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The role of universal stress protein Usp1413 in meropenem adaptive resistance and environmental stress responses in *Acinetobacter baumannii*

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ABSTRACT

Although various mechanisms of carbapenem-resistance have been identified in the nosocomial pathogen *Acinetobacter baumannii*, the critical process of resistance evolution and the factors involved in are not well understood. Herein, we identified a universal stress protein Usp1413 which played an important role in adaptive resistance of *A. baumannii* to meropenem (MEM). Based on RNA-Seq and genome sequencing, Usp1413 was not only one of the most downregulated USPs, but also the bare one having mutation of tyrosine and glycine inserted at the site of 229-230 (YG229-230) under the stimulation of MEM. Deletion of Usp1413 resulted in increased MEM resistance. In addition, Usp1413 affected the bacterial abilities of biofilm formation and swarm motility, as well as helped *A. baumannii* response to various environmental stresses. These effects of Usp1413 were achieved by regulating its interaction proteins, within the functions of YigZ family protein, acetyltransferase, and SulP family inorganic anion transporter. The insertion mutation of YG229-230 influenced both the expression of interaction proteins and the phenotypes of bacteria. Finally, the promotor region of Usp1413 was convinced by point mutations. Overall, our findings identified the universal stress protein Usp1413 as a contributor involved in MEM adaptive resistance and responded to numerous environmental stresses. This study provides novel insights into the mechanism of universal stress proteins in participating antibiotic resistance, and affords a potential target for controlling drug resistance development in *A. baumannii*.

1. Introduction

Acinetobacter baumannii is a significant nosocomial pathogen that can persist in stressful environments, such as desiccation and disinfection (Harding et al., 2018). It can be spread by air, water droplets, the peeling skin of colonized patients, and the hands of hospital workers (Raro et al., 2017). This microorganism can cause many kinds of infections including pneumonia, skin and soft tissue infections, wound infections, bacteremia, endocarditis, urinary tract infections (UTIs), and meningitis (Ibrahim et al., 2021).

Growing cases of *A. baumannii* infections are reported world widely due to its strong ability of obtaining drug resistance. In the last few decades, the rapid accumulation of resistance determinants in this bacterium to multiple classes of antibiotics has resulted in the less effective control by routine antimicrobials. Thus, carbapenems are recommended for the treatment of multidrug-resistant *A. baumannii* (MDRAB) due to their good activity and low toxicity. However, increasing emergence of resistance to carbapenems challenged this therapeutic option (Evans et al., 2013).

Several kinds of carbapenem resistance mechanisms in *A. baumannii* have been elucidated, including production of carbapenemases, alterations of outer membrane proteins, changes of drug action sites, overexpression of active efflux pumps, and formation of biofilms (Ibrahim, 2019; Bamunuarachchi et al., 2021). Since motility is recognized as a key factor in surface adherence and biofilm formation, it can also exacerbate the spread of infections associated with medical devices and

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promote the emergence of antibiotic resistance (Jeong et al., 2024). Whereas, the process that bacteria adapt to the stress of antibiotics provides a requisite for the development of the above mechanisms is unclear.

Adaptive resistance is a special and important form that is defined as resistance to one or more antibiotics induced by specific environmental signals, such as stress or subinhibitory level of antibiotics. Bacteria respond rapidly to antibiotic challenges by modulating gene expressions and can revert to the original state without long lasting signals. While, the increase in resistance may not fully recover after the removal of stimuli, thus resulting in a gradual increase in the minimal inhibitory concentration (MIC) over time. Therefore, adaptive resistance is proposed to facilitate the development of more effective and permanent mechanisms of resistance (Christaki et al., 2020).

Universal stress proteins (USPs) are widely spread proteins in nature. In the Pfam classification, USPs belong to the PF00582 superfamily (COG0589) and are present in a diverse set of organisms from archaea and bacteria to fungi and plants (Tkaczuk et al., 2013). These proteins physiologically function in regulations of cell growth and development, ion scavenging, cellular mobility and hypoxia responses. In addition, they are involved in cellular responses to abiotic and biotic stresses that include, but not limited to, heat shock, acid and high salinity environments, oxidants, DNA and macromolecular damages, nutrient starvation, uncouplers, pH and antibiotic stresses (Masamba and Kappo, 2021). Importantly, it has been shown that the expression of USPs is altered under the stress of antibiotics, and further affect the transcriptional process of bacteria (Kvint et al., 2003).

In this study, USPs of *A. baumannii* were found responding to the stress of meropenem (MEM), in which Usp1413 also showed genetic mutation after long-lasting MEM stimulation. Therefore, the function of adaptive resistance of Usp1413 was revealed. Its more activities involved in environmental stresses along with the regulation mechanisms were also investigated.

2. Materials and methods

2.1. Bacterial isolates and growth conditions

The carbapenem-susceptible *A. baumannii* strain AB5116 was used as a representative local clinical sensitive strain (whole genome accession number CP091173) (Han et al., 2022). Moreover, the serial stimulation method was used to obtained an MEM-resistant isolate AB5116MR (whole genome accession number JBEQCQ000000000) similar to the previous report (Gullberg et al., 2011), with some modifications. In brief, a single colony of AB5116 was cultured in LB broth supplemented with 0.5 × MIC of MEM (0.06 μg/mL; Aladdin, Shanghai, China), and incubated at 37 °C for 12 h. Then, the bacterial culture was 1:100 diluted into fresh LB broth containing the same concentration of MEM, followed by another 12 h incubation. Passages were continued, and the MIC was tested every two days from the colonies plating on LB agar medium. Upon the rise of MIC, a new 0.5 × MIC was executed accordingly until the MEM MIC reached to 32 μg/mL. *Escherichia coli* DH5α was obtained from TIAGEN Biotech Co. Ltd. (Beijing, China). Bacteria were cultured using Luria-Bertani (LB, Beijing Land Bridge Technology Co. Ltd., Beijing, China) medium, and the MIC testing was performed using Mueller-Hinton (MH, Beijing Land Bridge Technology Co. Ltd.) broth. For the selection of mutant strains, kanamycin (Aladdin) or carbenicillin (Aladdin) was supplemented in the medium at a final concentration of 50 μg/mL or 100 μg/mL, respectively. The bacteria were cultured at 37 °C and stored at -80 °C in LB broth with 20 % glycerol.

2.2. RNA extraction

For the purpose of identifying the expression of *A. baumannii* USP genes under the stress of MEM, bacteria were cultured and treated with 0.5 × MIC of MEM in the same way as we reported before Han et al. (2022). Samples without the treatment of MEM were set as control. To evaluate the expression of Usp1413-interaction proteins in different Usp1413 backgrounds, fresh cultures of tested strains were grown to an optical density of 600 nm (OD_{600}) at 1.0. DNA-free RNAs were extracted using RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany) and RNAprep Pure Cell/Bacteria Kit (TIAGEN Biotech Co. Ltd.) according to the manufacturer's instructions.

2.3. RNA sequencing

The conducting and analysis procedures of RNA sequencing were same as our previous report, with the raw RNA-Seq data in BioProject number PRJNA797559 (Han et al., 2022). The expression levels of USP genes were normalized by the fragments per kilobase of transcript per million mapped reads (FPKM). Variation in gene expression was determined between the samples treated and non-treated with MEM. USP genes were regarded as differentially expressed according to the standard that a fold change ≥ 2 and a false discovery rate (FDR) < 0.05.

