Antibiotic Susceptibility Profiles of *Escherichia coli* Strains Lacking Multidrug Efflux Pump Genes

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The contribution of seven known and nine predicted genes or operons associated with multidrug resistance to the susceptibility of *Escherichia coli* W3110 was assessed for 20 different classes of antimicrobial compounds that include antibiotics, antiseptics, detergents, and dyes. Strains were constructed with deletions for genes in the major facilitator superfamily, the resistance nodulation-cell division family, the small multidrug resistance family, the ATP-binding cassette family, and outer membrane factors. The agar dilution MICs of 35 compounds were determined for strains with deletions for multidrug resistance (MDR) pumps. Deletions in *acrAB* or *tolC* resulted in increased susceptibilities to the majority of compounds tested. The remaining MDR pump gene deletions resulted in increased susceptibilities to far fewer compounds. The results identify which MDR pumps contribute to intrinsic resistance under the conditions tested and supply practical information useful for designing sensitive assay strains for cell-based screening of antibacterial compounds.

Bacterial membrane transport systems function to take up essential nutrients, control cell homeostasis, export proteins, and control efflux of xenobiotic (including antibiotic) compounds (32). There are more than seven efflux systems in *Escherichia coli* that can export structurally unrelated antibiotics; these multidrug resistance efflux pump (MDR pump) systems contribute to intrinsic resistance for toxic compounds such as antibiotics, antiseptics, detergents, and dyes. They are of interest due to their unknown physiological roles (10), possible contribution to clinical resistance (20, 24), possible utility as antibacterial targets (20, 24), and potential value in cell-based screening for novel antibacterials (12).

In *E. coli*, seven different proton-dependent MDR pump systems have been identified in biological studies: AcrAB-TolC (22), EmrAB (21), MdfA (8), TehA (40), EmrE (33), AcrEF (15, 16), and EmrD (28). Others have been identified by comparative amino acid sequence analysis. All fall into three distinct families as compiled by Paulsen et al. (32): the major facilitator superfamily (MFS), the resistance nodulation-cell division (RND) family, and the small multidrug resistance (SMR) family. MFS members include *emrD, mdfA, emrB*, and predicted *emrY*. The RND family is comprised of at least *acrB, acrF*, predicted *yhiV*, and predicted *acrD*. The SMR family includes *emrE* (*mvrC*) and *tehA*, which is reported to have sequence identity to the SMR family (40).

In addition to proton-dependent systems, pumps that are ATP dependent have been identified in bacteria. One is LmrA, an ATP-binding cassette (ABC) MDR pump that has been identified in *Lactococcus lactis* that has homology to the eucaryotic P glycoprotein MDR1 (42). Putative ATP-dependent drug efflux pumps in *E. coli* have been identified by Paulsen et al. (31), including YhiG, MdlB, YbjZ, and MsbA.

Multidrug efflux systems in bacteria can consist of single gene products, such as NorA of *Staphylococcus aureus* or the multicomponent envelope translocases, such as MexA-MexB-OprM of *Pseudomonas aeruginosa* (19), that are found in gram-negative organisms and facilitate drug efflux across the gram-negative outer membrane (32). A well-studied example is the AcrA-AcrB-TolC MDR tripartite pump system of *E. coli* (9). This complex consists of an MDR pump, a membrane fusion protein (MFP), and an outer membrane factor (OMF). The MFP gene is located in an operon immediately upstream of the corresponding MDR pump gene. Examples of these gene pairs in *E. coli* are are *emrAB, acrAB, acrEF*, and the predicted *yhiVU* and *emrKY*. A second component is the outer membrane (OM) protein, such as the only known example in *E. coli*, TolC. It is the required third component for the AcrAB (9) and EmrAB (18) drug efflux systems.

Multidrug efflux pump systems and their substrates have, in part, been identified by experimental systems whereby the pump protein is overexpressed and substrates are identified by increased resistance to a panel of compounds. In a study of *E. coli* strains overexpressing *acrEF* (15), increased resistance was determined for compounds similar to the dyes, detergents, and antibiotic substrates of AcrAB. Strains overexpressing *emrE* (25, 44) identified the substrates methyl viologen, ethidium bromide, erythromycin, sulfadiazine, and tetraphenylphosphonium. Increased *tehAB* expression resulted in increased resistance to tetraphenylarsonium chloride, crystal violet, and proflavin (40). Overexpression of *mdfA* identified ethidium bromide, tetraphenylphosphonium, rhodamine, daunomycin, benzalkonium,

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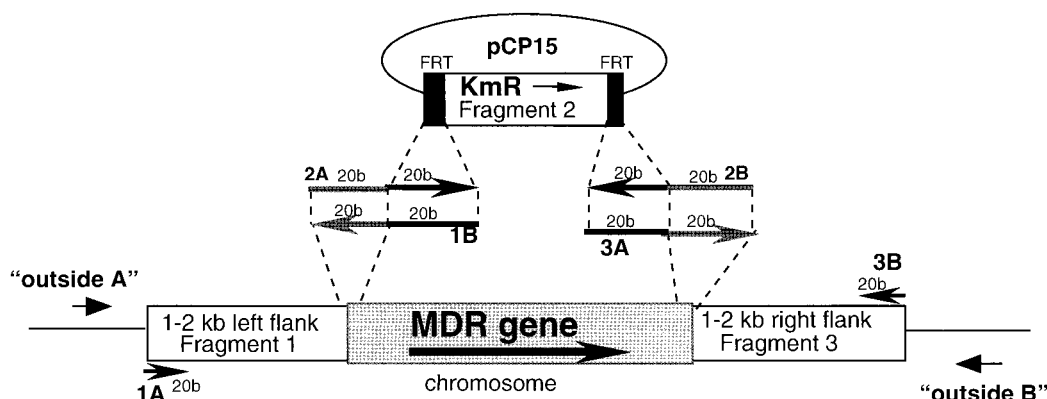‖ Present address: Cubist Pharmaceuticals, Inc., Cambridge, Mass.

FIG. 1. Location of primers for (i) the design of the overlap extension method and (ii) for verification of allelic exchange. Three amplification products were constructed into one product by an overlap extension method for subsequent use in MDR pump gene replacement with the Km^r-*FRT* fragment. Primers 1A and 1B amplify Fragment 1 from the chromosome. Primers 2A and 2B amplify the Km^r-*FRT* fragment from pCP15. Primers 3A and 3B amplify Fragment 3 from the chromosome. Primers "outside A" and "outside B" amplify chromosomal DNA to verify those strains for which the Km^r-*FRT* fragment has replaced the MDR pump gene.

rifampin, tetracycline, puromycin, chloramphenicol, erythromycin, some aminoglycosides, and fluoroquinolones (8).

Deletion of a multidrug pump sometimes results in increased susceptibility to the substrate of the pump. For example, *E. coli* overexpressing AcrAB demonstrates increased resistance to substrates such as bile salts, compared to an isogenic wild-type strain, and similarly, a strain with an *acrAB* deletion is more susceptible than the wild-type strain to the same compounds (23). However, although increased expression of AcrEF results in increased resistance to some AcrAB substrates, deletion of *acrEF* did not contribute to increased susceptibility to those substrates (15, 23). A comparison of the MICs for strains with deletions of different MDR pump genes can reveal the MDR pumps responsible for intrinsic resistance. Such a comparison can be useful to identify (i) potential physiological roles for a pump, (ii) contributors to clinical resistance, (iii) potential antibacterial targets, and (iv) MDR pumps that will result in susceptible strains useful for cell-based screening.

In this study, we determined which MDR pump genes, when deleted, result in strains with increased susceptibility to the approximately 35 compounds that have been previously reported as MDR pump substrates (8, 15, 16, 21, 22, 25, 28, 33, 40, 44). Predicted efflux genes whose substrate profiles are unknown were included in an attempt to functionally identify new MDR pump genes. Deletions of individual MDR pump genes and operons as well as deletions of entire families of MDR pump genes in one strain were studied. Sixteen genes or operons were selected and assessed for their contribution to intrinsic resistance to the compounds. All strains were tested side-by-side by an agar dilution MIC method so that the contribution of each MDR pump to susceptibility for each of the 35 compounds could be assessed. It was found that the major contributors to intrinsic resistance for the compounds tested were AcrAB and TolC.

MATERIALS AND METHODS

Bioinformatics methods. Amino acid sequences of a set of multidrug resistance efflux pumps were identified which included members of the MFS, RND, and SMR families, and homology search was performed using BLASTP (2)

against open reading frames of the *E. coli* MG1655 genome (5). The generated list was analyzed based on a set of criteria that included a probability score of $<10^{-20}$.

