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IcsA, a polarly localized autotransporter with an atypical signal peptide, uses the Sec apparatus for secretion, although the Sec apparatus is circumferentially distributed

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Summary

Asymmetric localization of proteins is essential to many biological functions of bacteria. *Shigella* IcsA, an outer membrane protein, is localized to the old pole of the bacillus, where it mediates assembly of a polarized actin tail during infection of mammalian cells. Actin tail assembly provides the propulsive force for intracellular movement and intercellular dissemination. Localization of IcsA to the pole is independent of the amino-terminal signal peptide (Charles, M., Perez, M., Kobil, J.H., and Goldberg, M.B., 2001, *Proc Natl Acad Sci USA* 98: 9871–9876) suggesting that IcsA targeting occurs in the bacterial cytoplasm and that its secretion across the cytoplasmic membrane occurs only at the pole. Here, we characterize the mechanism by which IcsA is secreted across the cytoplasmic membrane. We present evidence that IcsA requires the SecA ATPase and the SecYEG membrane channel (translocon) for secretion. Our data suggest that YidC is not required for IcsA secretion. Furthermore, we show that polar localization of IcsA is independent of SecA. Finally, we demonstrate that while IcsA requires the SecYEG translocon for secretion, components of this apparatus are uniformly distributed within the membrane. Based on these data, we propose a model for coordinate polar targeting and secretion of IcsA at the bacterial pole.

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Introduction

Spatial organization within cells is fundamental to cellular processes of prokaryotes and eukaryotes. Work over the past decade has demonstrated that certain individual bacterial proteins, including secreted proteins, are localized to specific spatial sites within the bacterial cell. Among Gram-negative bacteria, secreted proteins that localize to the cell poles include the *Shigella* actin assembly protein IcsA, which localizes to the old pole (Goldberg *et al*., 1993), chemotaxis protein complexes, which localize to the cell poles in *Escherichia coli* and *Caulobacter crescentus* (Alley *et al*., 1992; Maddock and Shapiro, 1993), the cell cycle-associated histidine kinases CckA, PleC, and DivJ of *C. crescentus*, which are localized to the pole at specific stages of the cell cycle (Jacobs *et al*., 1999; Wheeler and Shapiro, 1999) and the polar localization factor PodJ of *C. crescentus*, which provides positional information to both PleC and a pilus secretion factor (Viollier *et al*., 2002). The mechanisms by which these proteins localize to their specific sites are incompletely understood, but necessarily involve coordination of targeting with secretion.

Gram-negative bacteria have evolved several mechanisms of secreting proteins across the cytoplasmic membrane, including the Sec protein translocation pathway and the signal recognition particle (SRP) pathway, both of which utilize the SecYEG complex. All substrates for the Sec protein translocation pathway and many for the SRP pathway contain cleavable amino-terminal secretion signals (signal peptides) (Driessen *et al*., 2001; Mori and Ito, 2001). The cytosolic SRP complex, which consists of the protein Ffh (an orthologue of the mammalian SRP component SRP54) and a small RNA (4.5S RNA), binds hydrophobic domains within the nascent polypeptide early during translation and brings it to the membrane receptor FtsY, which catalyses insertion of the nascent chain into the SecYEG translocon (Luirink *et al*., 1994; Valent *et al*., 1995; Powers and Walter, 1997; Seluanov and Bibi, 1997; Valent *et al*., 1998). Certain proteins, typically cytoplasmic membrane proteins with large periplasmic loops, require the cooperative activity of both SRP and SecA for trans-

location (Neumann-Haefelin *et al*., 2000; Scotti *et al*., 2000).

Secreted proteins that are also asymmetrically localized raise unique questions relating to how spatial targeting is coordinated with secretion. Does asymmetric localization occur prior to or after secretion? Is the secretion apparatus involved in the generation of asymmetry of the secreted protein? Is the secretion apparatus asymmetrically localized or does it contain specialized asymmetrically localized components that participate in asymmetric secretion? The spatial localization of components of the SecAB secretion apparatus (a.k.a. SecA apparatus) and of components of the SRP apparatus within the cytoplasmic membrane has not been determined.

The only known example of asymmetrically localized secretion components is YidC, a membrane protein that is required for the membrane insertion of at least some SRP-secreted proteins (Houben *et al*., 2000; Scotti *et al*., 2000; Beck *et al*., 2001; Urbanus *et al*., 2001). Localization studies of a YidC–GFP fusion showed enhanced fluorescence at both bacterial poles and a less intense signal throughout the membrane (Urbanus *et al*., 2002), suggesting that secretion of proteins that require YidC might preferentially occur at the cell poles. Nonetheless, FtsQ, a bitopic membrane protein that participates in formation of the cell division septum at mid-cell, requires both SRP and YidC for membrane insertion (Tian *et al*., 2000; Urbanus *et al*., 2001; Buddelmeijer and Beckwith, 2002; Tian and Beckwith, 2002), suggesting that not all YidC substrates are ultimately localized to the poles.

The *Shigella* actin assembly protein IcsA is an outer membrane protein that is localized to the bacterial old pole (Goldberg *et al*., 1993). We have previously shown that a derivative of IcsA that lacks a signal peptide continues to localize to the old pole (Charles *et al*., 2001). This observation suggests both that targeting of IcsA to the pole occurs prior to its secretion across the cytoplasmic membrane and that its secretion across the cytoplasmic membrane occurs only at the pole (Charles *et al*., 2001). We were interested in defining the mechanism of IcsA secretion across the cytoplasmic membrane and in determining whether the secretion apparatus utilized by IcsA is also asymmetrically localized in the cell. We present evidence that IcsA secretion is SecA and SecYEG dependent, yet its localization to the pole is Sec independent, and the SecYEG translocon is circumferentially distributed in the cytoplasmic membrane. These data suggest that the restriction of IcsA secretion to the pole is independent of the secretion apparatus.

Results and discussion

IcsA is a member of the autotransporter family of secreted proteins of Gram-negative bacteria (Henderson *et al*., 1998), designated 'autotransporters' because their translocation across the outer membrane is not absolutely dependent on accessory factors. The mechanism by which IcsA and other autotransporter proteins are translocated across the cytoplasmic membrane has not been examined. Like the signal peptide of many autotransporters, the IcsA signal peptide is atypical for those of proteins known to be secreted by the Sec apparatus in both its long length (52 residues) and its overall amino acid composition (Fig. 1A and B). Whereas residues 26–52 of the IcsA signal peptide are similar in charge and hydrophobicity profile to Sec-secreted proteins, residues 1–25 are not and have no known function.

IcsA secretion across the cytoplasmic membrane is dependent on SecA

To test whether IcsA secretion is dependent on SecA, we examined the translocation of IcsA to the bacterial surface after depletion of SecA following shift of a SecA temperature-sensitive strain to the non-permissive temperature (Fig. 2). IcsA is unique to *Shigella*. Yet, as, in terms of its

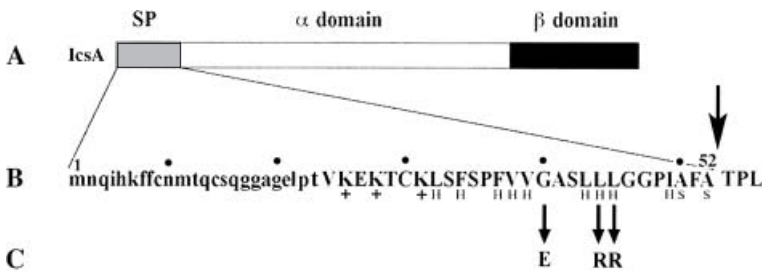

Fig. 1. IcsA signal peptide and domain organization.
A. Schematic representation of IcsA signal peptide (SP), α domain, which is exposed on the bacterial surface, and β domain, which mediates translocation of the α domain across the outer membrane.
B. Amino acid sequence of IcsA SP. Lower case lettering indicates stretch of residues that are atypical for Sec-secreted proteins. Upper case lettering indicates stretch of residues similar in charge and hydrophobicity profile to SPs of Sec-secreted proteins. +: positively charged residues; H: hydrophobic residues; S: small non-polar residues. Large arrow: signal peptidase cleavage site.
C. Site-directed mutations introduced into the hydrophobic core of the SP in this study (see text).

