Polar targeting of *Shigella* virulence factor IcsA in Enterobacteriacae and *Vibrio*

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Asymmetric localization is key to the proper function of certain prokaryotic proteins important to virulence, chemotaxis, cell division, development, motility, and adhesion. *Shigella* IcsA is localized to the old pole of the bacterium, where it mediates assembly of an actin tail inside infected mammalian cells. IcsA (VirG) is essential to *Shigella* intracellular motility and virulence. We used translational fusions between portions of IcsA and the green fluorescent protein (GFP) to determine the regions of IcsA that are necessary and sufficient for its targeting to the bacterial old pole. An IcsA-GFP fusion that lacks a signal peptide localized to the old pole, indicating that signal peptide-mediated secretion is not required for polar localization. Two regions within IcsA were required for localization of an IcsA-GFP fusion to the old pole. Further characterization of these regions indicated that amino acids 1–104 and 507–620 were each independently sufficient for polar localization. Finally, when expressed in *Escherichia coli*, *Salmonella typhimurium*, *Yersinia pseudotuberculosis*, and *Vibrio cholerae*, each of the two targeting regions localized to the pole, indicating that the mechanism of polar targeting used by IcsA is present generally among Enterobacteriacae and *Vibrio*.

The *Shigella* outer membrane protein IcsA (VirG) is localized to the old pole of the bacterium (1), where it mediates assembly of an actin tail at the pole of the bacterium inside infected mammalian cells (1, 2). Continuous assembly of an actin tail provides force that propels the bacterium through the cytoplasm of infected cells and into adjacent cells. In its human host, *Shigella* causes diarrhea and dysentery by infecting and spreading through the colonic epithelium, a process for which IcsA is a key virulence factor. Disruption of IcsA leads to loss of bacterial intracellular actin assembly, loss of cell-to-cell spread, and markedly reduced virulence in humans and animal models (2–6).

The asymmetric localization of IcsA is unusual but not unique among bacterial proteins. Certain proteins involved in chemotaxis, cell division, and development, as well as certain macromolecular surface structures, are also asymmetrically localized. Among Gram-negative bacteria, these include chemotaxis protein complexes, which are localized at the cell pole in *Escherichia coli* and *Caulobacter crescentus* (7, 8), the cell division inhibitor MinCD, which oscillates from one pole to the other in *E. coli* (9–12), and the cell cycle-associated histidine kinases CckA, PleC, and DivJ of *C. crescentus*, which are localized to the cell pole during specific stages of the cell cycle (13, 14). Bacterial flagella can be assembled exclusively on the old pole, as for *Vibrio* sp., *Campylobacter* sp., and *C. crescentus*, assembled on both poles (lophotrichous), or distributed randomly on the bacterial surface (peritrichous). Among other surface structures, type IV pili are present exclusively at the old pole of a variety of Gram-negative bacteria (15). Proper localization of these proteins and macromolecular structures is essential to important biological processes; yet the molecular mechanisms mediating their asymmetric localization are incompletely understood.

IcsA is a member of the autotransporter family of proteins, which are thought to be secreted across the inner membrane by the Sec apparatus and which mediate their own transport across the outer membrane (16, 17). Newly synthesized IcsA appears on the bacterial surface first at the old pole, suggesting that it is directly targeted to the pole (18). Once inserted in the outer membrane, it diffuses laterally along the sides of the rod-shaped cell away from the old pole toward the new pole (18). On the bacterial surface, IcsA is cleaved in a regulated fashion by the specific serine protease IcsP (SopA), which releases the amino-terminal domain of IcsA into the extracellular milieu (19, 20). IcsP cleavage of IcsA is important in the maintenance of a sharply polarized distribution of IcsA. In *icsP* mutants, IcsA is present in small amounts over the entire surface (19, 20).

Although IcsA is visualized on the bacterial surface first at the old pole, it is not known when targeting occurs. Targeting could theoretically occur at any stage of secretion or by rapid capping of protein in the outer membrane. We found that IcsA is targeted in the absence of signal peptide-mediated secretion and that two small regions within IcsA mediate its targeting. Further, we found that the molecular mechanism of targeting of IcsA is present generally among Enterobacteriacae and *Vibrio*.

Materials and Methods

Bacterial Strains and Plasmids. The strains used in this study are *Shigella flexneri* serotype 2a wild-type (wt) strain 2457T (21), *S. flexneri* MBG283, which is 2457T *icsA*::Ω (18), *E. coli* DH10B, *Salmonella typhimurium* 14028s (American Type Culture Collection), *Yersinia pseudotuberculosis* 126 (22), and *Vibrio cholerae* O395 (23).

