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Contribution of protein isoaspartate methyl transferase (PIMT) in the survival of *Salmonella* Typhimurium under oxidative stress and virulence

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ABSTRACT

The enteric pathogen *Salmonella* Typhimurium (ST) survives inside the oxidative environment of phagocytic cells. Phagocyte generated oxidants primarily target proteins and modify amino acids in them. These modifications render the targeted proteins functionally inactive. Conversion of Asp to *iso*-Asp is one of the several known oxidant mediated amino acids modifications. By repairing *iso*-Asp to Asp, protein-isoaspartyl methyltransferase (PIMT) maintains the activities of proteins and thus helps in cellular survival under oxidative stress. To elucidate the role of PIMT in ST survival under oxidative stress, we have constructed a *pimt* gene deletion strain (Δ*pimt* strain) of ST. The Δ*pimt* strain grows normally in various culture media *in vitro*. However, in comparison to wild type ST, the Δ*pimt* strain is found significantly ($p < 0.001$) more susceptible to H_2O_2 and hypochlorite (HOCl). Further, the Δ*pimt* mutant strain shows hypersusceptibility ($p < 0.001$) to INF-γ stimulated macrophages. This susceptibility is reversed by pharmacological inhibition of reactive oxygen species (ROS) but not reactive nitrogen species (RNS) production. Further, plasmid based complementation enhances the survival of Δ*pimt* mutant strain against oxidants *in vitro* and also inside the macrophages. In mice model, the LD_{50} for wild type ST and mutant Δ*pimt* has been 1.73×10^4 and 1.38×10^5, respectively. Further, the mutant strain shows reduced dissemination to spleen and liver in mice. Following infection with a mixture of wild type ST and the Δ*pimt* mutant (co-infection experiment), we recover significantly ($p < 0.001$) less numbers of mutant bacteria from the spleen and liver of mice.

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1. Introduction

Non-typhoidal *Salmonella* (NTS) infections are highly prevalent throughout the world. It is estimated that NTS infects about 93.8 million of cases every year, causing 155,000 deaths (Majowicz et al., 2010). *Salmonella enterica* subspecies *enterica* serovar Typhimurium (ST) and *Salmonella enterica* subspecies *enterica* serovar Enteritidis (SE) are two most important *Salmonella* serovars associated with food born infections in human being (Khoo et al., 2015; Yeung et al., 2014). Out of these two serovars, the ST is more prevalent throughout the world including in Indian sub-continent (Greig and Ravel, 2009). In human, ST causes gastroenteritis in mild infection and septicaemia to death in severe cases (McClelland et al., 2001).

ST resides and survives inside the macrophages and other phagocytic cells. Phagocyte generated oxidants constitute one of the most important stresses encountered by the bacteria inside the host. Phagocytes generate several reactive oxygen species (ROS) such as, superoxides (O^{2-}), hydroxyl ($^{\bullet}OH$), hydroperoxyl ($HOO^{\bullet}$) radicals, H_2O_2 and HOCl (Fang, 2011). Having membrane permeable abilities, the H_2O_2 and HOCl are very effective ROS in terms of oxidizing cellular macromolecules (Morales et al., 2012). ROS can oxidize all bio-molecules such as DNA, RNA, proteins and lipids of the bacteria (Cabiscol et al., 2010). ST overcomes the detrimental effects of the oxidants by three ways. First, by injecting type III effectors, ST inhibits NADPH assembly on the phagosomal membrane and interferes with the ROS production in host cells (Gallois et al., 2001). Second, the primary antioxidant enzymes of

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Table 1
Details of primers used in this study.

Name of primer	Primer sequence (5′-3′)
A	GATGTGGTTTCAGACTGGTTAGACAGCGTGGGAGTTGGCACGCAGTAGGCTGGAGCTGCTTC
B	CGTTTCGGCTTCATCAGGCGTAAGAGTGGGTGTTTGCAGGGCAAACATATGAATATCCTCCTTA
C	ATGAAGGCTACGTCTCCGTC
D	GTTGCACGGTTCTTACGTCAC
pimt complement For	TAG GAT CCATGGTAAGTGGACGTGTACAG
pimt complement Rev	CGCAAGCTTCTAGGCCAGCTCTCCCTTGAC

ST (such as, superoxide dismutases (SODs), catalases and peroxiredoxins) catalytically degrade ROS (Aussel et al., 2011). Third, the DNA and protein repair enzymes of ST help in the repair of damaged molecules (Buchmeier et al., 1995; Mahawar et al., 2011). The repairs of macromolecules become significantly important during respiratory burst *i.e.,* when bacteria enter in to the phagocytes and encounter large amounts of ROS.

Proteins are the key targets of oxidant mediated inactivation (Hawkins et al., 2003). Oxidation of the proteins leads to structural perturbation and consequently their functional inactivation. Proteases mediated degradation and removal of the damaged proteins, followed by the replacement via translational synthesis is the straight forward way to restore the protein functions (Truscott et al., 2011). Protein repair enzymes can reactivate damaged proteins without *de novo* synthesis (Mahawar et al., 2011). Many types of covalent modifications in amino acids have been reported. The example includes methionine sulfoxide, cysteic acid, *iso*-aspartate, chlorotyrosine and nitro-tyrosine formation (Cabiscol et al., 2010; Hawkins et al., 2003; Kern et al., 2005; Chondrogianni et al., 2014). But only a couple of repair enzymes have been so far reported. While methionine sulfoxide reductase (MSR) repairs oxidized methionine (methionine sulfoxide, Met-SO) to Met (Mahawar et al., 2011); the repair of altered asparatyl residues (*iso*-Asp) to Asp is catalyzed by protein isoaspartate methyl transferase (PIMT) (Vigneswara et al., 2006; Cimmino et al., 2008).

