

## Escherichia coli Gene Expression Responsive to Levels of the Response Regulator EvgA

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Received 8 January 2002/Accepted 22 August 2002

To investigate the function of the EvgA response regulator, we compared the genome-wide transcription profile of EvgA-overexpressing and EvgA-lacking *Escherichia coli* strains by oligonucleotide microarrays. The microarray measurements allowed the identification of at least 37 EvgA-activated genes, including acid resistance-related genes *gadABC* and *hdeAB*, efflux pump genes *yhiUV* and *emrK*, and 21 genes with unknown function. EvgA overexpression conferred acid resistance to exponentially growing cells. This acid resistance was abolished by deletion of *ydeP*, *ydeO*, or *yhiE*, which was induced by EvgA overexpression. These results suggest that *ydeP*, *ydeO*, and *yhiE* are novel genes related to acid resistance and that EvgA regulates several acid resistance genes. Furthermore, the deletion of *yhiE* completely abolished acid resistance in stationary-phase cells, suggesting that YhiE plays a critical role in stationary-phase acid resistance. The multidrug resistance in an *acrB* deletion mutant caused by EvgA overexpression was completely abolished by deletion of *yhiUV*, while the *emrKY* deletion had no effect on the increase in resistance by EvgA overexpression. In addition, EvgA overexpression did not confer resistance in a *tolC*-deficient strain. These results suggest that YhiUV induced by EvgA overexpression is functionally associated with TolC and contributes to multidrug resistance.

Bacteria have developed sophisticated signaling systems for adaptive responses to a variety of environments. One of the major mechanisms of signal transduction leading to specific gene expression is known as the two-component system and as its more complex variant, the phosphorelay system (11, 24, 34, 40). A typical two-component system consists of a sensor kinase and its cognate response regulator, which usually functions as a transcriptional factor. The sensor kinase receives an environmental signal, which induces autophosphorylation of a histidine residue. The phosphoryl group on the histidine residue is then transferred to an aspartate residue on the cognate response regulator, resulting in modulation of the expression of its target genes.

Genomic sequencing of various microorganisms has revealed the presence of many two-component regulatory systems in all species examined. The functions of many of these putative two-component systems remain unknown. The two-component system that consists of response regulator EvgA and sensor kinase EvgS is one such uncharacterized system in *Escherichia coli*. EvgA and EvgS are highly similar to BvgA and BvgS, respectively, which control the expression of adhesins, toxins, and other virulence factors in *Bordetella pertussis* (2, 31, 38). The *evgAS* operon is located adjacent to the *emrKY* operon, which codes for an efflux pump, and each operon is transcribed in the opposite direction. EvgA binds the intergenic region of *evgAS* and *emrKY* and regulates the expression of both operons (13, 36). Nishino and Yamaguchi (20) reported that overexpression of EvgA increases multidrug resistance in a drug-hypersusceptible strain which lacks the constitutive multidrug efflux pump genes *acrA* and *acrB* (18).

However, the physiological function of the EvgAS system is unknown.

The recently developed DNA microarray technique has allowed the parallel study of the expression of every gene in an organism. Ogura et al. (22) overexpressed three *Bacillus subtilis* response regulator gene products, DegU, ComA, and PhoP, in *B. subtilis* strains containing disruptive insertions in the cognate sensor kinase gene and identified regulon candidates of each response regulator by comprehensive analysis of gene expression by using DNA microarrays. The overproduction of the nonphosphorylated response regulators resulted in altered expression of the target genes in the absence of the environmental signals responsible for their phosphorylation. Kobayashi et al. (14) comprehensively analyzed regulon candidates of 24 *B. subtilis* response regulators by this strategy. This approach was applicable to the detection of the target genes of uncharacterized two-component systems for which environmental signals for activation were unknown.

In this study, we compared mRNA levels in EvgA-overexpressing strains to those in EvgA-lacking strains with oligonucleotide microarrays composed of 4,241 *E. coli* protein-encoding genes (open reading frames [ORFs]) and revealed the target gene candidates for the EvgAS system. Furthermore, we knocked out several genes induced by EvgA overexpression and identified novel genes related to acid resistance and multidrug resistance.

### MATERIALS AND METHODS

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**Bacterial strains and media.** *E. coli* wild-type strain MG1655 (ATCC 47076) was used. DH5 $\alpha$  competent cells (Invitrogen) were used for amplification of plasmids. Bacterial cells were grown in Luria-Bertani (LB) broth (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter, pH 7.0) or on LB agar medium (Bio 101). Antibiotics were added, when required, at the following final concentrations: carbenicillin, 100  $\mu$ g/ml; chloramphenicol, 20  $\mu$ g/ml for AcrB-producing strains and 5  $\mu$ g/ml for AcrB-deficient strains. LB agar was supplemented with 10% (wt/vol) sucrose, as required.

**Molecular biology techniques.** Chromosomal DNA was isolated with a DNeasy tissue kit (Qiagen). Plasmids were isolated with a HiSpeed plasmid purification kit or a QIAprep Miniprep kit (Qiagen). PCRs were performed in an MJ Research PTC-100 programmable thermal controller. PCRs for constructions of plasmids were performed with *Pfu* Turbo DNA polymerase (Stratagene) or Platinum Pfx DNA polymerase (Invitrogen) because of their high fidelity. Diagnostic PCRs were performed with *Z-Taq* polymerase (Panvera). PCR products were purified with a QIAquick PCR purification kit (Qiagen). Restriction endonucleases and alkaline phosphatase were obtained from New England Biolabs. The DNA-ligation kit was obtained from Panvera. Restriction fragments were purified with a QIAquick PCR purification kit or a MinElute reaction cleanup kit (Qiagen) or were isolated, as required, from agarose gels with a QIAquick gel extraction kit (Qiagen). Electroporation was performed with a Gene Pulser apparatus (Bio-Rad). All molecular biology techniques were carried out according to the manufacturer's instructions or as described by Sambrook et al. (27). Oligonucleotides were purchased from Operon.

**Construction of plasmids for EvgA expression and allelic exchange.** The EvgA overexpression plasmid was constructed as follows. The 884-bp *evg4* gene with the native promoter and EvgA-binding site, which is important to simulate the *evg4* promoter (36), was amplified by PCR from MG1655 by using primers 5'-TTCCCTTAAGCTCTAAGACTAAACCGTGGCTTGCAATAC-3' and 5'-TTCCCTGAATTCTTACCGATTTGTTACGTGCG-3'. These primers contain *Hind*III and *Eco*RI sites (underlined) suitable for cloning. The PCR products were digested with restriction enzymes and then were ligated into the *Hind*III and *Eco*RI sites of pUC19. Sequence analysis confirmed that no mutations had been introduced into the *evg4* gene. The resulting plasmid was designated pUCevgA. Another EvgA expression plasmid was constructed as follows. The 613-bp *evg4* gene was amplified by PCR from MG1655 with primers 5'-CGCGGATCCAACGCAATAATTATTGATGACCACCTCTTG-3' and 5'-TTCCTCTCAGTTAGCCGATTTGTTACGTGTCG-3'. These primers introduced *Bam*HI and *Pst*I sites (underlined) at the 5' and 3' ends of the PCR-generated *evg4* gene, respectively, and enabled the amplified gene to be inserted into the *Bam*HI and *Pst*I sites of the expression vector pQE80L (Qiagen) in the correct reading frame cloning. Sequence analysis confirmed the in-frame insertion and confirmed that no mutations had been introduced by PCR. The resulting plasmid, designated pQEevgA, expresses an EvgA fusion protein (composed of full-length EvgA with an amino-terminal Met-Arg-Gly-Ser-His-His-His-His-Gly-Ser) under transcriptional control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-regulated phage T5 promoter.

