

Genome Mining and Molecular Docking-Based Prediction of a Putative Antimicrobial Protein from Rhizospheric Bacteria

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Abstract

A bacterial strain was isolated from the rice rhizosphere through serial dilution and cultured on Yeast Extract Mannitol Agar (YEMA) medium. Biochemical characterization revealed a positive indole test, indicating the ability to metabolize tryptophan contribute to plant growth promotion. Molecular identification based on 16S rRNA gene sequencing revealed a close relationship with *Enterobacter hormaechei*, and the sequence was submitted under GenBank accession QB096036. The antimicrobial activity of the isolate against ciprofloxacin was investigated, while genomic analysis enabled the identification of a potential inhibitor protein. Molecular docking simulations were subsequently performed to evaluate the predicted interactions between the selected protein and its target. Overall, these findings suggest that the isolate may possess both plant growth-promoting potential and promising antimicrobial-related properties. Further quantitative IAA analysis, plant-based assays, and experimental validation of the predicted protein interactions are required to establish its agricultural and biotechnological applications.

Key words: Antimicrobial, PGPR, YEMA

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Introduction

The plant rhizosphere-the narrow region of soil directly influenced by root secretions-is an ecologically dynamic hotspot harbored by diverse microorganisms known as Plant Growth-Promoting Rhizobacteria (PGPR). Among cereal crops, rice (*Oryza sativa*) relies heavily on rhizospheric microbial networks to facilitate nutrient acquisition, enhance stress tolerance, and suppress soil-borne pathogens. Soil microorganisms from specific ecological niches produce a wide array of secondary metabolites, bioactive proteins, and phytohormones, such as indole-3-acetic acid (IAA), making them vital resources for bioprospecting new antimicrobial agents and biochemical tools (Zhou et al., 2021).

The rhizosphere contains a much larger microbial population than ordinary bulk soil. This increase in microbial density occurs because root exudates attract bacteria and stimulate their multiplication. Rhizospheric bacteria may be free-living, symbiotic, or associative in nature. Free-living bacteria survive independently in the soil, while symbiotic bacteria form close associations with plants. Associative bacteria remain attached to root surfaces and benefit plants without forming specialized structures. Common genera of

rhizospheric bacteria include *Rhizobium*, *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Bacillus*, and *Enterobacter*. These bacteria differ in their structure, metabolism, and functions, but all contribute to improving soil health and plant productivity. (Nelson, 2004).

To isolate and characterize potential functional microbes, culturing techniques paired with selective media-such as Yeast Extract Mannitol Agar (YEMA)-allow for the recovery of specific soil bacterial strains. Definitive taxonomical identification relies on 16S rRNA gene sequencing, which provides a precise phylogenetic benchmark for depositing novel sequences into public databases like GenBank. Alongside taxonomical profiling, biochemical characterizations such as the indole test provide crucial insights into metabolic capabilities, such as tryptophan catabolism and secondary metabolite biosynthesis (Sharma & Kaur, 2010).

Concurrently, the rapid global rise of antibiotic resistance has necessitated the exploration of novel microbial strains and target proteins capable of modulating antibiotic susceptibility or exhibiting targeted antimicrobial activity. Ciprofloxacin, a broad-spectrum fluoroquinolone widely used in clinical and agricultural settings, serves as a key

benchmark antibiotic for testing novel resistance mechanisms or competitive antimicrobial pathways. Furthermore, modern biological research bridges traditional *in vitro* assays with *in silico* computational workflows. Molecular docking simulations allow researchers to identify screening-derived inhibitor proteins directly from genomic data, predicting binding affinities and potential interactions with targeted microbial structures (Abada et al., 2014).

