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Effect of NlpE Overproduction on Multidrug Resistance in *Escherichia coli*▽

Kunihiko Nishino,[1,3]* Seiji Yamasaki,[1,2,4] 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 School of Pharmaceutical Sciences, Osaka University, Osaka 565-0871, Japan[4]

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NlpE, an outer membrane lipoprotein, functions during envelope stress responses in Gram-negative bacteria. In this study, we report that overproduction of NlpE increases multidrug and copper resistance through activation of the genes encoding the AcrD and MdtABC multidrug efflux pumps in *Escherichia coli*.

Multidrug efflux pumps cause serious problems in cancer chemotherapy and treatment of bacterial infections. Bacterial drug resistance is often associated with multidrug efflux pumps that decrease drug accumulation in the cell (15). Bacterial multidrug efflux pumps are classified into five families based on sequence similarity: major facilitator, resistance-nodulation-cell division (RND), small multidrug resistance, multidrug and toxic compound extrusion, and the ATP-binding cassette (2, 24). Of these, the RND family efflux pumps play major roles in both intrinsic and elevated resistance of Gram-negative bacteria to a wide range of compounds including β-lactams (15, 18). RND efflux pumps require two other proteins to function: a membrane fusion protein and an outer membrane protein. Many drug efflux pumps in *Escherichia coli* need TolC to function (7, 18). Bacterial genome sequencing enables us to trace drug resistance genes (22). There are many putative and proven drug efflux pumps in *E. coli*, and we have previously identified 20 such functional drug efflux pumps (19). Because many such efflux pumps have overlapping substrate spectra (19), 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. The currently available data show that multidrug efflux pumps are often expressed under precise and elaborate transcriptional control (8, 17). Expression of *acrAB*, which encodes the major AcrAB efflux pump, is subject to multiple levels of regulation. In *E. coli*, it is locally modulated by the repressor AcrR (13) and AcrS (11). At a more global level, it is modulated by stress conditions and global regulators such as MarA, SoxS, and Rob (14, 30). This example illustrates the complexity and diversity of the mechanisms regulating bacterial multidrug efflux pumps.

Stress responses in the bacterial cell envelope are transmitted to the cytoplasm in which gene expression is regulated in order to maintain the quality of the envelope. NlpE is anchored to the outer membrane through the lipid attached to its N-terminal cysteine and functions during envelope stress responses in Gram-negative bacteria. Adhesion to abiotic surfaces has been reported as an NlpE-dependent activation cue of the Cpx pathway (21). The Cpx envelope stress response is a two-component signal transduction pathway consisting of CpxA, an inner membrane histidine kinase, and CpxR, a cytoplasmic response regulator (5). Envelope stress responses play important roles in infection by many Gram-negative bacterial pathogens (25, 27). The expression level of NlpE is higher in a clinical, multidrug-resistant strain of the bacterium than that in a reference strain (28). In addition, overproduction of NlpE activates the Cpx pathway (29) and has been frequently utilized as a Cpx-specific activation signal in studies of the Cpx pathway (3, 6). However, the precise roles of NlpE in drug resistance are not clearly understood. In this study, we report that NlpE affects the multidrug resistance of *E. coli* by inducing the expression of drug efflux genes.

The expression of multidrug efflux genes is often regulated in a complex manner as described above. We therefore screened the genomic library of *E. coli* for genes that increased multidrug resistance levels in this organism. We screened a host strain (NKE96) lacking a functional *acrB* gene for identifying the regulatory elements involved in the expression of other multidrug resistance systems. A library was developed from the chromosomal DNA of the MG1655 strain (1), and then the recombinant plasmids were transformed into the Δ*acrB* strain NKE96. In one experiment, we found an 8-fold increase in oxacillin MIC against the transformant (data not shown). Introduction of the plasmid isolated from this strain into fresh Δ*acrB* cells resulted in the same oxacillin resistance phenotype; MIC was increased 8-fold over the recipient strain (data not shown).

