

Expression of the RND-Type Efflux Pump AdeABC in *Acinetobacter baumannii* Is Regulated by the AdeRS Two-Component System

Isabelle Marchand,¹ Laurence Damier-Piolle,¹ Patrice Courvalin,^{1*}
and Thierry Lambert^{1,2}

Unité des Agents Antibactériens, Institut Pasteur, Paris,¹ and Centre d'Etudes Pharmaceutiques,
Châtenay-Malabry,² France

Received 21 October 2003/Returned for modification 2 January 2004/Accepted 7 May 2004

The AdeABC pump of *Acinetobacter baumannii* BM4454, which confers resistance to various antibiotic classes including aminoglycosides, is composed of the AdeA, AdeB, and AdeC proteins; AdeB is a member of the RND superfamily. The *adeA*, *adeB*, and *adeC* genes are contiguous and adjacent to *adeS* and *adeR*, which are transcribed in the opposite direction and which specify proteins homologous to sensors and regulators of two-component systems, respectively (S. Magnet, P. Courvalin, and T. Lambert, *Antimicrob. Agents Chemother.* 45:3375–3380, 2001). Analysis by Northern hybridization indicated that the three genes were cotranscribed, although mRNAs corresponding to *adeAB* and *adeC* were also present. Cotranscription of the two regulatory genes was demonstrated by reverse transcription-PCR. Inactivation of *adeS* led to aminoglycoside susceptibility. Transcripts corresponding to *adeAB* were not detected in susceptible *A. baumannii* CIP 70-10 but were present in spontaneous gentamicin-resistant mutants obtained in vitro. Analysis of these mutants revealed the substitutions Thr153→Met in AdeS downstream from the putative His-149 site of autophosphorylation, which is presumably responsible for the loss of phosphorylase activity by the sensor, and Pro116→Leu in AdeR at the first residue of the α_5 helix of the receiver domain, which is involved in interactions that control the output domain of response regulators. These mutations led to constitutive expression of the pump and, thus, to antibiotic resistance. These data indicate that the AdeABC pump is cryptic in wild *A. baumannii* due to stringent control by the AdeRS two-component system.

Acinetobacter baumannii is a ubiquitous nonfermentative gram-negative bacterial species able to colonize patients in intensive care units. During the last 20 years this microorganism has become an important opportunistic nosocomial pathogen responsible for pneumonia, urinary tract infections, septicemia, and meningitis (9). Epidemic strains of *A. baumannii* are often multidrug resistant due to their capacity to acquire and accumulate resistance determinants. However, we recently reported that resistance to aminoglycosides, β -lactams, chloramphenicol, erythromycin, tetracyclines, and the dye ethidium bromide in clinical isolate BM4454 was due to overexpression of the AdeABC pump (22). The chromosomally encoded pump is a tripartite efflux machinery that belongs to the RND-type family (28). The AdeB protein contains 12 transmembrane segments and exhibits a high degree of identity (approximately 50%) with several RND proteins (27, 28). AdeA is homologous to membrane fusion proteins, whereas AdeC is most similar to the outer membrane protein OprM from *Pseudomonas aeruginosa*. The structural genes *adeA*, *adeB*, and *adeC* are contiguous and directly oriented, suggesting that they constitute an operon. They are preceded by two adjacent open reading frames, AdeR and AdeS, that are transcribed in the opposite direction and whose deduced products are closely related to proteins of two-component regulatory systems. Two-

component systems are signal transduction pathways in bacteria that respond to environmental conditions (16). They consist of a sensor kinase and its cognate response regulator. Signal transduction by the histidine protein kinase domain of the sensor and the response regulator domain of the transcriptional activator involves the reversible phosphorylation of each domain and the transfer of phosphoryl groups between these domains. The sensor monitors certain environmental conditions and, accordingly, modulates the active state of the response regulator, which controls gene expression. Two-component systems mediate adaptive responses to a broad range of environmental stimuli (16). However, they are an uncommon mode of regulation of drug efflux transporters, although these systems have recently been associated with RND-type multidrug exporters, such as MdtABC and YhiUV of *Escherichia coli* (7, 25, 26), RagCD of *Bradyrhizobium japonicum* (18), and SmeABC of *Stenotrophomonas maltophilia* (20). The aim of this work was to study the role of AdeRS in the regulation of expression of the AdeABC efflux pump of *A. baumannii* BM4454.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The bacterial strains and plasmids 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, Mich.). 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).

DNA manipulations. Plasmid DNA was prepared by the alkaline lysis method (29) or with a Wizard miniprep DNA kit (Promega, Madison, Wis.). *A. bau-*

