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Role of Histone-Like Protein H-NS in Multidrug Resistance of *Escherichia coli*

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The histone-like protein H-NS is a major component of the bacterial nucleoid and plays a crucial role in global gene regulation of enteric bacteria. It is known that the expression of a variety of genes is repressed by H-NS, and mutations in *hns* result in various phenotypes, but the role of H-NS in the drug resistance of *Escherichia coli* has not been known. Here we present data showing that H-NS contributes to multidrug resistance by regulating the expression of multidrug exporter genes. Deletion of the *hns* gene from the Δ*acrAB* mutant increased levels of resistance against antibiotics, antiseptics, dyes, and detergents. Decreased accumulation of ethidium bromide and rhodamine 6G in the *hns* mutant compared to that in the parental strain was observed, suggesting the increased expression of some drug exporter(s) in this mutant. The increased drug resistance and decreased drug accumulation caused by the *hns* deletion were completely suppressed by deletion of the multifunctional outer membrane channel gene *tolC*. At least eight drug exporter systems require TolC for their functions. Among these, increased expression of *acrEF*, *mdtEF*, and *emrKY* was observed in the Δ*hns* strain by quantitative real-time reverse transcription-PCR analysis. The Δ*hns*-mediated multidrug resistance pattern is quite similar to that caused by overproduction of the AcrEF exporter. Deletion of the *acrEF* gene greatly suppressed the level of Δ*hns*-mediated multidrug resistance. However, this strain still retained resistance to some compounds. The remainder of the multidrug resistance pattern was similar to that conferred by overproduction of the MdtEF exporter. Double deletion of the *mdtEF* and *acrEF* genes completely suppressed Δ*hns*-mediated multidrug resistance, indicating that Δ*hns*-mediated multidrug resistance is due to derepression of the *acrEF* and *mdtEF* drug exporter genes.

The emergence of bacterial multidrug resistance has become an increasing problem in the treatment of infectious diseases. Multidrug resistance often results from the overexpression of multidrug efflux transporters. Recent genome sequence analysis has revealed that bacteria have many intrinsic putative and proven drug transporter genes. We previously cloned all of the gene clusters encoding putative and known drug transporters of *Escherichia coli* and revealed that 20 genes actually encode the transporters of some drugs and/or toxic compounds (30). Since the substrate spectra of these multidrug transporters partially overlap, we are intrigued by the question of why bacteria, with their economically organized genomes, harbor such large sets of multidrug efflux genes. The key to understanding how bacteria utilize these multiple transporters lies in analysis of the regulation of transporter expression. In the present study, we analyzed the relationship between the regulation of drug transporters and the *E. coli* nucleoid-associated protein H-NS (histone-like nucleoid structuring protein).

H-NS, one of the most abundant proteins in the *E. coli* nucleoid, is widely distributed within gram-negative bacteria (4). H-NS was initially described as a transcription factor (10) and was later shown to play roles in the structure and function of chromosomal DNA (2, 40). H-NS is involved in the condensation of the bacterial chromosome and regulates the expression of many genes (~5% of the open reading frames of the *E. coli* genome). Most of these genes are related to bacterial adaptation to environmental conditions and/or virulence (9). H-NS modulates transcription through the formation of large nucleoprotein structures (6, 13, 39). Mutations in *hns* result in various phenotypes, because H-NS is involved in the regulation of a variety of genes. However, the role of H-NS in the drug resistance of *E. coli* is unknown. In this paper, we report that H-NS controls the multidrug resistance of *E. coli* by regulating the expression of drug exporter genes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this work were *E. coli* K-12 derivatives (Table 1). They were grown at 37°C in Luria-Bertani (LB) broth (34). Cells were rapidly collected for total RNA extraction when the cultures reached an optical density at 600 nm of 0.6.

RNA extraction. Total RNA from bacterial cultures was isolated by using an RNeasy Protect bacterial minikit and RNase-free DNase (both from Qiagen) in accordance with the manufacturer's instructions. The absence of genomic DNA in DNase-treated RNA samples was confirmed by inspecting nondenaturing agarose electrophoresis gels and also by performing PCR with primers known to target the genomic DNA. RNA concentrations were determined spectrophotometrically (35).

Determination of specific transcript levels by quantitative real-time reverse transcription-PCR (qRT-PCR). Bulk cDNA samples were synthesized from total RNA derived from *E. coli* cells by using TaqMan reverse transcription reagents (Perkin-Elmer [PE] Applied Biosystems) and random hexamers as primers. Specific primer pairs were designed with ABI PRISM Primer Express software (PE Applied Biosystems). *rrsA* of the 16S rRNA gene was chosen as the normalizing gene. Real-time PCR was performed with each specific primer pair by

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TABLE 1. Bacterial strains used in this study