Exposure of the cells to various stresses including oxidative stress increases formation of *iso*-Asp (Shimizu et al., 2005). During stress Asp residues in the proteins convert into L-succinimide. The hydrolysis of L-succinimide results in to abnormal *iso*-Asp and normal Asp in a ratio of ~3:1 (Vigneswara et al., 2006). The PIMT converts *iso*-Asp to *iso*-aspartyl methyl ester by transferring the methyl group from *S*-adenosyl methionine (SAM). These unstable methyl esters rapidly hydrolyze and again form succinimides. This cycle repeats several times and majority of *iso*-Asp get converted into Asp (DeVry and Clarke, 1999). The contribution of *pimt* in the survival of various organisms (such as, *E. coli*, *C. elegans*) under stress has been studied by mutation analysis (Visick et al., 1998; Yamamoto et al., 1998; O'Connor, 2006; Khare et al., 2009).

ST can survive in the oxidative environment of phagocytes (Leung and Finlay, 1991; Salcedo et al., 2001) and the active PIMT is present in this bacterium (Li and Clarke, 1992a). However, the role of *pimt* in the stress survival and virulence of ST is not known. In the present study, we have evaluated the contribution of *pimt* in the ST survival under oxidative stress. Further we have also assessed the role of *pimt* in the virulence of this bacterium. We have adopted a genetic approach and generated a *pimt* gene deletion strain of ST (Δ*pimt*). Subsequently, we have analyzed the susceptibility of Δ*pimt* strain to H_2O_2, HOCl and macrophages. Finally, we have evaluated the contribution of *pimt* in ST virulence in mouse model.

2. Material and methods

2.1. *Salmonella* Typhimurium and growth conditions

Salmonella Typhimurium strain E-2375 was procured from repository of National *Salmonella* Centre (Veterinary), IVRI, Izatnagar, India. The ST was cultured in LB broth or in Hektoen enteric agar (HEA). The antibiotic kanamycin (50 μg ml^{-1}) and ampicillin (100 μg ml^{-1}) were supplemented to the media for selection purposes, whenever required.

2.2. *Construction and confirmation of* pimt *gene deletion mutant of ST (*Δpimt*) and its complementation*

The *pimt* gene was deleted by one step gene inactivation method using lambda red recombination system (Datsenko and Wanner, 2000). In brief, the kanamycin cassette was amplified from plasmid pKD4 (using primer A and B, complete primer list provided in Table 1) and fused to the flanking regions of the *pimt* gene. The PCR product was digested with DpnI and transformed to lambda red recombinase expressing ST. Following confirmation of the deletion the kanamycin cassette was removed by pCP20 encoded FLP recombinase. The mutant (Δ*pimt*) colonies were confirmed by PCR using *pimt* test primers C and D (Table 1) and also by Western blot analysis described below. Plasmids pKD4 and pCP20 were a kind gift from Dr. Robert Maier, Dept. of Microbiology, University of Georgia, Athens, GA, USA.

The recombinant PIMT was produced in *E. coli* (Dixit et al., 2014) and isolated using Ni-NTA chromatography. The antibodies against purified PIMT were raised in rabbits commercially (Merck Life Sciences, India). The lysates of ST and Δ*pimt* mutant were resolved on SDS-gels and transferred to PVDF membrane. Free sites on the membrane were blocked by incubation of the membrane in skimmed milk solution. The membrane was then incubated in anti-PIMT antibodies (in PBS-T) for overnight. After several washes with PBS-T the blot was incubated in anti- rabbit IgG conjugated with alkaline phosphatase (Sigma) and developed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Harlow and Lane, 1988).

The complementation of the Δ*pimt* mutant was done in trans (transcomplementation) as described earlier (Allam et al., 2011; Trivedi et al., 2015). In brief, the *pimt* gene was cloned in pQE60 plasmid (Qiagen) at BamHI and HindIII sites. pQE 60 vector has T5 promoter and synthetic ribosomal binding site. This vector allows the translation of coding fragments to initiate at the original start codon. After confirmation the recombinant plasmid was transformed into Δ*pimt* strain (Trivedi et al., 2015). The complementation was confirmed by Western blot analysis using anti-PIMT antibodies.

2.3. In vitro *growth analysis of* Δpimt *mutant*

The growth of wild type ST and Δ*pimt* mutant were compared in LB broth or M9 or RPMI-1640 (supplemented with 10% FBS, 1x glutamax and 1 mM sodium pyruvate) media. In brief, the isolated colonies of ST and Δ*pimt* strains were grown overnight and diluted (@ 1: 100) in fresh LB broth or M9 or RPMI-1640 media and incubated on a shaker incubator (180 rpm) at 37 °C. The aliquots were withdrawn at various times post inoculation and the absorbances were recorded at 600 nm.

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