# An Extreme Thermophile, *Thermus thermophilus*, Is a Polyploid Bacterium[▽]

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An extremely thermophilic bacterium, *Thermus thermophilus* HB8, is one of the model organisms for systems biology. Its genome consists of a chromosome (1.85 Mb), a megaplasmid (0.26 Mb) designated pTT27, and a plasmid (9.3 kb) designated pTT8, and the complete sequence is available. We show here that *T. thermophilus* is a polyploid organism, harboring multiple genomic copies in a cell. In the case of the HB8 strain, the copy number of the chromosome was estimated to be four or five, and the copy number of the pTT27 megaplasmid seemed to be equal to that of the chromosome. It has never been discussed whether *T. thermophilus* is haploid or polyploid. However, the finding that it is polyploid is not surprising, as *Deinococcus radiodurans*, an extremely radioresistant bacterium closely related to *Thermus*, is well known to be a polyploid organism. As is the case for *D. radiodurans* in the radiation environment, the polyploidy of *T. thermophilus* might allow for genomic DNA protection, maintenance, and repair at elevated growth temperatures. Polyploidy often complicates the recognition of an essential gene in *T. thermophilus* as a model organism for systems biology.

The extreme thermophile *Thermus thermophilus* is a Gram-negative aerobic bacterium that can grow at temperatures ranging from 50°C to 82°C (33, 34). The genome sequences of two strains, HB27 and HB8, are available (13; see also http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome&cmd=Retrieve&dopt=Overview&list_uids=530). The genome of the HB27 strain consists of a chromosome (1.89 Mb) and a megaplasmid (0.23 Mb), while that of the HB8 strain includes a plasmid (9.3 kb) coupled with a chromosome (1.85 Mb) and a megaplasmid (0.26 Mb) (13; see also the NCBI website [above]). This organism has attracted attention as one of the model organisms for genetic manipulation, structural genomics, and systems biology (9, 44). In the case of the HB8 strain, the Structural and Functional Whole-Cell Project for *T. thermophilus* HB8, which aims to understand the mechanisms of all the biological phenomena occurring in the HB8 cell by investigating the cellular components at the atomic level on the basis of their three-dimensional (3-D) structures, is in progress (44). In addition to the stability and ease of crystallization of thermophilic proteins, natural competency and an established genetic engineering system add value to *T. thermophilus* HB8 as a model organism (12, 14, 23, 44). Thermostabilized resistances against antibiotics such as kanamycin (Km), hygromycin (Hm), and bleomycin (Bm), which were developed by directed evolution, have also encouraged the system (5, 6, 16, 29; Y. Koyama, unpublished data).

However, we had been puzzled about several gene disruptions in *T. thermophilus* HB8 that resulted from replacement with the drug resistance gene. Even if drug-resistant transformants were obtained, the target gene of the transformants had not often been deleted. The target gene, probably an essential gene, seemed to coexist with the drug resistance gene. A similar phenomenon has been reported in the deletion of the *recJ* gene in *Deinococcus radiodurans* (7). Repeated observation of this phenomenon suggested that *T. thermophilus* HB8 might possess multiple genomic copies. Many bacteria, including the most-studied bacteria *Escherichia coli* and *Bacillus subtilis*, essentially carry a single genomic copy per cell and are genetically haploid organisms (3, 10, 42, 43). On the other hand, several bacteria have been proposed to be polyploid, harboring multiple genomic copies per cell. They include *Buchnera* species (21, 22), *Blattabacterium* species (24), *Epulopiscium* species (1, 4), *Borrelia hermsii* (20), *Azotobacter vinelandii* (28, 35), *Neisseria gonorrhoeae* (41), *D. radiodurans* (11, 27), a few *Lactococcus lactis* laboratory strains (26), and many cyanobacteria (2, 25, 37). In particular, *D. radiodurans*, an extremely radioresistant bacterium, has been suggested to be closely related to the genus *Thermus* by comparative genomic analysis (13, 32). The radioresistant bacterium carries four genome copies per cell in the stationary phase and up to 10 copies per cell during exponential growth (11, 27). In contrast with this well-known polyploidy of *D. radiodurans*, no report on the genomic copy number of *Thermus* has been done, in spite of the attention it has received as a model organism. Therefore, in this paper, the potential polyploidy and the genomic copy number were first studied in *T. thermophilus* HB8.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *T. thermophilus* HB8 and its derivatives were grown at 70°C in TR medium as a rich medium (12) or at 55°C in synthetic medium as a nutritionally defined medium (40), and 1.5% gellan gum with 1.5 mM CaCl$_2$ and 1.5 mM MgCl$_2$ was added to TR medium for plates (12). Km (500 μg/ml), Hm (100 μg/ml), and/or Bm (20 μg/ml) were added to the medium when needed. *B. subtilis* strain BEST7003, in which a linearized pBR322 sequence was inserted into the *proB* gene, has been described previously (19). This genomic pBR322 sequence is called GpBR. *B. subtilis* strains were grown at 37°C in Luria-Bertani (LB) medium or at 20°C in Spizizen medium as a synthetic medium (39).

