Molecular characterisation of *Shigella flexneri* outer membrane protease IcsP

Elizabeth Ngoc Hoa Tran

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Thesis Amendments

Abstract

Page I, 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 "...an increased detection..."
Page 61, section 4.3.4.1, line 3:	should read "...unexpectedly solubilised by the Triton/$MgCl_2$..."
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 Nakata *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-IcsP. The differences seen in Figure 5.10B are reproducible and are not affected by the immunoblotting chemiluminescence substrate problem.
Page 85, line 5:	should read "...no effect on IcsP..."
Page 85, line 8:	should read "...that *virK* has no detectable effect on the structure of LPS."

Chapter 6 – Alternative substrates for IcsP

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 89, 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 OmpT..."

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, α_2-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 α_2AP 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

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List of Abbreviations

~	approximately
°C	degree
%	percentage
#	number
α	alpha
α_2AP	alpha$_2$-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
cm^2	cm square
CM	cytoplasmic membrane
Cml	chloramphenicol

CO_2	carbon dioxide
D-PBS	Dulbecco's PBS
DAPI	4', 6-diamidino-2-phenylindole dihydrochloride
DMEM	Dulbecco's MEM
DMF	dimethylformamide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	ethylene diamine tetra-acetic acid
Ef1, Ef2, Ef3	elution fractions 1, 2, 3
EIEC	enteroinvasive *E. coli*
FAE	follicular associated epithelium
FCA	Freund's complete adjuvant
FCS	foetal calf serum
Fig.	Figure
FITC	fluorescein isothiocyanate
FRT	FLP recognition target
Glc*N*Ac	*N*-acetylglucosamine
GTE	Glucose/Tris/EDTA
h; min; sec	hour (s); minutes (s); seconds (s)
HA	haemagglutinin
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIC	heat inactivated complement
His_6	6x histamine
His_6-PsaA	N-terminal His_6 tagged PsaA
$IcsP^{HA}$	HA epitope tagged IcsP
IcsP-His_6	C-terminal His_6 tagged IcsP
IF	immunofluorescence
IL-1β	interleukin-1β

IL-8	interleukin-8
IM	inner membrane
Ipa	invasion plasmid antigens
Ipg	invasion plasmid gene
IPTG	isopropyl-β-D-thiogalactopyranoside
Kan	kanamycin
kb	kilobase pairs
kDa	kilodaltons
L	litres
Lab	laboratory
LB	Luria-bertani
LPS	lipopolysaccharide
Lys	lysine
M; mM	molar; millimolar (s)
M-cells	Membraneous epithelial cells
mA	milli-amps
MEM	Modified Eagle's Media
mg; ml; mm	milligram (s); millilitre (s); millimetre (s)
MOPS	3-(N-Morpholino)-propanesulfonic acid
MQ	MilliQ
mxi-spa	membrane expression of Ipas-surface presentation of antigens
N-terminal	amino terminal
N-WASP	neural Wiskott-Aldrich syndrome protein
NaAc	sodium acetate
NEB	New England Biolabs
Ni-charged	nickel-charged
nm	nanometre
nt	nucleotide
Oag	O antigen

OD_{600}	optical density of 600 nm
OM	outer membrane
Omp	outer membrane protease
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Plg	plasminogen
^{R}	resistance
Rha	rhamnose
RI	refractive index
Rif	rifampicin
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SOE PCR	Splicing by Overlap Extension PCR
Strep	streptomycin
TBE	tris-borate-EDTA
TBS	tris buffered saline
TCA	trichloroacetic acid
Tet	tetracycline
Tris	tris (hydroxymethyl) aminomethane
TSP	tailspike protein
TBS	tris buffered saline
TTBS	tween tris buffered saline
TTSS	Type III secretion system
U	units

UV	ultraviolet
v/v	volume per volume
V	volts
VASP	vasodilator-stimulating phosphoprotein
VP^{-ve}	virulence plasmid negative
w/v	weight per volume
WM	whole membrane
X-Gal	5’-bromo-4-chloro-3-indolyl-β-D-galactopyranoside