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Avirulence of Rough Mutants of *Shigella flexneri*: Requirement of O Antigen for Correct Unipolar Localization of IcsA in the Bacterial Outer Membrane

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Mutations in the lipopolysaccharide (LPS) of *Shigella* spp. result in attenuation of the bacteria in both in vitro and in vivo models of virulence, although the precise block in pathogenesis is not known. We isolated defined mutations in two genes, *galU* and *rfe*, which directly affect synthesis of the LPS of *S. flexneri* 2a, in order to determine more precisely the step in virulence at which LPS mutants are blocked. The *galU* and *rfe* mutants invaded HeLa cells but failed to generate the membrane protrusions (fireworks) characteristic of intracellular motility displayed by wild-type shigellae. Furthermore, the *galU* mutant was unable to form plaques on a confluent monolayer of eucaryotic cells and the *rfe* mutant generated only tiny plaques. These observations indicated that the mutants were blocked in their ability to spread from cell to cell. Western immunoblot analysis of expression of IcsA, the protein essential for intracellular motility and intercellular spread, demonstrated that both mutants synthesized IcsA, although they secreted less of the protein to the extracellular medium than did the wild-type parent. More strikingly, the LPS mutants showed aberrant surface localization of IcsA. Unlike the unipolar localization of IcsA seen in the wild-type parent, the *galU* mutant expressed the protein in a circumferential fashion. The *rfe* mutant had an intermediate phenotype in that it displayed some localization of IcsA at one pole while also showing diffuse localization around the bacterium. Given the known structures of the LPS of wild-type *S. flexneri* 2a, the *rfe* mutant, and the *galU* mutant, we hypothesize that the core and O-antigen components of LPS are critical elements in the correct unipolar localization of IcsA. These observations indicate a more precise role for LPS in *Shigella* pathogenesis.

Bacteria of the genus *Shigella* are the major cause of bacillary dysentery in humans. The pathogenicity of these bacteria is centered on the ability of the organisms to penetrate the target mammalian cell, multiply within the cell, and spread intercellularly (19). In the human host, these properties lead to cell death and a severe inflammatory response in the epithelial lining of the large intestine. Isolation of *Shigella* mutants defective in one or more steps required for full virulence has been invaluable in helping to define the gene functions involved. It has been shown that a large (180- to 230-kbp) plasmid resides in virulent strains of shigellae and encodes the genes responsible for invasion and multiplication within mammalian cells (37). Subsequent work in the past decade has resulted in identification and characterization of a variety of genes which contribute to the pathogenic phenotype of *Shigella* spp. (for a recent review, see reference 9). One of these genes, *icsA*, encodes a protein essential for intercellular spread, which is present on the bacterial cell surface and secreted into the extracellular medium (2). IcsA catalyzes the polymerization of actin monomers in the host cell cytoplasm at one pole of the bacterium, resulting in the dramatic intracellular motility of *Shigella* organisms which is seen in infected mammalian cells (2, 8).

Mutations in a pathogen often create pleiotropic defects which can indirectly affect expression of functions required for virulence. Such is the case with mutations that affect certain nutritional requirements, which preclude survival of the organism in vivo. Another type of pleiotropic mutation affects lipopolysaccharide (LPS) synthesis in gram-negative pathogenic bacteria. Hence, rough mutants of many pathogens are no longer virulent. The same pleiotropic effect on virulence has been demonstrated for *Shigella flexneri* and *S. dysenteriae* 1 as well (29–31, 36, 48). While these reports showed a relationship between LPS structure and the pathogenicity of *Shigella* spp., many of these studies were performed with spontaneous mutants whose genetic defects were not well defined. In addition, comparisons of pathogenic ability did not always involve use of isogenic strains. This situation precluded the assignment of specific molecular roles in virulence to genes involved in LPS biosynthesis. More recently, Rajakumar et al. identified a series of mutations in the *rfb* locus of *S. flexneri* which result in defective cell-to-cell spread of the bacteria (32).

In the course of generating random chromosomal insertions of Tn*10* into a wild-type strain of *S. flexneri* 2a, we isolated a mutation in *galU*, a gene with a well-defined role in the LPS biosynthetic pathway. The mutation in *galU*, which encodes uridine diphosphoglucose (UDP-glucose) pyrophosphorylase, results in synthesis of an incomplete LPS core with no O antigen attached. This mutant strain of *S. flexneri* was tested in

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virulence assays that measure the different steps of invasion, intracellular multiplication, and intercellular spread. Our results confirm the earlier reports of attenuated virulence of rough mutants and show that this mutant is blocked in its ability to spread from cell to cell. We extend these reports through the analysis of an additional mutant with a defect in LPS biosynthesis to define a molecular mechanism underlying this defect in pathogenicity. Our results suggest a model which involves an interaction between IcsA and the O antigen and outer core of LPS that is essential for the correct localization of IcsA to one pole of the bacterium, where it acts as the motor for intracellular motility and intercellular spread of *Shigella* spp.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains used in these experiments are all derivatives of the wild-type *S. flexneri* 2a strain 2457T (19) with the exception of BS441, which is an *icsA* mutant derived from *S. flexneri* 5 strain M90T (2). BS484 was constructed by P1L4 transduction of *rfe*::Tn*10-48* from *Escherichia coli* 21548 (25) into 2457T with selection for tetracycline resistance. The rough LPS phenotype was confirmed as described below. All bacteria were grown at 37°C in tryptic soy broth (Difco Laboratories, Detroit, Mich.) unless otherwise stated.

Genetic techniques. Random transposon Tn*10* mutagenesis of 2457T was done as described by Silhavy et al. (42). Tn*10* insertions were routinely backcrossed into 2457T by P1L4 transduction to ensure the presence of a single transposon insertion in the otherwise wild-type background. Transduction mapping and preparation of lysates of the generalized transducing phage P1L4 were done by the methods of Miller (26). When selection was applied for inheritance of Tn*10*, the medium was supplemented with 5 μg of tetracycline per ml.

