

**ELUCIDATION OF STRUCTURE AND FUNCTION
OF CONSERVED HYPOTHETICAL PROTEINS
RELATED TO THERMAL STRESS RESPONSE
IN *Pedobacter cryoconitis* BG5 AND
Glaciozyma antarctica PI12**



MAKDI MASNODDIN

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UNIVERSITI MALAYSIA SABAH

**BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
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UNIVERSITI MALAYSIA SABAH

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IJAZAH: DOKTOR FALSAPAH

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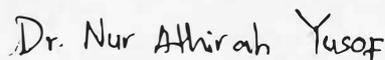
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MAKDI MASNODDIN



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MAKDI MASNODDIN
DZ1721007T

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Disahkan Oleh,



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NORAZLYNNE MOHD JOHAN
PUSTAKAWAN KANAN
UNIVERSITI MALAYSIA SABAH

(Tandatangan Pustakawan)



(Dr. Nur Athirah Yusof)
Penyelia Utama

DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, equations, summaries, and references, which have been duly acknowledged.

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DZ1721007T



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DEGREE : **DOCTOR OF PHILOSOPHY IN APPLIED SCIENCE**

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Signature

1. MAIN SUPERVISOR

Dr. Nur Athirah Yusof

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2. CO - SUPERVISOR

Prof. Clemente Michael Wong Vui Ling

A handwritten signature in black ink, consisting of stylized cursive letters, positioned above a horizontal line.

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Makdi Masnoddin

27 April 2022

ABSTRACT

The genome data of two native Antarctic microbes, *Pedobacter cryoconitis* (bacterium) and *Glaciozyma antarctica* (yeast), highlighted the presence of specific proteins with unique adaptive features. However, most of these proteins were designated as conserved hypothetical proteins (HPs), complicating efforts to understand their cellular functions. Consequently, we aim to identify the structural features of the conserved HPs that were ideal for their function in response to temperature stress. We posit that proteins that respond to thermal stress may have unique structural flexibility and stability that allows them to function under thermal stress, protecting host organisms against cold denaturation and heat aggregation. To address, an *in silico* analysis of HPs was conducted, followed by an *in vitro* approach whereby all selected HPs were cloned, expressed in *Escherichia coli*, purified, and subjected to crystal screening experiment. Purified recombinant proteins were assessed via colorimetric ATPase and citrate synthase aggregation assays and quantitative PCR (qPCR) gene expression analysis. Finally, the three-dimensional (3D) structures of the HPs were constructed and further refined for comparative structure analysis and function relationship clarification. Twelve *P. cryoconitis* and four *G. antarctica* conserved HPs with significant thermal stress response functions that met crystallisation criteria were identified through *in silico* analysis. All target genes were successfully amplified and cloned *in vitro*. Three *P. cryoconitis* (Pcbg5HP1, Pcbg5HP2, and Pcbg5HP12) recombinant proteins were overexpressed in the soluble forms at 16°C and subsequently purified using a two-step purification process. Three recombinant proteins from *G. antarctica* (GaHP2, GaHP3, and GaHP4) were overexpressed in soluble forms at 20°C, but only GaHP2 and GaHP3 were successfully purified. The ATPase assay showed protein activity at 4°C and 25°C for Pcbg5HP1, Pcbg5HP2, Pcbg5HP12, GaHP2, and GaHP3, which thus clarified that protein activity is maintained at low and moderate temperatures. Meanwhile, lower citrate synthase aggregation at 43°C in the presence of either Pcbg5HP1 or GaHP2 suggested the characteristics of chaperone-like activity. The qPCR analysis revealed that these genes were expressed constitutively when cells were exposed to temperatures below or above their optimal growth temperature, indicating their involvement in cellular processes associated with thermal stress. Initial crystal formation was observed when purified Pcbg5HP2 proteins were incubated at 4°C, and reagent optimization revealed the formation of a plate-shaped crystal in reagent 0.2 M potassium sodium tartrate tetrahydrate, 30% PEG/Ion, pH 7.4. This clarified the crystallisation potential of the HPs as predicted by bioinformatics analysis. Furthermore, comparative structural analysis demonstrated that the HPs exhibited cold-adapted traits, most notably increased flexibility in their 3D structures compared to their mesophilic or thermophilic counterparts. Concurrently, the presence of a disulphide bridge and aromatic clusters was attributed to Pcbg5HP1 and GaHP2's unusual protein stability and chaperone activity. Thus, this demonstrated that the HPs examined in this study adopted strategies to maintain a balance between molecular stability and structural flexibility, which contributed to their flexibility and ability to retain protein activities in an extreme environment. Conclusively, this study has established the structure-function relationships of the HPs produced by *P. cryoconitis* and *G. antarctica* and provided crucial experimental evidence indicating their importance in thermal stress response.

