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Title

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Running title

IcsA autochaperone region mutations

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Summary

The *Shigella flexneri* IcsA (VirG) protein is a polarly distributed autotransporter protein. IcsA functions as a virulence factor by interacting with the host actin regulatory protein, N-WASP, which in turn activates the Arp2/3 complex, initiating actin polymerisation. Formation of F-actin comet tails allows bacterial cell-to-cell spreading. Although various accessory proteins such as periplasmic chaperones and the BAM complex have been shown to be involved in the export of IcsA, the IcsA translocation mechanism remains to be fully elucidated. A putative autochaperone (AC) region (aa 634-735) located at the C-terminal end of the IcsA passenger domain, which forms part of the self-associating autotransporter (SAAT) domain, has been suggested to be required for IcsA biogenesis, as well as for N-WASP recruitment, based on mutagenesis studies. $IcsA_i$ proteins with linker insertion mutations within the AC region have a significant reduction in production and are defective in N-WASP recruitment when expressed in smooth lipopolysaccharide (S-LPS) *S. flexneri*. In this study, we have found that the LPS O-antigen (Oag) play a role in $IcsA_i$ production based on use of an *rmlD* (*rfbD*) mutant having rough LPS (R-LPS), and a novel assay in which Oag is depleted using tunicamycin treatment and then regenerated. In addition, we have identified a new N-WASP binding/interaction site within the IcsA AC region.

Introduction

Shigella flexneri is a Gram-negative bacterium that causes bacillary dysentery in humans by infecting and colonising the colonic epithelium (Philpott *et al.*, 2000). IcsA (VirG) is an essential virulence factor of *S. flexneri*. It is a polarly distributed 120 kDa outer membrane (OM) protein that interacts with N-WASP, resulting in F-actin comet tail formation and inter- and intracellular spreading of *S. flexneri* within the host intestinal epithelium (Bernardini *et al.*, 1989; Lett *et al.*, 1989; Makino *et al.*, 1986; Sansonetti *et al.*, 1991; Suzuki & Sasakawa, 2001).

IcsA is a member of the autotransporter (AT) family (Type Va secretion system), the largest family of secreted proteins in Gram-negative bacteria (Henderson *et al.*, 2004; Pallen *et al.*, 2003). Like other ATs, IcsA is composed of three major domains: an extended N-terminal signal sequence (amino acids [aa] 1-52), a functional passenger α-domain (aa 53-758), and a C-terminal translocation β-domain (aa 759-1102) that mediates the translocation of the passenger domain across the OM via the BAM (beta-barrel assembly machine) complex (Brandon *et al.*, 2003; Henderson *et al.*, 2004; Jain & Goldberg, 2007; Peterson *et al.*, 2010; Suzuki *et al.*, 1995). The involvement of the BAM complex and periplasmic chaperones such as DegP, Skp and SurA in IcsA translocation suggest that the term ‘autotransporter’ is no longer applicable (Ieva & Bernstein, 2009; Jain & Goldberg, 2007; Purdy *et al.*, 2007; Sauri *et al.*, 2009; Sklar *et al.*, 2007; Wagner *et al.*, 2009). A common feature of AT proteins is that the exported passenger domain can be cleaved and released into the external milieu or remain associated on the bacterial surface (Henderson *et al.*, 2004). In *S. flexneri*, IcsP (SopA) is a virulence plasmid encoded serine OM protease that cleaves surface IcsA at the Arg758-Arg759 bond position, releasing a ~85 kDa IcsA fragment into the culture supernatant (d'Hauteville *et al.*, 1996; Fukuda *et al.*, 1995; Steinhauer *et al.*, 1999). In spite of the diversity in sequence, function and

length, the passenger domains of most ATs possess a β-helical structure (Wells *et al.*, 2010).

IcsA has recently been classified as a member of the self-associating AT (SAAT) family, together with other *Escherichia coli* ATs such as Ag43, AIDA-1, TibA and *Haemophilus influenzae* Hap that mediate bacterial aggregation and biofilm formation (Klemm *et al.*, 2006; Meng *et al.*, 2011). In addition, a putative autochaperone (AC) region at the C-terminal of the IcsA passenger domain (aa 634-735), which is part of the self-associating domain, has been proposed to be required for IcsA biogenesis (May & Morona, 2008; Meng *et al.*, 2011). The AC region is well conserved among many ATs and is essential for AT secretion, folding and/or stability (May & Morona, 2008; Oliver *et al.*, 2003a; Oliver *et al.*, 2003b; Yen *et al.*, 2008). While the crystal structure of the full length IcsA passenger domain is not yet available, the IcsA AC region crystal structure (aa 591-758) has been determined at 2.0-Å resolution and features two coils of a right-handed parallel β-helix (Kuhnel & Diezmann, 2011). May & Morona (2008) have previously suggested that the IcsA AC region is also an N-WASP interacting region (N-WASP IR III), based on linker-insertion mutagenesis.

Lipopolysaccharide (LPS) is a major constituent of the Gram-negative bacteria OM and is comprised of lipid A that is anchored into the OM, the core sugar region and the O-antigen (Oag) polysaccharide repeat units that extends from the bacterial surface (Raetz & Whitfield, 2002). The intact LPS molecules are known as smooth LPS (S-LPS), while LPS molecules lacking the Oag, due to mutations affecting either the biosynthesis of Oag repeat units (eg. *rmlD*/*rfbD*) or their linkage to the core sugars, are known as rough LPS (R-LPS) (Van Den Bosch *et al.*, 1997). LPS molecules are synthesised at the inner membrane, and current models for the LPS biosynthesis pathway involve the MsbA ABC transporter and the LPS transport (Lpt) pathway (Narita & Tokuda, 2009; Raetz & Whitfield, 2002; Ruiz *et al.*, 2008; Ruiz *et al.*, 2009; Sperandeo *et al.*, 2008; Tran *et al.*,

2010). *S. flexneri* LPS has been suggested to be required to reinforce IcsA polar localisation and mask laterally located IcsA proteins (Morona & Van Den Bosch, 2003; Robbins *et al.*, 2001; Sandlin *et al.*, 1995). However, the effect of LPS Oag in IcsA translocation across the OM and its interaction with IcsA have not been reported.

The role of the IcsA putative AC/SAAT domain has not yet been fully investigated but we have previously shown via linker-insertion mutagenesis that this region may play a role in IcsA biogenesis (May & Morona, 2008). The production of AC $IcsA_i$ (with insertion mutations within the AC region) is significantly reduced in the S-LPS *S. flexneri* strain and the strains were defective in recruiting N-WASP. In this study, we analysed the $IcsA_i$ production in both S-LPS and R-LPS *S. flexneri* to investigate the role of the AC region/SAAT domain in IcsA biogenesis and N-WASP recruitment. We found that LPS Oag affects the production of $IcsA_i$. Tunicamycin was used to suppress Oag synthesis, and this restored $IcsA_i$ production. Hence, Oag synthesis directly impacts $IcsA_i$ biogenesis. In addition, we investigated the mechanism underlying this and found that DegP protease activity contributed to lack of $IcsA_i$ production in the S-LPS background. Finally, we identified a new N-WASP binding/interaction site within the previously identified N-WASP IR III (aa 508-730).

111 **Methods**

112 *Bacterial strains and plasmids*

113 The strains and plasmids used in this study are listed in Table 1.

114

115 *Growth media and growth conditions*

116 All strains used in this study were routinely grown in Luria Bertani (LB). *S. flexneri* strains
117 were grown from a Congo Red positive colony except virulence plasmid-cured strains as
118 previously described (Morona *et al.*, 2003). Bacterial cultures were cultured for 18 h,
119 diluted 1:20 and grown to mid-exponential phase (2 h) with aeration at 37°C (unless
120 otherwise stated). Where appropriate, antibiotics were used at the following
121 concentrations: ampicillin (Ap, 100 μg mL^{-1}), chloramphenicol (Cm, 25 μg mL^{-1}),
122 kanamycin (Km, 50 μg mL^{-1}), streptomycin (Sm, 100 μg mL^{-1}) or tetracycline (Tet, 10 μg
123 mL^{-1}).

124

125 *DNA methods*

126 *E. coli* K-12 DH5α was used for all cloning. DNA manipulation, PCR, transformation and
127 electroporation into *S. flexneri* were performed as previously described (Baker *et al.*, 1999;
128 Morona *et al.*, 1995).

129

130 *Construction of pBAD33::icsP*

131 The *icsP* gene was amplified by PCR from *S. flexneri* 2457T chromosomal DNA (GenBank
132 #AF386526) using primers (ET22/ET25) with *Kpn*I and *Hin*dIII restriction sites (Table S1).

The purified PCR product was subsequently cloned into the pBAD33 vector using the *Kpn*I and *Hin*dIII restriction sites.

Antibodies and antisera

Affinity purified rabbit polyclonal antibody to IcsA was produced as previously described (Van Den Bosch *et al.*, 1997). The anti-IcsA antibody was used at 1:100 in immunofluorescence (IF) assay or 1:1000 in Western immunoblotting (WB). The rabbit polyclonal anti-Skp (1:6000), rabbit polyclonal anti-SurA (1:10,000) and rabbit polyclonal anti-MBP-DegP antibodies are generous gifts from Thomas Silhavy (Princeton University), Carol Gross (UCSF) and Michael Ehrmann (University of Duisburg-Essen), respectively. Affinity purified rabbit polyclonal anti-IcsP antibody (1:1000) was produced by immunising a rabbit with purified IcsP-His_6 protein. The protein was overexpressed in *E. coli* M15 containing pREP4 and IPTG-inducible pQE60-*icsP*. This plasmid was constructed by cloning the *icsP* gene, which was PCR amplified from 2457T chromosomal DNA (GenBank #AF386526) using primers (ET9/ET10) with *Nco*I and *Bam*HI restriction sites (Table S1), and cloned into the corresponding sites in pQE60 (Qiagen).

IcsP protein induction

18 h bacterial cultures were diluted 1:20 into 10 mL LB supplemented with 0.3% (w/v) glucose and grown to mid-exponential phase with aeration at 37°C. Bacterial cultures were centrifuged (2,200 x*g*, 10 min), the supernatant was discarded and cells were washed twice with fresh LB prior to resuspension in 10 mL LB. Cultures were divided into 5 mL aliquots, 0.3% (w/v) glucose (final concentration) was added into one aliquot as the no

156 IcsP expression control, while 0.2% (w/v) L-arabinose (final concentration) was added into
157 another aliquot to induce IcsP expression for 1 h at 37°C.

158

159 *Preparation of whole cell lysate*

160 The equivalent of $5x10^8$ bacteria were centrifuged (2,200 x*g*, 2 min), resuspended in 100
161 µL of 1x sample buffer (Lugtenberg *et al.*, 1975), and heated at 100°C for 5 min prior to
162 SDS-PAGE.

163

164 *TCA precipitation of culture supernatant*

165 TCA precipitation of culture supernatant was performed as described previously (May &
166 Morona, 2008). The equivalent of $5x10^8$ bacteria were centrifuged, and the supernatant
167 was treated with 5% (w/v) ice-cold trichloroacetic acid (TCA), and incubated on ice. The
168 TCA precipitate was then collected by centrifugation (40,000 x*g*, 30 min, 4°C), and the
169 pellet washed with ice-cold acetone prior to recentrifugation for 5 min. The pellet was air-
170 dried, resuspended in 100 µL of 1x sample buffer, and heated at 100°C for 5 min prior to
171 SDS-PAGE.

172

173 *Western transfer and detection*

174 Western immunoblotting was performed as described previously (May & Morona, 2008)
175 with some modifications. Briefly, proteins were separated on SDS-7.5%, 12% or 15%-
176 PAGE gels and transferred to a nitrocellulose membrane. The membrane was blocked
177 with 5% (w/v) skim milk in TTBS (Tris-buffered saline, 0.005% (v/v) Tween-20) for 20 min
178 and incubated with desired primary antibody in TTBS containing 2.5% (w/v) skim milk for

18 h. After three washes in TTBS, the membrane was incubated with horseradish peroxidise-conjugated goat anti-rabbit secondary antibody (Biomediq DPC) for 2 h, followed by three washes in TTBS, then three times in TBS. The membrane was incubated with CPS3 chemiluminescence substrate (Sigma) for 5 min, followed by exposure of the membrane to X-ray film (Agfa). The film was developed using a Curix 60 automatic X-ray film processor (Agfa) or imaged with a Kodak Image Station 4000MM Pro (Carestream Molecular Imaging) to visualise the reactive bands.

Trypsin accessibility assay

Limited proteolysis was performed as described previously (May & Morona, 2008; Oliver *et al.*, 2003b) with modifications. The equivalent of 5 $x10^9$ bacteria were centrifuged, the supernatant discarded, and the pellet resuspended in 250 µL PBS. Bacterial suspensions were then treated with 0.1 µg trypsin mL^{-1} (from bovine pancreas; Roche) and incubated at 25°C to allow proteolysis. Aliquots were taken at different time points (0 min, 5 min, 15 min and 20 min) and supplemented with 1 mM phenylmethylsulphonyfluoride (PMSF; Sigma) to inhibit further proteolysis by trypsin. An equal volume of 2x sample buffer was added to each sample, heated at 100 °C for 5 min prior to SDS-PAGE and Western immunoblotting or storage at -20 °C.

LPS and silver staining

LPS samples were prepared as previously described (Murray *et al.*, 2003; Tsai & Frasch, 1982). Briefly, the equivalent of $1x10^9$ bacteria were centrifuged (2,200 x*g*, 2 min), resuspended in 50 µL lysing buffer (Papadopoulos & Morona, 2010) and incubated with 2.5 µg proteinase K mL^{-1} (Invitrogen) for 16 h at 56°C. The LPS samples were

electrophoresed on a SDS-15%-PAGE gel for 16-18 h at 12 mA, and the gel was stained with silver nitrate and developed with formaldehyde (Murray *et al.*, 2003).

