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Evolution of Carbapenem-Resistant *Acinetobacter baumannii* Revealed through Whole-Genome Sequencing and Comparative Genomic Analysis

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***Acinetobacter baumannii* is a globally important nosocomial pathogen characterized by an evolving multidrug resistance. A total of 35 representative clinical *A. baumannii* strains isolated from 13 hospitals in nine cities in China from 1999 to 2011, including 32 carbapenem-resistant and 3 carbapenem-susceptible *A. baumannii* strains, were selected for whole-genome sequencing and comparative genomic analysis. Phylogenetic analysis revealed that the earliest strain, strain 1999BJAB11, and two strains isolated in Zhejiang Province in 2004 were the founder strains of carbapenem-resistant *A. baumannii*. Ten types of AbaR resistance islands were identified, and a previously unreported AbaR island, which comprised a two-component response regulator, resistance-related proteins, and RND efflux system proteins, was identified in two strains isolated in Zhejiang in 2004. Multiple transposons or insertion sequences (ISs) existed in each strain, and these gradually tended to diversify with evolution. Some of these IS elements or transposons were the first to be reported, and most of them were mainly found in strains from two provinces. Genome feature analysis illustrated diversified resistance genes, surface polysaccharides, and a restriction-modification system, even in strains that were phylogenetically and epidemiologically very closely related. IS-mediated deletions were identified in the type VI secretion system region, the *csuE* region, and core lipooligosaccharide (LOS) loci. Recombination occurred in the heme utilization region, and intrinsic resistance genes (bla_{ADC} and $bla_{OXA\text{-}51\text{-}like}$ variants) and three novel $bla_{OXA\text{-}51\text{-}like}$ variants ($bla_{OXA\text{-}424}$, $bla_{OXA\text{-}425}$, and $bla_{OXA\text{-}426}$) were identified. Our results could improve the understanding of the evolutionary processes that contribute to the emergence of carbapenem-resistant *A. baumannii* strains and help elucidate the molecular evolutionary mechanism in *A. baumannii*.**

Acinetobacter baumannii is an important opportunistic pathogen that has caused severe nosocomial infections worldwide (1). Multidrug-resistant *A. baumannii* strains resistant to carbapenems have been increasingly reported worldwide, which raises serious concerns about the limited antimicrobial treatment options available (2). Our previous study indicated that the percentage of imipenem- and meropenem-resistant *A. baumannii* strains in China increased from 4.5% in 2003 to 61.7% in 2010 and from 4.5% in 2003 to 62.8% in 2010, respectively. In 2012, according to the Chinese Meropenem Surveillance Study (CMSS), the rates of *A. baumannii* susceptibility to imipenem and meropenem were 37.8% and 36.0%, respectively (3).

A. baumannii rapidly develops multidrug resistance due to the presence of mobile genetic elements (MGEs), such as insertion sequences (ISs), transposons, and resistance islands (4). Many recent studies have highlighted the diversity in the genomic location, architecture, and content of resistance islands, demonstrating the dynamic nature of *A. baumannii* antibiotic resistance mechanisms and the adaptive significance of these elements (5–7). However, the evolution of antibiotic resistance over time in relation to changes in the major clones in China has not yet been investigated. Moreover, only a few studies have focused on the genetic background differences among *A. baumannii* isolates collected from different locations at different times.

Comparative genomics studies help in the evaluation of the resistance mechanisms, pathogenicity, and evolution of bacterial pathogens at the genome-wide level (8). Despite the increased amount of research on *A. baumannii* epidemiology and evolution that has been performed (9–12), large gaps remain in our understanding of the evolutionary processes that contribute to multidrug resistance and genomic diversification of *A. baumannii*, especially in China. In the present study, a total of 35 representative *A. baumannii* strains isolated from 13 hospitals in nine cities in China from 1999 to 2011 were selected for comparative whole-genome sequencing to examine strain-level genetic diversity and gene content variation. Through the analysis of these strains with different backgrounds, we aimed to gain a better understanding of the evolution of *A. baumannii* resistance in China.

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MATERIALS AND METHODS

Strain isolation and genotypic and phenotypic characterization. The *A. baumannii* strains were collected from 13 hospitals in nine cities in China from 1999 to 2011. The isolates from 1999 to 2005 were selected from the 221 carbapenem-resistant *A. baumannii* (CRAB) strains evaluated in our previous study (13), and the isolates from 2011 were selected from 283 *A. baumannii* strains in the Chinese Antimicrobial Resistance Surveillance of Nosocomial Infections (CARES) collection (14). The isolates were subjected to additional analysis to characterize the antibiotic resistance phenotypes (see Table S1 in the supplemental material) and genotypes via multilocus sequence typing (MLST) (http://pubmlst.org/abaumannii/) (15). From this collection of isolates, 35 representative isolates consisting of the predominant clone in different locations and from different dates and sources were selected. To put the sequenced strains in a phylogenetic context and assess the shared and clade-specific gene contents, the genomes were compared with those of 17 reference *Acinetobacter* genomes available in NCBI as of January 2014.

DNA preparation, library construction, sequencing, and assembly. DNA was isolated with a DNA purification kit (Qiagen). Illumina sequencing libraries were prepared by using Nextera kits with indexed-encoded adapters from Illumina, according to the manufacturer's instructions. The libraries were pooled for sequencing on a MiSeq sequencer, and paired-end sequence reads representing 50- to 200-fold genome coverage were obtained. The Illumina sequence data were assembled using the SOAPdenovo (version 2.04) package. PCR amplification and Sanger sequencing were used to solve the ambiguity of the order and orientation of scaffolds.

Genome annotation. The assembled genome sequence was annotated by the NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAAP), using the programs Glimmer (version 3.0) for the identification of protein-coding genes (16), tRNAscan-SE for the identification of tRNA genes (17), and RNAmmer for the identification of rRNA genes (18). The ISs were identified using the IS Finder database (www-is-.biotoul.fr) (19). The origin of replication (*oriC*) and putative DnaA boxes were identified using the Ori-Finder system (20).

