Phylogenetic Analysis of Beta-Lactamases Reveals Distinct Evolutionary Patterns of Chromosomal and Plasmid-Encoded BLs and the Mosaic Role of VIM Linking NDM and IMP

Abstract
Beta-lactamases (BLs) are a major driver of antibiotic resistance in pathogens like *Acinetobacter baumannii*, Enterobacteriaceae and *Pseudomonas aeruginosa*. This study explores the structural stability, and functional divergence of metallo-beta-lactamases (MBLs) and AmpC through study of protein-protein interaction (PPI) network analysis, phylogenetic study and identification of conserved domains and motifs. The study also involves analysis of co-evolutionary dynamics of BLs with related genes. PPI analysis identified NagZ as central interacting partner for BLs in *P. aeruginosa* and Enterobacteriaceae, suggesting its pivotal role in BL expression, and highlighting its probable role as a potential drug target. In contrast, *A. baumannii* exhibited such a high diversity in protein interactions, that considering a single protein as topmost interacting partner was difficult. Phylogenetic analysis revealed strong co-evolutionary trends between functionally-related proteins such as NagZ, AmpC and AmpR. Plasmid-encoded MBLs and related proteins seem to exhibit higher HGT rates between *Acinetobacter* and Enterobacteriaceae. On the other hand, similar evolutionary patterns for chromosomally-encoded BLs like AmpC and related genes was found in *A. baumannii* and *P. aeruginosa*. Analysis of PFAM domains showed that catalytic and substrate binding domains are more conserved than accessory ones. Few domains were found to be conserved across all MBLs and thus carry the potential drug targets. Finally, inter-BL analysis revealed that VIM acts as an evolutionary link by acting like a mosaic between IMP and NDM as supported by intermediate GC content, shared sequence features, and functional domains.

1. Introduction

The emergence and spread of antibiotic resistance represent one of the most pressing challenges to modern medicine, with beta-lactamases (BLs) playing a central role (**Bush et al., 2016**). BLs are enzymes that hydrolyze the beta-lactam ring of the beta-lactam antibiotics such as penicillin, cephalosporin and carbapenem, rendering them ineffective and severely limiting treatment options for multidrug-resistant (MDR) bacterial infections **(Arer and Anurag Anand et al., 2024**). World Health Organization (WHO), in its 2017 priority pathogen list, identified carbapenem-resistant *Acinetobacter baumannii*, Enterobacteriaceae and *Pseudomonas aeruginosa*, as critical threats due to their ability to acquire and disseminate BL genes through both vertical inheritance and horizontal gene transfer (HGT) **(World Health Organization [WHO], 2017; World Health Organization [WHO], 2024).** We, thus, aimed to study the evolution and transfer patterns of BLs in and across these pathogens.
BLs are broadly classified into four molecular classes (A, B, C, and D) based on their amino acid sequences and catalytic mechanisms (**Anurag Anand et al., 2025a; Arer and Kar, 2023**). Class A, C, and D BLs are serine-based enzymes that utilize a serine residue at their active site to hydrolyze beta-lactams. In contrast, Class B BLs, also known as metallo-beta-lactamases (MBLs), are zinc-dependent and require divalent metal ions for activity (**Bush and Jacoby, 2010)**. MBLs, such as New Delhi metallo-beta-lactamase (NDM), Verona integron-encoded metallo-beta-lactamase (VIM), and imipenemase (IMP), are particularly concerning due to their plasmid borne-nature facilitating high HGT rates, broad substrate specificity and resistance to nearly all beta-lactams, including carbapenems (**Anurag Anand et al., 2024)**. AmpC-type BLs, which are majorly chromosomally encoded, confer resistance to cephalosporins and penicillins and are well known for their inducibility in response to antibiotic exposure **(Liu et al., 2022)**. Plasmid-encoded MBLs of several types have been largely identified in *P. aeruginosa*, *A. baumannii*, and Enterobacteriaceae, indicating extensive HGT between environmental and clinical bacterial reservoirs. In contrast, chromosomally encoded BLs like AmpC are more conserved within specific bacterial lineages, reflecting host-specific evolutionary pressures **(Imkamp et al., 2022; Schotte et al., 2024)**. The ability of pathogens to harbor both chromosomal and plasmid-encoded BLs further complicates treatment and increases the likelihood of multidrug resistance. Thus, it becomes important for us to identify conserved evolutionary patterns in order to target multiple BLs.
Recently, phylogenetic studies have been showing great success in analysis of several enzymes and their associated proteins by providing insights into the evolutionary pressures shaping these interactions and revealing conserved and divergent motifs that may serve as drug targets (**Salahuddin and Khan, 2014**). Therefore, this study aims to address the growing challenge of BL-mediated antibiotic resistance by studying evolutionary patterns, and identifying conserved and divergent motifs in BLs (**Anurag Anand et al., 2025b**). Through study of protein-protein interaction (PPI) network analysis, multiple sequence alignment (MSA), phylogenetic analysis and motif identification, we uncover co-evolutionary relationships and functional interdependence between BLs and associated proteins. We also evaluate the evolutionary clustering of beta-lactamases based on amino acid composition (AAC), atomic and bond composition (ABC), and physicochemical properties (APP), providing insights into the structural and functional mechanisms driving resistance **(Brandt and Barrangou, 2016)**.
Our findings reveal that *A. baumannii* exhibits a higher diversity of interacting proteins, reflecting a more complex and adaptable resistance strategy. This increased diversity is likely driven by its high capacity for HGT and its ability to integrate foreign genetic material, thereby expanding its repertoire of beta-lactamase-associated proteins (**Mei et al., 2019**). In contrast, *P. aeruginosa* and Enterobacteriaceae demonstrate more conserved interaction patterns, indicating a streamlined and specialized approach to resistance. This study identifies NagZ as the topmost interacting partner for beta-lactamases in *P. aeruginosa* and Enterobacteriaceae. NagZ plays a role in the regulation of BL expression. BL ex-

pression is regulated via a complex network of interacting proteins such as AmpR, AmpD, and NagZ. NagZ serves a role in AmpC expression through the AmpR-AmpD regulatory cascade and is also a central player in the peptidoglycan recycling pathway. Most importantly, our study suggests a strong co-evolutionary link between NagZ, AmpC, and AmpR indicating that these proteins have undergone coordinated genetic changes to enhance resistance efficiency under antibiotic pressure (**Bedhomme et al., 2017**). Further, we found that plasmid-encoded MBLs and related genes show similar evolutionary patterns for *Acinetobacter* and Enterobacteriaceae, whereas chromosomally-encoded BLs like AmpC and related show similar evolutionary patterns across *A. baumannii* and *P. aeruginosa*. Comparative analysis of AAC, ABC, and APP clusters using Principal Component Analysis (PCA) reveals distinct clustering patterns among NDM, VIM, IMP, and AmpC BLs. NDM and VIM form a cohesive cluster due to conserved hydrophobic and charged residues, reflecting their evolutionary stability and broad substrate specificity. In contrast, IMP shows greater structural divergence, indicating functional differentiation and adaptation to diverse environmental niches. GC content analysis further highlights differences in structural stability, with NDM exhibiting high GC content and lower mutational plasticity, while IMP displaying low GC content and higher evolutionary variability **(Brandt and Barrangou, 2016)**. VIM was found to lie between NDM and IMP in GC content analysis. Motif analysis using MEME and TOMTOM identifies conserved motifs and mutational hotspots across different beta-lactamase classes **(Anurag Anand et al., 2025a)**. Highly conserved motifs in NDM and VIM suggest a strong evolutionary link, while the partial conservation of motifs in IMP reflects a mix of conserved and functionally distinct regions. Mutational hotspots affecting Zn^{2} coordination and enzyme stability are identified, highlighting key structural vulnerabilities that could serve as drug targets. Lastly, inter-BL phylogenetic analysis combined with study of GC content, conserved domains and motifs reveals that VIM acts as an evolutionary link between IMP and NDM due to shared sequence physiochemical properties, motifs and functional domains from both the MBLs.

2. Methodology

2.1 Protein-protein interaction study using STRING

STRING database was utilized to search for interacting partners of BLs and related proteins **(Szklarczyk et al., 2025)**. By related proteins, we mean proteins which have domains similar to those lying within BLs. We not only identified the interacting partners of BLs and related proteins using STRING, but also extracted the protein sequence of these, for further analysis. The keyword used for protein search was 'beta-lactamases' along with organism-specific search filters: '*Pseudomonas aeruginosa* group', '*Acinetobacter baumannii'* and 'Enterobacteriaceae' for *P. aeruginosa*, *A. baumannii* and Enterobacteriaceae respectively. Topmost interacting partners found associated with most BLs and related proteins were identified. Those proteins which were found to interact with at least 5 BLs and related proteins were selected for studying their co-evolutionary dynamics with BLs and related proteins.

2.2 Selection of beta-lactamase sequences for BLAST

The sequence of NDM-1 was sourced from high quality PDB structure with PDB ID 8B1W, that can be accessed at https://doi.org/10.2210/pdb8b1w/pdb (**Bersani et al., 2023**). The sequences chosen for sequence analysis of VIM-2, and IMP-1 were obtained from NCBI accession ACY78406.1 and CRP48843.1, respectively. Subsequently, homologous sequences from NCBI were obtained using the NCBI BLASTP facility. These *P. aeruginosa* sequences were considered as the starting point.

2.3 Sequence similarity search of BLs, related proteins and their interacting partners

BLASTP was performed using NCBI BLAST facility (**Camacho et al., 2009**). For VIM, NDM and IMP, the topmost sequences were from *Pseudomonas* as expected. Thus, the sequences of *A. baumannii* and Enetrobacteriaceae were added when not found in top hits. This was done by performing BLASTP with organism-specific search. The keywords or filters used while performing organism-specific search were Taxid: 562 (*Escherichia coli*), Taxid: 91347 (Enterobacteriaceae) and Taxid: 470 (*A. baumannii).*

For BL sequences, related proteins and interacting partners identified from STRING, the sequences derived from STRING were subjected to BLASTP and the similarity search for all three organisms was done in a manner similar to above (like that done for VIM, NDM and IMP).

2.4 Multiple Sequence Alignment and Mutational Analysis

Multiple sequence alignment (MSA) for each of the protein datasets was performed. MEGA software (version 11) was used for the alignment, with the Muscle algorithm as the alignment method **(Edgar, 2004; Kumar et al., 2008; Tamura et al., 2021)**. The gap open penalty was set to -2.90, while the gap extension penalty was set at 0.00. To account for sequence hydrophobicity, a multiplier of 1.20 was applied. The algorithm was run for a maximum of 16 iterations, ensuring sufficient refinement in the alignment. UPGMA (Unweighted Pair Group Method with Arithmetic Mean) was used to guide the clustering of sequences **(Sokal and Michener, 1958)**. This method continued to be applied in subsequent iterations, maintaining consistency in alignment structure. To enhance alignment precision, the minimum diagonal length (Lambda) was set to 24. This systematic approach with MEGA 11 and the Muscle algorithm allowed us to produce multiple sequence alignments, which were subsequently visualized using itol (itol can be accessed at https://itol.embl.de/) (used for construction of phylogenetic tree using newick data extracted from MEGA analysis). These were further analyzed in the Jalview software (version 2.11.4.0) **(Waterhouse et al., 2009)**. Through Jalview, we

identified conserved domains present across all species in the study. Additionally, it enabled us to detect specific amino acid mutations occurring within these conserved domains. While studying the alignment of protein sequences using Jalview, color-coding proved useful in refining the alignment.
Sequence logos were then generated using the WebLogo software to visualize residue conservation across all aligned blocks in our MSA **(Crooks et al., 2004)**. These logos illustrate a highly conserved domain, with minimal mutations observed within the conserved region.

2.5 Phylogenetic analysis
Maximum likelihood (ML) tree was built to perform the phylogenetic analysis and study the evolutionary dynamics of the BLs, related proteins and their interacting partners **(Felsenstein, 1981)**. Our aim was to construct a phylogenetic tree using bootstrap sampling. We first prepared and aligned the DNA sequences of the taxa of interest. Next, we selected the best-fit substitution model using tools like MEGA, which informs the likelihood calculations. An initial ML tree was then constructed based on the full dataset, evaluating various topologies to maximize likelihood. We performed bootstrap analysis by resampling the original alignment 100 times to create pseudo-replicate datasets, reconstructing an ML tree for each replicate to assess clade consistency (**Russo et al., 2018**). Bootstrap support values were calculated for each node in the original tree, indicating the reliability of clades based on the proportion of supporting replicates. The combination of ML methods and bootstrap sampling provides a statistically robust framework for accurately estimating evolutionary relationships while measuring uncertainty, resulting in phylogenetic trees that reflect evolutionary relationships with greater confidence.
Where necessary, we reconstructed the tree using 5 other algorithms as well, namely, NJ (neighbor joining), ME (minimum evolution), UPGMA original (Unweighted Pair Group Method with Arithmetic Mean), UPGMA BCT (bootstrap consensus tree with 1000 times bootstrap sampling), and MP (minimum parsimony). Thus, instead of relying on a single evolutionary tree, insights were derived from 6 different algorithms, to enable efficiency and reliability of results.

2.6 Study of co-evolutionary dynamics
We assessed as to which interacting partners show co-evolutionary pattern with their respective BLs and related proteins. This was done by visually identifying how the genus and species divergence is occurring in the trees.

2.7 Study of closely and distantly related priority pathogens
We also assessed the phylogenetic relationships among priority pathogens for each protein in such a way so as to identify which two priority pathogens lie closer together and which one lays farther apart.

2.8 GC content analysis
GC content analysis was performed using Biopython for all sequences of a BL. This was done in order to account for stability of different BL types, and to compare the changes in nucleotide composition over the period of time within the group of a particular beta-lactamase.

