Analysis of a Complete Library of Putative Drug Transporter Genes in *Escherichia coli*

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The complete sequencing of bacterial genomes has revealed a large number of drug transporter genes. In *Escherichia coli*, there are 37 open reading frames (ORFs) assumed to be drug transporter genes on the basis of sequence similarities, although the transport capabilities of most of them have not been established yet. We cloned all 37 putative drug transporter genes in *E. coli* and investigated their drug resistance phenotypes using an *E. coli* drug-sensitive mutant as a host. *E. coli* cells transformed with a plasmid carrying one of 20 ORFs, i.e., *fsr*, *mdfA*, *yceE*, *yceL*, *ber*, *emrKY*, *emrAB*, *emrD*, *ydiY*, *yjiO*, *ydhE*, *acrAB*, *cuaA* (formerly *ybdE*), *yegMNO*, *acrD*, *acrEF*, *yhiUV*, *emrE*, *ydgFE*, and *yhiYZ*, exhibited increased resistance to some of the 26 representative antimicrobial agents and chemical compounds tested in this study. Of these 20 ORFs, *cuaA*, *yegMNO*, *ydgFE*, *yceE*, *yceL*, *ydiY*, and *yhiYZ* are novel drug resistance genes. Three ORFs, *fsr*, *acrB*, and *yhiUV* genes gave broader resistance spectra than previously reported.

Bacterial species that have developed clinical resistance to antimicrobial agents are increasing in numbers, and the mechanisms underlying their resistance are being studied. The active efflux of antibiotics is mediated by a family of transmembrane proteins frequently referred to as drug resistance translocases (3, 31, 39, 40, 53). The first drug-pumping protein to be reported was the plasmid-encoded tetracycline resistance Tet protein in 1980 (24).

Five families of drug extrusion translocases are currently identified based on sequence similarity (3, 31, 42, 53). These are the MF (major facilitator) family, SMR (small multidrug resistance) family, RND (resistance nodulation cell division) family, ABC (ATP-binding cassette) family, and recently identified MATE (multidrug and toxic compound extrusion) family (4). Membrane transporters of the MF family possess 12 to 14 transmembrane domains. For example, Bcr (*Escherichia coli*) (1), EmrB (*E. coli*) (19), EmrD (*E. coli*) (29), MdfA/Cmr (*E. coli*) (6, 32), NorA (*Staphylococcus aureus*) (58), QacA (*S. aureus*) (45), and Bmr (*Bacillus subtilis*) (30) are members of this family, and these systems mediate drug extrusion with different specificities. Transporters of the SMR family are rather small and usually possess four transmembrane domains. SMR or QacC (*S. aureus*) (11, 18), QacE (*Klebsiella aerogenes*) (35), and EmrE (*E. coli*) (57) belong to this family. EmrE seems to be organized as a homooligomer, most likely a trimer (28). Some proteins in this family appear to function as heterooligomers (14, 23). Transporters of the RND family are usually components of tripartite efflux systems facilitating extrusion of substrates directly into the external medium rather than into the periplasm. Besides the inner-membrane RND transporter, such systems contain a membrane fusion protein (MFP) and an outer membrane factor (OMF). AcrA-AcrB-ToIC (*E. coli*) (21) and MexA-MexB-OprM (*Pseudomonas aeruginosa*) (41) are examples of tripartite efflux systems, where AcrB and MexB are RND transporters, AcrA and MexA are MFPs, and ToIC and OprM are OMFs. An electrochemical potential gradient of H⁺ across cell membranes seems to be the driving force for drug efflux by the MF, SMR, and RND family transporters (9, 59). Transporters of the ABC family utilize ATP as an energy source. LmrA (*Lactococcus lactis*) (54) and MsrA (*S. aureus*) (44) are members of this family. Transporters of the MATE family have 12 predicted transmembrane segments, like members of the MF family. However, the MATE family transporters do not exhibit significant sequence similarity to any member of the MF family (4). NorM (*Vibrio parahaemolyticus*) (26) is a member of this family. NorM seems to be an Na⁺-driven multidrug efflux pump (25).

The last 5 years have seen impressive progress in the sequencing of the entire genomes of both prokaryotic and eukaryotic free-living organisms. As of June 2001, the complete genome sequences of 46 microbial species were publicly available (TIGR microbial database, http://www.tigr.org/tdb/mdbcomplete.html). The complete genome sequence of *E. coli* was determined by Blattner et al. in 1997 (2). Surprisingly, analysis of microbial genomes has revealed a large number of putative drug transporter genes (36–38, 48). In *E. coli*, in the five families, 37 putative drug transporter genes (19 MF, 3 SMR, 7 RND, 7 ABC, and 1 MATE) were found in the course of sequence annotation. The transport systems of these genes were classified according to the putative membrane topology, protein family, bioenergetics, and substrate specificity (38). However, the transport capability of the majority of them has not been established yet.

