

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

**Characterization of the secretins, large outer membrane
channels of Gram-negative bacteria**

A thesis presented in partial fulfillment of the requirements for the
degree of

Doctor of Philosophy

In

Biochemistry

at Massey University, Palmerston North

New Zealand

Sofia Khanum

2015

Dedicated to my mother

Acknowledgements

“In the name of Allah, the Most Gracious, the Most Merciful”

First of all I would like to acknowledge my supervisor Dr. Jasna Rakonjac for her continuous support and critical evaluation of my work throughout my PhD. Indeed it was due to her encouragement and innovative thinking that polished my skills and enabled me to do well. I would also like to thank my co-supervisor Dr. Gill Norris for her valuable feedback. A special thanks to my lab fellows Wesley Wen, and Julian Spagnuolo at Helipad. I would also like thank the staff at Institute of Fundamental Sciences; their cooperation and kindness will be treasured always.

I would like to extend my gratitude to my sweet friends Sadia Sattar, Shazrah Salam, Sadia Tahir and Amber Faisal for their continuous support, understanding, cooperation and care; they indeed made things easier for me at the time of stress by just being around apart from giving me valuable suggestions during my research and writing this thesis.

A special thanks to my family.

My parents remained a source of contentment for me always, particularly during this course of study. My parents’ continuous encouragement, support and suggestions for my study throughout these five years is remarkable. A special thanks to my parents for letting me pursue my dream of higher education.

This thesis would have never been possible without my loving husband Khurram Iqbal. You were always around at times I thought that it is impossible to continue, you helped me to keep things in perspective. I am grateful to you for your remarkable patience and unwavering love and support over the course of my research, and also for the countless days of daddy daycare to our daughters. My daughters Mahin and Mahrosh, I owe you lots and lots of fun hours. I couldn’t imagine doing my PhD without you; you really gave me the reason to continue.

Not to forget the support of my sisters Jamila, Anila along with the continuous encouragement of my brother Waseem. Lastly I would like to say thanks to my nephews Hannan, Ahmed, Ali and my niece Shiza for their lovely smiles in stressful days.

Abstract

Secretins, a family of large outer membrane channels, mediate secretion and/or assembly of virulence factors and/or complex proteinaceous structures, such as rods, type IV pili and filamentous bacteriophage. Secretins form large radially-symmetric channels composed of 12 to 14 identical subunits, with internal diameters of up to 10 nm, whose lumen is interrupted by a septum/plug structure that very likely represents a gate or a valve. The identity of septum in the primary sequence of secretins has not been determined as yet, however the cryo-EM and SPA analyses point to the C-terminal domain forming these structures, whereas mutagenesis specifically identified two regions in this domain, named GATE1 and GATE2, as having an important role in gating of the filamentous phage secretion system secretin pIV. However, it is not known whether these regions are also involved in gating of the secretins from type II and type III secretion systems.

In this work, twelve “leaky-gate” mutants in the secretin PulD from the type II secretion system (T2SS), selected from a random mutant library based on ability to utilise 829 Da oligosaccharide maltopentaose in the absence of maltoporin, were analysed in detail. Most of PulD leaky-gate mutants clustered in the GATE1 and GATE2 regions. All point mutants were positive for secretion of the cognate PulD substrate, enzyme Pullulanase (PulA), whereas a 5-residue in-frame deletion ($\Delta 477-481$) was negative. Two severely leaky GATE1 region mutants, G458S and $\Delta 477-481$, sensitised *E. coli* to all tested antibiotics whose molecular weight is too high to pass through porins: rifamycin SV (720 Da), bacitracin (1423 Da) vancomycin (1449 Da) and daptomycin (1621 Da). The GATE1 of this secretin is therefore a potential drug target, for design of molecules that can sensitise secretin-containing pathogenic Gram-negative bacteria to > 600 Da antibiotics and/or block the secretion of substrates, including virulence factors.

Engineered chimeras between PulD and pIV were used to probe functional compartmentalisation among the secretin domains and the segments involved in gating. This analysis showed that the N-terminal domains, GATE1 region and channel-forming secretin homology domain are interdependent with respect to function in secretion/assembly of the substrates, and to different extents for folding and multimerisation.

This work further analysed the gating properties of the type III secretion system (T3SS) secretins EscC and InvG. When expressed in *E. coli* K12, these secretins were naturally “leaky” and mistargeted to the inner membrane, resulting in growth retardation. The survival of *E. coli* expressing these secretins depended on the PspF, positive regulator of the key inner membrane stress response Psp. Therefore, in the T3SS secretin-expressing or toxin-secreting cells, PspF is a potential target for design of molecules that could kill T3SS-containing toxin-secreting Gram-negative bacteria.

