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Contact of *Shigella* with host cells triggers release of Ipa invasins and is an essential function of invasiveness

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The invasion of colonic epithelial cells by *Shigella*, an early essential step for causing bacillary dysentery, is mediated by the IpaB, IpaC and IpaD proteins. Secretion of the Ipa proteins from *Shigella* requires functions encoded by the *mxi* and *spa* loci. In this study, we show that contact between the bacteria and epithelial cell triggers release of the Ipa proteins into the external medium, which results in a rapid decrease in levels of Ipa proteins presented on the cell surface. When the bacteria were used to infect polarized Caco-2 cells, release of Ipa proteins occurred efficiently from bacteria interacting with the basolateral surface rather than with the apical surface. Moreover, the interaction of bacteria with components of the extracellular matrix, such as fibronectin, laminin or collagen type IV, also stimulates the release of Ipa proteins. The release of Ipa proteins from *Shigella* required the surface-located Spa32 protein encoded by one of the *spa* genes on the large plasmid.

Key words: Ipa invasins/*Shigella*/Spa32 protein/invasion/extracellular matrix

Introduction

Bacteria of the genus *Shigella* are the causative agents of shigellosis or bacillary dysentery, an infection confined to humans and primates. The early essential step leading to shigellosis is the invasion of colonic epithelial cells (LaBrec *et al.*, 1964), followed by intracellular bacterial multiplication and spread of invading bacteria into adjacent epithelial cells. Virulent shigellae possess a large plasmid which encodes various classes of virulence determinants such as those required for epithelial invasion, spreading into adjacent cells and regulation of the virulence genes (Hale, 1991; Sasakawa *et al.*, 1992).

Among the virulence proteins encoded by the large 230 kb plasmid of *S.flexneri*, IpaB, IpaC and IpaD, three immunogenic proteins encoded by the *ipaBCD* genes in the *ipa* operon, have been shown to play the most direct role in the invasion of epithelial cells (Buysse *et al.*, 1987; Baudry *et al.*, 1988; Venkatesan *et al.*, 1988; Sasakawa *et al.*, 1989). High *et al.* (1992) and Menard *et al.* (1993) have recently shown that the IpaB, IpaC and IpaD proteins are required for inducing actin polymerization at the site of bacterial attachment, a characteristic of cell entry by *Shigella* (Clerc *et al.*, 1987), and lysis of the phagocytic vacuoles by the bacteria. Several recent studies have shown that the IpaB, IpaC and IpaD proteins are secreted onto the cell surface and/or into the external medium (Mills *et al.*, 1988; Hromockyj and Maurelli, 1989; Andrews *et al.*, 1991; Andrews and Maurelli, 1992; Venkatesan *et al.*, 1992; Allaoui *et al.*, 1993; Sasakawa *et al.*, 1993). The ability of *S.flexneri* to secrete Ipa proteins has been shown to be crucial for invasiveness, since mutants incapable of secreting these proteins become non-invasive, even though they produce normal intracellular levels of Ipa proteins. These studies have indicated that the secretion of IpaB, IpaC and IpaD proteins requires the expression of other genes encoded by a 21 kb DNA segment, comprising the *mxi* and *spa* operons, which is located upstream of the *ipa* operon on the large plasmid. The *mxi* operon encodes *mxiHIJMEDCA* (Andrews *et al.*, 1991; Allaoui *et al.*, 1992, 1993; Andrews and Maurelli, 1992), while the *spa* operon encodes *spa47*, *spa13*, *spa32*, *spa33*, *spa24*, *spa9*, *spa29* and *spa40* (Venkatesan *et al.*, 1992; Sasakawa *et al.*, 1993), although the exact roles of the proteins in the secretion of Ipa proteins are still unclear. Recent studies have shown that the proteins encoded by *mxi* and *spa* operons share significant amino acid sequence homologies with other proteins involved in protein secretion in a number of non-related organisms including *Salmonella* (Spa), *Yersinia* (Ysc), *Bacillus* (Fli), *Caulobacter* (Fli), *Xanthomonas* (Hrp), *Pseudomonas* (Hrp) and *Eriwinia* (Mop) (Groisman and Ochman, 1993; Salmond and Reeves, 1993; Van Gijsegem *et al.*, 1993; Forsberg *et al.*, 1994). The protein export mechanisms of these proteins are different from two other protein secretion systems known as the sec-dependent protein export process and hemolysin protein export system, since these latter systems require a signal peptide sequence at the N-terminus and a transmembrane domain at the C-terminus respectively in the proteins to be secreted (Salmond and Reeves, 1993; Van Gijsegem *et al.*, 1993).

In the course of generating a series of merodiploid derivatives of Tn*5* insertion mutants of each of the *spa* genes, and testing for the Spa phenotype (surface presentation of invasion plasmid antigenes) (Venkatesan *et al.*, 1992) in colony immunoblots with three antibodies specific for each of the IpaB, IpaC and IpaD proteins, we found that one of the *spa* mutants defective in *spa32* expression was still able to present the IpaC protein and smaller amounts of IpaB and IpaD proteins (Sasakawa *et al.*, 1993; M.Watarai, unpublished observation). In this context, we undertook more detailed analysis of the involvement of Spa32 by constructing an in-frame deletion mutation in the *spa32* gene of *S.flexneri* 2a YSH6000T. We show here that the secretion of IpaB, IpaC and IpaD proteins from the bacteria into the culture supernatant

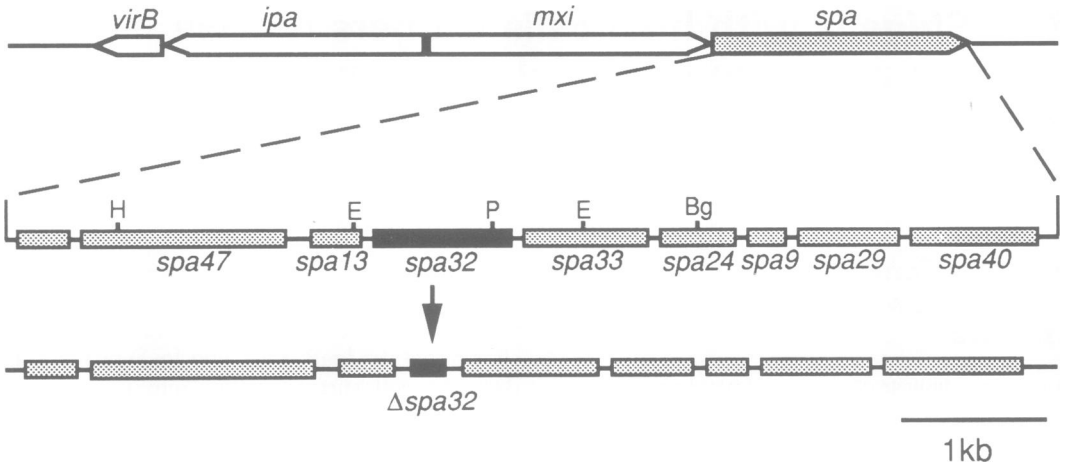

Fig. 1. Genetic organization of the *spa* operon on pMYSH6000 and the construction of an in-frame deletion in the *spa32* gene. The construction of plasmids used for generating the *spa32* in-frame deletion mutation is detailed in Materials and methods. The genetic organization of the *spa* genes was described previously (Venkatesan *et al.*, 1992; Sasakawa *et al.*, 1993). Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I.

apparently takes place by a process comprising surface exposition of the Ipa proteins and then release into the external medium, since the *spa32* mutant constructed was able to expose Ipa proteins on the cell surface at similar levels to those of the wild-type but failed to release into the culture supernatant. Subsequent experiments have indicated that the release of the Ipa proteins from the wild-type was triggered by contact with the epithelial cells. Interestingly, the release of the Ipa proteins from the bacteria in polarized colonic epithelial Caco-2 cells was shown to be greatly increased from bacteria infecting the basolateral surface compared with those interacting with the apical surface. The release of Ipa proteins was also mediated by interaction with extracellular matrix glycoproteins. Thus, these results suggest that the pathogen senses the site of bacterial entry into the host epithelial cell and then releases the Ipa proteins from the bacterial surface. The roles of the released Ipa proteins in the invasion process will also be discussed.

