk* in order to investigate the capability of the Cre/*lox* system in multiple markerless gene disruption. The next target, TTC1576, was located approximately 34.3 kbp from TTC1535 (Fig. 8a). The gene disruption plasmid for TTC1576 was constructed as described in Fig. 3b. Due to the lack of an appropriate restriction site in TTC1576 ORF, 45 bp of the coding region (between base positions 242 and 287) was replaced by the *Bgl*II site using inverse PCR to insert the *loxP–htk–loxP* cassette. The resultant plasmid, pDELloxTTC1576, was used as a template for PCR in order to amplify the gene disruption construct, ΔTTC1576::*loxP–htk–loxP*. The transformation of ST1Δ*htk* was performed using the purified PCR product, and I confirmed the complete replacement of the multiple copies of genomic TTC1576 by the gene

disruption construct using a PCR analysis for all three of the resultant Km-resistant transformants examined (Fig. 9a, lanes 2–4). The resultant double disruptant (ΔTTC1535*Kpn*I::*loxP,* ΔTTC1576::*loxP–htk–loxP*), designated as ST2, was transformed with pSH-Cre to remove *htk* from the *loxP–htk–loxP* cassette. Five independent ST2/pSH-Cre transformants were incubated at 50 °C to remove *htk*, as described in the Materials and methods. I found that all transformants were converted to Km-sensitive clones and their genomic DNAs were prepared for a PCR analysis. As shown in Fig. 9b (ΔTTC1576::*loxP*), a 1.9-kb DNA fragment corresponding to the size of ΔTTC1576::*loxP* was specifically amplified from all Km-sensitive clones (lanes 2–6). The loss of *htk* in all clones was also confirmed by PCR with *htk*-specific primers (Fig. 9b, *htk*, lanes 2–6). Therefore, I concluded that the removal of *htk* from the polyploid genome of the five ST2/pSH-Cre clones was successfully accomplished, and the resultant markerless double disruptant was designated as ST2Δ*htk*/pSH-Cre. The genotype of the ST2Δ*htk* strain was (ΔTTC1535*Kpn*I::*loxP,* ΔTTC1576::*loxP*).

While Cre-mediated recombination between two *loxP* sites in the same direction resulted in the removal of the sequence in the middle, it led to the inversion of the sequence between the two oppositely oriented *loxP* sites (Nagy 2000) such as those inserted in TTC1576 and TTC1535 in the chromosome of ST2Δ*htk* (see Fig. 8b); therefore, the genomic DNAs of the five ST2Δ*htk*/pSH-Cre clones were subjected to an additional PCR analysis for the amplification of DNA fragments specific to the two junctions of the inversion between the two *loxP* sites in ΔTTC1535*Kpn*I::*loxP* and ΔTTC1576::*loxP*, which was hereafter referred to as IN(TTC1535–1576) (Fig. 8c). As shown in Fig. 9b (IN(TTC1535–1576) left junction and right junction), 2.4-kb and 1.6-kb DNA fragments corresponding to the left and right junctions, respectively, were amplified for all five clones (lanes 2–6), indicating the occurrence of IN(TTC1535–1576).

Since the amplification of 1.9-kbp fragment corresponding to ΔTTC1576::*loxP* (Fig. 9b, ΔTTC1576::*loxP*, lanes 2–6) indicated the presence of the chromosome without IN(TTC1535–1576) in the genomic DNAs of the five ST2Δ*htk*/pSH-Cre clones, two possibilities were proposed: the chromosome with and without IN(TTC1535–1576) co-existed in a single cell of ST2Δ*htk*/pSH-Cre or the culture of ST2Δ*htk*/pSH-Cre clones consisted of a mixture of cells with the two individual genotypes. Even in the former case, cultivation without selection was previously proven to be effective for chromosome segregation in *T. thermophilus* (Ohtani et al. 2010). Thus, one of the ST2Δ*htk*/pSH-Cre clones was subjected to the plasmid curing process, as described in the Materials and methods, for the isolation of ST2Δ*htk* without IN(TTC1535–1576) and pSH-Cre. After three passages, a culture was plated on PY plates for an overnight incubation at 70 °C. Genomic DNA was prepared from six independent colonies, and a series of PCR analyses was performed. As a result (Fig. 9c), the DNA fragments derived from the left and right junctions of IN(TTC1535–1576) were specifically amplified for three clones (lanes 2, 5, and 7), whereas the 1.9-kb DNA fragment derived from genomic DNA without IN(TTC1535–1576) was specifically amplified for the remaining clones (lanes 3, 4, and 6). In addition, the 1.6-kb DNA fragment amplified by PCR using the primers 165/180 was confirmed to contain the right junction of IN(TTC1535–1576) by sequencing the PCR product using the primer 146 (Fig. 8c). These results indicate that the segregation/isolation of the two individual genotypes was achieved by the plasmid curing process. The absence of pSH-Cre among them was also confirmed by a PCR analysis (Fig. 9c, *hph5*, lanes 2–7), and thus the double markerless gene disruptant, (ΔTTC1535*Kpn*I::*loxP,* ΔTTC1576::*loxP*) without IN(TTC1535–1576) was established. The clones were conclusively designated as ST2Δ*htk*, whereas the clones possessing IN(TTC1535–1576) were designated as ST2Δ*htk*IN. In this experiment, I demonstrated that two distantly located genes were disrupted without leaving a selection marker

gene using the Cre/*lox*-based system, and also found the inversion of a 34.5-kbp chromosomal region between them.

