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# Contribution of Resistance-Nodulation-Cell Division Efflux Systems to Antibiotic Resistance and Biofilm Formation in *Acinetobacter baumannii*

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**ABSTRACT** *Acinetobacter baumannii* is a nosocomial pathogen of increasing importance due to its multiple resistance to antibiotics and ability to survive in the hospital environment linked to its capacity to form biofilms. To fully characterize the contribution of AdeABC, AdeFGH, and AdeIJK resistance-nodulation-cell division (RND)-type efflux systems to acquired and intrinsic resistance, we constructed, from an entirely sequenced susceptible *A. baumannii* strain, a set of isogenic mutants overexpressing each system following introduction of a point mutation in their cognate regulator or a deletion for the pump by allelic replacement. Pairwise comparison of every derivative with the parental strain indicated that AdeABC and AdeFGH are tightly regulated and contribute to acquisition of antibiotic resistance when overproduced. AdeABC had a broad substrate range, including  $\beta$ -lactams, fluoroquinolones, tetracyclines-tigecycline, macrolides-lincosamides, and chloramphenicol, and conferred clinical resistance to aminoglycosides. Importantly, when combined with enzymatic resistance to carbapenems and aminoglycosides, this pump contributed in a synergistic fashion to the level of resistance of the host. In contrast, AdeIJK was expressed constitutively and was responsible for intrinsic resistance to the same major drug classes as AdeABC as well as antifolates and fusidic acid. Surprisingly, overproduction of AdeABC and AdeIJK altered bacterial membrane composition, resulting in decreased biofilm formation but not motility. Natural transformation and plasmid transfer were diminished in recipients overproducing AdeABC. It thus appears that alteration in the expression of efflux systems leads to multiple changes in the relationship between the host and its environment, in addition to antibiotic resistance.

**IMPORTANCE** Increased expression of chromosomal genes for RND-type efflux systems plays a major role in bacterial multidrug resistance. *Acinetobacter baumannii* has recently emerged as an important human pathogen responsible for epidemics of hospital-acquired infections. Besides its remarkable ability to horizontally acquire resistance determinants, it has a broad intrinsic resistance due to low membrane permeability, endogenous resistance genes, and antibiotic efflux. The study of isogenic mutants from a susceptible *A. baumannii* clinical isolate overproducing or deleted for each of the three major RND-type pumps demonstrated their major contribution to intrinsic resistance and to the synergism between overproduction of an efflux system and acquisition of a resistance gene. We have also shown that modulation of expression of the structural genes for the efflux systems results in numerous alterations in membrane-associated cellular functions, in particular, in a decrease in biofilm formation and resistance gene acquisition.

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*Acinetobacter baumannii* has emerged as a clinically and epidemiologically important nosocomial pathogen, essentially because of its ability to persist in the hospital environment and its propensity to rapidly acquire antibiotic resistance mechanisms (1). Multidrug-resistant *A. baumannii* strains are frequently found to be responsible for epidemics of nosocomial infections, such as respiratory, bloodstream, urinary tract, skin, and soft tissue infections (1). In addition to its high capacity to acquire new genetic determinants, overproduction of resistance-nodulation-cell division (RND) efflux pumps with broad substrate specificity

has been shown to be associated with multidrug resistance (MDR) in *A. baumannii* (2). RND efflux systems, composed of an inner membrane protein (RND pump) linked by a periplasmic adaptor protein (PAP) to an outer membrane factor (OMF), can extrude a wide range of substrates often unrelated in structure (3). To date, three *Acinetobacter* drug efflux (Ade) RND systems, AdeABC (4), AdeFGH (5), and AdeIJK (6), have been characterized in *A. baumannii*. Overexpression of the AdeABC pump, primarily, and of AdeFGH plays a major role in acquired resistance (2, 7), whereas AdeIJK contributes to intrinsic resistance (6). Expression of each

pump is tightly regulated by a different mechanism: AdeABC by a two-component regulatory system, AdeRS (8), AdeFGH by the LysR-type transcriptional regulator AdeL (5), and AdeIJK by the TetR transcriptional regulator AdeN (9). The study of the relative contributions of these three most prevalent pumps in 14 clinical isolates outlined the high incidence of *adeABC* overexpression in MDR strains, resulting from a variety of point mutations in AdeRS, whereas only half of the strains had a slight increase in *adeFGH* expression and, curiously, none overexpressed *adeIJK* (7).

Involvement of these pumps in antibiotic efflux was demonstrated by the study of *in vitro* mutants of clinical isolates overproducing the pumps and of deletion mutants obtained by insertional inactivation of the structural genes for the pumps (4–6) and by the use of efflux inhibitors. Interpretation of data obtained with efflux inhibitors is difficult due to their lack of specificity, their own antibacterial activity, and the multiplicity of the pumps (10). Moreover, these studies provide limited, and sometimes discrepant, findings on the respective roles of the pumps because of lack of characterization of the parental strains, which are most often MDR clinical isolates expressing complex sets of resistant determinants (11).

In an attempt to better characterize the contribution of the RND efflux systems to intrinsic or acquired resistance of *A. baumannii*, we constructed from the susceptible and entirely sequenced clinical BM4587 isolate a set of isogenic mutants (i) overproducing each of the three RND efflux systems as a result of a single mutation in their cognate regulator or (ii) deleted in each efflux system following clean allelic replacement. Pairwise comparison of every point mutant or of its deleted counterpart with the parental strain led to precise characterization of the contribution of AdeABC and AdeFGH and of the contribution of AdeIJK to acquired and intrinsic antibiotic resistance, respectively. In addition, this report documents the consequences of the level of production of the pumps for biofilm formation, host motility, competence, and conjugation, i.e., with respect to persistence of this bacterial species and horizontal acquisition of resistance genes.

## RESULTS

**Genomic features of *A. baumannii* BM4587.** Clinical strain BM4587, isolated in 2009 in France, was previously screened for resistance determinants with a microarray and shown to be susceptible to antibiotics (12). Its genome is composed of a 3,913,120-bp circular chromosome with a 39.0% GC content similar to that of other sequenced *A. baumannii* isolates and of an 8,057-bp cryptic plasmid, pIP870. A total of 3,766 genes were annotated on the chromosome, including six rRNA clusters, 72 tRNAs, 3,665 predicted protein coding sequences (CDSs), and the three RND efflux operons, *adeABC*, *adeFGH*, and *adeIJK* (see Table S1 in the supplemental material). The AdeRS two-component regulatory system of AdeABC and the AdeL LysR-type transcriptional regulator of AdeFGH were transcribed in the opposite direction upstream from the corresponding operon, whereas the TetR-type transcriptional regulator AdeN was ca. 800 kb away from the AdeIJK operon (Fig. 1). *A. baumannii* BM4587 encodes seven RND efflux systems, but only AdeABC, AdeFGH, and AdeIJK have been functionally defined. The remaining four pumps are still putative.

In the phylogenetic tree obtained from a set of 133 core genomes of species of the genus *Acinetobacter* (13), BM4587 was

positioned in the clade of the 34 *A. baumannii* spp. (average nucleotide identity, 97.7%). It was close to AB0057 (14), AYE (15), and AB307-0294 (14), which belong to international Clonal Complex 1 (CC1) (13). By MultiLocus Sequence Typing (Pasteur scheme), the allelic profile of BM4587, 1-63-2-2-9-4-3, was shown to correspond to new Strain Type 621.

