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Disruption of IcsP, the major *Shigella* protease that cleaves IcsA, accelerates actin-based motility

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Summary

Shigella **pathogenesis involves bacterial invasion of colonic epithelial cells and movement of bacteria through the cytoplasm and into adjacent cells by means of actin-based motility. The *Shigella* protein IcsA (VirG) is unipolar on the bacterial surface and is both necessary and sufficient for actin-based motility. IcsA is inserted into the outer membrane as a 120-kDa polypeptide that is subsequently slowly cleaved, thereby releasing the 95-kDa amino-terminal portion into the culture supernatant. IcsP, the major *Shigella* protease that cleaves IcsA, was identified and cloned. It has significant sequence similarity to the *E. coli* serine proteases, OmpP and OmpT. Disruption of *icsP* in serotype 2a *S. flexneri* leads to a marked reduction in IcsA cleavage, increased amounts of IcsA associated with the bacterium and altered distribution of IcsA on the bacterial surface. The *icsP* mutant displays significantly increased rates of actin-based motility, with a mean speed 27% faster than the wild-type strain; moreover, a significantly greater percentage of the *icsP* mutant moves in the cytoplasm. Yet, plaque formation on epithelial monolayers by the mutant was not altered detectably. These data suggest that IcsA, and not a host protein, is limiting in the rate of actin-based motility of wild-type serotype 2a *S. flexneri*.**

Introduction

Shigella is a Gram-negative bacterium that causes diarrhoea and dysentery. Disease pathogenesis involves the invasion of colonic mucosal cells by induced phagocytosis, followed by rapid lysis of the phagocytic vacuole, releasing the bacterium into the host cytoplasm (LaBrec *et al*., 1964; Sansonetti *et al*., 1986; Clerc and Sansonetti, 1987; High *et al*., 1992). *Shigella* is unusual among enteric pathogens in that, once within the cytoplasm of host cells, it recruits host actin and other proteins to form a long 'actin tail' at one pole of the bacterium that propels it through the cytoplasm and into adjacent cells (Ogawa *et al*., 1968; Bernardini *et al*., 1989; Pal *et al*., 1989; Kadurugamuwa *et al*., 1991; Prevost *et al*., 1992; Sansonetti *et al*., 1994). The *Shigella* 120-kDa outer membrane protein IcsA (VirG) has been shown to be both necessary and sufficient for *Shigella* actin-based motility (Makino *et al*., 1986; Bernardini *et al*., 1989; Lett *et al*., 1989; Goldberg and Theriot, 1995; Kocks *et al*., 1995).

IcsA is localized to the pole of the *Shigella* surface at which the actin tail is formed (Goldberg *et al*., 1993). The carboxy-terminal 344 amino acids (known as the β-domain) are embedded within the outer membrane, while the amino-terminal 706 amino acids (known as the α-domain) extend from the bacterial surface into the surrounding environment (Suzuki *et al*., 1995). IcsA is slowly cleaved at the Arg–Arg bond at position 758–759 (Fukuda *et al*., 1995), close to the junction of the α and β-domains, such that approximately 20% as much IcsA is detected as a 95-kDa amino-terminal fragment in the culture supernatant as is detected as the full-length 120-kDa polypeptide on the bacterial surface in mid-exponential phase (M. B. Goldberg, unpublished observations). The 95-kDa amino-terminal fragment is also detectable in infected host cells (M. B. Goldberg, unpublished observations).

The role of IcsA cleavage in pathogenesis is unknown. Within infected cells, polyclonal sera to IcsA were shown to detect a protein in the actin tail, at a distance from the bacterial surface, suggesting that the cleaved amino-terminal portion of the protein might be functional within the tail (Goldberg *et al*., 1993). Subsequently, d'Hauteville *et al.* (1996) have shown that polyclonal sera cross-react with an unknown 70-kDa host protein, raising the question of whether or not IcsA is present in the tail. In addition, site-directed mutation of IcsA to change the first, the second or both arginines of the cleavage site to asparagines leads to loss of IcsA cleavage (Fukuda *et al*., 1995; d'Hauteville *et al*., 1996). d'Hauteville and Sansonetti (1992) reported that serotype 5 *S. flexneri* strains that express the Asp-759 or Asp-758/Asp-759 mutant form of IcsA on a multicopy plasmid spreads more rapidly through an epithelial

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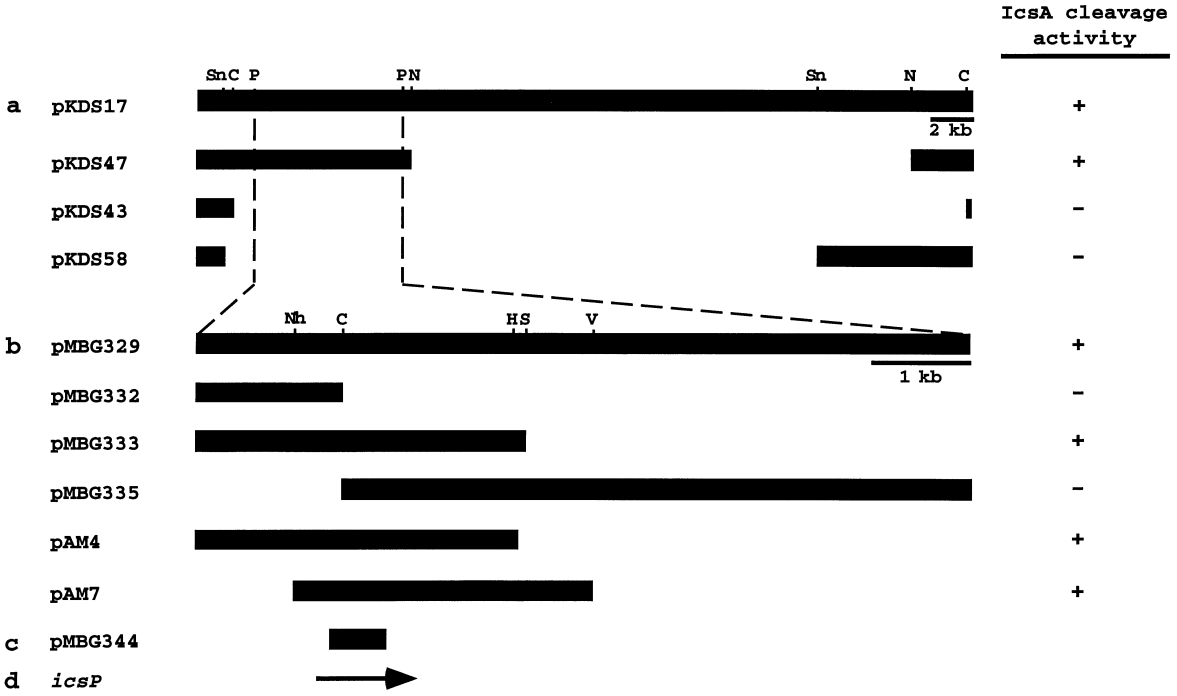

Fig. 1. Cloning strategy of *S. flexneri* genomic DNA encoding IcsA cleavage activity.
A. Maps of cosmid pKDS17 and subclones of pKDS17 in the cosmid cloning vector pYUB328.
B. Maps of subclones of the genomic insert from pKDS17 in the cloning vector pBC-KS$^+$.
C. Map of insert in suicide vector pMBG344, which was used to disrupt *icsP* on the virulence plasmid of wild-type strain 2457T.
D. Direction of transcription and delimitations of the coding sequence of *icsP*. pMBG331 contains the same insert as pMBG329, but in the opposite orientation. IcsA cleavage activity of each cosmid or plasmid was determined as described in the text. Restriction enzyme recognition sites: C, *Cla*I; H, *Hpa*I; N, *Nco*I; Nh, *Nhe*I; P *Pst*I; S, *Sma*I; Sn, *Sna*BI; V, *Eco*RV.

cell monolayer, whereas Fukuda *et al.* (1995) reported that a serotype 2a *S. flexneri* strain that expresses the Asp-759 mutant form of IcsA from an integrated single copy of the gene did not spread differently from the wild type. The reasons for these observed differences are unclear. Moreover, these non-cleavable forms of IcsA also have significant charge, and possibly structural alterations; the contributions of these alterations to the observed phenotypes is unknown.

