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The Cloning and Sequencing Analasis of Peptidylprolyl*cis trans*isomerases C (*ppiC)* gene of *Salmonella* Typhimurium

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***Salmonella enterica* subspecies *enterica* serovar Enteritidis (SE) and *Salmonella enterica subspecies enterica serovar* Typhimurium (ST) are the zoonotic *Salmonella* that colonizes in the intestine of poultry asymptomatically and are transmitted to human by consumption of contaminated foods like meat, eggs and egg products. The PPIases are enzymes that accelerate folding of proline-containing proteins by catalyzing the *cis-trans* isomerisation, which plays important roles in a variety of stress responses in bacterium. In this study, the Peptidyl prolyl *cis–trans* isomerases *C (ppiC)* gene from ST was cloned, sequenced, analyzed and submitted to gene bank (accession number KR054755). The retrieved sequence data analysis, suggested that gene belongs to the parvulin family of peptidyl-prolyl *cis*/*trans* isomerases (PPIases, EC 5.2.1.8) of ST. Entertainingly, the *ppiC* sequence shows about 100% -99% with other *Salmonella* serovars. The conclusion of this study effectively indicate that *ppiC* gene of *Salmonella* is highly conserved in most of the pathogenic *Salmonella spp.* as analogous to patterns of other serovars.**

Keywords: *Salmonella*, parvulin, PPIases C and *ppiC.*

Salmonella are well known for their impact on human and animal health as they are associated with a wide range of clinical problems, including typhoidal and non-typhoidal infections. Non-typhoidal *Salmonella* infections, mainly resulting from *S*. Enteritidis and *S*. Typhimurium, are commonly the result of the consumption of poultry derived food, mainly meat, eggs and egg products (Schroeder *et al.,* 2005, Zaidi *et al.,* 2006, Stephens *et al*., 2008). *Salmonella* species are responsible for a variety of acute and chronic diseases in poultry. Avian salmonellosis can develop as a result of infection with poultry-specific serovars, *S*. Gallinarum and *S*. Pullorum, causing systemic illness in birds as well as other *Salmonella* serotypes, including *S*. Typhimurium and *S*. Enteritidis and many others, which contribute to paratyphoid infections (Gast, 2007). The global burden of non-typhoidal salmonellosis was estimated to be about 93.8 million cases per year and of this 80.3 million are food borne (Majowicz *et al.,* 2010) which means more than 80 % of cases of *Salmonella* infection are foodborne. Various serotypes of *Salmonella* are implicating the food-borne infections, but the most prevalent serotypes isolated from humans are *S*. Typhimurium and S. Enteritidis (Graham*et al.,* 2000). In India some reports showed the predominance of *S*. Enteritidis during 1994-1996 (Verma *et al*., 2001) but later *S*.

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Typhimurium was found to be the predominant species (Singh, 2002).

Being intracellular the bacterium undergoes various stress conditions generated by the host. Various genes come in to play during stress conditions in the *Salmonella*. The Peptidylprolyl*cis–trans* isomerasesare the chaperonsencoded by various genes found to be involved in the mitigation of stress response. The molecular chaperone are the important proteins, facilitates correct folding and maturation of proteins in Gram-negative bacteria. Peptidyl-prolyl*cis/trans*isomerases (PPIases) catalyze the isomerization of prolyl peptide bonds. The four families of this class of enzymes are cyclophilins (Cyp), FK506-binding proteins (FKBPs), and pavilions which involved in protein folding (Lu et al., 2007). The Peptidylprolyl*cis–trans* isomerases are ubiquitous proteins and are found in both prokaryotic and eukaryotic cells (chou*et al.,* 1997). The first member of the parvulin subfamily was identified in *E coli* in 1994 and consists of only 92 amino acids, thus named parvulin (Rahfeld et al., 1994), whose gene was later designated *ppiC*. The PPIases are encoded by many different genes *fkpA, fklB, slyD, cypD, slpA, ppiA, ppiB, ppiC* and *surA*(Ramseier*et al.,* 2013).Parvulins constitute an important PPIase subfamily.SurA was the only known member of the parvulin class of PPIases in the periplasm of *E. coli*, however in ST *ppiC* and *ppiD also* member of parvulin class. Dartigalongue andraina (1998) reported that in *E. coli ppiD* is that its gene is regulated by both the Cpxtwo-component system and the σ^{32} heat shock factor,known to regulate the expression of cytoplasmic chaperones.

