Overexpression of Resistance-Nodulation-Cell Division Pump AdeFGH Confers Multidrug Resistance in *Acinetobacter baumannii*[▽]

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Acinetobacter baumannii is a major nosocomial pathogen which frequently develops multidrug resistance by acquisition of antibiotic resistance genes and overexpression of intrinsic efflux systems, such as the RND efflux pumps AdeABC and AdeIJK. A third RND system was characterized by studying spontaneous mutants BM4663 and BM4664, which were selected in the presence of chloramphenicol and norfloxacin, respectively, from the AdeABC- and AdeIJK-defective derivative *A. baumannii* BM4652. They exhibited enhanced resistance to fluoroquinolones, tetracycline-tigecycline, chloramphenicol, clindamycin, trimethoprim, sulfamethoxazole, sodium dodecyl sulfate, and dyes such as ethidium bromide, safranin O, and acridine orange. Comparison of transcriptomes of mutants with that of their parental strain, using a microarray technology, demonstrated the overexpression of three genes that encoded an RND efflux system, named AdeFGH. Inactivation of AdeFGH in BM4664 restored an antibiotic susceptibility profile identical to that of BM4652, indicating that AdeFGH was cryptic in BM4652 and responsible for multidrug resistance in its mutants. RNA analysis demonstrated that the three genes were cotranscribed. The *adeFGH* operon was found in 36 out of 40 *A. baumannii* clinical isolates, but none of the 22 isolates tested overexpressed the pump genes. Spontaneous MDR mutant BM4684, overexpressing *adeFGH*, was obtained from clinical isolate BM4587, indicating that *adeFGH* can be overexpressed in a strain harboring *adeABC-adeIJK*. An open reading frame, coding a LysR-type transcriptional regulator, named *adeL*, was located upstream from the *adeFGH* operon and transcribed in the opposite direction. Mutations in *adeL* were found in the three *adeFGH*-overexpressing mutants, suggesting that they were responsible for overexpression of AdeFGH.

Multidrug resistance by overexpression of chromosomally encoded efflux systems is common in Gram-negative bacteria, most often associated with members of the resistance-nodulation-cell division (RND) superfamily (20). These pumps consist of an efflux membrane transporter (RND), which interacts with a major fusion protein (MFP) and an outer membrane factor (OMF) for drug export through both the inner and the outer membranes. The RND systems are involved in various functions, including homeostasis of the cell, export of virulence determinants, and extrusion of toxic compounds (19). They generally exhibit a broad substrate spectrum, and their overexpression can diminish the activity of several unrelated drug classes.

Acinetobacter baumannii is typically associated with hospital-acquired infections and possesses an extraordinary propensity to develop multiresistance (6). In addition to acquired resistance determinants, carried by plasmids, transposons, or resistance islands (18), two RND efflux systems, AdeABC (15) and AdeIJK (3), have been reported to confer a multidrug-resistant (MDR) phenotype to the species. The *adeABC* operon is found in ca. 80% of clinical isolates and is naturally cryptic due to tight regulation of its expression by the two-component system

AdeRS (16). Overexpression of AdeABC can result from point mutations in AdeR-AdeS (16) or from the insertion of an *ISAbal* copy upstream from the operon (22). AdeIJK is constitutively expressed at low levels, leading to a weak decrease in activity of some antibiotics (3), but its overexpression can contribute to multidrug resistance (2). Three additional efflux systems belonging to other superfamilies have been described in *A. baumannii*: CraA, a pump of the major facilitator superfamily that confers intrinsic chloramphenicol resistance (21), AbeS, a small drug resistance efflux pump mostly involved in resistance to detergents and dyes (26), and AbeM, a system of the multidrug and toxic compound extrusion family (27). However, the role of AbeM in antibiotic resistance remains to be determined.

