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Characteristics of Oral *Acinetobacter* spp. and Evolution of Plasmid-Mediated Carbapenem Resistance in Bacteremia Patients with Hematological Malignancies

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Background: Patients with hematological malignancies are more susceptible to infections, leading to a poor prognosis. *Acinetobacter* colonization is a risk factor for secondary bacteremia.

Methods: Antibiotic susceptibility phenotypes and genomic characteristics of 48 oral *Acinetobacter* spp. and one bloodstream *Acinetobacter baumannii* from patients with hematological malignancies were analyzed by antimicrobial susceptibility tests and whole-genome sequencing. We conducted comparative genomic analysis of oral and blood isolates from the same patient.

Results: *A. baumannii* was the most common (72.92%, 35/48) *Acinetobacter* species in oral *Acinetobacter* spp. isolates. Seventeen different *A. baumannii* sequence types were identified using the Pasteur MLST scheme; however, the dominant global clones GC1 and GC2 were not present. Among the isolates, 46 (95.8%) were carbapenem-susceptible *Acinetobacter* spp. One patient treated with meropenem for 15 days developed *A. baumannii* bacteremia 46 days after the isolation of oral *A. baumannii* AOR07. Oral and bloodstream isolates from the same patient were closely related to only four non-synonymous mutations on the chromosome. The $bla_{\text{OXA-58}}$ gene was transferred between plasmids through XerCD-mediated recombination, leading to an elevated copy number, causing carbapenem resistance in bloodstream isolates.

Conclusion: Oral *Acinetobacter* spp. may cause secondary bacteremia. The amplification and transfer of $bla_{\text{OXA-58}}$ in the plasmids explained the increased carbapenem resistance in the bloodstream isolate.

Keywords: *Acinetobacter*, within-host evolution, oral colonization, bloodstream infection, hematological malignancies

Introduction

Acinetobacter spp. are important pathogens in hospital-acquired infections and have attracted greater attention owing to their ability to exhibit multidrug resistance (MDR). *Acinetobacter* spp. is widely distributed in water and soil. In humans, they can colonize various sites such as the skin, respiratory tract, and gastrointestinal tract.[1]

Patients with hematological malignancies are more likely to develop bacterial infections. Neutropenia can result from the malignancy itself or develop as a complication of chemotherapy, and the development of infections remains a major risk factor in these patients.[2] Another study indicated that patients with hematological malignancies and febrile neutropenia were at high risk of *A. baumannii* bacteraemia. The high prevalence of carbapenem-resistant *A. baumannii* has been associated with high mortality rates.[3] Colonization by MDR bacteria is thought to precede infection and is an important risk factor for subsequent infections.[4,5] *Acinetobacter* infections are closely related to colonisation, and several studies have shown that colonization of the respiratory or gastrointestinal tracts is a risk factor for secondary bacteremia.[6,7]

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Carbapenem-resistant *A. baumannii* is regarded by the World Health Organization as a critical pathogen for the development of novel antibiotics. The carbapenem-hydrolyzing class D oxacillinases, OXA-23, OXA-24, and OXA-58, are the most important factors for carbapenem resistance in *A. baumannii*.[8] *A. baumannii* was able to meet the evolutionary challenge of combating antimicrobial chemotherapy by acquiring pre-existing resistance determinants from the bacterial gene pool. This is achieved through the concerted activity of mobile genetic elements capable of relocating within or between DNA molecules. These elements include insertion sequences, transposons, and gene cassettes/integrons, and those that can be transferred between bacterial cells, such as plasmids and integrative conjugative elements.[9]

However, the characteristics of oral *Acinetobacter* spp. in patients with hematological malignancies have not been reported, and few studies on the resistance evolution of oral *Acinetobacter* have been conducted. Therefore, the objective of this study was to investigate whether there is an association between the source of bacteria in the blood of patients with *A. baumannii* bloodstream infections and their oral colonization. In this study, we analyzed the distribution and phenotypic characteristics of the antibiotic susceptibility of oral *Acinetobacter* in patients with hematological malignancies. Additionally, the genomic correlation between oral colonization and bloodstream infection isolates were analyzed in one patient with bacteremia. This study provides novel evidence for the evolution of carbapenem resistance in *A. baumannii* isolates from patients with hematological malignancies.