Results

Construction of a deletion mutation in the spa32 gene on the large plasmid of YSH6000T and the effect on secretion of IpaB, IpaC and IpaD proteins

To investigate the role of the *spa32* gene in the secretion of IpaB, IpaC and IpaD proteins or in bacterial entry into epithelial cells, an in-frame deletion mutation was constructed in the *spa32* gene. The resultant deletion mutation was designated *spa32–1* and the mutant was designated CS2585 (Figure 1). Examination for the ability to present IpaB, IpaC and IpaD proteins on the cell surface using whole-cell enzyme-linked immunosorbent assay (ELISA) with antibodies specific for IpaB, IpaC or IpaD revealed that CS2585 presented the Ipa proteins on the cell surface, as did YSH6000T (Figure 2). CS2585 was then checked together with YSH6000T and CS2586, a CS2585 derivative carrying pMAW200 (a cloned *spa32* gene), for its invasive capacity using Giemsa-stained monkey kidney epithelial (MK2) cell monolayers. The results showed that CS2585 failed to enter MK2 cells, but this capacity was restored in CS2586 to a level similar to that of YSH6000T (data not shown).

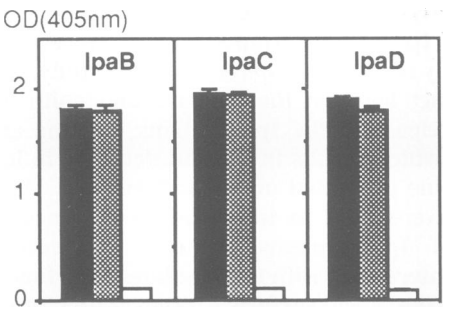

Fig. 2. Detection of cell surface-associated IpaB, IpaC and IpaD on CS2585 compared with that on YSH6000T and S325. Shown are levels of IpaB, IpaC and IpaD expressed on YSH6000T (black column), CS2585 (cross-hatched column) or S325 (white column) assayed by whole-cell ELISA with antibodies specific for IpaB, IpaC and IpaD. The bar above each column represents the standard deviation of triplicate samples.

To visualize the IpaB protein presented on the cell surface, CS2585, YSH6000T and S325 (*mxiA*::Tn*5*), grown to mid-log phase in brain–heart infusion (BHI) broth at 37°C, were immunostained with antibody specific for IpaB protein and analyzed for surface expression using immunofluorescence microscopy. IpaB was surface presented by CS2585 and YSH6000T but not at all by S325. The same was true for IpaC and IpaD proteins (data not shown). However, the capacity of CS2585 to secrete the IpaB, IpaC and IpaD proteins into the culture supernatant was impaired. Examination by immunoblotting with antibodies specific for IpaB, IpaC or IpaD showed that, while the production of the Ipa proteins in the whole-cell lysates of CS2585 was the same as that in YSH6000T (Figure 3A), their secretion into the culture supernatant was abolished (Figure 3B). As expected, the *mxiA*::Tn*5* mutant, S325, was unable to secrete the Ipa proteins onto the bacterial surface. These results suggested that, although the *spa32* mutant was able to expose IpaB, IpaC and IpaD proteins on the cell surface, the bacteria was incapable of releasing Ipa proteins into the external medium.

Surface location of the Spa32 protein

To assess the direct role of Spa32 in releasing the IpaB, IpaC and IpaD proteins, its cellular location was examined.

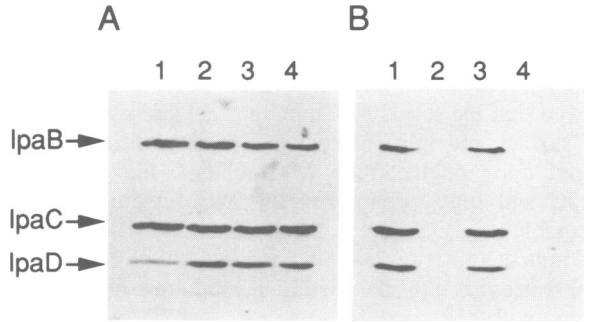

Fig. 3. Effect of the *spa32–1* mutation on the secretion of IpaB, IpaC and IpaD proteins. Immunoblot analysis of whole-cell lysates (**A**) and culture supernatants (**B**). IpaB, IpaC and IpaD proteins were detected by immunoblots with antibodies specific for IpaB, IpaC and IpaD proteins. Details are given in Materials and methods. Lanes 1, YSH6000T; 2, CS2585; 3, CS2586; 4, S325.

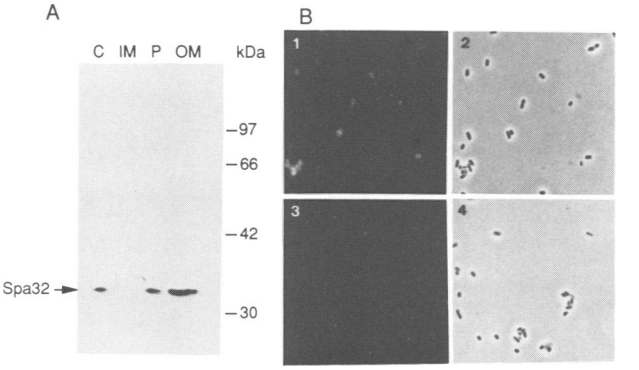

Fig. 4. Subcellular location of Spa32 protein. (**A**) Immunoblot of protein extracts from subcellular fractions of YSH6000T with anti-Spa32 antiserum. Lanes C, cytoplasm; IM, inner membrane; P, periplasm; OM, outer membrane. (**B**) Labeling of bacteria grown *in vitro*, with antibody specific for the Spa32 protein. Fluorescence microscopy of labeled bacteria (panels 1 and 3) and phase-contrast microscopy of the corresponding microscopic fields (panels 2 and 4) are shown. Panels 1 and 2, YSH6000T; 3 and 4, CS2585.

