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# Comprehensive Studies of Drug Resistance Mediated by Overexpression of Response Regulators of Two-Component Signal Transduction Systems in *Escherichia coli*

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**In *Escherichia coli*, there are 32 open reading frames (ORFs) that are assumed to be response regulator genes of two-component signal transduction systems on the basis of sequence similarities. We cloned all of these 32 ORFs into a multicopy expression vector and investigated whether or not they confer drug resistance via control of drug resistance determinants. Fifteen of these ORFs, i.e., *baeR*, *citB*, *cpxR*, *evgA*, *fimZ*, *kdpE*, *narL*, *narP*, *ompR*, *rcsB*, *rstA*, *torR*, *yedW*, *yehT*, and *dcuR*, conferred increased single- or multidrug resistance. Two-thirds of them conferred deoxycholate resistance. Five of them, i.e., *evgA*, *baeR*, *ompR*, *cpxR*, and *rcsB*, modulated the expression of several drug exporter genes. The drug resistance mediated by *evgA*, *baeR*, and *cpxR* could be assigned to drug exporters by using drug exporter gene knockout strains.**

Bacterial species that have developed resistance to antimicrobial agents are increasing in numbers. We previously found the interesting phenomenon that the overexpression of response regulators of bacterial two-component signal transduction systems confers drug resistance as a result of controlling the expression of some drug transporter genes (17, 18). Drug efflux plays a major role in intrinsic tolerance of bacteria to drugs and toxic compounds (14, 15). Previously, we cloned all of the putative intrinsic drug transporter open reading frames (ORFs) in *Escherichia coli* and investigated their drug resistance phenotypes (16).

Two-component systems are signal transduction pathways in prokaryotic organisms that respond to environmental conditions (11). A typical two-component system consists of two types of signal transducers, a sensor kinase and its cognate response regulator. The sensor kinase monitors some environmental conditions and accordingly modulates the phosphorylation state of the response regulator. The response regulator controls gene expression and/or cell behavior (7, 19).

In *E. coli*, 32 response regulators and 30 sensor kinases have been assumed to exist on the basis of the results of genome sequence analysis (10). As yet only a few two-component systems have been characterized (7). Recently, we found that the overexpression of *evgA* up-regulates the drug transporter genes *emrKY* and *yhiUV*. In addition, *baeR* up-regulates *mdtABC*, resulting in multidrug resistance (2, 13, 17, 18). Such response regulator-mediated drug resistance is a novel mechanism for acquiring multidrug resistance.

In this study, we surveyed whether it is a general phenomenon for bacteria that overexpression of response regulators confers drug resistance. We cloned all of the ORFs of the putative response regulators in *E. coli* into an expression vector and then investigated whether or not they confer drug resistance.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** *E. coli* W3104 (22) was used as a donor of chromosomal DNA. *E. coli* TG1 (21) and DH5α (Takara Shuzo Co., Kyoto, Japan) were used as cloning hosts. *E. coli* KAM3 (12), a derivative of K-12 that lacks a restriction system and *acrB*, was used for the drug susceptibility testing. *E. coli* cells were grown in 2× YT medium (20), supplemented with ampicillin (100 μg/ml) when necessary, under aerobic conditions at 37°C. Competent cells were prepared by the method of Hanahan (6). The pTrc99A vector was purchased from Amersham Pharmacia Biotech. pTrc6His was derived from the pTrc99A vector for the production of a C-terminal $His_6$ tag (16). The pQE70 vector was purchased from Qiagen.

**Drug susceptibility test.** The MICs of drugs were determined on YT (20) agar containing various drugs (chloramphenicol, tetracycline, minocycline, erythromycin, nalidixic acid, norfloxacin, enoxacin, kanamycin, fosfomycin, doxorubicin, novobiocin, rifampin, polymyxin B, acriflavine, crystal violet, ethidium bromide, rhodamine 6G, methyl viologen, tetraphenylphosphonium bromide [TPP], carbonyl cyanide *m*-chlorophenylhydrazone, benzalkonium, sodium dodecyl sulfate [SDS], and deoxycholate) at various concentrations. These agar plates were made by the twofold agar dilution method (16). Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the agar plates at 1, 0.1, or 0.01 mM as an inducer when we examined the susceptibility of *E. coli* cells. Ten thousand cells were inoculated on a test agar plate and incubated at 37°C for 16 h. Growth was then evaluated.