To investigate the power of prediction and to investigate the substrate specificity of potential drug transporters, first we cloned all 37 putative drug transporter genes of *E. coli* with native promoters and proximate genes into multicopy plasmids and then investigated their drug resistance phenotypes using an *E. coli* mutant lacking the major multidrug efflux system.
AcrAB as a host. We examined the susceptibility of *E. coli* cells harboring a multicopy plasmid carrying a putative drug transporter gene to 26 representative antimicrobial agents and chemical compounds well translocated by AcrAB or other major drug transporters. We found that 16 of 33 plasmids constructed conferred drug resistance on *E. coli* cells. The other 17 of the 33 multicopy plasmids carrying drug transporter open reading frames (ORFs) did not produce increased resistance to any compounds tested in this study. There is a possibility that these ORFs might not be expressed from their own native promoters or that their expression might be repressed by other ORFs that were cloned simultaneously. For this reason, we cloned these ORFs into an expression vector and then investigated their drug resistance phenotypes with induction by IPTG (isopropyl-β-D-thiogalactopyranoside). As a result, we identified several new drug resistance genes by using information on the complete genome sequence, and we showed that the substrate spectra of some previously identified transporters were more extensive than originally thought.

**MATERIALS AND METHODS**

**Bacteria and growth.** *E. coli* W3104 (56) was used as the donor of chromosomal DNA. *E. coli* TG1 (50) was used as the cloning host. *E. coli* KAM3 (26), a derivative of K-12 that lacks a restriction system and AcrAB, was used for drug susceptibility testing and expression confirmation. *E. coli* cells were grown in 2% YT medium (46), supplemented with ampicillin (100 μg/ml) when necessary, under aerobic conditions at 37°C. Competent cells were prepared by the method of Hanahan (12).

**Drug susceptibility test.** The MICs of drugs were determined on YT agar containing various drugs (chloramphenicol, tetracycline, minocycline, erythromycin, nalidixic acid, norfloxacin, enrofloxacin, kanamycin, vancomycin, fosfomycin, fosmidomycin, bicyclomycin, doxorubicin, rifampin, trimethoprim, acriflavine, crystal violet, ethidium bromide, rhodamine 6G, methylviologen, tetraphenylphosphonium bromide [TPP], carbonyl cyanide-3-chloro-7-oxobenzo-2-oxa-1,3-diazole [CCCP], benzalkonium, sodium dodecyl sulfate [SDS], and deoxycholate) at various concentrations, as indicated. These agar plates were made by the twofold agar dilution technique (33). We added 0.1 mM IPTG to agar plates when we examined the susceptibility of *E. coli* cells harboring pTrcHis plasmids that carry drug transporter ORFs under control of the trc promoter (see Tables 1 and 3). A total of 10° cells on a test agar plate were incubated at 37°C for 24 h, and then growth was evaluated.

**Construction of multicopy plasmid library containing putative drug transporter ORFs.** ORFs assumed to be drug transporter genes were cloned from *E. coli* chromosomal DNA of W3104 as follows. Chromosomal DNA from *E. coli* W3104 was isolated as described (46). ORFs were amplified with native promoters and peripheral genes by means of PCR using primers containing the restriction enzyme site that exists in the multicloning sites of the pTrc6His vector, and then the DNA fragments were ligated into the vector to produce an expression plasmid library (Table 1). Competent KAM3 cells were transformed with at least five of the constructed plasmids that were extracted from independent colonies, and then the susceptibilities of all transformants to various drugs were measured with induction by 0.1 mM IPTG, and protein expression was detected with antipolyhistidine antibodies.

