INSIGHTS INTO THE STRUCTURE AND FUNCTIONS OF THE ALKALIPHILIC *Bacillus lehensis* G1 ClpC PROTEIN IN ALKALINE ENVIRONMENT

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To *mama* who regularly questions without really understanding the answers.

To *ayah* whose thoughts I value for in sustaining this mere worldly life.

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May peace and blessing be upon who are reading.

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ABSTRACT

The heat shock protein 100 (Hsp100) ClpC is a member of the AAA+ protein superfamily that contributes to the maintenance of the cellular protein homeostasis by energy dependent proteolysis. The aim of this work was to elucidate the interaction of the alkaliphilic Hsp100 ClpC from *Bacillus lehensis* G1 (*Bl*-ClpC) and its adaptor protein MecA in order to postulate their functions. The ClpC N-terminal domain of *B. lehensis* (*Bl*-ClpCN) was crystallised and the structure was solved to 1.85 Å. The crystal structure of ClpCN was analysed and compared to that of the archetypal species *Bacillus subtillis*. Comparisons of the N-terminal ClpC-interacting and hexamerisation domains between both species showed that *B. lehensis* G1 had an insertion (of unknown function) of four amino acids at the loop between helix 4 and strand 2, which were located on the outer surface of the barrel-shaped molecule. Apart from being highly unique, this characteristic was conserved only in the newly-isolated alkaliphilic *B. lehensis* G1 and not in any of the previously-reported structures of N-terminal domains isolated from the Hsp100 family. Furthermore, information concerning the physicochemical basis of alkaliphilic ClpC is still unclear. Therefore, the biochemical properties of *Bl*-ClpC were characterised under varying pH, temperatures, salt concentrations, and metal ions. *Bl*-ClpC showed two features which were distinct from other proteins in the Hsp100 family which were high salt concentrations and mild acidic pH caused an increase in ATPase activity. *Bl*-ClpC activity was considered to be similar to that of a halophilic protein, which demonstrated increased activity in high concentrations of NaCl, which was a common characteristic of a highly acidic protein. pH-induced structural changes and *Bl*-ClpC stability have been investigated as well, and these changes correlated with ATPase activity modulations in different pH. There was little effect on the protein structure when the pH was lowered from 9 to 5. In alkaline pH (pH 9 - 11), the presence of an α-helical-dominated molten globule state was reported. The function of the unique four-residue insertion at the α4-β2 loop, which was absent in the *B. subtilis* ClpC orthologue, has been successfully elucidated by using the structure-guided mutation approach, whereby a deletion mutation devoid of residue 76-79 (Δ76-79) was constructed. Circular dichroism spectroscopy was used to evaluate the structural perturbations associated with the deletion. The results demonstrated that the precise configuration of the α4-β2 loop was important for maintaining the structure and function of *Bl*-ClpC. Δ76-79 led to severe destabilisation as well as unfolding of the secondary structure of the protein, all of which decreased ATPase activity. The optimum temperature for Δ76-79 was 25°C instead of 45°C for *Bl*-ClpC. These findings showed that the additional four residues at the α4-β2 loop were critical for *Bl*-ClpC's structure and function. Overall, *Bl*-ClpC exhibited distinct responses to salt stress and mild acidic pH, hence implying that environmental conditions and stress adaptations were important selective forces which gave rise to the divergence of Hsp100 ClpC from its alkaliphilic archetype.

