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Pyrimidine Biosynthesis Genes (*pyrE* and *pyrF*) of an Extreme Thermophile, *Thermus thermophilus*

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We have isolated uracil auxotrophic mutants of an extreme thermophile, *Thermus thermophilus*. A part of the pyrimidine biosynthetic operon including genes for orotate phosphoribosyltransferase (*pyrE*) and for orotidine-5′-monophosphate decarboxylase (*pyrF*) was cloned and sequenced. The *pyrE* gene can be a bidirectional marker for the gene manipulation system of the thermophile.

Improvement and analysis of thermostability of proteins are major concerns in protein engineering. Thermophilic bacteria can be a source of thermostable proteins. Combination of random mutation and screening of stabilized proteins in thermophiles is another way to produce thermostable enzymes. Thermostable mutant enzymes are screened in moderately thermophilic microorganisms (14, 17). We have recently succeeded in stabilizing an enzyme, 3-isopropylmalate dehydrogenase, in an extreme thermophile, *Thermus thermophilus* (26).

T. thermophilus can grow at temperatures of up to 85°C (18). Several vectors have been developed for the organism (10, 13, 16). Several genes have been used as markers in the organism. We cloned and analyzed a unique gene for the genetic handling marker in this work.

The orotate phosphoribosyltransferase and orotate decarboxylase genes in the pyrimidine biosynthetic pathway have unique characteristics. Strains deficient in one of these genes become resistant to the bactericidal compound 5-fluoroorotic acid (5-FOA), while all deficient strains are uracil auxotrophs. Thus, both the wild type and the deficient strains of the genes can be positively selected. These genes have been cloned, sequenced, and used as genetic markers in many microorganisms (3–9, 19–21, 23, 24, 28). One of the genes, *pyrE*, has been cloned from a closely related species, *Thermus flavus*, very recently (29). We have isolated uracil auxotrophs from *T. thermophilus* and cloned and analyzed the *pyrE* gene and a part of the *pyrF* gene.

Culture conditions and genetic transformation. *T. thermophilus* HB27 was cultured in rich medium (18) or minimum medium (27) at 70°C as previously reported. Media were solidified by the addition of 1% Gelrite (25). Uracil (0.05 mg/ml) and/or 5-FOA (0.5 mg/ml) were added to the media when required. *T. thermophilus* was genetically transformed as described previously (12, 25).

Isolation of uracil auxotrophs. A log-phase culture (10^8 cell per ml) was centrifuged and resuspended in the minimum medium. It was irradiated with a 15-W germicidal lamp (Toshiba) at a distance of 56 cm for 60 sec (25). The survival rate is about 2% under these conditions. The cells were centrifuged, resuspended in minimum medium supplemented with uracil (50 μg/ml), and then incubated at 70°C for 3 h. They were spread on the minimum medium plates containing 5-FOA and uracil and then incubated at 70°C for 2 days. Several 5-FOA-resistant colonies appeared on the plates. 5-FOA-resistant colonies were purified on 5-FOA plates, and stable mutants were isolated. Among 17 5-FOA-resistant colonies, 4 clones showed clear and stable uracil auxotrophy.

Enzyme activity measurements. For the preparation of cell extracts, a 2-day culture grown in minimum medium supplemented with uracil was harvested. One gram of the cells was resuspended in 10 ml of 50 mM Tris-HCl, pH 8.0, and disrupted by a Branson Sonifier. The lysate was centrifuged at 25,000 × *g* for 30 min at 4°C, and then the supernatant was stored at −80°C. Protein concentration was determined by the use of the commercial Lowry reagent, with bovine serum albumin as the standard.

Orotate phosphoribosyltransferase (OPRTase) and orotidine-5′-monophosphate decarboxylase (OMPdcase) activities were measured by the method of Lieberman et al. (15). The reaction was carried out at 50°C, and A_{295} or A_{285} was monitored for OPRTase or OMPdcase, respectively. Differential extinction coefficients −3.67 and −1.65 μmole^{-1} for OPRTase and OMPdcase, respectively, (2, 15) were employed for the reaction rate estimation.

