

Integrated Experimental and Computational Analyses of *Klebsiella pneumoniae* ClpK: Metal Ion Modulation, Vaccine Targeting, and Interaction Networks

By

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Preface

The experimental work described in this dissertation was carried out in the School of Life Science, University of KwaZulu-Natal, Pietermaritzburg, from January 2022 to December 2025, under the supervision of Dr. Thandeka Khoza. The studies represent original work by the author and none of this work has been submitted for the award of any degree or examination at any university. All authors of data and any other information have been acknowledged accordingly by reference.

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As the candidate's supervisor, I have approved this thesis for examination.

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Plagiarism Declaration

I, **Tehrim Ballim**, declare that:

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Abstract

Klebsiella pneumoniae is a Gram-negative opportunistic pathogen which has been classified by the World Health Organization as a critical priority organism underscoring the need for the development of new therapeutics and alternative interventions. The genetic diversity and horizontal transmission of antimicrobial resistance determinants in this pathogen demonstrate its capacity for rapid adaptation and persistence thus posing a significant global health challenge. To address the challenges caused by this pathogen, this thesis focused on the structural and functional characterisation of ClpK ATPase, together with complementary vaccine-based approaches aimed at disrupting its survival and stress response pathways.

ClpK is a thermotolerant caseinolytic ATPase implicated in protein quality control and stress tolerance. Biophysical and enzymatic characterization showed that ClpK is predominantly α -helical and moderately thermally stable, with ATP binding conferring conformational stabilization. Divalent cations modulated ClpK activity, with Mg^{2+} enhancing structural stability and Mn^{2+} promoting conformational flexibility and enzymatic activity. These findings provide critical insights into the metal ion-dependant regulation of ClpK and highlight its potential as a drug target. To exploit metal ion dependant regulation of this protein, and in pursuit of novel compounds and drug-binding mechanisms targeting this protein, structure-based virtual screening, ADMET analyses and molecular dynamics simulations were used to identify natural and synthetic inhibitors of ClpK. Among the screened drugs, ADEP3 and sclerotiamide derivatives exhibited the most favourable binding energetics and stable interactions under varying ionic conditions, highlighting their potential as ClpK inhibitors.

Targeting ClpK alone may not be sufficient to fully disrupt the pathogen's adaptive capacity; therefore, exploring its protein–protein interaction network provides an additional strategy for interfering with stress-regulated proteostasis mechanisms. Given the role of ClpK in bacterial stress response and protein homeostasis, identifying its interacting partners offers valuable insight into how this ATPase integrates within broader cellular survival pathways. Therefore, mapping its protein–protein interaction partners provide an additional avenue for interfering with stress-regulated proteostasis mechanisms to disrupt the pathogen's adaptive mechanisms. Computational analyses were employed to predict high-confidence ClpK interaction

partners involved in chaperone and stress response pathways. This study predicted several chaperones and stress response proteins as potential interacting partners for ClpK. This was in line with the role of ClpK in protein homeostasis. Molecular docking and dynamics simulations confirmed the stability of the predicted interactions, providing a basis for understanding the role of ClpK in bacterial stress response pathways. These findings expand the understanding of ClpK's role beyond its intrinsic ATPase activity, positioning it as part of a broader proteostasis network and reinforcing its potential as a multifaceted therapeutic target.

As part of a multi-tiered strategy to combat *K. pneumoniae*, a reverse vaccinology pipeline was applied to six outer membrane proteins of this pathogen, yielding seven multiepitope vaccine constructs. Five constructs demonstrated stable binding to TLR2 and TLR4 receptors, high predicted antigenicity, and global epitope coverage of ~75%. Codon optimization and high codon adaptation index values further supported the feasibility for expression in *Escherichia coli*.

A comprehensive exploration of ClpK as a multifunctional therapeutic target was undertaken using combined computational structural and biophysical approaches. The study characterised metal ion dependent regulation of ClpK, identified several promising inhibitors, mapped its interaction network with key chaperones and stress response proteins providing a mechanistic insight into ClpK's role in bacterial proteostasis and highlight its significance in stress adaptation and survival. Furthermore, the integration of a reverse vaccinology pipeline led to the rational design of multiepitope vaccine constructs, thereby advancing preventive intervention strategies against *K. pneumoniae*. Together, these complementary approaches bridge molecular understanding with translational application, contributing to the development of next-generation therapeutic and prophylactic solutions targeting this WHO-designated critical priority pathogen.

Declaration – Research Outputs

Publications

Published

Ballim T, Seele PP, Mpye KL, Khoza T. Using reverse vaccinology to construct multi-epitope subunit vaccine candidates for *Klebsiella pneumoniae*. Hum Immunol. 2025 Sep 13;86(5):111568. doi: 10.1016/j.humimm.2025.111568. Epub ahead of print. PMID: 40946484.

Submitted

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Ballim T, Khoza T. Computational Repurposing and Phytochemical Screening of Inhibitors Against ClpK from *Klebsiella pneumoniae*.

Ballim T, Achilonu, I, Khoza T. ClpK as a Chaperone Hub: Computational Exploration of its Protein Interaction Landscape.

Conference

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Date: 14-03-2026

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To my parents, Majid and Siffiyya Mehmood, thank you for your constant support, encouragement, and sacrifices that enabled me to pursue my goals.

To my husband, Zeyad, thank you for being my unwavering source of encouragement, especially in moments when I was close to giving up.

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List of Abbreviations

EGCG	(-)-Epigallocatechin-3-Gallate
ANS	8-anilino-1-naphthalenesulfonic acid
ADMET	absorption, distribution, metabolism, excretion, and toxicity
ADEPs	acyldepsipeptide antibiotics
ATP	Adenosine triphosphate
ADME	Adsorption, Distribution, Metabolism, Excretion
α	alpha
$^{\alpha}\text{C}$	Alpha carbon atoms
AMPs	antimicrobial peptides
AMR	antimicrobial resistance
Å	Armstrong
ann	artificial neural networks
AAA+	ATPases associated with diverse cellular activities
ABC	ATP-binding cassette
β	beta
CSE1034	beta-lactam and beta-lactamase inhibitor
ΔG	binding affinity
BBB	Blood brain barrier
BARNARDS	Burden of Antibiotic Resistance in Neonates from Developing Societies
cLogP	calculated LogP
CPS	capsular polysaccharides
CR- <i>Kp</i>	carbapenem-resistant <i>K. pneumoniae</i>
Clp	Caseinolytic
CDC	Centers for Disease Control and Prevention
CD	Circular dichroism
CD	circular dichroism
<i>cKp</i>	classical <i>K. pneumoniae</i>
ClpP	Clp protease
IGF/L motif	ClpP-binding tripeptide motif
CRISPR	Clustered regularly interspaced short palindromic repeats
CAI	Codon adaptation index
CHDF	continuous hemodiafiltration
cryo-EM	cryo-electron microscopy

CTL	cytotoxic T-lymphocyte
D-Ala-D-Ala	D-alanyl-D-alanine
°C	Degrees Celsius
K _d	dissociation constants
DFIRE	distance-scaled, finite ideal-gas reference state
PMX	endotoxin adsorption
EGCG	Epigallocatechin gallate
E-value	Expect value
ESBL	Extended-spectrum β -lactamase
XDR	extensively drug-resistant
FMT	Fecal Microbiota Transplant
Wzx	flippase
FFT	Fourier transformation
γ	Gamma
GI	Gastrointestinal
GOAP	Generalized Orientation-Dependent, All-Atom Statistical Potential
ΔG	Gibbs free energy
GMQE	Global Model Quality Estimation
g	Gram
GRAVY	Grand average of hydropathicity
GPU	Graphics processing unit
GC	guanine–cytosine
Hsp	Heat shock protein
RpoH	heat shock sigma factor σ^{32}
HSFs	heat shock transcription factors
HTL	helper T-lymphocyte
h	hour
HLA	human leukocyte antigen
hvKp	hypervirulent <i>K. pneumoniae</i>
IHME	Institute for Health Metrics and Evaluation
IFN γ	interferon-gamma
pI	isoelectric point
K	Kelvin
kcal/mol	Kilocalories per molar
kDa	Kilodaltons

LPS	lipopolysaccharides
MFS	major facilitator superfamily
MHC	major histocompatibility complex
$V_{\max}$	maximum velocity
θ_{MRE}	mean residue ellipticity
Mant-ATP	Methylantraniloyl-ATP
K_m	Michaelis-Menten constant
$\mu\text{g/mL}$	Microgram per millilitre
μL	Microlitre
μM	Micromolar
M	middle domain
θ_{mdeg}	millidegrees ellipticity
mg/mL	Milligram per millilitre
mL	Millilitre
mm	Millimetre
mM	Millimolar
min	Minute
M	Molar
MD	Molecular Dynamics
MM/GBSA	Molecular Mechanics/Generalized Born Surface Area
MWM	Molecular Weight Marker
mAb	Monoclonal antibodies
MATE	multidrug and toxic compound extrusion
MDR	multi-drug resistant
MESV	Multiepitope subunit vaccine
nm	Nanomolar
ns	Nanosecond
NCBI	National Center for Biotechnology Information
NBD	Nucleotide Binding Domain
NPT	number of particles, pressure, temperature
NVT	number of particles, volume, temperature
OPS	O polysaccharide
OPLS	Optimized Potentials for Liquid Simulations
Omp	Outer membrane proteins
PME	Particle Mesh Ewald
PRRs	pattern recognition receptor

PBC	periodic boundary conditions
PEPCK	phosphoenolpyruvate carboxykinase
PEG	polyethylene glycol
Wzy	polymerase
PSSM	position-specific scoring matrix
PRODIGY	PROtein BinDIng enerGY prediction
PDB	Protein Data Bank
proteostasis	Protein homeostasis
ProSA	Protein Structure Analysis
PLIP	Protein-Ligand Interaction Profiler
PPIs	protein-protein interactions
QMEAN	Qualitative Model Energy ANalysis
QSIs	Quorum sensing inhibitors
R _g	Radius of gyration
$\times g$	Relative centrifugal force
RND	resistance–nodulation–division
RNAi	RNA interference
RMSD	root mean square deviation
RMSF	root mean square fluctuations
σ^E	RpoE
NaCl	salt
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
sec	seconds
SMR	small multidrug resistance
NaCl	Sodium Chloride
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SASA	Solvent Accessible Surface Area
SVM	Super Vector Machine
TSA	Thermal shift assays
3D	three-dimensional
TLR	Toll-like receptor
TIP3P	transferable intermolecular potential with 3 points
TF	Trigger Factor
Tris	<i>tris</i> (hydroxymethyl)aminomethane
MrkA	type III fimbrial protein
T6SS	Type VI secretion system contractile sheath

units/L	Units per litre
units/mg	Units per milligram
VADAR	Volume, Area, Dihedral Angle Reporter
WSL	Windows Subsystem for Linux
WHO	World Health Organisation
Z	zinc

Chapter 1: Literature Review

1.1. Antibiotics and Resistance

The discovery of penicillin by Sir Alexander Flemming in 1928 marked the beginning of the modern antibiotic era, revolutionizing modern medicine and saving millions of lives worldwide (Sengupta *et al.*, 2013, Gould and Bal, 2013). Antibiotics are agents that inhibit or eliminate microorganisms and can be categorized based on their origin as natural compounds derived from microbes, semi-synthetic derivatives of natural products or fully synthetic compounds (Selvarajan *et al.*, 2023, Kourkouta *et al.*, 2018, Pancu *et al.*, 2021). During World War II, penicillin was widely used to control bacterial infections in soldiers, however the emergence of penicillin resistance in the late 1940s reduced its effectiveness (Lobanovska and Pilla, 2017). Antibiotic resistance refers to the ability of pathogens to survive in the presence of antibiotics designed to inhibit or kill them (Muteeb *et al.*, 2023). Penicillin resistance prompted the development of new beta-lactam antibiotics such as methicillin and ampicillin in the 1950s, thereby expanding treatment options (Spellberg and Gilbert, 2014, Sengupta *et al.*, 2013, Ventola, 2015). Unfortunately, beta-lactam resistance soon emerged as confirmed by the identification of methicillin-resistant *Staphylococcus aureus* in the United Kingdom and United States in 1962 and 1968, respectively (Sengupta *et al.*, 2013, Ventola, 2015). Despite significant advancements in the discovery, development, and use of antibiotics, pathogens have continually evolved mechanisms of resistance as illustrated in Figure 1-1.

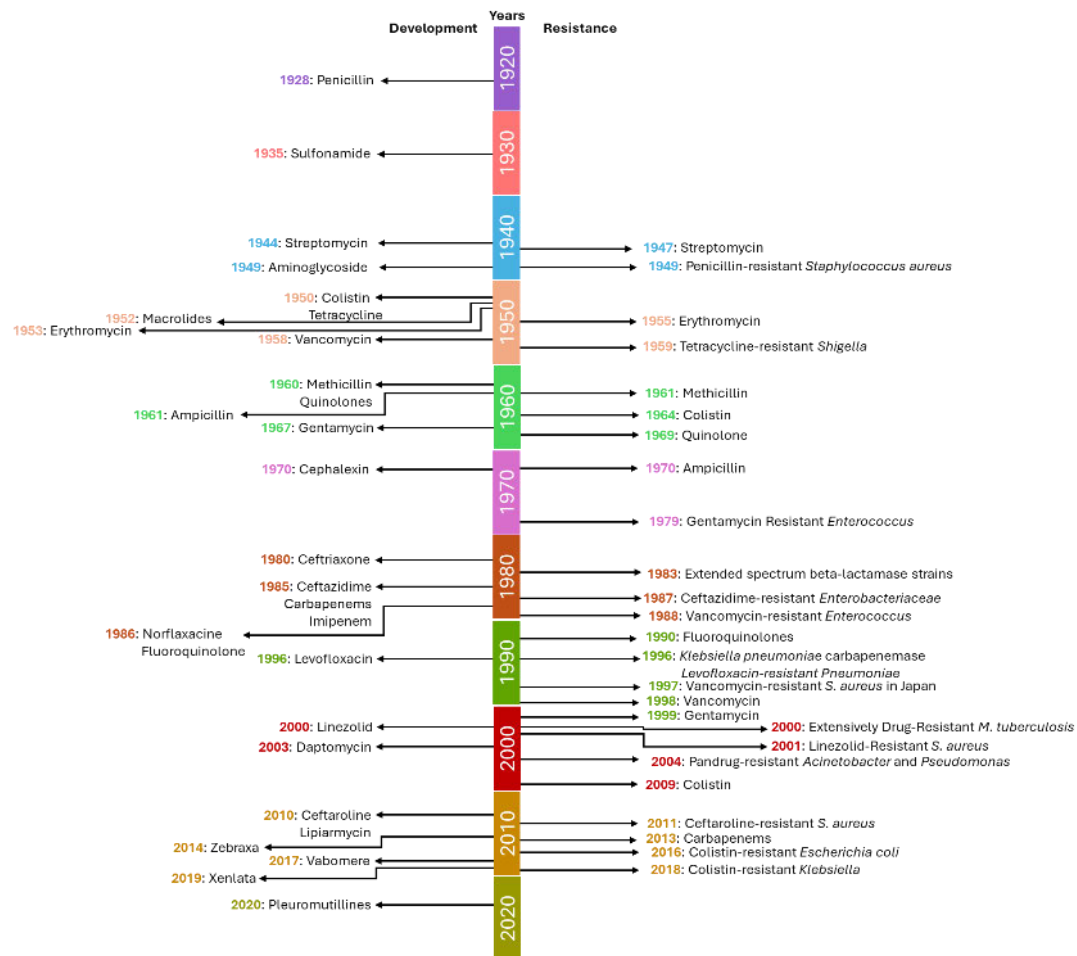


Figure 1-1: Timeline of antibiotic introduction and resistance. The year the antibiotic was developed is depicted on the left, and the year resistance emerged is depicted on the right. Adapted from De Oliveira Santos *et al.* (2022), Acharya and Wilson (2019), and Helmy *et al.* (2023).

The World Health Organization (WHO) has declared antimicrobial resistance (AMR) as a serious and urgent global public health concern (Bengtsson-Palme *et al.*, 2018, Urban-Chmiel *et al.*, 2022). In 2019, WHO reported that AMR was responsible for approximately 700 000 deaths annually, with projections suggesting that this figure is expected to rise to 20 million by 2050 (Watkins and Bonomo, 2016, Uddin *et al.*, 2021). These alarming statistics coupled with an increase in antibiotic resistant bacterial strains underscores the urgent need for novel therapeutic strategies and coordinated global action. In response, international health authorities have called for concerted efforts to develop and implement policies to promote responsible antibiotic use, enhance surveillance and monitoring of antibiotic resistance patterns, support research and development of new antibiotics, information sharing, and increasing public awareness and education (O'Neill, 2016, Tacconelli *et al.*, 2018, Laxminarayan *et al.*, 2013).

AMR develops due to a combination of natural microbial evolution and human-related factors. Microorganisms develop resistance mechanisms to survive antibiotic exposure through natural selection, where the selective pressure exerted by antibiotics favours the survival and spread of resistant strains (Acar and Röstel, 2001, Uddin *et al.*, 2021). Apart from the natural survival instinct of microorganisms to survive the action of antibiotics several human-related factors contribute to the acceleration and spread of resistance, including, antibiotic overuse, improper antibiotic prescription and use, self-medication, and lack of surveillance of resistance development (Uddin *et al.*, 2021, Chokshi *et al.*, 2019, Abdelrahman Hussain *et al.*, 2022). The interconnectedness of human and animal health also plays a significant role in the spread of antibiotic resistance. The use of antibiotics in livestock, particularly in sub-therapeutic doses and prolonged treatment periods, creates an environment that fosters the development and transmission of antibiotic-resistant bacteria (Abate and Birhanu, 2025). These resistant pathogens can then be transmitted to humans through the food chain, underscoring the direct link between animal agriculture practices and public health risks (Pandey *et al.*, 2024). This complex interplay highlights the importance of adopting a One Health approach, which recognises the intrinsic connection between human, animal, and environmental health in combatting antibiotic resistance (Laxminarayan *et al.*, 2013, Robinson *et al.*, 2016, World Health, 2017, Kumar *et al.*, 2021).

Antibiotic resistance may be broadly categorized into natural and acquired resistance (Chin *et al.*, 2023). Natural resistance refers to the inherent ability of bacterial species to resist specific antibiotics due to structural or physiological characteristics, including but not limited to lack of target sites, low cell membrane permeability, presence of efflux pumps (Cox and Wright, 2013, Reygaert, 2018, Urban-Chmiel *et al.*, 2022). For example, *Mycoplasma* spp. lacks a cell wall and are therefore intrinsically resistant to beta-lactam antibiotics such as penicillin and cephalosporin, which target cell wall synthesis (Waites Ken *et al.*, 2017). Natural resistance can be further subcategorized into intrinsic and induced resistance. Intrinsic resistance is genetically encoded and consistently expressed in all members of a bacterial species, independent of antibiotic exposure. These resistance determinants are typically located on the bacterial chromosome and not subject to horizontal gene transfer (Reygaert, 2018, Mancuso *et al.*, 2021). For example, it was identified that many opportunistic nosocomial

pathogens which are members of the *Serratia* genus contain genes that confer resistance to a broad spectrum of antibiotics, including beta-lactams, aminoglycosides, quinolones, macrolides, and polypeptide antimicrobials (Sandner-Miranda *et al.*, 2018). This phenomenon is also seen in other bacteria, such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterococcus* spp., *Klebsiella pneumoniae*, and *Staphylococcus epidermidis* (Li and Nikaido, 2009, Otto, 2009, Nikaido and Pagès, 2012). This constitutive expression of resistance genes, even in the absence of antibiotic pressure, contributes to the persistence and clinical challenge of intrinsic resistance mechanisms (Munita Jose and Arias Cesar, 2016, Uddin *et al.*, 2021).

In contrast, induced resistance refers to naturally occurring resistance genes that are only expressed in the presence of antibiotics. This adaptive mechanism allows bacteria to survive exposure to specific antibiotics by activating genes that inhibit the effect of antibiotics (Reygaert, 2018, Belay *et al.*, 2024). For example, in *P. aeruginosa* induced resistance is observed through the production of beta-lactamases, enzymes that break down beta-lactam antibiotics as they enter the periplasm, thereby rendering the antibiotics ineffective (Cox and Wright, 2013). This phenomenon is also seen in other bacteria, such as *E. coli*, *S. aureus*, and *K. pneumoniae*, where similar inducible resistance mechanisms have been reported (Jacoby and Munoz-Price, 2005, Fernández and Hancock, 2012, Uddin *et al.*, 2021). This inducible response highlights the dynamic nature of bacterial resistance, where exposure to antibiotics directly triggers the expression of resistance genes, contributing to the development and persistence of resistance within bacterial populations (Davies and Davies, 2010, Rodríguez-Martínez *et al.*, 2016).

Acquired resistance arises when previously susceptible bacteria develop the ability to withstand antibiotics through genetic mutation or horizontal gene transfer (Habboush and Guzman, 2023). This process occurs via two distinct pathways: primary (mutational) or secondary (horizontal gene transfer) resistance (Acar and Röstel, 2001, Urban-Chmiel *et al.*, 2022). Primary resistance is a result of spontaneous mutations that occur independently of antibiotic exposure. For instance, mutations in the *rpsL* or *rrs* genes of *Mycobacterium tuberculosis* alter ribosomal function, leading to streptomycin resistance (Maus Courtney *et al.*, 2005). Although this is a rare

occurrence, it does give the mutants an advantage in the presence of antibiotics; however, these genetic changes are non-transferable to other bacteria (Urban-Chmiel *et al.*, 2022). In contrast, secondary resistance arises from the acquisition of extrachromosomal materials such as plasmids that can be transferred amongst bacterial populations via conjugation, transformation, or transduction (Van Hoek *et al.*, 2011, Urban-Chmiel *et al.*, 2022). A study by Hardiman *et al.* (2016) highlighted the important role of horizontal plasmid transfer in the spread of multidrug resistant Gram-negative bacteria such as *K. pneumoniae* and *E. coli*. This genetic exchange can lead to cross-resistance when plasmids or transgenes are transferred between resistant and previously susceptible bacterial strains (Davies and Davies, 2010, Muteeb *et al.*, 2023). Once resistance is acquired, these traits can be passed down to subsequent bacterial generations through vertical transmission, leading to the emergence of bacterial populations resistant to multiple antibiotics (Muteeb *et al.*, 2023, Acar and Röstel, 2001).

1.2. Mechanisms of antibiotic resistance

The four main mechanisms of antibiotic resistance are: i) limited drug uptake, ii) drug target site modification, iii) drug inactivation, and iv) efflux pumps and these are illustrated in Figure 1-2. Limited drug uptake refers to the reduced entry of antibiotics into bacterial cells due to cell wall impermeability. Several key features facilitate this mechanism:

- a) **Lipopolysaccharide** on the outer membrane of Gram-negative bacteria act as a barrier to restrict the diffusion of hydrophobic antibiotics (Helmy *et al.*, 2023, Rosenfeld and Shai, 2006).
- b) Branched stem peptides within the **thick peptidoglycan layer** of Gram-positive bacteria have been implicated in beta-lactam antibiotic resistance by reducing drug penetration (Silhavy *et al.*, 2010).
- c) **Mycolic acid** layer found in the *Mycobacterium* species forms a waxy, hydrophobic coat that limits the permeability of drugs such as isoniazid and rifampin (Batt *et al.*, 2020).
- d) **Biofilms** are formed when bacteria cluster and produce a matrix to attach themselves to animate and inanimate objects (Helmy *et al.*, 2023, Vestby *et al.*, 2020). This acts as a physical barrier against antibiotics and serves as

an ideal ground for the transfer of antibiotic resistance through horizontal gene transfer (Helmy *et al.*, 2023).

- e) Membrane protein channels (**porins**) in the outer membrane of Gram-negative bacteria modulate the crossing of hydrophilic molecules such as beta-lactams into the cell (Helmy *et al.*, 2023). Resistance can arise via a decrease in porin expression as seen in the *Enterobacteriaceae* spp. resisting carbapenems. It can also arise from porin mutations that alter permeability and specificity, as observed in *Neisseria gonorrhoeae*, which adjusts porin selectivity to resist beta-lactam and tetracycline antibiotics (Helmy *et al.*, 2023, Reygaert, 2018).

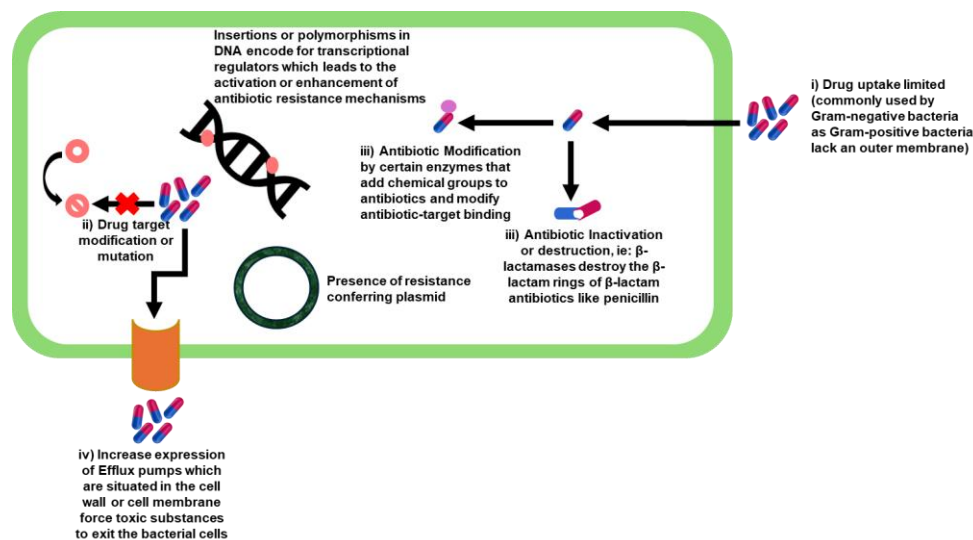


Figure 1-2: Broad overview of bacterial antibiotic resistance mechanisms. Adapted from Reygaert (2018), Mak *et al.* (2022), Zhang and Cheng (2022) and Munita and Arias (2016).

Drug target modification refers to the altering of binding sites via enzymatic reactions, target site replacement, or mutations in genes encoding target sites (Helmy *et al.*, 2023, Reygaert, 2018). In *S. aureus* and *Enterococcus*, vancomycin-resistance arises via the *vanA* gene cluster which encodes several proteins, including two key enzymes: VanH and VanA. VanH, a dehydrogenase, converts pyruvate to D-lactate, while VanA, a ligase, catalyzes the formation of D-alanyl-D-lactate or D-alanyl-D-serine (Stogios and Savchenko, 2020). These enzymatic modifications alter the terminal D-alanyl-D-alanine (D-Ala-D-Ala) moiety of peptidoglycan precursors (Kumar, 2017, Helmy *et al.*, 2023). The D-Ala-D-Ala sequence, located at the C-terminal end of the pentapeptide stem structure, plays an important role in cross-linking peptidoglycan chains during cell wall synthesis and is also the primary target for vancomycin binding (Stogios and Savchenko, 2020). Vancomycin inhibits cell wall synthesis by preventing cross-link

formation however, modifications to the D-Ala-D-Ala moiety reduces vancomycin's binding affinity, enabling vancomycin-resistant strains to evade the antibiotic's inhibitory effects (Ge *et al.*, 1999). Another form of target modification occurs in Gram-negative bacteria through alterations to DNA gyrase to decrease the efficiency of drugs such as fluoroquinolones that target nucleic acid synthesis (Reygaert, 2018).

Drug inactivation represents another major resistance mechanism, in which bacteria use enzymatic degradation or chemical modification to neutralize antibiotic molecules (Reygaert, 2018, Mak *et al.*, 2022, Munita Jose and Arias Cesar, 2016). In enzymatic degradation, bacteria produce specific enzymes that cleave or hydrolyse antibiotic molecules to neutralize antimicrobial activity (Vivekanandan *et al.*, 2025). A well-known example is the production of extended-spectrum beta-lactamases (ESBLs) by drug-resistant bacteria such as *E. coli* and *K. pneumoniae* which hydrolyse beta-lactam antibiotics, including penicillin, cephalosporins, and monobactams (Huang and Zhou, 2025, Gholipour *et al.*, 2014). Alternatively, bacteria may inactivate antibiotics via chemical modification, a mechanism in which functional groups are added to the antibiotic molecule thus reducing its ability to bind to its target site (Saleemi *et al.*, 2023). Transferases are key enzymes mediating this process and are responsible for resistance in mycobacterial species against drugs such as fluoroquinolones, isoniazid, and aminoglycosides (Saxena *et al.*, 2021). While these mechanisms rely on direct enzymatic or chemical modification of antibiotics, bacteria also possess other strategies that limit intracellular drug accumulation.

Efflux pumps are membrane-associated proteins that play an active role in the removal of foreign substances from bacterial cells and subsequently inhibit the accumulation of toxic compounds and antibiotics. As a result, these systems lower the effective concentration of antibiotics within the cell, contributing significantly to the development of multi-drug resistance (Helmy *et al.*, 2023, Vivekanandan *et al.*, 2025). There are five major families of efflux pumps namely, the major facilitator superfamily (MFS), resistance–nodulation–division (RND) family, small multidrug resistance (SMR) family, ATP-binding cassette (ABC) transporters, and multidrug and toxic compound extrusion (MATE) transporters. These classes are categorized based on protein sequence similarity, energy source, and overall structural organization (Helmy *et al.*, 2023, Reygaert, 2018, Cox and Wright, 2013, Vivekanandan *et al.*, 2025).

Furthermore, efflux systems may exhibit substrate specific or have broad-range activity. Among these classes, the MFS and RND families are the most extensively studied due to their prevalence and clinical importance (Helmy *et al.*, 2023, Vivekanandan *et al.*, 2025).

The MFS is the most widely distributed across microbial genomes and exhibits broad substrate specificities (Quistgaard *et al.*, 2016). In *E. coli*, for example, the MdfA transporter, a member of MFS family, facilitates the export of lipophilic, cationic, and neutral substrates (Nagarathinam *et al.*, 2018). In contrast, the RND superfamily is predominantly found in Gram-negative bacteria where it contributes to bacterial adaptation under harsh conditions, the expulsion of toxins and metabolites, biofilm formation, and quorum sensing (Vivekanandan *et al.*, 2025). A well characterized example is the AcrAB-TolC efflux system, a member of the RND family, which expels a wide range of antibiotics including tetracycline and chloramphenicol (Vivekanandan *et al.*, 2025).

The remaining three efflux families (SMR, ABC, and MATE) also play critical roles in antimicrobial resistance. The SMR family typically transports small, charged, and toxic compounds; for example, the AbeS efflux system in *Acinetobacter baumannii* confers resistance to chloramphenicol, ciprofloxacin, and erythromycin, while the Mmr efflux system in *M. tuberculosis* confers resistance to ofloxacin and rifampicin (Burata *et al.*, 2022, Thakur *et al.*, 2021, Srinivasan *et al.*, 2009, Machado *et al.*, 2017). ABC transporters hydrolyse ATP to energize transport, as seen in the MacAB-TolC and MsrA systems in *Enterobacteriaceae* and *S. aureus* respectively, which mediate macrolide resistance (Saier and Paulsen, 2001, Kobayashi *et al.*, 2001, Schindler and Kaatz, 2016). Finally, MATE transporters use Na⁺ or H⁺ gradients to remove toxic and xenobiotic compounds (Pastacaldi *et al.*, 2024). The most well-studied MATE transporter, NorM, which exports dyes, fluoroquinolones and aminoglycosides was first identified in *Vibrio parahaemolyticus* and later found in *Vibrio cholera* and *N. gonorrhoeae* (Spengler *et al.*, 2017).

Collectively, bacterial pathogens use both intrinsic and extrinsic mechanisms to survive antimicrobial stress. Intrinsic resistance involves limited drug intake, drug target modification, or drug inactivation, while acquired resistance relies on mechanisms such as drug target modification, drug inactivation, and efflux pumps

(Reygaert, 2018, Cox and Wright, 2013). Importantly, many bacterial pathogens use a combination of mechanisms to survive, this has contributed to the emergence of multi-drug resistant (MDR) organisms (Urban-Chmiel *et al.*, 2022, Chiş *et al.*, 2022).

1.3. *Klebsiella pneumoniae*

Klebsiella pneumoniae is one of the WHO-listed “ESK(C)APE” pathogens for which the development of alternative treatment options is urgently required (Mar’ah *et al.*, 2024, Mulani *et al.*, 2019). This multidrug-resistant group includes *Enterococcus faecium* (E), *S. aureus* or *Stenotrophomonas maltophilia* (S), *K. pneumoniae* (K) or *Clostridioides difficile* (C), *A. baumannii* (A), *P. aeruginosa* (P), and *Enterobacteriaceae* (E). These pathogens are responsible for various nosocomial infections which contribute significantly to global high mortality and morbidity rates (Mulani *et al.*, 2019, Denissen *et al.*, 2022). Following the declaration of the SARS-CoV-2 pandemic in 2020, more than 50% of severe infection cases were found to be coinfecting with bacterial species belonging mainly to the ESK(C)APE group highlighting their clinical relevance and persistence in healthcare settings (Loyola-Cruz *et al.*, 2023, Chen *et al.*, 2020). Similarly, the Centers for Disease Control and Prevention (CDC) has designated these organisms as urgent public health threats worldwide (Khasapane *et al.*, 2024).

K. pneumoniae is a Gram-negative, encapsulated, opportunistic pathogen belonging to the *Enterobacteriaceae* family. It is commonly found in water, soil and animals and is known to colonize medical devices (Mar’ah *et al.*, 2024, Janda and Abbott, 2021, Podschun *et al.*, 2001, Bengoechea and Sa Pessoa, 2018). Clinically it is associated with several infections including pneumoniae, urinary tract infections and wound infections (Podschun and Ullmann, 1998, Holt *et al.*, 2015). Strains of *K. pneumoniae* have been divided into two groups; namely classical *K. pneumoniae* (cKp) and hypervirulent *K. pneumoniae* (hvKp) (Abbas *et al.*, 2024, Salvador-Oke *et al.*, 2024). cKp have been identified to cause infections in immunocompromised patients, elderly patients, and neonates while hvKp causes infections in healthy and immunocompromised individuals (Abbas *et al.*, 2024). Initially, hvKp phenotypes were responsive to antibiotics however, over the years cKp and hvKp phenotypes merged and this resulted in certain hvKp strains having gained resistance to carbapenems and beta-lactam antibiotics (Liu *et al.*, 2014, Li *et al.*, 2013, Abbas *et al.*, 2024).

The pathogenicity and virulence of *K. pneumoniae* strains can be attributed to virulence factors such as capsules, lipopolysaccharide (LPS), adhesins, iron-scavenging systems, and biofilm formation (Kot *et al.*, 2023, Cruz-Córdova *et al.*, 2014). The capsule is a protective outer structure that protects *K. pneumoniae* and many other pathogenic bacteria against the host immune system (Huang *et al.*, 2022). There are 80 structurally different polysaccharide capsules produced by *K. pneumoniae* using the flippase (Wzx)/polymerase (Wzy) polysaccharide polymerization machinery (Wen and Zhang, 2015, Rendueles *et al.*, 2017, Schmid *et al.*, 2015, Huang *et al.*, 2022). The capsule interacts with the mucus layer thus allowing for successful colonization (Taylor and Roberts, 2002). Although the mechanism of capsule-mediated bacterial pathogenesis in *K. pneumoniae* infection is unknown, analogous mechanisms in other pathogens include electrostatic repulsion of phagocytes by *Neisseria meningitidis*, surface protein masking by *S. aureus*, and host glycan mimicry by *Campylobacter jejuni*, *Helicobacter pylori*, and *E. coli* (Taylor and Roberts, 2002, Wilkinson *et al.*, 1979, Huang *et al.*, 2022, De Jong *et al.*, 2022).

LPS are endotoxins which are components of the Gram-negative bacterial outer membrane and are made up of O-antigen, a core oligosaccharide and lipid A (Bulati *et al.*, 2021). There are 9 different *K. pneumoniae* O-antigens, with O1 being the most common (Paczosa Michelle and Meccas, 2016). The O-antigen binds to C3b, which is a component of the complement pathway, thus making *K. pneumoniae* with the full-length O-antigen less susceptible to complement-mediated killing (Paczosa Michelle and Meccas, 2016, Bulati *et al.*, 2021). Iron acquisition is another important virulence mechanism with *K. pneumoniae* containing three iron-scavenging systems that synthesize small iron-scavenging molecules called siderophores (Abbas *et al.*, 2024, Paczosa Michelle and Meccas, 2016). Enterobactin produced by both *cKp* and *hvKp*, is commonly identified among *K. pneumoniae* isolates (El Fertas-Aissani *et al.*, 2013, Paczosa Michelle and Meccas, 2016).

Adhesins such as fimbriae or pili play a key role in host colonization through the facilitation of bacterial adhesion to abiotic or epithelial surfaces (Riwu *et al.*, 2022). This facilitates the formation of biofilms on catheters and other hospital equipment which in turn results in a strain on the nosocomial environment as *K. pneumoniae* infections persist (Struve *et al.*, 2008, Paczosa Michelle and Meccas, 2016). Biofilm

associated bacteria are less affected by antimicrobial activity and host defence mechanisms (Guerra *et al.*, 2022, Guilhen *et al.*, 2016).

Antibiotic resistant *K. pneumoniae* have been associated with fitness costs that influence cellular metabolism, surface architecture, and virulence, with potential implications for vaccine susceptibility (Hafza *et al.*, 2018; Li *et al.*, 2024). Resistance mechanisms such as enzymatic antibiotic inactivation, target modification, reduced membrane permeability, and increased efflux pump activity cause metabolic and regulatory burdens that reduce growth efficiency and compromise stress response pathways (Li *et al.*, 2024). Therefore, antibiotic-resistant *K. pneumoniae* strains exposed to these fitness constraints may exhibit increased vulnerability to vaccine strategies targeting conserved and functionally indispensable antigens.

In 2018, the European Centre for Disease Prevention and Control reported that more than one third of *K. pneumoniae* isolates are resistant to at least one antimicrobial group (Bengoechea and Sa Pessoa, 2018). Additionally, many *K. pneumoniae* virulent and multidrug-resistant clones have access to elements related to horizontal gene transfer (such as plasmids) and antimicrobial resistance genes, therefore these pathogens can spread antibiotic-resistant genes to other Gram-negative bacteria (Bengoechea and Sa Pessoa, 2018, Lam *et al.*, 2018). Due to the emergence of antibiotic resistance over the years and the severity of infections, *K. pneumoniae* has been identified as an urgent threat to human health (Holt *et al.*, 2015). This urgency directly translates to the need to develop alternative therapies such as targeting important survival proteins.

1.4. Bacterial protein homeostasis and stress response

Protein homeostasis (proteostasis) is essential for bacterial survival and can be defined as the production and maintenance of cellular proteins in the correct conformation and concentration (Khodaparast *et al.*, 2021). Over time, bacteria have evolved controlled systems to monitor and maintain protein homeostasis, these include: i) independently folding newly synthesized proteins into their three-dimensional forms, ii) preventing aggregation of unfolded proteins, iii) degrading misfolded proteins, and iv) refolding or degrading resolubilized protein aggregates (Mogk *et al.*, 2011, Shukla and Narayan, 2025). Following protein synthesis on

ribosomes, nascent polypeptides interact with the ribosome-bound Trigger Factor (TF) which promotes efficient *de novo* protein folding by preventing co-translational misfolding (Bornemann *et al.*, 2014, Mogk *et al.*, 2011). Upon release from TF, the newly synthesized proteins either fold spontaneously or through the assistance of chaperones such as DnaK (Mogk *et al.*, 2011, Kramer *et al.*, 2009).

Bacteria are often subject to environmental stresses such as radiation, extreme temperatures, nutrient limitation, acid, antimicrobials, or oxidative stress (Mogk *et al.*, 2011, Dawan and Ahn, 2022, Kuzu *et al.*, 2025). Such conditions disrupt protein homeostasis by promoting non-native interactions that drive misfolding, while causing other proteins to unfold (Schramm *et al.*, 2020, Kuzu *et al.*, 2025). These unfolded and misfolded proteins then gather to form non-functional protein assemblies, referred to as protein aggregates, which disrupt protein homeostasis (Mogk *et al.*, 2018, Schramm *et al.*, 2020). To mitigate such effects, bacteria have evolved transcriptional, translational, and post-translational modifications to survive stressful environmental conditions, thus resulting in altered gene expression, protein activity, and cellular metabolism (Dawan and Ahn, 2022).

Stress proteins are important for survival and are upregulated under stressful conditions such as temperature fluctuations, antibiotic presence, and oxidative stress (Dalbanjan *et al.*, 2024). Depending on their regulatory context, these proteins are either stress-activated or dual-function, performing additional roles under non-stress conditions (Fang *et al.*, 2019, Wan *et al.*, 2020, Dalbanjan *et al.*, 2024). Among them, heat shock proteins (HSPs) represent a highly conserved family present in both prokaryotes and eukaryotes (Hu *et al.*, 2022). Under normal physiological conditions, HSPs constitute approximately 5–10% of total cellular proteins. However, during stress, activation of heat shock transcription factors (HSFs) leads to a rapid increase in HSP expression (Dalbanjan *et al.*, 2024, Murshid *et al.*, 2018). Functionally, HSPs serve as molecular chaperones that maintain proteostasis by refolding denatured proteins, dissociating aggregates, and targeting irreversibly misfolded proteins for degradation (Dalbanjan *et al.*, 2024) (Table 1-1). This adaptive function makes HSPs essential for bacterial survival, especially under stressful conditions.

Table 1-1: Function of commonly studied Heat shock proteins (HSPs) under stressful conditions.

Protein	Function	Reference
GroEL/GroES	Expression is regulated by the heat shock sigma factor $\sigma 32$ (RpoH). Complex prevents aggregation and refolding of denatured proteins in an ATP-dependent manner	(Dalbanjan <i>et al.</i> , 2024, Hu <i>et al.</i> , 2022, Singh <i>et al.</i> , 2024)
DnaK/DnaJ/GrpE	Works as a complex and is regulated by RpoH, prevents the aggregation of denatured proteins and assists in nascent protein folding in an ATP-dependent manner.	(Warnecke, 2012, Dalbanjan <i>et al.</i> , 2024)
Hsp33	Molecular chaperone that prevents protein aggregation and is regulated by oxidative stress response pathways.	(Krewing <i>et al.</i> , 2019)
Hsp60	Chaperonin that assists in protein folding, prevents misfolding, and ensures correct protein conformation	(Hu <i>et al.</i> , 2022, Singh <i>et al.</i> , 2024)
HspX	Expressed during nutrient starvation. Keeps bacteria dormant	(Verma <i>et al.</i> , 2022)
IbpA/IbpB	Protect aggregated proteins from thermal oxidative stress	(Miwa <i>et al.</i> , 2021)
HslVU/ClpYQ	Protease complex degrades misfolded and aggregated proteins in an ATP-dependent manner under heat stress	(Kebe <i>et al.</i> , 2019, Chang <i>et al.</i> , 2019)
degP	In response to heat and oxidative stress this protease and chaperone break down misfolded periplasmic proteins. During envelope stress, expression is regulated by the σE (RpoE) sigma factor	(Zarzecka <i>et al.</i> , 2019)
HfIC/HfIK	Expression regulated by RpoH during heat stress, part of regulatory complex of FtsH protease that breaks down misfolded membrane protein	(Yi <i>et al.</i> , 2022)
Lon	Expression regulated by stress response elements. Under antibiotic stress, it degrades regulatory and misfolded proteins in an ATP-dependent manner	(Kirthika <i>et al.</i> , 2023)
SecB	Ensures proper protein localization and function	(Sala <i>et al.</i> , 2014)
ClpP	Protease degrades misfolded, damaged, and regulatory proteins	(Alves França <i>et al.</i> , 2024)
ClpA, ClpX	Chaperone and unfoldase associates with ClpP to degrade regulatory and aggregated proteins	(Singh <i>et al.</i> , 2001)
ClpB	Disaggregates and refolds proteins	(Rosenzweig <i>et al.</i> , 2015)
ClpC, ClpM	Associates with ClpP to degrade non-native proteins	(Aljghami <i>et al.</i> , 2022, El Bakkouri <i>et al.</i> , 2010)
ClpD	Unfolds chloroplast proteins	(Bouchnak and Van Wijk, 2021)
ClpE	Expression induced by heat stress to disaggregate proteins and involved in regulation of class III heat shock genes	(Molière and Turgay, 2009)
ClpK	Allows bacteria to survive heat shock	(Bojer <i>et al.</i> , 2010)
ClpL	Refold proteins and heat stress survival	(Park <i>et al.</i> , 2015)
ClpV	Disassembles and remodels the T6SS contracted sheath	(Ho <i>et al.</i> , 2014)

1.5. ClpK as a potential drug target

Owing to the essential role of HSPs in maintaining proteostasis under stress, proteins belonging to the HSP100 family, particularly Caseinolytic (Clp) ATPases, have emerged as promising antimicrobial targets. Clp ATPases are members of the AAA+ (ATPases associated with diverse cellular activities) superfamily, which use ATP hydrolysis to mediate protein unfolding, disaggregation, and translocation into associated proteases for degradation (Zolkiewski *et al.*, 2012). These ATPases are important for bacterial survival under proteotoxic conditions, and their inhibition can disrupt proteostasis, leading to the accumulation of aggregated proteins and ultimately cell death (Ahyoung *et al.*, 2015, Schirmer *et al.*, 1996).

Clp ATPases contain highly conserved ATPase/AAA+ domains and typically function as hexameric rings composed of one (class II) or two (class I) nucleotide binding domains (NBDs) (Zolkiewski *et al.*, 2012, Thibault *et al.*, 2006, Snider *et al.*, 2008) (Figure 1-3). The NBDs are critical for stability, substrate recognition, ATP hydrolysis, and oligomerization in Clp ATPases (Walker *et al.*, 1982). Each NBD contains Walker A and B motifs essential for ATP binding and hydrolysis (Walker *et al.*, 1982, Maurizi and Xia, 2004, Wojtyra *et al.*, 2003).

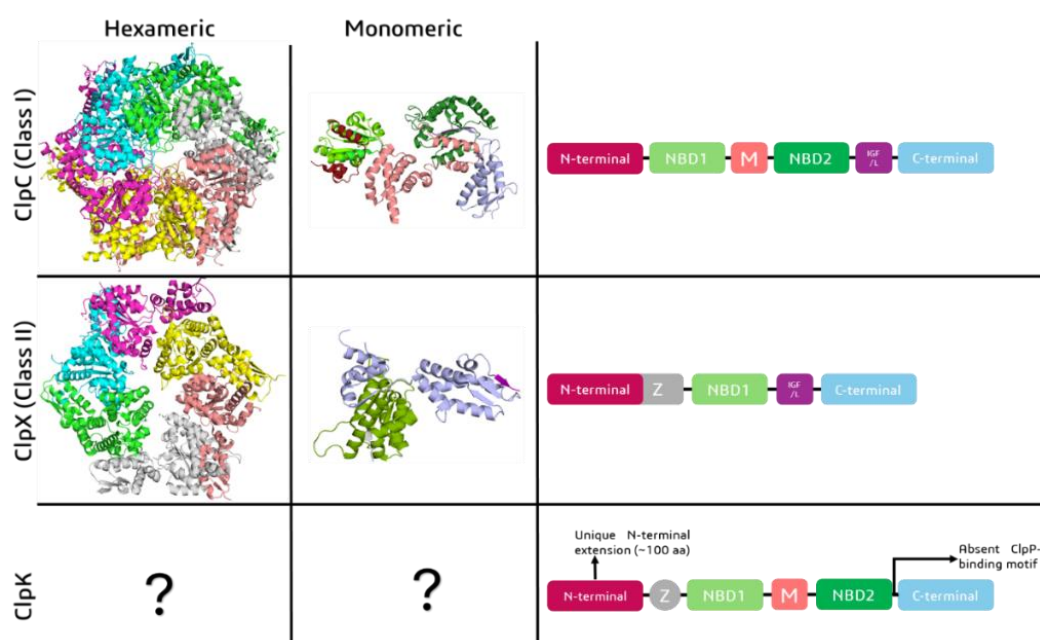


Figure 1-3: Domain organisation and 3D structures of Clp ATPases. Representative hexameric (right) and monomeric (left) 3D structures of Class I (ClpC; PDB: 7ABR) and Class II (ClpX; PDB: 3HWS) Clp ATPases. Class I and Class II Clp ATPases contain two and one nucleotide binding domains, respectively. The zinc (Z) motif is represented in grey. The middle domain (M) is represented in salmon. The ClpP-binding motif (IGF/L) is represented in purple (Bojer *et al.*, 2010, Miller *et al.*, 2018).

Among these, ClpK is a clinically significant member of the Clp ATPase family and was first isolated in *K. pneumoniae* C132-98 strains during a hospital outbreak in Denmark (Bojer *et al.*, 2010, Bojer *et al.*, 2013, Jørgensen *et al.*, 2016). The *clpK* gene was identified to be located on a conjugative plasmid conferring resistance to multiple antibiotics and was found to be situated on the heat resistant locus, suggesting an important role for ClpK in bacterial stress survival (Bojer *et al.*, 2010, Bojer *et al.*, 2013). Additionally, functional studies demonstrated that *clpK* deletion resulted in the loss of *K. pneumoniae* thermotolerance, suggesting that ClpK enables *K. pneumoniae* to survive high-temperature or hospital-related stress conditions (Bojer *et al.*, 2010, Bojer *et al.*, 2013).

Structurally, ClpK is a class I Clp ATPase containing two NBDs connected by an intermediate length linker region (Bojer *et al.*, 2013) (Figure 1-3). Additionally, ClpK displays several features that distinguish it from other Clp proteins such as ClpA, ClpB, or ClpC. These include a unique 100 amino acid N-terminal extension, absence of the ClpP-binding tripeptide motif (IGF/L motif), and presence of a zinc binding motif which is only present in Gram-positive bacteria (Wojtyra *et al.*, 2003, Lo *et al.*, 2001, Amor *et al.*, 2019). These unique structural features suggest that ClpK has evolved specialised mechanisms to ensure proteostasis in stressful environments, providing *K. pneumoniae* with enhanced survival capacity in hostile environments. Direct experimental evidence distinguishing the substrate-threading mechanisms of ClpK is currently lacking; however, based on its classification as a class I Clp ATPase and its structural similarity to ClpB and ClpX, ClpK is likely to employ a ratchet-like translocation.

Given the increase in the prevalence of antibiotic resistance and the important role of stress response systems such as ClpK in bacterial survival, designing therapies to target these systems is a compelling alternative to conventional antimicrobial strategies. By inhibiting essential enzymes such as ClpK, it is possible to sensitize pathogens to environmental and antibiotic-induced stress, thereby compromising their ability to survive in hostile conditions. This has therefore resulted in the development of alternative therapeutic strategies to target bacterial fitness and adaptability instead of solely inhibiting bacterial growth (La Guidara *et al.*, 2024, Singha *et al.*, 2024).

1.6. Alternative therapies to target bacteria

In recent years, growing attention has been directed towards the development of innovative therapeutic strategies aimed at overcoming antibiotic resistance through non-traditional mechanisms (Kumar *et al.*, 2021). These emerging approaches include the use of antimicrobial peptides (AMPs), hemofiltration, quorum-sensing inhibitors, RNA based therapies, monoclonal antibodies, phage therapies, faecal microbiota transplant, nanomaterials, phytopharmaceuticals, traditional medicinal systems, biofilm disruption agents, probiotics, postbiotics, synbiotics, and vaccines (Kumar *et al.*, 2021, Konwar *et al.*, 2022, La Guidara *et al.*, 2024, Singha *et al.*, 2024).

