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MECHANISMS OF RESISTANCE

Molecular Epidemiology and Mechanism of Sulbactam Resistance in *Acinetobacter baumannii* Isolates with Diverse Genetic Backgrounds in China

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ABSTRACT Sulbactam is a plausible option for treating *Acinetobacter* infections because of its intrinsic antibacterial activity against the members of the *Acinetobacter* genus, but the mechanisms of sulbactam resistance have not been fully studied in *Acinetobacter baumannii*. In this study, a total of 2,197 clinical *A. baumannii* isolates were collected from 27 provinces in China. Eighty-eight isolates with various MICs for sulbactam were selected on the basis of their diverse clonality and underwent multilocus sequence typing (MLST), antimicrobial susceptibility testing, and resistance gene screening. The copy number and relative expression of $bla_{\text{TEM-1D}}$ and *ampC* were measured via quantitative PCR and quantitative reverse transcription-PCR, respectively. The genetic structure of multicopy $bla_{\text{TEM-1D}}$ was determined using the whole-genome sequencing technology. The cefoperazone-sulbactam resistance rate of the 2,197 isolates was 39.7%. The rate of positivity for $bla_{\text{TEM-1D}}$ or IS*Aba1-ampC* in the sulbactam-nonsusceptible group (64.91% and 78.95%, respectively) was significantly higher than that in the sulbactam-susceptible group (0% and 0%, respectively; $P < 0.001$). The MIC of sulbactam ($P < 0.001$) varied considerably between the groups expressing *ampC* with or without upstream IS*Aba1*. Notably, the genetic structure of the multicopy $bla_{\text{TEM-1D}}$ gene in strain ZS3 revealed that $bla_{\text{TEM-1D}}$ was embedded within four tandem copies of the cassette IS*26*-$bla_{\text{TEM-1D}}$-Tn*3*-IS*26*. Therefore, $bla_{\text{TEM-1D}}$ and IS*Aba1-ampC* represent the prevalent mechanism underlying sulbactam resistance in clinical *A. baumannii* isolates in China. The structure of the four tandem copies of $bla_{\text{TEM-1D}}$ first identified may increase sulbactam resistance.

KEYWORDS *Acinetobacter baumannii*, sulbactam, $bla_{\text{TEM-1}}$, IS*Aba1-ampC*, multicopy

Acinetobacter baumannii is a notorious pathogen that causes severe nosocomial infections and exhibits a remarkable ability to develop multidrug or even pandrug resistance (1). In recent decades, the rate of multidrug-resistant *Acinetobacter baumannii* (MDRAB) strains has dramatically increased worldwide; therefore, treatment options are limited (2). Sulbactam is a plausible option for treating carbapenem-resistant *Acinetobacter baumannii* (CRAB) and MDRAB infections because of its intrinsic antibacterial activity against the members of the *Acinetobacter* genus (3, 4).

Sulbactam is a common β-lactamase inhibitor that is typically administered in combination with ampicillin or cefoperazone. Recent experimental and clinical studies have demonstrated that sulbactam has a promising effect against CRAB or MDRAB

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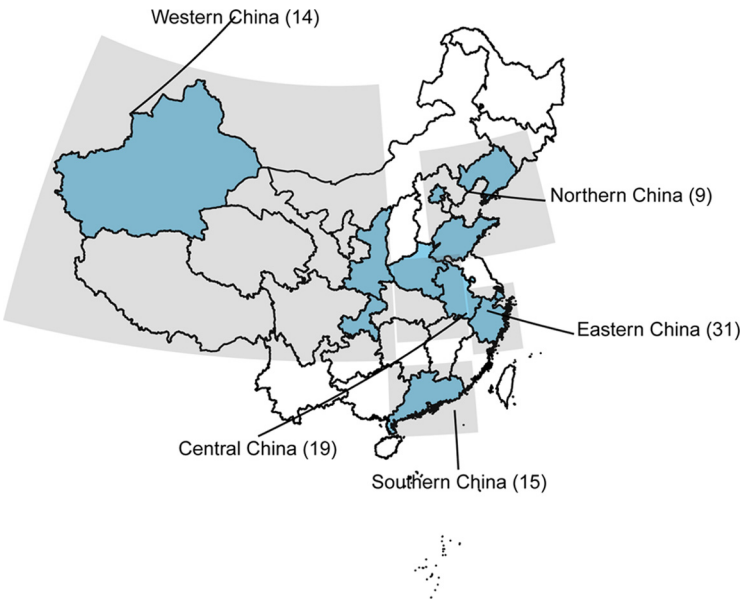

