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Growth Phase-Dependent Expression of Drug Exporters in *Escherichia coli* and Its Contribution to Drug Tolerance

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Drug exporters contribute to the intrinsic drug resistance in many organisms. Although there are at least 20 exporter genes in *Escherichia coli*, most of them apparently do not confer drug resistance in complex laboratory media except for the AcrAB, EmrE, and MdfA efflux systems. In this study, we comprehensively investigated the growth phase-dependent expression of drug exporter genes. The expression of *acrAB*, *emrAB*, *emrD*, *emrE*, *emrKY*, *mdfA*, and *ydgFE* is stable at moderate levels during any growth phase, whereas *mdtEF* promoter activity greatly increased with cell growth and reached the maximum level at the late stationary phase. The growth phase-dependent increase in *mdtEF* expression was also observed on quantitative reverse transcription-PCR analysis. As expected from the transporter expression, the stationary-phase cells actually showed MdtEF-dependent tolerance to drugs and toxic dyes. Growth phase-dependent elevation of *mdtEF* expression was found to be mediated by the stationary-phase σ factor *rpoS* and the RpoS-dependent signaling pathway, Hfq, GadY, and GadX. The induction level was decreased by *tnaAB* deletion, suggesting that indole sensing stimulates this process.

Bacterial intrinsic tolerance to a wide range of antimicrobial agents is often caused by active efflux systems, such as AcrAB in *Escherichia coli* and MexAB in *Pseudomonas aeruginosa* (17, 18, 23), and multidrug-resistant mutants due to the overexpression of efflux pumps have been isolated in clinical settings (15). Our previous studies revealed that there are at least 20 drug exporter genes in *E. coli* that confer drug resistance when they are overexpressed (19); however, the previous study showed that most of them do not contribute to drug tolerance in complex laboratory media, probably due to their low expression levels, except for *acrAB*, *emrE*, and *mdfA* (29). In that study, drug resistance was determined as MICs, which would not reflect the actual expression levels of drug exporters at different growth phases.

In *E. coli*, drug exporter gene expression is affected by various environmental stresses. For instance, the *acrAB* gene is known to be induced by ethanol, osmotic shock, oxidizing agents (11), and bile salts and fatty acids (24). Throughout bacterial growth, the bacterial cell density, nutrient conditions, pH, and other factors are changing. Therefore, it would be important to study the growth phase dependency of the expression of drug exporters that may facilitate understanding of drug exporter-mediated multidrug resistance at actual infection sites.

The growth phase-dependent expression of various genes in *E. coli* has been reported. For instance, quorum-sensing signal molecule autoinducer 2, which is produced and secreted into the culture medium at the logarithmic phase, influences the expression of type III secretion system-related genes (27) and motility-related genes (28). The σ factor, RpoS, and RpoS-dependent genes are known to be induced at the stationary phase (7). However, there is little information available on growth phase-dependent expression of drug exporter genes.

In this study, we comprehensively investigated the expression of the 20 drug exporter genes at different growth phases and their contribution to growth phase-dependent drug tolerance. We found that out of the 20 drug exporter genes in *E. coli*, the expression levels of the *emrA*, *emrD*, *emrK*, and *ydgF* genes are relatively stable at moderate levels without a significant change throughout the bacterial growth phase as well as those of the *acrA*, *emrE*, and *mdfA* genes. In contrast, *mdtEF* gene expression was significantly increased at the late cell growth phase, followed by *mdtEF*-dependent drug tolerance. A possible regulation scheme is discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are presented in Table 1. The construction of gene deletion mutants of *E. coli* MC4100 (2) was performed by the gene replacement method previously described, using the pKO3 plasmid (9). *E. coli* cells were cultured in Luria-Bertani (LB) medium, supplemented with appropriate antibiotics when necessary.

Construction of reporter plasmids. The reporter plasmids were constructed as follows. DNA fragments including the putative promoter region were amplified by PCR using the primers listed in Table 2. Chromosomal DNA of *E. coli* MG1655 (1) was used as a PCR template. The DNA fragments were cloned in front of the *lacZ* reporter gene in a single-copy pNN387 vector, which carries chloramphenicol resistance as a marker (3). Since the *emrAB*, *cusBA*, and *mdtEF* genes are transcribed in the *emrRAB* (10), *cusCFBA* (4), and *gadE-mdtEF* (14; our unpublished data) operons, respectively, *emrR*, *cusC*, and *gadE* promoter-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
E. coli strains		
MG1655	Wild type, chromosomal DNA used for PCR amplification	1
MC4100	F$^-$ *araD139*Δ(*argF-lac*)*U169 rpsL150*(Str^r) *relA1 flbB5301 deoC1 ptsF25 rbsR*	2
MC4100Δ*acrB*	Derivative of MC4100 that lacks *acrB*	5
MC4100Δ*acrB*Δ*mdtEF*	Derivative of MC4100Δ*acrB* that lacks *mdtEF*	5
MC4100Δ*evgSA*	Derivative of MC4100 that lacks *evgSA*	5
MC4100Δ*gadX*	Derivative of MC4100 that lacks *gadX*	5
MC4100Δ*hfq*	Derivative of MC4100 that lacks *hfq*	This study
MC4100Δ*mdtEF*	Derivative of MC4100 that lacks *mdtEF*	This study
MC4100Δ*rpoS*	Derivative of MC4100 that lacks *rpoS*	This study
MC4100Δ*tnaAB*	Derivative of MC4100 that lacks *tnaAB*	5
Plasmids		
pNN387	Single-copy vector, Cp^r,[a] NotI-HindIII cloning site upstream of promoterless *lacZ*	3
pNNacrA	pNN387 (*acrAB* gene promoter-*lacZ*)	This study
pNNacrD	pNN387 (*acrD* gene promoter-*lacZ*)	5
pNNacrE	pNN387 (*acrEF* gene promoter-*lacZ*)	This study
pNNbcr	pNN387 (*bcr* gene promoter-*lacZ*)	This study
pNNcusC	pNN387 (*cusCFBA* gene promoter-*lacZ*)	This study
pNNemrA	pNN387 (*emrRAB* gene promoter-*lacZ*)	This study
pNNemrD	pNN387 (*emrD* gene promoter-*lacZ*)	This study
pNNemrE	pNN387 (*emrE* gene promoter-*lacZ*)	This study
pNNemrK	pNN387 (*emrKY* gene promoter-*lacZ*)	5
pNNfsr	pNN387 (*fsr* gene promoter-*lacZ*)	This study
pNNgadE	pNN387 (*gadE mdtEF* gene promoter-*lacZ*)	5
pNNgadX	pNN387 (*gadX* gene promoter-*lacZ*)	This study
pNNgadY	pNN387 (*gadY* gene promoter-*lacZ*)	This study
pNNhfq	pNN387 (*hfq* gene promoter-*lacZ*)	This study
pNNmacA	pNN387 (*macAB* gene promoter-*lacZ*)	This study
pNNmdfA	pNN387 (*mdfA* gene promoter-*lacZ*)	This study
pNNmdtA	pNN387 (*mdtABC* gene promoter-*lacZ*)	5
pNNrpoS	pNN387 (*rpoS* gene promoter-*lacZ*)	This study
pNNyceE	pNN387 (*yceE* gene promoter-*lacZ*)	This study
pNNyceL	pNN387 (*yceL* gene promoter-*lacZ*)	This study
pNNydgF	pNN387 (*ydgFE* gene promoter-*lacZ*)	This study
pNNydhE	pNN387 (*ydhE* gene promoter-*lacZ*)	This study
pNNyidY	pNN387 (*yidY* gene promoter-*lacZ*)	This study
pNNyjiO	pNN387 (*yjiO* gene promoter-*lacZ*)	This study

[a] Cp^r is a chloramphenicol resistance marker.

fused *lacZ* are reporters for the respective exporter expression. The resulting plasmids were introduced into host cells for β-galactosidase activity measurements.