All *icsA* alleles fused to green fluorescent protein (GFP) shown in Fig. 1 were expressed under the control of the arabinose promoter in pBAD24 (24). $IcsA_{1–757}$-GFP, $IcsA_{\Delta 58–103}$-GFP, $IcsA_{\Delta 505–537}$-GFP, $IcsA_{\Delta 507–729}$-GFP, and $IcsA_{\Delta 725–757}$-GFP (Fig. 1*b*) were generated as follows. The coding sequence for IcsA residues 1–757, which includes the signal peptide and the entire α domain except for its carboxy-terminal-most residue (Arg^{758}), was amplified by PCR on *S. flexneri* 2457T and was cloned into pACYC184 (New England Biolabs), generating pMBG369. An *Nco*I site was inserted overlapping the ATG translation start codon, thereby changing Asn^2 to Asp^2. A *Bgl*II site was inserted at the 3′ end, thereby changing Ser^{756} to Arg^{756}. Intact *gfp* was amplified by PCR on pGFPmut2 (25) and was cloned as a translational fusion downstream of *icsA* in pMBG369, generating pMAC339. $IcsA_{1–757}$-GFP (Fig. 1*b*) was generated by cloning the fusion from pMBG339 into pBAD24. Deletions of IcsA residues 58–103, 507–729, and 725–757 were generated by reverse PCR on pMAC339 with insertion of a *Kpn*I site at the junction; this resulted in insertion of a glycine residue between residues 506 and 730 of $IcsA_{507–729}$-GFP. Deletion of IcsA residues 505–537 was generated similarly, except an *Xho*I site was inserted at the junction.

Abbreviations: GFP, green fluorescent protein; wt, wild type.

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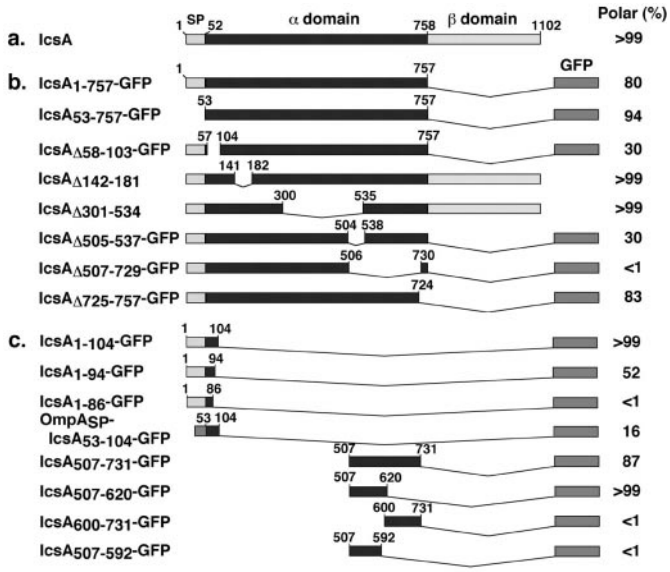

Fig. 1. Maps of IcsA and IcsA constructs used in this study. Schematic diagram of IcsA and IcsA-GFP translational fusion proteins and percentages of *icsA*$^-$ *Shigella* expressing each in which the protein is polarly localized. Native IcsA (*a*), IcsA α domain deletions (*b*), and other IcsA polypeptide fusions with GFP (*c*). Thin lines indicate gaps in IcsA sequence. SP, signal peptide.

Intact *gfp* was amplified by PCR on pGFPmut2 and was cloned into pSU19 (26), giving pMAC338. For $IcsA_{1–104}$-GFP, $IcsA_{507–731}$-GFP, $IcsA_{507–620}$-GFP, $IcsA_{600–731}$-GFP, $IcsA_{507–592}$-GFP, and $IcsA_{526–592}$-GFP (Fig. 1*c*), the coding sequences for the corresponding IcsA fragments were amplified as *Hin*dIII–*Bam*HI fragments and cloned into pMAC338 upstream of and in-frame with *gfp*. The *icsA-gfp* fusion sequences were then individually subcloned as *Bsp*HI–*Pst*I fragments into pBAD24. $IcsA_{53–757}$-GFP was constructed by separately cloning into pACYC184 *gfp* and the PCR-generated coding sequence for IcsA residues 53–757, and cloning the fusion into pBAD24; the coding sequence for Met-Ala was inserted upstream of the codon for IcsA residue Thr^{53}, and Ser^{756} was changed to Arg^{756}.

For $IcsA_{1–94}$-GFP and $IcsA_{1–86}$-GFP (Fig. 1*c*), the *gfp*-coding sequence was first amplified as a *Pst*I–*Hin*dIII fragment and cloned into pBAD24, and the IcsA sequences were amplified as *Bsp*HI–*Pst*I fragments and cloned upstream of and in-frame with *gfp*. The *Nco*I site within *gfp* had been previously eliminated (Altered Sites II, Promega), with no change in amino acid sequence. $OmpA_{SP}$-$IcsA_{53–104}$-GFP (Fig. 1*c*) was generated by nested PCR, by using oligonucleotides that contained the coding sequence for the *E. coli* OmpA signal peptide, and PCR products encoding *gfp* and the coding sequence of IcsA residues 53–104.

To test the localization of $IcsA_{507–620}$ in the presence of $IcsA_{1–104}$, the coding sequence for residues 507–620 fused to GFP was cloned as an *Eco*RI–*Xba*I fragment under the control of the *tac* promoter in pEXT22 (27). The coding sequence for residues 1–104 was cloned as an *Eco*RI–*Bam*HI fragment in-frame with glutathione *S*-transferase under the control of the *tac* promoter in pGEX-2T (Amersham Pharmacia). Two constructs in which IcsA residues 1–104 were expressed from the arabinose promoter were constructed by digesting the $IcsA_{1–104}$-GFP construct with either *Nde*I and *Xba*I or *Hin*dIII and *Xba*I, filling-in with T_4 polymerase, and religating, thereby deleting the first 77 residues of GFP or replacing *gfp* with 17 unrelated residues, respectively.