2.9 Statistical analysis for protein features and physiochemical properties
The amino acid composition (AAC), atoms and bonds composition (ABC) and various advanced physiochemical properties (APP/PCP) of the proteins were studied using the Pfeature software. Pfeature is a renowned web server for computing various features of proteins from their amino acid sequence **(Pande et al., 2023)**. The values for AAC, ABC and APP descriptors for each of the proteins were subjected to PCA to cluster the sequences into different groups on the basis of their overall similarity. The contribution of each amino acid to AAC, ABC and APP was then calculated.

2.10 PFAM domain analysis
Protein family (PFAM) domains with different structural and functional roles were identified and studied across different BLs **(Finn et al., 2016)**. Comparisons were made both within (between different sequences of a particular BL type) and across BLs (between different MBLs, and between MBLs and AmpC). This was done to get an idea of how the protein structures of beta-lactamases have evolved over the period of time.
Further, PFAM domain conservation scores were calculated based on the number of bacterial strains containing each domain. Percentages were assigned based on conservation levels across different bacterial groups. Heatmaps were built using matrix representation of these conservation values, for visualization. Heatmaps were generated using seaborn.heatmap().
The conservation percentage of PFAMs was calculated as follows:
Conservation Score = (Number of strains with the domain/Total strains in the group) × 100
Example: If 90% of Pseudomonas strains contains the Beta-lactamase domain of AmpC, then the conservation score for AmpC-Pseudomonas will be 90.

2.11 Motif identification and comparison
Repeatedly occurring motifs were identified using the MEME software. TOMTOM comparative analysis was then performed to see how these differ across different BLs **(Nystrom and McKay, 2021)**. Comparisons were made both within and across different BLs (between different MBLs, and between MBLs and AmpC). This was done to find out how beta-lactamases have evolved over the period of time.

2.12 Defining Similarity Scores based on motifs analysis

We used approximate similarity values based on the p-values and motif conservation analysis from TOMTOM. The similarity score was derived from the motif alignment p-values in the TOMTOM results. A lower p-value means stronger similarity between motifs, while a higher p-value suggests weaker similarity.

We used the following formula to convert p-values into similarity scores:

Similarity Score = 100 - (log10 (p-value) × 10)

The above formula ensures that highly conserved motifs (lower p-values) result in higher similarity scores, whereas weakly conserved motifs (higher p-values) result in lower similarity scores. This approach provides normalized similarity scores. Scores close to 100 indicate high conservation, whereas lower scores (closer to 50-60) indicate moderate or weak conservation.

3. Results and discussion

3.1 NagZ as the topmost interacting partner for BL and related proteins

For *P. aeruginosa*, 87 entries of BLs and related proteins were found for *P. aeruginosa* on STRING database. String interactions for all the proteins were extracted in the form of nodes. Redundancy was removed to make sure that each node is taken into account only once for evaluation. A combined list containing STRING IDs of the interacting partners of all BLs and related proteins was formulated and was further annotated with the names of the BLs and related proteins with which they interact.

For *A. baumannii*, 18 entries of BLs and related proteins were found, whereas 350 entries of BLs and related proteins were found for Enterobacteriacea.

For *P. aeruginosa*, NagZ was found to be the topmost interacting protein, showing interactions with 19 BLs and related proteins. Gpmi, polA, and rpoB were found to interact with 13 proteins each. RpoZ was found to interact with 12 whereas guaA was found to interact with 11 such proteins. The topmost interacting partners and the associated BLs and related proteins for *P. aeruginosa* have been mentioned in **Table 1.**

Table 1. Topmost interacting partners and the associated BLs and related proteins for *P. aeruginosa*

Gene name	Name of BLs and related proteins
nagZ	ampC - Beta-lactamase precursor; Product name confidence
	ampR - Transcriptional regulator AmpR
	ampD - Beta-lactamase expression regulator AmpD
	SEC41457.1 - Beta-lactamase class C
	SED55975.1 - CubicO group peptidase
	SED86930.1 - CubicO group peptidase
	SDG81131.1 - CubicO group peptidase
	SDH02584.1 - Beta-lactamase class C
	SDH53063.1 - MFS transporter, PAT family
	SDI24945.1 - CubicO group peptidase
	SDI46265.1 - CubicO group peptidase
	ELS26800.1 - Beta-lactamase class C
	ELS26603.1 - Beta-lactamase
	SDO75697.1 - CubicO group peptidase, beta-lactamase class C family
	SDO98540.1 - MFS transporter, PAT family, beta-lactamase
	SDP11108.1 - CubicO group peptidase
	MDS_1181 - Beta-lactamase
	Pmen_2038 - PFAM: beta-lactamase
	SEJ90567.1 - Glyoxylase, beta-lactamase superfamily II
gpmi	Pmen_1284 - PFAM: beta-lactamase domain protein
	Pmen_1856 - PFAM: beta-lactamase domain protein
	SEB92735.1 - Metallo-beta-lactamase family protein
	SDI51646.1 - Metallo-beta-lactamase family protein

Gene name	Name of BLs and related proteins
	SDI51743.1 - Metallo-beta-lactamase family protein
	ELS25187.1 - Metallo-beta-lactamase family protein
	SDN35178.1 - Metallo-beta-lactamase family protein
	MDS_1318 - Beta-lactamase domain-containing protein
	MDS_1962 - Beta-lactamase domain-containing protein
	Pmen_2196 - Exonuclease of the beta-lactamase
	SEJ69490.1 - Metallo-beta-lactamase family protein
	Ysh1 - Metallo-beta-lactamase family protein
	SEJ88278.1 - Metallo-beta-lactamase family protein
polA	Pmen_1284 - PFAM: beta-lactamase domain protein
	Pmen_1856 - PFAM: beta-lactamase domain protein
	SEB92735.1 - Metallo-beta-lactamase family protein
	SDI51646.1 - Metallo-beta-lactamase family protein
	SDI51743.1 - Metallo-beta-lactamase family protein
	ELS25187.1 - Metallo-beta-lactamase family protein
	SDN35178.1 - Metallo-beta-lactamase family protein
	MDS_1318 - Beta-lactamase domain-containing protein
	MDS_1962 - Beta-lactamase domain-containing protein
	Pmen_2196 - Exonuclease of the beta-lactamase
	SEJ69490.1 - Metallo-beta-lactamase family protein
	Ysh1 - Metallo-beta-lactamase family protein
	SEJ88278.1 - Metallo-beta-lactamase family protein
rpoB	Pmen_1284 - PFAM: beta-lactamase domain protein
	Pmen_1856 - PFAM: beta-lactamase domain protein
	SEB92735.1 - Metallo-beta-lactamase family protein
	SDI51646.1 - Metallo-beta-lactamase family protein
	SDI51743.1 - Metallo-beta-lactamase family protein
	ELS25187.1 - Metallo-beta-lactamase family protein
	SDN35178.1 - Metallo-beta-lactamase family protein
	MDS_1318 - Beta-lactamase domain-containing protein
	MDS_1962 - Beta-lactamase domain-containing protein
	Pmen_2196 - Exonuclease of the beta-lactamase
	SEJ69490.1 - Metallo-beta-lactamase family protein
	Ysh1 - Metallo-beta-lactamase family protein
	SEJ88278.1 - Metallo-beta-lactamase family protein
rpoZ	Pmen_1284 - PFAM: beta-lactamase domain protein
	Pmen_1856 - PFAM: beta-lactamase domain protein
	SEB92735.1 - Metallo-beta-lactamase family protein
	SDI51646.1 - Metallo-beta-lactamase family protein
	SDI51743.1 - Metallo-beta-lactamase family protein
	ELS25187.1 - Metallo-beta-lactamase family protein
	SDN35178.1 - Metallo-beta-lactamase family protein
	MDS_1318 - Beta-lactamase domain-containing protein
	MDS_1962 - Beta-lactamase domain-containing protein
	SEJ69490.1 - Metallo-beta-lactamase family protein
	Ysh1 - Metallo-beta-lactamase family protein
	SEJ88278.1 - Metallo-beta-lactamase family protein
guaA	SEC41457.1 - Beta-lactamase class C

Gene name	Name of BLs and related proteins
	SED55975.1 - CubicO group peptidase
	SED86930.1 - CubicO group peptidase
	SDG81131.1 - CubicO group peptidase
	SDH02584.1 - Beta-lactamase class C
	SDI24945.1 - CubicO group peptidase
	SDI46265.1 - CubicO group peptidase
	ELS26800.1 - Beta-lactamase class C
	ELS26603.1 - Beta-lactamase
	SDO75697.1 - CubicO group peptidase, beta-lactamase class C family
	SDP11108.1 - CubicO group peptidase

For *A. baumannii*, no such top contender was concluded as the topmost interacting partner, because the maximum number of BLs and related proteins with which any protein was interacting was found to be 2. This indicated high variation in diversity or a large variety of proteins present in *A. baumannii*, such that there is minimal similarity of interactions across the BLs.
For Enterobacteriaceae, NagZ was again found to be the topmost interacting protein, showing interactions with 42 BLs and related proteins, followed by guaA, ybeY, ampD and BssS.
The details of all the interacting partners and the associated BLs and related proteins for *P. aeruginosa*, *A. baumannii* and Enterobacteriaceae can be found in the **Supplementary Folder 1.**

3.2 Phylogenetic analysis of NagZ
The results for phylogenetic analysis of all the genes, including NagZ, in the form of maximum likelihood tree, can be found in **Supplementary Folder 2.** Phylogenetic analysis of NagZ revealed a distinct evolutionary separation within the studied bacterial taxa. The root node of the tree bifurcates into two primary branches: the first branch groups the Enterobacteriaceae family, forming a cohesive clade. The second branch further splits into two distinct clades, representing *Pseudomonas* and *Acinetobacter*.
The distinct clustering of Enterobacteriaceae within a singular clade suggests a conserved evolutionary path for NagZ within this family, potentially linked to their specific metabolic requirements or environmental adaptations. The separation of *Pseudomonas* and *Acinetobacter* into individual clades under the second branch may indicate unique selective pressures or functional specializations in NagZ across these genera.

3.3 Co-evolutionary dynamics of NagZ with associated BLs and related proteins
Our analysis of the co-evolutionary dynamics between NagZ and associated beta-lactamase-related proteins reveals notable phylogenetic correlations (**Table 2**). The branching patterns observed in the phylogenetic trees of NagZ and AmpC exhibit a strong similarity, suggesting potential co-evolution between these two proteins. This co-evolutionary trend was also evident for AmpR, meaning that these proteins share a similar evolutionary trajectory with NagZ. However, no such pattern was detected for glyoxylase, MFS transporter, or AmpD, indicating a lack of phylogenetic congruence between these proteins and NagZ.
The parallel branching patterns of NagZ with AmpC and AmpR suggest a co-evolutionary relationship, which may reflect a functional interdependence or complementary adaptation of these proteins within bacterial resistance mechanisms **(Table 2).** The phylogenetic trees showing evolutionary patterns of all these proteins can be seen in **Supplementary Folder 2.**

3.4 Evolutionary dynamics of phosphoglycerate mutase (gpmi)
The phylogenetic analysis of gpmi reveals a distinct divergence at the root, where the first branch consists of a mixed clade of Enterobacteriaceae and *A. baumannii* while the second branch contains *Pseudomonas* species. The mixed clade of Enterobacteriaceae and *Acinetobacter baumannii* may indicate shared functional pressures or evolutionary constraints acting on gpmi within these groups. The distinct *Pseudomonas* clade suggests unique adaptations potentially linked to its ecological niche or metabolic requirements. The results for phylogenetic analysis of all the genes, including gpmi, in the form of maximum likelihood tree, can be found in **Supplementary Folder 2.**

3.5 Co-evolutionary dynamics of gpmi with associated BLs and related proteins
The phylogenetic analysis of gpmi reveals an evolutionary pattern indicative of co-evolution with several beta-lactamase-related proteins. Gpmi displays a similar branching pattern with MDS_1962 (MBL fold metallo-hydrolase; PFAM: beta-lactamase domain protein, RNA-metabolising metallo-beta-lactamase), Pmen_1856 (MBL fold metallo-hydrolase; PFAM: beta-lactamase domain protein, RNA-metabolising metallo-beta-lactamase), Pmen_1284 (MBL fold metallo-hydrolase; PFAM: beta-lactamase domain protein; RNA-metabolising metallo-beta-lactamase), and Pmen_2196 (ligase-associated DNA damage response exonuclease; an exonuclease with a beta-lactamase domain). These co-clustering patterns suggest an evolutionary link between gpmi and the studied beta-lactamase domain proteins.

This parallel evolution may indicate shared functional or adaptive pressures, possibly linked to metabolic pathways or cellular processes in which both phosphoglycerate mutase (gpmi) and beta-lactamase-associated proteins play complementary roles. The co-evolutionary signal in these proteins suggests that gpmi may have evolved in concert with beta-lactamases (including MBLs), possibly enhancing or coordinating functions crucial for bacterial survival or resistance mechanisms. The phylogenetic trees showing evolutionary patterns of all these proteins can be seen in **Supplementary Folder 2.**

3.6 Evolution of rpoB , rpoZ , polA & guaA

Both *rpoB* and *rpoZ* exhibit a random distribution across the taxa studied, suggesting a lack of clear phylogenetic association or selective pattern. In contrast, *guaA* shows a closer phylogenetic association between *Pseudomonas* and Enterobacteriaceae, whereas *polA* shows a closer phylogenetic association between *Pseudomonas* and Acinetobacter, indicating potential shared evolutionary pressures or functional links between these groups for these specific genes. The random distribution of *rpoB* and *rpoZ* suggests that these genes may have evolved independently across taxa, potentially reflecting a functional independence from taxonomic constraints or ecological specialization.

