Screening and Quantification of the Expression of Antibiotic Resistance Genes in *Acinetobacter baumannii* with a Microarray[▽]

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An oligonucleotide-based DNA microarray was developed to evaluate expression of genes for efflux pumps in *Acinetobacter baumannii* and to detect acquired antibiotic resistance determinants. The microarray contained probes for 205 genes, including those for 47 efflux systems, 55 resistance determinants, and 35 housekeeping genes. The microarray was validated by comparative analysis of mutants overexpressing or deficient in the pumps relative to the parental strain. The performance of the microarray was also evaluated using in vitro single-step mutants obtained on various antibiotics. Overexpression, confirmed by quantitative reverse transcriptase PCR, of RND efflux pumps AdeABC, due to a G30D substitution in AdeS in a multidrug-resistant (MDR) strain obtained on gentamicin, and AdeIJK, in two mutants obtained on cefotaxime or tetracycline, was detected. A new efflux pump, AdeFGH, was found to be overexpressed in a mutant obtained on chloramphenicol. Study of MDR clinical isolates, including the AYE strain, whose entire sequence has been determined, indicated overexpression of AdeABC and of the chromosomally encoded cephalosporinase as well as the presence of several acquired resistance genes. The overexpressed and acquired determinants detected by the microarray could account for nearly the entire MDR phenotype of the isolates. The microarray is potentially useful for detection of resistance in *A. baumannii* and should allow detection of new efflux systems associated with antibiotic resistance.

Multidrug-resistant (MDR) strains of *Acinetobacter baumannii* have emerged in recent decades. This opportunistic pathogen is responsible for severe infections, particularly hospital-acquired pneumonia and bloodstream, urinary tract, and wound infections, and has become of worldwide concern (13). As in other bacterial species, multidrug resistance can be achieved by two mechanisms: (i) horizontal transfer of genetic information and (ii) mutation of endogenous genes. Acquired resistance determinants that are carried by plasmids (18, 28), transposons (23, 29), and integrons (33, 43) have been described for *Acinetobacter* spp. Determination of the genomic sequence of several *A. baumannii* strains has improved our knowledge of the ways in which *A. baumannii* can develop antibiotic resistance (1, 21, 38, 45). An 86-kb resistance island, AbaR1, found in strain AYE, contains as many as 25 antibiotic and 20 antiseptic and heavy metal resistance genes (16). Variants of this island are integrated at the same chromosomal locus in a significantly high proportion of MDR strains (37). In addition to these acquired resistance genetic elements, alterations in endogenous functions are involved in resistance, such as overexpression of chromosomally encoded β-lactamases ADC and OXA-51-like; loss of porins CarO and Omp33–36 contributing to carbapenem resistance; mutation in the GyrA and ParC fluoroquinolone targets; and overexpression of efflux systems (13).

Efflux systems are components of the bacterial membrane that are thought to play a role in homeostasis of the cell and extrusion of toxic compounds (32). They can also be involved in cell-to-cell communication via quorum-sensing systems (22) and in bacterial pathogenicity (32). Efflux pumps of the resistance nodulation cell division (RND) superfamily, widespread in gram-negative bacteria, confer multidrug resistance to their host when overexpressed (34). In most instances, the level of resistance remains moderate; however, reduced intracellular accumulation of antibiotics provides a delay for the selection of high-level resistance by drug inactivation or target alteration. Efflux thus can act in synergy with other mechanisms to achieve high-level resistance (34).

Two RND efflux systems, AdeABC and AdeIJK, have been characterized for *A. baumannii* (9, 24). They are composed of an efflux protein (AdeB or AdeJ) which interacts with a membrane fusion protein (AdeA or AdeI) and an outer membrane factor (AdeC or AdeK) to facilitate drug export across both the inner and the outer membranes. The *adeABC* operon is cryptic in approximately 80% of *A. baumannii* strains (24), and its overexpression leads to diminished susceptibility to aminoglycosides, cefepime, fluoroquinolones, chloramphenicol, and tetracycline-tigecycline. Consequently, the AdeABC efflux pump constitutes a major mechanism of multiple antibiotic resistance in *A. baumannii*, and its clinical significance has been established (31). AdeIJK is present in all *A. baumannii* strains and confers decreased susceptibility to β-lactams, fluoroquinolones, chloramphenicol, rifampin (rifampicin), erythromycin, clindamycin, and tetracycline-tigecycline. The toxicity observed with *Escherichia coli* and *A. baumannii* when the *adeIJK* operon was cloned and overexpressed suggests its effect on resistance is minimal (9). Another pump belonging to the MATE family, AbeM, has been characterized (39), but its role in the antibiotic resistance of *A. baumannii* remains to be

