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A New *Thermus-Escherichia coli* Shuttle Integration Vector System

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We established a *Thermus thermophilus* strain in which the *pyrE* gene (coding for orotate phosphoribosyltransferase of the pyrimidine biosynthetic pathway) was totally deleted. We also constructed an integration vector, which consisted of the *Escherichia coli* plasmid vector pBluescript and a 2.1-kb segment of the *T. thermophilus leu* operon sequence, for the integration of a foreign gene into a chromosome of the thermophile. *pyrE* and *leuB* genes were used as probes to test the integration vector. The integration vector pINV, bearing the *pyrE* gene, transformed the Δ*pyrE* strain at a frequency of 6×10^{-5} through a single crossover event. The *leuB* gene could also be used as another marker of the integration vector system. The vector could be integrated at the expected site. By digesting the chromosomal DNA of the *T. thermophilus* transformants with a unique restriction enzyme, the vector could be recovered into *E. coli* after the recircularization in vitro. The kanamycin nucleotidyltransferase gene could be successfully expressed in the thermophile by using pINV.

Thermus thermophilus is a gram-negative, aerobic microorganism which can grow at temperatures up to 85°C (17). The genetic manipulation of this thermophile has many potential applications for basic as well as applied research. For example, a heat-stable protease for which production was unsuccessful in *Escherichia coli* could be produced and secreted by using a host-vector system for *T. thermophilus* (24). Another example is selection for thermostable proteins in thermophiles. In the pioneering studies of Matsumura and Aiba (15) and Liao et al. (13), thermostable forms of kanamycin nucleotidyltransferase were selected in a moderate thermophile, *Bacillus stearothermophilus*. A thermostable chloramphenicol acetyltransferase was also selected in this moderate thermophile (25). Recently, we developed an integration vector system in *T. thermophilus* and selected for thermostable enzymes in this extreme thermophile (6, 22). By screening enzymes with elevated thermal stability in thermophiles, we successfully generated thermostable proteins, even though we had no knowledge of this structure.

Some autonomously replicating plasmid vectors for *T. thermophilus* have been constructed by several groups, using *trpB* (7), beta-galactosidase (9), or kanamycin resistance (12, 14) genes as selectable markers. However, a significant background was observed because of the high recombination frequency in the thermophile (5, 8). On the other hand, integration vectors have been shown to be very useful for stable expression (22, 26) or for gene analyses (11).

In many microorganisms and lower eukaryotes, genes in the pyrimidine biosynthetic pathway, orotate phosphoribosyltransferase and orotate decarboxylase genes, are used as genetic markers (1, 2, 4, 16, 18, 20). Recently we cloned the *pyrE* gene (orotate phosphoribosyltransferase gene) from *T. thermophilus* (27). The bactericidal compound 5-FOA (5-fluoroorotic acid) has been used to isolate *pyrE* mutants of microorganisms. While this compound is toxic to wild-type cells, *pyrE* strains become resistant and can be positively selected on 5-FOA plates. Using this strategy, we have obtained a Δ*pyrE* strain. We also report the construction of a *T. thermophilus-E. coli* shuttle integration vector system. The vector could be integrated into the chromosomal DNA of *T. thermophilus* by homologous recombination and could be recovered in *E. coli.*

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MATERIALS AND METHODS

Strains and media used. *T. thermophilus* MT106 (Δ*leuB*) has been described previously (22). *E. coli* JM109 [*recA1 endA1 gyrA96 thi supE44 relA1 hsdR17* Δ(*lac-proAB*) F′(*traD36 proAB lacI*^{q}*Z*ΔM15)] and HB101 [*supE44 hsdS20*(r_B^- m_B^-)*recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*] were used as hosts for genetic manipulations of plasmids. The rich growth medium and minimum medium for *T. thermophilus* have been described previously (23). Leucine and isoleucine (50 μg/ml each) were included in the leucine medium. Uracil (50 μg/ml) and 5-FOA (200 μg/ml) were included in the 5-FOA medium. The solidification of media was performed by mixing the double-strength media and 1% Gelrite solution after autoclave sterilization (21).

