

**Molecular Characterisation of  
*Shigella flexneri* IcsA  
and the  
Role of Lipopolysaccharide  
O-Antigen in Actin-based Motility**

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## Abstract

*Shigella* spp. cause bacillary dysentery through invasion of the colonic epithelium. *Shigella flexneri* IcsA (VirG) is a polarly localised, outer membrane (OM) protein that is essential for virulence. IcsA activates the host actin regulatory protein, neural Wiskott-Aldrich syndrome protein (N-WASP), which in-turn recruits the Arp2/3 complex that polymerises host actin. The resultant F-actin comet tails initiate bacterial actin-based motility (ABM) and intercellular spread. The N-terminal surface-exposed region of IcsA, referred to as the passenger domain (aa 53-758), is responsible for IcsA activity in ABM. A glycine-rich region (aa 140-307) within this passenger domain is involved in mediating N-WASP binding.

This thesis sought to conduct a comprehensive study of IcsA structure-function. Linker-insertion mutagenesis was undertaken to randomly introduce in-frame insertions of 5 aa within the IcsA passenger domain. Forty-seven linker-insertion mutants (IcsA<sub>i</sub>) mutants were isolated and expressed in *S. flexneri*  $\Delta$ icsA. The resultant strains were characterised for IcsA protein production, cell surface-expression and localisation, as well as intercellular spreading, F-actin comet tail formation, and the recruitment of N-WASP. Linker-insertions between aa 595-716 of IcsA affected production and lie in a region homologous to the putative auto-chaperone domain of *Bordetella pertussis* BrkA. Two mutant proteins (IcsA<sub>i532</sub> and IcsA<sub>i563</sub>) exhibited disrupted polar targeting, enabling refinement of the polar targeting region to aa 532-563. Twenty-two of the *S. flexneri* strains expressing IcsA<sub>i</sub> mutants were unable to spread from cell-to-cell; further characterisation revealed that nineteen strains were unable to form either F-actin comet tails or recruit N-WASP.

Since lipopolysaccharide O-antigen (LPS-Oag) on the bacterial surface has been shown to mask IcsA function, IcsA<sub>i</sub> mutants were expressed in rough LPS (R-LPS) strains (that lack the Oag component) to investigate the effect of LPS-Oag on IcsA:N-WASP

interactions. Mutants were identified that were unable to recruit N-WASP and induce F-actin comet tails when expressed in smooth LPS (S-LPS) *S. flexneri* strains but able to recruit N-WASP and form F-actin comet tails in a R-LPS background. These studies enabled identification of two novel functional regions (aa 330-381 and aa 508-730) involved in N-WASP interaction.

For the first time, a structural model of the IcsA passenger domain was created using the Robetta protein prediction server and IcsA was predicted to form a  $\beta$ -helical structure. However, not all IcsA<sub>i</sub> mutant phenotypes could not be clearly correlated to the model.

As LPS-Oag had been shown to mask IcsA function, LPS profiles at the bacterial pole (where IcsA is predominantly located) were investigated. A comparison of the LPS profiles of purified minicells (derived from the bacterial cell pole) and purified whole cells, indicated that LPS populations are uniformly distributed on the polar and lateral regions of the bacterium.

IcsA is a member of the autotransporter (AT) family of proteins. Another AT protein, IgA protease of *Neisseria gonorrhoea* forms oligomeric structures in the OM. *In situ* chemical cross-linking revealed that IcsA is able to form high molecular weight complexes. Moreover, IcsA<sub>i</sub> mutants were shown to exert a negative dominant effect on WT IcsA, providing evidence for IcsA:IcsA interactions in the OM.

In conclusion, studies conducted in this thesis revealed that multiple regions of IcsA interact with N-WASP, suggesting that IcsA has evolved to activate N-WASP in the presence of LPS-Oag and host actin regulatory proteins.

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 available for loan and photocopying.

Kerrie May

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# Abbreviations

Abbreviations acceptable to the American Society for Microbiology are used without definition in this thesis. Additional and frequently used abbreviations are defined when first used in the text, and are listed below.

Å	angstroms
A <sub>600</sub>	absorbance at 600 nm
aa	amino acid
ABM	actin-based motility
Ap	ampicillin
Ap <sup>R</sup>	ampicillin resistance
Arp	actin-related protein
AT	autotransporter
ATP	adenosine triphosphate
bp	base pairs
β-ME	β-mercaptoethanol
cfu	colony forming units
Cm	chloramphenicol
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride
DNA	deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle medium
DMF	dimethyl formamide
dNTP	deoxynucleoside triphosphate
dsDNA	double stranded deoxyribonucleic acid
DSP	Dithio-bis(succinimidylpropionate)
F-actin	filamentous actin
FCS	foetal calf serum
g	gravitational units