$IcsA_{\Delta142–181}$ and $IcsA_{\Delta301–534}$ (Fig. 1*b*) were expressed under the control of the native *icsA* promoter in a derivative of pBR322. They were generated by reverse PCR on pMBG235, a chloramphenicol-resistant derivative of pBR322 that carries the coding sequence for *icsA* and ≈500 bp of sequence upstream of *icsA* (28); *Bam*HI sites were inserted at the junction, with no alteration in the amino acid sequence. The sequence of each construct made in this study was verified by DNA sequencing. Deletion of IcsA residues 58–103 was performed by reverse PCR on pMBG472 (18) with insertion of a *Kpn*I site at the junction. The α domain of SepA was cloned as an *Nco*I–*Xho*I fragment upstream of and in-frame with *gfp* in pBAD24.

Bacterial Growth Conditions and Protein Analysis. All strains carrying constructs under the control of the arabinose promoter were grown overnight in M9 minimal media (29) containing 0.2% glycerol at 37°C. Each strain was diluted into the same media and grown at 37°C to OD_{600} 0.4–0.8, at which time L-arabinose was added to 0.2% for *Shigella* and *E. coli* strains and 2% for *Salmonella*, *Yersinia*, and *Vibrio* strains, and growth was continued at room temperature in the dark for 60 min. For all strains that express *icsA* from the native *icsA* promoter, growth was in tryptic soy broth rather than minimal media because the IcsA-specific protease IcsP, which is important to the maintenance of IcsA at the pole (19), is inactive upon growth in minimal media (29). After growth overnight at 37°C, these strains were back-diluted into tryptic soy broth and grown for 60 min at 30°C. To test the localization of $IcsA_{507–620}$ in the presence of $IcsA_{1–104}$, bacteria were grown in minimal media and were visualized at either 30 min and 1 h (arabinose-promoter constructs) or 2 1/2 and 4 h (glutathione *S*-transferase fusion construct) after induction. *Y. pseudotuberculosis* strains were grown at 28°C. Where appropriate, antibiotics were added to the following concentrations: ampicillin, 100 μg/ml; spectinomycin 100 μg/ml; and chloramphenicol, 25 μg/ml, except for *Yersinia* strains, for which ampicillin was added to 5 μg/ml.

IcsA on the bacterial surface was observed by indirect immunofluorescence by using antibody to IcsA (1). Osmotic shock was performed as described (30). Bacterial membranes were labeled by the addition of the dye FM4–64 (Molecular Probes), which fluoresces in the rhodamine wavelength, to the growth medium at a concentration of 0.2 μg/ml 60 min before induction with L-arabinose. Western blot analysis was performed by using antibody to IcsA (1) or to GFP (Molecular Probes) and enhanced chemiluminescence (Pierce).

Microscopy. For microscopy, bacteria were spotted onto a 1% agarose pad on a 15-well glass slide (ICN). The agarose was allowed to solidify in the dark for 5 min, the excess fluid was aspirated, and the bacteria were observed immediately. Fluorescence and phase microscopy was performed by using a Nikon TE300 microscope with Chroma Technology filters (Brattleboro, VT). Images were captured digitally by using a black and white Sensys charge-coupled device (CCD) camera and IP LAB software (Scanalytics, Billerica, MA). The color figure was assembled by capturing separately in the rhodamine and GFP wavelengths and subsequently digitally pseudocoloring the images.

Tabulation of Targeting Patterns. Tabulation of targeting patterns on fluorescent images was performed on 100 bacteria of each strain that had been previously randomly selected on phase images. To eliminate from tabulation individual cells in which the IcsA-GFP fusion construct might not be expressed, any cell in which the absolute level of fluorescence at mid-cell was less than two SDs below the mean absolute level at mid-cell of $IcsA_{1–757}$-GFP *Shigella* was not included in the tabulation (this represented <5% of cells for each strain). Tabulation of targeting patterns of native IcsA, $IcsA_{\Delta142–181}$, and $IcsA_{\Delta301–534}$ was performed similarly by using indirect immunofluorescence images of surface IcsA.

