

Functional Analysis of a Rickettsial OmpA Homology Domain of *Shigella flexneri* IcsA

MACARTHUR CHARLES,¹ JUANA MAGDALENA,¹ JULIE A. THERIOT,²
AND MARCIA B. GOLDBERG^{1*}

Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York 10461-1602,¹
and Department of Biochemistry, Stanford University School of Medicine,
Palo Alto, California 94305-5307²

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***Shigella flexneri* is a gram-negative bacterium that causes diarrhea and dysentery by invasion and spread through the colonic epithelium. Bacteria spread by assembling actin and other cytoskeletal proteins of the host into “actin tails” at the bacterial pole; actin tail assembly provides the force required to move bacteria through the cell cytoplasm and into adjacent cells. The 120-kDa *S. flexneri* outer membrane protein IcsA is essential for actin assembly. IcsA is anchored in the outer membrane by a carboxy-terminal domain (the β domain), such that the amino-terminal 706 amino acid residues (the α domain) are exposed on the exterior of the bacillus. The α domain is therefore likely to contain the domains that are important to interactions with host factors. We identify and characterize a domain of IcsA within the α domain that bears significant sequence similarity to two repeated domains of rickettsial OmpA, which has been implicated in rickettsial actin tail formation. Strains of *S. flexneri* and *Escherichia coli* that carry derivatives of IcsA containing deletions within this domain display loss of actin recruitment and increased accessibility to IcsA-specific antibody on the surface of intracytoplasmic bacteria. However, site-directed mutagenesis of charged residues within this domain results in actin assembly that is indistinguishable from that of the wild type, and in vitro competition of a polypeptide of this domain fused to glutathione *S*-transferase did not alter the motility of the wild-type construct. Taken together, our data suggest that the rickettsial homology domain of IcsA is required for the proper conformation of IcsA and that its disruption leads to loss of interactions of other IcsA domains within the amino terminus with host cytoskeletal proteins.**

The gram-negative bacterium *Shigella flexneri* causes diarrhea and dysentery by directly invading the colonic epithelium and eliciting a severe local inflammatory response. After inducing its entry into the cell, *S. flexneri* rapidly lyses the phagocytic vacuole and is released into the cytoplasm (6, 21, 27, 40). Within the cytoplasm, bacteria assemble actin on one pole of the bacterial body into tails that consist of bundled actin filaments. The continuous polymerization of actin at the junction of the bacterial body with the tail and the incorporation of the resulting actin filaments into the tail provide the force to propel the microorganism through the cytoplasm at speeds of 12 $\mu\text{m min}^{-1}$. Then, in a process that also requires actin assembly, bacteria induce cell surface protrusions, which enable bacterial spread into adjacent cells (23, 35, 37, 41).

IcsA, a 120-kDa *S. flexneri* outer membrane protein, has been shown to be essential and sufficient for actin tail formation (4, 17, 26, 30). A strain with a disruption of *icsA* invades cells and lyses the phagocytic vacuole but fails to induce polymerization of actin and remains confined to the cytoplasm (9). In addition, introduction of *icsA* into *Escherichia coli* confers upon this organism the ability to form actin tails in cytoplasmic extracts (17, 26).

IcsA is an 1,102-amino-acid protein that is unusual in that it is localized at one pole of *S. flexneri* (16). It is anchored in the outer membrane via a domain within the carboxy-terminal 344 amino acid residues (the β domain), thereby exposing its amino-terminal 706 amino acid residues (the α domain) on the

exterior of the bacillus (45). Once translocated across the outer membrane, IcsA is slowly cleaved by IcsP (SopA) between Arg⁷⁵⁸ and Arg⁷⁵⁹ to release the 95-kDa α domain into the growth medium or the cytosol (12, 13, 42). The specific domain(s) of IcsA that is required for actin assembly has not been identified, although deletion of a large region encompassing amino acids 320 to 507 leads to loss of actin recruitment on the surface of the bacillus (46).

Several other intracellular microorganisms have been shown to assemble actin tails that are similar to those assembled by *Shigella*: the gram-positive bacterium *Listeria monocytogenes* (8, 32, 48), certain members of the *Rickettsiaceae* family of small gram-negative bacteria (19), and vaccinia virus (7). The specific protein that mediates actin assembly on *L. monocytogenes*, ActA, has been extensively characterized, while those that mediate actin assembly on rickettsiae and vaccinia virus have not yet been definitively identified. ActA has been shown to be the sole *Listeria* factor required for this process (11, 25) and bears no significant sequence similarity to IcsA. ActA has two domains that appear to have distinct roles in actin tail formation: an amino-terminal domain (amino acid residues 128 to 151) that is required for actin recruitment and a domain containing a series of four proline-rich repeats (amino acid residues 263 to 390) that directly binds the vasodilator-stimulated phosphoprotein (VASP), a focal adhesion protein, which in turn recruits profilin-actin to the surface of the bacterium (5, 24, 29, 36, 43). Deletion of the actin recruitment domain leads to loss of actin association with ActA (29, 36), whereas deletion of the proline-rich repeats leads to slowing of the rate of actin assembly by approximately two-thirds (28, 33, 43). In addition, mounting evidence indicates that the Arp2/3 complex of seven

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461-1602. Phone: (718) 430-2118. Fax: (718) 430-8711. E-mail: mgoldber@aecom.yu.edu.