DNA manipulations. All routine DNA manipulations, i.e., plasmid preparation, subcloning, agarose gel electrophoresis, and Southern blotting, were done essentially as described by Sambrook et al. (19). Restriction endonucleases and DNA modification enzymes were purchased from Toyobo Biochemicals (Osaka, Japan) or Takara Shuzo (Kyoto, Japan) and used as recommended by the manufacturers.

Southern blotting was performed according to the protocol described by Sambrook et al. (19). DNA was capillary transferred to a nylon membrane (Hybond N+; Amersham) by using 0.4 N NaOH as a transfer buffer overnight. The probe used for hybridization was an *Nde*I-*Nde*I 0.55-kbp *pyrE* fragment, a *Kpn*I-*Kpn*I 1.7-kbp *pyrE* fragment, or pINV. Preparation of labeled probes and detection were performed with an enhanced chemiluminescence random prime labeling and detection system (Amersham).

Oligonucleotide-directed mutagenesis was performed by the method of Kun-

TABLE 1. Oligonucleotides used for site-directed mutagenesis

Oligonucleotide	Sequence[a]
INT-*pyrE*-N	5′-CAGGACGTCCATATGCCCCTACTCTAC-3′
TERM-*pyrE*-N	5′-GAGGAAGCGCATATGAGACCTCCTCC-3′
TERM-*pyrE*-V	5′-GGCAGCGAGGGATATCACCCTAGACC-3′
INT-*leuB*-N	5′-CCACCTTCATATGGTCCTCCTG-3′
TERM-*leuB*-V	5′-GTGCCGGATATCATCCCCATC-3′
KM1	5′-GCTCTAGAGGAGGAGCCATATGAATGGAC CAATAATAATGA-3′
KmEcoV	5′-GGGATATCGTTCAAAATGGTATGC-3′

[a] Restriction endonuclease recognition sites, *Nde*I and *Eco*RV, are underlined, and a ribosome binding site is shown by dots above the letters.

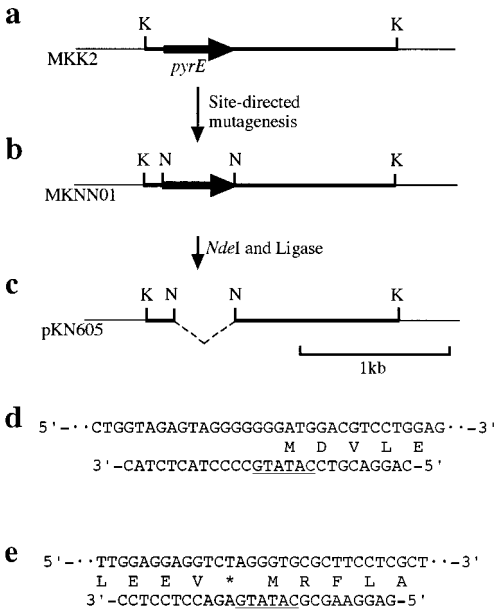

FIG. 1. Construction of Δ*pyrE* plasmid. Thick lines represent sequences originated from *T. thermophilus*. A 1.7-kb *Kpn*I fragment bearing the *pyrE* gene (26) was subcloned in M13mp18 (MKK2) (a) and two *Nde*I sites were introduced with the mutagenic primers INT-*pyrE*-N and TERM-*pyrE*-N as described in Materials and Methods (b). The 1.7 kb *Kpn*I fragment was recloned in pUC119, followed by *Nde*I digestion and self-ligation (c). The resultant plasmid, pKN605, was used to transform *T. thermophilus* MT106 (Δ*leuB*). A broken line indicates deletion. Sequences of the oligonucleotides used as mutagenic primers around the start codons of the *pyrE* gene (d) and the next open reading frame (e) are shown with the original nucleotide and amino acid sequences around the regions. An asterisk indicates a stop codon. The *Nde*I sites are underlined.

kel (10). Oligonucleotides used for site-directed mutagenesis and PCR amplification of the kanamycin resistance gene are listed in Table 1.