Whole-genome phylogeny construction. Multiple-sequence alignments of 52 genomes were performed using the Mugsy program (21). The tree was constructed on the basis of single nucleotide polymorphisms (SNPs) from the whole-genome alignment. This alignment included SNPs from 17 reference genomes and 35 genomes sequenced in this study.

eBURST analysis. eBURST analysis was employed to investigate the evolutionary relationships and clonal complexes (CCs) within the isolates, using the software on the eBURST website (http://eburst.mlst.net/v3/enter_data/single/), with statistical support for the complexes being assessed via the bootstrap resampling method with 1,000 resamplings (22). The analysis was performed using both stringent (a minimum of six shared alleles) and relaxed (a minimum of five shared alleles) grouping parameters.

Estimation of core genome and pan-genome size. A BLASTP search between every pair of protein sequences from each strain was performed. The PanOCT program was used to identify the orthologs with the BLASTP output (23).

Nucleotide sequence accession numbers. The data from this whole-genome shotgun project have been deposited at DDBJ/EMBL/GenBank under accession number PRJNA261018. The three novel bla_{OXA} types were designated $bla_{OXA-424}$ (GenBank accession number KM588352), $bla_{OXA-425}$ (GenBank accession number KM588353), and $bla_{OXA-426}$ (GenBank accession number KM588354) (http://www.lahey.org/Studies/).

RESULTS

A total of 35 representative *A. baumannii* strains, including 32 carbapenem-resistant *A. baumannii* (CRAB) strains and 3 carbapenem-susceptible *A. baumannii* strains, isolated from patients with hospital-acquired infections in 13 hospitals in nine cities in China from 1999 to 2011 were selected for whole-genome sequencing. The 32 CRAB isolates consisted of 1 isolate from 1999, 8 isolates from 2003 to 2005, and 23 isolates from 2011. Most of the isolates were collected in Beijing, China (14/35, 40%). The sources of these 35 isolates were sputum (20/35, 57.1%), blood (10/35, 28.6%), and abdominal fluid (5/35, 14.3%). The general characteristics of the 35 *A. baumannii* genomes are listed in Table S2 in the supplemental material.

Whole-genome phylogeny of the genus *Acinetobacter*. To facilitate detailed analysis of strain relationships, we developed a phylogeny based on single nucleotide polymorphisms (SNPs) from the whole-genome alignment to represent the ancestral relationships among 35 strains and 16 other *A. baumannii* strains (Fig. 1A). These included 12 multidrug-resistant *A. baumannii* strains (AYE, AB0057, ACICU, 1656-2, TYTH-1, TCDC-AB0715, BJAB07104, BJAB0715, BJAB0868, ZW85-1, MDR-TJ, and MDR-ZJ06), 2 susceptible strains (ATCC 17978 and AB307-0294), 1 community-acquired strain (D1279779), and 1 nonclinical strain (SDF) isolated from a human body louse. ADP1, a soil-living *Acinetobacter baylyi* strain, was used as the outgroup for comparison.

On the basis of the phylogenetic data, all the strains, along with nine previously reported Asian strains (including MDR-ZJ06, MDR-TJ, ZW85-1, BJAB07104, BJAB0715, and BJAB0868 from mainland China; TCDC-AB0715 and TYTH-1 from Taiwan, China; and 1656-2 from South Korea) were grouped together with ACICU, a strain of the global clone II (GC II) group. The susceptible strains were clustered on the edge of the evolutionary tree. Two groups of founder strains were identified among the CRAB strains on the basis of the phylogenetic tree. These included the earliest isolated strain (strain 1999BJAB11) and two strains from Zhejiang Province, China, isolated in 2004. Interestingly, the earliest isolated strain, 1999BJAB11, was separated from all of the other sequence type 92 (ST92) strains, suggesting that it may have a genome content different from that of the other ST92 strains.

The eBURST algorithm revealed one clonal complex (CC), CC1, and six singletons (Fig. 1B). CC1 contained ST92, which was identified to be a potential founder, with ST75, ST137, ST90, ST118, and ST138 radiating from it. All the isolates in CC1 were CRAB, and the three susceptible strains were clustered into singletons. The location and source of these isolates showed diversity in different STs.

Evolution of AbaR resistance islands. Ten types of AbaR resistance islands were identified in the 32 CRAB strains (Fig. 2A). Except for the type 6 AbaR island, the other types shared high homology. The type 10 AbaR island was the predominant type and was identified in 12 strains (12/32, 37.5%); it was 19.123 kb in length and contained the highest number of resistance genes, including *tniB*, *usp*, *sul*, *tetA*, *tetR*, *arsR*, and *aph*, similar to those noted in a strain from Japan with one different nucleotide (24). The type 5 AbaR island in 1999BJAB11 contained the largest number of genes, and the conjugal transfer gene *traA* was identified only in this isolate. The resistance genes *tetA*, *tetR*, *arsR*, *aphA*, and *strB* were not found in the type 1 AbaR island, while the sulfonamide resistance proteins dihydropteroate synthase and phosphoglucosamine mutase (GlmM) were detected in the type 2, 7, and 10 AbaR islands. GlmM can catalyze the conversion of glucosamine-6-phosphate to glucosamine-1-phosphate, which is an essential step in the formation of the cell wall precursor UDP-*N*-acetylglucosamine (25). In *Streptococcus gordonii*, mutations in GlmM appear to influence bacterial cell growth and morphology,

