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Authors
Sugawara, Etsuko
Nikaido, Hiroshi

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Properties of AdeABC and AdeIJK Efflux Systems of *Acinetobacter baumannii* Compared with Those of the AcrAB-TolC System of *Escherichia coli*

Etsuko Sugawara, Hiroshi Nikaido

Department of Molecular and Cell Biology, University of California, Berkeley, California, USA

Acinetobacter baumannii **contains RND-family efflux systems AdeABC and AdeIJK, which pump out a wide range of antimicrobial compounds, as judged from the MIC changes occurring upon deletion of the responsible genes. However, these studies may miss changes because of the high backgrounds generated by the remaining pumps and by β-lactamases, and it is unclear how the activities of these pumps compare quantitatively with those of the well-studied AcrAB-TolC system of *Escherichia coli*. We expressed *adeABC* and *adeIJK* of *A. baumannii*, as well as *E. coli acrAB*, in an *E. coli* host from which *acrAB* was deleted. The *A. baumannii* pumps were functional in *E. coli*, and the MIC changes that were observed largely confirmed the substrate range already reported, with important differences. Thus, the AdeABC system pumped out all β-lactams, an activity that was often missed in deletion studies. When the expression level of the pump genes was adjusted to a similar level for a comparison with AcrAB-TolC, we found that both *A. baumannii* efflux systems pumped out a wide range of compounds, but AdeABC was less effective than AcrAB-TolC in the extrusion of lipophilic β-lactams, novobiocin, and ethidium bromide, although it was more effective at tetracycline efflux. AdeIJK was remarkably more effective than a similar level of AcrAB-TolC in the efflux of β-lactams, novobiocin, and ethidium bromide, although it was less so in the efflux of erythromycin. These results thus allow us to compare these efflux systems on a quantitative basis, if we can assume that the heterologous systems are fully functional in the *E. coli* host.**

Acinetobacter species, similar to *Pseudomonas aeruginosa*, display high levels of multidrug resistance to a broad range of antimicrobial agents (1, 2). Our previous study (3) demonstrated that *Acinetobacter baumannii* produces an outer membrane with only a low-permeability porin or a slow porin (4), $OmpA_{Ab}$, as the major nonspecific diffusion channel. When the absolute penetration rates of hydrophilic β-lactams across the outer membrane were determined by an intact-cell assay, the permeability coefficient for cephaloridine (0.57×10^{-6} cm/s) in *A. baumannii* strain ATCC 17978 was indeed 2 orders of magnitude lower (3) than the values obtained in *Escherichia coli* strains producing only OmpF or OmpC (5.3×10^{-4} and 0.45×10^{-4}, respectively [5]). We also found in *A. baumannii* a high level of expression of the endogenous AmpC β-lactamase, whose V_{max} value for cephaloridine was 0.70 nmol/s/mg (dry weight) cells (3). These two factors, a low-permeability outer membrane and a significant expression of endogenous AmpC β-lactamase, certainly contribute to the high levels of resistance to β-lactams in *A. baumannii*, but an additional mechanism of active efflux is required in order to explain quantitatively the very high MIC values for these compounds as well as other antibiotics.

To date, three RND system pumps, two MFS system pumps, and one member each of the MATE and SMR families of pumps have been reported to be involved in antibiotic efflux in *A. baumannii* (6). Among the RND systems, the AdeABC system, which was the first member of the RND family discovered in *A. baumannii*, is controlled by a two-component regulatory system, *adeRS*, located upstream from the *adeABC* genes, and this operon is not expressed strongly in wild-type strains (7). Interestingly, the *adeABC* operon does not exist in all strains (only about 80% of clinical isolates contain this operon), and some strains are missing the gene for the outer membrane component, *adeC* (8). The gene inactivation approach showed that the AdeABC system is involved in aminoglycoside resistance in a clinical strain of *A. baumannii* (9). Furthermore, the overexpression of the AdeABC system was shown to increase resistance to aminoglycosides, cefepime, fluoroquinolones, chloramphenicol, and tetracycline-tigecycline (6). The AdeIJK system is another member of the RND family. It appears to be present in all *A. baumannii* strains and is known to pump out a broad range of antibiotics, including β-lactams, chloramphenicol, tetracyclines, and erythromycin (10). A gene inactivation study showed little evidence that the third system, AdeFGH, contributes to resistance; its overexpression was necessary to see its functions (6).

However, all the data currently available are based on the effect of efflux gene deletions on MIC values in *A. baumannii* strains, which have exceedingly low outer membrane permeability. Recent studies of efflux kinetics in our laboratory (11, 12) showed that the effect of efflux gene deletion on the MIC can sometimes be amplified very strongly by the presence of a low-permeability outer membrane. Furthermore, it is unclear if the *A. baumannii* efflux pumps are more effective than the pumps in the *Enterobacteriaceae*. We therefore expressed the two *A. baumannii* RND systems that show a strong effect on MICs, AdeABC and AdeIJK (6), as well as the well-studied *E. coli* AcrAB system, in a single com-

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Address correspondence to Hiroshi Nikaido, nhiroshi@berkeley.edu.

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TABLE 1 Oligonucleotides used in this study