FIG 1 Geographical distribution of the strains collected from 11 different provinces (shaded blue). The numbers in the parentheses represent the numbers of strains.

compared with that of colistin and imipenem-cilastatin (5, 6). However, a survey in the United States reported that the rate of resistance to ampicillin-sulbactam increased from 35.2% during the period from 2003 to 2005 to 41.2% during the period from 2009 to 2012 (7). Data from the Chinese surveillance system (CHINET) showed that the rate of *Acinetobacter* sp. resistance to cefoperazone-sulbactam increased from 25% to approximately 40% from 2005 to 2014 in China (8).

Limited studies have shown that the β-lactamase TEM-1 and an *Acinetobacter*-derived cephalosporinase (ADC-30) could confer sulbactam resistance to *A. baumannii* (9, 10). However, their partial contributions and other underlying mechanisms of sulbactam resistance have not been fully studied, especially in strains with a background different from that of *A. baumannii* as well as strains with different sulbactam MICs.

This study aimed to explore the epidemiological characteristics and contributions of known mechanisms of sulbactam resistance in *A. baumannii* strains with diverse genetic backgrounds in China, and we discovered the first clinical strain of *A. baumannii* with four tandem chromosomal copies of $bla_{\text{TEM-1D}}$ originating from China.

RESULTS AND DISCUSSION

Epidemiology of sulbactam-resistant isolates in Chinese hospitals. A total of 2,197 *A. baumannii* clinical isolates were collected from 27 provinces and areas representing a broad regional distribution in China. The resistance rate of all *A. baumannii* isolates was 39.7% for cefoperazone-sulbactam, 76.6% for imipenem, 78.5% for meropenem, 86.8% for amikacin, and 93.3% for cefepime. Compared with the rate of resistance to sulbactam in combination with other compounds in other countries during the same period, the rate of resistance in China was similar to that in the United States (41.2%) from 2009 to 2012 (7). However, the rate in China was slightly lower than that in Vietnam (66.7%) from 2009 to 2011 (11).

According to the temporary susceptibility breakpoint for sulbactam in *A. baumannii* (≤4 mg/liter), 57 and 31 isolates were obtained from the sulbactam-nonsusceptible and sulbactam-susceptible groups, respectively. The clinical information and sequence types (STs) for these isolates are provided in Table S2 in the supplemental material. These isolates were distributed in 11 geographically separated provinces in China (Fig. 1). The STs based on the Oxford and Pasteur schemes are listed in the Table S2. The MIC