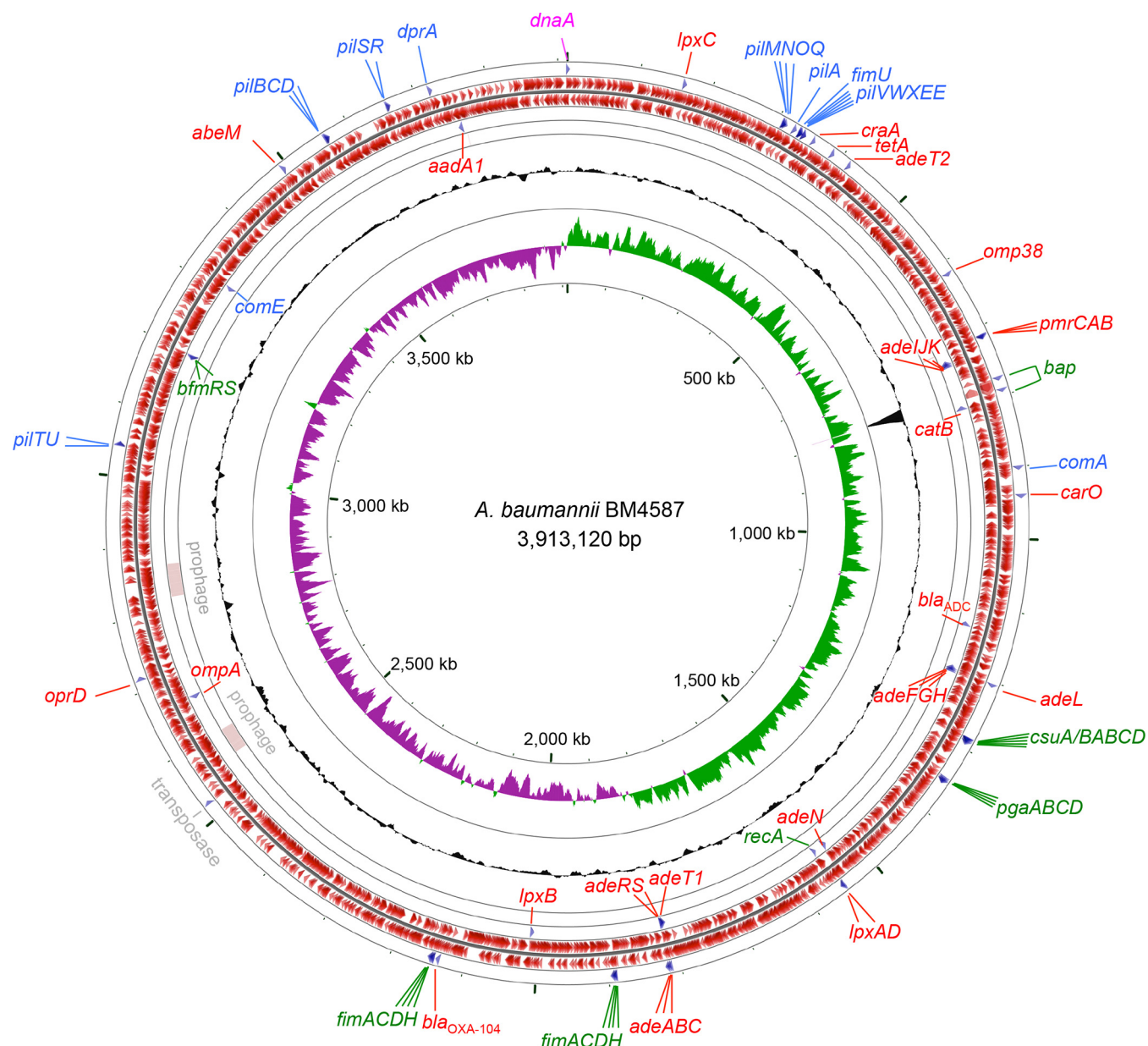
*A. baumannii* is known to have a plastic genome with numerous horizontally acquired mobile genetic elements. In contrast to published chromosome sequences, including our own study results (13), no pathogenicity or resistance islands, insertion sequences (IS), transposons, or integrons were found in BM4587. We detected only a truncated gene for a transposase (76% amino acid [aa] identity for 50% profile coverage with transposase ISRme19) which was not associated with an IS and two 51-kb and 41-kb putative prophages related to the rare podoviral bacteriophage YMC/09/02/B1251 ABA BP (16).

**Construction of isogenic mutants overproducing or deleted for Ade efflux pumps.** We generated *in vitro* from BM4587 spontaneous resistant mutants overproducing the three main RND efflux pumps on gradient agar plates containing gentamicin for *adeABC* and chloramphenicol for *adeFGH*, as previously described (17); the mutant overexpressing *adeIJK* was obtained previously on a cefotaxime gradient (12) (see Table S2 in the supplemental material).

The derivatives differed from the parental strain by a single point mutation in their respective regulators (see Table S2 in the supplemental material) which was confirmed by whole-genome sequencing. The two variants overexpressing *adeABC*, BM4688[ $\nearrow$ *adeABC*] AdeS<sub>R152K</sub> in the sensor and BM4689[ $\nearrow$ *adeABC*] AdeR<sub>A91V</sub> in the response regulator of the two-component regulatory system, had already-reported mutations. The AdeS<sub>R152K</sub> substitution near phosphorylatable histidine 149 is a mutational hot spot in clinical isolates overexpressing *adeB* (7), and AdeR<sub>A91V</sub> in the signal receiver domain was obtained *in vitro* (18). Overexpression of the pump was quantified by reverse transcription-quantitative PCR (RT-qPCR). We were able to detect *adeB* expression in the parental strain but at a very low level (0.01-fold less than that seen with *rpoB* or *secE* genes). The *adeG* expression was similarly low, less than 0.2-fold that seen with the *rpoB* and *secE*. These figures explain why there were no changes in the MIC values for both deletion mutants from those for the parental strain. In contrast, the *adeJ* gene was expressed at levels similar to (1.1-fold) those seen with *rpoB* and *secE*, which was consistent with the membrane protein data (Table 1). BM4688[ $\nearrow$ *adeABC*] presented expression that was increased 60-fold for *adeA*, 38-fold for *adeB*, and 10-fold for *adeC* compared to the levels seen with the parent, whereas BM4689[ $\nearrow$ *adeABC*] had expression levels of *adeABC* that were nearly 5 times higher (Fig. 2).

Two *adeFGH*-overexpressing derivatives, BM4690[ $\nearrow$ *adeFGH*] with an AdeL<sub>Q332stop</sub> mutation and BM4691[ $\nearrow$ *adeFGH*] with AdeL<sub>N334H</sub>, were obtained. Mutations in the C-terminal region of LysR-type regulators confer constitutive expression of the target genes (5). In the two mutants, *adeFGH* was greatly (ca. 80 to 750 times) overexpressed compared with the BM4587 results (Fig. 2).

The BM4666[ $\nearrow$ *adeIJK*] derivative has an *adeN*<sub>ΔC584</sub> mutation (9) and presented expression that had increased 13-fold for *adeI*, 6-fold for *adeJ*, and 10-fold for *adeK* (Fig. 2). AdeN is a transcriptional regulator belonging to the TetR family (9), and, as already mentioned, the coding gene is located 802 kb distant from the *adeIJK* operon (Fig. 1). To characterize potential global regulatory



**FIG 1** Graphical circular map of *A. baumannii* BM4587 chromosome. Circles display (from the outside) (1) genes for antibiotic resistance, motility, competence, biofilm formation, and a transposase in the clockwise direction; (2) predicted CDSs transcribed in the clockwise direction; (3) predicted CDSs transcribed in the counterclockwise direction; (4) genes for antibiotic resistance, motility, competence, and biofilm formation in the counterclockwise direction; (5) prophages; (6) Mol% G+C deviation (mean G+C content in a 1-kb window for overall mean G+C); and (7) positive (green) and negative (purple) G+C skew. CGView software (60) was utilized to construct the genome map. Genes indicated in red represent those encoding antibiotic resistance; blue, motility and competence; green, biofilm formation; gray, mobile elements; and magenta, the replication origin.

activity frequently associated with this type of transcriptional activator, Biolog phenotypic microarray analysis was carried out for BM4666 [ $\Delta$ adeIJK] in comparison with BM4587. This array measures bacterial respiration changes under different culture conditions. The responses to the various antibiotics were consistent with the decreased susceptibility of the mutant to the drugs (see below and Table 2). Otherwise, no significant changes were found under conditions of different carbon, nitrogen, phosphorus, sulfur, and peptide nitrogen sources and various osmolarity and pH levels (data not shown). Strain BM4666 [ $\Delta$ adeIJK] showed only a

slight stimulation of respiration in the presence of adenine, adenosine, or Tween 20. Of note, mutated AdeN resulted in moderate overexpression of the pump, as already reported for other *in vitro* mutants (9). Analysis of *adeN* loci in 34 *A. baumannii* genomes (13) showed that high deduced aa conservation, with only three strains in which AdeN was truncated, was associated with 4-fold, 3-fold, and 3-fold increased *adeJ* expression relative to BM4587 taken as a reference.

