Selection-free markerless genome manipulations in the polyploid bacterium *Thermus thermophilus*

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Abstract

A genome manipulation approach based on double-crossover homologous recombination was developed in the polyploid model organism *Thermus thermophilus* HB27 without the use of any selectable marker. The method was established and optimized by targeting the megaplasmid-encoded β -glucosidase gene *bgl*. When linear and supercoiled forms of marker-free suicide vector were used for transformations, the frequencies of obtaining apparent Bgl⁻ mutant were 10^{-5} and 10^{-3} , respectively; while the frequency could reach 10^{-2} when transformation with concatemer form of the same vector. All randomly selected Bgl⁻ colonies from the transformations were found to be true *bgl* knockout mutants. Thus, markerless gene deletion mutants could be constructed in *T. thermophilus* by the direct selection-free method. The functionality of this approach was further demonstrated by deletion of one chromosomal locus (TTC_0340–0341) as well as by generation of a reporter strain for the phytoene synthase promoter (*P_{prtB}*), homozygous mutants of the both targets could also be detected with a frequency of approximately 10^{-2} . During the genome modification process, heterozygous cells carrying two different alleles at a same locus (e.g., *bgl* and *pyrE*) could also be generated. However, in the absence of selection pressure, these strains could rapidly convert to homozygous strains containing only one of the two alleles. This indicated that allele segregation could occur in the heterozygous *T. thermophilus* cells, which probably explained the ease of obtaining homozygous gene deletion mutants with high frequency (10^{-2}) in the polyploid genomic background, as after the mutant allele had been introduced to the target region, allele segregation would lead to homozygous mutant cells. This marker-free genome manipulation approach does not require phenotype-based screens, and is applicable in gene deletion and tagging applications.

Keywords *Thermus thermophilus* · Polyploid · Marker-free genome manipulations · Allele segregation

Introduction

Thermus thermophilus has been established as a model organism for studying thermophilic bacteria, this is mainly due to the ease of laboratory cultivation and the presence of an efficient natural transformation system (Koyama et al. 1986; Hidaka et al. 1994). The genome of *T. thermophilus* HB27 contains a chromosome (1.89 Mb) and a megaplasmid (0.23 Mb), over the last years, several genetic manipulation

tools have been developed for this strain (de Grado et al. 1999; Cava et al. 2009; Fujita et al. 2015; Fujita and Misumi 2017). The most convenient method for the generation of knockout mutants in *T. thermophilus* is based on gene replacement with antibiotic resistance markers, i.e. kanamycin (Hashimoto et al. 2001), hygromycin (Nakamura et al. 2005) or bleomycin resistance genes (Brouns et al. 2005). This approach has inevitable disadvantages, e.g., the specific antibiotic resistance gene cannot be reused and polar effects for down-stream genes are possible. Alternative markerless gene deletion strategy has become attractive for research on *T. thermophilus*, several methods that rely on selection/counter-selection have been developed, in which the *pyrE* (Tamakoshi et al. 1999), *rpsL* (Blas-Galindo et al. 2007), *bgl* (Angelov et al. 2013), *crtB* (Fujita et al. 2015), *pheS* (Carr et al. 2015) or *codA* (Wang et al. 2016) gene was used as a counter-selectable marker, respectively. Some of these strategies could be used in wild-type background (e.g., the

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pheS, *codA*, *bgl* system), but the others were only applicable in corresponding mutant backgrounds (e.g., the *pyrE*, *rpsL*, *crtB* system). Nevertheless, this strategy requires two selectable markers, and notably, the frequency of spontaneous mutants that were resistant to counter-selection agent was considerably high (Blas-Galindo et al. 2007; Carr et al. 2015). Cre/loxP-based markerless gene disruption system has recently been reported (Togawa et al. 2018), however, it is only applicable for cells grown at the lowest growth temperature (50 °C), and undesired deletion or inversion of genomic regions might occur. Taken together, it seems that selection-free markerless genome manipulation methods are required for *T. thermophilus*.

