

BamA protein as the target of bacteriocin PA166 from *Pseudomonas* sp. strain 166

Yu Wang^a, Yue Ma^a, Xiaojia Fu^a, Lin Han^a, Xiaouo Zhao^b, Yanhui Chen^{a,*}, Haiyong Guo^{a,*}

^a Jilin Normal University, SiPing, Jilin 136000 China

^b Jilin Academy of Agricultural Sciences, ChangChun, Jilin 130033 China

*Corresponding authors, e-mail: cyh@jlnu.edu.cn, guohaiyong78@jlnu.edu.cn

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ABSTRACT: The bacteriocin PA166 protein is secreted by *Pseudomonas* sp. strain 166 as previously reported by our research group. This bacteriocin is characterized by its good stability, potent antibacterial activity and low toxicity, providing valuable insights and potential opportunities for the development of novel antimicrobial agents. On this basis, we further explored the target of bacteriocin PA166. Molecular docking results showed that bacteriocin PA166 could bind to the extracellular membrane protein BamA. The real-time quantitative reverse transcription PCR (RT-qPCR) result showed that after bacteriocin PA166 action on *Pasteurella multocida* ATCC43137 (*P. multocida* ATCC43137), the *bamA* gene expression of the bacteria was significantly reduced. The results of the checkerboard studies showed that the activity of bacteriocin PA166 was significantly reduced by the addition of the BamA protein, thus identifying BamA as the target of the antimicrobial activity of this bacteriocin. This research not only provides an idea for the development of new antimicrobial agents, but also a useful reference for understanding their mechanism.

KEYWORDS: BamA protein, bacteriocin PA166, antibacterial target, molecular docking, antibiotics

INTRODUCTION

Bacterial drug resistance is one of the most serious public health problems facing the world today, and Gram-negative bacteria are particularly susceptible to drug resistance. Outer membrane proteins have important biological functions in Gram-negative bacteria, not only playing a key role in the formation and stabilization of the outer membrane but also containing several potential antibacterial targets [1].

The beta-barrel assembly machinery (BAM) complex has been investigated as a potential target for the development of new antibiotics [2–5]. BAM is a Gram-negative bacterial outer membrane protein assembly machine that contains five components, BamA, BamB, BamC, BamD and BamE, in most of the Gram-negative bacteria [6]. Of these, BamA is the core component on which bacterial survival depends, is largely conserved among Gram-negative bacteria [7, 8] and is considered a potential therapeutic target for the development of therapies against infectious bacterial diseases [9]. It is both a natural recognition site for phages and a target for certain antimicrobial peptides [10, 11].

In 2019, researchers identified compounds with antimicrobial properties that target BamA, using a rational design approach to develop novel sequences of antimicrobial peptides that induce misfolding of outer membrane proteins, leading to antimicrobial effects [12, 13]. Anatol et al discovered that a group of chimeric peptidomimetic antibiotics exert bactericidal effects by binding to both lipopolysaccharides and the outer membrane protein BamA [14]. Additionally, Hart et al highlighted that the compound MRL-494 inhibits Gram-negative bacteria by binding to BamA

and disrupting the assembly of outer membrane proteins [3].

In our previous study, we found that PA166 exhibited good antibacterial activity against *P. multocida* ATCC43137 with a minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 2 µg/ml and 8 µg/ml, respectively. It also showed a favorable safety profile at 256 µg/ml, with a hemolysis rate of 1.36% and a survival rate of over 80% for both Vero and NR8383 cells. In addition, PA166 acted on the outer cell membrane to kill bacteria [15]. Therefore, in this study we found through molecular docking experiments that bacteriocin PA166 has the ability to bind to the outer membrane protein BamA. More importantly, we observed a significant decrease in the expression level of the *bamA* gene in *P. multocida* ATCC43137 exposed to bacteriocin PA166. To further confirm this finding, we performed a competitive binding assay which showed that the activity of bacteriocin PA166 was significantly reduced upon addition of BamA protein, confirming that BamA protein is the target of bacteriocin PA166.

