Expression and Degradation of SEp22, a Pathogenicity-Related Protein of *Salmonella* Dps, in *Salmonella enterica* serovar Enteritidis Isolated from the Poultry Farms in Japan

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We isolated and characterized a pathogenicity-related protein in *Salmonella enterica* serovar Enteritidis (SE) from poultry farms, and designated it as SEp22, which has recently been identified with *Salmonella* Dps, a DNA-binding protein (12, 13, Amano *et al.*, manuscript in preparation). Expression of SEp22 was shown to be transcriptionally regulated, because another SE strain without virulence was found to possess a full-length non-mutated gene of *sep22*, but had little expression of SEp22 mRNA and much lower levels of SEp22 protein compared to the virulent standard strain SE Cl#15-1. Besides, expression of SEp22 was connected with bacterial growth, showing reduced expression in the logarithmic phase but increased expression from the late logarithmic to stationary phases. These changes were slightly later than those in σ^{38} levels as well as SEp22 mRNA, suggesting that expression of SEp22 is under transcriptional control through RNA polymerase activity by σ^{38} . In addition, high levels of SEp22 in the stationary phase were rapidly reduced upon incubation of the bacteria in fresh medium. This reduction was dependent on bacterial concentration in the culture, temperature, and time of incubation, suggesting proteolytic degradation of SEp22 in growing bacteria.

Key words: *Salmonella enterica* serovar Enteritidis; Dps; growth phase; pathogenicity; transcriptional control

INTRODUCTION

Salmonella enterica serovar Enteritidis (SE) has been one of the most causative bacteria of food-borne diseases in Japan. Poultry is an important source for spreading SE, not only thorough food but also through environmental contamination (2). Epidemiological studies have revealed that the major vehicles of SE outbreaks in humans have included contaminated poultry meat and eggs (5). In our previous studies searching for factors and mechanisms underlying acquisition by environmental SE of pathogenicity to the mouse, we identified and characterized a pathogenicity-related protein of MW, of approximately 22 kDa, in SE isolated from poultry farms and designated it as SEp22. We analyzed 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 168 amino acids. The *sep22* gene was shown to be similar to *E. coli* *dps/pexB*, and subsequently to be identical to *Salmonella* *dps* (7). We also constructed *sep22*-defective *Salmonella*

mutants and examined the correlation between expression of SEp22 and pathogenicity to BALB/c mice using these mutants together with the wild-type parental strain (12, 13, Amano *et al.*, manuscript in preparation). In the course of our study on SEp22, Halsey *et al.* reported the involvement of *Salmonella* Dps in the pathogenicity of *Salmonella* infection in a gene-targeting study (7). However, regulation of SEp22 expression especially with respect to virulence of environmental isolates remains largely unknown. In this study, we show that the assessment of pathogenicity of *Salmonella* requires analysis of SEp22 at mRNA or protein level, because analysis at the gene level is insufficient and expression of SEp22 is transcriptionally regulated. Moreover, we show that SEp22 levels are regulated by bacterial growth not only in induction of the transcription and translation through the logarithmic to stationary phases, but also in the breakdown of the SEp22 protein in fresh medium, where it depends on bacterial concentration, and incubation time and temperature under growing conditions in the early logarithmic phase.

MATERIAL AND METHODS

Bacterial culture

Salmonella enterica serovar Enteritidis strains SE

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Cl#15-1 and SE Cl#16-1, obtained from CAF Laboratories (Fukuyama, Hiroshima, Japan), were used in this study. The bacteria were cultured in 100 ml of Luria-Bertani (LB) medium (Difco) in 300-ml Erlenmeyer flasks at 37°C overnight, with shaking at 150 strokes min⁻¹. They were harvested by centrifugation at 3,500 rpm for 20 min (Tomy EX-125) at 4°C, and were regrown in 15 ml of fresh LB in 50-ml tubes at 37°C for 1 hr, with shaking as above. Incubation was terminated by chilling on ice, and the cells were harvested by centrifugation. The cells were washed twice with ice-cold phosphate-buffered saline (PBS), pH 7.5, and were suspended in PBS until use. For quantitative analysis of SEp22 by SDS-PAGE/western blotting, different sets of Erlenmeyer flask cultures were prepared for each time point to obtain sufficient numbers of bacteria; the initial volume and concentration of bacteria in each flask were equal among the cultures, but the bacteria in the logarithmic phase were harvested from 2–4 flasks for each time point, while those in the stationary phase, from one.