T. thermophilus has been suggested to be polyploid which contains 4 to 5 genome copies under slow growth condition (Ohtani et al. 2010). There seems to be a contradiction between polyploidy and the ease with which homozygous gene deletion mutants can be generated in *T. thermophilus*. It is conceivable that during gene deletion process, heterozygous mutants containing both the wild-type and mutant alleles at the target gene locus would be possible, and the frequency of these strains would be higher than their counterparts. On the other hand, when a non-essential locus was targeted with a DNA fragment which carries an antibiotic resistant marker flanked by sequences from both sides of that locus, practically all of the antibiotic resistant colonies obtained are homozygous gene replacement mutants for that locus (Li et al. 2015; Ohtani et al. 2015). In this study, a versatile method which allows the introduction of mutations into the genome of *T. thermophilus* HB27 without using any marker for selection was developed. By transforming the wild-type strain with suicide vectors, which contained two homology arms of target genes and no selection marker for *T. thermophilus*, knockout and other mutants could be obtained with a frequency of approximately 10^{-2} . Further, it was found that heterozygous mutant cells could be formed during gene deletion process, however, this kind of mutants was genetically unstable, which could convert to homozygous cells carrying either the mutant or wild-type allele. The finding provides an explanation for the possibility of obtaining homozygous mutants with high frequencies in the polyploid *T. thermophilus* cells, and was utilized to optimize the selection-free markerless genome manipulation method.

Materials and methods

Bacterial strains and growth conditions

Escherichia coli DH5 α was used as a host strain for plasmid constructions and was grown at 37 °C in LB medium (contains 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl). *T. thermophilus* HB27 wild type (DSM 7039) and derivative

strains were grown at 60 °C or 70 °C in TB medium. TB medium contained 8 g/L trypticase peptone (BD Biosciences), 4 g/L yeast extract, and 3 g/L NaCl, and has a pH of 7.5. The growth media were supplemented with ampicillin (100 μ g/mL for *E. coli*), kanamycin (50 μ g/mL for *E. coli* and 20 μ g/mL for *T. thermophilus*), bleomycin ("Bleocin", Calbiochem, 15 μ g/mL for *E. coli* and 3 μ g/mL for *T. thermophilus*) or X-Gluc (5-bromo-4-chloro-3-indolyl- β -D-glucopyranoside, 100 μ g/mL) when necessary. All chemical reagents were purchased from Sigma-Aldrich.

Plasmid construction

All plasmids and strains used are listed in Table S1 and all primers used are listed in Table S2. For construction of the plasmids pUC- Δbgl and pUC- $\Delta 0340$ –0341, the flanking regions about 1 kbp of the *bgl* gene (TTP_0042, 1296 bp, encoding β -glucosidase, NCBI accession NO. WP_011174517.1) and TTC_0340–0341 loci (in total 1498 bp, NCBI accession NO.: WP_011172790.1 and WP_011172791.1, respectively) were, respectively, PCR amplified from *T. thermophilus* HB27, using primers that permitted to generate sufficient overlaps between each other; the two flanking regions of each gene locus were then cloned into *Xba*I digested pUC18 vector via Gibson Assembly method (New England Biolabs) (Gibson et al. 2009). For the construction of pUC- $\Delta crtB$::*bgl*, the complete *bgl* gene flanked by the upstream and downstream homology arms of *crtB* gene (TTP_0057, 870 bp, encoding phytoene synthase, NCBI accession NO. WP_011174502.1) was introduced into *Xba*I digested pUC18 vector via the same approach. For the generation of pJ- $\Delta pyrE$::*blm* and pJ- $\Delta pyrE$::*kat*, a 2.3 kbp *pyrFE* region (*pyrE*, 552 bp, NCBI accession NO. WP_011173763.1) was PCR amplified and cloned in pJET1.2 giving pJ-*pyrFE*; two *Nde*I sites were introduced to pJ-*pyrFE* through a mutagenesis tool (Change-IT Multiple Mutation Site Directed Mutagenesis Kit, Affymetrix, USA) at positions –3 and +551 relative to the ORF of the *pyrE* gene. The mutagenized pJ-*pyrFE* was then digested with *Nde*I to excise *pyrE*, and ligated with a bleomycin resistance marker *blm* (sequence data from Brouns et al. 2005) or a kanamycin resistance marker *kat* (sequence data from Hashimoto et al. 2001) via the Gibson Assembly method, resulting in pJ- $\Delta pyrE$::*blm* and pJ- $\Delta pyrE$::*kat*, respectively.

