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FEMS Microbiology Letters 221 (2003) 173–180

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Lipopolysaccharide O antigen chains mask IcsA (VirG) in *Shigella flexneri*

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Received 5 January 2003; received in revised form 9 February 2003; accepted 9 February 2003

First published online 2 April 2003

Abstract

Shigella flexneri 2a strain 2457T lipopolysaccharide (LPS) has O antigen (Oag) chains with two modal lengths (S-type and VL-type), and has IcsA apparently located at one pole on its cell surface. Treatment of Y serotype derivatives of 2457T and RMA696 (2457T wzz_{SF}) with Sf6 tailspike protein (TSP) resulted in hydrolysis of Oag chains, and an increase in detection of IcsA by indirect immunofluorescence staining on both the lateral and polar regions of the cell surface. Newly synthesised IcsA expressed from a pBAD promoter in a *S. flexneri* Y strain was also detected on both the lateral and polar regions of the cell when incubated with TSP prior to immunofluorescence staining. We conclude that IcsA is actually located on both lateral and polar regions of the *S. flexneri* cell surface, and that LPS Oag chains mask the presence of IcsA by hindering its detection with antibodies. These results have implications for the mechanism of IcsA export. They suggest that while IcsA export is predominantly targeted to the old cell pole, it can also occur on the lateral regions of the cell surface.

Keywords: Lipopolysaccharide; O antigen; Steric hindrance; Tailspike protein; IcsA

1. Introduction

Shigella flexneri bacteria cause bacillary dysentery in humans as a consequence of their ability to invade and replicate within intestinal epithelial cells, and to trigger the death of local macrophages by apoptosis resulting in an acute inflammatory response [1,2]. *S. flexneri* bacteria use actin-based motility (ABM) to spread and move inside the cytoplasm of infected cells, and spread to adjacent cells by means of bacteria-containing extrusions of the plasma membrane that penetrate into these cells [2]. The 116-kDa outer membrane protein IcsA (also called VirG) is essential for nucleating ABM [3,4], and is located at one of the cell poles [5–7]. Sequences located within the amino-terminal α domain target export of IcsA to the old cell pole [8–10], and an outer membrane protease (IcsP(SopA)) that slowly cleaves IcsA also contributes to its polar localisation [11–14]. In addition, *S. flexneri* mutants with altered lipopolysaccharide (LPS) structure have altered cell surface expression and localisation of IcsA, and reduced virulence [15–23].

LPS has three regions: lipid A, core sugars and O antigen (Oag) polysaccharide chains. Intact LPS molecules are referred to as smooth LPS, while LPS molecules without Oag chains are referred to as rough LPS (R-LPS). The LPS of *S. flexneri* 2a strain 2457T has Oag chains with two modal lengths. The S-type modal length (11–17 repeat units (RUs)) is determined by the chromosomally located wzz_{SF}, and the VL-type modal lengths (>90 RUs) by the pHS-2 plasmid located wzz_{pHS2} [15,23,24].

We have characterised *S. flexneri* 2457T mutants that had either R-LPS (RMA723, *rmlD*::Km^R) or whose LPS did not have S-type Oag chains (RMA696, wzz_{SF}::Km^R) [23]. Both were able to invade mammalian cells in tissue culture, unable to plaque on HeLa cell monolayers, and had a defect in F-actin comet tail formation indicating a defect in ABM. Like other R-LPS mutants RMA723 had high levels of circumferentially distributed IcsA on its cell surface (at both cell poles and lateral regions) [20,23], whereas RMA696 had reduced levels of IcsA on its cell surface. This was not due to decreased production of IcsA ([23]; Morona, R., Daniels, C. and Van Den Bosch, L., submitted for publication). Based on these observations,

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0378-1097/03/$22.00
doi:10.1016/S0378-1097(03)00210-6

we hypothesise that the LPS Oag chains mask IcsA in both the wild-type strain and the wzz_{SF} mutant thereby causing steric hindrance that affects the binding of antibodies to IcsA.