ABSTRAK

PENCIRIAN STRUKTUR DAN FUNGSI PROTEIN HIPOTETIK TERPULIHARA YANG BERKAITAN DENGAN TINDAK BALAS TERHADAP HABA DALAM *Pedobacter cryoconitis* BG5 DAN *Glaciozyma antarctica* PI12

Data genom dua mikroorganisma Antartika, Pedobacter cryoconitis (bakteria) dan Glaciozyma antarctica (yis), mengandung protein dengan ciri penyesuaian unik. Namun, sebahagian besar protein ini dikategorikan sebagai protein hipotetik (HP) terpulihara, yang merumitkan pemahaman tentang fungsinya. Oleh itu, kajian ini bertujuan untuk menentukan ciri struktur HP terpulihara yang berkaitan dengan fungsinya. Hipotesis kajian adalah protein ini mungkin mempunyai fleksibiliti dan kestabilan struktur tersendiri yang membolehkan ia terus berfungsi dan melindungi organisma perumah daripada suhu melampau. Analisis in silico telah dijalankan, kemudian analisis in vitro di mana semua HP yang dipilih telah diklon, diekspres dalam Escherichia coli, dituliskan, dan digunakan untuk eksperimen penghabluran. Protein rekombinan yang telah dituliskan, diuji melalui ujian kolorimetrik ATPase dan pengagregatan sitrat sintase, serta analisis kuantitatif ekspresi gen PCR (qPCR). Pada akhirnya, struktur tiga dimensi (3D) HP dibina dan dimurnikan untuk perbandingan struktur dan penentuan fungsi. Dua belas HP daripada P. cryoconitis dan empat HP daripada G. antarctica dengan fungsi berkaitan perubahan haba dan sesuai untuk penghabluran telah ditentukan melalui analisis in silico. Semua gen sasaran berjaya diamplifikasi dan diklon secara in vitro. Tiga protein rekombinan P. cryoconitis (Pcbg5HP1, Pcbg5HP2, dan Pcbg5HP12) telah diekspreskan secara larut pada 16°C dan dituliskan melalui purifikasi dua peringkat. Tiga protein rekombinan G. antarctica (GaHP2, GaHP3, dan GaHP4) berjaya diekspreskan dalam bentuk larut pada 20°C, tetapi hanya GaHP2 dan GaHP3 berjaya dituliskan. Ujian ATPase menunjukkan aktiviti protein Pcbg5HP1, Pcbg5HP2, Pcbg5HP12, GaHP2, dan GaHP3 pada 4°C dan 25°C. Ini menunjukkan protein tersebut kekal aktif pada suhu rendah dan sederhana. Agregasi sitrat sintase yang lebih rendah pada 43°C dengan kehadiran Pcbg5HP1 atau GaHP2 pula menunjukkan sifat seperti pengantar. Analisis qPCR menunjukkan gen-gen tersebut diekspres secara berterusan apabila sel terdedah kepada suhu di bawah atau di atas suhu pertumbuhan optimum, justeru mengesahkan ia terlibat dalam proses tindak balas terhadap perubahan haba. Pembentukan hablur dikesan pada protein tulen Pcbg5HP2 pada suhu inkubasi 4°C. Seterusnya pembentukan hablur berbentuk plat dihasilkan dengan optimasi bahan uji 0.2 M kalium natrium tartrat tetrahidrat, 30% PEG/Ion, pH 7.4. Ini mengesahkan potensi penghabluran HP yang diramalkan oleh analisis bioinformatik. Analisis struktur HP menunjukkan ciri-ciri penyesuaian pada suhu sejuk, terutamanya fleksibiliti yang tinggi pada struktur 3D berbanding protein mesofilik atau termofilik. Kewujudan ikatan disulfida dan kelompok aromatik pula boleh dikaitkan dengan kestabilan dan aktiviti chaperon bagi Pcbg5HP1 dan GaHP2. Ini menunjukkan bahawa HP-HP dalam kajian ini menggunakan strategi kestabilan molekul dan fleksibiliti struktur, yang menyumbang kepada fleksibiliti dan keupayaan mereka untuk mengekalkan aktiviti dalam persekitaran ekstrim. Kajian ini telah menyelesaikan hubungan antara struktur dan fungsi HP-HP yang dihasilkan oleh P. cryoconitis dan G. antarctica dan menghasilkan data eksperimen yang menunjukkan kepentingannya dalam tindak balas terhadap perubahan haba.