2.4. Sequences analyzes of USPs and Usp1413-interaction proteins

The DNA and protein sequences of USPs from AB5116 and AB5116MR were aligned using the Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi). Eighty protein sequences homologous to each USP protein were downloaded from NCBI. Then, the phylogenic tree was constructed using MEGA 11 (https://www.megasoftware.net) by the Neighbor-Joining (NJ) model, with 1000 Bootstrap replications, and further modified by iTOL (https://itol.embl.de). Multiple sequence alignments of USPs and Usp1413-interaction proteins were performed using Jalview (https://www.jalview.org/). The protein structures of Usp1413 and the mutated Usp1413 (MT1413) were analyzed by SWISS-MODEL (https://swissmodel.expasy.org/).

2.5. Real-time polymerase chain reaction (qPCR)

Real-time PCR was performed to validate the transcription levels of the target genes using the primers listed in Table S1. Total RNAs were reverse-transcribed to cDNAs using the PrimeScript RT Master Mix (Takara Biomedical Technology (Beijing) Co., Ltd., Beijing, China). qPCR was conducted in 20 μL with the utilization of TB Green Premix Ex Taq II (Takara) and the LightCycler 96 Instrument (Roche, Basel, Switzerland). The cycling condition was set as initial incubation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 55 °C for 30 s, and 72 °C for 30 s. The gene expression level was standardized relative to that of 16S rRNA. For determining the expressions of USP genes under the stress of MEM, their relative abundances were calculated in comparison with MEM-untreated cultures by the cycle threshold (ΔΔCt) method. In addition, the relative expression levels of Usp1413-interaction proteins from different Usp1413 background strains were examined in comparison with the wild type (WT) strain AB5116. All assays were carried out in triplicate.

2.6. In situ deletion of target genes

Markerless deletion mutants of the target genes were constructed in AB5116 according to the description of Tucker et al. (2014), with some modifications. Firstly, kanamycin insertion mutants, which carried the FRT-flanked gene replacement cassette, were obtained in the same way as we modified before Han et al. (2022). Thereafter, the FLP recombinase-carrying plasmid pAT03 was transformed into these insertion mutants. Positive colonies were cultured on solid agar supplemented with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Aladdin) to induce the expression of FLP recombinase, thus to eradicate the kanamycin cassette and simultaneously remove the unstable plasmid. The triple deletion mutant Δ1411Δ1412Δ2171 was

constructed by knocking out the adjacent two genes 1411 and 1412 first, and 2171 afterwards. The final deletion mutants were confirmed by PCR and Sanger sequencing.

2.7. Complementation of the deletion mutants

Genetic complementation of the deletion mutants was performed according to the method we used before Li et al. (2024). Briefly, the target genes from AB5116 and the liner pAT03 were amplified by PCR using 2 × Fast Pfu Master Mix (Novoprotein, Shanghai, China) and PrimeSTAR Max DNA polymerase (TaKaRa, Beijing, China), respectively. Then, the target fragments and the liner pAT03 were ligated using the Seamless Cloning Kit (Beyotime, Shanghai, China), followed by the transformation into DH5α. The recombinant plasmids were verified by PCR and sequencing, and were transformed into the competent deletion mutant cells. The positive strains were selected on LB agar containing carbenicillin, and were further confirmed by PCR. The expression of target genes was induced by IPTG.

2.8. Susceptibility testing of A. baumannii strains to MEM

The MEM sensitivity of Usp1413-related strains, including Usp1413 deletion mutant (Δ1413), two complementary strains harboring wild type Usp1413 (WT1413) and mutated Usp1413 (MT1413), and their parental strain AB5116, was tested by disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2024). Furthermore, the MIC of MEM for all the *A. baumannii* isolates used in this study was examined by broth macrodilution method (CLSI, 2024). All experiments were performed in triplicate.

2.9. Biofilm formation analysis

Biofilm formation assay was carried out as previously described (Li et al., 2024), with brief modifications. First, an overnight culture of *A. baumannii* was 1:100 diluted in LB broth supplemented with IPTG and incubated at 37 °C for another 3 h to obtain a fresh bacterial suspension. Then, the OD_{600} of the suspension was normalized to 0.2, which was equivalent to 10^8 CFU/mL bacteria. Thereafter, 200 μL of the adjusted bacterial suspension was seeded into a sterile 96-well polystyrene microtiter plate, followed by incubation at 37 °C for 24 h. Afterwards, planktonic bacteria were removed from the plate, and the latter was gently washed with phosphate buffered saline (PBS) and air-dried. The biofilm attached in the well was stained with 0.1 % crystal violet at 37 °C for 30 min, then gently rinsed with PBS twice. The remaining dye was solubilized in 200 μL of 95 % ethanol, and the biofilm biomass was quantified by measuring the OD_{570} of the supernatant. All assays were executed in triplicate.

2.10. Motility assay

Swarming motility of the Usp1413-related isolates was determined as reported by Liu et al. (2023), with minor modifications. Briefly, one colony was inoculated in the center of LB medium containing 0.25 % agar and 2 mM IPTG. Then, the plates were incubated at 37 °C for 14 h, and the diameter of growth was measured. The experiment was independently performed for three times.

2.11. Growth curve determination

Growth curves of the isolates were recorded as mentioned by Han et al. (2022). Overnight cultures of tested strains were inoculated in fresh LB broth supplemented with IPTG at a concentration of 5×10^5 CFU/mL. During the cultivation (200 rpm) at 37 °C, the OD_{600} value was measured per hour from 0 h to 24 h. Three independent experiments were performed.

2.12. Sensitivity assay of disinfectants

The disk diffusion method was utilized to evaluate the fitness of Usp1413-related strains when challenged with disinfectants such as 30 % H_2O_2 and NaClO according to previous report (Elhosseiny et al., 2015), with some modifications. 120 μL (approximate one swab) of fresh culture containing 10^8 CFU/mL bacteria was spread on IPTG-supplemented LB agar plate using a sterile swab. Thereafter, a sterile disk absorbing 15 μL of newly prepared 30 % H_2O_2 or NaClO was placed on the surface of the plate. After an overnight incubation at 37 °C, the diameter of the inhibition zone was measured. Experiments were repeated for three times.

2.13. Osmotic stress assessment

The role that Usp1413 played in responding to osmotic stress was investigated by determining the survival rate of Usp1413-related strains grown under different concentrations of NaCl (Elhosseiny et al., 2015). Briefly, overnight cultures of bacteria were adjusted to OD_{600} of 0.2, and the bacterial suspensions were 1:100 inoculated in LB broth containing 0 %, 0.9 %, and 2.5 % NaCl, as well as IPTG. After 3-h incubation at 37 °C with shaking at 200 rpm, the cultural suspensions were serially diluted to 10^{-4}. 100 μL of each dilution was spread on LB agar plates, and cultured overnight at 37 °C. The survival rate was calculated by dividing the CFU/mL of bacteria grown in LB broth supplemented with NaCl by that grown in the normal LB broth. Assays were carried out in triplicate.

2.14. Analysis of Usp1413 interaction proteins

The interaction proteins of Usp1413 were predicted using the online database STRING (https://cn.string-db.org/), which is effective for estimating the interactions of uncharacterized proteins. In order to improve the accuracy of prediction, three model strains in the database were all used for analysis, in which, ATCC17978 is a standard *A. baumannii* strain, AB030 is an extensively drug-resistant clinical strain recorded in the database before 2023, and BJAB07104 is a multidrug-resistant clinical strain recorded in the database after 2023. Three overlapping proteins were further investigated for their expressions and functions in the field of MEM adaptive resistance.