Clean-deletion mutants BM4717 [ $\Delta$ adeB], BM4718 [ $\Delta$ adeG], and BM4719 [ $\Delta$ adeJ], lacking an RND pump gene, were obtained



**TABLE 1** Proteins differentially expressed in *A. baumannii* isogenic mutants BM4689[ $\Delta adeABC$ ] and BM4666[ $\Delta adeIJK$ ] relative to BM4587

Location <sup>a</sup>	Protein	BM4689[ <i>ΔadeABC</i> ] vs BM4587				BM4666[ <i>ΔadeIJK</i> ] vs BM4587			
		Pept <sup>b</sup>	Score <sup>c</sup>	ANOVA ( <i>P</i> )	Fold <sup>d</sup>	Pept <sup>b</sup>	Score <sup>c</sup>	ANOVA ( <i>P</i> )	Fold <sup>d</sup>
RND efflux system									
BM4587v1_20947	AdeA	22	1577	4.47e-11	65.3				
BM4587v1_20948	AdeB	4	210	2.89e-09	40.4				
BM4587v1_20949	AdeC	17	991	5.66e-11	29.5				
BM4587v1_10691	AdeI					17	1524	1.27e-06	3.9
BM4587v1_10690	AdeJ					11	552	7.78e-04	3.8
BM4587v1_10689	AdeK					21	1786	3.56e-05	2.5
Cell motility/adhesion									
BM4587v1_21011	FimA, fimbrial adhesin protein					4	177	8.91e-04	−33.9
BM4587v1_20439	CsuA/B; putative secreted protein related to type I pili	5	378	1.69e-04	−2.5	5	754	1.19e-04	−3.2
BM4587v1_20442	CsuC; putative type I pilus usher pathway chaperone	8	373	3.48e-03	−2.8	7	321	2.19e-05	−6.7
BM4587v1_10582	Omp38, outer membrane protein A	5	190	1.83e-05	−2.9				
BM4587v1_21772	Outer membrane protein A family	3	125	2.29e-03	2.6				
Metabolism/amino acid transport/biosynthesis									
BM4587v1_20210	DABA-AT, diaminobutyrate-2-oxoglutarate aminotransferase	6	210	2.43e-05	−3.8	7	431	4.52e-04	−2.7
BM4587v1_21371	Amino acid ABC transporter periplasmic protein	3	103	1.42e-03	2.6				
BM4587v1_20299	TrpC, indole-3-glycerol-phosphate synthase	4	173	1.97e-06	−6.4	4	174	3.32e-03	−2.5
BM4587v1_20152	AspC, aspartate aminotransferase A	4	152	2.40e-05	−5.7	4	246	1.36e-03	−2.5
BM4587v1_10396	RES, type III restriction enzyme, res subunit					3	95	1.87e-03	−5.8
BM4587v1_22098	BetA, oxygen-dependent choline dehydrogenase	3	162	1.96e-03	2.6	3	130	1.23e-03	3.4
BM4587v1_10056	PurC, phosphoribosylaminoimidazole-succinocarboxamide synthase	5	352	2.13e-06	−3.0				
BM4587v1_22207	FabD, malonyl-CoA <sup>e</sup> -[acyl-carrier-protein] transacylase	4	489	2.10e-03	−8.8	4	173	4.59e-04	−3.6
BM4587v1_22458	FumA, fumarate hydratase					3	160	1.47e-03	−2.6
BM4587v1_20069	EtfA, electron transfer flavoprotein alpha-subunit	3	682	9.14e-05	−2.5				
DNA recombination									
BM4587v1_10194	Ssb, ubiquitous ssDNA <sup>f</sup> binding protein	4	158	6.86e-04	−2.4				

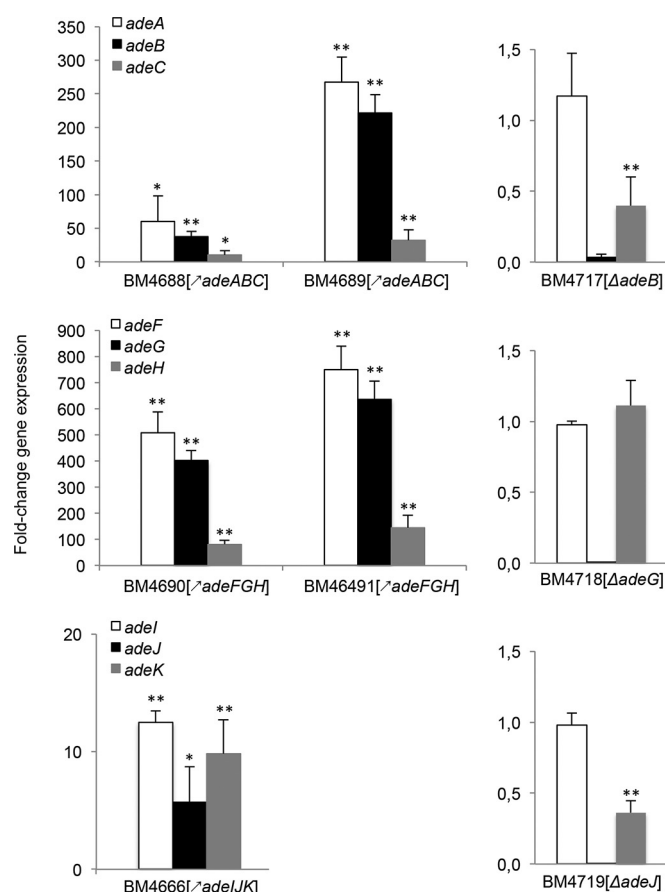
<sup>a</sup> Structural gene location in *A. baumannii* BM4587 chromosome.<sup>b</sup> Number of peptides for identification and quantification.<sup>c</sup> Confidence score for identification by Mascot software.<sup>d</sup> Fold change between the isogenic mutant and the parental strain; only values above a 2-fold change are indicated; negative values indicate underproduction.<sup>e</sup> CoA, coenzyme A.<sup>f</sup> ssDNA, single-stranded DNA.

by two-step allelic exchange (see Fig. S1 in the supplemental material) (19), and expression of the genes for the PAP, RND, and outer membrane protein (OMP) components was determined by RT-qPCR (Fig. 2). As expected, there was no expression of the deleted genes and the genes for the PAPs were expressed at levels similar to those in the parental strain. In contrast, the genes for OMPs were expressed at levels less than half of those of the parental strain in both the BM4717[ $\Delta adeB$ ] and BM4719[ $\Delta adeJ$ ] derivatives.

In contrast to report by others (10), we did not observe any alterations in the expression of the other Ade RND systems in the overproducing or deleted mutants for a single pump (data not shown).

***A. baumannii* BM4587 and isogenic mutants overproducing or deleted for Ade efflux pumps allow identification of antibiotic substrates.** Broad intrinsic resistance in *A. baumannii* is due to low membrane permeability, the presence of chromosomally encoded  $\beta$ -lactamases, and basal expression of efflux systems. MDR in this species results from horizontal gene transfer but also from overexpression of the pumps (1, 2).

Genes responsible for intrinsic and acquired resistance to antibiotics were searched for in the BM4587 genome (Fig. 1; see also Table S3 in the supplemental material). The genes for the two intrinsic  $\beta$ -lactamases of *A. baumannii*, *bla*<sub>ADC</sub> for an AmpC-type cephalosporinase (99% identical to ADC-52) and *bla*<sub>OXA-104</sub> for an OXA-51-type  $\beta$ -lactamase, were present. The *craA* gene for a



**FIG 2** Relative levels of expression of *ade* operons. Gene expression relative to that of *rpoB* was determined by RT-qPCR. Results are presented relative to parental BM4587 taken as a reference. Every isolate was tested in duplicate in two independent experiments. The bars represent the means and the error bars the standard deviations. Genes indicated in white represent those encoding periplasmic adaptor proteins; black, RND pumps; and gray, outer membrane factors. \*\*,  $P < 0.01$ ; \*,  $P < 0.05$  (Student *t* test).

chloramphenicol-specific major facilitator superfamily (MFS) efflux system was also found (20). An acquired *aadA1* gene for a streptomycin 3'-O-adenylyltransferase accounted for high-level streptomycin resistance of BM4587. In contrast, acquired gene *tetA* for an MFS pump did not confer resistance, as was the case for 6 of 14 *A. baumannii* isolates in our previous study (13).