An oligonucleotide-based DNA microarray has been developed to both quantify expression of chromosomal genes for efflux systems and detect acquired resistance determinants in *A. baumannii* (2). This powerful tool has been successfully used to detect overexpression of AdeABC and AdeIJK efflux pumps in isogenic mutants and allows study of clinical MDR strains by comparative analysis of their transcriptome relative to that of a reference strain. This is of particular interest because of the difficulty in evaluating the contribution of efflux to antibiotic resistance in clinical isolates. The microarray also enabled us to identify the role of a new RND efflux pump, AdeFGH, in conferring multidrug resistance in a single-step mutant obtained from the AdeABC- and AdeIJK-defective strain BM4652 (2). The aim of this work was to characterize

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Reference or source
<i>A. baumannii</i> strains		
BM4454	Wild-type strain, MDR	16
BM4587	Wild-type clinical isolate	2
BM4652	BM4454 Δ adeABC Δ adeIJK	3
BM4663	BM4652 spontaneous mutant obtained on chloramphenicol, MDR	This study
BM4664	BM4652 spontaneous mutant obtained on norfloxacin, MDR	This study
BM4679	BM4664 Δ adeFGH	This study
BM4684	BM4587 spontaneous mutant obtained on chloramphenicol, MDR	This study
Plasmids		
pUC18	Tra ⁻ Mob ⁻ Ap <i>lacZ</i> α	30
pAT520	pUC18 Ω adeF-ant(3'')-Ia-adeH	This study

^a Ap, ampicillin resistance; MDR, multidrug resistant; Mob⁻, nonmobilizable; Tra⁻, non-self-transferable.

the AdeFGH pump and the involvement of AdeL, a LysR-type transcriptional regulator, in its overexpression.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and antibiotic susceptibility testing. The bacterial strains used in this study are listed in Table 1. Cells were grown at 37°C in brain heart infusion (BHI) broth and agar (Difco Laboratories, Detroit, MI). Antibiotic susceptibility was tested by disk diffusion on Mueller-Hinton agar (Bio-Rad, Marnes-la-Coquette, France), and MICs were determined by the Etest procedure (AB Biodisk, Solna, Sweden). Susceptibility to ethidium bromide, safranin O, acridine orange, and sodium dodecyl sulfate (SDS) was screened for on BHI agar plates containing gradients of each drug (13).

Selection of MDR mutants. BM4652, in which both the AdeABC and AdeIJK systems are inactivated (3), and clinical isolate BM4587 (2) were grown on plates containing a gradient of cefotaxime, chloramphenicol, ciprofloxacin, erythromycin, imipenem, norfloxacin, rifampin, tetracycline, ticarcillin, or trimethoprim (28). Mutants were tested for antibiotic susceptibility by agar diffusion, and those resistant to several drug classes were selected for further analysis. MDR mutants BM4663 and BM4664 were obtained from BM4652 on chloramphenicol and norfloxacin, respectively, and BM4684 from BM4587 on chloramphenicol (Table 1).

DNA manipulations. *A. baumannii* genomic DNA was extracted as described previously (24). DNA amplification was performed in a GeneAmp PCR system 9700 (Perkin-Elmer Cetus, Norwalk, CT) with *Taq* (MPbio, Illkirch, France) or Phusion (Finnzymes, Espoo, Finland) DNA polymerase. PCR elongation times and temperatures were adjusted according to the expected size of the PCR products and the nucleotide sequences of the primers (Table 2), respectively. Sequence determination was carried out with a CEQ 2000 DNA analysis system automatic sequencer (Beckman Instruments, Inc., Palo Alto, CA).

RNA analysis. *A. baumannii* total RNA was isolated from exponentially growing cells (optical density at 600 nm [OD₆₀₀], 0.8 to 1.2) as described previously (2). cDNA synthesis was performed with avian myeloblastosis virus (AMV) reverse transcriptase (Roche Diagnostic GmbH, Mannheim, Germany) and real-time PCR using the SYBR PCR master kit (Roche) and gene-specific primers, 0.5 mM each (Table 2), according to the manufacturer's instructions. Amplification and detection of specific products were performed using the LightCycler sequence detection system (Roche) with the following cycle profile: 1 cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 58°C for 10 s, and 72°C for 30 s. Each experiment was performed in triplicate.

