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Phage Shock Protein G, a Novel Ethanol-Induced Stress Protein in *Salmonella typhimurium*

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Abstract Exposure to ethanol is a stress condition that *Salmonella typhimurium* often encounters during its life cycle. Food, beverage, drugs, and cosmetics have a long history of using alcohols to control pathogens. Ethanol is also commonly used for disinfecting medical instruments. This study was conducted to evaluate the ethanol stress variations on the protein profile, cell structure, and serologic features of *S. typhimurium*. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed the phage shock protein G (pspG), a new ethanol-induced stress protein in cells adapted to 10% ethanol. The result was confirmed by liquid chromatography–mass spectrometry. The maximum quantity of this 9.02-kDa protein was produced in 12.5% (v/v) of ethanol-treated cultures. Scanning electron microscopy has demonstrated new phenotypic characteristics in bacterial structure. The cells were unable to undergo binary fission. This phenomenon explains the tight attachment of bacteria in a colony. Overall, ethanol extreme stress induced expression of new proteins like PspG and repression of some other proteins in *S. typhimurium*. These induction and repression processes have inflicted dramatic changes on Salmonella behaviors.

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Introduction

Salmonella typhimurium is a facultatively anaerobic Gram-negative bacterium. During its life cycle, Salmonella can encounter various environmental stress stipulations, such as nutrient starvation, pH extremes, oxidative stress, osmotic pressure, heat shock, and ethanol stress [2, 13]. Ethanol and cleaners containing ethanol are used extensively as a preservative for biological specimens, in many medicines and drugs, and in parts of food processing plants to reduce or remove microorganisms on equipment [25]. Occasionally, low concentrations of enduring ethanol might be present on treated surfaces of instruments and in environmental niches that are not properly cleaned and sanitized. This provides an opportunity for pathogens to adapt and grow in environments with sublethal concentrations of ethanol [6].

The phage shock protein operon was first described in *Escherichia coli* [5] and is highly conserved in many Gram-negative bacteria including some pathogens. There is convincing evidence that the *psp* genes are involved in protecting bacterial cells during infectious courses; for example, *psp* mutants of *Yersinia enterocolitica* are severely attenuated for virulence during infection [8]. These mutants exhibit growth defects when the type III secretion system is expressed [9]. The *psp* operon is also upregulated during biofilm formation in *E. coli* [4]. Psp

proteins mediate the regulation of the *psp* operon [1, 26]. Transcription of the *psp* operon is compel by the σ^{54}-RNA polymerase [26], which is activated by the enhancer binding protein PspF [18] and facilitated by the integration host factor [17]. Induction of the *psp* operon was first depicted as a response of *E. coli* upon infection with filamentous phages [5]. Other more general stress conditions, including extreme heat shock, hyperosmotic stress, ethanol treatment, and uncoupling of proton motive force, induce *psp* as well [23]. Under stress conditions, PspA, PspD, and PspG (previously *yjbO*) deliver their effector functions at least in part by activating *ArcB/ArcA* through positive feedback [16]. The essential role of the *psp* system is unknown, although it has been proposed to be involved in sensing and responding to disintegration of the proton motive force [20]. The *pspG* is a member of the *pspF* regulon in *E. coli* and Salmonella that is tightly regulated in concert with the *psp* operon. Because the expression of the activator *pspF* is constant under all growth conditions, the key regulatory point under normal growth conditions is strong negative regulation imposed by *pspA*, whereas under inducing conditions, this regulation is lifted, leading to the suited expression of the *psp* operon and *pspG* [24]. It is striking that the *psp* operon and the *pspG* σ^{54} promoters, which are physically unlinked, are both regulated by *pspF* and *pspA* in exactly the same fashion. Further evidence to support this hypothesis has been reported [14].

The physiochemical and functional characterization of ethanol-stressed *S. typhimurium* has not yet been described. It is important to gain a better understanding of the mechanisms that *S. typhimurium* might possess to grow in this situation and to assess the level of safety hazard they might represent. In this study, production of phage shock protein G was reported for the first time to be induced by ethanol stress in *S. typhimurium*.

