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ARTICLE

Inducible *spy* Transcription Acts as a Sensor for Envelope Stress of *Salmonella typhimurium*

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Abstract

Salmonella enterica infects a broad range of host animals, and zoonostic infection threatens both public health and the livestock and meat processing industries. Many antimicrobials have been developed to target *Salmonella* envelope that performs essential bacterial functions; however, there are very few analytical methods that can be used to validate the efficacy of these antimicrobials. In this study, to develop a potential biosensor for *Salmonella* envelope stress, we examined the transcription of the *S. enterica* serovar *typhimurium spy* gene, the ortholog of which in *Escherichia coli* encodes Spy (spheroplast protein y). Spy is a chaperone protein expressed and localized in the periplasm of *E. coli* during spheroplast formation, or by exposure to protein denaturing conditions. *spy* expression in *S. typhimurium* was examined by constructing a *spy-gfp* transcriptional fusion. *S. typhimurium spy* transcription was strongly induced during spheroplast formation, and also when exposed to membrane-disrupting agents, including ethanol and the antimicrobial peptide polymyxin B. Moreover, *spy* induction required the activity of regulator proteins BaeR and CpxR, which are part of the major envelope stress response systems BaeS/BaeR and CpxA/CpxR, respectively. Results suggest that monitoring *spy* transcription may be useful to determine whether a molecule particularly cause envelope stress in *Salmonella*.

Keywords envelope stress, extracytoplasmic chaperone, *Salmonella*, Spy

Introduction

The species *Salmonella enterica* comprises more than 2,500 serovars, and causes one of the most frequently-reported foodborne disease, salmonellosis. *S. enterica* infects numerous animal hosts, and is considered as a significant public health threat worldwide. The severity of salmonellosis ranges from mild gastroenteritis to life-threatening typhoid, and depends on the serovar type and the species of infected host animal. Although many nontyphoidal serovars are only associated with relatively minor symptoms, they are still responsible for the largest burden of food borne disease in the world (Crump *et al*., 2015; Kirk *et al*., 2015). In addition, *S. enterica* infects a wide range of host mammals, causing significant zoonoses, which is particularly damaging to the livestock and meat processing industries (L Plym and Wierup, 2006). To control salmonellosis, a variety of antibiotics and chemicals have been widely used in veterinary medicine and in interventions for food processing. However, the emergence of multidrug-resistant *S. enterica* strains has

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increased levels of drug tolerance, and necessitates the development of new antimicrobials (Doyle, 2015; Wales *et al*., 2010). Many antimicrobial drugs or chemical preservatives target the *Salmonella* cell envelope, the disruption of which efficiently abolishes numerous essential functions. Despite the availability of these drugs, analytical methods validating whether a given chemical can cause damage to the *Salmonella* envelope are still lacking (Wolf and Mascher, 2016).

In this study, we examined whether expression of the *S. enterica* serovar *typhimurium spy* gene could be used as a biosensor for measuring envelope stress. Spy was originally reported to be exclusively expressed in the periplasmic space of *Escherichia coli* during spheroplast formation, and was therefore named spheroplast protein y (Hagenmaier *et al*., 1997). Further studies with *E. coli* revealed that Spy functions as a periplasmic chaperone with high flexibility and its expression is induced when cells are exposed to envelope stresses that result in protein misfolding, such as ethanol, indole, copper, zinc, and spheroplasting (Bury-Mone *et al*., 2009; Quan *et al*., 2011; Raffa and Raivio, 2002; Wang and Fierke, 2013). Indole treatment also increases *spy* transcription in *S. typhimurium* (Appia-Ayme *et al*., 2012). These studies strongly suggest that Spy is involved in maintaining protein homeostasis in the periplasmic space in response to various envelope stress conditions, and that measuring the induction of *spy* transcription may be useful for monitoring envelope stress in bacteria.

In this study, we investigated *spy* transcription in *S.* Typhimurium under several envelope stresses, and examined the effect of regulators for three major envelope stress signal transduction systems, RpoE, Cpx, and Bae, on *spy* transcription.

