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 recF gene in *Deinococcus radiodurans* (2). 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), *Borelia hermsii* (20), *Azotobacter vinelandii* (28, 35), *Neisseria gonorhoeae* (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 (90 g/liter) 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 BEST 7003, 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).
Plasmids and materials. The E. coli plasmids for the deletion of T. thermophilus HB8 genes (jrn, polX, TTHB090, and TTHB222) were provided by Karamitsu (Osaka University and RIKEN) (44). The plasmid contains a DNA fragment in which a Km resistance gene (highly thermostable kanamycin [HTK] or Kmr) (16) is sandwiched between the 500-bp fragments just upstream and downstream of the target gene. The Km' 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 (Hmr) (Koyama, unpublished) for T. thermophilus HB8 was kindly donated by Koyama (AIST), whereas a Bm resistance gene (high-temperature Streptoalloteichus hindustanus blmA [HTS] or Bmr) (5) was chemically synthesized by TaKaRa Bio. The LA Tag 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 CaCl₂ and 0.4 mM MgCl₂, 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 Kmr gene of the plasmid from Karamitsu was replaced with the Hm r and Km r or Hmr and Bmr 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 Scal and Ndel digestion.

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' or Bm' 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 ∆m r 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 (OD₆0₀) 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 (OD₆0₀ of 0.8) or until the stationary phase (OD₆0₀ 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 ∆m r 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 (OD₆0₀ of 0.8) or until the stationary phase (OD₆0₀ 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 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. 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. (B) The TTHB090 (i) or TTHB222 (ii) gene-null mutants to determine the quantity of the megaplasmid pTT27. In the ∆m r 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. The crosses indicate homologous recombination. (C) The TTHB090 (i) or TTHB222 (ii) gene-null mutants to determine the quantity of the megaplasmid pTT27. In the ∆m r 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. The crosses indicate homologous recombination. (D) The TTHB090 (i) or TTHB222 (ii) gene-null mutants to determine the quantity of the megaplasmid pTT27. In the ∆m r strain labeled by the Hm r and Km r marker, the TTHB090 or TTHB222 gene located on the pTT27 was deleted by replacement with the Hm r and Bm r marker. The top figure represents the pTT27 region around the gene, and the bottom figure represents a DNA fragment for deletion. The crosses indicate homologous recombination.
Furthermore, Southern analysis with the Hmr probe suggested in Fig. 3A), supported by the result of colony PCR in Fig. 2A.

The genomic DNAs were digested by SfiI for \textit{jrn} (A), Eco52I for \textit{polX} (B), and BamHI for TTHB090 (C) and TTHB222 (D). (A and B) Lane 1, \textit{\lambda/HindIII} digest marker; lane 2, mutant carrying the \textit{Hm'} and \textit{Km'} marker; lanes 3 and 4, the transformants of the \textit{Hm'} and \textit{Bm'} strain (corresponding to the strain in lane 5) by the DNA fragment carrying the \textit{Hm'} and \textit{Km'} marker; lane 5, the transformant of the wild-type strain by the DNA fragment carrying the \textit{Hm'} and \textit{Bm'} 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 \textit{\Delta jm} strain carrying the \textit{Hm'} and \textit{Bm'} marker (corresponding to the strain in lane 5 of panel A) was used as shown in Fig. 1B. Lane 1, \textit{\lambda/HindIII} digest marker; lanes 2 and 3, the transformants of the \textit{\Delta jm} strain by the DNA fragment carrying the \textit{Hm'} and \textit{Km'} marker; lane 4, the \textit{\Delta jm} 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 \textit{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 \textit{jrn} (A), Eco52I for \textit{polX} (B), and BamHI for TTHB090 (C) and TTHB222 (D). (A and B) Lane 1, \textit{\lambda/HindIII} digest marker; lane 2, mutant carrying the \textit{Hm'} and \textit{Km'} marker; lanes 3 and 4, the transformants of the \textit{Hm'}- and \textit{Bm'}-labeled mutant (corresponding to the strain in lane 5) by the DNA fragment carrying the \textit{Hm'} and \textit{Km'} marker; lane 5, the mutant carrying the \textit{Hm'} and \textit{Bm'} marker; lane 6, wild type. (C and D) Lane 1, \textit{\lambda/HindIII} digest marker; lane 2, the \textit{\Delta jm} strain; lanes 3 and 4, the transformant of the \textit{\Delta jm} strain by the DNA fragment, in which the TTHB090 or TTHB222 gene is replaced with the \textit{Hm'} and \textit{Km'} marker. The numbers along the left side of the gel represent the DNA fragment size of each marker (lane 1).

