

Comprehending the outer membrane protein modelling of pathogenic Gram-negative bacteria for *in silico* antibiotics screening

Rudi Nirwantono^{1,3,*}, Alam Ahmad Hidayat², Angelica Laura Kusnadi¹, Fitya Syarif Mozar⁴, Alyssa Imani⁴, and Bens Pardamean^{3,4}

¹Biotechnology Department, Faculty of Engineering, Bina Nusantara University, Indonesia 11480

²Mathematics Department, School of Computer Science, Bina Nusantara University, Indonesia 11480

³Computer Science Department, BINUS Graduate Program, Bina Nusantara University, Jakarta, Indonesia 11480

⁴Bioinformatics and Data Science Research Center, Bina Nusantara University, Jakarta, Indonesia 11480

Abstract. Pathogenic Gram-negative Bacteria (GNB) continue to become a major challenge in the modern public health, as the development of new antibacterials against pathogenic GNB is hindered by the emergence of multidrug-resistant (MDR) strains. Thus, the discovery of new antibacterial drugs to overcome the MDR bacteria must remain an ongoing effort. One of the promising strategies is to utilize computational molecular simulations to understand interactions between the target protein and various drug candidates. β -barrel assembly machinery subunit A (BamA) has recently gained attention as a potential target protein. Yet, the availability of the 3D structure of the protein from various species of GNB is still limited and hinders the drug discovery studies. In this study, a pipeline to construct BamA protein model from pathogenic MDR strains of *Klebsiella pneumoniae* HS11286 and *Shigella flexneri* A was established using Modeller 10.5, followed by refinement and models evaluations. The BamA sequences from *K. pneumoniae* and *S. flexneri* used in this pipeline shared about 91.01% and 99.75% identity, subsequently, with BamA from *Escherichia coli* K12. The final refined models of BamA proteins from both bacteria showed optimal structural characteristics, as reflected in all assessment parameters. Thus, the modelling pipeline performed in this preliminary study is promising to facilitate the *in-silico* screening study in searching for new antibiotics for combating Gram-negative pathogens.

1 Introduction

Pathogenic Gram-negative Bacteria (GNB) continue to become a significant challenge in the modern era due to the development of antimicrobial resistance against multiple antibiotics [1]. The World Health Organization (WHO) estimated that about 1.27 million fatal cases occurred globally due to drug-resistant infections in 2019 [2]. Moreover, about 10 million

* Corresponding author: rudi.nirwantono@binus.ac.id

annual mortal cases attributed to the AMR crisis are also projected to occur in 2050 [2,3]. The Centers for Disease Control and Prevention (CDC) identified the multidrug-resistant Gram-negative bacteria (MDR-GNB), including *Klebsiella pneumoniae* and *Shigella flexneri*, that are ineffective for the current antibiotic treatments, contributing more threats to the AMR issue [4,5].

On the other hand, the development of new antibacterials against pathogenic GNB is argued to be at the same pace as the emergence of multidrug-resistant (MDR) strains [6,7]. Recently, antimicrobial development has seen little or no progress due to the limited number of newly developed antibiotics. It leads to a complicated situation in medical practice due to limited options to effectively overcome infection caused by MDR-GNB [6]. Consequently, discovering new antibacterial drugs and novel drug targets for exploring promising strategies combating MDR-GNB remains an ongoing challenge.

The cell of GNB is covered by a semi-permeable outer membrane (OM) that highly depends on integral proteins to exchange molecules. It also provides an extra protector for GNB against antimicrobial substances by prohibiting them from penetrating the cell or providing an efflux pump for harmful substance removal, which supports the GNB to develop an extraordinary drug-resistance characteristic [8]. One of the most notable integral proteins in OM of GNB is the β -barrel assembly machinery (BAM) complex for its irreplaceable function as a helper for other outer membrane proteins (OMP) to fold into OM appropriately. During the protein translocation into the OM, the immature protein will be transported from the cytoplasmic space into the intermembrane space through the Sec export machinery. Several chaperones complex stabilizes the protein in its linear form in the intermembrane space [9]. Eventually, the β -barrel assembly machinery (BAM) complex regulates the insertion of the protein into OM by guiding the formation of amphipathic β -barrel structures [10,11].

