JOURNAL OF BACTERIOLOGY, Apr. 2002, p. 2319–2323 Vol. 184, No. 8
0021-9193/02/$04.00+0 DOI: 10.1128/JB.184.8.2319–2323.2002

EvgA of the Two-Component Signal Transduction System Modulates Production of the YhiUV Multidrug Transporter in *Escherichia coli*

Kunihiko Nishino and Akihito Yamaguchi*

Department of Cell Membrane Biology, Institute of Scientific and Industrial Research, Osaka University, Ibaraki, Osaka 567-0047, Faculty of Pharmaceutical Science, Osaka University, Suita, Osaka 565-0871, and CREST, Japan Science and Technology Corporation, Osaka 567-0047, Japan

Received 28 September 2001/Accepted 8 January 2002

Overexpression of the EvgA regulator of the two-component signal transduction system was previously found to modulate multidrug resistance of *Escherichia coli* by increasing efflux of drugs (K. Nishino and A. Yamaguchi, J. Bacteriol. 183:1455–1458, 2001). Here we present data showing that EvgA contributes to multidrug resistance through increased expression of the multidrug transporter *yhiUV* gene.

For many years, antibiotics have been effective in the treatment of many infectious diseases caused by a range of pathogens. The occurrence of antibiotic resistance, however, has transformed some previously treatable diseases into a new threat to public health. One of the mechanisms underlying antibiotic resistance involves the extrusion of the compounds by an efflux pump or carrier. The most intriguing mechanisms of drug extrusion are those that include a wide variety of structurally unrelated compounds as substrates for multidrug resistance (MDR) transporters (14, 23). MDR transporters are found in a variety of bacterial species (19, 20, 26). Recently we cloned 37 putative drug transporter genes of *Escherichia coli* and investigated their drug resistance phenotypes (15). During the course of that study, we found that the DNA locus including both the putative drug efflux transporter *emrKY* genes and the two-component signal transduction system *evgSA* genes conferred MDR to *E. coli*. We showed that the MDR phenomenon was not due to the *emrKY* genes but was due to the response regulator *evgA* gene (16).

Two-component systems are signal transduction pathways in prokaryotic organisms responding to environmental conditions (4, 18). A typical two-component system consists of two types of signal transducers, a sensory kinase and a response regulator. The sensory kinase monitors some environmental conditions and accordingly modulates the phosphorylation state of the response regulator. The response regulator regulates gene expression and/or cell behavior. The EvgSA two-component system is known to regulate the expression of the putative drug efflux transporter *emrKY* genes (5).

Effect of deletion of *yhiUV* on MDR induced by overexpression of the EvgA regulator. In our previous study (16), we found that overexpression of the gene regulator EvgA modulates MDR. *E. coli* KAM3 cells (13) harboring the pUCA plasmid carrying *evgA* showed drug resistance against deoxycholate (>32-fold compared to the no-plasmid control level), doxorubicin (64-fold), rhodamine 6G (16-fold), erythromycin (8-fold), crystal violet (8-fold), benzalkonium (8-fold), and sodium dodecyl sulfate (SDS) (4-fold) (Table 1). Since EvgA is known to positively regulate *emrKY* gene expression (5), we cloned *emrKY* into the pQE30 expression vector and observed that *E. coli* cells overexpressing EmrKY acquire resistance only to deoxycholate (eightfold) (Table 1). These results suggest a clear difference between the effects of overexpression of EmrKY and EvgA on MDR (16). One possibility is that EvgA regulates an additional, unknown multidrug efflux system(s), different from EmrKY. Previously, we cloned 37 known and putative drug transporter genes of *E. coli* and investigated the drug resistance activities (15). In those studies, we found that the drug resistance pattern conferred by overexpression of YhiUV is very similar to that conferred by EvgA (Table 1). In order to investigate whether EvgA regulates the expression of *yhiUV*, we constructed a *yhiUV* deletion mutant of *E. coli* KAM3. Construction of a chromosomal in-frame deletion mutant was performed by the gene replacement method as previously described (8), using plasmid pKO3ΔyhiUV (Table 2). The *yhiUV* deletion strain itself exhibited no alteration in drug susceptibility compared to the parental strain KAM3 (Table 1), probably because *yhiUV* are not expressed under normal conditions. This observation is in good agreement with the results of the systematic deletion of putative drug transporter genes in *E. coli* as reported by Sulavik et al. (27). In contrast to the case for the KAM3 strain, even when the cells were transformed with plasmid pUCA carrying the *evgA* gene, the mutant that lacks the *yhiUV* genes exhibited neither increased drug resistance (Table 1) (except for deoxycholate) nor increased drug efflux (Fig. 1). These observations indicate that EvgA-induced MDR is caused by stimulation of *yhiUV* gene expression. In regard to deoxycholate resistance, EvgA overexpression in the *yhiUV* deletion mutant caused a moderate increase (fourfold), which is probably due to EvgA-dependent EmrKY expression. Overexpression of either *evgA* or *yhiUV* resulted in different levels of resistance to some compounds, probably due to differences in *yhiUV* expression levels.