LPS depletion-regeneration assay

18 h bacterial cultures were diluted 1:20 into LB and grown to mid-exponential phase with aeration at 37°C. The bacterial strains were further diluted 1:20 into LB supplemented with 3 µg polymyxin B nonapeptide mL^{-1} (PBMN; Sigma) and 10 µg tunicamycin mL^{-1} (Sigma) in DMSO, and treated for 3 h with aeration at 37°C (depletion phase). Bacterial cultures were then centrifuged, washed twice with fresh LB to remove PMBN and tunicamycin residues, and further diluted 1:20 into LB prior to additional incubation at 37°C with aeration for another 3 h (regeneration phase). Samples were prepared for LPS analysis and Western immunoblotting.

Construction of a S. flexneri ΔicsA degP::Cm mutant strain

The construction of *S. flexneri* Δ*icsA degP*::Cm mutant strain was performed as previously described (Purins *et al.*, 2008). pRMA2802 [pCVD442-*degP*::Cm] was conjugated into RMA2041, and sucrose/Cm/Tet-resistant and Ap-sensitive colonies were isolated and named MYRM522. The *degP*::Cm mutation was confirmed by Western immnublotting and a temperature sensitivity phenotype.

Site-directed mutagenesis

224 Site-directed mutagenesis was performed according to the QuikChange® Lightning Site-
225 directed protocol (Stratagene). Specific primers with the desired substitutions were
226 designed (Table S1). Mutations were confirmed by DNA sequencing.

227

228 *Construction of the icsA*-TGA-*lacZ reporter*

229 The *Pst*I flanked *lacZ*-Km fragment from pKOK6.1 (Kokotek & Lotz, 1989; Murray *et al.*,
230 2003) was digested with *Pst*I and ligated into the compatible *Nsi*I site of pIcsA (Van Den
231 Bosch & Morona, 2003) (nt 221623 and nt 225138, GenBank #AF386526) to give the
232 P*icsA*-*lacZ* construct, pMYRM632. The transcriptional P*icsA*-TGA-*lacZ* reporter plasmid
233 was created by mutating an amino acid between the *icsA* sequence and *lacZ* sequence of
234 P*icsA*-*lacZ* into a stop codon (TGA) via site-directed mutagenesis, as described above,
235 with primer pairs listed in Table S1. The resultant construct was named pMYRM676. The
236 *Pst*I-*Sal*I fragment from pMYRM676 which contains the P*icsA*-TGA-*lacZ* fragment, was
237 subsequently cloned into the likewise digested pSU23 vector to obtain the transcriptional
238 reporter plasmid, pMYRM718.

239

240 *β-galactosidase assay*

241 The β-galactosidase assay was performed using the Miller protocol (Miller, 1972) with
242 some modifications. 18 h bacterial cultures were diluted 1:20 into 10 mL LB and grown to
243 mid-exponential phase with aeration at 37°C. The OD_{600} of bacterial culture was
244 measured, 1 mL sample was taken, centrifuged, the supernatant discarded and the pellet
245 resuspended in Z buffer. Cells were permeabilised by adding 20 µL 0.1% (w/v) SDS and
246 40 µL chloroform and mixed vigorously for 10 sec. 120 µL samples were dispensed into a
247 96 well microtitre tray in triplicates and 24 µL 7 mg ortho-Nitrophenyl-β-galactoside mL^{-1}

248 (ONPG) was added into each well. Samples were incubated at 37°C for 3 h and readings
249 (OD_{420} and OD_{550}) were taken at 2 min intervals. Units of activity were calculated as
250 previously described (Miller, 1972).

251

252 *Indirect immunofluorescence of whole bacteria*

253 Indirect IF labelling of bacteria was performed as described previously (May & Morona,
254 2008). Briefly, mid-exponential phase bacteria were fixed in 3.7% (v/v) formalin and
255 centrifuged onto poly-L-lysine-coated coverslips. Bacteria were incubated with anti-IcsA
256 antibody diluted 1:100 in PBS with 10% (v/v) foetal calf serum (FCS), washed with PBS
257 and labelled with Alexa 488-conjugated donkey anti-rabbit secondary antibody (Molecular
258 Probes) (1:100). Microscopy was performed using an Olympus IX-70 microscope with
259 phase-contrast optics using a 100x oil immersion objective. Fluorescence and phase-
260 contrast images were false colour merged using the Metamorph software program
261 (Version 7.7.3.0, Molecular Devices).

262

263 *Infection of tissue culture monolayers with S. flexneri* and IF labelling

264 Infection of HeLa cell monolayers and IF staining were performed as described previously
265 (May & Morona, 2008). Briefly, HeLa monolayers were inoculated with mid-exponential
266 phase bacteria and incubated for 1 h at 37°C, 5% CO_2. The infected monolayers were
267 washed three times with D-PBS and incubated with MEM containing gentamycin for a
268 further 1.5 h. Infected cells were then washed and fixed for 15 min in 3.7% (v/v) formalin,
269 incubated with 50 mM NH_4Cl in D-PBS for 10 min, and permeabilised with 0.1% (v/v)
270 Triton X-100 for 5 min. The infected cells were blocked with 10% (v/v) FCS in PBS and
271 incubated with anti-N-WASP antibody. After washing in PBS, coverslips were incubated

272 with Alexa Fluor 594-conjugated donkey anti-rabbit secondary antibody (Molecular
273 Probes) (1:100). F-actin was visualised by staining with Alexa Fluor 488-conjugated
274 phalloidin (2 U mL^{-1}), and 4',6'-diamidino-2-phenylindole (DAPI) (10 µg ml^{-1}) was used to
275 counterstain bacteria and cellular nuclei.

Results

IcsA$_i$ mutant production is restored in S. flexneri ΔicsA ΔrmlD

LPS is known to mask surface IcsA function (Morona *et al.*, 2003; Morona & Van Den Bosch, 2003). May & Morona (2008) also reported that S-LPS can affect N-WASP recruitment and F-actin comet tail formation, by expressing certain IcsA$_i$ (outside the AC region) and IcsA deletion mutants in a R-LPS *S. flexneri* strain. Hence, four previously identified IcsA$_i$ mutants with 5 aa insertion within the AC region (IcsA$_{i633}$, IcsA$_{i643}$, IcsA$_{i677}$, and IcsA$_{716}$) which had defects in protein production and N-WASP recruitment when expressed in *S. flexneri* Δ*icsA* S-LPS (RMA2041) (May & Morona, 2008), were re-examined in this study. The mutants were expressed in *S. flexneri* Δ*icsA* Δ*rmlD* R-LPS strain (RMA2043) and the IcsA$_i$ protein production was assessed by Western immunoblotting.

As previously reported, IcsA$_{i633}$, IcsA$_{i643}$ and IcsA$_{i677}$ were poorly detected while IcsA$_{i716}$ could not be detected when expressed in *S. flexneri* Δ*icsA* S-LPS strain (Fig. 1a, lanes 3, 5, 7 and 9). Surprisingly, IcsA$_i$ mutant production in *S. flexneri* Δ*icsA* Δ*rmlD* R-LPS strain was restored to a near wild-type (WT) levels (Fig. 1a, lanes 4, 6, 8, and 10), which were consistent with the results obtained by IF microscopy (Fig. 1b). IcsA$_{i716}$ protein expression on the surface of *S. flexneri* Δ*icsA* strain was poorly detectable (Fig. 1b(iii)), in comparison to the high level of IcsA$_{i716}$ detected on *S. flexneri* Δ*icsA* Δ*rmlD* strain (Fig. 1b(iv)); it was also noted that IcsA$_{i716}$ has a polar localisation, like IcsA$_{WT}$ (Fig. 1b(i,ii)). Similar results were obtained for IcsA$_{i633}$, IcsA$_{i643}$, and IcsA$_{i677}$ (data not shown). IcsA staining was not observed for RMA2041 (Δ*icsA*) and RMA2043 (Δ*icsA* Δ*rmlD*), indicating the anti-IcsA antibody is specifically targeting the IcsA protein (Fig. 1b(v,vi)).

rmlD complementation

301 The *rmlD* (also known as *rfbD*) gene encodes for dTDP-6-deoxy-L-mannose
302 dehydrogenase which is involved in the synthesis of the nucleotide sugar dTDP-rhamnose
303 that is the precursor for rhamnose in the *S. flexneri* Oag repeat units (Reeves *et al.*, 1996).
304 To determine if the effect of *rmlD* on the $IcsA_i$ expression is directly related to the
305 Δ*rmlD*::Km mutation, *S. flexneri* Δ*icsA* Δ*rmlD* R-LPS strains expressing either $IcsA_{WT}$, or
306 various $IcsA_i$ were complemented with *rmlD* carried by pRMA727. As a control, the same
307 *S. flexneri* strains were transformed with the empty vector, pACYC184. The LPS profile
308 and IcsA production of the resultant strains was subsequently determined. *S. flexneri*
309 Δ*rmlD* strains were successfully complemented by pRMA727 which restored their S-LPS
310 profile (Fig. 2a, lanes 2, 4, 6, 8 and 10), while strains that were transformed with
311 pACYC184 retained their R-LPS profile (Fig. 2a, lanes 1, 3, 5, 7 and 9). As seen in Fig. 2b,
312 *rmlD* complementation also reverted $IcsA_i$ production to very low level as seen in the S-
313 LPS background, compared to the R-LPS strains. $IcsA_{WT}$ production remained unaffected
314 in both S-LPS and R-LPS backgrounds. In another experiment, very low levels of $IcsA_i$
315 production was also observed in the *E. coli* K-12 *ompT*$^-$ UT5600 strains that were
316 complemented to produce S-LPS but had higher $IcsA_i$ protein expression in the R-LPS
317 background (Fig. S1). Hence, the presence and absence of Oag chains results in an
318 inverse $IcsA_i$ production in both *S. flexneri* and *E. coli* K-12 *ompT*$^-$ strain backgrounds.

319

320 *Effect of LPS Oag modulation on $IcsA_{i716}$ mutant production*

321 The *rmlD* complementation assay showed that LPS Oag synthesis affects $IcsA_i$ mutant
322 production in *S. flexneri*. To independently verify the effect of LPS Oag on $IcsA_i$ mutant
323 production, a novel LPS Oag depletion-regeneration assay was developed, as described
324 in the Methods, and performed on a *S. flexneri* Δ*icsA* strain expressing either $IcsA_{WT}$ or
325 $IcsA_{i716}$. Tunicamycin was used at a concentration of 10 μg mL^{-1} (in DMSO) in conjunction

with 3 μg polymyxin B nonapeptide mL^{-1} (PMBN) to inhibit Oag biosynthesis. The subsequent removal of tunicamycin and PMBN resulted in regeneration of Oags. Tunicamycin is a nucleoside antibiotic that inhibits the WecA enzyme (N-acetylglucosamine-1-phosphate transferase) which is involved in the first step of Oag biosynthesis (Alexander & Valvano, 1994; Brandish *et al.*, 1996). PMBN is a non-toxic peptide derivative of polymyxin B which helps to permeabilise the OM of Gram-negative bacteria and facilitates the entry of hydrophobic tunicamycin into the bacterial cell (Vaara & Vaara, 1983; Vaara, 1992). LPS silver staining showed that Oag biosynthesis was significantly inhibited by approximately 90% after 3 h of treatment with tunicamycin/PMBN (Fig. 3a, lanes 2 and 7), while the Oag production was restored upon the removal of tunicamycin/PMBN (Fig. 3a, lanes 3 and 8). Oag biosynthesis in strains treated with DMSO only (negative control) remained unchanged (Fig. 3a, lanes 4-5 and 9-10, respectively).

Western immunoblotting showed that $IcsA_{WT}$ protein was produced regardless of the tunicamycin/PMBN treatment (Fig. 3b, lanes 1-5). In contrast, while $IcsA_{i716}$ mutant production was hardly detectable when the strain was untreated (Fig. 3b, lane 6) or treated with DMSO (Fig. 3b, lanes 9-10), a full length 120 kDa $IcsA_{i716}$ band was readily detected when the LPS Oag biosynthesis was inhibited (Fig. 3b, lane 7). As expected, the $IcsA_{i716}$ band was no longer detectable when the LPS Oags were regenerated (Fig. 3b, lane 8). Overall, these results indicate that LPS Oags affect $IcsA_i$ mutant production in the S-LPS *S. flexneri*. The potential mechanism(s) involved were investigated as described below.

Effect of IcsP on $IcsA_i$ expression in S-LPS and R-LPS *S. flexneri*

The locations of the 5 aa linker-insertion mutations in the IcsA AC region are in close proximity to the IcsP cleavage site (aa 758-759). Therefore, we hypothesised that the IcsP

cleavage site of IcsA$_i$ may be altered and/or became more accessible to IcsP which may cleave IcsA$_i$ more readily in the S-LPS background. To test our hypothesis, IcsA$_{i716}$ was selected and the amount of IcsA' protein (cleaved form) in the culture supernatants of both S-LPS and R-LPS backgrounds was investigated. The cleaved form of IcsA$_{i716}$ was not detected in the S-LPS background (Fig. 4a, lane 6) but was observed in the R-LPS background (Fig. 4a, lane 8), at a similar level to that seen for IcsA$_{WT}$ protein in both backgrounds (Fig. 4a, lanes 2 and 4).

