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Regulation of O-antigen chain length is required for *Shigella flexneri* virulence

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Summary

It is shown that *Shigella flexneri* maintains genetic control over the modal chain length of the O-antigen polysaccharide chains of its lipopolysaccharide (LPS) molecules because such a distribution is required for virulence. The effect of altering O-antigen chain length on *S. flexneri* virulence was investigated by inserting a kanamycin (Km)-resistance cassette into the *rol* gene (controlling the modal O-antigen chain length distribution), and into the *rfbD* gene, whose product is needed for synthesis of dTDP-rhamnose (the precursor of rhamnose in the O-antigen). The mutations had the expected effect on LPS structure. The *rol*::Km mutation was impaired in the ability to elicit keratoconjunctivitis, as determined by the Serény test. The *rol*::Km and *rfbD*::Km mutations prevented plaque formation on HeLa cells, but neither mutation affected the ability of *S. flexneri* to invade and replicate in HeLa cells. Microscopy of bacteria-infected HeLa cells stained with fluorescein isothiocyanate (FITC)-phalloidin demonstrated that both the *rol*::Km and *rfbD*::Km mutants were defective in F-actin tail formation: the latter mutant showed distorted F-actin tails. Plasma-membrane protrusions were occasionally observed. Investigation of the location of IcsA (required for F-actin tail formation) on the cell surface by immunofluorescence and immunogold electron microscopy showed that while most *rol* mutant bacteria produced little or no cell-surface IcsA, 10% resembled the parental bacterial cell (which had IcsA at one cell pole; the *rfbD* mutant had IcsA located over its entire cell surface although it was more concentrated at one end of the cell). That the O-antigen chains of the *rol*::Km mutant did not mask the IcsA protein was demonstrated by using the endorhamnosidase activity of Sf6c phage to digest the O-antigen chains, and comparing untreated and Sf6c-treated cells by immunofluorescence with anti-IcsA serum.

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Introduction

Shigella flexneri and related serovars/species are the causative agents of bacillary dysentery in humans. The disease is characterized by an acute inflammatory response in the large intestine followed by ulceration of the bowel and accompanied by a mucoid, bloody diarrhoea (Sansonetti, 1994). The initial stages of infection are now believed to involve invasion of M cells overlying Peyer's patches, followed by intracellular bacterial replication. The bacteria are then able to infect local macrophages whose death is triggered by apoptosis. The spread to other cells is by formation of plasma membrane protrusions containing bacteria which can penetrate adjacent cells. After lysis of the protrusion's double membrane the bacteria are released into the cytoplasm, where further intracellular replication can occur. The inflammatory response is the result of an influx of polymorphonuclear cells which damage the epithelial cell layer and facilitate bacterial access to the baso-lateral surface of epithelial cells (Sansonetti, 1994).

Numerous genes and gene products which are directly or indirectly involved in the pathogenic process have been identified (Hale, 1991). The IpaB, IpaC and IpaD proteins are directly involved at early stages of invasion and release from the entry vacuole. IcsA is required for intracellular motility via polar actin deposition (Bernardi *et al*., 1989; Goldberg *et al*., 1993; Lett *et al*., 1989) and IcsB is required for lysis of the double membrane of the protrusion after intercellular spread (Allaoui *et al*., 1992). These loci are encoded by the virulence plasmid. A number of chromosomal loci (*vac* genes) affecting virulence have been recently identified and characterized (Okada *et al*., 1991a,b). Mutants affected in *vacJ* are defective in intercellular spread and have a phenotype similar to that of *icsB* mutants (Suzuki *et al*., 1994). Apart from proteins acting in *Shigella* virulence, a role for lipopolysaccharides (LPS) has been demonstrated (Okada *et al*., 1991a; Okamura and Nakaya, 1977; Okamura *et al*., 1983). *Shigella* mutants with either spontaneous or transposon insertion mutations which alter the structure of LPS are not affected in invasion or intracellular replication, but are affected in their ability to move within epithelial cells and in their ability to spread to adjacent cells in a monolayer. This phenotype