- i. **AMPs** are small bioactive molecules found in mammals, microorganisms, amphibians, and insects that exhibit a wide range of inhibitory effects against bacteria, fungi, parasites, and viruses (Huan *et al.*, 2020). Some AMPs target bacterial membranes through interaction with the outer membrane and surface lipids to promote membrane disruption while other AMPs target intracellular DNA, RNA, and essential proteins (Benfield and Henriques, 2020). Approximately 34 natural and synthetic AMPs have been identified to have potent activity against *K. pneumoniae* with many of them targeting the bacterial membrane (De Souza *et al.*, 2022).
- ii. **Hemofiltration** or renal replacement therapy removes toxic products, waste products, or excessive water from blood through convective solute transport (Kumar *et al.*, 2021). Clinical evidence supports its therapeutic potential in severe *K. pneumoniae* infections. For instance, Kamata *et al.* (2001) reported improved outcomes in a patient treated with endotoxin adsorption (PMX) and continuous hemodiafiltration (CHDF) treatment. More recently, Hajjej *et al.* (2016) described successful treatment of carbapenem-resistant *K. pneumoniae* (CR-Kp) septicaemia using continuous venovenous hemodiafiltration with amikacin and imipenem.
- iii. **Quorum sensing inhibitors (QSIs)** target bacterial communication systems that regulate virulence factors such as biofilm formation and siderophore production (Kumar *et al.*, 2021, Rémy *et al.*, 2018, Naga *et al.*, 2023). Natural and synthetic QSIs have shown potential in attenuating *K. pneumoniae* infection. For example, Liu *et al.* (2020) demonstrated that tea

polyphenols act as effective QSIs both *in vitro* and *in vivo*, while Rachma *et al.* (2022) found that *Aspergillus oryzae* inhibited biofilm formation, exopolysaccharides, and type III fimbrial protein (mrkA) expression *in vitro*, and increased the survival rate of *C. elegans* against *K. pneumoniae* infections *in vivo*.

- iv. **RNA based therapies** include antisense oligonucleotides, RNA interference (RNAi), ribozymes, synthetic sRNAs, (Clustered regularly interspaced short palindromic repeats) CRISPR guide RNAs and aptamers (Burnett and Rossi, 2012). These tools silence genes, degrade RNA, or block translation of key bacterial proteins (Sparmann and Vogel, 2023, Burnett and Rossi, 2012, Di Mauro *et al.*, 2024, Gao *et al.*, 2021). Notably, CRISPR-Cas9 was used in *K. pneumoniae* to restore carbapenem susceptibility (Ahmed *et al.*, 2024). Due to the instability and immunogenic characteristics of RNA, there has been staggered clinical progress in the development of RNA-based drugs and varying clinical results. However, with alterations in delivery methods and therapy optimization, RNA therapy has demonstrated promise in the treatment of viruses, cancer, and genetic disorders (Burnett and Rossi, 2012).
- v. **Monoclonal antibodies (mAb)** provide vaccine like immunity by binding to essential and non-essential antigens such as virulence factors (Baker *et al.*, 2020). MrkA was identified as a promising antibody therapeutic target in *K. pneumoniae* (Wang *et al.*, 2016). Although mAbs exhibit safety and long serum half-lives, challenges remain due to high production costs and limited antigen accessibility caused by the bacterial capsule and biofilm formation (La Guidara *et al.*, 2024).
- vi. **Phage therapies** employ bacteriophages (viruses that infect only bacterial cells) to lyse bacterial cells. While monophages target specific receptors, polyphages recognize multiple receptors and allow broader host range (Rios *et al.*, 2016, Kumar *et al.*, 2021, Singha *et al.*, 2024). Recent studies, including Rotman *et al.* (2024) used the *Klebsiella* PhageBank to design effective phage cocktails, while Gan *et al.* (2022) demonstrated phage-mediated suppression of early-stage *K. pneumoniae* infections. Phage PG14 has been shown to disrupt preformed biofilms to allow antibiotic penetration (Mulani *et al.*, 2022).

- vii. **Faecal Microbiota Transplant (FMT)** therapy includes the transfer of faecal microbiota from healthy individuals to reconstitute healthy gut microbiota, combat dysbiosis and resist pathogens (Kumar *et al.*, 2021). Several successful case studies have been reported worldwide, including treatment of *K. pneumoniae* infections in South Africa, Poland, and Portugal (Ueckermann *et al.*, 2020, Martínez Pizarro, 2021, Biliński *et al.*, 2016). Despite these successes, risks such as the transfer of pathogens in faecal matter, faecal toxicity due to the presence of toxins, waste and inconsistent reproducibility highlight the need for refined FMT therapy methodologies (Khan *et al.*, 2022).
- viii. **Nanomaterials** can act as carriers for antimicrobial substances or have antibacterial properties of their own such as cell wall disruption, host immune response modulation, and reactive oxygen species generation (Kumar *et al.*, 2021). Silver and copper nanoparticles have been shown to be effective against *K. pneumoniae* in combination with imipenem and other antibiotics (Fontoura *et al.*, 2023, Valadbeigi *et al.*, 2023). Additionally, neutrophil membrane-coated nanoparticles enhance targeted drug delivery and have shown efficacy in the inhibition of *K. pneumoniae*-induced pneumonia (Liu *et al.*, 2023). However, large scale production challenges and limited toxicity data limit the full-scale development of nanoparticle-based therapies (Xuan *et al.*, 2023)
- ix. **Traditional medicinal systems** such as Ayurveda and Traditional Chinese Medicine are important for the discovery of medicinal plants and the development of new compounds. Herbal medicines are effective, have lower side effects and are cost effective, thus making them promising alternative drug candidates. Flavonoids are found in most plants and exert antioxidant, anticarcinogenic, anti-inflammatory, antibacterial, immune-stimulating, and antiviral activity (Khan *et al.*, 2013). For instance, Apigenin and Kaempferol, flavonoids derived from different plant sources have been explored for their potential to target *K. pneumoniae* (Singha *et al.*, 2024). Several flavonoids have also been investigated for their ability to inhibit ATPase activity, particularly in microbial systems, mitochondria and HSPs (Table 1-2).

Table 1-2: Flavonoids with known effects on ATPase activity.

Flavonoids	ATPase Targets	Mechanism/ Effect	Reference
Quercetin	HSP70, HSP90, mitochondrial ATPases, Gastric H ⁺ , K ⁺ -ATPase, mitochondrial ATP synthase	Inhibits ATPase activity; binds ATP-binding site, down-regulates expression	(Wu <i>et al.</i> , 2023, Aalinkeel <i>et al.</i> , 2008, Murakami <i>et al.</i> , 1999, Althaher and Alwahsh, 2023)
Epigallocatechin gallate (EGCG)	Hsp70, Hsp90, DNA gyrase	Inhibits chaperone and ATPase activity, impairs co-chaperone association	(Zininga <i>et al.</i> , 2017, Li <i>et al.</i> , 2009, Yin <i>et al.</i> , 2009, Nie <i>et al.</i> , 2018)
Kaempferol	Hsp70, Gastric H ⁺ , K ⁺ -ATPase, mitochondrial ATP synthase	Reduced expression in under heat stress, inhibits ATPase activity	(Liu <i>et al.</i> , 2022, Murakami <i>et al.</i> , 1999, Althaher and Alwahsh, 2023)
Fisetin	ATP-binding cassette (ABC) transporters mitochondrial ATP synthase, Gastric H ⁺ , K ⁺ -ATPase, ATP-binding cassette (ABC) transporters	Inhibit transporter ATPase activity	(Yarla and Ganapaty, 2013)
Apigenin	ATP-binding cassette (ABC) transporters mitochondrial ATP synthase, Gastric H ⁺ , K ⁺ -ATPase, ATP-binding cassette (ABC) transporters	Inhibit ATPase activity	(Althaher and Alwahsh, 2023, Murakami <i>et al.</i> , 1999, Saeed <i>et al.</i> , 2015)
Luteolin	ATP synthase	Impairs ATP production, inhibits ATPase activity	(Chagas <i>et al.</i> , 2025)
Genistein	Tyrosine kinase	Inhibit ATP binding and hydrolysis	(Akiyama <i>et al.</i> , 1987)

- x. **Biofilm disruption agents** degrade the biofilm matrix and enhance antibiotic penetration (Singha *et al.*, 2024). The agents used to disrupt or inhibit biofilms can range from herbs to biocides to AMPs and much more. For example, phytochemical 1,8-cineole and nonantibiotic adjuvant EDTA along with beta-lactam and beta-lactamase inhibitor (CSE1034) disrupts and eradicates *K. pneumoniae* biofilms (Vazquez *et al.*, 2022, Chaudhary and Payasi, 2012). Benzoic acid derivatives, and cobalt sulphate inhibit biofilm formation (Rohatgi and Gupta, 2023, Chhibber *et al.*, 2013).
- xi. **Probiotics** are bacteria or fungi that are consumed to promote a healthy balance of bacteria in the body. The use of probiotics together with antibiotics results in the reduction of infection severity and duration (Zavišić *et al.*, 2023). Probiotic species *Lactobacillus*, *Bacteroidetes*, and *Actinobacteria* have been associated with decreased *K. pneumoniae* levels (Yan *et al.*, 2021). **Postbiotics**, the metabolic by-products of probiotics, exhibit antibacterial and antibiofilm activities through organic acids and bacteriocins (Mishra *et al.*, 2024).
- xii. **Vaccines** reduce antibiotic usage and decrease the transmission of bacterial determinants (Mullins *et al.*, 2023). There are different types of

vaccines that can be developed, these include live attenuated vaccines, inactivated whole cell vaccines, polysaccharide and lipopolysaccharide (LPS)-based vaccines, protein-based vaccines, and ribosomal vaccines. While no licensed *K. pneumoniae* vaccines exist, promising candidates such as KP-Ag1, a fusion antigen vaccine, and mHla-EpiVac, a multiepitope subunit vaccine, have demonstrated protective immune responses in preclinical models (Chen *et al.*, 2024, Assoni *et al.*, 2021, Liao *et al.*, 2024).

With an advanced and vested interest in the use of alternative therapies to combat antibiotic resistance, there are challenges that are yet to be overcome. These challenges include:

- i. Identifying different mechanisms to target,
- ii. Safety and efficacy concerns,
- iii. Time-consuming and expensive to develop robust pre-clinical models,
- iv. Lack of collaboration and knowledge sharing and
- v. Lack of public education and misinformation (Singha *et al.*, 2024).

1.7. Objectives

Combatting AMR is associated with the One Health approach which is the combined attempt of different disciplines to provide optimal health for humans, animals, and the environment (Fletcher, 2015, Kumar *et al.*, 2021). *K. pneumoniae* is a multi-drug-resistant pathogen that causes infections in immunocompromised patients, neonates and some strains cause infections in healthy individuals. An increase in antibiotic resistant strains results in high morbidity and mortality rates which in turn causes a strain on the healthcare and agricultural system. It is therefore imperative to investigate alternative targets or use alternative therapies to hinder or inhibit the growth of this pathogen. The objectives of this study included:

1. Model and validate the 3D structure of ClpK to enable subsequent structure-based analyses.
2. Characterize the molecular interactions of ClpK with ATP and divalent metal ions (Mg^{2+} , Ca^{2+}) through molecular docking and molecular dynamics simulations.

3. Overexpress and purify recombinant ClpK, followed by biophysical characterization to assess its structural stability and activity in the presence of ATP and metal ions (Mg^{2+} , Mn^{2+} , Ca^{2+}).
4. Identify and evaluate potential inhibitory ligands through *in silico* docking and dynamic simulations of ClpK–ligand complexes, including analysis of magnesium-dependent binding interactions.
5. Design and evaluate multi-epitope subunit vaccines targeting *Klebsiella pneumoniae* using a reverse vaccinology and immunoinformatic pipeline.

1.8. References

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Chapter 2: Unveiling the Role of Divalent cations in *Klebsiella pneumoniae* ClpK: Experimental and Computational Perspectives.

2.1. Abstract

Klebsiella pneumoniae is one of the four leading pathogens responsible for antimicrobial resistance-associated deaths in the African region. This opportunistic bacterium causes life-threatening infections, especially in immunocompromised individuals, newborns, and the elderly. Its ability to survive harsh environmental conditions through various virulence mechanisms poses a significant therapeutic challenge. Among these mechanisms is ClpK, a thermotolerant chaperone belonging to the Clp ATPase family, which plays an important role in bacterial stress tolerance and survival. However, the role of divalent cations in modulating the structural and functional properties of ClpK remains poorly understood. Therefore, this study used a combined computational and experimental approach to investigate the impact of divalent cations (Mg^{2+} , Mn^{2+} and Ca^{2+}) on ClpK stability, ATPase activity, and conformational dynamics. A homology model generated using the AlphaFold server demonstrated that ClpK adopts a predominantly α -helical structure. Molecular docking indicated energetically favoured ATP binding at both nucleotide binding domains, with dual occupancy enhancing α -helical content and overall protein stability. Experimentally, ClpK was cloned, expressed, purified, and characterised for ATPase activity under varying conditions of pH, ATP concentrations, and the presence of Mg^{2+} , Mn^{2+} and Ca^{2+} ions. Optimal ATPase activity was observed at pH 6, with kinetic analyses revealing moderate ATP affinity. Spectroscopic analyses showed that Mg^{2+} strongly stabilised ClpK in both *apo* and ATP-bound forms, whereas Mn^{2+} induced greater conformational changes, correlating with enhanced enzymatic activity. These findings offer critical insights into the metal ion-dependant regulation of ClpK and lay the groundwork for the rational design of inhibitors that disrupt ATP or metal ion interactions to combat antimicrobial resistance in *K. pneumoniae*.

2.2. Introduction

Antimicrobial resistance (AMR) is a growing global health crisis. According to the Institute for Health Metrics and Evaluation (IHME), of the 4.71 million deaths associated with drug-resistant infections in 2021, 1.14 million deaths were directly attributed to AMR (Naghavi *et al.*, 2024, IHME, 2019). Alarming, this number is projected to increase by over 300% between 2025 and 2050, with AMR-related deaths expected to reach approximately 39 million (IHME, 2019). A 2023 IHME report further highlighted that AMR caused more deaths in the African region than HIV/AIDS and malaria combined. The report identified *Klebsiella pneumoniae* alongside *Streptococcus pneumoniae*, *Escherichia coli*, and *Staphylococcus aureus* as one of four priority pathogens responsible for over 100,000 deaths (Anon, 2023, IHME, 2023).

K. pneumoniae is a Gram-negative, opportunistic, nosocomial pathogen capable of causing life-threatening infections, including pneumonia, bloodstream infections, liver abscesses, and urinary tract infections (Kot *et al.*, 2023, Podschun and Ullmann, 1998). Its increasing prevalence among immunocompromised individuals has contributed to rising mortality and morbidity rates (Verani *et al.*, 2024, Abbas *et al.*, 2024). Critically, *K. pneumoniae* demonstrates a remarkable ability to acquire and transmit antibiotic resistance genes, facilitating the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains (Li *et al.*, 2023). Due to its rapidly evolving resistance profile, the World Health Organization (WHO) has classified *K. pneumoniae* as a “critical” priority pathogen, underscoring the urgent need for alternative therapeutic strategies (Abbas *et al.*, 2024, Kot *et al.*, 2023, Murugaiyan *et al.*, 2022).

Among the mechanisms aiding bacterial survival under stress, heat shock protein (Hsp) 100 family chaperones, particularly caseinolytic (Clp) ATPases, play an important role in bacterial stress response, protein homeostasis, and virulence (Schirmer *et al.*, 1996, Maurizi and Xia, 2004, Hoskins *et al.*, 2000). These ATPases are part of the conserved ATPases associated with diverse cellular activities (AAA+) superfamily and are categorized into the ClpA and ClpB/Hsp104 subfamilies. The ClpA subfamily mediates proteolysis in concert with ClpP whereas the ClpB/Hsp104 subfamily facilitates protein resolubilisation under stressful conditions (Ahyoung *et al.*, 2015, Maurizi and Xia, 2004). Beyond protein quality control, Clp ATPases have been

linked to bacterial pathogenesis, with ClpP-dependent proteolysis contributing to virulence in several clinically significant pathogens (Frees *et al.*, 2007, Butler *et al.*, 2006, Brötz-Oesterhelt and Sass, 2014). Consequently, Clp ATPases and ClpP have emerged as promising targets for anti-virulence drug development (Motiwalla *et al.*, 2022, Brötz-Oesterhelt and Sass, 2014).

ClpK is a thermotolerant Clp ATPase identified in several *Klebsiella* species and is associated with enhanced survival under heat and stress conditions (Bojer *et al.*, 2013, Motiwalla *et al.*, 2021, Bojer *et al.*, 2010). ClpK contains two nucleotide-binding domains (NBDs) characterized by Walker A and Walker B motifs, which are essential for adenosine triphosphate (ATP) hydrolysis and metal ion coordination (Maurizi and Xia, 2004, Motiwalla *et al.*, 2021). Divalent cations are vital for bacterial survival, acting as structural components of metalloproteins, enzyme cofactors, and regulators of oxidative and environmental stress responses (Porcheron *et al.*, 2013). Among these, Mg^{2+} , Mn^{2+} and Zn^{2+} are biologically important metal ions (Costa *et al.*, 2023; Sissi & Palumbo, 2009; Vicogne *et al.*, 2023). Mg^{2+} stabilises negative charges of phosphate groups and facilitates nucleophilic attacks, thereby coordinating ATP binding and hydrolysis (Sissi & Palumbo, 2009). Mn^{2+} can essentially be used as a substitute for Mg^{2+} in certain enzymatic reactions and is frequently implicated in oxidative stress resistance and enzymatic robustness under extreme conditions (Vicogne *et al.*, 2023). In contrast, Zn^{2+} functions primarily as a structural and regulatory cofactor, contributing to protein stability, domain organisation, and conformational control rather than direct catalysis (Costa *et al.*, 2023). Collectively, these divalent cations are central to maintaining proteostasis and enzymatic efficiency under stress, and perturbation of metal-ion homeostasis can critically impair ATPase-driven processes essential for bacterial viability. Therefore, it is important to understand how these ions affect ClpK structure, function, and activity.

While Clp proteins such as ClpA, ClpB, and ClpX have been extensively studied in pathogens like *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Mycobacterium tuberculosis*, and *Bacillus subtilis*, little is known about the structural and functional roles of ClpK (Capestany *et al.*, 2008, Zolkiewski *et al.*, 2012, Park *et al.*, 2015). In particular, the influence of divalent cations on ClpK stability, ATPase activity, and structural dynamics has not been elucidated. This study aims to fill this knowledge gap

by employing both *in silico* and *in vitro* approaches to evaluate the impact of divalent cations on ClpK structure, function, and ATP-mediated stabilization.

2.3. Material and Methods

2.3.1. Over-expression of Recombinant ClpK

ClpK was expressed as per previous protocols (Motiwala *et al.*, 2021). Briefly, the *clpK* gene (Uniprot ID: E0W6V3) was cloned into the pColdI vector using BamHI and SalI restriction sites, and the recombinant plasmid (pColdI-ClpK) was synthesized by GenScript (NJ, USA). Chemically competent *Escherichia coli* BL21 cells were transformed using a heat-shock protocol with pColdI-*clpK*, plated on 2xYT agar supplemented with ampicillin (100 µg/mL) and incubated overnight at 37 °C. Single colonies were picked and screened via colony PCR using pColdI-specific primers. For expression, overnight cultures of transformed *E. coli* were grown in LB medium containing ampicillin (100 µg/mL) at 37 °C (16 h), then diluted 1:100 in fresh LB and grown to OD₆₀₀ 0.4–0.6. Cultures were cold-shocked on ice (30 min), induced with 0.25 mM IPTG, and incubated at 15 °C for 24 h. Cells were harvested by centrifugation (6800 × g, 15 min), resuspended in lysis buffer (20 mM sodium phosphate, 0.2 M NaCl, pH 5), and lysed by sonication (15 min, 30 s on/20 s off, 50% amplitude). The lysate was centrifuged (6800 × g, 15 min) to separate soluble and insoluble fractions, which were analysed by 12.5% SDS-PAGE under reducing conditions (Laemmli, 1970).

2.3.2. Purification of ClpK

ClpK was purified in two steps: (1) polyethylene glycol (PEG) 6000 precipitation and (2) Ni²⁺-affinity chromatography. The soluble fraction containing the N-terminus 6× His tagged ClpK was subjected to sequential PEG precipitation by gradually increasing PEG concentration to 2.5%, 10%, 20%, and 30% (w/v), each followed by incubation (30 min, stirring) and centrifugation (6800 × g, 30 min). Pellets were stored at 4 °C and later dissolved in binding buffer (20 mM sodium phosphate, 0.2 M NaCl, 20 mM imidazole, pH 7.4). Fractions were analysed by reducing 12.5% SDS-PAGE (Laemmli, 1970). Pellets from 2.5% and 10% PEG steps were further purified using a 5 mL HisTrap HP column (GE Healthcare) pre-equilibrated with binding buffer. After washing, bound proteins were eluted with 200 mM imidazole in elution buffer (20 mM

sodium phosphate, 0.2 M NaCl, pH 7.4). Eluted fractions were analysed via SDS-PAGE (Laemmli, 1970).

2.3.3. Enzyme activity

ATP hydrolysis by ClpK was measured using the Malachite Green Phosphate Assay Kit (MAK307, Sigma-Aldrich) as per the manufacturer's instructions. ClpK (0–3 μ M) in Tris-HCl buffer (pH 7.5) was incubated with 1 mM ATP for 30 minutes. After dilution, working reagent was added and the mixture was incubated for an additional 30 min at room temperature. Inorganic phosphate release was quantified spectrophotometrically at 620 nm using the GloMax® Discover microplate reader (Promega, USA). All reactions were performed in triplicate. To further characterize enzymatic activity, ATPase assays were conducted using 1) varying ATP concentrations, 2) different pH conditions (Table 2S-1), and 3) in the presence or absence of 5 mM divalent cations (Mg^{2+} , Ca^{2+} , Mn^{2+}). Kinetic parameters (Michaelis-Menten constant (K_m) and maximum velocity (V_{max})) were calculated using the Lineweaver-Burk plot generated with the ggplot2 package in RStudio (Wickem, 2016). Relative activity under different conditions was calculated as:

$$\text{Relative Activity (\%)} = \frac{\text{Activity at given condition}}{\text{Maximum activity}} \times 100 \quad (1)$$

2.3.4. Far UV-CD spectroscopy

Far-UV circular dichroism (CD) spectroscopy was used to analyse the secondary structure of purified ClpK (2 μ M in ddH₂O) in the presence and absence of 0.1 mM ATP and 8M urea. Spectra were recorded on a Jasco J-1500 spectropolarimeter at 20 °C using a quartz cuvette (pathlength: 1 mm), with seven accumulations collected from 250 to 180 nm (bandwidth: 2.5 nm, data pitch: 0.2 nm and response time: 1 sec). To assess the influence of divalent cations, 5 mM $MgCl_2$, $CaCl_2$, or $MnCl_2$ was added to the samples. Buffer contributions were subtracted from the final spectrum. The CD spectra were recorded in millidegrees ellipticity (θ_{mdeg}) and converted to mean residue ellipticity [θ_{MRE}] ($deg.cm^2.dmol^{-1}$) using the equation:

$$[\theta_{MRE}] = \frac{100 \times \theta_{mdeg}}{c \times l} \quad (2)$$

where c represents protein concentration (mM), n represents the number of protein amino acid residues, and l represents the pathlength (cm). The Dichroweb analysis program, CONTIN-LL, was used to deconvolute spectra data and derive the fraction of amino acids involved in α -helical, β -strand and loop formation (Lobley *et al.*, 2002).

2.3.5. Fluorescence spectroscopy studies

2.3.5.1. Tryptophan fluorescence

Tertiary structural changes in ClpK were assessed using intrinsic tryptophan fluorescence. ClpK (2 μ M) was prepared in Tris-HCl buffer with or without 1 mM ATP and 5 mM MgCl₂, or CaCl₂, or MnCl₂. Emission spectra (250 – 500 nm) were recorded upon excitation at 295 nm using the Jasco FP-6300 spectrofluorometer (excitation/emission bandwidths: 2.5 nm; data pitch: 1 nm; scan speed: 200 nm/min) at 20 °C in a 10 mm quartz cuvette. All samples were measured in triplicates, averaged and corrected for buffer background.

2.3.5.2. ANS fluorescence

Extrinsic fluorescence using 8-anilino-1-naphthalenesulfonic acid (ANS) was used to probe hydrophobic surface exposure of ClpK (2 μ M). Samples were prepared in Tris-HCl buffer with or without 1 mM ATP and 5 mM of MgCl₂, CaCl₂, or MnCl₂, and incubated in the dark. Fluorescence emission spectra (400 – 600 nm) were recorded upon excitation at 390 nm (bandwidths: 5 nm excitation, 10 nm emission, scan speed: 200 nm/min) using a Jasco FP-6300 fluorescence spectrophotometer at 20 °C and a 10 mm quartz cuvette. Spectra were background-subtracted and averaged from triplicates.

2.3.6. Thermal stability of ClpK

ClpK thermal stability was assessed using SYPRO orange dye as a fluorescent probe. ClpK (10 ng/ μ L) was tested under various conditions in Tris-HCl buffer. First, dye and protein concentration ranges were optimised using varying SYPRO orange (1X–20X) and ClpK concentrations. Stability was then evaluated under different pH conditions (Table 2S-1), salt (NaCl) concentrations and in the presence of 1 mM ATP and 5 mM MgCl₂, CaCl₂, or MnCl₂. All samples were run in triplicate. Thermal denaturation was monitored using the QuantStudio 5 Real-Time PCR System with a continuous

temperature ramp from 25 and 99 °C at a rate of 0.5 °C/min. Data was analysed using the Quantstudio™ Design and Analysis Software v. 1.3.1.

2.3.7. ClpK Modelling and Analysis

The three-dimensional (3D) structure of ClpK protein (Uniprot ID: E0W6V3) was predicted using AlphaFold, which generates high-accuracy models based on primary amino acid sequence (Jumper *et al.*, 2021). The predicted model was refined using the Galaxy Refine server to improve side chain packing and overall structural quality (Heo *et al.*, 2013). MolProbity (Chen *et al.*, 2010, Chen *et al.*, 2015), VADAR (Volume, Area, Dihedral Angle Reporter) version 1.8 (Willard *et al.*, 2003), Protein Structure Analysis (ProSA) web server (Wiederstein and Sippl, 2007), ERRAT module on the Saves v1.6 server and the SWISS MODEL structure assessment tool (Waterhouse *et al.*, 2018) were used for structural validation. MolProbity provides a comprehensive assessment of structural geometry through all-atom contact analysis and dihedral-angle validation (Chen *et al.*, 2010, Chen *et al.*, 2015). VADAR integrates over 15 algorithms to analyse secondary structures, torsion angles, and solvent accessibility (Willard *et al.*, 2003). ProSA-web evaluates model quality based on statistical potential (Wiederstein and Sippl, 2007). ERRAT assesses non-bonded atomic interactions, while the SWISS-MODEL assessment tool examines overall model geometry and stereo chemistry (Waterhouse *et al.*, 2018).

2.3.8. Protein-Ligand Preparation and Docking

The 3D structure of ATP (CID: 5957) was downloaded in .sdf format from the PubChem database. The homology model of ClpK and ATP were prepared for docking using the Dock Prep tool in UCSF Chimera, and saved in .mol2 format (Pettersen *et al.*, 2004). Molecular docking was performed using the AutoDock Vina extension in UCSF Chimera (Eberhardt *et al.*, 2021, Trott and Olson, 2010, Pettersen *et al.*, 2004). Targeted docking was performed at the nucleotide-binding domains (NBD1 and NBD2) of ClpK using the following grid parameters:

- **NBD1:** Centre (X = -2.47413, Y = -2.17765, Z = 30.2718); Size (X = 69.5754, Y = 46.4482, Z = 40.2351)
- **NBD2:** Centre (X = 15.0416, Y = -9.4847, Z = -23.9084); Size (X = 55.8811, Y = 36.4722, Z = 62.3181)

Protein-ligand interactions were visualised and analysed using Discovery Studio Visualizer and the Protein-Ligand Interaction Profiler (PLIP) (Salentin *et al.*, 2015, Biovia, 2023).

2.3.9. Molecular Dynamic Simulations

Molecular dynamic (MD) simulations were performed on a GPU-enabled Linux (Ubuntu) workstation using the Desmond molecular dynamics simulation engine integrated into Maestro v12.2 (Schrödinger). The top-ranked ClpK:ATP complexes at the NBD1 and NBD2 sites, and apo-ClpK, were analysed in the presence of metal ions at 300 K. System setup was conducted using the System Builder module in Desmond, which solvated the complexes using the TIP3P explicit water model and applied the OPLS_2005 force field. Each system was embedded in an orthorhombic simulation box with 10 Å buffer from the solute to the box edges and box angles set at 90°. Systems were neutralized with counter ions, and physiological ionic strength was mimicked by adding 0.15 M NaCl or MgCl₂ or CaCl₂. Prior to the production run, each system underwent energy minimization followed by an eight-stage relaxation protocol. Stages 1–7 comprised the equilibration stage, while stage 8 constituted the production MD simulation (500 ns) under NPT conditions. Stage 1 involved system recognition and initialization. Brownian Dynamics simulations under NVT conditions at 10 K were performed for 100 ps (stage 2) and 12 ps (stage 3), with restraints applied to solute heavy atoms. Pocket solvation was skipped (stage 5). Stages 4, 6 and 7 were performed under NPT conditions at 10 K for 12, 12 and 24 ps, respectively. No restraints were placed on heavy atoms at stage 7. The final production stage (stage 8) was performed at 300 K.

2.3.10. Post Dynamic analysis

Post Dynamic analysis was performed using trajectories derived from the MD simulations in Schrodinger Maestro v12.2. The Simulation Quality Analysis module was employed to assess the overall simulation quality, including system stability indicators such as total energy, temperature, pressure, and volume. The Simulation Interaction Diagram tool was used to compute the root mean square deviation (RMSD) of the protein backbone alpha carbon atoms (α C), ligand RMSD relative to the receptor, and root mean square fluctuations (RMSF) of individual residues. In addition,

the Simulation Events Analysis module was used to evaluate the radius of gyration (Rg) to assess protein compactness and folding dynamics through the simulation trajectory.

2.3.11. Statistical analysis

Data was analysed using RStudio (version 2025.05.1, Build 513). The Kruskal-Wallis test was used to investigate the significant differences in RMSD and Rg values across different ClpK systems. The data did not meet the assumptions of normality; therefore, the non-parametric Kruskal-Wallis test was used for analysis (Theodorsson-Norheim, 1986). The Dunn's test with Benjamini-Hochberg correction for multiple testing was used to conduct post-hoc pairwise comparisons to control the false discovery rate (Benjamini and Hochberg, 1995). Boxplots with jitter were generated for visualization using the ggplot2 package (version 3.5.2). The rcompanion package (version 2.5.0) was used to generate compact letter displays, facilitating the clear presentation of statistically significant differences between groups following Dunn's test. All statistical tests were two-tailed, with a significance threshold set to $p < 0.05$.

2.4. Results

2.4.1. ClpK expression and purification using PEG precipitation and affinity chromatography

ClpK was overexpressed in *E. coli* using pCold1 expression vector under the conditions of 0.25 mM IPTG, at 15 °C, a 24 h induction time and yielded approximately 1.12 g/L of wet cell pellet. The SDS-PAGE analysis of the cell lysate showed a significant yield of soluble protein (~105 kDa), which was purified using a two-step purification. Resuspended pellets obtained after the addition of 2.5% (Lane 4, Figure 2-1A) and 7.5% (Lane 6, Figure 2-1A) PEG were used for affinity chromatography purification (Figure 2-1B, Figure 2-1C, Figure 2-1D). The protein concentration was estimated spectrophotometrically as ~1.29 mg/mL.

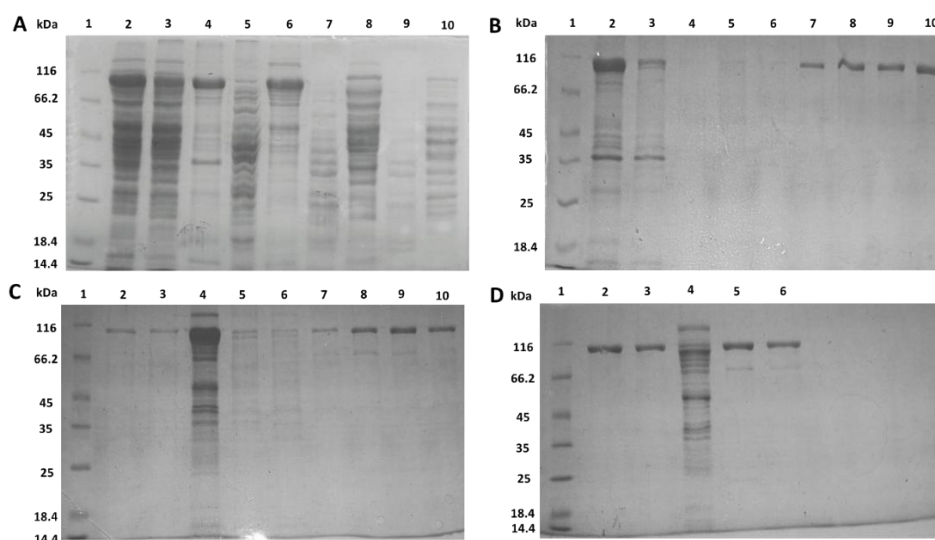


Figure 2-1: SDS-PAGE analysis of ClpK purification using PEG precipitation and affinity chromatography. A) PEG precipitation of ClpK from *E. coli* lysate. Lane 1: Molecular Weight Marker (MWM), Lane 2: Supernatant (Sn), Lane 3: 2.5% PEG Sn, Lane 4: 2.5% PEG Pellet (P), Lane 5: 7.5% PEG Sn, Lane 6: 7.5% PEG P, Lane 7: 20% PEG Sn, Lane 8: 20% PEG P, Lane 9: 30% PEG Sn, and Lane 10: 30% PEG P. B) Affinity chromatography of resuspended 2.5% PEG pellet. Lane 1: MWM, Lane 2: Resuspended 2.5% PEG P, Lane 3: Flow through, Lane 4: Unbound, Lane 5: wash with 20% elution buffer, Lane 6-10: elution with 40% elution buffer. C) Affinity chromatography of resuspended 7.5% PEG pellet. Lane 1: MWM, Lane 2: elution with 40% elution buffer, Lane 3: elution with 100% elution buffer., Lane 4: Resuspended 7.5% PEG P, Lane 5: Flow through, Lane 6: Unbound, Lane 7: wash with 20% elution buffer, Lane 8-10: elution with 40% binding buffer. D) Elution profile of affinity chromatography. Lane 1: MWM, Lane 2-3: elution with 40% binding buffer, Lane 4-6: elution with 100% binding buffer. Unstained Protein MWM (Product number: 26610). Affinity chromatography elutions were collected as 2 mL samples.

2.4.2. Enzyme activity of ClpK

The enzymatic activity of ClpK was investigated by measuring the amount of inorganic phosphate released during ATP hydrolysis (Kress *et al.*, 2009, Maurizi and Xia, 2004, Schirmer *et al.*, 1996). A phosphate standard curve was established with concentrations ranging from 0 to 40 μ M, and absorbance was measured at 620 nm (Figure 2S-1). The ATPase activity of ClpK was determined to be concentration dependent as enzymatic activity increased from 0 ± 0.048 units/L to 5.02 ± 0.102 units/L with an increase in protein concentration from 0 to 3 μ M (Figure 2-2).

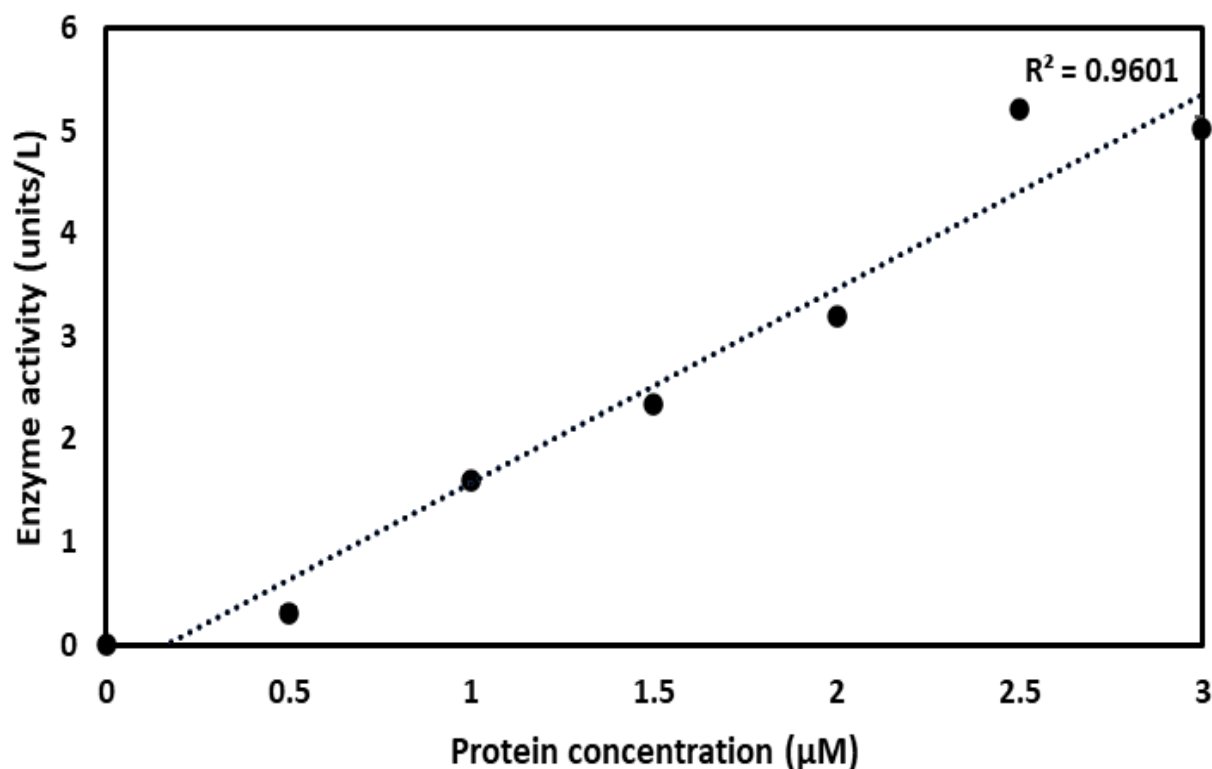


Figure 2-2: ATPase activity of ClpK at increasing concentrations. ATPase activity was determined by incubating ClpK at varying concentrations (0 – 3 μM) in Tris buffer containing 1mM ATP (pH 7.5) for 30 minutes. The release of phosphate ions was determined at 620 nm and the absorbance values were used to calculate enzyme activity. Each data point is an average of three independent measurements and error bars represent standard deviation. R^2 : 0.9601.

Kinetic parameter analysis yielded a Michaelis-Menten constant (K_M) of 0.270 mM and a maximal velocity (V_{max}) of 5.82 units/L, indicating that ClpK exhibits moderate affinity for ATP under the assay conditions. An increase in enzymatic activity was observed with an increase in ATP concentrations ranging from 0.25 mM to 1 mM (Figure 2-3), consistent with Michaelis-Menten kinetics. Moderate K_M suggests that ATP concentrations are within the physiological range found in prokaryotic cells (~ 1 to 5 mM) (Mempin *et al.*, 2013), while V_{max} further supports moderate catalytic efficiency under the assay conditions. Collectively, these findings are consistent with a classical ATP-dependant enzymatic mechanism of ClpK.

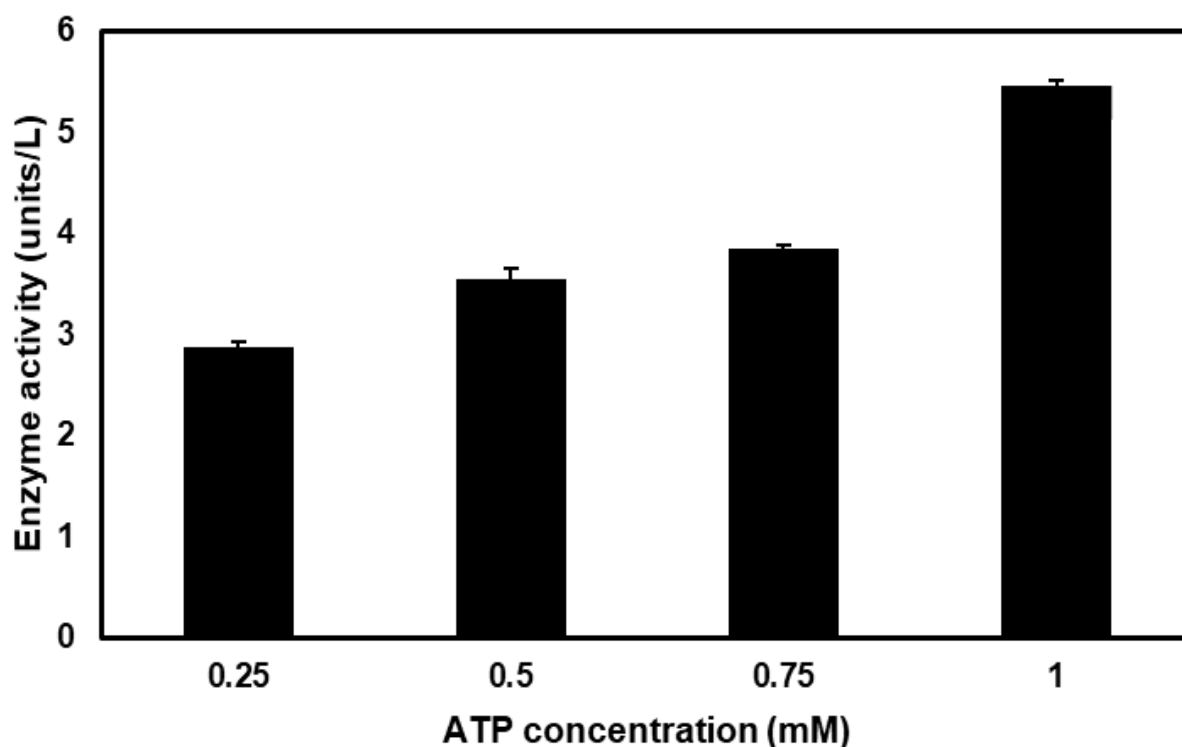


Figure 2-3: ClpK ATPase Activity at Varying ATP Concentrations. ATPase activity was determined by incubating 3 μ M ClpK with varying ATP concentrations (0.25 to 1 mM) in Tris buffer (pH 7.5) for 30 minutes. The release of phosphate ions was determined at 620 nm and the absorbance values were used to calculate enzyme activity. Each data point is an average of three independent measurements and error bars represent standard deviation.

Enzyme activity varied significantly with changes in pH (Figure 2-4). ClpK exhibited optimal activity at pH 6 (4.76 ± 0.0613 units/L) and maintained high relative activity (100 to 80.70%) from pH 6 to 10. In contrast, reduced activity was observed under acidic conditions (pH 2 to 5), with relative activity decreasing from 68.91% to 33.94% and reaching 50.16% at pH 5. This reduction in enzymatic activity at pH 5 is consistent with ClpK's isoelectric point ($pI = 5.61$), where protein solubility decreases and the likelihood of aggregation increases (Pace *et al.*, 2009). These findings suggest that ClpK functions optimally in mildly acidic to alkaline environments, consistent with its role in bacterial stress response.

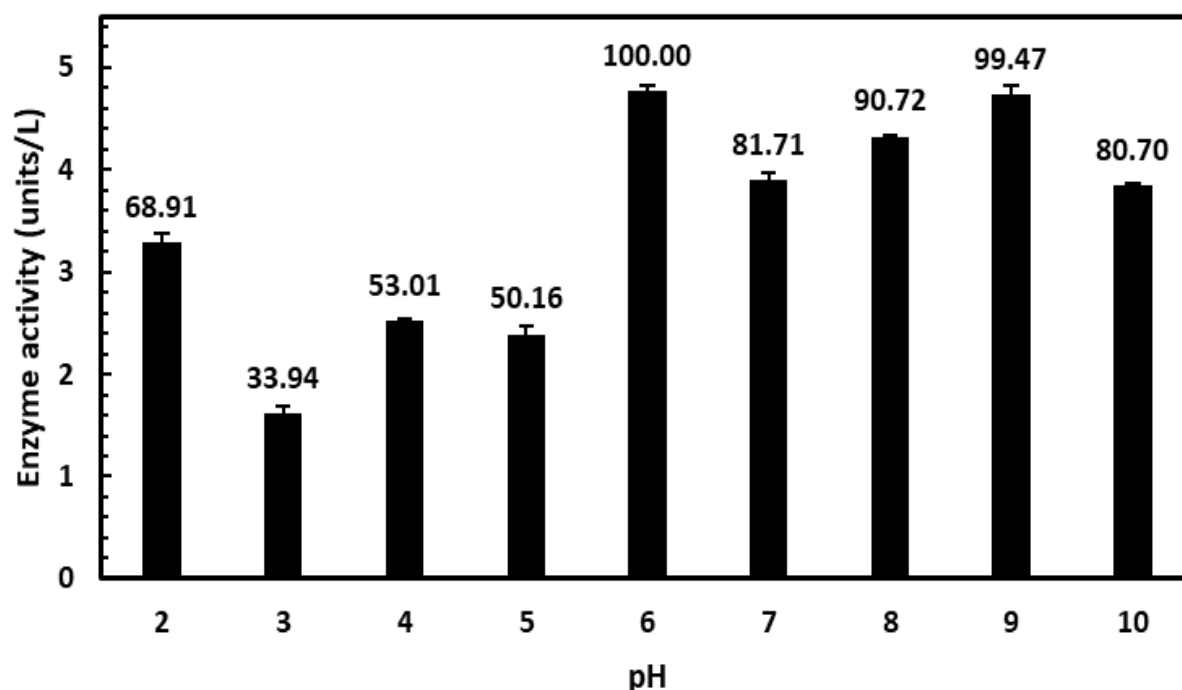


Figure 2-4: ClpK ATPase Activity measured across a range of pH values. ATPase activity was determined by incubating 3 μ M ClpK in various buffers with pH ranging from 2 to 10 with 1 mM ATP for 30 minutes. The release of phosphate ions was determined at 620 nm. Each data point is an average of three independent measurements and error bars represent standard deviation. Relative activity (%) is represented at the top of each bar.

ClpK exhibited the highest activity in the presence of Mn^{2+} (6.15 ± 0.048 units/L), indicating its potential as an effective cofactor (Figure 2-5). Mg^{2+} supported moderate enzymatic activity (5.19 ± 0.054 units/L), whereas the lowest enzymatic activity was observed with Ca^{2+} (4.19 ± 0.038 units/L). Interestingly, the control (no metal ions added) displayed slightly higher activity (4.27 ± 0.025 units/L) than that observed in the presence of Ca^{2+} , indicating that Ca^{2+} may exert an inhibitory effect on ClpK function. These findings highlight the importance of divalent metal ions in modulating ClpK activity, with Mg^{2+} and Mn^{2+} acting as essential cofactors for optimal catalytic activity.

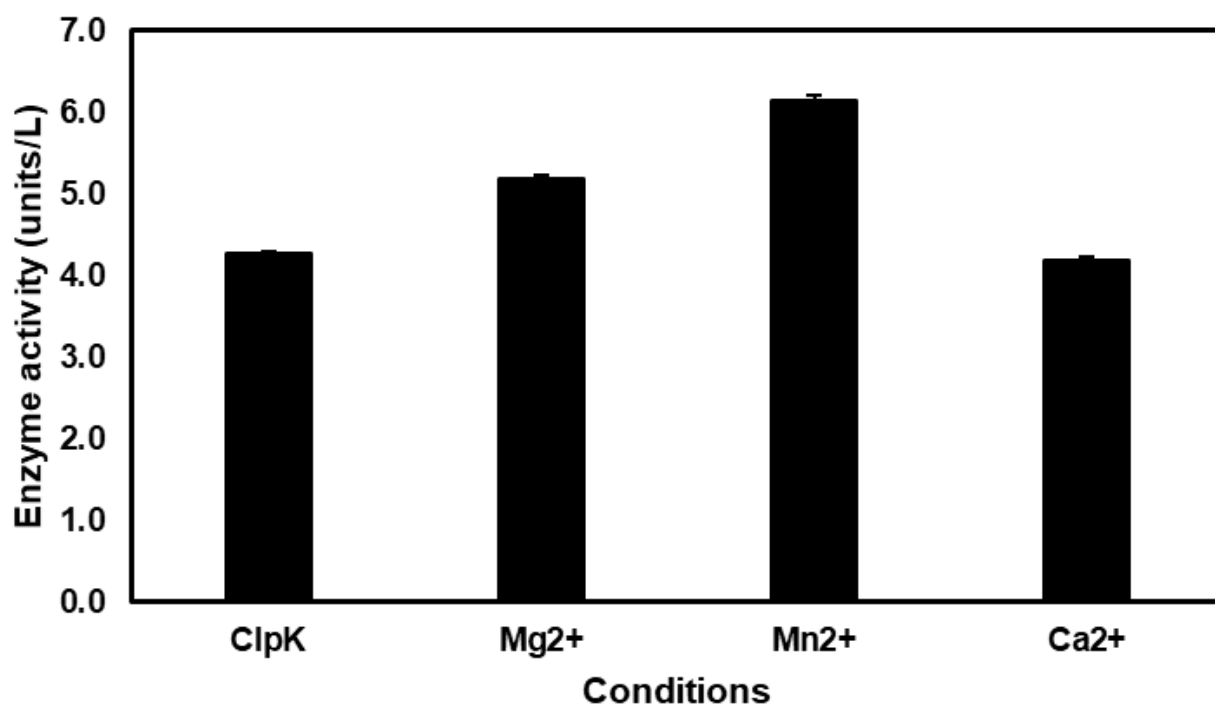


Figure 2-5: ClpK ATPase Activity measured in the presence of metal ions. ATPase activity was determined by incubating 3 μ M ClpK with 1 mM ATP and 5 mM metal ions (Mg^{2+} , Mn^{2+} , and Ca^{2+}) for 30 minutes. The release of phosphate ions was determined at 620 nm and the absorbance values were used to calculate enzyme activity. Each data point is an average of three independent measurements and error bars represent standard deviation.

2.4.3. Spectroscopical Analysis

2.4.3.1. Far UV-CD spectroscopy

The secondary structure of ClpK was investigated using CD spectroscopy in the presence and absence of ATP and divalent cations (Figure 2-6). The CD spectrum of ClpK alone exhibited a characteristic α -helical profile with a maximum at ~ 190 nm and negative minima at around 205 and 220 nm (Figure 2-6A). Upon ATP binding, an increase in ellipticity was observed at both minima, suggesting enhanced helical content or protein stabilisation (Figure 2-6A). In the presence of Mg^{2+} and Ca^{2+} , minimal spectral changes were observed (Figure 2-6B, Figure 2-6D). However, Mn^{2+} induced a marked spectral shift especially around 205 nm (Figure 2-6C), suggesting a substantial conformational rearrangement (Figure 2-6C). Quantitative analysis (Figure 2-6E) showed that ClpK alone exhibited 26.15% α -helical content, which increased with ATP (34.25%) and Mg^{2+} (28.7%), while Mn^{2+} reduced α -helical content to 17.1% and increased disorder to 43.9%. ATP with Mn^{2+} further reduced α -helicity to 14.25% and increased disorder (49.2%), indicating major conformational

rearrangement. These results suggest that ATP stabilises ClpK through increased α -helicity, while Mn^{2+} induces conformational flexibility, aligning with enhanced enzymatic activity observed in Figure 2-5.