FIG. 4. Multiple chromosomes in \textit{T. thermophilus} HB8. The genomic DNAs of the \textit{jrn} (A)- or \textit{polX} (B)-null mutants of \textit{T. thermophilus} HB8 were analyzed by Southern hybridization with a probe for the \textit{Hm'} marker, as described in the legend to Fig. 3. Lane 1, \textit{\lambda/HindIII} digest marker; lane 2, the mutant carrying the \textit{Hm'} and \textit{Km'} marker; lanes 3 and 4, the transformants of the \textit{Hm'}- and \textit{Bm'}-labeled strain (corresponding to the strain in lane 5) by the DNA fragment carrying the \textit{Hm'} and \textit{Km'} marker; lane 5, the mutant carrying the \textit{Hm'} and \textit{Bm'} marker; lane 6, wild type. Open and closed arrowheads indicate DNA fragments containing the \textit{Hm'} and \textit{Km'} and \textit{Hm'} and \textit{Bm'} 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 \textit{Hm'} and \textit{Km'} and \textit{Hm'} and \textit{Bm'} 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.
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 \textit{T. thermophilus} HB8 was compared with the lower signal corresponding to the Hm\(^r\) gene of \textit{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 copy number per cell was determined in \textit{T. thermophilus} HB8. \textit{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 \(\Delta jrn\) strain mentioned above (corresponding to lane 5 in Fig. 4A) was used as \textit{T. thermophilus} HB8. \textit{B. subtilis} was grown in the Spizizen medium at the low temperature of 20°C for slow growth. \textit{T. thermophilus} HB8 was grown in the synthetic medium at 55°C as described in Materials and Methods. The defined number of \textit{T. thermophilus} HB8 cells was mixed with that of \textit{B. subtilis} cells at various rates, and both genomic DNAs were extracted from the mixtures. \textit{T. thermophilus} HB8 and \textit{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 \textit{T. thermophilus} HB8 was compared with the lower signal corresponding to the Hm\(^r\) gene of \textit{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. 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The amount of each chromosomal copy number per cell was determined in \textit{T. thermophilus} HB8. \textit{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 \(\Delta jrn\) strain mentioned above (corresponding to lane 5 in Fig. 4A) was used as \textit{T. thermophilus} HB8. \textit{B. subtilis} was grown in the Spizizen medium at the low temperature of 20°C for slow growth. \textit{T. thermophilus} HB8 was grown in the synthetic medium at 55°C as described in Materials and Methods. The defined number of \textit{T. thermophilus} HB8 cells was mixed with that of \textit{B. subtilis} cells at various rates, and both genomic DNAs were extracted from the mixtures. \textit{T. thermophilus} HB8 and \textit{B. subtilis} used for this study possess a unique Hm\(^r\) sequence per chromosome. 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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' and Bm' marker, the TTHB090 or TTHB222 gene was replaced by an Hm' and Km' 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' and Bm' marker on the chromosome (top) was almost equal to that of the Hm' and Km' 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' at the *jrn* gene coexisted in a cell with that labeled by Km' 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 shatttered 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 was 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 polI. 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 factor. In any case, the results in Fig. 4 and 5 are impossible in a haploid cell, and multiple recombinational copies would make these phenomena possible.

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