Reporter gene assay. To investigate the growth phase-dependent transcription of various reporter constructs, each bacterial strain was grown at 37°C in LB broth containing 15 mg/liter chloramphenicol until the optical density at 600 nm (OD_{600}) reached 0.4 (early logarithmic phase), 0.8 (late logarithmic phase), 3.0 (early stationary phase), or 6.5 (late stationary phase). When we determined the effect of indole on the transcription of *mdtEF*, cells were grown at 37°C in LB broth until the OD_{600} reached 0.8 with 15 mg/liter chloramphenicol and 1 mM indole or only with 15 mg/liter chloramphenicol and the solvent (dimethyl sulfoxide) as a control. β-Galactosidase activity in cell lysates was assayed using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate as described by Miller (16), with a slight modification.

Quantitative real-time RT-PCR. Quantitative real-time reverse transcription-PCR (RT-PCR) was performed as follows. Cells were grown at 37°C in LB broth until the absorbance at 600 nm reached 0.8 (logarithmic phase), 3.0 (early stationary phase), or 6.0 (late stationary phase). The purification of total RNAs and the synthesis of cDNAs were performed by the methods described previously (6). The specific primer pairs are listed in Table 3. Real-time PCR was performed with each specific primer pair, using SYBR green PCR master mix (PE Applied Biosystems). The *E. coli rrsA* gene was chosen as a control for normalization of the cDNA loading in each PCR. The reactions were performed with an ABI PRISM 7000 sequence detection system (PE Applied Biosystems).

Drug tolerance assay. Each bacterial strain, grown at 37°C in LB broth until OD_{600} reached 0.8 (logarithmic phase) or 6.5 (stationary phase), was diluted to an OD_{600} of 0.1. Then, growth was measured in the presence or absence of crystal violet or kanamycin.

Survival assay. Each bacterial strain was grown at 37°C in LB broth until the OD_{600} reached 0.6 (logarithmic phase) or 6.5 (stationary phase). The stationary-phase cells were diluted to an OD_{600} of 0.6 with fresh medium, and then crystal violet was added to each bacterial cell culture (final concentration, 50 or 200 mg/liter). After incubation for 30 min at 37°C, aliquots of the cell cultures were spread on YT agar plates. After overnight incubation, the numbers of colonies were determined and percent survival was calculated in comparison with that of untreated cells.

Indole production assay. The extracellular indole concentration was determined by high-performance liquid chromatography (HPLC). The *E. coli* strain was cultured at 37°C and then pelleted by centrifugation at 20,000 × *g*. The resulting supernatant was extracted twice with ethyl acetate. The ethyl acetate phase was loaded onto a Symmetry C_{18} column (5 μm, 4.6 by 150 mm; Waters Corp.) attached to an L2130 HPLC system (HITACHI). The loaded samples were eluted with acetonitrile-H_2O (1:1) at the flow rate of 0.8 ml/min. Then the indole peak was detected relative to the absorbance at the wavelength of 276 nm and was identified by the corresponding peak of the purified indole (Sigma). The indole concentration was calculated from the ratio of the detection peak area to the standard peak one.

RESULTS

The growth phase-dependent expression of the intrinsic drug exporter genes in *Escherichia coli*. In order to profile the growth phase-dependent expression levels of intrinsic drug ex-

TABLE 2. Oligonucleotides used for plasmid construction

Oligonucleotide	Oligonucleotide sequence (5′ to 3′)	Length (bp)
acrA_primerPF	CGCGCGGCCGCAGAGTGGATCGCCAGGGAA	400
acrA_primerPR	CGCAAGCTTATGTAAACCTCGAGTGTCCGA	
acrD_primerPF	GCGGCGGCCGCAACGCGCGGAACGGCTAGG	276
acrD_primerPR	GCGAAGCTTTAAAAGAGGACCTCGTGTTTC	
acrE_primerPF	CGCGCGGCCGCGATTAATTATTCAGGAAAT	400
acrE_primerPR	CGCAAGCTTTACTATTCCTCAAAAAACCAA	
bcr_primerPF	CGCGCGGCCGCGGTGCTGATGACTGATGAT	400
bcr_primerPR	CGCAAGCTTAACGGGCTCCTGAAAGTCATT	
cusC_primerPF	CGCGCGGCCGCCGGCAACCTGAAACTGACT	550
cusC_primerPR	CGCAAGCTTAGGCTCATAATTTCTGGTGAT	
emrD_primerPF	CGCGCGGCCGCTTCTATAATATCACTGTAC	600
emrD_primerPR	CGCAAGCTTTATCACGGATGCTTTTATAAA	
emrE_primerPF	CGCGCGGCCGCGCTGAAAGTGGAATGTATC	450
emrE_primerPR	CGCAAGCTTAGCATATTCTTTCCTGTTCAA	
emrK_primerPF	CGCGCGGCCGCTCCCTTTGCAATGAAGCAT	408
emrK_primerPR	CGCAAGCTTTATTATCTCTCATTTCTCATA	
emrR_primerPF	CGCGCGGCCGCGTTACTAGTTGGCGTGGCG	400
emrR_primerPR	CGCAAGCTTTTGGGTATGACCTCATTAATT	
fsr_primerPF	CGCGCGGCCGCCGTTTTTTGCGCCGCCAGA	400
fsr_primerPR	CGCAAGCTTAGGAAAGTCACTTTTTCAGGG	
gadE_primerPF	CGCGCGGCCGCTTACCCCGGTTGTCACCCG	798
gadE_primerPR	CGCAAGCTTAACTTGCTCCTTAGCCGTTAT	
gadX_primerPF	GCGGCGGCCGCATTGCCCAGCAGAACAGC	403
gadX_primerPR	GCGAAGCTTTAGTTGACTTAATATTACATA	
gadY_primerPF	GCGCGGCCGCGATTATCCCTTATATTTCATAC	1,389
gadY_primerPR	GCAAGCTTAAAAACCCGGCATAGGGGAC	
hfq_primerPF	GCGGCGGCCGCCGCATTGAGCAGCGTTTTC	400
hfq_primerPR	GCGAAGCTTTCTCTCTTTTCCTTATATGC	
macA_primerPF	CGCGCGGCCGCCTCCAGCAATTGCTTACGG	450
macA_primerPR	CGCAAGCTTAAATTTTCTGAATACTCCA	
mdfA_primerPF	CGCGCGGCCGCGTCCCATCGTCCAGGACAC	550
mdfA_primerPR	CGCAAGCTTGCAATTTCTTCGCCAATAATAATCGCGC	
mdtA_primerPF	GCGGCGGCCGCAGCTTATGACTAAGAGCAC	321
mdtA_primerPR	GCGAAGCTTCGTTAAGAGTTTCTCTTCCTG	
rpoS_primerPF	GCGGCGGCCGCGATACCGTGGAGGCCGTG	1,395
rpoS_primerPR	GCGAAGCTTAAGGTGGCTCCTACCCGTG	
yceE_primerPF	CGCGCGGCCGCCACCGGATCATGATTACGG	500
yceE_primerPR	CGCAAGCTTAGCAATCCGCTGTTGGTGCGC	
yceL_primerPF	CGCGCGGCCGCATAACAGTGGCTTTCGTCG	400
yceL_primerPR	CGCAAGCTTTTCCCCTCCCGGGAAATAAAA	
ydgF_primerPF	CGCGCGGCCGCGCTTGTCCCCGTTTTTTCT	550
ydgF_primerPR	CGCGGATCCATATATACGACAGAGAAATCA	
ydhE_primerPF	CGCGCGGCCGCGTCAAAACTGACATGGTTG	400
ydhE_primerPR	CGCAAGCTTGTGAACACCTTTTATTTGTAG	
yidY_primerPF	CGCGCGGCCGCTGCTGTTAACCTTCCTGCC	400
yidY_primerPR	CGCAAGCTTGGGCTAAAGCGTCCTGATAGT	
yjiO_primerPF	CGCGCGGCCGCTGCTGGTCGTGAAGTTTGT	500
yjiO_primerPR	CGCAAGCTTAACAAACAACTCCTTGTCCGG	

