Molecular characterisation of

*Shigella flexneri* outer membrane protease IcsP

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Discipline of Microbiology and Immunology

The School of Molecular and Biomedical Science

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Thesis Amendments

Abstract
Page 1, para 2, line 3: should read "...found that icsP in both..."
Page VI, abbreviations list: should include HEPES abbreviation "4-(2-hydroxyethyl)1-piperazineethanesulfonic acid"

Chapter 1 – Literature Review
Page 8, line 11: should read "...Gram-negative..."
Page 9, section 1.5.2, line 3: should read "...zoonosis of plague caused..."
Page 10, para 2, line 7: should read "...cleave colicins A..."
Page 11, section 1.5.5, line 3: should read "...detected in culture supernatants..."
Page 17, section 1.7, line 1: should read "...discovered to affect intracellular..."
Page 18, section 1.7.3, para 1, line 6: should read "...to encode proteins required for maximal...",
Page 18, section 1.7.3, para 2, line 3: should read "...which has 36% identity..."
Page 18, section 1.7.3, para 2, line 5: should read "...encodes the enzyme..."

Chapter 2 – Materials and Methods
Page 40, section 2.11.4, line 1: should read "...(-4 μl) were labelled..."
Page 45, section 2.14.1.1, line 1: should read "...bacteria were centrifuged..."

Chapter 3 – Characterisation of IcsP
Fig 3.7 legend, line 3: should read "...second agarose layer..."
Fig 3.8 legend, line 6: should read "...second agarose layer..."
Page 47, section 3.1, line 14: should read "...form plaques and F-actin comet tails..."
Page 48, para 2, line 6: should read "...ETRM22 (Section 5.6) and ETRM108 (data not shown) using anti-icsP..."
Page 52, section 3.4.2, line 3: should read "...with FITC-phalloidin..."

Chapter 4 – Surface distribution of IcsP
Page 54, section 4.1, para 2, line 9: should read "...increased detection..."
Page 61, section 4.3.4.1, line 3: should read "...unexpectedly solubilised by the Triton/MgCl2..."
Page 61, section 4.3.4.1, line 6: should read "...in the soluble and the insoluble fractions (Table 4.1, and data not shown)."
Page 70, section 4.6, line 3: should read "Experiments whereby the LPS of S. flexneri 2a 2457T was labelled with..."
Page 70, section 4.6, line 5: should read "...(Fig. 3.9A)."

Chapter 5 – Effect of virK and rmlD mutations on IcsP and S. flexneri virulence
Page 76 vs Page 85: The effect of the mutation used in the Nakate et al. (1992) study is speculated upon here as one of two possible differences. The virK mutation used in this study is unlikely to have a polar effect as it is a deletion. Proving the absence of a polarity effect does not change the results observed. Further experiments are also beyond the scope of this thesis and mutations affecting other genes in the operon would also need to be made.
Page 78, section 5.3, line 10: should read "...little or no detectable effect on the structure of LPS...",
Page 81, section 5.5, para 1, line 3: should read "...attributed to an effect...",
Page 81, section 5.5, para 2, line 1: should read "...Figure 5.10..."
Page 82, section 5.6, line 6: should read "...Figure 5.10C..."
Page 83, section 5.7, line 4: should read "...Figure 5.12"
Page 83, section 5.7, line 13: should read "...have no effect on IcsP..."
Page 84, section 5.9 conclusions: The data shown in Figure 5.11 is correct and reproducible. No effect on IcsA cleavage (Fig. 5.12) was observed which is consistent with the results in Figure 5.11. A problem with the immunoblotting chemiluminescence substrate was encountered during the course of
this thesis, and this was resolved by switching to a different substrate from a new supplier. This problem only affected immunoblotting with anti-LcsP. The differences seen in Figure 5.10B are reproducible and are not affected by the immunoblotting chemiluminescence substrate problem. should read “...no effect on LcsP...” should read “...that virK has no detectable effect on the structure of LPS.”

Chapter 6 – Alternative substrates for LcsP
Page 89, line 2: should read “...showed resistance...” Page 89, line 5: should read “...experiment was performed twice...” Page 89, para 2, line 6: should read “...experiment was performed twice...” Page 90, section 6.3.2, line 1: should read “...sequence similarity to the...” Page 90, line 2: should read “...experiment was performed twice...” Page 94, section 7.1, line 9: should read “...shares most similarity to OmpF...”