Effect of EvgA on expression of the MDR transporter YhiUV. The data presented above suggest that the level of the *yhiUV* transcripts may be increased by EvgA overproduction. To test this hypothesis, total cellular RNA was isolated from pUC119- or pUCA-carrying KAM3 cells. Northern blot analysis using the *yhiU* probe DNA shows that the *yhiUV* mRNA is

* Corresponding author. Mailing address: Institute of Scientific and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki-shi, Osaka 567-0047, Japan. Phone: 81-6-6879-8545. Fax: 81-6-6879-8549. E-mail: akihito@sanken.osaka-u.ac.jp.

TABLE 1. Resistance of *E. coli* cells harboring a plasmid carrying *evgA*, *yhiUV*, or *emrKY*

Compound	MIC (μg/ml)[a] for strain:							
	KAM3	KAM3/ pUCA	KAM3ΔyhiUV	KAM3ΔyhiUV/ pUCA	KAM3/ pUCyhiUV	KAM3/ pQE30emrKY[b]	KAM3/ pUCA-D52A	KAM3/ pUCA-D54A
Doxorubicin	3.13	**200**	3.13	3.13	**25**	3.13	**25**	**>200**
Erythromycin	3.13	**25**	3.13	3.13	**25**	3.13	3.13	**50**
Crystal violet	1.56	**12.5**	1.56	1.56	**3.13**	1.56	3.13	**12.5**
Rhodamine 6G	6.25	**100**	6.25	6.25	**100**	6.25	6.25	**100**
Benzalkonium	3.13	**25**	3.13	3.13	**6.25**	3.13	3.13	**25**
SDS	100	**400**	100	100	**200**	100	100	**>400**
Deoxycholate	1,250	**>40,000**	1,250	**5,000**	**5,000**	**10,000**	1,250	**>40,000**

[a] Boldface indicates values significantly different from control values. Drug resistance of cells was determined by a sequential twofold dilution method on YT (24) agar plates as previously described (15).
[b] IPTG (1 mM) was added to agar plates.

detected in the EvgA-overproducing cells but not in the host KAM3 cells (Fig. 2), indicating that the overproduction of EvgA stimulates *yhiUV* transcription.

Determination of drug transporter transcript levels by quantitative real-time PCR. In our previous study, we found that 20 intrinsic drug transporter genes conferred drug resistance (15). We investigated the changes of the expression levels of these transporter genes (listed in Table 3, except for *acrAB*) and the *evgA*-induced *yfdX* gene (16) when EvgA was overproduced in *E. coli* KAM3 (lacking *acrAB*). Quantitative real-time reverse transcription-PCR was used to verify the expression changes. Total RNA was purified from KAM3 cells harboring pUC119 or pUCA, using RNAprotect Bacteria Reagent (Qiagen) and the SV total RNA isolation system (Promega). cDNA samples were synthesized from total RNA by using TaqMan reverse transcription reagents (PE Applied Biosystems) and random hexamers. Specific primer pairs were designed with the ABI PRISM Primer Express software (PE Applied Biosystems), and then real-time PCR was performed with each specific primer pair using SYBR Green PCR Master Mix (PE Applied Biosystems) and run on an ABI PRISM 7000 sequence detection system (PE Applied Biosystems). The results are shown in Table 3. Increases of 470-fold in *yhiU* expression, 28-fold in *emrK* expression, and 3,200-fold in *yfdX* expression were observed upon *evgA* amplification.