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TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristic(s)	Reference or source
CIP 70-10	Susceptible reference strain	3
BM4546	CIP 70-10 AdeS$_{T153M}$ spontaneous mutant, MDR	25
BM4547	CIP 70-10 AdeR$_{P116L}$ spontaneous mutant, MDR	25
BM4454	Clinical strain overexpressing AdeABC	24
BM4579	BM4454 $\Delta adeIJK$	9
BM4651	BM4454 $\Delta adeABC$	9
BM4652	BM4454 $\Delta adeABC$ $\Delta adeIJK$	9
BM4587	Susceptible clinical strain	This study
BM4665	BM4587 spontaneous mutant obtained on gentamicin, MDR	This study
BM4666	BM4587 spontaneous mutant obtained on cefotaxime, MDR	This study
BM4667	Susceptible clinical strain	This study
BM4668	BM4667 spontaneous mutant obtained on tetracycline, MDR	This study
AYE	MDR clinical strain	16
BM4675	MDR clinical strain	This study
BM4676	MDR clinical strain	This study

determined. Analysis of the genome sequence of strain AYE revealed the presence of 41 other genes putatively associated with efflux (16).

Microarrays are powerful tools for large-scale screening of the genomic or transcriptomic content of a cell population. Several genomic microarrays have been developed to detect acquired antibiotic resistance genes (5) and transcriptomic microarrays have been used to compare spontaneous mutants with the parental strain, highlighting the role of overexpression of efflux genes in antibiotic resistance (14).

Overexpression of efflux systems is an important factor leading to antibiotic resistance but is not easily detectable at both the phenotypic level, because of the low level of resistance conferred, and the genotypic level, because the regulatory mutations most often remain unknown. To study the mechanisms of multidrug resistance in *A. baumannii*, particularly those involving efflux, we have developed a microarray that allows quantification of the expression of resistance genes, including intrinsic and acquired determinants.

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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 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).

Production of MDR mutants. Spontaneous MDR mutants BM4665 and BM4666 were obtained from wild-type strain BM4587, and mutant BM4668 was obtained from strain BM4667, on gradient plates (40) containing either gentamicin, cefotaxime, or tetracycline (Table 1). Colonies growing at concentrations higher than the normal MIC were tested for antibiotic susceptibility by diffusion, and those exhibiting resistance to several drug classes were selected for further studies.

DNA manipulations. *A. baumannii* genomic DNA was extracted as described previously (36). Amplification of DNA was performed in a GeneAmp PCR system 9700 (Perkin-Elmer Cetus, Norwalk, CO) with *Taq* (MPbio, Illkirch,

France) or Phusion (Finnzymes, Espoo, Finland) DNA polymerase. Amplification of large DNA fragments was achieved using the Expand Long Template PCR system (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's recommendations. PCR elongation times and temperatures were adjusted according to the expected sizes 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 isolation. RNA was isolated as described previously (27). Briefly, exponentially growing cells (optical density at 600 nm of 0.8 to 1.2) were harvested, and the pellet was resuspended in 12.5 mM Tris–5 mM EDTA–10% glucose. Cells were sheared mechanically in the presence of acid phenol (pH 4.6) and glass beads (0.2- to 0.3-mm diameter; Sigma-Aldrich, St. Louis, MO) using a Fastprep apparatus (Bio 101). After centrifugation, Trizol reagent (Invitrogen, Leek, The Netherlands) was added to the supernatant. Total RNA was extracted twice with chloroform-isoamyl alcohol (24:1 [vol/vol]), precipitated in 0.7 volume of isopropanol and washed twice with 70% ethanol. Treatment with RNase-free DNase (Ambion, Austin, TX) was performed according to the manufacturer's recommendations. RNA was quantified by determining absorbance at 260 and 280 nm; its purity and integrity were analyzed on agarose gels.

Microarray construction. Oligonucleotide probes (70-mer) were designed with ArrayOligoSelector (4) to represent each open reading frame annotated as an *A. baumannii* coding sequence in the GenBank database (http://www.ncbi.nlm.nih.gov/) using the complete genome sequence of AYE as a reference. BLAST analysis of probes against themselves or the AYE genome enabled nonspecific binding effects to be eliminated. The 205 probes selected were synthesized and spotted in triplicate on UltraGAPS glass slides (Corning Life Sciences, Corning, NY) using a Chipwriter Pro Virtek arrayer (Bio-Rad, Hercules, CA). After printing, arrays were treated as recommended by the slide manufacturer.

Synthesis of cDNA and microarray hybridization. cDNAs were synthesized from 10 μg of total RNA and labeled with Cy3 or Cy5 cyanin (GE Healthcare, Uppsala, Sweden) using the SuperScript indirect cDNA labeling system (Invitrogen). Labeled cDNAs of the studied and reference strains were mixed at equal concentrations (250 pmol of cyanin) and hybridized on the microarray. Slides were scanned (GenePix 4000 A scanner; Axon), and images were analyzed (GenePix5 software; Axon).