Assays for the rough phenotype. Slide agglutination tests with *S. flexneri* 2a typing serum (Difco Laboratories) were performed on purified colonies grown overnight on tryptic soy broth plus 1.5% agar at 37°C. Sensitivity to rough phenotype-specific bacteriophages was measured on eosin methylene blue agar base (Difco) containing 0.1% glucose, 0.5% NaCl, 0.5% yeast extract (Difco), and 10 mM $MgSO_4$. The enzyme assay for UDP-glucose pyrophosphorylase activity was performed by the method of Hansen et al. (10).

Isolation and analysis of bacterial LPS. Proteinase K-treated whole-cell lysates were prepared from 18-h cultures by the procedure of Hitchcock and Brown (11). Approximately 1 μg of LPS was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 13% isocratic gel in Tris-glycine buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS [pH 8.3]) at a constant current of 30 mA per gel for 4 h at 10°C. The gel was fixed overnight in 40% ethanol–5% acetic acid and then silver stained (44).

Virulence assays. The HeLa cell invasion assay was performed as previously described with minor modifications (13). To preserve the membrane protrusions (fireworks) induced in the HeLa cell membranes by intracellular movement of the bacteria, infected cells were fixed with a solution of 0.8% glutaraldehyde–2.0% formaldehyde in phosphate-buffered saline for 5 min at 4°C before being stained with Giemsa. Bacterial invasion was scored as (number of HeLa cells invaded/total number of HeLa cells counted) × 100. A minimum of 200 cells in random fields were counted for each assay plate.

The plaque assay, used to determine intercellular spread, was performed as originally described by Oaks et al. (28). The Sereny test (40) was used as an additional means of assessing a strain's ability to invade, multiply intracellularly, and spread from cell to cell.

Protein preparation and immunoblot analysis. Whole-cell and supernatant protein extracts were prepared as previously described (1, 12). The relative amounts of proteins produced by the different strains were estimated from the intensity of the signals produced on Western immunoblot analysis. Western blotting was performed essentially as previously described (4), with a rabbit IcsA antiserum (8) and subsequent visualization by enhanced chemiluminescence (ECL; Amersham Life Science, Arlington Heights, Ill.).

Labelling of bacteria grown in vitro and bacteria infecting HeLa cells. Bacteria grown in vitro were labeled as previously described (8) with a mouse-derived monoclonal antibody against IcsA (HybridoLab, Institut Pasteur, Paris, France). For labelling of bacteria in HeLa cells, cells were prepared and infected as previously described (5, 27). After fixation with 3.7% paraformaldehyde in phosphate-buffered saline for 20 min at room temperature, cells were permeabilized with 0.1% Triton X-100 in Tris-buffered saline for 30 min at room temperature and then blocked with 10 mg of bovine serum albumin per ml in the same buffer for 10 min at room temperature. Polymerized actin was labeled as previously described (27) with rhodamine-conjugated phalloidin (Molecular Probes, Inc., Eugene, Oreg.). Fluorescence microscopy was performed with a Diaphot 200 microscope (Nikon, Inc., Melville, N.Y.). Confocal microscopy was performed with a Nikon confocal microscope with a 60× Planapo lens (Nikon) and standard filters (Bio-Rad Laboratories, Inc., Hercules, Calif.). The distance between adjacent sections was 0.5 μm.

DNA sequencing and data analysis. Previous work by Hromockyj et al. (14) localized the *galU* gene to a 1.8-kbp *Acc*I-*Eco*RI fragment which also contains the *virR*/*hns* gene, a negative regulator of *Shigella* virulence. This fragment was isolated from a digest of *S. flexneri* chromosomal DNA and cloned into pBR329 to yield pAEH122. DNA-sequencing reactions were performed by the double-stranded dideoxy-chain termination method (35) with a Sequenase 7-deaza-dGTP DNA sequencing kit (United States Biochemical, Cleveland, Ohio) and [^{35}S]dATP (Amersham). pUC/M13 universal and reverse primers (United States Biochemical) and oligonucleotides synthesized from sequence data were used with pAEH122 as the template. The University of Wisconsin Genetics Computer Group DNA software package (7) was used to assemble and analyze the nucleotide sequence data generated.

To determine the site of Tn*10* insertion in the *galU* gene, the regions spanning the 5′ and 3′ termini of *galU* and the transposon were amplified from BS109 with oligonucleotide primers complementary to the ends of the transposon (5′-GAATTGGTAAAGAGAGTCGTG-3′), from the 5′ region upstream of *galU* (5′-GCTGACTGCGCTTGATGTTG-3′) and at the 3′ end (5′-GGATAACAC CGATACGGATG-3′). The PCR products, of 985 and 406 bp, respectively, were purified and sequenced from one end of the transposon toward the *galU* gene.

Nucleotide sequence accession number. The nucleotide sequence of the *galU* coding region and adjacent sequence from *S. flexneri* 2457T has been assigned the GenBank accession number L32811.

TABLE 1. Cotransduction mapping of Tn*10* insertion in BS109

Donor	Recipient	Selected marker	Unselected marker	Cotransduction frequency (%)
BS109	BS124[a]	Trp$^+$	Tc^r, Gal$^-$	51
		Tc^r	Trp$^+$	71
		Tc^r	Gal$^-$	100
		Tc^r	Sensitivity to rough-specific phage	100
2457T	BS109	Gal$^+$	Tc^s	100
		Gal$^+$	Resistance to rough-specific phage	100

[a] *S. flexneri trp-1 thr-1.*

RESULTS

Isolation and identification of a Tn*10* insertion in *galU*. In the course of creating a bank of random Tn*10* insertions in a λ-sensitive strain of *S. flexneri* 2a, we had previously identified an insertion which resulted in a rough phenotype (24). The rough nature of this mutant strain, BS109, was confirmed by its newly acquired sensitivity to several "rough"-specific phages (e.g., Br10, Br60, and C21) and the characteristic broad diffuse pellet which formed on gravity sedimentation of an overnight culture in tryptic soy broth left at room temperature for several hours. BS109 also failed to agglutinate in typing serum specific for *S. flexneri* serotype 2a. Since rough mutants of pathogenic microorganisms generally are nonvirulent, we sought to precisely identify the genetic defect in this mutant in order to ascertain how the mutation might alter virulence. Mutations in a variety of different genetic loci can lead to a rough phenotype (33). In addition to altering LPS biosynthesis, the mutation in strain BS109 caused the strain to be unable to utilize galactose as the sole carbon source. Enzymatic reactions involved in galactose utilization are also required for LPS synthesis in *Shigella* spp. Thus, on the basis of the phenotype it causes, the mutation in BS109 was either in *galE* (UDP-galactose-4-epimerase) or in *galU* (UDP-glucose pyrophosphorylase). We therefore first determined whether the Tn*10* insertion mapped to the *galKTE* operon or to the *trp* operon, which is closely linked to *galU*. Transductional analysis with bacteriophage P1L4 showed close linkage of the Tn*10* marker with *trp* (Table 1). The cotransduction frequency indicated a genetic linkage similar to that seen between *galU* and *trp* in *E. coli* K-12 (41).

