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# AdeIJK, a Resistance-Nodulation-Cell Division Pump Effluxing Multiple Antibiotics in *Acinetobacter baumannii*▽

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**We have identified a second resistance-nodulation-cell division (RND)-type efflux pump, AdeIJK, in clinical isolate *Acinetobacter baumannii* BM4454. The *adeI*, *adeJ*, and *adeK* genes encode, respectively, the membrane fusion, RND, and outer membrane components of the pump. AdeJ belongs to the AcrB protein family (57% identity with AcrB from *Escherichia coli*). mRNA analysis by Northern blotting and reverse transcription-PCR indicated that the genes were cotranscribed. Overexpression of the cloned *adeIJK* operon was toxic in both *E. coli* and *Acinetobacter*. The *adeIJK* genes were detected in all of the 60 strains of *A. baumannii* tested. The two latter observations suggest that the AdeIJK complex might contribute to intrinsic but not to acquired antibiotic resistance in *Acinetobacter*. To characterize the substrate specificity of the pump, we have constructed derivatives of BM4454 in which *adeIJK* (strain BM4579), *adeABC* (strain BM4561), or both groups of genes (strain BM4652) were inactivated by deletion-insertion. Determination of the antibiotic susceptibility of these strains and of BM4652 and BM4579, in which the *adeIJK* operon was provided in *trans*, indicated that the AdeIJK pump contributes to resistance to β-lactams, chloramphenicol, tetracycline, erythromycin, lincosamides, fluoroquinolones, fusidic acid, novobiocin, rifampin, trimethoprim, acridine, safranin, pyronine, and sodium dodecyl sulfate. The chemical structure of these molecules suggests that amphiphilic compounds are the preferred substrates. The AdeABC and AdeIJK efflux systems contributed in a more than additive fashion to tigecycline resistance.**

*Acinetobacter baumannii* is a gram-negative, nonfermentative bacillus that has emerged as a major nosocomial infectious agent over the last 20 years. It is responsible for pneumonia, bacteremia, urinary tract infections, septicemia, and meningitis (3). Clinical isolates of *Acinetobacter* are often resistant to a wide range of antimicrobials because of the capacity of this species to acquire and accumulate resistance determinants. Multidrug resistance in this bacterial genus often renders *Acinetobacter* infections difficult to eradicate, and thus combination therapy is required for treatment (3).

Multiresistance to antibiotics in gram-negative bacteria is due to the association of various mechanisms, including low membrane permeability, drug inactivation, target bypass or alteration, and active efflux. To date, two efflux systems have been described in *Acinetobacter* (5, 14). AdeABC, identified in *A. baumannii* BM4454, is responsible for resistance to aminoglycosides, tetracycline, erythromycin, chloramphenicol, trimethoprim, and fluoroquinolones (14). The AdeDE efflux pump has been reported to confer resistance to amikacin, ceftazidime, chloramphenicol, ciprofloxacin, erythromycin, meropenem, rifampin, and tetracycline in *Acinetobacter* genomic DNA group 3 (5, 6). These pumps belong to the resistance-nodulation-cell division (RND) family and are involved in the export of a wide spectrum of compounds.

RND systems are ubiquitous in gram-negative bacteria. The pumps form tripartite complexes composed of a periplasmic membrane fusion protein, an inner membrane RND transporter, and an outer membrane factor (OMF) (18, 24). The three components are most often encoded by operons, as is the case of the AdeABC pump (15). In other instances, the structural gene for the OMF is not part of the gene cluster, as for AdeDE (5).

In this study, we have identified and characterized the *adeI*, *adeJ*, and *adeK* genes from *A. baumannii* BM4454, which encode a three-component RND efflux system consisting of AdeI, which is similar to membrane fusion proteins; AdeJ, which is similar to RND proteins; and AdeK, which is similar to OMFs. The three genes are widely distributed in *A. baumannii* species and are cotranscribed. We show that this system contributes to resistance to numerous antibiotics, including β-lactams, chloramphenicol, tetracycline, erythromycin, lincosamides, fluoroquinolones, fusidic acid, novobiocin, rifampin, and trimethoprim. Moreover, the AdeIJK pump can extrude acridine, pyronine, safranin, and sodium dodecyl sulfate (SDS) but not ethidium bromide.