Materials and Methods

Bacterial Strain and Preparation of Ethanol-Stressed Cells

Salmonella enterica serovar typhimurium strain ATCC 14028 was obtained from Persian Type Culture Collection (PTCC 1186) in Tehran, Iran. It was maintained in Trypticase soy agar (TSA; Difco, France) at 37°C, in which it formed smooth colonies. Eight aliquot tubes (10 mL) of working cultures (10^8 CFU/mL) were supplemented with ethanol (Merck, Germany) to final concentration of 2.5% (v/v). After 60 min of adaptation to this concentration, the bacteria were harvested from the first tube with 5 min of centrifugation at 5000 *g*. The harvested cells were inoculated into tripticase soy broth (TSB) and maintained for 18 h at 37°C for subsequent examinations. This process was continued for the seven other tubes with concentrations of 5%, 7.5%, 10%, 12.5%, 15%, 17.5%, and 20% (v/v) ethanol, in order. Cultures grown in untreated TSB for 18 h at 37°C were used as controls.

Protein Profile Analysis by SDS-PAGE

Stress-recovered cells were centrifuged for 5 min at 5000 *g* and pellets were resuspended in 30 mM Tris-HCl, pH 8.1. The suspensions were centrifuged for 10 min at 10,000 g. Pellets were vortexed in 200 µL of 20% sucrose in Tris-HCl. These cells were resuspended again in phosphate buffer (pH 7) and incubated on ice with 33 mg/L lysozyme for 30 min and then sonicated (Branson Sonifier 250, microtip) for 20 s. Soluble and insoluble fractions were separated by centrifugation for 15 min at 15,000 *g*. One hundred microliters of the supernatants were mixed in 3× Laemmli Upper Gel (3LUG) sample buffer as described previously [3]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed according to the method of Laemmli on 12% polyacrylamide gels [21] as follows: Cell extracts (7.5 µL) were mixed with 7.5 µL of Laemmli dye solution (Bio-Rad) supplemented to contain 10% (v/v) mercaptoethanol and heated in a water bath at 95°C for 5 min. After cooling to 25°C, the mixture was deposited on 12% polyacrylamide gels and separated with a mini-Protean II (Bio-Rad) set at 20 mA constant current for the two gels for 15 min and then the current was raised to 30 mA for 40 min. The gels were stained with colloidal Coomassie blue overnight and destained with a solution containing 1% acetic acid and 1% glycerol. A low-molecular-weight marker (Amersham Biosciences Co., Piscataway, NJ) was run concurrently as a marker.

Identification of Proteins by LC-MS

Protein bands of concern were definitely excised from Coomassie blue-stained gels on which about 10 µg of protein was separated. The gel pieces were cut into small cubes, washed several times with 100 µL of water for 30 min, and then washed three times with 100 µL of acetonitrile–water at a ratio of 1:1 for 15 min. To shrink the gel and to extract the leftover water, pure acetonitrile was added for 10 min. After removal of the acetonitrile, 50 µL of digestion buffer [50 mM *N*-methylmorpholine (pH 8.1)] and 0.5 µg of trypsin were supplemented. Trypsin digestion of the protein was performed at 37°C for 10 h. The supernatant containing the generated peptides was recovered, and the gel pieces were extracted twice with 0.1% trifluoroacetic acid for 30 min. The volume of the combined extracts was reduced to 5 µL in a Speedvac concentrator. Liquid chromatography-mass spectrometry (LC-MS) and collision-induced fragment ion

(CID) spectra were recorded on a Finnigan LCQ ion trap MS equipped with an electrospray ionization source as described previously [11]. The Sequest algorithm compares the measured fragment ion spectra of all selected peptides to the predicted spectra of tryptic peptides that are contained in protein databases (NCBI, OWL, and NRDB) and that exhibit the same molecular weight. Identification of multiple peptides derived from the same protein and evaluation of their cross-correlation scores resulted in unambiguous recognition of the protein.

