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SopA, the outer membrane protease responsible for polar localization of IcsA in *Shigella flexneri*

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Summary

The spreading ability of *Shigella flexneri*, a facultative intracellular Gram-negative bacterium, within the host-cell cytoplasm is the result of directional assembly and accumulation of actin filaments at one pole of the bacterium. IcsA/VirG, the 120 kDa outer membrane protein that is required for intracellular motility, is located at the pole of the bacterium where actin polymerization occurs. Bacteria growing in laboratory media and within infected cells release a certain proportion of the surface-exposed IcsA after proteolytic cleavage. In this study, we report the characterization of the *sopA* gene which is located on the virulence plasmid and encodes the protein responsible for the cleavage of IcsA. The deduced amino acid sequence of SopA exhibits 60% identity with those of the OmpT and OmpP outer membrane proteases of *Escherichia coli*. The construction and phenotypic characterization of a *sopA* mutant demonstrated that SopA is required for exclusive polar localization of IcsA on the bacterial surface and proper expression of the motility phenotype in infected cells.

Introduction

Shigella flexneri, a Gram-negative enteric bacillus, causes dysentery in humans. Bacteria invade colonic epithelial cells and trigger an inflammatory cascade leading to destruction of the mucosa (Sansonetti *et al*., 1995). Based on *in vitro* assays, invasion of an epithelial cell monolayer can be divided into three major steps: (i) entry into epithelial cells by bacterial-induced phagocytosis (Clerc and Sansonetti, 1987; Adam *et al*., 1995); (ii) lysis of the phagocytic vacuole and intracellular growth (Sansonetti *et al*., 1986); and (iii) bacterial spreading inside the cell cytoplasm and from cell to cell (Makino *et al*., 1986; Bernardini *et al*., 1989; Sansonetti *et al*., 1994).

Whereas entry into epithelial cells requires many different proteins, most of which are encoded by a 200 kb virulence plasmid (for a review, see Parsot and Sansonetti, 1995), the ability to spread within the intracellular compartment and from cell to cell (the Ics phenotype) is mainly dependent on expression of the *icsA*/*virG* gene, which is also located on the virulence plasmid (Makino *et al*., 1986; Lett *et al*., 1988; Bernardini *et al*., 1989). IcsA is a 120 kDa outer membrane protein which is required for actin polymerization at the bacterial surface. Directional assembly and accumulation of short actin filaments into an F-actin comet tail leads to bacterial movement and colonization of the cytoplasm of infected cells (Bernardini *et al*., 1989; Theriot, 1995). An *icsA* mutant is unable to generate the actin-based movement and infect adjacent cells. This mutant forms microcolonies near the nucleus of infected cells, which confirms that accumulation of actin filaments provides the motive force for intracellular movement. The Ics phenotype is a prerequisite for causing dysentery because *icsA* mutants showed a considerably attenuated virulence in monkeys (Sansonetti *et al*., 1991). In the process of intracellular dissemination, bacteria interact at the inner face of the plasma membrane with components of the cell intermediate junctions (i.e. E-cadherins) and induce the formation of membrane protrusions (Sansonetti *et al*., 1994). Engulfment of these protusions by adjacent cells and lysis of the cellular membranes allow *Shigella* to gain access to the cytoplasm of new cells without release into the extracellular environment (Allaoui *et al*., 1992).

Surface presentation and anchorage of IcsA to the outer membrane of the bacterium are achieved by a mechanism similar to that described for translocation of the IgA protease of *Neisseria gonorrhoeae* (Klauser *et al*., 1993; Suzuki *et al*., 1995). Accordingly, IcsA can be divided in three portions: the signal peptide (residues 1–52), the central domain ($IcsA_{\alpha}$, residues 53–758), and the C-terminal domain ($IcsA_{\beta}$, residues 759–1102). After removal of the signal peptide during export to the periplasm, the portion corresponding to $IcsA_{\alpha}$ is translocated across the outer membrane by the $IcsA_{\beta}$ domain and is exposed at the bacterial surface. Unusual among bacterial proteins, the surface localization of IcsA is asymmetrical. In dividing bacteria, IcsA is present in both sister bacteria at the pole opposite to the septation furrow. Inside infected cells, it

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is localized at the pole of the bacterium where actin polymerization occurs (Goldberg *et al.*, 1993). The mechanism underlying the polar distribution of IcsA is not yet known. In addition, cleavage of a certain amount of membrane-bound IcsA leads to release of $IcsA_{\alpha}$ in broth culture or inside infected cells (Goldberg *et al.*, 1993; 1994). This cleavage occurs between the two arginine residues within the sequence SSRRASS (Fukuda *et al.*, 1995) which has also been described as a PKA phosphorylation site (d'Hauteville and Sansonetti, 1992).

Previous studies have shown that membrane-bound IcsA is sufficient to induce actin polymerization and to promote bacterial motility (Fukuda *et al.*, 1995; Kocks *et al.*, 1995; Goldberg and Theriot, 1995). However, using a strain producing a non-cleavable form of IcsA encoded by a recombinant plasmid, we have recently shown that lack of cleavage leads to localization of IcsA over the entire bacterial surface and to the formation of abnormal F-actin comet tails in infected cells (d'Hauteville *et al.*, 1996). This suggested that cleavage and release of $IcsA_{\alpha}$ were necessary to achieve the polar localization of IcsA on the bacterial surface and to optimize the motility phenotype. To investigate further the role of proteolysis of a certain proportion of IcsA on the surface distribution of the remaining pool of IcsA protein and in intracellular movement, we have identified the gene encoding the protease responsible for secretion of $IcsA_{\alpha}$. This protein, SopA (for *Shigella* outer membrane protease), is homologous to the OmpT and OmpP outer membrane proteases of *Escherichia coli*. Analysis of a *sopA* mutant indicated that production of SopA is essential to achieve exclusive polar localization of IcsA. Within the intracellular compartment, actin is polymerized on the entire surface of the *sopA* mutant. As a consequence, this mutant is impaired in its capacity to assemble a polar actin tail and to move intracellularly. Therefore, SopA appears to be essential for polar localization of IcsA and for actin-based motility inside infected cells.

