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The making of a gradient: IcsA (VirG) polarity in *Shigella flexneri*

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Summary

The generation and maintenance of subcellular organization in bacteria is critical for many cell processes and properties, including growth, structural integrity and, in pathogens, virulence. Here, we investigate the mechanisms by which the virulence protein IcsA (VirG) is distributed on the bacterial surface to promote efficient transmission of the bacterium *Shigella flexneri* from one host cell to another. The outer membrane protein IcsA recruits host factors that result in actin filament nucleation and, when concentrated at one bacterial pole, promote unidirectional actin-based motility of the pathogen. We show here that the focused polar gradient of IcsA is generated by its delivery exclusively to one pole followed by lateral diffusion through the outer membrane. The resulting gradient can be modified by altering the composition of the outer membrane either genetically or pharmacologically. The gradient can be reshaped further by the action of the protease IcsP (SopA), whose activity we show to be near uniform on the bacterial surface. Further, we report polar delivery of IcsA in *Escherichia coli and Yersinia pseudotuberculosis*, suggesting that the mechanism for polar delivery of some outer membrane proteins is conserved across species and that the virulence function of IcsA capitalizes on a more global mechanism for subcellular organization.

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Introduction

Shigella flexneri is a facultative intracellular pathogen that causes diarrhoea and dysentery. A key to its pathogenicity is its method of intra- and intercellular spread: upon invading a host cell, it nucleates host actin filaments at one of its poles to form a comet-like tail, which propels the bacterium forward until it contacts the host cell membrane and pushes into a neighbouring cell (Makino *et al*., 1986; Bernardini *et al*., 1989; Goldberg *et al*., 1994). The 120 kDa outer membrane protein IcsA (VirG) is both necessary and sufficient for actin nucleation and actin-based motility (Makino *et al*., 1986; Bernardini *et al*., 1989; Lett *et al*., 1989; Goldberg and Theriot, 1995; Kocks *et al*., 1995). In the absence of IcsA, intercellular spread is abolished (Makino *et al*., 1986; Lett *et al*., 1989).

In order to achieve robust unidirectional actin-based motility, IcsA is expressed on the bacterial surface in a unipolar fashion, i.e. its concentration is highest at one end of the rod-shaped bacterium (Goldberg *et al*., 1993; Suzuki *et al*., 1995). The carboxy-terminus of IcsA is predicted to form a β-barrel structure that anchors the protein in the outer membrane, whereas the functional amino-terminus lies exposed on the bacterial surface (Goldberg *et al*., 1993). The outer membrane protease IcsP (SopA), which shares homology with the *E. coli* K-12 proteases OmpP and OmpT (Fukuda *et al*., 1995; Egile *et al*., 1997; Shere *et al*., 1997), cleaves the amino-terminus of IcsA to release a 95 kDa N-terminal fragment into the surrounding environment (Nakata *et al*., 1992; 1993; Goldberg *et al*., 1993; 1994). The only known substrate for IcsP is IcsA (Steinhauer *et al*., 1999). The biological role of this proteolysis is not completely understood.

It has been demonstrated recently by indirect immunofluorescence that, when IcsA transcription is induced in a *ΔicsAΔicsP* background, IcsA is first detectable at one pole and, over time, becomes more broadly distributed along the sides of the bacterium (Steinhauer *et al*., 1999). In the absence of IcsP, a polar '*cap*' region is still apparent, but IcsA is distributed more broadly in the membrane than in wild-type cells (Shere *et al*., 1997).

A polar cap has also been observed when IcsA is expressed in *E. coli* strain ATM379, demonstrating that no other *Shigella*-specific virulence factors are required for its unipolar targeting (Sandlin and Maurelli, 1999). However, this phenomenon appears to be dependent on the presence of an intact lipopolysaccharide (LPS). Mutations

that cause truncation of the LPS in *S. flexneri* lead to increased uniformity of the IcsA distribution across the bacterial length, even in the presence of IcsP (Sandlin *et al*., 1995; 1996; van den Bosch *et al*., 1997). In addition, when IcsA is expressed in *E. coli* K-12, which lacks the O antigen of LPS (Nikaido and Vaara, 1987), a visually uniform distribution is observed (Goldberg and Theriot, 1995). Bacteria with a non-unipolar distribution of IcsA on their surface exhibit erratic, non-unidirectional actin-based motility in *Xenopus* egg cytoplasmic extracts (Goldberg and Theriot, 1995).

These observations have led to the formulation of the following hypothesis (Sandlin and Maurelli, 1999; Steinhauer *et al*., 1999): IcsA is delivered strictly to one pole of the bacterium, where it is inserted into the outer membrane. Subsequently, membrane-bound IcsA diffuses laterally, forming a gradient that decreases in concentration with distance from the secreting pole. IcsP protease activity may help to maintain this gradient (Steinhauer *et al*., 1999).

This hypothesis predicts that: (i) steady-state IcsA distribution should follow a characteristic curve determined by the rate of polar protein secretion and the rate of lateral diffusion; (ii) alteration in the rate of lateral diffusion in the outer membrane should alter the shape of this curve; and (iii) the presence of IcsP should sharpen this curve in a predictable manner. Our experiments confirm these quantitative predictions and measure the distinct contributions of lateral diffusion and proteolysis of IcsA using both genetic and pharmacological methods. Furthermore, we find that the establishment of polar IcsA localization is similar in several Gram-negative species, suggesting the existence of an evolutionarily conserved polar secretory apparatus for IcsA and possibly for IcsP and other proteins.

Results

IcsA concentration decreases exponentially along the bacterial length

Many observations have confirmed the unipolar distribution of IcsA in wild-type *S. flexneri* (Goldberg *et al*., 1993; 1994; Sandlin *et al*., 1995; Sandlin and Maurelli, 1999; Steinhauer *et al*., 1999). This unipolar gradient could be generated by delivery of IcsA to the outer membrane exclusively at one pole, followed by lateral diffusion along the sides of the bacterium, as has been suggested (Steinhauer *et al*., 1999). If this were true, and IcsA diffuses from its point of secretion, then the one-dimensional distribution of IcsA along the bacterial length should approximately fit an exponential decay function originating at the secreting pole (Fig. 1).