Materials and Methods

Sample Collection Procedure for Oral Swabs

This is a retrospective study. After the patient has rinsed their mouth with water to remove food particles and any external debris, the examiner then instruct them to slightly open their mouth and use the swab (Copan, Italy) to gently scrape the buccal mucosa, typically in the area between the upper and lower teeth. When collecting the sample, the examiner should carefully remove the oral swab without touching the swab tip with their hands. The swabs were inoculated onto Columbia blood agar, MacConkey agar, and chocolate agar plates, and incubated overnight at 35°C in a 5% CO_2 incubator.

Isolates

Acinetobacter isolates were collected from oral swabs between January and November 2020 in a teaching hospital with a bed capacity of more than 1400, and a total of 48 isolates were obtained from 37 patients. We performed whole-genome sequencing (WGS) of oral and bloodstream samples from patients who subsequently developed bloodstream infections. The isolates were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Bruker Daltonics, Germany).

Antimicrobial Susceptibility Tests

MICs of the antimicrobials were determined using the broth microdilution method. The results were interpreted according to CLSI M100 29th edition (2019).[10] Tigecycline susceptibility results were interpreted according to the US Food and Drug Administration for Enterobacteriaceae guidelines (https://www.fda.gov/drugs/development-resources/tigecycline-injection-products). The antimicrobial agents tested were ceftazidime, meropenem, levofloxacin, colistin E, minocycline, trimethoprim/sulfamethoxazole, and tigecycline. Reference isolates *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control standards.

WGS and Sequence Analysis

Genomic DNA was purified from overnight bacterial cultures using a Genomic-tip 100/G (Qiagen, Hilden, Germany). Libraries for short-read sequencing were sequenced using an Illumina MiSeq system. De novo genome assembly of all isolates in this study was performed using ABySS v2.1.5.[11] Libraries for long-read sequencing were sequenced using the *RS* II DNA sequencing system (Pacific Biosciences, Menlo Park, CA, USA). Hybrid assembly of short and long reads was performed using Unicycler v.0.4.8 to obtain the complete genome sequences.[12] The genomes were annotated using

Prokka software[13] and Rapid Annotation using the Subsystems Technology (https://rast.nmpdr.org/).[14] Single-nucleotide variant analysis was performed and filtered using the Genome Analysis Toolkit software with default mapping parameters.[15] For phylogenetic analysis, the core genome was extracted using the Roary software[16] and IQ-TREE was used to construct a phylogenetic tree.[17] COG functional categories were identified using eggNOG-Mapper with the default parameters (http://eggnog-mapper.embl.de/).[18] The multilocus sequence typing (MLST) for *A. baumannii* was analyzed using PubMLST (https://pubmlst.org/organisms/acinetobacter-baumannii) with default parameters.[19] Antimicrobial resistance genes were identified using ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) with the default parameters.[20] Plasmids were typed using the Acinetobacter Typing Database.[21] The p*dif* sites in plasmids were located by first identifying a sequence matching the less variable XerD binding site (5′-ATTTAACATAA-3′) and then examining the sequence 6 bp in either direction for a site resembling the XerC binding site (5′-TTATGCGAAAT-3′) of *Acinetobacter*.[22] All annotations were visualized using iTOL (https://itol.embl.de/).[23]

Quantitative Real-Time PCR

Total RNA was extracted from cells in the mid-log growth phase using RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA was reverse transcribed to cDNA using the PrimeScript RT reagent kit (TaKaRa Bio) and quantified via real-time PCR with TB Green Premix Ex Taq II (TaKaRa Bio) for genes, using an ABI PCR system (Thermo Fisher Scientific). Relative gene expression was calculated from the comparative threshold cycle method ($2^{-\Delta\Delta Ct}$), and the *rpoB* gene was used as an internal control for normalization. The primer sequences are listed in Table S1.