The results for phylogenetic analysis of all the genes, in the form of maximum likelihood tree, can be found in **Supplementary Folder 2.**

Further, the phylogenetic relationship of proteins across *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Escherichia coli* has been summarized via **Table 2.**

Table 2. Phylogenetic relationship of genes/proteins among different priority pathogens

Gene/Protein	Closely related	Comparatively distantly related
NagZ	*Acinetobacter baumannii, Pseudomanas aeruginosa*	*Escherichia coli*
AmpC	*Acinetobacter baumannii, Pseudomanas aeruginosa*	*Escherichia coli*
AmpR	*Acinetobacter baumannii, Pseudomanas aeruginosa*	*Escherichia coli*
AmpD	*Acinetobacter baumannii, Escherichia coli;* *Acinetobacter baumannii, Pseudomanas aeruginosa*	*Escherichia coli from Pseudomanas aeruginosa*
MDS 1318	*Acinetobacter baumannii, Pseudomanas aeruginosa*	*Escherichia coli*
polA	*Acinetobacter baumannii, Pseudomanas aeruginosa*	*Escherichia coli*
Cubico	*Acinetobacter baumannii, Escherichia coli*	*Pseudomanas aeruginosa*
Gpmi	*Acinetobacter baumannii, Escherichia coli*	*Pseudomanas aeruginosa*
MFS	*Acinetobacter baumannii, Escherichia coli*	*Pseudomanas aeruginosa*
Glyoxylase	*Acinetobacter baumannii, Escherichia coli*	*Pseudomanas aeruginosa*
MDS 1962	*Acinetobacter baumannii, Escherichia coli*	*Pseudomanas aeruginosa*
Pmen 1856	*Acinetobacter baumannii, Escherichia coli*	*Pseudomanas aeruginosa*
Pmen 2196	*Acinetobacter baumannii, Escherichia coli*	*Pseudomanas aeruginosa*
Pmen 1284	*Acinetobacter baumannii, Escherichia coli*	*Pseudomanas aeruginosa*
ELS25187.1	*Escherichia coli, Acinetobacter baumannii*	*Pseudomanas aeruginosa*
guaA	*Pseudomanas aeruginosa, Escherichia coli*	*Acinetobacter baumannii*
SDI51646	*Pseudomanas aeruginosa, Escherichia coli*	None
SDI51743	*Pseudomanas aeruginosa, Escherichia coli*	None

SDN35178	*Escherichia coli, Pseudomanas aeruginosa*	*Acinetobacter baumannii*
SEB92735	random	random
SEJ69490.1	*Acinetobacter baumannii, Pseudomanas aeruginosa*	None
Ysh.1	random	random
rpoz	random	random
rpob	random	random

The phylogenetic trees showing evolutionary patterns of different BLs and related proteins can be seen in **Supplementary Folder 2.**

3.7 Chromosomally-encoded BLs vs plasmid-encoded BLs

We found that chromosomally-encoded BLs exhibit a closer evolutionary relationship between *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (as indicated by similar evolutionary patterns of AmpC, NagZ and AmpR) (**Figure 1. (a)**). On the other hand, plasmid-encoded MBLs, in contrast to chromosomally-encoded ones, demonstrated a higher similarity between *Acinetobacter* species and Enterobacteriaceae (as indicated by similar evolutionary dynamics several MBL domain containing proteins) (**Figure 1. (b)**).

The observed close relationship of chromosomally-encoded BLs between *A. baumannii* and *P. aeruginosa* highlights a potential co-evolutionary adaptation that may be driven by similar ecological niches and antibiotic selection pressures. This evolutionary convergence suggests that both species may be experiencing similar challenges in their environments, potentially leading to the development of analogous resistance mechanisms. In contrast, the increased similarity of plasmid-encoded MBLs between *Acinetobacter* and Enterobacteriaceae *i*ndicates a significant role for horizontal gene transfer in the dissemination of resistance traits. The ability of plasmids to facilitate rapid transfer of resistance genes underscores the adaptive advantage conferred to these bacteria in the face of antibiotic pressure.

The higher rate of plasmid exchange between *Acinetobacter* and Enterobacteriaceae likely underpins the rapid dissemination of carbapenem resistance. For instance, MBL-encoding plasmids originating in Enterobacteriaceae may be transferred to *Acinetobacter*, transforming non-resistant strains into carbapenem-resistant strains. Probably, this is why the carbapenem-resistant strains of *Acinetobacter baumannii* and Enterbacteriaceae still lie in the critical category (owing to their greater prevalence) whereas those of *P. aeruginosa* lie in high category of the updated priority pathogen list of WHO that was released in 2024. Clearly, *Acinetobacter baumannii* and Enterbacteriaceae are driven heavily by HGT of MBLs across them.

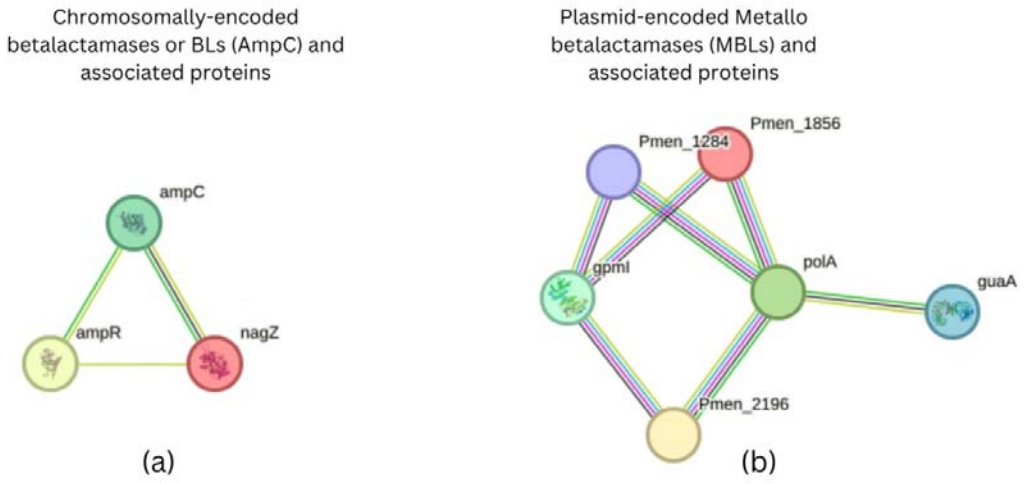


Figure 1. (a) Interaction network of proteins where *Acinetobacter baumannii* and *Pseudomanas aeruginosa* are closely related. **(b)** Interaction network of proteins where *Acinetobacter baumannii and* Enterobacteriaceae are closely related.

Overall, this study also indicates that Acinetobacter is participating more actively in acquiring genes via HGT. It is found to be closely associated with both *P. aeruginosa* as well as *Enetrobacteriaceae.* This is why *Acinetobacter* has

high diversity or variety of interacting partners for BLs, unlike *Pseudomonas and* Enterobacteriaceae where there is occurrence of common interacting partners for several BLs. *Enterobacteriaceae* and *Pseudomonas* show higher divergence for most enzymes in the BL pathway, indicating considerable functional divergence.
Lastly, nagZ and gpmi among others, have the ability to act as potential drug targets in order to combat drug-resistance in *P. aeruginosa* and Enterbacteriaceae, due to their higher interaction ability BLs and related proteins.

3.8 Insights from BLASTP results of NDM, IMP and VIM

BLASTP results of NDM indicating top 5 hits of each priority pathogen show that NDM is conserved across *P. aeruginosa*, *A. baumannii* and *E. coli* (**Table 3.**). The high percent identity values (99–100%) in the BLASTP results indicate that the NDM is highly conserved among the three pathogens. This suggests that the enzyme's structure and function are retained across these species, which may enhance the stability and effectiveness of NDM in conferring antibiotic resistance. The very low E-values (ranging from 5.0E-178 to 1.0E-177 for *A. baumannii* and *P. aeruginosa*, and similarly low for E. coli) confirm the statistical significance of these hits. These E-values show that the matches to NDM in the database are highly significant and unlikely to be due to random similarity, further supporting the conclusion that the enzyme is well conserved across these species.

Table 3. BLASTP results for NDM indicating top 5 hits for each Priority Pathogen

Description	**Scientific name**	**E-value**	**Percent identity**
TPA: NDM family subclass B1 metallo-beta-lactamase [*Acinetobacter baumannii*]	*Acinetobacter baumannii*	5.0E-178	100
subclass B1 metallo-beta-lactamase NDM-2 [*Acinetobacter baumannii*]	*Acinetobacter baumannii*	8.0E-178	100
subclass B1 metallo-beta-lactamase NDM-40 [*Acinetobacter baumannii*]	*Acinetobacter baumannii*	1.0E-177	100
subclass B1 metallo-beta-lactamase NDM-42 [*Acinetobacter baumannii*]	*Acinetobacter baumannii*	1.0E-177	100
metallo-beta-lactamase NDM [*Acinetobacter baumannii*]	*Acinetobacter baumannii*	1.0E-177	100
TPA: NDM family subclass B1 metallo-beta-lactamase [*Escherichia coli*]	*Escherichia coli*	7.0E-178	100
NDM family subclass B1 metallo-beta-lactamase [*Escherichia coli*]	*Escherichia coli*	8.0E-178	100
TPA: NDM family subclass B1 metallo-beta-lactamase [*Escherichia coli*]	*Escherichia coli*	3.0E-177	99.59
TPA: NDM family subclass B1 metallo-beta-lactamase [*Escherichia coli*]	*Escherichia coli*	7.0E-177	99.17
Chain A, SUBCLASS B1 METALLO-BETA-LACTAMASE NDM-9 [*Escherichia coli*]	*Escherichia coli*	7.0E-177	99.59
TPA: NDM family subclass B1 metallo-beta-lactamase [*Pseudomonas aeruginosa*]	*Pseudomonas aeruginosa*	1.0E-177	100
subclass B1 metallo-beta-lactamase NDM-64 [*Pseudomonas aeruginosa*]	*Pseudomonas aeruginosa*	3.0E-177	100
NDM family subclass B1 metallo-beta-lactamase [*Pseudomonas aeruginosa*]	*Pseudomonas aeruginosa*	1.0E-177	99.59
TPA: NDM family subclass B1 metallo-beta-lactamase [*Pseudomonas aeruginosa*]	*Pseudomonas aeruginosa*	6.0E-177	99.59

BLASTP results for IMP revealed similar distribution indicating the conserved nature of the MBL across *P. aeruginosa*, *A. baumannii* and *E. coli* (**Table 4.**).
BLASTP results for VIM showed conserved nature only across *Pseudomonas* and Enterobacteriaceae, but not *A. baumannii*, as no significant hit was found in its case (**Table 4.**). The selective conservation of VIM in *Pseudomonas* and Enterobacteriaceae, with its absence in *A. baumannii*, suggests a differential distribution and reliance on distinct carbapenemase types across these critical pathogens. *Pseudomonas* and Enterobacteriaceae appear to be the primary carriers of VIM, possibly due to genetic compatibility and environmental adaptation, whereas *A. baumannii* may rely on alternative resistance mechanisms. This distribution highlights the complexity of antibiotic resistance and emphasizes the need for pathogen-specific approaches in treatment and surveillance.

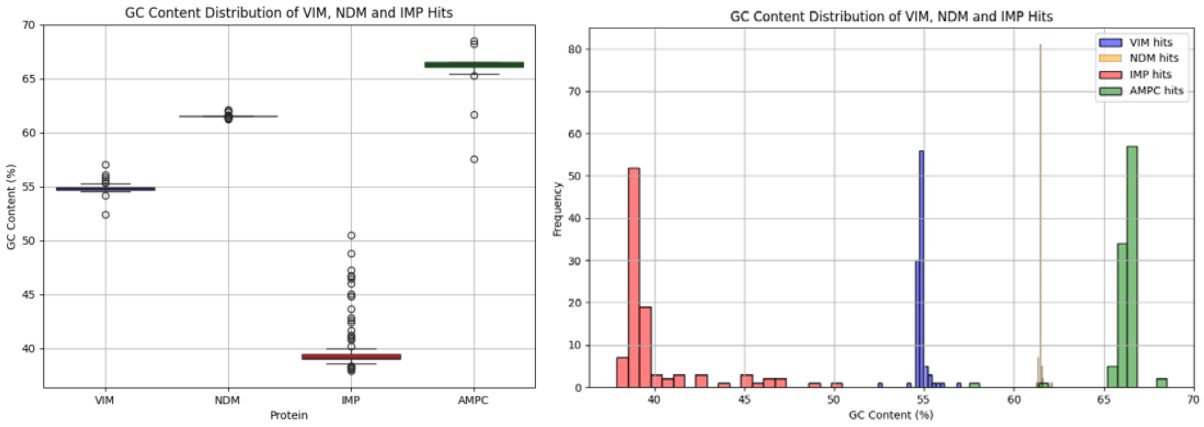
Table 4. BLASTP results for IMP and VIM indicating top 5 hits for each PP

