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H-NS Modulates Multidrug Resistance of *Salmonella enterica* Serovar Typhimurium by Repressing Multidrug Efflux Genes *acrEF*▽

Kunihiko Nishino,[1,3]* Mitsuko Hayashi-Nishino,[2] and Akihito Yamaguchi[2,4]

Laboratory of Microbiology and Infectious Diseases[1] and Department of Cell Membrane Biology,[2] Institute of Scientific and Industrial Research, Osaka University, Osaka 567-0047, Japan; PRESTO, Japan Science and Technology Corporation, Tokyo 102-0075, Japan[3]; and Graduate School of Pharmaceutical Sciences, Osaka University, Osaka 565-0871, Japan[4]

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Screening of *Salmonella* mutants for the ability to increase β-lactam resistance has led to the identification of a mutation in *hns*, which codes for the histone-like nucleoid structuring protein (H-NS). In this study, we report that H-NS modulates multidrug resistance through repression of the genes that encode the AcrEF multidrug efflux pump in *Salmonella enterica* serovar Typhimurium.

Bacterial multidrug efflux pumps confer resistance to a wide range of antibiotics, dyes, and biocides. In gram-negative bacteria, pumps belonging to the resistance-nodulation-cell division family are especially effective in conferring resistance (14). For example, high-level fluoroquinolone resistance in *Salmonella enterica* serovar Typhimurium phage type DT204 has been shown to be largely attributable to multiple target gene mutations and active efflux by the AcrAB-TolC system (3, 4). Recent studies have shown that *S. enterica* has nine functional drug efflux pumps (15). Because many of these multidrug transporters 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 efflux pumps lies in the regulation of pump expression. Currently available data show that multidrug efflux pumps are often expressed under precise and elaborate transcriptional control (16). Expression of *acrAB*, which encodes the major AcrAB efflux pump, is subject to multiple levels of regulation. In *S. enterica*, it was reported that mutation in the *acrR* repressor contributes to the overexpression of *acrAB* and increases resistance to multiple drugs (20). Recent reports showed that the RamA activator (2, 13, 22) and RamR (1) repressor are also involved in the regulation of *acrAB*.

However, few data are available on the regulation of *S. enterica* multidrug efflux genes other than *acrAB*. We therefore screened for *S. enterica* mutations that increase multidrug resistance levels in this organism. The *S. enterica* strains used in this study were derived from wild-type strain ATCC 14028s (9). We used a host strain (NKS175) lacking a functional *acrB* gene in the screening in order to identify regulatory elements involved in the expression of other multidrug resistance systems. NKS175 was subjected to transposon mutagenesis with the EZ-Tn5<R6Kγ*ori*/KAN-2>Tnp Transposome kit (Epicentre) according to the manufacturer's instructions. Briefly, the transposome complex was electroporated into NKS175 and then approximately 10,000 colonies were screened. Cells were plated on LB agar medium (21) containing 25 μg/ml kanamycin and inhibitory concentrations of various drugs. In one experiment, the medium contained 8 μg/ml oxacillin, which had an MIC of 2 μg/ml against NKS175. When a mutant colony that grew on this medium was purified and reexamined, we indeed found an eightfold increase in the oxacillin MIC (16 μg/ml) against the transposon insertion mutant (data not shown).

Sequencing determined that the transposon was inserted into the coding sequence of *hns*. H-NS belongs to a family of small, abundant, nucleoid-associated proteins of gram-negative bacteria that have the ability to bind DNA, and it has been shown to act as a transcriptional repressor (7). It seemed possible that deletion of *hns* might be causing the transcriptional activation of genes involved in oxacillin resistance. To test whether deletion of *hns* confers oxacillin resistance on the *S. enterica* NKS175 strain, a nonpolar deletion was made in the *hns* gene with the lambda Red system (5). The oxacillin MICs for cells lacking *hns* (NKS291) was eight times higher than that for NKS175 cells (16 versus 2 μg/ml) (Table 1), suggesting that the deletion of the H-NS regulator indeed conferred oxacillin resistance on *S. enterica*. We investigated the effect of *hns* deletion on the susceptibility of *S. enterica* to other toxic compounds. Various drugs were tested, including common substrates of multidrug efflux pumps, and we found that *hns* deletion increased the resistance of the NKS175 strain to cloxacillin, nafcillin, cefamandole, methylene blue, and rhodamine 6G (Table 1). These results indicate that the deletion of H-NS induces multidrug resistance in *S. enterica*. It has been reported that many multidrug efflux systems need the membrane channel TolC for their function (8, 10, 17). To determine whether H-NS-mediated multidrug resistance is attributable to the TolC-dependent drug efflux pump(s), we investigated the effect of *tolC* deletion on the drug resistance of the *hns* deletion-containing cells. MICs against NKS419 were the same as those against NKS174, indicating that the *tolC* deletion completely inhibited H-NS-mediated multidrug resistance (Table 1). This result indicates that H-NS-mediated multidrug resistance is attributable to increased functioning of a TolC-dependent drug efflux pump.