We also investigated the effect of the presence and absence of IcsP in virulence plasmid-cured (VP^{-ve}) S-LPS and R-LPS backgrounds. Results consistent with the above were obtained when various IcsA$_i$ proteins were expressed in VP^{-ve} S-LPS and VP^{-ve} R-LPS *S. flexneri* strain carrying pBAD33::*icsP*. IcsA$_i$ production in *S. flexneri* VP$^-$ S-LPS remained extremely low and was not restored to IcsA$_{WT}$ levels despite the absence of IcsP (Fig. 4b). In contrast, *S. flexneri* VP$^-$ R-LPS background showed production of IcsA$_i$ proteins at WT level (except IcsA$_{i634}$ which showed slightly lower levels of protein production than IcsA$_{WT}$) when IcsP was absent (Fig. 4c). Interestingly, the IcsA$_i$ proteins appeared to be more sensitive to IcsP cleavage than IcsA$_{WT}$ as a reduction in the full length IcsA$_i$ mutant proteins (120 kDa) was observed when IcsP was induced (Fig. 4c, lanes 4, 6, 8, and 10). The IcsA$_{i643}$ mutant in particular, appeared to be more sensitive to IcsP cleavage compared to the other IcsA$_i$ proteins (Fig. 4c, lane 6). The levels of the cleaved form of IcsA$_i$' (85 kDa) that remained associated with bacterial cells were comparable to IcsA'$_{WT}$ (Fig. 4c). These data suggest that IcsP does not have a major role in reducing IcsA$_i$ production in the S-LPS background and most IcsA$_i$ proteins are more sensitive to IcsP cleavage than IcsA$_{WT}$, possibly due to a change in protein conformation.

icsA promoter activity

To determine if the *icsA* promoter activity is upregulated in the R-LPS background and contributing to the high $IcsA_i$ mutant expression level, we created a transcriptional reporter plasmid pMYRM718 [P*icsA*-TGA-*lacZ*], encoding an *icsA* promoter fusion with *lacZ* in pSU23, as described in the Methods. Both S-LPS and R-LPS *S. flexneri* strains carrying either $pIcsA_{WT}$ or $pIcsA_{i716}$ were transformed with pMYRM718, to give strains MYRM734 (S-LPS + $pIcsA_{WT}$ + P*icsA*-TGA-*lacZ*), MYRM721 (R-LPS + $pIcsA_{WT}$ + P*icsA*-TGA-*lacZ*), MYRM722 (S-LPS + $pIcsA_{i716}$ + P*icsA*-TGA-*lacZ*) and MYRM723 (R-LPS + $pIcsA_{i716}$ + P*icsA*-TGA-*lacZ*), respectively. Each strain was grown to mid-exponential phase and β-galactosidase levels were assayed. LacZ activity was measured in the presence of either $IcsA_{WT}$ or $IcsA_{i716}$ because the presence of a misfolded protein might create a feedback loop to activate *icsA* promoter activity or gene expression. The data show that there was no significant difference in *icsA* promoter activity between MYRM734 and MYRM721 (in the presence of $IcsA_{WT}$), while the *icsA* promoter activity was slightly higher in MYRM722 than MYRM723 (in the presence of $IcsA_{i716}$) (*$P < 0.5$) (Fig. 5). The data suggest that the very low $IcsA_i$ protein production in the S-LPS background is unlikely due to an effect of transcriptional level, but may be due to a post-transcriptional effect, such as increased proteolysis.

$IcsA_{i716}$ mutant production in a S. flexneri degP::Cm ΔicsA S-LPS strain

Since the OM protease, IcsP, did not appear to cause the low $IcsA_i$ mutant production in S-LPS *S. flexneri,* we hypothesised that the translocation of $IcsA_i$-periplasmic intermediate across the OM is retarded in such a way that prolonged exposure to periplasmic proteases, such as DegP, degrades the $IcsA_i$-periplasmic intermediate. DegP is a temperature regulated bifunctional periplasmic protein that may act as a chaperone or a protease at 37 °C but as a chaperone at low temperature (Spiess *et al.*, 1999). A S-LPS *S.*

401 *flexneri degP*::Cm Δ*icsA* mutant strain was created as described in the Methods and the
402 strain was subsequently complemented with pIcsA$_{WT}$ or pIcsA$_{i716}$. The resultant strains
403 expressing either IcsA$_{WT}$ or IcsA$_{i716}$ were grown to mid-exponential phase at 30°C or 37°C
404 and the production of IcsA was assessed by Western immunoblotting. Protein production
405 of IcsA$_{i716}$ was partly restored in the Δ*degP* background at both 30°C and 37°C (Fig. 6,
406 lanes 5 and 6), in comparison to IcsA$_{WT}$ (Fig. 6, lanes 3 and 4). The data suggest that in
407 the presence of LPS Oag, DegP protease is largely responsible for degrading the IcsA$_i$
408 mutant protein.

409

410 *Expression of DegP, Skp and SurA periplasmic chaperones in S-LPS and R-LPS S.*
411 *flexneri*

412 The expression of periplasmic chaperones such as DegP, Skp and SurA are regulated by
413 the σ^E envelope stress response or the Cpx two-component signal transduction system
414 (Duguay & Silhavy, 2004; Raivio & Silhavy, 2001; Rhodius *et al.*, 2006). In comparison to
415 a *S. flexneri* Δ*icsA* S-LPS strain, the *S. flexneri* Δ*icsA* Δ*rmlD* R-LPS strain (which has the
416 *rmlD* gene deleted) might be under stress and hence, may have upregulated periplasmic
417 chaperone expression. Therefore, the expression level of DegP, Skp and SurA in both S-
418 LPS and R-LPS *S. flexneri* (in the presence or absence of IcsA$_{WT}$ or IcsA$_{i716}$), were
419 examined. The production of DegP, Skp and SurA was not upregulated in the R-LPS
420 background (Fig. S2) even in the presence of IcsA$_{i716}$ protein, indicating a lack of cell
421 envelope stress response. However, this does not exclude that their chaperone activity is
422 not being upregulated in the R-LPS strain. Likewise, DegP proteolysis activity in the S-LPS
423 background may be upregulated in the presence of IcsA$_i$ mutant protein.

424

425 *Effect of AC region insertion mutations on IcsA$_i$ functionality in S. flexneri* Δ*icsA* Δ*rmlD*

426 The N-WASP recruitment ability of various IcsA$_i$ AC mutants expressed by R-LPS *S.*
427 *flexneri* was investigated to determine if the proteins retained their function. R-LPS *S.*
428 *flexneri* expressing IcsA$_{WT}$ or various IcsA$_i$ were used to infect HeLa cells and N-WASP
429 recruitment was detected as described in the Methods. Except the IcsA$_{i716}$ mutant (Fig. 7f),
430 IcsA$_{i633}$, IcsA$_{i643}$ and IcsA$_{i677}$ (Fig. 7d) (Data for IcsA$_{i633}$, IcsA$_{i643}$ not shown) mutants
431 recruited N-WASP and formed F-actin comet tails within HeLa cells, suggesting that
432 IcsA$_{i633}$, IcsA$_{i643}$ and IcsA$_{i677}$ have WT protein conformation and are functional. In contrast,
433 despite the high level of IcsA$_{i716}$ protein detected on the bacterial surface of LB grown cells
434 (Fig. 1b), neither N-WASP nor F-actin comet tails were detected (Fig. 7f), suggesting that
435 an N-WASP binding/interacting site could be located at aa 716-717. Alternatively, IcsA$_{i716}$
436 might be highly misfolded, thus, masking the N-WASP binding/interacting sites and
437 inhibiting F-actin comet tail formation. Data for S-LPS *S. flexneri* expressing IcsA$_{WT}$,
438 IcsA$_{i677}$, and IcsA$_{i716}$ are shown for comparison (Fig. 7a, c and e).

439

440 *Potential effect of insertion mutations on IcsA$_i$ protein folding*

441 As the 5 aa linker-insertion mutations might result in altered IcsA$_i$ protein conformations
442 and affect N-WASP recruitment ability, the protein conformation of IcsA$_i$ was investigated.
443 *S. flexneri* Δ*icsA* Δ*rmlD* expressing either IcsA$_{WT}$ or IcsA$_i$ were subjected to 1 µg trypsin
444 mL^{-1} over a period of time (0 min, 5 min, 15 min and 20 min). A misfolded protein could be
445 more sensitive to trypsin degradation, giving a different proteolysis profile than IcsA$_{WT}$ in
446 Western immunoblotting. The results of limited proteolysis assay (Fig. 8) suggest that all
447 tested IcsA$_i$ mutants do not have major difference to the IcsA$_{WT}$ profile. In spite of the
448 similar proteolysis profiles, IcsA$_{i643}$ and IcsA$_{i716}$ seem to be more sensitive to trypsin
449 degradation, as the overall IcsA band profile intensity for these IcsA$_i$ mutants was
450 significantly reduced after 15 min of trypsin digestion (Fig. 8, lanes 11 and 19), whilst
451 IcsA$_{i633}$ and IcsA$_{i677}$ had a WT proteolysis profile (Fig. 8, lanes 5-8 and 13-16,

452 respectively). The data suggest that the IcsA$_i$ mutants do not have major change in
453 conformation. Furthermore, our laboratory has recently shown that the *S. flexneri* IcsA
454 protein is able to self-associate and form a complex in the OM (May *et al.*, 2012). Thus,
455 DSP chemical cross-linking was performed to examine if the IcsA$_i$ mutants retain their self-
456 association ability. The results showed that all tested IcsA$_i$ mutants formed high molecular
457 weight proteins like IcsA$_{WT}$ (data not shown), indicating that the mutants possess WT
458 protein conformation, which is consistent with the results obtained from the trypsin
459 accessibility assay, and the ability to recruit N-WASP (except for IcsA$_{i716}$).

460

Discussion

The IcsA (VirG) protein of *S. flexneri* is a Type Va autotransporter protein (Henderson *et al.*, 2004) that allows *S. flexneri* to spread between the host intestinal epithelial cells (Bernardini *et al.*, 1989; Goldberg *et al.*, 1993; Goldberg, 2001; Lett *et al.*, 1989). IcsA hijacks the host actin regulatory protein, N-WASP, and forms F-actin comet tails at one pole of the bacterium that propel *Shigella* in the opposite direction (Bernardini *et al.*, 1989; Brandon *et al.*, 2003).

The translocation mechanism of various OMPs or ATs has been studied extensively in *E. coli* (Ieva *et al.*, 2011; Peterson *et al.*, 2010; Ruiz-Perez *et al.*, 2010; Soprova *et al.*, 2010; Walton *et al.*, 2009). However, the detailed mechanisms of IcsA biogenesis have not been fully elucidated. In this study, N-WASP recruitment ability and the role of the AC region in IcsA biogenesis was investigated in both S-LPS and R-LPS backgrounds, and differs markedly from other studies on AC regions in this regard.

The restoration of AC $IcsA_i$ mutant production in R-LPS *S. flexneri* allowed us to examine N-WASP recruitment ability, and $IcsA_{i716}$ which possesses 5 aa insertion at the Aro_{716}-X_{717}-Aro_{718} motif was identified as defective in N-WASP recruitment. This suggests that either residues 716-717 is an N-WASP binding/interaction site, or $IcsA_{i716}$ is severely misfolded and masked the N-WASP binding sites. Both $IcsA_{i643}$ and $IcsA_{i716}$ demonstrated a slightly increased sensitivity to trypsin degradation but had a proteolysis profile similar to the WT (Fig. 8). This could be due to the presence of arginine residue in the inserted 5 aa sequence (Table S2), which is a trypsin cleavage site. However, $IcsA_{i643}$ is able to recruit N-WASP despite the increased sensitivity to trypsin. This result implies that any alteration in protein conformation is limited. Thus, $IcsA_{i716}$ which has a similar proteolysis profile to $IcsA_{i643}$ is likely to also possess a near WT conformation. Moreover, high molecular $IcsA_i$ complexes were detected in DSP chemical cross-linking (data not shown), and suggests

that all $IcsA_i$ AC mutants are able to self-associate and have a conformation similar to WT. Taken together, all $IcsA_i$ AC mutants do not have major alteration in protein conformation. The data also suggest that an N-WASP binding/interacting site is located at residues 716-717. This has been confirmed by site-directed mutagenesis of aa 716-717, and functional complementation of an N-WASP interacting region II mutant IcsA protein (Teh and Morona, unpublished data).

Mutations within the IcsA AC region significantly affected $IcsA_i$ production in the *S. flexneri* S-LPS background (May & Morona, 2008) but not in the R-LPS background (Fig. 1). This finding was supported by *rmlD* complementation assay which generated the same phenotype (Fig. 2), as well as expression of various $IcsA_i$ mutants in the *E. coli* K-12 *ompT*⁻ UT5600 strain (with or without S-LPS) (Fig. S1). These data demonstrate that the presence of LPS Oag affects biogenesis of $IcsA_i$ proteins with mutations within the AC region, independent of *S. flexneri*-specific virulence determinants, as the $IcsA_i$ phenotype was reproduced in VP^{-ve} *S. flexneri* and in UT5600 strain backgrounds. In addition, we showed that the very low level of $IcsA_i$ production in the S-LPS background was not due to extensive IcsP cleavage. Therefore, to directly investigate the effect of LPS Oag on $IcsA_i$ mutant production, an LPS depletion-regeneration assay was undertaken. We demonstrated that the depletion of Oag restored $IcsA_i$ mutant production and the effect was reversible (Fig. 3). The data suggests that the presence of Oag affects IcsA biogenesis in an unknown mechanism and possibly plays an unidentified new role in the IcsA translocation system. LPS Oag has never been shown to interact with either the exported IcsA or IcsA-periplasmic intermediate.

Our findings show that the *icsA* promoter activity was not downregulated in S-LPS *S. flexneri* in the presence of $IcsA_{WT}$ or $IcsA_i$ AC mutant, indicating the differences in $IcsA_i$ production between the S-LPS and R-LPS backgrounds was not due to a general effect on

511 the transcription of AC IcsA$_i$. These data suggest that a post-transcriptional effect is
512 involved in affecting AC IcsA$_i$ production in the S-LPS strain background.