LIST OF CONTENTS

	Pages
TITLE	i
DECLARATION	ii
CERTIFICATION	iii
ACKNOWLEDGEMENT	iv
ABSTRACT	v
ABSTRAK	vi
LISA OF CONTENTS	vii
LIST OF TABLES	x
LIST OF FIGURES	xi
LIST OF SYMBOLS	xiv
LIST OF ABBREVIATIONS	xv
LIST OF APPENDICES	xvi
CHAPTER 1 : INTRODUCTION	
1.1 Research Background	1
1.2 Problem Statement	4
1.3 Research Objectives	5
1.4 Expected Outcome	6
CHAPTER 2 : LITERATURE REVIEW	
2.1 The Antarctic Psychrophiles, <i>Pedobacter cryoconitis</i> and <i>Glaciozyma antarctica</i>	7
2.1.1 <i>P. cryoconitis</i>	8
2.1.2 <i>G. antarctica</i>	10
2.2 Conserved Hypothetical Proteins (HPs)	13
2.3 Thermal Stress Protein in Psychrophiles	15
2.4 Gene Expression Analysis by Reverse Transcriptase Quantitative Polymerase Chain Reaction	19
2.5 Gene Cloning and Recombinant Protein Expression in <i>E. coli</i> system	21
2.6 Protein Crystallization	24
2.7 Structure and Function Annotations	27
2.8 Comparative Structure Modelling	30
2.9 Structural Features of Thermal Stress Protein	34

CHAPTER 3 : METHODOLOGY

3.1	Screening for Conserved HPs Related to Thermal Stress Response in <i>P. cryoconitis</i> and <i>G. antarctica</i>	38
3.2	Cloning of the Genes Coding for the Selected HPs	40
3.2.1	Cultivation of Microorganism and Isolation of Genomic DNA and Total RNA	40
3.2.2	Genes Amplification by Polymerase Chain Reaction (PCR)	41
3.2.3	Construction of Recombinant Plasmid by Ligation Independent Cloning (LIC)	43
3.2.4	Transformation of Recombinant Plasmid into <i>E. coli</i>	44
3.2.5	DNA Sequencing for Insert Verification	45
3.3	Bioinformatic Analysis of the Selected HPs	45
3.4	Protein Expression and Purification	46
3.4.1	Protein Expression	46
3.4.2	Protein Purification	48
3.5	Gene Expression Analysis and Protein Assay	49
3.5.1	Quantitative Reverse Transcription PCR (RT-qPCR)	49
3.5.2	Malachite Green Colorimetric Assay	51
3.5.3	Thermal Unfolding Assays using Citrate Synthase	52
3.6	Crystal Screening and Optimisation	53
3.7	Protein Structure Construction	54
3.8	Comparative Structure Analysis	55

CHAPTER 4 : RESULTS

4.1	Selected Conserved HPs Related to Thermal Stress Response in <i>P. cryoconitis</i> and <i>G. antarctica</i> .	57
4.2	Gene Cloning into <i>E. coli</i> Expression System	58
4.2.1	Cultivation of <i>P. cryoconitis</i> and <i>G. antarctica</i>	59
4.2.2	Genomic DNA Extraction and Total RNA Extraction	60
4.2.3	PCR Amplification of the Selected Genes related to Thermal Stress Response	62
4.2.4	Sequence Verification of <i>P. cryoconitis</i> and <i>G. antarctica</i> Genes	66
4.3	Functional Annotation and Physicochemical Analysis of the Selected HPs	70
4.3.1	Functional Annotation of the Selected Conserved HPs in <i>P. cryoconitis</i> and <i>G. antarctica</i>	70
4.3.2	Physicochemical Analysis of the Conserved HPs in <i>P. cryoconitis</i> and <i>G. antarctica</i>	74
4.4	Expression and Purification of Recombinant Protein	75