MATERIALS AND METHODS

Bacteria, chemicals, and reagents

Spin Column Bacteria Total RNA Purification Kit, unstained protein marker, *E. coli* DH5α and *E. coli* BL21 (DE3) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Mueller-Hinton Broth (MHB) was procured from Coolaber Technology Co., Ltd. (Beijing, China). PrimeScript™ RT Reagent Kit with gDNA Eraser was purchased from Takara Biomedical Technology Co., Ltd. (Beijing, China).

Bacteriocin PA166 was produced by *Pseudomonas* sp. strain 166 during cultivation in LB broth at 16 °C for 48 h. The cell-free fermentation supernatant was subjected to ammonium sulfate precipitation, followed by Sephadex G-75 gel filtration and Q-Sepharose ion-exchange chromatography, resulting in purified bacteriocin PA166.

RT-qPCR analysis

RT-qPCR analysis was performed following the protocol described in a previous study [16]. Specifically, RNA was extracted from *P. multocida* ATCC43137 using the Spin Column Bacteria Total RNA Purification Kit. The experiment was divided into two groups: one group was treated with 2 µg/ml bacteriocin PA166 and the other group served as an untreated control. The specific procedures were as follows, *P. multocida* ATCC43137 was cultured until the optical density at 600 nm (OD_{600nm}) reached 0.5, and 1 ml of bacterial culture was collected. The treatment group was supplemented with bacteriocin PA166 to a final concentration of 2 µg/ml, while the control group received an equal volume of sterile PBS instead of PA166. Both groups were then incubated at 37 °C for 4 h to ensure consistent treatment conditions. RNA was diluted to the same concentration and reverse transcribed to cDNA. Reverse transcription was performed using the PrimeScript™ RT Reagent Kit with gDNA Eraser. The 16S rRNA gene was used as the internal reference gene and the group without bacteriocin PA166 treatment as the control group. The forward primer sequence was 5'-TGATCCTGGGCTCAGGATGA-3' and the reverse primer sequence was 5'-TTCGCTCGACTTGCATGTA-3'. Cycling conditions were as follows: first, the polymerase was activated at 95 °C for 30 s; followed by 40 cycles of amplification, each cycle consisting of denaturation at 95 °C for 5 s and annealing at 60 °C for 34 s. The $2^{-\Delta\Delta Ct}$ method was used to calculate the changes in gene expression levels. The experiment was performed in triplicate.

Homology modeling and molecular docking

Homology modeling and molecular docking analyses were carried out in accordance with a prior investigation [17]. In this study, several bioinformatics tools and methods were used to construct and analyze the BamA amino acid sequence and protein model of bacteriocin PA166. Firstly, suitable templates were searched on the SwissModel online server (<https://swissmodel.expasy.org/interactive/>) and combined with the 5D0O and 1KAP structures in the Protein Data Bank as references, the BamA and bacteriocin PA166 amino acid sequences were homology modeled. The protein structures were then processed using the Protein Preparation Wizard tool on the Maestro 11.9 platform, including removal of water of crystallization, addition of hydrogen atoms and repair of chemical bonds and peptides. The prepared proteins were then

subjected to energy minimization and geometry optimization to ensure stability and accuracy. Finally, to assess the quality of the constructed protein models, the SAVES 6.0 online server software (<https://servicesnmbi.ucla.edu/SAVES/>) was used for model evaluation to ensure that the resulting protein models had good structural and functional predictive values.

Molecular docking of BamA with bacteriocin PA166 was performed and visualized using the HDOCK software. Except for the number of runs, which was set to 100 for the experiment, all docking parameters were kept at their default values. The docking results obtained were grouped into clusters using the Root Mean Square Deviation criteria, and the conformations within each cluster were ranked according to their energy levels. Subsequently, molecular docking validation was performed using HADDOCK (<https://rascar.science.uu.nl/haddock2.4/>), followed by binding affinity calculations employing PRODIGY (<https://rascar.science.uu.nl/prodigy/>).