Bacterial strains and media. *E. coli* strains W3110 (13), MG1655 (5), JC7623 (4), DH5α (Life Technologies, Bethesda, Md.), and V355 (36) were used. Luria broth (LB), LB agar, and Mueller-Hinton agar were obtained from Difco (Detroit, Mich.). Antibiotics used for strain constructions and selection were obtained from Sigma (St. Louis, Mo.) and were used at the following concentrations in LB or LB agar: kanamycin, 40 μg/ml; ampicillin, 100 μg/ml; and chloramphenicol, 20 μg/ml.

Allelic exchange method for construction of deletion strains. Methods for DNA recombinant techniques were done according to Ausubel et al. (3). DNA amplification was performed using PCR with Elongase Supermix (Life Technologies), according to the instructions of the manufacturer. Gene splicing by the overlap extension method of Horton et al. (11) using PCR was done to create DNA fragments for subsequent steps. Oligonucleotides were purchased from Life Technologies.

For each gene or tandem group of genes chosen for deletion, DNA fragments were produced that consisted of the Tn5 kanamycin resistance determinant (Km^r) flanked by the yeast 2μm Flp-recombination target (*FRT*) sites and further flanked by ca. 2 kb of MG1655 genomic DNA that normally flanks the designated gene to be disrupted. Briefly, the overlap extension method is as follows, and a schematic of primers and fragments generated is shown in Fig. 1. The Km^r-*FRT* DNA (Fig. 1, Fragment 1) was amplified from pCP15 (a kind gift from Wilfried Wackernagel) (7) using primers 2A and 2B (Fig. 1). The two chromosomal fragments 1 and 3 (Fig. 1) were amplified by PCR from MG1655 using primers 1A and 1B and 3A and 3B, respectively. All three DNA amplification products, fragments 1, 2, and 3 (Fig. 1), were purified from bands resolved by agarose gel electrophoresis using the Qiaex gel extraction purification method (Qiagen, Valencia, Calif.) per the instructions of the manufacturer. The three DNA amplification products were combined in an Elongase Supermix PCR mixture containing primers 1A and 3B, and the reaction was performed according to the manufacturer's instructions. Resulting amplified products of predicted sizes were purified, as described above, for subsequent manipulation.

In the second step, the resulting overlap-extension DNA fragments for each gene were ligated to pBR322 (6) using T4 DNA ligase (Life Technologies). An exception was that the *emrAB*- and *acrAB*-related DNA fragments were ligated to pT7Blue (Novagen, Madison, Wis.) instead of pBR322. Ligated products were introduced into *E. coli* DH5α, and the correct plasmid recombinants were determined by restriction-digestion analysis.

The third step involved the introduction of plasmid recombinants into the *recBC sbcBC* strain JC7623 by CaCl₂-mediated transformation with selection for colonies that were resistant to 40 μg of kanamycin/ml. In one case (*emrAB*), the *recD* strain V355 was used with linearized DNA. Both JC7623 and V355 are strains that facilitate allelic exchange by homologous recombination (30, 35) of incoming plasmid and chromosomal DNA, represented by fragments 1 and 3 (Fig. 1). Also, kanamycin-resistant colonies were screened for those that were sensitive to 100 μg of ampicillin (the antibiotic resistance marker for pBR322

and pT7Blue) per ml. Plasmids used for this step were ColE1 plasmids that are unstable in JC7623, and thus this strain facilitates loss of the plasmid backbone following an allelic exchange event (4). The kanamycin-resistant and ampicillin-sensitive colonies represent successful allelic exchange strains that had lost the plasmid DNA and contained the Km^r-FRT DNA, replacing the gene slated for deletion.

In the fourth step, verification of an allelic exchange for each gene was done by PCR (see Table 3). Analysis using oligonucleotides located outside primers 1A and 3B (Fig. 1, outside primers A and B) were used to amplify genomic DNA from wild-type and experimental samples. The calculation for the predicted size (bp) shown below in Table 3 was based on the following equation: predicted size = (distance in MG1655 contig between location of primers 1A and 3B) − (size of deleted gene or distance in MG1655 contig between regions of primers 1B and 3A homologous to MG1655) + (size of the Km^r-FRT fragment, 1,485 bp).

Finally, the Km^r-linked gene deletion strains were transduced to W3110 by P1 transduction using standard procedures (37) to create the group of W3110 isogenic strains listed below in Table 4.

Oligonucleotide sequences for 1A, 1B, 2A, 2B, 3A, and 3B (diagrammed in Fig. 1) used to construct each gene deletion by the overlap extension method are listed in Table 1 according to the respective MDR pump gene. This table also contains the outside A and outside B (Fig. 1) nucleotide sequences used to verify allelic exchanges.

As opposed to the deletion strains described above, the *acrAB* and *emrAB* deletion strains were constructed differently. The *acrAB* and *emrAB* genes with flanking 2 kbp of DNA were amplified by PCR (primers from 5′ to 3′ are GCCCACCAGCAGTAGAGGGGAAGTAACAGA and GCCCACCAGCCTT CAGGACGGCAATCTGCT for *acrAB* and GCCCACCAGCGTGTTAAGAA GCCCATCAGT and GCCCACCAGCTCCGCAATTACTATCGTTTC for *emrAB*). These fragments were cloned into the pT7Blue cloning vector by blunt-end ligation using T4 DNA ligase. Another amplification was done using the recombinant plasmid as template with primers located near and directed away from the start and end of *acrA* or *emrA* (primers from 5′ to 3′ were CTTGTT GGGCCTGTTTGTCG and CTTCGCGCCCGTTACCATGATTTAGCCGCA AGAATGAAGA for *acrAB* and TGCTGGGGCTGGTGTTGGTTT and GGTC CTGACTTTGGTCGCGACCCTATCGCTACGGCAATAA for *emrAB*). This product was blunt-end ligated to the purified Km^r-FRT SmaI/HinDII fragment from pCP15 that was treated with T4 DNA polymerase. These products were introduced into strain DH5α via transformation, and recombinant clones were obtained. The resulting constructs were used for subsequent allelic exchange into *E. coli* JC7623 or V355 as described above. Verification of the allelic exchange was done as described above by PCR amplification using outside primers A and B that were from 5′ to 3′ CGCGGGGTATTCAGAGACTCA and CAGACGCA AATCCGCGATGCG for *acrAB* and CATTCTGGCAAGCGAAGTGCGGTG ATA and CCGCAAATTAAGCAATCAGCAACCGTT for *emrAB*.

Because Km^r-marked deletions were flanked by FRT sites, it was possible to remove the Km^r-FRT DNA directly from the chromosome, leaving a deletion marked by a single FRT site by the method of Cherepanov and Wackernagel (7). Briefly, the pCP20 plasmid (7) that is temperature sensitive for replication and contains the yeast 2μm Flp recombinase was introduced into Km^r-FRT-marked deletion strains. Introduction of the plasmid by transformation was performed using selection for chloramphenicol resistance at 30°C. The plasmid expressing Flp recombinase facilitated the FRT site-specific recombination and subsequent loss of the chromosomal Km^r-FRT cassette. Culturing of the strain at 42°C in the absence of antibiotic selection produced strains cured for pCP20. The resulting strains were chloramphenicol sensitive and kanamycin sensitive. Then another Km^r-marked MDR pump deletion was introduced by P1 transduction, and again the Km^r determinant was removed. Reiteration of this method resulted in multiple unmarked gene deletions in one strain. Verification of the loss of the Km^r marker was done by PCR using outside primers as described above.

Susceptibility testing. Agar dilution antimicrobial susceptibility tests were performed as outlined in the NCCLS standard M7-A4 (29), using Mueller-Hinton agar with the modification that concentrations of the initial compounds (shown below) may differ from those recommended. Experiments were repeated at least twice. The inoculum *E. coli* cultures consisted of 2 × 10^4 bacteria/2-μl spot on Mueller-Hinton agar media containing serial dilutions of the compounds listed below. The plates were incubated for 16 to 20 h at 37°C. The lowest concentration of antibiotic that completely inhibited growth was identified as the MIC. A ≥4-fold difference in susceptibility of the deletion strain versus the isogenic W3110 parent strain was considered significant. The following compound stock solutions were prepared in water unless indicated otherwise, and the compound class is listed in parentheses: clotrimazole (imidazole; Sigma) at 0.5 mg/ml in 50% ethanol; ampicillin (β-lactam; Sigma) at 100 mg/ml; chloramphen-