The sequencing of the plasmid revealed an insertion containing the complete coding sequence of *nlpE* and a partial sequence of *yaeJ*. It seemed likely that in cells carrying this plasmid, overexpressed NlpE caused the transcriptional activation of genes involved in oxacillin resistance. Full-length wild-type *nlpE* was cloned in the pHSG398 vector to obtain p*nlpE* (Table 1). Oxacillin MICs for NKE96 cells harboring p*nlpE* were eight times higher (8 versus 1 μg/ml) than for cells harboring the pHSG398 vector (Δ*acrB*-vector) (Table 1). This suggests that the NlpE produced by this plasmid conferred oxacillin resistance on *E. coli*. During the screening, we also

* Corresponding author. Mailing address: Laboratory of Microbiology and Infectious Diseases, 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 *E. coli* strains to toxic compounds

Strain	Genotype	MIC (μg/ml)[a]										
		OXA	CLOX	NAF	FAM	ATM	CAR	SBPC	CRMN	KAN	NOV	DOC
NKE96	Δ*acrB*	1	1	2	0.063	0.063	1	2	0.031	2	4	5,000
NKE154	Δ*acrB*-vector	1	1	2	0.063	0.063	1	2	0.031	2	4	5,000
NKE155	Δ*acrB*-p*nlpE*	**8**	**16**	**16**	**0.25**	**0.25**	**8**	**16**	**0.25**	**8**	**16**	**40,000**
NKE127	Δ*acrB cpxAR*	0.5	1	1	0.063	0.063	1	1	0.031	1	4	2,500
NKE158	Δ*acrB cpxAR*-vector	0.5	1	1	0.063	0.063	1	1	0.031	1	4	2,500
NKE159	Δ*acrB cpxAR*-p*nlpE*	0.5	1	1	0.063	0.063	1	1	0.031	1	4	2,500
NKE128	Δ*acrB tolC*	0.5	0.25	1	0.063	0.063	1	1	0.031	1	2	156
NKE160	Δ*acrB tolC*-vector	0.5	0.25	1	0.063	0.063	1	1	0.031	1	2	156
NKE161	Δ*acrB tolC*-p*nlpE*	0.5	0.25	1	0.063	0.063	1	1	0.031	**2**	2	156
NKE126	Δ*acrB acrD*	0.5	1	1	0.063	0.063	1	1	0.031	2	4	5,000
NKE156	Δ*acrB acrD*-vector	0.5	1	1	0.063	0.063	1	1	0.031	2	4	5,000
NKE157	Δ*acrB acrD*-p*nlpE*	**2**	**2**	**4**	**0.13**	0.063	**2**	**2**	**0.063**	**4**	**8**	**10,000**
NKE1365	Δ*acrB mdtABC*	1	1	1	0.063	0.063	1	2	0.031	2	4	5,000
NKE1366	Δ*acrB mdtABC*-vector	1	1	1	0.063	0.063	1	2	0.031	2	4	5,000
NKE1367	Δ*acrB mdtABC*-p*nlpE*	**4**	**4**	**4**	**0.13**	**0.25**	**4**	**8**	**0.13**	**8**	**8**	**10,000**
NKE1316	Δ*acrB acrD mdtABC*	0.5	0.5	1	0.063	0.063	1	1	0.031	2	2	2,500
NKE1368	Δ*acrB acrD mdtABC*-vector	0.5	0.5	1	0.063	0.063	1	1	0.031	2	2	2,500
NKE1369	Δ*acrB acrD mdtABC*-p*nlpE*	0.5	0.5	1	0.063	0.063	1	1	0.031	**4**	2	2,500

[a] MIC determinations were repeated at least three times. Values in boldface are larger than those of a corresponding parental strain harboring the pHSG398 vector. OXA, oxacillin; CLOX, cloxacillin; NAF, nafcillin; FAM, cefamandole; ATM, aztreonam; CAR, carbenicillin; SBPC, sulbenicillin; CRMN, carumonam; KAN, kanamycin; NOV, novobiocin; DOC, deoxycholate.

yielded the already known drug resistance activator BaeR (data not shown). We investigated the effect of *nlpE* overexpression on the susceptibility of *E. coli* to other toxic compounds. Various drugs were tested, including common substrates of multidrug efflux pumps, and we found that *nlpE* overexpression increased the resistance of the NKE96 strain to cloxacillin, nafcillin, cefamandole, aztreonam, carbenicillin, sulbenicillin, carumonam, kanamycin, novobiocin, and deoxycholate (Table 1). These results indicate that the overexpression of NlpE induces multidrug resistance in *E. coli*.

