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Distribution and molecular profiling of class 1 integrons in MDR *Acinetobacter baumannii* isolates and whole genome-based analysis of antibiotic resistance mechanisms in a representative strain

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ABSTRACT

The class 1 integron is an important driver of the nosocomial dissemination of multidrug-resistant (MDR) bacteria, such as *Acinetobacters*. In this study, we characterized the gene cassette arrays of class 1 integrons in *Acinetobacter baumannii*, where the detailed structure of these integrons for 38 clinical strains was analyzed. The results showed that there are three types of gene cassette arrays that are carried by different class 1 integrons, among them the *aac(6′)-IId-catB8-aadA1* array was the most prevalent. For detailed analysis of the integron structure, whole genome sequencing was carried out on strain AB16, and it was found that a single integron on its chromosome has a partial Tn*21* transposon in its 5′ flanking region and two complete copies of the insertion element IS*26* in both the 5′ and 3′ flanking regions, indicating that the integron could be acquired by horizontal gene transfer. Furthermore, there is one resistance island AbaR22, one *bla* gene containing a transposon, four intrinsic resistant genes and one efflux pump that together confer six types of antibiotic resistance.

1. Introduction

An integron is a site-specific recombination system capable of integrating into the bacterial genome. It carries many modular structures called gene cassettes that confer drug resistance (Fluit and Schmitz, 2004). The integron can be divided into three parts according to the sequence conservation: the 5′-conserved region, the variable region and the 3′-conserved region. The 5′-conserved region contains a promoter, P_C, and an *intI* gene encoding integrase; the variable region always includes several gene cassettes; and the 3′-conserved region consists of sequences derived from transposons, such as those found in class 1 integrons: *qacEΔ*, *sul1* and *orf5* (Norrby, 2005). According to the sequence identity of the *int* gene, five different classes of integrons have been identified. Although class 1 integrons are the most widespread and clinically important class, all five classes are associated with antibiotic resistance. Class 1 integrons are found in Gram-negative bacteria and occasionally in Gram-positive bacteria (Cambray et al., 2010). They are associated with Tn*402*-like transposons and can be further embedded in larger transposons, such as Tn*21*. Therefore, class 1 integrons are easily transferred among a diverse range of bacteria. Class 2 integrons are associated with Tn*7*, which can insert itself into conjugative plasmids with low frequency. However, a mutation in codon 179 frequently occurs in *intI2*, which produces a non-functional protein and subsequently results in the low diversity of class 2 integron gene cassettes. Class 3 integrons have a similar structure to class 1 integrons, and they are also Tn*402*-like elements. Nevertheless, the class 3 integrons have a different sequence of *attI* sites than class 1 integrons. This discrepancy results in the class 3 integrase recognizing fewer *attC* sites, and therefore, it is less prevalent in the clinical environment

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than class 1 integrons (Collis et al., 2002). The other two classes of integrons are not as frequent, and they are only found in *Vibrio* species: the SXT element integron, which exists in *Vibrio cholerae* (Hochhut et al., 2001) and is associated with VCR repeats (*V. cholera* repeats) (Hall, 2012), and the *dfrA1*-containing integron carried by the pRSV1 plasmid of *Aliivibrio salmonicida* (Cambray et al., 2010). Thus, the class 1 integrons are important vehicles for the horizontal transfer of antibiotic resistance genes. For example, in *Acinetobacter baumannii*, these integrons often contain efflux pump genes, beta-lactam resistance genes and aminoglycosides resistance genes (Gootz and Marra, 2008).

A. baumannii is an important opportunistic pathogen and can cause severe nosocomial infections, with high mortality rates due to its extensive antibiotic resistance (Perez et al., 2007). Most *A. baumannii* strains were susceptible to antibiotics before the 1970s (Gootz and Marra, 2008); however, with the increased use of antibiotics, the multidrug-resistant (MDR) strains rapidly emerged, and up to 57% of the strains have demonstrated MDR in a recent study (De Francesco et al., 2013). This rapid increase of MDR is not only due to the intrinsic resistant genes carried by these strains but also to their outstanding capacity to acquire resistant elements from other bacteria. Class 1 integrons are important in enabling the *A. baumannii* genome to capture and accumulate many antibiotic resistance genes.

