

The Expression Levels of Outer Membrane Proteins STM1530 and OmpD, Which Are Influenced by the CpxAR and BaeSR Two-Component Systems, Play Important Roles in the Ceftriaxone Resistance of *Salmonella enterica* Serovar Typhimurium[▽]

Wensi S. Hu,* Hung-Wen Chen, Rui-Yang Zhang, Chung-Yi Huang, and Chi-Fan Shen

Department of Biotechnology and Laboratory Science in Medicine, School of Biomedical Science and Engineering, National Yang-Ming University, Taipei, Taiwan

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Significant increases in STM3031, STM1530, and AcrD protein levels and significant decreases in OmpC and OmpD protein levels are present when the ceftriaxone-resistant *Salmonella enterica* serovar Typhimurium R200 strain is compared with the ceftriaxone-susceptible strain 01-4. AcrD is known to be involved in drug export, and STM3031 seems to play a key role in ceftriaxone resistance. Here, we examine the roles of STM1530, OmpC, and OmpD in ceftriaxone resistance. An *ompD* gene deletion mutant showed 4-fold higher ceftriaxone resistance than 01-4. An *ompC* gene deletion mutant showed 4-fold higher cephalothin and erythromycin resistance than 01-4, but there was no effect on ceftriaxone resistance. However, a *stm1530* deletion mutant did show >64-fold lower ceftriaxone resistance than R200. Moreover, the STM3031 protein was significantly decreased in R200(Δ *stm1530*) compared to R200. STM3031 expression has been shown to be influenced by the two-component system regulator gene *baeR*. CpxR seems to modulate BaeR. A *cpxA-cpxR* gene deletion mutant showed >2,048-fold lower ceftriaxone resistance than R200. The outer membrane protein profile of R200(Δ *cpxAR*) showed significant decreases in STM3031 and STM1530 compared to R200, while OmpD had returned to the level found in 01-4. Furthermore, the *stm3031*, *stm1530*, and *ompD* mRNA levels were correlated with their protein expression levels in these strains, while decreases in the mRNA levels of the efflux pump *acrB*, *acrD*, and *acrF* genes were found in R200(Δ *cpxAR*). Findings similar to those for R200(Δ *cpxAR*) were found for R200(Δ *baeSR*). These results, together with those for STM3031 and the fact that STM1530 is an outer membrane protein, suggest that STM1530 and OmpD are influenced by the CpxAR and BaeSR two-component systems and that this contributes to *S. enterica* serovar Typhimurium ceftriaxone resistance.

Salmonella enterica serovar Typhimurium is a bacterial pathogen that causes gastroenteritis in humans. Resistance to multiple antimicrobial agents is common among *S. enterica* serovar Typhimurium strains. Emergence of resistance to a variety of antibiotics in *Salmonella* has been found in many areas of the world and is a potentially serious public health problem. Although extended-spectrum cephalosporins and fluoroquinolones have proved effective, resistance to these agents has been reported (20, 28, 36). The mechanism of resistance to extended-spectrum cephalosporins is mainly via the acquisition of multidrug-resistant plasmids that encode a variety of extended-spectrum and AmpC-type beta-lactamases (1, 9, 13, 23). In general, there have been fewer reports that have found a reduction in permeability to a β -lactam drug or an increase in drug efflux to be the resistance mechanism in *Salmonella* (3, 10, 12).

The bacterial envelope is the front line for most external

stress conditions that may alter envelope components and thus induce an extracytoplasmic stress response. In *Escherichia coli*, a number of response pathways are induced in response to envelope stress (5, 31). These systems are involved in the biogenesis, maintenance, and repair of the bacterial envelope and thus contribute to cell surface integrity. In addition, they modulate key bacterial life functions, such as motility, colony formation, and biofilm formation (4, 11). These are found in nearly every sequenced bacterial genome. In response to environmental change, the signal is transduced from a sensor histidine kinase to a cognate response regulator through phosphoryl transfer (19, 35). Although studies have shown that two-component signal transduction systems do contribute to antibiotic resistance, only a few reports have shown that CpxAR or BaeSR is associated with antibiotic resistance (14, 15).

It has been shown that outer membrane proteins participated in antibiotic resistance either by decreasing permeability or by increasing export of drug. A reduction in the permeation of antibiotics is generally related to a decrease in porin expression or an alteration in the porin structure (27). For example, a lack of porin OmpK35 is found in most extended-spectrum beta-lactamase-expressing *Klebsiella pneumoniae* clinical iso-

* Corresponding author. Mailing address: Department of Biotechnology and Laboratory Science in Medicine, School of Biomedical Science and Engineering, National Yang-Ming University, 155, Li-Nong St., Sec. 2, Peitou, Taipei 112, Taiwan. Phone: 886-2-2826-7151. Fax: 886-2-2826-4092. E-mail: huws@ym.edu.tw.