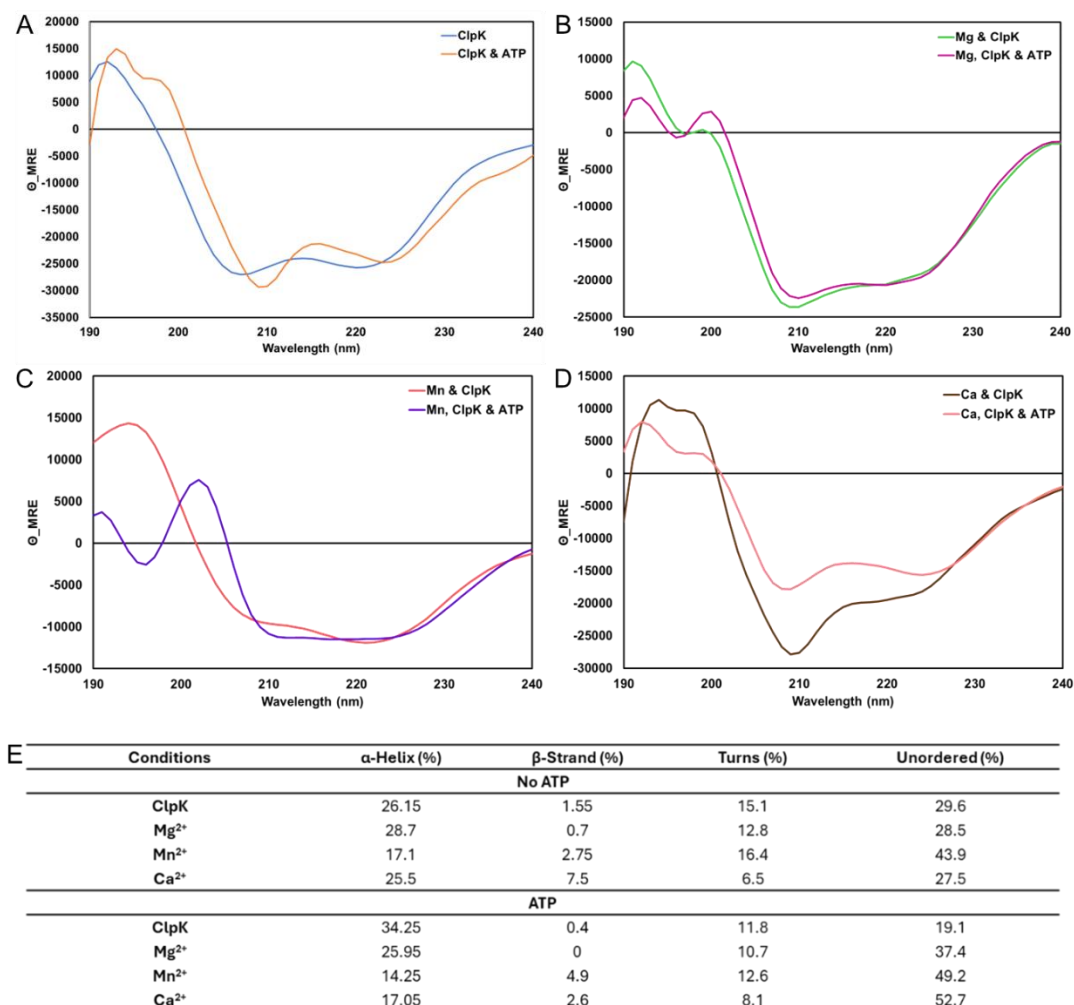


Figure 2-6: Far-UV CD Analysis of ClpK in the Presence and Absence of ATP and Divalent Cations. A) ClpK in the absence (blue) and presence of ATP (orange). B) ClpK in the presence of Mg^{2+} with (purple) and without ATP (green). C) ClpK in the presence of Mn^{2+} with (red) and without ATP (blue). D) ClpK in the presence of Ca^{2+} with (brown) and without ATP (pink). Each spectrum represents an average of seven accumulated spectra derived at 20°C using a 1 mm path length quartz cuvette. E) Influence of divalent cations on the secondary structural elements of ClpK in the presence and absence of ATP as predicted using the DichroWeb server.

ClpK was substantially unfolded in the presence of 8 M urea, with helical content decreasing from 26.15% to 2.7% (Figure 2-6E, Figure 2S-2-E). ATP provided a modest stabilising effect (Figure 2S-2-A) increasing α -helicity to 3.15% under denaturing conditions. A complete loss of defined minima was observed in the presence of Mg^{2+} or Ca^{2+} (Figure 2S-2-B, 2S-2-D), suggesting that these ions offered no protection against urea-induced unfolding. In contrast, distinguishable features

observed between 210 and 230 nm suggest that Mn^{2+} assists in retaining protein secondary structure or promotes an alternative conformation under denaturing conditions (Figure 2S-2-C).

2.4.3.2. Tryptophan Fluorescence

In the native state, ClpK exhibited strong intrinsic fluorescence, which significantly decreased upon denaturation, indicating the loss of tertiary structure (Figure 2-7A). ATP binding under native conditions resulted in a moderate decrease in fluorescence intensity, suggesting subtle conformational changes. As shown in Figure 2-7A and 2-7B, the fluorescence spectra of ClpK alone, or in the presence of ATP or Mg^{2+} were similar. However, the ClpK–ATP– Mg^{2+} complex showed a decrease in fluorescence, implying conformational changes due to Mg^{2+} -mediated coordination at the active site. In contrast, Mg^{2+} increased fluorescence intensity under denaturing conditions, suggesting that ClpK adopts a partially unfolded or intermediate state with exposed tryptophans. In the presence of Mn^{2+} (Figure 2-7C), ATP reduces fluorescence in the native state, consistent with conformational change. Upon denaturation, the combination of Mn^{2+} and ATP markedly increased fluorescence intensity suggesting tryptophan exposure in a partially unfolded or intermediate state. In the presence of Ca^{2+} (Figure 2-7D), ATP addition decreased fluorescence, indicating subtle conformational changes. However, fluorescence intensity decreased in the presence and absence of ATP under denaturing conditions, indicating unfolding and tryptophan exposure. These results demonstrate that ATP and divalent cations modulate the structural dynamics of ClpK. Mg^{2+} moderately stabilises ClpK, while Mn^{2+} induced pronounced conformational changes, especially in the presence of ATP, highlighting its significant role in altering the ClpK tertiary structure.

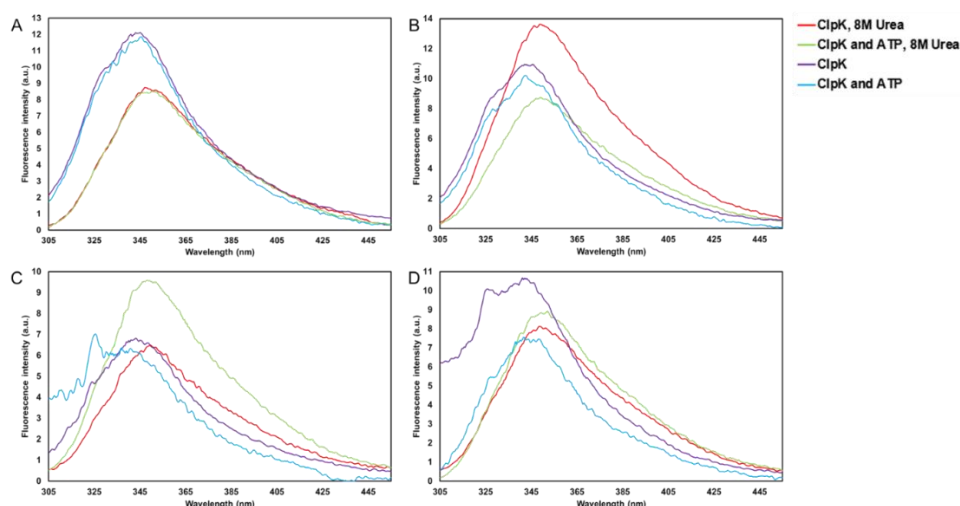


Figure 2-7: Intrinsic fluorescence spectroscopy of ClpK in the presence and absence of ATP and divalent cations. A) ClpK alone. B) ClpK in the presence of Mg^{2+} . C) ClpK in the presence of Mn^{2+} . D) ClpK in the presence of Ca^{2+} .

2.4.3.3. ANS Fluorescence Spectroscopy

ANS fluorescence showed that ATP binding to ClpK resulted in a clear hypsochromic shift of emission maxima accompanied by a reduction in fluorescent intensity (Figure 2-8), indicating conformational tightening that reduces ANS-accessibility to hydrophobic surfaces. The binding of divalent cations to ClpK resulted in increased ANS fluorescence, suggesting that Mg^{2+} , Mn^{2+} , and Ca^{2+} induce conformational changes that expose hydrophobic regions of ClpK. The combination of Mg^{2+} or Mn^{2+} with ATP resulted in higher fluorescence intensity and a more pronounced hypsochromic shift (Figure 2-8B, 2-8C), highlighting their role in ATP-dependant structural rearrangement. In contrast, minor differences in fluorescence were observed in the presence of Ca^{2+} in the ATP-bound and unbound forms (Figure 2-8D), implying possible interference with ATP binding by occupying ATP-binding sites. Elevated fluorescence intensity in the presence of Ca^{2+} indicated conformational changes resulting in the exposure of hydrophobic residues. Overall, these results indicate that ATP and divalent cations modulate ClpK structure, with Mg^{2+} and Mn^{2+} promoting ATP-dependent conformational tightening, whereas Ca^{2+} induces partial structural destabilisation. Similar observations were made in the presence of Mant-ATP (data not shown).

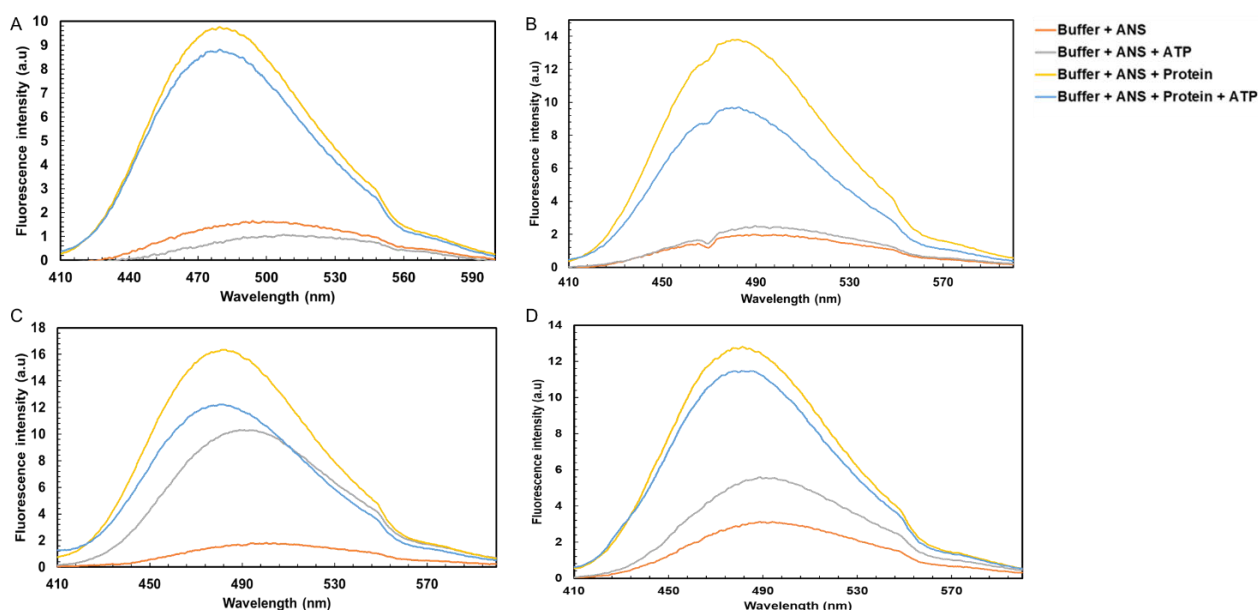


Figure 2-8: Tertiary structure analysis of ClpK in the presence and absence of ATP and divalent cations using ANS fluorescence spectroscopy. A) ClpK alone. B) ClpK in the presence of Mg^{2+} . C) ClpK in the presence of Mn^{2+} . D) ClpK in the presence of Ca^{2+} .

2.4.4. Thermal shift analysis

Thermal shift assays (TSA) using SYPRO orange were used to evaluate the thermal stability of ClpK. A clear, well-defined melting peak was obtained using 10X dye and 10 ng/ μ L ClpK (Figure 2S-3 and 2S-4). ClpK stability was evaluated under various conditions, including different pH levels, varying NaCl concentrations and in the presence of ATP and divalent cations. ClpK exhibited high thermal stability and defined melting transitions from pH 6 to 10, indicating that ClpK maintains structural integrity in neutral to alkaline environments (Figure 2-9A). These findings align with enzymatic activity profiles, supporting the functional relevance of ClpK's stability under physiological pH. The lowest melting temperature (T_m) was observed at pH 5 (56.8 ± 0.7 °C), which correlated closely to ClpK's isoelectric point ($pI = 5.61$), where reduced solubility and increased aggregation are expected. A concentration dependent increase in thermal stability was observed with increasing NaCl concentrations: the T_m increased from 62.1 ± 0.1 °C at 0 M NaCl to 66.4 ± 0.1 °C 1 M NaCl (Figure 2-9B). This indicates that ClpK maintains structural integrity under physiologically relevant ionic strengths, supporting its functional stability in diverse cellular environments. ATP alone moderately increased melting temperature from 62.1 ± 0.1 °C (apo-ClpK) to 62.8 ± 0.2 °C, indicating mild structural stabilisation. In contrast, divalent cations (Mg^{2+} , Mn^{2+} , and Ca^{2+}) alone slightly decreased the T_m , suggesting a minor destabilising

effect. However, when combined with ATP, these cations increased T_m to ~63 °C, indicating a cooperative stabilizing effect (Figure 2-9C). These findings suggest that while divalent cations alone may perturb ClpK structure, their presence alongside ATP enhances conformational stability, potentially reflecting a more physiologically relevant, catalytically competent state.

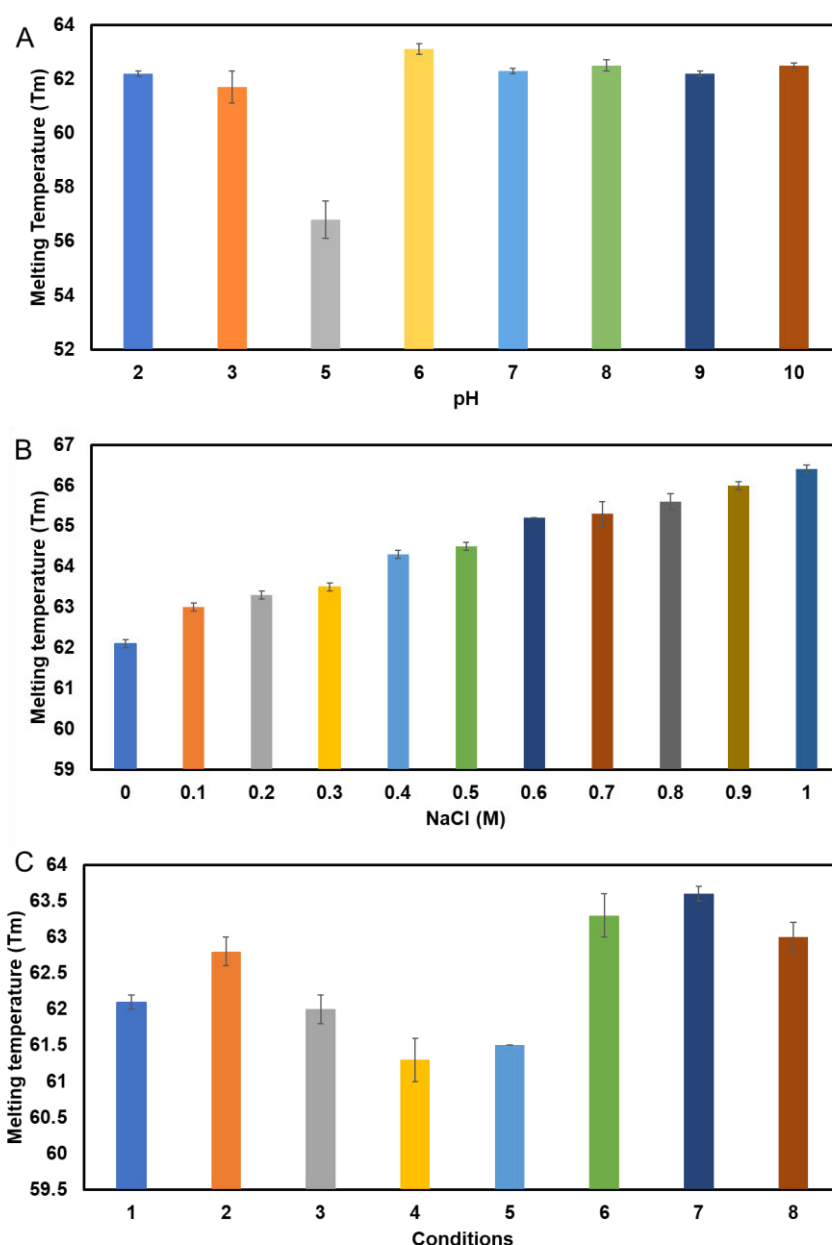


Figure 2-9: Melting temperature analysis of ClpK using thermal shift assay. A) Effect of varying pH levels. B) Effects of varying NaCl concentrations. C) Thermal stability of ClpK in the presence of ATP and divalent cations. Conditions: 1- ClpK, 2- ClpK & ATP, 3- ClpK and Mg^{2+} , 4- ClpK and Mn^{2+} , 5- ClpK and Ca^{2+} , 6- ClpK, ATP and Mg^{2+} , 7- ClpK, ATP and Mn^{2+} , and 8- ClpK, ATP and Ca^{2+} . Melting temperatures were extrapolated from the first derivative of the fluorescence curves $d(\text{Fluorescence})/dT$ and are presented with standard deviation error bars. All experiments were performed in triplicates and average melting temperatures were plotted against temperature (°C).

2.4.5. ClpK Homology Modelling

The 3D structure of ClpK was predicted using the AlphaFold server and refined with Galaxy Refine (Figure 2-10). Model validation showed a Rama Z-score of 0.53 ± 0.23 , ERRAT overall quality factor of 95.02, ProSA Z-score of -13.52, and MolProbity score of 1.32, indicating high structural reliability. Additionally, 98.53% of residues were in favourable regions of the Ramachandran plot (Figure 2-10A). Collectively, these results confirm that the ClpK model is of high quality and suitable for downstream *in silico* analysis.

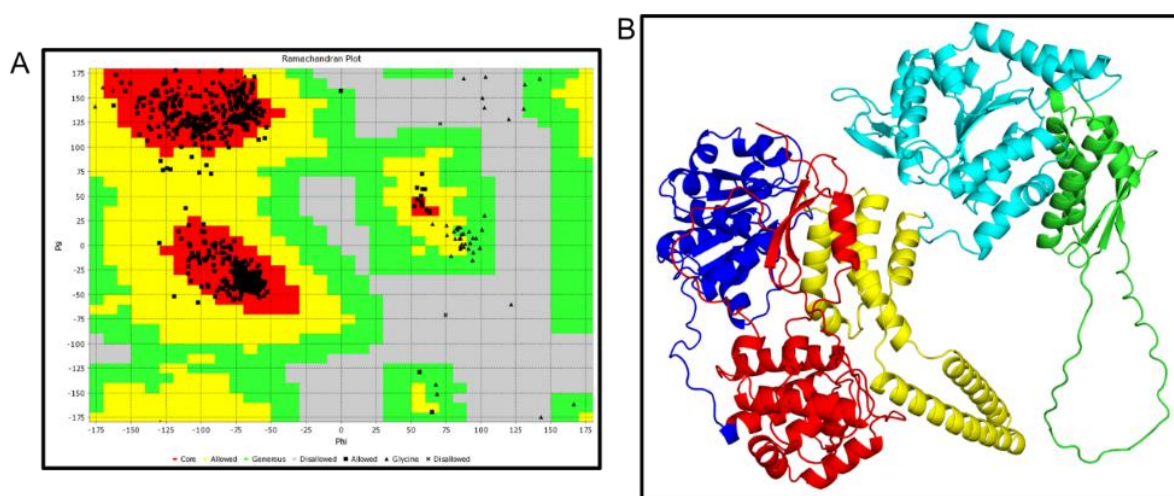


Figure 2-10: ClpK structure modelled using the AlphaFold server and refined using the Galaxy Refine server. A) Ramachandran plot obtained from the VADAR v1.8 server. Core regions (red), allowed regions (yellow), generous regions (green), and disallowed regions (grey). B) Five domains: N-terminal domain (residues 1–244; red); Nucleotide Binding Domain (NBD) 1 (residues 245–445; blue); the D1-small domain (residues 446–590; yellow); NBD2 (residues 591–814; cyan); and the D2-small domain/ C-terminal domain (residues 815–952; green) coloured as per Lee *et al.* (2003) and Motiwala *et al.* (2021).

2.4.6. ClpK-ATP molecular docking

Molecular docking was used to investigate ATP binding to ClpK's two NBDs (NBD1: residues 302 – 449, NBD2: 642 – 785) using Autodock Vina (Figure 2-11). Predicted binding energies were -7.6 (NBD1) and -8.8 kcal/mol (NBD2), indicating strong binding affinity. Interactions included carbon hydrogen bonds, van der Waal interactions, conventional hydrogen bonds, and pi-alkyl bonds (Figure 2-11), collectively supporting stable ATP positioning within the binding pockets.

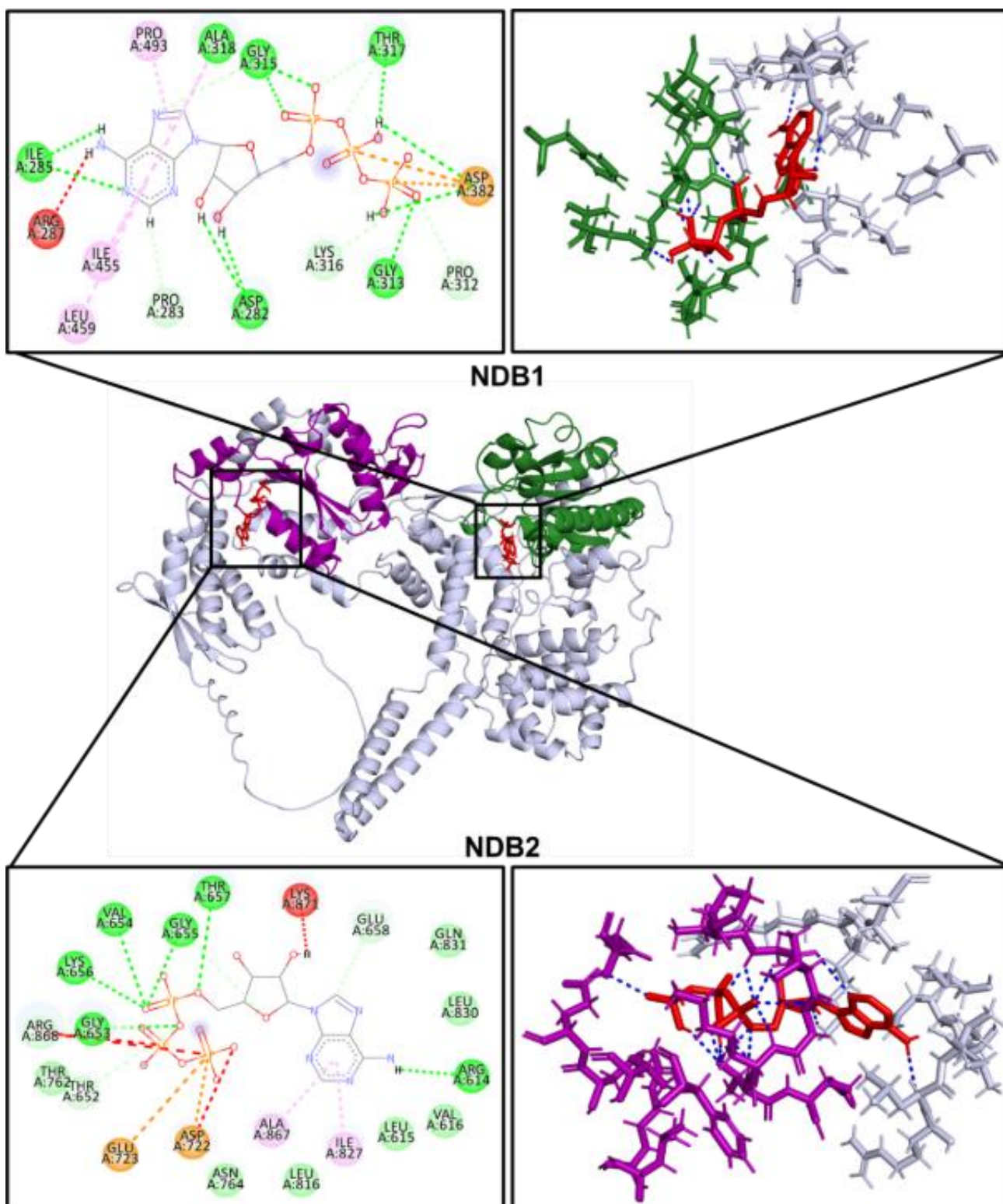


Figure 2-11: Binding sites of ATP on ClpK. Nucleotide-binding domain (NDB) 1 is coloured green and nucleotide-binding domain 2 is coloured purple. ATP is represented in red. The 2D interaction diagram was obtained from the BIOVIA Discovery Studio Visualiser. Light green represents carbon hydrogen bonds, medium green represents van der Waal interactions, bright green represents conventional hydrogen bonds, orange represents attractive charges, light purple represents Pi-Alkyl bonds and red represents unfavourable bonds.

2.4.7. ClpK-ATP molecular simulation analysis

Molecular dynamics simulations were conducted to evaluate the effect of ATP and divalent cation (Ca^{2+} and Mg^{2+}) binding on ClpK stability. Unbound ClpK exhibited a potential energy of ~ -543365 kcal/mol, while binding of Ca^{2+} and Mg^{2+} lowered potential energies, with the ClpK- Mg^{2+} complex reaching ~ -606887 kcal/mol, indicating stronger stabilisation by Mg^{2+} . ATP bound ClpK showed similar potential energies to the unbound state; however, in the presence of Mg^{2+} , ATP-bound complexes stabilised further to ~ -606886.463 kcal/mol and ~ -607072.587 kcal/mol, for NBD1 and NBD2, respectively (Table 2-1). These results suggest that Mg^{2+} stabilises ClpK in both the ATP-bound and unbound states.

Table 2-1: Total and Potential Energy Profiles of ClpK in the absence and presence of ATP and metal ions (Ca^{2+} and Mg^{2+}). Values represent mean energy $\pm$ standard deviation (kcal/mol).

Structure	Total energy	Potential energy
ClpK alone		
ClpK	-445044.131 ± 176.574	-543364.807 ± 152.251
Ca^{2+}	-496551.290 ± 173.349	-594601.894 ± 148.876
Mg^{2+}	-508579.133 ± 175.055	-606886.897 ± 151.095
ATP bound to NBD1		
ClpK	-445017.992 ± 178.082	-543355.043 ± 154.582
Ca^{2+}	-496542.007 ± 176.867	-594609.684 ± 153.272
Mg^{2+}	-508563.359 ± 178.594	-606886.463 ± 155.038
ATP bound to NBD2		
ClpK	-445140.455 ± 183.571	-543503.508 ± 160.530
Ca^{2+}	-496664.733 ± 176.270	-594753.418 ± 152.447
Mg^{2+}	-508712.148 ± 176.391	-607072.587 ± 152.206
ATP bound to both NBD1 and NBD2		
ClpK	-445106.233 ± 180.073	-543476.337 ± 156.677
Ca^{2+}	-496587.563 ± 174.424	-594692.419 ± 149.953
Mg^{2+}	-508614.492 ± 180.652	-606974.830 ± 156.875

Protein compactness was assessed by analysing radius of gyration (R_g) over 500 ns (Figure 2-12). Apo-ClpK showed stable R_g values (58.83 ± 0.22 Å), while Ca^{2+} and Mg^{2+} ions resulted in a slight reduction of the R_g values to 58.72 ± 0.22 Å and 58.76 ± 0.22 Å, indicating increased structural compactness. ATP binding induced minor structural changes, while ClpK- Mg^{2+} showed a more compact conformation in

comparison to ClpK- Ca^{2+} . Kruskal-Wallis analysis confirmed significant differences in Rg distributions across ClpK systems (Kruskal-Wallis chi-squared = 70.85, $p < 0.0001$), with Dunn's post-hoc analysis showing significant differences especially in the presence of ATP and Mg^{2+} systems compared to the ATP-bound and apo forms. These findings indicate that ATP and Mg^{2+} impact ClpK conformational stability.

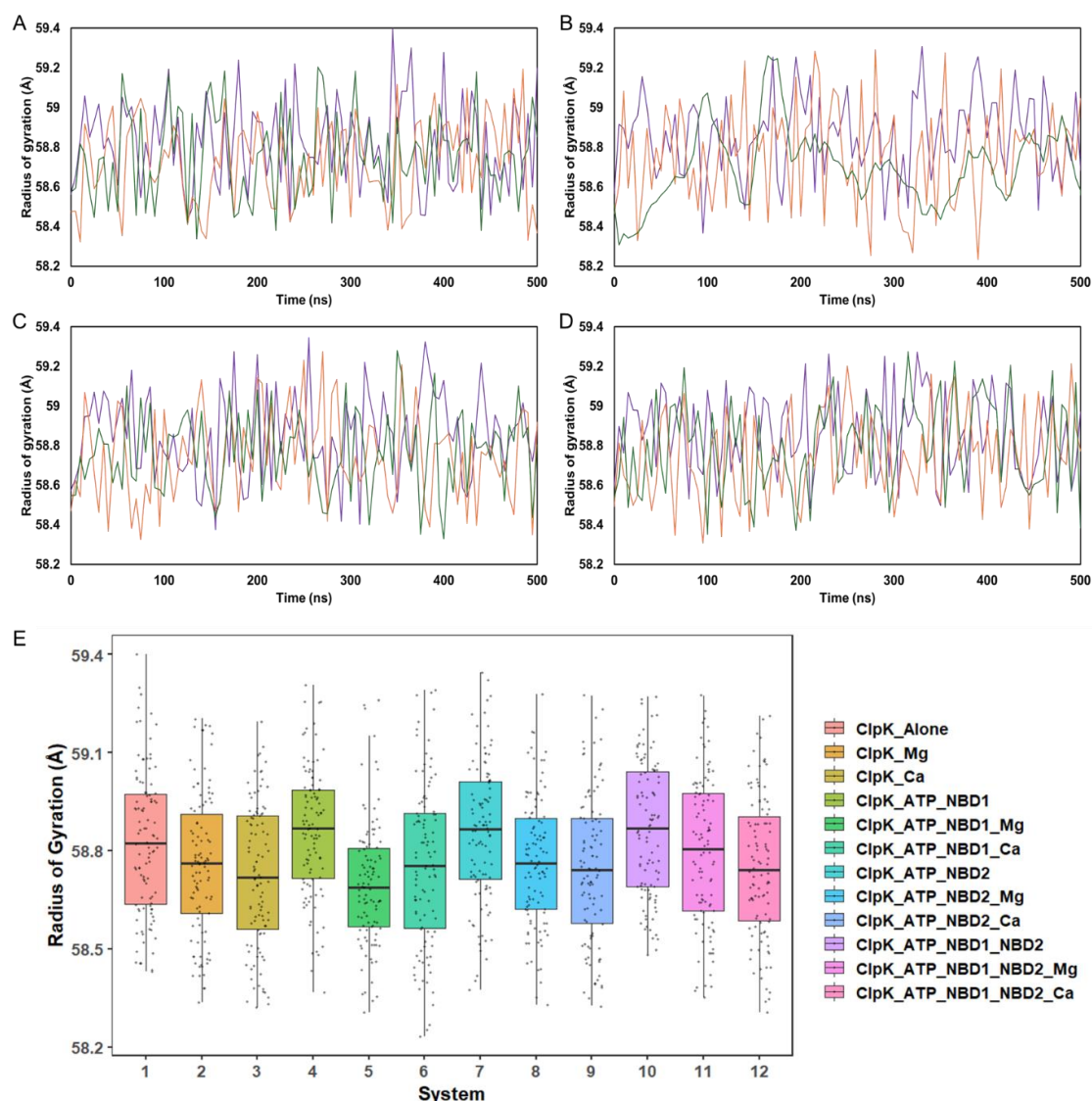


Figure 2-12: Trajectory analysis of the Rg values of ClpK-apo and ClpK-ATP complex in the presence of metal ions (Ca^{2+} and Mg^{2+}). ClpK in the absence of metal ions (purple), ClpK in the presence of Ca^{2+} (orange), and ClpK in the presence of Mg^{2+} (green). A) ClpK-apo in the presence and absence of metal ions. B) ClpK with ATP bound to NDB1. C) ClpK with ATP bound to NDB2. D) ClpK with ATP bound to NDB1 and NDB2. E) Boxplot illustrating the distribution of Rg across systems.

RMSD and RMSF analyses were performed to investigate the stability of ClpK under varying conditions. Apo-ClpK showed high RMSD fluctuations (11.85 ± 1.91 Å), indicating structural instability (Figure 2-13). RMSD decreased in the presence of Ca^{2+}

(9.91 ± 1.09 Å), while it increased slightly in the presence of Mg^{2+} (13.09 ± 1.89 Å). ATP binding to NBD1 alone resulted in high RMSD (13.31 ± 2.28 Å), which decreased in the presence of Mg^{2+} (12.01 ± 1.71 Å) but remained elevated in the presence of Ca^{2+} (12.76 ± 2.32 Å) (Figure 2-13B). In addition, ATP binding to NBD2 alone showed high RMSD (13.11 ± 3.20 Å), stabilising upon addition of Ca^{2+} (10.70 ± 1.55 Å) and Mg^{2+} (11.31 ± 1.92 Å) (Figure 2-13C). ATP binding to NBD1 and NBD2 led to lower, stabilised RMSD (10.30 ± 1.52 Å), indicating intrinsic stability that reduced ion-dependent effects (Figure 2-13D). Kruskal-Wallis analysis confirmed significant RMSD distributions across ClpK systems (Kruskal-Wallis chi-squared = 466.48, $p < 0.0001$), with Dunn's post-hoc tests identifying multiple significant differences in RMSD distributions (Figure 2-13E). These results indicate that ATP binding and divalent cations influence ClpK conformational stability during simulation.

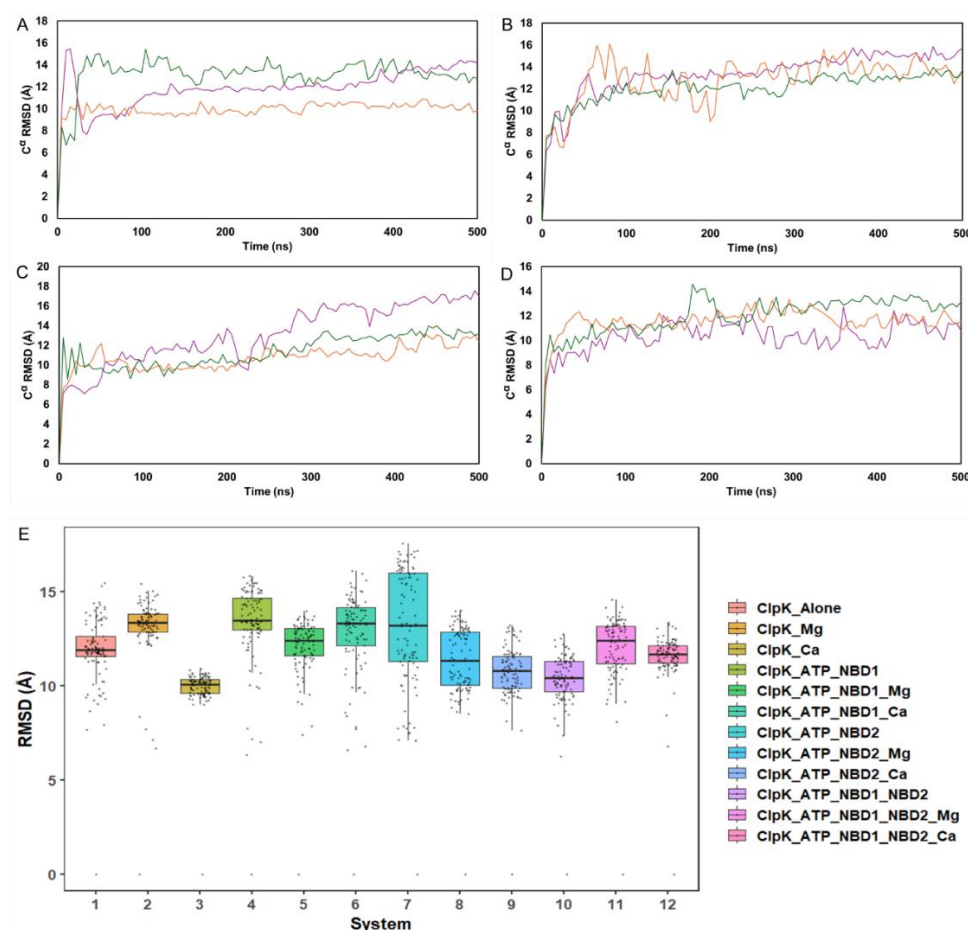


Figure 2-13: Trajectory analysis of the RMSD values of ClpK-apo and ClpK-ATP complex in the presence of metal ions (Ca^{2+} and Mg^{2+}). ClpK in the absence of metal ions (purple), ClpK in the presence of Ca^{2+} (orange), and ClpK in the presence of Mg^{2+} (green). A) ClpK-apo in the presence and absence of metal ions. B) ClpK with ATP bound to NBD1. C) ClpK with ATP bound to NBD2. D) ClpK with ATP bound to NBD1 and NBD2. E) Boxplot illustrating the distribution of RMSD across systems.

Residue flexibility was investigated using RMSF analysis (Figure 2-14). Apo-ClpK showed the highest fluctuations ($4.74 \pm 3.12 \text{ \AA}$), particularly residues 50 to 100 (N-terminal domain), 500 to 600 (D1 small domain and partially NDB2) and 800 to 950 (C-terminal domain). Fluctuations decreased in the presence of divalent cations, with Ca^{2+} providing greater stabilisation than Mg^{2+} (Figure 2-14A), consistent with RMSD observations. When ATP was bound to NBD1, fluctuations were suppressed in the absence of ions, however Ca^{2+} increased fluctuations particularly in the C-terminal domains (Figure 2-14B). When ATP was bound to NBD2, fluctuations increased towards the C-terminal domain in the absence of ions, aligning with observed RMSD results indicating destabilisation (Figure 2-14C). Dual ATP binding to NBD1 and NBD2 resulted in lower RMSF compared to the apo and single-site ATP-bound forms (Figure 2-14D). Indicating that dual ATP binding results in greater structural stability compared to either single-site binding or apo form, regardless of metal ion presence.

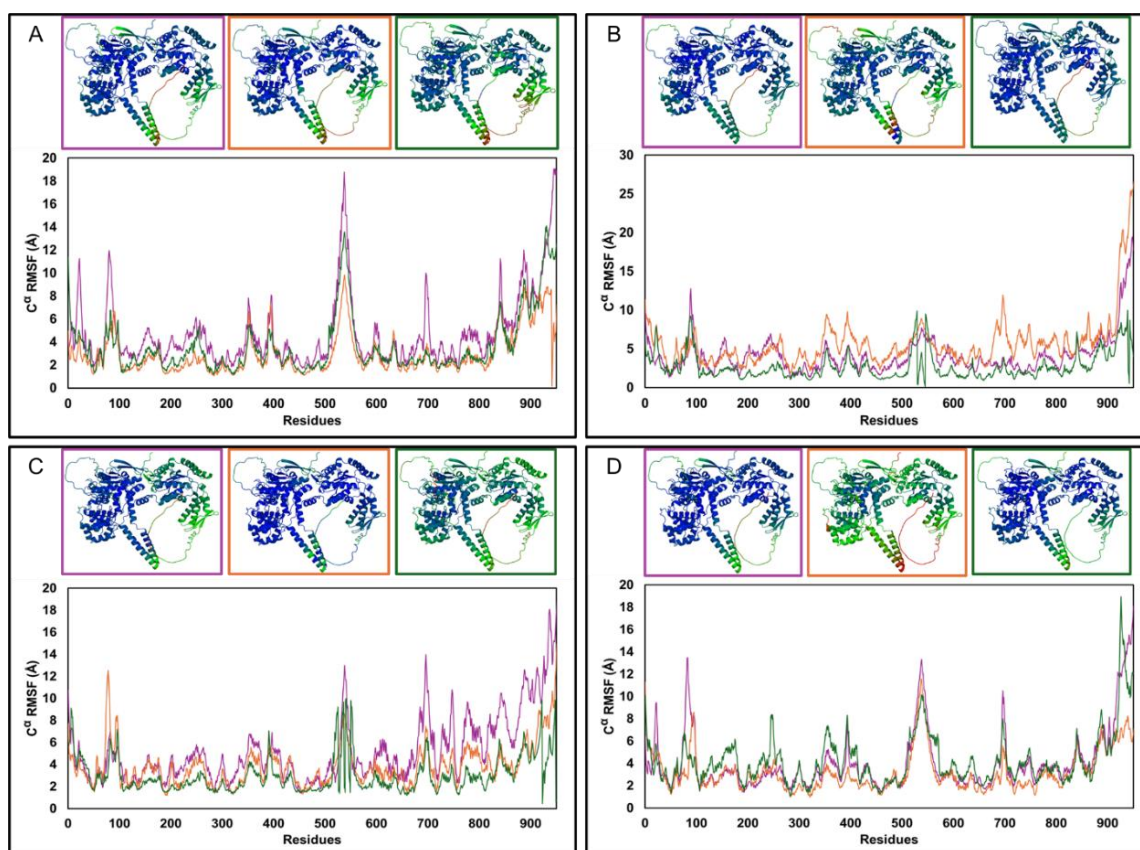


Figure 2-14: Trajectory analysis of the RMSF values of ClpK-apo and ClpK-ATP complex in the presence of metal ions (Ca^{2+} and Mg^{2+}). ClpK in the absence of metal ions (purple), ClpK in the presence of Ca^{2+} (orange), and ClpK in the presence of Mg^{2+} (green). A) ClpK-apo in the presence and absence of metal ions. B) ClpK with ATP bound to NBD1. C) ClpK with ATP bound to NBD2. D) ClpK with ATP bound to NBD1 and NBD2. The structures of ClpK were coloured as per RMSF using PyMol using a blue-green-red (minimum to maximum) colour scheme.

2.5. Discussion

Klebsiella pneumoniae is a critical priority pathogen responsible for severe, multidrug-resistant infections worldwide, contributing to high mortality and morbidity rates (Tacconelli *et al.*, 2018, Martin and Bachman, 2018). It poses a major challenge to public health and exerts a significant burden on healthcare funding due to the excessive use of healthcare systems and resources (De Kraker *et al.*, 2016). To address this challenge, it is important to identify the biophysical characteristics of key virulence and survival mechanisms to unlock novel applications and inform the rational design of inhibitors with therapeutic potential. This study focused on the experimental and computational exploration of the role of divalent cations in the structural and functional regulation of *K. pneumoniae* ClpK, an ATPase involved in thermotolerance and stress adaptation.

ClpK displayed protein concentration-dependent ATPase activity (Figure 2-2) with moderate ATP affinity (Michaelis constant (K_M) = 0.270 mM) indicative of optimal function within the physiological ATP concentration range (0.5 to 5 mM) typical of cellular environments (Huang *et al.*, 2010). Interestingly, ClpK retained activity across a broad pH range and demonstrated tolerance to mildly acidic environments (Figure 2-4). This suggests that ClpK can adapt to acidic environments and survive metabolic acidosis that occurs upon host infection or stress exposure (Torres *et al.*, 2017). Similarly, ClpB from *Corynebacterium crenatum* demonstrated optimal activity at mildly acidic pH suggesting that Clp ATPases can persist at acidic pH ranges (Huang *et al.*, 2020). Although ClpK may be active in acidic environments, thermal stability data suggests that the ClpK structure is compromised under such conditions, as indicated by the decreased thermal stability observed in the acidic pH range (Figure 2-9A). A comparable observation was reported for ClpC1 in *Mycobacterium tuberculosis* where ATPase activity fluctuated with environmental pH and temperature suggesting that conformational changes occurred under non-optimal conditions (Kar *et al.*, 2008). Moreover, the thermal stability of ClpK increased in the presence of increasing salt concentrations, highlighting the importance of electrostatic interactions in maintaining protein thermal stability (Figure 2-9B). Salt ions can shield repulsive charges on the protein surface, thereby reducing unfavourable electrostatic

interactions and promoting a more stable, folded conformation (Kumar and Nussinov, 2002).

A major focus of this study was to investigate the effect of divalent cations (Mg^{2+} , Mn^{2+} and Ca^{2+}) and ATP in modulating ClpK structure and stability. Metal ions enhance protein structural stability in the conformation required for biological function and play an important role in the catalytic processes of enzymes (Glusker, 1991). The baseline melting temperature of ClpK (~62 °C) increased slightly in the presence of ATP and further in the presence of both ATP and metal ions (Figure 2-9), suggesting a cooperative stabilizing effect. Structural analysis (Figure 2-6 and 2-10) confirmed that ClpK is mainly α -helical, with ATP binding increasing helicity, further supporting the role of ATP promoting structural stability. ANS-binding data further revealed that ATP induced subtle conformational rearrangements, which may reflect domain closure or functional activation (Figure 2-7). This is consistent with the behaviour of related Clp ATPases such as ClpX which uses energy from the hydrolysis of ATP to drive conformational changes that unfold and translocate target proteins into the ClpP peptidase for degradation (Stinson *et al.*, 2013). The presence of Mg^{2+} and Mn^{2+} enhanced ATP-induced changes indicating that these divalent cations stabilise the ATP-bound state of ClpK. The role of Mg^{2+} in supporting ATP-binding to ClpK was further enforced through Mant-ATP fluorescence experiments. In the presence of ATP, fluorescence intensity of tryptophan decreases, once again indicating domain closure or functional activation as observed in the ANS-binding study. The protective effects of Mg^{2+} and Mn^{2+} observed in the presence of urea suggest that these ions may help preserve the functional conformation of ClpK under cellular stress. This is important given the role of ClpK in survival under stressful environmental conditions (Bojer *et al.*, 2013, Bojer *et al.*, 2010). In contrast, Ca^{2+} had a less pronounced effect on ClpK stability, highlighting the ion-specific nature of these interactions.

Furthermore, the interaction of ATP with ClpK was investigated using molecular docking, a computational approach that allows for the investigation of protein-ligand interactions and binding mode prediction (Rao *et al.*, 2021). Docking results showed that ATP binding to both nucleotide binding domains provided the greatest structural stability in comparison to ATP bound to a single NBD or ClpK-apo (Figure 2-11). This highlights the cooperative nature of ClpK-ATP interactions. Additionally, MD

simulations showed that Mg^{2+} ions enhanced ClpK stability as observed by the lower potential energies, reduced RMSD and RMSF values, and compact radius of gyration across simulations (Table 1, Figure 2-12 – 2-14). This suggests that Mg^{2+} ions stabilise ClpK in the ATP bound and unbound form. In cells, Mg^{2+} ions are the most abundant divalent cations and form complexes with phosphate-containing species such as ATP (Sissi and Palumbo, 2009). The stabilisation of apo-ClpK in the presence of Mg^{2+} suggests that these ions can bind to ClpK even in the absence of ATP, potentially interacting with the Walker B motif, which is known to coordinate metal ions (Motiwala *et al.*, 2021).

ATPase activity was enhanced in the presence of Mn^{2+} (Figure 2-5), highlighting a potential co-factor dependency that influences ClpK activation during stress response. Studies have shown that Mn^{2+} increases chaperonin-assisted protein folding efficiency in GroES and enhances the chaperonin activity of ClpL (Diamant *et al.*, 1995, Park *et al.*, 2015). In contrast, major conformational changes and disorder were observed in the presence of Mn^{2+} suggesting that Mn^{2+} preserves protein secondary structure or promotes an alternative conformation under denaturing conditions (Figure 2-6 – 2-8). For example, a study on *Saccharomyces cerevisiae* phosphoenolpyruvate carboxykinase (PEPCK) showed that Mn^{2+} conferred significant protection against urea-induced denaturation suggesting that Mn^{2+} may stabilize protein conformation and make it more resistant to urea-induced unfolding (Encinas *et al.*, 2002). There was no significant structural impact in the presence of Mg^{2+} , the secondary structure was preserved, and ATP binding was supported, reinforcing the physiological relevance of the cations. Notably, the identification of metal ion-specific effects on the conformation and activity of ClpK has important implications for targeting bacterial stress-response pathways. This could potentially reveal vulnerability of ClpK that could be exploited in anti-virulence or antibiotic potentiation strategies.

This study provides a foundation for future studies that could combine kinetic, mutational, and structural approaches to clarify the precise mechanisms by which divalent cations affect ClpK's activity and regulation. Additional work should focus on validating these findings *in vivo*, especially under infection-mimicking conditions, and exploring the impact of ClpK mutations on metal coordination and ATPase function. Structure-guided drug discovery targeting ClpK's nucleotide-binding domains or metal-

binding sites could open new avenues for combating multidrug-resistant *K. pneumoniae* strains. In conclusion, this study sheds light on the interplay between ATP, metal ions, and ClpK structural dynamics, thereby enhancing our understanding of bacterial stress adaptation and providing insights for therapeutic intervention.

2.6. Supplementary

Table 2-S1 Buffer systems from pH 2 to pH 10 prepared in deionized water and adjusted to desired pH with NaCl or NaOH. All buffers were filtered and stored at 4°C until use.