**Construction of an expression plasmid library of the response regulator ORFs.** ORFs assumed to be regulatory genes of two-component systems were cloned as follows. Chromosomal DNA from *E. coli* W3104 was isolated as described previously (20). ORFs were amplified by PCR with forward primers containing an *Nco*I site that included the initiation codons of the response regulator genes (except for *uvrY*) and reverse primers containing the translation termination codons of these genes. A forward primer containing an *Eco*RI site was used for *uvrY*. The amplified fragments were inserted into the pTrc99A vector and cut with *Nco*I (*Eco*RI for *uvrY*) and *Bam*HI (*Pst*I for *kdpE* and *yjdG*). The Shine-Dalgarno sequence supplied by the vector was placed at a correct distance from the ATG codon. The overexpressed proteins were expected to have exactly the same sequence as native proteins except that the nonconserved second amino acids from the N terminus have been changed. Competent KAM3

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TABLE 1. Drug resistance of *E. coli* cells harboring pTrc plasmids carrying putative response regulator ORFs

| Drug | No gene (0.01, 0.1, 1) | MIC (μg/ml) for strain carrying gene, with indicated IPTG concn (mM)[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *evgA* | | | *baeR* | | *cpxR*[b] | | *dcuR* | | |
| | | 0.01 | 0.1 | 1 | 0.01, 0.1 | 1 | 0.01 | 0.1, 1 | 0.01 | 0.1 | 1 |
| Erythromycin | 3.13 | **12.5** | **25** | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | **6.25** | **6.25** |
| Kanamycin | 3.13 | 3.13 | 3.13 | 1.56 | 3.13 | 3.13 | 3.13 | **12.5** | 3.13 | 3.13 | 3.13 |
| Fosfomycin | 1.56/0.78[d] | 1.56 | 0.78 | 0.78 | 1.56/0.78[d] | 0.78 | 1.56 | 0.78 | 1.56 | 0.78 | 0.78 |
| Doxorubicin | 3.13 | **100** | **25** | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 |
| Novobiocin | 0.78 | **3.13** | 0.78 | 0.78 | **6.25** | **6.25** | 0.78 | **3.13** | 0.78 | **3.13** | **3.13** |
| Crystal violet | 0.78 | **3.13** | **1.56** | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | **1.56** |
| Ethidium bromide | 25 | **50** | **50** | 25 | 25 | 25 | 12.5 | 12.5 | 25 | 25 | 25 |
| Rhodamine 6G | 6.25 | **100** | **25** | 3.13 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 |
| Methyl viologen | 100 | 100 | 50 | 50 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| TPP | 6.25 | **25** | **25** | 6.25 | 6.25 | 6.25 | 3.13 | 6.25 | 6.25 | 6.25 | 6.25 |
| Benzalkonium | 3.13 | **25** | **12.5** | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 |
| SDS | 50 | **400** | 50 | 50 | **100** | **100** | 50 | 50 | 50 | 100 | **100** |
| Deoxycholate | 1,250 | **>40,000** | 1,250 | 1,250 | **>40,000** | 1,250 | 1,250 | **>40,000** | 1,250 | **20,000** | **20,000** |

[a] Values in boldface are larger than values for the control strain KAM3 harboring pTrc99A. IPTG at 0.01, 0.1, or 1 mM was added to the agar plates. pTrcarcA, pTrcatoC, pTrcb2381, pTrcbasR, pTrccheB, pTrccheY, pTrccreB, pTrcglnG, pTrchydG, pTrcphoB, pTrcphoP, pTrcrssB, pTrcuhpA, pTrcuvrY, pTrcyfhA, pTrcygiX, and pTrcylcA did not affect the MICs. The MICs of chloramphenicol, tetracycline, minocycline, nalidixic acid, norfloxacin, enoxacin, rifampin, polymyxin B, acriflavine, and carbonyl cyanide *m*-chlorophenylhydrazone did not change.