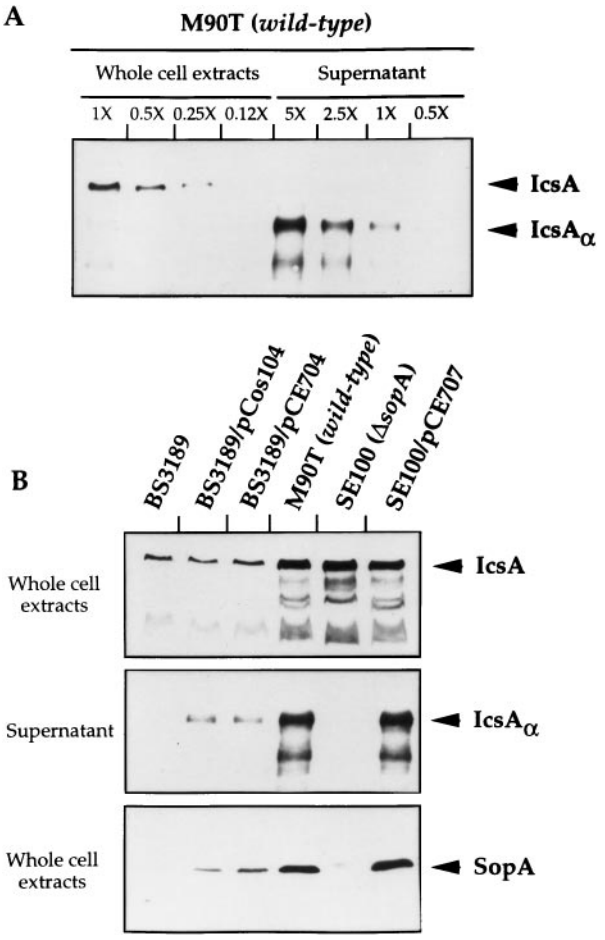
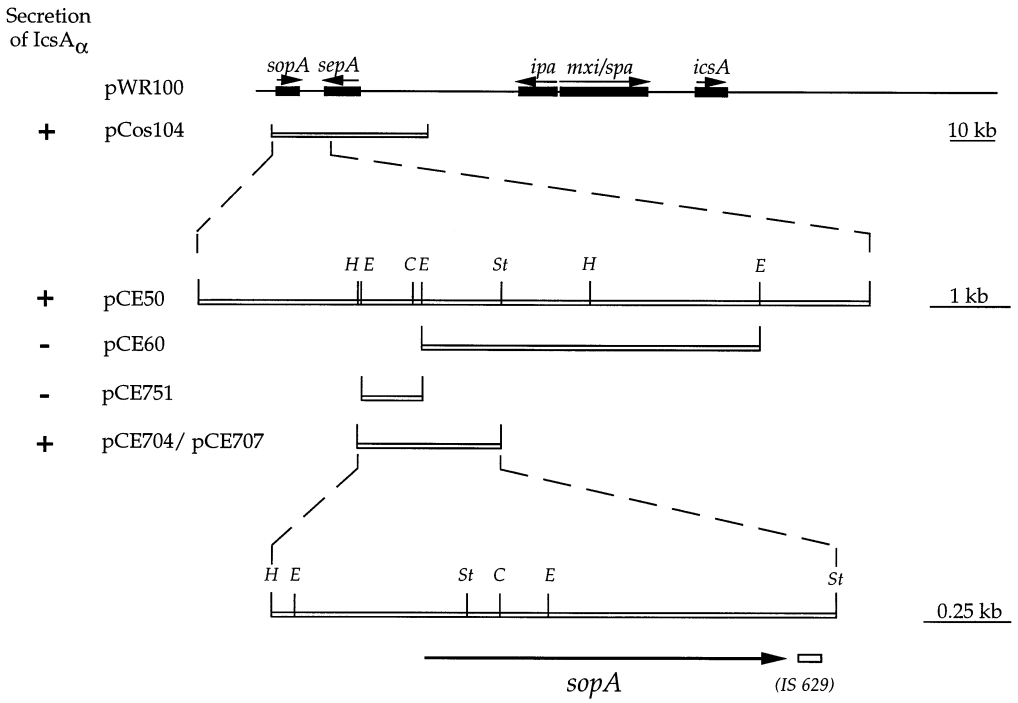


Fig. 1. Secretion of $IcsA_{\alpha}$. Whole cell extracts and culture supernatants of derivatives of BS3189 (the virulence plasmid-cured strain expressing *icsA* from pHS3189) and of derivatives of M90T (wild type) were analysed by SDS–PAGE and immunoblotting using anti-IcsA and anti-SopA antisera. Samples of culture supernatants were prepared from a volume of culture five times larger than that used to prepare samples of whole cell extracts.
A. Estimation of the amount of $IcsA_{\alpha}$ secreted by M90T (wild type). Twofold dilutions of samples of whole cell extract and culture supernatant of M90T were analysed using an anti-IcsA antiserum.
B. Production of IcsA and SopA and secretion of $IcsA_{\alpha}$ by BS3189 and its derivatives harbouring pCos104 or pCE704, M90T (wild type), SE100 (the *sopA* mutant) and its derivative harbouring plasmid pCE707 ($SopA^{+}$).