Recently, Egile *et al.* (1997) have reported the identification and targeted disruption of a *S. flexneri* serotype 5 wild-type strain M90T virulence plasmid gene (*sopA*) that encodes a protease of IcsA. They report that the disruption of *sopA* leads to alteration of the surface distribution of IcsA: IcsA was present over the entire surface of the mutant bacilli, although relatively more IcsA was present on one pole than elsewhere on the surface. Further, they report that a strain carrying a disruption of *sopA* forms abnormally short bacterial protrusions from the surface of infected HeLa cells and abnormally small plaques on Caco-2 cell monolayers.

Using *S. flexneri* serotype 2a wild-type strain 2457T, we have also identified and constructed a targeted deletion of a gene that encodes for IcsA cleavage activity, *icsP*, which is identical to *sopA*. We have then characterized the strain containing the targeted disruption in terms of IcsA expression, bacterial intracytoplasmic motility and plaque formation on tissue culture cell monolayers. We observe differences from those reported by Egile *et al.* (1997) in terms of the ability of the mutant to form surface protrusions from infected cells and to form plaques on cell monolayers.

Results

Identification of a gene encoding an IcsA protease

A cosmid library of *S. flexneri* serotype 2a wild-type strain 2457T genomic DNA was introduced into MBG344, an *ompT*$^-$ derivative of *E. coli* strain MC1061 that carries *icsA* on the plasmid pMBG226. Supernatant proteins from 192 individual colonies of the resultant library were screened by enzyme-linked immunosorbent assay (ELISA) for increased amounts of IcsA. Twelve individual colonies were selected from the 192, and supernatant protein preparations from each of these were evaluated by Western blot for the presence or absence of cleavage of IcsA. The cosmid pKDS17 was identified as one that produced cleaved IcsA (seen as a 95-kDa band) in the culture supernatant (Fig. 1A, and Fig. 2, lane 3, arrowhead). The *S. flexneri* genomic DNA contained on pKDS17 was

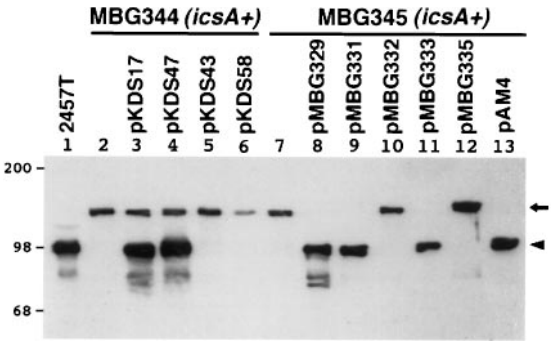

Fig. 2. Assay of IcsA cleavage activity in *E. coli*. Western blot of supernatant protein preparations of *Shigella* wild-type strain 2457T and of cosmid pKDS17 and various subclones of pKDS17 in MBG344 (lanes 3–6) or MBG345 (lanes 8–13), using IcsA antiserum and enhanced chemiluminescence. Lane 1, wild-type strain 2457T (*icsA*$^+$ *icsP*$^+$); lane 2, MBG344 (*icsA*$^+$); lane 3, MBG344 (*icsA*$^+$) carrying pKDS17 (*icsP*$^+$); lane 4, MBG344 (*icsA*$^+$) carrying pKDS47 (*icsP*$^+$); lane 5, MBG344 (*icsA*$^+$) carrying pKDS43 (*icsP*$^-$); lane 6, MBG344 (*icsA*$^+$) carrying pKDS58 (*icsP*$^-$); lane 7, MBG345 (*icsA*$^+$); lane 8, MBG345 (*icsA*$^+$) carrying pMBG329 (*icsP*$^+$); lane 9, MBG345 (*icsA*$^+$) carrying pMBG331 (*icsP*$^+$); lane 10, MBG345 (*icsA*$^+$) carrying pMBG332 (Δ*icsP*); lane 11, MBG345 (*icsA*$^+$) carrying pMBG333 (*icsP*$^+$); lane 12, MBG345 (*icsA*$^+$) carrying pMBG335 (Δ*icsP*); lane 13, MBG345 (*icsA*$^+$) carrying pAM7 (*icsP*$^+$). Arrow, uncleaved 120-kDa form of IcsA. Arrowhead, cleaved 95-kDa form of IcsA. Apparent molecular masses of standard proteins run in parallel are indicated in kilodaltons at left.

subcloned, and supernatant protein preparations of subclones were evaluated for the presence or absence of IcsA cleavage activity in strain MBG344 (Fig. 1A and B, and Fig. 2). An approximately 2.4 kb *Nhe*I–*Sma*I DNA segment was found sufficient to encode the IcsA cleavage activity. Southern blot analysis of DNA prepared from *Shigella* strains 2457T (wild-type) and BS103, which is 2457T cured of the virulence plasmid, revealed that the 2.4 kb DNA fragment is located on the virulence plasmid (data not shown).

Sequence analysis of icsP

The 2.4 kb DNA segment that encoded IcsA cleavage activity was completely sequenced. It contains an open reading frame of 981 bp that begins with the codon ATG and was given the name *icsP*. The sequence TAAAGT, similar to the *E. coli* consensus −10 box, is found between positions −15 and −10. The sequence TTGGCA, similar to the *E. coli* consensus −35 box, is found between positions −36 and −31. And, the sequence GTAAG, similar to the *E. coli* consensus ribosome-binding site sequence, is found between positions −11 and −7. There is no open reading frame on the complementary strand or immediately upstream or downstream of the coding sequence and, beginning 35 bp downstream of the TGA stop site, is a Tn*629* element. Thus, *icsP* appears not to be part of an operon, and the targeted disruption of *icsP* (described below) should not have polar effects on any downstream or complementary strand genes.

The deduced amino acid sequence reveals a 327-amino acid polypeptide that has 58% sequence identity to each of the *E. coli* proteases, OmpP and OmpT, 42% sequence identity to the *Salmonella typhimurium* protease E precursor, PrtA (also called PgtE), and 40% sequence identity to the *Yersinia pestis* fibrinolysin precursor Pla (also called ColY) (Fig. 3). Except for the addition of the first 11 amino acids of the IcsP sequence, IcsP is identical to SopA, which was recently identified by Egile *et al.* (1997).