TABLE 3. Oligonucleotides used for quantitative real-time PCR

Gene	Sequence of primer (5′ to 3′)	
	Forward	Reverse
gadX	TTTATACCGCTGCTTCTG AACGT	GTGTCCACTCATGG GCGATATTA
gadY	GCAAGCTTAAAAACCCG GCATAGGGGAC	AGAGCACAAAGTTT CCCGTG
hfq	CAAGCACGCGATTTCTA CTGTT	CACCGGCGTTGTTA CTGTGA
mdtE	CCCCCGGTTCGGTCAA	GGACGTATCTCGGC AACTTCAT
rpoS	TCGCAGGGAGCCA CACA	TGAATAACCAATCT CACCAAGGTAAA
rrsA	CGGTGGAGCATGTGGT TTAA	GAAAACTTCCGTGG ATGTCAAGA

porter genes, we examined their reporter enzyme activities at different growth phases using single-copy plasmids containing *lacZ*-fused gene promoters. Because the *emrAB*, *cusBA*, and *mdtEF* genes are transcribed as a part of the operons of *emrRAB* (10), *cusCFBA* (4), and *gadE-mdtEF* (14 and our unpublished data), respectively, *emrR*, *cusC*, and *gadE* promoter-fused *lacZ* constructs were used as reporters for these three genes. Each bacterial strain was grown until the OD_{600} reached 0.4 (early logarithmic phase), 0.8 (late logarithmic phase), 3.0 (early stationary phase), or 6.5 (late stationary phase), and then β-galactosidase activities were measured. Among the 20 exporter genes, only expression of the *mdtE* gene greatly increased with growth cessation (Fig. 1). Compared with the late logarithmic phase, 14- and 41-fold increases in the *mdtE* expression were detected at the early and late stationary phases,

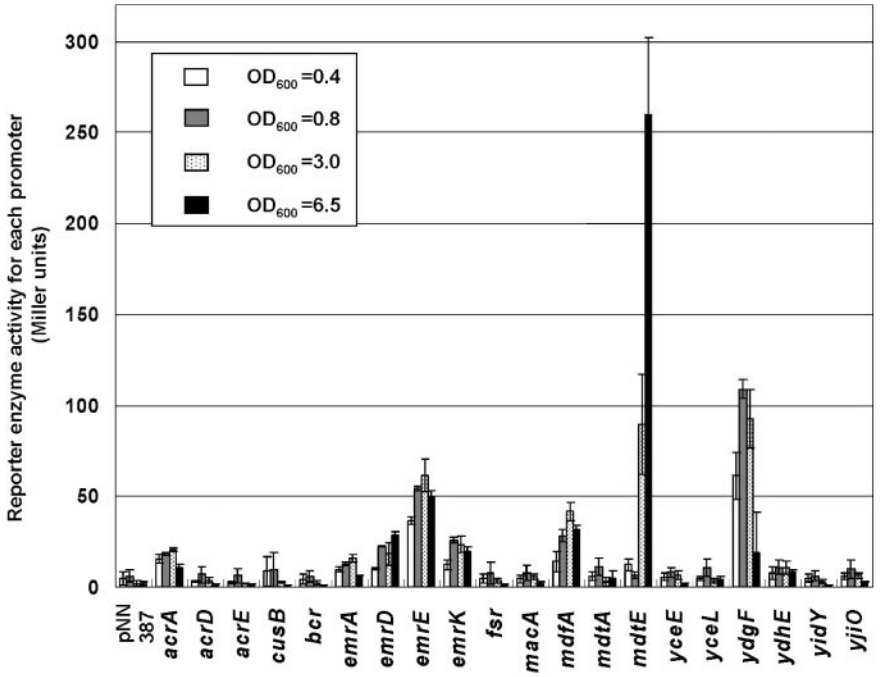

FIG. 1. Expression of the 20 drug exporter genes at different growth phases. The expression of the drug exporter genes was determined by the β-galactosidase reporter enzyme assay. Single-copy reporter plasmids were transformed into *E. coli* strain MC4100. *E. coli* cells were cultured until the OD_{600} reached 0.4 (early logarithmic phase), 0.8 (late logarithmic phase), 3.0 (early stationary phase), or 6.5 (late stationary phase), and then β-galactosidase activity was measured. pNN387 indicates the vector control.

respectively. No other exporter genes showed such increases in expression at the stationary phase.

At the logarithmic phase, the gene that showed the highest expression level was *ydgF* (109 Miller units); however, YdgF confers low-level drug resistance only to deoxycholate and sodium dodecyl sulfate (SDS), even when overproduced from a multicopy plasmid (19). The *ydgF*-knockout strain did not show hypersensitivity to drugs, including deoxycholate and SDS. The *emrE*, *mdfA*, and *acrA* genes showed relatively high expression levels at the logarithmic phase, next to that of *ydgF*. The deletion of these genes is known to increase drug susceptibility (29). The expression level of *mdtE* at the logarithmic phase was lower than those of *emrE*, *mdfA*, and *acrA*. In addition, RND (resistance nodulation cell division)-type multidrug exporter genes *acrE*, *acrD*, and *mdtABC*, which cause high multidrug resistance when they are overexpressed (19), showed very low expression levels throughout the cell growth. Therefore, it seems that the drug tolerance of *E. coli* cells under laboratory conditions mainly reflects the expression levels of these drug exporters at the logarithmic phase except for *ydgF*.

On the other hand, at the late stationary phase, the expression level of *mdtE* (260 Miller units) was the highest out of those of the 20 drug exporter genes. The second highest was that of *emrE*, but the level of its activity (50 Miller units) was far lower than that of *mdtE*. Such a growth phase-dependent increase in *mdtE* gene expression was also confirmed by determination of transcripts by quantitative RT-PCR analysis, although the maximum level was observed at the early stationary phase with respect to the mRNA level. The *mdtE* gene transcripts showed 380- and 76-fold increases at the early and late stationary phases, respectively, compared to the logarithmic phase. This indicates that the promoter activity of *mdtEF* is highest at the early logarithmic phase. Although the reporter enzyme was accumulated during the stationary phase, the amount of mRNA of *mdtEF* was gradually decreased, probably due to its high turnover rate. In summary, MdtEF is greatly induced at the stationary phase and contributes the intrinsic drug tolerance.