Parvulins proteins have also been reported earlier from Arabidopsis thaliana (Landrieu *et al.,* 2000), Digitalis lanata (Metzner*et al.,* 2001) and Malus domestica (yao *etal.,* 2001) and the activity is modulated by stress conditions, thus suggesting that it may also be involved in stress adaptation.*surA*gene mutant ST was protected against virulent challenge with the parental strain and to deliver a heterologous antigen to the murine immune system (Sydenham *et al.,* 2000). Humphreys *et al.* (2003) showed that *SurA* and to a lesser extent *fkpA*, are important for the survival of *S.* Typhimurium in the mouse.However, the studies on the *ppiC* gene of ST hardly noticed. The current study was conducted to clone the *ppiC* gene of *Salmonella* Typhimurium in pET28C vector followed by sequencing and analyzing the gene with different virulent serovars of *Salmonella*.

MATERIALS AND METHODS

Bacterial strain

Poultry isolate *Salmonella* Typhimurium (ST) strain 3232 was procured from the Microbial Type Culture Collection and Gene Bank (MTCC) Chandigarh, India was used in the study. The culture was revived by growing in LB broth at 37 °C overnight and the isolated colonies were obtained by streaking on Hektoen Enteric Agar (HEA) plates. The isolated colonies were confirmed through purity, morphology and biochemical characteristics as per standard protocol. In addition, ST is also further performed by serotyping at National *Salmonella* Centre, Indian Veterinary Research Institute, Izatnagar, India.

Genomic DNA isolation

The single isolate colony of *Salmonella* Typhimurium inoculate in LB broth and grown at 37 °C, 180 rpm overnight. The overnight grown culture was pelletted (3 ml) for genomic DNA isolation. The DNA was isolated bypurelinkgenomic DNA isolation kitfollowing the manufacturer's protocol (Invitrogen, USA). The concentration and quality of the genomic DNA were assessed by UV absorbance and electrophoresis on 1 % agarose gel.

PCR amplification of *ppiC*gene form *Salmonella* Typhimurium

The primers were designed by using Oligo Analyzer software targeting the PPIasesC (*ppiC*) (STM3910) DNA sequence (NCBI-Gene ID: 1255436 Region: 4118944 - 4119225).PCR reaction was performed in a total volume of 100 µl containing 50 ng of DNA, 10 pmol of each primer (Table 1), 200 µM dNTP, 10 µl of 10X PCR buffer, 1.5 mM $MgCl_2$ and2.5 Uof *Taq* DNApolymerase. The final volume was made with nuclease-free water. The PCR cycling conditions consists of initial denaturation at 95°C for 5 min, followed by 30 cycles each of denaturation (95°C for 30 s), annealing (52 °C for 30 s) and extension (72°C for 30 s) and it was ended with a final extension at (72ºC for 10 min.). Finally, the amplified DNA of *ppiC* gene was visualized by

electrophoresis on 1% agarose gel and *ppiC* a gene fragments were purified from gel purified using QIA quick gel extraction kit (Qiagen, Germany) using manufacturers protocol.

The purified PCR product was digested with restriction endonuclease enzyme *NheI* for 5 h at 37°Cfollowed *BamHI* for 1 h at 37°C. Finally, the enzymes were inactivated by incubating at 80°C for 10 min. The RE digested *ppiC* PCR product was again gel purified. In the similar manner the pET28c vector was also digested with *NheI* and *BamHI* restriction enzymes to generate complementary overhangs regions. The RE digested vector was then dephosphorylated at 37 °C for 30 min using shrimp alkaline phosphatase enzyme followed by heat inactivation at 80 °C for 10 min. The digested and dephosphorylated pET28c was gel purified.

