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Original Article

Characterization of *stm3030* and *stm3031* genes of *Salmonella enterica* serovar Typhimurium in relation to cephalosporin resistance

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Abstract *Background/purpose:* The outer membrane protein STM3031 had been shown to confer *Salmonella enterica* serovar Typhimurium resistance to ceftriaxone. In this study, the STM3030 was increased in strain R200 and decreased in strain R200(Δ*stm3031*). How *stm3030* and *stm3031* contributing to antibiotic resistance was investigated.
Methods: The level of STM3030 protein in R200(Δ*stm3031*) were compared between 01–4, R200, and R200(Δ*stm3031*) by 2-DE analysis. The *stm3030* gene deleted strain, R200(Δ*stm3030*), was generated by the one-step inactivation chromosome gene method. The various antibiotic susceptibility of strains 01–4, R200, R200(Δ*stm3031*) and R200(Δ*stm3030*) were determined by agar dilutions assays and E-test. The co-transcription of *stm3031* and *stm3030* were determined by RT-PCR. The promoter activities of these two genes fused with LacZ were determined. The binding of the regulatory protein BaeR on the promoter of both genes was detected by EMSA. The interaction between STM3030 and STM3031 proteins was determined by GST pull-down assay.
Results: Strain R200(Δ*stm3030*) displayed a 32- to 64-fold reduction in resistance to cephalosporin drugs. Transcription analyses revealed that *stm3030* and *stm3031* are independent genes and that the promoter of *stm3030* is stronger than that of *stm3031*. The regulator BaeR binds to the promoter region of *stm3031* but not that of *stm3030*. The STM3031 decreased in R200(Δstm*3030*) compared to R200 by western blot analysis. The pull-down assay revealed that STM3030 and STM3031 bind to each other.
Conclusion: Our data indicate that STM3030 has a chaperone-like activity and may modulate or stabilize STM3031, leading to resistance of *S. enterica* serovar Typhimurium to cephalosporin drugs.

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Introduction

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a leading cause of foodborne illness in humans. Over the last 10 years, *S.* Typhimurium isolates that are resistant to fluoroquinolones and third-generation cephalosporins have been reported in Taiwan.[1–3] Resistance of *S.* Typhimurium to these drugs is mainly due to the production of plasmid-encoded AmpC β-lactamases.[4,5]

Several two-component systems (TCSs) have been identified in bacteria. These systems contribute to envelope stress response, metal resistance, and drug resistance of *Escherichia coli* and *Salmonella*.[6] Among them, the TCS BaeSR responds to a wide range of environmental stresses such as spheroplasting and exposure to indole, tannin, zinc, or cooper.[7–9] It also mediates drug resistance by regulating the expression of genes encoding drug transporters in *Salmonella*.[10] In a previous study, the outer membrane protein STM3031 (Ail/OmpX-like) was determined to be involved in ceftriaxone resistance, and its production in *S.* Typhimurium was found to be regulated by BaeR.[11] The finding that deletion of the *stm3031* gene results in a >64-fold reduction in resistance to ceftriaxone indicates the role of STM3031 in ceftriaxone resistance.[12]

In this study, the STM3030 was increased in strain R200 and decreased in strain R200(Δ*stm3031*). In addition, *stm3030* is located only 46 bp downstream of *stm3031*, we investigated whether these two genes are co-transcribed and whether the mRNA of *stm3030* is co-regulated with *stm3031*. The binding of the regulator BaeR to the promoters of both genes and the role of STM3030 in antibiotic resistance were also examined.

Methods

Bacterial strains and culture conditions

S. Typhimurium strain 01-4 is a clinical isolate from Taiwan and is susceptible to ceftriaxone. *S.* Typhimurium strain R200 is a ceftriaxone-resistant strain derived from strain 01-4 by multistep resistance selection.[13] Mutant strains 7F2,[11] R200(Δ*stm3031*),[12] and R200(Δ*stm3030*) were generated from strain R200. *E. coli* JM109 and DH5α were used for cloning and expression. Cells of *S.* Typhimurium and *E. coli* were grown at 37 °C in Luria–Bertani broth or tryptic soy broth (Difco) containing appropriate antibiotics.