(An initial report of this work was presented at the 44th Interscience Conference on Antimicrobial Agents and Chemotherapy [L. Damier et al., 44th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1-1202, 2004].)

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Multiresistant strain BM4454, isolated from a patient with a urinary tract infection and overexpressing the AdeABC pump, and susceptible *A. baumannii* CIP 70-10 and CIP 70-21 have

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TABLE 1. Oligonucleotides used in this study

| Primer | Sequence[a] |
|---|---|
| D1 | 5′-(C/A)G(A/T)CC(A/T)GT(A/T)TT(T/C)GC(A/T)TGGGT-3′ |
| D2 | 5′-AC(A/T)AC(A/T)AT(A/T)GC(A/G)TC(A/G)TC(A/T)AC-3′ |
| AP1 | 5′-CATACGTTATGCCGTTACAGCTTC-3′ |
| AP2 | 5′-CCATGAGAAACTGATTGCAGCTC-3′ |
| AP3 | 5′-GTAGATCAGGCTCAAGCTGGTG-3′ |
| AP15 | 5′-GCATGCCATGGGTATGATGTCGGCTAAGCTTTG-3′ |
| AP16 | 5′-TCCGCTCGAGTTATTGCTTTTTAAGTTCAGCAC-3′ |
| Ori1 | 5′-GCCACATGTGATCGTAGAAATATCTATGA-3′ |
| Ori2 | 5′-GCCACATGTCGGATTTTAACATTTTGCG-3′ |
| AP21 | 5′-GCGATAAAGTCATTGTTGATGGTG-3′ |
| AP8r | 5′-CAGCATAGAGCACGCCAGAGAAG-3′ |
| AP29 | 5′-CTTTCTTTGGTGGTACAACAGGTG-3′ |
| AP7r | 5′-CGCAAGTGCAGACACCGCAATG-3′ |
| S1 | 5′-TCCCAAGCTTGGCTAACCAAATCCAGTC-3′ |
| S2r | 5′-TCCCAAGCTTACTGTCCAAACCTAGTGAG-3′ |
| C1 | 5′-CAGCTCTAGATTTACGGACTGCTACGC-3′ |
| C2r | 5′-CAGCTCTAGAGTAAGCCTTGTTGTGCAGC-3′ |
| AACf | 5′-TACTGCAGTTGACACAGTGCCGTTGATCGTGCTATG-3′ |
| AACr | 5′-ATTCTAGACTCATGAGCTCAGCCAATCGACTG-3′ |

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

been described previously (4, 14). Fifty-eight clinical isolates of *A. baumannii* from our laboratory collection were studied. *Escherichia coli* TOP10 and DH5α (Invitrogen, Groningen, The Netherlands) were used as hosts for gene cloning and protein overexpression. *E. coli* AG100A (Δ*acrAB*) was used to overexpress AdeIJK (20). The strains were grown at 37°C in brain heart infusion broth and agar or in LB medium (Difco Laboratories, Detroit, MI).

**Susceptibility testing.** Antibiotic susceptibility was tested by disk diffusion on Mueller-Hinton agar (Bio-Rad, Marnes-la-Coquette, France). The MICs of antibiotics were determined on solid medium by the E-test procedure (AB Biodisk, Solna, Sweden) or by agar dilution with $10^4$ CFU/spot (7). Isopropyl-β-D-thiogalactopyranoside (IPTG), 0.005 mM, was added to test the susceptibility of BM4652 and BM4579 harboring plasmid pAT807.

**DNA manipulations.** *A. baumannii* genomic DNA was extracted as described previously (1). Plasmid DNA was prepared by boiling (8) or with a Wizard minipreps DNA kit (Promega, Madison, WI). Digestion of DNA by restriction endonucleases was carried out according to the supplier's recommendations. DNA fragments were purified from agarose gels with a QIAquick gel extraction kit (Qiagen, Hilden, Germany). Ligation, transformation, and agarose gel electrophoresis were performed as described previously (26). Sequence determination was carried out with a CEQ 2000 DNA Analysis System automatic sequencer (Beckman Instruments, Inc., Palo Alto, CA) according to the manufacturer's recommendations.