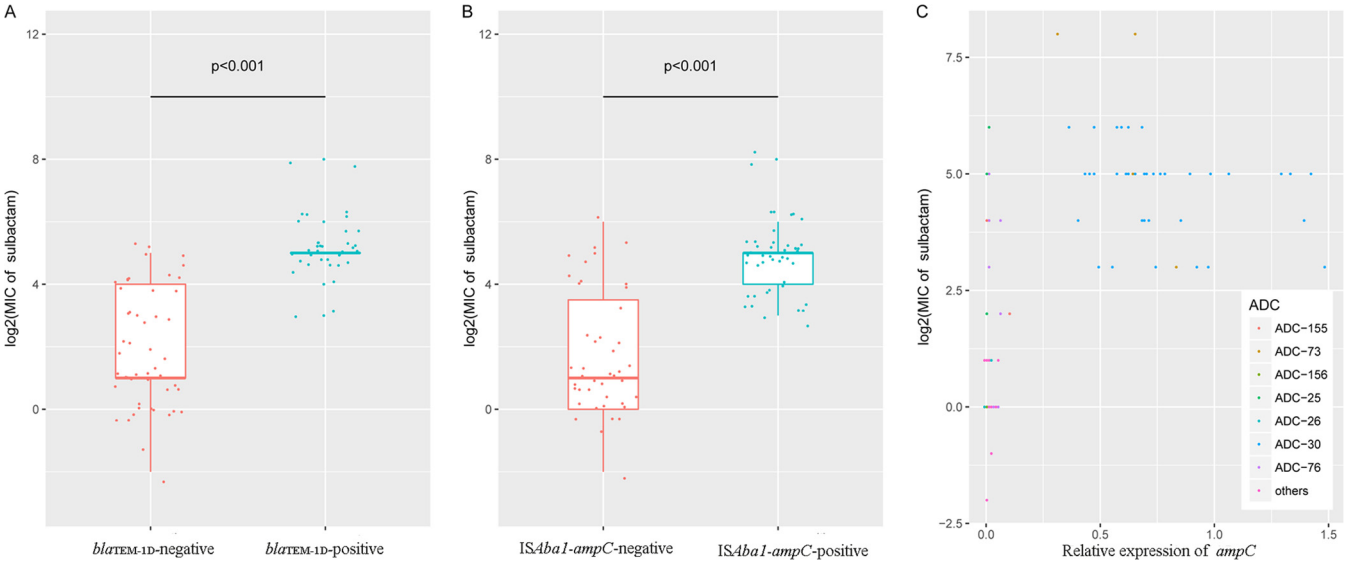

FIG 2 Roles of bla_{TEM-1D} and IS*Aba1-ampC* in sulbactam resistance. (A) Comparison of the MICs of sulbactam (in milligrams per liter) between the groups harboring bla_{TEM-1D} or not; (B) comparison of the MICs of sulbactam between the groups with or without IS*Aba1-ampC*; (C) comparison of sulbactam resistance and the relative expression of *ampC* between the different ADC protein variants.

of most of these strains was 32 mg/liter, and the MICs of sulbactam ranged from 0.25 mg/liter to 256 mg/liter.

Prevalence and contribution of bla_{TEM-1D} to sulbactam resistance. Of the 88 clinical strains in the study, 37 (42.05%) isolates tested positive for bla_{TEM-1D}. All bla_{TEM-1D}-positive strains were identified in the group without susceptibility to sulbactam. A statistically significant difference in the rate of positivity for bla_{TEM-1D} was noted between the sulbactam-nonsusceptible group and the sulbactam-susceptible group (64.91% versus 0%, $P < 0.001$). Furthermore, when all strains were divided into two groups (strains with bla_{TEM-1D} and strains without bla_{TEM-1D}), the MIC of sulbactam for strains with bla_{TEM-1D} was significantly increased compared with that for strains without bla_{TEM-1D} ($P < 0.001$) (Fig. 2A). These results indicate that the presence of bla_{TEM-1D} could play an important role in the development of sulbactam resistance in *A. baumannii*, which is consistent with the findings of a previous study (9).

To evaluate the correlation between the sulbactam MIC and bla_{TEM-1D} expression, we performed quantitative reverse transcription-PCR (qRT-PCR) to assess bla_{TEM-1D} expression. The results are presented in Table S2. In general, a moderate association between the expression of bla_{TEM-1D} and the MIC of sulbactam ($\log_2$) was observed in the *A. baumannii* strains ($r = 0.541$; $P < 0.001$), although this correlation was smaller than that reported in a previous study ($r = 0.92$) (9). Additionally, the levels of bla_{TEM-1D} expression were similar for all bla_{TEM-1D}-positive strains ($P > 0.05$) except strain ZS3, which exhibited an increased relative expression of bla_{TEM-1D} (11.38 ± 4.76) compared with that of the other strains ($P < 0.001$). Consistent with this finding, the sulbactam MIC of strain ZS3 was the highest (256 mg/liter) among all the clinical isolates tested. However, for most of the isolates, the MICs of sulbactam for the bla_{TEM-1D}-positive strains, except for strain ZS3, ranged from 8 to 256 mg/liter, and these strains presented the same level of bla_{TEM-1D} expression, suggesting that additional mechanisms contribute to sulbactam resistance in *A. baumannii*. For example, overexpression of other β-lactamases, mutations of penicillin-binding proteins, inactivation or downregulation of porins, and overexpression of the efflux pump are common factors that lead to β-lactam resistance in *A. baumannii* (1, 10, 12).

