— Review —

SEp22, *Salmonella* Dps, a Key Molecule Bearing Both Pathogenicity and Resistance to Environmental Stresses in *Salmonella*

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We isolated and characterized a pathogenicity-related protein in *Salmonella* Enteritidis (SE) from poultry farms, and designated it as SEp22, which was identified later as *Salmonella* Dps, a DNA-binding protein from starved cells. Expression of SEp22 was regulated by bacterial growth in Luria-Bertani (LB) medium, showing reduced expression in the logarithmic phase but increased expression from the late logarithmic to stationary phases, which was linked to the expression of σ^S both in protein as well as mRNA levels. In addition, induction of SEp22 required nutritional factors in LB or casamino acids during overnight incubation in M9 minimal medium, or even through short-term exposure to H_2O_2 for 5–15 min. Induction of SEp22 was also remarkable after sudden addition of H_2O_2 and exposure of the bacteria to the drying protocol. The roles of SEp22 in the pathogenicity of *Salmonella* in mice, the survival of *Salmonella* exposed to H_2O_2, or that under dry-stress were proved not only by the difference among *Salmonella* clones from environmental isolates with different levels of SEp22 expression, but also by *sep22*-gene-depleted mutants that originated from SECl#15-1, a wild type virulent strain with high levels of SEp22. These results suggest that SEp22 is a key molecule that is responsible for pathogenesis as well as environmental stress-resistance. In this review, the diverse roles of SEp22/Dps in *Salmonella* are also described, suggesting the importance of this protein in the bacterial stress responses in infection and survival, as well as in the regulation of bacterial growth through aerobic metabolism. Recent progress in SEp22/Dps research at the molecular levels is also discussed.

Key words —— *Salmonella* Enteritidis, Dps, pathogenicity, resistance to environmental stresses, H_2O_2-resistance, dry-resistance

INTRODUCTION

Salmonella is one of the most causative bacteria of food-borne diseases in Japan[1)] and other countries,[2)] and is detected not only in food but also in natural environments including river water, soil or air-borne dust. Among *Salmonella* spp., *Salmonella enterica* serover Enteritidis (*S*. Enteritidis; SE) has been the most dominant species detected, and is frequently found in contaminated foods such as eggs, chicken meat, and even vegetables including cabbages, tomatoes, and pimentos. It has also been the cause of mass food-poisoning in Japan and U.S.A., which might be because SE shows rather strong virulence in humans among *Salmonella* species that cause food-poisoning. Poultry is an important source for spreading SE, not only thorough food but also through environmental contamination.[3)] Epidemiological studies have revealed that the major vehicles of SE outbreaks in humans have included contaminated poultry meat and eggs.[4)] In order to control the spread of SE to foods, it is of a great importance to understand the characteristics of SE that allow it to survive in natural environments under various conditions, and also to understand the mechanisms underlying the spread of SE in environments as well as in clinical cases.

It is also important to remind ourselves that the virulence and pathogenicity of *Salmonella* are

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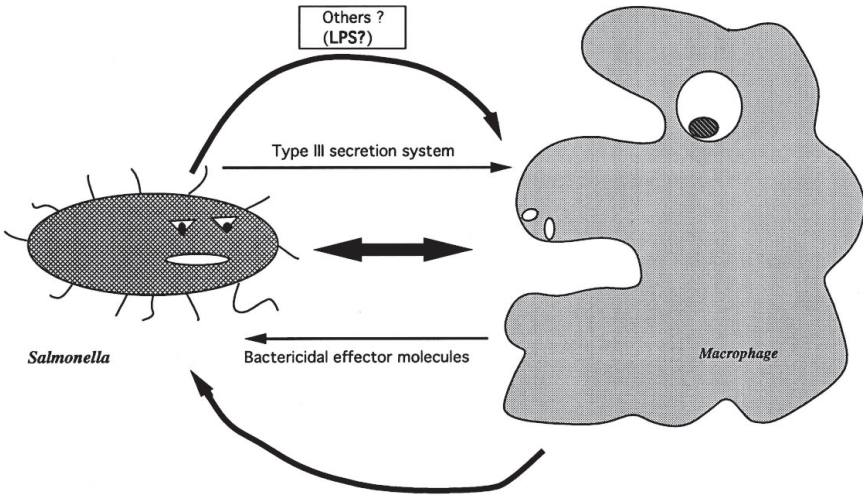


Fig. 1. *Salmonella*-macrophage Interaction (a Model)

Table 1. Expression of SEp22 and *invA*, Pathogenicity in Mice, and Resistance to H_2O_2 and to the Drying Protocol in SE Environmental Isolates

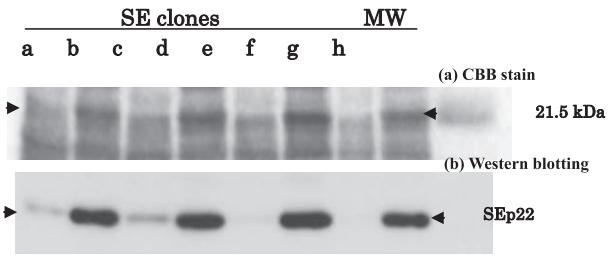
Clone	SEp22[a)]	*invA*[b)]	pathogenicity[c)]	H_2O_2-resist[d)]	dry-resist[e)]
SECl#4-1	±	+	−	−	±
SECl#7-1	+++	+	+	+	+++
SECl#14-1	±	+	−	−	±
SECl#15-1	+++	+	+	+	+++
SECl#16-1	±	+	−	−	±
SECl#23-1	+++	+	+	+	+++
SECl#26-1	++	−	−	N/A	N/A
SECl#28-1	−	+	−	−	−
SECl#40*	+++	+	+	+	+++