Based on the drug MIC changes with respect to the pump mutants (Table 2), ampicillin for AdeABC and ticarcillin and aztreonam for AdeIJK were weakly extruded. All cephalosporins were substrates for AdeIJK, and ceftazidime and cefepime were also substrates for AdeABC. The three carbapenems were substrates for AdeABC, but only meropenem was a substrate for AdeIJK. Of note, against the  $\Delta$ adeJ variant, but not against the  $\Delta$ adeB and  $\Delta$ adeG variants, the MICs of most  $\beta$ -lactams were lower than those against parental BM4587, indicating basal expression of AdeIJK, responsible for intrinsic resistance to these drugs, in the latter strain. These data confirm our earlier reports based on overexpressing and single-deletion or double-deletion derivatives (4, 6, 9) and are in good agreement with those obtained by others (11, 21–23) except for meropenem-AdeIJK in a single study (11). That discrepancy could have resulted from the study of an MDR strain

producing a carbapenemase (11) masking the moderate effect of efflux on carbapenems. No overexpression of *ampC* or OXA-51 was detected in strain BM4666[ $\Delta$ adeIJK]. RT-qPCR experiments were carried out to determine if the expression of intrinsic *ampC* and *bla*<sub>OXA-51-like</sub> genes was altered by the upregulation of the various efflux systems. No overexpression of *ampC* or of *bla*<sub>OXA-51-like</sub> was detected in the BM4666[ $\Delta$ adeIJK] and BM4690[ $\Delta$ adeFGH] strains. There was a significant 2-fold-lower level of production of both  $\beta$ -lactamases in strain BM4689[ $\Delta$ adeABC]. Therefore, the increase in the MICs of carbapenems against the *adeABC*-overexpressing mutant was not the result of overexpression of the structural genes for the enzymes but was due solely to efflux of the drugs.

All aminoglycosides were substrates for AdeABC. Importantly, overexpression of the pump resulted in strain BM4689[ $\Delta$ adeABC] being categorized as clinically resistant to gentamicin, netilmicin, and tobramycin (24, 25).

Strain BM4587 was susceptible to nalidixic acid, norfloxacin, and ciprofloxacin; these quinolones were substrates for the three RND pumps.

Tetracycline and tigecycline were substrates for the AdeABC and AdeIJK pumps and minocycline for AdeIJK only. This was not found in another study for minocycline-AdeABC and tetracycline-AdeIJK (22) and could have been due to the fact that the experiments were carried out in different bacterial hosts, *A. baumannii* and *Escherichia coli*, which differ drastically in their outer membrane permeability (26).

Although macrolides and lincosamides are thought to be inactive against Gram-negative bacteria because of impermeability associated with the outer membrane (27), erythromycin and clindamycin were good substrates for AdeABC and AdeIJK. The MICs of both drugs against strain BM4719[ $\Delta$ adeJ] were largely decreased, indicating that efflux contributes significantly to intrinsic resistance to these two classes of drugs in *A. baumannii*.

Chloramphenicol was a substrate for the three RND pumps. The MIC of the drug against parental BM4587 dropped from 64  $\mu$ g/ml to 16  $\mu$ g/ml for strain BM4719[ $\Delta$ adeJ], indicating that efflux contributes more to intrinsic resistance to chloramphenicol than the MFS pump CraA.

*Acinetobacter* spp. are intrinsically resistant to low levels of trimethoprim and sulfonamides (27) due to AdeIJK activity; they were also substrates for AdeFGH. The AdeIJK system was also responsible for intrinsic resistance to fusidic acid and, to a much lesser extent, to rifampin.

Colistin, which acts at the surface of the cells, was not a substrate for any of the pumps.

The susceptibility of strains BM4717[ $\Delta$ adeB] and BM4718[ $\Delta$ adeG] was similar to that of parent strain BM4587, indicating that both the AdeABC system and the AdeFGH system are tightly regulated and are expressed only following induction by as-yet-unknown signals or mutations in the cognate regulatory system (5, 8). There was good correlation between the level of AdeABC expression and that of resistance to the substrate drugs, since the MICs of substrate antibiotics against strain BM4689[ $\Delta$ adeABC] were 1- to 4-fold higher than against strain BM4688[ $\Delta$ adeABC], which overexpressed *adeB* at a level 4 times lower (data not shown).

Of note, deletion mutant BM4719[ $\Delta$ adeJ] had increased susceptibility to several antibiotic classes,  $\beta$ -lactams, quinolones, tetracyclines, MLS, phenicols, antifolates, fusidic acid, and the biocide triclosan (Table 2), consistent with constitutive production of

TABLE 2 Antimicrobial susceptibility of *A. baumannii* BM4587 and derivatives

Antimicrobial agent	MIC <sup>a</sup> (μg/ml)						
	BM4587	BM4689 [ <i>ΔadeABC</i> ]	BM4717 [ <i>ΔadeB</i> ]	BM4690 [ <i>ΔadeFGH</i> ]	BM4718 [ <i>ΔadeG</i> ]	BM4666 [ <i>ΔadeIJK</i> ]	BM4719 [ <i>ΔadeJ</i> ]
<b>β-Lactams</b>							
Ampicillin	8	<b>32</b>	8	16	8	8	8
Ticarcillin	8	8	8	4	8	<b>32</b>	2
Aztreonam	32	16	32	16	32	<b>128</b>	4
Cephalothin	128	128	128	64	128	<b>512</b>	128
Cefuroxime	16	16	16	32	16	<b>64</b>	2
Ceftriaxone	4	4	4	2	2	<b>16</b>	4
Ceftazidime	4	<b>16</b>	4	4	4	8	1
Cefepime	1	<b>16</b>	1	0.5	1	<b>4</b>	0.5
Imipenem	0.125	<b>0.5</b>	0.125	0.125	0.125	0.125	0.125
Meropenem	0.5	<b>2</b>	0.5	0.5	0.5	<b>2</b>	<b>0.125</b>
Doripenem	0.25	<b>4</b>	0.25	0.25	0.25	0.5	0.125
<b>Aminoglycosides</b>							
Streptomycin	32	<b>1024</b>	32	32	32	32	32
Neomycin B	2	<b>16</b>	2	2	2	2	2
Kanamycin	4	<b>32</b>	4	4	4	4	4
Tobramycin	2	<b>16</b>	2	2	2	2	2
Amikacin	2	<b>16</b>	2	2	2	2	2
Gentamicin	1	<b>32</b>	1	1	1	1	1
Netilmicin	1	<b>128</b>	1	1	1	1	1
<b>Quinolones</b>							
Nalidixic acid	4	8	4	<b>32</b>	2	8	1
Norfloxacin	2	<b>32</b>	2	<b>16</b>	2	<b>16</b>	<b>0.25</b>
Ciprofloxacin	0.125	<b>1</b>	0.125	<b>1</b>	0.125	<b>0.5</b>	<b>0.016</b>
<b>Polymyxin</b>							
Colistin <sup>b</sup>	0.5	0.5	0.5	0.5	0.5	0.5	0.5
<b>Tetracyclines</b>							
Tetracycline	0.5	<b>4</b>	0.5	0.5	0.5	<b>2</b>	<b>0.063</b>
Minocycline	0.25	0.25	0.25	0.25	0.125	0.5	<b>0.015</b>
Tigecycline	0.125	<b>2</b>	0.125	0.25	0.125	<b>0.5</b>	<b>0.031</b>
<b>Macrolide-lincosamide</b>							
Erythromycin	64	<b>256</b>	64	128	64	128	8
Clindamycin	512	<b>2048</b>	512	1024	512	1024	<b>16</b>
<b>Phenicol</b>							
Chloramphenicol	64	<b>256</b>	64	<b>512</b>	64	<b>256</b>	<b>16</b>
<b>Antifolates</b>							
Trimethoprim	16	32	16	<b>64</b>	16	<b>128</b>	2
Sulfadoxine	128	128	128	<b>1024</b>	128	256	8
<b>Miscellaneous</b>							
Fusidic acid	128	128	256	128	256	<b>512</b>	<b>8</b>
Rifampin	1	1	1	1	1	2	0.5
Triclosan	4	4	4	8	4	8	1

<sup>a</sup> MICs were determined by microdilution according to the CLSI guidelines (54). Data in bold indicate values  $\geq 4$  times the parental strain value.

<sup>b</sup> Microdilution MICs of colistin were determined according to the CLSI guidelines with the addition of Tween 80 at a final concentration of 0.002%.

AdeIJK and with a major contribution of that pump to the broad intrinsic resistance of *A. baumannii*.