**RESULTS AND DISCUSSION**

**Cloning and analysis of MF- and MATE-type drug transporter ORFs.** In the MF family, there are 19 ORFs on the chromosomal DNA of *E. coli* that can be assumed to be drug transporters on the basis of sequence similarities (Table 1). Among these transporters, EmrB, EmrY, YebQ, and YegB possess 14 hydrophobic regions, and Fsr, MdfA (Cmr), YdeA, Bcr, EmrD, YceE, YdeF, YdhC, YidY, YieO, YajR, YceL, YnfM, and YdiM possess 12 hydrophobic regions, which may be transmembrane domains, as judged from hydrophy analysis according to Eisenberg et al. (7) (data not shown). All of these transporters possessing 14 hydrophobic regions have the glycine-rich motif (motif C) in transmembrane segment V that is conserved in the MF-type drug transporters (13, 42). Transporters possessing 12 hydrophobic regions have the GXXXY(R/K)XGR(R/K) motif (motif A) in the putative cytoplasmic loop between transmembrane segments II and III that is conserved in the MF-type drug transporters (42, 55). Therefore, it is likely that these transporters are members of the MF family. In the MATE family, only *ydiE* had been identified as a multidrug resistance (MDR) gene (26).

In these two families, EmrA/B (19), Fsr (8), MdfA (Cmr) (6, 32), Bcr (1), EmrD (29), *yjiO* (6), and *ydhE* (26) had been identified as drug exporters, and *ydeA* (5) had been reported as an exporter of 1-arabinose and IPTG, although the transport capabilities of EmrY, YebQ, YegB, YceE, YdeF, YdhC, YidY, YieO, YajR, YceL, YnfM, and YdiM have not been established yet. We amplified these 20 ORF clusters with the endogenous promoters and some peripheral ORFs from the chromosomal DNA of *E. coli* W3104 by PCR. The PCR fragments were cloned into multicopy *pUC* vectors (Table 1). The MICs of 24 different compounds for *E. coli* KAM3 cells harboring these plasmids were measured. We used a broad range of toxic compounds, including representative cationic dyes, antimicrobial agents, antiseptics, anticancer drugs, uncouplers, and detergents that are well translocated by AcrAB or other major drug transporters. *E. coli* KAM3, which lacks AcrAB, showed hypersensitivity to these compounds (Table 2). The MICs were measured without IPTG so that the ORFs were expected to be expressed under the control of the endogenous promoters.

In the MF family, nine different plasmids (*pUCemrAB*, *pUCfes*, *pUCmefA*, *pUCbcr*, *pUCemD*, *pUCydeE*, *pUCemKY*, *pUCyegMNOB*, and *pUCyceE*) conferred drug resistance on *E. coli* KAM3 cells (Table 2). *pUCemAB* conferred resistance to deoxycholate (32-fold the wild-type level), CCCP (8-fold), rhodamine 6G (2-fold), methylviologen (2-fold), and SDS (2-fold). Thus, EmrB, which is a putative transmembrane protein, seems to be a multidrug transporter, as reported by Lomovskaya and Lewis (19). *emrA* encodes a protein containing a single hydrophobic domain and a C-terminal hydrophilic...
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<tr>
<th>Plasmid</th>
<th>Relevant characteristic</th>
<th>Origin</th>
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<tbody>
<tr>
<td>pUC118</td>
<td>Vector; Ap&lt;sup&gt;r&lt;/sup&gt;; multiple cloning site in lacZ</td>
<td>Takara Shuzo Co.</td>
</tr>
<tr>
<td>pUC119</td>
<td>Vector; Ap&lt;sup&gt;r&lt;/sup&gt;; multiple cloning site in lacZ</td>
<td>Takara Shuzo Co.</td>
</tr>
<tr>
<td>pTrc99A</td>
<td>Expression vector; Ap&lt;sup&gt;r&lt;/sup&gt;; multiple cloning site downstream of trc promoter</td>
<td>Amersham Pharmacia Biotech</td>
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Plasmids carrying MF-type transporter ORFs