**Cloning of the *adeIJK* gene cluster.** With degenerate primers D1 and D2 (Table 1), designed from conserved regions of RND genes, a 1.2-kb fragment was amplified from BM4454 total DNA. The flanking sequences were obtained by thermal asymmetric interlaced PCR with arbitrary degenerate primers and gene-specific primers (13). The four random primers AD1, AD2, AD3, and AD4 were as previously described (13). Three nested primers, AP1, AP2, and AP3 (Table 1; Fig. 1), specific for internal *adeB* sequences, were used successively in individual PCRs in combination with each of the random primers as previously described (9). The PCR products were purified and sequenced. The flanking regions were reamplified independently by PCR, and the products were resequenced.

**Computer analysis of sequence data.** Amino acid sequences were analyzed using the GCG sequence analysis software package (version 10.1; Genetics Computer Group, Madison, WI). The GenBank and protein databases were screened for sequence similarities.

**RNA analysis.** *A. baumannii* total RNA extracted from an exponential-phase culture (optical density at 600 nm = 0.6) with a FastRNA Pro Blue kit (Q-biogen, Montreal, Canada) was treated with RNase-free DNase (Roche, Mannheim, Germany) for 60 min at 37°C, digested with proteinase K (Eurobio, Les Ulis, France), extracted with phenol, and precipitated. Reverse transcription-PCR was