* Corresponding author. Mailing address: Unité des Agents Antibactériens, Institut Pasteur, 25, rue du Docteur Roux, 75724 Paris Cedex 15, France. Phone: (33) 1 45 68 83 20. Fax: (33) 1 45 68 83 19. E-mail: pcourval@pasteur.fr.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Reference or source
<i>A. baumannii</i>		
BM4454	Wild strain, MDR	22
BM4542	BM4454 <i>adeB</i> ::pAT794	22
BM4543	BM4454 <i>adeC</i> ::pAT798, MDR	This work
BM4544	BM4454 <i>adeR</i> ::pAT799	This work
BM4545	BM4454 <i>adeS</i> ::pAT800, MDR	This work
CIP 70-10	Reference strain	10
BM4546	CIP 70-10 AdeS _{T153M} spontaneous mutant, MDR	This work
BM4547	CIP 70-10 AdeR _{P116L} spontaneous mutant, MDR	This work
BM4548	Wild strain	This work
Plasmid		
pUC18	Tra ⁻ Mob ⁻ Ap, <i>lacZ</i> α	36
pAT794	pUC18Ω979-bp fragment of <i>adeB</i> from BM4454	22
pAT798	pUC18Ω630-bp fragment of <i>adeC</i> from BM4454	This work
pAT799	pUC18Ω375-bp fragment of <i>adeR</i> from BM4454	This work
pAT800	pUC18Ω621-bp fragment of <i>adeS</i> from BM4454	This work
pKK232-8	Tra ⁻ Mob ⁻ Ap, <i>cat</i> promoterless	11
pAT804	pKK232-8Ω809-bp fragment upstream from <i>adeC</i>	This work
pAT805	pKK232-8Ω228-bp fragment upstream from <i>adeC</i>	This work

^a Tra⁻, non-self-transferable; Mob⁻, nonmobilizable; Ap, ampicillin resistance, *cat*, chloramphenicol resistance gene; MDR, multidrug resistance due to overexpression of the AdeABC pump.

mannii genomic DNA was extracted as described previously (5). Digestion of DNA by restriction endonucleases, ligation, transformation, and agarose gel electrophoresis were performed as described previously (29). DNA fragments were extracted from agarose gels with a QIAquick gel extraction kit (Qiagen, Inc., Chatsworth, Calif.). PCR was performed in a GeneAmp PCR system 2400 (Perkin-Elmer Cetus, Norwalk, Conn.) with *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.), according to the recommendations of the manufacturers. PCR products were purified with a QIAquick PCR purification kit (Qiagen). Nucleotide sequencing was carried out with a CEQ 2000 DNA analysis system automatic sequencer (Beckman Instruments, Inc., Palo Alto, Calif.), according to the recommendations of the manufacturer.

Search for a promoter for *adeC* gene. The 228- and 809-bp fragments located upstream from the initiation codon of *adeC* were amplified with primer pairs AdeCpless1-AdeCpless3r and AdeCpless2-AdeCpless3r, respectively (Table 2).

The PCR products were cloned at the BamHI site of pKK232-8 (Amersham Pharmacia Biotech, Uppsala, Sweden), leading to plasmids pAT804 and pAT805 (Table 1), respectively. The orientations of the inserts were determined by sequencing. *Escherichia coli* Top10 harboring plasmid pKK232-8, pAT804, or pAT805 was grown in BHI broth containing ampicillin (100 µg/ml) to an optical density at 600 nm of 0.7. The cells were washed, treated with lysozyme, and sonicated. After centrifugation at 100,000 × *g* for 45 min, the supernatant was used to determine chloramphenicol acetyltransferase activity. Formation of 5-thio-2-nitrobenzoate was measured at 37°C in the presence and absence of chloramphenicol, as described previously (4).

Insertion-inactivation of *ade* genes. Insertion-inactivation of the *adeC*, *adeR*, and *adeS* genes was performed as described previously (22). Briefly, a fragment internal to the *adeC*, *adeR*, or *adeS* gene was amplified with specific primer pairs C-am and C-av, R-am and R-av, and S-am and S-av, respectively (Table 2). The PCR products were cloned into SmaI-linearized pUC18 DNA and transformed into *E. coli*. The cloned fragments were sequenced, and the recombinant plasmids were introduced into strain BM4454 by electrotransformation. Since pUC18 is a suicide vector in *A. baumannii*, the transformants stably resistant to tetracycline should be the result of a homologous recombination event. Total DNA from these clones was screened for insertion by PCR with the M13 reverse and M13 (-20) forward primers and two primers complementary to the regions flanking the inserts in the BM4454 chromosome. The resulting derivatives with an inactivated *adeB*, *adeC*, *adeR*, or *adeS* gene were designated BM4542 (22), BM4543, BM4544, and BM4545, respectively (Table 1).

Computer analysis of sequence data. Nucleotide sequence data were analyzed with the Clustal W program (31). Amino acid sequences were analyzed at the websites of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST) and the European Molecular Biology Laboratory (www.smart.embl-heidelberg.de/). The GenBank and protein databases were screened for sequence similarity.

RNA isolation and Northern analysis. The *A. baumannii* strains were grown to an optical density at 600 nm of 0.6, and total RNA was extracted as described previously (37). The mRNA was depleted of rRNA by use of a MICROBExpress kit (Ambion, Austin, Tex.); and equal amounts (10 µg) were electrophoresed on a 1.2% agarose-formaldehyde gel, transferred onto a nylon membrane, and hybridized as described previously (37). To generate the probes, PCR products corresponding to the genes of interest (Fig. 1) were treated with the Megaprime DNA labeling system (Amersham Biosciences, Orsay, France).

RT-PCR. Reverse transcription (RT)-PCR was performed as described previously (29). Briefly, RT was carried out for 45 min at 50°C with 0.5 µg of *A. baumannii* RNA as the template and 20 pmol of primer RS-av in a 20-µl reaction mixture. An aliquot of the cDNA (5 µl of the RT reaction mixture) was amplified by PCR with 20 pmol each of primers RS-av and RS-am. The PCR products (10 µl) were separated on an agarose gel and transferred onto a Hybond N⁺ membrane (Amersham Biosciences), followed by hybridization with probes specific for the *adeR* and *adeS* genes (Fig. 1).

