This report describes efficient plasmid uptake by the thermophile Geobacillus kaustophilus HTA426 by means of a ternary conjugation system, which was used to construct thermophilic DNA libraries for G. kaustophilus and to identify the genes for orotidine-5'-phosphate decarboxylase by *in vivo* functional screening. The results indicate that the conjugation system is useful in constructing *G. kaustophilus* libraries, which are practical in identifying thermophile genes.

**Key words:** Geobacillus kaustophilus; orotidine-5' phosphate decarboxylase; pyrF; ternary conjugation; functional screening

Thermophilic enzymes have several advantages with regard to utility in biotechnological applications.1,2) These enzymes, along with their genes, are commonly identified in thermophiles by protein purification techniques or data mining of thermophile genomes. *In vivo* functional screening of thermophilic DNA libraries is also a proven method to identify thermostable enzyme genes. Although *Escherichia coli* cells are usually used as library hosts, we assume that thermophiles serve as more efficient hosts, because thermostable enzymes often exhibit negligible activity at moderate temperatures. In addition, thermophile genes are some times insufficiently expressed in *E. coli*, which has codons and promoter sequences different from those of thermophiles. Early studies identified thermostable kanamycin nucleotidyltransferase genes in DNA libraries for *Thermus thermophilus*3) and *Bacillus stearothermophilus*,4) suggesting that thermophiles can be used as alternative tools for identifying thermostable enzyme genes by *in vivo* functional screening.

*Geobacillus kaustophilus* HTA426 is an aerobic, Gram-positive, thermophilic bacterium that grows between 42 °C and 74 °C, with an optimum growth temperature of 60 °C.5,6) It can take up plasmid DNA by conjugative DNA transfer from *E. coli* BR4087) and can express various heterologous genes,8) suggesting that the HTA426 strain can serve as an efficient host of thermophile DNA libraries. However, whereas conjugative transfer from *E. coli* BR408 to *G. kaustophilus* is very efficient, plasmid transformation of *E. coli* BR408 is extremely inefficient even by electroporation. This makes it difficult to construct large DNA libraries for *E. coli* BR408, and consequently for *G. kaustophilus*.

In this study, we constructed *G. kaustophilus* MK244, which is deficient in restriction–modification (R–M) genes. The R–M system plays important roles in defending the bacterial host against transformation by exogenous DNA, such as that of bacteriophages and foreign plasmids, by cleaving only at exogenous DNA.9) Thus a deficiency of it generally facilitates bacteria transformation by way of foreign plasmids. Among the four types of R–M systems, type II systems consist of a DNA endonuclease and methylase. The methylase adds a methyl group to the nucleobases of endogenous DNA at specific sites. The endonuclease subsequently cuts exogenous DNA at those sites, but it does not cleave the methylated endogenous DNA. Type I and III systems comprise three and two protein subunits respectively. They recognize specific sites to cleave unmethylated DNA while catalyzing the complete methylation of hemimethylated DNA. Type IV systems cleave the DNA that carries heterologously modified nucleobases.

*G. kaustophilus* HTA426 harbors two sets of type I R–M genes (*GK1380, GK1381, and GK1382, and GK0343, GK0344, and GK0346; Fig. 1A). The *GK1380–GK1382* genes are apparently part of the *GK1378–GK1390* gene cluster, which includes three type IV R–M genes: *GK1378, GK1379, and GK1390*. In addition, the large plasmid pHTA426 harbors one set of type II genes: *GKP08 and GKP09*. We constructed *G. kaustophilus* MK244 from strain MK72 (∆*pyrF* ∆*pyrR*)20) by executing global deletion of the *GK1378–GK1390* and the *GK0343–GK0346* genes (Fig. 1A). The experimental details are described in Supplemental Methods (see *Biosci. Biotechnol. Biochem.* Web site). Relevant descriptions of bacterial
strains and plasmids are summarized in Supplemental Table S1. The correctness of the deletions was confirmed by Southern blot and PCR (Fig. 1B). Although strain MK244 still harbored type II R–M genes, we assumed that it accepts E. coli DH5α-derived DNA, because E. coli DH5α harbors the dam gene, which is responsible for DNA methylation to circumvent the restriction barrier.7)