Statistical Analysis

The statistical analysis was conducted using GraphPad Prism 8 software, and comparisons between the two groups were performed using a two-tailed unpaired Student's *t*-test. The threshold for statistical significance was set at a p-value of less than 0.05. *** means $p < 0.001$.

Results

Characteristics of Clinical Acinetobacter Isolates in Oral Swabs of Patients with Hematological Malignancies

A total of 48 oral *Acinetobacter* isolates were obtained from 37 patients. Among these isolates, *A. baumannii* accounted for the largest proportion (72.92%, 35/48). In addition, five *A. pittii*, three *A. junii*, two *A. calcoaceticus*, one *A. nosocomialis*, one *A. bereziniae*, and one *A. ursingii* isolates were obtained (Figure 1). Two oral isolates were obtained from 11 patients during hospitalization, and 90.9% (10/11) shared the same species between the two isolates. However, in patient P6, the two isolates belonged to different species: *A. pittii* and *A. baumannii*.

There were 17 types defined by the Pasteur MLST scheme and 23 types according to the Oxford MLST scheme; however, the dominant global clones, GC1 and GC2, were not present (Figure 1). The most common ST was the Pasteur-type ST40/Oxford type ST373 (4, 11.43%). Among the patients with two isolates, 72.7% (8/11) had two isolates of the same MLST type. However, the isolates from the same patient had different MLST types according to the Oxford MLST scheme in two patients (P22 and P32), and different Pasteur types in P22. Moreover, the *gdhB* gene in Oxford MLST scheme was missing in two isolates (AOR03 and AOR46) (Figure 1).

We analyzed seven antibiotics: ceftazidime (CAZ), meropenem (MEM), levofloxacin (LEV), colistin E (COL), minocycline (MNO), trimethoprim/sulfamethoxazole (TMP/SMZ), and tigecycline (TGC) (Table 1 and Figure 1). Of the 48 isolates, 42 (87.5%) were susceptible to all seven antibiotics and only six isolates (12.5%) were resistant to at least one antibiotic. There were two meropenem resistant *A. baumannii* isolates, AOR49 and AOR50, which were both Pasteur type ST2034/Oxford type ST710 and were isolated from the same patient P37. Five *A. baumannii* isolates (AOR03, AOR07, AOR26, AOR49, and AOR50) were resistant to TMP/SMZ, while only one *A. pittii* isolate (AOR33) was resistant to levofloxacin.

