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Isolation and Whole-genome Sequence Analysis of the Imipenem Heteroresistant *Acinetobacter baumannii* Clinical Isolate HRAB-85

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ABSTRACT

Objectives: Heteroresistance is a phenomenon in which there are various responses to antibiotics from bacterial cells within the same population. Here, we isolated and characterised an imipenem heteroresistant *Acinetobacter baumannii* strain (HRAB-85).
Methods: The genome of strain HRAB-85 was completely sequenced and analysed to understand its antibiotic resistance mechanisms. Population analysis and multilocus sequence typing were performed.
Results: Subpopulations grew in the presence of imipenem at concentrations of up to 64 μg/mL, and the strain was found to belong to ST208. The total length of strain HRAB-85 was 4,098,585 bp with a GC content of 39.98%. The genome harboured at least four insertion sequences: the common IS*Aba1*, IS*Aba22*, IS*Aba24*, and newly reported IS*Aba26*. Additionally, 19 antibiotic-resistance genes against eight classes of antimicrobial agents were found, and 11 genomic islands (GIs) were identified. Among them, GI3, GI10, and GI11 contained many ISs and antibiotic-resistance determinants.
Conclusions: The existence of imipenem heteroresistant phenotypes in *A. baumannii* was substantiated in this hospital, and imipenem pressure, which could induce imipenem-heteroresistant subpopulations, may select for highly resistant strains. The complete genome sequencing and bioinformatics analysis of HRAB-85 could improve our understanding of the epidemiology and resistance mechanisms of carbapenem-heteroresistant *A. baumannii*.

Introduction

Acinetobacter baumannii is an opportunistic pathogen with increasing relevance in a variety of nosocomial infections, such as ventilator-associated pneumonia, central line-associated bloodstream infections, urinary tract infections, surgical-site infections, and other types of wound infections, particularly in immunocompromised patients or in patients in the intensive care unit (ICU) (Peleg et al., 2008). The ability of these organisms to accumulate diverse mechanisms of resistance limits the available therapeutic agents, makes the infection difficult to treat, and is associated with a greater risk of death (Kim et al., 2014). Although carbapenems, such as imipenem (IPM) and meropenem (MPM), are recommended as last-resort antimicrobial therapies against *A. baumannii* infections, carbapenem resistance in *A. baumannii* (CRAB) has been emerging in many parts of the world, and the resistance rate has increased to about 30% or higher (Pogue et al., 2013).

Previously, resistance to carbapenems and other antibiotics in *A. baumannii* or other bacteria was thought to be homogeneous within a culture. However, the phenomenon of heteroresistance (also known as “heterogeneous antibiotic resistance” or “heterogeneous resistance”) has been observed in staphylococci and has been applied to describe the phenomenon in infections where

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subpopulations of seemingly isogenic bacteria exhibit a range of susceptibilities to a particular antibiotic (El-Halfawy and Valvano 2015). This phenomenon is more common in gram-positive staphylococci, particularly in the vancomycin-susceptible *Staphylococcus aureus* (VSSA) and vancomycin-intermediate *Staphylococcus aureus* (VISA) (Hiramatsu et al., 1997; Wong et al., 1999; Marchese et al., 2000; Khosrovaneh et al., 2004). Very recently, this phenomenon was found to occur in some gram-negative bacterium, such as *Escherichia coli* (Baquero et al., 1985), *Pseudomonas aeruginosa* (Pournaras et al., 2007; Ikonomidis et al., 2008; Hermes et al., 2013), *Klebsiella pneumoniae* (Pournaras et al., 2010; Tato et al., 2010), and *Acinetobacter baumannii* (Pournaras et al., 2005; Ikonomidis et al., 2009; Savini et al., 2009; Lee et al., 2011). Since more resistant subpopulations may be selected during antimicrobial therapy, these subpopulations may lead to treatment failure and persistent infection with increased mortality rates. However, this phenomenon, which further complicates the study of antibiotic resistance, is poorly characterised, and its clinical relevance is uncertain.

Accordingly, in this study, we isolated and characterised an IPM heteroresistant *A. baumannii* strain and aimed to improve our understanding of its antibiotic resistance mechanisms by sequencing and analysing the complete genome of this strain.

Methods

Bacterial isolates and antimicrobial susceptibility testing

The *A. baumannii* strain HRAB-85 was isolated from the sputum sample of an 83-year-old female in the 307th Hospital of PLA in Beijing, China, who had severe pneumonia and chronic obstructive pulmonary disease (COPD). Five days after the administration of Imipenem-Cilastatin, the consecutive derived *A. baumannii* strain lost susceptibility to Imipenem rapidly. The strain was identified using the Vitek 2 System (BioMerieux Vitek, Inc., Hazelwood, MO, USA) and cultured at 37 °C in Luria-Bertani broth overnight. The minimum inhibitory concentration (MIC) was determined in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI) and was confirmed by the Vitek 2 System with 24 antibiotics: ampicillin, amikacin, ampicillin/sulbactam, piperacillin, piperacillin/tazobactam, cefazolin, cefuroxime, cefuroxime ester, cefotetan, ceftazidime, aztreonam, IPM, MPM, gentamycin, ciprofloxacin, ceftriaxone, cefepime, cotrimoxazole, levofloxacin, nitrofurantoin, polymyxin B, tobramycin, sulfamethoxazole, and tigecycline. *Pseudomonas aeruginosa* ATCC 27853 was used as a control.