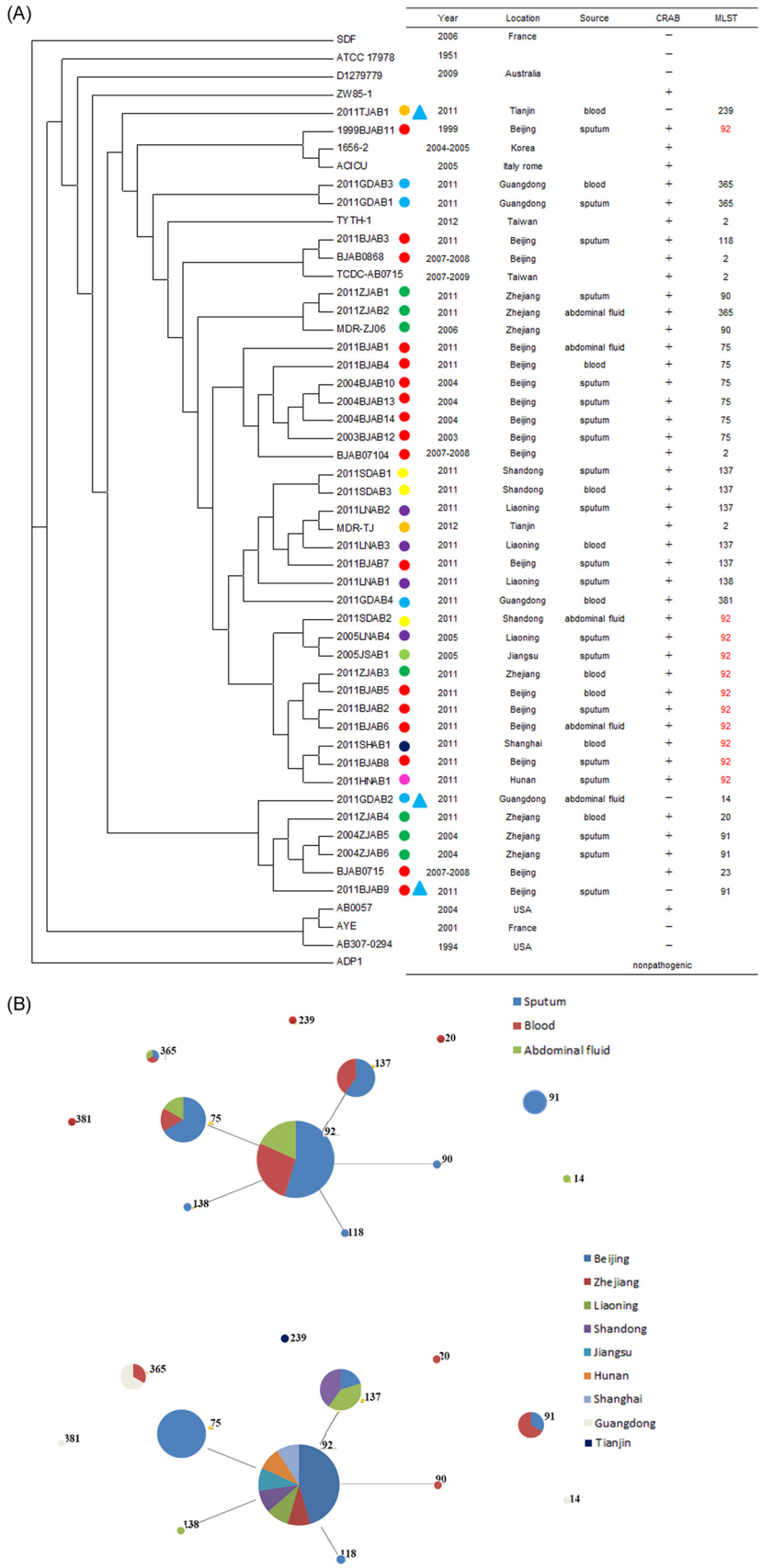

FIG 1 (A) Whole-genome phylogeny of the genomes of the 35 *A. baumannii* strains evaluated in this study and 17 sequenced *A. baumannii* genomes. The phylogeny tree was constructed on the basis of SNPs and was rooted with *A. baylyi* ADP1. Colored circles, the different locations of the strains isolated; blue triangles, the strains were susceptible to carbapenems. The predominant MLST, ST92, is highlighted in red. (B) Results of eBURST analysis conducted to assign CCs to the 35 *A. baumannii* strains utilizing seven loci. The CCs are indicated by circles, and the predicted clonal ancestors are shown by the central circles. Numbers indicate the MLST type. The sizes of the points are proportional to the number of isolates assigned to each MLST type.

