

Computational Annotation and Experimental Validation of Hypothetical Proteins as Novel Drug Targets in *Klebsiella pneumoniae* HS11286

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ABSTRACT

Background: *Klebsiella pneumoniae* is a significant pathogen causing severe infections, especially among immunocompromised individuals. The emergence of Multidrug-Resistant (MDR) strains has heightened public health concerns. This study investigates the potential of Hypothetical Proteins (HPs) in *K. pneumoniae* HS11286 as new drug targets through functional annotation and experimental validation. **Materials and Methods:** We utilized bioinformatics tools to analyze 974 HPs for their functions, subcellular localization, and drug target potential. Techniques such as subtractive genomics and virulence prediction were applied to identify essential and non-toxic proteins. Structural modeling and druggability assessments were conducted on selected HPs. The role of these proteins in antibiotic resistance was experimentally validated using Polymyxin B and the PmrD protein. **Results:** From the 974 HPs, 732 were successfully classified functionally, with 30 identified as potential drug targets. Experiments revealed that PmrD significantly increases *K. pneumoniae*'s resistance to Polymyxin B, highlighting its involvement in resistance mechanisms. **Conclusion:** Our study offers a detailed functional annotation of HPs in *K. pneumoniae* HS11286 and uncovers several promising drug targets. These findings are crucial for developing new therapies against MDR *K. pneumoniae*. Future work should focus on further validating these targets through molecular docking and high-throughput screening for potential clinical applications.

Keywords: Computational Annotation, Drug Targets, Experimental Validation, Hypothetical Proteins, *Klebsiella pneumoniae*, Subcellular Localization.

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INTRODUCTION

Klebsiella pneumoniae is a Gram-negative pathogen responsible for a wide range of infections, including pneumonia, septicemia, and urinary tract infections, particularly in immunocompromised patients.¹ The emergence of Multidrug-Resistant (MDR) strains, especially those producing Extended-Spectrum β -Lactamases (ESBLs) and carbapenemases, has become a significant global health concern.^{2,3} The HS11286 strain, isolated from a sputum sample in Shanghai, China in 2011, is a notable example of a Carbapenemase-Producing *K. pneumoniae* (CPE) strain with a high level of antibiotic resistance, making it a critical target for research.⁴⁻⁶ The rapid spread of MDR *K. pneumoniae* strains has

severely limited treatment options, leading to increased morbidity, mortality, and healthcare costs.^{7,8} Despite advances in genomic research, a significant portion of the *K. pneumoniae* proteome, particularly Hypothetical Proteins (HPs) with unknown functions, remains uncharacterized. These HPs may play crucial roles in bacterial survival, pathogenesis, and antibiotic resistance, yet their potential as drug targets remains largely unexplored.^{9,10} This study aims to computationally annotate and experimentally validate the functions of HPs in *K. pneumoniae* HS11286 to identify novel drug targets. By leveraging bioinformatics tools, we seek to classify these proteins, predict their subcellular localization, and assess their potential as therapeutic targets. Additionally, we aim to validate the role of selected HPs in antibiotic resistance through experimental approaches.

Using a combination of sequence retrieval, functional prediction, subcellular localization analysis, subtractive genomics, and structural modeling, we systematically analyzed 974 HPs from the *K. pneumoniae* HS11286 genome. Experimental validation was performed using Polymyxin B and the PmrD protein to assess the role of selected HPs in antibiotic resistance. This integrated



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approach provides a comprehensive framework for identifying and validating potential drug targets in *K. pneumoniae*.

MATERIALS AND METHODS

Bacterial Strain and Culture Medium

The *Klebsiella pneumoniae* HS11286 strain was utilized in this study. The bacteria were cultured in Mueller-Hinton (MH) medium under standard laboratory conditions.

Chemicals and Reagents

Polymyxin B (Sigma-Aldrich) was served as a positive control drug for antibacterial activity assays. Other reagents included sterile distilled water, Phosphate-Buffered Saline (PBS), and Dimethyl Sulfoxide (DMSO) for drug dilution.

Experimental Materials

96-well plates (Corning) for bacterial growth and drug susceptibility assays. Micropipettes (Eppendorf) for precise liquid handling. Incubator (Thermo Scientific) set at 37°C for bacterial culture. Microplate reader (BioTek) for measuring Optical Density (OD) at 600 nm to assess bacterial growth.

Methods

The list of tools used throughout this functional annotation of *K. pneumoniae* shows in Table 1. These tools are available in online as open source where it can use anytime without the restriction of time and cost. In the table above, each website link to the tools is provided together with the name of the tools. Workflow of computational pipeline for annotating and finding drug targets among HPs was showed in Figure 1.

Sequence Retrieval and Preprocessing

The complete proteome of *Klebsiella pneumoniae* HS11286 was retrieved from the National Center for Biotechnology Information (NCBI). A total of 1,694 Hypothetical Proteins (HPs) were identified, and their sequences were obtained in FASTA format from UniProt. Redundant sequences were removed using the Cluster Database at High Identity with Tolerance (CD-HIT) tool, resulting in 974 non-redundant HPs for further analysis.

Functional Prediction

Functional domains of the 974 HPs were predicted using multiple bioinformatics tools: CDART: Identified conserved domains through evolutionary distance analysis. InterProScan: Classified protein families and predicted functional sites. SVM-Prot: Predicted protein domains using a support vector machine-based approach. Pfam: Provided comprehensive domain and family classification.