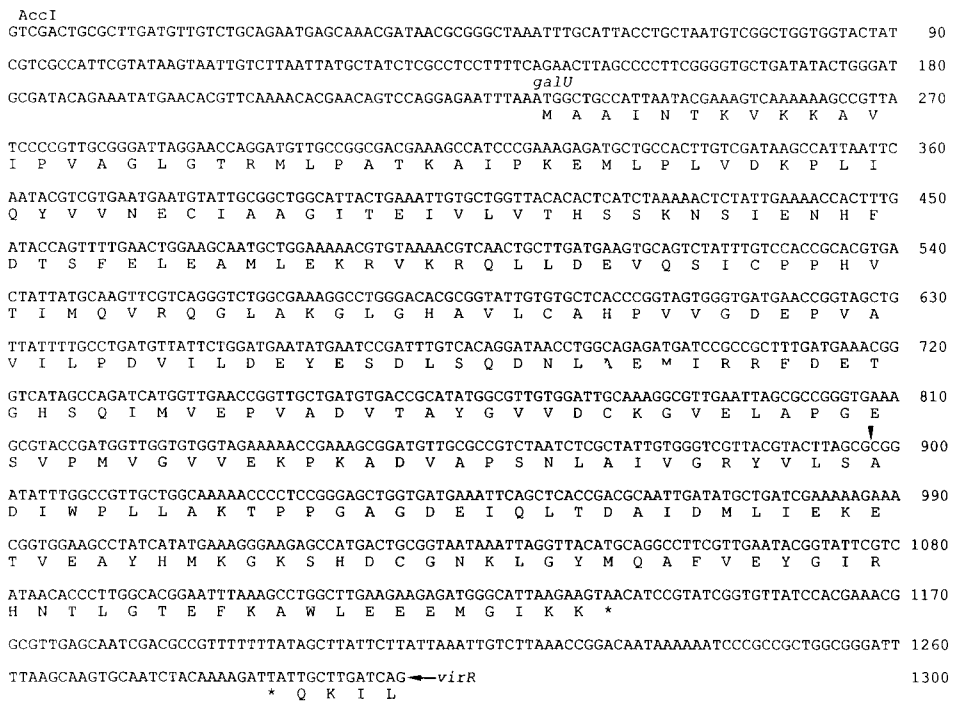

FIG. 1. Nucleotide sequence of the *S. flexneri* 2a *galU* gene and the flanking DNA. The *galU* sequence was determined from the 1.8-kb *Acc*I-*Eco*RI fragment cloned in pAEH122. The single-letter amino acid designation for the *galU* open reading frame and the extreme carboxy-terminal end of the *virR* gene is shown. The arrowhead indicates the site of the Tn*10* insertion in the *galU* gene in BS109.

Thus, the mutation which created the rough phenotype in BS109 was identified as *galU*::Tn*10*. Biochemical evidence that insertion of the transposon had altered expression of the *galU* gene in BS109 was provided by the absence of detectable UDP-glucose pyrophosphorylase activity (data not shown).

The results of the P1L4 transduction experiments also demonstrated that resistance to tetracycline was 100% linked with the rough and galactose-negative phenotypes (Table 1), thereby confirming that only a single insertion of Tn*10* in BS109 was responsible for the observed phenotype. In addition, selection for spontaneous reversion to galactose utilization always resulted in loss of resistance to tetracycline (data not shown), which is consistent with the presence of a single Tn*10* insertion in BS109.

Cloning and sequencing of the *galU* gene. Previous work on the virulence gene regulator *virR* demonstrated its close genetic linkage with *galU*. Both *galU* and *virR* are encoded on a 1.8-kbp *Acc*I-*Eco*RI fragment from the *S. flexneri* 2a chromosome (14). This fragment was cloned from 2457T into pBR329 to yield pAEH122, and the ability of pAEH122 to complement the *galU* mutation in BS109 was confirmed. The complete nucleotide sequence of the *galU* gene was determined from pAEH122 and is shown in Fig. 1. The *galU* and *virR* genes are transcribed in a convergent orientation, with the 3′ end of *galU* ending 143 bp from the 3′ end of the *virR* gene. A search of the GenBank data base revealed that the *galU* sequence of *S. flexneri* is similar to that reported for *E. coli* K-12 (45). Weissborn et al. recently presented definitive proof that the *galU* gene is the structural gene for UDP-glucose pyrophosphorylase (49). Similarity in nucleotide sequence was also noted for open reading frame 2.8 in *Salmonella typhimurium* (15). Strong similarity was seen at the NH_2 terminus among the amino acid sequences of *galU*, the UDP-glucose pyrophosphorylase gene (*gtaB*) of *Bacillus subtilis* (46), and the UDP-glucose pyrophosphorylase gene (*celA*) of *Acetobacter xylinum* (3). The site of the Tn*10* insertion in BS109 was in the *galU* open reading frame between bases 897 and 898 (Fig. 1) from the *Acc*I site of pAEH122.

Behavior of *galU*::Tn*10* mutant in assays for virulence. Several in vitro and animal models are available to assess the different steps in *Shigella* pathogenesis. Invasion can be measured in tissue culture by infecting semiconfluent monolayers of HeLa cells. The wild-type strain 2457T is highly efficient at invasion and reproducibly invaded more than 90% of the monolayer after the 2-h assay (Fig. 2a). The *galU* mutant BS109 was also highly efficient at invasion of mammalian cells in tissue culture. However, we observed that BS109 failed to form fireworks in infected cells (Fig. 2b). Since formation of fireworks is characteristic of intracellular motility of *S. flexneri*, these results suggested that the block in virulence caused by the mutation in *galU* is at the level of cell-to-cell spread of the bacteria.

