Coordinated expression and immunogenicity of an outer membrane protein from *Salmonella enterica* serovar Typhi under iron limitation, oxidative stress and anaerobic conditions

V. Chanana¹, S. Majumdar², P. Ray³, M. Sharma³ & P. Rishi^{1,*}

¹Department of Microbiology, Panjab University, Chandigarh, 160014, India; ²Department of Experimental Medicine and Biotechnology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, 160012, India; ³Department of Medical Microbiology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India

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Summary

Successful pathogens overcome the environmental stresses by the coordinated expression of various genes and eventually proteins. Since, the surface of the microbe is likely to come in contact with the host initially, an attempt was made to identify the outer membrane proteins (OMPs), if any, which may get expressed under more than one environmental conditions simulating the *in vivo* ones. In the present study, *Salmonella enterica* serovar Typhi was grown under iron-limited, oxidative stress as well as anaerobic conditions and the OMP profiles were compared. A 69 kDa OMP was found to express with enhanced intensity under the selected stress conditions in comparison to normal conditions. The phenotypic similarity among the proteins was assessed on the basis of their molecular weight, cross reactivity and HPLC. The protein expressed under oxidative stress and anaerobic conditions reacted with the antibodies raised against iron-regulated outer membrane protein (IROMP), indicating the sharing of at least some of the epitopes. A single peak observed after subjecting the pooled 69 kDa protein sample and appearance of a single band on SDS-PAGE thereafter, confirmed the purity and phenotypic similarity of the 69 kDa OMP. Reactivity of pooled 69 kDa protein with 85% of sera from typhoid patients revealed the *in vivo* expression of this protein. The results of this study indicate the coordination of this phenotype under iron stress, oxidative stress and anaerobic conditions. In view of the expression of the 69 kDa protein under the selected stress conditions and their *in vivo* immunogenicity, these findings may be relevant for the better understanding of the host–microbe interactions and for the further development of diagnostic and preventive strategies.

Introduction

Typhoid fever is a human disease caused by *Salmonella enterica* serovar Typhi, a facultative, intracellular pathogen. This disease remains a major public health problem due to significant

morbidity and mortality worldwide and therefore, continues to be a priority of the World Health Organization. After *S. typhi* is ingested through contaminated food and/or water, the organism migrates to distal ileum. Presumably, *S. typhi*, then adheres to and invades the intestinal epithelium, migrates to the underlying Peyer's patches and disseminates throughout the reticuloendothelial system [1]. However, little is known about the

*To whom correspondence should be addressed. Fax: +91-172-2541770; E-mail: rishipraveen@yahoo.com

bacterium–host interactions that occur at each of these steps in the pathogenesis of *S. typhi*.

Like other enteric pathogens, *Salmonella typhi* encounters iron limitation during the disease process, as the human body has different iron withholding defense systems, preventing the organism from acquiring essential iron for surviving inside the host [2]. Besides iron limitation, availability of oxygen, is yet another stress encountered by *S. typhi* while using molecular oxygen for respiration or oxidation of nutrients to obtain energy and within the intracellular milieu of macrophage-phagolysosomes as well. This results in continuous generation of reactive oxygen species, such as anion radical (O_2^-), hydrogen peroxide (H_2O_2) and highly reactive hydroxyl radicals (OH^-) [3]. Anaerobic conditions are also frequently encountered by pathogens invading the gastrointestinal tract due to low/limiting oxygen conditions prevalent in the small intestine [4, 5]. However, the regular interaction of the host and the pathogen causes the latter to express certain regulons for sensing the host environments and develop survival strategies against the environmental stresses [2, 6]. Although, a number of genes including *fur*, *sox R/S*, *mar A*, *oxy R* have been identified, for combating these stresses [7–10], there are not many studies indicating the identification and localization of the proteins involved in pathogenesis under these conditions.

There is some indication from the existing literature that if stress tolerance occurs in a bacterium when exposed to a single stressor, cross protection can be manifested across multiple stresses and general tolerance of these environments is increased [10, 11]. Iron responsive regulator Fur has been found to be directly or indirectly involved in regulation of oxidative stress defense [12]. Iron regulation has been reported to confer resistance against oxidative stress too, as the organisms are also exposed to extracellular oxygen metabolites of stimulated polymorphonuclear phagocytes in the infected gut. The limited availability of iron in mucosal secretions, in tissues and within the phagocytes, due to the bound iron is well known. The link between iron metabolism and oxidative stress is further demonstrated by the results that the primary regulators of the oxidative stress response in *E. coli* i.e. OxyR and SoxRS induce the synthesis of Fur and that the *E. coli fur* mutant is hypersensitive to oxidative stress [3, 9]. It

is clear from these studies that the pathogens evolve strategies to overcome the stresses by coordinated expression of genes and proteins. However, very little is known about the coordination of phenotype(s) expression and availability of iron and oxygen particularly with respect to outer membrane proteins.