*A clinical isolate from a patient of sporadic diarrhea. The other bacterial clones in this table are environmental isolates from poultry farms in Japan. *a*) SEp22 was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)/Western blotting. *b*) *invA* was determined by RT-PCR. *c*) Pathogenicity of the bacteria was estimated by lethality after oral administration of 1×10^8 cfu of each bacterial clone to BALB/c mice. *d*) H_2O_2-resistance was assayed by colony-formation of each bacterium after exposure of 0.1–10 mM H_2O_2. *e*) Dry-resistance was assayed by the drying protocol. In this table, the extents of each phenotype are relatively expressed as negative (−), sometimes positive (±), positive (+), strongly positive (++), and very strongly positive (+++). N/A means "not assayed."

tightly linked to its behavior in the host after infection: As a food-poisoning bacterium, *Salmonella* usually invades from gut epithelium through M cells in the neighboring Peyer's patches, or through intestinal epithelial cells directly, where polymorphonuclear leukocytes (PMNs) and macrophages phagocytose them.[5)] The macrophages play major roles in elimination of these *Salmonella* from the host by exerting bactericidal activity including phagocytosis, secretion of lysozyme or other lysosomal enzymes, and the production of reactive oxygen species (ROIs) such as superoxide anion ($O_2{\cdot}^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH{\cdot}$), singlet oxygen ($^1O_2$), nitric oxide ($NO{\cdot}$), or peroxynitrite ($ONOO^-$).[6)] Among them, H_2O_2 plays central roles in bactericidal activity, because it readily penetrates through bacterial membranes and is converted to $OH{\cdot}$ in the presence of Fe^{2+} by the Fenton reaction.

To cope with these attacks and effector molecules produced by macrophages, *Salmonella* has a counter system to attack host cells (Fig. 1); one of the most important systems is the Type III secretion system (T3SS) which is composed of a series of molecules necessary for injection of virulence factors through a needle-like structure. Among them, *invA* encodes an inner membrane protein that forms the apparatus of T3SS.[7)]

So far, the virulence of bacteria has been estimated by the presence of *invA*, *spv*[8)] or enterotoxin genes. However, as shown in Table 1, the pathogenicity of *Salmonella* clones is not entirely



SE clones used are:
a: SECl#4-1, b: SECl#7-1, c: SECl#14-1, d: SECl#15-1,
e: SECl#16-1, f: SECl#23-1, g: SECl#28-1, h: SECl 40
Arrowheads show the positions of SEp22.

Fig. 2. Expression of SEp22 in SE Clones

consistent with the presence of *invA*. Therefore, *invA* is a necessary factor for acquisition of virulence that is required to attack the host. Apparently, another defense factor for protection of the bacteria from certain effector molecules which are derived from the immune phagocyte system like ROIs, is important. This is an adequate factor that is closely linked to the pathogenicity of the bacteria during infection of the host. Therefore it is important to consider both the offensive and defensive factors of *Salmonella* when estimating the pathogenicity in the host.

In our studies searching for the factors and mechanisms underlying acquisition of pathogenicity in the mice by environmental SE clones, we recognized one protein band of MW of approximately 22 kDa, that was evident in all of the cell extracts of the virulent clones but not in the avirulent clone [Fig. 2(a) Coomassie brilliant blue (CBB) stain]. This experiment lead me to discover SEp22, which will be described in the following section.

ISOLATION AND CHARACTERIZATION OF SEp22 PROTEIN

Isolation of SEp22

More than 30 SE clones, isolated from poultry farms (CAF Laboratories; Fukuyama, Japan), were individually cultured in 100 ml of Luria-Bertani (LB) medium in 300-ml Erlenmeyer flasks at 37°C overnight, with shaking at 150 strokes min^{-1}. The cells were harvested by centrifugation at 4120 × *g* for 20 min at 4°C, washed with ice-cold phosphate-buffered saline (PBS), and disrupted with 0.1 g of glass beads (Sigma) in a cell beater (BIO 101 Savant, Fast Prep™ FP120) and centrifuged at 40300 × *g* for 5 min at 4°C. The resultant supernatant was collected as bacterial cell extract, which was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Terai *et al.*).[9] As shown in Fig. 2(a), SECl#4-1, #14-1, #16-1, and #28-1 showed only faint bands of CBB-staining around 22 kDa, while SECl#7-1, #15-1, and #23-1 as well as SECl#40, a clinical isolate strain, all showed clear bands in the corresponding MW. We designated this protein band as SEp22, and then tried to purify it using a large scale culture of SECl#15-1, because infection of these SE strains into BALB/c mice orally resulted in two distinct classifications; *i.e.*, a strongly virulent group (SECl#7-1, #15-1, and #23-1 as well as SECl#40), and a completely avirulent group (SECl#4-1, #14-1, #16-1, and #28-1), which showed good correlation with the expression of SEp22.

One liter culture of SECl#15-1 in LB medium in an Erlenmeyer flask at 37°C overnight yielded a sufficient number of stationary phase cells, which were washed with PBS, disrupted, and the resultant cell extract was mixed with ammonium sulfate solution to 50% saturation. SEp22 was not precipitated by 50% saturation but most of the other cell proteins were precipitated and separated from SEp22. Dialysis of the supernatants of the 50% ammonium sulfate saturation against 20 mM Tris-HCl, pH 7.5, resulted in precipitation of SEp22 aggregates to almost homogeneity.