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IcsP     MDISTKKVEFSMKLKFFVLALCVPAIFTTHATTNYPLFI----PDNISTDISLGSLSGKTKERVYHPKEGGRKISQLDWKYSNATIVRGG  86
OmpP                •QT•LLAIM•AA•VV•SSQEASASDF•G-----EK•••E•N••T••••••••••E•E•••••V••••••T•••A•LK•A  75
OmpT                •RA•LLGIV•TT•IAISSF•S•ETLS•T---------NA•••••T••••••••••LAE•••••V••••••FN••A•IK•A  75
PrtA MKTSSPLQITSEYEDKRNEKTCYCVMMI•V•SESVYAESA•••PDVS••SVT•SL•V•V•N••SR•L••D-TDT•••L••••••IK•VATLQ•D  93
Pla                MKKSSIVATIITILSLGSANAASSQLI•NIS------SFTVAA•T•M••••SH•ML•DA-•T••••••••••IK•VA•LK•D  76

  87 IDWKLIPKVSFGVSGWTTLGNQKASMVDKDWNNSNTPQVWTDQSWHPNTHLRDANEFELNLKGWLLNNLDYRLGLIAGYQESRYSFNAMGGSYI 180
  76 VN•E•N•WL•V•AA•••••NSRGGN•••Q••MD•G••GT•••E•R••D•R•NY••••D••V•••FLKES••••AIM••••••••••••T••T•• 169
  76 •N•D•M•QI•I•AA•••••GSRGGN•••Q••MD•SN•GT•••E•R••D•Q•NY••••D••I••••••EPN•••••M••••••••••T•R••••• 169
  94 LS•EPYSFMTLDAR•••S•ASGSGH•••H••MS•EQ•-G•••R•I••D•SVNY•••YD••V•••••QGDN•KA•VT•••••T•F•WT•R••••• 186
  77 •S•DPYSFLTLNAR•••S•ASGSGN•D•Y••MNE•QSE-•••H•S••A•NVNH•••YD••V•••••QDEN•KA•IT•••••T•F•WT•T••••S 169

 181 YSENGGSRNKKGAHPSGERTIGYKQLFKIPYIGLTANYRHENFEFGAELKYSGWVLSSDTDKHYQTE--TIFKDEIKNQNYCSVAANIGYYVTP 272
 170 ••••••F••ET••L•DKIKV•••••H•••••V•••G•••YD•••••GAF••••••RG••N•E••VRQ--•T•RSKVI••••Y•••V•A•••I•• 261
 170 ••SEE•F•DDI•SF•N•••A•••••R••M••••••GS••Y•D••L•GTF••••••E•••N•E••DPGKRITYRSKV•D•••Y•••V•A•••••• 263
 187 •-D•••••RYI•NF•H•V•G•••S•R•EM•••••AGD••IND••CNVLF•••D••NAH•N•E••MRK--LT•REKTE•SR•YGASIDA•••I•S 274
 170 •NNGA----YT•NF•K•V•V•••N•R•SM•••••AGY••IND••LN•LF•F•D••RAH•N•E••MRD--LT•REKTSGSR•YGTVI•A•••••• 257

 273 SAKFYIE-GSRNYISNKKGDTSLYEQSTNISGTIKNSASIEYIGFLTSAGIKYIF 326
 262 E••V••••-•VWSRLT•••••••••DR•D•TSEHNN•G•G••NYN•I•T••L••T• 315
 264 N••V•V•-•AW•RVT••••N••••DHNN•T•DYS••G•G••NYN•I•T••L••T• 317
 275 N••IFA•FAYSK•EEG•G•TK•                                  296
 258 N••VFA•FTYSK•DEG•G•TQTIDKN•GDSVSIGGDA•G•SNKNYTVT••LQ•R• 312
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Fig. 3. Amino acid sequence identity of IcsP to known proteins. Complete amino acid sequences are shown. OmpP, *E. coli* outer membrane protein P; OmpT, *E. coli* outer membrane protein T; PrtA, *S. typhimurium* protease E precursor; Pla, *Y. pestis* fibrinolysin precursor. Dots indicate amino acids that are identical to the amino acid in the corresponding aligned position of IcsP. Dashes indicate gaps that were introduced into the sequence to optimize alignment.

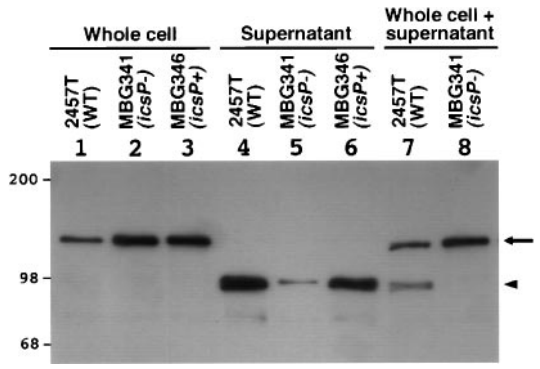

Fig. 4. Reduction of IcsA cleavage in *icsP* deletion strain MBG341. Western blot of whole cell protein preparations (lanes 1–3), supernatant protein preparations (lanes 4–6) and combinations of the whole cell protein and supernatant protein preparations derived from a predetermined volume of logarithmic-phase bacterial culture (lanes 7 and 8), using IcsA antiserum and enhanced chemiluminescence. Lanes 1, 4 and 7, wild-type strain 2457T (*icsA*$^+$ *icsP*$^+$); lanes 2, 5 and 8, *icsP* deletion strain MBG341 (*icsA*$^+$ Δ*icsP*); lanes 3 and 6, *icsP* deletion carrying *icsP in trans* on pAM4 (strain MBG346) (*icsA*$^+$ *icsP*$^+$). Arrow, uncleaved 120-kDa form of IcsA. Arrowhead, cleaved 95-kDa form of IcsA. Apparent molecular masses of standard proteins run in parallel are indicated in kilodaltons at left.

Disruption of icsP *in* S. flexneri *leads to a marked reduction in cleavage of IcsA*

Disruption of *icsP* in *S. flexneri* wild-type strain 2457T was performed, yielding strain MBG341. Analysis of IcsA expression in *icsP* mutant strain MBG341 shows markedly reduced amounts of IcsA in the culture supernatant (Fig. 4, lane 4 vs. lane 5), indicating loss of most of the IcsA cleavage activity. Of note, there is a very small amount of cleaved (95 kDa) IcsA in the supernatant of *icsP* mutant strain MBG341, which indicates that a second protease that cleaves IcsA less efficiently exists in *S. flexneri*. Transformation of pAM4, a subclone of pKDS17 that contains *icsP* (Fig. 1B), into *icsP* mutant strain MBG341 (to give strain MBG346) restored cleavage activity of IcsA (Fig. 4, lane 6), confirming both that the disruption in MBG341 was of *icsP* and that the cleavage activity encoded on the 2.4 kb fragment was active in *S. flexneri.* The cleavage activity seen for MBG346 is slightly less than that seen for wild-type 2457T, suggesting that the expression of IcsP encoded on pAM4 is different from native IcsP expression in 2457T.

The total amount of IcsA produced by each strain was evaluated by combining the whole cell protein and supernatant protein preparations derived from a predetermined volume of logarithmic phase bacterial culture (Fig. 4, lanes 7 and 8). Bands on a Western blot loaded in an identical fashion to lanes 7 and 8 of Fig. 4 and performed with ^{125}I-conjugated protein A (to give a more linear response than obtained with enhanced chemiluminescence) were analysed for band density by scanning densitometry. Using this technique, the total amount of IcsA detected for *icsP* mutant strain MBG341 is approximately the same as that detected for wild-type strain 2457T (data not shown).