Microarray experiment. A microarray developed to quantify the expression of genes for putative efflux systems in *A. baumannii* was used for transcriptomic study (2). cDNAs of BM4664 and BM4652 were synthesized from 10 µg of total RNA, labeled with Cy3 or Cy5 cyanin (GE Healthcare, Uppsala, Sweden) using the SuperScript indirect cDNA labeling system (Invitrogen), mixed at equal concentrations (250 pmol of cyanin), and hybridized on the microarray. Slides

TABLE 2. Oligonucleotides used in this study

Primer	Sequence (5' → 3') ^a	Position ^b
L1	AGGAGTGTGCGTGTGGATC	−30−17
L2	GAAATCGGCATCGGTGCTG	794−776
F1	GAATTCGCGCAAACAGTTTGACTG	9−30
F2 ^c	TTATATCCACTGGGTTCGTGCCTGTCA	750−717
	AGGCAAGTCCCATATAGACAGGTAC	
F3	GGTGTGACCAAGATAAACG	943−962
F4	GTGAATTTGGCATAGGGACG	1150−1131
G1	TTCATCTAGCCAAGCAGAAG	2520−2539
G2	GTGTAGTGCCACTGGTTACT	2969−2988
G3	ATGTGGGCTAGCTAACGGC	3171−3153
H1 ^d	GATCACCAAGGTAGTCGGCAAATAAC	262−283
	CTAAAAGCGGTGGCAGCAA	
H2	GGATCCAACCTCGCTTAAACTGAAGA	1030−1005
	TTCA	
H3	CGATCAGCAAATTCAGGCTC	1230−1249
H4	GCTTGCAATGATTTGGCTGC	1410−1391
rpoB for	TCCGCACGTAAAGTAGGAAC	1960−1979
rpoB rev	ATGCCGCCTGAAAAAGTAAC	2114−2095

^a The restriction sites introduced into the oligonucleotides are underlined.

^b Coordinates refer to the first base of each gene.

^c The first 27 bp are specific for the 5' part of *ant*(3'')-Ia.

^d The first 25 bp are specific for the 3' part of *ant*(3'')-Ia.

were scanned (GenePix 4000 A scanner; Axon), and data were analyzed as described previously (2). Three independent biological replicates and a dye swap were performed, leading to six hybridizations per experiment. A spot was considered differentially expressed when the Bonferroni method-adjusted *P* value was lower than 0.05.

Insertion-inactivation of *adeFGH*. Fragments internal to the *adeF* and *adeH* genes from BM4652 were amplified with primers F1/F2 and H1/H2, respectively (Table 2). The first 27 and 25 bp of F2 and H1, respectively, are complementary to an *ant*(3'')-Ia streptomycin-spectinomycin resistance cassette so that the *adeF* and *adeH* fragments when mixed with the *ant*(3'')-Ia cassette allow amplification of an *adeF-ant*(3'')-Ia-*adeH* sequence using primers F1 and H2 (Fig. 1). F1 and H2 possess a BamHI and an EcoRI restriction site, respectively. The *adeF-ant*(3'')-Ia-*adeH* fragment digested by both enzymes was ligated into BamHI-EcoRI-linearized pUC18, generating plasmid pAT520, which was introduced into competent cells of strain BM4664 by electroporation. Since pAT520 does not stably replicate in *A. baumannii*, the transformants selected for streptomycin-spectinomycin resistance and screened for ticarcillin susceptibility should result from a double recombination event leading to partial deletion-inactivation of *adeFGH*. The genetic construction was confirmed by PCR and sequencing of the BM4579 transformant.