Characterization of SEp22

We analyzed the sequences of *N*-terminal 22 amino acids of SEp22, and performed cloning of the *sep22* gene covering full length coding of nucleotide sequences corresponding to 167 amino acids (Fig. 3). The *sep22* gene was shown to be similar to *Escherichia coli* (*E. coli*) *dps*/*pexB*, which was first reported by Almiron *et al.*,[9] and we subsequently showed[10] *sep22* to be identical to *Salmonella dps*.[11] SEp22 contains a proposed DNA-binding domain at the *N*-terminal ($_5$**K**-X-X-**K**-X-**K**) as *E. coli* DNA-binding proteins from starved cells (Dps) does. In addition, a characteristic aggregate formation of SEp22 during dialysis of the 50% ammonium sulfate supernatant fraction suggested an automatic crystallization of SEp22 under low salt concentration. In fact, analysis of the crystal structure of SEp22, based on our research, has been reported,[12] showing a dodecameric shell with 23 symmetry, together with the ferroxidase center of a single iron ion per pro-

1	6	11	16	21	26	31	36	41	46
MSTAK	LVKTK	ASNLL	YTRND	VSESD	KKATV	ELLNR	QVIQF	IDLSL	ITKQA
HWNMR	GANFI	AVHEM	LDGFR	TALTD	HLDTM	AERAV	QLGGV	ALGTT	QVINS
KTPLK	SYPLD	IHNVQ	DHLKE	LADRY	AVVAN	DVRKA	IGEAK	DEDTA	DIFTA
ASRDL	DKFLW	FIESN	IE						

Fig. 3. Amino Acid Sequence of SEp22

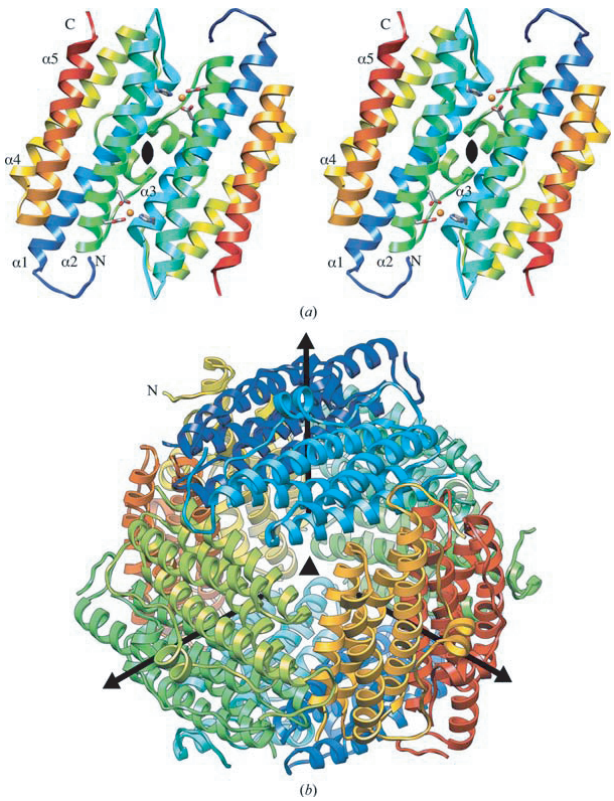


Fig. 4. Crystal Structure of SEp22

Stereoview of the dimer (a) and dodecamer (b) structure of SEp22 is shown.[12)]

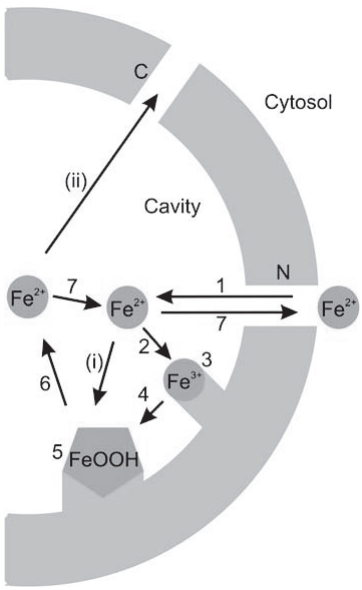


Fig. 5. Iron Entry, Oxidation, Mineralization and Reutilization in SEp22/Dps

The pathway of iron movement in Dps is composed of: (1) entering of Fe^{2+} into the dodecamer through the *N*-terminal pores, (2) binding of Fe^{2+} to the ferroxidase site, (3) oxidization of Fe^{2+} to Fe^{3+}, (4) move of Fe^{3+} to the nucleation site, (5) starting of mineralization with the formation of FeOOH at the nucleation site, (6) neutralization of iron by reducing FeOOH back to Fe^{2+}, and (7) transport of Fe^{2+} back to cytosol through *N*-terminal pores. Alternatively, (i) Fe^{2+} flows to the cavity where it might also get oxidized on the surface of the growing mineral, or (ii) iron is transported out of the protein via the *C*-terminal pores. (Transferred and modified from the model by Haikarainen *et al.*[17)])

tomer (Fig. 4). These results show that SEp22 is actually *Salmonella* Dps, which has a structure similar to that of other bacterial species such as *E. coli*,[13)] *Halobacterium salinarum*[14)] and so on.

SEp22/Dps AS AN IRON-BINDING, FERRITIN-LIKE PROTEIN

Because SEp22 was assigned to *Salmonella* Dps, which has been identified as a ferritin-like protein partly because of its ferroxidase activity, or more specifically, its ability to oxidize bound ferrous ions to the ferric state,[15)] extensive study has been done on whether SEp22 has iron-binding ability at the ferroxidase center (FOC) inside the dodecamer structure of this ferritin-like protein, Dps.[16)] X-ray diffraction analysis of the SEp22 crystals without or with iron-soaking revealed that iron ion was bound to the FOCs between the acidic side chains of D78 and E82 of one protomer and the basic side chains of H51, K48 and H63 of the other protomer in the iron-soaked SEp22 crystals.[12)] Dps-like proteins, including SEp22, are known to be widely distributed among bacterial species, and all Dps-like proteins have common characteristic three-

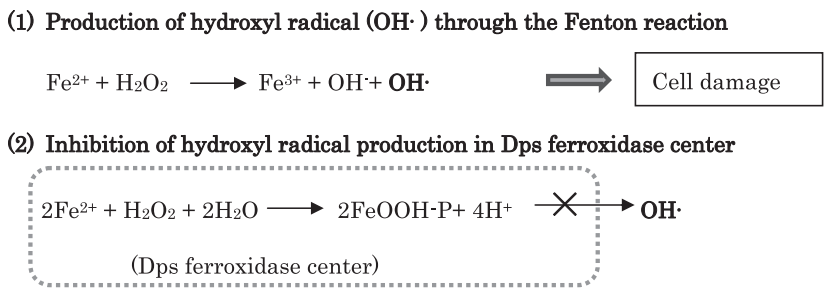


Fig. 6. Inhibition of Hydroxyl Radical Formation by the Fenton Reaction in Dps Ferroxidase Center

