Acid Induced Outer Membrane Phenotype in *Salmonella typhi*-a Gene Product of *fliC*

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Abstract

Salmonella enterica has been reported to have evolved different strategies to subvert normal host cellular functions which allow it to enter into and proliferate within the host cells. Although low pH of stomach is a formidable barrier to infections, but majority of the *Enterobacteriaceae* family members exhibit strong innate resistance to low pH by expressing certain gene products involved in acid tolerance responses (ATR). In this brief report, we have isolated and characterized an over expressed outer membrane protein of *Salmonella enterica* serovar Typhi Ty2 grown under acidic conditions (pH 4.5). The protein band was excised and subjected to peptide mass fingerprinting (MALDI-TOF). The fragments obtained were BLAST using MASCOT search engine and it was identified as flagellin (*fliC*) gene product (53.2 kDa) consisting of 506 amino acid residues. Up-regulation of flagellin protein may influence the functional capacity of *Salmonella enterica* during the course of infection. Further, an attempt to knockout this gene may help in confirming the exclusive role of *fliC* gene product in the biological implications such as apoptosis, inflammation, cytokine production, adhesion and invasion which would help in better understanding of the host-pathogen interactions.

Keywords: *Salmonella typhi*, Outer-membrane Protein, Acid tolerance response, *fliC*, MALDI-TOF.

1. INTRODUCTION

During establishment of gut infection, enteric organisms are exposed to inorganic as well as organic acid stress in the gut [1]. On exposure to inorganic acidity in the stomach as well as intracellular milieu of macrophages, the acid tolerance response (ATR) has been reported to control the expression of proteins that serves as an adaptive mechanism for the survival of the organism under acid stress [2,3]. Several pathogens including *V. cholerae*, *Escherichia coli*, as well as *Salmonella enterica* serovar Typhimurium have been shown to endure the acidic stress through the induction of acid stress proteins [4-6]. The paucity of the information on stress induced proteins of serovar Typhi is surprising in view of *Salmonella* pathogenicity being multiphasic and multifactorial.

A number of genes including *PhoP/Q* regulon, *Fur* gene (iron stress), *adi* operon regulating arginine decarboxylase in *Salmonella* [7-9], *ToxRT* of *V. cholera* [10], *RpoS* and lysine-cadaverine antiporter (*CadB*) gene of *E. coli* [11,12] have been identified for combating acid stress. However, there are not many studies indicating the identification and localization of the gene products playing a role in ATR and involved in the pathogenesis under acid stress conditions. Our earlier study had shown an enhanced expression of 55 kDa (approximately) phenotype in *Salmonella enterica* serovar Typhi under acidic conditions [13]. This protein was found to be reactive with the sera of typhoid patients and also had the potential to induce apoptosis in macrophages [14]. This prompted us to characterize this protein in order to identify the gene encoding this phenotype which might be playing a potential role in ATR in serovar Typhi.

2. MATERIALS AND METHODS

2.1 Bacterial strain

Standard strain of *Salmonella enterica* serovar Typhi Ty2, procured from Central Research Institute (CRI), Kasauli, India was used in present study. This strain has been used in our previous

studies [15-17] and is being maintained in our laboratory for the past several years. The strain was identified and characterized biochemically and serologically. Strain was maintained in 10% glycerol broth at -80°C and fresh sub-culture was done for every new experiment.

2.2 Growth conditions

Bacterial cells were grown under normal (neutral pH) as well as inorganic acid stress conditions according to the method of Foster and Hall [18]. Briefly, seed culture was prepared by transferring a single colony of *Salmonella enterica* serovar Typhi Ty2 in nutrient broth for 24 hr. at 37 °C. 1% fresh seed culture was inoculated in nutrient broth having pH 4.5 to induce acidic stress.