[b] pTrccpxR conferred amikacin resistance (MIC of 1.56 μg/ml for KAM3 versus 6.25 μg/ml for KAM3/pTrccpxR).

[c] The growth of the KAM3 strain carrying pTrcfimZ was inhibited with 1,250 μg of deoxycholate per ml; however, the growth recovered with 10,000 to 40,000 μg/ml.

[d] The fosfomycin MIC for host and *baeR* cells was 1.56 μg/ml with 0.01 and 1 mM IPTG and 0.78 μg/ml with 0.1 mM IPTG.

cells were transformed with at least three of the constructed plasmids that had been extracted from independent colonies, and then the susceptibilities of all transformants to various drugs were measured after induction by IPTG.

**Transcriptional analysis of putative drug transporter genes.** Cells were grown at 37°C in Luria-Bertani broth containing ampicillin until the absorbance at 600 nm reached 0.8. Total RNA was then purified by using the RNAprotect bacterial reagent (Qiagen) and the SV total RNA isolation system (Promega), with a slightly revised protocol. cDNA samples were synthesized from the purified total RNA by using TaqMan reverse transcription reagents (PE Applied Biosystems) and random hexamers as primers. Specific primer pairs were designed with ABI PRISM Primer Express software (PE Applied Biosystems) for putative drug transporter genes. Real-time PCR was performed with each specific primer pair, using SYBR Green PCR master mix (PE Applied Biosystems). Equal amounts of cDNA, derived from RNA samples, were used as templates in the amplification reactions. *E. coli rrsA* was chosen as the control for the normalization of cDNA loading in each PCR. The reactions were performed with an ABI PRISM 7000 sequence detection system (PE Applied Biosystems), during which the fluorescence signal due to SYBR Green intercalation was monitored to quantify the double-stranded DNA product formed after each PCR cycle. The threshold cycle (Ct) is the first cycle for which a statistically significant increase in the amount of the PCR product is detected. Ct values are thus inversely proportional to the amounts of the RNA species in the original RNA samples. The Ct value was determined for each amplification reaction. ΔCt between samples was calculated for each tested gene. Since the PCR products doubled with each amplification cycle, the fold difference in the initial concentration of each transcript equals $2^{\Delta Ct}$.

**Construction of *acrD*, *mdtABC*, *acrAB*, and *tolC* deletion mutants.** *acrD* and *mdtABC* deletion mutants of *E. coli* KAM3 were constructed by the gene replacement method as previously described, using plasmids pKO3ΔacrD and pKO3ΔmdtABC (9). *acrAB* and *tolC* deletion mutants of *E. coli* TG1 were constructed by the same method, using pKO3ΔacrAB and pKO3ΔtolC.

## RESULTS

**Identification of the response regulators conferring drug resistance.** The 32 ORFs of putative response regulators were cloned under control of the *trc* promoter. Strain KAM3 (12), a derivative of K-12 that lacks a restriction system and *acrB*, was used as the host cell. The expression of the response regulators was induced with IPTG at concentrations of 0.01, 0.1, and 1 mM.

As shown in Table 1, 15 response regulator genes conferred drug resistance to various degrees, indicating that response regulator-mediated drug resistance has great potential for bacterial drug resistance. Among them, the *evgA* gene conferred the most significant resistance to wide range of toxic compounds, such as erythromycin, doxorubicin, novobiocin, crystal violet, rhodamine 6G, TPP, benzalkonium, SDS, and deoxycholate. The *baeR* gene conferred significant novobiocin and deoxycholate resistance and low-level SDS resistance. It should be noted that eight different regulator genes (*evgA*, *baeR*, *cpxR*, *dcuR*, *ompR*, *rcsB*, *narP*, and *yehT*) conferred deoxycholate resistance.