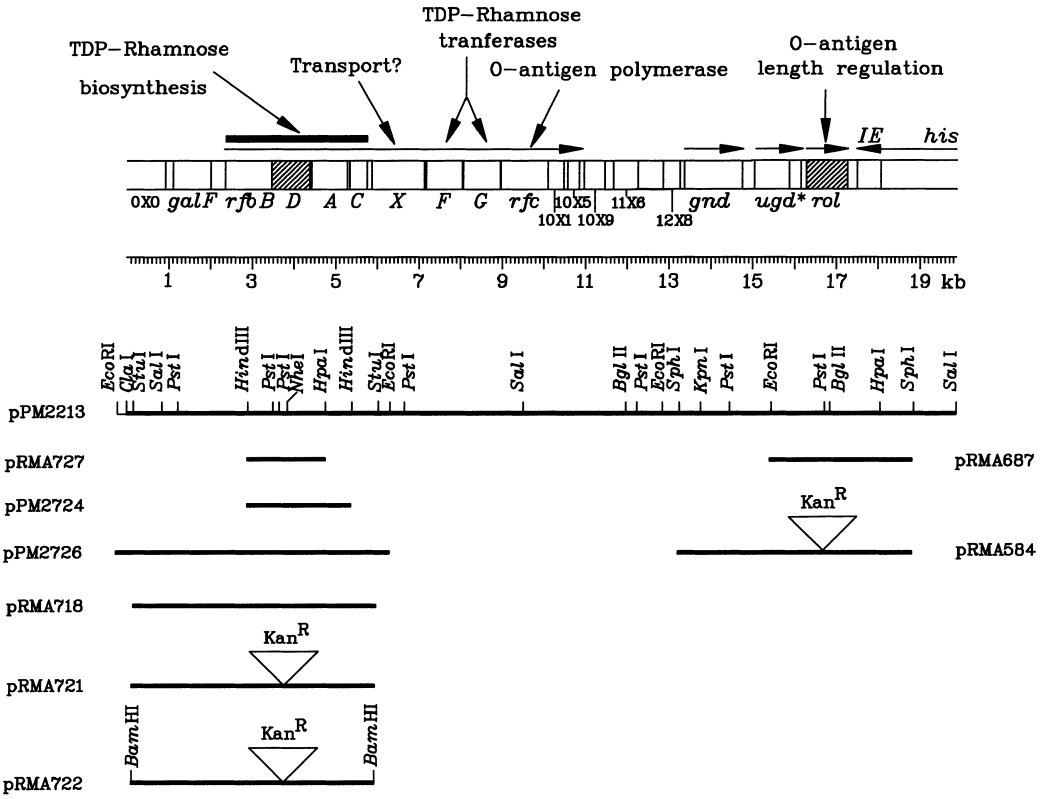

Fig. 1. The *S. flexneri rfb* region and plasmids used. The plasmids used were constructed as follows. Plasmids pPM2213, pPM2724, pPM2726 and pRMA584 have been described previously (Macpherson *et al*., 1991; 1994). Plasmid pRMA687 was constructed by subcloning the *Eco*RI–*Sph*I fragment shown from pRMA520 (Morona *et al*., 1995) between the corresponding sites in pBR322. Plasmid pRMA727 was constructed from pPM2724 by deletion of an *Hpa*I–*Eco*RV fragment. Plasmid pRMA718 was constructed by cloning the *Stu*I–*Stu*I fragment shown from pPM2726 into the *Hin*dII site of pUC1318 (Kay and McPherson, 1987). A kanamycin-resistance cassette (Km), derived from pUC4K (Pharmacia) and cloned as a *Pst*I fragment into the *Pst*I site of pUC1318, was excised as an *Xba*I fragment and cloned into a unique *Nhe*I site located in the *rfbD* gene of pRMA718 to give plasmid pRMA721. Plasmid pRMA722 was constructed by excising the insert in pRMA721 as a *Bam*HI fragment and cloning it into a unique *Bam*HI site in pCACTUS (Morona *et al*., 1995). The functions determined by various genes are indicated in the upper part of the Figure. Only the regions inserted in the plasmids are shown. Gene symbols were as described previously (Macpherson *et al*., 1994; Morona *et al*., 1994; 1995).

is exhibited by strains with mutations in *rfb* (blocking O-antigen production), *rfa* and *galU* mutations (affecting synthesis of the LPS core sugars), and *rfe* (blocking synthesis of O-antigen and enterobacterial common antigen (ECA)) (Okada *et al*., 1991a; Sandlin *et al*., 1995). The *rfb*::Tn*5* mutations are reported to result in altered IcsA production (Rajakumar *et al*., 1994). However, strains with a *galU* or an *rfe* mutation have unaltered IcsA production but have altered distribution of IcsA on the surface of *S. flexneri* cells relative to the polar location found in the wild-type strain (Sandlin *et al*., 1995).

Analysis of clones containing the cloned *rfb* region of *S. flexneri* identified a linked function involved in the control of LPS O-antigen chain length (Macpherson *et al*., 1991). This control of modal distribution of LPS O-antigen chain lengths was recently shown to be due to the *rol* gene (Morona *et al*., 1995). In this communication, it is shown that this distribution is required for virulence and provides a biological function for the Rol protein. The *rol* mutant was defective in intracellular movement and intercellular spread, and expressed reduced amounts of IcsA on its cell surface. A strain with a defined, non-polar insertion mutation in *rfbD* was also constructed to allow direct comparison with the *rol* mutant strain, and to clarify the effect of having LPS with no O-antigen chains on virulence-related properties.

Results

Construction of S. flexneri rol*::Km and* rfbD*::Km mutants*

S. flexneri 2a strain 2457T was used to construct a *rol*::Km mutant derivative (strain RMA696) using plasmid pRMA584 (Fig. 1) as detailed in the *Experimental procedures*. Southern hybridization with a digoxigenin (DIG)-labelled DNA fragment containing *rol* confirmed an insertion of the correct size (data not shown). The LPS produced by RMA696

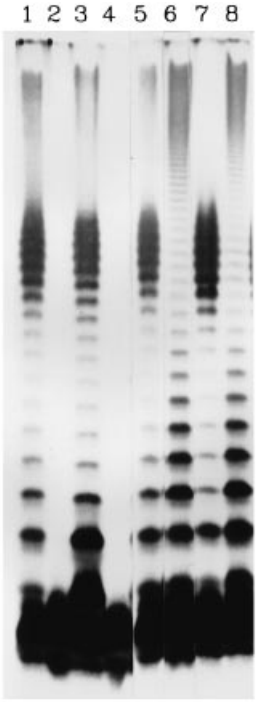

Fig. 2. Analysis of lipopolysaccharides of *S. flexneri* mutants. LPS samples were prepared by proteinase K treatment of the indicated strains, then electrophoresed in an SDS/20% polyacrylamide gel which was then silver stained. The strains in each lane were: lane 1, 2457T; lane 2, RMA723; lane 3, RMA728; lane 4, RMA725; lane 5, 2457T; lane 6, RMA696; lane 7, RMA729; lane 8, RMA730. Each lane contained $\approx 2 \times 10^8$ bacteria.

lacked the modal O-antigen chain length distribution of the parental strain (Fig. 2, lane 6) and the *rol*::Km defect could be complemented by pRMA687 (Fig. 2, lane 7).

RfbD is the last enzyme in the pathway for synthesis of dTDP-rhamnose, a precursor used in synthesis of the *S. flexneri*, rhamnose-containing, O-antigen tetrasaccharide repeat unit (Macpherson *et al*., 1994). Strain RMA723, lacking O-antigen and hence producing rough LPS (Fig. 2, lane 2), was constructed using pRMA722 to insert a kanamycin-resistance cassette into the *rfbD* gene (Fig. 1). Southern hybridization with a DNA fragment containing *rfbD* confirmed that it had an insertion of the correct size (data not shown). The *rfbD*::Km mutation in strain RMA723 could be complemented by introducing either plasmid pPM2726 or plasmid pPM2724 (Fig. 1 and data not shown). A further plasmid (pRMA727), which contained *rfbD* as the sole intact open reading frame (Fig. 1) also fully complemented the *rfbD*::Km mutation (Fig. 2, lane 3). The lack of polarity of the kanamycin-resistance cassette inserted in *rfbD* on *rfbA* and other distal *rfb* genes suggests that a promoter may be located in the intergenic region between *rfbD* and *rfbA* (Macpherson *et al*., 1994).

