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Molecular cloning and structural analyses of the *pyrF* gene for orotate monophosphate (OMP) decarboxylase from extremely halophilic archaeon *Haloarcula japonica* strain TR-1

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Synopsis

The orotate monophosphate decarboxylase gene (*pyrF*) in the pyrimidine biosynthetic pathway have unique characteristics. In this study, the *pyrF* gene was found to encode a polypeptide consisting of 277 amino acid residues, showing 96.0 % identity to that of *H. marismortui*. Southern hybridization of the *pyrF* gene region with partial sequence probe revealed that single copy number of the gene is distributed on the chromosome of *H. japonica* genome. By phylogenetic analyses of the *H. japonica* PyrF, the amino acid sequences of the enzyme is relatively conserved in other archaeal strains including methanogens and thermophiles. Alignments of several sequences containing *pyrF* gene showed that the genetic organization of *pyrF* gene was not conserved in several haloarchaeal strains expect for *H. marismortui*.

Key word: *Haloarcula japonica*, *pyrF*, pyrimidine biosynthesis, 5-FOA, auxotroph

1. Introduction

Until recently, a long-established dogma in biology was that all life on earth is conventionally divided into two kingdoms: eukaryotes and prokaryotes. However, 16S rDNA analyses in the 1970s indicated that the prokaryotic world is not a single coherent entity, but, instead, is divided into two distinct groups, the eubacteria and archaea [1].

Halophilic archaea survive in environments containing molar concentrations of salts. The high external salt condition is balanced by an equally high internal salt concentration. Thus, halophilic archaea offer the opportunity to study specific interactions of biomolecules (i.e. DNA and proteins) in the presence of high osmolarity. The progress of research on extremely halophilic archaeon, *Haloarcula japonica*, had been constantly hampered by the limitation of available tools for genetic manipulation. The orotate monophosphate decarboxylase gene (*pyrF*) in the pyrimidine biosynthetic pathway have unique characteristics. Strains deficient of the gene become resistant to the bactericidal compound 5-fluoroorotic acid (5-FOA), while all deficient strains are uracil auxotrophs (Fig. 1). Thus, both the wild type and the deficient strains of the gene can be positively selected. The gene have been cloned, sequenced, and used as genetic markers in many microorganisms [2,3]. Thus, as a prerequisite for future establishing *H. japonica* gene manipulation system, we report here the cloning *pyrF* gene form *H. japonica*.

2. Materials and Methods

2.1 Strain and culture conditions

The *H. japonica* strains used in this study are listed in Table 1. The *H. japonica* cells were grown at 37°C with vigorous shaking in a rich medium, containing (per litter): 200 g of NaCl, 20 g of $MgSO_4 \cdot 4H_2O$, 3.0 g of trisodium citrate·$2H_2O$, 3.0 g of KCl, 10 g of yeast extract, 7.5 g of casamino acid and a mixture of trace elements (0.0218 g of $MnCl_2 \cdot 4H_2O$ and 0.486 g of $FeCl_3 \cdot 6H_2O$ per 100ml), and pH was adjusted to 7.3 with NaOH. For investigation of tryptophan auxotrophy, minimal medium was used, containing (per litter): 200 g of NaCl, 20 g of $MgSO_4 \cdot 4H_2O$, 3.0 g of trisodium citrate·$2H_2O$, 3.0 g of KCl, 6.0 g of Tris base per litter and a mixture of trace elements (0.0218 g of $MnCl_2 \cdot 4H_2O$ and 0.486 g of $FeCl_3 \cdot 6H_2O$ per 100ml), and pH was adjusted to 7.3 with HCl. When necessary, 5-FOA(0.25 mg/ml; Wako, Osaka, JAPAN) and uracil(Nacalai, Osaka, JAPAN) were added to minimal medium. *E. coli* was grown at 37°C in Luria-Bertani media (LB; yeast extract, 5g/l; tryptone, 10g/l; NaCl, 10g/l) supplemented with 50μg/ml ampicillin for growth of strains containing plasmids.

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2.2 DNA manipulations

H. japonica genomic DNA was isolated with Microbial DNA isolation kit (MoBio) as recommended by the manufacture. The plasmids used in this study are listed in Table 1. Plasmids were subcloned and maintained in E. *coli* JM109, and isolated using AccuPrep Plasmid Mini Extraction Kit (BIONEER).

2.3 Construction of plasmid for pyrF disruption by degenerate PCR

The *pyrF* from *H. japonica* genomic DNA was amplified by degenerate PCR using primers pyrF1s and pyrF1as (Table 2). The degenerate PCR was performed with the degenerate primers by using 25 thermal cycles consisting 94°C for 30 sec, 45°C for 30 sec, and 72°C for 1.5 min. The PCR product was cloned into cloning vector pT7Blue-2 (Novagen), after which competent *Eschericha coli* JM109 cells were transformed. Plasmids were isolated using AccuPrep Plasmid Mini Extraction Kit (BIONEER).