513 The presence of a transient soluble IcsA-periplasmic intermediate has previously been
514 reported (Brandon & Goldberg, 2001) and periplasmic chaperones such as DegP, Skp and
515 SurA are required for proper IcsA presentation (Purdy *et al.*, 2002; Purdy *et al.*, 2007).
516 DegP which is also a protease was reported to degrade unproductive or misfolded OMPs
517 in the periplasm (Kolmar *et al.*, 1996; Soprova *et al.*, 2010). As shown in Fig. 6, IcsA$_i$
518 protein production was restored by a Δ*degP* mutant in the S-LPS background, suggesting
519 that IcsA$_i$-periplasmic intermediate was sensitive to DegP degradation in the presence of
520 LPS Oag. In addition, we showed that the DegP expression level (as well as Skp and
521 SurA) remained the same between S-LPS and R-LPS backgrounds even in the presence
522 of IcsA$_i$ AC protein. It is possible that DegP could be less active as a protease in the R-
523 LPS background or vice versa in the S-LPS background. However, there are no reports of
524 DegP having LPS-dependent activity. Hence, it is unclear how S-LPS may be affecting
525 DegP and hence, IcsA$_i$ AC mutant protein production.

526 We speculate that an early interaction occurs between the Skp-IcsA$_i$ complex with
527 transiting S-LPS or R-LPS molecules in the periplasm, via the putative LPS binding site on
528 the outer surface of Skp (Korndorfer *et al.*, 2004; Walton & Sousa, 2004). This interaction
529 may promote dissociation of IcsA$_i$ from Skp due to a weak interaction caused by the IcsA$_i$
530 AC mutations. The released and unprotected IcsA$_i$ mutant might then be rapidly
531 aggregated and become degraded by DegP. On the other hand, binding of R-LPS
532 molecule to the Skp-IcsA$_i$ complex would not promote IcsA$_i$ dissociation; this could be a
533 function of Oag polysaccharide chains. Interestingly, Oag polysaccharides are a substrate
534 for bacteriophage tailspike protein (TSP) that have a right-handed parallel β-helix structure
535 (Steinbacher *et al.*, 1996) which is similar to the predicted IcsA structure (Kuhnel &
536 Diezmann, 2011), and that of other ATs (Emsley *et al.*, 1996; Johnson *et al.*, 2009; Khan

537 *et al.*, 2011; Otto *et al.*, 2005). Another possibility is that a third unknown factor which is
538 affected by Oag is involved in influencing $IcsA_i$ interaction with chaperones. Nonetheless,
539 this hypothetical model warrants further investigation, as interaction between Skp and LPS
540 has not been shown *in vivo*, and no data suggesting Skp binding to AC region of IcsA
541 have been reported.

542 Even though $IcsA_{i716}$ production was restored in the *S. flexneri* Δ*degP* strain, the degree of
543 $IcsA_{i716}$ restoration was not 100% (Fig. 6), and this could be due to the location of the
544 insertion mutation. Residue Y716 is part of the Aro-X-Aro motif which is a preferential SurA
545 binding site (Bitto & McKay, 2003; Xu *et al.*, 2007) and based on the IcsA AC crystal
546 structure, residue Y716 is predicted to be located at the beginning of the first anti-parallel
547 β-strand which appears to be exposed to the external medium (Fig. S3). Direct interaction
548 between IcsA and SurA has not been reported, and if residues 716-718 were a SurA
549 binding site, we speculate that the 5 aa insertion mutation might have altered this potential
550 SurA binding site and reduced the chaperone protection from SurA during the
551 translocation of IcsA passenger domain across the OM, hence, reducing $IcsA_{i716}$
552 production. Again, the assumption of interaction between SurA and IcsA AC region
553 requires further investigations. Alternatively, $IcsA_{i716}$ could be degraded by other
554 periplasmic protease such as DegQ. In comparison, triple Aro-X-Aro motif mutations in the
555 *E. coli* AT EspP passenger domain were required to observe a reduction in the EspP
556 secretion (Ruiz-Perez *et al.*, 2009), suggesting that the effect of Aro-X-Aro motif in IcsA is
557 more significant than in EspP.

558 We have demonstrated for the first time that the presence of LPS Oag affects protein
559 production of IcsA. Nevertheless, LPS Oag does not affect $IcsA_{WT}$ biogenesis but affects
560 biogenesis of IcsA that has mutational alteration in the AC region, which is a novel finding
561 that has never been reported before in IcsA or other autotransporters. As suggested
562 above, we speculate that LPS, Skp and SurA interaction with IcsA may be altered by the

AC region mutations, and this remains to be investigated. Collectively, our data suggest an alternative role for the AC domain and that it affects interactions with chaperones during export. Our findings provide new insights into IcsA biogenesis. In addition, we have identified a new N-WASP binding/interaction site within the AC region which narrows down the previously reported N-WASP interacting region (aa 508-730) (May & Morona, 2008) and this is the subject of further study (Teh and Morona, unpublished data).

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References

Alexander, D. C. & Valvano, M. A. (1994). Role of the *rfe* gene in the biosynthesis of the *Escherichia coli* O7-specific lipopolysaccharide and other O-specific polysaccharides containing N-acetylglucosamine. *J Bacteriol* **176**, 7079-7084.

Baker, S. J., Gunn, J. S. & Morona, R. (1999). The *Salmonella typhi* melittin resistance gene *pqaB* affects intracellular growth in PMA-differentiated U937 cells, polymyxin B resistance and lipopolysaccharide. *Microbiology* **145 (Pt 2)**, 367-378.

Bartolome, B., Jubete, Y., Martinez, E. & de la Cruz, F. (1991). Construction and properties of a family of pACYC184-derived cloning vectors compatible with pBR322 and its derivatives. *Gene* **102**, 75-78.

Bernardini, M. L., Mounier, J., d'Hauteville, H., Coquis-Rondon, M. & Sansonetti, P. J. (1989). Identification of *icsA*, a plasmid locus of *Shigella flexneri* that governs bacterial intra- and intercellular spread through interaction with F-actin. *Proc Natl Acad Sci U S A* **86**, 3867-3871.

Bitto, E. & McKay, D. B. (2003). The periplasmic molecular chaperone protein SurA binds a peptide motif that is characteristic of integral outer membrane proteins. *J Biol Chem* **278**, 49316-49322.

Bolivar, F., Rodriguez, R. L., Betlach, M. C. & Boyer, H. W. (1977). Construction and characterization of new cloning vehicles. I. Ampicillin-resistant derivatives of the plasmid pMB9. *Gene* **2**, 75-93.

Brandish, P. E., Kimura, K. I., Inukai, M., Southgate, R., Lonsdale, J. T. & Bugg, T. D. (1996). Modes of action of tunicamycin, liposidomycin B, and mureidomycin A: inhibition of phospho-N-acetylmuramyl-pentapeptide translocase from *Escherichia coli*. *Antimicrob Agents Chemother* **40**, 1640-1644.

Brandon, L. D. & Goldberg, M. B. (2001). Periplasmic transit and disulfide bond formation of the autotransported *Shigella* protein IcsA. *J Bacteriol* **183**, 951-958.

Brandon, L. D., Goehring, N., Janakiraman, A., Yan, A. W., Wu, T., Beckwith, J. & Goldberg, M. B. (2003). IcsA, a polarly localized autotransporter with an atypical signal peptide, uses the Sec apparatus for secretion, although the Sec apparatus is circumferentially distributed. *Mol Microbiol* **50**, 45-60.

d'Hauteville, H., Dufourcq Lagelouse, R., Nato, F. & Sansonetti, P. J. (1996). Lack of cleavage of IcsA in *Shigella flexneri* causes aberrant movement and allows demonstration of a cross-reactive eukaryotic protein. *Infect Immun* **64**, 511-517.

Davison, J., Heusterspreute, M., Chevalier, N., Ha-Thi, V. & Brunel, F. (1987). Vectors with restriction site banks. V. pJRD215, a wide-host-range cosmid vector with multiple cloning sites. *Gene* **51**, 275-280.

Duguay, A. R. & Silhavy, T. J. (2004). Quality control in the bacterial periplasm. *Biochim Biophys Acta* **1694**, 121-134.

Emsley, P., Charles, I. G., Fairweather, N. F. & Isaacs, N. W. (1996). Structure of *Bordetella pertussis* virulence factor P.69 pertactin. *Nature* **381**, 90-92.

Fukuda, I., Suzuki, T., Munakata, H., Hayashi, N., Katayama, E., Yoshikawa, M. & Sasakawa, C. (1995). Cleavage of *Shigella* surface protein VirG occurs at a specific site, but the secretion is not essential for intracellular spreading. *J Bacteriol* **177**, 1719-1726.

Goldberg, M. B., Barzu, O., Parsot, C. & Sansonetti, P. J. (1993). Unipolar localization and ATPase activity of IcsA, a *Shigella flexneri* protein involved in intracellular movement. *Infect Agents Dis* **2**, 210-211.

Goldberg, M. B. (2001). Actin-based motility of intracellular microbial pathogens. *Microbiol Mol Biol Rev* **65**, 595-626, table of contents.

Guzman, L. M., Belin, D., Carson, M. J. & Beckwith, J. (1995). Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* **177**, 4121-4130.

Henderson, I. R., Navarro-Garcia, F., Desvaux, M., Fernandez, R. C. & Ala'Aldeen, D. (2004). Type V protein secretion pathway: the autotransporter story. *Microbiol Mol Biol Rev* **68**, 692-744.

Ieva, R. & Bernstein, H. D. (2009). Interaction of an autotransporter passenger domain with BamA during its translocation across the bacterial outer membrane. *Proc Natl Acad Sci U S A* **106**, 19120-19125.

Ieva, R., Tian, P., Peterson, J. H. & Bernstein, H. D. (2011). Sequential and spatially restricted interactions of assembly factors with an autotransporter beta domain. *Proc Natl Acad Sci U S A* **108**, E383-391.

Jain, S. & Goldberg, M. B. (2007). Requirement for YaeT in the outer membrane assembly of autotransporter proteins. *J Bacteriol* **189**, 5393-5398.

Johnson, T. A., Qiu, J., Plaut, A. G. & Holyoak, T. (2009). Active-site gating regulates substrate selectivity in a chymotrypsin-like serine protease the structure of *Haemophilus influenzae* immunoglobulin A1 protease. *J Mol Biol* **389**, 559-574.

Khan, S., Mian, H. S., Sandercock, L. E., Chirgadze, N. Y. & Pai, E. F. (2011). Crystal structure of the passenger domain of the *Escherichia coli* autotransporter EspP. *J Mol Biol* **413**, 985-1000.

Klemm, P., Vejborg, R. M. & Sherlock, O. (2006). Self-associating autotransporters, SAATs: functional and structural similarities. *Int J Med Microbiol* **296**, 187-195.

Kokotek, W. & Lotz, W. (1989). Construction of a lacZ-kanamycin-resistance cassette, useful for site-directed mutagenesis and as a promoter probe. *Gene* **84**, 467-471.

Kolmar, H., Waller, P. R. & Sauer, R. T. (1996). The DegP and DegQ periplasmic endoproteases of *Escherichia coli*: specificity for cleavage sites and substrate conformation. *J Bacteriol* **178**, 5925-5929.

Korndorfer, I. P., Dommel, M. K. & Skerra, A. (2004). Structure of the periplasmic chaperone Skp suggests functional similarity with cytosolic chaperones despite differing architecture. *Nat Struct Mol Biol* **11**, 1015-1020.

Kuhnel, K. & Diezmann, D. (2011). Crystal structure of the autochaperone region from the *Shigella flexneri* autotransporter IcsA. *J Bacteriol* **193**, 2042-2045.

Lett, M. C., Sasakawa, C., Okada, N., Sakai, T., Makino, S., Yamada, M., Komatsu, K. & Yoshikawa, M. (1989). *virG*, a plasmid-coded virulence gene of *Shigella flexneri*: identification of the virG protein and determination of the complete coding sequence. *J Bacteriol* **171**, 353-359.

Lugtenberg, B., Meijers, J., Peters, R., van der Hoek, P. & van Alphen, L. (1975). Electrophoretic resolution of the "major outer membrane protein" of *Escherichia coli* K12 into four bands. *FEBS Lett* **58**, 254-258.

Makino, S., Sasakawa, C., Kamata, K., Kurata, T. & Yoshikawa, M. (1986). A genetic determinant required for continuous reinfection of adjacent cells on large plasmid in *S. flexneri* 2a. *Cell* **46**, 551-555.

May, K. L. & Morona, R. (2008). Mutagenesis of the *Shigella flexneri* autotransporter IcsA reveals novel functional regions involved in IcsA biogenesis and recruitment of host neural Wiscott-Aldrich syndrome protein. *J Bacteriol* **190**, 4666-4676.

May, K. L., Grabowicz, M., Polyak, S. W. & Morona, R. (2012). Self-association of the *Shigella flexneri* IcsA autotransporter protein. *Microbiology* **158**, 1874-1883.

Meng, G., Spahich, N., Kenjale, R., Waksman, G. & St Geme, J. W., 3rd (2011). Crystal structure of the *Haemophilus influenzae* Hap adhesin reveals an intercellular oligomerization mechanism for bacterial aggregation. *EMBO J* **30**, 3864-3874.

Miller, J. (1972). Experiments in molecular genetics. *Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY*.

Morona, R., Mavris, M., Fallarino, A. & Manning, P. A. (1994). Characterization of the rfc region of *Shigella flexneri*. *J Bacteriol* **176**, 733-747.