The promoter and the copy number are two common parameters associated with the mRNA expression of resistance genes. Therefore, the promoter region was also amplified and sequenced. Promoter P4 was the only promoter type in all bla_{TEM-1D}-

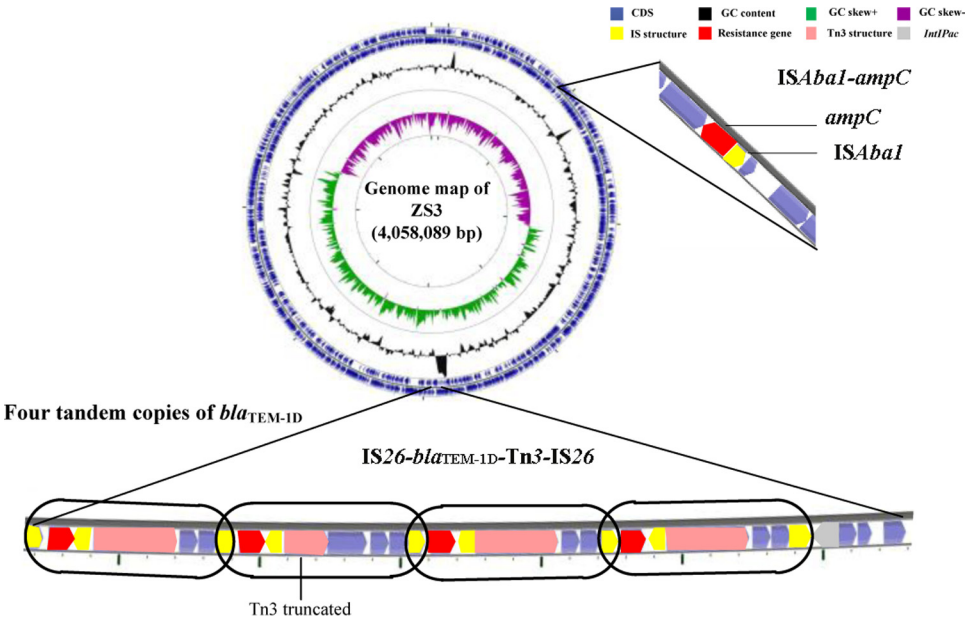

FIG 3 Circular map of the *A. baumannii* ZS3 genome and the gene environment of multiple copies of bla_{TEM-1D} and IS*Aba1-ampC*. The genome of *A. baumannii* ZS3 is represented by the outer blue circle. The GC content is represented by the black circle, and the GC skewed circle is the inner circle represented in green and purple. Blue arrows, coding sequences (CDS); red arrows, resistance genes; yellow rectangles, insertion sequence (IS) structures; pink arrows, Tn*3* structures; gray arrow, IntIPac. The bla_{TEM-1D} repeat fragment is highlighted with a black frame.

positive strains. P4, which is upstream of the bla_{TEM} genes, contributes to increases in the MICs of β-lactams for *Escherichia coli* according to the findings of a previous study (13). Regarding the bla_{TEM-1D} copy number, all bla_{TEM-1D}-positive strains except strain ZS3 possessed one copy of bla_{TEM-1D}; strain ZS3 carried at least two copies of bla_{TEM-1D}. Therefore, the multicopy bla_{TEM-1D} gene is a likely factor contributing to the high level of sulbactam resistance in strain ZS3 because it increases the production of the β-lactamase TEM-1D.

Structure and mechanism of multicopy bla_{TEM-1D}. To investigate the structure and mechanism of multicopy bla_{TEM-1D}, the whole genome of strain ZS3 (which carries multiple copies of bla_{TEM-1D}) was sequenced using the single-molecule real-time (SMRT) sequencing technology. Interestingly, the bla_{TEM-1D} gene was identified in the chromosome of strain ZS3 and embedded within a long quartic-duplicated fragment (7.6 kb) (Fig. 3). The duplicated fragment consisted of a cluster of genes (IS*26*-bla_{TEM-1D}-Tn*3*-IS*26*), which is equivalent to that noted in other strains with single copies of bla_{TEM-1D}. Four contiguous duplicated units were arranged together in the same direction and shared the same internal element, namely, IS*26*.

