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Research Idea

Targeting *Proteus mirabilis* BAM Complex Proteins for Development of Novel Antibiotics

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Abstract

Urinary tract infections (UTIs) are frequent hospital-acquired infection, with *Escherichia coli* and *Proteus mirabilis* accounting for 90% of complicated UTIs. Emergence of multidrug-resistant (MDR) bacteria have complicated the treatment UTIs. *P. mirabilis* related UTIs has been associated with the production of urinary stones and long-term infections in patients with catheters. *P. mirabilis* and other uropathogens constitute a largely unexplored pathogen group. The pathogen is resistant to most antibiotics as a result of its impermeable outer membrane (OM). The β-barrel assemble machinery folds and inserts outer membrane proteins; however, there are no antibiotics targeting the OM assemble in clinical use currently. Therefore, this study seeks to identify drugs that will inhibit the activity of *P. mirabilis* B complex proteins and also determine their effects on *P. mirabilis* OM biogenesis. This would be achieved by screening approved drugs against the *P. mirabilis* Bam complex using computer-based *in silico* screening and cellular-based assays. First, the binding effects of drugs on *P. mirabilis* B complex proteins will be determined using docking algorithms. The antimicrobial and antivirulence activity of selected drugs from *in silico* analysis will be screened against MDR *P. mirabilis*. Finally, the effect of active drug(s) on the OM biogenesis of wild-type *P. mirabilis* and mutant *P. mirabilis* will be determined

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using peptide nucleic acids (PNA). Western lot analysis will be used to determine the abundance of proteins involved in OM biogenesis. Successful completion of this study will lead to the identification of novel antibiotics against MDR *P. mirabilis* and associated mechanisms while providing the foundation for future research endeavours on other uropathogens.

Keywords

rinary tract infections, *Proteus mirabilis*, antibiotics, urinary stones, outer membrane, β-barrel assemble machinery, Peptide Nucleic Acid

Introduction

Proteus spp. are Gram-negative rod-shaped bacteria belonging to the Enterobacterales order and the Morganellaceae family. They are found naturally in the guts of animals and humans, and their existence in the surroundings is attributed to faecal contamination (O'Hara et al. 2000). There are six species within its genus and *P. mirabilis* is the most common (O'Hara et al. 2000). Ninety percent of clinical isolates of *P. mirabilis* are urinary tract infections (UTIs) associated (Schaffer and Pearson 2016). UTIs are among the most frequent bacterial illnesses, annually affecting over 150 million individuals globally, in male children, older men and women of all ages (Flores-Mireles et al. 2015, Stamm and Norrby 2001). *P. mirabilis* is predominantly responsible for UTIs in type 2 diabetes patients and indwelling catheters. It is the main causative organism for complicated UTIs (Armbruster et al. 2018). They complicate UTIs by expressing urease, leading to the formation of kidney stones (Schaffer and Pearson 2016). The kidney stones serve as a nidus for non-*P. mirabilis* bacteria to establish UTI and thus leading to treatment failure (Torzewska and Rozalski 2014). , the WHO classified *P. mirabilis* as a critical pathogen due to its resistance to third-generation cephalosporin (e.g. ceftriaxone) and last line of antibiotics against resistant bacteria such as carbapenems (Shrivastava et al. 2018).

Global public health is seriously threatened by antimicrobial resistance (AMR). The spread of AMR is aided by the misuse of antibiotics (Wang et al. 2021). *Proteus mirabilis* AMR is attributed to reduced expression of penicillin-binding proteins or via carbapenemase and cephalosporinases (Mata et al. 2010). Aside the enzymatically mediated AMR, the outer membrane (OM) of *P. mirabilis* plays an essential role in AMR (Girlich et al. 2020). This is because most antibiotics are designed to target intracellular processes in Gram-negative bacteria. However, the OM in Gram-negative bacteria restricts the permeability of many antibiotics (e.g. imipenem) (Delcour 2009). The significance of the OM barrier in antibiotic sensitivity is demonstrated by the development of drug-resistant strains in a wide variety of bacterial species due to alterations in the lipid or protein composition of the OM (Delcour 2009).