Primer extension. Primer extension was carried out as described previously (12). Briefly, the EA-adeA oligonucleotide was end labeled with [³²P]ATP and polynucleotide kinase, purified, and annealed to 25 µg of total RNA extracted from strain BM4454 for the extension reaction. The sample, together with the corresponding sequencing reaction, was run on a 6% polyacrylamide-urea sequencing gel.

TABLE 2. Oligonucleotides used in this study

Primer	Sequence	Position ^a
C-am	5'-CCC AAC CAT TGG TGT AAC G	8110-8128
C-av	5'-GAA CAT CCG TGC TTT AGC	8722-8739
R-am	5'-GGC ATG AGT GTT ATT CGG	3169-3186
R-av	5'-CTC AGA GTG TAT ATA AAC GC	2812-2831
S-am	5'-TTC AAC AAG AAG ATT GGA CC	2355-2374
S-av	5'-CTT GCT CAA TAC GAC GG	1754-1770
RS-av	5'-TCT AGC AGA GAG GTC GC	2192-2209
RS-am	5'-ATA CCG ACA CTC ATA CCG	2839-2846
EA-adeA	5'-CTT TAG CCG GTG GTG GCT CAG C	3491-3534
AdeCpless1	5'-TGC <i>GGA TCC</i> TAT CAG AAG TTG TAA CG ^b	7001-7017
AdeCpless2	5'-CGC <i>GGA TCC</i> AGT TCA GAA ACT CAA CA ^b	7582-7598
AdeCpless3r	5'-CGC <i>GGA TCC</i> GTA TTC TCC AAA TAA AGT ^b	7792-7809

^a The positions refer to the 10,627-bp sequence of BM4454 (GenBank accession no. AF370885).

^b The BamHI site is italicized; the oligonucleotide anneals downstream from the restriction site.

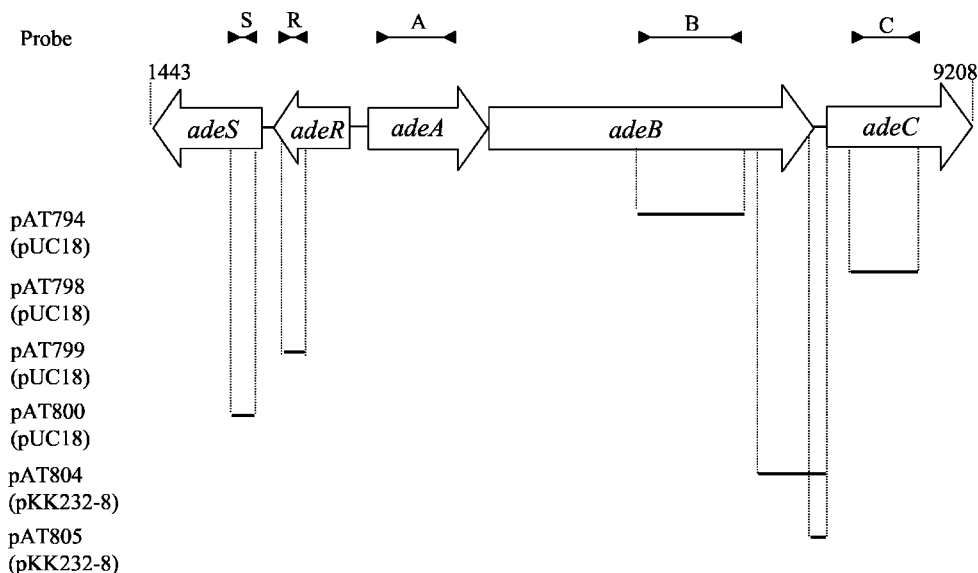


FIG. 1. Schematic representation of the *ade* gene cluster of BM4454 and recombinant plasmids. Open arrows represent coding sequences and indicate the direction of transcription. Closed arrowheads indicate the positions and orientations of the primers used to generate PCR fragments (represented by thin lines), which were used as probes: probe A (positions 3477 to 4369), probe B (positions 6197 to 7175), probe C (positions 8110 to 8737), probe R (positions 2548 to 2846), and probe S (positions 2193 to 2512) (all positions are according to accession no. AF370885 in GenBank). The inserts in recombinant plasmids are represented by horizontal thick lines, and the vectors are indicated in parentheses under the name of the plasmid.

Nucleotide sequence accession number. The 2,824-bp sequence of *A. baumannii* CIP 70-10 has been deposited in the GenBank data library under accession number no. AY426969.