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**Plasmids and materials.** The *E. coli* plasmids for the deletion of *T. thermophilus* HB8 genes (*jrn*, *polX*, TTHB090, and TTHB222) were provided by Kuramitsu (Osaka University and RIKEN) (44). The plasmid contains a DNA fragment in which a Km resistance gene (highly thermostable kanamycin [HTK] or Km$^r$) (16) is sandwiched between the 500-bp fragments just upstream and downstream of the target gene. The Km$^r$ gene can be clipped out with KpnI and PstI enzymes. *E. coli* plasmid pCISP401 derived from pBR322 has been described previously (18). An Hm resistance gene (Hm$^r$) (Koyama, unpublished) for *T. thermophilus* HB8 was kindly donated by Koyama (AIST), whereas a Bm resistance gene (high-temperature *Streptoalloteichus hindustanus* blmA [HTS] or Bm$^r$) (5) was chemically synthesized by TaKaRa Bio. The LA Taq hot-start version for PCR and restriction enzymes were from TaKaRa Bio.

**Transformation and gene deletion.** Transformation and gene deletion of *T. thermophilus* HB8 were carried out using the following procedures (12). An overnight culture was diluted 1:60 with TR medium supplemented with 0.4 mM CaCl$_2$ and 0.4 mM MgCl$_2$ and shaken at 70°C for 2 h. Four hundred microliters of this culture was mixed with a 50-μl DNA solution. The mixture was shaken at 70°C for 1 h and spread on plates. The plates were incubated overnight at 70°C. To delete the *T. thermophilus* HB8 genes in this experiment, the Km$^r$ gene of the plasmid from Kuramitsu was replaced with the Hm$^r$ and Km$^r$ or the Hm$^r$ and Bm$^r$ marker, as shown in Fig. 1. To achieve proper substitution by two homologous recombinations as shown in Fig. 1, 1 μg of the plasmid was used for transformation after being linearized by ScaI and NdeI digestion.

*B. subtilis* BEST7003 was transformed with a pCISP401+Hm$^r$ plasmid, in which the Hm$^r$ amplified by PCR was inserted into an EcoRI site of pCISP401 (Fig. 1C), as described previously (18). The pCISP401+Hm$^r$ plasmid is not a plasmid for *B. subtilis* but rather an *E. coli* plasmid harboring the chloramphenicol gene for *B. subtilis*. Therefore, as shown in Fig. 1C, the tetracycline-sensitive and chloramphenicol-resistant transformant necessarily resulted in possessing the Hm$^r$ gene within the GpBR region on the chromosome and was used for quantitation of ploidy as a haploid control.

**Southern hybridization.** Genomic DNAs were prepared by a liquid isolation method (36), digested by a restriction enzyme, and used for Southern hybridization analyses. Probes for the analyses were prepared by using an internal region of Hm$^r$, Km$^r$, Bm$^r$, or the deleted genes amplified by PCR as a template for the DIG high prime DNA labeling kit (Roche). In this paper, the results with the Km$^r$ or Bm$^r$ probe are not shown. Anti-DIG-alkaline phosphatase Fab fragments and CDP-Star were used for detection according to the manufacturer's instructions (Roche).

**Chromosome separation kinetics.** The Δ*jrn* strain carrying both chromosomes labeled by the Hm$^r$ and Km$^r$ or the Hm$^r$ and Bm$^r$ marker was precultured overnight at 70°C in TR medium containing both Km and Bm. The cells were harvested, washed with the antibiotic-free TR medium, and then inoculated into the TR medium for the following assay. To determine the chromosome separation kinetics, the culture at 70°C was sampled at regular intervals, and the number of viable cells was determined by counting the colonies on the antibiotic-free TR plate. The drug resistance of each colony was characterized by restreaking on the plate containing Km or Bm.