The *cpxR* and *dcuR* genes (the *yjdG* gene was redesignated *dcuR* [5]) conferred high deoxycholate resistance and moderate novobiocin resistance. *cpxR* also conferred moderate kanamycin and amikacin resistance. In the case of the *dcuR* gene, low-level erythromycin, crystal violet, and SDS resistances were additionally observed (Table 1). In contrast to the cases for *evgA* and *baeR*, *cpxR* and *dcuR* showed the maximum resistance with a high IPTG concentration.

The *ompR* and *rcsB* genes conferred high deoxycholate resistance and low-level fosfomycin resistance. In addition, *ompR* also conferred low-level SDS resistance, and *rcsB* conferred low-level kanamycin and methyl viologen resistance. The *ompR* gene conferred the maximum resistance with a high IPTG concentration, while *rcsB* showed maximum resistance with a low IPTG concentration (Table 1).

The *narP* and *yehT* genes conferred moderate deoxycholate and crystal violet resistance. *narP* also conferred low-level methyl viologen and SDS resistance, and *yehT* conferred low-level fosfomycin resistance.

The eight regulator genes described above are all clearly related to deoxycholate resistance to various degrees; however, the case of *fimZ* is complicated. The growth of cells carrying the multicopy *fimZ* gene was inhibited with 1,250 μg of deoxycholate per ml (host level), whereas growth was again observed with more than 10,000 μg/ml. This phenomenon suggests the

TABLE 1—*Continued*

| MIC (μg/ml) for strain carrying gene, with indicated IPTG concn (mM)[a] | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *ompR* | | | *rcsB* | | | *narP* | | | *yehT* | | | *fimZ* (0.01, 0.1, 1) |
| 0.01 | 0.1 | 1 | 0.01 | 0.1 | 1 | 0.01 | 0.1 | 1 | 0.01 | 0.1 | 1 | |
| 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 |
| 3.13 | 3.13 | 3.13 | **6.25** | **6.25** | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | **12.5** |
| 1.56 | **1.56** | **3.13** | 1.56 | **1.56** | 0.78 | 1.56 | 0.78 | 0.78 | 1.56 | **1.56** | **3.13** | 1.56 |
| 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 |
| 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 |
| 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | **1.56** | **1.56** | **1.56** | 0.78 | 0.78 | **1.56** | 0.78 |
| 25 | 25 | 25 | 25 | 25 | 12.5 | 25 | 25 | 25 | 25 | 25 | 25 | 25 |
| 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 |
| 100 | 100 | 100 | **200** | **200** | 100 | **200** | 100 | 100 | 100 | 100 | 100 | 100 |
| 6.25 | 6.25 | 6.25 | 6.25 | 3.13 | 3.13 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 |
| 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 |
| 50 | **100** | **100** | 50 | 50 | 50 | **100** | **100** | 50 | 50 | 50 | 50 | 50 |
| 1,250 | **10,000** | **40,000** | **20,000** | 1,250 | 1,250 | **5,000** | **5,000** | **5,000** | **2,500** | **5,000** | **5,000** | **1.250**[c] |

presence of some unknown *fimZ*-dependent deoxycholate adaptation mechanism. In addition, *fimZ* conferred moderate (fourfold) resistance to kanamycin.

The six other regulator genes conferred some drug resistance without deoxycholate resistance. The *kdpE* gene conferred resistance to kanamycin (fourfold) and methyl viologen (twofold). The *narL* gene conferred resistance to kanamycin (twofold), doxorubicin (twofold), novobiocin (twofold), and benzalkonium (twofold). Although the resistance spectrum of *narL* was broad, the individual levels were low. The *yedW* gene conferred kanamycin-specific low-level resistance (twofold). The resistance levels conferred by *kdpE*, *narL*, and *yedW* increased with increasing concentration of IPTG.

The *citB* and *torR* genes conferred fosfomycin-specific resistance with a low concentration of IPTG. The *rstA* genes conferred resistance to crystal violet (twofold) and fosfomycin (twofold) with high (1 mM) and intermediate (0.1 mM) concentrations of IPTG, respectively. It is not clear why *rstA* could not simultaneously confer crystal violet and fosfomycin resistance.