Binding of the EvgA protein to the DNA fragment containing the *yhiU* promoter region. If EvgA regulates transcription of *yhiUV* directly, it may bind to the *yhiU* promoter region. In order to test this possibility, we constructed plasmid pQE30evgA, which encodes a His_6-tagged EvgA (His_6-EvgA) under control of the T5 promoter. The His_6-EvgA protein was overproduced in *E. coli* M15(pREP4, pQE30evgA) and purified by Ni-chelating affinity beads (Qiagen). The binding of His_6-EvgA to the *yhiU* and *emrK* promoter regions was tested by using the electrophoretic mobility shift assay. DNAs of 338 bp containing the *yhiU* or *emrK* promoter regions were amplified by PCR and used as target DNAs for the mobility shift assay. The *macA* (6) promoter region was also amplified as a negative control. Electrophoretic mobility shift assay showed that both the *pyhiU* and *pemrK* DNA fragments exhibited changes in mobility in the presence of 5 μg of His_6-EvgA (Fig. 3), indicating that EvgA directly binds to both the *yhiU* and *emrK* promoter regions.

Effect of a mutation at the phosphorylation site of EvgA. In a two-component regulatory system, a sensor kinase phosphorylates a conserved aspartic acid in a response regulator (25). The phosphorylation is a trigger for a signal transduction event. A phospho-accepting domain of the response regulator is composed of a short consensus motif, ΔΔD (where Δ is a nonpolar amino acid such as I, L, M, or V) (12). The EvgA gene regulator also contains the conserved motif ^{50}IID^{52}, and the aspartate (D) 52 is thought to be a phosphorylation site (29). Another adjacent aspartate, Asp54 in EvgA, which does not seem to be a phosphorylation site, was used as a control in the following experiments. We replaced Asp52 and Asp54 individually with alanine by the Kunkel method (7). The mutant *evgA* genes were cloned into pUC119 (31), and the resulting plasmids were named pUCA-D52A and pUCA-D54A, respectively. Total cell proteins from *E. coli* KAM3 cells harboring the constructed plasmids were separated on an SDS-polyacrylamide gel, and EvgA expression was detected by Coomassie brilliant blue staining (data not shown). KAM3 cells transformed by pUCA-D52A were no longer multidrug resistant, except for having resistance to doxorubicin, whereas cells transformed by pUCA-D54A showed resistance similar to that conferred by pUCA (Table 1). pUCA-D52A showed moderate doxorubicin resistance (eightfold), possibly due to phosphorylation of another amino acid in place of Asp52. When the *evgA*-D52A mutant gene was cloned into pQE30, the cells harboring the mutant plasmid showed gradually increasing moderate MDR depending on the IPTG (isopropyl-β-D-thiogalactopyranoside) concentration (data not shown). Doxorubicin and rhodamine 6G efflux activities of cells carrying pUCA-D52A were significantly lower than those of cells carrying pUCA (data not shown). Thus, phosphorylation of EvgA is required for modulation of MDR.

YhiUV and EmrKY transporters require the outer membrane channel TolC for their function. The YhiV and EmrY transporter proteins belong to the resistance-nodulation-cell division and major facilitator superfamily transporter families, respectively. YhiU and EmrK both belong to the membrane fusion protein family. AcrA and EmrA, which are typical membrane fusion proteins, show 70 and 65% sequence similarity to YhiU and EmrK, respectively. Membrane fusion protein-dependent drug transporters in general depend on the multifunctional outer membrane channel TolC for their function (3, 6,