RESULTS AND DISCUSSION

Transcriptional analysis of *adeABC* and *adeRS* genes. To analyze the transcription of the *adeABC* gene cluster, total RNA extracted from BM4454 cells was analyzed by Northern hybridization with probes specific for regions internal to every gene of the cluster (Fig. 1). A transcript of approximately 6 kb, which cohybridized with probes *adeA*, *adeB*, and *adeC*, was detected, indicating that the three genes are cotranscribed (Fig. 2). However, a stronger signal of approximately 4.5 kb, detected with the *adeA*- and *adeB*-specific probes, showed that the main transcript corresponded to the *adeAB* genes (Fig. 2). A transcript of approximately 1.4 kb, which corresponds to the size of *adeC*, was also detected (Fig. 2). These data suggest the independent transcription of *adeC*, consistent with the presence of a hairpin structure ($\Delta G = -12.9$ kJ) in the *adeB-adeC* intergenic region. Similarly, it has been established that the *smeABC* multidrug efflux operon of *S. maltophilia* generates a transcript that corresponds to the SmeC outer membrane protein (20). To test this hypothesis, a promoter for the *adeC* gene was searched for by cloning the 228- and 809-bp fragments upstream from the initiation codon of the *adeC* gene upstream from the promoterless *cat* reporter gene of plasmid pKK232-8 (11), generating pAT804 and pAT805, respectively (Table 1). Expression of the *cat* gene was tested for indirectly by determining the chloramphenicol MICs and was tested for directly by assaying enzymatic activity in *E. coli* Top10 harboring pKK232-8, pAT804, or pAT805. Chloramphenicol acetyltransferase activity could not be detected by either technique (data not shown). The presence of the *adeC* transcript could thus be

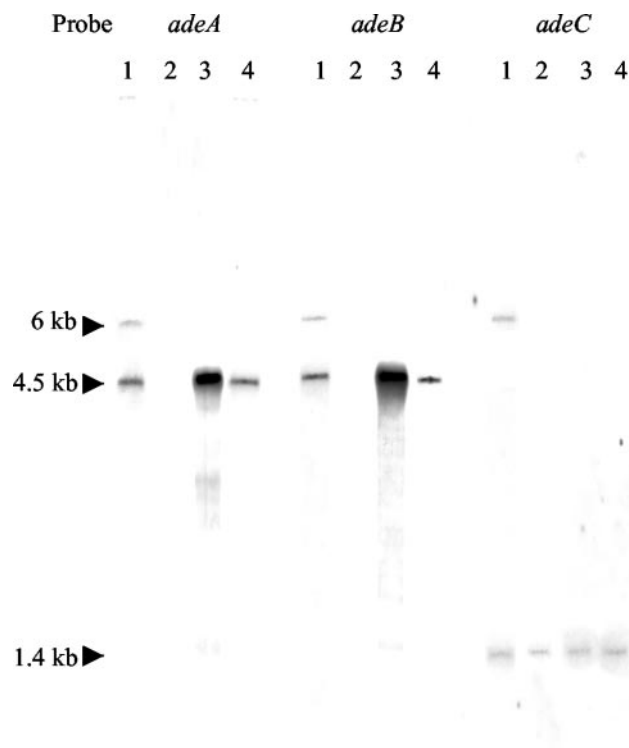


FIG. 2. Transcription analysis of the *adeA*, *adeB*, and *adeC* genes by Northern hybridization. Total RNA depleted of 16S and 23S RNA from BM4454 (lane 1), CIP 70-10 (lane 2), BM4546 (CIP 70-10 AdeS_{T153M}) (lane 3), and BM4547 (CIP 70-10 AdeR_{P116L}) (lane 4) was hybridized with the probes indicated at the top. The sizes of the transcripts relative to RNA molecular size marker I (Boehringer) are indicated.

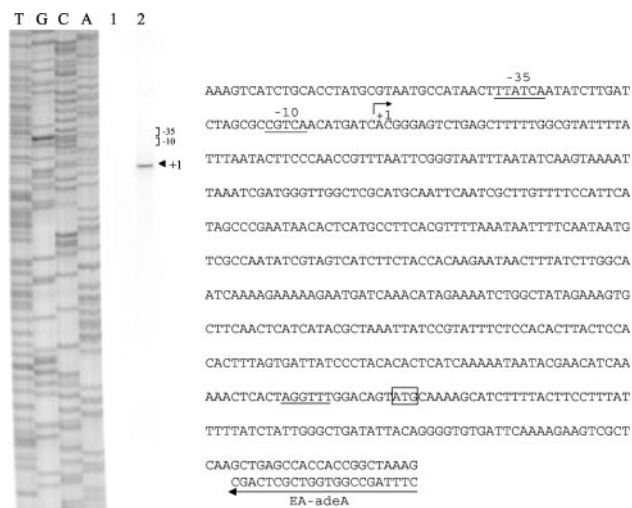


FIG. 3. Identification of the transcriptional start site for the *adeABC* operon in BM4454 by primer extension analysis. (Left panel) Lanes T, G, C, and A, results of sequencing reactions performed with primer EA-adeA; lane 1, control without RNA; lane 2, primer elongation product obtained with oligonucleotide EA-adeA and 25 μ g of total RNA from BM4454 (arrowhead); (right panel) sequence from nucleotide positions 2951 to 3539 according to numbering of the sequence with GenBank accession no. AF370885. The +1 transcriptional start site for *adeABC* and the potential -35 and -10 promoter sequences are indicated and underlined. The ATG start codon of the *adeA* gene is boxed, and the ribosome-binding site is underlined.

accounted for by cleavage of the *adeABC* mRNA into two mRNAs, *adeAB* and *adeC* mRNAs, which displayed differences in their stabilities.