icol (Sigma) at 25 mg/ml in ethanol; florfenicol (Schering-Plough Research Institute) at 12.5 mg/ml in ethanol; puromycin (lipophilic basic compound; Sigma) at 100 mg/ml; methotrexate (Calbiochem-Novabiochem, La Jolla, Calif.) at 10 mg/ml in alkaline water; erythromycin (macrolide; Sigma) at 100 mg/ml in methanol; novobiocin (Sigma) at 50 mg/ml in 50% methanol; fusidic acid sodium salt (steroidal antibiotic; Aldrich Chemical Co., Inc., Milwaukee, Wis.) at 100 mg/ml in methanol; tetracycline (Sigma) at 10 mg/ml in methanol; ciprofloxacin (fluoroquinolone) at 10 mg/ml; norfloxacin (fluoroquinolone; Sigma) at 10 mg/ml in dimethyl sulfoxide; nalidixic acid sodium salt (quinolone precursor; Aldrich) at 50 mg/ml; rifampin (Sigma) at 20 mg/ml in methanol; streptomycin sulfate (Sigma) at 500 mg/ml; sulfacetamide (Sigma) at 500 mg/ml; sodium dodecyl sulfate (detergent; Sigma) at 200 mg/ml; deoxycholate sodium salt (bile salt; Sigma) at 100 mg/ml; sodium cholate (bile salt; Sigma) at 100 mg/ml; sodium taurodeoxycholate (bile salt; Sigma) at 100 mg/ml; sodium oxalate (dicarboxylic acid; Aldrich) at 25 mg/ml; proflavin (intercalator; Sigma) at 100 mg/ml; crystal violet (intercalator; Aldrich) at 50 mg/ml; acriflavin (intercalator; Sigma) at 200 mg/ml; ethidium bromide (intercalator; Sigma) at 10 mg/ml; cetyltrimethyl-ammonium bromide (quaternary amino compound; Aldrich) at 100 mg/ml; dequalinium chloride (quaternary amino compound; Aldrich) at 25 mg/ml; benzalkonium chloride (quaternary amino compound; Sigma) at 500 mg/ml; tetraphenylphosphonium chloride (lipophilic quaternary amino compound; Aldrich) at 100 mg/ml; tetraphenylarsonium chloride (lipophilic quaternary amino compound; Aldrich) at 100 mg/ml; rhodamine 6G (lipophilic quaternary amino compound; Sigma) at 20 mg/ml; daunomycin (redox cycling drug; Sigma) at 10 mg/ml; plumbagin (redox cycling drug; Sigma) at 20 mg/ml in methanol; methyl viologen (redox cycling drug; Sigma) at 100 mg/ml; and carbonyl cyanide-chlorophenyl hydrazone (CCCP) (uncoupler of proton motive force; Sigma) at 8 mM in 50% methanol–20% dimethyl sulfoxide.

RESULTS

Identification of MDR pump genes. In order to conduct a comprehensive survey on the effect of the loss of MDR pump genes on susceptibility to toxic compounds, genes encoding 25 *E. coli* multidrug efflux pumps, OMFs, and an MFP were deleted (Table 2). These genes include known and predicted MDR genes as well as predicted OMF and MFP elements that were identified by bioinformatics analysis as described in Materials and Methods. Specifically, for the OMF class of genes, OprM—the *P. aeruginosa* OM component of the MexAB multidrug resistance pump (19)—identified YlcB (45% identity and 62% amino acid similarity over 455 residues), YjcP (26% identity and 47% similarity over 466 residues), and YohG (27% identity and 45% similarity over 345 residues). For additional MF family proteins, the *Neisseria gonorrhoeae* MtrC lipoprotein open reading frame (32) was used to identify *yegM* (32% identity and 50% similarity over 378 residues). The gene *yegM* is located immediately upstream, in operon-like form, of two genes that show sequence similarity to the hypothetical RND family efflux pumps identified by Paulsen et al. (31): *yegN* (30% identity and 53% similarity over 1,027 residues with *acrD*) and *yegO* (29% identity and 52% similarity over 1,031 residues with *acrD*). MtrC was homologous to YbjY (28% identity and 45% sequence similarity over 332 residues), which is proximal to *ybjZ*, a gene that encodes a putative ABC MDR pump. YbjZ is a putative ABC drug efflux transporter (31). Although most bacterial MDR pumps identified are proton motive force-dependent efflux genes, the identification of *ybjY* immediately upstream of a putative ABC transporter prompted us to include this putative multidrug resistance-like pump in the study.

Construction of *E. coli* strains with deletions for multidrug efflux genes. Contiguous MDR pump genes oriented in the same direction can constitute a multidrug efflux operon (Table 2, genetic context of MDR pump genes). We chose to delete

TABLE 1. Oligonucleotides used to construct and verify MDR deletion strains

Gene(s)	Name	Oligonucleotide sequence (5′ to 3′)
tolC	1A	AAAAAAAAGCGGCCGCTAGCTGAATATGACCGCACAGCAA
tolC	1B	AACTTCAGAGCGCTTTTGAAGCTTGCTCAGGCCGATAAGAATGG
tolC	2A	CCATTCTTATCGGCCTGAGCAAGCTTCAAAAGCGCTCTGAAGTT
tolC	2B	GTGGTAGTGCGTGCGGATGTCCCGGGGATCTTGAAGTTCCTATT
tolC	3A	AATAGGAACTTCAAGATCCCCGGGACATCCGCACGCACTACCAC
tolC	3B	AAAAAAAAGCGGCCGCGGATCCGTGGATAAAACCGAGACATA
tolC	Outside A	AGCGGTATCAGGTTGGAAAC
tolC	Outside B	TACCTCGACCATGAAGGTGG
yjcP	1A	AAAAAAAAGCGGCCGCGATCGATGCCTGCTGGTGATTCTGAT
yjcP	1B	AACTTCAGAGCGCTTTTGAAGCTTCTTACGTACCAGGGCACAGC
yjcP	2A	GCTGTGCCCTGGTACGTAAGAAGCTTCAAAAGCGCTCTGAAGTT
yjcP	2B	GACGACGGGACCTGCCTGATCCCGGGGATCTTGAAGTTCCTATT
yjcP	3A	AATAGGAACTTCAAGATCCCCGGGATCAGGCAGGTCCCGTCGTC
yjcP	3B	AAAAAAAAGCGGCCGCGTCGACCGAACGGCAAGAAGTAGTGAC
yjcP	Outside A	TACAGGCACCAATCCACCAC
yjcP	Outside B	TCCGGTCAAAATCATGGTCG
yohG	1A	AAAAAAAAGCGGCCGCGATCGATCCTTTAATCCGCTGACCATA
yohG	1B	AACTTCAGAGCGCTTTTGAAGCTTCTCCATATCCGCCGAAAAGT
yohG	2A	ACTTTTCGGCGGATATGGAGAAGCTTCAAAAGCGCTCTGAAGTT
yohG	2B	GGATTCTGGCTTCGCTGACGCCCGGGGATCTTGAAGTTCCTATT
yohG	3A	AATAGGAACTTCAAGATCCCCGGGCGTCAGCGAAGCCAGAATCC
yohG	3B	AAAAAAAAGCGGCCGCGTCGACATAACGCCGTAAAACAAGAC
yohG	Outside A	ACCAAGGTAAAGCAGGTAGC
yohG	Outside B	TCAACACCCCAGATGAGATG
ylcB	1A	AAAAAAAAGCGGCCGCTAGCGTCCAGGTGCCCCAGATGCT
ylcB	1B	AACTTCAGAGCGCTTTTGAAGCTTAATGGCAGAAGTTTACAAGG
ylcB	2A	CCTTGTAAACTTCTGCCATTAAGCTTCAAAAGCGCTCTGAAGTT
ylcB	2B	CAAATAAAGAACGCTCGGCACCCGGGGATCTTGAAGTTCCTATT
ylcB	3A	AATAGGAACTTCAAGATCCCCGGGTGCCGAGCGTTCTTTATTTG
ylcB	3B	AAAAAAAAGCGGCCGCGGATCCTCGCCCGCGACCTGACACTT
ylcB	Outside A	CCGCTACCTTCACCTTTTCG
ylcB	Outside B	CGGTGCCATCTTCGAAAATG
acrEF	1A	AAAAAAAAGCGGCCGCGTCGACCATCAAAAGTCGCATCGTCT
acrEF	1B	AACTTCAGAGCGCTTTTGAAGCTTCAGTTCGCTATCCTACAAAT
acrEF	2A	ATTTGTAGGATAGCGAACTGAAGCTTCAAAAGCGCTCTGAAGTT
acrEF	2B	TACGAAGAAGATTGCCAGCACCCGGGGATCTTGAAGTTCCTATT
acrEF	3A	AATAGGAACTTCAAGATCCCCGGGTGCTGGCAATCTTCTTCGTA
acrEF	3B	AAAAAAAAGCGGCCGCCACGTGAAACCTCGGAAAGCATAAC
acrEF	Outside A	TTGCCGACCCACCATATAAC
acrEF	Outside B	GATAGACCGACATGGTCATG
yhiUV	1A	AAAAAAAAGCGGCCGCTAGCTATTGCCGGTTTGTTCTGTGT
yhiUV	1B	AACTTCAGAGCGCTTTTGAAGCTTGTTTTCCGCCGATTTGTCAT
yhiUV	2A	ATGACAAATCGGCGGAAAACAAGCTTCAAAAGCGCTCTGAAGTT
yhiUV	2B	CCACTACAACGAAAAAGACCCCCGGGGATCTTGAAGTTCCTATT
yhiUV	3A	AATAGGAACTTCAAGATCCCCGGGGGTCTTTTTCGTTGTAGTGG
yhiUV	3B	AAAAAAAAGCGGCCGCGGATCCTTTTTCTTGAGGATGAGCAC
yhiUV	Outside A	GTGTATCAGTTTCCCGTTCG
yhiUV	Outside B	GTGTCAAGGACACGCTTTC
acrD	1A	AAAAAAAAGCGGCCGCTAGCATGTGCCCAGAATAAGTAAC
acrD	1B	AACTTCAGAGCGCTTTTGAAGCTTTGGGGCGATCAATAAAGAAA
acrD	2A	TTTCTTTATTGATCGCCCCAAAGCTTCAAAAGCGCTCTGAAGTT
acrD	2B	ACAAAGAACAGCGGCACGAACCCGGGGATCTTGAAGTTCCTATT
acrD	3A	AATAGGAACTTCAAGATCCCCGGGTTCGTGCCGCTGTTCTTTGT
acrD	3B	AAAAAAAAGCGGCCGCGGATCCGGTCGGCAGCGTTCACACAT
acrD	Outside A	GGGATGAAAATGGTGTTGGC
acrD	Outside B	GTTGAAATCACGATGCGGGG
yegM yegNO	1A	AAAAAAAAGCGGCCGCTAGCCCAGTCTGGAAACGGCATCT
yegM yegNO	1B	AACTTCAGAGCGCTTTTGAAGCTTGTCTGGAATCGTCTGAATGA
yegM yegNO	2A	TCATTCAGACGATTCCAGACAAGCTTCAAAAGCGCTCTGAAGTT
yegM yegNO	2B	CTCATTACCAGTCCGCCGACCCCGGGGATCTTGAAGTTCCTATT
yegM yegNO	3A	AATAGGAACTTCAAGATCCCCGGGGTCGGCGGACTGGTAATGAG
yegM yegNO	3B	AAAAAAAAGCGGCCGCTAGCCCAGAATGGGTCGCCGTGTA
yegM yegNO	Outside A	CATGAGGAGAATTATCCAGC
yegM yegNO	Outside B	TTTACCGGTGCCAGTAAACC
emrD	1A	AAAAAAAAGCGGCCGCTAGCCCCCAGCATACCCAGCGACA
emrD	1B	AACTTCAGAGCGCTTTTGAAGCTTGTTTCTTTGCCTTTTCATTA
emrD	2A	TAATGAAAAGGCAAAGAAACAAGCTTCAAAAGCGCTCTGAAGTT
emrD	2B	GAAACAGCATCCCGGCACCGCCCGGGGATCTTGAAGTTCCTATT