The OM is made up of β-barrel proteins that facilitate the binding and folding of the β-barrel complex (BAM complex) (Ricci and Silhavy 2019). The B complex is made up of five

components Bam ABCDE, with Bam A and D as the only essential protein conserved in all Gram-negative bacteria (Kim et al. 2012). The BAM complex is a new drug target for developing novel antibiotics (Hart et al. 2019, Li et al. 2020). This is due to its essential role in the formation of OM proteins and therefore a good target against drug resistant *P. mirabilis*. Currently, there are no antibiotics that inhibit the B complex proteins.

On the surface of Gram-negative bacteria are adhesion proteins which aid in the interaction between the pathogen and host by initiating infections and coloniing host cells Vaca et al. 2019. In *P. mirabilis*, the mannose-resistant *Proteus*-like A (*mrpA)* is the main structural subunit of the *mrp* operon Bahrani and Mobley 1994 and together with *Proteus mirabilis* fimbria *(pmfA*) contribute to biofilm formation, infection of the bladder and coloniation of the kidney Schaffer and Pearson 2016. Recent studies have shown that inhibition of these adhesion protein is essential for identifying novel antivirulence drugs against multidrug resistant Gram-negative bacteria Dehbanipour and Ghalavand 2022, Pecoraro et al. 2023.

Drug repurposing and repositioning are effective in the development of new and effective drugs to circumvent the challenges of AMR. This approach saves time and its cost-effective (Rudrapal et al. 2020). In comparison to traditional drug discovery programmes, repurposing drugs ha the potential to reduce the high financial expenses, prolonged development time and higher risk of failure (Xue et al. 2018). Therefore, this study would leverage *in silico* informatic tools with complementary drug assays for *P. mirabilis* associated UTIs.

Objectives

The research objectives include screening for drugs with activity against *P. mirabilis* B complex proteins using *in silico* computer-based method; determination of antimicrobial and antivirulence activities of selected drugs on *P. mirabilis* using *in vitro* drug assays; and the effects of identified drugs on the OM biogenesis of wild-type and mutant *P. mirabilis* would be determined. These objectives will be achieved as described (Fig. 1).

Background

Urinary Tract Infections (UTI)

UTI is a serious health problem caused by bacteria. Most hospitals regularly observe this globally, with 150 million cases annually (Stamm and Norrby 2001b). Uncomplicated UTIs often affect healthy people without neurological or anatomical urinary tract complications. These infections are divided into cystitis or pyelonephritis. Cystitis risk factors include female gender, vaginal infection, a previous UTI, diabetes, obesity, sexual activity and genetics (Foxman 2014). "Complicated UTIs" refer to UTIs caused by conditions that compromises the host immune system, such as urinary tract obstruction, neurological illness or immunosuppression, renal failure or transplantation, pregnancy, or the of calculi

or indwelling catheters or other drainage devices (Levison and Kaye 2013, Lichtenberger and Hooton 2008).

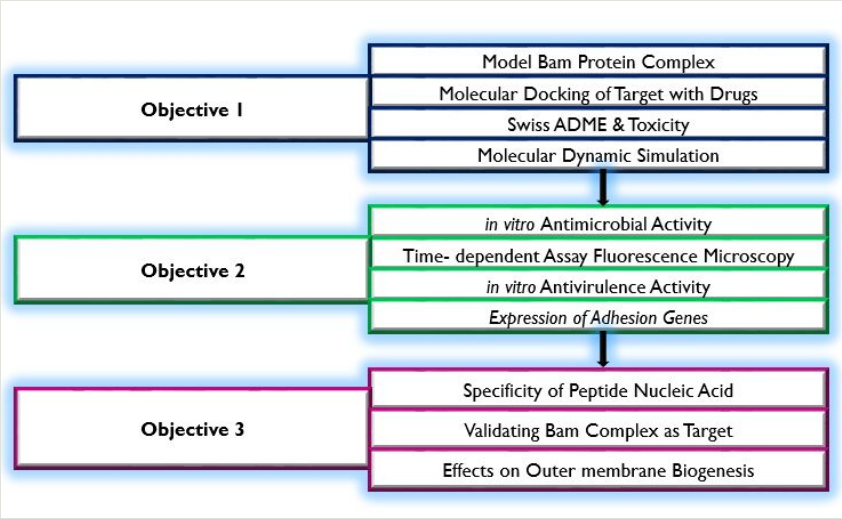

Figure 1. doi
Study Workflow.