To construct plasmids for allelic exchange, approximately 1-kb fragments flanking the gene or tandem group of genes to be disrupted were amplified from MG1655 genomic DNA in two separate PCRs by using primer pair No and Ni or primer pair Ci and Co, respectively. The two PCR products were combined and amplified by PCR using primers No and Co. Resulting amplified products were digested with restriction enzymes and then were cloned into pKO3 (17) or pKOV (25), constructed from pKO3 by removing the extra *NoI* site and adding a stuffer. Table 1 lists oligonucleotide sequences for No, Ni, Ci, and Co used to construct plasmids for deletion of each gene or tandem group of genes. The Ni and Ci primers were designed such that the ends of the PCR products facing the 5' and 3' ends of the gene to be disrupted contained a sequence suitable for in-frame fusion of the 5' and 3' flanking regions by annealing. Each gene or tandem group of genes was completely deleted, leaving only the start codon of the first ORF of a tandem group of genes and the stop codon of the last ORF, except in the case of deletions of *ydeP*, *b1500*, *ydeO*, *yegR*-b2084-*yegZ*, *sly-yhfF*, *hdeB*, *yiiS*, *yjE*, and *emrKY* in which the 9, 51, 15, 33, 9, 132, 93, 111, and 6 nucleotides after the start codon were left, respectively. These exceptions were designed to minimize polar effects or to obtain primers with adequate melting temperatures. The No and Co primers contain restriction enzyme cutting sites suitable for cloning. pKO3 and pKOV were used for insertion of *NoI-Sal*I- and *NoI-Bam*HI-digested fragments, respectively.

**Gene deletion and screening.** Each plasmid for deletion was electroporated into cells, and they were allowed to recover for 1 h at 30°C with aeration. The cells were plated on LB plates containing chloramphenicol and were incubated at 43°C overnight to isolate integrants. The integrants were streaked on LB plates containing chloramphenicol and were incubated at 43°C for 12 h to prevent contamination of the parent strain. The purified integrants were subsequently grown in drug-free LB for 9 h for a second allelic exchange, serially diluted, plated on an LB plate supplemented with 10% (wt/vol) sucrose, and incubated at 30°C for 24 h. Chloramphenicol-susceptible and sucrose-resistant clones were selected and subjected to PCR for verification of gene deletion. The verification was done by direct amplification of genomic DNA from each colony by using the primer pair No and Co. A wild-type or deletion genotype was diagnosed according to the size of the PCR product.

**RNA isolation, mRNA enrichment, and labeling.** A single colony of *E. coli* harboring plasmid was inoculated in 1 ml of LB broth containing carbenicillin and was grown overnight with aeration at 37°C. The next day 30 ml of LB broth was inoculated with 0.1 ml of the overnight culture and was grown at 37°C with aeration to an optical density at 600 nm of 0.2. The expression of His-tagged EvgA was induced by adding 1 mM IPTG to the LB broth before inoculation of the culture, when required. Total RNA was isolated from the cells by extraction with hot acid phenol:chloroform. Briefly, samples of culture were transferred directly into acid phenol:chloroform (5:1; Ambion) at 65°C to ensure rapid lysis and inactivation of RNases. Two additional acid phenol:chloroform extractions were performed, followed by ethanol precipitation. The pellet was then resuspended in diethyl pyrocarbonate-treated water and was purified with an RNeasy Mini Kit (Qiagen). Isolated RNA was resuspended in diethyl pyrocarbonate-treated water, quantitated on the basis of its absorption at 260 nm, visualized on a denaturing polyacrylamide gel to check quality, and stored at -20°C until further use.

Enrichment of mRNA was done as described in the GeneChip expression analysis technical manual (Affymetrix). In brief, a set of oligonucleotide primers specific for either 16S or 23S rRNA was mixed with total RNA. After annealing at 70°C for 5 min, Moloney murine leukemia virus reverse transcriptase (New England Biolabs) was added to synthesize cDNA strands complementary to the two rRNA species. The cDNA strand synthesis allows for selective degradation of the 16S and 23S rRNAs by RNase H (Epicentre Technologies). Treatment of the RNA-cDNA mixture with DNase I (Amersham Pharmacia Biotech) removed the cDNA molecules and oligonucleotide primers, which resulted in an RNA preparation that was enriched for mRNA.

For direct labeling of RNA, 20 µg of enriched RNA was fragmented at 95°C for 30 min in a total volume of 88 µl of T4 polynucleotide kinase buffer (New England Biolabs). After being cooled to 4°C, 2 µl of 5 mM γ-S-ATP (Roche Molecular Biochemicals) and 10 µl of a 10-U/µl concentration of T4 polynucleotide kinase (New England Biolabs) were added to the fragmented RNA and the reaction mixture was incubated at 37°C for 50 min. To inactivate T4 polynucleotide kinase, the reaction mixture was incubated for 10 min at 65°C and the RNA was subsequently ethanol precipitated to remove excess γ-S-ATP. After centrifugation the RNA pellet was resuspended in 96 µl of 30 mM MOPS (morpholinepropanesulfonic acid, pH 7.5), and 4 µl of a 50 mM polyethylene oxide-iodoacetyl biotin (Pierce Chemical) solution was added to introduce the biotin label. The reaction mixture was incubated at 37°C for 1 h, and the labeled RNA was subsequently ethanol precipitated and washed twice with 70% ethanol to remove unreacted biotin compounds. The labeled RNA was quantitated on the basis of the absorption at 260 nm and was hybridized to an *E. coli* genome array (Affymetrix).

**Microarray hybridization, scanning, and analysis.** Hybridization was done as described in the GeneChip expression analysis technical manual. The hybridization solution contained 100 mM MES (*N*-morpholinoethanesulfonic acid), 1 M NaCl, 20 mM EDTA, and 0.01% Tween 20, pH 6.6 (referred to as 1× MES). In addition, the solution contained 0.1 mg of herring sperm DNA (Promega)/ml, 0.5 mg of acetylated bovine serum albumin (BSA; Invitrogen)/ml, and 50 pM control oligonucleotide B2 (Affymetrix). Hybridization was carried out at 45°C for 16 h with mixing on a rotary mixer at 60 rpm.

Following hybridization, the sample solution was removed and the array was washed and stained in a GeneChip fluidics station (Affymetrix). In brief, to enhance the signals 10 µg of streptavidin (Vector Laboratories)/ml and 2 mg of BSA/ml in 1× MES were used as the first staining solution. After the streptavidin solution was removed, an antibody mix was added as the second stain, containing 0.1 mg of goat immunoglobulin G (Sigma-Aldrich)/ml, 5 µg of biotinylated anti-streptavidin antibody (Vector Laboratories)/ml, and 2 mg of BSA/ml in 1× MES. Nucleic acid was fluorescently labeled by incubation with 10 µg of streptavidin R-phycerythrin conjugate (Molecular Probes)/ml and 2 mg of BSA/ml in 1× MES.

The arrays were scanned at 570 nm with a resolution of 3 µm with a Gene-Array scanner (Affymetrix). Data analysis was performed by using Affymetrix Microarray Suite 5.0 software. The software calculates change calls, change *p* values (statistical significance for change calls), and signal log ratio. Signal log ratio is the change in expression level for a transcript between a baseline and an experiment array, expressed as the log<sub>2</sub> ratio. Arrays were globally scaled to a target signal of 500 and were normalized. The default parameters for change calls were used.

**Susceptibility testing.** The antibacterial activities of the agents were determined by the broth microdilution method by following the recommendations of the National Committee for Clinical Laboratory Standards (19) with modifications as follows. LB broth was used instead of cation-adjusted Mueller-Hinton broth, and initial concentrations of the compounds may differ from those rec-