Buffer	pH
0.1 M HCl-KCl	2
0.05 M Citrate	3
0.05 M Acetate	4
0.05 M Sodium Phosphate	5-6, 9
0.1 M Tris-HCl	7-8
0.1 M Glycine	10

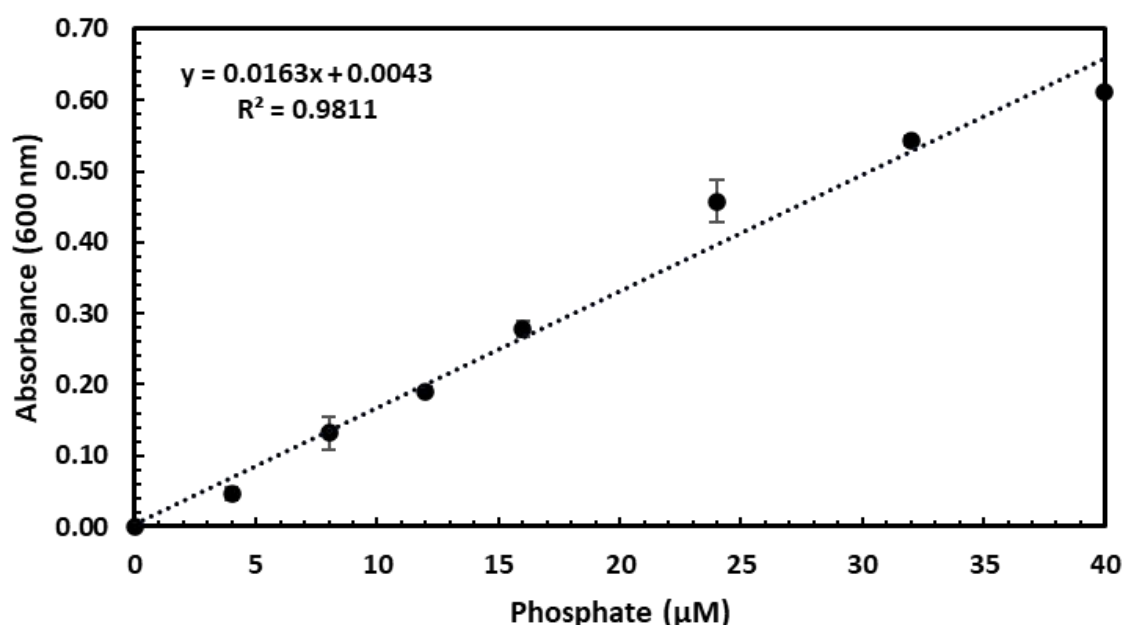


Figure 2S-1: Phosphate standard curve. Varying concentrations (0, 4, 8, 12, 16, 24, 32, and 40 μM) of phosphate was used to generate the standard curve. The experiment was performed in triplicates. The R2 value obtained was 0.9811 and the equation of the graph was $y = 0.0163x + 0.0043$.

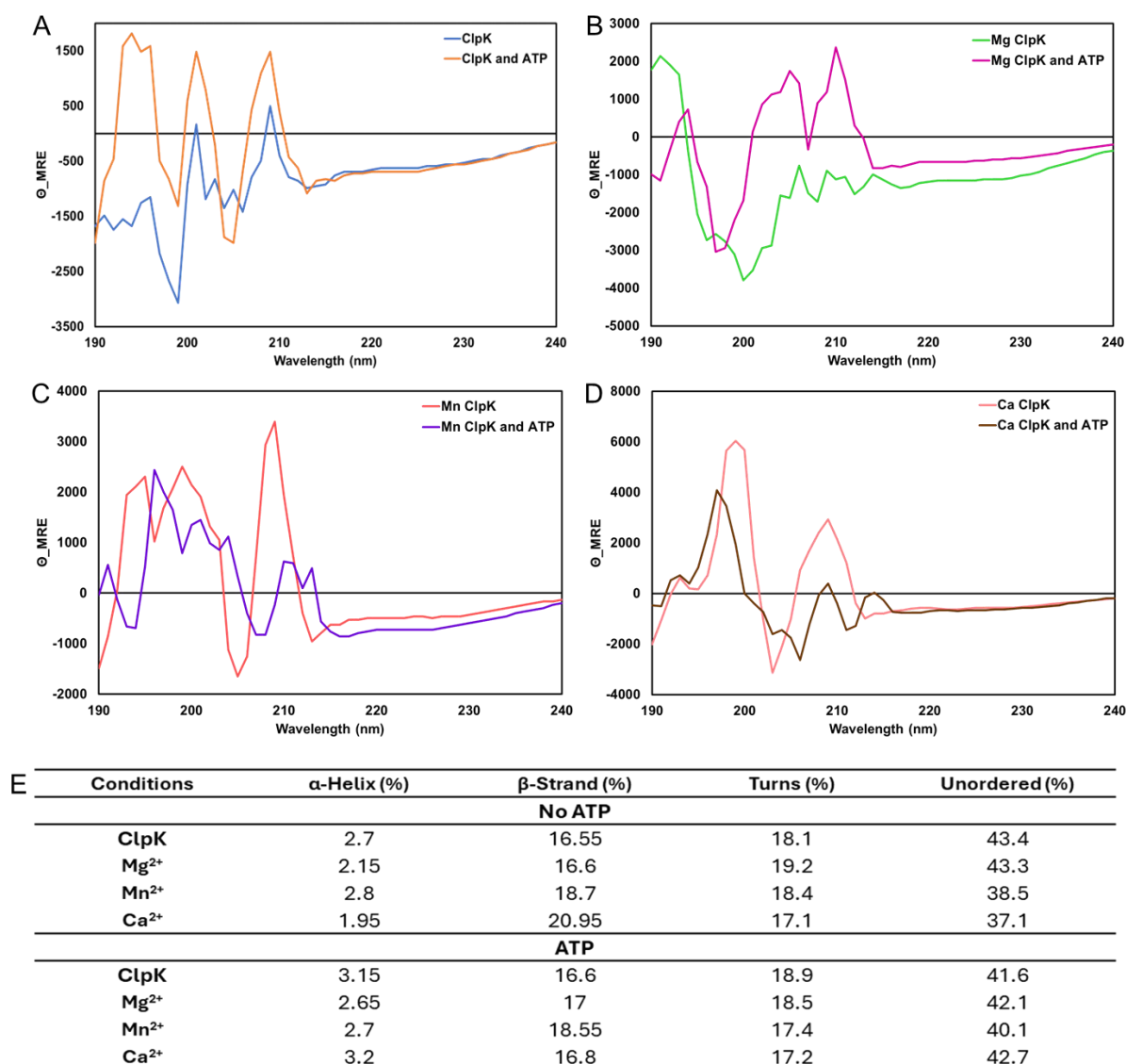


Figure 2S-2: Far-UV CD Analysis of ClpK with 8 M Urea in the Presence and Absence of ATP and Divalent Cations. A) ClpK with 8 M urea in the absence (blue) and presence of ATP (orange). B) ClpK with 8 M urea in the presence of Mg²⁺ with (purple) and without ATP (green). C) ClpK with 8 M urea in the presence of Mn²⁺ with (red) and without ATP (blue). D) ClpK with 8 M urea in the presence of Ca²⁺ with (brown) and without ATP (pink). Each spectrum represents an average of seven accumulated spectra derived at 20°C using a 1 mm path length quartz cuvette. E) Influence of 8 M urea on the secondary structural elements of ClpK in the presence and absence of ATP and divalent cations as predicted using the DichroWeb server.

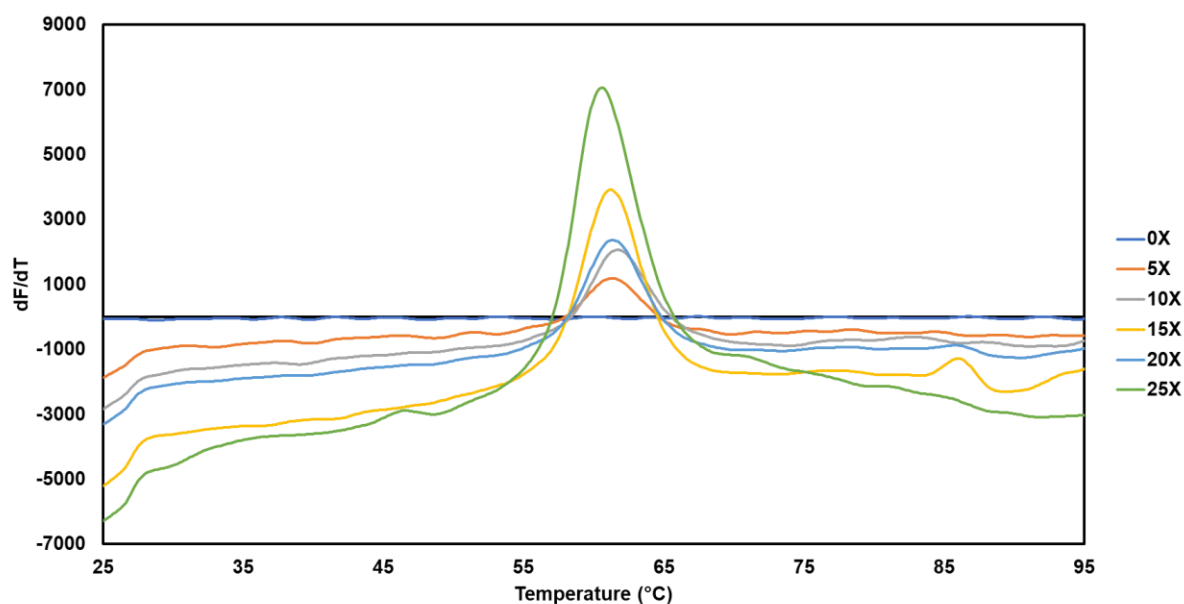


Figure 2S-3: Derivative melt curves from thermal shift analysis of ClpK with varying SYPRO orange concentrations. SYPRO orange concentrations ranging from 0X to 25X were investigated in the presence of ClpK (10 ng/ μ L) and a final reaction volume of 25 μ L. The experiments were done in triplicates and average d(Fluorescence)/dT was plotted against temperature ($^{\circ}$ C).

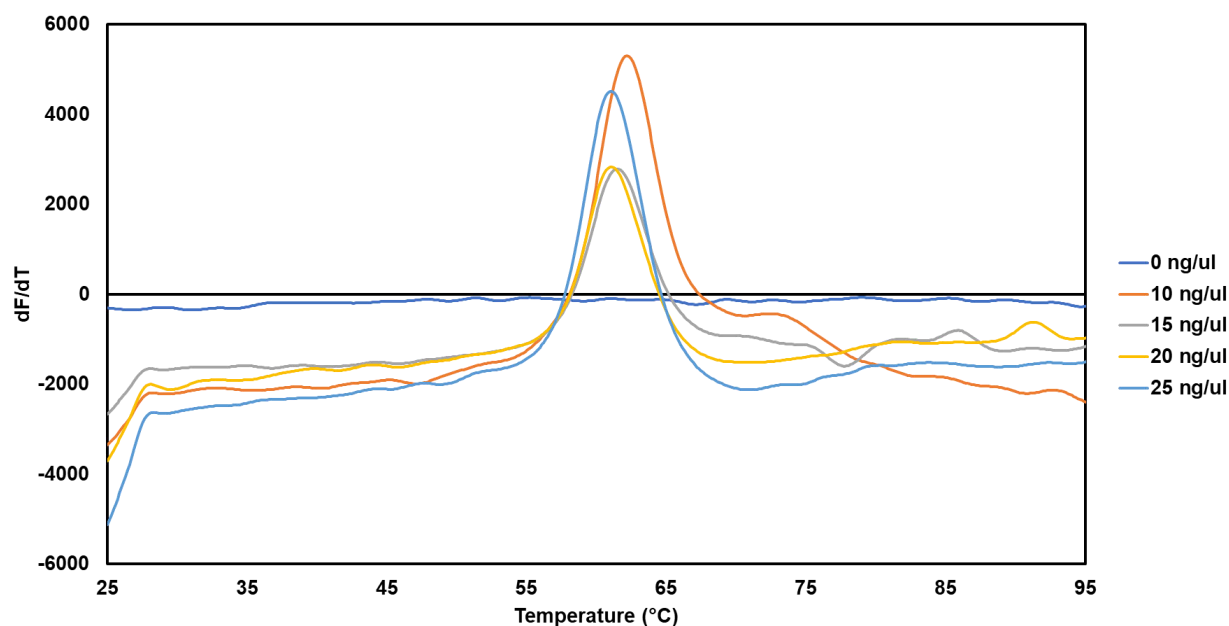


Figure 2S-4: Derivative melt curves from thermal shift analysis of ClpK with varying protein concentrations. Protein concentrations ranging from 0 ng/ μ L to 25 ng/ μ L were investigated in the presence of 10X SYPRO orange and a final reaction volume of 25 μ L. The experiments were done in triplicates and average d(Fluorescence)/dT was plotted against temperature ($^{\circ}$ C).

2.7. References

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Chapter 3: ClpK as a Chaperone Hub: Computational Exploration of its Protein Interaction Landscape.

3.1. Abstract

Protein-protein interaction networks provide a foundation for understanding the molecular mechanisms within cells and are increasingly recognized as promising targets for therapeutic intervention. However, the experimental elucidation of these interactions is complicated by the complexity and dynamic nature of protein interaction networks. *In silico* methods have become invaluable allowing researchers to identify potential interactions and focus on those that may be biologically significant to guide experimental validation and drug discovery. This study employed computational modelling, visualization, and network analysis to investigate the interactions of ClpK, a Clp ATPase integral to protein homeostasis and thermotolerance of *Klebsiella pneumoniae*. Potential interacting partners of ClpK were predicted using the STRING database with ClpB used as a structural proxy for ClpK. The proteins with edge confidence scores greater than 0.8 were selected for molecular docking and molecular dynamics simulations to assess interaction stability and binding affinities. Molecular docking using the ClusPro server confirmed stable interactions between ClpK and its predicted partners, which were further characterised using PDBsum and Hawkdock to visualise binding interfaces and estimate binding free energies MM-GBSA calculation. Among the predicted complexes, KPN_01323 had the highest interaction score, highlighting it as the most promising candidate for further investigation. Molecular dynamics simulations further confirmed the stabilisation of putative partners upon interaction with ClpK supporting the robustness of this interaction. Collectively, these findings provide insight into the ClpK interaction network and establish a framework for future *in vitro* and *in vivo* studies contributing to elucidate the biological role and therapeutic potential of ClpK.

3.2. Introduction

Bacteria depend on an ATP dependent protease system to regulate protein turnover and eliminate misfolded or dysfunctional proteins, particularly under environmental stress conditions (Queraltó *et al.*, 2023, Xu *et al.*, 2024). The Clp/Hsp100 family of molecular chaperones forms a key component of this system, functioning in both protein disaggregation and targeted proteolysis under stressful conditions (Maurizi and Li, 2001). This family consists of Clp ATPase and the caseinolytic proteolytic subunit (ClpP), which together assemble into the caseinolytic protein (Clp) complex responsible for ATP-dependent protein degradation (Bouchnak and Van Wijk, 2021, Brötz-Oesterhelt and Sass, 2014). Clp ATPases are classified into two classes; Class I ATPase which contain two ATP binding domains separated by a spacer region (e.g.: ClpA, ClpB, ClpK, ClpC) and Class II ATPases which contain a single ATP-binding domain (e.g.: ClpY and ClpX) (Motiwala *et al.*, 2021, Queraltó *et al.*, 2023, Yu *et al.*, 2018).

ClpK belongs to Class I ATPase and has been associated with heat stress survival in *K. pneumoniae* (Bojer *et al.*, 2010, Bojer *et al.*, 2013, Motiwala *et al.*, 2022). Interestingly, ClpK is considered a unique Clp ATPase among Gram-negative bacteria because it contains an intermediate-size linker domain resembling that of ClpC and its structural homologs, ClpE and ClpL (Bojer *et al.*, 2010). This feature suggests that ClpK may employ specialized molecular interactions that differentiate its functional behaviour from other Clp homologs. Consistent with this observation, adaptor proteins such as MecA and ClpS have been shown to interact with other Clp ATPases to enhance proteolysis and modulate substrate recognition (Wahl *et al.*, 2014, Dougan *et al.*, 2002). In addition to Clp ATPase-adaptor interaction, chaperone activity is also critical for maintaining protein quality control. For example, the molecular chaperone DnaK interacts with ClpB under stress conditions to deliver misfolded substrates to the proteolytic complex for degradation (Schlieker *et al.*, 2004). Therefore, such protein-protein interactions are essential for maintaining bacterial proteostasis, survival, stress tolerance, and virulence in pathogens such as *Staphylococcus aureus* and *Mycobacterium tuberculosis*, where Clp systems have been identified as promising drug targets (Ollinger *et al.*, 2012, Frees *et al.*, 2014).

Among these systems, Clp ATPases such as ClpA, ClpX and ClpC contain the ClpP interaction motif – IG(F/L) which mediates their association with the ClpP peptidase to form a proteolytic complex responsible for protein degradation (Maurizi and Xia, 2004, Queraltó *et al.*, 2023). While these interactions are well characterized, the interaction partners and structural basis of ClpK-mediated associations with adaptor proteins or chaperones remains unexplored. Furthermore, no crystal structures, cryo-electron microscopy (cryo-EM) data, or experimentally validated interaction models for *K. pneumoniae* ClpK are available in structural databases. This lack of structural and functional insight represents a major gap in understanding how ClpK mediates substrate recognition, binding, and protein degradation during cellular stress. Addressing this gap is critical for exploiting ClpK as a potential antimicrobial target, especially in the context of rising multidrug resistance in *K. pneumoniae*. Elucidating these interactions is crucial for defining ClpK's cellular role and aid in the rational design of inhibitors that disrupt key chaperone-protease interactions.

Given the importance of protein-protein interactions (PPIs) in regulating Clp ATPase function, mapping the interaction network of ClpK is essential for understanding its biological mechanisms. Computational PPI prediction tools such as Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) and deep-learning models now enable the identification of potential interaction partners, complementing experimental approaches such as co-immunoprecipitation or X-ray crystallography (Farooq *et al.*, 2021). The STRING database integrates both known and predicted PPIs from diverse sources, including experimental data, computational prediction methods, and public text collections (Szklarczyk *et al.*, 2023, Ding and Kihara, 2018). It then assigns interaction scores based on evidence, allowing for the identification of high-confidence interaction partners for a given protein (Szklarczyk *et al.*, 2023). To explore the structural basis of these interactions, protein structure prediction platforms such as AlphaFold and SWISS-MODEL are then widely used (Jumper *et al.*, 2021, Schwede *et al.*, 2003). AlphaFold uses deep learning to generate high-accuracy 3D models of proteins based on their amino acid sequences, while SWISS-MODEL uses homology-based modelling for reliable structure prediction in the absence of crystal structures (Jumper *et al.*, 2021, Waterhouse *et al.*, 2024). To predict and analyse protein–protein binding modes, molecular docking tools such as ClusPro and HADDOCK are used. These tools simulate interactions between two or more protein structures by

considering complementarity in shape, electrostatics, and known binding residues, thereby predicting plausible interaction interfaces and binding affinities (De Vries *et al.*, 2010, Kozakov *et al.*, 2017).

In this study the ClpK interaction network was first investigated using the STRING database, subsequently, homology models of ClpK and its putative interaction partners were modelled successfully for structural and functional analysis. These models were then subjected to docking and molecular dynamics simulations, which provided insights into the structural basis and stability of these interactions, laying the groundwork for future experimental validation and inhibitor development.

3.3. Materials and Methods

3.3.1. STRING Interaction Analysis

In the absence of ClpK in the STRING database (version 12; <https://string-db.org>), the closest homolog, ClpB, was used to construct the PPI network. The interactions were predicted based on combined scores from experimental data, co-expression, neighbourhood, gene fusion, co-occurrence, and text mining. A high-confidence score threshold (>0.7) was applied to filter predicted associations. The resulting network was visualized using STRING's integrated viewer, and nodes were annotated according to their predicted functional partners.

3.3.2. Protein 3D structures

The 3D structure of ClpK was retrieved from the AlphaFold Protein Structure Database, which uses deep learning approaches to generate high-confidence models based on homology and multiple sequence alignments (Jumper *et al.*, 2021). The model with the highest predicted Local Distance Difference Test (pLDDT) score was selected for subsequent analysis. The 3D structures of ClpK-interacting proteins were constructed using the SWISS MODEL server. The amino acid sequences of the interacting proteins were retrieved from the Uniprot database and submitted to the SWISS-MODEL workspace for comparative modelling. The most suitable templates were selected based on sequence identity, GMQE (Global Model Quality Estimation), and Qualitative Model Energy ANALysis (QMEAN) scoring functions. Only models with high structural confidence and favourable stereochemical properties were used for molecular docking and interaction analyses.

3.3.3. Protein-protein docking and analysis

Molecular docking of ClpK and its putative interacting partners was performed using the ClusPro 2.0 server. Blind docking was performed without prior specification of the binding interface. The cluster with the highest number of members was selected for further analysis as the most probable binding conformation. The PDBSum server was used to analyse protein-protein interactions (Laskowski *et al.*, 2018). The HawkDock server was used to perform Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) free energy calculations to providing an estimate of the binding stability of the docked protein–protein complex (Zhang *et al.*, 2025). These analyses were further complemented using the PRODIGY server to predict the binding affinity (ΔG) of the protein-protein interactions and help validate biological interaction plausibility (Xue *et al.*, 2016).

3.3.4. Molecular dynamics simulation and post dynamics analysis

Molecular dynamics (MD) simulations were performed on a Linux (Ubuntu) workstation using the GPU-enabled Desmond simulation engine integrated within Maestro v12.2. Prior to simulation, each complex was prepared using the System Builder module in Desmond. This module solvated the systems using the TIP3P explicit solvent model and the OPLS_2005 force field. The complexes were embedded in an orthorhombic box, with a 10 Å buffer between the outermost atom of the solute and box edges, and box angles set at 90°. Counter ions were added to neutralize the system, and 0.15 M NaCl was included to mimic physiological strength. The solvated system was then energy minimized before proceeding to the simulation phase. The MD protocol consists of eight stages, with stages 1–7 serving as equilibration and stage 8 as the production run. In stage 1, the system topology and parameters were initialized. Stages 2 and 3 involved Brownian dynamic simulations under NVT conditions at 10 K, lasting 100 ps and 12 ps, respectively, with restraints applied to the solute's heavy atoms. Pocket solvation (stage 5) was skipped. Stages 4, 6 and 7 were conducted under NPT conditions at 10 K, lasting 12, 12 and 24 ps, respectively. Restraints on heavy atoms were maintained in stages 4 and 6 but removed in stage 7. Finally, stage 8 comprised the production phase, during which as unrestrained 200 ns MD simulation was performed at a constant temperature. Trajectories derived from the MD simulation studies were used for post dynamic analysis using Schrodinger Maestro v12.2. The

simulation interaction diagram algorithm was used to analyse the root mean square deviation (RMSD) of the alpha carbon atoms ($^{\alpha}\text{C}$) of the complexed and apo proteins. The simulation events analysis algorithm was used to analyse the radius of gyration (R_g) of the complex and apo proteins.

3.4. Results

3.4.1. Identification of ClpK Interacting Proteins

Interaction networks were constructed from the STRING database using ClpB from *Klebsiella pneumoniae* MGH78578 as a proxy for ClpK due to its highest sequence identity (44.3%; Figure 3-1A) amongst other homologs. A high-confidence homology-based association was inferred with an Expect value (E-value) of 7.5×10^{-188} , suggesting strong evolutionary conservation and supporting the reliability of predicted ClpK interactions. The resulting interaction network revealed ClpB exhibits dense connectivity with multiple chaperones and protease-related proteins (Figure 3-1B). In this analysis, many ClpB-associated partners were supported primarily by functional association evidence, particularly co-expression and curated database annotations, rather than confirmed physical binding. Notably, strong associations were predicted with molecular chaperones DnaK, GrpE, ClpP, and DnaJ, all of which are integral components of the cellular protease machinery. The edge confidence scores of these interactions ranged from 0.839 to 0.976, suggesting evidence for functional linkage (Szklarczyk *et al.*, 2023). It is important to note that STRING network edges represent protein–protein associations, signifying a shared or related biological function. These associations do not necessarily imply direct physical binding but may reflect participation in the same biological pathway or process (Szklarczyk *et al.*, 2023). Of the identified proteins, ClpP belongs to the Clp protease system and is more likely to represent physical interactions, given the well-established ATPase–protease docking mechanism. In contrast, heat shock proteins, chaperones, or stress-response regulators identified in the network are more appropriately classified as functional partners, reflecting coordinated involvement in protein quality control.

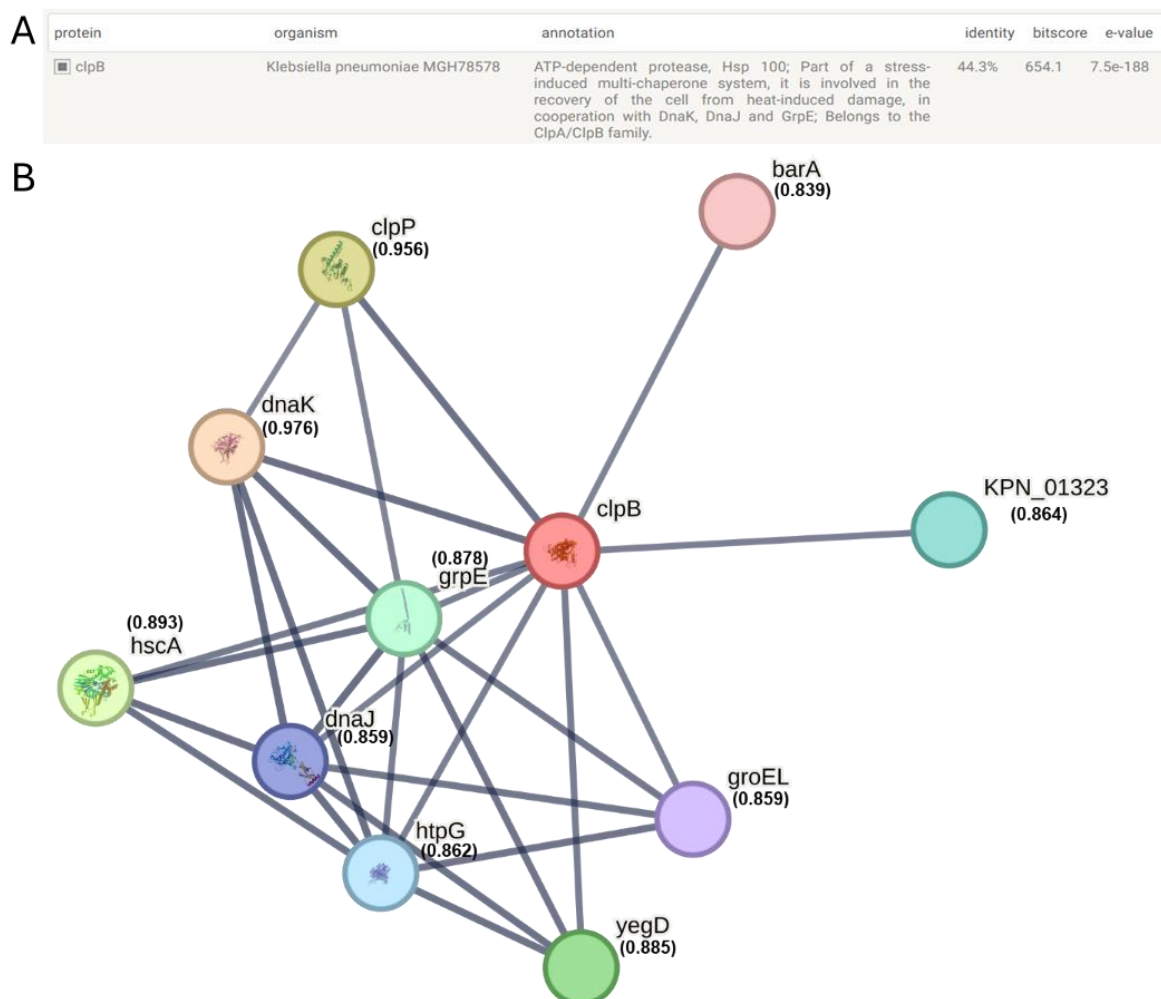


Figure 3-1: Predicted protein-protein interaction network of ClpK. A) *Klebsiella pneumoniae* MGH78578 ClpB was used as a proxy for ClpK due to its highest sequence identity. B) The network depicts predicted ClpK protein-protein interactions, identified using the STRING database. Each node represents a protein, and edges indicate functional associations based on experimental data, text mining, co-expression, and computational prediction. The key interacting proteins are clpP (Uniprot ID: A6T5I0), dnaK (Uniprot ID: A6T4F4), dnaJ (Uniprot ID: A6T4F5), grpE (Uniprot ID: A6TCM1), htpG (Uniprot ID: A6T5N6), groEL (Uniprot ID: A6TH28), hscA (Uniprot ID: A6TCE7), barA (Uniprot ID: A6TD58), KPN_01323 (Uniprot ID: A6T834), and yegD (Uniprot ID: A6TBG8). The edge confidence scores are black, bold and in brackets.

3.4.2. Modelling of ClpK and its putative interacting partners

Structural models of ClpK and its putative interacting partners were generated using the SwissModel server (Figure 3-2A). Model quality was successfully evaluated prior to multiple validation metrics analysis using the Molprobit score, percentage of residues in Ramachandran favoured regions and QMEANDisCo global scores (Figure 3-2B). The MolProbit score is generated using a composite measure in which lower values correspond to models of superior structural quality (Chen *et al.*, 2010). The clash score, rotamer and Ramachandran evaluations were combined into a single

composite metric, where lower scores indicate higher structural quality (Chen *et al.*, 2015). For all the modelled proteins, more than 80% of the residues fell within the favoured regions of the Ramachandran plot. Additionally, the QMEANDisCo global score, which ranges from 0 to 1 and reflects both global and per-residue model quality, was close to 1 for all the models, indicating high structural reliability (Studer *et al.*, 2019).

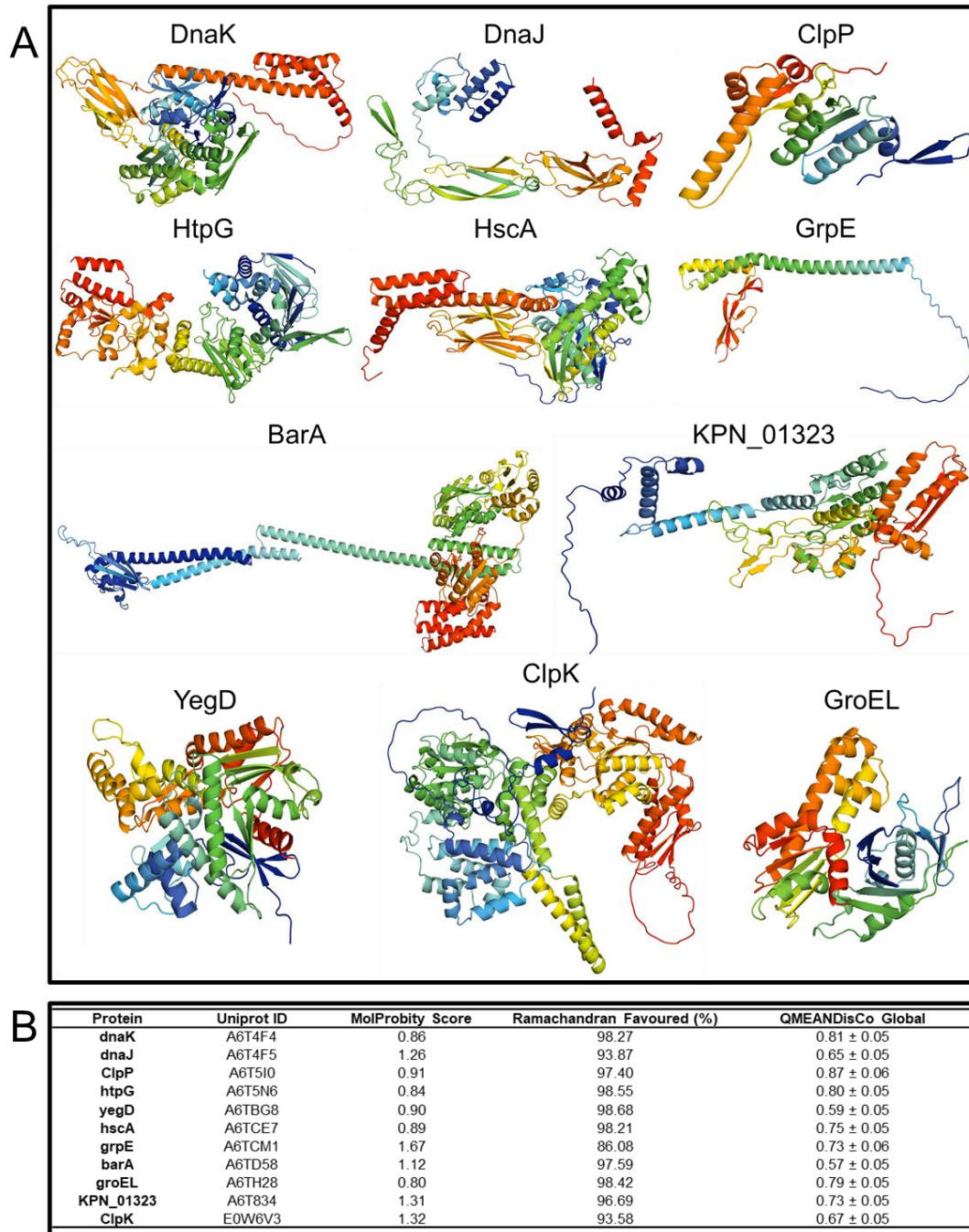


Figure 3-2: Homology modelling of ClpK and predicted interacting partners. A) The models were generated using the SwissModel server and visualised using PyMol (Schrödinger, 2015, Schwede *et al.*, 2003). B) Summary of the structural validation parameters for the modelled proteins.

3.4.3. Docking analysis of ClpK with putative interaction partners

Interactions between ClpK and its predicted interacting partners were analysed through rigid-body protein-protein docking using the ClusPro 2.0 server. This server uses the blind rigid PIPER docking algorithm based on fast Fourier transformation (FFT) correlation to efficiently sample low-energy conformations using pairwise interaction potentials (Kozakov *et al.*, 2017). Top-ranking blind-docking models (Table 3-1) were selected based on cluster sizes and energy parameters, with larger clusters and lower weighted scores representing the most probable binding conformations. Among the predicted complexes, ClpP, DnaJ and BarA had the lowest energy binding scores (-1341.3, -1396.5 and -1565.6, respectively), indicating a strong likelihood of stable interaction with ClpK.

Table 3-1: Blind docking showing models with the highest cluster size and lowest weighted scores.

Protein	Members	Representative	Weighted Score
DnaK	31	Center	-701.7
		Lowest Energy	-810.5
GrpE	35	Center	-934.1
		Lowest Energy	-997.0
GroEL	36	Center	-818.7
		Lowest Energy	-885.0
HscA	37	Center	-930.3
		Lowest Energy	-1019.6
YegD	46	Center	-1015.3
		Lowest Energy	-1015.3
KPN_01323	48	Center	-1215.5
		Lowest Energy	-1223.0
BarA	53	Center	-1467.4
		Lowest Energy	-1565.6
DnaJ	69	Center	-1358.0
		Lowest Energy	-1396.5
HtpG	59	Center	-1106.6
		Lowest Energy	-1106.6
ClpP	100	Center	-1134.4
		Lowest Energy	-1341.3

The docking poses revealed variable binding orientations and interface regions, suggesting different potential interaction modes for each partner (Figure 3-3). It was observed that the putative partners bind near of the N-terminal domain, except HscA and GroEL which bind in the proximity of the C-terminal domain. These predicted complexes suggest a range of potential binding modes suggesting functional diversity among the putative interactors.

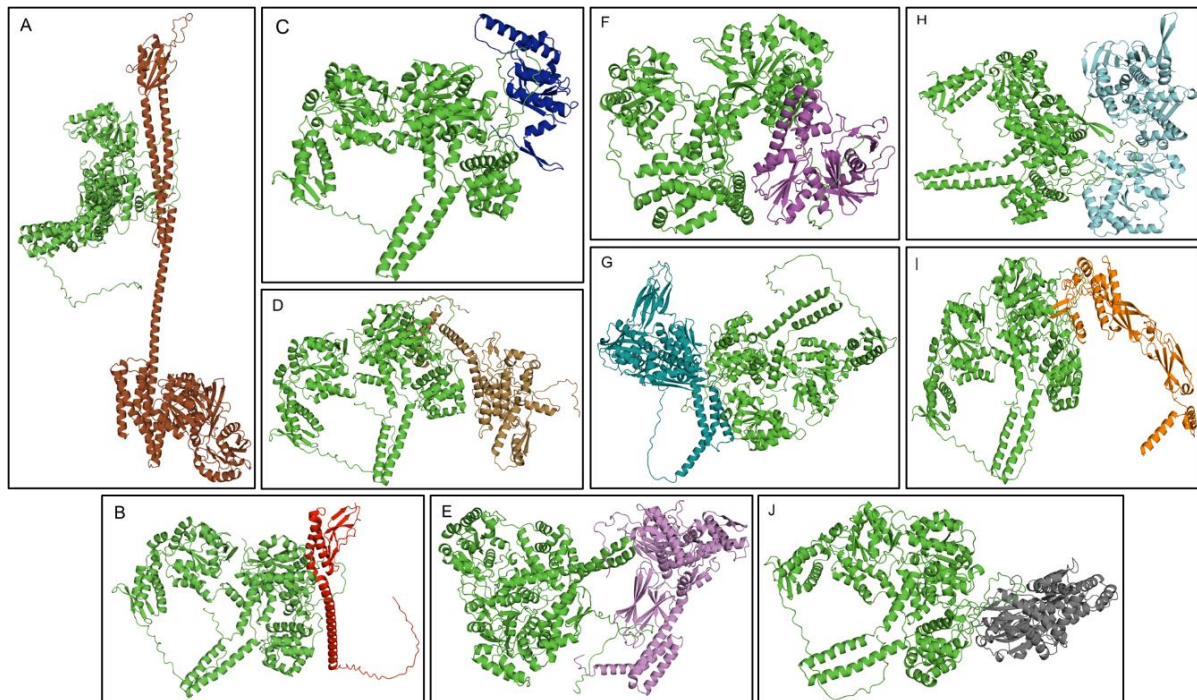


Figure 3-3: ClpK- putative partner docking complexes. ClpK was coloured green and is presented docked with A) barA (brown), B) grpE (red), C) ClpP (blue), D) KPN_01323 (light brown), E) hscA (violet), F) groEL (purple), G) DnaK (teal), H) htpG (light blue), I) DnaJ (orange), and J) YegD (grey). The ClpK complexes were visualised using PyMol.

Analysis of predicted interaction interfaces identified key residues mediating the stability of ClpK-partner complexes (Figure 3-4). The complexes were primarily stabilised by a combination of salt bridges, hydrogen bonds, and non-bonded contacts, which collectively contribute to protein–protein interaction specificity, stability, and recognition (Donald *et al.*, 2011). Recurrent interaction hotspots were observed at both terminal regions. Lys9, Tyr54, Arg63, Arg78, and Glu94 were identified as common residues across many interacting partners at the N-terminal domain, suggesting a conserved electrostatic surface involved in substrate or co-chaperone interaction. At the C-terminal domain, Glu551, Arg560, Lys570, and His574 were identified as common interacting residues. This indicates a conserved regulatory interaction

interface. Collectively, these findings support the presence of structurally conserved terminal hotspots that mediate functional coupling within the Clp stress-response network. KPN_01323 exhibited the highest number of intermolecular interactions, whereas DnaK displayed the fewest. This trend is consistent with the binding energy scores presented in Table 3-1 where DnaK had the weakest predicted binding affinity and KPN_01323 was among the proteins with the strongest interaction energies. KPN_01323's high interaction count highlights its potential as physiologically relevant binding partner of ClpK. These findings provide structural rationale to prioritise ClpK-KPN_01323 as a candidate for future *in vitro* studies, especially in the context of ClpK's role in stress response and protein homeostasis.

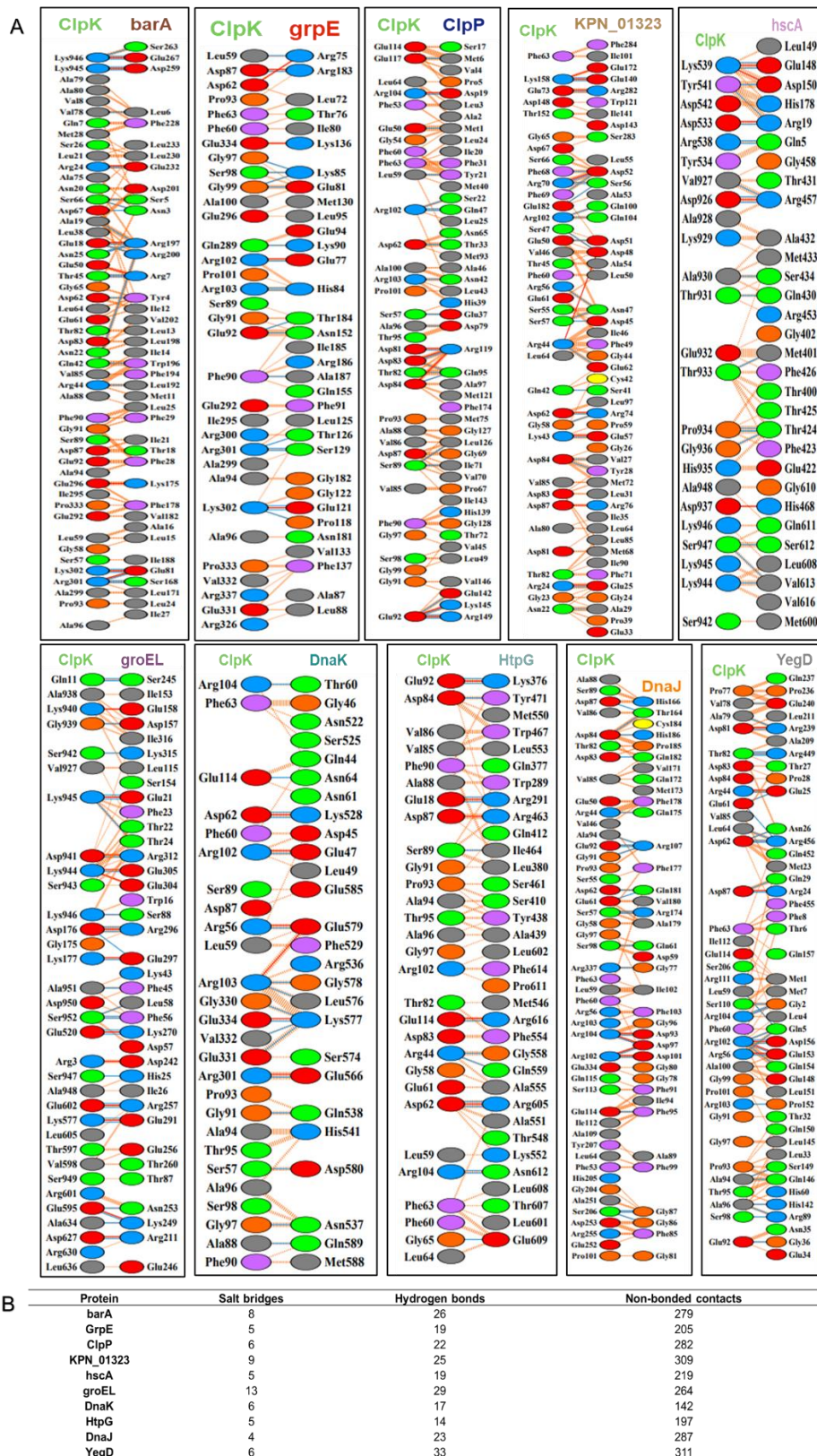


Figure 3-4: Residue interactions at the interface of ClpK and its putative binding partners. A) The interaction diagram is coloured by residue type where red lines represent salt bridges; blue lines represent hydrogen bonds and orange dashes represent non-bonded contacts. B) Number of salt bridges, hydrogen bonds, and non-bonded contacts for each complex.

The binding affinities and interaction energies between ClpK and its putative protein partners were evaluated based on Gibbs free energy (ΔG) values, dissociation constants (K_d), and binding free energies (Table 3-2). KPN_01323 had the strongest predicted interaction with ClpK as shown by the low ΔG values (-18.6 kcal/mol), and high K_d (7.5×10^{-14} M). This correlates with the binding free energy scores observed in Table 3-1 and the interactions observed in Figure 3-4. In contrast, DnaK had the lowest ΔG values (12.4 kcal/mol) and K_d (1.8×10^{-9} M), indicating relatively lower binding stability. These findings suggest that ClpK may interact with specific protein partners, possibly reflecting functional relevance in protein quality control or stress response pathways.

Table 3-2: Summary of ClpK and putative protein partners interaction energetics.

Protein	ΔG (kcal/mol) ¹	K_d (M) ¹	Binding free energy (kcal/mol) ²
BarA	-17.3	6.8×10^{-13}	-170.54
ClpP	-15.8	7×10^{-12}	-174.96
DnaJ	-16.4	2.5×10^{-12}	-186.09
DnaK	-12.4	1.8×10^{-9}	-39.43
GroEL	-12.5	1.6×10^{-9}	-84.37
GrpE	-13.5	2.9×10^{-10}	-96.91
HscA	-12.4	1.9×10^{-9}	-62.69
HtpG	-14.0	1.3×10^{-10}	-72.86
KPN_01323	-18.6	7.5×10^{-14}	-134.25
YegD	-17.7	3.4×10^{-13}	-174.21

¹PRODIGY server

²HawkDock server – MM-GBSA

3.4.4. MD simulation analysis of ClpK complexes

RMSD analyses over the 200 ns MD simulation was used to evaluate the conformational stability of both the apo proteins and their ClpK-bound complexes. Overall, the apo forms displayed greater structural fluctuations compared to the bound states (Figure 3-5). In particular, greater fluctuations in the apo state were observed for barA (32.16 ± 12.47 Å) and grpE (24.37 ± 12.47 Å) indicative of significant structural flexibility in their unbound conformations (Figure 3-5A). In contrast, complex formation with ClpK resulted in reduced RMSD fluctuations (Figure 3-5B). This suggests that complex formation with ClpK may contribute to enhanced structural stability, except for DnaK where the fluctuations are indicative of increased structural flexibility.

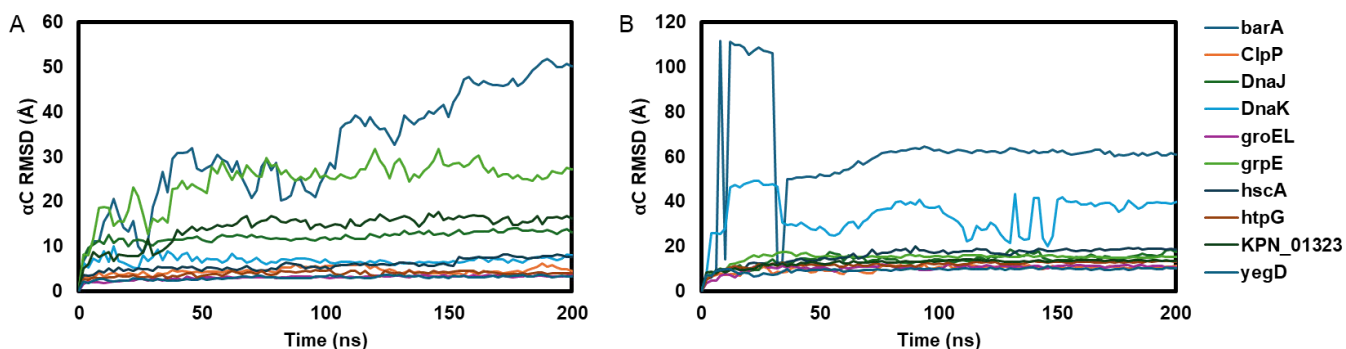


Figure 3-5: RMSD analysis profiles of alpha-carbon (α C) atoms of the putative partners. A) RMSD trajectories of apo forms. B) RMSD trajectories of the ClpK-partner complexes over 200 ns.

To evaluate the overall compactness and structural integrity of the putative ClpK-interacting partners, the Rg was calculated over the 200 ns MD simulation (Figure 3-6). In the apo state, the Rg trajectories remained relatively stable with only minor fluctuations, suggesting that the proteins maintained consistent folding and compactness throughout the simulation (Figure 3-6A). Similarly, the ClpK-bound complexes exhibited comparable Rg stability (Figure 3-6B), indicating that ClpK binding did not induce major conformational expansion or unfolding (Motiwala *et al.*, 2025). Interestingly, a slight increase in Rg was observed for all the complexes except barA, in comparison to their apo counterparts. For BarA (95.52 ± 0.20 Å), a slight decrease was observed in comparison to the apo form (105.84 ± 0.31 Å). This indicates subtle conformational adjustments to accommodate binding. None of the trajectories exhibited drastic deviations or signs of structural destabilization. Overall, this analysis indicates that proteins retain their compact structure upon ClpK interaction, supporting the stability and integrity of the predicted complexes.

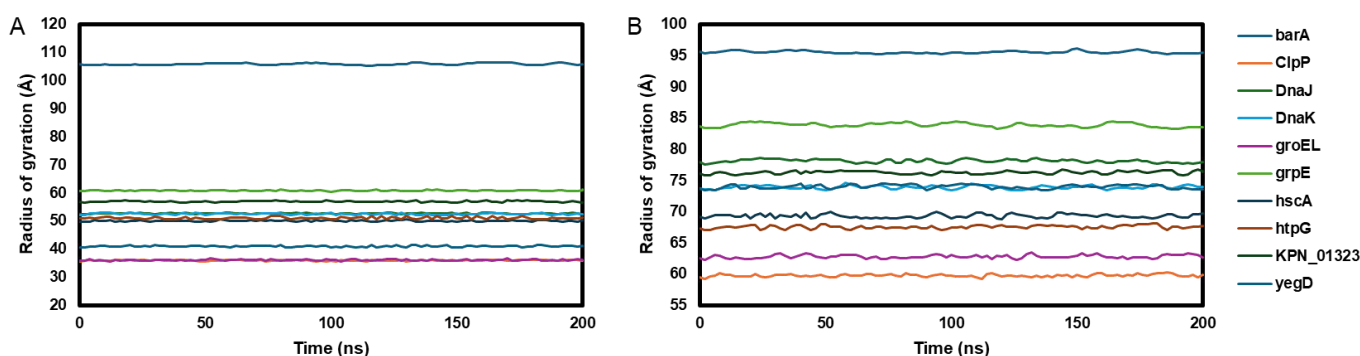


Figure 3-6: Radius of gyration (Rg) analysis of the putative partners. A) Rg trajectories of the apo proteins over 200 ns. B) Rg trajectories of ClpK-partner complexes.

3.5. Discussion

ClpK is a thermotolerant ATPase found across various *Klebsiella* species and has been associated with enhanced heat resistance and improved survival under stressful conditions (Bojer *et al.*, 2013, Motiwala *et al.*, 2021). Despite its role in thermotolerance and protein quality control, ClpK remains poorly characterized, and therefore there is limited evidence pertaining to its PPIs. It is important to understand the significance of biological and molecular processes of PPIs within cells for identifying novel antimicrobial targets (Shahid *et al.*, 2021, Murakami *et al.*, 2017). This study represents a computational effort to predict and characterise potential ClpK interaction partners, combining network-based inference, molecular docking, and molecular dynamics simulations to investigate the nature and stability of these associations.

The STRING database was used to derive interaction networks using ClpB as a proxy for ClpK (Figure 3-1), owing to their structural and functional similarity, an approach supported by previous structural and functional comparisons (Motiwalda *et al.*, 2021). The resulting high-confidence network included ClpP, BarA, KPN_01323, GroEL, GrpE, DnaK, HscA, DnaJ, HtpG and YegD as potential ClpK interaction partners. These proteins are known to participate in proteostasis, stress adaptation, and signal regulation pathways (Straus *et al.*, 1990, Susin *et al.*, 2006, Lupoli *et al.*, 2018, Mangla *et al.*, 2023). Docking analyses revealed that all ClpK-partner complexes formed energetically favourable and stable interactions mediated by hydrogen bonds, salt bridges, and non-bonded contacts (Figure 3-4), indicating the formation of stable protein-protein interactions (Donald *et al.*, 2011).