Serological Tests

Serological tests using polyvalent A, group B, and factor 4, 5, and 12 somatic (O) and flagellar (H) *Salmonella* antisera (Hi Media, India) were performed before and after the induction of ethanol stresses to evaluate the serological variations of recovered bacteria as described previously [22].

Scanning Electron Microscopy

Well-isolated colonies of stressed *S. typhimurium* grown in TSA were processed after growth for at least 1 day at 37°C. The colonies were washed in 0.9% NaCl solution and centrifuged for 10 min at 4000 *g*. The resulting pellets were mixed with 45 mL of phosphate buffer and fixed in 3% glutaraldehyde in phosphate-buffered saline (PBS; pH 7.4). The samples were postfixed with 1% osmium tetroxide, dehydrated with ethanol, critical point-dried, and coated with gold–palladium alloy as described elsewhere [7]. Finally, samples were examined with a Hitachi S-4700 scanning electron microscope (Hitachi Scientific Inst., Gaithersburg, MD) at 2.7 to 5 kV acceleration.

Results

Protein Profile Analysis by SDS-PAGE

Protein profiles of *S. typhimurium* were examined after exposure to ethanol concentrations between 0.0% and 20% (v/v). There were different patterns of protein expression after recovery from different ethanol concentrations (Fig. 1). After the stress was induced by 10% (v/v) ethanol, the phage shock protein G was revealed in the point of 9 kDa and its quantity was elevated in 12.5% (v/v) ethanol concentration, but it was decreased in 15% and disappeared completely in 20% (v/v) ethanol cultures.

Identification of Proteins by LC-MS

To analyze the *pspG* regulon of *S. typhimurium*, whole-cell proteins isolated from overnight cultures of (1) untreated and (2) ethanol-stressed *S. typhimurium* ATCC 14028, were subjected to SDS-PAGE. However, clear differences were seen upon comparison of the gels obtained from untreated cells and stress-recovered bacteria. The proteins, identified by LC-MS, are listed in Table 1. The production of phage shock protein G is significantly enhanced in stressed cells.

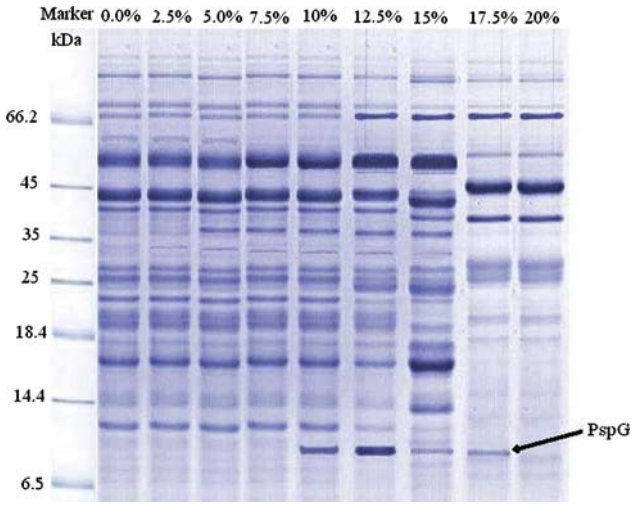

Fig. 1 Protein profiles of *S. typhimurium* recovered from different concentrations of ethanol. The position of the phage shock protein G lane which was induced by ethanol stress has been indicated by the arrow. The Amersham marker shows the molecular weights of protein bands. Molecular masses of size markers are given in kilodaltons

Serological Tests

By induction of ethanol stress at 15% (v/v), the agglutination reaction with somatic antigen was very weak and lower than stressed cells at 12.5% concentration or lower. The cells recovered from 17.5% and 20% (v/v) ethanol-treated cultures did not react with polyvalent O antiserum. Flagellar reaction with H polyvalent antiserum was only seen in 10% (v/v) ethanol concentration (Table 2).

Scanning Electron Microscopy

After recovery of bacterial cells from different concentrations of ethanol stresses, the scanning electron micrographs revealed drastic phenotypic changes in *S. typhimurium*. Recovered cells were attached together very tightly in an amorphous manner with coalescence in the neighboring bacterial cell walls, as shown in Fig. 2. Bacterial size variations were readily observed.