Drug tolerance mediated by up-regulation of *mdtEF* at the stationary phase. In order to determine whether the growth phase-dependent induction of *mdtEF* contributes to drug tolerance, cell growth was compared in the presence of drugs after the stationary phase and in logarithmic-phase cells being diluted to the same density with fresh medium. If cells at two different phases have different susceptibilities to the drug, the growth rate must reflect their initial viability. At first, the growth rate was compared in the Δ*acrB* background because the high-performance housekeeping drug exporter AcrAB may mask the contribution of MdtEF. MC4100Δ*acrB* and MC4100Δ*acrB*Δ*mdtEF* cells were first grown to the logarithmic phase (OD_{600} of 0.8) or to the stationary phase (OD_{600} of 6.5) in the absence of drugs, and then the cells were diluted to the same density with fresh medium. The growth was monitored in the absence or presence of several drugs, dyes, detergents, and antiseptics. Our previous studies revealed that MdtEF confers resistance to erythromycin, doxorubicin, crystal violet, ethidium bromide, rhodamine 6G, tetraphenylphosphonium bromide (TPP), benzalkonium, SDS, and deoxycholate when overexpressed (19). We used these compounds for the drug tolerance assay. In addition to these compounds, kanamycin, nalidixic acid, and norfloxacin, which are not substrates of MdtEF, were used as negative controls (19). In Fig. 2A and B,

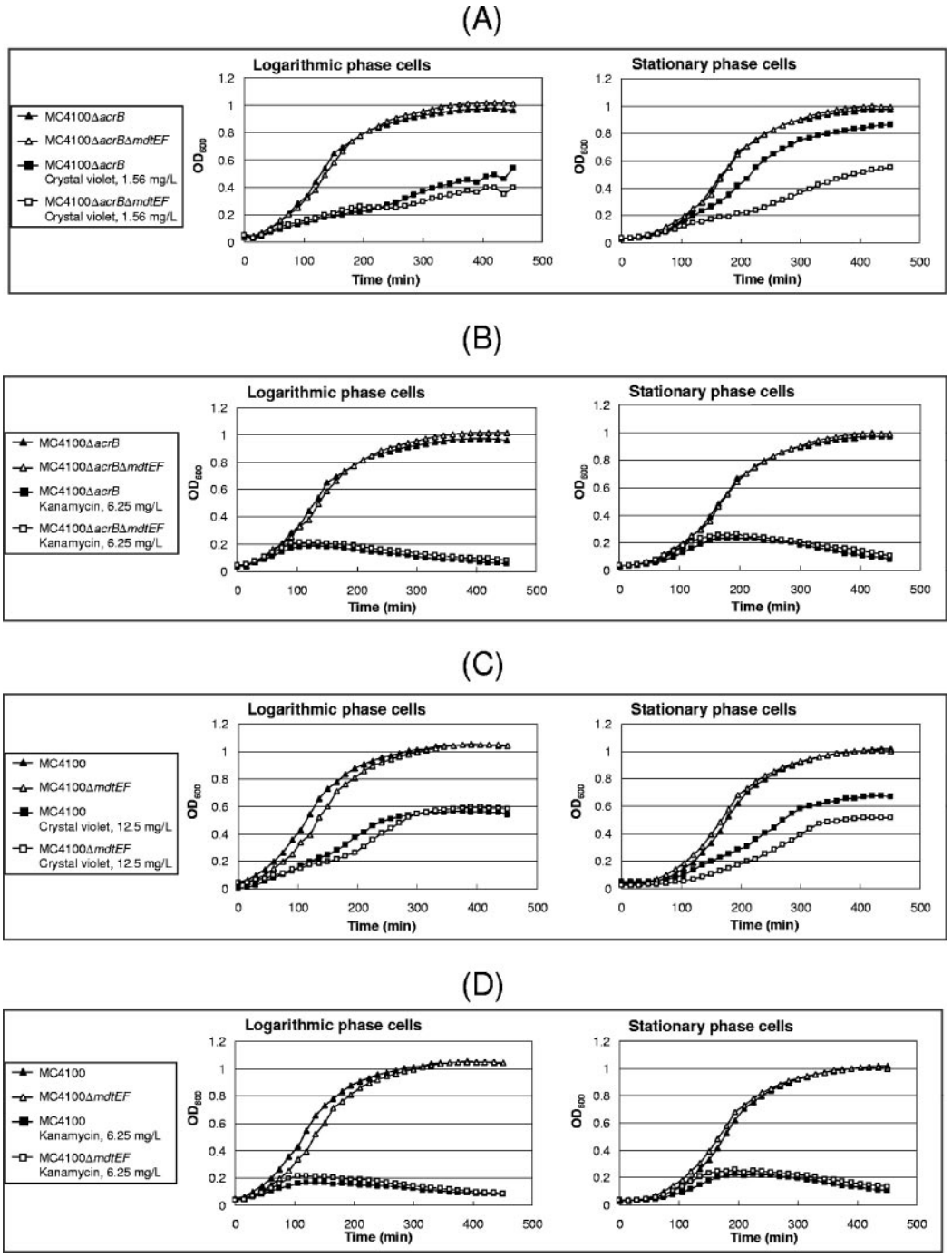

FIG. 2. Growth phase dependence of drug tolerance. Each strain (MC4100Δ*mdtEF*, MC4100Δ*acrB*, and MC4100Δ*acrB*Δ*mdtEF*) was grown until the OD_{600} reached 0.8 or 6.5 and then diluted to an OD_{600} of 0.1 with fresh medium. Cell growth was monitored in the absence or presence of drugs. (A) MC4100Δ*acrB* and MC4100Δ*acrB*Δ*mdtEF* with or without crystal violet. (B) MC4100Δ*acrB* and MC4100Δ*acrB*Δ*mdtEF* with or without kanamycin. (C) MC4100 and MC4100Δ*mdtEF* with or without crystal violet. (D) MC4100 and MC4100Δ*mdtEF* with or without kanamycin.

the growth curves in the presence or absence of crystal violet and kanamycin are shown as examples for MdtEF substrates and negative controls, respectively. All logarithmic-phase and stationary-phase cells grew at about the same rate in the absence of drugs, while the growth of logarithmic-phase cells was greatly retarded with 1.56 mg/liter crystal violet or 6.25 mg/liter kanamycin (Fig. 2A and B). Although the growth of MC4100Δ*acrB*Δ*mdtEF* stationary-phase cells was also greatly retarded in the presence of crystal violet, the growth of MC4100Δ*acrB* stationary-phase cells was significantly recovered in the presence of crystal violet (Fig. 2A). Because MC4100Δ*acrB* stationary-phase cells did not exhibit growth recovery in the presence of kanamycin (Fig. 2B), which is not a substrate of MdtEF, the drug tolerance of the stationary-

phase cells to crystal violet was certainly due to MdtEF in the Δ*acrB* background. Similarly, we observed *mdtEF*-dependent drug tolerance of the stationary-phase cells to erythromycin, doxorubicin, rhodamine 6G, ethidium bromide, TPP, benzalkonium, SDS, and deoxycholate, but not to nalidixic acid or norfloxacin (data not shown).

Then, in order to determine whether MdtEF contributes to drug tolerance even in the presence of the *acrB* gene, we measured the growth of wild-type MC4100 and MC4100Δ*mdtEF*. In the presence of 1.56 mg/liter crystal violet, both logarithmic- and stationary-phase cells showed full growth independent of *mdtEF*. However, in the presence of high concentration (12.5 mg/liter) of crystal violet, an increase in the *mdtEF*-dependent drug tolerance of the stationary-phase cells of the wild-type strain was observed (Fig. 2C), although the degree of the relative increase in drug tolerance (MC4100/MC4100Δ*mdtEF*) was lower than that in the Δ*acrB* background. In contrast, wild-type stationary-phase cells did not exhibit tolerance to kanamycin (Fig. 2D). These observations indicate that MdtEF actually contributes to the multidrug tolerance of *E. coli* at the stationary phase.