In this study, a bacterial isolate was retrieved from the rice rhizosphere, purified on YEMA medium, and taxonomically identified via 16S rRNA gene sequencing (GenBank Accession: QB 096036). Following physiological testing and phenotypic evaluation of its antimicrobial profile against ciprofloxacin, genomic mining was conducted to identify a potential inhibitor protein. Subsequent molecular docking studies were performed to elucidate its structural binding mechanics and elucidate the bio-protective and antimicrobial mechanism of this rhizospheric strain (Schwede et al, 2003).

Materials and Methods

To perform serial dilution for isolating the rhizospheric bacterium, 1 g of soil tightly adhering to the rice roots was collected and suspended in 9 mL of sterile distilled water (or phosphate-buffered saline) in a sterile test tube, yielding the initial 10^{-1} soil suspension. The mixture was vortexed vigorously for 2 to 3 minutes to detach bacterial cells from the root soil matrix. Under strict aseptic conditions, 1 mL of this uniform suspension was transferred to a second tube containing 9 mL of sterile diluent to prepare the 10^{-2} dilution, and this stepwise tenfold dilution process was sequentially repeated through to 10^{-6} . Aliquots of 100 μ L from selected higher dilutions (10^{-4} , 10^{-5} , and 10^{-6}) were then spread-plated in duplicate onto sterile Yeast Extract Mannitol Agar (YEMA) plates using a sterile glass spreader. The plates were inverted and incubated at 28-30°C for 24 to 48 hours until well-isolated bacterial colonies appeared for morphotypic selection and pure culture generation (Ben-David & Davidson, 2014).

Indole test

The indole test is used to assess whether bacteria can metabolize tryptophan by producing the enzyme tryptophanase, which breaks down tryptophan into indole. The test was conducted using tryptone broth with the following composition (gm/liter and pH 7.5 ± 0.2). Tryptone: 10g, Sodium chloride: 5g

Tryptone broth was prepared, and 5 mL was dispensed into each test tube. The tubes were then inoculated with the bacterial isolates and incubated for 48 hours. After the incubation period, 1 mL of Kovac's reagent was added to each tube and mixed gently. The appearance of a cherry-red color

indicated a positive result for indole production (Mac Fadden, 2000).

Genomic DNA Isolation

The sample was collected and homogenized thoroughly in a mortar with 1 mL of extraction buffer, and the homogenate was transferred to a 2-mL microfuge tube. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and mixed gently by shaking. The tubes were centrifuged at 14,000 rpm for 15 min at room temperature, and the upper aqueous phase was transferred to a fresh tube. An equal volume of chloroform:isoamyl alcohol (24:1) was added, mixed thoroughly, and centrifuged at 14,000 rpm for 10 min at room temperature. The resulting upper aqueous phase was transferred to a new tube. DNA was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 7.0) and 0.7 volume of isopropanol, followed by incubation at room temperature for 15 min. The samples were then centrifuged at 14,000 rpm for 15 min at 4 °C. The resulting DNA pellet was washed twice with 70% ethanol, followed by a brief wash with 100% ethanol, and air-dried. The DNA was subsequently dissolved in TE buffer (10 mM Tris-Cl, pH 8.0; 1 mM EDTA). For RNA removal, 5 μ L of DNase-free RNase A (10 mg/mL) was added to the DNA preparation (Moore.D., 1994).

PCR Amplification of the 16S rRNA Gene

For amplification of the bacterial 16S rRNA gene, 0.15 μ g of extracted genomic DNA was used as the template. The PCR reaction was performed using 10 pM of each forward and reverse primer. The reaction mixture contained the required template DNA, primers, dNTPs, Taq DNA polymerase, and PCR assay buffer.

