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NOTES

Global Analysis of Genes Regulated by EvgA of the Two-Component Regulatory System in *Escherichia coli*

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The response regulator EvgA controls expression of multiple genes conferring antibiotic resistance in *Escherichia coli* (K. Nishino and A. Yamaguchi, J. Bacteriol. 184:2319-2323, 2002). To understand the whole picture of EvgA regulation, DNA macroarray analysis of the effect of EvgA overproduction was performed. EvgA activated genes related to acid resistance, osmotic adaptation, and drug resistance.

Bacteria have developed signaling systems for eliciting a variety of adaptive responses to their environment. These adaptive responses are often mediated by two-component regulatory systems, generally consisting of a sensor kinase and a response regulator (1, 17, 35, 36, 45). In a previous study, Nishino and Yamaguchi found that the EvgAS two-component system modulates drug resistance of *Escherichia coli* by regulating the expression of drug transporters (32, 33). The response regulator EvgA modulates the expression of *emrKY* (20), which encodes a bile salt-specific exporter (31, 33), and *yhiUV*, which encodes a multidrug exporter (31, 32). Overexpression of EvgA in the background of a deficiency of *E. coli* major multidrug exporter AcrB (25) confers drug resistance against antibiotics, dyes, and bile salts (33). EvgA also significantly regulates the expression of *yfdX*, whose function is unknown (32, 33). However, the physiological role of the EvgAS system is unknown.

We hypothesized that EvgA must control the expression of a wide range of genes. *E. coli* macroarrays have been successfully used to quantify the entire complement of individual mRNA transcripts (5, 7, 44, 46). Therefore, in order to reveal the whole picture of the EvgA-controlled genes, macroarray analysis of the effect of EvgA overproduction was employed in this study.

Effect of overexpression of *evgA* on gene expression. DNA macroarrays, which contain most of the genomic open reading frames of *E. coli* (8), allowed comprehensive studies on EvgA-controlled *E. coli* gene expression. The strain NK1230 has a single copy of *evgA* in its chromosome and harbors a mock plasmid, pUC119, while NK1231 bears high-copy-number plasmid pUCA, which carries the *evgA* gene (Table 1). The growth rates of the two strains were indistinguishable (data not shown). The comprehensive transcript profiles of these two strains prepared from exponential-phase cells were compared as follows. Cells were grown at 37°C in Luria-Bertani (LB) medium (41) and were rapidly collected for total RNA extraction when the culture reached an optical density at 600 nm of 0.6. Total RNA was isolated by using an RNeasy Protect Bacteria Mini kit (Qiagen). ^{33}P-labeled cDNAs were prepared from RNA extracted from NK1230 and NK1231 by using cDNA-labeling primers (Sigma-Genosys). Labeled cDNAs were hybridized to the Panorama *E. coli* gene arrays (Sigma-Genosys), and phosphorimager files and autoradiograms were obtained according to the manufacturer's instructions as described previously (7, 46). The increased *evgA* gene dosage in NK1231 resulted in a 41.8-fold elevation of cognate *evgA* transcripts, and the expression of 23 genes (open reading frames) was elevated more than fourfold while the expression of 3 genes was repressed by a factor of at least 4 (Table 2).

Known genes in EvgA regulon. In previous studies, Nishino and Yamaguchi reported that overproduction of EvgA increases the expression of *yhiUV*, *emrKY*, and *yfdX* (32, 33). In the DNA macroarray analysis, the enhancement of the gene expression of *yhiU*, *yhiV*, and *yfdX* was 1.8-, 4.4-, and 54.4-fold, respectively. Significant enhancement of *emrK* and *emrY* was not observed in the macroarray analysis. Therefore, we reinvestigated the EvgA-dependent induction of these genes by quantitative real-time reverse transcription-PCR (qRT-PCR) as follows. Bulk cDNA samples were synthesized from total RNA derived from *E. coli* cells by using TaqMan reverse transcription reagents (PE Applied Biosystems) and random hexamers as primers. A real-time PCR was performed with each specific primer pair (Table 3) by using SYBR Green PCR Master Mix (PE Applied Biosystems). *rrsA* of the 16S rRNA gene was chosen as the normalizing gene. The reactions were run on an ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems).

