

# An O antigen can interfere with the function of the *Yersinia pseudotuberculosis* invasin protein

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## Summary

*Escherichia coli* strains harbouring the *Yersinia pseudotuberculosis* *inv* gene are able to enter cultured mammalian cells. We show here that this property is not shared by all enteric bacteria, since *Shigella flexneri* 2a cured of its virulence-associated plasmid and harbouring the *inv* gene is unable to enter mammalian cells efficiently. Mapping studies showed that the region of the chromosome responsible for this phenotype includes *rfaB*, a locus involved in the production of O antigen. *S. flexneri* 2a strains that express O antigen were unable to enter mammalian cells, even though invasin was efficiently expressed and localized, showing that this structure interferes with invasin activity. The O antigen either masks invasin or sterically hinders the ability of the mammalian cell receptor to bind this protein.

## Introduction

*Yersinia pseudotuberculosis* is an enteroinvasive bacterium that is able to enter normally non-phagocytic cultured mammalian cells (Bovallius and Nilsson, 1975; for a review, see Miller *et al.*, 1988). One of the pathways for uptake into mammalian cells is promoted by invasin, the product of the *inv* locus (Isberg, 1989; Miller and Falkow, 1988). Invasin is expressed on the surface of *Escherichia coli* strains harbouring *inv*, and expression of this 103 kDa protein is sufficient to convert the normally innocuous *E. coli* K12 strain into a microorganism that can efficiently enter mammalian cells (Isberg *et al.*,

1987). Uptake of bacteria into mammalian cells via the *inv* pathway is initiated by the binding of the carboxyl terminal region of invasin to at least one member of the  $\beta_1$  chain integrin family of cell-adhesion molecules found on the host cell surface (Isberg and Leong, 1990; Leong *et al.*, 1990). The results from these studies imply that any microorganism that efficiently localizes invasin on its surface should bind and enter cultured mammalian cells encoding the appropriate receptors.

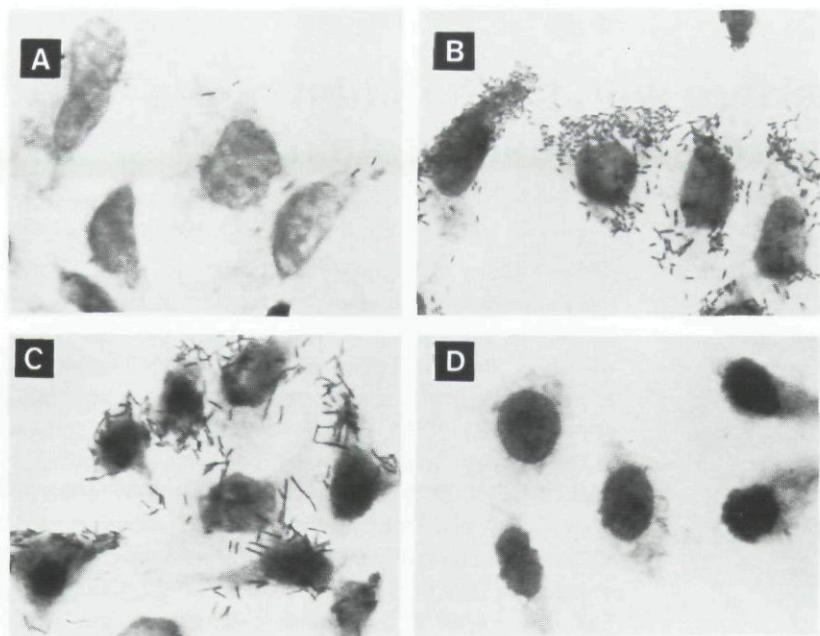
Enteric bacteria other than enteropathogenic *Yersinia* species are able to penetrate cultured cells, although the factors involved in the uptake process are less well-characterized (Maurelli *et al.*, 1985; Elsinghorst *et al.*, 1989; Galan and Curtiss, 1989). One such virulent bacterium is *Shigella flexneri* (LaBrec *et al.*, 1964; Maurelli and Sansonetti, 1988). As is true of all *Shigella* species, uptake of *S. flexneri* into cultured cells requires the presence of a large virulence-associated plasmid that contains a contiguous 37 kb region required for cellular invasion (Sansonetti *et al.*, 1982; Hale *et al.*, 1983). This plasmid, as well as three isolated segments of the *Shigella* chromosome, is required for expression of virulence in animal models, and it is these four regions of DNA that primarily distinguish *Shigella* species from *E. coli* K12 (Sansonetti *et al.*, 1983). *S. flexneri* clearly has the potential to enter mammalian cells when expressing the appropriate genes.

The extensive sequence homology between *E. coli* K12 and *S. flexneri* 2a suggests that genes expressed in *E. coli* should be efficiently expressed in *S. flexneri* as well. Therefore the introduction of the *inv* locus into a *Shigella* strain cured of its virulence-associated plasmid should allow the microorganism to efficiently enter mammalian cells. Surprisingly, we found that *S. flexneri* strains harbouring *inv* were defective for entry into cultured cells in spite of the fact that invasin is efficiently synthesized in this species. This report describes the nature of the defect and the identification of a host factor that influences the function of invasin.