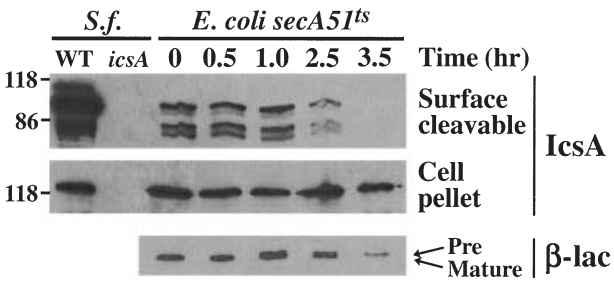

Fig. 2. IcsA secretion after SecA depletion. SecA was depleted by growth of *E. coli secA51*^{ts} at the non-permissive temperature (42°C). Western blot analysis of distribution of IcsA into a surface exposed fraction or the cell pellet after depletion of SecA, using antibody to IcsA or β-lactamase. Time indicated is hours of growth at the non-permissive temperature prior to harvesting cells. 0 h, cells harvested immediately prior to the shift to the non-permissive temperature. β-Lactamase blot is of cell pellets prepared from proteins grown in parallel. Pre: pre-protein; mature: mature protein; β-lac: β-lactamase. Surface cleavable IcsA was isolated by incubation of intact cells with proteinase K (see text). Apparent molecular masses of standard proteins run in parallel are indicated in kilodaltons at the left.

genetics, *Shigella* is felt to be an *Escherichia coli* (Pupo *et al*., 2000; Jin *et al*., 2002; Wei *et al*., 2002), and because all aspects of IcsA localization and secretion that have been studied are essentially identical in *Shigella* and *E. coli* (Suzuki *et al*., 1995; Sandlin and Maurelli, 1999; Charles *et al*., 2001), we performed these and certain other studies in *E. coli*. For these studies, *icsA* was expressed from the *icsA* promoter on a low-copy plasmid in *E. coli* MM52, which is *secA51*^{ts} (Oliver and Beckwith, 1981), and in which the gene encoding OmpT, an *E. coli* surface protease that cleaves surface-localized IcsA, had been disrupted. The SecA51^{ts} protein is competent for protein secretion at 30°C (the permissive temperature) but not at 42°C (the non-permissive temperature) (Oliver and Beckwith, 1981).

We assessed IcsA secretion in IcsA^{+} *E. coli secA51*^{ts} *ompT* by examining relative amounts of IcsA that were localized to the bacterial surface. Cells were grown to mid-log at the permissive temperature and then shifted to the non-permissive temperature for predetermined lengths of time. To be certain that IcsA localized on the bacterial surface at the time of harvest represented predominantly protein that had been secreted during growth at the non-permissive temperature, 30–90 min prior to harvesting each sample, intact bacteria were pre-treated with proteinase K to proteolytically remove IcsA expressed on the surface and then were resuspended in fresh media (*Experimental procedures*). At the predetermined times, newly secreted IcsA exposed on the surface was removed by treatment with proteinase K, after which proteins recovered from the culture supernatant and the cell pellet were analysed by Western blot analysis (Fig. 2). IcsA was expressed on the surface of *E. coli secA51*^{ts} *ompT* up to 1 h after the shift to the non-permissive temperature in an amount approximately equivalent to that on the surface of the same strain during growth at the permissive temperature (Fig. 2, top panel). However, at 2.5 h after the shift to the non-permissive temperature, the amount of IcsA exposed on the surface was markedly reduced, and at 3.5 h after the shift to the non-permissive temperature, there was no discernible IcsA exposed on the cell surface. Growth of the parent strain, which carries the wild-type *secA* allele, at 42°C did not lead to a reduction in IcsA secretion (data not shown). β-Lactamase, which requires SecA for secretion, was used as a control for SecA depletion: at 1.0, 2.5, and 3.5 h after temperature shift, the ratio of pre-protein to mature β-lactamase progressively increased (Fig. 2, bottom panel), consistent with progressive depletion of SecA. To confirm that the profile of IcsA seen in the culture supernatant was not a function of protein degradation within the cell, we analysed the amount of IcsA in the cell pellets (Fig. 2, middle panel) and found that it was similar at all times under both permissive and non-permissive conditions. These data suggest that IcsA secretion is dependent on SecA.

IcsA secretion across the cytoplasmic membrane is dependent on SecY

Essentially all proteins secreted by the SecAB pathway and many proteins secreted by the SRP pathway traverse the cytoplasmic membrane by passage through a channel, the core of which consists of a heterometric complex formed by SecYEG. To examine whether IcsA secretion might require the SecYEG translocon, we employed a genetic approach based on the observation that certain mutations in the signal peptides of proteins normally dependent on SecY for translocation lead to loss of translocation in the presence of wild-type SecY, but permit rescue of translocation in strains carrying the *prlA4* mutant allele of *secY* (Bankaitis and Bassford, 1985; Prinz *et al*., 1996). We reasoned that if IcsA requires the SecYEG translocon for secretion, the IcsA derivatives that contain these signal peptide mutations might be incompetent for secretion across the cytoplasmic membrane in the *prlA* wild-type (WT) background, but their secretion might then be restored in the *prlA4* suppressor strain (Prinz *et al*., 1996). We separately replaced each of three uncharged amino acid residues present in the hydrophobic core of the IcsA signal peptide with charged, hydrophilic amino acid residues: G40E, L44R and L45R (Fig. 1C). We then tested whether IcsA derivatives that contain mutant signal peptides (Fig. 1C) are present or absent on the bacterial surface in a *prlA* wild type (*secY* wild type) or *prlA4* suppressor background.

As determined by indirect immunofluorescence of intact

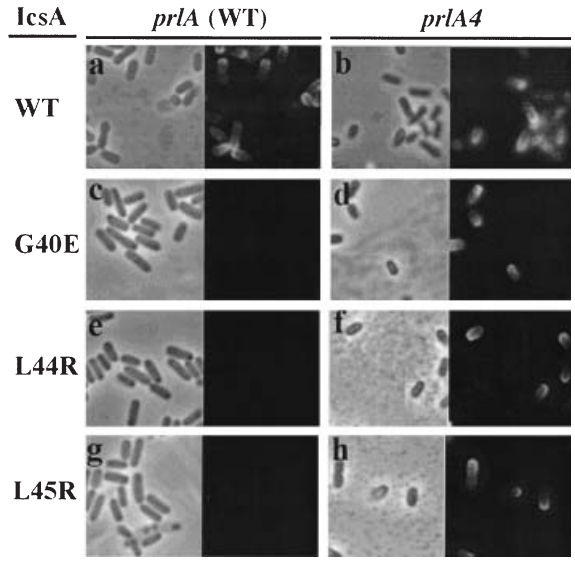

Fig. 3. Secretion of IcsA in *prlA4* background. IcsA detected on the surface of intact bacteria that carry the *prlA* or *prlA4* allele of *secY*. IcsA signal peptide derivative is indicated at the left. Indirect immunofluorescence (right panels) and phase (left panels) micrographs. IcsA derivative: WT, wild-type; G40E, L44R and L45R, signal peptide mutant derivatives.

bacteria, in *prlA* WT *Shigella*, the IcsA signal peptide mutant proteins were undetectable on the bacterial surface, whereas wild-type IcsA was present (Fig. 3, left panels). In contrast, in the *prlA4* suppressor background, surface localization of the IcsA signal peptide mutant proteins was restored (Fig. 3, right panels). Levels of expression of the IcsA signal peptide mutant proteins were similar to the level of expression of wild-type IcsA (data not shown), indicating that the observed differences in amounts of the IcsA signal peptide mutant proteins on the bacterial surface was not due to differential expression of the proteins.

Because a portion of secreted IcsA is cleaved from the bacterial surface by the *Shigella*-specific IcsA protease (IcsP), the presence of a 94 kDa cleaved IcsA polypeptide in the culture supernatant serves as an indication of IcsA secretion. Therefore, we examined the amount of the IcsA signal peptide mutant proteins in the culture supernatant in each strain background by Western blot analysis. In the *prlA* wild-type background, the amount of the cleaved portion of the IcsA signal peptide mutant proteins found in the culture supernatant was reduced compared with that of wild-type IcsA expressed in the same background, whereas in the *prlA4* suppressor strain, the amount of the cleaved portion of the IcsA signal peptide mutant proteins found in the supernatant fraction was restored to wild-type levels (data not shown). Taken together, these data are consistent with IcsA secretion across the cytoplasmic membrane being dependent on SecY, an essential component of the SecYEG translocon, as is the case for other proteins whose secretion is SecA dependent. However, we point out that *prlA* mutants are capable of exporting Sec-dependent proteins that are completely missing their signal sequences (Prinz *et al*., 1996). Therefore, it is conceivable that proteins ordinarily exported by another pathway can be handled by a *prlA*-altered translocon when their signal sequences are defective, although no such instance has been reported thus far.

IcsA secretion appears to be independent of specific components of the SRP pathway

Because certain proteins require the cooperative activity of SRP and SecA for secretion across the cytoplasmic membrane (Neumann-Haefelin *et al*., 2000; Scotti *et al*., 2000), we examined in parallel whether IcsA secretion was dependent on components of the SRP secretion apparatus. IcsA was expressed in *E. coli* strains carrying a point mutation in either of two genes that encode components of the SRP secretion apparatus, *ftsY* or *ffh* (mutant alleles *ftsY70* and *ffh-77* respectively) (Tian and Beckwith, 2002). Of note, because these strains express the *E. coli* surface protease OmpT, which cleaves IcsA, thereby releasing a 94 kDa fragment into the culture supernatant (Goldberg *et al*., 1993 and M. B. Goldberg, unpubl. data), secretion of IcsA can be assessed by analysis of cleaved IcsA recovered from the culture supernatant. For *ftsY70* and *ffh-77 E. coli* and the parent strain, the amount of IcsA recovered from the culture supernatant, the absence of IcsA associated with the cell pellet and the overall level of IcsA expression were similar, suggesting that the *ftsY70* and *ffh-77* mutations had minimal or no effect on IcsA secretion (Fig. 4). The presence of the mutations in the strains was verified either by DNA sequencing (*ftsY70*) or examining the effect on subcellular localization of a *malF–lacZ* fusion (*ffh-77*) (data not shown), which is known to require SRP for membrane insertion (Tian *et al*., 2000).

