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Molecular Cloning of the pyrE Gene from the Extreme Thermophile Thermus flavus

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Mutants of the extreme thermophile Thermus flavus in the pyrimidine biosynthetic pathway (Pyr−) were isolated by resistance to 5-fluoroorotic acid. The pyrE gene, which codes for the orotate phosphoribosyltransferase, was cloned by recombination with one of the isolated Pyr− T. flavus mutant strains. It was subcloned by complementation of an Escherichia coli pyrE mutant strain and was sequenced. The deduced polypeptide sequence extends over 183 amino acids. Several independent Pyr− mutations were mapped within the pyrE locus by recombination with fragments of the cloned gene.

Interest in thermophilic microorganisms has increased in recent years from both academic and industrial perspectives. Fundamental biological questions about thermophiles can address adaptation to extreme living conditions and can give insights into the mechanisms underlying thermophily and the nature of thermostable proteins. One of the best-studied groups of thermophiles is the genus Thermus. Many genes have been isolated from these gram-negative aerobic extreme thermophiles; however, only a few have proven useful as genetic markers (8, 11). Since no antibiotic resistance markers in the genus Thermus had been reported previously, thermostabilized mutants of kan gene derived either from a moderate thermophile Bacillus gene (18) or from the Staphylococcus aureus kan gene (16) were introduced into Thermus strains (13, 14, 17, 26).

On the basis of the existence of selections for either the loss or the restoration of gene function, genes of the uracil biosynthetic pathway have been successfully used for genetic manipulations in procaryotes and lower eucaryotes (3, 25, 28). Mutations in genes coding for either orotate phosphoribosyltransferases (OPRT) (designated URA5 or URA10 for yeasts and pyrE for procaryotes) or orotidine-5′-phosphate decarboxylase (designated URA3 for yeasts and pyrF for procaryotes) confer resistance to 5-fluoroorotic acid (5-FOA) and can be directly selected on 5-FOA-containing media (1, 12). Additionally, the pyr genes can be used in a direct biosynthetic selection for their presence in Pyr− mutant strains and serve as convenient genetic markers.

Here, we describe the selection of mutants in the uracil biosynthetic pathway in Thermus strains, the cloning and sequencing of the pyrE gene from Thermus flavus coding for an OPRT, and the genetic mapping of a variety of T. flavus Pyr− mutations using subclones of the pyrE gene.

Bacterial strains and growth. Escherichia coli strains BW322 (Hfr [PO45 of Hfr KL 16; serA, lzyA], λ− relA rfa-210: Tn10 pyrE70 spoT1 thi-1) (E. coli Genetic Stock Center), DH5α[ F −80lacZAM15 Δ(lacZYA-argF)U169 endA1 recA1 hsdR17 mK− mB−] deoR thi-1 supE44 λ− pyrA96 relA1] (Bethesda Research Laboratories), and MC1060 [F − hsdR X1488 leuB6 Δ(lacPOZY)74, galE15 galK16 relA spoT1 trpC9830 pyrF74:: Tn5 rpsL150] (2) were grown in Luria-Bertani (LB) and SOC-rich media or in minimal M9 medium supplemented with the appropriate amino acids or antibiotics (23) at 37°C and were transformed by electroporation (Gene Pulse [Bio-Rad]) according to the manufacturer’s instructions. Complex broth medium for growth of T. flavus AT62 (ATCC no. 33923) (TT) has been described in the American Type Culture Collection catalog as medium 697. TT agar medium additionally contained 15 g of Bacto Agar (Difco) per liter. Transformation medium (TM) for T. flavus has been described by Koyama et al. (10). The minimal agar medium (Tmin) described by Yeh and Trela (27) was used with or without the addition of 0.2% Casamino Acids for T. flavus auxotrophs.

Selection of Pyr− mutant strains and their reversion frequencies. UV light-induced or spontaneous Pyr− mutant strains of various Thermus strains were isolated. For selection of UV light-induced mutants, 100 µl from an overnight culture of Thermus cells was spread on TT agar and mutagenized face down on a UV transilluminator (305 nm) for time periods of between 2 s and 2 min, with approximately 20 s leading to optimal mutation rates for all strains. After 2 days of growth at 65°C, the surviving colonies were harvested by the addition of 1 ml of TT broth to the plates and by replating of dilutions of the mixture onto TT agar supplemented with 500 µg of 5-FOA (Sigma Chemicals, St. Louis, Mo.) per ml. The minimal concentrations of 5-FOA necessary to eliminate background growth were found to be 500 µg/ml for T. flavus and 300 µg/ml for T. aquaticus and T. thermophilus.

