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Virulence Increasing of *Salmonella typhimurium* in Balb/c Mice After Heat-Stress Induction of Phage Shock Protein A

Alireza Shoae Hassani · Nour Amirmozafari · Amir Ghaemi

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Abstract *Salmonella typhimurium* is a potentially intracellular pathogen and is responsible for thousands of reported cases of acute gastroenteritis and diarrhea each year. Although many successful physiological and genetic approaches have been taken to conclude the key virulence determinants encoded by this organism, the total number of uncharacterized reading frames observed within the *S. typhimurium* genome suggests that many virulence factors remain to be discovered. This study was conducted to evaluate the role of heat induced phage shock protein A (PspA), in the pathogenicity of *S. typhimurium*. The stress proteins detected on sodium dodecyl sulfate-polyacrylamide gel electrophoresis were identified specifically by immunoblotting with polyclonal antibody against PspA. PspA was produced in response to heat stress at 45°C and it was over-expressed at 65°C. At this temperature, the stressed bacterial cells producing PspA were more virulent (16 folds greater) to female 6–8 week-old Balb/c mice. Correspondency between decrease in LD_{50} and increase in PspA production during heat stress and lower pathogenicity in non-producing cells that emerged during stress at 55°C represents PspA as an important virulence factor in heat stressed *S. typhimurium*.

A. S. Hassani
Department of Microbiology, Fars Science and Research Branch of IAU, Shiraz, Fars, Iran

A. S. Hassani · A. Ghaemi
Young Researchers Club (YRC) of Science and Research Branch of Islamic Azad University, Tehran, Iran

N. Amirmozafari
Department of Microbiology, Iran University of Medical Sciences, Tehran, Iran

A. Ghaemi (✉)
Faculty of Medicine, Golestan University of Medical Sciences, Gorgan, Iran
e-mail: ghaem_amir@yahoo.com

Introduction

Salmonella typhimurium is the leading cause of salmonellosis, a severe form of food poisoning that can be life threatening in elderly, very young, and immunocompromised patients [7]. This bacterium is closely related to *Salmonella typhi*, the causative agent of typhoid fever and is a serious concern in Africa, South America, Mexico, and parts of Asia [30]. In spite of decrease in the rate of typhoid fever, the incidence of non-typhoidal salmonellosis is on the increase [16].

Pathogenicity of *S. typhimurium* is complex and highly integrated process that can not be attributed to any single protein activity [35]. Many studies have used biologically relevant models of infection to analyze the molecular basis of infection [32]. Protein profiling, as performed in other bacterial systems, is a useful approach for obtaining a universal overview of the proteins present in a system under differing conditions [11, 17, 24]. This understanding is essential for developing effective strategies to struggle infection as well as for revealing new therapeutic targets.

Following a brief heat stress at high temperatures, transient synthesis of a set of highly conserved stress proteins happens [6]. Exposure to heat has been recognized as a primary method for preserving foods, typically resulting in the production of either a pasteurized or a commercially sterile food product [33].

The phage shock protein operon (*pspABCDE*) was first described in *Escherichia coli* [4] and is highly conserved in many Gram negative bacteria embracing several pathogens. The *psp* genes are involved in protecting the bacterial cell throughout infectious processes. For instance, *psp* mutants of *Yersinia enterocolitica* are severely virulent attenuated during infection [8]. These bacteria exhibit growth defects when the type III secretion system is expressed [9]. The *psp* operon is also up-regulated during swarming in *S. typhimurium* [36] and also during biofilm formation in *E. coli* [3]. Regulation of Psp proteins intervene with *psp* operon [1, 37]. Transcription of the *psp* operon is driven forward by the σ^{54}-RNA polymerase [37], which is activated by the enhancer binding protein called PspF [19] and facilitated by integration host factor [18]. Induction of the *psp* operon was first depicted as a response upon infection with filamentous phages [4]. Other more general stresses including extreme heat shock, hyperosmotic shock, ethanol treatment, and uncoupling of proton motive force influence the induction of *psp* [25, 32]. Genetic analysis showed that the operon consists of four or five genes [5]. The first gene, *pspA*, is copiously expressed upon induction. This gene encodes a 25.4 kDa protein, which is peripherally associated with the cytosolic membrane, although a proportion of this protein is localized in the cytoplasm. Phage shock protein A has a negative regulatory role in expression of the operon [12].

