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Methionine sulfoxide reductase A (MsrA) contributes to *Salmonella* Typhimurium survival against oxidative attack of neutrophils

Raj Narayan Trivedi[a,1], Pranjali Agarwal[b,1], Manoj Kumawat[b], Pavan Kumar Pesingi[c], Vivek Kumar Gupta[d,2], Tapas Kumar Goswami[a], Manish Mahawar[b,*]

[a] *The Immunology Section, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122, India*
[b] *Division of Biochemistry, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122, India*
[c] *Division of Veterinary Public Health, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122, India*
[d] *Animal Health Division, CIRG, Makhdoom, Mathura, India*

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ABSTRACT

Salmonella Typhimurium (ST) must evade neutrophil assault for infection establishment in the host. Myeloperoxidase generated HOCl is the key antimicrobial agent produced by the neutrophils; and methionine (Met) residues are the primary targets of this oxidant. Oxidation of Mets leads to methionine sulfoxide (Met-SO) formation and consequently compromises the protein function(s). Methionine sulfoxide reductase A (MsrA) reductively repairs Met-SO to Mets. In this manner, MsrA maintains the function(s) of key proteins which are important for virulence of ST and enhance the survival of this bacterium under oxidative stress. We constructed *msrA* gene deletion strain (Δ*msrA*). The primers located in the flanking regions to Δ*msrA* gene amplified 850 and 300 bp amplicons in ST and Δ*msrA* strains, respectively. The Δ*msrA* strain grew normally in *in vitro* broth culture. However, Δ*msrA* strain showed high susceptibility ($p < 0.001$) to very low concentrations of HOCl which was restored (at least in part) by plasmid based complementation. Δ*msrA* strain was hypersensitive (than ST) to the granules isolated from neutrophils. Further, the Δ*msrA* strain was significantly ($p < 0.05$) more susceptible to neutrophil mediated killing.

1. Introduction

Salmonella Typhimurium causes self limiting to severe gastroenteritis in normal individuals and life threatening infections in old aged and immunocompromised persons. ST not only survives but replicates inside the phagocytes. The phagocyte respiratory burst generates copious amount of oxidants. These oxidants are critical components of host innate immune responses. To survive and establish infection in the host, the pathogens must have evolved strategies to overcome these oxidants. The situation is even worse for intracellular pathogens like *Salmonella* as they are first taken up in the confined environment of phagosome and then subjected to oxidants bombardment. Superoxides (O_2^-), H_2O_2, hydroxyl radicals (•OH) and HOCl are some important reactive oxygen species (ROS) generated by the phagocytes (Fang, 2011; Slauch, 2011). *Salmonella* tackle the situation in three ways. First, by injecting type III effector proteins, it interferes with the NADPH oxidase assembly, and thus impede with the oxidant production. Second, primary antioxidants (like catalases, superoxide dismutases and peroxyredoxins) of *Salmonella* directly detoxify the oxidants (Aussel et al., 2011). Third, the protein and DNA repair enzymes repair damaged molecules (Denkel et al., 2011; Buchmeier et al., 1995). •OH and HOCl are considered as most reactive ROS. These oxidants have abilities to oxidize lipids, DNA, RNA, proteins, *etc.*, and thus, exert bactericidal effect. Amino acids (free or protein bound) are the prime targets of oxidants. Strong oxidants like HOCl can damage variety of amino acid residues. However the sulphur containing amino acids (Mets and Cys) are the principal targets of HOCl (Hawkins et al., 2003).

Oxidation of Met residue converts it into Met-SO, compromising the protein function(s). Msr reductively repairs Met-SO to Met by thioredoxin–thioredoxin reductase system (Boschi-Muller et al., 2008). Msr mediated repair enhances the cell survival under oxidative stress in two ways. First, it restores the function of Met-SO containing proteins. Second, the Met residues (either free or in protein bound form) serve as sink for oxidants; initially they get oxidized when cell get exposed to large quantities of oxidants *i.e.*

* Corresponding author.
E-mail address: manishbiochemistry@gmail.com (M. Mahawar).
[1] These authors contributed equally to this work.
[2] Current address: Joint Director (CADRAD), IVRI, Izatnagar, Uttar Pradesh 243 122, India.

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Table 1
The primer sets used in study.

S N	Primer sequences	Primer name	Purpose
a.	5′CGTTACAACCTTAACGCCAATTAAC GCTTTCGGGAGAATAGCTCGTAGGCT GGAGCTGCTTC 3′	MsrA deletion Forward	To delete *msrA* gene from *S.* Typhimurium
b.	5′AGGGTTCATTTTCAACTCCGACAAGTT CCCCCTACGCCAGCGTCGCATATGAATA TCCTCCTTA 3′	MsrA deletion Reverse	To delete *msrA* gene from *S.* Typhimurium
c.	5′ AGATACATTAAT-GTTGTTATT	MsrA deletion test Forward	To confirm *msrA* gene deletion from *S.* Typhimurium
d.	5′ GTAACGTTTAAT-GAAAACCG	MsrA deletion test Reverse	To confirm *msrA* gene deletion from *S.* Typhimurium
e.	5′ TAGGATCCATGAGTTTATTTGA TAAAAAACATCTG 3′	MsrA comp BamHI Forward	To complement Δ*msrA*
f.	5′ CGCAAGCTTTCACGCGTCAGGC GGCAGG3′	MsrA comp HindIII Reverse	To complement Δ*msrA*

during respiratory burst and subsequently get repaired by Msr (Luo and Levine, 2009; Abulimiti et al., 2003; Le et al., 2009).