Cloning of *ppiC* gene

The ligation of the gel purified *ppiC* gene in pET28c expression vector achieved by mixing them in 3:1 ratio followed by incubation at room temperature for 1 h and then at 4°C for 16 h. The ligation reaction was transformed to *E.coli* DH5á strain competent cells and dispensed on agar plate containing 30 µg/ mL of kanamycin. The plates were incubated at 37 °C for overnight. A number of recombinant colonies were assayed by colony PCR. After selecting the recombinant clones, the recombinant pET28c_ppiC plasmid was extracted from overnight grown culture. The recombinant plasmids were digested by *NheI* and *BamHI*RE enzymes to confirm insert release. The PPIases*C* gene cloned in pET28c plasmid was confirmed by sequencing (GCC biotech).

The sequencing results were assembled and analyzed. Further, the nucleotide sequence was subjected to nucleotide BLAST to compare for sequence similarities with other sequences available in the NCBI database. The final sequence was aligned with the available PPIasesC (*ppiC*) gene of *S.enterica* serovar Typhimurium str. LT2. The sequence was submitted to NCBI Genbank (http://www.ncbi.nlm.nih.gov/nuccore/KR054755).

RESULTS AND DISCUSSION

Poultry isolate *Salmonella* Typhimurium strain (3232) was used in present study showed characteristic biochemical, morphological and serological characteristics. On HEA plate, the isolate produced smooth, black centered colonies with greenish periphery (Fig. 1).The ST colonies were confirmed through biochemical and serotyping (Phagoo*et al.*, 2015).

The genomic DNA of *S.enterica* serovar Typhimurium extracted using a commercial kit showed a concentration of 40 ng/ µL and good quality on agarose gel (data are not shown). The full gene-coding region of the PPIases*C* gene of *S.* Typhimurium was amplified using designed specific primers (Table -1). The size of PCR amplified product on agarose gel electrophoresis for PPIases*C* (*ppiC*) gene was 282 bp (Fig. 2). The PCR products were cloned into a pET28c expression vector and this resulted in a recombinant pET28c_ppiC plasmid that was transformed successfully into *E. coli* bacteria. The recombinant plasmids from the colonies were PCR positive against the *ppiC* gene.

The recombinant pET28c_ppiC plasmids were confirmed by double digestion with *NheI* and *BamHI* restriction enzymes. Release of the 282 bp amplified product from recombinant vectors by RE digestion further confirmed the gene cloning (Fig. 3). The sequence thus obtained was submitted to GenBank, National Centre for Biotechnology information under the accession no. KR054755. Nucleotide sequence analysis showed that the amplified genes possessed the correct reading frame with compared to other *Salmonella* serovar.

The obtained sequence was subjected to nucleotide BLAST search revealed a high degree of similarity with other *Salmonella* serovar (Table2).

Table 1. Primers used in present study

S. No	Primer name	Primer Sequences (5' to 3')	Purpose
1.	PPIase C_For	TATAT**GCTAGC**ATGGCAAAAATGGCAGCAG(31)	Amplification of PPIases C(*ppiC*)gene from ST
2.	PPIase C_Rev	TATAT**GCTAGC**TTATTTACGATACAATACCTTA(34)	

Maximum identity was obtained with *Salmonella* Typhimurium. The percent of identity with *Salmonella* Typhimurium LT2 *ppiC* gene was 100% (Fig. 4). The obtained sequence showed high similarity with different serovar of *Salmonella* strains like 33676, YU39, CDC2011K-0870, ATCC 13311, CFSAN002064, CFSAN001921, STMD1, CFSAN001588,CT18, and ATCC 9120. The *ppiC* gene is highly conserved in most of the pathogenic serovar of *Salmonella* as evidenced from the sequence analysis (Table 2).