TABLE 1. Oligonucleotides used to construct and verify deletion strains

Gene(s)	Name	Oligonucleotide sequence (5' to 3') <sup>a</sup>	Enzyme <sup>b</sup>
<i>evgAS</i>	No	aaggaaaaaaggccgc <u>CTGAATACGTGCTGAAGCGACTTC</u>	
	Ni	ggagccgtat <u>ttaCATAGATTATCCCTTGCAATGAAGC</u> ATC	
	Ci	gcaaggaaataatctat <u>TAAATAGCGGCCCAACATGTT</u> C	
	Co	cgcacgc <u>atgtcgacCGCTAAAAGATGCTCCTGCCTAC</u>	
<i>acrB</i>	No	aaggaaaaaaggccgc <u>CATCAGTAAGCAAGAGTACGATCAGG</u>	
	Ni	gattcacat <u>gttgtatcaCATGTCTTAACGGCTCCTGTT</u>	
	Ci	gagccgttaagacat <u>tgatACAACGTTAATCAGATATT</u> CGC	
	Co	cgcacgc <u>atgtcgacTAAAGGTGTCATCCAGATATT</u> CGTC	
<i>appCBA</i>	No	aaggaaaaaaggccgc <u>GCGTGCCCATGAGCGAAC</u> AAC	
	Ni	gctcttttat <u>gcatACATGCGCACTCCTGTAGGC</u>	
	Ci	ggagtgc <u>gaTGTATGCATAAAAAAGAGCATT</u> CAGTTACCTG	
	Co	cgcgatcc <u>AAGCGTTGAACGTGGTCTTG</u>	BamHI
<i>gadC</i>	No	aaggaaaaaaggccgc <u>AGTTGTTATGGACCCGAAACGC</u>	
	Ni	ccattc <u>agacgttacATATTATCCCCCTAAACGGTATT</u> CTG	
	Ci	gttttagggataat <u>tgAACCATGTCATGAATGGTTATAAG</u> ACAGG	
	Co	cgcacgc <u>atgtcgacGCTAACACCAAATCGACTCCC</u>	
<i>ydeP-b1500-ydeO</i>	No	aaggaaaaaaggccgc <u>AAACCTGGTACAAGGCTCAGC</u>	
	Ni	ctcgtag <u>caaataatcaCATCTACTTATCCTGTGTGAAATG</u> ATTATTAGG	
	Ci	acacaggata <u>agttagatTGATTATTGCTAACGAGTAGTCAACCAC</u>	
	Co	cgcacgc <u>atgtcgacCGTTGTTATCTCCGGTACCG</u>	
<i>ydeP</i>	No	aaggaaaaaaggccgc <u>AAACCTGGTACAAGGCTCAGC</u>	
	Ni	aaatgaga <u>aggaggatTTTTTCTTCATCTACTTATCCTGTGTG</u> GG	
	Ci	gataaggat <u>gatgaaaaaaTAATGCCTCTCATTTCTGCTG</u>	
	Co	cgcacgc <u>atgtcgacCTTCAACAGGTTCACTGATGTACAG</u>	
<i>b1500</i>	No	aaggaaaaaaggccgc <u>GGACGTAGCGAGATTGACATGC</u>	
	Ni	ccattct <u>gaaatacTCGCATGCAATTCAAATATGTTATTAGCGG</u>	
	Ci	tttgaat <u>gcatgcGAGTATTTCAGAAATGGGTCGATTG</u>	
	Co	cgcacgc <u>atgtcgacGCAAGTCATAAATATGTGATGGATGTC</u>	
<i>ydeO</i>	No	aaggaaaaaaggccgc <u>CATATAACCAAGGGCATCCGT</u> CATC	
	Ni	ctcgtag <u>caaataatcaAGAACAAACGAGCGACATT</u> TTATCTCC	
	Ci	tcgctgttgttc <u>TTGATTATTGCTAACGAGTAGTCACCAC</u>	
	Co	cgcacgc <u>atgtcgacCGTTGTTATCTCCGGTACCG</u>	
<i>yegR-b2084-yegZ</i>	No	aaggaaaaaaggccgc <u>CATCGTTAAATTACGCGTT</u> CGGAC	
	Ni	gataaaa <u>aaacatcaACGACACTCTCTAGTGTAAATATAAATGGC</u>	
	Ci	actagagat <u>gtcgacTGATGTTTATTTATCTGTGTTGTAAGGG</u>	
	Co	cgcacgc <u>atgtcgacTTTACCGCAAAGCTATCGATG</u> CG	
<i>yfdXWUVE</i>	No	aaggaaaaaaggccgc <u>CACACAGCTTGCATTAAACATGCTG</u>	
	Ni	cagcacat <u>ttaCATACTTACCTCATTCTTAAACAGTTT</u> AGAAAACG	
	Ci	agaatgaggta <u>gtatTAATGTAAGATGTGCTGGTAAATAGCTC</u>	
	Co	cgcacgc <u>atgtcgacATCGCAGGAAGAACAAACA</u> ACTG	
<i>slp-yhiF</i>	No	aaggaaaaaaggccgc <u>TACCAATGCTGCATGCAGTGAATG</u>	
	Ni	gttagcc <u>gttacatGTCATGTTACTATCCTTATCAAC</u>	
	Ci	agtaacat <u>gaacatgacaTGAATTTCAGGCTACGGTGAGTC</u>	
	Co	cgcacgc <u>atgtcgacCGTTGGCAGCCAATGAATCC</u>	
<i>yhiD</i>	No	aaggaaaaaaggccgc <u>CTAAATTGCACTATCTGATGCATCTG</u> TAACTC	
	Ni	ctgccac <u>agacattACATATAAAATGCAAAAGGAGCAGCAAGATG</u>	
	Ci	ccttttg <u>cattttatatTAATCTGTGTGGCAGCGTAGC</u>	
	Co	cgcacgc <u>atgtcgacGCTTCCCTGATCCAGTGA</u> ACTATCG	
<i>hdeB</i>	No	aaggaaaaaaggccgc <u>CAATGGCAAAATGCCAGCTC</u>	
	Ni	ggtat <u>cgccacattACCCATTCAATATAGAGATTATATGAA</u> CAGGAGG	
	Ci	tcttatatt <u>gtatggTAAAGGTGGCGATACCGTTACTTTAAATG</u>	
	Co	cgcacgc <u>atgtcgacCAACGGAA</u> GTATTCTGGAGAAACTG	
<i>hdeA</i>	No	aaggaaaaaaggccgc <u>GTTGAAATGGCTAATTGCCATAGAGT</u> G	
	Ni	cattccc <u>gaaattACATGTAATATCCTCAACTATAAAGTGAAGAGC</u>	
	Ci	gttgaggat <u>ttagcATGTAATTCCGGGAATGCGTTACATC</u>	
	Co	cgcacgc <u>atgtcgacTGCTGCTTAAACCA</u> GTGAGC	
<i>hdeD-yhiE</i>	No	aaggaaaaaaggccgc <u>CCTCATTAAATTCCGGCAAGTCATTAGATG</u> C	
	Ni	ttgcttat <u>gtctgactaCATAGAACCCCTATAAAATTAAGAAGAAA</u> ATCC	
	Ci	taggggttgt <u>ctatAGTCAGGACATAAGCAACTGAAATTGATG</u>	
	Co	cgcacgc <u>atgtcgacCGTAAGAAATCTGCACCGACT</u> GC	
<i>hdeD</i>	No	aaggaaaaaaggccgc <u>CCTCATTAAATTCCGGCAAGTCATTAGATG</u> C	
	Ni	caaccgggtaatt <u>ACATAGAACCCCTATAAAATTAAGAAGAAA</u> ATCC	
	Ci	taggggttgt <u>ctatAGTCAGGACATAAGCAACTGAAATTGATG</u>	
	Co	cgcacgc <u>atgtcgacCGTAAGAAATCTGCACCGACT</u> GC	
<i>yhiE</i>	No	aaggaaaaaaggccgc <u>GAGTTGGTATCGTCAGCGTT</u> CAATG	
	Ni	ttgcttat <u>gtctgactaCATAACTGCTCCATTAGCCGTTATCG</u>	
	Ci	ctaggaga <u>caagttagTAGTCAGGACATAAGCAACTGAAATTGATG</u>	
	Co	cgcacgc <u>atgtcgacCGTAAGAAATCTGCACCGACT</u> GC	