Table of Contents

Chapter 1: Literature Review	1
1.1 Introduction.....	1
1.2 Secretion Systems	1
1.2.1 Type II secretion system (T2SS).....	2
1.2.2 Type III secretion system (T3SS)	5
1.2.3 Type IV pilus biogenesis system (T4PBS)	10
1.2.4 Filamentous phage secretion system (FPSS)	11
1.3 The role of bacterial secretion systems in pathogenesis	15
1.4 Secretins	17
1.4.1 Secretins: the N-terminal domain(s)	20
1.4.2 Secretin homology (or C-terminal) domain	21
1.4.3 Secretin targeting to the outer membrane	22
1.4.4 Structural similarities and differences within the secretin family.....	25
1.4.5 Gating of the secretins.....	30
1.5 Envelope stress responses induced by secretins	32
1.5.1 Phage shock protein (Psp) response.....	33
1.5.2 The Rcs phosphorelay	36
1.6 Aims of the project.....	39
Chapter 2: Materials and Methods	42
2.1 Media	42
2.2 Bacterial Strains	42
2.3 Plasmids and phage.....	42
2.4 Construction of plasmids.....	43
2.4.1 Construction of phagemid pYMK01 and pIV-PulD chimeric secretins ...	43
2.4.2 Construction of plasmids expressing type III secretins (InvG and EscC)	44

2.5 Mutagenesis	59
2.5.1 <i>In vivo</i> random mutagenesis of gene <i>escC</i>	59
2.5.2 <i>In vitro</i> random mutagenesis of gene <i>escC</i>	59
2.6 Antibiotic sensitivity assays	60
2.7 Protein extraction, electrophoresis and detection.....	61
2.7.1 Protein extraction, electrophoresis and detection	63
2.8 Pulullanase secretion assays.....	63
2.9 Phage assembly and secretion assay	64
2.10 Protein purification for antibody production.....	65
2.11 Synthetic lethality assays	66
2.12 Statistical analysis	66
Chapter 3: Characterization of PulD leaky mutants.....	67
3.1 PulD leaky mutations correspond to those in pIV	67
3.2 Characterization of leaky mutants.....	72
3.3 Sensitization of GATE1 leaky mutants G458S and Δ 477-481 to antibacterials..	79
3.4 Outer membrane targeting and multimerization of leaky GATE1 mutants G458S and Δ 477-481	82
3.5 Conclusions	86
Chapter 4: Probing the functional compartmentalisation and domain-domain interactions of secretins pIV and PulD	87
4.1 Approach to identify the exchangeable domains	87
4.2 Phagemid-based complementation assay for assessing the functionality of pIV and derived chimeras.....	94
4.3 Stability, targeting and multimerisation of pIV-PulD N-C chimeras.	103
4.4 Stability, targeting and multimerisation the PulD-pIV GATE1 swap chimeras	109
4.5 Conclusions	115

Chapter 5: Gating of EscC and InvG, secretins of type III secretion systems.....	117
5.1 Characterisation of the EscC secretin	117
5.1.1 Phenotypic effect of EscC expression in <i>E. coli</i> K12	117
5.1.2 Effect of the EscC expression on sensitivity to large antibiotics	118
5.1.3 Subcellular targeting of EscC in <i>E. coli</i> K12	120
5.1.4 Effect of EscVN coexpression with EscC on <i>E. coli</i> K12	123
5.1.5 Construction of leaky gate mutant libraries of <i>escC</i>	124
5.2 InvG, type III secretion system secretin of Salmonella Typhimurium	130
5.2.1 Phenotypic effect of InvG or InvGH expression	130
5.2.2 InvG targeting in <i>Escherichia coli</i>	136
5.3 Stress responses required for survival of <i>E. coli</i> K12 expressing EscC and InvG	138
5.4 Conclusions	142
Discussion.....	143
6.1 PulD C-terminal domain has pIV-homologous GATE1 and GATE2 regions...	143
6.2 GATE1 pIV-PulD swap chimeras provide insight into the secretin homology domain function and structure.....	147
6.3 N-C pIV-PulD chimeras.....	151
6.3 Secretins of T3SS are naturally leaky	157
6.4 Conclusions	158
6.5 Future directions.....	160
References... ..	161

List of Tables

Table 2.1 List of strains used in this study.....	46
Table 2.2 List of plasmids.....	49
Table 2.3 List of phages.....	54
Table 2.4 List of oligonucleotides.....	55
Table 3.1 Summary of PulD mutants and their phenotypes.....	74
Table 4.1 Permeability of pIV-PulD chimeras to Van and maltopentaose.....	108
Table 5.1 Transformation and plating efficiency of EscC-RCA product.....	125
Table 5.2 Sequence analysis of EscC-RCA mutants.....	126

