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The unipolar *Shigella* surface protein IcsA is targeted directly to the bacterial old pole: IcsP cleavage of IcsA occurs over the entire bacterial surface

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Summary

***Shigella flexneri* is an intracellular pathogen that is able to move within the cytoplasm of infected cells by the continual assembly of actin onto one pole of the bacterium. IcsA, an outer membrane protein, is localized to the old pole of the bacterium and is both necessary and sufficient for actin assembly. IcsA is slowly cleaved from the bacterial surface by the protease IcsP (SopA). Absence of IcsP leads to an alteration in the distribution of surface IcsA, such that the polar cap is maintained and some IcsA is distributed along the lateral walls of the bacillus. The mechanism of unipolar localization of IcsA and the role of IcsP in its unipolar localization are incompletely understood. Here, we demonstrate that cleavage of IcsA occurs exclusively in the outer membrane and that IcsP is localized to the outer membrane. In addition, we show that IcsA at the old pole is susceptible to cleavage by IcsP and that native IcsP is active at the pole. Taken together, these data indicate that IcsP cleaves IcsA over the entire bacterial surface. Finally, we show that, immediately after induction from a tightly regulated promoter, IcsA is expressed exclusively at the old pole in both the *icsP*$^-$ *icsA*$^-$ and the *icsA*$^-$ background. These data demonstrate that unipolar localization of IcsA results from its direct targeting to the pole, followed by its diffusion laterally in the outer membrane.**

Introduction

Shigella flexneri is a facultative intracellular pathogen that, after entry into host cells, is able to move within the cell cytoplasm and into adjacent cells by the continual assembly of actin onto one pole of the bacterium. *S. flexneri* enters cells by a process of induced phagocytosis (LaBrec *et al*., 1964; Clerc and Sansonetti, 1987). Shortly after entry, it lyses the phagocytic vacuole (Sansonetti *et al*., 1986; High *et al*., 1992) and is thereby released into the cytoplasm. Within the cytoplasm, it assembles a tail at one pole that consists of bundled filaments of host cell actin (Bernardini *et al*., 1989). The ongoing assembly of actin filaments into the tail at its junction with the bacterial pole provides the force that propels the bacterium forward through the cytoplasm (Theriot *et al*., 1992). The *S. flexneri* 120 kDa outer membrane protein IcsA (VirG) is both necessary and sufficient for actin tail assembly (Bernardini *et al*., 1989; Pal *et al*., 1989; Goldberg and Theriot, 1995; Kocks *et al*., 1995). IcsA is unusual among outer membrane proteins in that it is localized to a single pole of the bacillus (Goldberg *et al*., 1993), that pole at which actin assembly occurs (Goldberg *et al*., 1993). Data on the localization of IcsA on dividing bacteria and on the direction of actin-based movement of dividing bacteria and their daughters demonstrate that the pole at which IcsA is localized is always the old pole (Goldberg *et al*., 1993; 1994).

The molecular mechanism by which IcsA is localized to one pole is incompletely understood. IcsA is a 1102-amino-acid polypeptide, of which the carboxy-terminal 344 amino acids (the β-domain) serve as an anchor in the outer membrane, with the 95 kDa amino-terminal portion of the mature protein (the α-domain) exposed at the bacterial surface (Suzuki *et al*., 1995). IcsA is cleaved at the Arg-758–Arg-759 bond by the protease IcsP (SopA), which bears sequence similarity to bacterial serine proteases (Fukuda *et al*., 1995; Egile *et al*., 1997; Shere *et al*., 1997). Cleavage of IcsA occurs both under *in vitro* culture conditions and in infected cells (Goldberg *et al*., 1993). Upon disruption of *icsP*, IcsA cleavage is markedly reduced, such that less than 10% of the amount of the cleaved fragment of IcsA that is found in the culture supernatant of wild-type *S. flexneri* is found in the culture supernatant of the *icsP*$^-$ strain (Egile *et al*., 1997; Shere *et al*., 1997). The sequence similarity of IcsP to bacterial serine proteases, in conjunction with the identification of the IcsA cleavage site and the known specificity of the target site of certain serine proteases as Arg–Arg, Arg–Lys, Lys–Arg and Lys–Lys bonds (Sugimura and Nishihara, 1988; Kaufmann *et al*., 1994), suggests that IcsP cleaves IcsA directly.

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The expression of IcsA on wild-type *Shigella* is maximal during exponential growth and markedly reduced during stationary phase, such that 75% of stationary-phase bacteria have no IcsA on the surface (Goldberg *et al*., 1994). Cleavage of IcsA by IcsP is such that, in mid-exponential growth phase, approximately 80–85% of IcsA is present on the bacterial surface and approximately 15–20% can be recovered as the 95 kDa amino-terminal fragment in the culture supernatant (M. B. Goldberg, unpublished observations). Thus, it appears that either the cleavage of IcsA by IcsP is a slow process (i.e. the K_m is high) or some IcsA is protected from cleavage, perhaps by virtue of its subcellular localization or by virtue of some as yet unidentified post-translational modification.

Two models for the mechanism of unipolar localization of IcsA have been proposed (Fig. 1). In the first, IcsA is targeted directly to one pole of the bacterium, where it is translocated across the inner membrane and inserted into the adjacent outer membrane (Fig. 1A). In the second model, IcsA is not targeted to a pole directly, but is instead translocated across the inner membrane in a uniform manner and is subsequently or simultaneously cleaved from all sites except one pole (Fig. 1B). As IcsA is cleaved by IcsP, it was of interest to examine the localization of IcsA on *icsP*$^-$ *S. flexneri*. We demonstrated that, on the surface of the *icsP*$^-$ mutant derived from serotype 2a strain 2457T, IcsA maintains a unipolar cap, but is also found over the rest of the bacterial surface (Shere *et al*., 1997). Egile *et al*. (1997) also observed that, on the surface of their *icsP*$^-$ (*sopA*$^-$) mutant, which was derived from serotype 5 strain M90T, IcsA was most prominent at the pole. These results indicated that either (i) IcsA is directly targeted to the pole and, when not cleaved, is able to diffuse laterally within the outer membrane; or (ii) IcsA is inserted into the outer membrane in a more uniform manner and is not cleaved by IcsP only at the pole. Lack of cleavage at the pole might theoretically result from either modification of polar IcsA such that it cannot be cleaved or selective absence or inactivity of IcsP at the pole. If the former were true, then phosphorylation might be important to the regulation of IcsA cleavage, as the protease cleavage site lies within the sequence Ser-756–Ser–Arg–Arg–Ala–Ser–Ser-762, a recognition site for serine–threonine phosphatases, and IcsA can be phosphorylated *in vitro* (d'Hauteville and Sansonetti, 1992).