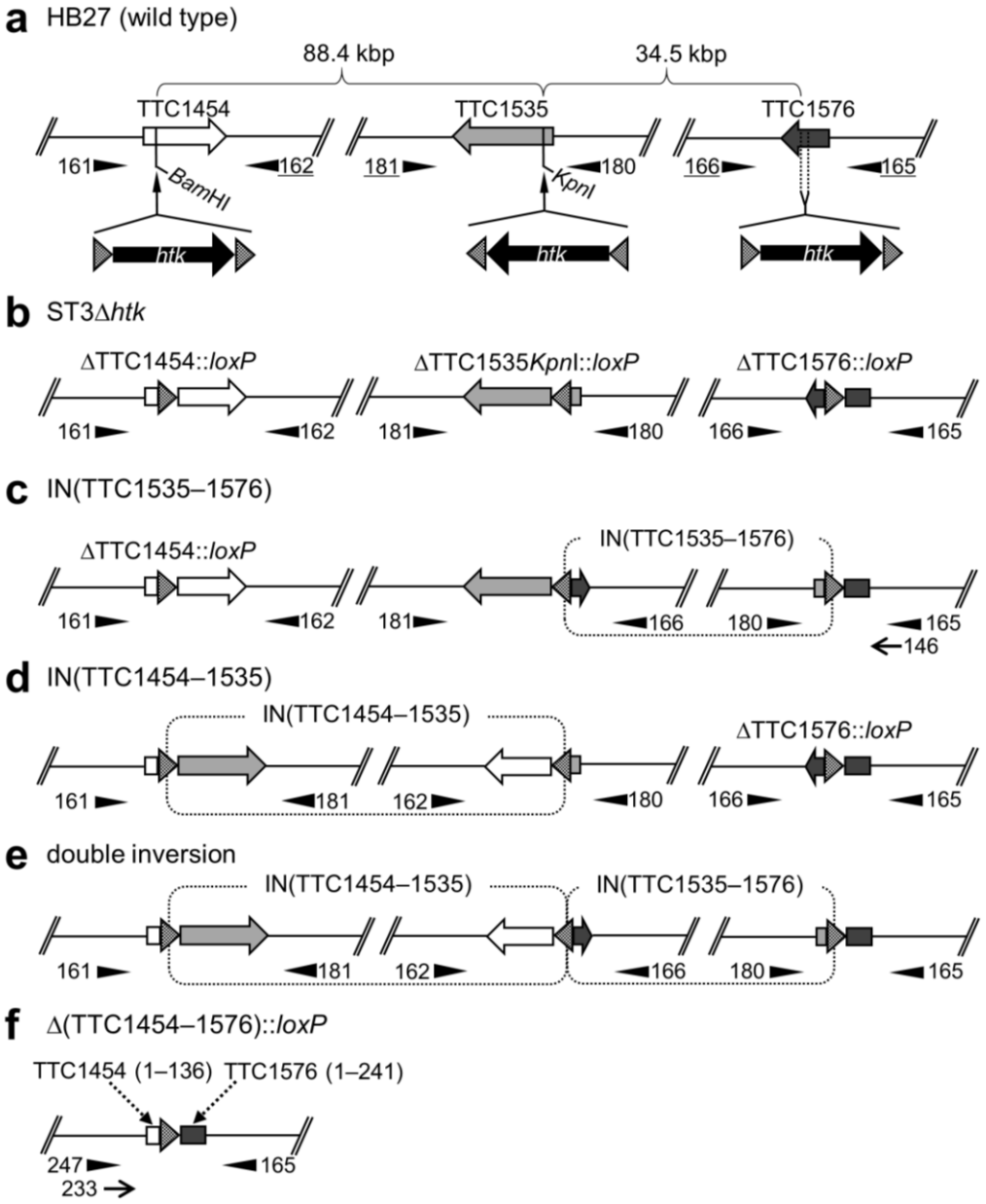

Fig. 8 Schematic representations of the chromosomal location of the three disruption target genes and possible Cre-mediated chromosomal rearrangements. **a** HB27 (wild-type). The physical distances between the genes are shown above the map. The disruption cassette was inserted at the *Kpn*I and *Bam*HI sites of TTC1535 and TTC1454, respectively. In order to insert the cassette in the TTC1576 ORF, inverse PCR was performed to replace 45 bp of the coding region by a *Bgl*II site (see Fig. 4). **b** ST3Δ*htk*. Triple markerless gene disruptant without any chromosomal rearrangements. **c** IN(TTC1535–1576). Inversion between the two *loxP* sites in TTC1535 and TTC1576. **d** IN(TTC1454–1535). Inversion between the two *loxP* sites in TTC1454 and TTC1535. **e** Double inversion. Co-occurrence of IN(TTC1535–1576) and IN(TTC1454–1535). **f** Δ(TTC1454–1576)::*loxP*. Deletion of the chromosomal region between the two *loxP* sites in TTC1454 and TTC1576. Numbers in *parentheses* are the base positions of each coding region. The positions of the primers used for PCR analyses are indicated by *filled triangles*. The *underlined* primers in **a** are located outside of each cloned region in the gene disruption construct. Primers 146 and 233 (*thin arrows*) in **c** and **f**, respectively, were used for DNA sequencing. *Shaded triangles* show the orientation of the *loxP* sites.

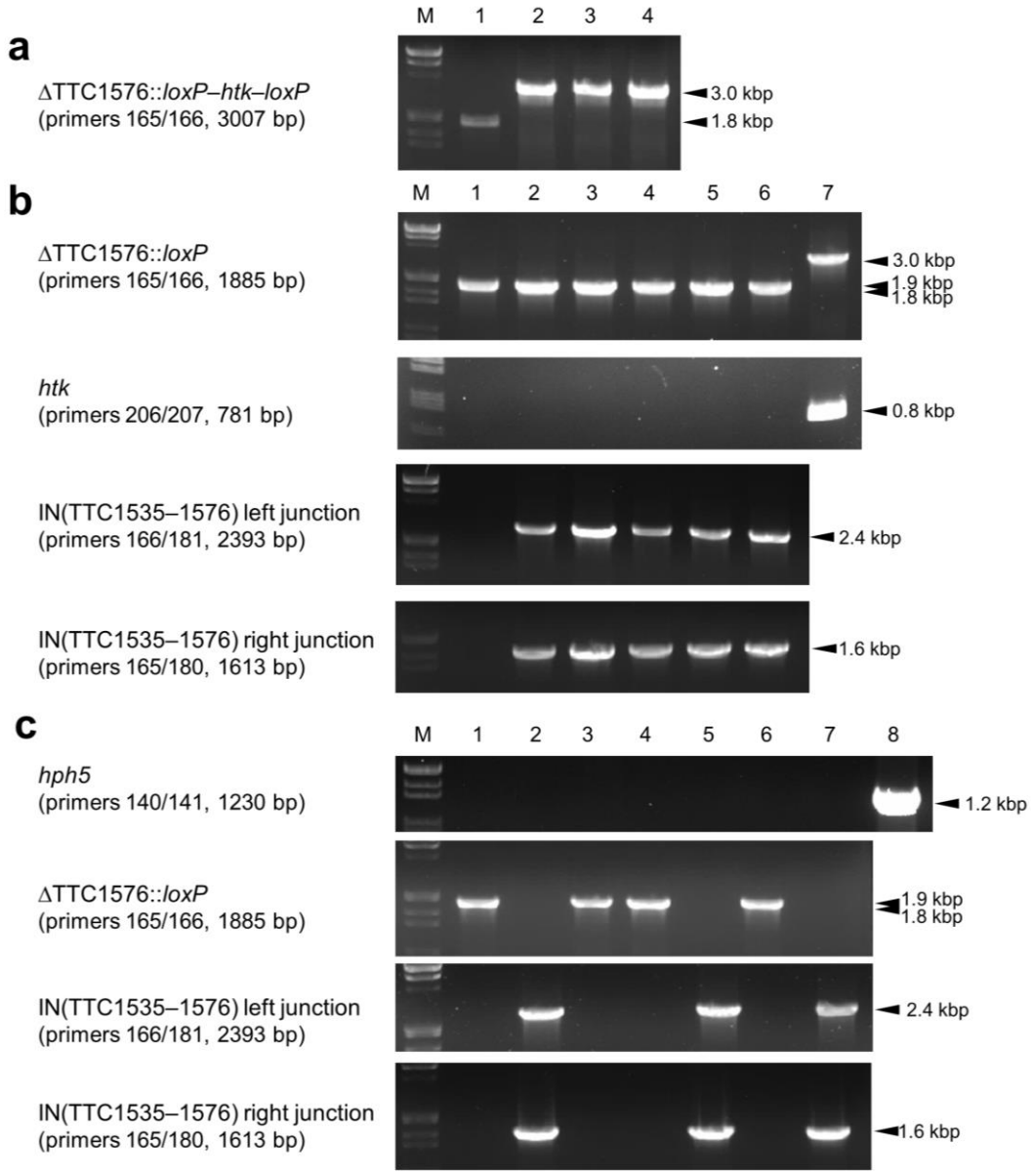

Fig. 9 PCR analyses of the double gene disruptants. The genomic regions analyzed by PCR are indicated on the *left*. Each primer set and the expected lengths (bp) of the objective PCR products are also indicated in *parentheses*. The sizes (kbp) of the PCR products are indicated on the *right*. M indicates the lambda DNA/*Eco*RI+*Hin*dIII size marker. **a** Analysis of the gene disruption of TTC1576 using a *loxP–htk–loxP* cassette. The expected length of the PCR product for HB27 is 1846 bp. *Lane 1* HB27, *lanes 2–4* Km-resistant clones. **b** Analyses of the removal of *htk* and predicted junctions of inversion in Km-sensitive ST2 clones. *Lane 1* HB27; *lanes 2–6* Km-sensitive ST2 clones; *lane 7* ST2. **c** Analyses for the absence of pSH-Cre and segregation/isolation of the ST2Δ*htk* strain. *Lane 1* HB27; *lanes 2–7* ST2Δ*htk* candidate clones isolated from a third-passage culture of ST2Δ*htk*/pSH-Cre; *lane 8* ST2/pSH-Cre.

Construction of a triple markerless gene disruptant

To further elucidate the efficacy of the Cre/*lox*-based system, triple gene disruption was performed for ST2Δ*htk* in the same manner. The next target gene TTC1454 was located approximately 86.8 kb from TTC1535 (Fig. 8a). The ST2Δ*htk* strain was transformed with the gene disruption construct, ΔTTC1454::*loxP–htk–loxP*, which was amplified from pDELloxTTC1454 (Fig. 3c). As shown in Fig 10a, all of the four Km-resistant transformants examined were identified as TTC1454 disruptants by a PCR analysis using primers 161/162 (lanes 2–5). The triple gene disruptants were designated as the ST3 strain and their genotype was (ΔTTC1454::*loxP–htk–loxP*, ΔTTC1535*Kpn*I::*loxP,* ΔTTC1576::*loxP*). ST3 was transformed with pSH-Cre to remove *htk* by Cre-mediated recombination. Five independent ST3/pSH-Cre transformants were incubated at 50 °C, as described in the Materials and methods. I found that all transformants were converted to Km-sensitive clones (#1–5) and their genomic DNAs were prepared for a PCR analysis. As shown in Fig. 10b (ΔTTC1454::*loxP*), a 1.9-kb DNA fragment corresponding to the size of ΔTTC1454::*loxP* was specifically amplified from them, except for clone 4 (lane 5). Since the PCR analysis with *htk*-specific primers resulted in no detectable DNA band for any clone (Fig. 10b, *htk*, lanes 2–6), the Km-sensitive clones were tentatively designated as ST3Δ*htk*/pSH-Cre. The genotype of the ST3Δ*htk* strain was (ΔTTC1454::*loxP*, ΔTTC1535*Kpn*I::*loxP*, ΔTTC1576::*loxP*).

