Vietnam Journal of Biotechnology **23**(1): 125-136, 2025. DOI: 10.15625/vjbt-21792

INSIGHTS INTO ANTIMICROBIAL RESISTANCE GENOTYPE AND POTENTIAL VIRULENT TRAITS OF AN EXTENSIVELY DRUG-RESISTANT *Acinetobacter baumannii* SEQUENCE TYPE ST2

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Received: 26.10.2024
Accepted: 09.01.2025

ABSTRACT

Carbapenem-resistant *Acinetobacter baumannii* has been ranked as the priority 1 pathogen and is urgently needed for the development of new antimicrobials. Understanding the genetic determinants associated with antibiotic resistance and virulence can help to control the resistant evolution, decide on treatment and have appropriate prevention methods. The present study aimed to characterize the genomic features of an extensively drug-resistant (XDR) *A. baumannii* sequence type ST2. Phenotypic-drug susceptibility testing was conducted against 28 antibiotics. Whole genome sequencing was performed, followed by an analysis of Clusters of Orthologous Genes (COG), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, multilocus sequence typing (MLST), genetic determinants associated with resistance and virulence, and mobile genetic elements. *A. baumannii* VD610 was resistant to 26 antibiotics and identified as an extensively antibiotic-resistant phenotype. The genome size of *A. baumannii* VD610 was 3,765,945 bp, comprising a circular chromosome and two plasmids. The COG annotation identified 3012 genes that could be classified into 22 functional categories. There were 1644 genes mapped to the KEGG pathways. This strain was assigned to the sequence type ST2 by the Pasteur MLST scheme, and harbored 32 antibiotic-resistant genes responsible for aminoglycosides, β-lactams, quinolones, phenicols, tetracyclines, fosfomycins, antifolates, erythromycin, and streptogramin resistance, in which *blaOXA-23* and *blaOXA-66* are responsible for carbapenem resistance. The virulome of *A. baumannii* VD610 consists of 36 virulence genes which are crucial for its pathogenicity. Our findings provide the genetic features of Vietnamese XDR *A. baumannii* sequence type ST2, which can be a reference for further study.

Keywords: *Acinetobacter baumannii*, extensively drug resistance, antibiotic-resistant genes, sequence type ST2, virulence genes.

INTRODUCTION

Acinetobacter baumannii, one of the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species), is a highly opportunistic pathogen and commonly causes nosocomial-hospital acquired infection (Santajit and Indrawattana, 2016). The pathogen has now evolved multidrug-resistant phenotypes resistant to the best available antibiotics for treating multi-drug resistant (MDR) bacteria including third-generation cephalosporins and carbapenems recognized as last-resort antibiotics (Hamidian and Nigro, 2019). In 2017, the World Health Organization (WHO) published a list of the critical pathogens that need developing new antibiotics in which carbapenem-resistant *A. baumannii* is classified as priority 1. It has been reported that different genotypes of MDR *A. baumannii* strains have acquired antibiotic resistance independently and followed by international spread (Zarrilli *et al*., 2013). Thus, the lack of a vaccine against *A. baumannii* and the increasing global prevalence of carbapenems resistance underline the need to monitor drug-resistant genetic determinants of this pathogen in all countries (Hamidian and Nigro, 2019; Zarrilli *et al*., 2013).

Although *A. baumannii* possesses various natural antibiotic resistance mechanisms, this pathogen is frequently acquired by antibiotic-resistant determinants mediated by mobile genetic elements (MGEs) (Hamidian and Nigro, 2019; Zarrilli *et al*., 2013). *A. baumannii* could acquire a large amount of foreign DNA, which could play a role in antimicrobial resistance and pathogenesis (Leal *et al*., 2020). Genetically, *A. baumannii* naturally possesses various efflux pump-coding genes that are associated with resistance to antibiotic groups. *A. baumannii* often possesses a chromosomal *blaOXA-51* or *blaOXA-51*-like gene (for oxacillinase) and an ADC (Acinetobacter-derived cephalosporinase, AmpC-type β-lactamase), which can hydrolyze a wide range of β-lactam antibiotics (Hamidian and Nigro, 2019; Zarrilli *et al*., 2013). Nevertheless, these enzymes have a low hydrolytic activity towards carbapenems – the last antibiotic to treat multidrug-resistant Gram-negative bacteria. Genes *blaOXA-23*, *blaOXA-58* and *blaOXA-143* were commonly detected in carbapenem-resistant *A. baumannii* strains. In addition, the presence of metallo-carbapenemases such as *blaNDM*, *blaIMP*, *blaKPC* and *blaVIM* was also reported in carbapenems-resistant *A. baumannii* (Hamidian and Nigro, 2019; Zarrilli *et al*., 2013).