Results

Characterization of the sopA *gene*

Wild-type *Shigella* cells produce both the membrane-bound and secreted forms of IcsA. The amount of $IcsA_{\alpha}$ which is present in the culture supernatant depends on the stage of growth and can represent up to 50–80% of the amount of surface-bound IcsA (Goldberg *et al.*, 1993; 1994; Fukuda *et al.*, 1995). As shown in Fig. 1A, the amount of $IcsA_{\alpha}$ detected in the culture medium of M90T, a wild-type isolate of *S. flexneri*, in the exponential phase of growth corresponds to approx. 25–50% of IcsA. In contrast, expression of *icsA* from the recombinant plasmid pHS3189 in BS176, a derivative of M90T which has been cured of the virulence plasmid pWR100, did not lead to secretion of $IcsA_{\alpha}$ (Fig. 1B). This suggested that the protease responsible for cleavage of IcsA is encoded by the virulence plasmid. To test this hypothesis, 10 overlapping cosmids encompassing the pWR100 plasmid were independently introduced into the derivative of BS176 containing pHS3189 (strain BS3189) and the presence of $IcsA_{\alpha}$ in the culture supernatant of the recombinant strains was analysed by SDS–PAGE and immunoblotting using anti-IcsA antibodies. These cosmids are part of a library previously constructed by inserting 40 kb fragments of pWR100, generated by partial digestion with *Sau*3A, into



MKLKFFVLALCVPAIFTTHATTNYPLFIPDNISTDISLGSLSGKTKERVYHPKEGGRKISQLDWKYSNATIVRGGIDWKL

IPKVSFGVSGWTTLGNQKASMVDKDWNNSNTPQVWTDQSWHPNTHLRDANEFELNLKGWLLNNLDYRLGLIAGYQESRYS

FNAMGGSYIYSENGGSRNKKGAHPSGERTIGYKQLFKIPYIGLTANYRHENFEFGAELKYSGWVLSSDTDKHYQTETIFK

DEIKNQNYCSVAANIGYYVTPSAKFYIEGSRNYISNKKGDTSLYEQSTNISGTIKNSASIEYIGFLTSAGIKYIF

Fig. 2. Cloning of the *sopA* gene. Black boxes and arrows at the top of the figure represent the position and direction, respectively, of transcription of the *sopA* gene and different loci on the virulence plasmid pWR100. Open bars show the DNA fragments of pWR100 inserted in each recombinant plasmid. The position of selected restriction sites are indicated: *C*, *Cla*I; *H*, *Hin*dIII; *E*, *Eco*RI; *S*, *Sty*I. Presence (+) or absence (−) of IcsA$_\alpha$ in the culture supernatant of derivatives of BS3189 harbouring each recombinant plasmid is indicated directly in front of each plasmid. The deduced amino acid sequence of SopA is shown at the bottom of the figure. Residues which are conserved in the sequence of the *E. coli* OmpT and OmpP proteases are individually underlined; the proposed signal sequence of SopA has a solid underline.

the *Bam*HI site of pJB8 (Maurelli *et al.*, 1985). Three overlapping cosmids led to secretion of an IcsA$_\alpha$ protein, the size of which was identical to that of the protein secreted by the wild-type strain. Cosmid pCos104 was selected for further analysis (Fig. 1B).

Construction of deletion derivatives of pCos104 and analysis of secretion of IcsA$_\alpha$ by BS3189 harbouring these derivatives allowed us to localize the gene responsible for cleavage of IcsA, designated *sopA*, on the 1881 bp *Hin*dIII–*Sty*I fragment carried by plasmid pCE704 (Fig. 1B and Fig. 2). Analysis of the sequence of this fragment revealed the presence of a 989 bp open reading frame (ORF), from position 567 to 1556 (GenBank Accession Number U73461). The second ATG codon within this ORF (position 612) was preceded by a potential ribosome-binding site (5′-AGTAGA-3′) and appeared as the probable translation start codon of *sopA*. Accordingly, the *sopA* gene would encode a 315-residue polypeptide with a calculated M_r of 36 kDa. The deduced N-terminal sequence of SopA (Fig. 2) exhibited features which are characteristic of a signal sequence, which suggested that the protein was processed during export into the periplasm. SDS–PAGE analysis of whole cell extracts of the wild type and recombinant strains expressing the cloned gene and immunoblotting using anti-SopA antibodies indicated that the size of the SopA protein was approx. 28 kDa (Fig. 1B). No other ORF was detected upstream or downstream from *sopA*, which suggested that this gene belongs to a monocistronic transcription unit. Nucleotide sequence analysis revealed that, 34 bp downstream from the *sopA* stop codon, the complementary strand was 95% identical to the first 156 nucleotides of the insertion sequence IS*629*. The *sopA* gene is located ≈12 kb downstream from *sepA* (Benjelloun-Touimi *et al.*, 1995) and is transcribed in the opposite direction (Fig. 2). On pWR100, the *icsA* gene is located 100 kb away from the *sopA* gene,

15 kb downstream from the *spa* operon (C. Parsot, Z. Benjelloun-Touimi, B. Demers and C. Egile, unpublished results).

The deduced amino acid sequence of SopA exhibited strong similarity (60% identity) throughout the sequence with those of the *E. coli* outer membrane proteases OmpT (Grodberg and Dunn, 1988; Sugimura and Nishihara, 1988) and OmpP (Kaufmann *et al*., 1994) (Fig. 2). This sequence similarity suggests that SopA is located in the outer membrane and is a serine protease.

SopA is required for cleavage of IcsA

To test the role of SopA in cleavage of IcsA in wild-type *Shigella*, the *sopA* gene was inactivated on pWR100 by integration of a suicide plasmid containing an internal fragment of *sopA* (see the *Experimental procedures*). Whole cell extracts and culture supernatants of the wild type (M90T) and *sopA* (SE100) strains were analysed by SDS–PAGE and immunoblotting using anti-IcsA and anti-SopA antibodies (Fig. 1). Secretion of $IcsA_{\alpha}$ was abolished in the *sopA* mutant and could be restored by pCE707, a derivative of pCE704 which expressed the *sopA* gene. This indicated that SopA is the protease responsible for cleavage of IcsA in *Shigella*. Secretion of Ipa proteins, which is acheived by a type III secretion machinery (Ménard *et al*., 1994), was not affected in the *sopA* mutant (data not shown).