Wild-type *S. flexneri* 2a strain 2457T were fixed during exponential growth, when IcsA expression is high (Goldberg

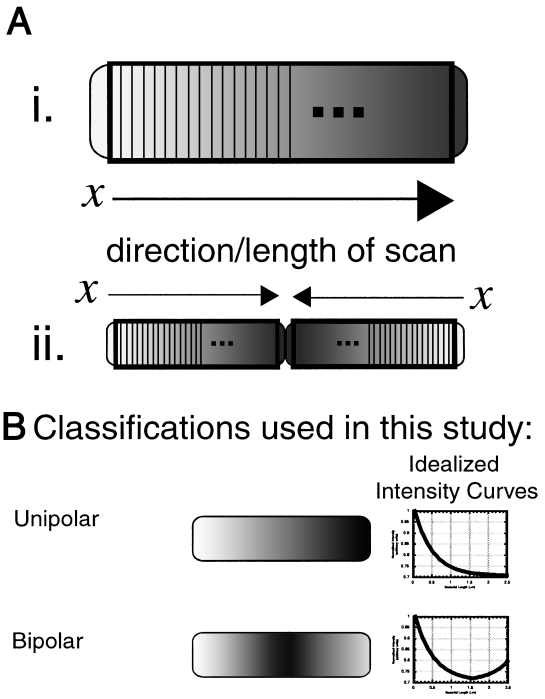

Fig. 1. Diagrammatic representation of the quantification procedure used in Figs 2–4 and 6.
A. Measurements of average intensity were collected along the long (x) axis of the bacterium (vertical lines). For septating bacteria (ii), each sister cell was considered individually, with its end-points as one end of the cell and the septum. Peaks of fluorescence intensity were never observed at the septum; hence, all bacteria with a visible septum were counted as two unipolar sister cells.
B. Idealized curves for two bacteria: one unipolar and the other bipolar.

et al., 1994). Bacteria were then labelled using a monoclonal antibody raised against the amino-terminus (95 kDa fragment) of IcsA, and the resulting fluorescence distributions were imaged (Fig. 2). By plotting the average fluorescence intensity across a rectangle approximating the bacterial surface against the bacterial long axis (Fig. 1A), we obtained a one-dimensional distribution profile of unproteolysed IcsA for each bacterium (Fig. 2). In the cases in which a bacterium was dividing and a septum was visible, each sister cell was measured individually from the pole to the septum (Fig. 1A). The rounded tips of the poles (three to five pixels) were excluded to reduce variance caused by bacterial shape.

Eighty-five per cent of these intensity curves ($n = 113$) could be well fitted by an exponential curve, exhibiting a correlation coefficient (R) of 0.90 or greater for the equation:

$$I(x) = a + be^{-x/\lambda} \quad (1)$$

where I represents fluorescence intensity, x the distance along the bacterial axis and a, b and λ are constants. The average decay constant (λ) for these curves was 0.52 μm (Table 1). (The decay constant is defined here as the characteristic length for the value of x for which $I = 1/e(I_0)$,

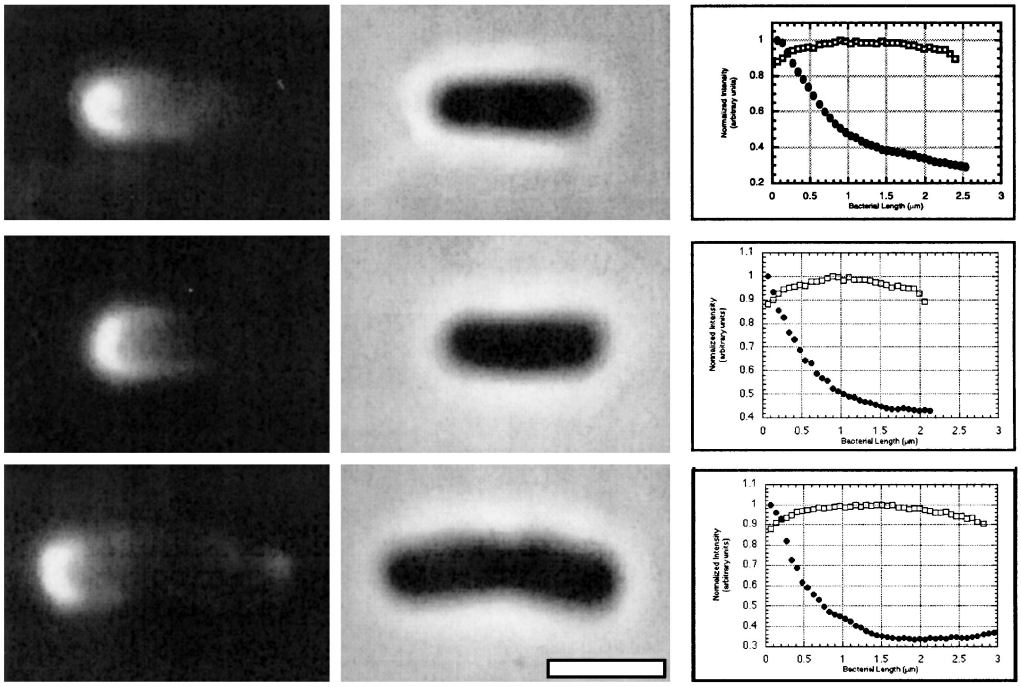

Fig. 2. Wild-type strain 2475T *S. flexneri* 2a displays an exponential gradient of IcsA.
Left. Indirect immunofluorescence for IcsA.
Middle. Phase contrast.
Right. Quantification of normalized fluorescence intensity as a function of bacterial length (truncated to 3 μm for initial slope comparison). Dark circles, anti-IcsA intensity; light circles, TRITC intensity on independently labelled bacteria of similar length. Bar = 2 μm.

where I_0 is the highest intensity, at the bacterial pole. A smaller value of λ indicates a steeper gradient.) Representative curves are shown in Fig. 2.

In some cases, the IcsA concentration appeared to be higher at both poles than in the centre of the bacterium. We termed these bacteria 'bipolar', whereas those with a monotonically decreasing concentration of IcsA are designated 'unipolar' (Fig. 1B). Bacteria whose distributions did not exhibit exponential decay across the entire bacterial length were usually mildly bipolar (Fig. 1B): although both poles exhibited peak fluorescence intensities, one peak was significantly (> 50%) brighter than the other (data not shown). When measurement was limited to the first or last third of the bacterium, 94% of the intensity curves for both poles were well fitted by an exponential function ($R > 0.90$, $n = 47$ bacteria), suggesting that some bacteria delivered IcsA to both poles, although in unequal amounts. Bipolar distribution of IcsA on dividing bacteria has been noted previously (Goldberg *et al.*, 1993). The targeting of IcsA to the pole that will become the old pole of the daughter cell appears to occur before the completion of septation.

Performing the same analysis on *S. flexneri* unspecifically labelled with 10 μM tetramethylrhodamine isothiocyanate

Table 1. Decay constants (λ) indicate that IcsA diffuses from the pole at varying rates in multiple strains of three different bacterial species.