This is the first study to discover that four tandem copies of bla_{TEM-1D} are detected in the chromosome of *A. baumannii*. The mechanism of tandem gene amplification was revealed in a previous review (14). Gene duplication and amplification (GDA) were achieved by nonequal homologous recombination, in which the two flanking IS*26* elements provided homology for recombination. The IS*26* element was formed at the joint between the amplified units.

Chromosomal gene amplification can lead to increased antibiotic resistance by increasing the expression of a modifying or degrading enzyme, which explains why strain ZS3 exhibited a higher level of bla_{TEM-1D} gene expression (11.38 ± 4.76) and the highest sulbactam MIC (256 mg/liter). However, the correlation between the copy number and the MIC of sulbactam could not be verified because of a lack of a sufficient number of strains harboring multiple copies of the bla_{TEM-1D} gene.

Prevalence of IS*Aba1*-bla_{ADC} and contribution of IS*Aba1*-bla_{ADC} to sulbactam resistance. All 88 clinical strains tested possessed the ADC β-lactamase, and 41

(46.59%) isolates harbored ADC-30 (*ampC* allele 2). In addition to ADC-30, other types of ADC protein variants were found, including ADC-76 (*ampC* allele 10 [*ampC*-10], 12.50%), ADC-155 (*ampC*-36, 5.68%), ADC-25 (*ampC*-19, 5.68%), ADC-26 (*ampC*-56, 5.68%), ADC-73 (*ampC*-20, 4.55%), ADC-156 (*ampC*-52, 3.41%), and others. Of these, some novel *ampC* alleles and ADC protein variants (such as ADC-154, -155, -156, and -157) were identified and submitted to the GenBank database (accession and ID numbers are listed in Table S2 in the supplemental material). Notably, ADC-30 and ADC-73 were found only in the sulbactam-nonsusceptible group, whereas ADC-76, ADC-155, and ADC-25 were detected in the sulbactam-nonsusceptible and sulbactam-susceptible groups. The other ADC protein variants were present only in the sulbactam-susceptible group.

The correlation between the *ampC* alleles and STs based on the Pasteur multilocus sequence typing scheme is listed in Table S3, and the single *ampC* allele type was not included. The predominant allele, *ampC*-2, was present in the isolates of clonal complex 2 (CC2) and ST215. Additionally, isolates from CC2 had other *ampC* alleles, alleles 2, 19, 20, and 28, which were similar to those found in a previous study (15). In addition, *ampC* allele 10 was detected in all CC10 isolates (ST10 and ST23), but also in two isolates of other STs. The linkages between the *ampC* alleles and STs could provide the foundation for searching for clinically important clones of *A. baumannii*. ADCs, which are also known as AmpC cephalosporinases, are inherent to *A. baumannii* strains and are chromosomally encoded (1, 16, 17). Furthermore, the basal level of *ampC* expression does not reduce the efficacy of expanded-spectrum cephalosporins (18), and the relationship between *ampC* expression and the efficacy of sulbactam remains unknown.

To assess the correlation between the sulbactam MIC and *ampC* expression, we measured *ampC* expression via qRT-PCR. The results illustrated that the relative expression of *ampC* was significantly lower in the sulbactam-susceptible group than in the sulbactam-nonsusceptible group ($P < 0.001$). The level of *ampC* expression was moderately correlated with the MIC of sulbactam ($\log_2$) in the *A. baumannii* isolates ($r = 0.553$; $P < 0.001$). In Fig. 2C, the dots which represent ADC-30 and ADC-73 are located in the top center and are clearly separated from the dots for the other ADC protein variants. The MIC of sulbactam ($\log_2$) and the level of *ampC* expression in the ADC-30 and ADC-73 groups were increased compared with those in the groups of the remaining ADC protein variant types ($P < 0.001$). In addition, previous studies have shown that the AmpC β-lactamase, which is located on the chromosome of *A. baumannii*, is noninducible, in contrast to other Gram-negative organisms, because of the absence of the *ampR* gene (1, 17, 19); therefore, induction experiments with sulbactam were not performed.