The outer membrane proteins of bacteria function as the dynamic interface between the bacterium and its surroundings and are involved in the maintenance of cell structure, binding a variety of substances adhesion to other cells, and regulation of transport of both nutrients and bactericidal agents [13]. There is a vast amount of information about OMPs and their roles in virulence, inflammation, immunogenicity and anti-microbial resistance [14–16]. However, the knowledge about the expression of OMPs of the bacteria, in reference to coordination of iron and oxygen availability is largely not available. The present study was therefore; carried out to evaluate the coordinated expression of signal sensing phenotype(s) in serovar Typhi under the environmental conditions to mimic the *in vivo* environmental milieu.

Materials and methods

Animals

Young male New Zealand white rabbits (1–2 kg in weight) used for raising anti-sera, were procured from Central Animal House, Panjab University, Chandigarh. Care and use of laboratory animals was followed, in accordance with the guidelines of the institutional ethical committee.

Microorganisms

The standard strain of *S. enterica* serovar Typhi (Ty2) procured from CRI Kasauli, India, was used in the present study. The strain was checked for its purity and was characterized biochemically as well as serologically. It was maintained in 10% glycerol broth and also stored as lyophilized ampules.

Growth conditions

Bacteria was grown on nutrient agar plates and single colony was used to prepare seed culture. 0.1% inoculum from seed culture was

used for growing the bacteria under stress conditions.

Iron stress

Cells were grown in nutrient broth under iron-depleted conditions created by adding different concentrations of iron chelator 2,2'-dipyridyl (100, 150, 200 and 250 μM) in accordance with the method originally described by Fernandez-Beros et al. [17]. Iron content of the media before and after chelation was measured by the method of Carter [18]. The concentration of dipyridyl that could induce the maximum expression of IR-OMP(s) was chosen for the subsequent experiments. Iron-sufficient conditions were created by adding ferrous sulphate in growth medium using the concentration comparable to the concentration of the iron chelator.

Oxidative stress

For the oxidative stress, *S. Typhi* cells were exposed to different concentrations of hydrogen peroxide (10, 15 and 20 mM) in accordance with the method of Ozkanca et al. [19]. The cells were grown in nutrient broth and hydrogen peroxide was added to the culture medium in the early logarithmic phase. The cells were allowed to grow overnight at 37 °C, 150 rpm and were harvested in the stationary phase. The concentration of H_2O_2 that could induce the maximum expression of oxidative stress induced OMP(s) was chosen for the subsequent experiments.

Anaerobic stress

The bacteria were cultured by growing in nutrient broth, under anaerobic conditions by the method of Schimann and Shope [20]. Anaerobic conditions were maintained by placing the culture in Mackintosh anaerobic chamber in the presence of 85% nitrogen, 10% H_2 and 5% CO_2 . The chamber was kept at 37 °C overnight and the cells were harvested.

Combined stress

The bacteria were subjected to the combined stress of iron and oxygen by growing the cells in nutrient broth under iron depleted conditions created by

the addition of 2,2'-dipyridyl as described earlier and *S. Typhi* cells were then exposed to hydrogen peroxide (15 mM) in the early logarithmic phase of growth as described by the method of Ozkanca et al. [19]. The cells were allowed to grow overnight at 37 °C, 150 rpm and were harvested in the stationary phase.

Extraction of OMPs

To study the expression of stress-induced proteins, OMPs were prepared according to the method described by Chander et al. [21]. Briefly, cells were grown under selected stress conditions as described earlier. Bacterial growth was harvested and the pellet obtained was suspended in 20 mM Tris-HCl buffer (pH 7.6) containing 5 mM MgCl_2 . Cells were harvested twice and suspended in the same buffer containing 2 mM phenyl methyl sulphonyl fluoride (PMSF, Sigma, USA). Cells were then disrupted by ultrasonication and undisrupted material was removed by low speed centrifugation. Supernatant was then ultracentrifuged (Beckman Coulter, Optima™ XL-100K Ultracentrifuge, USA) at $100,000 \times g$ for 60 min at 4 °C and the pellet was suspended in 1% sodium lauryl sarcosyl (ICN, USA) (in 20 mM Tris-HCl buffer, pH 7.6) for 2 h at 37 °C. Detergent insoluble OMP fraction was collected by ultracentrifugation at the same speed.