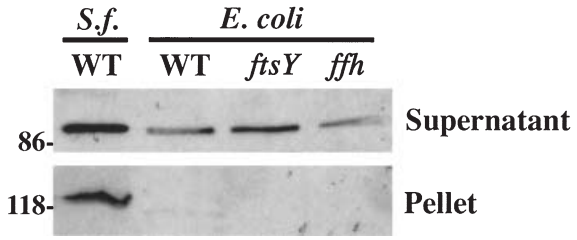

Fig. 4. Secretion of IcsA in SRP mutant *E. coli*. Secretion of IcsA into the culture supernatant in wild-type (WT) or SRP mutant *ftsY* or *ffh*. Western blot analysis of IcsA in culture supernatant (top panel) and cell pellet (bottom panel) using antibody to IcsA. *S. f.*: *Shigella flexneri* control. Apparent molecular masses of standard proteins run in parallel are indicated in kilodaltons at the left.

While these results suggest that IcsA does not use the SRP pathway, they should be interpreted with some caution, as the *ffh* and *ftsY* mutations used in this study have relatively weak effects on proteins exported by the SRP pathway. Although these data indicate that IcsA does not absolutely require FtsY or Ffh for secretion, they do not eliminate the possibility that IcsA could utilize these or other SRP components for secretion under certain conditions.

IcsA secretion across the cytoplasmic membrane is independent of the SRP-associated secretion protein YidC

YidC, a cytoplasmic membrane protein, is required for the membrane insertion of at least some SRP-secreted proteins (Houben *et al*., 2000; Scotti *et al*., 2000; Beck *et al*., 2001; Urbanus *et al*., 2001). Whether YidC is involved in the secretion of any SRP-independent proteins is unknown. Recent localization studies of a YidC–GFP fusion showed enhanced fluorescence at both bacterial poles (Urbanus *et al*., 2002), suggesting that secretion of proteins that require YidC might preferentially occur at the cell poles. Although the data presented above indicates that FtsY and Ffh are not absolutely required for IcsA secretion, as IcsA secretion across the cytoplasmic membrane appears to occur at the pole (Charles *et al*., 2001), we sought to directly test whether YidC might be required.

We examined IcsA secretion after YidC depletion using an OmpT$^+$ *E. coli* strain that carries a chromosomally integrated copy of *yidC* under the control of the arabinose promoter and a disruption of native *yidC* (Samuelson *et al*., 2000), thereby allowing YidC depletion by growth in the absence of L-arabinose. As with the *ftsY70* and *ffh-77* strains, we assessed IcsA secretion by analysis of cleaved IcsA recovered from the culture supernatant. Because *yidC* is essential, YidC depletion leads to a decrease in growth rate (Samuelson *et al*., 2000). To control for cell density effects on IcsA expression and secretion (Goldberg *et al*., 1994), the arabinose-containing YidC replete culture was harvested at approximately the same OD_{600} as the glucose-containing YidC depleted culture. To be certain that IcsA localized on the bacterial surface at the time of harvest represented protein that had been secreted only after significant depletion of YidC, 1 h prior to harvesting each sample, supernatant proteins, which would include secreted IcsA, were removed by centrifugation and washing of the cells. We found that the amount of IcsA recovered from the culture supernatant was similar in the YidC replete and YidC depleted cells (Fig. 5A, top panel). Moreover, no detectable IcsA was associated with the cell pellets under either condition (Fig. 5A, middle panel), and the overall level of IcsA expression was similar for both conditions. YidC depletion of cells grown in glucose was verified by Western blot analysis of cell pellets for YidC (Fig. 5A, bottom panel). These data indicate that IcsA secretion is not absolutely dependent on YidC. Thus, if YidC proves to be essential to the polar secretion of certain polarly localized proteins, it is clearly not the only mechanism by which proteins can be secreted at the pole.

Whereas IcsA secretion is not absolutely dependent on YidC, it remains possible that YidC is involved in the localization of IcsA to the pole in association with its secretion. To test this, we examined in YidC replete and YidC depleted cells the localization of a fragment of IcsA ($IcsA_{1\text{-}757}$), which we have previously shown localizes a GFP fusion to the pole (Charles *et al*., 2001). $IcsA_{1\text{-}757}$-GFP localized to the poles similarly in YidC-replete and YidC-depleted cells (Fig. 5B), indicating that IcsA does not directly require YidC for its localization to the pole. The observed elongated morphology of the YidC-depleted cells likely reflects inhibition of cell division as a result of inefficient membrane insertion of the cell division protein FtsQ, which has been shown to be a YidC-dependent process (Urbanus *et al*., 2001).

Taken together, these data demonstrate that, despite its unusual signal peptide, IcsA requires SecA, the ATPase that drives the movement of substrate proteins across the

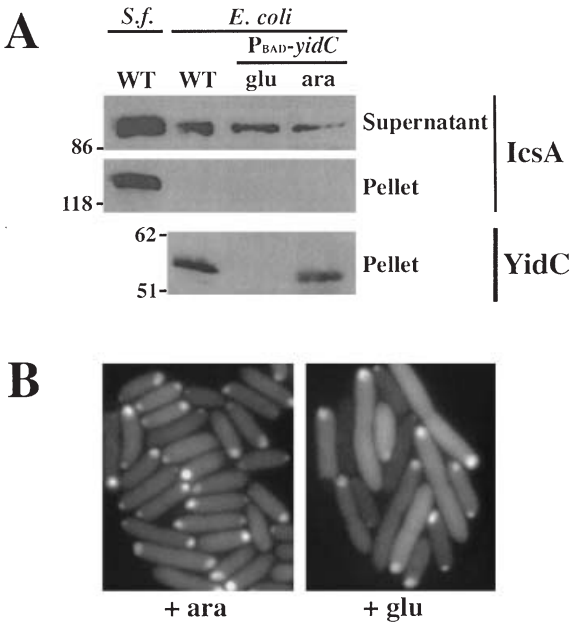

Fig. 5. IcsA secretion and polar localization after YidC depletion.
A. Secretion of IcsA into the culture supernatant after depletion of YidC by growth in the absence of arabinose. Western blot analysis of IcsA in culture supernatant (top panel) and cell pellet (middle panel), using antibody to IcsA, and YidC in cell pellet (bottom panel), using antibody to YidC. *S. f.*: *S. flexneri* wild-type (WT). P_{BAD}-*yidC*, YidC depletion strain JS7131. glu, YidC depleted cells; ara, YidC replete cells. Apparent molecular masses of standard proteins run in parallel are indicated in kilodaltons at the left.
B. Distribution of $IcsA_{1\text{-}757}$-GFP in YidC replete (+ara) or YidC depleted (+glu) cells. Fluorescence micrographs of live cells.

cytoplasmic membrane, for secretion (Fig. 2). These data also suggest that IcsA secretion is dependent on SecY (Fig. 3), which together with SecE and SecG, forms the cytoplasmic membrane channel (translocon) through which SecA assists in translocaton of substrate proteins. These data suggest that other autotransporters with atypical signal peptides probably use the SecAB pathway for secretion.

The Sec machinery is distributed in the cytoplasmic membrane in a circumferential fashion

A derivative of IcsA that lacks a signal peptide continues to localize to the old pole (Charles *et al*., 2001), suggesting that IcsA localization to the pole may occur in the absence of engagement of the secretion apparatus and prior to secretion. Secretion of IcsA at the pole might occur either by utilization of a secretion apparatus that is localized to the pole or that contains certain components which are localized to the pole, or by restricting IcsA engagement of a uniformly distributed secretion apparatus to polar sites. Having demonstrated that IcsA requires the Sec apparatus for secretion, we were interested in defining its spatial distribution. To address this, we generated and expressed translational fusions of SecY–GFP and GFP–SecE from chromosomal integrants and examined the distribution of each fusion protein *in vivo*.

To generate the translational SecY–GFP fusion construct, we fused the entire coding sequence of *secY* in frame to that of *gfp*, thereby placing GFP at the extreme carboxy terminus of SecY. When expressed from a plasmid, this fusion protein complemented the growth defect of *E. coli* MC4100 *secY24*^{ts} (Baba *et al*., 1994) at the non-permissive temperature (data not shown), suggesting that protein expressed from the *secY*::*gfp* fusion protein is functional. To generate a *secY*::*gfp* fusion that would be expressed at native levels, the *secY*::*gfp* fusion and a downstream *aph* cassette was introduced by double allelic exchange onto the chromosome of *E. coli* replacing native *secY* (*Experimental procedures*) (Yu *et al*., 2000). That the fusion was integrated into the correct locus was confirmed by PCR analysis (data not shown). Growth of the strain was similar to that of the wild-type parent strain, again suggesting that the SecY–GFP fusion is functional.

