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Impact of Hfq on the intrinsic drug resistance of *Salmonella enterica* serovar Typhimurium

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Salmonella enterica is an important enteric pathogen, and its various serovars cause both systemic and intestinal diseases in humans and domestic animals. The emergence of multidrug-resistant strains of *Salmonella*, leading to increased morbidity and mortality, has further complicated its management. Hfq is an RNA chaperon that mediates the binding of small RNAs to mRNA and assists in post-transcriptional gene regulation in bacteria. Although Hfq is related to important phenotypes including virulence in *Salmonella*, its role in the drug resistance of this organism is unknown. The aim of this study was to investigate the role of Hfq in intrinsic drug resistance of *S. enterica* serovar Typhimurium. *hfq* Mutant was susceptible to acriflavine. Although there is a relationship between the production of the AcrB multidrug efflux pump and Hfq in *Escherichia coli*, the deletion of the drug efflux *acrB* did not impair the effect of *hfq* deletion on *Salmonella* susceptibility. In contrast, the deletion of another drug efflux gene, *smvA*, impaired the effect of *hfq* deletion on acriflavine susceptibility. These results indicate that Hfq regulates the intrinsic drug resistance, and it may influence drug susceptibility by regulating SmvA in *Salmonella*.

Keywords: drug efflux system, drug resistance, Hfq, *Salmonella*, small RNA

INTRODUCTION

Salmonella causes a variety of diseases in humans, ranging from gastroenteritis to bacteremia and typhoid fever (Scherer and Miller, 2001). In the 1990s, the prevalence of multidrug-resistant *Salmonella enterica* increased dramatically in the United Kingdom, the United States, and Canada (Hosek et al., 1997; Threlfall et al., 1997; Glynn et al., 1998; Ng et al., 1999). Many other countries have also documented outbreaks associated with drug-resistant *Salmonella* in poultry, beef, and pork (Davies et al., 1996; Cody et al., 1999; Grein et al., 1999; Molbak et al., 1999; Villar et al., 1999). Emerging resistance in *Salmonella* has been observed in both humans and animals, and thus, this is a potentially serious public health problem (Cloeckaert and Chaslus-Dancla, 2001; Piddock, 2002). Drug resistance in bacteria is often associated with multidrug efflux pumps that decrease cellular drug accumulation (Nikaido, 1996; Zgurskaya and Nikaido, 2000).

The phenomenon of multidrug resistance is associated with the ability of pumps to expel from cells multiple drugs with different modes of action. Multidrug resistance is a serious problem in treatment of human ailments caused by pathogenic bacteria, fungi, parasites, and cancer. Functional studies identified multidrug efflux pumps classified in five families: ATP-binding cassette (ABC), the major facilitator (MFS), resistance-nodulation-cell division (RND), small multidrug resistance, and multidrug and toxic compound extrusion families (Brown et al., 1999; Putman et al., 2000; Paulsen et al., 2001). The sequencing of bacterial genomes enables us to trace putative drug resistance genes (Paulsen et al., 1998, 2000). There are many putative and proven drug efflux pumps in the *Salmonella* genome. We previously demonstrated that *S. enterica* serovar Typhimurium has nine functional drug efflux pumps (Nishino et al., 2006). In addition to these pumps, it has been reported that SmvA is an important efflux pump for acriflavine and related compounds (Villagra et al., 2008). Because many of these multidrug efflux pumps have overlapping substrate spectra, it is intriguing that bacteria, with their economically organized genomes, harbor such large sets of multidrug efflux genes. The key to understanding how bacteria utilize these multiple pumps lies in the regulation of pump expression. Currently, available data indicate that multidrug efflux pumps are often expressed under precise and elaborate transcriptional control (Ahmed et al., 1994; Lomovskaya et al., 1995; Brooun et al., 1999; Grkovic et al., 2002).

The Hfq protein is a conserved RNA chaperone protein first characterized as a host factor (HF-1) for phage Qβ RNA replication (Franze de Fernandez et al., 1968) and subsequently shown to be widely distributed in the bacterial kingdom with multiple homologs in the annotated genomic database (Brennan and Link, 2007). As a bacterial homolog of the eukaryotic and archaeal Sm/LSm proteins, Hfq is known largely for its global post-transcriptional regulation by binding AU-rich sequences of target mRNA and facilitating pairing between sRNAs and mRNAs (Moller et al., 2002; Zhang et al., 2002; Valentin-Hansen et al., 2004; Waters and Storz, 2009). Most Hfq homologs are known to function as homohexamers with two independent RNA-binding motifs (Brennan and Link, 2007), and *hfq* mutants exhibit pleiotropic phenotypes (Tsui et al., 1994). In recent years, Hfq has been established as an important virulence factor in bacterial pathogens (Hansen and Kaper, 2009). Deletion of *hfq* has long been known to impair the expression of σ^S (Brown and Elliott, 1996), a general stress sigma factor essential for *Salmonella* virulence in mice (Fang et al., 1992). *hfq* mutation was also revealed to attenuate

the ability of *Salmonella* to invade epithelial cells, secrete virulence factors, infect mice, and survive inside cultured macrophages (Sittka et al., 2007). Transcriptomic analysis revealed that Hfq controls the expression of *Salmonella* genes in several horizontally acquired pathogenicity islands (SPI-1, -2, -4, -5), two sigma factor regulons, and the flagellar gene cascade (Sittka et al., 2008). However, the role Hfq in the drug resistance of *Salmonella* is unknown.