Among the predicted interacting partners for ClpK, ClpP is a caseinolytic protease that functions in conjunction with Clp ATPases through IG(F/L), a conserved interaction motif, to facilitate the degradation of damaged, misfolded, or regulatory proteins (Aljghami *et al.*, 2022, Maurizi and Xia, 2004, Queraltó *et al.*, 2023). The inclusion of ClpP within the predicted network supports earlier evidence that ClpK contributes to thermotolerance via proteolytic regulation (Bojer *et al.*, 2013). Structural modelling revealed that ClpK did not interact with this motif directly, suggesting a non-canonical interaction mechanism distinct from other Clp ATPases. This divergence could reflect evolutionary adaptation, allowing ClpK to interact flexibly with ClpP or alternative partners under stress. Experimental validation of this non-canonical mode of

interaction through site-directed mutagenesis or co-immunoprecipitation would be valuable to confirm this hypothesis.

Several other predicted partners, namely, GroEL, GrpE, DnaK, DnaJ, HtpG and YegD are molecular chaperones essential for protein folding, disaggregation, and stress response (Thirumalai *et al.*, 2020, Harrison, 2003, Calloni *et al.*, 2012, Silberg *et al.*, 2004, Muskopf *et al.*, 2018, Garcie *et al.*, 2016, Itoh *et al.*, 1999). Their association with ClpK reinforces the view that this ATPase may act as a chaperone hub, coordinating multiple arms of the bacterial heat-shock response. The presence of BarA, a highly conserved histidine kinase which regulates the Csr system, suggests a broader regulatory dimension in ClpK function, possibly linking stress sensing to proteolytic activity (Alvarez *et al.*, 2021). Interestingly, the inclusion of KPN_01323, a component of the Type VI secretion system contractile sheath (T6SS), was unexpected and points toward a potential role of ClpK in bacterial competition and pathogenesis (Cherrak *et al.*, 2019). Interestingly, ClpK exhibited the strongest and most stable predicted interaction with KPN_01323. Binding energy and interaction energy analyses further supported the strength of ClpK-KPN_01323 interaction, with this complex displaying the most favourable energetics among all tested partners (Table 3-2). Given the novelty and predicted stability of this interaction, further *in vitro* experimental validation to explore the functional relevance of this interaction in *K. pneumoniae* is warranted.

MD simulations over 200 ns confirmed the stability of the ClpK-partner complexes. RMSD profiles showed that all complexes equilibrated rapidly and maintained structural integrity throughout the simulation period (Figure 3-5), while Rg analysis demonstrated consistent compactness across both apo and bound forms (Figure 3-6). These results suggest that ClpK-partner binding does not induce large-scale conformational rearrangements or destabilization, which aligns with ClpK's proposed role as a thermotolerant ATPase capable of maintaining structure under fluctuating conditions (Motiwala *et al.*, 2025). The observed stabilization of key complexes, particularly ClpK-KPN_01323 and ClpK-ClpP, supports their potential physiological relevance and provides structural bases for experimental follow-up.

In conclusion, this bioinformatics study proposes the first prediction of PPIs of ClpK with ten putative interacting partners. These findings provide valuable insight into the

potential functional network of ClpK, especially its role in stress response and protein homeostasis in *Klebsiella* species, particularly *K. pneumoniae*. It is important to further characterise these predicted interactions through experimental validation methods such as enzyme assays, co-immunoprecipitation, or pull-down assays. Overall, this study provides a foundation for further *in vitro* and *in vivo* investigations that will contribute to understanding the biological role of ClpK and its potential as a therapeutic target.

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Chapter 4: Computational Repurposing and Phytochemical Screening of Inhibitors Against ClpK from *Klebsiella pneumoniae*.

4.1. Abstract

Over the years, the increase in antibiotic resistant *Klebsiella pneumoniae* has highlighted the urgent need for the identification of novel therapeutic compounds and targets. ClpK is a caseinolytic ATPase that plays an important role in protein quality control and thermotolerance, making it a promising drug target. This study used *in silico* methods including ADMET screening, molecular docking, and molecular dynamics simulations to evaluate binding efficiency and dynamic stability of selected natural and synthetic compounds with ClpK under varying ionic conditions. ADMET screening of synthetic and natural compounds excluded those violating Lipinski's rule of 5 or showing predicted toxicity. Sclerotiamide P was identified as the top binder (-10.9 kcal/mol), while compound D3 had the weakest binding (-4.5 kcal/mol). The top ten compounds with the most favourable binding scores were then further investigated using molecular dynamics simulations in the presence of NaCl and MgCl₂. Trajectory and MM-GBSA binding analyses confirmed that ClpK-ligand complexes remained stable in both ionic environments, with negative binding free energy values indicative of favourable interactions. Overall, the study highlighted ADEP3 and sclerotiamide derivatives as promising ClpK inhibitors and provides a foundation for further experimental validation.

4.2. Introduction

The Gram-negative pathogen, *Klebsiella pneumoniae* has drawn the attention of many researchers following its classification as a critical priority by the World Health Organization (WHO) (Prestinaci *et al.*, 2015, WHO, 2014, WHO, 2017). This opportunistic pathogen accounts for 30% of Gram-negative nosocomial infections with a mortality rate of more than 50% (Podschun and Ullmann, 1998, Li *et al.*, 2023a). It can infect wounds, soft tissues, the respiratory tract and urinary tract of newborns, elderly, and immunocompromised patients (Bagley, 1985, Li *et al.*, 2023b). *K. pneumoniae* has displayed resistance to major classes of antibiotics, including carbapenems, cephalosporins, aminoglycosides, and fosfomycin (Li *et al.*, 2023b, Daya *et al.*, 2022). This increasing antibiotic resistance is attributed to various mechanisms including enzymatic antibiotic modification or inactivation, biofilm formation, and expression of antibiotic specific efflux pumps (Li *et al.*, 2023b, Mulani *et al.*, 2019).

The growing threat of antibiotic resistance underscores the urgent need to develop alternative therapeutic strategies to combat *K. pneumoniae* (Mulani *et al.*, 2019). One such approach involves the design of inhibitors that selectively target proteins essential for its survival. Among these key survival proteins, are Caseinolytic (Clp) ATPases, which use the energy from ATP hydrolysis to drive the refolding or degradation of misfolded and aggregated proteins, thus maintaining protein quality control (Motiwala *et al.*, 2022, Culp and Wright, 2017, Carroni *et al.*, 2017, Lairson *et al.*, 2008). Inhibiting Clp ATPases compromises protein homeostasis by increasing the level of misfolded or aggregated proteins, ultimately resulting in cell toxicity and death (Gersch *et al.*, 2015). Targeting these proteins therefore offers an opportunity to disrupt critical pathways without affecting host systems.

Clp ATPases share the canonical Walker A and B motifs, which are essential for ATP binding and hydrolysis. The Walker A motif contains the GXXXXGK[T/S] consensus sequence which forms the phosphate loop (delToro *et al.*, 2016). Lysine is conserved and stabilises interactions with ATP β - and γ -phosphates, while the terminal threonine or serine coordinates Mg^{2+} ions essential for nucleotide binding and catalysis. Mutations within this region typically impair ATP binding and abolish enzymatic activity (Seraphim and Houry, 2020; Bouchnak and Van Wijk, 2021, delToro *et al.*, 2016). The

Walker B motif includes a conserved hydrophobic hhhhDE sequence, in which the hydrophobic residues stabilise the nucleotide-binding pocket, the aspartate coordinates Mg^{2+} , and the glutamate acts as the catalytic base required for ATP hydrolysis (Han et al., 2025). Mutations to this motif result in substrate trapping, where proteins bind to the chaperone but cannot be released resulting in proteotoxicity and cell death (Bouchnak and Van Wijk, 2021, Rei Liao and Van Wijk, 2019). The strict conservation of these motifs across bacterial species highlights their important role in Clp ATPase function and is consequently attractive for rational inhibitor design. Small-molecule inhibitors that disrupt ATP binding or hydrolysis at these sites could selectively inactivate Clp ATPases and protein homeostasis, providing a promising therapeutic target (Berg et al., 2007). Binding of inhibitors at the Walker A site could potentially prevent ATP binding and hexamer activation, resulting in the loss of ATPase activity (Pezza et al., 2002). Beyond the Walker A motif, the Walker B motif plays an equally important role in the catalytic cycle of AAA^+ ATPases by facilitating ATP hydrolysis. Inhibition of Walker B catalytic glutamate allows ATP binding but inhibits hydrolysis (Futamata et al., 2020).

In silico protein-ligand docking is an effective and valuable technique to explore how candidate ligands might interact with proteins (Mfeka et al., 2025). By simulating binding events, docking allows for the evaluation of both the preferred orientation of ligands within the active or binding site and their relative binding affinities (Afriza et al., 2018, Shamsara, 2018). This approach, compared to experimental methods, is cost-effective and time-efficient, allowing for high-throughput screening of multiple ligands against target proteins (Afriza et al., 2018). To date, several natural and synthetic compounds targeting essential proteins in clinically relevant pathogens have been developed or isolated (Chaturvedi and Shrivastava, 2005, Plotniece et al., 2023, Xuan et al., 2023), including inhibitors of Clp ATPases (such as ClpC, and ClpX) and Clp proteases (ClpP) (Table 4-1).

Table 4-1: Mode of action of the compounds targeting Clp ATPases and ClpP.

Compound	Mode of Action	References
Dihydrothiazepine 334	Disrupts the hexameric state of methicillin-resistant <i>Staphylococcus aureus</i> (MRSA) ClpX, inhibits ATPase activity and results in the collapse of the ClpXP proteolytic complex.	(De Campos <i>et al.</i> , 2023, Fetzer <i>et al.</i> , 2017)
Thiophene 336	Blocks ClpX ATPase activity in <i>S. aureus</i>	(Fetzer <i>et al.</i> , 2017)
Synthetic peptidomimetic boronate inhibitors	Irreversibly inhibits ClpP in methicillin-resistant <i>S. aureus</i> .	(Bhandari <i>et al.</i> , 2018)
Phenyl ester Compounds	Inhibits ClpP activity via covalent modification of the active site.	(Ishikawa <i>et al.</i> , 2024, Bhandari <i>et al.</i> , 2018, Hackl <i>et al.</i> , 2015)
Armeniaspirols	Competitive inhibition of ClpXP and ClpYQ in <i>Bacillus subtilis</i> and disrupts the regulation of key proteins such as FtsZ, DivIVA, and MreB.	(Labana <i>et al.</i> , 2021)
Acyldepsipeptides	Displace ClpX ATPase by binding to the specific IGF and IGL motifs that are critical for ClpP and Clp ATPase interaction.	(Moreno-Cinos <i>et al.</i> , 2019, Kirstein <i>et al.</i> , 2009)
Hydantoins	Hydantoin 1 and 286 bind to the fully assembled ClpXP complex and inhibits protease activity.	(Fetzer <i>et al.</i> , 2019)
Rufomycin B	Inhibit <i>M. tuberculosis</i> ClpC1 and results in the modulation of protein degradation of intracellular proteins. Binds to a highly acidic region of the ClpC1 ATPase complex of <i>M. tuberculosis</i>	(Choules <i>et al.</i> , 2019)
Lassomycin	and stimulates ATPase activity without stimulating protein degradation.	(Gavrish <i>et al.</i> , 2014)
Cyclomarin A	Binds to the regulatory subunit of the <i>Mycobacterium</i> Clp protease complex resulting in elevated proteolysis and cell death.	(Schmitt <i>et al.</i> , 2011)
Ecumicin	Inhibits ClpC1 proteolytic activity whilst simultaneously stimulating ATPase activity.	(Choules <i>et al.</i> , 2019)
Sclerotiamides	Non-peptide-based natural product activator of ClpP resulting in excessive protein degradation.	(Lavey <i>et al.</i> , 2016)

ClpK is a thermotolerant Clp ATPase, first identified in *K. pneumoniae*, with later studies confirming its presence across multiple *Klebsiella* species (Bojer *et al.*, 2010, Bojer *et al.*, 2012, Motiwala *et al.*, 2021). To date, inhibitors specifically targeting ClpK remain largely unexplored. To address this knowledge gap, previously identified compounds and plant derived flavonoids such as quercetin, (-)-Epigallocatechin-3-Gallate (EGCG), and kaempferol which have been identified to inhibit other Clp

ATPases, heat shock proteins and mitochondrial ATPases (Khan *et al.*, 2013, Zininga *et al.*, 2017, Zanini *et al.*, 2007) were investigated. Candidate compounds were subjected to ADMET (absorption, distribution, metabolism, excretion, and toxicity) analysis to assess pharmacokinetic and safety profiles. The filtered compounds were then docked to ClpK, and the resulting interactions were evaluated by free-binding energy calculations, molecular dynamics simulations and post-dynamic analyses.

4.3. Materials and Methods

4.3.1. ClpK Modelling and Analysis

ClpK was modelled using the AlphaFold server, which predicts three-dimensional (3D) structures using the primary protein sequence (Jumper *et al.*, 2021, Schwede *et al.*, 2003, Ko *et al.*, 2012). The modelled structure was evaluated using the MolProbity, VADAR (Volume, Area, Dihedral Angle Reporter) version 1.8, Protein Structure Analysis (ProSA) web server, ERRAT module on the Swiss v1.6 and the SWISS MODEL structure assessment tool (Williams *et al.*, 2018, Wiederstein and Sippl, 2007, Waterhouse *et al.*, 2018, Willard *et al.*, 2003, Colovos and Yeates, 1993). ClpK binding sites were identified using the DoGSiteScorer module on the ProteinPlus server (<https://proteins.plus/>, Accessed 12 August 2024).

4.3.2. Ligand Preparation

Both small and large ligands were included to explore different potential binding interactions within the ATP-binding region of the Clp ATPase. Small ligands were used to assess interactions within the conserved nucleotide-binding motifs, while larger ligands were included to evaluate whether extended compounds could engage additional surrounding residues or adjacent pockets that may enhance inhibitory potential. The ligands dihydrothiazepine 334 and 336 were drawn using the ChemSketch software while the ADEP1 structure was derived from PDB ID: 3KT and sketched on the ChemSketch software using the SMILES obtained from the Protein Data Bank (PDB). Additional ligands namely, ADEP2 to ADEP 5, Armeniaspirols, phenyl ester compounds (AV126, AV145, AV167, AV170), synthetic peptidomimetic boronate inhibitors (D3, E2, G2, U1), and Hydantoin derivatives were drawn using image identification on the KingDraw software. All ligand structures were saved as .mol files. Sclerotiamide (CID: 10647785), Sclerotiamide P (CID: 170989817), Sclerotiamide L (CID: 170989811), Sclerotiamide N (CID: 170989813), Cyclomarin A

(CID: 10772429), Lassomycin (CID: 132528125), Ecumicin (CID: 101894301), Rufomycin B (CID: 76871757), Sclerotiamide B (CID: 146682810), Quercetin (CID: 5280343), Epigallocatechin gallate (EGCG) (CID: 65064), Kaempferol (CID: 5280863), Fisetin (CID: 5281614), Apigenin (CID: 5280443), Luteolin (CID: 5280445) and Genistein (CID: 5280961) were downloaded in the .sdf format from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>, Accessed 29 July 2024).

4.3.3. Ligand ADMET and Toxicity Analysis

The ProTox 3.0 server was used to predict the toxicity of the small ligands to ensure that they were suitable for use in humans (Banerjee *et al.*, 2024). The SWISSADME server (<http://www.swissadme.ch/>; Accessed 30 July 2024) was used to determine the pharmacodynamics of the small ligands through ADME (Adsorption, Distribution, Metabolism, Excretion) analysis (Daina *et al.*, 2017). The pkCSM server was used to predict ADMET properties of the large ligands (Pires *et al.*, 2015).

4.3.4. Molecular Docking Studies

The ligands selected for analysis were prepared using the LipPrep module on the Maestro Schrodinger software (Yang *et al.*, 2021). Molecular docking was performed using the AutoDock Vina program integrated on the PyRx software (Ayodele *et al.*, 2023). Briefly, the prepared protein was loaded into the PyRx software and made into a macromolecule for docking analysis. The ligands were then loaded into the PyRx software and converted into ligands for docking and saved as .pdbqt files. The grid (Center: X = - 1.1473, Y = -1.1450, Z = -3.1984, Dimensions: X = 93.9220, Y = 105.3030, Z = 107.8333) was set to cover the entirety of ClpK and docking was performed. ClpK-ligand interactions were visualised using Discovery Studio Visualiser 2023 v24.1.0.23298 and PyMol (Schrödinger, 2015).

4.3.5. Molecular Dynamics Simulations using GROMACS

Molecular dynamics (MD) simulations are computational methods used to predict how protein atoms or other organic systems move over time (Hollingsworth and Dror, 2018). MD simulations were performed using GROMACS, an open-source and widely used software package for biomolecular simulation (Abraham *et al.*, 2015). The Graphics processing unit (GPU)-accelerated version of GROMACS v2024.2 was installed on a Windows Subsystem for Linux (WSL) 2. Simulations were performed for

100 ns on ClpK alone and selected ClpK-ligand complexes in the presence of either 0.15 M NaCl and 0.05 M MgCl₂, to assess structural stability and interaction dynamics. The simulation workflow was adapted based on established protocols available on the official GROMACS tutorial website (<http://www.mdtutorials.com/gmx/>) and is briefly summarised in 4.3.5.1. and 4.3.5.2 (Lemkul, 2018).

4.3.5.1. ClpK MD Simulation in the Absence of Ligands

The refined ClpK model was subjected to MD simulation using GROMACS. The initial structure file (protein.pdb) was processed using the pdb2gmh module to generate the topology, position restraint and post-processed (protein.gro) structure files. The all-atom Optimized Potentials for Liquid Simulations (OPLS) forcefield was used, and solvent model specified as SPC/E. A triclinic simulation box was generated with a minimum distance of 1.0 nm between ClpK and box edges. Solvation was performed using transferable intermolecular potential with 3 points (TIP3P) water model with the predefined SPC216 configuration. The system was then neutralised, and ions were added (either 0.15 M NaCl or 0.05 M MgCl₂). Energy minimisation was performed using the steepest descent algorithm for 50000 steps to relieve steric clashes and optimize the initial geometry. The system was then equilibrated in two steps: NVT (number of particles, volume, temperature) and NPT (number of particles, pressure, temperature). The 100 ps NVT ensemble was used to stabilise the temperature at 300 K. The 100 ps NPT ensemble was used to stabilise the pressure at 1 bar. A production MD was subsequently performed for 100 ns using a 2 fs time step. The Leap-frog integrator was used to solve the equations of motion and Verlet cut-off scheme was used to evaluate short-ranged non-bonded interactions. Temperature coupling was controlled using the modified Berendsen thermostat, and pressure coupling was controlled using the Parrinello-Rahman barostat. Long-range electrostatic interactions were calculated using the Particle Mesh Ewald (PME) method.

4.3.5.2. ClpK-Ligand Bound MD simulations

To prepare the ClpK-ligand complexes for MD simulations, ClpK and ligand files were saved as separate files. ClpK structure was processed using the Charmm36-jul2022 force field and solvated using the TIP3P water model. The resulting ClpK_processed.gro file was visualised in PyMol to ensure successful model processing. Ligand preparation was performed using Open Babel and Networkx v2.3.

A Perl script for ligand topology generation was obtained from the GROMACS tutorial site (http://www.mdtutorials.com/gmx/complex/02_topology.html). The ligand.pdb file was converted to ligand.mol2 using Open Babel. The ligand.mol2 file manually edited using a text editor to ensure consistent naming of the ligand (renamed to "LIG") across the header and atom records, and the bond order was verified. The edited ligand_fix.mol2 file was submitted to the CHARMM General Force Field server (CGenFF) (<https://app.cgenff.com/homepage>) to generate topology and parameter files (ligand.str). The generated ligand_ini.pdb file was converted to ligand.gro, which was visualised in PyMol to ensure correct formatting and structure. The processed ClpK and ligand files were then combined using a text editor tool and visualised in PyMol to verify proper merging of the complex. The system topology file (topol.top) was manually updated to include ligand topology and parameters. A triclinic simulation box was defined with a 1.0 nm buffer around the complex, and system was solvated using the TIP3P water model. The solvated system was visualised using PyMol to confirm that the ClpK-ligand complex was centred within the box. The system was then neutralised and either 0.15 M NaCl or 0.05 M MgCl₂ was added. Visual inspection using PyMol confirmed the presence of ions. Energy minimisation was performed to relieve any steric clashes. Prior to equilibration, the ligand was restrained and temperature coupling groups were defined. A position restrain topology file for the ligand was appended to the end of the system topology file. The index.ndx file was examined to confirm successful definition of ClpK, ligand, and solvent groups. Equilibration parameter files (nvt.mdp, md.mdp and npt.mdp) were adapted from the GROMACS tutorial and modified to reflect the custom groups. NVT equilibration was performed for 100 ps at 300 K, followed by NPT equilibration for 100 ps at 1 bar. A 100 ns production MD simulation was then performed to evaluate structural dynamics and protein-ligand complex stability.

4.3.6. Molecular Dynamic Simulation Post-run Analysis

Following the MD simulations, trajectory post-processing was performed to correct for periodic boundary conditions (PBC), which cause the protein to appear broken or diffused across the unit cell. For the apo-ClpK simulation the following analyses were conducted: root mean square deviation (RMSD), root mean square fluctuation (RMSF), potential energy, and radius of gyration (Rg). For the ClpK-ligand complexes, additional analyses were conducted to investigate the impact of ligand binding, these

included: RMSD, RMSF, Rg, potential energy, hydrogen bond analysis, Solvent Accessible Surface Area (SASA), and Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) (Bondi, 1964).

4.4. Results

4.4.1. Clpk Modelling and Binding Pocket Identification

A homology model of ClpK (Figure 4-1A) was generated using the AlphaFold server. The model was evaluated for structural quality using structural parameters (Figure 4-1B) and displayed favourable stereochemical properties with 98.53% of residues in the most favoured region of the Ramachandran plot. Remaining parametric scores confirmed that the predicted ClpK structure was structurally reliable and suitable for further *in silico* analysis.

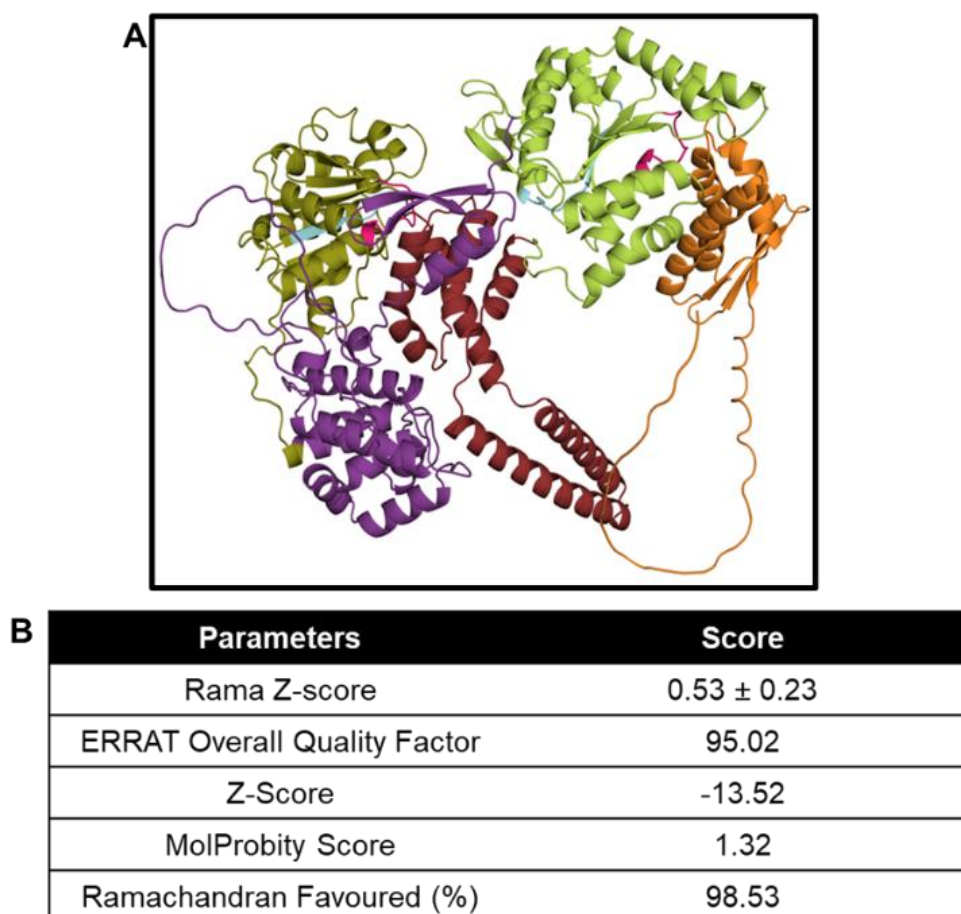


Figure 4-1: ClpK modelled using the AlphaFold server. The structure was visualised and coloured using PyMol (Schrödinger, 2015). Five domains: N-terminal domain (residues 1–244; purple); Nucleotide Binding Domain (NBD) 1 (residues 245–445; olive); the D1-small domain (residues 446–590; maroon); NBD2 (residues 591–814; lime); and the D2-small domain/ C-terminal domain (residues 815–952; orange). The Walker A and Walker B motifs were coloured hot pink and cyan, respectively.

To assess ClpK druggability prior to ligand screening, potential binding pockets were predicted using the DoGSiteScorer module on the ProteinPlus server, which identifies cavities based on a difference of Gaussian filter. A total of 29 potential binding sites were identified, of which 14 binding sites had a druggability score greater than 0.5. Among these 5 models exhibited a SimpleScore greater than 0.5, indicating favourable size, shape, and flexibility for ligand binding (Figure 4-2A, 4-2B) (Volkamer *et al.*, 2010). These pockets contained hydrophobic regions that facilitate protein-protein or protein-molecule interactions (Jernigan *et al.*, 2022), as well as hydrogen acceptors and donors that further facilitate ligand binding (Zhao and Huang, 2011) (Figure 4-2A). Collectively, these features confirmed ClpK as a druggable target suitable for subsequent docking analyses.

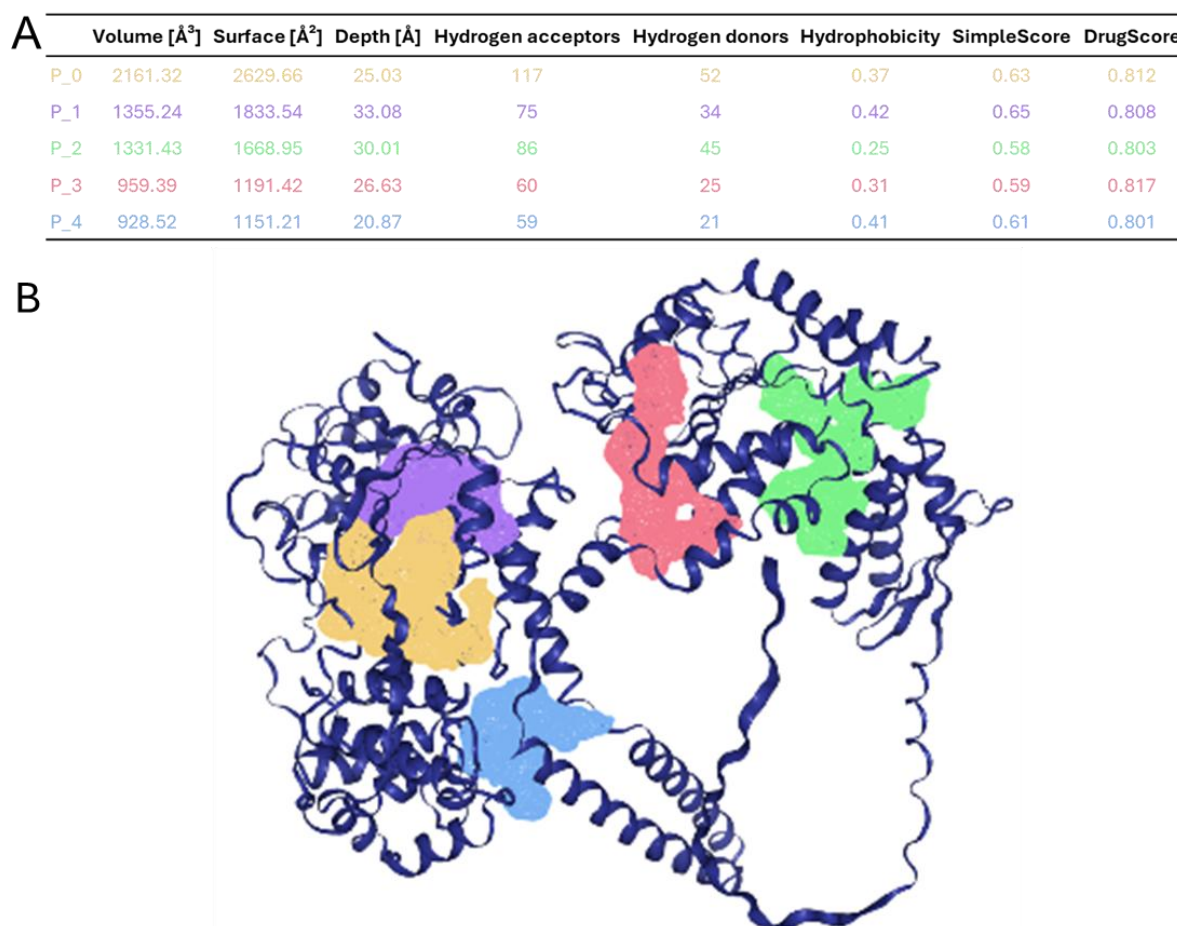


Figure 4-2: Prediction of the potential binding pockets in ClpK. A) Quantitative properties of the predicted binding pockets. B) Visual representation of the top five predicted binding pockets on the ClpK. Each pocket is colour coded to correspond with the data in panel A: P_0 (yellow), P_1 (purple), P_2 (green), P_3 (red), and P_4 (blue). The ClpK backbone is shown in a cartoon representation as navy blue.

4.4.2. ADMET Analysis

To assess oral bioavailability and drug likeliness, the compounds were analysed according to Lipinski's 'rule of five,' which outlines key physicochemical criteria for drug-like behaviour (Varma *et al.*, 2006). According to these rules, a compound is considered drug-like if it meets at least two of the following four criteria; i) molecular weight less than 500, ii) calculated LogP (cLogP) value less than 5, iii) less than 10 H-bond acceptors and iv) less than 5 H-bond donors (Ali *et al.*, 2023). The cLogP value indicates the hydrophilic (negative values) or lipophilic nature of a compound (Silakari and Singh, 2021). All selected small ligands satisfied these criteria, indicating potentially suitability for oral administration (Table 4-2).

Table 4-2: ADME properties of small ligands predicted using the SWISSADME server.

Compound	Molecular Weight (g/mol)	cLogP	H-bond Donor	H-bond Acceptor	Lipinski's Rule
334	357.43	3.87	2	2	4
336	347.45	4.29	1	1	4
G2	180.24	2.59	0	2	4
U1	300.44	5.18	0	2	3
AV126	374.41	3.56	0	6	4
AV145	353.44	2.89	1	5	4
AV170	374.38	3.26	0	7	4
D3	248.36	4.21	0	2	4
E2	230.26	2.56	0	3	4
Armeniaspirols					
Armeniaspirol-A	384.25	3.72	1	4	4
Armeniaspirol -B	384.25	3.59	1	4	4
Armeniaspirol -C	398.28	3.89	1	4	4
5-chloro-armeniaspirols	418.70	4.26	1	4	4
Acyldepsipeptide antibiotics (ADEPs)					
ADEP1	718.84	1.73	3	8	3
ADEP2	798.92	3.10	3	10	3
ADEP3	798.92	2.90	3	10	3
ADEP4	770.86	2.75	3	10	3
ADEP5	843.91	1.71	4	13	2
IDR-10001	744.83	2.49	3	10	3
IDR-10011	790.92	2.64	3	10	3
Hydantoin					
Mephentyoin	218.25	1.48	1	2	4
(+)-Hydantocidin	218.16	-2.33	5	6	4
Azimilide	457.95	2.71	0	6	4
Dantrolene	314.25	0.94	1	6	4
Fosphenytoin	362.27	0.69	3	6	4
Hydantoin-1	481.99	4.13	1	3	4
Hydantoin-286	432.49	3.42	3	4	4
Iprodion	330.17	2.42	1	3	4
Nifurtoinol	268.18	-0.83	1	7	4
Nilutamide	317.22	1.66	1	7	4
Nitrofurantoin	238.16	-0.50	1	6	4
Phenytoin	252.27	1.81	2	2	4
Sorbinil	236.20	0.99	2	4	4
Flavonoids					
Quercetin	302.24	1.23	5	7	4
Epigallocatechin gallate (EGCG)	458.37	0.95	8	11	2
Kaempferol	286.24	1.58	4	6	4
Fisetin	286.24	1.55	4	6	4
Apigenin	270.24	2.11	3	5	4
Luteolin	286.24	1.73	4	6	4
Genistein	270.24	2.04	3	5	4

The selected small ligands demonstrated distinct pharmacokinetic and toxicity profiles, as determined by SWISSADME and ProTox 3.0 analyses. Variations were observed in the predicted bioavailability, absorption, synthetic accessibility, BBB permeability, and toxicity indicating key differences in druggability and safety. The bio-availability score estimates how much of an orally administered compound reaches the blood; a value closer to 1 indicates better bioavailability (Martin, 2005). Among the test ligands, acyldepsipeptide antibiotics (ADEPs) and epigallocatechin gallate (EGCG) had a bioavailability score of 0.17, indicating poor bioavailability, whereas the other ligands had scores >0.5, suggesting good bioavailability (Daina *et al.*, 2017). Gastrointestinal (GI) absorption was high, except for ADEPs, EGCGs and (+)-hydantocidin. Synthetic accessibility scores were less than 5 for all except ADEPs, suggesting ease of synthesis. BBB permeability was observed for nine ligands, including G2 and D3. Toxicity classification ranged from Class 1 (most toxic, fatal if swallowed, ≤ 5 mg/kg) to Class 6 (non-toxic, > 5000 mg/kg); armeniaspirols A - C were predicted to be highly toxic and were excluded from further analysis (Table 4-3) (Ali *et al.*, 2023, Alazmi and Motwalli, 2021).

Table 4-3: ADMET properties of small ligands predicted using the SWISSADME and ProTox 3.0 server.

Compound	Bioavailability score	Gastrointestinal (GI) absorption	Synthetic accessibility	Blood brain barrier (BBB) permeability	LD50 (mg/kg)	Toxicity class
334	0.55	High	4.19	No	1000	4
336	0.55	High	4.21	No	1000	4
G2	0.55	High	3.29	Yes	8000	6
U1	0.55	High	3.26	Yes	2100	5
AV126	0.55	High	3.05	No	4500	5
AV145	0.55	High	3.30	No	500	4
AV170	0.55	High	2.75	No	1000	4
D3	0.55	High	3.71	Yes	5000	5
E2	0.55	High	3.05	Yes	2000	4
Armeniaspirols						
Armeniaspirol-A	0.55	High	4.49	Yes	4	1
Armeniaspirol -B	0.55	High	4.75	Yes	4	1
Armeniaspirol -C	0.55	High	4.86	Yes	4	1
5-chloro-armeniaspirols	0.55	High	4.48	Yes	360	4
Acyldepsipeptide antibiotics (ADEPs)						
ADEP1	0.17	Low	7.13	No	200	3
ADEP2	0.17	Low	7.40	No	200	3
ADEP3	0.17	Low	7.40	No	200	3
ADEP4	0.17	Low	7.31	No	200	3
ADEP5	0.17	Low	7.67	No	200	3
IDR-10001	0.17	Low	7.14	No	200	3
IDR-10011	0.17	Low	7.27	No	200	3
Hydantoins						
Mephénytoin	0.55	High	2.19	Yes	440	4
(+)-Hydantocidin	0.55	Low	4.16	No	2000	4
Azimilide	0.55	High	4.18	Yes	1000	4
Dantrolene	0.55	High	3.27	No	2300	5
Fosphenýtoin	0.56	High	2.92	No	500	4
Hydantoin-1	0.55	High	3.91	No	1300	4
Hydantoin-286	0.55	High	3.52	No	200	3
Iprodion	0.55	High	2.57	Yes	3500	5
Nifurtoinol	0.55	High	3.19	No	1000	4
Nilutamide	0.55	High	2.33	No	625	4
Nitrofurantoin	0.55	High	3.10	No	1000	4
Phenytoin	0.55	High	1.83	Yes	150	3
Sorbinil	0.55	High	3.27	No	5000	5
Flavonoids						
Quercetin	0.55	High	3.23	No	159	3
Epigallocatechin gallate (EGCG)	0.17	Low	4.20	No	1000	4
Kaempferol	0.55	High	3.14	No	3919	5
Fisetin	0.55	High	3.16	No	159	3
Apigenin	0.55	High	2.96	No	2500	5
Luteolin	0.55	High	3.02	No	3919	5
Genistein	0.55	High	2.87	No	2500	5

Among the large ligands, only Sclerotiamide derivatives fully complied with Lipinski's rule of five, while other ligands violated one or more criteria. Maximum tolerated doses were less than 5 mg/kg/day, indicating high oral toxicity and suggesting the need for non-oral administration (Ali *et al.*, 2023). Most ligands were lipophilic ($\log P > 0$), supporting their formulation for non-oral routes, such as injections (Han *et al.*, 2021). Sclerotiamides demonstrated $\log BB \geq -1$, while others showed poor penetration ($\log BB < -1$). Sclerotiamide L was predicted to be toxic and excluded from subsequent analysis (Table 4-4).

Table 4-4: ADMET analysis of the large ligands predicted using the pkCSM server.

	Molecular Weight (g/mol)	LogP	H-bond Donor	H-bond Acceptor	Lipinski's Rule	Max. tolerated dose (human) (mg/kg/day)	Intestinal absorption (human) (%)	BBB permeability (log BB)	AMES toxicity
Rufomycin B	1012.263	5.18	6	11	0	3,43	46.464	-2.112	No
Lassomycin	1880.283	7.24	31	24	0	2,74	0	-8.923	No
Cyclomarin A	1043.317	3.63	7	12	1	3,85	45.419	-2.376	No
Ecumicin	1600.066	3.92	10	18	1	2,74	0	-3.652	No
Sclerotiamides									
Sclerotiamide B	477.561	2.37	2	5	4	0,063	93.807	-0.639	No
Sclerotiamide L	477.561	3.46	2	5	4	0,17	98.277	-0.898	Yes
Sclerotiamide N	445.519	3.18	2	4	4	0,081	93.912	-0.62	No
Sclerotiamide P	463.534	2.33	2	5	4	0,11	98.431	-0.673	No
Sclerotiamide	463.534	1.71	3	5	4	0,086	96.097	-0.665	No

4.4.3. Molecular Docking Studies

Molecular docking analysis revealed binding affinities ranging from -10.9 kcal/mol (Sclerotiamide P) to -4.5 kcal/mol (compound D3) indicating varying binding strengths (Table 4-5). The top ten compounds with the most favourable (negative) binding affinities were selected for subsequent *in silico* analysis, including molecular dynamics simulation and binding free energy calculations.

Table 4-5: Molecular docking scores for ClpK-ligand complexes as obtained using AutoDock Vina on the PyRx software.

Compound	Binding affinity (kcal/mol)
Sclerotiamide P	-10.9
Sclerotiamide	-10.5
ADEP3	-10.2
Sclerotiamide N	-10.1
ADEP1	-9.9
Hydantoin-1	-9.1
Sclerotiamide B	-9.0
Epigallocatechin gallate (EGCG)	-9.0
ADEP5	-8.9
ADEP2	-8.5
IDR-10011	-8.4
Hydantoin-286	-8.4
Fisetin	-8.4
336	-8.2
IDR-10001	-8.2
Fosphenytoin	-8.2
Phenytoin	-8.1
Kaempferol	-8.1
334	-7.9
Quercetin	-7.9
Dantrolene	-7.7
Nilutamide	-7.7
Apigenin	-7.7
Luteolin	-7.7
Rufomycin B	-7.6
ADEP4	-7.5
Azimilide	-7.5
Genistein	-7.5
ADEP4	-7.5
AV126	-7.4
Iprodion	-7.4
Cyclomarin A	-7.3
5-chloro- armeniaspirols	-7.0
Nifurtinol	-7.0
AV145	-6.9
Nitrofurantoin	-6.7
AV170	-6.5
Mephenytoin	-6.5
Lassomycin	-6.5
Ecumicin	-6.5
Sorbinil	-6.5
(+)-Hydantocidin	-6.4
U1	-6.2
E2	-5.5
G2	-5.0
D3	-4.5

The binding interactions of the top 10 ligands and ClpK were visualised in Figure 4-3. All ligands formed multiple stabilising contacts with ClpK, including hydrogen bonds, π - π stacking, amide- π , and hydrophobic interactions, consistent with their docking affinities. Sclerotiamides and ADEP5 predominantly bind within Pocket 0 (N-terminal domain, NBD1, and D1-small domain), EGCG and ADEP1 bind within Pocket 3 (D1-small domain and NDB2 region), hydantoin and ADEP3 interacts in Pocket 1 (primarily engaging NBD1), and ADEP2 in Pocket 4 (making contacts within the N-terminal domain) (Figure 4-2, Figure 4-3). The following residues participated in multiple ligand interactions: PRO283, THR462, ASP282, LEU108, PHE197, VAL198, and GLY279, each involved in five or more ligands, suggesting their role as core binding sites thus suggesting their potential role in ligand recognition and stabilisation.

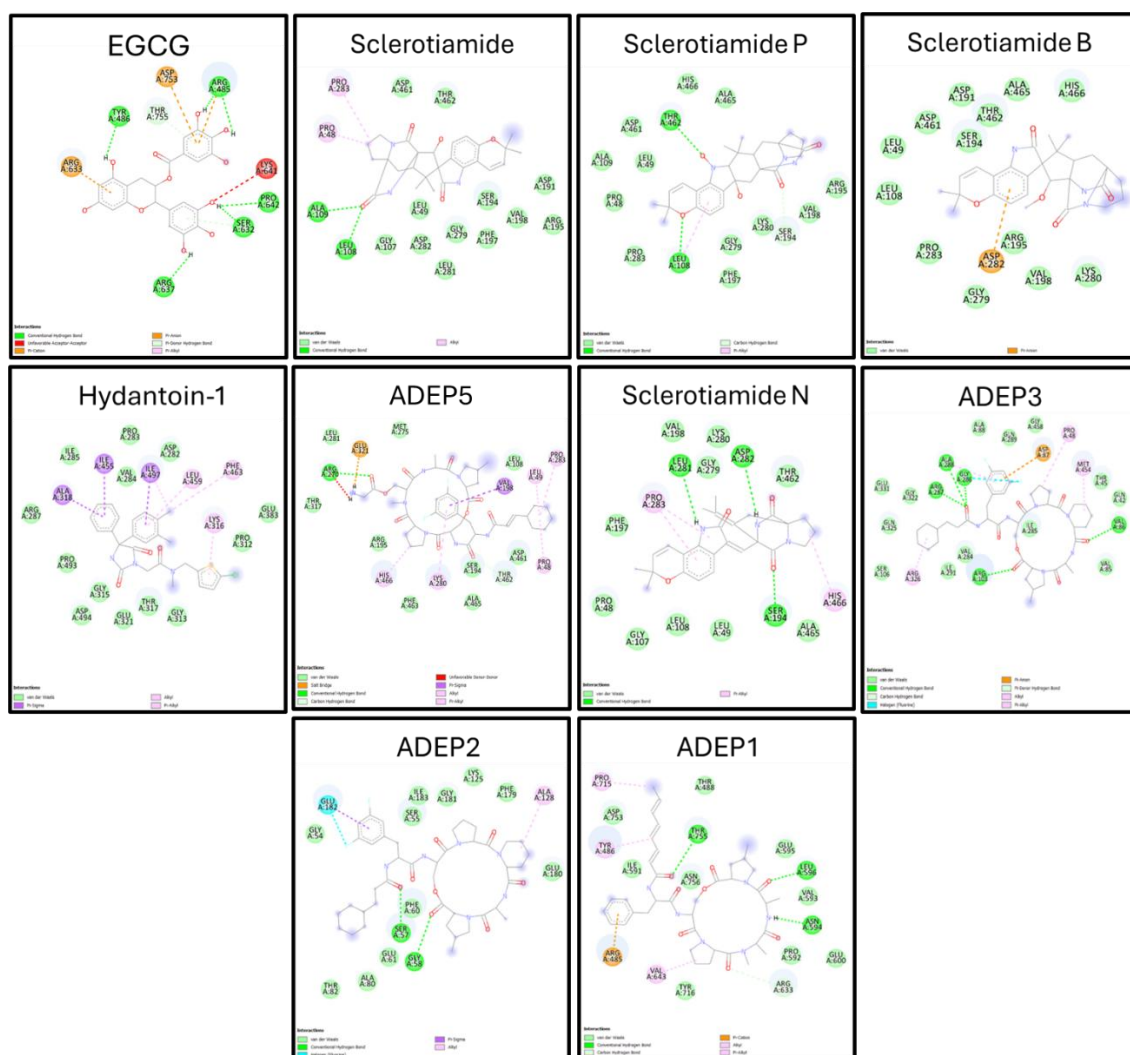


Figure 4-3: Molecular interaction of ClpK with the top 10 ligands exhibiting the strongest binding affinities. Hydrogen bonds are represented by green balls and sticks, π - π /amide- π interactions are represented by violet balls and sticks, π -alkyl/alkyl interactions are presented by pink balls and sticks, π -sulphur bonds are represented by gold balls and sticks, and carbon-hydrogen bonds are represented as light green balls and sticks.

4.4.3. Molecular Dynamics Simulations

MD simulations of 100 ns were performed to investigate ClpK-ligand interactions, stability, and conformational changes. The potential energy of a system indicates the energetic stability of the apo- and complexed ClpK structures (Ajao *et al.*, 2012). The average potential energy of apo-ClpK was observed to be -2813010.92 kJ/mol in the presence of NaCl, and this increased slightly to -2859433.80 kJ/mol in the presence of MgCl₂, indicating a stable and well-equilibrated system under both ionic conditions. The increase in potential energy observed in the presence of compounds is expected as ligand binding would result in an increase in the systems total energy (Figure 4-4). Despite these differences, the potential energy remains stable over the 100 ns simulation period suggesting that the systems reached equilibration and maintained energetic stability.

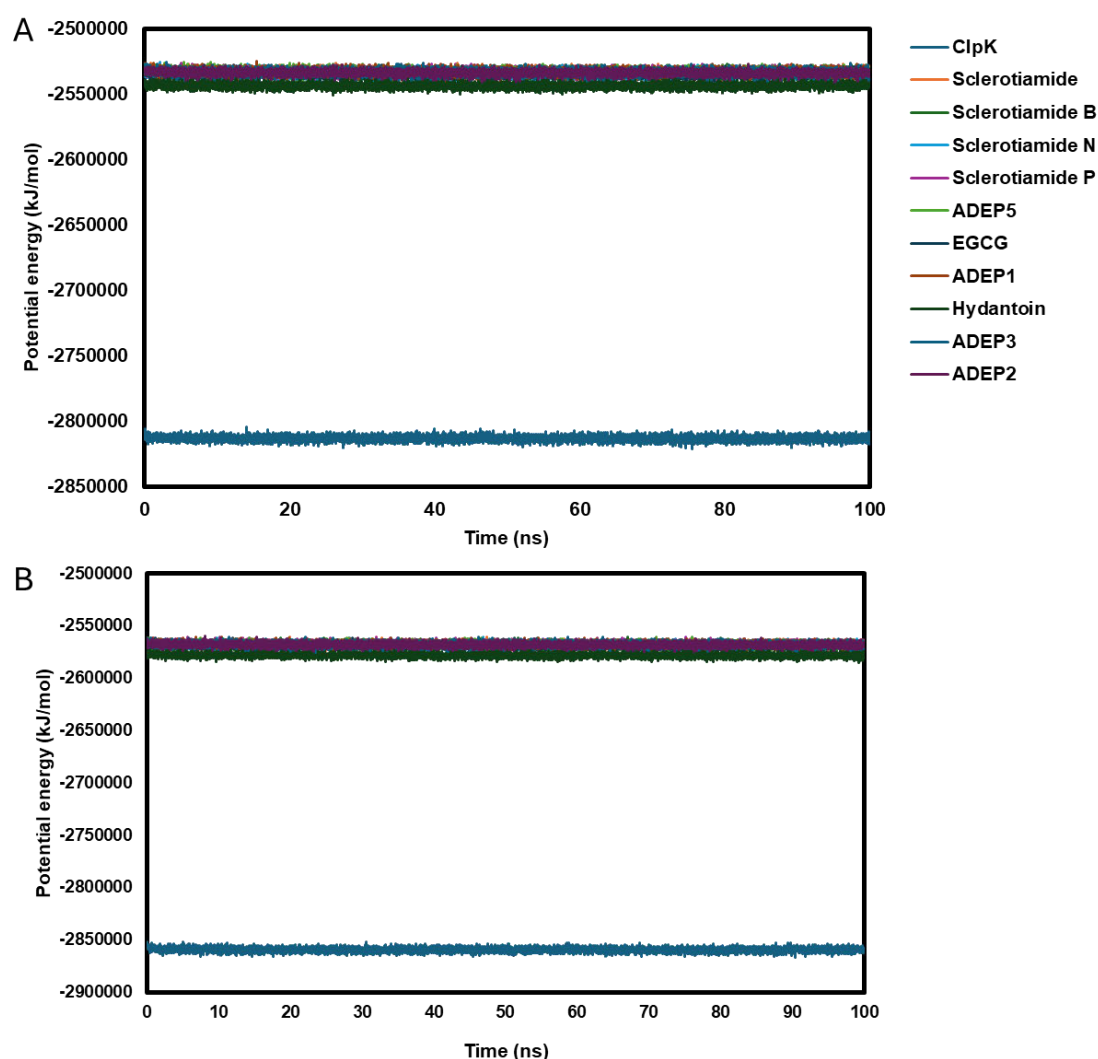


Figure 4-4: Potential energy (kJ/mol) profiles of apo-ClpK and ClpK-compound complexes analysed over 100 ns. In the presence of A) NaCl and B) MgCl₂.

RMSD analysis showed an average of 0.941 nm for apo-ClpK in NaCl, increasing to 1.21 nm in MgCl_2 , indicating greater structural deviations and enhanced flexibility in the presence MgCl_2 (Figure 4-5). Ligand binding increased RMSD in NaCl (1.01 nm to 1.62 nm), with ClpK-Sclerotiamide N showing the highest deviation (1.62 nm). In MgCl_2 , some complexes, notably ClpK-Sclerotiamide-B (1.14 nm) and ClpK-ADEP3 (1.06 nm), exhibited decreased RMSD, indicating stable ligand binding, while others, such as ClpK-ADEP2, displayed increased RMSD up to 1.76 nm, suggesting destabilisation (Figure 4-5).