The degree of enhancement of expression of *yhiU*, *yhiV*, *yfdX*, *emrK*, and *emrY* was 250, 67, 1,600, 15, and 12, respectively (Table 4). The degree of induction measured by macroarray analysis was obviously lower than that measured by

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TABLE 1. Strains and plasmids

Strain or plasmid	Genotype or description	Reference or source
E. coli		
KAM3	Derivative of K-12 that lacks *acrAB*	16a, 26
NK1230	KAM3/pUC119	This study
NK1231	KAM3/pUCA	This study
Plasmid		
pUCA	*Hin*dIII-*Sal*I fragment containing *evgA* with a 366-bp upstream flanking sequence cloned into pUC119	32

qRT-PCR, probably because the dynamic range of the former analysis is narrower than that of the latter measurement. The order of the degree of the enhancement was consistent between assays except for that of *yhiU*. The unexpectedly low induction of *yhiU* gene expression measured by the macroarray analysis might be due to the inefficient primers for *yhiU* used in this analysis. The detection limit of the enhancement in the macroarray analysis was also poorer than was the case with qRT-PCR, because the former method could not detect the EvgA-dependent enhancement of *emrKY* genes.

Enhanced expression of genes near *evgAS*. Amplification of the *evgA* gene elevated the expression of genes located near the *evgA* gene in the *E. coli* chromosome (Fig. 1A). That is, two genes (*emrKY*) upstream and six genes (*yfdXWUVE* and *ypdI*) downstream of *evgAS* were controlled by EvgA. These genes, except for *ypdI*, are transcribed in the opposite direction from that of *evgAS*. The *emrKY* transcript expression was increased as described above. The *yfdXWUVE* and *ypdI* transcripts were increased 54-, 6.8-, 1.0-, 3.0-, 4.0-, and 1.9-fold, respectively, in macroarray analysis. qRT-PCR showed increased expression of *yfdXWUVE* and *ypdI* by a factor of 1,600, 1,300, 890, 500, 170, and 30, respectively. These values were again larger than the values obtained from the macroarray analysis, while the orders of degree were roughly consistent with each other, except for that of *yfdU*, which might be due to the inefficient primer for *yfdU* in the macroarray analysis. It was previously reported that EvgA binds upstream of *emrK*, and this region contains the inverted repeat sequence TTCTTAC-GTAA GAA (20). By using the SEARCH PATTERN utility (http://genolist.pasteur.fr/Colibri/) we also found that the upstream region of *yfdW* contains the same sequence (TTCTTAC-GTA AGAA) (Table 5). A similar sequence is also located in the upstream regions of *yfdE* (TTCTTCA-GTAAGAA), *yfdX* (TTCTTGC-GTAAGAT), and *ypdI* (ATCTTAC-GCAAG

TABLE 2. *E. coli* genes whose relative expression levels were increased or decreased by *evgA* amplification