TABLE 1. Strains and plasmids

Bacterial strain or plasmid	Relevant characteristics	Reference or source
<i>S. flexneri</i> strains		
M90T	Wild-type serotype 5	39
SC560	M90T Δ icsA:: Ω Sp ^f	9
MAC1000	SC560 carrying <i>icsA</i> on pMBG235, Sp ^f Amp ^r	This study
MAC1003	SC560 carrying <i>icsA3</i> on pMAC103, Sp ^f Amp ^r	This study
MAC1007	SC560 carrying <i>icsA7</i> on pMAC107, Sp ^f Amp ^r	This study
MAC1008	SC560 carrying <i>icsA8</i> on pMAC108, Sp ^f Amp ^r	This study
MAC1009	SC560 carrying <i>icsA9</i> on pMAC109, Sp ^f Amp ^r	This study
MAC1010	SC560 carrying <i>icsA10</i> on pMAC210, Sp ^f Amp ^r	This study
MAC1014	SC560 carrying <i>icsA14</i> on pMAC214, Sp ^f Amp ^r	This study
MAC1015	SC560 carrying <i>icsA15</i> on pMAC215, Sp ^f Amp ^r	This study
MAC1016	SC560 carrying <i>icsA16</i> on pMAC216, Sp ^f Amp ^r	This study
MAC1017	SC560 carrying <i>icsA17</i> on pMAC217, Sp ^f Amp ^r	This study
<i>E. coli</i> strains		
BL21	F ⁻ <i>ompT</i> [<i>lon</i>] <i>hsdS_B</i> (<i>r_B</i> ⁻ <i>m_B</i> ⁻) <i>gal dcm</i>	44
MC1061	F ⁻ <i>araD139</i> Δ (<i>ara-leu</i>)7696 <i>galE15 galK16</i> Δ (<i>lac</i>)X74 <i>rpsL</i> (Str ^r) <i>hsdR2</i> (<i>r_K</i> ⁻ <i>m_K</i> ⁺) <i>mcrA mcrB1</i>	31
MBG263	MC1061 <i>ompT</i> ::Km	17
MAC2000	MBG263 carrying <i>icsA</i> on pMBG235, Km ^r Amp ^r	This study
MAC2003	MBG263 carrying <i>icsA3</i> on pMAC103, Km ^r Amp ^r	This study
MAC2009	MBG263 carrying <i>icsA9</i> on pMAC109, Km ^r Amp ^r	This study
MAC2016	MBG263 carrying <i>icsA16</i> on pMAC216, Km ^r Amp ^r	This study
MAC2017	MBG263 carrying <i>icsA17</i> on pMAC217, Km ^r Amp ^r	This study
Plasmids		
pMBG226	pBR322 containing <i>icsA</i> from <i>S. flexneri</i> M90T, Amp ^r Cm ^r	This study
pMBG235	pMBG226 with elimination of the <i>Bam</i> HI site, Amp ^r Cm ^r	This study
pGEX-2T	<i>gst</i> gene fusion vector, Amp ^r	Pharmacia
pMBG282	pGEX-2T with <i>icsA</i> 1912–2088 ^a inserted at its <i>Bam</i> HI and <i>Eco</i> RI sites	This study
pMAC103	pMBG235 containing <i>icsA3</i> , which is <i>icsA</i> Δ (1909–2091)	This study
pMAC107	pMBG235 containing <i>icsA7</i> , which is <i>icsA</i> Δ (1474–2175):: <i>ompA</i> 2671–2868 ^b	This study
pMAC108	pMBG235 containing <i>icsA8</i> , which is <i>icsA</i> Δ (1474–2175):: <i>ompA</i> 2128–2436	This study
pMAC109	pMBG235 containing <i>icsA9</i> , which is <i>icsA</i> Δ (1474–2175):: <i>ompA</i> 2320–2454	This study
pMAC210	pMBG235 containing <i>icsA10</i> , which is <i>icsA</i> 2032 GATACAGATGGCGAC 2047 changed to 2032 GCTACAGCTGGCGCT 2047	This study
pMAC214	pMBG235 containing <i>icsA14</i> , which is <i>icsA</i> 1972 GACTATACAAAA 1983 changed to 1972 GCCTATACAGCT 1983	This study
pMAC215	pMBG235 containing <i>icsA15</i> , which is <i>icsA</i> 1996 GACCAGAATAAAA 2007 changed to 1996 GCCCAGAATGCG 2007	This study
pMAC216	pMBG235 containing <i>icsA16</i> , which is <i>icsA</i> Δ (1909–1992)	This study
pMAC217	pMBG235 containing <i>icsA17</i> , which is <i>icsA</i> Δ (2020–2091)	This study

^a Nucleotide designations for *icsA* (*virG*) are those in GenBank accession no. M22802.

^b Nucleotide designations for *R. rickettsiae ompA* are those in GenBank accession no. M31227.

proteins is also required for actin assembly on *L. monocytogenes* (49, 50).

Among the *Rickettsiaceae*, spotted-fever group rickettsiae (*Rickettsia rickettsii*, *R. conorii*, and *R. akari*) form actin tails in the cytoplasm of infected cells, whereas typhus-group rickettsiae do not. Since spotted-fever group rickettsiae express the 190-kDa outer membrane protein OmpA and typhus-group rickettsiae lack OmpA, it has been suggested that OmpA may have a role in rickettsial actin tail formation (19, 20).

In this context, we were interested in defining the IcsA domain(s) that is essential to its function in actin assembly. We identified a domain of IcsA that bears significant sequence similarity to a repeated domain of rickettsial OmpA. We therefore analyzed the role of this domain in translocation of IcsA to the bacterial surface and in actin assembly.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cell lines. The bacterial strains and plasmids used in this study are listed in Table 1. All strains carrying *icsA* alleles are derived from *S. flexneri* serotype 5 wild-type strain M90T (39) or *E. coli* MC1061 (31). *Shigella* strains were grown in tryptic soy broth, and *E. coli* strains were grown in

Luria-Bertani broth. Where appropriate, antibiotics were used at the following concentrations: ampicillin, 100 μ g/ml; spectinomycin, 100 μ g/ml; kanamycin, 40 μ g/ml. HeLa and L2 cells used for plaque assays and *Shigella* intracellular motility assays were maintained in minimal essential medium supplemented with 10% fetal bovine serum and 1% nonessential amino acids.

Construction of in-frame deletions within *icsA*. Plasmid pMBG235 (*icsA* WT [wild-type]) contains the entire coding sequence for *icsA*, as well as 523 bp of flanking DNA upstream of the translational start site and 56 bp of flanking DNA downstream of the translational stop codon. pMAC103 (*icsA3*), pMAC216 (*icsA16*), and pMAC217 (*icsA17*) were generated by reverse PCR on pMBG235 DNA, using oligonucleotide primer pairs 5'-CTGTGGATCCTTTATCTGCAC TTAG-3' and 5'-CTTACTGGATCCACTATCTGGCA-3', 5'-ACCTGTGGAT CCTTTATCTGCACCTTA-3' and 5'-GCTATGGATCCGACCAGAATAAATTG-3', and 5'-CCAGACTGGATCCGTAGATCAATTA-3' and 5'-CTGAACCTAC TGGATCCACTATTCT-3', respectively. To construct the three site-directed mutants (*icsA10*, *icsA14*, and *icsA15*), reverse PCR was carried out with pMBG235 as a template and three different oligonucleotide primer pairs. pMAC210 (*icsA10*) was made with 5'-CTGGAATGATACAGATGGCGCTAG CCATG-3' and 5'-TCCATGGCTAGCGCCAGCTGTAGATTC-3', thus creating *Nhe*I ends and resulting in the following three amino acid substitutions: Asp⁴⁸⁷ to Ala, Asp⁴⁸⁹ to Ala, and Asp⁴⁹¹ to Ala. pMAC214 (*icsA14*) was made with 5'-GACTATACAGCTAGCTATATCAGTGAC-3' and 5'-CTGATATAG CTAGCTGTATAGGCAATAG-3' to create *Nhe*I ends and resulted in the following two amino acid substitutions: Asp⁴⁶⁷ to Ala and Lys⁴⁷⁰ to Ala. pMAC215 (*icsA15*) was generated with 5'-GTGACCAGAACGCGTTGATCTACGGTT