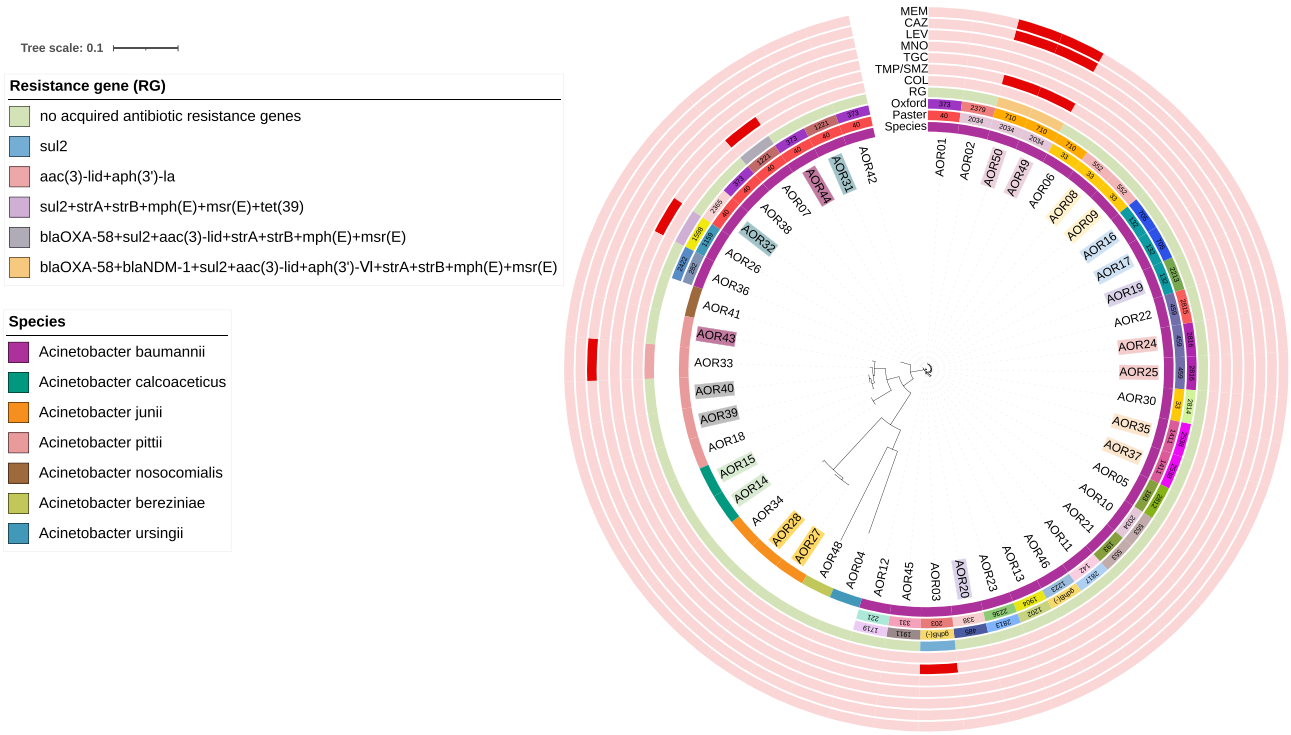


Figure 1 Phylogenetic analysis of *Acinetobacter* isolates collected from oral in hematological malignancies patients. The outer color of the trees denoted different species, Pasteur and Oxford MLST types, acquired resistance genes, and antimicrobial susceptibility results for seven antibiotics. Isolates from the same patient were shown with the same background color. The detailed Pasteur and Oxford MLST type of *A. baumannii* was listed, the *gdhB*(-) was utilized to indicate the lack of the *gdhB* gene in the Oxford MLST scheme of AOR03 and AOR46. Light pink represented the isolate was susceptible to antibiotics, while red represented resistance.

Distribution of Antibiotic Resistance Genes in Oral Acinetobacter Isolates

Using the core genome, we analyzed the phylogenetic and antibiotic resistance gene characteristics of the 48 oral isolates. Isolates of the same species clustered together and *A. baumannii* was divided into two clades. We compared the acquired antibiotic resistance genes among these isolates (Figure 1). Among the 48 isolates, *A. baumannii* AOR07 harboured $bla_{\text{OXA-58}}$. AOR49 and AOR50, which were collected from the same patient, P37, carried the $bla_{\text{OXA-58}}$ and $bla_{\text{NDM-1}}$. Five TMP/SXZ-resistant isolates (AOR03, AOR07, AOR26, AOR49, and AOR50) carried the *sul2* gene. Five isolates (AOR07, AOR26, AOR33, AOR49, and AOR50) harbored at least one aminoglycoside resistance gene. Four isolates (AOR07, AOR26, AOR49, and AOR50) harbored macrolide resistance genes *mph(E)* and *msr(E)*. The tetracycline resistance gene *tet*(39) was identified in one *A. baumannii* isolate, AOR26.