In this study, we demonstrate that Hfq affects drug susceptibilities in *Salmonella*. In addition, we reveal that SmvA and not the AcrB drug efflux system contributes to the Hfq-mediated drug resistance of *Salmonella*, whereas it has been reported that AcrB contributes to the Hfq-mediated drug resistance of *Escherichia coli*. Our data suggest that Hfq plays an important role in controlling drug susceptibility against acriflavine and that the SmvA efflux pump is involved in this susceptibility in *Salmonella*.

MATERIALS AND METHODS

BACTERIAL STRAINS, PLASMIDS, AND GROWTH CONDITIONS

The bacterial strains and plasmids used in this study are listed in **Table 1**. The strains of *S. enterica* serovar Typhimurium used in this study were derived from the wild-type strain ATCC 14028s (Fields et al., 1986). Bacterial strains were grown at 37°C in Lysogeny Broth (LB). Ampicillin was added to the growth medium at a final concentration of 100 μg/ml for plasmid maintenance.

CONSTRUCTION OF GENE DELETION MUTANTS

The Δ*acrB* (NKS148) and Δ*tolC* (NKS174) mutants were constructed as described previously (Horiyama et al., 2010). To construct Δ*hfq* and Δ*smvA* mutants, gene disruption was performed as described by Datsenko and Wanner (2000). The following oligonucleotide primers were used for the construction of the mutants: *hfq*-P1 (GAAAGGTTCAAAGTACAAATAAGCATATAAGGAAAAGAGAGTGTAGGCTGGAGCTGCTTC); *hfq*-P2 (ATTATCCGACGCCCCCGACATGGATAAACAGCGCGTGAACCATATGAATATCCTCCTTAG); *smvA*-P1 (CTGGACAAGCGTCCAAATTTGAGTTTTTGAAGGGAGAGTTGTGTAGGCTGGAGCTGCTTC); and *smvA*-P2 (CCAGCTAGCGCATTAAGCGCTTATCTCACCAGGCGTTATGCATATGAATATCCTCCTTAG). The chloramphenicol resistance gene *cat*, flanked by Flp recognition sites, was amplified by PCR using the primers listed above. The resulting PCR products were used to transform the recipient ATCC 14028s strain that harbors the plasmid pKD46, which expresses Red recombinase. The chromosomal structure of the mutated *loci* was verified by PCR. *cat* was eliminated using the plasmid pCP20, as described previously (Datsenko and Wanner, 2000). To construct the Δ*acrB*Δ*hfq*, Δ*tolC*Δ*hfq*, and Δ*smvA*Δ*hfq* double mutants, the deletions were transferred to strains by P22 transduction as described by Davis et al. (1980).

Table 1 | *Salmonella* strains and plasmids used in this study.

Strain or plasmid	Characteristics	Source or reference
ATCC 14028s	*Salmonella enterica* serovar Typhimurium wild-type	Fields et al. (1986)
NKS798	Δ*hfq*	This study
NKS148	Δ*acrB*	Horiyama et al. (2010)
NKS799	Δ*acrB* Δ*hfq*	This study
NKS174	Δ*tolC*	Horiyama et al. (2010)
NKS802	Δ*tolC* Δ*hfq*	This study
NKS771	Δ*smvA*	This study
NKS1390	Δ*smvA* Δ*hfq*	This study
NKS1396	Δ*smvA* Δ*hfq*/vector	This study
NKS1395	Δ*smvA* Δ*hfq*/p*smvA*	This study
Vector	pBR322, ColE1-type vector, TCR ApR	Takara Bio, Inc.
Plasmid	p*smvA*, *smvA* gene cloned into pBR322, Ap^R	This study

PLASMID CONSTRUCTION

smvA was amplified from ATCC 14028s genomic DNA by using the primers GCGCATGCCATTCGTTCAACTTACCGAGG and GCGTCGACGGAAATGGACTCCCCCTGCC, which introduced *Sph*I and *Sal*I sites (underlined in the primer sequences above). The fragment was cleaved with *Sph*I and *Sal*I and then cloned into the corresponding sites of pBR322, resulting in p*smvA* (**Table 1**).

DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATION OF TOXIC COMPOUNDS

The antibacterial activities of various agents were determined on LB agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl) plates containing nalidixic acid, acriflavine, rhodamine 6G, benzalkonium, oxacillin, cefamandole, sodium dodecyl sulfate, or norfloxacin (Sigma, St. Louis, MO, USA) at various concentrations as indicated in **Table 2**. Agar plates were made by the twofold agar dilution technique, as described previously (Horiyama et al., 2010). To determine minimum inhibitory concentrations (MICs), bacteria were grown in LB broth at 37°C overnight, diluted into the same medium, and then tested at a final inoculum size of 1×10^5 cfu/μl using a multipoint inoculator (Sakuma Seisakusyo, Tokyo, Japan) after incubation at 37°C for 20 h. The MIC was the lowest concentration of a compound that inhibited cell growth.

RESULTS AND DISCUSSION

Hfq AFFECTS THE INTRINSIC DRUG SUSCEPTIBILITY OF *SALMONELLA*

To investigate the role of Hfq in drug susceptibilities, *hfq* was deleted from *S. enterica* serovar Typhimurium strain ATCC 14028s, as described in the Section "Materials and Methods." Δ*hfq* mutant was more sensitive to acriflavine (64-fold) than the wild-type strain (**Table 2**). The MICs of nalidixic acid, rhodamine 6G, benzalkonium, oxacillin, cefamandole, sodium dodecyl sulfate, and norfloxacin were the same for Δ*hfq* mutant as those for the wild-type strain. These data indicate that Hfq affects the intrinsic acriflavine resistance of *Salmonella*.

AcrB IS NOT INVOLVED IN Hfq-MEDIATED DRUG SUSCEPTIBILITY OF *SALMONELLA*

In *E. coli*, it has been demonstrated that the AcrB multidrug efflux pump is involved in Hfq-mediated multidrug resistance (Yamada et al., 2010). To investigate whether AcrB in *Salmonella* is also involved in Hfq-mediated drug susceptibility of this organism, we measured MICs of several toxic compounds against Δ*acrB*

Table 2 | Susceptibility of *Salmonella* strains to toxic compounds.

Strain	MIC (μg/ml)							
	NAL	**ACR**	**R6G**	**BENZ**	**OXA**	**FAM**	**SDS**	**NFLX**
Wild-type	4	4096	4096	64	1024	0.5	>32768	0.25
Δ*hfq*	4	**64**	4096	64	1024	0.5	>32768	0.25
Δ*acrB*	**1**	**64**	**8**	**4**	**2**	**0.125**	**128**	**0.031**
Δ*acrB* Δ*hfq*	**1**	**16**	**8**	**4**	**2**	**0.125**	**128**	**0.031**
Δ*tolC*	**0.5**	**32**	**8**	**4**	**0.5**	**0.125**	**32**	**0.031**
Δ*tolC* Δ*hfq*	**0.5**	**8**	**8**	**4**	**0.5**	**0.125**	**32**	**0.031**
Δ*smvA*	4	**64**	4096	64	1024	0.5	>32768	0.25
Δ*smvA* Δ*hfq*	4	**64**	4096	64	1024	0.5	>32768	0.25
Δ*smvA* Δ*hfq*/vector	4	**64**	4096	64	N.D.	N.D.	>32768	0.25
Δ*smvA* Δ*hfq*/p*smvA*	4	4096	4096	64	N.D.	N.D.	>32768	0.25

NAL, nalidixic acid; ACR, acriflavine; R6G, rhodamine 6G; BENZ, benzalkonium; OXA, oxacillin; FAM, cefamandole; SDS, sodium dodecyl sulfate; NFLX, norfloxacin.
Values in bold are smaller than those of the wild-type strain.
MIC determinations were repeated at least three times. Shown is one of the three experiments, which gave same results.
N.D., not determined, because vectors have an ampicillin resistance cassette.

mutant (**Table 2**). The Δ*acrB* mutant was sensitive to nalidixic acid (fourfold), acriflavine (64-fold), rhodamine 6G (512-fold), benzalkonium (16-fold), oxacillin (512-fold), cefamandole (fourfold), sodium dodecyl sulfate (>256-fold), and norfloxacin (eightfold). Although Δ*acrB* mutant was as sensitive to acriflavine as Δ*hfq* mutant, the drug susceptibility pattern for other compounds was very different among these mutants. Δ*acrB*Δ*hfq* double mutant was more sensitive to acriflavine (fourfold) than Δ*acrB* mutant, indicating that the deletion of *acrB* did not impair the effect of *hfq* deletion on *Salmonella* susceptibility. Based on these data, it was suggested that factors other than AcrB may be involved in the Hfq-mediated drug susceptibility of *Salmonella* because the drug susceptibility pattern of Δ*acrB* was very different from that of Δ*hfq*, and the deletion of *hfq* from Δ*acrB* mutant made this strain more sensitive to acriflavine.