## Results

### *Identification of a region of the *S. flexneri* chromosome responsible for the invasion defect*

The *Y. pseudotuberculosis* *inv* locus harboured on plasmid pRI203 (Isberg and Falkow, 1985) was introduced



**Fig. 1.** Penetration-deficient *S. flexneri* is unable to bind HEp-2 cells. Bacterial strains harbouring the plasmid pRI203 (*inv*<sup>+</sup>), treated as in the legend to Table 1, were incubated at 37°C for 90 min with HEp-2 cells grown on glass coverslips. The coverslips were then washed, fixed, and treated with Giemsa stain prior to microscopic observation. Displayed here are the *S. flexneri* strains FB1 (P1<sup>R</sup>) (A) and FB14 (P1<sup>R</sup>) (B) as well as the *E. coli* strains LE392 (pRI203 *Inv*<sup>+</sup>) (C) and LE392 (*Inv*<sup>-</sup>) (D). Note that few bacteria are associated with the mammalian cells in Panel A, even though FB1 harbours an intact *inv* locus.

into a *S. flexneri* 2a strain that had been cured of its virulence-associated plasmid (Sansonetti *et al.*, 1983). A strain so constructed, FB1, was tested for its ability to enter cultured HEp-2 cells using a standard gentamicin-killing assay to quantitate intracellular bacteria (*Experimental procedures*). The *S. flexneri* strain FB1 was approximately 20- to 40-fold less efficient in terms of entry than two different *E. coli* strains harbouring pRI203 *Inv*<sup>+</sup> (Table 1B), although it was more proficient in terms of cellular penetration than the parental *S. flexneri* strain M4243A<sub>1</sub>. This defect in entry was apparently due to an inability of the *inv*-encoding bacteria to bind the mammalian cell receptor, since Giemsa-stained fixed preparations of HEp-2 cells incubated with the *S. flexneri* strain showed few bacteria associated with the mammalian cells (Fig. 1).

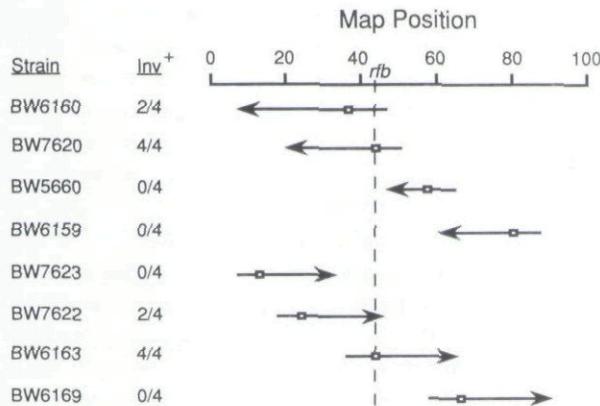
The chromosomal region responsible for the low level of entry found in the *Shigella* strain was mapped in order to determine the nature of the defect. These experiments were facilitated by the high degree of homology between *S. flexneri* and *E. coli*, which allows for efficient inter-species recombination (Falkow *et al.*, 1963). This approach has been used extensively in the past, and has been particularly useful for the mapping of temperature-sensitive mutations as well as for identifying regions of the *Shigella* chromosome that are critical for virulence (Low, 1973; Sansonetti *et al.*, 1983). The invasion-defective FB1 was subjected to interrupted matings with a series of *E. coli* K12 Hfr strains containing origins of transfer at

**Table 1.** Phage P1-resistant *S. flexneri* strains harbouring the *inv* locus are able to penetrate HEp-2 cells.

A. P1 <sup>R</sup> colonies picked	Inv <sup>+</sup>	Inv <sup>-</sup>
22	22	0
B. Strain	Entry efficiency (%)	
MC4100	0.019 ± 0.006	
LE392	0.01	
MC4100(pRI203)	19.0 ± 2.0	
LE392(pRI203)	13.1	
M4243A <sub>1</sub>	0.005 ± 0.003	
FB1	0.34 ± 0.03	
FB11	0.48 ± 0.06	
FB14	10.5 ± 2.1	
FB17	6.5 ± 1.4	

A. P1-resistant derivatives of FB1 were isolated as described (*Experimental procedures*). The mutants were then tested in the gentamicin survival assay for the ability to penetrate HEp-2 cells, using FB11 and MC4100 (pRI203) as control *Inv*<sup>-</sup> and *Inv*<sup>+</sup> strains, respectively (*Experimental procedures*). For the control *Inv*<sup>+</sup> strain, survival in the presence of gentamicin was approximately 19% of input bacteria, while for the control *Inv*<sup>-</sup> strain, survival was approximately 0.4% of input. P1-resistant mutants were judged to be *Inv*<sup>+</sup> if survival in the presence of gentamicin was at least 10-fold higher than that found for the control *Inv*<sup>-</sup> strain.

B. Bacteria growing in mid-logarithmic phase were assayed for the ability to enter HEp-2 cells as described (*Experimental procedures*). M4243A<sub>1</sub> is the parental *S. flexneri* 2a strain cured of its virulence plasmid. FB14 and FB17 are spontaneous P1-resistant mutants picked among the 22 derivatives assayed in (A). The data shown here are from a representative experiment. The entry efficiency was the mean of four determinations for each strain, except in the case of LE392 derivatives, for which the average of two determinations was used.



**Fig. 2.** The function of invasin is restored in *S. flexneri* strains carrying the *rfb* region of *E. coli* K12. FB1, a *S. flexneri* 2a strain cured of the virulence plasmid and harbouring pRI203 (*inv*<sup>+</sup>), was mated for one hour with a series of *E. coli* K12 Hfr strains containing Tn10 insertions near their origins of transfer. Tet<sup>R</sup> Cm<sup>R</sup> recombinants were selected and four colonies from each mating were screened for the ability to penetrate HEp-2 cells (Experimental procedures). Denoted above are Hfr strain numbers, the map position on the *E. coli* chromosome of each Hfr, the direction of transfer of each Hfr, and the site of Tn10 insertion transferred to *S. flexneri*. Arrowhead: origin and direction of transfer; open box: site of Tn10 insertion; Inv<sup>+</sup>: number of *S. flexneri* recombinants that are able to penetrate HEp-2 cells at similar efficiency to *E. coli* K12 strain harbouring invasin. Lines between arrowheads and boxes are regions of chromosome transferred prior to transfer of Tn10.

diverse sites (Fig. 2). Each Hfr strain harboured a Tn10 (tetracycline-resistance) insertion downstream from the origin of transfer (Fig. 2), allowing direct selection for transfer of *E. coli* DNA near the site of insertion of the drug-resistance element. Inasmuch as *E. coli* strains harbouring *inv* are able to enter mammalian cells efficiently, recombinants from some of these crosses should contain *E. coli* chromosomal DNA that rescues the entry defect.