4.5	Gene Expression Analysis and Protein Assay	86
4.5.1	RT-qPCR Analysis	86
4.5.2	ATPase Activity of Purified Recombinant Proteins	89
4.5.3	Thermal Unfolding Assay of Purified Recombinant Proteins	91
4.6	Crystallization of Recombinant Proteins, Pcbg_HP2 and Pcbg5HP12	92
4.7	The 3D Structures of <i>P. cryoconitis</i> and <i>G. antarctica</i> HPs	95
4.8	Analysis of the 3D Structures of <i>P. cryoconitis</i> and <i>G. antarctica</i> HPs in Relation to Their Homologs	98

CHAPTER 5 : DISCUSSION

5.1	Functional Annotation and Physicochemical Analysis of the Selected Conserved HPs in <i>P. cryoconitis</i> and <i>G. antarctica</i> .	114
5.2	Expression, Purification, and Protein Assay	119
5.3	Gene Expression Analysis of <i>Pcbg5HP1</i> , <i>Pcbg5HP2</i> , <i>Pcbg5HP12</i> , <i>GaHP2</i> , <i>GaHP3</i> , and <i>GaHP4</i> under heat-shock and cold-shock conditions	124
5.4	3D Structures Analysis of <i>Pcbg5HP1</i> , <i>Pcbg5HP2</i> , <i>Pcbg5HP12</i> , <i>GaHP2</i> , <i>GaHP3</i> , and <i>GaHP4</i>	126
5.5	Comparative 3D Structural Elucidation and Analysis of <i>Pcbg5HP1</i> , <i>Pcbg5HP2</i> , <i>Pcbg5HP12</i> , <i>GaHP2</i> , and <i>GaHP4</i>	130
5.6	General Discussion	139

CHAPTER 6 : CONCLUSION **140**

REFERENCES **142**

APPENDICES **173**

LIST OF TABLES

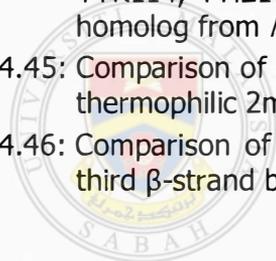
	Pages
Table 3.1: Primer design for PCR amplification of selected <i>P. cryoconitis</i> genes.	42
Table 3.2: Primer design for PCR amplification of selected <i>G. antarctica</i> genes.	43
Table 3.3: Mixtures of SDS-PAGE gel	47
Table 3.4: Primer sequences for qPCR analysis of <i>P. cryoconitis</i> and <i>G. antarctica</i> genes expression by quantitative real-time PCR.	50
Table 4.1: Sequence Analysis of <i>P. cryoconitis</i> and <i>G. antarctica</i> Raw Genome Data for Conserved HPs related to Thermal Stress Response.	58
Table 4.2: The BLAST search results for the selected HPs from <i>P. cryoconitis</i> against the NCBI non-redundant (nr) database	71
Table 4.3: The BLAST search results for the selected HPs from <i>G. antarctica</i> against the NCBI non-redundant (nr) database	72
Table 4.4: Functional annotation of <i>P. cryoconitis</i> hypothetical proteins by the InterProScan tool	73
Table 4.5: Functional annotation of <i>G. antarctica</i> HPs by the InterProScan tool	74
Table 4.6: The physicochemical properties of <i>P. cryoconitis</i> and <i>G. antarctica</i> HPs retrieved by the Protparam tool	75
Table 4.7: Summary of templates used by Phyre2 server for the 3D structure construction of <i>P. cryoconitis</i> and <i>G. antarctica</i> overexpressed protein	97
Table 4.8: Summary of Structural Quality Assessment Results of the Superimposed 3D Structure of <i>P. cryoconitis</i> and <i>G. antarctica</i> HPs with templates retrieved from DALI server	98
Table 4.9: Analysis of intra protein interactions in Pcbg5HP1 and 1pl8-C, 1e3j-A, and 3m6i-B homologs	106
Table 4.10: Intra protein interactions analysis in Pcbg5HP2 and 2jl1 chain A, 3e48 chain B, and 2zcv chain A homologs	107
Table 4.11: Analysis of intra protein interactions in Pcbg5HP12 and homologs 3cex chain A, 4x8d chain A, and 1rxq-B	109
Table 4.12: Comparative analysis of intra and inter-protein interactions between GaHP2 and its homologs, 3zjh chain B, 2w31 chain A, and 1or4 chain A	110
Table 4.13: Analysis of intra protein interactions between GaHP4 and its homologs, 2mo0 chain A, 5udz chain A, and 3aqq chain A	112