Materials and Methods

Bacterial strains and culture conditions

Wild-type (WT) parental strain, *S. typhimurium* 14028S, and its isogenic gene mutant strains were used. Luria–Bertani (LB) broth (Difco, USA) was used for bacterial culture. For broth culture, strains were cultured in LB broth at 37°C with shaking. To construct gene knockout mutants, the polymerase chain reaction (PCR)-mediated one-step gene mutation using the λ Red recombinase was used, as described previously (Datsenko and Wanner, 2000). *baeR* and *cpxR* deletion mutant strains were constructed using the following primer pairs: (*baeR*) F: 5'-aacacgccgc gcattttgat tgttgaagat gaacccaagc gtgtaggctg gagctgcttc-3' and R: 5'-caccccgtag accgcgcgta taaatgactg ttcggcatcc catatgaata tcctccttag-3'; and (*cpxR*) F: 5'-gttagt tgat gatgaccgag agctgacttc cctgttaaaa catatgaata tcctcct-tag-3' and R: 5'-aaaccacggg tgaccgtctt tgcgttccgg cagtttg-cgggtgtaggctg gagctgcttc-3'. Primer pairs, F: 5'-cgtcaatatt gtacacgcgc-3' and R: 5'-acagaaacgt catctcacc-3' (*baeR*), and F: 5'-gatgtgttgc cgtcaacgta-3' and R: 5'-actggctgga gctcttact-3' (*cpxR*) were used for PCR confirmation. Final selection of mutants was performed as described previously (Park *et al*., 2015).

Construction of *spy-gfp* transcriptional fusion and measurement of green fluorescence protein (GFP) fluoresence

To construct *spy* transcriptional fusion, we employed a promoter probe vector plasmid pFPV25, which contains a GFP reporter gene (gfp_{mut3}) reporter gene (gift from Raphael Valdivia) (Valdivia and Falkow, 1996). A DNA fragment containing the *spy* promoter region was amplified from *S. typhimurium* chromosomal DNA by PCR using Pfu polymerase (iNtRON Biotech., Korea) with primer pairs F: 5'-tatgagctct gctatatcat gctgttgta-3' and R: 5'-tat-ggatcca ggacggctat agaattctctg-3'. The purified PCR products were digested with SacI and BamHI (NEB, USA), and then ligated with SacI/BamHI-digested pFPV25. A single clone was finally selected following DNA sequence confirmation of insert region in pFPV25. *S. typhimurium* harboring the *spy-gfp* fusion plasmid was cultured overnight and then diluted 1:1000 in fresh LB medium. The culture was grown to log phase (OD_{600}~ 0.4), and then cells were treated with 4% ethanol or polymyxin B (1 ug/mL) for 1 h. After washing with phosphate buffered saline, the fluorescence intensity of bacterial cells (adjusted to OD_{600} = 1.0) in a black 96 well plate (SPL, Korea) was measured with excitation at 485 nm and emission at 535 nm using a DTX 880 microplate reader (Beckman Coulter, USA).

Salmonella spheroplast formation

Method used for spheroplast formation was modified from a previously described strategy (Birdsell and Cota-Robles, 1967). Briefly, overnight cultures of WT *S. typhimurium* were diluted 1:4 into fresh LB broth and cultured to mid-log phase (OD_{600}~ 0.8). Cells were collected by centrifugation (5,000 rpm for 20 min at room temperature) were washed once with 10 mM Tris buffer (pH 8.0). Following centrifugation as above, the cell pellet was re-

suspended with 10 mM Tris buffer (pH 8.0) containing sucrose (0.5 M) and incubated for 10 min before the addition of lysozyme (200 μg/mL). The cells were incubated for 10 min, and then an equal volume of Tris buffer containing EDTA (20 mM) was added and the cells were incubated at 37°C for a further 4 h.

Fluorescence microscopy

Salmonella cells harboring *spy-gfp* fusion for microscopic observation were washed once with 10 mM Tris buffer (pH 8.0) before analysis. Cell aliquots were dropped onto microscope slides and coated with 1% agarose, and then analyzed using a Zeiss Axio A1 microscope (Zeiss, Germany) with an oil-immersion 100X objective lens. Images were captured with a charge coupled device camera.