Cloning and expression of the *bamA* gene

The *bamA* gene was amplified from *P. multocida* ATCC43137 genomic DNA using the following forward primer: 5'-GCGGATCCATGAAAACTTTTAATTG-3' and reverse primer: 5'-GTAAGCTTTTAGAACGTCCCACCAATG-3'. The PCR reaction was performed as follows: an initial denaturation at 98 °C for 5 min, then 94 °C for 1 min, 53 °C for 1 min, 72 °C for 130 s for 28 cycles, and a final extension step at 72 °C for 10 min.

The amplified PCR product and the pET-28a vector were digested with BamHI and HindIII enzymes, respectively. The digestion was performed at 37 °C for 12 h. The *bamA* gene was then ligated into the pET-28a vector to produce pET-28a-BamA. The ligation products were transformed into DH5α cells, and the plasmids were extracted. Finally, the recombinant plasmid pET-28a-BamA was further transformed into BL21 cells for expression.

BamA expression and purification

Protein expression and purification were performed following the procedures described in a previous study [18]. Transformed cells were inoculated into liquid LB medium containing a final concentration of 300 µg/ml kanamycin and cultured until the OD_{600} reached 0.5. Then 1 mmol/l isopropyl-β-D-thiogalactopyranoside (IPTG) was added and the cells were cultured at 37 °C, 150 rpm for 3 h. The cells were harvested by centrifugation at 8,000 g for 30 min and washed three times with PBS buffer. The cells were then disrupted by sonication and centrifuged at 8,000 g for 30 min at 4 °C to remove cell debris and intact cells. BamA protein was purified by nickel ion affinity chromatography. The molecular mass of BamA was visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using

a 12% resolving gel.

Effect of different concentrations of BamA on the antibacterial activity of bacteriocin PA166

To evaluate the effect of BamA on the antibacterial activity of bacteriocin PA166, a checkerboard assay was performed as described previously [19]. Briefly, 50 μ l of BamA (at concentrations of 4 μ g/ml, 16 μ g/ml, and 64 μ g/ml) and 50 μ l of bacteriocin PA166 (final concentration of 2 μ g/ml) were combined in MHB supplemented with 5% fetal bovine serum in a 96-well microtiter plate. *P. multocida* ATCC43137 was grown to an OD₆₀₀ of 0.6, then diluted to a final concentration of 10⁶ CFU/ml. A 100 μ l aliquot of this bacterial suspension was added to each well. Following incubation at 37°C for 18 h, the MIC [20] of bacteriocin PA166 under varying BamA concentrations were determined by measuring the OD₆₀₀ using a microplate reader (Shanghai, China). All experiments were performed in triplicate.

Statistical analysis

Statistical analyses were performed using one-way ANOVA with GraphPad Prism 9.0 software. Post hoc comparisons between group means were conducted using Tukey's multiple comparison test. Quantitative data are expressed as mean \pm standard error of the mean (SEM). Statistical significance is indicated as * p < 0.05 and ** p < 0.01.

RESULTS

Analysis of primer specificity

The specificity of the primers was confirmed by melting curve analysis. The results showed that each primer pair produced a single distinct melting peak at the expected annealing temperature (Fig. 1), with no amplification observed in the negative control lacking template cDNA.

Expression of the *bamA* gene in *P. multocida* ATCC43137 as determined by RT-qPCR

We analyzed the transcription of the *bamA* gene in *P. multocida* ATCC43137. Fig. 2 showed a significant decrease in *bamA* gene expression after treatment with bacteriocin PA166 compared to the control.

Homology modeling

To better understand the interaction between BamA and bacteriocin PA166, we predicted the three-dimensional structures of BamA and bacteriocin PA166 based on their amino acid sequences. The molecular structure of BamA was shown in Fig. 3a. The Ramachandran map showed that 89.7% of the amino acids (604 residues) were in the most favored regions and 9.8% of the amino acids (66 residues) were in additional allowed regions. In total, 99.9% of the

residues were in the most favored and additional allowed regions, suggesting that the overall structure was quite reasonable (Fig. 3b). Furthermore, the Global Model Quality Estimate (GMQE) value of the model is 0.75, indicating a high reliability of the overall structure prediction.