Population analysis and stability of the heterogeneous phenotype

Population analyses were performed by the methods described by Ikonomidis et al., (2009) and Pournaras et al., (2010), with some modifications. Briefly, subpopulations were yielded by spreading approximately 10^8 bacterial CFU on Mueller-Hinton agar plates with IPM in serial dilutions at concentrations ranging from 0.5 to 64 mg/L and incubating the plates for 48 h. The analysis was performed three times, and the mean numbers of viable CFU were estimated and plotted on a semilogarithmic graph. *P. aeruginosa* ATCC 21636 was used as a control for the population analysis experiments. The frequency of appearance of heteroresistant subpopulations in the presence of the highest drug concentration was calculated by dividing the number of colonies that grew on the antibiotic-containing plate by the colony counts from the same bacterial inoculum that grew on antibiotic-free plates (Pournaras et al., 2010). The stability of IPM MICs for three distinct colonies grown at the highest drug concentration was determined by agar dilution after seven daily subcultures in antibiotic-free medium.

Multilocus sequence typing (MLST)

The MLST scheme described by Bartual was performed according to the *Acinetobacter baumannii* MLST (Oxford) database (http://pubmlst.org/abaumammii) (Bartual et al., 2005). The assembled sequences of seven housekeeping gene sequences (*gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi*, and *rpoD*) were aligned using BLAST, and the aligned sequences were then extracted by comparing them to allele profiles in the *A. baumannii* MLST (Oxford) database.

DNA extraction, whole-genome sequencing, and annotation

The genomic DNA of HRAB-85 was extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Total DNA was then subjected to quality control by agarose gel electrophoresis and quantified by Qubit2.0 fluorometric quantification (Thermo Fisher Scientific, MA, USA). The genome of HRAB-85 was sequenced by Single Molecule, Real-Time (SMRT) technology. Sequencing was performed at the Beijing Novogene Bioinformatics Technology Co., Ltd. SMRT Analysis 2.3.0 was used to filter low quality reads, and the filtered reads were assembled to generate one contig without gaps.

The HRAB-85 genome was assembled by using GeneMarkS (Besemer et al., 2001) (http://topaz.gatech.edu/). Gene prediction was performed with an integrated model combining the GeneMarkS generated (native) and heuristic model parameters. tRNA genes were predicted with tRNAscan-SE (Lowe and Eddy 1997), rRNA genes were predicted with rRNAmmer (Lagesen et al., 2007), and sRNAs were predicted by BLAST against the Rfam database (Gardner et al., 2009). Repetitive sequences were predicted using RepeatMasker (Saha et al., 2008) (http://www.repeatmasker.org/). Tandem repeats were analysed using Tandem Repeat Finder (Benson 1999) (http://www.pathogenomics.sfu.ca/islandviewer/resources.php). Functional classification was performed by aligning predicted proteins to the Clusters of Orthologous Groups (COG) database (Tatusov et al., 1997; Tatusov et al., 2003). All predicted genes were compared to a nonredundant (nr) protein database in NCBI using BLASTX, with E values of $\leq 1e^{-5}$ and identity of $\geq 30\%$. Metabolic pathways were analysed by a single-directional best-hit method on the KEGG web server (http://www.genome.jp/kegg/).

The mobile genetic elements in the HRAB-85 genome sequences were detected by the following online tools and/or open-access databases and manual examinations: MobilomeFINDER for tRNA/tmRNA gene-related genomic islands (GIs) (Ou et al., 2007), IslandViewer for the island-like regions (Dhillon et al., 2013), and IS Finder for insertion sequence (IS) elements (Siguier et al., 2006). PHAST (Zhou et al., 2011a, 2011b) was used for prophage prediction (http://phast.wishartlab.com/), and CRISPRFinder (Grissa et al., 2007) was used for CRISPR identification.

Phylogenetic tree

The phylogenetic tree was generated by TreeBeST (Nandi et al., 2010) using the method of PhyML, with 1,000 bootstraps for orthologous genes detected from gene family analysis. Genomic data used in the phylogenetic tree were downloaded from the NCBI database, including complete genome sequences of the *A. baumannii* isolates AB0057 (CP001182.1), AB307-0294 (CP001172.1), AYE (CU459141.1), ACICU (CP000863.1), XH386 (CP010779.1), MDR-TJ (CP003500.1), MDR-ZJ06 (CP001937.1), TCDC-AB0715 (CP002522.2), TYTH-1 (CP003856.1), ATCC 17978