In the cells, ferrous ion (Fe^{2+}) is oxidized by hydrogen peroxide (H_2O_2) at the molar ratio of 1 : 1 through the Fenton reaction and hydroxyl radical (OH·) is produced as shown in (1). However, in the presence of Dps, ferrous ion is incorporated into Dps, then bound to the ferroxidase center and subsequently reacted with hydrogen peroxide at the molar ratio of 2 : 1, forming ferric peroxide complex (FeOOH-P) at the binding site (P), which inhibit hydroxyl radical formation as shown in (2). (Transferred and modified from the model by Calhoun and Kwon[16]).

dimensional architecture of spherical dodecamers with a hallow central cavity where FOC exists, containing the several conserved core amino acids (H51, H63, D78, E82 and W52 in case of *E. coli*) in the neighborhood.[17]

As for the processes of iron uptake, binding and oxidation in SEp22/Dps, the 23 symmetry of the SEp22/Dps dodecamer (Fig. 4) creates 4 threefold axes through the protein leading to 8 trimeric interfaces, formed at the end of the *C*- or *N*-termini of the monomers creating channels, which connect the exterior of the dodecamer to the internal cavity of this protein.[16, 17] The pathway is composed of iron entry inside the dodecamer, binding of iron to the ferroxidase center, oxidation of iron at the ferroxidase center, nucleation, mineralization, iron re-utilization by reduction to ferrous iron, and transport back to cytosol (Fig. 5).[17]

Dps was reported to bind and chelate iron ion and thus inhibit production of highly toxic hydroxyl radical (OH·) by the Fenton reaction, as shown in Fig. 6. This inhibition proceeds at the FOC of Dps, which prefers H_2O_2 to O_2 as an oxidization agent for ferrous iron, harboring catalase-like activity to Dps. In this sense, Dps is not comparable to ferritin, in which ferrous iron is oxidized preferentially not by H_2O_2 but by O_2.[16, 18]

SEp22/Dps AS A DNA-BINDING PROTEIN

Because the finding of Dps from *E. coli* starved cells by Almiron *et al.*[9] was accompanied by characterization of this protein as a DNA-binding protein, subsequent studies concerning Dps in other laboratories as well as their own one have focused on both the structure and function of the DNA-Dps complex. Intensive structural research, especially using with X-ray crystallography, has revealed a highly conserved structural fold of proteins within the Dps protein family isolated from different organisms.[12, 16, 17, 19–25] However, most of these studies mainly showed structural features common to other ferritins characterized in bacteria, including the paradigmatic ferritin (FtnA) and the heme-containing bacterioferritin (Bfr), which are composed of at least 12 subunits folded into four-helix bundles and assembled into spherical protein shells as Dps is.[13, 26] *E. coli* Dps has a very compact and stable structure with protrusion of the highly flexible and lysine-rich *N*-terminus (Fig. 3) of each of its monomers,[17] which has been believed to be responsible for Dps-DNA co-crystallization as well as Dps self-aggregation in solution.[26] The DNA-binding ability of Dps was initially examined by addition of Dps to supercoiled or linear plasmid DNA, which resulted in very stable Dps-DNA complex formation.[9] Although heating of Dps at 65°C prior to addition of DNA failed to form a Dps-DNA complex, the Dps-DNA complex itself is, once formed, stable against heating at 100°C, deoxyribonuclease (DNase) I digestion, and treating with 5% Triton X-100 or 5% Brij-58, but is dissociated with 30% formamide or 2% SDS. The binding of *E. coli* Dps to DNA showed no apparent sequence specificity, because Dps-bound DNA reveals no clear footprint after DNase I digestion.[9]

N-terminal deletion mutants of Dps revealed the importance of *N*-terminus lysine residue multiplicity in the formation of large Dps-DNA complexes: wild-type Dps, carrying three positively charged lysine residues at its *N*-terminus, forms large Dps-DNA complexes *in vitro* at physiolog-

ical pH, while Dps mutants, possessing only one of the three terminal lysine residues, are also able to bind DNA but are unable to sequester and condense it. However, mutants lacking all three terminal lysine residues are unable to bind or condense DNA.[26] In addition, the binding of Dps to DNA in *E. coli* cells was also examined by electron microscopy and the results revealed DNA-Dps co-crystallization in the Dps-overexpressed cells as well as the starved ones.[27] This highly organized co-crystallization process is produced with closed super-coiled plasmids, linear double-stranded DNA, as well as single-stranded RNA molecules *in vitro*, and the DNA-Dps co-crystallization proceeds very rapidly within seconds, by addition of DNA to Dps solution.[27] Because immediate sequestration of vital macromolecules like DNA in intracellular crystalline assemblies might be an effective and efficient way of protection of the molecule,[27] Dps seems no doubt to play an important role in biodefense mechanisms in bacterial survival and environmental stress responses. Furthermore, Dps-DNA complex formation has been shown to be involved in the architecture of bacterial nucleoids by atomic force microscopy,[26, 28] providing further proof that protein-protein interactions play a major role in generic DNA protection[29] and reduction of mutation frequency.[30]

However, DNA does not seem to bind directly to the surface of Dps, because the spherical surface of this protein has no apparent DNA-binding motifs and is negatively charged, which might repel binding of negatively charged DNA.[13] Frenkiel-Krispin *et al.*[29] proposed that DNA-Dps complex formation relies on ion bridges formed by Mg^{2+}. The similarity between packing of Dps dodecamers in protein crystals and that within Dps-DNA complexes suggest a close relationship between Dps self-aggregation and DNA condensation.[26] Dps self-aggregation forms a crystal lattice, creating a line of lysine-rich *N*-termini with holes derived from 3 adjacent Dps molecules,[13] where DNA might be threaded through. Wolf *et al.*[27] also suggested a similar mechanism of DNA threading through the Dps crystalline structure. The important role of the *N*-termini in Dps-DNA interaction is also supported by studies on Dps family members that do not possess positive residues at *N*-termini, such as *Listeria innocua* ferritin, *Bacillus anthracis* Dlp, and *Helicobacter pylori* HP-NAP, showing no DNA binding ability.[31–33]