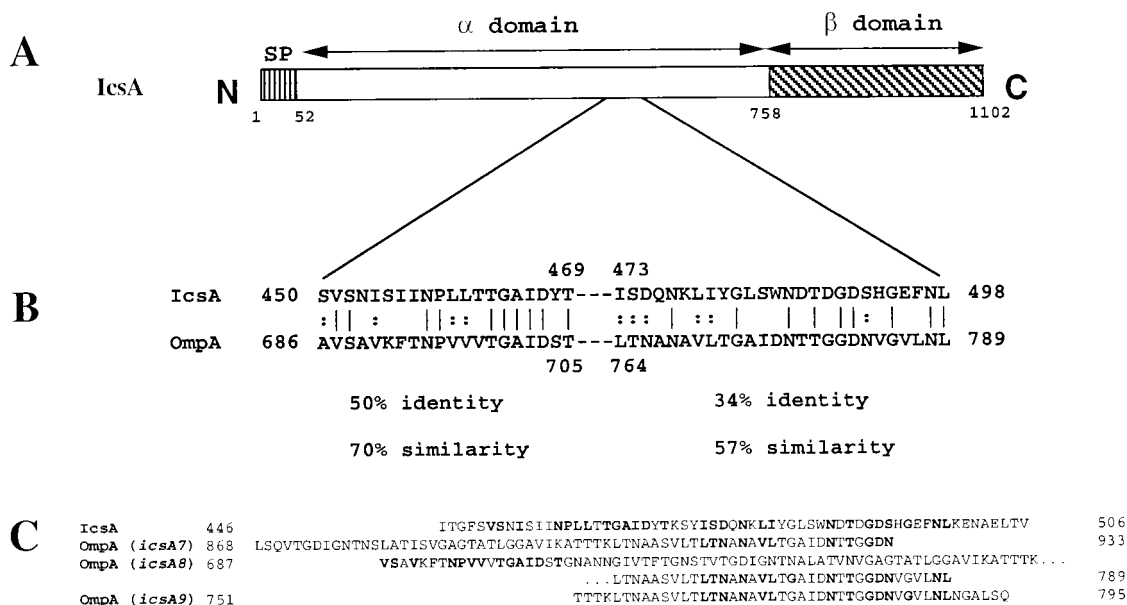


FIG. 1. Schematic of IcsA and sequence alignment of IcsA with the rickettsial homology domain. (A) Schematic of IcsA. SP and vertically striped bar, signal peptide; open bar, α domain; diagonally striped bar, β domain. (B) Sequence alignment of IcsA amino acids 450 to 498 with *R. rickettsii* OmpA amino acids 686 to 789. Dashes represent gaps introduced in the sequence to optimize alignment. (C) Sequence alignment of IcsA amino acids 446 to 506 with the three *R. rickettsii* OmpA domains exchanged for it in this study. Amino acids in boldface type are conserved between IcsA and the OmpA sequences. Amino acid designations for IcsA are those in GenBank accession no. M22802, and those for OmpA are those in GenBank accession no. M31227.

T-3' and 5'-CCGTAGATCAACGCGTTCTGGGCACTGA-3' to generate *Mlu*I ends and resulted in the following two amino acid substitutions: Asp⁴⁷⁵ to Ala and Lys⁴⁷⁷ to Ala. The absence of the deleted segment or the presence of the point mutations in each case was confirmed by DNA sequencing.

To construct pMAC107 (*icsA7*), a region encompassing *R. rickettsii ompA* bp 2671 to 2868 was amplified by PCR with oligonucleotide primers 5'-GGAGCG GTGATTAGATCTACTACG-3' and 5'-CTATATCCCAGATCTTTGACTT AAC-3', thereby generating *Bgl*II ends. This fragment was inserted into pMAC103 at the *Bam*HI site in *icsA3*, thus resulting in insertion of rOmpA amino acids 868 to 933 after IcsA amino acid 507 with insertion of an Arg residue and a Ser residue. The presence of an insert was confirmed by restriction digestion and PCR analysis, and the orientation and nucleotide sequence were verified to be correct by DNA sequencing. pMAC108 (*icsA8*) and pMAC109 (*icsA9*) were made similarly, except with oligonucleotide primers 5'-CGACTA AAATAACGAGATCTGTGCA-3' and 5'-GACTTAAAGATCTATTTAAAT TTAACACA-3'. To construct pMBG282 (*gst::icsA67*), a fragment encompassing *icsA* bp 1897 to 2105 was amplified by PCR with oligonucleotide primers 5'-G CAGATAAAAGATCTACAGGTTTCAG-3' and 5'-GCCAGAATAGTGAAT TCAGTAAGTTC-3', thus generating a *Bgl*II and an *Eco*RI end. This fragment was ligated into the cloning vector pGEX-2T (Pharmacia) at its *Bam*HI and *Eco*RI sites. The resulting gene fusion codes for a 34-kDa protein (*GST::IcsA67*) consisting of glutathione *S*-transferase (*GST*) and IcsA amino acids 447 to 505 with a thrombin cleavage site (LVPRGS) between the two polypeptides and six additional amino acids at the carboxy terminus (EFIVTD). Stop codons in each of the three reading frames are located downstream of the coding sequence of the gene fusion. The fusion protein was expressed in *E. coli* BL21, and purification was carried out according to the manufacturer's recommendations. DNA isolations, transformation, and cloning were performed by standard methods (38).

Protein preparation and analysis. Whole-cell and supernatant extracts were prepared as described previously (1, 22). Protein extracts, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, were transferred to nitrocellulose and probed with the monoclonal antibody VIF8, which recognizes an epitope within the α domain of IcsA, or with affinity-purified IcsA antiserum (16). The VIF8 monoclonal antibody was synthesized according to standard procedures by using IcsA that had been purified as previously described (16). Visualization was performed by enhanced chemiluminescence (Amersham).

IcsA localization and cell infection assays. Labeling for IcsA on the surface of bacteria grown in vitro was performed essentially as described previously (16). Labeling for IcsA and actin on *S. flexneri*-infected HeLa or L2 cell monolayers was performed essentially as previously described (16, 18). Plaque assays were performed essentially as described previously (42) with the exception that, where appropriate, ampicillin was added to the agarose overlay at a final concentration of 250 μ g/ml. Monolayers were photographed 48 h after infection. Speeds of motile intracytoplasmic bacteria were determined as previously described (42).

Motility assays in *Xenopus laevis* egg cytoplasmic extracts. Motility assays were performed as described previously (17). For competition studies, 8 μ l of rhodamine-conjugated monomeric actin in G buffer (5 mM Tris [pH 8.0], 0.2 mM CaCl₂, 0.2 mM ATP, 0.5 mM dithiothreitol [DTT]) was mixed with 3.2 μ l of Kron buffer (0.05 mg of catalase per ml, 0.10 mg of glucose oxidase per ml, 2.5 mg of glucose per ml, 0.5 mM DTT). The following were then combined in a small Eppendorf tube: 5.5 μ l of extracts; 0.7 μ l of the rhodamine-conjugated actin mixture to a concentration of 40 μ g/ml; 0.5 μ l of phosphate-buffered saline (PBS), *GST*, or *GST::IcsA67*; and 0.5 μ l of a suspension of formaldehyde-fixed bacteria in XB buffer (100 mM KCl, 0.1 mM CaCl₂, 2 mM MgCl₂, 5 mM EGTA, 10 mM K HEPES [pH 7.7], 50 mM sucrose). Following incubation on ice for 1 h, 0.5 μ l of the assay mixture was spotted on a glass slide, covered with a 22- by 22-mm coverslip, sealed with nail polish, and examined by phase and fluorescence microscopy on a Nikon Diaphot 200 microscope with epifluorescence optics using standard rhodamine filters (Chroma, Brattleboro, Vt.). Between 250 and 350 individual bacteria were examined for each concentration of polypeptide per experiment and were evaluated for an association with polymerized actin in the form of clouds and tails.