Similar to the double disruptant, I investigated Cre-mediated chromosomal rearrangements by a series of PCR analyses using sets of primers to amplify each junction of possible inversions and a deletion (Fig. 8c–f). The results shown in Fig. 10b indicated that each multicopy chromosome of clones 3 (lane 4) and 4 (lane 5) was composed of the respective single genotype. Clone 3 was identified as my objective genotype (ΔTTC1454::*loxP,* ΔTTC1535*Kpn*I::*loxP,* ΔTTC1576::*loxP*) because a 2.1-kbp DNA fragment derived from

genomic DNA without any chromosomal rearrangements was specifically amplified by PCR only when using the primers 180/181 (lane 4). On the other hand, a 1.9-kbp DNA fragment derived from the right junction of the inversion, IN(TTC1454–1535), (Fig. 8d), was specifically amplified for clone 4 (lane 5). The genomic DNA of the other clones was a mixture of all possible patterns of chromosomal rearrangements because, in addition to the 2.1-kbp DNA fragment (no inversion), the junctions of each inversion and large chromosomal deletion described in Fig. 8 c-f were amplified for all of these clones (Fig. 10b, lanes 2, 3, and 6). These results indicate that the Cre/*lox* system has the potential to induce the inversion (88.4 kbp) and deletion (122.9 kbp) of chromosomal regions in *T. thermophilus* HB27. In fact, the 1.8-kb DNA fragment amplified by PCR using the primers 165/247 (Fig. 10b, Δ(TTC1454–1576)::*loxP*, lanes 2, 3, and 6) was confirmed to contain the junction of the 122.9-kbp deletion, Δ(TTC1454–1576)::*loxP*, by sequencing the PCR product using the primer 233 (Fig. 8f). The pSH-Cre curing of clone 3 was performed in the same manner, and the absence of pSH-Cre was confirmed for 6 independent clones by a PCR analysis (Fig. 10c, lanes 3–8). Cre-mediated chromosomal rearrangements were not induced during the plasmid curing process (data not shown). As a result, the established triple markerless gene disruptant (ΔTTC1454::*loxP,* ΔTTC1535*Kpn*I::*loxP,* ΔTTC1576::*loxP*) was definitively designated it as ST3Δ*htk*. The results presented here demonstrate that the triple markerless gene disruptant was efficiently produced by the Cre/*lox*-based system. This is the first example of the disruption of three genes located distantly on the chromosome of *T. thermophilus* in the wild-type background without leaving behind a selectable marker.

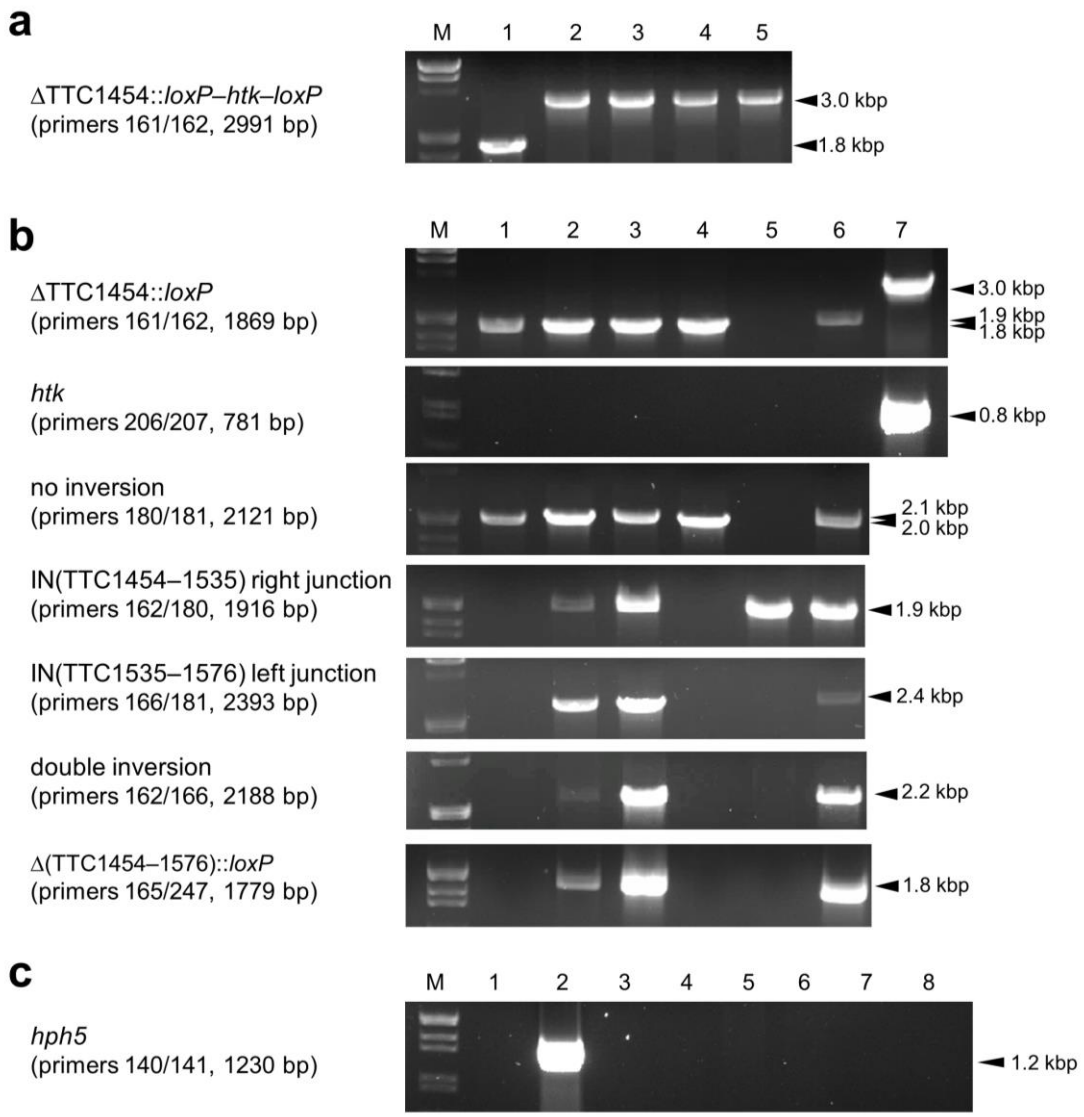

Fig. 10 PCR analyses of the triple gene disruptants. The genomic regions analyzed by PCR are indicated on the *left*. Each primer set and the expected lengths (bp) of the objective PCR products are also indicated in *parentheses*. The sizes (kbp) of the PCR products are indicated on the *right*. M indicates the lambda DNA/*Eco*RI+*Hin*dIII size marker. **a** Analysis of the gene disruption of TTC1454 using a *loxP–htk–loxP* cassette. The expected length of the PCR product for HB27 is 1785 bp. *Lane 1* HB27, *lanes 2–5* Km-resistant clones. **b** Analyses of the removal of *htk* and the junctions of the predicted chromosomal rearrangements inTTC1454, TTC1535, and TTC1576 triple disruptants. The predicted inversions and deletion are indicated in Fig. 8. Genomic DNA without any chromosomal rearrangements (no inversion) was examined by the amplification of ΔTTC1535*Kpn*I::*loxP*. Double inversion indicates the co-occurrence of IN(TTC1454–1576) and IN(TTC1535–1576). *Lane 1* HB27; *lanes 2–6* Km-sensitive ST3 clones; *lane 7* ST3. **c** Analysis for the absence of pSH-Cre from the ST3Δ*htk* strain. *Lane 1* HB27; *lane 2* ST3/pSH-Cre; *lanes 3–8* ST3Δ*htk* candidate clones isolated from a third-passage culture of ST3Δ*htk*/pSH-Cre.

Markerless gene disruption by irreversible Cre/*lox* recombination using a *lox66–htk–lox71* cassette

In experiments for the markerless disruption of multiple genes, I found that each *loxP* site generated after the removal of *htk* was responsible for Cre-mediated chromosomal rearrangements during subsequent gene disruptions. In order to minimize this genetic instability, the mutant *lox* sites, *lox66* and *lox71*, were used to suppress undesired chromosomal rearrangements in other organisms (Albert et al. 1995; Lambert et al. 2007; Kovács et al. 2010). The *lox72* site (Fig. 7b) produced by Cre-mediated recombination between *lox66* and *lox71* exhibited markedly reduced binding affinity for Cre, and thus allowed for repeated gene disruption. In order to evaluate its performance at 50 °C in *T. thermophilus*, I constructed another disruption cassette, *lox66–htk–lox71* (Fig. 1a). Using the *loxP–htk–loxP* and *lox66–htk–lox71* cassettes, I constructed a set of gene disruption plasmids for TTC1535 (Fig. 3d) and TTC1537 (Fig. 3e), namely, pDELloxTTC1535 and pDELloxTTC1537 with a *loxP–htk–loxP* cassette, and pDELmloxTTC1535 and pDELmloxTTC1537 with a *lox66–htk–lox71* cassette. As shown in Fig. 11a, all of the disruption cassettes in the four plasmids were in the same direction relative to the two genes on the chromosome, which, in principle, led to an undesired intergenic deletion between the *lox* sites via Cre-mediated recombination.