Virulence of S. flexneri RMA696 (rol::Km) in the Serény test

The Serény test (Sereny, 1957; Hartman *et al*., 1991) was used to assess the virulence of the *S. flexneri rol*::Km mutant strain RMA696. The parent strain 2457T caused a strong keratoconjuctivitis score (+++) 3 d after inoculation of $3–5 \times 10^8$ bacteria. In contrast, at the same dose and time-point, the isogenic *rol*::Km mutant (RMA696) was weakly positive (+). A control strain (RMA720), selected as Congo Red negative and hence lacking the *Shigella* virulence plasmid, was also tested and gave no reaction during the course of the assay. This result showed that maintainance of a modal chain length for the LPS O-antigen is required for full *S. flexneri* virulence in this model; the *rol*::Km mutation is attenuating despite having smooth LPS and unaltered LPS core sugar structure.

Infection of HeLa cells with S. flexneri rol*::Km and* rfbD*::Km mutants*

HeLa cell monolayers were infected with 2457T, RMA696 and RMA723. No difference was observed between the strains in terms of the proportion of HeLa cells invaded (data not shown). The ability of these strains to invade and grow within HeLa cells was further investigated by quantifying the invasion and intracellular bacterial growth in the presence of gentamicin and using plate counts. The results showed that all strains invaded to the same extent, and replicated at a similiar rate during the course of the assay (data not shown). Hence the *rol*::Km and *rfbD*::Km mutants are not defective in their ability to invade and replicate within HeLa cells. This is consistent with previous studies of rough LPS mutants (Okada *et al*., 1991a; Okamura and Nakaya, 1977; Rajakumar *et al*., 1994; Sandlin *et al*., 1995).

To determine if RMA696 and RMA723 were defective in intracellular movement and intercellular spread, as they were not invasion- and growth defective, their ability to form plaques on HeLa cells was assayed (Oaks *et al*., 1985). The parent strain, *S. flexneri* 2457T, formed plaques (2–3 mm in diameter) at high efficiency on the HeLa cell monolayer (Fig. 3A). In contrast, the *rol*::Km mutant did not give any plaques, even at high multiplicities of infection, and did not disturb the monolayer (Fig. 3B). The plaque-forming ability could be restored by complementing the *rol*::Km mutation with pRMA687 (Fig. 3C). This implied that the *rol*::Km mutation affected intracellular movement and/or intercellular spread of the bacteria. The *rfbD*::Km mutant also failed to form plaques (Fig. 3D); microplaques or foci of infection, reported by others, were not detected (Okada *et al*., 1991a; Sandlin *et al*., 1995). Introduction of pRMA727 into RMA723 restored its ability to form plaques (Fig. 3E).

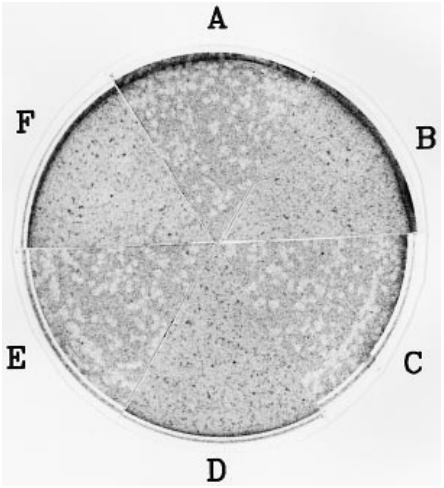

Fig. 3. Plaque formation on HeLa cells by *S. flexneri* strains. The indicated strains were plated at varying dilutions onto HeLa cell monolayers. A composite of infected HeLa monolayers is shown. The HeLa cells in each sector were infected with the following strains: A, 2457T; B, RMA696; C, RMA729; D, RMA723; E, RMA728; F, RMA720.

F-actin polymerization within HeLa cells

The ability of *S. flexneri* to move within infected cells and to spread to uninfected cells is correlated with the ability to form F-actin tails or comets (Bernardi *et al*., 1989; Goldberg *et al*., 1993). Intracellularly located 2457T, RMA696 and RMA723 were examined for their ability to form F-actin tails. HeLa monolayers were infected with these strains, and after 3 h of infection the cells on coverslips were fixed and stained with fluorescein isothiocyanate (FITC)-phalloidin to detect F-actin. The bacteria were detected by staining with anti-group antigen [3,4] serum, which reacts with the O-antigen, or antiserum raised to the rough LPS of RMA723 (*rfbD*::Km mutant). The latter was needed since RMA723 lacks O-antigen (and hence group antigen [3,4]) and is not stained by the highly specific anti-group antigen [3,4] serum used. Figure 4A shows that 2457T was able to form F-actin tails, and bacteria containing protrusions extending from the HeLa cell plasma membrane were readily seen. In contrast, HeLa cells infected with RMA696 (*rol*::Km) (Fig. 4C) produced few bacteria containing protrusions, but they were occasionally observed on some infected cells; although the infected HeLa cells had many bacteria, only the occasional cell had bacteria with F-actin tails. Strain RMA723 (*rfbD*::Km) was similar to RMA696 (Fig. 4E) in that infected HeLa cells formed few protrusions, and but a few of the bacteria within the cells had distorted F-actin tails. The observations are consistent with the results of Goldberg and Theriot (1995), who reported that an IcsA-producing *Escherichia coli* K-12 strain, which does not produce O-antigen, was able to form F-actin tails and was motile despite having IcsA located over its entire cell surface.