Data analysis. Script analyses were developed with the software R (R Development Core Team; http://www.R-project.org). For each spot, the fluorescence value corresponded to the median pixel intensity within the spot and no background was subtracted. Three independent biological replicates and a dye swap for each replicate to overcome cyanin bias were performed. Loess normalization (46) was applied to all spots, on a slide-by-slide basis. To determine differentially expressed genes from replicates pooling log$_2$ ratios, a paired t test was performed. Low numbers of observations per spot and of spots per slide (615) did not permit calculation of a specific variance and clusters of spots with equal variance, respectively. For these reasons, it was assumed that the variance of the log$_2$ ratios was the same for all spots, and spots displaying extreme specific variance (too small or too large) were excluded from the statistical analysis (17). A spot was considered differentially expressed when the Bonferroni method-adjusted P value was lower than 0.05. The mean and standard deviation were calculated for genes corresponding to significantly differentially expressed spots and with a change of < -1.5 or > 1.5.

Quantitative reverse transcriptase (qRT) PCR. cDNA synthesis was performed for 60 min at 42°C using 0.1 to 0.2 μg of RNA, 0.8 μl of avian myaloblastosis virus reverse transcriptase (Roche Diagnostic GmbH, Mannheim, Germany), and 2 μl of random primer according to the manufacturer's instructions. Real-time PCR was performed with a 20-μl reaction volume containing 2 μl of cDNA, 1× SYBR PCR master mix (Roche), and gene-specific primers, 0.5 mM each (Table 2). Amplification and detection of specific products were performed using the LightCyler sequence detection system (Roche) with the following cycle profile: 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s, 58°C for 10 s, and 72°C for 30 s.

Microarray data accession number. The microarray data obtained in this study were deposited in ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) according to MIAME standards under accession number E-MEXP-2254.

RESULTS AND DISCUSSION

Design of the microarray. An oligonucleotide-based DNA microarray was developed to compare the expression levels of efflux genes of an isogenic MDR mutant and its parental strain or those of clinical isolates and a reference strain. The

TABLE 2. Oligonucleotides used in PCR and qRT-PCR

Gene	Primer	Sequence (5′→3′)	Position[a]	Size (bp)
rpoB	rpoB Rev	ATGCCGCCTGAAAAAGTAAC	1960–1979	154
	rpoB For	TCCGCACGTAAAGTAGGAAC	2114–2095	
adeA	adeA For	ATCGCTAACAAAGGCTTGAA	1024–1043	159
	adeA Rev	CGCCCCCTCAGCTATAGAA	1183–1163	
adeB	adeB For	CTTGCATTTACGTGTGGTGT	2907–2926	168
	adeB Rev	GCTTTTCTACTGCACCCAAA	3075–3056	
adeC	adeC For	TACACATGCGCATATTGGTG	1157–1176	117
	adeC Rev	CGTAAAATAACTATCCACTCC	1274–1244	
adeI	adeI For	CAAATGCAAATGTAGATCTTGG	511–532	210
	adeI rev	AAACTGCCTTTACTTAGTTG	702–721	
adeJ	adeJ For	GGTCATTAATATCTTTGGC	1142–1160	221
	adeJ Rev	GGTACGAATACCGCTGTCA	1363–1345	
adeK	adeK For	TTGATAGTTACTTGACTGTTC	1264–1285	162
	adeK Rev	GGTTGGTGAACCACTGTATC	1407–1426	
ampC	ampC For	CAGTAATTCAGAACAGATTGTG	927–947	183
	ampC Rev	GCGCTCTTCATTTGGAATAC	1110–1091	
adeS	adeS For	TATGAAAAGTAAGTTAGGAAT	1–19	1,072
	adeS Rev	TTAGTTATTCATAGAAATTTT	1073–1053	
adeR	adeR For	AATGCTTAATACACTGACTTTAGCCGGTGG	−265–−236	1,429
	adeR Rev	TCTAGCAGAGAGGTCGC	1101–1084	
IS*Aba1*	IS*Aba1*a[b]	ATGCAGCGCTTCTTTGCAGG	462–471	379
	IS*Aba1*b[b]	AATGATTGGTGACAATGAAG	822–841	
IS*Aba4*	IS*Aba4*a[c]	ATTTGAACCCATCTATTGGC	140–159	611
	IS*Aba4*b[c]	ACTCTCATATTTTTTCTTGG	751–732	
bla~OXA-23~	oxa23+	ATGAATAAATATTTTACTTG	1–20	821
	oxa23−	TTAAATAATATTCAGCTGTT	822–803	
tniA	tniA For	CAATGTGGTTGTCGGTTATGA	54–74	162
	tniA Rev	ATTCAAACTCTCAGTTAGCGT	216–196	
ATPase	ATPase3′	AATGGCGGTACATCAATATGT	1215–1195	492
	ATPase5′	GAAACTCTTGAAGTTGCAAGT	723–743	

[a] Coordinates refer to the first base of each gene.
[b] From reference 20.
[c] From reference 8.