To assess the relative abundance of breakdown products that might be responsible for complementation, we performed Western blot analysis of whole cell lysates from a *secY* wild-type strain (MC4100), the temperature sensitive *secY24*^{ts} strain after growth at the permissive and non-permissive temperatures, and the chromosomal *secY*::*gfp* integrant (Fig. 6A and B). Antibodies to SecY revealed a protein migrating at 50 kDa that was present in the wild-type strain and temperature-sensitive strain after growth at the permissive temperature, but only weakly present in the temperature-sensitive strain after growth at the non-permissive temperature and absent in the chromosomal *secY*::*gfp* integrant (Fig. 6A), indicating lack of full-length native SecY in the *secY*::*gfp* integrant. The SecY–GFP fusion protein was detected as an 86 kDa protein by both antibody to SecY (Fig. 6A) and antibody to GFP (Fig. 6B), without significant breakdown. These results indicate that the full-length fusion is probably the dominant species responsible for the complementation of the *secY* deletion and represents the vast majority, if not all, of the observed GFP signal.

We examined the spatial distribution of SecY–GFP protein expressed from the native promoter of the chromosomally integrated *secY::gfp* in live bacteria. The GFP signal was distributed uniformly and circumferentially, in a pattern consistent with membrane localization (Fig. 6C).

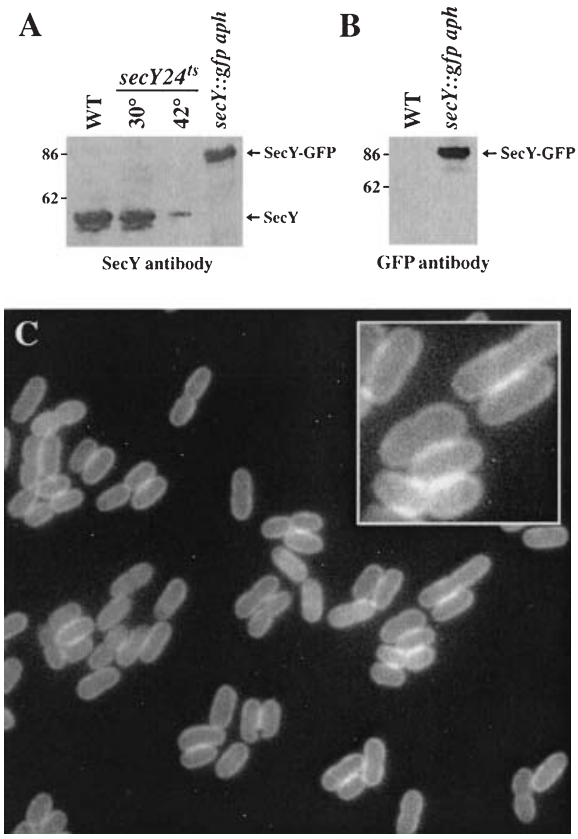

Fig. 6. Characterization and localization of SecY–GFP fusion.
A. Expression of SecY and SecY–GFP in membrane preparations of the wild-type strain MC4100 (WT), the temperature sensitive *secY24*^{ts} strain at the permissive (30°C) and non-permissive (42°C) temperatures, and the chromosomal *secY::gfp* integrant (*secY::gfp aph*). Western blot using antibody to SecY.
B. Expression of SecY–GFP in membrane preparations of the chromosomal *secY::gfp* integrant (*secY::gfp aph*). Western blot using antibody to GFP. Apparent molecular masses of standard proteins run in parallel are indicated in kilodaltons at the left (A and B).
C. Distribution of SecY–GFP expressed from chromosomal *secY::gfp* integrant. Fluorescence micrographs of live cells. Inset, higher magnification of same.

This suggests that the SecYEG translocon is distributed circumferentially and is not enhanced at the bacterial pole or mid-cell.

In parallel, we examined the distribution of SecE, another essential component of the SecYEG translocon, using a translational GFP–SecE fusion in which GFP is fused to the extreme N-terminus of SecE. The *gfp*::*secE* fusion was expressed under the control of a partly disabled *pTrc* promoter [pDSW204 (Weiss *et al*., 1999)]. This plasmid was introduced in single copy into PS288, a *secE* deletion strain complemented for SecE function from the high-copy plasmid pJS65 (see *Experimental procedures*). Complementation was initially verified by testing the ability of the resulting strain to segregate the plasmid in the presence but not absence of 1 mM IPTG (data not shown). Additionally, we assessed function of the fusion protein by complementation for growth in a strain, NWG107, which had lost pJS65. Induction of the fusion with 25 or 100 μM IPTG complemented growth to wild-type rates, but induction with 10 μM IPTG led to slower than wild-type growth rates. In the absence of IPTG, the strain was unable to grow (data not shown). These complementation results suggested that the GFP–SecE fusion protein is functional, although it remained possible that a breakdown product of the fusion, rather than the full-length fusion, was responsible for complementation.

To assess the relative abundance of breakdown products that might be responsible for complementation, we performed Western blot analysis of whole cell lysates from a *secE* wild-type strain and NWG107, a *secE* deletion strain carrying the *gfp*::*secE* fusion (Fig. 7A). Antibodies recognizing the essential C-terminal third of SecE (Schatz *et al*., 1991) revealed a protein migrating at 17 kDa that was present in the wild-type strain, but absent from NWG107 (Fig. 7A, left panel), indicating the lack of full-length SecE protein in NWG107. The GFP–SecE fusion protein was detected as a 44 kDa band on Western blots probed with anti-GFP antibodies (Fig. 7A, right panel). Expression of the fusion protein was induced in a dose-dependent manner by incubation in the presence of IPTG. Unfortunately, the fusion could not be seen on SecE blots due to the presence of a prominent non-specific band at the same molecular weight. The only significant breakdown product was a band at 28 kDa that was detected by both SecE and GFP antibodies. Although it is theoretically possible that this breakdown product alone is responsible for complementation, it is present at a very low level compared with the levels of both wild-type SecE and the GFP–SecE fusion protein. These results indicate to us that the full-length fusion is most probably the dominant species responsible for the complementation of the *secE* deletion and represents the vast majority, if not all, of the observed GFP signal.

We examined the spatial distribution of GFP–SecE in bacteria (Fig. 7B). The chromosomally integrated *gfp*::*secE* was induced with 0, 10, 25 or 100 μM IPTG for 1 h at 37°C, at which time the cells were fixed. At each level of induction, the GFP signal was uniform and circumferential (Fig. 7B), in a pattern consistent with membrane localization. The intensity of the signal increased with increasing concentrations of IPTG, but the distribution appeared the same for all concentrations. In the absence of induction, only a faint diffuse signal, consistent with low levels of bacterial autofluorescence, was detected (Fig. 7B, 0 μM IPTG). The distribution of GFP–SecE was indistinguishable from that of SecY-GFP (Fig. 6C), consistent with the presence of the described SecYEG heterotrimeric complex. Since the signal from GFP alone does not generate a membrane pattern (data not shown), the localization of the GFP signal generated in the *secY*::*gfp* strain and *gfp*::*secE* strain to the membrane necessarily indicates that the membrane-associated GFP signals represent fusion protein. We believe that the localization of

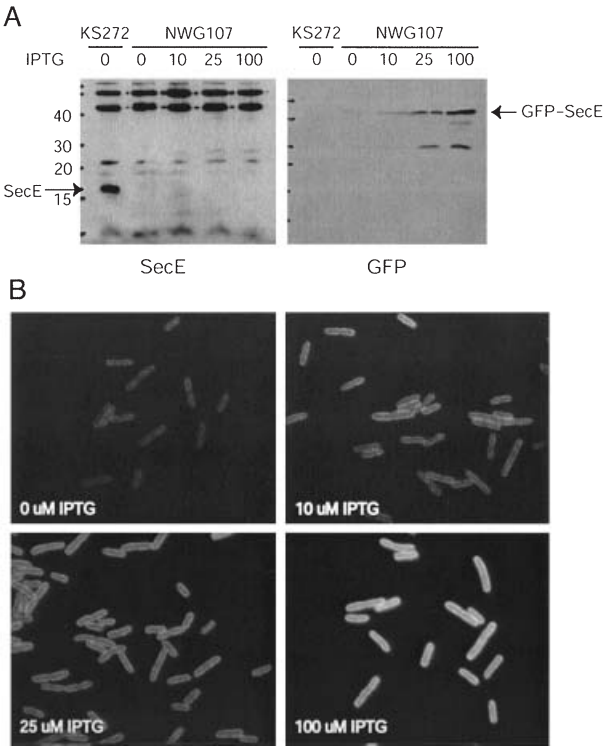

Fig. 7. Characterization and localization of GFP–SecE fusion.
A. Expression of SecE and GFP–SecE from the wild-type strain (KS272) and the *secE* deletion strain carrying the *gfp::secE* fusion (NWG107). Concentration of IPTG (in uM) used during induction of *gfp::secE* in NWG107 is indicated above the lanes. Apparent molecular masses of standard proteins run in parallel are indicated in kilodaltons at the left.
B. Distribution of GFP–SecE in the *secE* deletion strain carrying the *gfp::secE* fusion (NWG107) after growth in various concentrations of IPTG. Fluorescence micrographs of fixed cells. The exposure time was 500 ms for all images.

the SecY-GFP and the GFP–SecE reflect those of native SecY and native SecE respectively.