Continued on following page

TABLE 1—Continued

Gene(s)	Name	Oligonucleotide sequence (5' to 3') <sup>a</sup>	Enzyme <sup>b</sup>
<i>yhiUV</i>	No	aaggaaaaaaggccGCTTTGGCAGTGAAAGATAATCAGC	
	Ni	cttactctcatatcacgttaCATTTAGTCCTGAAAATTCTGAGAAATAACG	
	Ci	tcagggactaaatGTAACGTGTAAATGAGAGTAAGGTGAACATG	
	Co	cgccgat <u>CITCAATGAATCCGGGGTAAATT</u> CAC	<i>Bam</i> HI
<i>yiiS</i>	No	aaggaaaaaaggccgc <u>T</u> TA <u>CTGCAGCACGAGTGAGATTG</u>	<i>Not</i> I
	Ni	ccctccagat <u>CATAGGGCACCTCTGTATATT</u> CGC	
	Ci	cagagtgcc <u>tatGGATCTGGAGGGGCAAG</u> TTG	
<i>yjdE</i>	Co	cgacgc <u>atgtcgacGGATTCTTGCTGATGAAG</u> TG	<i>Sa</i> I
	No	aaggaaaaaaggcc <u>CGCTTTTTGCTGTTATT</u> TCTG	<i>Not</i> I
	Ni	tgaccacata <u>acc</u> CTAATTAA <u>ACTCCTGCGAAGGCGAG</u>	
<i>emrKY</i>	Ci	cgcaggat <u>taattatGAGGT</u> TATGTGGTCATTGTCACC	
	Co	cgacgc <u>atgtcgacGGTTAGTTCACGCGGGTAGC</u>	<i>Sa</i> I
	No	aaggaaaaaaggcc <u>GTTGGGACTTATGTA</u> CTGCAATCAAAG	<i>Not</i> I
<i>tolC</i>	Ni	ccctcc <u>tttatcatca</u> CTGTTCACTATTATCTCATTCTCATAGATG	
	Ci	agagata <u>atgtgaaca</u> GT <u>GATGATAAAAGGAGGGGTTA</u> AGC	
	Co	cgacgc <u>atgtcgacCAAAGCATTCCTGAAACTGCTGC</u>	<i>Sa</i> I
<i>tolC</i>	No	aaggaaaaaaggcc <u>TGCTAACAGTATCGCAACCAGTC</u>	<i>Not</i> I
	Ni	tcgtcg <u>atcatca</u> CATT <u>CCCTGTGGTAAGCAGTATT</u> AGC	
	Ci	caccaca <u>aggaa</u> tg <u>GATGACGACGACGGGGC</u>	
	Co	cgacgc <u>atgtcgacCTCGTATAGTGACGTTGGCGTATC</u>	<i>Sa</i> I

<sup>a</sup> Sequences complementary to the amplified regions are represented by capital letters, and newly added sequences are represented by lowercase letters. Incorporated cutting sites for restriction enzymes (No and Co) and start and stop codons of the gene or tandem group of the genes to be disrupted (Ni and Ci) are underlined.

<sup>b</sup> Restriction enzyme for cutting the amplified fragment.

ommended. The expression of His-tagged EvgA was induced by adding 1 mM IPTG to the LB broth before inoculation of cells, when required. The lowest concentration of agent that completely inhibited growth was identified as the MIC. A fourfold-or-more difference in MICs was considered significant. All agents used for susceptibility testing were obtained from Sigma-Aldrich.

**Acid resistance assay.** To determine the acid resistance of exponential-phase cells, a single colony of *E. coli* harboring plasmid was inoculated in 1 ml of LB broth containing carbenicillin and was grown overnight with aeration at 37°C. The next day 20 ml of LB broth was inoculated with 0.1 ml of the overnight culture and was grown at 37°C with aeration. The expression of His-tagged EvgA was induced by adding 1 mM IPTG to the LB broth before inoculation of the culture, when required. When the cultures reached a cell density of  $2 \times 10^8$  CFU/ml, 50 µl of the culture was transferred to 2 ml of phosphate-buffered saline (PBS) (pH 7.2) and to 2 ml of warmed LB broth (pH 2.5, adjusted with HCl). Some experiments involving a challenge by dilution into Vogel-Bonner minimal E medium (39) containing 0.4% glucose (EG; pH 2.5, adjusted with HCl) were also performed. To determine the acid resistance of stationary-phase cells, a single colony of *E. coli* was inoculated into 1 ml of LB broth and was grown overnight with aeration at 37°C. The overnight (24-h) stationary-phase cultures were diluted 1:1,000 into PBS and warmed LB broth (pH 2.5). The number of CFU per milliliter in PBS was determined by plating serial dilutions in PBS buffer (pH 7.2) on LB agar and was used as initial cell populations. The LB broth (pH 2.5) inoculated with *E. coli* was incubated further at 37°C, and the number of CFU per milliliter in LB broth (pH 2.5) was determined as described above and was used as final cell populations. Percent acid survival was then calculated as the number of CFU per milliliter remaining after the acid treatment divided by the initial number of CFU per milliliter at time zero. Two or three repetitions were performed for each experiment. Percent survival values were converted to logarithmic values ( $\log_{10}x$ , where  $x$  equals the percent survival) for calculation of geometric means and standard errors.

## RESULTS

**Microarray analysis of EvgA regulon.** To identify the target gene candidates for EvgA, we constructed *evgAS* deletion strains and EvgA expression plasmids and then compared the genome-wide transcription profiles of strains overexpressing and lacking EvgA by using oligonucleotide microarrays. In MG1655 cultured in rich medium the EvgA response regulator might be in an active form, resulting in the expression of some EvgA-regulated genes. To eliminate basal expression of EvgA regulon genes, a markerless in-frame *evgAS* deletion mutant, *ΔevgAS*, was constructed from MG1655. Nishino and Yamagu-

chi (20) reported that the overexpression of EvgA confers multidrug resistance in an *E. coli acrAB*-deficient strain. To investigate this phenomenon, we also constructed a *ΔacrB* strain from MG1655 and then constructed a *ΔacrBΔevgAS* strain from the *ΔacrB* strain. The pUC19 vectors, pUCevgA and pQEevgA, which are constitutive and conditional EvgA expression plasmids, respectively, were transformed into the *ΔevgAS* and *ΔacrBΔevgAS* strains by electroporation. Table 2 lists susceptibilities of constructed transformants lacking *acrB*. The *ΔacrBΔevgAS* strain harboring pUCevgA exhibited elevated resistance to erythromycin, novobiocin, sodium dodecyl sulfate (SDS), deoxycholate, and rhodamine 6G as reported for a *ΔacrAB* strain (20). The *ΔacrBΔevgAS* strain harboring pQEevgA showed elevated resistance to these agents in the presence of IPTG, suggesting that the His-tagged EvgA functions as EvgA.

The comprehensive transcript profiles of the *ΔevgAS* and *ΔacrBΔevgAS* strains bearing plasmid pUCevgA were compared to those of *ΔevgAS* and *ΔacrBΔevgAS* strains bearing plasmid pUC19, respectively. The comprehensive transcript profiles of the *ΔevgAS* and *ΔacrBΔevgAS* strains bearing plasmid pQEevgA grown in the presence of IPTG were also compared to those in the absence of IPTG. Altogether, four comparisons were performed with eight microarray data sets. The complete data set can be found at <http://arep.med.harvard.edu/cgi-bin/ExpressDBecoli/EXDStart>. Seventy-nine of the ORFs showed a change call of increase at all four comparisons, and 24 of the ORFs showed a change call of decrease at all four comparisons. Table 3 lists 37 of the 79 ORFs and 5 of the 24 ORFs that had an average  $\log_2$  ratio of greater than 2 or less than -2 over four comparisons (i.e., more than a fourfold change). The Microarray Suite 5.0 software uses statistical algorithms to calculate change *p* values (statistical significance for change calls). Among the 42 genes listed in Table 3 the least significant *p* value was 0.002211, suggesting that changes in expression levels of these genes are significant. Thus, these

TABLE 2. Drug susceptibilities of constructed strains

Strain	IPTG <sup>a</sup>	MIC ( $\mu\text{g/ml}$ ) of <sup>b</sup> :								
		ERY	NB	DOX	CV	EtBr	SDS	DOC	BEN	R6G
$\Delta acrB\Delta evgAS/pUC19$	—	4	2	ND <sup>c</sup>	ND	ND	62.5	2,000	ND	8
$\Delta acrB\Delta evgAS/pUCevgA$	—	64	8	ND	ND	ND	>16,000	>32,000	ND	128
$\Delta acrB\Delta evgAS/pQEevgA$	—	4	2	ND	ND	ND	62.5	2,000	ND	8
$\Delta acrB\Delta evgAS/pQEevgA$	+	64	8	ND	ND	ND	>16,000	>32,000	ND	128
$\Delta acrB$	—	8	2	4	1	16	125	4,000	4	8
$\Delta acrB\Delta yhiUV$	—	4	2	2	1	16	125	2,000	4	4
$\Delta acrB\Delta emrKY$	—	8	2	4	1	16	125	4,000	4	8
$\Delta acrB\Delta yhiUV\Delta emrKY$	—	4	2	2	1	16	125	2,000	4	4
$\Delta acrB/pUCevgA$	—	64	8	>128	4	64	>16,000	>32,000	16	256
$\Delta acrB\Delta yhiUV/pUCevgA$	—	4	2	2	1	16	125	4,000	4	4
$\Delta acrB\Delta emrKY/pUCevgA$	—	64	8	>128	4	64	>16,000	>32,000	16	256
$\Delta acrB\Delta yhiUV\Delta emrKY/pUCevgA$	—	4	2	2	1	16	125	4,000	4	4

<sup>a</sup> MICs were determined in the absence (—) or presence (+) of 1 mM IPTG.<sup>b</sup> ERY, erythromycin; NB, novobiocin; DOX, doxorubicin; CV, crystal violet; EtBr, ethidium bromide; DOC, deoxycholate; BEN, benzalkonium; R6G, rhodamine 6G.<sup>c</sup> ND, not determined.

genes are target gene candidates for the EvgA response regulator.