Transformation experiments

The transformation experiments of *T. thermophilus* were essentially performed as described by de Grado et al. (1999). pUC- Δbgl , pUC- $\Delta 0340$ –0341 were transformed into the wild-type strain, and pUC- $\Delta crtB$::*bgl* was transformed in the Δbgl background. The plasmid preparations used for transformations were performed using the

GenElute™ Plasmid Midiprep Kit (Merck). The transformation experiments with different forms of DNA were performed by addition of 10 µg of plasmid DNA into 1 mL exponentially-growing cell culture. The plasmid DNAs had been enzymatically treated to obtain linear, relaxed or open circular (*oc*) and concatemer (*con*) forms. For the preparation of the linear and concatemer forms, 20 µg (1 µg/µL) pUC- Δbgl was digested with *NotI*, followed by heat inactivation of the enzyme, giving linear form; 10 µg of the linearized DNA was then treated with 5 U of T4 ligase (Thermo Scientific) followed by incubation at 16 °C for 16 h, and due to the high DNA concentration, the *con* form was obtained. Topoisomerase IV from *E. coli* (Inspiral, Norwich, UK) was used to obtain the *oc* plasmid form as described by Kato et al. (1992). Native plasmid was treated at the same way but omitting the enzyme in the steps and represented the supercoiled (*sc*) form. For each plasmid form, three independent transformations were carried out.

Isolation and identification of marker-free deletion mutants

To obtain *bgl* gene clean deletion mutants, dilutions of the transformation reactions were plated on TB agar plates supplemented with X-Gluc, and after incubation at 70 °C for 2 days, yellow colonies (Bgl^-) were selected. PCR (with primers flanking the target region) and Southern blot were then used to determine the genotype of these colonies, from which Δbgl mutants were identified. For the Southern blot, a 663 bp biotin-labelled PCR fragment of the TTP0043 ORF was used as a probe, genomic DNA was digested with *BamHI* (the deleted *bgl* region contains one *BamHI* site); to obtain $\Delta 0340\text{--}0341$, the transformation mixtures were also diluted and streaked on TB plates. 96 colonies were randomly picked out and grown in a 96-well plate, genomic DNAs were isolated by pooling method (8 colonies in one pool). PCR was then used to determine the strain pool which contained the $\Delta 0340\text{--}0341$ allele. Sequentially, the specific colony containing the mutant allele was sorted out by the same PCR method. The colony was re-grown and re-streaked in TB medium without selection, from which homozygous $\Delta 0340\text{--}0341$ mutants were identified. For Southern blot confirmation of the $\Delta 0340\text{--}0341$ mutant, a 959-bp DNA sequence located in the downstream of TTC_0340–0341 was used as template; genomic DNA was digested with *SacII*. For the generation of $\Delta crtB::bgl$, the transformation reactions were streaked on TB-X-Gluc plates, followed by incubation under light. The colonies projecting blue color were then analyzed by PCR, from which homozygous $\Delta crtB::bgl$ mutants were isolated.

Determination of frequency of obtaining gene deletion mutants

The frequency of obtaining apparent Δbgl mutants was defined as the ratio of yellow colonies (Bgl^-) to the total viable counts on TB agar plates supplemented with X-Gluc. Statistic analysis of the transformation frequency data was performed with the Student's *t* test.