To do this, we prepared a Spa32-specific polyclonal antiserum (VRS-32) by immunizing rabbits with Spa32 protein prepared as described in Materials and methods. The outer and inner membrane proteins, cytoplasmic and periplasmic proteins extracted from YSH6000T were subjected to SDS–PAGE and immunoblotted with VRS-32 antibody. The results showed that a band corresponding to the 32 kDa protein was detected in the outer membrane and also, to a lesser extent, in the periplasm and cytoplasm, but not in the inner membrane (Figure 4A). Subsequent examination by immunofluorescence for cell surface staining with VRS-32/goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugated IgG revealed strong immunofluorescence on YSH6000T but not on CS2585 (Figure 4B). Furthermore, the culture supernatant protein extracts from YSH6000T analyzed by immunobloting with VRS-32 revealed that Spa32 was not present in the medium (see below), indicating that Spa32 exists as a surface-exposed outer membrane protein.

spa32–1 mutant fails to elicit actin polymerization at the site of epithelial cells around the attached bacteria

Shigella attached to the host plasma membrane induced actin polymerization around the site of bacterial attachment, a process essential for bacterial entry requiring IpaB, IpaC and IpaD proteins (Clerc *et al.*, 1987; Menard *et al.*, 1993). Hence, we asked whether or not the defect in *spa32* expression also affected the induction of the actin polymerization. CS2585 or YSH6000T infecting MK2 cell islets were stained with rhodamine–phalloidin and rabbit anti-*S.flexneri* lipopolysaccharide/goat anti-rabbit FITC-conjugated IgG and analyzed for the induction of actin polymerization by immunofluorescence and confocal laser scanning microscopy. Approximately 20–30% of YSH6000T attached to the edge of peripheral cells induced actin polymerization around the site of the bacterial attachment, but CS2585 did not at all (data not shown). Interestingly, when YSH6000T attached to MK2 cell islets was stained with rhodamine–phalloidin and rabbit anti-IpaC/goat anti-rabbit FITC-conjugated IgG, we noted that the bacteria which induced actin polymerization possessed a barely detectable immunofluorescence signal for IpaC protein on the cell surface, while the bacteria without induction of actin polymerization had a strong immunofluorescence signal (Figure 5), suggesting that the bacteria interacting with the site of entry were releasing Ipa from their surface.

Epithelial cell contact triggers release of IpaB, IpaC and IpaD proteins from the bacteria

Based on the above observation (Figure 5) and on the fact that contact of *Shigella* with epithelial cells is a prerequisite for efficient invasion, we reasoned that the release of Ipa proteins is an active event in *Shigella*, probably triggered by bacterial contact with the target cell. Hence, we allowed attachment of YSH6000T, CS2585 or S325 following centrifugation for 10 min at 700 *g* onto semiconfluent MK2 cell monolayers, and measured the levels of IpaB, IpaC and IpaD proteins presented on the cell surface, in the cytoplasm and in the tissue culture medium (MEM) at 0, 5, 10 and 20 min using ELISA with antibody specific for IpaB, IpaC or IpaD (see Materials and methods). Amounts of IpaB, IpaC and IpaD proteins of YSH6000T present on the cell surface and in the cytoplasm before centrifugation (at –10 min) were shown to be at a ratio of 1:2 (Figure 6, panels A, B and C). The levels of Ipa proteins exposed on the cell surface immediately after centrifugation (0 min) were greatly reduced, to 20% of those of the levels at –10 min (100%), whilst levels of Ipa proteins in the cytoplasm at 0 and 5 min were not significantly changed but were reduced at 10 min to levels corresponding to 80% of those at –10 min. In agreement with this, the increase in levels of Ipa proteins appearing in the MEM at 0 and 5 min apparently correlated with the decreased levels on the cell surface (Figure 6, panels A, B and C). Examination of levels of the IpaB, IpaC and IpaD proteins of CS2585 in the three compartments revealed that they were expressed on the cell surface and in the cytoplasm at similar levels to those of YSH6000T, but that they were not altered upon contact with MK2 cells (Figure 6, panels D, E and F). As expected, levels of Ipa proteins in the cytoplasm of S325 were not changed at all upon contact with MK2 cells (Figure 6, panels G, H and I). We also measured levels of Spa32 protein present on the cell surface, in the cytoplasm and in the

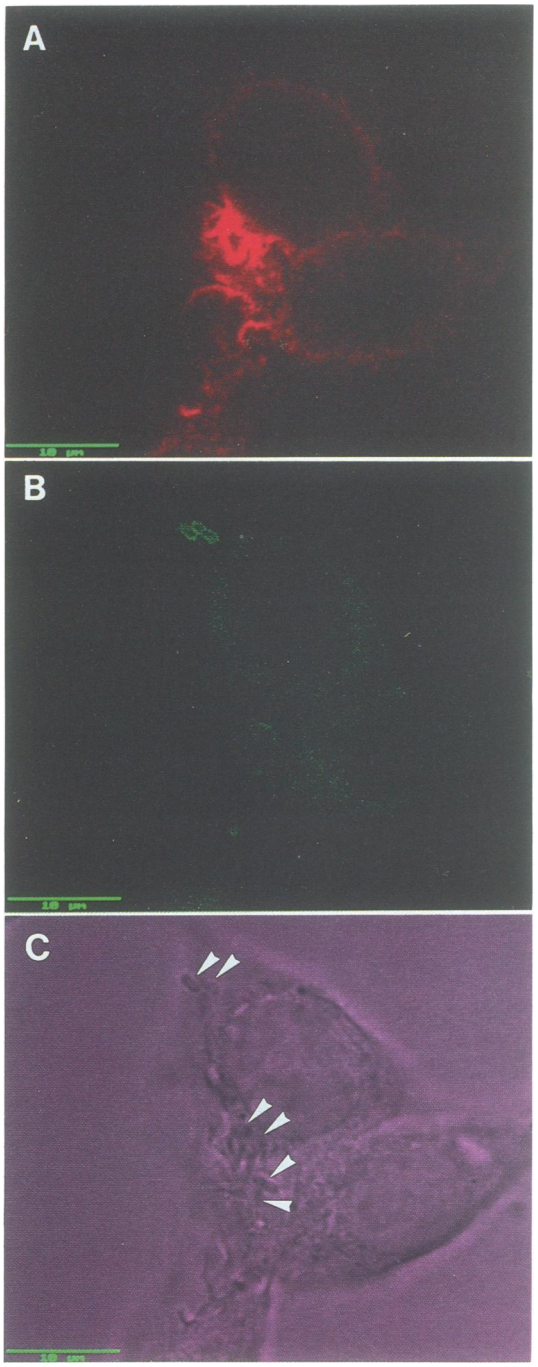

Fig. 5. Actin polymerization and disappearance of surface IpaC protein. Rhodamine–phalloidin (**A**) and rabbit anti-IpaC/goat anti-rabbit FITC-conjugated IgG (**B**). Differential interference contrast of the corresponding microscopic field (**C**). Arrowheads indicate the positions of YSH6000T bacterial particles.

culture supernatant of YSH6000T by ELISA with VRS-32 antibody, since it has been shown to be exposed on the cell surface (Figure 4) and involved in release of Ipa proteins into the culture supernatant (Figures 3 and 6). The results showed that Spa32 present on the cell surface was not reduced upon contact with MK2 cells, and that the Spa32 protein was not released into MEM (Figure 6, panel J). To confirm that the appearance of Ipa proteins in MEM upon epithelial cell contact did not result from destruction of YSH6000T, levels of OmpC, an outer membrane protein, and IpgC, a cytoplasmic protein (Menard *et al.*, 1994a), were measured by ELISA with antibody specific for OmpC or IpgC protein. The results showed that the levels of OmpC protein expressed on the cell surface and IpgC protein in the cytoplasm were not altered upon contact with MK2 cells, whilst levels of OmpC and IpgC proteins in the MEM were at barely detectable levels (Figure 6, panels K and L). These results thus indicated that Ipa proteins exposed on the cell surface were released into the external medium shortly after contact with epithelial cells.