**EvgA overexpression confers acid resistance to exponential-phase cells.** Five of the genes whose expression was induced by EvgA overexpression code for proteins that are known to provide protection against acid stress in *E. coli*. These are the *gadA*, *gadBC*, and *hdeAB* genes. *gadA* and *gadB* code for isozymes of glutamate decarboxylase, which catalyze the conversion of glutamate to  $\gamma$ -aminobutyrate (30). *gadC* is located downstream of *gadB* and is predicted to code for a glutamate:  $\gamma$ -aminobutyrate antiporter (10). The GadA and GadB decarboxylases and the GadC antiporter are proposed to function together to help maintain a near-neutral intracellular pH when cells are exposed to extremely acidic conditions (29). HdeA and HdeB are predicted to be periplasmic proteins. HdeA may act as a chaperone and may prevent the aggregation of periplasmic proteins denatured at low pH (7). HdeB is predicted to be a structural homologue of HdeA and to form heterodimers with HdeA (7). The fact that EvgA overexpression induced these acid protection genes prompted us to study the effect of EvgA overexpression on resistance to acidic stress. Exponential-phase  $\Delta evgAS$  strains harboring pUC19, pUCevgA, and pQEevgA grown in LB broth (pH 7.0) and an exponential-phase  $\Delta evgAS$  strain harboring pQEevgA grown in LB broth (pH 7.0) containing IPTG were incubated in LB broth (pH 2.5) for 1 h, and percent survival values were determined as described in Materials and Methods. The percent survival values of EvgA-lacking strains were reduced to less than 0.01% in 1 h, while EvgA overexpression strains exhibited elevated resistance to acidic conditions (Fig. 1). A time-course experiment was performed with MG1655 bearing pQEevgA (Fig. 2). In the absence of IPTG the percent survival value was reduced to less than 0.01% within 30 min. In the presence of IPTG 1.7% of the cells survived after 4 h of exposure to acidic conditions. These results indicate that EvgA overexpression confers acid resistance to exponential-phase *E. coli*.

Bacteria have acid pH neutralization mechanisms based on the production of cytoplasmic amino acid decarboxylases and their cognate amino acid antiporters. Each system requires its cognate amino acid during acid challenge to allow the cells to survive. In *E. coli* glutamate decarboxylases GadA and GadB

(30), glutamate:  $\gamma$ -aminobutyrate antiporter GadC (10) and arginine decarboxylase AdiA (32) have been identified. To confirm whether an acid resistance system(s) induced by EvgA overexpression requires amino acid or not, exponential-phase MG1655 harboring pQEevgA grown in LB broth (pH 7.0) containing IPTG was incubated in EG (pH 2.5) for 1 h and percent survival values were determined as described in Materials and Methods. EvgA overexpression conferred acid resistance in EG to the same level as that in LB broth (Fig. 1, bar 8). Thus, acid resistance conferred by EvgA overexpression includes amino acid-independent mechanism(s).

**Identification of novel genes related to acid resistance.** To identify genes responsible for the acid resistance caused by EvgA overexpression, we constructed deletion mutants of the genes that were induced more than eightfold on average by EvgA overexpression, transformed pQEevgA to the constructed mutants, and then tested acid resistance in the presence of IPTG. MG1655 harboring pQEevgA showed acid resistance in the presence of IPTG despite having a native *evgS* gene (Fig. 2 and 3). Therefore, MG1655 was used as the parent strain. Markerless in-frame *appCBA*, *gadC*, *ydeP*-b1500-*ydeO*, *yegR*-b2084-*yegZ*, *yfdXWUVE*, *sly-yhiF*, *yhiD*, *hdeB*, *hdeA*, *hdeD-yhiE*, *yhiUV*, *yiiS*, and *yjDE* deletion mutants were constructed from MG1655. Because GadC is an essential component of the glutamate-dependent acid resistance system, constructions of *gadB* and *gadA* deletion strains were omitted. Exponential-phase cultures of each strain harboring pQEevgA grown in LB broth (pH 7.0) containing IPTG were incubated in LB broth (pH 2.5) for 1 h, and percent survival values were determined as described in Materials and Methods. Although the deletions of *gadC* and *hdeA* decreased the acid resistance to 34 and 46% survival (Fig. 3, bars 3 and 13), respectively, these strains still showed more than 1,000-fold higher resistance than EvgA-lacking strains. The deletions of *hdeD-yhiE* and *ydeP*-b1500-*ydeO* decreased survival to 0.9% and to less than 0.01% (Fig. 3, bars 14 and 4), respectively. The deletions of *yfdXWUVE* slightly decreased survival to 56% (Fig. 3, bar 9), while the deletions of the other genes had no effect on survival. To identify genes responsible for the acid resistance in *ydeP*-b1500-*ydeO* and *hdeD-yhiE*, markerless in-frame *ydeP*, b1500, *ydeO*, *hdeD*, and *yhiE* deletion strains were constructed from

TABLE 3. Genes most affected by overexpression of EvgA

Expression level and b no.	Gene <sup>a</sup>	Gene description <sup>b</sup>	Log <sub>2</sub> ratio <sup>c</sup>			
			Mean	ΔevgAS/ pUCevgA	ΔevgAS/ pQEevgA	ΔacrBΔevgAS/ pUCevgA
<b>Increased expression</b>						
b0978	<i>appC</i>	Probable third cytochrome oxidase, subunit I	5.8	6.1	6.4	3.2
b0979	<i>appB</i>	Probable third cytochrome oxidase, subunit II	6.5	7.3	6	5.5
b0980	<i>appA</i>	Phosphoanhydride phosphorylase, pH 2.5 acid phosphatase, periplasmic	4.1	4.3	3.7	3.7
b1330	<i>ynlA</i>	ORF, hypothetical protein	2.2	2.4	2.6	0.9
b1492	<i>gadC</i>	Acid sensitivity protein, putative transporter	5.0	6.1	5.5	4.1
b1493	<i>gadB</i>	Glutamate decarboxylase isozyme	8.1	8.5	7.8	7.9
b1499	<i>ydeO</i>	Putative ARAC-type regulatory protein	5.6	5.2	5.6	5.8
b1500		ORF, hypothetical protein	6.3	7	3.6	6.4
b1501	<i>ydeP</i>	Putative oxidoreductase, major subunit	6.9	5.5	8.4	6.2
b1608	<i>rstA</i>	Response transcriptional regulatory protein (RstB sensor)	2.0	2.5	2.3	1
b1826	<i>yobG</i>	ORF, hypothetical protein	2.2	2.8	2.4	1.2
b2083	<i>yegZ</i>	ORF, hypothetical protein	5.7	7.5	5.6	4.1
b2084		ORF, hypothetical protein	3.0	3.5	3.4	1.5
b2085	<i>yegR</i>	ORF, hypothetical protein	4.8	5.5	6	3.6
b2368	<i>emrK</i>	Multidrug resistance protein K	3.1	3.5	2.5	2.2
b2369	<i>evgA</i>	Putative positive transcription regulator (sensor EvgS)	7.9	8.6	6.4	9.2
b2371	<i>yfdE</i>	Putative enzyme	4.7	4.9	4.8	2.8
b2372	<i>yfdV</i>	Putative receptor protein	6.0	6.4	5.1	5.8
b2373	<i>yfdU</i>	Putative enzyme	5.5	6.4	5.4	3.9
b2374	<i>yfdW</i>	Putative enzyme	9.2	8.5	9.7	8.2
b2375	<i>yfdX</i>	ORF, hypothetical protein	8.2	8.2	10	5.4
b3238	<i>yhcN</i>	ORF, hypothetical protein	2.2	4.9	1.7	1.1
b3491	<i>yhiM</i>	ORF, hypothetical protein	2.5	3.5	3.1	1.6
b3506	<i>slp</i>	Outer membrane protein induced after carbon starvation	5.8	7.3	5.9	4.4
b3507	<i>yhiF</i>	ORF, hypothetical protein	4.1	4.4	4.8	2
b3508	<i>yhiD</i>	Putative transport ATPase	4	7.2	2.8	2.4
b3509	<i>hdeB</i>	ORF, hypothetical protein	8.3	9.8	8.7	5.8
b3510	<i>hdeA</i>	ORF, hypothetical protein	6.1	6.6	5.6	5.7
b3511	<i>hdeD</i>	ORF, hypothetical protein	4.6	5.1	5.3	3.3
b3512	<i>yhiE</i>	ORF, hypothetical protein	7.3	6.5	8.3	6.5
b3513	<i>yhiU</i>	Putative membrane protein	3.3	3.1	4	1.3
b3514	<i>yhiV</i>	Putative transport system permease protein	3.4	4.5	2.8	1.3
b3517	<i>gadA</i>	Glutamate decarboxylase isozyme	7.3	6.8	7.9	7.8
b3922	<i>yiiS</i>	ORF, hypothetical protein	3.2	3.5	4.1	1.2
b4111	<i>proP</i>	Low-affinity transport system, proline permease II	2.2	3	2.7	0.6
b4113	<i>basR</i>	Transcriptional regulatory protein, member of two-component regulatory system	2.4	1.5	4.1	0.6
b4115	<i>yjdE</i>	Putative amino acid/amine transport protein, cryptic	3.4	5	5.1	0.9
<b>Decreased expression</b>						
b0553	<i>nmpC</i>	Outer membrane porin protein; locus of qsr prophage	-2.6	-5.7	-2.3	-0.2
b2167	<i>fruA</i>	PTS system, fructose-specific transport protein	-2.7	-3.8	-3.1	-1.2
b2168	<i>fruK</i>	Fructose-1-phosphate kinase	-2.5	-3.2	-2.6	-1.7
b2169	<i>fruB</i>	PTS system, fructose-specific IIA/fpr component	-2.5	-4	-2	-2.1
b2943	<i>galP</i>	Galactose-proton symport of transport system	-2.1	-1.7	-2.8	-0.8