Description	Scientific Name	E-value	Percent Identity
IMP Results			
subclass B1 metallo-beta-lactamase IMP-61	*Acinetobacter baumannii*	3.00E-176	99.59
metallo-beta-lactamase IMP	*Acinetobacter baumannii*	1.00E-175	99.19
subclass B1 metallo-beta-lactamase IMP-55	*Acinetobacter baumannii*	7.00E-174	97.97
subclass B1 metallo-beta-lactamase IMP-5	*Acinetobacter baumannii*	2.00E-166	93.09
subclass B1 metallo-beta-lactamase IMP-2	*Acinetobacter baumannii*	4.00E-150	85.37
subclass B1 metallo-beta-lactamase IMP-66	*Escherichia coli*	9.00E-177	99.59
subclass B1 metallo-beta-lactamase IMP-52	*Escherichia coli*	4.00E-176	99.19
IMP family subclass B1 metallo-beta-lactamase	*Escherichia coli*	2.00E-168	95.92
subclass B1 metallo-beta-lactamase IMP-59	*Escherichia coli*	3.00E-168	95.53
TPA: IMP family subclass B1 metallo-beta-lactamase	*Escherichia coli*	4.00E-168	95.53
Beta-lactamase IMP precursor	*Pseudomonas aeruginosa*	0	100
subclass B1 metallo-beta-lactamase IMP-79	*Pseudomonas aeruginosa*	2.00E-177	99.59
subclass B1 metallo-beta-lactamase IMP-70	*Pseudomonas aeruginosa*	2.00E-177	99.59
subclass B1 metallo-beta-lactamase IMP-76	*Pseudomonas aeruginosa*	5.00E-177	99.59
IMP family subclass B1 metallo-beta-lactamase	*Pseudomonas aeruginosa*	6.00E-177	99.59
VIM Results			
VIM family subclass B1 metallo-beta-lactamase	*Escherichia coli*	0	99.25
metallo-beta-lactamase VIM	*Pseudomonas aeruginosa*	0	100
VIM protein	*Pseudomonas aeruginosa PA21_ST175*	0	99.62
VIM family beta-lactamase	*Pseudomonas aeruginosa*	0	99.62
VIM metallo-beta-lactamase	*Pseudomonas aeruginosa*	0	99.25
subclass B1 metallo-beta-lactamase VIM-17	*Pseudomonas aeruginosa*	0	99.25

3.9 GC Content analysis

The GC content analysis of the three MBLs, NDM, VIM, and IMP, revealed significant differences, indicating distinct evolutionary origins (**Figure 2.**). Each MBL exhibited nearly consistent GC content across different species, suggesting that these genes retain their nucleotide composition when transferred to new bacterial hosts. NDM and VIM were more conserved across different species unlike IMP (**Figure S1, S2 and S3 of Supplementary Folder 3**). However, GC content of IMP was found to vary in several cases, indicating its lesser conserved nature as compared to VIM and NDM.

Figure 2 (a) and (b) show that NDM has a thin range of GC content percentage over which the sequences have been distributed, showing its highly conserved nature and lower variability as compared to the other MBLs taken into study. This indicates lower rates of HGT for NDM. On the other hand, IMP shows maximum variability out of the three MBLs, indicative of higher rates of HGT.



(a) **(b)**

Figure 2. GC Content comparison of different BLs

Additionally, IMP was shown to possess lowest GC content out of all BLs taken for study. The average GC content for IMP was 40.07%, for VIM was 54.85%, for NDM was 61.51% and for AmpC was found to be 66.18%. The GC content analysis of AmpC can be seen in **Figure S4 of Supplementary Folder 3.** A comparison of the same with MBLs can be seen in **Figure 2.** An interesting observation found was that lower GC content was shown to promote variability of the enzyme (IMP and VIM). This is in line with the fact that higher GC content promotes stability (NDM).
Figure 2 (a) clearly shows NDM to have less evolutionary capacity currently due to high GC content reflecting stability. Figure 2 (b) clarifies NDM to be less evolved due to less spread of sequences (showing less variability) and a sharp peak for GC content.
Two important observations were: (i) GC distribution of NDM and AmpC was found to be overlapping, and (ii) GC distribution of VIM lies between IMP and NDM. This forced us to question- Is there a link in evolvability of BLs from AmpC to NDM? Or is AmpC contributing a part of its genome to NDM? On the other hand, is VIM acting as an evolutionary link between IMP and NDM? To answer these questions, we had finally run the alignment for all MBLs together, followed by Pairwise sequence similarity mapping (PaSiMap). The same was done to compare MBLs and AmpC against each other.

In detail, to check if VIM acts as an evolutionary link between IMP and NDM, the following was done. Sequences were aligned using MUSCLE on MEGA. The alignment was then subjected to the Maximum Likelihood algorithm for making the phylogenetic tree. The phylogenetic tree was finally visualized and edited using the itol server. The aligned sequences were also subjected to pairwise sequence similarity mapping (PaSiMap) which resulted in clustering of the BLs in different groups.

To find evolutionary links between MBLs and AmpC, following was done. Sequences were aligned using MUSCLE on MEGA. The alignment was then subjected to the Maximum Likelihood algorithm for making the phylogenetic tree. The phylogenetic tree was finally visualized and edited using the itol server. The aligned sequences were also subjected to pairwise sequence similarity mapping (PaSiMap) which resulted in clustering of the BLs in different groups.

In case of PaSiMap, we found VIM to lie between IMP and NDM, further indicating that VIM could act as an evolutionary link between the two (**Figure S1 of Supplementary Folder 4**). The same could be seen in the direct maximum likelihood tree (without bootstrapping; direct trees are indicative of raw sequence features and composition and show inherent evolutionary relationships based purely on sequence data) which showed VIM and its homologs to lie between IMP and NDM (**Supplementary Folder 4**).

Although in Maximum Likelihood trees constructed for MBLs, we found that VIM lies between IMP and NDM, when the Maximum Likelihood tree was constructed by including AmpC alongside MBLs, we found that AmpC separates VIM from NDM and IMP (**Supplementary Folder 4)**. However, this might occur due to changes in evolutionary distance calculations, potential long branch attraction (LBA), and differences in evolutionary rates between AmpC (Class C) and MBLs (Class B). AmpC likely acts as a distant outgroup, forcing a reorganization of MBL relationships, shifting

VIM's position relative to NDM and IMP. Therefore, we reconstructed the tree using 5 other algorithms, namely, NJ (neighbor joining), ME (minimum evolution), UPGMA (unweighted pair group method with arithmetic mean) original, UPGMA BCT (bootstrap consensus tree with 1000 times bootstrap sampling), and MP (minimum parsimony). Thus, total 6 algorithms were used to study the inter-BL relationship of the enzymes in-depth. The evolutionary tree or pattern outputted by different phylogeny driving algorithms can be seen in **Supplementary Folder 4.**

While direct UPGMA tree is representative of sequence composition and raw sequence features, bootstrapping helps in validation of evolutionary relationships by in-depth analysis of the sequences. The UPGMA tree output directly indicates intrinsic similarity. Intrinsic similarity in the context of a UPGMA tree refers to the natural similarity between sequences based on their genetic composition, without any external statistical validation (like bootstrapping). In direct UPGMA tree, VIM was found between NDM and IMP but in bootstrap consensus tree (BCT), we found IMP to lie between the two (**Supplementary Folder 4)**. However, this doesn't rule out the fact VIM might act as an evolutionary link between NDM and IMP. This simply suggests that these three beta-lactamase families are closely related, and their exact evolutionary order is sensitive to small changes in data. We must also understand that if an enzyme is to act as an evolutionary link between other two, it can't simply be understood from their relationship in a phylogenetic tree. Even if an enzyme doesn't lie between the other two, might anyhow share characteristics of them both by acting as a mosaic **(Milani and Chauhan, 2023; Truty et al., 2023)**. Thus, VIM could be a mosaic sequence having acquired characteristics from both NDM and IMP via HGT, recombination or other mechanisms. To further test our hypothesis we studied if VIM shares protein features, motifs and domains from both IMP and NDM or not.

3.10 Protein features: carbapenem resistant vs non-carbapenem resistant BLs

3.10.1 Amino Acid Composition

Table 5. Amino Acid Composition

P.C.	AmpC	IMP	VIM	NDM
PC1 +ve	R, L, A, E, P, D, W, F	K, N, F, I, Y, E, C, S	R, P, A, G, D, C, H, W, L, V	C, R, W, Q, G, E, Y, L, F, P
PC1 -ve	K, V, T, N, Y, M, C, G, S, H, I	A, R, L, X, Q, G, P, H, T, M, V, D	K, I, N, Y, S, Q, F, M, E, T	N, T, K, A, S, H, I, M, W, D
PC2 +ve	Q, L, P, C, N, W, P, S	Q, T, I, D, M, H, N, Y	E, V, S, Y, G, A, R	P, L, E, M, Q, T, I, N
PC2 -ve	E, G, I, F, M, R, V, D, K	E, P, G, X, V, K, F, R, T, C, L, A	W, Q, L, C, T, K, F, H, M, P, N, I, D	D, A, G, H, Y, R, W, S, C, V, F, K

The clustering of bacterial species based on contribution of each of the residues to the principal components can be visualized in PCA scatter plots contained in **Supplementary Folder 5.**

Principal component analysis (PCA) revealed distinct patterns in amino acid contributions across AmpC, NDM, VIM, and IMP **(Table 5).**
PC1 +ve contributions reflect that VIM and NDM sequences are clustered distinctly based on similar amino acids. Similarly, PC2 +ve contributions reflect that AmpC and NDM sequences are clustered based on similar residues, re-emphasizing that there could be a link in evolvability of BLs from AmpC to NDM.

3.10.2 Atom and Bond Composition

PCA was further utilized to reveal distinctive atomic and bond compositions that contribute to clustering of bacterial species **(Table 6).** PC2 +ve contributions show that NDM shares features from AmpC and VIM as seen in previous findings as well. It shared the double bond of AmpC and C-atom of VIM. PC1 +ve contributions showed high variability or diverse set of bonds for IMP, indicating that each could be affected leading to distinct clustering of different bacterial species, further re-emphasizing its least stable nature.

Table 6. Atoms and Bonds Composition

P.C.	AmpC	IMP	VIM	NDM
PC1 +ve	Total bonds, Single bond, Double bond, H-bond, C atom	Double bond, Total bonds, H-bond, H atom, Single bond, O atom, C atom	Total bonds, H bond, Single bond, Double bonds, S atom, N atom	Total bonds, H-bond, S.1 bond, Double bond
PC1 -ve	S atom, N atom, H atom	N atom	H atom, O atom, C atom	H atom, C atom, N atom
PC2 +ve	O atom, Double bond	H atom, O atom, Single bond	N atom, C atom, S atom, H bond	C atom, O atom, Double bond
PC2 -ve	H atom, N atom, S atom, C atom, H bond, S bond, Total bonds	N atom, C atom, H-bond, Total bonds, Double bond	H atom, O atom, Double bond, Triple bond, Single bond	N atom, Single bond, H atom, H-bond, S.1 bond, Total bonds

3.10.3 Advanced Physicochemical Properties

PCA of advanced physicochemical properties (Z-descriptors) helped us identify the distribution of unique attributes across enzymes **(Table 7)**. Here, lipophilicity (Z1), bulk (Z2), polarity / charge (Z3) and electronic effects (Z4 and Z5) all were taken into account.

Positive contributors to PC1 included Z5 (electronegativity) for all enzymes and Z1 (lipophilicity) for AmpC, IMP and VIM. However, only VIM and NDM show Z3 and Z5 as large contributors in PC1. This suggests that VIM shares features from both NDM and IMP. Reliance of NDM on Z5 and Z3 (electronic properties) suggest specialization for electronic interactions. For PC2, AmpC relied on Z1, emphasizing hydrophobic interactions. NDM displays versatility with multiple descriptors (Z1, Z3, Z4, Z5), enabling adaptation to diverse environments.

Overall, Z-descriptors reveal differences in resistance strategies. NDM's reliance on Z5 and Z3 indicates specialized electronic interactions, potentially linked to its broad-spectrum resistance. AmpC's focus on Z1 suggests a hydrophobic interaction-driven mechanism, while VIM and IMP's use of Z2 and Z5 points to both steric/bulk and electronic adaptations for inhibitor resistance.

Table 7. Advanced physiochemical properties

P.C.	**AmpC**	**IMP**	**VIM**	**NDM**
PC1 +ve	Z2, Z5, Z1, Z4	Z1, Z2, Z4, Z5	Z5, Z3, Z1, Z4	Z5, Z3
PC1 -ve	Z3	Z3	Z2	Z2, Z4, Z1
PC2 +ve	Z1	Z1	Z4, Z2, Z5	Z1, Z3, Z4, Z5
PC2 -ve	Z5, Z3, Z4, Z2	Z4, Z2, Z3, Z5	Z1, Z3	Z2

Finally, these carbapenem-resistant BLs (VIM, NDM, IMP) demonstrate unique structural features compared to the carbapenem-non-resistant beta-lactamase AmpC. The presence of sulfur and oxygen atoms in VIM and NDM highlights their capability for diverse catalytic interactions, enabling resistance to carbapenems. IMP's nitrogen-centric composition further supports its adaptation for specific antibiotic challenges. In contrast, AmpC's reliance on hydrophobic interactions, as reflected by its Z1-positive contributions, aligns with its role in resisting cephalosporins rather than carbapenems. These differences underscore the specialized evolutionary paths that equip resistant enzymes with broader substrate adaptability and advanced catalytic mechanisms.

Moreover, plasmid-encoded beta-lactamases (VIM, NDM, IMP) exhibit enhanced versatility due to their ability to spread horizontally across bacterial populations. This is reflected in their broader physicochemical adaptability, including reliance on Z5 (electronegativity) and Z3 (electronic properties). These traits enable them to confer resistance to a wide range of beta-lactam antibiotics, including carbapenems, in diverse environments. On the other hand, the chromosomally-encoded AmpC demonstrates a narrower focus, with adaptations emphasizing hydrophobic interactions and structural stability, as seen in its reliance on Z1 and fewer versatile residues. These distinctions highlight the evolutionary and functional advantages plasmid-encoded enzymes have in promoting antibiotic resistance.

3.11 PFAM Domain Analysis of Beta-Lactamases (BLs)

The PFAM domain analysis helped us identify conserved protein domains. These domains define the functional and structural characteristics of BLs. Figure 3 shows the visual representation of PFAM domain distribution for different beta-lactamases.