Discussion

This study has demonstrated the phenotypic changes in *S. typhimurium* in the extreme stresses induced by ethanol,

Table 1 Ethanol-induced proteins in *S. typhimurium* that were identified by LC-MS[a]

Ethanol stress regulation	Protein	Molecular mass (kDa)[a]	Gene
Expression[b]	GroEL (chaperone Hsp60)	57.29	mopA
	*PspG (phage shock protein G)	9.02	pspA
	OmpD (outer membrane porin)	39.68	nmpC
	RfaL (LPS ligase)	29.90	rfaL
	PotD (spermidine/putrescine-binding periplasmic protein)	39.00	potD
	N-Acetylneuraminate lyase (aldolase) subunit (NanA)	32.46	nanA
	Glycerol dehydrogenase	38.74	gldA
	Thiol peroxidase	18.03	tpx
	Putative oxidoreductase	27.87	ucpA
Repression[c]	Putative ManNAc-6P epimerase	24.00	nanE
	OsmY (hyperosmotically and carbon starvation-inducible protein)	21.45	osmY
	LuxS (quorum-sensing protein)	19.31	luxS
	Purine nucleoside phosphorylase	25.98	deoD
	*FliC (flagellin)	51.61	fliC
	YgaU (putative LysM domain protein)	16.12	ygaU
	Putative outer membrane protein	46.98	STM4242
	Periplasmic L-asparaginase II (AnsB)	36.93	ansB
	YbdQ (function unknown)	15.90	ybdQ
	YciF (putative cytoplasmic protein)	18.65	yciF

Note: Cellular proteins from *S. typhimurium* grown to the stationary phase were analyzed

[a] Theoretical molecular masses of the *S. typhimurium* proteins were calculated from the corresponding amino acid sequences deposited in the NCBI protein database (http://www.ncbi.nlm.nih.gov/Entrez) by using ExPASy proteomics tool Compute Mw (http://www.expasy.ch)

[b] Proteins show higher expression levels in 12% ethanol-stressed *S. typhimurium*

[c] Proteins show higher expression levels in untreated (control) *S. typhimurium*

Table 2 Serological characteristics of stressed *S. typhimurium* recovered from different concentrations of ethanol

Ethanol concentration Antiserum	2.5%	5%	7.5%	10%	12.5%	15%	17.5%	20%
Somatic polyvalent (O)	+	+	+	+	+	+	−	−
Flagellar polyvalent (H)	+	+	+	+	−	−	−	−

+ = agglutination; − = no agglutination

the organic solvent and disinfectant so often used in the food, drug and cosmetic industries.

Inability to swim in semisolid media (SIM) occurred due to the loss of flagella by stress induced by 12.5% (v/v) ethanol cultures. This result was supported by an agglutination test that was performed using *S. typhimurium* polyvalent H antiserum (Table 2). The result showed lack of any flagellar structure on the surface of stressed cells. LC-MS results revealed the repression of FliC protein (Flagellin) in this concentration of ethanol (Table 1).

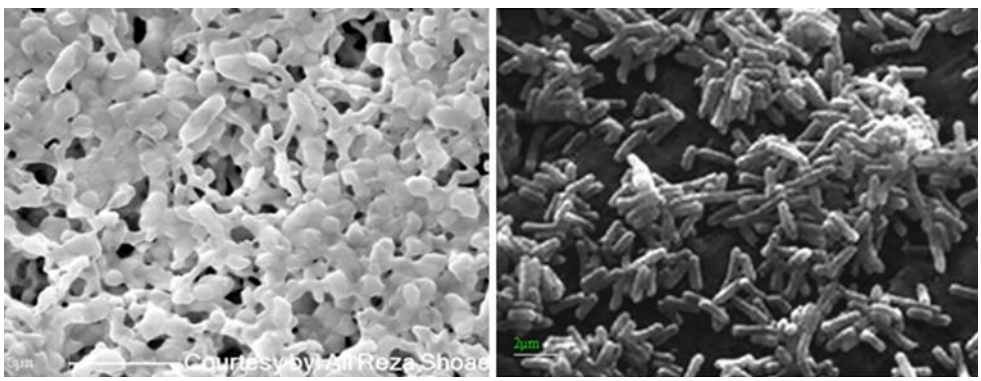

Fig. 2 Electron micrographs of *S. typhimurium* after recovery from 12% (v/v) ethanol. The stress-recovered cells are located very tightly in an amorphous manner with coalescence in the neighboring bacterial cell walls (left); the control untreated *S. typhimurium* cells (right)

