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## Molecular Characterization and Sequence Analysis of Trehalose Biosynthesis Genes in *Escherichia coli* from Fish

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**Abstract:** Trehalose, a non-reducing disaccharide is a major compatible solute, maintains fluidity of membranes and protects the biological structure of the organisms under stress. In this study, Trehalose-6-Phosphate Synthase (TPS) and Trehalose-6-Phosphate Phosphatase (TPP) genes encoding for trehalose biosynthesis were PCR amplified from genomic DNA of *Escherichia coli* isolated from Rohu (*Labeo rohita*) collected from local fish market of Cochin, Kerala, India. The amplified genes were cloned and nucleotide sequences were determined. The sequencing results showed that TPS and TPP genes contain 1, 424 and 801 bp long ORF encoding 474 and 266 amino acids, respectively. *In silico* sequence analysis revealed that the TPS and TPP sequences of *E. coli* were conserved in many eubacteria. In conclusion, the nucleotide sequence of TPS and TPP genes of this study make known to have a high degree of similarity with other organisms. The phylogenetic tree analysis of nucleotide and amino acid sequences of TPS and TPP of our isolate also reveals the maximum similarity with other organisms.

**Key words:** PCR, electrophoresis, cloning, transformation, recombinant clones, sequencing

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### INTRODUCTION

Trehalose is a nonreducing disaccharide, having two glucose units are linked in a,  $\alpha$ -1, 1-glycosidic linkage. Present in a wide variety of organisms, including bacteria, yeast, fungi, insects, invertebrates, lower and higher plants, where it serves as a source of energy and carbon (Kaasen *et al.*, 1994). Trehalose has the remarkable ability to protect plants from damage (Roser, 1991). In yeast and plants, it serves as a signaling molecule to direct or control certain metabolic pathways (Hohmann, 2002). In addition, trehalose also protects the proteins and cellular membranes from inactivation or denaturation caused by variety of stress conditions including desiccation, heat, cold and oxidation (Leslie *et al.*, 1995). In *E. coli*, trehalose biosynthesis reactions are catalyzed by Trehalose-6-Phosphate Synthase (TPS) and Trehalose-6-Phosphate Phosphatase (TPP), encoded by the *otsA* and *otsB* gene, respectively (Hohmann, 2002; Leslie *et al.*, 1995). *Escherichia coli* synthesize trehalose only when exposed to high osmolarity (Giaever *et al.*, 1988; Dimbier *et al.*, 1992; Kaasen *et al.*, 1992; Strom *et al.*, 1986). This is different from *Saccharomyces cerevisiae*, in which trehalose is synthesized by a large multisubunit complex with the catalytic activities of both *otsA* and *otsB* (Styrbold and Strom, 1991; Cabib and Leloir, 1958; Londesborough and Vuorio, 1991).

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In mycobacteria and corynebacteria, trehalose is an integral component of various glycolipids that are important cell wall structures. Trehalose stabilizes enzymes, foods, cosmetics and pharmaceuticals at high temperatures (Vandercammen *et al.*, 1989; Carninci *et al.*, 1998; Colaco *et al.*, 1992). Due to its desirable physical and chemical characteristics, commercial production of trehalose is anticipated. The aim of the present study is the identification, characterization and *in silico* analysis of the trehalose biosynthesis genes (TPP and TPS) in *Escherichia coli* isolated from fish.

## MATERIALS AND METHODS

### Bacterial Strain, Growth Conditions and DNA Isolation

*Escherichia coli* TCJAR023 was isolated from a finfish; Rohu (*Labeo rohita*) procured from local fish markets in Cochin, India. Microbial identification and biochemical characterization of *E. coli* was carried out as per [U.S. Food and Drug Administration (USFDA)] methods. *Escherichia coli* was grown aerobically in Luria-Bertani (LB) broth medium containing 1% tryptone, 0.5% yeast extract, 0.5% NaCl and incubated at 37°C. Genomic DNA of *E. coli* was prepared as described by Ausubel *et al.* (1994).