2. Materials and methods

2.1. Growth media and growth conditions

Strains were routinely grown in Luria–Bertani (LB) broth and agar as previously described [23]. Unless otherwise stated, *S. flexneri* bacteria were grown from a Congo red agar [23] positive colony in LB for 16 h, then diluted 1 in 50 into fresh LB and grown for 2 h. Under these growth conditions, IcsA protein was almost entirely in the intact 116-kDa form. Antibiotics used were: ampicillin (Ap), 50 μg ml^{-1}; chloramphenicol (Cm), 25 μg ml^{-1}; kanamycin (Km), 50 μg ml^{-1}; and tetracycline (Tc), 10 μg ml^{-1}. Unless stated otherwise, strains were grown at 37°C.

2.2. Bacterial strains and plasmids

The bacterial strains and plasmids used are described in Table 1. Virulence plasmid cured (VP-ve) derivatives of *S. flexneri* strains were isolated on Congo red agar as white colonies and re-streaked until pure. Polymerase chain reaction (PCR) with oligonucleotides #2156 (5′-gccgaattcaacggaatcttttcagggg-3′) and #2170 (5′-gcggatcccccatgtgatttgcctcg-3′) which amplify *icsA* was used confirm the absence of the virulence plasmid.

2.3. DNA methods

DNA manipulations, transformation, PCR, and electroporation into *S. flexneri* were performed as recently described [29,30]. DH5α was used for all cloning.

2.4. Construction of pRMA2217 and gtrII::Tc^R mutant strains

Plasmid pRMA2217 was obtained as follows. A 4-kb *Bam*HI fragment containing *gtrII* was sub-cloned from plasmid pRMM264 into *Bam*HI digested pCACTUS, a temperature sensitive suicide vector, resulting in pRMA2216. A 2.5-kb *Bam*HI fragment containing *tetAR*(B) (Tc resistance cassette (Tc^R)) from pSBA383 was then cloned into a unique *Bgl*II located within *gtrII* on pRMA2216 resulting in pRMA2217. Allelic exchange was performed as described by Morona et al. [26]. Colonies were confirmed to be Tc resistant and Cm sensitive, and PCR was used to confirm the presence of the insertion mutation.

2.5. Construction of pBAD-IcsA

Plasmid pBAD-IcsA was obtained by sub-cloning from pRMA920 an *Eco*RI-*Sal*I fragment that has *icsA* without its native promoter, and the *Eco*RI site upstream of the ribosomal binding site and the *Sal*I site downstream of the stop codon, into *Eco*RI-*Sal*I digested pBAD30. Production of the 116-kDa IcsA after arabinose induction was confirmed by Western immunoblotting.

Table 1
Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics	Reference/source
Escherichia coli K-12		
DH5α	Cloning host	Gibco-BRL
Shigella flexneri		
2457T	*Shigella flexneri* 2a wild type (VP-positive)	[23]
RMA696	2457T *wzz*::Km^R	[23]
RMA723	2457T *rmlD*::Km^R	[23]
RMA2161	RMA723 VP-ve	This study
RMA2218	2457T *gtrII*::Tc^R	This study
RMA2219	RMA696 *gtrII*::Tc^R	This study
RMA2238	RMA2218 VP-ve	This study
RMA2230	RMA2161 [pBAD-IcsA]	This study
RMA2234	RMA2238 [pBAD-IcsA]	This study
Plasmids		
pRMA920	*icsA* gene cloned in pTTQ181;Ap^R	R. Morona, C. Daniels and L. Van Den Bosch, submitted for publication
pBAD30	pBAD promoter vector, Ap^R	[25]
pBAD-IcsA	pBAD30 with *icsA* gene	This study
pCACTUS	Suicide vector, Cm^R	[26]; Laboratory collection
pSBA383	Source of *tetAR*(B) cassette (Tc^R)	[27]
pRMM264	Source of SfII phage *gtrII* gene; Cm^R	[28]
pRM2216	*gtrII* gene cloned in pCACTUS	This study
pRM2217	pCACTUS with *gtrII*::Tc^R; Cm^R	This study

2.6. Arabinose induction of IcsA expression

S. flexneri strains harbouring pBAD-IcsA were grown in 30 ml LB containing Ap and 0.5% (w/v) glucose for 2 h. Bacteria were harvested by centrifugation (IEC Centra 4×, 5000 rpm, 10 min), washed once with 30 ml LB, resuspended in 30 ml LB, a 10-ml sample was taken ($t = 0$ min), and arabinose was added to 0.2% (w/v). The bacteria were grown with aeration and 10-ml samples were taken at 30 min and 60 min. All samples were immediately placed at 4°C, centrifuged (4°C, Sigma centrifuge, 5000 rpm, 10 min), and formalin fixed.