Uropathogens and Antimicrobial Resistance

Bacteria and fungi are common causative pathogens of UTIs, which might be uncomplicated complicated. Complicated UTIs are caused by *E. coli*, *Klebsiella pneumonia* , *Staphylococcus saprophyticus*, group B streptococcus (GBS), *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *P. mirabilis*, *S. aureus and Candida* species. Uncomplicated UTIs are caused by Uropathogenic *E. coli*, *Enterococci*, *K. pneumonia*, GBS, *P. aeruginosa*, *Candida* species and *S. aureus (Levison and Kaye 2013*). *P. mirabilis* is a model organism for urease producing and urease-negative bacteria like *E. coli* for studying infection stones, polymicrobial UTIs and catheter associated urinary tract infection (CAUTIs). *P. mirabilis* and other ureaseproducing bacteria have not been extensively studied. Inherent colistin and imipenem resistance are implicated in *P. mirabilis* infections. *P. mirabilis* isolates develop imipenem resistance by losing porins, downregulating penicillin-binding protein expression or acquiring multiple AMR genes Fig. 2. Non-β-lactam resistance including resistance to drugs used in UTI treatment such as cephalosporins have been indicated. There is increasing emergence of MDR *P. mirabilis*, with attendant public health challenges. This has necessitated the search for novel antibiotics against *P. mirabilis* in treating UTIs.

Drug Repurposing (DR)

DR involves identifying new uses for experimental, approved, abandoned and discontinued drugs to treat diseases. It is an innovative strategy that replaces traditional drug discovery, as it reduces time, money, safety issues and the likelihood of failure associated with

traditional drug discovery. DR can be done experimentally or by *in silico* approach. The experimental approach involves screening drugs for therapeutic effects using biological assays. It requires *in vitro* or/and *in vivo* screening of drugs against the cellular components or drug targets. In the *in silico* approach, available chemical libraries from databases are virtually screened against protein targets using bioinformatics tools. Bioactive molecules are identified based on the molecular interaction of drugs with these targets (Talevi 2018).

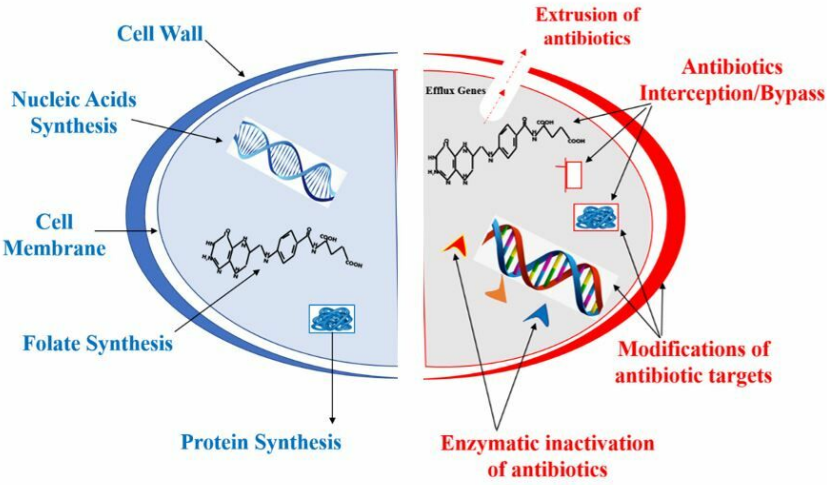

Figure 2. doi

General targets of common antibiotics (eft side with blue labels) and resistance mechanisms (ight side with red labels) Abiola 2019.

Computational versus Experimental DR

Compared to experimental approaches, *in silico* DR reduces time and risk of failure and is also cost-effective. The drawback in the experimental approach is that it requires accurate details about drug targets, drugs and disease phenotypes (Rosa and Santos 2020). Currently, researchers use the two approaches (mixed approach) to identify the therapeutic purposes of current drugs. In the mixed approach, computational results are validated through pre-clinical biological research and clinical studies. Applying both approaches gives a robust and accurate way of discovering new drugs, which is more efficient than serendipity (Turanli et al. 2018). This study will use the two approaches to identify potential drugs that can efficiently inhibit *Proteus mirabilis* Bam complex proteins.