Subcellular Localization Prediction

Subcellular localization of the HPs was predicted using: CELLO: A two-level support vector machine tool for bacterial and eukaryotic localization. PSLpred: Specifically designed for Gram-negative bacteria. TMHMM and HMMTOP: Predicted transmembrane helices and membrane protein propensity. SignalP: Identified signal peptides involved in non-classical secretory pathways.

Subtractive Genomics

To identify essential and non-toxic HPs, we performed a stepwise filtering process:

Non-homology to human proteins: The 974 HPs were compared against the human proteome (RefSeq GRCh38) using BLASTp (E-value cutoff: 10^{-4}), excluding any HP with >30% sequence identity to human proteins to ensure minimal off-target effects.

Essentiality screening: Remaining HPs were analyzed against the Database of Essential Genes (DEG) using an E-value threshold of 10^{-100} and coverage >50% to confirm bacterial essentiality. Virulence and druggability: Essential HPs were further filtered by: VirulentPred score >0.7 (indicating high virulence potential), DrugBank druggability classification (presence of known drug-binding domains). This pipeline ensured the selection of targets that are essential for bacterial survival, non-homologous to humans, and pharmacologically tractable.

Virulence Prediction

The virulence potential of the HPs was assessed using the VirulentPred tool, which classified proteins as virulent or non-virulent based on their sequence features.

Structural Modeling

Three-dimensional structures of the selected HPs were modeled using the Phyre2 server, which employs homology detection and advanced modeling techniques to predict protein structures and ligand-binding sites. Model quality was assessed by: Ramachandran plot analysis (via PROCHECK) to evaluate backbone torsion angles; QMEAN score (Z-score >-4.0 considered acceptable); Template identity threshold (>30% sequence identity to known structures). Models with >90% residues in favored regions (Ramachandran) and QMEAN Z-score > -3.0 were retained for further analysis.

Drug Target Prediction

The druggability of the HPs was evaluated using the DrugBank database, which contains over 8,000 drug entries, including small molecules and biologics. Proteins with known drug interactions were classified as putative drug targets.

Experimental Validation

Bacterial Culture: *Klebsiella pneumoniae* HS11286 was cultured in Mueller-Hinton (MH) medium at 37°C until reaching the logarithmic growth phase.

Concentration Selection: Polymyxin B concentrations (0-100 µg/mL) were chosen based on:

Clinical breakpoints for *K. pneumoniae* (EUCAST guidelines). Preliminary dose-response experiments showing 50% inhibition at ~25 µg/mL. PmrD protein concentration (100 µg/mL) was optimized to: Match physiological levels in resistant strains. Avoid non-specific growth effects (validated in control experiments)

Antibacterial Activity Assay: Bacterial suspensions were adjusted to an appropriate concentration and inoculated into 96-well plates. Polymyxin B and PmrD protein were added at varying

concentrations (0-100 µg/mL), a control group without drugs was included and bacterial growth was monitored using a microplate reader.

Control Experiments: To rule out non-specific effects, the following controls were included: Negative control: Bacteria cultured without Polymyxin B or PmrD protein. Solvent control: Bacteria treated with DMSO (the solvent for Polymyxin B) at the highest concentration used. Protein control: Bacteria treated with PmrD protein (100 µg/mL) alone to exclude growth effects from the protein itself. Positive control: Bacteria treated with Polymyxin B alone (without PmrD).

Growth Measurement: Plates were incubated at 37°C for 24 hr, and bacterial growth was monitored by measuring OD at 600 nm using a microplate reader, and the bacterial growth inhibition rate was calculated for each treatment group.