This study was conducted to assess the role of phage shock protein A, on pathogenesis strength of *S. typhimurium* after exposure to different degrees of heat stresses.

Materials and Methods

Media, Organism, and Preparation of Cultures

Salmonella typhimurium strain ATCC 14028 (PTCC 1186) was obtained from Persian Type Culture Collection in Tehran, Iran. It was routinely cultured on Trypticase soy agar (TSA, Difco, France) and incubated at 37°C. Trypticase soy broth (TSB, Difco, France) was used to assess the ability of *Salmonella typhimurium* growth after recovery from different degrees of heat stresses. Nine aliquot tubes (10 ml) of working cultures (10^8 CFU/ml) were heat stressed by immersion (3 cm above medium level in bottle) into 40°C water bath. After 10 min, one of the tubes was removed from water bath and the remaining eight tubes were remained. For heat adapted cells, the temperature was elevated to 45°C, and after 10 min, this process were repeated at 50, 55, 60, 65, and 70°C. Heat stressed cells were prepared after incubation of the cultures at 40°C for 18 h. Culture grown in TSB for 18 h at 37°C was used as control [39].

Protein Profile Analysis by SDS-PAGE

For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, stress recovered cells were centrifuged 5 min in 5,000*g* and the pellets were resuspended in Tris–HCl 30 mM (pH 8.1). This suspension was centrifuged for 10 min at 10,000*g*. The pellets were vortexed in 200 µl Sucrose 20% in Tris–HCl and were resuspended in phosphate buffer (pH 7). The suspension was incubated on ice with 33 mg/l lysozyme for 30 min and then disrupted by sonication for 20 s. Soluble and insoluble fractions were separated by centrifugation for 15 min at 15,000*g*. One hundred microliters of the supernatant was mixed in 3LUG sample buffer [2] and SDS-PAGE analysis was performed on 15% polyacrylamide gel electrophoresis as described [23]. Gels were stained by silver staining for visualization.

Immunoblotting

After blotting onto nitrocellulose filters (0.45 µm, Schleicher and Schuell) using a Mini Trans Blot Cell (Bio-Rad Laboratories), immunoincubations were performed essentially [21] using rabbit polyclonal antibodies against PspABCDE (Urogentec-Belgium).

Pathogenesis Evaluation of Stressed Bacteria in Balb/c Mice

Forty female 6–8 week-old Balb/c mice (purchased from Pasteur Institute, Tehran, Iran) were chosen to inoculate the recovered bacteria orally. Groups of five mice were housed in polypropylene cages with sterilized bedding under controlled temperature (25°C), and relative humidity (55%). The LD_{50}s were determined as follows: groups of five mice were infected orally with graded doses of bacteria ranging over 5 logs. The LD_{50} studies were performed with the control and stressed *S. typhimurium* by use of five animals per dose. The challenge inocula were enumerated by viable counts. Orally infected mice were observed for 3 weeks to estimate the LD_{50} as described previously [31]. All experimental procedures were carried out according to the standards set forth in the Guide for the Care and Use of Laboratory Animals [29].

Results

SDS-PAGE Analysis and Immunoblotting

Protein profiles of *S. typhimurium* after heat stresses at 40–70°C revealed different patterns. After stress induction at 40°C, the phage shock protein A were not observed in

protein samples but at 45°C the narrow protein band was seen in this lane (Fig. 1). At 50, 55, and at 60°C, the quantity of PspA protein was increased, respectively. At 65°C, the bacteria had maximum expression of PspA, but this band was disappeared in recovered bacteria at 70°C.

Surprisingly a colony of the heat stressed bacteria did not produce pspA after stress at 50°C and even at 65°C, in spite that other colonies had produced maximum PspA (Fig. 2). These heat stressed cells had similar protein patterns and just had a difference in PspA production; in the other word they were only PspA deficient. In fact the spontaneously mutation in *pspA* gene inhibited the production of PspA protein in temperatures higher than 50°C.