The model of IcsA translocation proposed by Suzuki *et al*. (1995) involves insertion of the carboxy-terminal β-domain into the outer membrane in the form of a barrel-shaped pore, followed by displacement of the amino-terminal α-domain through the pore. If IcsA is targeted directly to one pole of the bacterium (model depicted in Fig. 1A), then cleavage of IcsA would probably occur only after IcsA translocation across the outer membrane, in which case IcsP would be predicted to be located in the outer membrane with its serine protease site exposed at the bacterial surface. If IcsA is translocated across the inner membrane in a uniform manner and is cleaved at all sites except one pole (model depicted in Fig. 1B), then IcsP could theoretically be located either in the membrane or in the periplasm and would be predicted to be inactive or absent at the pole. To distinguish between the proposed models of IcsA unipolar localization and to characterize the subcellular localization and function of IcsP further, the present study was undertaken.

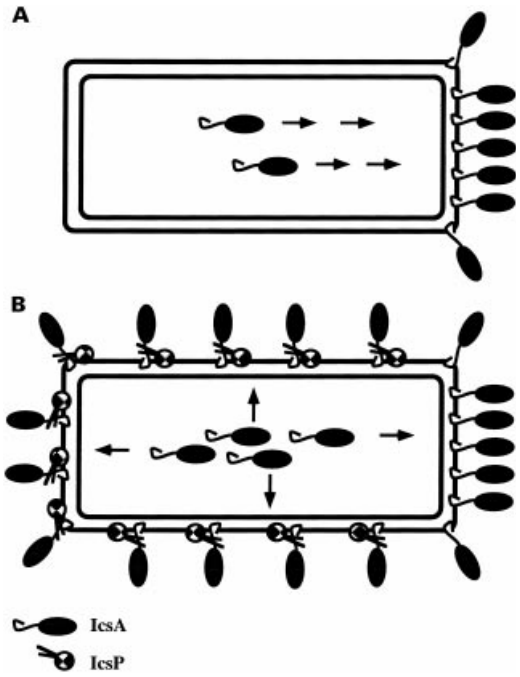

Fig. 1. Two models for the mechanism of unipolar localization of IcsA in *S. flexneri*.
A. Direct targeting of IcsA to one pole.
B. Uniform insertion of IcsA into the outer membrane in conjunction with IcsP-mediated cleavage of IcsA from all sites except one pole.

Results

Presence of the cleaved β-domain of IcsA in the outer membrane

Cleavage of IcsA either during or subsequent to its translocation across the outer membrane would be predicted to lead to the localization of the cleaved carboxy-terminal β-domain of IcsA within the outer membrane, whereas cleavage of IcsA before its translocation would be predicted to lead to the localization of the cleaved carboxy-terminal β-domain of IcsA exclusively within the periplasm or inner membrane. Bacterial proteins from wild-type and *icsA*$^-$ *S. flexneri* were prepared and fractionated (see *Experimental procedures*) and were examined by both

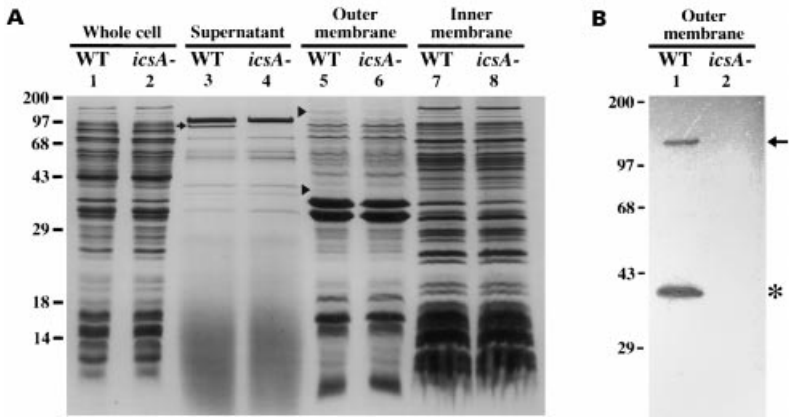

Fig. 2. Subcellular localization of the cleaved β-domain of IcsA.
A. Coomassie-stained SDS–PAGE gel. Whole-cell (lanes 1 and 2), supernatant (lanes 3 and 4), outer membrane (lanes 5 and 6) and inner membrane (lanes 7 and 8) protein preparations of wild-type *S. flexneri* strain M90T (odd-numbered lanes) and *icsA*⁻ strain SC560 (even-numbered lanes). Short arrow, cleaved α-domain of IcsA. Arrowheads, mature IcsA and cleaved β-domain of IcsA.
B. Western blot analysis of outer membrane protein preparations of wild-type *S. flexneri* strain M90T (lane 1) and *icsA*⁻ strain SC560 (lane 2), probed with antiserum to the β-domain of IcsA. Arrow, mature IcsA (after cleavage of signal peptide). Asterisk, cleaved β-domain of IcsA. Apparent molecular masses of standard proteins run in parallel are indicated in kilodaltons on the left.

Coomassie staining of SDS–PAGE and Western blot analysis using antiserum to the β-domain of IcsA (Fig. 2). In outer membrane fractions, two bands that were present in wild-type *S. flexneri* were absent from the *icsA*⁻ strain (Fig. 2A, lane 5 versus lane 6, arrowheads): a large band at approximately 120 kDa, which corresponds to mature IcsA (after cleavage of the signal peptide) and a small band migrating at approximately 35 kDa. This smaller band migrated at approximately the mass predicted for the cleaved β-domain of IcsA (38 kDa), which is a 344-amino-acid polypeptide. As expected, in supernatant fractions, a 95 kDa band that corresponds to the cleaved α-domain of IcsA was present in wild-type *S. flexneri* and was absent from the *icsA*⁻ strain (Fig. 2A, lane 3 versus lane 4, short arrow). Both the 120 kDa and the 35 kDa bands in outer membrane fractions were recognized specifically by antiserum to the IcsA β-domain, with the signal from the 35 kDa band being stronger than that from the 120 kDa band (Fig. 2B, arrow and asterisk respectively), indicating that significant cleavage of IcsA by IcsP occurs in the outer membrane.