Strain	λ (μm)	% of WT λ	SD (μm)	*n* with $R > 0.90$	% Total
S. flexneri 2a	0.52	100	0.16	96	85
MBG264, log phase	0.69	133	0.67	72	55
MBG264, log phase, one pole	0.79	152	0.47	101	82
MBG264, stationary phase	0.89	171	0.65	105	85
BS520	0.90	173	0.63	83	72
BS520 + chlorpromazine	0.59	113	0.25	80	92
YPIIIpIB71(pHS3199)	0.66	127	0.41	108	96
YPIIIpIB71(pHS3199)(picsP1)	0.48	92	0.53	75	56

Measurements of the brighter pole only were performed for strains exhibiting a high degree of bipolarity and reflect 25–33% of bacterial length. *n* with $R > 0.90$, number of bacteria included in average. % of WT λ, comparison of λ value with that of *S. flexneri* 2a. A larger λ indicates a shallower slope. % total, percentage of total bacteria represented by average.

(TRITC) yielded uniform distributions (i.e. near-linear read-outs), demonstrating that the exponential decay is not a function of bacterial shape (Fig. 2, white circles). This quantitative description of IcsA distribution is of limited use in comparing absolute concentrations of IcsA among different samples, but represents a powerful method for determining relative distribution shapes.

Escherichia coli *exhibit growth-dependent IcsA polar secretion in the absence of IcsP*

The distribution of IcsA expressed in *E. coli* K-12 strains appears to be near uniform on the bacterial surface (Goldberg and Theriot, 1995). To determine whether this distribution is a result of a non-polar mechanism of IcsA secretion or altered mobility of IcsA in the outer membrane after secretion, we examined the IcsA distribution quantitatively in the IcsA-expressing *E. coli* K-12 strain MBG264(pHS3199) (Goldberg and Theriot, 1995). IcsP is not expressed by these bacteria, and *ompT* has been disrupted. These bacteria can undergo actin-based motility in *Xenopus* extract, but their movement is often non-unidirectional – sideways or spiralling – apparently as a result of their poor IcsA polarity (Goldberg and Theriot, 1995).

Figure 3A shows the distribution of IcsA on these bacteria, which appeared nearly uniform to the eye. However, quantitative analysis revealed that IcsA was actually distributed in a bipolar fashion, with one pole significantly brighter than the other (Fig. 3A). This bipolarity is far more prevalent in these bacteria than in wild-type *S. flexneri* 2a but, as in the latter case, the IcsA at most *E. coli* poles (first or last quarter of the bacterium) fits an exponential decay (Table 1, Fig. 3A). In most cases, the bipolarity was sufficiently mild that intensity curves could still be well fitted by an exponential function: 56% of bacteria fit an exponential decay for their entire length ($R > 0.90$), with an average decay constant of 0.69 μm (Table 1). There was no obvious correlation between decay constant and bacterial length. Bacteria whose polar distributions were non-exponential often exhibited IcsA puncta at random points along the surface, possibly indicating IcsA aggregation. These puncta were apparent in about 58% of bacteria, although their intensities were often not sufficient to disrupt the curve fit significantly. Regardless of whether the concentration is high at one pole or two, the exponential decay of IcsA from the pole(s) in the absence of IcsP is consistent with the hypothesis that the protein is being delivered strictly to the pole and is subsequently diffusing laterally through the membrane.

We found that, although *S. flexneri* and MBG264 (pHS3199) exhibit similar exponential growth rates by optical density, the percentage of bacteria with visually apparent septa is significantly greater for MBG264 (pHS3199) (51% versus 37%). Differences in the cell cycles of these bacteria may correlate with the fraction of bipolar bacteria observed. To test this hypothesis, we also analysed MBG264(pHS3199) in stationary phase (Fig. 3B) and found that 85% of these bacteria exhibit unipolar exponential decay ($R > 0.90$), with 18% septating. However, we were surprised to find that the decay constant for these bacteria was 0.89 μm (Table 1), significantly greater ($P < 0.001$) than that for MBG264(pHS3199) in exponential growth (by Student's *t*-test, Fig. 3C). This shallower slope indicates that diffusion can change with growth phase.

Fluorescence polarization studies have shown that membrane fluidity varies with *E. coli* growth phase in other strains (Souzu, 1986). We suspected that the variation in decay constants between stationary and exponential growth may result from such a phenomenon and hence wished to test directly the effect of outer membrane fluidity on IcsA distributions in *S. flexneri*.

IcsA gradient slope depends on S. flexneri*'s outer membrane fluidity*

A second prediction of the model in which delivery to one pole is followed by lateral diffusion of the protein is that altering membrane fluidity will affect the steady-state protein distribution. Previous work has demonstrated that changing the outer membrane composition of *S. flexneri* perturbs the distribution of IcsA (Sandlin *et al.*, 1995; 1996; van den Bosch *et al.*, 1997). In those studies, the alteration in the LPS structure in the outer membrane resulted in differing degrees of IcsA polarization.

We measured the IcsA gradient in the *S. flexneri* strain BS520, in which the O side-chain repeats (made up of saccharide polymers) are not synthesized, leaving only the LPS core in the outer membrane (Sandlin *et al.*, 1996; Fig. 4A). BS520 is otherwise isogenic with *S. flexneri* 2a 2457T. In these cells, 72% of the intensity curves could be well fitted by the exponential decay in eqn 1 (Table 1), but their average decay constant (0.90 μm, SD = 0.63) was significantly greater ($P < 0.001$) than that of the wild-type strain (0.52 μm, SD = 0.16) by Student's *t*-test (Fig. 4C, Table 1), indicating a shallower gradient. Most bacteria whose distributions did not fit an exponential decay were mildly bipolar.

It is possible that a specific interaction between IcsA or IcsP and the O side-chain results in this loss of polarity. However, other studies have noted that a decrease in the length of the O side-chain also results in increased general membrane fluidity in other Gram-negative bacteria with different O side-chains (Rottem and Leive, 1977; Yeh and Jacobs, 1992), and side-chain length appears to be more important than sugar content in proper polarity on *S. flexneri* (Sandlin *et al.*, 1996). We therefore hypothesized that the broader distribution of IcsA on BS520

may result simply from increased lateral diffusion in this strain. In order to test this, we decreased membrane fluidity in the mutant using an independent pharmacological method. We incubated BS520 in 25 μM chlorpromazine, which has been shown to decrease membrane fluidity in *E. coli* (Tanji *et al*., 1992), and found that wild-type IcsA distribution was restored (Fig. 4B), with 92% of the bacteria displaying an exponential decay (Table 1). The