Whole genome sequencing (WGS) technologies and advances in bioinformatic tools have provided insight into antimicrobial resistance and virulence determinants in bacterial pathogens. These tools allow screening and phylogenomic investigations of epidemic strains, which are important for understanding the transmission and epidemiology of infectious diseases in each nation (Jauneikaite *et al*., 2023; Popovich and Snitkin, 2017). In Vietnam, *A. baumannii* is a major pathogen associated with nosocomial infections, resulting in significant health and economic burdens. The prevalence of carbapenem-resistant *A. baumannii* was found to be up to 78% in Vietnam (Le *et al*., 2015). Although several studies have sporadically reported nevertheless, molecular characteristics of carbapenem-resistant *A. baumannii* strains from many Vietnamese healthcare settings were not completely investigated (Hoang *et al*., 2019; Le *et al*., 2015; Tada *et al*., 2015; Nguyen *et al*., 2017). Here, we conducted the whole genome sequencing of a Vietnamese carbapenem-resistant *A. baumannii* strain VD610 sequence type ST2 to identify the resistome and virulome. This information is important for better understanding the resistance evolution and pathogenicity of MDR *A. baumannii* sequence type ST2 in Vietnam.

MATERIALS AND METHODS

Bacterial isolation

In the framework of the Drug Resistance in South East Asia Project (DRISA), a bacterial strain *A. baumannii* VD610 isolated from a patient in Viet Duc Hospital, Hanoi, was kindly provided by the Laboratory of Antimicrobial Resistance, National Institute of Hygiene and Epidemiology, Hanoi, Vietnam. This strain was identified as *A. baumannii* by using a VITEK mass spectrum system (Biomerieux, USA). The bacterial stock was prepared in a Tryptic Soy Broth (TSB, Sigma) medium supplied with 50% glycerol and stored at -30^{o}C for further experiments.

Antibiotic susceptibility testing

The antimicrobial susceptibility testing was performed based a disk diffusion assay against 28 antibiotics (SirScan/i2a Diagnostics, France) including Aminoglycosides (amikacin (30 µg), gentamicin (10 µg), tobramycin (10 µg), netilmicin (10 µg)), Antifolates (trimethoprim & sulphamethoxazole (25 µg)), *β*-lactams (ampicillin (10 µg), amoxicillin & clavulanic acid (30 µg), temocillin (30 µg), piperacillin (30 µg), piperacillin & tazobactam (36 µg), ticarcillin (75 µg), ticarcillin & clavulanic acid (85 µg), ceftazidime (10 µg), cefotaxime (10 µg), cefepime (30 µg), cephalexin (30 µg), cefoxitin (30 µg), cefpodoxime proxetil (10 µg), aztreonam (30 µg), imipenem (10 µg), ertapenem (10 µg)), Quinolones (nalidixic acid (30 µg), ciprofloxacin (5 µg), ofloxacin (5 µg), levofloxacin (5 µg)), tetracyclines (tetracycline (30 µg)), Phenicols (chloramphenicol (30 µg)), and Phosphonic acids (fosfomycin (200 µg)). *Escherichia coli* ATCC 29522 was included as a control for all experiments. The results were interpreted based on the guidelines of the Clinical and Laboratory Standards Institute (CLSI) version M100, 2020.

DNA isolation and whole-genome sequencing

Total genomic DNA of *A. baumannii* strain VD610 was extracted using the Bacterial Genomic DNA Isolation Kit (Norgen Biotek Corp., Thorold, Ontario, Canada) following the manufacturer's protocol. The quality and quantity of DNA were measured by a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and visualized on an agarose gel electrophoresis. Then, the genomic DNA of *A. baumannii* VD610 was sequenced on the BGISEQ-500 platform in a paired-end 150 bp mode at Beijing Genomics Institute (BGI), China.