Recent studies on the mode of entry of *S.flexneri* into polarized colonic Caco-2 epithelial cells have indicated that the bacteria can enter the polarized epithelial cells more efficiently from the basolateral pole than that from the apical pole (Mounier *et al.*, 1992; Perdomo *et al.*, 1994). YSH6000T was thus used to infect polarized Caco-2 cells grown on permeable filters either treated with EGTA or not, thus allowing the bacteria to infect from the basolateral surface (+EGTA) or from the apical surface (–EGTA) (Mounier *et al.*, 1992). Invasive capacity and the release of IpaB, IpaC and IpaD proteins were measured. The results showed that YSH6000T infecting the Caco-2 cells treated with EGTA gave rise to 3.7×10^3 times higher invasion capacity than that infecting the epithelial cells without EGTA treatment (Figure 7A). In agreement with this, examination for released Ipa proteins using the cell-free ELISA revealed that the bacteria infecting the Caco-2 cells treated with EGTA yielded 6-fold high amounts of IpaB, IpaC and IpaD proteins in the MEM compared with the bacteria infecting the epithelial cells without EGTA treatment (Figure 7B). As expected, CS2585 was unable to release Ipa proteins even when incubated with the Caco-2 cells treated with EGTA (Figure 7B). These results strongly suggested that the release of Ipa proteins from *Shigella* occurred *in vivo*, in which the bacterial interaction with the site of entry into the epithelial cells would be critical.

Bacterial contact with the extracellular matrix promotes the release of IpaB, IpaC and IpaD proteins from YSH6000T

The above results prompted us to test whether extracellular matrix glycoproteins (ECMs) such as collagen type IV, fibronectin or laminin could trigger the release of Ipa proteins, since such ECMs are abundant on the basolateral surface of the polarized epithelial cells. To test this possibility, YSH6000T or CS2585 suspended in phosphate-buffered saline (PBS) was added to microtiter wells previously coated with either collagen type IV, fibronectin or laminin. After promoting attachment of the bacteria to the wells by centrifugation, the levels of released IpaB, IpaC and IpaD proteins were measured using the cell-free ELISA. As shown in Table I, YSH6000T, but not CS2585, attached to the wells released IpaB, IpaC and IpaD proteins into the PBS, while YSH6000T attached to wells coated with gelatin or bovine serum albumin (BSA) did not. Indeed, the levels of Ipa proteins released showed a 26-fold increase in the bacterial contact with collagen type IV compared with that with BSA (Table I).

Released Ipa proteins elicit invasion by SC2585

In order to investigate the roles of the released IpaB, IpaC and IpaD proteins in the invasion process, the capacity to

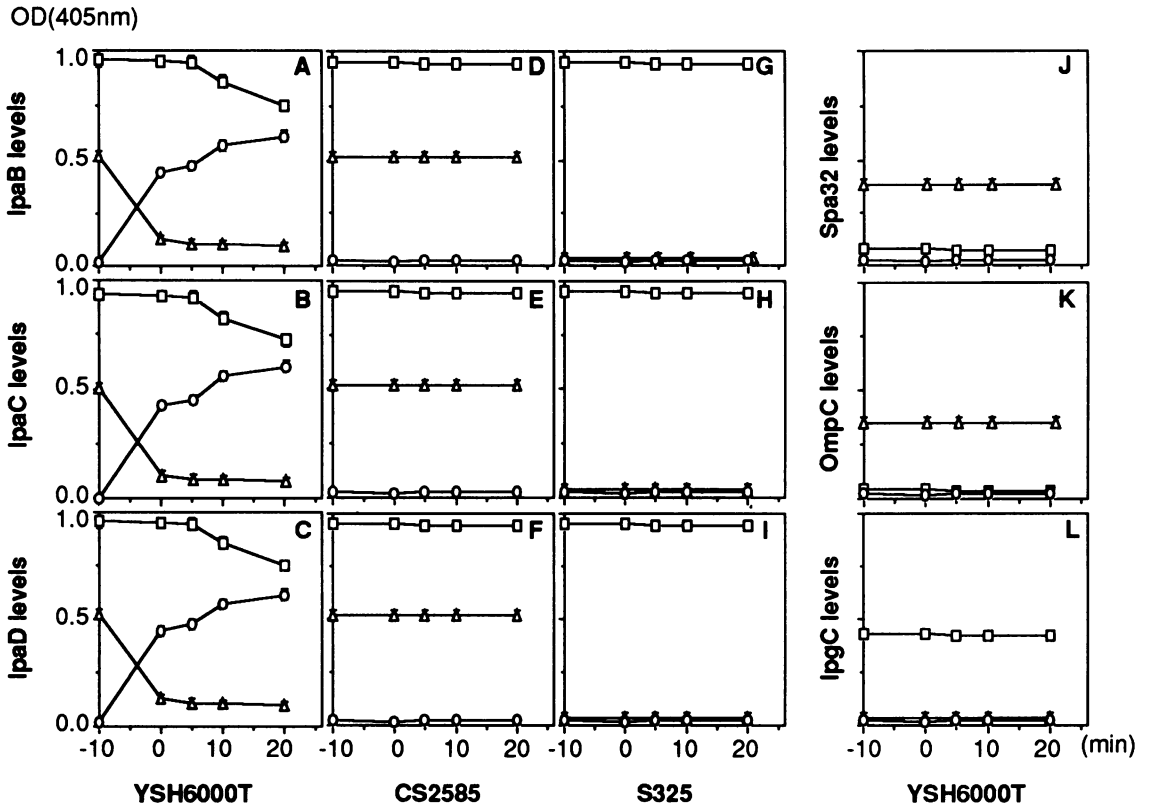

Fig. 6. Epithelial cell contact-induced Ipa release. Changes in levels of IpaB, IpaC and IpaD proteins present in the cytoplasm, or on the cell surface and in the tissue culture supernatant (MEM), upon contact with MK2 cells were measured by ELISA using antibody specific for IpaB, IpaC or IpaD as described in Materials and methods. Changes in levels of Spa32, OmpC and IpgC proteins present in the three compartments of YSH6000T upon contact were measured by ELISA using antibody specific for Spa32, OmpC or IpgC. The vertical scale represents absorbance as measured at 405 nm using an ELISA reader model 450 (Bio-Rad), while the horizontal scale indicates time after centrifugation (min). Panels: **A, B, C, J, K** and **L**, YSH6000T; **D, E** and **F**, CS2585 (*spa32–1*); **G, H** and **I**, S325 (*mxiA*::Tn*5*). Symbols: △, cell surface ELISA; □, cytoplasm ELISA; ○, supernatant ELISA. Each value represents the average of three experiments. The bar indicates the standard deviation.