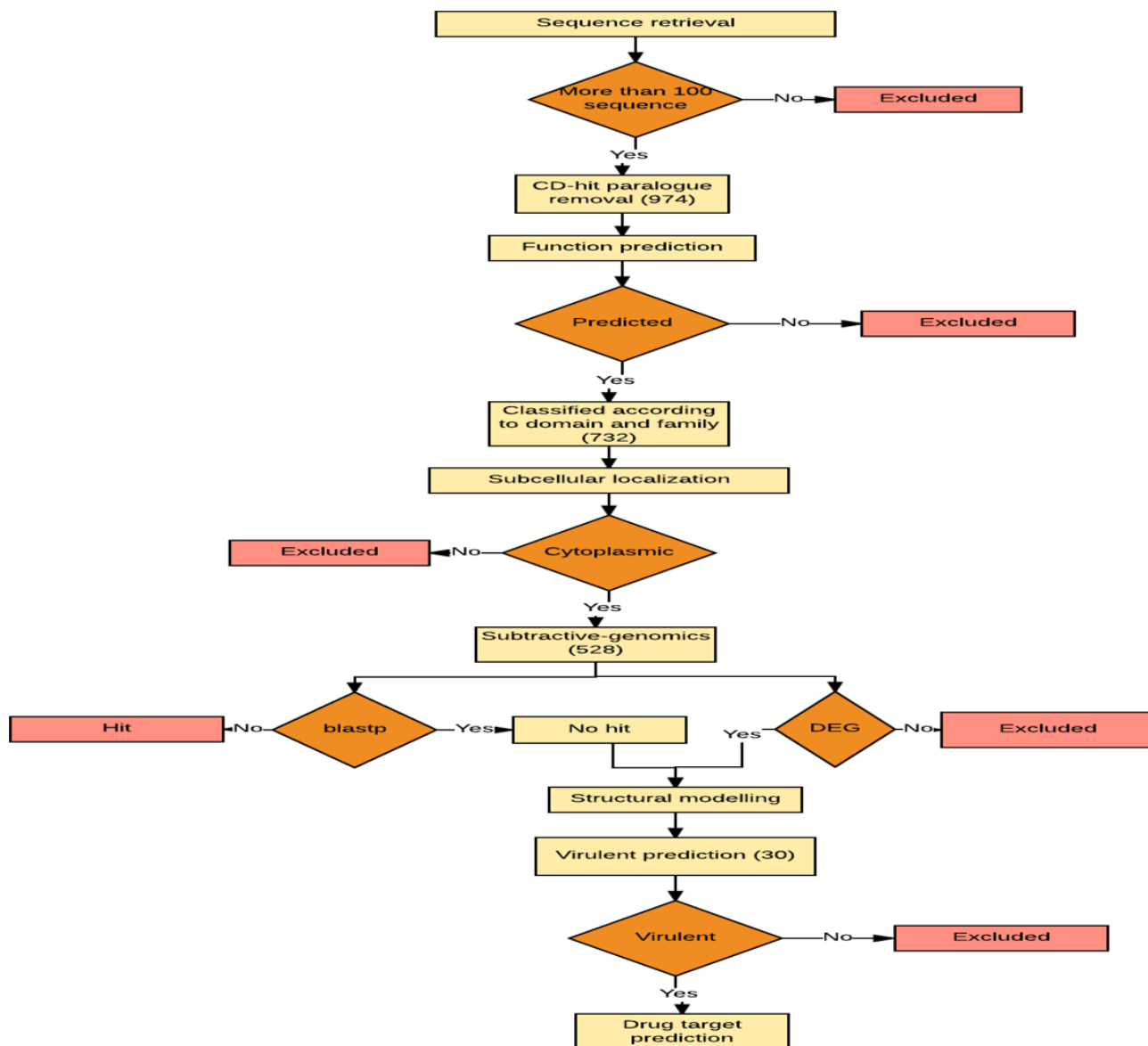


Figure 1: Workflow of computational pipeline for annotating and finding drug target among HPs.

Table 1: List of tools.

Sl. No.	Tools	URL
1.	NCBI	https://www.ncbi.nlm.nih.gov/
2.	Uniprot	http://www.uniprot.org/
3.	CD-HIT	www.bioinformatics.org/cd-hit/
4.	CDART	https://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi
5.	Interproscan	http://www.ebi.ac.uk/Tools/pfa/iprscan/
6.	SVMprot	http://bidd2.nus.edu.sg/cgi-bin/svmprot/svmprot.cgi
7.	Pfam	http://pfam.sanger.ac.uk/
8.	PSLPred	http://www.imtech.res.in/raghava/pslpred/
9.	CELLO	http://cello.life.nctu.edu.tw/
10.	TMHMM	http://www.cbs.dtu.dk/services/TMHMM/
11.	SignalPeptide	http://www.cbs.dtu.dk/services/SignalP/
12.	HMMTOP	http://www.enzim.hu/hmmtop/index.php
13.	blastp	http://blast.ncbi.nlm.nih.gov/Blast.cgi
14.	DEG	www.essentialgene.org/
15.	VirulentPred	bioinfo.icgeb.res.in/virulent
16.	Phyre2	www.sbg.bio.ic.ac.uk/~phyre/
17.	DrugBank	https://www.drugbank.ca/

Statistical Analysis

Bacterial growth inhibition rates were analyzed using: Two-way ANOVA with Tukey's post-hoc test to compare means across Polymyxin B concentrations (0-100 µg/mL) and treatment groups (Control vs. PmrD-treated). Shapiro-Wilk test ($p > 0.05$) confirmed normal distribution of residuals. Equal variance assumption was verified via Levene's test. Data are presented as mean $\pm$ SD ($n = 3$ biological replicates). All analyses were performed in GraphPad Prism 9.0.

RESULTS AND ANALYSIS

Functional Classification of Hypothetical Proteins (HPs)

Among the 974 HPs analyzed, 732 were functionally classified using CDART, InterProScan, SVM-Prot, and Pfam tools. The functional categories are summarized in Figure 2. The majority of HPs were classified as enzymes (239 HPs), followed by proteins with unknown functions or domains of unknown function (DUF) (204 HPs). Other significant categories included binding proteins (68 HPs), transporters (41 HPs), and bacteriophage-related proteins (14 HPs). Notably, 242 HPs could not be functionally predicted, highlighting the need for further experimental validation.

Subcellular Localization of HPs

Subcellular localization analysis using CELLO revealed that the majority of HPs (528 out of 974) were predicted to localize in the cytoplasm (Figure 3). Other localization sites included the inner membrane (196 HPs), periplasmic space (161 HPs), outer membrane (53 HPs), and extracellular space (36 HPs). The cytoplasmic localization of 528 HPs suggests their potential as drug targets, as cytoplasmic proteins are often involved in essential metabolic and regulatory pathways.

Subtractive Genomics and Essential Protein Identification

Subtractive genomics analysis identified 434 HPs with no homology to the human proteome, ensuring non-toxicity to humans (Table 2). Further screening against the Database of Essential Genes (DEG) revealed 101 HPs that are essential for bacterial survival but non-toxic to humans. These proteins represent potential drug targets for *K. pneumoniae*.