**Quantitation of ploidy.** *B. subtilis* possessing the Hm$^r$ sequence was grown in Spizizen medium at 20°C until the stationary phase at an optical density at 600 nm (OD$_{600}$) of 0.7. On the other hand, *T. thermophilus* HB8 possessing the Hm$^r$ and Bm$^r$ marker was grown in synthetic medium at 55°C until exponential growth (OD$_{600}$ of 0.8) or until the stationary phase (OD$_{600}$ of 1.7). The cell density was determined with a bacterial-counting chamber and by counting colonies on plates. The *B. subtilis* cells were mixed with a specific amount of the *T. thermophilus* HB8 cells at various ratios, and from the mixtures, genomic DNAs were prepared as described above. The genomic DNAs were digested by KpnI and used for Southern analyses with the Hm$^r$ probe. The signals corresponding to the Hm$^r$ sequence were detected and quantified using the Molecular Imager FX (Bio-Rad).

**Determination of the pTT27 copy number.** In the Δ*jrn* strain carrying the Hm$^r$ and Bm$^r$ marker, a TTHB090 or TTHB222 gene was replaced by the Hm$^r$ and Km$^r$ marker. The resultant strain was grown in the synthetic medium at 55°C until exponential growth (OD$_{600}$ of 0.8) or until the stationary phase (OD$_{600}$ of 1.7). Genomic DNA was prepared from each culture, digested by KpnI, and used for Southern analyses with the Hm$^r$ probe. Detection and quantification were performed as described above.

# RESULTS

***T. thermophilus* HB8 is polyploid.** To validate polyploidy in *T. thermophilus* HB8, we tested whether or not two different
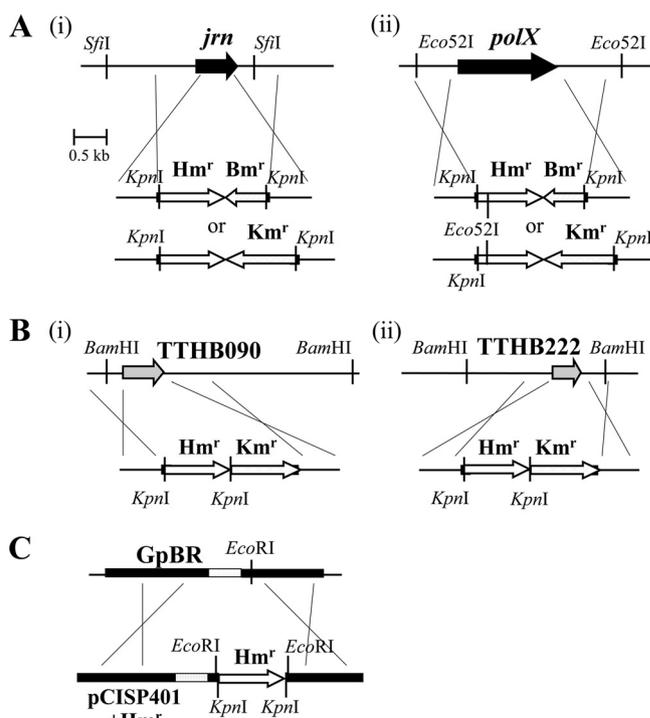


FIG. 1. Constructs for analyses of *T. thermophilus* HB8 polyploidy. (A) The *jrn* (i) and the *polX* (ii) gene-null mutants in *T. thermophilus* HB8. The gene was deleted by replacement with the Hm$^r$ and Km$^r$ or the Hm$^r$ and Bm$^r$ marker. The top figure represents the chromosomal region around the *jrn* gene, and the bottom figures represent the DNA fragments used for deletion. The crossings indicate homologous recombination. (B) The TTHB090 (i) or TTHB222 (ii) gene-null mutants to determine the quantity of the megaplasmid pTT27. In the Δ*jrn* strain labeled by the Hm$^r$ and Bm$^r$ marker, the TTHB090 or TTHB222 gene located on the pTT27 was deleted by replacement with the Hm$^r$ and Km$^r$ marker. The top figure represents the pTT27 region around the gene, and the bottom figure represents a DNA fragment for deletion. (C) *B. subtilis* possessing the Hm$^r$ sequence used for *T. thermophilus* HB8. This strain was used as a haploid control for the quantitation of the chromosomal copy number of *T. thermophilus* HB8 in Fig. 6. The top figure represents the chromosomal region around the pBR322 sequence (GpBR) of BEST7003, and the bottom figure represents the pCISP401 (pBR322 derivative) plasmid cloning Hm$^r$. The Hm$^r$ amplified by PCR was inserted into the EcoRI site of pCISP401, and two KpnI sites were introduced just inside the resultant two EcoRI sites by PCR primers. The top and bottom shaded regions represent the tetracycline and chloramphenicol resistance genes for *B. subtilis*, respectively.