Along with other cells, macrophages and neutrophils are two critical cell types which recruit in response of *Salmonella* infection (Yang et al., 2002; Coates and McColl, 2001; Fierer et al., 2002). Neutrophils in particular come early at the infection site (Kirby et al., 2002). The antibacterial repertoire of neutrophils consists of reactive oxygen species (ROS), antibacterial peptides (lysozymes, calprotectin, *etc.*) and neutrophil extracellular traps (NETs) (Brinkmann et al., 2004; Segal, 2005; Nauseef and Borregaard, 2014). The ROS (HOCl in particular) is the main antibacterial arsenal of neutrophils. Several reports suggest that ST can sustain neutrophil mediated killing (Geddes et al., 2007; Loetscher et al., 2012). By repairing both protein bound and free Met-SO in the *Salmonella* MsrA contributes to intra macrophage survival and virulence in mouse model (Denkel et al., 2011). However, its role in the resistance of *Salmonella* against neutrophil is not known.

In current study, by employing genetic approach we generated an *msrA* gene deletion strain of ST (Δ*msrA*). We assessed the contribution of ST MsrA in combating reagent HOCl stress. We further investigated the role of MsrA in ST resistance against granules extracted from neutrophils and neutrophil culture.

2. Materials and methods

2.1. Bacterial strains and culture

Salmonella enterica subspecies *enterica* serovar Typhimurium (ST) strain E 2375 (a field poultry isolate) was procured from the National *Salmonella* Center (Veterinary type) repository, Division of Bacteriology and Mycology, Indian Veterinary Research Institute, Izatnagar, India. The cultures were streaked on Hektoen Enteric (HE) agar. Isolated colonies were inoculated in 10 ml LB broth and incubated overnight at 37 °C/180 rpm. The cultures were diluted in fresh media and grown up to an OD_{600} of 0.6–0.8 (mid log phase). For all assays ST was cultured in this manner (if changes made in specific experiments they are discussed there itself). For selection purposes kanamycin and ampicillin were included in the media @ 50 and 100 μg/ml, respectively.

2.2. Generation of msrA gene deletion strain of ST and its complementation

msrA gene from ST was deleted by one step gene inactivation protocol (Datsenko and Wanner, 2000). Plasmids pKD4 and pCP20 were a kind gift from Dr. Robert Maier, Dept. of Microbiology, UGA, Athens, USA. The genomic location of *msrA* gene and schematic of the gene knock out method is shown in Fig. 1a and primers are listed in Table 1. In brief, the flaking regions of *msrA* gene were fused with the kanamycin cassette. *msrA* gene was then replaced by kanamycin cassette. Following confirmation, the antibiotic cassette was removed by flp recombinase.

The Δ*msrA* strain was transcomplemented and the complemented strain was referred as Δ*msrA* + p*msrA*. Briefly, the *msrA* gene was cloned in to pQE60 plasmid at BamHI and HindIII sites (Allam et al., 2011). The cloned plasmid was then transformed in to Δ*msrA* mutant strain. The positive colony was selected by PCR and confirmed by Western blot analysis using anti-MsrA antibodies. In Western blots ST and Δ*msrA* were served as positive and negative controls. The primer sequences used for complementation are given in Table 1. Anti-MsrA antibodies were custom synthesized commercially from Merck (India).

2.3. Growth curve analysis

Isolated colonies of ST and Δ*msrA* strains were inoculated in LB broth and grown at 37 °C/180 rpm in an orbital shaker. The strains were subcultured @ 1:100 in 50 ml LB media and grown for 12 h. Aliquots were withdrawn at hourly intervals and the optical densities were measured at 600 nm.

2.4. Sensitivities of ST, ΔmsrA and complemented strains to HOCl

The mid log phase grown cultures were pelleted, washed and suspended in PBS at the OD_{600} of 0.8. The suspensions were exposed to different concentrations of HOCl (0.1 and 0.2 mM) for 2 h. The excess HOCl was quenched by L-methionine (10 mM final) for 10 min. The suspensions were then serially diluted and plated on HEA plates. Plates were incubated at 37 °C for overnight and colony-forming unit per millilitre were calculated.

2.5. Isolation of granules from neutrophils

The granules were extracted according to the protocol of Riley and Robertson (1984) with minor changes. About 5×10^8 neutrophils were suspended in 3 ml of 25% sucrose solution. The suspension was stored on ice and lysed by sonication. Two bursts of 20 s each with an interval of 60 s were given (using Qsonica, USA sonicator @ 50% of maximum amplitude). Unbroken cells were removed by centrifugation at $126 \times g$ for 10 mins and supernatant was transferred in a new tube. The granules were recovered by centrifuging the supernatant at $20{,}000 \times g$ for 30 min at 4 °C. Extracts were prepared by incubating the granules in high salt buffer (0.1 M Na_2HPO_4 pH 7.0–1.0 M NaCl) for 2 h on ice with intermittent mixing. The debris was removed by centrifugation at $6000 \times g$ for an hour at 4 °C. Protein concentration was determined by Pierce® BCA protein assay kit (Thermo Scientific, USA).