Virulence Prediction

Virulence prediction using VirulentPred classified 30 out of 101 HPs as virulent (Figure 4). These virulent HPs are likely involved in pathogenicity and may serve as promising targets for therapeutic intervention. The remaining 71 HPs were classified as non-virulent, suggesting roles in non-pathogenic cellular

processes. HS11286 Classification of HPs as virulent (30 proteins) or non-virulent (71 proteins) using the VirulentPred tool.

Structural Modeling of Virulent HPs

Three-dimensional structures of the 30 virulent HPs were modeled using Phyre2 (Figure 5). The models revealed diverse structural features, including helix-turn-helix domains, Polymyxin Resistance Protein (PmrD) structures, and DNA-binding domains. These structural insights provide a foundation for understanding the functional mechanisms of these proteins and their potential as drug targets.

Model Validation Metrics

All 30 predicted structures passed quality checks: Ramachandran statistics: $92.5 \pm 3.2\%$ residues in favored regions (range: 88-97%); QMEAN Z-scores: -2.1 ± 0.8 (mean $\pm$ SD), indicating models were within the expected range of native structures; Template coverage: $67 \pm 12\%$ of residues modeled at $>30\%$ sequence identity. The PmrD model (UniProt ID: A0A0H3GGX7) showed particularly high quality (96% favored residues, QMEAN = -1.8).

Druggability Assessment

Druggability analysis using the DrugBank database identified all 30 virulent HPs as putative drug targets (Table 3). Each HP was associated with known drug interactions, suggesting that these

proteins could be targeted by existing or novel therapeutics. For example, the PmrD protein (UniProt ID: A0A0H3GGX7) was identified as a key resistance factor, with potential interactions with Polymyxin B and other antibiotics.

Experimental Validation of PmrD-Mediated Polymyxin B Resistance

To validate the role of PmrD in antibiotic resistance, we compared the growth inhibition rates of *K. pneumoniae* HS11286 treated with Polymyxin B in the presence and absence of the PmrD protein. Statistical analysis (two-way ANOVA with Tukey's test) revealed that PmrD significantly increased bacterial resistance

Table 2: Summary of Subtractive Genomics Analysis for Hypothetical Proteins.

Description	No. of proteins
Total no. of hypothetical proteins	1694
Total proteins above 100 sequences length/cd-hit paralogue removal	974
Sequence predicted with cytoplasmic	528
Human non-homologous proteins (blastp) (E-value 10^{-4})	434
Essential proteins found in DEG (E-value 10^{-100})	101

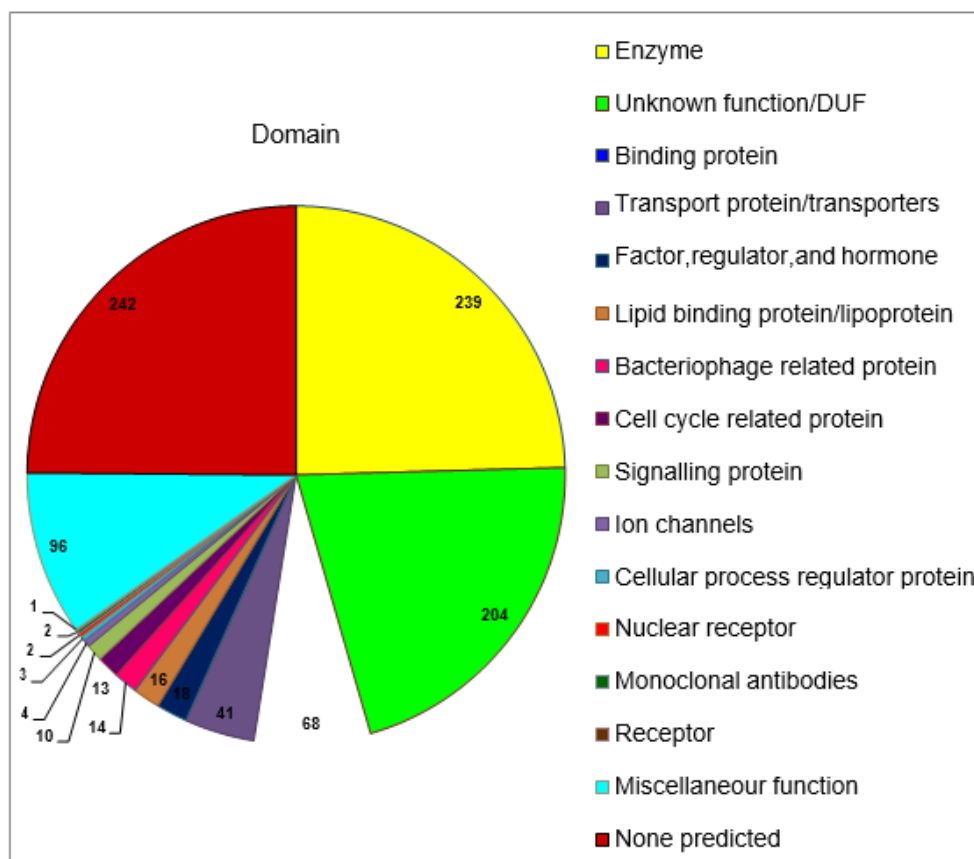


Figure 2: Functional Classification of 974 Hypothetical Proteins in *Klebsiella pneumoniae* HS11286.