We have not directly examined the distribution of SecG, the third component of the SecYEG complex, but it is reasonable to assume that SecG is has the same circumferential distribution as SecY and SecE. Nor have we examined the localization of SecD, SecF or YajC, integral membrane protein components of the secretion apparatus that control insertion and de-insertion of SecA into the translocon (Duong and Wickner, 1997). Because certain solubilization procedures lead to the isolation of an intact SecYEGDFYajC complex (Duong *et al*., 1997), we predict that the distribution of SecD, SecF and YajC will be identical to that of SecY and SecE. Finally, SecA, which engages SecY during translocation of substrate proteins (Eichler *et al*., 1997; Matsumoto *et al*., 1997; Ramamurthy and Oliver, 1997; van der Does *et al*., 1998), is found both associated with the membrane fraction and in the cytosol (Cabelli *et al*., 1991; Chun and Randall, 1994). We expect that the membrane-associated portion of SecA will have the same distribution as that we have observed here for SecY and SecE.

In conjunction with SecA, the SecYEG translocon mediates the secretion of a large number of proteins. Whereas most substrates of this secretion apparatus are known or thought to be circumferentially distributed in the membrane or periplasm, certain substrates are known to be asymmetrically distributed. Within the latter category are components of the type II secretion system of Gram-negative bacteria (Pugsley *et al*., 1990; Sandkvist, 2001; Scott *et al*., 2001), the chemosensory receptor Tsr (Gebert *et al*., 1988; Maddock and Shapiro, 1993) and now IcsA, all of which localize to the cell pole. On the other hand, at least one substrate of SRP and YidC, FtsQ, localizes to the septum (Tian *et al*., 2000; Urbanus *et al*., 2001; Tian and Beckwith, 2002).

As described above, our published data suggest that secretion of IcsA across the cytoplasmic membrane occurs at the pole (Charles *et al*., 2001). Both an amino-terminal region of IcsA (residues 1–104) that includes the signal peptide and a distinct internal region of IcsA (residues 506–620) that lacks a signal peptide localize to the bacterial pole (Charles *et al*., 2001). We visualized both SecY and the amino-terminal region of IcsA (residues 1–104) in single cells, by tagging each with a different, yet compatible, fluorophore. SecY was fused to YFP in pLDB435, and the amino terminal region of IcsA was fused to enhanced CFP (ECFP) (*Experimental procedures*). Visualization in live bacteria of both fluorophores demonstrated uniform and circumferential localization of the YFP signal (SecY) and polar localization of the ECFP signal (IcsA) (Fig. 8). Taken together, our data suggest that IcsA secretion occurs at the pole via a secretion apparatus that is not polar, but rather is circumferentially distributed in the membrane.

Polar localization of IcsA is independent of SecA

The observation that a derivative of IcsA that lacks a signal peptide localizes to the pole suggests that IcsA localizes to the pole prior to secretion (Charles *et al*., 2001). As a corollary to this, we propose that IcsA engages the Sec apparatus only after it has localized to the pole. If this proposal is correct, then even in the absence of SecA or the SecYEG translocon, IcsA would accumulate at the pole. We tested the dependence of IcsA polar localization on components of the Sec apparatus by examining the distribution of IcsA–GFP fusions that were expressed after depletion of SecA or SecY.

In these studies, we examined the localization of a GFP fusion to either (i) the amino-terminal region of IcsA (residues 1–104), which includes the signal peptide and is sufficient to localize a GFP fusion to the pole, or (ii) the signal peptide and entire α domain of IcsA (residues 1–757), which contains both regions of IcsA that are able to localize a GFP fusion to the pole (Charles *et al*., 2001). The localization of IcsA–GFP fusions was determined in SecA and SecY temperature-sensitive strains [MM52, which is *secA51*^{ts} (Oliver and Beckwith, 1981), and MPH107, which is *secY24*^{ts} (Baba *et al*., 1994)] and the wild-type strain MC4100 after shift to the non-permissive temperature (Fig. 9). For these studies, expression of the *icsA*::*gfp* fusions was from the arabinose promoter and was induced after 0, 1.0, 2.5 or 3.5 h of growth at the non-permissive temperature. Each IcsA–GFP fusion localized to the cell pole in each Sec depletion strain at all times after shift to the non-permissive temperature in a pattern identical to its polar localization in the wild-type strain

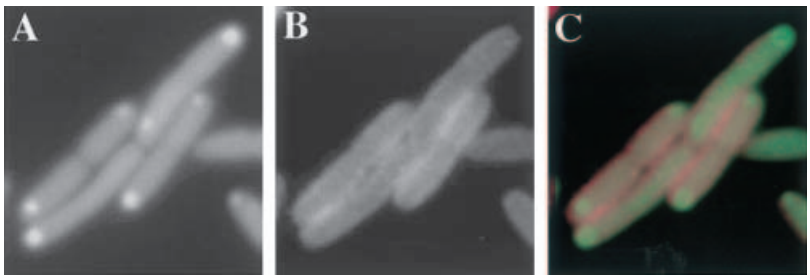

Fig. 8. Localization of SecY and IcsA. Distribution of IcsA$_{1\text{-}104}$-ECFP (A) and SecY-YFP (B). Fluorescence micrographs of live cells. (C) Overlay of images in panels (A) and (B), after pseudo-coloration of ECFP to green and YFP to red in Adobe Photoshop.

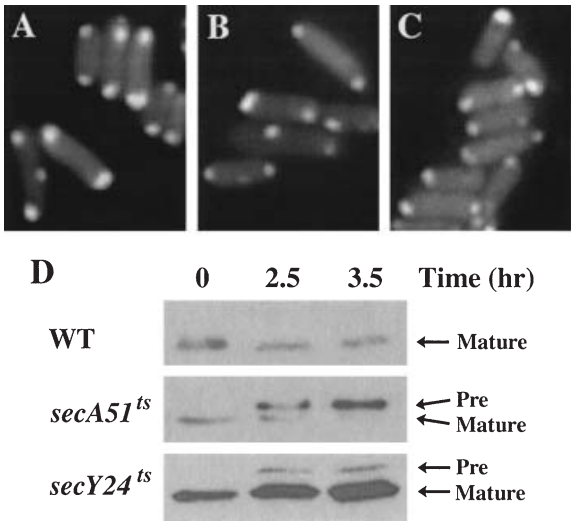

Fig. 9. IcsA localization in SecA- or SecY-depleted cells.
A–C. Localization of $IcsA_{1-104}$-GFP after 3.5 h of growth at the non-permissive temperature (42°C) in the wild-type strain MC4100 (A), the temperature sensitive *secA51*^{ts} strain (B) and the temperature sensitive *secY24*^{ts} strain (C).
D. Western blot analysis using antibody to β-lactamase. Time indicated is hours of growth at the non-permissive temperature prior to inducing IcsA-GFP expression. 0 h, cells in which IcsA-GFP induction was performed prior to the shift to the non-permissive temperature. Pre: pre-protein; mature, mature protein. Localization of $IcsA_{1-757}$-GFP was indistinguishable from localization of $IcsA_{1-104}$-GFP in each strain under each condition (data not shown).

either after shift to the non-permissive temperature or during growth at the permissive temperature (Fig. 9A–C, and data not shown). β-Lactamase, which requires SecA and SecY for secretion, was used as a control for SecA or SecY depletion. In the SecA depletion strain, the ratio of pre-protein to mature β-lactamase increased with growth at the non-permissive temperature (Fig. 9D, and data not shown), consistent with significant depletion of SecA. In the SecY-depletion strain, the shift from pre-protein to mature β-lactamase was only partial, suggesting that functional SecY was present even after 3.5 h of depletion. Thus, whereas these data suggest that SecA is not required for polar localization of IcsA, they are inconclusive about the requirement for SecY. However, as in the absence of functional SecA, nascent polypeptides are unable to engage the SecYEG translocon, polar localization of IcsA–GFP in the SecA depletion strain is also consistent with SecY not being required for localization of IcsA to the pole. In the context of other data presented in this paper, these results suggest that IcsA localization to the pole is independent of its interaction with SecA, as well as perhaps with all components of the secretion apparatus, and is independent of its secretion.