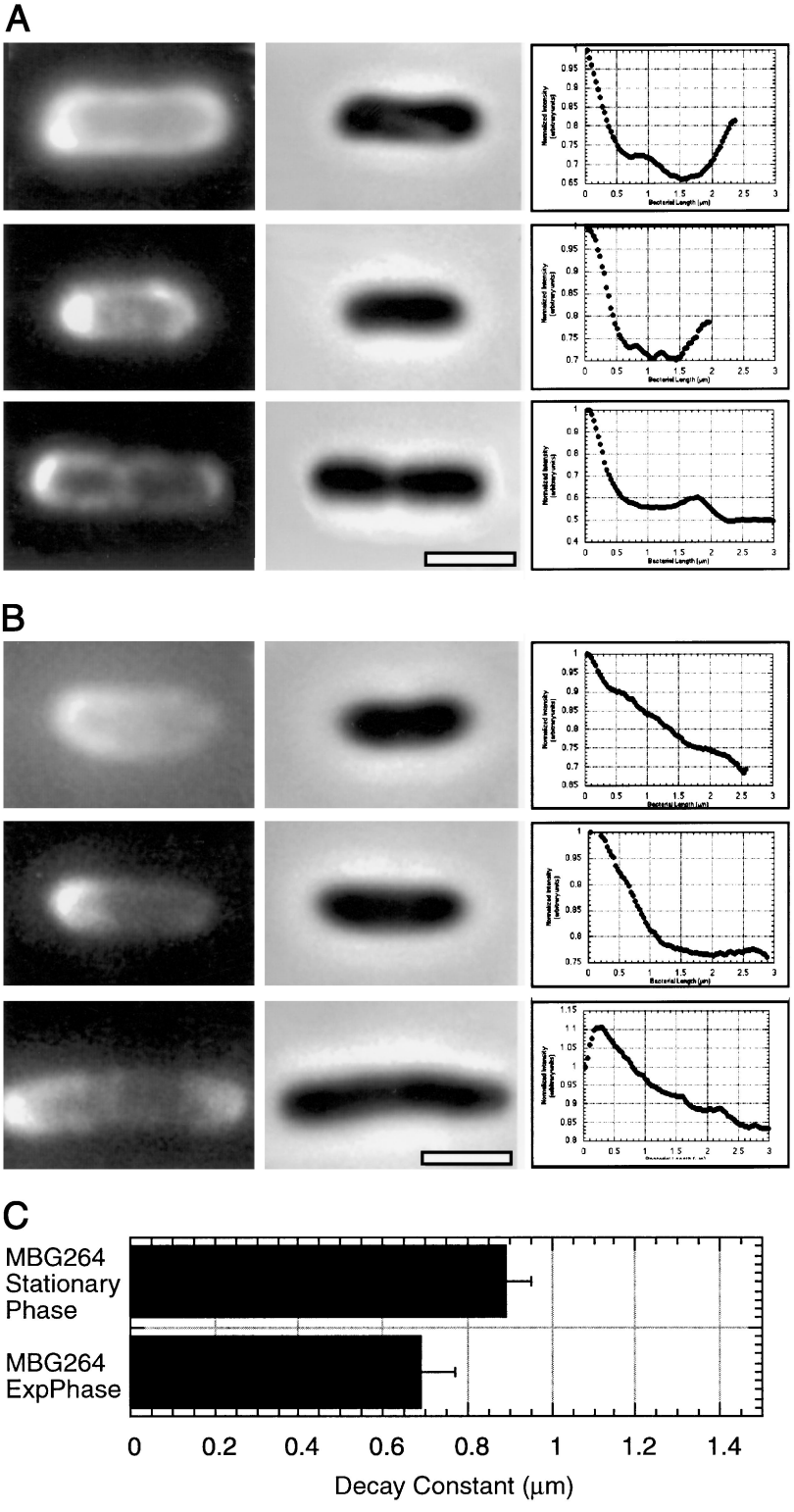

Fig. 3. *E. coli* K-12 displays growth phase-dependent, bipolar exponential gradients of IcsA.
A. Log-phase *E. coli* K-12 strain MBG264 (pHS3199) expressing IcsA.
B. Stationary-phase *E. coli* K-12 strain MBG264 (pHS3199) expressing IcsA.
A and B. Left, indirect immunofluorescence for IcsA; middle, phase contrast; right, quantification of normalized fluorescence intensity as a function of bacterial length (truncated to 3 μm for initial slope comparison).
C. Average decay constants (error bars are standard error of the mean).

average decay constant for chlorpromazine-rescued BS520 was not significantly different from that of wild-type *S. flexneri* ($P = 0.29$ by Student's *t*-test; Fig. 4C).

This pharmacological rescue of the LPS defect is consistent with the hypothesis that lateral diffusion of IcsA away from the pole is dependent only on the fluidity of the outer membrane and not on specific molecular interactions between IcsA and LPS.