Pathogenesis Evaluation of Stressed Bacteria in Balb/c Mice

Lethal dose of untreated *S. typhimurium* for 50% of female 6–8 week- old Balb/c mice inoculated orally was 6×10^3 CFU. The LD_{50} was calculated for two groups of these bacteria. One group that had maximum expression of PspA at 65°C and the second group who lost their ability to express PspA. Death of inoculants mice occurred between

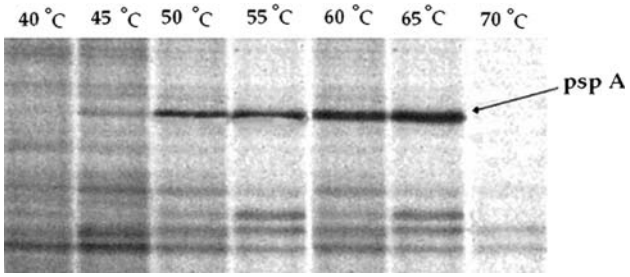

Fig. 1 PspA protein lanes of *Salmonella typhimurium* ATCC14028 recovered from heat stress. Maximum expression of PspA (25.4 kDa) was seen at 65°C stressed bacteria. SDS-PAGE was followed by immunoblotting with antiserum directed against PspA (dilution, 1:1,000). Blots were developed with 4-chloro-1-naphthol-H_2O_2 as the substrate. The positions of molecular mass marker proteins are indicated at the right (in kDa)

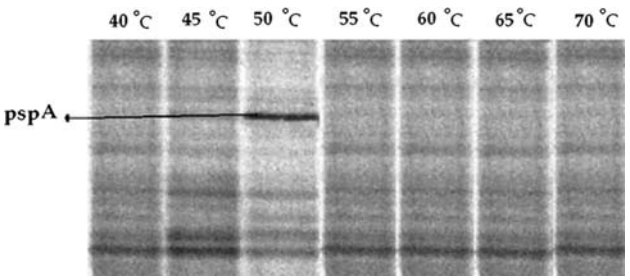

Fig. 2 PspA protein lane of some *Salmonella typhimurium* ATCC14028 cells that recovered from different degrees of heat stresses. A mutation in *pspA* gene inhibited the production of PspA protein in temperatures higher than 50°C

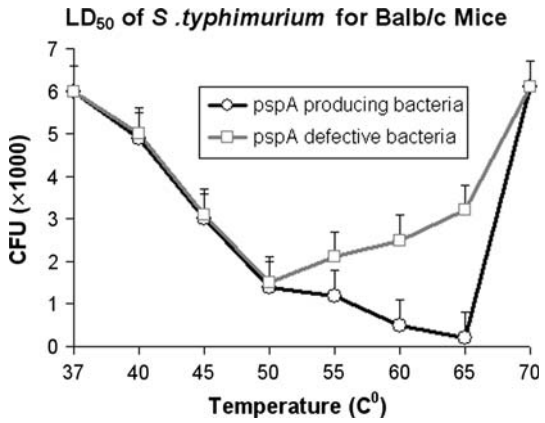

Fig. 3 A 16-fold difference between LD_{50}s of PspA producing and nonproducing *Salmonella typhimurium* cells to Balb/c mice

8 and 11 days after oral inoculation. After induction of heat stress by different temperatures, the LD_{50} calculated for every dose of stress and maximum pathogenesis was observed after recovery from stress at 65°C by LD_{50} of 200 CFU for oral inoculation that shows 30 folds raised virulence by these organisms in contrast to the control bacteria. The bacterial cells that did not produce PspA protein had 16 folds lower virulence in mice in contrast to PspA producing colonies. The results are shown in Fig. 3.