REGULATION OF SEp22/Dps EXPRESSION

The first finding of SEp22 was based on the different expression of stationary phase proteins among SE clones from environmental isolates which were pre-incubated in LB medium overnight.[10] Because SEp22 was proved to be *Salmonella* Dps[10] as described in the previous section, the expression and induction of SEp22 and that of Dps should occur similarly, if the experimental conditions are controlled. Almiron *et al.*[9] also described the induction of Dps in *E. coli* cells during incubation in LB medium at the stationary phase but not at the mid-exponential phase. However, subsequent studies on *E. coli* Dps and related Dps seemed to have paid much more attention to the expression of Dps in starved cells cultured in minimal medium like M63[9, 16] as well as the molecular structure of Dps and regulation of gene expression.[16] It remains largely unknown whether nutrient starvation in M63 medium, which means depletion of glucose, induces the same signals for induction of SEp22/Dps during culture in LB medium or not. To address this question, we used M9 minimal medium containing 0.4% glucose, in which SE did not induce SEp22 after incubation overnight even when the cells reached the stationary phase. However, addition of 10% (v/v) LB medium to M9 medium turned out to induce SEp22 during overnight culture. A similar effect on SEp22 induction was obtained by addition of casamino acids, although the effect was dose-dependent.[34] These results imply the involvement of unknown regulatory mechanisms concerning nutrient factors and their depleting effects.

Expression of SEp22 during culture in LB medium was growth phase-dependent, regulated at the transcriptional level, and dependently on σ^s-expression (Fig. 7).[10] It has also been shown that Dps expression is highly dependent on the growth phase: In the stationary phase, σ^s, the stationary phase-specific sigma factor, controls the expression of *dps* mRNA,[35] as was the case of *sep22* in SE.[10] In the *dps* promoter, consensus sequences for the histone-like integration factor (IHF) are present, and heterodimeric IHF protein was shown to be required for the induction of σ^s-dependent *dps* mRNA in the starved cells.[35] In the exponentially growing cells, H_2O_2 induces OxyR activation,[36] leading to the activation of another sigma factor, σ^{70}, that binds to *dps* promoter and stimulates gene expres-

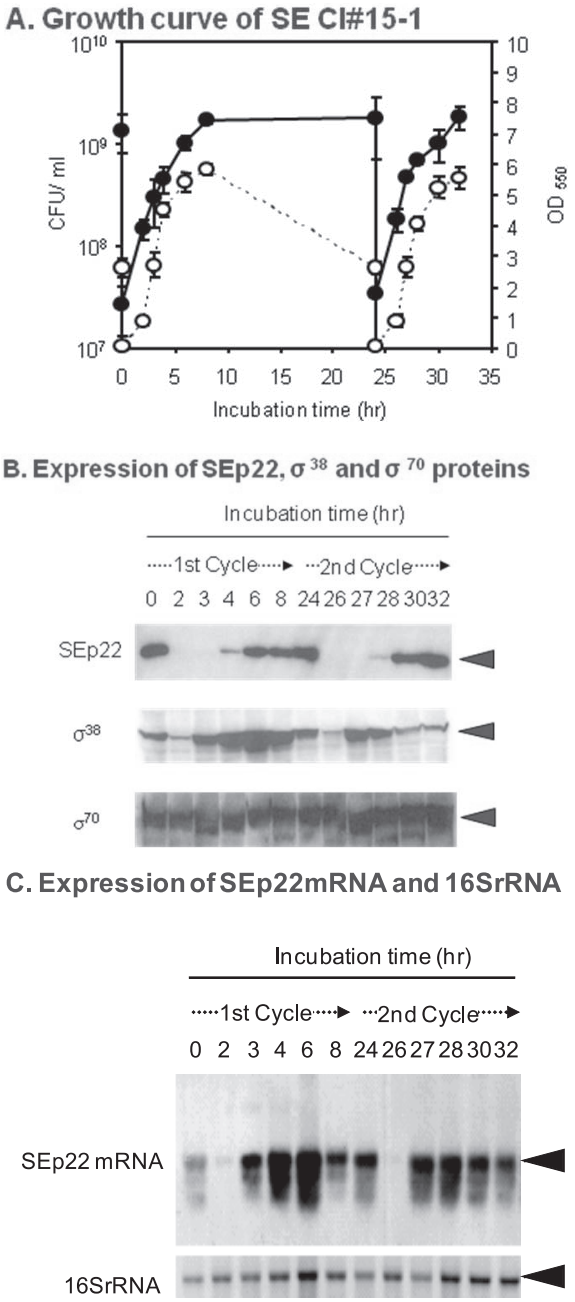


Fig. 7. Expression of SEp22 in the Course of Cell Growth in LB Medium

(A) Growth curve of SECl#15-1 in LB medium. Bacteria numbers are shown as CFU ml^{-1}(●) and OD_{550}(○). An overnight culture of SECl#15-1 in the stationary phase was diluted and incubated at 37°C, with shaking at 150 strokes min^{-1} (1st cycle). On the second day, dilution and culture of the bacterium was repeated (2nd cycle). (B) Expression of (a) SEp22, (b) σ^{38} (σ^{S}) and (c) σ^{70} proteins in SECl#15-1 in the growth phases during culture (hr). Western blot analysis was performed with specific antibodies against SEp22, σ^{38}, or σ^{70}. Panels (a), (b) and (c) correspond to the proteins with MW of 19 kDa (SEp22), 38 kDa (σ^{38}) and 70 kDa (σ^{70}), respectively. (C) Expression of SEp22 mRNA (upper panel) and 16S rRNA (lower panel). Equal amounts of total RNA (1.5 μg) were loaded in each lane and northern blots of SEp22 mRNA are shown in the upper panel. In the lower panel, the EtBr-stained 16S rRNA bands are shown. [Cited from Ref. 8) with modification.]