Primer	Sequence[a]
1	5′-CGGAATTCATGCAAAAGCATCTTTTACTTCCTTTATTTTTATCTATTGGG-3′
2	5′-TCCCCCGGGCTTAATTGAATTGCTGCCGCGGTAGCAGGCTTACCA-3′
3	5′-TCCCCCGGGTGCTAACGCCGTGAAAACTGCCGAAGTTGTTCGAGC-3′
4	5′-GCTCTAGATTAGACTTTTGATATTCCTCCTCCTAAAACTTTATA-3′
5	5′-GTTTAACTTTAAGAAGGAGATATAGGATCCGAGCTCCGTCGACAAGCTTGCGGC-3′
6	5′-GCCGCAAGCTTGTCGACGGAGCTCGGATCCTATATCTCCTTCTTAAAGTTAAAC-3′
7	5′-CTCCGTCGACAAGCTTCCCGGGGCACTCGAGCACC-3′
8	5′-GGTGCTCGAGTGCCCCGGGAAGCTTGTCGACGGAG-3′
9	5′-CGGGATCCATGATGTCGGCTAAGCTTTGGGCACCAGCCCTTACT-3′
10	5′-ACGCGTCGACCAGAAGACCGATTGCCAATACCATAGCGAACAT-3′
11	5′-ACGCGTCGACGACGCCATTGTTGTAGTCGAAAACGTTGAACGT-3′
12	5′-TCCCCCGGGTTATTGCTTTTTAAGTTCAGCACTAGATGGTTG-3′
13	5′-CGGGATCCATGAACAAAAACAGAGGGTTTACGCCTCTGGCGGTC-3′
14	5′-TCCCCCGGGTCAATGATGATCGACAGTATGGCTGTGCTCGAT-3′
15	5′-GCTCTAGATTAATGGTGATGGTGATGGTGAGATGAGATTTTTTTCTTAGAGGAAAATAGCTTTTCTAC-3′
16	5′-TCCCCCGGGTTAATGGTGATGGTGATGGTGCGATTTATGCTCCTGAGTGTTTATGGTTTTTGG-3′
17	5′-CGGGATCCGGATGAACAAAAACAGAGGGTTTACGCCTCTGGCGGTC-3′
18	5′-TCCCCCGGGTCAATGATGATGATGATGATGATCGACAGTATGGCTGTGCTCGAT-3′
19	5′-GCTCTAGAATGCAAAAAGTATGGTCTATTTCAGGTCGTAGC-3′
20	5′-GCTCTAGATTATTGCTTTTTAAGTTCAGCACTAGATGGTTGGTG-3′
21	5′-GCTCTAGATCAGTGGTGGTGGTGGTGGTGTGGTTGTGCCCCTTCAGCTATAGAAGTTTTGGTTTCTAC-3′
22	5′-TCCCCCGGGTTAGTGGTGGTGGTGGTGGTGTGCATTTGAAGCTGCTTTCTGTTCTGCTTGAGG-3′
23	5′-TCCCCCGGGTTAGTGGTGGTGGTGGTGGTGAGACTTGGACTGTTCAGGCTGAGCACCGCTTGCGGC-3′

[a] The restriction sites introduced into the oligonucleotides are underlined.

mon *E. coli* host and tried to compare the MIC increases due to the expression of these three RND systems so that the substrate preference of various pumps can be compared without prejudice. We show below that both of the *A. baumannii* RND efflux systems can be expressed in a functional form in *E. coli* and present the results of their comparison with the AcrAB system.

MATERIALS AND METHODS

Bacterial strains. AG100AΩ (Δ*acrAB*::*spc*) is a Δ*acrAB* derivative of AG100 [K-12 *argE3 thi-1 rpsL xyl mtl* Δ(*gal-uvrB*) *supE44*] (13). AG100AΩ (Δ*lacY*) was constructed by transducing the *lacY*::*kan* gene from strain JW0334-1 [Δ*lacY784*::*kan* Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-3*) λ⁻ *rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*; obtained from the Coli Genetic Stock Center, Yale University], with transductants being selected with 35 μg/ml kanamycin. The presence of the *kan* gene within the *lacY* gene of AG100AΩ (Δ*lacY*) was confirmed by sequencing of the chromosomal DNA. FHU-100 (MC4100 Δ*ara* Δ*acrAB* Δ*tolC*::*kan*) was obtained from R. Misra. *A. baumannii* strains ATCC 17978 (3) and BM4454 (9, 10) were obtained from P. Courvalin.

Cloning of the *adeABC*, *adeIJK*, and *acrAB* genes. The *adeABC* and *adeIJK* genes were cloned by PCR amplification from *A. baumannii* strain BM4454 (9). For the *adeABC* operon, two DNA fragments, *adeA* through the SmaI site in *adeB* (2,077 bp) and the SmaI site of *adeB* through *adeC* (3,701 bp), were amplified by an upstream primer containing an EcoRI site (primer 1) (primer sequences are listed in Table 1) and a downstream primer with a SmaI site (primer 2) and by an upstream primer with a SmaI site (primer 3) and a downstream primer with an XbaI site (primer 4), respectively, by using PfuUltra high-fidelity DNA polymerase (Agilent Technologies). These two DNA fragments were cloned into pBluescript (+/−) separately and sequenced, producing pBluescript-*adeA*-SmaI and pBluescript-SmaI-*adeC*, respectively. The amplicon consisting of *adeA* through the SmaI site in *adeB* (2077 bp), obtained by restriction enzyme treatment with EcoRI and SmaI, was cloned into the plasmid pBluescript-SmaI-*adeC*, producing the entire *adeABC* operon in pBluescript (+/−). Finally, the *adeABC* operon, obtained by cutting with EcoRI and NotI, was cloned into pKY9790, which is a 5.1-kb medium-copy-number (about 15 to 20 per cell) vector with the pBR322 origin, a chloramphenicol marker, the *lacI* gene, and *Ptac* promoter I (14).

For the cloning of the *adeIJK* gene into pKY9790, two restriction sites, the EcoRI site and the NotI site of pKY9790, were changed to a BamHI site and a SmaI site, respectively, by site-directed mutagenesis using primers (for the change of EcoRI to BamHI, primer 5 and primer 6; for the change of the NotI site to the SmaI site, primer 7 and primer 8), producing pKY9790 (EcoRI/BamHI, NotI/SmaI). For cloning of the *adeIJK* operon, two DNA fragments, *adeI* through the SalI site in *adeJ* (2,484 bp) and SalI of *adeJ* through *adeK* (3,416 bp), were amplified by an upstream primer containing BamHI site (primer 9) and a downstream primer with a SalI site (primer 10) and by an upstream primer with a SalI site (primer 11) and a downstream primer with a SmaI site (primer 12), respectively, by using PfuUltra high-fidelity DNA polymerase. These two DNA fragments were cloned into pSportI separately and sequenced, producing pSportI-*adeI*-SalI and pSportI-SalI-*adeK*, respectively. The DNA fragment consisting of *adeI* through the SalI site in *adeJ*, after restriction enzyme treatment with BamHI and SalI, was cloned into plasmid pSportI-SalI-*adeK*, producing the entire *adeIJK* operon in pSportI. Finally the *adeIJK* operon, obtained by cutting with BamHI and SmaI, was moved into pKY9790 (EcoRI/BamHI, NotI/SmaI).