RESULTS AND DISCUSSION

Substrate specificity of AdeFGH pump. Spontaneous MDR BM4663 and BM4664 were selected on chloramphenicol and norfloxacin, respectively, from BM4652 (Δ adeABC Δ adeIJK), which had been derived from BM4454 by inactivation of RND efflux systems AdeABC and AdeIJK (3). The mutants had a similar phenotype, i.e., high-level resistance to chloramphenicol, clindamycin, fluoroquinolones, and trimethoprim and decreased susceptibility to tetracycline-tigecycline and sulfonamides (Table 3); susceptibility to β -lactams, erythromycin, and rifampin was not affected. The impact against most aminoglycosides could not be assessed due to the presence of a kanamycin or an apramycin resistance cassette to inactivate the *adeABC* and *adeIJK* gene clusters (3). However, the activity of amikacin, which is not affected by the corresponding APH(3')-I and AAC(3)-VI enzymes, remained unchanged, suggesting that aminoglycosides are not substrates for the pump. BM4663 and BM4664 also demonstrated, com-

FIG. 1. Schematic representation of the *adeFGH* operon of *A. baumannii* BM4664 and derivative BM4679. Open arrows indicate coding sequences and direction of transcription. The streptomycin-spectinomycin *ant*(3'')-Ia resistance gene is hatched. Black arrows indicate the position and orientation of the primers used.

pared with parental strain BM4652, increased resistance to ethidium bromide, safranin O, acridine orange, and SDS (data not shown). Furthermore, the mutants exhibited a higher level of resistance to trimethoprim and sulfamethoxazole than BM4454, which possesses AdeIJK and overexpresses AdeABC (15).

Overexpression of AdeFGH in MDR mutants. Multidrug resistance resulting from *in vitro* single-step selection can be due either to a decrease in membrane permeability or, more likely, to increased efflux. In addition, diminished susceptibility to tigecycline is known to result from overexpression of RND efflux systems in *A. baumannii* (22), *Pseudomonas aeruginosa* (4), and enterobacteria (8, 23). Comparison of the transcriptome of BM4664 with that of BM4652, using a microarray which enables quantification of the expression of all putative efflux genes in *A. baumannii* (2), revealed overexpression in BM4664 of three adjacent open reading frames (ORFs) annotated as encoding putative MFP (CT025800) (2.9-fold in-

crease), RND efflux protein (CT025801) (3.8-fold), and OMF (CT025802) (3.1-fold) (7). The microarray did not detect any other gene differentially expressed. Overexpression of the three ORFs, respectively named *adeF*, *adeG*, and *adeH*, was confirmed in BM4663 and BM4664 by quantitative reverse transcriptase PCR (RT-PCR) using primers F3/F4, G2/G3, and H3/H4 (Table 2). Increases of more than 300-fold and 200-fold in *adeFGH* expression in BM4663 and BM4664, respectively, were observed. The *adeFGH* operon was thus considered responsible for the MDR phenotype of the mutants.

A third spontaneous mutant, BM4684, was selected on chloramphenicol from clinical strain BM4587, which harbors functional *adeABC* and *adeIJK* efflux gene clusters (2). BM4684 had an MDR phenotype similar to those of BM4663 and BM4664 (Table 3), and quantitative RT-PCR indicated overexpression of more than 200-fold of the *adeFGH* genes whereas expression of *adeABC* or *adeIJK* remained unchanged. These data

TABLE 3. Antibiotic susceptibility of *A. baumannii* strains

Antimicrobial agent	MIC (μg/ml) for strain:						
	BM4454	BM4652 (<i>ΔadeABC ΔadeIJK</i>)	BM4663 ^a (<i>ΔadeABC ΔadeIJK</i>)	BM4664 ^a (<i>ΔadeABC ΔadeIJK</i>)	BM4679 (<i>ΔadeABC ΔadeIJK ΔadeFGH</i>)	BM4587	
Ticarcillin	8	0.75	0.75	0.75	0.75	8	12
Cefepime	3	0.25	0.125	0.19	0.25	0.75	1
Aztreonam	16	1.5	2	1.5	1.5	12	16
Imipenem	0.25	0.125	0.25	0.25	0.125	0.19	0.38
Chloramphenicol	>256	32	>256	>256	16	96	>256
Tetracycline	32	0.75	3	2	0.75	1.5	3
Minocycline	0.75	0.064	0.75	0.5	0.064	0.064	0.25
Tigecycline	3	0.064	1.5	1	0.064	0.094	0.38
Erythromycin	16	1.5	2	1.5	1.5	6	6
Clindamycin	>256	2	>256	>256	2	>256	>256
Rifampin	2	2	3	2	2	1.5	4
Norfloxacin	>256	12	>256	>256	12	1.5	12
Ciprofloxacin	>32	1.5	>32	>32	2	0.094	0.38
Moxifloxacin	12	0.75	12	12	0.5	0.032	0.19
Amikacin	4	1.5	1.5	1.5	0.75	3	3
Trimethoprim	8	0.75	>32	>32	0.75	16	>32
Sulfamethoxazole	2	1	16	16	1	4	32
Co-trimoxazole	0.064	0.047	0.5	0.5	0.047	0.19	0.75