LIST OF FIGURES

	Pages
Figure 2.1: Protein crystallisation technique using vapor diffusion	26
Figure 3.1: Workflow used for the selection of HPs from <i>P. cryoconitis</i> and <i>G. antarctica</i> for structure and function determination	39
Figure 3.2: The aLICator pLATE51 (Thermo Scientific) expression vectors	44
Figure 3.3: An optimisation grid that manipulated precipitant concentration and pH of buffer	54
Figure 3.4: Flowchart depicting the analysis of the tertiary protein structures of <i>P. cryoconitis</i> and <i>G. antarctica</i> HPs using several bioinformatic tools	56
Figure 4.1: <i>Pedobacter cryoconitis</i> spirillum-shaped cells observed under 1000X oil immersion after 3 days of culture in LB broth at 20°C	59
Figure 4.2: <i>G. antarctica</i> cells grown in YPD Broth at 12°C after seven days of culture	60
Figure 4.3: Agarose gel electrophoresis analysis of the extracted <i>P. cryoconitis</i> genomic DNA for gene amplification	61
Figure 4.4: Agarose gel electrophoresis of RNA samples isolated from <i>G. antarctica</i> and <i>P. cryoconitis</i> cells using GENEzol™ Reagent	61
Figure 4.5: Agarose gel electrophoresis analysis of <i>P. cryoconitis</i> genes annealed at 45°C	62
Figure 4.6: Agarose gel electrophoresis analysis of <i>P. cryoconitis</i> genes amplified at 50°C	63
Figure 4.7: Agarose gel electrophoresis analysis of <i>P. cryoconitis</i> genes annealed at 55°C	64
Figure 4.8: Agarose gel electrophoresis analysis of <i>G. antarctica</i> genes amplification at 62°C annealing temperature	65
Figure 4.9: Agarose gel electrophoresis analysis of <i>G. antarctica</i> genes amplification at 60°C annealing temperature	65
Figure 4.10: Transformant colonies of <i>E. coli</i> BL21 (DE3) harbouring the recombinant plasmid pLATE51 grown on LB agar supplemented with 100 g/mL ampicillin after a 16-hour incubation period at 37 °C	66
Figure 4.11: Agarose gel electrophoresis analysis of PCR indicating positive colony harbouring <i>P. cryoconitis</i> genes amplified using specific primer pairs	67
Figure 4.12: Agarose gel electrophoresis analysis of PCR indicating positive colony harbouring <i>G. antarctica</i> genes amplified using specific primer pairs	67
Figure 4.13: Agarose gel electrophoresis analysis of recombinant plasmid containing insert	68