RESULTS

Sequence similarity of IcsA amino acids 446 to 506 to rickettsial OmpA repeats. IcsA has been shown to be anchored in the outer membrane by a domain within the carboxy-terminal 344 amino acids (β domain); as a result, the amino-terminal 706 amino acids of the mature protein (α domain) are exposed on the bacterial surface (45, 46) and thus constitute the region of the protein likely to be involved in interactions with host proteins. To aid in defining functional domains of IcsA, we compared the α domain of IcsA against the Swiss-Prot, EMBL, and GenBank databases. Sequence alignment reveals significant similarity of IcsA amino acids 446 to 506 to two discontinuous domains of the rickettsial outer membrane protein OmpA (Fig. 1B). Spotted-fever group *Rickettsiae* (*R. rickettsii*, *R. conorii*, and *R. akari*), like *S. flexneri*, form actin tails in the cytoplasm of cells, whereas typhus-group *Rickettsiae* do not. Since spotted-fever group *Rickettsiae* express OmpA and typhus-group *Rickettsiae* lack OmpA, it has been suggested that OmpA may have a role in rickettsial actin tail formation (19, 20). Significantly, the region that contains these two dis-

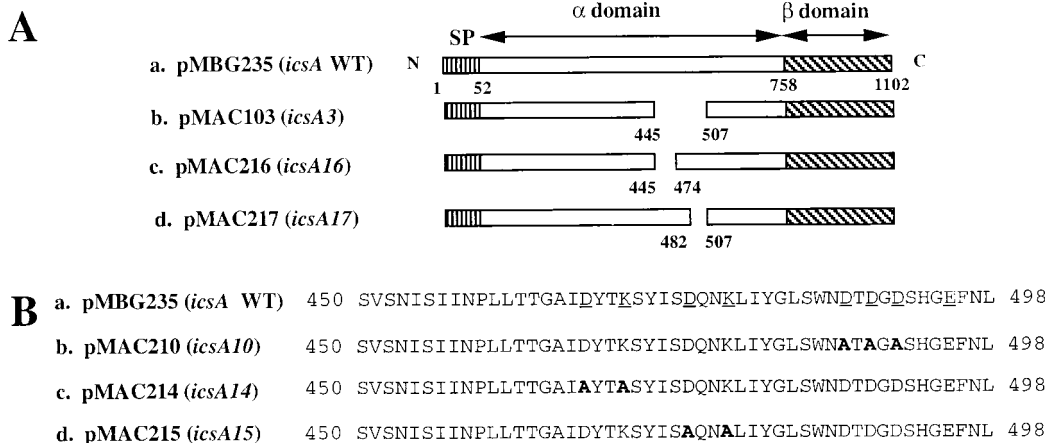


FIG. 2. Schematic of IcsA constructs made in this study. (A) Schema of internal in-frame deletions of IcsA. a, *icsA* WT, which encodes wild-type IcsA; b, *icsA3*, which encodes a deletion of amino acids 446 to 506, inclusive; c, *icsA16*, which encodes a deletion of amino acids 446 to 473, inclusive; d, *icsA17*, which encodes a deletion of amino acids 483 to 506, inclusive. Gaps represent deleted amino acids. Amino acid designations are those in GenBank accession no. M22802. SP and vertically striped bar, signal peptide; open bar, α domain; diagonally striped bar, β domain. (B) Alignment of IcsA amino acids 450 to 498 with the site-directed mutations within this domain. a, *icsA* WT, which encodes the wild-type amino acid sequence of IcsA (charged amino acids are underlined); b, *icsA10*, which encodes the amino acid substitutions Asp⁴⁸⁷ to Ala, Asp⁴⁸⁹ to Ala, and Asp⁴⁹¹ to Ala; c, *icsA14*, which encodes the amino acid substitutions Asp⁴⁶⁷ to Ala and Lys⁴⁷⁰ to Ala; d, *icsA15*, which encodes the amino acid substitutions Asp⁴⁷⁵ to Ala and Lys⁴⁷⁷ to Ala. Alanine residues indicated in boldface type replace the charged residues underlined in line a.

continuous domains is repeated 13 times in *R. rickettsii* OmpA, 14 times in *R. conorii* OmpA, and 11 times in *R. akari* OmpA (2, 14, 15). Within IcsA, the domain is not repeated. Of note, IcsA bears no sequence similarity to *L. monocytogenes* ActA.

As shown in Fig. 1A, two discontinuous regions within the rickettsial OmpA repeat bear similarity to sequences within IcsA amino acids 446 to 506. One, OmpA amino acids 686 to 705, shares 50% sequence identity and 70% sequence similarity with IcsA amino acids 450 to 469; the other, OmpA amino acids 764 to 789, shares 34% sequence identity and 50% sequence similarity with IcsA amino acids 473 to 498.

We constructed a series of in-frame deletions that encompass all or portions of the coding region for the rickettsial homology domain of IcsA on plasmid pMBG235 (Fig. 2A). *icsA3* contains a deletion that closely encompasses the rickettsial homology domain, while *icsA16* and *icsA17* contain deletions that roughly encompass the coding sequence for the amino-terminal and carboxy-terminal halves of the rickettsial homology domain, respectively. All *icsA* alleles constructed in this study contain the native *icsA* promoter, signal sequence, and coding region for the transmembrane domain.

Expression of *icsA* constructs in *S. flexneri* and *E. coli* grown in vitro. IcsA is inserted into the outer membrane as a 120-kDa protein. Once in the outer membrane, it is slowly cleaved by IcsP (SopA) between Arg⁷⁵⁸ and Arg⁷⁵⁹, thereby releasing the 95-kDa α domain into the culture supernatant or host cell cytosol (10, 12, 13, 42). To determine whether the deletions within IcsA altered either the amount of IcsA present or cleavage by IcsP, protein samples prepared from approximately equivalent numbers of bacteria of each construct in both *S. flexneri* SC560 and *E. coli* MBG263 were analyzed by Western blotting.

As expected, *S. flexneri* strains M90T (wild type) and MAC1000 (*icsA*⁺ [i.e., the wild-type *icsA* allele on pMBG235]) exhibited strong bands corresponding to the full-length 120-kDa protein in whole-cell extracts (Fig. 3A, lanes 1 and 3) and the cleaved 95-kDa polypeptide in supernatant extracts (Fig. 3B, lanes 1 and 3). MAC1016 (*icsA16*) gave bands in both whole-cell extracts (Fig. 3A, lane 6) and supernatant extracts (Fig. 3B, lane 6) that were approximately as strong as those

seen for M90T and MAC1000. Each of the other two constructs, MAC1003 (*icsA3*) and MAC1017 (*icsA17*), produced less protein in both whole-cell extracts (Fig. 3A, lanes 4 and 7) and supernatant extracts (Fig. 3B, lanes 4 and 7). For each construct, the apparent molecular masses of the observed bands correspond to the expected sizes of the deletion constructs. The relative faintness of the bands for MAC1003 and MAC1017 suggest that the mutant protein produced by alleles *icsA3* and *icsA17* might be slightly less stable than the native protein. To verify that the relative faintness of the signal was not due to partial loss of the epitope by the IcsA monoclonal antibody, Western blot analysis was also performed with a polyclonal antiserum and gave similar results (data not shown).

When expressed in *E. coli*, the level of protein expression from each of the mutant alleles was no different than that from the wild-type allele (Fig. 3C, lanes 3, 5, and 6 versus lane 1), suggesting that polypeptides expressed from *icsA3* and *icsA17* might be more stable in *E. coli* than in *S. flexneri*. The apparent molecular mass of each of the mutant constructs was appropriate. Since IcsA is not cleaved from the surface of *E. coli* MBG263, we did not examine supernatant protein preparations from the constructs in these strains.

Localization of IcsA on the surface of *S. flexneri* and *E. coli* is not altered by deletions within the rickettsial homology domain of IcsA. IcsA is distributed unipolarly on the surface of *S. flexneri* (16) and circumferentially on *E. coli* MBG263 (17). To assess whether the deletions involving the rickettsial homology domain affected surface localization of IcsA, indirect immunofluorescence was performed on each strain. IcsA is present on the surface of *S. flexneri* SC560 carrying the alleles *icsA3*, *icsA16*, and *icsA17* and is in the same unipolar distribution that it is on wild-type *S. flexneri* (Fig. 4 and data not shown). For the strains carrying the *icsA3*, *icsA16*, and *icsA17* alleles, up to 60 to 75% of the bacteria did not have detectable IcsA on the surface. Individual bacteria that had IcsA on the surface appeared qualitatively to have less surface IcsA than did individual wild-type organisms, as indicated by a less intense signal on indirect immunofluorescence (data not shown).