<sup>a</sup> Gene names are taken from <http://bmb.med.miami.edu/EcoGene/EcoWeb/>.

<sup>b</sup> Gene descriptions are taken from the Affymetrix Expression Analysis Sequence Information Database.

<sup>c</sup> Log<sub>2</sub> ratio of transcript levels for the EvgA-overexpressing strain to those for the EvgA-lacking strain. ΔevgAS/pUCevgA, expression in ΔevgAS/pUCevgA versus that in ΔevgAS/pUC19; ΔevgAS/pQEevgA, expression in ΔevgAS/pQEevgA in the presence of IPTG versus that in the absence of IPTG; ΔacrBΔevgAS/pUCevgA, expression in ΔacrBΔevgAS/pUCevgA versus that in ΔacrBΔevgAS/pUC19; ΔacrBΔevgAS/pQEevgA, expression in ΔacrBΔevgAS/pQEevgA in the presence of IPTG versus that in the absence of IPTG. A negative value denotes a decrease in transcription in the EvgA-overexpressing strain compared to that in the EvgA-lacking strain.

MG1655 and were tested as described above. The deletions of *ydeP* and *ydeO* decreased survival to 0.7 and 0.08%, respectively (Fig. 3, bars 5 and 7), while the deletion of b1500 slightly decreased survival to 35% (Fig. 3, bar 6). The deletion of *yhiE*

decreased survival comparable to that of *hdeD-yhiE* (Fig. 3, bar 16), while the deletion of *hdeD* had no effect on survival (Fig. 3, bar 15). Altogether, EvgA overexpression induced five genes, *gadA*, *gadB*, *gadC*, *hdeA*, and *hdeB*, known to provide

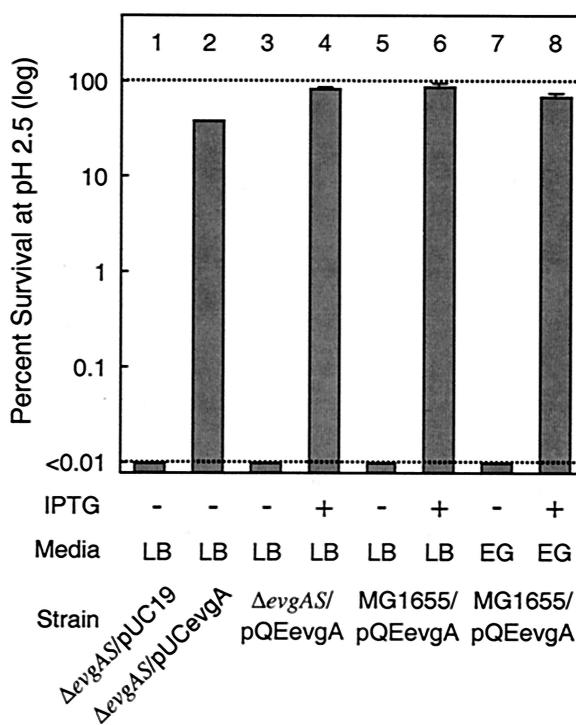


FIG. 1. Acid resistance of EvgA overexpression strains. Various strains were grown to mid-log phase in LB broth (pH 7.0) with or without IPTG. Cells were diluted 40-fold into LB broth (pH 2.5) or EG (pH 2.5) and were incubated for 1 h at 37°C. Initial cell densities ranged from  $1.2 \times 10^6$  to  $1.6 \times 10^7$  CFU/ml. Error bars represent standard errors of the means.

acid protection and at least three novel acid resistance genes, *ydeP*, *ydeO*, and *yhiE*. These results suggest that EvgA is involved in acid resistance.

*E. coli* in stationary phase can remain viable for several hours at pH 2.5 (9). In fact, 71% of MG1655 cells grown in LB broth (pH 7.0) to stationary phase survived even after 4 h of exposure in LB broth (pH 2.5) (Fig. 2). We also tested whether or not the genes induced by EvgA overexpression were involved in stationary-phase acid resistance. Each deletion strain grown in LB broth (pH 7.0) to stationary phase was incubated in LB broth (pH 2.5) for 2 h, and percent survival values were determined as described in Materials and Methods. The  $\Delta gadC$  and  $\Delta hdeA$  mutants showed four- and twofold decreases in acid resistance (Fig. 4, bars 4 and 14), respectively. Survival of the  $\Delta hdeD-yhiE$  and  $\Delta yhiE$  mutants was more than 4 log units lower than that of MG1655 (Fig. 4, bars 15 and 17), suggesting that YhiE is involved in stationary-phase acid resistance. The other deletion mutants showed survival rates comparable to that of MG1655.

**Identification of the efflux pump responsible for multidrug resistance.** Thirty-seven drug transporter and seven membrane fusion protein (MFP) genes have been identified in the *E. coli* genome (15, 35, 37; also see <http://www-biology.ucsd.edu/~ipaulsen/transport/>). The transporter genes are classified into the ABC (ATP-binding cassette) family (7 genes), MFS (major facilitator superfamily) (18 genes), RND (resistance nodulation cell division) family (6 genes), SMR (small multidrug resistance) family (4 genes), and MATE (multidrug and toxic

compound extrusion) family (2 genes) (23). To identify the efflux pump(s) responsible for the multidrug resistance caused by EvgA overexpression, we compared the expression data of these efflux pump genes. The expressions of three efflux pump genes, *emrK*, *yhiU*, and *yhiV*, were significantly induced by EvgA overexpression, as shown in Table 3. The *yhiU* and *yhiV* genes form an operon and code for MFP and an RND family transporter, respectively (Fig. 1). The *emrK* gene coding for MFP is located in an operon immediately upstream of the *emrY* gene coding for an MFS transporter (Fig. 1). The expression of *emrY* was induced 3.0- to 9.8-fold, although only one of four change calls was increase, with the others called no change because their *p* values were only marginally significant (*P* = 0.0031 to 0.036; default threshold value, 0.0025). The induction of the *emrKY* operon by EvgA overexpression is consistent with the result reported by Kato et al. (13). The other 40 genes showed no significant change in expression by EvgA overexpression. Therefore, YhiUV and EmrKY are candidate contributors to the multidrug resistance.