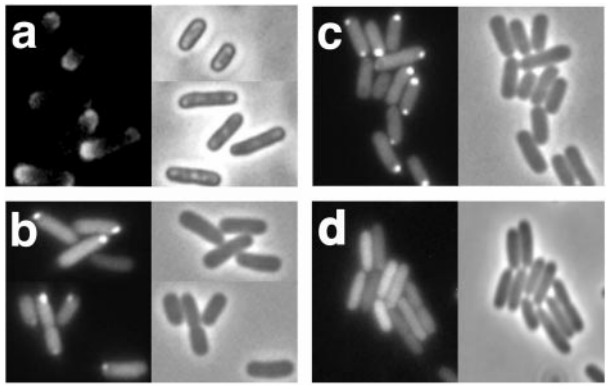

Fig. 2. Polar localization of IcsA and intact IcsA α domain constructs and the diffuse localization of the autotransporter SepA. Fluorescence micrographs of IcsA (indirect immunofluorescence) and IcsA-GFP and SepA-GFP fusion proteins (direct fluorescence) and phase micrographs of corresponding fields. Surface IcsA on wt *Shigella* (*a*), $IcsA_{1–757}$-GFP in *icsA*$^-$ *Shigella* (*b*), and $IcsA_{53–757}$-GFP in *icsA*$^-$ *Shigella* (*c*). The IcsA-GFP fusions were never seen to oscillate from pole to pole (data not shown). (*d*) $SepA_\alpha$-GFP in virulence plasmid-cured (*sepA*$^-$) *Shigella*.

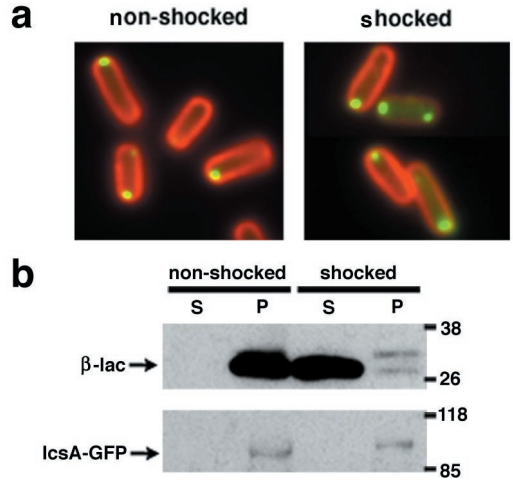

Fig. 3. Localization of $IcsA_{53–757}$-GFP after osmotic shock of bacteria. (*a*) Fluorescence micrographs of $IcsA_{53–757}$-GFP (green) within bacterial membranes (red) in *icsA*$^-$ *Shigella* after osmotic shock (*Right*) or no shock (*Left*). (*b*) Western blot analysis. Release of β-lactamase into the supernatant (S) with osmotic shock but not without. Association of $IcsA_{53–757}$-GFP with the bacterial pellet (P) after either osmotic shock or no shock.

Results

Targeting of IcsA Is Independent of Secretion. IcsA is a member of the autotransporter family of secreted proteins of Gram-negative bacteria. It displays the domain structure characteristic of members of this family, which consists of a functionally active domain (the α domain, residues 53–758) flanked on its amino-terminal end by a leader peptide (residues 1–52) and on its carboxy-terminal end by an outer membrane translocation domain (the β domain, residues 759-1102) (Fig. 1*a*) (16, 17). Because the amino-terminal leader peptide contains features typical of known Sec secretion signals, secretion across the cytoplasmic membrane is thought to be mediated by the Sec apparatus. Once in the periplasm, the β domain of the proprotein is thought to form a β barrel channel in the outer membrane, consisting of amphipathic antiparallel β sheets, through which the α domain is threaded to the bacterial surface (17, 31). IcsA is the only member of this family known to be distributed asymmetrically.

The β domains of the autotransporters are highly conserved in both sequence and function, whereas the α domains are quite divergent (16). Given the conservation of the β domain and the observation that IcsA is the only autotransporter family member known to be asymmetrically localized, we reasoned that the β domain of IcsA would likely not be required for its polar localization. To test this, we replaced the β domain with the GFP ($IcsA_{1–757}$-GFP, Fig. 1*b*) and expressed the construct in *icsA*$^-$ *Shigella* (strain MBG283). $IcsA_{1–757}$-GFP was present as discrete fluorescent foci at the bacterial pole, with generally one dot of fluorescence per cell (Fig. 2*b*). Fluorescent dots were polarly distributed in 80% of cells (Fig. 1*b*). This pattern is similar to that seen for full-length native IcsA on the surface of wt *Shigella*, which localizes to the bacterial pole on >99% of cells, as determined by indirect immunofluorescence (Figs. 1*a* and 2*a*). Expression of GFP alone from the parent vector (pBAD24) caused diffuse fluorescence in the bacteria (data not shown). To exclude the possibility that polar targeting is a general feature among autotransporters, a fusion of the α domain of the *Shigella* autotransporter SepA to GFP was examined; it showed diffuse fluorescence (Fig. 2*d*). That the polarized fluorescent accumulations of $IcsA_{1–757}$-GFP (and other constructs, below) are not inclusion bodies was further supported by expression in the presence of native IcsA (see below) and the absence of polar refractile bodies on phase microscopy (Fig. 2). Taken together, these results indicate that the β domain is not required for targeting.

Because the β domain mediates translocation of the α domain across the outer membrane, the polarized localization of $IcsA_{1–757}$-GFP suggested that targeting was occurring before insertion of the protein into the outer membrane. To determine whether targeting required signal peptide-mediated secretion, we constructed a fusion that lacked a signal peptide and contained only the α domain fused to GFP ($IcsA_{53–757}$-GFP, Fig. 1*b*) and would thereby be incompetent for signal peptide-mediated secretion across the cytoplasmic membrane. When expressed in *icsA*$^-$ *Shigella*, $IcsA_{53–757}$-GFP was present as discrete fluorescent foci at the bacterial pole in 94% of cells (Figs. 1*b* and 2*c*), similar to the pattern of expression of native IcsA and $IcsA_{1–757}$-GFP. These results indicate that targeting is independent of the signal peptide-mediated secretion pathway.