TABLE 2. Bacterial strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Genotype or characteristics[a]	Reference or source
E. coli strains		
W3104	Wild type, used as the donor of chromosomal DNA for PCR amplification	30
TG1	*supE hsd*Δ5 *thi* Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15], used as the cloning host	28
DH5α	*recA endA1 hsdR17 supE4 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* (ϕ80d*lacZ*ΔM15), used as the cloning host	24
M15	*recA*$^+$ *uvr*$^+$ F$^-$ *mtl gal ara lac* (pREP4), used for overexpression of hexahistidine-tagged EvgA	Qiagen
CJ236	*dut-1 ung thi-1 relA1*/pCJ105(Cm^r F′), used for mutagenesis by the Kunkel method (7)	7
ZK796	Tet^r; same as MC4100 but *tolC*::Tn*10*	32
KAM3	Derivative of TG1 that lacks a restriction system and *acrAB*	13
KAM3ΔyhiUV	Derivative of KAM3 that lacks *yhiUV*	This study
KAM3ΔevgS	Derivative of KAM3 that lacks *evgS*	This study
Plasmids		
pUC119	Vector; Ap^r; multiple cloning site in *lacZ*	31
pQE30	His expression vector; Ap^r; multiple cloning site downstream of T5 promoter	Qiagen
pKO3	*repA*(Ts) Cm^r *sacB*$^+$	8
pKO3ΔyhiUV	*Bam*HI-*Bam*HI fragment for *yhiUV* deletion cloned into pKO3	This study
pKO3ΔevgS	*Bam*HI-*Bam*HI fragment for *evgS* deletion cloned into pKO3	This study
pUCA	*Hin*dIII-*Sal*I fragment containing *evgA* (gene regulator of two-component system) with 366-bp upstream flanking sequence cloned into pUC119 to be in the same orientation as the lactose promoter; Ap^r	16
pUCA-D52A	D52A derivative of pUCA	This study
pUCA-D54A	D54A derivative of pUCA	This study
pQE30emrKY	*Sph*I-*Pst*I fragment containing *emrKY* (MFP/ MFS transporter) genes cloned into pQE30; Ap^r	16
pQE30evgA	*Sph*I-*Pst*I fragment containing *evgA* gene cloned into pQE30; Ap^r	16
pUCyhiUV	*Pst*I-*Bam*HI fragment containing *yhiUV* (MFP/ RND transporter) genes with 180-bp upstream flanking sequence cloned into pUC119 to be in the same orientation as the lactose promoter; Ap^r	15
Oligonucleotides[b]		
evgA-D52A	CCGGGGATGTCGACAGCAATGATGACG, mutagenic primer for D52A of *evgA*	
evgA-D54A	CCGGGGATAGCGACGTCAATGATGACG, mutagenic primer for D54A of *evgA*	
yhiU-No	CGCGGATCCCAGTTCAAAATTATGCAACTGATTCTG, used for crossover PCR for the in-frame deletion of *yhiUV*	
yhiU-Ni	CACGCAATAACCTTCACACTCCAAATTTATAACCATTTTAGTCCCTGAAAATTCTTGAG, used for crossover PCR for the in-frame deletion of *yhiUV*	
yhiV-Co	CGCGGATCCCGTCAAATTCCTCTGCATACTATTGC, used for crossover PCR for the in-frame deletion of *yhiUV*	
yhiV-Ci	GTTATAAATTTCGAGTGTGAAGGTTATTGCGTGTAACGTGTAAATGAGAGTAAGGTTGA, used for crossover PCR for the in-frame deletion of *yhiUV*	
evgS-No	CGCGGATCCGGGTGGAAACACTTAAGCCTGA, used for crossover PCR for the in-frame deletion of *evgS*	
evgS-Ni	CACGCAATAACCTTCACACTCCAAATTTATAACCATGTGGTTAGCCGATTTTGTTAC, used for crossover PCR for the in-frame deletion of *evgS*	
evgS-Co	CGCGGATCCCATGGCACCTTTTGATGTTTTCAATACT, used for crossover PCR for the in-frame deletion of *evgS*	
evgS-Ci	GTTATAAATTTGGAGTGTGAAGGTTATTGCGTGTAAATAGCGGCTCCCACAATGTTC, used for crossover PCR for the in-frame deletion of *evgS*	
yhiUpr-F	CTCTCTACCGCCAGCAATGCCCGC, used for amplification of *yhiU* probe	
yhiUpr-R	CCCGTAATCGGCGAGGTGACATTCGCG, used for amplification of *yhiU* probe	
evgAF	GGGGCATGCAACGCAATAATTATTG, used for amplification of *evgA* cloned into pQE30	
evgAR	CCCCTGCAGTTAGCCGATTTTGTTACGTTGT, used for amplification of *evgA* cloned into pQE30	
macAPF	ACATTGAGATTAGGCCAGGGAAAGTTCG, used for amplification of *macA* promoter region	
macAPR	TCCGGGTCATTAACTTCAACGAAATATCAA, used for amplification of *macA* promoter region	
emrKPF	AGAAAATCTGAGCTTCCTTAAG, used for amplification of *emrK* promoter region	
emrKPR	CTGTTCCACTATTATCTCTCATTTC, used for amplification of *emrK* promoter region	
yhiUPF	TCAGGACATAAGCAACTGAAATTG, used for amplification of *yhiU* promoter region	
yhiUPR	TTTAGTCCCTGAAAATTCTTGAG, used for amplification of *yhiU* promoter region	

[a] Ap, ampicillin; Cm, chloramphenicol; MFP, membrane fusion protein; MFS, major facilitator superfamily; RND, resistance-nodulation-cell division family.
[b] Introduced restriction sites used for cloning are underlined.