On the basis of these results, primer extension was performed to determine the transcriptional start site for *adeABC* by using primer EA-adeA, whose sequence is complementary to the 5' end of *adeA* (Table 2). The DNA fragment generated allowed the positioning of the transcriptional start site (Fig. 3) and suggested the presence of -35 TTATCA (positions 2999 to 3004; GenBank accession no. AF370885) and -10 CGTCA motifs, which were separated by 17 bp, as the promoter. The -10 sequence did not display homology with those recognized by the main σ factors but contained the CGwC consensus sequence recognized by *Bacillus subtilis* σ^x (15). This factor belongs to the family of extracytoplasmic function (ECF) σ factors that are cotranscribed with anti- σ factors located in the cytoplasmic membrane and are released upon interaction with an extracytoplasmic signal (15). Several ECF factors have been described in *B. subtilis* and *P. aeruginosa* (15), but they have not yet been described in *A. baumannii*. Moreover, promoters recognized by certain transcriptional activators, such as some belonging to the two-component system family, lack a clear -35 sequence.

Since Northern hybridization performed with the *adeS*- and *adeR*-specific probes was not sensitive enough to reveal an mRNA (data not shown), transcription of the *adeR* and *adeS* genes was analyzed by RT. Internal *adeS*-specific primer RS-av was used to produce a cDNA which was amplified with the same oligonucleotide and RS-am, whose sequence is specific for a region located in *adeR* (Table 2). A PCR product of the expected size of 650 bp was obtained, and the product cohy-

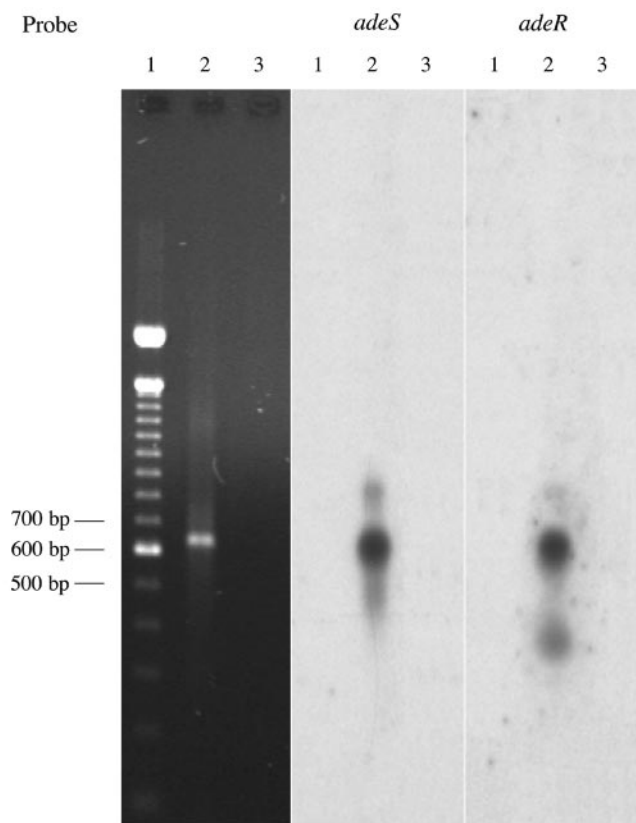


FIG. 4. Transcription analysis of *adeR* and *adeS* genes. (Left) Agarose gel electrophoresis of the product obtained by RT-PCR with primers RS-av and RS-am and corresponding Southern hybridizations with probes specific for *adeS* (center) and *adeR* (right). Lane 1, 100-bp DNA ladder (Gibco BRL). Incubations were carried out in the presence (lane 2) or in the absence (lane 3) of reverse transcriptase.

bridized with the *adeS*- and *adeR*-specific probes (Fig. 4). Altogether, these results demonstrate that the structural genes for the AdeABC efflux pump and the two genes for the regulatory system, *adeR* and *adeS*, are located in two divergently transcribed operons.

Inactivation of *adeA*, *adeB*, and *adeC* genes in BM4454. Disruption of the *adeB* gene in BM4454 is responsible for the loss of multiple-antibiotic resistance by the host (22). However, the insertion could have had a polar effect on the transcription of the downstream *adeC* gene. To study the contribution of this gene to resistance, we constructed strain BM4543 (BM4454 *adeC*::pAT798), in which *adeC* was inactivated by insertion. This derivative displayed resistance to the various substrates of the AdeABC pump similar to that of parental strain BM4454, indicating that *adeC* is not essential for resistance. Some efflux gene clusters, such as *mexXY* from *P. aeruginosa* and *acrAB* from *E. coli*, do not encode an outer membrane protein (2, 21, 24). To form a functional tripartite complex, MexXY recruits OprM, whereas AcrA associates with TolC (14), a multifunctional outer membrane channel (17, 30, 38). That AdeC is not required for resistance suggests that AdeAB can utilize another outer membrane constituent. The AdeK outer membrane protein associated with the AdeIJK RND efflux pump

TABLE 3. MICs of gentamicin and kanamycin for strains expressing or not expressing the AdeABC pump

<i>A. baumannii</i> strain	MIC ($\mu\text{g/ml}$)	
	Gentamicin	Kanamycin
BM4454	12	4
BM4542 (BM4454 <i>adeB</i> ::pAT794)	0.25	0.5
BM4543 (BM4454 <i>adeC</i> ::pAT798)	12	4
BM4544 (BM4454 <i>adeR</i> ::pAT799)	0.25	0.5
BM4545 (BM4454 <i>adeS</i> ::pAT800)	0.5	1
CIP 70-10	1	2
BM4546 (CIP 70-10 AdeS _{T153M})	12	4
BM4547 (CIP 70-10 AdeR _{P116L})	12	4

recently identified in BM4454 also (L. Damier-Piolle et al., unpublished data) could be a candidate. In contrast, the OprM outer membrane protein of *P. aeruginosa* plays a crucial role in the intrinsic multidrug resistance conferred by MexAB (19). The SmeC outer membrane protein is also necessary for the resistance conferred by the *smeABC* multidrug efflux operon in *S. maltophilia* (20).