To confirm that targeting was not occurring in the periplasm, we examined the localization of $IcsA_{53–757}$-GFP after osmotic shock of bacteria in which $IcsA_{53–757}$-GFP had been expressed (Fig. 3). Osmotic shock causes release of soluble periplasmic contents into the surrounding buffer. Discrete fluorescent foci at the poles of the osmotically shocked cells were no different in appearance or localization from those of nonshocked cells (Fig. 3*a*). The release of β-lactamase (encoded by the expression vector) into the culture supernatant confirmed that soluble periplasmic contents had been specifically and efficiently released from osmotically shocked cells, whereas $IcsA_{53–757}$-GFP remained associated with the cell pellet (Fig. 3*b*). The bacterial membranes, visualized in the red spectrum by the fluorescent membrane dye FM4–64, are clearly located outside the green dots (Fig. 3*a*), further suggesting that the polar dots are in the bacterial cytoplasm.

Two Regions of IcsA Are Necessary for Polar Targeting. To identify the region(s) within IcsA that was mediating its localization to the pole, we examined in cells the distribution of a series of IcsA-GFP constructs that contain internal deletions (Fig. 1*b*). Two nonadjacent regions appeared to be involved. The deletion of amino acids 58–103, amino acids 507–729, or the overlapping amino acids 505–537 led to a marked decrease in the percentage of cells that had polarly localized fluorescent dots. Instead, these cells had diffuse fluorescence (Figs. 1*b* and 4 *a* and *b*). Deletion

of amino acids 58–103 led to a 70% decrease in polar localization, deletion of amino acids 507–729 led to a complete loss of polar localization, and deletion of amino acids 505–537 led to a 70% decrease in polar localization, whereas deletion of other portions of the α domain did not significantly alter the frequency of polar localization (Fig. 1*b*). Thus, amino acids 58–103 and 507–729 are each required for polar localization of IcsA. Verification that $IcsA_{\Delta 58-103}$-GFP and $IcsA_{\Delta 507-729}$-GFP were expressed as full-length fusion proteins and at levels comparable to other IcsA-GFP constructs was performed by Western blot analysis. Native IcsA, $IcsA_{1-757}$-GFP, $IcsA_{53-757}$-GFP, $IcsA_{\Delta 58-103}$-GFP, and $IcsA_{\Delta 506-730}$-GFP were expressed at approximately equivalent levels, whereas $IcsA_{1-104}$-GFP and $IcsA_{506-620}$-GFP (see below) were expressed at ≈5- to 8-fold higher levels (data not shown). There was no correlation between level of expression and ability to localize to the pole.

Shigella expressing IcsA that contains a deletion of amino acids 509–729 has previously been shown to distribute uniformly on the bacterial surface (32). To test whether amino acids 58–103 are also necessary for polar localization of intact IcsA, we deleted them from full-length IcsA. Using a polyclonal antiserum, the protein expressed by this construct could not be detected on the surface of *Shigella*, and by Western blot, the protein was largely degraded (data not shown). Thus, we were unable to directly test the role of this region in the polar localization of full-length IcsA.

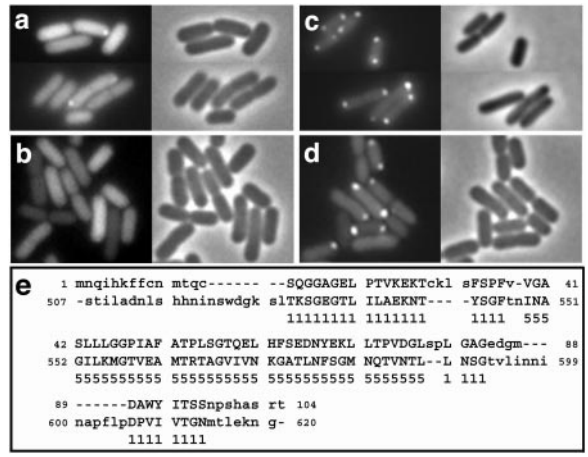

Fig. 4. Regions of IcsA that mediate its polar localization. (*a–d*) Direct fluorescence micrographs of IcsA-GFP fusion proteins expressed in *icsA*$^-$ *Shigella* and phase micrographs of corresponding fields. $IcsA_{\Delta 58-103}$-GFP (*a*), $IcsA_{\Delta 507-729}$-GFP (*b*), $IcsA_{1-104}$-GFP (region 1 targeting sequence) (*c*), and $IcsA_{507-731}$-GFP (*d*) (region 2 targeting sequence). (*e*) DIALIGN regional alignment of IcsA residues 1–104 (region 1 targeting sequence) and 507–620 (region 2 targeting sequence). Significant alignment is indicated by capitalization of residue designations, with the extent of alignment [from 1 (weakly significant) to 5 (highly significant)] shown below the sequence.