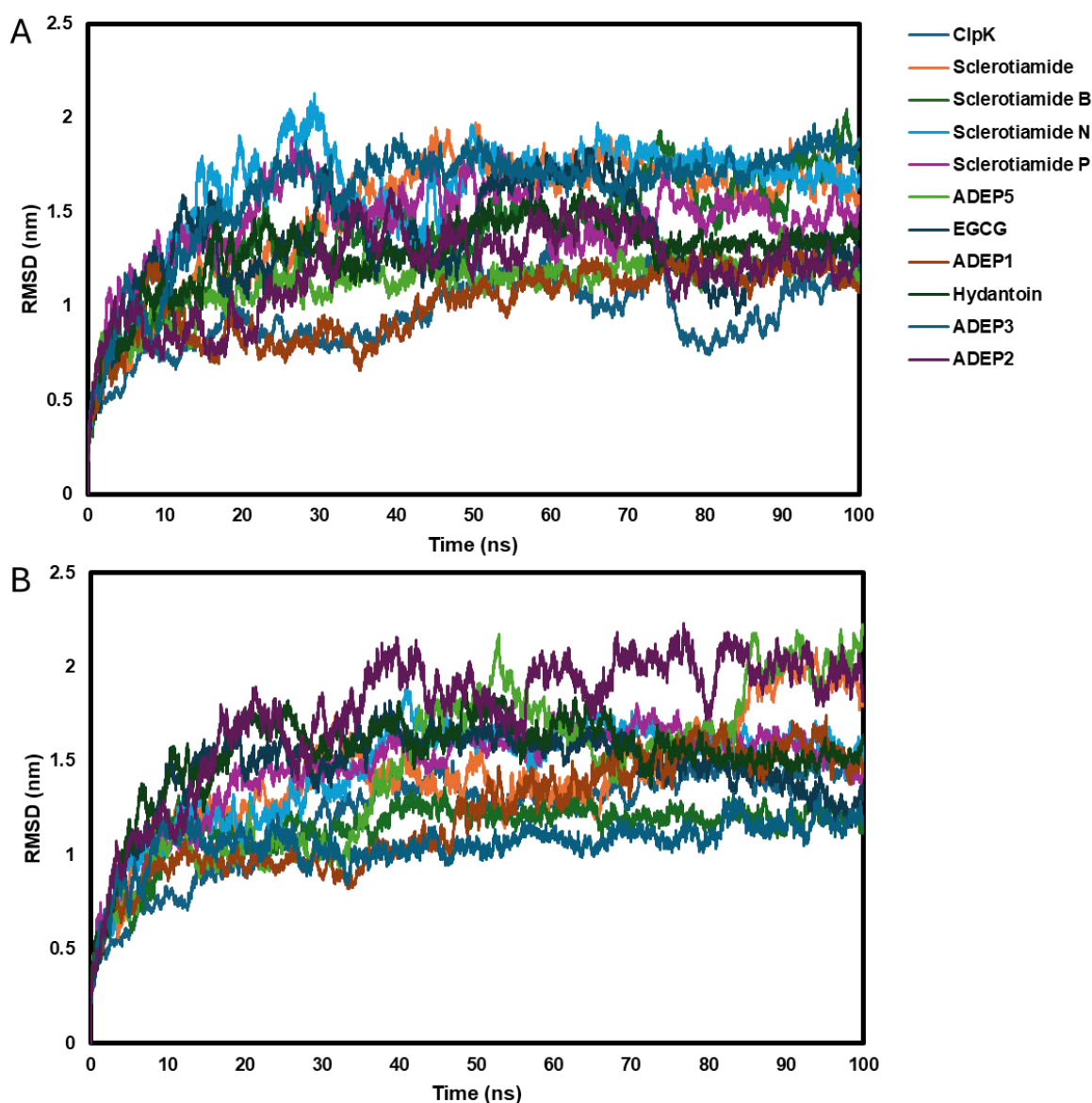


Figure 4-5: RMSD profiles of apo-ClpK and ClpK-compound complexes over 100 ns molecular dynamics simulations. Simulations were run in the presence of A) NaCl and B) MgCl_2 .

Residue flexibility over the 100 ns simulation was assessed using RMSF (Figure 4-6). For apo-ClpK, average RMSF increased slightly from 0.37 nm in NaCl to 0.38 nm in MgCl_2 , consistent with the RMSD results, indicating that MgCl_2 affects structural stability. Ligand binding increased RMSF, ranging from 0.42 nm to 0.64 nm in NaCl and 0.41 nm to 0.62 nm in MgCl_2 . In NaCl, fluctuations were distributed across N-terminal, middle, and C-terminal domains, with Sclerotiamide N exhibiting the highest RMSF (0.64 nm), reflecting destabilisation. In MgCl_2 , fluctuations were primarily at the C-terminal, with ADEP5 (0.62 nm) and ADEP2 (0.61 nm) complexes showing higher RMSF, corresponding to the destabilising effect observed in RMSD analysis.

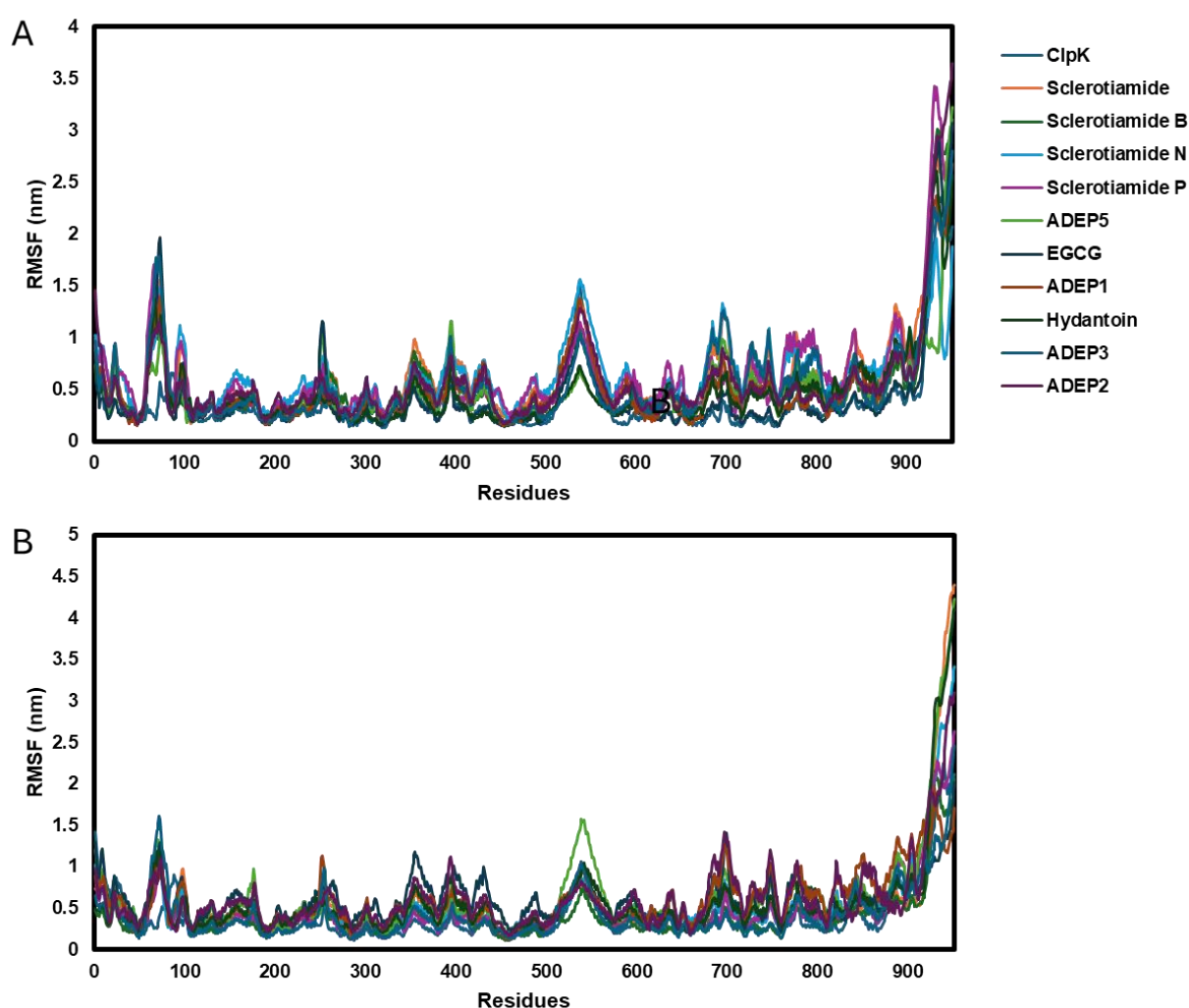


Figure 4-6: RMSF profiles of apo-ClpK and ClpK-compound complexes analysed over 100 ns. In the presence of A) NaCl and B) MgCl_2 .

The Rg was used to assess the structural compactness of ClpK (Figure 4-7). Apo-ClpK maintained stable Rg values of 3.83 nm in NaCl and 3.85 nm in MgCl₂. In NaCl, ligands such as Sclerotiamide B, ADEP1, ADEP5, and Hydantoin reduced Rg, indicating tighter conformations. In contrast, Sclerotiamide, Sclerotiamide N, Sclerotiamide P, EGCG, ADEP3 and ADEP2 showed increased Rg, suggesting loosening or partial unfolding. In MgCl₂, most ligands maintained Rg values comparable to apo. However, Sclerotiamide B, ADEP1 and ADEP3 showed decreased Rg, while Sclerotiamide P, Hydantoin and ADEP2, suggesting Mg²⁺-dependent differences in ligand-induced relaxation.

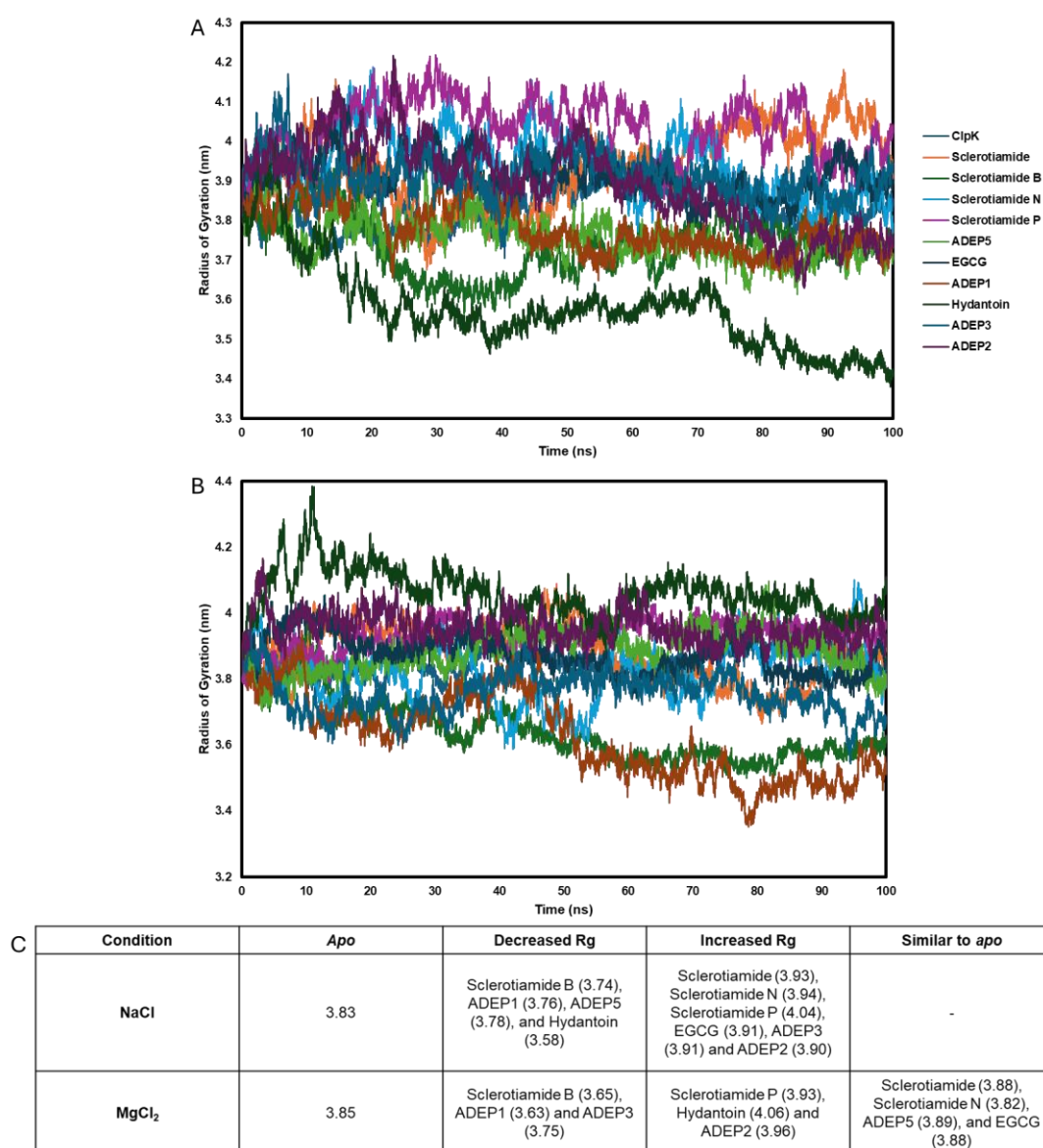


Figure 4-7: Radius of gyration (Rg) profiles of apo-ClpK and ClpK-compound complexes analysed over 100 ns. In the presence of A) NaCl and B) MgCl₂. C) Mean Rg values (nm) of ClpK in apo and ligand bound states.

The SASA represents the portion of the protein that is exposed to surrounding solvent molecules and is important for protein solvation and compactness analysis (Borjian Boroujeni *et al.*, 2021). The average SASA of apo-ClpK was observed to be 514.25 nm²/N in the presence of NaCl and 504.86 nm²/N in the presence of MgCl₂, indicating a slightly more compact conformation under MgCl₂ conditions. A lower SASA indicates reduced solvent exposure, this is associated with tighter protein packing and correlates with the slight increase in Rg observed in Figure 4-6 in the presence of MgCl₂. In the presence of ligands, an increase in average SASA was observed under both ionic conditions. This increase could be attributed to ligand induced conformational changes in the surface-exposed regions of ligand binding sites which may cause protein unfolding or expansion of the surface area (Figure 4-8). Additionally, increased SASA values indicating structural relaxation and therefore a decrease in protein stability (Borjian Boroujeni *et al.*, 2021).

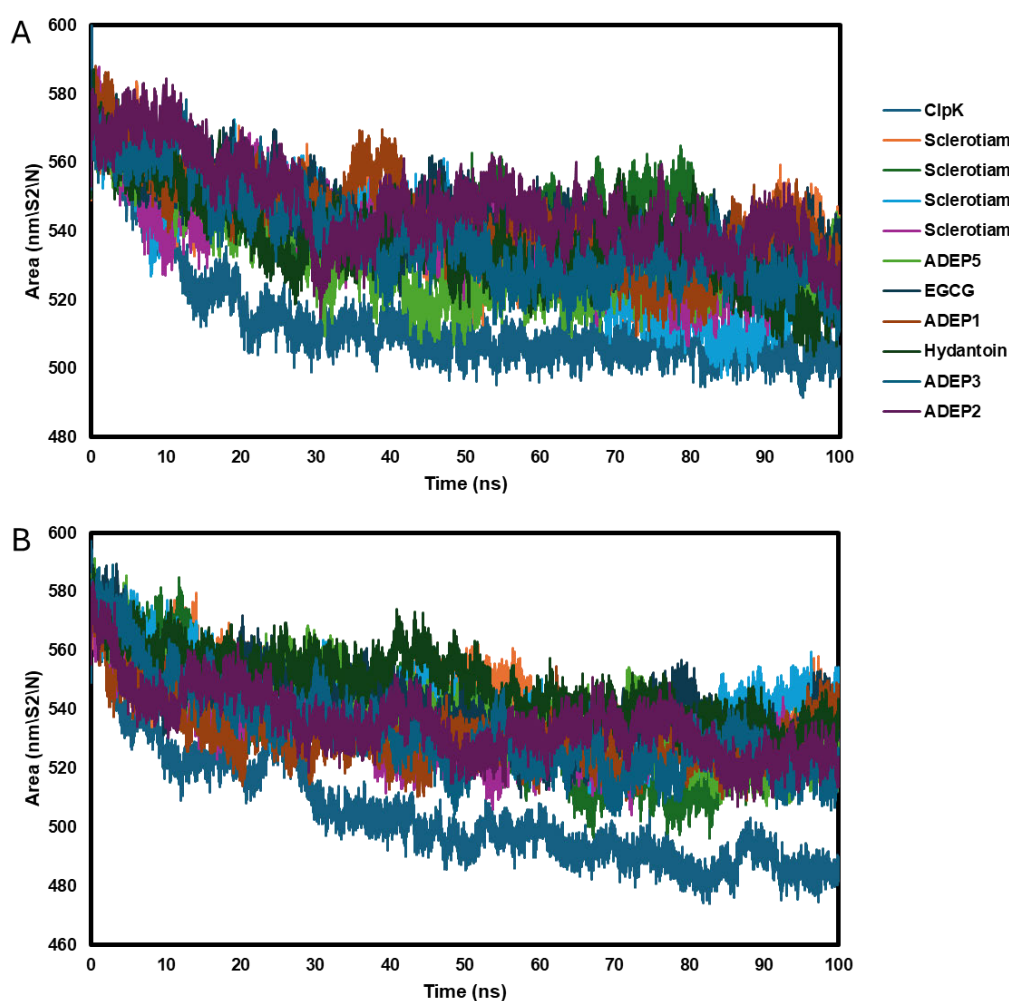


Figure 4-8: SASA profiles of apo-ClpK and ClpK-compound complexes analysed over 100 ns. In the presence of A) NaCl and B) MgCl₂.

The number of hydrogen bonds between proteins and ligands can be investigated to evaluate interaction stability (Molaakbari *et al.*, 2024). To evaluate the dynamic nature of these interactions, the number and persistence was investigated in the presence of NaCl and MgCl₂ (Figure 4-9). A high diversity and fluctuation of hydrogen bonds is observed in the presence of NaCl (Figure 4-9A), however in the presence of MgCl₂ the hydrogen bonds are more stable and persistent (Figure 4-9B). The MD simulations indicate that the ionic environment and ligand binding influence the global structural dynamics of ClpK.

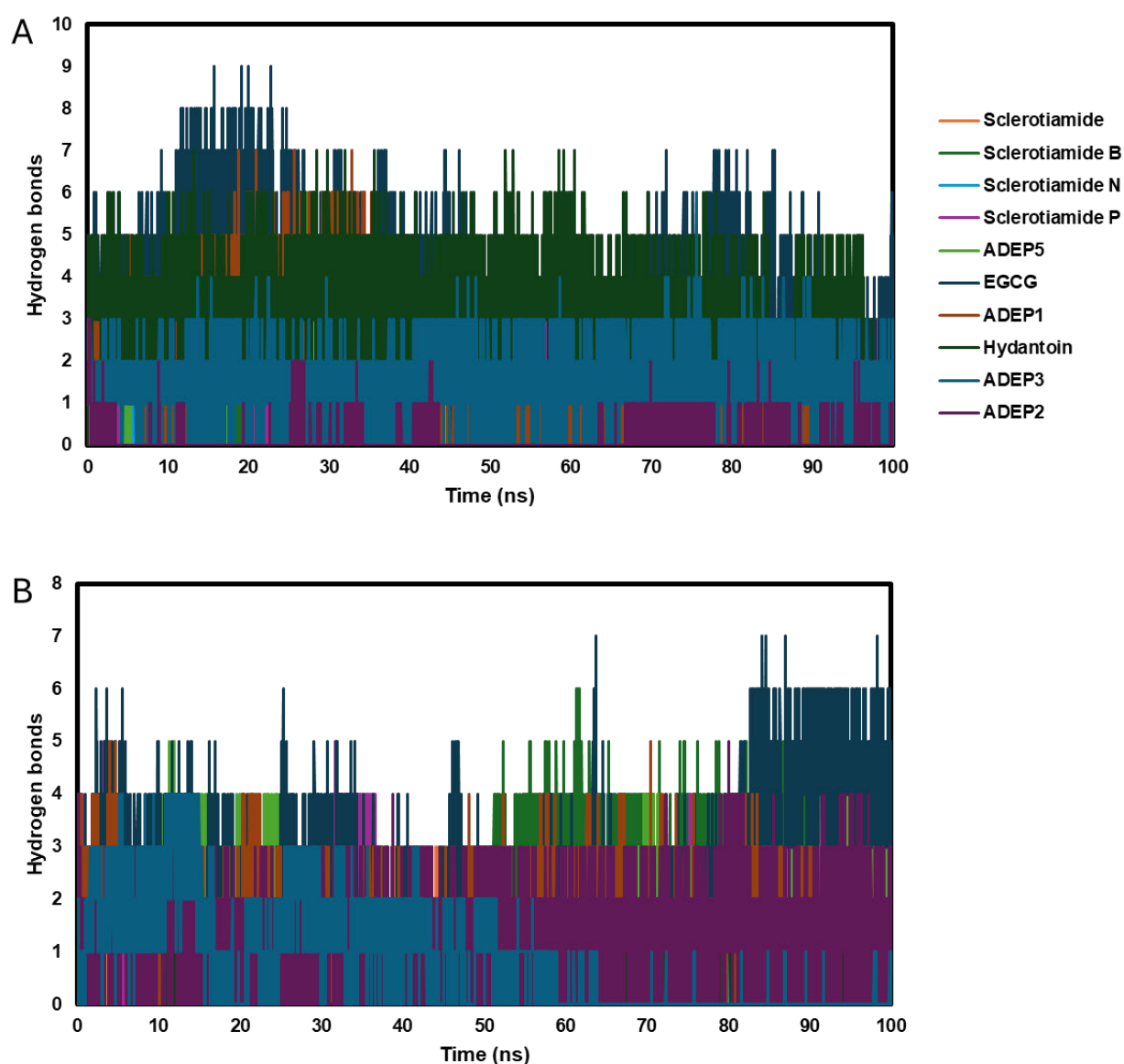


Figure 4-9: Hydrogen bond count between ClpK and compounds over the course of molecular dynamic simulations. The number of hydrogen bonds formed was investigated in the presence of A) NaCl and B) MgCl₂.

Table 4-6 summarises the binding free energies values for the ClpK-compound complexes in the presence of NaCl and MgCl₂, as calculated using MM-GBSA. Negative values indicate favourable binding. In the presence of NaCl, ADEP3 (-41.39 kcal/mol) had the lowest binding energy, followed by Sclerotiamide (-35.65 kcal/mol) and ADEP1 (-35.43 kcal/mol). Similarly, in the presence of MgCl₂, ADEP3 (-42.59 kcal/mol) remained the most favourable binder, followed by ADEP2 (-34.03 kcal/mol) and Sclerotiamide B (27.70 kcal/mol). Interestingly, ADEP3 had the lowest binding affinity in the presence of both NaCl and MgCl₂ suggesting that ADEP3 may be the most stable and effective binder to ClpK among the tested compounds. The binding energies of some compounds varied significantly between some compounds.

Table 4-6: Molecular Mechanics Generalized Born Surface Area (MM-GBSA) analysis of the binding free energy of ClpK and compounds.

	NaCl (kcal/mol)	MgCl ₂ (kcal/mol)
ADEP1	-35.43	-26.28
ADEP2	-17.26	-34.03
ADEP3	-41.39	-42.59
ADEP5	-22.17	-27.13
EGCG	-23.93	-23.24
Hydantoin	-31.50	-16.07
Sclerotiamide	-35.65	-20.93
Sclerotiamide B	-16.76	-27.70
Sclerotiamide N	-17.38	-17.12
Sclerotiamide P	-24.70	-20.34

4.5. Discussion

K. pneumoniae is a multidrug resistant, WHO-designated priority pathogen that urgently requires the development of new therapeutic strategies. The ClpK ATPase, implicated in protein homeostasis and stress tolerance, represents a potential target for therapeutic development (Shrivastava *et al.*, 2018, Abbas *et al.*, 2024). In this study, *in silico* approaches were used to investigate the binding efficiency and dynamic stability of selected natural and synthetic compounds with ClpK under varying ionic conditions.

Binding site prediction in the ClpK homology model revealed 14 potential binding pockets, five of which were classified as druggable based on the pocket size and SimpleScore metrics (Figure 4-2). Computational binding sites prediction provides a valuable first step in drug discovery however, they may be limited by data quality and standardisation challenges (Stratiichuk *et al.*, 2025). To explore the functional significance of these predicted sites, molecular docking and molecular-dynamics simulations were subsequently performed to evaluate ligand binding, interaction

stability, and potential inhibitory effects. A preliminary screening of natural and synthetic compounds (Tables 3-2 – 3-4) enabled the exclusion of molecules with unfavourable ADMET characteristics, refining the selection for downstream docking and dynamics studies. Docking analysis identified several high affinity compounds, mainly the sclerotiamide derivatives and ADEP analogues (Table 4-4) and this is consistent with findings that reported sclerotiamide activates ClpP, while ADEP can act as both a ClpP activator and inhibitor (Vass and Chien, 2016, Lavey *et al.*, 2016).

To further characterise binding, ligand interaction profiling was conducted for the top ten compounds. Compounds were observed to form hydrogen bonds, π - π stacking, and hydrophobic interactions with residues within key functional domains of ClpK including, the N-terminal domain, NBD1, D1-small domain, and NBD2 (Figure 4-3). These interactions reinforced binding stability and specificity with hydrogen bonds orientating the ligands, π - π stacking enhancing affinity and hydrophobic contacts enhancing stability (Madushanka *et al.*, 2023, Brylinski, 2018, Ferenczy and Kellermayer, 2022). Additionally, the involvement of multiple domains is advantageous for drug development potentially offering avenues for the development of compounds that disrupt ClpK's interdomain interaction and ATPase function (Ferrari *et al.*, 2003).

The structural dynamics of these multi-domain interactions were investigated using 100 ns MD simulations with NaCl and MgCl₂ to explore how these ionic conditions effect structural stability and flexibility. Since ionic environments can alter protein electrostatics, conformational flexibility, and ligand stability, this comparison can provide an insight into the physiochemical factors that can influence inhibitor binding (Schönichen *et al.*, 2013). The potential energy profiles of apo-ClpK and ligand-bound complexes remained stable throughout the simulations (Figure 4-4). Suggesting that complexes were energetically and thermodynamically stable, allowing for the reliable interpretation of structural and dynamic parameters (Ajao *et al.*, 2012).

RMSD trajectories (Figure 4-5) revealed distinct stability profiles under the two ionic conditions. In the presence of NaCl, the average RMSD values increased upon ligand binding, with the sclerotiamide N complex exhibiting the highest deviation. In contrast, systems exhibited slightly higher RMSD fluctuation in MgCl₂ suggesting enhanced overall flexibility. This indicates that the stronger electrostatic influence of Mg²⁺ ions may perturb intramolecular interactions and promote local rearrangements in the

protein backbone (Schauss *et al.*, 2021). This observation is consistent with findings reported by Shao *et al.* (2022), where simulations of protein 1BBL in the presence of MgCl_2 also showed increased RMSD values, indicating enhanced protein dynamics at moderate salt concentrations. In the presence of MgCl_2 , sclerotiamide B and ADEP3 complexes maintained stable ligand binding, while ADEP2 increased average RMSD values suggesting destabilisation. Overall, RMSD values showed that most complexes reached equilibrium within the 100 ns timeframe.

RMSF analysis (Figure 4-6) was consistent with the RMSD findings and provided an insight into local dynamics. A moderate increase in residue level fluctuations were observed in the presence of NaCl, with the binding of sclerotiamide N exhibiting the highest fluctuation. In contrast, elevated peaks were observed in the presence of MgCl_2 particularly in the C-terminal region. Increased fluctuations observed for the ADEP2 and ADEP5 complexes indicated that specific ligand interactions may induce local destabilisation in the presence of MgCl_2 . Complementary analysis of Rg (Figure 4-7) further investigated the influence of ions on the overall structural organisation. ClpK-ligand complexes maintained relatively consistent Rg values in the presence of NaCl, indicating preserved structural compactness and stable folding (Motiwala *et al.*, 2025). In contrast, higher Rg values with more pronounced fluctuations were observed in the presence of MgCl_2 indicating increased structural flexibility. This corresponds with the observed RMSD trends which indicate that the Mg^{2+} ions promote a more relaxed conformational state. However, all complexes reached equilibrium by the end of the 100 ns simulation time, indicating overall complex stability under both ionic conditions suggesting that ionic composition modulates ClpK-ligand interactions.

The ion-dependent difference in protein-ligand interaction stability were further investigated through SASA analysis (Figure 4-8). The average SASA of apo-ClpK decreased in the presence of MgCl_2 suggesting that a more compact protein conformation is promoted under these conditions. This is also observed for the ClpK-ligand complexes where lower SASA values in the presence of MgCl_2 suggest reduced solvent exposure and enhances structural compactness. In contrast, in the presence of NaCl higher and more fluctuating SASA values are observed therefore indicating structural relaxation (Borjian Boroujeni *et al.*, 2021). This variability can further be supported through hydrogen bonding analyses (Figure 4-9). It was observed that NaCl

promoted more diverse and fluctuating hydrogen bond patterns suggesting dynamic and transient interactions. In contrast, stable and persistent hydrogen bonds were observed in the presence of MgCl_2 suggesting stronger electrostatic stabilisation and reduced conformational mobility. These structural observations were consistent with MM-GBSA binding free energy results (Table 4-5) where the negative binding free energy values of all complexes indicated that the compounds bind to ClpK under both ionic conditions, supporting their potential biological relevance. Additionally, these findings highlight the importance of carefully considering buffer composition in molecular simulations and drug screening workflows due to the influence ionic interactions can have on predicted binding behaviours.

In this study, ten compounds demonstrated favourable docking scores, stable binding interactions, and thermodynamically viable complexes during 100 ns molecular dynamics simulations under both NaCl and MgCl_2 conditions. These compounds included Sclerotiamide derivatives, ADEP analogues, EGCG and hydantoin. Importantly, many of these compounds have been identified previously as modulators of bacterial proteostasis, strengthening their potential for repurposing against *K. pneumoniae*. Favourable binding free energy and stable ligand retention were observed in the presence of ADEP3, suggesting that this compound may disrupt ATP cycling or interferes with domain coordination within ClpK. ADEP2 also exhibited high binding affinity but increased sensitivity was observed in the presence of Mg^{2+} . This suggests possible interference with Mg^{2+} -dependent ATP binding or hydrolysis at the Walker motifs. This interpretation is supported by the established mechanism of ADEPs, which are well-characterised ClpP-targeting antibiotics which displace the ClpX ATPase by binding to the IGL/F motifs to disrupt ClpP binding, resulting in the accumulation of misfolded proteins, cellular toxicity, and bacterial death (Moreno-Cinos et al., 2019, Kirstein et al., 2009). Similarly, sclerotiamide has been shown to activate ClpP and induce excessive protein degradation (Lavey et al., 2016). In this study, Sclerotiamide B remained structurally stable in the presence of both NaCl and MgCl_2 ,

suggesting sustained binding that may stabilise a non-productive ClpK conformation. In contrast, Sclerotiamide N induced moderate conformational fluctuations suggesting that the molecule may disrupt interdomain communication necessary for coordinated substrate processing. Hydantoins have been reported to bind to the ClpXP complex

and inhibit protease activity (Fetzer et al., 2019). Stable interactions within functional domains were also observed in the presence of EGCG suggesting that the compound may restrict the ATPase machinery. EGCG has primarily been investigated in eukaryotic systems, however it has been reported to bind to the C-terminal of HSP90 to prevent protein dimerization and inhibit chaperone function (Moses et al., 2015).

While these compounds have not yet been directly investigated against ClpK in *K. pneumoniae*, their established activity against Clp systems in other bacterial species provides mechanistic precedent for targeting AAA+ proteostasis machinery. Given that ClpK contributes to stress tolerance and survival in hostile environments, including heat and potentially antibiotic stress, inhibition or dysregulation of its ATPase function may sensitize *K. pneumoniae* to host immune pressures or existing antibiotics. Therefore, the identification of stable ClpK–ligand complexes in this study not only supports structural feasibility but also suggests a viable therapeutic strategy based on repurposing proteostasis-targeting molecules for multidrug-resistant Gram-negative pathogens.

In conclusion, this study provides a foundation for future *in vitro* studies that focus on validating the predicted interactions. To validate the *in silico* findings, it is important to assess ClpK ATPase activity in the presence of these ligands. Based on the docking affinities, MM-GBSA binding free energies, stability across ionic conditions and favourable MD parameter, Sclerotiamide B, Sclerotiamide N, ADEP3, ADEP2 and EGCG could be prioritised as the top five candidates for further evaluation. These experiments should be performed under varying physiological conditions, including different pH levels and salt concentrations to reflect the dynamic intracellular environment. Additionally, the impact of these compounds on ClpK thermal stability would offer insights into their potential to function as stabilisers or destabilisers of ClpK. Collectively, these studies would help confirm the biological relevance of the predicted interactions and further support the development of ClpK as a promising therapeutic target against *K. pneumoniae*.

4.6. References

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Chapter 5: Using reverse vaccinology to construct multi-epitope subunit vaccine candidates for *Klebsiella pneumoniae*.

5.1. Abstract

Klebsiella pneumoniae is a Gram-negative clinically relevant pathogen responsible for causing nosocomial infections. This bacterium is resistant to a spectrum of antibiotics and has been listed under “critical” priority for research and development of new antibiotics and alternative strategies. Reverse vaccinology was used to construct multiepitope vaccine candidates against six *K. pneumoniae* outer membrane proteins. Predicted B- and T-cell epitopes were evaluated using various bioinformatic tools to confirm their antigenic and non-allergenic nature. Additionally, the global population coverage of the epitopes was observed to be 75% as per *in silico* analysis. These epitopes were then used for the design and *in silico* characterization of seven multiepitope vaccine candidates. Molecular docking and simulation studies demonstrated that 5 out of the 7 vaccine candidates formed strong stable interactions with TLR2 and TLR4. Furthermore, *in silico* immunization simulations demonstrated the ability of these candidates to elicit effective immune responses. Lastly, high codon adaptation Index values ensured that the candidates can be expressed in the *Escherichia coli* K12 host system following codon optimized multiepitope cloning. Importantly, the high antigenicity and global coverage of the combined construct represent a novel combination not previously reported. Based on our findings, the designed multiepitope vaccine candidates have potential as a vaccination strategy against *K. pneumoniae*.

5.2. Introduction

Klebsiella pneumoniae is a Gram-negative, opportunistic pathogen responsible for severe nosocomial infections, including pneumoniae, sepsis, and urinary tract infections (Sharma *et al.*, 2023, Della Rocca *et al.*, 2022, Zhang *et al.*, 2021). From 2014 to 2019, *K. pneumoniae* was responsible for 26% of the invasive neonatal infections in South Africa (Magobo *et al.*, 2023). The Burden of Antibiotic Resistance in Neonates from Developing Societies (BARNARDS) network identified *K. pneumoniae* as being accountable for 10% of total sepsis deaths and was the main cause of neonatal sepsis in low- and middle-income countries (Assoni *et al.*, 2021, Sands *et al.*, 2021). Also, the World Health Organization (WHO) has recognized the antimicrobial resistance (AMR) capabilities of *K. pneumoniae* and has listed this antibiotic-resistant pathogen under the “critical” priority for research and development of new antibiotics and alternative strategies (Zhang *et al.*, 2021). Limited treatment options mean that infections caused by *K. pneumoniae* are associated with high mortality and morbidity rates. Therefore, it has become increasingly important to investigate alternative therapies to reduce the burden of these pathogens on the healthcare sector (Navon-Venezia *et al.*, 2017).

Vaccines are a successful and cost-effective way of improving health and living standards which can be used to control the spread of diseases and are one of the most effective investments in the health care sector (Rodrigues and Plotkin, 2020, Seib *et al.*, 2012). According to the WHO, approximately 800 000 lives are saved per year in the African region due to vaccines (World Health, 2018). To our knowledge, there is no approved commercially available vaccine for *K. pneumoniae*. Initially, inactivated, and whole cell *K. pneumoniae* showed potential as vaccine candidates. However, both these vaccine candidates cause adverse effects such as fever and septic shock due to the presence of lipopolysaccharides (LPS) and capsular polysaccharides (CPS) (Shahbazi *et al.*, 2023, Chen *et al.*, 2024, Cuscino *et al.*, 2022). Furthermore, immunization with one strain of *K. pneumoniae* does not ensure protection against different bacterial strains due to genetic and antigenic diversity (Cuscino *et al.*, 2022, Umitaibatin *et al.*, 2023, Holt *et al.*, 2015). Consequently, they were not approved for clinical applications (Chen *et al.*, 2024, Cuscino *et al.*, 2022). Inactivated vaccines (such as the Polio and Influenza vaccines) require multiple doses to be administered to confer protective immunity (Yadav *et al.*, 2014, Chen *et al.*, 2024,

Cuscino *et al.*, 2022). This can be time consuming and disadvantageous in areas where people do not have access to healthcare facilities (Yadav *et al.*, 2014, Health and Human, 2022). In 2021, a *K. pneumoniae* tetravalent bioconjugate vaccine (Kleb4V) which includes O antigen-polysaccharides of the most predominant *K. pneumoniae* serotypes entered Phase I/II in 2021 (Wantuch *et al.*, 2023). This O polysaccharide (OPS) vaccine was promising as four out of the eight *K. pneumoniae* OPS have been indicated to cover more than 80% of clinical isolates worldwide (Chen *et al.*, 2024). However, the full outcomes of the trial are yet to be published.

Over the past decade, technological advancement has led to the development and application of reverse vaccinology in vaccine related research. This method uses *in silico* analysis to identify antigenic bacterial proteins that have a potential to be used as vaccine candidates (Cuscino *et al.*, 2022, Martinelli, 2022, Da Silva *et al.*, 2023). Researchers are using reverse vaccinology to target conserved peptides from rapidly mutating bacteria (Tsai *et al.*, 2011, Umitaibatin *et al.*, 2023). This approach is advantageous as it does not use a live attenuated pathogen, therefore the chances of reversion to the virulent strain is abolished. Furthermore, live attenuated vaccines may retain residual pathogenicity, making them unsuitable for immunocompromised patients (Whitaker *et al.*, 2019). In such cases, alternative vaccine options, such as epitope-based vaccines provide long-lived immunity without compromising safety (Kalita *et al.*, 2020). While reverse vaccinology offers numerous advantages, it also has significant limitations. Limited knowledge of vaccine immunology means that protective immunity is the rate-limiting step of reverse vaccinology. Furthermore, this approach cannot be used to identify non-protein or CD1-restricted antigens such as polysaccharides or glycolipids which are important components of successful vaccines (Rappuoli, 2000). A fusion antigen, KP-Ag1 was identified through reverse vaccinology to provide protective immunity against *K. pneumoniae* infection in pneumonia murine models and further highlighted its potential as a vaccine candidate through humoral and cellular immune responses in mice (Chen *et al.*, 2024).

To design a multiepitope vaccine, the first step involves the identification of a specific target protein such as a surface protein (Martinelli, 2022, Da Silva *et al.*, 2023). Like other Gram-negative bacteria, the cell envelope of *K. pneumoniae* contains three layers, namely, the outer membrane, the peptidoglycan cell wall, and the inner

membrane (Silhavy *et al.*, 2010, Duong *et al.*, 1997). Porins are conserved pore-forming channel proteins located on the outer membrane surface, comprising of antiparallel beta-strands organized to form beta-barrels. These beta-barrels are hollow, water-filled channels allowing the transport of molecules such as iron, nutrients, and antibiotics across lipid bilayer membrane into the cell (Della Rocca *et al.*, 2022, Belousov *et al.*, 2023, Prajapati *et al.*, 2021). Porins play an important role in bacterial virulence by mediating the adhesion of bacterial cells to host cells (Della Rocca *et al.*, 2022, Sharma *et al.*, 2022). These surface proteins are more likely to be recognized by various elements of the immune system and are thus promising drug targets and vaccine candidates. Zhang *et al.* (2021) identified OmpC, OmpW, and YfaZ porins as surface proteins that can induce an immune response in two *K. pneumoniae* infection models. Two other porins, OmpK35 and OmpK36, expressed by extended-spectrum beta-lactamase (ESBL) producing *K. pneumoniae*, and have been associated with mediating carbapenem resistance in *K. pneumoniae* by limiting carbapenem influx (Wong *et al.*, 2022, Tsai *et al.*, 2011). OmpF was studied in various pathogens and was found to induce bactericidal antibody responses in *Salmonella typhi* and *Escherichia coli* (Wang *et al.*, 2017).

A dual-compartment strategy is presented in this thesis through targeting intracellular stress-response machinery (ClpK - chapter 2 to 4) while simultaneously enhancing host immune recognition at the bacterial surface. ClpK inhibition potentially compromises intracellular protein quality control, thus reducing the pathogens ability to withstand host-mediated stresses. Simultaneously, vaccination targeting surface-exposed OMPs potentially enhances immune recognition and promotes antibody- and complement-mediated clearance.

In this study, the above mentioned porins (namely, OmpK35, OmpK36, OmpW, OmpF, OmpC and YfaZ) of *K. pneumoniae* were identified based on their immunogenic potential and ability to elicit an immune response. Among these, porins such as OmpK35, OmpK36 and YfaZ remain underexplored in combinatorial vaccine constructs. The porins were then characterized using a reverse vaccinology approach. Seven multi-epitope vaccine candidates containing T- and B-cell epitopes were designed and analysed for antigenic potential using molecular docking, molecular dynamic analysis, and immunization simulations.

5.3. Materials and Methods

5.3.1. Protein sequence retrieval and analysis

The protein sequences of OmpK36, OmpC, OmpF, YfaZ, OmpW, and OmpK35 from *K. pneumoniae* were downloaded from NCBI Protein Database (<https://www.ncbi.nlm.nih.gov/protein/>, accessed 10 June 2024). The sequences of the three proteins (OmpC: QDX70306.1, OmpW: QEX42815.1, YfaZ: QDX64613.1) identified by Zhang *et al.* (2021) were also downloaded and included in analysis. The downloaded sequences were aligned using the Clustal Omega server to finalize the sequences to be used for further analysis (Sievers *et al.*, 2011). The sequences were then uploaded to the VaxiJen v2.0 server using a threshold of 0.4 for antigenicity prediction, target organism; bacteria (Doytchinova and Flower, 2007).

5.3.2. B-cell, CTL and HTL epitope prediction

The conservancy of the sequences selected from the six protein groups was analysed using the Clustal Omega server (Sievers *et al.*, 2011). To identify linear B-cell epitopes, the machine learning-based ABCPred server was used within a 0.51 threshold (Saha and Raghava, 2006). The NetCTL 1.2 server was used to identify 9-mer cytotoxic T-lymphocyte (CTL) epitopes from the chosen protein sequences, using the default threshold of 0.75. This server predicts peptide major histocompatibility complex (MHC) class I binding using artificial neural networks (ann) to predict MHC/peptide binding for 12 MHC class I supertypes (Larsen *et al.*, 2007). The server IEDB MHC-II calculates binding capabilities of peptides from the chosen sequence towards the alleles and uses percentile rank which is inversely related to binding affinities (Khalid *et al.*, 2022). The IEDB MHC-II server was used to predict helper T-lymphocyte (HTL) epitopes related to a reference set of seven human leukocyte antigens (HLA), namely; HLA-DRB1*03:01, HLA-DRB1*07:01, HLA-DRB1*15:01, HLA-DRB3*01:01, HLA-DRB3*02:02, HLA-DRB4*01:01, and HLA-DRB5*01:01 (Reynisson *et al.*, 2020). The top ten highest scoring epitopes obtained from each server were further assessed. Threshold values for the various servers were selected based on literature-reported standards and guidelines provided by the respective developers.

5.3.3. B-cell, CTL and HTL epitope analysis

The selected HTL epitopes were assessed using IFN-epitope, an online tool which identifies the HTL epitopes that stimulate interferon-gamma (IFN γ) cytokines (Dhanda *et al.*, 2013). The server uses a mixed model which includes motifs and Super Vector Machine (SVM) models. The AllerTop v. 2.0 server, VaxiJen 2.0, IEDB Class I immunogenicity and Toxinpred tools were used to assess the allergenicity, antigenicity, immunogenicity, and toxicity of the selected epitopes (B-cell, HTL, and CTL) (Dimitrov *et al.*, 2014, Doytchinova and Flower, 2007, Calis *et al.*, 2013). The population coverage of these peptides was assessed using the IEDB Population coverage analysis tool (Bui *et al.*, 2006). Calculations for different areas including MHC classes were done separately and averaged using Excel. Threshold values for the various servers were selected based on literature-reported standards and guidelines provided by the respective developers.

5.3.4. Multiepitope subunit vaccine (MESV) construction

The chosen high affinity B-cell, CTL and HTL epitopes were connected in a sequence wise manner using connecting sequences known as 'linkers' to construct multiepitope subunit vaccine (MESV) candidates (Figure 5-1) (Martinelli, 2022, Khalid *et al.*, 2022). To avoid redundancy in the combined construct, care was taken to exclude identical or overlapping epitopes derived from different proteins. Inserting the AAY and KK sequences between the CTL and B-cell epitopes respectively, increases epitope presentation while the insertion of the glycine rich linkers; GP GPG or GGGGS, between the HTL epitopes allows for a better simulation of the HTL immune response (Khalid *et al.*, 2022, Tarrahimofrad *et al.*, 2021).

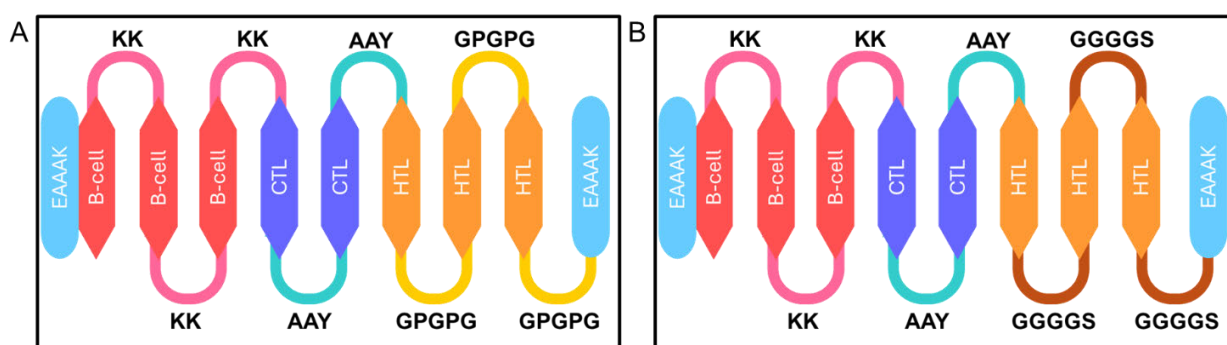


Figure 5-1: Schematic representation of the assembly of the final vaccine candidates. Schematic representations of the linkers used for the construction of the six vaccine candidates: (A) OmpK35, OmpF, and OmpW, and (B) OmpK36, OmpC, and YfaZ.

The B-cell, CTL and HTL epitopes with the highest affinity from each protein group was used to construct a combination multiepitope vaccine (Figure 5-2). AAY and KK sequences were used to link the CTL and B-cell epitopes respectively, while GGGGS sequence was used to link the HTL epitopes.

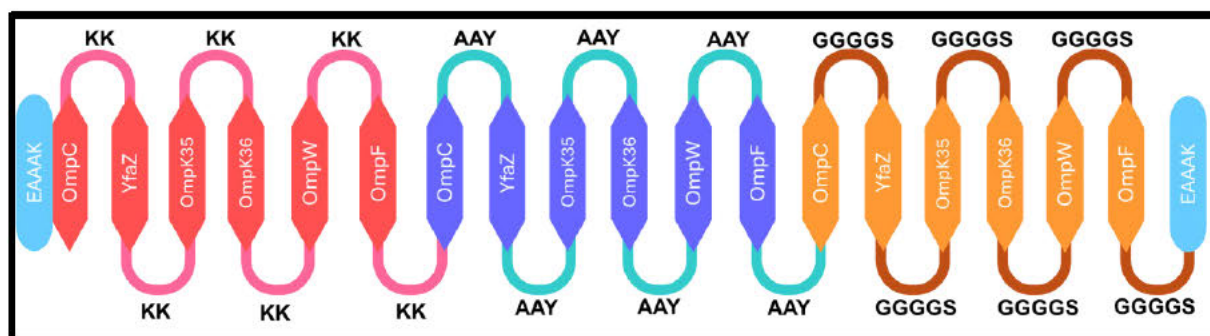


Figure 5-2: Schematic representation of the assembly of the combination multiepitope vaccine. Schematic representations of the linkers used for the construction of the six vaccine candidates and the position of the B-cell, CTL and HTL epitopes with the highest antigenicity identified from the six protein groups. Red represents the B-cell epitopes, blue represents the CTL epitopes and orange represents the HTL epitopes.

5.3.5. MESV primary sequence analysis

The non-allergenic characteristics of the vaccine candidates was assessed using the AllerTop v2.0 server, wherein the protein allergenicity is determined through primary sequence analyses (Dimitrov *et al.*, 2014). The antigenicity of the constructs was assessed using the Vaxijen server (Doytchinova and Flower, 2007). To ensure that there was no cross reactivity in the constructs, Blastp was used to analyse each vaccine candidate against the human proteome (taxid id. 9606) (Altschul *et al.*, 1990). The physicochemical properties of the vaccine constructs were analysed using the ExPASy ProtParam server (Gasteiger *et al.*, 2005). Vaccine solubility was confirmed using the SOLpro tool available through the SCRATCH suite (Magnan *et al.*, 2009).

5.3.6. MESV secondary and tertiary structure analysis

The secondary structure of the MESV candidates was predicted using the PSIPRED server (Mcguffin *et al.*, 2000). The three-dimensional structure of the designed candidates was determined using the GalaxyWEB TBM server to better understand their biological functioning (Ko *et al.*, 2012). Modelled structures were then refined using the GalaxyRefine server which is integrated on the GalaxyWEB server (Ko *et al.*, 2012). MolProbity, VADAR version 1.8, ProSA-web server, Saves v1.6 and the SWISS MODEL evaluation tools were then used to validate the 3D structures of the

candidates (Williams *et al.*, 2018, Wiederstein and Sippl, 2007, Waterhouse *et al.*, 2018, Willard *et al.*, 2003, Colovos and Yeates, 1993). Finally, the visualization and colour coding for the tertiary structure of candidates was done using the PyMol software (Schrödinger, 2015).

5.3.7. Screening linear and discontinuous B-cell epitopes

The Ellipro webserver was used to identify the linear and discontinuous B-cell epitopes in the candidate vaccines (Ponomarenko *et al.*, 2008). This webserver determines residue protrusion index, protein shape estimation, and neighbouring residues cluster formation to identify potential B-cell epitopes. The score predicted for each epitope is based on the Protrusion Index value. Default parameters of 0.5 and 6 were used for the minimum score and maximum distance parameters, respectively (Banerjee *et al.*, 2020). The linear and discontinuous B-cell epitopes were visualized and coloured using the PyMol software (Schrödinger, 2015).