Genome analysis and analysis

The raw reads were firstly checked by FastQC v.2.0, followed by trimming with Trimmomatic v.0.39 (Bolger *et al*., 2014) and *de novo* assembly using SPAdes v.3.14.1 (Bankevich *et al*., 2012). The results were compared with the genome of a reference strain, *A. baumannii* AB30 by QUAST-5.0.2 (Gurevich *et al*., 2013). The genomic annotation was conducted for assembled reads using a Bakta tool (Schwengers *et al*., 2021). Subsequently, a Cluster of Orthologous Genes (COGs) analysis was conducted for classifying prokaryote protein sequences according to functional categories using COGclassifier v.10.5 (https://github.com/moshi4/COGclassifier/). Functional and biological systems were analyzed using The Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa and Goto, 2000). The sequence type of the *A. baumannii* strain VD610 was identified by multilocus sequence typing analysis (MLST) of seven housekeeping genes, *cpn60, gltA, gpi, gyrB, recA, rpoD* and *gdhB*, following the Pasteur scheme, database on PubMLST (Jolley *et al*., 2018). The draft genome sequence of *A. baumannii* strain VD610 was registered on GenBank, NCBI (Bioproject: PRJNA857185, BioSample: SAMN29620987).

Detection of genetic determinants associated with resistance and virulence

Antibiotic-resistant genes of *A. baumannii* strain VD610 were detected using ResFinder 4.0 (Bortolaia *et al*., 2020) and CARD-RGI 5.1.0 (Alcock *et al*., 2020). Virulence genes were detected using the Virulent Factor Database (VFDB) (Chen *et al*., 2005). The chromosome of this strain was visualized using Proksee (Grant *et al*., 2023). Mobile genetic elements carrying antibiotic-resistant genes were also predicted using MobileElementFinder (Johansson *et al*., 2021).

RESULTS AND DISCUSSION

Phenotypic antibiotic-resistant profile

A. baumannii VD610 exhibited an extensively drug resistant (XDR) phenotype with resistance to 26 antibiotics tested except for levofloxacin and aztreonam (Table 1). In Vietnam, *A. baumannii* is a major pathogen causing hospital-acquired infections, particularly in intensive-care units. Although, the proportion of MDR *A. baumannii* was often very high (50-92%) (Diep *et al*., 2023; Hoang *et al*., 2019; Nguyen *et al*., 2017), the prevalence of XDR *A. baumannii* phenotypes is unknown in Vietnam. Extensive resistance to carbapenem antibiotics is considered to be a sign of XDR bacteria, and carbapenem-resistant *A. baumannii* is now causing serious problems worldwide. Previous studies have reported that the prevalence of XDR phenotypes of *A. baumannii* is very high in European countries (64.6%) and Asian and American countries (73.1–80.6%) (Hu *et al*., 2016; Mirzaei *et al*., 2020; Nowak *et al*., 2017). XDR *A. baumannii* infections are very difficult to treat, resulting in mortality rates. Developing new antibacterial drugs and evaluating their clinical performance will be essential to provide new treatments for XDR *A. baumannii* infections in Vietnam.

Table 1. Phenotypic antibiotic-resistant profile of *A. baumannii* VD610

No.	Antibiotic group	Antibiotic	ZOI (mm)	Result
1	Aminoglycosides	Gentamicin	0	R
2		Tobramycin	0	R
3		Amikacin	0	R
4		Netilmicin	0	R
5		Piperacillin	0	R