The other 17 regulator genes (*arcA*, *atoC*, *b2381*, *basR*, *cheB*, *cheY*, *creB*, *glnG*, *hydG*, *phoB*, *phoP*, *rssB*, *uhpA*, *uvrY*, *yfhA*, *ygiX*, and *ylcA*) conferred no drug resistance irrespective of the IPTG concentration.

In our previous study, we found that EvgA and BaeR up-regulate the expression of drug exporter genes such as *emrKY*, *yhiUV*, and *mdtABC* (13, 17, 18). Therefore, we next analyzed the relationship between overexpression of response regulators and up-regulation of expression of the drug exporter genes.

**Determination of mRNA levels of drug exporters by quantitative real-time PCR.** In our previous study, 37 putative drug exporter genes were found in the course of sequence annotation. We found that 20 intrinsic putative drug exporter genes actually conferred drug resistance when they were expressed from multicopy plasmids (16). In this study, we investigated the regulator gene-dependent changes in the amounts of the mRNAs of all of these drug exporter genes by quantitative real-time reverse transcription-PCR. The IPTG concentration that gave the maximum MIC was chosen. The results are shown in Table 2. Out of the 32 response regulator genes, only five (*evgA*, *baeR*, *cpxR*, *ompR*, and *rcsB*) caused significant increases (more than fourfold in comparison with the basal levels) in the mRNA levels of some drug exporter genes. None of the other 27 regulator genes affected the mRNA levels of drug exporter genes.

Sixty-, 7.6-fold, and 4.1-fold increases in the *yhiU*, *emrK*, and *bcr* mRNA levels, respectively, were observed upon *evgA* amplification (Table 2). In the case of *baeR*, a 530-fold increase in the *mdtA* mRNA level was observed. In addition, we newly found that BaeR also controls the *acrD* mRNA level (15-fold). The *cpxR* and *rcsB* genes modified the *acrD* (4.1-fold) and *macA* (4.6-fold) mRNA levels, respectively. On the other hand, the *ompR* gene regulated numerous exporter genes. The genes significantly (more than 4-fold) up-regulated by *ompR* were *acrD* (10-fold), *emrE* (6.7-fold), *emrA* (6.5-fold), and *bcr* (4.3-fold), while the expression levels of a number of other genes tended to increase by a factor of three or twofold.

No other response regulators conferring drug resistance significantly affected the expression of drug exporter genes.

**Contributions of AcrD and MdtABC to multidrug resistance mediated by overexpression of BaeR, CpxR, and OmpR.** First, we investigated whether overexpression of *acrD* confers drug resistance in KAM3 cells (Table 3). Elkins and Nikaido reported that the AcrD system depends on the membrane fusion protein AcrA (4). KAM3 cells harboring plasmid pQE70BH carrying *acrB* showed the same drug resistance levels as TG1 cells (Table 3). When *acrD* was expressed from a multicopy plasmid in KAM3 cells, it conferred resistance to deoxycholate, SDS, and novobiocin. However an *acrAB* deletion mutant of TG1 showed no increase in resistance even when *acrD* was overexpressed (Table 3). In addition, a *tolC* deletion mutant also showed no resistance increase when *acrD* was overexpressed. Thus, it was confirmed that AcrD functions in cooperation with AcrA and TolC. It is clear that as a result of AcrB and AcrD overexpression, KAM3 cells retain intact AcrA protein.