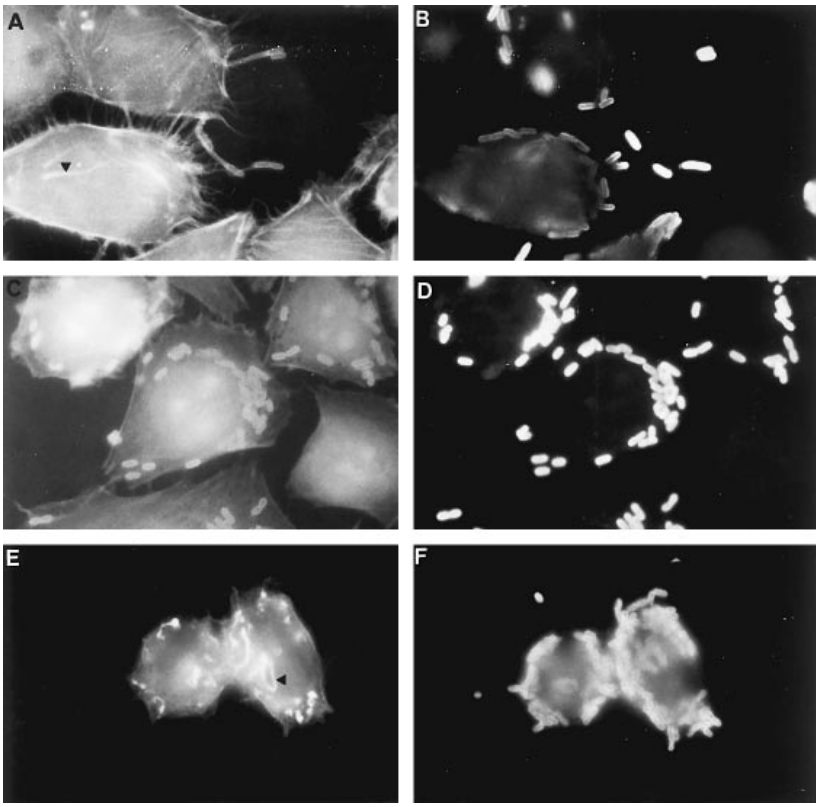

Fig. 4. Dual fluorescence staining of *S. flexneri* strains in HeLa cells to detect F-actin. Semi-confluent HeLa monolayers were infected with 2457T (A and B), RMA696 (C and D), and RMA723 (E and F). The infected cells were stained with FITC-phalloidin to detect polymerized F-actin (A, C, E). Bacteria were detected by staining with either rabbit anti-O-antigen, group antigen [3,4] antiserum (B, D), or rabbit anti-rough LPS serum (F), followed by a TRITC-conjugated goat anti-rabbit immunoglobulin secondary antibody. Arrowheads indicate F-actin tails (A) or distorted F-actin tails (E). Images were photographed with a 100× objective.

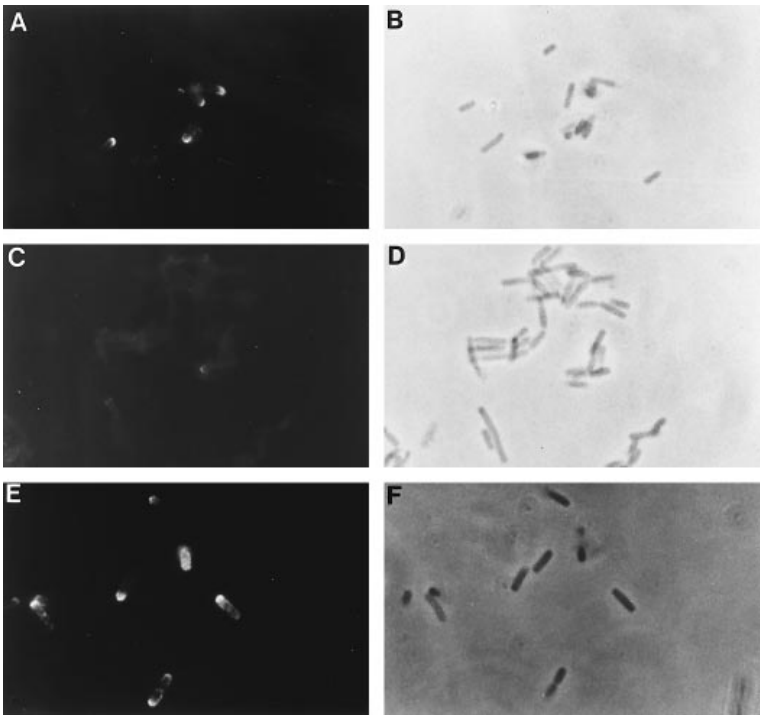

Fig. 5. Detection of IcsA on *S. flexneri* cells by indirect immunofluorescence. Cells of *S. flexneri* strains (2457T (A and B), RMA696 (C and D), RMA723 (E and F)) were stained with an anti-IcsA serum, and bound antibodies were detected with an FITC-conjugated goat anti-rabbit immunoglobulin secondary antibody (A, C, E). Panels B, D, and F are phase-contrast images of the fluorescence images in panels A, C, and E. Images were photographed with a 100× objective.

Cell-surface expression of IcsA

The experimental evidence indicated that the *rol*::Km and *rfbD*::Km mutations affected the ability of the bacteria to move intracellularly and spread intercellularly, and that F-actin tail formation was altered. As the IcsA protein has been shown to be essential for intracellular movement and intercellular spread (Bernardi *et al*., 1989; Lett *et al*., 1989), and initiation of F-actin tail formation (Goldberg and Theriot, 1995), we used an antiserum specific for IcsA to determine if its production was altered by the *rol*::Km and *rfbD*::Km mutations. The distribution of IcsA on the cell surfaces of 2457T, RMA696 (*rol*::Km) and RMA723 (*rfbD*::Km) was determined by indirect immunofluoresence and immunogold electron microscopy. Figure 5A shows that, as expected (Goldberg *et al*., 1993; 1994), a large proportion (80%) of 2457T bacteria bound anti-IcsA antibodies predominantly at cell poles. Most cells in the *rol*::Km mutant (RMA696) population showed little or no staining with anti-IcsA serum (Fig. 5C) and ≈10% had polar staining resembling the cells of the wild-type strain. The *rfbD*::Km mutant (RMA723) bacteria were stained by the anti-IcsA serum over the entire cell surface (Fig. 5E). The localization of IcsA on individual bacteria was assessed by immunogold electron microscopy. Nearly all the cells in the 2457T population had colloidal gold particles at the cell poles (Fig. 6A). In contrast, most RMA696 cells were either weakly labelled at one cell pole (45%) or were not labelled at all (45%) (Fig. 6B), and the remainder were labelled at one cell end and resembled wild-type cells. RMA723 cells bound colloidal-gold particles all over their cell surface, but one pole was usually more heavily labelled (Fig. 6C).