9). In order to investigate the role of TolC in the YhiUV and EmrKY systems, the TolC-deficient strain *E. coli* ZK796 (32) was transformed with pUCyhiUV and pQE30emrKY. The resulting transformed cells showed no increase in resistance (MICs for ZK796 with no plasmid, pUCyhiUV, or pQE30emrKY are 3.13 μg/ml [doxorubicin], 3.13 μg/ml [erythromycin], 1.56 μg/ml [crystal violet], 6.25 μg/ml [rhodamine 6G], 3.13 μg/ml [benzalkonium], 25 μg/ml [SDS], and 156 μg/ml [deoxycholate]), indicating that YhiUV and EmrKY also require TolC for their function.

Conclusions. Numerous studies on the regulation of multidrug transporter gene expression have been performed with *E. coli*. For example, expression of the EmrAB multidrug pump (9) is controlled by EmrR, a MarR type of repressor protein

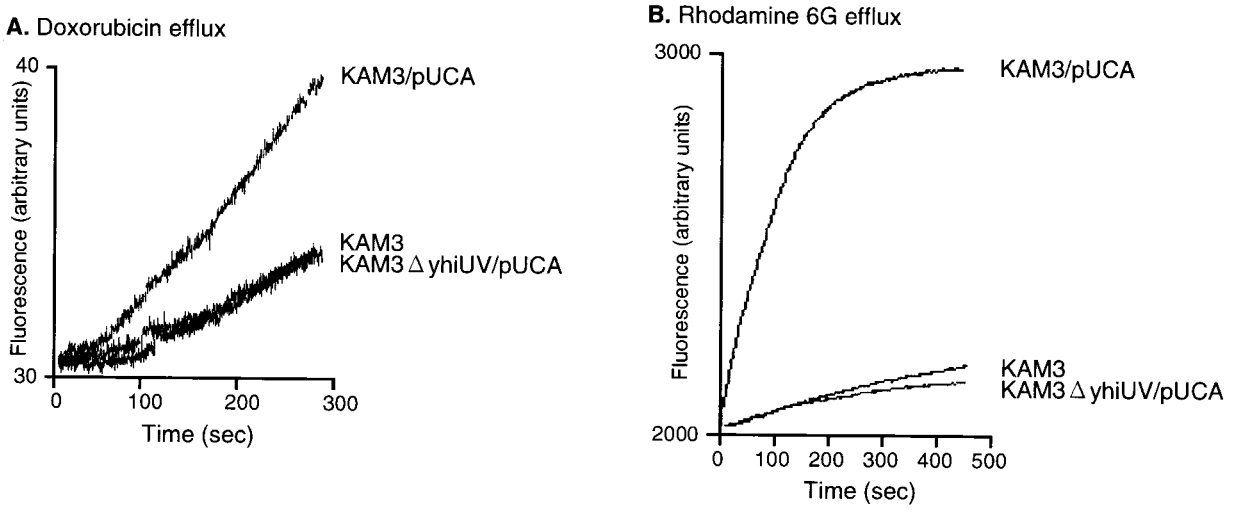

FIG. 1. Active efflux of doxorubicin (A) and rhodamine 6G (B) from *E. coli* KAM3 and KAM3ΔyhiUV cells overproducing EvgA. Active efflux of doxorubicin and rhodamine 6G from *E. coli* cells was measured as previously described (16). In order to obtain maximal preloading of the fluorophore, the cells were preincubated with 11.5 μM doxorubicin (A) or 1 μM rhodamine 6G (B) in the presence of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (40 μM) at 37°C for 1 h. The cells were then centrifuged and resuspended in the same medium containing 25 mM glucose without fluorescent drugs and carbonyl cyanide *m*-chlorophenylhydrazone, followed by fluorescence measurement. Doxorubicin transport was measured with excitation at 478 nm and emission at 591 nm. Rhodamine 6G transport was measured with excitation at 529 nm and emission at 553 nm.

(10). The transcription of the *acrAB* operon in *E. coli* is regulated by the repressor AcrR (11). Gel mobility shift assays and *lacZ* transcriptional fusion proteins suggested that the general stress-enhanced transcription of *acrAB* is mediated primarily by global regulatory pathways such as the *mar* regulon and that a major function of AcrR is that of a specific secondary modulator (11).