GenBank database was screened for *A. baumannii* gene sequences since no annotated genome was available at the beginning of the study. A total of 205 genes was selected for the microarray. The list of genes and corresponding probes is available at ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MEXP-2254. The probes were designed to detect the 47 efflux-related genes of strain AYE (16), which comprise six RND (including *adeABC* and *adeIJK*), seven MF, and two MATE systems; 10 genes for outer membrane proteins, e.g., those encoding the pore-forming protein HMP-AB (19), the 33- to 36-kDa protein (10), and CarO (30); and 8 genes involved in biofilm formation, notably the *csu* operon (41) and its two-component regulatory system (42). Fifty-five antibiotic resistance genes from gram-negative bacteria were added, several of them not yet described for *A. baumannii*. They represent the most clinically relevant resis-

tance mechanisms, i.e., the chromosomal cephalosporinase, class A β-lactamases, metallo-β-lactamases and oxacillinases, including carbapenemases; aminoglycoside modifying enzymes (11 acetyl-, 4 nucleotidyl-, and 3 phosphotransferases), 16S RNA methylases, tetracycline efflux pumps and ribosomal protection proteins, rifampin ADP ribosyltransferase (Arr-2), chloramphenicol acetyltransferases and efflux pumps, and plasmid-borne quinolone resistance gene *qnr*. Genes conferring resistance to arsenic (*ars*) and mercury (*mer*) heavy metals were also included. Finally, genes involved in mobility or transfer of genetic elements, such as integrases, insertion sequences, or transposases, and several genes present in the 86-kb resistance island of strain AYE, such as *trxB*, *lspA*, and *uspA*, were added. These sequences should enable detection of the backbone structures of mobile genetic elements carrying antibiotic resistance genes.

TABLE 3. Differential quantification of gene expression in isogenic strains of *A. baumannii*

Strain		Change (fold)[a]						
Parent	Mutant	*adeA*	*adeB*	*adeC*	*adeI*	*adeJ*	*adeK*	*aac(3)-IVa*
BM4454	BM4579	NS	NS	NS	−3.64	−3.60	NS	NA
	BM4651	−4.21	−3.25	1.84	NS	NS	NS	3.56
	BM4652	−1.17[b]	−1.50	1.38[b]	−1.80	−2.70	NS	12.88
CIP70-10	BM4546	2.17	2.74	NS	NS	NS	NS	NS
	BM4547	NS	NS	NS	NS	NS	NS	NS
BM4587	BM4665	1.79	3.04	2.09	NS	NS	NS	NA
	BM4666	NS	NS	NS	1.65	2.35	1.16[b]	NS
BM4667	BM4668	NS	NS	NS	1.53	2.42	1.04[b]	NS

[a] Positive and negative values correspond to genes with, respectively, increased or decreased expression in the mutant strain in comparison with the parental strain. NS, not statistically significant. NA, not applicable.
[b] Not statistically significant by the microarray but confirmed by qRT-PCR.

Thirty-five housekeeping genes, such as *gyrA*, *parC* and *rpoB*, as well as genes used for an MLST scheme (2) and a multilocus PCR typing method (15), and 20 genes linked to metabolism functions were added to avoid normalization bias due to the low density of the chip.

Validation of the microarray. Microarray validation was first performed by comparing strain BM4454, which overexpresses the AdeABC efflux system (24), with derivative mutants BM4579, BM4651, and BM4652, in which *adeIJK*, *adeABC*, and both systems, respectively, had been inactivated by insertion-deletion (9). Comparative analysis of the data obtained with BM4454 and BM4579 (BM4454 Δ*adeIJK*) revealed that, as expected, the *adeI* and *adeJ* genes were not expressed in the mutant (Table 3). Since integration of the cassette partially deletes the *adeI* and *adeJ* genes but not *adeK*, no differences were observed for *adeK* expression. These results were confirmed by qRT-PCR using specific primers (Table 2). Comparison of the BM4454 and BM4651 (BM4454 Δ*adeABC*) transcriptomes confirmed inactivation of *adeA* and *adeB* in the latter. The *aac(3)-IVa* cassette that was inserted to inactivate this system was confirmed to be expressed in BM4651. Similarly, *adeC* was overexpressed due to a transcriptional fusion with *aac(3')-IVa* in the mutant (Table 3) (9). The expression of other efflux genes was not affected by inactivation of *adeABC*. Comparative analysis of the transcriptomes of BM4454 and BM4652 (BM4454 Δ*adeABC* Δ*adeIJK*) confirmed the inactivation of *adeA*, *adeB*, *adeI*, and *adeJ* and overexpression of *aac(3)-IVa* and *adeC*. Differential expression of *adeA* and *adeC* was not found to be statistically significant by the microarray but was confirmed by qRT-PCR (Table 3).