As described above, it has been reported that overproduction of NlpE activates the Cpx pathway (29). In order to determine whether NlpE-mediated multidrug resistance depends on the CpxAR two-component signal transduction pathway, we constructed a deletion mutant of the *cpxAR* genes. The deletion was made by using the lambda Red system (4). In the Δ*acrB cpxAR* strain, overexpression of *nlpE* conferred no drug resistance (Table 1), indicating that NlpE conferred multidrug resistance of *E. coli* in a Cpx-dependent manner.

A major mechanism of bacterial multidrug resistance is active drug efflux. The results described above indicate that the expression of a multidrug efflux pump may be induced by *nlpE* overexpression. RND efflux pumps need two other proteins for their function: a membrane fusion protein and an outer membrane channel. It has been reported that many drug efflux systems need the membrane channel TolC for their function (7, 18). In order to determine whether NlpE-mediated multidrug resistance also depends on the TolC-dependent drug efflux pump(s), we investigated the effect of *tolC* deletion on the drug resistance in cells overexpressing *nlpE*. Deletion of *tolC* from the Δ*acrB* strain increased susceptibility to cloxacillin and deoxycholate (Table 1), which is in good agreement with a previous report (18). The *tolC* deletion inhibited NlpE-mediated multidrug resistance (Table 1). This result indicates that

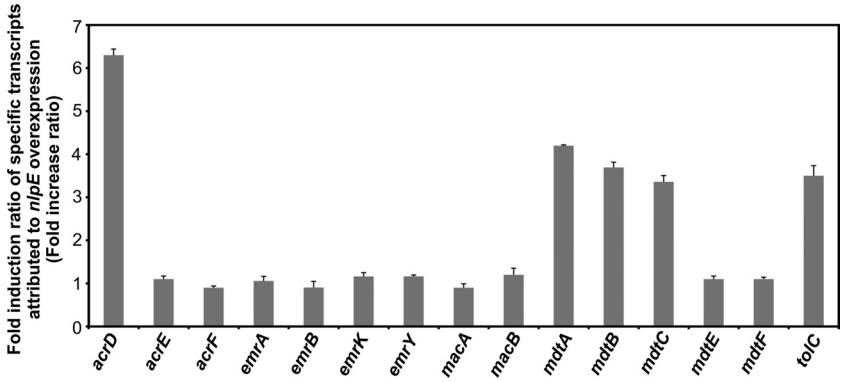

FIG. 1. Effect of NlpE on the expression levels of drug efflux and outer membrane channel genes. The level of the mRNA transcript was determined by qRT-PCR. The fold change ratio was calculated by dividing the expression level of the gene in the NKE155 strain by that in the NKE154 strain. The data correspond to mean values of three independent experiments. Error bars represent standard deviation.