G-actin	globular actin
GFP	green fluorescent protein
GRR	glycine rich repeat
h	hour(s)
HMW	high molecular weight
IcsA <sub>i</sub>	IcsA linker-insertion mutant
IcsA <sub>Δ</sub>	IcsA deletion mutant
IF	immunofluorescence
IL	interleukin
IM	inner membrane
IPTG	isopropyl-β-D-thiogalactopyranoside
Kb	kilobases
kDa	kilodaltons
Km	kanamycin
Km <sup>R</sup>	kanamycin resistance gene or phenotype
L	litre
LB	Luria Bertani medium
LPS	lipopolysaccharide
M	molar
m	metre
M cells	membranous epithelial cells
min	minutes
MOPS	3-(N-morpholino) propane sulfonic acid
mRNA	messenger ribonucleic acid
NA	nutrient agar
NEB	New England Biolabs
nt	nucleotide
N-WASP	neural Wiskott-Aldrich syndrome protein
Oag	O antigen (O polysaccharide)



OM	outer membrane
OMPs	outer membrane proteins
ON	overnight
ORF	open reading frame
PAGE	polyacrylamide electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMN	polymorphonuclear
R-LPS	rough lipopolysaccharide
rpm	revolutions per minute
RT	room temperature
RUs	(Oag) Repeat units
SAP	shrimp alkaline phosphatase
sec	second
SD	standard deviation
SDS	sodium dodecylsulphate
S-LPS	smooth lipopolysaccharide
Sm	streptomycin
ss	signal sequence
S-type	short Oag modal length
Tc	tetracycline
Tc <sup>R</sup>	tetracycline resistance cassette or phenotype
TCA	trichloroacetic acid
TTSS	type III secretion system
VL-type	VL Oag modal length
WASP	Wiskott-Aldrich syndrome protein
WT	Wild-type
X-gal	5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside
$\Delta$	Deletion mutation

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## Corrigenda

Page 1, line 9 and elsewhere - “*Shigellae*” should be written “Shigellae”.

Page 2, line 5 - “Genetic and DNA sequence analyses of *Shigella* spp. ...”

Page 3, line 3 - “During this process, the bacteria become localised within an endocytic vacuoles.”

Figure legend 1.4, line 1 - “A schematic diagram of IcsA functional domains.”

Page 10, line 10 - “...detected with anti-IcsA antisera throughout the length of F-actin tails formed by *Shigella*”

Page 14, line 17 - “...the most closely related to IcsA based on sequence identity (~29% and ~ 24%, respectively).”

Page 16, line 7 - “The status of ATs in the periplasm is not known.”

Page 17, line 14 and elsewhere - “Oomen *et al.*, 2002” should read “Oomen *et al.*, 2004”.

Page 34, line 23 - “...*virK::Tn10* mutant had WT levels of *icsA* mRNA...”

Figure legend 3.5 and elsewhere - “Pannel” should be written “panel”.

Figure legend 4.2 - “Alex594 and FITC-phalloidin images were false colour merged...”

Page 74, line 25 - “A *S. flexneri rmlD* strain is...”

Page 74, lines 16-18 - “One population of F-actin tails displayed WT morphology (Figure 4.2) whilst the other population appeared thicker, and more heavily labeled with FITC-phalloidin (refer to Figure 4.4).”

Page 78, line 13 - “...were transformed into *S. flexneri ΔicsA ΔrmlD* strain...”

Page 79, lines 12-14 - “Additionally, it was necessary to verify that IcsA<sub>i</sub> proteins (identified in the previous section that were able to induce F-actin comet tail formation in a R-LPS background) were able to recruit N-WASP.”

Page 80, line 12 - “...an altered proteolysis profile did not correlate with...”

Page 80, lines 21-22 - “...perhaps to an even greater extent...”

Figure 4.11, lines 9-10 - “...polar localisation region #2 (aa 507-620, orange); and a putative auto-chaperone region (aa 634-735, white)...”

Page 83, lines 18-21 - “In this chapter, various *in silico* analysis tools were employed to create a structural model for the IcsA passenger domain, and correlations were made between these structure predictions and data from linker-mutagenesis and function studies (Chapters Three and Four).

Page 86, lines 22-23 - “The first  $\alpha$ -helix (aa 260-270) was predicted to lie within the GRR #5...”

Page 88, line 17 - “These loop regions could possibly...”

Page 97, line 4 - “IcsA<sub>i677</sub> possesses a linker-insertion...”

Page 98, line 17 - “...may be explained by two hypotheses.”

Page 98, line 20 - “...IcsA is still detected more readily at the poles...”

Figure legend 7.2, line 2 - “The chromosomal mutation of *minD* was confirmed...”

Page 104, line 13 - “7.5 Analysis of LPS of minicell-producing mutants...”

Page 122, line 18 - “Based on X-ray crystallography data...”

Page 123, lines 17-18 - “As well as identifying novel N-WASP interacting regions, LPS-Oag has been shown...”