For the cloning of the *acrAB* genes into pKY9790 (EcoRI/BamHI, NotI/SmaI), the *acrAB* genes were amplified by an upstream primer containing a BamHI site (primer 13) and a downstream primer with a SmaI site (primer 14) by using an Expand long-template PCR system (Roche). The amplicon was cloned into pKY9790 (EcoRI/BamHI, NotI/SmaI), after restriction enzyme treatment with BamHI and SmaI. These expression plasmids, pKY-*adeABC*, pKY-*adeIJK*, and pKY-*acrAB*, were transformed into an *E. coli* strain, AG100AΩ (Δ*acrAB*::*spc*) or AG100AΩ (Δ*lacY*) (Δ*acrAB*::*spc lacY*::*kan*).

Construction of pKY-*adeAB*-6His, pKY-*adeIJ*-6His, and pKY-*acrAB*-6His. To check for the expression of the proteins AdeB, AdeJ, and AcrB in the *E. coli* host, we constructed pKY-*adeAB*-6His, pKY-*adeIJ*-6His, and pKY-*acrAB*-6His. This was carried out by amplifying the *adeAB*,

adeIJ, and *acrAB* sequences with the upstream primers 1, 9, and 13, respectively, and downstream primers coding for a hexahistidine or a tetrahistidine sequence, primers 15, 16, and 18, respectively, followed by their insertion into pKY9790. (Because the C terminus of AcrB ends in His-His, the addition of an exogenous tetrahistidine sequence produces a hexahistidine tail in this case.)

For *acrAB*, a low-copy-number vector, pHSG576 (15), was also used. Here the *acrAB* genes were amplified by an upstream primer containing a BamHI site (primer 17) and a downstream primer with a SmaI site and tetrahistidines (primer 18) by using the Expand long-template PCR system (Roche). The amplicon was cloned into pHSG576, after restriction enzyme treatment with BamHI and SmaI.

Construction of pKY-*adeAB-adeK*. For cloning of the *adeAB-adeK* genes into pKY9790, the *adeK* gene was amplified by an upstream primer with an XbaI site (primer 19) and a downstream primer with an XbaI site (primer 20) by using PfuUltra high-fidelity DNA polymerase. The amplicon was cloned into pKY-*adeAB*-6His, after restriction enzyme treatment with XbaI, producing pKY-*adeAB*-6His-*adeK*.

All the expression plasmids mentioned in this and the preceding two sections were sequenced to confirm that there was no error during amplification and cloning.

Construction of pKY-*adeA*-6His, pKY-*adeI*-6His, pKY-*acrA*-6His, and pHSG-*acrA*-6His. To check for the expression of periplasmic adaptor proteins AdeA, AdeI, and AcrA, we constructed pKY-*adeA*-6His, pKY-*adeI*-6His, and pKY-*acrA*-6His, respectively, as well as pHSG576-*acrA*-6His. This was carried out by amplifying the *adeA*, *adeI*, and *acrA* genes with the upstream primers 1, 9, 13, and 17 and downstream primers coding for the hexahistidine sequence, primers 21, 22, 23, and 23, respectively, followed by their insertion into appropriate vectors.

MIC determination. MIC values were determined with 96-well microtiter plates using a standard 2-fold broth microdilution method with LB broth containing 5 mM $MgCl_2$. The expression vector was introduced into *E. coli* strain AG100AΩ, AG100AΩ (Δ*lacY*), or FHU-100 (Δ*acrAB* Δ*tolC*) by electroporation. The transformants were selected on LB medium-chloramphenicol (10 μg/ml) plates. Cells containing a plasmid-borne efflux pump system were cultured in 3 ml of LB broth containing 5 mM $MgCl_2$, 1% glucose, and 10 μg/ml chloramphenicol at 37°C with shaking, and at late exponential phase (optical density [OD] = 1.0) the culture was diluted to an OD at 600 nm (OD_{600}) of 0.01 and used as the inoculum. A microdilution assay was done using a series of 200 μl of LB broth supplemented with 5 mM $MgCl_2$, different concentrations of IPTG (isopropyl-β-D-thiogalactopyranoside), and 2-fold serial dilution of different drugs, which were prepared in the 96-well microtiter plates. Ten thousand cells from the inoculum described above were added, and the plates were incubated overnight at 37°C.

Preparation of crude cell extract and inner membrane fraction. Cells containing plasmid-borne *adeABC*, *adeIJK*, or *acrAB* genes and the vector only as a control were grown in LB medium containing 5 mM $MgCl_2$, 1% glucose, and 10 μg/ml chloramphenicol until early stationary phase (OD_{600} = 1) at 37°C with shaking. These cells were diluted (1/100 dilution) into 50 ml of LB medium–5 mM $MgCl_2$ medium and were cultured at 37°C with shaking until the A_{600} reached about 1.0. Cells from 50 ml culture were washed and resuspended in 2 ml 20 mM HEPES-NaOH buffer, pH 7.5, containing 1 mM phenylmethanesulfonyl fluoride and were disintegrated with a French press. The crude cell extract was obtained after the removal of unbroken cells by low-speed centrifugation.

The membrane fraction was collected by high-speed centrifugation for 45 min at 60,000 rpm with a Beckman TLA100.2 ultracentrifuge. After removal of the supernatant, Sarkosyl (final concentration, 0.5%) was added to the pellet in 60 μl of the same buffer to solubilize mainly the inner membrane proteins. The suspension was centrifuged for 45 min at 60,000 rpm with the Beckman TLA100.2 ultracentrifuge to separate the inner membrane proteins from the Sarkosyl-insoluble fraction containing outer membrane fragments.