Analysis of OMP profile

The protein content in each preparation was estimated by the method of Markwell and Tolbery [22]. The proteins were separated on SDS-PAGE as described by Laemmli [23]. The molecular weight of the protein(s) expressed with enhanced intensity under iron stress, oxidative stress as well as anaerobic stress conditions was determined by calculating the relative front (Rf) values in comparison to that of standard molecular weight markers [phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa) and carbonic anhydrase (29 kDa)]. Densitometric analysis of stress induced OMPs was determined using GENE TOOL software on GEL DOCUMENTATION (Syngene, USA). For this, one of the protein bands in the marker lane was taken as 1. Then the protein of interest expressed with

enhanced intensity was quantified as how many times of the marker band.

Elution of stress induced OMP from polyacrylamide gels

Stress induced OMPs were eluted out of the preparative gel by the method of Hager and Burgess [24]. Briefly, OMPs extracted from pathogen grown under different stress conditions as described earlier, were resolved on SDS-PAGE and the region expressing stress induced OMP was excised from the gel. Gel section was chopped into small pieces and crushed. Proteins were eluted by incubating the crushed mass of gel with elution buffer for overnight at 4 °C. The mixture was centrifuged and the supernatant was then transferred to a fresh vial. Four volumes of ice-cold acetone was added to the gel eluate and the sample was allowed to precipitate for 30 min at -20 °C. The precipitation was then centrifuged (27,000 × g, 10 min) and supernatant was drained out. The recovery of the protein was checked on SDS-PAGE by silver staining.

Phenotypic similarity amongst the stresses induced proteins

Phenotypic similarity amongst the stress induced proteins was determined on the basis of (i) *Identical molecular weight*: for this, the bands corresponding to the protein of interest were excised and eluted from respective gels [24] as described before. The three proteins were then pooled and run on the SDS-PAGE again. The gel was silver stained by the method of Budowle et al. [25] (ii) *Cross reactivity*: for this, the anti-serum against iron regulated outer membrane protein (IROMP) was raised in rabbits according to Claire et al. [26]. The titre of the antibodies was checked by ELISA [27]. Cross reactivity of oxidative and anaerobic stress induced proteins with IROMP was assessed using anti-IROMP anti-serum and anti-rabbit IgG HRP conjugate (Dako Inc. USA) by Western blotting [28]; and (iii) *HPLC*: for this, the selected stress induced outer membrane proteins were pooled and subjected to high performance liquid chromatography (HPLC) (Shimadzu). Analytical details for HPLC included; Column – Shim-pack DIOL-150, 7.9 mm ID × 25 cm; Mobile phase – 0.2 M sodium sulfate

in 0.01 M phosphate buffer, pH 7.0; Flow rate – 1.0 ml/min; Temperature – 22 °C; Detector – 280 nm.

In-vivo expression of the stress-induced phenotype

In-vivo immunogenicity of the selected stress-induced OMP was detected by Western blotting, using sera from 20 typhoid fever patients (Widal positive sera). The sera from six apparently healthy individuals were used as controls. For this, outer membrane proteins extracted from *S. Typhi* grown under iron stress, oxidative stress as well as anaerobic stress were resolved on SDS-PAGE. Resolved proteins were transferred onto the nitrocellulose paper (NCP) as described previously [28] at 200 mA for 3 h. The NCP containing the transferred antigen (OMPs) was blocked with 5% (w/v) skimmed milk in PBS for 2 h at 37 °C. Thereafter, NCP was incubated with 1:1500 diluted anti-human IgG HRP conjugate (Dako Inc. USA) for 1 h at 37 °C. Strips were then washed with washing solution for 3–4 times and then with PBS. The developing was done with 3,3'-diaminobenzidine (DAB) (Sigma Chemical Co. USA) acting as a substrate (5 mg DAB in 10 ml PBS with 5 µl H₂O₂). The reaction was stopped with double distilled water.

Results

Analysis of outer membrane proteins

A number of proteins were found to be expressed by *S. Typhi* as assessed by SDS-PAGE. On analyzing the protein profiles, it was interesting to note that a protein having molecular weight of 69 kDa (determined by calculating R_f value) was getting expressed with high intensity under all the selected stress conditions. On comparing the expression of this protein under different concentrations of dipyrityl, it was found that the expression was maximum at 200 µM, than at 150 or 250 µM concentration of dipyrityl (Figure 1). On exposing the bacteria to different concentrations of H₂O₂ (10, 15 and 20 mM), the expression of the 69 kDa OMP was found to be highest at 15 mM concentration of H₂O₂ (Figure 2). Enhanced expression of the 69 kDa phenotype was also observed when *S. Typhi* was grown