The data from the copy number assay demonstrated that all isolates possessed only one copy of *ampC*. Interestingly, the insertion element IS*Aba1*, associated with ADC-30 (*ampC*-2) in a previous study (10), was identified only upstream of *ampC*-2 and *ampC*-20 and was not identified to be present in combination with the other *ampC* alleles in this study. The surrounding region of *ampC* with IS*Aba1* is presented in Fig. 3. The rate of IS*Aba1-ampC*-positive strains in the sulbactam-nonsusceptible group was significantly increased compared with that in the sulbactam-susceptible group (78.95% versus 0%, $P < 0.001$). To explore the role of IS*Aba1*, we determined the differences between the relative expression of *ampC* and the MIC of sulbactam in groups with and groups without IS*Aba1*. There was a notable difference in the expression of *ampC* ($P < 0.001$) and the MIC of sulbactam ($\log_2$) ($P < 0.001$) (Fig. 2B) between the two groups.

A previous study illustrated that IS*Aba1* provides strong promoter sequences (20), which results in *ampC* overexpression and could explain why in our study the expression of *ampC*-2 and *ampC*-20 with IS*Aba1* was significantly increased compared with that of the other allele types of *ampC* without IS*Aba1*. Accordingly, *ampC*-2 overexpression contributed to sulbactam resistance in *A. baumannii*, as described in a previous study (10). In addition, other *ampC* alleles, such as *ampC*-20, could also exert this effect with the IS*Aba1* upstream sequence. The role of *ampC* in sulbactam resistance is related

to the presence of IS*Aba1*, which can regulate *ampC* overexpression. Therefore, exploring a method for detecting IS*Aba1-ampC* rather than detecting ADC-30 alone is suitable for surveillance of sulbactam resistance in clinical *A. baumannii* isolates.

In summary, both bla_{TEM-1D} and IS*Aba1-ampC* are prevalent factors involved in the sulbactam resistance mechanism of *A. baumannii* isolates, based on their polyclonal background in China. This report is the first to identify four tandem copies of bla_{TEM-1D} located on the *A. baumannii* chromosome, and the multiplication of bla_{TEM-1D} may enhance the sulbactam resistance level by increasing the expression of the β-lactamase TEM-1D. IS*Aba1*, which is associated with *ampC*, plays a key role in sulbactam resistance by upregulating *ampC* expression.

MATERIALS AND METHODS

Strains. A total of 2,197 nonduplicate clinical *A. baumannii* isolates were collected from 64 central Chinese hospitals in 27 provinces from January 2009 to September 2010 (21). Eighty-eight isolates were selected from the nationwide survey program based on their different types according to multilocus sequence typing (MLST), which was performed using the Oxford scheme and the Pasteur scheme, as previously described (22, 23). Several novel alleles and profiles were assigned using the PubMLST database (http://pubmlst.org/abaumannii/). All isolates were identified to the species level using a PCR targeting the 16S-23S rRNA gene intergenic spacer region and the partial RNA polymerase β-subunit (*rpoB*) gene (24–26).

Antimicrobial susceptibility testing. The MICs of sulbactam were determined using the disk diffusion method and the broth microdilution method according to the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) (27). On the basis of the CLSI susceptibility breakpoint for ampicillin-sulbactam ($\leq$8/4 mg/liter) in *A. baumannii*, we adopted $\leq$4 mg/liter as the temporary susceptibility breakpoint because of the lack of a clinical breakpoint for sulbactam alone (27). The interpretive criterion of susceptibility for ampicillin-sulbactam (zone diameter $\geq$ 15 mm) in *A. baumannii* was adopted as the breakpoint for cefoperazone-sulbactam in the disk diffusion method.

PCR and sequencing. Isolates were screened for the presence of bla_{TEM} and *ampC* by PCR using the bla_{TEM}-specific-C1/C2 and bla_{ADC}-F/R primers (see Table S1 in the supplemental material) (28). For the positive strains, a second PCR was performed using the primers TEM Promo F/R and IS*Aba1*-bla_{ADC}-F/bla_{ADC}-R to amplify the full-length sequence containing the promoter region (29). The alleles of *ampC* and the ADC protein variants were identified in the database for *ampC* alleles in *A. baumannii*, which is available at the PubMLST platform for *A. baumannii* (http://pubmlst.org/abaumannii/) (15). The novel alleles and variants have been submitted to the GenBank database.