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<td>pUCyajR</td>
<td>2.0-kb <em>KpnI</em>-HindIII fragment containing <em>yajR</em> (putative transporter) gene cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<td>pUCfsr</td>
<td>1.8-kb <em>EcoRI</em>-SphI fragment containing <em>fsr</em> (fosmidomycin transporter) gene cloned into pUC118, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>pUCmdfA</td>
<td>1.6-kb <em>KpnI</em>-HindIII fragment containing <em>mdfA</em> (multidrug transporter) gene cloned into pUC118, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pUCycCE</td>
<td>1.4-kb <em>SphI</em>-BanHI fragment containing <em>yeeE</em> (putative transporter) gene cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<td>pUCycEL</td>
<td>1.7-kb <em>SphI</em>-BanHI fragment containing <em>yeeL</em> (putative transporter) gene cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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<tr>
<td>pUCydeA</td>
<td>1.4-kb <em>SphI</em>-BanHI fragment containing <em>ydeA</em> (t-arabinose and IPTG transporter) gene cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pUCydeF</td>
<td>1.7-kb <em>SphI</em>-BanHI fragment containing <em>ydeF</em> (putative transporter) gene cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pUCynfM</td>
<td>2.5-kb <em>SphI</em>-BanHI fragment containing <em>ynfM</em> (putative regulator) and -M (putative transporter) genes cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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<tr>
<td>pUCydhC</td>
<td>2.4-kb <em>SphI</em>-BanHI fragment containing <em>ydhB</em> (putative regulator) and -C (putative transporter) genes cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pUCydiM</td>
<td>1.7-kb <em>SphI</em>-BanHI fragment containing <em>ydiM</em> (putative transporter) gene cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pUCyebQ</td>
<td>2.8-kb <em>BanHI</em>-EcoRI fragment containing <em>yebQ</em> (putative regulator) and <em>yebR</em> (putative transporter) genes cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pUCbcr</td>
<td>2.3-kb <em>Accl</em>-KpnI fragment containing <em>ydeA</em> (putative regulator and <em>bcr</em> (bicyclomycin transporter) genes cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pUCemrKY</td>
<td>7.5-kb <em>SphI</em>-BanHI fragment containing <em>emrK/Y</em> (two-component system)</td>
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<td>pUCemrAB</td>
<td>3.9-kb <em>SphI</em>-BanHI fragment containing <em>emrA</em> (regulator), -A (MFP), and -B (multidrug transporter) genes cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pUCemrD</td>
<td>2.0-kb <em>HindIII</em>-SacI fragment containing <em>emrD</em> (multidrug transporter) gene cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pUCyidY</td>
<td>2.7-kb <em>PstI</em>-BanHI fragment containing <em>yidY</em> (putative transporter) and -Z (putative regulator) genes cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pUCyieO</td>
<td>2.2-kb <em>PstI</em>-BanHI fragment containing <em>yieO</em> (hypothetical protein) and -O (putative transporter) genes cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pUCyjiO</td>
<td>2.8-kb <em>SphI</em>-BanHI fragment containing <em>yjiO</em> (putative transporter) and -N (hypothetical protein) genes cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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Plasmids carrying MATE-type transporter ORFs

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<th>Relevant characteristic</th>
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<td>pUCydhE</td>
<td>1.6-kb <em>PstI</em>-BanHI fragment containing <em>ydhE</em> (multidrug transporter) gene cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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Plasmids carrying RND-type transporter ORFs

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<td>pUCacAB</td>
<td>6.0-kb <em>PstI</em>-Smal fragment containing <em>acrR</em> (regulator), -A (MFP), and -B (multidrug transporter) genes cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pUCcusA</td>
<td>9.0-kb <em>SphI</em>-EcoRI fragment containing <em>cusS</em> (formerly ybcZ; sensor), <em>cusR</em>/C/F/B (ybcA/B/C/D; regulator)/putative OMP/putative binding protein/putative MFP), and <em>cusA</em> (ydeB; putative metal ion or drug transporter) genes cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pUCyegMNQOB</td>
<td>14.2-kb <em>SphI</em>-EcoRI fragment containing <em>yegK/L</em> (putative two-component system), <em>yegM/N/O/B</em> (putative MFP/putative RND transporter/putative RND transporter/putative MF transporter), and <em>bceS/R</em> (putative two-component system) genes cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pUCacD</td>
<td>3.5-kb <em>HindIII</em>-SacI fragment containing <em>acrD</em> (drug transporter) gene cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pUCacrEF</td>
<td>5.9-kb <em>SphI</em>-SalI fragment containing <em>emrR</em> (regulator), <em>acrE/F</em> (MFP/multidrug transporter) genes cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pUCyhiUV</td>
<td>4.7-kb <em>PstI</em>-BanHI fragment containing <em>yhiUV</em> (putative MFP/putative transporter) genes cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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Plasmids carrying SMR-type transporter ORFs