For analysis of the expression levels of periplasmic adaptor proteins, cells containing a plasmid-borne *adeA*-6His, *adeI*-6His, or *acrA*-6His gene or the vector only as a control were grown under the same conditions described above. We used the crude cell extract prepared by French press treatment, followed by low-speed centrifugation to remove unbroken cells.

SDS-PAGE and immunoblotting. All samples were dissolved in sample buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, and 0.01% bromphenol blue in 0.125 M Tris-HCl, pH 6.8). Samples corresponding to 10 μg of the inner membrane protein were separated by SDS-PAGE with 8% (wt/vol) acrylamide in the running gel. For immunoblotting, the proteins were transferred onto an Immobilon-P polyvinylidene difluoride membrane (Sigma) by electrophoretic blotting at 4°C for 1 h at 100 V. The membrane was blocked for 1 h with 5% dry milk in TNT buffer (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.05% Tween 80), followed by treatment for 1 h with primary antibody (monoclonal antibody against tetrahistidine; Qiagen) at a 1/1,000 dilution in 3% bovine serum albumin-TNT buffer. After washing the membrane three times with TNT buffer, the membrane was treated for 1 h with secondary antibody conjugated to alkaline phosphatase (Bio-Rad) at a 1/3,000 dilution in 1% dry milk in TNT buffer. After washing the membrane three times with TNT buffer, the histidine-tagged proteins were visualized with 5 ml color development solution containing 0.4 mg/ml nitroblue tetrazolium and 0.2 mg/ml 5-bromo-4-chloro-3-indolylphosphate in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$. All incubations and washings in the immunoblotting procedure were carried out at room temperature with agitation.

RESULTS AND DISCUSSION

The *adeABC* and *adeIJK* genes are expressed and functional in *E. coli*. Comparison of amino acid sequences between the corresponding components of AdeABC and AdeIJK from *A. baumannii* and AcrAB-TolC from *E. coli* showed a relatively high degree of identity (AdeB and AcrB, 49% identity; AdeJ and AcrB, 58% identity) among efflux pump proteins. The degree of identity among the membrane fusion proteins (AdeA and AcrA, 38% identity; AdeI and AcrA, 38% identity) was somewhat less, and these values became even lower among the outer membrane components (AdeC and TolC, 20% identity; AdeK and TolC, 20% identity).

A recent molecular dynamics simulation study of the binding of various substrates to the distal binding pocket of AcrB revealed important residues in this area, on the basis of the frequency of their contribution to binding (16). Here we compared these 14 residues in AcrB to the corresponding residues in AdeB and AdeJ (Table 2). Most residues were similar in AdeJ; exceptions were the R620V and I278D substitutions. Other substitutions resulted in amino acids with similar properties, as with S180G and I277F. However, in AdeB, I277, V612, F615, and R620 of AcrB were replaced by F, I, W, and A, respectively, suggesting that AdeB may have a range of substrates significantly different from the AcrB substrates.

The *adeABC* and *adeIJK* genes, as well as the *acrAB* genes as a reference, were cloned into medium-copy-number plasmid pKY9790 in the same orientation as the *Ptac* promoter and expressed in *E. coli* AG100AΩ (Δ*lacY*), a strain lacking the *acrAB* genes. These plasmid-borne genes were apparently expressed in the *E. coli* host, as they strongly increased the MIC values of several antibiotics, and the MICs were further increased by IPTG induction (Table 3). The outer membrane channel proteins were essential for this resistance (Table 3), suggesting that at least these and likely all components of the *A. baumannii* tripartite systems are needed for drug efflux. It is thus likely that with plasmids containing the entire efflux operons, all three components are indeed

TABLE 2 Residues in distal pocket having a higher contribution to substrate binding in AcrB than the corresponding residues in AdeB and AdeJ[a]

Residue in AcrB	Residue in:	
	AdeB	AdeJ
F136	F	F
V139	L	V
Q176	Q	Q
F178	F	F
G179	G	G
S180	A	G
I277	F	F
I278	A	D
Y327	Y	Y
F610	T	F
V612	I	V
F615	W	F
R620	A	V
F628	F	F

[a] Data for AcrB are from reference 16.

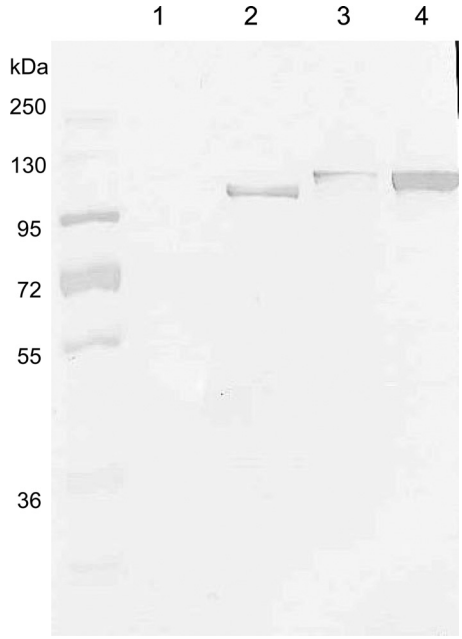

FIG 1 Expression levels of AdeB, AdeJ, and AcrB in the inner membrane fraction of AG100AΩ without induction. The inner membrane proteins (10 μg) in the sample buffer prepared as described in Materials and Methods were separated by SDS-PAGE with 8% (wt/vol) acrylamide in the running gel. Western immunoblots using antitetrahistidine antibody show the expression of RND pump proteins. Lane 1, AG100AΩ containing pKY9790; lane 2, AG100AΩ containing pKY-*adeAB*-6His; lane 3, AG100AΩ containing pKY-*adeIJ*-6His; lane 4, AG100AΩ containing pKY-*acrAB*-6His.

expressed to produce the resistance levels seen in Table 3. Finally, when the pKY plasmids carrying the His-tagged forms of the transporters (pKY-*adeAB*-6His, pKY-*adeIJ*-6His, and pKY-*acrAB*-6His) were used, we found that the AdeB and AdeJ proteins were clearly expressed, as assessed by Western blotting with a monoclonal antibody against tetrahistidine (Fig. 1), although their expression levels were somewhat lower than the AcrB expression level.