Transformation of *T. thermophilus*. To obtain uracil auxotrophs, *T. thermophilus* MT106 was genetically transformed as described by Koyama et al. (8) with a slight modification (22). The transformation mixture was incubated at 70°C overnight and spread on 5-FOA plates. The transformation frequency of the integration vectors was determined as described previously (8) without modification.

RESULTS AND DISCUSSION

Deletion of the *pyrE* gene from *T. thermophilus* MT106. The *pyrE* gene encodes orotate phosphoribosyltransferase of the pyrimidine biosynthetic pathway. In our previous work, we constructed a Δ*leuB* strain (MT106) from wild-type *T. thermophilus* HB27 by means of homologous recombination (22). We used the same method to obtain Δ*pyrE* strains. Plasmid pOM17 contains the 5.0-kb *Bam*HI fragment that encodes the pyrimidine biosynthetic operon cloned from *T. thermophilus* HB27 (27). A 1.7-kb *Kpn*I fragment was subcloned into M13 (MKK2). *Nde*I sites were introduced at the start codons of the *pyrE* gene and of the next open reading frame by oligonucleotide-directed mutagenesis. Oligonucleotide primers INT-*pyrE*-N and TERM-*pyrE*-N (Table 1) were used for the mutagenesis. The *Kpn*I fragment was recloned in pUC119. The *pyrE* gene was deleted by removing the *Nde*I-*Nde*I fragment (Fig. 1).

The resultant plasmid, pKN605, was used to transform a leucine auxotroph, *T. thermophilus* MT106 (22). The transformants were screened on minimum-medium Gelrite plates containing 5-FOA and uracil. 5-FOA-resistant uracil auxotrophs (KT7 and KT8) were selected. Chromosomal DNA was prepared from strain KT8 and analyzed by Southern hybridization (Fig. 2A and B). A 1.7-kbp *Kpn*I-*Kpn*I fragment can be seen in