*E. coli*-*A. baumannii* shuttle plasmid pCM88 confers resistance to carbapenems by production of  $\beta$ -lactamase OXA-23 and to gentamicin by production of an AAC(3)-I (unpublished data). This plasmid was electrotransformed into BM4587 and its derivatives overexpressing the three efflux systems, and the MICs of antibiotics for the transformants were determined (see Ta-

ble S4 in the supplemental material). There was no consequence of overexpression of the AdeFGH and AdeIJK pumps with respect to the levels of resistance of the host strains (data not shown). In contrast, the MICs of the carbapenems were 2-fold to 4-fold more elevated against strain BM4689[*ΔadeABC*]/pCM88, and the gentamicin MICs against that strain increased from 2,048 μg/ml to more than 4,096 μg/ml. Since AdeABC is the only system that can extrude the carbapenems and the aminoglycosides, these data in-

indicate an effect that is synergistic between efflux and other resistance mechanisms.

**Overexpression of *adeABC* and *adeIJK* alters bacterial membrane composition.** The proteomic analysis was performed on cell envelope proteins and membrane or membrane-associated proteins from both inner and outer membranes, obtained from 4-h bacterial cultures as previously described (28). Comparative study of the envelope proteins of *A. baumannii* BM4587 and of the mutants (Table 1) confirmed overproduction of the AdeA, AdeB, and AdeC proteins (65-, 40-, and 29-fold changes, respectively) in strain BM4689 [ $\nearrow adeABC$ ] and of AdeI, AdeJ, and AdeK (3.9, 3.8, and 2.5, respectively) in strain BM4666 [ $\nearrow adeIJK$ ]. These data were in good agreement with those given by RT-qPCR analysis (Fig. 2). In the parental strain, only AdeA and AdeC proteins from the AdeABC system were detected, whereas all three proteins from the AdeIJK system were easily detected (data not shown). These observations reinforce the notions that AdeABC is expressed at a low level in the susceptible parent strain, its expression being under stringent control (8), and that AdeIJK is constitutively expressed (6). Besides the overproduction of these Ade proteins, we noticed underproduction of several interesting proteins in the mutants (Table 1): (i) the CsuA/B and CsuC proteins underproduced in both mutants are part of the CsuA/BABCDE type I pilus system required for attachment to abiotic surfaces and for biofilm formation (28, 29); (ii) in strain BM4666 [ $\nearrow adeIJK$ ], a fimbrial protein also involved in biofilm formation (30) was extremely underproduced (34-fold); and (iii) in strain BM4689 [ $\nearrow adeABC$ ], production of the major OmpA protein, which plays a partial role in biofilm formation on plastic and is essential for bacterial adherence to A549 epithelial cells (31), was decreased nearly 3-fold. Finally, both mutants underproduced the diaminobutyrate-2-oxoglutarate aminotransferase (DABA-AT) involved in biosynthesis of diaminopropane (DAP), a polyamine required for *A. baumannii* surface-associated motility (32). Taken together, these data suggest possible impairment in adhesion, biofilm formation, and motility of the mutants overexpressing *adeABC* and *adeIJK*.

**Overexpression of Ade efflux pumps diminishes biofilm formation but not motility.** *A. baumannii* displays several forms of motility on wet surfaces, including twitching motility at the agar-petri dish interface, which is, in part, driven by type 4 pili (T4P) (33), and the form of surface motility called “swarming”; both types have been shown to depend on the synthesis of 1,3-diaminopropane (32). There was no difference between parental BM4587 and the mutants in the extent of motility. All the strains moved with spreading zones of similar sizes at the agarose surface and at the interface between agarose and the polystyrene of the petri dish (see Fig. S2 in the supplemental material). Formation of biofilms by *A. baumannii* BM4587 and the mutants on a solid support and at the air-liquid interface was quantified by a crystal violet colorimetric assay (Fig. 3). Consistent with the results of proteomic analyses, overexpression of the efflux pumps resulted in a decrease of biofilm and pellicle formation at 48 h. The phenomenon was clearly highlighted by experiments performed in 24-well plates (Fig. 3, white bars) that concomitantly took into account the formation of pellicles and biofilms on the walls of the well. If the impact on the BM4666 [ $\nearrow adeIJK$ ] mutant remained moderate, with a 36% decrease in biofilm formation, the impact on the other mutants was much more important, with 59%, 63%, and 82% decreases for mutants BM4688 [ $\nearrow adeABC$ ],

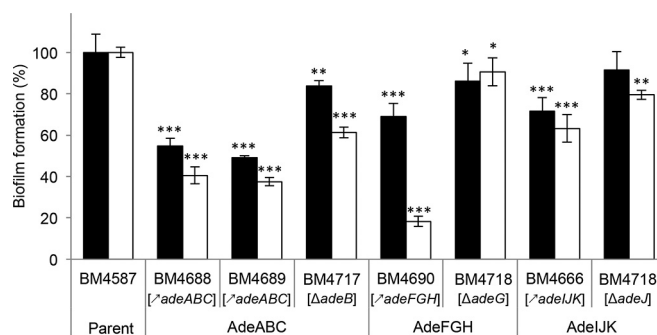
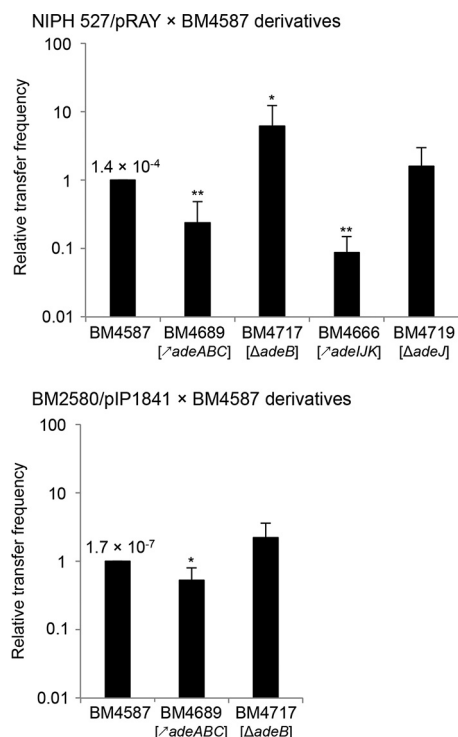


FIG 3 Biofilm and pellicle formation by *A. baumannii* BM4587 and its derivatives. Black bars, biofilms formed on Calgary device pegs; white bars, biofilms and pellicles formed on 24-well plates. Percentages indicate 48-h biofilm formation relative to parental BM4587 results taken as a reference. Experiments were performed least three times independently, and results are presented as means  $\pm$  SE. \*\*\*,  $P < 0.0001$ ; \*\*,  $P < 0.001$ ; \*,  $P < 0.05$ . Numbers in parentheses indicate fold increase in overexpression of the pump.

BM4689 [ $\nearrow adeABC$ ], and BM4690 [ $\nearrow adeFGH$ ], respectively. Deletion of the genes for the RND efflux pump in the BM4718 [ $\Delta adeG$ ] and BM4719 [ $\Delta adeJ$ ] mutants restored the biofilm and pellicle phenotypes of parent BM4587 (Fig. 3). The  $\Delta adeB$  mutant still had a significant defect of 39% in biofilm formation, thus presenting behavior similar to that of *E. coli* and *Salmonella enterica*, in which the deletion or inhibition of their efflux pumps was associated with decreased biofilm formation (34, 35).

**Influence of overexpression of Ade efflux pumps on natural transformation.** The emergence of MDR *A. baumannii* relies, in part, on acquisition of resistance determinants by horizontal gene transfer. This can occur via conjugation, transduction, or transformation. Since DNA uptake by transformation involves membrane structures such as T4P and takes place during adhesion and twitching motility (33, 36), increased expression of efflux systems could alter cell competence. Motility and DNA uptake are associated with T4P and are abolished by inactivation of *pilT*, which encodes the T4P retraction ATPase, and of ComEC, a DNA uptake channel. The genes for all key T4P components and competence factors are present in the chromosome of strain BM4587 (Fig. 1; see also Table S3 in the supplemental material). The transformation frequencies of the parental strain and of the mutant derivatives during twitching motility were determined using genomic DNA of *A. baumannii* mutant 179, which has a kanamycin resistance gene cassette inserted in the sulfite reductase gene (36). Strain BM4587 was naturally transformable at a very low rate,  $3.0 \times 10^{-10}$  ( $\pm 1.4 \times 10^{-10}$ ), and the transformation frequencies of the pump mutants were similarly low. Under these conditions, competence was detected in only 10 of 28 clinical isolates with various transformation rates (from  $1 \times 10^{-4}$  to  $6 \times 10^{-8}$ ) and only while twitching on semisolid medium was occurring (36). Similarly, only three of nine fully sequenced *A. baumannii* strains possessing all the competence genes (13) had transformation efficiencies higher than  $10^{-8}$ . To study the influence of *adeABC* overexpression on transformation, we performed selection on gentamicin from the most transformable strain, *A. baumannii* clinical NIPH 67 (13), an *adeABC*-overexpressing mutant. This derivative had the same mutation, AdeS<sub>R152K</sub>, as the BM4688 [ $\nearrow adeABC$ ] mutant and displayed expression of *adeB* that was 31-fold increased relative to that of NIPH 67. Its transformation frequency





**FIG 4** Effect of overproduction or deletion of Ade efflux pumps on plasmid transfer. Conjugation experiments were carried out in liquid (top) or on solid (bottom) medium. Relative plasmid transfer frequency data represent the ratio of transconjugants per donor to that of parental BM4587 used as a recipient. The data are the means and standard deviations of the results of at least seven independent experiments. \*\*,  $P < 0.001$ ; \*,  $P < 0.05$ .