Surface distribution of IcsA on icsP *mutant strain MBG341*

A mutant form of IcsA that is not cleaved because of an arginine to asparagine mutation at position 759 of the IcsA cleavage site in serotype 2a *S. flexneri* was shown to distribute normally on the bacterial surface (Fukuda *et al*., 1995), while a mutant form that is not cleaved as a result of arginine to asparagine mutations at both position 758 and position 759 in serotype 5 *S. flexneri* was shown to distribute along the sides and septum of the bacteria, while maintaining polar reinforcement (d'Hauteville *et al*., 1996). Loss of cleavage of surface IcsA as a result of disruption of *icsP* might also be expected to lead to an altered distribution of IcsA on the bacterial surface. The recently reported disruption of *sopA* (which is identical to *icsP*) in serotype 5 *S. flexneri* led to distribution of IcsA over the entire bacterial surface, with maintenance of relatively greater amounts of IcsA at one pole (Egile *et al.*, 1997).

To explore the surface localization of IcsA on our serotype 2a *icsP* deletion mutant, indirect immunofluorescence on *icsP* mutant strain MBG341 and wild-type strain 2457T was performed using a polyclonal antibody to IcsA that recognizes only the 95-kDa portion of the 120-kDa form or the isolated 95-kDa fragment of IcsA (M. B. Goldberg, unpublished results). Indirect immunofluorescence shows that, in addition to the unipolar cap of IcsA that is seen on wild-type *Shigella* (Fig. 5A and B, arrow), *icsP* mutant strain MBG341 has IcsA distributed in an irregular punctate manner over the entire bacterial surface (Fig. 5C and D, arrowheads). This distribution is similar to that reported for the *sopA* (which is identical to *icsP*) mutant in serotype 5 *S. flexneri*, although the IcsA along the sides of the bacillus appears to be localized in a more punctate fashion. Since preparations of whole cell proteins indicate that the IcsA that is associated with the bacterium is the uncleaved 120-kDa form (Fig. 4, lanes 1 and 2), the additional IcsA on the surface of strain *icsP* mutant strain MBG341 is the 120-kDa form.

icsP *mutant strain MBG341 displays accelerated actin-based motility*

IcsA is the only *Shigella* protein necessary to permit actin-based motility (Goldberg and Theriot, 1995; Kocks *et al*., 1995). Since a quantitative increase and/or altered distribution of surface IcsA might alter the dynamics of motility, both rates of movement of motile intracytoplasmic bacteria

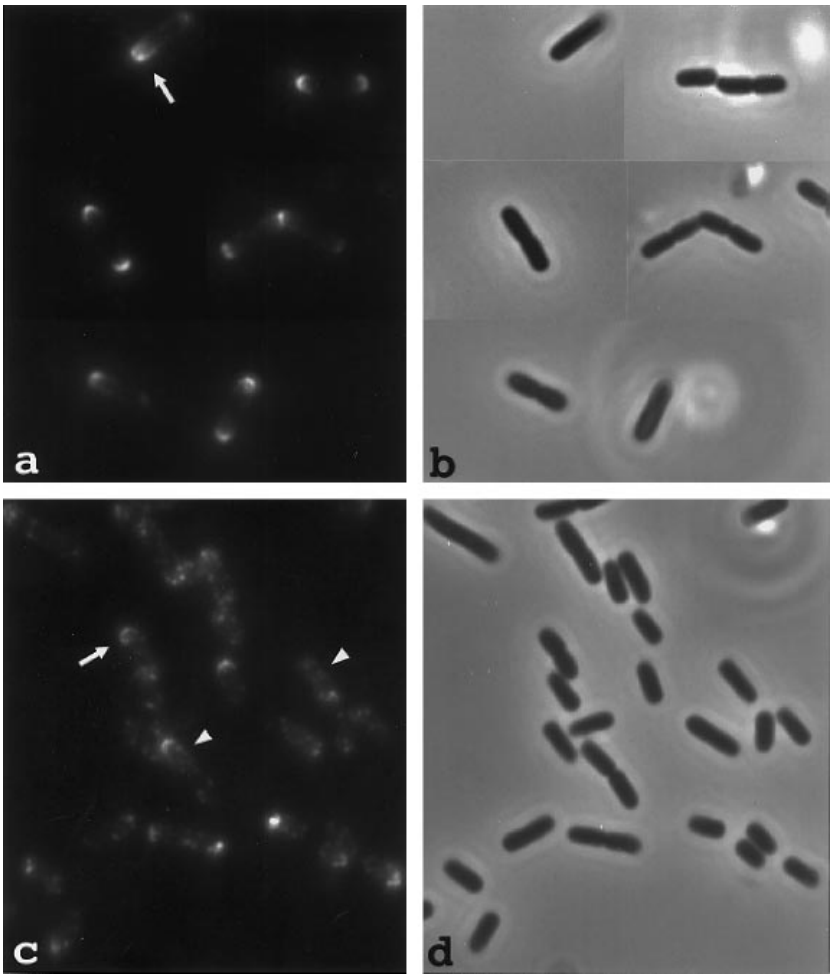

Fig. 5. IcsA on the surface of the *icsP* deletion strain MBG341. Indirect immunofluorescence of extracellular wild-type 2457T (a and b) and *icsP* deletion MBG341 (c and d), using IcsA antiserum. Indirect immunofluorescence images (a and c) and phase-contrast images of the same microscopic fields (b and d respectively). Arrows, unipolar cap of IcsA on bacterial pole. Arrowheads, punctate distribution of IcsA over entire surface of *icsP* deletion strain MBG341.

and percentages of intracytoplasmic bacteria that were motile were determined for *icsP* mutant strain MBG341 and wild-type 2457T. *icsP* mutant strain MBG341 moved significantly faster than wild-type strain 2457T, with a mean rate of movement of $15.6 \pm 8.6\,\mu m\,min^{-1}$ (mean ± SD, $n = 98$), compared with $12.3 \pm 7.7\,\mu m\,min^{-1}$ (mean ± SD, $n = 55$) for the wild-type strain 2457T (unpaired *t*-test, $P = 0.02$) (Table 1). Significantly more *icsP* mutant MBG341 than wild-type 2457T moved at speeds faster than $10\,\mu m\,min^{-1}$ (chi-square test, $P = 0.02$). The range of speeds attained by *icsP* mutant strain MBG341 was 2.8–40.7 $\mu m\,min^{-1}$, compared with 2.1–31.8 $\mu m\,min^{-1}$ for wild-type strain 2457T (Table 1). In addition, the distribution of bacterial speeds was shifted to slightly faster speeds (Fig. 6). The percentage of *icsP* mutant MBG341 moving in any 1-min window of observation was significantly greater than the percentage of wild-type strain 2457T moving in any 1-min window of observation, with a mean of 33.4 ± 10.7 (mean ± SD, $n = 12$) for *icsP* mutant strain MBG341 compared with 23.2 ± 9.5 (mean ± SD, $n = 13$) for 2457T (unpaired *t*-test, $P = 0.02$) (Table 1). Thus, the mean rate of movement of *icsP* mutant strain MBG341 was 27% faster than that of the wild-type strain, the fastest MBG341

Table 1. Motility characteristics of *icsP* mutant strain MBG341 and wild-type strain 2457T.

	Rate of motility ($\mu m\,min^{-1}$)			Percentage of bacteria moving		
Strain	*n*	Mean ± SD[a]	Range	*n*	Mean ± SD[b]	Range
2457T	55	12.3 ± 7.7	2.1–31.8	13	23.2 ± 9.5	8.6–40.0
MBG341	98	15.6 ± 8.6	2.8–40.7	12	33.4 ± 10.7	13.3–48.4

a. $P = 0.02$.
b. $P = 0.02$.