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<td>pUCemrE</td>
<td>1.2-kb <em>SmaI</em>-BanHI fragment containing <em>emrE</em> (drug transporter) gene cloned into pUC118, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pUCydFE</td>
<td>1.1-kb <em>SalI</em>-BanHI fragment containing <em>ydfE</em> (putative transporter) genes cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pUCsugE</td>
<td>1.0-kb <em>PstI</em>-Smal fragment containing <em>sugE</em> (putative transporter) gene cloned into pUC119, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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domain. This gene is needed for emrB-modulated drug resistance (19). The gene far was believed to code for resistance to a single drug, fosmidomycin (8). pUC5r indeed increased resistance to fosmidomycin (32-fold), but also unexpectedly increased slightly (2-fold) the MIC of trimethoprim and CCCP and increased resistance to some uncouplers. This gene also conferred resistance to some toxic compounds, than reported by Morita et al. (26). Although bcr had been reported to codes for resistance to a single drug, bicyclomycin (1), this gene seems to confer a moderate increase in resistance to some other compounds. pUCemrD conferred resistance to SDS (2-fold) and benzalkonium (2-fold). Naroditskaya et al. (29) reported that the emrD gene confers resistance to some uncouplers. This gene also conferred resistance to detergents. pUCydhE conferred increased resistance to TPP (32-fold), deoxycholate (32-fold), norfloxacin (8-fold), enoxacin (8-fold), doxorubicin (8-fold), trimethoprim (4-fold), chloramphenicol (2-fold), fosfomycin (2-fold), ethidium bromide (2-fold), and benzalkonium (2-fold), indicating that this gene confers resistance to a far broader range of compounds, including toxic compounds, than reported by Morita et al. (26). pUCemrKY conferred resistance to doxorubicin (32-fold), rhodamine 6G (16-fold), benzalkonium (8-fold), erythromycin (4-fold), crystal violet (4-fold), TPP (4-fold), deoxycholate (4-
yegMNOB carries both RND- (yegB yegM/N/O) and MF-type (cpUCfsr) conferred fosmidomycin resistance (MIC, 0.39 g/ml). pUCyjiO, pUCcusA, pUCsugE, pUCmdlAB, pUCyddA, pUCyojIH, and pUCyhiH did not alter MIC values.


SDS (100 g/ml), ethidium bromide (25 g/ml), acriflavin (12.5 g/ml), crystal violet (12.5 g/ml), benzalkonium (25 g/ml), and 1,2,500 >40,000 >40,000 >40,000 5,000 5,000

Deoxycholate 1,250 1,250 1,250 1,250 1,250 50 50 50 100 100

SDS 50 50 50 50 50 6.25 6.25 6.25 3.13 3.13


Methylviologen 100 100 100 100 100 3.13 3.13 3.13 3.13 3.13


Crystal violet 1.56 1.56 1.56 1.56 1.56 1.56 1.56 1.56 1.56 1.56

Acriflavin12.5 12.5

Novobiocin 1.56 1.56 1.56 1.56 1.56 1.56 1.56 1.56 1.56 1.56

Doxorubicin 3.13 3.13

Fosfomycin 1.56 1.56 1.56 1.56 1.56

Enoxacin 0.05 0.05 0.05 0.05 0.05

Noroxacin0.025 0.025


Chloramphenicol 0.39 0.39 0.39 0.39 0.39 0.39 0.39 0.39 0.39 0.39

TABLE 2. Drug resistance of E. coli cells harboring plasmids carrying putative drug transporter ORFs.
fold), fosfomycin (2-fold), novobiocin (2-fold), acriflavine (2-fold), ethidium bromide (2-fold), methylviologen (2-fold), and SDS (2-fold). The insert region of this plasmid carries four ORFs (\textit{evgS}, \textit{evgA}, \textit{emrK}, and \textit{emrY}) (Table 1). In our previous study (33), we found that overexpression of \textit{evgA}, which is a response regulator of a two-component system, confers multidrug resistance by stimulating the expression of several MDR transporters. Although the expression of \textit{emrK/Y} is also regulated by \textit{evgA} (15), \textit{emrK/Y} only conferred deoxycholate resistance (33). LB (plasmid) conferred resistance to deoxycholate (32-fold), novobiocin (16-fold), SDS (4-fold), nalidixic acid (2-fold), norfloxacin (2-fold), and fosfomycin (2-fold). This plasmid carries eight ORFs in its insert region (Table 1), and this region contains both MF- (\textit{yegB}) and RND-type (\textit{yegN/O}) transporter ORFs.

We cloned \textit{yegB} into an expression vector (pTrcHyegB) and investigated the resulting drug resistance phenotype. \textit{yegB} expression could be detected (Fig. 1), but this gene did not confer resistance to any of the compounds listed in Table 3. Identification of the drug resistance gene in this locus is discussed later. pUCyegE conferred fosfomycin resistance (4-fold) on \textit{E. coli} (Table 2). We also cloned \textit{yceE} into an expression vector (pTrcHyceE). Expression of \textit{yceE} could be detected (Fig. 1), and pTrcHyceE conferred resistance to fosfomycin (4-fold) and deoxycholate (2-fold) in the presence of IPTG (Table 3). Deoxycholate resistance of pUCyceE might not be detectable because the expression level of \textit{yceE} was lower than that of pTrcHyceE. \textit{yceE} seems to be a novel resistance gene.