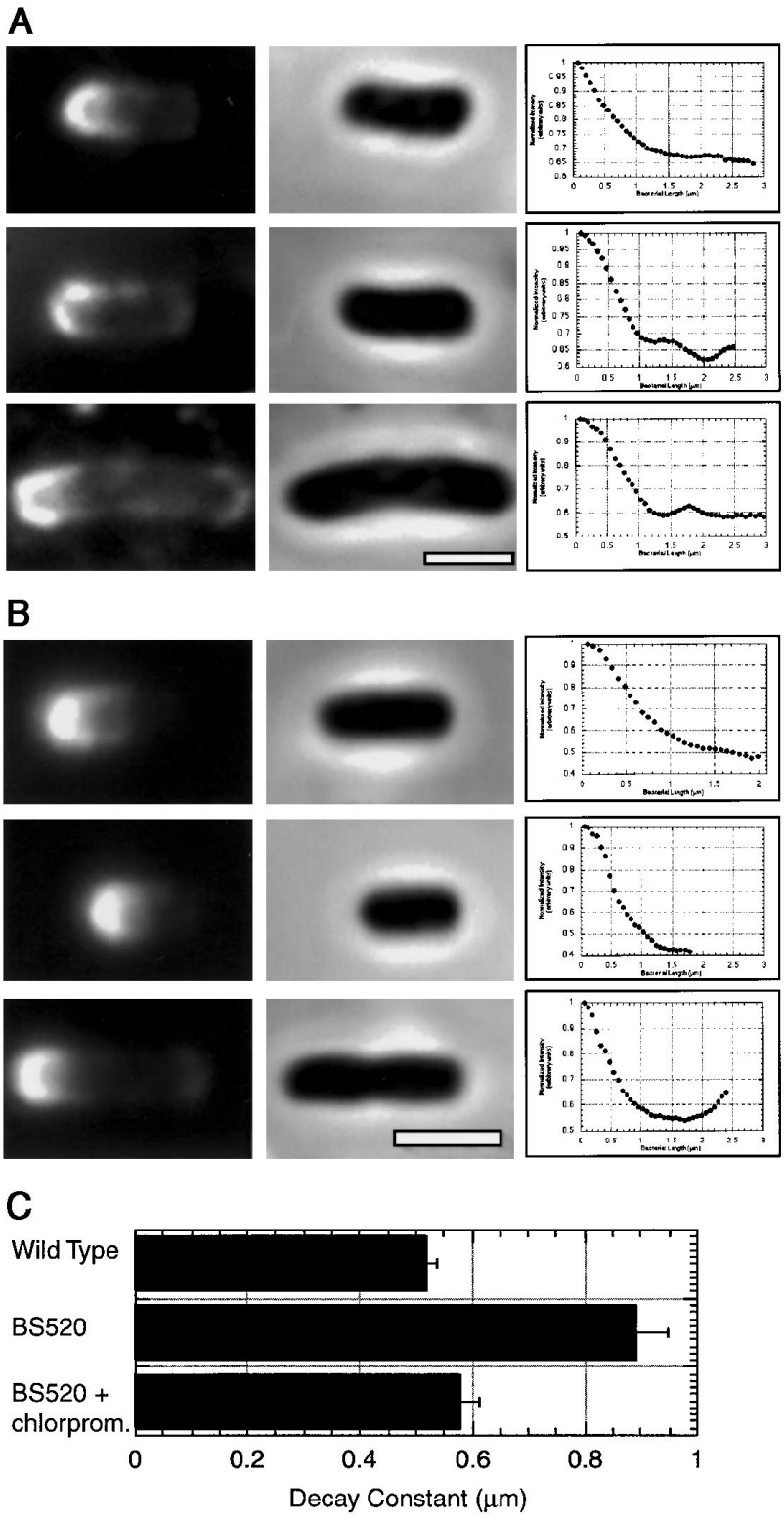

Fig. 4. Alterations in LPS structure and membrane fluidity alter the shape of the IcsA gradient in *S. flexneri*.
A. BS520 *S flexneri*.
B. BS520 *S flexneri* grown in the presence of 25 μM chlorpromazine.
A and B. Left, indirect immunofluorescence for IcsA; middle, phase contrast; right, quantification of normalized fluorescence intensity as a function of bacterial length (truncated to 3 μm for initial slope comparison). Bar = 2 μm.
C. Average decay constants (error bars are standard error of the mean).

Polar delivery of IcsA generates a gradient that is sharpened by IcsP in Yersinia pseudotuberculosis

An IcsA polar gradient can be generated in the absence of the protease IcsP (Fig. 3), or altered in its presence by alteration in membrane fluidity (Fig. 4). In order to probe the role of IcsP specifically, we transformed the heterologous Gram-negative pathogen *Y. pseudotuberculosis* YPIIIpIB71 with either pHS3199 (to express IcsA) or both pHS3199 and picsP1 (to express both IcsA and IcsP; Fig. 5). *Y. pseudotuberculosis* expressing IcsA alone displayed an exponential gradient of surface IcsA originating at one pole, with 96% of bacteria fitting eqn 1 (Table 1, Fig. 6). When IcsP was also present, only 56% of the bacteria fitted an exponential decay (Table 1), but the decay constant was significantly decreased from 0.66 μm to 0.48 μm ($P < 0.001$ by Student's *t*-test' Fig. 6, Table 1). The population of YPIIIpIB71(pHS3199)(picsP1) that did not exhibit an exponential decay often exhibited punctate staining at random positions along the bacterium (apparent in about 8% of the bacteria), similar to that observed in MBG264(pHS3199). In this comparison, membrane fluidity, and hence the rate of IcsA diffusion, has presumably not changed, but the presence of the protease significantly increased the slope of the IcsA gradient.

IcsP activity is distributed in a near-uniform fashion in Y. pseudotuberculosis

It has been shown that, when IcsP is overexpressed in wild-type *S. flexneri* 2a, IcsA at the pole is proteolytically processed (Steinhauer *et al*., 1999). Thus, IcsP must be capable of acting at the pole. However, this does not preclude the possibility of non-uniform protease activity under normal circumstances. In order to map the location of protease activity at the bacterial surface, we compared quantitatively the distributions of IcsA in the presence or absence of IcsP.

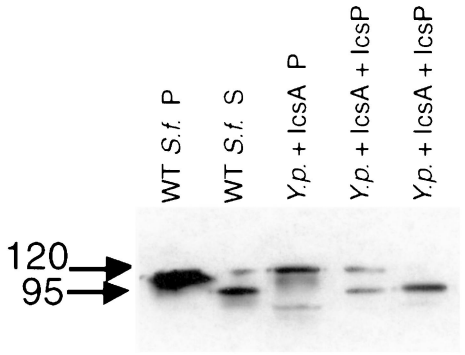

Fig. 5. Western blot showing expression of IcsA and IcsP in *Y. pseudotuberculosis*. Lane 1, wild-type *S. flexneri* 2a pellet. Lane 2, supernatant. The N-terminal cleaved fragment is apparent as a 95 kDa band. Lane 3, YPIIIpIB71(pHS3199) pellet. Note that non-IcsP-dependent degradation is apparent. Lane 4, YPIIIpIB71(pHS3199)(picsP1) pellet. Lane 5, YPIIIpIB71(pHS3199) (picsP1) supernatant.

At each point on the bacterial surface, the concentration (C) of IcsA over time is governed by the following: (i) the rate of secretion of IcsA at that point; (ii) lateral diffusion of the protein from other sites of secretion; (iii) removal of IcsA by IcsP; and (iv) degradation of IcsA by other housekeeping mechanisms. At steady state, the distribution of IcsA does not change with time. We therefore model the steady-state distribution of IcsA on the bacterial surface with the equation:

$$S(x) + DC''(x) - k_1C(x)[\text{IcsP}] - k_2C(x) = 0. \quad (2)$$

where x is the distance along the bacterial axis, S the source of new IcsA, D the diffusion coefficient for IcsA in the outer membrane, k_1 the rate constant for the proteolysis of IcsA by IcsP, and k_2 the constant rate at which IcsA is degraded by non-IcsP-dependent housekeeping mechanisms (assumed to be uniform and constant). At steady state, the distribution of IcsA does not change with time; thus, the contributions of these factors must add to zero. As we and others (Steinhauer *et al*., 1999) have demonstrated that IcsA is delivered at only one pole, $S(x)$ is non-zero only at that pole. For the distribution of IcsA on the rest of the bacterium, eqn 2 can be rearranged:

$$\frac{C''(x)}{C(x)} = \frac{(k_2 + k_1[\text{IcsP}])}{D}. \quad (3)$$

We calculated the ratio of $C''(x)/C(x)$ in terms of IcsA immunofluorescence intensity in the YPIIIpIB71(pHS3199)(picsP1) strain. Any spatial variation in this ratio must result from spatial variation in IcsP activity (Fig. 7A). We found that this distribution was highly uniform, although IcsP activity was apparently slightly higher at the pole from which IcsA originated. To reduce noise and compensate for bacterial shape, we calculated the same ratio for YPIIIpIB71(pHS3199) (Fig. 7B) and subtracted this background from YPIIIpIB71(pHS3199)(picsP1) (Fig. 7C). This subtraction also presumably removes the effect of non-IcsP-dependent housekeeping mechanisms. The slight increase in IcsP activity at the pole was more evident, whereas the overall distribution of protease activity remained near uniform.