The PCR master mix consisted of high-fidelity DNA polymerase, 0.5 mM dNTPs, 3.2 mM $MgCl_2$, and PCR enzyme buffer. For the PCR reaction, 1 μ L of genomic DNA, 2 μ L of 16S forward primer, 2 μ L of 16S reverse primer, 4 μ L of dNTPs (2.5 mM each), and 10 μ L of 10 $\times$ Taq DNA polymerase assay buffer were used. The final reaction volume was adjusted appropriately with nuclease-free water and DNA polymerase according to the manufacturer's instructions ((Weisburg et al., 1991). After amplification, sequenced at Cytogene Research and Development Laboratory, Lucknow. A similarity search was performed through BLAST, followed by sequence alignment, and the tree was built in MEGA11 software (<https://www.megasoftware.net/>) by applying the UPGMA method by generating 1000 bootstrap replicates (Pataranutaporn et al., 2026).

Anti-microbial Activity:

Procedure:

The antimicrobial activity of Ciprofloxacin was evaluated against the microbe, PDK-1. Four different concentrations of Ciprofloxacin: 150ppm, 100ppm, 50ppm and 25ppm were prepared to

check its antimicrobial property against PDK-1. The activity was evaluated by the Disc Diffusion Method. The process for the same is as follows: Major components of Muller Hinton Media in g/l at pH 7.0

For this test, Initially the Mueller Hinton Agar (MHA) media was prepared as per the standard composition given by HI media that is 38 g of the media was dissolved in 1L of water and the media was autoclaved at 121°C and 15psi for 15 minutes using autoclave. After sterilization, media was poured in sterile glass petri dishes in the laminar air flow (Toshiba, India) using the aseptic techniques, each plate was poured with 25ml of the culture media. The plates were allowed to solidify in laminar, the bacterial inoculum was uniformly spread using sterile cotton swab on a sterile Petri dish MH agar. Ten minutes after spreading, Discs with 4 different concentrations i.e. 150ppm, 100ppm, 50ppm and 25ppm were placed using sterile forceps. The samples were allowed to diffuse into the media and then the plates were sealed with paraffin and incubated at 37°C for 24hrs. The plates had 5 discs -ve was a negative control (Distilled Water) C1 for 150ppm, C2 for 100ppm, C3 for 50ppm and C4 for 25ppm. Next day after incubation the plates were observed for the clear zone around the well called as the zone of inhibition, and the diameter of these zones was measured in mm. (Bauer et al., 1959)

Genome Mining

The identified *Enterobacter hormaechei* genome was used for visualization and RGI Resistance Gene Identifier analysis (Comprehensive Antibiotic Resistance Database (CARD) analysis.

Molecular Modeling & Docking Study

Comparative homology modelling was employed to develop 3 D structure of Protein on easy modeller v 4.0. First Template was generated for *E. coli* and then it was used for *Enterobacter hormaechei* (Arab et al., 2023; Fig4). The structure generate was validated by Ramachandra plot (Ramachandran et al., 1963).

Genome of *Enterobacter hormaechei* (GCF_001729725.1) was analyzed using online package and a protein AcrD of super family RND was found as multidrug efflux RND transporter permease which has antimicrobial activity against Ciprofloxacin. Interaction study was first performed between AcrD of *E. coli* and Gentamycin and the between AcrD gene of *Enterobacter hormaechei* and Ciprofloxacin (Fig5).

Results

The isolate was further subjected to the Tryptophan utilization test to evaluate its ability to metabolize tryptophan-containing compounds. The development of a characteristic color reaction confirmed that the isolate was TRP positive. This result suggests that the bacterium possesses metabolic pathways involved in tryptophan

metabolism and may produce indole-related compounds such as Indole Acetic Acid (IAA). IAA is one of the major phytohormones involved in plant growth promotion, root elongation, and stimulation of nutrient uptake. Therefore, the positive TRP test indicates the possible PGPR potential of the isolate and its beneficial role in enhancing plant growth and development.



Fig. 1. Indole

Test

The sequence analysis demonstrated high similarity with members of the genus *Enterobacter*. Further phylogenetic analysis was performed to determine the evolutionary relationship of the isolate with related bacterial strains. The phylogenetic tree revealed that the isolate clustered closely with *Enterobacter hormaechei* (Accession: QB 096036) indicating a close evolutionary and taxonomic relationship. The branching pattern and sequence similarity strongly supported the identification of the isolate as a strain closely related to *Enterobacter hormaechei*.