Table 1 Antimicrobial Susceptibility Profiles of Oral *Acinetobacter* spp. Isolates

Antimicrobial Agent	Resistant (%)	Susceptible (%)	MIC Range	MIC_{50}	MIC_{90}
CAZ	4.17	95.83	0.5–>128	4	8
MEM	4.17	95.83	<=0.25–16	<=0.25	1
LEV	2.08	97.92	<=0.125–8	<=0.125	0.25
MNO	0	100	<=0.125	<=0.125	<=0.125
TGC	0	100	<=0.064–0.5	0.125	0.25
TMP/SMZ	10.42	89.58	<=0.125–8	0.25	4
COL	0	100	0.25–2	0.5	1

Table 2 Genetic Changes Identified on Chromosome Between Oral and Bloodstream *A. Baumannii* from the Same Patient

Mutation Type	Base Position in AOR07	Protein	Gene Function	Mutation
SNV	280,556	–	Intergenic	A/T
SNV	1,818,738	YbiH	Transcriptional regulator YbiH, TetR family	V65E
Frameshift mutation	1,942,875	Gp2	Phage/plasmid replication protein, II/X family	Interrupted in AOR07
Frameshift mutation	3,802,820	IS*Aba45*	IS*3* family transposase	Interrupted in AOR07

Oral and Bloodstream Isolates from the Same Patient Were Closely Related

One patient developed bacteremia 46 days after oral isolation of *A. baumannii*. The patient was treated with meropenem for 15 days before the positive blood culture. The susceptibility phenotypes of the oral isolates, AOR07 and AOR07-BL, were similar, except for meropenem. The MIC of meropenem increased 8-fold in AOR07-BL (8 mg/L). Both the oral isolate AOR07 and the bloodstream isolate AOR07-BL were Oxford type ST 1221 and Pasteur type ST40. Additionally, both isolates harbored the same antibiotic resistance genes.

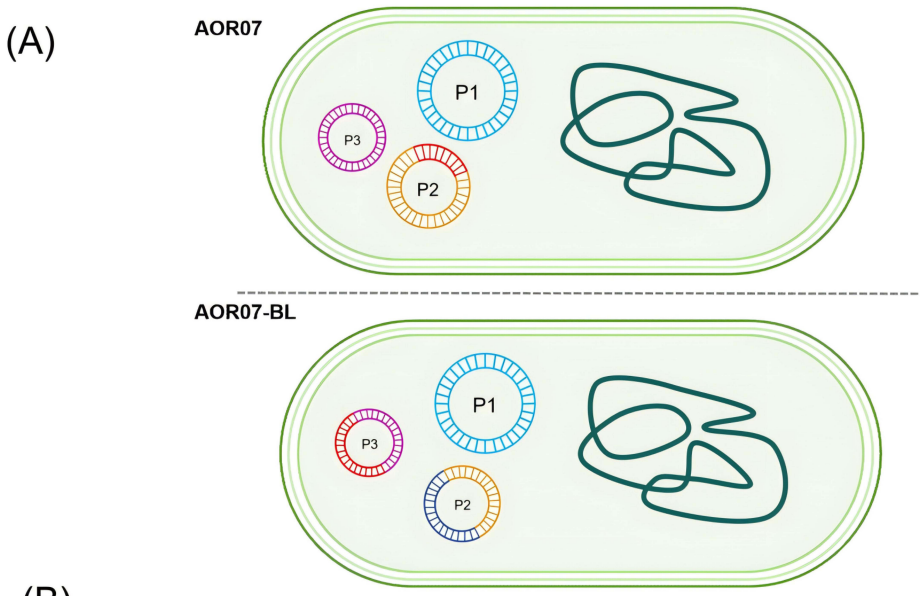
Four non-synonymous mutations were observed between the AOR07 and AOR07-BL chromosomes (Table 2). The first single nucleotide variant (SNV) occurred in the intergenic region; base A from the oral cavity was mutated to base T during bloodstream infection, and its downstream target was the type II secretion system-related gene *gspN*. The second SNV occurred in the *ybiH* gene, encoding a member of the TetR family of transcriptional regulators upstream of *YbhG*, a membrane-related component of the ABC efflux pump, which is mutated from base A in the oral cavity to base T in bloodstream infection. This SNV causes an amino acid missense mutation from valine to glutamate at position 65 in YbiH. The third SNV occurred in the phage replication initiation protein Gp2 and caused a frameshift mutation in its amino acid sequence in AOR07. The fourth SNV occurred in IS*Aba45*, which was located upstream of membrane-type matrix metallopeptidase-1 (*mmp*). In AOR07, IS*Aba45* lacked a single base, resulting in a frameshift mutation in its amino acid.