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lates (7). Enhanced export of a drug is usually related to an increase in transporter efflux pump activity. For example, the efflux pump AcrAB-TolC, which belongs to the RND family, is known to play an important role in multidrug resistance in *E. coli* and *Salmonella* (3, 10, 12, 21). As part of this efflux pump, TolC is an outer membrane protein and has been suggested to cooperate with a number of transporters, such as AcrD and MdtABC, to expel drugs from bacteria (2, 24, 26). In a previous study, we showed that significant increases in the levels of outer membrane protein STM3031 and putative outer membrane protein STM1530 were present in the ceftriaxone-resistant strain R200 (18). Of these proteins, STM3031 has been suggested to play a key role in ceftriaxone resistance, possibly by reducing permeability via a decreased OmpD level and by enhancing export via an increase in AcrD efflux pump activity (18). Furthermore, the expression of this protein is influenced by *baeR*, the regulator gene of a two-component system of *S. enterica* serovar Typhimurium (17). In *E. coli*, CpxR is believed to act as a modulator of other extracytoplasmic response proteins, especially BaeR and PspF, rather than as a stand-alone regulator (5). In addition, bioinformatics analysis predicts that CpxR is likely to be a transcription factor that affects *stm3031* gene expression (data not shown). Thus, in this study, we investigated the role of OmpD and STM1530 in ceftriaxone resistance; furthermore, we also examined whether the expression of these two proteins is influenced by the CpxAR and/or the BaeSR two-component signal transduction systems of *S. enterica* serovar Typhimurium.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. enterica* serovar Typhimurium strain 01-4 is a ceftriaxone-susceptible strain that was isolated in Taiwan. *S. enterica* serovar Typhimurium strain R200 is a ceftriaxone-resistant strain that was generated from parental strain 01-4. *S. enterica* serovar Typhimurium strain, R200(Δ *stm3031*) was generated from strain R200. All these strains have been described previously (18). Strains R200(Δ *stm1530*), R200(Δ *cpxAR*), and R200(Δ *baeSR*) were generated from strain R200. Strains 01-4(Δ *ompC*) and 01-4(Δ *ompD*) were generated from strain 01-4. All the above deletion mutants were created using the one-step inactivation of chromosomal genes method that is described below. The plasmid pGEM-T Easy (Promega) was used as the cloning vector. The plasmids pKD46 and pKD4 was used to generate strains R200(Δ *cpxAR*), R200(Δ *baeSR*), R200(Δ *stm1530*), 01-4(Δ *ompC*), and 01-4(Δ *ompD*). *E. coli* and *S. enterica* serovar Typhimurium were routinely grown in Luria broth (LB) and tryptic soy broth (TSB) at 37°C for 18 h, respectively. Bacterial cultures were supplemented, when necessary, with an appropriate amount of antibiotic.

Antibiotics for susceptibility testing. The MICs for the various antibiotics were determined by the agar dilution method as recommended by the Clinical and Laboratory Standards Institute (25). The antibiotics used for the agar dilution method were tetracycline (TET), streptomycin (STR), gentamicin (GEN), chloramphenicol (CHL), novobiocin (NOV), erythromycin (ERY), nalidixic acid (NAL), rifampin (RIF), cephalothin (CEF), cefamandole (FAM), and ampicillin (AMP) (Sigma-Aldrich Corp., St. Louis, MO). An Etest strip was used, according to the manufacturer's recommendations (AB Biodisk, Solna, Sweden), to measure the resistance to ceftriaxone (CRO) of strains 01-4 and R200, as well as their isogenic strains.

Construction of various gene deletion mutants. The procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of *E. coli* and *S. enterica* serovar Typhimurium were carried out as described previously (17). Each specific gene deletion mutant was constructed by the one-step inactivation of chromosomal genes method of Datsenko and Wanner (6). To construct the *S. enterica* serovar Typhimurium R200(Δ *stm1530*) mutant, a Red recombinase expression plasmid, pKD46-apra, was transformed into strain R200 by electroporation. For the construction of plasmid pKD46-apra, the apramycin resistance gene was amplified by PCR using plasmid pVK173T as template DNA and primer set ApaLI-apra-F and AhdI-apra-R. The amplified product was

cloned into plasmid pGEM-T Easy to form pGEM-apra. The 1,200-bp ApaLI-AhdI fragment containing an apramycin resistance gene from pGEM-apra was directionally inserted into pKD46 that had been cut with ApaLI/AhdI to form pKD46-apra. Then, pKD46-apra was introduced into *S. enterica* serovar Typhimurium strain R200 cells by electroporation, and bacteria were selected on LB agar plates containing apramycin. A 1,579-bp DNA fragment containing the first 40 bp of the 5' end of the *stm1530* gene, a kanamycin resistance gene, and the last 40 bp of the 3' end of the *stm1530* gene was amplified by PCR using pKD4 as template DNA and primer set *stm1530*-kan-F/*stm1530*-kan-R. R200 cells containing pKD46-apra were induced by 0.2% arabinose; then the 1,579-bp PCR product was introduced by electroporation, and bacteria were selected on a LB agar plate with kanamycin. The R200(Δ *stm1530*) mutant was verified by PCR using two sets of primers, primer set 1, *stm1530*-F/*stm1530*-R, and primer set 2, NcoI-*stm1530*-F/XbaI-*stm1530*-R. The construction of the R200(Δ *cpxAR*) and R200(Δ *baeSR*) mutants was similar to that of the R200(Δ *stm1530*) mutant, except for the primers used to generate the gene deletion mutants and for verification of mutants. The primer set used to generate R200(Δ *cpxAR*) was *cpxAR*-kan-F/*cpxAR*-kan-R, and the primer sets used for verification were primer set 1, NcoI-*cpxAR*-F/XbaI-*cpxAR*-R, and primer set 2, NcoI-*cpxR*-F/XbaI-*cpxR*-R. The primer set used for generation of R200(Δ *baeSR*) was *baeSR*-kan-F/*baeSR*-kan-R, and the primer sets used for verification were primer set 1, NcoI-*baeSR*-F/XbaI-*baeSR*-R, and primer set 2, NcoI-*baeR*-F/XbaI-*baeR*-R. The construction of the 01-4(Δ *ompC*) and 01-4(Δ *ompD*) mutants was similar to that of the R200(Δ *stm1530*) mutant, except that the plasmid pKD46-apra was introduced into *S. enterica* serovar Typhimurium strain 01-4. The primer set used for generation of 01-4(Δ *ompC*) was *ompC*-kan-F/*ompC*-kan-R, and the primer sets used for verification were primer set 1, *kan*-F/*kan*-R, and primer set 2, NcoI-*ompC*-F/XbaI-*ompC*-R. The primer set used for generation of 01-4(Δ *ompD*) was *ompD*-kan-F/*ompD*-kan-R, and the primer sets used for verification were primer set 1, *kan*-F/*kan*-R, and primer set 2, NcoI-*ompD*-F/XbaI-*ompD*-R. All primers are listed in Table 1.

Preparation of outer membrane proteins and SDS-PAGE. Outer membrane fraction preparation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for protein analysis were performed as described previously (17, 18). The detection of STM3031 protein or STM1530 protein was by Western blotting as described previously (18).