Expression of the *sopA* gene from the recombinant plasmid pCE707 in the *sopA* mutant did not increase secretion of $IcsA_{\alpha}$ as compared to the wild type. Immunoblot analysis using anti-SopA antibodies indicated that SopA was not overproduced in the *sopA* mutant carrying pCE707 (Fig. 1B). Similar amounts of SopA were produced in derivatives of BS3189 harbouring pCE707 or pCE704, which differ only by the resistance gene carried by the vector part, but a higher amount of SopA was produced by pCE707 in the *sopA* mutant than in BS3189, the virulence plasmid-cured strain. This suggested that expression of *sopA* might be regulated by a gene carried by the virulence plasmid.

Role of SopA in the surface distribution of IcsA

To investigate the role of SopA in the distribution of IcsA, we analysed by immunolabelling with an anti-IcsA antiserum the localization of IcsA at the surface of the wild-type and *sopA* strains. IcsA was detected at the surface of approx. 50% of the wild-type bacteria and its localization was restricted to one pole of the bacterium (Fig. 3, panels 1 and 2). In contrast, IcsA was present at the surface of all bacteria carrying the *sopA* mutation and was localized on the entire surface, with some polar reinforcement (Fig. 3, panels 3 and 4). Both phenotypic changes were reverted by introduction of pCE707 into the *sopA* mutant (Fig. 3, panels 5 and 6). These results showed that the cleavage of IcsA by SopA was necessary to achieve exclusive polar localization of IcsA.

To determine whether SopA is the only virulence plasmid-encoded protein required to achieve polar localization of IcsA, we analysed by immunofluorescence the distribution of IcsA at the surface of BS3189, which expressed *icsA* from a recombinant plasmid, and its derivative harbouring a plasmid encoding SopA. The localization of IcsA at the surface of BS3189 (Fig. 3, panels 7 and 8) was similar to that observed for the *sopA* mutant, i.e. all bacteria were labelled and labelling occurred over the entire bacterial body with some polar location. In contrast, expression of SopA from pCE704 in BS3189 (Fig. 3, panels 9 and 10) gave rise to a pattern of labelling similar to that observed for the wild-type strain. Therefore, virulence plasmid-encoded proteins other than SopA do not appear to be necessary to achieve the polar distribution of IcsA.

Analysis of the intracellular phenotype of the sopA *mutant*

To characterize the invasive capacity of the *sopA* mutant, HeLa cells were infected with the wild-type strain or the *sopA* mutant and, after 2 h of infection, cells were stained with Giemsa (Fig. 4) or labelled for F-actin with nitrobenzoxadiazole (NBD)–phallacidin (Fig. 5). Wild-type bacteria displayed an intracellular localization characteristic of the Ics phenotype: they were spread throughout the entire cell cytoplasm (Fig. 4, A and B) with many bacteria associated with long polar-orientated assembled actin tails, the rest of the bacteria being devoid of actin filaments (Fig. 5A). Some bacteria were present inside protusions. In contrast, *sopA* mutants remained clustered at the periphery of the cell and formed very few protusions (Fig. 4, C and D). These bacteria remained in close contact with the inner face of the host-cell membrane, suggesting that they were trapped inside the actin subcortical network, or the actin-rich focal adhesion plaques (Fig. 5, B and C). Moreover, actin polymerization occurred over the entire bacterial surface (Fig. 5C) and the few F-actin comet tails produced by the *sopA* mutant were curved, shorter and thicker than the ones assembled by the wild type (Fig. 5D). Thus, the *sopA* mutant was not impaired in its ability to enter HeLa cells but was clearly affected in its spreading capacity. This was confirmed in the plaque-forming assay carried out on confluent monolayers of Caco-2 cells. In this test, both the wild-type strain and the *sopA* mutant formed an equivalent number of plaques, but the diameter of those caused by the mutant was half the diameter of those formed by the wild type (Fig. 6). Complementation of the *sopA* mutant by the recombinant plasmid pCE707 gave