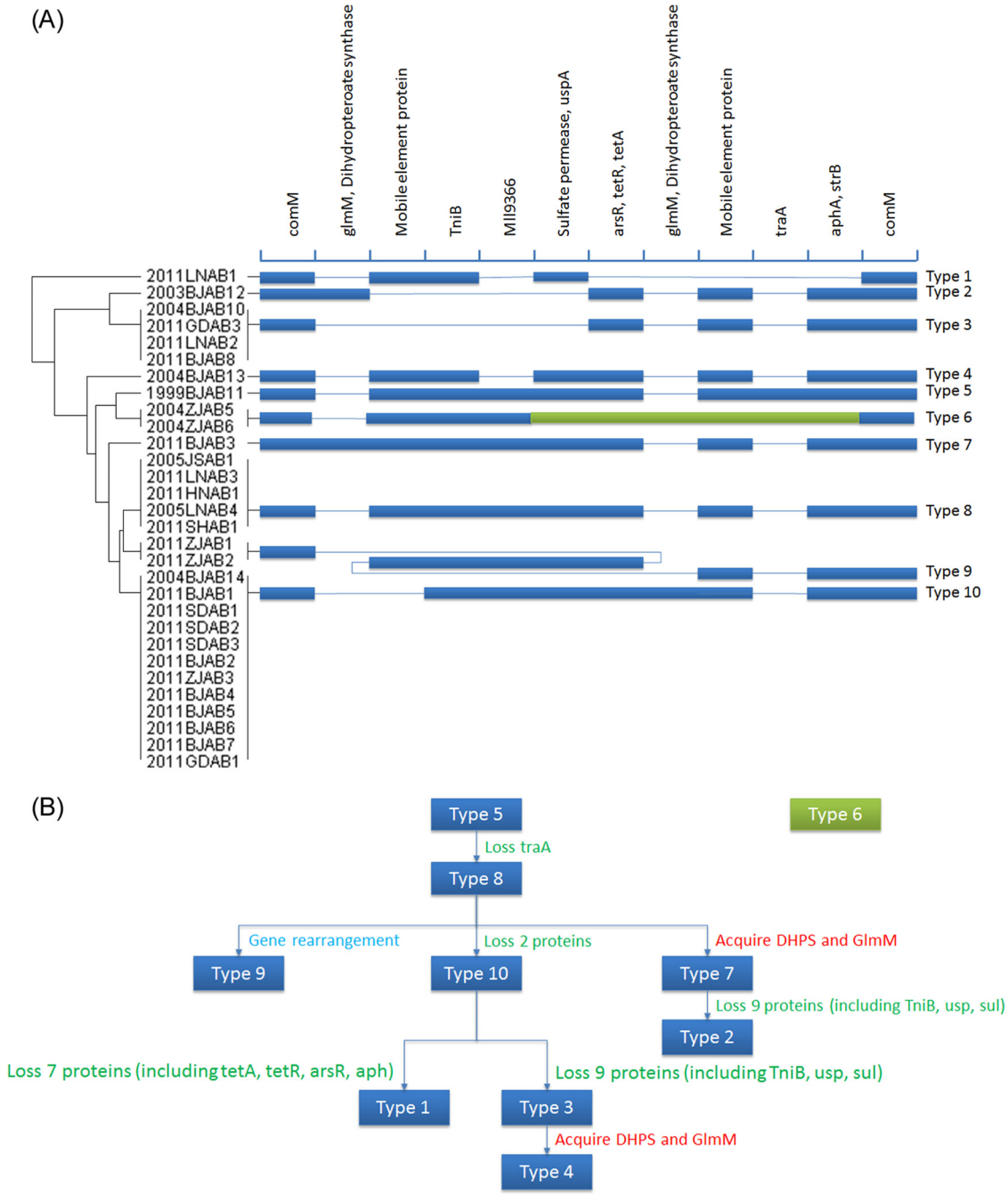

FIG 2 (A) Structure and distribution of the AbaR resistance islands. (B) Evolutionary ideograph of the AbaR islands in the evolution of CRAB. DHPS, dihydropteroate synthase.

biofilm formation, and sensitivity to penicillins (26). The function of GlmM in *A. baumannii* is currently not well-known. The mobile element protein was not observed in type 3 and 10 AbaR islands. Two strains isolated in Zhejiang Province in 2011 contained the type 9 AbaR island, which showed a gene rearrangement different from that of the type 8 AbaR island. Two Zhejiang strains collected in 2004 had the type 6 AbaR island, which included the two-component response regulator, the osmosensitive K^+ channel histidine kinase KdpD, methyl viologen resistance protein SmvA, an RND multidrug efflux transporter acriflavine resistance protein, and RND efflux system membrane fusion protein CmeA. These genes were not observed in the other nine types of AbaR islands. The evolutionary ideograph of the AbaR islands according to time is shown in Fig. 2B. The type 5 AbaR island in the earliest strain (1999BJAB11) contained the largest number of genes, and with genome evolution, gene acquisition, gene loss, and gene rearrangement were detected in strains isolated from 2003 to 2011. The type 6 AbaR island in two Zhejiang strains was relatively independent of the AbaR island types found in other strains during AbaR island evolution.