2.3 Extraction and analysis of OMP profile

The OMP profile was analyzed by SDS-PAGE as described by us earlier [18]. The SDS-PAGE had a resolving gel of 10% acrylamide gel with 4% stacking gel. The gel was subjected to electrophoresis at 30V initially (for stacking gel) and 70V (for separating gel). The molecular weight of the protein(s) expressed with enhanced intensity under acidic stress condition was determined by calculating the R_f-values in comparison to that of Standard molecular weight markers. The 55 kDa (approx.) acid induced protein (AIP) was excised and subjected to peptide mass fingerprinting (MALDI-TOF).

2.4 Western Blotting

Before performing western blotting, the concentration of primary antibody and secondary antibody was standardized by checker board titration using ELISA.

To confirm the over-expression of 55 kDa (approx.) AIP, the resolved OMP profile was electrophoretically transferred on to polyvinylidene fluoride (PVDF) membrane (0.45 µ pore size) [19] at 25 V for 30 min on a semi-dry blot apparatus (BIORAD Inc.). Transfer efficacy of the protein profile was checked by staining the PVDF with Ponceau-S followed by immediate de-staining with distilled water. The PVDF containing the transferred protein bands was blocked with 10% (w/v) skimmed milk in PBS for

2 h at 37°C. The PVDF was then washed thrice with washing solution containing 0.05% (v/v) Tween-20 in PBS. Thereafter, PVDF was incubated with 1:100 diluted Widal positive sera (dilution made in PBS) for 2 h at 37°C. The membrane was washed with washing solution for 3 times, followed by washing with PBS. Further, incubation was carried out in 1:500 diluted anti-human IgG HRP conjugate (Banglore Genei, India) for 2 h at 37°C. PVDF membrane was again washed with washing solution thrice and then with PBS. Developing was done with 3, 3'-diaminobenzidine (DAB) (Sigma chemicals, USA) acting as substrate (5mg DAB in 10 ml PBS with 5µl H₂O₂). The reaction was stopped with double distilled water.

2.5 Peptide mass fingerprinting (PMF)

The initial separation of proteins followed by *in situ* proteolytic digestion of the AIP in the SDS gel matrix was conducted according to the method of Shevchenko *et al* [20]. Briefly, AIP band was carefully excised from the gel and placed in a siliconized tube. The gel pieces were covered with 200 µl of 200 mM ammonium bicarbonate containing 40% acetonitrile and incubated at 37 °C for 30 minutes to remove the stain and SDS. The step was repeated for complete removal of the stain and SDS. Gel pieces were dried under vacuum system (Speed VacTM, Eppendorf AG, Germany) for approximately 20 minutes. Proteolytic digestion of protein present in dried gel was carried out by adding 30 µl of trypsin solution (0.4 µg of trypsin) along with 50 µl of 40 mM ammonium bicarbonate in 9% acetonitrile and incubated at 37 °C overnight under gentle shaking. The peptides were extracted with a 1:1 mixture of acetonitrile and 50 mM ammonium bicarbonate couple of times and the recovered extracts were pooled, transferred to a fresh siliconized tube and lyophilized to dryness.

Samples were prepared by acidification of the lyophilized peptides by trifluoroacetic acid (TFA) (2:1) and 1 µl aliquot was mixed on the MALDI-MS target with 1 µl of a solution of the matrix α -Cyano-4-hydroxycinnamic acid (CHCA) in acetonitrile/0.1% TFA (2:1).

MALDI-MS was performed with Kratos Probe matrix-assisted laser desorption-ionization–

time of flight (MALDI-TOF) mass spectrometer (Voyager- DE STR 4402, Applied Biosystems USA). Spectra were recorded at an acceleration voltage of 20 kV, using a deflection pulse of 1 µs to remove low mass matrix ions. Approximately 100 single laser shots were accumulated in the resultant spectrum. MS/MS data was interpreted using MASCOT [21] search programme (Matrix Science, MA, USA).