Cell extract was prepared from each uracil auxotrophic mutant. OPRTase and OMPdcase activities of the mutant and wild-type strains were tested and are listed in Table 1. OPRTase activity was markedly decreased in strains FOR1,

TABLE 1. OPRTase and OMPdcase activities of the wild type and the uracil auxotrophic strains of *T. thermophilus*

Strain	Enzyme activity (U/mg of protein)[a]	
	OPRTase	OMPdcase
HB27 (wild type)	0.090	0.398
FOR1 (*pyrE-1*)	0.005	0.535
FOR4 (*pyrF-4*)	0.084	0.004
FOR13 (*pyrE-13*)	0.008	0.679
FOR17 (*pyrE-17*)	0.004	0.414

[a] A unit of enzyme is defined as the amount that catalyzes 1 μmole of substrate per h. The values are averages of duplicated measurements.

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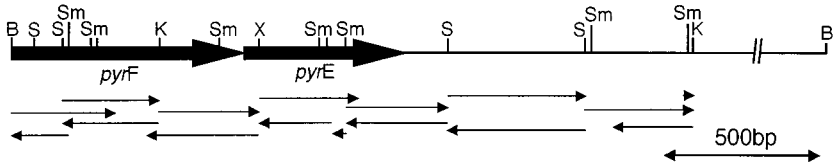

FIG. 1. Sequencing strategy and physical map of pOM17. Restriction endonuclease sites: B, *Bam*HI; S, *Sac*I; Sm, *Sma*I; K, *Kpn*I; X, *Xba*I.

FOR13, and FOR17. OMPdcase activity of these strains was higher than that of the wild-type strain. OMPdcase activity was decreased in strain FOR4.

Cloning of the *pyrE* gene. *T. thermophilus* shows a high frequency of natural transformation (12). The transformation frequencies of the mutant strains were determined with wild-type chromosomal DNA. The uracil requirement of the mutants was returned to autotrophy by genetic transformation with wild-type chromosomal DNA (Table 2). Table 2 also shows transformation frequencies for the wild-type chromosomal DNA digested with restriction enzymes. Transformation frequency did not significantly decrease after treatment with *Bam*HI. The DNA digested with other enzymes showed markedly decreased frequencies of transformation.

One of the uracil auxotrophic mutants of *T. thermophilus*, FOR13, was used to clone the *pyrE* gene in *Escherichia coli*. The technique used to clone the *trp* gene (11) was employed. Chromosomal DNA was digested with *Bam*HI, fractionated by electrophoresis in low-melting-point agarose gel, and recovered. The transformation efficiency of each fraction was tested with the mutant FOR13. A fraction containing 3 to 8 kb of DNA fragments showed the highest transformation frequency. The fragments were inserted into the *Bam*HI site of the pUC19 plasmid vector. The DNA was used to transform *E. coli* HB101. The *E. coli* colonies were replicated on minimum medium Gelrite plates that were coated with *T. thermophilus* FOR13 cells. The Gelrite plates were incubated at 70°C for 1 day. Some colonies of *T. thermophilus* were detected on the Gelrite plates. The *E. coli* colonies at the position corresponding to the colonies of transformed FOR13 cells were picked and analyzed. Four clones harbored the plasmid with about 6 kb of DNA insert. One of the plasmids, pOM17, was analyzed further.

The plasmid pOM17 showed the ability to transform uracil auxotrophic strains of *T. thermophilus* (Table 2). Thus, the *pyrE* and *pyrF* genes are expected to be at least partially encoded by the cloned fragment.

Sequence analysis of the cloned DNA fragment. The DNA fragment cloned in pOM17 was digested with the restriction enzymes indicated in Fig. 1 and subcloned into bacteriophage M13. Sets of partially deleted fragments were also prepared from the subclones with a Kilo-Sequence-Deletion-Kit (Takara Shuzo). DNA sequence was determined by the dideoxy-chain-termination method with a DNA sequencer (ABI 370A).