The molecular structure of bacteriocin PA166 was shown in Fig. 3c. The Ramachandran map showed that 83.9% of the amino acids (348 residues) were in the most favored regions and 14.7% of the amino acids (61 residues) were in additional allowed regions. In total, 99.5% of the residues were in the most favored and additional allowed regions, suggesting that the overall structure was quite reasonable (Fig. 3d). Furthermore, the model's GMQE value of 0.79 indicates a high level of reliability in the overall structural prediction.

Molecular docking

Docking results confirmed bacterial PA166 binds BamA (Fig. 4a). The results of the interaction analysis were shown in Table 1. The binding score of bacteriocin PA166 with the BamA protein was calculated to be -312.96. The Haddock validation results are consistent with those obtained from Hdock. According to PRODIGY analysis, the binding energy between the two is -18.9 kcal/mol. This low binding energy highlighted the stability and specificity of the complex formed. Detailed interaction analysis revealed that bacteriocin PA166 involved several amino acid residues, including THR-418, GLN-470, TYR-468, ASN-157, ASN-93, GLU-161 and ASN-483, which are critical for the formation of hydrogen bonds and other stabilizing non-covalent interactions. Similarly, the BamA protein contributed residues such as HIS-257, SER-190, SER-162, ARG-35, SER-36 and SER-424, which complemented the PA166 binding sites. These interactions created a robust network of hydrogen bonds, electrostatic attractions and hydrophobic contacts that collectively enhanced the stability and affinity of the complex. The interaction surface plot (Fig. 4b) illustrated the spatial complementarity between PA166 and BamA, indicating a high degree of structural compatibility. This strong fit underscored the potential functional role of PA166 in targeting and inhibiting BamA, which may be critical for its antibacterial activity.

Expression and purification of BamA protein

The recombinant plasmid pET-28a-BamA was heterologously expressed in BL21 as an N-terminal 6 \times His tag. Under IPTG induction, the recombinant bacteria expressed a protein of approximately 110 kDa (Fig. 5a). The BamA protein was purified by nickel ion affinity chromatography. SDS-PAGE analysis revealed a single band with apparent molecular weights of approximately 110 kDa (Fig. 5b).

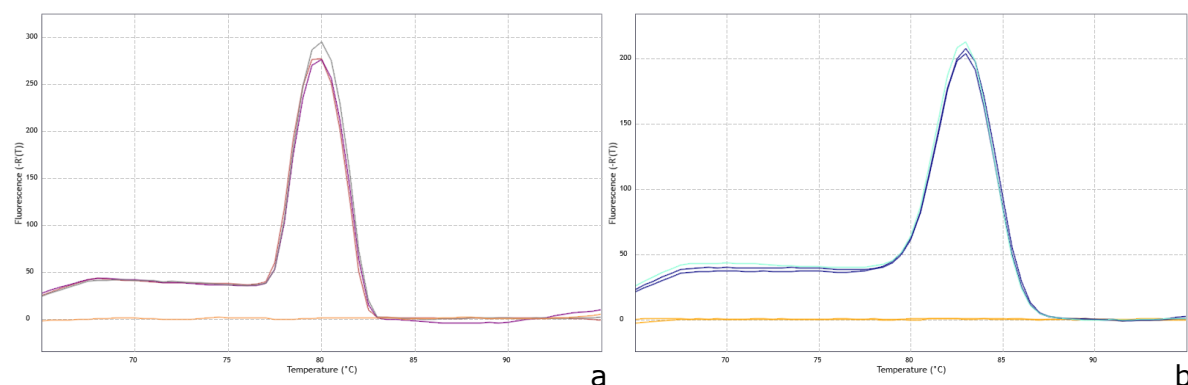


Fig. 1 Melting curve analysis of RT-qPCR products. (a) Melting curve of the *bamA* gene; (b) Melting curve of the 16S rRNA gene.

Table 1 Docking results of two targets.