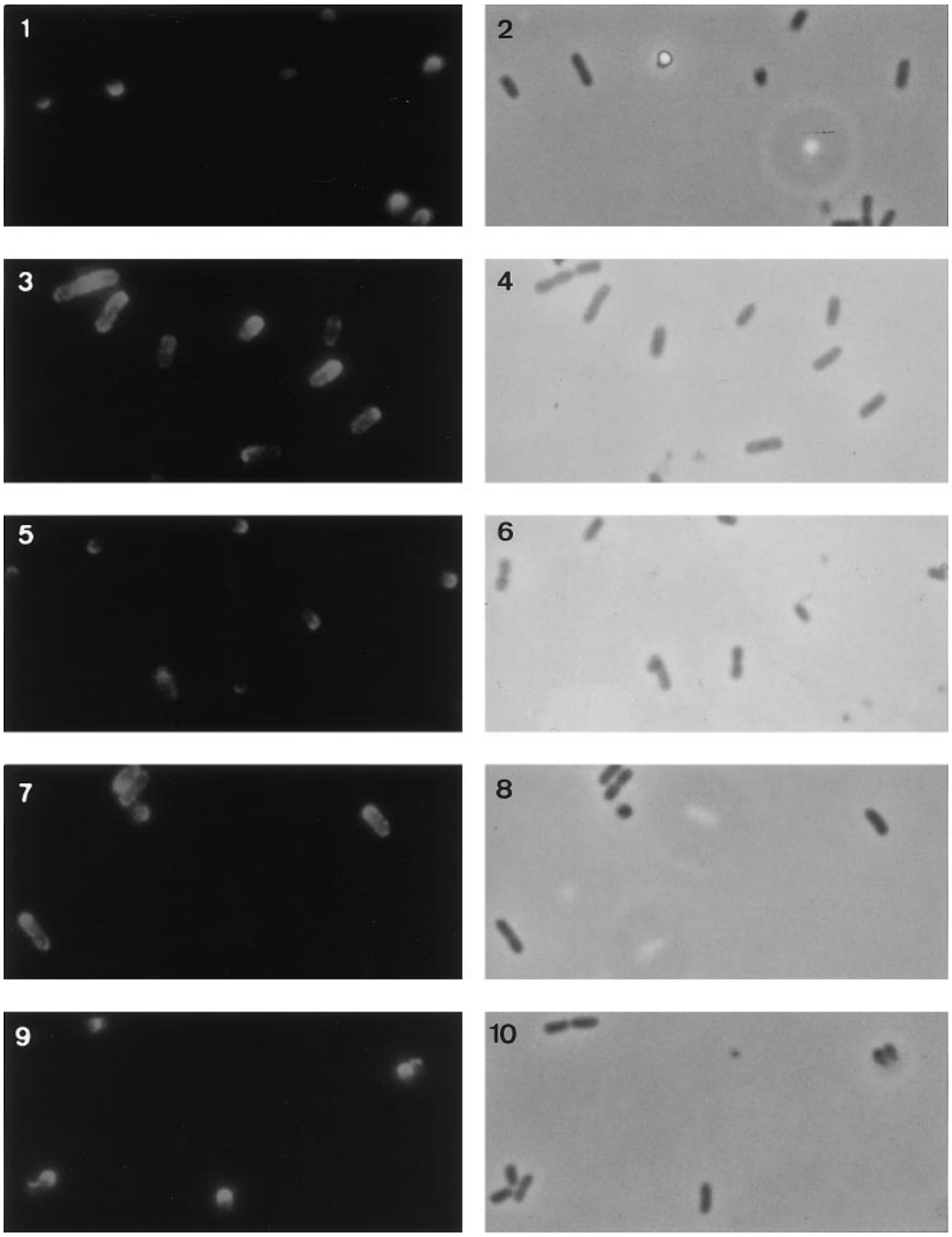


Fig. 3. Surface distribution of IcsA. Panels 1, 3, 5, 7 and 9 show the immunofluorescence labelling using an anti-IcsA antiserum and panels 2, 4, 6, 8 and 10 show the corresponding phase-contrast images. Panels: 1 and 2, M90T (wild type); 3 and 4, SE100 (*sopA*); 5 and 6, SE100 harbouring pCE707 (SopA$^+$); 7 and 8, BS3189 (the virulence plasmid-cured strain expressing *icsA* from a recombinant plasmid); 9 and 10, BS3189 harbouring pCE704 (SopA$^+$).

rise to plaques the size of which was similar to those produced by the wild-type strain.

Discussion

The physiological relevance of cleavage of a certain proportion of IcsA and secretion of IcsA$_\alpha$ were hitherto unknown. Previous evidence indicated that presentation of IcsA at the bacterial surface, without subsequent cleavage, was sufficient to elicit actin polymerization. Production of IcsA in *E. coli* could promote the F-actin-mediated motility of some bacteria in cytosolic extracts of *Xenopus*

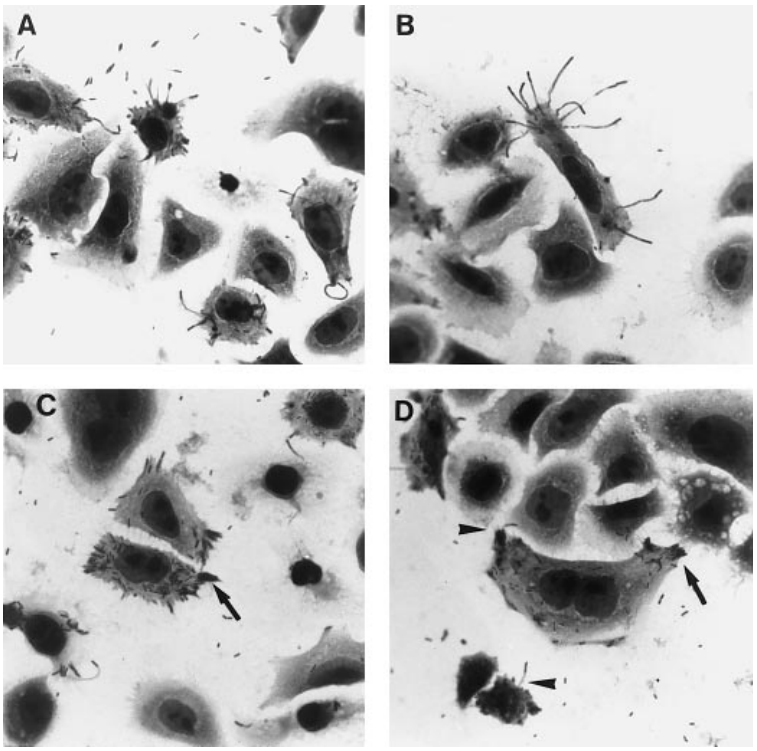


Fig. 4. Giemsa staining of HeLa cells infected by *Shigella* strains. Cells infected by M90T (wild type) (A and B) or SE100 (*sopA*) (C and D) were fixed and stained with Giemsa. Arrows indicate *sopA* mutant bacteria caught in peripherial zones of the cell and arrowheads indicate small protusions induced by the *sopA* mutant.

oocytes (Kocks *et al.*, 1995; Goldberg and Theriot, 1995). In addition, a strain of *Shigella flexneri* that was unable to secrete $IcsA_{\alpha}$, due to elimination of the cleavage site by site-directed mutagenesis, was still motile intracellularly (d'Hauteville and Sansonetti, 1992; Fukuda *et al.*, 1995). However, we have recently shown that lack of cleavage led to circumferential localization of IcsA on the bacterial surface, both in culture and intracellularly, and to aberrant