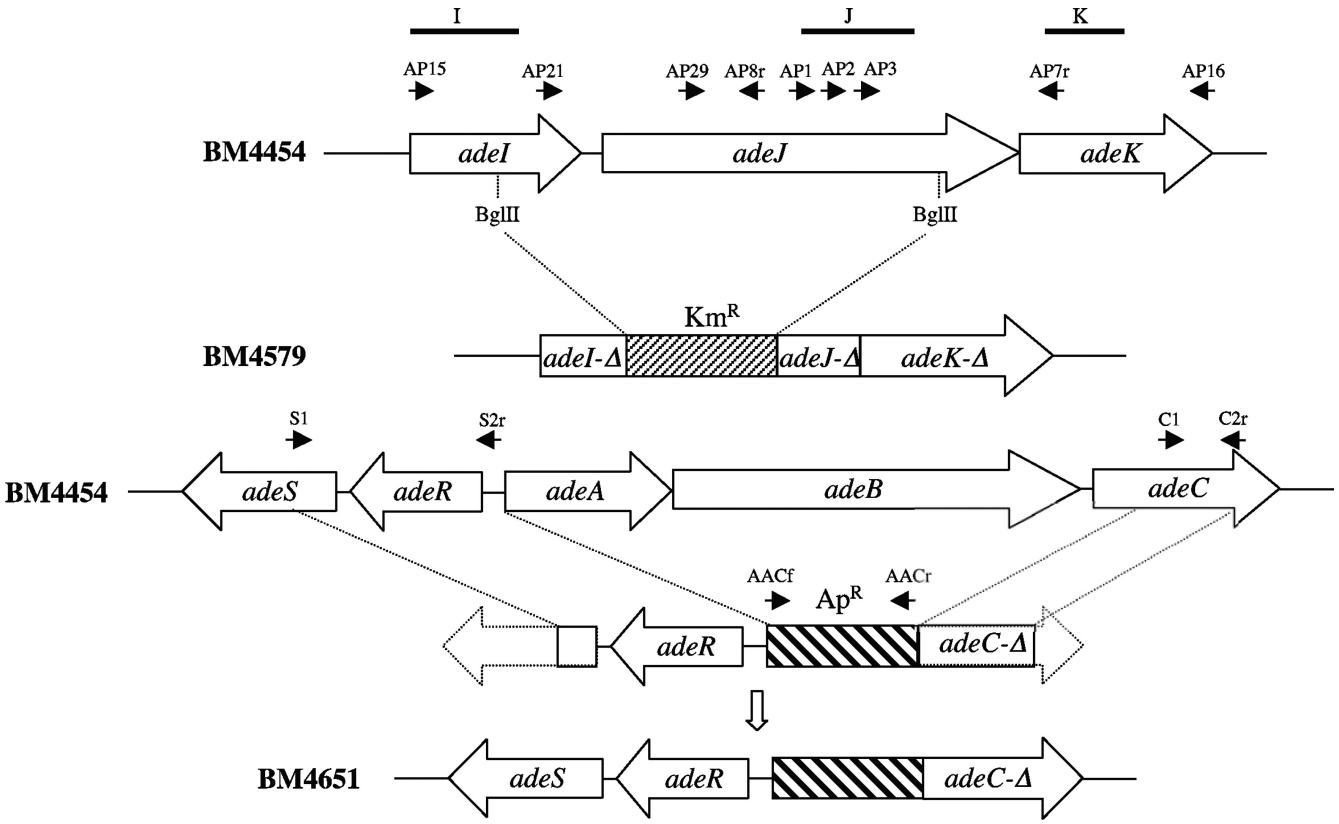


FIG. 1. Schematic representation of the *adeIJK* and *adeABC* operons from *A. baumannii* BM4454 and derivatives BM4579 and BM4651. Open arrows represent coding sequences and indicate the sense of transcription. The regions corresponding to the kanamycin and apramycin resistance genes ($Km^R$ and $Ap^R$, respectively) are hatched. Black arrows indicate the positions and orientations of the primers used. The probes are represented by thick black horizontal lines.

TABLE 2. Identities of AdeI, -J, and -K with related proteins

| Protein and related protein[a] | % Identity |
|---|---|
| AdeI | |
| AdeI from *A. baumannii* AYE (CAJ77872.1) and ATCC 17978 (ABO13128.1) | 100 |
| AdeX from *Acinetobacter* genomic DNA group 3 (ABB30248.1) | 97 |
| RND family drug transporter from *A. baylyi* ADP1 (CAG69658.1) | 80 |
| MDR protein from *Geobacter sulfurreducens* (AAR36069.1) | 49 |
| AdeJ | |
| AdeJ from *A. baumannii* AYE (CAJ77871.1) and ATCC 17978 (ABO13149.1) | 99 |
| AdeY from *Acinetobacter* genomic DNA group 3 (ABB30249.1) | 97 |
| RND family drug transporter from *A. baylyi* ADP1 (CAG69659.1) | 88 |
| RND family drug transporter from *Caulobacter* sp. strain K31 (EAU11518.1) | 61 |
| AdeK | |
| AdeK from *A. baumannii* AYE (CAJ77869.1) and ATCC 17978 (ABO13150.1) | 100 |
| AdeZ from *Acinetobacter* genomic DNA group 3 (ABB30250.1) | 97 |
| Outer membrane protein from *A. baylyi* ADP1 (CAG69660.1) | 88 |
| AdeC from *A. baumannii* BM4454 (AAL14441.1) | 61 |

[a] Accession numbers are in parentheses.

performed with the Titan One Tube PCR System (Roche). For Northern hybridization, the probes were prepared with the Megaprime DNA labeling system (Amersham Biosciences, Orsay, France). The 0.7-kb *adeI* and 0.4-kb *adeK* probes were located in the 5′ moiety of the corresponding genes, whereas the *adeJ* probe (0.7 kb) was complementary to the 3′ portion of the gene. Twenty micrograms of RNA was separated by electrophoresis on a 1% agarose-formaldehyde gel, transferred onto a nylon membrane, and hybridized with *adeIJ* and *adeIK* probes at 65°C in Church buffer (0.5 M sodium phosphate [pH 7.2], 7% SDS, 1 mM EDTA [pH 8.0], 1% bovine serum albumin). The membrane was washed twice and autoradiographed.

**Construction of expression plasmids pAT806, pAT807, and pAT810.** A PCR product of 5,900 bp, corresponding to the *adeIJK* genes, was amplified with primers AP15 and AP16, which introduced NcoI and XhoI restriction sites, respectively (Table 1). The restricted fragment was ligated into pTrc99A DNA digested with NcoI and SalI, leading to recombinant plasmid pAT806. The sequence of the insert was determined. The same PCR product was inserted into the NcoI-XhoI-linearized pBAD/Myc-HisA vector (Invitrogen), generating pAT810. A PCR product containing the replication origin of cryptic plasmid pWH1277 from *A. lwoffii* DSM30013 (11) was amplified with primers Ori1 and Ori2, each containing a PciI restriction site (Table 1). The PciI-restricted DNA fragment was cloned into PciI-linearized pAT806, generating shuttle expression plasmid pAT807, which was electrotransferred into *A. baumannii* BM4454 and CIP70-10.