Strain	Genotype	Reference or source
DH5α	*recA endA1 hsdR17 supE4 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* (ϕ80 *lacZ*ΔM15)	34
W3104	Wild type	41
W3104Δ*acrAB*	Derivative of W3104 that lacks *acrAB*	15
W3104Δ*acrAB*Δ*emrKY*	Derivative of W3104Δ*acrAB* that lacks *emrKY*	This study
W3104Δ*acrAB*Δ*mdtEF*	Derivative of W3104Δ*acrAB* that lacks *mdtEF*	This study
W3104Δ*acrAB*Δ*acrEF*	Derivative of W3104Δ*acrAB* that lacks *acrEF*	This study
W3104Δ*acrAB*Δ*acrEF*Δ*emrKY*	Derivative of W3104Δ*acrAB* that lacks *emrKY*	This study
W3104Δ*acrAB*Δ*acrEF*Δ*mdtEF*	Derivative of W3104Δ*acrAB*Δ*acrEF* that lacks *mdtEF*	This study
W3104Δ*acrAB*Δ*acrEF*Δ*mdtEF*Δ*emrKY*	Derivative of W3104Δ*acrAB*Δ*acrEF*Δ*mdtEF* that lacks *emrKY*	This study
W3104Δ*acrAB*Δ*tolC*	Derivative of W3104Δ*acrAB* that lacks *tolC*	This study
W3104Δ*hns*	Derivative of W3104 that lacks *hns*	This study
W3104Δ*acrAB*Δ*hns*	Derivative of W3104Δ*acrAB* that lacks *hns*	This study
W3104Δ*acrAB*Δ*hns*Δ*tolC*	Derivative of W3104Δ*acrAB*Δ*hns* that lacks *tolC*	This study
W3104Δ*acrAB*Δ*hns*Δ*emrKY*	Derivative of W3104Δ*acrAB*Δ*hns* that lacks *emrKY*	This study
W3104Δ*acrAB*Δ*hns*Δ*mdtEF*	Derivative of W3104Δ*acrAB*Δ*hns* that lacks *mdtEF*	This study
W3104Δ*acrAB*Δ*hns*Δ*acrEF*	Derivative of W3104Δ*acrAB*Δ*hns* that lacks *acrEF*	This study
W3104Δ*acrAB*Δ*hns*Δ*acrEF*Δ*emrKY*	Derivative of W3104Δ*acrAB*Δ*hns*Δ*acrEF* that lacks *emrKY*	This study
W3104Δ*acrAB*Δ*hns*Δ*acrEF*Δ*mdtEF*	Derivative of W3104Δ*acrAB*Δ*hns*Δ*acrEF* that lacks *mdtEF*	This study
W3104Δ*acrAB*Δ*hns*Δ*acrEF*Δ*mdtEF*Δ*emrKY*	Derivative of W3104Δ*acrAB*Δ*hns*Δ*acrEF*Δ*mdtEF* that lacks *emrKY*	This study
W3104Δ*acrAB*Δ*hns*Δ*evgAS*	Derivative of W3104Δ*acrAB*Δ*hns* that lacks *evgAS*	This study
W3104Δ*acrAB*Δ*hns*Δ*acrEF*Δ*evgAS*	Derivative of W3104Δ*acrAB*Δ*hns*Δ*acrEF* that lacks *evgAS*	This study
W3104Δ*acrAB*Δ*ydeO*	Derivative of W3104Δ*acrAB* that lacks *ydeO*	This study
W3104Δ*acrAB*Δ*hns*Δ*ydeO*	Derivative of W3104Δ*acrAB*Δ*hns* that lacks *ydeO*	This study

using SYBR Green PCR Master Mix (PE Applied Biosystems). Reactions were performed with an ABI PRISM 7000 sequence detection system (PE Applied Biosystems); during the reactions, the fluorescence signal due to SYBR Green intercalation was monitored to quantify the double-stranded DNA product formed in each PCR cycle.

Susceptibility testing. The antibacterial activities of the agents were determined on L agar (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) plates containing various compounds (oxacillin, erythromycin, novobiocin, doxorubicin, acriflavine, crystal violet, ethidium bromide, methylene blue, rhodamine 6G, tetraphenylphosphonium bromide, benzalkonium chloride, sodium dodecyl sulfate, and sodium deoxycholate) at various concentrations, as indicated. Agar plates were made by the twofold agar dilution technique recommended by the Japan Society of Chemotherapy (11, 12). Organisms were tested at a final inoculum size of 10^5 CFU/spot, with the use of a multipoint inoculator (Sakuma Seisakusyo, Tokyo, Japan), and were incubated at 37°C for 18 h in air. MICs of drugs and toxic compounds were determined as the concentrations that severely inhibited bacterial cell growth.

Construction of in-frame deletion mutants. To construct gene deletion mutants from *E. coli* W3104 cells (41), precise in-frame deletions were generated by crossover PCR. Four sets of oligonucleotide primers (designations ending in -No, -Ni, -Ci, and -Co [Table 2]) were used for each gene. The fragment containing the deletion was then cloned into the *Bam*HI site of the pKO3 vector (18), a gene replacement vector that contains a temperature-sensitive origin of replication and markers for positive and negative selection for chromosome integration and excision. The deletion was introduced into the chromosome by use of the pKO3 gene replacement protocol, as described previously (18).

Observation of drug accumulation in *E. coli* cells. *E. coli* cells were spotted onto L-agar plates containing a low concentration of ethidium bromide (1 μg/ml) or rhodamine 6G (0.5 μg/ml) at a final inoculum size of 10^5 CFU/spot, by use of a multipoint inoculator (Sakuma Seisakusyo), and were incubated at 37°C for 18 h in air. Drug accumulation in *E. coli* cells was observed as the fluorescence of ethidium bromide or rhodamine 6G in cells under UV light, by use of an Electronic U.V. Transilluminator FAS-II (TOYOBO, Osaka, Japan).

RESULTS

Deletion of the *hns* gene increases resistance to multiple antibiotics and toxic compounds. Deletion of *hns* did not change the drug susceptibility of wild-type *E. coli* W3104 (41) (Table 3), because the intrinsic multidrug efflux transporter AcrAB masks the effect of *hns* deletion (Table 3). In addition, qRT-PCR analysis did not detect any changes in the expression levels of *acrA* and *acrB* in the *hns* deletion strain relative to the wild-type strain (data not shown). We therefore used a host strain lacking the *acrAB* gene (W3104Δ*acrAB*) (15). AcrAB is constitutively expressed in *E. coli* and is largely responsible for the intrinsic resistance of *E. coli* to dyes, detergents, and most lipophillic antibiotics (38). *E. coli* W3104Δ*acrAB* showed hypersensitivity to these compounds (Table 3). Deletion of *hns* increased the drug resistance of the *acrAB* deletion strain to multiple structurally unrelated compounds such as antibiotics, antiseptics, dyes, and detergents, as shown in Table 3.