Subsequently, in order to confirm the increase in drug tolerance at the stationary phase, the viability of the cells was measured after short exposure to bactericidal compounds. MC4100Δ*acrB* and MC4100Δ*acrB*Δ*mdtEF* cells were first grown to the logarithmic phase (OD_{600} of 0.6) or to the stationary phase (OD_{600} of 6.5). Then the stationary-phase cells were diluted to the same density as the logarithmic-phase cells (OD_{600} of 0.6) with fresh medium. Both types of cells were exposed to 50 mg/liter crystal violet. After incubation for 30 min at 37°C, the survival rate was calculated as described in Materials and Methods (Fig. 3A). The logarithmic-phase cells of MC4100Δ*acrB* and MC4100Δ*acrB*Δ*mdtEF* showed very low survival rates (0.7% and 1.6%, respectively). On the other hand, the MC4100Δ*acrB* stationary-phase cells showed very high viability (101%), whereas MC4100Δ*acrB*Δ*mdtEF* cells still exhibited low viability (2.2%) at the stationary phase. In the $acrB^+$ background, both logarithmic- and stationary-phase cells were fully viable in the presence of 50 mg/liter of crystal violet independent on the presence or absence of *mdtEF*. However, in the presence of 200 mg/liter of crystal violet, the viability of the stationary-phase cells (57%) was significantly higher than that of the logarithmic-phase cells (4%) and the viability of the stationary-phase Δ*mdtEF* cells (25%) was significantly lower than the wild-type cells. These results indicated that the induction of *mdtEF* gene expression at the stationary phase contributes to the drug tolerance, while in the high drug concentration, the drug resistance mechanisms other than MdtEF also partly contribute to the stationary-phase drug tolerance.

The effect of the *evgSA* and *tnaAB* gene deletions on the growth phase-dependent induction of *mdtEF* gene expression. Previously, we found that the EvgSA two-component system positively controls *mdtEF* expression (20). In order to determine whether the growth phase-dependent *mdtEF* induction is controlled by the EvgSA system, the expression level of *mdtEF* was measured in the Δ*evgSA* background. It was found that the level of induction of *mdtEF* at the stationary phase was not affected by *evgSA* deletion (Fig. 4A), indicating that the growth

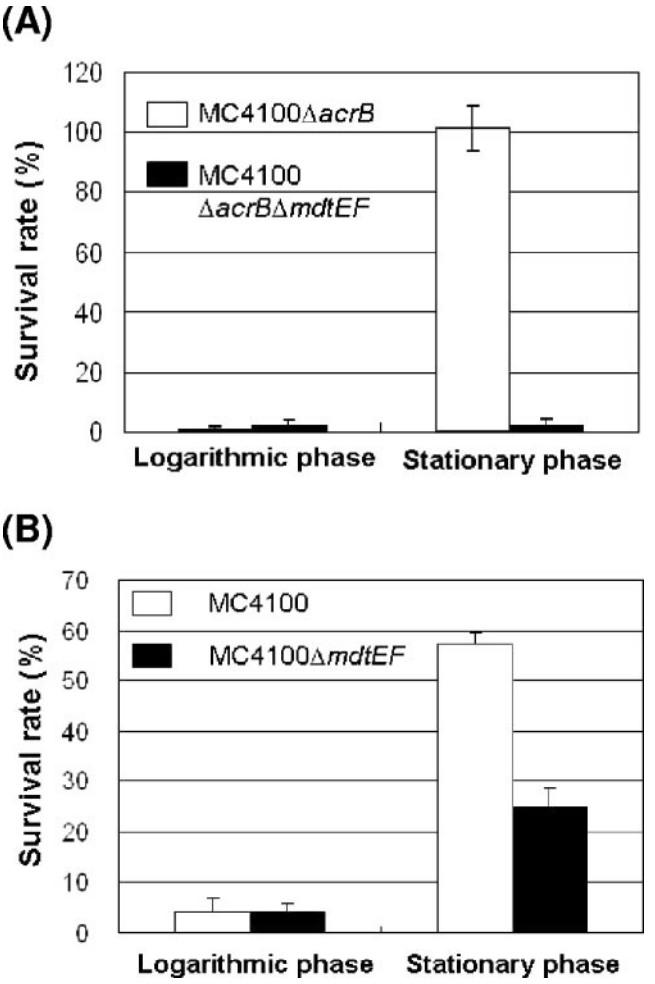

FIG. 3. Growth phase-dependent increase in cell viability with the bactericidal drugs. (A) Each bacterial strain (MC4100Δ*acrB* and MC4100Δ*acrB*Δ*mdtEF*) was grown at 37°C in LB broth until the OD_{600} reached 0.6 (logarithmic phase) or 6.5 (stationary phase). The stationary-phase cells were diluted to an OD_{600} of 0.6. Then crystal violet was added to each bacterial cell culture (final concentration, 50 mg/liter). After incubation for 30 min at 37°C, viability was measured as described in Materials and Methods. (B) Each bacterial strain (MC4100 and MC4100Δ*mdtEF*) was grown at 37°C in LB broth until an OD_{600} of 0.6 (logarithmic phase) or 6.5 (stationary phase). Then the bacterial cell viability with medium containing crystal violet (200 mg/liter) was measured as described above.

phase-dependent regulation of *mdtEF* was not mediated by the EvgSA system.

We also previously reported that indole, which is a toxic metabolite synthesized from tryptophan by a tryptophanase, TnaA (26), up-regulates *mdtEF* expression (5). The extracellular indole concentration in wild-type cells increased with cell growth and reached about 500 μM after 24 h of culture, whereas there was no detectable level of indole in the culture medium of Δ*tnaAB* cells even after 24 h of culture (Fig. 5). In order to determine the contribution of indole to the expression level of *mdtEF*, we measured the growth phase-dependent *mdtEF* induction in the Δ*tnaAB* background. The *tnaAB* deletion significantly decreased the level of induction of *mdtEF* at the stationary phase to about 65% of the wild-type level (Fig. 4B).