Gene[a]	b no.[b]	Functional classification[c]	Known or predicted function	Effect of EvgA on gene expression (fold change)
Increased expression				
hdeA[d,e]	b3510	Not known	Protein regulated by H-NS, chaperone	84.3
yfdX	b2375	Not known	Protein regulated by EvgA	54.4
hdeB[d,e]	b3509	Not known	Protein regulated by H-NS, predicted chaperone	42.2
evgA[e]	b2369	Regulation	Regurator of EvgAS two-component system	41.8
gadA[d,e]	b3517	Metabolism	Glutamate decarboxylase-alpha	41.4
ydeP[e]	b1501	Metabolism	Putative reductase	15.3
yjfL	b4184	Cell structure	Putative membrane protein	9.2
yfdW[e]	b2374	Metabolism	Putative formyl-coenzyme A transferase	6.8
msyB	b1051	None	Acidic protein suppresses mutants lacking function of protein export	6.5
dps[e]	b0812	Adaptation (starvation)	Stress response DNA-binding protein	6.3
yhiE[e]	b3512	Regulation	Putative regulator	6.2
ybjR	b0867	Metabolism	Putative amidase	5.5
asr	b1597	Adaptation (stress)	Acid shock RNA controlled by *phoBR*	5.3
yfdM	b2359	Extrachromosomal (phage)	Putative transferase	5.2
gadC (*xasA*)[d,e]	b1492	Transport	Predicted amino acid transporter	5.2
ypeC	b2390	None	Function unknown	5.2
ycaC	b0897	Metabolism	Putative cysteine hydrolase	5.0
yahO	b0329	None	Function unknown	4.7
yfdO	b2358	Extrachromosomal (phage)	Putative replication protein	4.6
yhiV	b3514	Transport	Multidrug transport protein	4.4
ybaS	b0485	Metabolism	Putative glutaminase	4.3
mgtA	b4242	Transport	Magnesium transporter	4.1
yfdE	b2371	Metabolism	Putative formyl-coenzyme A transferase	4.0
Decreased expression				
tnaA	b3708	Metabolism	Tryptophan deaminase	7.1
tnaL	b3707	Metabolism	Tryptophanase leader peptide	5.0
glpQ	b2239	Metabolism	Glycerophosphodiester phosphodiesterase	4.5

[a] Based on information from the EcoGene database (http://bmb.med.miami.edu/ecogene/ecoweb/) (40).
[b] Blattner number. Based on information from the *E. coli* Genome Project database (http://www.genome.wisc.edu/) (8).
[c] Based on information from the Gene ProtEC database (http://genprotec.mbl.edu/) (38, 39).
[d] Previously shown to be important for acid survival.
[e] Expression of these genes are increased in the H-NS-deficient strain (18).

TABLE 3. Primer pairs used to compare transcript levels

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
emrK	GCGCTTAAACGTACGGATATTAAGA	ACTGTTTCGCCGACCTGAAC
emrY	GCTTTCTGGGAGCATCAACAG	CAGTAACAGGGATCGCAATGG
evgA	TTCTTGTTTCGATAAGGAGTCGAGTT	TGCTATTTCCCCTTCTCTCTCAAC
fimA	GGTGTGCAGATCCTGGACAGA	CGGAATGGTATTGGTTCCGTTA
fimF	GGGTTTACTGGCGTTGCAGAT	GAAGCCGCTGACACCGTATT
fimH	CGATGTTTCTGCTCGTGATGTC	GTTGTGCCGGAGAGGTAATACC
fimI	ACCGGTGCCTTTTGTTATTCAT	ACCGTGAAACGCCACACCTA
fliA	TTAGGGATCGATATTGCCGATT	CGTAGGAGAAGAGCTGGCTGTT
fliC	TCAGGCGGTACACCTGTTCAG	GCAGCGTAAAGATTGCCATTT
gadA	CGGCTTCGAAATGGACTTTG	TGGGCAATACCCTGCAGTTT
gadX	TTTATACCGCTGCTTCTGAACGT	GTGTCCACTCATGGGCGATATTA
hdeA	CTGCCAGTTGTGAGCAATGC	CTGCAGTTGGCTGGAAGGAT
hdeB	CACTGGTGAACGCACAATCTG	CTTCATGCAGCATCCACCAT
hdeD	ATGAAAGGCAGCTGGCTACAG	CCAGTGTGGAAACCAGCGTTA
hns	GCTGATCGCTGACGGTATTGA	GCTTTGGTGCCAGATTTAACG
rrsA	CGGTGGAGCATGTGGTTTAA	GAAAACTTCCGT GGATGTCAAGA
tolC	CCGGGATTTCTGACACCTCTT	TTTGTTCTGGCCCATATTGCT
yfdE	ATCAACCCGCGCCTCATAT	GCTCATTGCCTGAATGATGGT
yfdU	TGCCAATGTCTATGGCGAAAG	CGTGTGCCAATAACCAATTCA
yfdV	GAGTTCAGTGCCGAAATTGCTT	TGTTCGCTGTTCAAATGACATG
yfdW	GGCAACGAGTCACCATGTCA	AGACGCTGCTGGTCACGTAA
yfdX	ACGTCACGCATCGCATATAAAC	AGCAAGTTCAGCAAACCCAGAA
yhiE	TCATATCCTTGATGAAGAAGCGATT	GCGCTTTAGCTTTTAGTTTACTGATG
yhiU	CCCCCGGTTCGGTCAA	GGACGTATCTCGGCAACTTCAT
yhiV	TTACCGTCAGCGCTACCTATCC	GCCATCAAGCCCATTCATATTT
yhiW	CGAGAGTATTCTGCTGCTGGAGA	AGTAATGGCAAACTGTCAGCTCAT
ypdI	GGTCTCTATTATGGCATGCAATGT	ATCGCCTGTGATGGCTGAAG