2.7. Formalin fixation of bacteria

Bacteria washed twice in saline by centrifugation, resuspended in 5 ml 2% (w/v) paraformaldehyde (Sigma) in saline (formalin–saline), and incubated at 37°C for 1 h with agitation every 15 min. The bacteria were then washed three times in saline, and resuspended in 1 ml saline.

2.8. Treatment with Sf6 TSP

Formalin fixed bacteria in saline (0.1 ml, $\sim 2 \times 10^9$) were mixed with (and without) 3 μg of purified Sf6 phage tailspike protein (TSP), incubated at 37°C for 30 min, then washed three times (1 min at 15 000 rpm (Heraeus Biofuge 15)) in either Milli-Q water (Millipore) (for LPS analysis by SDS–PAGE) or phosphate buffered saline (PBS) (for immunofluorescence microscopy), and resuspended in 50 μl of either Milli-Q water or PBS as required. The Sf6 phage TSP used was a highly purified preparation [31].

2.9. Analysis of LPS

LPS was prepared by proteinase K (Roche) treatment of whole cell lysates, electrophoresed on SDS–20% polyacrylamide gels and detected by silver staining as described previously [30,32].

2.10. Preparation of rabbit anti-IcsA antibody

Generation of the rabbit anti-IcsA antibody was performed as described elsewhere ([23]; Morona, R., Daniels, C. and Van Den Bosch, L., submitted for publication). Western immunoblotting showed that the antibody was reactive with epitopes located along the length of the amino-terminal α domain, but not with the carboxy-terminal β domain (data not shown).

2.11. Indirect immunofluorescence (IF) staining and confocal microscopy

The method used was recently described [23,33]. In brief, formalin fixed bacteria on coverslips were incubated with anti-IcsA (1:100 in PBS with 10% foetal calf serum (FCS)), washed, then incubated with fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit antibody (Silenus) in PBS with 10% FCS. Propidium iodide (PI) (10 μg ml^{-1}, Sigma) was included with the secondary antibody to counter-stain bacteria. The coverslips were mounted on glass microscope slides with Mowiol 4-88 (Calbiochem) containing 20 μg ml^{-1} *p*-phenylenediamine (Sigma). FITC and PI images were collected simultaneously with a Bio-Rad MRC-600 confocal laser scanning microscope