2.15. Identification of the Usp1413 promoter region

The promoter region of Usp1413 was predicted using the online softwares BDGP (https://www.fruitfly.org/seq_tools/promoter.html) and BPROM (http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb). To confirm the region and function of the promoter, deletion mutants and complementary strains were constructed as mentioned above. To ascertain the positions of −35 and −10 boxes, complementary strains harbouring mismatched mutations at these sites were manipulated by overlapping PCR. The expression of Usp1413 was determined in all these mutants by qPCR to validate the promoter region.

2.16. Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0 (San Diego, CA, USA) and SPSS 22.0 (SPSS Inc., Chicago, IL, USA). To analyze the differences in the relative expression levels of the target genes, bacterial growth under various stress conditions, bacterial ability of biofilm formation and swarming motility, one-way ANOVA was used. Analysis of bacterial growth curves was carried out using two-way ANOVA. A P value < 0.05 was considered statistically significant for all tests.

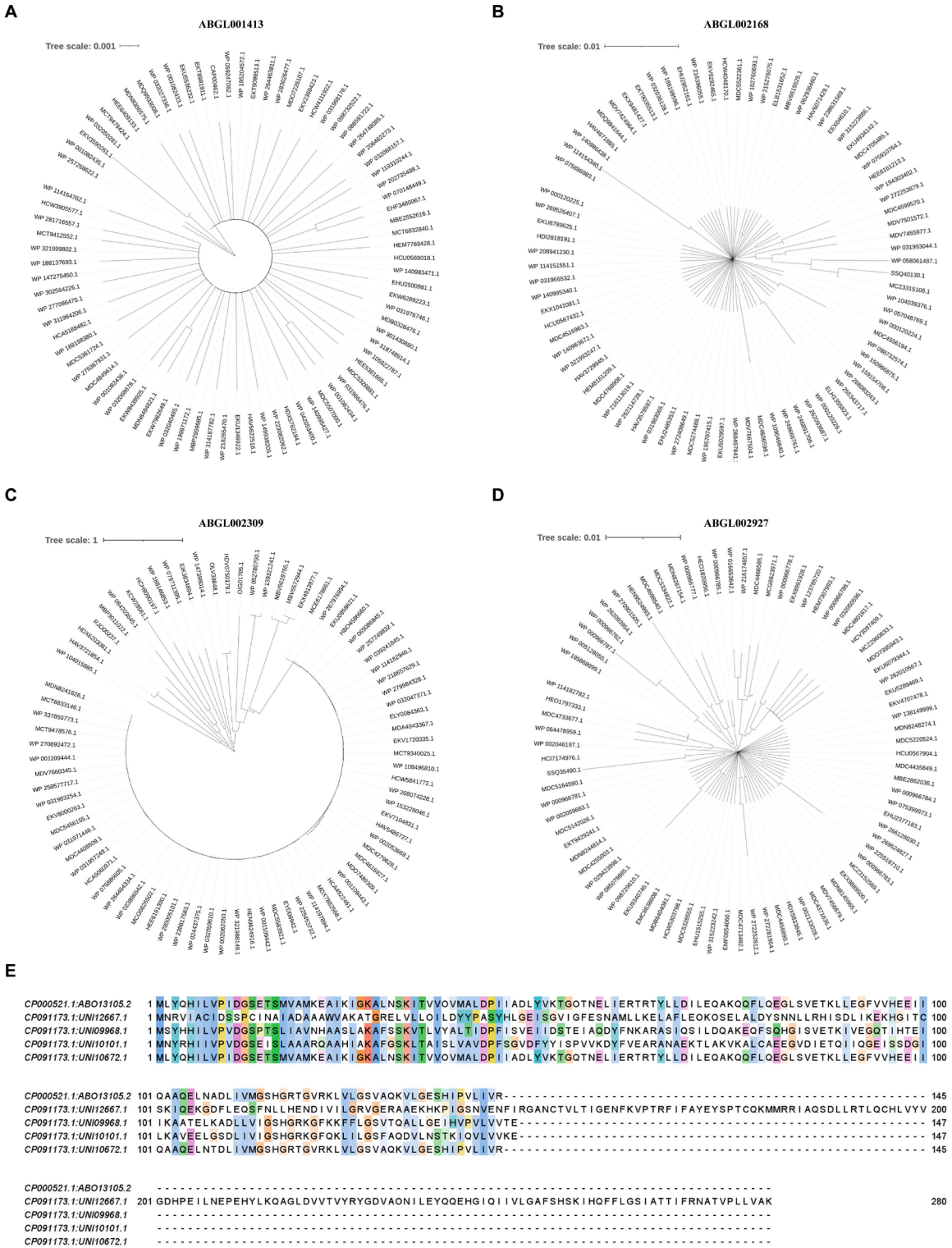

Fig. 1. Homology analysis of four USP proteins. Phylogenic trees of USP proteins homologous to those encoded by (A) ABGL001413, (B) ABGL002168, (C) ABGL002309, and (D) ABGL002927 were constructed by NJ model in MEGA 11, with the Bootstrap replications as 1000. (E) Comparative analysis of four USP proteins from AB5116 with UspA from *A. baumannii* using Jalview. The first line is UspA of ATCC17978, followed by the proteins encoded by ABGL001413, ABGL002168, ABGL002309, and ABGL002927.

3. Results

3.1. Decreased expression and gene mutation occurred in Usp1413 under MEM stress

According to the annotation of National Center for Biotechnology Information (NCBI), there were four USP genes in AB5116, namely ABGL001413 (denoted according to the original sequencing data, but was annotated as L2M54_07165 in GenBank), ABGL002168 (L2M54_10880), ABGL002309 (L2M54_11590) and ABGL002927 (L2M54_14715). The amino acid sequences of them were highly conserved among diverse *A. baumannii* strains (Fig. 1A-D). Out of the four USPs, the amino acid sequence of ABGL002927 showed the highest homology (99 %) with UspA of ATCC 17978 (Elhosseiny et al., 2015) (Fig. 1E).

To investigate the role of the four USP genes in carbapenem resistance in *A. baumannii*, we observed the response of them under the stress of MEM. We firstly compared the expression of the four genes between the MEM-treated and non-treated groups in the RNA sequencing results published by our group before (Han et al. 2022). All of these four USP genes were significantly downregulated in MEM-treated AB5116 cultures, with the fold change > 2 and the false discovery rate (FDR) < 0.05 (Fig. 2A). Furthermore, the RT-qPCR assay validated these transcriptome results, that after treatment with $0.5 \times$ MIC of MEM for 0.5 h, the relative transcript levels of ABGL001413, ABGL002168, ABGL002309, and ABGL002927 dropped to 0.519, 0.785, 0.557, and 0.761, respectively (Fig. 2B). The changed expression of a series of USPs indicated for the first time that USPs might involve in the response to MEM pressure in *A. baumannii* at early time.

In addition, the potential of USPs in responding to long-lasting MEM stimulation was further explored by determining the whole genome backgrounds of AB5116 and the AB5116-generated MEM-resistant strain AB5116MR. Out of the four USP genes, ABGL001413 was the most exceptional one that it not only showed the most significant decreased expression under the stress of MEM, but was the unique gene having mutation after serial MEM stimulation (Table 1). This mutation resulted in the insertion of two amino acids, tyrosine and glycine, at the site of 229-230 (YG229-230). According to the three-dimensional (3D) protein models constructed by SWISS-MODEL, Usp1413 and mutated Usp1413 matched with USPs in various bacteria. As shown from Fig. 2C, the insertion of YG229-230 was in the externally exposed position, which might influence the function of the protein in some extent. Therefore, we furtherly focused on Usp1413 and mutated Usp1413 to elucidate their effects on MEM resistance of *A. baumannii*.