\textit{E. coli} cells harboring multicopy plasmids carrying either \textit{yajR}, \textit{yceL}, \textit{ydeA}, \textit{ydeF}, \textit{ynfM}, \textit{ydhC}, \textit{ydiM}, \textit{yebQ}, \textit{yidY}, \textit{yieO}, or \textit{yjiO} did not show any resistance to the compounds tested in this study (Table 2). Since there is a possibility that these ORFs are not expressed from their native promoters, we cloned these ORFs into the pTrc6His expression vector. The expression of all these ORFs except \textit{ynfM} could be detected (Fig. 1). We found again that \textit{yceL}, \textit{yidY}, and \textit{yjiO} conferred drug resistance (Table 3). \textit{yceL} conferred resistance to norfloxacin (2-fold) and enoxacin (2-fold), \textit{yidY} conferred chloramphenicol resistance (2-fold), and \textit{yjiO} conferred resistance to acriflavine (4-fold), chloramphenicol (2-fold), norfloxacin (2-fold), ethidium bromide (2-fold), and TPP (2-fold), indicating that this gene confers resistance to a broader range of compounds than previously reported (6). \textit{ydeA} has been reported to be an L-arabinose and IPTG exporter (5), but this gene did not confer resistance to any of the compounds tested in this study. pUCynfM and pUCyebQ conferred hypersensitivity to acriflavine and trimethoprim, respectively (Table 2). However, when \textit{ynfM} or \textit{yebQ} was expressed alone from an expression vector, these genes did not confer the hypersensitivity phenotype (Table 3). In the case of the pUC plasmid library, these genes were cloned simultaneously with putative regulatory gene \textit{ynfL} or \textit{kdgR}, respectively (Table 1). Thus, it seems that overexpres-

![FIG. 1. Expression of putative drug transporter ORFs. Twenty-eight putative drug transporter ORFs were cloned into pTrc6His expression vectors to produce hexahistidine-tagged proteins under control of the trc (trp/lac hybrid) promoter. E. coli KAM3 cells harboring the constructed plasmids were cultured in 2×YT medium in the presence of 0.1 mM IPTG for induction of protein expression. The cells were harvested and then disrupted by sonication. Total cell proteins were separated on an SDS-polyacrylamide gel, and protein expression was detected by Western blot analysis with an antipolyhistidine antibody.](http://journals.asm.org/doi/abs/10.1128/JB.00985-17?journalCode=jb)
sion of ynjL or kdgR in the induction of acriflavin or trimethoprim would be deleterious to cell growth.

In summary, we found two novel multidrug resistance determinants (yceE and yceL) and one chloramphenicol resistance gene (yidY). Two drug-specific determinants, yfi and ber, were revealed to be multidrug resistance organisms. In addition, emrD, yjiO, and ydhE conferred broader resistance than hitherto believed.

**Cloning and analysis of RND-type drug transporter ORFs.**

In the RND family, there are seven ORFs on the chromosomal DNA of *E. coli* that can be assumed to be drug transporters on the basis of sequence similarities. AcrB, AcrD, AcrF, YhiV, CusA (formerly YbdE), YegN, and YegO possess 12 hydrophobic regions, which may be transmembrane domains, as judged from hydropathy analysis according to Eisenberg et al. (7) (data not shown). AcrD, AcrF, YhiV, CusA, YegN, and YegO exhibit sequence similarity to AcrB at 76, 84, 79, 41, 47, and 48% similarity and 66, 77, 71, 20, 28, and 28% identity, respectively, in the amino acid sequences.

Of these seven ORFs, AcrA/B (21), AcrD (43), and AcrE/F (16, 22; J. Xu, M. L. Nilles, and K. P. Bertrand, Abstr. 93rd Gen. Meet. Am. Soc. Microbiol. 1993, abstr. K-169, p. 290, 1993) had been identified as drug exporters. It was reported that yidUV shows homology to acrAB (20, 22) and yidUV confers octane resistance on *E. coli* (52), but deletion of this gene pair does not increase the susceptibility of *E. coli* (49).

Recently, the yhdE gene was renamed cusA (27) and has been shown to be involved in the efflux of copper (10). The transport capabilities of YegN and YegO have not been reported yet.