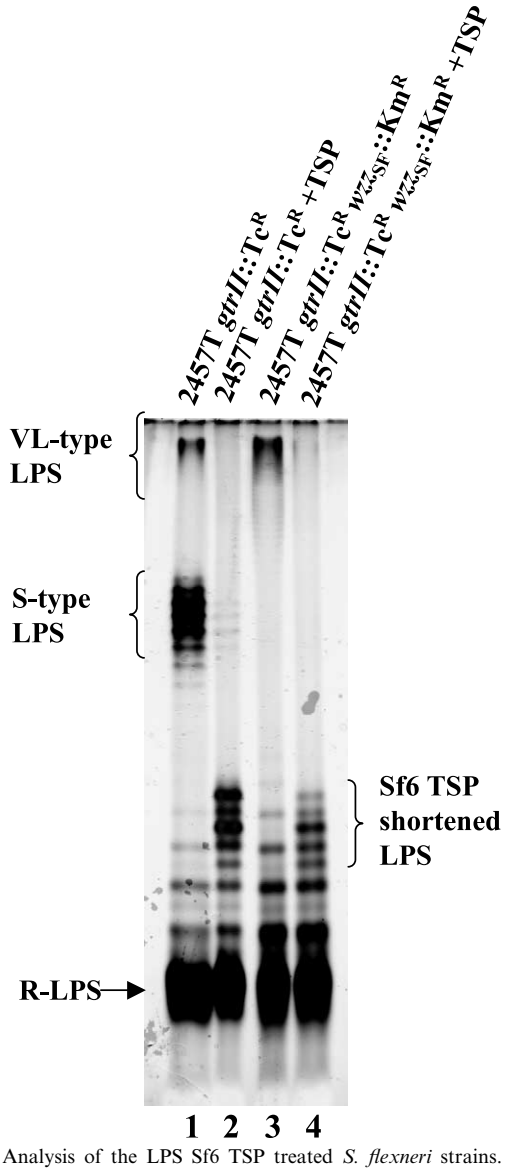

Fig. 1. Analysis of the LPS Sf6 TSP treated *S. flexneri* strains. *S. flexneri* strains were incubated with and without Sf6 TSP, treated with proteinase K, and their LPSs were electrophoresed on an SDS–20% polyacrylamide gel and detected by silver staining. The strains in each lane are: 1 and 2, 2457T *grtII*::Tc^R [RMA2218]; 3 and 4, 2457T *grtII*::Tc^R *wzz*::Km^R [RMA2219]. R-LPS, LPS lacking Oag chains; S-type LPS, LPS with 11–17 Oag RUs; VL-type LPS, LPS with Oag chains with >90 Oag RUs. The positions of truncated LPS Oag chains arising due to TSP endorhamnosidase activity are indicated on the right-hand side of the image.

using a 100× oil immersion lens, and false colour merged using Confocal Assistant 4.02. Each image shown is from a single plane.

3. Results

3.1. Construction of S. flexneri Y serotype derivatives

To test our hypothesis that Oag chains mask detection of IcsA, we investigated if removal of *S. flexneri* Oag chains by in situ enzymatic hydrolysis affected IcsA detection by indirect IF staining. This was accomplished using Sf6 bacteriophage TSP that has endorhamnosidase activity on the Oag polysaccharides of *S. flexneri* Y serotype [34]; Sf6 TSP is not active on the Oag of serotype 2a strains such as 2457T that we have used in previous studies [23].

We used pRMA2217 to construct Y serotype derivatives of 2457T and 2457T wzz_{SF}::Km^R [RMA696] by inactivating the *gtrII* gene which determines the 2a serotype [28] with a Tc^R cassette as described in Section 2.4. 2457T *gtrII*::Tc^R [RMA2218] and 2457T *gtrII*::Tc^R *wzz*::Km^R [RMA2219] were Y serotype, and behaved identically to their respective parental strain with respect to HeLa cell monolayer plaquing ability.

3.2. Effect of Sf6 TSP treatment on detection of IcsA

2457T *gtrII*::Tc^R and 2457T *gtrII*::Tc^R *wzz*::Km^R were grown to early exponential phase, formalin fixed and incubated with and without Sf6 TSP. Analysis of the LPS by SDS–PAGE and silver staining showed that TSP treatment resulted in marked shortening of the LPS Oag chains (> 90% of the LPS Oag chains were shortened) (Fig. 1).

The TSP treated and control samples were then subjected to IF staining to detect cell surface IcsA. Untreated 2457T *gtrII*::Tc^R and 2457T *gtrII*::Tc^R *wzz*::Km^R bacteria had polarly localised IcsA (approximately 40% and 30% of bacteria ($n = 100$), respectively; Fig. 2a,c), with none (< 1.0%) having IcsA on lateral cell surfaces. After TSP treatment, IcsA could be detected on the lateral and polar regions of both strains (approximately 90% of 2457T *gtrII*::Tc^R and 2457T *gtrII*::Tc^R *wzz*::Km^R bacteria), and staining of IcsA became more intense (Fig. 2b,d). Furthermore, following TSP treatment the number of unstained bacteria decreased from approximately 60% to 10% for 2457T *gtrII*::Tc^R, and from approximately 70% to 10% for 2457T *gtrII*::Tc^R *wzz*::Km^R. These results support our hypothesis that Oag chains mask and interfere with detection of IcsA by antibody. The data also indicate that some IcsA is located on the lateral regions of the cell sur-