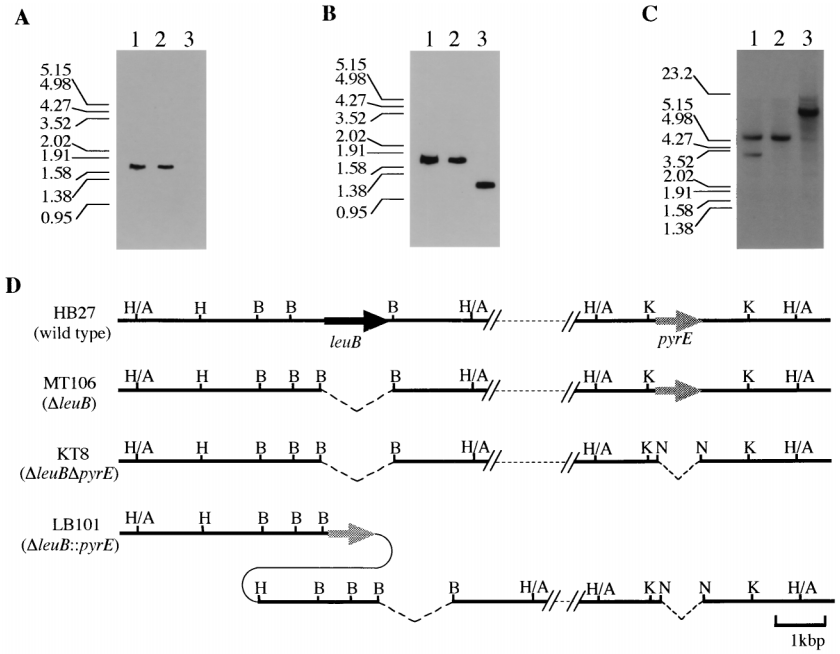

FIG. 2. Southern blot analysis of chromosomal DNA. (A) DNA was digested with *Kpn*I, electrophoresed on 1% agarose gel, transferred to a nylon membrane, and hybridized with the *Nde*I-*Nde*I *pyrE* 0.55-kbp fragment as a probe. Lane 1, HB27 (wild type); lane 2, MT106 (Δ*leuB*); lane 3, KT8 (Δ*leuB*Δ*pyrE*). Positions of size markers are shown by horizontal lines and their sizes are given in kilobase pairs. (B) Reprobing with the *Kpn*I-*Kpn*I *pyrE* 1.7-kbp fragment. Lane 1, HB27 (wild type); lane 2, MT106 (Δ*leuB*); lane 3, KT8 (Δ*leuB*Δ*pyrE*). (C) DNA was digested with *Hpa*I and *Afl*II, electrophoresed on 0.8% agarose gel, transferred to a nylon membrane, and hybridized with pINV. Lane 1, MT106 (Δ*leuB*); lane 2, KT8 (Δ*leuB*Δ*pyrE*); lane 3, LB101 (Δ*leuB*::*pyrE*, one of the transformants by pINV). (D) Restriction maps of the strains, as determined by Southern hybridization analysis. Deletion of the *leuB* locus was described and analyzed in a previous paper (22). The *leu* operon fragment was extended in LB101 compared with those in MT106 and KT8. The thin solid line represents an *E. coli* cloning vector, pBluescript. Restriction sites: H, *Hin*dIII; B, *Bam*HI; K, *Kpn*I; N, *Nde*I; H/A, *Hpa*I or *Afl*II (only the distance between the two H/A sites is reliable for the H/A sites). Broken lines indicate deletion (––––) and separation of these two loci in the chromosome (-----). Black and shaded arrows indicate the *levB* and *pyrE* genes, respectively.