Spontaneous 5-FOA-resistant mutants were also obtained by spreading T. flavus cells onto TT agar with a 5-FOA concentration gradient. This gradient developed by diffusion of 0.5 ml of 5-FOA (1 mg/ml) out of the center of the plate.

All 5-FOA-resistant mutants were purified and checked for their uracil requirement. Reversion frequencies were determined by plating serial dilutions of cultures with an optical density at 600 nm of 0.5 onto Tmin Casamino Acids agar with or without the addition of uracil (40 µg/ml). The six mutant strains of T. flavus (with reversion frequencies in parentheses), TGF13 (2 × 10−4), TGF14 (3.7 × 10−4), TGF30 (1.6 × 10−4), TGF35 (7.5 × 10−4), TGF46 (6.7 × 10−4), and TGF53 (1.6 × 10−4) were chosen for further use.

Construction of gene library. A λ-ori cosmid, Lorisit 6 (5), was used as the vector and E. coli DH5α was used as the host for the construction of a gene library from T. flavus. Genomic
DNA was prepared by treatment of *T. flavus* cells with 2% sodium dodecyl sulfate in 50 mM glucose–25 mM Tris-HCl (pH 8)–30 mM EDTA at 42°C for 5 min and for another 10 min in the presence of proteinase K (0.1 mg/ml), after which extraction with phenol-chloroform-isoamyl alcohol and ethanol precipitation were performed. The cloning of size-fractionated, Sau3A-digested (restriction endonucleases from New England BioLabs, Beverly, Mass.) chromosomal DNA of *T. flavus* was achieved by using the HindIII-BamHI and EcoRV-BamHI arms of the cosmid (23). Size fractionation was obtained through 10 to 40% sucrose gradients, and the fractions were analyzed by conventional electrophoresis. Fractions with average fragment sizes of 35 to 45 kb were used for cloning.

Purified insert (300 ng of DNA) was dephosphorylated (calf intestinal alkaline phosphatase [Boehringer Mannheim Biochemicals, Indianapolis, Ind.]) to avoid cloning of nonneighboring DNA fragments, was ligated (T4 DNA ligase [New England BioLabs]) to an equimolar amount of cosmid arms in 10-μl ligation mixtures, and the fractions were analyzed by conventional electrophoresis. Fractions with average fragment sizes of 35 to 45 kb were used for cloning. Purified insert (300 ng of DNA) was dephosphorylated (calf intestinal alkaline phosphatase [Boehringer Mannheim Biochemicals, Indianapolis, Ind.]) to avoid cloning of nonneighboring DNA fragments, was ligated (T4 DNA ligase [New England BioLabs]) to an equimolar amount of cosmid arms in 10-μl ligation mixtures, and the fractions were analyzed by conventional electrophoresis. Fractions with average fragment sizes of 35 to 45 kb were used for cloning.

**Screening for pyrE-containing region.** Thermophile genes do not always complement *E. coli* mutations because of expression barriers or low level of activity in *E. coli* at 37°C (15). To avoid this, the *T. flavus* λ cosmid library was screened for recombination with the Pyr− mutation in *T. flavus* TGF35. Individual cosmid clones were grown in 96-well microtiter plates, and miniscale cosmid DNA preparations were made by an alkaline lysis protocol (23) carried out in small scale. To screen these DNA samples, a spot transformation technique similar to the one reported by Koyama and Furakawa (9) was used. A 50-ml culture of TM broth was inoculated with 1.6 ml of a *T. flavus* TGF35 overnight culture, and the mixture was incubated at 65°C with aeration for 4 h to achieve a state of natural competence (10). This cell culture was concentrated sixfold, and 100 μl of it was spread on Tmin Casamino Acids medium lacking uracil. DNA was applied onto the lawn of competent cells by using a 48-prong replicator. Of 672 recombinant cosmids tested, 28 were found to transform TGF35 to prototrophy. Two cosmids (2A6 and 6F6) were chosen for further characterization.