Effect of deletion of *hns* on drug accumulation in *E. coli* cells. One of the major mechanisms of bacterial multidrug resistance is active drug efflux. Therefore, we investigated drug efflux in the *hns*-deficient mutant. *E. coli* W3104Δ*acrAB* and W3104Δ*acrAB*Δ*hns* cells were spotted onto agar plates containing 1 μg of ethidium bromide/ml or 0.5 μg of rhodamine 6G/ml, and the plates were then incubated at 37°C for 18 h. Since the concentrations of the drugs were eightfold lower than their MICs for W3104Δ*acrAB*, these compounds did not inhibit cell growth (Fig. 1A and C). Accumulation of these drugs in *E. coli* cells was observed from the fluorescence of ethidium bromide (Fig. 1B) and rhodamine 6G (Fig. 1D) under UV light. As shown in Fig. 1B and D, *hns* deletion resulted in a drastic decrease in fluorescence, clearly indicating the active efflux of these drugs from Δ*hns* cells.

Effect of *tolC* deletion on the effect of *hns* deletion. The results described above indicate that the expression of a multidrug exporter(s) may be increased by *hns* deletion. In a previous study, we revealed that at least 20 intrinsic drug efflux transporters are encoded in the *E. coli* chromosome (30). Among these, RND (resistance nodulation cell division)-family transporters play major roles in both intrinsic and elevated resistance of gram-negative bacteria to a wide range of noxious

TABLE 2. Oligonucleotides used for construction and verification of deletion strains

Oligonucleotide	Oligonucleotide sequence (5′ to 3′)
acrA-No	CGCGGATCCATTCGCATTTGTGGAATATAATCTCCATCA
acrA-Ni	CACGCAATAACCTTCACACTCCAAATTTATAACCATATGTAAACCTCGAGTGTCCG
acrB-Ci	GTTATAAATTTGGAGTGTGAAGGTTATTGCGTGTGATACAACGTGTAATCACTAAGGCC
acrB-Co	CGCGGATCCATGGAAAAAACTTACTGACCTGGAC
emrK-No	CGCGGATCCTGGATACCGTTAACTCCGGGG
emrK-Ni	CACGCAATAACCTTCACACTCCAAATTTATAACCACTATTATCTCTCATTTCTCATAGAT
emrY-Ci	GTTATAAATTTGGAGTGTGAAGGTTATTGCGTGTGATGATAAAAGGAGGGGGTTATAGCG
emrY-Co	CGCGGATCCTGTTGGCGGTGGTCCTGGTGG
mdtE-No	CGCGGATCCCAGTTCAAAATTATGCAACTGATTCTG
mdtE-Ni	CACGCAATAACCTTCACACTCCAAATTTATAACCATTTTAGTCCCTGAAAATTCTTGAG
mdtF-Ci	GTTATAAATTTGGAGTGTGAAGGTTATTGCGTGTAACGTGTAAATGAGAGTAAGGTTGA
mdtF-Co	CGCGGATCCCGTCAAATTCCTCTGCATACTATTGC
acrE-No	CGCGGATCCCGTCGTCTTGCTTACGCCAT
acrE-Ni	CACGCAATAACCTTCACACTCCAAATTTATAACCATTACTATTCCTCAAAAAACCAAAAG
acrF-Ci	GTTATAAATTTGGAGTGTGAAGGTTATTGCGTGTAAATCAGAAACATAAAGGCGCTTTCG
acrF-Co	CGCGGATCCCGTCGTCTTGCTTACGCCAT
tolC-No	CGCGGATCCTCATCCCGGCAACCATCTC
tolC-Ni	CACGCAATAACCTTCACACTCCAAATTTATAACCATTCCTTGTGGTGAAGCAGTAT
tolC-Ci	GTTATAAATTTGGAGTGTGAAGGTTATTGCGTGTGATGACGACGACGGGG
tolC-Co	CGCGGATCCGCTGGATTGCTGGGCC
hns-No	CGCGGATCCCTCCCTTACGAAGCCTTGCATAATCC
hns-Ni	CACGCAATAACCTTCACACTCCAAATTTATAACCATTGTAGTAATCTCAAACTTATAT
hns-Ci	GTTATAAATTTGGAGTGTGAAGGTTATTGCGTGTAATCTTTTGTAGATTGCACTTGC
hns-Co	CGCGGATCCGTTGAATTAGCGCCGGGTGAAAGCGTAC
evgA-No	CGCGGATCCGAAAACGCAATAAATAAAACTACCGCC
evgA-Ni	CACGCAATAACCTTCACACTCCAAATTTATAACCATAGATTATTCCCTTTGCAATGA
evgS-Ci	GTTATAAATTTGGAGTGTGAAGGTTATTGCGTGTAAATAGCGGCTCCCACAATGTTC
evgS-Co	CGCGGATCCCATGGCACCTTTTGATGTTTTCAATACT
ydeO-No	CGCGGATCCTATTCCGGTTGAATTAGAACC
ydeO-Ni	CACGCAATAACCTTCACACTCCAAATTTATAACCATTTTATCTCCTTAAAACAATAAAGT
ydeO-Ci	GTTATAAATTTGGAGTGTGAAGGTTATTGCGTGTGATTATTTGCTAACGAGTAGTCAACC
ydeO-Co	CGCGGATCCGTCGGCGTAAAGTCTGAGAAA

compounds (22, 26, 27). RND transporters need two other proteins for their function: a membrane fusion protein (MFP) and an outer membrane channel. In *E. coli*, all of the five RND drug exporter systems (AcrAB, AcrD, AcrEF, MdtEF, and MdtABC) require the common outer membrane channel TolC for their functions (5, 7, 8, 25, 29–31). (YhiUV has been renamed MdtEF according to the systematic nomenclature available at the EcoGene website [33; http://bmb.med.miami.edu