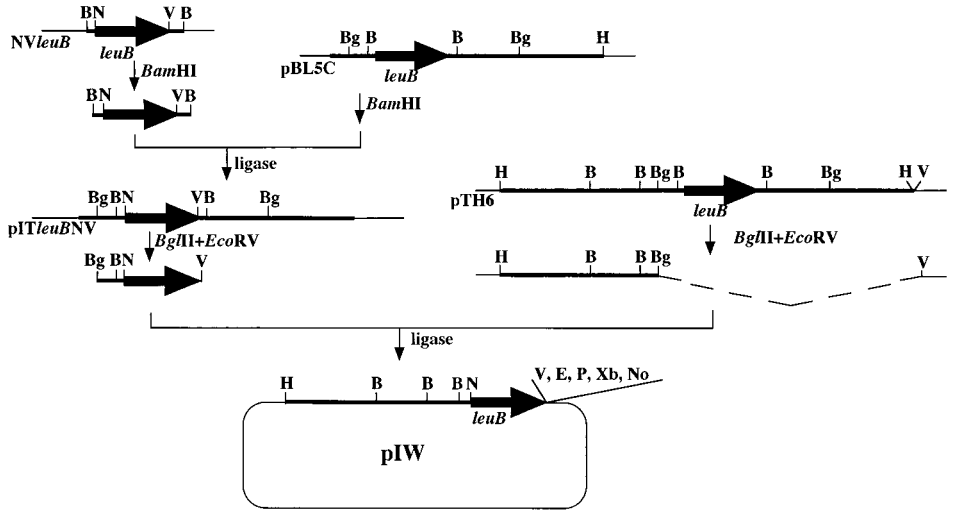

FIG. 3. Construction of integration vectors pIW and pINV. The thin lines represent *E. coli* cloning vectors. Thick lines represent sequences originated from *T. thermophilus*. A broken line indicates deletion. Solid arrows indicate the *levB* gene of *T. thermophilus*. A 1.2-kbp *Bam*HI fragment including the *T. thermophilus leuB* gene was subcloned in M13. An *Nde*I site and an *Eco*RV site were introduced at the initiation codon and immediately downstream of the termination codon of the *leuB* gene (NV*leuB*) with the primers INT-*leuB*-N and TERM-*leuB*-V, respectively. Construction of pBL5C was described previously (22). The *Bam*HI fragment of pBL5C including *leuB* was replaced with the *Bam*HI fragment of NV*leuB* (pIT*leuB*NV). Plasmid pTH6, which consists of pBluescript and a 5.8-kbp *Hin*dIII fragment including the *leuB* gene of *T. thermophilus* HB8 (23), was digested with *Bgl*II and *Eco*RV and then ligated with a *Bgl*II-*Eco*RV fragment of pIT*leuB*NV. Cloning sites of pIW originated from pBluescript. The restriction sites that remain unique in pIW are shown at the top of the box. pINV was constructed by replacing the *Nde*I-*Eco*RV *leuB* gene fragment with the *Nde*I-*Eco*RV *pyrE* gene fragment (not shown). The primer TERM-*pyrE*-V was used to introduce an *Eco*RV site at the 3′ end of the *pyrE* gene. The same sites as in pIW are unique in pINV. Restriction sites: E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; N, *Nde*I; Bg, *Bgl*II; V, *Eco*RV; P, *Pst*I; Xb, *Xba*I; No, *Not*I.

the wild-type (HB27) and Δ*leuB* (MT106) strains, while the fragment is lost in strain KT8. A 1.7-kbp *Kpn*I-*Kpn*I fragment, which encodes the *pyrE* gene in HB27 and MT106, was shortened to about 1 kbp in KT8. The results show that the *pyrE* gene is deleted in strain KT8.

Construction of vectors pIW and pINV and transformation efficiency of *T. thermophilus* KT8. pIW and pINV, the expression vectors for the *leuB* and *pyrE* genes, respectively, were constructed as shown in Fig. 3. pIW and pINV are based on the *E. coli* vector pBluescript and carry the *leuB* (3-isopropylmalate dehydrogenase gene) and *pyrE* gene cassettes, respectively, downstream of the *leu* operon. They lack replication origins for *T. thermophilus* and can, therefore, be kept in the thermophile only after integration into a *T. thermophilus* chromosome.

The Δ*leuB*Δ*pyrE* strain, KT8, was transformed with circular pIW or pINV and spread on minimum-medium plates supplemented with uracil or leucine plus isoleucine, respectively. The transformation efficiency was about 4×10^{-5} or 6×10^{-5} for pIW and pINV, respectively (Fig. 4). The isolated transformants maintained the marker genes even after 40 generations in nonselective medium. The plasmid structures and the transformation efficiencies are summarized in Fig. 4. The transformation efficiency with pIW or pINV was lower than that with pBL5C. Since both pIW and pINV transformed KT8 at similar efficiencies, the low efficiency was not due to the characteristics of the marker genes. One possible explanation is that the integration event depends strictly on the lengths of the homologous sequences. In fact, pBL5C has a 3.0-kb homologous sequence, whereas the homologous sequence in pIW or pINV is 2.0 kb. Alternatively, two homologous regions, one upstream and the other downstream of the inserted genes, may be necessary for a high efficiency of recombination.

Molecular analysis of the integration site. To confirm the integration site of pINV in KT8, one of the transformants with pINV, LB101, was analyzed by Southern hybridization after digestion of its chromosomal DNA with the restriction endonucleases *Hpa*I and *Afl*II, neither of which cleaves pINV, followed by hybridization with pINV as a probe (Fig. 2C). A 3.2-kb fragment can be seen in the Δ*leuB* (MT106) strain, while the fragment is lost in strain KT8, which shows again that the *pyrE* gene was completely deleted in KT8. A 5.5-kb fragment,