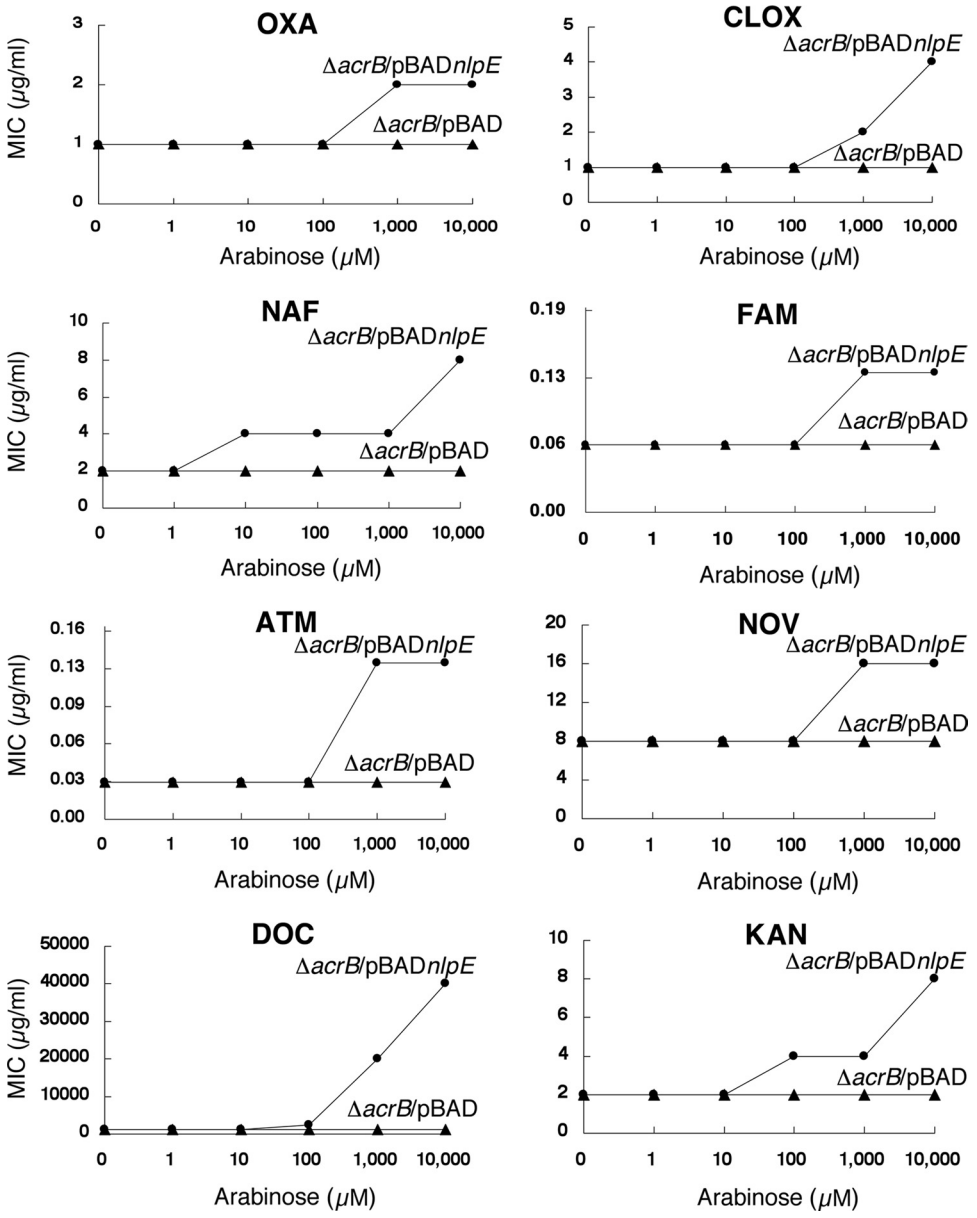

FIG. 2. Multidrug resistance enhanced by the induction of NlpE using arabinose. MICs of oxacillin (OXA), cloxacillin (CLOX), nafcillin (NAF), cefamandole (FAM), aztreonam (ATM), novobiocin (NOV), deoxycholate (DOC), and kanamycin (KAN) to NKE1355 (Δ*acrB*-pBAD) and NKE1359 (Δ*acrB*-pBAD*nlpE*) strains were measured under several concentrations (0 to 10,000 μM) of arabinose. MIC determinations were repeated at least three times.

NlpE-mediated multidrug resistance is attributable to increased functioning of a TolC-dependent drug efflux pump.

In order to determine which drug efflux pump shows increased expression when *nlpE* is overexpressed, we used quantitative reverse transcription-PCR (qRT-PCR) to investigate changes in the levels of drug efflux gene mRNAs dependent on *nlpE* overexpression. Total RNAs were isolated from exponential-phase cultures of NKE154 (Δ*acrB*-vector) and NKE155 (Δ*acrB*-p*nlpE*) strains, and cDNA samples were synthesized using TaqMan reverse transcription reagents (PE Applied Biosystems) with random hexamers as primers. Real-time PCR of cDNAs was performed with each specific primer pair using SYBR green PCR master mix (PE Applied Biosystems) as described previously (20). The changes in the expression levels of seven TolC-dependent-type drug efflux pump systems, including *acrD*, *acrEF*, *emrAB*, *emrKY*, *macAB*, *mdtABC*, *mdtEF*, and the outer membrane channel *tolC* genes were measured. The expression levels of *acrD*, *mdtABC*, and *tolC* were significantly increased by *nlpE* overexpression (Fig. 1). This increased expression was not observed in the Δ*cpxAR* strain (data not shown), indicating that NlpE stimulates these genes via the Cpx pathway.

In order to determine whether multidrug resistance mediated by *nlpE* overexpression is due to increased expression of *acrD* and/or *mdtABC*, we investigated the effects of deleting these genes on NlpE-mediated multidrug resistance (Table 1).