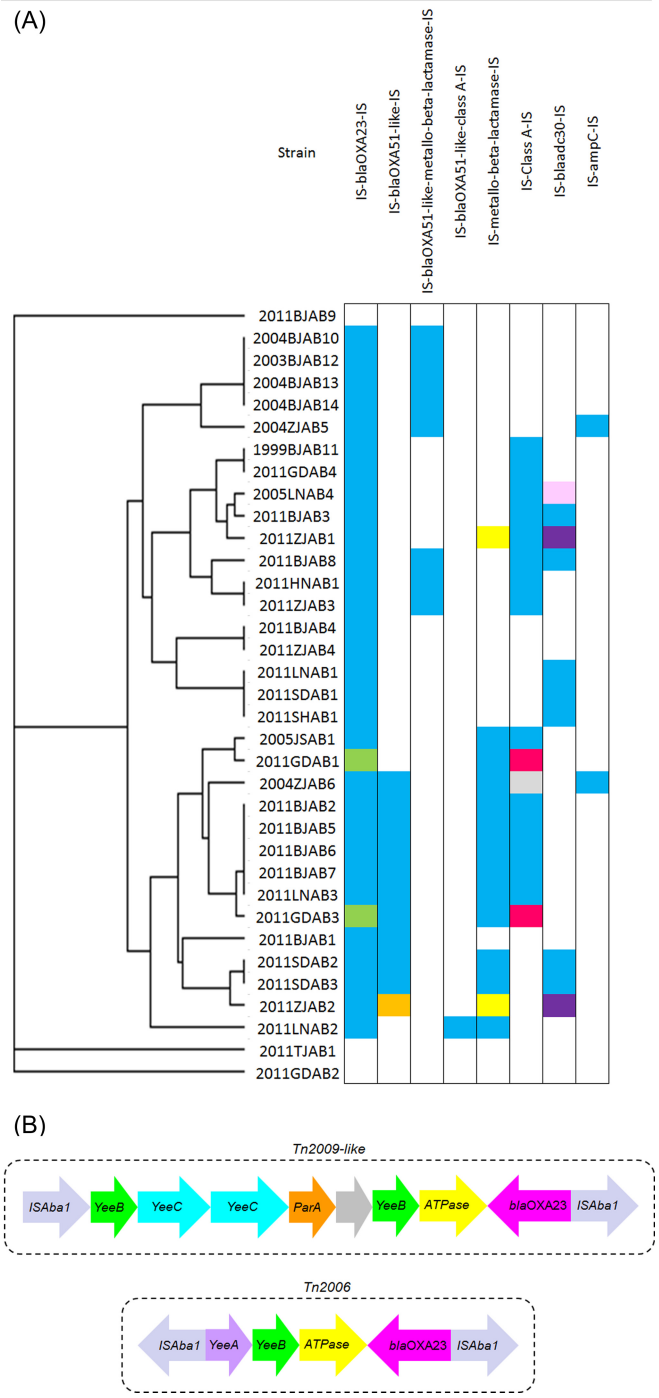

FIG 3 Transposons containing drug resistance gened in different *A. baumannii* strains. (A) Blue and green, IS*Aba1*; orange, IS*Aba1* and IS*Aba16*; yellow, IS*Aba14* and IS*Aba16*; gray, IS*Aba20* and IS*Aba1*; rose red, IS*Aba1* and IS*Aba13*; pink, IS*Aba1* and IS*Aba18*; purple, IS*Aba1* and IS*Aba14*; blank areas, the absence of transposons containing drug resistance genes. (B) Structures of transposons containing bla_{OXA23}, Tn*2009*-like (blue in panel A) and Tn*2006* (green in panel A).

Variations in transposon-associated antibiotic resistance genes. The transposons containing drug resistance genes varied in the different *A. baumannii* strains (Fig. 3A). The $bla_{OXA-51-like}$ gene was located in three types of transposons. Eleven strains possessed an IS–$bla_{OXA-51-like}$–IS transposon, and 10 of them were flanked by two IS*Aba1* elements. One strain, 2011ZJAB2, was flanked by IS*Aba1* and IS*Aba16*, and eight strains possessed IS*Aba1*–$bla_{OXA-51-like}$–metallo-beta-lactamase–IS*Aba1* transposons. Only one strain, 2011LNAB2, possessed an IS*Aba1*–$bla_{OXA-51-like}$–class A beta-lactamase–IS*Aba1* transposon. IS*Aba1*-metallo-beta-lactamase-IS*Aba1* was detected in 11 stains, but in 2 strains from Zhejiang isolated in 2011, metallo-beta-lactamase was flanked by IS*Aba14* and IS*Aba16*. IS*Aba1*–class A beta-lactamase–IS*Aba1* was identified in 13 strains. In one strain from Zhejiang isolated in 2004, IS*Aba20* and IS*Aba1* were detected on both sides of a class A beta-lactamase, and in two strains from Guangdong Province, China, isolated in 2011, IS*Aba1* and IS*Aba13* were detected. In seven strains isolated in 2011, bla_{ADC-30} was located in IS*Aba1*–bla_{ADC-30}–IS*Aba1*, but in 2005LNAB4, bla_{ADC-30} was flanked by IS*Aba1* and IS*Aba18*, and in two strains from Zhejiang isolated in 2011, bla_{ADC-30} was flanked by IS*Aba1* and IS*Aba14*. Furthermore, in two strains from Zhejiang isolated in 2004, IS*Aba1-ampC*-IS*Aba1* was identified.

The bla_{OXA-23}-containing resistance island was identified in all 32 CRAB strains (Fig. 3B). In most of the sequenced strains (30/32, 93.8%), bla_{OXA-23} was located in a transposon, which showed 99% identity to Tn*2009*. Tn*2009* was initially identified on the chromosome of *A. baumannii* MDR-ZJ06 from China, had a length of 8,421 bp, and was flanked by two IS*Aba1* elements. Tn*2009* was noted to carry the bla_{OXA-23} gene, the truncated DEAD/DEAH box helicase gene, the ATPase gene, the *yeeC* gene, and the *yeeB* gene. However, in two isolates from Guangzhou, China, obtained in 2011, bla_{OXA-23} was found in transposon Tn*2006*, which was shorter than Tn*2009*. Tn*2006* contained the ATPase gene, the *yeeA* gene, and the *yeeB* gene and was flanked by two IS*Aba1* elements.

Pan-genome: core and accessory genes. All the sequenced *A. baumannii* strains contained a genetically highly homogeneous core genome that encodes proteins involved in DNA replication, transcription, and translation as well as many metabolic pathways. By using the best reciprocal BLAST matches, we identified 2,830 core genes/proteins and 1,206 accessory genes/proteins among all the 35 *A. baumannii* isolates. Graphing of the numbers of core genome and pan-genome genes as a function of the number of strains sequenced revealed that the slope for the core gene number was approaching an asymptote, whereas the pan-genome gene number continued to expand even after the compilation of 35 genomes (Fig. 4A).

Variation in intrinsic chromosomal resistance genes related to carbapenem resistance. The phylogenetic distribution of different alleles of $bla_{OXA-51-like}$ and bla_{ADC} showed evidence of recombination and mutation. The presence of an upstream insertion element, IS*Aba1*, suggested that the entire region was replaced by homologous recombination. The bla_{ADC} gene was always associated with an upstream IS*Aba1* oriented to allow overexpression of the gene, except in the three susceptible strains (Fig. 4B). The sequences of 19 strains were identical to the sequence of the extended-spectrum bla_{ADC-30} variant (blue), and the sequences of 3 strains showed 99% identity to the bla_{ADC-30} sequence (dark blue). Five strains contained bla_{ADC-25} (yellow), and one strain carried bla_{ADC-56} (purple). The sequences of one susceptible strain, 2011BJAB9, and three resistant strains showed 99% identity to the bla_{ADC-53} sequence (orange), while the sequence of bla_{ADC} in 1999BJAB1 showed 99% identity to that of bla_{ADC-29}. The sequences of the suscep-