A more rigorous assay for virulence is the plaque assay, since plaque formation depends on the ability of the bacteria to invade, multiply intracellularly, and then spread intercellularly. A marked difference in virulence between 2457T and BS109 was observed in this assay. BS109 failed to form plaques, even when the period of incubation was extended to 72 h (Fig. 3b). High multiplicities of BS109 also failed to yield plaques.

An even more stringent test of the ability of the bacteria to invade, multiply intracellularly, and then spread intercellularly is the Sereny test of bacterial infection within the corneal epithelial tissues of the eye of a guinea pig. Similar to the results obtained with the plaque assay, BS109 failed to provoke a positive reaction in the Sereny test. To confirm that the defect in virulence was due only to the mutation in *galU* caused by the insertion of Tn*10*, a lysate of P1L4 was prepared on the wild-type parent 2457T and used to transduce BS109 to *galU*$^+$.

All seven transductants that were selected for their ability to utilize galactose as a carbon source and reacquired sensitivity to tetracycline regained full virulence as measured in the HeLa invasion assay and Sereny test (data not shown).

Virulence phenotype of an *rfe* mutant. We sought to determine the minimal structure of LPS necessary for expression of intercellular motility of *S. flexneri* 2a. A *galU* mutant synthesizes an incomplete core since it is unable to make UDP-glucose and therefore cannot incorporate the first glucose residue into the outer core (33). Glucose, galactose, and *N*-acetylglucosamine (GlcNAc) complete the core of *S. flexneri* (43). GlcNAc is also the first residue of the tetrasaccharide repeat common to the O antigen of all serotypes of *S. flexneri* (21). In the first step of O-antigen synthesis in *S. dysenteriae* 1 and *E. coli*, the product of the *rfe* gene is required to transfer GlcNAc to the lipid carrier (16, 34). An *rfe* mutant of *S. flexneri* would therefore express a complete core but be unable to synthesize the O antigen (Fig. 4). The *rfe*::Tn*10-48* mutation in *E. coli* 21548 was moved into 2457T by P1 transduction and selection for resistance to tetracycline. The phenotype of the *rfe* mutant, BS484, was confirmed by analysis of the LPS structure (Fig. 5). Examination of silver-stained gels demonstrated that BS484 did not produce the typical LPS structure seen in the wild-type parent, 2457T. The BS484 component migrated with the first component of 2457T, showing that it contains a complete core but does not add the O side chain. By comparison, the BS109 component migrated with the R_d *Salmonella* LPS marker, which consists of lipid A and terminates in the core with the addition of heptose (data not shown). This size is consistent with the predicted LPS structure of a *galU* mutant.

BS484 was examined in the virulence assays used above, and its behavior was compared with that of 2457T (the wild-type parent) and BS109 (the *galU* mutant). In the HeLa cell invasion assay, although BS484 was invasive, it behaved similarly to BS109 in that it failed to generate fireworks in the infected cells. In contrast, in the plaque assay, BS484 behaved in a manner intermediate between that of BS109 and that of 2457T. BS484 formed very small plaques with irregular edges (Fig. 3c and d). The plaque-forming efficiency of BS484 was 50 to 70% that of 2457T. An exact determination was difficult because of the small size and diffuse edges of the plaques. In the Sereny test, BS484 was consistently negative and failed to provoke an inflammatory reaction.

Localization of IcsA on LPS mutants. The *Shigella* surface protein IcsA has previously been shown to be located at one pole of the bacterium and to be essential for actin tail formation, intracellular movement, and cell-to-cell spread (2, 8, 20, 23). We therefore examined the surface localization of IcsA on BS109 and BS484 to determine whether their abnormal movement phenotypes might be explained by aberrant localization of IcsA. As shown in Fig. 6, the distribution of IcsA on the surface of BS109 was distinctly different from the typical unipolar distribution seen on the wild-type strain 2457T (Fig. 6a to d). IcsA was located over the entire surface of BS109 in an irregular blotchy pattern. Labelling of the septum of BS109 was also observed, whereas it was not observed in the wild type.