Table 2. Results of nucleotide blast showing comparison of PPIase Cgene of *S.enterica* serovar Typhimurium strain 3232 with other *Salmonella* serovar

No.	Description	Max score	Total score	Query cover	Ident	Accession
1.	Salmonella *enterica* subsp. enteric serovar Typhimurium strain 33676, complete genome	521	521	100 %	100 %	CP012681.1
2.	Salmonella *enterica* subsp. *enterica* strain YU39, complete genome	521	521	100 %	100 %	CP011428.1
3.	Salmonella *enterica* subsp. *enterica* serovar Typhimurium str. CDC 2011K-0870, complete genome	521	521	100 %	100 %	CP007523.1
4.	Salmonella *enterica* subsp. *enterica* serovar Typhimurium strain ATCC 13311, complete genome	521	521	100 %	100 %	CP009102.1
5.	Salmonella *enterica* subsp. *enterica* serovar Heidelberg str. CFSAN002064, complete genome	521	521	100 %	100 %	CP005995.1
6.	Salmonella *enterica* subsp. *enterica* serovar Typhimurium str. DT2, complete genome	521	521	100 %	100 %	HG326213.1
7.	Salmonella *enterica* subsp. *enterica* serovar Typhimurium var. 5- str. CFSAN001921, complete genome	521	521	100 %	100 %	CP006048.1
8.	Salmonella typhimurium fragment STMD1	521	521	100 %	100 %	AF233324.1
9.	Salmonella *enterica* subsp. *enterica* serovar Cerro str. CFSAN001588, complete genome	516	516	100 %	99 %	AF233324.1
10.	Salmonella *enterica* subsp. *enterica* serovar Paratyphi A strain CMCC50093 genome	516	516	100 %	99 %	CP011967.1
11.	Salmonella *enterica* subsp. *enterica* serovar Typhi str. CT18, complete chromosome	516	516	100 %	99 %	AL513382.1
12.	Salmonella *enterica* subsp. *enterica* serovar Typhi Ty2, complete genome	516	516	100 %	99 %	AE014613.1
13.	Salmonella *enterica* subsp. *enterica* serovar Sloterdijk str. ATCC 15791,complete genome	516	516	100 %	99 %	CP012349.1
14.	Salmonella *enterica* subsp. *enterica* serovar Enteritidis strain OLF-SE9-10012, complete genome	516	516	100 %	99 %	CP009091.1
15.	Salmonella *entericaa* subsp. *enterica* serovar Pullorum str. ATCC 9120, complete genome	505	505	100%	99 %	CP012347.1

Fig. 1. *S.typhimurium* (3232) growth on HE agar and LB agar plates. *S*. Typhimurium strain 3232 (ST) was sub-cultured and grown in LB-broth media. After that broth culture was streaked on HE agar and LB agar plates. The isolated colonies (indicated by arrow) showed typical morphology of *salmonella*.

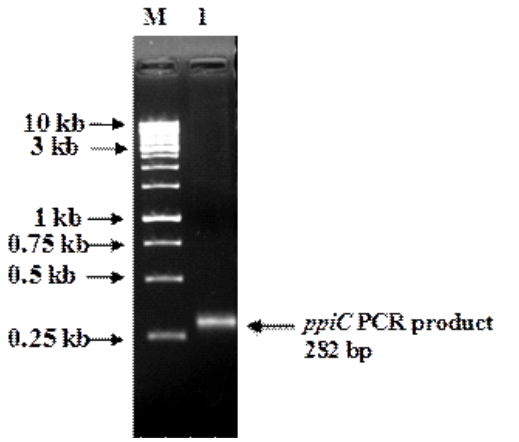


Fig. 2. PCR amplification of *ppiC* gene. Electrophoresis *ppiC* gene PCR product was performed on 1 % agarose gel. Lane M is 1 kb DNA ladder. Lane 1 is expected *ppiC* PCR product 282 bp (marked by arrow)

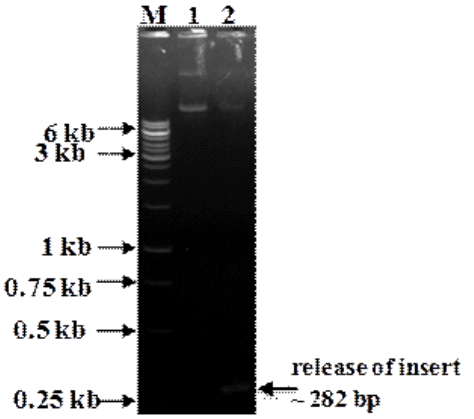
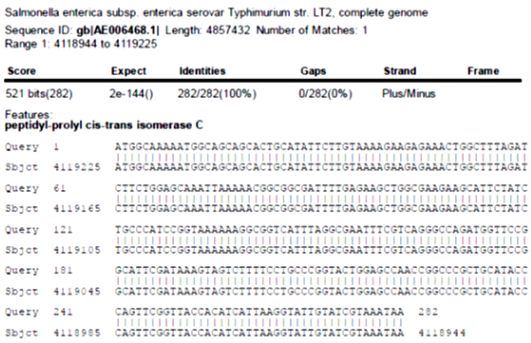