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed different patterns of protein profiles in stressed *S. typhimurium.* The result shows that adoptive responses by ethanol stress is a usual mode of stress protection. Production of phage shock protein G, which is a 9-kDa protein in the outer membrane of *S. typhimurium*, was determined after treatment with 10% (v/v) ethanol for the first time. PspG production was overexpressed after treatment in 12.5% ethanol (Fig. 1). Minimum expression of PspG was seen in treated cultures in 17.5% (v/v) ethanol. The phage shock protein G was not produced by stress induced lower than less than 10% and more than 17.5% (v/v) ethanol concentrations (Fig. 1). Results of LC-MS confirmed positive regulation of pspG and production of this phage shock protein in the mentioned range of ethanol stress (Table 1).

In 2001, Darwin and Miller confirmed high levels of *pspG* expression in *S. typhimurium* cells infecting epithelial cells and in *Shigella flexneri* infecting macrophages and epithelial cells [9]. Similarly, in 2003, Eriksson and colleagues have shown that the *pspABCDE* operon and *pspG* are among the 25 most highly upregulated genes in *S. typhimurium* infecting macrophages [12].

The appearance of PspG in the stationary phase of *S. typhimurium* cells exposed to ethanol [10% (v/v) and higher] presumably protect cells against environmental challenge conditions and enable the cells to grow under the stress caused by ethanol. In 2000, Zimmer and colleagues reported that *psp* response is linked with various virulence-related genes in a wide range of enteric bacteria [28]. This is in contrast to the response of *E. coli* to stresses such as nitrogen limitation [27] and specialized growth conditions [19, 24], which causes substantial changes on a transcriptional level.

Ethanol is an amphiphilic compound that functions as a general membrane perturbant to repress cell growth. Reduction of growth rate, prevention of cell division, and cell death of *S. typhimurium*, exposed to ethanol was seen clearly.

Scanning electron micrographs revealed the different phenotypes of *S. typhimurium* after recovery from 12% (v/v) ethanol concentration. Cellular sizes of these bacteria were about 1 μm in length and therefore much smaller if compared with control cells about 3 μm in length (Fig. 2). Additionally, the lack of cellular fission between bacterial cell walls was clear. The reason for this tight attachment between adjacent cells is probably due to the inability for binary fission and deficiency of newly reproduced bacteria (daughter cells) to separate into parts from mother cells. The lower growth rate of the treated bacteria and changes in cell wall composition of the ethanol-treated bacteria have made their colonies on TSA much smaller than control cultures.

In 1976, Ingram explained that the membrane fatty acid compositions were changed in *E. coli* 0157:H7 as an adaptation response to the presence of ethanol [15]. The effects of alcohols of various chain lengths on the fatty acid composition of *E. coli* K-12 were investigated too. In 1984, Dombek and Ingram showed that membranes from cells grown in the presence of ethanol are more rigid than those from control cells due to a decline in the lipid-to-protein ratio [10]. This change was apparently to compensate for the fluidizing effect of ethanol and the ethanol-induced increase in membrane $C_{18:1}$ fatty acid, which occurs during growth and regulation of the lipid-to-protein ratio. This change in the plasma membrane might be an important adaptive response of *E .coli* to growth in the presence of ethanol.

In conclusion, this investigation revealed, for the first time, the phage shock protein G expression in ethanol-stressed *S. typhimurium*. This protein is apparently produced to help the bacterial cells during stress. It is therefore stipulated that processes that use ethanol for decreasing bacterial populations in industries or disinfecting instruments in hospitals should be done carefully because any surviving bacterial cells could be adopted with ethanol and spread in environments to cause contamination problems.

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