TABLE 3. Susceptibilities of *E. coli* strains to toxic compounds

Strain	MIC (μg/ml)[a] of:												
	OXA	EM	NOV	DXR	ACR	CV	EBR	MB	R6G	TPP	BENZ	SDS	DOC
W3104	**>256**	**128**	**>512**	**>128**	**256**	**128**	**512**	**>512**	**256**	**512**	**64**	**>512**	**>40,000**
W3104Δ*hns*	**>256**	**64**	**>512**	**>128**	**256**	**128**	**512**	**>512**	**256**	**512**	**64**	**>512**	**>40,000**
W3104Δ*acrAB*	1	4	32	8	16	1	16	8	4	8	4	64	5,000
W3104Δ*acrAB*Δ*emrKY*	1	4	32	8	16	1	16	8	4	8	4	64	5,000
W3104Δ*acrAB*Δ*mdtEF*	1	4	32	8	16	1	16	8	4	8	4	64	5,000
W3104Δ*acrAB*Δ*acrEF*	1	4	32	8	16	1	16	8	4	8	4	64	5,000
W3104Δ*acrAB*Δ*acrEF*Δ*emrKY*	1	4	32	8	16	1	16	8	4	8	4	64	5,000
W3104Δ*acrAB*Δ*acrEF*Δ*mdtEF*	1	4	32	4	16	1	16	8	4	8	4	64	5,000
W3104Δ*acrAB*Δ*acrEF*Δ*mdtEF*Δ*emrKY*	1	4	32	4	16	1	16	8	4	8	4	64	5,000
W3104Δ*acrAB*Δ*tolC*	0.5	2	2	2	8	1	8	8	4	8	4	16	156
W3104Δ*acrAB*Δ*hns*	**64**	**32**	**128**	**>128**	**64**	**128**	**512**	**512**	**256**	**256**	**32**	**>512**	**>40,000**
W3104Δ*acrAB*Δ*hns*Δ*tolC*	0.5	2	2	2	8	1	8	8	4	8	4	32	156
W3104Δ*acrAB*Δ*hns*Δ*emrKY*	**64**	**32**	**128**	**>128**	**64**	**128**	**512**	**512**	**256**	**256**	**32**	**>512**	**>40,000**
W3104Δ*acrAB*Δ*hns*Δ*mdtEF*	**64**	**16**	**128**	**>128**	**64**	**64**	**512**	**512**	**256**	**256**	**16**	**>512**	**>40,000**
W3104Δ*acrAB*Δ*hns*Δ*acrEF*	**4**	**16**	32	**>128**	32	**8**	**64**	**32**	**128**	8	8	128	10,000
W3104Δ*acrAB*Δ*hns*Δ*acrEF*Δ*emrKY*	**4**	**16**	32	**>128**	32	**8**	**64**	**32**	**128**	8	8	128	10,000
W3104Δ*acrAB*Δ*hns*Δ*acrEF*Δ*mdtEF*	1	2	16	2	16	1	16	8	4	8	4	64	2,500
W3104Δ*acrAB*Δ*hns*Δ*acrEF*Δ*mdtEF*Δ*emrKY*	1	2	16	2	16	1	16	8	4	8	4	64	2,500
W3104Δ*acrAB*Δ*hns*Δ*evgAS*	**32**	**32**	**128**	**>128**	**64**	**128**	**512**	**512**	**256**	**256**	**32**	**>512**	**>40,000**
W3104Δ*acrAB*Δ*hns*Δ*acrEF*Δ*evgAS*	2	**16**	32	**>128**	32	**8**	**64**	16	**128**	8	8	128	10,000
W3104Δ*acrAB*Δ*ydeO*	1	4	32	8	16	1	16	8	4	8	4	64	5,000
W3104Δ*acrAB*Δ*hns*Δ*ydeO*	**64**	**32**	**128**	**>128**	**64**	**128**	**512**	**512**	**256**	**256**	**32**	**>512**	**>40,000**

[a] MIC determinations were repeated at least three times. Boldfaced values differ from the MIC of W3104Δ*acrAB* by a factor of more than 4. Abbreviations: OXA, oxacillin; EM, erythromycin; NOV, novobiocin; DXR, doxorubicin; ACR, acriflavine; CV, crystal violet; EBR, ethidium bromide; MB, methylene blue; R6G, rhodamine 6G; TPP, tetraphenylphosphonium bromide; BENZ, benzalkonium chloride; SDS, sodium dodecyl sulfate; DOC, sodium deoxycholate.

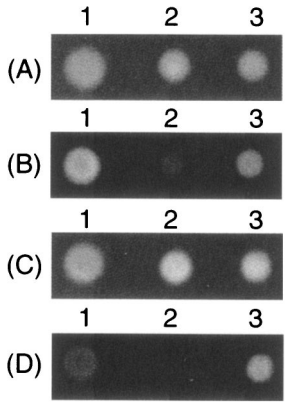

FIG. 1. Effects of deletion of *hns* and *tolC* on drug accumulation in *E. coli* cells. Strains W3104Δ*acrAB* (lanes 1), W3104Δ*acrAB*Δ*hns* (lanes 2), and W3104Δ*acrAB*Δ*hns*Δ*tolC* (lanes 3) were spotted onto L-agar plates containing 1 μg of ethidium bromide/ml (A and B) or 0.5 μg of rhodamine 6G/ml (C and D). After incubation at 37°C for 18 h, *E. coli* colonies were observed under visible white light (A and C) and UV light (B and D).

/ecogene/ecoweb/].) In addition, two major facilitator superfamily drug transporter systems (EmrAB and EmrKY) and one ABC drug transporter system (MacAB) also need TolC for their functions (15, 16, 19, 31).

In order to determine whether or not *hns* deletion-mediated multidrug resistance is due to the TolC-dependent drug exporter(s), we investigated the effect of *tolC* deletion on the drug resistance of the Δ*hns* strain. Deletion of *tolC* from strain W3104Δ*acrAB* increased the susceptibilities of cells to some compounds, particularly novobiocin, sodium dodecyl sulfate, and sodium deoxycholate. This increase is probably due to prevention of the leaking of compounds through TolC or inactivation of some TolC-dependent drug exporter(s). *tolC* deletion completely inhibited *hns* deletion-mediated multidrug resistance. *tolC* deletion from W3104Δ*acrAB*Δ*hns* increased susceptibilities to all the compounds listed in Table 3. *tolC* deletion restored the accumulation of ethidium bromide and rhodamine 6G in the Δ*hns* strain (Fig. 1B and D, lanes 3). These results indicated that *hns* deletion-mediated multidrug resistance is due to increased expression of a TolC-dependent drug exporter(s) caused by *hns* deletion.