RT-PCR. Total RNA was isolated from 1×10^9 cells of *S. enterica* serovar Typhimurium using an RNeasy minikit (Qiagen) according to the manufacturer's instructions. The concentration and the quality of the RNA in each sample were determined by measuring their absorbance at 260 nm. All RNAs were adjusted to a concentration of 100 ng μ l⁻¹. The detection of *S. enterica* serovar Typhimurium 16S rRNA was employed as an internal control. The reverse transcriptase PCR (RT-PCR) protocol was carried out with the SuperScript first-strand synthesis system (Invitrogen). The primers used for the RT-PCR analysis are listed in Table 1.

Overexpression and purification of STM1530 protein and antibody production. A DNA fragment containing the *stm1530* gene was amplified by PCR from the R200 strain using primers NcoI-*stm1530*-F and XbaI-*stm1530*-R (Table 1). The PCR product was ligated into the pGEM-T Easy vector to construct pGEM-1530. The NcoI/XbaI DNA fragment containing the *stm1530* gene was then subcloned into similarly digested pBAD/Myc-His and transformed into *E. coli* JM109. The resulting *E. coli* strain harboring pBAD/Myc-His-1530 was cultured in 100 ml LB and then induced with arabinose; after this the protein was identified by SDS-PAGE. The recombinant protein 6 \times His-STM1530 was then purified by affinity chromatography using nickel-nitrilotriacetic acid resin (Qiagen) and SDS-PAGE. The purified recombinant 6 \times His-STM1530 protein was finally sent to LTK BioLaboratories (Taiwan) and used to immunize a rabbit in order to generate polyclonal antibodies against the STM1530 protein.

Fractionation into outer membrane, periplasm, and inner membrane fractions. Using the various *Salmonella* strains, the separation of the bacterial cells into cytoplasmic, periplasmic, inner membrane, and outer membrane fractions was carried out as previously described (18).

Western blotting. The procedure for detection of STM3031 and STM1530 by Western blotting was as previously described (18). The primary polyclonal rabbit antibodies, anti-STM3031 and anti-STM1530, were diluted 1/625,000 and 1/20,000, respectively.

RESULTS

Construction of the 01-4(Δ *ompC*) and 01-4(Δ *ompD*) mutants and determination of their susceptibility to various antibiotics. On the basis of the findings that there were decreased

TABLE 1. Sequences of primers designed for this study

Function and gene	Primer	Sequence (5' → 3') ^a	Reference or source	
Gene deletion from R200 <i>Δstm1530</i>	stm1530-kan-F	ATGAAAAAAGTAGTGGTGTATCTGCGGTAGCCGCAGCCGATT GTGTAGGCTGGAGCTGC	This study	
	stm1530-kan-R	TCAAAACTCGTAGACTAAGCCGAGCGCCACGGTGTGTCTATG GGAATTAGCCATGGTCC	This study	
	stm1530-F	CGTCTCGGTTTTGCTGGTTTTGG	18	
	stm1530-R	GCCGTCATTTTTACCCTGATACTGC	18	
	NcoI-stm1530-F	CCATGGCGATGAAAAAAGTAGTGGTGTATCTG	This study	
	XbaI-stm1530-R	TCTAGAGCAAACCTCGTAGACTAAGCCG	This study	
	<i>ΔcpxAR</i>	cpxAR-kan-F	ATGAATAAAAATCCTGTAGTTGATGATGACGAGAGCTGAGTGT AGGCTGGAGCTGCTTC	This study
		cpxAR-kan-R	TTAGGTTGCTGTGACAGCGGTAGCCACAGCGTAAGCCGCGAC ATGGGAATTAGCCATGGTCC	This study
		NcoI-cpxAR-F	CCATGGCGATGAATAAAAATCCTGTAGTTGATGAT	This study
		XbaI-cpxAR-R	TCTAGAGCGGTTGCTGTGACAGCGGTA	This study
<i>ΔbaeSR</i>	NcoI-cpxR-F	CCATGGCGATGAATAAAAATCCTGTAGTTGATGAT	This study	
	NcoI-cpxR-R	TCTAGAGCTGAAGCGGAAACCATCAGAT	This study	
	XbaI-cpxR-R	TCTAGAGCTGAAGCGGAAACCATCAGAT	This study	
	baeSR-kan-F	ATGAAAGTCTGGCGGCCCGGAATCACCGGCAAGCTGTTTCGTG TAGGCTGGAGCTGCTTC	This study	
	baeSR-kan-R	TACCAGGCGACACGCATCCGTTCCAGCGATATCCCACCGAC ATGGGAATTAGCCATGGTCC	This study	
	NcoI-baeSR-F	CCATGGCGATGAAAGTCTGGCGGCCCGG	This study	
	XbaI-baeSR-R	TCTAGAGCTACCAGGCGACACGCATCC	This study	
	XbaI-baeR-F	CCATGGCGATGAATAAAAATCCTGTAGTTGATGAT	This study	
XbaI-baeR-R	TCTAGAGCTACCAGGCGACACGCATCC	This study		
Gene deletion from 01-4 <i>ΔompC</i>	ompC-kan-F	ATGAAAGTTAAAGTACTGTCCCTCCTGGTACCAGCTCTGCGTG TAGGCTGGAGCTGCTTC	This study	
	ompC-kan-R	TTAGAACTGGTAAACCAGACCCAGCGCTACGATGTCGTCGGAC ATGGGAATTAGCCATGGTCC	This study	
	kan-F	GACAGGATGAGGATCGTTTTC	This study	
	kan-R	CCTTCTATCGCCTTCTTGAC	This study	
	NcoI-ompC-F	CCATGGCGATGAAAGTTAAAGTACTGTCCCTC	This study	
	XbaI-ompC-R	TCTAGAGCGAACTGGTAAACCAGACCCAG	This study	
	<i>ΔompD</i>	ompD-kan-F	ATGAAACTTAAGTTAGTGGCAGTGGCAGTACTCCCTGTTGG CAGCAGGCGTTGGTGTAGGCTGGAGCTGCTTC	This study
		ompD-kan-R	TTAGAACTGGTAGTTCAGACCAACAGCAACGATGTTGTCGGTA GACACTTTAGCCATGGGAATTAGCCATGGTCC	This study
		kan-F	Sequence described above	This study
		kan-R	Sequence described above	This study
NcoI-ompD-F	CCATGGCGAACTTAAGTTAGTGGCAGTGG	This study		
XbaI-ompD-R	TCTAGAGCGAACTGGTAGTTCAGACCAAC	This study		
Apramycin gene for pKD46	ApaLI-apra-F	GTGCACATCCCCGGGGACCTGCA	This study	
	AhdI-apra-R	GACTCCCCGTCGTGTTGCCCCAGCAATCAGC	This study	
<i>stm1530</i> gene expression	NcoI-stm1530-F	Sequence described above	This study	
	XbaI-stm1530-R	Sequence described above	This study	
RT-PCR	<i>stm3031</i>	stm3031-F	TGCAAGCAGGGAGTAATAACGGGT	
		stm3031-R	TCACTTGGATACGCCAGTCCCAT	
	<i>stm1530</i>	stm1530-F	Sequence described above	
		stm1530-R	Sequence described above	
	<i>acrA</i>	acrA-F	TATCGTGAACCTGACGGTGA	
		acrA-R	ACCAATACGACCGCTAATCG	
	<i>acrB</i>	acrB-F	GTTTCGACCTAACGGTCTGGA	
		acrB-R	AACATGCGGTATTTTCGCTTC	
	<i>acrD</i>	acrD-F	TCCGGCCAAATTGAATAGTT	
		acrD-R	TCGGAACCGTCTGTATTAAC	
	<i>acrF</i>	acrF-F	TATCTGGCTGGATGCGAATCTGCT	
		acrF-R	ACTTTGCCGAACCTTCCGGATCT	
	<i>tolC</i>	tolC-F	CACCCAGTACGACGATAGCA	
		tolC-R	CTGCTGATGGAGGCGTAAAT	
	<i>ompC</i>	ompC-F	TCGCAGCCTGCTGAACCAGAAC	
ompC-R		ACGGGTTGCGTTATAGGTCTGAG	18	