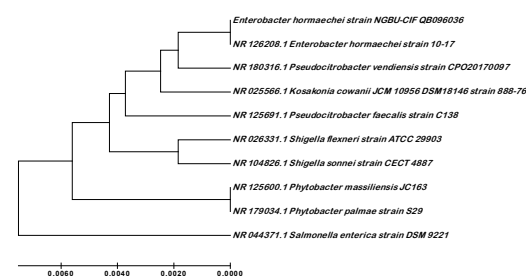


Fig. 2. Phylogenetic

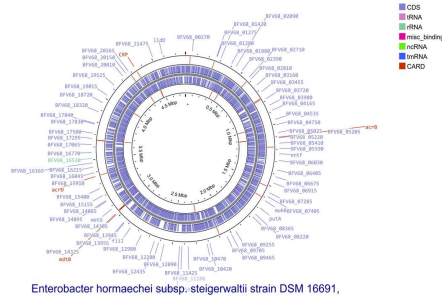
Analysis of NGBU-CIF

The antimicrobial activity of Ciprofloxacin against CIF was assessed using the disc diffusion method. Mueller Hinton Agar (MHA) was prepared, sterilized, and poured into sterile petri dishes. The bacterial inoculum was spread uniformly, and discs containing Ciprofloxacin concentrations of 150ppm (C1), 100ppm (C2), 50ppm (C3), and 25ppm (C4) were placed on the media. A negative control (distilled water) was also included. After incubation at 37°C for 24 hours, the plates were observed for zones of inhibition around the discs. The diameter of these zones was measured in millimeters, indicating the antimicrobial efficacy of Ciprofloxacin at different concentrations.

Fig.3. Antimicrobial test against Ciprofloxacin
Table 1. Inhibition Zone Against the Ciprofloxacin

Observation						
S . N o .	B a c t e r i a	Conc entration (ppm)	Zone of Inhibition (mm)			
			R 1	R 2	R 3	Mean ± S. D.
1	C I F	150	27	27	27	27.0 ± 0.06
2		100	22	21.5	21	21.8 ± 0.26
3		50	16	15.8	16	15.9 ± 0.15
4		25	8	8.5	8	8.6 ± 0.15
5	Neg . Co ntrol (D W)	-	N il	N il	N il	Nil

Genome Mining



Enterobacter hormaechei subsp. steigerwaltii strain DSM 16691.

Fig.4. Enterobacter hormaechei genome visualization with RGI genes

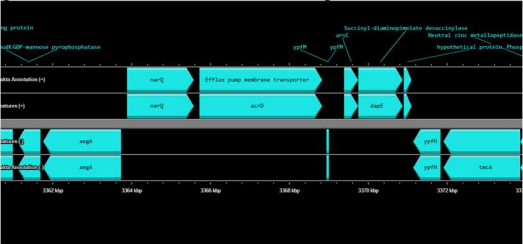


Fig 5. Representative acrD region Template (3,365,717 bp-3,368,830 bp)

The Resistance Gene Identifier (RGI) analysis using the Comprehensive Antibiotic Resistance Database (CARD) identified several antimicrobial resistance-associated genes and determinants in *Enterobacter hormaechei*. Multiple efflux pump components and related systems were identified, including acrA, acrB, acrD, mdfA, msbA, KpnE, KpnF, mdtB, mdtC, emrB, adeF, and AcrAB-TolC, which may contribute to the export of antimicrobial compounds and reduced susceptibility to different antibiotics. Further AcrD was used for bioinformatics based characterization (Acc. No. EPS8793618.1).