**Overexpression of the *adeIJK* genes.** *E. coli* DH5α and AG100A harboring expression plasmid pAT806 and *E. coli* TOP10 harboring pAT810 were grown in LB broth containing 75 μg/ml ampicillin. *A. baumannii* BM4454 and CIP70-10 containing pAT807 were grown in brain heart infusion broth containing 80 μg/ml ticarcillin. The cultures were incubated at 37°C until the optical density at 600 nm reached 0.5 to 0.6, and expression of the *adeIJK* genes was induced for 4 h at 37°C by addition of 0.005, 0.05, 0.1, or 1 mM IPTG for strains containing pAT806 and pAT807 or by addition of arabinose (0.00002 to 0.2%) for *E. coli* TOP10/pAT810. Overexpression of AdeIJK in crude extracts was screened for by SDS-polyacrylamide gel electrophoresis.

**Inactivation of efflux genes.** The 5,900-bp *adeIJK* product amplified with primers AP15 and AP16 (Fig. 1) was subcloned into the pCR-Blunt vector (Invitrogen) and subsequently into BamHI-XhoI-linearized pBluescriptSK$^+$. The resulting plasmid, pAT808, was digested by BglII to delete a 3,646-bp fragment internal to the *adeIJ* genes and purified on agarose gel. The BamHI-kanamycin resistance cassette from the pUC-4K vector (Amersham Biosciences, Uppsala, Sweden) was cloned into BglII-linearized pAT808, generating plasmid pAT809, which was introduced into BM4454 by electrotransformation. Since pAT809 does not replicate in *A. baumannii*, the transformants selected for kanamycin resistance and screened for ticarcillin susceptibility should be the result of a double homologous recombination event leading to partial deletion-inactivation of *adeIJ*. The genetic construction was confirmed by PCR and sequencing in transformant BM4579. A 1,116-bp *adeSR* product amplified from BM4454 DNA with primers S1 and S2r (Table 1) was cloned into pCR-Blunt. The resulting plasmid was digested with PstI and XbaI to clone a PstI-XbaI apramycin cassette obtained by PCR of the *aac(3)-IV* gene with its promoter (accession number X01385) with primers AACf and AACr (Table 1). The plasmid was linearized by XbaI to clone an XbaI intragenic *adeC* PCR fragment obtained from BM4454 with primers C1 and C2r (Table 1). The construction was verified by PCR and sequencing and introduced by electroporation into strains BM4454 and BM4579, leading to BM4651 and BM4652, respectively. Deletion in the *adeABC* genes in these derivatives was confirmed by PCR analysis.

**Nucleotide sequence accession number.** The 8,281-bp sequence of *A. baumannii* BM4454 has been deposited in the GenBank data library under accession no. AY769962.

## RESULTS AND DISCUSSION

**Detection and cloning of an RND efflux system.** With degenerate primers D1 and D2 (Table 1), designed to anneal to sequences deduced from those at the N terminus and in the conserved D motif of the RND protein family, respectively, a 1.2-kb fragment was amplified from BM4454 total DNA and sequenced. Comparison of the deduced sequence with those in the SwissProt protein database revealed 46 to 62% identity with internal portions of other members of the RND family in various genera. The sequences flanking the PCR product were obtained by thermal asymmetric interlaced PCR, and three open reading frames (ORFs) were identified. The first ORF, *adeI*, of 1,251 bp, encoded the 416-residue AdeI protein, which was similar to membrane fusion proteins (Table 2). The second, *adeJ*, of 3,177 bp, encoded AdeJ, of 1,058 amino acids, which was similar to RND efflux proteins (Table 2). The third ORF (1,455 bp), designated *adeK*, specified the 484-residue AdeK protein, which belonged to the outer membrane protein family. The organization of the three adjacent genes suggests that they formed an operon, similar to the AdeABC efflux system (15).

Transmembrane topology of AdeJ was predicted with the MEMSAT program of the PSIPRED server. As expected, AdeJ contained 12 transmembrane domains (TMDs) and two large periplasmic loops between TMDs 1 and 2 and TMDs 7 and 8. Comparison of the AdeJ sequence with those of AcrB and MexB showed 58% identity; the most conserved regions corresponded to the TMDs, whereas greater variability was observed among sequences of the loops.