The nucleotide and deduced amino acid sequences are shown in Fig. 2. Three open reading frames (ORFs) were found in the region. The second ORF showed significant homology to OPRTase genes of other microorganisms. Figure 3 shows the alignment of the deduced amino acid sequences of these OPRTase genes. The similarity between the *pyrE* gene of *T. thermophilus* and the OPRTase genes of other species is lower than the mutual similarities between the genes of the other species. Nevertheless, significant homology was observed between the *T. thermophilus* gene and the OPRTase genes of the other microorganisms. The homologies of the amino acid and nucleotide sequences of the *pyrE* genes between *T. thermophilus* and *T. flavus* (29) were 100 and 98.5%, respectively.

Recently, the three-dimensional structure of the OPRTase of *Salmonella typhimurium* has been reported by Scapin et al. (22). They have reported active-site amino acid residues. Most of them are conserved and found in *T. thermophilus* OPRTase. They are indicated by dots in Fig. 3. Some residues (i.e., Lys-73, Arg-99, Lys-100, and Gln-141 in *S. typhimurium* numbering) could not be assigned in the *T. thermophilus* sequence by the alignment. These residues may be replaced by residues at different sites or may not be essential for the enzyme function.

The *pyrF* gene could also be aligned with the genes from other organisms. Although the homologies of the sequences are less than that of the *pyrE* genes, a conserved sequence could be seen in the stretch that is marked in Fig. 4.

Another ORF was found downstream of the *pyrE* gene. We could not find the homologous gene from known pyrimidine biosynthetic genes or from other genes with known sequences. However, the ORF may still be related to the gene in the pyrimidine biosynthetic pathway, because the sequence conservation of the gene may not be as high as can be seen in the *pyrE* and *pyrF* genes.

T. thermophilus is capable of incorporating DNA from outside of the cell and shows a high frequency of genetic transformation (12). Accordingly, for the analysis and expression of foreign genes, it is essential to delete the homologous gene

TABLE 2. Genetic transformation frequency of uracil auxotrophic mutant strains of *T. thermophilus*

Donor DNA[a]	Amt (ng)	Transformation frequency (%) of *T. thermophilus* mutants[b]			
		FOR1 (*pyrE*)	FOR4 (*pyrF*)	FOR13 (*pyrE*)	FOR17 (*pyrE*)
HB27	100	2.4	0.19	2.7	0.92
HB27 (*Bam*HI)	200	0.10	0.023	0.19	0.043
HB27 (*Kpn*I)	200	0.0018	$<3.8 \times 10^{-5}$	$<3.8 \times 10^{-5}$	4.6×10^{-4}
HB27 (*Sac*I)	200	$<1.5 \times 10^{-4}$	$<3.8 \times 10^{-5}$	$<3.8 \times 10^{-5}$	$<7.6 \times 10^{-5}$
HB27 (*Sma*I)	200	0.0038	$<3.8 \times 10^{-5}$	$<3.8 \times 10^{-5}$	$<7.6 \times 10^{-5}$
pOM17	100	9.8	0.91	5.0	7.2

[a] Chromosomal DNA of *T. thermophilus* HB27 was used for transformation directly or after treatment with respective restriction enzyme.
[b] % of total recipient CFU.