Determination of the amounts of TolC-dependent drug exporter transcripts by qRT-PCR. In order to determine which drug exporters' expression is increased by *hns* deletion, we investigated *hns* deletion-dependent changes in the amounts of mRNAs of drug exporter genes by qRT-PCR. Total RNAs from exponential-phase cultures of W3104Δ*acrAB* and W3104Δ*acrAB*Δ*hns* were isolated, and cDNA samples were then synthesized by using TaqMan reverse transcription reagents (PE Applied Biosystems) and random hexamers as primers. Then real-time PCR of the cDNAs was performed with each specific primer pair by using SYBR Green PCR Master Mix (PE Applied Biosystems). The expression levels of TolC-dependent drug exporter genes (except for AcrAB), typical TolC-independent drug exporter genes (*mdfA*, *emrE*, and *mdtK* [*ydhE* has been renamed *mdtK* according to the systematic nomenclature available at the EcoGene website]), and the *tolC* gene in W3104Δ*acrAB*Δ*hns* were compared with those in W3104Δ*acrAB*. The results are shown in Table 4. The expression levels of three exporter genes (*acrE*, *mdtE*, and *emrK*) were significantly increased (more than fourfold in comparison with basal levels) by *hns* deletion: 4.1-, 12-, and 6.7-fold increases were observed for *acrE*, *mdtE*, and *emrK*, respectively. Deletion of *hns* did not increase the expression levels of other drug exporter genes or of the *tolC* gene (Table 4).

TABLE 4. Fold induction of specific transcripts attributed to *hns* deletion as determined by qRT-PCR

Gene	Fold change
TolC-dependent transporter genes	
acrD	1.0
acrE	4.1
mdtE	12.0
mdtA	0.5
emrK	6.7
macA	2.3
emrA	0.5
TolC-independent transporter genes	
mdfA	0.7
emrE	1.1
mdtK	1.5
Outer membrane channel gene (*tolC*)	0.9

Effects of deletion of drug exporter genes on *hns* deletion-mediated multidrug resistance. In order to determine whether or not *hns* deletion-mediated multidrug resistance is due to increased expression of the *acrEF*, *mdtEF*, and/or *emrKY* drug exporter genes, we investigated the effects of these gene deletions on drug resistance levels of W3104Δ*acrAB* and W3104Δ*acrAB*Δ*hns* (Table 3). When the *acrEF*, *mdtEF*, and *emrKY* genes were deleted one by one or simultaneously from W3104Δ*acrAB*, resistance levels did not change, suggesting that these genes are not expressed under normal conditions. Single deletion of *emrKY* or *mdtEF* did not change the increased multidrug resistance of W3104Δ*acrAB*Δ*hns*. On the other hand, deletion of *acrEF* from W3104Δ*acrAB*Δ*hns* drastically decreased the levels of *hns* deletion-mediated multidrug resistance, except for resistance to erythromycin, doxorubicin, and rhodamine 6G, indicating that *hns* deletion-mediated drug resistance is mainly due to AcrEF. However, this strain still retained some resistance to several compounds. That is, strain W3104Δ*acrAB*Δ*hns*Δ*acrEF* showed decreased but significant resistance to oxacillin, erythromycin, doxorubicin, crystal violet, ethidium bromide, methyl viologen, and rhodamine 6G. The remaining drug resistance pattern was similar to that conferred by overproduction of MdtEF (YhiUV) (30). Double deletion of *acrEF-mdtEF* from W3104Δ*acrAB*Δ*hns* completely prevented *hns* deletion-mediated multidrug resistance, clearly indicating that *hns* deletion-mediated multidrug resistance is due to increased expression of these two drug exporter genes. The reason why the single deletion of *mdtEF* from W3104Δ*acrAB*Δ*hns* did not change *hns* deletion-mediated resistance levels may be that increased expression of AcrEF masks the effect of *mdtEF* deletion. Deletion of *emrKY* from W3104Δ*acrAB*Δ*hns*Δ*acrEF* and W3104Δ*acrAB*Δ*hns*Δ*acrEF*Δ*mdtEF* did not affect the drug susceptibilities of these strains.