Protein1	Protein2	Binding energy (kcal/mol)	Contact sites (protein1)	Contact sites (protein2)	Combination type
bacteriocin PA166	BamA	-312.96	THR-418, GLN-470, TYR-468, ASN-157, ASN-93, GLU-161, ASN-483	HIS-257, SER-190, SER-162, ARG-35, SER-36, SER-424	Salt bridge, Hydrogen bond, Hydrophobic interaction

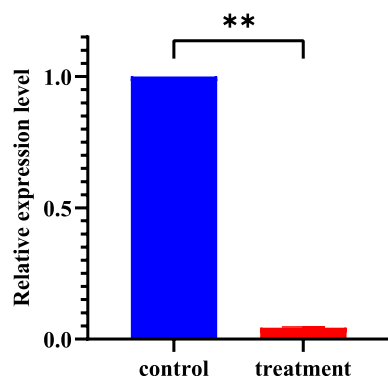


Fig. 2 Determination of *bamA* gene expression. Data are expressed as mean \pm SEM. * $p < 0.05$ and ** $p < 0.01$, significant differences were determined by Student's *t*-test for comparison with the control group.

Effect of different concentrations of BamA on the antibacterial activity of bacteriocin PA166

The effect of BamA on the antimicrobial activity of bacteriocin PA166 was verified by checkerboard studies. As shown in Fig. 6, the antimicrobial activity of bacteriocin PA166 was increased by the addition of different concentrations of BamA in a dose-dependent manner. The 2 $\mu\text{g/ml}$ bacteriocin PA166 against *P. multocida* ATCC43137 was increased to 4 $\mu\text{g/ml}$, 64 $\mu\text{g/ml}$ and 128 $\mu\text{g/ml}$ for BamA concentrations of 4 $\mu\text{g/ml}$,

16 $\mu\text{g/ml}$ and 64 $\mu\text{g/ml}$, respectively.

DISCUSSION

For Gram-negative bacteria, the outer membrane is an additional hurdle that antibiotics must overcome in their quest to eliminate the bacterium [13]. The outer membrane is an important organelle of Gram-negative bacteria and consists of four main components: lipopolysaccharides, phospholipids, β -barrel proteins and lipoproteins [21]. In addition, outer membrane proteins are indispensable components of bacterial cells and are involved in a variety of microbe-related functions. Changes in the composition of outer membrane proteins can alter bacterial pathogenicity and susceptibility to antibiotics, and Gram-negative bacterial outer membrane proteins are attractive drug targets.

The outer membrane, an indispensable feature of Gram-negative bacteria, harbors several potential targets for antibacterial action, such as BamA, which plays a crucial role in the folding and insertion of trans-membrane β -barrel proteins. BamA, which promotes the folding and insertion of outer membrane proteins, was the central component of the important BAM complex. Impaired BamA function results in defects in outer membrane proteins and impaired folding, leading to bacterial death [6, 22]. We therefore analyzed the transcription of the *bamA* gene in *P. multocida* ATCC43137 and found that *bamA* gene expression was significantly reduced after treatment with bacteriocin