Discussion

This study was performed at the temperatures that occur frequently in the food, drug, and cosmetic industrial processes. Thus, one can readily envision that the continued high level synthesis of general stress proteins in heat adapted and actively growing cells can yield cellular levels sufficient for protection. Therefore, the heat triggered induction of many general stress regulons is likely to be of ecophysiological pertinence for the growth of *S. typhimurium* [38].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was revealed production of new stress proteins especially a 25.4 kDa protein. The nature of this protein confirmed as PspA by means of immunoblotting (Fig. 1). The role of the psp system is unknown, although Kleerebezem et al. [21] claimed that Psp system has been proposed to sense and respond to dissipation of the proton motive force. Recently, Maxson and Darwin [26–28] found that specific induction of *Y. enterocolitica pspA* operon expression was seen subsequent to over-expression of secretins and some cytosolic membrane proteins, and upon disruption of the F_0F_1-ATPase.

The maximum production of pspA was induced in stress at 65°C, and minimal expression of this protein was observed at 45°C. This protein was not produced at 40 and

at 70°C (Fig. 1). In 2003, Eriksson et al. [15] discovered that *psp* genes are among the most highly up-regulated genes in *S. typhimurium* during macrophage infection and they showed *pspA* operon is induced during macrophage infection. As the macrophage infection is a stress condition (oxidative stress) [15]; therefore, we can induce this protein by other stress conditions like heat stress in high temperatures.

PspA directly oppose a variety of inducing stimuli by switching the cell to anaerobic respiration and fermentation and by down-regulating motility, thereby subtly adjusting and maintaining energy employment and proton motive force [20]. Under normal growth conditions, PspA and PspD control the activity rank of *ArcB/ArcA* system that senses the redox or metabolic state of the cell, whereas under stress conditions PspA and PspD deliver their effector functions at least in part by activating *ArcB/ArcA* through positive feedback [20]. So, PspA plays an important role in surviving *S. typhimurium* in harsh conditions.

Also we found a colony of stressed *S. typhimurium* that did not produce PspA. In the first stage of stress induction, these cells expressed PspA at 50°C but they were unable to express PspA at 55°C. The process of stress induction continued at 70°C. At this temperature, the recovered bacteria did not produce PspA (Fig. 2). Furthermore, the recovered bacteria subjected once again to heat stress in different temperatures were unable to produce PspA. Probably due to the heat stress, a mutation was occurred in *pspA* gene, because all the other characteristics of these bacteria such as their protein patterns were similar to those which can express the PspA protein. On the other hand, an increase in PspA synthesis could happen only by mutations that abolished export nearly completely [10].

Increase in PspA production has showed direct relationship with virulence of the bacteria in Balb/c mice. Before expression of pspA at 37°C, the lethal dose for 50% of orally inoculated Balb/c mice was 6×10^3 CFU and after PspA production at 45°C, the LD_{50} of these bacteria decreased 50% to 3×10^3 CFU. This process of virulence elevation was continued up to 65°C at which LD_{50} decreased to 200 CFU. In the other word, there was a 30 folds increase in virulence as a result of maximum production of pspA by stress at this temperature (Fig. 3). Induction of stress at 70°C increased the LD_{50} to 6.8×10^3 CFU that was even higher than LD_{50} of the untreated bacteria due to no production of pspA and other stress proteins such as RfaL, PhoQ and etc. [22].

By comparing the pathogenicity of PspA producing and non-producing cells, the important role of pspA as a virulence factor is confirmed. The LD_{50} of pspA non-producing bacteria at 45°C decreased to 3.1×10^3 CFU but there was no production of PspA protein at 55°C and higher temperatures (Fig. 2), therefore at 65°C, the LD_{50} was raised to 3.2×10^3 CFU. This amount of LD_{50} is 16 folds higher than LD_{50} of pspA producing *S. typhimurium* at this temperature.

Dworkin and Elderkin confirmed that the peripheral cytoplasmic membrane protein PspA acts as a negative regulator, by directly interacting with PspF and inhibiting its activity [12–14]. Furthermore, when the *pspA* mutant was grown under hyper-osmotic shock conditions, it grew to a consistently lower density [34].