Morona, R., van den Bosch, L. & Manning, P. A. (1995). Molecular, genetic, and topological characterization of O-antigen chain length regulation in *Shigella flexneri*. *J Bacteriol* **177**, 1059-1068.

Morona, R., Daniels, C. & Van Den Bosch, L. (2003). Genetic modulation of *Shigella flexneri* 2a lipopolysaccharide O antigen modal chain length reveals that it has been optimized for virulence. *Microbiology* **149**, 925-939.

Morona, R. & Van Den Bosch, L. (2003). Lipopolysaccharide O antigen chains mask IcsA (VirG) in *Shigella flexneri*. *FEMS Microbiol Lett* **221**, 173-180.

Murray, G. L., Attridge, S. R. & Morona, R. (2003). Regulation of *Salmonella typhimurium* lipopolysaccharide O antigen chain length is required for virulence; identification of FepE as a second Wzz. *Mol Microbiol* **47**, 1395-1406.

Mutalik, V. K., Nonaka, G., Ades, S. E., Rhodius, V. A. & Gross, C. A. (2009). Promoter strength properties of the complete sigma E regulon of *Escherichia coli* and *Salmonella enterica*. *J Bacteriol* **191**, 7279-7287.

Narita, S. & Tokuda, H. (2009). Biochemical characterization of an ABC transporter LptBFGC complex required for the outer membrane sorting of lipopolysaccharides. *FEBS Lett* **583**, 2160-2164.

Oliver, D. C., Huang, G. & Fernandez, R. C. (2003a). Identification of secretion determinants of the *Bordetella pertussis* BrkA autotransporter. *J Bacteriol* **185**, 489-495.

Oliver, D. C., Huang, G., Nodel, E., Pleasance, S. & Fernandez, R. C. (2003b). A conserved region within the *Bordetella pertussis* autotransporter BrkA is necessary for folding of its passenger domain. *Mol Microbiol* **47**, 1367-1383.

Otto, B. R., Sijbrandi, R., Luirink, J., Oudega, B., Heddle, J. G., Mizutani, K., Park, S. Y. & Tame, J. R. (2005). Crystal structure of hemoglobin protease, a heme binding autotransporter protein from pathogenic *Escherichia coli*. *J Biol Chem* **280**, 17339-17345.

Pallen, M. J., Chaudhuri, R. R. & Henderson, I. R. (2003). Genomic analysis of secretion systems. *Curr Opin Microbiol* **6**, 519-527.

Papadopoulos, M. & Morona, R. (2010). Mutagenesis and chemical cross-linking suggest that Wzz dimer stability and oligomerization affect lipopolysaccharide O-antigen modal chain length control. *J Bacteriol* **192**, 3385-3393.

Peterson, J. H., Tian, P., Ieva, R., Dautin, N. & Bernstein, H. D. (2010). Secretion of a bacterial virulence factor is driven by the folding of a C-terminal segment. *Proc Natl Acad Sci U S A* **107**, 17739-17744.

Philpott, D. J., Edgeworth, J. D. & Sansonetti, P. J. (2000). The pathogenesis of *Shigella flexneri* infection: lessons from in vitro and in vivo studies. *Philos Trans R Soc Lond B Biol Sci* **355**, 575-586.

Purdy, G. E., Hong, M. & Payne, S. M. (2002). *Shigella flexneri* DegP facilitates IcsA surface expression and is required for efficient intercellular spread. *Infect Immun* **70**, 6355-6364.

Purdy, G. E., Fisher, C. R. & Payne, S. M. (2007). IcsA surface presentation in *Shigella flexneri* requires the periplasmic chaperones DegP, Skp, and SurA. *J Bacteriol* **189**, 5566-5573.

Purins, L., Van Den Bosch, L., Richardson, V. & Morona, R. (2008). Coiled-coil regions play a role in the function of the *Shigella flexneri* O-antigen chain length regulator WzzpHS2. *Microbiology* **154**, 1104-1116.

Raetz, C. R. & Whitfield, C. (2002). Lipopolysaccharide endotoxins. *Annu Rev Biochem* **71**, 635-700.

Raivio, T. L. & Silhavy, T. J. (2001). Periplasmic stress and ECF sigma factors. *Annu Rev Microbiol* **55**, 591-624.

Reeves, P. R., Hobbs, M., Valvano, M. A. & other authors (1996). Bacterial polysaccharide synthesis and gene nomenclature. *Trends Microbiol* **4**, 495-503.

Rhodius, V. A., Suh, W. C., Nonaka, G., West, J. & Gross, C. A. (2006). Conserved and variable functions of the sigmaE stress response in related genomes. *PLoS Biol* **4**, e2.

Robbins, J. R., Monack, D., McCallum, S. J., Vegas, A., Pham, E., Goldberg, M. B. & Theriot, J. A. (2001). The making of a gradient: IcsA (VirG) polarity in *Shigella flexneri*. *Mol Microbiol* **41**, 861-872.

Ruiz-Perez, F., Henderson, I. R., Leyton, D. L., Rossiter, A. E., Zhang, Y. & Nataro, J. P. (2009). Roles of periplasmic chaperone proteins in the biogenesis of serine protease autotransporters of *Enterobacteriaceae*. *J Bacteriol* **191**, 6571-6583.

Ruiz-Perez, F., Henderson, I. R. & Nataro, J. P. (2010). Interaction of FkpA, a peptidyl-prolyl cis/trans isomerase with EspP autotransporter protein. *Gut Microbes* **1**, 339-344.

Ruiz, N., Gronenberg, L. S., Kahne, D. & Silhavy, T. J. (2008). Identification of two inner-membrane proteins required for the transport of lipopolysaccharide to the outer membrane of *Escherichia coli*. *Proc Natl Acad Sci U S A* **105**, 5537-5542.

Ruiz, N., Kahne, D. & Silhavy, T. J. (2009). Transport of lipopolysaccharide across the cell envelope: the long road of discovery. *Nat Rev Microbiol* **7**, 677-683.

Sandlin, R. C., Lampel, K. A., Keasler, S. P., Goldberg, M. B., Stolzer, A. L. & Maurelli, A. T. (1995). Avirulence of rough mutants of *Shigella flexneri*: requirement of O antigen for correct unipolar localization of IcsA in the bacterial outer membrane. *Infect Immun* **63**, 229-237.

Sansonetti, P. J., Arondel, J., Fontaine, A., d'Hauteville, H. & Bernardini, M. L. (1991). OmpB (osmo-regulation) and *icsA* (cell-to-cell spread) mutants of *Shigella flexneri*: vaccine candidates and probes to study the pathogenesis of shigellosis. *Vaccine* **9**, 416-422.

Sauri, A., Soprova, Z., Wickstrom, D., de Gier, J. W., Van der Schors, R. C., Smit, A. B., Jong, W. S. & Luirink, J. (2009). The Bam (Omp85) complex is involved in secretion of the autotransporter haemoglobin protease. *Microbiology* **155**, 3982-3991.

Sklar, J. G., Wu, T., Kahne, D. & Silhavy, T. J. (2007). Defining the roles of the periplasmic chaperones SurA, Skp, and DegP in *Escherichia coli*. *Genes Dev* **21**, 2473-2484.

Soprova, Z., Sauri, A., van Ulsen, P., Tame, J. R., den Blaauwen, T., Jong, W. S. & Luirink, J. (2010). A conserved aromatic residue in the autochaperone domain of the autotransporter Hbp is critical for initiation of outer membrane translocation. *J Biol Chem* **285**, 38224-38233.

Sperandeo, P., Lau, F. K., Carpentieri, A., De Castro, C., Molinaro, A., Deho, G., Silhavy, T. J. & Polissi, A. (2008). Functional analysis of the protein machinery required for transport of lipopolysaccharide to the outer membrane of *Escherichia coli*. *J Bacteriol* **190**, 4460-4469.

Spiess, C., Beil, A. & Ehrmann, M. (1999). A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. *Cell* **97**, 339-347.

Steinbacher, S., Baxa, U., Miller, S., Weintraub, A., Seckler, R. & Huber, R. (1996). Crystal structure of phage P22 tailspike protein complexed with *Salmonella* sp. O-antigen receptors. *Proc Natl Acad Sci U S A* **93**, 10584-10588.

Steinhauer, J., Agha, R., Pham, T., Varga, A. W. & Goldberg, M. B. (1999). The unipolar *Shigella* surface protein IcsA is targeted directly to the bacterial old pole: IcsP cleavage of IcsA occurs over the entire bacterial surface. *Mol Microbiol* **32**, 367-377.

Suzuki, T., Lett, M. C. & Sasakawa, C. (1995). Extracellular transport of VirG protein in *Shigella*. *J Biol Chem* **270**, 30874-30880.

Suzuki, T. & Sasakawa, C. (2001). Molecular basis of the intracellular spreading of *Shigella*. *Infect Immun* **69**, 5959-5966.

Tran, A. X., Dong, C. & Whitfield, C. (2010). Structure and functional analysis of LptC, a conserved membrane protein involved in the lipopolysaccharide export pathway in *Escherichia coli*. *J Biol Chem* **285**, 33529-33539.

Tsai, C. M. & Frasch, C. E. (1982). A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal Biochem* **119**, 115-119.

Vaara, M. & Vaara, T. (1983). Sensitization of Gram-negative bacteria to antibiotics and complement by a nontoxic oligopeptide. *Nature* **303**, 526-528.

Vaara, M. (1992). Agents that increase the permeability of the outer membrane. *Microbiol Rev* **56**, 395-411.

Van Den Bosch, L., Manning, P. A. & Morona, R. (1997). Regulation of O-antigen chain length is required for *Shigella flexneri* virulence. *Mol Microbiol* **23**, 765-775.

Van Den Bosch, L. & Morona, R. (2003). The actin-based motility defect of a *Shigella flexneri rmlD* rough LPS mutant is not due to loss of IcsA polarity. *Microb Pathog* **35**, 11-18.

Wagner, J. K., Heindl, J. E., Gray, A. N., Jain, S. & Goldberg, M. B. (2009). Contribution of the periplasmic chaperone Skp to efficient presentation of the autotransporter IcsA on the surface of *Shigella flexneri*. *J Bacteriol* **191**, 815-821.

Walton, T. A. & Sousa, M. C. (2004). Crystal structure of Skp, a prefoldin-like chaperone that protects soluble and membrane proteins from aggregation. *Mol Cell* **15**, 367-374.

Walton, T. A., Sandoval, C. M., Fowler, C. A., Pardi, A. & Sousa, M. C. (2009). The cavity-chaperone Skp protects its substrate from aggregation but allows independent folding of substrate domains. *Proc Natl Acad Sci U S A* **106**, 1772-1777.

Wells, T. J., Totsika, M. & Schembri, M. A. (2010). Autotransporters of *Escherichia coli*: a sequence-based characterization. *Microbiology* **156**, 2459-2469.

Xu, X., Wang, S., Hu, Y. X. & McKay, D. B. (2007). The periplasmic bacterial molecular chaperone SurA adapts its structure to bind peptides in different conformations to assert a sequence preference for aromatic residues. *J Mol Biol* **373**, 367-381.

Yen, Y. T., Kostakioti, M., Henderson, I. R. & Stathopoulos, C. (2008). Common themes and variations in serine protease autotransporters. *Trends Microbiol* **16**, 370-379.

Zalucki, Y. M. & Jennings, M. P. (2007). Experimental confirmation of a key role for non-optimal codons in protein export. *Biochem Biophys Res Commun* **355**, 143-148.

887 **Table 1: Bacterial strains and plasmids**

Strain or plasmid	Relevant characteristics [#]	Reference or source
***E. coli* K-12**		
DH5α	*F-(80dlacZ M15) (lacZYA-argF) U169 hsdR17(r-m+) recA1 endA1 relA1 deoR*	Gibco-BRL
DH5	*F-, rec A1, end A1, hod R17 (rk-, mk+), sup E44, lambda -, thi 1, gyrA, rel A1*	Laboratory collection
M15	Nal^s Str^s Rif^s *thi⁻ lac⁻ ara⁺ gal⁺ mtl⁻ F⁻ recA⁺ uvr⁺ lon⁺*	Qiagen
S-17	Conjugative strain	Laboratory collection
RMA2802	S-17-1 λ pir [pCVD442 + *degP*::Cm]	(Purins *et al.*, 2008)
MC4100	*F-araD139 A(argF-lac)U169 rpsL150 deoCI relAI thiA ptsF25 flbB5301*	(Zalucki & Jennings, 2007)
JGS190	MC4100 *ara*$^+$ Δ*skp zae*::*Tn*10	(Sklar *et al.*, 2007)
MG1655	K-12 (MG1655) *rph*-1	(Mutalik *et al.*, 2009)
CAG41291	MG1655 *surA*::Km^R	Carol Gross
S. flexneri		
2457T	*S. flexneri* 2a wild type	Laboratory collection
RMA2041	2457T Δ*icsA*::Tc^R	(Van Den Bosch & Morona, 2003)
RMA2090	RMA2041 [pIcsA]	(Van Den Bosch & Morona, 2003)
RMA2043	RMA2041 Δ*rmlD*::Km^R	(Van Den Bosch & Morona, 2003)
RMA2107	RMA2043 [pIcsA]	(Van Den Bosch & Morona, 2003)
RMA2159	Virulence plasmid-cured 2457T	Laboratory collection
RMA2161	RMA2159 *rfbD*::Km^R	Laboratory collection
KMRM109	RMA2041 [pIcsA$_{i633}$]	(May & Morona, 2008)
KMRM114	RMA2041 [pIcsA$_{i643}$]	(May & Morona, 2008)
KMRM134	RMA2041 [pIcsA$_{i677}$]	(May & Morona, 2008)
KMRM147	RMA2041 [pIcsA$_{i716}$]	(May & Morona, 2008)
MYRM275	RMA2043 [pIcsA$_{i643}$]	This study
MYRM276	RMA2043 [pIcsA$_{i677}$]	This study
MYRM277	RMA2043 [pIcsA$_{i716}$]	This study
MYRM289	RMA2043 [pIcsA$_{i633}$]	This study
MYRM293	RMA2041 [pBR322]	This study
MYRM294	RMA2043 [pBR322]	This study
MYRM296	RMA2159 [pBAD33::*icsP*]	This study
MYRM297	RMA2161 [pBAD33::*icsP*]	This study
MYRM298	MYRM296 [pIcsA]	This study
MYRM299	MYRM297 [pIcsA]	This study
MYRM303	MYRM296 [pIcsA$_{i633}$]	This study
MYRM304	MYRM296 [pIcsA$_{i643}$]	This study
MYRM305	MYRM296 [pIcsA$_{i677}$]	This study
MYRM306	MYRM296 [pIcsA$_{i716}$]	This study
MYRM310	MYRM297 [pIcsA$_{i633}$]	This study
MYRM311	MYRM297 [pIcsA$_{i643}$]	This study
MYRM312	MYRM297 [pIcsA$_{i677}$]	This study
MYRM313	MYRM297 [pIcsA$_{i716}$]	This study
MYRM373	UT5600 [pJRD215 + pIcsA]	This study
MYRM374	UT5600 [pJRD215 + pBR322]	This study
MYRM378	UT5600 [pJRD215 + pIcsA$_{I633}$]	This study
MYRM379	UT5600 [pJRD215 + pIcsA$_{I643}$]	This study