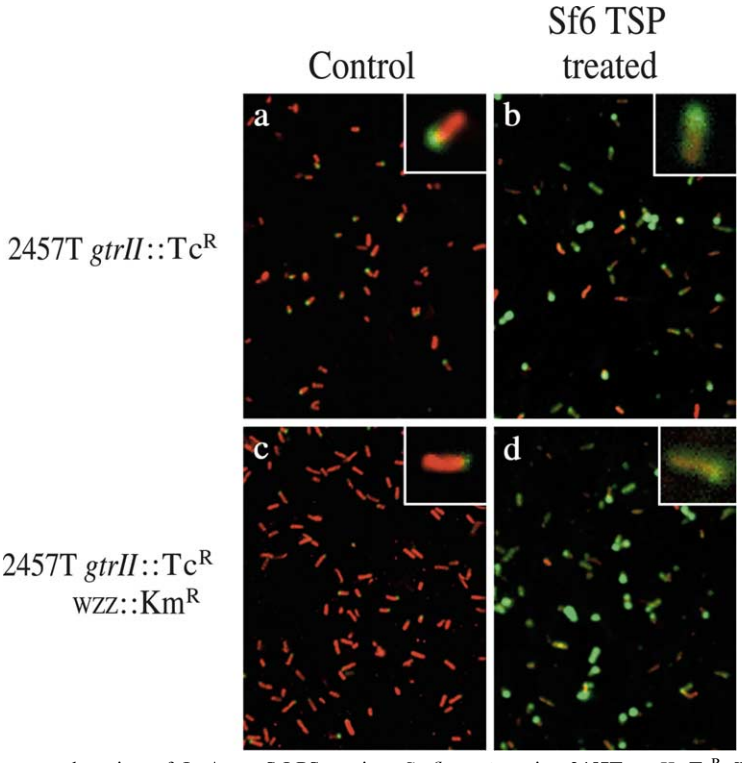

Fig. 2. Effect of Sf6 TSP treatment on detection of IcsA on S-LPS strains. *S. flexneri* strains 2457T *gtrII*::Tc^R [RMA2218] and 2457T *gtrII*::Tc^R *wzz*::Km^R [RMA2219] were incubated with and without Sf6 TSP, and stained to detect IcsA by with a rabbit anti-IcsA antibody and an FITC conjugated secondary antibody (seen here as green), and *S. flexneri* bacteria were detected by counter-staining with propidium iodide (seen here as red). Images are: a, 2457T *gtrII*::Tc^R [RMA2218] untreated; b, 2457T *gtrII*::Tc^R [RMA2218] TSP treated; c, 2457T *gtrII*::Tc^R *wzz*::Km^R [RMA2219] untreated; d, 2457T *gtrII*::Tc^R *wzz*::Km^R [RMA2219] TSP treated. Within each image, an enlargement of a typical bacterium is shown. The experiment was performed at least three times and representative results are shown.

face in addition to the cell poles in both *S. flexneri* wild-type and wzz_{SF} mutant bacteria.

3.3. *Effect of TSP treatment on detection of newly synthesised* IcsA

We also investigated the effect of TSP treatment on detection of newly synthesised IcsA. For these experiments plasmid pBAD-IcsA having *icsA* under control of the arabinose inducible pBAD promoter was constructed as described in Section 2.5. A VP-ve derivative of 2457T *gtrII*::Tc^R carrying pBAD-IcsA [RMA2234] was induced with arabinose, and samples were taken at 30 min and 60 min after induction, formalin fixed, and IcsA was detected by IF staining. IcsA could be detected at the cell poles of 2457T VP-ve *gtrII*::Tc^R (pBAD-IcsA) at 60 min post induction (approximately 15% of bacteria; Fig. 3c) but could not be detected prior to induction (Fig. 3a,d) or 30 min post induction (Fig. 3b). However, after TSP treatment, IcsA was detected at the cell poles of 2457T VP-ve *gtrII*::Tc^R (pBAD-IcsA) bacteria at 30 min post induction (approximately 10% of bacteria; Fig. 3e), and at 60 min post induction it was detected on the lateral region and the cell pole of approximately 50% of bacteria and at the cell pole only of approximately 20% of bacteria (Fig. 3f). Our data show that IcsA is targeted to the cell pole, as re-