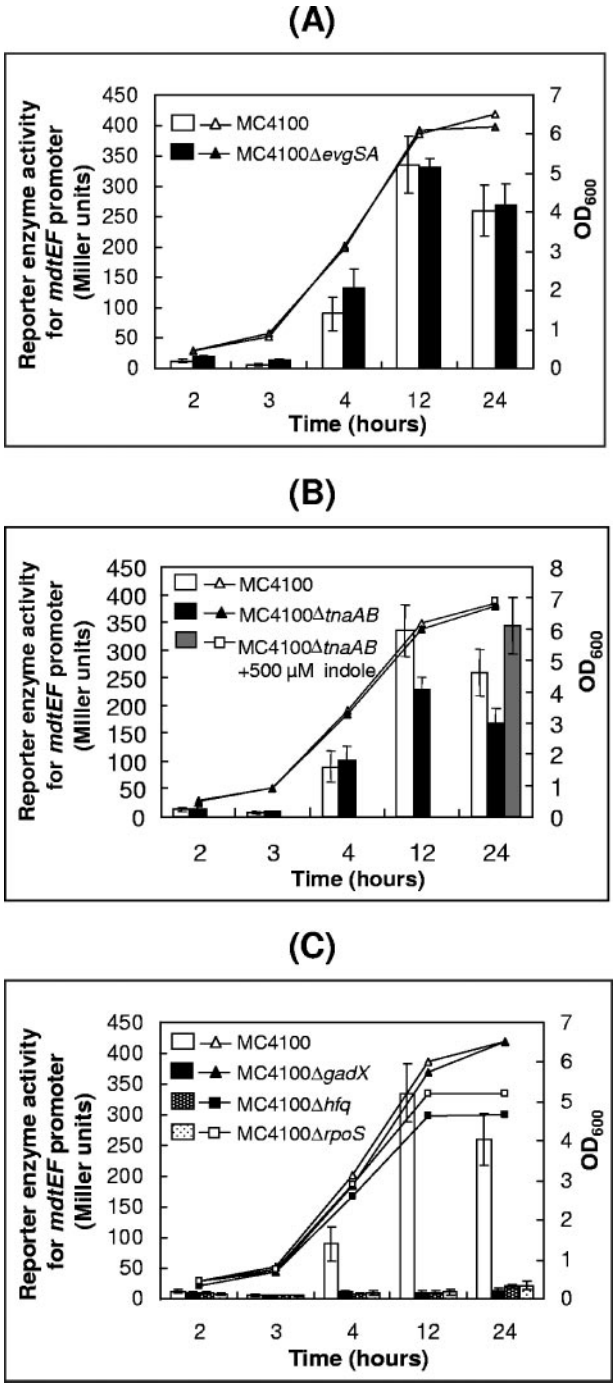

FIG. 4. Effects of deletion of *mdtEF* regulatory genes on the expression of the *gadE-mdtEF* genes. The expression of *gadE-mdtEF* in the wild type (MC4100) or each gene deletion mutant (MC4100Δ*evgSA*, MC4100Δ*tnaAB*, MC4100Δ*gadX*, MC4100Δ*hfq*, and MC4100Δ*rpoS*) was determined by means of the β-galactosidase reporter enzyme assay. *E. coli* cells cultured for 2, 3, 4, 12, and 24 h were collected, followed by β-galactosidase activity measurement. (A) Effect of *evgSA* deletion. (B) Effect of *tnaAB* deletion and addition of indole (500 μM). (C) Effect of deletion of the *gadX*, *hfq*, and *rpoS* genes. At least three independent experiments were performed in each case.

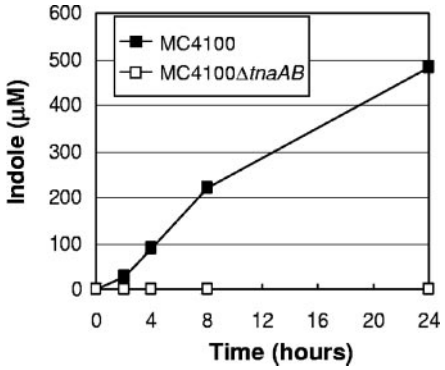

FIG. 5. Indole accumulation with cell growth. The extracellular indole concentrations of the wild-type and Δ*tnaAB* strains were measured by HPLC analysis as described in Materials and Methods. Black squares, MC4100 (wild type); white squares, MC4100Δ*tnaAB*.

When 500 μM indole was added in the medium, the expression level of *mdtEF* in the Δ*tnaAB* strain was restored to almost the same level as the stationary-phase *tnaAB*$^+$ strain (Fig. 4B). Thus, indole plays some role in the growth phase-dependent induction of *mdtEF*.

The effect of the *gadX*, *hfq*, and *rpoS* genes on growth phase-dependent induction of *mdtEF* gene expression. Since indole induces the expression of *mdtEF* via transcriptional regulator GadX (5), we examined whether the growth phase-dependent induction of *mdtEF* is mediated by *gadX*. In the *gadX* deletion mutant (MC4100Δ*gadX*), growth phase-dependent induction of *mdtEF* was completely abolished (Fig. 4C). Recently, a small-RNA regulator, GadY, which binds to Hfq protein, was found to be induced at the stationary phase in a sigma factor RpoS-dependent manner (22). The overexpression of GadY enhances the mRNA level of *gadX* (22). Therefore, we then deleted the *hfq* and *rpoS* genes. In the resultant strains, the growth phase-dependent induction of *mdtEF* was completely abolished, like on the *gadX* deletion. Thus, the growth phase-dependent *mdtEF* induction is mediated by the RpoS-GadY-(Hfq)-GadX signaling pathway.

Then we investigated the effect of *rpoS*, *hfq*, and *gadX* deletion on drug tolerance of *E. coli*. As shown in Fig. 6, the deletion of these genes greatly reduced drug tolerance of the stationary-phase cells to crystal violet in the Δ*acrB* background. The deletion of these genes did not affect the crystal violet sensitivity of the logarithmic-phase cells.

Growth phase-dependent expression of *rpoS*, *hfq*, and *gadX*. In order to characterize the RpoS-GadY(Hfq)-GadX signaling pathway, we measured the growth phase-dependent changes in the expression of the *rpoS*, *gadY*, *hfq*, and *gadX* genes by means of the β-galactosidase reporter assay. As shown in Fig. 7A, the expression of *rpoS*, *gadY*, and *gadX* showed growth phase dependence and the maximum expression was observed at the late stationary phase, except in the case of *gadX*, of which the expression levels at the early and late stationary phases were almost equal to each other within experimental error. On the other hand, the expression level of *hfq* did not show such significant growth phase dependence. Compared with the expression levels at the early logarithmic phase (OD_{600} of 0.4), the expression levels of *rpoS*, *gadY*, and *gadX* at the late sta-