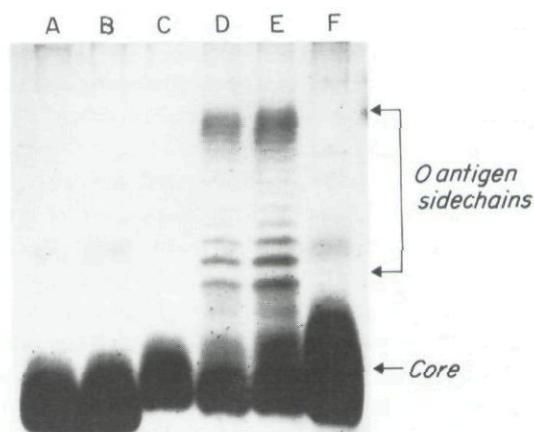
*S. flexneri* FB1 (Cm<sup>R</sup>) was mated with 16 different Hfr (Tet<sup>R</sup>) strains for one hour, and Tet<sup>R</sup> Cm<sup>R</sup> recombinants were selected. Recombinants were then assayed for the ability to enter mammalian cells. Twelve of the crosses yielded recombinants that retained the parental *S. flexneri* defect and were inefficient at entering mammalian cells. Examples of four such crosses are shown in Fig. 2, and represent recombinants derived from the majority of the *E. coli* chromosome. Four other crosses, however, did yield recombinants that had become proficient at entering mammalian cells to an extent comparable to *E. coli* strains harbouring *inv* (Fig. 2). Linkage to the zed::Tn10 insertion at 43 min on the map seemed particularly tight, because when this marker was selected in matings with BW6163 (Fig. 2) only one Tet<sup>R</sup>Cm<sup>R</sup> recombinant out of 40 screened retained the entry phenotype of the parental *Shigella* organism (data not shown). Therefore *Shigella* hybrids in which the chromosome around 43 min had been

replaced with *E. coli* sequences were able to enter mammalian cells efficiently.

#### Entry-proficient *Shigella* derivatives are defective for production of O antigen

Previous studies have shown that three regions of chromosomal DNA distinguish virulent *Shigella* species from innocuous *E. coli* strains (Sansonetti *et al.*, 1983). One of these regions is near the zed::Tn10 insertion and is due to the *S. flexneri* *rfb* locus, encoding O antigen (Sansonetti *et al.*, 1983). In order to determine if the recombinants that entered mammalian cells no longer synthesized *S. flexneri* 2a O antigen, lipopolysaccharide (LPS) was isolated from a variety of strains and analysed by gel electrophoresis (Fig. 3). *Shigella* recombinants that were able to enter HEp-2 cells yielded O-antigen profiles that were similar to those of *E. coli* K12, indicating that *Shigella* O antigen was not produced (Fig. 3, compare lanes C and F). The one recombinant that we were able to obtain from crosses with BW6163 (zed::Tn10) which remained defective for cellular penetration, on the other hand, retained an O-antigen profile that was identical to that of the parental *Shigella* strain (Fig. 3, compare lanes D and E). These results implicated the *Shigella* O antigen as an antagonist of cellular penetration.

For further support of the connection between *Shigella* O-antigen production and the defect in cellular penetration, we sought to isolate spontaneous single-step mutations in *S. flexneri* unable to produce O-antigen, and to determine if such mutants were able to enter mammalian cells efficiently. To this end, we isolated spontaneous *S. flexneri* mutants that were resistant to phage



**Fig. 3.** Penetration-proficient *S. flexneri* strains harbouring the *inv* locus lack the O side chain. LPS was isolated from *S. flexneri* derivatives as described (Westphal and Jann, 1965; Experimental procedures), fractionated by electrophoresis on a 13% SDS-polyacrylamide gel (Sansonetti *et al.*, 1983), and visualized by silver staining. Displayed are: A, FB17 (Inv<sup>+</sup>); B, FB14 (Inv<sup>+</sup>); C, FB12 (Inv<sup>+</sup>); D, FB11 (Inv<sup>-</sup>); E, FB1 (Inv<sup>-</sup>); and F, *E. coli* strain LE392 (pRI203 Inv<sup>+</sup>). FB17 and FB14 are spontaneous P1-resistant derivatives of FB1.

P1vir, a phage that adsorbs to the LPS core structure (Nishijima *et al.*, 1981; Coleman and Deshpande, 1985). Mutants that are resistant to phage P1vir should be unable to synthesize a complete core oligosaccharide to which the O side chain is linked, and thus should be defective for production of O antigen on the bacterial cell surface. Twenty-two such P1<sup>R</sup> mutants were isolated and tested for their ability to enter mammalian cells. In contrast to the parental *S. flexneri* strain, each of the phage-resistant mutants was able to enter mammalian cells efficiently (Table 1A). The entry efficiency of two such mutants is displayed in Table 1B. The *S. flexneri* P1<sup>R</sup> strains harbouring inv were 15- to 25-fold more efficient at entering mammalian cells than the parental P1<sup>S</sup> strain (Table 1B, FB14 and FB17) and were comparable in terms of entry efficiency to the *E. coli* strain harbouring inv (Table 1B, MC4100(pRI203)). These two spontaneous mutants were then analysed for their ability to produce O antigen by gel electrophoresis (Fig. 3). Neither mutant synthesized an O side chain and, as expected, each had a core region with an apparent molecular weight that was smaller than that of the parental *Shigella* core oligosaccharide (Fig. 3, compare lanes A and B with lane E). We conclude that we can cure *S. flexneri* strains harbouring inv of their entry deficiency by isolating mutants resistant to phage P1, and that such mutants do not synthesize O antigen.