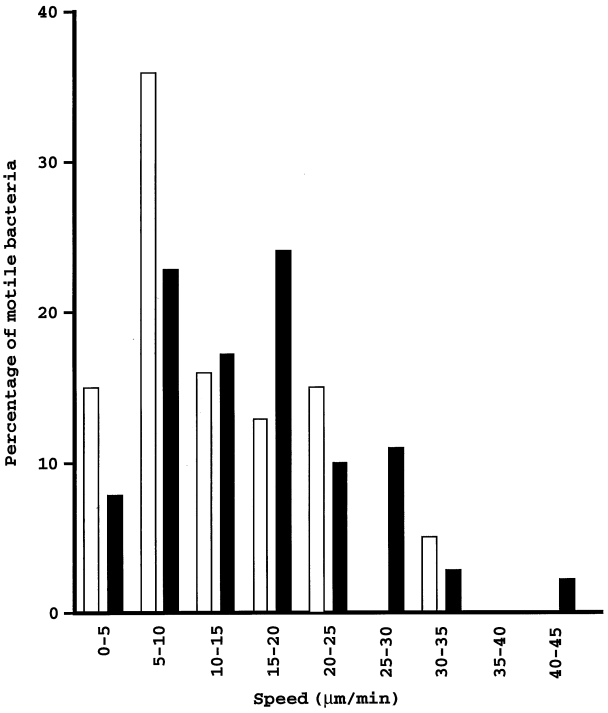

Fig. 6. Speed distribution of moving wild-type strain 2457T (□) and *icsP* deletion strain MBG341 (■) in infected PtK2 cells.

organisms moved 28% faster than the fastest wild-type organisms and 23% more MBG341 were moving than wild-type.

Evaluation of actin tails formed by icsP *mutant strain MBG341*

To characterize the alterations in actin-based motility of *icsP* mutant strain MBG341 further, bacterial actin tails were evaluated by direct immunofluorescence after fixation and labelling of polymerized actin with rhodamine-conjugated phalloidin. The general shape and contour of the tails appeared similar for the two strains (Fig. 7). However, on *icsP* mutant MBG341 having tails, the polymerized actin frequently extended far up the lateral sides of the bacterium, whereas this was rarely seen with wild-type 2457T. Representative examples of this are shown in Fig. 7. This alteration in distribution of actin on the surface of intracytoplasmic *icsP* mutant MBG341 is consistent with the altered distribution of bacterial surface IcsA seen by indirect immunofluorescence on extracellular MBG341.

Protrusion formation by icsP *mutant strain MBG341 is similar to that of wild-type*

The spread of *Shigella* through mammalian cell monolayers involves the formation of membrane-bound protrusions by intracytoplasmic bacteria from the surface of the infected cell, followed by engulfment and uptake of bacteria within the tips of these protrusions by adjacent cells (Bernardini *et al*., 1989; Pal *et al*., 1989; Kadurugamuwa *et al*., 1991; Prevost *et al*., 1992; Sansonetti *et al*., 1994). The process of protrusion formation appears to be actin dependent, since bacteria within protrusions have actin tails that extend from the bacterium at the protrusion tip down the length of the protrusion towards the cell body.

Since disruption of *icsP* caused alteration in the surface distribution of IcsA and acceleration of intracytoplasmic actin-based motility, it was of interest to examine the nature of protrusions formed by bacteria in infected cells for *icsP* mutant strain MBG341 compared with wild-type strain 2457T. Bacterium-induced cell surface membrane-bound protrusions, as observed by time-lapse microscopy and direct immunofluorescence of fixed samples, appeared morphologically similar for the two strains.

Plaque formation on epithelial monolayers by icsP *mutant strain MBG341 is similar to that of wild-type*

Since the disruption of *icsP* and the resultant increase in IcsA on the bacterial surface leads to accelerated rates of bacterial movement within the cytoplasm, it was of interest

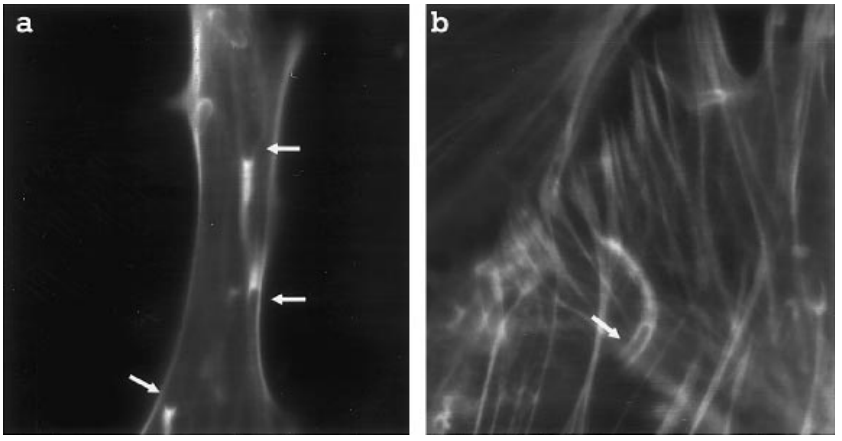

Fig. 7. Actin tail formation by *icsP* deletion strain MBG341. Representative examples of direct immunofluorescence of actin tails in HeLa cells infected with wild-type strain 2457T (a) or *icsP* deletion strain MBG341 (b), using rhodamine phalloidin. Arrows indicate extension of actin along lateral sides of MBG341, but not wild-type strain 2457T.

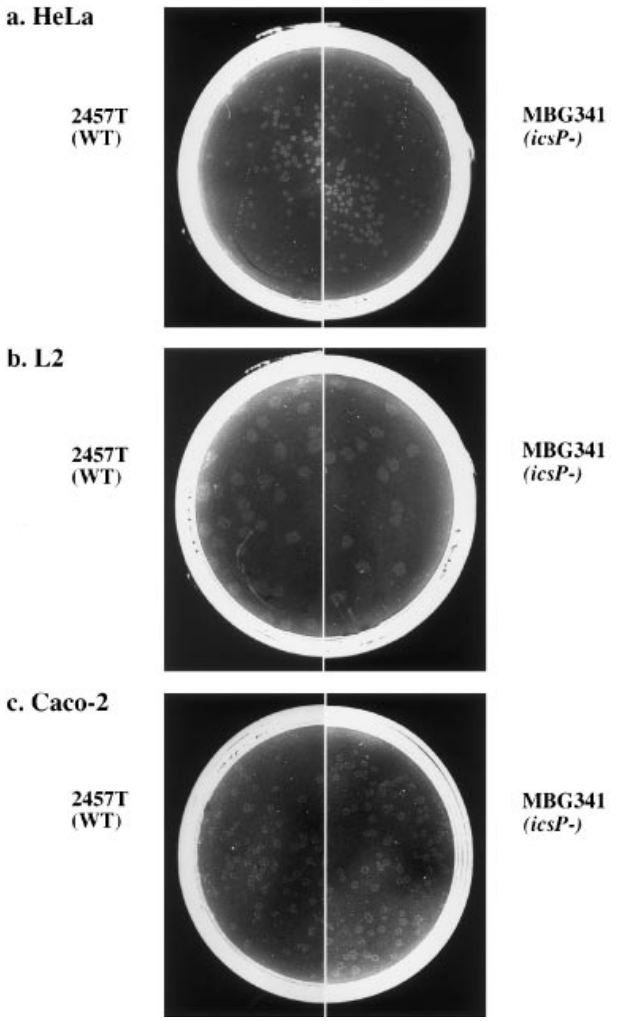

Fig. 8. Bacterial plaque formation by *icsP* deletion strain MBG341 (right) and wild-type strain 2457T (left) on monolayers of HeLa cells (a), L2 cells (b) and Caco-2 cells (c). Petri dishes were photographed after 48 h of infection.

to examine whether the ability of the bacteria to spread from cell to cell would be altered compared with the wild-type strain. Bacterial plaque formation was examined for *icsP* mutant strain MBG341 and wild-type strain 2457T on monolayers of the human epithelial-derived cell line HeLa (Fig. 8A), the rat fibroblast cell line L2 (Fig. 8B) and the polarized human colonic carcinoma-derived cell line Caco-2 (Fig. 8C). For each of the three cell lines, there was no significant difference seen in the size of plaque formed by *icsP* mutant strain MBG341 compared with wild-type strain 2457T.