In *Escherichia coli*, the BAM complex consists of BamA, BamB, BamC, BamD, and BamE. Among them, BamA is the only integral subunit in the complex that is embedded into OM with its β -barrel domain. Lately, this subunit has been explored as a drug target due to its key roles in OMP assembly. Two novel promising antibiotics, aerobactin and dynobactins, have been studied and have shown strong inhibitory activity against BamA function. They can also selectively kill GNB pathogens [12,13].

Despite providing a promising drug target, the 3D crystal structures of these pathogenic MDR-GNB have not yet been available in the Protein Data Bank (PDB database). On the hand, a massive drug screening can only be achieved when the correct 3D crystal structures are available [14]. Hence, the generation of the 3D structure of BamA from pathogenic MDR-GNB can initiate and foster the *in-silico* screening for potential drug targets.

Recently, computational structure predictors are increasingly reliable for modelling putative proteins. Homology modelling, which relies on a specific existing crystal structure as a template to model putative homologous proteins, is widely used. Meanwhile, *de novo* modelling such as AlphaFold only utilizes the primary sequence of amino acid information to predict tertiary structures [15]. However, homology modelling typically yields higher quality structures than the *de novo* modelling if high-quality templates of the given protein are available in the database, such as PDB [16]. Several studies demonstrated the success of computational 3D protein modelling, including *Bacillus cereus* M4 metalloprotease protein [14], *Pasteurella multocida* S-Adenosylmethionine-Dependent Methyltransferase [17], and RD7 region from *Mycobacterium tuberculosis* [18]. Moreover, the same strategy can be also employed to study the mutation effect on the structural change of bacterial N-glycosylation reporter protein [19]. The generated models can subsequently be used to study drug-protein interaction or other dynamic properties in protein studies.

Therefore, this study employed the homology modeling approach to generate the 3D structure of putative β -barrel assembly machinery subunit A (BamA) from multidrug-

resistant *Klebsiella pneumoniae* and *Shigella flexneri* strains. Various model assessments were conducted to check the quality of the models. Finally, the quaternary structure of the enzyme was displayed and compared with existed enzyme from *Escherichia coli* K12 using Pymol software.

2 Method

2.1 BamA Sequence Retrieval and Template Preparation

The 3D modelling was performed using homology modelling using the query sequence and the template obtained from protein database (NCBI database and PDB database) (**Fig.1**). The protein sequences used in this study were from *Klebsiella pneumoniae* HS11286 and *Shigella flexneri* A. Both sequences were retrieved from the Whole Genome Sequencing (WGS) of Multidrug Resistant strains of *Klebsiella pneumoniae* HS11286 (GeneBank Assession: CP003200.1) and *Shigella flexneri* A (GenBank Assession: CP097840.1) according to the annotation reports provided by the authors [20,21]. The sequences of BamA from both bacteria were then downloaded and compared with the sequence of BamA from *Escherichia coli* K12, which it became the first BamA as drug target studied by Kaur et al. [12,13]. The template search was then performed against the PDB database in the www.rcsb.org platform, with the BamA protein sequence from each species used as the query sequences.

2.2 Homology Modelling and Model Selection

The generation of the three-dimensional (3D) structure of BamA from *Klebsiella pneumoniae* HS11286 and *Shigella flexneri* A was performed using a homology modeling approach. The homology modeling was based on the sequence homology between the query sequence and the sequence owned by the protein crystal structure in the database (template protein) [22]. In other words, the construction of a 3D model of the query sequence was based on the conserved primary structure (amino acid sequence) of the query sequence with its template protein.