6		Ampicillin	0	R
7		Temocillin	0	R
8		Ticarcillin	0	R
9		Ceftazidime	0	R
10	β-lactams (Penicillins, Cephalosporins, Carbapenems, Monobactams)	Cefotaxime	0	R
11		Cefepime	7	R
12		Cephalexin	0	R
13		Cefoxitin	0	R
14		Cefpodoxime proxetil	0	R
15		Imipenem	11.5	R
16		Ertapenem	0	R
17		Aztreonam	16	S
18	β-lactams combinations	Piperacillin & tazobactam	8	R
19		Ticarcillin & clavulanic acid	0	R
20		Amoxicillin & clavulanic acid	0	R
21	Quinolones	Nalidixic acid	0	R
22		Ciprofloxacin	7	R
23		Ofloxacin	7	R
24		Levofloxacin	19	S
25	Antifolates	Trimethoprim & sulfamethoxazole	10	R
26	Tetracycline	Tetracycline	0	R
27	Phenicol	Chloramphenicol	14	R
28	Fosfomycin	Fosfomycin	0	R

Interpretation: R - Resistant, S - Sensitive, ZOI: zone of inhibition.

Genomic features of *A. baumannii* strain VD610

The draft genome of *A. baumannii* strain VD610 was approximately 3.77 Mb in length with a GC content of 38.9% (Table 2). The genome consists of 3,510 coding sequences, in which 3,375 genes are encoded for proteins with functional assignments and 135 hypothetical proteins.

Table 2: Genomic features of the genome of *A. baumannii* strain VD610

Genomic features	Values
Genome size	**3,765,945 bp**
G+C content	38.9%
Number of coding sequences (CDSs)	3,510

Protein with functional assignments	3,375
Hypothetical proteins	135
Protein with KEGG assignments	1,922
Genes assigned to COGs	2,076
Number of tRNA	60
Number of rRNA	4
N50 value	140,356 bp
MLST	ST2
Plasmids	2

Among 3,510 predicted coding sequences, 3,012 genes were classified into 22 functional groups based on COGs analysis (Figure 1). The majority COG category was associated with metabolism pathways (8 functional groups, 55.3%), followed by cellular processes and signaling (8 functional groups, 21.7%), and information storage and processing (4 functional groups, 23.0%). The main functional groups were E (amino acid transport and metabolism, n = 475, 17.5%), K (transcription, n = 251, 9.3%), J (translation, ribosomal structure and biogenesis, n = 233, 8.6%), and I (lipid transport and metabolism, n = 227, 8.4%). In addition, 306 (general and unknown functions, 11.3%) genes were poorly characterized, which are subjected to further analysis.