genes can coexist at the same position on the chromosome. To do this, we examined whether a drug resistance gene displacing a nonessential gene was replaced by or could coexist with another drug resistance gene. The *jrn* gene encoding junction RNase was selected as a nonessential gene (31). As shown in Fig. 1Ai, *T. thermophilus* HB8 was transformed with a DNA fragment carrying an Hm$^r$ and Km$^r$ or an Hm$^r$ and Bm$^r$ marker. Resultant transformants on TR plates containing Km or Bm were checked by colony PCR with primers located outside the *jrn* gene. In Fig. 2A, the amplified fragment for the transformant (lane 2 for Hm$^r$ and Km$^r$ and lane 5 for Hm$^r$ and Bm$^r$) was different from that for the wild type (lane 6), implying that the *jrn* gene could be replaced by the marker. Then, the replacement was confirmed by sequencing of the amplified
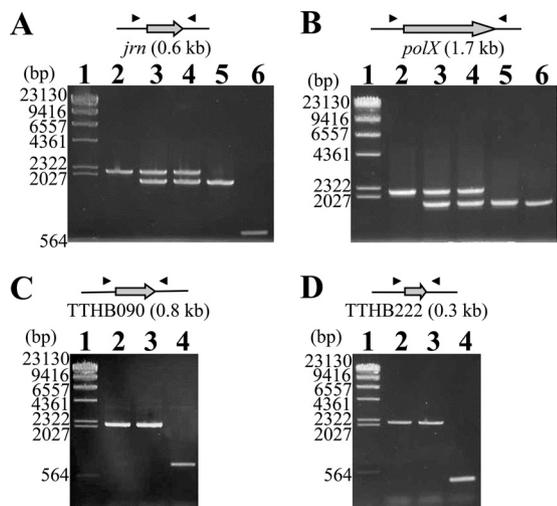
FIG. 2. Confirmation of transformants by colony PCR. Colony PCR of the transformants of *T. thermophilus* HB8 was performed with primers located outside the deleted gene. The black triangles in the upper figures indicate the primers. The results of the *jrn* and the *polX* gene-null mutants are shown in panels A and B, respectively. Lane 1, λ/HindIII digest marker; lane 2, the transformant of the wild-type strain by the DNA fragment carrying the Hm[r] and Km[r] marker; lanes 3 and 4, the transformants of the Hm[r] and Bm[r] strain (corresponding to the strain in lane 5) by the DNA fragment carrying the Hm[r] and Km[r] marker; lane 5, the transformant of the wild-type strain by the DNA fragment carrying the Hm[r] and Bm[r] marker; lane 6, the wild-type strain. The results of the TTHB090 and the TTHB222 gene-null mutants are shown in panels C and D, respectively. For deletion of these genes, the Δ*jrn* strain carrying the Hm[r] and Bm[r] marker (corresponding to the strain in lane 5 of panel A) was used as shown in Fig. 1B. Lane 1, λ/HindIII digest marker; lanes 2 and 3, the transformants of the Δ*jrn* strain by the DNA fragment carrying the Hm[r] and Km[r] marker; lane 4, the Δ*jrn* strain. All of these amplified DNA fragments were checked by DNA sequencing. The numbers along the left side of the gel represent the DNA fragment size of each marker (lane 1).
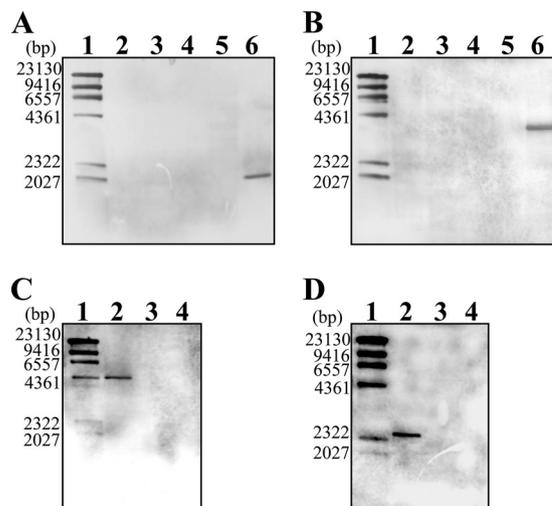