ABSTRAK

Protein renjatan haba 100 (Hsp100) ClpC merupakan ahli kumpulan superfamili protein AAA+ yang menyumbang kepada pengekalan homeostasis protein sel melalui proteolisis bersandarkan tenaga. Kajian ini bertujuan untuk menjelaskan interaksi alkalofilik Hsp100 ClpC daripada *Bacillus lehensis* G1 (*Bl*-ClpC) dengan penyesuainya protein MecA bagi mencadangkan fungsi-fungsinya. Domain N-terminal ClpC pada *B.lehensis* dihablurkan dan strukturnya telah diselesaikan kepada 1.85 Å. Struktur hablur dianalisis dan dibandingkan dengan spesies arkitipal *Bacillus subtillis*. Perbandingan domain interaksi dan heksamerisasi N-terminal ClpC antara kedua-dua spesies menunjukkan *B. lehensis* G1 mengalami penyisipan (tidak diketahui fungsinya) empat asid amino pada gelung heliks 4 dan lembar 2 yang terletak pada permukaan luar molekul berbentuk tong itu. Selain daripada sifatnya yang unik, ciri ini terpulihara hanya pada *B. lehensis* G1 alkalofilik yang baharu terasing dan tiada pada struktur domain N-terminal yang diasingkan daripada famili Hsp100 yang pernah dilaporkan sebelum ini. Selain itu, maklumat berkenaan asas fizikokimia ClpC alkalofilik masih tidak jelas. Oleh itu, sifat biokimia *Bl*-ClpC dicirikan melalui bacaan pH, suhu, kepekatan garam dan ion logam yang berbeza. *Bl*-ClpC menunjukkan dua sifat yang jelas berbanding protein lain dalam famili Hsp 100: kepekatan garam yang tinggi dan pH sedikit berasid yang menyebabkan peningkatan aktiviti ATPase. Aktiviti *Bl*-ClpC dianggap menyamai protein halofilik yang menunjukkan peningkatan aktiviti pada kepekatan NaCl yang tinggi iaitu satu ciri protein yang sangat berasid. Perubahan struktur dan kestabilan *Bl*-ClpC disebabkan pH tinggi juga dikaji, dan perubahan ini berkorelasi dengan modulasi aktiviti ATPase pada pH yang berbeza. Hanya terdapat sedikit kesan ke atas struktur protein ketika pH diturunkan dari 9 ke 5. Keadaaan pH beralkali (pH 9 - 11) dilaporkan menyebabkan kehadiran keadaan globul lebur terdominasi α-heliks. Fungsi penyisipan empat residu unik pada gelung α4-β2 yang tiada pada ortolog *B. subtilis* ClpC berjaya dihuraikan menggunakan pendekatan mutasi berpandukan struktur, dimana mutasi delesi yang melibatkan residu 76-79 (Δ76-79) dijalankan. Spektroskopi edaran dikroisme digunakan untuk menilai gangguan struktur yang berkaitan dengan penghapusan residu. Hasil kajian menunjukkan bahawa konfigurasi gelung α4-β2 yang tepat adalah penting untuk struktur dan fungsi *Bl*-ClpC. Δ76-79 menyebabkan ketidakstabilan yang kritikal dan pembukaan lipatan struktur sekunder protein, yang mengurangkan aktiviti ATPase. Walau bagaimanapun, suhu optimum Δ76-79 dikurangkan kepada 25 °C, berbanding dengan *Bl*-ClpC, iaitu pada 45 °C. Keputusan mencadangkan bahawa empat residu tambahan dalam gelung α4-β2 adalah berperanan penting didalam menentukan struktur dan fungsi *Bl*-ClpC. Secara keseluruhannya, *Bl*-ClpC mempamerkan tindakbalas jelas terhadap tegasan garam dan pH sedikit berasid, membuktikan bahawa keadaan persekitaran dan adaptasi tegasan adalah daya selektif yang penting bagi meningkatkan penyimpangan Hsp100 ClpC dari sifat arkitip alkalofiliknya.

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LIST OF SYMBOLS / ABBREVIATIONS

2D	-	two-dimensional
3D	-	three-dimentional
A280	-	absorbance at optical density of 280 nm
A600	-	absorbance at optical density of 600 nm
AAA+	-	ATPases Associated with a variety of cellular Activities
Ala or A	-	alanine
ADP	-	Adenosine 5'-diphosphate
AEX	-	anion exchange chromatography
Arg or R	-	arginine
Asn or N	-	asparagine
Asp or D	-	aspartic acid
Atm	-	pressure unit atmosphere
ATP	-	Adenosine 5'-triphosphate
ATPase	-	ATP hydrolase
Ba	-	*Bacillus amyloliquefaciens*
Bc	-	*Bacillus coahuilensis*
B. clausii	-	*Bacillus clausii*
B. lehensis	-	*Bacillus lehensis*
Bl-ClpC	-	*Bacillus lehensis* ClpC
Bl-ClpCN	-	*Bacillus lehensis* ClpC N-terminal domain
B. subtilis	-	*B. subtilis*
Bs-ClpC	-	*B. subtilis* ClpC
Bs-ClpCN	-	*B. subtilis* ClpC N-terminal domain
BLAST	-	Basic Local Alignment Search Tool
C	-	carbon