The workflow of the homology modeling in this work is summarized in **Fig.1**. The 3D modeling was performed in Modeller 10.6 software with Python 3.11.5 framework. A crystal structure from *Escherichia coli* K12 (PDB assession: 5D0O) from the PDB database was used as a template protein to construct the 3D models of BamA protein from *Klebsiella pneumoniae* HS11286 and *Shigella flexneri* A due to its high similarity with both queries sequence. The model generation was started with the file extension conversion from the FASTA file to the ALI file using a script. In Modeller 10.6, the **Alignment** command was used to align the sequence from the query with the template. Then, the default algorithm via **AutoModel** command, which operates based on the satisfaction of spatial restraints approach, was used to generate ten different models. The model generation was based on alignment-guided construction under CHARMM22 force field implementation to estimate the geometry (angles and distances) of the modeled protein based on the geometrical structure of the template. Finally, the **assess_dope** command was used to generate the DOPE score of each model. The best model with the most negative DOPE score was then selected for downstream analysis.

2.3 Model Optimization and Evaluation

The model refinement was performed to reduce the free energy of the model to its lowest and optimize the structural geometry. In this study, Galaxy Refine Server was utilized in the

model refinement [25,26]. The structural optimization using Galaxy Refine Server was based on molecular dynamics simulation using Gromacs software. The selected model from the previous step was uploaded onto the server, and the server provided the result.

The optimized model based on MolProbity score, Clash score, poor rotamers, and Rama favoured parameters was then downloaded and evaluated based on several parameters including stereochemical accuracy (QMEANDisCo Global on SwissModel), the rotary angel of each amino acid (ProCeck on <https://saves.mbi.ucla.edu/>), and the Z-score position of the model among existing PDB structures. The calculation was conducted by uploading the optimized model onto these web servers and collecting the calculation result. Geometrical changes before and after model refinement were assessed visually using PyMol software.

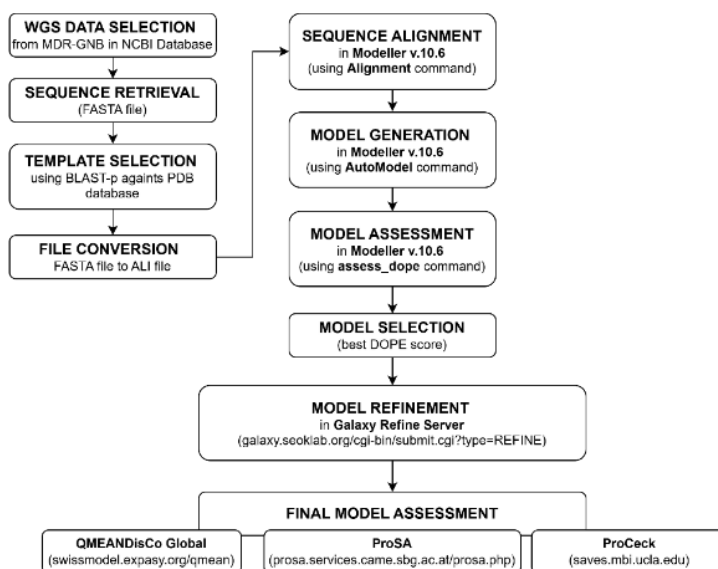


Fig. 1. Pipeline of the homology modelling to generate a 3D model of a protein using Modeller 10.6.

3 Results and Discussion

Based on the template selection in PDB using the primary sequence of BamA from *Klebsiella pneumoniae* HS11286 (AFI95226.1) and *Shigella flexneri* A (UVE88604.1) as the searching query, the crystal structure of BamA from *Escherichia coli* K12 (PDB assession: 5D0O; 2.90 Å) was selected to become the template for homology modeling. The sequence alignment revealed that the sequence identities of the template with the BamA sequence from *Klebsiella pneumoniae* and *Shigella flexneri* were 91.01 % and 99.75%, respectively (Table 1). Meanwhile, the query coverage between them was 100% for all queries.