I attempted to generate the TTC1535 disruptant in the wild-type background using a ΔTTC1535::*lox66–htk–lox71* construct derived from pDELmloxTTC1535. The transformation, identification of gene disruption (Fig. 12a, lanes 2–5), and introduction of pSH-Cre were successfully performed in the same manner as the methods using a *loxP–htk–loxP* cassette. In order to remove *htk* from the resultant ΔTTC1535::*lox66–htk–lox71* strain designated as ST4, 16 colonies of ST4/pSH-Cre streaked on PY/Hm plates were incubated at 50 °C for five days. Each single-colony isolate of the 16 clones was restreaked on PY and PY/Km plates and incubated at 70 °C overnight. However, in contrast to the high efficiency of the experiments

using the *loxP–htk–loxP* cassette, in which 100% of the Km-resistant clones examined were converted to Km-sensitive clones, all 16 clones grew on PY/Km plates. A PCR analysis was performed on seven clones that showed poorer growth than the others on PY/Km plates, and the results obtained indicated that a 2.9-kbp DNA fragment corresponding to the size of ΔTTC1535::*lox66–htk–lox71* was amplified for all seven clones (Fig. 12b, lanes 3–9), while a putative 1.7-kbp DNA fragment for ΔTTC1535::*lox72* was observed for one clone (Fig. 12b, lane 3). These results suggest that Cre-mediated recombination occurred between *lox66* and *lox71* in *T. thermophilus* under my experimental conditions, but with lower efficiency than that between two *loxP* sites. Since the mutant *lox* sites represented a marked reduction in the capacity for recombination, the 16 clones on the PY/Hm plate were subjected to an additional round of single colony isolation. In the second cycle on a fresh PY/Hm plate at 50 °C for five days, I found that four of the clones not only grew poorly, but exhibited distinct sensitivity to Km on the PY/Km plate. The four corresponding clones on the PY plate were subjected to a PCR analysis, and the results obtained suggested that each genomic DNA included ΔTTC1535::*lox66–htk–lox71* and ΔTTC1535::*lox72* (Fig. 12c, lanes 3–6). In order to obtain my objective ΔTTC1535::*lox72* strain by chromosome segregation/isolation, 16 independent colonies derived from a frozen stock of clone 1 (Fig. 12c, lane 3) was subjected to a PCR analysis. The results obtained revealed that a 1.7-kbp DNA fragment derived from ΔTTC1535::*lox72* was specifically amplified for two clones (Fig. 12d, lanes 5 and 14). I confirmed that the DNA sequence of the junction in the PCR fragment was completely identical to the *lox72* sequence (Fig. 7b) produced by Cre-mediated recombination between the mutant *lox* sites on the *lox66–htk–lox71* cassette. After the plasmid curing process for the two clones, I confirmed the absence of pSH-Cre and *htk* in their genomic DNAs by a series of PCR analyses (data not shown); therefore, the markerless gene disruptant, ΔTTC1535::*lox72* designated as

ST4Δ*htk* was established.

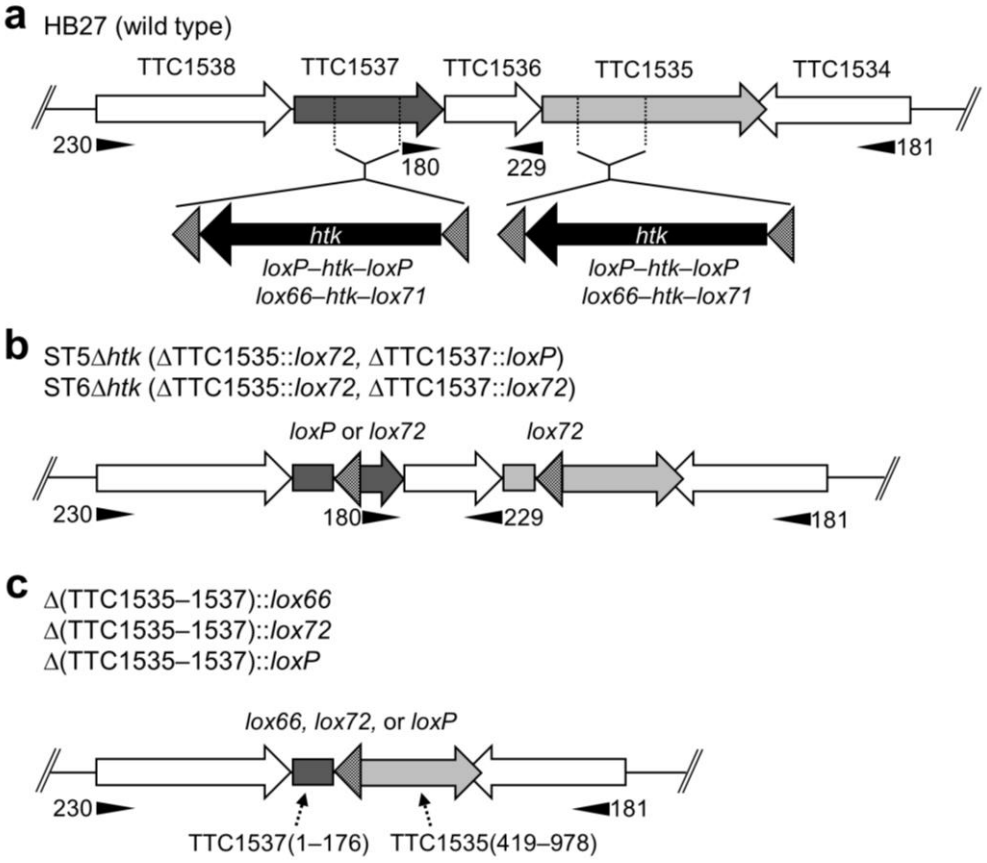

Fig. 11 Schematic representations of the gene disruption of TTC1535 and TTC1537 using *loxP–htk–loxP* and *lox66–htk–lox71* cassettes. **a** The chromosomal locus of TTC1534-TTC1538 in *T. thermophilus* HB27. In order to insert the indicated cassettes in the ORF of TTC1535 and TTC1537, inverse PCR was performed to replace 300 bp and 280 bp of the respective coding regions by a *Bgl*II site. **b** ST5Δ*htk* and ST6Δ*htk* were constructed by the removal of the *htk* gene from ΔTTC1537::*loxP–htk–loxP* and ΔTTC1537::*lox66–htk–lox71* in the ΔTTC1535::*lox72* background, respectively. **c** Δ(TTC1535–1537)::*lox*. Deletion of the chromosomal region between the two *lox* sites in TTC1535 and TTC1537. Cre-mediated recombination between *lox72* and *loxP* and between two *lox72* sites described in **b** generated *lox66* and *lox72*, respectively. Numbers in parentheses are the base positions of each coding region. The positions of the primers used for PCR analyses are indicated by *filled triangles*. The 181 primer is located outside of the cloned region in both gene disruption constructs for TTC1535 and TTC1537. *Shaded triangles* show the orientation of the *lox* sites.