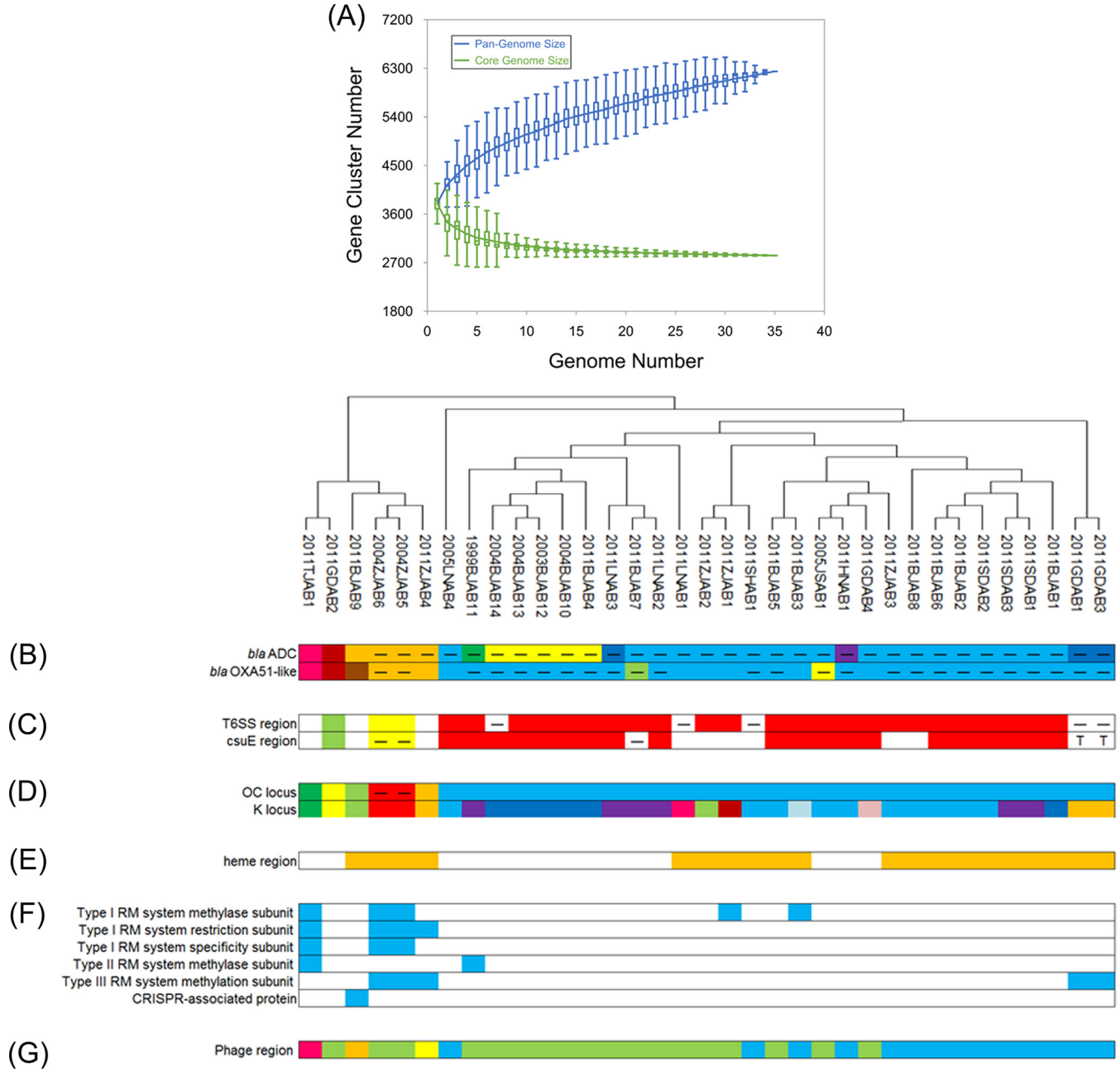

FIG 4 Genome features of the 35 *A. baumannii* strains. (A) Number of total features in the core genome and pan-genome as a function of the number of strains sequenced. An average of 500 random permutations of the genome order is presented for the pan-genome and core genome content; the error bars represent the standard deviations of these results. (B) Intrinsic chromosomal β-lactamase alleles. Different colors, the different types; blue, bla_{ADC} is an ADC30 variant and a bla_{OXA66} variant; horizontal bars, the presence of an upstream IS*Aba1*. (C) Variation in the presence of a T6SS gene cluster and a *csuE* gene cluster. Different colors, the different types; blank areas, the region is absent; horizontal bars, IS*Aba1* or IS*Aba13* insertion locations within each locus; T, transposase insertion locations within each locus. (D) Surface polysaccharide variants for the OC and capsular polysaccharide (K) loci. Different colors, the different types; horizontal bars, the IS*Aba1* insertion locations within each locus. (E) Variant region showing recombination around the heme utilization region. Orange, the heme region is present; blank areas, the heme region is absent. (F) RM system. Blue, the subunit is present; blank areas, the subunit is absent. (G) Phage content refers to the phage-related region located in strain TYTH-1. Blue, both the phages are present; green, the first phage is present; other colors, different phages are present (refer to the text for details).

tible strains 2011GDAB2 and 2011TJAB1 showed 98% and 99% identity to those of bla_{ADC-52} and bla_{ADC-63}, respectively.

The $bla_{OXA-51-like}$ variants were different in the resistant strains and susceptible strains. The most common $bla_{OXA-51-like}$ variant in resistant strains was bla_{OXA-66}, which was detected in 27 resistant strains (27/35, 77.1%), and bla_{OXA-68} was detected in 3 resistant strains from Zhejiang. One unusual variant, $bla_{OXA-234}$, was detected in a resistant strain from Jiangsu Province, China, in 2005. Furthermore, three novel variants were identified, including $bla_{OXA-425}$ in the resistant strain 2011BJAB7 and $bla_{OXA-424}$ and

$bla_{OXA-426}$ in the susceptible strains 2011GDAB2 and 2011BJAB9, respectively. bla_{OXA-69} was detected in the susceptible strain 2011TJAB1. In most CRAB strains (25/32, 78%), $bla_{OXA-51-like}$ variants were associated with an upstream IS*Aba1* element, but no upstream IS*Aba1* element was detected in any of the three susceptible strains.