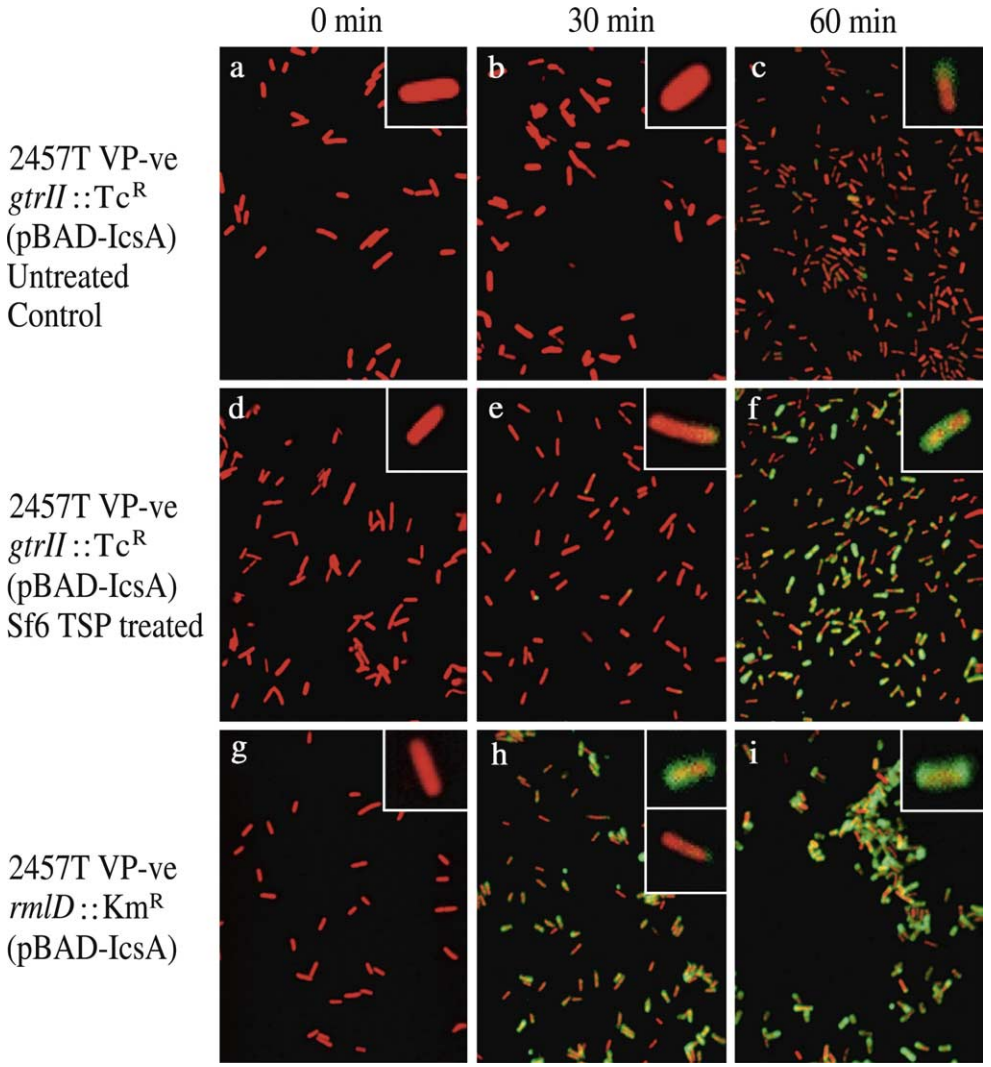

Fig. 3. Effect of Sf6 TSP treatment on detection of newly synthesised IcsA. *S. flexneri* strains 2457T VP-ve, *gtrII*::Tc^R (pBAD-IcsA) [RMA2234] and 2457T VP-ve, *rmlD*::Km^R (pBAD-IcsA) [RMA2230] were grown in LB with glucose, washed, and *icsA* expression was induced by addition of arabinose. Samples were taken prior to induction (0 min), and at 30 min and 60 min post induction. After formalin fixation, 2457T VP-ve, *gtrII*::Tc^R (pBAD-IcsA) [RMA2234] samples were incubated with and without Sf6 TSP. Cell surface IcsA was detected by indirect IF staining with a rabbit anti-IcsA antibody and an FITC conjugated secondary antibody (seen here as green), and *S. flexneri* bacteria were detected by counter-staining with propidium iodide (seen here as red). The images are: 2457T VP-ve, *gtrII*::Tc^R (pBAD-IcsA) [RMA2234] untreated, (a) 0 min, (b) 30 min, (c) 60 min; 2457T VP-ve, *gtrII*::Tc^R (pBAD-IcsA) [RMA2234] TSP treated, (d) 0 min, (e) 30 min, (f) 60 min; 2457T VP-ve, *rmlD*::Km^R (pBAD-IcsA) [RMA2230], (g) 0 min, (h) 30 min, (i) 60 min. Within each image, an enlargement of a typical bacterium (two for (h)) is shown. The experiment was performed a least three times and representative results are shown.

ported by Steinhauer et al. [35], but that IcsA is also exported at the lateral regions of the cell surface but can only be detected there after TSP treatment.

3.4. Detection of newly synthesised IcsA on the cell surface of a R-LPS mutant

S. flexneri mutants such as RMA723 [2457T *rmlD*::Tc^R] whose LPS lacks Oag chains (R-LPS) have IcsA localised on polar and lateral regions of the cell surface. We compared detection of newly synthesised IcsA in the R-LPS strain 2457T VP-ve *rmlD*::Tc^R (pBAD-IcsA) [RMA2230] with