Enzyme assays

The exponentially growing cells were collected, and their β -glucosidase activities were measured using pNP- β -glucoside as a substrate, according to the method described in (Ohta et al. 2006). The assays were performed at 80 °C for 60 min in phosphate buffer (pH 7.0). For each strain, three independently grown cultures were assayed.

Construction of a stable heterozygous strain and allele segregation experiment

The allele exchange vectors pJ- $\Delta pyrE::kat$ and pJ- $\Delta pyrE::blm$ were linearized by *HindIII*, respectively, then transformed into *T. thermophilus* HB27, generating two homozygous strains, i.e., $\Delta pyrE::kat$ and $\Delta pyrE::blm$, which carried either the *kat* or the *blm* resistance marker at the *pyrE* gene locus. Sequentially, linearized pJ- $\Delta pyrE::blm$ was transformed into the $\Delta pyrE::kat$ strain, by selecting on TB agar plates supplemented with both kanamycin and bleomycin, the heterozygous strain KB01 was obtained. Southern blot and DNA sequencing were used to confirm the genotype of the heterozygous strain. For the Southern blot, biotin-labelled *pyrF* gene was used as a probe, genomic DNAs were digested with *BamHI*, homozygous strains $\Delta pyrE::kat$ and $\Delta pyrE::blm$ were used as controls. The allele segregation experiment was performed as follows: KB01 was initially grown in 5 mL of TB medium supplemented with both antibiotics for 12 h; the cells were then washed three times by 1 × PBS buffer and inoculated in 50 mL antibiotic-free TB medium (time point 0). Growth was continued for 72 h at 70 °C with agitation. Samples (2 mL) were taken at various time points as indicated in the results, and were used to determine the fraction of each phenotype by plating the cells on antibiotic-free TB plates and re-streaking 50 colonies on plates supplemented with bleomycin or kanamycin.

Results

Direct and selection-free deletion of the *T. thermophilus bgl* gene

Wild-type *T. thermophilus* HB27 cells express β -glucosidase (Bgl), which is encoded by the megaplasmid-located gene *bgl* (TTP_0042). It has been previously shown that the *T. thermophilus* Δbgl strain shows low Bgl activity and that the gene can be used as a reporter in gene expression studies (Ohta et al. 2006). Thus, it is easy to distinguish Bgl[−] from Bgl⁺ colonies on agar plates supplemented with indicator substrate X-Gluc. For the deletion of the *bgl* gene, a suicidal vector containing fused upstream and downstream homology regions of *bgl*, designated pUC- Δbgl was used (see “Materials and methods”). After transformation with a series of different amount of untreated plasmid preparations (supercoiled) and plating on selection-free TB-X-Gluc agar plates, the observed maximal frequency of apparent Bgl[−] colonies reached 10^{-3} per viable count (Fig. 1a). Under the same transformation condition but without the addition of DNA, the frequency of obtaining spontaneous Bgl[−] mutants was lower than 10^{-8} . Eight apparent Bgl[−] colonies were randomly selected, PCR and Southern blot were used to determine the genotype. In all cases tested, the Bgl[−] cells were found to carry the expected *bgl* deletion, and no suicide plasmid DNA as well as no wild-type *bgl* allele was present (Fig. 1b, c). This indicated that in these colonies, the wild-type *bgl* allele had been completely exchanged by the Δbgl allele carried by the suicide vector pUC- Δbgl . The Δbgl mutants kept stable pheno- and genotype in non-selective medium. The intracellular β -glucosidase activities in these Δbgl mutant cells were approximately 10 folds lower than that in the wild type (Fig. 1d), which is consistent with the determined genotypes and in agreement with the previous reports (Ohta et al. 2006). The residual low activity in the mutants is probably due to unspecific cleavage of the substrate by family 2 glycoside hydrolases encoded by ORFs TTP_0220–0222 (Moreno et al. 2002; Park and Kilbane 2004). The above results indicated that practically all apparent Bgl[−] colonies obtained from the transformation with supercoiled pUC- Δbgl are true *bgl* gene clean deletion mutants, resulting from double-crossover event between pUC- Δbgl and the homologous genomic DNA sequence. Taken together, these experiments showed that gene clean-deletion mutants could be obtained at a considerably high frequency in the polyploid *T. thermophilus* cells by the direct selection-free method.