Adjustment of the expression levels of pump genes. In order to compare the activity of the AdeABC and AdeIJK systems with that of AcrAB-TolC, we tried to adjust the expression levels of these pump proteins through IPTG induction to a level comparable to the level of AcrB expression. In order to avoid the creation of a heterogeneous population by the use of suboptimal levels of IPTG, we used a mutant from which the *lacY* gene was deleted (see reference 17). An examination of the levels of the AdeB and AdeJ proteins by immunoblotting indicated that the cells induced with IPTG produced significantly more AdeB and AdeJ than the cells grown without IPTG (Fig. 2). Expression of AdeABC with 10 μM IPTG did not inhibit growth; the doubling time was 25.4 min, which was identical to the doubling time of AG100AΩ (Δ*lacY*) containing the vector alone. Use of 10 and 20 μM IPTG also increased the MIC values of AdeABC-producing cells (Table 3), but 50 μM IPTG led to the loss of the resistant phenotype (not shown); presumably, such a strong overexpression of AdeABC was toxic to the host *E. coli* cells. Similarly, expression of AdeIJK with 5 μM IPTG was nontoxic, as the doubling time was not increased, and this increased the MIC values (Table 3). However, 10 μM IPTG was the maximum concentration that could be used for the induction of AdeIJK, and higher concentrations resulted in growth inhibition, presumably because their overexpression was toxic, as described earlier (10). Because of this toxicity, we added 1% glucose to the preculture medium, as described in Materials and Methods, and cells at an OD_{600} of 1.0 instead of overnight cultures were used for MIC tests.

For the comparison of AdeABC with AcrAB-TolC, an examination of the levels of AdeB and AcrB by immunoblotting indicated that the cells expressing plasmid-borne *adeAB* genes with 10 μM IPTG and the cells expressing the *acrAB* genes without IPTG produced similar level of the transporters AdeB and AcrB (Fig. 3,

TABLE 3 MICs for AG100AΩ (Δ*lacY*) expressing plasmid-borne *adeABC* and *adeIJK* with different concentrations of IPTG[a]

Antibiotic	MIC (μg/ml) for AG100AΩ (Δ*lacY*) expressing the following plasmid with IPTG at the indicated concn (μM):										
	pKY9790			pKY-*adeABC*				pKY-*adeIJK*			
	0	10	20	0	10	20	pKY-*adeAB*, 10	0	5	10	pKY-*adeIJ*, 5
Oxacillin	2	2	2	50	200	200	2	100	400	800	1
Cloxacillin	4	4	4	50	100	100	4	200	400	800	2
Nitrocefin	2	2	2	15.6	62.5	125	4	62.5	125	250	2
Ethidium bromide	20	20	20	200	400	400	ND[b]	100	200	200	ND

[a] The MICs of *E. coli* AG100AΩ (Δ*lacY*) containing plasmid-borne *adeABC* and *adeIJK* with different concentrations of IPTG in the growth medium were determined as described in Materials and Methods.
[b] ND, not done.

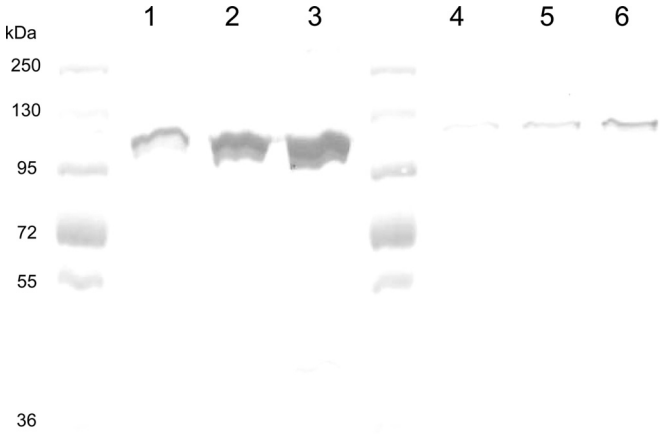

FIG 2 IPTG induces the expression of AdeB and AdeJ. The inner membrane proteins (10 μg) were prepared from AG100AΩ (Δ*lacY*) containing pKY9790-borne *adeAB*-6His and *adeIJ*-6His genes grown with the concentrations of IPTG specified below and were separated by SDS-PAGE. The expression levels of the AdeB and AdeJ proteins were determined by using immunoblotting with a monoclonal antibody against tetrahistidine, as described in Materials and Methods. Lane 1, AG100AΩ (Δ*lacY*) containing pKY-*adeAB*-6His without IPTG; lane 2, AG100AΩ (Δ*lacY*) containing pKY-*adeAB*-6His with 10 μM IPTG; lane 3, AG100AΩ (Δ*lacY*) containing pKY-*adeAB*-6His with 20 μM IPTG; lane 4, AG100AΩ (Δ*lacY*) containing pKY-*adeIJ*-6His without IPTG; lane 5, AG100AΩ (Δ*lacY*) containing pKY-*adeIJ*-6His with 5 μM IPTG; lane 6, AG100AΩ (Δ*lacY*) containing pKY-*adeIJ*-6His with 10 μM IPTG.