2.4 Southern blot

Genomic DNA was digested with *Aat*II, *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Kpn*I, *Mlu*I, *Nru*I, *Pst*I, *Pvu*II, *Sac*I, *Sal*I, and *Sty*I, fractionated by electrophoresis on 0.7% agarose gel and transferred to Hybond-N$^+$ nylon membranes (Amersham Biosciences UK Limited). Hybridization probes were generated by PCR with the primer set pyrF1s and pyrFas from plasmids carrying *pyrF* of *H. japonica*, and labeled using the DIG Labeling Kit (Roche Diagnostics GebH, Mannheim, Germany

Table 1 Strains and plasmids used in this study

Strain or plasmid	Characteristics	Sorce or reference
Microorganisms		
Haloarcula japonica TR-1	Wild type	
Escherichia coli JM109	*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*(rK- mK+), *e14-* (*mcrA-*), *supE44*, *relA1*, Δ(*lac-proAB*)/F'[*traD36*, *proAB+*, *lacIq*, *lacZ*ΔM15]	purchased by TaKaRa
Plasmids		
pT7Blue-2 T-Vector	Cloning vector containing ampicillin resistance gene	purchased by Novagen

Table 2 Degenerate primers used in this study

Primer	Sequence	Mer
pyrF1s	5'-TCNGTNGGNCTNGAYCCNGAYCC-3'	23
pyrF1as	5'-GCNCCNACNCCNGGNACNAGRAA-3'	23

Fig. 1 Pathways for the biosynthesis of pyrimidine nucleoside ribophosphates in *H. marismortui*

The enzymes are idetified by their corresponding gene designations as follows [4]: *arcB*, ornithine carbamoyltransferase; *argG*, argininosuccinate synthase; *argH*, argininosuccinate lyase; *carB*, carbamoyl phosphate synthase large subunit; *cdd*, cytidine deaminase; *dcd*, deoxycytidine triphosphate deaminase; *glnA*, glutamine synthetase; *ndk*, nucleoside diphosphate kinase; *surE*, acid phosphatase SurE; *pyrC*, dihydroortase; *pyrD*, dihydroortate oxidase; *pyrE*, orotate phosphribosyltransferase; *pyrF*, OMP decarboxylase; *pyrG*, CTP synthetase; *pyrH*, UMP kinase; *pyrI*, aspartate carbamoyltransferase regulatory subunit; *udp*, uridine phosphorylase; *upp*, uracil phosphoribosyltransferase.

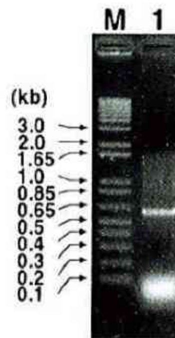

Fig. 2 Degenerate PCR amplification of *pyrF* gene in *H. japonica* using pyrF1s and pyrF1as primers. M; marker, 1; *pyrF* gene amplified using the degenerate primers, PCR amplification was electrophoresed on 1.0% agarose gels.

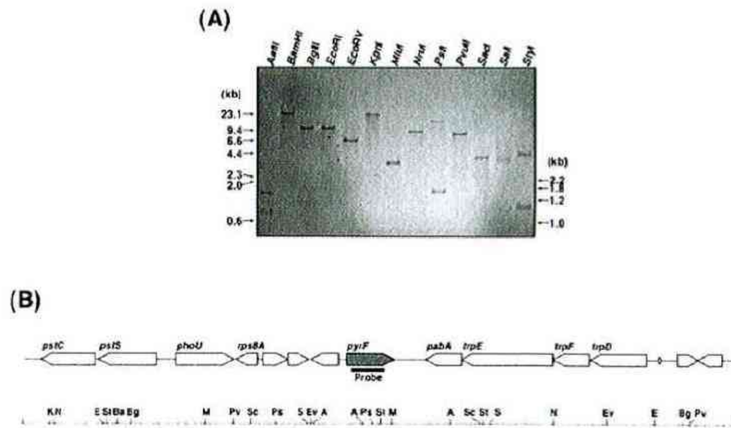

Fig. 3 Southern blot analyses using the *pyrF* probe

A) Generic DNA extracted from strain TR-1 was digested completely with *Aat*II, *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Kpn*I, *Mlu*I, *Nru*I, *Pst*I, *Pvu*II, *Sac*I, *Sal*I, and *Sty*I, electrophoresed on 0.7% agarose gels, and transferred to a nylon membrane and hybridized with *pyrF* probe. The size marker (kb) is shown on the left and right sides.