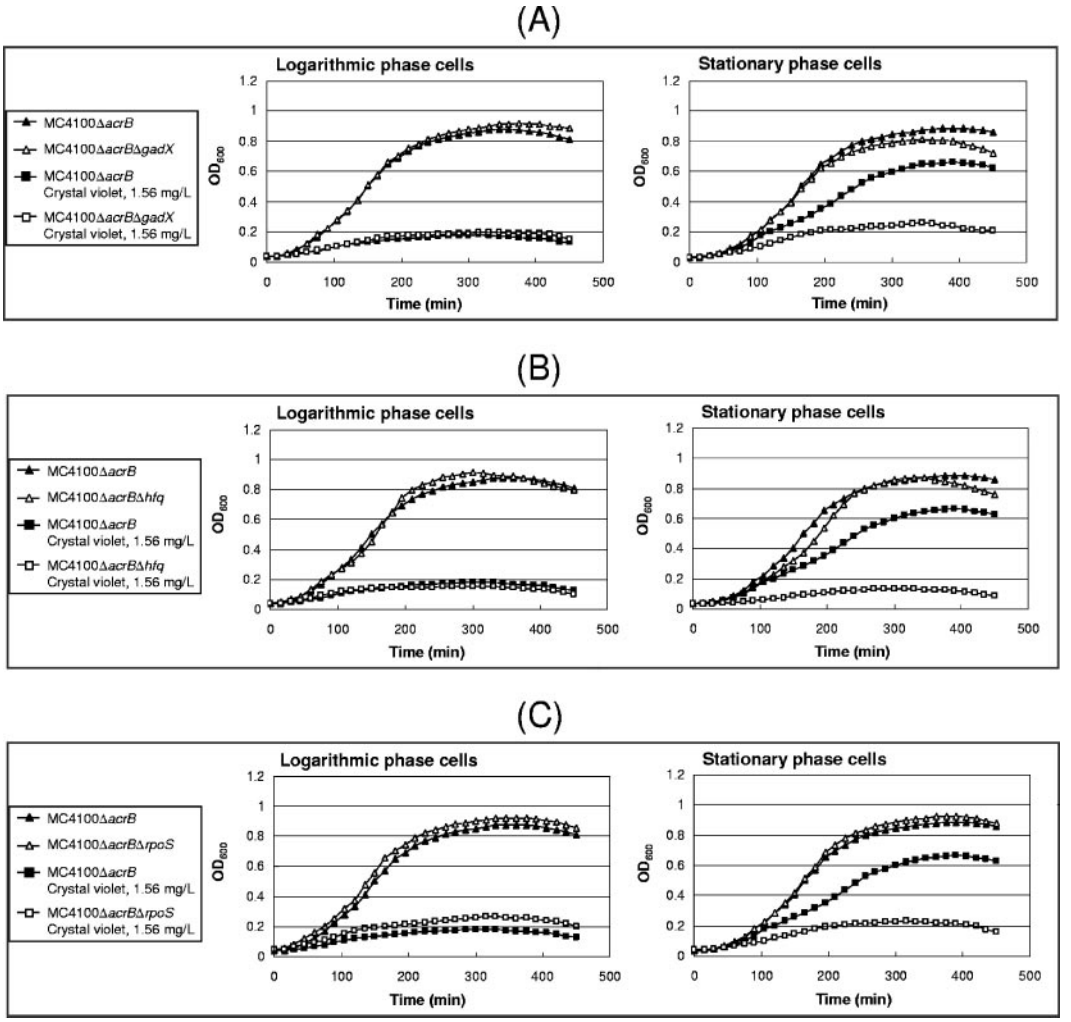

FIG. 6. Effect of deletion of the RpoS-dependent signaling pathway on drug tolerance of the stationary-phase cells. Each strain (MC4100, MC4100Δ*acrB*, MC4100Δ*acrB*Δ*gadX*, MC4100Δ*acrB*Δ*hfq*, and MC4100Δ*acrB*Δ*rpoS*) was grown until an OD_{600} of 0.8 or 6.5 and then diluted to an OD_{600} of 0.1 with fresh medium. Cell growth was monitored in the absence or presence of crystal violet. (A) MC4100Δ*acrB* and MC4100Δ*acrB*Δ*gadX*. (B) MC4100Δ*acrB* and MC4100Δ*acrB*Δ*hfq*. (C) MC4100Δ*acrB* and MC4100Δ*acrB*Δ*rpoS*.

tionary phase were increased by factors of 8.8, 2.5, and 3.6, respectively. That the degree of increase in the expression levels of regulator genes was relatively lower than that of *mdtEF* (41-fold) may be due to the fact that GadX acts as a dimer in transcriptional regulation. In summary, the growth phase-dependent control of *mdtEF* was mediated through modification of the amounts of RpoS-dependent small-RNA GadY and transcriptional regulator GadX. One exception is that, although *rpoS*, *gadY*, and *gadX* expression was increased from early to late log phase (Fig. 7A), the *mdtEF* expression was slightly decreased during this period (Fig. 1). The slight decrease of *mdtE* expression might be within the experimental deviation; however, there may be a possibility of the postexponential activation of RpoS.

The growth phase-dependent expression of *rpoS*, *gadY*, and *gadX* was confirmed on quantitative PCR analysis. At the early stationary phase, the mRNA of *mdtE* was drastically increased (380-fold), and those of *rpoS*, *gadY*, and *gadX* were also moderately increased (2.5-, 18-, and 23-fold, respectively), whereas a significant change in the *hfq* mRNA level was not observed (Table 4).

GadX, Hfq, and RpoS are essential for indole-induced *mdtEF* expression, and the expression is induced via *rpoS*, *gadY*, and *gadX* up-regulation. We investigated the relationship between the induction of *mdtEF* expression by indole and the RpoS-GadY(Hfq)-GadX signaling pathway. At first, indole production was measured in wild-type and *tnaAB*, *evgSA*, *gadX*, *hfq*, and *rpoS* deletion mutant cells. After a 24-h culture of these cells, the indole concentration in the culture medium of the respective deletion mutants was the same as that of the wild type, except in the case of the Δ*tnaAB* mutant (data not shown), in which indole production was not observed (Fig. 5), indicating that RpoS-GadY(Hfq)-GadX signaling does not affect indole production.

Then the *mdtEF* reporter gene expression by these strains was measured in the presence or absence of externally added indole. In the wild-type and *evgSA* deletion mutant, the expression was similarly increased by the addition of 1 mM indole,