The mechanisms of asymmetric localization of secreted proteins in bacteria remain incompletely understood. Compelling models for membrane insertion, followed by diffusion and subsequent capture of a protein at the pole in *Bacillus subtilis* (Rudner *et al*., 2002) or for direct secretion via a polar Type II secretion apparatus in *Vibrio cholerae* (Scott *et al*., 2001) have been presented. In the first case, asymmetric spatial localization of the membrane protein occurs after membrane insertion: SpoIVFB is inserted into the *B. subtilis* membrane, diffuses in the membrane and is captured in the asymmetric engulfing septal membrane by binding to SpoIVFA, which is restricted to the septal membrane (Rudner and Losick, 2002; Rudner *et al*., 2002). In the second case, the *V. cholerae* cytoplasmic membrane type II secretion component EpsM is localized to the old pole, suggesting that secretion of type II substrates occurs at the old pole (Scott *et al*., 2001).

Data presented here indicate that polar localization of IcsA occurs independent of SecA (Fig. 9B and D), which in conjunction with previously published data (Charles *et al*., 2001), suggest that polar localization of IcsA is independent of its secretion. Thus, it appears that at least three distinct paradigms exist for asymmetric localization of secreted proteins: (i) insertion into the membrane at any site, followed by diffusion and capture at an asymmetric site; (ii) secretion via an asymmetrically distributed secretion apparatus, exemplified by *V. cholerae* type II secretion substrates; and (iii) directed secretion at an asymmetric site via a circumferentially distributed secretion apparatus, exemplified by *Shigella* IcsA. Although the distribution of most components of the SRP apparatus has not yet been determined, the polarized distribution of YidC (Urbanus *et al*., 2002) suggests that a fourth paradigm, in which one or more specialized components of a circumferentially distributed secretion apparatus is required for the asymmetric secretion of a subset of substrates, may also exist.

We are left with the question of how IcsA is secreted exclusively at the old pole of the bacillus when the secretion apparatus it utilizes is found throughout the cytoplasmic membrane. Based on our data, we present a working model for this intriguing question (Fig. 10). We have shown previously that IcsA residues 1–104, which include the signal peptide, are sufficient to localize a GFP fusion to the pole (Charles *et al*., 2001), suggesting that within this region is a domain that interacts with an as yet unknown structure at the pole. We propose that, in the context of IcsA pre-protein, the IcsA signal peptide has two conformations: one that is able to participate in the recognition of a polar structure but unable to engage the secretion apparatus, and a second that is able to engage the secretion apparatus. We hypothesize that, in the cytoplasm, IcsA pre-protein maintains the first conformation, but that upon binding to the polar structure, it undergoes a conformational switch to the second conformation. Thus, IcsA pre-protein would be unable to engage the secretion

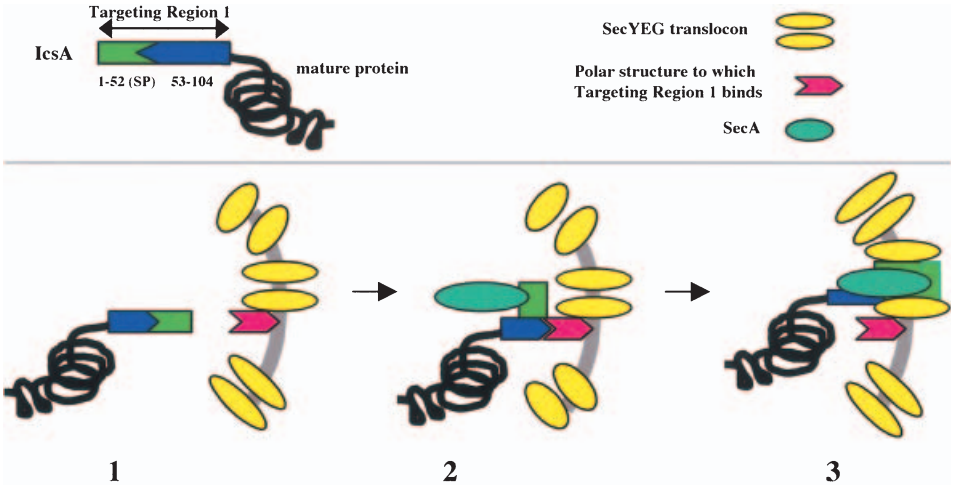

Fig. 10. Proposed mechanism of coordination of IcsA secretion with its polar localization. 1, In the cytoplasm, the amino terminus of IcsA preprotein, which contains the SP within Targeting Region 1, is in a conformation that blocks interaction with the Sec secretion apparatus. 2, Upon binding to a polar target (red hexagon), the conformation of the IcsA amino terminus changes, such that the hydrophobic region within the SP (green bar) is able to interact with SecA (turquoise oval). 3, SecA then guides IcsA into the SecYEG translocon (paired yellow ovals).

apparatus until it has bound the polar structure. The role of the second IcsA Targeting Region (residues 506–620) in coordinating IcsA polar localization with secretion is unclear. We have previously proposed a model in which IcsA Targeting Region 1 (residues 1–104) functions to establish polarity of the nascent protein and Targeting Region 2 maintains polarity (Charles *et al*., 2001), which is consistent with the model proposed here. In an alternative model, the affinity of IcsA for the polar structure would be sufficiently high so as to preclude significant interactions with the secretion apparatus except at the pole. Future experiments will distinguish between these models. Whether other asymmetrically distributed Sec substrates restrict their secretion to the pole or utilize similar mechanisms is currently unknown.

Experimental procedures

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Strains were constructed using standard molecular and genetic techniques (Sambrook *et al*., 1989). *S. flexneri* strains were grown in tryptic soy broth (TCS) and *E. coli* strains in Luria–Bertaini (LB) broth, NZY media or M9 minimal media (Miller, 1992). NZY differs from LB in that tryptone is replaced by NZ amine A (Quest International) and the NaCl concentration is 8 g l^{-1} (Miller, 1992). *Shigella* strains carrying plasmids expressing P_{BAD}-*icsA* (pMBG472) and P_{BAD}-*icsA* containing site-directed mutations in *icsA (*pLDB414, pLDB416 and pLDB417) were grown in TCS supplemented with 0.2% glycerol with or without 0.2% L-arabinose as described (Steinhauer *et al*., 1999). *E. coli* strains expressing *secY*::*gfp*, *secY*::*yfp* or *icsA*::*ecfp* were grown in M9 media containing 0.2% glucose or 0.2% glycerol as described (Charles *et al*., 2001), supplemented or not with 0.2% L-arabinose. The *E. coli* strain expressing *pTrc gfp*::*secE* was grown in NZY media, supplemented or not with IPTG. Where appropriate, antibiotics were used at the following concentrations: ampicillin, 100 μg ml^{-1}; kanamycin, 40 μg ml^{-1}; spectinomycin, 25 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1}; and tetracycline, 15 μg ml^{-1}.

Construction of IcsA derivatives with defective signal peptides.

To generate a secretion-defective form of IcsA, the upstream region of IcsA (bases 51–877, GenBank Accession No. M22802) was subcloned as a *Sph*I–*Xba*I fragment into pALTER-1 (Promega). Gly[40], Leu[44] and Leu[45] were individually changed to Glu, Arg and Arg, respectively, by site-directed mutagenesis according to the manufacturer's instructions (Altered Sites II *in vitro* Mutagenesis System, Promega) using primers 5′-GAGCAACAGGGATGCTTCAACAA CAAAAGGAG-3′, 5′-TATTGGCCCCCCGAGCCTCAGGGAT GCACCAAC-3′ and 5′-AGCTATTGGCCCCCCGCGCAACAG GGATGCACC-3′ respectively. A *Nhe*I site was introduced upstream of the *icsA* ATG start site for cloning purposes, using primer 5′-GAATTTGATTCATGCACTATGCTAGCAG TAAGTGGTTGAT-3′. Each construct was verified by sequencing, and each altered *Nhe*I–*Xba*I fragment was then exchanged into pMBG472 (Steinhauer *et al*., 1999) to generate plasmid pLDB414, pLDB416 or pLDB417, respectively; in these constructs, *icsA* is expressed under the control of the arabinose promoter. Each of these constructs and pMBG472 were introduced into *S. flexneri* MBG283 (*icsA*) and LDB376 *(prlA4 icsA*).

Construction of secY::gfp *fusion plasmids and strains*

To construct a plasmid-borne translational fusion of *secY* to

Table 1. Strains and plasmids used in this study.