^a Overexpresses *adeFGH*.

FIG. 2. Analysis of the *adeL-adeF* intergenic region. Start codons of *adeL* and *adeF* genes are in bold; arrows represent transcripts and indicate direction of transcription; promoters for *adeL* and *adeFGH*, predicted by the BProm software, are indicated on each strand. +1, in bold, start of transcription; -10 and -35 regions are in italics and indicated by bold lines; the typical TTA-N₇-TAA sequence of the LTTR box is boxed.

indicate that AdeFGH-mediated resistance can be selected in a strain containing *adeABC* and *adeIJK*.

Relationship of AdeFGH with other efflux systems. Comparison of the 407-, 1,060-, and 484-amino acid sequences of AdeF, AdeG, and AdeH, respectively, with those in the GenBank database revealed a low degree of identity with AdeABC and AdeIJK (<40%) and a high degree of identity with putative efflux transporters and already-characterized RND systems, in particular BpeEF-OprC from *Burkholderia pseudomallei* (55, 79, and 53% for the MFP, RND, and OMF proteins, respectively) and CeoAB-OpcM from *Burkholderia cenocepacia* (54, 79, and 50%). BpeEF-OprC extrudes chloramphenicol and trimethoprim (11), whereas CeoAB-OpcM exports, in addition, ciprofloxacin (17). These substrate ranges are consistent with the phenotypes of BM4663, BM4664, and BM4684, all of which were highly resistant to chloramphenicol, trimethoprim, and ciprofloxacin. Transmembrane topology of the AdeG transporter was predicted with the TMHMM program of the ExPASy server. As expected, AdeG contained 12 transmembrane domains (TMDs) and two large periplasmic loops between TMDs 1 and 2 and TMDs 7 and 8, typical of RND proteins.

Inactivation of *adeFGH* gene cluster in BM4664. To confirm the role of AdeFGH overexpression in multidrug resistance of BM4664, the pump was inactivated by partial replacement of the structural genes for the system with an *ant(3'')-Ia* streptomycin-spectinomycin resistance cassette following homologous recombination (Fig. 1). The double crossing-over was confirmed by PCR and sequencing of derivative BM4679 ($\Delta adeABC \Delta adeIJK \Delta adeFGH$) (Fig. 1). BM4679 had restored susceptibility to drugs and dyes that are substrates for AdeFGH, confirming the contribution of AdeFGH to multidrug resistance in BM4664 and the broad-substrate spectrum of the pump (Table 3). Furthermore, BM4679 ($\Delta adeABC \Delta adeIJK \Delta adeFGH$) had the same level of resistance as BM4652 ($\Delta adeABC \Delta adeIJK$), indicating that AdeFGH is not expressed in BM4652 and thus does not contribute to intrinsic resistance of *A. baumannii* (Table 3), an observation confirmed by quantitative RT-PCR (data not shown).

Transcriptional analysis of the *adeFGH* gene cluster. Transcription of the *adeFGH* gene cluster was analyzed by RT-PCR and Northern hybridization. Products with the expected size

were obtained by amplification of *adeFG*, *adeGH*, and *adeFGH* overlapping fragments from cDNA of BM4664, using primers F3/G3, G2/H4, and F3/H2, respectively, whereas no amplification was obtained for the *adeLF* overlapping fragment using primers L2/F4 (Table 2). Genes of RND efflux systems are typically organized in operons, and our data indicate that the *adeFGH* genes were cotranscribed.