3.2. Usp1413 participated in adaption to MEM stress

To uncover the mechanism of Usp1413 in responding to the stress of MEM, its coding gene was knocked out from AB5116. As noticed from the disk diffusion assay, the size of the inhibition zone obtained by the Usp1413-absent mutant (Δ1413) was significantly shrunk compared with AB5116, representing increased resistance to MEM. In addition, the sensitivity of the complementary strain expressing wild type Usp1413

Table 1
Gene analysis of USPs between AB5116 and AB5116MR.

Gene name	Sequence length (bp)	Mutation type	Mutation position
ABGL001413	843	Insertion	g.685~690insTACGGC *
ABGL002168	444	None	None
ABGL002309	444	None	None
ABGL002927	438	None	None

* Mutation occurred in AB5116MR after serial stimulation of MEM to AB5116.

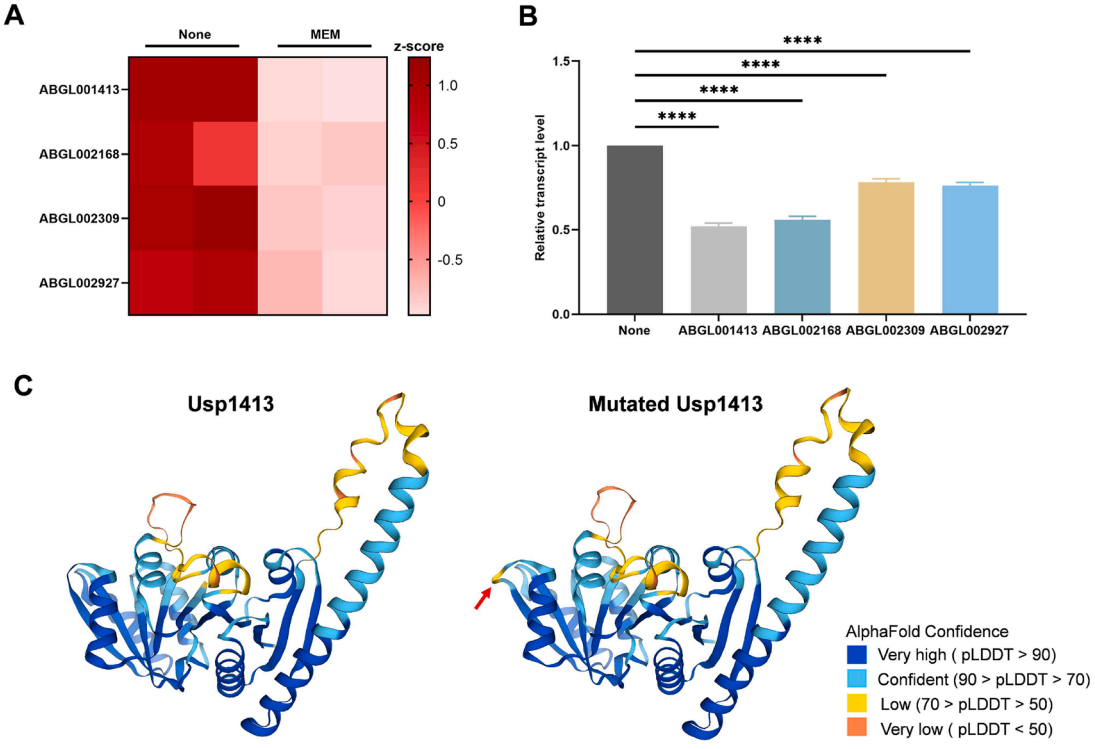

Fig. 2. Analysis of USPs' gene expression and mutation. (A) Expressional heatmap of four USP genes from RNA-Seq results. The fragments per kilobase of transcript per million mapped reads (FPKM) values were standardized based on the Z-score using the formula $Z = (x - \mu)/\sigma$. (B) Changed expressions of four USP genes under the stress of meropenem (MEM). The experiment was repeated in triplicate, and the results were shown as means ± SD. ****, $P < 0.0001$. (C) Structural prediction of Usp1413 and mutated Usp1413 by SWISS-MODEL workspace using AlphaFold2 method. Arrow indicates the insertion site of tyrosine and glycine. The pLDDT values of Y229 and G230 are 68 and 79, respectively.

(WT1413) closely reached to that of AB5116, whereas that expressing mutated Usp1413 (MT1413) was partially recovered (Fig. 3A). These findings were further confirmed by the MIC results. The deletion of ABGL001413 resulted in the rise of MEM MIC by 4-fold, which went up from 0.125 μg/mL to 0.5 μg/mL. The susceptibility of Usp1413-complemented strain restored to the level of the isogenic parent. While, the MIC value of the complementary strain harboring MT1413 was slightly elevated to 0.25 μg/mL (Table 2).

3.3. *Usp1413 involved in biofilm formation and swarming motility*

Biofilm is a prominent virulence factor that is also reported involving in antibiotic resistance in various bacteria (Trubenová et al., 2022; Mendes et al., 2023). Therefore, the biofilm formation ability of the above isolates was assessed. As shown in Fig. 3B, Δ1413 displayed significant reduced biofilm biomass in comparison with the wild type strain. While, strong capability of biofilm formation was observed in the two complementary strains, with WT1413 in a higher level (Fig. 3B).

Considering that swarming motility is a crucial behavior conferring biofilm-associated antibiotic resistance (Lai et al., 2009), we investigated this phenotype in our strains. Loss of Usp1413 resulted in poor ability of *A. baumannii* to migrate across semisolid surfaces with the swarm zone at 5 mm ($P < 0.0001$). However, two strains complemented by ectopic expression of WT1413 and MT1413 could in some extent restore this behavior (vs. Δ1413, $P < 0.0001$), in which, the WT1413-complemented strain migrated further (15 mm) than the MT1413-complemented strain (13 mm) ($P = 0.0236$) on the semisolid agar (Fig. 3C). Based on these results, Usp1413 was presumed to multifacetedly participate in adaption to MEM stress.

3.4. *Usp1413 contributed to the response of environmental stresses in A. baumannii*

USPs have been affirmed to resist environmental and physiological stresses (Nyström and Neidhardt, 1994). In order to investigate the effect of Usp1413 on *A. baumannii* growth and the response to environmental stresses, we examined the growth curves of our strains, as well as their sensitivity to the external pressures, such as oxidative and osmotic stresses. The Δ1413 mutant displayed growth disadvantage at logarithmic phase compared to AB5116 (5h, $P = 0.0022$). And the growth rates of two complementary strains were consistent with the level of the WT strain (Fig. 4A).