Table 1. Sequence comparison of BamA from *Klebsiella pneumoniae* HS11286 (AFI95226.1) and *Shigella flexneri* A (UVE88604.1) with BamA from *Escherichia coli* K12

Species	Identity	Query Cover	Total length	Region	Note	Accession
<i>Klebsiella pneumoniae</i> HS11286	91.01%	100%	809 aa	PRK11067	MDR	AFI95226.1
<i>Shigella flexneri</i> A	99.75%	100%	810 aa	PRK11067	MDR	UVE88604.1

By employing the procedure mentioned in sections 2.2, the generation of 3D models of BamA protein from *Klebsiella pneumoniae* HS11286 (AFI95226.1) and *Shigella flexneri* A (UVE88604.1) was successfully carried out. The program selected the best model out of 20 models generated for each bacterium in the pipeline, with the most negative DOPE score. The BamA protein model from *Klebsiella pneumoniae*, subsequently called KpBamA, has a DOPE score of -77289, while BamA from *Shigella flexneri* or SfBamA was -78097. However, the DOPE score from the model is still higher than from the template, which was -78911, reflecting that the quality of both models was slightly lower compared to the template. The deviation of amino acid positioning in protein geometry commonly affects the energy entropy in the models and causes a lower score in the modelled protein [23]. The models of KpBamA and SfBamA are displayed in Fig.2.

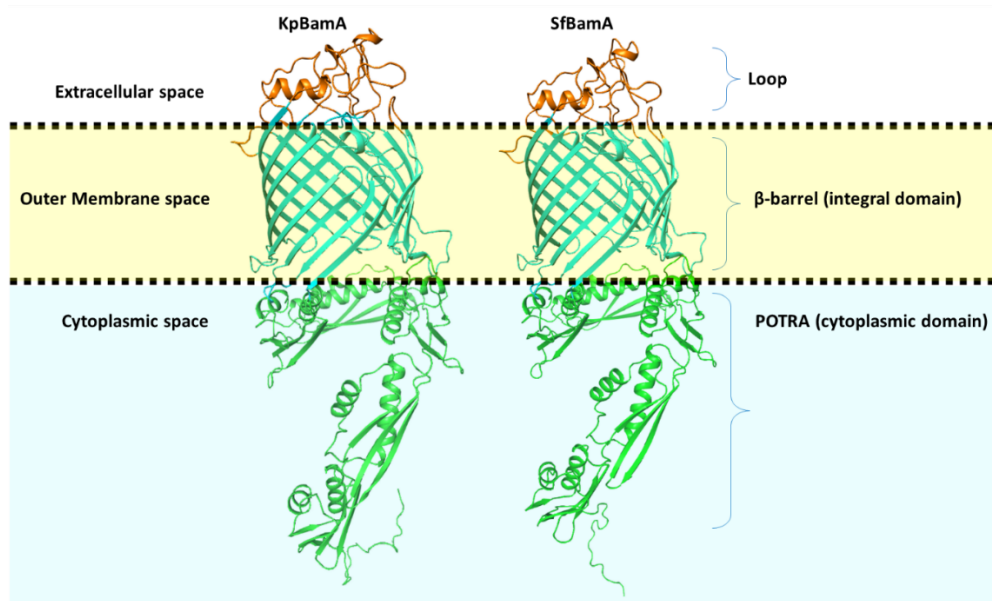


Fig. 2. The optimized 3D structure of BamA from Multidrug Resistant (MDR) strains of *Klebsiella pneumoniae* HS11286 (KpBamA) and *Shigella flexneri* A (SfBamA) modelled using Modeller 10.2 based on homology modeling with BamA from *Escherichia coli* K12 (Accession: 5D0O) as the template. KpBamA and SfBamA are homolog and show two main domains, integral (transmembrane β -barrel) (cyan color) and cytoplasmic (green color) (Polypeptide Translocation-Associated / POTRA) domains, with loops (orange color) in extracellular space. The red color is the binding site of antibiotic darobactins according to crystal structure of BamA from *Escherichia coli* K12 (Accession: 7NRE).