Involvement of *adeL* in expression of the *adeFGH* operon. An ORF for a putative LysR-type transcriptional regulator (LTTR) and named *adeL* was located upstream from the *adeFGH* operon and transcribed in the opposite direction (Fig. 1). The 337-amino-acid deduced sequence of AdeL exhibited homology with those of other LTTRs, in particular the CeoR regulator (56% identity). Thus, the AdeL-AdeFGH system appears to have an organization similar to that of the CeoR-CeoAB-OpcM efflux system from *B. cenocepacia* (17). The HelixTurnHelix program (<http://mobyle.pasteur.fr/>) predicted the presence of a helix-turn-helix (HTH) DNA-binding motif between residues 11 and 32, typical of the LTTR family. Sequence analysis of the *adeL-adeF* intergenic region using BProm software (Softberry, Inc., Mt. Kisco, NY) predicted the presence of overlapping promoters for *adeL* and *adeFGH* expression (Fig. 2). This region includes a TTA-N₇-TAA motif typical of a LTTR box which is implicated in DNA binding by LTTR (14).

The sequences of *adeL* and of the putative promoter region of *adeFGH* in the three mutants were compared with those of the parental strain. No mutations were detected in the putative promoter region or in the LTTR box upstream from the *adeFGH* operon, but mutations in *adeL* were found in both strains. In BM4663, a thymidine was inserted at position 981 of *adeL*, introducing a premature stop codon resulting in deletion of the 11 C-terminal residues, whereas an A-to-C mutation leading to a valine-to-glycine substitution at position 139 of AdeL was present in BM4664. A threonine-to-lysine substitution was detected at position 319 of AdeL in BM4684. Mutations in the C-terminal region of LTTRs have been reported to confer a constitutive phenotype (10, 12) by alteration of oligomerization and interaction with the RNA polymerase (5). Thus, deletion of the 11 C-terminal residues and a threonine-to-lysine substitution in BM4684 of AdeL could be responsible for overexpression of *adeFGH* in BM4663 and

BM4684, respectively. In BM4664, the V139G substitution occurred in the putative signal recognition domain of AdeL and probably conferred, as already reported for several LTTRs (14), a signal-independent phenotype.

Distribution and expression of the *adeFGH* operon in clinical isolates. The structural genes for the pump and the putative regulator are present and conserved in the genomes of the seven sequenced *A. baumannii* strains (1, 9, 25, 29). The *adeG* gene was detected by PCR using primers G1/G3 in 40 out of 44 *A. baumannii* strains from our laboratory collection. In the four *adeG*-negative strains, PCR using L2/L1, F1/F4, and H1/H4 (Table 2 and Fig. 1) showed the presence of *adeL* and *adeF* whereas *adeH* was lacking. These data suggest that the AdeFGH system is intrinsic to the *A. baumannii* species, although *adeGH* has been lost for some.

AdeFGH conferred high-level resistance to clindamycin when overexpressed. Quantitative RT-PCR was performed to measure the level of expression of *adeG* in isolates exhibiting high-level resistance to clindamycin. None of the 22 selected clinical strains expressed the efflux gene, indicating that AdeFGH is cryptic in *A. baumannii*. However, the number of isolates studied is too low to determine the incidence of AdeFGH-mediated multidrug resistance in *A. baumannii*.

Increased expression of AdeFGH in *A. baumannii* is an additional mechanism for high-level resistance to fluoroquinolones and decreased susceptibility to tigecycline and is therefore of a clinical relevance. The presence of the *adeFGH* operon in ca. 90% of the strains studied and overexpression likely due to point mutation in *adeL* suggest that this event could occur in clinical strains. Molecular and biochemical studies of the transcriptional regulator AdeL should allow better understanding of the mechanism of AdeFGH expression in *A. baumannii*.

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