To evaluate the plasmid uptake efficiency of strain MK244, first we examined the binary conjugative transfer of pUCG18T, an E. coli–Geobacillus shuttle plasmid7) from E. coli DH5α to G. kaustophilus MK244. This was mediated by plasmid pUB307.11) The donor, E. coli DH5α [pUCG18T, pUB307] (where denotes the plasmid-carrier state), and the recipient, MK244, were grown in Luria–Bertani (LB) medium. The cells were mixed and incubated at 37°C for 6 h to allow conjugation, and the resulting cells were incubated at 60°C on LB plates supplemented with 5 mg/L of kanamycin to isolate transconjugants. As expected, G. kaustophilus MK244 accepted pUCG18T transferred from E. coli DH5α; transfer efficiency, (1.3 ± 0.8) × 10−5 recipient−1, whereas strains HTA426 and MK72 did not ( < 1 × 10−7). We further examined ternary conjugation among E. coli DH5α [pUB307], E. coli DH5α [pUCG18T], and G. kaustophilus MK244. In ternary conjugation, it is possible that pUB307 first transfers from E. coli DH5α [pUB307] to E. coli DH5α [pUCG18T], generating E. coli DH5α [pUB307 and pUCG18T], and that this strain subsequently transfers pUCG18T to G. kaustophilus MK244. This procedure allowed more efficient transfer than binary conjugation: (6.4 ± 4.3) × 10−4. Transfer efficiency was comparable to that from E. coli BR408 [pUCG18T] to G. kaustophilus HTA426.7) In addition to E. coli DH5α [pUB307], E. coli DH5α [pKK2013]12) served as a mediator for ternary conjugation: (2.1 ± 1.5) × 10−4, but neither HTA426 nor MK72 served as recipients (< 1 × 10−7).

By ternary conjugation, we constructed three DNA libraries for G. kaustophilus MK244. Plasmid pGKE73 (Fig. 2), which is pUCG18T carrying the Pp3704 promoter, which is functional in G. kaustophilus,9) was used as DNA vector. DNA samples were partially digested with Sau3AI (average length, 3 kb) and cloned in the BamHI site of pGKE73. The resulting plasmids in E. coli DH5α (about 1 × 105 clones) were transferred to G. kaustophilus MK244 using E. coli DH5α [pUB307] as conjugation mediator, yielding the MK244 libraries. One library (5 × 105 clones) was constructed using total DNA of soil thermophiles, which was cultivated by aerobic incubation of a soil sample at the Hakozaki campus of Kyushu University (Hakozaki, Fukuoka, Japan) in LB medium at 60°C for 24 h. The other two libraries (1 × 105 clones in each) were constructed using Bacillus caldolyticus DSM 40513) and Geobacillus subterraneus DSM 1355214) chromosomes.

G. kaustophilus MK244 is auxotrophic for uracil due to a deficiency in its pyrF gene. To demonstrate that the MK244 library is effective for identifying thermophile genes by in vivo functional screening, we isolated 105
clones that were prototrophic for uracil from the 10^6 cells of the soil thermophile library. Among the positive clones, insert DNA sequences of 17 clones (Fig. 3A) were analyzed to confirm that the clones harbor plasmid pMK307 (4/17 clones; GenBank ID: AB823007), pMK308 (2/17 clones; AB823008), or pMK311 (11/17 clones; AB823009). The sequences were identical to each other although the lengths were different, and they shared the pyrF gene, the gene product of which showed sequence similarity to the PyrF proteins of Geobacillus species (identity: Geobacillus sp. WCH70, 81%; Geobacillus sp. Y4.1MC1, 78%). These results indicate that these fragments originated from only a single thermophile, probably a Geobacillus species (identity: G. kaustophilus sp. WCH70, 81%; G. subterraneus sp. Y4.1MC1, 78%).

These results are not evidence that the G. kaustophilus libraries have advantages over E. coli libraries for identifying thermophile genes, but they suggest that the G. kaustophilus libraries are practical for the identification of thermophile enzyme genes, especially of Geobacillus species, by in vivo functional screening, and that the ternary conjugation system is useful for library construction in G. kaustophilus.

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### References