Fig 3: NheI and BamHI digestion of ppiA-pET28c clones. 1 % Agarose gel electrophoresis of RE digested clones. Lane M is 1 kb DNA ladder. Lane 1 is uncut and Lane 2 is cut recombinant plasmids. Released *ppiC* band is shown by arrow.


Salmonella enterica subsp. enterica serovar Typhimurium str. LT2, complete genome
Sequence ID: **gb|AE006468.1|** Length: 4857432 Number of Matches: 1
Range 1: 4118944 to 4119225

Score	Expect	Identities	Gaps	Strand	Frame
521 bits(282)	2e-144()	282/282(100%)	0/282(0%)	Plus/Minus	

Features:
peptidyl-prolyl cis-trans isomerase C

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Query  1        ATGGCAAAAATGGCAGCAGCACTGCATATTCTTGTAAAAGAAGAGAAACTGGCTTTAGAT
Sbjct  4119225  ATGGCAAAAATGGCAGCAGCACTGCATATTCTTGTAAAAGAAGAGAAACTGGCTTTAGAT
Query  61       CTTCTGGAGCAAATTAAAAACGGCGGCGATTTTGAGAAGCTGGCGAAGAAGCATTCTATC
Sbjct  4119165  CTTCTGGAGCAAATTAAAAACGGCGGCGATTTTGAGAAGCTGGCGAAGAAGCATTCTATC
Query  121      TGCCCATCCGGTAAAAAAGGCGGTCATTTAGGCGAATTTCGTCAGGGCCAGATGGTTCCG
Sbjct  4119105  TGCCCATCCGGTAAAAAAGGCGGTCATTTAGGCGAATTTCGTCAGGGCCAGATGGTTCCG
Query  181      GCATTCGATAAAGTAGTCTTTTCCTGCCCGGTACTGGAGCCAACCGGCCCGCTGCATACC
Sbjct  4119045  GCATTCGATAAAGTAGTCTTTTCCTGCCCGGTACTGGAGCCAACCGGCCCGCTGCATACC
Query  241      CAGTTCGGTTACCACATCATTAAGGTATTGTATCGTAAATAA  282
Sbjct  4118985  CAGTTCGGTTACCACATCATTAAGGTATTGTATCGTAAATAA  4118944
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Fig. 4. Sequence analysis of peptidyl-prolyl *cis-trans* isomerase C (*ppi*C) from positive clones similar to 100% homology: Query- PPIase *C* sequence obtained by sequencing, subject-retrieved PPIase *C* sequence of *S.* Typhimurium LT2 strain from NCBI

Salmonella replicates inside the hostile niche of macrophages, and surviving the host depends upon its ability to defend / neutralize phagocyte generated oxidants. Under stress the reactivation of damaged proteins would be energetically favorable than new synthesis. Therefore, bacteria would go for repair the damaged proteins rather than their *de novo* synthesis (Visick and Clarke 1995).PPIases play a fundamental role in catalyzing the correct folding of many proteins that are non functional in a variety of biological functions and stress (Gething and Sambrook, 1992).

CONCLUSION

It may be concluded that ppiases catalyze the peptidyl-prolyl *cis-trans* isomerization, the rate-limiting step of protein folding, which is essential for newly synthesized protein to get its correct

functional form. They can also catalyze the isomerization of that protein which defeat during the oxidative stress and physiological operation (Fischer and Aumuller, 2004). The PpiC enzyme is responsible for the refold of the proteins to save its functions by repairing and also highly conserved in nucleotides sequences among the pathogenic *Salmonella* serovar. The similarity in the sequences of the cloned product with other *Salmonella* serovar suggests its importance in the utility of *ppiC*.

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