The Two Regions Necessary for Polar Targeting Overlap Regions That Are Independently Sufficient for Targeting. To test whether either or both of the regions shown above to be required for targeting could independently mediate targeting, each was fused directly to GFP; in the case of the amino-terminal region, amino acids 1–57 were also included because, when omitted, the fusion protein was unstable (data not shown). Remarkably, both $IcsA_{1-104}$-GFP and $IcsA_{507-731}$-GFP localized to the bacterial pole (in >99% of cells and in 87% of cells, respectively; Figs. 1*c* and 4 *c* and *d*), indicating that each region is independently sufficient for polar targeting. These observations further suggest that the loss of targeting that occurred in $IcsA_{\Delta 58-103}$-GFP and $IcsA_{\Delta 507-729}$-GFP (above) was due to loss of residues specifically involved in targeting and not merely the result of altered protein conformation. Thus, paradoxically, in the context of an intact α domain, both regions are required for targeting, whereas independently, each is sufficient for targeting.

Smaller regions within $IcsA_{1-104}$ and $IcsA_{507-731}$ were tested for their ability to localize to the pole. A construct in which the IcsA signal peptide was replaced with the OmpA signal peptide ($OmpA_{SP}$-$IcsA_{53-104}$-GFP) localized to the pole in only 16% of cells (Fig. 1*c*), indicating that residues within the IcsA signal peptide, or the signal peptide in its entirety, are required for polar localization by isolated $IcsA_{1-104}$, or that the OmpA signal peptide is interfering with the ability of $IcsA_{53-104}$ to target.

Truncation of the carboxy-terminal end of $IcsA_{1-104}$ to IcsA residue 94 ($IcsA_{1-94}$-GFP) led to polar localization in only 52% of cells, and truncation to residue 86 ($IcsA_{1-86}$-GFP) led to complete loss of polar localization (Fig. 1*c*). For $IcsA_{507-731}$, a construct in which the amino-terminal end of the region had been truncated to IcsA residue 600 ($IcsA_{600-731}$-GFP) failed to localize to the pole, whereas a construct in which the carboxy-terminal end had been truncated to IcsA residue 620 ($IcsA_{507-620}$-GFP) localized to the pole in >99% of cells (Fig. 1*c*). Thus, the smallest fragments of IcsA tested that mediated polar localization were residues 1–104 and 507–620. We designated $IcsA_{1-104}$ as the "region 1" targeting sequence and $IcsA_{507-620}$ as the "region 2" targeting sequence.

Both $IcsA_{\Delta 507-729}$-GFP and $IcsA_{1-104}$-GFP contain the region 1 targeting sequence and lack the region 2 targeting sequence. However, $IcsA_{\Delta 507-729}$-GFP was diffuse in the cell, whereas $IcsA_{1-104}$-GFP localized to the pole. The implication of these data are that sequences within IcsA residues 105–506 (which is present in the first construct, but absent in the second construct) inhibit the targeting behavior of targeting region 1.

Two constructs that contained all of region 2 but distinct portions of region 1 showed different targeting patterns. $IcsA_{53-757}$-GFP, which lacks the signal peptide but contains the amino acids 53–104, localized to the pole in 94% of cells, and $IcsA_{\Delta 58-103}$-GFP, which contains the signal peptide but lacks amino acids 58–103, localized to the pole in 30% of cells (Fig. 1*b*). These data indicate that region 2 is able to mediate polar localization efficiently in the absence of the signal peptide portion of region 1 and only poorly in the absence of residues 58–103 of region 1.

After osmotic shock, $IcsA_{507-620}$-GFP and other IcsA-GFP fusion constructs that lack signal peptides localized within the bacterial membranes and remained associated with the cell pellet (Fig. 3 and data not shown). Among constructs that contain signal peptides, ≈60% of $IcsA_{\Delta 57-104}$-GFP and 5–10% of $OmpA_{SP}$-$IcsA_{53-104}$-GFP were released into the surrounding buffer after osmotic shock (data not shown). Other signal peptide-containing constructs were not detected in the surrounding buffer after osmotic shock (data not shown), suggesting that these fusion proteins either were inefficiently secreted across the inner membrane or were tethered to the membrane.

We quantified unipolar vs. bipolar distributions for those constructs that showed polarized dots in >80% of cells and for native IcsA on wt *Shigella*. In mid-exponential growth phase, native IcsA and each of these IcsA-GFP fusions showed a bipolar distribution in approximately one-half of the cells (range 43–69% of cells). The relative number of cells having bipolar vs. unipolar foci of fluorescence did not correlate with the level of expression of the construct.

BLAST searches of each of these peptide sequences did not yield highly significant similarity to known sequences. However, regional alignment of the two targeting regions by using the algorithm DIALIGN (33) demonstrated significant similarity over their entire lengths (Fig. 4*e*), suggesting that they may share

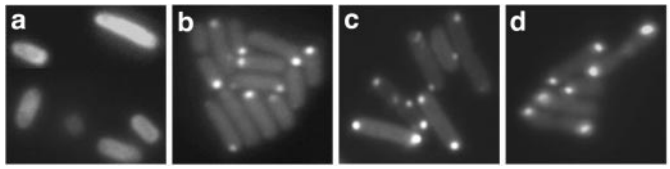

Fig. 5. Interference of native IcsA with IcsA-GFP fusion proteins. Fluorescence micrographs of IcsA-GFP fusion proteins in *icsA*$^-$ (*b* and *d*) and wt (*a* and *c*) *Shigella*. $IcsA_{53–757}$-GFP (*a* and *b*) and $IcsA_{507–620}$-GFP (*c* and *d*) (region 2 targeting sequence).

regional features essential to a common mechanism of polar targeting.