Our results suggest that phosphorylation of EvgA is required for modulation of MDR. Although it was reported that EvgA is phosphorylated by the EvgS sensor protein as determined by phosphotransfer analysis using radiolabeled ATP (21), EvgA also can be phosphorylated by acetylphosphate without EvgS (22). Recently, we have constructed an *evgS*-deficient strain of *E. coli* KAM3 and observed that overproduction of EvgA in this strain also causes MDR (data not shown). Thus, in cells

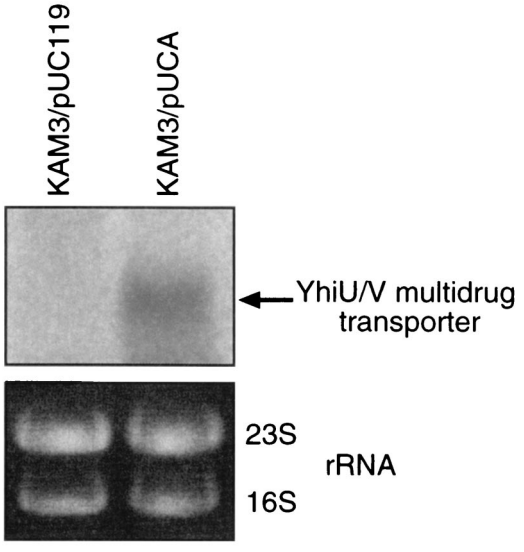

FIG. 2. Northern blot analysis of mRNA from *E. coli* cells. KAM3 cells harboring pUC119 or pUCA were grown in Luria-Bertani medium to an optical density at 600 nm of 0.8 at 37°C, and the RNA was isolated by using the SV total RNA isolation system (Promega). The membranes (Hybond-N; Amersham Pharmacia Biotech) were hybridized at high stringency (68°C) with the probe for *yhiU* labeled with [α-^{32}P]dCTP by random priming. The arrow indicates the main transcript. Each lane contains 15 μg of total RNA.

TABLE 3. Fold induction of transcripts attributed to *evgA* amplification as determined by amplification of cDNA samples

Gene	Fold change
acrE	0.9
acrD	1.0
bcr	3.0
cusB	1.6
emrA	1.6
emrD	1.3
emrE	1.2
emrK	28
fsr	2.3
mdfA	1.0
ybjY	1.1
yceE	1.6
yceL	1.0
ydgF	1.5
ydhE	1.2
yegM	1.2
yhiU	470
yidY	1.2
yjiO	0.9
yfdX	3,200

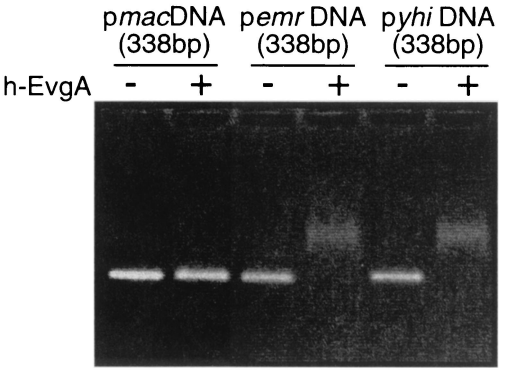

FIG. 3. Gel mobility shift profile of EvgA-responsive DNA fragments. DNA fragments of 338 bp including the *mac*, *emr*, and *yhi* promoter regions were prepared by PCR. The binding reaction was done in a total volume of 10 μl of binding buffer (20 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, and 10% glycerol). Each reaction mixture contained 5 μg of His_6-EvgA (+) or bovine serum albumin as a negative control (−) as well as 1 μg of DNA. After they were incubated at 37°C for 30 min, the mixtures were put onto a 4% NuSieve 3:1 (BioWhittaker Molecular Applications) agarose gel, and electrophoresis was done. The gel was stained with ethidium bromide and photographed under UV illumination.

overexpressing EvgA, it may be phosphorylated by an EvgS-independent process.

During recent years, it has been found that two-component systems regulate a number of bacterial drug resistance pathways. VncSR in *Streptococcus pneumoniae* (17) and VanSR in enterococci regulate vancomycin resistance (1). ArlSR in *Staphylococcus aureus* regulates the MDR pump NorA (2), and we identified EvgSA as the regulator of the MDR pump YhiUV in this study. To the best of our knowledge, this is the first case of a two-component system that directly regulates the expression of MDR transporters in gram-negative bacteria. These findings indicate that two-component system-controlled MDR might become a new threat for chemotherapy against bacterial pathogens in the near future.

We thank George M. Church for plasmid pKO3, Hiroshi Nikaido for strain ZK796, and Tomofusa Tsuchiya for strain KAM3. We thank Eitan Bibi for critically reading the manuscript.

K. Nishino is supported by a research fellowship from the Japan Society for the Promotion of Science for Young Scientists. This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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