Involvement of *adeRS* in expression of *adeABC* genes. The predicted products of the *adeR* and *adeS* genes showed substantial similarity with transcriptional activators and sensor kinases, respectively, that work together to regulate target gene expression in response to stimuli. To assess the role of *adeRS* in the regulation of *adeABC* expression, the *adeR* and *adeS* genes of *A. baumannii* BM4454 were disrupted by insertion of a suicide plasmid following homologous recombination, and the resulting mutants were tested for their antimicrobial susceptibilities. In order for the insertion not to exert a polar effect on the *adeABC* operon, recombination was performed upstream from the transcriptional start site for *adeABC*. As expected, inactivation of *adeR* in BM4544 (BM4454 *adeR*::pAT799) and of the *adeS* gene in BM4545 (BM4454 *adeS*::pAT800) led to susceptibility to aminoglycosides (Table 3) and to other substrates for the pump, a result which confirmed the role of *adeRS* in the control of expression of the efflux genes.

Thus, the *adeS* gene appears to be essential for expression of the *adeABC* operon in that strain. These experiments did not allow the determination of the role of AdeR alone, since inactivation of *adeR* in BM4544 could have a polar effect on *adeS*. In two-component regulatory systems it has been established that the sensor kinase autophosphorylates at an internal histidine (the H box) in response to a stimulus and that the phosphate group is then transferred to an aspartate residue of the response regulator. The phosphorylated regulator may also be dephosphorylated by the phosphatase activity of the sensor (8, 34). The histidine kinases are bifunctional, in that they phosphorylate and dephosphorylate their cognate response regulator (34), which leads to a switch between these two activities and directs the state of the regulators, thus governing expression of the genes on which they act. The observation that BM4545 (BM4454 *adeS*::pAT800) was susceptible to aminoglycoside could have resulted from the loss of AdeS kinase activity.

Analysis of *adeABC* gene expression in CIP 70-10 resistant mutants. Since it is likely that expression of AdeABC in clinical isolate BM4454 is secondary to an alteration in AdeRS, we

analyzed this regulatory system in *A. baumannii* CIP 70-10. This reference strain is susceptible to antibiotics and harbors the *adeABC* and *adeRS* genes, as evidenced by PCR (data not shown) and sequencing (GenBank accession no. AY426969). The AdeR regulator from CIP 70-10 shared 98% amino acid identity with that from BM4454, and the AdeS sensor shared 96.7% amino acid identity with that from BM4454, although the latter protein was shorter by 4 amino acids. The proteins contained the conserved motifs previously reported for histidine kinases and response regulators (32), and inside these motifs, no differences were detected between the sequences from the two strains. Spontaneous one-step mutants of CIP 70-10 exhibiting a multidrug resistance phenotype indistinguishable from that of BM4454 were selected on BHI agar containing 4 μg of gentamicin per ml. Nine mutants were obtained, and sequence analysis of their *adeRS* operons showed two mutations. The first one, Thr153 \rightarrow Met, which was detected in six mutants, was located in the kinase; and derivative BM4546 (CIP 70-10 AdeS_{T153M}) was selected for further studies. The second mutation, Pro116 \rightarrow Leu, which was detected in three strains, was located in the response regulator; and BM4547 (CIP 70-10 AdeR_{P116L}) was studied further. Total RNA from CIP 70-10, BM4546 (CIP 70-10 AdeS_{T153M}), and BM4547 (CIP 70-10 AdeR_{P116L}) cells was analyzed by Northern hybridization with probes specific for every gene of the *adeABC* operon (Fig. 1). The *adeAB* transcript was detected only in the two mutants (Fig. 2), whereas the *adeC* transcript was present in CIP 70-10 and its two derivatives (Fig. 2). These data indicate (i) that multidrug resistance in the mutants involves the AdeABC efflux system and (ii) that resistance is due to point mutations in the two-component system. As opposed to BM4454, the *adeABC* transcript was not detected in CIP 70-10 or its derivatives (Fig. 2). Although the aminoglycoside resistance levels of BM4546 (CIP 70-10 AdeS_{T153M}) and BM4547 (CIP 70-10 AdeR_{P116L}) were similar (Table 3), the amount of *adeAB* mRNA was higher in BM4546 (Fig. 2). There was thus no obvious relationship between the level of resistance and the quantities of the *adeA* and *adeB* transcripts. In BM4546 (CIP 70-10 AdeS_{T153M}), the mutation was located in the H box of the sensor, the motif which contains the conserved histidine residue (His-149), which is the site of autophosphorylation. The Thr153 \rightarrow Met substitution, located 4 amino acids downstream, was associated with a constitutive resistance. Substitutions at the corresponding positions of EnvZ (T247 \rightarrow R), PhoR (T220 \rightarrow N), and VanS_B (T237 \rightarrow K) result in a defect in phosphatase activity but not a defect in kinase activity (1, 6, 35). The constitutive phenotype associated with the AdeS Thr153 \rightarrow Met mutation may therefore also be due to a similar defect in AdeR dephosphorylation. In BM4547 (CIP 70-10 AdeR_{P116L}), the mutation took place at the 3' end of the CheY-homologous receiver region of the response regulator. Response regulators usually contain two domains separated by a flexible linker, and the N-terminal receiver domain modulates the activity of the C-terminal effector domain (3, 32, 33). It has been established in this family of proteins that phosphorylation of the aspartic acid involved in phosphate transfer of the N terminus induces rearrangements within the active site, leading to a larger-scale conformational change of the protein which most often enhances the affinity of its C terminus for specific DNA regions. The