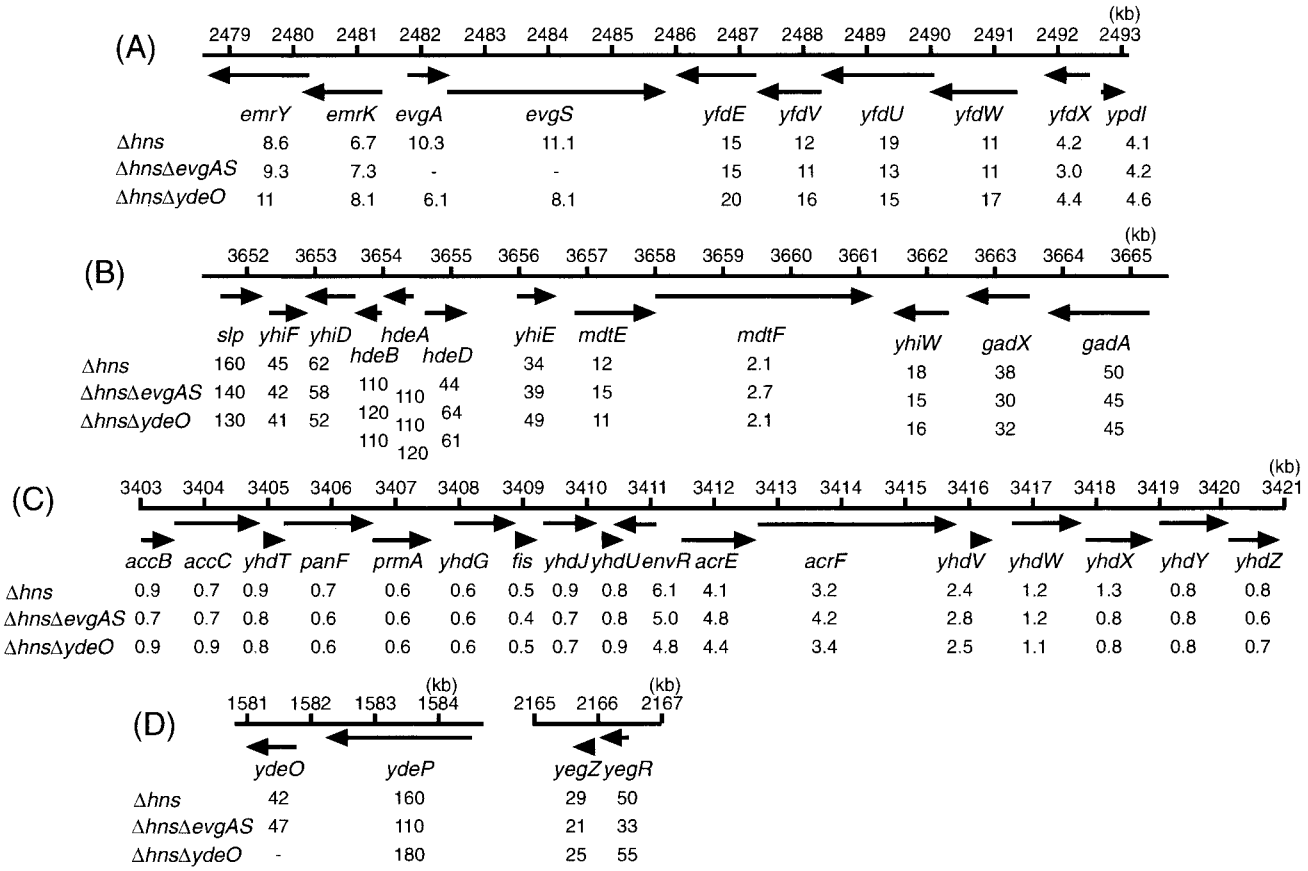

FIG. 2. Effects of deletion of *hns*, *evgAS*, and *ydeO* on the expression levels of genes near *emrKY*, *mdtEF*, and *acrEF*. (A) Gene clusters around *emrKY*; (B) gene clusters around *mdtEF*; (C) gene clusters around *acrEF*; (D) genes regulated by the EvgA response regulator. Arrows indicate the direction of transcription. Total RNAs from exponential-phase cultures of W3104Δ*acrAB*, W3104Δ*acrAB*Δ*hns*, W3104Δ*acrAB*Δ*hns*Δ*evgAS*, and W3104Δ*acrAB*Δ*hns*Δ*ydeO* were isolated, and the expression level of each gene was then determined by qRT-PCR. Values below diagrams are fold changes in mRNA levels from those in W3104Δ*acrAB*, as determined by qRT-PCR. Minus signs indicate gene deletion. Positions on *E. coli* chromosomal DNA (given above the diagrams in kilobase pairs) correspond to those on the Colibri website (http://genolist.pasteur.fr/Colibri/).

Effects of *hns* deletion on the expression levels of other genes located near *emrKY*, *mdtEF*, and *acrEF*. We investigated the effects of *hns* deletion on the expression levels of genes located near *emrK*, *mdtE*, and *acrE* by qRT-PCR analysis. The results are shown in Fig. 2. *hns* deletion increased the expression of genes near *emrK* (Fig. 2A). Expression of *emrY*, *emrK*, *evgA*, *evgS*, *yfdE*, *yfdV*, *yfdU*, *yfdW*, *yfdX*, and *ypdI* increased by factors of 8.6, 6.7, 10, 11, 15, 12, 19, 11, 4.2, and 4.2, respectively. *hns* deletion also increased the expression of genes near *mdtE* (Fig. 2B). Expression of *slp*, *yhiF*, *yhiD*, *hdeB*, *hdeA*, *hdeD*, *yhiE*, *mdtF*, *yhiW*, *gadX*, and *gadA* increased by factors of 160, 45, 62, 110, 110, 44, 34, 2.1, 18, 38, and 50, respectively. The effects of *hns* deletion on the expression of genes around *acrE* were lower than those on the expression of genes around *emrK* and *mdtE* (Fig. 2C). Deletion of *hns* increased the expression of one gene upstream of *acrE* (*envR*) and two downstream genes (*acrF* and *yhdV*) by factors of 6.1, 3.2, and 2.4, respectively. It is thought that *envR* is a repressor of the *acrEF* operon (3, 14, 30). However, although the expression level of *envR* was increased by *hns* deletion, the expression level of *acrEF* was also increased. This result indicates that the Δ*hns* effect overcomes the inhibitory effect of EnvR.

In a previous study, it was found that the gene clusters shown in Fig. 2A and B are positively regulated by the EvgA response regulator of the two-component signal transduction system (28). The expression level of *evgA* was increased by *hns* deletion (Fig. 2A). In order to determine whether or not the increased expression of drug exporter genes caused by *hns* deletion (Fig. 2A and B) is due to increased expression of the *evgAS* two-component system, we deleted *evgAS* from W3104Δ*acrAB*Δ*hns*. Deletion of *evgAS* from the *hns* deletion strain affected neither the increased expression levels of these genes (Fig. 2) nor the *hns* deletion-mediated multidrug resistance levels, even in the *hns-acrEF* deletion strain (Table 3).