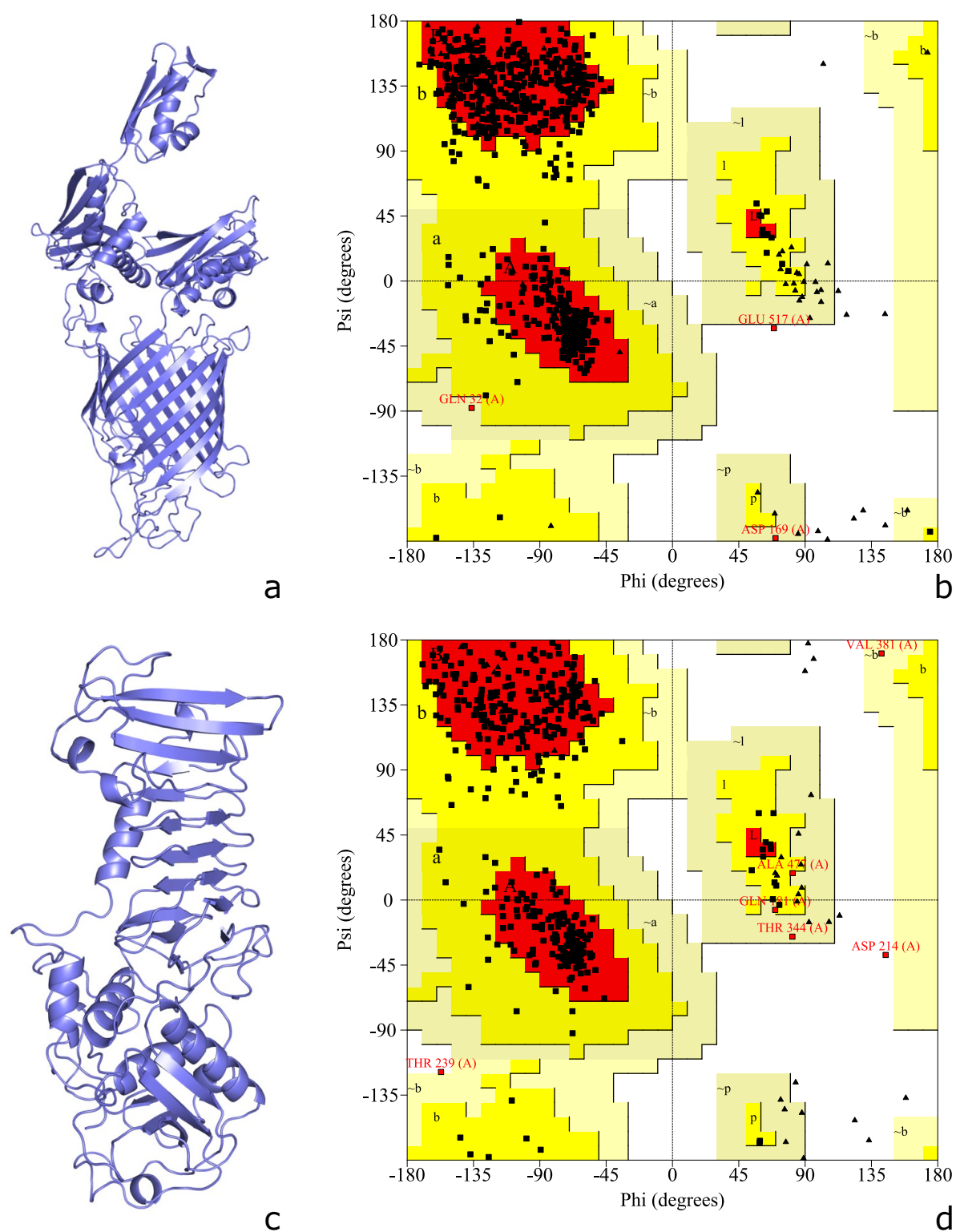


Fig. 3 Homology modeling of bacteriocin PA166 and BamA. (a) The 3D structure and electrostatic surfaces of BamA; (b) Ramachandran plot of the BamA model; (c) The 3D structure and electrostatic surfaces of bacteriocin PA166; (d) Ramachandran plot of the bacteriocin PA166 model.