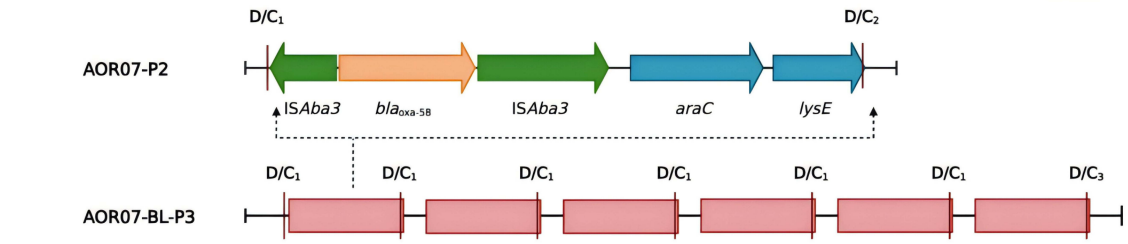
Evolution of Plasmid/Extrachromosomal Elements Resulted in Carbapenem Resistance

Three plasmid/extrachromosomal elements (P1, P2, and P3) were identified in the two isolates; however, no identical plasmids were found in the NCBI public database (Table 3). The GC content of the plasmids ranged from 34.03% to 41.07%, and all plasmids belonged to R3-T3 in the Rep_3 family. P1, a 133-kb plasmid, is present in both isolates, with its genome sequence highly conserved between them; in contrast, the sequences of P2 and P3 differed. Resistance genes *msr(E), mph(E), sul2, strA, strB*, and *aac(3)-IId* were located on plasmid 2 of AOR07 and AOR07-BL. Moreover, a 3.8 kb segment of P2 in AOR07 was lost in the P2 of AOR07-BL (Figure 2A). This segment contains a copy of the resistance gene $bla_{\text{OXA-58}}$. We also found sequence inversions flanking $bla_{\text{OXA-58}}$ in P2 of AOR07-BL, which showed structural changes in the plasmids during evolution. $Bla_{\text{OXA-58}}$ was identified in P2 of AOR07 and P3 of AOR07-BL. However, six copies of $bla_{\text{OXA-58}}$ were consecutively arranged in P3 of

Table 3 Information of Chromosome and Plasmid/Extrachromosomal Elements (P1, P2 and P3) in Oral and Bloodstream *A. Baumannii* from the Same Patient

Location	AOR07				AOR07-BL			
	Replicon Type	Length	GC Content	Resistance Genes	Replicon Type	Length	GC Content	Resistance Genes
P1	R3-T3	133,577	41.07%	–	R3-T3	133,579	41.07%	–
P2	R3-T3	90,740	39.87%	$bla_{\text{OXA-58}}$, *msr(E), mph(E), sul2, strA, strB, aac(3)-IId*	R3-T3	87,371	40.17%	*msr(E), mph(E), sul2, strA, strB, aac(3)-IId*
P3	R3-T3	8,571	35.47%	–	R3-T3	31,683	34.03%	$bla_{\text{OXA-58}}$





Sequence ID	*pdif* site: XerD-XerC
D/C_1	ATTTAACATAATGGCTGTTATACGAAAC
D/C_2	ATTTAACATAATGGTGGTTATACGCAAT
D/C_3	ATTTAACATAATGGGCGTTATCCGAAAT