TABLE 1—*Continued*

Gene(s)	Name	Oligonucleotide sequence (5′ to 3′)
emrD	3A	AATAGGAACTTCAAGATCCCCGGGCGGTGCCGGGATGCTGTTTC
emrD	3B	AAAAAAAAGCGGCCGCGTCGACCCGGTCAGGAAGGAAAAGAT
emrD	Outside A	CCAGCACGTCATCAACATCC
emrD	Outside B	TCCGCAGTTACCAATGGAGT
mdfA	1A	AAAAAAAAGCGGCCGCTAGCATCGTGATGGAAAATGTGGA
mdfA	1B	AACTTCAGAGCGCTTTTGAAGCTTCGTAAAGCACCAGACAGAGA
mdfA	2A	TCTCTGTCTCGGTGCTTTACGAAGCTTCAAAAGCGCTCTGAAGTT
mdfA	2B	GACAGCCACAAAATTCCGTTCCCGGGGATCTTGAAGTTCCTATT
mdfA	3A	AATAGGAACTTCAAGATCCCCGGGAACGGAATTTTGTGGCTGTC
mdfA	3B	AAAAAAAAGCGGCCGCTCCGGAGTTGCTGCTGATAGGTGTTG
mdfA	Outside A	AAGCGGGCTCAGTGACCATA
mdfA	Outside B	TTTATAGCCTGGGCACGCTG
emrKY	1A	AAAAAAAAGCGGCCGCTAGCGAGCCATTTTCATCCGTCAT
emrKY	1B	AACTTCAGAGCGCTTTTGAAGCTTAAACTACCGCCAATAAAGAA
emrKY	2A	TTCTTTATTGGCGGTAGTTTAAGCTTCAAAAGCGCTCTGAAGTT
emrKY	2B	AAAACCGTAAGTAAGATAAACCCGGGGATCTTGAAGTTCCTATT
emrKY	3A	AATAGGAACTTCAAGATCCCCGGGTTTATCTTACTTACGGTTTT
emrKY	3B	AAAAAAAAGCGGCCGCGGATCCGCCGTGTTTGTCGCCTATTA
emrKY	Outside A	AAGAATGTCCCCCATAACGC
emrKY	Outside B	ATTGGCAACCCTATCACTGC
emrE	1A	AAAAAAAAGCGGCCGCTAGCACGCGCATTACTGGTTGGTT
emrE	1B	AACTTCAGAGCGCTTTTGAAGCTTGTTGTACCAATGACCTCTGC
emrE	2A	GCAGAGGTCATTGGTACAACAAGCTTCAAAAGCGCTCTGAAGTT
emrE	2B	AATGTGGTGTGCTTCGTGACCCCGGGGATCTTGAAGTTCCTATT
emrE	3A	AATAGGAACTTCAAGATCCCCGGGGTCACGAAGCACACCACATT
emrE	3B	AAAAAAAAGCGGCCGCGGATCCTTTGTCACGTATTTTCAGTA
emrE	Outside A	GACATAATGCAGGCCTTCAC
emrE	Outside B	CCCCCAACCCAATTATTTGC
tehAB	1A	AAAAAAAAGCGGCCGCTAGCAATCCTCGCTTTATGTCCTT
tehAB	1B	AACTTCAGAGCGCTTTTGAAGCTTGTCCCCAACACAATACCAAA
tehAB	2A	TTTGGTATTGTGTTGGGGACAAGCTTCAAAAGCGCTCTGAAGTT
tehAB	2B	TTCATTGTATTTCACCCTCTCCCGGGGATCTTGAAGTTCCTATT
tehAB	3A	AATAGGAACTTCAAGATCCCCAGAGGGTGAAATACAATGAA
tehAB	3B	AAAAAAAAGCGGCCGCGGATCCTCTGTCCCCGCTTTTTGAAT
tehAB	Outside A	GGAGTGCGTATGTTTCCAGA
tehAB	Outside B	CTGATGTACCACTTACCGCA
ybjY ybjZ	1A	AAAAAAAAGCGGCCGCGCTAGCTCGTATCGGGTTATTTTGCTCGG
ybjY ybjZ	1B	TCAGAGCGCTTTTGAAGCTTCTTCTTCACGGTTTTCCGCT
ybjY ybjZ	2A	AGCGGAAAACCGTGAAGAAGAAGCTTCAAAAGCGCTCTGA
ybjY ybjZ	2B	TCTACTGGATCCAGTCGTGCCCCGGGGATCTTGAAGTTCC
ybjY ybjZ	3A	GGAACTTCAAGATCCCCGGGGCACGACTGGATCCAGTAGA
ybjY ybjZ	3B	AAAAAAAAGCGGCCGCGTCGACGGCACATCGCCCTGAACAATTC
ybjY ybjZ	Outside A	CAGGGACTTTCCGCAATGGTACA
ybjY ybjZ	Outside B	TTAGGTTAGCCGCATCGACCTGAC

the contiguous MDR pump genes, with the rationale that such putative operon gene products may associate to form multicomponent MDR efflux systems. Moreover, all of the MFP genes were immediately upstream of a pump (except for *yhiUV*, which has the MFP located downstream), which is a configuration that is seen in the best-studied *E. coli* pumps, *acrAB* and *emrAB*. Therefore, 16 unique deletions representing 25 individual gene deletions were made, and the specific genes deleted are shown in Table 2. The corresponding nucleotide deletion locations in the *E. coli* contigs (5) are shown in Table 3. *E. coli* W3110 strains with deletions for MDR pump genes or operons had a chromosomal deletion replaced by the Tn5 kanamycin resistance determinant, which was itself flanked by the yeast 2μm Flp recombination target *FRT* sites (Km^r-*FRT* fragment). In addition to the 16 deletion strains, 5 other strains (Table 4; HS230, HS276, HS235, HS236, HS275, and HS238) were constructed that had multiple operon or gene deletions. These strains had deletions for operons or

genes of the same OMF, MFS, RND, or SMR MDR pump families. They were constructed by initially deleting the chromosomal Km^r-*FRT* fragment in vivo using the method of Cherepanov and Wackernagel (7). A new Km^r-*FRT*-marked MDR pump deletion locus of the same MDR pump family was introduced by P1 transduction. This marker was removed as described above, and another marked deletion was introduced by P1 transduction. This iterative process was repeated such that many MDR pump deletions were introduced into a single strain. The construction of all strains listed in Table 4 is described in Materials and Methods. All deletion strains were verified as correct by analyzing the chromosomal locus by PCR amplification using oligonucleotide primers located outside the region of DNA used to make the deletion constructs. The outside A and B oligonucleotides used for PCR for each deletion strain are shown in Table 1, and the corresponding wild-type and mutant predicted PCR product sizes are depicted in Table 3. The PCR product size experimental data for