Discussion

The deduced amino acid sequence of IcsP (SopA) indicates that it is a unique protease. It has significant sequence similarity to two *E. coli* proteases, OmpP and OmpT. OmpT selectively cleaves at bonds consisting of the acidic residues arginine and lysine: Arg–Arg, Arg–Lys, Lys–Arg or Lys–Lys; with more rapid cleavage at an Arg–Lys bond than at a Lys–Lys bond (Sugimura *et al*., 1988). It is active only as an endopeptidase, not cleaving bonds at the termini of polypeptides. The substrate specificity of OmpP is similar (Kaufmann *et al*., 1994). This substrate specificity profile probably applies to IcsP as well, since the proteolytic cleavage site on IcsA is the Arg–Arg bond between amino acids 758 and 759 (Fukuda *et al*., 1995); thus, the mechanism of substrate site recognition is probably similar for the three proteases. These observations, combined with sequence similarities, suggest common evolutionary origins for these proteases. Of note, there are no Lys–Arg or Lys–Lys bonds in IcsA; there is a single Arg–Lys bond between amino acids 1025 and 1026, which is in the membrane-spanning β core region of the polypeptide and, therefore, may not be accessible to IcsP.

Based on inhibitor profiles, OmpT is thought to depend on a serine residue for catalytic activity (Hollifield *et al*., 1978; Grodberg and Dunn, 1988). The similar substrate specificity of OmpP and OmpT has led investigators to conclude that OmpP is also a serine protease (Kaufmann *et al*., 1994). Each contains several serine residues, but neither's serine active site has been identified and neither contains the conserved sequences flanking a serine residue that is seen in other classes of microbial and pancreatic serine proteases (Delbaere *et al*., 1975; Grodberg and Dunn, 1988; Rawlings and Barrett, 1994). IcsP has 31 serine residues, 13 of which are conserved among IcsP, OmpP and OmpT and would therefore be good candidates for the active site.

OmpP and OmpT are both outer membrane proteases. OmpT is known to cleave the *E. coli* ferric enterobactin receptor (Hollifield *et al*., 1978). No other natural substrate for OmpT and no natural substrate for OmpP has been identified. Of note, when IcsA is expressed in OmpT$^+$ strains of *E. coli*, it is cleaved from the surface (Goldberg *et al*., 1993; Nakata *et al*., 1993), and a 21 kb region of the *E. coli* chromosome that contains *ompT* is absent from the *Shigella* chromosome (Nakata *et al*., 1993). The *E. coli* strains used in this study are *ompT*$^-$; OmpP is known to be expressed in several *E. coli* strains (Kaufmann *et al*., 1994), but it is not known whether it is expressed in the *E. coli* strains used in this study or in *Shigella*. Given the similarities in substrate specificity of OmpT and OmpP, it is possible that OmpP would cleave IcsA. Further, an OmpP or OmpP-like protease might be present in *Shigella*, accounting for the small amount of IcsA cleavage that occurs in the *icsP* mutant (Fig. 4, lane 5).

Based on the pattern of OmpP cleavage by trypsinization of intact bacteria, the 155 amino-terminal residues of OmpP are exposed at the bacterial surface,

which suggests that OmpP is anchored in the membrane at the carboxy-terminus and that its active site is located in the exposed amino-terminus (Kaufmann *et al*., 1994). The topology of OmpT has not been determined. Taken together, the sequence similarities among IcsP, OmpP and OmpT and the observation that the substrate for IcsP, IcsA, is an outer membrane protein suggest that IcsP is an outer membrane protein with functional domains organized similarly to those of OmpP.

The mechanisms by which IcsA localizes to a single pole on the surface of *Shigella* is poorly understood. Two alternative hypotheses are: (1) that IcsA is directly targeted to the pole; and (2) that IcsA is inserted in the outer membrane over the entire bacterial surface and is selectively cleaved everywhere except at the pole (d'Hauteville *et al*., 1996). Data presented here suggest that each hypothesis is at least partially true. IcsP is the major *Shigella* protease that cleaves IcsA. Its disruption results in: (1) maintenance of a polar cap of IcsA, suggesting that IcsA is largely targeted to the pole; and (2) additional punctate accumulations of IcsA over the entire bacterial surface, indicating that some IcsA is inserted in regions distant from the pole (Fig. 5). These data suggest that the primary path for IcsA insertion in the outer membrane involves its targeting directly to the pole, but that a portion of the synthesized IcsA nevertheless becomes inserted in the outer membrane at sites away from the pole, and that IcsP functions to proteolyse the surface IcsA that is not at the pole. The surface localization of IcsP and why cleavage of IcsA by IcsP appears to be limited at the pole remain to be determined.

The IcsA protease mutant recently described by Egile *et al.* (1997) in *S. flexneri* serotype 5 strain M90T background displays a slightly different surface distribution of IcsA than that reported here. It was described as localization over 'the entire surface, with polar reinforcement' (Egile *et al*., 1997). The protease mutant studied in the present work is different in that it is serotype 2a *S. flexneri*. The two mutants were constructed using almost identical genetic approaches. The variation in surface IcsA between the two mutants may be a result of serotype differences, since IcsA on the surface of wild-type serotype 5 strain M90T extends farther down the sides of the bacillus than on wild-type serotype 2a strain 2457T (M. B. Goldberg, unpublished observations). Furthermore, alteration of the IcsA cleavage site in a serotype 5 strain resulted in the distribution of IcsA along the sides of the bacilli, with the maintenance of polar reinforcement (d'Hauteville *et al*., 1996), while a similar mutation in a serotype 2a strain did not result in the alteration of IcsA distribution compared with the wild-type strain (Fukuda *et al*., 1995). Taken together, these observations suggest that the molecular mechanisms of unipolar localization of IcsA may differ slightly between the two serotypes.

The serotype 2a IcsP protease mutant described in this study forms protrusions from the surface of infected PtK2 and HeLa cells that appear normal and plaques on HeLa, L2 or Caco-2 cell monolayers that are identical to those formed by the parent wild-type serotype 2a strain (Fig. 8). In contrast to our findings, Egile *et al.* (1997) recently reported that their serotype 5 IcsA protease mutant formed very few protrusions in infected HeLa cells and formed significantly smaller plaques than the parent wild-type serotype 5 strain on Caco-2 cells. The reasons for these differences are probably related to differences between the two serotypes, since serotype 5 wild-type strain M90T forms slightly smaller plaques than serotype 2a wild-type strain 2457T on HeLa and Caco-2 cell monolayers (M. Charles, A. Manessis and M. B. Goldberg, unpublished results), which suggests that the complex molecular mechanism of cell-to-cell spread may differ slightly between the two serotypes. It seems likely that, subsequent to the evolutionary divergence of the two serotypes, minor differences in the genomes have arisen.