Localization of Targeting Regions in the Presence of Native IcsA. We then tested the localization of IcsA-GFP fusions in the presence of native IcsA. The entire α domain fused to GFP ($IcsA_{53–757}$-GFP), the region 1 targeting sequence fused to GFP ($IcsA_{1–104}$-GFP), and the region 2 targeting sequence fused to GFP ($IcsA_{507–620}$-GFP) were individually introduced into a *Shigella* background that was wt with respect to IcsA. Although $IcsA_{53–757}$-GFP localized to the pole in *icsA*$^-$ *Shigella*, it was diffuse in *icsA*$^+$ *Shigella* (Fig. 5 *a* and *b*), suggesting that native IcsA interferes with interactions of $IcsA_{53–757}$-GFP with a putative target at the pole. Furthermore, this experimental observation strongly suggests that $IcsA_{53–757}$-GFP interacts with the same polar target as native IcsA, thereby validating our interpretation of the IcsA-GFP fusion protein localization data (above). In contrast, $IcsA_{1–104}$-GFP and $IcsA_{507–620}$-GFP each localized to the pole in *icsA*$^+$ *Shigella*, as well as in *icsA*$^-$ *Shigella* (Fig. 5 *c* and *d*, and data not shown), which could result from the 5- to 8-fold higher levels of expression of these constructs and/or higher affinity of these regions for the polar target than that of native IcsA.

Localization of Targeting Region 2 in the Presence of Targeting Region 1. To explore whether the two targeting regions are recognizing the same structure at the pole, we examined whether the expression of targeting region 1 would displace targeting region 2 from the pole. Three different nonfluorescent region 1 expression constructs were induced at the same time as $IcsA_{507–620}$-GFP, induced 30 min before $IcsA_{507–620}$-GFP, or not induced. In each case, the expression of the region 1 construct did not cause displacement of the GFP foci from the pole. These data suggest that either regions 1 and 2 bind distinct structures at the pole or that the affinity of region 2 is significantly greater than the affinity of region 1 for a structure that is bound by both.

Targeting of IcsA Constructs in Other Enterobacteriacae and *Vibrio*. IcsA is present only in *Shigella*, and no homologs have been identified in any other organism. To explore whether the targeting mechanism used by IcsA in *Shigella* was more universally present among Enterobacteriacae and *Vibrio*, we placed the region 1 and 2 targeting fusions ($IcsA_{1–104}$-GFP and $IcsA_{507–620}$-GFP) into *E. coli*, *S. typhimurium*, *Y. pseudotuberculosis*, and *V. cholerae* and analyzed the pattern of fluorescence for each. Surprisingly, each of the constructs was targeted in each of the other Enterobacteriacae and *V. cholerae* in a manner similar to its pattern of targeting in *Shigella* (Fig. 6, and data not shown). Moreover, because the region 2 targeting sequence lacks a signal peptide, polar localization is occurring independent of signal peptide-mediated secretion. These data suggest that the mechanism of IcsA targeting is universally present among these organisms.

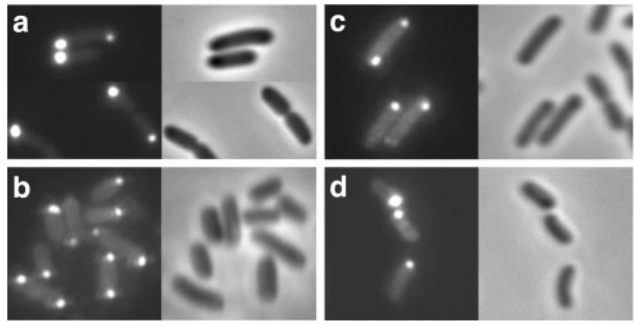

Fig. 6. Localization of IcsA region 2 targeting sequence fusion ($IcsA_{507–620}$-GFP) in other Enterobacteriacae and *Vibrio*. Direct fluorescence and phase micrographs of corresponding fields. *E. coli* (*a*), *S. typhimurium* (*b*), *Y. pseudotuberculosis* (*c*), and *V. cholerae* (*d*).

Discussion

Among bacteria, spatial localization of selected proteins is key to many cellular functions. IcsA is localized at the old pole on the surface of *Shigella*, where it mediates the assembly of an actin tail (1). Here we show that two discrete regions within IcsA are involved in the targeting of IcsA to the pole.