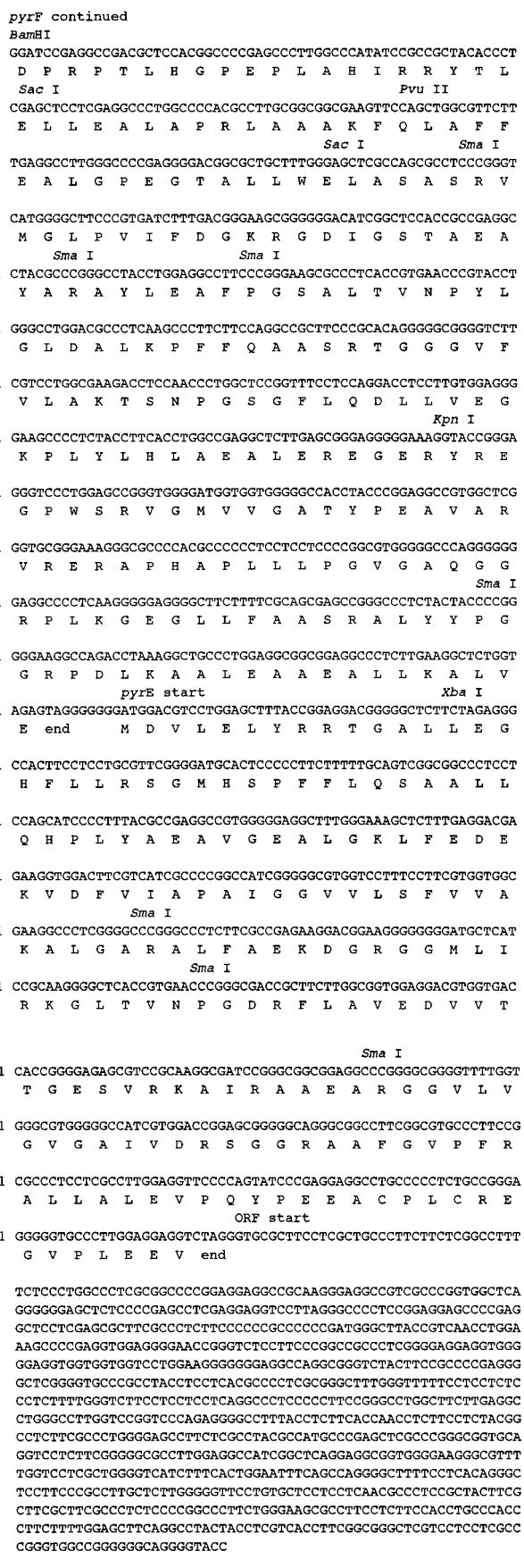

FIG. 2. Nucleotide and translated amino acid sequences of *pyrE* and *pyrF* genes of an extreme thermophile, *T. thermophilus*. Nucleotide sequence from the left *Bam*HI site to the right *Kpn*I site shown in Fig. 1 is presented.

from the genome of the thermophile to avoid unnecessary recombination. On the other hand, the high recombination frequency can be a useful characteristic for the development of integration and expression vectors. We have recently developed an integration and expression vector with the leucine operon (26).

The *pyrE* and *pyrF* genes have especially useful characteristics as discussed above. The screening of the mutants of one of these genes is easily done by positive isolation of the mutants on 5-FOA plates. Accordingly, the *pyrE-pyrF* gene fragment cloned here can be used as a useful integration and expression vector for the foreign gene in *T. thermophilus*. After the gene of interest is inserted in the *pyrE* gene, the *pyrE* gene with the guest gene can be integrated in the *pyrE* locus of the *T. thermophilus* chromosome. The *T. thermophilus* with the chromosome integrated with the disrupted *pyrE* gene can easily be isolated on 5-FOA plates as 5-FOA-resistant colonies. Thus, the fragment can be a more convenient vector than the *leu* vector. We have already succeeded in the integration and expression of the foreign gene in the *pyrE* gene and the preliminary results have been presented (1).