Validation was also performed by studying AdeABC-overexpressing mutants BM4546 and BM4547 obtained from strain CIP 70-10 following point mutations in *adeS* and *adeR*, respectively (Table 1) (25). The two-component system AdeRS controls the expression of *adeABC*, AdeR being a transcriptional regulator and AdeS a histidine kinase sensor (25). The regulatory operon is located upstream from *adeABC* and transcribed in the opposite direction. Overexpression of *adeA* and *adeB* in BM4546, but not in BM4547, was observed (Table 3). qRT-PCR showed 10- and 4-fold increases in *adeB* expression in BM4546 and BM4547, respectively. These results are in agreement with BM4546 having an amount of *adeB* mRNA

larger than that in BM4547 as detected by Northern hybridization (25), despite the fact that the two mutants exhibit similar resistance phenotypes. No changes in the expression of the *adeR* and *adeS* genes were detected, indicating that the two-component system was not self-regulated. Expression of several housekeeping genes, such as *cpn60* and *gltA* in BM4546 and *recA* in BM4547, was modified, suggesting collateral effects due to AdeRS mutations or AdeABC overexpression. However, no differences in growth levels were observed between the strains (data not shown).

Detection of *adeABC* and *adeIJK* overexpression. BM4665, a spontaneous resistant mutant of susceptible clinical isolate BM4587 obtained on gentamicin (Table 1), was found to be less susceptible to aminoglycosides (12-fold increase in MICs), fluoroquinolones, chloramphenicol, and tetracycline-tigecycline (Table 4). No changes in β-lactam susceptibility were observed, except for cefepime. Strain BM4665 overexpressed the *adeABC* operon (1.8- to 3-fold increase) (Table 3) but no other efflux genes, and the MDR phenotype of BM4665 was in agreement with overexpression of the pump (24). A 10- to 30-fold increase in expression of *adeABC* was found by qRT-PCR. Overexpression of the AdeABC system can be due either to mutations in the *adeRS* two-component system (25) or to the insertion of IS*Aba1* upstream from the *adeABC* operon (35). In order to elucidate the overexpression of *adeABC* in BM4665, the sequence of *adeRS* was determined, revealing a Gly-to-Asp substitution at position 30 of AdeS. This residue is located in the periplasmic input domain of the sensor. Mutations in this domain of several histidine protein kinases, such as NarX (7), BvgS (26), and VanS$_D$ (11), have been described and lead to constitutive expression of the regulated genes. Thus, the G30D substitution is most likely responsible for overexpression of AdeABC in BM4665.

Spontaneous MDR mutants BM4666 and BM4668 obtained from BM4587 and BM4667 on cefotaxime and tetracycline, respectively (Table 1), had similar resistance profiles characterized by decreased susceptibility to tetracycline-tigecycline, fluoroquinolones, chloramphenicol, co-trimoxazole, rifampin, and to a lesser extent, β-lactams, whereas aminoglycosides were not affected (Table 4). This phenotype was distinct from that associated with overexpression of AdeABC, suggesting another mechanism. Comparative microarray experiments re-

TABLE 4. Antibiotic susceptibility of *A. baumannii*

Strain	MIC (μg/ml)[a]																		
	TIC	TIM	CAZ	CTX	FEP	ATM	IPM	CHL	TET	MIN	TIG	RIF	NOR	CIP	SXT	GEN	TOB	AMK	NET
BM4454	8	12	6	4	3	16	0.25	>256	32	0.75	3	2	>256	>32	0.064	6	4	8	16
BM4587	6	8	4	3	0.75	12	0.19	96	1.5	0.064	0.094	1.5	1.5	0.094	0.094	1	0.75	4	1.5
BM4665	2	4	3	4	8	6	0.5	>256	3	0.125	1.5	1.5	12	0.5	0.125	12	16	64	64
BM4666	16	12	12	6	2	32	0.19	>256	8	0.19	0.38	3	4	0.38	0.25	1.5	1	6	1.5
BM4667	12	12	8	4	1.5	32	0.25	96	3	0.064	0.125	2	1.5	0.125	0.25	1.5	1.5	4	2
BM4668	24	16	16	8	3	48	0.25	>256	8	0.125	0.5	3	4	0.38	0.5	1.5	2	8	2
CIP 70-10	24	16	6	12	1.5	48	0.25	48	1	0.047	0.125	4	2	0.125	0.094	1.5	2	6	2
AYE	>256	>256	>256	>256	>256	>256	1	>256	64	0.5	1.5	16	>256	>32	>32	>256	64	96	48
BM4675	>256	>256	>256	>256	8	32	2	>256	32	8	1.5	>32	>256	>32	>32	24	12	96	32
BM4676	>256	>256	>256	32	32	32	>32	128	12	1	1.5	2	192	24	0.125	>256	96	>256	12