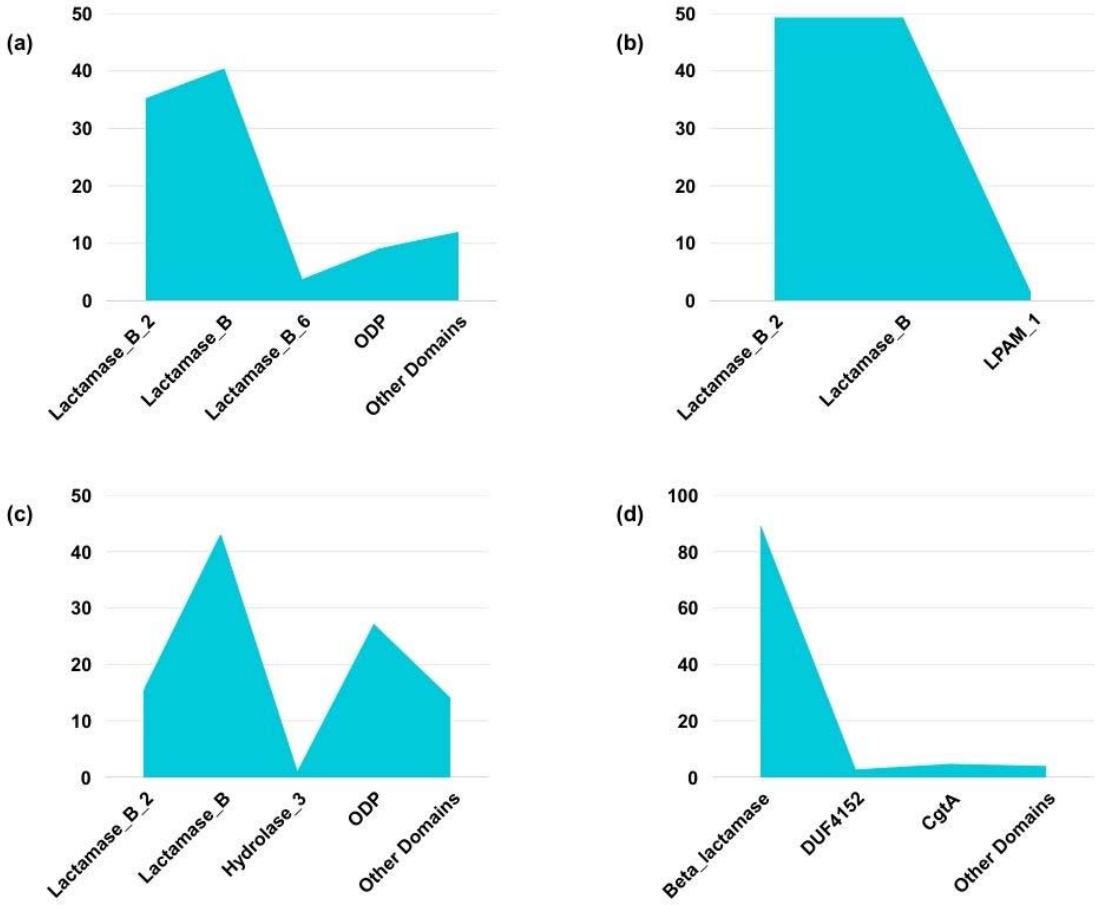


Figure 3. Distribution of major PFAM domains for different beta-lactamases. (a)**VIM** Beta-Lactamases: Dominated by Lactamase_B (40.31%) and Lactamase_B_2 (35.14%), with ODP (9.04%) playing a notable role. (b) **NDM** Beta-Lactamases: Highly conserved, with Lactamase_B and Lactamase_B_2 each comprising 49.3%. (c) **IMP** Beta-Lactamases: Shows more diversity, with Lactamase_B (42.93%), ODP (27.02%), and other minor domains. (d)**AmpC** Beta-Lactamases: Mostly Beta-lactamase domain (89.17%), with CgtA (4.46%) and DUF4152 (2.55%) suggesting functional links to glycosylation. It is to note that the figure here highlights the major domains in BLs. However, few other domains are also present which have been discussed in detail further.

Figure 3 once again depicts NDM to be less evolved or more conserved to due fewer accessory domains than other MBLs.

The tables showing frequency percentage of domains in sequences of each of the BLs in respective clusters have been discussed in detail in **Supplementary Folder 6.** In addition to this, **Table 8.** summarizes the PFAM domain analysis for different BLs.

Table 8. Comparative PFAM Domain Analysis of Beta-Lactamases

Feature	NDM	VIM	IMP	AmpC
Core Domains	Lactamase_B, Lactamase_B_2	Lactamase_B, Lactamase_B_2	Lactamase_B, Lactamase_B_2, ODP	Beta-lactamase, Beta-lactamase_2
Structural Variability	Low (Highly conserved)	High	Moderate	Low
Unique Domains	No	DUF6491, RelB_transactiv	Hydrolase_3, DUF1138, DUF1846	CgtA, DUF4152
Presence of ODP Domain	No	Yes	Yes	No
Presence of DUF Domains	No	Yes (Higher variability)	Yes (Moderate variability)	Yes (Lower variability)
Association with Cell Wall Metabolism	Indirect	Indirect	Potential (via Hydrolase_3)	Direct (CgtA association)

We found that NDM is the most conserved beta-lactamase, containing only Lactamase_B and Lactamase_B_2 without additional domains, indicating structural optimization for function. VIM and IMP show greater domain diversity, with VIM exhibiting DUF domains and regulatory elements (RelB_transactiv), while IMP includes Hydrolase_3 and additional DUFs, suggesting functional adaptability and evolutionary divergence. Additionally, the ODP domain is exclusive to metallo-beta-lactamases (VIM, IMP), indicating a potential role in substrate specificity or structural stabilization. AmpC is structurally distinct from MBLs, lacking the ODP domain and instead containing CgtA (linked to glycosylation and cell wall interactions), emphasizing a different evolutionary pathway and functional role.
Overall, plasmid-encoded beta-lactamases (VIM, NDM, IMP) exhibit more domain variability, possibly enhancing adaptability to diverse antibiotics, while chromosomally encoded AmpC remains more conserved, relying on hydrophobic interactions rather than diverse catalytic elements.

3.12 Cluster-wise PFAM Analysis of Beta-Lactamases
We further correlated our results of PFAM analysis with clusters that were previously derived using pfeature. The cluster-wise distribution of PFAM domains for each of the BLs can be studied in detail in **Supplementary Folder 7. Table 9** shows summarized results for cluster-wise PFAM analysis of BLs.

Table 9. Cluster-wise PFAM Analysis of Beta-Lactamases Based on Amino Acid Composition (AAC), Atoms and Bonds Composition (ABC), and Advanced Physicochemical Properties (APP)

BL Type	Cluster Type	Cluster	Dominant PFAM Domains (%)	Important Accessory Domains (%)
VIM	AAC	Cluster 1	Lactamase_B (31.03%), Lactamase_B_2 (25%)	ODP (26.95%), DUF1138 (1.41%),
		Cluster 2	Lactamase_B (39.39%), Lactamase_B_2 (33.47%)	ODP (14.69%), Lipase_3 (0.61%)
	ABC	Cluster 1	Lactamase_B (39.39%), Lactama-	ODP (14.69%)

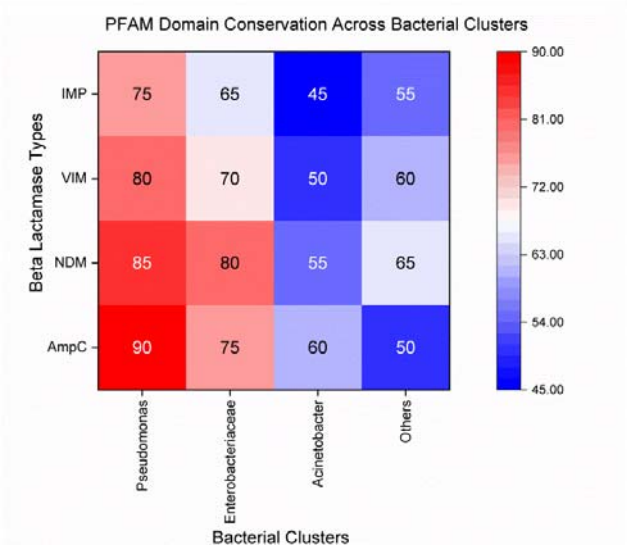
			se_B_2 (33.47%)	
		Cluster 2	Lactamase_B (29.93%), ODP (24.95%), Lactamase_B_2 (24.51%)	Lactamase_B_6 (6.51%)
	APP	Cluster 1	Lactamase_B (39%), Lactamase_B_2 (33%), ODP (15.7 %)	Lactamase_B_6 (2.52 %), RelB_transactiv (1.55%)
		Cluster 2	Lactamase_B (29.81%), Lactamase_B_2 (24.54%), ODP (24.31 %)	Lactamase_B_6 (6.65 %), DUF1138 (1.83%), Peripla_BP_4 (1.1%)
NDM	AAC	Cluster 1	Lactamase_B (49.3%), Lactamase_B_2 (49.3%)	LPAM_1 (1.4%)
		Cluster 2	Lactamase_B (50%), Lactamase_B_2 (50%)	None
	ABC	Cluster 1	Lactamase_B (49.3%), Lactamase_B_2 (49.3%)	LPAM_1 (1.33%)
		Cluster 2	Lactamase_B (50%), Lactamase_B_2 (50%)	None
	APP	Cluster 1	Lactamase_B (49.33%), Lactamase_B_2 (49.33%)	LPAM (1.33 %)
		Cluster 2	Lactamase_B (50%), Lactamase_B_2 (50%)	None
IMP	AAC	Cluster 1	Lactamase_B (42.93%), ODP (27.02%)	Hydrolase_3, DUF1138, Phage_Coat_A
		Cluster 2	Lactamase_B (37.9%), ODP (26.61%)	Hydrolase_3 (2.82%), DUF2242, DUF7189
	ABC	Cluster 1	Lactamase_B (39.95%), ODP (29.10%)	DUF1138, Myb_DNA-binding
		Cluster 2	Lactamase_B (40.27%), ODP (25.10%), Lactamase_B_2 (14.01%)	Hydrolase_3, DUF7189, CH
	APP	Cluster 1	Lactamase_B (42.37%), ODP (29.24%), Lactamase_B_2 (13.98%)	Hydrolase_3 (1.27%), Phage_Coat_A (1.27%)
		Cluster 2	Lactamase_B (37.62%), ODP (24.05%), Lactamase_B_2 (15.23%)	Hydrolase_3 (2.6%), Acetyltransf_4 (1.9%), T3RM_EcoP15I_C (1.4%), DUF7189 (1.2%)
AmpC	AAC	Cluster 1	Beta-lactamase (89.17%)	CgtA (4.46%)
		Cluster 2	Beta-lactamase (73.61%)	CgtA (15.28%), DUF domains (2-3%)
	ABC	Cluster 1	Beta-lactamase (85.31%), Beta-lactamase2 (2.10%)	CgtA (7.69%)
		Cluster 2	Beta-lactamase (82.56%), DUF4152 (4.65%)	CgtA (8.13%)

	APP	Cluster 1	Beta-lactamase (81.61%), CgtA (11.49%)	DUF4152 (1.15%)
		Cluster 2	Beta-lactamase (85.91%), Lower CgtA (5.63%)	Additional DUFs (2.11%)

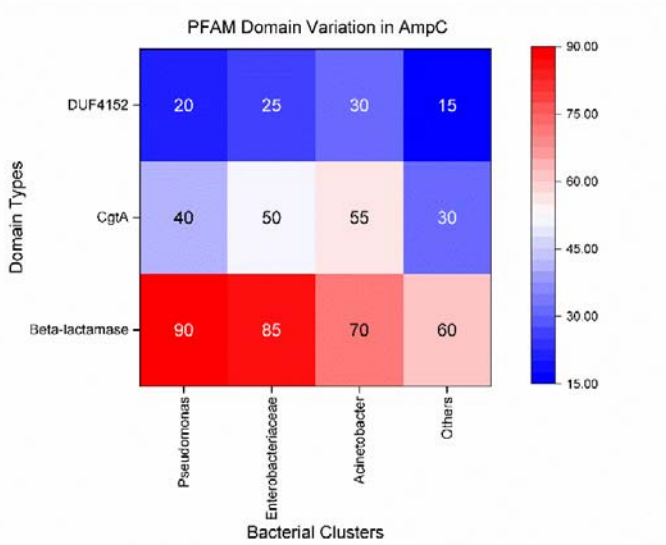
The cluster-wise PFAM analysis as summarized in the Table 9 shows that two clusters of the same BL vary significantly in both the core Beta-lactamase domains as well as accessory domains, except for NDM. In case of NDM, the Beta-lactamase domain distribution is same across different clusters, even though the accessory domains vary in their percentage. Thus, NDM is the most conserved across all cluster types, with minimal structural or physicochemical variability. VIM and IMP display greater diversity, particularly in ODP domains, suggesting higher adaptability to different environments. AmpC remains structurally conserved but still exhibits some considerable variability.