Figure 4.14: The alignment result of <i>Pcbg5HP1</i> gene with <i>P. cryoconitis</i> genome data	69
Figure 4.15: SDS-PAGE analysis of <i>Pcbg5HP1</i> expression in <i>E. coli</i> BL21 (DE3) at 16°C	76
Figure 4.16: SDS-PAGE analysis of <i>Pcbg5HP2</i> and <i>Pcbg5HP12</i> soluble expression in <i>E. coli</i> BL21 (DE3)	77
Figure 4.17: SDS-PAGE analysis of <i>Pcbg5HP1</i> , <i>Pcbg5HP2</i> , and <i>Pcbg5HP12</i> expression in <i>E. coli</i> BL21 (DE3) at 25°C	78
Figure 4.18: SDS-PAGE analysis of <i>Pcbg5HP1</i> , <i>Pcbg5HP2</i> , and <i>Pcbg5HP12</i> expression in <i>E. coli</i> BL21 (DE3) at 37°C	79
Figure 4.19: SDS-PAGE analysis of <i>GaHP2</i> and <i>GaHP3</i> soluble expression in <i>E. coli</i> BL21 (DE3) at 20°C	80
Figure 4.20: SDS-PAGE analysis of <i>GaHP4</i> soluble expression in <i>E. coli</i> BL21 (DE3)	81
Figure 4.21: Protein elution profile and SDS-PAGE analysis of purified <i>Pcbg5HP1</i>	82
Figure 4.22: Protein elution profile and SDS-PAGE analysis of purified <i>Pcbg5HP2</i>	83
Figure 4.23: Protein elution profile and SDS-PAGE analysis of purified <i>Pcbg5HP12</i>	84
Figure 4.24: Protein elution profile and SDS-PAGE analysis of purified <i>GaHP2</i>	85
Figure 4.25: Protein elution profile and SDS-PAGE analysis of purified <i>GaHP3</i>	86
Figure 4.26: Gene expression analysis of <i>Pcbg5HP1</i> , <i>Pcbg5HP2</i> , and <i>Pcbg5HP12</i>	87
Figure 4.27: Gene expression analysis of <i>GaHP2</i> , <i>GaHP3</i> , and <i>GaHP4</i> . Data are representative of three biological replicates and were expressed as the mean±SD	89
Figure 4.28: Phosphate concentration of <i>P. cryoconitis</i> (<i>Pcbg5HP1</i> , <i>Pcbg5HP2</i> , and <i>Pcbg5HP12</i>) and <i>G. antarctica</i> (<i>GaHP2</i> and <i>GaHP3</i>) recombinant proteins measured at 620 nm after ATPase/GTPase Colorimetric Assay at 4°C and room temperature (25°C)	90
Figure 4.29: Heat-induced denaturation of citrate synthase	91
Figure 4.30: Crystallization of recombinant <i>Pcbg5HP2</i> and <i>Pcbg5HP12</i> proteins using the PEG/Ion I and II Screens kit	93
Figure 4.31: Crystal growth of <i>Pcbg5HP2</i> recombinant proteins optimised by adjusting the precipitant concentration and buffer pH	95
Figure 4.32: The 3D structure of successfully expressed recombinant proteins from <i>P. cryoconitis</i> and <i>G. antarctica</i> constructed using the Phyre2 server and refined using the ModRefiner algorithm	96
Figure 4.33: <i>Pcbg5HP1</i> structural comparison with templates obtained from the DALI server	100
Figure 4.34: <i>Pcbg5HP2</i> structural comparison with templates obtained from the DALI server	101

Figure 4.35: Pcbg5HP12 structural comparison with templates obtained from the DALI server	102
Figure 4.36: GaHP2 structural comparison with templates obtained from the DALI server	103
Figure 4.37: GaHP4 structural comparison with templates obtained from the DALI server	104
Figure 4.38: The amino acids in GaHP2's non-superimposed loop with high energy content are highlighted in red	105
Figure 4.39: Proportion of amino acids with a high energy content detected in the Pcbg5HP1 non-superimposed loop	105
Figure 4.40: Comparison of the disulphide bridge in Pcbg5HP1 and its homolog 3m6i	107
Figure 4.41: Comparison of the aromatic-aromatic interaction in Pcbg5HP2 and 2jl1 homolog	108
Figure 4.42: Comparison of the aromatic-aromatic interaction in Pcbg5HP12 and 4x8d homolog	109
Figure 4.43: The aromatic-aromatic interaction takes place at heme binding sites in GaHP2 and its protoglobin (3zjh) homolog	111
Figure 4.44: Strong aromatic-aromatic interaction between PHE20 to TYR114, PHE195, and TYR187 in the protoglobin (3zjh) homolog from <i>M. acetivorans</i>	112
Figure 4.45: Comparison of aromatic-aromatic interaction in GaHP4 and thermophilic 2mo0 homolog	113
Figure 4.46: Comparison of loop structure connecting the second and third β -strand between GaHP4 and 2mo0 homolog	113



LIST OF SYMBOLS

°C	-	Degree Celsius
kDa	-	Kilo Dalton
mL	-	Mili liter
μL	-	Micro liter
μM	-	Micro gram
mM	-	Mili molar
mg	-	Mili gram
nm	-	Nano meter
mA	-	Mili ampere
pH	-	Potential of hydrogen
bp	-	Base pairs
kb	-	Kilo base pairs



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LIST OF ABBREVIATIONS

HP	-	Hypothetical proteins
3D	-	Three-dimensional
2D	-	Two-dimensional
1D	-	One-dimensional
FPLC	-	Fast protein liquid chromatography
SEC	-	Size exclusion chromatography
NCBI	-	National Center for Biotechnology Information
BLAST	-	Basic Local Alignment Search Tool
IPTG	-	Isopropyl β -D-1-thiogalactopyranoside
DUFs	-	Domain of unknown function
HMM	-	Hidden Markov Model
pI	-	Isoelectric point
ROS	-	Reactive oxygen species
SDS-PAGE	-	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
TEMED	-	Tetramethylethylenediamine
RAST	-	Rapid Annotation using Subsystem Technology
LIC	-	Ligation Independent Cloning (LIC)
LB	-	Luria-Bertani
UV/VIS	-	UltraViolet-Visible