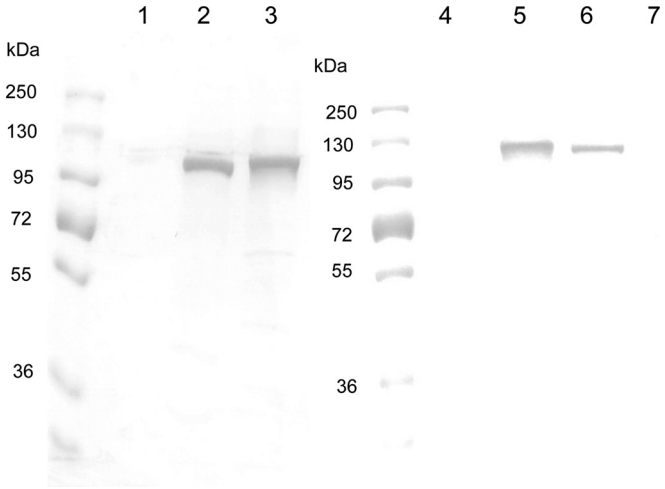

FIG 3 Adjustment of AcrB protein expression to a level comparable to the level of AdeB or AdeJ protein expression. (Lanes 1 to 3) In order to express similar levels of AdeB and AcrB proteins, AG100AΩ (Δ*lacY*) containing pKY-*adeAB*-6His was induced with 10 μM IPTG (lane 2) and the same strain containing pKY-*acrAB-4His* was grown without IPTG (lane 3); AG100AΩ (Δ*lacY*) containing pKY9790 alone was included in lane 1; (lanes 4 to 6) to express similar levels of AdeJ and AcrB, cells containing pKY-*adeIJ*-6His were induced with 5 μM IPTG (lane 5) and cells containing pHSG-*acrAB*-4His were induced with 500 μM IPTG (lane 6); cells containing pKY9790 alone (lane 4) and pHSG576 alone (lane 7) were included as controls. The inner membrane proteins (10 μg) were separated by SDS-PAGE, and the expression levels of the AdeB, AdeJ, and AcrB proteins were analyzed by using immunoblotting with monoclonal antibody against tetrahistidine.

lanes 2 and 3). However, the level of AdeJ protein in the cells expressing plasmid-borne *adeIJ* genes with 5 μM IPTG was significantly less than that of AcrB expressed from pKY-*acrAB* without induction (not shown). In order to create a situation in which the expression level of the AcrB protein is similar to that of the AdeJ protein, the *acrAB* genes were cloned into a low-copy-number plasmid, pHSG576. The level of AcrB protein (expressed from pHSG-*acrAB* with 500 μM IPTG) was similar to that of the AdeJ protein (expressed from pKY-*adeIJ* with 5 μM IPTG) (Fig. 3, lanes 5 and 6), thus making the functional comparison of AcrB and AcrJ possible. This induction condition did not affect the growth of cells expressing plasmid-borne *acrAB* genes (doubling times, 24 min with and without 500 μM IPTG).

We also checked the expression levels of periplasmic adaptor proteins (PAPs) by using 6His-tagged PAP genes expressed from pKY-*adeA*-6His, pKY-*adeI*-6His, pKY-*acrA*-6His, and pHSG-*acrA*-6His in AG100AΩ (Δ*lacY*). Determination of the levels of AdeA and AcrA by immunoblotting indicated that the cells expressing plasmid-borne *adeA* genes with 10 μM IPTG and the cells expressing the *acrA* genes without IPTG produced similar levels of the PAPs AdeA and AcrA (Fig. 4, lanes 2 and 3). The level of the AcrA protein (expressed from pHSG-*acrA* with 500 μM IPTG) was also similar to that of the AdeI protein (expressed from pKY-*adeI* with 5 μM IPTG) (Fig. 4, lanes 5 and 4). The expression conditions did not affect the growth of cells expressing genes borne by plasmids pKY-*adeA*, pKY-*adeI*, pKY-*acrA*, and pHSG-*acrA* (doubling times, 24, 27, 27, and 24 min, respectively).

Functional comparison of AdeABC and AdeIJK with AcrAB-TolC in the same host environment. The MIC values for various drugs in *E. coli* AG100AΩ (Δ*lacY*) expressing plasmid-borne *adeABC*, *adeIJK*, and *acrAB* genes under our induction conditions described above are summarized in Table 4. Because the expression levels of AdeB and AcrB are similar in columns 2 to 4 of Table 4, we can compare the function of AdeABC with that of AcrAB-TolC, if we assume that the former system is fully functional in the *E. coli* host. AdeABC appears to excrete a broad range of compounds similar to those excreted by AcrAB-TolC, but the efficiency of AdeABC for benzylpenicillin, cloxacillin, oxacillin, nitrocefin, novobiocin, and ethidium bromide efflux was much lower than the efficiency of AcrAB-TolC. However, AdeABC could excrete cefepime, tetracycline, minocycline, and ciprofloxacin better than AcrAB-TolC (Table 4).

Columns 5 to 7 of Table 4 show the consequences of AdeIJK expression, and comparison with the consequences of AcrAB expression shown in columns 2 to 4 shows that AdeIJK are expressed at a similar but lower level. The expression of plasmid-borne *adeIJK* genes remarkably increased the MIC levels of cloxacillin, oxacillin, nitrocefin, novobiocin, and ethidium bromide, in spite of the low level of expression, and the effect was much stronger than that of expression of the *acrAB* genes (Table 4). These results suggest that AdeIJK is a system optimized for the efflux of more lipophilic agents, consistent with the replacement of R620 in the AcrB distal binding site with valine in AdeJ (Table 2). Possibly, the introduction of phenylalanine at I277 of AcrB could also contribute to this effect, but the side chain of I278 is facing outward from the binding pocket in AcrB, and it is doubtful if its replacement by aspartate would affect ligand binding. AdeIJK produced only a modest or no increase in the MIC of relatively hydrophilic agents, such as ampicillin, cephaloridine, cephalothin, cefepime, tetracycline, minocycline, and fluoroquinolones. It also produced only a