We cloned these ORFs with some peripheral ORFs into multicopy plasmids (Table 1) and investigated their drug resistance phenotypes (Table 2). Among them, pUCacrAB, pUCacrD, pUCacrEF, pUCyhiUV, and pUCyegMNOB conferred drug resistance. pUCacrAB conferred increased resistance to doxorubicin (>64-fold), novobiocin (64-fold), rhodamine 6G (64-fold), TPP (64-fold), trimethoprim (>32-fold), ethidium bromide (>32-fold), deoxycholate (>32-fold), novobiocin (8-fold), ethidium bromide (8-fold), norfloxacin (8-fold), enoxacin (8-fold), crystal violet (8-fold), nalidixic acid (8-fold), and rifampin (2-fold). This broad specificity is the same as that reported previously (20, 22, 34, 31). pUCacrD conferred resistance to deoxycholate (>32-fold), SDS (>8-fold), chloramphenicol (8-fold), tetracycline (8-fold), norfloxacin (8-fold), enoxacin (8-fold), crystal violet (8-fold), nalidixic acid (8-fold), and rifampin (2-fold). This broad specificity is the same as that reported previously (20, 22, 34, 31). pUCacrD conferred resistance to deoxycholate (>32-fold), SDS (>8-fold), chloramphenicol (8-fold), tetracycline (8-fold), norfloxacin (8-fold), enoxacin (8-fold), crystal violet (8-fold), nalidixic acid (8-fold), and rifampin (2-fold).

When AcrD was cloned into an expression vector, it slightly increased (2-fold) the MIC of acrD (4-fold), and kanamycin (2-fold). When AcrD was cloned into an expression vector, it slightly increased (2-fold) the MIC of acrD (4-fold), and kanamycin (2-fold). When AcrD was cloned into an expression vector, it slightly increased (2-fold) the MIC of acrD (4-fold), and kanamycin (2-fold). When AcrD was cloned into an expression vector, it slightly increased (2-fold) the MIC of acrD (4-fold), and kanamycin (2-fold). When AcrD was cloned into an expression vector, it slightly increased (2-fold) the MIC of acrD (4-fold), and kanamycin (2-fold). When AcrD was cloned into an expression vector, it slightly increased (2-fold) the MIC of acrD (4-fold), and kanamycin (2-fold).
deoxycholate (>32-fold), novobiocin (32-fold), acriflavine (32-fold), ethidium bromide (32-fold), TPP (32-fold), chloramphenicol (16-fold), minocycline (16-fold), crystal violet (16-fold), benzalkonium (16-fold), SDS (>8-fold), tetracycline (8-fold), enoxacin (8-fold), nalidixic acid (4-fold), norfloxacin (4-fold), and methylobilogen (2-fold) (Table 3) than pUCacrEF. This is because envR acts as a repressor of acrEF. pUCyhiUV conferred resistance to rhodamine 6G (16-fold), erythromycin (8-fold), doxorubicin (8-fold), ethidium bromide (4-fold), TPP (4-fold), SDS (4-fold), deoxycholate (4-fold), crystal violet (2-fold), and benzalkonium (2-fold), indicating that YhiV is a multidrug resistance determinant. YhiU seems to be a membrane fusion protein (MFP) like AcrA. Although pUCcusA did not show any resistance to the compounds tested in this study (Table 2), when cusA (ybDE) was cloned into an expression vector with the putative MFP ORF cusB (ycD) (pTrcHcusAB), this plasmid conferred fosfomycin resistance (2-fold) in the presence of IPTG (Table 3), pUCyegMNOB conferred resistance to deoxycholate (32-fold), novobiocin (16-fold), SDS (4-fold), nalidixic acid (2-fold), norfloxacin (2-fold), and fosfomycin (2-fold) as described above. This plasmid contains eight ORFs, including three putative drug transporter ORFs, yeg-N (RND), -O (RND), and -B (MF) (Table 1). We separately cloned these three transporter ORFs into an expression vector (pTrcHyeg-N, -O, or -B) and then investigated their drug resistance phenotypes in the presence of IPTG (Table 3). The expression of YegN and YegB was detectable, while YegO was not detectable with an antipolyhistidine antibody (Fig. 1), possibly because the C terminus of YegO undergoes processing and the histidine tag may be removed. Out of three expression plasmids, only pTrcHyegO conferred drug resistance to deoxycholate (4-fold), nalidixic acid (2-fold), norfloxacin (2-fold), fosfomycin (2-fold), and SDS (2-fold), but lacked novobiocin resistance. Since YegM exhibits 47% similarity and 28% identity to the AcrA membrane fusion protein and the other ORFs (yegK/L and baeS/R) included in pUCyegMNOB are not transporter ORFs, there is a possibility that a multicomplex(es) formation (for example, YegM/YegO) may act as a transporter. When the yegMNO genes were cloned together into vector pUC, this plasmid conferred a drug resistance phenotype comparable to that of pUCyegMNOB, but the plasmid carrying the yegM/N genes did not confer resistance. (S. Nagakubo, K. Nishino and A. Yamaguchi, unpublished data). Thus, resistance conferred by pUCyegMNO seems to be due to overexpression of MFP and RND genes. Partially, overexpression of yegO is responsible for the drug resistance in this complex.