Referring to the chemical pressures, we firstly determined the function of Usp1413 in responding to H_2O_2. An enlarged inhibition zone around the H_2O_2 disk was observed for the Usp1413 knockout mutant, inferring that it was more sensitive to 30 % H_2O_2 than the other three strains. There was no difference between the two complemented strains in the sensitivity to H_2O_2; however, the inhibition zone of the complemented strain containing MT1413 remained larger than that of WT1413 (Fig. 4B). Similar results were recorded for the susceptibility to NaClO, which is a common disinfectant used in hospitals (Fig. 4C). Subsequently, the physiological role of Usp1413 involved in diverse osmotic pressures was determined by evaluating the survival rate of four strains grown under low osmolality (0 % NaCl), isosmotic pressure (0.9 % NaCl), and high osmolality (2.5 % NaCl) conditions. No matter in which condition, Δ1413 mutant exhibited the weakest surviving ability. In comparison, two complemented strains grew exuberantly without NaCl; whereas, they showed diminished growth performance with 0.9 % and 2.5 % NaCl. Of note, the complementary strain expressing MT1413 always grew less than that expressing WT1413 at all culture conditions

Table 2
MIC results of the strains used in this study.

Strain	MIC (μg/mL)
AB5116	0.125
AB5116Δ1413	0.5
AB5116Δ1413+WT1413	0.125
AB5116Δ1413+MT1413	0.25
AB5116Δ1411	0.5
AB5116Δ1411+1411	0.125
AB5116Δ1412	0.25
AB5116Δ1412+1412	0.125
AB5116Δ2171	0.5
AB5116Δ2171+2171	0.125
AB5116Δ1411Δ1412Δ2171	0.5

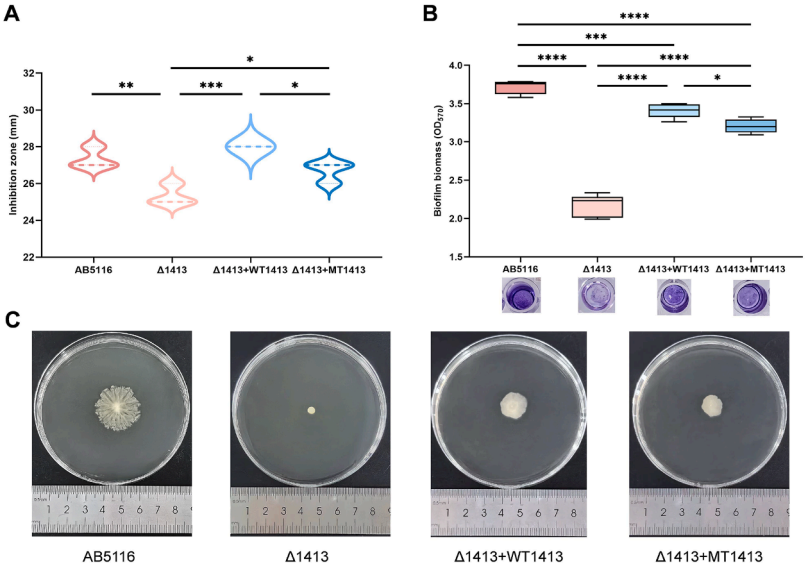

Fig. 3. The roles that Usp1413 played in MEM adaptive resistance, biofilm formation, and swarming motility. (A) MEM susceptibilities of AB5116 and Usp1413-related strains, including Usp1413-deletion mutant (Δ1413), wild type Usp1413 (WT1413) and mutated Usp1413 (MT1413) complemented strains, determined by disk diffusion method. The diameters of the inhibition zone were recorded after cultivation at 37 °C for 24 h. (B) Biofilm forming abilities of AB5116 and Usp1413-related strains detected by crystal violet staining method. The biofilm biomass was measured at optical density of 570 nm (OD_{570}) after 24 h incubation. (C) Representative results of swarming motility of AB5116 and Usp1413-related strains. The migration zone of bacteria on the semisolid surfaces were monitored after incubation for 14 h. IPTG was supplemented in all the medium. The experiments were repeated in triplicate, and the results were shown as means ± SD. ns, $P > 0.05$, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$.

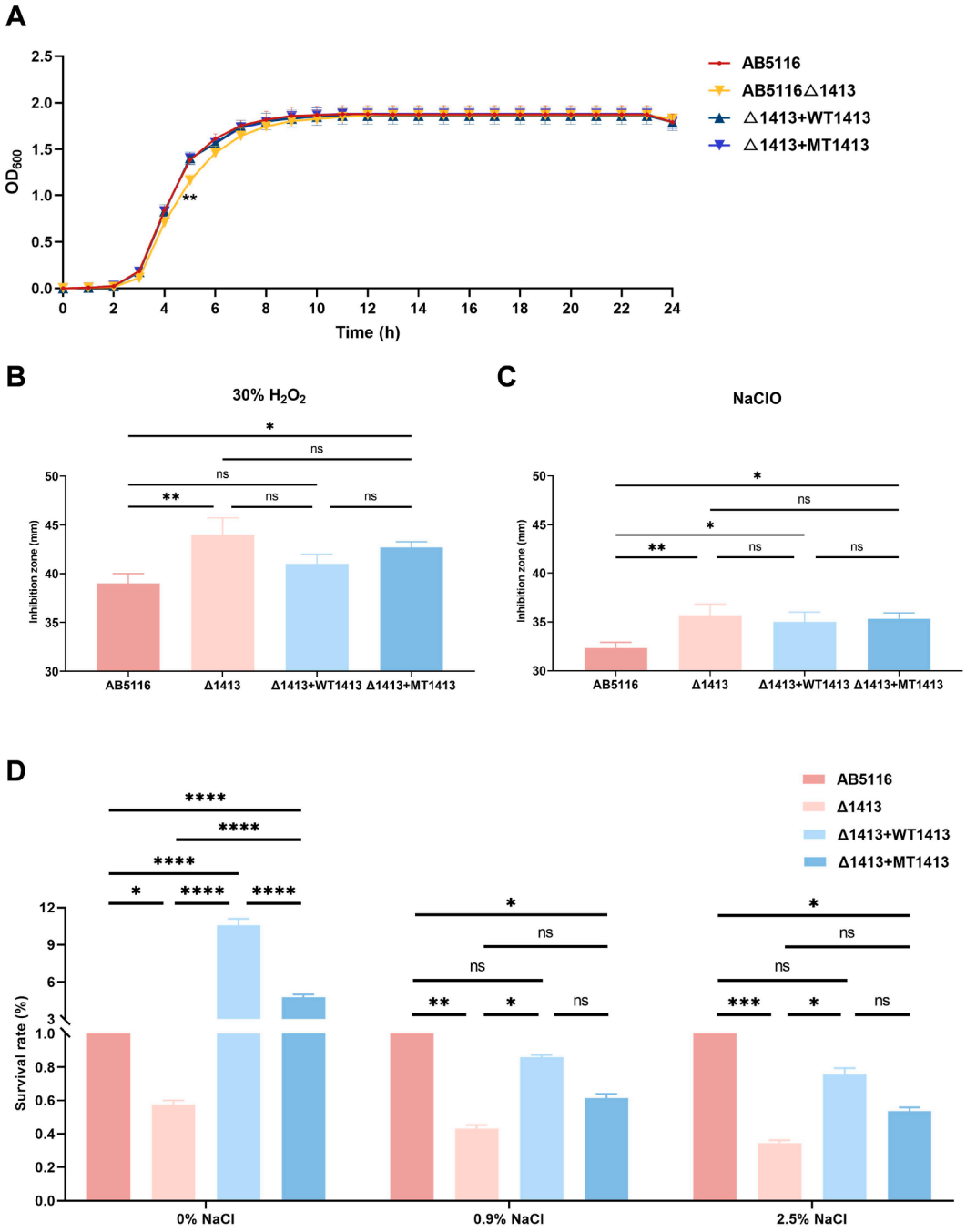

Fig. 4. Effects of Usp1413 on bacterial growth and environmental stresses in *A. baumannii*. (A) The growth curves of AB5116 and Usp1413-related strains. Bacteria were cultured in LB broth at 37 °C with shaking at 200 rpm. The OD_{600} of the bacterial cultures was recorded per hour until 24 h. (B) and (C) indicate the growth of AB5116 and Usp1413-related strains under the stresses of 30 % H_2O_2 and NaClO, respectively. The diameters of the inhibition zones around the detergent-containing disks were measured after incubation at 37 °C for 24 h. (D) Growth of AB5116 and Usp1413-related strains under various osmotic stresses. Bacteria grown within different concentrations of NaCl were serially diluted and spread on LB agar plates. Survival rate was calculated by dividing the CFU/mL of bacteria grown under osmotic stresses by that in control medium. IPTG was supplemented in all the medium, and all experiments were repeated in triplicate. The results were shown as means ± SD. ns, $P > 0.05$, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$.

with different NaCl concentrations (Fig. 4D).