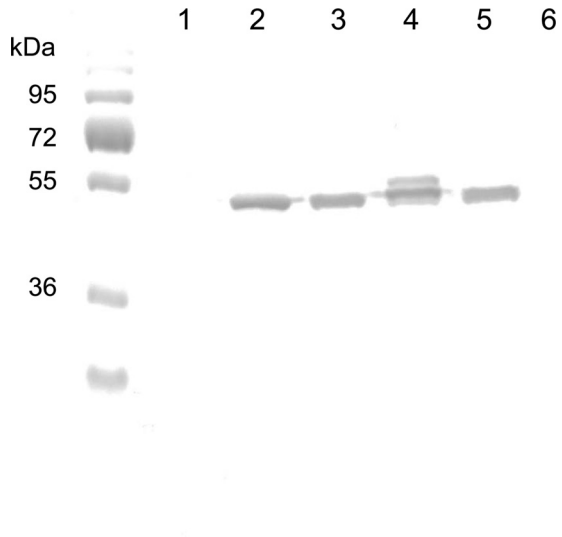

FIG 4 Expression levels of periplasmic adaptor proteins (PAPs) in AG100AΩ (Δ*lacY*). Plasmid-borne 6His-tagged PAP genes were expressed under the same conditions used in the assay whose results are presented in Fig. 3. Cells containing pKY-*adeA*-6His were induced with 10 μM IPTG (lane 2), and those containing pKY-*acrA*-6His were grown without IPTG (lane 3). Cells containing pKY-*adeI*-6His were induced with 5 μM IPTG (lane 4), and cells containing pHSG-*acrA*-6His were induced with 500 μM IPTG. Cells containing pKY9790 alone (lane 1) and pHSG576 alone (lane 6) were included as controls. The crude cell extracts (5 μg of protein) were separated by SDS-PAGE, and the expression levels of the AdeA, AdeI, and AcrA proteins were analyzed by using immunoblotting with a monoclonal antibody against tetrahistidine.

little increase in the MIC of erythromycin, a large, hydrophobic agent.

MIC changes due to efflux gene deletions in *A. baumannii* have been measured (6). Although these values have the advantage of being determined in the proper host strain with a very low outer membrane permeability (3), they have a major drawback as a measure of the capacity of individual efflux systems: because this species contains many efflux pumps, the effect of deletion of a single system can be masked by the activity of all other remaining systems. The first study of the RND efflux system in *A. baumannii* was performed by using an AdeABC-overproducing clinical isolate (9) and showed that the inactivation of this system produces a large (4- to 32-fold) decrease in the MICs of aminoglycosides, cefotaxime, tetracycline, erythromycin, chloramphenicol, trimethoprim, and fluoroquinolones, suggesting that this is a pump with an extremely wide substrate specificity. It is reassuring that the comparison of the Δ*adeIJK* and Δ*adeIJK* Δ*adeABC* strains, where the contribution from the remaining efflux systems is less (6), largely confirmed this conclusion, although there was no change in the cefotaxime MIC upon the deletion of the *adeABC* system. Another study (9) also failed to confirm the effect of AdeABC on β-lactam efflux. In contrast, our present plasmid-borne expression study (Table 4) showed us that AdeABC pumps out all β-lactams tested. β-Lactam efflux was likely masked by the highly expressed β-lactamase activity in *A. baumannii* but became evident in *E. coli*, where the endogenous β-lactamase is expressed only at a very low level (18). In quantitative terms, however, our results (Table 4) showed that the efflux of lipophilic β-lactams, novobiocin, and ethidium bromide by AdeABC was less than that by AcrAB-TolC when AdeABC and AcrAB-TolC were used at comparable levels, although AdeABC was more effective in pumping out tetracycline and minocycline, a result that is consistent with a significant difference in the amino acid sequences within the distal binding pocket (Table 2).

The deletion of *adeIJK* from the same clinical strain of *A. baumannii* showed that it pumps out β-lactams, chloramphenicol, and tetracyclines but not fluoroquinolones or macrolides (10). In the same study, we could also compare the MIC values of a Δ*adeABC* strain with those of a Δ*adeABC* Δ*adeIJK* double mutant, where the background contribution of AdeABC was removed (10). This comparison confirms the conclusions presented above and, in addition, shows that fluoroquinolones and macrolides are indeed substrates of AdeIJK. The findings of our heterologous expression study are consistent with these conclusions and, in addition, show that AdeIJK is even more powerful than the equivalent level of AcrAB-TolC in pumping out lipophilic β-lactams, novobiocin, and ethidium bromide (Table 4). Thus, although the expression level of the AdeJ protein was very low compared with that of AdeB and AcrB, the AdeIJK system, surprisingly, increased the MICs for cloxacillin, oxacillin, and nitrocefin by a factor of more than 100, suggesting that this pump is very efficient for these compounds, even at a low expression level. In contrast, the expression level of the AdeIJK system did not increase the level of resistance to erythromycin (or tetracyclines or fluoroquinolones) much in comparison with that of AcrAB, which was rather effective (Table 4). In *A. baumannii*, AdeIJK has a strong effect on the MIC values of these compounds (6), possibly, again, because of

TABLE 4 Comparison of MIC changes due to the expression of plasmid-borne *adeABC*, *adeIJK*, and *acrAB* genes

Antibiotic	MIC[a] (μg/ml) for AG100AΩ (Δ*lacY*) expressing RND pump genes from:					
	pKY9790			pHSG576 and pKY9790		
	pKY9790	pKY-*adeABC*	pKY-*acrAB*	pHSG576	pKY-*adeIJK*	pHSG-*acrAB*
Ampicillin	2	8	16	2	4	8
Benzylpenicillin	16	16	64	16	32	32
Cloxacillin	4	100	1,000	2	400	100
Oxacillin	2	200	1,000	2	400	100
Cephaloridine	2	8	8	4	8	4
Cephalothin	2	8	8	4	8	4
Cefepime	0.03	0.5	0.1	0.03	0.06	0.03
Nitrocefin	2	62	500	2	125	62
Erythromycin	20	640	1,000	20	40	125
Rifampin	16	32	32	32	16	32
Novobiocin	8	25	2,000	16	200	50
Tetracycline	4	100	25	4	4	6
Minocycline	1	50	25	2	4	6
Kanamycin	4	4	4	4	4	4
Gentamicin	2	2	2	2	2	2
Ciprofloxacin	0.03	0.5	0.25	0.03	0.06	0.06
Norfloxacin	0.25	2.0	2.5	0.12	0.30	0.31
Ethidium bromide	20	400	1,500	25	200	50

[a] MICs for various antibiotics due to the expression of plasmid-borne *adeABC*, *adeIJK*, and *acrAB* genes were analyzed in *E. coli* AG100AΩ (Δ*lacY*), and changes in the MICs for kanamycin and gentamicin were analyzed in AG100AΩ. In order to adjust the levels of expression of the efflux pumps, the *adeABC* genes were induced with 10 μM IPTG and the *adeIJK* genes were induced with 5 μM IPTG. The *acrAB* genes in a low-copy-number plasmid were induced with 500 μM IPTG. MIC tests were performed as described in Materials and Methods.