It is important to note that, although this method is useful for visualizing the distributions of protease activity, no absolute rate constants or values for IcsP concentration can be reliably derived, as immunofluorescence efficiency varies from sample to sample.

Our finding suggests that IcsP may be delivered to (or activated at) the same pole as IcsA, but at a much slower

rate. It then presumably diffuses laterally in the outer membrane and has a much longer half-life than IcsA. The slight exponential decay present in YPIIIpIB71(pHS3199) (Fig. 7B) suggests that non-IcsP-dependent housekeeping degradation of IcsA may also be slightly higher at the IcsA-enriched pole.

We can use the same equation to estimate the difference in *D*, the diffusion coefficient for IcsA in the

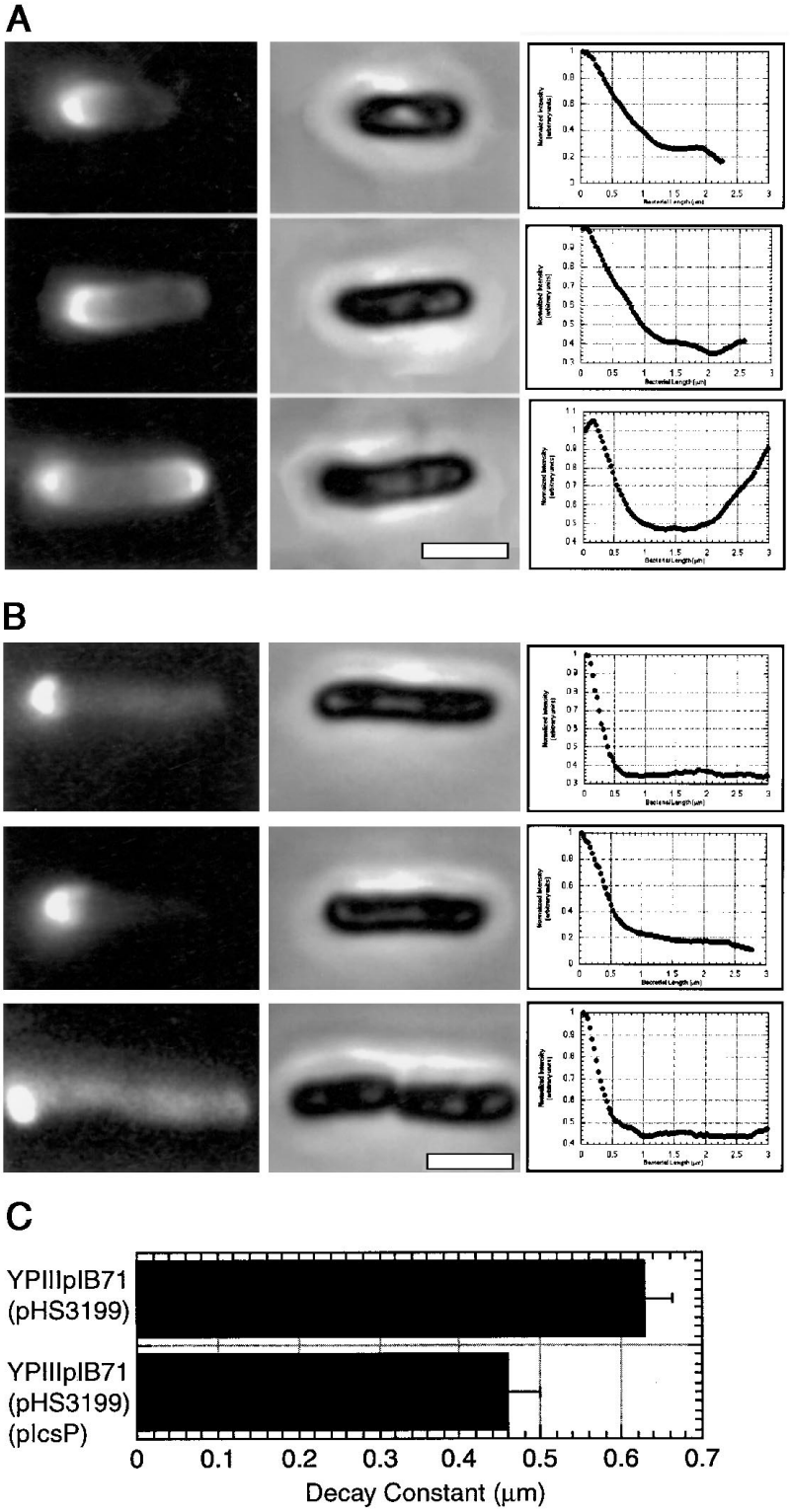

Fig. 6. IcsP sharpens the IcsA concentration gradient in *Y. pseudotuberculosis*.
A. YPIIIpIB71(pHS3199).
B. YPIIIpIB71(pHS3199) (picsP1).
A and B. Left, indirect immunofluorescence for IcsA; middle, phase contrast; right, quantification of normalized fluorescence intensity as a function of bacterial length (truncated to 3 μm for initial slope comparison). Bar = 1.9 μm.
C. Average decay constants (error bars are standard error of the mean).