#### Invasin is efficiently expressed and functions normally in extracts of *S. flexneri*

It is clear that the defect in entry of *S. flexneri* strains harbouring inv could be due to (i) depression in synthesis of invasin; (ii) improper localization of the protein; (iii) a block in the activity of invasin; or (iv) the presence of O antigen sterically hindering the animal cell receptor from interacting with surface-localized invasin. These possibilities were investigated in order to determine the nature of the entry defect caused by expression of O antigen.

The plasmid pDV7 was introduced into a series of strains in order to assay for expression and extracytoplasmic localization of invasin. This plasmid harbours the complete inv gene fused at its last codon to the reading frame corresponding to the mature *E. coli* phoA sequence, encoding alkaline phosphatase. The relative levels of expression of the inv locus in each strain could be easily determined by measuring the alkaline phosphatase activity of the resulting inv-phoA hybrid (Hoffman and Wright, 1985; Manoil and Beckwith, 1985). Furthermore, active alkaline phosphatase requires that the hybrid be extracytoplasmically localized, so any reduction in activity could also indicate defective localization (Maoil and Beckwith, 1987). As can be seen in Table 2, expression of the inv-phoA fusion was slightly lower in *S. flexneri* than in *E. coli* (compare LE392 (pDV7) with FB21). This effect,

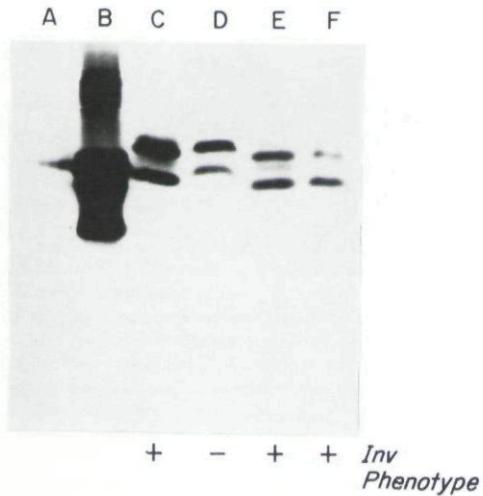
however, was clearly not the cause of inefficient entry, since the two P1<sup>R</sup> *S. flexneri* mutants that were entry-proficient and synthesized no O antigen were indistinguishable from their entry-deficient parent with regard to alkaline phosphatase activity (Table 2, compare FB21 with FB24 and FB27). Therefore, deficits in synthesis or extracytoplasmic localization of invasin do not appear to be the causes of inefficient entry. In support of this contention is the analysis of steady-state protein levels of native invasin, as determined by probing with immunoblots (Fig. 4). In concert with the assay of alkaline phosphatase fusions, there does not appear to be a reduction in the amount of invasin synthesized in *S. flexneri* strains harbouring O antigen (Fig. 4, compare lane D with E and F), with both the P1<sup>S</sup> and P1<sup>R</sup> derivatives expressing similar levels of the native invasin protein.

The ability to express invasion efficiently and export it out of the cytoplasm does not necessarily mean that the protein synthesized in the penetration-deficient *S. flexneri* strains retains bona fide invasin activity. It is conceivable that the presence of the O antigen could interfere with proper folding of the protein and prevent the cell-binding domain of invasin from assuming the necessary structure for binding the integrin receptor. To investigate this possibility, membranes were prepared from sonicates of *S. flexneri* strains harbouring the inv locus, and the ability of invasin to bind animal cells was tested. Equal amounts of total membrane protein from each strain were gel fractionated, and a filter replica of the gel was probed with live HEp-2 cells to assay for mammalian cell attachment to invasin (Isberg and Leong, 1988). Invasin synthesized by the *S. flexneri* strain containing O antigen was able to bind mammalian cells when it was extracted from the membrane (Fig. 5, lane D). As was true for all strains harbouring inv, multiple bands of cell attachment could be observed (Isberg and Leong, 1988; Fig. 5, lanes B to F), and the bands that appeared were dependent on the presence of invasin (Fig. 5, lane A). Although the presence of O antigen

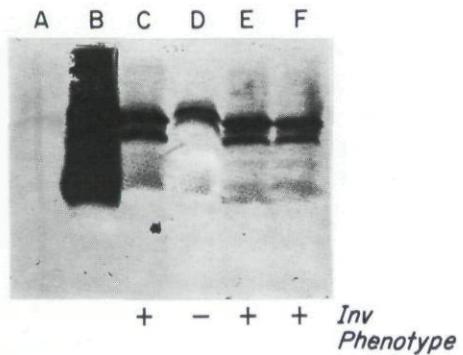
**Table 2.** The O-antigen phenotype does not affect expression of invasin in *S. flexneri*.

Strain	Alkaline phosphatase activity
LE392	1.06
LE392(pDV7)	31.6
FB1(pRI203)	0.62
FB21	22.6
FB24(P1vir <sup>R</sup> )	18.2
FB27(P1vir <sup>R</sup> )	17.2

Strains harbouring pDV7, a plasmid containing an inv-phoA gene fusion, were assayed for alkaline phosphatase activity under the control of the inv regulatory signals. Alkaline phosphatase assay and units of activity were as described (Maoil and Beckwith, 1985). Note that FB21 is a phage P1-sensitive *S. flexneri* derivative, whereas FB24 and FB27 are phage P1-resistant.