[a] AMK, amikacin; ATM, aztreonam; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; CLI, clindamycin; CTX, cefotaxime; ERY, erythromycin; FEP, cefepime; GEN, gentamicin; IPM, imipenem; MIN, minocycline; NET, netilmicin; NOR, norfloxacin; RIF, rifampin; SXT, co-trimoxazole; TET, tetracycline; TIC, ticarcillin; TIG, tigecycline; TIM, ticarcillin-clavulanic acid; TOB, tobramycin.

vealed overexpression of *adeI* and *adeJ* (1.5- and 2.4-fold increases, respectively) in the MDR mutants (Table 3), which was confirmed by qRT-PCR, indicating a 2.5- to 5-fold increase in the expression of the *adeIJK* gene set in the two mutants. Determination of the sequence of the *adeIJK* operon and of the upstream flanking region containing its putative promoter did not reveal any mutation, and regulation of expression of *adeIJK* remains unknown. The level of overexpression of *adeIJK* was systematically lower than that observed for *adeABC* in microarray as well as in qRT-PCR experiments. *trans*-complementation of BM4579 (BM4454 Δ*adeIJK*) with plasmid-borne *adeIJK* indicated that overexpression of AdeIJK was toxic for the host (9). Taken together, these observations suggest that AdeIJK is tightly regulated and can be overexpressed only within narrow limits. The fact that the BM4666 and BM4668 spontaneous mutants overexpress *adeIJK* at a low level suggests that these types of mutations can occur in clinical strains. The role of this efflux system in antibiotic resistance of clinical isolates remains to be determined.

Another mutant from BM4652 (BM4454 Δ*adeABC* Δ*adeIJK*) obtained on chloramphenicol exhibited an MDR phenotype. Comparison of the transcriptome of the mutant with that of the parental strain using the microarray revealed overexpression (2.9- to 3.8-fold) of a new RND efflux system, AdeFGH, that could be responsible for this phenotype and is currently under study.

Detection of antibiotic resistance genes in clinical isolates. Comparison by the microarray of AYE with susceptible reference strain CIP 70-10 indicated that genes *adc* for the chromosomal cephalosporinase and *adeABC* for the efflux system were overexpressed and that, among the 70 genes of the AbaR1 resistance island, 19 out of the 33 printed on the microarray were expressed, e.g., *bla*$_{VEB}$, *ant(2'')-Ia*, *cat*, *arr-2*, *aac(3)-Ia*, *floR*, *merA*, IS*1999*, *intI1*, and *sul1* (Table 5). Sequencing of the PCR product obtained with the IS*Aba1*b and amp Rev primers (Table 2) confirmed the presence of a copy of IS*Aba1* upstream from *adc* that likely provides a strong promoter (20) for enhanced production of cephalosporinase in AYE. Expression of the chromosomally located *bla*$_{OXA-51-type}$ was not detected, and analysis of the AYE genome sequence did not show the presence of IS*Aba1* upstream from the structural gene for the β-lactamase that is required for overexpression leading to carbapenem resistance (44). Fourteen genes of the resistance island were not detected by the microarray prob-

ably due to lack of, or weak, expression. All the resistance genes present in AYE except *tet*(A) and *bla*$_{OXA-10}$ were detected; *tet*(A) transcription is repressed by TetR in the absence of tetracycline (6), while *bla*$_{OXA-10}$, part of a class 1 integron that also contains *bla*$_{VEB}$, *ant(2'')-Ia*, *arr-2*, *cmlA5*, and *ant(3'')Ia*, was weakly expressed, although it possesses its own promoter (12). Within an integron, the expression of promoterless, cassette-associated resistance genes is influenced markedly by their position in the cassette array (12). A constant decrease in the expression of genes *bla*$_{VEB}$, *ant(2'')-Ia*, and *arr-2*, depending on the distance from the promoter borne by IS*1999*, was found by the microarray (Fig. 1). The microarray detected the expression of other genes in AbaR1, such as the mercury resistance operon and *intI1* and *qacEΔ1-sul1*, that comprise the 5′ and 3′ conserved sequences of the class 1 integron, respectively (Table 5). Combination of (i) overexpression of the cephalosporinase and AdeABC efflux system and (ii) the presence of acquired resistance genes, most of them carried by the 86-kb island, resulted in multiple antibiotic resistance of the AYE host.