TABLE 2. Known and predicted *E. coli* MDR pump proteins

MDR component	Assigned family	GenBank contig accession no. (and ORF no. in contig)[b]	Reference or source	Genetic context[a]
TolC	OMF	AE000385_0008	9	*tolC*
YjcP	OMF	AE000481_0007	This study; homology to *P. aeruginosa* OprM	*yjcP*
YohG	OMF	AE000303_0004	This study; homology to *P. aeruginosa* OprM	*yohG*
YlcB	OMF	AE000162_0003	This study; homology to *P. aeruginosa* OprM	*ylcB*
AcrB	RND	AE000152_0008	22	*acrAB*
AcrF	RND	AE000405_0007	16	*acrEF*
YhiU	RND	AE000427_0010	32	*yhiUV*
AcrD	RND	AE000334_0001	27	*acrD*
YegN	RND	AE000297_0008	31	*yegMNO*
YegO	RND	AE000297_0009	31	*yegMNO*
EmrD	MF	AE000445_0002	28	*emrD*
MdfA	MF	AE000186_0007	8	*mdfA*
EmrB	MF	AE000353_0005	21	*emrAB*
EmrY	MF	AE000325_0001	32	*emrKY*
EmrE	SMR	AE000160_0004	33	*emrE*
TehA	SMR	AE000240_0004	40	*tehAB*
YbjZ	ABC	AE000189_0008	31	*ybjYZ*
AcrA	MFP	AE000152_0007	22	*acrAB*
AcrE	MFP	AE000405_0006	16	*acrEF*
YhiV	MFP	AE000427_0011	32	*yhiUV*
YegM	MFP	AE000297_0007	This study; homology to *N. gonorrhoeae* MtrC	*yegMNO*
EmrA	MFP	AE000353_0004	21	*emrKY*
EmrK	MFP	AE000325_0002	32	*emrKY*
YbjY	MFP	AE000189_0007	This study; homology to *N. gonorrhoeae* MtrC	*ybjYZ*
TehB	NA[c]	AE000240_0005	40	*tehAB*

[a] The location of the MDR genes that are contiguous to and oriented in the same transcriptional direction as other MDR genes.
[b] Last four digits shown correspond to the open reading frame (ORF) in the contig.
[c] NA, not applicable.

the wild-type and mutant strains are seen in Fig. 2. The predicted sizes in Table 3 are the same as the experimental PCR product sizes seen in Fig. 2, providing strong evidence that the deletion strains are correct.

Susceptibility testing results. MICs of 35 compounds were determined for the newly constructed deletion strains. The agar dilution MIC results for *E. coli* strain W3110 and each of its 22 isogenic null mutants are shown in Table 4. Results were considered significant when there was a ≥4-fold difference between deletion strains and the wild-type control, *E. coli* W3110 (Table 4). The data are grouped by gene families, e.g., OMF and MDR pump families. For the OMF family, a deletion in *tolC* (strain HS151) resulted in a strain with increased susceptibility to 29 of the 35 compounds tested; single deletions in *yjcP* (strain HS154) or *yohG* (strain HS157) resulted in strains with increased susceptibility to puromycin, acriflavin, and tetraphenylarsonium chloride. In contrast, a strain with a deletion in another family member, *ylcB* (strain HS208), showed no difference versus W3110 for any of the compounds tested. Strain HS230, with all four genes in this OMF class deleted, showed a susceptibility profile comparable to that of the single *tolC* deletion strain HS151.

Another group of genes deleted are members of the RND family: a deletion in *acrAB* resulted in a strain with increased susceptibility to 25 of the 35 compounds (Table 4, compare strains HS832 and W3110). Strains containing single deletions of either *acrEF*, *yhiUV*, *acrD*, or *yegMNO* (strains HS212, HS193, HS215, and HS199) demonstrated no alteration in susceptibility compared to W3110 for all compounds tested. Twenty-four of these compounds resulted in increased susceptibility when tested on strains with a deletion in *tolC*. When all

five deletions of the RND class were combined in one strain (strain HS276), there were eight compounds to which the strain showed increased susceptibility above that of the *acrAB*-deleted strain. For these eight compounds, susceptibility appeared to be dependent on multiple RND efflux systems.

TABLE 3. Location and evidence for MDR deletions in *E. coli*[a]

Operon	*E. coli* contig accession no.	Location of operon in contig (nt)[b]	Location of deletion in contig (nt)	Predicted amplification product (kbp)[c]	
				WT[d]	Deletion strain
tolC	AE000385	5769–7256	5810–7206	5.7	5.8
yjcP	AE000481	9588–8122	8153–9502	5.8	5.9
yohG	AE000303	3588–2392	2512–3490	5.4	5.9
ylcB	AE000162	2377–3750	2402–3648	5.6	5.8
acrAB	AE000152	9033–4668	4721–8937	8.5	5.7
acrEF	AE000405	3660–7933	3585–7872	9.5	6.5
yhiUV	AE000427	8349–12644	8432–12593	8.5	5.7
acrD	AE000334	112–3225	139–3163	7.6	6.0
yegMNO	AE000297	7262–14856	7317–14737	11.5	5.6
emrD	AE000445	1091–2281	1114–2029	5.2	5.7
mdfA	AE000186	5562–6794	5637–6720	5.2	5.5
emrAB	AE000353	1902–4629	2018–4562	6.5	5.5
emrKY	AE000325	2785–84	149–2716	6.7	5.5
emrE	AE000160	1663–1995	1718–1974	4.5	5.7
tehAB	AE000240	1932–3514	1990–3411	5.5	5.5
ybjYZ	AE000189	8123–11208	8157–11189	7.1	5.5

[a] Evidence for replacement of operon with the Km[r] marker, based on predicted amplification product sites.
[b] nt, nucleotides.
[c] Amplification products were predicted based on location of primers located outside the DNA necessary for gene replacement (see Materials and Methods).
[d] WT, wild type.

TABLE 4. Susceptibility of *E. coli* MDR gene deletion strains to toxic compounds