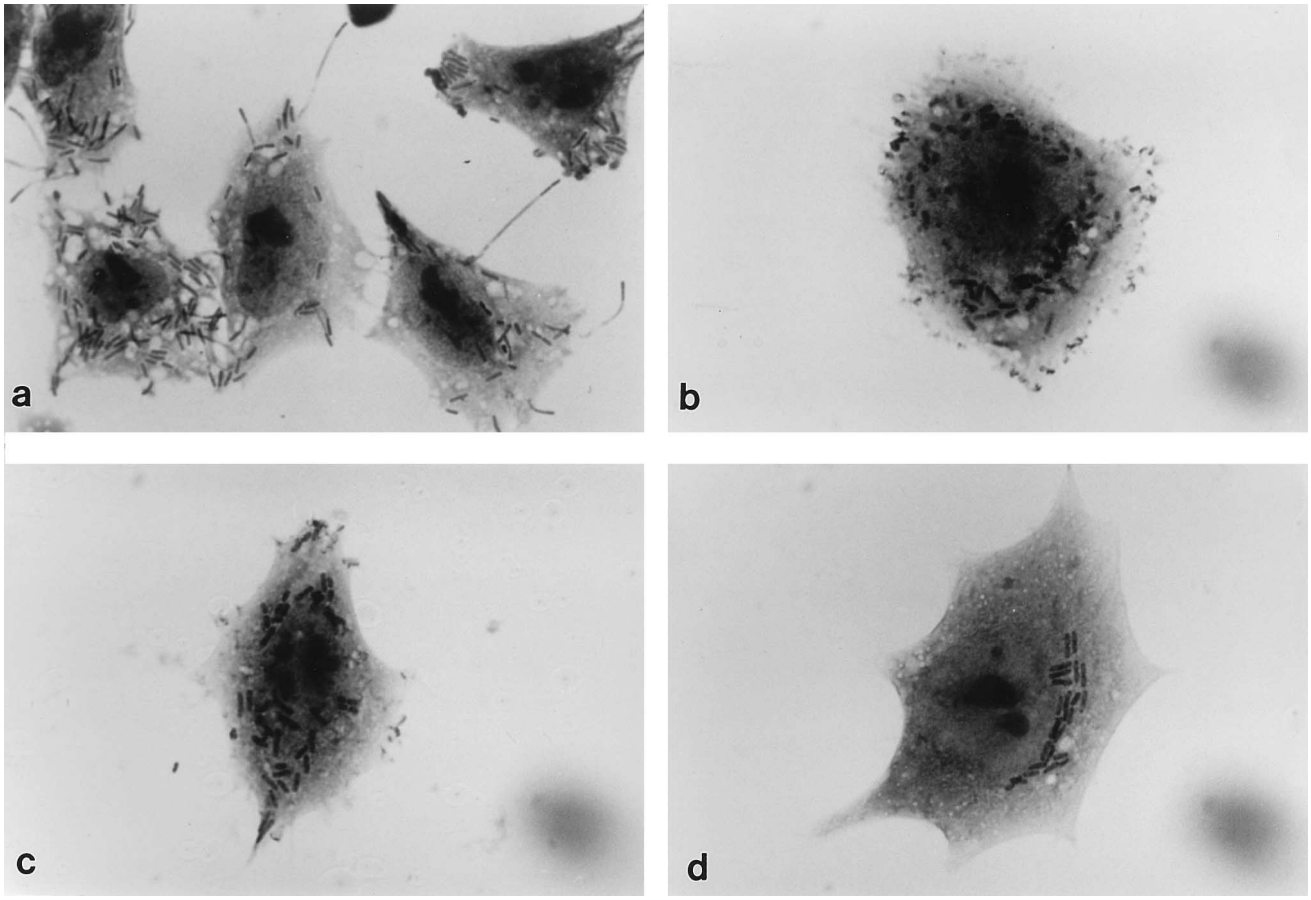

FIG. 2. Effects of the *galU*, *rfe*, and *icsA* mutations on HeLa cell invasion. HeLa cells were infected with the wild-type parent, 2457T (a); the *galU* mutant, BS109 (b); the *rfe* mutant, BS484 (c); and the *icsA* mutant, BS441 (d). After a 2-h invasion at 37°C, the infected cells were washed, fixed, and stained with Giemsa.

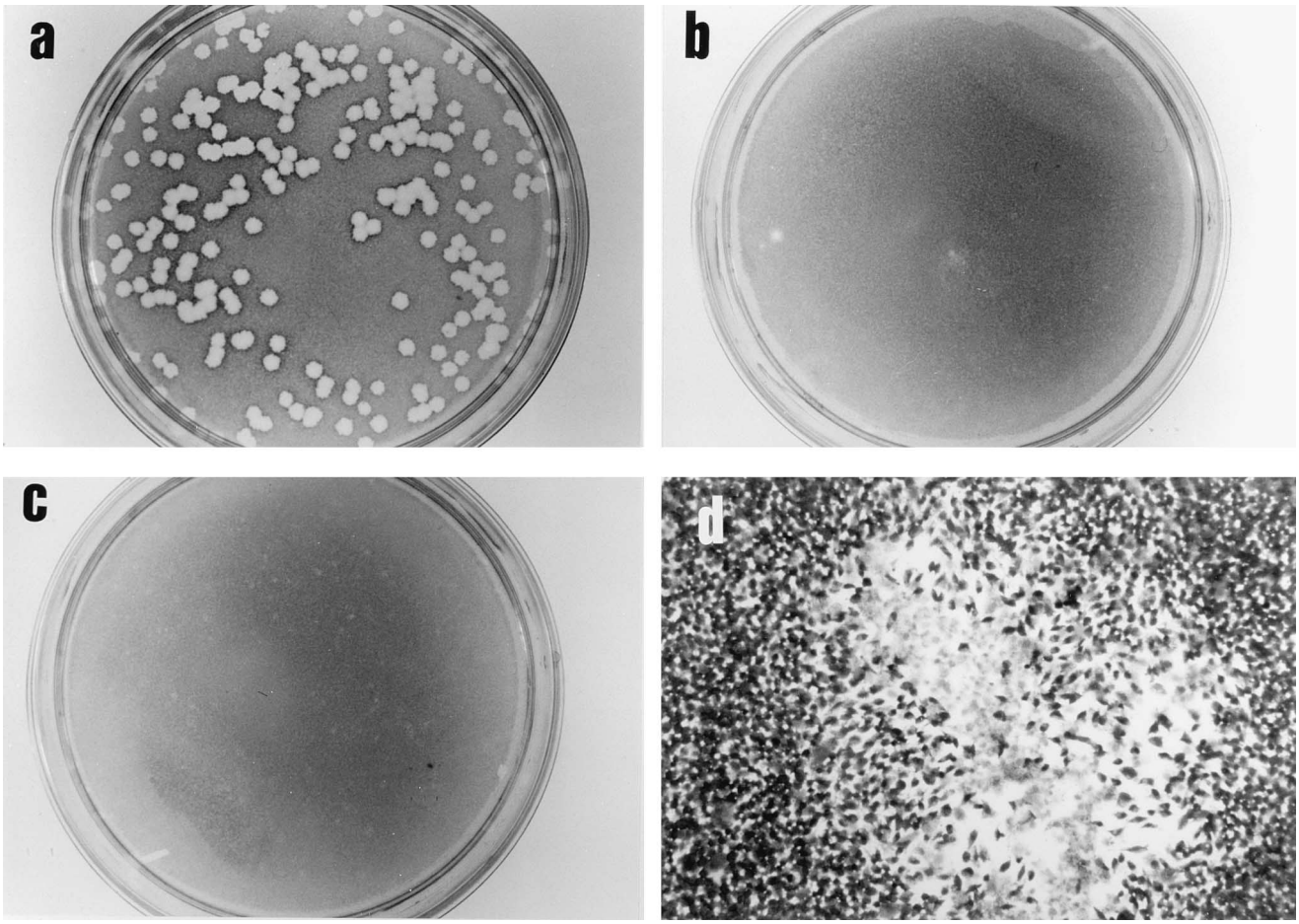

FIG. 3. Effects of the *galU* and *rfe* mutations on plaque formation in L2 cell monolayers. The monolayers were stained with neutral red at 48 h postinfection. (a) Wild-type parent, 2457T; (b) *galU* mutant, BS109; (c) *rfe* mutant, BS484; (d) ×10 magnification of a single plaque of BS484.