The expression of the *icsA* alleles on the surface of *E. coli* MBG263 is shown in Fig. 5. As for *E. coli* carrying the wild-

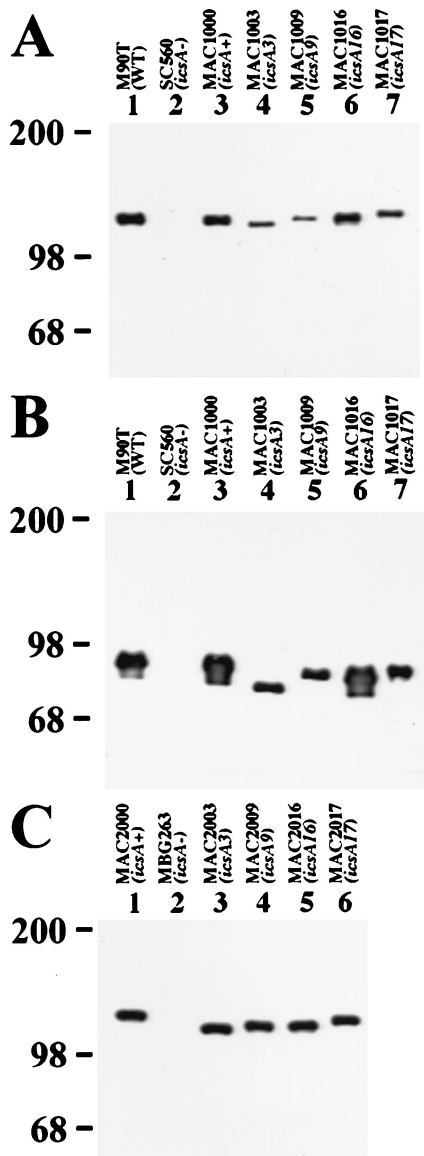


FIG. 3. Expression of IcsA constructs in *S. flexneri* SC560 (A and B) and *E. coli* MBG263 (C). Western blot analysis of whole-cell extracts (A and C) and supernatant extracts (B) was performed with monoclonal antibody to IcsA. For each panel, approximately equivalent amounts of protein preparations were loaded in each lane. Apparent molecular masses are indicated, in kilodaltons.

type allele of *icsA* (MAC2000) (Fig. 5A and B), *E. coli* carrying each of the mutant alleles displayed IcsA distributed circumferentially on the bacterial surface (Fig. 5C to F and data not shown). In *E. coli* MBG263, more than 90% of the bacteria observed for each strain displayed IcsA on the surface. In addition, the intensity of IcsA on the surface of MAC2003 (*icsA3*), MAC2016 (*icsA16*), and MAC2017 (*icsA17*) was approximately the same as that on MAC2000 (*icsA* WT).

Deletion of the rickettsial homology domain of IcsA results in loss of plaque formation in HeLa and L2 cell monolayers. The ability of a *Shigella* strain to enter and spread between mammalian cells can be assessed by its ability to form plaques on cell monolayers (34). Plaques result when *S. flexneri* successfully enter cells, multiply within them, and spread by actin-based motility into cells that are immediately adjacent and, subsequently, into the next layer of adjacent cells. SC560

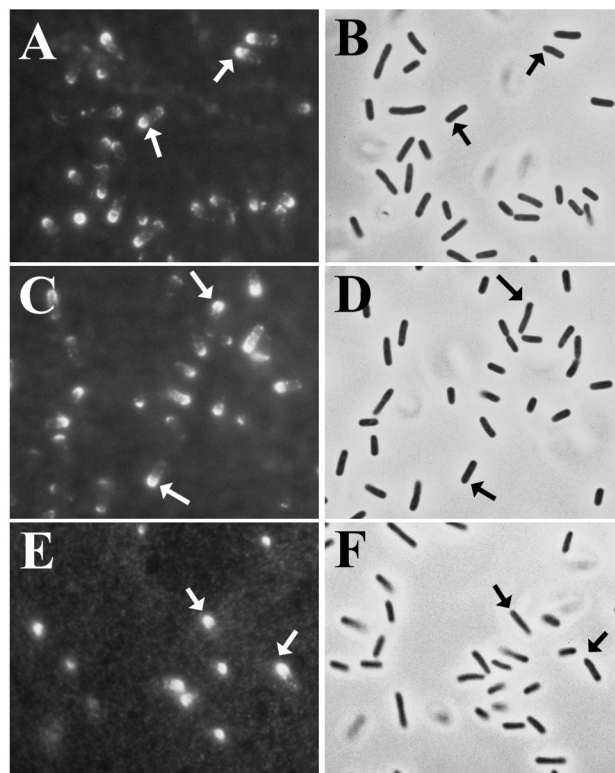


FIG. 4. Localization of IcsA on the surface of *S. flexneri* cells expressing IcsA constructs. Indirect immunofluorescence (A, C, and E) and phase-contrast (B, D, and F) micrographs are shown. (A and B) M90T (WT). (C and D) MAC1000 (*icsA* WT). (E and F) MAC1003 (*icsA3*). Arrows indicate unipolar localization of IcsA on bacteria. MAC1016 (*icsA16*) and MAC1017 (*icsA17*) each had a labeling pattern that was indistinguishable from that of MAC1003 (data not shown).

(*icsA*), while capable of entering cells and dividing within them, is unable to recruit actin and does not form plaques (10). To evaluate whether MAC1003 (*icsA3*) could form plaques, HeLa or L2 cell monolayers were infected with this strain. Unlike MAC1000 (*icsA* WT) (Fig. 6A, panel c, and 6B, panel c) and wild-type strain M90T (Fig. 6A, panel a, and 6B, panel a), MAC1003 (*icsA3*) (Fig. 6A, panel d, and 6B, panel d) did not form plaques on HeLa or L2 cells, indicating that it was either unable to enter cells or, once within the cell, unable to spread into adjacent cells. Of note, the plaques formed by wild-type strain M90T are larger than those formed by MAC1000 (*icsA* WT) (Fig. 6A and B, panels a versus panels c).

Deletions within the rickettsial homology domain of IcsA abrogate actin tail formation and actin-based motility in *S. flexneri*. To determine whether MAC1003 (*icsA3*) did not form plaques because of impairment in actin-based motility, HeLa cells were infected with this strain and the association of intracellular bacteria with actin was characterized. As expected, both the wild-type strain M90T (Fig. 7A and B) and MAC1000 (*icsA* WT) elaborated actin tails (Fig. 7C and D) and formed cell surface protrusions (data not shown). In contrast, MAC1003 (*icsA3*) gained access to the cytoplasm, but was not associated with actin and did not form tails (Fig. 7E and F) or cell surface protrusions (data not shown). In addition, while both MAC1016 (*icsA16*) and MAC1017 (*icsA17*) also gained access to the cytoplasm, neither was associated with actin or formed actin tails in the cytoplasm of HeLa cells (data not shown).