BM4675 and BM4676, two other unrelated MDR clinical isolates, were analyzed. BM4675 was highly resistant to fluoroquinolones, tetracyclines, rifampin, chloramphenicol, co-trimoxazole, and β-lactams and moderately resistant to aminoglycosides and exhibited decreased susceptibility to tigecycline (Table 4). The only antimicrobial agent tested that conserved in vitro activity was imipenem (Table 4). Comparison of the transcriptome of BM4675 with that of reference strain CIP 70-10 indicated that genes *adc* for the chromosomal cephalosporinase and *adeABC* for the efflux system were overexpressed (Table 5). As in AYE, a copy of IS*Aba1* was found upstream from *adc*, which probably provides the promoter for its expression. The experiment enabled detection of the β-lactam *bla*$_{TEM-like}$ and tetracycline *tet*(B) resistance genes. As with *tet*(A), regulation by the *tetR* repressor does not allow expression of *tet*(B) in the absence of tetracycline. Thus, constitutive expression of *tet*(B) in BM4675 could result from a lack of or a nonfunctional *tetR*. Overexpression of the ADC cephalosporinase and of AdeABC, combined with production of TEM and Tet(B), accounts for part of the resistance phenotype of BM4675 (Table 4). Other alterations, such as mutations in type II topoisomerases, diminished permeability, or acquired genes not spotted on the microarray, probably combined to produce the multidrug resistance phenotype.

TABLE 5. Differential quantification of gene expression in clinical isolates compared to CIP 70-10

Function or gene	Description	Change (fold)[a]		
		AYE	BM4675	BM4676
Efflux				
adeA	MFP	2.84	5.42	3.94
adeB	RND	2.80	3.99	3.58
adeC	OMF	2.28	2.28	2.72
adeI	MFP	NS	NS	1.73
adeJ	RND	NS	NS	1.83
adeK	OMF	NS	NS	NS
APC 41_138	D-Serine/D-alanine/glycine transport protein	2.08	NS	NS
MFS 12_508	MFS	NS	NS	1.53
RND 29_167	RND	2.10[b]	NS	NS
Outer membrane protein				
omp25	Putative outer membrane protein	1.72	NS	NS
omp33–36	33- to 36-kDa outer membrane protein	−1.62	NS	1.84
omp34	34-kDa outer membrane protein	2.22	NS	14.36
ompA	Outer membrane protein A	−3.01	−2.32	NS
Housekeeping and metabolism				
bfmS	Sensor kinase component of a two-component regulatory system (biofilm)	NS	NS	1.59
cpn60	60-kDa chaperonin	−2.00	NS	NS
csuA/B	Putative pilus subunit, biofilm operon	NS	−1.72	1.84
fusA	Translation elongation factor EF-G	NS	NS	1.68
gltA	Citrate synthase	NS	NS	1.54
ppa	Inorganic pyrophosphatase	−2.73	NS	NS
rpoC	DNA-directed RNA polymerase beta subunit	−1.97	NS	NS
Antibiotic resistance				
adc	Chromosomal cephalosporinase	22.99	5.73	5.72
bla_{OXA-23}	Class D carbapenemase	NS	NS	29.61
bla_{TEM-1}	β-Lactamase	NS	1.61	NS
bla_{VEB-1}	Extended-spectrum β-lactamase	3.51	NS	NS
aac(3)-Ia	Aminoglycoside adenylyltransferase	2.62	NS	NS
ant(2'')-Ia	Aminoglycoside nucleotidyltransferase	2.51	NA	29.39
aph(3')-VIa	Aminoglycoside phosphotransferase	NA	NS	4.04
arr-2	Rifampin ADP-ribosylating transferase	1.76	NS	NS
catI	Chloramphenicol acetyltransferase	3.79	NS	NS
cmlA	Chloramphenicol efflux pump	1.73	NS	NS
floR	Chloramphenicol efflux pump	1.66	NS	NS
sul1	Sulfonamide resistance	4.10	NS	NS
tet(B)	Tetracycline efflux pump	NS	1.87	NS
Heavy metal resistance				
HMD	Heavy metal detoxification	2.50	NS	NS
merA	Mercuric ion reductase	1.79	NS	NS
merR	Mercury resistance operon regulatory protein	1.96	NS	NS
Gene mobility and island				
groEL/int1	Fusion GroEL/integrase	2.71	NS	NS
intI1	Integrase	2.32	NS	NS
IS1999	Insertion sequence	5.26	NS	NS
IS6100	Insertion sequence	1.87	NS	NS
ISAba1	Insertion sequence	NS	1.83	NS
lspA	Lipoprotein signal peptidase	1.81	NS	1.56
qacEdelta1	Quaternary ammonium compound resistance protein	6.19	NS	NS
resolvase 1_318	Resolvase	2.56	NS	NS
tniA	Transposase	NS	2.99	1.59
tnpM	Transposase	1.74	2.38	NS
uspA	Universal stress protein	NS	NS	1.50

[a] Positive and negative values correspond to genes with increased and decreased expression, respectively, in the clinical isolate in comparison with CIP 70-10. NS, not statistically significant. NA, not applicable.