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LIST OF APPENDICES

	Pages
Appendix A: RAST Analysis for the Conserved HPs related to Thermal Stress Response in <i>P. cryoconitis</i>	173
Appendix B: Transcriptomic Analysis for the Conserved HPs related to Thermal Stress Response in <i>G. antarctica</i>	174
Appendix C: The Alignment Result of Recombinant Plasmid Inserts against <i>P. cryoconitis</i> Genome Data	175
Appendix D: The Alignment Result of Recombinant Plasmid Inserts against <i>G. antarctica</i> Genome Data	187
Appendix E: Genes Identification Information	192
Appendix F: The Amino Acid Sequences Arranged in Genbank/GB Format of the Selected HPs from <i>P. cryoconitis</i> and <i>G. antarctica</i>	193



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CHAPTER 1

INTRODUCTION

1.1 Research Background

Antarctica is a fascinating region with a diverse range of climatic extremes and is generally considered the centre of the Earth's climate and marine circulation systems (Convey & Peck, 2019). Two previously discovered and characterised native Antarctic microbes, *Pedobacter cryoconitis* and *Glaciozyma antarctica*, have generated research interest due to their exceptional capacity to thrive in the extreme Antarctic ecosystem (Margesin et al., 2003; Turchetti et al., 2011). According to past studies, the facultatively psychrophilic *P. cryoconitis* culture can grow at temperatures ranging from 1 to 25 °C (Wong et al., 2011). Meanwhile, the psychrophilic yeast *G. antarctica* has been observed to be temperature tolerant between -12°C and 20°C (Boo et al., 2013; Soon et al., 2018).

As a means of surviving and adapting to the Antarctic climate, these extremophiles produce a wide range of biologically important proteins, particularly those involved in the thermal stress response (Song et al., 2017; Yusof et al., 2016; Wong et al., 2011). Many interesting discoveries about *P. cryoconitis* have been made regarding genes encoding resistance to cold stress and heavy metals, as well as industrially valuable enzymes (Lee et al., 2016). Meanwhile, genomic analysis of *G. antarctica* revealed numerous protein-coding genes associated with cold tolerance, such as antifreeze proteins and fatty acid desaturases (Firdaus-Raih et al., 2018). However, despite the availability of whole genome sequences for both microorganisms, no detailed description of their stress response mechanisms has been documented (Wong et al., 2013, 2019). This problem is complicated by the fact that 35% of the protein-coding genes in *P. cryoconitis* (Lee et al., 2016) and

37% of the protein-coding genes in *G. antarctica* (Firdaus-Raih et al., 2018) were classified as hypothetical proteins (HPs). Furthermore, 82 of the 319 transcripts that are currently unique to *G. antarctica* have been identified as having unknown functions (Bharudin et al., 2018; Wong et al., 2019). These functionally unknown proteins may be involved in significant aspects of this microorganism's biological function. Previous research has demonstrated that a set of proteins with unknown functions is vital in the physiological regulation and cold adaptation of psychrophilic microorganisms (Koh et al., 2017; Teoh et al., 2021). Similarly, recent research suggested that the HPs in *P. cryoconitis* and *G. antarctica* were important in the early stages of cold and freeze stress, though these findings have yet to be validated (Soon et al., 2018; Wong et al., 2019). This presents an opportunity for new discoveries to be made in order to gain a better understanding of their distinctive properties for adaptation mechanisms.

Proteins are versatile macromolecules that are required for the cellular adaptation system to function properly. Although psychrophiles share basic cold-adaptation strategies, different species have been shown to adopt different approaches for tolerating and surviving thermal stressors (Boo et al., 2013; Collins & Margesin, 2019; Firdaus-Raih et al., 2018). As every protein is unique, characterization at the physiological and biochemical level is critical for unravelling their molecular mechanisms and realising their full biotechnological potential. Several discoveries have been made primarily as a result of structural biology research, including drug design, vaccine development, and the discovery of industrially important enzymes (Eisenstein et al., 2000; Maveyraud & Mourey, 2020; Muhammed & Aki-Yalcin, 2019). More specifically, structural and functional analysis of HPs from a variety of pathogenic species has resulted in a greater understanding of disease mechanisms, diagnostics, symptom treatment, and vaccine development (Islam et al., 2015; Sen & Verma, 2020). By elucidating the structure and function of the HPs, researchers will gain knowledge about new protein pathways and cascades, allowing us to better understand the protein mosaic and determine protein-protein interactions.