Subcellular localization of IcsP

The observed restriction of the carboxy-terminal portion of cleaved IcsA to the outer membrane suggested that its peptidase was probably located in the outer membrane or the periplasm. *Escherichia coli* OmpP and OmpT, to each of which IcsP bears 58% sequence identity (Egile *et al*., 1997; Shere *et al*., 1997), have been shown to be outer membrane proteins (Hollifield *et al*., 1978; Grodberg and Dunn, 1988; Sugimura and Nishihara, 1988; Kaufmann *et al*., 1994). Previous work has demonstrated that IcsP is present in whole-cell protein preparations (Egile *et al*., 1997). To characterize the subcellular localization of IcsP further, bacterial proteins from wild-type and *icsP*⁻ *S. flexneri* were prepared and fractionated (see *Experimental procedures*) and were examined by Western blot analysis using affinity-purified antiserum to IcsP (Fig. 3). A band specific to the wild-type strain, migrating at approximately 33 kDa (arrow), which corresponds to the predicted size of IcsP, was seen in outer membrane and whole-cell protein fractions, but was absent from other fractions. A second non-specific band migrated at approximately 43 kDa.

Substrate specificity of IcsP

In the plaque assay, which assesses the ability of *Shigella* strains to spread from cell to cell through a tissue culture monolayer, we observed no differences between the *icsP*⁻ mutant and the wild type (Shere *et al*., 1997). The lack of attenuation in this assay, in conjunction with the relatively common occurrence of the cleavage recognition sites of serine proteases (Arg–Arg, Arg–Lys, Lys–Arg and Lys–Lys bonds) raised the possibility that cleavage of IcsA might not serve a specific role in pathogenesis and might instead result from its being an 'innocent bystander' to IcsP activity of a different purpose. To address whether proteins other than IcsA were substrates for IcsP, banding patterns of whole-cell protein and culture supernatant protein preparations from biotinylated cultures were compared

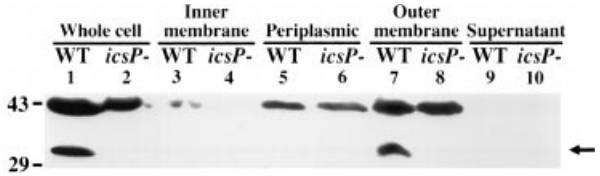

Fig. 3. Subcellular localization of IcsP. Western blot using antiserum to IcsP. Whole-cell (lanes 1 and 2), inner membrane (lanes 3 and 4), periplasmic (lanes 5 and 6), outer membrane (lanes 7 and 8) and supernatant (lanes 9 and 10) protein preparations of wild-type *S. flexneri* strain 2457T (odd-numbered lanes) and *icsP*⁻ strain MBG341 (even-numbered lanes). Protein prepared from approximately the same volume of bacterial culture was loaded into each lane. Arrow, IcsP. Apparent molecular masses of standard proteins run in parallel are indicated in kilodaltons on the left.

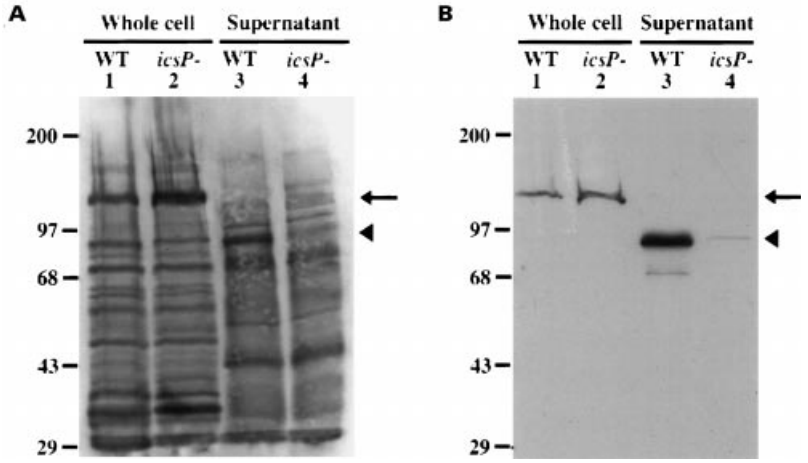

Fig. 4. Surface-exposed proteins of *S. flexneri* that are substrates for cleavage by IcsP. Whole-cell (lanes 1 and 2) and supernatant protein (lanes 3 and 4) preparations from wild-type strain 2457T (lanes 1 and 3) and *icsP*$^-$ strain MBG341 (lanes 2 and 4) after biotinylation of surface-exposed proteins, separated on a 7.5% polyacrylamide gel.
A. Autoradiogram of biotinylated proteins.
B. Western blot probed with antiserum to IcsA.
The amount of protein loaded into each lane containing supernatant proteins was prepared from an ≈16-fold larger volume of bacterial culture to that loaded into each lane containing whole-cell proteins. Arrow, mature IcsA. Arrowhead, cleaved and secreted α-domain of IcsA. Apparent molecular masses of standard proteins run in parallel are indicated in kilodaltons on the left.

for the *icsP*$^-$ strain MBG341 and the parent wild-type strain (Fig. 4). While multiple (more than 35) bands were biotinylated in the whole-cell protein preparations (Fig. 4A, lanes 1 and 2), IcsA is the only band that was visibly more intense in the preparation from MBG341 (*icsP*$^-$) than in the preparation from wild type (Fig. 4A, lane 2 versus lane 1, arrow), as shown by its recognition by antiserum to IcsA (Fig. 4B, lanes 1 and 2, arrow). Furthermore, in supernatant protein preparations, IcsA was the only band that was distinctly less intense in the preparation from MBG341 (*icsP*$^-$) than in the preparation from wild type (Fig. 4A, lane 4 versus lane 3, arrowhead), as shown by its recognition by antiserum to IcsA (Fig. 4B, lanes 3 and 4, arrowhead). Of note, a small amount of cleaved IcsA is present in the supernatant preparation from MBG341 (*icsP*-) (Fig. 4A and B, lane 4), which was expected, as disruption of *icsP* has been shown to eliminate its cleavage from the bacterial surface incompletely (Shere *et al*., 1997). To ensure that lower molecular weight proteins that would not be visualized on the 7.5% SDS–PAGE gel shown in Fig. 4 were not targets of IcsP, the same protein preparations were examined on a 12.5% gel: no additional bands that were specifically more intense in the whole-cell preparation from MBG341 or less intense or absent in the supernatant preparation from MBG341 were seen (data not shown). These data suggest that, under the growth conditions used, IcsA is the major, and possibly the only, *S. flexneri* target of IcsP.