IS-mediated T6SS and *csuE* region deletions. There were multiple instances of chromosomal gene loss that might have possibly been mediated by an IS adjacent to the deletion (Fig. 4C). Two large deletions adjacent to IS*Aba1* elements have been previously described (10), including a 40-kbp region encoding the entire type VI secretion system (T6SS) and an ~20-kbp region of adhesion genes (*csuE*) involved in aspartate metabolism. In the present study, three different types of T6SS regions were noted, and eight strains lacked a T6SS region. Among these eight strains, in five strains, deletion was mediated by an IS insertion. It is worth noting that, except for the IS*Aba1* insertion in three strains isolated in 2011, an IS*Aba13* insertion was detected in two strains from Guangdong isolated in 2011. Similarly, three different types of *csuE* regions were also noted, and 12 strains lacked this region. Among these 12 strains, the deletion was mediated by an IS insertion in 3 strains and a transposase insertion in 2 strains. Except for an IS*Aba1*-mediated deletion in 2011BJAB7, this study is the first to identify an IS*Aba125* insertion, which was found in two strains from Zhejiang isolated in 2004, and a transposase insertion, which was found in the *csuE* region in two strains from Guangdong isolated in 2011.

Variation in surface polysaccharide synthesis and heme utilization region. The 35 strains showed substantial variation in the content and organization of loci involved in surface polysaccharide synthesis (Fig. 4D). The core lipooligosaccharide (LOS) loci (outer core [OC] locus) (27) consisted of one predominant type (blue), which was present in most of the strains (29/35, 82.9%); however, we also identified several variations in the three susceptible strains and one resistant strain. In two strains from Zhejiang isolated in 2004, IS*Aba20* insertions were detected. With regard to capsular (K) loci, 12 variations were identified. One predominant type (blue) existed in 10 strains (28.6%), whose sequences were most closely related to the ACICU sequence (99.5% identity at the nucleotide sequence level).

The heme utilization region, containing several genes involved in heme utilization, was more common in strains collected in 2011. However, this region was absent in 1999BJAB1 and most of the strains collected from 2003 to 2005, except for two strains from Zhejiang isolated in 2004. Among the 23 strains collected in 2011, the heme utilization region was noted in 18 strains, and their sequences were 96% identical to the sequence of the ACICU heme utilization region (Fig. 4E).

Variation in the RM system. The restriction-modification (RM) system is a major participant in the coevolutionary interaction between mobile genetic elements (MGEs) and their hosts through the regulation of horizontal gene transfer (HGT). In the present study, six strains that clustered with two strains from Zhejiang isolated in 2004 contained more RM system genes (Fig. 4F). In the susceptible strain 2011TJAB1 and two resistant strains from Zhejiang isolated in 2004, we identified a type I RM system methylase subunit, a restriction subunit, and a specificity subunit. Furthermore, strains 2011ZJAB1 and 2011BJAB3 had only a type I RM system methylase subunit, and strain 2011ZJAB4 had only a type I RM system restriction subunit. A type II RM system methylase subunit was detected in strain 1999BJAB11 and susceptible strain 2011TJAB1, whereas a type III RM system methylation subunit was found in three Zhejiang strains and two Guangdong strains. A CRISPR-associated protein was detected in only one susceptible strain, 2011BJAB9.

Variation in phage-related regions. Phage-related regions are another source of variability contributing to the difference among *A. baumannii* strains. Two predominant types of phage-related regions were located within the same chromosomal locations, corresponding to 1.11 to 1.16 Mbp in strain ACICU (ACICU_00997 to ACICU_01077) and 1.45 to 1.53 Mbp in strain TYTH-1 (M3Q_1334 to M3Q_1458) (Fig. 4G). One primary variant typified by the completed reference genome of strain TYTH-1 consisted of two probable phage insertion events flanked by phage integrases (blue), whereas another variant possessed only the second of the two phage elements (green). Two susceptible strains (2011TJAB1 and 2011BJAB9) and strain 2011ZJAB4 had phage-related regions different from those of the two main variants, and all these strains were susceptible to tigecycline.

DISCUSSION

Despite increased research on *A. baumannii* evolution, the development of multidrug resistance and the genomic diversification of *A. baumannii* are not yet clear, especially in China. In the present study, we have reported the whole-genome sequences and on the comparative genomic analysis of 35 representative *A. baumannii* strains isolated from 1999 to 2011 in 13 hospitals in nine cities in China, including 32 CRAB and 3 carbapenem-susceptible *A. baumannii* strains with different genotypes and phenotypes. Data for these genomes represent a significant addition of genomic data from the genus and could serve as a valuable resource for future genomic studies.

It has been indicated that the AbaR resistance islands have evolved into diverse types through multiple events of insertion, deletion, and recombination (6, 28). The predominance of AbaR4-type resistance islands among the CRAB isolates has been reported in South Korea (29) and Taiwan (30). However, the evolution of AbaR resistance islands has not yet been investigated, especially in China. In the present study, it was found that the type 6 AbaR island in two Zhejiang strains collected in 2004 is different from the previously identified AbaR islands and may thus be a novel AbaR island. The type 6 AbaR island was noted to comprise the two-component response regulator, osmosensitive K^+ channel histidine kinase KdpD, methyl viologen resistance protein SmvA, an RND multidrug efflux transporter acriflavine resistance protein, and RND efflux system membrane fusion protein CmeA. These regulators and resistance-related proteins in the type 6 AbaR island may enhance the strain's adaptability and antibiotic resistance, which should be further studied.