Figure 1: Analysis of outer membrane protein preparations following inorganic acid stress by SDS-PAGE (10%). *Lane 1:* molecular weight standard marker. *Lane 2:* OMPs extracted at pH-4.5, 55kDa phenotype is marked with an arrow. *Lane 3:* OMPs extracted under normal conditions (pH-7.0).

3. RESULTS

3.1 Analysis of outer-membrane protein

In order to assess the OMP profile under stress condition, bacterial cells were grown under acid stress conditions (pH-4.5) and a number of proteins were found to be expressed by *S. typhi* as assessed by SDS-PAGE. On analyzing the protein profile, protein of 55kDa (as determined by Rf value) was found to be expressed with high

intensity. The GENE TOOL software of GEL DOCUMENTATION (Syngene, USA) revealed that, the protein expression of 55 kDa increased 0.4 fold in inorganic acid stress as compared to control (Figure-1). Western blot analysis of the protein profile (Figure-2) clearly reveals the reactivity (a distinct band) of the over-expressed protein with the widal positive serum (lanes: 2-6), which was not observed in the control lanes (i.e. with the protein expressed under normal conditions) (lanes: 1&7).

Figure 2: Western blot analysis showing reactivity of control and acid induced OMPs with widal positive serum. *Lane 1&7:* OMPs of *Salmonella typhi* grown under normal conditions (pH-7.0). *Lane 2-6:* OMP of acid induced (pH-4.5) *Salmonella typhi* recognized by typhoid patient serum on PVDF membrane.

3.3 Characterization of AIP by peptide mass fingerprinting (PMF)

Figure-3 shows the peaks generated from acquired product ion spectra and the peaks were then searched against the Swiss-Prot bacterial protein database containing 18,379 entries of *Salmonella* proteins sequence, using MASCOT software (Matrix Science, MA, USA). The peptide tolerance and MS/MS tolerance were set at 1.0 Da and 0.8 Da, respectively. One missed cleavage per

peptide was allowed. For detection, the proteins with resulting MASCOT scores ≥ 50 were selected. Then, the assigned peptides that have minimum peptide scores of ≥ 10 were listed (Table-1). The reliabilities of the proteins were then calculated from the real positive rates of the assigned peptides.

3.3 Protein sequence of AIP

The peptide fragments obtained by peptide mass fingerprinting (PMF) were analyzed using swiss-prot protein database and the resulted peptide sequence obtained was identified as “FLAGELLIN (*fliC* region present in outer membrane)” of *Salmonella enterica* subsp. *enterica* serovar Typhi Ty2 (NCBI Locus ID: AAO68596). The protein comprises of **506 amino acid residues**, having a molecular weight of **53238 Daltons** and calculated pI value of 4.1. The sequence of the protein identified also contains the matched peptide fragments obtained by PMF (Figure-4).

4. DISCUSSION

In order to survive, bacteria are able to regulate protein expression rapidly and efficiently in response to environmental signals [22] such as pH-variation. Enteric pathogens survive low pH of stomach with gastric acid secretion and high pH of intestine with pancreatic secretion by expressing acid stress proteins [23].

Earlier, report from our laboratory has shown that *Salmonella enterica* serovar Typhi elicits acid tolerance response by over expressing 55 kDa (approx) OMP. In the present study, we demonstrate for the first time that 53.2 kDa OMP of *Salmonella enterica* serovar Typhi is a FliC subunit-a component of flagellar motor complex located in the periplasmic space of the bacterium. It is possible that pH-variation 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. Chart *et al.* [24] have also suggested FliC to be acid shock protein in case of *Salmonella enterica* serovar Enteritidis. These findings indicate that *fliC* gene product may be a conserved protein in different

Salmonella species and its up-regulation may influence the virulence of the organism during the

course of infection.

Figure 3: MALDI-TOF MS analysis of trypsin digested 1D-GE band (55 kDa approx.) from acid induced outer membrane protein profile of *Salmonella typhi*.