Construction of mutant R200(Δ*stm3030*)

The one-step inactivation of chromosomal genes was carried out as described previously.[14] To generate *S.* Typhimurium R200(Δ*stm3030*), strain R200, which carries the lambda Red recombinase expression plasmid pKD46-apra, was mutated, and mutants were selected on LB agar plates containing apramycin.[15] In brief, a DNA fragment containing the first 100 bp of the 5′ end of *stm3030*, the kanamycin resistance gene, and the last 100 bp of the 3’ end of *stm3030* was amplified by PCR from pKD4.[14] The amplified PCR product was introduced into strain R200/pKD46-apra by electroporation, and transformants were selected on LB agar plates containing kanamycin. The Δ*stm3030* mutation in R200 was verified by PCR and further confirmed by DNA sequencing.

Antibiotic susceptibility testing

The MICs of a number of antibiotics were determined by the agar dilution method as recommended by the Clinical and Laboratory Standards Institute.[16] The following antibiotics were tested: cephalothin (CEF), cefoxitin (CFT), ceftriaxone (CRO), meropenem (MEM), imipenem (IPM), tetracycline (TET), streptomycin (STR), gentamicin (GEM), chloramphenicol (CHL), novobiocin (NOV), erythromycin (ERY), nalidixic acid (NAL), and rifampin (RIF) (Sigma–Aldrich Corp., St. Louis, MO). Susceptibility of strains 01–4, R200, R200(Δ*stm3031*), and R200(Δ*stm3030*) to ceftriaxone was determined by the E-test (AB Biodisk Solna, Sweden).

Fractionation of outer membrane and periplasm proteins for two-dimensional gel electrophoresis (2-DE) and Western blotting

Periplasmic and outer membrane proteins of various *Salmonella* strains were fractionated according to the method of Gauthier et al.[17] Sample preparation, 2-DE, and Western blotting were carried out as previously described.[12] Proteins were identified by Q-TOF mass spectrometry. The outer membrane fraction was used for Western blotting with anti-STM3031 antibody, which was produced as described previously.[12]

RNA isolation and RT-PCR analysis

Total RNA of *S.* Typhimurium was isolated using the RNeasy Mini kit (Qiagen). All RNA samples were adjusted to 100 ng/μl and treated with RNase-free DNase I (New England Biolabs.). Each RNA sample was reverse transcribed to cDNA using the SuperScript III reverse transcription kit (Invitrogen). RT-PCR was performed in triplicate using the cDNA as templates with appropriate primers. The 16S rRNA of *S.* Typhimurium was also analyzed by RT-PCR in an identical manner to serve as the internal control.

Promoter fusion to *lacZ* and *β-galatosidase* assay (Miller assay)

DNA fragments of 324 bp (P-stm3031) and 170 bp (P-stm3030) containing the promoter regions of *stm3031* or *stm3030* genes, respectively, were amplified from total DNA of *S.* Typhimurium. Both fragments were digested with XbaI and HindIII and then cloned into plasmid pKM005, thus fusing the promoter region of *stm3031* or *stm3030* to *lacZ*. To measure β-galatosidase activity, the resulting plasmids pKM-stm3031 and pKM-stm3030 were separately transformed into *E. coli* DH5α. ONPG was used as the substrate for β-galatosidase.[18] Three representative colonies were picked and examined for enzyme activity. Each value was calibrated with respect to bacterial growth density.