The frequent detection of transposons and ISs carrying resistance genes in bacteria shows that recombination flexibility exists during the acquisition of resistance (4). In the present study, each strain contained multiple transposons or ISs, which gradually tended to diversify in the process of evolution. It must be noted that some of these ISs or transposons are novel and that most of the novel IS element insertions or transposons were mainly found in strains from Zhejiang or Guangdong Province of China. The resistance genes or elements may spread through a transposon- or IS-mediated mechanism, and the diversification of transposons or

ISs in the process of evolution increases the possibility of acquisition of novel resistance genes or elements.

Recombination has been reported to contribute to the change in the *A. baumannii* genome (28). Furthermore, the heme utilization region was also highly variable among the *A. baumannii* strains, and it was more common in strains collected in 2011 in the present study, thus making it difficult to assess whether this region was present in a common ancestor and lost by some strains or was gained and subsequently transferred via recombination to different lineages, as initially observed. In addition, evidence of recombination events in the allelic distribution of bla_{ADC} and $bla_{OXA\text{-}51\text{-}like}$ variants was also noted. In all CRAB strains, the bla_{ADC} gene was associated with an upstream IS*Aba1* oriented to allow overexpression of the bla_{ADC} genes. However, in all three susceptible strains, no upstream IS*Aba1* was detected. In addition, the $bla_{OXA\text{-}51\text{-}like}$ variants were different in the resistant strains and susceptible strains. Four uncommon $bla_{OXA\text{-}51\text{-}like}$ genes were identified, including $bla_{OXA\text{-}234}$ and a novel bla_{OXA}-type, $bla_{OXA\text{-}425}$, in CRAB strains and novel bla_{OXA} types $bla_{OXA\text{-}424}$ and $bla_{OXA\text{-}426}$ in two susceptible strains. In most CRAB strains (25/32, 78%), $bla_{OXA\text{-}51\text{-}like}$ variants were associated with an upstream IS*Aba1*, but no upstream IS*Aba1* was detected in any of the three susceptible strains. Furthermore, the $bla_{OXA\text{-}23}$-containing resistance island was identified in all 32 CRAB strains and absent in all susceptible strains. It is very likely that $bla_{OXA\text{-}234}$ or $bla_{OXA\text{-}425}$ contributes to carbapenem resistance in *A. baumannii*, as demonstrated previously with $bla_{OXA\text{-}23}$, $bla_{OXA\text{-}24}$, and $bla_{OXA\text{-}58}$, and $bla_{OXA\text{-}234}$ or $bla_{OXA\text{-}425}$ was also associated with an upstream IS*Aba1*, which has been shown to promote overexpression of OXA (31).

Despite extensive research on the virulence potential of *A. baumannii*, little is known about its true pathogenic potential or virulence repertoire. *A. baumannii* thrives in hospital settings largely due to its persistence on abiotic surfaces (32). One mechanism for *A. baumannii* persistence in hospital settings is the presence of a putative tip adhesion gene, *csuE*. The *csuE* gene is involved in pilus and biofilm formation (33), and its presence has been associated with the persistence of *A. baumannii* on abiotic surfaces, such as plastic and glass (33). The resistant strains were found to contain two different types of *csuE*. Most of the strains contained the shortest-type *csuE*, which was not observed in 10 resistant strains. The second type of *csuE* was detected only in two Zhejiang strains isolated in 2004 and comprised IS*Aba125* and a gene cluster containing 16 proteins that were not present in the shortest type. T6SS is widespread among Gram-negative bacteria and can be used for toxicity against other bacteria and eukaryotic cells. T6SS has been implicated in the interaction between bacteria as well as between bacteria and their hosts. In *Vibrio cholerae*, T6SS confers toxicity toward other bacteria, providing a means of interspecies competition to enhance environmental survival (34). Furthermore, *Pseudomonas aeruginosa* has been found to activate T6SS during infection in patients with cystic fibrosis (35) and also use the T6SS-delivered toxins to actively kill competing bacteria (36, 37). In *A. baumannii* strains, T6SS is conserved and plays a role in competition with other bacterial species (38). In the present study, three different types of T6SS regions were noted, with one type being predominantly detected. Two of the three susceptible strains did not exhibit this region, and only one strain presented a unique type of T6SS region which was different from the T6SS regions noted in the carbapenem-resistant strains. Two resistant strains collected in 2004 in Zhejiang exhibited the third type of T6SS region, which contained more genes than the predominant type, including genes for VgrG, ATP synthase, and transcriptional regulators.

A. baumannii showed a strong ability to acquire foreign DNA, such as DNA encoding drug resistance and pathogenicity, which allows the bacterium to acquire genetic diversity and overcome antibiotic selection pressure. The flow of genetic information between the bacterial cells by HGT drives bacterial evolution, and RM systems are the key moderators of this process (39). The presence of DNA for a RM system may act as a selfish genetic element to ensure its dissemination and/or may function in defense against bacteriophages and the hindrance of lateral gene transfer (40). In the present study, on the basis of the phylogenetic tree, two groups of strains with different founder strains were identified among the CRAB strains. Furthermore, six strains that clustered with two strains from Zhejiang isolated in 2004 contained more RM system genes. The transfer of resistance genes may facilitate the rapid spread of these genes among *A. baumannii* strains, so the antimicrobial susceptibility profile of this pathogen should be closely monitored. We speculate that new antibiotics with a strong ability to interfere with the horizontal transfer of resistance genes or elements carrying them may contribute to the effective treatment of *Acinetobacter* infections in the future.

In conclusion, in the present study, we analyzed the genomes of 35 representative *A. baumannii* isolates from China. Extensive variation in the gene content was found even among strains that were phylogenetically and epidemiologically very closely related. In the process of evolution, the genome of *A. baumannii* gradually tended to diversify. Several mechanisms contributed to this diversity, including IS-mediated deletions, genome-wide homologous recombination, transfer of mobile genetic elements, and mobilization of transposons or ISs. Thus, the present study improves our understanding of the evolutionary processes that contribute to the emergence of CRAB in China. Future large-scale sampling across different areas and time scales is still needed to fully understand the evolution of *A. baumannii* and its drug resistance development and trends.

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