Chapter 7 – Discussion
Page 107, line 1: should read “...by van der Ley et al. ...” Page 107, section 7.6, line 5: should read “...plaque assay, and...”
Abstract

*Shigella* is a genus of Gram-negative bacteria responsible for bacillary dysentery in humans. *Shigella flexneri* type 2a in particular is responsible for the majority of incidents in developing countries. The *S. flexneri* protease IcsP, is a member of the Omptin family of outer membrane (OM) proteases which cleaves IcsA, a polarly localised OM protein required for *Shigella* virulence. Mutations in *icsP* have been shown to effect the observed distribution of IcsA, however the significance of IcsP in *Shigella* virulence is incompletely understood.

In this study, aspects of IcsP biology were investigated. *S. flexneri* 2457T and M90T *icsP* mutants were constructed to investigate the role of IcsP in *Shigella* intercellular spread, and it was found that *icsP* in both *S. flexneri* backgrounds did not appear to be essential for cell-to-cell spread in human cervical cancer HeLa cells, but enhanced cell-to-cell spread in monkey kidney CV-1 cells (as determined by plaque assays). Complementation with *icsP* returned the mutant phenotype to wild-type. The results suggest IcsP does play a role in *Shigella* intercellular spread.

The 2457T *icsP* mutant was subsequently complemented with an altered *icsP* gene encoding a haemagglutinin epitope tagged IcsP (IcsP$^{HA}$) to determine the distribution of IcsP on the cell surface. In both *S. flexneri* and *E. coli* K-12 possessing smooth and rough lipopolysaccharide (LPS), the distribution of IcsP$^{HA}$ was found to be punctate across the cell surface. Deconvolution analysis revealed that IcsP distribution was punctate and banded in both LPS backgrounds. A smooth LPS *E. coli* K-12 *yfdI* mutant strain expressing IcsP$^{HA}$ was also constructed, and experiments involving treatment of this strain with bacteriophage Sf6 tail spike protein suggested that LPS O antigen chains masked IcsP in smooth LPS strains. During these studies, double-labelling of IcsP$^{HA}$ and LPS in a *S. flexneri* 5a M90T strain revealed a helical distribution of LPS in this strain. Overall, the results suggest IcsP has a punctate, banded distribution across the cell surface.
The effect of virK and rmlD mutations on IcsP was then investigated by constructing a virK, rmlD and virK/rmlD double mutant in S. flexneri 2457T. Western immunoblotting showed no change in IcsP expression levels in either the virK, rmlD or virK/rmlD mutants compared to wild-type. Surprisingly, the virK mutant showed no change in IcsA expression levels by Western immunoblotting and plaque assays (using HeLa and CV-1 cells) suggested that virK was not essential for Shigella intercellular spread (contradicting the published data on this gene). No effect was also observed on IcsP expression level or on IcsP’s ability to cleave IcsA into culture supernatants.

Finally alternative substrates for the protease activity of IcsP were investigated against known Omptin substrates (plasminogen, α2-antiplasmin, complement, protamine and colicins). However, IcsP appeared to have no effect on these substrates as determined by proteolytic cleavage assays and antimicrobial assay. Interestingly, Plg cleavage by rough LPS S. flexneri, and α2AP cleavage by both smooth and rough LPS S. flexneri, was observed.
Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University library, being made available in all forms of media, now or hereafter known.

Elizabeth Ngoc Hoa Tran
Acknowledgements

I would firstly like to thank my supervisor, Dr. Renato Morona. Thank you for giving me the opportunity to do my PhD with you, and for teaching me so much about microbiology and research. I have grown and developed much as a scientist under your guidance and I am grateful for your patience, understanding and knowledge. Thank you secondly to Luisa Van Den Bosch, for your patience in teaching me all the techniques in the lab, and for looking out for me like a daughter.