In contrast, the distribution of IcsA on BS484 followed a pattern that was intermediate between that seen on the wild-type and that seen on BS109 (Fig. 6e and f), extending farther down the body of the bacterium than on the wild type but not becoming circumferential. Thus, the abnormal surface distribution of IcsA on BS109 and BS484 may contribute to their inability to form plaques and to induce conjunctivitis in the Sereny test.

Actin polymerization by LPS mutants. Since the intracellular movement and cell-to-cell spread of *Shigella* organisms have previously been shown to be based on tails of polymerized actin that form on the bacterial pole that contains IcsA (2, 8, 20, 23), we examined the ability of the LPS mutants to form polar actin tails and accumulate polymerized actin in infected HeLa cells. As expected, in cells infected with the wild-type strain 2457T, both actin tails and fireworks were observed (Fig. 7a and b). In cells infected with either BS109 or BS484, no actin tails or fireworks from the cell surface were observed (Fig. 7c to f). Instead, a halo of polymerized actin was seen surrounding the organisms. Thus, the distribution of polymerized actin associated with the bacteria directly reflects the distribution of IcsA on the bacterial surface.

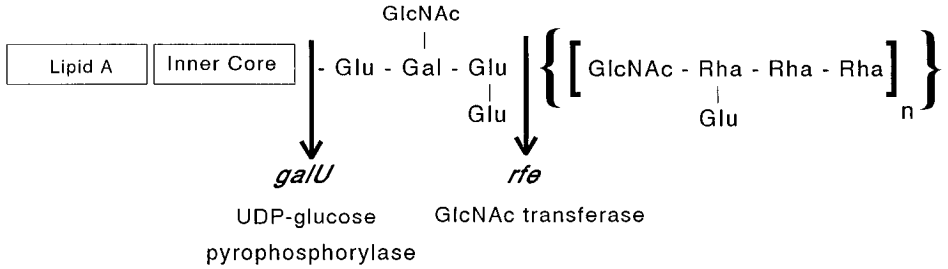

FIG. 4. Schematic diagram of the LPS structure of *S. flexneri* 2a. The arrows indicate the points of truncation in the LPS structure as a result of mutations in *galU* and *rfe*. The point of linkage of the O antigen to the core has not yet been determined experimentally, but the O antigen is presumed to be attached to one of the hexose sugars of the outer core. Abbreviations: Glu, glucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Rha, rhamnose; *n*, number of O-antigen repeat units.

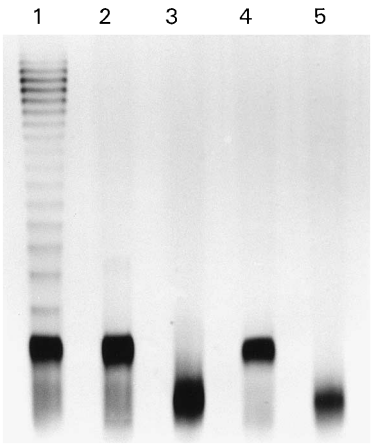

FIG. 5. SDS-PAGE analysis of *S. flexneri* 2a LPS mutants. Samples were prepared from proteinase K-treated whole-cell lysates, electrophoresed on a 13% polyacrylamide gel, and then silver stained. Lanes: 1, wild-type parent, 2457T; 2, *rfe* mutant, BS484; 3, *galU* mutant, BS109; 4, BS484; 5, BS109. The samples were prepared from cultures harvested at the same A_{650}. Lanes 1 to 3 contain equivalent amounts of sample. Lanes 4 and 5 were underloaded (they contain one-third of the amount of sample relative to lanes 1 to 3) to increase the resolution of the core and lipid A components in the mutants.

IcsA expression and processing in the LPS mutants. Since the surface localization of IcsA on BS484, while different from that on the wild type, remained essentially polar, we explored other factors that might contribute to the abnormal behavior of this strain in the plaque assay and Sereny test. We examined whether the expression and processing of IcsA in the two LPS mutants were similar to those in the wild type. The full-length 120-kDa form of IcsA that is found in whole-cell and membrane preparations is normally cleaved to release a 95-kDa form into culture supernatants (8). As shown in Fig. 8, Western blot analysis of whole-cell protein preparations demonstrates that approximately equivalent amounts of the 120-kDa form of IcsA is produced in both LPS mutants and the wild-type strain (Fig. 8, lanes 1 to 3). However, while the cleaved 95-kDa form was secreted into the supernatant of the two mutants, it was found in significantly smaller amounts than in the wild type, suggesting that it is not normally processed (lanes 4 to 6). This abnormal processing of IcsA may contribute to the abnormal phenotypes of the mutants.

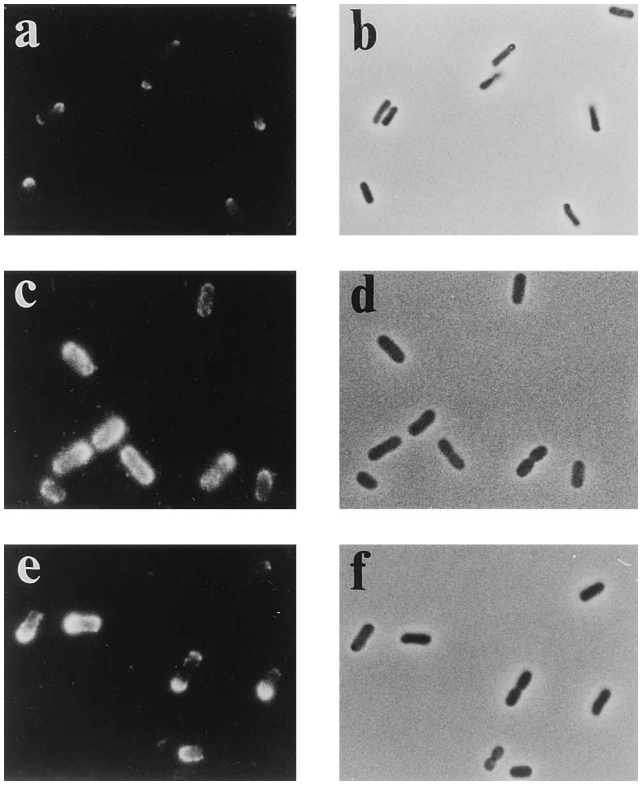

FIG. 6. Labelling of bacteria grown in vitro with monoclonal antibody to IcsA. Fluorescence microscopy of labelled bacteria (panels a, c, and e) and phase-contrast microscopy of the corresponding field (panels b, d, and f, respectively) are shown. (a and b) Wild-type 2457T; (c and d) *galU* mutant, BS109; (e and f) *rfe* mutant, BS484.