One possible explanation for the inability of MAC1003

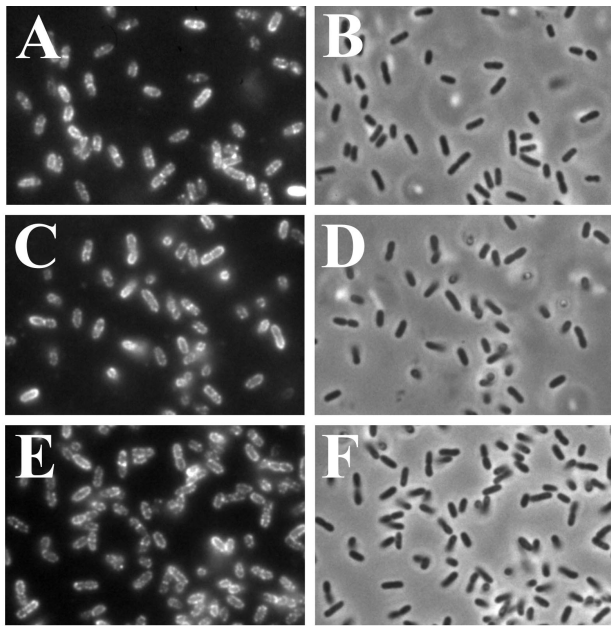


FIG. 5. Localization of IcsA on the surface of *E. coli* cells expressing IcsA alleles. Indirect immunofluorescence (A, C, and E) and phase-contrast (B, D, and F) micrographs are shown. (A and B) MAC2000 (*icsA* WT). (C and D) MAC2003 (*icsA3*). (E and F) MAC2009 (*icsA9*). MAC2016 (*icsA16*) and MAC2017 (*icsA17*) each had a labeling pattern that was indistinguishable from that of MAC2000 (data not shown).

(*icsA3*), MAC1016 (*icsA16*), and MAC1017 (*icsA17*) to associate with actin could be that the bacteria observed in the cytoplasm lacked surface IcsA. To ascertain whether the inability of MAC1003 (*icsA3*), MAC1016 (*icsA16*), and MAC1017 (*icsA17*) to recruit actin was attributable to reduced levels of surface IcsA, we evaluated the ability of the mutant constructs to recruit actin in the *E. coli* MBG263 background, since more than 90% of MBG263 cells carrying these alleles had levels of IcsA on their surfaces comparable to those on *E. coli* carrying the wild-type allele (*icsA* WT) (Fig. 5). We have previously shown that *X. laevis* egg cytoplasmic extracts support actin-based motility of IcsA-expressing *E. coli* MBG263 (15). Unlike MAC2000 (*icsA* WT), which moved at an average rate of $12.9 \pm 7.8 \mu\text{m}/\text{min}$ in *Xenopus* egg cytoplasmic extracts (Fig. 8B), MAC2003 (*icsA3*), MAC1016 (*icsA16*), and MAC1017 (*icsA17*) did not associate with actin or move (Fig. 8A and data not shown).

Deletion of the rickettsial homology domain of IcsA leads to relative unmasking of IcsA within the host cell cytosol. As mentioned above, a possible explanation for the inability of MAC1003 (*icsA3*), MAC1016 (*icsA16*), and MAC1017 (*icsA17*) to associate with actin in the cytosol would be a lack of IcsA on the surface of these strains in this setting. We therefore labeled MAC1003-infected cells for IcsA by using affinity-purified polyclonal antiserum. Approximately 70 to 75% of the observed intracellular bacteria displayed fluorescent label. Surprisingly, the signal was consistently more intense on MAC1003 (*icsA3*) (Fig. 9G and H) than on wild-type M90T (Fig. 9A and B) or MAC1000 (*icsA* WT) (Fig. 9E and F). This suggests that the mutant IcsA on MAC1003 was more accessible to the antibody than native IcsA on M90T or MAC1000 (*icsA* WT). Consistent with this, the signal from IcsA⁺ extracellular bacteria labeled with the same antiserum is significantly more

intense than that from intracellular bacteria of any of the strains examined (data not shown).

Replacement of IcsA amino acids 446 to 506 with the rickettsial repeat domain does not restore actin tail assembly. To examine whether the rickettsial repeat domain that bears sequence similarity to IcsA amino acids 446 to 506 could functionally complement the similar sequence of IcsA, the coding sequence of each of three distinct repeat sequences from *R. rickettsii* OmpA was exchanged for that for amino acids 446 to 506 of IcsA, creating alleles *icsA7*, *icsA8*, and *icsA9* (Fig. 1C).

The rickettsial repeats exchanged for IcsA amino acids 446 to 506 differ in length (Fig. 1C); *icsA7*, *icsA8*, and *icsA9* are predicted to encode fusion proteins of 122, 126, and 120 kDa, respectively. On Western blot analysis, the *S. flexneri* strains carrying these alleles, strains MAC1007 (*icsA7*), MAC1008 (*icsA8*), and MAC1009 (*icsA9*), exhibited bands corresponding to the predicted masses of the fusion proteins that were fainter than the bands corresponding to wild-type IcsA (Fig. 3A and B, lane 5, and data not shown versus Fig. 3A and B, lanes 1 and 3). For each of these constructs in *S. flexneri*, a significantly lower percentage of bacteria expressed IcsA on the surface than for MAC1000 (*icsA* WT). None of these constructs in *S. flexneri* formed plaques on HeLa or L2 cell monolayers or associated with actin in the cytoplasm of infected HeLa cells (data not shown).

The *E. coli* MBG263 strains carrying these alleles, MAC2007 (*icsA7*), MAC2008 (*icsA8*), and MAC2009 (*icsA9*), exhibited bands corresponding to the predicted masses of the fusion proteins that were of the same approximate intensity as the bands corresponding to wild-type IcsA (Fig. 3C, lane 4, and

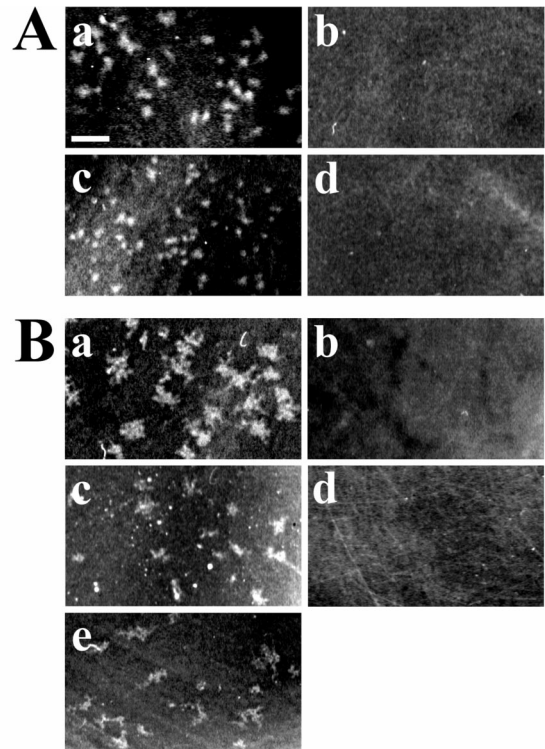


FIG. 6. Plaque formation of *S. flexneri* cells expressing IcsA constructs. (A) HeLa cell monolayers. a, M90T (WT); b, SC560 (*icsA*); c, MAC1000 (*icsA* WT); d, MAC1003 (*icsA3*). (B) L2 cell monolayers. a to d, same as in panel A; e, MAC1010 (*icsA10*). Plaques formed by MAC1014 (*icsA14*) and MAC1015 (*icsA15*) were not significantly different from those formed by MAC1010 (*icsA10*). Petri dishes were photographed 48 h after infection. Bar, 2 mm.