CCP4	-	Collaborative Computational Project Number 4
CD	-	circular dichroism
CEX	-	cation exchange chromatography
Clp	-	Caseinolytic protein
CTD	-	C-terminal domain
CTD-MecA	-	C-terminal domain of MecA
Cu^{2+}	-	cuprum ion
CV	-	column volume
Da	-	Dalton
dH_2O	-	distilled water
DNA	-	deoxyribonucleic acid
DMSO	-	dimethyl sulphoxide
DSC	-	differential scanning calorimetry
DTT	-	Dithiothreitol
E.coli	-	*Escherichia coli*
EDTA	-	ethylenediamine tetraacetic acid
Fe^{2+}	-	ferrous ion
FPLC	-	fast protein liquid chromatography
g	-	gram
GC	-	Guanine and cytosine
Gln or Q	-	glutamine
Glu or E	-	glutamic acid
Gly or G	-	glycine
h	-	hour
His or H	-	histidine
HCl	-	hydrocloric acid
His-tag	-	hexahistidine tag
Hsps	-	heat shock protein(s)
HT	-	highthroughput
I or Ile	-	isoleucine
IEX	-	ion exchange chromatography
IMAC	-	Immobilised metal affinity chromatography
IPTG	-	Isopropyl-β-D-thiogalactopyranoside
JCSG	-	Joint Center for Structural Genomic

K	-	Kelvin
Kcal	-	kilo calorie
kDa	-	kilodalton
l	-	litre
L or Leu	-	leucine
LB	-	Luria-Bertani
LBA	-	Luria-Bertani for autoinduction
Lys or K	-	lysine
m	-	mili
M	-	molar
mdeg	-	millidegrees
mg	-	miligram
Mg^{2+}	-	magnesium ion
$MgSO_4$	-	magnesium sulphate
MG	-	molten globule
min	-	minute
ml	-	milliliter
mm^3	-	millimeter cubic
mM	-	millimolar
Mn^{2+}	-	manganase ion
MOPS	-	3-(N-morpholino) propanesulfuric acid
MR	-	molecular replacement
MRE	-	Mean residues ellipticity
MW	-	molecular weight
Na^+	-	sodium ion
NaCl	-	sodium chloride
NBD	-	nucleotide binding domain
NEB	-	New England Biolabs
NCBI	-	The national Center for Biotechnology Information
Ni^{2+}	-	Nickel ion
Ni-NTA	-	Nickel-nitrilo triacetic acid
NTD	-	N-terminal domain
ng	-	nanogram
nl	-	nanoliter

nm	-	nanometer
nmol	-	nanomolar
O_2	-	Oxygen
OD	-	optical density
OE	-	Overlapping extension
OH	-	hydroxyl
ORF	-	Open reading frame
P_i	-	Inorganic phosphate
pI	-	Isoelectric point
PCR	-	polymerase chain reaction
PDB	-	protein data bank
PEG	-	polyethylene glycol
pH	-	potential of hydrogen
PMSF	-	phenylmethylsulfonyl fluoride
Pro or P	-	proline
RMSD	-	root mean square deviation
rpm	-	revolutions per minute
RSM	-	response surface methadology
RT	-	room temperature (22 ± 3 °C)
s	-	Second
SDS	-	sodium dodecyl sulphate
PAGE	-	polyacrylamide gel
SEC	-	size exclusion chromatography
Ser or S	-	serine
sp.	-	species
Thr or T	-	threonine
T_m	-	melting temperature
Trp or W	-	tryptophan
Tyr or Y	-	tyrosine
Tris	-	Tris(hydroxymethyl)aminomethane
U	-	unit
UV	-	Ultraviolet (light)
UV-VIS	-	Ultraviolet-visible (light)
V	-	volt