Strain	Relevant genetic markers or features	Reference or source
S. flexneri		
2457T	Wild-type serotype 2a	LaBrec *et al.* (1964)
LDB376	MBG283 *prlA4* (tet^R $spec^R$)	This study
MBG283	2457T *icsA*::Ω ($spec^R$)	Steinhauer *et al.* (1999)
E. coli		
CSF107	DHB4 *prlA4*::Tn*10* (tet^R)	Prinz *et al.* (1996)
DH10B	F-*mcrA* Δ(*mrr-hsdRMS-mcrBC*) Δ*lacX74 deoR recA1 endA1 araD139*Δ(*ara, leu*)*7697 galU galK rpsL nupG*	Gibco BRL
DY363	Δ*lacU169 nadA*::Tn*10 gal490* λ*cI857* Δ(*cro-bioA*)	Yu *et al.* (2000)
HPT300	MC4100 Δ*ara714 ftsY70 zhg*::Tn*10* (tet^R)	Tian and Beckwith (2002)
HPT405	MC4100 Δ*ara714 ffh-77 pheA3141*::Tn*10* (kan^R)	Tian and Beckwith (2002)
JP313	MC4100 Δ*ara714*	Tian and Beckwith (2002)
JS7131	MC1060 *attB::R6Kori* P_{BAD}-*yidC* ($spec^R$)	Samuelson *et al.* (2000)
KS272	F- Δ*lacX74 galE galK thi rpsL* Δ*phoA* (*Pvu*II)	Strauch and Beckwith (1988)
LDB534	MM52 *ompT*::Tn*10* (kan^R)	This study
LDB544	MC4100 *secY*::*gfp aph* (kan^R)	This study
MBG263	MC1061 *ompT*::*kan* (kan^R)	Goldberg and Theriot (1995)
MBG357	JP313 *ftsY*$^+$ *ffh*$^+$ Δ(λ*att-lom*)::kan P_{MAL} *malE malF-lacZ102*	This study
MBG358	HPT405 Δ(λ*att-lom*)::*bla* P_{MAL} *malE malF-lacZ102*	This study
MC4100	F- *araD*139 *relA1 thi rpsL150 flbB5301 (lacU139*) *deoC7 ptsF25*	Pogliano and Beckwith (1994)
MM52	MC4100 *secA51*^{ts} *srl*::Tn*10* (tet^R)	Oliver and Beckwith (1981)
MPH107	F$^-$ *secY24*^{ts} *zhd33*::Tn*10 phoR phoA proC tsx*::Tn*5* (tet^R str^R)	Baba *et al.* (1994)
NWG107	PS288 Δ λ *att*::pNG10 (pJS65 lost) (str^R)	This study
PS288	MC1000 *phoA* Δ*Pvu*II *pcnB80 zad*1::Tn*10 secE*Δ*19-111* pJS65 (str^R)	Unpublished
Plasmids		
pACYC177	Cloning vector (amp^R kan^R)	New England Biolabs
pALTER-1	*In vitro* mutagenesis cloning vector (tet^R)	Promega
pARJ57	pLDB435 *secY*::*yfp* (amp^R)	This study
pARJ60	pSU20 with *Sph*I–*Hin*dIII *secY*::*yfp* insert from pARJ57 (cm^R)	This study
pAWY4	pGZ119EH *icsA*1_{-757}::*gfp* (cm^R)	This study
pBAD24-*icsA*$_{1-104}$::*gfp*	P_{BAD}-*icsA*$_{1-104}$::*gfp* (amp^R)	Charles *et al.* (2001)
pBAD24-*icsA*$_{1-757}$::*gfp*	P_{BAD}-*icsA*$_{1-757}$::*gfp* (amp^R)	Charles *et al.* (2001)
pDSW207	pDSW204 *Ptrc gfp* (fusion expression vector) (amp^R)	Weiss *et al.* (1999)
pDSW208	pDSW204 *Ptrc gfp* (fusion expression vector) (amp^R)	Weiss *et al.* (1999)
pGZ119EH	*Ptac* expression vector (cm^R)	Lessl *et al.* (1992)
pJS51	pBluescript-II KS+ M13$^+$ with 0.8 kb *Eag*I–*Kpn*I insert from pBRU containing the *secE* gene under the native promoter	Schatz *et al.* (1989); Schatz *et al.* (1991)
pJS65	pJS51 *bla*::Tn*phoA* (kan^R)	(Schatz *et al.*, 1991)
pLBD416	pMBG472 *icsA* $Leu^{44}Arg$ (amp^R kan^R)	This study
pLDB414	pMBG472 *icsA* $Gly^{40}Glu$ (amp^R kan^R)	This study
pLDB417	pMBG472 *icsA* $Leu^{45}Arg$ (amp^R kan^R)	This study
pLDB435	pDSW208 *secY::gfp* (amp^R)	This study
pMBG270	pBR322 with *Sph*I–*BamH*I insert containing *icsA* (amp^R)	Magdalena and Goldberg (2002)
pMBG472	pBAD18 *icsA aph* (amp^R kan^R)	Steinhauer *et al.* (1999)
pNG10	pDSW207 *gfp::secE* (amp^R)	This study
pSU20	*Ptac* expression vector (cm^R)	Bartolome *et al.* (1991)
pTW4	pBAD24-*icsA*$_{1-104}$::*ecfp* (amp^R)	This study

gfp, *secY* was amplified by PCR using MC4100 chromosomal DNA as template and primer pair 5′-GTAGCCCATTGCTAAACAACCGGGATTAGAT-3′ and 5′-CGGGC*CTGCAGGTTGTTGTTGTT*TCGGCCGTAGCC-3′. Primer sites *Nco*I and *Pst*I used for subsequent cloning are underlined and the complementary strand to the linker codons (Asn–Asn–Asn–Asn–Leu–Gln) is shown in italics. The *Nco*I- and *Pst*I-digested PCR product was cloned into pDSW204 at the same sites to generate plasmid pLDB434.5, placing *secY* under the control of a partly disabled *pTrc* promoter [pDSW204 (Weiss *et al.*, 1999)]. A *Pst*I–*Hin*dIII fragment containing *gfp* was isolated from pDSW208 (Weiss *et al.*, 1999) and was ligated into pLDB434.5 at the same sites to generate an in frame fusion of *secY* with *gfp*, designated pLDB435. The resulting construct encodes an intact SecY polypeptide fused at its carboxy terminus via the linker to intact GFP.

To replace the chromosomal copy of *secY* with a *secY*::*gfp* translational fusion, *secY*::*gfp* was amplified by PCR using pLDB435 as template and the primer pair 5′-CATTTGTACCAGGAATTCGTCCGGGAGA-3′ and 5′-CCTGGTCGACAAGCTTATTTGTATAGTTCATCCATGCC-3′. Primer sites *Eco*RI and *Sal*I are underlined; the *Eco*RI site is native to *secY* near its 3′ end. The *Eco*RI- and *Sal*I-digested PCR product was cloned into pALTER-1 (Promega) at the same sites to generate plasmid pLDB456. The kanamycin resistance gene (*aph*) was amplified using pACYC177 (Accession No. X06402) as template and the primer pair 5′-CCTGGTCGACCATGAACAATAAAACTGTCTGCTTAC-3′ and 5′-CCTGACGCGTCCGCCGTCCCGTCAAGTCAGCGTAATGC-3′. Primer sites *Sal*I and *Mlu*I are underlined. The *rpmJ* gene, which is located immediately downstream and in the same orientation as *secY*, was amplified from 28 bp upstream of

its translation start site to 22 bp downstream of the stop codon by PCR using MC4100 chromosomal DNA as template and the primer pair 5′-CCTGACGCGTGTCGC CCGAGAAGTTACGGAGAG-3′ and 5′-CCTGGAATTCG CAAAGAAAAATATGCGAAAAAATCAGCC-3′. Primer sites *Mlu*I and *Eco*RI are underlined. Amplified *aph* and *rpmJ* DNA were digested with *Mlu*I and ligated; the ligated product was digested with *Sal*I and *Eco*RI and cloned into pALTER-1 (Promega) at the same sites to generate pLDB457. Plasmids pLDB456 and pLDB457 were each digested with *Eco*RI, treated with alkaline phosphatase and then digested with *Sal*I. The resulting *Eco*RI–*Sal*I inserts were isolated from each plasmid by agarose gel purification and the two inserts were then ligated at their *Sal*I sites. The resulting ligation product was cloned into pALTER-1 at the *Eco*RI site to generate pLDB542. That pLDB542 contained the *secY*::*gfp* fusion, the *aph* gene and the *rpmJ* genes in the correct order and orientation was verified by DNA sequencing.

The *secY*::*gfp, aph* and *rpmJ* construct was exchanged into the chromosome at the native *secY rpmJ* locus via homologous recombination (Yu *et al*., 2000). pLDB542 was digested with *Eco*RI and the linear DNA fragment containing *secY*::*gfp*, *aph* and *rpmJ* was isolated by agarose gel purification. Linear DNA was electroporated into *E. coli* strain DY363 as described by Yu *et al*. (2000) and homologous recombination events were selected by growth on LB agar containing kanamycin. The kanamycin-resistant locus was then introduced into MC4100 via generalized P1 transduction to generate *E. coli* strain LDB544. Verification that the construct had appropriately integrated at the *secY rpmJ* locus was performed by PCR.