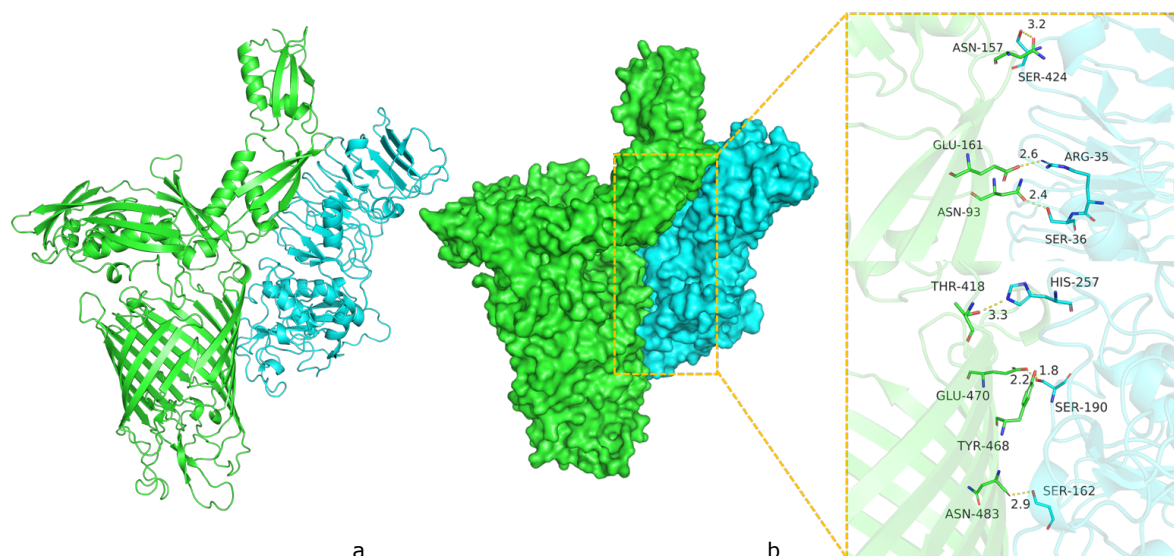


Fig. 4 Molecular docking of bacteriocin PA166 and BamA. (a) The binding mode of the complex between bacteriocin PA166 (blue) and BamA (green); (b) bacteriocin PA166 and BamA proteins are represented by the surface model; yellow lines indicate hydrogen bonds or salt bridges.

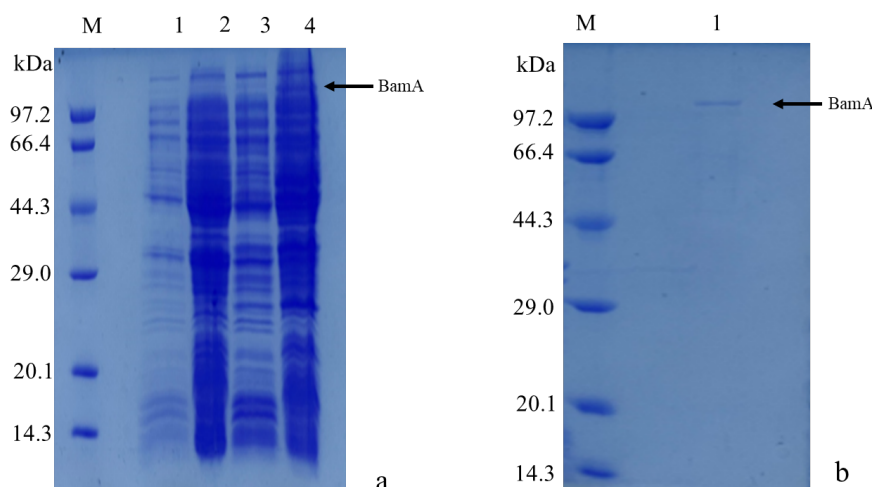


Fig. 5 Expression and purification of BamA protein. (a) SDS-PAGE analysis showing the expression of pET-28a-BamA; M: molecular standard marker; 1: pET-28a vector before induction; 2: pET-28a vector after induction; 3: pET-28a-BamA recombinant bacteria before induction; 4: pET-28a-BamA recombinant bacteria after induction; (b) SDS-PAGE analysis of the purified recombinant BamA; M: molecular standard marker; 1: purified BamA.

PA166. This phenomenon may be due to the inhibition of BamA function by PA166, which in turn triggers a negative feedback regulatory mechanism in the cell to lower the expression of the *bamA* gene to reduce the synthesis of outer membrane proteins, thereby alleviating cellular stress and maintaining homeostasis.

Beta-barrel outer membrane proteins are the major protein components of the outer membrane of Gram-negative bacteria and these proteins play key roles in cell structure, morphology, nutrient acquisition,

colonization, invasion and defense against external toxic threats such as antibiotics [23]. Molecular docking results indicate that bacteriocin PA166 can bind to BamA, an outer membrane protein of *P. multocida* ATCC43137, forming various interaction modes with the contact residues, including hydrogen bonds, salt bridges, and hydrophobic interactions. These interactions are critical for stabilizing the complex and may ultimately lead to BamA misfolding, thereby contributing to the antimicrobial activity of PA166. Hy-