In order to assess whether AcrD and MdtABC contribute to *baeR*-, *ompR*-, and *cpxR*-mediated multidrug resistance, each of the drug exporter genes was deleted from the chromosome of *E. coli* KAM3 (Table 4). The *acrD* deletion strain itself exhibited no alteration in drug susceptibility compared to the parental strain KAM3, probably because *acrD* is not expressed

TABLE 1—*Continued*

| MIC (μg/ml) for strain carrying gene, with indicated IPTG concn (mM)[a] | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *kdpE* | | | *narL* | | *yedW* | | *citB* | | *torR* | | | *rstA* | | |
| 0.01 | 0.1 | 1 | 0.01, 0.1 | 1 | 0.01 | 0.1, 1 | 0.01 | 0.1, 1 | 0.01 | 0.1 | 1 | 0.01 | 0.1 | 1 |
| 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 |
| 3.13 | **6.25** | **12.5** | 3.13 | **6.25** | 3.13 | **6.25** | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 |
| 1.56 | 0.78 | 0.78 | 1.56 | 0.78 | 1.56 | 0.78 | **6.25** | 0.78 | **6.25** | **3.13** | 0.78 | 1.56 | **1.56** | 0.78 |
| 3.13 | 3.13 | 3.13 | 3.13 | **6.25** | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 1.56 | 3.13 | 3.13 | 3.13 | 3.13 |
| 0.78 | 0.78 | 0.78 | 0.78 | **1.56** | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 |
| 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | **1.56** |
| 25 | 12.5 | 25 | 25 | 25 | 25 | 25 | 12.5 | 12.5 | 25 | 12.5 | 12.5 | 25 | 25 | 25 |
| 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 |
| 100 | **200** | **200** | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | 3.13 | 3.13 | 6.25 | 3.13 | 6.25 | 6.25 | 6.25 | 6.25 |
| 3.13 | 3.13 | 3.13 | 3.13 | **6.25** | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 |
| 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| 1,250 | 625 | 625 | 1,250 | 1,250 | 1,250 | 1,250 | 1,250 | 1,250 | 1,250 | 1,250 | 1,250 | 1,250 | 1,250 | 1,250 |

under normal conditions. The *mdtABC* deletion strain itself also exhibited no alteration in drug susceptibility compared to the parental strain KAM3 except for a slight increase in deoxycholate resistance. In both the *acrD* and *mdtABC* deletion strains, *baeR*-mediated deoxycholate and SDS resistance was not affected; *baeR*-mediated novobiocin resistance was not affected in the *acrD* deletion strain but was significantly decreased in the *mdtABC* deletion strain. In the *acrD mdtABC* double deletion strain, *baeR* overexpression did not cause any drug resistance (Table 4). Thus, both the AcrD and MdtABC multidrug exporters contribute to *baeR*-mediated multidrug resistance. In the *acrD* deletion strain, *cpxR* overexpression did not cause significant deoxycholate resistance, although the moderate novobiocin and kanamycin resistance was not affected. *cpxR*-mediated novobiocin and kanamycin resistance may be due to other drug resistance determinants. The level of *ompR*-mediated deoxycholate resistance was not changed even when *acrD* was deleted.

## DISCUSSION

In this study, we found that the 15 out of 32 putative response regulator genes of two-component signal transduction systems in *E. coli* conferred drug resistance when they were overexpressed in an *acrB*-free background. Among these regulator genes, five genes (*evgA*, *baeR*, *cpxR*, *ompR*, and *rcsB*),

TABLE 2. Induction of transcripts attributed to response regulator gene amplification, as determined by amplification of cDNA samples