evoke invasiveness from non-invasive mutants such as CS2585, S325 and N1411 (*ipaB*::Tn*5*) was examined. To test this, MEM used for the attachment of YSH6000T to MK2 cell monolayers which contained released Ipa proteins (equivalent to the MEM at 0 min in Figure 6) was filtered to remove bacteria, and the filtrate was then added to CS2585, S325 and N1411. Each of the bacteria were then added to the MK2 cells and examined for invasiveness within the Giemsa-stained infected cells (see Materials and methods). Observation of the Giemsa-stained infected MK2 cells revealed that addition of the MEM to CS2585 restored its invasiveness, since the percentage of infected MK2 cells determined was 22 times higher than that with plain MEM (Table II). Under the same conditions, however, neither S325 nor N1411 was internalized into the epithelial cells. To confirm the activity of the released Ipa proteins within the MEM, antibodies specific for IpaB, IpaC and IpaD proteins were added to the MEM which was then examined for its ability to restore invasion capacity to CS2585. The data revealed that the restored invasiveness of CS2585 was almost completely abolished (Table II). These results implied that the surface-expressed Ipa proteins, together with those able to be released, were essential for the bacterial invasion.

Discussion

In this study we have provided evidence suggesting that the presentation of IpaB, IpaC and IpaD proteins on the cell surface of *Shigella* is not sufficient to elicit invasiveness, but that their ability to be released into the external medium is also required. This was first noticed during characterization of the non-invasive *spa32* mutant (CS2585) constructed in this study, since it had the ability to present IpaB, IpaC and IpaD proteins onto the cell surface at levels similar to those of the wild-type YSH6000T, but failed to release the Ipa proteins into the culture supernatant. Interestingly, although the capacity of *Shigella* to secrete Ipa proteins into the culture supernatant when it is grown *in vitro* has been noted previously (Mills *et al.*, 1988; Hromockyj and Maurelli, 1989; Andrews *et al.*, 1991; Andrews and Maurelli, 1992; Venkatesan *et al.*, 1992; Allaoui *et al.*, 1993; Sasakawa *et al.*, 1993), this property seemed to be an active event triggered by contact with epithelial cells. Indeed, the tissue culture medium (MEM) used for the infection of MK2 cell monolayers by YSH6000T contained significant amounts of IpaB, IpaC and IpaD proteins, whereas the cell-free MEM used for YSH6000T without attachment to the MK2 cells or that used for CS2585 with attachment to MK2 cells possessed barely detectable amounts of the

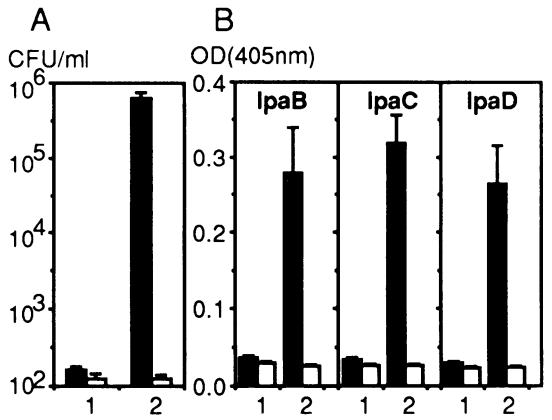

Fig. 7. Invasion capacity of YSH6000T infecting polarized Caco-2 cells either treated with EGTA or not and the levels of released IpaB, IpaC and IpaD proteins. (**A**) The invasion capacity of YSH6000T and CS2585. (**B**) The levels of released IpaB, IpaC and IpaD proteins from YSH6000T and CS2585. Each lane 1 represents the Caco-2 cells without EGTA treatment, while each lane 2 represents the Caco-2 cells treated with EGTA. The black and white columns indicate the Caco-2 cells infected with YSH6000T and SC2585 respectively. The leftmost panel shows the invasive capacity as determined using the gentamicin-protection assay. The bar above each column represents the standard deviation of triplicate samples.

Ipa proteins. In order to examine whether the surface-exposed or cytoplasmic Ipa proteins were primarily involved in the cell contact-triggered release, the kinetics of changes in the levels of IpaB, IpaC and IpaD on the cell surface, in the cytoplasm and culture supernatant (MEM) of YSH6000T upon contact with MK2 cells were assayed using ELISA with antibody specific for IpaB, IpaC or IpaD. The levels of Ipa proteins expressed on the cell surface at 0 min were greatly decreased after the contact, while Ipa proteins in the cytoplasm were not substantially decreased, even 10 min after contact (see Figure 6, panels A, B and C). Since increased levels of each of the Ipa proteins in the MEM immediately after contact (at 0 and 5 min) apparently correlate with the decreased levels on the cell surface, the Ipa proteins initially released from YSH6000T could be those that are the surface-exposed Ipa proteins rather than those in the cytoplasm. The levels of surface-exposed Ipa proteins of the *spa32* mutant (CS2585) were not altered at all upon MK2 cell contact, and the bacteria were unable to release Ipa proteins into the external medium (Figure 6, panels D, E and F), indicating that Spa32 is involved in release of Ipa proteins from the cell surface. In these experiments, the cell surface, the cytoplasm and the culture supernatant of YSH6000T were checked for their OmpC and IpgC protein content, since OmpC and IpgC are present on the cell surface and in the cytoplasm, respectively (Menard *et al.*, 1994a). Our data revealed that levels of the OmpC protein present on the cell surface and of the IpgC protein in the cytoplasm were not altered at all upon contact with MK2 cells. This indicated that release of Ipa proteins did not result from destruction of the bacteria during the centrifugation but, rather, that this phenomenon was an Ipa-specific event triggered by contact with epithelial cells.

Menard *et al.* (1994b) have shown recently that the release of Ipa proteins into the external medium can also be induced upon contact with HeLa cells, although the

Table I. Bacterial contact with ECM promotes release of IpaB, IpaC and IpaD proteins

Matrix	Contact-induced Ipa releasing activity[a]		
	IpaB	IpaC	IpaD
Fibronectin	0.357(0.032)	0.368(0.029)	0.329(0.031)
Laminin	0.450(0.026)	0.467(0.025)	0.418(0.022)
Collagen type IV	0.582(0.036)	0.588(0.029)	0.521(0.024)
Gelatin	0.021(0.006)	0.024(0.005)	0.019(0.002)
BSA	0.022(0.004)	0.021(0.005)	0.020(0.003)
PBS	0.018(0.007)	0.020(0.006)	0.021(0.005)

[a]Values represent amounts of released IpaB, IpaC and IpaD proteins measured using the cell-free ELISA (see Materials and methods). Standard deviation of triplicate samples for each assay is shown in parentheses.

released Ipa proteins were mainly derived from the cytoplasm. In addition, the authors indicated that ~90% of Ipa proteins are present in the cytoplasm (Menard *et al.*, 1994a). Our results differ in this regard, since, as mentioned above, we observed that nearly one third of the whole-cell Ipa proteins were present on the surface, and that the surface Ipa proteins were released immediately after the contact with epithelial cells (see Figure 6). However, it is possible that this discrepancy in observations between the two studies reflects the different growth conditions used; Menard *et al.* utilized M9 minimum medium supplemented with nicotinic acid, glucose and amino acids except methionine (1994b), while this study used BHI (brain heart infusion) broth, one of the richest media (see Materials and methods). It has generally been observed that the production of IpaB, IpaC and IpaD proteins of *S.flexneri* is greatly increased when grown up in a rich medium such as BHI broth. Accordingly, the levels of Ipa proteins present on the surface of YSH6000T grown in BHI broth were 4-fold higher than those grown in M9 minimum medium as determined by whole-cell ELISA with antibody specific for IpaB, IpaC and IpaD (M.Watarai, unpublished results). Consequently, the extent of the decrease in the levels of Ipa proteins present on the surface of the bacteria grown in M9 minimum medium upon contact with MK2 cells became apparently smaller than those grown in BHI broth (M.Watarai, unpublished results).