In vivo glutathione transferase (GST) pull-down assay

To generate a fusion protein of glutathione transferase (GST) and STM3030, a DNA fragment containing the *stm3030* gene was amplified. The amplified fragment was digested with XbaI and HindIII and inserted downstream of the GST gene in plasmid pGEX-KG (ATCC® 77103™), generating pGST-stm3030, which was then transformed into *S.* Typhimurium R200. In *vivo* GST pull-down assay was performed as described.[19–21] R200 total cell lysate containing GST or recombinant GST-STM3030 was incubated with 20 μl of glutathione-agarose (Sigma–Aldrich, Inc.) at 4 °C. After being rotated for 2 h and washed four times with wash buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1 mM EDTA, pH 7.4) to remove unbound proteins, the bound GST or recombinant GST-STM3030 proteins were eluted from the resin with elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 9.0). The eluted proteins were fractionated by 12% SDS-PAGE, transferred onto a nitrocellulose membrane, and subjected to Western blotting with the anti-STM3031 antibody. Proteins reacted with the antibody were visualized by enhanced chemiluminescence (ECL), according to manufacturer's instructions (Amersham Pharmacia Biotech).

Expression and purification of recombinant BaeR protein for electrophoretic mobility shift assay (EMSA)

A DNA fragment containing the *baeR* gene was amplified by PCR from strain R200 and inserted into plasmid pQE-80L (QIAGEN) to generate pQE-80L-His-BaeR, which contains the 6xHis tag at the N-terminus of BaeR. *E. coli* DH5α cells harboring pQE-80L-His-BaeR was cultured in LB broth and induced with IPTG. The recombinant protein 6xHis-BaeR was isolated by affinity chromatography using nickel-charged resin (Invitrogen) and eluted from the resin by competition with 200 mM imidazole in a buffer containing 0.5 M NaCl and 20 mM Tris-HCl (pH 7.9). The imidazole was then removed by dialysis in a solution containing 0.5 M NaCl, 20 mM Tris-HCl (pH 7.9).

Electrophoretic mobility shift assay (EMSA)

DNA fragments, 287 bp for *acrA*, 208 bp for *acrD*, 237 bp for *stm3031*, and 204 bp for *stm3030*, were amplified by PCR using the chromosomal DNA of *S.* Typhimurium R200 as template. These DNA fragments were labeled with biotin and purified using the Qiaquick nucleotide removal kit (QIAGEN). Approximately 50 nmole of each biotin-labeled DNA fragment was incubated with increasing concentrations (0–150 nmole) of the His-tag purified BaeR protein. EMSA was performed using the LightShift™ Chemiluminescent EMSA Kit (Thermo Scientific™) according to manufacturer's instructions.

Results

Effect of *stm3031* deletion on the STM3030 protein level

We have previously demonstrated that the *stm3031* gene located 46 bp upstream of *stm3030* is significantly overexpressed in the ceftriaxone-resistant strain R200 of *S.* Typhimurium.[12] This strain was derived from the ceftriaxone-susceptible strain 01-4 by multistep resistance selection, which generated several strains with different levels of resistance to ceftriaxone. In this study, the abundance of the STM3030 protein was found to be increased in strain R200 and decreased in the *stm3031* knockout strain, R200(Δ*stm3031*), with 2-DE analysis (Fig. 1A).

Construction of mutant R200(Δ*stm3030*) and determination of its susceptibility to various antibiotics

To investigate whether *stm3030* is also involved in antibiotic resistance, an *stm3030* gene deletion mutant, R200(Δ*stm3030*) was generated from the R200 strain (Fig. 1B). MICs of this strain together with the 01–4, R200, and R200(Δ*stm3031*) strains were determined. As shown in Table 1, strain R200(Δ*stm3030*), similar to strain R200(Δ*stm3031*), showed a 64-fold reduction in resistance to ceftriaxone compared to strain R200. Furthermore, strain R200(Δ*stm3030*) had a 32-fold and a 64-fold reduction in resistance to cephalothin and cefoxitin, respectively. However, strain R200(Δ*stm3031*) had only an 8-fold reduction in resistance to cephalothin and cefoxitin. R200(Δ*stm3030*) and R200(Δ*stm3031*) both had a 2- to 16-fold reduction in resistance to meropenem, tetracycline, streptomycin, chloramphenicol, erythromycin, and nalidixic acid. Compared to the ceftriaxone-resistant strain R200, both R200(Δ*stm3030*) and R200(Δ*stm3031*) had no change in resistance to imipenem, gentamicin, and novobiocin. R200(Δ*stm3031*) had a 2-fold reduction in susceptibility to rifampin, but R200(Δ*stm3030*) had the same level of susceptibility to rifampin as the wild type. These results suggest that *stm3030*, similar to *stm3031*, has a strong effect on cephalosporin resistance of *S.* Typhimurium.