FIG. 3. Confirmation of null mutants by Southern analysis. The null mutants of *T. thermophilus* HB8 as shown in Fig. 2 were confirmed by Southern hybridization analysis with an inside probe for each gene. The genomic DNAs were digested by SfiI for *jrn* (A), Eco52I for *polX* (B), and BamHI for TTHB090 (C) and TTHB222 (D). (A and B) Lane 1, λ/HindIII digest marker; lane 2, the mutant carrying the Hm[r] and Km[r] marker; lanes 3 and 4, the transformants of the Hm[r]- and Bm[r]-labeled mutant (corresponding to the strain in lane 5) by the DNA fragment carrying the Hm[r] and Km[r] marker; lane 5, the mutant carrying the Hm[r] and Bm[r] marker; lane 6, wild type. (C and D) Lane 1, λ/HindIII digest marker; lane 2, the Δ*jrn* strain; lanes 3 and 4, the transformant of the Δ*jrn* strain by the DNA fragment, in which the TTHB090 or TTHB222 gene is replaced with the Hm[r] and Km[r] marker. The numbers along the left side of the gel represent the DNA fragment size of each marker (lane 1).

the chromosome. If *T. thermophilus* HB8 was haploid, two markers would never coexist at the same locus on the chromosome in a cell. The transformants derived from the single colony were grown in a liquid medium containing both antibi-

fragment. Complete deletion of the gene was confirmed by Southern hybridization with the *jrn* internal probe (lanes 2 and 5 in Fig. 3A), supported by the result of colony PCR in Fig. 2A. Furthermore, Southern analysis with the Hm[r] probe suggested that the marker was located at a single locus on the chromosome (lanes 2 and 5 in Fig. 4A). Next, the Δ*jrn* strain carrying the Hm[r] and Bm[r] marker was transformed by the DNA fragment used for the strain carrying the Hm[r] and Km[r] marker and then spread on TR plates containing Km or both Km and Bm. As shown in Table 1, transformants grew not only on the Km plates but also on the plates containing both Km and Bm. Forty percent of transformants were estimated to exhibit both resistances. To test the presence of both markers, the transformants on the plates containing both Km and Bm were checked by colony PCR as described above. As shown in lanes 3 and 4 of Fig. 2A, two fragments were amplified, and they exhibited sizes similar to that of the fragment observed in the Δ*jrn* strain carrying the Hm[r] and Km[r] or the Hm[r] and Bm[r] marker (lanes 2 and 5). Sequencing of them indicated that the upper fragments in lanes 3 and 4 were identical to that in lane 2, and the lower fragments were identical to that in lane 5. In the transformants on the plates containing both Km and Bm, the Hm[r] and Km[r] and Hm[r] and Bm[r] markers were suggested to be located at the same locus (corresponding to the *jrn* gene) on
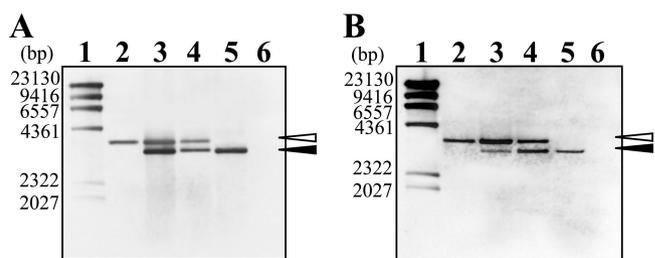


FIG. 4. Multiple chromosomes in *T. thermophilus* HB8. The genomic DNAs of the *jrn* (A)- or *polX* (B)-null mutants of *T. thermophilus* HB8 were analyzed by Southern hybridization with a probe for the Hm[r] marker, as described in the legend to Fig. 3. Lane 1, λ/HindIII digest marker; lane 2, the mutant carrying the Hm[r] and Km[r] marker; lanes 3 and 4, the transformants of the Hm[r]- and Bm[r]-labeled strain (corresponding to the strain in lane 5) by the DNA fragment carrying the Hm[r] and Km[r] marker; lane 5, the mutant carrying the Hm[r] and Bm[r] marker; lane 6, wild type. Open and closed arrowheads indicate DNA fragments containing the Hm[r] and Km[r] and Hm[r] and Bm[r] markers, respectively. The numbers along the left side of the gel represent the DNA fragment size of each marker (lane 1). The ratios of signal intensity between DNA fragments containing the Hm[r] and Km[r] and Hm[r] and Bm[r] markers were estimated to be approximately 1:3 for lane 3 in panel A, 2:5 for lane 4 in panel A, 5:1 for lane 3 in panel B, and 3:2 for lane 4 in panel B.