As the *rol*::Km mutation in RMA696 seemed to reduce production of IcsA protein, we compared IcsA production by RMA696 with the *rfbD*::Km mutant (RMA723) and the wild-type strain (2457T) by Western immunoblotting of whole-cell lysates, outer membranes, and culture supernatant proteins by using anti-IcsA serum (data not shown). Each strain produced IcsA protein (the 120 kDa form in the whole cells and outer membrane, and the 95 kDa form in the culture supernatant (Goldberg *et al*., 1993; Fukuda *et al*., 1995; Venkatesan *et al*., 1992)). No consistent difference in the amount of IcsA produced by the strains could be determined by the technique used. The IcsA produced by 2457T, RMA696, and RMA723 was located on the cell surface as shown by treatment of intact bacteria with proteinase K and Western immunoblotting (data not shown).

Use of Sf6 phage to probe the cell-surface location of IcsA

Although IcsA was poorly detectable on the cell surface of RMA696 by immuno-microscopy, this strain did produce IcsA which was readily detected by Western immunoblotting. Additionally, IcsA was detected at one cell pole in the wild-type strain, but was detected over the entire cell surface of the *rfbD*::Km mutant, RMA723. In order to

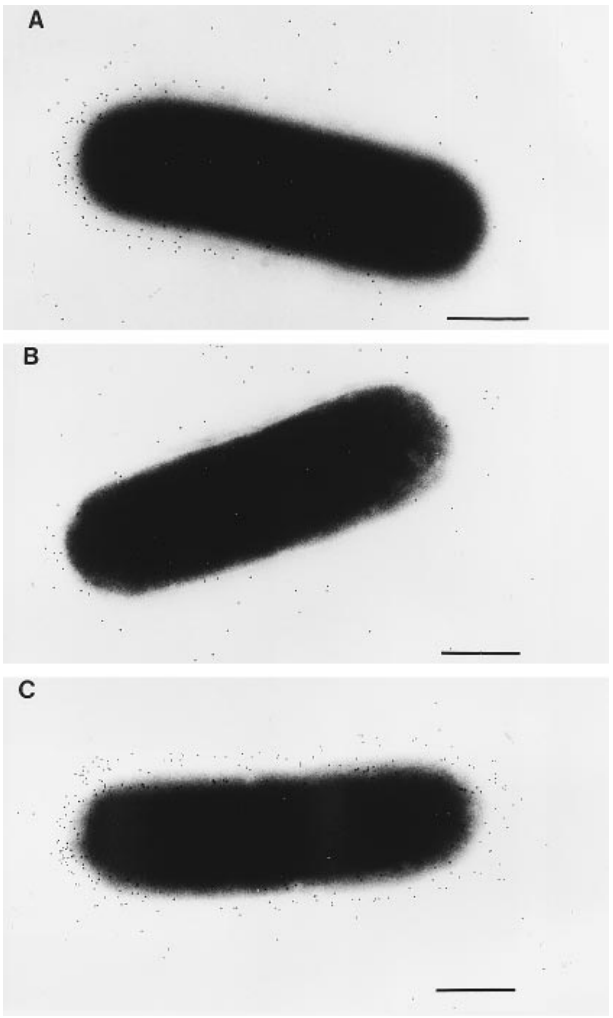

Fig. 6. Detection of IcsA on *S. flexneri* cells by immunogold electron microscopy. Mid-exponential phase cultures of the indicated *S. flexneri* strains were sequentially incubated with rabbit anti-IcsA serum, and with protein A-gold (10 nm), and viewed in a Philips CM100 transmission electron microscope. The strains in each panel are: A, 2457T; B, RMA696; C, RMA723. Colloidal gold particles can be seen as black dots. The lines repesent 500 nm.

investigate if the lengths of the O-antigen chains of the LPS molecules were in some way affecting the ability of anti-IcsA antibodies to bind to IcsA (Van der Ley *et al*., 1986), advantage was taken of the specific endorhamnosidase activity of the phage Sf6 tail spikes to degrade the Y serotype O-antigen *in situ* (Lindberg *et al*., 1978). Figure 7 shows the effect on LPS after incubation of formalin-fixed cells of SFL4 (*S. flexneri* Y serotype) and its isogenic *rol*::Km mutant derivative (RMA690) with phage Sf6c. In both cases (Fig. 7, lanes 5 and 6) the long O-antigen chains associated with the LPS molecules were removed. The residual LPS molecules migrate at a different position relative to those in the untreated control. This is expected, as the Sf6 endorhamnosidase does not cleave between the O-antigen tetrasaccharide repeats but instead recognizes a larger unit (Lindberg *et al*., 1978). Comparison of untreated and Sf6c-treated RMA690 showed no differences in the detection of IcsA by indirect immunofluorescence staining (Fig. 8, E and F). Hence, masking of IcsA by O-antigen chains is unlikely to explain the poor staining observed with anti-IcsA serum. Unmasking of cell-surface antigens by Sf6c treatment could be confirmed since RMA690, which does not react with anti-RMA723 rough LPS serum (Fig. 8C), did so after treatment with Sf6c (Fig. 8D). This indicated that the O-antigen chains prevent LPS-core specific antibodies from reaching their ligand. Correspondingly, treatment of RMA690 with Sf6c resulted in loss of staining with antiserum to O-antigen (Fig. 8B). When this experiment was performed with the wild-type parent strain (SFL4) similar results to those seen with RMA690 were obtained (data not shown); Sf6c phage treatment had no effect on the detection of IcsA at the cell poles. This suggested that the polar location of IcsA in wild-type strain *S. flexneri* is unlikely to be due to O-antigen masking of IcsA at other sites on the cell surface.