Pro116→Leu mutation in AdeR of BM4547 (CIP 70-10 AdeR_{P116L}) corresponds to position 113 in the conventional numbering based on the CheY structure (32). This mutation occurs in the first amino acid of the α_5 helix just downstream from the VIB turn involving the VKPF conserved residues at the end of β_5 (32). In this motif, K109 (K112 in AdeR) is absolutely conserved in response regulators homologous to CheY. Moreover, it has been established for PhoB that the β_5 - α_5 loop and the α_5 helix from the receiver domain are required to propagate the phosphorylation-triggered signal from the receiver domain to the output domain (3, 13). The C-terminal domain of PhoB belongs to the winged-helix-turn-helix family of transcription factors (23). Phosphorylation of PhoB relieves the inhibition of DNA binding of the C terminus, which is otherwise constitutively active for transcription. In the absence of phosphorylation, the α_5 helix is responsible for inhibition of the PhoB effector. The Pro116→Leu substitution in the α_5 helix of AdeR from BM4547 (CIP 70-10 AdeR_{P116L}) led to constitutive transcription of *adeABC* by possibly affecting the ability of the α_5 helix to silence the activity of the DNA-binding domain.

The nature of the signal and the mechanism of AdeRS activation in BM4454 remain unknown. Sequence alignment of AdeR and AdeS from clinical isolate BM4454 and drug-susceptible strains *A. baumannii* BM4548 and CIP 70-10 suggests that the G136V substitution in the output domain of the regulator could enhance the affinity of the effector for its specific DNA target. Unfortunately, the very low transformation efficiency of CIP 70-10 precludes the critical testing of this hypothesis by site-specific mutagenesis. As already mentioned, two-component systems have so far only rarely been shown to be involved in the modulation of expression of genes mediating resistance by efflux. It is therefore all the more interesting that multidrug-resistant derivatives could be one-step regulatory mutants.

ACKNOWLEDGMENTS

We thank T. Msadek for helpful discussions.

This work was supported by an unrestricted grant from Pfizer Inc. to P.C.