| Gene[a] | Induction (fold)[b] by: | | | | |
|---|---|---|---|---|---|
| | *baeR* | *cpxR* | *evgA* | *ompR* | *rcsB* |
| *acrD* | 15 | 4.1 | 1.7 | 10 | 2.0 |
| *acrE* | 0.3 | 1.4 | 0.5 | 3.3 | 0.6 |
| *bcr* | 1.3 | 1.6 | 4.1 | 4.3 | 2.9 |
| *cusB* | 0.4 | 1.1 | 0.7 | 3.3 | 1.3 |
| *emrA* | 1.0 | 2.5 | 2.0 | 6.5 | 2.5 |
| *emrD* | 0.7 | 0.9 | 1.1 | 2.6 | 1.5 |
| *emrE* | 0.5 | 1.1 | 1.1 | 6.7 | 1.0 |
| *emrK* | 0.4 | 1.6 | 7.6 | 2.5 | 1.2 |
| *fsr* | 0.3 | 1.0 | 1.3 | 2.5 | 0.8 |
| *mdfA* | 0.9 | 2.0 | 1.4 | 3.1 | 2.5 |
| *macA* | 0.7 | 2.4 | 2.0 | 3.1 | 4.6 |
| *yceE* | 0.3 | 2.1 | 0.9 | 3.3 | 0.9 |
| *yceL* | 0.3 | 1.4 | 0.5 | 1.5 | 0.5 |
| *ydgF* | 0.7 | 1.4 | 3.3 | 1.0 | 2.2 |
| *ydhE* | 1.1 | 1.2 | 1.3 | 3.2 | 1.3 |
| *mdtA* | 530 | 3.4 | 2.5 | 1.4 | 3.3 |
| *yhiU* | 0.2 | 0.8 | 60 | 1.5 | 0.9 |
| *yidY* | 0.2 | 1.5 | 0.5 | 3.0 | 0.5 |
| *yjiO* | 0.2 | 1.3 | 0.3 | 2.4 | 0.7 |
| *acrA* | 0.4 | 0.8 | 0.4 | 0.6 | 0.3 |
| *acrB* | 0.5 | 0.7 | 0.5 | 0.5 | 0.2 |

[a] The amounts of mRNAs were measured in KAM3 cells as the host, except for *acrA* and *acrB* mRNAs, which were measured in TG1 cells. Five regulator genes which up-regulated drug transporter genes at least fourfold are shown.
[b] Values correspond to $2^{\Delta Ct}$, as described in Materials and Methods.

TABLE 3. Drug resistance of *E. coli* cells harboring plasmids carrying *acrB* or *acrD* in *acrAB*, *acrB*, or *tolC* deletion mutant

| Drug | MIC (μg/ml) for strain with indicated transporter overexpression[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | KAM3 (*acrA*$^+$ *acrB tolC*$^+$) | | | TG1 (*acrA*$^+$ *acrB*$^+$ *tolC*$^+$) (None) | TG1ΔacrAB (*acrA acrB tolC*$^+$) | | | TG1 ΔtolC (*acrA*$^+$ *acrB*$^+$ *tolC*) | |
| | None | AcrB[b] | AcrD[c] | | None | AcrB | AcrD | None | AcrD |
| Acriflavine | 12.5 | **100** | ND[d] | 400 | 12.5 | 12.5 | ND | ND | ND |
| Crystal violet | 0.78 | **25** | ND | 25 | 0.78 | 0.78 | ND | ND | ND |
| Enoxacin | 0.05 | **0.20** | ND | 0.20 | 0.05 | 0.05 | ND | ND | ND |
| Erythromycin | 3.13 | **50** | ND | 50 | 3.13 | 3.13 | ND | ND | ND |
| Ethidium bromide | 12.5 | **>400** | ND | >400 | 12.5 | 12.5 | ND | ND | ND |
| Minocycline | 6.25 | **12.5** | ND | 12.5 | 6.25 | 6.25 | ND | ND | ND |
| Nalidixic acid | 0.39 | **3.13** | ND | 3.13 | 0.39 | 0.39 | ND | ND | ND |
| Norfloxacin | 0.05 | **0.10** | ND | 0.10 | 0.05 | 0.05 | ND | ND | ND |
| Novobiocin | 1.56 | **100** | **3.13** | 100 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 |
| SDS | 50 | **>400** | **400** | >400 | 50 | 50 | 50 | 25 | 25 |
| Tetracycline | 1.56 | **3.13** | ND | 3.13 | 1.56 | 1.56 | ND | ND | ND |
| TPP | 6.25 | **>400** | ND | >400 | 6.25 | 6.25 | ND | ND | ND |
| Deoxycholate | 1,250 | **>40,000** | **20,000** | >40,000 | 1,250 | 1,250 | 1,250 | 78 | 78 |
| Benzalkonium | 3.13 | **50** | ND | 50 | 3.13 | 3.13 | ND | ND | ND |
| Rhodamine 6G | 6.25 | **>400** | ND | >400 | 6.25 | 6.25 | ND | ND | ND |