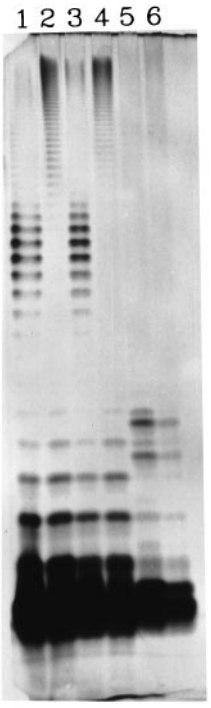

Fig. 7. Effect of Sf6c phage treatment of *S. flexneri* strains on LPS. *S. flexneri* strains SFL4 (Y serotype) and RMA690 (isogenic *rol*::Km derivative) were incubated with and without Sf6c phages. LPS samples were prepared by proteinase K treatment, and electrophoresed on a SDS-20%-polyacrylamide gel which was then silver stained. The cells in lanes 1 and 2 were not treated with formaldehyde. The samples in each lane were: lane 1, SFL4; lane 2, RMA690; lane 3, SFL4; lane 4, RMA690; lane 5, SFL4, Sf6c treated; lane 6, RMA690, Sf6c treated. Each lane contained $\approx 2 \times 10^8$ bacteria.

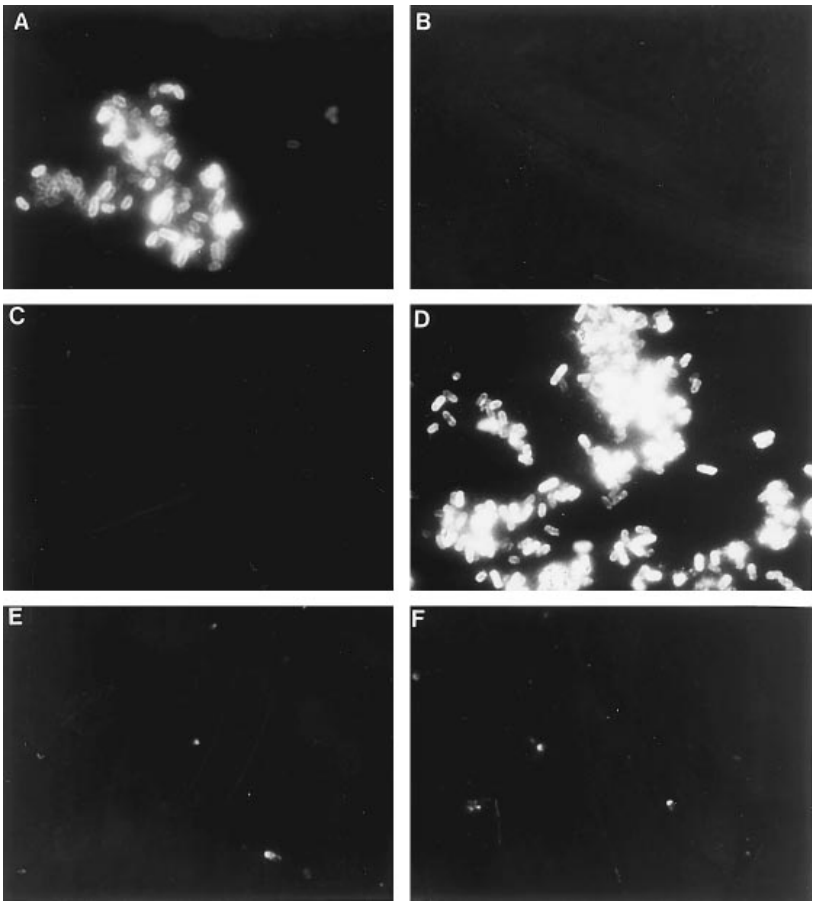

Fig. 8. Effect of Sf6c phages on detection of IcsA and O-antigen on RMA690 bacterial cells. *S. flexneri* strain RMA690 (Y serotype, *rol*::Km) was incubated without (A, C, E) and with (B, D, F) Sf6c phages. Antigens produced on the cell surface were then detected by indirect immunofluorescence.
A and B. Primary antibody, mouse monoclonal antibody Y5 detecting O-antigen and secondary antibody, FITC-conjugated goat anti-mouse immunoglobulin.
C and D. Primary antibody, rabbit anti-rough LPS serum and secondary antibody, FITC-conjugated goat anti-rabbit immunoglobulin.
E and F. Primary antibody, rabbit anti-IcsA antiserum and secondary antibody, FITC-conjugated goat anti-rabbit immunoglobulin.
Each field had several hundred bacterial cells. The images were photographed with a 100× objective.

Discussion

The *rol* gene of *S. flexneri* encodes a 36.5 kDa protein which is anchored into the cytoplasmic membrane by hydrophobic membrane-spanning domains located near its amino- and carboxy-termini, and the majority of the protein is located in the periplasmic space (Morona *et al*., 1995). The only phenotype previously ascribed to mutations in the *rol* gene was that the LPSs produced by the strains no longer have a modal distribution of O-antigen polysaccharide chains (most chains being 12–17 tetrasaccharide repeat units in length). Experiments described here demonstrate that the biological significance for maintaining a modal LPS O-antigen chain length distribution is that it is required for *S. flexneri* virulence. Although the data relate to the use of model systems, we cannot, of course, rule out the possibility that the observed effect may not be relevant to infection of humans. Nevertheless, the data on the *rol*::Km and *rfbD*::Km mutants, together with those of others using other mutants affecting LPS synthesis, show that O-antigen has a very important role in pathogenesis rather than only acting passively in the evasion of host defence mechanisms (Grossman *et al*., 1987; Joiner *et al*., 1982a,b; Liang-Takasaki *et al*., 1982). The data presented demonstrate that the mere presence of an O-antigen is insufficient for virulence, and that maintenance of a modal LPS O-antigen chain length distribution is essential for full virulence. The *rol* mutation seemed to affect the intracellular and intercellular motility of *S. flexneri*, and we showed that most cells (90%) in the *rol* mutant population had reduced IcsA on the cell surface. Although we have shown that the production of one cell-surface protein involved in virulence is altered, we cannot rule out the possibility that other proteins are also affected (see below).