B Complex Protein

B complex is the central machinery that assembles the unfolded OM proteins to create a highly impermeable barrier via the sur A chaperone (Diederichs et al. 2020). The complex comprises of five proteins Bam ABCDE, with Bam A and D as the only essential genes

(Gentle et al. 2005, Wu et al. 2005b). In the periplasm, the Bam D recruits the OM proteins while the Bam A folds and inserts OM proteins into the OM Fig. 3. However, non-folded OM proteins are degraded by DegP (rotease). The functions of Bam BCE are unclear. However, it has been shown that these proteins improve kinetics of Bam A and D (Wang et al. 2021). Small molecules which prevent OM proteins from binding to Bam A could lead to new treatments for Gram-negative infections (Lehman and Grabowicz 2019). MRL-494 is the first non-peptide small molecule inhibitor of Bam A, with activity against resistant bacterial strains (Hart et al. 2019). Darobactin is another antibacterial drug recently show to target Bam A. This drug showed antimicrobial activity against sensitive and resistant pathogens in an animal model of infections (Imai et al. 2019). By specifically targeting the B complex, nitazoxanide was observed to interfere with the B complex assembly (Psonis et al. 2019). Aside screening these known inhibitors of *E. coli* Bam complex proteins, this study will use selected chaperone inhibitors and off-target inhibitors to identify effective antibiotics against resistant *P. mirabilis*.

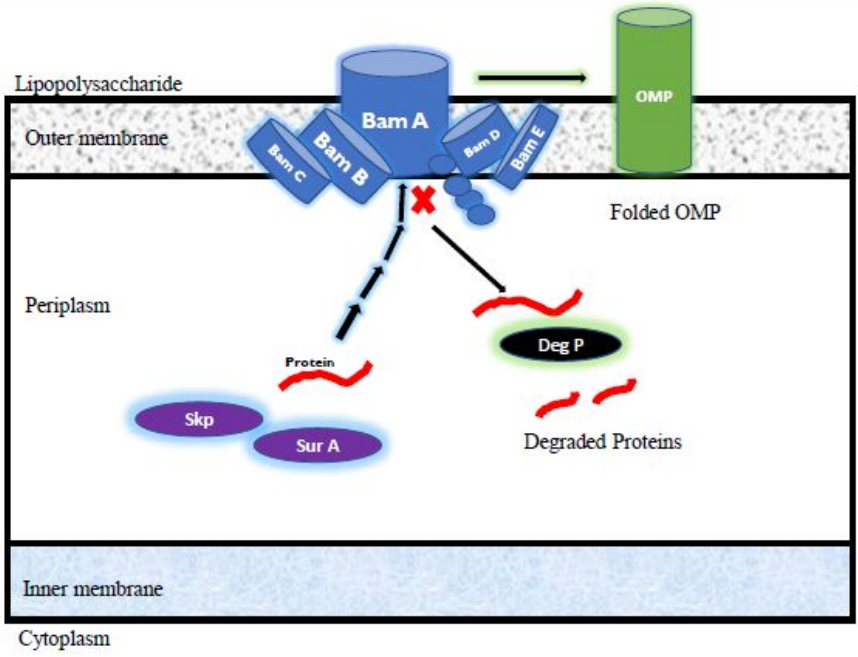

Figure 3. doi

Figure shows the formation of outer membrane proteins as adapted from Knowles et al. (2009) .