Several aspects of the results presented here initially appear paradoxical. First, the same two regions that are required for polar localization of the intact α domain of IcsA are each independently sufficient for polar localization. Deletion of either amino acids 58–103 or amino acids 507–729 from the intact amino terminal portion of IcsA ($IcsA_{\Delta 58–103}$-GFP or $IcsA_{\Delta 507–729}$-GFP) led to significant loss of polar localization, yet residues 1–104 (targeting region 1) and 507–620 (targeting region 2) were each independently sufficient to mediate localization to the pole ($IcsA_{1–104}$-GFP and $IcsA_{507–620}$-GFP). Second, sequences within IcsA residues 105–506 appear to inhibit the targeting behavior of targeting region 1. Both $IcsA_{\Delta 507–729}$-GFP and $IcsA_{1–104}$-GFP contain the region 1 targeting sequence and lack the region 2 targeting sequence, yet $IcsA_{\Delta 507–729}$-GFP was diffuse in the cell, whereas $IcsA_{1–104}$-GFP localized to the pole. And third, replacement of the IcsA signal peptide with the OmpA signal peptide led to loss of polar localization ($IcsA_{1–104}$-GFP was polar; $OmpA_{SP}$-$IcsA_{53–104}$-GFP was diffuse), whereas $IcsA_{53–757}$-GFP, which lacks the IcsA signal peptide, was polar.

We suggest a model in which targeting region 1 (IcsA residues 1–104) functions to establish the initial interaction with the putative polar target, and targeting region 2 (IcsA residues 507–620) subsequently serves to maintain that interaction. We propose that as the amino terminus of the protein is translated, targeting region 1 directs localization of the nascent protein to the old pole. Then, concomitant with translation of the ''inhibitory region'' of residues 105–506, the folding of IcsA may lead to a conformational change in or masking of targeting region 1 such that it becomes less able to interact with the putative polar target. However, this is immediately followed by translation of targeting region 2, which is independently able to interact with the putative polar target and thereby permits the nascent protein to remain at the pole. Targeting regions 1 and 2 may recognize distinct structures at the pole.

This model is consistent with the apparent paradoxes listed above. Thus, in isolation, targeting region 1 (residues 1–104) is able to localize to the pole, but, in the presence of residues 105–506, it is not. In the context of the intact α domain of IcsA, targeting region 2 is able to mediate some polar localization even in the absence of portions of targeting region 1, as seen with $IcsA_{53–757}$-GFP and $IcsA_{\Delta 59–103}$-GFP, which were polarly localized in 94% and 30% of cells, respectively. In contrast, in the

same context, targeting region 1 appears incapable of maintaining polar localization in the absence of targeting region 2, as seen with $IcsA_{\Delta 507-729}$-GFP. This model would predict that upon translation $IcsA_{\Delta 507-729}$-GFP is initially found at the pole and only subsequently becomes diffuse; our current assays are insufficiently sensitive to determine whether this is occurring.

A remarkable observation is that the IcsA targeting regions localize to the pole in a variety of Enterobacteriacae, including *E. coli*, *S. typhimurium*, and *Y. pseudotuberculosis*, and in *V. cholerae* (Fig. 6). This is consistent with the previous observation that native IcsA localizes to one pole on the surface of *E. coli* that express wt lipopolysaccharide (34). These observations indicate that the mechanism by which IcsA is polarly localized is present widely among Enterobacteriacae and in *Vibrio*. Conservation of this mechanism suggests that polar proteins in Enterobacteriacae other than *Shigella* and in *Vibrio* may be targeted to the pole by the same mechanism. It is not known whether IcsA is targeted to the pole in organisms other than those described herein.

Flagella of *Vibrio* sp. and *Campylobacter* sp., are assembled exclusively on the old pole. For successful flagellar assembly, the protein components of the flagellum and flagellar basal body must all be directed to the pole. It is possible that IcsA and the flagellar components use the same mechanism for polar localization. Similarly, type IV pili, which are adhesins expressed by a diverse group of Gram-negative bacteria, are assembled exclusively on the old pole (15). Interestingly, several components of the type II secretion machinery of the general secretory pathway are also required for type IV pilus biogenesis, suggesting that the two systems may also use the same physical structures in the cell. The type II secretion machinery permits certain proteins to cross the inner membrane by using the Sec apparatus and the outer membrane via specific terminal structures. Although the distribution in the cell of type II secretion machinery is unknown, it is conceivable that it is localized to the old pole and contains structural elements that are also used in IcsA targeting.

Chemotaxis protein complexes (7, 8), the cell division inhibitor MinCD (9–12), and *C. crescentus* cell-cycle histidine kinases CckA, PleC, and DivJ (13, 14) are all localized to the cell poles. The pattern of localization of MinCD differs from that of IcsA in that it oscillates between the two poles (9–12), and that of CckA differs in that it is localized to both poles simultaneously and its polar localization is restricted to a specific period of the cell cycle (the early predivisional cell) (13). PleC and DivJ are each localized to one cell pole, but unlike IcsA, their localization to the pole is only transient (14). The differences in these patterns of targeting may involve altogether different mechanisms of targeting or may involve similar mechanisms of targeting with variable means of exclusion from the new pole and different means of temporal regulation or anchoring at the pole. Further analysis will be required to identify the apparatus involved in IcsA targeting and to determine whether IcsA shares a common targeting mechanism with known or as yet unknown polarly localized proteins of Enterobacteriacae, *Vibrio*, or other bacteria, including those described above.

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