MYRM380	UT5600 [pJRD215 + pIcsA$_{I677}$]	This study
MYRM381	UT5600 [pJRD215 + pIcsA$_{I716}$]	This study
MYRM578	UT5600 [pRMA154 + pIcsA]	This study
MYRM383	UT5600 [pRMA154 + pBR322]	This study
MYRM387	UT5600 [pRMA154 + pIcsA$_{I633}$]	This study
MYRM388	UT5600 [pRMA154 + pIcsA$_{I643}$]	This study
MYRM389	UT5600 [pRMA154 + pIcsA$_{I677}$]	This study
MYRM390	UT5600 [pRMA154 + pIcsA$_{I716}$]	This study
MYRM522	RMA2041 *degP*::Cm^R	This study
MYRM675	MYRM522 [pIcsA]	This study
MYRM525	MYRM522 [pIcsA$_{i716}$]	This study
MYRM526	MYRM522 [pBR322]	This study
MYRM734	RMA2090 [pMYRM718]	This study
MYRM721	RMA2107 [pMYRM718]	This study
MYRM722	KMRM147 [pMYRM718]	This study
MYRM723	MYRM277 [pMYRM718]	This study
MYRM724	RMA2107 [pACYC184]	This study
MYRM725	MYRM289 [pACYC184]	This study
MYRM726	MYRM275 [pACYC184]	This study
MYRM727	MYRM276 [pACYC184]	This study
MYRM728	MYRM277 [pACYC184]	This study
MYRM729	RMA2107 [pRMA727]	This study
MYRM730	MYRM289 [pRMA727]	This study
MYRM731	MYRM275 [pRMA727]	This study
MYRM732	MYRM276 [pRMA727]	This study
MYRM733	MYRM277 [pRMA727]	This study
Plasmids		
pACYC184	Low copy number; P15A *ori*; Tc^R, Cm^R	NEB
pBAD33	Arabinose-inducible pBAD promoter vector; Cm^R	(Guzman *et al.*, 1995)
pBAD33::*icsP*	*icsP* gene cloned into pBAD33; Cm^R	This study
pBR322	Medium copy no.; ColE1 *ori*; Ap^R, Tc^R	(Bolivar *et al.*, 1977)
pIcsA	*icsA* gene cloned into pBR322; Ap^R	(Van Den Bosch & Morona, 2003)
pIcsA$_{i633}$	pIcsA with 5 amino acids insertion at aa633; Ap^R	(May & Morona, 2008)
pIcsA$_{i643}$	pIcsA with 5 amino acids insertion at aa643; Ap^R	(May & Morona, 2008)
pIcsA$_{i677}$	pIcsA with 5 amino acids insertion at aa677; Ap^R	(May & Morona, 2008)
pIcsA$_{i716}$	pIcsA with 5 amino acids insertion at aa716; Ap^R	(May & Morona, 2008)
pJRD215	Broad-host-range cosmid cloning vector; Km^R, Sm^R; *mob*, plasmid mobilisation functions	(Davison *et al.*, 1987)
pRMA154	pJRD215$_{ClaI\text{-}Kpn1}$-pPM2213$_{Cla1\text{-}Kpn1}$ Km^R, Sm^R; encodes *galF*, *rfb* locus, *rfc* and *gnd*, Oag biosynthesis genes from *S. flexneri*	(Morona *et al.*, 1994)
pKOK6.1	Derivative of pKOK6 with inverse direction of the *lacZ*/Kan cassette in pKOK6 and has additional stop codons inserted into upstream of *lacZ* via the *Bam*HI site; Cm^R, Km^R	(Kokotek & Lotz, 1989; Murray *et al.*, 2003)
pQE60	Expression vector with a C-terminal His$_6$ tag; Ap^R	Qiagen
pQE60::*icsP*	*icsP* gene cloned into pQE60; Ap^R	This study
pRMA727	*rmlD* (*rfbD*) gene cloned into pACYC184; Cm^R	(Van Den Bosch *et al.*, 1997)
pSU23	Medium copy no.; P15A *ori;* Cm^R	(Bartolome *et al.*, 1991)
pMYRM632	*icsA* promoter region transcriptionally fused to *lacZ*-Km; Ap^R, Cm^R, Km^R	This study
pMYRM676	Derived from pMYRM632 with a stop codon inserted between *icsA* and *lacZ*; Ap^R, Cm^R, Km^R	This study
pMYRM718	*Pst*I-*Sal*I fragment containing *icsA* promoter-TGA-	This study

	lacZ-Km derived from pMYRM676, cloned into pSU23; Cm^R, Km^R

888 # Tc^R, Tetracycline resistant; Km^R, Kanamycin resistant; Ap^R, Ampicillin resistant; Cm^R, Chloramphenicol
889 resistant; Sm^R, Streptomycin resistant.

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Figure Legends

Figure 1. IcsA$_i$ expression and localisation in S-LPS and R-LPS *S. flexneri*. (a) Whole cell lysates from mid-exponential phase cultures of the indicated *S. flexneri* strains were subjected to Western immunoblotting with anti-IcsA antibody. S= S-LPS; R = R-LPS. IcsA$^-$ = IcsA deletion control. The 120 kDa band corresponds to the full length IcsA and the 85 kDa band corresponds to the cleaved form (IcsA'). (b) IF microscopy to detect IcsA$_{WT}$ or IcsA$_i$ surface distribution. Mid-exponential phase cultures of the indicated *S. flexneri* strains were formalin fixed and labelled with anti-IcsA antibody and then goat anti-rabbit Alexa Fluor-488 secondary antibody. The while arrow indicates S-LPS *S. flexneri* expressing low levels of IcsA$_{i716}$ (KMRM147). Scale bar = 10 μm.

Figure 2. *rmlD* complemented *S. flexneri* strains expressing IcsA$_{WT}$ or IcsA$_i$. Whole cell lysates from mid-exponential phase cultures of indicated the *S. flexneri* strains were prepared as described in the Methods. (a) LPS samples (equivalent to 1 x 10^9 bacteria) were prepared, electrophoresed on a SDS-15%-PAGE gel and silver stained as described in the Methods. (b) Whole cell lysates were electrophoresed on a SDS-12%-PAGE gel and subjected to Western immunoblotting with anti-IcsA antibody.

Figure 3. LPS depletion-regeneration assay. Mid-exponential phase S-LPS *S. flexneri* expressing IcsA$_{WT}$ or IcsA$_{i716}$ were treated with 10 μg tunicamycin mL^{-1} or DMSO and 3 μg polymyxin B nonapeptide mL^{-1} (PMBN) for 3 h at 37°C, followed by removal of tunicamycin/PMBN/DMSO and allowed to grow for another 3 h to regenerate LPS Oag as described in the Methods. (a) LPS samples (equivalent to 1 x 10^9 bacteria) were prepared, electrophoresed on a SDS-15%-PAGE gel and silver stained as described in the Methods. U = Untreated; D = Degeneration phase; R = Regeneration phase. (b) Whole cell lysates

were electrophoresed on a SDS-12%-PAGE gel, and subjected to Western immunoblotting with anti-IcsA antibody.

Figure 4. Effect of IcsP on IcsA$_i$ production levels. (a) Tricholoroacetic acid-precipitated culture supernatants from mid-exponential phase cultures of the indicated *S. flexneri* strains. P = Whole cell lysates; SN = Supernatant. (b, c) Mid-exponential phase cultures of the indicated *S. flexneri* strain expressing IcsA$_{WT}$ or IcsA$_i$ mutants were treated with 0.2% (w/v) L-arabinose to induce IcsP expression as described in the Methods. Whole cell lysates were prepared, electrophoresed on a SDS-12%-PAGE gel and subjected to Western immunoblotting with anti-IcsA and anti-IcsP. (b) Western blot analysis of IcsA$_i$ mutant and IcsP expression in virulence plasmid-cured S-LPS strain. (c) Western blot analysis of IcsA$_i$ mutant and IcsP expression in virulence plasmid-cured R-LPS strain. The positions of the bands corresponding to IcsA, IcsA$'$ and IcsP are shown.

Figure 5. β-galactosidase assay on S-LPS and R-LPS *S. flexneri* strains. S-LPS and R-LPS *S. flexneri* strains expressing IcsA$_{WT}$, S-LPS and R-LPS *S. flexneri* strains expressing IcsA$_{i716}$ that are carrying pSU23-P*icsA*-TGA-*lacZ* were grown to mid-exponential phase at 37°C and the β-galactosidase activity was measured as described in the Methods. Data represent the means of three independent experiments. Error bars represent standard error of the mean (*P < 0.05). S-LPS IcsA$_{WT}$ = MYRM734; R-LPS IcsA$_{WT}$ = MYRM721; S-LPS IcsA$_{i716}$ = MYRM722; R-LPS IcsA$_{i716}$ = MYRM723.

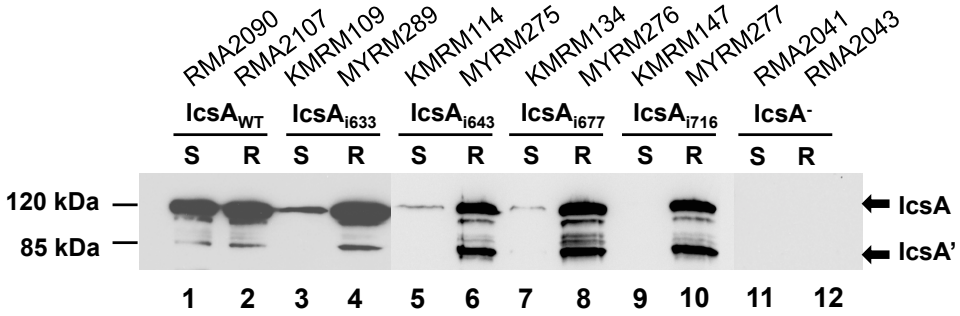
Figure 6. IcsA production in *S. flexneri degP*::Cm Δ*icsA*. Whole cell lysates from mid-exponential phase cultures (grown at 30°C or 37°C) of the indicated *S. flexneri* strains

were electrophoresed on a SDS-12%-PAGE gel and subjected to Western immunoblotting with anti-IcsA antibody.

Figure 7. N-WASP recruitment and F-actin comet tail formation by intracellular S-LPS and R-LPS *S. flexneri* strains. HeLa cells infected with *S. flexneri* expressing $IcsA_{WT}$, $IcsA_{i677}$ or $IcsA_{i716}$ were labelled with anti-N-WASP and Alexa Fluor 594-conjugated donkey anti-rabbit secondary antibody (red) to detect N-WASP, F-actin was labelled with Alexa Fluor 488-phalloidin (green), HeLa cells and bacteria was labelled with DAPI (blue) as described in the Methods. Strains were assessed in two independent experiments. Arrows indicate N-WASP recruitment and F-actin comet tail formation. Insert shows an enlarged view for greater clarity. Scale bar = 10 μm.

Figure 8. Trypsin accessibility of $IcsA_i$ mutants. Mid-exponential phase cultures of the indicated R-LPS *S. flexneri* strains expressing $IcsA_{WT}$ or $IcsA_i$ mutants were treated with 0.1 μg trypsin mL^{-1} at 25°C. Aliquots were taken at 0 min, 5 min, 15 min and 20 min and supplemented with 1 mM phenylmethylsulphonylfluoride (PMSF) to inhibit further proteolysis. Whole cell lysates were electrophoresed on a SDS-12%-PAGE gel and subjected to Western immunoblotting with anti-IcsA antibody.