**Fig. 4.** Invasin is synthesized in strains encoding the *S. flexneri* 2a O antigen. Membrane extracts were isolated from bacterial strains harbouring invasin derivatives, as described (*Experimental procedures*). Aliquots of each extract containing 40 µg of membrane protein were fractionated on a 10% SDS-polyacrylamide gel, transferred to Immobilon filters, and subjected to immunopropbing with 1B-10, a monoclonal antibody that recognizes the mammalian cell-binding domain of invasin (Leong *et al.*, 1990). Bacterial strains analysed were: A, MC1000, an *E. coli* strain not producing invasin; B, SW5AA2Q (pJL213), an *E. coli* strain that overproduces a carboxyl terminal fragment of invasin; C, LE392 (pRI203); D, FB1, (a phenotypically Inv<sup>-</sup> *S. flexneri* 2a derivative harbouring pRI203); E, FB14 (a spontaneous phage P1-resistant, phenotypically Inv<sup>+</sup>, *S. flexneri* 2a derivative); and, FB17 (a spontaneous phage P1-resistant, phenotypically Inv<sup>+</sup>, *S. flexneri* 2a derivative).



**Fig. 5.** Invasin is proficient for mammalian cell binding in extracts made from *S. flexneri* 2a. Extracts containing 100 µg of membrane protein were fractionated electrophoretically on 10% polyacrylamide gels containing SDS, and transferred to Immobilon filters (Towbin *et al.*, 1979). The filter replicas were then probed with live HEp-2 cells, and bands of cell attachment to invasin were detected as described (Isberg and Leong, 1988; *Experimental procedures*). Membrane extracts were made from: A, MC1000, an *E. coli* strain without an intact *inv* locus; B, SW5AA2Q (pGP1/pRI253), an *E. coli* strain that overproduces invasin under the control of the phage T7 RNA polymerase promoter; C, LE392 (pRI203) Inv<sup>+</sup>; D, FB11 (a phenotypically Inv<sup>-</sup> *S. flexneri* 2a derivative harbouring pRI203); E, FB14 (a spontaneous phage P1-resistant, phenotypically Inv<sup>+</sup>, *S. flexneri* 2a derivative); and F, FB17 (a spontaneous phage P1-resistant, phenotypically Inv<sup>+</sup>, *S. flexneri* 2a derivative).

seemed to interfere with proper migration of proteins on sodium dodecyl sulphate (SDS) gels (Fig. 5, compare lane D with lanes E and F), it was clear that active invasion protein was present in the membranes of O-antigen-containing, invasion-incompetent *Shigella*.

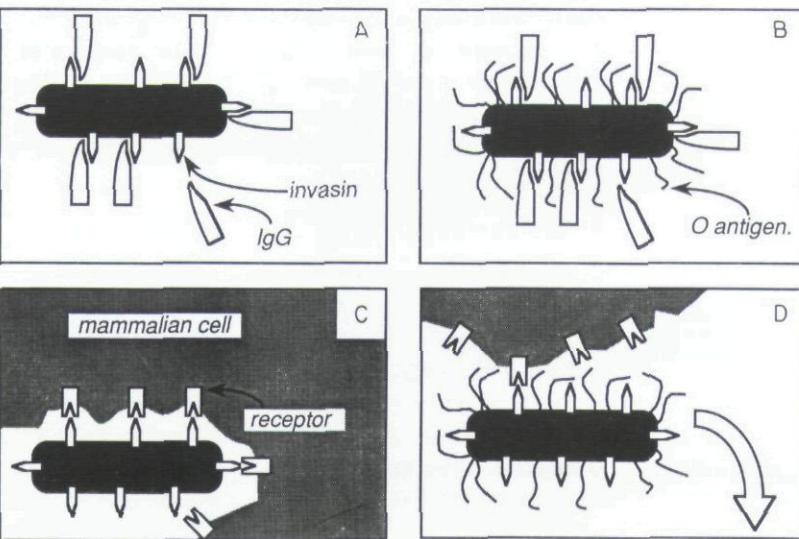
#### Invasin is properly localized on the surface of *S. flexneri*

To demonstrate directly that invasin is properly localized in *Shigella* strains harbouring *inv* and proficient at synthesizing O antigen, strains were analysed by quantitative indirect immunofluorescent probing of intact bacteria in order to determine the relative amount of surface-localized invasin. Logarithmically growing bacterial cells were washed and incubated with a mixture of monoclonal antibodies directed against the carboxyl terminal 472 amino acids of invasin (*Experimental procedures*). The amount of antibody bound to the cell surface was then detected by probing with antiserum containing fluorescein isothiocyanate (FITC)-linked anti-mouse IgG followed by fluorometric quantification (Table 3). As had been observed with alkaline phosphatase assays (Table 2), relatively more invasin was detected on the surface of *E. coli* than on the surface of *S. flexneri* strains (Table 3). There was no correlation, however, between the amount of surface-exposed invasin and the ability of a *Shigella* strain to enter animal cells. For instance, penetration-deficient FB11 yielded a somewhat higher level of invasin surface exposure than the penetration-proficient FB17, which does not synthesize O antigen (Table 3). These results indicated that the presence of *Shigella* O antigen does not interfere with the export and localization of invasin on the bacterial cell surface. The most likely explanation for the defective entry properties of the *S. flexneri* strain is that the

**Table 3.** Accessibility of antibody to surface-exposed invasin is not increased by the absence of O antigen.

Strain	Phenotype	Relative fluorescence intensity
LE392	Inv <sup>-</sup>	0.14 ± 0.06
LE392(pRI203)	Inv <sup>+</sup>	4.48 ± 0.31
M4243A <sub>1</sub>	Inv <sup>-</sup>	<0.05
FB1(P1 <sup>S</sup> )	Inv <sup>-</sup>	2.04 ± 0.43
FB1(P1 <sup>S</sup> )	Inv <sup>-</sup>	2.72 ± 0.14
FB14(P1 <sup>R</sup> )	Inv <sup>+</sup>	3.00 ± 0.72
FB17(P1 <sup>R</sup> )	Inv <sup>+</sup>	2.46 ± 0.25