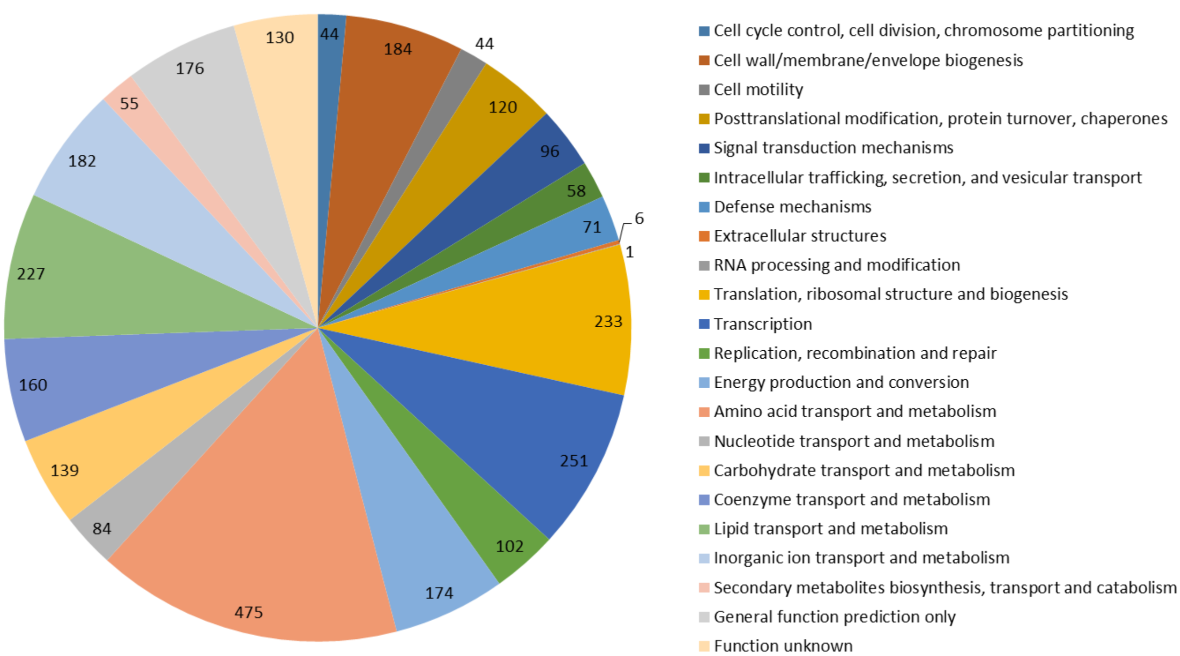

Figure 1. Predicted functional genes in *A. baumannii* strain VD610 based on COG analysis

Analysis of KEGG annotations revealed 1644 functional categories corresponding to 19 metabolic pathways. The main protein families: signaling and cellular processes (n = 261), protein families: genetic information processing (n = 253), carbohydrate metabolism (n = 168), carbohydrate metabolism (n = 161) and amino acid metabolism (n = 139). Nevertheless, 278 genes were unclassified (Figure 2). Furthermore, *A. baumannii* VD610 belonged to a sequence type ST2 under the Pasteur MLST scheme. This genotype is classified as international clone II and is widely distributed worldwide, including in Asian and Southeast Asian countries (Baleivanualala *et al*., 2023; Khuntayaporn *et al*., 2021; Kumkar *et al*., 2022). A recent study reported that *A. baumannii* ST2 was associated with intra- and inter-hospital transmission (Baleivanualala *et al*., 2023; Baleivanualala *et al*., 2024). Moreover, *A. baumannii* sequence type ST2 is often associated with XDR phenotypes and high virulence, resulting in high mortality (Morgado *et al*., 2023; Upmanyu *et al*., 2022). Therefore, genomic surveillance is necessary to better monitor the epidemiology and evolution of XDR *A. baumannii* strains in Vietnam.