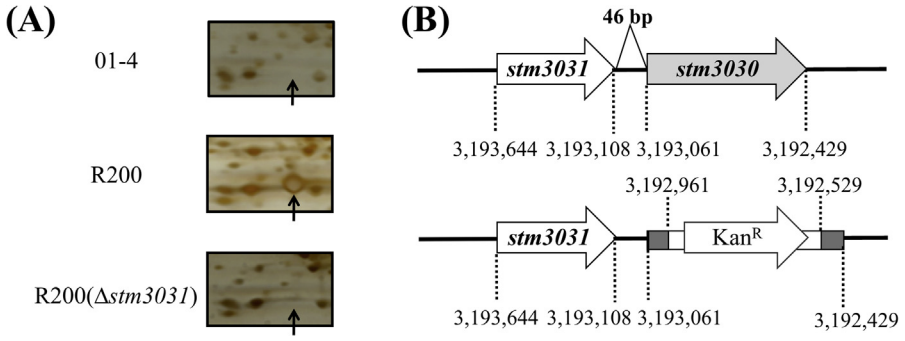

Figure 1. 2-DE analysis of periplasmic proteins of S. Typhimurium strains 01—4 (ceftriaxone susceptible), R200 (ceftriaxone resistant), and R200(Δ*stm3031*) (A). Arrows indicate locations of STM3030. (B) Top: schematic representation of *stm3030* and *stm3031* loci. (B) Bottom: kanamycin resistance gene inserted into *stm3030*. Open boxes flanking the kanamycin-resistance gene represent positions of the *stm3030* gene, and shaded boxes denote the two 100 bp regions immediately adjacent to the 5′ and 3′ end of *stm3030*. Numbers shown are nucleotide positions of the S. Typhimurium genome (GenBank accession no. NC_003197).

Table 1 Antibiotic MICs of *S. enterica* serovar Typhimurium strains 01—4, R200, R200(Δ*stm3031*), and R200(Δ*stm3030*).

Strain	β-lactam antibiotics MICs[a] (μg/ml)					Other antibiotics MICs[a] (μg/ml)							
	CEF	FOX	CRO[b]	MEM[b]	IPM[b]	TET	STR	GEM	CHL	NOV	ERY	NAL	RIF
01_4	1	1	0.047	0.047	4	16	64	1	128	>256	128	4	8
R200	256	256	>256	0.125	0.125	8	128	1	128	256	32	2	8
R200(Δ*stm3031*)	32	32	4	0.016	0.125	4	32	1	16	256	2	0.5	4
R200(Δ*stm3030*)	8	4	4	0.016	0.125	4	64	1	16	256	2	0.5	8

[a] CEF, cephalothin; FOX, cefoxitin; CRO, ceftriaxone; MEM, meropenem; IPM, inipenem; TET, tetracycline; STR, streptomycin; GEM, gentamicin; CHL, chloramphenicol; NOV, novobiocin; ERY, erythromycin; NAL, nalidixic acid; RIF, rifampin.
[b] . Antibiotic MICs were determined by E-test.