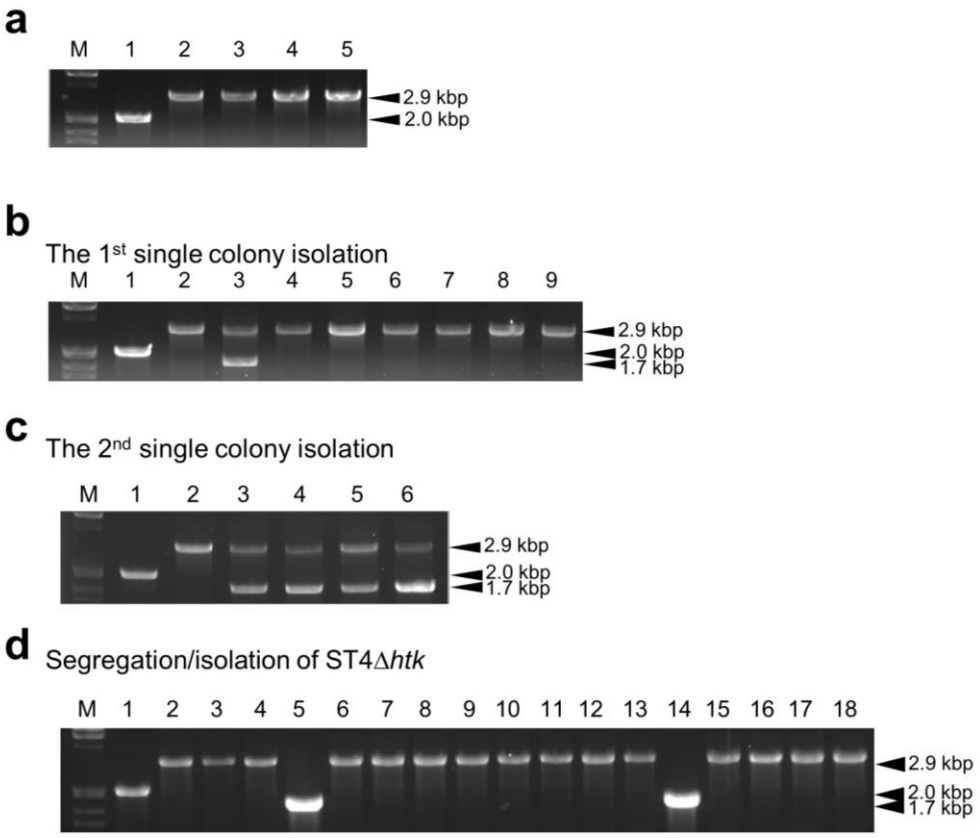

Fig. 12 PCR analyses of TTC1535 disruptants using the *lox66–htk–lox71* cassette. The TTC1535 locus was analyzed by PCR using the primers 180/181. The expected lengths of the PCR products for the wild type, ΔTTC1535::*lox66–htk–lox71*, and ΔTTC1535::*lox72* are 1995 bp, 2857 bp, and 1735 bp, respectively. **a** Analysis of the gene disruption of TTC1535 using a *lox66–htk–lox71* cassette. *Lane 1* HB27, *lanes 2–6* ST4. **b** Analysis of the removal of *htk* from ST4 after the first cycle of single colony isolation at 50 °C. The seven ST4/pSH-Cre clones that exhibited moderate sensitivity to Km were analyzed. *Lane 1* HB27; *lane 2* ST4; *lanes 3–9* Km-sensitive ST4/pSH-Cre clones. **c** Removal of *htk* after the second cycle of single colony isolation at 50 °C. The four ST4/pSH-Cre clones that exhibited distinct sensitivity to Km were analyzed. *Lane 1* HB27; *lane 2* ST4; *lanes 3–6* Km-sensitive ST4/pSH-Cre clones. **d** Segregation/isolation of ST4Δ*htk*. The 16 single-colony isolates derived from a frozen stock of one of the Km-sensitive ST4/pSH-Cre clones were analyzed for the identification of a single genotype of ΔTTC1535::*lox72*. *Lane 1* HB27; *lane 2* ST4; *lanes 3–18* ST4Δ*htk*/pSH-Cre candidate clones. The sizes (kbp) of the PCR products are indicated on the *right*. M indicates the lambda DNA/*Eco*RI+*Hin*dIII size marker.

Double gene disruption using a *lox66–htk–lox71* cassette

In order to clarify whether *lox72* is inactive during subsequent markerless gene disruption, ST4Δ*htk* was subjected to the disruption of TTC1537. The transformation, identification of gene disruption using the *loxP–htk–loxP* cassette (Fig. 13a, top, lanes 1 and 2) and *lox66–htk–lox71* cassette (Fig. 13b, top, lanes 1 and 2), and introduction of pSH-Cre were performed in the same manner. As a result, I obtained two strains (ΔTTC1535::*lox72*, ΔTTC1537::*loxP–htk–loxP*) and (ΔTTC1535::*lox72*, ΔTTC1537::*lox66–htk–lox71*) designated as ST5 and ST6, respectively, with pSH-Cre. All six independent ST5/pSH-Cre clones grown on PY/Hm plates at 50 °C were Km-sensitive, and the removal of *htk* from their chromosomes was identified by a PCR analysis (Fig. 13a, top, lanes 3–8). When I investigated the deletion of an intergenic region between *loxP* and *lox72*, designated as Δ(TTC1535–1537)::*lox66* (Fig. 11c), in these clones, a 2.0-kbp DNA fragment derived from the deletion was detected for one clone by a PCR analysis (Fig. 13a, bottom, lane 7).

On the other hand, the removal of *htk* from the ΔTTC1537::*lox66–htk–lox71* region of ST6/pSH-Cre required two cycles of single colony isolation at 50 °C. Among the resultant 16 independent clones examined, one exhibited significant and another seven had moderate sensitivity to Km. A PCR analysis to identify the removal of *htk* was performed for the eight Km-sensitive clones, and the results obtained suggested that a 1.7-kbp DNA fragment derived from ΔTTC1537::*lox72* was specifically amplified for clones 1, 6, and 7 (Fig. 13b, top, lanes 3, 8, and 9). A PCR analysis resulted in no detectable band corresponding to the deletion of an intergenic region between two *lox72* sites, designated as Δ(TTC1535–1537)::*lox72* (Fig. 11c) for these 3 clones (Fig. 13b, bottom, lanes 3, 8, and 9), whereas the *htk*-derived DNA fragment was detectable for clone 1 (data not shown). Taken together, five clones of ST5Δ*htk*/pSH-Cre and two clones of ST6Δ*htk*/pSH-Cre were established in this experiment. Plasmid curing was

successfully performed (data not shown) to produce ST5Δ*htk* and ST6Δ*htk* (Fig. 11b), respectively.

As a control for the above experiments, double gene disruption was performed for TTC1535 and TTC1537 by the *loxP–htk–loxP* cassette in the same manner. The markerless disruption of TTC1535 was carried out using the gene disruption construct amplified from pDELloxTTC1535 (Fig. 3d). The gene disruption (Fig. 14a, lanes 1 and 2) and the removal of *htk* (Fig. 14a, lanes 3–10) were successfully performed. The two strains, ΔTTC1535::*loxP–htk–loxP* and ΔTTC1535::*loxP* were designated as ST7 and ST7Δ*htk*, respectively. pSH-Cre was removed from ST7Δ*htk*, and then the strain was subjected to the disruption of TTC1537 using the gene disruption construct amplified from pDELloxTTC1537 (Fig. 3e). The transformation, identification of gene disruption (Fig. 14b, top, lanes 1 and 2), and introduction of pSH-Cre were performed in the same manner. As a result, a double gene disruptant (ΔTTC1535::*loxP*, ΔTTC1537::*loxP–htk–loxP*), designated as ST8, with pSH-Cre was obtained. Eight independent ST8/pSH-Cre clones were grown on PY/Hm plates at 50 °C, and the resultant Km-sensitive clones were analyzed by PCR to identify the removal of *htk*. While the PCR analysis using the primers 229/230 resulted in no detectable band corresponding to the size of ΔTTC1537::*loxP* for all clones (Fig. 14b, top, lanes 3–10), a 2.1-kbp DNA fragment derived from the intergenic deletion, Δ(TTC1535-1537)::*loxP* was detected for all clones (Fig. 14b, bottom, lanes 3–10). These results indicated that the chromosomal region between the *loxP* sites in ΔTTC1535::*loxP* and ΔTTC1537::*loxP* was completely removed in these clones, and thus the construction of the objective double markerless gene disruptant was unsuccessful. In contrast, an objective double gene disruptant was efficiently produced using a *lox66–htk–lox71* cassette. Among the six Km-sensitive ST4 clones, an intergenic deletion between the *lox72* and *loxP* sites occurred partially in the chromosomal DNA of only one clone (Fig. 13a, bottom, lane 7), whereas no

such deletion between the two *lox72* sites was observed in any of the eight clones examined (Fig. 13b, bottom, lanes 3–10). Taken together, the results presented here indicate that a *lox66–htk–lox71* cassette is of considerable utility in the construction of multiple gene disruptants of *T. thermophilus*.