Results and Discussion

spy transcription is strongly induced in *S. typhimurium* upon spheroplast formation

To examine changes in *spy* transcription in *S.* Typhimurium during spheroplast formation, we constructed a *spy-gfp* transcriptional fusion plasmid. This type of operon fusion can be useful for monitoring Spy expression because GFP protein expressed by the *spy* promoter remains in cytoplasm of spheroplast cells. Natural Spy protein is exclusively expressed in periplasmic space, without any membrane association, and can diffuse out of the cell when outer membrane of spheroplasts were osmotically lysed during spheroplasting (Birdsell and Cota-Robles, 1967; Hagenmaier *et al*., 1997). To determine whether *spy* transcription was induced during spheroplast formation in *S. typhimurium*, the morphology and fluorescence intensity of *spy-gfp* containing cells were examined by fluorescence microscopy. Fig. 1(A) clearly shows that WT *S.* Typhimurium spheroplasts were strongly fluorescent. The method used for spheroplast formation in this study essentially involves sequential exposure to high osmotic pressure induced by sucrose, peptidoglycan lysis by lysozyme, and then membrane weakening by EDTA. Of note, cells with rounded morphology were conspicuously fluorescence after all processes for spheroplast formation were completed. Given the fact that removal of cell wall can create rounded spheroplasts, *spy* transcription in *S. typhimurium* is likely triggered by changes in membrane rigidity. While *E. coli* can form spheroplasts without the addition of EDTA (Birdsell and Cota-Robles, 1967), we did not observe rounded spheroplasts and *spy-gfp* expression in the absence of EDTA. Although it is not known whether induction of *spy* transcription in *E. coli* spheroplasts requires EDTA treatment in addition to sucrose and lysozyme, our result suggests that there may be differ-

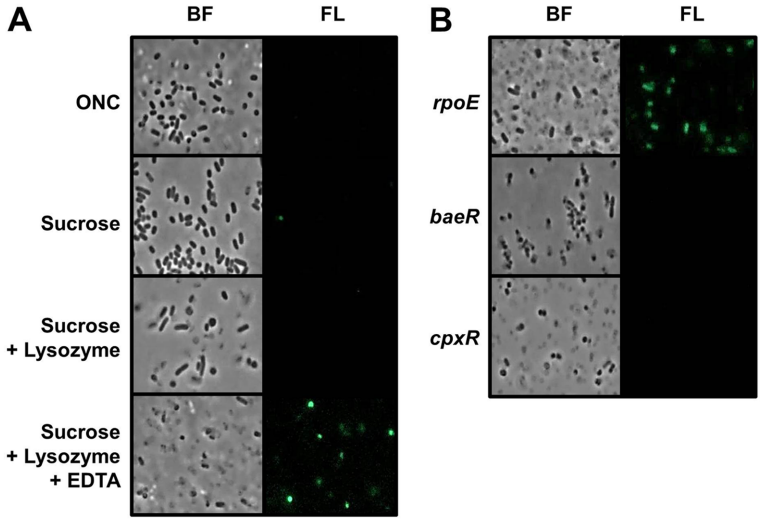

Fig. 1. Induction of *spy-gfp* transcription during spheroplast formation in *S. typhimurium*. (A) WT *S. typhimurium* harboring the *spy-gfp* fusion was visualized at each stage for spheroplast formation, including during cumulative treatments with sucrose, lysozyme, and EDTA, as described in the Materials and Methods. Notably, to normalize the incubation time for all samples taken thrughout the 4-h process, all cells at each stage of spheroplast formation were partly isolated and incubated for 4 h prior to microscopy. (B) *rpoE*, *baeR*, and *cpxR* mutant *S. typhimurium* harboring the *spy-gfp* fusion were visualized after completion of spheroplasting process. All paired images for bright field (BF) and fluorescence (FL) microscopy were taken from the same samples. Data are representative of at least three independent experiments.

ences in the envelope architecture of two species.