Preparation of bacterial cell extracts

The washed cells were disrupted with 0.1 g of glass beads (Sigma) in a cell beater (BIO 101 Savant, Fast Prep™ FP120) and centrifuged at 15,000 rpm for 5 min at 4°C. The resultant supernatant was collected as bacterial cell extract. Aliquots of the cell extracts containing the same amounts of protein were subjected to SDS-PAGE followed by western blot analysis. Protein concentrations of the cell extracts were determined with the Protein assay reagent (Bio-Rad) with bovine serum albumin as a standard.

SDS-PAGE/western blotting

SE extracts of the same protein amount were loaded on 5–20% polyacrylamide Slab gels (e-PAGEL™; ATTO, Tokyo), and electrophoresis was performed according to the discontinuous buffer system of Laemmli (9). After electrophoresis, the separated proteins were electrotransferred to PVDF membranes (Immobilon P™; Millipore), which were blocked with 30 mg ml⁻¹ milk casein (Snow Brand, Sapporo, Japan) in the rinse buffer (0.1% Triton X-100 in 10 mM Tris-HCl, 0.1 mM EDTA, 0.8% NaCl, pH 7.5), then reacted with a polyclonal anti-SEp22 antibody raised in rabbits, anti-σ³⁸ or anti-σ⁷⁰ antibody (generously donated by Dr. Akira Ishihama, The National Institute of Genetics) at 37°C for 2 hrs. The membrane was washed three times with the rinse buffer and incubated with HRP-conjugated anti-rabbit IgG (Cell Signaling) for 1 hr at room temperature. Finally,

immune complexes on the membrane were reacted with luminol and H₂O₂, according to the manufacture's protocol (Cell Signaling).

Quantitative analysis of SEp22

Quantitation of chemiluminescence associated with each protein was performed by a LAS1000 bioimage analyzer (Fuji Film) and the image was analyzed with Multi Gauge software (version 2.2; Fuji Film). For estimation of the levels of SEp22 in each cell extract, the quantitative determination was repeated at least three times with independent cell extracts from different experiments.

Sequence analysis of sep22 gene

To amplify the *sep22* gene with its putative promoter region from SE genomic DNAs, the following PCR primers were designed from the flanking sequence of the *dps* gene of *Salmonella enterica* serovar Typhimurium: 5'-ATGCAGAATATCACCTCGTT-3' as a forward primer and 5'-GAGGGGAGTACAAGACGTG-3' as a reverse primer. PCR conditions with *LA Taq* polymerase (TaKaRa) were 95°C for 2 min, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min, and a final extension at 72°C for 10 min. The PCR product of the expected size (972 bp) was subjected to direct sequencing (SciMedia; Tokyo) by using the same primers used in the PCR amplification and the following two additional primers: 5'-AGTTGCTTAATCGTCAGGT-3' and 5'-CTAACGCCACACCGCCAA-3'.