Study Rationale

P. miralbilis is a critical pathogen threatening human health, especially for UTI patients. The last-resort treatments for MDR-bacteria, third-generation cephalosporins and carbapenems, have not been effective (Shrivastava et al. 2018). Drug resistance occurs

partly because the OM of Gram-negative bacteria becomes less permeable to antibiotics over time. The B complex is required for proper OM formation since it is responsible for folding and inserting OM proteins. The BAM complex is a promising new target for generating novel antibiotics (Psonis et al. 2019), which is relevant as the emergence of MDR is increasing. Therefore, identifying drug targets that interfere with the activity of the BAM complex could help in the development of next generation of effective antibiotics. Presently, no antibiotics in clinical use disrupt the OM assembly of Gram-negative bacteria. Uropathogens such as *P. mirabilis* and other urease producing bacteria that have been implicated in life-threatening infections such as UTIs are not commonly studied (Norsworthy and Pearson 2017). Apart from the traditional drug discovery strategy, drug repurposing is an effective drug development approach against *P. mirabilis*-related UTIs. This study will use the drug repurposing approach, as it is cost-effective and accelerates the drug discovery process compared to the traditional methods.

Impact

This study will enhance better understanding of the function of B Complex proteins in *P. mirabilis*. The *ex-vivo* assay of field isolates will provide a broader perspective of the bacterium's *in vivo* behaviour. , the study will provide additional insights into alternative therapeutic approach to design future antibiotics (such as antibacterial, antivirulence and antisense drugs). Finally, the prioritiation of the B complex proteins and elucidation of drugs' mechanism of action will add more value to the knowledge of drug development.

Implementation

Research Question 1: Are there drugs which inhibit the activity of *Proteus mirabilis* B complex proteins?

OBJECTIVE 1: Screen for drugs with activity against *P. mirabilis* B complex proteins.

Homology Modelling Structures of *P. mirabilis* B ABCDE

B Complex protein sequence templates will be obtained from National Centre for Biotechnology Information (NCBI). Homology models will be generated based on the pairwise alignment of template and target sequences. Homology models of Bam A-E will be built in Modeller 10.2 utiliing templates with significant levels of sequence identity and coverage. DOPE scores will be used to rank models. ProSA-web, PROCHECK and ERRAT will be used to evaluate the quality of the modelled proteins.

Ligands Preparation and Molecular Docking

The 3D structures of about 3000 inhibitors (1500 on-target and 1500 off-target inhibitors) with their respective IDs will be retrieved from the PubChem database https://pubchem.ncbi.nlm.nih.gov/ and African Natural Product Database (ANPD - http://african-compounds.org/anpdb/. PyRx version 0.8 (AutoDock Vina - https://pyrx.sourceforge.io/

downloads will be used for docking experiments. The structure of the drug will be converted to pdbqt format and energy minimied using MMFF94 forcefield and Conjugate Gradient. Blind docking will be used, since the binding pockets of targets are unknown. PyMol will be used to display the docked structures. -8.0 kcal/mol will be binding energy cut-off point. The docked complexes will be loaded into LigPLOT+v1.45 to produce the ligand 2D interaction.

Pharmacokinetics Profiling

The SwissADME web resource (http://www.swissadme.ch/) will be used to determine the drug-likeness and pharmacokinetics properties of hit compounds as described by Daina et al. (2017). The OSIRIS property explorer (DataWarrior version 4.7.2) will be used to determine the toxicity of compounds (Sander et al. 2015).

Molecular Dynamics (MD) Simulation

To assess the stability of the system, MD simulation will be performed on the binding modes of the docked complexes as described by Gurung et al. (2016).

OBJECTIVE 2: determine the antimicrobial and antivirulence activity of selected drugs against *Proteus mirabilis*.

Strains of Microorganisms: *Proteus mirabilis* resistant catheters isolates will be obtained from patients (men and women - 60 years old) in the intensive care unit (ICU). These samples were characteried by typing *UreR* gene with PCR and conventional biochemical assays by the AbiMosi Bacteria Culture of the Department of Biochemistry, Cell and Molecular Biology. The strains are resistant to more than one antibiotic (ciprofloxacin, ceftazidime, colistin and imipenem) used in treating UTIs. The resistant strain *P. mirabilis* (ATCC BAA-3089) will be used as a control strain. For all drug assays, gentamicin and meropenem would be used as control antibiotics, as they are antibiotics of choice for treating UTIassociated infections in the study area.