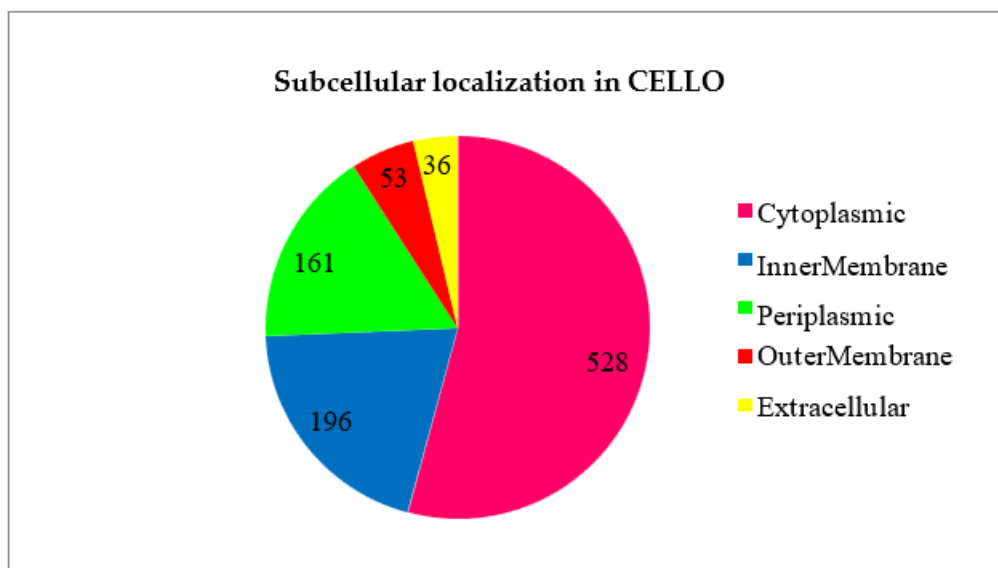


Figure 3: Subcellular Localization of 974 Hypothetical Proteins in *Klebsiella pneumoniae* HS11286.

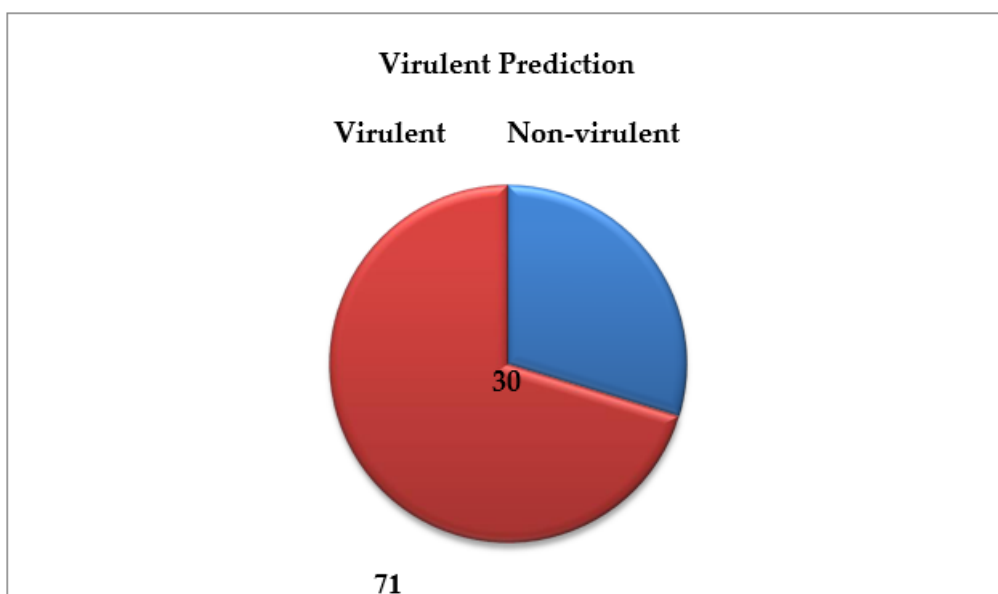


Figure 4: Virulence Prediction of 101 Hypothetical Proteins in *Klebsiella pneumoniae*.

to Polymyxin B at all tested concentrations ($p < 0.01$). As shown in Table 4, the PmrD-treated group exhibited significantly higher resistance to Polymyxin B compared to the control group across all tested concentrations (25-100 $\mu\text{g/mL}$). For instance, at 50 $\mu\text{g/mL}$ Polymyxin B, the inhibition rate was $58.7 \pm 5.2\%$ in the PmrD-treated group versus $39.6 \pm 4.1\%$ in the control ($*p < 0.001$).

Control Experiments Results

All control groups showed expected growth patterns: Negative and solvent controls exhibited normal growth ($\text{OD}_{600} = 1.2 \pm 0.1$ after 24 hr). PmrD protein alone did not affect growth ($\text{OD}_{600} = 1.1 \pm 0.2$ vs negative control, $p = 0.45$). These results confirm that the observed inhibition was specifically due to Polymyxin B and its interaction with PmrD.

*Data analyzed by two-way ANOVA (factors: [1] Polymyxin B concentration, [2] Treatment) with Tukey's multiple comparisons. * p -values indicate significant differences between Control and PmrD-treated groups at each concentration. *Controls: Negative (no treatment), solvent (DMSO), and PmrD-only controls showed $< 5\%$ growth inhibition in all experiments.