Overall, this investigation revealed that PspA protein were over-expressed after heat stress in *S. typhimurium* and it has direct effect on enhancing bacterial virulence. Therefore, thermal treatment that causes a decrease in bacterial populations employed in many industries, may lead to the generation of stress recovered bacteria be even more virulent than the wild type cells.

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References

1. Adams H, Teertstra W, Koster M, Tommassen J (2002) PspE (phage-shock protein E) of *Escherichia coli* is a rhodanese. FEBS Lett 518:173–176
2. Ausable FM (ed) (1989) Current protocols in molecular biology. Wiley Press, New York
3. Beloin C, Valle J, Latour-Lambert P, Faure P, Kzreminski M, Balestrino D, Haagensen J, Molin S, Prensier G, Arbeille B, Ghigo JM (2004) Global impact of mature biofilm lifestyle on *Escherichia coli* K-12 gene expression. Mol Microbiol 51: 659–674
4. Brissette JL, Russel M, Weiner L, Model P (1990) Phage shock protein, a stress protein of *Escherichia coli*. Proc Natl Acad Sci USA 87:862–866
5. Brissette JL, Weiner L, Ripmaster TL, Model P (1991) Characterization and sequence of the *Escherichia coli* stress induced *psp* operon. J Mol Biol 220:35–48
6. Cassens RG (1994) Meat preservation: preventing losses and assuring safety. Food and Nutrition Press, Inc, Trumbull
7. Cossart P, Sansonetti PJ (2004) Bacterial invasion: the paradigms of enteroinvasive pathogens. Science 304:242–248
8. Darwin AJ, Miller VL (1999) Identification of *Yersinia enterocolitica* genes affecting survival in an animal host using signature tagged transposon mutagenesis. Mol Microbiol 32:51–62
9. Darwin AJ, Miller VL (2001) The *psp* locus of *Yersinia enterocolitica* is required for virulence and for growth in vitro when the Ysc type III secretion system is produced. Mol Microbiol 39:429–445
10. DeLisa MP, Lee P, Palmer T, Georgiou G (2004) Phage shock protein PspA of *Escherichia coli* relieves saturation of protein export via the Tat pathway. J Bacteriol 186:366–373
11. DelVecchio VG, Wagner MA, Eschenbrenner M, Horn TA, Kraycer JA, Estock F, Elzer P, Mujer CV (2002) *Brucella* proteomes a review. J Vet Microbiol 90:593–603
12. Dworkin J, Jovanovic G, Model P (2000) The PspA protein of *Escherichia coli* is a negative regulator of s54-dependent transcription. J Bacteriol 182:311–319