stm3030 and *stm3031* are independent genes in *S.* Typhimurium

As *stm3031* is located only 46 bp upstream from *stm3030* (Fig. 1B) and both genes have similar effect on cephalosporin resistance, *stm3031* and *stm3030* were analyzed to determine whether they are co-transcribed. For this purpose, RNA of R200 was extracted and converted to cDNA. Primer 3031(F), which anneals to the *stm3031* gene, and primer 3030(R), which anneals to the *stm3030* gene (Fig. 2A), were used to perform PCR using genomic DNA, cytoplasmic RNA, and cDNA of R200 as templates. As shown in the left panel of Fig. 2A, the 278-bp PCR product was generated from the genomic DNA, but not from cytoplasmic RNA and cDNA. To prove that the RNA sample was well prepared, the primer set for the 16S rRNA gene was used for RT-PCR, and the expected 200-bp PCR product was generated from both genomic DNA and cDNA (Fig. 2A right panel). These results indicated that the failure to produce the 278-bp PCR product from the cDNA template was not due to poor RNA quality and that *stm3031* and *stm3030* are independent genes. To confirm this possibility, promotors of *stm3030* and *stm3031* were separately fused to the *lacZ* gene, generating pKM-stm3030 and pKM-stm3031 and assayed for their activity. As shown in Fig. 2B, the Miller units of the transformants containing pKM005, pKM-stm3030, or pKM-stm3031 were 10.2, 2378.6, and 319.6, respectively. These results demonstrated that *stm3031* and *stm3030* have their own promoters and that the promoter activity of *stm3030* is 7.45-fold (2378.6 versus 319.6 Miller units) higher than that of *stm3031*. The mRNA levels of *stm3030* in strain R200(Δ*stm3031*) and *stm3031* in strain R200(Δ*stm3030*) were similar to those of strain R200 (Fig. 2C), suggesting that *stm3030* and *stm3031* do not belong to the same operon.

Expression of *stm3031* but not *stm3030* is regulated by BaeR of the TCS BaeSR

We had previously demonstrated the involvement of *stm3031* and *baeR* in ceftriaxone resistance of S. Typhimurium.[11,12] The decrease in ceftriaxone resistance was seen in strains R200(Δstm*3030*) and R200(Δstm*3031*) (Table 1). To test whether the expression of *stm3031* and *stm3030* is controlled by the regulator BaeR of the TCS BaeSR, EMSA was performed to determine whether the purified BaeR protein can bind to biotin-labeled DNA fragments containing the promoter regions of *stm3031* and *stm3030* genes. As BaeR does not bind to the promoter region of *acrA* but can bind to that of *acrD*,[6] binding of BaeR to these two promoter regions was performed to serve as negative and positive controls, respectively. The sizes of biotin-labeled DNA fragments were 287 bp for *acrA*, 208 bp for *acrD*, 237 bp for *stm3031*, and 204 bp for *stm3030*. As shown in Fig. 3, the 237-bp DNA fragment containing the promoter region of *stm3031* was shifted with increasing amounts (10—150 nmole) of the BaeR protein. No DNA band-shift was