Three typical envelope stress response systems have been identified in *E. coli* and *Salmonella* species (Rowley *et al*., 2006). The alternative sigma factor, RpoE, directs transcription of many genes involved in envelope homeostasis, and two other envelope response systems, Bae and Cpx, both comprising a membrane histidine kinase and a cognate response regulator protein, also promote transcription of specific sets of genes in response to envelope stress. To examine the roles of these systems in *spy* transcription, we introduced *spy-gfp* fusion plasmid into *S. typhimurium* mutants lacking *rpoE, baeR,* or *cpxR*. Fig. 1B shows effects of these mutations on *spy* transcription during spheroplasting. More spheroplast cells were fluorescent in *rpoE* mutant than in WT, whereas no fluorescent cells were detected in *baeR* and *cpxR* mutant strains. These results demonstrate that the induction of *spy* transcription in *S. typhimurium* spheroplasts requires Bae and Cpx systems, and that elimination of RpoE-derived envelope protection can increase the spheroplast population, inducing *spy* transcription.

Envelope stress caused by ethanol and polymyxin B also increases *S. typhimurium spy* transctiption in Bae- and Cpx-dependent manner

In *E. coli*, *spy* transcription is induced by exposure to membrane-disrupting agents, including indole and ethanol (Raffa and Raivio, 2002; Srivastava *et al*., 2014). To examine whether *spy* transcription in *S. typhimurium* is also induced under membrane stress conditions, *spy-gfp* fusion-containing *Salmonella* were exposed to 4% ethanol or the membrane-damaging antibiotic polymyxin B. As shown in Fig. 2, *S. typhimurium* treated with 4% ethanol or polymyxin B showed an approximately 2- or 3-fold increase in *spy* transcription, respectively, compared with that of untreated cells. In microscopic analysis, the ethanol- or polymyxin B- treated cells mostly showed strong fluorescence, while untreated cells did not (data not shown). As was observed for spheroplasting, *spy* transcription was enhanced in *rpoE* mutant under both envelope stress conditions, confirming that *spy* transcription can be increased in the absence of the envelope protection systems. Again, mutations in *baeR* and *cpxR* abolished the induction of *spy* transcription in ethanol- and polymyxin B- treated *S. typhimurium*. As was observed in *E. coli* (Srivastava *et al*., 2014), *spy* transcription in *S. typhimurium* spheroplasts and in cells exposed to 4% ethanol required both the BaeR and CpxR regulators. Both regulators were also required for induction of *spy* transcription in polymyxin B-treated cells. Thus, regulatory elements for *spy* gene transcription appear to be conserved between the two species.

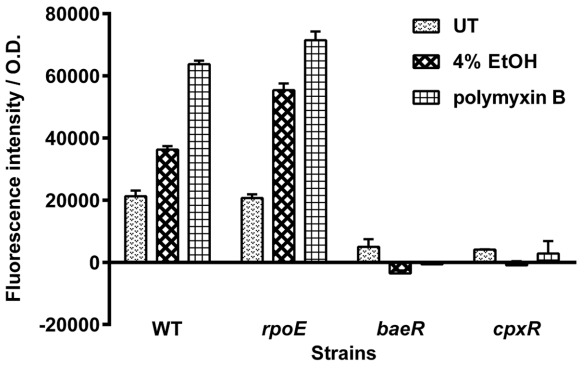

Fig. 2. Induction of *spy-gfp* transcription in *S. typhimurium* by exposure to ethanol and polymyxin B. Fluorescence intensity of *S. typhimurium* harboring the *spy-gfp* fusion was measured following treatment with 4% ethanol or polymyxin B (1 μg/mL) for 1 h. An untreated control (UT) was also included. Data shown are the means±standard deviation from three independent cultures.

In conclusion, this study has clearly demonstrated for the first time that *spy* transcription can be induced in *S. typhimurium* by exposure to envelope stresses, including ethanol, polymyxin B, and spheroplasting, and is dependent on the envelope stress response regulators BaeR and CpxR. Homology analysis showed that the *spy* genes are conserved amongst all serovars of *S. enterica,* including pathogens causing zoonosis (data not shown). Spy, which functions as a broad-spectrum chaperone, is exclusively expressed in the periplasmic space under envelope stress conditions, which are sensed by two major envelope response systems. Therefore, it is plausible that *Salmonella* strains harboring the *spy-gfp* fusion constructed in this study could be used as a whole-cell biosensor for screening the efficacy of new antimicrobials that specifically target the *Salmonella* cell envelope.

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