DISCUSSION

The attenuated nature of *Shigella* rough mutants has been observed for many years. These mutants were often spontaneously derived and genetically uncharacterized, thus making it difficult to assign a specific role in pathogenesis to the components of the bacterial LPS. The importance of the *Shigella* somatic antigen in virulence was demonstrated by Sansonetti et al. (36) through a genetic approach which involved introduction of regions of the *S. flexneri* chromosome into the chromosome of *E. coli* K-12 by conjugation. However, the large tracts of DNA transferred and the unknown end points of recombination made it difficult to precisely identify the genes responsible for the alteration in virulence.

We also took a genetic approach to determine the role of the LPS in the virulence of *Shigella* spp. The isolation of a defined mutation in *galU* provided us with the opportunity to analyze the nature of a specific LPS biosynthesis defect in *Shigella* spp. on virulence. The absence of fireworks in HeLa cells infected with the *galU* mutant, BS109, and the inability of this strain to form plaques on confluent monolayers suggested that the *galU* mutation resulted in a block in the ability of the bacteria to spread intercellularly. The altered virulence phenotype of the *rfe* mutant, BS484, further supported the hypothesis that LPS played a key role in expression of intercellular motility.

Cell-to-cell spread is essential for *Shigella* virulence in animal models and is dependent on the expression of IcsA (2). IcsA is unusual in that it is one of the few bacterial membrane proteins that is located as a cap over one pole of the bacterium (8). An intuitive assumption is that an asymmetric distribution of actin on the bacterial surface is a prerequisite for initiation of bacterial movement. Unipolar localization of IcsA would permit the protein to interact directly with host cell actin and enable the formation of the characteristic actin tail associated with motility of the bacterium in the cytoplasm of infected eucaryotic cells. Data presented here suggest that unipolar localization of IcsA is necessary for actin-based motility. We determined that the *galU* mutant expresses IcsA on its surface in a diffuse circumferential pattern and that this aberrant pattern is likely to be responsible for the inability of the bacterium to spread intercellularly. Although the IcsA expressed by the *galU* mutant was still capable of polymerizing actin, actin tails and cellular protrusions failed to form and the bacterium remained blocked in the cytoplasm.

Since the mutation in *galU* resulted in defective localization of IcsA, we sought to determine the minimal structure of LPS necessary for the correct unipolar localization of IcsA. A mutant of *S. flexneri* 2a defective in the *rfe* gene also formed no detectable protrusions from the membranes of infected HeLa