Continued on following page

TABLE 1—Continued

Function and gene	Primer	Sequence (5' → 3') ^a	Reference or source
<i>ompD</i>	ompD-F	GCAACCGTACTGAAAGCCAGGG	
	ompD-R	GCCAAAGAAGTCAGTGTACGGT	
<i>vacJ</i>	vacJ-F	TTTCCTGCAGGGCGATCCTTATCA	
	vacJ-R	CTGCCGTAGAACGGAAGTTGCATA	
<i>psd</i>	psd-F	AAACGTGCCGTTGCGGAACCTTATC	
	psd-R	ATTCTTTGTTCGTCGCTGCGTG	
16S RNA	16S RNA-F	TAACCGCAGCAATTGACGTTACCC	
	16S RNA-R	TCTACAAGACTCAAGCCTGCCAGT	

^a Restriction enzyme sites are underlined.

levels of OmpC and OmpD in strain R200, we investigated whether OmpC and/or OmpD were involved in resistance to ceftriaxone. The *ompC* gene and *ompD* gene deletion mutants, 01-4(Δ *ompC*) and 01-4(Δ *ompD*), were independently generated from the 01-4 strain, and each strain's MICs for a number of different antibiotics were then determined. As shown in Table 2, strain 01-4(Δ *ompD*) showed a 4-fold elevation in resistance to ceftriaxone, cefamandole, and cephalothin compared to the ceftriaxone-susceptible strain, 01-4 (0.047 μ g ml⁻¹ versus 0.19 μ g ml⁻¹, 2 μ g ml⁻¹ versus 8 μ g ml⁻¹, and 4 μ g ml⁻¹ versus 16 μ g ml⁻¹, respectively). For the same strain, there was also a 2- to 4-fold elevation in resistance to streptomycin, erythromycin, and chloramphenicol. However, there was no effect on resistance to ampicillin, tetracycline, gentamicin, novobiocin, nalidixic acid, and rifampin. For strain 01-4(Δ *ompC*), there was no effect on resistance to any of the tested antibiotics, except for a 4-fold elevation in resistance to cephalothin and erythromycin. Thus, these results suggest that the decrease in the level of the putative outer membrane porin precursor OmpD is involved in ceftriaxone resistance and may also participate in resistance to cephalothin and cefamandole.

Construction of R200(Δ *stm1530*) and determination of this mutant's susceptibility to antibiotics. To examine the role of the *stm1530* gene on antibiotic susceptibility, an *stm1530* gene deletion mutant, R200(Δ *stm1530*), was generated from the R200 strain, and the MICs of this strain to a number of antibiotics were then determined. As shown in Table 3, strain R200(Δ *stm1530*) showed a >64-fold reduction in resistance to ceftriaxone compared to strain R200 (>256 μ g ml⁻¹ versus 4 μ g ml⁻¹). When cephalosporins other than ceftriaxone were examined, strain R200(Δ *stm1530*) showed a >16-fold reduction in resistance to cephalothin and cefamandole compared to strain R200. In contrast, using the same strains, there was only

a 2- to 4-fold reduction in resistance to streptomycin, gentamicin, and chloramphenicol and no effect on resistance to ampicillin, tetracycline, novobiocin, erythromycin, nalidixic acid, and rifampin. These results suggest that the putative outer membrane protein STM1530 plays an important role in the *S. enterica* serovar Typhimurium ceftriaxone resistance.

Outer membrane protein profile of R200(Δ *stm1530*). The outer membrane proteins from *S. enterica* serovar Typhimurium 01-4, R200, and R200(Δ *stm1530*) were compared by SDS-PAGE analysis. Figure 1A shows that two protein bands, corresponding to molecular sizes of 40 and 39 kDa, were significantly altered. In strain R200(Δ *stm1530*), the 40-kDa band, which had previously been identified to be the putative outer membrane protein STM1530, became undetectable compared to the level in strain R200 (lanes 2 and 4); and the 39-kDa band, which had previously been identified to be the putative outer membrane porin precursor OmpD, was returned to the level found in the 01-4 strain (lanes 1 and 4). The changes in these two proteins are exactly the same as those previously described when the R200(Δ *stm3031*) strain was compared to R200 (lane 3). Furthermore, as shown in Fig. 1B, the STM3031 protein was significantly decreased in strain R200(Δ *stm1530*) (lane 4) compared to strain R200 (lane 2) by Western blot analysis. It has previously been reported that the STM1530 protein was significantly decreased in strain R200(Δ *stm3031*), and this was further confirmed by Western blot analysis in this study (Fig. 1C, lanes 2 and 3). Thus, these results suggest that the levels of expression of STM3031 and STM1530 are influenced by each other.