v/v	-	volume per volume
w/v	-	weight per volume
w/w	-	weight per weight
X-ray diffraction	-	Roentgen diffraction
Zn^{2+}	-	zinc ion
α	-	alpha
Å	-	1 Angström = 0.1 nm
β	-	beta
°C	-	Degree Celcius
Cα	-	alpha carbon
µ	-	micro
µm	-	micrometer
pK_a	-	Ionization constant
-	-	minus
%	-	Percentage
µl	-	microlitre
µg/ml	-	microgram per litre
µM	-	micromolar
1X	-	One time
µmol	-	micromole
~	-	Approximate value
Ψ	-	Psi
Δ76-79	-	*Bl*-ClpC with deletion at amino acid 76-79
Δ*Cp*	-	change in heat capacity
Δ*H*	-	change in enthalpy

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CHAPTER 1

INTRODUCTION

1.1 Background

Owing to several reasons, there has been a resurgent interest in the industrial and academic potentials of alkaliphilic microorganisms. Fundamentally, there is a desire to understand the biological pathways which are mostly influenced by an alkaline external pH apart from the adaptations required for an organism to thrive in alkaline conditions. From the industrial point of view, the search for enzymes that are capable of catalysing reactions in conditions above pH 9 has burgeoned due to the proven utility of alkali-tolerant proteases, hydrolases and lipases. Intriguingly, progress has also been witnessed in the incorporation of live microorganisms into the alkaline environment of cement-based construction materials. This innovative use of encapsulated bacteria can facilitate self-repair (Jonkers *et al.*, 2010) and enhance compressive strengths of concrete as well as mortar (Sung-Jin *et al.*, 2012). Jonkers (2010) has shown that alkaliphilic *Bacillus pasteurii* spores along with calcium lactate, when inserted into a concrete mixture, gave rise to a self-healing product. These bacteria grow in small cracks and undergo biomineralisation, hence producing calcite and seal the damage. As such, there is a great interest to understand the way by which alkaliphilic bacteria cope with the stresses imposed by high pH. This is the first study to examine the differences between stress-induced heat shock protein (hsp) systems of alkaliphilic *Bacilli* and that of a related neutrophilic species.

The Caseinolytic protein C (ClpC) is a member of the Hsp100/Clp ATPase associated with various cellular activities (AAA+) family, which form hexameric ATP-dependent protein-unfolding units that are conserved throughout eubacteria (Weibezahn *et al.*, 2004). *Bacillus subtilis* ClpC (*Bs*-ClpC) is a well-characterised stress-induced chaperone that exists as part of a larger proteolytic machine – the ClpCP protease – which is responsible for degrading aggregated or denatured proteins (Wang *et al.*, 2011; Turgay *et al.*, 1998). The ClpC molecule is a 91 kDa protein consisting of five separate domains: (1) a small N-terminal protein interaction domain; (2) an AAA^+ ATPase domain; (3) a coiled-coil M-domain; (4) a second AAA^+ ATPase domain; as well as (5) a C-terminal domain that couples to Clp Protease (ClpP). The appearance of the protease resembles two barrels joined together (Figure 1.1A). The first barrel has a six-fold symmetry and is built from six ClpC molecules in a complex which contains six MecA adaptor proteins (see Figure 1.1A and B, Wang *et al.*, 2011). ATP hydrolysis within the ClpC ATPase sites is coupled with the unfolding of substrate proteins in the barrel's interior. These facilitate either the refolding or degradation of the said proteins following their translocation to the associated tetradecameric barrel. Generally, the system is multi-functional and plays a major role in preventing the accumulation of detrimental cellular aggregates that form under chemical or thermal stress. However, ClpCP also regulates (1) competence by controlling the levels of the specific transcription factor ComK (Turgay *et al.*, 1998); as well as (2) sporulation through an unknown mechanism (Persuh *et al.*, 2002). ClpC is therefore a vital and interesting molecular marker which is involved in both stress-survival and sporulation.