Bacterial strains were grown to  $A_{600} = 0.6$ , probed with a mixture of monoclonal antibodies directed against invasin, and processed for indirect immune fluorescence analysis with FITC-linked anti-mouse IgG, as described (Leong *et al.*, 1990; *Experimental procedures*). The amount of fluorescence, which quantifies the accessibility of surface-localized invasin to the anti-invasin monoclonal antibodies, was determined in a microtitre fluorometer. Relative fluorescence intensity is defined in the *Experimental procedures*. Results are means of quadruplicate determinations ± standard deviations.



**Fig. 6.** Model for antagonism of cellular penetration by *S. flexneri* O antigen. The black rod represents a bacterium. Antibody is able to bind invasin in either the absence (Panel A) or the presence (Panel B) of O antigen. Multivalent binding of invasin to the mammalian cell surface allows stable bacterial association with the host cell and subsequent internalization (Panel C). The presence of *S. flexneri* O antigen, however, inhibits this multivalent binding and the bacterium cannot stably associate with the mammalian cell surface (denoted by an arrow in Panel D). Further details are described in the Discussion.

O antigen sterically interferes with the ability of the mammalian cell receptor to bind invasin-encoding bacteria.

## Discussion

We have shown that not all enteric bacteria harbouring the *Y. pseudotuberculosis* *inv* locus are able to enter mammalian cells efficiently. *S. flexneri* strains harbouring pRI203 (*inv*<sup>+</sup>) were approximately 30-fold less efficient at entering HEp-2 cells than comparable *E. coli* strains (Table 1). This defect did not exist in either recombinant or mutant *S. flexneri* strains that were unable to synthesize O antigen. The presence of O antigen did not interfere markedly with the synthesis, localization or *in vitro* cell binding activity of invasin. Rather, it would appear that O antigen physically prevented the mammalian cell receptor from interacting with a sufficient number of invasin molecules to establish a stable bacterium–host cell interaction.

An explanation for the inhibition of invasin-mediated cellular entry must take into account the seeming paradox that the *S. flexneri* O side chain does not block the binding of antibody to invasin on intact bacteria, yet the mammalian cell is unable to adhere to the microorganism. One clear difference between antibody- and receptor-binding invasin is that antibodies can bind independently of other antibody molecules to invasin, whereas the binding of integrin receptors may be constrained by their location on the mammalian cell surface. The binding of an antibody to invasin on the bacterial cell surface (Fig. 6, panel A) is either a univalent or a bivalent process. Apparently, invasin protrudes sufficiently from the bacterial cell surface to allow recognition by IgG, even in the presence of *Shigella* O antigen (Fig. 6, Panel B). Binding of the

bacterium to the mammalian cell surface, on the other hand, is a multivalent process (Fig. 6, Panel C), and the membrane-localized integrin protein does not have the same kind of freedom to move in three dimensions as a soluble IgG molecule. Probably there is a critical number of invasin molecules that must be ligated to receptors for stable binding of the microorganism to the host cell. If the number of contact sites falls below this critical number, then the microorganism may not stably associate with the mammalian cell (Fig. 6, Panel D). The presence of O antigen may allow the binding of a few invasin molecules to integrin receptors, but this interaction could cause either the migration or a change in conformation of the O antigen in response to the close association of the mammalian cell surface, such that further ligand–receptor interactions are blocked by steric hindrance (Fig. 6, Panel D). As a result of the newly redistributed O antigen which now masks invasin, not enough receptors are bound and the bacterium fails to associate stably with the host cell.

The mere presence of an O antigen on the bacterial cell surface is probably not sufficient to prevent invasin-mediated bacterial entry. It seems likely that the length of the O antigen side chain and its density on the bacterial cell surface are important factors in determining whether invasin–receptor interaction will be inhibited. This hypothesis is based on the fact that the parental *Y. pseudotuberculosis* organism synthesizes an O antigen when the bacteria are grown at 28°C (Gorshkova *et al.*, 1980), yet the microorganism enters mammalian cells efficiently. As would be expected, polyacrylamide gel electrophoresis of the *Y. pseudotuberculosis* III LPS indicates a much lower level of O antigen side chain relative to LPS core than was seen with *S. flexneri* 2a (data not shown).

Two previous studies on invasive microorganisms and

the role of LPS have an important bearing on this work. First, it has been shown that the intracellular bacterium *Coxiella burnetti* undergoes a phase variation in which the highly virulent Phase I organism has very large amounts of LPS on its surface. Antibody raised against bacteria growing in the second (less virulent) phase cannot recognize proteins on the surface of the Phase I organism because of steric hindrance by LPS (Hackstadt, 1988). Inasmuch as antibody is able to recognize invasin on the surface of *Shigella*, it would appear that the length or density of LPS on *Coxiella* is greater than that on *Shigella*, or that invasin protrudes considerably from the bacterial cell surface, facilitating recognition by antibody. The second report indicated that an intact LPS structure is actually required for efficient entry by *Salmonella choleraesuis* (Finlay *et al.*, 1988). In this latter work, insertion mutants were isolated that were defective for entry into cultured MDCK cells. Many of these mutants affected LPS structure. Although it is not clear what positive role LPS could play in the entry process, it should be noted that mutations affecting LPS biosynthesis could adversely affect the biogenesis of outer-membrane components, and changes in LPS structure could result in improper localization or even lack of expression (Beher *et al.*, 1980) of surface proteins required for cellular entry.