B) Structure and restriction enzyme sites of *pyrF* surrounding region

A: *Aat*II, Ba: *Bam*HI, Bg: *Bgl*II, E: *Eco*RI, Ev: *Eco*RV, K: *Kpn*I, M: *Mlu*I, N: *Nru*I, Ps: *Pst*I, Pv: *Pvu*II, Sa: *Sac*I, S: *Sal*I, St: *Sty*I

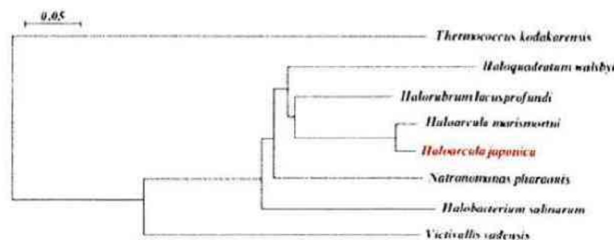

Fig. 4 Phylogenic tree of *pyrF* gene in halophilic archaea.

Phylogenic tree was constructed based on amino acid sequences of *pyrF* gene using the neighbor-joining algorism.

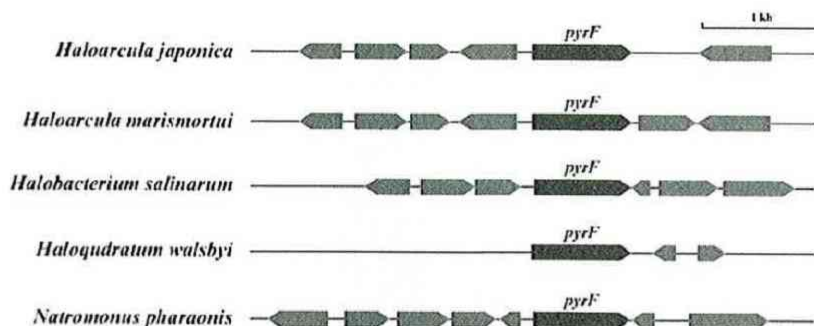

Fig. 5 Comparison of *pyrF* gene structure in *Halobacterium* archaea
Sequences of each *pyrF* region were colored by dark gray.

3. Results and discussion

At first, to clone a portion of the pyrF gene, a highly conserved region of amino acids in PyrF found in several archaeal strains was used as the basis for preparing a pair of degenerate primers for PCR (Table 2). The 589 bp PCR product was cloned into pCR2.1 vector and sequenced (Fig. 2). The DIG-labeled insert was used as a probe to screen and isolate the DNA clone containing the *pyrF* gene from lambda EMBL3 library. A positive clone with 16kb insert was further subcloned and sequenced. The *pyrF* gene was found to encode a polypeptide consisting of 277 amino acid residues, showing 96.0 % identity to that of *H. marismortui*. Southern hybridization was also performed to detect cloned gene on the chromosome, and estimate copy number of the gene. The analysis of the *pyrF* gene region with partial sequence probe revealed that single copy number of the gene is distributed on the chromosome of *H. japonica* genome (Fig. 3A). The structure of the gene was also shown in Fig. 3B. By phylogenetic analyses of the *H. japonica* PyrF, the amino acid sequences of the enzyme is relatively conserved in other archaeal strains including methanogens and thermophiles (Fig. 4). To compare genetic organization of *pyrF* gene of the strain with other archaeal strains, alignments of several sequences containing the gene were performed. The genetic organization of *pyrF* gene was not conserved in many archaeal strains expect for *H. marismortui* (Fig. 5). The gene is very useful to construct auxotroph of *H. japonica* for genetic manipulation [5].

Construction of *pyrF* disruptant plasmid and the positive screening with the transformants are being in progress in *H. japonica*.

4. References

1) A. Yamagishi, T. Tanimoto, T. Suzuki, and T. Oshima, Pyrimidine biosynthesis genes (*pyrE* and *pyrF*) of an extreme thermophile, *Thermus thermophilus*, Appl. Environ. Microbiol., 62 (6), 2191-2194 (1996)

2) A. Bouia, F. Bringel, L. Frey, A. Belarbi, A. Guyonvarch, B. Kammerer, and J. –C. Hubert, Cloning and structure of the *pyrE* gene of *Lactobacillus plantarum* CCM 1904, FEMS Microbiol. Lett., 69, 233-238 (1990)

3) F. P. Buxton, and A. Radford, Cloning of structural gene for orotidine 5'- phosphate carboxylase of *Neurospora crassa* by expression in *Escherichia coli*, Mol. Gen. Genet., 190, 403-405 (1983)

4) K. F. Jensen, J. N. Larsen, L. Schack, and A. Sivertsen, Studies on the structure and expression of *Eschericha coli pyrC*, *pyrD*, and *pyrF* using the cloned genes, FEBS, 140, 343-352 (1984)

5) T. Sato, T. Fukui, H. Atomi, and T. Imanaka, Improved and versatile Transformation system Allowing multiple genetic manipulations of the hyperthermophilic archaeon *Thermococcus kodakaraensis*, Appl. Environ. Microbiol., 71 (7), 3889-3899 (2005)