TABLE 1. Transformation efficiencies

| DNA[a] | Cell | No. of viable cells (CFU/ml)[b] | Transformants in CFU/ml (%)[c] | |
|---|---|---|---|---|
| | | | Km | Km and Bm |
| Δjrn | WT | $1.2 \times 10^5$ | $5.2 \times 10^2$ (0.43) | |
| | Δjrn carrying the Hm$^r$ and Bm$^r$ marker | $1.1 \times 10^5$ | $6.6 \times 10^2$ (0.60) | $2.6 \times 10^2$ (0.24) |
| ΔpolX | WT | $2.1 \times 10^5$ | $8.7 \times 10^2$ (0.41) | |
| | ΔpolX carrying the Hm$^r$ and Bm$^r$ marker | $1.8 \times 10^5$ | $1.5 \times 10^3$ (0.83) | $4.1 \times 10^2$ (0.23) |

[a] One microgram of the plasmid for replacement by the Hm$^r$ and Km$^r$ marker (Fig. 1A). To achieve proper substitutions by two homologous recombinations as shown in Fig. 1A, the plasmid linearized by ScaI and NdeI digestion was used.
[b] Number of CFU formed on TR plates.
[c] Number of CFU formed on TR plates containing Km or both Km and Bm. Parenthetical values represent transformants versus viable cells.

otics, and then genomic DNA was prepared and used for Southern hybridization analyses. As shown in Fig. 4A, the analysis using the Hm$^r$ probe indicated that the transformants could possess both chromosomes in which the jrn gene was replaced by the Hm$^r$ and Km$^r$ or Hm$^r$ and Bm$^r$ marker. Furthermore, the different signal intensities between the Hm$^r$ and Km$^r$ and Hm$^r$ and Bm$^r$ markers imply that three or more copies of the chromosome could exist (Fig. 4A, lanes 3 and 4).

To examine chromosome separation, the Δjrn strain carrying both Km and Bm markers (the strain in lane 3 of Fig. 4A) was cultured in TR liquid medium without antibiotics and then spread on antibiotic-free plates. The drug resistance of the resultant colonies was characterized by restreaking on each plate containing Km or Bm. If the Hm$^r$ and Km$^r$ and Hm$^r$ and Bm$^r$ markers are on the same single chromosome for any reason, the Bm and Km resistances would be genetically linked and therefore would be expected to be segregated together. As shown in Fig. 5, however, 40% of the cells lost one resistance after 1 h, compared with 4% at 0 h. The strain exhibited both Bm and Km resistances as long as both antibiotics were added, whereas one resistance seemed to be lost gradually once the addition of the antibiotics was ended. Therefore, it is reasonable to conclude that the two markers are not on the same single chromosome but on separate chromosomes, suggesting that T. thermophilus HB8 might carry more than one chromosomal copy.

These results were not specific to the jrn gene, since the same results were observed for the polX gene encoding DNA polymerase X (30) (Fig. 4B). The polX and jrn genes are located at opposite sides of the circular T. thermophilus HB8 chromosome (at the 1.097 and 0.194 positions on a 1.850-Mb genome). T. thermophilus HB8 must be a polyploid bacterium.

**Chromosomal copy number.** The number of copies of the chromosome per cell was determined in T. thermophilus HB8. B. subtilis labeled by the Hm$^r$ gene as shown in Fig. 1C was used as a haploid control, whereas the Hm$^r$- and Bm$^r$-labeled Δjrn strain mentioned above (corresponding to lane 5 in Fig. 4A) was used as T. thermophilus HB8. B. subtilis was grown in the Spizizen medium at the low temperature of 20°C for slow growth. T. thermophilus HB8 was grown in the synthetic medium at 55°C as described in Materials and Methods. The defined number of T. thermophilus HB8 cells was mixed with that of B. subtilis cells at various rates, and both genomic DNAs were extracted from the mixtures. T. thermophilus HB8 and B. subtilis used for this study possess a unique Hm$^r$ sequence per chromosome. The amount of each chromosomal

DNA could be estimated by Southern analysis detecting the Hm$^r$ sequence on the chromosome. In Fig. 6, the upper signal corresponding to the Hm$^r$ and Bm$^r$ marker of T. thermophilus HB8 was compared with the lower signal corresponding to the Hm$^r$ gene of B. subtilis. Both during exponential growth and in the stationary phase, the upper signals were about the same as or slightly weaker than the lower ones in the 1:4 lane. In the 1:6 lane, however, the lower signals were more intensive than the upper ones. The measured intensities of the upper and lower signals were calculated to give agreement at a ratio of the cell number between 1:4 and 1:5. Therefore, the chromosomal
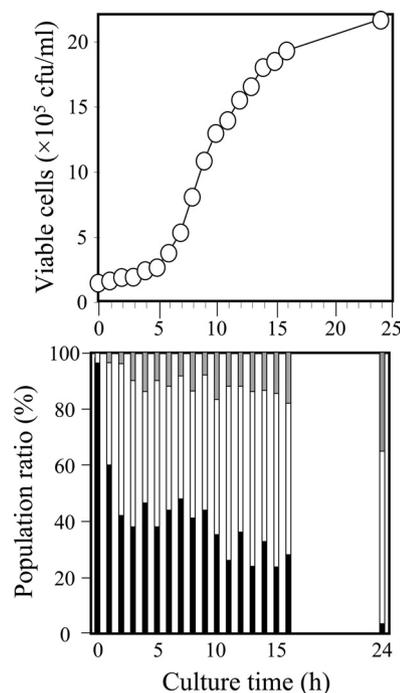