STM1530 is indeed an outer membrane protein. As shown in Fig. 2, by Western blot analysis, the STM1530 protein is highly abundant in the outer membrane fraction of strain R200 (lane 1), but only a very small, almost undetectable, amount is

TABLE 2. MICs for *Salmonella enterica* serovar Typhimurium strains 01-4, 01-4(Δ *ompC*), and 01-4(Δ *ompD*)

Strain	MIC (μ g ml ⁻¹) of the following drug ^a :											
	CEF	FAM	CRO ^b	AMP	TET	STR	GEM	NOV	ERY	NAL	RIF	CHL
01-4	4	2	0.047	>512	8	128	1	>512	128	4	16	256
01-4(Δ <i>ompC</i>) ^c	16	2	0.047	>512	8	128	1	>512	512	4	16	256
01-4(Δ <i>ompD</i>) ^d	16	8	0.19	>512	8	256	1	>512	512	4	16	512

^a CEF, cephalothin; FAM, cefamandole; CRO, ceftriaxone; AMP, ampicillin; TET, tetracycline; STR, streptomycin; GEM, gentamicin; NOV, novobiocin; ERY, erythromycin; NAL, nalidixic acid; RIF, rifampin; CHL, chloramphenicol.

^b The MIC was determined by Etest.

^c An *ompC* gene deletion mutant strain derived from 01-4.

^d An *ompD* gene deletion mutant strain derived from 01-4.

TABLE 3. MICs for *Salmonella enterica* serovar Typhimurium strains R200, R200($\Delta baeSR$), R200($\Delta cpxAR$), and R200($\Delta stm1530$)

Strain	MIC ($\mu\text{g ml}^{-1}$) of the following drug ^a :											
	CEF	FAM	CRO ^b	AMP	TET	STR	GEM	NOV	ERY	NAL	RIF	CHL
R200	>256	>256	>256	>512	8	256	1	>512	4	1	16	64
R200($\Delta baeSR$) ^c	8	4	0.25	>512	2	128	0.5	256	2	0.5	8	16
R200($\Delta cpxAR$) ^d	8	4	0.125	>512	2	16	0.25	>512	2	1	8	16
R200($\Delta stm1530$) ^e	16	16	4	>512	8	64	0.5	>512	4	1	16	32

^a CEF, cephalothin; FAM, cefamandole; CRO, ceftriaxone; AMP, ampicillin; TET, tetracycline; STR, streptomycin; GEM, gentamicin; NOV, novobiocin; ERY, erythromycin; NAL, nalidixic acid; RIF, rifampin; CHL, chloramphenicol.

^b The MIC was determined by Etest.

^c A *baeSR* gene deletion mutant strain derived from R200.

^d A *cpxAR* gene deletion mutant strain derived from R200.

^e An *stm1530* gene deletion mutant strain derived from R200.

present in strain 01-4 (lane 2), and the protein is absent in strain R200($\Delta stm1530$) (data not shown). Although the STM1530 protein was detectable in the inner membrane fraction (lane 5) of strain R200, this is likely to be due to incomplete separation of the membrane fractions, which has been reported previously (18). Thus, we conclude that STM1530 is indeed located in the outer membrane.

Comparison of the susceptibilities of strains R200, R200($\Delta cpxAR$), and R200($\Delta baeSR$) to various antibiotics. To examine whether the CpxAR two-component system, which is involved in the bacterial envelope stress response, contributes to drug susceptibility, a mutant strain containing a double deletion of *cpxA* and *cpxR*, R200($\Delta cpxAR$), was generated from the R200 strain. The MICs of this strain for a number of antibiotics were then determined. As shown in Table 3, strain R200($\Delta cpxAR$) showed a >2,048-fold reduction in resistance to ceftriaxone compared to strain R200 (>256 $\mu\text{g ml}^{-1}$ versus 0.125 $\mu\text{g ml}^{-1}$). When other cephalosporin drugs were tested, strain R200($\Delta cpxAR$) was found to have >32-fold and >64-fold reductions in resistance to cephalothin and cefamandole,

respectively, compared to strain R200. In contrast, for the same strains, there were only 2- to 16-fold reductions in resistance to tetracycline, streptomycin, chloramphenicol, gentamicin, erythromycin, and rifampin. Furthermore, there was no effect on resistance to ampicillin, novobiocin, and nalidixic acid. Our previous results have shown that expression of STM3031 is influenced by the regulator gene *baeR*, which is part of an *S. enterica* serovar Typhimurium two-component system gene pair (17). On the basis of this, a deletion mutant of the *baeS* sensor and *baeR* regulator genes, R200($\Delta baeSR$), was generated. As shown in Table 3, strain R200($\Delta baeSR$) shows a >1,024-fold reduction in resistance to ceftriaxone compared to strain R200 (>256 $\mu\text{g ml}^{-1}$ versus 0.25 $\mu\text{g ml}^{-1}$). When the other cephalosporin drugs are considered, strain R200($\Delta baeSR$) showed >32-fold and >64-fold reductions in resistance to cephalothin and cefamandole, respectively, compared to strain R200. In contrast, for the same strains, there was only a 2- to 4-fold reduction in resistance to tetracycline, streptomycin, chloramphenicol, gentamicin, novobiocin, erythromycin, nalidixic acid, and rifampin. Furthermore, there was