Effect of the topology of the suicidal vector on the frequency of *bgl* knockouts

Using the above gene deletion protocol as a convenient assay, the apparent frequencies of obtaining *bgl* gene

Fig. 1 Deletion of the *bgl* gene in *T. thermophilus* using direct selection-free method. **a** Observed frequency of obtaining Bgl[−] colonies after transformation with different amounts of native preparations of the suicide vector pUC- Δbgl . The mean and SD were from three independent transformations. **b**, **c** Confirmation of the Δbgl genotype of 8 apparent Bgl[−] colonies by PCR (**b**) and Southern blot (**c**). The in silico predicted sizes of the wild-type and clean-deleted *bgl* regions are indicated with arrows on the right side of each image. **d** Intracellular β -glucosidase activity (Miller units) of the 8 Δbgl mutants and the wild-type strain. Shown are the mean and SD from three independent assays

knockouts with different forms of the pUC- Δbgl plasmid were measured. Prior to transformation, one plasmid preparation was split and subjected to enzymatic modifications which yielded linear, relaxed or open circular (*oc*) and concatemer (*con*) forms. The native plasmid preparation was considered to consist mainly of the supercoiled form (*sc*), although small amounts of the other plasmid forms were also present. The results of these transformations are summarized in Fig. 2. The linear form was found to yield significantly fewer Bgl[−] colonies than the other DNA types ($P = 1.20 \times 10^{-4}$, $n = 14$, t test between the linear and *con* groups), while the frequency of the *sc* and *oc* forms was the same ($P = 0.78$, $n = 14$). Significantly more Bgl[−] mutants were observed when the *T. thermophilus* HB27 cells were transformed with the *con* DNA form as compared to the *sc* or *oc* form ($P = 1.69 \times 10^{-3}$, $n = 14$, t test between the *oc* and *con* groups), which reached 10^{-2} .

Fig. 2 Frequency of obtaining apparent Δbgl colonies after transformation with different forms of the pUC- Δbgl vector. The data are the mean and SD of three independent measurements; *linear* linearized plasmid, *sc* supercoiled, *oc* open circular, *con* concatemers

Theoretically, the observed high frequency of *bgl* knockouts could be the result of a much higher growth rate of the Δbgl cells, which perhaps could outgrow the wild type in the 2 h incubation step after DNA addition during the transformation procedure. To test this, a 1:1 mixture of the Δbgl and the wild-type strains were grown in TB medium and followed the viable cell counts of each strain by plating on agar plates supplemented with X-Gluc. The ratio of the counts of the two strains remained constant, even after 8 h of incubation ($P=0.44$, $n=6$, t test for the significance of the variation between the time points 0 and 8 h), which clearly showed that the high frequency of obtaining Δbgl mutants was not due to growth rate effects. Taken together, this experiment succeeded in increasing the frequency of obtaining knockout mutants generated via the selection-free method.

Allele segregation in heterozygous cells

During the process of generating *bgl* knockouts by the direct method, some transformants were found to show intermediate phenotype on TB-X-Gluc plate, i.e. the color of the colonies was between yellow and blue (Fig. 3a). The genotypes of 9 “intermediate” colonies were determined by Southern blot. As shown in Fig. 3b, the Δbgl signal was not from pUC- Δbgl vector integration event, thus the tested colonies were carrying both the wild-type and Δbgl alleles simultaneously at the *bgl* locus, i.e. they were heterozygous wt/ Δbgl mutants. Upon growing and re-streaking the heterozygous colonies on TB-X-Gluc plates, progeny colonies showing either yellow (Bgl⁻) or blue color (Bgl⁺) were observed (Fig. 3c). Southern blot analysis showed that the yellow

colonies only contained the Δbgl allele, and the blue ones only contained the wild-type allele (Fig. 3d). These results indicated that allele segregation had occurred in the heterozygous cells.