**Distribution of the *adeIJK* genes.** The presence of the *adeIJK* genes was detected by PCR in susceptible *A. baumannii* CIP

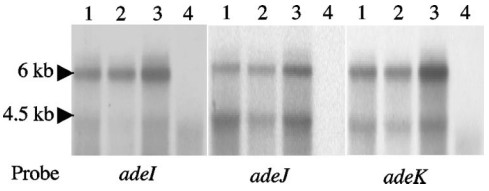


FIG. 2. Analysis of the transcription of the *adeIJK* genes by Northern hybridization. Total RNAs from BM4454 (lane 1), CIP 70-10 (lane 2), CIP 70-21 (lane 3), and BM4579 (lane 4) were hybridized with the probes indicated at the bottom. The sizes of the transcripts relative to RNA molecular weight marker I (Roche) are indicated at the left.

70-10 and CIP 70-21 and in 58 *A. baumannii* clinical isolates from our laboratory collection (data not shown), suggesting that the AdeIJK pump is intrinsic to this species.

**Analysis of *adeIJK* transcripts.** Transcription of the *adeIJK* gene cluster was analyzed by reverse transcription-PCR and Northern hybridization. Primer AP8r, specific for an internal fragment of *adeJ*, was used to produce cDNA from total RNA of *A. baumannii* BM4454, CIP 70-10, and CIP 70-21 (Fig. 1; Table 1). The cDNA was amplified with the same primer and AP21, which is complementary to an internal sequence of *adeI* (Fig. 1; Table 1). Similarly, with cDNA generated with primer AP7r, an *adeJK* fragment was amplified with primers AP29 and AP7r, which are complementary to *adeJ* and *adeK*, respectively (Fig. 1; Table 1). Products of the expected sizes were obtained from the three strains (data not shown). Amplification of the overlapping *adeIJ* and *adeJK* fragments indicated that the *adeIJK* genes are cotranscribed. These results were confirmed by Northern hybridization with probes specific for each of the *adeI*, *adeJ*, and *adeK* genes (Fig. 1 and 2). A transcript of approximately 6 kb, which cohybridized with the three probes, was detected, confirming cotranscription of the three genes. Another transcript of ca. 4.5 kb that hybridized with the *adeJ* and *adeK* probes was also detected. This transcript could correspond to a degradation product of the *adeIJK* transcript or could be generated by an independent transcriptional start site.

**Overexpression of the *adeIJK* genes in *E. coli* and *A. baumannii*.** To identify the substrates of the pump, the *adeIJK* gene cluster was expressed under the control of the P*trc* inducible promoter of plasmid pAT806 in *E. coli* DH5α and in AG100A, a strain lacking the *acrAB* genes (20). Expression was induced with 0.005, 0.05, 0.1, or 1 mM IPTG, and the growth of the bacteria was monitored for 4 h. The induction conditions from 0.05 to 1 mM IPTG were lethal for the cells, whereas growth was inhibited by 0.005 mM. The *adeIJK* cluster was thus cloned into *E. coli* TOP10 in the pBAD/*Myc*-HisA vector under the control of the more tightly regulated *araBAD* promoter. Induction of *adeIJK* expression was tested with several arabinose concentrations. Addition of 0.2, 0.02, or 0.002% arabinose in the culture medium prevented bacterial growth, but addition of 0.0002 or 0.00002% did not (data not shown). SDS-polyacrylamide gel electrophoresis analysis of crude extracts obtained from cultures induced with 0.0002% arabinose did not reveal any overproduced protein (data not shown), and the antibiotic susceptibility of the strain was not affected at this concentration, as tested by disk diffusion.

To study overexpression of the *adeIJK* genes in *Acinetobacter*, shuttle plasmid pAT807 was introduced by electrotransformation into BM4454 and CIP 70-10 and expression was induced with 0.005, 0.1, or 1 mM IPTG. In BM4454(pAT807), induction markedly inhibited bacterial growth, whereas in CIP 70-10(pAT807), growth inhibition was less pronounced. For both strains, no differences in antibiotic susceptibility were observed by disk diffusion on agar containing 0.005 mM IPTG, whereas at 0.1 and 1 mM IPTG, growth was inhibited and susceptibility to ticarcillin was restored because of the loss of plasmid pAT807. Similar growth inhibition was observed when pAT807 was introduced into Δ*adeIJK* derivatives BM4652 and BM4579 in the presence of IPTG. These observations suggest that overexpression of the AdeIJK pump could be toxic in both *E. coli* and *A. baumannii*.