13. Elderkin S, Jones S, Schumacher J, Studholme D, Buck M (2002) Mechanism of action of the *Escherichia coli* phage shock protein PspA in repression of the AAA family transcription factor PspF. J Mol Biol 320:23–37
14. Elderkin S, Bordes P, Jones S, Rappas M, Buck M (2005) Molecular determinants for PspA mediated repression of the transcriptional activator PspF. J Bacteriol 187:3238–3248
15. Eriksson S, Lucchini S, Thompson A, Rhen M, Hinton JC (2003) Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. Mol Microbiol 47:103–118
16. Gomez TM, Motarjemi Y, Miyagawa S, Kaferstein FK, Stohr K (1997) Foodborne salmonellosis. World Health Stat 50:81–89
17. Hecker M, Volker U (2004) Towards a comprehensive understanding of *Bacillus subtilis* cell physiology by physiological proteomics. Proteomics 4:3727–3750
18. Jovanovic G, Model P (1999) In vivo and in vitro activities of the *Escherichia coli* σ54 transcription activator, PspF, and its DNA binding mutant, PspF HTH. J Mol Biol 285:469–483
19. Jovanovic G, Weiner L, Model P (1996) Identification, nucleotide sequence, and characterization of PspF, the transcriptional activator of the *Escherichia coli* stress induced psp operon. J Bacteriol 178:1936–1945
20. Jovanovic G, Lloyd LJ, Stumpf MP, Mayhew AJ, Buck M (2006) Induction and function of the phage shock protein extracytoplasmic stress response in *Escherichia coli*. J Biol Chem 281:21147–21161
21. Kleerebezem M, Crielaard W, Tommassen J (1996) Involvement of stress protein PspA (phage shock protein A) of *Escherichia coli* in maintenance of the proton motive force under stress conditions. EMBO J 15:162–171
22. Kwan LY, Isaccson RE (1998) Identification and characterization of a phase nonfimbrial *Salmonella typhimurium* gene that alters O-antigen production. Infect Immun 66(12):5725–5730. doi: 0019-9567
23. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
24. Lipton MS, Pasa-Tolic L, Anderson GA, Anderson DJ, Auberry DL, Battista JR, Daly MJ, Fredrickson J, Hixson K, Kostandarithes H, Masselon C, Markillie L, Moore R, Romine MF, Shen Y, Stritmatter E, Tolic N, Udseth H, Venkateswaran A, Wong KK, Zhao R, Smith RD (2002) Global analysis of the *Deinococcus radiodurans* proteome by using accurate mass tags. Proc Natl Acad Sci USA 99:11049–11054
25. Lloyd LJ, Jones SE, Jovanovic G, Gyaneshwar P, Rolfe MD, Thompson A, Hinton JC, Buck M (2004) Identification of a new member of the phage shock protein response in *Escherichia coli*, the phage shock protein G (PspG). J Biol Chem 279:55707–55714
26. Maxson ME, Darwin AJ (2004) Identification of inducers of the *Yersinia enterocolitica* phage shock protein system and comparison to the regulation of the *RpoE* and *Cpx* extracytoplasmic stress responses. J Bacteriol 186:4199–4208
27. Maxson ME, Darwin AJ (2005) Improved system for construction and analysis of single copy beta-galactosidase operon fusions in *Yersinia enterocolitica*. Appl Environ Microbiol 71:5614–5618
28. Maxson ME, Darwin AJ (2006) *PspB* and *PspC* of *Yersinia enterocolitica* are dual function proteins: regulators and effectors of the phage shock protein response. Mol Microbiol 59:1610–1623
29. National Institutes of Health (1985) Guide for the care and use of laboratory animals. National Institutes of Health publication no 85±23. National Institutes of Health, Bethesda, MD
30. Pang TZ, Bhutta A, Finlay BB, Altwegg M (1995) Typhoid fever and other salmonellosis: a continuing challenge. Trends Microbiol 3:253–255
31. Reed LJ, Muench H (1938) A simple method of estimating fifty percent end point. Am J Hyg 27:493–499
32. Shoae Hassani A, Malekzadeh F, Amirmozafari N, Hamdi K, Ordouzadeh N, Ghaemi A (2009) Phage shock protein G, a novel ethanol induced stress protein in *Salmonella typhimurium*. Curr Microbiol 58(3):239–244
33. Suutari M, Laakso S (1994) Microbial fatty acids and thermal adaptation. Crit Rev Microbiol 20:285–328
34. Vrancken K, Mellaert LV, Anné J (2008) Characterization of the *Streptomyces lividans* PspA Response. J Bacteriol 190: 3475–3481
35. Wallis TS, Galyov EE (2000) Molecular basis of *Salmonella* induced enteritis. Mol Microbiol 36:997–1005
36. Wang Q, Frye JG, McClelland M, Harshey RM (2004) Gene expression patterns during swarming in *Salmonella typhimurium*: genes specific to surface growth and putative new motility and pathogenicity genes. Mol Microbiol 52:169–187
37. Weiner L, Brissette JL, Model P (1991) Stress induced expression of the *Escherichia coli* phage shock protein operon is dependent on sigma 54 and modulated by positive and negative feedback mechanisms. Genes Dev 5:1912–1923
38. Yousef AE, Juneja VK (2002) Microbial stress adaptation and food safety. CRC Press, Boca Raton, pp 178–183
39. Yuk HG, Marshall DL (2003) Heat adaptation alters *Escherichia coli* O157:H7 membrane lipid composition and verotoxin production. Appl Environ Microbiol 69:5115–5119