We investigated drug efflux activity in the *hns*-deficient mutant. The rhodamine 6G efflux activities of *S. enterica* NKS175

* Corresponding author. Mailing address: Institute of Scientific and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki, Osaka 567-0047, Japan. Phone: 81-6-6879-8546. Fax: 81-6-6879-8549. E-mail: nishino@sanken.osaka-u.ac.jp.

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TABLE 1. Susceptibility of *Salmonella* strains to β-lactams and toxic compounds

Strain	Genotype	MIC (μg/ml)[a]					
		OXA	CLOX	NAF	FAM	MB	R6G
ATCC 14028s	Wild type	**512**	**512**	**>512**	**1**	**>512**	**>512**
NKS175	Δ*acrB*	2	4	8	0.063	16	8
NKS291	Δ*acrB hns*	**16**	**128**	**64**	**0.25**	**512**	**32**
NKS174	Δ*tolC*	0.5	0.5	1	0.063	16	8
NKS419	Δ*tolC hns*	0.5	0.5	1	0.063	16	8
NKS176	Δ*acrEF*	**512**	**512**	**>512**	**0.5**	**>512**	**>512**
NKS422	Δ*acrB acrEF*	1	4	4	0.063	16	8
NKS416	Δ*acrB acrEF hns*	1	4	4	0.063	16	8

[a] OXA, oxacillin; CLOX, cloxacillin; NAF, nafcillin; FAM, cefamandole; MB, methylene blue; R6G, rhodamine 6G. Values in bold are larger than those of the Δ*acrB* strain. MIC determinations were repeated at least three times.

(Δ*acrB*) and NKS291 (Δ*acrB hns*) were measured as described previously (18). Briefly, exponential-phase cultures were harvested and washed with 100 mM potassium phosphate buffer (pH 7.5) and then the cells were incubated with 1 μM rhodamine 6G and 40 μM carbonyl cyanide *m*-chlorophenylhydrazone at 37°C for 1 h. These cells were then resuspended in the same buffer containing 25 mM glucose and subjected to fluorescence measurement. As shown in Fig. 1A, rapid efflux of rhodamine 6G from Δ*acrB hns* cells was observed as an increase in fluorescence intensity. On the other hand, no significant efflux was observed in Δ*acrB* mutant cells. This result clearly indicates that *hns* deletion induces an active efflux system.

It has been reported that *S. enterica* has nine drug efflux systems, including AcrAB, AcrD, AcrEF, EmrAB, MacAB, MdfA, MdsABC, MdtABC, and MdtK (15). In order to determine which drug efflux pump shows increased expression when *hns* is deleted, we used quantitative reverse transcription-PCR to investigate changes in the levels of drug efflux gene mRNAs dependent on *hns* deletion. Total RNAs were isolated from exponential-phase cultures of the wild-type and Δ*hns* mutant strains, and cDNA samples were synthesized with TaqMan reverse transcription reagents (PE Applied Biosystems) with random hexamers as primers. Real-time PCR of the cDNAs was performed with each specific primer pair and SYBR green PCR Master Mix (PE Applied Biosystems) as described previously (15). The expression levels of drug efflux pump genes and *tolC* in the Δ*hns* mutant were compared with those in the wild-type strain. The results are shown in Fig. 1B. Expression of *acrE* and *acrF* was significantly increased (more than 10-fold). The expression of *mdsC*, *macA*, and *macB* was slightly increased in the Δ*hns* mutant.

In order to determine whether multidrug resistance mediated by *hns* deletion is due to increased expression of *acrEF*, we investigated the effects of deleting these genes on H-NS-mediated multidrug resistance. In the Δ*acrB acrEF* mutant strain, deletion of *hns* conferred no drug resistance (Table 1), whereas it conferred multidrug resistance on the Δ*acrB* mutant strain. Together, these data indicate that the multidrug resistance conferred by H-NS deletion is due to derepression of the *acrEF* multidrug efflux genes.