Finally, the results of this study indicate that virulent invasive *Shigella* strains have very special problems when

they penetrate host cells. Entry of *S. flexneri* into host cells is probably mediated by a receptor-ligand interaction similar to the binding of invasin to its integrin receptor. It is clear that the O antigen synthesized in such strains can have a profound negative effect on the ability of the mammalian cell to recognize the microorganism, and *Shigella* must overcome this antagonism. A straightforward way of facilitating recognition of a cell-binding protein would be to build a protein complex protruding from the surface of the bacterial cell that excludes O antigen sufficiently to allow recognition by the mammalian cell. It is interesting to note that invasive *Shigella* species encode several novel immunogenic outer-membrane proteins (*ipa* gene products; Buysse *et al.*, 1987; Maurelli and Sansonetti, 1988) that are candidates for participating in such a complex, and that at least two of these proteins seem to be critical for host-cell recognition (T. L. Hale, personal communication; N. High and P. Sansonetti, personal communication). Perhaps *Shigella* species have evolved such a structure to overcome the steric-hindrance effect of O antigen. Future studies should shed light on the nature of this recognition process.

## Experimental procedures

### Bacterial strains and growth conditions

HEP-2 cells were maintained in RPMI medium containing 5% newborn calf serum. Bacterial strains were grown in L-broth (Miller, 1972) containing appropriate antibiotics, as described (Isberg *et al.*, 1987).

Bacterial strains are described in Table 4. All *Shigella* strains are derived from a single *S. flexneri* 2a strain (M4243A<sub>1</sub>) that had been cured of the large *Shigella* virulence plasmid conferring invasiveness and intracellular growth in cultured human cells (Sansonetti *et al.*, 1982). The plasmid pRI203 is a pBR325 derivative containing a 4.5 kb *Bam*H insert that encompasses the *Y. pseudotuberculosis* *inv* gene (Isberg *et al.*, 1987). The plasmid pDV7 contains an *inv-phoA* hybrid in which intact invasin is fused at its last amino acid to a sequence at the amino-terminal end of mature alkaline phosphatase (Hoffman and Wright, 1985). This plasmid was constructed by placing the *phoA* fragment of pCH2 (Hoffman and Wright, 1985) downstream from the 3' end of *inv* in the plasmid vector pEMBL18 (Dente *et al.*, 1983). The two genes were then fused by an oligonucleotide-generated deletion, as described (Boyd *et al.*, 1987). pJL213 and pRI253 were constructed as described (Isberg and Leong, 1988).

Mutations of *S. flexneri* 2a that were defective for synthesis of O antigen side chain were isolated as spontaneous mutants of FB1 resistant to killing by phage P1vir. Fresh overnight cultures of FB1 (0.1 ml) were plated on L-agar plates containing 100 µg ml<sup>-1</sup> ampicillin that were seeded with approximately 5 × 10<sup>8</sup> p.f.u. of P1vir. Colonies, which arose at a frequency of approximately 1 per 10<sup>6</sup> cells plated, were purified once on identical medium and twice more in the absence of phage, before verifying phage resistance by cross-streaking against high-titre stocks of P1vir.

Matings were performed between *S. flexneri* 2a and *E. coli* Hfr strains containing Tn10, as described (Miller, 1972). Briefly,

**Table 4.** Bacterial strains.

Strain <sup>a</sup>	Relevant genotype	Source
<i>S. flexneri</i> 2a		
M4243A <sub>1</sub>	Plasmid cured	Sansonetti <i>et al.</i> (1982)
FB1	Plasmid cured/pRI203 ( <i>inv</i> <sup>+</sup> )	Sansonetti <i>et al.</i> (1982)
FB11 <sup>b</sup>	FB1 <i>zed</i> ::Tn10 <i>Inv</i> <sup>-</sup>	This study
FB12 <sup>b</sup>	FB1 <i>zed</i> ::Tn10 <i>Inv</i> <sup>+</sup>	This study
FB14	FB1 P1 <sup>R</sup>	This study
FB17	FB1 P1 <sup>R</sup>	This study
FB21	FB1 (pDV7)/cured of pRI203	This study
FB24	FB14 (pDV7)/cured of pRI203	This study
FB27	FB17 (pDV7)/cured of pRI203	This study
<i>E. coli</i>		
BW5660	HfrPK19 <i>srlC</i> ::Tn10	B. Bachmann
BW6159	HfrKL14 <i>iv</i> <sup>+</sup> ::Tn10	B. Bachmann
BW6160	HfrBroda8 <i>zdh</i> ::Tn10	B. Bachmann
BW6163	HfrKL16 <i>zed</i> ::Tn10	B. Bachmann
BW6169	HfrAB313 <i>argA</i> ::Tn10	B. Bachmann
BW7620	HfrKL99 <i>zed</i> ::Tn10	B. Bachmann
BW7622	HfrKL96 <i>trp</i> ::Tn10	B. Bachmann
BW7623	HfrBroda7 <i>purE</i> ::Tn10	B. Bachmann
LE392	<i>supE</i> , <i>supF</i> , <i>hsdRS14</i>	
MC1000	<i>F</i> <sup>-</sup> $\Delta$ ( <i>ara-leu</i> ), $\Delta$ ( <i>lac</i> ) X74, <i>rpsL</i> , <i>galE</i> , <i>galK</i>	

**a.** All *S. flexneri* strains were derived from M4243A<sub>1</sub> (Sansonetti *et al.*, 1982), which was cured of the virulence plasmid that promotes entry into mammalian cells.