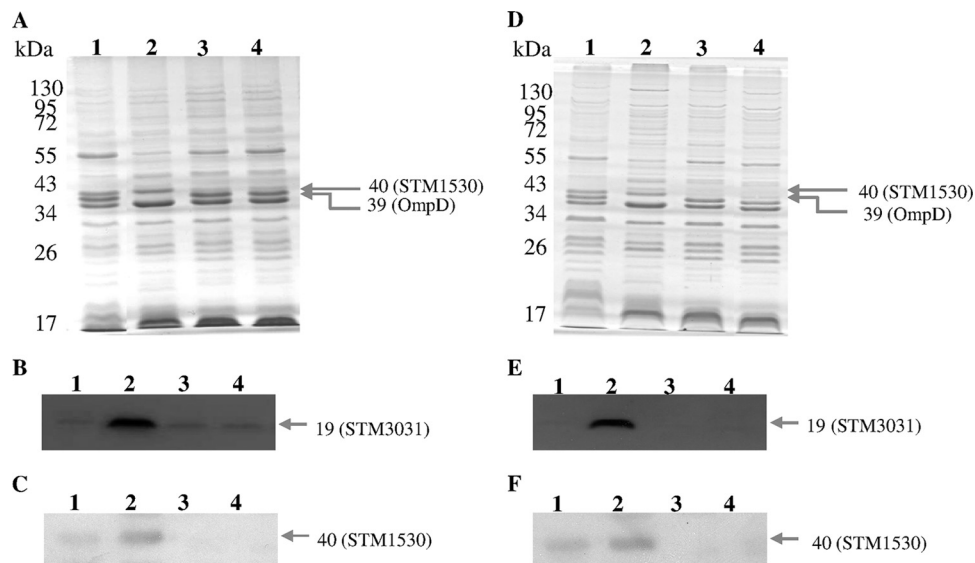


FIG. 1. Comparison of the outer membrane protein profiles of different strains of *S. enterica* serovar Typhimurium. Each sample was resolved by 12% SDS-PAGE. The proteins were visualized by Coomassie blue staining (A and D) and Western blot analysis after probing with anti-STM3031 antibodies (B and E) and anti-STM1530 antibodies (C and F). (A to C) Lanes 1, strain 01-4; lanes 2, strain R200; lanes 3, strain R200($\Delta stm1530$); lanes 4, strain R200($\Delta baeSR$). (D to F) Lanes 1, strain 01-4; lanes 2, strain R200; lanes 3, R200($\Delta baeSR$); lanes 4, R200($\Delta cpxAR$). The arrows indicate significantly changed proteins. Molecular size standards are shown on the left.

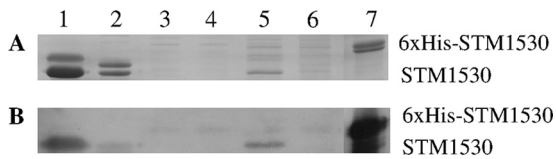


FIG. 2. STM1530 is localized on the outer membranes of different strains of *S. enterica* serovar Typhimurium. The bacteria were fractionated into periplasmic, outer membrane, and inner membrane fractions by the procedure described in Materials and Methods. Each sample (6 μ g of protein) was resolved by 12% SDS-PAGE. (A) Coomassie blue staining; (B) Western blot analysis after probing with anti-STM1530 antibodies. Lanes 1, 3, and 5 outer membrane, periplasmic, and inner membrane fractions of strain R200, respectively; lanes 2, 4, and 6, outer membrane, periplasmic, and inner membrane fractions of strain 01-4, respectively; lanes 7, recombinant protein 6 \times His-STM1530, which acts as a positive control.

no effect on resistance to ampicillin. Taken together, these results suggest that both of the CpxAR and the BaeSR two-component signal transduction pathways seem to be directly or indirectly involved in the control of ceftriaxone sensitivity/resistance, as well as expression of several membrane proteins

known or implicated in resistance to ceftriaxone. These two pathways may also be involved in cephalothin and cefamandole resistance.

Outer membrane profiles of R200(Δ baeSR) and R200(Δ cpxAR). The outer membrane proteins from *S. enterica* serovar Typhimurium strains 01-4, R200, R200(Δ baeSR), and R200(Δ cpxAR) were compared by SDS-PAGE analysis. As shown in Fig. 1D, OmpD in strains R200(Δ baeSR) and R200(Δ cpxAR) was returned to the level found in strain 01-4 (lanes 1, 3, and 4). In contrast, significant decreases in STM3031 (Fig. 1E) and STM1530 (Fig. 1F) were found in strains R200(Δ baeSR) and R200(Δ cpxAR) (lanes 3 and 4) compared to R200 (lane 2). Taken together, these results suggest that the outer membrane proteins STM3031 and STM1530 both play important roles in ceftriaxone resistance and that the expression levels of both proteins are influenced by the BaeSR and CpxAR two-component systems, which are participants in the envelope stress response of *S. enterica* serovar Typhimurium.

Determination of mRNA levels of altered proteins in R200(Δ baeSR) and R200(Δ cpxAR) by RT-PCR. When the results for R200 shown in Fig. 3 are compared, significant de-