sion via σ^{70}-RNA polymerase.[37] This pathway is redox-sensitive,[36] and thus could be activated by such reagents and chemicals that activate peroxide-formation, such as antibiotics.[38]

Recently, down-regulation of Dps expression was reported: selective repression of *dps* promotor was found in *E. coli* by Fis and H-NS through inhibition of transcription of *dps* gene.[38] Fis represses by trapping RNA polymerase containing σ^{70} at the promoter, while H-NS functions by displacing RNA polymerase containing σ^{70}, but not RNA polymerase containing (σ^{S}) σ^{38}. Because Fis levels are high in exponentially growing cells but decrease to near zero when the cells enter stationary phase, down-regulation of Dps expression might be explained by the functions of Fis in exponentially growing cells. On the contrary, the repression by H-NS can be overcome by binding of σ^{38} that also recognizes *dps* promoter. These results also suggest mechanisms by which Dps-dependent condensation of bacterial chromosome occurs in the course of cell growth from exponentially growing phase to stationary phase.[16, 39]

Accumulation of Dps during stationary phase was suggested to be due to the post-transcriptional help of ClpAP: ClpAP is an ATP-dependent protease with molecular chaperon activity, and it increased Dps levels through indirectly maintaining the ongoing translation of *dps* mRNA in the prolonged stationary phase, although it did not digest Dps protein in this period.[40] Another effect of ClpAP on translation of Dps was suggested to be due to proteolytic degradation of translational repressor of Dps during starvation of the cells, which resulted in accumulation of Dps.

In addition to the translational regulation, SEp22/Dps levels are post-translationally regulated by proteolysis. During re-incubation in fresh LB medium of SE which were pre-cultured overnight to the stationary phase, SEp22 decreased rapidly with time and reached a non-detectable level at the exponentially growing phase.[10] This decrease of SEp22 was dependent on cell density, and incubation time and temperature, and was sensitive to sodium azide (data not shown).[10] These results imply the presence of energy-dependent proteolysis of SEp22 during the exponential growth phase. Similar results were reported concerning Dps degradation: Dps was shown to be degraded directly by ClpXP and ClpAP during exponential growth,[41] and ClpXP-mediated degradation of Dps occurred upon re-entry

into the logarithmic phase.[40] Recent studies have revealed that Dps was a target of N-end rule degradation in *E. coli*,[40] and that ClpS played a role as an adapter protein for ClpAP-mediated degradation of Dps.[42] The N-rule degradation pathway, a major pathway of protein degradation in *E. coli*, requires the primary structure of target proteins at their *N*-terminal residues.[43] ClpS targets a truncated form of Dps (Dps6-167) and interacts directly with Leu6 at the *N*-terminal (Fig. 3) to recruit the protein for ClpAP protease.[41] On the other hand, ClpXP recognizes the *N*-terminal tail of Dps for degradation, and thus *N*-terminal cleavage of Dps decreases ClpXP-mediated turnover.[44]

Furthermore, recent studies by Hanna *et al.* showed the presence,[45] purification and cloning[46] of glycosylated form of Dps in *Sarmonella* Typhimurium (*S*. Typhimurium). It is a kind of post-translational modification and probably a new type of regulation of Dps, although the physiological meaning and importance of glycosylation of Dps remain largely unknown.

Taken together, Dps expression is regulated at the transcriptional, translational, and post-translational levels through a variety of regulators.[16]

SEp22/Dps AS A PATHOGENICITY-RELATED FACTOR

Salmonella is one example of intracellular parasitism that invades, survives and multiplies in the phagocytic vacuoles of macrophages, which allows this bacterium to persist in the host and sometimes to cause latent infections.[47, 48] In these processes of *Salmonella* infection, the mononuclear phagocyte systems, especially macrophages, plays major roles in exerting bactericidal activity toward *Salmonella* (Fig. 1). Among the effector molecules, ROIs show the central, and the strongest activity to kill the bacterium in the early stages of the infection. As SEp22/Dps is rapidly induced by exposure of H_2O_2,[9, 10, 16] it seems feasible to consider that SEp22/Dps plays some of the key roles in acquiring resistance to immune attack from the host. In fact, Halsey *et al.*[11] showed the oxidative stress resistance and virulence in mice was endowed to *S*. Typhimurium by Dps, showing that a *dps* gene deletion mutant clone of *Salmonella* was highly susceptible to H_2O_2, which was restored in the revertant clone with pBAD-*dps* transformation, and that the *dps* gene deletion mutant showed much reduced virulence to mice than the wild type *Salmonella* after intraperitoneal infection. Similar results were obtained in our laboratory with SE clones.[10]

Induction of *dps* gene expression in *S*. Typhimurium was examined after infection to the macrophages *in vitro*: Infection of wild type, virulent strain of *S*. Typhimurium to J774A.1 macrophage-like cells resulted in sudden elevation of *dps* gene expression at 2 hr after the infection, and a similar increase in *spv* genes, essential genes of virulence for establishment of a systemic infection of *Salmonella*, was also observed.[49] These results seem to suggest a very important response of *Salmonella* during infection into macrophages (Fig. 1): Dps is not an offense factor of *Salmonella* but rather a defense factor of the bacterium, as described in the previous sections, to protect *Salmonella* from harmful ROIs produced by macrophages and neutrophils. On the other hand, *spv* is an offense factor and is closely related to the virulent plasmid expression.[50] The expression of both the *dps* and *spv* genes, which is regulated under the control of RpoS (σ^S), seems to induce a co-ordinated response of *Salmonella* of the host, implying these genes are needed for survival and growth in macrophages. In this sense, Dps is a pathogenicity-related, or even pathogenic, factor, although it does not seem proper to call it a virulence factor.