The effect of the *rfbD*::Km mutation on strain 2457T, as determined in a number of assays of virulence properties, was similar to that reported for a *galU*::Tn*10* mutant which has a deep rough LPS because of the absence of the glucose form of the core sugar region (Sandlin *et al*., 1995). Paradoxically, the phenotype of the *rfbD*::Km mutant was different from that reported for strain 2457T with an *rfe*::Tn*10* mutation (Sandlin *et al*., 1995). The latter mutation resulted in a rough LPS, lacking O-antigen, as it is unable to incorporate *N*-acetyl-glucosamine into the O-antigen because of a deficiency in acyl carrier lipid:*N*-acetyl-glucosamine transferase activity. The *S. flexneri*

rfe::Tn*10* mutant was still able to form plaques, albeit of reduced size, and had IcsA partly distributed over its cell surface. The difference in phenotype may be explained either by differences in methodology, or by the fact that the *rfe* mutant used was leaky (it could be detected by immunofluorescence staining with an O-antigen-specific antiserum) (Sandlin *et al*., 1995).

One novel observation made in this study with RMA723 (*rfbD*::Km), and to a lesser extent with RMA696 (*rol*::Km), was that the strains were still able to form F-actin tails when growing in HeLa cells, and that the infected HeLa cells occasionally produced protrusions containing bacteria. This contrasts with the observations of others (Sandlin *et al*., 1995) who did not find F-actin tails or protrusions associated with the LPS-defective mutants. However, the observations described here are in agreement with the recent *in vitro* studies of Goldberg and Theriot (1995), who found that a polar location for IcsA was not needed for actin tail formation and motility. The results are also supported by the recent report (d'Hauteville *et al*., 1996) that *S. flexneri* producing a proteolytically non-cleavable mutant form of IcsA expressed the protein over the entire cell surface, and that such cells often had F-actin tails and movement perpendicular to the length of the cells resembling those reported in this study (Fig. 4). Although the location of IcsA on the bacteria growing inside HeLa cells has not been analysed, it was observed that ≈10% of the cells in an *in vitro*-grown culture of RMA696 expressed IcsA on their cell surface (see also for RMA690, Fig. 8). The presence of this subpopulation of cells in the RMA696 population is consistent with RMA696 giving a low-grade Serény reaction, and a low level of F-actin and protrusion formation in infected HeLa cells.

The effects of the *rol*::Km and *rfbD*::Km mutations are similar in many respects as they both affect localization of the IcsA protein on the cell surface. The mechanism whereby this occurs is unknown. In the absence of O-antigen, IcsA is located all over the bacterial cell, although immunogold electron microscopy showed that IcsA was more concentrated at one pole. d'Hauteville *et al*. (1996) recently reported that a mutated form of IcsA which is not released into the cell-culture supernatant is not polarly localized but is distributed over the cell surface. These authors speculated that proteolytic cleavage of IcsA is involved in determining its asymmetric distribution in the wild-type cell. We suggest that O-antigen affects this cleavage by altering either the conformation of IcsA at the cleavage site, or the production/activity of a protease active on IcsA. Alterations to the modal O-antigen chain length distribution, as caused by the *rol* mutation, could act on IcsA by a similar mechanism. IcsA is an autotransporter and is exported via an IgA1 protease-like export mechanism (Suzuki *et al*., 1995); an outer membrane-localized intermediate is integral to this process. It will be interesting to determine the effects of LPS mutations on IcsA export kinetics.

Proteins homologous to Rol have been detected in *E. coli* and *Salmonella enterica* serovars (Bastin *et al*., 1993; Batchelor *et al*., 1992). However, the construction and characterization of *rol* mutants in these bacterial species have not been reported. DNA sequencing of genes involved in biosynthesis of capsule and other polysaccharides has detected open reading frames with regions having sequence similarity with Rol (Bik *et al*., 1995 and data not shown). Hence, the Rol function may be relatively widespread. It will be interesting to determine if mutations in these genes affect the virulence of the respective bacteria. The maintenance of a modal LPS O-antigen chain length distribution is fairly ubiquitous amongst Gram-negative bacteria and probably represents an important adaption related to the functioning of various components of the bacterial cell surface. A clear example of this is found in *Aeromonas* species, in which the LPS O-antigen chains have almost uniform chain length and interact with the S-layer protein to anchor the surface array to the cell (Belland and Trust, 1985).

Experimental procedures

Bacterial strains, bacteriophages, and growth media

E. coli K-12 strain DH5α was used for routine cloning. *S. flexneri* strains SFL4 (Y serotype) and 2457T (2a serotype) were obtained from Dr A. B. Hartmann (Walter Reed Army Institute of Medical Research, Washington, DC). The construction of RMA696 (2457T *rol*::Km) and RMA723 (2457T *rfbD*::Km) is described below, and RMA690 (SFL4 *rol*::Km) was constructed using the same method. RMA720 was selected as a white colony of 2457T on Congo Red agar. RMA729 is RMA696 with pRMA687, RMA728 is RMA723 with pRMA727, RMA730 is RMA696 with pBR322, and RMA725 is RMA723 with pACYC184. Unless otherwise indicated, all bacterial strains were grown at 37°C in Luria Broth (LB) or LB agar (Morona *et al*., 1995). *S. flexneri* strains to be used in HeLa cell plaque assays were grown for 18 h at 37°C in Penassay Broth (Difco) and subcultured 1/50 in fresh Penassay broth and grown for 1.5 h at 37°C. Congo Red agar was Tryptic Soy Broth (Difco) with 1.5% (w/v) Bacto agar (Difco) and 0.05 (w/v) Congo Red (Ajax Chemicals). *S. flexneri* strains were routinely streaked on Congo Red agar to monitor retention of the virulence plasmid. The antibiotics ampicillin, kanamycin, and chloramphenicol were used at 50, 50, and 25 μg ml^{-1}, respectively. Bacteriophage Sf6c, described recently, was propagated on *S. flexneri* Y serotype strain PE577 (Morona *et al*., 1994). The phage was purified by banding in a CsCl equilibrium centrifugation gradient, and, after dialysis against TM (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$) buffer, was stored at 4°C.