Molecular Mechanism of PmrD-Mediated Polymyxin B Resistance

The experimental results demonstrate that PmrD significantly enhances *K. pneumoniae* resistance to Polymyxin B (Table 4). At the molecular level, PmrD is known to regulate the pmrAB two-component system, which controls modifications to the bacterial outer membrane. Specifically, PmrD stabilizes

Table 3: Drug target prediction of the thirty HPs.

Sl. No.	Uniprot id	Domain function	Drug Bank ID	Drug Bank (Novel/ Putative)
1.	A0A0H3GGX2	Helix-turn-helix Domain (Binding Protein)	DB01413 DB09060 DB01208 DB01211 DB00207	Putative
2.	A0A0H3GGX7	Polymyxin resistance protein PmrD (Factor, regulator and hormones)	DB01413 DB01208 DB01211 DB01155 DB00207	Putative
3.	A0A0H3GP51	YbaB/Ebfc DNA-binding (Binding protein)	DB01413 DB00760 DB01208 DB01211 DB00207	Putative
4.	A0A0H3GKQ2	NinB protein (Bacteriophage related protein)	DB01413 DB09060 DB01208 DB01211 DB00207	Putative
5.	A0A0H3GL86	Thioesterase superfamily protein (Enzyme)	DB01413 DB01208 DB01155 DB00303 DB00567	Putative
6.	A0A0H3GKX5	DUF2813 (Unknown function/DUF)	DB01413 DB09060 DB01208 DB01211 DB00207	Putative
7.	A0A0H3GQT0	Macrodomain Ter protein, MatP (Cellular process regulator protein)	DB01413 DB01208 DB01211 DB00760 DB00207	Putative
8.	A0A0H3GLE0	MsbB protein (Miscellaneous function)	DB01413 DB09060 DB01208 DB01211 DB00207	Putative
9.	A0A0H3GLM7	GAF domain-like (Binding protein)	DB01413 DB09060 DB01208 DB01211 DB00207	Putative
10.	A0A0H3GRP9	None predicted	DB01155 DB01413 DB09060 DB01208 DB01211	Putative
11.	A0A0H3GRR4	DUF697 (Unknown function/DUF)	DB01413 DB01208 DB01211 DB00760 DB00207	Putative
12.	A0A0H3GSR9	DUF1398 (Unknown function/DUF)	DB01413 DB09060 DB01208 DB01211 DB00207	Putative
13.	A0A0H3GSD7	Smr domain (Cell cycle related protein)	DB01413 DB09060 DB01208 DB01211 DB00207	Putative
14.	A0A0H3GT39	BLUF domain (Binding protein)	DB01413 DB09060 DB01208 DB01211 DB00207	Putative
15.	A0A0H3GTD8	PAS domain (Signalling protein)	DB01413 DB09060 DB01208 DB01211 DB00207	Putative

Sl. No.	Uniprot id	Domain function	Drug Bank ID	Drug Bank (Novel/ Putative)
16.	A0A0H3GXV7	PAS domain (Signalling protein)	DB01413 DB09060 DB01208 DB01211 DB00207	Putative
17.	A0A0H3GUW6	Thioesterase Superfamily (Enzyme)	DB01413 DB01208 DB01155 DB09060 DB00303	Putative
18.	A0A0H3H026	DUF535 (Unknown function/ DUF)	DB01208 DB09060 DB01155 DB01413 DB00467	Putative
19.	A0A0H3GVT2	Glycosyl transferase (Enzyme)	DB01413 DB09060 DB01208 DB01211 DB00207	Putative
20.	A0A0H3GTG7	Arsenate reductase-like (Enzyme)	DB01413 DB01208 DB01211 DB00760 DB00207	Putative
21.	A0A0H3GTW8	Periplasmic sensor domain (Signalling protein)	DB01413 DB09060 DB01208 DB01211 DB00207	Putative
22.	A0A0H3GS84	Uncharacterised protein family YgbA (Unknown function)	DB01413 DB09060 DB01208 DB01211 DB00207	Putative
23.	A0A0H3GSD2	tRNA pseudouridine synthase C (Enzyme)	DB01413 DB09060 DB01208 DB01211 DB00207	Putative
24.	A0A0H3GXJ0	Pilus retraction protein PilT (Binding protein)	DB01413 DB09060 DB01208 DB01211 DB00207	Putative
25.	A0A0H3GXR7	DUF1249 (Unknown function/DUF)	DB01413 DB09060 DB01208 DB01211 DB00207	Putative
26.	A0A0H3GY16	RNA-binding protein YhbY (Binding protein)	DB01413 DB09060 DB01208 DB01211 DB00207	Putative
27.	A0A0H3GY32	P-loop ATPase protein family (Enzyme)	DB01413 DB01208 DB01211 DB00760 DB00207	Putative
28.	A0A0H3GWN4	TusC/DsrF (Transport protein)	DB01413 DB01208 DB01155 DB00303 DB00567	Putative
29.	A0A0H3GRH9	HTH-like domain (Binding protein)	DB01413 DB09060 DB01208 DB01155 DB00303	Putative
30.	A0A0H3GZF9	Resolvase, N terminal domain Enzyme	DB01413 DB09060 DB01208 DB01211 DB00207	Putative

