Polarity and Secretion of *Shigella flexneri* IcsA: A Classical Autotransporter

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Abstract

The classical autotransporter IcsA is an essential virulence factor for the enteropathogen *Shigella flexneri* as it provides adherence properties and allows intra- and intercellular spreading in the colonic mucosa. IcsA is an outer membrane surface protein that specifically hijacks host-cell actin recruiting and polymerising complexes allowing actin polymerisation as a form of actin-based motility (ABM). Importantly, since IcsA is localised specifically at one end of the bacterium (the pole), the resulting ABM is unidirectional which is a requirement for efficient *S. flexneri* dissemination. However, the molecular mechanisms that generate IcsA polarity remain poorly understood. Furthermore, IcsA is a member of the autotransporter family of secreted virulence factors (Type Va). Although many steps in the autotransporter pathway have been elucidated, it is still poorly understood how these diverse proteins are efficiently translocated to the bacterial cell surface. As such, this thesis investigates the two arms of IcsA biogenesis: (1) polar targeting and (2) autotransport.

Regarding IcsA polarity, it was identified here that the IcsA-specific outer membrane protease IcsP localises to the septa of dividing *S. flexneri* and to the opposing pole relative IcsA. The concentration of IcsP was higher at the septum than the pole showing a life cycle dependent distribution of IcsP. This provides the basis of a model where IcsP is important during division of *S. flexneri* for setting up (and the continued maintenance of) IcsA polarity by the proteolysis of misdirected IcsA. Further, multiple previous reports have suggested that the *S. flexneri* lipopolysaccharide O-antigen surface structure can influence IcsA polarity by augmenting membrane fluidity or by asymmetric masking of IcsA surface exposure. These notions were tested here resulting in data that clearly refutes these models and agues simply that IcsA exposure is masked symmetrically over the bacterial cell surface. Finally, IcsA itself contains polar targeting (PT) regions that direct it to the pole by an, as yet, unclear mechanism. Examination of these regions revealed that the central PT (cPT) is most important in polarity augmentation and contains critical polarity targeting function residues.

Regarding IcsA autotransport, a highly conserved but uncharacterised autotransporter motif was scrutinized for potential biogenesis functions and was designated in this work as the passenger-associated transport repeat (PATR). It was found that the PATR plays a critical role in the efficient secretion of IcsA to the cell surface. Strikingly, bioinformatics analyses revealed that the PATR delineates an important separate autotransporter sub-type with unique functions, composition, and architecture.

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'For Mum, who protected me'

Thesis Style and Layout

This Thesis is submitted in the style of a 'Thesis by Publication'. As such, the results chapters are replaced by four Research Articles (In chronological publishing order Chapters 3 to 6). In Chapter 1: 'Introduction', the research is framed and the Aims of my Doctoral studies are outlined. Chapter 2 outlines the base 'Materials and Methods' used throughout candidature, with nuances and changes specific to each article described in the methods subsection of the respective publication.

Author contributions for each publication are stated in preceding 'Statements of Authorship', and the objectives of the article with respect to my thesis are outlined in 'Purpose of Article' subheadings. Article chapters retain the section order layout style of the journal publisher. Subheadings starting with 'Article …' indicate subdivisions that were included in the publication. Article supplementary data is included in this thesis (and [S] after the figure or table number). Please note that formatting may be slightly different between articles (i.e. US versus Australian spelling). This is a result of complying with the specific journal requirements. Important data that was collected in relation to a publication (but was not included in the article due to its peripheral nature) are marked with 'Additional Results' subheadings (and [A] after the figure number).

Chapter 7: 'Conclusions' draws together the outcomes of the presented publications, argues how the data aligns with the current mechanisms and models, and describes how this research leads to intriguing questions for future investigations.