In the Δ*acrB acrD* strain, overexpression of *nlpE* slightly conferred *E. coli* resistance to oxacillin, cloxacillin, nafcillin, cefamandole, carbenicillin, sulbenicillin, carumonam, kanamycin, novobiocin, and deoxycholate; however, these drug resistance levels were lower than those in the Δ*acrB*-p*nlpE* strain. This result indicates that AcrD is partially responsible for the NlpE-modulated multidrug resistance. AcrD was also fully responsible for aztreonam resistance (Table 1). In the Δ*acrB mdtABC* strain, overexpression of *nlpE* conferred resistance to oxacillin, cloxacillin, nafcillin, cefamandole, aztreonam, carbenicillin, sulbenicillin, carumonam, kanamycin, novobiocin, and deoxycholate. These resistance levels were lower than in the Δ*acrB*-p*nlpE* strain except for aztreonam and kanamycin, indicating that MdtABC partially contributes to the NlpE-mediated multidrug resistance. In the Δ*acrB acrD mdtABC* strain, overexpression of *nlpE* conferred no drug resistance (Table 1) except for slight increased resistance to kanamycin (2-fold increase). Together, these data indicate that the multidrug resistance conferred by NlpE is due to increased expression of both the *acrD* and *mdtABC* multidrug efflux genes.

Recent characterization of the Cpx regulon in *E. coli* strain MC4100 by Price and Raivio (23) showed that overexpression of *nlpE* from the cloning vector pBR322 did not increase the expression levels of *acrD* and *mdtABC*; however, we found that p*nlpE* generated from the high-copy-number cloning vector pHSG398 clearly increases the expression levels of these genes and AcrD-MdtABC-dependent multidrug resistance in *E. coli*. These results suggest that high levels of NlpE overproduction may be required to induce the multidrug resistance phenotype. To test this hypothesis, the *nlpE* gene was cloned into pBAD33 (10) to produce the plasmid pBAD*nlpE*. In this plasmid, *nlpE* is under the control of an arabinose-inducible P_{BAD} promoter. The multidrug resistance phenotype of the Δ*acrB*-pBAD*nlpE* strain was investigated under different concentrations of arabinose (from 1 to 10,000 μM) (Fig. 2). MICs of several drugs against the Δ*acrB*-pBAD*nlpE* strain increased under a high concentration of arabinose (Fig. 2). Results presented in Fig. 2 support our hypothesis that NlpE enhances the drug resistance level of *E. coli* in a dose-dependent manner.

In this study, we performed a genome-wide search for a regulator of multidrug resistance in *E. coli* by random shotgun cloning. We found NlpE, which increases resistance to oxacillin, cloxacillin, nafcilllin, cefamandole, aztreonam, carbenicillin, sulbenicillin, carumonam, kanamycin, novobiocin, and deoxycholate by upregulating *acrD* and *mdtABC*. NlpE-modulated kanamycin resistance was partially dependent on AcrD, but not on MdtABC, probably because aminoglycoside is the specific substrate for AcrD (19, 26). Slightly increased resistance to kanamycin (2-fold increase) was observed in Δ*acrB tolC*-p*nlpE* and Δ*acrB acrD mdtABC*-p*nlpE* strains, whereas it was not observed in the Δ*acrB cpxAR*-p*nlpE* strain. This suggests the possibility that NlpE may also affect other kanamycin resistance factors through the Cpx pathway in addition to AcrD. Because it was reported that NlpE is involved in copper tolerance (9), we tested effect of *nlpE* overexpression on the susceptibility of *E. coli* to $CuSO_4$. NlpE slightly increased resistance to copper (MIC of 4 mM for NKE154 versus 6 mM for NKE155). This phenotype was not observed in NKE159, NKE161, and NKE1369, suggesting that NlpE-mediated copper resistance was due to increased expression of *acrD* and *mdtABC* via the Cpx pathway. This is reminiscent of the copper and zinc resistance mechanism by the AcrD and MdtABC multidrug efflux systems regulated by the BaeSR two-component signal transduction system in *Salmonella enterica* (16). In this study, it was also revealed that NlpE enhances multidrug resistance of *E. coli* in a dose-dependent manner. Structural study suggests that unfolded NlpE is plausibly related to activation of the Cpx pathway (12). Thus, it is possible that overproduction of NlpE may have resulted in an increased amount of unfolded protein and then it stimulated multidrug resistance via the Cpx pathway. Previously, a relationship between NlpE and biofilm formation was also highlighted by a proteome analysis of *Acinetobacter baumannii* (28). We found the importance of NlpE as a drug resistance factor through the induction of the multidrug efflux genes in this study. 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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