>EPS8793618.1 multidrug efflux RND transporter permease AcrD [Enterobacter hormaechei]
MANFFIDRPIFAWVLAILLCLTGVLAITSLPVE
QYPDLAPPNVRLITANYPGASQTLNVTQVI
EQNMT
GLDNLMYMSSQSSATGQATVTLSTAGTDPD
EAVQQVQNQLQSALRKLPQAVQNQGVTVRK
TGDTNITI
AFVSTDGSMQDIADYVASNIQDPLSRING
VGDIDAYGSQYSMRIWLDPNKLSVQMTAK
DVTDAIESQ
NAQIAVGQLGGTPSVDNQALNATINSQSLLO
TPEQFRNITLRVNQDGSEVRLGDVATVEMGA
EKYDYLSR
FNGNAASGLGVKLASGANEMATAQRVLERL
DELSHYFPHGLEYKVAYETTSFVKASIEDVV
KTLLEAIAL
VFLVMYLFQNFRAITLIPTIAPVTVLLGTFAV
LYAFGYSINTLTMFAMVLAIGLLVDDAIVVV
ENVERIM
SEGLSPREATRSMGQIQGALVGAMVLSA
VFIPMAFFGGTTGAIYRQFSITIVSAMVLSVLV
AMILTP
ALCATLLKPLHKGEHHGQKGGFGWFNRMFN
RNAARYEAGVGKVLHRSVRWMVVVYVLLLG
GMVFLFLRLPT
SFLPLEDRGMFITSVQLPSGSTQQQTLKVVQK
VENYFHTQEKNVSVFSTVGSGPGGNGQN
VARMFVRL
KDWDQRDSDTGSSFAIERATKAFNKIKEARV
FASSPPAISGLSSAGFDMELQDHAGAGHDA
LMADRDK
LLELAGKDPQLTRVRHNGLLDDSPQLQVDIDQ
RKAQALGVSIIDINDTLQTAWGSSVNDFM
DRGRVKKVY
VQSAKYRMLPDDINRWYVRNNTGGMVPFS
AFATSRWETGSPRLERYNGYSALEIVGEAAP
GVSTGTAMD
IMEKL VQQLPTGFGLEWTAMSYQERLSGAQ
APALYALSLLVFLCLAALYESWSVPFSVML
VPLGVIGA
LLATWMRGLENDVYFQVGLLTVIGLSAKNAI
LIVEFANEMNAKGHELMAATLHACRQRLRPI
LMTSLAFV
FGVLPMATSSGAGSSSQHAVGTGVMGGMIS
ATILAIYFVPLFFVLVRRRFLPKDKPE

Active site residues identified using BIOVIA Discovery Studio 2019 used for defining the binding site based on comparison with known active site residues of Gentamicin bound aminoglycoside efflux pump AcrD.

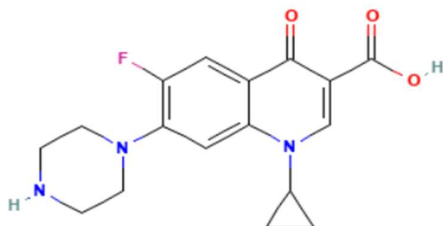


Fig. 6. Ligand 2D

Ciprofloxacin is a broad-spectrum fluoroquinolone antibiotic widely used against a variety of Gram-negative and Gram-positive bacterial pathogens. It primarily exerts its antibacterial activity by inhibiting DNA gyrase (DNA topoisomerase II) and topoisomerase IV, thereby interfering with bacterial DNA replication, transcription, and chromosome segregation. Ciprofloxacin is particularly effective against many members of the Enterobacteriaceae, including *Enterobacter* species.