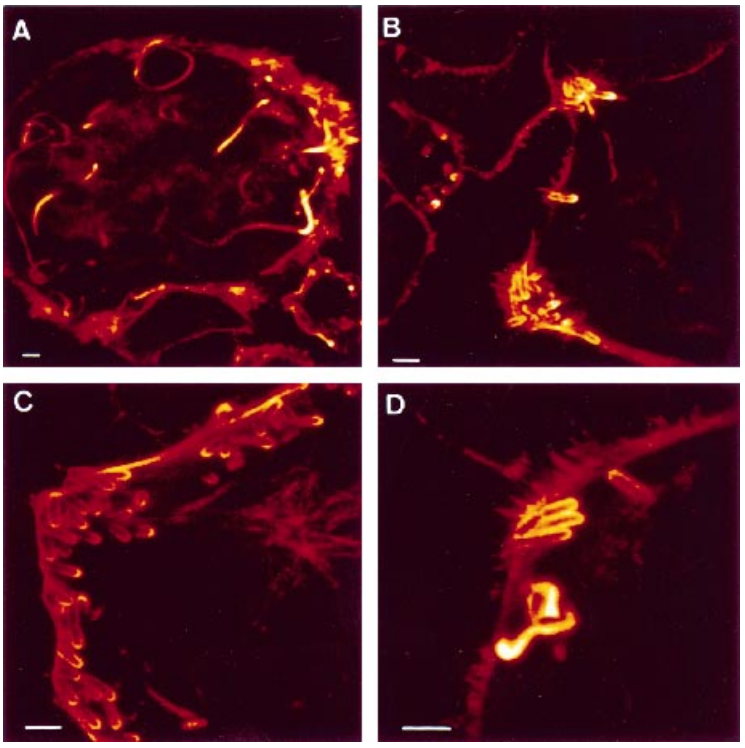


Fig. 5. F-actin labelling of HeLa cells infected by *Shigella* strains. Cells infected by M90T (wild type) (A) or SE100 (*sopA*) (B, C and D) were fixed and labelled for F-actin with NBD–phallacidin. The bars represent 5 μm.

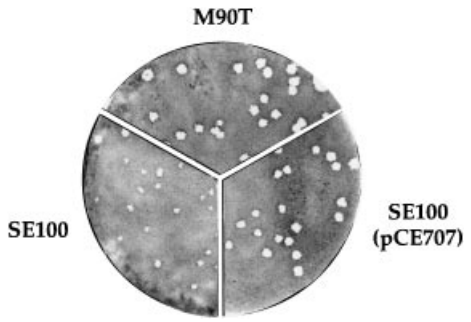


Fig. 6. Formation of plaques on confluent monolayers of Caco-2 cells. Cell monolayers were infected by M90T (wild type), SE100 (*sopA*), and SE100 harbouring pCE707 (SopA$^+$) for 2 h, overlayed with gentamicin-containing soft agar, incubated at 37°C for 48 h, fixed, and stained with Giemsa.

actin polymerization and abnormal movement (d'Hauteville *et al.*, 1996). This suggested that polar localization of IcsA might result from proteolytic cleavage of a certain amount of the surface-exposed protein and that this cleavage was essential to the fine-tuning of actin-based motility. Here, we present the characterization of SopA, the protease responsible for cleavage of IcsA, and demonstrate the importance of this cleavage in the polar localization of IcsA and the intracellular motility of *Shigella*.

A strong similarity (60% identity) was detected between the sequence of SopA and those of OmpT and OmpP. OmpT is a major outer membrane protease that has a trypsin-like activity specific for Arg–Arg bonds (Grodberg and Dunn, 1988; Sugimura and Nishihara, 1988). OmpP is 73% identical to OmpT and has the same specificity (Kaufmann *et al.*, 1994). The sequence similarity observed between OmpT, OmpP, and SopA suggests that SopA is a serine protease located in the outer membrane. This is consistent with the activity of SopA, which cleaves surface-exposed IcsA, presumably between two Arg residues. Nakata *et al.* (1993) have shown that expression of OmpT in *Shigella* led to total degradation of IcsA. Similarly, we have observed that expression of OmpP from a recombinant plasmid in *Shigella* led to complete degradation of IcsA (C. Egile, unpublished results). The partial cleavage of IcsA by SopA, as opposed to its degradation induced by OmpT and OmpP, might result from either a lower level of production of the protein or a lower specific activity of SopA. *Shigella* spp. do not have *ompT* due to a chromosomal deletion (Nakata *et al.*, 1993) and it is not known whether *ompP* is present. The fact that *sopA* is located at some distance from *icsA* on the virulence plasmid suggests that these two genes have been acquired independently. Addition of *sopA* has probably provided a selective advantage to *Shigella* spp. by improving their intracellular motility.

The identification of the *sopA* gene allowed us to assess the role of cleavage and release of $IcsA_\alpha$ on surface distribution of IcsA by comparing the wild type and *sopA* strains, as well as virulence plasmid-cured strains expressing IcsA in either the absence (BS3189) or the presence of SopA (BS3189 with pCE704). Both BS3189 and the *sopA* mutant exhibited a circumferential repartition of IcsA, with some polar enrichment, and expression of SopA in both strains caused a polar localization of IcsA. The amount of SopA that is produced by M90T was too low to allow determination of the surface distribution of the protein. Given the very high sequence similarity between SopA and OmpP, SopA, similar to OmpP, is probably uniformly distributed on the bacterial surface (Kaufmann *et al.*, 1994). Therefore, the polar localization of IcsA appears to result from the conjunction of two phenomena: (i) targeting of the protein to the entire bacterial surface with an enhancement at the pole opposite to the division septum; and (ii) cleavage by SopA of a low amount of IcsA over the entire bacterial surface. This proteolytic activity achieves the exclusive polar localization of IcsA by removing the proteins which are present on the sides of the bacterium. The mechanism involved in the accumulation of IcsA at the bacterial pole in the absence of SopA is not known. Such an accumulation occurs even in the strain which has been cured of the virulence plasmid, which suggests that no virulence plasmid-encoded genes are involved in this process. An intrinsic stability of IcsA could be sufficient to lead to an accumulation of the protein in the older portion of the bacterial surface, without any requirement for targeting proteins. Alternatively, the C-terminal anchor domain of IcsA might have a higher affinity for components that are characteristic of the polar area of the bacterium, the periseptal annulus. Drastic bacterial surface modifications, such as the lack of expression of the O-side chains of LPS, also impair polar localization of IcsA, secretion of $IcsA_\alpha$, and actin-based motility (Rajakumar *et al.*, 1994; Sandlin *et al.*, 1995). The OmpT protease has been proposed to be active as a trimer and its trimerization might be LPS dependent, as demonstrated for other outer membrane proteins (Sen and Nikaido, 1991). Therefore, in rough mutants, the abnormal surface repartition of IcsA could be due to an effect on the trimerization of SopA.