( $6 \times 10^{-9} \pm 3 \times 10^{-9}$ ,  $P < 0.05$ ) was 4.1-fold lower than that of parental strain NIPH 67 ( $2 \times 10^{-8} \pm 9 \times 10^{-9}$ ), suggesting that overexpression of *adeABC* reduces DNA uptake by the host.

**Plasmid transfer is diminished by overexpression of *adeABC* and *adeIJK* in the recipient.** In the genome of *A. baumannii* BM4587, there were no type IV secretion systems which are involved in conjugation and the pIP870 resident plasmid is cryptic and not self-transferable. Conjugation was carried out using as a donor *A. baumannii* NIPH 527 harboring the 6-kb mobilizable pRAY plasmid conferring resistance to kanamycin (37) which is widely distributed in *A. baumannii* (38). Since strain NIPH 527 has the MPF<sub>I</sub> conjugation machinery integrated in its chromosome (13), it can mobilize the Tra<sup>+</sup> Mob<sup>+</sup> pRAY plasmid. In liquid medium, the frequency of transfer to strain BM4587 was  $1.4 \times 10^{-4}$  and was significantly ( $P < 0.05$ ) reduced to strain BM4689 [ $\Delta adeABC$ ] ( $2.5 \times 10^{-5}$ ) and strain BM4666 [ $\Delta adeIJK$ ] ( $1.2 \times 10^{-5}$ ) (Fig. 4, top panel). The frequency of transfer to strain BM4717 [ $\Delta adeB$ ] was significantly increased ( $5.7 \times 10^{-4}$ ,  $P < 0.01$ ), but the frequency of transfer to strain BM4719 [ $\Delta adeJ$ ] ( $2.0 \times 10^{-4}$ ) remained unchanged. *A. baumannii* BM4587 and its derivatives overexpressing or deleted for *adeABC* were also used as recipients in filter-mating experiments performed with *A. baumannii* BM2580 harboring pIP1841, a plasmid self-transferable to various *Acinetobacter* spp., such as *A. baumannii*, *A. haemolyticus*, and *A. Iwoffii* (39). The frequency of transfer of pIP1841 to BM4587 was  $1.5 \times 10^{-7}$  ( $\pm 1.2 \times 10^{-7}$ ) (Fig. 4, bottom panel). The efficiency of conjugation to strain BM4689 [ $\Delta adeABC$ ] was

half that seen with the parent strain, whereas that to strain BM4717 [ $\Delta adeB$ ] was 2.2-fold higher. Taken together, these data indicate a decrease in conjugation efficiency for both plasmid transfer types in the *adeABC*-overexpressing derivative.

## DISCUSSION

The antibiotic substrate range of the three RND efflux systems was determined by comparing the phenotype of strain BM4587 with those of single-mutation derivatives overexpressing or deleted for each pump (Table 2). The AdeABC mutants were representatives of the clinical isolates, since they harbored the mutations in AdeR and AdeS that are the most commonly found in clinical settings and which conferred similar levels of overexpression of the pump (7). To the best of our knowledge, no clinical strains with high-level production of *adeFGH* have been reported, whereas *adeIJK* is rarely overexpressed (7, 21; unpublished data on a collection of 25 entirely sequenced *A. baumannii* strains described in reference 13). The data indicate that AdeABC, which was found in 80% of *A. baumannii* isolates, could extrude a wide range of antibiotics, including the majority of  $\beta$ -lactams and aminoglycosides, the fluoroquinolones, tetracyclines-tigecycline, the macrolides-lincosamides, and chloramphenicol. The mutant deleted for *adeB* had MICs of all antibiotics indistinguishable from those against parental BM4587, confirming that this pump is tightly regulated by the AdeRS two-component system (8) and does not contribute to intrinsic resistance of *A. baumannii*. Antibiotic efflux occurs only when the pump is overexpressed and, for certain drug classes, such as aminoglycosides, can result in clinical resistance. In addition to the excellent correlation between the data obtained following overexpression of the pumps and that obtained following deletion, which reinforces our conclusions on the substrate specificities of this system, there was also a good relationship between the level of expression of AdeABC and that of resistance which could lead to differences in clinical categories for doripenem and tobramycin of mutants overexpressing *adeABC* at various levels (Fig. 2 and data not shown). This implies that one should exert caution in assigning substrates to a pump using a single overproducing variant.

Importantly, we observed that overexpression of *adeABC*, which extrudes carbapenems and aminoglycosides, combined with enzymatic resistance to these two classes of drugs, contributes in a more than additive fashion to the level of resistance of the host (see Table S4 in the supplemental material).

The AdeIJK system, which is present in all strains (6, 13), had an extremely broad substrate specificity. The MICs of the antibiotics that were substrates for AdeIJK were always lower against the *adeJ*-deleted derivative than against parental BM4587. This indicates that synthesis of AdeIJK is constitutive, as also demonstrated by analysis of membrane proteins (Table 1), and that the pump is responsible for intrinsic resistance of *A. baumannii* to all major classes of antibiotics, including  $\beta$ -lactams, fluoroquinolones, tetracyclines, MLS, phenicols, antifolates, and fusidic acid.

While proteomic analysis of bacterial membranes confirmed the constitutive expression of AdeIJK, we highlighted, in mutants overexpressing *adeABC* and *adeIJK*, the concomitant underexpression of proteins belonging to chaperone-usher (CU) pilus assembly systems (Table 1): (i) the CsuA/B and CsuC proteins, which are part of the CsuA/BABCD type I pilus system, the expression of which is activated by the BfmRS transcriptional regulatory system (29, 40), and (ii) the FimA fimbrial protein. Pilus

systems are known to have a major role in establishing the initial stages of biofilm formation, promoting initial adhesion and surface colonization but also formation of microcolonies (41). Both pilins CsuA/B and FimA were detected in the matrix of different *A. baumannii* pellicles (42), and the disruption of both CU systems results in a severe decrease in biofilm formation on abiotic surfaces (29, 30). Furthermore, the OmpA major outer membrane protein, also underexpressed in the BM4689[ $\Delta$ adeABC] mutant, is an adhesin required for bacterial adherence to epithelial cells and contributes to the process of biofilm formation (31). Therefore, reduced production of these proteins correlated well with the decrease in formation of biofilm and pellicle by the mutants overexpressing the efflux pumps (Fig. 3). It may be noted that such a negative correlation between overexpression of efflux systems leading to antibiotic resistance and decreases in biofilm formation has recently been observed in a transcriptomic analysis of imipenem-selected *A. baumannii* (43). Conversely, a high capacity of MDR clinical isolates of *A. baumannii* to form biofilm and to adhere to respiratory epithelial cells has been previously demonstrated (44). The contribution of Ade efflux systems to the decrease in biofilm formation remains difficult to explain. But it has been shown in *P. aeruginosa* that biofilm formation and antibiotic resistance could be controlled antagonistically via the Roc two-component systems. The *cupC* genes for Cup fimbriae (assembled by the chaperone-usher pathway) contributing to biofilm formation would be upregulated by these regulators, whereas the *mexAB* and *OprM* genes would be concomitantly downregulated (41). The proteomic analysis also demonstrated a reduction of the expression of DABA-AT, involved in biosynthesis of DAP in the mutants overexpressing *adeABC* and *adeIJK* (Table 1). In *Yersinia pestis*, the absence of polyamines reduces drastically the expression of the HmsR and HmsS hemin storage proteins that are involved in poly-N-acetylglucosamine polysaccharide synthesis and the expression of HmsT, a diguanylate cyclase responsible for the synthesis of the secondary messenger c-di-GMP (cyclic diguanosine-5'-monophosphate), a key regulator in biofilm formation (45, 46). Thus, in mutants of *Y. pestis* and *Vibrio cholerae* deficient in synthesis of putrescine and norspermidine polyamines, respectively, biofilm formation is severely impaired (47, 48). It appears that, similarly, reduced synthesis of DAP in *A. baumannii* may contribute to the decrease in biofilm formation.