The three-dimensional (3D) protein structures that mediate biochemical interactions must be evaluated to understand biological processes at the system

level (Hauri et al., 2019). For structural determination experiments, a high level of soluble protein amenable to downstream processing is required. Heterologous expression Implementing a suitable expression host, such as *Escherichia coli*, is one of the most effective strategies to optimise the production of high-quality pure proteins (Ahmad et al., 2018). Subsequent protein purification is then accomplished by a fast protein liquid chromatography (FPLC) system through the two-step purification of recombinant proteins via nickel affinity chromatography followed by size exclusion chromatography (SEC) (Kim et al., 2015). Due to the capability of X-ray crystallography to provide extremely detailed structural information, it has become indispensable in protein structure determination (Maveyraud & Mourey, 2020). However, crystallisation is a difficult process with a low success rate, and not all proteins crystallise (Holcomb et al., 2017). In the absence of an experimentally determined structure, comparative or homology modelling can generate a useful 3D model of a protein that is linked to at least one known protein structure. To achieve this ambitious target, expensive and time-consuming structural determination experiments are complemented with theoretical approaches. Bioinformatics aids in the prediction of structures from genomic data and comparative structural modelling. Recent advances in omics technologies have provided an excellent insight into the molecular basis behind cold adaptation processes, enabling the theoretical assessment of several physicochemical parameters to identify the function and composition of previously uncharacterized proteins (Li et al., 2011; Naqvi et al., 2015). Thus, by combining structural knowledge of proteins with functional annotation tools, previously uncharacterized proteins can be elucidated (Jez, 2017).

The present study was designed to evaluate the conserved HPs associated with thermal stress responses in *P. cryoconitis* and *G. antarctica* in order to establish a better understanding of their adaptation mechanisms. While gene sequences provide important information, they are devoid of information on uncharacterized proteins, making it challenging to determine functionally significant sequences. Moreover, there is a limited number of studies addressing the properties of thermal stress proteins produced by psychrophilic microorganisms (Song et al., 2017; Wong et al., 2019). To date, there are no reports that focus on the functional and structural analysis of conserved HPs in *P. cryoconitis* and *G.*

roadblocks in the study of HPs, such as the limitation of publicly available public databases and the costly and labour-intensive experimental procedures for structure determination. The findings of this work will provide a new perspective and scientific approach to understand how proteins behave in terms of flexibility, stability, and dynamic conformations under thermal stress.

1.3 Research Questions

As mentioned, the following research questions arise:

1. Do the hypothetical proteins found in the genomes of *P. cryoconitis* and *G. antarctica* play an important role in thermal adaptation?
2. How are the hypothetical proteins different from other known proteins?
3. Do the hypothetical proteins acquire certain characteristics in the structure that allow them to function at extreme temperatures?

1.4 Research Objectives

The purpose of this research is to elucidate the function and structure of HPs involved in the thermal stress response in *P. cryoconitis* and *G. antarctica*, as well as to understand the relationship between protein molecular architecture and function under cold-adapted conditions. To accomplish these goals, molecular biology techniques will be integrated with omics technology. The scope of the study comprises the *in silico* analysis of existing genomic data and the collection of *in vitro* experimental data for protein characterization. In an attempt to achieve the research target, the main objectives are:

- 1 To screen for conserved hypothetical proteins related to thermal stress.
- 2 To conduct *in vitro* analysis of the selected hypothetical proteins and resolve their functions.
- 3 To determine the relationship between the protein's molecular structure and its function.

1.5 Expected Outcome

The conserved HPs that respond to thermal stress may have specific structural attributes that contribute to their flexibility and stability. This enhanced their capacity to maintain their activities under thermal stress, thus protecting host organisms against cold denaturation and heat aggregation. The results of this research will add to our understanding of the cold-adapted protein's structural and functional properties, thus revealing its real potential for biotechnological applications.



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