In this study, we analyzed 39 *A. baumannii* clinical isolates. The antibiotic resistance phenotypes and population structure of these isolates were investigated, and the molecular characteristics of class 1 integrons in the MDR isolates were analyzed. To gain insight into the genetic environment of a prevalent class 1 integron, as well as other resistance gene content and context, the whole genome of a representative strain was sequenced and analyzed.

2. Materials and methods

2.1. Bacterial isolates and antimicrobial susceptibility testing

From September to November 2012, 39 *A. baumannii* strains were isolated from various wards at the 306th Hospital of People's Liberation Army in Beijing, China (Supp. Table 1). Identification of isolates and antimicrobial susceptibility testing were carried out using the automated microbiology system. The minimum inhibitory concentration (MIC) of 11 antimicrobial agents was determined according to the recommendations given by the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute, 2011). The reference strains *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality controls.

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2.2. PCR amplifications, restriction enzyme analysis and sequencing analysis

Two multiplex PCRs (mPCRs) were performed as described by Turton et al. (2007) to define the sequence groups. The PCRs were designed to selectively amplify alleles of the *ompA*, *csuE* and $bla_{\text{OXA-51-like}}$ genes, and sequence groups were determined according to the different combinations of amplicons obtained in the two mPCRs. The PCR conditions were 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 57 °C for 45 s and 72 °C for 1 min, with a final extension at 72 °C for 10 min.

The variable region of class 1 integron was amplified in a PCR reaction using the following primers: 5′-GGCATCCAAGCAGCAAG-3′ (Var1-F) and 5′-AAGCAGACTTGACCTGA-3′ (Var1-R) (Levesque et al., 1995). The PCR amplification conditions were as follows: 94 °C for 5 min and 30 cycles of 94 °C for 30 s (denaturation), 55 °C for 45 s (annealing) and 72 °C for 5 min (extension), with a final extension at 72 °C for 10 min. PCR amplicons were analyzed by electrophoresis in 1% agarose gel prepared in 1× TAE buffer and visualized using ultraviolet light following staining with ethidium bromide. PCR positive products were digested with the restriction enzyme *Sau* 3A I (Takara), and PCR products with different restriction enzyme digestion patterns were sequenced on both strands by a primer walking sequence strategy using an ABI 3730 automated DNA sequencer. Sequences were assembled using the SeqMan program in the DNAStar software package and aligned using BLAST (Basic Local Alignment Search Tool) in the NCBI (National Center for Biotechnology Information) database to determine the gene cassettes within the variable region of class 1 integrons.

2.3. DNA extraction and whole genome sequencing

AB16 genomic DNA was extracted using the TIANamp bacteria DNA kit (Tiangen Biotech (Beijing) Co., Ltd.) according to the manufacturer's instructions. The genomic DNA was fragmented by ultrasonication, and the DNA fragments 300–500 bp in length were subjected to the whole genome sequencing workflow of the Genome Analyzer IIx system (Illumina).

2.4. Genome sequence analysis

The raw sequencing reads were filtered using the DynamicTrim and LengthSort Perl scripts within SolexaQA and assembled using SOAPdenovo version 1.05, with a K-mer size of 51. Class 1 integron and its adjoining sequence were mapped using the AbGRI2-1 sequence (GeneBank accession number JX869489). The antibiotic resistance island (AbaR) inserted in the *comM* gene was mapped using the sequences of the *comM* gene and the AbaRs sequences identified in MDR-ZJ and MDR-TJ, two prevalent strains isolated in China (GeneBank accession numbers CP001937 and CP003500). The *bla*-containing transposon was mapped using Tn*2009*, which exists in MDR-ZJ. Other antibiotic resistance-associated genes in the AB16 genome were investigated using BLAST against the Antibiotic Resistance Genes Database (ARDB) (Liu and Pop, 2009). Primer pairs were designed to close the gaps within the integron, resistance island or transposon by sequencing PCR products using an ABI 3730 automated DNA sequencer. For large PCR amplicons, restriction enzyme digestion and a subcloning technique were used before ABI 3730 sequencing. Insertion sequence (IS) elements were identified using the ISfinder database (https://www-is.biotoul.fr//) (Siguier et al., 2006).