3.5. *Usp1413 helped A. baumannii adapt to MEM stress by regulating the interaction proteins*

To investigate whether there are some Usp1413-associated factors involved in the process of bacterial adaptation to MEM, we looked for the interaction proteins of Usp1413 using the online software STRING based on three model strains from the database, including AB030 (GenBank accession number CP009257), ATCC17978 (CP000521) and BJAB07104 (CP003846). Usp1413 showed 98.2 % similarity in protein sequence among the above strains (Fig. S1). And there were eight, nine, and ten interaction proteins in strains AB030, ATCC17978 and BJAB07104, respectively (Fig. 5A–C). Of these proteins, three were common and highly conserved in the investigated strains, as well as in AB5116 (Table 3, Fig. S1). Thus, we further examined their expression in different Usp1413 backgrounds. As shown in Fig. 5D, the transcriptional level of three interaction proteins was notably reduced in the absent of

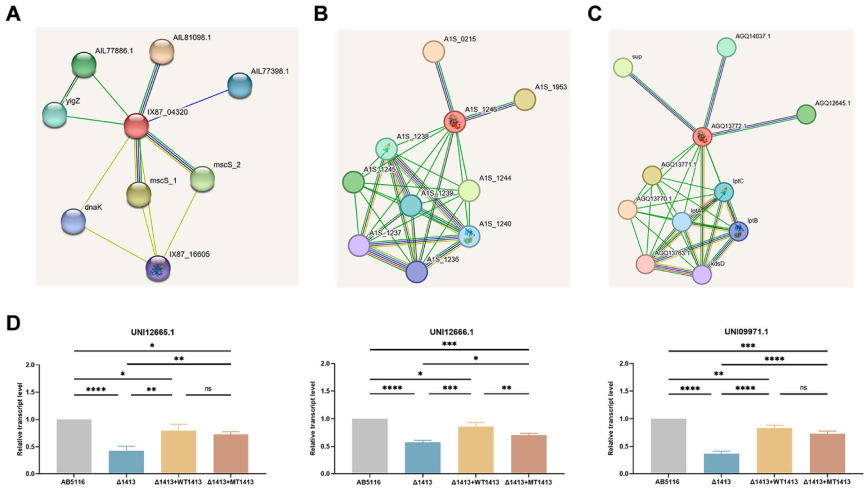

Fig. 5. The prediction and confirmation of Usp1413 interaction proteins. (A-C) The interaction proteins of Usp1413 were predicted by STRING database based on three model strains, including AB030, ATCC17978 and BJAB07104, respectively. The red spheres show the proteins corresponding to Usp1413, and others represent the interaction proteins. (D) The relative expression levels of three common interaction proteins, which are UNI12665.1, UNI12666.1, and UNI09971.1, were determined in AB5116 and Usp1413-related strains. The experiment was repeated in triplicate, and the results were shown as means ± SD. ns, $P > 0.05$, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$.

Table 3
Three common interaction proteins of Usp1413 from different strain backgrounds.

AB5116 Gene name	AB5116 Protein name	AB030 Protein name	ATCC17978 Protein name	BJAB07104 Protein name	Information
ABGL001411	UNI12665.1	AIL77887.1/*yigZ*	ABO11672.2	AGQ13770.1	YigZ family protein
ABGL001412	UNI12666.1	AIL77886.1	ABO11673.2	AGQ13771.1	Acetyltransferase
ABGL002171	UNI09971.1	AIL81098.1	ABO12380.2	AGQ14037.1	SulP family inorganic anion transporter

Usp1413, and was reinstated in the presence of both WT1413 and MT1413, with the latter at a slightly lower level.

To understand the extent of each interaction protein participating in MEM adaptive resistance, their coding genes were deleted from AB5116 separately and ternary. Compared with the strains expressing the three genes, all the single deletion mutants showed increased MIC (Table 2), diminished biofilm biomass, and compromised swarming motility. These changes were more obvious in the triple mutant, which coincided with the phenotype of Δ1413 mutant (Fig. 6). In the three coding genes, ABGL001411 and ABGL002171 were more likely to influence the MIC, and ABGL001412 involved more in the capabilities of biofilm formation and swarming motility. These results demonstrated that the three interaction proteins were regulated by Usp1413 to engage in the MEM adaption process.

3.6. *Determination of Usp1413 promoter region*

Promoter regions are intrinsic DNA elements located upstream of genes. Correct mapping of promoters is a critical step when studying the regulatory mechanism of Usp1413. The most possible regions are shown in Fig. 7A, in which, the grey bar was predicted by BDGP and the blue letters were predicted by BPROM. Region 1 and region 2 were considered as the –35 box and –10 box, respectively. In order to verify their regulatory funtion, we firstly checked the expression level of Usp1413 in the promotor deletion mutant. As expected, loss of the whole grey region dramatically declined the expression of ABGL001413 relative to AB5116. While, when we complemented the full promoter region along with ABGL001413 gene into its parental deletion mutant (strain ΔTotal1413+Total1413), the expression restored considerably. Similar as the promotor lacking mutant, point mutation in region 1 (–35 box) downregulated the transcription of Usp1413 hugely, more than that in region 2 (–10 box) (Fig. 7B).

4. Discussion

The global infection of resistant *A. baumannii* remains one of the most serious public health issues. Both of the World Health Organization (WHO) and the European Centre for Disease Prevention and Control (ECDC) consider CRAB as one of the highest priorities (Mancuso et al., 2023). Environmental carbapenem stress is a crucial trigger that forces *A. baumannii* to be CRAB. The ability of adaptive resistance helps this bacterium survive these antibiotics in the very early stage (Fernández and Hancock, 2012; Motta et al., 2015), and may provide prerequisite for the effect of other resistance mechanisms. Therefore, identifying targets involved in *A. baumannii* adaptive resistance may support the control of this deadly pathogen. USPs are important regulatory stress proteins which exist across various of species (Luo et al., 2023). They are pivotal factors associated with the survival or persistence of numerous pathogens under stressful conditions (Masamba and Kappo, 2021). However, their relationship with antibiotic resistance is rarely reported.