Interestingly, it is also indicated from the present study that under acidic conditions, *S. typhi* expresses 53.2 kDa phenotype with much higher

intensity (0.4 times) as compared to the expression under normal condition [13]. Confirmation of over-expression of this protein by western blotting

showing immuno-dominance also indicates the *in-vivo* immunogenicity of the protein. The enhanced expression may be due to the modification of the pre-existing protein under acid shock rather than getting synthesized *de-novo*. The same phenotype

has earlier been reported to be expressed under the heat shock conditions as well [13]. This may be attributed to the presence of common sensing mechanism in response to different environmental stress conditions.

Table 1: Table showing the peptides sorted by residue number according to their respective fragments

Start – End Residues	Observed Monoisotopic Mass fingerprint	M _r (expected)	M _r (calculated)	Partial Amino Acid Sequence
137-161	2655.2878	2654.2805	2655.3603	K.VLAQDNTLTIQVGANDGETIDIDL K.E
173-190	1992.4242	1991.4169	1991.0211	K.LNVQDAYTPKETAVTVDK.T
228-236	1100.7907	1099.7834	1099.5186	K.DGQYYLDVK.G
255-274	2100.4574	2099.4501	2100.1062	K.VNIDTTDKTPLATAEATAIR.G
337-349	1524.5208	1523.5135	1524.6079	K.MGDDFYAATYDEK.T
423-434	1284.7985	1283.7912	1284.7038	K.IDAALAQVDTLR.S

1 MAQVINTNSL SLLTQNNLNK SQSALGTAIE RLSSGLRINS AKDDAAGQAI
51 ANRFTANIKG LTQASRNAND GISIAQTTEG ALNEINNNLQ RVRELAVQSA
101 NGTNSQSDLD SIQAEITQRL NEIDRVSGQT QFNGVKVLAQ DNTLTIQVGA
151 NDGETIDIDL KEISSKTLGL DKLNVQDAYT PKETAVTVDK TTYKNGTDPI
201 TAQSNTDIQT AIGGGATGVT GADIKFKDGQ YYLDVKGAS AGVYKATYDE
251 TTKKVNIDTT DKTPLATAEA TAIRGTATIT HNQLAEVTKE GVDTTTVAAG
301 LAAAGVTGAD KDNTSLVKLS FEDKNGKVID GGYAVKMGDD FYAATYDEKT
351 GAITAKTTTY TDGTGVAQTG AVKFGGANGK SEVVTTATDGK TYLASDLDKH
401 NFRTGGELKE VNTDKTENPL QKIDAALAQV DTLRSDLGAV QNRFNSAITN
451 LGNTVNNLSS ARSRIEDSDY ATEVSNMSRA QILQQAGTSV LAQANQVPQN
501 VLSLLR

Figure 4: Protein sequence identified, unique for flagellin subunit of 506 amino acid residues (fragments corresponding to amino acids are marked in red), Sources NCBI BLAST.

Flagellins from gram negative bacteria have been reported to be required for invasion, cytokine production and systemic inflammation [25-28]. It has also been indicated that mutants of *Salmonella typhimurium* that fail to express *fliC* are defective in expressing a subset of Sip (*Salmonella* invasive proteins) and fail to invade host cells [29]. In another study, it has been observed that non-

flagellated mutants of *Salmonella enteritidis* show 50 fold reduction in invasion of host cells but no reduction in bacterial adherence [30]. Therefore, it may be possible that the pH regulated expression of flagellin subunit in *S. typhi* may be playing an important role not only in making the bacteria to survive under acidic shock but also by contributing to the enhanced virulence of the

pathogen under the ever-changing diverse stress conditions offered by the host.

Scanty information regarding interaction of *fliC* wild type and *fliC* deficient *Salmonella enterica* serovar Typhi Ty2 mutant with the host cells [31] has prompted us to construct a *fliC* mutant of *S. typhi* which would help us in deciphering various aspects of host parasite interactions.

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