### Polymerase Chain Reaction

The trehalose biosynthesis genes were amplified by using gene specific primers. The PCR reaction was done in a final volume of 50 µL that contained 0.5 µM each of forward and reverse primers; 1.0 µL of crude genomic DNA; 200 µM of dNTPs; 1X Pfu buffer; 2.5 mM MgSO<sub>4</sub>; 1.25U Pfu DNA polymerase (MBI Fermentas, USA) and autoclaved Millipore water. The PCR was performed using a PTC-150 Mini cyclor (MJ Research, USA) with the following conditions; initial denaturation at 94°C for 3 min, followed by 30 repeated cycles of 94°C for 30 sec, 50°C for 1 min and 72°C for 2 min and final extension at 72°C for 5 min. The PCR amplified product was analyzed on 1.5% agarose gel along with DNA ladder (MBI Fermentas) and documented using a gel documentation system (Alpha Imager 1220, Alpha Innotech Corporation, San Leandro, CA, USA).

### Cloning of PCR Product

The amplified gene fragments were purified with Perfectprep Gel Cleanup Kit (Eppendorf, Germany) and cloned into pTZ57R/T (MBI Fermentas), according to the manufacture's instructions. The cloned inserts were transformed into the *E. coli* JM109 and plated on LB agar containing ampicillin (100 µg mL<sup>-1</sup>), IPTG (50 µM) and X-gal (80 µg mL<sup>-1</sup>). The plates were incubated at 37°C and the transformants were selected by blue white selection. The white recombinant colonies were selected and inoculated in 5 mL LB broth containing ampicillin. The plasmids were isolated from the overnight culture by alkaline lysis method (Sambrook and Russell, 2001).

### Characterization of the Recombinant Plasmid

The recombinant plasmid was double digested with restriction enzymes, *EcoRI* and *HindIII* for (TPS) and *SmaI* and *HindIII* for (TPP). The reaction mixture contained recombinant plasmid 2 µL, enzyme buffer (10X) 2 µL, each restriction enzyme (10U µL<sup>-1</sup>) 0.5 µL and volume up to 20 µL with autoclaved Millipore water. The reaction mixture was incubated overnight at 37°C in a water bath. The digested products were analyzed on 1.5% agarose gel. The clone with the correct insert as judged by size was sequenced on an ABI PRISM 377 genetic analyzer (Applied Biosystems, Perkin Elmer Co., Foster City, CA, USA).

### ***In silico* Sequence Analysis**

The nucleotide sequences obtained were compared against database sequences using BLAST provided by NCBI (<http://www.ncbi.nlm.nih.gov>) and were aligned and clustered using CLUSTAL-X version 1.81 program (Thompson *et al.*, 1997). The output alignments were imported into the GeneDoc program (<http://www.psc.edu/biomed/genedoc/>) and BioEdit version 7.05 program ([www.mbio.ncsu.edu/BioEdit/](http://www.mbio.ncsu.edu/BioEdit/)) to calculate the percent identities among the nucleotide and amino acid sequences. The molecular masses and the theoretical pI values of the polypeptides were predicted using the ProtParam tool (<http://www.expasy.org/tools/protparam.html>).

## **RESULTS AND DISCUSSION**

The TPS and TPP genes encode the trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, respectively. Together these proteins constitute the trehalose biosynthetic pathway. The TPS and TPP genes were PCR amplified and is encoded by polynucleotides of 1,424 and 801 bp (Fig. 1). The TPS and TPP genes encodes proteins of 474 and 266 amino acids with the calculated molecular masses of 53,611 and 29,177 Da, based on the *in silico* estimates. After PCR amplification, the products were purified from the agarose gel and cloned into pTZ57R/T cloning vector. The white colonies were selected and screened for the presence of insert, which also exhibited the specific product by PCR amplification. The recombinant transformants with TPS and TPP genes were also confirmed by double digestion with restriction enzymes. The nucleotide sequence of inserts TPS and TPP genes were submitted to GenBank and have been given accession nos. FJ895834 and FJ823259.

Homology search of the genes and deduced amino acid sequence were performed using BLAST. The nucleotide sequence of TPS and TPP genes matches significantly with the trehalose biosynthesis genes from other organisms. The TPS and TPP sequences from *E. coli* TCJAR023 were analyzed with reported nucleotide and amino acid sequences of other eubacteria *viz.*, *Shigella sonnei* (GenBank accession No. CP000038), *Pseudomonas savastanoi* (AY308799), *Salmonella enterica* (AE017220) using Clustal W software. *In silico* analysis of the nucleotide sequences of TPS and TPP genes showed a high degree of similarity with other eubacteria. The amino acids analysis revealed that the TPS gene encoded protein belongs to the trehalose-6-phosphate synthase family. It had partial homology with phosphate synthase family from other bacteria: *S. enterica*, 86% identity; *P. savastanoi*, 99% identity and *S. sonnei* with 100% identity. *In silico* sequence analysis