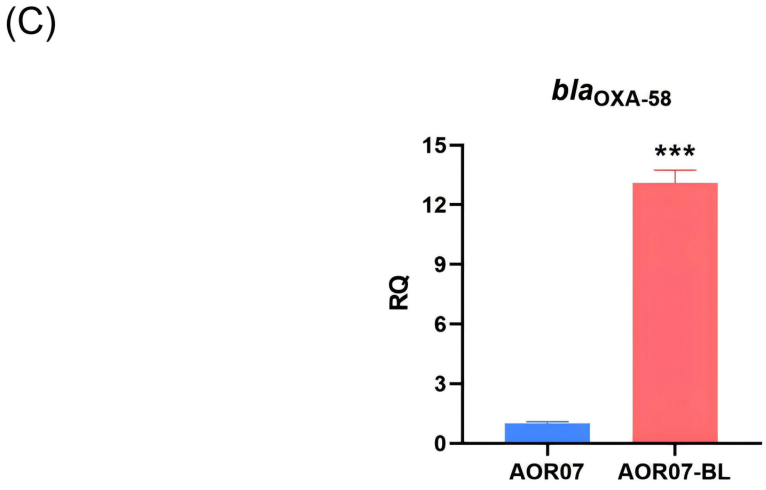


Figure 2 Evolution of plasmid/extrachromosomal elements within the host. (**A**) Changes in the content of plasmids/extrachromosomal elements between oral and bloodstream *A. baumannii* isolates from the same patient. The chromosome is depicted in green. P1, P2 and P3 indicate the plasmids/extrachromosomal elements. The red color indicated the single or multiple copies of ΔIS*Aba3*-bla_{OXA-58}-IS*Aba3*-araC-lysE clusters. The dark blue color indicated the inverted regions. (**B**) Linearized map of regions containing the bla_{OXA-58} resistance determinant module in AOR07-P2 compared to AOR07-BL-P3. Arrows indicate the extent and direction of genes. Vertical bars indicate *pdif* sites. The sequences of the *pdif* sites that flank the bla_{OXA-58} module are shown in the table at the bottom of the figure. The bases highlighted in red are different. (**C**) Relative gene expression of bla_{OXA-58} between oral and bloodstream *A. baumannii* isolates from the same patient (***$p<0.001$).

AOR07-BL, which may be responsible for the high MICs of meropenem and imipenem in AOR07-BL. The genetic environment surrounding bla_{OXA-58} in these two isolates revealed an imperfect IS*Aba3* and an intact copy of IS*Aba3* downstream. Manual inspection of the region surrounding ΔIS*Aba3*-bla_{OXA-58}-IS*Aba3*-araC-lysE in P3 of AOR07-BL revealed that the p*dif* sites (D/C_1, D/C_2 or D/C_3) flanked these genes. The segment containing the bla_{OXA-58} resistance determinant module located between the flanking p*dif* sites was 3824 bp in size, and the flanking sites were in the same orientation (XerD-XerC) (Figure 2B). The transcriptional expression of bla_{OXA-58} was upregulated 13-fold in the AOR07-BL cells (Figure 2C). Amplification and overexpression of bla_{OXA-58} in plasmids explains the increased carbapenem resistance observed in the bloodstream isolate AOR07-BL.

Discussion

Several previous studies based on whole-genome sequencing have suggested that *Acinetobacter* isolates from colonization and infection have similar genetic characteristics, suggesting that colonization is the source of infection.[24–26] *Acinetobacter* colonization is closely related to infection and understanding the characteristics of oral colonization in patients with hematological malignancies is helpful in the prevention and control of nosocomial infections. The proportion of *Acinetobacter* spp. in the oral microbiome is low in the normal population but high in hospitalized patients with hematological malignancies, which may be related to their immunocompromised status and use of multiple antibiotics.[27,28] However, few studies have characterized oral *Acinetobacter* spp. and their genomic associations with subsequent bloodstream infections.