that detected after TSP treatment of the smooth LPS strain 2457T VP-ve *gtrII*::Tc^R (pBAD-IcsA) as described above. After arabinose induction, IcsA was detected on the cell surface of 2457T VP-ve *rmlD*::Tc^R (pBAD-IcsA) at 30 min post induction with labelling occurring on lateral regions and the cell pole of approximately 50% of bacteria and at the cell pole only of approximately 20% of bacteria (Fig. 3h). At 60 min post induction, labelling was more intense, and IcsA was detected on lateral regions and cell pole of most bacteria (approximately 75% of bacteria) (Fig. 3i). A difference was seen in the intensity of IcsA labelling at 30 min post induction between 2457T VP-ve *rmlD*::Tc^R (pBAD-IcsA) cells (Fig. 3h) and TSP treated 2457T VP-ve *gtrII*::Tc^R (pBAD-IcsA) cells (Fig. 3e). This difference may be due to the complete absence of Oag chains in the R-LPS mutant (2457T VP-ve *rmlD*::Tc^R (pBAD-IcsA)) and the incomplete removal of Oag chains by TSP treatment of the smooth LPS strain (2457T VP-ve *gtrII*::Tc^R (pBAD-IcsA)) (Fig. 1, lane 2). Alternatively, arabinose induction may be more effective in the R-LPS strain. However, the localisation of newly exported IcsA in TSP treated smooth LPS bacteria is similar to that in R-LPS bacteria consistent with masking of IcsA by LPS Oag chains.

4. Discussion

Characterisation of *S. flexneri* mutants with altered LPS structure revealed that LPS Oag had an effect on IcsA production, localisation, and function in ABM, however, the mechanisms whereby altered LPSs affect IcsA are poorly understood. Based on our previous studies on *S. flexneri* 2457T *rmlD* and *wzz*$_{SF}$ mutants [23], we hypothesised that the Oag chains mask IcsA on the cell surface and interfere with its detection by antibodies.