W3110 pump deletion strain	Strain no.	MDR family	MIC (µg/ml)														
			AMP	CAM	FLOR	CLO	PUR	ERM	METH	NOV	CPRO	NOR	NAL	RIF	FUS	SM	SUL
W3110	HS414		12.5	6.25	6.25	>32	100	50	>640	100	0.01	0.004	3.125	5	400	1.95	2,000
Δ*tolC*::Km[r]	HS151	OMF	6.25	**0.78**	**0.78**	**1**	**1.56**	**1.56**	**20**	**0.78**	**0.0025**	**0.001**	**0.39**	10	**3.125**	1.95	2,000
Δ*yjcP*::Km[r]	HS154	OMF	12.5	6.25	6.25	>32	**25**	25	>640	100	0.01	0.004	3.125	5	400	1.95	2,000
Δ*yohG*::Km[r]	HS157	OMF	12.5	6.25	3.12	>32	**25**	50	>640	100	0.01	0.004	3.125	5	400	1.95	2,000
Δ*ylcB*::Km[r]	HS208	OMF	6.25	6.25	6.25	>32	100	50	>640	100	0.01	0.002	6.25	2.5	400	1.95	2,000
Δ*yjcP* Δ*yohG* Δ*ylcB* Δ*tolC*::Km[r]	HS230	OMF	**3.12**	**0.78**	**0.78**	**1**	**1.56**	**1.56**	**20**	**0.78**	**0.0025**	**0.001**	**0.39**	10	**3.125**	1.95	2,000
Δ*acrAB*::Km[r]	HS832	RND	**3.12**	**0.78**	**0.78**	16	**1.56**	**1.56**	**80**	**1.56**	**0.0025**	0.004	**1.56**	2.5	**3.125**	1.95	2,000
Δ*acrEF*::Km[r]	HS212	RND	6.25	6.25	6.25	>32	100	50	>640	100	0.01	0.004	6.25	5	400	1.95	2,000
Δ*yhiUV*::Km[r]	HS193	RND	6.25	6.25	6.25	>32	100	50	>640	100	0.01	0.004	6.25	5	400	1.95	2,000
Δ*acrD*::Km[r]	HS215	RND	6.25	6.25	6.25	>32	100	50	>640	100	0.01	0.004	6.25	5	400	1.95	2,000
Δ*yegMNO*::Km[r]	HS199	RND	12.5	6.25	6.25	>32	100	50	>640	100	0.02	0.004	6.25	5	800	3.9	2,000
Δ*acrEF* Δ*acrAB* Δ*yhiUV* Δ*acrD* Δ*yegMNO*::Km[r]	HS276	RND	**1.56**	**0.78**	**0.78**	**2**	**1.56**	**1.56**	**20**	**0.39**	**0.0025**	**0.001**	**1.56**	2.5	**3.125**	1.95	2,000
Δ*emrD*::Km[r]	HS196	MF	6.25	6.25	6.25	>32	100	50	>640	100	0.01	0.004	3.125	2.5	400	1.95	2,000
Δ*mdfA*::Km[r]	HS221	MF	6.25	6.25	6.25	>32	100	50	>640	100	0.01	0.004	6.25	2.5	400	1.95	2,000
Δ*emrD* Δ*mdfA*::Km[r]	HS235	MF	6.25	6.25	6.25	>32	100	50	>640	100	0.01	0.004	3.125	2.5	400	1.95	2,000
Δ*emrAB*::Km[r]	HS833	MF	12.5	6.25	6.25	>32	100	50	>640	100	0.01	0.004	6.25	5	400	1.95	2,000
Δ*emrKY*::Km[r]	HS205	MF	6.25	6.25	6.25	>32	100	50	>640	100	0.01	0.004	6.25	5	400	1.95	2,000
Δ*emrKY*	HS236	MF	6.25	6.25	6.25	>32	100	50	>640	100	0.01	0.004	6.25	5	400	1.95	2,000
Δ*emrKY* Δ*emrAB* Δ*emrD* Δ*mdfA*::Km[r]	HS275	MF	6.25	6.25	6.25	>32	100	50	>640	100	0.01	0.004	6.25	5	400	1.95	2,000
Δ*emrE*::Km[r]	HS218	SMR	6.25	6.25	6.25	>32	100	50	>640	100	0.01	0.004	6.25	2.5	400	1.95	2,000
Δ*tehAB*::Km[r]	HS202	SMR	12.5	6.25	6.25	>32	100	50	>640	**400**	0.01	0.004	6.25	10	400	1.95	2,000
Δ*emrE* Δ*tehAB*::Km[r]	HS238	SMR	6.25	6.25	6.25	>32	100	50	>640	100	0.01	0.004	6.25	5	400	1.95	2,000
Δ*ybjYZ*::Km[r]	HS273	ABC	6.25	6.25	6.25	>32	100	50	>640	100	0.01	0.004	6.25	2.5	400	1.95	2,000

[a] Values in bold differ from the W3110 control strain value by ≥4-fold.
[b] Abbreviations: AMP, ampicillin; CAM, chloramphenicol; FLOR, florfenicol; CLO, clotrimazole; PUR, puromycin; ERM, erythromycin; METH, methotrexate; NOV, novobiocin; CIPRO, ciprofloxacin; NOR, norfloxacin; NAL, nalidixic acid; RIF, rifampin; FUS, fusidic acid; SM, streptomycin; SUL, sulfacetamide; TET, tetracycline; DOC, deoxycholate; CHO, sodium cholate; TDOC, sodium taurodeoxycholate; OXAL, sodium oxalate; PROF, proflavin; CV, crystal violet; ACR, acriflavin; EBR, ethidium bromide; TPP, tetraphenylphosphonium; R6G, rhodamine 6G; TPAC, tetraphenylarsonium chloride; DEQC, dequalinium chloride; BENZ, benzalkonium chloride; DAUN, daunomycin; PLUM, plumbagin; VIOL, methyl viologen.

Single, double, and quadruple deletions of the MF family were constructed. Strains with single deletions in *emrD, emrAB,* and *emrKY* (strains HSHS196, HS833, and HS205) showed no alternations in susceptibility to the 35 compounds when compared to W3110. In contrast, a strain with a single deletion of *mdfA* (strain HS221) resulted in increased susceptibility to ethidium bromide and benzalkonium chloride. Strains with two pump deletions were also created. A strain with *emrD* and *mdfA* deleted (strain HS235) had the same susceptibility profile as a single *mdfA* deletion. The strain with deletions for *emrAB* and *emrKY* (strain HS236) had susceptibility data similar to W3110. A deletion of all four MF family loci resulted in MICs comparable to those for the strain with a deletion for *mdfA* alone (compare strain HS275 to strain HS221), suggesting that among the four members there is no additive or synergistic effect of multiple deletion in this family.

Two members of the SMR family were deleted and analyzed. A strain with a deletion of *emrE* (strain HS218) displayed increased susceptibility to acriflavin, ethidium bromide, and methyl viologen, while a strain with a deletion of *tehAB* (a possible SMR family member) (strain HS202) resulted in increased resistance to novobiocin. Deletions of both genes in one strain (strain HS238) showed the same susceptibility profiles as single deletions of *emrE* and *tehAB* for the compounds tested, with the exception of novobiocin, for which the profile was unchanged from wild type.

A putative ABC transporter and the MF component located immediately upstream were also deleted. The strain with deletion of *ybjYZ* (strain HS273) showed no differences in MICs of any compound compared to MICs for W3110.

DISCUSSION

E. coli strains were constructed with null mutations in efflux pump genes. Efflux genes were deleted singly or by groups according to the class of pump (RND, MF, SMR, ABC) or MDR multicomplex component (OM). The susceptibility profiles for each of the isogenic *E. coli* strains were determined using 35 compounds, including antibiotics, antiseptics, detergents, and dyes that have been previously identified by other investigators as substrates for the known MDR pumps. This study was designed to systematically identify the contribution of efflux pump genes for intrinsic susceptibility to broad classes of compounds. By and large, deletions of *tolC* (OM family) and *acrAB* (RND family) resulted in the greatest increase in susceptibility for most of the compounds tested. They each individually accounted for increased susceptibility to ≥25 of the 35 compounds (Table 4).

The data support the concept that TolC functions with and without AcrAB in its contribution to intrinsic resistance. Multicomponent gram-negative pump systems are comprised of a pump, an MFP, and an OMF. Two examples are AcrA(MFP)-