To confirm the involvement of the YhiUV and EmrKY efflux systems in the multidrug resistance caused by EvgA overexpression, *yhiUV* and/or *emrKY* deletion mutants were isolated from the  $\Delta acrB$  strain and then were transformed with pUCevgA. The  $\Delta acrB\Delta evgAS$  strains harboring pUC19 and pUCevgA had almost the same susceptibilities to the agents tested as the  $\Delta acrB$  strains harboring pUC19 and pUCevgA, respectively (Table 2). These results suggest that the level of EvgAS expressed in the  $\Delta acrB$  strain has no effect on susceptibility to these agents. Therefore, we isolated the deletion strains from  $\Delta acrB$  instead of  $\Delta acrB\Delta evgAS$ . Table 2 shows susceptibilities of constructed strains to erythromycin, novobiocin, doxorubicin, crystal violet, ethidium bromide, SDS, deoxycholate, benzalkonium, and rhodamine 6G. The  $\Delta acrB\Delta yhiUV$  strains showed no change in susceptibilities by EvgA overexpression, while the  $\Delta acrB\Delta emrKY$  strain bearing

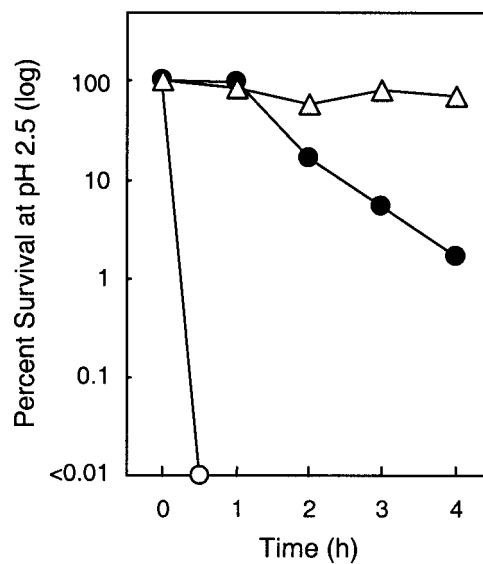


FIG. 2. Acid resistance as a function of exposure time. The percentage of survival of exponential-phase MG1655/pQEevgA grown in the absence (○) or presence (●) of IPTG and stationary-phase MG1655 (△) after acid exposure in LB broth (pH 2.5).

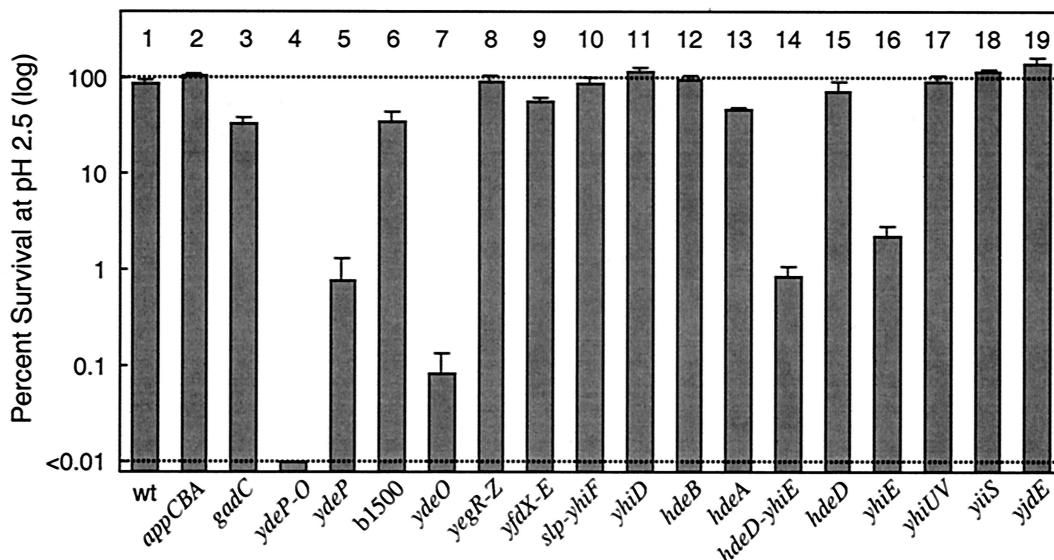


FIG. 3. Acid survival of exponential-phase cells. Various deletion mutants harboring pQEvgA were grown to mid-log phase in LB broth (pH 7.0) containing IPTG. Cells were diluted 40-fold into LB broth (pH 2.5) and were incubated for 1 h at 37°C. Initial cell densities ranged from  $7.4 \times 10^5$  to  $1.3 \times 10^7$  CFU/ml. Error bars represent standard errors of the means. wt, wild-type MG1655; ydeP-O, ydeP-b1500-ydeO; yegR-Z, yegR-b2084-yegZ; yfdX-E, yfdXWUVE.

pUCevgA showed the same level of resistance as the  $\Delta acrB$  strain bearing pUCevgA. These results suggest that *yhiUV* is essential for the multidrug resistance caused by EvgA overexpression and that *emrKY* is not involved in the resistance.

The *tolC* gene encoding the multifunctional outer-membrane channel showed a change call of increase at all four comparisons, while it was induced only 1.2- to 2.5-fold. The *tolC* homologues *yjcP*, *yohG*, and *ylcB* (35) showed no significant change in expression by EvgA overexpression. In order to investigate the role of TolC in the multidrug resistance caused by YhiUV induction, a markerless in-frame *tolC* deletion mu-

tant,  $\Delta tolC$ , was constructed from the  $\Delta acrB$  strain and was transformed with pUCevgA. The *tolC*-deficient strain harboring pUCevgA showed no increase in resistance (data not shown). These results suggest that YhiUV requires TolC for its efflux activity.

## DISCUSSION

By measuring gene expression in isogenic EvgA-lacking and EvgA-overexpressing *E. coli* strains with DNA microarrays, we identified various target gene candidates for the EvgA re-

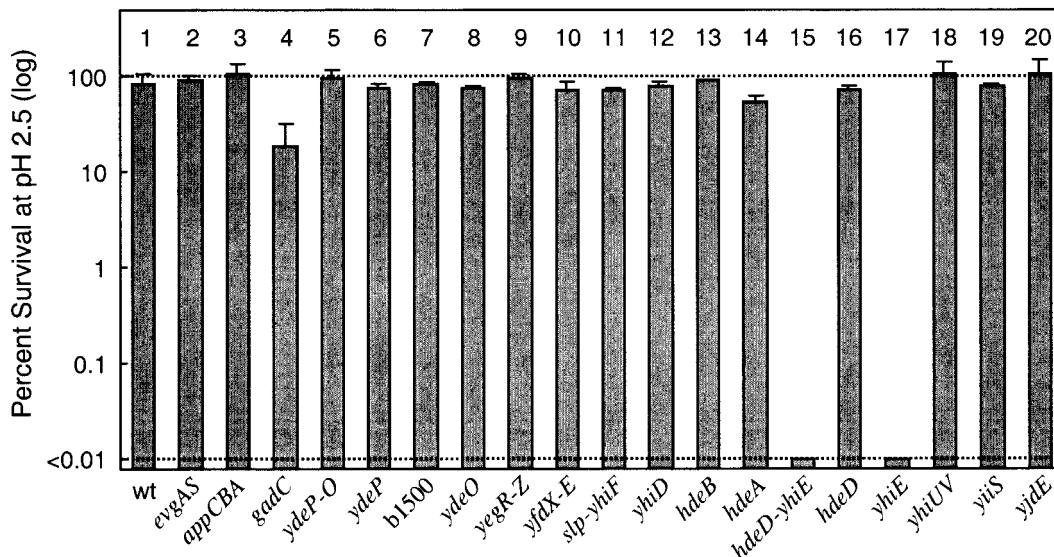


FIG. 4. Acid survival of stationary-phase cells. Various deletion mutants were grown overnight in LB broth (pH 7.0). Cells were diluted 1,000-fold into LB broth (pH 2.5) and were incubated for 2 h at 37°C. Initial cell densities ranged from  $2.3 \times 10^6$  to  $9.5 \times 10^6$  CFU/ml. Error bars represent standard errors of the means. wt, wild-type MG1655; ydeP-O, ydeP-b1500-ydeO; yegR-Z, yegR-b2084-yegZ; yfdX-E, yfdXWUVE.

sponse regulator. Of 37 genes whose expression was highly up-regulated by EvgA overexpression, 21 code for uncharacterized or hypothetical proteins. In contrast to the relatively large number of up-regulated genes, only five genes were down-regulated by EvgA overexpression.