In *Listeria monocytogenes*, another facultative intracellular bacteria, actin-based motility is due to ActA. This protein is also asymmetrically distributed at the bacterial surface, although with a less-restricted pattern, i.e. it is found at one pole and on the sides of the bacterium but is excluded from the new pole following cell division. The molecular mechanism of polarization of ActA is also unknown (Kocks *et al.*, 1993). Apart from structures such as pili and flagella, few other bacterial proteins are known to exhibit a polar localization: a haemagglutinin of *Myxococcus xanthus*, the maltose-binding protein of *E. coli*, and the chemosensory apparatus of *Caulobacter crescentus*

(reviewied in Shapiro, 1993; Maddock *et al*., 1993). McpA, an integral membrane signal transducer of this chemotaxis complex, is targeted to the flagellated pole of the predivisional cell. During differentiation of this swarmer pole into a stalked pole, McpA is removed from the swarmer pole by proteolysis, and new molecules are targeted to the new swarmer pole of the predivisional cell. Two different mechanisms contribute to asymmetric distribution of McpA: degradation of the old McpA during the swarmer-to-stalked differentiation, and targeting of newly synthesized McpA to the swarmer pole (Maddock *et al*., 1993). Similar mechanisms are required for the polar localization in the predivisional cell of the motor protein of the flagellum, FliF (Jenal and Shapiro, 1996). Accordingly, proteolysis of the laterally distributed proteins does not lead to the polar distribution but is involved in the transient loss of the asymmetry. In contrast, the exclusive polar localization of IcsA is due to the removal of laterally distributed proteins.

The *sopA* mutant was dramatically affected in its ability to move within the intracellular compartment. Actin polymerization occurred over the entire bacterial surface and bacteria established a seemingly stable association with the actin subcortical network and the structures forming adhesion plaques. It is probable that the coating of the entire bacterial body by F-actin caused a glue-like effect leading to entrapment of bacteria in structures that are themselves essentially composed of F-actin. Thus, polar location of IcsA on the surface of wild-type bacteria appears to be required for asymmetric accumulation of F-actin, in order to generate a unidirectional force allowing bacteria to escape from peripheral zones of infected cells. In contrast, opposite forces generated by actin polymerization on the entire surface on the *sopA* mutant lead to bacterial immobilization. Although the *sopA* mutant was severely affected in its intracellular movement, as evaluated by actin staining after 2 h of infection, the size of plaques produced by this mutant after 48 h was reduced by only approx. 50% as compared to those produced by the wild-type strain. There is no linear correlation between the speed of the movement of intracellular bacteria and the size of plaques. For example, deletion of the entire proline-rich repeats in the *L. monocytogenes* ActA protein can reduce the speed of the actin-based intracellular movement down to 29% of that observed with the wild-type ActA but lead to a decrease of only 61% in the size of the plaques (Smith *et al*., 1996). Such mutations also have a marked effect on the virulence in infected mice.

Previous investigations using cell-free models of motility suggested that polar localization of the bacterial protein required for actin polymerization was essential for efficient motility of intracellular pathogens. Expression of *icsA* in an *E. coli* strain led to the peripheral localization of IcsA protein with some polar preference. In cytoplasmic extracts of *Xenopus* oocytes, actin polymerization occurred in a large proportion of bacteria (74%), but formation of an F-actin comet tail and actin-based motility were observed with only 10% of these recombinant bacteria (Kocks *et al*., 1995). Smith *et al*. (1995) have shown that an ActA–LytA hybrid protein, which had been uniformly coated on the surface of *Streptococcus pneumoniae*, could induce actin nucleation in cytoplasmic extracts. However, formation of a F-actin comet tail and *S. pneumoniae* motility were observed only when the hybrid protein became distributed unipolarly after bacterial division.

In the present study, we have demonstrated that establishment of IcsA polarity is an essential factor for *Shigella* motility within infected cells and that the polar distribution of IcsA on the bacterial surface involves cleavage and release of a certain proportion of the surface-exposed protein by an outer membrane protease of the OmpT family. Although proteases homologous to OmpT are produced by other Gram-negative enteropathogens, such as *Salmonella typhimurium* (Grodberg and Dunn, 1989) and *Yersinia pestis* (Sodeinde and Goguen, 1989), there is as yet no direct demonstration of a role in pathogenicity for members of this family of proteases.

Experimental procedures

Bacterial strains, plasmids, and growth media

S. flexneri strains used in this study are derivatives of the wild-type strain M90T of serotype 5 (Sansonetti *et al*., 1982). *E coli* NM554 was used for cloning into conventional vectors, DH5αλpir was used as a host for derivatives of the suicide plasmid pGP704 (Miller and Mekalanos, 1988), SM10λpir was used to transfer suicide plasmids to *S. flexneri*, and BL100 was used to express a His-tagged SopA protein. *S. flexneri* was grown in tryptic–soy broth and *E. coli* in Luria (L) broth or 2× yeast–tryptone (YT)-broth. Antibiotics were used at the following concentrations: ampicillin, 100 μg ml^{-1}; tetracyclin, 5 μg ml^{-1}; chloramphenicol, 35 μg ml^{-1} and streptomycin, 100 μg ml^{-1}.