Antisera

An affinity-purified, rabbit polyclonal anti-IcsA serum was obtained from Professor P. J. Sansonetti (Institut Pasteur,

Paris) (Goldberg *et al*., 1993). A second rabbit anti-IcsA serum, which behaved like the affinity-purified anti-IcsA serum, was obtained by hyperimmunizing a rabbit with IcsA purified by two cycles of SDS–PAGE. The protein was overexpressed in a derivative of RMA723 (cured of the virulence plasmid) harbouring a plasmid with *icsA* under the control of an IPTG-inducible promoter. This plasmid was constructed by cloning *icsA,* amplified by the polymerase chain reaction (PCR) from pD10 (Lett *et al*., 1989) using primers with suitable restriction enzyme sites, into pTTQ181 (Stark, 1987) (data not shown). Polyclonal antisera to detect O-antigen (type antigen II, group antigen 3,4) were purchased from Denka Seiken. The mouse monoclonal antibody MASF-Y5, which detects the group antigen [3,4] of the O-antigen, was a hybridoma supernatant obtained from Dr N. Carlin (Stockholm). A polyclonal antiserum to *S. flexneri* rough LPS was raised by hyperimmunization of a rabbit with heat-killed (2 h, 100°C) cells of strain RMA723.

DNA methods

Preparation of plasmid and chromosomal DNA, restriction enzyme digestion, agarose gel electrophoresis, and DNA ligation, transformation and electroporation were performed as described (Morona *et al*., 1995). DNA fragments were isolated from agarose gels using Geneclean II (BIO101 Inc.), and Southern hyridization was performed at high stringency with DNA probes random primer-labelled with DIG-UTP (Boehringer Mannheim) according to Sambrook *et al*. (1989). Detection of bound probe was by chemiluminescence (Boehringer Mannheim).

Construction of chromosomal mutations

Strains in which the kanamycin-resistance cassette was inserted in the chromosomal *rol* (RMA696) or *rfbD* (RMA723) genes were constructed by use of plasmids based on a chloramphenicol-resistance-conferring, temperature-sensitive suicide vector (pCACTUS) also carrying the *sacB* gene (C. Clark *et al*., in preparation) as described by Morona *et al*. (1995). In brief, plasmid pRMA584 or pRMA722 was electroporated into a *S. flexneri* strain, and kanamycin- and chloramphenicol-resistant colonies were selected at 30°C. These were grown at 42°C with kanamycin selection, then plated at 37°C on LB agar lacking NaCl but containing 5% (w/v) sucrose and kanamycin (to select colonies in which the plasmid had been lost but in which an allelic exchanged had occurred). The presence of the *S. flexneri* virulence plasmid was monitored throughout the procedure by plating on Congo Red agar.

Lipopolysaccharide methods

The production of O-antigen was routinely assessed by agglutination tests on glass slides, using bacteria in a saline suspension and specific polyclonal O-antigen antisera. LPS profiles were examined by SDS–PAGE and silver staining (Tsai and Frasch, 1982) as described recently (Morona *et al*., 1995) using proteinase K-treated cells (Hitchcock and Brown, 1983).

Infection of HeLa cells

Semi-confluent monolayers of HeLa cells on glass coverslips were infected with *S. flexneri* strains essentially as described by Hale and Formal (1981). After 3 h, the monolayers were washed with phosphate-buffered saline (PBS), fixed with 0.8% (v/v) glutaraldehyde, 2.0% (w/v) formaldehyde in PBS, and either stained with Giemsa for examination by light microscopy or stained for immunofluorescence microscopy. For quantification of intracellular growth, replica infected monolayers were lysed at various times with Triton X-100 in PBS prior to plating on LB agar to enumerate bacterial growth. The ability of *S. flexneri* strains to form plaques on HeLa cells was determined as described by Oaks *et al*. (1985).

Double fluorescence staining of F-actin and bacteria in HeLa cells

After fixation, infected HeLa monolayers on coverslips were treated with 0.1% (v/v) Triton X-100 in PBS for 3 min to permeabilize their plasma membranes. Coverslips were incubated for 20 min in a 1/100 dilution of antiserum to either O-antigen or rough LPS to detect bacteria. After three washes in PBS, bound immunoglobulin was detected by incubation for 20 min with a 1/100 dilution of goat anti-rabbit tetramethylrhodamine B isothiocyanate (TRITC)-conjugated immunoglobulin (Sigma Chemical Co.). F-actin was detected by staining the coverslips with 2 μg ml^{-1} FITC conjugated with phalloidin (Sigma) in PBS. Coverslips were washed three times in PBS, mounted in Moviol 4-88, and sealed with clear nail polish. Infected cells and bacteria were photographed with Kodak TMAX400 film using an Olympus fluorescence microscope also equipped with a phase-contrast condensor.

Labelling of bacteria for indirect immunofluorescence and immunogold microscopy

Bacterial culture (1.0 ml) grown to mid-exponential phase was centrifuged (Heraeus Biofuge 15, 20 800 $\times$ *g* for 1 min), washed in saline, and resuspended in 0.1 ml of PBS. Antisera to IcsA, O-antigen, or rough LPS were added at a 1/100 dilution. After incubation for 20 min the cells were washed twice with PBS, resuspended in 0.1 ml of PBS, and an FITC-conjugated secondary antibody (goat anti-rabbit or goat anti-mouse (Sigma)) was added at a 1/100 dilution. After incubation for a further 20 min, the cells were centrifuged, washed thrice with PBS, washed once with water, centrifuged, and resuspended in 30 μl of Milli-Q water; 1 μl was air-dried on a glass slide and mounted as described above. Immunogold electron microscopy was performed as described by Attridge *et al*. (1990). Bacteria (in PBS with 0.1 mg ml^{-1} BSA) were treated with rabbit anti-IcsA serum (1/150 dilution), then with protein A-gold (10 nm, ICN) directly on Formvar-coated, nickel grids which had also been lightly coated with carbon. Specimens were visualized using a Philips CM100 transmission electron microscope.

Treatment of bacteria with bacteriophage Sf6c

Bacterial culture (10 ml) was washed twice, resuspended in 5 ml of saline, and then fixed in 1% (w/v) formaldehyde for

1 h at 37°C, with shaking every 15 min. The bacteria were then washed again three times and resuspended in saline; 0.1 ml of bacteria ($\approx 1 \times 10^{10}$) was mixed with 1×10^{11} plaque-forming units of Sf6c phage diluted in LB. After incubation at 37°C for 30 min, the cells were centrifuged, washed twice in saline, and resuspended in 0.2 ml of saline. The cells were used for LPS analysis by SDS–PAGE or indirect immunofluorescence.

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