TABLE 5 MICs of *E. coli* AG100AΩ (Δ*acrAB* Δ*lacY*) and FHU-100 (Δ*acrAB* Δ*tolC*) expressing plasmid-borne efflux genes

Antibiotic	MIC (μg/ml) for the following host strain in which the indincated efflux system genes were expressed:										
	AG100AΩ (Δ*acrAB* Δ*lacY*)[a]						FHU-100 (Δ*acrAB* Δ*tolC*)[b]				
	None	*adeAB*	*adeABC*	*adeABK*	*adeIJ*	*adeIJK*	None	*adeAB*	*adeABC*	*adeIJ*	*adeIJK*
Ampicillin	2	4	4	4	2	4	4	8	8	4	4
Cloxacillin	2	4	160	50	2	400	1	1	100	1	512
Oxacillin	1	2	160	100	1	400	1	1	50	1	256
Cephaloridine	2	4	8	8	2	8	4	4	4	4	4
Cephalothin	2	2	8	8	4	8	8	8	8	8	8
Cefepime	0.03	0.03	0.50	0.50	0.03	0.06	0.03	0.03	0.12	0.03	0.03
Nitrocefin	2	4	64	64	2	250	2	2	32	4	256
Erythromycin	20	80	640	640	40	40	10	10	320	10	20
Novobiocin	8	8	32	16	10	100	2	2	16	2	128
Tetracycline	4	4	200	100	4	4	2	2	32	2	2
Minocycline	1	2	50	25	4	8	2	2	16	2	4
Ciprofloxacin	0.03	0.05	0.50	0.25	0.02	0.05	0.02	0.02	0.16	0.02	0.04
Norfloxacin	0.25	0.25	2.50	1.25	0.2	0.4	0.1	0.1	0.4	0.1	0.4

[a] The MICs for AG100AΩ (Δ*acrAB* Δ*lacY*) were determined using a standard 2-fold broth microdilution method with LB broth containing 5 mM $MgCl_2$ and 10 μM IPTG for the *adeAB*, *adeABC*, and *adeABK* genes and 5 μM IPTG for the *adeIJ* and *adeIJK* genes. The genes were cloned in the vector pKY9790.
[b] The MICs for FHU-100 (Δ*acrAB* Δ*tolC*) were determined under the same conditions described in footnote *a* for AG100AΩ (Δ*acrAB* Δ*lacY*), but without IPTG.

the synergy with the low permeability of the outer membrane. Finally, we were able to see that the AdeIJK system is indeed an efficient efflux pump for a broad range of compounds, similar to the AcrAB-TolC system (Table 4).

One weakness of the heterologous expression assay in an *E. coli* host is the difficulty in detecting aminoglycoside efflux. This is because aminoglycosides, being basic and very hydrophilic, presumably penetrate through the trimeric porin channels of *E. coli* extremely rapidly. Because increases in MICs require the synergistic interaction between the outer membrane permeability barrier and RND-type pumps (19), aminoglycoside efflux is nearly impossible to detect in *E. coli* by the measurement of MICs and required an assay with a reconstituted transporter (in this case, AcrD) (20). Indeed, increases in aminoglycoside MICs were not detected in the *E. coli* host (Table 4), although MIC values were clearly decreased upon the deletion of *adeABC* (9) and increased by the overexpression of *adeABC* (6) in *A. baumannii*, which has an outer membrane with a very low permeability (3).

Outer membrane components for the AdeAB system. The *adeABC* operon is not present in all *A. baumannii* strains, and the gene for the outer membrane protein (*adeC*) was not found in almost 41% of clinical isolates carrying *adeRS-adeAB* (8), suggesting that AdeC is not the only outer membrane channel that could function with the AdeAB system and AdeAB could recruit another outer membrane channel. This was further supported by the observation that the deletion of the *adeC* gene in *A. baumannii* did not alter the multidrug-resistant phenotype (7). As *adeIJK* genes exist in all strains of *A. baumannii*, it seemed possible that AdeAB may form a complex with AdeK and may function as a multidrug efflux pump. Therefore, the *adeAB* genes were linked to the *adeK* gene, and plasmid-borne *adeAB-adeK* genes were expressed in AG100AΩ. The expression of plasmid-borne *adeAB-adeK* genes produced an MIC profile almost identical to that produced by expression of the *adeABC* genes (Table 5), indicating that the outer membrane components (AdeC and AdeK) are interchangeable.

We also examined if TolC could complement the function of AdeC or AdeK by expressing plasmid-borne *adeAB* genes or *adeIJ* genes alone in *E. coli* AG100AΩ (Δ*acrAB*). The expression of the *adeAB* genes alone did not change the MICs for various drugs, suggesting that AdeAB could not interact with TolC as a functional efflux pump system. A similar experiment was performed for the AdeIJ-AdeK system. The expression of plasmid-borne *adeIJ* genes alone did not show any changes in MIC levels for the drugs tested, suggesting that three components (AdeIJ-AdeK) are necessary for the function.

When a host strain (FHU-100) also lacking TolC, in addition to AcrAB, was used for a similar experiment with the plasmid-borne *adeABC*, the results were rather similar to those obtained with the Δ*acrAB* host strain (Table 5), although the MIC values were often somewhat lower, presumably because of the absence of IPTG induction. However, the plasmid carrying *adeIJK* was, unexpectedly, unstable in FHU-100, and the growth rate of the host strain (doubling time, 26.5 min) was affected by the presence of plasmid-borne *adeIJK* (doubling time, 34.6 min). We were able to detect increases in MIC levels only by using a freshly transformed colony of FHU-100 containing plasmid-borne *adeIJK*, which appeared small and thin. Although MIC tests were performed without IPTG induction, plasmid-borne *adeIJK* increased the MIC values to values similar to those obtained with the Δ*acrAB* host strain (Table 5). These results confirm that both the AdeAB and AdeIJ systems were fully using their own cognate outer membrane channel proteins, AdeC and AdeK, in the *E. coli* host strains.

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