To further support the suggestion, a conditionally stable heterozygous strain KB01 was constructed (see “Materials and methods”). Southern blot and DNA sequencing were used to confirm the genotype of the KB01 strain, which excluded the possibility of vector integration and existence of any copy of wild-type *pyrE* allele, and explicitly showed that in KB01 the *pyrE* gene was completely replaced by both the *kat* and *blm* resistance markers simultaneously (Fig. 3e). Using TB medium supplemented with both kanamycin and bleomycin, the heterozygous state of the KB01 strain could be stably maintained. To detect and quantify allele segregation in *T. thermophilus* heterozygous cells, KB01 was grown in the absence of selection pressure for 72 h. The culture was sampled at various time points, and the changes of the fraction of each phenotype (Kat^RBlm^R, Kat^RBlm^S, and Kat^SBlm^R) in the population were scored by spreading the samples on antibiotic-free plates and restreaking 50 colonies for each time point on plates containing bleomycin or kanamycin. As expected, during growth without selection pressure, the fraction of heterozygous cells (as determined by the resistance phenotype) decreased and that of homozygous cells increased apparently (Fig. 3f). The data from the above experiments further confirmed that the polyploid *T. thermophilus* cells could form heterozygous state, which would convert to homozygous state under the growth conditions without selection pressure. Together, the allele segregation mechanism probably explains the ease with which to obtain homozygous gene deletion mutants with high frequency in the polyploid *T. thermophilus* cells as observed in Fig. 2.

Application of the direct and selection-free gene exchange method to other genomic loci

It was of interest to test whether the above high efficient gene clean deletion method, was applicable to target other genes/loci than *bgl*. In addition, since the *bgl* gene is located on the megaplasmid, it was not clear if chromosomal sequences can be deleted with comparably high frequency. To test this, a suicide vector that targeted two continuous genes (ORF TTC_0340 and TTC_0341, encoding putative alpha/beta hydrolases) was used. The *con* form of this plasmid was transformed in the wild-type strain and appropriate dilutions of the transformation mixture were streaked on non-selective agar plates. 96 colonies were selected and distributed into 12 pools (i.e. 8 colonies in one pool) which were subjected to PCR detection of the $\Delta 0340$ – $\Delta 0341$ allele. One pool was found to contain the $\Delta 0340$ – $\Delta 0341$ allele (Supplementary Fig. S1a), and from which two colonies were determined to contain the mutant allele in a heterozygous state (Fig.

Fig. 3 Allele segregation in heterozygous *T. thermophilus* cells. **a** Phenotype of heterozygous *wt/Δ-bgl* colonies on TB-X-Gluc plate. Intermediate colonies (heterozygous, *Bgl*^{+/−}) are pointed with arrows, yellow colonies (homozygous, *Bgl*^{−/−}) are indicated with triangles. **b** Genotype of 9 colonies showing intermediate phenotype were determined by Southern blot. **c, d** The resultant phenotype (**c**) and genotype (**d**) of the heterozygous *wt/Δ-bgl* mutant after growing without selection and re-streaking on TB-X-Gluc plate. *wt-bgl*: genotype of the blue colonies, *Δ-bgl*: genotype of the yellow colonies. **e** Genotype confirmation of the stable heterozygous strain KB01 by Southern blot. The expected sizes are indicated with arrows. **f** Allele segregation kinetics of the KB01 strain. Shown are changes of the fraction of each phenotype (*Kat*^R*Blm*^R—grey bar, *Kat*^R*Blm*^S—black bar and *Kat*^S*Blm*^R—white bar) in the population

S1b). One of the two colonies was grown without selection for 16 h, and the culture was plated on selection-free plates. PCR and Southern blot showed that the resultant colonies were homozygotes containing either the mutant or wild-type 0340–0341 allele (Fig. 4a, b). Therefore, the allele segregation property in heterozygous cells contributed to simplify the marker-free deletion method. As when the mutant allele was found in a heterozygous state with the wild-type allele, it was sufficient to grow the cells from such colonies in liquid medium and re-streak on selection-free agar plates to obtain homozygous gene deletion mutants. The above example also showed that this method could be used in genomic loci without the need to perform any phenotype-based screens.