In addition to leading to an increase in the amount of IcsA on the bacterial surface, disruption of *icsP* described here in serotype 2a leads to a 28% increase in the rate of the fastest moving organisms and a 27% increase in mean rates of intracytoplasmic motility (Table 1). These data suggest that the concentration of IcsA is limiting in the molecular interactions that occur in the generation of force that propels the bacterium. The observation that bacterially associated actin on motile organisms extends further along the lateral sides of the bacilli (Fig. 7), where punctate accumulations of IcsA are seen on extracellular bacteria, demonstrates that additional surface IcsA is able to recruit additional actin into the tail, further supporting this conjecture. Rates of motility were not determined for the serotype 5 IcsA protease mutant and so cannot be compared with those of the serotype 2a mutant described here.

Increased surface IcsA on the *icsP* mutant also leads to a 23% increase in the percentage of intracytoplasmic bacteria that move (Table 1), suggesting that the threshold of actin accumulation required to initiate and maintain movement is more easily attained. This is consistent with previous observations that increasing levels of surface IcsA during bacterial division correlate with the initiation of bacterial movement (Goldberg *et al*., 1994).

In the *Listeria monocytogenes* system, certain alterations in the localization of ActA, the surface protein necessary for actin-based motility, are detrimental to motility: when covered over the entire surface with ActA, *Streptococci*, which are almost spherical, are not able to form actin tails, whereas, when covered with ActA over only one hemisphere of the surface, they are able to form actin tails (Smith *et al*., 1995). In the present study, we did not observe a detrimental effect on *Shigella* motility as a result

Table 2. Strains and plasmids.

Bacterial strain or plasmid	Genotype	Source
S. flexneri strains		
2457T	Wild-type serotype 2a	(LaBrec *et al*., 1964)
BS103	2457T cured of the virulence plasmid pSF2a140	(Maurelli *et al*., 1985)
BS109	2457T *galU*::Tn*10*	(Maurelli *et al*., 1985)
MBG341	2457T *icsP*::pMBG344	This study
MBG346	2457T *icsP*::pMBG344 carrying *icsP* on pAM4	This study
E. coli strains		
MC1061	F$^-$ *araD139* Δ(*ara–leu*)*7696 galE15 galK16* Δ(*lac*)*X74 rpsL* (Str^r) *hsdR2* (r_K^- m_K^+) *mcrA mcrB1*	(Meissner *et al*., 1987)
χ2764	F$^-$ λ (cI857 *b2 redβ3 S7*)Δ(*gpt-proA*)*62 leuB1 glnV44 ara-14 galK2 lacY1 hsdS20 rpsL20 xyl-5 mtl-1 recA13*	(Jacobs *et al*., 1993)
MBG263	MC1061 *ompT*::km	(Goldberg and Theriot, 1995)
MBG344	MC1061 *ompT*::km carrying *icsA* on pACYC177	This study
MBG345	MC1061 *ompT*::km carrying *icsA* on a Tet^r derivative of pACYC177	This study
Plasmids and cosmids		
pAM4	pBC-KS$^+$ containing *icsP*$^+$ 3.3 kb *Pst*I–*Hpa*I fragment of pMBG329 insert, Cm^r	This study
pAM7	pBC-KS$^+$ containing *icsP*$^+$ 3.1 kb *Nhe*I–*Eco*RV fragment of pMBG329 insert, Cm^r	This study
pBC-KS$^+$	Cloning vector, Cm^r	Pharmacia
pCVD442	Suicide vector, Am^r	(Mobley *et al*., 1993)
pKDS17	Cosmid cloning vector pYUB328 containing *icsP*$^+$ 2457T genomic DNA, Am^r	This study
pKDS47	*icsP*$^+$ *Nco*I deletion subclone of pKDS17, Am^r	This study
pKDS43	*icsP*$^-$ *Cla*I deletion subclone of pKDS17, Am^r	This study
pKDS58	*icsP*$^-$ *Sna*BI deletion subclone of pKDS17, Am^r	This study
pMBG226	pACYC177 containing *icsA*, Km^r Cm^r	This study
pMBG297	Tet^r derivative of pACYC177 containing *icsA*	This study
pMBG329	pBC-KS$^+$ containing *icsP*$^+$ 8 kb *Pst*I fragment of pKDS17, Cm^r	This study
pMBG331	Same as pMBG329, except that *Pst*I fragment is in opposite orientation within pBC-KS$^+$, Cm^r	This study
pMBG332	pBC-KS$^+$ containing *icsP*$^-$ 1.5 kb *Pst*I–*Cla*I fragment of pMBG329 insert, Cm^r	This study
pMBG333	pBC-KS$^+$ containing *icsP*$^+$ 3.4 kb *Pst*I–*Sma*I fragment of pMBG329 insert, Cm^r	This study
pMBG334	pBC-KS$^+$ containing *icsP*$^+$ 4.1 kb *Eco*RV–*Pst*I fragment of pMBG329, Cm^r	This study
pMBG335	pBC-KS$^+$ containing icsP$^-$ 6.5 kb *Cla*I–*Pst*I fragment of pMBG329 insert, Cm^r	This study
pMBG344	Suicide vector pCVD442 containing internal fragment of *icsP*, Am^r	This study
pYUB328	Cosmid cloning vector, Am^r	(Balasubramanian *et al*., 1996)

of the altered surface distribution of IcsA that accompanied disruption of *icsP*. While it seems logical that strict unipolar localization of IcsA might lead to more efficient actin-based motility, both the faster rates observed for the *icsP* mutant and the prior observation that *E. coli* that express IcsA uniformly on their surfaces are capable of normal actin-based motility in cytoplasmic extracts (Goldberg and Theriot, 1995) establish that unipolarity of IcsA is not essential to directional actin-based motility. Perhaps, the *icsP* mutant would display aberrant motility in cell lines or hosts that were not examined in this study, where IcsA affinity for its substrate may differ. Proper surface localization of IcsA may enable the bacteria to move in straighter trajectories, and the *icsP* mutant bacteria may move less straight than the wild type; further analysis will address this. It remains possible that IcsP functions in pathogenesis by cleaving another surface protein and that IcsA is an innocent bystander, although it seems remarkable that, were this the case, IcsA would not have evolved resistance to cleavage by alteration of the cleavage site. It seems more likely that IcsP cleavage of IcsA in serotype 2a *S. flexneri* functions in a step in pathogenesis that has not yet been fully evaluated.

Experimental procedures

Bacterial strains, plasmids, cell lines and media

The bacterial strains and plasmids used in this study are listed in Table 2. *E. coli* strains were grown in L broth, and *S. flexneri* strains were grown in tryptic soy broth. Where appropriate,

antibiotics were added to the following final concentrations: ampicillin, 100 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1}; kanamycin, 30 μg ml^{-1}; and tetracycline, 12.5 μg ml^{-1}.

PtK2 (potoroo kidney epithelial) cells used for motility studies were maintained in Dulbecco's modified Eagle medium (DMEM, Gibco) with low glucose and 10% fetal calf serum. HeLa cells used for plaque assays and the labelling of actin tails and L2 cells used for plaque assays were maintained in minimal essential medium with 1% non-essential amino acids and 10% fetal calf serum. Caco-2 cells used for plaque assays were maintained in minimal essential medium with Earle's balanced salt solution, 1% non-essential amino acids and 20% fetal calf serum.