The KpBamA and SfBamA models displays a C-terminal transmembrane β -barrel domain consisting of 16 antiparallel β strands. The structure is highly conserved among all BamAs [12,24]. It forms a hollowed pocket in the center of the structure at which the top of the barrel is domed by three extracellular loops (eL4, eL6, and eL7). It also has lateral gate between by β 1 and β 16. Conversely, the periplasmic N-terminal domain consists of five polypeptide translocation-associated (POTRA) domains extending from the barrel. According to Noinaj et al. [24], the periplasmic domain is pivotal in protein-protein interaction with other Bam subunits (BamB, BamC, BamD, and BamE).

The models' refinement using Galaxy Refine Server also resulted in better structural configuration for both the KpBamA and SfBamA models. These were reflected in the lower DOPE scores of refined models compared with unrefined models. The DOPE score of refined

KpBamA reduced from -77289 to -83244, while in SfBamA declined from -78097 to -84150 (**Fig. 3a**). The final models eventually had better quality base on DOPE scope since their score were lower than score from template (-78911). **Fig.4**. also revealed that DOPE scores of each amino acid residue in the refined models (blue lines) were lower than the unrefined models (red lines) and even from the template (green lines), indicating a better geometrical structure of each amino acid in the refined models. A molecular dynamic-based optimization on a server-based Gromacs platform successfully optimized the geometry of each amino acid by amino acid's side-chain repacking in the model and offered the minimum energy state [25]. The optimum configuration state may reflect the native state of the protein in the cellular settings so that it supports the downstream analysis, such as drug docking analysis [28,29].

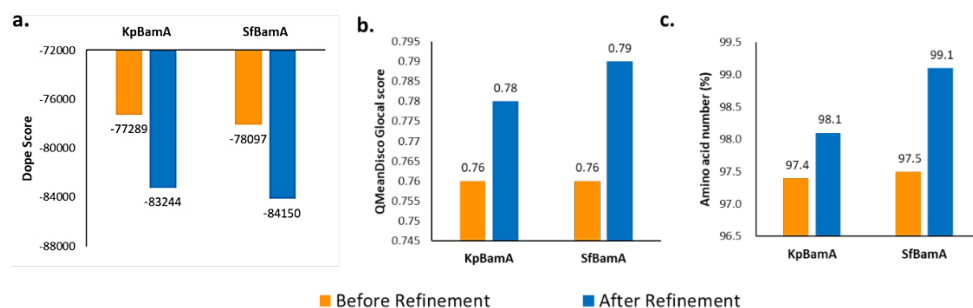


Fig. 3. Scoring Comparison between before (orange bar) and after (blue bar) model refinement. All parameters display an improvement score in both KpBamA and SfBamA, including for DOPE score (a), QMEANDisco Global Score (b), and the number of amino acid residues in favourable area of Ramachandran plot (c).

In addition, **Fig. 3b** also indicated that the quality of the KpBamA and SfBamA models is better according to the QMEANDisco Global score. The QMEANDisco Global parameter indicated the expected pairwise $C\alpha$ - $C\alpha$ distances and provided error estimation. The QMEANDisco Global scores of the KpBamA model are 0.76 for the unrefined and 0.78 for the refined model, while for the SfBamA model are 0.76 for the unrefined and 0.79 for the refined model, suggesting that the quality of the residue in the refined models are slightly improved [30]. Overall, all unrefined and refined models are higher than the minimum cut-off score of 0.6, indicating high quality structures.