In this study, we performed a genome-wide search for a regulator of multidrug resistance in *S. enterica* by random insertion and found that H-NS downregulates the expression of *acrEF*. We initially found by random insertion that the mutation in *hns* conferred oxacillin resistance on the NKS175 strain. Then we investigated the susceptibility of the strain lacking *hns* to various drugs, including common substrates of multidrug efflux pumps, and found that H-NS modulates *S. enterica* resistance to oxacillin, cloxacillin, nafcillin, cefamandole, methylene blue, and rhodamine 6G (Table 1). The DNA-binding protein H-NS is a global transcriptional regulator that specifically silences horizontally acquired virulence genes in *Salmonella* (12). This process is achieved through interactions between H-NS and AT-rich DNA regions with low specificity (6).

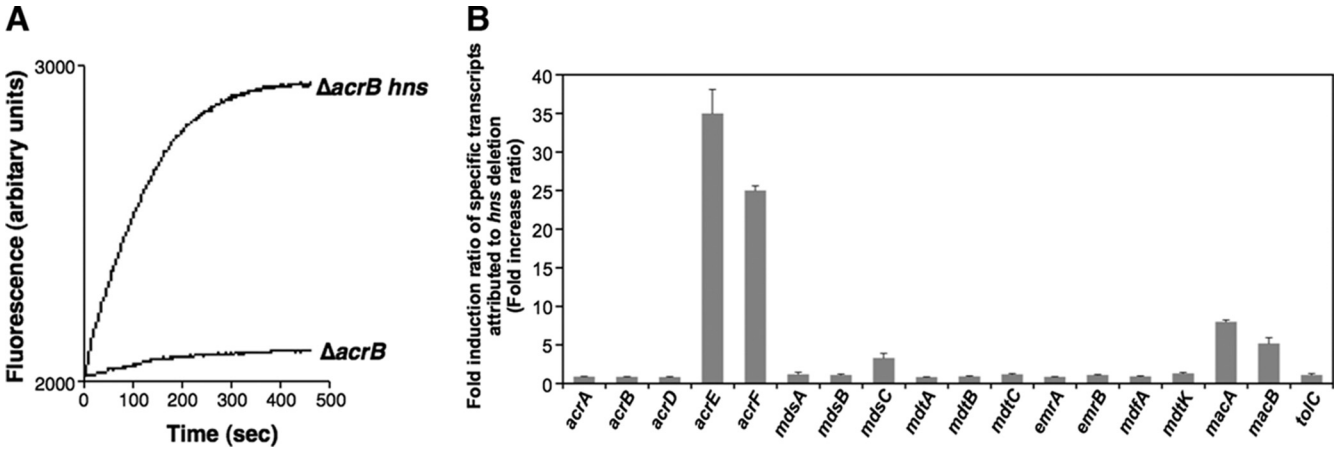

FIG. 1. Effect of *hns* deletion on efflux activity and of drug efflux gene expression levels. (A) Rhodamine 6G efflux activity in *S. enterica* cells. Energy-starved cells of *S. enterica* NKS175 (Δ*acrB*) and NKS291 (Δ*acrB hns*) were loaded with rhodamine 6G, and efflux activities were monitored with an F-2000 fluorescence spectrophotometer (Hitachi, Japan). Rhodamine 6G efflux was measured with excitation at 529 nm and emission at 553 nm. (B) Changes in drug efflux and outer membrane channel gene expression. The mRNA transcript level was determined by quantitative real-time PCR. The *n*-fold change ratio was calculated by dividing the expression level of the gene in the NKS285 (Δ*hns* mutant) strain by that in the ATCC 14028s (wild-type) strain. The data correspond to mean values of three independent experiments. Error bars represent the standard deviation.

For the intergenic region located upstream of *acrE* in which H-NS was found to repress expression to 1/35 of the original level, the average GC content is 31.7%, whereas the average GC content of the entire *S. enterica* serovar Typhimurium LT2 genome is 52.2% (11). It should be noted that this intergenic region harbors a 100-bp AT-rich DNA region located 295 to 196 bp upstream of the start codon of *acrE*, which has a GC content of 16.0%. The AcrF efflux pump is highly homologous to AcrB in *S. enterica* with an 81.4% amino acid identity and similar substrate specificities (15). We found that *acrEF* is repressed by H-NS, whereas *acrAB* is not and is constitutively expressed. Because overexpression of *acrEF* confers an effect similar to that of *acrAB* (15), H-NS represses *acrEF* and might confer a fitness advantage on this organism. AcrEF might be required for drug resistance in *S. enterica* when AcrAB does not function. Indeed, in a fluoroquinolone-resistant mutant selected from *S. enterica* serovar Typhimurium DT204 *acrB* mutant cells, an insertion sequence (IS*1* or IS*10*) was found integrated upstream of the *acrEF* operon, which increased the expression level of *acrEF* (19). Further investigation of the regulation of multidrug efflux systems in several natural environments such as those inside hosts is needed to elucidate the biological significance of their regulatory networks.

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