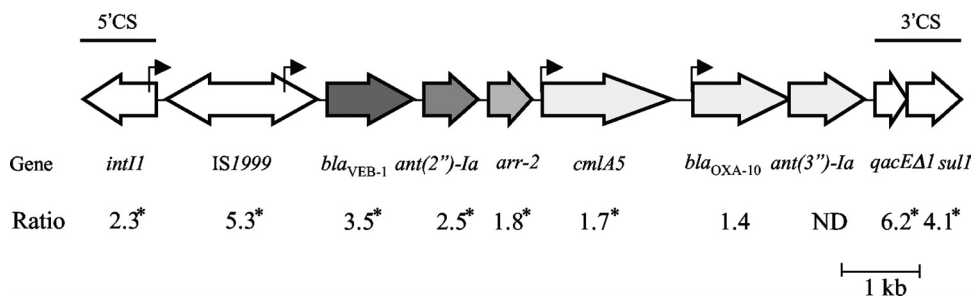[b] Statistically significant but not confirmed by qRT-PCR.

FIG. 1. Differential gene expression in the class 1 integron of the AbaR1 island from strain AYE. The ratio was determined by comparing the expression levels of every gene between strains AYE and CIP 70-10. 5'CS and 3'CS, 5' and 3' conserved sequences of class 1 integron; *, statistically significant; ND, not determined. Arrows indicate coding sequences and sense of transcription. Bent arrows represent the promoters for the gene cassettes, with IS*1999* bringing a strong promoter. Decreased intensity in gray indicates decreased overexpression from bla_{VEB-1} to bla_{OXA-10}.

BM4676 had high-level resistance to imipenem that was not restored by EDTA, suggesting the presence of a class D carbapenemase. The strain was also resistant to β-lactams, chloramphenicol, fluoroquinolones, and tetracycline and had decreased susceptibility to minocycline and tigecycline. Moderate-level resistance was observed for netilmicin, whereas the strain was highly resistant to the other aminoglycosides tested (Table 4). Comparative transcriptome analysis with reference strain CIP 70-10 indicated overexpression of chromosomal genes *adc* for the cephalosporinase and *adeABC* for the efflux system (Table 5). Weak overexpression of *adeI* and *adeJ*, confirmed by qRT-PCR, was observed (Table 5). As in BM4675, a copy of IS*Aba1* was found upstream from *adc*. The microarray also enabled detection of production of OXA-23 carbapenemase and of ANT(2'')-Ia and APH(3')-VIa aminoglycoside nucleotidyl- and phosphotransferase (Table 5). The presence of a copy of IS*Aba4* upstream from bla_{OXA-23}, located by PCR using IS*Aba4*b and oxa23- primers (Table 2), was presumably responsible for enhanced expression of this gene, as previously demonstrated (8). As observed for BM4675, the overexpressed and acquired determinants detected by the microarray could account for nearly the entire MDR phenotype of BM4676 (Table 4).

The microarray also detected the expressed transposase *tnpM* and *tniA* genes in BM4675 and the *tniA*, *lspA*, and *uspA* genes, coding for a transposase, a lipoprotein signal peptide, and a universal stress protein in BM4676, respectively (Table 5). These genes are part of the AbaR1 resistance island of AYE (16). PCR experiments using the ATPase3' and tniA Rev primers (Table 2) indicated that, in both strains, *tniA* was inserted at the 3' end of an ATPase gene which has been proposed to be a potential "hot spot" for integration of genomic islands in *A. baumannii* (37). No amplification products were obtained when long-range PCR was performed between *tniA* and the 5' end of the ATPase gene using the tniA For and ATPase5' primers (Table 2), possibly because either the 5' end of the ATPase gene had been deleted or the primers were too distant. None of the antibiotic resistance genes described for the original AbaR1 island were detected with BM4675 and BM4676.

Differential expression of several genes coding for outer membrane proteins was observed with the three clinical iso-

lates relative to CIP 70-10 (Table 5). These changes could impact membrane permeability and account for susceptibility decrease, in particular with imipenem, either alone or in combination with efflux systems and β-lactamase production.

Other genes, in particular housekeeping genes *cpn60*, *ppa*, and *rpoC* in AYE and *fusA* and *gltA* in BM4676, were found to be differentially expressed (Table 5). Modification of expression of these genes could reflect the cost of antimicrobial resistance for host fitness, as already proposed (14).

Microarray technology is a powerful tool that can be designed for comparative genomics (presence or absence of a gene) or for transcriptomics (level of gene expression). The microarray described in this study is a combination of both approaches since it enabled us to quantify expression of chromosomal genes and to detect acquired determinants that are expressed. It represents a useful tool to screen for resistance, in particular by overexpression of efflux systems, which is difficult to detect phenotypically and genotypically. In addition, the microarray allowed detection of a new pump, AdeFGH, involved in the antibiotic resistance of *A. baumannii*. The large panel of antibiotic resistance genes spotted on the microarray, including some that have not yet been detected in *A. baumannii*, enabled detection of most of the mechanisms that are responsible for the multidrug resistance phenotype of numerous clinical isolates of this species.

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