Deletion or inhibition of efflux pumps in *E. coli* and *S. enterica* has been associated with a significant decrease of biofilm formation (34, 35, 49). However, in *A. baumannii*, biofilm formation by the mutants lacking RND pump genes *adeG* and *adeJ* was very similar to that of the parental strain. Only the  $\Delta$ adeB mutant presented a significant defect in biofilm formation (Fig. 3). In *S. enterica*, this defect was demonstrated to be related to repression of curli biosynthesis (50), but *A. baumannii* BM4587 lacks the homologous *cgs* genes involved in the curli biosynthesis pathway. Of note, exposure of *P. aeruginosa* to efflux inhibitors does not result in a significant reduction of biofilm formation (34). The impact of expression or inhibition of efflux systems on biofilm formation may be dependent on the bacterial species.

In addition to efflux, emergence of MDR *A. baumannii* relies on the ability to acquire antibiotic resistance genes by horizontal transfer. *A. baumannii* possesses the complete set of genes required for transformation (13), but natural competence in association with T4P production and twitching motility in a clinical isolate was found only recently (33). DNA uptake was further

demonstrated in 10 of 28 clinical isolates with various transformation rates (from  $1 \times 10^{-4}$  to  $6 \times 10^{-8}$ ) but only while the organisms were moving on semisolid media (36). Strain BM4587 was not transformable under similar conditions, although twitching motility was observed. Of note, there were no differences in two types of motility, twitching and surface mobility, between parental BM4587 and the mutants, although proteomic analysis of the BM4689[ $\Delta$ adeABC] and BM4666[ $\Delta$ adeIJK] mutants demonstrated underexpression of the DABA-AT involved in biosynthesis of DAP a polyamine required for *A. baumannii* surface-associated motility (32). The impact of the level of AdeABC pump production on competence was thus studied with another pair of isogenic strains, and the results demonstrated a diminution of transformation in the overexpressing mutant. Of note, a 2.4-fold decrease of an Ssb single-stranded DNA binding protein was observed in the membrane of the BM4689[ $\Delta$ adeABC] mutant (Table 1). In Gram-positive bacteria, protein SsbB is increasingly expressed during competence for genetic transformation (51).

These observations indicate that the inducing conditions for competence in *A. baumannii* are still poorly understood. Exposure of some bacterial species to certain antibiotics, in particular, bactericidal drugs, induces competence (52). Overexpression of efflux pumps, as a result of increases in the MICs of a large number of antibiotics, allow cells to survive in the presence of the drugs, providing them with a longer period of time to become competent and, ultimately, MDR by acquisition of foreign resistance genes. The influence of antibiotics on *A. baumannii* natural transformation remains to be studied. The biological cost of overproduction of the various efflux systems and the putative consequences with respect to the virulence of the host are currently under study.

In two types of plasmid transfer, mobilization and conjugation, overexpression of *adeABC* and *adeIJK* by the recipient resulted in reproducible reduction of transfer frequencies (Fig. 4). It thus appears that, if overexpression of pumps contributes to MDR by efflux, it decreases acquisition of foreign DNA by both transformation and conjugation. Alternatively, if acquisition of exogenous resistance genes occurs first, then efflux contributes in a synergistic manner, quantitatively and qualitatively, to resistance of the host.

In conclusion, our report stresses that, to be truly informative on the substrate ranges and the contribution of efflux systems to acquired and intrinsic resistance to antibiotics, the work (i) should be carried out on the bacterium of interest, rather than on a surrogate species, because of the impact of variations in levels of outer membrane permeability on the resistance phenotype, (ii) should be carried out on isogenic single-mutant derivatives overexpressing and deleted for each pump derived from a susceptible strain and (iii) should be carried out on those harboring various mutations reflecting the genetic events most commonly found in clinical isolates because of the important contribution of the levels of expression of the structural genes for the various systems, and (iv) should address the putative synergism between the pumps and the other mechanisms of resistance to a given substrate.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are described in Table S2 in the supplemental material. Bacteria were grown using brain heart infusion (BHI) or Luria-Bertani (LB) broth or agar at 37°C. Antibiotic susceptibility was determined by disk diffusion on Mueller-Hinton (MH) agar (53), and the

MICs were determined by microdilution in cation-adjusted MH broth according to the CLSI guideline (54).

**Selection of mutants overproducing RND efflux pumps.** Spontaneous mutants were obtained from susceptible clinical isolate *A. baumannii* BM4587 (12) on BHI gradient plates (17) containing 0 to 64  $\mu\text{g/ml}$  of gentamicin for *adeABC* and 0 to 1,000  $\mu\text{g/ml}$  chloramphenicol for *adeFGH*. Colonies growing at concentrations higher than the MIC were tested for antibiotic resistance by disk diffusion, and those resistant to several drug classes were selected for RT-qPCR analysis to quantify the expression level of each RND pump.

**Whole-genome sequencing.** Bacterial whole-genome sequencing was performed with Solexa single-read sequencing technology using a HiSeq 2000 system (Illumina) as previously described (9).

**OGM of *A. baumannii* BM4587.** A whole-genome restriction map of BM4587 was produced through optical genome mapping (OGM) technology (55) using a MapCard kit and an enzyme kit-NcoI on an Argus system (OpGen Inc.). The map was aligned with the *in silico* restriction map predicted from contigs, allowing amplification of PCR products to cover the gaps in the sequence (see Fig. S3 in the supplemental material). During gap coverage, we found a ca. 21-kb CDS between positions 774542 and 795637. By megablasting the 3,249-bp 5' end and the 5,736-bp 3' end, a biofilm-associated protein-encoding gene with repeated motifs, *bap* (56), was found, and its size was estimated to be 21,095 bp by OGM.

**Annotation and comparative genome analysis.** CDSs were first predicted using AMIGene (Annotation of Microbial Genomes) software (57). Each CDS was then submitted to automatic functional annotation, and, as a final step, manual validation of automatic annotations was performed using the Mage web interface as previously described (15).

**DNA manipulations and recombinant DNA techniques.** *A. baumannii* genomic DNA was extracted using a Wizard genomic DNA purification kit (Promega). DNA amplification was performed in a GeneAmp 9700 PCR system (PerkinElmer Cetus) with Phusion High-Fidelity DNA polymerase (Thermo Scientific), and the PCR products were cloned in pCR-Blunt vector with *E. coli* NEB5 $\alpha$  as the host strain. Nucleotide sequencing was carried out with a ceq 8000 DNA analysis system automatic sequencer (Beckman Instruments).

**Construction of *A. baumannii* deleted for *adeB*, *adeG*, and *adeJ*.** The *adeB*, *adeG*, or *adeJ* gene was deleted by overlapping PCR and recombination according to the *sacB*-base strategy (see Fig. S1 in the supplemental material) (19). Approximately 0.5-kb fragments upstream and downstream from the target genes were amplified from *A. baumannii* BM4587 genomic DNA using primer pairs *adeB1/adeB2* and *adeB3/adeB4*, *adeG1/adeG2* and *adeG3/adeG4*, or *adeJ1/adeJ2* and *adeJ3/adeJ4* (see Table S5 and Fig. S1 in the supplemental material), linked by overlapping PCR with primer pairs *adeB1/adeB4*, *adeG1/adeG4*, or *adeJ1/adeJ4*, generating ca. 1-kb fragments that were cloned in pCR-Blunt with selection on kanamycin (50  $\mu\text{g/ml}$ ). After sequencing of the inserts, the plasmids were digested by BamHI and NsiI ( $\Delta\text{adeB}$  and  $\Delta\text{adeG}$ ) or BamHI and PstI ( $\Delta\text{adeJ}$ ) and the resulting fragments were ligated to BamHI/NsiI-linearized pKNG101, generating pAT744, pAT745, and pAT746, respectively, which were introduced into *E. coli* DH5 $\alpha$ pir (58) with selection on streptomycin (50  $\mu\text{g/ml}$ ). The recombinant plasmids were transferred to *A. baumannii* BM4587 by triparental mating. *E. coli* DH5 $\alpha$ pir harboring pAT744, pAT745, or pAT746, *E. coli* HB101/pRK2013 (see Table S2) (59), and recipient *A. baumannii* BM4587 were grown separately overnight at 37°C in LB supplemented with streptomycin for selection of the donors and kanamycin for the helper plasmid. Aliquots of 0.5 ml of the donor and helper strains were centrifuged, and the pellets were suspended to an optical density at 600 nm ( $\text{OD}_{600}$ ) of 4 in LB. Equal volumes were mixed, and 50- $\mu\text{l}$  volumes were spotted on MH agar in duplicate and incubated at 37°C for 2 h. Fifty microliters of the recipient strain was plated on the spots, and conjugation was performed overnight at 37°C. Bacteria were collected and resuspended in 1 ml of LB, and 50- $\mu\text{l}$  aliquots were spread on LB agar plates containing streptomycin (300  $\mu\text{g/ml}$ ) plus fosfomycin (60  $\mu\text{g/ml}$ ) for counterselection of the *E. coli* strains and incubated at 28°C