3.13 Final interpretation of PFAM domain distribution across MBLs and AmpC

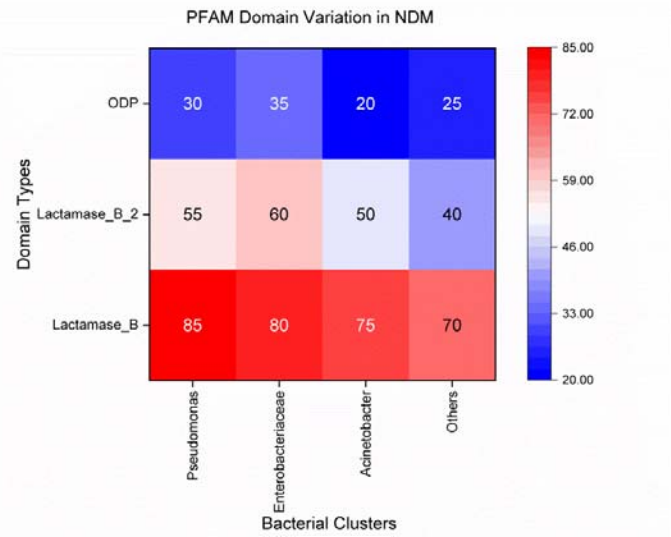
A final conservation matrix for PFAMs across all priority pathogen was obtained for all enzymes and can be studied in **Table S1 of Supplementary Folder 8**. **Figure 4a** shows the heatmap for the same. The heatmap was generated using the PFAM domain conservation scores, which were assigned based on the presence of each domain in different bacterial groups.



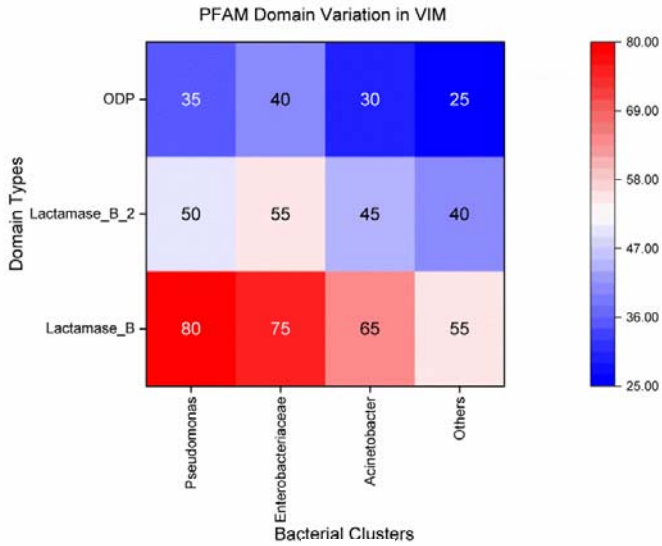
(a)



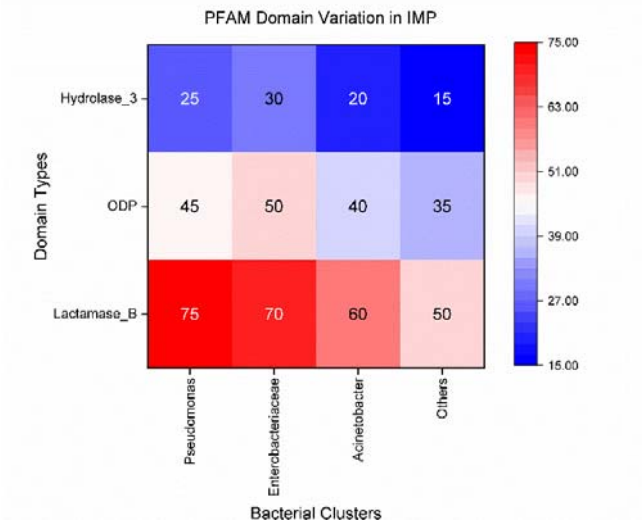
(b)



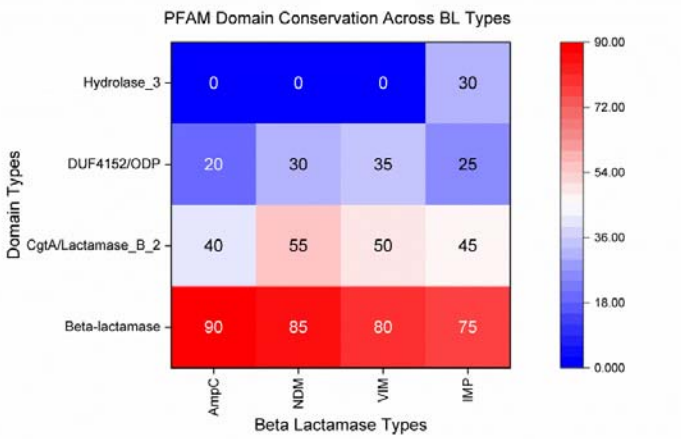
(c)



(d)



(e)



(f)

Figure 4. PFAM domain analysis for different BLs. (a) PFAM domains conservation matrix for all BLs across different bacteria. (b) PFAM domains conservation matrix for AmpC across different bacteria. (c) PFAM domains conservation matrix for NDM across different bacteria. (d) PFAM domains conservation matrix for VIM across different bac-

teria. (e) PFAM domains conservation matrix for IMP across different bacteria. (f) Conservation matrix of key domains across all BLs.

AmpC domain variations across bacterial clusters were studied by calculating conservation scores and heatmap. The Beta-lactamase, CgtA, and DUF4152 domains in AmpC were checked across different bacterial clusters. The final conservation matrix for AmpC can be seen in **Table S2 of Supplementary Folder 8. Figure 4b** shows the heatmap for the same. Similarly, NDM domain variations across bacterial clusters were studied. Lactamase_B, Lactamase_B_2, and ODP domains of NDM beta-lactamases were checked across bacterial groups. The conservation percentage was computed. The final conservation matrix for NDM can be seen in **Table S3 of Supplementary Folder 8. Figure 4c** shows the heatmap for the same. Further, a heatmap of VIM domain variations across bacterial clusters was generated. Lactamase_B, Lactamase_B_2, and ODP domains in VIM beta-lactamases were analyzed similarly. The final conservation matrix for VIM can be seen in **Table S4 of Supplementary Folder 8. Figure 4d** shows the heatmap for the same. A heatmap of IMP domain variations across bacterial clusters was also generated. Lactamase_B, ODP, and Hydrolase_3 domains in IMP beta-lactamases were analyzed. The final conservation matrix for IMP can be seen in **Table S5 of Supplementary Folder 8. Figure 4e** shows the heatmap for the same.
Figure 4a clearly shows that PFAM domains lying within IMP are least conserved across different bacteria, followed by VIM, NDM and finally AmpC. A closer look at **Figure 4b-f** shows that the core lactamase domains are most conserved in *P. aeruginosa*, followed by Enterobacteriaceae. On the other hand, *Acinetobacter* shows high domain variability.
Finally, a comparative study of PFAM domain conservation across all BL types was performed. The conservation percentage of key PFAM domains across all beta-lactamases (ampc, NDM, VIM, IMP) was recorded. The final conservation matrix of key domains across all BLs can be seen in **Table S6 of Supplementary Folder 8. Figure 4f** shows the heatmap for the same. The results highlight that the core lactamase domains are more conserved within the BLs as compared to accessory domains.

3.14 Motif Analysis of Beta-Lactamases: Insights from MEME and TOMTOM Discovery

The motif analysis conducted using MEME and TOMTOM provided significant insights into the structural and functional conservation of BLs. The analysis revealed that while some motifs were highly conserved across different beta-lactamase classes, others displayed notable sequence variations that could reflect functional divergence.
The results from TOMTOM showing overlapping of motifs across different BLs can be found in the form of a tabular output in **Supplementary Folder 9.**

3.14.1 **AmpC vs. IMP: Conservation and Divergence in Functional Regions**
Comparison of AmpC and IMP motifs highlighted multiple regions of sequence conservation, with significant statistical relevance indicated by low p-values and E-values. Several motifs were found in the same orientation (+) in both enzymes, suggesting conserved structural or functional regions that may contribute to similar enzymatic activities. One of the strongest motif matches was FAACTC in AmpC and FAAGTT in IMP, with a p-value of 0.0133, indicating a conserved functional region. Another highly significant alignment was observed between MLSGVRNLLISALLLGAGNCM (AmpC) and MKKLFLLLLFL (IMP), which showed strong similarity with an E-value of 0.018, suggesting evolutionary conservation in sequence structure.
Overlapping regions, particularly spanning residues 6-19, further suggested that certain peptide segments in AmpC and IMP may perform similar functional roles in substrate binding or catalysis. Despite these similarities, some motifs displayed differences in specific amino acid residues, indicating functional divergence. Such variations may account for the mechanistic differences between AmpC, which is a serine beta-lactamase, and IMP, which functions as a metallo-beta-lactamase (MBL) requiring metal ion coordination for hydrolysis.

3.14.2 **AmpC vs. NDM: Structural and Functional Conservation in Active Site Motifs**
The comparison between AmpC and NDM motifs revealed strong motif conservation, particularly in regions associated with enzymatic activity. One of the most significant matches was DRAASMLS (AmpC) and KLSTALAAALMLSGC (NDM), with a p-value of 0.012, suggesting conservation in a functionally important domain. Another notable match was observed between DRAASMLS (AmpC) and MELPNIMHPVAKLSTALAAALMLSGCMPG (NDM), which exhibited an E-value of 0.038, further supporting evolutionary conservation of sequence elements.
Several functionally conserved domains were identified, including a strong similarity between LSLSDKASQHLPALKGSAFDHISVLQLGTYTAGGLPLQFPDEADSADKML (AmpC) and WVEPATAPNFGPLKVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKS (NDM) (p-value = 0.001). This alignment suggested that these enzymes share common catalytic or structural features, potentially influencing substrate binding and hydrolysis mechanisms. Interestingly, many motifs aligned with minimal offset, reinforcing the idea that AmpC and NDM may have evolved from a common ancestral beta-lactamase. However, regions of sequence variability were also present, suggesting adaptations in enzymatic activity and substrate specificity that differentiate AmpC from NDM.

3.14.3 **AmpC vs. VIM: Evolutionary Links and Divergent Functional Adaptations**

The motif comparison between AmpC and VIM beta-lactamases further highlighted conserved sequence regions, supporting a possible evolutionary link between these enzymes. A highly significant match was observed between AAAQLRAVVDAAVKPLMQQQG (AmpC) and AEDTLGLPVRAAVVT (VIM), with a p-value of 0.00079, indicating strong conservation in an enzymatic region. Another motif pair, AADRLEALVDAAVQPVMQQQD (AmpC) and AEDTLGLPVRAAVVT (VIM), displayed moderate sequence conservation (p-value = 0.028).
Structural and functional conservation was further supported by motif alignments in substrate-binding regions, with sequences such as IPGLAVAVTVNGKAHYFNYGVASKETGQPVSEBTLFEIGSVSKTFTATLA (AmpC) and KLAKEQGYEVPNPILDELTTL (VIM) (p-value = 0.031) showing similarities in enzymatic function. Another motif alignment, IPGMAVAITHKGQRHYFNYGVASKETGQAVTEDTLFEIGSVSKTFTATLG (AmpC) vs. KLAKEQGYEVPNPILDELTTL (VIM), further confirmed structural similarity (p-value = 0.010). However, sequence variability in certain motifs, such as VRVPADQMARYAQGY (AmpC) and MPRASKQQARYAVGRCLMLWSSNDVTQQGSRPKTKLCRTHP (VIM) (p-value = 0.041), suggested some degree of functional divergence, potentially contributing to differences in enzymatic activity and antibiotic resistance mechanisms.

3.14.4 **IMP vs. Other Beta-Lactamases: Conserved and Unique Motif Features**
When comparing IMP to other beta-lactamases, several motifs exhibited significant similarity, reinforcing potential structural and functional overlaps. A particularly strong match was identified between DVTFATDTTFTVGKQ (IMP) and FAACTC (AmpC) (p-value = 0.03), indicating moderate similarity in sequence elements. A highly conserved region was observed in MRIAIRGCAALMIGAIANAQA (IMP) and MRFTITALALLLIGQ (AmpC), with an extremely low p-value of 0.00016, suggesting evolutionary conservation in functionally significant regions.
In contrast, motif comparisons between IMP and NDM revealed a mixture of conservation and divergence. A notable alignment between ASNGLI (NDM) and ASNKSIQPTAEASAD (IMP) (p-value = 0.004) suggested partial conservation in substrate-binding regions. However, some motif matches exhibited significantly lower p-values (e.g., 6.5E-17), highlighting regions of functional similarity that may contribute to beta-lactam hydrolysis.

3.14.5 **NDM vs. VIM: High Motif Conservation and Shared Functional Features**
NDM and VIM exhibited some of the highest motif conservation, suggesting a strong evolutionary relationship among metallo-beta-lactamases. One of the most conserved motifs was EVFYPGAGHTMDNIVVWLPQQKILFGGCLVKSLQAKDLGNTADADLNSWP (VIM) and WVEPATAPNFGPLKVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKS (NDM), with a p-value of 7.9E-16, indicating a highly conserved functional region. Similarly, a sequence alignment between ASNGLI (NDM) and FPSNGLIVETGKGLVLIDTAWGEEQTEEL (VIM) (p-value = 4.7E-5) suggested partial conservation in substrate recognition domains.
The strong conservation of motifs in NDM and VIM indicates that these enzymes likely share a common structural framework, which may explain their similar catalytic mechanisms. However, sequence variations in certain motifs suggest subtle functional adaptations, potentially contributing to differences in substrate affinity and antibiotic resistance profiles.
The final motif conservation matrix for all BLs can be seen in **Table 10.**

Table 10. Similarity matrix based on motif analysis (higher values mean more conservation)

Query ↓ / Target →	**Motifs of AmpC**	**Motifs of IMP**	**Motifs of NDM**	**Motifs of VIM**
Similarity to AmpC motifs	100	85	80	88
Similarity to IMP motifs	85	100	90	92
Similarity to NDM motifs	80	90	100	96
Similarity to VIM motifs	88	92	96	100

A closer look at **Table 10** reveals that motifs of AmpC, IMP and NDM are most similar to motifs of VIM than other enzymes. This further re-emphasize the fact that VIM shares maximum features from different BLs, especially the MBLs and therefore could be the evolutionary link between NDM and IMP.