3. Results

3.1. Identification of sequence groups

Different sequence groups have been defined according to the fragments yielded by multiplex PCRs. Previous studies have described sequence groups 1–14 (Karah et al., 2012). In this study, the 39 strains are assigned to five different sequence groups. Thirty three strains belong to three known sequence groups: 17 strains to group 1, 13 strains to group 4 and 3 strains to group 5. The remaining 6 strains are not known to belong to any of the sequence groups and are assigned to two novel groups (group 15 and 16) (Table 1). Among the five sequence groups from this study, group 1 is the most prevalent in our clinical isolates (17/39), followed by group 4 (13/39). No strains were assigned to group 2, which corresponds to GC1, a globally prevalent strain (Hamidian and Hall, 2011).

Table 1
Combinations of amplicons obtained in the mPCRs used to define sequence groups.

Sequence groups[a]	Group 1 PCR			Group 2 PCR			Number of strains (n = 39)
	csuE (702 bp)	bla_{OXA-51} (559 bp)	*ompA* (355 bp)	*csuE* (580 bp)	*ompA* (343 bp)	bla_{OXA-51} (162 bp)	
1	+	+	+	–	–	–	17
4	–	+	+	–	–	–	13
5	–	–	+	–	–	–	3
15	+	–	–	–	–	–	1
16	–	–	–	–	–	–	5

[a] The sequence groups are PCR-based groups, which have been defined according to the combinations of the PCR amplicons.

3.2. Antibiotic susceptibilities

Antibiotic resistant susceptibility testing was carried out according to the international standard of the Clinical Laboratory Standards Institute (CLSI). Among the 39 strains, resistance to 11 antibiotic drugs, belonging to seven antibiotic classes, was detected, and the non-susceptible rates were 34/39 to gentamicin, 32/39 to amikacin, 37/39 to imipenem, 35/39 to ciprofloxacin, 33/39 to lavo-ofloxacin, 38/39 to piperacillin-tazobactam, 39/39 to ceftriaxone, 38/39 to ceftazidime, 38/39 to cefepime, 2/39 to polymyxin B and 35/39 to tetracycline (Table 2). According to the standardized definition of MDR strains (Magiorakos et al., 2012), strain AB21 is the only non-MDR strain, which is susceptible to all the tested drugs except piperacillin-tazobactam, ceftriaxone and ceftazidime. The remaining strains are MDR strains that show resistance to at least one antibiotic drug in at least three antibiotic classes.

3.3. Variable regions of class 1 integrons

The variable region of the class 1 integron is a region that can repeatedly insert gene cassettes at the *attI* site. Primers Var1-F and Var1-R were used to amplify and sequence the variable region of 38 strains, 23 (61%) of which carried the class 1 integron. The amplicons exhibited 3 different lengths: 2.3 kb in 16 strains, 2.5 kb in 6 strains and 3.0 kb in 1 strain (Table 3). Amplicons of the same length were identified by fingerprinting using *Sau*3A I. The 2.3 kb PCR amplicon is most commonly found in these strains (Table 3).

The 2.3 kb amplicon contains *aac(6′)-IId*, *catB8* and *aadA1*; the 2.5 kb amplicon harbors gene cassettes *aacC1*, *orfP*, *orfQ* and *aadA1*; and the 3.0 kb amplicon includes *aacC1*, *orfP*, *orfP*, *orfQ* and *aadA1*. Among these gene cassettes, *aacC1*, *aadA1* and *aac(6′)-IId* confer resistance to aminoglycosides; *catB8* contains the group B chloramphenicol acetyltransferase gene; and *orfP* and *orfQ* encode proteins of unknown function. The structures of the three types of gene cassette arrays are shown in Fig. 1.

3.4. The aac(6′)-IId-catB8-aadA1 integron is flanked by active IS26

The *aac(6′)-IId-catB8-aadA1* cassette was frequently found in the integrons characterized in this study; therefore, we selected strain AB16, containing this integron, for whole genome sequencing. The genome data are deposited in NCBI AVPO00000000 (Supp. Table 2).

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The genome sequence of AB16 revealed that the integron includes the outer ends of IRi and IRt, and there is an incomplete Tn*21* at the 5′ end and a partial copy of IS*6100* at the 3′ end (Fig. 2). This integron-containing segment is flanked by two inverted copies of IS*26*, which may mediate the transfer of the entire integron.