(a)


RMA2090
RMA2107
KMRM109
MYRM289
KMRM114
MYRM275
KMRM134
MYRM276
KMRM147
MYRM277
RMA2041
RMA2043
IcsA$_{WT}$
IcsA$_{i633}$
IcsA$_{i643}$
IcsA$_{i677}$
IcsA$_{i716}$
IcsA^{-}
S R S R S R S R S R S R
120 kDa
85 kDa
IcsA
IcsA'
1 2 3 4 5 6 7 8 9 10 11 12

(b)

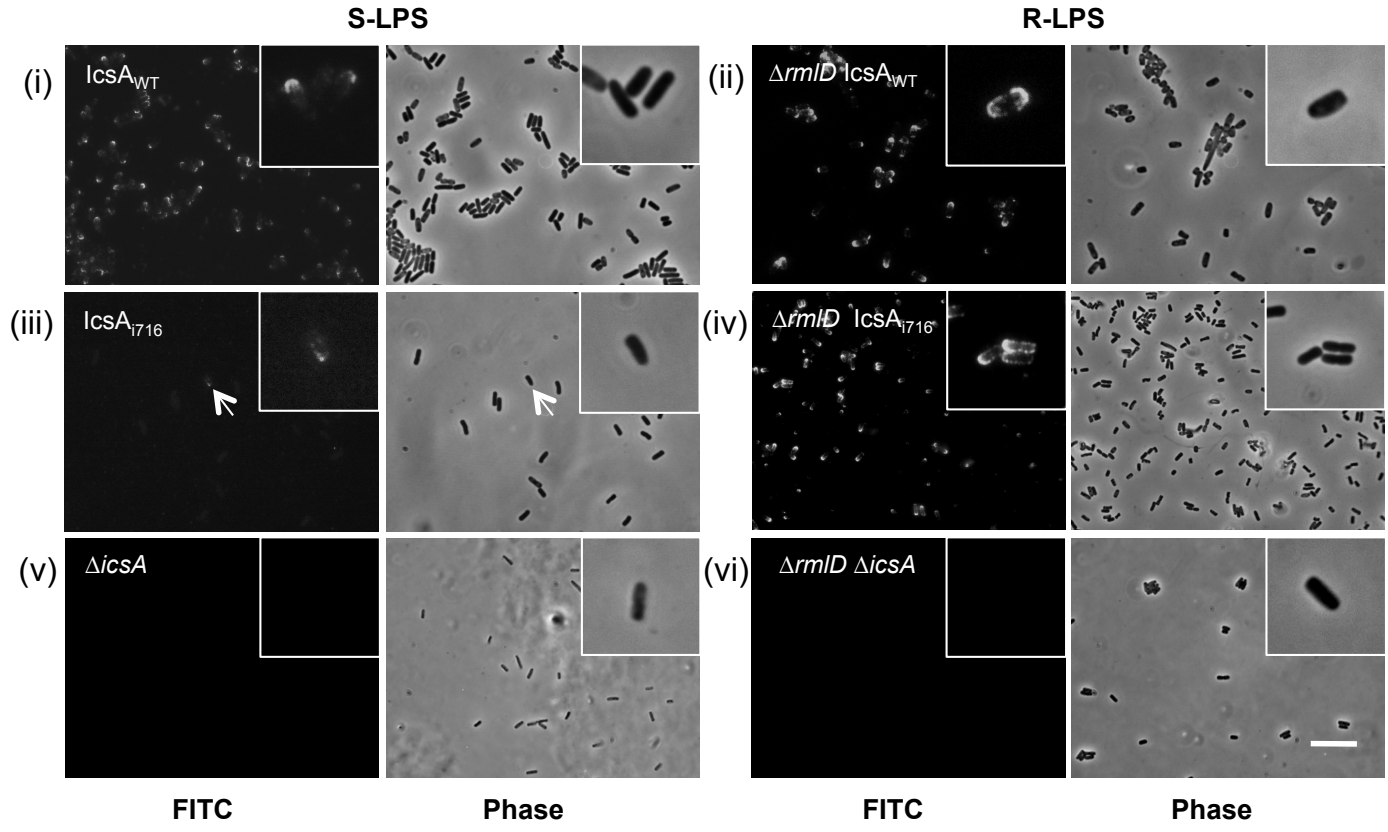

S-LPS
R-LPS
(i) IcsA$_{WT}$
(ii) Δ*rmlD* IcsA$_{WT}$
(iii) IcsA$_{i716}$
(iv) Δ*rmlD* IcsA$_{i716}$
(v) Δ*icsA*
(vi) Δ*rmlD* Δ*icsA*
FITC
Phase
FITC
Phase

Figure 1

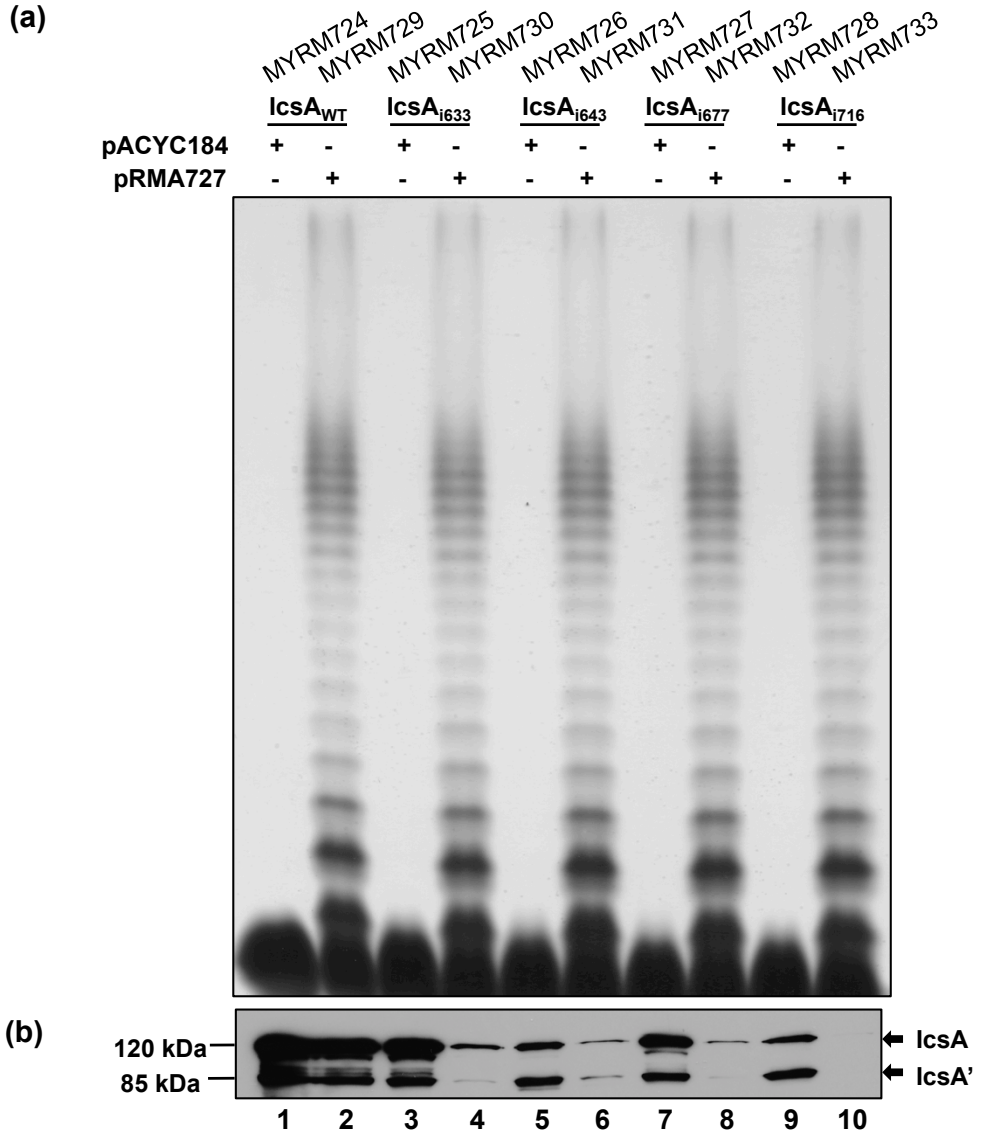

(a)
MYRM724
MYRM729
MYRM725
MYRM730
MYRM726
MYRM731
MYRM727
MYRM732
MYRM728
MYRM733
IcsA$_{WT}$
IcsA$_{i633}$
IcsA$_{i643}$
IcsA$_{i677}$
IcsA$_{i716}$
pACYC184 + - + - + - + - + -
pRMA727 - + - + - + - + - +
(b)
120 kDa
85 kDa
IcsA
IcsA'
1 2 3 4 5 6 7 8 9 10

Figure 2

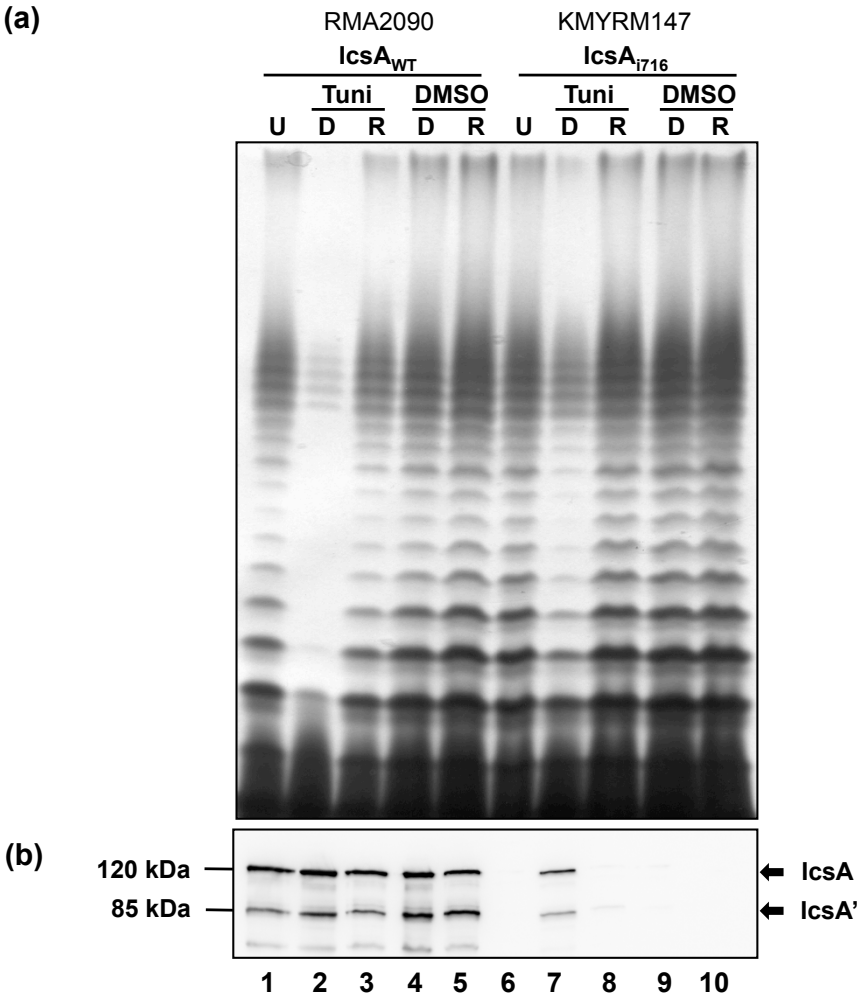

(a)
RMA2090
KMYRM147
IcsA$_{WT}$
IcsA$_{i716}$
Tuni
DMSO
U D R D R U D R D R
(b)
120 kDa
85 kDa
IcsA
IcsA'
1 2 3 4 5 6 7 8 9 10