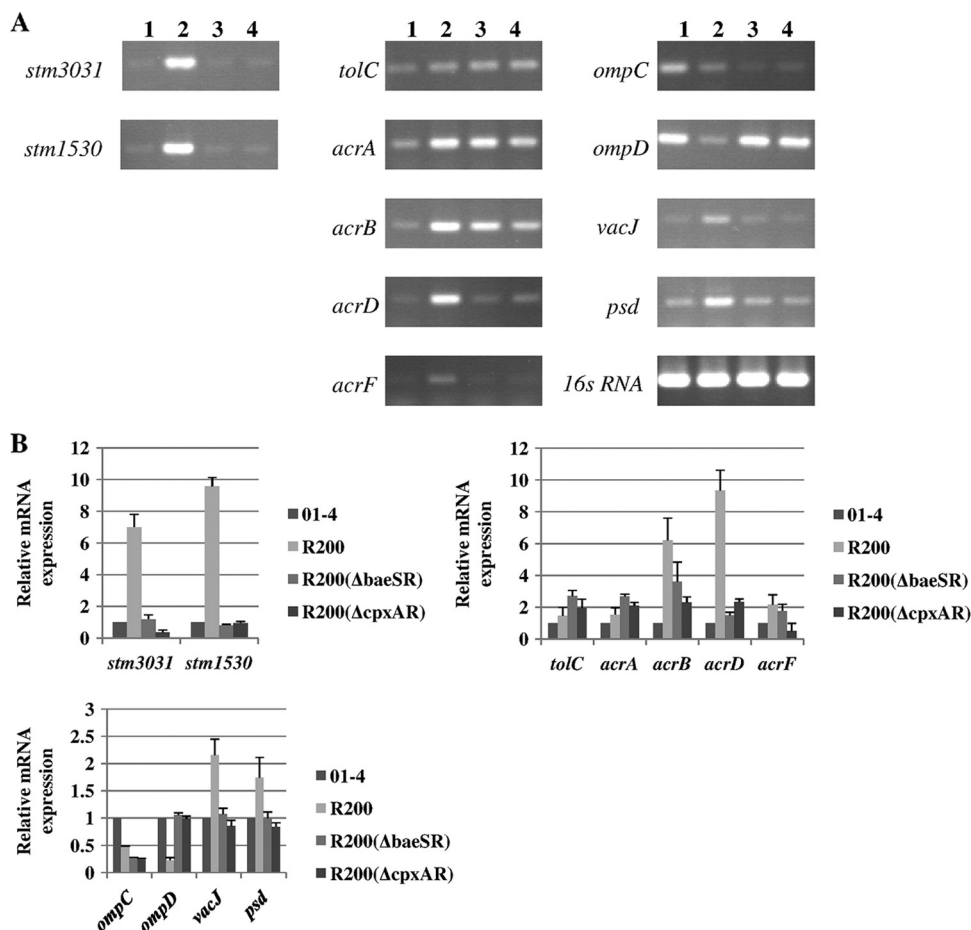


FIG. 3. Quantification of the transcription levels of the genes encoding the various proteins with altered protein expression levels. (A) RT-PCR analysis of the mRNA expression levels of various genes. Lane 1, strain 01-4; lane 2, strain R200; lane 3, strain R200(Δ baeSR); lane 4, strain R200(Δ cpxAR). (B) Relative mRNA expression levels (n -fold) determined from the results shown in panel A. The expression level of each mRNA in strain 01-4 represents 1-fold. The expression of the 16S RNA gene was used as an internal control. Each bar represents the average value of three independent experiments. Error bars are standard deviations.

creases in the mRNA levels of STM3031 and STM1530 and significant increases in the mRNA levels of OmpD for strains R200($\Delta baeSR$) and R200($\Delta cpxAR$) were detected; thus, the changes in translation levels of these proteins are mirrored by changes in the transcription levels of the same genes. In addition, the mRNA levels of *ompC*, *vacJ*, *psd*, and *acrD* in strains R200($\Delta baeSR$) and R200($\Delta cpxAR$) were decreased relative to strain R200. The changes in the mRNA level pattern of the above seven genes in strains R200($\Delta baeSR$) and R200($\Delta cpxAR$) are similar to those previously found in strain R200($\Delta stm3031$). Among RND-type transporter genes other than *acrD*, the mRNA level of *acrF* was also significantly decreased in strain R200($\Delta cpxAR$), which resulted in a level lower than that in the 01-4 strain but not significantly changed relative to that in strain R200($\Delta baeSR$). In contrast, the mRNA level of *acrB* was decreased in strains R200($\Delta baeSR$) and R200($\Delta cpxAR$), but this decrease was significantly greater in R200($\Delta cpxAR$) than R200. The mRNA levels of the other components in the RND-type transporter gene group, *acrA* and *tolC*, were higher for strains R200($\Delta baeSR$) and R200($\Delta cpxAR$) than strain R200. These changes in the mRNA patterns of strains R200($\Delta baeSR$) and R200($\Delta cpxAR$) across these four genes are different from those previously found in strain R200($\Delta stm3031$). Furthermore, the mRNA levels of the *baeS* and *baeR* genes were decreased in R200($\Delta cpxAR$); in contrast, the mRNA levels of the *cpxA* and *cpxR* genes were decreased in R200($\Delta baeSR$) (data not shown). These results suggest that the BaeSR and the CpxAR two-component systems influence each other and that these two pathways are sensitive to and respond to ceftriaxone, an extracytoplasmic agent that is likely to induce cellular stress. They do this by upregulating the levels of STM3031, STM1530, and AcrD and downregulating the level of OmpD; the result of these changes is an increase in *S. enterica* serovar Typhimurium ceftriaxone resistance.

DISCUSSION

In our previous study, we showed that a transposon inserted into the regulator gene *baeR*, which is part of a two-component system found in *S. enterica* serovar Typhimurium, resulted in a >4-fold reduction in resistance to ceftriaxone compared to ceftriaxone-resistant strain R200 (17). This is further supported in this study by the finding that deletion of both the sensor and regulator genes of the two-component system *baeS* and *baeR* results in a strain, R200($\Delta baeSR$), that shows a >1,024-fold reduction in resistance to ceftriaxone (Table 3). In addition, a 2,048-fold reduction in resistance to ceftriaxone was found in strain R200($\Delta cpxAR$) (Table 3), which supports the hypothesis that the CpxAR two-component system also contributed to ceftriaxone resistance. Upon introduction of pBAD-*baeSR* into R200($\Delta baeSR$) to give R200($\Delta baeSR$)/pBAD-*baeSR*, there was a partial restoration of the ceftriaxone resistance (6-fold), as estimated by Etest MIC (data not shown). A similar finding whereby there was a partial restoration of ceftriaxone resistance (16-fold) was found for R200($\Delta cpxAR$) on complementation with pBAD-*cpxAR* to give R200($\Delta cpxAR$)/pBAD-*cpxAR* (data not shown). The fact that there was not complete restoration of ceftriaxone resistance might be due to the slight increase in the levels of

STM3031 and STM1530 in both R200($\Delta baeSR$)/pBAD-*baeSR* and R200($\Delta cpxAR$)/pBAD-*cpxAR* strains compared to R200 (data not shown). However, there are still other possibilities, too. First, it is well-known that autoregulation is a common feature of many regulatory pathways that control stress responses. For example, activation of the CpxAR pathway is tightly controlled by both autoamplification and feedback inhibition mechanisms, the function of which is to turn the CpxAR pathway on and off very rapidly (30). A second possibility is that there is cross talk or cross-regulation between the various bacterial two-component signal pathways involved in ceftriaxone resistance; for example, cross talk is known to occur between EnvZ-OmpR and CpxA-CpxR (34), and cross-regulation is also known to occur between ArcB-ArcA and EnvZ-OmpR (22).