AA) (Fig. 1A and Table 5). This fact suggests that EvgA may directly regulate *yfdE*, *yfdW*, *yfdX*, and *ypdI* as well as *emrK*.

Enhanced expression of genes near *yhiUV*. Amplification of the *evgA* gene elevated the expression of genes located near *yhiUV* in the *E. coli* chromosome (Fig. 1B). That is, seven genes (*hdeABD*, *slp*, and *yhiDEF*) upstream and three genes (*gadAX* and *yhiW*) downstream of *yhiUV* were controlled by EvgA. Regulation of *yhiUV* by EvgA was described above. The *gadAX*, *hdeABD*, *slp*, and *yhiDEFW* transcripts were increased 41-, 3.6-, 84-, 42-, 2.7-, 2.0-, 1.2-, 6.2-, 1.9-, and 2.8-fold, respectively, in macroarray analysis. qRT-PCR showed increased expression of *gadAX*, *hdeABD*, and *yhiEW* by a factor of 320, 13, 480, 530, 120, 400, and 17, respectively. These values were again larger than the values obtained from the macroarray analysis, while the orders of degree were roughly consistent between assays. We searched for a putative EvgA-binding motif in the upstream region of them. As a result, we found the motif in the upstream regions of *gadA*, *hdeA*, *hdeD*, *slp*, *yhiD*, *yhiW*, *yhiE*, and *yhiU* (Fig. 1B and Table 5). This fact suggests that these genes may be controlled directly by EvgA. The *gadA* gene encodes a glutamate decarboxylase (Table 2) (5, 10), and *hdeA* encodes a chaperone-like protein (13). Both *gadA* and *hdeA* play important roles in acid survival. *slp* encodes a carbon starvation-inducible lipoprotein that stabilizes the outer membrane (2). The functions of *hdeD* and *yhiDWEU* are not well understood.

Genes downregulated by EvgA. Amplification of *evgA* caused a decrease in expression of 26 genes by a factor of 2 or more (3 genes that were decreased in expression by a factor of 4 or more are listed in Table 2). Of these genes, 12 were involved in metabolism (*ansB* [factor of 2.9], *aspA* [3.8], *frdA* [2.5], *garD* [2.0], *glpQ* [4.5], *narG* [2.7], *pflB* [2.2], *sgaE* [2.9], *tdcD* [2.1],

TABLE 4. Fold induction of specific transcripts attributed to *evgA* amplification as determined by probing of macroarrays and amplification of cDNA samples