To pursue further the biological significance of the release of Ipa proteins observed *in vivo*, we investigated the invasion of polarized colonic epithelial Caco-2 cells, which when grown on permeable filters treated with EGTA prior to bacterial infection were more readily invaded than those without EGTA treatment (Mounier *et al.*, 1992). Such treatment of the Caco-2 cells opens the epithelial junctions and permits the infecting bacteria to move down to the basolateral pole of the cells where the bacteria are internalized efficiently (Mounier *et al.*, 1992). Therefore, the capacity of YSH6000T to release IpaB, IpaC and IpaD proteins into the tissue culture medium was measured by infecting polarized epithelial cells either treated with EGTA or not. As shown in Figure 7B, bacteria infecting the Caco-2 cells treated with EGTA released greater amounts of Ipa proteins than those infecting cells without EGTA treatment. We noted that YSH6000T treated with 100 μM EGTA did not release Ipa proteins into the

Table II. Capacity of released Ipa proteins to promote the internalization of non-invasive mutants into MK2 cells

Strain	No treatment			Immunoprecipitated by anti-IpaB, C and D antibodies (%)[a]		
	MEM	MEM with released Ipa[b]	Induction[c]	MEM	MEM with released Ipa[d]	Induction
YSH6000T (wild-type)	24.3	24.0	0.99	24.3	24.4	1.00
CS2585 (*spa32–1*)	0.6	14.3	21.38	0.5	0.6	1.20
S325 (*mxi*::Tn*5*)	0.6	0.8	1.13	0.6	0.6	1.00
N1411 (*ipaB*::Tn*5*)	0.6	0.6	1.00	0.6	0.6	1.00
YSH6200 (no plasmid)	0.5	0.6	1.20	0.5	0.6	1.20

[a]Percentage of infected cells 2 h after infection. Cells invaded by more than five bacteria were considered to be infected.
[b]The MEM used as the tissue culture medium for the attachment of YSH6000T to MK2 cells.
[c]The induction index corresponds to the ratio of infected cells obtained following invasion of the bacteria in MEM with released Ipa proteins over that in MEM.
[d]The MEM containing the released Ipa proteins was immunoprecipitated by adding antibodies specific for IpaB, IpaC and IpaD followed by incubation with protein A–Sepharose beads.

external medium (M.Watarai, unpublished results). We thus presume that the pathogen can sense the entry site on epithelial cells and release its Ipa proteins accordingly. In agreement with this hypothesis, YSH6000T attached to the edge of peripheral cells, in zones identical to basolateral poles (Mounier *et al.*, 1992), often induced actin polymerization around the site of bacterial attachment and revealed a barely detectable immunofluorescence signal for IpaC on the surface, while the bacteria without induction of the actin polymerization possessed a strong immunofluorescence signal (see Figure 5). Similar findings were also noted for the IpaB and IpaD proteins on YSH6000T (M.Watarai, unpublished observations).

The bacterial response in releasing the Ipa proteins upon interaction with the basolateral pole of the polarized Caco-2 cells raised the possibility that the ECMs, i.e. collagen type IV, fibronectin or laminin, could be host factors responsible for triggering the release of Ipa protein from the infecting bacteria as these cell components are abundant at the basolateral surface. Indeed, YSH6000T displayed the release of significant amounts of IpaB, IpaC and IpaD proteins into the medium following contact with microtiter wells previously coated with either collagen type IV, fibronectin or laminin (Table I). This reaction displayed a degree of specificity, as bacterial attachment to wells coated with gelatin (a denatured form of collagen), or BSA did not stimulate the release of Ipa proteins. Although the mechanisms underlying the release of Ipa proteins triggered by ECM or the epithelial cells still remain to be elucidated, these results may account for the efficient invasion from the basolateral surface of polarized epithelial cells compared with that from the apical surface (Mounier *et al.*, 1992).

It was of great interest to ask whether or not the released IpaB, IpaC and IpaD proteins could promote entry of non-invasive mutants into MK2 cell monolayers, since recent genetic studies have indicated that the Ipa proteins take part in at least two different aspects of the bacterial entry process, namely actin polymerization and lysis of the endocytic vacuole (Clerc *et al.*, 1987; Allaoui *et al.*, 1992; High *et al.*, 1992). Accordingly, MEM containing released Ipa proteins obtained from the attachment of YSH6000T to MK2 cells was examined for its capacity to promote the entry of CS2585, S325 (*mxiA*::Tn*5*) or N1411 (*ipaB*::Tn*5*) bacteria into the epithelial cells. The results showed that the addition of Ipa-containing MEM restored invasion capacity to CS2585, but not to S325 or N1411 (Table II). Since S325 and N1411 cannot present the Ipa proteins on their cell surface, these results suggested that released Ipa proteins, together with those associated with the bacterial surface, were required for invasion. Importantly, CS2585 was unable to induce actin polymerization around the site of bacterial contact with the epithelial cell. Furthermore, a recent study by Menard *et al.* (1994a) has shown that IpaB and IpaC proteins released into the external medium can form complexes. It is thus likely that the release of Ipa proteins is required for forming such a complex, which may interact with putative receptors on the basolateral surface, allowing the bacteria to be efficiently internalized. The Ipa proteins associated with the cell surface may contribute at a later stage in which they could disrupt the endocytic vacuole. Alternatively, the two forms of the Ipa proteins, cell-free (released) and cell-associated forms, may be required for the whole invasion process. Clearly, we must await further studies to elucidate the molecular interaction between the Ipa proteins and the putative receptor(s) which lead to actin polymerization in the invasion process.