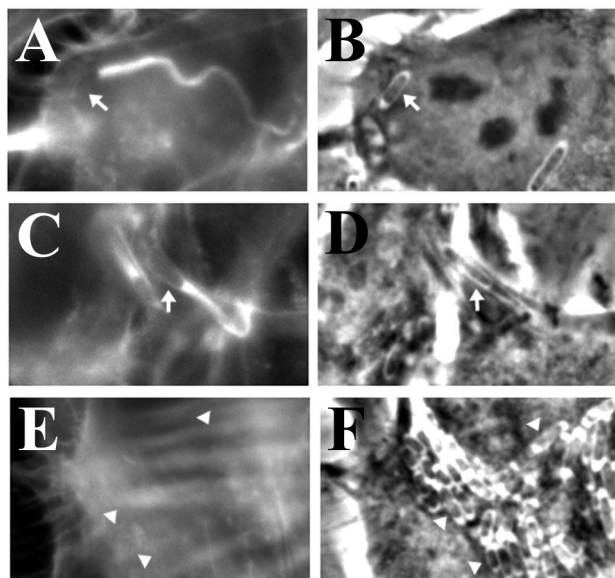


FIG. 7. Actin tail formation of *S. flexneri* expressing IcsA constructs. Rhodamine-phalloidin signal (A, C, and E) and phase-contrast (B, D, and F) micrographs are shown. (A and B) *S. flexneri* M90T (WT). (C and D) MAC1000 (*icsA* WT). (E and F) MAC1003 (*icsA3*). Arrows indicate bacteria with tails; arrowheads indicate bacteria lacking tails.

data not shown versus Fig. 3C, lanes 1 and 3). Each of these strains expressed IcsA on the bacterial surface to the same extent and in the same circumferential distribution as did MAC2000 (*icsA* WT). However, none either associated with actin or moved in *Xenopus* egg cytoplasmic extracts (data not shown).

To evaluate potentially critical contact residues within IcsA amino acids 446 to 506, clusters of charged residues, which are likely to be exposed on the surface of the protein and therefore to be involved in protein-protein interactions, were identified and subjected to site-directed mutagenesis. This strategy has been successfully applied in a number of cases (3, 51). Three sets of mutations were introduced in the region (Fig. 2B): the first replaced amino acids Asp⁴⁸⁷, Asp⁴⁸⁹, and Asp⁴⁹¹ with alanines (giving *icsA10*); the second replaced Asp⁴⁶⁷ and Lys⁴⁷⁰ with alanines (*icsA14*); and the third replaced Asp⁴⁷⁵ and Lys⁴⁷⁸ with alanines (*icsA15*). *S. flexneri* strains carrying the resultant *icsA* alleles were no different from *S. flexneri* carrying the wild-type allele (MAC1000) in any of the assays performed: they expressed protein at wild-type levels on Western blot analysis, expressed IcsA on the surface of approximately the same percentage of bacteria and at approximately the same intensity, formed plaques on HeLa and L2 cell monolayers that were not significantly different from MAC100 (Fig. 6B, panel c versus panel e), formed actin tails in HeLa cells, and moved in the cytoplasm of HeLa cells at speeds comparable to MAC1000 (*icsA* WT) (data not shown).

Competition of the rickettsial homology domain polypeptide in actin recruitment assays. To further examine the role of IcsA amino acids 446 to 506 in actin-based motility, a polypeptide that consists of GST fused to amino acid residues 447 to 505 of IcsA (GST::IcsA67) was used in competition assays with MAC2000 (*icsA* WT) in the *Xenopus* egg cytoplasmic extracts. The polypeptide is greater than 90% pure (Fig. 10). As shown in Table 2, the fusion protein had no significant effect on either (i) the percentage of bacteria associated with actin clouds, which are accumulations of polymerized actin that is not or-

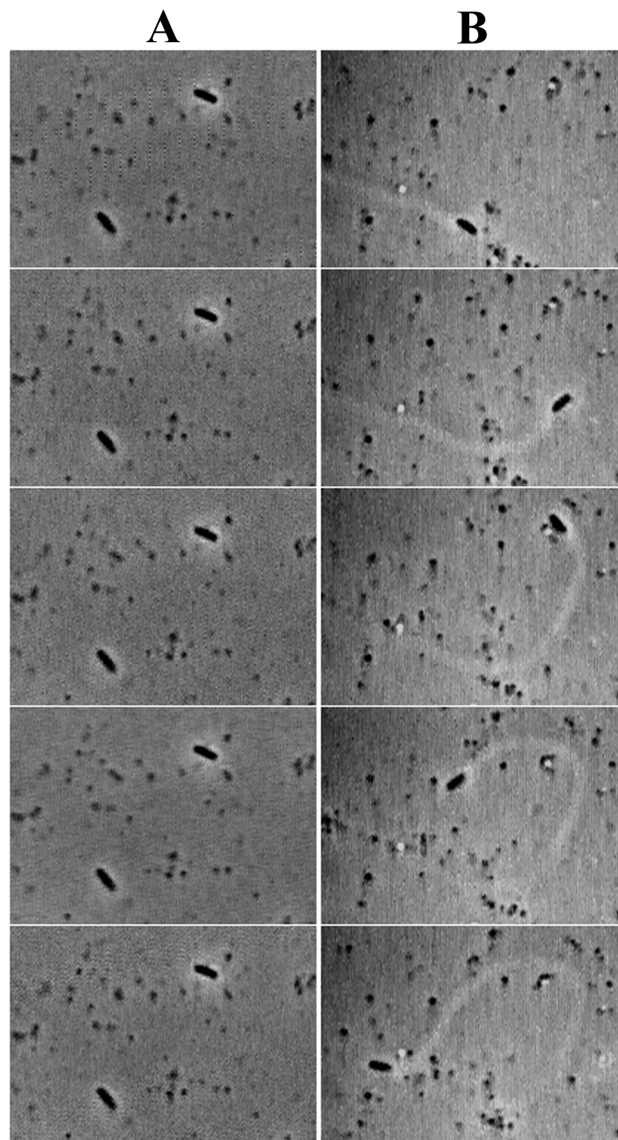


FIG. 8. Motility assay of *E. coli* MBG263 carrying IcsA constructs. Phase-contrast time-lapse images in *Xenopus* egg cytoplasmic extracts of MAC2003 (*icsA3*) (A) and MAC2000 (*icsA* WT) (B) are shown. The actin tail is seen as a phase-lucent streak behind moving MAC2000. Sequential frames are separated by 30-s intervals. Images of MAC2016 (*icsA16*) and MAC2017 (*icsA17*) were indistinguishable from those of MAC2003.

ganized into a bundle, or actin tails or (ii) the percentage of bacteria assembling actin tails. Further, the IcsA-derived peptide alone (after removal of GST) had no effect on actin polymerization in these assays (unpublished data).