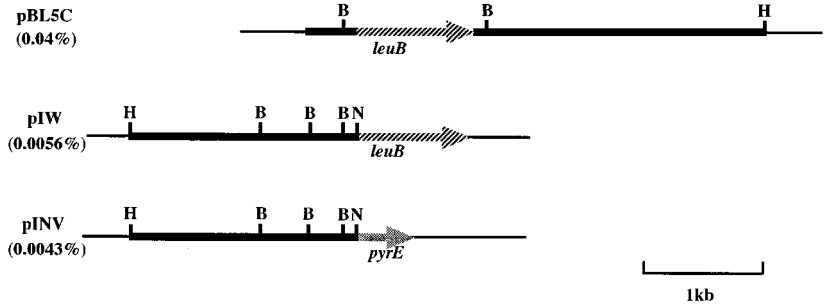

FIG. 4. Restriction maps of pBL5C, pIW, and pINV. Transformation efficiency, i.e., the number of transformed cells per total number of cells (about 5×10^7 cells/ml), of *T. thermophilus* KT8 with each vector is indicated in parentheses. KT8 was transformed with 10 μg of circular plasmid DNA/ml as described in Materials and Methods. Restriction sites: B, *Bam*HI; H, *Hin*dIII; N, *Nde*I.

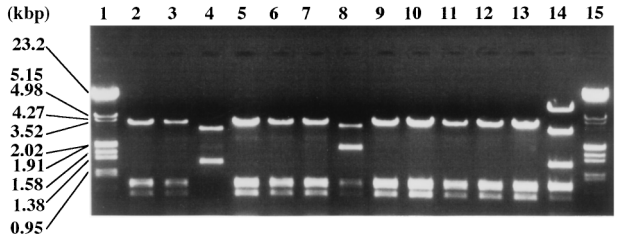

FIG. 5. Agarose gel electrophoresis of restriction enzyme-digested DNA of recovered plasmids. Plasmids recovered in *E. coli* from *T. thermophilus* transformants were digested with *Bam*HI and analyzed by agarose gel electrophoresis (lanes 3 to 14). Lane 2, pINV digested with *Bam*HI; lanes 1 and 15, lambda DNA digested with *Eco*RI plus *Hin*dIII.

which contains a *leu* operon, can be seen in MT106 and KT8, but disappeared in LB101 while a longer, 12-kb band emerged. The results show that pINV is integrated within the *leu* operon region in strain LB101. However, additional recombination may have occurred in minor populations of cells, which can be seen as smeared bands above and below the 12-kb band in Fig. 2C.

Recovery of the integrated vectors into *E. coli*. The fact that the vector was integrated through a single crossover event permits its recovery into *E. coli* (3). The *Hin*dIII site is one of the unique restriction sites in pINV. Chromosomal DNA from LB101 was digested with *Hin*dIII, and then the digest was religated at a low DNA concentration. *E. coli* was then transformed with the ligated DNA. Twelve *E. coli* ampicillin-resistant colonies were obtained. Restriction digests of the plasmids isolated from the colonies are shown in Fig. 5. Nine of them (Fig. 5, lanes 3, 5–7, and 9–13) had the same structure as pINV (Fig. 5, lane 2). The others had deleted or additional sequences, which are probably due to unexpected rearrangements that occurred in *T. thermophilus* or in *E. coli* during the recovery of the plasmid. The result indicates that the system described here allows stable chromosomal integration of the vectors at the cognate locus and that the integrated gene can be recovered into *E. coli* efficiently and easily.

Expression of a foreign gene by using the integration vector. Although we previously developed an integration vector for *T. thermophilus* (22), the vector was applicable only to *leuB* genes. New vectors, pIW and pINV, were designed to overcome this problem. A kanamycin nucleotidyltransferase gene was used to determine if a foreign gene can be cloned and expressed in the restriction site(s) of the multiple cloning sites of pINV. A kanamycin resistance gene (13) was amplified by PCR with the primers KM1, containing a ribosome binding site, and KmEcoV, treated with T4 DNA polymerase (Table 1), and was cloned into the *Eco*RV site of pINV. KT8 was transformed with the resultant plasmid, pINVK, and plated on minimum-medium plates without kanamycin. Eight randomly selected colonies from among many transformants which could grow without uracil were tested for kanamycin resistance. All of the colonies were able to grow on nutrient medium plates containing 40 μg of kanamycin/ml at 60°C. This experiment shows that the *pyrE* gene of pINV can be used as a marker for proper integration of the vector and that the integration vector can be used for expression of foreign genes in *T. thermophilus.*

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