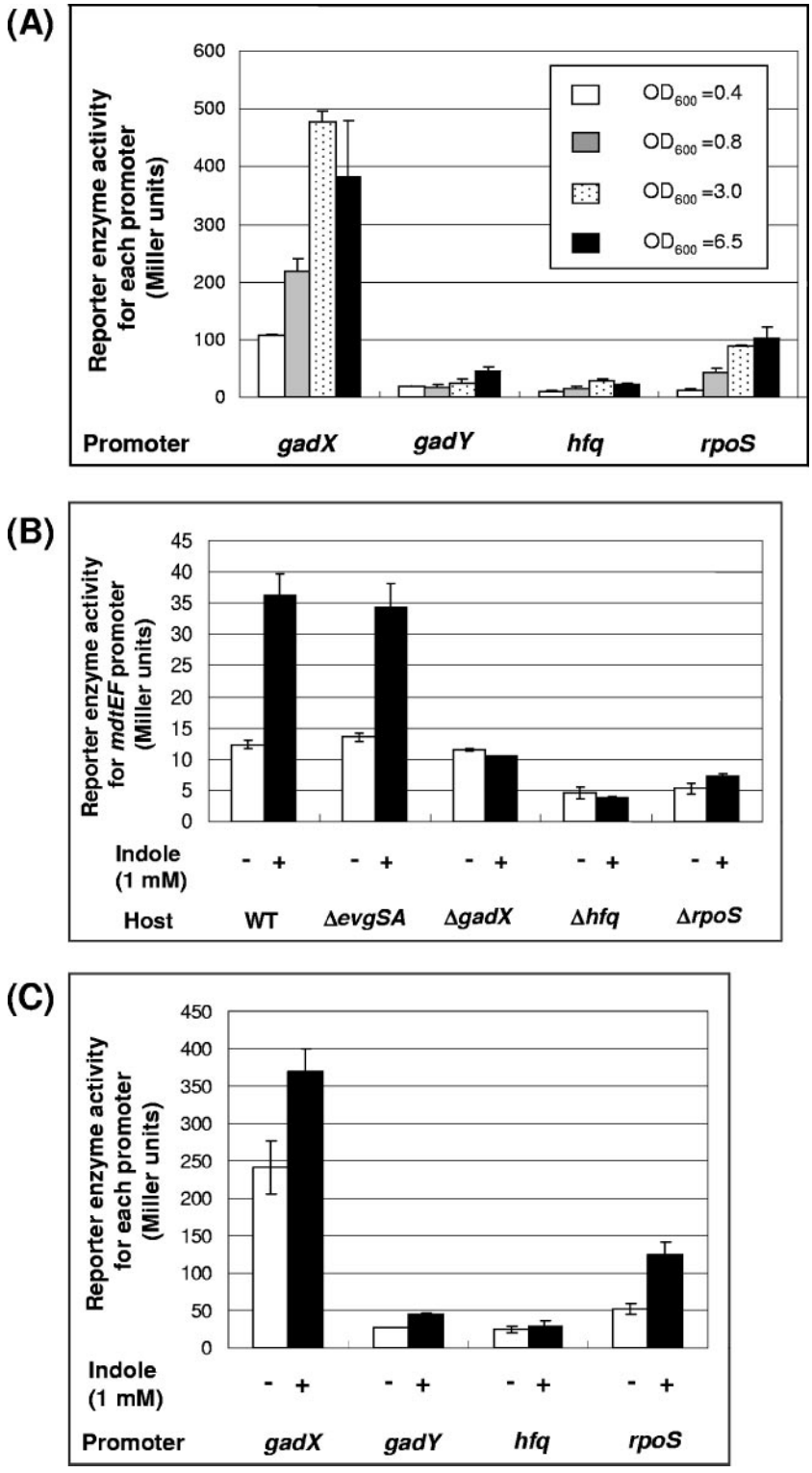

FIG. 7. Expression levels of *rpoS*, *hfq*, *gadY*, and *gadX* and effect of their deletion on induction of *mdtEF* genes by indole. (A) The growth phase-dependent expression of the *rpoS*, *hfq*, *gadY*, and *gadX* genes was determined by means of the β-galactosidase reporter enzyme assay. *E. coli* cells were cultured until the OD_{600} reached 0.4 (early logarithmic phase), 0.8 (late logarithmic phase), 3.0 (early stationary phase), or 6.5 (late stationary phase), and then β-galactosidase activity was measured. (B) Effect of deletion of *mdtEF* regulatory genes on the induction of *mdtEF* by indole. The wild-type and mutant strains (Δ*evgSA*, Δ*gadX*, Δ*hfq*, and Δ*rpoS*) were grown until the OD_{600} reached 0.8 in LB broth with (black bars) or without (white bars) 1 mM indole. The β-galactosidase activity of the *lacZ* fusion of the *gadE-mdtEF* promoter was measured. (C) Effect of indole on expression of *rpoS*, *hfq*, *gadY*, and *gadX*. The expression of the *rpoS*, *hfq*, *gadY*, and *gadX* genes was determined by means of the β-galactosidase reporter enzyme assay. *E. coli* cells were grown until the OD_{600} reached 0.8 in LB broth with (black bars) or without (white bars) 1 mM indole, and then the β-galactosidase activity of the *lacZ* fused to each promoter was measured.

TABLE 4. Induction of *mdtE*, *rpoS*, *hfq*, *gadY*, and *gadX* gene transcripts attributed to the growth phase, as determined by amplification of cDNA samples

Gene	Fold change from stationary phase vs logarithmic phase[a]
mdtE	380
rpoS	2.5
hfq	0.66
gadY	18
gadX	23

[a] Values indicate the fold change in the transcript level of cells cultured up to an OD_{600} of 3.0 (early stationary phase) compared to that of cells cultured up to an OD_{600} of 0.8 (logarithmic phase).

whereas when the *gadX*, *hfq*, or *rpoS* gene was deleted, the expression of *mdtEF* was no longer increased by indole at all (Fig. 7B); therefore, the induction of *mdtEF* by indole is also mediated by the RpoS-GadY(Hfq)-GadX signaling pathway.

The effects of indole on the expression levels of *rpoS*, *hfq*, *gadY*, and *gadX* were examined by means of the reporter gene assay. The expression of these genes was also increased by 1 mM indole, except in the case of *hfq* (Fig. 7C). These results indicate that indole controls *mdtEF* gene expression via increasing the amounts of RpoS, GadY, and GadX. Quantitative PCR analysis of *rpoS*, *hfq*, *gadY*, and *gadX* gave similar results (data not shown).

DISCUSSION

In this study, we comprehensively investigated the growth phase-dependent expression of drug exporter genes in *E. coli*. We found that out of the 20 drug exporter genes, only the expression of *mdtEF* greatly increased with cell growth (Fig. 1 and Table 4). The induction of *mdtEF* expression actually conferred drug tolerance to *E. coli* at the stationary phase (Fig. 2 and 3). The growth phase-dependent activation of the *mdtEF* promoter was mediated by the RpoS-GadY(Hfq)-GadX signaling pathway and enhanced by indole (Fig. 4 and 7). As expected, the deletion of *rpoS*, *hfq*, and *gadX* caused the lack of the MdtEF-dependent drug tolerance of the stationary-phase cells.

Schellhorn et al. (25) reported that *yhiUV*, which is an old name for *mdtEF*, is one of the RpoS-dependent genes that are induced at the stationary phase. Our previous study revealed that YhiUV is a multidrug exporter system (20), and thus it was renamed MdtEF (21). In this study, we revealed that the growth phase-dependent expression of *mdtEF* confers drug tolerance at the stationary phase. In addition, we revealed that the growth phase regulation of *mdtEF* is mediated by RpoS-dependent small-RNA GadY and transcriptional regulator GadX, which is the same signaling pathway as indole signaling.

Sulavik et al. reported that most drug exporter genes did not contribute to drug tolerance under laboratory conditions, except for *acrAB*, *emrE*, and *mdfA*, in an exporter gene knockout experiment (29). The results of this study indicate that the drug hypersensitization of exporter gene deletion mutants reflects the expression levels of drug exporter genes at the logarithmic phase, except in the case of *ydgF*, which is a weak exporter having a very narrow substrate range. Since the expression level of *mdtEF* is very low at the logarithmic phase, on deletion of the *mdtEF* gene, no hypersensitization might be seen on MIC measurement. On the other hand, at the stationary phase, MdtEF is a major drug exporter and certainly confers drug tolerance, although the contribution of AcrAB to the stationary-phase drug tolerance is still significant.

As for the possibility that MdtEF confers tolerance against indole, experimental detection was difficult because the indole toxicity is very low. However, the fact that the Δ*mdtEF* mutant showed a somewhat reduced indole concentration in the stationary-phase medium and increased accumulation of indole in the cells when indole was externally added (data not shown) suggests the possibility that MdtEF plays some role in indole export.

The *mdtEF* genes are cotranscribed with acid response regulator *gadE*, which is encoded upstream of *mdtEF* in the same operon. The *gadE* expression is controlled as a response to acid stress (22, 30, 31, 32). However, in our experiments, the pH of the medium at the stationary phase was moderately alkaline (about pH 8.5). Therefore, the signal causing growth phase-dependent induction must be different from acid stress. We examined the effect of alkaline pH on *mdtE* expression by means of quantitative PCR analysis. The expression level of *mdtE* was not altered when MC4100 was cultured until the logarithmic phase (OD_{600} of 0.8) in the LB broth at pH 7.0 or in the LB broth that had been prepared to pH 8.5 (data not shown).

In our previous study, we reported that 2 mM indole added to the culture medium significantly induced the expression of *mdtE*, *acrD*, *acrE*, *emrK*, *yceL*, and *cusB* at the logarithmic phase, whereas when the indole concentration was 1 mM, only *mdtE* induction was significant (5). The concentration of indole in the MC4100 culture medium at the stationary phase was around 500 μM with the complex laboratory medium. That is the reason why the induction of the drug exporters other than *mdtE* by intrinsic indole at the stationary phase was not observed in this study. Of course, this fact does not exclude the possibility that indole-dependent drug exporter genes other than *mdtEF* may be actually induced and play some roles at infection sites due to the high local concentration of indole produced by other bacteria.

In *P. aeruginosa*, *N*-(3-oxododecanoyl)-L-homoserine lactone and *N*-(butyryl)-L-homoserine lactone are known as quorum-sensing signal molecules at the stationary phase, while *E. coli* does not produce acylhomoserine lactones. On the other hand, the indole concentration increases with cell growth as a by-product of pyruvate production in *E. coli* cells (33) (Fig. 5). Besides, it has been reported that indole regulates biofilm formation by *E. coli* and is associated with the virulence of *Haemophilus influenzae* (12, 13). Our results confirmed the finding of Wang et al. (33) that indole acts as a stationary-phase signal molecule.

It is well known that the drug resistance of pathogens at the sites of infection is generally higher than that under laboratory conditions (8). We believe that elucidation of the mechanisms underlying the growth phase-dependent induction of multidrug exporter genes is important for understanding such acquired drug resistance mechanisms at the site of infection.

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