REFERENCES

- Aiba, H., F. Nakasai, S. Mizushima, and T. Mizuno. 1989. Evidence for the physiological importance of the phosphotransfer between the two regulatory components, EnvZ and OmpR, in osmoregulation in *Escherichia coli*. *J. Biol. Chem.* **264**:14090–14094.
- Aires, J. R., T. Köhler, H. Nikaido, and P. Plésiat. 1999. Involvement of an active efflux system in the natural resistance of *Pseudomonas aeruginosa* to aminoglycosides. *Antimicrob. Agents Chemother.* **43**:2624–2628.
- Allen, M. P., K. B. Zumbrennen, and W. R. McCleary. 2001. Genetic evidence that the α_5 helix of the receiver domain of PhoB is involved in interdomain interactions. *J. Bacteriol.* **183**:2204–2211.
- Arthur, M., C. Molinas, and P. Courvalin. 1992. The VanS-VanR two-component regulatory system controls synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. *J. Bacteriol.* **174**:2582–2591.
- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y.
- Baptista, M., F. Depardieu, P. Reynolds, P. Courvalin, and M. Arthur. 1997. Mutations leading to increased levels of resistance to glycopeptide antibiotics in VanB-type enterococci. *Mol. Microbiol.* **25**:93–105.
- Baranova, N., and H. Nikaido. 2002. The BacSR two-component regulatory system activates transcription of the *yegMNOB* (*mdtABCD*) transporter gene cluster in *Escherichia coli* and increases its resistance to novobiocin and deoxycholate. *J. Bacteriol.* **184**:4168–4176.
- Batchelor, E., and M. Goulian. 2003. Robustness and the cycle of phosphorylation and dephosphorylation in a two-component regulatory system. *Proc. Natl. Acad. Sci. USA* **100**:691–696.
- Bergogne-Bérézin, E., and K. J. Towner. 1996. *Acinetobacter* spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. *Clin. Microbiol. Rev.* **9**:148–165.
- Bouvet, P. J. M., and P. A. D. Grimont. 1986. Taxonomy of the genus *Acinetobacter* with the recognition of *Acinetobacter baumannii* sp. nov., *Acinetobacter haemolyticus* sp. nov., *Acinetobacter johnsonii* sp. nov., and *Acinetobacter junii* sp. nov. and emended descriptions of *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii*. *Int. J. Syst. Bacteriol.* **36**:228–240.
- Brosius, J. 1984. Plasmid vectors for the selection of promoters. *Gene* **27**:151–160.
- Casadewall, B., P. E. Reynolds, and P. Courvalin. 2001. Regulation of expression of the *vanD* glycopeptide resistance gene cluster from *Enterococcus faecium* BM4339. *J. Bacteriol.* **183**:3436–3446.
- Ellison, D. W., and W. R. McCleary. 2000. The unphosphorylated receiver domain of PhoB silences the activity of its output domain. *J. Bacteriol.* **182**:6592–6597.
- Fralick, J. A. 1996. Evidence that TolC is required for functioning of the Mar/AcrAB efflux pump of *Escherichia coli*. *J. Bacteriol.* **178**:5803–5805.
- Helmann, J. D. 2002. The extracytoplasmic function (ECF) sigma factors. *Adv. Microb. Physiol.* **46**:47–110.
- Koretke, K. K., A. N. Lupas, P. V. Warren, M. Rosenberg, and J. R. Brown. 2000. Evolution of two-component signal transduction. *Mol. Biol. Evol.* **17**:1956–1970.
- Koronakis, V., A. Sharff, E. Koronakis, B. Luisi, and C. Hughes. 2000. Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. *Nature* **405**:914–919.
- Krummenacher, P., and F. Narberhaus. 2000. Two genes encoding a putative multidrug efflux pump of the RND/MFP family are cotranscribed with an *rpoH* gene in *Bradyrhizobium japonicum*. *Gene* **241**:247–254.
- Li, X.-Z., H. Nikaido, and K. Poole. 1995. Role of *mexA-mexB-oprM* in antibiotic efflux in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **39**:1948–1953.
- Li, X.-Z., L. Zhang, and K. Poole. 2002. SmeC, an outer membrane multidrug efflux protein of *Stenotrophomonas maltophilia*. *Antimicrob. Agents Chemother.* **46**:333–343.
- Ma, D., D. N. Cook, M. Albert, N. G. Pon, H. Nikaido, and J. E. Hearst. 1993. Molecular cloning and characterization of *acrA* and *acrE* genes of *Escherichia coli*. *J. Bacteriol.* **175**:6299–6313.
- Magnet, S., P. Courvalin, and T. Lambert. 2001. Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. *Antimicrob. Agents Chemother.* **45**:3375–3380.
- Martinez-Hackert, E., and A. M. Stock. 1997. Structural relationships in the OmpR family of winged-helix transcription factors. *J. Mol. Biol.* **269**:301–312.
- Mine, T., Y. Morita, A. Kataoka, T. Mizushima, and T. Tsuchiya. 1999. Expression in *Escherichia coli* of a new multidrug efflux pump MexXY from *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **43**:415–417.
- Nishino, K., and A. Yamaguchi. 2001. Overexpression of the response regulator *evgA* of the two-component signal transduction system modulates multidrug resistance conferred by multidrug resistance transporters. *J. Biol.* **183**:1455–2001.
- Nishino, K., and A. Yamaguchi. 2002. EvgA of the two-component signal transduction system modulates production of the YhiUV multidrug transporter in *Escherichia coli*. *J. Biol.* **184**:2319–2323.
- Paulsen, I. T., M. H. Brown, and R. A. Skurray. 1996. Proton-dependent multidrug efflux systems. *Microbiol. Rev.* **60**:575–608.
- Saier, M. H., Jr. 1994. Computer-aided analyses of transport protein sequences: gleaned evidence concerning function, structure, biogenesis, and evolution. *Microbiol. Rev.* **58**:71–93.
- Sambrook, J., and D. Russell. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Thanabalu, T., E. Koronakis, C. Hedges, and V. Koronakis. 1998. Substrate-induced assembly of a contiguous channel for protein export from *E. coli*: reversible bridging of an inner-membrane translocase to an outer membrane exit pore. *EMBO J.* **17**:6487–6496.
- Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**:4673–4680.
- Volz, K. 1993. Structural conservation in the CheY superfamily. *Biochemistry* **32**:11741–11753.
- Walthers, D., V. K. Tran, and L. Kenney. 2003. Interdomain linkers of homologous response regulators determine their mechanism of action. *J. Bacteriol.* **185**:317–324.

34. West, A. H., and A. M. Stock. 2001. Histidine kinases and response regulator proteins in two-component signaling systems. *Trends Biochem. Sci.* **26**:369–376.
35. Yamada, M., K. Makino, M. Amemura, H. Shinagawa, and A. Nakata. 1989. Regulation of the phosphate regulon of *Escherichia coli*: analysis of mutant *phoB* and *phoR* genes causing different phenotypes. *J. Bacteriol.* **171**:5601–5606.
36. Yanisch-Perron, C., J. Viera, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103–119.
37. Yarchuck, O., N. Jacques, J. Guillerez, and M. Dreyfus. 1992. Interdependence of translation, transcription and mRNA degradation in the *lacZ* gene. *J. Mol. Biol.* **226**:581–596.
38. Zgurskaya, H. I., and H. Nikaido. 2000. Multidrug resistance mechanisms: drug efflux across two membranes. *Mol. Microbiol.* **37**:219–225.