Sf6 TSP treatment of strains either constitutively producing IcsA (2457T *gtrII*::Tc^R [RMA2218] and 2457T *gtrII*::Tc^R *wzz*$_{SF}$::Km^R [RMA2219]) or producing newly synthesised IcsA (2457T VP-ve *gtrII*::Tc^R (pBAD-IcsA) [RMA2234]) showed that IcsA could be detected on lateral regions of the cell surface on these bacterial cells. Hence, in addition to its known polar localisation, IcsA is also present on the lateral regions of the cell surface in both the wild-type and *wzz*$_{SF}$ mutant bacteria but cannot be detected due to the Oag chains preventing antibody binding to IcsA. These results have implications for understanding the phenotypic effect of the *wzz*$_{SF}$ and *rmlD* mutations, and the mechanism of IcsA export.

We reported that a *S. flexneri wzz*$_{SF}$ mutant (RMA696), unable to produce S-type Oag chains but still producing VL-type Oag chains, had reduced levels of IcsA and defective ABM [23]. Based on the effect of TSP treatment on detection of IcsA in 2457T *gtrII*::Tc^R *wzz*$_{SF}$::Km^R [RMA2219], we propose that the apparent decrease in cell surface IcsA seen in RMA696 [2457T *wzz*$_{SF}$::Km^R] is likely to be caused by the VL-type Oag chains masking IcsA and interfering with its detection by antibodies. It also seems likely that the VL-type Oag chains interfere with IcsA's function in nucleating ABM and is the mechanism responsible for the virulence defect caused by inactivation of the *wzz*$_{SF}$ gene. This is supported by the observation that *S. flexneri* strains unable to produce VL-type Oag chains do not have an ABM defect ([15]; Morona, R., Daniels, C. and Van Den Bosch, L., submitted for publication).

A model to explain IcsA export and polar localisation has been described in which LPS Oag chains act to restrict diffusion of IcsA away from the pole to lateral regions of the cell [35]. According to this model, the absence of Oag chains in R-LPS mutants results in increased diffusion IcsA and its circumferential localisation on the cell surface in these mutants. Additionally, it has been shown in an *Escherichia coli* hybrid system that Oag contributes to polar localisation of IcsA [36]. In this study, we have shown that newly synthesised IcsA can be detected on lateral regions of the cell. An alternative model to explain the role of Oag in polar localisation of IcsA is that although IcsA is predominantly exported to the cell pole, export also occurs at the lateral regions of the cell but is not detected due to the presence of LPS Oag chains. In R-LPS mutants, the absence of Oag chains allows IcsA to be detected circumferentially on the cell surface by indirect IF staining.

Since our data show that Oag chains can mask IcsA, it is intriguing that IcsA can be detected at all at the old cell pole. One possibility is that as IcsA is predominantly exported at the old cell poles, its high concentration at this site enhances its detection. In support of this hypothesis, we have previously shown by immunogold labelling that IcsA is more concentrated at one of the cell poles on the cell surface of the R-LPS *S. flexneri rmlD* mutant (RMA723) [23]. Alternatively, the old cell pole may have relatively more R-LPS and LPS with very short Oag chains than the lateral regions. However, as the distribution of LPS molecules on the cell surface of *S. flexneri* bacteria is unknown, this possibility remains to be investigated.

The masking of IcsA by LPS Oag chains in *S. flexneri* is not without precedent. Oag chains have been shown to

mask the presence of heterologous Oag chains co-expressed on the cell surface of *Salmonella* and *Shigella* bacteria [37,38]. LPS Oag chains have also been reported to mask *E. coli* outer membrane proteins, such as PhoE [39,40], the *Yersinia pseudotuberculosis* Inv protein [41], and can also interfere with HeLa cell invasion by enteropathogenic *E. coli* [42]. Hence, the topology of the outer membrane of *S. flexneri* strains such as 2457T must be finely balanced. While Oag chains are needed for IcsA to function in ABM, their length distribution must be such that they do not interfere with its activity.

Acknowledgements

This work was supported by a Project Grant from the National Health and Medical Research Council of Australia to R.M. Elizabeth Anderson is thanked for technical support. Robert Seckler and Ulrich Baxa are thanked for generously supplying Sf6 TSP. Judy Morona is thanked for reading the manuscript.

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