5.3.8. TLR and vaccine construct docking

The structures of human Toll-like receptor (TLR) -2 (PDB ID: 6NIG) and TLR-4 (PDB ID: 4G8A) was retrieved from the Protein Data Bank (PDB), the structures were then prepared for docking by using the Molegro molecular viewer to delete ligands. The ClusPro 2.0 server was then used to investigate the interactions of the vaccine candidates (OmpW, OmpK35, OmpK36, OmpF, YfaZ and combined) with TLR-2 and TLR-4 (Kozakov *et al.*, 2017). The LZerD Web Server was used to investigate the interactions of the vaccine candidate (OmpC) with human TLR-2 and TLR-4 as the size of the construct exceeded the limit of protein size accepted by the ClusPro server (Christoffer *et al.*, 2021). Both, ClusPro and LZerD internally generate and assess multiple docking conformations based on different scoring functions. The final selected models were chosen from among the top ranked poses based on their interaction energies and biological relevance. This approach provides confidence in the robustness of the predicted docking poses. Thereafter, the docked structures were visualized using PyMol to observe the bond interactions between the vaccine-receptor complexes. The PROtein BinDIng enerGY prediction (PRODIGY) web server (<https://bianca.science.uu.nl/prodigy/>, accessed 23 August 2024) was used to predict the binding free energy and dissociation constant of the docked complexes at 37 °C (Honorato *et al.*, 2021, Vangone and Bonvin, 2015, Xue *et al.*, 2016). Docking

interactions between the vaccine constructs and TLR2/4 were visualized using the PDBsum server (Laskowski *et al.*, 2018).

5.3.9. Molecular dynamics simulations

Molecular dynamics (MD) simulations are important to determine the stability of the vaccine candidates and vaccine-receptor complexes (Khalid *et al.*, 2022, Chatterjee *et al.*, 2023) and were thus performed using the Desmond package from Schrodinger. Protein molecules were dissolved in orthorhombic water boxes with TIP3P water molecules with dimensions set at 10 Å. The NPT ensemble (300 K and 1 bar pressure) was used, and MD simulations were performed for 200 ns. Analysis of simulations was accomplished by examining the root mean squared deviation (RMSD), root mean squared fluctuation (RMSF), and radius of gyration (Rg).

5.3.10. Immune response simulations

Understanding the magnitude of immune response elicited by the candidate vaccine is a critical aspect to evaluate, and the C-ImmSim web server was used which exploits a position-specific scoring matrix (PSSM). For vaccination, the duration between dose 1 and dose 2 should be at least 4 weeks apart (Dey *et al.*, 2022). Therefore, the server was set so three injections containing 1000 vaccine proteins were administered at time steps 1, 84 and 168 (1 time step = 8 h in real life), for a total of 250 simulation steps. The remaining simulation settings were left at default.

5.3.11. Codon optimization and *in silico* cloning

Finally, the Vector Builder Codon Optimization tool (<https://en.vectorbuilder.com/tool/codon-optimization.html>, Accessed 19 July 2024) was used to ensure that there was conformity between the codons of the host vector and the gene of interest. This optimization is important to ensure that there are high activity levels after protein purification (Khalid *et al.*, 2022). The designed constructs were optimized according to the *Escherichia coli* strain K-12 due to its survival capabilities and fast reproduction rates (Biselli *et al.*, 2020). The Geneious Prime software was used to add two restriction sites, to the N- and C-terminus of the protein to insert it into the pET-28a (+) vector.

5.4. Results

5.4.1. Prediction and analysis of CTL and HTL epitopes

CTLs bind to the MHC-I and are responsible for the clearance of intracellular pathogens (Pathak *et al.*, 2022, Elliott and Haurum, 2001). Table 5-1 shows the non-toxic and non-allergenic CTL epitopes identified from the OmpW, OmpK35, OmpK36, OmpF, OmpC and YfaZ sequences mined from the NCBI database (Table 5-S1) using the NetCTL 1.2 server. Epitope allergenicity and toxicity analysis is vital as vaccines should not elicit an undesired immune response, primarily they should be non-allergenic and non-toxic to ensure vaccine safety and biocompatibility (Martinelli, 2022, Pathak *et al.*, 2022). The antigenicity scores of the selected CTL epitopes were greater than the threshold of 0.4. Antigenicity is defined as the ability of an antigen to interact with B- or T-cell receptors (Zhang and Tao, 2015). The immunogenicity scores of the majority of the selected epitopes were greater than zero, thus indicating the potential of these epitopes to elicit a cell-mediated immune response (Zhang and Tao, 2015).

Table 5-1: Antigenicity and immunogenicity of the non-toxic and non-allergenic CTL epitopes identified from the OmpW, OmpK35, OmpK36, OmpF, OmpC and YfaZ sequences.

OmpC	Antigenicity	Immunogenicity
KTVGDVDLY	0.8587	0.1202
SAADAAEIY	0.6434	0.27221
SAANAAEIY	0.6746	0.24338
DTYTQTDVY	0.7715	0.00444
KSVDGDQTY	1.6323	0.01452
KSDNTSWTR	0.4613	0.09238
QTTKTRLAF	1.0359	-0.10438
GAAHAAEIY	0.8075	0.28244
DVVALGLVY	0.4924	0.08863
EVYSGGLKY	0.8526	-0.23813
ETYTGGLKY	0.9239	-0.0326
DAWTAGLKY	0.7940	0.0456
GTAGAAEIY	1.1807	0.28399
YfaZ	Antigenicity	Immunogenicity
QTGGVGAGY	1.9772	0.17002
HSDNDGDSV	1.5450	-0.02375
DSAGLGMGY	1.4244	-0.0605
HSDNDGDRV	1.4174	0.10315
DSVGLGMGY	1.1303	-0.0598
HSDNDGDGV	2.1506	0.09271
YTDVQAGLG	0.8956	-0.00511
VTGEAGEHY	1.1725	0.28515
KIGDSFRLY	1.4716	0.01962
QTGDVGAGY	1.6088	0.15824
QAGGVGAGY	1.8614	0.17002
NSVGLGMGY	0.9596	-0.0598
HSDNDGDNV	1.3285	0.06913
QTGGVGVGY	1.8177	0.17184
SIHVFGEY	0.4543	0.30224
OmpK35	Antigenicity	Immunogenicity
SSDDTTYAR	1.2457	0.1236
DTRSDTTY	2.1356	-0.05175
DTSSNDTTY	1.5882	-0.15015
OmpK36	Antigenicity	Immunogenicity
KSVDGDQTY	1.6323	0.01452
DVVALGLVY	0.4924	0.08863
GAAHAAEIY	0.8075	0.28244
SSDDTTYAR	1.2457	0.1236
GTSVTYDIF	0.8369	0.11864
OmpW	Antigenicity	Immunogenicity
RLDPWVFMF	2.7515	0.2468
VSSDNVLGF	0.7429	0.01162
AAGQVGLDY	0.7357	-0.02986
OmpF	Antigenicity	Immunogenicity
QNMEFVLQY	0.7581	0.11957
SSDDTTYAR	1.2457	0.1236
YTDNYMTGR	0.9085	-0.11565

Helper T-cells bind to MHC-II and play a significant role in the induction of host humoral and cellular immune responses (Pathak *et al.*, 2022, Alexander *et al.*, 1998). Table 5-2 shows the non-toxic, non-allergenic HTL epitopes identified from the OmpW, OmpK35, OmpK36, OmpF, OmpC and YfaZ sequences mined from the NCBI database (Table 5S-1) using the IEDB MHC-II server. The antigenicity of the selected epitopes was greater than the 0.4 threshold, indicating potential for inducing an immune response. All the selected HTL epitopes had positive immunogenicity scores (greater than 0), making them suitable candidates for vaccine construction. The interferon gamma (IFN- γ) score of 89% of the selected HTL epitopes were indicated

as being positive using the IFN-epitope server. This implies that the majority of the selected HTL epitopes can initiate the expression of IFN- γ which plays an important role in the activation of macrophages and natural killer cells (Wu *et al.*, 2014).

Table 5-2: Antigenicity, immunogenicity, and IFN- γ induction score of the non-toxic and non-allergenic HTL epitopes identified from the OmpW, OmpK35, OmpK36, OmpF, OmpC and YfaZ sequences.

OmpC	Antigenicity	Immunogenicity	Allele	IFN-γ induction
GTRFRFAADYYLFDD	1.1633	0.49092	HLA-DRB1*07:01	2
SSYYDLDMGISFGAA	0.5699	0.01964	HLA-DRB3*01:01	0.4486
DNSFTHNAGISTDDV	0.7052	0.23492	HLA-DRB3*02:02	2
YPQTYNATRAGSLGF	0.5028	0.04367	HLA-DRB1*07:01	0.4860
SDNFLQSRANGVATY	0.5214	0.04076	HLA-DRB1*07:01	1
NKGVGDGATYVRLGF	1.7302	0.3004	HLA-DRB1*03:01	3
KINLVDDNRFTSAAG	0.5443	0.16619	HLA-DRB1*03:01	0.5426
SAKYDANNIYAAMVY	0.4990	0.10655	HLA-DRB3*02:02	0.3758
YGQWEYNMDASNVEG	1.1963	0.08774	HLA-DRB3*02:02	0.3949
YGQWEYNVQANNTET	1.1417	0.31726	HLA-DRB3*02:02	1
DDNSFTRNAGISTDD	0.6147	0.11802	HLA-DRB3*02:02	3
AAEYQTYNATRFSG	0.4392	0.10844	HLA-DRB5*01:01	0.4620
YGQWEYNVQANNTES	1.1140	0.31726	HLA-DRB3*02:02	1
GLKYNANRVYLATQF	0.4136	0.02989	HLA-DRB3*02:02	4*
SNYYDLDMGISFGAA	0.6672	0.01964	HLA-DRB3*01:01	0.4576
NAAEIYNNNGNKLDL	0.9288	0.04785	HLA-DRB3*02:02	33*
SSYYDLDIGISFGAA	0.7787	0.32024	HLA-DRB3*01:01	0.4410
TAGLKYDANDIYLAT	0.7487	0.00086	HLA-DRB3*01:01	1*
SSYYDLDMGINFGAA	0.6075	0.17444	HLA-DRB3*01:01	0.4689
SSYYVLDMGISFGAA	0.5143	0.03824	HLA-DRB3*01:01	0.5002
SSYYDLDMGISFGGA	0.5369	0.01658	HLA-DRB3*01:01	0.4280
SSYYALDMGISFGAA	0.5522	0.03614	HLA-DRB3*01:01	-0.2295*
TVGAKYDANNIYLAA	0.8410	0.02349	HLA-DRB3*02:02	1*
DFGLRPFVGNQTKG	0.9886	0.01446	HLA-DRB1*15:01	2*
YfaZ	Antigenicity	Immunogenicity	Allele	IFN-γ induction
GVNVALTDSIHVFGE	0.5258	0.2836	HLA-DRB1*07:01	0.4876
VEDYVEANAGVRLNV	1.2034	0.31986	HLA-DRB5*01:01	0.4665
SGVKDYVEANAGVRL	0.9672	0.11898	HLA-DRB5*01:01	0.4562
GVRLNALPSLNIEAG	1.5294	0.07028	HLA-DRB1*15:01	2
VKNYVEANAGVRLNV	1.0676	0.31056	HLA-DRB5*01:01	0.4510
GVRLDVLPSLNIEAG	1.5798	0.10028	HLA-DRB1*15:01	1
FEDYVEANAGVRLNV	1.0995	0.31986	HLA-DRB5*01:01	0.5013
VFKPLHVNAGYRVIE	0.5020	0.20226	HLA-DRB3*02:02	0.4897
SSGIDSYQEANVGAS	1.0595	0.10864	HLA-DRB1*15:01	1
SSGIDSYQEANVGAN	1.0729	0.10864	HLA-DRB1*15:01	1
LGPFLATVGGKGIYT	0.5785	0.21756	HLA-DRB1*07:01	0.5444
VEDDVEANAGVRLNV	1.6934	0.3459	HLA-DRB3*02:02	0.4460
VEDYVEANVGVRNLN	1.1993	0.32196	HLA-DRB5*01:01	0.4933
IEDYVEANAGVRLNV	1.1448	0.31986	HLA-DRB5*01:01	0.4998
GAGYNLEVGPVMLNA	1.4420	0.01184	HLA-DRB5*01:01	1
OmpK35	Antigenicity	Immunogenicity	Allele	IFN-γ induction
YGQWEYNMDASNVEG	1.1963	0.08774	HLA-DRB3*02:02	0.3881
SAKYDANNIYAAMVY	0.4990	0.10655	HLA-DRB3*02:02	0.3700
SAKYDANNIYAAMVY	0.5171	0.10858	HLA-DRB3*02:02	0.3999
OmpK36	Antigenicity	Immunogenicity	Allele	IFN-γ induction
SDNFLQSRANGVATY	0.5214	0.04076	HLA-DRB1*07:01	1
GKDIEGYGDQDLLKY	0.9789	0.08562	HLA-DRB1*15:01	0.4745
YGQWEYNVQANNTET	1.1417	0.31726	HLA-DRB3*02:02	1
YGQWEYNMDASNVEG	1.1963	0.08774	HLA-DRB3*02:02	0.3881
DNSFTHNAGISTDDV	0.7052	0.23492	HLA-DRB3*02:02	2
DDNSFTRNAGISTDD	0.6147	0.11802	HLA-DRB3*02:02	3
OmpW	Antigenicity	Immunogenicity	Allele	IFN-γ induction
QSKVRPYVGAGINYT	0.6176	0.2695	HLA-DRB1*15:01	3
GLDYLINRDWLLNMS	0.5982	0.27438	HLA-DRB3*02:02	0.5721
AGLDYELDQNWMLNA	1.0918	0.04158	HLA-DRB3*01:01	1
SSSKLRPYIGAGVNY	0.8604	0.01816	HLA-DRB1*15:01	0.4785
SKVRPYIGAGVNYTT	0.6250	0.33845	HLA-DRB1*15:01	1
OmpF	Antigenicity	Immunogenicity	Allele	IFN-γ induction
VNYRIIDDDRSAAGV	1.0149	0.35383	HLA-DRB1*03:01	0.5033
YGQWEYNMDASNVEG	1.1963	0.08774	HLA-DRB3*02:02	0.3949

5.4.2. Prediction and analysis of B-cell epitopes

B-cell epitopes play an important role in initiating adaptive immune system response and are important vaccine components which boost CTL antitumor immune response (Krishnamoorthy and Karuppasamy, 2023, Ghaffar *et al.*, 2024). The B-cell epitope scores predicted by the ABCPred server range between 0 and 1. A score closer to 1 suggests that the identified sequences are likely to be epitopes (Wang *et al.*, 2016). Table 5-3 shows the non-allergenic and non-toxic B-cell epitopes identified from the OmpW, OmpK35, OmpK36, OmpF, OmpC and YfaZ sequences using the ABCPred server. The scores of the identified B-cell epitopes were close to 1, confirming the suitability of the chosen B-cell epitopes for vaccine construction. Furthermore, and based on the obtained antigenicity and immunogenicity scores (Table 5-3), these epitopes were antigenic with a potential to initiate a cell-mediated immune response.

Table 5-3: Antigenicity, and immunogenicity of the non-toxic and non-allergenic B-cell epitopes identified from the OmpW, OmpK35, OmpK36, OmpF, OmpC and YfaZ sequences.

OmpC	Position	Score	Antigenicity	Immunogenicity
LFDWDNHYAEGANNTT	105	0.95	0.8909	0.49112
ASAADAAEIYNKNGNK	16	0.88	1.1727	0.1758
EGANSWTRLGFAGLKF	89	0.87	0.5204	0.21983
ELTGYGQWEYNIKANT	70	0.95	0.6927	0.22244
NFEVVAQYQFDDFNLP	287	0.81	0.7095	0.1786
NFEVVAPYQFDFGLRP	283	0.96	1.6628	0.32752
PEFGGDTYGSDNFLQS	130	0.95	1.1318	0.08126
FGGDGDTYGSDNFLQS	134	0.90	1.3707	0.04248
SGSNRTLDQRANTLNN	215	0.88	1.3877	0.03159
QLTGYGQWEYNVQANN	70	0.91	0.6124	0.227
AGVATDDQAAVGIVYQ	263	0.80	0.5736	0.32833
GDSYTYADNFMAGRAN	134	0.93	0.7888	0.10972
GDTYTYADNFMAGRAN	134	0.92	0.7830	0.17602
MLTGYGQWEYNVQANN	70	0.90	0.4844	0.227
AGSGEGTNNGGARKLA	179	0.88	2.5485	0.10044
QITGYGQWEYNIQANN	70	0.91	0.6826	0.3164
AGTAGAAEIYNKDGNGK	16	0.92	1.4342	0.21407
YfaZ	Position	Score	Antigenicity	Immunogenicity
DGSQTGGVGAGYNLEV	58	0.86	2.5019	0.11729
CAAVNLHGEAGAEFTN	22	0.89	1.0529	0.52407
GDSVGLGMGYNFNVGP	62	0.81	1.7847	0.05769
CTAVNLHGEAGAEFTN	22	0.87	1.0308	0.52407
NLHGEAGVEFTNLSAS	26	0.88	1.0132	0.3692
AGAEFTNLSASFGAGE	31	0.83	0.6736	0.16277
CAAVNLHGESGAFTN	22	0.92	1.1314	0.32487
GDSVGLGMGYNFNLGP	62	0.82	1.8435	0.01349
LGDADSGLSFNNGWAR	39	0.87	0.4667	0.11258
SSGIDSYQEANVGASW	125	0.95	0.8301	0.02678
SQTGDVGAGYNLEVGP	60	0.86	1.8689	0.34079
SQAGGVGAGYNLEVGP	60	0.80	1.9915	0.35229
SSGIDSYQEANVGANW	125	0.96	0.8871	0.11966
PLSIQAGYRYLNLGAK	145	0.89	0.5856	0.107
GVAFPVGGGVNVALTD	95	0.57	0.9412	0.38315
OmpK35	Position	Score	Antigenicity	Immunogenicity
GKMVGEHVWTTNGDTS	37	0.89	0.9822	0.54504
AGAANAAEIYNKNGNK	17	0.87	1.2639	0.1479
OmpK36	Position	Score	Antigenicity	Immunogenicity
QLTGYGQWEYNVQANN	70	0.91	0.6124	0.227
PEFGGDTYGSDNFLQS	117	0.95	1.1318	0.08126
GDSYTYADNFMAGRAN	134	0.93	0.7888	0.10972

Table 5-3 continued...

OmpK36	Position	Score	Antigenicity	Immunogenicity
GKMVGEHVWTTNGDTS	29	0.89	0.9822	0.54504
QLTGYGWWEYNVQANN	70	0.92	0.4366	0.5555
OmpW	Position	Score	Antigenicity	Immunogenicity
VSTRDPWVFMFSAGY	195	0.83	1.3666	0.27467
GAAGQVGLDYLINRDW	34	0.81	1.0322	0.18375
LMAQYYFRTPEDKLRP	103	0.85	1.0515	0.01732
QQSISTRDPWVFMFS	190	0.85	1.7301	0.29362
GMAGQVGLDYLINRDW	152	0.86	0.8480	0.18375
OmpF	Position	Score	Antigenicity	Immunogenicity
YGFYWQYRITGGENEWS	73	0.92	0.8010	0.73362
GKMVGEHVWTTNGDTS	37	0.89	0.9822	0.54504
GEHVWTTNGDTSDDT	6	0.84	1.6271	0.14429
TPAATIPMPYARIGL	3	0.85	0.6437	0.23141

5.4.3. Epitope population coverage

The application of computational methods in the design of vaccines act as a primer to overcoming the human leukocyte antigen (HLA) associated heterogeneity (Krishnamoorthy and Karuppasamy, 2023, Prawiningrum *et al.*, 2022). The IEDB population coverage server was used to analyse the population coverage of the HTL, CTL and B-cell epitopes identified for vaccine construction. Figure 5-3 shows that the identified epitopes cover 75% of the world's population. However, the epitopes of the combination vaccine construct only cover 50% of the world's population. This suggests global applicability and efficacy in targeting diverse genetic backgrounds, it also indicates that the combination vaccine may have to be administered along with one or more of the other vaccines to be efficient. In this study, Central America has the lowest coverage between 26% and 40% for the single and combined vaccines respectively, while Southeast Asia had the highest coverage of more than 75% for the six single vaccines. It was observed that South Africa had a low population cover for HLA Class II. This observation, along with the low population coverage of epitopes in Central America could be attributed to the diverse population heredity which owes to their ancestry (Sarvmeili *et al.*, 2024). The population coverage of the selected epitopes indicate that they are suitable candidates for vaccine construction as they can be used for a diverse population group.

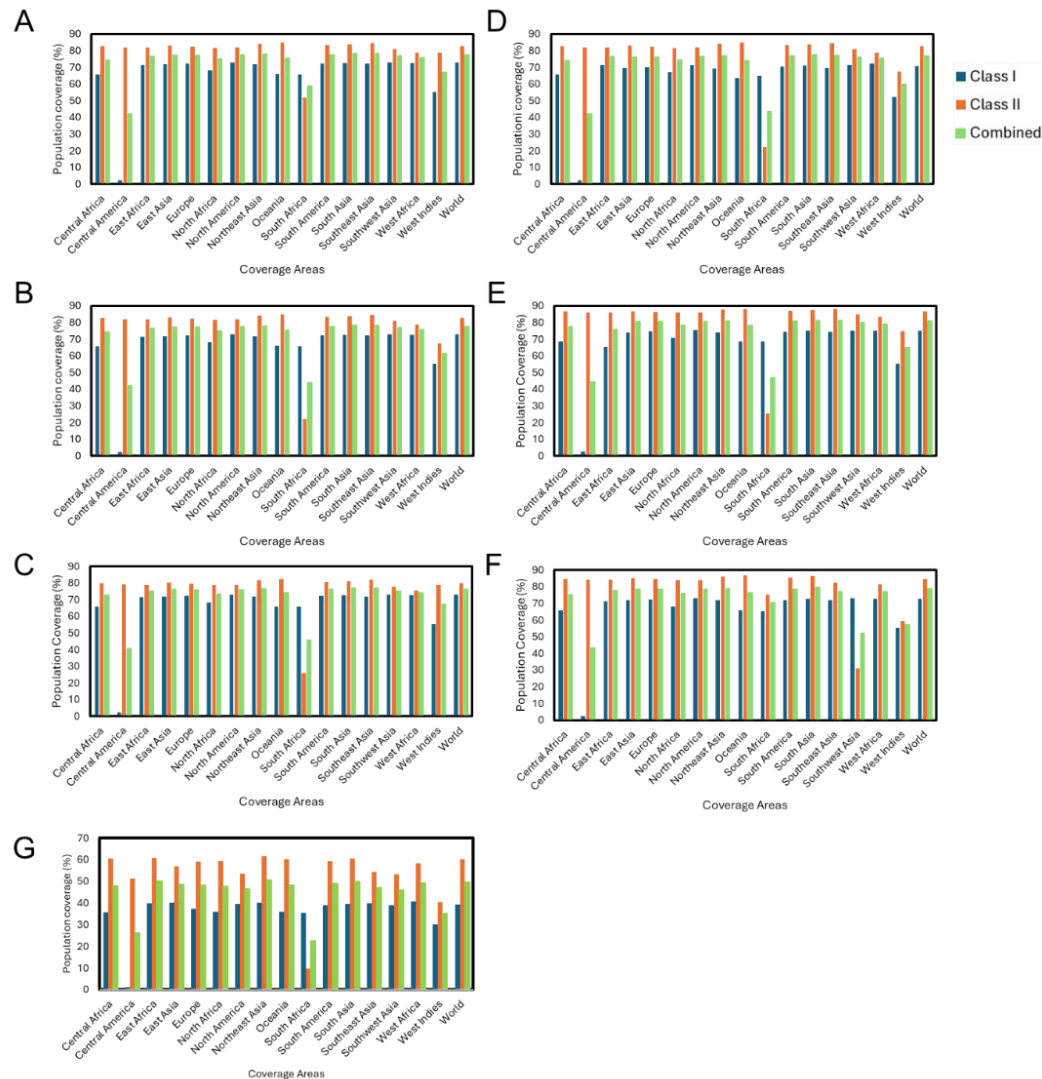


Figure 5-3: Population analysis coverage report of selected epitopes around the world. A) OmpC epitopes. B) OmpK35 epitopes. C) OmpW epitopes. D) YfaZ epitopes. E) OmpK36 epitopes. F) OmpF epitopes. G) Combination vaccine epitopes. Blue bars represent HLA Class I, orange bars represent HLA Class II and green bars represent the average of the two HLA classes.

5.4.4. Vaccine candidate physicochemical analysis

The identified CTL, HTL and B-cell epitopes were combined using AAY, GGGGS/GPGPG, and KK linkers, respectively to construct seven vaccine candidates. Linkers are important in the development of multiepitope vaccines as they contribute to increased expression, stability and folding of the vaccine construct (Rouzbahani *et al.*, 2022). The solubility of the vaccine candidates was predicted to be greater than 0.7 using the SOLpro server, indicating that the vaccine candidates are soluble (Table 5-4). Solubility influences the distribution, stability, administration, storage conditions, and the success of vaccination programs amongst other factors (Sarvmeili *et al.*,

2024). Therefore, as per the solubility criteria, the constructs are suitable vaccine candidates.

Table 5-4: Antigenicity, solubility, and physicochemical properties of the non-allergenic and non-toxic vaccine constructs.

Properties	OmpC	YfaZ	OmpK35	OmpK36	OmpW	OmpF	Combined
Antigenicity	1.2899	1.3957	1.0830	1.2484	1.0281	1.0633	1.6713
Solubility	0.992	0.996	0.852	0.802	0.887	0.918	0.780
Molecular weight (kDa)	98.56	75.03	14.93	29.44	25.66	17.30	32.39
pI	5.02	4.97	4.88	4.48	9.50	5.39	4.48
Instability index	31.91	25.02	32.03	28.05	20.47	11.43	23.41
Aliphatic index	49.42	68.97	38.80	43.36	70.34	42.15	45.94
Grand average of hydropathicity	-0.575	-0.246	-0.839	-0.799	-0.345	-0.870	-0.784

Additionally, the Expasy ProtParam was used to investigate the physicochemical properties of the vaccine candidates (Table 5-4). This provides a general perspective about the charge, stability, as well as a generalized characterisation of the vaccine constructs (Ghaffar *et al.*, 2024). The molecular weight of the constructs is below 110 kDa (Table 5-4), thus indicating that these constructs are all suitable vaccine candidates (Rouzbahani *et al.*, 2022, Barh *et al.*, 2013). The pI of the combination, OmpC, YfaZ, OmpK35, OmpK36 and OmpF vaccine range between 4 and 6, therefore these constructs are acidic in nature while the pI of 9.50 for the OmpW construct suggests that it is basic in nature. The instability index of the vaccine constructs is less than 40, meaning the constructs are stable (Sarvmeili *et al.*, 2024). The aliphatic index highlights the thermostability of the constructs, and in comparisons the YfaZ and OmpW constructs are more thermostable at aliphatic indices greater than 65. Based on the negative Grand average of hydropathicity (GRAVY) scores, the vaccine constructs are hydrophilic in nature and would therefore interact well with water and blood (Krishnamoorthy and Karuppasamy, 2023, Sarvmeili *et al.*, 2024) (Table 4). The estimated half-life of the vaccine constructs in mammalian reticulocytes (*in vitro*) is 1 h, in yeast (*in vivo*) it is approximately 30 min, and in *E. coli* (*in vivo*) it is greater than 10 h. This indicates that the constructs are stable in different development phases (Sarvmeili *et al.*, 2024). In addition, there was no similarity observed between the constructs and human proteome (NCBI Blastp), suggesting the unlikelihood of undesirable side effects following vaccine administration.

5.4.5. Analysing secondary and tertiary vaccine construct

The secondary structure of proteins is important for its folding and can be predicted using the PSIPred tool. Figure 5-4 shows that the vaccine constructs are mainly beta-stranded and none of the vaccine constructs are predicted to be transmembrane proteins therefore challenges in their expression are unanticipated (Sarvmeili *et al.*, 2024). The tertiary structure of the constructs is shown in Figure 5-4, these structures were constructed and refined using the Galaxy Web server followed by validation using the ProSA, MolProbity and Swiss-Model servers. The Rama Z-score was below 2 for all the vaccine constructs, implying normality in the protein backbone of the vaccine constructs (Sobolev *et al.*, 2020) (Table 5-5). Four out of the six vaccine constructs (YfaZ, OmpC, OmpF and OmpK35) have a Ramachandran favoured score greater than 90% and two vaccine constructs (OmpW and OmpK36) have a Ramachandran favoured score greater than 85% (Figure 5-4, Table 5-5). The QMeanDisCo Global score is the average per residue score used to measure the overall quality of protein structures and was estimated to be more than 0.2 (Studer *et al.*, 2019). This score was expected since no specific template was used to model these constructs (Table 5-5). The ERRAT quality factor score of all the constructs are greater than 50 indicating that the models are of good quality (Hossain *et al.*, 2020). The Z-score of the constructs ranged from -2.65 to -0.76, which was within the range of scores for similar size proteins with the exception of OmpC and YfaZ (Figure 5-3, Table 5-5). The MolProbity scores range from 1.56 to 2.29, these scores lie slightly above the range (-4 to 2) of acceptable scores (Chen *et al.*, 2010) (Table 5-5). Therefore, the tertiary structural assessments confirm that the vaccine constructs are of adequate quality, warranting further analysis.

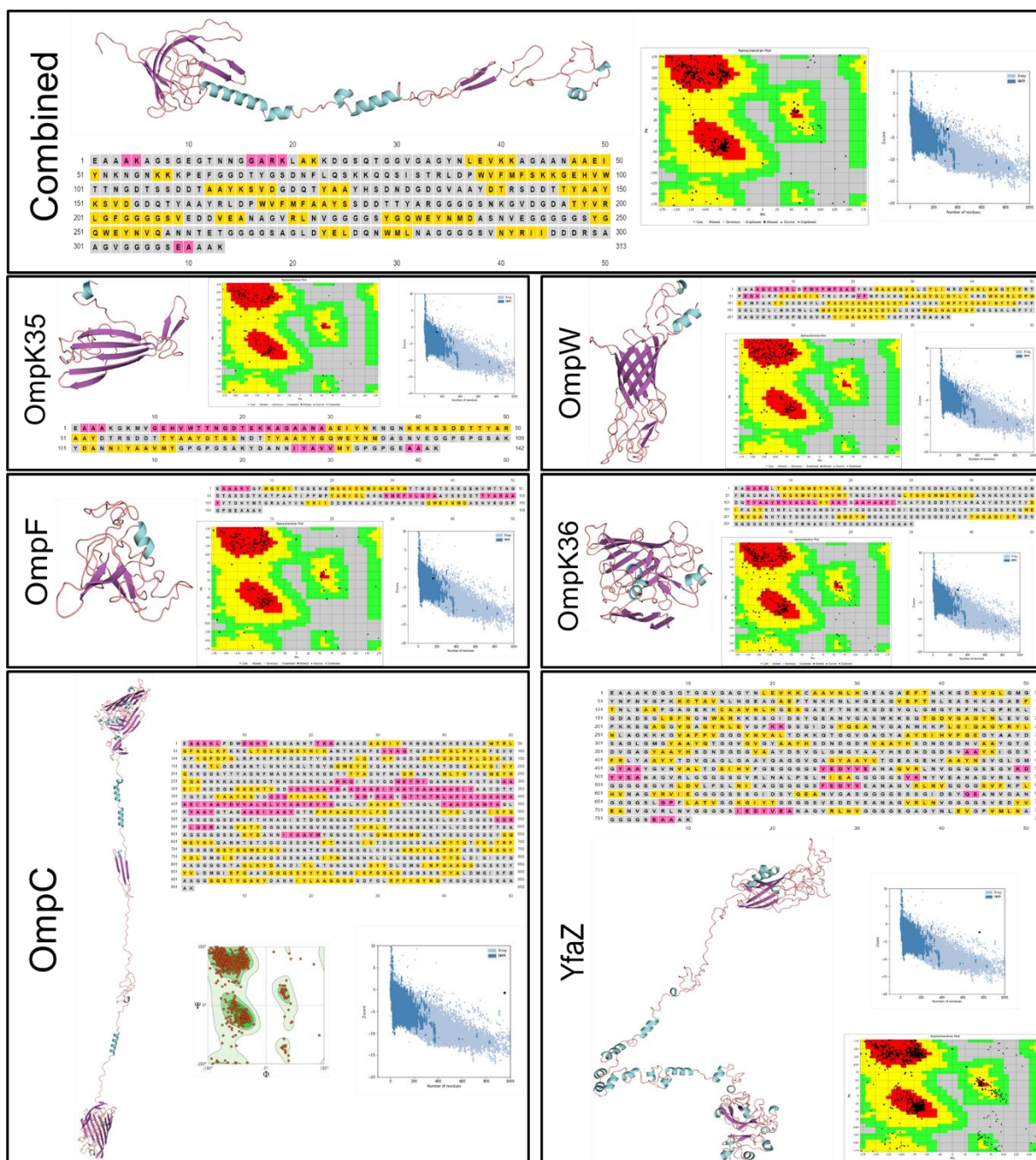


Figure 5-4: Secondary and tertiary structure of the seven multiepitope vaccine constructs. The secondary structure of the constructs was analysed using the PSIPred server, the yellow blocks represent the β -strands and pink blocks represent α -helices. Tertiary structures were modelled and refined using the GalaxyWEB server and visualised using PyMol. The cartoon representations display the 3D structure of each construct with helices (light blue), β -strands (purple), and loops (pink). Structure validation was performed using VADAR, ProSA and Swiss-Model servers. The Ramachandran plot show the ϕ – ψ angle distribution. For all the constructs, expect OmpC, in the Ramachandran plot, red regions represent most favoured; yellow/green regions are allowed or generously allowed; gray regions are disallowed. The Ramachandran plot for OmpC shows dark green regions as being the most favoured, the light green being allowed or generously allowed and the white regions are disallowed. ProSA z-score plots show the overall quality of the predicted structures compared to known crystal structures. Points falling within the shaded region represent acceptable models.

Table 5-5: Structure validation of the seven vaccine candidates evaluated using the MolProbity, ProSA, SAVES v6.1 and Swiss-Model servers.

	OmpK36	YfaZ	OmpC	OmpF	OmpW	OmpK35	Combined
Rama Z-score	-2.83±0.44	-1.61±0.25	-2.16±0.23	-2.27±0.54	-2.23±0.49	-1.81±0.55	-1.25 ± 0.40
Ramachandran favoured	87.77%	93.93%	92.95%	92.31%	88.89%	93.57%	94.86%
QMEANDisCo Global	0.28±0.05	0.26±0.05	0.28±0.05	0.28±0.07	0.32±0.05	0.26±0.07	0.23±0.05
ERRAT Quality Factor	60.85	83.89	63.86	59.02	77.33	75.21	77.17
Z-Score	-1.18	-2.3	-0.76	-2.58	-2.65	-1.45	-3.14
MolProbity Score	2.02	1.79	1.87	1.56	2.29	2.00	1.65

5.4.6. Prediction of Linear and Conformational B-cell epitopes

For the initiation of a humoral response, it is important for vaccine constructs to have epitopes for B-cell lymphocytes to stimulate antibody production (Khalid *et al.*, 2022). The ElliPro Webserver was used to identify the continuous and linear B-cell epitopes on the vaccine constructs (Table 5-6, Figure 5-5). Both linear and discontinuous B-cell epitopes were identified on the constructs confirming that these vaccine candidates have the potential to elicit an immune response and therefore should be further investigated *in vitro* and *in vivo*. Interestingly, many of the linear and discontinuous epitopes overlap in all the constructs (Figure 5-5).

Table 5-6: Linear and discontinuous B-cell epitopes identified on the vaccine constructs using the ElliPro Webserver.

Vaccine Construct	Linear	Score Range	Discontinuous	Score Range
OmpC	25	0.52 – 0.909	4	0.593 – 0.737
OmpF	5	0.52 – 0.835	2	0.643 – 0.703
OmpK35	6	0.572 – 0.804	3	0.623 – 0.777
OmpK36	11	0.512 – 0.768	4	0.521 – 0.667
OmpW	10	0.564 – 0.817	5	0.64 – 0.929
YfaZ	11	0.537 – 0.849	6	0.51 – 0.837
Combined	10	0.515 – 0.873	8	0.512 – 0.774

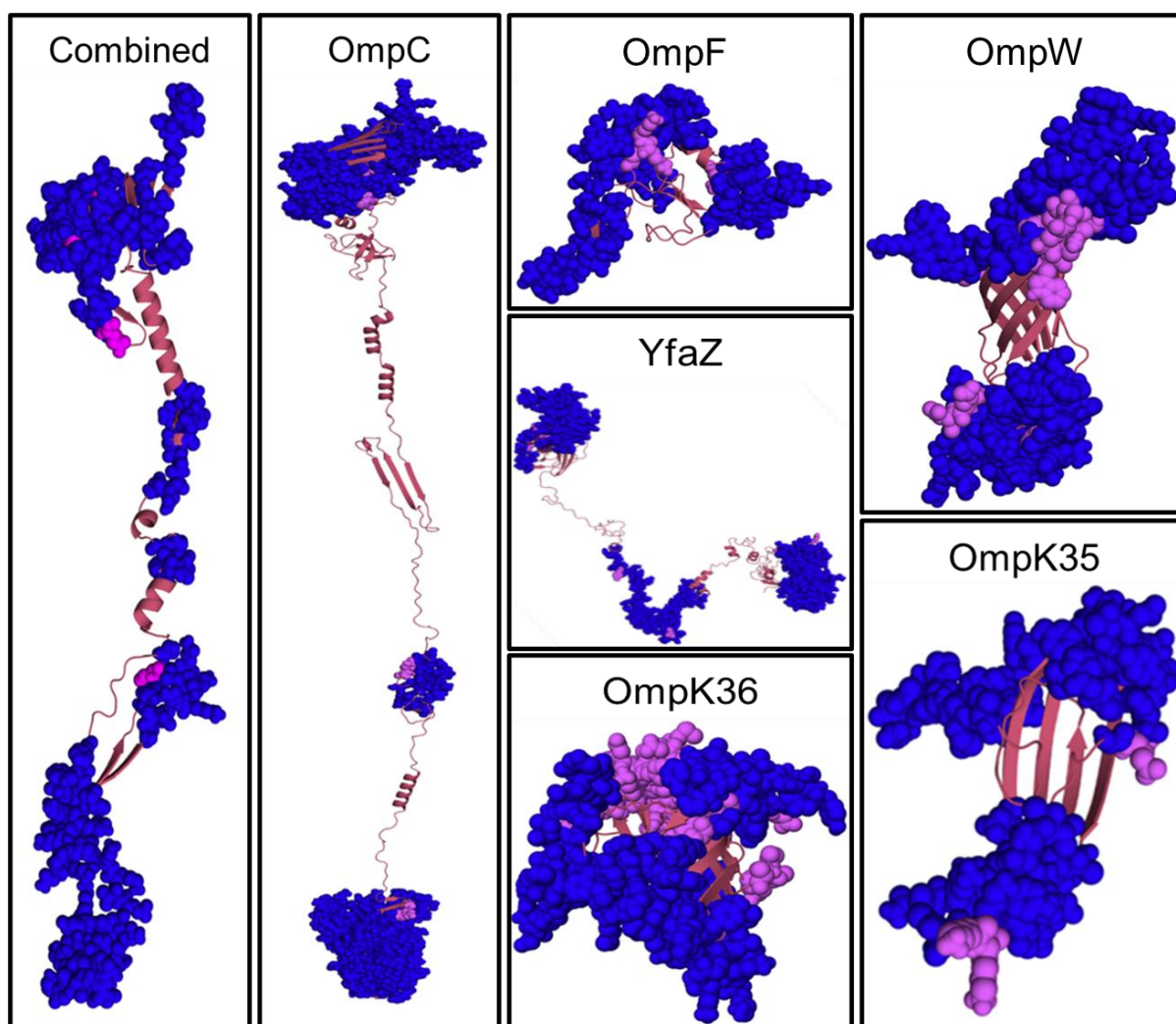


Figure 5-5: 3D representation of the linear and discontinuous epitopes identified using the Ellipro server. The vaccine construct is coloured brown; linear epitopes are represented as purple spheres and discontinuous epitopes are represented as blue spheres.

5.4.7. Molecular Dynamics Simulation of vaccine constructs

MD simulations are performed to validate protein stability in biological systems (Banerjee *et al.*, 2020). Simulations were run for 200 ns and post-dynamic analyses were performed using Desmond on the Schrodinger Maestro suite. The Rg was calculated to estimate the compactness of the vaccine constructs around its axes (Dey *et al.*, 2023, Behmard *et al.*, 2020). The Rg fluctuations of the constructs lie within 1 Å, indicating that the constructs are compact and therefore stable (Figure 5-6A, Figure 5-6B). The RMSD plot illustrated the stability over 200 ns with mild fluctuations after 50 ns, again highlighting the constructs stability (Figure 5-6C, Figure 5-6D). And this was consistent with the average RMSF values of OmpW, OmpF, OmpK35 and OmpK36 of less than 3.5 Å (Figure 5-6E). It was interesting to observe that YfaZ, OmpC and combined vaccines have substantial fluctuation and average RMSF values

of 20.3 Å, 31.7 Å, and 10.05 Å, respectively (Figure 5-6F). These fluctuations can be attributed to the loop region of the constructs that do not have a stable tertiary structure (Figure 5-6F).

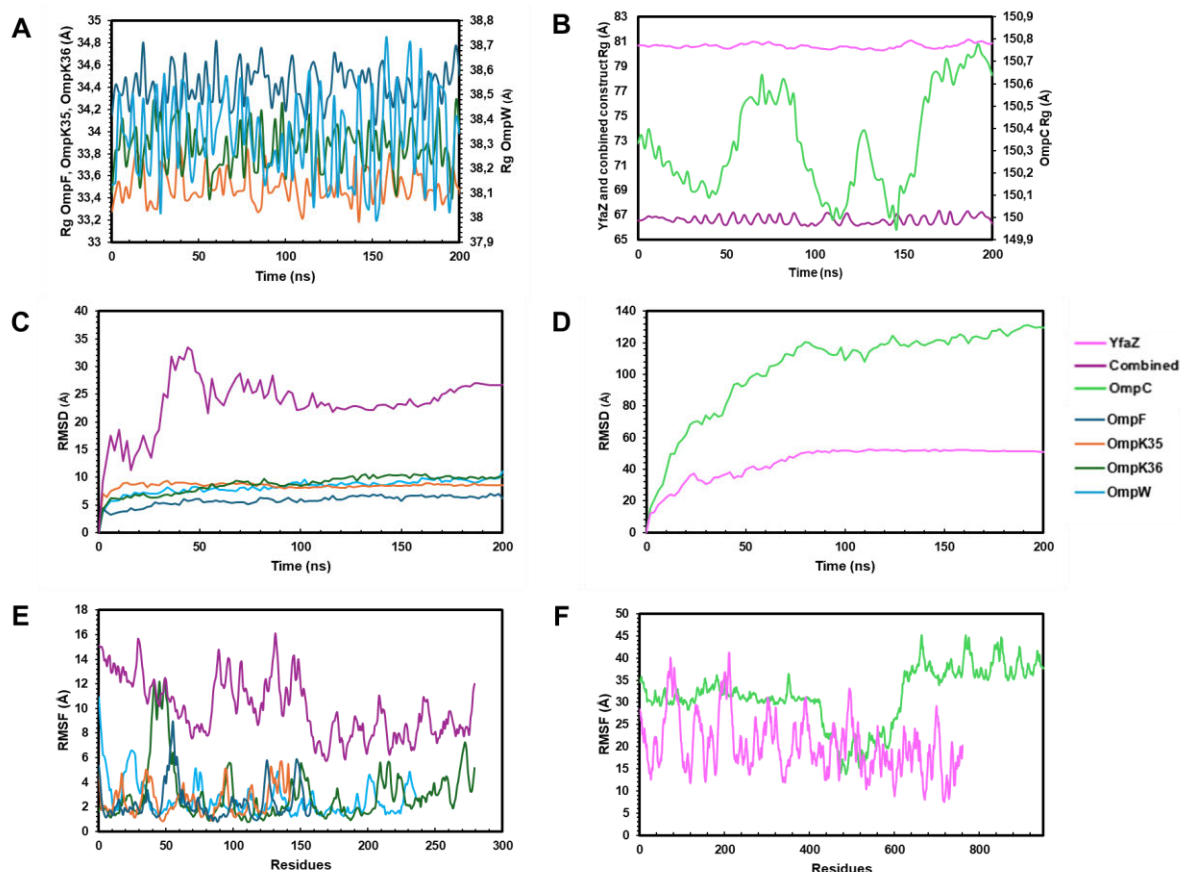


Figure 5-6: Molecular dynamics simulation of the seven designed vaccine constructs. A) Radius of gyration (Rg) of OmpF, OmpW, OmpK35 and OmpK36 constructs. B) Rg of OmpC, combined and YfaZ constructs. C) root-mean-square-deviation (RMSD) of OmpF, OmpW, OmpK35, combined and OmpK36 constructs. D) RMSD of OmpC, and YfaZ constructs. E) root mean squared fluctuations (RMSF) of combination, OmpF, OmpW, OmpK35 and OmpK36 constructs. F) RMSF of OmpC and YfaZ constructs.

5.4.8. Molecular Docking of MESVs with immune receptors

Upon entry of *K. pneumoniae* into the lungs, TLRs which are pattern recognition receptor (PRRs) for early detection of pathogens are activated and trigger the release of cytokines and chemokines that then activate neutrophils (Wieland *et al.*, 2011, Umitaibatin *et al.*, 2023). Upon *K. pneumoniae* infection, the expression of TLR2 and TLR4 increases in the epithelial pathways (Liang *et al.*, 2022, Bengoechea and Sa Pessoa, 2018). TLR2 forms heterodimers with TLR1 or TLR6, by transmitting signals, these heterodimers play an important role in resisting pathogens (Liang *et al.*, 2022). TLR4 can sense LPS on the outer membrane of Gram-negative pathogens and is an

important TLR in the recognition of *K. pneumoniae* (Wieland *et al.*, 2011). Docking of the vaccine constructs with TLR2 and TLR4 using the ClusPro and LZerD protein-protein docking server suggested that the constructs interacted with the receptors (Figure 5-7A). However, it was important to conduct further analysis to confirm the interaction (non-covalent) and investigate its nature. Subsequently, the PDBsum server was used to identify molecular interactions between the vaccine constructs and TLRs (Figure 5-7A).

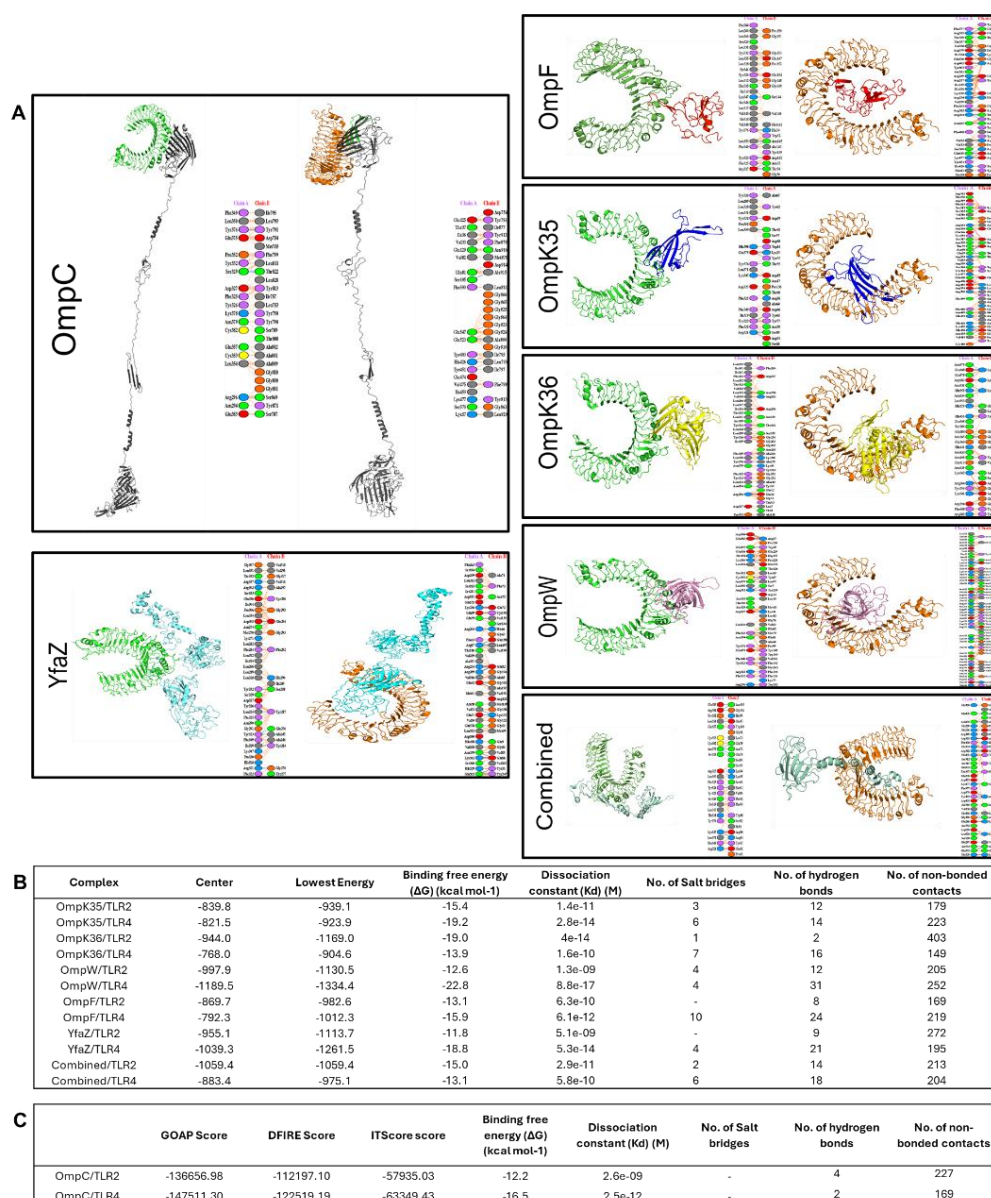


Figure 5-7: Molecular interaction of vaccine constructs with TLR2 (green) and TLR4 (orange). A) Docking was performed using the ClusPro and LZerD docking server. Interaction analysis for each docked complex was obtained from the PDBsum server. B) Scores obtained from the ClusPro server. C) Scores obtained from the LZerD server. PRODIGY server was used to obtain the binding free energy and dissociation constant. The number of salt bridges, hydrogen bonds and non-bonded contacts were obtained from the PDBsum server. Analysis was performed using the PRODIGY and PDBsum servers.

All the constructs except the OmpF and YfaZ formed salt bridges with TLR2, whereas OmpC did not form salt bridges with neither TLR2 nor TLR4 (Figure 5-7B, Figure 5-7C). Salt bridges are strong, non-covalent interactions that contribute to protein stability, hence their presence in protein-protein interactions indicates a stable interaction (Spasov *et al.*, 2023, Bosshard *et al.*, 2004). Therefore, the absence of the salt bridges in the four complexes suggests that these interactions are weak. The complex with the lowest binding free energy was OmpW/TLR4 and this coincided with the results obtained from the PDBsum server which identified the complex as having the highest number of salt bridges, hydrogen bonds, and non-covalent contacts (Figure 5-7B). The Generalized Orientation-Dependent, All-Atom Statistical Potential (GOAP), distance-scaled, finite ideal-gas reference state (DFIRE), and ITScore indicated that the OmpC vaccine construct interacts better with the TLR4 proteins since the complex score for OmpC/TLR4 is lower than that of the OmpC/TLR2 interaction (Figure 5-7B, Figure 5-7C). However, the binding energy score and the number of bonds suggests that the OmpC/TLR2 complex is more stable than the OmpC/TLR4 complex. Overall, the observed interactions between the vaccine constructs and the TLRs, including their nature, supports their suitability as vaccine candidates with the potential to interact with components of the immune system and therefore elicit an immune response.