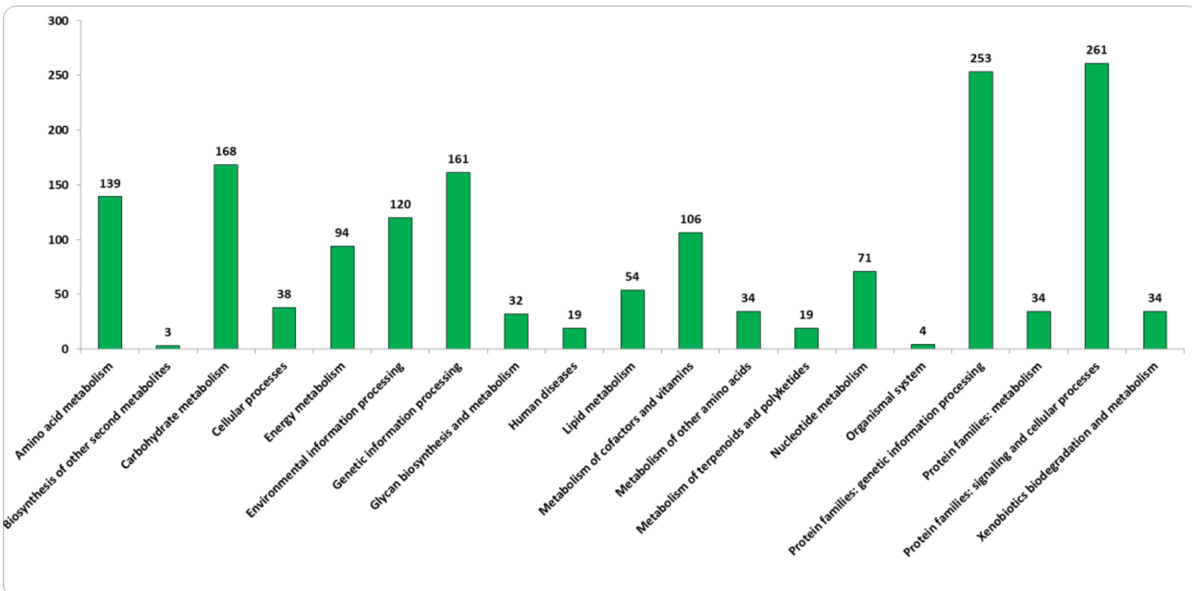

Figure 2. KEGG function of predicted genes found in *A. baumannii* VD610

Resistome and virulomes in *A. baumannii* VD610

A. baumannii VD610 possessed 32 antibiotic-resistant genes (Figure 3), of which the majority were antibiotic efflux pumps, including 13 genes that belonged to RND antibiotic efflux pumps (*ade*A, *ade*B, *ade*C, *ade*F, *ade*G, *ade*H, *ade*L, *ade*I, *ade*K, *ade*R, *ade*S, *ade*J and *ade*N), 5 genes encoded for MFS antibiotic efflux pumps (*aba*F, *aba*Q, *amv*A, *tet*R and *tet*B), 1 gene encoded MATE transporter (*abe*M), 1 gene for SMR antibiotic efflux pump (*abe*S) and 1 gene for ABC-F ATP-binding cassette ribosomal protection protein (*msr*E) (Abdi *et al*., 2020; Verma *et al*., 2021). The presence of these genes confirmed the resistance to aminoglycosides, β-lactams, quinolones, antifolates, phenicols and fosfomycin in *A.*

baumannii VD610. In addition, this strain acquired eleven antibiotic-resistant genes responsible for resistance to certain antibiotics. Specifically, *A. baumannii* VD610 carried *aph(3")-Ib*, *aph(6)-Id* and *armA*, which are responsible for resistance to aminoglycosides (gentamicin, tobramycin, amikacin and netilmicin) (Tada *et al*., 2020). This strain was resistant to almost *β*-lactams and *β*-lactamase inhibitors by possessing *blaADC-25*, *blaTEM-1D*, *blaOXA-23* and *blaOXA-66* (Hamidian and Nigro, 2019; McCarthy *et al*., 2021). It is well known that TEM-1 and ADC-25 *β*-lactamases are associated with resistance to penicillin, cephalosporin, and *β*-lactam/*β*-lactamase inhibitor combinations (McCarthy *et al*., 2021). The OXA-23 and OXA-66 belonged to the class D carbapenem-hydrolyzing oxacillinases that are responsible for carbapenem resistance (Evans and Amyes, 2014; McCarthy *et al*., 2021). Finally, *A. baumannii* VD610 was resistant to lincosamides, streptogramins and oxazolidinones (*mphE, msrE*), sulfonamides (*sul2*) and tetracycline (*tetB*). These findings are in agreement with the phenotypic XDR profile of *A. baumannii* VD610. Notably, 31 antibiotic-resistant genes were located on the chromosome (Figure 2), while only *sul*2 was detected on the plasmid. In agreement with previous studies, *blaOXA-23* responsible for carbapenem resistance was predominant among MDR and XDR *A. baumannii* strains in several regions worldwide. The co-occurrence of *blaTEM-1D*, *blaOXA-23* and *blaOXA-66* on its chromosome suggested that this clone acquired these genes under high drug selection pressure for a long time. Furthermore, *aph(3")-Ib* and *aph(6)-Id*) (aminoglycosides resistance), and *tet*B (tetracycline resistance) were found along with an insertion sequence ISVsa3 (family IS91), while *arm*A, *mph*E and *msr*E were detected within an insertion sequence ISAba24 (family IS66), suggesting that *A. baumannii* VD610 could acquire these ARGs through HGTs (Baleivanualala *et al*., 2023; Baleivanualala *et al*., 2024; McCarthy *et al*., 2021). Thus, MGEs insertion sequences play a crucial role in the propagation of ARGs among bacterial communities.