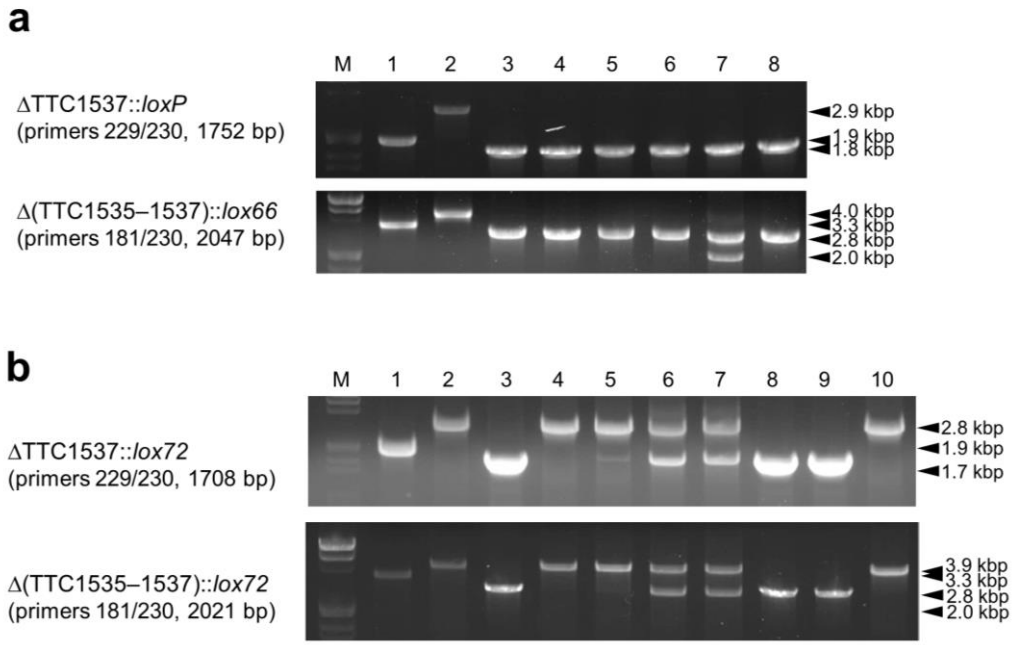

Fig. 13 PCR analyses of double gene disruptants using *loxP–htk–loxP* and *lox66–htk–lox71* cassettes. The genomic regions analyzed by PCR are indicated on the *left*. Each primer set and the expected lengths (bp) of the objective PCR products are also indicated in *parentheses*. The sizes (kbp) of the PCR products are indicated on the *right*. M indicates the lambda DNA/*Eco*RI+*Hin*dIII size marker. **a** Analyses of the markerless disruption of TTC1537 in ST4Δ*htk* using the *loxP–htk–loxP* cassette. (*Top*) Replacement of TTC1537 with ΔTTC1537::*loxP–htk–loxP*, and the removal of *htk* from ST5. (*Bottom*) Analysis of the intergenic deletion between ΔTTC1535::*lox72* and ΔTTC1537::*loxP*. *Lane 1* HB27; *lane 2* ST5; *lanes 3–8* Km-sensitive ST5/pSH-Cre clones. **b** Analyses of the markerless disruption of TTC1537 in ST4Δ*htk* using the *lox66–htk–lox71* cassette. (*Top*) Replacement of TTC1537 with ΔTTC1537::*lox66–htk–lox71*, and removal of *htk* from ST6. (*Bottom*) Analysis of the intergenic deletion between ΔTTC1535::*lox72* and ΔTTC1537::*lox72*. *Lane 1* HB27; *lane 2* ST6; *lanes 3–10* Km-sensitive ST6/pSH-Cre clones. The expected lengths of the other PCR products for each experiment are as follows: **a** (*top*) HB27 and ΔTTC1537::*loxP–htk–loxP* are 1947 bp and 2874 bp, respectively, **a** (*bottom*) HB27, ΔTTC1537::*loxP–htk–loxP*, and ΔTTC1537::*loxP* are 3298 bp, 3965 bp, and 2843 bp, respectively, **b** (*top*) HB27 and ΔTTC1537::*lox66–htk–lox71* are 1947 bp and 2830 bp, respectively, **b** (*bottom*) HB27, ΔTTC1537::*lox66–htk–lox71*, and ΔTTC1537::*lox72* are 3298 bp, 3921 bp, and 2799 bp, respectively.

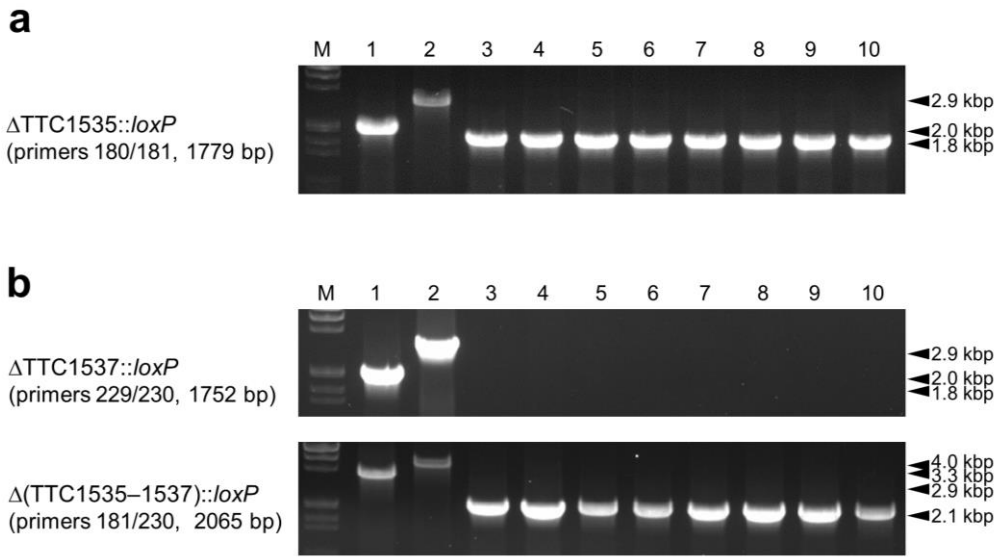

Fig. 14 PCR analyses of the double gene disruptant using the *loxP–htk–loxP* cassette. The genomic regions analyzed by PCR are indicated on the *left*. Each primer set and the expected lengths (bp) of the objective PCR products are also indicated in *parentheses*. The sizes (kbp) of the PCR products are indicated on the *right*. M indicates the lambda DNA/*Eco*RI+*Hin*dIII size marker. **a** Analysis of the markerless disruption of TTC1537 using the *loxP–htk–loxP* cassette. *Lane 1* HB27; *lane 2* ST7; *lanes 3–10* Km-sensitive ST7/pSH-Cre clones. **b** Analyses of the markerless disruption of TTC1537 in ST7Δ*htk* using the *loxP–htk–loxP* cassette. (*Top*) Replacement of TTC1537 with ΔTTC1537::*loxP–htk–loxP*, and the removal of *htk* from ST8. (*Bottom*) Analysis of the intergenic deletion between ΔTTC1535::*loxP* and ΔTTC1537::*loxP*. *Lane 1* HB27; *lane 2* ST8; *lanes 3–10* Km-sensitive ST8/pSH-Cre clones. The expected lengths of the other PCR products for each experiment are as follows: **a** HB27 and ΔTTC1535::*loxP–htk–loxP* are 1995 bp and 2901 bp, respectively, **b** (*top*) HB27 and ΔTTC1537::*loxP* are 1947 bp and 2874 bp, respectively, **b** (*bottom*) HB27, ΔTTC1537::*loxP–htk–loxP*, and ΔTTC1537::*loxP* are 3298 bp, 4009 bp, and 2887 bp, respectively.

Discussion

I herein described the construction of a Cre/*lox*-based system for the markerless gene disruption of the *T. thermophilus* HB27 strain, which is the first example of the application of the Cre/*lox* system to an extreme thermophile. In order to achieve this, I produced two genetic tools, a *loxP–htk–loxP* cassette and the *cre*-expressing plasmid pSH-Cre, which functioned in two steps: the disruption of a target gene by the conventional method with a *loxP–htk–loxP* cassette, and removal of the selection marker gene, *htk*, by *loxP*-site specific recombination using Cre recombinase encoded by pSH-Cre. I herein demonstrated the construction of the triple gene disruptant, ST3Δ*htk* (ΔTTC1454::*loxP,* ΔTTC1535*Kpn*I::*loxP,* ΔTTC1576::*loxP*) using this system. To the best of my knowledge, this is the first example of the disruption of three genes located distantly on the chromosome of *T. thermophilus* in a wild-type background. In every experiment for the removal of *htk* in the construction of the ST3Δ*htk* strain, I found that all of the Km-resistant clones incubated at 50 °C became Km-sensitive clones and completely lacked *htk*. These results indicate that the Cre/*lox*-based system was sufficiently effective for the markerless disruption of multiple target genes in *T. thermophilus*.