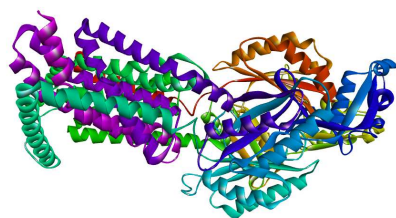


Fig. 7. 3D Model of *Enterobacter hormaechei* Resistance protein D (AcrD) is an efflux pump that belongs to the resistance-nodulation-cell division (RND) superfamily

The three-dimensional (3D) structure of the AcrD protein was modeled using BIOVIA Discovery Studio 2019. The protein sequence of AcrD was used to generate a reliable structural model based on suitable structural templates available in the Protein Data Bank (PDB). The generated model was subjected to structural refinement and energy minimization to obtain a stable conformation. The predicted 3D structure was subsequently used for further structural analysis and molecular docking studies to investigate the potential interactions of AcrD with selected ligands (Fig 7).

The stereochemical quality of the predicted AcrD protein structure was evaluated using a Ramachandran plot. The analysis comprised 1032 residues, of which 852 residues (93.6%) were located in the most favored regions, while 54 residues (5.9%) were present in additionally

allowed regions. Only 4 residues (0.4%) were located in generously allowed regions, and no residues (0.0%) were detected in the disallowed regions. Among the non-glycine and non-proline residues, 100% (910 residues) were represented in the analysis (Fig 8)

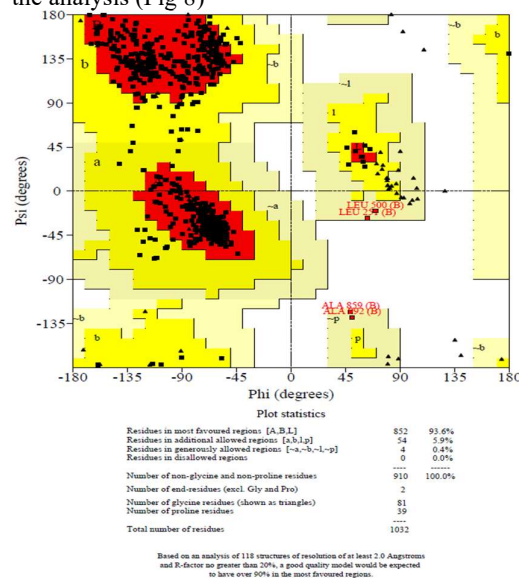


Fig 8. Stereochemical property Validation
Molecular docking analysis of AcrD with ciprofloxacin

Molecular docking analysis was performed to investigate the interaction of ciprofloxacin with the multidrug efflux RND transporter permease AcrD (EPS8793618.1) of *Enterobacter hormaechei*. The AcrD protein comprises 1037 amino acid residues and was used as the receptor for molecular docking with ciprofloxacin. The docked complex was subsequently analyzed using BIOVIA Discovery Studio 2019 to identify the residues involved in ligand recognition and stabilization.

The interaction analysis revealed that ciprofloxacin was positioned within a defined binding pocket of AcrD and interacted with multiple surrounding amino acid residues. A total of 14 residues were located in the predicted interaction region, including ASP37, PRO100, ASP99, THR98, GLY97, PRO41, PRO40, ALA39, ASP134, GLY133, THR132, THR135, SER295 and GLY296. The interaction pattern comprised several types of non-covalent interactions, indicating that the binding of ciprofloxacin was stabilized by a combination of electrostatic, hydrophobic and weak polar interactions (Fig 9).