Expression of multidrug transporter genes is frequently regulated by both global and specific mechanisms. The fact that overexpression of the *adeIJK* operon appeared to be toxic in both *E. coli* and *Acinetobacter* could suggest that expression of the gene cluster is subject to tight regulation. Since no ORFs coding for regulatory proteins were found in the vicinity of the *adeIJK* genes, expression does not seem to be specifically regulated but could be subject to control at a global level.

**Inactivation of the *adeIJ* and *adeABC* genes.** To characterize its function, the AdeIJK pump was inactivated in BM4454 by partial replacement of the *adeIJ* genes with a kanamycin resistance cassette following homologous recombination (Fig. 1). The double crossing over was confirmed by PCR and sequencing of derivative BM4579 (Δ*adeIJK*). Northern analysis of BM4579 (Δ*adeIJK*) total RNA indicated that the deletion-insertion had a polar effect on the expression of the downstream *adeK* gene, based on the lack of an *adeK* transcript (Fig. 2). To determine the substrate range of the AdeIJK pump, the *adeABC* genes were inactivated by an apramycin resistance cassette in parental BM4454, generating strain BM4651 (Δ*adeABC*) (Fig. 1), and in BM4579 (Δ*adeIJK*), leading to BM4652 (Δ*adeABC* Δ*adeIJK*). These constructs were confirmed by PCR.

**Substrate specificity of the AdeIJK pump.** The antibiotic susceptibility of BM4454 and derivatives BM4579 (Δ*adeIJK*), BM4651 (Δ*adeABC*), BM4652 (Δ*adeABC* Δ*adeIJK*), BM4579/pAT807 (*adeIJK*), and BM4652/pAT807 (*adeIJK*) was studied by disk diffusion (data not shown), and MICs were determined by agar dilution or by E-test (Table 3). The activity of aminoglycosides could not be assessed because of the presence of kanamycin or apramycin resistance cassettes in the mutant derivatives. Strain BM4579 (Δ*adeIJK*) was more susceptible than parental strain BM4454 to a wide range of antibiotics, including β-lactams, chloramphenicol, tetracyclines, and erythromycin, but not azithromycin, lincosamides, or rifampin, and also slightly to fluoroquinolones. Fusidic acid, novobiocin, and trimethoprim were also found to be substrates for AdeIJK (data not shown). Strain BM4651 (Δ*adeABC*) was less resistant to chloramphenicol, tetracyclines, erythromycin, and fluoroquinolones, as already reported (14), and was exquisitely susceptible to azithromycin. The contrasting behaviors of the two macrolides is surprising since azithromycin derives from erythromycin by the incorporation of a methyl-substituted nitrogen in the lactone ring. The susceptibility of the BM4652 (Δ*adeABC* Δ*adeIJK*) double mutant to chloramphenicol, tetracyclines, erythromycin, clindamycin, and fluoroquinolones that were substrates for both pumps was increased compared

TABLE 3. MICs of various drugs for *A. baumannii* BM4454 and derivative strains

| Antimicrobial agent | MIC[a] (μg/ml) for strain: | | | | | |
|---|---|---|---|---|---|---|
| | BM4454 | BM4652 (Δ*adeABC* Δ*adeIJK*) | BM4652/pAT807 (*adeIJK*) | BM4579 (Δ*adeIJK*) | BM4579/pAT807 (*adeIJK*) | BM4651 (Δ*adeABC*) |
| Ticarcillin | 8 | 1 | >256 | 1 | >256 | 8 |
| Cefotaxime | 8 | 1.5 | 3 | 1.5 | 3 | 8 |
| Imipenem | 0.5 | 0.25 | 0.25 | 0.25 | 0.25 | 0.5 |
| Chloramphenicol | 256 | 16 | 96 | 64 | 96 | 128 |
| Tetracycline | 48 | 0.5 | 6 | 4 | 6 | 8 |
| Minocycline | 1.5 | 0.064 | 0.75 | 0.25 | 1 | 0.5 |
| Tigecycline | 4 | 0.047 | 0.75 | 1.5 | 2 | 0.5 |
| Erythromycin | 12 | 1.5 | 3 | 8 | 12 | 4 |
| Azithromycin | 96 | 1.5 | 3 | 96 | 96 | 1.5 |
| Clindamycin | >256 | 2 | 24 | 48 | 48 | >256 |
| Rifampin | 4 | 2 | 2 | 2 | 2 | 4 |
| Moxifloxacin | 12 | 0.5 | 1.5 | 12 | 16 | 3 |
| Pefloxacin | 48 | 4 | 12 | 24 | 48 | 24 |
| Levofloxacin | 12 | 0.38 | 2 | 6 | 8 | 3 |