In a phenotypic analysis of the three markerless gene disruptants (ST1Δ*htk*, ST2Δ*htk*, and ST3Δ*htk*), only ST3Δ*htk* showed a slightly increased sensitivity to H_2O_2 compared to wild type (data not shown). The fact that a TTC1454 single disruptant showed a H_2O_2 sensitivity comparable to that of ST3Δ*htk* suggested that the disruption of TTC1454, which encodes a DNA glycosylase MutM (Henne et al. 2004), was responsible for the H_2O_2 sensitivity in ST3Δ*htk*. The result was in agreement with the observation in *T. thermophilus* HB8 strain that *mutM* disruptant is sensitive to H_2O_2 compared to wild type (Fukui et al. 2011). These results indicated that insertion of the 90-bp *loxP* site (Fig. 7a) is sufficient for the disruption of a target gene and that the removal of the coding region is not necessarily required (see Fig. 3c).

Besides the removal of the marker gene, I found undesired large chromosomal rearrangements, as exemplified by the 88.4-kbp inversion, IN(TTC1454–1535), and the 122.9-kbp deletion, Δ(TTC1454–1576)::*loxP*. According to the general strategy in other organisms (Lambert et al. 2007; Leibig et al. 2008; Yan et al. 2008; Kovács et al. 2010), I constructed another gene disruption cassette, *lox66–htk–lox71*, which used mutant *lox* sites to prevent undesired chromosomal rearrangements. The efficiency of the double gene disruption of closely located TTC1535 and TTC1537 was significantly greater with the *lox66–htk–lox71* cassette than with the *loxP–htk–loxP* cassette. Double gene disruption performed using the *loxP–htk–loxP* cassette resulted in 100% of the intergenic deletion, Δ(TTC1535–1537)::*loxP*, whereas I succeeded in producing an objective double gene disruptant using a *lox66–htk–lox71* cassette, even when second gene disruption was performed using a *loxP–htk–loxP* cassette.

I found that a *lox66–htk–lox71* cassette was valuable for markerless gene disruption; however, the removal of *htk* was less efficient than that with a *loxP–htk–loxP* cassette. My results demonstrated that inducing the removal of *htk* from a *lox66–htk–lox71* cassette required two cycles of single colony isolation at 50 °C. Although I was unable to directly construct the ΔTTC1535::*lox72* strain by two cycles of single colony isolation at 50 °C (Fig. 12), *htk* was completely removed in 12.5% (2/16) of the clones in the case of ΔTTC1537::*lox66–htk–lox71* after the second single colony isolation at 50 °C (Fig. 13b, top). Following the same procedure, I performed markerless gene disruption of TTC0366, TTC0784, and TTC1584 using *lox66–htk–lox71*; the *htk* gene was removed from 25.0% (4/16), 31.3% (5/16), and 12.5% (2/16) of the respective gene disruptants (data not shown). Collectively, these results indicate that the two cycles of single colony isolation at 50 °C are sufficient for markerless gene disruption using a *lox66–htk–lox71* cassette. Since the decrease observed in the efficiency of recombination between *lox66* and *lox71* was not described in previous studies using mesophiles

(Albert et al. 1995; Suzuki et al. 2005; Lambert et al. 2007; Leibig et al. 2008; Yan et al. 2008; Kovács et al. 2010), it may be specific to my experimental conditions, namely, temperature. Although Cre-mediated recombination was efficiently performed between two native *loxP* sites, even at 50 °C, my results indicate that the activity of Cre is reduced at this temperature.

While several other methods for the markerless disruption of multiple genes have been reported in *T. thermophilus* (Tamakoshi et al. 1999; Angelov et al. 2013; Carr et al. 2015; Wang et al. 2016), the Cre/*lox*-based system has some advantages; the wild type may be directly used as a parental strain, and the processes of the disruption of genes and removal of selectable markers are highly efficient. Even in the case of the *lox66–htk–lox71* cassette, the screening of 16 colonies is sufficient for isolating the objective gene disruptant. However, the most significant advantage of the Cre/*lox*-based system is its freedom from counter-selection in the procedure of markerless gene disruption. Most of the currently available methods are based on counter-selection for the removal of each selection marker (Tamakoshi et al. 1999; Angelov et al. 2013; Carr et al. 2015; Wang et al. 2016), which may also select clones that contain spontaneous mutations conferring resistance to counter-selection agents. The possibility of selecting these mutants has been reported in the *pheS*-based method (Carr et al. 2015). Therefore, repeated counter-selection, which is essential for multiple gene disruption, may lead to the accumulation of mutations somewhere in the genome. In this regard, this may not occur in the Cre/*lox*-based system because potential markerless clones were selected by sensitivity, not resistance to Km. In practice, a spontaneous Km-resistant colony was not observed during the construction of the triple gene disruptant, ST3Δ*htk*.

The Cre/*lox* system has been used in a broad spectrum of biological species for genetic manipulations (Nagy 2000); however, *T. thermophilus* may be one of the most suitable organisms for utilizing it because its genomic DNA has a high GC content and its higher

temperature for optimal growth. Even though the random occurrence of a 34-bp *loxP* site was expected to be very rare, the significant capacity of the mutant *lox* sites reported in many bacterial species (Suzuki et al. 2005; Lambert et al. 2007; Leibig et al. 2008; Yan et al. 2008; Kovács et al. 2010) suggested that natural sequences homologous to a *loxP* site have the potential to induce aberrant recombination by Cre. The GC content of *T. thermophilus* (69.4%) (Henne et al. 2004) differed from that of *loxP* (46.7%), and the weaker activity of Cre at 50 °C suggested in this study may be valuable for precise recombination between the *loxP* sites artificially introduced into the genome. The elimination/inactivation of Cre after the removal of *htk* is also likely to be important for the identification of distinct phenotypes because the expression of *cre* itself resulted in abnormal phenotypes in mice and several plant species without the introduction of *loxP* sites into their genomes (Schmidt et al. 2000; Coppoolse et al. 2003). In this respect, the inactivation of Cre in *T. thermophilus* may be easily accomplished by growing pSH-Cre-bearing strains at the optimal growth temperature.

The rapid development of genetic tools has resulted in significant advances in research to understand *T. thermophilus* (Averhoff 2006). However, further studies are needed on the genetics of this relatively new model organism. As of 2004, there were 736 genes with unknown functions in the genome of the *T. thermophilus* HB27 strain, and 488 of them had no substantial similarity to entries in the database comprised of all publicly available sequence data (Henne et al. 2004). The *in vivo* functions of these genes have yet to be elucidated in order to understand the physiology of *T. thermophilus* in more detail. Previous studies reported that multiple gene disruptions have played a significant role in identifying the functions of genes in *T. thermophilus* (Sakai et al. 2008; Nakane et al. 2012), and thus the development of an easy-to-use and highly efficient multiple gene disruption method will facilitate genetic analyses on this extreme thermophile. In the present study, I demonstrated that the Cre/*lox* system may be

used for *in vivo* DNA manipulation in *T. thermophilus*, which opens up the possibility of applying a number of Cre/*lox*-based techniques widely used in mesophiles for extreme thermophiles. I consider the results presented here to be a powerful addition to the genetic toolbox to study not only *T. thermophilus*, but also other extreme thermophiles.

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References

Albert H, Dale EC, Lee E, Ow DW (1995) Site-specific integration of DNA into wild-type and mutant *lox* sites placed in the plant genome. Plant J 7:649–659.

Angelov A, Li H, Geissler A, Leis B, Liebl W (2013) Toxicity of indoxyl derivative accumulation in bacteria and its use as a new counterselection principle. Syst Appl Microbiol 36:585–592.

Aoki K, Itoh T (2007) Characterization of the ColE2-like replicon of plasmid pTT8 from *Thermus thermophilus*. Biochem Biophys Res Commun 353:1028–1033.

Averhoff B (2006) Genetic systems for *Thermus*. Methods Microbiol 35:279-308.

Blas-Galindo E, Cava F, López-Viñas E, Mendieta J, Berenguer J (2007) Use of a dominant *rpsL* allele conferring streptomycin dependence for positive and negative selection in *Thermus thermophilus*. Appl Environ Microbiol 73:5138–5145.

Brouns SJJ, Wu H, Akerboom J, Turnbull AP, de Vos WM, van der Oost J (2005) Engineering a selectable marker for hyperthermophiles. J Biol Chem 280:11422–11431.

Buchholz F, Ringrose L, Angrand PO, Rossi F, Stewart AF (1996) Different thermostabilities of FLP and Cre recombinases: implications for applied site-specific recombination. Nucleic Acids Res 24:4256–4262.

Buchholz F, Angrand PO, Stewart AF (1998) Improved properties of FLP recombinase evolved by cycling mutagenesis. Nat Biotechnol 16:657–662.

Carr JF, Danziger ME, Huang AL, Dahlberg AE, Gregory ST (2015) Engineering the genome of *Thermus thermophilus* using a counterselectable marker. J Bacteriol 197:1135–1144.