Antimicrobial Activity of Selected Drugs on *Proteus mirabilis*

Overnight cultures of *P. mirabilis* strains (10^8 CFU/ml expressed as (Number of colonies x Dilution Factor)/Volume of culture plated) will be inoculated into 10 ml of Mueller Hinton (MH) broth at 37^{o}C and adjusted to Optical Density (OD) 600 nm of 0.5 MacFarland standard. About 50 µl of the culture will be transferred into each well of a 96-well plate containing LB broth (100 µl). Ten elected drugs from objective 1 (Artenimol, Dasatinib) at different concentrations (200 µg/ml-1000 µg/ml) will be introduced into the wells, incubated (24 h, 37^{o}C, 180 rpm) and OD measured with microplate reader (OD 600 nm) and minimum inhibition concentration (MIC) determined as described by Wiegand et al. (2008). Briefly, the MIC would be expressed as percentage OD of test strain to OD of control and the percentage OD < 10 would be assigned the MIC at specific concentration.

Effect of Drugs on *Proteus mirabilis* Using Fluorescent Microscopy

The effect of the varying concentrations of drugs on *P. mirabilis* after incubation for 4 and 8 h will be determined via live/dead staining technique according to Das et al. (2018). The cell medium will be centrifuged and discarded, the pellet rinsed and resuspended with Phosphate Buffer Saline (PBS). The treatment and control samples will be incubated at room temperature in the dark for 15 min after SYTO 9 and propidium iodide (PI) slide seeding and examined by a fluorescence microscope (40x magnification). Morphological changes as compared to the untreated controls would be determined and quantified.

Antivirulence Activity of Selected Drugs on *P. mirabilis*

HeLa cells will be seeded in 96-well plate with/without selected drugs (at different concentrations), infected with *P. mirabilis* and incubated (5% CO_2, 37°C, 2 h). Non-adherent bacterial cells will be removed from the wells by washing three times with PBS. Cell cultures will be treated with 0.025% Triton X-100 (5 minutes, 37°C, 5% CO_2) to separate and lyse the cell monolayer. The cell lysates will be diluted ten-fold and inoculated onto Trypticase Soy Agar (TSA). After incubation (24 h, 37°C), the colony forming unit (CFU/ml) will be calculated as described by Hazan et al. (2012).

Effect of Drugs on Expression of adhesion genes (*mrpA* and *pmfA*)

10-100 cells/ml overnight cultures of *P. mirabilis* will be treated with ten selected drugs at 37^{o}C and incubated at different time points (2, 8, 16, 24 and 48 h). Total RNA will be extracted from bacteria cells using RNAeasy extraction kit and cDNA synthesis synthesied with reverse-transcriptase. qRT-PCR will be conducted with 20 µl reaction consisting 2× Master Mix (SYBR Green Ampliqon), RNAase-free double distilled water, Primers (10 pM) and cDNA (5 ng/µl). The cycling condition will be: denaturation (95^{o}C, 10 minutes), denaturation (95^{o}C, 15 seconds, 44 cycles), annealing and elongation (54^{o}C, 60 seconds). The internal control will be 16S rRNA and the primers designed using the Primer 3 tool. Triplicate samples will be analyed and the $2^{-\Delta\Delta CT}$ method will be used to determine the fold change in expression of *mrpA* and *pmfA* genes, as described by Livak and Schmittgen (2001).

Research Question 2: How does the drug interact and inhibit *P. mirabilis* B complex?

OBJECTIVE 3: determine the effect of active drugs on wild-type and mutant *P. mirabilis* outer membrane biogenesis. This objective determines changes in the OM pathway in wild-type and mutant *P. mirabilis* as a result of drug treatment. The expression of B complex proteins will be down modulated using Peptide Nucleic Acid (PNA) to validate the mode of action of drugs. Gentamicin and meropenem would be used as control antibiotics, as they are antibiotics of choice for treating UTIs associated infections in the study area.

Antisense PNA Design

Specific antisense PNA for B ABCDE genes in *P. mirabilis* will be synthesied by semi-automated parallel synthesis using the ABIMED robot described by Good et al. (2001), with cell permeating peptide $(KFF)_3K$. Candidate antisense sequences will be checked for uniqueness in sequences using BLASTN.