TABLE 4—*Continued*

						MIC (µg/ml)													
TET	DOC	CHO	TDOC	SDS	OXAL	PROF	CV	ACR	EBR	TPP	R6G	TPAC	CTAB	DEQC	BENZ	DAUN	PLUM	VIOL	CCCP
1.25	>3,200	>12,800	>12,800	>12,800	>3,200	100	25	400	800	1,600	1,280	1,600	200	160	62.5	>80	40	50	26
0.156	**200**	**1,600**	**400**	**50**	>3,200	**6.25**	**0.78**	**3.12**	**3.12**	**6.25**	**2.5**	**1.56**	**25**	**25**	**2**	**0.625**	**10**	50	**1.6**
1.25	>3,200	>12,800	>12,800	>12,800	>3,200	50	25	**100**	400	800	1,280	**400**	100	160	31.2	>80	40	50	13
1.25	>3,200	>12,800	>12,800	>12,800	>3,200	50	25	**100**	400	800	1,280	**400**	100	160	31.2	>80	40	50	13
1.25	>3,200	>12,800	>12,800	>12,800	>3,200	100	25	400	400	1,600	1,280	1,600	200	160	62.5	>80	80	50	26
0.156	**200**	**1,600**	**400**	**50**	>3,200	**12.5**	**0.78**	**3.12**	**3.12**	**6.25**	**2.5**	**3.125**	**25**	**2.5**	**2**	**0.625**	**10**	50	**1.6**
0.156	**1,600**	>12,800	>12,800	**100**	>3,200	**12.5**	**0.78**	**3.12**	**3.12**	**6.25**	**2.5**	**3.125**	**2.5**	**2.5**	**2**	**0.625**	**10**	50	13
1.25	>3,200	>12,800	>12,800	>12,800	>3,200	100	25	400	400	1,600	1,280	1,600	200	160	62.5	>80	80	50	13
1.25	>3,200	>12,800	>12,800	>12,800	>3,200	100	25	400	400	1,600	1,280	1,600	200	160	62.5	>80	80	50	13
1.25	>3,200	>12,800	>12,800	>12,800	>3,200	100	25	400	400	1,600	1,280	1,600	200	160	62.5	>80	80	50	13
1.25	>3,200	>12,800	>12,800	>12,800	>3,200	100	25	400	800	1,600	1,280	1,600	200	160	62.5	>80	80	50	52
0.156	**1,600**	**6,400**	**400**	**100**	>3,200	**6.25**	**0.78**	**3.12**	**1.56**	**6.25**	**2.5**	**1.56**	**25**	**2.5**	**2**	**0.625**	**10**	**25**	26
1.25	>3,200	>12,800	>12,800	>12,800	>3,200	100	25	400	400	1,600	1,280	1,600	200	160	62.5	>80	80	50	13
1.25	>3,200	>12,800	>12,800	>12,800	>3,200	100	25	400	**200**	1,600	1,280	1,600	100	160	**15.6**	>80	**>80**	50	13
1.25	>3,200	>12,800	>12,800	>12,800	>3,200	100	25	400	**200**	1,600	1,280	1,600	100	160	**15.6**	>80	80	50	13
1.25	>3,200	>12,800	>12,800	>12,800	>3,200	100	25	400	800	1,600	1,280	1,600	200	160	62.5	>80	40	50	13
1.25	>3,200	>12,800	>12,800	>12,800	>3,200	100	25	400	400	1,600	1,280	1,600	200	160	62.5	>80	80	50	13
1.25	>3,200	>12,800	>12,800	>12,800	>3,200	100	25	400	400	1,600	1,280	1,600	200	160	62.5	>80	80	50	13
1.25	>3,200	>12,800	>12,800	>12,800	>3,200	100	25	**200**	**200**	1,600	1,280	1,600	200	160	**15.6**	>80	80	50	13
1.25	>3,200	>12,800	>12,800	>12,800	>3,200	100	25	**50**	**200**	1,600	1,280	1,600	100	160	62.5	>80	80	**6.25**	13
1.25	>3,200	12,800	>12,800	>12,800	>3,200	100	25	400	800	1,600	1,280	1,600	200	160	62.5	>80	80	100	26
1.25	>3,200	>12,800	>12,800	>12,800	>3,200	100	25	**50**	**200**	1,600	1,280	1,600	100	160	62.5	>80	80	**6.25**	13
1.25	>3,200	>12,800	12,800	>12,800	>3,200	100	25	400	400	1,600	1,280	1,600	200	160	62.5	>80	80	50	13

AcrB(pump)-TolC(OMF) of *E. coli* (9) and the MexA(MFP)-MexB(pump)-OprM(OMF) of *P. aeruginosa* (19). The *acrAB* and *tolC* mutant strains have overlapping substrate susceptibility profiles, supporting their interaction as a tripartite pump system. None of the other OM components showed similar overlapping profiles with other strains with deletions for different classes of pumps. For example, *yjcP* and *yohG* mutants showed increased sensitivities to two compounds for which no mutant strains other than *acrAB* showed increased susceptibility. On the other hand, it was evident that TolC also functions independently of AcrA-AcrB. This is supported by (i) the strain deleted for *tolC* showed hypersusceptibility to more compounds than any of the single-locus-deletion strains tested; (ii) for 7 of 24 compounds, the MIC for the *tolC* strain was lower than that for the *acrAB*-deletion strain; and (iii) the *tolC* strain was susceptible to five more compounds than was the *acrAB*-deletion strain.

This study identifies those genes (*tolC, acrAB, yjcP, yohG, mdfA,* and *emrE*) that contribute to intrinsic resistance for the compounds studied here. First, consistent with previous studies (9, 18, 43), the absence of *tolC* in *E. coli* accounts for increased susceptibility to many classes of compounds (29 of the 35 compounds tested), and it is known that *acrAB* mutant strains are more sensitive to antibiotics, detergents, and dyes (22). Second, we have found that in addition to TolC, two putative members of the OM family also affect intrinsic resistance. Deletion of *yjcP* and *yohG* resulted in a fourfold increase in susceptibility to puromycin, acriflavin, and tetraphenylarsonium chloride. These data support the idea that these two genes are OM components. Third, the *mdfA* null mutant dis-

played increased susceptibility to two (ethidium bromide and benzalkonium chloride) of the 12 compounds (the other compounds were chloramphenicol, erythromycin, tetraphenylphosphonium, puromycin, tetracycline, daunomycin, rhodamine 6G, rifampin, ciprofloxacin, and norfloxacin) identified by Edgar and Bibi (8). Interestingly, the two compounds for which the null mutant displayed increased susceptibility are those for which the strain overexpressing *mdfA* displayed the highest increases in resistance compared to the wild-type control (8). Finally, it is known that strains that overexpress *emrE* demonstrate increased resistance to ethidium bromide and methyl viologen. Here, the *emrE* null mutant displayed increased susceptibility to these two compounds and also to acriflavin.

In contrast to the deletion strains mentioned above, there are others for which no increase was seen in susceptibility. They are strains in which individual deletions are in *emrAB, emrD, tehAB, acrEF, acrD, ylcB, yhiUV, yegMNO,* and *ybjYZ.* First, although *E. coli* strains overexpressing *emrAB* are resistant to nalidixic acid and CCCP (21) (compounds tested in this study), the *emrAB*-deletion strain was not found to exhibit susceptibility to these compounds. Second, whereas an *emrD* mutant has been shown to be sensitive to CCCP by the efficiency-of-plating method used by Naroditskaya et al. (28), such a result was not identified here. However, it is plausible that the discrepancy in results can be attributed to the use here of a less sensitive agar dilution MIC method compared to the more sensitive efficiency-of-plating method used by Naroditskaya et al. (28). Third, the *tehAB* deletion had little effect on susceptibility and in fact showed increased resistance to novobiocin, a phenomenon that may be related to the fact that
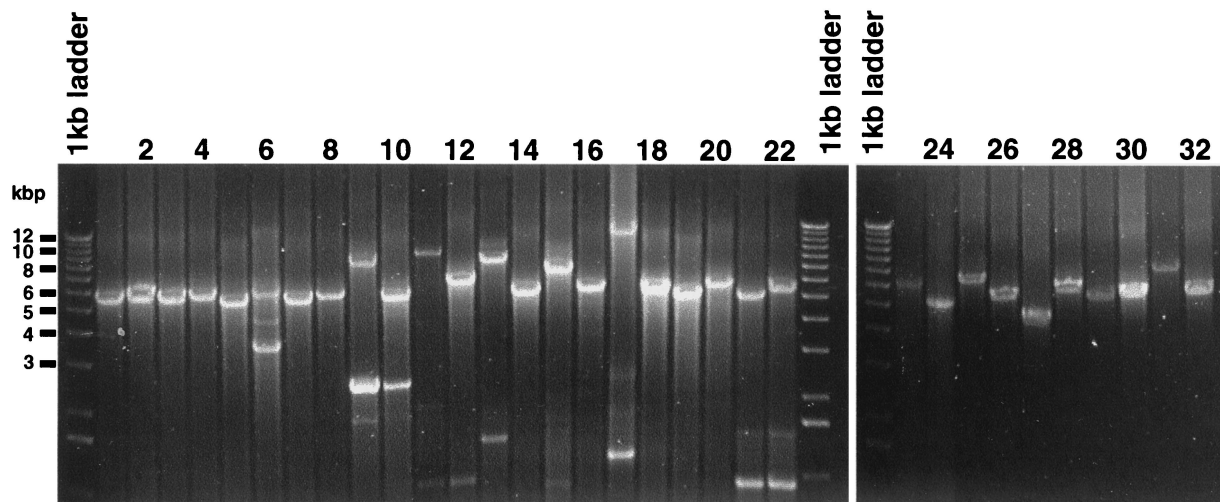
FIG. 2. Evidence for successful allelic exchange of the Km^r-*FRT* determinant for the genes or operons used in this study, as assessed by ethidium bromide-stained PCR products following electrophoresis in a 0.7% agarose gel. PCRs using oligonucleotides located outside the operons deleted (outside primers in Table 1) were used with genomic DNA templates of the wild type (W3110) or of the specific mutant Km^r strain. PCR products were amplified using *tolC* primers with genomic W3110 (lane 1) and with HS151 (lane 2); *yjcP* primers with W3110 (lane 3) and HS154 (lane 4); *yohG* primers with W3110 (lane 5) and HS157 (lane 6); *ylcB* primers with W3110 (lane 7) and HS208 (lane 8); *acrAB* primers with W3110 (lane 9) and HS832 (lane 10); *acrEF* primers with W3110 (lane 11) and HS212 (lane 12); *yhiUV* primers with W3110 (lane 13) and HS193 (lane 14); *acrD* primers with W3110 (lane 15) and HS215 (lane 16); *b2074yegNO* primers with W3110 (lane 17) and HS199 (lane 18); *emrD* primers with W3110 (lane 19) and HS196 (lane 20); *mdfA* primers with W3110 (lane 21) and HS221 (lane 22); *emrAB* primers with W3110 (lane 23) and HS833 (lane 24); *emrKY* primers with W3110 (lane 25) and HS205 (lane 26); *emrE* primers with W3110 (lane 27) and HS218 (lane 28); *tehAB* primers with W3110 (lane 29) and HS202 (lane 30); and b0878*ybjC* primers with W3110 (lane 31) and HS273 (lane 32). The two *tehAB* 5.5-kbp bands seen for both wild-type and mutant strains in lanes 29 and 30 do not distinguish mutant from wild-type alleles. The wild-type product was distinguished from the Δ*tehAB*::Km^r PCR product upon digestion with *Hin*dII; the Δ*tehAB*::Km^r PCR product seen in lane 30 was digested to two fragments of size 2 and 3.5 kbp, whereas the wild-type product seen in lane 29 remained a 5.5-kbp undigested product as expected (data not shown).