Gene	Fold change by[a]:	
	Probing	Amplification
Increased expression		
yfdX	54	1,600
yfdW	6.8	1,300
yfdU	1.0	890
hdeA	84	780
hdeB	42	530
yfdV	3.0	500
yhiE	6.2	400
gadA	41	320
yhiU	1.8	250
yfdE	4.0	170
evgA	42	148
hdeD	2.7	120
yhiV	4.4	67
ypdI	1.9	30
yhiW	2.8	17
emrK	1.0	15
gadX	3.6	13
emrY	1.1	12
tolC	1.4	2.2
hns	1.1	1.1
Decreased expression		
fimA	2.9	76
fimI	3.2	40
fimF	2.8	36
fimH	2.7	10
fliA	2.2	5.5
fliC	2.2	4.5

[a] Amplification results were obtained by RT of bulk RNA followed by PCR amplification. PCR was performed with each specific primer pair (see Table 2). Probing results were obtained with ^{33}P-labeled cDNA hybridized to DNA macroarrays.

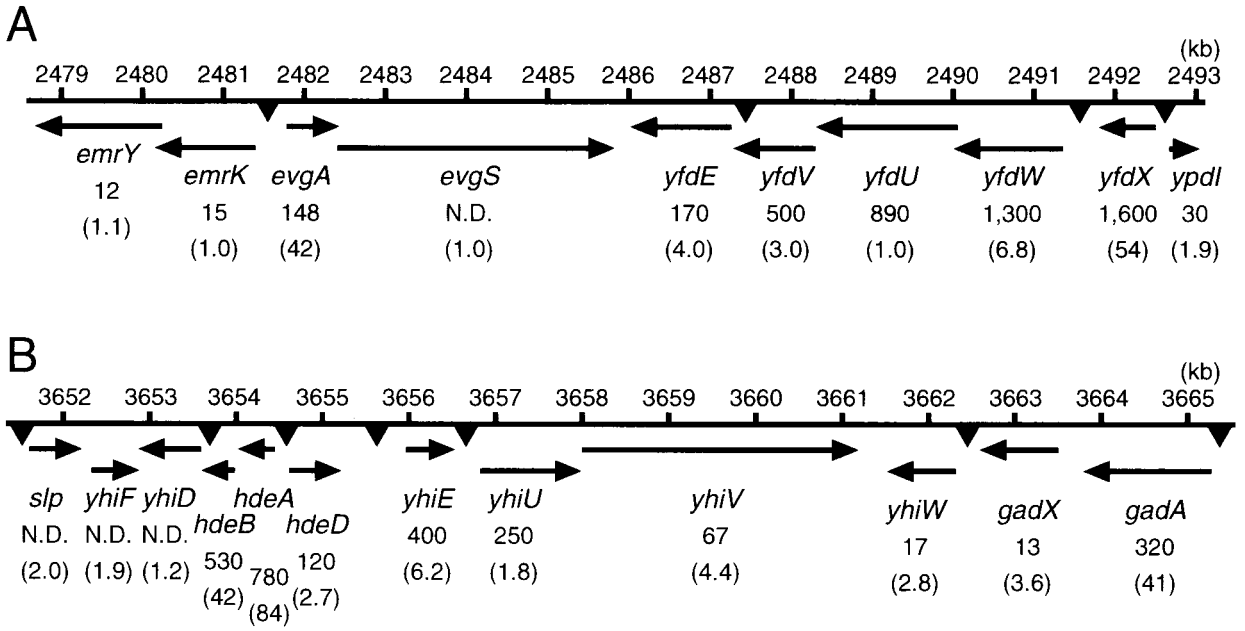

FIG. 1. Two clusters of EvgA-regulated operons and genes. (A) Gene clusters near the *evgAS* two-component regulatory system. (B) Gene clusters near the *yhiUV* multidrug exporter system. Arrows denote the direction of transcription. Putative EvgA-binding motifs are indicated by triangles. Expression changes of genes detected by real-time qRT-PCR are indicated under the gene names. Numbers in parentheses indicate the expression level changes detected by macroarray analyses. Kilobase pairs (kb) indicate the position on *E. coli* chromosomal DNA as annotated on the Coribri website (http://genolist.pasteur.fr/Colibri/). N.D., not determined.