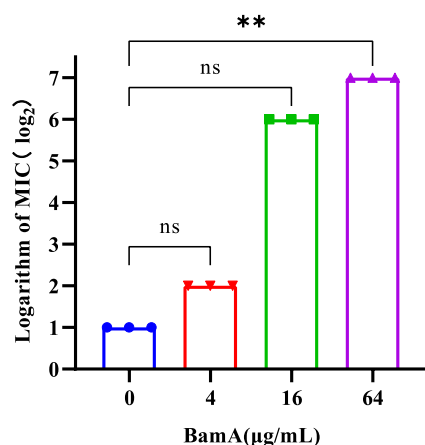


Fig. 6 Effect of different concentrations of BamA on the antibacterial activity of bacteriocin PA166. Data are the mean \pm SEM, $n = 3$. ** $p < 0.01$, ns = not significant.

drogen bonds and hydrophobic interactions are crucial for maintaining the stability of the interactions [24]. At the same time, salt bridges also have a significant effect on the stability and solubility of proteins [25]. Molecular docking predictions were primarily carried out using HDock, which demonstrates high accuracy in modeling peptide-protein interactions. However, its rigid docking approach may not fully account for conformational changes that occur during binding. To enhance the reliability of the predicted binding modes, we further validated the results using HADDOCK. In addition, we employed PRODIGY to estimate the binding affinities of the complexes, thereby providing further support for the stability of the interactions. Previous studies have shown that antimicrobial peptides such as 11pep and D-11pep can bind to specific β -strands (e.g., $\beta 1$, $\beta 9$, $\beta 15$, and $\beta 16$) of BamA, leading to protein misfolding and antimicrobial activity [13]. Matano et al showed that the target of bacteroidetocins is an aspartic acid residue located at the beginning of loop 3 of the outer ring of the BamA protein [26]. Molecular docking showed that the designed antimicrobial peptides were able to polymerize BamA, causing misfolding of the outer membrane protein and exerting an antimicrobial effect [27].

Darobactin exhibits potent inhibitory activity against clinically important Gram-negative pathogens by compromising the integrity of the bacterial outer membrane through its action on BamA [2]. Ghequire et al also discovered that heterologous expression of the *bamA* gene renders resistant strains sensitive to LlpA [10]. Our competition binding assay revealed a significant reduction in the antimicrobial activity of bacteriocin PA166 upon addition of varying concentrations of BamA. This compelling evidence supports the view that BamA is likely to be a specific target of bacteriocin PA166. Hart et al [3] also suggested

that the compound MRL-494 inhibits the synthesis of outer membrane proteins in Gram-negative bacteria by specifically binding to BamA and exerting inhibitory effects. A thorough understanding of the different factors that affect BamA-mediated insertion and folding of outer membrane proteins will facilitate the rational design of novel antibiotic compounds targeting the BAM complex and hopefully advance our efforts to address the current antimicrobial resistance crisis [28]. The interaction between bacteriocin PA166 and BamA disrupts outer membrane protein folding and compromises membrane integrity, leading to cell death. By targeting BamA, a core component of the BAM complex, PA166 overcomes the outer membrane barrier, a major challenge for conventional antibiotics. With its potent antibacterial activity, low toxicity and stability, PA166 shows promise as a novel therapeutic, either alone or in combination with other antibiotics. However, challenges remain in clinical development, including optimizing pharmacokinetics, assessing toxicity and improving bioavailability. Overcoming these could make PA166 a valuable tool against resistant infections.

CONCLUSION

In this study, molecular docking and RT-qPCR analyses demonstrated that PA166 targets BamA, with checkerboard assays further validating that exogenous BamA attenuates the antimicrobial efficacy of PA166, conclusively identifying BamA as the functional target of PA166. These results provide valuable insight into the mechanism of action of PA166 and highlight its potential as a promising candidate for the development of novel antibacterial agents, particularly in addressing the growing challenge of antibiotic resistance. However, this study has some limitations. As molecular docking is a predictive technique, the results require further validation. Future research will use techniques such as gene knockout, immunoprecipitation or isothermal titration to fully elucidate the mechanism of bioactivity, which will be critical to advance PA166 towards clinical application and contribute to the development of new therapeutic strategies against multidrug resistant pathogens.

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