Figure 1. Analysis of outer membrane protein preparations following iron stress at different concentrations of dipyrindyl by SDS-PAGE (10%). Lane M, Molecular weight standards; lane 1, OMPs extracted under normal conditions (nutrient broth, 150 rpm, 37 °C overnight); lane 2, OMPs extracted at 200 μ M concentration of dipyrindyl; lane 3, OMPs extracted at 100 μ M concentration of dipyrindyl; lane 4, OMPs extracted at 150 μ M concentration of dipyrindyl; lane 5, OMPs extracted at 250 μ M concentration of dipyrindyl.

under anaerobic conditions (Figure 3). However, this protein was not found to be expressed when the cells were grown under normal and iron-sufficient conditions. On subjecting serovar Typhi to the combined stress of iron and oxygen, enhanced expression of 69 kDa OMP was again evident (Figure 4) and the protein profile observed in this case was identical to the OMP profile observed in the presence of iron stress (Figure 1), oxidative stress (Figure 2) and anaerobic stress (Figure 3). The GENE TOOL software on GEL DOCUMENTATION (Syngene, USA) revealed that the protein expression of 60 kDa increased by 3.6-fold in iron stress, 3.2-fold in oxidative stress and 3.4-fold in anaerobic stress conditions, as compared to the control.

Phenotypic similarity

As the molecular weight is always apparent, the phenotypic similarity among the proteins was assessed on the basis of their identical molecular weight, cross reactivity and HPLC. When the

Figure 2. Analysis of outer membrane protein preparations following oxidative stress at different concentrations of hydrogen peroxide (H_2O_2) by SDS-PAGE (10%). Lane M, Molecular weight standards; lane 1, OMPs extracted under normal conditions (nutrient broth, 150 rpm, 37 °C overnight); lane 2, OMPs extracted at 10 mM concentration of hydrogen peroxide; lane 3, OMPs extracted at 20 mM concentration of hydrogen peroxide; lane 4, OMPs extracted at 15 mM concentration of hydrogen peroxide.

protein expressed under all the stress conditions were eluted separately, pooled and analyzed on SDS-PAGE by silver staining, a single band appeared at the same position as in the unpooled samples indicating the similarity of this protein (in terms of identical molecular weight under the selected conditions) (Figure 5). Western blot analysis revealed the cross reactivity (Figure 6) of oxidative-stressed and anaerobic-stressed protein with anti-IROMP anti-serum (raised against the iron-stressed OMP shown in Figure 1). On analyzing the HPLC chromatogram, a single peak with a small shoulder was observed (Figure 7a). Therefore, the fractions containing the peak were pooled and were again subjected to SDS-PAGE followed by silver staining. A single band observed thereafter confirmed the purity of the pooled protein (Figure 7b).

In vivo expression

The *in vivo* expression of this stress induced OMP(s) was revealed indirectly by the *in vivo* immunogenicity indicated by the reactivity of these proteins with sera of typhoid patients. Out of 20 typhoid patients sera (Widal-positive) tested, 17

Figure 3. Analysis of outer membrane protein preparations of *S. Typhi*, grown under anaerobic conditions, by SDS-PAGE (10%). Lane M, Molecular weight standards; lane 1, OMPs extracted under normal conditions (nutrient broth, 150 rpm, 37 °C overnight); lane 2 & 3, OMPs extracted under anaerobic conditions.

sera were recognized by all the three stress-induced OMP(s) (Figure 8). In contrast, none of the six normal sera from apparently healthy individuals reacted with these proteins.

Discussion

The pathogen is exposed to a variety of host environmental conditions that may influence the functional capacity. Such environmental cues include acidic pH, elevated temperature, oxidative stress, different osmotic strengths and the presence of cationic peptides as well as the availability of ions and nutrients. As the infection proceeds, the expression of the virulence determinants varies with time and anatomical sites [6]. Therefore, our

Figure 4. Analysis of outer membrane protein preparations of *S. Typhi* subjected to the combined stress of iron (200 μ M dipyridyl) and oxidative (15 mM H_2O_2) by SDS-PAGE (10%). Lane M, Molecular weight standards; lane 1, OMPs extracted under normal conditions (nutrient broth, 150 rpm, 37 °C overnight); lane 2 & 3, OMPs extracted from *S. Typhi* subjected to the combined iron and oxidative stress.

hypothesis is to identify the set of proteins, which may get expressed under different environmental conditions with an ultimate aim to develop a preventive intervention.