FIG. 5. Chromosome separation kinetics. The Δjrn strain carrying both chromosomes labeled by the Hm$^r$ and Km$^r$ or the Hm$^r$ and Bm$^r$ marker was cultured at 70°C in TR medium as described in Materials and Methods. At 0 h of culture time, the cells precultured in the presence of both Km and Bm were added to fresh antibiotic-free medium. The number of viable cells (top) was determined by counting the colonies on the antibiotic-free TR plate, and the drug resistance of each colony (bottom) was characterized by restreaking on the plate containing Km or Bm. In the bottom panel, the resistance only to Km or Bm is shown in white and gray, respectively, and that to both is shown in black.
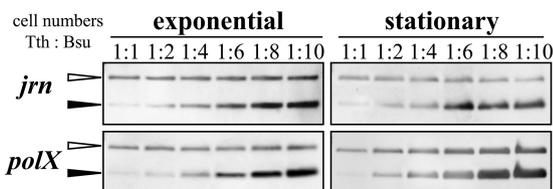
FIG. 6. Quantitation of chromosomal copy number of *T. thermophilus* HB8. The defined number of *T. thermophilus* HB8 Δ*jrn* or Δ*polX* cells during exponential growth and in the stationary phase was mixed with that of *B. subtilis* cells as a haploid control at different ratios (1:1 to 1:10), and genomic DNAs of both cells were prepared from the mixtures. The genomic DNA mixtures were digested by KpnI and used for Southern analyses with a probe for the Hm^r marker. As shown in Fig. 1A and C, *T. thermophilus* HB8 and *B. subtilis* are labeled by the Hm^r and Bm^r and the Hm^r markers, respectively. Open and closed arrowheads indicate the Hm^r and Bm^r and the Hm^r signals, respectively.



FIG. 7. Number of copies of the megaplasmid pTT27. Genomic DNA from the ΔTTHB090 or ΔTTHB222 strain (as described in the legend to Fig. 1B) during exponential growth or in the stationary phase was prepared, digested by KpnI, and used for Southern analyses with a probe for the Hm^r marker. In the figure, ×2 and ×4 mean that 2- and 4-fold amounts of the digested DNAs were applied for electrophoresis. The strain possesses the Hm^r and Bm^r and the Hm^r and Km^r markers replacing the *jrn* gene on the chromosome and the TTHB090 gene on the pTT27, respectively. Open and closed arrowheads indicate the Hm^r and Km^r signal on pTT27 and the Hm^r and Bm^r signal on the chromosome, respectively.

copy number in *T. thermophilus* HB8 is about four or five times that of *B. subtilis*. The same analysis was carried out on the *polX* gene (Fig. 6). The estimate for the *polX* gene was also four or five copies of chromosome per cell both during exponential growth and in the stationary phase.

**Copy number of pTT27.** The copy number of the pTT27 megaplasmid was also estimated on the basis of the chromosomal copy number. For the estimation, TTHB090 and TTHB222 genes encoding a hypothetical protein were selected. They are located at the 0.080 and 0.227 positions on the 0.257-Mb pTT27, respectively. In the Δ*jrn* strain in which chromosomal DNA was previously labeled by the Hm^r and Bm^r marker, the TTHB090 or TTHB222 gene was replaced by an Hm^r and Km^r marker, as shown in Fig. 1B. Absolute replacement was confirmed by sequencing of the amplified fragment by colony PCR and Southern analyses as described for Δ*jrn* and Δ*polX* (Fig. 2 and 3). These strains were grown in the synthetic medium at 55°C and used for the analysis. In Fig. 7, the result of ΔTTHB090 showed that the signal intensity of the Hm^r and Bm^r marker on the chromosome (top) was almost equal to that of the Hm^r and Km^r marker on pTT27 (bottom), suggesting that the number of copies of pTT27 is almost the same as that of the chromosome. The result of ΔTTHB222 also agreed with this view.