RNA extraction and northern blot analysis

Total RNA was isolated from SE cells at different time points of growth by resuspending them in TRIzol reagent (Invitrogen) and sonicating for 5 min at 200 W. It was then subjected to purification using the SV Total RNA Isolation System (Promega), incorporating DNase treatment. Total RNA samples (1.5 or 2 µg) were separated in a non-denaturing 1.3% agarose gel using DynaMaker RNA Easy Measurement N (BioDynamics Laboratory Inc., Tokyo). Transfer of separated RNAs to a positively charged nylon membrane (BrightStar-Plus; Ambion, Inc., Texas, USA) and hybridization with a probe were performed using the NorthernMax^R Kit (Ambion) according to the manufacturer's instructions. The DNA probe was prepared by PCR amplification of a 459 bp *dps* fragment using the primer set 5'-AAAACAAAAGCGTCTAATCT-3' and 5'-CCACAGGAATTTATCGAGGT-3', and labeled with Biotin (BrightStar^R Psoralen-Biotin; Ambion). The biotin-labeled probe was detected using the BrightStar^R

BioDetect™ Kit (Ambion) according to the manufacturer's instructions, and was visualized by exposure to X-ray film (Fujifilm). The intensity of the *dps* band was quantitated relative to that of the EtBr-stained 16S rRNA band in the gel before the blotting, using Scion image Beta 4.02.

RESULTS

Comparison of sep22 gene from different SE clones

We isolated a variety of SE strains from poultry farms that were either pathogenic or non-pathogenic to BALB/c mice. Among them, we showed that SEp22 (+) killed mice infected orally, while SEp22 (–) strains did not. We suggest that these differences in the virulence of SE strains is closely related to the expression of SEp22 protein (12, 13, Amano *et al.*, manuscript in preparation). In this study, we examined whether there was a difference at the gene level, comparing the sequences of *sep22* gene of SE Cl#15-1 as a SEp22 (+) strain and that of SE Cl#16-1 as a SEp22 (–) strain by PCR cloning. The strains have the same *sep22* gene structure in the coding sequences (data not shown), however, expression of SEp22 mRNA was greatly different between these two clones. The amount of mRNA in SE Cl#15-1 was more than 6.83 times higher than that in SE Cl#16-1, based on the northern blot analysis (Fig. 1). Similar results were obtained by semi-quantitative RT-PCR analyses of SEp22 mRNA and 16S rRNA, which showed that the ratio of SEp22 mRNA/ 16S rRNA in SE Cl#15-1 was 8.81 ± 0.57 times greater than that in SE Cl#16-1 (data not shown). These results suggest that expression of SEp22 is transcriptionally regulated.

Growth phase-dependent expression of SEp22

To examine the effects of the proliferation of the bacterium on the appearance of SEp22, we quantitated the amounts of SEp22 protein in *Salmonella* extracts, together with those of sigma factors (σ^{38} and σ^{70}) at different time points in the growth phases. SEp22 decreased soon after the culture in fresh medium started, followed by increase after the bacterial growth entered into the late logarithmic phase with the maximal expression in the stationary phase (Fig. 2 A, B). On the other hand, expression of σ^{38} also showed cyclic changes similar to SEp22, but always 1-2 hrs in advance of those in SEp22 (Fig. 2B). Unlike those of σ^{38} , σ^{70} levels remained unchanged throughout the culture (Fig. 2B). Northern blot analysis revealed that increase of SEp22 mRNA always preceded SEp22 protein increase by 1-2 hrs, but SEp22 mRNA began to decrease after the culture entered the stationary phase (Fig. 2C). These results

Fig. 1. Northern blot analysis of SE Cl#15-1 and SE Cl#16-1. Total RNA of each cell (2 μ g) was loaded in each lane, electrophoresed, and northern blot analysis was performed as described in the text. On the left, EtBr-stained 16S rRNA bands are shown, and on the right, northern blots of SEp22 mRNA are shown.

suggest that expression of SEp22 is transcriptionally induced in accordance with the progression of the growth phase to the stationary phase, probably through σ^{38} .

In addition, it should be noted that SEp22 decreased in the cells very rapidly when stationary phase bacteria were cultured in fresh medium, and it was observed at 2 and again at 26 hrs, two hours after bacteria were diluted and transferred to new medium (Fig. 2A). This was not the dilution of the bacterial cell extract loaded on the SDS-gels, but the actual disappearance of SEp22 from the cell extracts in the logarithmic phases, because the same amounts of the cell extracts were loaded, separated and analyzed by SDS-PAGE/western blotting. These results suggest that expression of SEp22 is down-regulated post-translationally probably through rapid digestion by protease(s).