It is well known that the expression of numerous genes is affected by more than one stress response pathway in *E. coli*, and this suggests that redundancy in these pathways probably also occurs in *Salmonella*. Nonetheless, a recent study using transcriptome analyses of *E. coli* showed that induction of specific genes via multiple pathways is not that common (5). Among the pathways where it does occur, an example is the two-component system regulators BaeR and CpxR; in *E. coli* these have been found to control several drug export systems that might be important to the export of toxic compounds during extracytoplasmic stress (16). The control is exerted via the binding of either one or the other, or both, of the regulators, namely, BaeR or CpxR, to the promoter region of the efflux pump *acrD* or to the promoter region of the operon *mdtABC* (16). A similar finding whereby BaeR increases multidrug resistance by directly binding to the promoter regions of *acrD* and *mdtABC*, thereby regulating AcrD and MdtABC, has also been found in *S. enterica* serovar Typhimurium (26).

In this study, the finding that there was a significant reduction in the *acrD* mRNA level, together with a drastic decrease in ceftriaxone resistance in R200($\Delta baeSR$) and R200($\Delta cpxAR$) (Fig. 3), provides additional evidence supporting the hypothesis that the BaeSR/CpxAR systems might upregulate AcrD and that this results in increased ceftriaxone resistance in *Salmonella*. Two possible mechanisms can be proposed for the above results. One is that BaeSR and CpxAR respond to ceftriaxone independently. Alternatively, one of these two pathways may be the main signaling system that responds to ceftriaxone, and after this signaling occurs, the other pathway functions as a modulator. Recent studies have suggested that CpxR may play an accessory or modulator role relative to other extracytoplasmic stress responders such as BaeR and PspF rather than acting as a stand-alone regulator (5). The results in this study showed that expression of one sensor and regulator pair, BaeSR or CpxAR, influences expression of the other one; this opens up the possibility that either pathway may function as a modulator of the other pathway. This final mechanism is the one that best explains the present findings.

Our previous studies have shown that both OmpD and OmpC are highly reduced at the protein level in the ceftriaxone-resistant strain R200 (18). OmpD is one of the most abundant outer membrane proteins of *S. enterica* serovar Typhimurium and is regulated by a multiplicity of environmental factors, including anaerobiosis, lower pH, and catabolite repression (33). As shown in Table 1, strain 01-4($\Delta ompD$)

showed a 4-fold elevation in resistance to ceftriaxone compared to strain 01-4; however, there was no difference in MIC value when *ompC* was deleted to give strain 01-4(Δ *ompC*). In addition, a return in OmpD protein level to that of strain 01-4 paralleled a significant decrease (at least 64-fold) in ceftriaxone resistance in four mutants, strains R200(Δ *stm3031*), R200(Δ *stm1530*), R200(Δ *baeSR*), and R200(Δ *cpxAR*) (Fig. 1A and 1D and Table 3) (18). This suggests that OmpD is highly likely to be functionally involved in allowing ceftriaxone to enter *Salmonella* because an increase in OmpD protein parallels a reduction in ceftriaxone resistance in these four mutants. Thus, OmpD would seem to also play an important role in the ceftriaxone resistance of *S. enterica* serovar Typhimurium.

Furthermore, we have confirmed in this study that the putative outer membrane protein STM1530 is indeed an outer membrane protein. Although the function of this protein remains unknown, a >64-fold reduction in resistance to ceftriaxone in strain R200(Δ *stm1530*) suggests that this outer membrane protein plays a key role in ceftriaxone resistance. Protein structure prediction using the protein homology/analogy recognition engine (PHYRE) search tool suggests that STM1530 is a porin similar to *Klebsiella pneumoniae* OmpK36, *E. coli* PhoE, and *E. coli* OmpF. It has been reported that in *S. enterica* serovar Typhimurium, the porin OmpD together with a putative inner membrane efflux pump, YddG, is involved in resistance to methyl viologen and aromatic amino acids (8, 32). In addition, it has been suggested that OmpD in *S. enterica* serovar Typhimurium interacts with the periplasmic protein YdeI/OmdA to increase resistance to antimicrobial peptides (29). On the basis of the above models, we suggest that overexpression of the STM1530 protein in strain R200 may function in conjunction with a periplasmic protein(s) and/or an inner membrane efflux pump(s) to export ceftriaxone in a manner similar to that by OmpD. Interestingly, the level of STM3031 protein is significantly decreased in strain R200(Δ *stm1530*) (Fig. 1B). In contrast, the level of STM1530 protein is significantly decreased in strain R200(Δ *stm3031*) (Fig. 1C). Thus, it would seem likely that both of these outer membrane proteins, STM3031 and STM1530, play important roles in the ceftriaxone resistance of *S. enterica* serovar Typhimurium.

In conclusion, we identified the BaeSR and CpxAR two-component pathways to be important signal systems that are involved in conferring ceftriaxone resistance on *S. enterica* serovar Typhimurium and that they do this by controlling the levels of expression of STM3031, STM1530, OmpD, and AcrD. To our knowledge, this is the first evidence showing that STM1530 is an important protein involved in ceftriaxone resistance. Further studies are under way to explore how STM3031 and STM1530 influence each other, to explore whether STM3031 and STM1530 are directly or indirectly regulated by BaeR and/or CpxR, and to discover how these regulatory mechanisms affect ceftriaxone resistance in *S. enterica* serovar Typhimurium.

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