tdcE [2.4], *tnaA* [7.1], and *tnaL* [5.0]), and 7 were involved in motility (fimA [factor of 2.9], fimF [2.8], fimH [2.7], fimI [3.2], fliA [2.2], fliC [2.2], and yebV [2.2]). These data suggest that overexpression of *evgA* may repress the motility of *E. coli*. We also investigated the expression level changes of *fimAFHI* and *fliAC* by qRT-PCR (Table 4). qRT-PCR showed decreased expression of all of these genes, and decreased values were larger than the values obtained from the macroarray analysis, just as in the case of genes whose expression was increased by EvgA.

Effect of EvgA on acid survival. The list of upregulated genes (Table 2) contains several genes encoding proteins induced at low pH. That is, *gadC* (formerly called *xasA*) encodes a probable GABA/glutamate antiporter (11, 16), and *gadA* and *gadB* (*gadB* transcript expression was increased 3.8-fold) encode two glutamate decarboxylases (Table 2) (5, 10). The *hdeA*-encoded protein has been proposed to have a chaperone-like function under extremely acidic conditions (13). Recently, Hommais et al. reported that *gadX* (formerly called *yhiX*; *gadX* transcript expression was increased 3.6-fold) plays a role in the control of

TABLE 5. Putative EvgA-binding motifs in EvgA-regulated genes that are located near *evgAS* or *yhiUV*[a]

Base pairs from start codon	Gene	Pattern sequence
−172	*gadA*	TTATTACGATAATAA
−269	*yfdW*	TTCTTACAGTTGTAAGAA
−563	*yfdE*	TTCTTCAGGAGCGGATGGTAAGAA
−200	*emrK*	TTCTTACTAATCCTACAGGCGTAAGAA
−242	*evgA*	TTCTTACGCCTGTAGGATTAGTAAGAA
−968	*hdeD*	TTCTTACAGTATTCGATCACTTTAGGAA
−152	*yhiD*	TTCCTAAAGTGATCGAATACTGTAAGAA
−127	*yfdX*	TTCTTGCGATAATAACTACAACTGTAAGAT
−564	*yhiU*	TACTTATGGTAAACACTTGCCCCATAAGAA
−198	*ypdI*	ATCTTACAGTTGTAGTTATTATCGCAAGAA
−542	*slp*	TTCATACAATGACATATTAAAATATCAGCAAGAA
−736	*yhiX*	CTCTTACCAGGTTGCCGCTTATCTGGCGGATGAA
−410	*yhiW*	TTTTTACGGCACTGACCGTTCTGCGGAAGGAATAA
−279	*hdeA*	TTGTTGCCTTATCTATATATAACATAGAACCACCCTATAAAATTAAGAA
−99	*yhiE*	TTCTTATAGGCGTTTACTATATTGAACAACGATTCGGACAAGGATGTAAATA

[a] No more than a total of three mismatches were allowed for the entire motif (TTCTTAC-GTAAGAA), and no more than two mismatches were allowed per half. The search was restricted to regions within 1,000 bp upstream of start codons. Putative EvgA-binding motifs are underlined.

genes induced by low pH (18). This prompted us to study the effect of *evgA* overexpression on resistance to acidic stress. The overnight (20-h) cultures in LB medium (pH 6.5) were diluted 1:1,000 (dilution to 3×10^6 CFU/ml) into prewarmed LB (pH 2.0). Acid challenge was carried out for 2 h at 37°C. Viable cell counts were determined at 2 h after the acid challenge, and then the percentage of survival was calculated as 100 times the number of CFU per milliliter remaining after acid treatment divided by the initial number of CFU per milliliter at time zero. The normal *E. coli* cells showed a low level of resistance (0.1% survival) to acid stress. A large increase in resistance (7.9% survival) was measured in *evgA*-overexpressing cells compared to that measured with the normal cells (a nearly 80-fold increase).