Recently, it was reported that the gene cluster shown in Fig. 2B is positively regulated by *ydeO* (23) and that the level of *ydeO* expression is increased by *hns* deletion (Fig. 2D). Therefore, we investigated the effect of *ydeO* deletion. Deletion of *ydeO* affected neither the increased expression of genes shown in Fig. 2B nor *hns* deletion-mediated multidrug resistance. These data, together with those for *evgAS* deletion, clearly indicate that the *hns* deletion-mediated increase in the expression of drug exporter genes is independent of EvgAS- and YdeO-mediated regulation.

DISCUSSION

In this study, we found that H-NS represses the expression of some TolC-dependent multidrug exporter genes and that, as a result, deletion of the *hns* gene confers multidrug resistance on the *acrAB*-deficient strain. In addition, qRT-PCR analysis revealed that expression of genes located near the *mdtEF* and *emrKY* exporter systems was increased by *hns* deletion. This observation is in good agreement with the microarray data of Hommais et al. (9).

Previously, Sulavik et al. constructed *E. coli* strains with deletions of putative drug exporters and outer membrane channels (38). They reported that deletion of *acrAB* increased the drug susceptibility of *E. coli* cells, whereas deletion of the other drug exporter genes increased *E. coli* drug susceptibility slightly or not at all, indicating that most drug exporter genes are not expressed under normal conditions. Therefore, studies on the regulation of these drug exporter genes are necessary to gain further insights into the physiological roles of multidrug exporters.

We previously found that overexpression of *evgA*, which is a response regulator of the two-component regulatory system, conferred multidrug resistance on *E. coli* cells (31, 32). Later, Masuda and Church reported that overexpression of the *ydeO* regulatory gene also conferred multidrug resistance on *E. coli* (23). The *hns* deletion-mediated increase in expression of drug exporter genes is independent of such transcriptional regulator-mediated upregulation. *hns* deletion-mediated regulation is more global than two-component system-mediated regulation.

Ma et al. reported that the expression of *acrAB* is induced by fatty acids, sodium chloride, and ethanol (21). Lomovskaya et al. reported that the *emrAB* drug exporter gene is induced by salicylic acid and 2,4-dinitrophenol (20). In addition, it has been reported that the expression of *mdtEF* (*yhiUV*) is controlled by RpoS (1, 37), a conserved alternative sigma factor that is needed for *E. coli* to survive stresses such as heat shock (17, 24), oxidative stress (17, 24), osmotic challenge (24), and near-UV light (36). Thus, the regulation of drug exporter genes is closely related to stress responses. Hommais et al. suggested that the control of gene expression by H-NS has a strong relationship with the maintenance of intracellular homeostasis (9). In this study, we found that H-NS represses the expression of *acrEF* and *mdtEF*. Thus, it was revealed that H-NS-mediated maintenance of intracellular homeostasis has a close relationship with the expression of drug exporter genes.

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REFERENCES

1. **Altuvia, S., D. Weinstein-Fischer, A. Zhang, L. Postow, and G. Storz.** 1997. A small, stable RNA induced by oxidative stress: role as a pleiotropic regulator and antimutator. Cell **90:**43–53.
2. **Atlung, T., and H. Ingmer.** 1997. H-NS: a modulator of environmentally regulated gene expression. Mol. Microbiol. **24:**7–17.
3. **Blattner, F. R., G. Plunkett III, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao.** 1997. The complete genome sequence of *Escherichia coli* K-12. Science **277:**1453–1474.
4. **Bloch, V., Y. Yang, E. Margeat, A. Chavanieu, M. T. Auge, B. Robert, S. Arold, S. Rimsky, and M. Kochoyan.** 2003. The H-NS dimerization domain defines a new fold contributing to DNA recognition. Nat. Struct. Biol. **10:** 212–218.
5. **Elkins, C. A., and H. Nikaido.** 2002. Substrate specificity of the RND-type multidrug efflux pumps AcrB and AcrD of *Escherichia coli* is determined predominantly by two large periplasmic loops. J. Bacteriol. **184:**6490–6498.
6. **Falconi, M., B. Colonna, G. Prosseda, G. Micheli, and C. O. Gualerzi.** 1998. Thermoregulation of *Shigella* and *Escherichia coli* EIEC pathogenicity. A temperature-dependent structural transition of DNA modulates the accessibility of *virF* promoter to transcriptional repressor H-NS. EMBO J. **17:** 7033–7043.
7. **Fralick, J. A.** 1996. Evidence that TolC is required for functioning of the Mar/AcrAB efflux pump of *Escherichia coli.* J. Bacteriol. **178:**5803–5805.
8. **Hirakawa, H., K. Nishino, T. Hirata, and A. Yamaguchi.** 2003. Comprehensive studies on the drug resistance mediated by the overexpression of response regulators of two-component signal transduction systems in *Escherichia coli.* J. Bacteriol. **185:**1851–1856.
9. **Hommais, F., E. Krin, C. Laurent-Winter, O. Soutourina, A. Malpertuy, J. P. Le Caer, A. Danchin, and P. Bertin.** 2001. Large-scale monitoring of pleiotropic regulation of gene expression by the prokaryotic nucleoid-associated protein, H-NS. Mol. Microbiol. **40:**20–36.
10. **Jacquet, M., R. Cukier-Kahn, J. Pla, and F. Gros.** 1971. A thermostable protein factor acting on in vitro DNA transcription. Biochem. Biophys. Res. Commun. **45:**1597–1607.
11. **Japan Society of Chemotherapy.** 1981. Method for the determination of minimum inhibitory concentrations (MICs) of aerobic bacteria by the agar dilution method. Chemotherapy (Tokyo) **29:**76–79.
12. **Japan Society of Chemotherapy.** 1992. Method for the determination of minimum inhibitory concentrations (MICs) by the micro-broth dilution method. Chemotherapy (Tokyo) **40:**184–189.
13. **Jordi, B. J., and C. F. Higgins.** 2000. The downstream regulatory element of the *proU* operon of *Salmonella typhimurium* inhibits open complex formation by RNA polymerase at a distance. J. Biol. Chem. **275:**12123–12128.
14. **Kobayashi, K., N. Tsukagoshi, and R. Aono.** 2001. Suppression of the hypersensitivity of the *Escherichia coli acrB* mutant to organic solvents by integrational activation of the *acrEF* operon with the IS1 or IS2 element. J. Bacteriol. **183:**2646–2653.
15. **Kobayashi, N., K. Nishino, T. Hirata, and A. Yamaguchi.** 2003. Membrane topology of ABC-type macrolide antibiotic exporter MacB in *Escherichia coli.* FEBS Lett. **546:**241–246.
16. **Kobayashi, N., K. Nishino, and A. Yamaguchi.** 2001. Novel macrolide-specific ABC-type efflux transporter in *Escherichia coli.* J. Bacteriol. **183:**5639–5644.
17. **Lange, R., D. Fischer, and R. Hengge-Aronis.** 1995. Identification of transcriptional start sites and the role of ppGpp in the expression of *rpoS*, the structural gene for the sigma S subunit of RNA polymerase in *Escherichia coli.* J. Bacteriol. **177:**4676–4680.
18. **Link, A. J., D. Phillips, and G. M. Church.** 1997. Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. J. Bacteriol. **179:**6228–6237.
19. **Lomovskaya, O., and K. Lewis.** 1992. Emr, an *Escherichia coli* locus for multidrug resistance. Proc. Natl. Acad. Sci. USA **89:**8938–8942.
20. **Lomovskaya, O., K. Lewis, and A. Matin.** 1995. EmrR is a negative regulator of the *Escherichia coli* multidrug resistance pump EmrAB. J. Bacteriol. **177:**2328–2334.
21. **Ma, D., D. N. Cook, M. Alberti, N. G. Pon, H. Nikaido, and J. E. Hearst.** 1995. Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli.* Mol. Microbiol. **16:**45–55.
22. **Ma, D., D. N. Cook, J. E. Hearst, and H. Nikaido.** 1994. Efflux pumps and drug resistance in gram-negative bacteria. Trends Microbiol. **2:**489–493.
23. **Masuda, N., and G. M. Church.** 2003. Regulatory network of acid resistance genes in *Escherichia coli.* Mol. Microbiol. **48:**699–712.
24. **McCann, M. P., C. D. Fraley, and A. Matin.** 1993. The putative sigma factor KatF is regulated posttranscriptionally during carbon starvation. J. Bacteriol. **175:**2143–2149.
25. **Nagakubo, S., K. Nishino, T. Hirata, and A. Yamaguchi.** 2002. The putative response regulator BaeR stimulates multidrug resistance of *Escherichia coli* via a novel multidrug exporter system, MdtABC. J. Bacteriol. **184:**4161–4167.