DISCUSSION

Shigella spp., like *L. monocytogenes*, spotted-fever group *Rickettsiae*, and vaccinia virus, are able to recruit host cell monomeric actin into a tail that consists of filamentous actin bundled in a parallel array, with the barbed end of the filaments uniformly directed towards the bacterium. Monomeric actin is incorporated into the filaments at the junction of the bacterium with the tail (47). The mechanism of actin tail formation can therefore be presumed to involve recruitment of

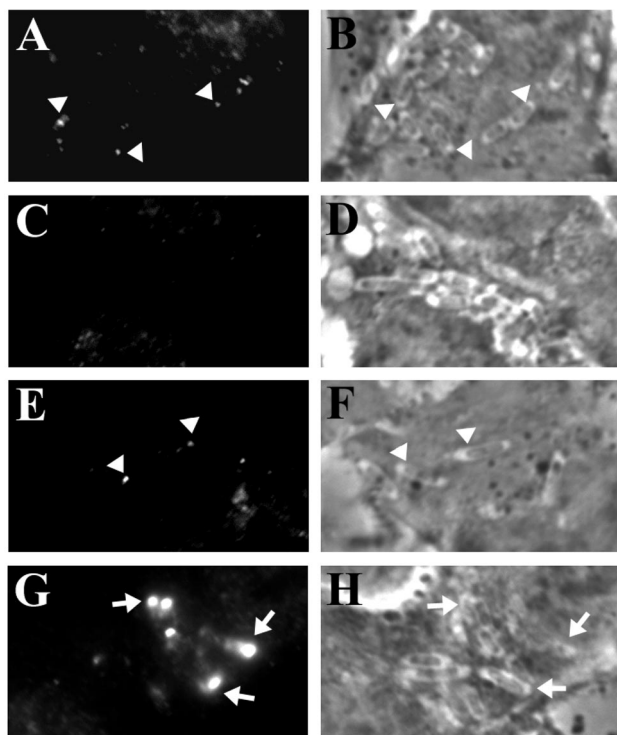


FIG. 9. Immunolabeling of IcsA on intracellular *S. flexneri* strains carrying IcsA alleles. Indirect immunofluorescence micrographs (A, C, E, and G), with affinity-purified polyclonal antiserum to IcsA, and phase-contrast micrographs (B, D, F, and H) are shown. (A and B) M90T (WT). (C and D) SC560 (*ics4*). (E and F) MAC100 (*ics4* WT). (G and H) MAC1003 (*icsA3*). The apparent output of the immunofluorescence signal was normalized for panels A, C, E, and G by setting the background nonspecific signals to equivalent levels. Arrows indicate intense signal from IcsA on MAC1003; arrowheads indicate significantly weaker signal from IcsA on M90T and MAC1000.

monomeric actin to the bacterial pole, nucleation of this actin, orientation of the nuclei such that the barbed end faces the bacterium, polymerization from the barbed end while protecting it from barbed end capping proteins, and bundling of the filament into a parallel array. We have previously shown that the *S. flexneri* outer membrane protein IcsA is located unipolarly on the bacterium at the site of the forming tail (16), and we and others have shown that IcsA expressed in *E. coli* is

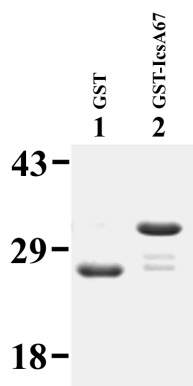


FIG. 10. Purified IcsA rickettsial homology domain used in competition assays. A Coomassie-stained sodium dodecyl sulfate-polyacrylamide gel of GST (lane 1) and GST::IcsA67 (lane 2) is shown. Apparent molecular masses are indicated, in kilodaltons.

TABLE 2. Competition assay with IcsA rickettsial homology domain polypeptide fused to GST

Competitor	Concn (μ M)	Accumulation of polymerized actin ^a	Assembly of actin tail ^b
GST::IcsA67	35	55 \pm 16	22 \pm 8.3
GST::IcsA67	0.35	66 \pm 12	18 \pm 8.4
GST::IcsA67	0.035	71 \pm 15	12 \pm 8.6
GST	35	34 \pm 23	17 \pm 15
PBS		73 \pm 7.6	24 \pm 7.4

^a Values are percentages (mean \pm standard deviation) of bacteria associated with actin clouds or actin tails in six separate experiments.

^b Values are percentages (mean \pm standard deviation) of bacteria with actin tails in six separate experiments.

sufficient to permit actin tail formation in cytoplasmic extracts (17, 26). Thus, IcsA is able to induce the specific series of steps outlined above.

In this work, we have identified a region of IcsA that is necessary for the interaction of IcsA with host cytoskeletal factors involved in actin assembly on *S. flexneri*. Deletion of the domain of amino acid residues 446 to 506 totally eliminates actin-based motility of either *S. flexneri* or *E. coli* strains that express the IcsA deletion construct. The mutant *Shigella* strain does not form actin tails or cell surface protrusions, does not recruit actin into a cloud at the bacterial surface, and does not form plaques on cell monolayers, indicating an inability to spread from cell to cell. Most striking, however, is the relative unmasking of the IcsA on the surface of *S. flexneri* strains expressing the mutant allele when in the cytoplasm of infected cells (Fig. 9). This suggests that native IcsA is made relatively inaccessible to antibody by its interactions with host cell factors, while the mutant allele is accessible by virtue of few or no such interactions.

These observations suggest that the deletion of amino acids 446 to 506 of IcsA either (i) leads to loss of a domain that directly interacts with key host cell factors or (ii) disrupts the native conformation of IcsA so as to impair the interaction of the IcsA domain that binds key host cell factors with those factors. On the basis of data presented here, we favor the latter. First, with the exception of the IcsA construct encoded by *icsA16*, all of the IcsA deletion constructs examined in this study are less stably expressed in *S. flexneri* and presented in lower amounts of the bacterial surface (Fig. 3 to 5). Second, alanine mutagenesis of clusters of charged residues within the region, which would be predicted to be on the surface of the protein in this region and therefore likely to interact with outer proteins, resulted in maintenance of the wild-type phenotype. Third, each of the small nonoverlapping deletions within amino acid residues 446 to 506 encoded by *icsA16* and *icsA17* has the phenotype of the larger deletion, which suggests that the intact rickettsial homology domain may be required for proper spacing or proximity of other domains. And finally, in vitro competition of a polypeptide of the region fused to GST did not alter the motility of the wild-type construct (Table 2). These data provide further refinement on the large region, encompassing amino acids 320 to 507, that was reported by Suzuki et al. to be required for actin recruitment (46).

IcsA is thought to cross the bacterial cytoplasmic membrane via the Sec secretion machinery, and subsequent translocation of the α domain of IcsA to the bacterial surface has been proposed to occur through a pore formed by 13 passages of the β domain through the outer membrane (45). We observed a moderate decrease in the surface presentation of most of the mutant deletion constructs expressed in *S. flexneri*. Notably,

when expressed in *E. coli*, the amount of IcsA on the bacterial surface was not detectably decreased from those for native IcsA. IcsA was nevertheless unable to induce actin-based motility. The differences in levels of expression on the bacterial surface between the *S. flexneri* background and the *E. coli* background suggest that the mechanism of translocation of IcsA to the bacterial surface may differ in *S. flexneri* and *E. coli*.

Despite the sequence similarity between IcsA amino acids 446 to 506 and the repetitive domains of the rickettsial OmpA proteins, exchange of these domains did not yield a functional form of IcsA. One possible explanation for this is that the exchange of the rickettsial domain did not reconstitute the protein to its native conformation, as suggested by the observation that the levels of expression on the bacterial surface and the level of protein in whole-cell preparations did not return to those seen with wild-type IcsA. The presence of homologous domains in rickettsial OmpA suggests that the function served by these domains may be generalizable among a group of outer membrane proteins. How the function of the multiple copies of the domain that are present in rickettsial OmpA would differ from the function of the single copy that is present in IcsA is unclear.

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