Effect of EvgA on survival under high osmolarity. Several genes induced by EvgA are involved in the response to high osmolarity. That is, the *osmC* gene, which is induced by high osmolarity (14), showed an increased mRNA level in the *evgA*-overexpressing cells. The *osmY* gene expression is also a locus of hyperosmotic stress response (34). The *ompA* deletion mutant is significantly more sensitive than that of its parent strain to acidic environment and high osmolarity (47), and the expression of this gene was induced by EvgA overproduction. Nakashima et al. reported that the expression of OmpX is affected by both the medium osmolarity and pressure (29). *osmCY* and *ompAX* transcript expressions were increased 2.7-, 3.1-, 2.4-, and 3.1-fold, respectively, in macroarray analysis. We analyzed the role of EvgA in the response to osmotic stress. *E. coli* cells were grown overnight in LB medium. Cells were diluted 1:1,000 in fresh LB medium supplemented with 3 M NaCl for 1 h at 37°C and then were plated on LB. Viable cells were counted after 16 h at 37°C. A 6.5-fold increase in resistance to high ionic strength was measured in the *evgA*-overexpressing cells (55.7% survival) compared to that measured in the normal cells (8.7% survival).

Conclusions. This work investigated the utility of the macroarray analysis in determining the global effects of *evgA* gene dosage amplification. In this study, we found a lot of genes whose EvgA dependence was not known previously. We discovered that the increased expression of several genes in the EvgA-overexpressing cells resulted in a better ability of cells to survive at low pH and high osmolarity than that of normal cells.

EvgA affects the expression of gene clusters located near *emrKY* and *yhiUV*. Since these two clusters contain several EvgA-binding motifs (Fig. 1), most of these are probably controlled directly by EvgA. EmrKY and YhiUV drug exporters need the outer membrane protein TolC for their function, like some other drug transporters of *E. coli* (16a, 21, 28, 30). Also, EvgA overproduction moderately increased the expression of *tolC* (Table 4).

Recently it was reported that expression of the gene cluster located near *yhiUV* is also increased by a deficit of H-NS protein (18). H-NS is a nucleoid-associated protein that is required for the organization of chromosomal DNA (3, 6, 15, 48), and it also functions as a transcription factor (18, 19). We found that there are several overlapping genes whose expression levels were increased both by EvgA overproduction and by the lack of H-NS (indicated in Table 2) (18). Overproduction of EvgA did not change the expression level of *hns* detected either by macroarray or by qRT-PCR analysis (Table 4), but the expression level of *evgA* is increased in the H-NS-deficient strain compared to that of its wild-type parent strain (18). This overlap might be due to EvgA overproduction in the H-NS-deficient strain.

Ma et al. reported that the expression of the *acrAB* multidrug exporter system is induced by treatment with fatty acids, sodium chloride, or ethanol (24). Lomovskaya et al. reported that the *emrAB* multidrug exporter system is induced by treatment with salicylic acid or 2,4-dinitrophenol (23). It was reported that the expression of *yhiUV* is controlled by RpoS (4, 43), a conserved alternative sigma factor, that is needed for *E. coli* to survive stresses associated with starvation, such as heat shock (22, 27), oxidative stress (22, 27), osmotic challenge (27), and near-UV light (42). These events indicate that there is a strong relationship between regulation of drug transporters and stress responses. EvgS may sense some kind of stress. Bock and Gross suggested that EvgS is connected with the oxidation status of the cell via the link to the ubiquinone pool (9). Recently, information about factors that affect the expression of *evgAS* was obtained from microarray studies. Specifically, it was found that addition of autoinducer 2, a signaling pheromone of quorum sensing, represses the expression of *evgS* (12) and that the superoxide-generating agent paraquat represses the expression of *evgA* (37).

Further investigation of the natural signal that activates EvgS is needed in order to understand the biological significance of the EvgS-EvgA signal transduction system and may provide further insights into the role of multidrug transporters in the physiology of the cell.

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