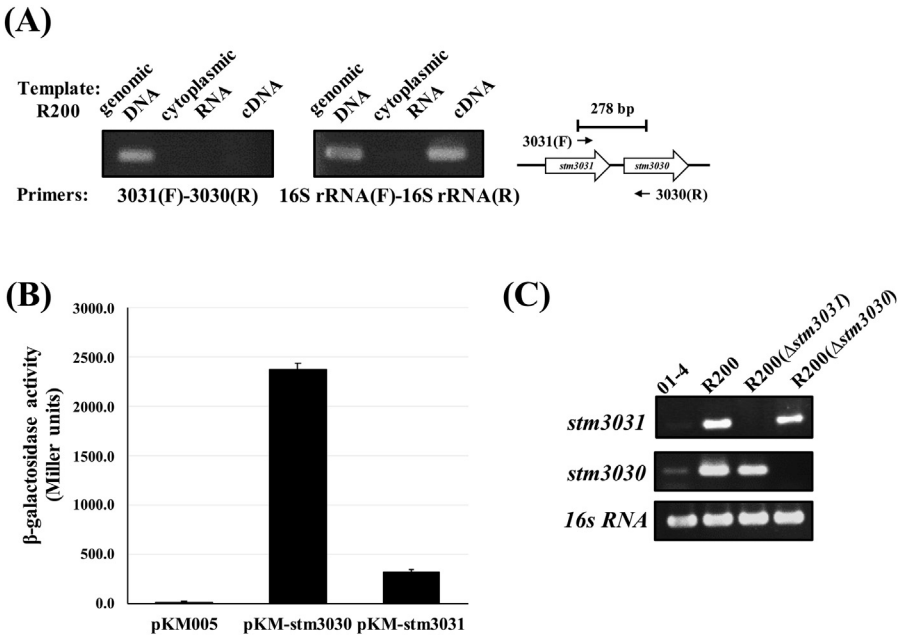

Figure 2. Independent relationship between *stm3030* and *stm3031*. (A) PCR was performed with the primer set 3031(F)-3030(R) to amplify the 278-bp DNA fragment containing *stm3030* and *stm3031*. The 16S rRNA was employed as the internal control. The templates were genomic DNA, cytoplasmic RNA, and cDNA of *S.* Typhimurium R200. The lack of the 278-bp PCR product from cDNA indicates that *stm3030* and *stm3031* are not co-transcribed. (B) The activity of *stm3030* and *stm3031* promoters determined by the β-galactosidase assay. (C): Expression levels of *stm3031* and *stm3030* in 01—4, R200, R200(Δ*stm3031*), and R200(Δ*stm3030*) determined by RT-PCR.

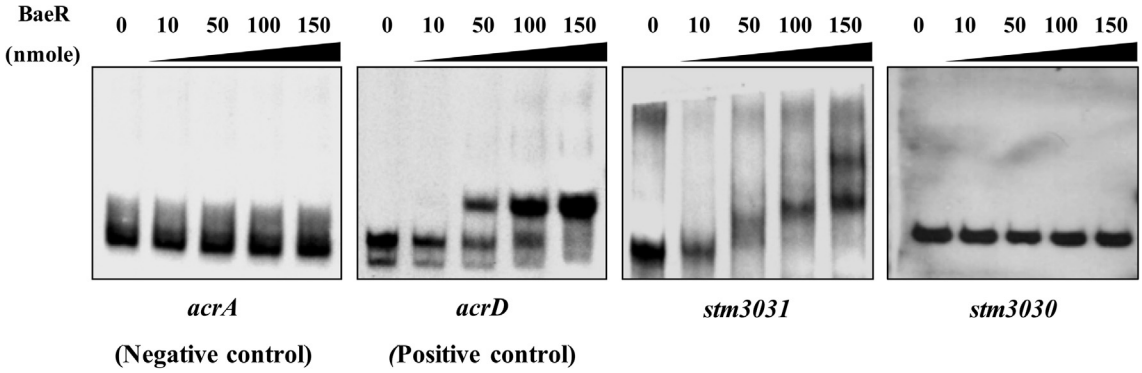

Figure 3. Electrophoretic mobility shift assay (EMSA) for BaeR binding. Approximately 50 nmole each of biotin-labeled DNA fragments containing the promoter region of *acrA*, *acrD*, *stm3030*, or *stm3031* was incubated with increasing concentrations of the His-tag purified BaeR protein. Binding of BaeR to the promoter regions of *acrA* and *acrD* was performed to serve as negative and positive controls, respectively.

observed for the 204-bp DNA fragment containing the promoter region of *stm3030*. These results indicate that the expression of the *stm3031* gene, but not the *stm3030* gene, is regulated by the regulator protein BaeR of the TCS BaeSR.

STM3030 and STM3031 interact with each other

As seen in Fig. 1A, the level of the STM3030 protein is significantly decreased in R200(Δ*stm3031*), suggesting that the STM3031 protein can affect the levels of the STM3030 protein. STM3031 in the outer membrane fraction of strains R200, R200(Δ*stm3031*), and R200(Δ*stm3030*) was compared by Western blotting using anti-STM3031 antibody. As shown in Fig. 4A, the protein level of STM3031 was significantly decreased in strain R200(Δstm*3030*) similar to that of strain R200(Δ*stm3031*), compared to strain R200. We hypothesized that STM3030 binds to and stabilizes STM3031. To test this hypothesis, we investigated whether STM3031 and STM3030 can interact with each other using the glutathione-S-transferase (GST) pulldown assay *in vivo*. Plasmids pGST-only and pGST-stm3030, which contains *stm3030* fused to the GST gene, were separately introduced into strain R200. Proteins in the cell lysates of the transfromants were immobilized on glutathione sepharose