26. **Nikaido, H.** 1996. Multidrug efflux pumps of gram-negative bacteria. J. Bacteriol. **178:**5853–5859.
27. **Nikaido, H.** 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science **264:**382–388.
28. **Nishino, K., Y. Inazumi, and A. Yamaguchi.** 2003. Global analysis of genes regulated by EvgA of the two-component regulatory system in *Escherichia coli.* J. Bacteriol. **185:**2667–2672.
29. **Nishino, K., J. Yamada, H. Hirakawa, T. Hirata, and A. Yamaguchi.** 2003. Roles of TolC-dependent multidrug transporters of *Escherichia coli* in resistance to β-lactams. Antimicrob. Agents Chemother. **47:**3030–3033.
30. **Nishino, K., and A. Yamaguchi.** 2001. Analysis of a complete library of putative drug transporter genes in *Escherichia coli.* J. Bacteriol. **183:**5803–5812.
31. **Nishino, K., and A. Yamaguchi.** 2002. EvgA of the two-component signal transduction system modulates production of the *yhiUV* multidrug transporter in *Escherichia coli.* J. Bacteriol. **184:**2319–2323.
32. **Nishino, K., and A. Yamaguchi.** 2001. Overexpression of the response regulator *evgA* of the two-component signal transduction system modulates multidrug resistance conferred by multidrug resistance transporters. J. Bacteriol. **183:**1455–1458.
33. **Rudd, K. E.** 2000. EcoGene: a genome sequence database for *Escherichia coli* K-12. Nucleic Acids Res. **28:**60–64.
34. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
35. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Sammartano, L. J., R. W. Tuveson, and R. Davenport.** 1986. Control of sensitivity to inactivation by H_2O_2 and broad-spectrum near-UV radiation by the *Escherichia coli katF* locus. J. Bacteriol. **168:**13–21.
37. **Schellhorn, H. E., J. P. Audia, L. I. Wei, and L. Chang.** 1998. Identification of conserved, RpoS-dependent stationary-phase genes of *Escherichia coli.* J. Bacteriol. **180:**6283–6291.
38. **Sulavik, M. C., C. Houseweart, C. Cramer, N. Jiwani, N. Murgolo, J. Greene, B. DiDomenico, K. J. Shaw, G. H. Miller, R. Hare, and G. Shimer.** 2001. Antibiotic susceptibility profiles of *Escherichia coli* strains lacking multidrug efflux pump genes. Antimicrob. Agents Chemother. **45:**1126–1136.
39. **Tupper, A. E., T. A. Owen-Hughes, D. W. Ussery, D. S. Santos, D. J. Ferguson, J. M. Sidebotham, J. C. Hinton, and C. F. Higgins.** 1994. The chromatin-associated protein H-NS alters DNA topology in vitro. EMBO J. **13:**258–268.
40. **Williams, R. M., and S. Rimsky.** 1997. Molecular aspects of the *E. coli* nucleoid protein, H-NS: a central controller of gene regulatory networks. FEMS Microbiol. Lett. **156:**175–185.
41. **Yamamoto, T., M. Tanaka, C. Nohara, Y. Fukunaga, and S. Yamagishi.** 1981. Transposition of the oxacillin-hydrolyzing penicillinase gene. J. Bacteriol. **145:**808–813.