IcsA cleavage upon overexpression of IcsP

A key feature of the second model of IcsA unipolar localization (Fig. 1B) is that IcsA at the old pole is not cleaved by IcsP. To address whether polar IcsA was resistant to cleavage by IcsP, *icsP* was placed into the wild-type strain under the control of an inducible promoter. Two such constructs were made: the first consisted of the intact coding sequence of *icsP* under the control of the IPTG-inducible promoter (strain MBG352), and the second consisted of the *icsP* coding sequence fused at its carboxy-terminus to a six-histidine tag, also under the control of the IPTG-inducible promoter (strain MBG350). Each of these constructs was transformed into the wild-type strain that contained an episomal copy of the *lacI* gene (contained on plasmid pREP4).

To verify both that, upon IPTG induction, IcsP expression increased significantly and that, under non-inducing conditions, IcsP expression was minimal, the amount of IcsP in whole-cell protein preparations from cultures of MBG350 that had been induced was compared with that from cultures that had not been induced, using the histidine-tagged derivative of IcsP (Fig. 5A). As shown by Western blot probed with antibody to the histidine tag, which has relatively high affinity for the histidine tag, at 2 h after induction with 0.01 mM IPTG during exponential growth, a band that corresponds in size to the IcsP–histidine tag fusion protein was present in protein preparations from IPTG-induced cultures (Fig. 5A, lane 2), whereas none was detected in preparations from non-induced cultures (Fig. 5A, lane 1).

Using the same induction conditions, the amounts of IcsA in whole-cell and supernatant protein preparations was examined in wild-type *S. flexneri* carrying the intact coding sequence of *icsP* under the control of the same IPTG-inducible promoter (strain MBG352) (Fig. 5B). At 45 min after induction, the amount of IcsA seen in whole-cell protein preparations was markedly reduced compared with that from non-induced cultures grown in parallel (Fig. 5B, lane 2 versus lane 1) and, at 2 h after induction, almost no IcsA was seen in whole-cell protein preparations (Fig. 5B, lane 3). Integrated density values, performed on an immunoblot prepared in parallel to that shown in Fig. 5, showed the presence of 67% as much protein in the 120 kDa IcsA band in whole-cell proteins from 45 min of

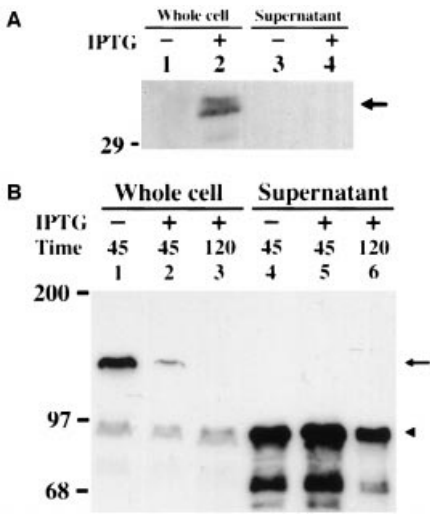

Fig. 5. Cleavage of IcsA upon overexpression of IcsP.
A. Overexpression of IcsP. Western blot probed with antiserum to the histidine tag on a tagged form of IcsP. Whole-cell (lanes 1 and 2) and supernatant (lanes 3 and 4) protein preparations of cultures grown in the presence or absence of IPTG induction of expression of a histidine-tagged form of IcsP (strain MBG350). Arrow, IcsP.
B. Cleavage of IcsA. Western blot probed with antiserum to IcsA. Whole-cell (lanes 1–3) and supernatant (lanes 4–6) protein preparations of cultures grown in the presence or absence of IPTG induction of IcsP expression (strain MBG352). Protein preparations were harvested at the times indicated (in min) after the addition (or not) of IPTG. The amount of protein loaded into each lane containing supernatant proteins was prepared from an ≈fivefold larger volume of bacterial culture to that loaded into each lane containing whole-cell proteins. All lanes are taken from the same blot. The lower bands in lanes 4–6 (migrating at ≈70 kDa) represent degradation products of the IcsA α-domain. Arrow, mature IcsA. Arrowhead, cleaved and secreted α-domain of IcsA. Apparent molecular masses of standard proteins run in parallel are indicated in kilodaltons on the left.

induction (Fig. 5B, lane 2) and 4% as much protein in the corresponding band in whole-cell proteins from 2 h of induction (Fig. 5B, lane 3) as in the corresponding band in whole-cell proteins from the non-induced preparation (Fig. 5B, lane 1). In addition, at 45 min after induction, the amount of IcsA seen in supernatant protein preparations (a 95 kDa band) was slightly increased compared with that in supernatant protein preparations from non-induced cultures grown in parallel (Fig. 5B, lane 5 versus lane 4); the integrated density value for the IcsA band in supernatant proteins at 45 min of induction (Fig. 5B, lane 5) had 15% more protein than the corresponding band in supernatant proteins from non-induced cultures (Fig. 5B, lane 4). There was a significant reduction in IcsA in the supernatant protein preparations after 2 h of induction (Fig. 5B, lane 6); this probably represents degradation of the cleaved extracellular fragment. The amount of full-length IcsA in whole-cell protein preparations and of the 95 kDa cleaved fragment in supernatant protein preparations from proteins prepared under non-inducing conditions is similar to that of the wild-type strain alone (data not shown).

Surface distribution of IcsA upon overexpression of IcsP

The observed marked decrease in IcsA in whole-cell protein preparations upon IPTG-induced overexpression of IcsP suggested that much or all of logarithmic-phase surface IcsA might be susceptible to cleavage by IcsP. To address specifically whether IcsA at the old pole was susceptible to cleavage of IcsP, the surface localization of IcsA on cells that were overexpressing IcsP was determined. As can be seen in Fig. 6, overexpression of IcsP led to the total absence of IcsA on the bacterial surface (Fig. 6C and D), while the surface distribution of IcsA on the same strain grown in the absence of IPTG (Fig. 6A and B) was qualitatively identical to that on the wild-type strain (Fig. 7G–I). Thus, polar IcsA is not protected from cleavage by IcsP.