Coppoolse ER, de Vroomen MJ, Roelofs D, Smit J, van Gennip F, Hersmus BJM, Nijkamp HJJ, van Haaren MJJ (2003) Cre recombinase expression can result in phenotypic aberrations in plants. Plant Mol Biol 51:263–279.

Faraldo MM, de Pedro MA, Berenguer J (1992) Sequence of the S-layer gene of *Thermus thermophilus* HB8 and functionality of its promoter in *Escherichia coli*. J Bacteriol 174:7458–7462.

Fujita A, Sato T, Koyama Y, Misumi Y (2015) A reporter gene system for the precise measurement of promoter activity in *Thermus thermophilus* HB27. Extremophiles 19:1193–1201.

Fukui K, Wakamatsu T, Agari Y, Masui R, Kuramitsu S (2011) Inactivation of the DNA repair genes *mutS*, *mutL* or the anti-recombination gene *mutS2* leads to activation of vitamin B_1 biosynthesis genes. PLoS One 6(4): e19053. https://doi.org/10.1371/journal.pone.0019053

Henne A, Brüggemann H, Raasch C, Wiezer A, Hartsch T, Liesegang H, Johann A, Lienard T, Gohl O, Martinez-Arias R, Jacobi C, Starkuviene V, Schlenczeck S, Dencker S, Huber R, Klenk HP, Kramer W, Merkl R, Gottschalk G, Fritz HJ (2004) The genome sequence of the extreme thermophile *Thermus thermophilus*. Nat Biotechnol 22:547–553.

Hiratsu K, Shiotani S, Makino K, Nunoshiba T (2013) Construction of a *supF*-based system for detection of mutations in the chromosomal DNA of *Arabidopsis*. Mol Genet Genom 288:707–715.

Hoess RH, Abremski K (1985) Mechanism of strand cleavage and exchange in the Cre-*lox* site-specific recombination system. J Mol Biol 181:351–362.

Hoseki J, Yano T, Koyama Y, Kuramitsu S, Kagamiyama H (1999) Directed evolution of thermostable kanamycin-resistance gene: a convenient selection marker for *Thermus thermophilus*. J Biochem 126:951–956.

Kovács ÁT, van Hartskamp M, Kuipers OP, van Kranenburg R (2010) Genetic tool development for a new host for biotechnology, the thermotolerant bacterium *Bacillus coagulans*. Appl Environ Microbiol 76:4085–4088.

Koyama Y, Hoshino T, Tomizuka N, Furukawa K (1986) Genetic transformation of the extreme thermophile *Thermus thermophilus* and of other *Thermus* spp. J Bacteriol 166:338–340.

Lambert JM, Bongers RS, Kleerebezem M (2007) Cre-*lox*-based system for multiple gene deletions and selectable-marker removal in *Lactobacillus plantarum*. Appl Environ Microbiol 73:1126–1135.

Leibig M, Krismer B, Kolb M, Friede A, Götz F, Bertram R (2008) Marker removal in staphylococci via Cre recombinase and different *lox* sites. Appl Environ Microbiol 74:1316–1323.

Leis B, Angelov A, Li H, Liebl W (2014) Genetic analysis of lipolytic activities in *Thermus thermophilus* HB27. J Biotechnol 191:150–157.

Nagy A (2000) Cre recombinase: the universal reagent for genome tailoring. Genesis 26:99–109.

Nakamura A, Takakura Y, Kobayashi H, Hoshino T (2005) *In vivo* directed evolution for thermostabilization of *Escherichia coli* hygromycin B phosphotransferase and the use of the gene as a selection marker in the host-vector system of *Thermus thermophilus*. J Biosci Bioeng 100:158–163.

Nakane S, Nakagawa N, Kuramitsu S, Masui R (2012) The role of the PHP domain associated with DNA polymerase X from *Thermus thermophilus* HB8 in base excision repair. DNA Repair (Amst) 11:906–914.

Ohta T, Tokishita S, Imazuka R, Mori I, Okamura J, Yamagata H (2006) β-Glucosidase as a reporter for the gene expression studies in *Thermus thermophilus* and constitutive expression of DNA repair genes. Mutagenesis 21:255–260.

Ohtani N, Tomita M, Itaya M (2010) An extreme thermophile, *Thermus thermophilus*, is a polyploid bacterium. J Bacteriol 192:5499–5505.

Oshima T, Imahori K (1974) Description of *Thermus thermophilus* (Yoshida and Oshima) comb. nov., a nonsporulating thermophilic bacterium from a Japanese thermal spa. Int J Syst Bacteriol 24:102–112.

Sakai T, Tokishita S, Mochizuki K, Motomiya A, Yamagata H, Ohta T (2008) Mutagenesis of uracil-DNA glycosylase deficient mutants of the extremely thermophilic eubacterium *Thermus thermophilus*. DNA Repair (Amst) 7:663–669.

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Schmidt EE, Taylor DS, Prigge JR, Barnett S, Capecchi MR (2000) Illegitimate Cre-dependent chromosome rearrangements in transgenic mouse spermatids. Proc Natl Acad Sci USA 97:13702–13707.

Short JM, Fernandez JM, Sorge JA, Huse WD (1988) λ ZAP: a bacteriophage λ expression vector with *in vivo* excision properties. Nucleic Acids Res 16:7583–7600.

Sternberg N, Hamilton D (1981) Bacteriophage P1 site-specific recombination: I. Recombination between *loxP* sites. J Mol Biol 150:467–486.

Suzuki N, Nonaka H, Tsuge Y, Inui M, Yukawa H (2005) New multiple-deletion method for the *Corynebacterium glutamicum* genome, using a mutant *lox* sequence. Appl Environ Microbiol 71:8472–8480.

Takayama G, Kosuge T, Sunamura S, Matsui I, Ishikawa K, Nakamura A, Hoshino T (2004) Use of a *Thermus thermophilus* host-vector system for expression of genes from the hyperthermophilic archaeon *Pyrococcus horikoshii*. J Japanese Soc Extrem 3:28-36.

Tamakoshi M, Yaoi T, Oshima T, Yamagishi A (1999) An efficient gene replacement and deletion system for an extreme thermophile, *Thermus thermophilus*. FEMS Microbiol Lett 173:431–437.

Wang L, Hoffmann J, Watzlawick H, Altenbuchner J (2016) Markerless gene deletion with cytosine deaminase in *Thermus thermophilus* strain HB27. Appl Environ Microbiol 82:1249–1255.

Yan X, Yu HJ, Hong Q, Li SP (2008) Cre/*lox* system and PCR-based genome engineering in *Bacillus subtilis*. Appl Environ Microbiol 74:5556–5562.

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119.

Yokoyama S, Hirota H, Kigawa T, Yabuki T, Shirouzu M, Terada T, Ito Y, Matsuo Y, Kuroda Y, Nishimura Y, Kyogoku Y, Miki K, Masui R, Kuramitsu S (2000) Structural genomics projects in Japan. Nat Struct Mol Biol 7:943–945.

Academic achievements

Publication

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Oral presentations

[1] ○塩谷詩織、外川陽一郎、布柴達男、平津圭一郎．高度好熱菌 *Thermus thermophilus* の突然変異検出系の構築．変異機構研究会第２９回夏の学校、あうる京北（京都府立ゼミナールハウス)、２０１６年９月

[2] ○外川陽一郎、塩谷詩織、布柴達男、平津圭一郎．高度好熱菌 *Thermus thermophilus* の *supF* 突然変異検出系の構築．日本農芸化学会２０１７年度大会、京都女子大学、２０１７年３月

[3] ○外川陽一郎、布柴達男、平津圭一郎．Cre/*lox* システムを用いた高度好熱菌 *Thermus thermophilus* HB27 のマーカーレス多重遺伝子破壊法の開発．変異機構研究会第３０回夏の学校、あうる京北（京都府立ゼミナールハウス)、２０１７年９月

Poster presentations

[1] ○塩谷詩織、外川陽一郎、布柴達男、平津圭一郎．高度好熱菌 *Thermus thermophilus* の突然変異検出系の構築．日本環境変異原学会第４５回大会、つくば国際会議場、２０１６年１１月

[2] ○塩谷詩織、外川陽一郎、布柴達男、平津圭一郎．高度好熱菌 *Thermus thermophilus* の突然変異検出系の構築 II．日本分子生物学会第３９回年会、パシフィコ横浜、２０１６年１２月

[3] ○外川陽一郎、布柴達男、平津圭一郎. Cre/*lox* システムを用いた高度好熱菌 *Thermus thermophilus* HB27 のマーカーレス多重遺伝子破壊法の開発．生命科学系学会合同年次大会　日本分子生物学会第４０回年会、神戸ポートアイランド、２０１７年１２月