It has been shown that the *T. thermophilus* phytoene synthase gene *crtB* (TT_P0057) is under light-dependent transcriptional control (Takano et al. 2011). Thus, a reporter *T.*

thermophilus strain in which the *crtB* gene replaced with the *bgl* gene was created. It was achieved by transforming the *con* form of the pUC- Δ *crtB*::*bgl* plasmid to the *T. thermophilus* Δ *bgl* strain, and a phenotype screen based on the known response of *PcrtB* to illumination was performed. It was expected that in Δ *bgl* Δ *crtB*::*bgl* strain, the expression of the *bgl* ORF should be light-inducible. In agreement with the data obtained for the knockout mutants, approximately 1 out of 100 colonies from the transformation mixture turned blue on TB-X-Gluc agar plates only after light exposure, indicating a successful allelic exchange. The genotype of 6 of these blue colonies was verified by PCR, which showed that *crtB* was completely replaced with *bgl* (Fig. 4c). This experiment indicated that the described method was useful also for performing precise genome modifications other than deletions.

Discussion

A genome manipulation method based on direct double-crossover homologous recombination was created, it was initially established and optimized using the *bgl* gene as the target for deletion, and then utilized in the TTC_0340–0341 and TTP_0057 loci. Stable homozygous clean deletion mutants could be obtained at a frequency of 10^{-2} when the concatemer (*con*) plasmid forms were used for transformation. After the mutant allele had been introduced in the genome by transformation, cells would become heterozygous, however, subsequent allele segregation in these cells would lead to homozygous mutant cells, this should be the main reason for the ease of obtaining homozygous gene deletion mutants in the polyploid *T. thermophilus* cells. The other properties contributing to the high frequency of obtaining knockout mutants are that *T. thermophilus* is able to uptake DNA and execute homologous recombination efficiently. In fact, *T. thermophilus* has been shown to be the microorganism which possesses the highest efficient natural transformation system known to date (Salzer et al. 2014), the DNA uptake efficiency of *T. thermophilus* HB27 has been estimated at approx. $40 \text{ kbp} \times \text{s}^{-1} \times \text{cell}^{-1}$ (Schwarzenlander and Averhoff 2006). It could be expected that compared with monomeric *sc* or *oc* plasmid molecules the concatemer form had led to the uptake of a larger number of mutant allele copies per cell (Fig. 2). The linear plasmid form generated the lowest frequency of knockout mutants (10^{-5}). A possible explanation is that degradation of the free ends by exonucleases occurred severely in the linear DNA, which might decrease the length of the homology regions needed for recombination. A similar frequency of obtaining targeted gene deletion mutants with linearized plasmids has been reported in the presence of selection for other genes in *T. thermophilus* (Schwarzenlander and Averhoff 2006).

Fig. 4 Application of the selection-free gene exchange method in *T. thermophilus* TTC_0340–0341 and TTP_0057 loci. **a**, **b** Heterozygous Δ 0340–0341 cells were re-grown and re-streaked on TB plate, from which 4 colonies were randomly selected and the genotypes were determined by PCR (**a**) and Southern blot (**b**). **c** Genotype determination of 6 apparent Δ *crtB*::*bgl* colonies by PCR. The expected sizes are indicated with arrows on the left side of each image

The phenomenon that the linear plasmid form would yield lower transformation frequency was also observed in some other bacteria, for example, in *Lactobacillus plantarum*, the covalently closed circular plasmid DNA is capable of electroporating at a frequency 500-fold higher than with linearized molecule (Thompson et al. 1997); in *Bacillus subtilis*, Ohse et al. (1997) reported that the transformation efficiency was zero for the linearized plasmid form.