Susceptibility of IcsA to cleavage by IcsP at the pole

As overexpression of IcsP might lead to its improper localization, the experiments described above, while demonstrating that polar IcsA can be cleaved by IcsP, are not informative about whether native IcsP is active at the pole. To address this issue, we examined IcsA localization on stationary-phase bacteria, based on the following rationale. We have shown previously that the percentage of

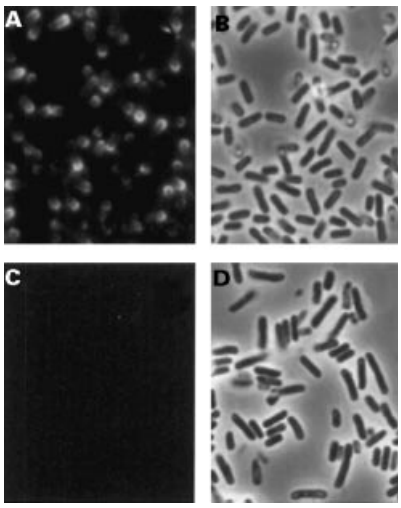

Fig. 6. IcsA on the surface of bacteria after overexpression of IcsP. Indirect immunofluorescent labelling with antiserum to IcsA (A and C) and corresponding fields by phase-contrast (B and D) microscopy.
A and B. MBG352 grown in the absence of IPTG.
C and D. MBG352 grown in the presence of IPTG induction of IcsP expression.

stationary-phase bacteria that have IcsA on the surface is markedly less than the percentage of logarithmic-phase bacteria that have IcsA on the surface (Goldberg *et al*., 1994). IcsA on the surface of those stationary-phase bacteria that express IcsA is in the same unipolar distribution as that on the surface of logarithmic-phase bacteria (Goldberg *et al*., 1994). As bacteria shift into stationary phase, loss of IcsA at the old pole could occur either (i) by IcsP-mediated cleavage of IcsA at the pole; or (ii) by diffusion of IcsA laterally in the outer membrane, followed by its cleavage by IcsP at sites distant from the pole. To distinguish between these two possibilities, we examined both the percentage of bacteria with IcsA present on the surface and the distribution of surface IcsA for stationary-phase *icsP*$^-$ bacilli. Eighty nine ± four per cent (mean ± SD) of stationary-phase MBG341 bacilli had detectable IcsA on the surface, while only 31 ± 3% of stationary-phase wild-type strain 2457T bacilli had detectable IcsA on the surface (representative fields shown in Fig. 7D–F versus A–C respectively). IcsA on the surface of stationary-phase *icsP*$^-$ bacilli was distributed in an asymmetrical fashion, with maintenance of a cap of IcsA on one pole, IcsA extending down the lateral sides of the bacilli and relatively less IcsA on the distal pole (Fig. 7D–F). Thus, the maintenance of a polar cap of IcsA indicates that, on stationary-phase bacteria, a significant percentage of surface IcsA remains at the pole and has not diffused laterally in the membrane. This, in conjunction with the observation that a significant percentage of stationary-phase wild-type *S. flexneri* bacilli have no IcsA on the surface, indicates both that the cleavage that occurs on wild-type bacteria is mediated by IcsP and that IcsP cleaves IcsA from the bacteria pole. Of note, we have shown previously that, on the surface of logarithmic-phase *icsP*$^-$ bacilli, IcsA maintains a polar cap with some IcsA present over the surface of the rest of the bacillus (Fig. 7J–L) (Shere *et al*., 1997). The distribution of IcsA shown here for stationary-phase *icsP*$^-$ bacilli appears to be slightly more delocalized from the pole; this may in part be caused by differences in both the shape of the bacillus and the level of IcsA expression between logarithmic and stationary phases of growth (Goldberg *et al*., 1994).

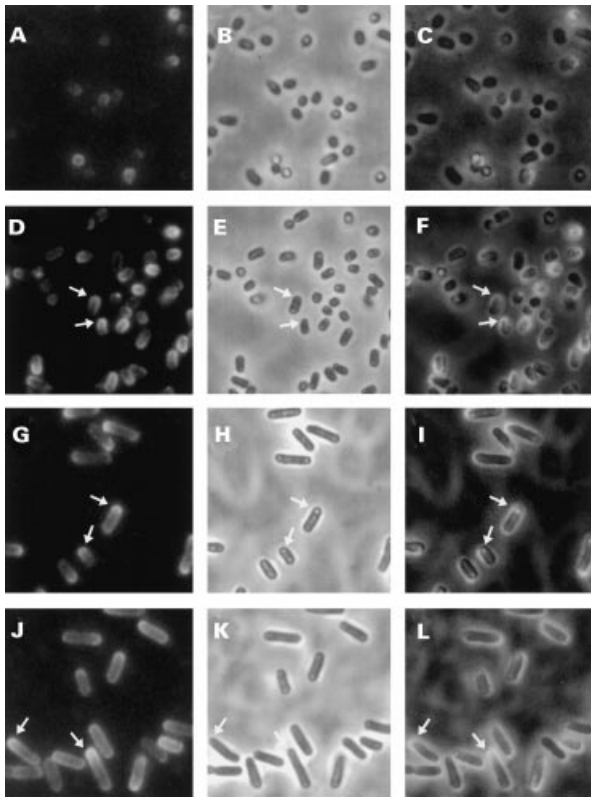

Fig. 7. IcsA distribution on the surface of *S. flexneri* strains in stationary phase. Indirect immunofluorescent labelling with antiserum to IcsA (A, D, G and J); corresponding fields by phase-contrast microscopy (B, E, H and K); and images derived from the superimposition of the indirect immunofluorescent labelling image on the phase-contrast image (C, F, I and L). Wild-type strain 2457T (A–C and G–I) or *icsP*$^-$ strain MBG341 (D–F and J–L) in stationary phase (A–F) or logarithmic phase (G–L) of growth. Arrows, polarly localized IcsA.

Surface distribution of IcsA after expression from a tightly regulated promoter

To further evaluate the mechanism of IcsA localization at the pole, *icsA* was placed under the control of the tightly regulated arabinose promoter, and this construct was introduced into both the *icsA*$^-$ and the *icsA*$^-$ *icsP*$^-$ background of *Shigella* (strains MBG353 and MBG354 respectively). The surface distribution of IcsA was then determined on logarithmic-phase bacteria grown under non-inducing conditions, and at 20, 40 and 60 min after induction. As shown in Fig. 8, under non-inducing conditions, no IcsA is seen on the bacterial surface (A, a–c, and B, a–c). Also at 20 min after induction, no IcsA is seen on the bacterial surface (data not shown). At 40 min after induction, IcsA is seen in a markedly polar distribution on the surface of both strain MBG354 (*icsA*$^-$ *icsP*$^-$ P_{BAD}-*icsA*$^+$) (arrows, Fig. 8A, d–f) and strain MBG353 (*icsA*$^-$ P_{BAD}-*icsA*$^+$) (arrows, Fig. 8B, d–f). At 60 min after induction, a polar cap of IcsA is seen on the surface of both strain MBG354 (arrows, Fig. 8A, g–i) and strain MBG353 (arrows, Fig. 8B, g–i); however, some IcsA is also seen in a punctate distribution over the rest of the surface of strain MBG354. Thus, in the absence of IcsP, IcsA is first seen at the pole and appears along the sides of the bacillus only after additional time has elapsed. These data indicate that IcsA is targeted directly to the pole.