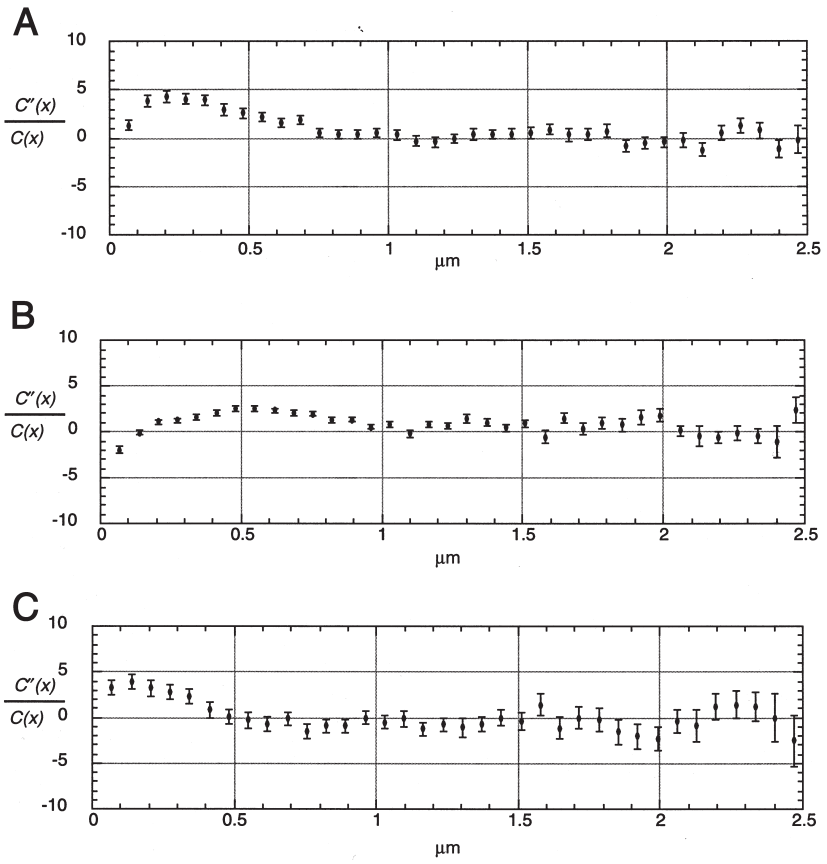

Fig. 7. The ratio $C''(x)/C(x)$ plotted as a function of bacterial length (see eqn 3), plotted from greatest [IcsA] to least [IcsA], shows near-uniform surface IcsP activity.
A. YPIIIpIB71(pHS3199) (picsP1), $n = 164$.
B. YPIIIpIB71(pHS3199), $n = 168$. Error bars are the standard error of the mean. To reduce noise, each neighbouring set of three pixel intensity values was averaged to generate these curves.
C. YPIIIpIB71(pHS3199) trace subtracted from YPIIIpIB71(pHS3199) (picsP1) trace.

outer membrane, between the wild-type *S. flexneri* 2a and BS520 strains. Assuming a perfect exponential fit:

$$\frac{C''(x)}{C(x)} = \frac{1}{\lambda^2} = \frac{(k_2 + k_1[\text{IcsP}])}{D}. \quad (4)$$

Assuming that $k_2 + k_1$[IcsP] is the same for the wild-type and BS520 strains,

$$\frac{D_{\text{WT}}}{D_{\text{BS520}}} = \frac{\lambda^2_{\text{WT}}}{\lambda^2_{\text{BS520}}}. \quad (5)$$

Solving this ratio gave us $D_{\text{WT}}/D_{\text{BS520}} = 0.34$. Thus, the rate of IcsA diffusion through the outer membrane of BS520 was approximately threefold faster than in wild-type *S. flexneri* 2a.

Discussion

Our results are consistent with the hypothesis that IcsA polarity is generated and maintained by at least three ongoing biological processes: (i) delivery of IcsA to a single bacterial pole; (ii) diffusion of the protein in the outer membrane, which is governed by membrane fluidity; and (iii) near-uniform proteolysis by the protease IcsP, which can sharpen the gradient of IcsA.

Our one-dimensional mathematical description of the model for IcsA distribution if IcsA is delivered to the membrane exclusively at the pole (eqn 1) is merely an approximation, as the bacterium is a three-dimensional object. However, given the high consistency with which IcsA distributions can be fitted accurately by an exponential decay distribution, we consider this approximation sufficient within the resolution limits of light microscopy and acceptable error.

Previous studies were also consistent with the hypothesis that IcsA is delivered to the pole (Sandlin and Maurelli, 1999; Steinhauer *et al.*, 1999), but these experiments did not exclude the alternative possibility that IcsA is delivered to the bacterial surface in a uniform fashion followed by rapid diffusion and retention at the pole, either by self-aggregation or by association with other proteins. The steady-state mathematical prediction of such a model is very similar to eqn 1 and cannot be ruled out by examining wild-type bacteria alone. However, if this alternative model were true, increasing the membrane fluidity should lead to a sharper gradient, as uniformly outputted molecules would diffuse more quickly to the trapping pole. Conversely, decreased membrane fluidity should lead to a shallower gradient. Our experiments show the opposite result, solely consistent with the model of diffusion from a single source – the bacterial pole.

The cross-species preservation of unipolar delivery suggests a common mechanism in Gram-negative

bacteria for the localization of at least some outer membrane proteins. It is possible that such a mechanism is specific to IcsA or some localization '*tag*' region in IcsA analogous to the targeting domains of differentially localized eukaryotic proteins. Indeed, mutants in the alpha-domain (near the carboxy-terminus) of the protein fail to localize in a polar fashion (Suzuki *et al*., 1996). It is also possible that polar delivery of outer membrane proteins is the default pathway for all or most outer membrane proteins. As IcsA is targeted to the old pole in at least two Gram-negative organisms other than *Shigella*, it is probable that the molecules responsible for its polar targeting have a more global function. Further examination of other outer membrane proteins with high turnover rates in all three species should help to distinguish among these possibilities.

This study has directly addressed the effect of membrane fluidity on IcsA diffusion from the pole. It has been shown previously that variations in LPS composition of *S. flexneri* affect polarity (Sandlin *et al*., 1995; 1996; Van den Bosch, 1997). We show here that the gradient-shallowing effect of missing O side-chains can be rescued by independent pharmacology (Fig. 4), arguing that IcsA distribution depends only on its ability to diffuse through the outer membrane. It has been hypothesized that O antigens interact to form interlocking microdomains on the bacterial surface (Rottem and Leive, 1977; Yeh and Jacobs, 1992), which might inhibit diffusion of IcsA through the membrane.

Growth rate and septum formation can clearly affect the bacterial decision to deliver IcsA to either one or both poles, as demonstrated here by *E. coli* strain MBG264. In exponential phase, the bacteria are mostly mildly bipolar for IcsA but, in stationary phase, most bacteria are unipolar (Fig. 3). This apparent need to ensure that both sister cells will have polarized protein also argues for a polar delivery mechanism conserved across species, as non-*Shigella* species can have no evolutionary preference for the polar distribution of IcsA. Furthermore, previous work has shown that the lambda receptor is distributed in an exponential gradient originating either from the pole or from the septum, depending on *E. coli*'s stage in the growth cycle, suggesting that secretory localization is a concern for at least one other protein in another species (Ryter *et al*., 1975).

Other work has shown IcsA-expressing *E. coli* to be unipolar in exponential growth (Sandlin and Maurelli, 1999), in contrast to our results showing a high frequency of bipolarity, but this may be attributed to strain-dependent differences in cell cycle, as both *S. flexneri* and *Y. pseudotuberculosis* are unipolar in exponential growth in this study. In addition, strain-dependent differences in LPS structure are likely to affect the steady-state distributions. The *E. coli* strain (MBG264(pHS3199)) used in this study is a K-12 derivative, and one of the major distinguishing features of such strains is their truncated LPS sugars (Nikaido and Vaara, 1987). The more elaborated LPS may play a role in the apparent unipolarity of non-K-12 strains such as ATM379 (Sandlin and Maurelli, 1999). The data presented here suggest that increased length of O antigen leads to decreased membrane fluidity, decreased diffusion of IcsA in the outer membrane and a more polar distribution of IcsA.