The finding that the release of Ipa proteins from *Shigella* is triggered by contact with epithelial cells was similar to the recent study of the release of YopE cytotoxin from *Yersinia* (Rosqvist *et al.*, 1994), since *Yersinia* attached to mammalian cells was able to sense the contact and then induce expression and polarized transfer of the YopE protein into the eukaryotic cell. Indeed, recent studies have indicated that the sequences of Spa proteins Spa47, Spa13, Spa32, Spa33, Spa24, Spa9, Spa29 and Spa40 (Venkatesan *et al.*, 1992; Sasakawa *et al.*, 1993), have significant homologies to the Ysc proteins YscN, YscO, YscP, YscQ, YscR, YscS, YscT and YscU, respectively, of *Y.pseudotuberculosis* (Allaoui *et al.*, 1994; Bergman *et al.*, 1994) or those of *Y.pestis* (Fields *et al.*, 1994). Furthermore, the genetic organization of the *spa* and *ysc* regions is similar. Thus it is possible that *Shigella* has evolved a mechanism for Ipa secretion similar to that of Yop proteins in *Yersinia*, although the mode of bacterial entry into the mammalian cells and the fate of the invading bacteria are different for the two pathogens. The deduced amino acid sequence of Spa32 showed only 16% identity to YscP of *Y.pseudotuberculosis,* and this homology was the lowest among the Spa and Ysc proteins. The significance of this low homology in terms of common function

is not yet clear. We have observed that Spa32 exists on the bacterial surface (Figure 4) without being released into the medium (Figure 6, panel J), and that it is a disulfide-containing protein whose correctly folded structure seems to be required for its presentation on the outer membrane (M.Watarai, unpublished results), suggesting that Spa32 takes part in the release of Ipa proteins from the cell surface. Although it is not clear at present how the Spa32 protein mediates the release of IpaB, IpaC and IpaD proteins from the cell surface through interaction with epithelial cells, Spa32 function is clearly crucial for *Shigella* to initiate the invasion process upon contact with target epithelial cells.

Materials and methods

Bacterial strains and plasmids

Shigella flexneri 2a YSH6000T, a spontaneous tetracycline-, chloramphenicol-, ampicillin- and streptomycin-sensitive derivative of YSH6000, and YSH6200, a large plasmid-less derivative of YSH6000, were described previously (Sasakawa *et al.*, 1986; Sakai *et al.*, 1988; Nakata *et al.*, 1992). S325 and N1411 were *mxiA*::Tn*5* and *ipaB*::Tn*5* derivatives of YSH6000 respectively (Sasakawa *et al.*, 1988, 1989). CS2585 contained an in-frame deletion in the *spa32* gene derived from YSH6000T (see below). SY327λ*pir* was used as the host for a suicide plasmid, pCVD442 (Donnenberg and Kaper, 1991), while SM10λ*pir* (Simon *et al.*, 1983) was used to transfer pCVD442 into YSH6000T. pMAW200 was a derivative of pMW119 (Nippongene) containing a 1539 bp *Eco*RI segment carrying the *spa32* gene from pMYSH6000, the 230 kb plasmid of YSH6000. pBluescriptIISK+ (Stratagene) was routinely used as the vector for cloning *spa32*.

Construction of an in-frame deletion mutation in spa32 on pMYSH6000

A 3606 bp *Hin*dIII–*Bgl*II segment containing the *spa32* gene obtained from pMYSH6000 was cloned in pBluescriptIISK+ and the resultant plasmid was designated pMAW101. A DNA fragment encompassing nucleotides from position 1581 upstream of the 5′ end of *spa32* through to nucleotide 68 downstream from the 5′ end (Sasakawa *et al.*, 1993) was amplified by PCR using primers 5′-GAAATTGTTGAAGAGCTAG-3′ and 5′-TAACTGCAGATAGTTTCTCACATTTTTCTAT-3′. The latter primer contained a *Pst*I site. The amplified DNA, which comprised 1656 bp, was digested by *Hin*dIII and *Pst*I and then used to replace the *Hin*dIII–*Pst*I segment on pMAW101; the resulting plasmid was designated as pMAW102. The 3106 bp *Sal*I–*Sac*I segment on pMAW102 was subcloned into the *Sal*I and *Sac*I sites on pCVD442 and the resulting plasmid was named pMAW201. pMAW201 was introduced into SM10λ*pir* followed by transfer of the plasmid into YSH6000T using ampicillin selection. To eliminate the vector portion integrated into pMYSH6000, purified trans-conjugants were spread onto LN agar (Sasakawa and Berg, 1982) plates without NaCl but with 5% sucrose and incubated at 30°C. The resulting sucrose-resistant colonies were tested for ampicillin sensitivity, indicative of the loss of the suicide vector. One of the ampicillin-sensitive colonies thus selected was confirmed to contain an in-frame deletion in the *spa32* gene, by sequencing of the PCR-amplified 380 bp segment. The mutation was designated *spa32–1* and the resulting mutant CS2585.

Invasion assay

The conventional invasion assay with MK2 cell monolayers was used as described previously (Sasakawa *et al.*, 1986; Lett *et al.*, 1989) as was the gentamicin-protection assay (Small *et al.*,1987).

Detection of IpaB, IpaC and IpaD proteins presented on the bacterial surface

IpaB, IpaC and IpaD proteins presented on the cell surface were measured by a modified ELISA (Andrews *et al.*, 1991). Bacteria grown to mid-log phase in BHI broth at 37°C were harvested and suspended in PBS to an adsorbance at 600 nm of 5.0. The bacterial suspension was added to wells of 96-well microtiter plates (Costar) and incubated for 2 h at room temperature. After the wells were emptied of each fraction, 5% BSA was applied and incubated for 30 min. After two washes with PBS–0.05% Tween 20 (PBST), anti-IpaB, IpaC or IpaD antibody was added to each well and incubated for 2 h at 37°C. After three washes with PBST, horseradish peroxidase-conjugated protein A was added and incubated for 1 h at 37°C. After three washes with PBST, the substrate, 2,2′-azino-di(3-ethylbenzthiazoline sulfonic acid) diammonium salt was added. Absorbance was measured at 405 nm using an ELISA reader model 450 (Bio-Rad).

Bacteria grown *in vitro* were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature on a coverslip. After fixation, the bacteria were incubated with antibody specific for IpaB, IpaC or IpaD proteins and stained with a second antibody of goat anti-rabbit FITC-conjugated IgG.

Detection of IpaB, IpaC and IpaD proteins in the whole-cell lysate or culture supernatant

Protein extracts from culture supernatants and whole bacterial cells were prepared as follows: 1 ml of bacterial culture grown to mid-log phase in BHI broth at 37°C was centrifuged and the resulting supernatant passed through a 0.45 μm pore-size filter (Millipore, Amersham). Trichloroacetic acid (TCA) was added to 0.6 ml of the supernatant to a final concentration of 6%, and the precipitated proteins were dissolved in 60 μl of sodium dodecylsulfate (SDS) sample buffer [2% SDS, 4% 2-mercapthoethanol, 10% glycerol and 0.1 M Tris–HCl (pH 6.8)] and boiled for 5 min. The bacterial pellet from the centrifugation step was suspended in 1 ml of cold water and one third of a volume of 24% TCA was added. After centrifugation, the precipitated pellet was dissolved in 100 μl of SDS sample buffer and boiled for 5 min. Fifteen μg of each extract was subjected to an SDS–polyacrylamide gel (10%) electrophoresis (SDS–PAGE). The protein bands separated by SDS–PAGE were transferred to a nitrocellurose membrane and detected using antibody specific for IpaB, IpaC or IpaD proteins according to the methods previously described (Adler *et al.*, 1989).