Discussion

Data presented here indicate that IcsA is targeted directly to the old pole of *Shigella* and that IcsP-mediated cleavage

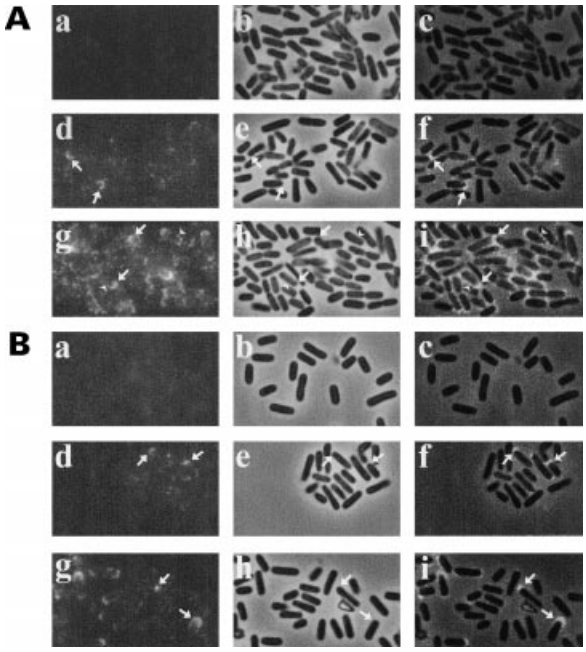

Fig. 8. Localization of IcsA on the bacterial surface after induction of expression from the tightly regulated arabinose promoter.
A. MBG354 (*icsA*$^-$ *icsP*$^-$ P_{BAD}-*icsA*$^+$).
B. MBG353 (*icsA*$^-$ P_{BAD}-*icsA*$^+$).
A and B. Indirect immunofluorescent labelling with antiserum to IcsA (a, d and j); corresponding fields by phase-contrast microscopy (b, e and h); and images derived from the superimposition of the indirect immunofluorescent labelling image on the phase-contrast image (c, f and i). a–c, non-inducing conditions; d–f, at 40 min of induction; and g–i, at 60 min of induction. Arrows, polarly localized IcsA. Arrowheads, IcsA localized along the sides of the bacillus.

of IcsA occurs over the entire bacterial surface. The presence of a prominent cap of IcsA on the pole of logarithmic-phase bacteria suggests that IcsP-mediated cleavage of IcsA occurs significantly more slowly than insertion of IcsA into the polar outer membrane during this phase of growth. The same is not true during stationary phase, however, when IcsA expression is reduced (Goldberg *et al*., 1994), leading to an almost complete loss of IcsA from the surface of the wild-type strain (Fig. 7).

Data presented here demonstrate that IcsP is localized to the outer membrane. Consistent with the localization of IcsP to the outer membrane are data presented here that demonstrate that, after IcsP-mediated cleavage, a significant amount of the carboxy-terminal membrane anchor (β-domain) of IcsA is localized to the outer membrane. Taken together, these data suggest that cleavage of IcsA at its Arg-758–Arg-759 bond occurs either after it has been translocated to the outer membrane or during translocation. Based on the significant primary sequence similarity of IcsP to *E. coli* OmpP and OmpT, we have proposed previously that the active site of IcsP would be within the amino-terminal portion of the polypeptide and the membrane anchor at the carboxy-terminus (Shere *et al*., 1997). This orientation of IcsP within the outer membrane would enable it to cleave IcsA exposed on the bacterial surface.

The predicted amino-terminus of presecretory IcsP contains the sequence 19-Leu–Ala–Leu–Cys-22 (GenBank accession no. AF001633), which is characteristic of the consensus sequence for the site recognized by signal peptidase II (lipoprotein signal peptidase or LspA), Leu–X–Y–Cys (Wu, 1987). Before the site can be cleaved by signal peptidase II, the cysteine must be modified to glycerylcysteine; after cleavage, which occurs at the Y–Cys peptide bond, the glycerylcysteine is generally further modified by the addition of a fatty acid to the free amino group and two fatty acids to the glyceryl residue (Pugsley, 1993). Signal peptides that are recognized by signal peptidase II also lack the glycine or proline residue that is found at or around position −6 of signal peptides cleaved by signal peptidase I (LepB) (von Heijne, 1989); the predicted amino acid sequence of IcsP lacks glycine and proline residues in this region. Sorting of lipoproteins to the outer membrane is determined by a specific sorting signal that permits the interaction of the protein with the periplasmic lipoprotein carrier protein P20 (Yamaguchi *et al*., 1988; Pugsley and Kornacker, 1991; Poquet *et al*., 1993; Pugsley, 1993; Matsuyama *et al*., 1995). The sorting signal consists of the amino acid residue at position +2, i.e. the residue that immediately follows the modified cysteine. The presence of an aspartate in position +2 leads to maintenance of the lipoprotein in the inner membrane, whereas the presence of any other amino acid in this position leads to sorting of the lipoprotein to the outer membrane (Yamaguchi *et al*., 1988; Gennity and Inouye, 1991; Pugsley and Kornacker, 1991; Poquet *et al*., 1993; Pugsley, 1993; Matsuyama *et al*., 1995). The amino acid residue at position +2 of IcsP is valine, which predicts the sorting of IcsP to the outer membrane, consistent with what was observed experimentally (Fig. 3).

Taken together, our observations indicate that IcsP cleaves IcsA over the entire bacterial surface: (i) IcsA at the old pole is susceptible to cleavage by IcsP, as demonstrated by the absence of IcsA on the old pole of bacteria that are overexpressing IcsP; and (ii) IcsP cleaves IcsA at the old pole, as demonstrated by the absence of IcsA on the old pole of the majority of IcsP$^+$ stationary-phase organisms and its presence on the old pole of the essentially all IcsP$^-$ stationary-phase organisms.