3.15 Finding mutations of critical importance

The alignment of protein sequences as analyzed using Jalview software, can be seen in **Figure S1a of Supplementary Folder 10.** In the VIM protein, positions 175 to 440 are largely conserved across all species, with only minimal mutations observed within this range. Before position 175, no consistent alignment was found, with gaps occurring frequently. Similarly, gaps were also observed between positions 440 and 443. Mutations involving one or two amino acids within conserved columns are detailed in Table **S1 of Supplementary Folder 10**. Sequence logos generated using the WebLogo software to visualize residue conservation across all aligned blocks in our MSA can be seen in **Figure S1b of Supplementary Folder 10.** These logos illustrate a highly conserved domain spanning positions 175 to 440, with minimal mutations observed within this conserved region. Thus, mutations are clustered in both central and terminal regions, often affecting polar/non-polar or charged/uncharged transitions, indicative of selective pressure on functionally critical residues in the active site and structure stabilization.

Similarly, the alignment of NDM protein sequences as analyzed using Jalview software, can be seen in **Figure S2a of Supplementary Folder 10.** In the NDM protein, positions 30 to 271 are largely conserved across all species, with only minimal mutations observed within this range. Before position 30, no consistent alignment was found, with gaps occurring frequently. Similarly, gaps were also observed between positions 272 and 292. Mutations involving one or two amino acids within conserved columns are detailed in **Table S2 of Supplementary Folder 10.** Sequence logos for the same can be seen in **Figure S2b of Supplementary Folder 10.** These logos illustrate a highly conserved domain spanning positions 30 to 271, with minimal mutations observed within this conserved region. Thus, mutational hotspots are distributed throughout, showing frequent polar/non-polar, charged/uncharged exchanges, likely driven by selective forces for broad substrate adaptation and resistance phenotype maintenance.

The alignment of IMP protein sequences as analyzed using Jalview software, can be seen in **Figure S3a of Supplementary Folder 10.** In the IMP protein, positions 65 to 285 are largely conserved across all species, with only minimal mutations observed within this range. Before position 65, no consistent alignment was found, with gaps occurring frequently. Similarly, gaps were also observed between positions 286 and 300. Mutations involving one or two amino acids within conserved columns are detailed in **Table S3 of Supplementary Folder 10**. Sequence logos for the same have been depicted in **Figure S3b of Supplementary Folder 10.** These logos illustrate a highly conserved domain spanning positions 65 to 285, with minimal mutations observed within this conserved region. Thus, we can see extensive spread from early to late sequence positions with multiple changes in charge, polarity, or aromaticity, reflecting adaptation across environmental niches and possibly compensatory mutations to maintain enzyme efficacy.

Similar to the above, the alignment of AmpC protein sequences can be studied from **Figure S4a of Supplementary Folder 10.** Key mutated positions include 75, 82, 85, 105, 111, 135, 137, 193, 197, 243, 255, 263, 329, 359, 373, 380, 392. Thus, several conserved positions undergo substitutions in AmpC sequences, notably at aromatic, polar, and charged sites, indicating hotspots in regions related to substrate recognition or enzyme structural integrity. Mutations in AmpC involving one or two amino acids within conserved regions are detailed in **Table S4 of Supplementary Folder 10.** Sequence logos generated to visualize residue conservation across all aligned blocks in our MSA can be seen in **Figure S4b of Supplementary Folder 10.**

Upon closer examination of the mutated residues, it was found that in VIM and NDM, several non-polar and uncharged polar residues were substituted by positively charged residues like Arginine, Lysine and Histidine. On the other hand, charged residues were substituted by non-polar and uncharged polar residues in IMP and AmpC. Additionally, in VIM and NDM, the negatively charged residues like glutamate and aspartate were substituted by non-polar or positively charged residues. In contrast, in IMP, it was observed at few instances that positively charged residues were replaced by negatively charged residues. Thus, VIM and NDM undergo similar types of mutation, further indicating that VIM is closer to NDM.

3.16 The dual mechanism of HGT and evolution

Overall, our findings suggest a dual mechanism of HGT and evolution (both intrinsic and extrinsic). While intrinsic evolution occurs as a result of chromosomal adaptations and co-evolution with regulatory proteins (e.g., NagZ with AmpC and AmpR), extrinsic evolution encompasses plasmid-mediated HGT, allowing rapid dissemination of resistance genes across diverse bacterial genera. The dual mechanism of HGT and evolution of BLs has been visually represented in **Figure 5a-b.**

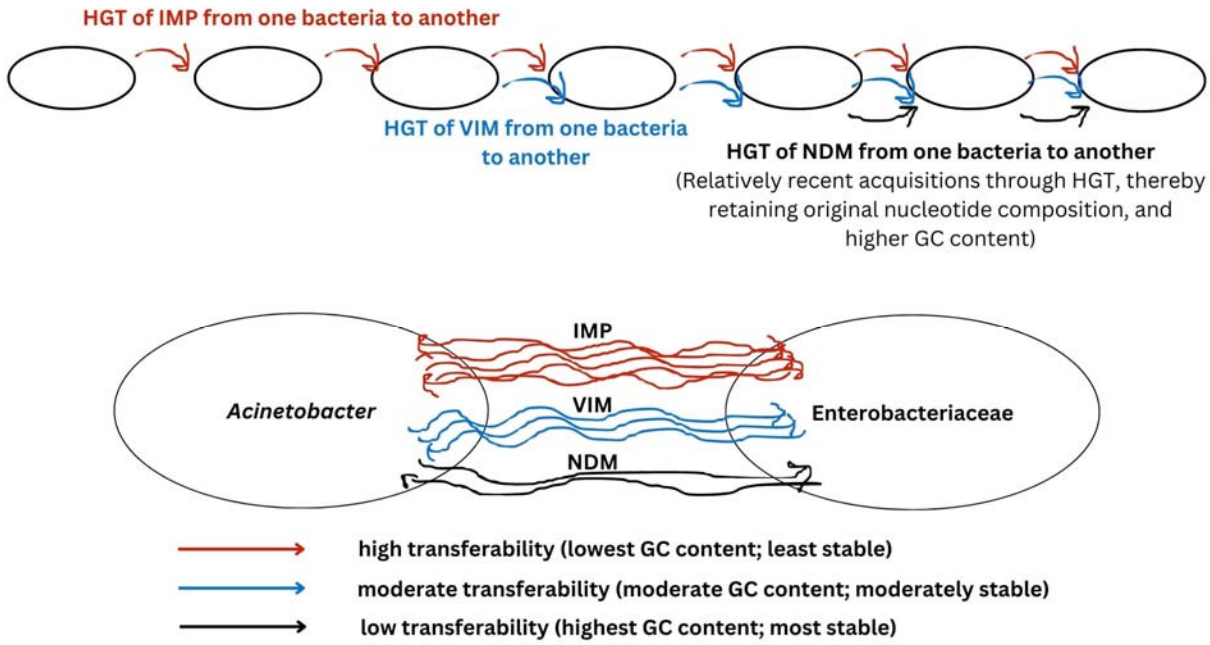


Figure 5(a). Horizontal gene transfer (HGT) of plasmid-encoded beta-lactamases from one bacteria to another.

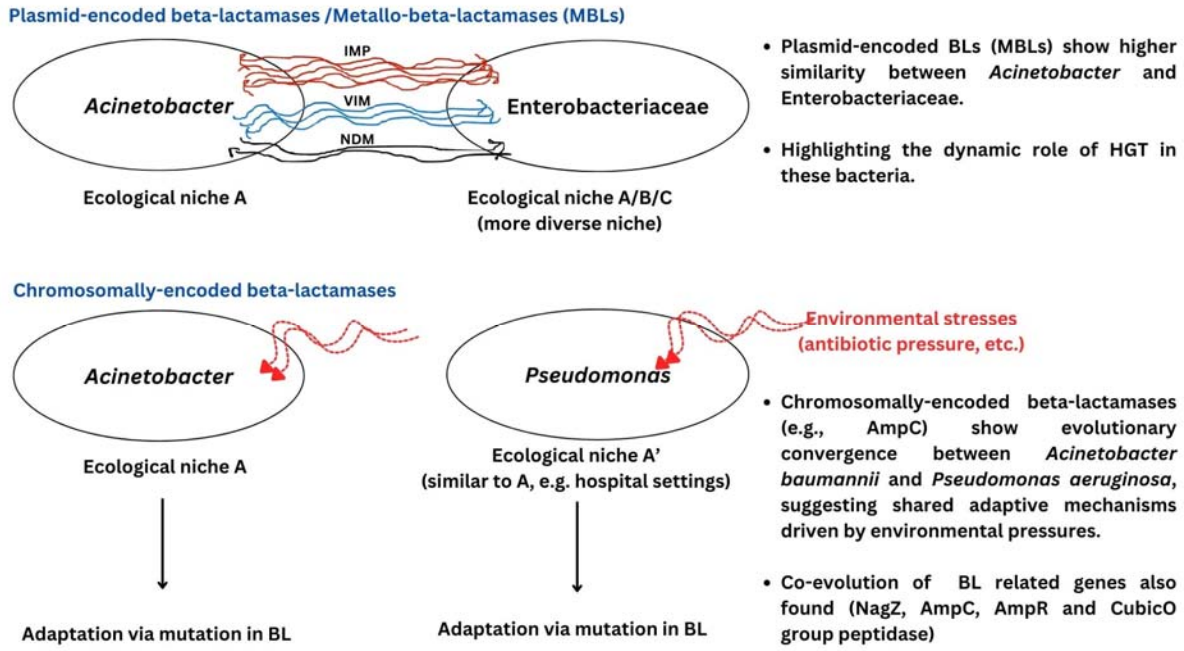


Figure 5(b). Plasmid-encoded BLs and chromosomally-encoded BLs show evolutionary trends different from each other

Our findings suggest higher transferability for IMP, followed by VIM and NDM due to their increasing GC content and thereby stability, respectively (**Figure 5 a).**

The **Figure 5(b)** shows that plasmid-encoded MBLs and related genes show similar evolutionary patterns for *Acinetobacter* and Enterobacteriaceae, whereas chromosomally encoded BLs like AmpC and related show similar evolutionary patterns across *A. baumannii* and *P. aeruginosa*. This clearly indicates that *Acinetobacter* is involved in both HGT (with Enterobacteria, in case of plasmid encoded-BLs) and ecological adaptation via mutation (similar to *Pseu-*

domonas, in case of chromosomally-encoded BLs), thereby highlighting the dual mechanism of HGT and evolution in *Acinteobacter*. We anticipate that similar mechanisms might be occurring in other bacteria as well. This also re-establishes the finding that there is more diversity in *Acinteobacter*, due to both HGT and chromosomal mutations.

3.16.1 Extrinsic Evolution

3.16.1.1 Plasmid-Mediated HGT

Thus, the study highlights the significant role of plasmid-encoded metallo-beta-lactamases (MBLs) such as NDM, VIM, and IMP in facilitating HGT. Plasmids seem to enable the rapid transfer of resistance genes between *Acinetobacter* and Enterobacteriaceae, enhancing the spread of carbapenem resistance. The increased similarity of plasmid-encoded MBLs between these genera indicates a higher rate of plasmid exchange, which supports the dissemination of antibiotic resistance traits through mobile genetic elements like integrons and transposons **(Noel et al., 2022; Yao et al., 2024).**

3.16.1.2 GC content and transferability of BLs

Genes with lower GC content are often more prone to mutations and recombination events. This genomic plasticity can facilitate integration into diverse bacterial genomes, enhancing the likelihood of horizontal transfer **(Hayek, 2013).** The differential GC content of MBLs (e.g., low GC in IMP and high GC in NDM) indicates distinct evolutionary origins and possibly varied pathways of HGT. The stability of GC content across different species in case of NDM suggests that these genes retain their original nucleotide composition when transferred to new hosts, supporting the notion of relatively recent acquisitions through HGT.

3.16.1.3 Evolutionary Flexibility

The study indicates that lower GC content promotes enzyme variability, as seen with IMP and VIM, while higher GC content like in NDM provides stability. This flexibility could mean that IMP is not only transferred more frequently but also adapts more readily to new host environments through mutations (**Mori et al., 2021**; **Michiels et al., 2016).** Additionally, in environments with strong antibiotic pressure, genes with lower GC content might accumulate mutations that help in evolving resistance, further promoting their transfer among bacteria **(Hayek et al., 2013).**

3.16.1.4 Evidence from BLASTP and Phylogenetic Analysis

The BLASTP results showed that IMP is conserved across *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Escherichia coli*, demonstrating its widespread presence and suggesting active HGT. The phylogenetic analysis also pointed to the evolutionary transition of IMP beta-lactamases from marine bacteria to clinical pathogens, highlighting the role of environmental reservoirs in contributing to HGT.

The lower GC content of IMP not only reflects its evolutionary origins but also implies: higher HGT rates (easier incorporation into diverse bacterial genomes), broader host range (facilitating the spread of carbapenem resistance across different genera), and adaptive potential (through increased mutational flexibility, IMP might evolve new resistance traits more readily than NDM or VIM).

3.16.1.5 Environmental Reservoirs and Evolutionary Divergence

The phylogenetic analysis of IMP beta-lactamases suggests an evolutionary transition from marine bacteria to clinical pathogens, indicating that environmental reservoirs play a crucial role in introducing resistance genes into pathogenic bacteria. This evolution likely involves both vertical inheritance and HGT, contributing to the broad host range of IMP.