Preparation of antibody

A fusion protein of Spa32 tagged with six histidine residues at the N-terminus was constructed using the QIAexpress system with pQE-30 plasmid (Qiagen). The fusion Spa32 protein purified by Ni-NTA chromatography (Qiagen) was used to immunize rabbits. A peptide encompassing residues 139–155 (LKIKAQSYLDAIQDIKE), which corresponded to the C-terminal 17 amino acids of the IpgC protein (Sasakawa *et al.*, 1989), was synthesized, and antiserum specific for the oligopeptide was obtained by immunization of rabbits with the peptide coupled to keyhole limpet hemocyanin using benzidine. Anti-OmpC serum was obtained from T.Mizuno (Nagoya University).

Location of Spa32

Bacterial culture of YSH6000T grown to mid-log phase (100 ml) was pelleted and washed in 10 mM HEPES buffer (pH 7.5) containing 1 mM phenylmethylsufonyl fluoride (PMSF), 1 mM *o*-phenanthrene and 0.2 mM 2-mercaptoethanol and then disrupted by passing through a French press at 12 000 kg/cm^2. The cleared lysate thus obtained was pelleted down at 40 000 r.p.m. for 2 h at 4°C in a Type 60 Ti rotor (Beckman). The total membrane pellet was suspended in 1 ml of the same HEPES buffer, and inner and outer membranes were separated by discontinuous sucrose gradient centrifugation (Osborn *et al.*, 1972). Periplasmic proteins were released from cells by the cold osmotic shock procedure of Neu and Chou (1967), while the cytoplasmic fraction was subsequently obtained from the bacterial debris as described by Maagd and Lugtenberg (1986). The efficiency of the fractionation were assayed by measuring levels of the cytoplasmic enzyme marker malate dehydrogenase (Kitto, 1969), inner membrane marker NADH oxidase (Osborn *et al.*, 1972), outer membrane marker 2-keto-3-deoxyoctonate (Karkhanis *et al.*, 1978) and the periplasmic enzyme marker alkaline phosphatase (Michaelis *et al.*, 1983). The fractionated proteins were analyzed by SDS–PAGE, and the Spa32 content of the fraction was assayed by immunoblotting with antibody specific for Spa32.

Assay for contact-induced Ipa release

Levels of IpaB, IpaC and IpaD proteins present on the cell surface, in the cytoplasm and the tissue culture medium (MEM) of YSH6000T, CS2585 or S325 attached to MK2 cells were measured by ELISA using antibody specific for IpaB, IpaC or IpaD proteins (Andrews *et al.*, 1991). Semiconfluent MK2 cell monolayers (~2×10^5 cells/ml) in 35 mm plastic tissue culture dishes (Falcon) were treated with cytochalasin D (500 μg/ml) for 45 min prior to attachment of the bacteria (Menard *et al.*, 1994b). Bacteria grown up to the mid-log phase in BHI broth at 37°C were centrifuged and resuspended in 2 ml MEM containing cytochalasin D (500 μg/ml) to contain ~100 bacteria per MK2 cell. The bacterial

suspension was added onto the MK2 cells monolayers, centrifuged at 700 *g* for 10 min at room temperature and incubated for 0, 5, 10 and 20 min at 37°C. The supernatants were passed through a 0.22 μm pore-size filter (Millipore), and the resulting cell-free MEM was used for supernatant ELISA. To obtain the bacteria attached to the MK2 cells, the epithelial cells with attached bacteria used above were suspended in 10 ml of MEM and pipeted several times to detach the bacteria. The suspensions were then centrifuged at 700 *g* for 4 min to spin down MK2 cells, and the supernatants were centrifuged at 6000 *g* for 10 min at 4°C. The bacteria thus collected were suspended in 2 ml of PBS and used for cell surface ELISA. To assay the levels of Ipa proteins present in the cytoplasm, the MK2 cells with attached bacteria were prepared in the same manner as above. One ml of pronase medium (250 μg/ml of trypsin and 50 μg/ml of proteinase K) was then added and the mixture incubated for 10 min at 37°C to detach bacteria from MK2 cells and to degrade extracellular proteins (Menard *et al.*, 1994b). After the incubation, the medium was added into 10 ml MEM containing 1 mM PMSF, 30 μg/ml of trypsin inhibitor, 0.7 μg/ml of pepstatin and 1 mM EDTA, centrifuged at 700 *g* for 4 min to eliminate MK2 cells and subsequently suspended in 300 μl of 1% SDS, heated at 100°C for 5 min, incubated at 4°C and centrifuged at 13 000 *g* for 30 min at 4°C. The resulting supernatant was added into PBS giving a volume of 2 ml, and used for the cytoplasm ELISA. Levels of Spa32, OmpC and IpgC present on the cell surface, in the cytoplasm and culture supernatant of YSH6000T were assayed by the same ELISA methods as above but using antibody specific for Spa32, OmpC or IpgC.

Release of IpaB, IpaC and IpaD proteins from bacteria in contact with Caco-2 cells was performed as follows. Polarized Caco-2 cell monolayers on a 25 mm BioCoat Cell Culture Insert-Matrigel (Becton Dickinson Labware) were inoculated with bacteria and centrifuged for 10 min at 700 *g*. Immediately after the centrifugation, the supernatants were passed through a 0.22 μm pore-size filter (Millipore), and the resulting cell-free tissue culture medium (MEM) was added to wells of 96-well microtiter plates (Costar). The amounts of IpaB, IpaC and IpaD proteins contained in the MEM were measured by ELISA using antibody specific for IpaB, IpaC or IpaD protein.

ECM-induced Ipa release

Fifty μl of extra solution cellular matrix proteins [collagen type IV from human placenta (Cosmo Bio), fibronectin from human plasma (Upstate Biotechnol. Inc.) or laminin from Engelbreth–Holm–Swarm mouse tumor (Becton Dickinson Labware)] at a concentration of 200 μg/ml in PBS was coated onto 96-well microtiter plates (Costar) by incubation for 2 h at room temperature after which 100 μl of the bacterial suspension in PBS at an A_{600} of 1.0 was added. After centrifugation at 700 *g* for 10 min, the supernatants were passed through a 0.22 μm pore-size filter (Millipore). The amounts of IpaB, IpaC and IpaD proteins contained in the tissue culture medium were measured by ELISA using antibody specific for IpaB, IpaC or IpaD protein.

Culture and infection of polarized Caco-2 cells on permeable filters

The polarized Caco-2 cell monolayers were used after 6 days on the permeable filters described above. The confluent monolayers were treated with 100 μM EGTA [ethylene glycobis (β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid] in Ca^{2+}-free Krebs–Ringer solution, pH 7.4 (Mounier *et al.*, 1992), for 1 h prior to infection of bacteria and throughout the incubation period.

Double immunofluorescence staining of F-actin and infecting bacteria

F-actin aggregation and the bacteria within the MK2 cells were visualized by fluorescence microscopy as described by Clerc *et al.* (1987). Bacteria were labeled by indirect immunofluorescence with rabbit antiserum raised against the *S.flexneri* 2a lipopolysaccharide or IpaC and a goat anti-rabbit FITC-conjugated IgG as the second antibody. F-actin was visualized by staining the cells with rhodamine-labeled phalloidin.

Fluorescence microscopy

The specimens were analyzed using an epifluorescence microscope (Nikon, Diaphot) or a confocal laser scanning microscope equipped with dual detectors and an argon–krypton (Ar/Kr) laser for simultaneous scanning of two different fluorochromes (MRC-1000, Bio-Rad).

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