Studies on the host factor concerning the induction of *dps* gene expression in *S*. Typhimurium during infection to macrophages were performed using Toll-like receptor 4 (TLR4)- and NADPH oxidase-deficient mice.[51] Compared with TLR4-deficient mice, bone marrow-derived macrophages in the presence of TLR4 resulted in the induction of *dps* gene in *S*. Typhimurium. In addition, the *dps* gene-deletion mutant of *S*. Typhimurium failed to increase the bacterial number to about 1/100 of the wild type clone at 3 days after administration of these bacteria to C57BL/6 mice. Similar results were obtained from the experiments using $gp91^{Phox}$-deficient mice, *i.e.*, NADPH oxidase-deficient mice, and wild type as well as *dps* gene-deletion mutant of *S*. Typhimurium. These results show that intracellular survival of *S*. Typhimurium in normal macrophages requires Dps expression in the bacterium, and suggest that a TLR4-signaling cascade(s) links NADPH oxidase activity in macrophages to induce fully bactericidal activity.

REGULATORY ROLES OF SEp22/Dps IN ENVIRONMENTAL STRESS-RESISTANCE OF *Salmonella*

Although extensive studies have revealed that Dps not only functions as an iron-binding ferritin-like protein,[17] but also works as a DNA-binding protein,[16] or more accurately, a DNA-guard protein. Because iron is an essential metal for most of cells in both prokaryotic and eukaryotic organisms, it is very important for these cells to manage the various effects of redox changes of iron and of the resultant ROIs.[16] One of the most remarkable changes that cause Dps expression is nutritional deprivation and oxidative stress, as described in the previous section. However, it remains largely unknown whether Dps has a pleiotropic effect in the regulation of gene and/or protein expression during times of starvation or environmental stresses such as oxidative stresses.

To address this question, we analyzed environmental isolates of SE, with various levels of SEp22/Dps, and examined whether these clones are pathogenic, or resistant to H_2O_2, heat-shock, ultraviolet (UV)-irradiation, or other chemical stresses. We found dry-resistance was closely linked to the SEp22/Dps level,[52] showing that the clones expressing high levels of SEp22/Dps were resistant to the drying protocol[53] but those with little or extremely low levels were sensitive to the dry-stress (Fig. 8). In addition, all of the *sep22*-deletion mutants that originated from the wild type, virulent strain, showed high susceptibility toward the dry-stress (Fig. 9). Induction of SEp22 expression during the drying procedure in the wild type strain also supported the importance and involvement of SEp22 in the acquisition of the dry-resistance.[52] Dry-resistance is one of the remarkable characteristics of *Salmonella* in an environment, and is recognized as an important point for food-safety control.[54] It is interesting that lactoferrin, which is known as an iron-binding protein in mammals,[55] functions not only as an iron-supplier but also as a bio-protective material against infection, and shows strong inhibitory effects toward the acquisition of dry-resistance by various *Salmonella* species.[56] Although the mechanisms underlying the inhibitory effects of lactoferrin remain largely unknown, it may be due to the iron-binding property of lactoferrin that competes with the iron-capturing activity of SEp22/Dps.[12]

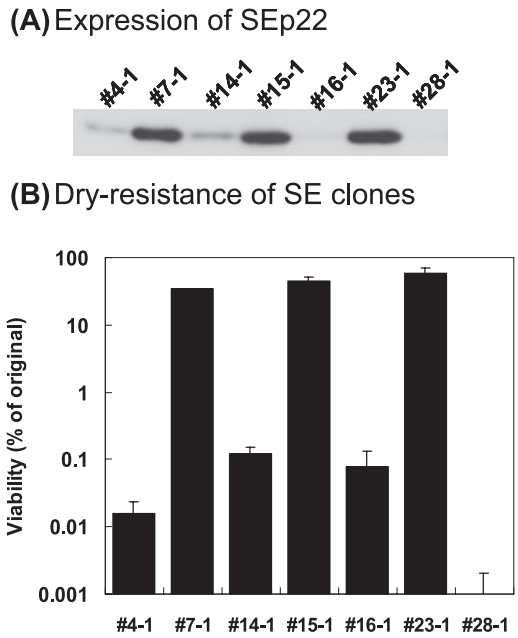


Fig. 8. Dry-resistance of SE Clones with Varied Expression Levels of SEp22/Dps[52]

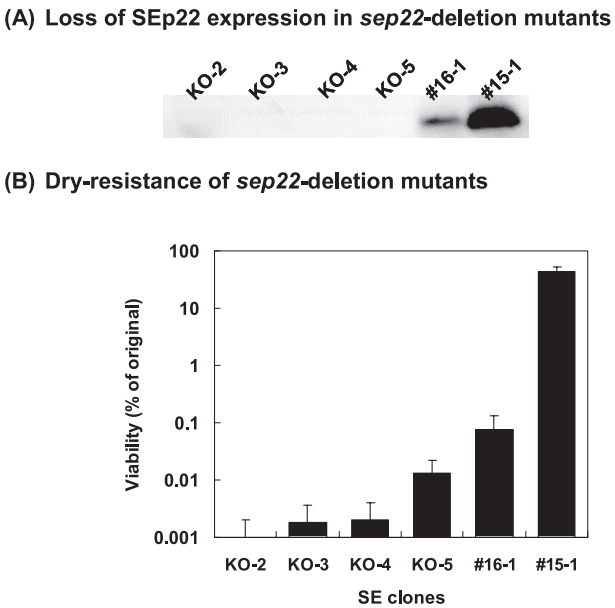


Fig. 9. Dry-resistance of *sep22*-deletion Mutants[52]

Another way to determine the regulatory roles of Dps in the stress responses is to investigate changes in protein expression patterns, showing a dramatic difference in the proteomes of the parental and *dps*-deleted mutant strains, and also to examine the pleiotropic phenotype of mutants lacking a functional *dps* gene, as discussed by Calhoun and Kwon.[16] Dps has a DNA-binding property, although the sequence-specificity of DNA has not