## DISCUSSION

**Polyploidy of *T. thermophilus*.** In spite of the absence of discussion of the ploidy of *Thermus* species, *T. thermophilus* HB8 was believed to be haploid, as are *E. coli* and *B. subtilis*. However, the bacterium was found to be polyploid. Under a slow growth condition such as at 55°C in the synthetic medium, the HB8 strain was estimated to have four or five copies of the chromosome per cell both during exponential growth and in the stationary phase (Fig. 6), and as many pTT27 megaplasmids were determined to exist as the number of chromosomes in a cell (i.e., four or five copies per cell) (Fig. 7). Agreement between the number of copies of the chromosome and of the pTT27 megaplasmid might imply that some cooperating mechanisms regulating both copy numbers exists in *T. thermophilus* HB8 cells. The HB8 strain also harbors the pTT8 plasmid at 8 copies per chromosome, as has already been reported (15). In
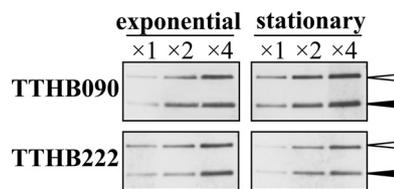
consequence, the HB8 strain turns out to contain approximately 30 to 40 copies of the plasmid pTT8 per cell.

*T. thermophilus* HB27, another representative strain of *T. thermophilus*, also seems to be polyploid, as its chromosome labeled by Bm^r at the *jrn* gene coexisted in a cell with that labeled by Km^r at the gene (data not shown). Other *Thermus* species remain to be analyzed. However, as the closely related *D. radiodurans* and related cyanobacteria are well known to be polyploid cells, it seems likely that *Thermus* species might be generally polyploid.

**Physiological function of bacterial polyploidy.** In *D. radiodurans*, multiple genome copies contribute to the reassembly of chromosomes shattered by exposure to desiccation and ionizing radiation (38, 45). The homologous recombinations between chromosomal fragments promote the reassembly. As is the case for *D. radiodurans* in a radiation environment, the polyploidy of *T. thermophilus* might allow for genomic DNA protection, maintenance, and repair at elevated growth temperatures. The *recA* inactivation in *T. thermophilus* HB27 has been reported to show extremely low viability depending on the growth temperature (8). Therefore, high growth temperatures could damage the genomic DNAs, and multiple genome copies would assist DNA repair by RecA. The cyanobacteria, which contain extremophiles such as thermophiles, psychrophiles, halophiles, xelophiles, and so on, also include many polyploid cells. Is the genomic polyploidy correlated with extreme environmental conditions causing DNA damage? It remains an interesting question whether hyperthermophilic bacteria such as *Thermotoga* or *Aquifex* species are haploid or polyploid. From another perspective, genomic polyploidy also has the potential to harbor accidental mutations to encourage adaptation to environmental changes. However, the potential of polyploidy often complicates the recognition of an essential gene in *T. thermophilus*. In the genetics of *T. thermophilus*, it should be carefully confirmed whether the gene in this bacterium was perfectly deleted or disrupted.

**Chromosome separation and homologous recombination.** The chromosome separation mechanism in *T. thermophilus* HB8 is not understood. It is even unclear whether identical chromosomes are separated equally into two daughter cells or the chromosomes are randomly distributed. Although it has been less well known how multiple identical chromosomes in

bacteria may be separated, in cyanobacteria, it has been suggested to be random (17, 37). In the absence of antibiotics, as shown in Fig. 5, *T. thermophilus* HB8 cells carrying both of the chromosomes labeled by the different markers tended to lose one of the two markers immediately. If each identical chromosome were separated into daughter cells, both of the markers would be retained in a cell. Therefore, the chromosome separation in *T. thermophilus* HB8 might also be a random process. However, this is not a definitive demonstration of random distribution of chromosomes in the HB8 strain because homologous recombination between chromosomes is likely to occur. In *T. thermophilus* HB8, replacement of a nonessential gene on all of four or five chromosomes by the drug resistance gene was established easily, as shown for *jrn* or *polX*. However, the result in Table 1 means that in at least 30 to 40% of transformants, all of four or five chromosomes are not necessarily replaced with the foreign marker. Completion of the replacement on all chromosomes is likely facilitated by subsequent homologous recombination between chromosomes. The previous report that the absence of RecA in *T. thermophilus* HB27 apparently results in a dramatic and generalized increase in the mutation rate (8) also implies that the high-frequency recombination between chromosomes could be involved in DNA repair. Therefore, this probable recombination between chromosomes might make it more difficult to monitor the chromosome separation in *T. thermophilus* HB8. For instance, in Fig. 5, although the number of viable cells after 1 h is at most 1.1-fold that at 0 h, as many as 40% of the cells have already lost one resistance after 1 h. In any case, the results in Fig. 4 and 5 are impossible in a haploid cell, and multiple chromosomal copies would make these phenomena possible.

## ACKNOWLEDGMENTS

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