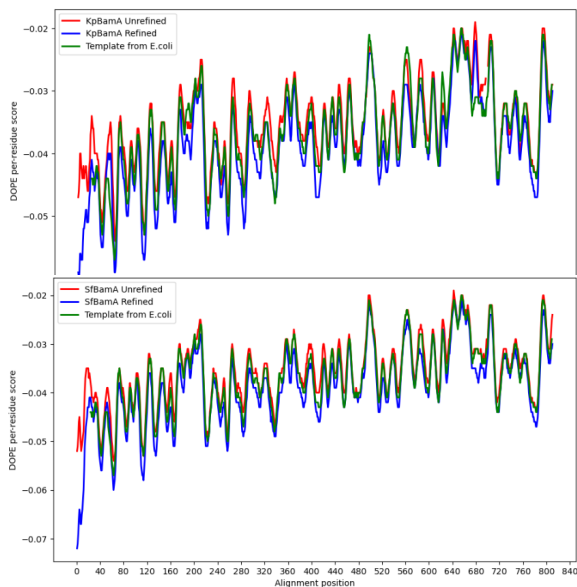


Fig. 4. The graphs display the comparison between DOPE score of each amino acid residues from before (red lines) and after model refinement (blue lines) of KpBamA model (a) and SfBamA model (b). The DOPE score from template is also shown in the graphs (green lines).

Further, a better repacking of amino acid side-chain is also indicated by the number of amino acids in favorable areas in Ramachandran plot (**Fig. 3c**). More number of amino acids in favorable indicates that the torsional angles (the phi (ϕ) angle, and the psi (ψ) angle) distribution of each amino acid residue in a protein's backbone were optimal for certain secondary structure, including α helix and β sheet [32]. The model refinement increased the number of amino acids in favorable areas by 1.1% in KpBamA from 97.4% to 98.5% and 1.6% in SfBamA from 97.5% to 99.1%. The results indicate that high quality models were achieved according to amino acid geometry.

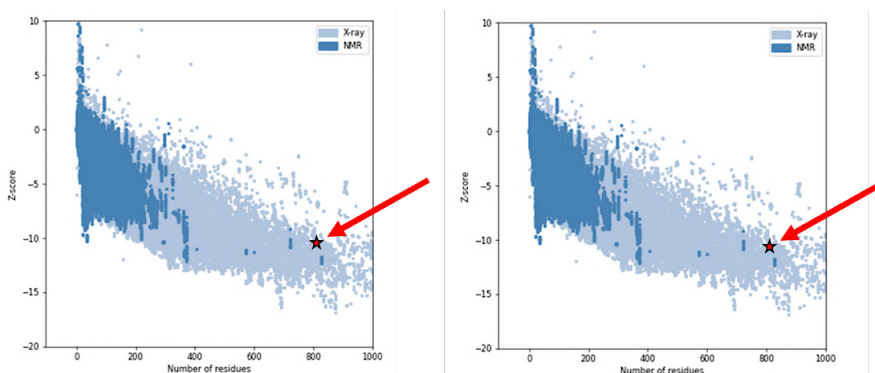


Fig. 5. The figure shows the position of the model (red star with red arrow) of KpBamA (a) and SfBamA (b) among identified 3D structure of other proteins in PDB database according to its Z-score.

Furthermore, the ProSA score indicated the model was relatively good, with a Z-score of -10.41 and -10.65 for KpBamA and SfBamA, subsequently. The Z-score-residue length ratio for both models reflected the position of the model of a protein in the ProSA analysis plot compared to all native proteins existing in the PDB database [29]. **Fig. 5** shows the position of the model among existing PDB structures, indicating that the model was considerably still in the range characteristic of a native protein.