Construction of a genomic library of S. flexneri *wild-type strain 2457T*

A genomic library of *S. flexneri* DNA was constructed essentially as described previously (Balasubramanian *et al*., 1996). Genomic DNA from *S. flexneri* wild-type strain 2457T, which includes both chromosomal and virulence plasmid DNA, was partially digested with *Sau*3AI and was ligated to *Bam*HI arms of pYUB328, which had been previously prepared by digestion of pYUB328 with *Xba*I, dephosphorylation of the *Xba*I ends and digestion with *Bam*HI. The ligation mixture was packaged into lambda phage heads using the GigaPack Gold II packaging mix (Stratagene). The temperature-sensitive *E. coli* strain χ2764 was then infected with the lambda phage heads containing the ligation DNA, and colonies containing circularized cosmid vectors were isolated by selection on ampicillin and pooled. Subsequently, induction of phage was performed by heat shock after allowing bacteria to grow for one doubling time. The resultant phage lysate was used to introduce the cosmid library into *ompT*$^-$ *icsA*$^+$ *E. coli* strain MBG344 by transfection.

ELISA identification of S. flexneri *DNA containing IcsA cleavage activity*

A total of 192 individual colonies of the cosmid library in MBG344 were then screened by ELISA for increased levels of IcsA in the culture supernatant, as follows. Wells of 96-well microtitre plates that had been precoated with sodium bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$) were inoculated with individual colonies in 200 μl of L broth and were incubated overnight at 37°C. Bacteria were pelleted by centrifugation of the microtitre plate at 1000 × *g* for 20 min. Culture supernatant from each well (175 μl) was transferred to a well in a second precoated microtitre plate, and the second plate was incubated overnight at 4°C. Wells were then washed with washing solution [phosphate-buffered saline (PBS) containing 0.05% Tween 20] and blocked with blocking buffer (3% bovine serum albumin in PBS). Wells were incubated with affinity-purified IcsA antiserum (Goldberg *et al*., 1993) for 2 h at room temperature, washed with washing solution and then incubated with a 1:10 000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antiserum for 1 h at room temperature. Visualization was performed by adding to each well 50 μl of substrate, which is a solution of 0.4 mg ml^{-1} *o*-phenylenediamine dihydrochloride (Sigma) in 0.0514 M Na_2PO_4, 0.0243 M citric acid, pH 5.0, to which 1.2×10^{-4}% (v/v) H_2O_2 was added just before use. Relative cleavage of substrate was quantitated at 450 nm using a microtitre plate reader. Individual colonies with significantly elevated levels of substrate cleavage were selected for further evaluation.

Molecular and genetic techniques

Genetic procedures and DNA analysis, including DNA preparation, restriction enzyme digestion, agarose gel electrophoresis, ligation, bacterial transformation and conjugation, were performed according to standard procedures (Sambrook *et al*., 1989). DNA sequencing was performed using fluorescent cycle sequencing with dye-labelled terminators in an automated ABI377 sequencer (Perkin-Elmer, Foster City, CA, USA). Analysis of DNA sequence was performed using the Wisconsin Package software, Version 8 (Genetics Computer Group, Madison, WI, USA). Sequence data have been submitted to GenBank (accession number AF001633).

Subcloning of the portion of the *S. flexneri* genomic DNA within cosmid pKDS17 that contains IcsA cleavage activity was performed as follows. First, deletions were made within the genomic insert by digestion with various restriction enzymes and religation (Fig. 1A). Each of these deletion constructs was assayed for IcsA cleavage activity by transformation into MBG344, an *ompT*$^-$ *E. coli* strain carrying *icsA*, and evaluation for the presence or absence of cleaved IcsA in the culture supernatant by Western blot (Figs 1 and 2). Based on mapping of the various deletion constructs, the activity was localized to the DNA fragment between approximately 2.5 kb and 10 kb of the genomic insert in pKDS17. This was subcloned as a *Pst*I fragment in each orientation into the cloning vector pBC-KS$^+$ (Pharmacia), creating plasmids pMBG329 and pMBG331. Plasmids containing deletions within the genomic DNA in pMBG329 and pMBG331 were then constructed and assayed for IcsA cleavage activity following transformation into *ompT*$^-$ *icsA*$^+$ *E. coli* strain MBG345 (Figs 1 and 2). In this manner, the IcsA cleavage activity was localized to a 2.4 kb *Nhe*I–*Sma*I DNA fragment.

Targeted deletion of *icsP* on the virulence plasmid of *S. flexneri* wild-type strain 2457T was performed as follows. A 498-bp fragment internal to the coding sequence of *icsP* (extending from 123 bp to 620 bp of the open reading frame) was amplified by polymerase chain reaction, cloned into the ampicillin-resistant suicide vector pCVD442 (yielding plasmid pMBG344) and transformed into *E. coli* strain SY327λ*pir*. Thus, even if the methionine at amino acid position 12, and not that at position 1, is the true start codon, the amplified fragment lies completely within the open reading frame. pMBG344 was then transformed into *E. coli* strain SM10λ*pir* and subsequently introduced by conjugation into the tetracycline-resistant *S. flexneri* strain BS109. Transconjugants were selected by plating on ampicillin and tetracycline, and integration of the vector into *icsP* was verified by Southern blotting. The ampicillin-resistant locus was transduced into *S. flexneri* wild-type strain 2457T using P1L4 phage, and proper integration of the vector into *icsP* was again verified by Southern blotting.

Protein preparation and analysis

Whole cell and supernatant protein extracts were prepared as

described previously (Hovde *et al*., 1988; Allaoui *et al*., 1992 respectively). Western blots not used for scanning densitometry were performed using affinity-purified IcsA antiserum prepared as described previously (Goldberg *et al*., 1993) and enhanced chemiluminescence (Amersham). Western blots on which levels of protein expression were quantified by scanning densitometry were performed with the same primary antiserum and ^{125}I-conjugated Protein A as a secondary antibody to give a linear response. Scanning densitometry was performed with a Molecular Dynamics laser scanning densitometer using ImageQuant software.

Motility assays and surface labelling for IcsA

Time-lapse microscopic imaging and determinations of bacterial speed were performed on semiconfluent monolayers of PtK2 cells infected with *icsP* mutant strain MBG341 or wild-type strain 2457T, as described previously (Goldberg, 1997), except that images were recorded at one every 5 s for 5-min periods. Percentages of intracytoplasmic bacteria that were motile were determined as described previously (Goldberg *et al*., 1994). Surface labelling for IcsA was performed as described previously (Goldberg *et al*., 1993). Fluorescence microscopy was performed on an Olympus IX70 fluorescent microscope with a Photometrics CH1 cooled CCD camera, using Oncor Image software.

Plaque assays

Plaque assays to evaluate cell-to-cell spread of bacteria were performed as described previously (Oaks *et al*., 1985), with slight modification. Bacteria were placed onto confluent monolayers of HeLa, L2 or Caco-2 cells in 60-mm plastic Petri dishes at a multiplicity of infection of 2–5 bacteria per 1000 cells. The Petri dishes were centrifuged at $700 \times g$ for 10 min and incubated at 37°C for 80 min. The medium was then aspirated off the cells, an overlay of 6 ml of 0.5% agarose in DMEM containing 40 μg ml^{-1} gentamicin was placed on top of the cells and the monolayers were placed at 37°C. Monolayers were evaluated for bacterial plaque formation at 24 and 48 h. To assess for potential loss of the integrated suicide vector from *icsP* mutant strain MBG341 within plaques, bacteria were isolated from plaques at 48 h and evaluated for maintenance of ampicillin resistance. It was found that 100% of plaque isolates of MBG341 maintained ampicillin resistance, indicating that the integrated suicide vector had been maintained.

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