Fig. 2. Growth phase-dependent changes of the expression of SEp22, σ^{38} and σ^{70} proteins and SEp22 mRNA in SE Cl#15-1. (A) Growth curve of SE Cl#15-1 in LB medium. Bacteria numbers are shown as CFU ml⁻¹ (●) and OD₅₅₀ (○). An overnight culture of SE Cl#15-1 in the stationary phase was diluted and the concentration at OD₅₅₀ was adjusted to 0.05 with fresh LB medium, followed by incubation of SE 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). The results are from a representative experiment of three independent ones, and are shown as means $\pm$ SD. (B) Expression of (a) SEp22, (b) σ^{38} and (c) σ^{70} proteins in SE Cl#15-1 at different time points in the growth phases are shown at the top of the figure as incubation times (hrs). Western blot analysis was performed with specific antibodies against SEp22, σ^{38} , or σ^{70} , following SDS-PAGE of the same amount of each bacterial cell extract, as described in the text. 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 μ g) 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.

Degradation of SEp22

As shown in Fig. 2B, SEp22 protein became undetectable in the first 2 hrs after the bacteria started growth in fresh medium, showing rapid degradation of

Fig. 3. Time course of SEp22 degradation in the regrowing cells. SE Cl#15-1 cultured overnight was diluted to OD₅₅₀ = 1.0 with fresh LB medium followed by incubation at 0, 25, 30 or 37°C for various times up to 60 min. The growth was stopped by chilling the bacteria on ice, followed by washing with ice-cold PBS and the bacterial cell extracts were prepared as described in the text. Analysis of SEp22 by SDS-PAGE and western blotting was performed as described in the legend to Fig. 2. The upper figure shows a representative result of western blot and the lower one, relative amounts of SEp22 protein analyzed from three independent experiments, shown as means $\pm$ SD, * P < 0.05 by Student's t -test, compared with the control at 0°C, at 0 time.

SEp22 took place in the stationary phase. We next focused on the degradation of SEp22 because the regulatory mechanisms of Dps degradation remain largely unknown, especially under physiological conditions. First we examined the time-course of SEp22 degradation. Stationary phase bacteria, cultured overnight, were adjusted a concentration showing OD₅₅₀ = 1.0 and recultured in fresh medium at various temperatures for 0–60 min (Fig. 3). The degradation of SEp22 at 37°C proceeded rapidly, within 10 min, while that at 4°C showed little change even after 60 min. Changing the incubation temperature to 30°C or 25°C resulted in slower degradation than that at 37°C, but SEp22 was still significantly decreased from the initial

Fig. 4. Effects of cell density in the culture on the degradation of SEp22. Experiments were performed as described in the legends to Figs. 2 and 3, except the conditions for the cell density (OD_{550}) of stationary SE for re-incubation varied from 0.5 to 4.0 OD_{550} at 37°C for 60 min. On the right side is the negative control incubated at 0°C for 60 min at $OD_{550}=1.0$. The upper figure shows a representative result of western blotting and the lower one, relative amounts of SEp22 protein analyzed from three independent experiments, shown as means $\pm$ SD, * $P \leq 0.05$ by Student's *t*-test, compared with the control at 0°C, at 0 time.

level with time. These results show that SEp22 is temperature-dependently degraded in fresh medium during incubation.

We next estimated the effect of cell density on the degradation of SEp22. When stationary phase bacteria showing $OD_{550} = 0.5, 1.0, 2.0$ or 4.0 were cultured in fresh medium at 37°C for 60 min, SEp22 was degraded almost entirely at $OD_{550} = 0.5$ and 1.0, while it remained in significant quantities at $OD_{550} = 2.0$ or 4.0 (Fig. 4). Under the conditions of incubation of SE grown to the stationary phase described in the legend of Fig. 2, SEp22 disappeared much more rapidly from the cells during incubation at $OD_{550} = 0.05$ than at $OD_{550} = 0.5$ or 1.0 (Figs. 3, 4; precise data not shown). Therefore, the degradation of SEp22 proceeds more rapidly in low density bacteria than in high density ones.