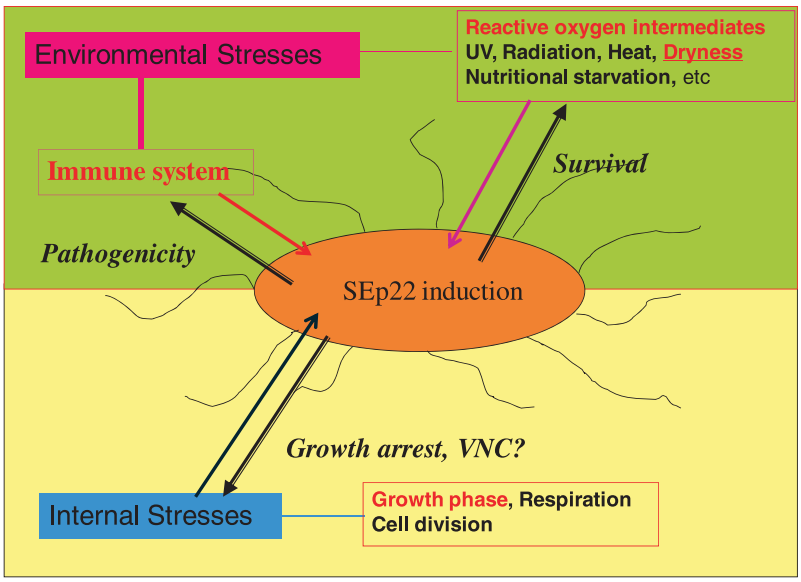


Fig. 10. SEp22/Dps Induction by Internal and Environmental Stresses in *Salmonella*

been reported.[9] However, the binding of Dps to DNA should have some effect on cell functions and gene expression. Proteome analysis seems to provide good clues for understanding the regulatory roles of Dps, using the sets of parental and mutant strains grown under various conditions, such as exponentially growing and stationary phases,[9] or stress-treated and non-treated states.[57] In addition, DNA microarray analysis seems to be useful to monitor changes at the mRNA levels between wild type and *dps*-deletion mutants under the various conditions as described above. Anyway, if the useful clue, either protein or gene, becomes apparent, possible targets of regulation will be identified, leading to a breakthrough in future studies of SEp22/Dps in biology and pathology, as well as application of the findings to microbial regulation in many fields including food-safety, infection control, and agriculture.[58]

However, it is possible, as pointed out by Calhoun and Kwon,[16] that Dps does not have any special role in the pleiotropic regulation of other genes or phenotypes, but instead merely binds and environs DNA at the stationary phase or under starved conditions for DNA protection and survival.[15, 30, 59, 60] It is worth conducting studies on Dps to determine the regulatory role of Dps, because Dps may be considered as a histone-like protein showing many similarities with the eukaryotic histone.[61, 62] Histones are well known not as a still player surrounding DNA but as an active molecule that undergoes covalent modifications in response to cell behavior, and also make DNA accessible or inaccessible to transcriptional machinery, finally leading to regulation of gene expression.[63]

SEp22/Dps AS A COMMON REGULATOR OF *Salmonella* SURVIVAL IN THE NATURAL ENVIRONMENT AND INFECTION: CONCLUDING REMARKS AND FUTURE PERSPECTIVES

SEp22 was initially discovered as a pathogenicity-related protein of SE, and was then assigned as *Salmonella* Dps.[10] As shown in this review and others,[16, 17] Dps is a protein normally associated with stress resistance during stationary phase, as originally found and described by Almiron *et al.*[9] However, Dps is also recognized as a H_2O_2-resistant protein during exposure to oxidative stress in the exponential growth phase.[35] Because expression of SEp22/Dps is regulated primarily at the transcriptional level through OxyR regulon and σ^S, it seems rational toassume that there are common pathways and regulators for the induction of Dps under different culture conditions and exposure to stresses which activate these transcriptional factors.

As shown in Fig. 10, *Salmonella* and other bacteria exist under stressful conditions, both environmentally and internally: environmental stresses include ROIs, UV, radiation, heat, dryness, nutritional

starvation, and other stresses such as chemicals and antibiotics. During infection of a host, effector molecules from the host immune phagocyte system, such as ROIs, acidic pH in phagolysosomes, lysosomal acid hydrolases, and lysozymes (Fig. 1), also become stresses for the invading pathogens. *Salmonella* copes with most of these stresses from the environment by inducing SEp22/Dps, which protects DNA not only by detoxifying iron by chelation and subsequent trapping ferroxide in the center of Dps to avoid hydroxyl radical formation, but also binding and sequestration of DNA to stabilize it.[16] On the other hand, SEp22/Dps is also induced by internal, *i.e.*, intracellular stresses, including the growth phase, respiration as well as cell division, which have close relationships to oxygen radical production or chromosomal rearrangement. Because Dps is a major DNA-binding protein in stationary phase cells and also in stress-exposed cells, the expression of Dps is under σ^S control. As described in the previous section, down-regulation of Dps expression by H-NS and Fis is closely linked to the growth phases.

It is interesting to summarize the circumstances surrounding the bacterium in Fig. 10 with regard to SEp22/Dps, because both environmental stresses and internal stresses are factors common to the induction of (sometimes the reduction of) SEp22/Dps, and because SEp22/Dps combats these stresses in order to survive and grow. In this sense, SEp22/Dps is a key molecule that plays essential roles in both the environmental stress-resistance and pathogenicity of *Salmonella*. Our recent study suggests that accumulation of dry-resistant *Salmonella* clones in poultry farms is accompanied increase in SEp22 expression in these cells through repeated dry procedure. Furthermore, maintaining viability in the VNC (viable but non-culturable) state of *Salmonella*, obtained during prolonged incubation in M9 minimal medium, is also linked to SEp22 expression. Future studies on SEp22/Dps should examine the molecular information of SEp22/Dps and the phenotypes of *sep22*/*dps* gene-deletion mutants, and perform careful observation and detailed analysis of natural environmental isolates with various levels of SEp22/Dps proteins.

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