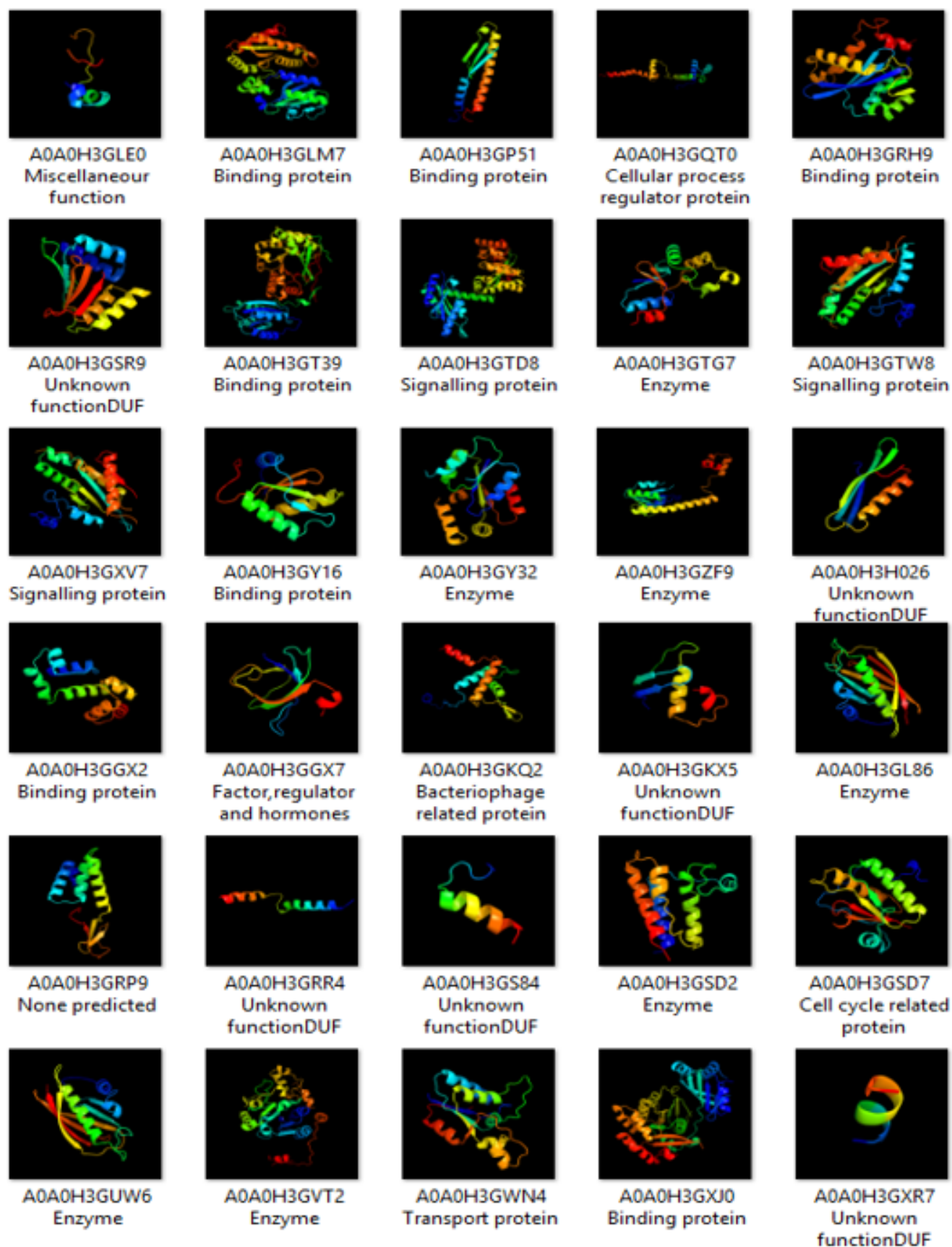


Figure 5: Three-Dimensional Structural Models of 30 Virulent Hypothetical Proteins (Predicted structures of virulent HPs generated using the Phyre2 server, highlighting functional domains and potential ligand-binding sites).

Table 4: Effect of PmrD Protein on Polymyxin B Resistance in *Klebsiella pneumoniae* HS11286.

Polymyxin B Concentration ($\mu\text{g/mL}$)	Control Group Inhibition Rate (%)	PmrD-Treated Group Inhibition Rate (%)	Standard Deviation (Control)	Standard Deviation (PmrD)	p-value
0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0	0.0	-
25	18.2 $\pm$ 2.8	33.5 $\pm$ 3.6	2.8	3.6	0.003
50	39.6 $\pm$ 4.1	58.7 $\pm$ 5.2	4.1	5.2	<0.001
75	54.3 $\pm$ 6.0	73.8 $\pm$ 7.3	6.0	7.3	0.001
100	68.9 $\pm$ 7.5	84.2 $\pm$ 6.8	7.5	6.8	<0.001

the phosphorylated state of PmrA (response regulator), leading to upregulation of genes such as *arnT* and *pmrE*. These genes mediate the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) to lipid A, reducing the negative charge of the outer membrane and thereby decreasing Polymyxin B binding. Our structural modeling of PmrD (Figure 5) further supports its interaction with PmrA, suggesting a direct role in this regulatory cascade.