It has been suggested that polyploid extreme prokaryotes generally possess efficient homologous recombination (HR) systems, which will facilitate repairment of double-strand breaks triggered by radiation, desiccation or other extreme conditions (Soppa 2013, 2014, 2017; Zerulla and Soppa 2014). In *Deinococcus radiodurans* and *Halobacterium salinarum*, the ionizing radiation-shattered chromosomal fragments could be rapidly reassembled due to homologous recombination among multiple broken genome copies (Kottemann et al. 2005; Zahradka et al. 2006; Slade et al. 2009). In *T. thermophilus*, the *recA* gene (encoding one of the recombinases for HR) deletion mutant had extremely low viability with the increase of growth temperature (Castán et al. 2003). This indicated that *T. thermophilus* might be suffered from frequent double-strand DNA breaks during growth at high temperature, and in response, the polyploid cells would provide efficient HR system for DNA repair. Taken together, the prominent DNA uptake rate in combination with the efficient homologous recombination system of *T. thermophilus* also allow the observed high frequency of obtaining gene deletion mutants in this bacterium.

During the deletion of the *bgl* gene, heterozygous mutant containing both the mutant and wild-type alleles at the *bgl* locus simultaneously were observed, and this kind of mutant was unstable, since after growing and re-streaking, colonies that were homozygous for either the wild-type or mutant allele were obtained (Fig. 3a–d), which indicated that allele segregation had occurred in the heterozygous cells. Through constructing a stable heterozygous *T. thermophilus* strain (KB01) followed by performing allele segregation kinetics experiment (Fig. 3e, f), the above suggestion was strengthened. This allele segregation phenomenon has also been observed in the HB8 strain (Ohtani et al. 2010). The intrinsic mechanism of the allele segregation in *T. thermophilus* has not been elucidated, however, it is possibly caused either by random chromosome segregation or gene conversion, which will both result in homogeneity in the progeny. Random segregation of chromosome copies has indeed been proposed for some polyploid prokaryotes, such as the euryarchaeotal species *Methanococcus jannaschii* (Malandrin et al. 1999), and the cyanobacterial species *Anabaena* sp. 7120 (Hu et al. 2007) and *Synechocystis* sp. (Schneider et al. 2007) based on DNA content analysis in dividing cells. On the other hand, allele equalization via gene conversion has also been demonstrated in polyploid methanogenic and halophilic archaea,

i.e., *Methanococcus maripaludis* and *Haloferax volcanii* (Hildenbrand et al. 2011; Lange et al. 2011). Nevertheless, the property could be well utilized in the described selection-free gene deletion method, when deletion of the non-essential genes producing phenotypes which are not easy to be detected, it is sufficient to obtain colonies carrying the mutant allele first, and then grow the cells of such colonies without selection pressure to promote formation of homozygous knockout mutants.

In conclusion, based on the merits of *T. thermophilus*, i.e., the efficient natural competence, the high homologous recombination frequency, and the ability that heterozygous cells can be converted to homozygous cells, a selection-free genome manipulation method was created. The main advantages of this approach are (i) to date, it is the most direct and simplest method compared with all other reported genome manipulation systems in *T. thermophilus*, as no selection and counterselection markers or other elements are needed, which will shorten the genome manipulation procedure to a great extent; (ii) it can be used in the wild-type background, i.e., no prior genetic modifications are necessary; (iii) spontaneous mutants are impossible to be detected using this protocol, as the mutants can be determined based on genotypes directly other than phenotypes. Further, apart from its use in *T. thermophilus*, it is possibly used in other polyploid bacteria or archaea, such as *H. volcanii*, as long as they have efficient homologous recombination systems and/or the ability to convert heterozygotes into homozygotes.

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Compliance with ethical standards

Conflict of interest There are no conflicts of interest.

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