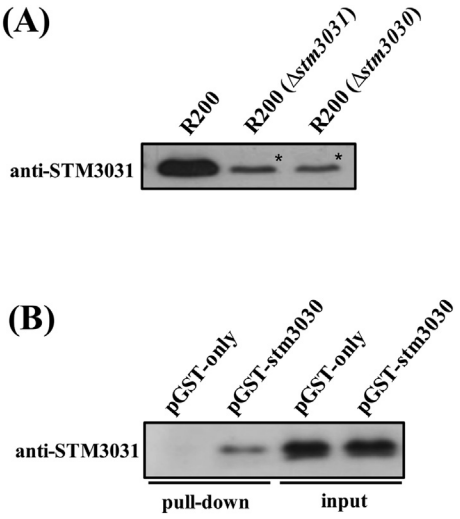

Figure 4. Interaction between STM3030 and STM3031. (A) Outer membrane proteins were isolated and analyzed by Western blotting using anti-STM3031 antiserum. *: nonspecific bands. (B) GST pull-down assay was performed. Total cell lysates of R200/pGST and R200/pGST-stm3030 were mixed with glutathione beads, and the bound proteins were recovered by centrifugation and analyzed by immunoblotting using anti-STM3031 antibody. The input sample was total cell lysate of R200/pGST-only or R200/pGST-stm3030; STM3030 was present in both samples.

beads, and the bound proteins were analyzed by Western blotting using anti-STM3031 antiserum. As shown in Fig. 4B, a band was present in the lane of pGST-stm3030 (pull-down) indicating that the GST-STM3030 fusion protein bound to STM3031, and thus the GST pull-down product (GST-STM3030/STM3031 complex) reacted with the anti-STM3031 antibody. As expected, there was no band in the lane of pGST-only (pull-down). The input samples (total cell lysate of R200/pGST-only or R200/pGST-stm3030) in pGST-only and pGST-stm3030 lanes are shown as gel loading control. This result suggests that STM3031 and STM3030 can bind to each other.

Discussion

Our previous studies showed that the outer membrane protein STM3031 (Ail/OmpX-like) plays a role in ceftriaxone resistance of *S.* Typhimurium by increasing the activity of the AcrD efflux pump and reducing the levels of the OmpD porin.[12] It has been shown that AcrD is an RND-type efflux pump that confers *S.* Typhimurium resistance to various beta-lactam drugs,[22] and that OmpD affects drug permeability.[23]

Results of our previous study also suggest that the expression of *stm3031* is affected by the regulatory gene *baeR* of the BaeSR TCS in *S.* Typhimurium.[11] To investigate whether such effect is mediated by regulator binding, the binding of BaeR to the *stm3030* promoter was examined by EMSA. As shown in Fig. 3, BaeR failed to bind the *stm3030* promoter, indicating that the expression of *stm3030* is not directly regulated by BaeSR. STM3030 belongs to the YfdX protein family and shares a 99% amino acid identity with STY3178 from *Salmonella typhi*.[24] It has been shown that the expression of *yfdX* in *E. coli* is positively regulated by the TCS EvgAS.[25] Whether the *stm3030* gene is regulated by EvgAS or other types of TCSs remains to be determined.

As STM3030 is highly homologous to STY3178, which is an ATP independent chaperone,[26] the possibility that STM3030 acts as a chaperone was examined. Our GST-pull down assay revealed that STM3030 can interact with STM3031 (Fig. 4B), suggesting that STM3030 can function as a chaperon. We also identified a non-specific protein (Fig. 4A) to be OmpX, which shares a 31% amino acid identity with STM3031. Since STM3031 was originally identified as an Ail/OmpX-like protein, it is possible that STM3031 may have functions similar to those of the *E. coli* outer membrane protein OmpX, which mediates binding of YebF to OmpF/C to import ions and protein toxins.[27] The observation that STM3030 and STM3031 can bind to each other suggests that STM3030 may stabilize STM3031 thus conferring *S.* Typhimurium drug resistance. Our findings would aid in the understanding of bacterial resistance to cephalosporin drugs.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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