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List of Abbreviations

～ approximately
°C degree
% percentage
# number
α alpha
α₂AP alpha₂-antiplasmin
β beta
γ gamma
λ lambda
µg; µl; µm microgram (s); microliter (s); micrometre (s)
aa amino acid
3D 3-dimensional
ABM actin based motility
Amp ampicillin
Anti-Pla anti-plasminogen
Arg arginine
Arp2/3 actin related protein 2/3
Av average
bp base pairs
C-terminal carboxyl-terminal
CAT# catalogue number
Ch. 3, 4, 5, 6 chapter 3, 4, 5, 6
cm centimetres
cm² cm square
CM cytoplasmic membrane
Cml chloramphenicol
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
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<tr>
<td>D-PBS</td>
<td>Dulbecco’s PBS</td>
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<tr>
<td>DAPI</td>
<td>4’, 6-diamidino-2-phenylindole dihydrochloride</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s MEM</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleoside triphosphate</td>
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<tr>
<td>EDTA</td>
<td>ethylene diamine tetra-acetic acid</td>
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<tr>
<td>Ef1, Ef2, Ef3</td>
<td>elution fractions 1, 2, 3</td>
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<tr>
<td>EIEC</td>
<td>enteroinvasive E. coli</td>
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<td>FAE</td>
<td>follicular associated epithelium</td>
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<td>FCA</td>
<td>Freund’s complete adjuvant</td>
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<td>FCS</td>
<td>foetal calf serum</td>
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<td>Fig.</td>
<td>Figure</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>FRT</td>
<td>FLP recognition target</td>
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<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
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<td>GTE</td>
<td>Glucose/Tris/EDTA</td>
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<tr>
<td>h; min; sec</td>
<td>hour (s); minutes (s); seconds (s)</td>
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<tr>
<td>HA</td>
<td>haemagglutinin</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
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<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<tr>
<td>HIC</td>
<td>heat inactivated complement</td>
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<td>His₆</td>
<td>6x histamine</td>
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<td>His₆-PsaA</td>
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<td>IcsP&lt;sup&gt;HA&lt;/sup&gt;</td>
<td>HA epitope tagged IcsP</td>
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<td>IcsP-His₆</td>
<td>C-terminal His₆ tagged IcsP</td>
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<td>IF</td>
<td>immunofluorescence</td>
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<tr>
<td>IL-1β</td>
<td>interleukin-1β</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>IL-8</td>
<td>interleukin-8</td>
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<td>IM</td>
<td>inner membrane</td>
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<tr>
<td>Ipa</td>
<td>invasion plasmid antigens</td>
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<td>Ipg</td>
<td>invasion plasmid gene</td>
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<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
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<tr>
<td>Kan</td>
<td>kanamycin</td>
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<tr>
<td>kb</td>
<td>kilobase pairs</td>
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<td>kDa</td>
<td>kilodaltons</td>
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<td>L</td>
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<td>Lab</td>
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<tr>
<td>LB</td>
<td>Luria-bertani</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>Lys</td>
<td>lysine</td>
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<td>M; mM</td>
<td>molar; millimolar (s)</td>
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<tr>
<td>M-cells</td>
<td>Membraneous epithelial cells</td>
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<tr>
<td>mA</td>
<td>milli-amps</td>
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<td>MEM</td>
<td>Modified Eagle’s Media</td>
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<td>mg; ml; mm</td>
<td>milligram (s); millilitre (s); millimetre (s)</td>
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<tr>
<td>MOPS</td>
<td>3-(N-Morpholino)-propanesulfonic acid</td>
</tr>
<tr>
<td>MQ</td>
<td>MilliQ</td>
</tr>
<tr>
<td>mxi-spa</td>
<td>membrane expression of Ipas-surface presentation of antigens</td>
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<tr>
<td>N-terminal</td>
<td>amino terminal</td>
</tr>
<tr>
<td>N-WASP</td>
<td>neural Wiskott-Aldrich syndrome protein</td>
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<td>New England Biolabs</td>
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<td>Ni-charged</td>
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<td>nt</td>
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<td>O antigen</td>
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<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>optical density of 600 nm</td>
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<td>OM</td>
<td>outer membrane</td>
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<td>Omp</td>
<td>outer membrane protease</td>
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<td>phosphate buffered saline</td>
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<td>rpm</td>
<td>revolutions per minute</td>
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<tr>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>SDS-PAGE</td>
<td>SDS polyacrylamide gel electrophoresis</td>
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<tr>
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<tr>
<td>TBS</td>
<td>tris buffered saline</td>
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<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
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<tr>
<td>Tet</td>
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<tr>
<td>Tris</td>
<td>tris (hydroxymethyl) aminomethane</td>
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<tr>
<td>TSP</td>
<td>tailspike protein</td>
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<tr>
<td>TBS</td>
<td>tris buffered saline</td>
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<td>X-Gal</td>
<td>5’-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
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