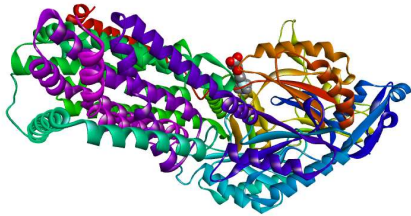


Fig. 9. Docked complex structure

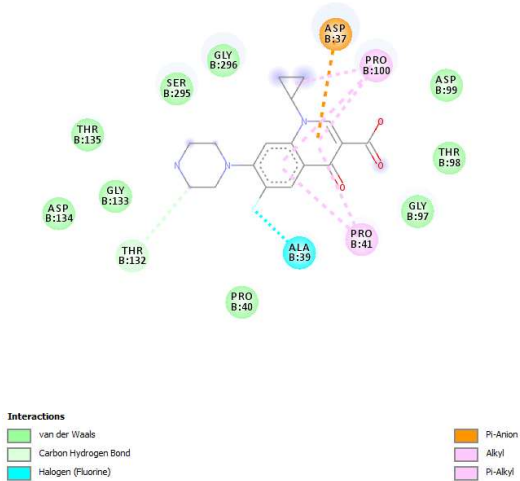


Fig. 10. Asp99 and Pro100 residues were identified as of known interacted active site residues SwissDock analysis was performed to further evaluate the interaction between ciprofloxacin and the AcrD multidrug efflux RND transporter permease of *Enterobacter hormaechei*. Six representative docking models were obtained, with calculated binding affinities ranging from -5.916 to -6.796 kcal/mol. Among the predicted complexes, Model 1 exhibited the highest predicted binding affinity (-6.796 kcal/mol), followed by Model 2 (-6.782 kcal/mol) and Model 3 (-6.630 kcal/mol). Models 4, 5 and 6 showed binding affinities of -6.595 , -6.352 and -5.916 kcal/mol, respectively (Table 2).

Table 2. Interaction Result Ciprofloxacin Docking score

Model	Calculated affinity (kcal/mol)
1	-6.796
2	-6.782
3	-6.630
4	-6.595
5	-6.352
6	-5.916

Conclusion

The present study demonstrated the potential plant growth-promoting characteristics of the bacterial isolate, obtained from the rice rhizosphere. The positive tryptophan utilization test indicated its ability to metabolize tryptophan and suggested the possible production of indole-related compounds, including indole-3-acetic acid (IAA), which may contribute to root development and plant growth promotion. Molecular identification based on 16S rRNA gene sequence analysis and phylogenetic relationships revealed that the isolate was closely related to *Enterobacter hormaechei* (NCBI accession QB 096036). Furthermore, the antimicrobial susceptibility of CIF strain to ciprofloxacin was evaluated using the disc diffusion method at different concentrations to assess the antibiotic's inhibitory effect on bacterial growth. Overall, these findings indicate that Isolates have potential applications in plant growth promotion.

The three-dimensional structural modeling of the AcrD protein using BIOVIA Discovery Studio 2019 provided a structural framework for further computational investigation of its potential biological function and molecular interactions. The stereochemical validation using the Ramachandran plot demonstrated good structural quality, with 93.6% of residues located in the most favored regions, 5.9% in additionally allowed regions, and only 0.4% in generously allowed regions. Importantly, no residues were observed in the disallowed regions, indicating favorable backbone conformations and supporting the reliability of the predicted model. The validated structure can therefore serve as a useful basis for subsequent molecular docking studies to investigate the potential interactions of AcrD with selected ligands. However, further computational validation and experimental studies are necessary to confirm the structural accuracy and biological significance of the predicted interactions.

Molecular docking analysis revealed that ciprofloxacin could interact with the multidrug

efflux RND transporter permease, AcrD of *Enterobacter hormaechei*. The predicted binding pose identified a potential interaction pocket involving 14 amino acid residues, including ASP37, PRO100, ASP99, THR98, GLY97, PRO41, PRO40, ALA39, ASP134, GLY133, THR132, THR135, SER295, and GLY296. The observed interaction pattern suggested that non-covalent forces, including electrostatic, hydrophobic, and weak polar interactions, may contribute to the stabilization of the AcrD-ciprofloxacin complex. These findings provide preliminary structural insights into the possible interaction between ciprofloxacin and AcrD. However, molecular docking alone cannot establish whether ciprofloxacin is effectively transported by AcrD or determine the antibiotic susceptibility of the isolate. Further studies, including molecular dynamics simulations, binding-affinity validation, and experimental efflux assays, are required to confirm the predicted interactions and clarify the role of AcrD in ciprofloxacin resistance in *E. hormaechei*.

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