In this study, four USPs were identified from our local clinical isolate AB5116. All their expressions were enormously diminished under the stress of MEM, suggesting their potential role in reacting to MEM pressure instantly in *A. baumannii*. Moreover, long lasting stimulation of sublethal concentrations of MEM was performed to determine the importance of the USP genes in drug-resistance evolution, which was similar as the study manipulated by Liu et al. (2023). Interestingly, out of these four genes, not only did Usp1413 show the most significant lowered expression under the instant MEM stress, but it was also the only gene which had mutation after serial stimulation of MEM. The Usp1413 is highly conserved among different *A. baumannii* strains, indicating that it could be a plausible target for combating adaptive resistance and reducing the stress capacity of bacteria to the environment. Therefore, we furtherly focused on elucidating the function of Usp1413 in various aspects of stress response including the reaction to antibiotics.

Up to date, UspA is the most particular universal stress protein

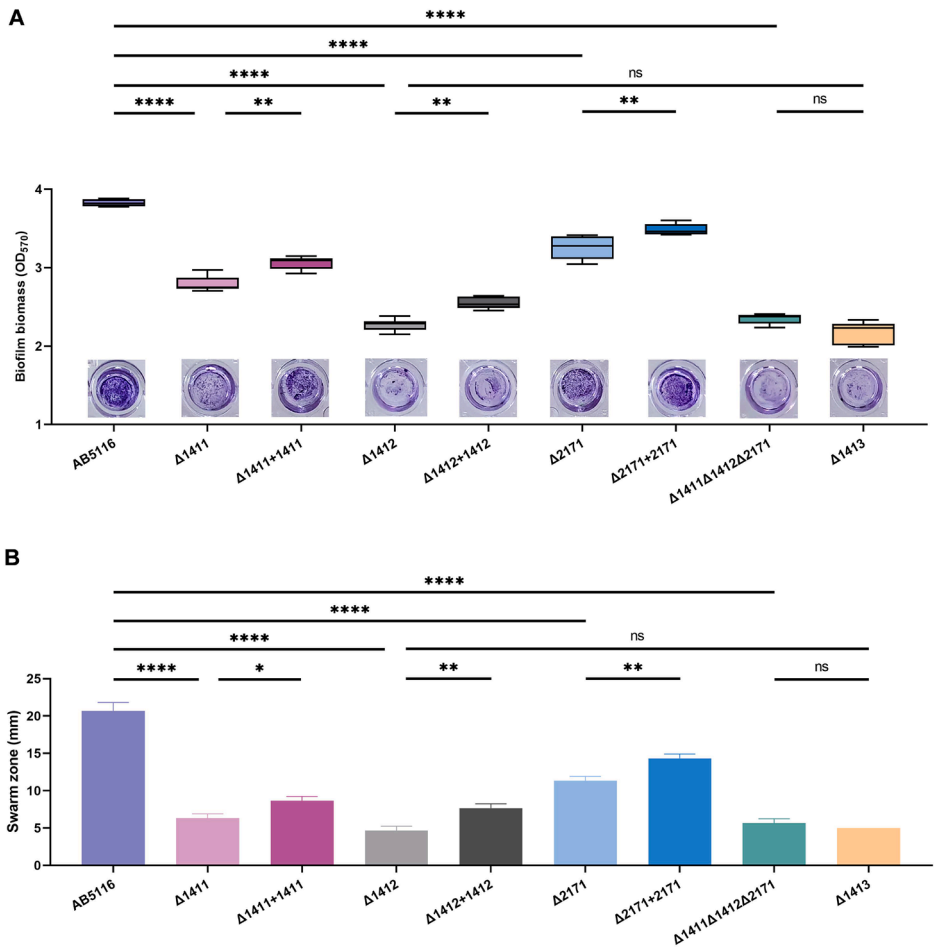

Fig. 6. Effects of Usp1413-interaction proteins on biofilm formation and swarming motility. (A) Biofilm formation abilities of Usp1413-interation protein mutants compared with AB5116 and Δ1413. The biofilm biomass was measured at OD_{570} after 24 h incubation. (B) Swarming abilities of Usp1413-interation protein mutants compared with AB5116 and Δ1413. The swarm zone of bacteria on the semisolid surfaces were recorded after incubation for 14 h. IPTG was supplemented in all the medium, and the experiments were repeated in triplicate. The results were shown as means ± SD. ns, $P > 0.05$, *, $P < 0.05$, **, $P < 0.01$, ****, $P < 0.0001$.

widely investigated in bacteria (Yan et al., 2024). The existence of UspA was found phenotypically related to drug resistance, e.g., an AbaR-like resistance island, which is a transposition module harboring UspA, was identified in *A. baumannii* (Bonnin et al., 2012). However, the expression of UspA under the stimulation of antibiotics is controversial in different bacteria. In several bacteria such as *E. coli* and *Staphylococcus aureus*, UspA was expected to protect bacteria from the harm of antibiotics by overexpression (Attia et al., 2013; Bandyopadhyay and Mukherjee, 2022). Whereas, Kashyap et al. discovered that UspA in *A. baumannii* was significantly downregulated after the treatment of tobramycin and colistin (Kashyap et al., 2021), which is similar to our findings. Although the expressions of Usp genes are opposite in *A. baumannii* compared with other bacteria, the protective function is consistent, since the diminished expression of Usp1413 under the stress of MEM could further raise the antibiotic adaptation in AB5116. In addition, the mutation occurred in Usp1413 enhanced MEM resistance in *A. baumannii*. The resistance-adapted function of Usp1413 may be achieved by regulating the interaction proteins including UNI12665.1 (YigZ family protein) and UNI09971.1 (SulP family inorganic anion transporter), on account of the fact that loss of them resulted in enhanced MEM resistance at the similar level of Δ1413 strain. A recent report highlighted the importance of YigZ, a protein of unknown function, in early biofilm formation in *E. coli* (Holden et al., 2021). Furthermore, substitution in the putative sulfate transporter SulP resulted in an elevated daptomycin MIC in *Enteroccoccus faecium*, demonstrating its possibility in bacterial antibiotic resistance (Wardal et al., 2023). Indeed, the complementation of WT1413 successfully restored the expression of both YigZ and SulP family proteins, which consequently retrieved the bacterial sensitivity to MEM.

Except of the influence on MEM resistance, Usp1413 also participated in the biological process of biofilm formation and motility, which are important contributors to bacterial virulence. It is a common finding in various bacteria that deletion of USP genes leads to poor biofilm formation (Chen et al., 2006; Samanta et al., 2020; Lv et al., 2023). Similarly, reduced biofilm in Usp1413-absent strain demonstrated its significant contribution to the form of biofilm in *A. baumannii*. There are some clues that swarming behavior is an integrated part of biofilm formation (Verstraeten et al., 2008; Mea et al., 2021). Furthermore, the ability of bacterial motility could even influence the shape of biofilms (Tolker-Nielsen, 2015). Indeed, losing of Usp1413 greatly weakened *A. baumannii*'s swarming motility, which was in accordance with the change of biofilm. The weaker the bacteria could migrate on the surface of the semi-solid agar, the less the biofilm was constructed. Additionally, Usp1413 may influence biofilm formation and motility by regulating its downstream protein UNI12666.1, which is an acetyltransferase. Recent studies manifest that acetyltransferases are important to promote the form of biofilms in numerous bacteria (Lammers, 2021; Ma et al., 2021; Bertrand et al., 2022; Li et al., 2023). They are also confirmed to positively regulate bacterial motility (Castaño-Cerezo et al., 2014; Lammers, 2021; Fang et al., 2022). Moreover, a novel acetyltransferase, Dpa, is found directly affecting the motility and biofilm of *A. baumannii* (Armalytė et al., 2023). Likewise, among the three interaction proteins,