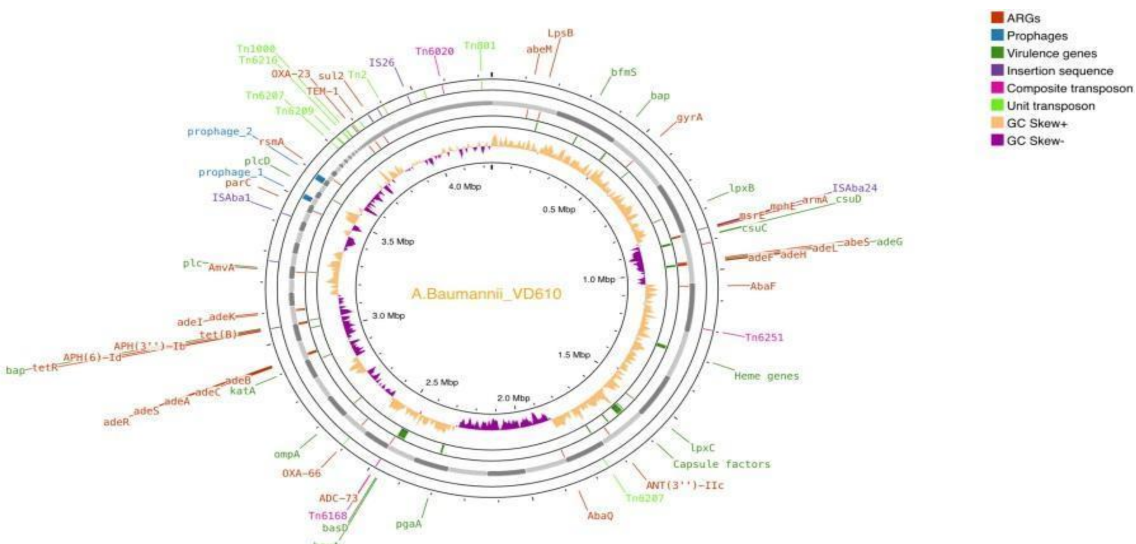

Figure 3. Distribution of antibiotic-resistant genes (ARGs), virulence genes and mobile genetic elements on the chromosome of *A. baumannii* VD610

A total of 36 virulence factors were identified in the genome of *A. baumannii* VD610 (Figure 3). The major virulence determinants include adherence (*omp*A), biofilm formation (*ade*FGH, CsuDE), the poly-β-1,6-N-acetylglucosamine polysaccharide (*pga*ABC), phospholipase enzyme (*pl*C, *pl*D), immune evasion (LPS and capsule), iron uptake (Heme genes), quorum sensing regulation (*aba*R, *bfm*S), serum resistance (*pbp*G), ATP-dependent Clp protease proteolytic subunit (*clp*P), aldehyde dehydrogenase (*ald*A), and stress adaptation (*kat*A) (Chen *et al*., 2005; Kumkar *et al*., 2022). These virulence factors play a crucial role in colonization of host niches to cause diseases. Notably, the presence of genes involved in biofilm formation is also responsible for antibiotic resistance in clinical *A. baumannii* VD610. In *A. baumannii*, biofilm formation is regulated by several genes, including *omp* and *csu*A/BABCDE and the *aba* quorum sensing system. Furthermore, biofilm-mediated *A. baumannii* infections are associated with medical devices, and they are extremely difficult to treat. Therefore, understanding the regulatory mechanisms of biofilm formation in *A. baumannii* may have a potential strategy to control the transmission and emergence of MDR strains in healthcare settings.

CONCLUSION

The present study reported *A. baumannii* strain VD610 belonged to sequence type ST2 and exhibited extensively drug-resistant phenotypic and genotypic profiles. Markedly, the co-existence of *blaOXA-23, blaOXA-66* and *bla-TEM-1* in the chromosome of *A. baumannii* VD610 underlines the resistance acquisition is very dynamic. This strain possessed insertion sequences carrying antibiotic-resistant genes which underline the role of mobile genetic elements in the propagation and transmission of ARGs in the bacterial community. Nevertheless, more comprehensive studies on the evolutionary relation can potentially reveal new insight into the antibiotic resistance and pathogenic mechanisms of *A. baumannii* ST02 for better control of the dissemination and transmission of this pathogen in healthcare settings and community.

ACKNOWLEDGMENTS

This research was financially supported by MICH project, Emerging Research Group of USTH, 2024 - 2026. We would like to thank LMI DRISA, IRD and NIHE for their support.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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