5.4.9. Molecular Dynamics simulation of vaccine constructs

MD simulations were performed using the Desmond suite on the Maestro Schrodinger software to analyze the biophysical interactions between the vaccine constructs and the TLR2/4 immune receptors. The Rg plots for the vaccine constructs docked to TLR2 ranged between 1 to 2 Å over 200 ns, indicating that the complexes are stable and compact (Figure 5-8A, Figure 5-8B). The RMSD plots show that the OmpC_2, OmpW_2, and OmpK36_2 complexes stabilize rapidly. In comparison, the OmpF_2, combination_2, OmpK35_2, and YfaZ_2 complexes took a longer time and only partially stabilized after 150 ns (Figure 5-8C, Figure 5-8D).

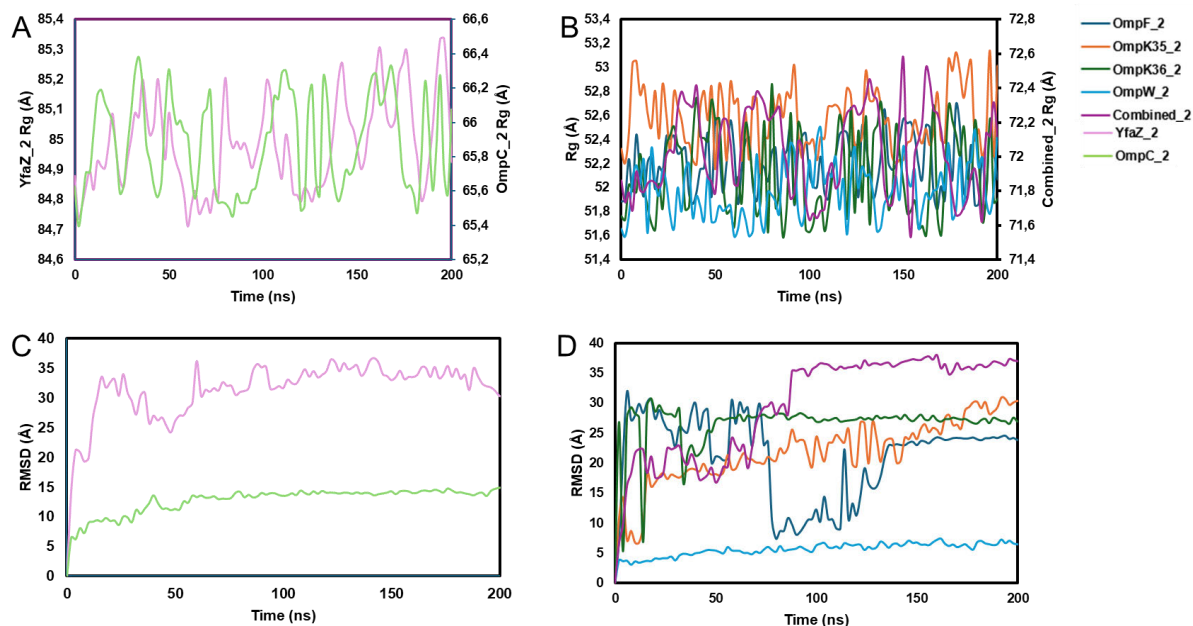


Figure 5-8: Molecular dynamics simulation of the six vaccine constructs docked with TLR2. A) Radius of Gyration (Rg) of the YfaZ and OmpC and B) OmpF, OmpW, combination, OmpK35, and OmpK36 constructs docked to TLR2. C) Root Mean Square Deviation (RMSD) of the YfaZ and OmpC and B) OmpF, OmpW, OmpK35, combination, and OmpK36 constructs docked to TLR2.

The Rg plots for the vaccine constructs and TLR4 ranged between 1 to 4 Å, which represents the complexes stableness and compactness (Figure 5-9A, Figure 5-9B). However, this range of fluctuations is greater than those observed with the constructs complexed to TLR2, thus the construct-TLR2 complexes are more stable. The RMSD plots show that the OmpC_4 and YfaZ_4 complexes stabilize rapidly (Figure 5-9C). Contrastingly, the OmpF_4, OmpW_4, OmpK35_4, combination_4, and OmpK36_4 complexes do not completely stabilize, fluctuating throughout the simulation, depicting variations in the nature of the interactions throughout the duration of their contacts (Figure 5-9D).

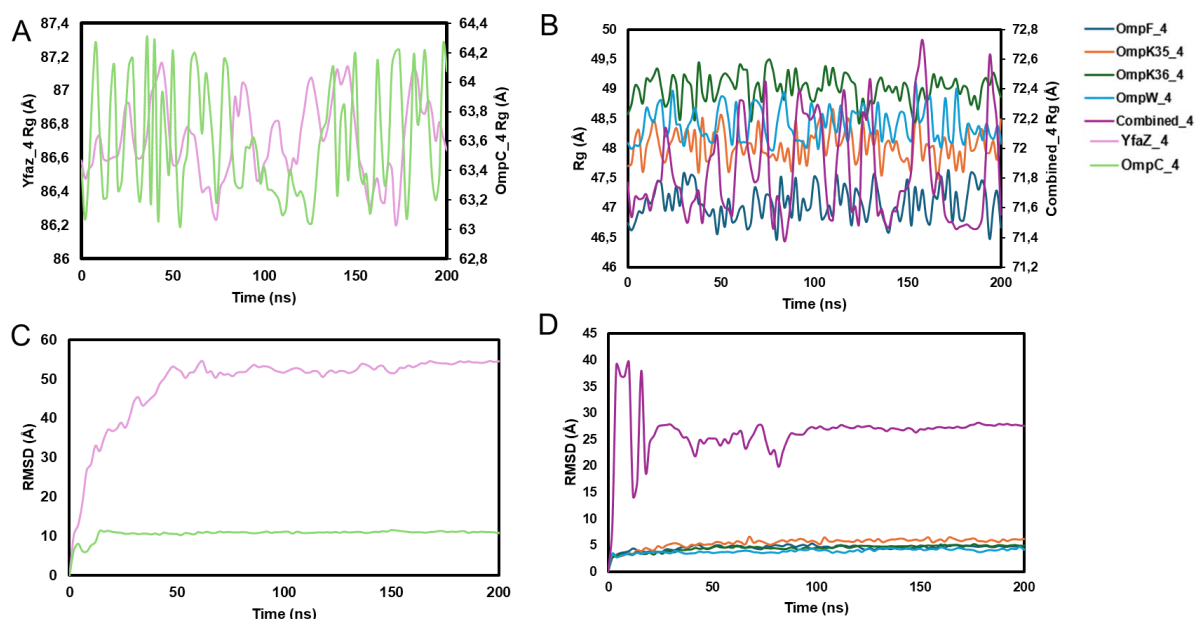


Figure 5-9: Molecular dynamics simulation of the six vaccine constructs docked with TLR4. A) Radius of Gyration (Rg) of the YfaZ and OmpC and B) OmpF, OmpW, OmpK35, combination, and OmpK36 constructs docked to TLR4. C) Root Mean Square Deviation (RMSD) of the YfaZ and OmpC and B) OmpF, OmpW, OmpK35, combination, and OmpK36 constructs docked to TLR4.

5.4.10. Immune simulation

The immunogenic profiles of the six vaccine constructs showed similar profiles in their immune cell induction (Figure 5-10). For OmpK35, OmpK36 and OmpC the secondary and tertiary immune response (IgG1, IgG2, IgG + IgM) was greater than the primary immune response (IgM), whereas OmpW, OmpF, combination and YfaZ displayed high IgM concentrations in the primary response (Figure 5-10A). The production of B-cells is long-lasting, demonstrating isotype switching ability and memory cell development for all the vaccine constructs (Figure 5-10B) (Banerjee *et al.*, 2020). The high levels of IFN- γ and IL-2 produced in the immune simulation suggests strong potency for producing the cytokine. Additionally, the increase in IFN- γ and IL-2 levels are important for viral replication inhibition and inducing cellular immunity (Rouzbahani *et al.*, 2022). The activation of macrophages was identified by the increase in levels of IL-12 and IL-18 following the first injection (Umitaibatin *et al.*, 2023) (Figure 5-10C). The *in-silico* immunization shows that the investigated constructs can lead to activation of cytotoxic T-cells (Figure 5-10D) and is important to ensure robust and effective protection against *K. pneumoniae*. No unwanted responses were initiated as represented by the low danger signal in the inset plot in Figure 5-10C justifying the

safety of the vaccine constructs. Overall, the results obtained from the C-ImmSim server indicates that the vaccine constructs can induce sufficient immune response without eliciting undesirable response and are therefore suitable vaccine candidates.

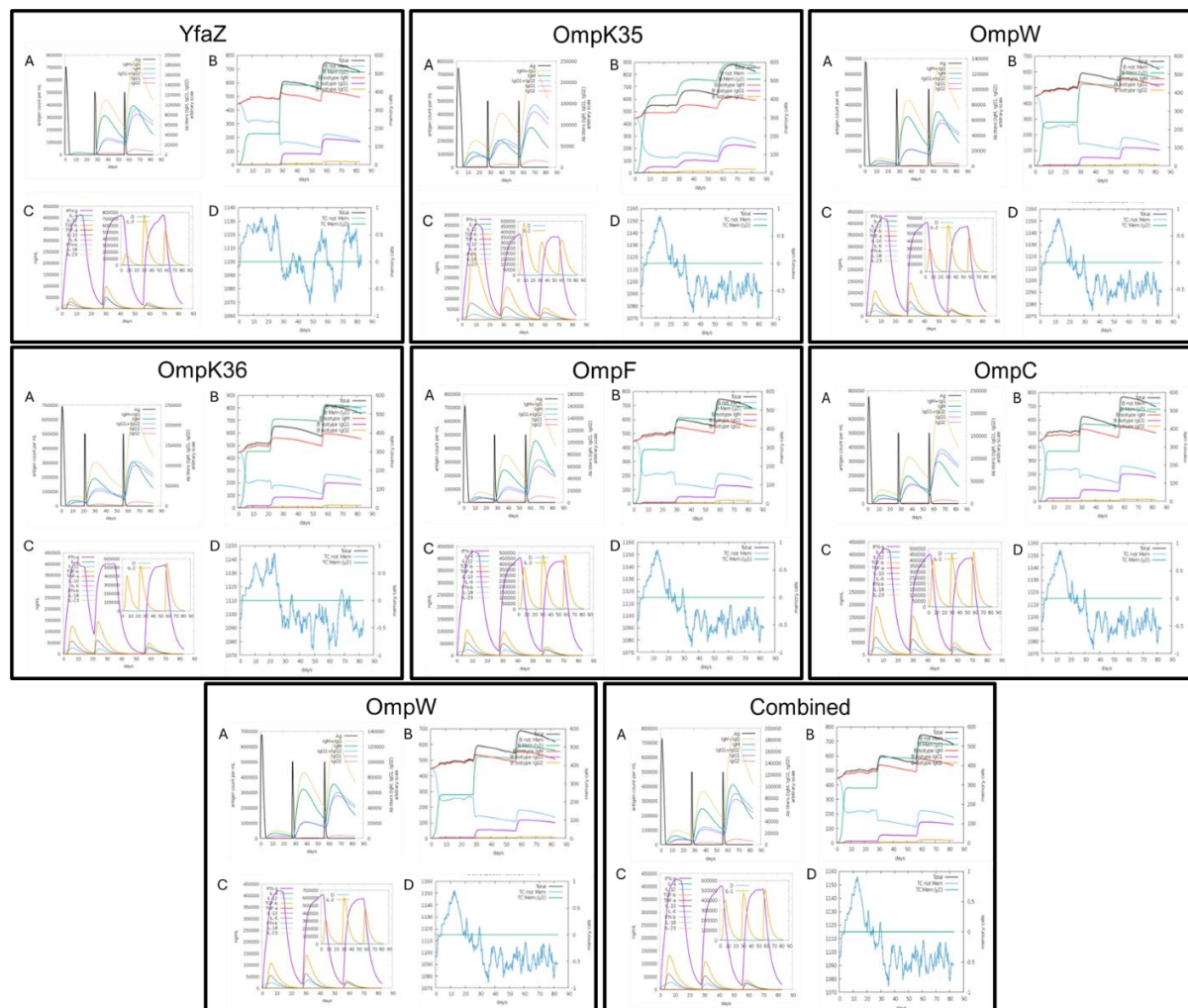


Figure 5-10: *In silico* immune simulation of vaccine constructs investigated using the C-ImmSim server. Top graph represents the Antigen and immunoglobulin response, and the bottom graph represents the production of cytokine and interleukins evaluated using Simpson index.

5.4.11. Vaccine construct codon optimization and *in silico* cloning

Prior to *in silico* cloning, the Vector Builder Codon Optimization tool was used to optimize vaccine design for expression in the *E. coli* K12 host system. The Codon adaptation index (CAI) value of the vaccine constructs was greater than 0.9 and the ideal GC (guanine–cytosine) content was greater than 55% for all the vaccine constructs (Figure 5-11). Both these values fall within the acceptable range of 0.8 to 1.0, and 30 to 70% for the CAI value and GC content, respectively, supporting that the designed constructs all have a high likelihood of protein expression (Dey *et al.*, 2022,

Banerjee *et al.*, 2020). Finally, the adapted codon sequences were inserted into the pET-28a (+) vector using the Geneious Prime software to present the design of the recombinant plasmid (Figure 5-11).

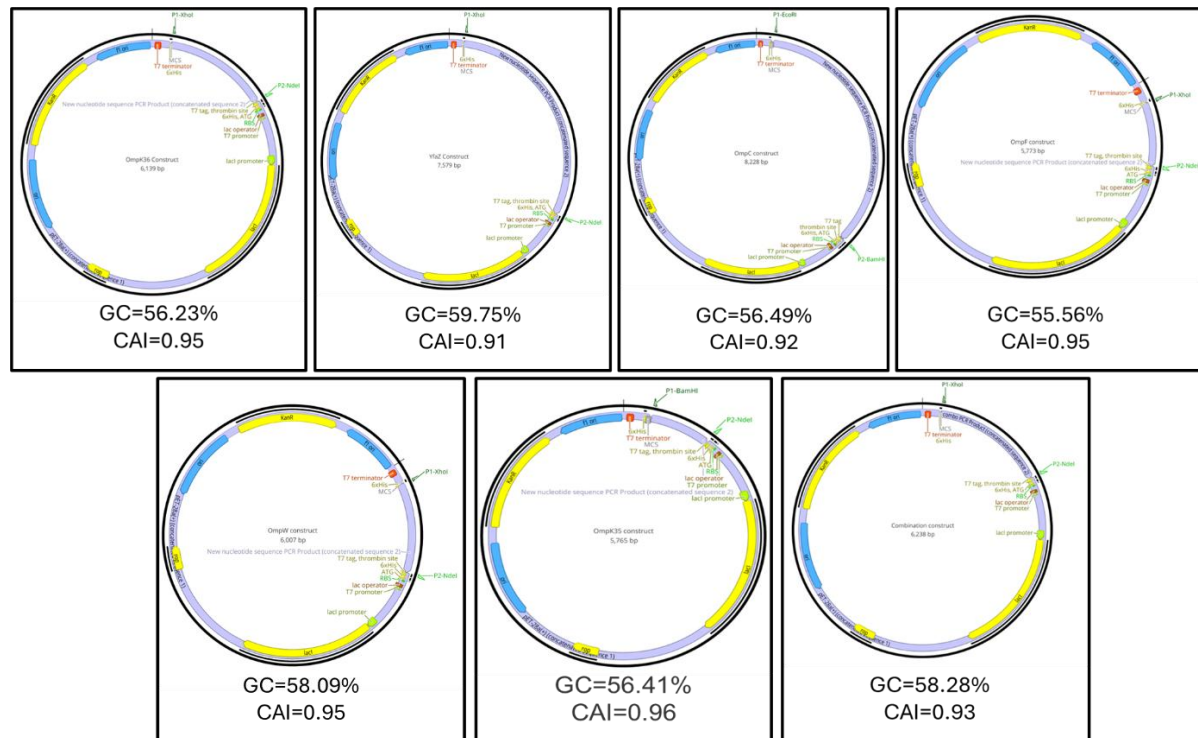


Figure 5-11: *In silico* restriction cloning of the adapted codon sequences into the pET28a (+) expression vector using Geneious Prime. The vector backbone is represented with a black circle and the gene encoding the vaccine is represented in purple as New nucleotide sequence PCR product. The restriction enzymes used for the respective clones are displayed in green.

5.5. Discussion

The overuse of antibiotics has resulted in antibiotic resistant bacteria and alternative therapies like vaccines provide an effective preventative approach (Nahian *et al.*, 2025). The COVID-19 pandemic led researchers to delve further into novel vaccine development strategies including reverse vaccinology due to limitations imposed by the pandemic which did not allow for swift research development and manufacturing of much needed vaccines (Khalid and Poh, 2023, Rappuoli *et al.*, 2021). Leveraging on the use of bioinformatics and computational analysis of pathogen genomes, reverse vaccinology is advantageous as multiple epitopes can be simultaneously targeted thus, enhancing vaccine efficacy and effectiveness against highly diverse and rapidly evolving antibiotic-resistant strains (Rappuoli, 2000, Rappuoli *et al.*, 2016, Herrera, 2021, Khalid and Poh, 2023, Nahian *et al.*, 2025). Thus, the current study

used immunoinformatics strategies to generate multi-epitope vaccines against *K. pneumoniae*.

The immunogenicity and protective efficacy of Omps such as OmpA, OmpK36, and OmpK17 have been evaluated. However, the typical beta-barrel transmembrane structure of Omps makes them difficult-to-express and prepare in large scale (Liao *et al.*, 2024). This study used 6 outer membrane surface proteins (OmpC, OmpW, OmpF, OmpK35, OmpK36 and YfaZ) which play a role in antibiotic resistance and have essential immunogenic properties and are therefore ideal for the development of multi epitope vaccines. Our strategy was to ensure that the host immune system identifies the most antigenic protein sequences on the pathogen upon host-pathogen interaction (Zhang *et al.*, 2021, Wong *et al.*, 2022, Tsai *et al.*, 2011, Wang *et al.*, 2017). However, some clinically relevant beta-lactamase producing strains of *K. pneumoniae* lack or express low levels of OmpK35, this contributes to the antibiotic resistance of ESBL producing *K. pneumoniae* (Hernández-Allés *et al.*, 1999, Tsai *et al.*, 2011). Therefore, it becomes increasingly important to develop multiple vaccines against the pathogen to maximize chances of priming the immune system for pathogen identification and inhibition (Tsai *et al.*, 2011). The importance of these proteins in eliciting an immune response indicates that they are recognized by the immune system, therefore suitable candidates for multiepitope vaccine construction.

Various servers were used to identify HTL, CTL and B-cell epitopes from the six proteins, the epitopes were then screened for their toxicity, allergenicity and antigenicity (Table 5-1, Table 5-2, Table 5-3). Population coverage analysis of the selected epitopes revealed that the six individual vaccines covered a wider population range as compared to the combined vaccine (Figure 5-3). The selected epitopes were then linked using suitable linkers (Figure 5-1, Figure 5-2), and following relevant assessments of the vaccine constructs, they were mainly thermostable, antigenic, and non-allergenic (Table 5-4).

Evaluation of the quality of the modelled structures (Table 5-5) and MD simulation studies (Figure 5-6) confirmed that the constructs were stable, compact, and ordered. RMSD plots indicated that constructs achieved equilibrium after ~50 ns with minor deviations. The combined construct, YfaZ and OmpC exhibited higher RMSF values attributed to loop flexibility. Rg plots showed consistent compactness, suggesting

thermal and conformational stability. Molecular docking provided insights into the interaction of the constructs with TLR2 and TLR4 (Figure 5-7) which are of interest because of their increased expression levels in the epithelial pathways following *K. pneumoniae* infection. Although all the constructs interacted with TLR2/4, OmpF and YfaZ did not form salt bridges with TLR2, also, OmpC did not form salt bridges with neither TLR2 nor TLR4, indicating the potential weakness of these interactions (Figure 5-7). Furthermore, MD simulations provided insights into the stability and dynamics of the vaccine-receptor complexes (Figure 5-8, Figure 5-9). The interactions were observed to stabilize over the 200 ns simulation period, which implies stability in the interactions of the constructs with TLR2 and TLR4. The decrease observed between the RMSD of unbound and TLR-bound states can be attributed to the formation of hydrogen and hydrophobic bonds. The decrease in Rg values upon complex formation confirms the conformational changes induced in the TLR receptors to result in internal signalling and an immunological response (Shahraki *et al.*, 2025). *In silico* cloning studies suggested the potential expression of the vaccines in a microbial expression system and this poses the constructs as viable vaccine candidates against *K. pneumoniae*.

Further investigation with the C-imm sim server confirmed the ability of these constructs to activate immunoglobulins, T-cells, B-cells, and a low danger signal (Figure 5-10) substantiating the prospective use of the constructs in immunocompromised patients and infants. The importance of these proteins in eliciting an immune response indicates that they are recognized by the immune system, hence these candidates are proposed for multiepitope vaccine construction. The combined construct is unique in that it integrates top-scoring B-cell, CTL and HTL epitopes from six outer membrane proteins which have not been previously combined into a single vaccine construct. This design strategy enhances epitope diversity and immunogenic coverage to offer broader protection across genetically diverse human populations.

Several recent studies have used Omps such as OmpA, OmpK36, OmpF, and OmpW in the design of multiepitope subunit vaccines against *K. pneumoniae*, for example the AK36 vaccine and mHla-EpiVac (Li *et al.*, 2025). The AK36 vaccine, designed based on OmpK36 and OmpA, demonstrated significant protection in murine sepsis models

through robust humoral and cellular responses (Babu *et al.*, 2017). mHla-EpiVac which was designed using ferric enterobactin protein (FepA), OmpA, and OmpW, showed strong immunogenicity and protective potential (Liao *et al.*, 2024). Compared to these studies, our approach integrates a broader set of immunodominant epitopes predicted from conserved Omps, optimized for population coverage and MHC-binding affinity, potentially enhancing global applicability. However, unlike prior *in vivo* validations, our study is limited to *in silico* predictions and modelling, which, although cost-effective and rapid, require experimental validation to confirm immunogenicity and efficacy. Despite these limitations, our work advances the field by proposing a rationally designed, epitope-focused MESV with optimized structural and immunological features, offering a valuable foundation for subsequent experimental evaluation (Liao *et al.*, 2024).

In conclusion, due to the urgency for mitigating *K. pneumoniae* antibiotic resistance, and the severity of the infections, a coordinated multi-layered strategy to weaken, expose and neutralize *K. pneumoniae* is timely and justified. Earlier chapters focused on inhibition of the internal stress-response chaperone machinery ClpK, thereby compromising bacterial survival under hostile conditions. In contrast, this chapter uses OMPs for the rational design of multiepitope vaccines through computational approaches, thus helping control the disease. Epitope vaccines are constructed with only the antigenic portions of the pathogen that elicit a direct immune response. The computational studies undertaken in this study show that the multiepitope vaccine constructs are stable and show good efficacy and safety against *K. pneumoniae* in humans. Despite *in silico* studies providing a valuable insight into construct design, structure prediction, and molecular interactions, they cannot fully replicate the complexity of biological systems. Therefore, anticipated future studies will prioritise the validation of the designed constructs through laboratory-based experimentation resulting into a potential vaccine candidate for preclinical and clinical testing.

5.6. Supplementary

Table 5-S1: List of the NCBI sequences sharing common epitopes as identified by the respective databases.

NCBI sequences	Epitopes
OmpC, B-cell epitopes	
QDX70306.1, CAK6612449.1	LFDWDNHYAEGANNTT
UKL99967.1, KAA8854169.1, WP_248961503.1, WP_231821751.1, WP_149781330.1, WP_149796246.1, WP_192846906.1, WP_122021035.1, WP_122008057.1, WP_327778688.1, WP_331432422.1, WP_314231259.1	ASAADAAEIYNKNGNK
WQD07879.1, RNT56230.1, UYL76072.1, XAE07659.1, WIC34936.1, WFA37008.1, UZL70618.1, UUU74782.1, UWX52356.1, UQY73146.1, RYI04097.1, RRE67929.1, RRE85280.1, AYJ98535.1, WP_282851359.1, WP_301362654.1, WP_301692008.1, WP_309353650.1, WP_310967208.1, WP_285282094.1, WP_285282002.1, WP_326395400.1, WP_318042278.1, WP_322207347.1, WP_323790420.1, WP_330190358.1, WVU15307.1, WP_331320517.1, WP_334612587.1, WP_334676376.1, WP_311747608.1, WP_085288266.1, WP_099451410.1, WP_110232093.1, WP_064765141.1, WP_048328035.1, WP_048291620.1, WP_020804449.1, UMJ19653.1, WP_124049030.1, WP_117265175.1, WP_080851244.1, WP_080784230.1, WP_065953963.1, WP_064158758.1, WP_040089309.1, WP_124043043.1, WP_117099241.1, WP_117073183.1, WP_117058983.1, WP_114472826.1, WP_114266799.1, WP_109226703.1, WP_094340598.1, WP_070182456.1, WP_040153802.1, WP_117026893.1, WP_116986644.1, WP_104442942.1, WP_102094005.1, WP_101997843.1, WP_100756959.1, WP_095884909.1, WP_088426917.1, WP_101529042.1, WP_087794523.1, WP_087760599.1, WP_085787659.1, WP_048279902.1, WP_130948364.1, WP_117147203.1, WP_117084541.1, WP_038991833.1, WP_117066895.1, WP_040244965.1, WP_040213520.1, WP_117095469.1, WP_117082071.1, WP_114501475.1, WP_114266103.1, WP_109043042.1, WP_103556088.1, WP_099718471.1, WP_117043665.1, WP_116773334.1, WP_117079246.1, WP_143829948.1, WP_143861016.1, WP_143992893.1, TYY07783.1, WP_064181404.1, WP_117057121.1, WP_087826851.1, WP_102023965.1, WP_102084901.1, WP_110225613.1, WP_114265044.1, WP_114266681.1, WP_117028972.1, WP_149999044.1, WP_150330949.1, WP_131874964.1, WP_134913655.1, WP_135713153.1, WP_134872670.1, WP_156731239.1, WP_159179712.1, WP_159211604.1, WP_159214465.1, WP_159224285.1, WP_159310559.1, WP_161271940.1, WP_160596539.1, WP_169329557.1, WP_170911869.1, WP_069196821.1, WP_186956682.1, WP_186983828.1, WP_192953767.1, WP_195494341.1, WP_182140822.1, WP_181239263.1, WP_142499497.1, WP_071067762.1, WP_084456119.1, WP_160558054.1, WP_156718871.1, WP_227343305.1, WP_223815227.1, WP_202020262.1, WP_229578996.1, WP_229540628.1, WP_228366205.1, UDD03693.1, WP_221016328.1, WP_221013082.1, WP_220152806.1, WP_219686246.1, WP_203402299.1, WP_201143203.1, WP_200539696.1, WP_200536208.1, WP_200533953.1, WP_243228511.1, WP_243212684.1, WP_210531032.1, WP_243201952.1, WP_243201689.1, WP_241506567.1, WP_237437859.1, WP_211671646.1, WP_211570310.1, WP_232580047.1, WP_231798485.1, WP_211585664.1, QMB91690.1, WP_263747397.1, WP_265924158.1, WP_270479957.1, WP_274094135.1, WP_274162382.1, WP_257066233.1, WP_250177994.1, WP_256868802.1, WP_248720701.1, WP_248887609.1, WP_248889683.1, WP_248943871.1, WP_251336013.1, WP_255076420.1, WP_255158326.1, WP_341799107.1, WP_163600026.1, WP_336271608.1, WP_346802830.1, WP_341971372.1, WXF77254.1, WP_347073066.1, WP_334683112.1, QAV78775.1, TWY79052.1, RRY40163.1, RLK91389.1, RMA03070.1, RMA17957.1, WLX27860.1, WLE51170.1, PXH72497.1, QWZ04123.1, QLB30097.1, QIF85576.1	EGANSWTRLGFAGLKF
WFA62900.1, QZO55406.1, QVR15640.1	ELTGYGQWEYNIKANT
UZL56251.1, UNT75751.1, WP_087775943.1, WP_149998991.1, WP_156728612.1, WP_200786702.1, UYK36726.1, PXI21960.1, QEP92281.1, WP_336820264.1, WP_257945851.1, UON21488.1, WP_117042627.1, WP_117092516.1, WP_049184262.1, WP_064188382.1, WP_015958668.1, WP_289554875.1	NFEVVAQYQFDDFNLP
SAS52961.1	NFEVVAPYQDFGLRP
THB56057.1, OSH93659.1, WP_211667291.1, WP_243397069.1, OVH93769.1, WP_159164455.1, WP_259677994.1, WP_318245143.1, WP_265776627.1, WP_317617258.1, WP_343218342.1, RIU78125.1, WP_227522615.1, WJU19241.1, ODO76005.1, WP_283008440.1, WP_196530590.1, WP_213491968.1, WP_318656123.1, SVM67749.1, SWY93610.1, WP_219716381.1, WP_265921688.1, WP_289501189.1, TMZ80829.1, QJM66675.1, WP_261640214.1, QGV95675.1, WP_193402161.1, WP_223325905.1, WP_211779225.1, WP_181241426.1, WP_159306338.1, WP_159215382.1, WP_110179909.1, WP_032439413.1, OGE60177.1, WP_234288258.1, SAS75217.1, SXT92881.1, STU35004.1, STU52529.1, STT94564.1, WP_277458831.1, WP_208928306.1, PLH81922.1, WP_117052451.1, SAS82013.1, SXW35097.1, SWG63216.1, WP_213438824.1, SXO01807.1, UJH88160.1, SVS06406.1, SSJ80593.1, UMH99987.1, KAB0295101.1, PLM80144.1, OSP35175.1, AOF11941.1, WP_340057090.1, WP_282822495.1, WP_279511071.1, WP_275256022.1, WP_247134940.1, WP_240026578.1, WP_238901708.1, WP_234267732.1, WP_234245825.1, WP_226651105.1, WP_153591630.1, WP_152280478.1, WP_264002543.1, QLT69985.1, WP_130627977.1, WP_277458183.1, WP_221012470.1, SYJ58699.1, WP_248259695.1, WP_086551331.1, WP_094611202.1, WP_155367669.1, WP_159183680.1, WP_325926962.1, UZL30959.1, SXO68517.1, SWF25576.1, WP_109231237.1, WP_211779174.1, RCJ97741.1, WP_023284069.1, WP_263741913.1, WP_231539665.1, WP_278315587.1, QGV94718.1, SLR24913.1, PLH05903.1, PBD65156.1, OZZ66614.1, WP_319851953.1, WP_149795910.1, WP_229301417.1, RXH90690.1, WP_081541261.1, WP_087874780.1, UMK12827.1, SYT74814.1, SSH09621.1, WP_219191795.1, SYH38205.1, SAV85257.1, WP_031593320.1, WOE00935.1, WOD94257.1, UMI53619.1, WKA18962.1, POK54666.1, UUUW02157.1, TNO06337.1, SXP18006.1, WP_253969632.1, WP_290361651.1, SWT44453.1, SWM22522.1, SXW18119.1, WP_340196434.1, WP_331237020.1, WP_316570244.1, WP_289804118.1, WP_242743341.1, UYA13039.1, WP_211560577.1, WP_198090383.1, WP_180851984.1, WP_187789829.1, WP_193427526.1, ORT65953.1, WP_257965156.1, WP_110249385.1, OSP65732.1, WP_200784053.1, WP_206552284.1, WP_208952819.1, SVP70297.1, VGJ27147.1, VGM07034.1, VGM49658.1, VGD51647.1, VGA52765.1, WP_099130593.1, SWP10486.1, UOQ16333.1, SWN41200.1, WP_319835255.1, WP_309598403.1, WP_301696809.1, WP_282812839.1, WPC53863.1, WP_094317181.1, SLR16842.1, PLP27273.1, WP_301116071.1, SWX92750.1, SYQ01434.1, SWG05188.1, UKL16235.1, WP_275265796.1, WP_117255657.1, WP_348616457.1, WP_229537864.1, WP_289490647.1, RBZ52481.1, UY547971.1, WP_264254725.1, AWS76028.1, QGV98684.1, SYQ76118.1, SSK77223.1, WP_238640587.1, WP_271039833.1, WP_213225483.1, WP_206641001.1, WP_338858070.1, WP_104469851.1, WP_117029872.1, SSN52783.1, WP_152238079.1, OVG18711.1, WP_023278788.1	PEFGGDTYGSDFNLQS

WP_180160369.1, WP_282812068.1, WP_211562604.1, WP_128306440.1, WP_117241917.1, WP_141407503.1, WP_142483158.1, WP_153587453.1, WP_259542593.1, STW63726.1, STW83625.1, QPP81436.1, SWP71078.1, SYR84348.1, WP_218791990.1, STT31211.1, STT70889.1, SYN59671.1, SSH68140.1, WP_283000881.1, SYA25916.1, SXL34051.1, SYO47099.1, SWM94440.1, SYU21238.1, SXH17249.1, SXP40726.1, RLL06471.1, RNN44747.1, SYC18800.1, RNT62415.1, RNQ16913.1, RNR53438.1, RNS27073.1, QPB95286.1, VUH07807.1, RNT79630.1, VUI92928.1, CAB1213883.1, RRE85511.1, RYL55180.1, QND03424.1, WP_279497573.1, STW51842.1, WP_318593092.1, WP_211668644.1, SAX07249.1, GMX25281.1, QPG81129.1, WP_257301274.1, WP_182920142.1, WP_142470809.1, WP_096812370.1, WP_117087397.1, WP_323631001.1, QWZ03931.1, SYR85775.1, WP_117066688.1, WP_071556197.1, APV24287.1, SLO77468.1, GMW43809.1, WP_107357812.1, WP_116439487.1, SAR51253.1, PLM60024.1, WP_277467211.1, WP_255041356.1, WP_264930213.1, WP_130943320.1	
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UGN04833.1	SGSNRTLDQRANTLNN
CED76676.1, WP_164484984.1, WP_115490335.1, WP_256939959.1, WP_181238624.1, WP_302009521.1, WP_234738824.1, WP_175285060.1, RNQ99650.1, RNR98840.1, UHA83140.1, WP_125329014.1, WP_302005111.1, WP_253969580.1, WP_155066213.1, SWV97426.1, WP_179382854.1, WP_179382565.1, WP_211786665.1, WP_336406914.1, POI54364.1, WBT34898.1, WP_275862030.1, UYS53439.1, WP_192930073.1, UKL30986.1, WP_248142311.1, WP_211538396.1, WP_211565367.1, SVO29024.1, WP_179377432.1, WP_117044767.1, RCX46825.1, WP_176326763.1, WP_163594031.1, SUY89028.1, QLR66988.1	QLTGYGQWEYNVQANN
CAK6610105.1, CAL0388291.1	AGVATDDQAAVGIVYQ
PAW96996.1, WP_117093624.1, WP_338783487.1, WP_117039222.1, WP_064174943.1, WP_219705341.1, WP_210500541.1, WP_267718165.1, QWZ05048.1, GAA6568479.1, RMA05321.1, GMW58476.1, GMX16646.1, QGV90219.1, WP_263003685.1, WP_254541391.1, WP_250178208.1, WP_241866776.1, WP_241362171.1, WP_218783923.1, WP_200536244.1, WP_223370440.1, WP_203135181.1, WP_171482341.1, WP_159205173.1, WP_135715345.1, WP_070182388.1, WP_085163388.1	GDSYTYADNFMAGRAN
RLL08050.1	GDTYTYADNFMAGRAN
WLX82359.1	MLTGYGQWEYNVQANN
AVO78979.1	AGSGEGTNNGGARKLA
UYK36410.1	QITGYGQWEYNIQANN
WP_117249824.1	AGTAGAAEIYNKDGNK
OmpC, Cytotoxic T-cell Lymphocyte epitopes	
QDX70306.1, CAK6612449.1	KTVGDVDLY
WP_314231259.1, WP_331432422.1, WP_327778688.1, WP_122021035.1, WP_192846906.1, WP_149796246.1, WP_149781330.1, WP_231821751.1, WP_248961503.1, KAA8854169.1, UKL99967.1, WP_122008057.1	SAADAAEIY
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OmpW, HTL	
QEX42815.1, VTM53222.1, STT66979.1, STT02980.1, WP_048983352.1, WP_117254174.1, STT49126.1, STX08194.1, WP_264278190.1, WP_264237781.1, WP_180846922.1, QCC81588.1, WP_326410716.1, WP_096608902.1, QGV84249.1, QGV99371.1, RRF84835.1, WP_310972259.1, TYX80781.1, WYK64190.1, WP_160806104.1, SVK53449.1, WP_169517852.1, VGL86951.1, WP_217961205.1, WP_334677459.1, WP_255070816.1, WP_202685932.1, WP_048335168.1, WP_085843487.1, SSG44124.1, WP_069354422.1, XAX68422.1, QHU71794.1, UZJ22105.1, WP_103505323.1, WJU36809.1, WP_064177339.1, WQG33052.1, UMX78243.1, PHY75122.1, QIV45128.1, KAA8855943.1, WP_125034562.1, WP_104468292.1, WQD11364.1, WRT86441.1, WFB70208.1, QPW00766.1, WP_211659469.1, RTR21194.1, WP_130951121.1, WP_334656360.1, WP_305828941.1, WP_240264954.1, WP_326413491.1, WP_318670942.1, TYW73548.1, WP_275341037.1, WP_274897068.1, WDA29381.1, UZK97897.1, WP_264254758.1, WP_064161227.1, WP_255066372.1, USB49868.1, WP_248941482.1, WP_243207073.1, WP_229540736.1, WP_223390840.1, WP_221777144.1, WP_220998538.1, WP_219658558.1, WP_069333877.1, WP_219155589.1, WP_217800207.1, WP_185775716.1, WP_217966865.1, WP_080884362.1, WP_213493658.1, GKM74534.1, WP_204745682.1, WP_200543991.1, WP_198077422.1, WP_148825174.1, WP_180863782.1, WP_159220179.1, WP_152659822.1, WP_148831906.1, WP_150074352.1, WP_142490149.1, WP_109223298.1, WP_071068009.1, WP_110245829.1, WP_105178138.1, WP_064081402.1, WP_110180015.1, WP_023283574.1, WP_047694588.1, WP_117034509.1, WP_048978139.1, WP_058229112.1, WP_085820106.1, WP_058343217.1, WP_069345385.1, SSL13738.1, GKJ20357.1, PLN05328.1, WP_284117140.1, WP_268888161.1, WP_265702295.1, WP_257952029.1, WP_163599965.1, WP_255065584.1, WP_253991244.1, WP_241502613.1, WP_114473729.1, WP_221016763.1, WP_101974283.1, WP_117269670.1, WP_064173929.1, WP_069339145.1	QSKVRPYVAGINYT
SQC86617.1, UGM02811.1, WP_087834026.1, WP_073545900.1, WP_117254792.1	GLDYLINRDWLLNMS
SSN05715.1	AGLDYELDNQWMLNA
SSH02388.1	SSSKLRPYIGAGVNY
GJL09055.1	SKVRPYIGAGVNYTT
OmpF, B-cell	
GJG77703.1	YGFWQYRITGGENEWS
VTM79273.1, CAA0305814.1, VGM18299.1, VCZ31425.1, VCZ38058.1, VCZ36521.1, VGL91232.1	GKMGVEHVWTTNGDTS
VCZ53886.1, VUJ92435.1	GEHVWTTNGDTSSDDT
SVU11736.1	TPAATIPMPYARIGL
OmpF, HTL	
GJG77703.1	VNYRIIDDRSAAGV
VCZ31425.1, VCZ53886.1, VGM18299.1, VCZ38058.1, VCZ36521.1, VGL91232.1, SVU11736.1, CAA0305814.1, VUJ92435.1, VTM79273.1	YGQWEYNMDASNVEG

5.7. References

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Chapter 6: General Discussion and conclusion

Antimicrobial resistance in *K. pneumoniae* is a growing global health concern, with the WHO classifying it as a critical priority pathogen for the development of new therapeutics and alternative interventions (Kot *et al.*, 2023, Podschun and Ullmann, 1998). This thesis addresses this challenge through a multifaceted approach which combines structure-based inhibitor discovery with reverse vaccinology. Together, these studies propose strategies to target the internal machinery of *K. pneumoniae* that enables it to survive stressful environments and to prevent infection through immune system engagement. The central focus is ClpK, a thermotolerant caseinolytic ATPase implicated in bacterial stress tolerance and proteostasis, as well as complementary pathways contributing to *K. pneumoniae* virulence and persistence.

ClpK was studied using *in silico* and *in vitro* methods to assess its structural stability and thermotolerance in the presence of ATP and divalent metal ions. Experimental analysis following protein expression and purification confirmed protein concentration dependent ATPase activity (Figure 2-2). ClpK was observed to have a Michaelis constant (K_m) of 0.270 mM suggesting that the protein operates effectively at physiological ATP concentrations (Figure 2-3). These observations suggest that the activity of ClpK has been optimised for environments such as those encountered during host infection, highlighting its potential as a target for *K. pneumoniae* inhibition (Bojer *et al.*, 2012, Bojer *et al.*, 2010).

Mechanistic insights into ClpK function were further revealed through metal-ion studies. Mg^{2+} increased ClpK stability, while Mn^{2+} further enhanced ATPase activity, suggesting a potential role in modulating ClpK function and regulate the broader stress-response network rather than ClpK in isolation. CD analysis in the presence of 8 M urea showed that ATP conferred partial protection against denaturation and Mn^{2+} either assisted in retaining protein secondary structure or promoted alternative conformations under denaturing (8 M urea) conditions (Figure 2S-2). These observations align with studies that have shown Mn^{2+} to increase chaperonin-assisted protein folding efficiency as well as stabilising protein conformations under denaturing (8 M urea) conditions (Encinas *et al.*, 2002, Diamant *et al.*, 1995, Park *et al.*, 2015). Additionally suggesting that Mn^{2+} may influence the chaperonin activity of ClpK. Fluorescence spectroscopy further supported this interpretation by demonstrating that

both Mg^{2+} and Mn^{2+} stabilised ClpK in the presence and absence of ATP and urea (Figure 2-6, 2-7). Moreover, thermal stability assays revealed that ClpK remained folded at temperatures exceeding 60 °C under varying NaCl concentrations and pH conditions, reinforcing its thermotolerant nature. The observed ClpK stability suggests that ClpK plays a functional in protecting *K. pneumoniae* during heat or oxidative stress which may occur inside the host or in a clinical environment. Chapter 2 clearly demonstrated the effects of metal ions on the conformation and activity of ClpK representing a potential vulnerability that could be exploited in anti-virulence or antibiotic potentiation strategies in response to the mechanisms *K. pneumoniae* uses to withstand host-induced stress and the growing antimicrobial-resistance challenge it poses. In addition, it is important to note that many ATP-dependent chaperones use divalent cations such as Mg^{2+} for nucleotide binding and hydrolysis. This suggests that metal availability may influence conformational cycling and substrate processing across the proteostasis machinery. Although ClpP is not directly metal-activated, its proteolytic activity depends on association with divalent cation dependent ATPase partners. Therefore, fluctuations in intracellular metal concentrations may modulate the dynamics of the entire stress complex, affecting protein refolding, degradation efficiency, and survival under stress.

Building on the biochemical findings, computational analyses were used to investigate how ClpK interacts with small-molecule ligands and putative protein partners, thereby providing insight into its binding behaviours and functional associations within the bacterial stress response network. Molecular docking and MD simulation analysis showed that, ATP binding to both nucleotide binding domains provided the greatest structural stability in comparison to ATP bound to a single NBD or ClpK-apo (Figure 2-11). This highlights the cooperative nature of ClpK-ATP interactions. Lower potential energies, reduced RMSD and RMSF values, and compact Rg across simulations showed that Mg^{2+} ions enhanced ClpK stability in the presence of ATP (Table 2-1, Figure 2-12 – 2-13). The observed cooperativity between nucleotide binding domains suggests that ClpK activity is structurally regulatory and not just catalytic, allowing coordinated proteostasis under thermal and oxidative stress. Collectively, these results provide a basic understanding for how ATP cooperativity and Mg^{2+} contribute to ClpK stability highlighting their importance for ClpK activity in physiological environments.

A library of non-toxic, Lipinski-compliant molecules were screened for interaction with ClpK. Sclerotiamide derivatives and ADEP analogues, demonstrated the highest binding affinity and favourable MM-GBSA binding free energy scores across different ClpK domains (Table 4-5, 4-6). Sclerotiamide, Sclerotiamide P, Sclerotiamide B, Sclerotiamide N, ADEP5, Hydantoin and ADEP3 bind near NBD1, with PRO283 and ILE285 being key interacting residues (Figure 4-3). These residues are part of the ATP binding site therefore ligand binding at this position could disrupt or compete with ATP interaction, thus modulating ClpK activity. In a similar manner, ADEP2 binds to LYS656 in NBD2 which further supports the hypothesis that these compounds could interfere with ClpK enzymatic activity.

Given that these compounds bind to residues in conserved ATPase domains shared across the Clp ATPase family, inhibitors targeting these domains may exhibit cross-reactivity with other bacterial Clp ATPases. This could enhance broad-spectrum antibacterial activity; however, selectivity of these compounds should be considered. Humans lack ClpK however, mitochondrial proteostasis depends on the ClpXP complex. Due to the structural conservation of ATP-binding mechanisms, ATP-competitive inhibitors may pose a theoretical risk of off-target mitochondrial interference. Additionally, broad inhibition of Clp ATPases across bacterial species could impact beneficial commensal flora, potentially leading to dysbiosis. Future inhibitor development should therefore prioritise pathogen-selective interactions to minimise host and microbiome disruption while preserving therapeutic efficacy.

Furthermore, this predicted mode of interference is consistent with established inhibitory mechanisms observed in other Clp-family proteins, where several ADEP derivatives are known to target the bacterial ClpP protease and interfere with its normal interaction with Clp-ATPases in *Escherichia coli* and *Bacillus subtilis*. These compounds displace ClpX ATPase by binding to the specific IGF and IGL motifs that are critical for ClpP and Clp ATPase interaction (Moreno-Cinos *et al.*, 2019, Kirstein *et al.*, 2009). In a similar manner, sclerotiamide and its derivatives have been identified to activate ClpP resulting in excessive protein degradation (Lavey *et al.*, 2016). These previous successes in inhibiting ClpP and other Clp ATPases using ADEPs and sclerotiamides indicate druggability of Clp proteins and support their potential for future inhibitor development.

MD simulations confirmed the dynamic stability of these protein-ligand complexes which were characterised by key stabilising interactions including hydrogen bonds, π - π stacking, and hydrophobic contacts (Figure 4-4 – 4-7). These high affinity interactions are unlikely to be incidental and reflect inhibitory potential that warrants *in vitro* investigation of inhibitor efficiency using ATPase assays, thermal folding analysis and crystallization. Previously, ADEPs and sclerotiamide derivatives have successfully disrupted proteolytic machinery in other bacteria therefore, their predicted interaction with ClpK suggests a plausible strategy to destabilise stress tolerance in *K. pneumoniae*. These inhibitors may function as anti-virulence modulators, sensitising bacteria to host immunity, or conventional antibiotics. The outcome of these inhibitory studies will contribute to novel therapeutic strategies aimed at reducing persistence and burden of pathogenic *K. pneumoniae* in clinical settings.

ClpK and putative protein partner docking further contextualized ClpK in the broader proteostasis system. The predicted interaction of ClpK with chaperones such as ClpP, DnaJ and DnaK suggested that ClpK does not function alone, instead it functions as part of a cooperative stress response complex (Table 3-1). Docking interface analysis showed the presence of stabilizing interactions, including hydrogen bonds and salt bridges, confirming that these proteins form energetically favored interactions with ClpK (Figure 3-4). This observation was strengthened by MD simulation analyses that confirmed the dynamic stability of protein-protein interactions (Figure 3-5 - 3-6). The protein-protein interactions described in this work enhances our understanding of the molecular role of ClpK and highlights its potential as a drug target to impair the survival mechanism of *K. pneumoniae*. Future work can focus on validating the interactions between ClpK and its putative protein partners using *in vitro* techniques such as co-immunoprecipitation or pull-down assays.

The study then pivoted towards host-directed control through the design of subunit vaccines against *K. pneumoniae* using immunoinformatics and reverse vaccinology. Six outer membrane proteins (OMPs), namely, OmpK35, OmpK36, OmpW, OmpF, OmpC and YfaZ, were identified and screened for conserved non-allergenic, and antigenic epitopes that are identified by host immune system B- and T-cells. OMPs provide immunological leverage points, allowing antibody- and complement-mediated clearance. Based on these analyses, six vaccine constructs were designed, each

incorporating epitopes from a single protein. Additionally, one combined vaccine construct incorporating epitopes from all six proteins was designed to broaden antigenic coverage, overcome strain heterogeneity and maximize the range of *K. pneumoniae* strains targeted by the vaccine. Structural modelling, and MD analyses suggested that the vaccine constructs were stable (Figure 5-6). Furthermore, molecular docking of the constructs with TLRs suggested strong immune recognition and activation with the global population coverage of the epitopes observed to be 75% (Figure 5-3, 5-7 – 5-9). This supports the broad applicability of the designed vaccine constructs however, epitope expression, antigenicity and protective efficacy must be investigated *in vitro* and *in vivo* to confirm vaccine construct antigenicity and efficacy.

The targets discussed in this thesis, although distinct, operate in mechanistically complementary compartments of the bacterial cell. ClpK is an intracellular chaperone that allows the pathogen to survive under hostile host conditions. In contrast, OMPs identified in Chapter 5 function at the host-pathogen interface and act as immunologically accessible vaccine targets. ClpK inhibition potentially compromises intracellular protein quality control, thus reducing the pathogens ability to withstand host-mediated stresses. Simultaneously, vaccination targeting surface-exposed OMPs potentially enhances immune recognition and promotes antibody- and complement-mediated clearance. Therefore, these strategies align within a dual-compartment therapeutic framework.

In conclusion, ClpK is confirmed to be structurally resilient under stress and likely plays a central role in protein quality control and thermotolerance. The identification of strong binding compounds opens avenues for experimental validation of ClpK inhibition. Its potential interactions with chaperones suggest that it is a component of the *K. pneumoniae* stress response system. Lastly, the rational design of multiepitope vaccine constructs provides a safe and broad-spectrum approach to immunization. However, these are limited as they rely on predictive models that simplify biological complexity. Therefore, experimental validation is the next important step to confirm the biological relevance of computational predictions as well as strengthen the translational potential of ClpK inhibiting compounds, interacting proteins and the vaccine constructs. This thesis contributes new knowledge on the elucidation of ClpK structural features and interaction networks under stressful conditions. A dual-

compartment strategy is presented, targeting intracellular stress-response machinery while simultaneously enhancing host immune recognition at the bacterial surface. Inhibition of ClpK may sensitize *K. pneumoniae* to immune effector mechanisms triggered by OMP-based vaccination, providing a mechanistic basis for therapeutic synergy. Through the combination of structural biology, computational pharmacology and immunoinformatics, this work highlights the importance of interdisciplinary approaches in the search for new solutions for antibiotic resistant bacterial infections.

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