Generation of mutant *P. mirabilis*

P. mirabilis culture in LB broth with previouslystated antibiotics and different concentrations (2 µM, 4 µM, 6 µM, 8 µM, 10 µM) of gene-specific PNA will be co-culture and incubated at 37^{o}C overnight. For positive control, *P. mirabilis* will be cultured with an antisense PNA as a positive control while a non-specific antisense PNA as negative control. Bacterial cells will be lysed and analyed by estern lotting to confirm the specificity of antisense PNA as described by Wu et al. (2005a). Specific antibodies (anti-Bam A, anti-Bam B, anti-Bam C, anti-Bam D and anti-Bam E) will be used to probe the B complex genes.

MIC of Active Drugs against wild-type and mutant *P. mirabilis*

Overnight cultures of Wild-type and mutant *P. mirabilis* strains (10^8 CFU/ml expressed as Number of colonies x Dilution Factor)/Volume of culture plated) will be inoculated into LB broth (10 ml) at 37^{o}C and adjusted to Optical Density (OD) 600 of 0.5 MacFarland standard. About 50 µl of the culture will be pipetted into each well of a 96-well plate containing LB broth (100 µl). Serial dilutions of four active drugs will be prepared in the broth in a final assay volume of about 100 µl. About 50 µl of bacterial culture will be added to the seriallydiluted drugs and incubated (37^{o}C, 24 h). The bacterial growth will be monitored using microplate reader and MIC determined as previously indicated.

Sample Preparation for Western Iot Analysis

Using the same sample from above, 25 µl of the samples will be reconstituted with 1U benzonase at an OD of 600 and incubated (27^{o}C, 10 min). The samples will be mixed with 25 µl β-mercaptoethanol supplemented with Laemilli sample buffer. The samples will be boiled for 10 min to measure total OMP levels in estern blot analysis (Kielkopf et al. (2020)). Bradford Protein Assay will be used to quantify the protein concentration as described by Kielkopf et al. (2020). For the western blot analysis, protocols described by Mahmood and Yang (2012) would be used. This would involve separating the samples on SDS PAGE, transfer to a Polyvinylidene fluoride membrane and marking target protein with relevant antibody.

Abundance of OM Protein in both wild-type and mutant *P. mirabilis*

Samples from bacteria strains will be loaded on a sodium dodecyl sulphate polyacrylamide gel electrophoresis at 120 V as described by Wu et al. (2005a). Protein samples will be transferred to a nitrocellulose membrane and blocked in non-fat dry milk (5%) and incubated (4^{o}C, 2 h). embrane will be incubated overnight with primary antibodies (anti-Bam A, anti-LptD, anti-DegP, anti-OmpF, anti-OmpC, anti-Lam B) in tris(hydroxy

methyl)aminomethane (ris)-buffered saline Tween 20 at 4^{0}C for 2 h. The membrane will be washed and incubated with secondary antibody (anti-rabbit horse radish peroxidase) at 4^{0}C. β-actin will be used as the loading control. The signals will be detected using chemical luminescence (using luminol as substrate and copper ions as catalyst).

DATA ANALYSIS

All experiments will be repeated thrice with average and standard deviations calculated. One-way analysis of variance (ANOVA) will be used for comparative mean analysis as indicated by Tukeys Post hoc analysis in Graph Pad Prism. The docked complex of Bam A with a ligand is shown in Fig. 4.

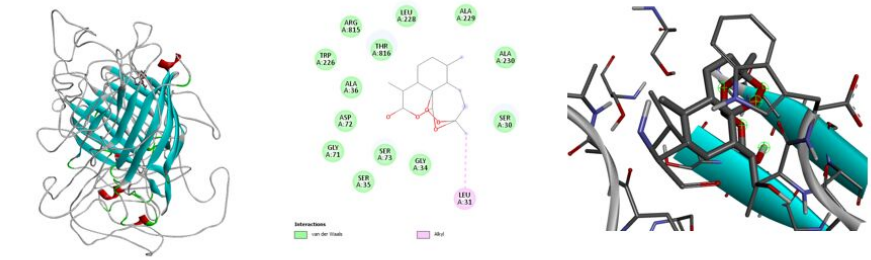

Figure 4. doi

This figure shows the 3D and 2D Docked Model of Bam A protein

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Hosting institution

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Conflicts of interest

The authors have declared that no competing interests exist.

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