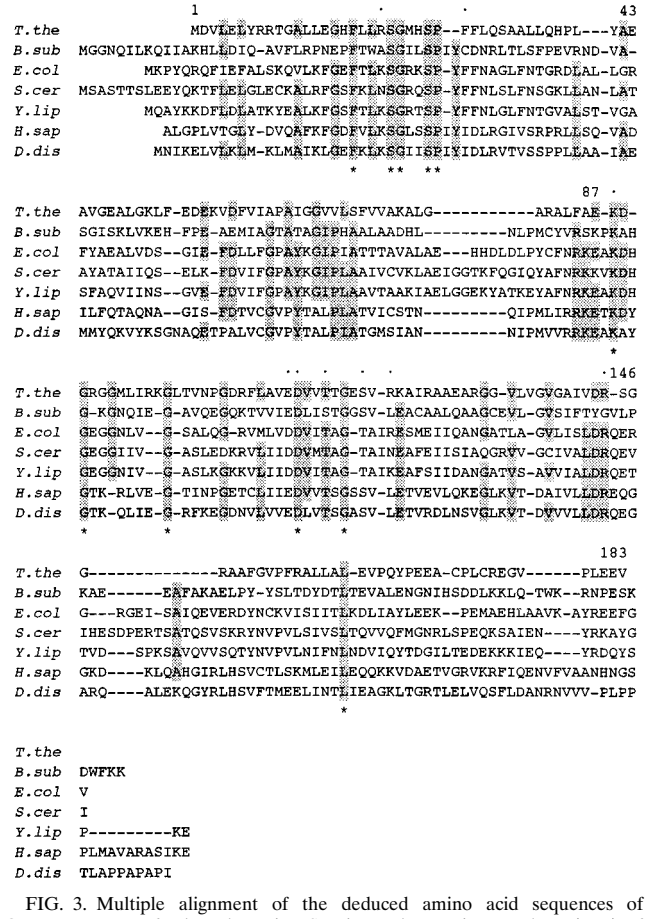

FIG. 3. Multiple alignment of the deduced amino acid sequences of OPRTase genes of selected species. Species and accession numbers (PIR) of sequences are *Thermus thermophilus* (D83330, DDBJ), *Bacillus subtilis* (A30492), *Escherichia coli* (A00580), *Saccharomyces cerevisiae* (S48223), *Yarrowia lipolytica* (S44156), *Homo sapiens* (A30148, A60258), and *Dictyostelium discoideum* (S03826). Relatively conserved residues are shaded. Conserved residues are marked by asterisks. Conserved residues of reaction center are dotted.

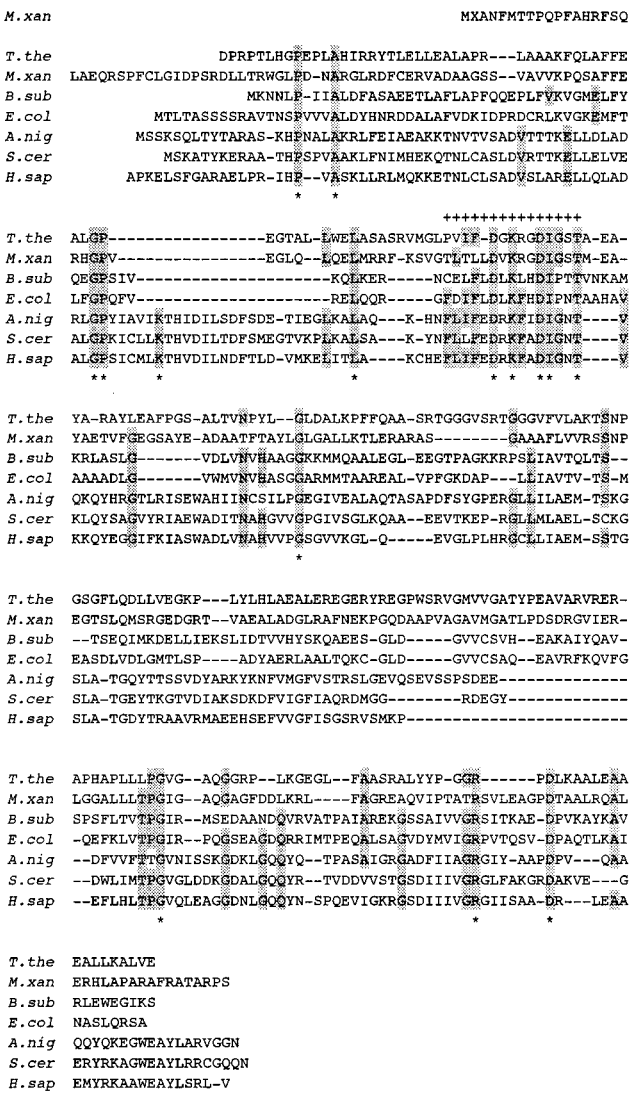

FIG. 4. Multiple alignment of the deduced amino acid sequences of OMPdcase genes of selected species. Species names are listed in the legend of Fig. 3. *Aspergillus niger* and *Mixococcus xanthus* are also included. Accession numbers (PIR) of the sequences are B41768, I39845, A28440, S03652, A01082, A25665, A30148, and A60258 (from top). Relatively conserved residues are shaded. Conserved residues are marked by asterisks. Especially conserved region is indicated by +.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number D83330.

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