Further analysis of the *A. baumannii* genome revealed that this type of integron-containing structure is widespread in the global clone II strains, including MDR-ZJ, MDR-TJ, TCDC and WM99c. However, the integrons of the global clone II strains contain different gene cassette arrays. MDR-ZJ, similar to AB16, has a gene cassette array of *aac(6′)-IId-catB8-aadA1*. WM99c contains an *aacC1-orfP-orfP-orfQ-aadA1* array, which is also found in our clinical MDR *A. baumannii* strains (Table 3). MDR-TJ contains a similar array as in WM99c, but it includes three consecutive copies of *orfP*. *A. baumannii* strain TCDC contains two class 1 integrons with gene cassette arrays of *aacA4-catB8-aadA1* and *aacC1-orfP-orfP-orfQ-aadA1*. Additionally, AB16, MDR-ZJ, MDR-TJ and WM99c, share the same integron-containing structure insertion location, which suggests a possible novel insertion hotspot (Nigro et al., 2013).

3.5. AB16 has other elements that confer drug resistance

The AB16 strain has a genome island, AbaR, in the chromosomal *comM* gene (Fig. 3). This genome island shares 99% similarity with AbaR22 (Zhou et al., 2011). AbaR22 contains two copies of Tn*6022*, which make up the backbone of most AbaRs in GC2 strains, except for AbaR2 (Huang et al., 2012). The tetracycline efflux pump and

Table 2
Susceptibility of *A. baumannii* to antibiotics.

Antibiotic classes	Antibiotic drugs	Resistant	Intermediate	Susceptible
Aminoglycosides	Gentamicin (CN)	33	1	5
	Amikacin (AK)	32	0	7
Antipseudomonal carbapenems	Imipenem (IPM)	36	1	2
Antipseudomonal fluoroquinolones	Ciprofloxacin (CIP)	34	1	4
	Lavo-ofloxacin (LEV)	24	9	6
Antipseudomonal penicillins and β-lactamase inhibitors	Piperacillin-tazobactam (TZP)	35	3	1
Extended-spectrum cephalosporins	Ceftriaxone (CTR)	37	2	0
	Ceftazidime (CAZ)	35	3	1
	Cefepime (FEP)	36	2	1
Polymyxins	Polymyxin B (PB)	2	0	37
Tetracyclines	Tetracycline (TE)	31	4	4

Table 3
Distribution of class 1 integrons in multidrug-resistant *A. baumannii* strains.

Length of variable region/kb	Gene cassette arrays	Number of strains	Percentage in MDR strains %
2.3	*aac(6′)-IId-catB8-aadA1*	16	42
2.5	*aacC1-orfP-orfQ-aadA1*	6	16
3.0	*aacC1-orfP-orfP-orfQ-aadA1*	1	3

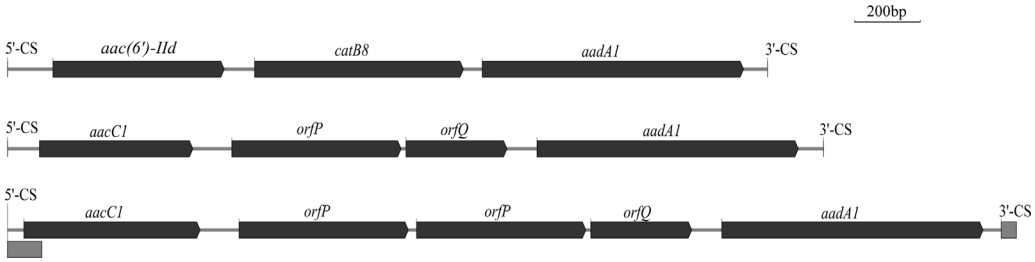

Fig. 1. Gene cassette arrays of class 1 integrons in MDR *A. baumannii* strains.

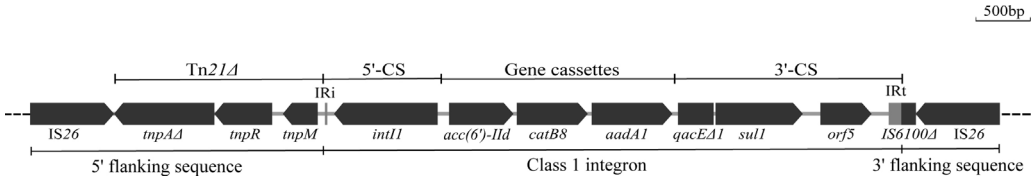

Fig. 2. Structures of the class 1 integron and its flanking regions in AB16.

its regulator gene, *tetA* and *tetR*, and the streptomycin resistance genes, *strA* and *strB*, are found in AbaR22 (Zhou et al., 2011; Huang et al., 2012). A small mobile element, ISCR, is also found in AbaR22. ISCR is a member of the IS*91* family, and it can use rolling-circle transposition for genetic rearrangement (Toleman et al., 2006), a function that is important for the transfer of antibiotic resistance. AB16 also contains a transposon, Tn*2009*, which harbors a bla_{OXA-23} gene. This transposon has been previously described in MDR-ZJ (Zhou et al., 2011).