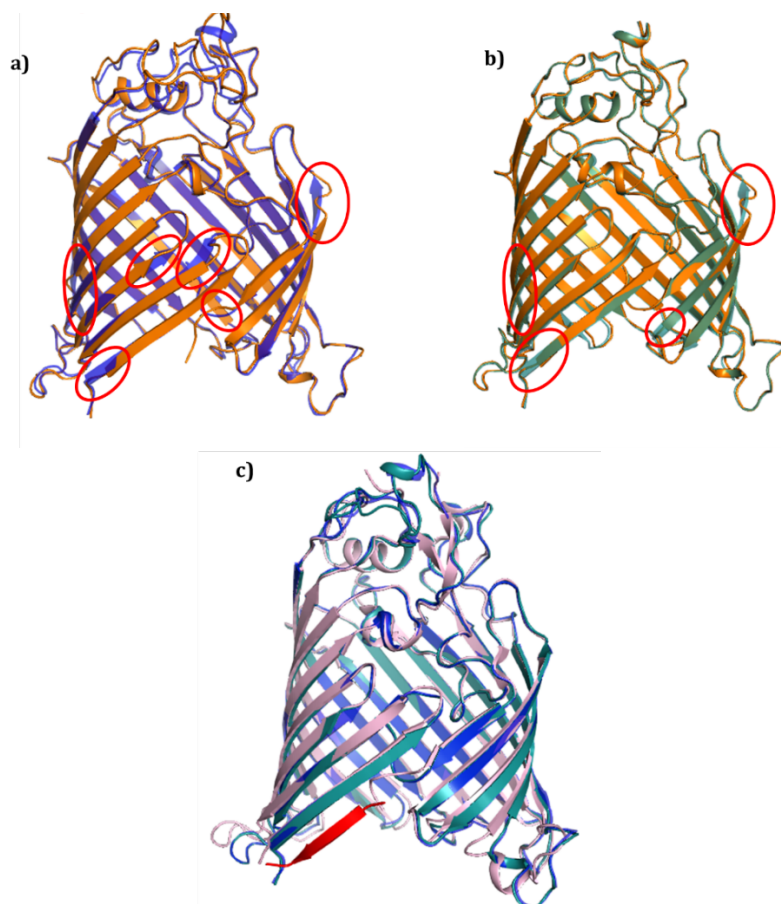


Fig. 6. The figures shows the transmembrane domain of KpBamA (a) and SfBamA (b) in comparison between refined (blue & green models) and unrefined model (orange model) with the highlights on the structural alterations (red circles). The refined models of KpBamA and SfBamA were also superimposed with crystal structure of BamA from *Escherichia coli* K12 (Accession: 7NRE) (c) with antibiotic darobactins bound to it.

Eventually, **Fig. 6a** and **6b** disclose the visualization of KpBamA and SfBamA to compare before and after model refinement, focusing on the transmembrane domain. This domain is common target for drug candidates such as darobactin, dynobactin [12,13,30], MRL-494 [31], VUF15259 [32], PTB1 and PTB2 [33] that shared different binding poses to the domain. The structural comparison shows a high similarity before and after the refinement. However, some structural alterations (red circles) were also revealed due to model refinement, especially in $\beta 1$, $\beta 2$, $\beta 3$, $\beta 5$, and $\beta 15$. In addition, the superimposition between models and template of BamA from *Escherichia coli* K12 shows some slight differences, occurring only due to the difference in the amino acid at certain positions, including in the loops.

4 Conclusion

The result of this study revealed that the BamA sequences from multidrug-resistant (MDR) strains of *Klebsiella pneumoniae* HS11286 (AFI95226.1) and *Shigella flexneri* A (UVE88604.1) shared about 91.01% and 99.75% sequence identity with BamA from *Escherichia coli* K12. Those proteins were also successfully modelled using Modeller 10.2, employing the crystal structure of BamA from *Escherichia coli* K12 as the modelling template. The structure is known to be conserved and consists of a transmembrane domain with 16 antiparallel β -sheet and a periplasmic domain with 5 Polypeptide Translocation-Associated (POTRA) domains.

The final refined models of BamA proteins from both bacteria (KpBamA and SfBamA) showed optimal structural characteristics, as reflected in all assessment parameters. After model refinement, the model's evaluation parameters, including the DOPE score, QMEANDisco Global score, and Ramachandran plot. Furthermore, both models' Z-score over sequence length (ProSA parameter plot) is also optimal, suggesting that the models are in the range characteristic of the native protein. All parameters indicated that all refined models were considered good and ready for downstream analysis, including drug target analysis using molecular docking approach followed by molecular dynamic analysis.

Although the pipeline lacks a pipeline to estimate B-factor, which is important to understand the flexible part in the model geometry, the modeling pipeline carried out in this preliminary study is promising to facilitate the *in-silico* screening study in searching for new antibiotics to combat Gram-negative pathogens. The parameters, including binding affinity and stability, collected from the future drug discovery studies, could be compared with the existing references for promising drug candidates.

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