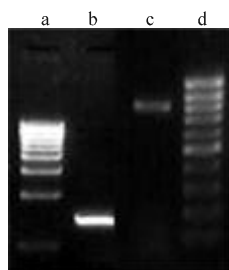


Fig. 1: Agarose gel electrophoresis of PCR products of TPS and TPP. Lane a: 1 kb DNA marker, Lane b: TPS amplicon 1,425 bp, Lane c: TPP amplicon 801 bp, Lane d: 100 bp DNA marker

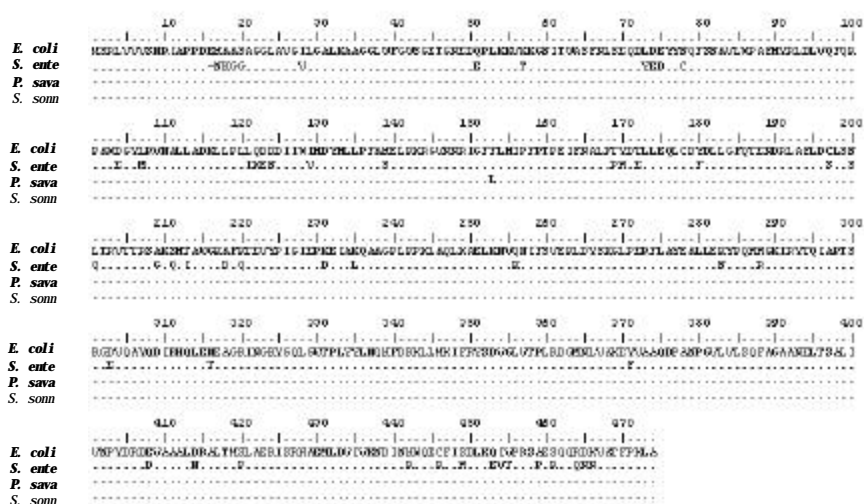


Fig. 2: Alignment of the amino acid sequence of TPS gene from *E. coli* TCJAR023 with sequences from *Salmonella enterica*, *Pseudomonas savastanoi* and *Shigella sonnei*. Dots indicate amino acid identity and letters represents amino acid substitutions

also revealed an amino acid substitution at position 153 in *P. savastanoi*, resulted in the substitution of phenylalanine (F) with leucine (L) residue. In *S. enterica*, various amino acid substitutions were observed in N-terminal, middle and C-terminal regions (Fig. 2). The amino acid analysis of TPP gene suggests that the encoded protein belongs to the trehalose-6-phosphatase family. The TPP of *E. coli* TCJAR023 had highly conserved regions with phosphate phosphatase from other bacteria. *S. enterica* sequence has 73% identity; 98% identity and *S. sonnei* with 99% identity. The sequence analysis of TPP revealed various amino acid substitutions with that of other eubacteria. Upon analysis a total of two amino acid substitutions were found in *S. sonnei* at positions 124 and 208, resulted in the amino acid substitution of threonine (T) with alanine (A). In *P. savastanoi*, a total of four amino acid substitutions were observed at positions 8, 116, 124 and 208 resulted in the substitution of threonine (T) with proline (P); glutamine (Q) with arginine (R); threonine (T) with alanine (A) and alanine (A) with valine (V) residue, respectively. In *S. enterica*, various amino acid substitutions were observed in N-terminal, middle and C-terminal regions as predicted in TPS (Fig. 3).

Phylogenetic tree based on evolutionary distances was constructed from nucleotide and amino acid sequences of TPS and TPP genes using MEGA software (Molecular Evolutionary Genetics Analysis, version 3.1) (Kumar *et al.*, 2004) using the neighbor-joining method. The tree at nucleotide and amino acid level of TPS and TPP (data not shown) reveals the phylogenetic similarity of TPS and TPP genes from *E. coli* TCJAR023 with other organisms. The bacterial species switched to different clusters for TPS and TPP genes at nucleotide and amino acid level indicating divergence among the organisms and the degree of divergence between the two genes.