strains overexpressing *tehAB* are more susceptible to methyl viologen and dequalinium chloride (40). Fourth, the data here are consistent with studies indicating that single *acrEF* and *acrD* null mutants have not been shown to increase susceptibility in *E. coli* K-12 (23). However, for *acrD*, Rosenberg and Nikaido have recently shown that a deletion in *acrD* decreased MICs of aminoglycosides (amikacin, gentamicin, neomycin, kanamycin, and tobramycin) by a factor of 2 to 8 (34). The aminoglycosides kanamycin and neomycin could not be tested here because the Tn*5* Km^r marker that confers resistance to these two compounds is present in all strains. More work is needed to identify the contributions of the MDR pumps for susceptibility to both aminoglycosides and also to other compounds not studied here. Finally, for the putative pumps *ylcB*, *yhiUV, yegMNO*, and *ybjYZ*, we were unable to identify a functional contribution to intrinsic resistance.

There can be a number of reasons why strains with deletions for single pumps do not show susceptibility to those substrates for which strains overexpressing the same pump genes show resistance. MDR pumps, such as EmrAB, AcrEF, EmrD and TehAB (8, 15, 25, 40, 44), have been identified by their ability to confer resistance to compounds when overexpressed in bacteria. In this study, strains with deletions for *acrAB* and *emrE* had increased susceptibility to specific compounds for which overexpression reportedly confers resistance. However, strains with deletions for *emrAB, acrEF, emrD*, and *tehAB* did not. There are a number of possible explanations for this. First, for those MDR pumps where no clear effect on intrinsic resistance was seen, it is possible that the set of growth conditions em-

ployed here is not sufficient to detect their contribution. Other growth conditions may be necessary. Second, since pumps have overlapping substrate profiles (e.g., *acrAB* and *acrEF* [25]), an MDR pump that is active in the cell can mask the effect of the deletion of another MDR pump. An example may be the AcrA-AcrB-TolC pump, which is the primary efflux system in *E. coli* under the conditions tested. A deletion in *acrAB* or *tolC* would be necessary to identify the contribution of other pumps with overlapping substrate profiles. This was in fact shown to be the case when *emrAB* was identified as contributing to bile salt susceptibility in an *acrAB* mutant background by Thanassi et al. (39). This may also be the case for *emrAB, acrEF, emrD*, and *tehAB* and others. Third, these MDR pumps are normally poorly expressed and therefore a deletion may have little effect—an idea previously suggested by Ma et al. for *acrEF* (23). Fourth, a corollary to the previous explanation may be that the true inducer of the pump (such as bile salts, as postulated for AcrAB) is not present, and so the pump is not expressed in the presence of its alternative pump substrates. An example of this explanation is found for two *Bacillus* pumps. *Bacillus subtilis bmr* and *blt* have similar substrate specificities (e.g., to rhodamine) when overexpressed but have different inducers. Rhodamine is the inducer of *bmr* but not of *blt* (1). Whereas rhodamine both induces and is effluxed by *bmr*, it does not induce expression of *blt* and thus is not effluxed. Perhaps *emrAB, acrEF, emrD*, and *tehAB* are like *blt*, where the inducer is not present in the list of compounds used in this study. One possible way to identify inducers of these pumps is to treat

E. coli with many structurally unrelated compounds and look for increased expression of these genes.

The effect on susceptibility for strains with multiple pump knockouts was assessed only with MDR pumps of the same family (OM, RND, MF, and SMR) in an initial effort to identify possible new combinations of deletions that may lead to a susceptible phenotype. No additive or synergistic effects on susceptibility were seen for the strains with deletions for all genes comprising the OM, MF, or SMR families. However, the strain with deletions for genes in the RND family led to increased susceptibility above levels seen for the *acrAB*-deletion strain alone, indicating that another MDR pump in this family contributes to intrinsic resistance. A recent report by Lee et al. (17) indicated that simultaneous expression of single-component (MdfA or CmlA) and multicomponent (AcrAB) MDR pump genes results in resistance to antibiotics that is greater than the additive resistance of each MDR pump expressed singly. Perhaps for the RND family the AcrD single-component pump contributes to resistance with AcrAB. The strains constructed in this study are a resource that can be used to construct other novel strains to assess the interplay among efflux pumps for intrinsic resistance.

Mutations leading to increased expression of efflux genes can contribute to increased resistance to antibiotics, such as quinolone resistance in *norA* mutants of *S. aureus* (14). Conversely, inhibition of MDR pumps can lead to increased susceptibility (24), such as is seen for the 5′-methoxyhydnocarpin inhibition of NorA (38). The notion of inhibiting efflux pumps in order to potentiate the efficacy of antibiotics was described for *S. aureus* by Stermitz et al. (38) when *S. aureus* had increased susceptibility to berberine and NorA substrates in the presence of the NorA inhibitor, 5′-methoxyhydnocarpin. Lomovskaya et al. (20) and Hsieh et al. (12) reported that the potential effect of inhibiting MDR pumps in combination with fluoroquinolone treatment would result in increased fluoroquinolone susceptibility for *P. aeruginosa* and *S. aureus*, respectively. Moreover, other studies suggest that a pump inhibitor combined with an antimicrobial would decrease the likelihood of the emergence of strains clinically resistant to fluoroquinolones (20, 24). This study suggests that an inhibitor specific for the dominant efflux system identified here, AcrA-AcrB-TolC, would result in an increase in susceptibility to a broad spectrum of toxic compounds including antibiotics, detergents, and dyes. Such an inhibitor could potentially be used in conjunction with current antimicrobials that are substrates for the AcrA-AcrB-TolC pump. The strains generated in this study could also be used to identify those *E. coli* MDR pumps that contribute to resistance to any given antimicrobial, and subsequent identification of a compound that inhibits that MDR pump could be used with the particular antimicrobial for effective cotherapy.

Efflux pump deletion strains can yield sensitive bacterial strains that are useful for cell-based screening of compound libraries to identify novel new antimicrobials (12). The susceptible strains can be used to detect lower concentrations of antibiotics in compound libraries than those for wild-type *E. coli* strains. Of the 16 deletion strains studied here, the most sensitive strains for cell-based screening are the *acrAB* or *tolC* deletion strains. This study shows that highly susceptible *E. coli* strains may be obtained for cell-based screening if other efflux pump genes are disrupted in combination with an *acrAB* or *tolC* deletion. Conceivably, non-MDR pump alleles that affect susceptibility, such as the *E. coli lpxA, firA*, or *rfa* alleles that increase sensitivity to multiple antibiotics (41), can also be used in combination with the efflux pump mutants to produce strains hypersusceptible to broad classes of compounds. Systematic deletions of MDR pumps, similar to those in this study, in organisms other than *E. coli* can be performed to generate gram-negative, gram-positive, or fungal-sensitive strains for cell-based antimicrobial screens.

Use of bioinformatics, genetics, and susceptibility testing will continue to identify new MDR pumps and additional substrates. For example, MDR pumps such as YdhE, a homolog of the NorM MDR pump from *Vibrio parahaemolyticus*, can be added for study (26), and newer substrates such as the recently identified substrate indole for the AcrEF pump can be tested (15). Thus, the systematic approach used in this study is an initial step to determine the relative contributions to intrinsic resistance of many efflux pumps. Specifically, this approach identified AcrAB-TolC as the dominant MDR pump among the 16 MDR pumps evaluated for the 20 different classes of compounds assessed.

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