These data are inconsistent with the model of unipolar localization of IcsA in which IcsA is inserted into the outer membrane uniformly and is subsequently or simultaneously cleaved from all sites except one pole (Fig. 1B). Rather, the data presented clearly support a modified model of direct targeting of IcsA to the old pole (Fig. 9).

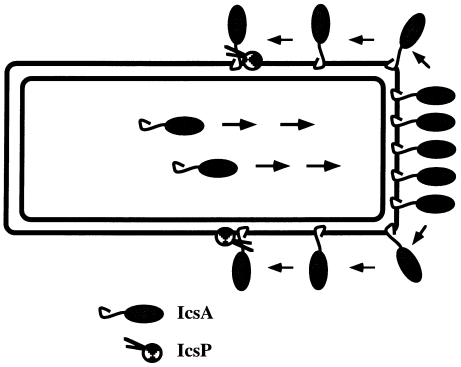

Fig. 9. Model for the mechanism of unipolar localization of IcsA in *S. flexneri*.

These data would further predict that IcsP would localize uniformly over the bacterial surface; unfortunately, we were unable to test this with our antibody to IcsP or the antibody to the histidine tag.

This model suggests that outer membrane-anchored IcsA may not be covalently bound to any structure at the old pole. On *S. flexneri* strains that carry mutations in lipopolysaccharide biosynthetic genes, leading to truncations in the lipopolysaccharide, surface IcsA is not restricted to the pole, but rather extends down the sides of the bacilli (Sandlin *et al*., 1995; 1996). In view of this, the alteration in the distribution of IcsA on the surface of these strains would have important implications for the fluidity properties of outer membranes. Among the *S. flexneri* strains carrying mutations in lipopolysaccharide that have been examined for IcsA distribution, the more truncated the mutant lipopolysaccharide, the less tightly restricted the distribution of IcsA is to the pole and the farther it extends down the sides of the bacillus (Sandlin *et al*., 1995; 1996). If the proposed model for unipolar localization of IcsA is correct, then the observed alterations in distribution on the surface of the lipopolysaccharide mutants suggest that outer membranes that contain truncated lipopolysaccharide allow more rapid diffusion of certain proteins than wild-type outer membranes, perhaps as a result of alteration in intermolecular electrostatic interactions.

It is not yet possible to distinguish whether the polar targeting of IcsA occurs in the cytoplasm or in the periplasm. IcsA has sequence motifs that suggest that it may be a member of a class of autotransporter proteins (Henderson *et al*., 1998) that do not require accessory proteins for translocation across the outer membrane (e.g. *Neisseria gonorrhoeae* and *Haemophilus influenzae* IgA proteases). Members of this family of proteins are translocated across the inner membrane as preproproteins. Within the periplasm, the carboxy-terminus of the proprotein inserts into the outer membrane and forms a channel through which the amino-terminus passes, thereby exposing the amino-terminus on the bacterial surface (Klauser *et al*., 1993). Subsequently, the amino-terminus is autoproteolytically cleaved from the bacterial surface. While several features of this pathway appear to apply to IcsA as well (Suzuki *et al*., 1995), one notable difference is that IcsA is not autoproteolytic, but rather is cleaved by IcsP. Whether a periplasmic chaperone or accessory proteins are required for IcsA secretion is unknown.

Upon translocation into the outer membrane at the pole, IcsA is anchored in the membrane by its carboxy-terminal domain, with the amino-terminal domain exposed at the bacterial surface. We propose that, subsequent to its insertion into the outer membrane, two processes occur: (i) IcsA diffuses laterally within the outer membrane, such that some drifts down the sides of the bacillus towards the septum; and (ii) IcsP slowly cleaves IcsA at all sites on the bacterial surface. As the insertion of IcsA is occurring exclusively at the pole, cleavage by IcsP, despite occurring at all sites on the surface, would result in a unipolar distribution of IcsA.

Experimental procedures

Bacterial strains and media

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in L broth, and *S. flexneri* strains were grown in tryptic soy broth. Expression of *Shigella* strains carrying the *icsA* expression plasmid pMBG372 (P_{BAD}-*icsA*$^+$) was performed in M9 medium in the presence of 0.2% glycerol with or without 0.2% L-arabinose. Where appropriate, antibiotics were added to the following final concentrations: ampicillin, 100 μg ml^{-1}; and kanamycin, 25–45 μg ml^{-1}.

Construction of icsP *expression plasmids*

icsP expression plasmids pMBG358, pMBG359 and pMBG403 were each derived from the cloning vector pQE-60 (Qiagen), which contains an *E. coli* phage T5 promoter with two *lac* operator sites immediately upstream of a multiple cloning site and a six-histidine tag. *icsP* DNA was amplified by polymerase chain reaction (PCR) using pAM7 as template and the following pairs of oligonucleotides as primers: for the insert in pMBG358, 5′-CGTTTATCTTATAAAGTAAGTCGACCATGGAC-3′ and 5′-TAGGATGGGATCCTTTTTTATTCCTGCTC-3′; for the insert in pMBG359, 5′-CGTTTATCTTATAAAGTAAGTCGACCATGGAC-3′ AND 5′-TCAAAAAATATACTTGGATCCTGCGGAAG-3′; and for the insert in pMBG403, 5′-CGTTTATCTTATAAAGTAAGTCGACCATGGAC-3′ and 5′-GAAAACGTAATCAACTCGGGGATCCAATTC-3′. The PCR product ends were digested with *Sal*I and *Bam*HI, and the resulting DNA fragments were cloned into pACYC184. The *Nco*I–*Bam*HI (pMBG358) or *Nco*I–*Bgl*II (pMBG359 and pMBG403) fragment that contains *icsP* DNA was then cloned from the pACYC184-derived plasmid into pQE-60. This placed the *icsP* DNA in frame with the six-histidine tag in pMBG358 and pMBG359, and out of frame with the six-histidine tag in pMBG403.

Table 1. Strains and plasmids used in this study.