Quantitative PCR (qPCR) and quantitative reverse transcription-PCR (qRT-PCR). To detect the bla_{TEM} and *ampC* copy numbers, we performed qPCR using a ViiA7 real-time PCR system (Life Technologies) and a SYBR Premix *Ex Taq* PCR kit (TaKaRa Bio, Japan). Primers targeting the bla_{TEM} (primers RT TEM-F/R), *ampC* (primers qPCR for ADC-F/R), and *rpoB* (primers qPCR for *rpoB*-F/R) genes were used as previously described and are listed in Table S1 (9, 10, 30). Each reaction mixture contained a total volume of 10 μl with 2 ng of genomic DNA as a template, 100 nmol/liter of each primer, and 1$\times$ SYBR green PCR master mix (Applied Biosystems, Carlsbad, CA, USA). Based on its complete genome sequence (GenBank accession number CP014541), strain XH856 possesses a single copy of bla_{TEM} and *ampC* and was used as the control strain. The relative copy number was calculated using the comparative threshold cycle (C_T) method ($2^{-\Delta\Delta CT}$ method) (31, 32). Triplicate samples were included in each run, and all qPCRs were performed three times. The definition of multiple copies relies on the one-sample *t* test, as noted in a previous study (33).

The expression of bla_{TEM} and *ampC* was quantified using the same primers used for the qPCR to detect the copy numbers of bla_{TEM} and *ampC*. RNA was extracted with the RNAprotect Bacteria reagent and an RNeasy minikit (Qiagen, Valencia, CA, USA) and was reverse transcribed into single-stranded cDNA with random hexamer transcriptase (TaKaRa, Japan). Each isolate was tested three times. The level of expression of bla_{TEM} and *ampC* relative to that of the *rpoB* gene was calculated as previously described (31).

Whole-genome sequencing and annotation. *A. baumannii* strain ZS3 was cultured to the midlogarithmic phase in 50 ml of LB broth at 37°C. DNA was extracted using a QIAamp DNA minikit (Qiagen, Valencia, CA) and was further purified using a PowerClean DNA cleanup kit (Mo Bio Laboratories, Carlsbad, CA) according to the manufacturer's recommendations. The genome of the strain was sequenced by the Tianke Company (Zhejiang, China). Single-molecule real-time (SMRT) sequencing reads were generated using a PacBio RS II platform (Pacific Biosciences, Menlo Park, CA) and subsequently *de novo* assembled and resequenced using the PacBio Hierarchical Genome Assembly Process workflow available in the SMRT Analysis (version 2.3.0)/Quiver software package.

The assembled genome was annotated using the NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAAP) and manually assessed by use of the BLASTX (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and InterProScan (34) programs. Antimicrobial resistance genes were detected by use of the ResFinder tool (https://cge.cbs.dtu.dk/services/ResFinder/). Whole genomes were compared using the Artemis comparison tool (version 11.1.1). *In silico* MLST analyses of the whole-genome sequencing data were performed using the BacWGSTdb server (35). Graphical maps, sequence features, base composition plots, analysis results, and sequence similarity plots were generated using the CGView server (http://stothard.afns.ualberta.ca/cgview_server/) (36).

Statistical analysis. Categorical variables were compared using chi-square analysis or Fisher's exact test as appropriate. The normal distribution of continuous variables was determined using the Kolmogorov-Smirnov test and histograms. Continuous variables with normally distributed data were compared by analysis of variance (ANOVA) or a *t* test, whereas Wilcoxon rank-sum tests or Kruskal-Wallis tests were used for nonnormally distributed data. Correlations between the variables were determined using Spearman's correlation test.

Accession number(s). The genome of strain *A. baumannii* ZS3 had been submitted to GenBank under accession number CP021496, and the accession numbers of the *ampC* alleles and the ID numbers of the ADC protein variants are listed in Table S2.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01947-17.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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We have no competing interests to declare.

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