List of Figures

Figure 1.1 Hypothetical model of T2SS of Gram-negative bacteria and its mechanism of action.	4
Figure 1.2 Organization of basal body and needle sub-structure of T3SS.....	8
Figure 1.3 Schematic presentation of the type IV pilus biogenesis system (T4PBS).13	
Figure 1.4 Schematic presentation of filamentous phage secretion system (FPSS)..	14
Figure 1.5 Modular organization of the secretin domains and their structures.....	18
Figure 1.6 Comparison of the secretin three dimensional structures obtained by cryo-EM and single particle analyses.	28
Figure 1.7 Schematic presentation of the Psp response.	35
Figure 1.8 Schematic presentation of the Rcs signal transduction pathway.	37
Figure 3.1 Alignment of PulD and pIV leaky mutations.	70
Figure 3.2 Detection of PulA and PulD by western blotting.	75
Figure 3.3 Secreted pullulanase (PulA) enzymatic activity.	77
Figure 3.4 GATE1 mutants sensitise <i>E. coli</i> to high molecular weight antibiotics and detergent deoxycholate (DOC).	80
Figure 3.5 Outer membrane targeting and multimerization of PulD mutants.....	84
Figure 3.6 Characterization of the multimers formed by the PulD GATE1 leaky mutants $\Delta 477-481$ and G458S.	85
Figure 4.1 Alignment of pIV and PulD.....	90
Figure 4.2 Schematic presentation of PulD and pIV domain organization and pIV-PulD chimeras.	91
Figure 4.3 Alignment of GATE1 region of pIV, pIV(PulDGATE1), PulD(pIVGATE1) and PulD.	93
Figure 4.4 Schematic presentation of “phagemid-phage” complementation system for testing the function of pIV-PulD chimeras.	96
Figure 4.5 R484 (helper phage) and phagemid titers.	101
Figure 4.6 PulA secretion by pIV-PulD chimeras.	102
Figure 4.7 Detection of pIV-PulD chimeric secretins monomers and multimers....	106
Figure 4.8 Targeting of pIV-PulD N-C chimeric secretins.	107
Figure 4.9 Outer membrane targeting and multimerization of PulD mutants.....	111
Figure 4.10 PulD _(pIVGATE1) chimeric secretins failed to sensitise <i>E. coli</i> to high molecular weight antibiotics and detergent deoxycholate (DOC).	113

Figure 5.1 Plating efficiencies of the cells expressing EscC in the presence of large antibiotics and DOC.....	119
Figure 5.2 Subcellular localization of EscC.....	122
Figure 5.3 Alignment of EscC with pIV, showing the GATE regions as mapped in pIV and the newly obtained EscC mutations.	127
Figure 5.4 Plating efficiencies of EscC mutants in the presence of large antibiotics and DOC.	129
Figure 5.5 Plating efficiency of cells expressing InvG.....	132
Figure 5.6 Relative plating efficiency of cells expressing InvG in the presence of large antibiotics and DOC.....	133
Figure 5.7 Antibiotic and deoxycholate sensitivity assay of <i>S. typhimurium</i> LT2..	135
Figure 5.8 Subcellular localization of InvG.....	137
Figure 5.9 Estimation of cell viability with overnight cultures of null mutants of <i>pspA</i> , <i>pspF</i> , <i>rcaA</i> and <i>rcaB</i> expressing InvG/InvH, EscC and empty vector as control using drop method technique.	140
Figure 5.10 Plating efficiencies of InvG and EscC expressing stress response in null mutants.....	141
Figure 6.1 Proposed outer membrane topology model of PulD with transmembrane β -strands (red arrow) surrounding the two GATE regions.	150
Figure 6.2 Model of PulD secretin homology (C-terminal) domain predicted by I-TASSER (Yang et al., 2015).....	154

List of Abbreviations

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ARFs	ADP ribosylation factors
ATPase	Adenosine triphosphatase
Bac	Bacitracin
Bae	Bacterial adaptive response
Bp	Base pairs
cAMP	Cyclic AMP
CDS	Coding sequence
CFU	Colony forming unit
Cm	Chloramphenicol
Cpx	Conjugative pilus expression response
Cryo-EM	Cryo Electron Microscopy
CtxAB	Cholera toxin AB
Da	Dalton
DNA	Deoxyribonucleic acid
DNS	3,5-dinitrosalicylic acid
DOC	Deoxycholate
EHEC	Enterohaemorrhagic <i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
EOP	Efficiency of plating
EPEC	Enteropathogenic <i>Escherichia coli</i>
ESRs	Envelope stress responses
ETEC	Enterotoxigenic <i>Escherichia coli</i>
FPSS	Filamentous phage secretion system
GST	Glutathione S-transferase
IPTG	Isopropyl β -D-1-thiogalactopyranoside
Kn	Kanamycin
LEE	Locus of enterocyte effacement
MIC	Minimal inhibitory concentration
MW	Molecular weight

OD	Optical density
PCR	Polymerase chain reaction
Pfu	Plaque forming unit
Pi	Inorganic phosphate
PKA	Protein kinase A
Psp	Phage shock protein response
R	Resistant
Rcs	Regulation of capsular synthesis
RF	Replicative form
RpoE	RNA polymerase, extracytoplasmic stress sigma factor
S	Sensitive
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide
SecYEG	General secretory translocon
SPA	Single particle analysis
SPI	Salmonella pathogenicity island
spp.	species
sv.	serovar
T2SS	Type II secretion system
T3SS	Type III secretion system
T4PBS	Type IV pilus biogenesis system
Tat	Twin-arginine translocon
TCA	Trichloroacetic acid
TEM	Transmission electron microscope
Tet	Tetracycline
Van	Vancomycin
WT	Wild type
Ysa-PI	Yersinia pathogenicity islands