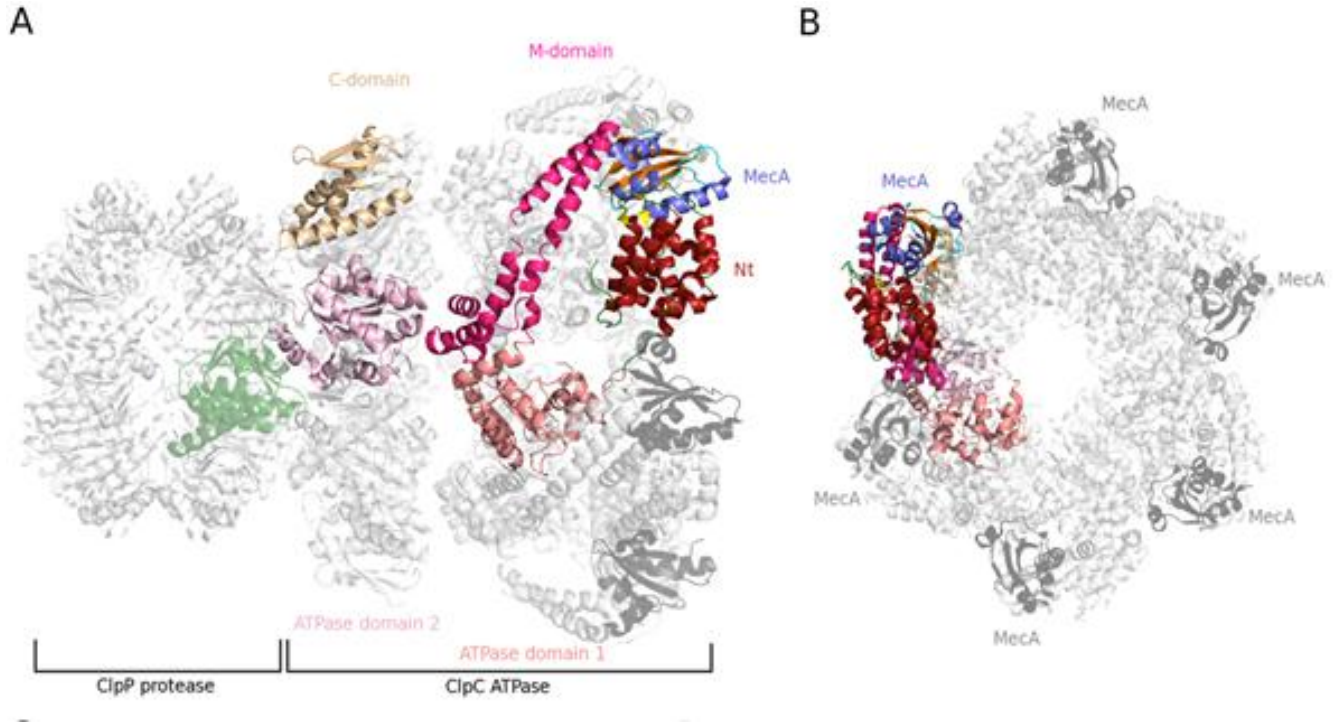

Figure 1.1 The ClpC-MecA hexameric structure (A) Model of the *Bs*-ClpC hexameric assembly based on the work of Wang *et al*. (2011). The ClpC "barrel" is shown in a complex with the ClpP tetradecameric protein ring (left of the image in green). A single chain of the ClpC hexamer is displayed with its five domains coloured and labelled. The associated MecA adapter molecule is coloured with α-helices in *teal*, β-strands in *orange* and loop regions in *cyan*. The symmetry related monomers are transparent. (**B)** The hexameric assembly and internal channel of the barrel through a 90° clockwise rotation about the y-axis relative to Figure 1.1A.