Finally, we have addressed the role of the IcsA-specific protease IcsP by expressing it in *Y. pseudotuberculosis*. We found a dramatic sharpening of the polar distribution of IcsA when we co-expressed IcsP, which is expected if there is a uniform distribution of a protease whose target protein is exponentially distributed on the bacterial surface. This demonstrates that, although the polarity of IcsA is initially established by polar delivery followed by lateral diffusion governed by the properties of the outer membrane, its gradient can be sharpened by the protease IcsP. The use of specific proteases to sharpen or refine protein gradients originally established by diffusion is observed in other biological systems, including the establishment of pheromone gradients during yeast mating (Barkai *et al*., 1998).

Our quantitative analysis of IcsA distribution allows us to demonstrate that IcsP protease activity is in fact almost uniform, as has generally been assumed (Steinhauer *et al*., 1999). However, we find that IcsP activity is slightly higher at the IcsA-rich end of the bacterium, suggesting that IcsP delivery may also be polarized, although its production is slower (given its shallower slope) and/or its half-life in the outer membrane is longer than that of IcsA.

We have quantified the effects of several major bacterial inputs on a single output: the polar distribution of IcsA. The cross-species reproducibility of these effects suggests that, in evolving its virulence, *S. flexneri* has capitalized on more global underlying mechanisms for generating subcellular organization. Further study will yield a greater understanding of the machinery behind these mechanisms and the prokaryotic cell that they maintain.

Experimental procedures

Bacterial strains and growth conditions

All *S. flexneri* strains were derived from serotype 2a wild-type strain 2457T (LaBrec *et al*., 1964). Strain BS520 was generously provided by Dr Anthony Maurelli. *E. coli* MBG264 is a K-12 strain that is F$^-$ *araD139 lacU169 rpsL thi pyrC46 gyrA thyA his fla ompT*::Kan (Goldberg and Theriot, 1995). pHS3199 is a pBR322-derived vector that encodes *icsA* (d'Hauteville and Sansonetti, 1992; Goldberg and Theriot, 1995). *S. flexneri* strains (Sandlin *et al*., 1996) were grown in trypticase soy broth overnight with 45 μg ml^{-1} kanamycin followed by a 1:100 dilution and 180 min

incubation at 37°C with agitation for log-phase bacteria. MBG264(pHS3199) was grown overnight at 37°C in Luria–Bertani (LB) broth with 30 μg ml^{-1} carbenicillin, diluted 1:100 in fresh broth and grown for 180 min at 37°C with agitation for log-phase bacteria. The *Y. pseudotuberculosis* strain YPIII-pIB71, an lcr mutant that does not secrete the *Yersinia* outer membrane proteins (Yops) (Bolin and Wolf-Watz, 1984), containing pHS3199 was grown overnight in 2× YT broth and 30 μg ml^{-1} carbenicillin at 26°C with agitation. The overnight culture was diluted 1:100 in fresh broth and grown for 180 min at 26°C for log-phase bacteria.

IcsP *cloning*

Plasmid DNA isolation, restriction enzyme digests, ligations and electroporations were performed essentially as described previously (Sambrook *et al*., 1989). *icsP* DNA was amplified from *S. flexneri* 2457T by polymerase chain reaction (PCR) using the following pairs of oligonucleotides: 5′-TCTGATTG ATTTCAT-3′ and 5′-CCGAAGTCCGTCAACG-3′. The PCR products were digested with *Bam*HI and *Sph*I, and the resulting fragments were cloned into pACYC184 (New England Biolabs) to create picsP1.

Western blotting

Shigella and *Yersinia* strains were grown as described for log-phase bacteria. Secreted proteins were precipitated with 10% trichloroacetic acid, and whole bacteria were subjected to SDS–PAGE. The proteins were transferred to nitrocellulose by standard Western blotting procedures (Harlow and Lane, 1988). Western blot analysis was performed using an affinity-purified rabbit IcsA antiserum (Goldberg *et al*., 1993), with subsequent visualization by enhanced chemiluminescence (ECL; Amersham). The samples of supernatant protein extract loaded onto the gel were prepared from approximately 20 times the culture volume compared with the samples of whole-cell protein extract loaded onto the same gel.

Generation and characterization of IcsA monoclonal antibody

Hybridomas were generated to full-length IcsA, purified as described previously (Goldberg *et al*., 1993). Ascites containing monoclonal antibodies were generated by the injection of $\approx 1 \times 10^7$ hybridoma cells intraperitoneally in SCID mice that had been primed with 2, 6, 10, 14-tetramethylpentadecane (Pristane). Ascites were recovered, precipitated in 30% or 50% ammonium sulphate and resuspended in PBS. Antibodies were bound to a GammaBind Plus sepharose (Pharmacia) column, eluted with 0.1 M glycine, pH 3.0, and neutralized with 2 M Tris, pH 8.0. Fractions containing specific antibody were identified from among protein-containing fractions by enzyme-linked immunosorbent assay (ELISA) against purified IcsA. Specificity for IcsA was confirmed by Western blot and surface labelling of intact bacteria. Antibody VIF8, which was used in this study, was shown to recognize an epitope within the 95 kDa portion of IcsA that is exposed on the bacterial surface.

Immunofluorescence

Bacteria were grown to log phase as described, centrifuged at 5000 r.p.m., resuspended in PBS and air dried for $\approx$ 5 min on poly L-lysine-coated coverslips. Bacteria were fixed with 3% formaldehyde in PBS followed by incubation with a monoclonal antibody to the IcsA N-terminus (VIF8) diluted 1:100 in PBS and 2% bovine serum albumin (BSA) for 1 h. They were then incubated with a Texas red-conjugated anti-mouse secondary antibody (Calbiochem) diluted 1:100 in PBS and 2% BSA for 30 min and mounted onto slides. Imaging was performed on an Axioplan 2 microscope (Zeiss), and images were collected with a Micromax:512BFT cooled CCD camera (Princeton Instruments) connected to an Optiplex computer (Dell) using METAMORPH software (Universal Imaging). Published images were adjusted for optimized contrast; however, quantitative measurements were made on absolute data.

Quantification

The METAMORPH 'linescan' function was performed on each bacterium with a width equal to the width of the bacterium. For septating bacteria, the linescan end-points were defined as the bacterial end and the septum. The readout for each point is recorded as the average fluorescence intensity of the pixels on a line perpendicular to the bacterial long axis; these values can be plotted against pixels to give the average fluorescence distribution across the bacterial length, excluding the rounded tips of the poles (three to five pixels). The decay constant λ of each curve was obtained by fitting these values by exponential regression to eqn 1. Only curves with a correlation value > 0.90 for the regression were included in this analysis.

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