**b.** *S. flexneri-E. coli* hybrid constructed as described in the Experimental procedures.

bacterial strains were grown to mid-logarithmic phase in L-broth containing the appropriate antibiotics, washed twice in phosphate-buffered saline (PBS) and resuspended in the original volume of L-broth in the absence of antibiotics. Aliquots (0.1 ml) of recipient and donor were mixed, and matings were allowed to proceed for one hour at 37°C in stationary 13 × 150 mm culture tubes. The tubes were vortexed for one minute each, and aliquots were streaked for single colonies on L-agar containing 30 µg ml<sup>-1</sup> chloramphenicol and 20 µg ml<sup>-1</sup> tetracycline to select for transfer of *E. coli* chromosomal markers. No further attempts were made to interrupt the matings. Single colonies were purified twice and tested for the ability to enter HEp-2 cells using the gentamicin survival assay (see below).

#### Bacterial binding and entry into mammalian cells

The binding of bacteria to HEp-2 cells was measured as described (Isberg, 1989). Entry into mammalian cells was measured in a fashion similar to that described previously (Isberg and Falkow, 1985). Tissue culture dishes (24-well) (Bellco) were seeded with 5 × 10<sup>4</sup> HEp-2 cells in RPMI 1640 medium containing 5% newborn calf serum. After incubation for 36 h at 37°C in a 5% CO<sub>2</sub> atmosphere, the wells were washed twice with PBS and incubated with RPMI 1640 containing 20 mM HEPES (pH = 7.0), 0.4% bovine serum albumin (BSA) and no serum. Bacteria were grown to mid-logarithmic phase in appropriate media and placed on ice to titre for viable counts. Approximately 5 × 10<sup>6</sup> bacteria were immediately added to the HEp-2 cell monolayers, and incubated for 3 h at 37°C in a 5% CO<sub>2</sub> atmosphere. To measure intracellular entry, the wells were washed once in PBS and filled with RPMI 1640 containing 20 mM HEPES (pH = 7.0), 0.4% BSA and 50 µg ml<sup>-1</sup> gentamicin, as described. After allowing 90 min for killing of extracellular bacteria, the monolayers were washed three times in PBS, lysed in 0.5 ml of 1% Triton X-100, and viable counts surviving gentamicin treatment were titrated on L-agar containing 100 µg ml<sup>-1</sup> ampicillin.

#### Isolation and analysis of bacterial LPS

LPS was isolated essentially as described (Westphal and Jann, 1965). Bacterial cultures were grown overnight in 250 ml of L-broth, pelleted, washed in distilled water, and pelleted once again. The pellet was weighed before being resuspended in prewarmed 68°C distilled water at a volume equal to five times the cell-pellet weight. At this time, an equal volume of 68°C phenol was added, and the extraction proceeded in a shaking 68°C water bath for 15 min. Tubes containing the extract were equilibrated in a 10°C water bath, and the phases were separated by centrifugation at 5000 × g for 30 min at 25°C. The aqueous phases were pooled and dialysed against six changes of distilled water at room temperature. The dialysed LPS was then pelleted by centrifugation at 29 000 r.p.m. in a Beckman 50ti angle rotor for 2 h at 25°C and resuspended in 0.5 ml of distilled water before sonication and storage at 4°C. LPS was analysed by loading 6 µl of a 1:18 dilution of the extracted material on a standard 13% SDS-polyacrylamide gel (Laemmli, 1970). Detection of LPS was by silver staining with a commercially available kit, according to the manufacturer's specification (BioRad).

#### Quantification of surface-exposed invasin by indirect immunofluorescence

Surface exposure of invasin was quantified as described (Leong et al., 1990). Bacterial strains were grown in 10 ml L-broth (containing 100 µg ml<sup>-1</sup> ampicillin if the strain harboured pRI203) to an A<sub>600</sub> = 0.6 before being washed three times in IF buffer (2 mM MgCl<sub>2</sub>, 150 mM NaCl, 10 mM HEPES, pH = 7.0) and resuspended in 0.5 ml of IF buffer containing 1 mg ml<sup>-1</sup> BSA. The bacteria were allowed to incubate with a mixture of anti-invasin monoclonal antibodies for 2 h at 37°C with rotation, and then the bacteria were processed for immunofluorescence, as described (Leong et al., 1990), except that the FITC-linked second antibody was allowed to incubate with the bacteria overnight at 4°C. Bacterial-surface-associated fluorescence was measured after extraction in base (Leong et al., 1990) in a Flow Instruments Fluoroskan II microtitre fluorometer using an excitation filter of 485 nm and an emission filter of 538 nm. Wells containing extraction buffer alone (Leong et al., 1990) were used as blanks. Relative fluorescence intensity was expressed as the number of fluorescence units per A<sub>600</sub> unit of cells extracted.

#### Preparation and analysis of membrane extracts

Bacterial membranes were isolated from late-logarithmic cultures (A<sub>600</sub> = 1.0) grown in 50 ml of L-broth. Pelleted cells were washed once in 10 mM HEPES (pH = 8.0), resuspended in 1.5 ml of the same buffer containing 150 µg ml<sup>-1</sup> phenyl methyl sulphonyl fluoride, and disrupted by sonication on ice. Membrane material was pelleted in a Beckman TL-100 centrifuge at 90 000 r.p.m. for 30 min, resuspended in identical buffer, pelleted once more as above, and resuspended in 200 µl of 10 mM Tris-HCl (pH = 8.0). Preparations were stored at -70°C until further analysis.

Membrane preparations were analysed by immunopropbing and assaying for the ability of HEp-2 cells to attach to invasin. For each assay, membrane preparations containing identical amounts of proteins (as determined by Lowry et al., 1951) were loaded onto SDS-polyacrylamide gels after solubilization in standard SDS-containing loading buffer (Laemmli, 1970). After fractionation, proteins were electrotransferred to Immobilon filters (Millipore, Towbin et al., 1979) and either immunopropbed or analysed for the ability to support HEp-2 cell attachment to invasin species (Isberg and Leong, 1988).

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