Further genome analysis of AB16 has identified additional antibiotic resistance genes (Table 4). The class A β-lactamase gene *tem-1* confers resistance to cephalosporin and penicillin. The *sul2* gene encodes sulfonamide-resistant dihydropteroate synthase. Furthermore, mutations of Ser83Leu in *gyrA* and Ser80Ile in *parC* are responsible for quinolone resistance.

Efflux-pump-mediated resistance to antimicrobials is generally associated with the major facilitator superfamily (MFS) and the resistance-nodulation-division (RND) family in *A. baumannii* (Vila et al., 2007). Five efflux-pump-related genes were found in AB16 (Table 4). The *tetA* and *tetR* genes, which belong to the MFS family, are responsible for tetracycline resistance. The *adeA*, *adeB* and *adeC* genes, which belong to the RND family, are three components of an efflux pump, and its overexpression can confer resistance to aminoglycosides, β-lactams, chloramphenicol, erythromycin, tetracycline and ethidium bromide (Vila et al., 2007).

Porins also play a role in *A. baumannii* antibiotic resistance by decreasing outer membrane permeability (Vila et al., 2007). However, *carO* and *oprD* are identical to genes found in MDR-TJ, and therefore, they are complete and not disrupted. Thus, porin changes appear to make little contribution to drug resistance in AB16 (Huang et al., 2012).

4. Discussion

A. baumannii multidrug resistance contributes to its persistence in the hospital setting (Lockhart et al., 2007). Recent data from China showed that 61.3% of patients carried MDR *Acinetobacter* (Zheng et al., 2013). In this study, 38 MDR strains were identified among 39 *A. baumannii* clinical isolates (97%), which demonstrates a more serious situation of the multidrug resistance. The majority of the strains were sensitive to polymyxin B, regarded as a last resort against MDR *A. baumannii*, most likely because polymyxin B has not been widely used in China; thus, the corresponding selection pressure has not been established on this bacteria species.

Globally, sequence group 1 is the most widespread strain (Higgins et al., 2010), and the majority of our clinical strains (44%)

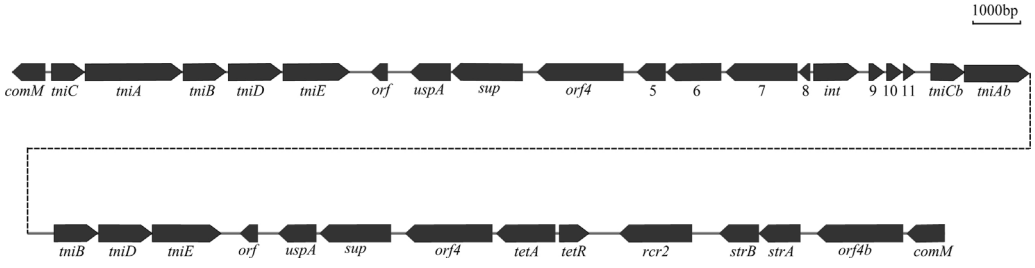

Fig. 3. Structure of genome island in AB16.

Table 4
Antimicrobial resistance-associated genes in the AB16 genome.

Drug class	Enzyme class, description	Coding gene	Locus tag
β-Lactamases	Class D β-lactamase	*bla*$_{OXA-23}$(Tn*2009*)	C1451_6081_5260
	Class A β-lactamase	*tem-1*	C1375_927_67
Aminoglycosides	Aminoglycoside N-acetyltransferase	*aac(6′)-IId* (*in*tegron)	C1361_1318_821
	Aminoglycoside	*strA*(AbaR island)	scaffold32_59208_60053
	O-phosphotransferase	*strB*(AbaR island)	scaffold32_60059_60889
	Aminoglycoside O-adenyltransferase	*aadA1*(integron)	C1391_26_805
Chloramphenicol	Group B chloramphenicol acetyltransferase	*catB8*(integron)	C1361_728_96
Sulfonamides	Sulfonamide-resistant dihydropteroate synthase	*sul1* (*in*tegron)	C1391_1310_2149
		sul2	C1351_19_885
Fluoroquinolones	gyrA mutation (Ser-83→Leu)	*gyrA*	scaffold11_93765_96479
	parC mutation(Ser-80→Ile)	*parC*	scaffold15_18902_21121
Efflux pumps	MFS family	*tetA*(AbaR island)	scaffold32_64262_65479
		tetR(AbaR island)	scaffold32_64180_63557
	RND family	*adeA*	scaffold44_45275_46465
		adeB	scaffold44_46465_49572
		adeC	scaffold44_49649_51046