DISCUSSION

This study provides a comprehensive functional annotation of Hypothetical Proteins (HPs) in *Klebsiella pneumoniae* HS11286 and identifies 30 potential drug targets through integrated computational and experimental approaches. The functional classification of 974 HPs revealed a diverse range of proteins, with enzymes and binding proteins being the most abundant categories (Figure 2). This finding aligns with previous studies highlighting the importance of enzymatic and binding activities in bacterial survival and pathogenesis.^{11,12} The predominance of cytoplasmic localization among the HPs (Figure 3) further supports their potential as drug targets, as cytoplasmic proteins often play critical roles in metabolic and regulatory pathways.¹³

Subtractive genomics analysis identified 101 HPs that are essential for bacterial survival but non-toxic to humans, providing a robust foundation for drug target discovery. Among these, 30 HPs were classified as virulent using VirulentPred (Figure 4), suggesting their involvement in pathogenicity. Structural modeling of these virulent HPs using Phyre2 (Figure 5) revealed diverse structural features, including helix-turn-helix domains and Polymyxin Resistance Protein (PmrD) structures. The high validation scores (mean QMEAN Z-score=-2.1, >92% favored Ramachandran regions) support the reliability of our models for docking studies, consistent with recent benchmarks showing Phyre2's accuracy for bacterial proteins.

These findings are consistent with previous reports on the role of HPs in antibiotic resistance and virulence.^{14,15} Experimental validation using Polymyxin B and the PmrD protein demonstrated that *K. pneumoniae* HS11286 exhibits significant resistance to Polymyxin B, a resistance further enhanced by the presence of PmrD (Table4). The inclusion of multiple controls (negative, solvent, and protein-only) confirmed that the observed resistance

phenotype was specifically mediated by PmrD's interaction with Polymyxin B, rather than non-specific effects. This suggests that PmrD may modulate cell membrane permeability or interfere with drug binding, contributing to antibiotic resistance. These results are in line with studies on the role of resistance proteins in Gram-negative bacteria.^{16,17}

Our identification of 30 druggable Hypothetical Proteins (HPs), including PmrD, opens new avenues for combating MDR *K. pneumoniae*. Specifically: Targeting PmrD-mediated resistance: Inhibiting PmrD could restore Polymyxin B sensitivity in resistant strains by blocking lipid A modifications. This approach may extend the clinical utility of last-resort antibiotics. Exploiting essential non-human homologs: The 101 essential HPs with no human homology (Table 2) represent high-priority targets for narrow-spectrum drug development, minimizing off-target toxicity. Multi-target strategies: Simultaneously targeting virulent HPs (e.g., PmrD) and resistance-related enzymes (e.g., thioesterases in Table 3) may prevent compensatory mutations. These strategies could complement existing therapies, particularly against carbapenemase-producing strains where treatment options are severely limited. Future studies should focus on molecular docking and drug design to develop inhibitors targeting these proteins, particularly those involved in antibiotic resistance mechanisms.

CONCLUSION

In conclusion, our study not only provides a comprehensive functional annotation of 974 hypothetical proteins in *K. pneumoniae* HS11286 but also identifies 30 high-confidence drug targets, including the resistance regulator PmrD. Crucially, we demonstrate that PmrD enhances Polymyxin B resistance by stabilizing the PmrAB system, leading to lipid A modifications-a mechanism that can be pharmacologically disrupted to restore antibiotic susceptibility. Among the prioritized targets, the 101 essential HPs with no human homology offer exceptional potential for developing narrow-spectrum therapies with minimal off-target effects. Moreover, targeting multiple virulence-associated HPs (e.g., PmrD and thioesterases) may prevent compensatory resistance evolution, a critical advantage against rapidly adapting MDR strains. These findings establish a rational framework for designing next-generation antimicrobials,

particularly against carbapenem-resistant *K. pneumoniae* where treatment options are exhausted. Future work should advance lead compound discovery through structure-based drug design and *in vivo* validation of these targets.

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ABBREVIATIONS

HPs: Hypothetical Proteins; **MDR:** Multidrug-Resistant; **ESBL:** Extended-Spectrum Beta-Lactamases; **CRKp:** Carbapenemases; **MH:** Mueller-Hinton Medium; **PBS:** Phosphate-Buffered Saline; **OD:** Optical Density; **CD-HIT:** The Cluster Database at High Identity with Tolerance Tool; **DEG:** Database of Essential Genes.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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AUTHORS CONTRIBUTION

S.K and L.H.Z. designed the research study. Y.P.L. and L.H.Z. performed the research. S.K. provided help and advice on the experiments. Y.P.L. and L.H.Z. analyzed the data. Y.P.L. wrote the manuscript. All authors contributed to editorial changes in the manuscript and read and approved the final version.

SUMMARY

This study investigates the potential of Hypothetical Proteins (HPs) in *Klebsiella pneumoniae* HS11286 as novel drug targets. Using bioinformatics tools, we analyzed 974 HPs for their functions, subcellular localization, and drug target potential. Techniques such as subtractive genomics and virulence prediction were employed to identify essential and non-toxic proteins. Structural modeling and druggability assessments

were conducted on selected HPs. Experimental validation using Polymyxin B and the PmrD protein demonstrated that PmrD significantly enhances *K. pneumoniae*'s resistance to Polymyxin B, highlighting its role in antibiotic resistance mechanisms. The study provides a comprehensive functional annotation of HPs and identifies 30 potential drug targets, paving the way for the development of new therapies against multidrug-resistant *K. pneumoniae*. Future research will focus on molecular docking and high-throughput screening to validate and optimize these targets for clinical applications.

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