3.16.2 Intrinsic Evolution

3.16.2.1 Chromosomal vs. Plasmid Evolution

Chromosomally-encoded beta-lactamases (e.g., AmpC) show evolutionary convergence between *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, suggesting shared adaptive mechanisms driven by environmental pressures **(Lister, 2002).** In contrast, plasmid-encoded MBLs show higher similarity between *Acinetobacter* and Enterobacteriaceae, highlighting the dynamic role of HGT in these bacteria **(Da Silva & Domingues, 2016)**

3.16.2.2 Co-Evolutionary Dynamics

The analysis of NagZ and its associated beta-lactamase-related proteins (e.g., AmpC, AmpR) demonstrates co-evolutionary trends. Phylogenetic congruence suggests functional interdependence, possibly reflecting concurrent adaptation to antibiotic pressures. The lack of phylogenetic congruence with proteins like glyoxylase and AmpD indicates independent evolutionary paths, hinting at selective pressures specific to certain proteins.

3.16.2.3 Similar adaptive mechanisms in *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, and biofilm formation

Acinetobacter baumannii and *Pseudomonas aeruginosa* are generally known to form more robust and mature biofilms compared to many members of the Enterobacteriaceae family **(Gedefie et al., 2021; Papa et al., 2018).** This might be linked to their close proximity in phylogenetic tree for NagZ, AmpC and AmpR, which are seen to co-evolve. *Pseudomonas aeruginosa* uses a complex regulatory network, including quorum sensing (las, rhl, and pqs systems) and cyclic-

di-GMP signaling, to form thick, structured biofilms. It produces extracellular polymeric substances (EPS), including alginate, which contributes to the biofilm matrix **(De Kievit et al., 2001).** *Acinetobacter baumannii* forms biofilms on abiotic surfaces, often associated with hospital environments. The Bap (biofilm-associated protein) and Csu pili play critical roles in biofilm initiation and maturation **(Tomaras et al., 2003).** Many members of the Enterobacteriaceae family, like Escherichia coli, *Klebsiella pneumoniae*, and *Proteus mirabilis*, can also form biofilms. However, they typically do not produce biofilms as mature and resilient as *P. aeruginosa* and *A. baumannii*, especially in clinical and environmental contexts **(Ezzariga et al., 2025).** E. coli can form biofilms, particularly in the case of uropathogenic strains (UPEC), but the structure and resistance of these biofilms vary widely among strains **(Soto et al., 2007).** As compared to Enterobacteriaceae, both *P. aeruginosa* and *A. baumannii* excel on abiotic surfaces, giving them an advantage in healthcare settings (e.g., catheters, ventilators) **(Abdallah et al., 2014; (Anurag Anand et al., 2025c)**. The EPS produced by *P. aeruginosa* and *A. baumannii* often have stronger adhesive and protective properties. Biofilms of *P. aeruginosa* and *A. baumannii* are highly resistant to antibiotics and host immune responses, which is a major clinical challenge (**Gedefie et al., 2021; Papa et al., 2018**). Thus, while Enterobacteriaceae can form biofilms, *P. aeruginosa* and *A. baumannii* generally form more robust and clinically challenging biofilms. The similar ecological niche or environmental stresses associated with both these bacteria may be the reason they show evolutionary convergence in case of chromosomally encoded BLs like AmpC and related genes (NagZ and AmpR). Overall, there is a possibility that *Pseudomonas and Acinetobacter* might be sharing other resistance patterns as well.

3.16.2.4 Evidence from Motif Conservation and Mutational Hotspots

The presence of conserved motifs and mutational hotspots within beta-lactamases, as shown by MEME and TOMTOM analyses, suggests an evolutionary strategy where critical functional domains are maintained while allowing mutations at non-essential sites (**Table 10**). This could contribute to adaptive resistance while preserving enzymatic functionality.

Figure 6(a) highlights the mechanism of co-evolution as a result of functional inter-dependence, whereas **Figure 6(b)** highlights the adaptive nature of motifs and domains in response to antibiotic pressure.

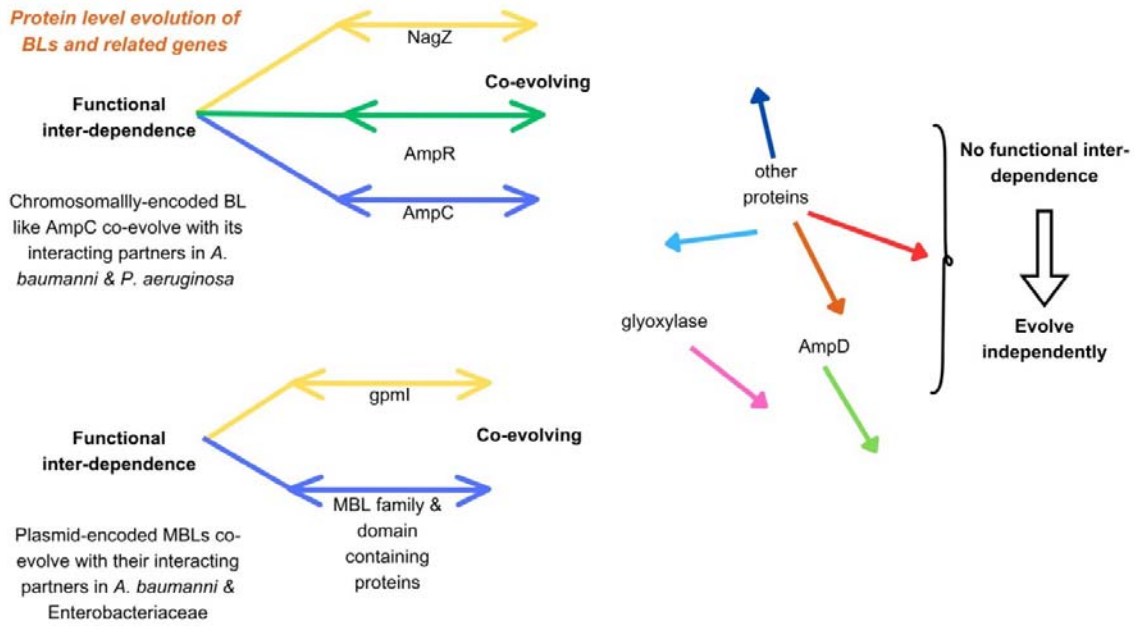


Figure 6(a). Emergence of co-evolution as a result of functional inter-dependence

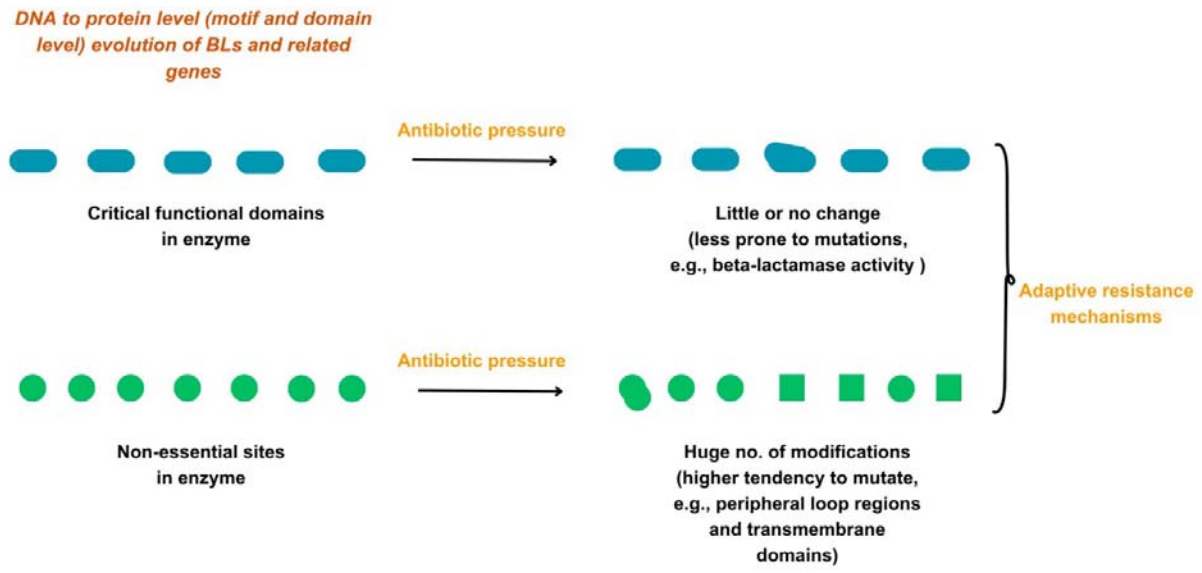


Figure 6(b). The adaptive nature of motifs and domains in response to antibiotic pressure

3.17 VIM as an evolutionary link between IMP and NDM

The variety of shared characteristics between VIM and IMP and VIM and NDM is indicative of VIM acting as an evolutionary link between the other two. Phylogenetic trees and PaSiMap analysis show that VIM lies between NDM and IMP. GC content analysis showed VIM to have average GC content between NDM and IMP. Comparative analysis of AAC, ABC, and APP using Principal Component Analysis (PCA) reveals distinct clustering patterns among NDM, VIM, IMP, and AmpC beta-lactamases, but with similar positive contributors between VIM and NDM, and between VIM and IMP. Further, PFAM domains and motifs are conserved maximum in NDM but least in IMP, with VIM lying in between them, suggestig a strong evolutionary link. Thus, VIM seems to be acting as a mosaic, having acquired characteristics from both NDM and IMP via HGT, recombination or other mechanisms **(Milani and Chauhan, 2023; Truty et al., 2023)**.

4. Conclusion and future perspectives

This study presents an integrated analysis of the co-evolutionary dynamics, structural stability, and functional divergence of beta-lactamases (BLs) and their associated proteins in priority pathogens, mainly, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and Enterobacteriaceae. By combining phylogenetic, statistical, and sequence analyses with STRING-based protein-protein interaction (PPI) networks, we uncovered key insights into the molecular mechanisms driving beta-lactam resistance. NagZ emerges as the primary interacting partner for BLs in *P. aeruginosa* and Enterobacteriaceae, highlighting its critical role in the regulatory cascade leading to beta-lactamase expression. The strong co-evolutionary trends between NagZ, AmpC, and AmpR, emphasize functional interdependence and shared evolutionary pressures among these proteins. In contrast, *A. baumannii* exhibits a diverse range of interactions, indicating a more complex and varied resistance mechanism. Plasmid-encoded MBLs (e.g., NDM, VIM, IMP) display higher horizontal gene transfer (HGT) rates, particularly between *Acinetobacter* and Enterobacteriaceae, contributing to the widespread dissemination of carbapenem resistance across these organisms. Conversely, chromosomally encoded BLs exhibited closer evolutionary relationships between *Pseudomonas* and *Acinetobacter*. The GC content analysis indicated that lower GC content, as seen in IMP, correlates with higher mutational plasticity, while higher GC content, as in NDM, is associated with structural stability. Principal Component Analysis (PCA) of amino acid composition and physicochemical properties demonstrate similar positive contributors between VIM and NDM, and between VIM and IMP. This clustering highlights the adaptive mechanisms of BLs under selective pressures, optimizing enzymatic catalysis and substrate affinity. Motif analysis using MEME and TOMTOM uncovered varying conservation across BL classes. Identifying mutational hotspots in VIM, IMP, NDM, and AmpC provides valuable insights for designing targeted inhibitors. Finally, inter-BL analysis revealed that VIM can be understood as an evolutionary link between IMP and NDM due to presence of features shared from NDM as well as IMP, such as GC content, PFAM domains and structural and functional motifs.

In the light of these findings, we propose several therapeutic strategies. Given the strong co-evolutionary link between NagZ, AmpC, and AmpR, designing small-molecule inhibitors targeting NagZ could suppress the regulatory cascade leading to beta-lactamase expression. This offers a novel therapeutic strategy to combat resistance in *Pseudomonas aeruginosa* and Enterobacteriaceae. The high HGT rates observed for plasmid-encoded MBLs, particularly between *Acinetobacter* and Enterobacteriaceae, highlight the need for targeted interventions to curb the spread of resistance genes. Inhibitors targeting mobile genetic elements could effectively reduce the dissemination of resistance. Enzymes with lower GC content, such as IMP, exhibit higher mutational plasticity, making them more susceptible to structural disruptions. This insight can guide the development of drugs targeting these vulnerabilities, particularly in highly mutable BLs. Given the distinct evolutionary bifurcation patterns observed in BLs, CRISPR-based strategies could selectively disable plasmid-encoded MBLs while preserving essential chromosomally encoded functions. Since *Acinetobacter baumannii* exhibits high genetic plasticity, targeted genomic surveillance can track emerging mutations and guide adaptive treatment strategies. The finding that VIM may serve as an evolutionary link between IMP and NDM, can help to better understand the evolution and emergence of new beta-lactamase variants. Additionally, targeting components conserved across VIM, NDM and IMP (such as common PFAM domains) can help target multiple beta-lactamases at once which is a practical solution to the real-life problems. Lastly, findings such as the selective conservation of VIM in *Pseudomonas* and Enterobacteriaceae, with its absence in *A. baumannii*, suggesting a differential distribution and reliance on distinct carbapenemase types across these critical pathogens indicate that there is much more to explore in BL evolution.

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6. Author contributions

A.A.A.: Conceptualization, Data mining and curation, Data Analysis, Methodology, Writing-original draft, review and editing; V.Y.: Data mining and curation, Data Analysis, Methodology; K.K.: Data Analysis, Methodology; P.C.S.: Methodology; S.A.: Methodology; S.K.S.: Conceptualization, Formal Analysis, Supervision, Investigation, Resources, Writing-review & editing.

7. Conflict of interests

The authors declare no conflict of interest.

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9. Data availability

All the data can be found in the supplementary folders. Any additional data shall be made available on request.

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