By constructing a series of mutants lacking genes induced by EvgA overexpression we identified at least three novel acid-resistance-related genes, *ydeP*, *ydeO*, and *yhiE*. We cannot exclude the possibility that an additional mutation occurred during the steps of deletion strain construction which affected the acid resistance phenotype. However, cognate deletion strains isolated by different experiments, i.e.,  $\Delta ydeP$ -b1500-*ydeO*,  $\Delta ydeP$ , and  $\Delta ydeO$  and  $\Delta hdeD$ -*yhiE* and  $\Delta yhiE$ , showed similar phenotypes, strongly suggesting that *ydeP*, *ydeO*, and *yhiE* are involved in acid resistance. The deletions of *ydeP*-b1500-*ydeO* and *yhiE* decreased acid resistance caused by EvgA overexpression more than those of *gadC* and *hdeA*, suggesting that *ydeP*-b1500-*ydeO* and *yhiE* are more critical for acid resistance induced by EvgA overexpression. In contrast, the deletion of *yhiE* decreased the acid resistance in stationary-phase cells cultured in LB broth (pH 7.0) more than those of *gadC* and *hdeA*, suggesting that *yhiE* is more critical for acid resistance induced in stationary phase. Deletion of *ydeP*-b1500-*ydeO* or *yhiE* decreased acid resistance caused by EvgA overexpression drastically, suggesting that both are essential for the EvgA-induced acid resistance. The deletions of *ydeP*, b1500, and *ydeO* decreased acid resistance, though the degree of percent change in survival by each deletion varied. Although we designed primers to minimize polar effects, the possibility that deletion of *ydeP* or b1500 affects the expression of downstream *ydeO* cannot be excluded.

*YhiE* is similar to *YhiF*, whose deletion has no effect on acid resistance; however, no homologue has been reported for other species. *YdeP* is a putative oxidoreductase that has a homology to the  $\alpha$  subunit of *E. coli* formate dehydrogenase H (E value, 2e-31). *YdeO* is a member of the AraC/XylS family of transcriptional regulators (8). Thirty-seven genes of the AraC/XylS regulators are on the MG1655 chromosome (<http://www.eez.csic.es/arac-xyls/>). *YdeO* is more similar to *YhiW*, *AppY*, *AdiY*, and *GadX* than the other AraC/XylS regulators. *AppY* regulates the *appCBA* operon (5), which was induced by EvgA overexpression. *GadX* regulates the acid resistance genes *gadA*, *gadBC*, and *hdeABD* (12), which were also induced by EvgA. *AdiY* regulates another acid resistance gene, *adiA* (33). The expression of *gadX* was 1.7- to 4.9-fold induced by EvgA overexpression, and the changes were significant in all four comparisons (<http://arep.med.harvard.edu/cgi-bin/ExpressDBcoli/EXDStart>). *yhiW*, *appY*, and *adiY* showed no significant change by EvgA overexpression except that the expression of *yhiW* was significantly induced 28-fold by IPTG in the  $\Delta evgAS$  strain harboring pQEevgA. Exponential-phase MG1655 harboring pQEevgA grown in LB broth (pH 7.0) containing IPTG also showed acid resistance in EG (pH 2.5) (Fig. 1, bar 8), suggesting that acid resistance conferred by EvgA overexpression includes an amino-acid-independent mechanism(s). Further study is needed to elucidate the functions of *YdeP*, *YdeO*, and *YhiE*.

The expression of *gadABC* (6), *hdeAB* (4, 41), *slp* (1), and *appCBA* (5) was increased in stationary phase. Selinger et al. (28) compared the transcriptome of exponential- and station-

ary-phase cells. Of 37 genes listed in Table 3 only 8 were significantly induced in stationary phase (*gadC*, b2084, *emrK*, *yfdX*, *hdeAB*, *yhiE*, and *gadA*) (28). In particular, the expression of *yhiE*, which is essential for acid resistance in stationary phase (Fig. 4), was induced 52-fold in stationary phase (28). The  $\sigma^S$ -dependent oxidative system (16), which is an amino-acid-independent acid resistance system, might be attributable to *YhiE*.

Exposure to short-chain fatty acids, such as acetate, at neutral or nearly neutral pH has been shown to increase acid survival of *E. coli*. Arnold et al. (3) identified 26 acetate-induced genes by microarray analysis. Of the 37 genes listed in Table 3 only 7 genes, *gadA*, *gadB*, *gadC*, *hdeA*, *hdeB*, *hdeD*, and *slp*, were induced by acetate, suggesting that the EvgAS two-component system is not directly related to acetate-induced acid tolerance. Our data strongly suggest that the EvgAS system is involved in acid shock response, although further study is needed to elucidate it.

Gene knockout experiments suggest that *YhiUV* induced by EvgA overexpression confers multidrug resistance in functional association with *TolC*. Very recently, Nishino and Yamaguchi (21) also reported that EvgA contributes to multidrug resistance through increased expression of *YhiUV*, and *YhiUV* transporter requires *TolC* for its function. They also reported that EvgA overexpression in the *yhiUV* deletion mutant caused a fourfold decrease in susceptibility to deoxycholate, which seems to be due to EvgA-dependent *EmrKY* expression. We found a twofold decrease in susceptibility to deoxycholate by EvgA overexpression in our  $\Delta yhiUV$  mutant, while the twofold decrease in susceptibility was also found with the  $\Delta yhiUV\Delta emrKY$  mutant (Table 2). Therefore, the moderate decrease in susceptibility to deoxycholate is not due to *EmrKY*. The  $\Delta acrB$  strain showed hypersusceptibility, and the  $\Delta acrB$  strain harboring pUCevgA exhibited elevated resistance to these agents (Table 2), while MG1655 harboring pUCevgA exhibited no change in drug susceptibility (data not shown). Thus, the physiological role of EvgA appears to be the control of acid resistance genes rather than control of multidrug resistance.

Analysis with the motif-finding program AlignACE (26) identified a highly conserved 18-bp sequence in upstream regions of *ydeP*, b1500, *yegR*, *evgA/emrK*, *yfdX*, and *yfdW* (unpublished data). The 18-bp consensus sequence consists of 5'-TT CPyTACA-3' and its inverted repeat 5'-TGTAPuGAA-3' separated by two random bases. Gel mobility shift (36) and DNase I footprinting (13) analyses have shown that EvgA binds to the 27- and 33-bp regions located upstream of *evgA/emrK*, respectively. Tanabe et al. (36) and Kato et al. (13) proposed that the 5'-TTCTTA-3' sequence and its inverted repeat, 5'-TAAGAA-3', separated by a 15-bp spacer sequence, were important for EvgA binding. The inverted repeats that we found are included in both 27- and 33-bp regions that they identified, while the inverted repeats that they proposed are completely included in the 27-bp region but not in the 33-bp regions. EvgA overexpression induced the expression of *ydeO*, an AraC/XylS regulator, indicating that some of the genes induced by EvgA overexpression might be directly induced by *YdeO* instead of EvgA. Therefore, further experiments are needed to determine the EvgA-binding sequences.

The nucleoid protein H-NS is involved in the negative con-

trol of *gadA* and *gadBC* transcription during exponential phase, while the alternative sigma factor RpoS is responsible for Gad expression in stationary phase (6). Hommais et al. (12) compared the proteome and transcriptome of an H-NS-deficient strain and its parent strain and reported that the expression of *evgA* is induced by H-NS deletion. They also raised the possibility that H-NS could initiate a new kind of regulation by modulating the expression level of response regulators. Of 30 genes induced more than eightfold by EvgA overexpression, 18, including *ydePO* and *yhiE*, are also induced by H-NS deletion. Induction of these genes by H-NS deletion might be the secondary effect of the EvgA induction. Overexpression of either EvgA or GadX induced the expression of *gadA*, *gadBC*, and *hdeABD*. It is evident that a complex regulatory cascade exists that consists of H-NS, EvgA, GadX, and YdeO.

#### ACKNOWLEDGMENTS

We thank Wayne Rindone for construction of the microarray database and for loading data on it. We thank Jay Shendure for help with AlignACE analysis. We thank Daniel H. Levin and Doug Selinger for careful reading of the manuscript.

This work was supported by grants from the Department of Energy and the National Science Foundation to G.M.C.

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