belong to this group. In addition to the extensive global dissemination of groups 1 and 2 in more than 30 countries (Karah et al., 2012), CC92, a representative of sequence group 1, has become the major clone spread throughout China (Li et al., 2013; Ruan et al., 2013). This national spread of CC92 is possibly because sequence group 1 has advantages in acquiring resistance determinants and surviving in the nosocomial environment in China. Furthermore, we found an increase in our MDR population in sequence group 4: 33% versus 10% in a previous study (Towner et al., 2008), showing a tendency of increasing sequence group 4 MDR strains in Beijing.

The high prevalence of class 1 integrons among multidrug-resistant *A. baumannii* clinical strains has been confirmed globally (Turton et al., 2005; Asadollahi et al., 2011; Poonsuk et al., 2012). In China, class 1 integrons are also detected in >50% of antibiotic-resistant *A. baumannii* strains (Zhang et al., 2010; Zhong et al., 2012). Furthermore, a high proportion of the class 1 integron gene cassette array *aac(6′)-IId-catB8-aadA1* is observed (Zhong et al., 2012; Chen et al., 2013). In this study, approximately 61% of the 38 MDR strains contain class 1 integrons, most of which include the *aac(6′)-IId-catB8-aadA1* array. Further study of the gene cassette array transfer mechanism is required, with a focus on the prevalence of the *aac(6′)-IId-catB8-aadA1* cassette array in the clinical setting.

Class 1 integrons contain cassettes that usually do not include a promoter. Therefore, they are commonly transcribed from P_C, a promoter located upstream of the cassette arrays, which leads to considerably decreased expression levels of the downstream gene cassettes (Hall, 2012). Therefore, the class 1 integron typically contains no more than 6 gene cassettes (Gillings et al., 2008). Furthermore, the integrons found in this study contained 3–5 gene cassettes, which is characteristic of class 1 integrons. The most frequently reported resistant type is β-lactam (Norrby, 2005). Although gene cassettes in class 1 integrons encode various types of resistance, no gene cassette was found in this study that confers β-lactam resistance. Moreover, in the study presented here, gene cassettes encoding aminoglycoside-modifying enzymes were the most prevalent. This suggests a close relationship between aminoglycoside resistance and our MDR isolates.

The mobile element IS*6100Δ* exists in the 3′-CS of the class 1 integron in AB16, and the Tn*21Δ* found in its 5′ flanking sequence showed a subsequent association with Tn*21*. However, it is the flanking IS*26* elements that appear to be vital for transfer of the class 1 integron. A similar structure of the class 1 integron and its flanking sequence has been found in a previous study (Nigro et al., 2013). This supports our view that the structure of the class 1 integron in *A. baumannii* is becoming conserved, and its movement is mediated by the IS*26* insertion/deletion.

The genomic analysis of AB16 provides an overview of its various antibiotic resistance mechanisms. *A. baumannii* acquires multiple resistance mechanisms via integrons, transposons and plasmids (Camp and Tatum, 2010). A class 1 integron, transposon Tn2009 and a mosaic resistance island all contain antibiotic resistance genes. No plasmid has been found in this strain. Intrinsic resistance mechanisms generally fall into three categories: antimicrobial-modifying enzymes, impaired entry or active efflux through the bacterial outer membrane, and mutations that alter targets or cellular functions (Rice, 2006; Gootz and Marra, 2008; Maragakis and Perl, 2008). These three categories of resistance mechanisms are all found in AB16: the genes of *tem1* and *aac(6′)-IId* belong to the first category, the RND family efflux pump AdeABC reduces the access of antimicrobial agents to bacterial targets, and finally, mutations in *gyrA* and *parC* genes belong to the third category. All these classes of resistance mechanisms contribute to the emerging multi-drug resistance of *A. baumannii* strains.

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