[a] Values in boldface are larger than the control strain values.
[b] AcrB His tagged at the C terminus was overexpressed under control of the T5 promotor in the pQE70 vector.
[c] AcrD His tagged at the C terminus was overexpressed under control of the *trc* promotor in the pTrc6His vector (16).
[d] ND, not determined.

significantly up-regulated the expression of several drug exporter genes. None of them up-regulated the expression of *acrAB*. Although this study is based on the artificial overproduction of response regulators, such a system is thought to often mimic the physiological phosphorylation response (1, 3).

We determined that the resistance mediated by *baeR* was due to two kinds of multidrug exporters, AcrD and MdtABC, although Baranova and Nikaido reported that *baeR* overexpression conferred no drug resistance in an *mdtABC* deletion strain, AG100AΔ*yegMNOB*::*cat*. In their study, AcrD might not function because the AG100A strain has lost the *acrA* gene (2).

With regard to *ompR*, the expression of many drug exporter genes was up-regulated. However, deletion of the *acrD* gene, which is the gene most highly controlled by OmpR among these exporter genes, did not affect the *ompR*-mediated drug resistance. Thus, the *ompR*-mediated drug resistance might be due to drug resistance determinants other than drug exporters.

In any case, the drug resistance mediated by *evgA*, *baeR*, and *cpxR* was due to the up-regulation of exporter gene expression. In contrast, *rcsB*-mediated deoxycholate resistance was not assigned to the *macAB* gene stimulated by *rcsB*. MacAB confers only macrolide-specific resistance (8).

None of the other 10 drug resistance-related regulator genes significantly affected exporter gene expression. These regulator genes, including *rcsB*, confer drug resistance via stimulation of drug resistance determinants other than exporters.

In this study, we revealed that expression of the *acrD* gene was controlled by numerous regulator genes, *baeR*, *cpxR*, and *ompR*, indicating that the AcrD system may be important for two-component system-mediated bacterial environmental adaptation. Our results indicate that response regulator overproduction is a possible mechanism for novel multidrug resistance of pathogenic bacteria.

TABLE 4. Drug resistance of *E. coli* cells harboring pTrc plasmids carrying *baeR*, *cpxR*, or *ompR*

| Strain | Genotype | | Overexpressed regulator | MIC (μg/ml)[a] | | | |
|---|---|---|---|---|---|---|---|
| | *acrD* | *mdtABC* | | Deoxycholate | SDS | Novobiocin | Kanamycin |
| KAM3 | + | + | | 1,250 | 50 | 0.78 | 3.13 |
| KAM3ΔacrD | − | + | | 1,250 | 50 | 0.78 | 3.13 |
| KAM3ΔmdtABC | + | − | | 5,000 | 100 | 0.78 | 3.13 |
| KAM3ΔacrDΔmdtABC | − | − | | 1,250 | 50 | 0.39 | 3.13 |
| KAM3/baeR | + | + | BaeR | **>40,000** | **200** | **25** | 3.13 |
| KAM3ΔacrD/baeR | − | + | BaeR | **>40,000** | **200** | **25** | 3.13 |
| KAM3ΔmdtABC/baeR | + | − | BaeR | **>40,000** | **200** | **6.25** | 3.13 |
| KAM3ΔacrDΔmdtABC/baeR | − | − | BaeR | 1,250 | 50 | 0.39 | 3.13 |
| KAM3/cpxR | + | + | CpxR | **>40,000** | 50 | **3.13** | **12.5** |
| KAM3ΔacrD/cpxR | − | + | CpxR | 1,250 | 50 | **3.13** | **12.5** |
| KAM3/ompR | + | + | OmpR | **40,000** | **100** | 0.78 | 3.13 |
| KAM3ΔacrD/ompR | − | + | OmpR | **40,000** | 100 | 0.78 | 3.13 |

[a] Values in boldface are larger than the control strain values.

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