In our study, the oral isolates were mainly *A. baumannii* and not the predominant global clones GC1 and GC2. *Acinetobacter* spp. isolated from the oral cavity were highly susceptible to carbapenems, and only two isolates were resistant to carbapenems. In China, bla_{OXA-23} is the most common resistance gene among carbapenem-resistant *A. baumannii* strains.[29] However, bla_{OXA-23} was not detected in any of the oral or bloodstream isolates included in this study. AOR07 is susceptible to carbapenems despite carrying one copy of bla_{OXA-58}. However, the bloodstream isolate AOR07-BL, which was isolated from the same patient as AOR07, showed carbapenem resistance owing to an increase in the copy number and expression of bla_{OXA-58}. Previous studies have indicated that the overexpression of bla_{OXA-58} driven by IS*Aba3*, is associated with imipenem resistance in a clinical *A. baumannii* isolate.[30] Plasmids harboring carbapenem resistance genes are important contributors to the spread of carbapenem resistance in *A. baumannii* infections.[31] Gene duplication is a major source of genomic evolution.[32] Changes in the plasmid and bla_{OXA-23} copy numbers have been reported during the experimental evolution of *A. baumannii*.[33] Multi-copy bla_{OXA-58} is a source of high resistance to carbapenems in *A. baumannii*.[34] In this study, we found that, after treatment with carbapenems, the isolate from the patient showed increased carbapenem resistance through transfer via XerCD-mediated recombination and an increase in the copy number of bla_{OXA-58} in the plasmids.

Oral and bloodstream isolates from the same patient were closely related, and mutations in *YbiH, Gp2*, and IS*Aba45* were found on the chromosomes during within-host evolution. YbiH is an uncharacterized transcription factor in *Escherichia coli* that belongs to the TetR family. The YbiH operon includes, besides the *ybiH* gene itself, the *ybhG* gene, which encodes an inner membrane protein of unknown function, and *ybhFSR*, which encodes an ABC-type transporter of unknown function. A previous study has shown that the transcription factor YbiH regulates a set of genes that affect the sensitivity of *E. coli* to cefoperazone and chloramphenicol.[35] However, the role of YbiH in *A. baumannii* has not been elucidated. However, few studies have been conducted on phage evolution during host evolution. A recent study found that prophage regions are conserved, and no sequence changes were observed among the three *A. baumannii* strains collected before and during phage treatment.[36] In our study, a frameshift mutation was identified in the phage replication initiation protein Gp2; however, no sequence change was observed in the predicted prophage regions of the two isolates (data not shown). However, the role of prophages in the within-host evolution of *A. baumannii* requires further study.

Our study has some limitations. First, our study included only two isolates from one patient that showed oral-to-blood evolution; therefore, its representativeness was limited. Studies with larger sample sizes are necessary to determine the generalizability of these conclusions. Additionally, the mechanisms underlying within-host evolution of chromosomes and plasmids require further investigation.

Our research demonstrated that the oral *Acinetobacter* spp. might be a source of bacteremia in patients with hematological malignancies. The use of carbapenem antibiotics may have contributed to the plasmid evolution and amplification of the bla_{OXA-58}-bearing *dif* module, leading to the development of carbapenem resistance.

Data Sharing Statement

Complete nucleotide sequences of oral *Acinetobacter* spp. and one *A. baumannii* isolate from the blood were submitted to GenBank under the accession numbers PRJNA789055 and PRJNA868510, respectively. Detailed information is provided in Table S2.

Ethics Approval and Informed Consent

The study complies with the Declaration of Helsinki and this study was reviewed and approved by the Ethical Review Committee of Peking University People's Hospital (No. 2018PHB187). The need for informed consent was waived because the collected medical records and patient information were anonymized for this observational study. The isolates were obtained from patients as part of routine hospital procedure and were leftover specimens after clinical testing.

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Disclosure

The authors have no competing interests to declare that are relevant to the content of this article.

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