Figure 3

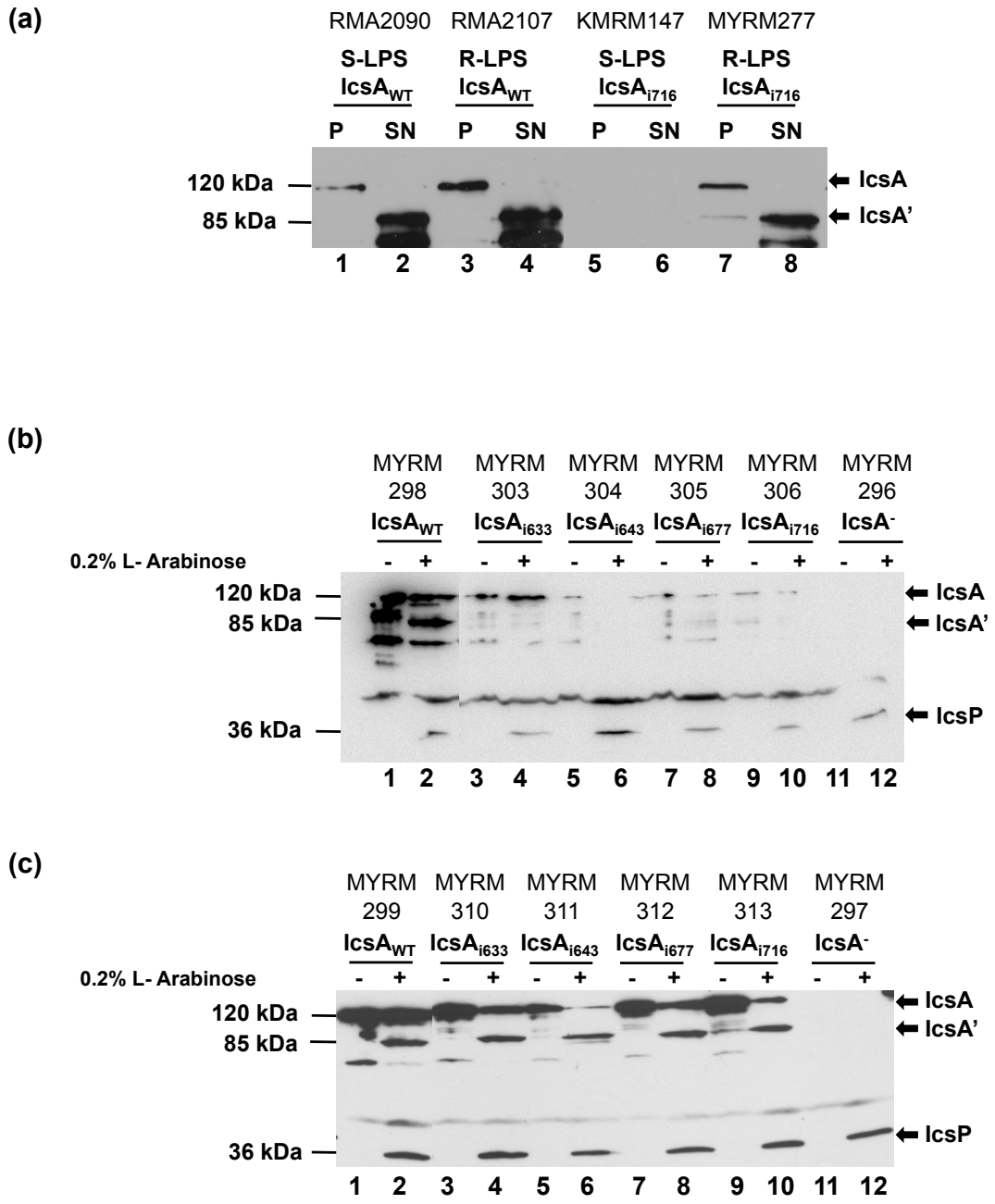

(a)
RMA2090
RMA2107
KMRM147
MYRM277
S-LPS
IcsA$_{WT}$
R-LPS
IcsA$_{WT}$
S-LPS
IcsA$_{i716}$
R-LPS
IcsA$_{i716}$
P SN P SN P SN P SN
120 kDa
85 kDa
IcsA
IcsA'
1 2 3 4 5 6 7 8
(b)
MYRM 298
MYRM 303
MYRM 304
MYRM 305
MYRM 306
MYRM 296
IcsA$_{WT}$
IcsA$_{i633}$
IcsA$_{i643}$
IcsA$_{i677}$
IcsA$_{i716}$
IcsA^{-}
0.2% L- Arabinose
- + - + - + - + - + - +
120 kDa
85 kDa
36 kDa
IcsA
IcsA'
IcsP
1 2 3 4 5 6 7 8 9 10 11 12
(c)
MYRM 299
MYRM 310
MYRM 311
MYRM 312
MYRM 313
MYRM 297
IcsA$_{WT}$
IcsA$_{i633}$
IcsA$_{i643}$
IcsA$_{i677}$
IcsA$_{i716}$
IcsA^{-}
0.2% L- Arabinose
- + - + - + - + - + - +
120 kDa
85 kDa
36 kDa
IcsA
IcsA'
IcsP
1 2 3 4 5 6 7 8 9 10 11 12

Figure 4

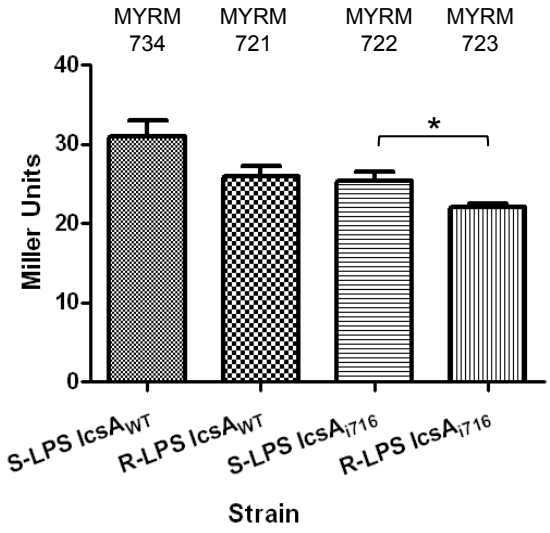

MYRM 734
MYRM 721
MYRM 722
MYRM 723
40
30
20
10
0
Miller Units
*
S-LPS IcsA$_{WT}$
R-LPS IcsA$_{WT}$
S-LPS IcsA$_{i716}$
R-LPS IcsA$_{i716}$
Strain

Figure 5

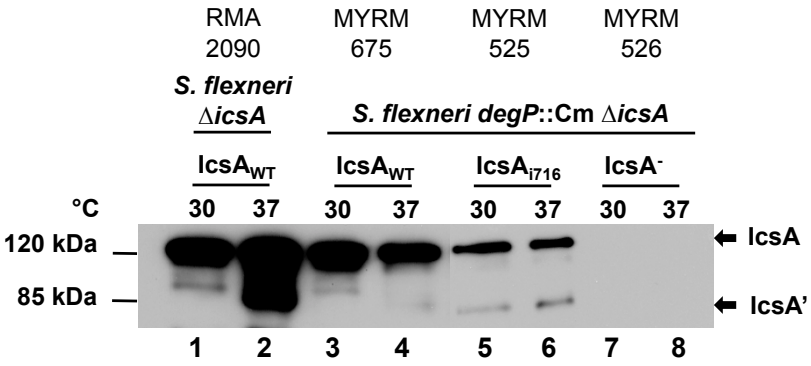

RMA 2090
MYRM 675
MYRM 525
MYRM 526
S. flexneri ΔicsA
S. flexneri degP::Cm ΔicsA
IcsA$_{WT}$
IcsA$_{WT}$
IcsA$_{i716}$
IcsA^{-}
°C
30 37 30 37 30 37 30 37
120 kDa
85 kDa
IcsA
IcsA'
1 2 3 4 5 6 7 8

Figure 6

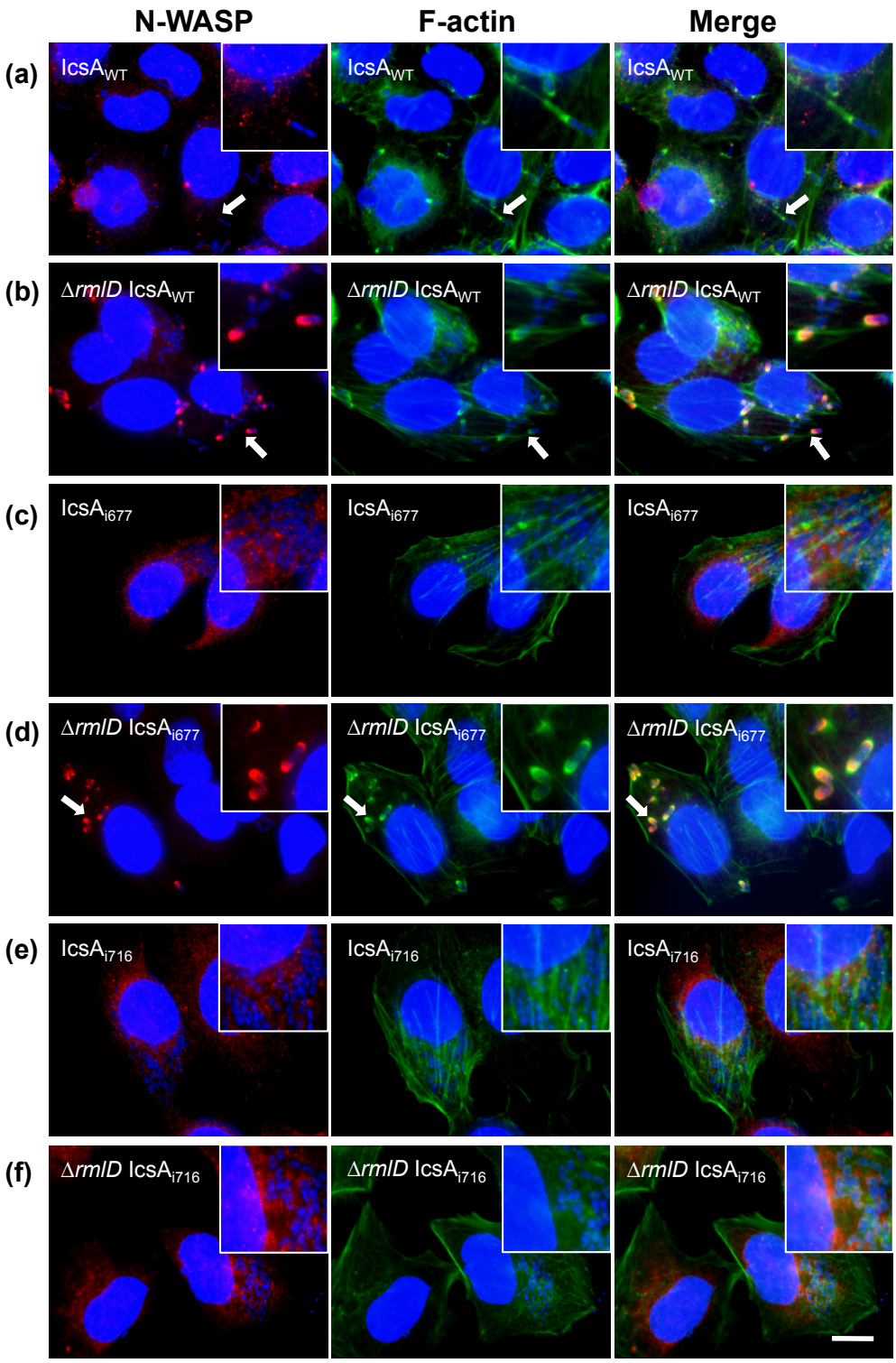

N-WASP
F-actin
Merge
(a)
IcsA$_{WT}$
(b)
Δ*rmlD* IcsA$_{WT}$
(c)
IcsA$_{i677}$
(d)
Δ*rmlD* IcsA$_{i677}$
(e)
IcsA$_{i716}$
(f)
Δ*rmlD* IcsA$_{i716}$

Figure 7

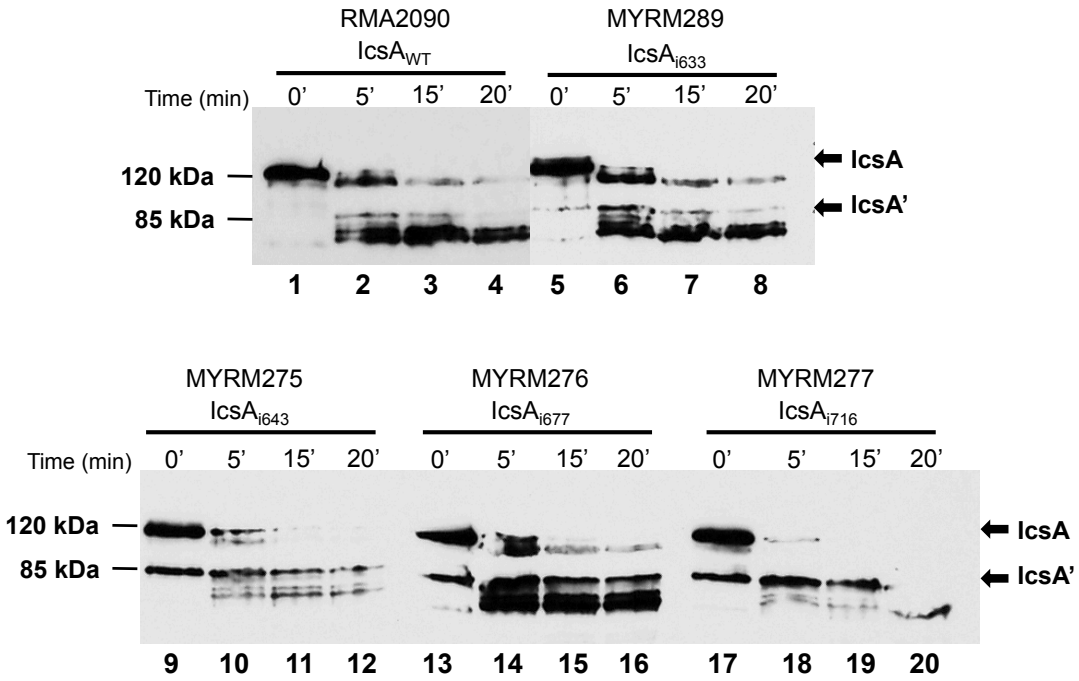

RMA2090
IcsA$_{WT}$
MYRM289
IcsA$_{i633}$
Time (min)
0’ 5’ 15’ 20’ 0’ 5’ 15’ 20’
120 kDa
85 kDa
IcsA
IcsA’
1 2 3 4 5 6 7 8
MYRM275
IcsA$_{i643}$
MYRM276
IcsA$_{i677}$
MYRM277
IcsA$_{i716}$
Time (min)
0’ 5’ 15’ 20’ 0’ 5’ 15’ 20’ 0’ 5’ 15’ 20’
120 kDa
85 kDa
IcsA
IcsA’
9 10 11 12 13 14 15 16 17 18 19 20

Figure 8

Supplementary Material

Absence of O-antigen suppresses *Shigella flexneri* IcsA autochaperone region mutations

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Table S1. Oligonucleotides used in this study

Primer	Oligonucleotide sequence (5' – 3') [#]	Target	Nucleotide positions
lacZ_TGA_F	tcttccaacccctctcatgca**t**g**a**ggggctagaga	IcsA-TGA-*lacZ*	- [‡]
lacZ_TGA_R	tctctagcccc**t**c**a**tgcatgagaggggttggaaga	IcsA-TGA-*lacZ*	- [‡]
ET9	ggccatggacatttcaaccaaaaaag	*icsP*	220267 [§]
ET10	gcggatccaaaaatatactttatacctgcg	*icsP*	221244 [§]
ET22	gcggtaccataaagtaagaagatcatggac	16bp upstream *icsP*	220251 [§]
ET25	gggaagctttcaaaaaatatactttatacctg	*icsP*	221247 [§]

[#] Underlined and bolded sequences indicate the nucleotides that undergo site-directed mutagenesis
[‡] Contact authors for the DNA sequence
[§] Nucleotide positions based on the sequence of the *S. flexneri* 2a strain 301 virulence plasmid pCP301 – GeneBank accession # AF386526