Strain	Genotype	Reference or source
Shigella flexneri strains		
M90T	Wild-type serotype 5a	Sansonetti *et al*. (1982)
2457T	Wild-type serotype 2a	LaBrec *et al*. (1964)
SC560	M90T pWR100 *icsA*::Ω	d'Hauteville and Sansonetti (1992)
MBG283	2457T pWR100 *icsA*::Ω	This study
MBG341	2457T pWR100 *icsP1*	Shere *et al*. (1997)
MBG347	MBG283 *icsP1*	This study
MBG350	2457T carrying pMBG359 and pREP4	This study
MBG352	2457T carrying pMBG403 and pREP4	This study
MBG353	MBG283 carrying pMBG472 (P_{BAD}-*icsA*$^+$)	This study
MBG354	MBG347 carrying pMBG472 (P_{BAD}-*icsA*$^+$)	This study
E. coli strains		
BL21	*F*$^-$ *ompT hsdS*	Grodberg and Dunn (1988)
MBG348	BL21 carrying pMBG358 and pREP4	This study
Plasmids		
pACYC177	Cloning vector, Am^r Km^r	New England Biolabs
pACYC184	Cloning vector, Cm^r Tet^r	New England Biolabs
pQE-60	Expression vector; *E. coli* phage T5 promoter with *lac* operator sites, Am^r	Qiagen
pREP4	Vector carrying *lacI*, Km^r	Qiagen
pAM7	Vector carrying *icsP* and flanking DNA	Shere *et al*. (1997)
pMBG358	pQE-60 carrying *icsP* fragment that encodes amino acids 1–194, fused at the carboxy-terminus to a six-histidine tag, Am^r	This study
pMBG359	pQE-60 carrying *icsP* fragment that encodes all but the five most carboxy-terminal amino acids of IcsP, fused at the carboxy-terminus to a six-histidine tag, Am^r	This study
pMBG403	pQE-60 carrying intact *icsP*, Am^r	This study
pMAL-p2	Cloning vector for MalE fusions	New England BioLabs
pMBG171	pMAL-p2 containing *malE*::*icsA* (basepairs 3375–3879) (GenBank accession no. M22802)	This study
pMBG47	pBAD18-*icsA*$^+$ *aph*, Am^r Km^r	This study

Construction of IcsA *expression strains*

icsA was placed under the control of the arabinose promoter as follows. First, *icsA* DNA was amplified by PCR using 2457T genomic DNA as template and the following pair of oligonucleotides as primers: 5′-ATCAACCACTTACTGCTAGCATAG-TGGATG-3′ and 5′-CGGTGGATCCCAGAGGCATGCAGG-AC-3′. The PCR product ends were digested with *Nhe*I and *Bam*HI, and the resulting DNA fragment was cloned into pACYC177. The *Nhe*I–*Sph*I fragment that contains *icsA* DNA was then cloned from the pACYC184-derived plasmid into a derivative of pBAD18 (Guzman *et al*., 1995) that had previously had a kanamycin cassette (*aph*) inserted into its *Nsi*I site, thereby generating pMBG472.

The *icsA*$^-$ *icsP*$^-$ strain MBG347 was constructed by P1L4 transduction of the ampicillin-resistant locus from MBG341 into MBG283. Strains MBG353 and MBG354 were generated by transformation of pMBG472 into strains MBG283 and MBG347 respectively.

Antibodies

Rabbit antiserum to IcsA was prepared and used as described previously (Goldberg *et al*., 1993). Mouse antibody Tetra·His to the histidine tag was purchased from Qiagen and used according to the manufacturer's recommendations.

Antibody to the β-domain of IcsA was prepared as follows. The 1.1 kb fragment containing *icsA* from the *Hin*cII site at basepair 3375 (GenBank accession no. M22802) to a cloned *Pst*I site located downstream of the *icsA* coding sequence was cloned into pMAL-p2 (New England BioLabs), thereby fusing the coding sequence for *malE* carried on pMAL-p2 to IcsA amino acids 935–1102, which lies completely within the IcsA β-domain. The construct was transformed into JM04, and expression of fusion protein was induced with 1 mM IPTG. Antiserum to Triton X-100 insoluble fractions, which contained the fusion protein, was raised in New Zealand white rabbits. Antibody to the fusion protein was obtained by affinity purification of the antiserum, as described previously (Goldberg *et al*., 1993).

Antibody to IcsP was generated as follows. The *icsP* expression plasmid pMBG358, which encodes a fusion protein that consists of the amino-terminal 194 amino acids of IcsP fused to a six-histidine tag, was constructed as described above. The expression of fusion protein was induced by growth of strain MBG348, which carries pMBG358, overnight at room temperature in the presence of 0.05 mM IPTG. Bacteria were harvested by centrifugation and lysed by sonication. Protein was resuspended in 6 M urea, and the fusion protein was purified by Ni^{++}-NTA-Sepharose affinity chromatography according to the manufacturer's protocol (Qiagen).

Protein preparation and analysis

Whole-cell, membrane and supernatant proteins were prepared from bacteria grown in tryptic soy broth as described

previously (Hovde *et al*., 1988; Bernardini *et al*., 1989; Allaoui *et al*., 1992). Membrane proteins were prepared by harvesting bacteria in mid-to-late logarithmic phase of growth, sonicating the bacteria in 10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, and removing debris by centrifugation at 1400 × *g* for 10 min at 4°C. Membranes were pelleted by centrifugation at 16 000 × *g* for 60 min at 4°C. Membranes were resuspended in 2% Triton X-100, 10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, and inner membranes were separated from outer membranes by centrifugation at 16 000 × *g* for 60 min at 4°C. Periplasmic proteins were prepared according to the TEX protocol described by Thorstenson *et al*. (1997). Proteins were separated by SDS–PAGE. Western blots were performed using one of the antibodies described above and enhanced chemiluminescence (Amersham or Pierce). Integrated density measurements were performed using a ChemiImager 4000 and Alpha Ease software (Alpha Innotech) and selecting signals that were all within the linear range.

Biotinylation of bacterial surface proteins

Logarithmic-phase bacteria were grown at 37°C in the presence or absence of EZ-Link Sulpho-NHS-LC-Biotin (Pierce) (0.5 mg ml^{-1}) in Hank's balanced salt solution for 30 min. Bacteria were pelleted by centrifugation, resuspended in fresh media and grown for an additional 90 min before harvesting for the preparation of protein extracts as described above. Protein extracts were separated by SDS–PAGE and transferred to nitrocellulose. Labelling of biotinylated proteins was performed with avidin and biotinylated horseradish peroxidase, which were obtained from the Vectastain Elite ABC kit (Vector Laboratories) and used according to the manufacturer's recommendations, followed by visualization using enhanced chemiluminescence (Amersham).

Surface labelling for IcsA

Surface labelling for IcsA was performed as described previously (Goldberg *et al*., 1993). Fluorescent microscopy was performed on a Nikon Diaphot 200 fluorescent microscope. Determination of the percentage of stationary-phase bacteria that were labelled was performed essentially as described previously (Goldberg *et al*., 1994); three separate microscopic fields, each containing more than 180 bacteria, were counted per strain.

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