Molecular cloning procedures

DNA analysis, plasmid constructions, and electroporation of *E. coli* and *S. flexneri* were performed according to standard procedures (Maniatis *et al*., 1982). Nucleotide sequence of both DNA strands were determined by the dideoxy chain-termination procedure. Amplification of DNA fragments by the polymerase chain reaction (PCR) was performed using 10 ng of plasmid DNA template, 1 μM of each primer, and 400 μM of each deoxynucleotide triphosphate in a 50 μl reaction containing Vent DNA polymerase buffer and Vent DNA polymerase (New England Biolabs). After 5 min of denaturation at 95°C, 2 U of Vent DNA polymerase were added. Twenty-five cycles of 1 min of denaturation at 95°C, 1 min of primer annealing at 55°C, and 1 min of primer extension at 72°C were carried out in a thermocycler.

Protein analysis

Whole cell and supernatant extracts were prepared as described previously (d'Hauteville *et al*., 1996). Ten millilitres of a culture in exponential-growth phase was centrifuged at 5000 × *g* for 10 min and the culture supernatant was passed through a 0.2 μm filter. Proteins present in the filtrate were precipitated by addition of 10% trichloroacetic acid and resuspended in Laemmli sample buffer (Laemmli, 1970). Whole cell protein extracts were prepared from 1 ml of bacterial culture by centrifugation at 5000 × *g* and solubilization of the pellet in Laemmli sample buffer. Samples of culture supernatant and whole cell extracts were boiled for 5 min and analysed by SDS–PAGE. After electrophoresis, proteins were transferred onto a nitrocellulose membrane. Immunoblotting procedures were carried out with rabbit polyclonal antiserum raised against IcsA or SopA (Towbin *et al*., 1979). Horseradish peroxidase-labelled goat anti-rabbit antibodies were used as secondary antibodies and visualized by enhanced chemiluminescence.

Cloning of the sopA *gene*

BS3189 is a derivative of the virulence plasmid-cured strain BS176 which harbours plasmid pHS3189 expressing *icsA*. This plasmid, which contains a 7.6 kb *Eco*RI fragment of pWR100 including the *icsA* gene, is a derivative of pACYC184 and confers resistance to tetracycline. Cosmid pCos104 is part of a set of 10 cosmids from a library previously constructed by inserting fragments of pWR100, obtained by partial digestion with *Sau*3A, into the *Bam*HI site of pJB8, a derivative of pBR322 (Maurelli *et al*., 1985). These cosmids, which confer resistance to ampicillin, are compatible with pHS3189.

Digestion of pCos104 by *Sal*I and *Nde*I, filling in of protruding ends with Klenow fragment of DNA polymerase, and religation gave rise to plasmid pCE50. Plasmids pCE60 and pCE751 were constructed by subcloning 3.5 kb and 0.96 kb *Eco*RI fragments from pCE50 in pBluescript-II SK+ (Stratagene), respectively. Similarly, a 1.9 kb *Hin*dIII–*Sty*I fragment from pCE50 was cloned in pBluescript-II SK+ to give rise to pCE704 and in pBCSK+ (Stratagene) to give rise to pCE707.

Construction of the sopA *mutant*

An internal fragment of *sopA*, extending from codon 50 to codon 250, was amplified by PCR and cloned into the *Sal*I site of a derivative of the suicide vector pGP704 (Miller and Mekalanos, 1988) to give rise to plasmid pCES6. This plasmid was transferred by conjugation into M90T-Sm (SC302) (Allaoui *et al*., 1992) and transconjugants were selected on plates containing streptomycin (Sm) and ampicillin. Integration of pCES6 into the *sopA* locus present on pWR100 was confirmed by Southern blot analysis and the corresponding strain was designated SE100.

Preparation of the anti-SopA antiserum

An anti-SopA serum was raised in rabbits after immunization with a His-tagged SopA protein. In the first step, a *Nde*I site was introduced at the *sopA* translation start codon and the resulting *sopA* gene, carried by a 1.2 kb *Nde*I–*Bam*HI fragment, was cloned into pET19b+ (Novagen). The resulting plasmid, pCEXSOP, encodes a recombinant SopA protein which contains 10 His residues at its N-terminal extremity and is expressed under control of the T7 promoter. A high level of expression of the His_{10}–SopA recombinant protein was obtained in strain BL100 after 3 h of induction in the presence of IPTG (1 mM) at 30°C and the protein was purified using the following procedure. Bacteria were harvested by centrifugation at 5000 × *g* and sonicated in buffer A (20 mM Tris, 100 mM NaCl, pH 8). The extract was centrifuged at 10 000 × *g* for 10 min and the pellet, which contained the recombinant protein, was resuspended in buffer B (buffer A containing 8 M urea). After clarification at 100 000 × *g* for 30 min, the supernatant was loaded onto a 2 ml column of Talon IMAC resin (Clontech). The His_{10}–SopA fusion protein was eluted in buffer C (buffer B containing 100 mM imidazole) and dialysed against buffer A. Rabbit immunization with the purified protein was performed as previously described (Goldberg *et al*., 1993). The anti-SopA antiserum was adsorbed successively against whole bacterial lysates of *S. flexneri sopA* mutant and *E. coli* BL100.

Cell infection and fluorescence labelling

Culture and infection of HeLa and Caco-2 cells were performed as previously described (d'Hauteville *et al*., 1996). Bacteria in the exponential phase of growth were immunolabelled as described previously (d'Hauteville *et al*., 1996). Briefly, 100 μl of a bacterial suspension at an OD_{600} of 0.5 was diluted fivefold in PBS and incubated for 30 min in the presence of an anti-IcsA rabbit polyclonal serum, used at a 1/100 dilution. Fluorescence labelling was performed with donkey anti-rabbit rhodamine antibodies (Amersham). For labelling of F-actin, HeLa cell infections were performed on glass coverslips in 35 mm Petri dishes as described previously (d'Hauteville *et al*., 1996). Infected HeLa cells were fixed in PBS containing 3.7% paraformaldehyde for 20 min and labelled with NBD–phallacidin (Sigma Chemical Co.). Labelled preparations were observed using a conventional fluorescence microscope (BH2-RFCA, Olympus Optical Co.) or a confocal laser scanning microscope (Wild Leitz Instruments GmbH). The plaque assay on confluent monolayers of Caco-2 cells was performed as previously described (Oaks *et al*., 1985).

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