for 3 days. Colonies were isolated, and single homologous recombination events were screened for by PCR with primer pairs *adeB7/adeB4* and *adeB1/adeB8* or primer pairs *adeG7/adeG4* and *adeG1/adeG8* or primer pairs *adeJ4/adeJ7* and *adeJ1/adeJ8*. Transconjugants were then plated on LB agar containing 10% sucrose at 28°C for 24 to 48 h to induce expression of *sacB* and to select bacteria susceptible to sucrose. Deletion of the target genes was confirmed by PCR with primer pairs *adeB7/adeB8*, *adeB7/adeB6*, and *adeB5/adeB8* or primer pairs *adeG7/adeG8*, *adeG7/adeG6*, and *adeG5/adeG8* or primer pairs *adeJ7/adeJ8*, *adeJ7/adeJ6*, and *adeJ5/adeJ8*.

**RNA isolation and RT-qPCR.** *A. baumannii* total RNA was extracted from exponentially grown bacteria ( $\text{OD}_{600}$ , 0.8 to 0.9) using TRIzol reagent (Invitrogen). RNA samples were treated with Turbo DNA-free kit (Applied Biosystems) to remove any genomic DNA carryover. Expression of genes *adeB*, *adeG*, and *adeJ* was quantified by RT-qPCR as previously described (12) using LightCycler RNA amplification kit SYBR green I (Roche Diagnostic) with the following cycle profile: 1 cycle at 95°C for 30 s, followed by 45 cycles at 95°C for 5 s, 56°C for 10 s, and 72°C for 20 s. The expression level of the *rpoB* gene of *A. baumannii* BM4587 was used as a reference (12). Each experiment was performed independently in duplicate at least twice.

**Proteomic analyses.** Proteins from bacterial membranes of strains BM4587, BM4689[ $\Delta\text{adeABC}$ ], and BM4666[ $\Delta\text{adeIJK}$ ] were extracted from 4-h cultures as previously described (29). Briefly, after the bacterial pellets were washed twice with sterile phosphate-buffered saline (PBS), they were sonicated with a Branson Sonifier (Branson Ultrasonic) and incubated 30 min at 37°C in lysis buffer (0.7% [wt/vol] Triton-X-100, 50 mM Tris-HCl (pH 7.4)). Unbroken cells were eliminated by centrifugation at 10,000  $\times g$  for 10 min at 4°C, and the supernatant was centrifuged at 100,000  $\times g$  for 45 min at 4°C. The (insoluble) pellet containing the cell envelope proteins was resuspended in 20 mM Tris-HCl (pH 7.4). Total protein content was determined by the Bradford assay. Protein separation on polyacrylamide gels, protein digestion by trypsin, and peptide extraction were performed as previously described (29). Peptide extracts were separated by the use of nano-liquid chromatography (nanoLC; Easy-nLC), and mass spectrometry analyses were performed on an LTQ-Orbitrap Elite system (Thermo Scientific) by injecting 1  $\mu\text{l}$  of sample. For each strain, three independent extractions of membrane proteins were performed and two technical replicates of each extract were analyzed. For protein quantification and identification, we used Progenesis LC-MS software (Nonlinear Dynamics), retaining the same statistical parameters as previously described for peptides (42). The merged peak list was searched against the *A. baumannii* AYE database (<http://www.ncbi.nlm.nih.gov/protein>) using a local version of Mascot (version 2.2; Matrix Science). Protein fold change was taken into account when the value was above 2. At least 3 peptides were used for protein identification and quantification.

**Biofilm assays.** Biofilm formation by *A. baumannii* BM4587 and its derivatives was examined either on pegs using a Calgary device (Innovotech, Edmonton, Canada) for solid-support biofilms (42) or in 24-well plates for formation of biofilms together with pellicles (42). Biofilm biomass was quantified after 48 h of growth at 37°C by crystal violet staining as previously described (42). Assays were performed in triplicate at least, and data are expressed as means  $\pm$  standard errors of the means (SE). Results were analyzed using Prism Graph Pad 5 software by a one-way analysis of variance (ANOVA) method to assess significant differences of biofilm growth between the strains.

**Motility and transformation.** Motility and transformation experiments were carried out as previously described (36). For both tests, plates were composed of 0.5% agarose, 5 g/liter tryptone, and 2.5 g/liter NaCl. The inoculum was stabbed into the semisolid medium to enable growth at the surface and at the interface between the agar and the bottom of the petri dish, and the plates were sealed with Parafilm to prevent drying. For the interface growth (twitching), the medium was allowed to air-dry for 5 min before the stabbing. Formation of a biofilm at the interface was visualized by crystal violet staining. For transformation, a suspension of a



single colony in 20  $\mu$ l of PBS was mixed with an equal volume of transforming DNA (400 to 800 ng/ $\mu$ l of DNA from kanamycin-resistant *A. baumannii* 179) (36) and stabbed into the motility plate (seven times, with 2  $\mu$ l of the mixture used for each stabbing) and the Parafilm-sealed plates were incubated for 18 h at 37°C. The bacteria were then flushed from the motility medium with 1 ml of sterile PBS and plated on BHI agar containing 30  $\mu$ g/ml of kanamycin. The number of CFU was determined for calculation of transformation rates. Effective transformation was determined on multiple colonies by PCR with 179-F and 179-R primers (36).

**Plasmid transfer.** Spontaneous rifampin-resistant mutants from *A. baumannii* BM4587 and derivatives were isolated on LB agar containing 100  $\mu$ g/ml of rifampin and used as recipients (see Table S2 in the supplemental material). For the liquid method, *A. baumannii* NIPH 527 with conjugative-system MPF<sub>1</sub> integrated in the chromosome and harboring kanamycin-resistant plasmid pRAY (38) was used as a donor. Equal amounts of overnight cultures of the donor and of the recipient were mixed, incubated in LB for 5 h, and diluted. Transconjugants were selected by plating on LB agar containing rifampin (50  $\mu$ g/ml) and kanamycin (50  $\mu$ g/ml), purified, and tested by disk-agar diffusion. Plasmids were extracted from randomly selected transconjugants and digested by BamHI or HindIII, and the size of the resulting fragments was determined. For the standard filter method, *A. baumannii* BM2580 harboring self-transferable plasmid pIP1841 (39) was used as a donor. Equal amounts of donor and recipient overnight cultures in LB were harvested, washed with an equal volume of LB, and mixed on a mating filter (Millipore) placed on LB agar. After 5 h of mating at 37°C, cells were recovered and plated on LB agar with antibiotics selective for donors (kanamycin at 50  $\mu$ g/ml), recipients (rifampin at 50  $\mu$ g/ml), and transconjugants (kanamycin at 50  $\mu$ g/ml and rifampin at 50  $\mu$ g/ml). The frequency was calculated as the number of transconjugants per donor.

**Nucleotide sequence accession number.** The assembled sequence of *A. baumannii* BM4587 was submitted to the GenBank database and is accessible under accession number JNOT000000000.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00309-15/-/DCSupplemental>.

Figure S1, PDF file, 0.1 MB.  
Figure S2, PDF file, 1.2 MB.  
Figure S3, PDF file, 0.1 MB.  
Table S1, DOCX file, 0.1 MB.  
Table S2, DOCX file, 0.05 MB.  
Table S3, DOCX file, 0.1 MB.  
Table S4, DOCX file, 0.1 MB.  
Table S5, DOCX file, 0.1 MB.

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