Publications

Peer Reviewed Research Articles

Tran ENH*, **Doyle MT***, Morona R (2013) LPS unmasking of *Shigella flexneri* reveals preferential localisation of tagged outer membrane protease IcsP to septa and new poles. *PLoS ONE* 8(7): e70508. (* equal authorship)

Doyle MT, Grabowicz M, May KL, Morona R (2015) Lipopolysaccharide surface structure does not influence IcsA polarity. *FEMS Microbiology Letters* 362 (8), fnv042

Doyle MT, Tran ENH, Morona R (2015) The passenger-associated transport repeat promotes virulence factor secretion efficiency and delineates a distinct autotransporter subtype. *Molecular Microbiology* 97(2): 315-329.

Doyle MT, Grabowicz M, Morona R (2015) A small conserved motif supports polarity augmentation of *Shigella flexneri* IcsA. *Microbiology-SGM* doi: 10.1099/mic.0.000165.

Abbreviations

a.a.	amino acid
ABM	actin-based motility
Ap^R	ampicillin resistance cassette
AT	autotransporter
BAM	β-Barrel Assembly Machinery
BSG	buffered saline gelatin
CM	cytoplasmic membrane
Cml^R	chloramphenicol resistance cassette
cPT	central polar targeting region
DMEM	Dulbecco's modified eagles medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
D-PBS	Dulbecco's phosphate buffered saline
DSP	dithiobis(succinimidyl propionate)
DTRM	domain targeted random mutagenesis
ESS	extended signal sequence
F-actin	filamentous actin
FAE	follicle-associated epithelia
FCS	foetal calf serum
g	force of gravity
GA	Gibson assembly
GFP	green fluorescent protein
GL	grey level
GRR	glycine rich repeats
GST	glutathione S-transferase
HRP	horse radish peroxidase
HS	hot-spot
IF	immunofluorescence
IM	inner membrane
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
Km^R	kanamycin resistance cassette
LB	Luria Bertani broth / Lysogeny broth
LPA	large polar aggregate
LPS	lipopolysaccharide
MEM	modified eagles medium
MQ	MilliQ H_2O
mRNA	messenger RNA
Mw	molecular weight
NC	non-cleavable
nPT	N-terminal polar targeting region
ns	not significant
N-WASP	Neural Wiskott Aldrich Syndrome Protein
OA	oligo annealing
Oag	O-antigen
OD_{600}	optical absorbance at 600 nm
OM	outer membrane
OMP	outer membrane protein
PAGE	polyacrylamide gel electrophoresis
PAP2	type 2 phosphatidic acid phosphatase
PATR	passenger-associated transport repeat
PbH1	parallel β-helix type 1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGN	peptidoglycan
PK	proteinase K
PL	pertactin-like
PLL	pectin lyase like
PMBN	polymixin B nonapeptide
PMN	polymorphonuclear leukocytes
PMSF	phenylmethanesulfonyl fluoride
POMP	polymorphic outer membrane protein
POTRA	polypeptide transport associated
PT	polar targeting region
QC	QuikChange mutagenesis
QD	quantum dot
RBS	ribosome binding site
RDA	randomly distributed aggregate
R-LPS	rough lipopolysaccharide
RNA	ribonucleic acid
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
ShET	*Shigella* enterotoxin
^{S}LPS	short type lipopolysaccharide
S-LPS	smooth lipopolysaccharide
SOE	splicing by overlap extension
SPATE	serine protease AT of the enterobacteriae
SRL	*Shigella* resistance locus
SRP	signal recognition particle
SS	signal sequence
T3SS	Type III secretion system
TBS	tris buffered saline
TCA	trichloroacetic acid
Tc^R	tetracycline resistance cassette
TS	tryptic soy
TSP	tail-spike protein
TTBS	tris buffered saline plus tween
v/v	volume per volume
VC	vacuolating cytotoxin
^{VL}LPS	verly long type lipopolysaccharide
VP	virulence plasmid
w/v	weight per volume
WIP	WASP inhibiting protein
WM	whole membrane
WT	wild-type
X-gal	5-bromo-5-chloro-3-indolyl-β-D-galactoside
β-ME	β-mercaptoethanol

Contents