DISCUSSION

SE has been one of the most causative bacteria of food-borne diseases and the accurate detection of the pathogenic bacteria is important for the prevention of food poisoning. SE has several virulence factors whose detection has been dependent on genetic analyses like PCR (11, 14). For their detection, the *invA* gene and *spvC* gene have been most commonly used as markers (6). Dps is the DNA-binding protein from starved cells, and is one of the most abundant proteins in stationary-phase *Escherichia coli* (1, 4). We isolated and characterized SEp22 in SE from poultry farms, which had recently been identified with *Salmonella* Dps (Amano *et al.*, manuscript in preparation).

In this study, we showed that not only the SEp22 (+) strain but also the SEp22 (-) strain have the same *sep22* gene, and suggest that the expression of SEp22 through the growth phases was both transcriptionally regulated for induction and post-translationally regulated for reduction. The results of growth phase-dependent expression of SEp22 suggest coordinated expression of σ^{38} and SEp22 mRNA through regulation of RNA polymerase activity (Fig. 2). As for Dps in *E. coli*, its expression was shown to be regulated by the stationary-phase sigma-factor, RpoS (σ^{38}), and OxyR and IHF (4). As *E. coli* enters the stationary phase, more than 180,000 Dps molecules were reported to be accumulated within a single organism, making Dps the most abundant protein in the cell (1, 3, 4, 10, 15). These reports seem to be in good agreement with our results for SEp22 in the present study. However, it is not known whether or not a threshold SEp22 amount determines the pathogenicity of *Salmonella* in the mouse. In our preliminary experiments, expression of very low amounts of SEp22 protein in environmental isolates including SE Cl#16-1 (Fig. 1) failed to show pathogenicity to mouse, while wild-type, SEp22 (+) strain including SE Cl#15-1 (Fig. 1) showed very strong virulence. We performed the experiments using *sep22*-defective mutants originated from SE Cl#15-1, a pathogenic parental strain, and showed that SEp22 expression is closely correlated with pathogenicity of *Salmonella* to mice (12, 13, Amano *et al.*, manuscript in preparation). Further studies are in progress with revertants to one of the *sep22*-defective mutants to examine whether or not higher or moderate expression of SEp22 protein might recover the pathogenicity.

The results shown in Figs. 3 and 4 imply the degradation of SEp22 is regulated by some kind of cellular metabolism in fresh medium, because energy

metabolism in the bacteria is active when cultured at low cell density due to good aeration, while it becomes reduced in bacteria cultured at high cell density.

The presence of Dps homologs in a wide variety of microorganisms, though with molecular divergence, suggests their importance for microorganisms' survival under many kinds of stressful environments. In most organisms Dps serves as an iron-binding protein and protects the organism from oxygen stresses (7, 8, 10, 15). In addition, Halsey *et al.* reported the contribution of *dps* to *Salmonella* stress resistance, survival in macrophages, and virulence in mice (7). Previously, we reported that the expression of SEp22 protein was closely related to the virulence of SE (12, 13). In this study, we showed that the expression of SEp22 protein was transcriptionally regulated probably through σ^{38} and was regulated by growth phases (Figs. 1, 2), and that the degradation of SEp22 was influenced by temperature and cell-density (Figs. 3, 4), suggesting the requirement of energy metabolism in the degradation of SEp22.

The results of the present study might provide some clues for clarifying the relationship between the regulatory mechanisms underlying the pathogenicity of *Salmonella* to the host and those underlying the survival and growth of *Salmonella* in various environments through SEp22 expression. Further studies are now in progress for improving the immunological detection methods for a rapid and sensitive assay of SEp22 which would help in the prevention of *Salmonella* food poisoning.

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