[a] MICs were determined by E-test, except for ticarcillin and SDS, where they were determined by dilution in agar.

to that of the single mutants (Table 3). This indicates a cumulative effect of the pumps, as already reported for other efflux systems in *Pseudomonas aeruginosa* (12). Interestingly, inactivation of both AdeABC and AdeIJK led to susceptibility to clindamycin and pefloxacin. The overlapping substrate profiles of the two pumps suggest that if AdeABC or AdeIJK is impaired, the remaining system could export at least some of the toxic compounds.

Since detergents and basic dyes can be substrates for efflux pumps, the MICs of SDS, acridine, ethidium bromide, pyronine, and safranin were also determined (data not shown) and indicated that these compounds, except ethidium bromide, were exported by AdeIJK. On the contrary, ethidium bromide is a substrate for AdeABC (14).

Taken together, these results indicate that, as commonly observed for RND efflux systems (18), the AdeIJK pump has a very wide substrate range very similar to those of the AcrAB-TolC and MexAB-OprM systems (12, 19), amphiphilic compounds being preferred substrates. X-ray crystallographic analyses have been recently performed to reexamine the basis of multidrug efflux of AcrB (16). It has been established that the three TMDs of AcrB form a large cavity that spans the cytoplasmic membrane and is surrounded by many hydrophobic amino acids (29). Ligands bind to a number of these residues, increasing the range of potential drug-protein interactions. Interestingly, sequence comparison of AdeJ and AcrB showed that amino acids (Phe 386, Phe 388, Phe 458, Phe 459, Leu 25, Lys 29, Asp 99, Asp 101) recognized as binding sites were conserved, which is consistent with the similarity observed at the substrate level.

**Synergistic contribution of AdeIJK and AdeABC to tigecycline resistance.** The glycylcycline tigecycline, the 9-*t*-butylglycylamido derivative of minocycline, has a broad spectrum of activity against gram-positive and -negative bacteria, including *Acinetobacter* spp. (23). Recently, overproduction of AdeABC has been reported to be associated with decreased susceptibility of *A. baumannii* to this antibiotic (21, 22, 25). We have tested the contribution of the two Ade pumps to resistance to the tetracyclines and the glycylcycline (Table 3). Compared to that for the BM4454 parental strain, the MICs for the three insertion mutants BM4651 (Δ*adeABC*), BM4579 (Δ*adeIJK*), and BM4652 (Δ*adeABC*, Δ*adeIJK*) showed 8-, 3-, and 85-fold reductions, respectively. Moreover, these data indicated that, in contrast to other antibiotics that are also substrates for the two efflux systems (Table 3), both pumps contribute in a more than additive fashion to tetracycline, minocycline, and tigecycline resistance. As already noted, glycyl substitution does not overcome efflux by RND family pumps (28).

The structural genes for the AdeIJK pump were detected by PCR in all of the *A. baumannii* strains tested and in the genomes of *A. baumannii* AYE and ATCC 17978 (10, 27), in contrast to AdeABC, which was recovered in 24 out of the 27 strains studied (unpublished data). Absence of the latter efflux system among members of this species has been reported (17). On the contrary, proteins very similar to AdeIJK are present in other *Acinetobacter* species such as *Acinetobacter* genomic group 3 and *A. baylyi* ADP1 (2, 5) (Table 2). These observations suggest that AdeIJK may have a more important physiological role than AdeABC and is likely to be responsible, at least in part, for the intrinsic low-level resistance to various antibiotics in *Acinetobacter*. However, the toxicity observed when this system was overexpressed suggests that its contribution to acquired antibiotic resistance may be limited.

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