**Subcloning of the pyrE gene.** Both pyrE- and pyrF-type mutants can be selected with 5-FOA. To find out which mutation was present in the *T. flavus* TGF35 strain and to reduce the size of the Pyr−-complementing DNA fragment, the insert of cosmid 2A6 was subcloned into pUC19. *E. coli* BW322 (pyrE) and MC1066 (pyrF) were used as recipients for transformation. Cosmid 2A6 was partially digested with Sau3A, dephosphorylated to avoid ligation of nonneighboring pieces, and cloned into the BamHI site of pUC19. By selection on minimal medium lacking uracil, only BW322 transformants grew, indicating that the DNA cloned from *T. flavus* contained the pyrE gene. Small-scale plasmid DNA preparations from 40 individually picked clones were made by standard protocols (23) and were characterized by restriction analysis. Plasmid pVUF10 contained the smallest insert (4 kb), and its physical map is shown in Fig. 1. The 1.7-kb *Kpn*I fragment was further sub-
Cloned into the KpnI site of pUC19. This pVUF10.5 plasmid was still able to complement the E. coli pyrE mutation. The smallest T. flavus DNA fragment able to complement the E. coli mutant BW322 was found to be the 900-bp KpnI-XhoI insert of pVUF10.5.

**Determination and analysis of the nucleotide sequence of the pyrE gene.** The 1.7-kb KpnI fragment of subclone pVUF10.5 was cloned in both orientations into the Bluescript II SK+ plasmid and designated pTG-F1-5 and pTG-R1-8, respectively (Fig. 1). We have sequenced 1,710 nucleotides corresponding to the KpnI fragment within a region of 600 bp upstream and 900 bp downstream of the pyrE gene. The nucleotide sequence of the 1.7-kb KpnI fragment of subclone pVUF10.5 was cloned in both orientations into the Bluescript II SK+ plasmid and designated pTG-F (forward) or pTG-R (reverse). Unidirectional deletions were generated from pTG-F and pTG-R by using exonuclease III and S1 nuclease, (reverse). The single-letter amino acid code is used.

![Figure 2](https://example.com/figure2.png) FIG. 2. Nucleotide sequence of the T. flavus pyrE gene and putative pyrE coding region. The nucleotide sequence of the 1.7-kb KpnI insert from pVUF10.5 and the deduced pyrE amino acid sequence are shown. Arrows indicate regions of dyad symmetry. The proposed 5-PRPP binding site (bs) is marked.

**Genetic analysis of the pyrE region of T. flavus.** To characterize the pyrE region of T. flavus chromosome, we constructed six T. flavus pyrE mutants with different DNA fragments containing the pyrE gene region. Chromosomal T. flavus DNA; cosmids 2A6 and 6F6; plasmids pVUF10, pVUF10.5, and pVUF10.5.1, and the deletion plasmids generated for sequencing were used for these transformations. A 50-μl volume of competent cell cultures prepared as described above was mixed with 1 μg of plasmid or 5 μg of chromosomal DNA, and the mixture was incubated at 65°C during shaking for 1 h, diluted, and plated on T_{min} Casamino Acids agar. The results are shown in Fig. 1. Both of the original cosmid clones were able to restore the gene in all mutated strains. The mutations in the Thermus strains TGFI4, TGF35, and TGF53 were localized to a degree of accuracy of a few hundred base pairs. TGF30 probably carries either a double mutation or a deletion. The mutations in TGF31 and TGF46 do not seem to be located within a region of 600 bp upstream and 900 bp downstream of the pyrE gene. This observation may be explained by a regulatory nature of these mutations and needs further investigation.

The new pyrE marker should be a useful genetic tool for chromosomal insertion of heterologous DNA into T. flavus, especially because of the possibility of positive selection for inactivation of the gene. Since we were able to complement the pyrE mutation in E. coli, the protein must have functional similarities to the E. coli ORP. This makes the pyrE gene convenient for applications in E. coli-Thermus shuttle vector development.

**Nucleotide sequence accession number.** The sequence data presented here have been submitted to the GenBank database and were assigned accession number U27180.

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**REFERENCES**