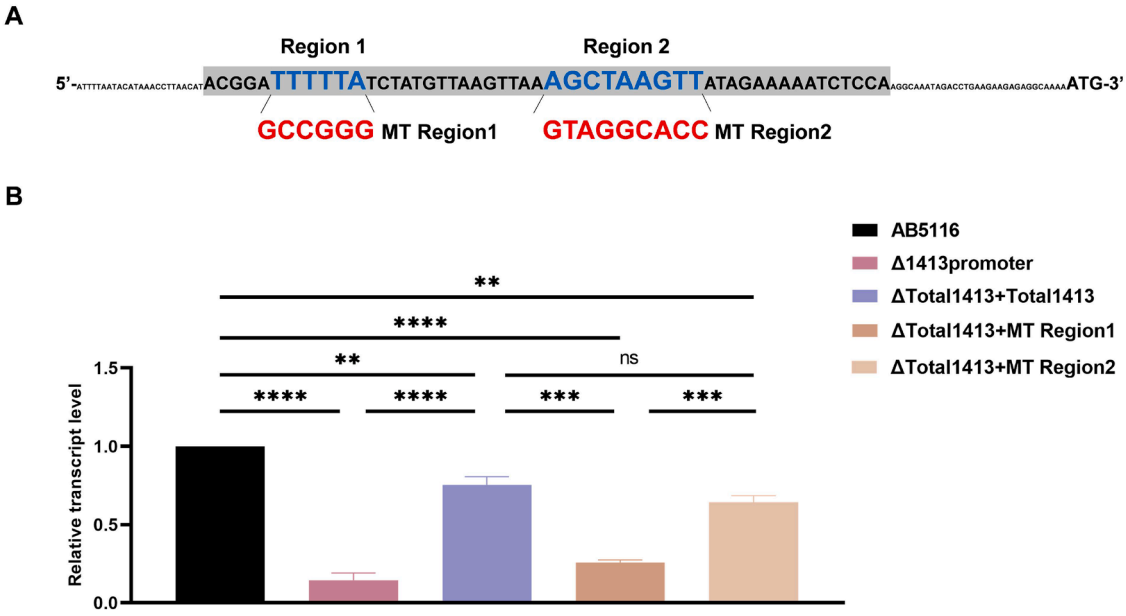

Fig. 7. Analysis of Usp1413 promoter region. (A) Prediction and mutation sites of Usp1413 promoter region. The grey bar was the promoter region predicted by BDGP. Region 1 and region 2 labeled in blue letters were predicted by BPROM. Mutations were constructed in region 1 and region 2 as marked in red letters. (B) The expression levels of ABGL001413 in various strains with different backgrounds of promoter region. The strain Δ1413promoter was a deletion mutant lacking of the whole grey bar. The strain ΔTotal1413+Total1413 was a complementary strain expressing the whole promoter region and ABGL001413 base on their deletion. The strain ΔTotal1413+MT Region1 was a complementary strain expressing the mutated promoter region 1 and ABGL001413 base on their deletion. The strain ΔTotal1413+MT Region2 was a complementary strain expressing the mutated promoter region 2 and ABGL001413 base on their deletion. The assay was repeated in triplicate, and the results were shown as means ± SD. ns, $P > 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$.

UNI12666.1 deletion mutant (Δ1412) displayed the closest phenotype with Δ1413 strain in terms of both biofilm and motility, indicating its role in these functions. Intriguingly, the triple knockout mutant performed same as the Usp1413-absent strain in all aspects of MIC value, biofilm formation and swarming motility. These consequences convinced that Usp1413 had an effect on MEM resistance adaptation and virulence through downregulating three interaction proteins.

USPs are not only involved in antibiotic resistance and virulence, but also have been reported to play a more extensive protective role in bacteria (O'Connor and McClean, 2017). Usp1413 placed important effect on protecting *A. baumannii* from the pressure of H_2O_2 that the absence of Usp1413 made bacteria more sensitive to H_2O_2, which was similar to the finding of Elhosseiny et al. in UspA (Elhosseiny et al., 2015). In addition to H_2O_2, disinfectants are major stresses imposed to bacteria in hospitals. Daer et al. revealed that monochloramine treatment on *E. coli* led to notable downregulation of universal stress response genes including UspA (Daer et al., 2021). In *A. baumannii*, the protective role of Usp1413 against NaClO was observed in our study, since losing of the USP gene impaired the resistance to this disinfectant. Besides, NaCl is an important material required for bacterial reproduction, metabolism, as well as maintaining intracellular and extracellular osmotic pressures. While, high salinity could be a kind of environmental stress, under which USPs play a key role for bacteria to survive. For example, deletion of UspA made *E. coli* defective in growth under hyperosmotic shock (Nyström and Neidhardt, 1994). Moreover, the Δ*saUspA* mutant of *Sulfolobus acidocaldarius* grew remarkably slower than the wild type strain at 400 mM NaCl (Ye et al., 2020). In agreement with these studies, Usp1413 was found critical for *A. baumannii* to remain alive not only in high osmotic stress, but also in low osmolality and isosmotic conditions.

Notably, the mutated Usp1413, which had tyrosine and glycine inserted at the site of 229-230 after the long-lasting stimulation of MEM, endowed stronger ability of resisting MEM pressure to *A. baumannii*. This variation was at the externally exposed position in the protein, thus might be a contributory factor in the evolutionary procedure of drug resistance and the spread of resistant bacteria (Baker et al., 2013). Nevertheless, in compensation, the mutant exhibited less capability in biofilm formation and motility. Meanwhile, it showed damaged persistence to the environmental stresses including H_2O_2, disinfectants and osmotic. This phenomenon is unanimous with the observation in other bacteria that counteracting fitness cost is accompanied with antibiotic resistance acquisition (Björkholm et al., 2001; Alame Emane et al., 2021; Eger et al., 2022). Additionally, the promoter region of Usp1413 was confirmed, so that provided evidence for further investigation of Usp1413 regulation.

To summarize, the USPs in *A. baumannii* could respond to the challenge of MEM. In which, Usp1413 exerted special protective effect on bacteria opposed to this antibiotic by acquiring insertion mutation. Both lowered expression and mutation of Usp1413 improved adaptive resistance of *A. baumannii* to MEM. Whereas, on contrary to the enhanced resistance, compromised biological processes were noticed, including motility, biofilm formation, and tolerance to unfavorable environments. Thus, Usp1413 is possible to be an attractive target for the development of novel antimicrobials to control the infection of *A. baumannii*. Further work will be performed to investigate the coactivity of Usp1413 with other USPs, revealing the common role of USPs in *A. baumannii*.

CRediT authorship contribution statement

Sirui Zhang: Conceptualization, Methodology, Data curation, Software, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Jingdan Wang:** Conceptualization, Methodology, Data curation, Software, Formal analysis, Validation, Writing – original draft. **Rong Yu:** Methodology, Investigation. **Haiping Liu:** Resources, Methodology. **Shuyan Liu:** Visualization, Formal analysis. **Kai Luo:** Formal analysis, Investigation. **Jin'e Lei:** Resources, Investigation. **Bei Han:** Resources, Supervision. **Yanjiong Chen:** Resources, Supervision. **Shaoshan Han:** Conceptualization, Methodology. **E Yang:** Methodology, Validation. **Meng Xun:** Methodology, Validation. **Lei Han:** Conceptualization, Funding acquisition, Supervision, Project

administration, Methodology, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2024.100332.

Data availability

Data will be made available on request.

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