Several forms of stresses increase the rate of synthesis of stress genes and subsequently proteins expressed under different stress conditions. In response to the generation of resistance mechanisms in bacteria when exposed to a particular stress, cross protection has also been shown to manifest across multiple stresses [10, 11]. Baillon et al. [29] have shown that iron regulated proteins of *Campylobacter jejuni* are involved in its defense

Figure 5. Analysis of stress induced 69 kDa OMP under different conditions by SDS-PAGE (10%) after chemical elution. Lane M, Molecular weight standards; lane 1, IROMP; lane 2, oxidative stress outer membrane protein; lane 3, anaerobic stress outer membrane protein; lane 4, OMP extracted under iron and oxidative combined stress; lane 5, pool of the above stress proteins.

against oxidative stress. Rocha et al. [30] have shown that response to oxidative stress in anaerobically grown *B. fragilis* induces the synthesis of at least 28 proteins in the presence of H_2O_2 that confers protection to the organism against the hydrogen peroxide induced oxidative damage. Pathogens have been reported to combat these stress conditions through the induction of various iron-regulated proteins [31, 16], oxidative stress proteins [19, 32] and anaerobic stressed proteins [33]. However, the coordination of phenotype(s) and their immunodominance under iron and oxygen availability have not been studied in detail. The present study revealed the expression, phenotypic similarity and immunodominance of the 69 kDa signal sensing phenotype under these stress conditions. This protein may be one of the factors, which may help the organism to combat the stresses offered by the host.

Enhanced expression of the 69 kDa OMP in all the selected stresses is suggestive of the hypothesis that stress may be responsible for a signal, which induces the synthesis of this protein, which then crosses the inner membrane to become associated with outer membrane. Fernandez-Beros et al. [17] and Baumler et al. [34] have also reported that iron regulated proteins observed in their study were also associated with the outer membrane. Cross reactivity of anti-IROMP anti-sera with both the oxidative and anaerobic stressed proteins

Figure 6. Western blot analysis showing the cross-reactivity of IROMP with oxidative, anaerobic and combined (iron and oxidative) stress induced protein using anti-IROMP antibodies. Lane 1, Iron stress protein; lane 2, oxidative stress protein; lane 3, anaerobic stress protein; lane 4, combined (iron and oxidative) stress protein.

indicates the sharing of atleast some of the epitopes expressed on these proteins under the stressed conditions. A single peak after subjecting the pooled 69 kDa protein further confirmed the phenotypic similarity among the 69 kDa OMP expressed under all the selected stress conditions and its purity. The ability of the organism to switch on a common regulatory network suggest that there is a class of 'stress regulated' genes that are regulated by common sensing mechanism in response to different environmental signals [6, 35]. The results of the present study indicating the overlapping expression of proteins under different stress conditions are in agreement with earlier studies of Chander et al. [21] and Ramamoorthy and Schoel-Meeker [36]. The results suggest that expression of proteins does not depend on single

Figure 7. (a) Auto-scaled HPLC chromatogram of 69 kDa stress protein. (b) Silver staining of purified 69 kDa protein after HPLC.

Figure 8. Immuno detection of *S. Typhi* stress induced outer membrane proteins with serum from patients with typhoid fever. lane 1, Normal outer membrane proteins; lane 2, iron stress proteins (IROMPs); lane 3, oxidative stress proteins; lane 4, combined (iron and oxidative) stress proteins. lane 5, anaerobic stress proteins; →, indicates 69 kDa protein; ---→, indicates porins and other proteins.

environmental stimulus in host but there may be an interaction among stimuli. Expression of 69 kDa phenotype under iron, oxidative and anaerobic stress conditions and their cross reactivity raises the question to confirm their identity in terms of commonality. Hence, our future goal is to compare their N-terminal sequences and to evaluate their genetic expression under the selected conditions.

The *in-vivo* expression of this stress protein is indicated indirectly by its reactivity with the sera of typhoid fever patients. These results are in agreement with the earlier studies where IROMPs in the serovar Typhi were found to be immunogenic *in-vivo* [17]. Non-reactivity of two sera observed in our study can be due to the undefined stage of illness at the time of sample collection. Although the sera collected from patients suffering with pyrexia (but widal test negative) did not react with this protein, but sera from non-typhi *Salmonella* bacteremia patients along with a larger number of typhoid patient sera need to be explored to determine the specificity and the diagnostic utility of this protein.

In view of the expression of 69 kDa OMP under iron limitation, oxidative stress as well as anaerobic conditions and its reactivity with the typhoid patient sera, it may be an important factor, induced during the evolution of salmonellosis. This finding may be relevant for the better understanding of the disease pathophysiology and for the future developments of diagnostic and preventive strategies.

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