**Cloning and analysis of SMR-type drug transporter ORFs.**

There are three ORFs that can be assumed to be genes encoding ABC-type drug exporters on the basis of sequence similarities. MdlA, MdlB, YddA, YojI, YojH, and YhiH each lack six hydrophobic putative transmembrane regions, and YbjZ possesses four hydrophobic regions. Among them, YhiH has two nucleotide-binding domains, and the others have a single nucleotide-binding domain. None of these has yet been reported to have transport capability. We cloned all of these ORFs with some peripheral ORFs into pUC vectors (Table 1), and the resulting drug resistance was investigated (Table 2). Only pUCybjYZ conferred increased resistance on E. coli KAM3. This plasmid confers erythromycin-specific resistance (8-fold). However, YbjZ has no sequence homology to MsrA (44), which is an ABC-type macrolide-specific exporter in gram-positive cocci, except for the nucleotide-binding domain. We also cloned seven putative ABC-type ORFs into expression vectors (pTrcHyeg-MdlA, -mdlB, -ybI, -yddA, -yojI, -yojH, and -yhiH). None of them conferred resistance to any of the compounds listed in Table 3, although their expression was detected (Fig. 1). Overexpression of ybI alone also did not confer drug resistance. It seems that ybI and ybjZ make a complex for the drug resistance phenotype, and this gene pair exhibited resistance against macrolides composed of 14- and 15-membered lactones, such as clarithromycin, oleandomycin, and azithromycin, in addition to erythromycin (17a). Thus, ybjYZ is a novel macrolide resistance determinant. This is the first experimentally identified ABC-type drug resistance gene in gram-negative bacteria.

In this study, the 37 putative drug exporter genes in E. coli were all cloned into multicopy vectors and expression vectors, and the resistance phenotypes against 26 structurally different compounds were investigated. As a result, we found that 20 genes (11 MF, 2 SMR, 6 RND, and 1 ABC) conferred drug resistance. Among them, we identified seven novel drug resistance determinants, yceE, yceL, yidY, ydgFE, yegO, cusa (ybDE), and ybjYZ (3 MF, 1 SMR, 2 RND, and 1 ABC), their drug resistance capabilities having not yet been reported. When yegMNO were expressed together, this complex gave broader resistance spectra than yegO alone. The ybjZ gene is the first case of an experimentally identified ABC drug ex-
porter gene in gram-negative bacteria. In addition, the fsr, bcr, yjiO, ydhE, acrD, and yhiUV genes gave broader resistance spectra than previously reported.

The remaining 17 of the 37 putative drug transporter ORFs did not confer resistance to the compounds tested in this study on *E. coli*. The possible reasons are as follows. (i) These ORFs might confer resistance to compounds other than those tested in this study. (ii) These ORFs might need complex formation with other ORFs for drug resistance. (iii) These ORFs might be unrelated to drug resistance. Currently, Sulavik et al. reported antibiotic susceptibility profiles of *E. coli* strains lacking multidrug efflux pump genes (49). They reported that deletion of three known MDR pump genes (*acrAB, mdIA*, and *emtE*) and three OMF genes (*tolC, yjcp, and yohG*) resulted in strains with increased susceptibility to some compounds. However, they reported that deletion of other known and predicted MDR pump genes, including *yhiUV, yegMNO*, and *ybyYZ*, which were identified as drug resistance determinants in our study, did not increase susceptibility to any compounds. Thus, the method of overexpression analysis in this study is useful for identifying drug resistance factors underlying chromosomal DNA. The drug exporter ORF expression library established in this study is a genetic resource for bacterial drug resistance, and we believe that the library should be useful for the future development of chemotherapy.

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