Trehalose (1- $\alpha$ -D-glucopyranosyl-1- $\alpha$ -D-glucopyranoside), a non-reducing disaccharide is a major compatible solute, which maintains fluidity of membranes and protects the biological structure of organisms under stress. Trehalose represents the predominant class of osmolytes in aerobic chemoheterotrophic eubacteria (Kaasen *et al.*, 1992). Trehalose has

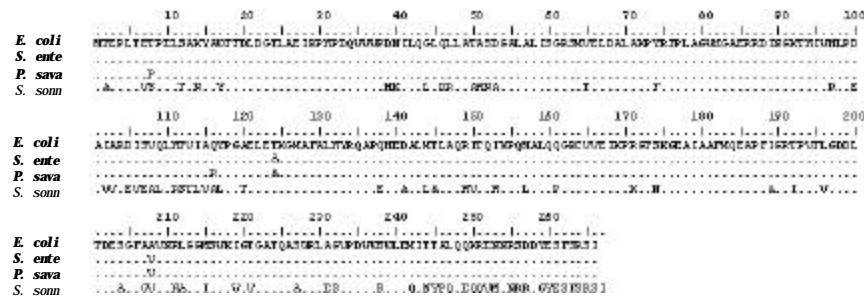


Fig. 3: Alignment of the amino acid sequence of TPP gene from *E. coli* TCJAR023 with sequences from *Salmonella enterica*, *Pseudomonas savastanoi*, *Shigella flexneri* and *Shigella sonnei*. Dots indicate amino acid identity and letters represents amino acid substitutions

many potential applications in biotechnology including increased stress tolerance of important crops, stability of recombinant proteins, etc., as well as in the food industry (Schiraldi *et al.*, 2002). The biosynthetic pathway for trehalose comprises two steps, the first being the conversion of glucose from UDP-glucose to glucose-6-phosphate to form trehalose-6-phosphate and UDP. This is followed by de-phosphorylation of trehalose-6-phosphate to trehalose and orthophosphate. The trehalose biosynthesis involved two genes TPS and TPP. The presence of these two genes was confirmed by PCR amplification of genomic DNA from *E. coli* TCJAR023. The fragment sizes of 1,424 and 801 bp were obtained in amplification. Restriction analysis also showed the similar pattern. Finally, the sequences of the genes were confirmed by sequencing. The translated protein of TPS and TPP genes has 474 and 266 amino acids, with the isoelectric points of 6.37 and 5.38, respectively. The predicted molecular weights were 53 and 29 kDa, respectively. The amino acids analysis of TPS gene reveals that the encoded protein belongs to the trehalose-6-phosphate synthase family. It had minimum conserved regions with phosphate synthase from other eubacteria. The amino acid analysis also showed that the amino acid sequence of *S. sonnei* and *P. savastanoi* has the maximum identity of 100 and 99%, respectively with that of *E. coli* TCJAR023. The sequence analysis of TPS revealed that amino acid residues of *E. coli* TCJAR023 were well conserved with *S. sonnei* amino acids. These results revealed that, the catalytic activity of trehalose-6-phosphate synthase was similar in both organisms. The amino acid analysis of TPP gene suggests that the encoded protein belongs to the trehalose-6-phosphatase family. It had highly conserved regions with phosphate phosphatase from other bacteria. The amino acid analysis suggests that *S. sonnei* has the maximum identity of 99% with the amino acid sequence of *E. coli* TCJAR023. The other organism's also accounted considerable similarity; *P. savastanoi* with 98% and *S. enterica* with 73% identity. The sequence analysis revealed that, the amino acid residues of TPP in *E. coli* TCJAR023 were partially conserved with *S. sonnei*, *S. enterica* and *P. savastanoi* sequences.

Based on phylogenetic analysis, it was found that the *E. coli* TCJAR023 and *S. sonnei* were clustered together whereas, *P. savastanoi* and *S. enterica* form separate clusters for both in nucleotide and amino acid sequences of TPS gene. The phylogenetic tree analysis of amino acid sequences from TPP gene showed that *S. sonnei* and *P. savastanoi* were clustered together while, *E. coli* TCJAR023 and *S. enterica* form separate clusters in nucleotide and amino acid level. From the phylogenetic grouping of nucleotide and amino acid sequence between the different organisms, it was inferred that *E. coli* TCJAR023 had

comparatively higher conserved sequences both at nucleotide and amino acid level for the genes involved in trehalose biosynthesis.

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