To date, many of the best-characterised alkaliphilic species belong to the *Bacillus* genus. Thus, they are a useful tool for comparing alkali-extremophile adaptions with well-studied neutrophilic species such as *B. subtilis, B. cereus* and *B. megaterium*. The entire genome sequence is now available for alkaliphilic (Takami *et al.*, 2000; Veith *et al.*, 2004) and neutrophilic (Eppinger *et al.*, 2011) species, hence allowing the execution of comparative genomic studies to examine the ways by which some branches of the *Bacillus* evolutionary tree acquired alkaliphilic adaptations (Takami *et al.*, 2000). To contribute to this analysis, the complete genome of *B. lehensis* G1 has been sequenced by the Malaysia Genome Institute (MGI) (Noor *et al.*, 2014). This research was aimed to build on the genomic data of *B. lehensis* G1. As such, the adaptive alterations in the Hsps coded by the same were assessed to identify those which were speciation-related and vital for survival in high-pH environments. It is hypothesised that the adaptation of *B. lehensis* to alkaline conditions appears to have been possible with only minor changes within the protein environment in its stress-response machinery.This include by changing a lot of difference parameter within the protein environment as such changing the ionic strength of the buffer. This study may provide more insight into the structural and functional adaptations of alkaliphiles.

1.2 Problem statement

Heat shock proteins are known to be highly common in extremophilic organisms. However, a knowledge gap exists regarding the roles of the structure and physicochemistry of Hsp100 ClpC in the stress response mechanism of alkaliphilic *Bacilli*. In fact, the sole crystal structure of heat shock proteins that has been solved thus far is that of the neutrophilic *Bs*-ClpC (Wang *et al.*, 2011). Nevertheless, the low resolution (6.9Å) employed to assess the hexameric complex comprising ClpC (full length) and MecA has failed to provide an in-depth comprehension of ClpC's mechanism of action. (1) As such, the structures of both *Bl*-ClpC (stress protein) and MecA (adaptor protein) of *B. lehensis* G1 needed to be explicated in an attempt to understand the stress-related functions as well as regulation of extremophilic stress proteins. (2) This in turn would facilitate discussions about the origins and functions of the Hsp in terms of evolution. There is currently no published study on the structure of alkaliphilic ClpC and the effects of stressful conditions on its activity. Protein crystallisation has become a leading technique for a detailed understanding of the structure of the protein at the atomic level. Therefore, X-ray crystallography will give a clearer insight into the relationship between the structure and functions of the said protein.

1.3 Objectives and novelty

The most basic biological activity of live cells is protein-protein interactions, which can be used to predict the functions of the proteins. As such, researches into the *Bl*-ClpC-MecA interaction were likely to yield reasonable postulates regarding the said functions. Based on this concept, X-ray crystallography technique has been

used to allow an in-depth comprehension of the atomic structures of proteins. This in turn has provided greater clarity of the proteins' structure-function association.

The novelty and rationale of the of the study is that, a) biochemical studies on MecA-dependent ClpC have not yet characterised the protein with respect to the influence of the environmental stressors on its ATPase activity. Also, little has been deciphered regarding its 2D structure stability and physicochemical properties, making it an attractive candidate in the understanding of this class of protein. b) This study is the first to describe the structure of an intracellular stress-response protein of an alkaliphile. C) Finally, the conservation of the protein sequence of *B. lehensis* G1 MecA only occurred in alkaliphilic *Bacilli*. Hence, the aim of this research was to bring to light previously-unknown aspects of the MecA of this alkaliphilic *Bacillus* species. A structural biology technique was employed to provide molecular data for proving the connection between the MecA-ClpC of *B. lehensis* G1 as well as *B. subtilis.*

1.4 Scopes of study

This study aims to elucidate the structure and functions of *B. lehensis* ClpC (*Bl*-ClpC) using appropriate strategies. Hence, the following scopes were outlined to achieve the objective:

I. Cloning, expression, and purification of ClpC and MecA in *E. coli* expression system.
II. Determining the 3D structures of ClpC and MecA through protein crystallisation.
III. Studying the effects of environmental stressors including a) pH, b) salt concentration, c) temperature, d) metal exposure and e) MecA concentration on the regulation of the ATPase activity of *Bl*-ClpC.
IV. Investigating the effects of various pH on the 2D structure stability of proteins.
V. Studying the potential possibilities of the *Bl*-ClpC in light of the combined effects of dual extremities of salt and temperature with pH, on ATPase activity by response surface methodology analysis.
VI. Structure-guided mutation construction, cloning, expression, and purification of the mutants.
VII. Investigating the effects of beneficial mutations towards pH and other environmental stressors through activity assays and secondary structure stability, as well as thermostability.

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