Construction of gfp::secE *fusion plasmid and strains*

secE was amplified by PCR using primer pair NWG0017, 5′-GCAGCAGAATTCAACAACAACAGTGCGAATACCGAAGC TCAAGGA-3′, and NWG0018, 5′-GCAGCAAAGCTTAA ACGCCTGAACGACGTACCA-3′. Primer sites *Eco*RI and *Hin*dIII are underlined. The amplified product was digested with *Eco*RI and *Hin*dIII and ligated into the same sites of pDSW207 (Weiss *et al*., 1999) to place *secE* under the control of a partly disabled *pTrc* promoter [pDSW204 (Weiss *et al*., 1999)] and create pNG10, which contains a three Asn linker between GFP and SecE. pNG10 was integrated in the chromosome of a wild-type strain using lambda InCh, as described in Boyd *et al*. (2000). The fusion was then introduced by P1 transduction into PS288 by selecting clones resistant to 25 μg ml^{-1} ampicillin. The final assay strain, NWG107, was produced by growth in 1 mM IPTG lacking kanamycin and screening for kan^{S} clones that had lost plasmid pJS65. Lambda integration and *secE* deletion of the final strain were confirmed by PCR as described previously (Schatz *et al*., 1991; Boyd *et al*., 2000).

Expression of an icsA::gfp *fusion in the YidC depletion strain*

Expression of YidC in the YidC depletion strain JS7131 is dependent on arabinose. To permit selective induction of the translational IcsA$_{1\text{-}757}$–GFP fusion in JS7131, the coding sequence for the fusion was subcloned as a *Pst*I–*Eco*RI fragment (Charles *et al*., 2001) and was ligated into the same sites in pGZ119EH (Lessl *et al*., 1992), thereby placing it under the control of an IPTG-inducible promoter. Expression of the IcsA$_{1\text{-}757}$–GFP fusion was induced by growth in 1 mM IPTG.

Expression of icsA::gfp *fusions after depletion of SecA or SecY*

E. coli MC4100, MM52 (*secA51^{ts}*) and MPH107 (*secY24^{ts}*), each carrying pBAD24-*icsA*$_{1\text{-}104}$::*gfp* or pBAD24-*icsA*$_{1\text{-}757}$::*gfp*, were grown overnight in M9 media supplemented with 0.2% glycerol. Cultures were back-diluted 1:50 into fresh media and grown to the early exponential phase at 30°C (the permissive temperature). Aliquots were then shifted to 42°C (the non-permissive temperature for *secA51^{ts}* and *secY24^{ts}*) and grown at that temperature for 1.0, 2.5 or 3.5 h, at which time growth was continued for an additional 30 min in the presence of 0.1% arabinose to induce expression of the *icsA*::*gfp* fusion proteins. Glucose was then added to a final concentration of 0.4%, and cells were visualized microscopically for localization of the IcsA–GFP fusions.

Construction of plasmids for localization of both SecY and IcsA in single cells

Localization studies were performed in *E. coli* DH10B using a plasmid-borne YFP derivative of *secY*::*gfp*, expressed from an IPTG-inducible promoter, in combination with a plasmid-borne fusion of the amino-terminal 104 residues of IcsA to enhanced CFP (ECFP), under the control of an arabinose-inducible promoter. A T203Y mutation was introduced into *gfp*, thereby changing it to *yfp* (pALTER, Promega). The *yfp* allele was exchanged into pLDB435 to generate a translational *secY*::*yfp* fusion, creating pARJ57. The *secY*::*yfp* allele was cloned into a second IPTG-inducible vector, pSU20 (Bartolome *et al*., 1991), to create pARJ60. The *ecfp* allele was amplified by PCR from pECFP (Clontech) and cloned in frame and downstream of the coding sequence of IcsA$_{1\text{-}104}$ under the control of the arabinose promoter in pBAD24 (Charles *et al*., 2001), thereby creating pTW4. Each construct was verified by sequencing.

Protein preparation and analysis

Whole cell (Bernardini *et al*., 1989), membrane (Brandon and Goldberg, 2001) and culture supernatant (Allaoui *et al*., 1992) proteins were prepared as previously described. Western blot analysis was performed using rabbit IcsA antiserum (Goldberg *et al*., 1993), β-lactamase antibody (5 Prime ⇒ 3 Prime), GFP antibody (Clontech), YidC antibody (gifts of M. Mueller and R. E. Dalbey), SecY antibody (laboratory collection) or SecE antibody (laboratory collection) and enhanced chemiluminescence (Pierce Chemical Co.).

To determine the effect of SecA depletion on IcsA secretion, *E. coli* expressing IcsA were grown at 30°C to the mid-exponential phase (OD$_{600}$ of 0.4) and then shifted to 42°C for up to 3.5 h. To eliminate from analysis IcsA that had been secreted during growth at the permissive temperature, cells

were pre-treated with proteinase K as follows: cells were incubated in pre-warmed media containing 20 μg ml^{-1} proteinase K (Sigma) for 10 min, recovered by centrifugation at room temperature, washed in pre-warmed media and recovered by centrifugation again, then resuspended in pre-warmed media (42°C) without proteinase K. Cells harvested at early times (0.5 and 1.0 h) were subjected to pre-treatment with proteinase K in pre-warmed media (30°C) prior to shifting to 42°C and were then grown at 42°C until the harvest time. Cells harvested at late times (2.5 and 3.5 h) were subjected to pre-treatment with proteinase K in pre-warmed media (42°C), beginning at 90 min prior to the designated harvest time. After completion of proteinase K pre-treatment, these cells were incubated at 42°C for an additional 60 min. IcsA that had been translocated to the bacterial surface during this 60 min 42°C incubation was recovered as follows: the bacterial culture was incubated in the presence of 2.5 μg ml^{-1} proteinase K at 42°C for 10 min, then proteinase K activity was quenched by addition of *N*-α-*p*-TOSYL-L-arginine methyl ester (TAME) to a final concentration of 50 mM, and whole cell and supernatant proteins were prepared.

In the analysis of the requirement of YidC for secretion of IcsA, the YidC depletion strain JS7131 (Samuelson *et al*., 2000) was used. This strain, which contains a deletion in native *yidC* and a copy of *yidC* under the control of the arabinose promoter integrated into the chromosome at the *attB* locus (Samuelson *et al*., 2000), was maintained by growth in the presence of 0.2% arabinose. Bacteria grown in the presence of arabinose were recovered from growth media by centrifugation and resuspended in LB containing either arabinose or glucose. The glucose-containing culture was grown for an additional 3–4 h before analysis, as this duration of growth in the absence of arabinose has previously been shown to lead to essentially complete depletion of YidC (Samuelson *et al*., 2000). Because YidC is essential for growth, YidC depletion was assessed by growth rate. To control for cell density effects on IcsA expression and secretion (Goldberg *et al*., 1994), the arabinose-containing culture was harvested at approximately the same OD_{600} as the glucose culture. To be certain that IcsA localized on the bacterial surface at the time of harvest represented protein that had been secreted only after significant depletion of YidC, 1 h prior to harvesting each sample, supernatant proteins, which would include secreted IcsA, were removed by centrifugation and washing of the cells. Because reversion occurs at a low but significant rate, at each stage of manipulation of the culture, bacteria were streaked out on LB agar containing arabinose and six individual colonies were replica plated onto media lacking arabinose; at no time did we detect any reversion.

Microscopy

For direct fluorescence microscopy, overnight cultures were back-diluted, expression of GFP, ECFP or YFP protein fusions was induced and live exponential phase bacteria were spotted onto a 1% agarose pad on a 15-well glass slide (ICN), as described (Charles *et al*., 2001). For microscopy of the YidC depletion strain, YidC depletion was performed as described above prior to induction of expression of the IcsA–GFP fusion. Fluorescence and phase microscopy was performed using a Nikon TE300 microscope with Chroma Technology filters. Images were captured digitally using a black and white Sensys charge coupled device (CCD) camera and IP Laboratory software (Scanalytics). Colour figures were assembled by separately capturing the YFP and ECFP signals and digitally pseudo-colouring the images. For indirect immuno-fluorescence microscopy of surface localized IcsA, cells were fixed on cover slips and antibody labelling and imaging were performed as previously described (Goldberg *et al*., 1993).

For imaging of the GFP–SecE fusion, cells were grown overnight in NZY at 37°C in the presence of 100 μM IPTG. Cells were diluted 1:100 in fresh media containing 100 μM and grown to an OD_{600} of 0.5. Cells were washed in fresh media and inoculated at 1:50 into media containing 0, 10, 25 or 100 μM IPTG and grown for 2.5 h. Cells were fixed in a formaldehyde, gluteraldehyde solution (Chen *et al*., 1999) and immobilized on an agarose cushion as described previously (van Helvoort and Woldringh, 1994). Fluorescence microscopy was performed using a Zeiss Axioskop 2 microscope with Chroma Technology filters. Images were captured digitally using a black and white Hamamatsu Photonics charge coupled device (CCD) camera and Improvision Openlab software.

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Note added in proof

Sijbrandi *et al.* [(2003) *J Biol Chem* **278:** 4674–4659] have recently demonstrated that the *E. coli* autotransporter Hemoglobin protease (Hbp) requires both SRP and the Sec apparatus for secretion.

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