TolC IS NOT INVOLVED IN THE Hfq-MEDIATED DRUG SUSCEPTIBILITY OF *SALMONELLA*

TolC is a major outer membrane channel, and a variety of inner membrane and accessory protein interact with TolC to expel structurally diverse molecules. We previously identified that seven drug efflux systems, AcrAB, AcrD, AcrEF, MdsAB, MdtABC, EmrAB, and MacAB, in *Salmonella* that require TolC to function (Horiyama et al., 2010). To investigate whether TolC-dependent type drug efflux systems are involved in Hfq-mediated drug susceptibility, we measured MICs of compounds against Δ*tolC* mutant (**Table 2**). Δ*tolC* mutant was sensitive to nalidixic acid (eightfold), acriflavine (128-fold), rhodamine 6G (512-fold), benzalkonium (16-fold), oxacillin (2048-fold), cefamandole (fourfold), sodium dodecyl sulfate (>1024-fold), and norfloxacin (eightfold). The susceptibilities of Δ*tolC* mutant to oxacillin and sodium dodecyl sulfate were higher than those of the Δ*acrB* mutant probably because TolC-dependent type efflux systems other than AcrB are involved in the efflux of these compounds. The deletion of *hfq* from Δ*tolC* mutant made this strain more sensitive to acriflavine (fourfold), meaning that the TolC-dependent type drug efflux systems are not involved in the Hfq-mediated drug susceptibility of *Salmonella*.

INVOLVEMENT OF SmvA EFFLUX PUMP IN THE Hfq-MEDIATED ACRIFLAVINE SUSCEPTIBILITY

Among the tested compounds, Δ*hfq* mutant was specifically susceptible to acriflavine (**Table 2**) as mentioned above. Because it has been reported that SmvA is an important efflux pump for acriflavine (Villagra et al., 2008), we hypothesized that SmvA may be involved in the Hfq-mediated acriflavine susceptibility of *Salmonella*. Similarly, as Δ*hfq* mutant, Δ*smvA* mutant was more sensitive to acriflavine (64-fold) than the wild-type strain (**Table 2**). This phenotype is in good agreement with a previous report (Villagra et al., 2008). MIC of acriflavine against Δ*smvA*Δ*hfq* double mutant was similar to that against Δ*smvA* mutant, indicating that deletion of *smvA* impaired the effect of *hfq* deletion on acriflavine susceptibility. Moreover, p*smvA*, which expressed *smvA*, conferred acriflavine resistance to Δ*smvA*Δ*hfq* double mutant. MIC of acriflavine against Δ*smvA*Δ*hfq*/p*smvA* strain is similar to that against the wild-type strain (**Table 2**). Taken together, these results indicated that Hfq regulates the intrinsic acriflavine resistance of *Salmonella* and SmvA plays an important role in this resistance because the drug susceptibility pattern of Δ*smvA* was same as that of Δ*hfq*, and the deletion of *hfq* from Δ*smvA* mutant did not change the acriflavine susceptibility of this strain.

In this study, we investigated the role of Hfq in the drug susceptibility of *S. enterica* serovar Typhimurium ATCC 14028s and found that Hfq plays a role in its intrinsic acriflavine resistance and that SmvA efflux pump is involved in this resistance. Interestingly, Δ*hfq* mutant of *Salmonella* was specifically sensitive to acriflavine among the tested compounds. This phenotype is very different from Δ*hfq* mutant of *E. coli* W3104 or MC4100 (Yamada et al., 2010). In case of *E. coli*, Δ*hfq* mutant was susceptible to various compounds including acriflavine, benzalkonium, cefamandole, chloramphenicol, crystal violet, nalidixic acid, novobiocin, oxacillin, and rhodamine 6G because Hfq positively

regulates the production of the AcrB drug efflux pump (Yamada et al., 2010). However, AcrB was considered not to be involved in the Hfq-mediated intrinsic acriflavine resistance of *Salmonella*. These observations suggest the differential regulation of genes by Hfq between *E. coli* and *Salmonella*. Indeed, transcriptomic analysis revealed that Hfq controls the *Salmonella* gene expression in several horizontally acquired pathogenicity islands (SPI-1, -2, -4, -5) that are not present in *E. coli* (Sittka et al., 2008). Unlike the AcrAB drug efflux system, which is widely distributed throughout all Enterobacteriaceae, homologs of SmvA are not found in *E. coli* and *Shigella* spp. Villagra et al. (2008) suggested that acriflavine is a substrate for both AcrB and SmvA efflux pumps, but SmvA pump plays the major role in the efflux of acriflavine in *Salmonella*. This may explain why SmvA and not AcrB drug efflux system contributes to the Hfq-mediated drug resistance of *Salmonella*.

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