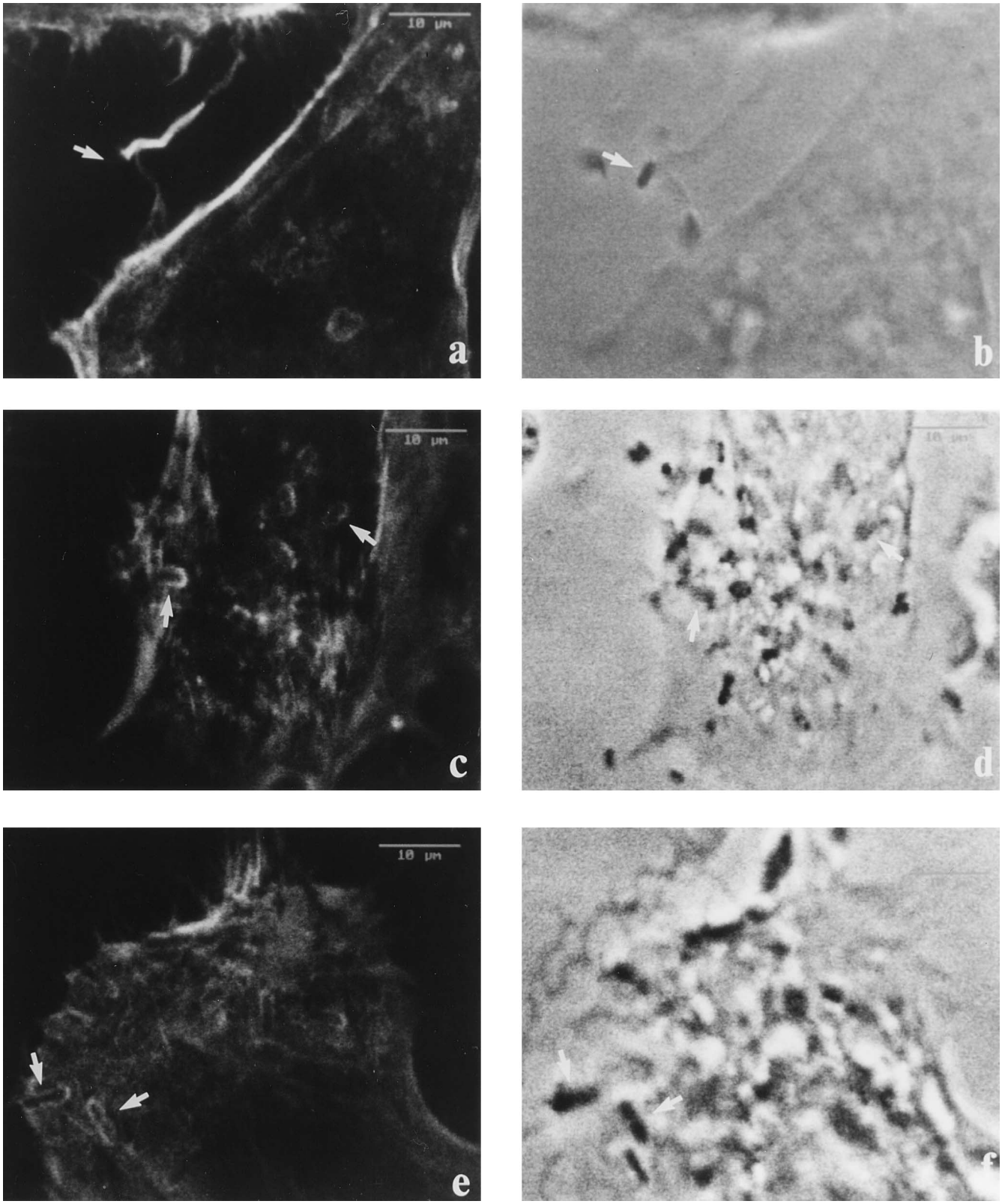

FIG. 7. Labelling of polymerized actin in semiconfluent HeLa cell monolayers infected with *S. flexneri* 2457T, BS109, or BS484. Labelling with rhodamine-conjugated phalloidin (which labels polymerized actin) was observed by confocal microscopy (panels a, c, and e). Each image represents a single z-plane. Phase-contrast images of the corresponding fields are shown in panels b, d, and f, respectively. (a and b) Wild-type 2457T; (c and d) *galU* mutant, BS109; (e and f) *rfe* mutant, BS484. Arrows indicate a bacterium at the tip of a protrusion (panels a and b) and some intracytoplasmic bacteria (panels c to f). Bars, 10 µm.

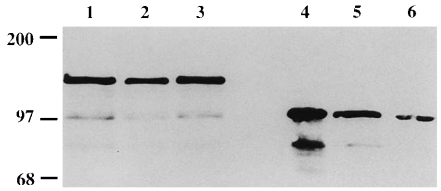

FIG. 8. Western blot analysis of whole-cell and supernatant protein preparations with anti-IcsA antiserum. Lanes: 1, wild-type 2457T whole-cell proteins; 2, *galU* mutant BS109 whole-cell proteins; 3, *rfe* mutant BS484 whole-cell proteins; 4, 2457T supernatant proteins; 5, BS109 supernatant proteins; 6, BS484 supernatant proteins. The samples were prepared from the same culture volumes at the same A_{600}. Apparent molecular masses are indicated in kilodaltons.

cells. However, the *rfe* mutant had a virulence phenotype intermediate between those of the *galU* mutant and the wild-type parent in that it produced tiny, irregularly shaped plaques. Furthermore, the *rfe* mutant appeared to localize IcsA in an intermediate fashion, with IcsA extending farther down the body of the bacterium than on the wild type. These results are compatible with our model that an intact outer core and O antigen are required for proper unipolar localization, processing, and function of IcsA in *S. flexneri.*

The behavior of the mutants described here is similar to that of a series of chromosomal mutants described by Okada et al. (29). These workers screened random Tn*5* insertion mutants of *S. flexneri* 2a in the plaque assay to identify mutants defective in plaque formation. Of the 50 plaque-negative mutants characterized, 36 were rough mutants with either altered core structures or O-antigen defects. Six of these mutants were recently shown to have insertions in the *rfb* locus encoding the enzymes required for biosynthesis of the rhamnose residues of the O antigen (32). These results further support our hypothesis that the LPS is a critical element in proper unipolar localization of IcsA in the membrane.

An intact LPS is known to be necessary for the proper processing and insertion of certain outer membrane proteins in *E. coli*. Deep rough mutants have reduced amounts of the major outer membrane proteins (18). In vitro, the trimeric porin OmpF interacts with intact LPS or outer membrane bilayers and will become inserted into the bilayer and trimerized, while OmpF that interacts with LPS from deep rough mutants will not trimerize (38, 39). A similar interaction with LPS has been demonstrated for PhoE, another trimeric porin (6). How LPS carries out these processes is unknown, but it is possible that LPS functions similarly in the proper insertion of IcsA into the *Shigella* outer membrane. Since LPS is distributed circumferentially, the nature of how it might function to restrict the distribution of IcsA to a single pole is more obscure. One model would be that an additional factor interacts with an IcsA-LPS complex in such a way as to locate the protein to one pole.

The role of *galU* in expression of IcsA in *Shigella* spp. is somewhat different from its role in expression of secreted proteins in other bacteria. For example, *galU* mutants of *E. coli* show altered expression of flagellin (17) and *galU* mutants of *E. coli* and *Erwinia chrysanthemi* fail to secrete alpha-hemolysin and proteases, respectively (47). Thus, it was reasonable to ask whether a *galU* mutation in *Shigella* spp. interfered with expression and/or secretion of IcsA and led to the altered virulence phenotype. Both BS109 and BS484 synthesized amounts of IcsA comparable to that synthesized by the wild-type strain. However, they secreted far smaller amounts of IcsA into the extracellular medium. Although the aberrant localization of IcsA on the surface of these mutants is sufficient to provide a logical mechanistic explanation for their lack of motility, altered secretion of IcsA may also be a factor in their deficiencies in movement and cell-to-cell spread. Alternatively, the inability of the bacteria to correctly localize IcsA may be directly responsible for the secretion defects we observed.

The significance of these studies is twofold. They address a specific aspect of *Shigella* virulence, and they may provide insight into the more general question of asymmetric localization of proteins in other prokaryotes such as the chemoreceptor complexes in *E. coli* and *Caulobacter crescentus* and the attachment proteins of *Bradyrhizobium japonicum* (22). We have demonstrated that LPS plays a role in the correct trafficking of IcsA on the bacterial cell surface, the essential factor for intracellular motility. Unfortunately, little is known about how bacteria segregate proteins asymmetrically in the cell membrane. As pointed out by Maddock et al. (22), unipolar localization of proteins in bacteria requires a mechanism for asymmetrically distributing the proteins, as well as a mechanism for maintaining the asymmetric location of these proteins. Thus, we can consider two possible models to explain the role of LPS in the unipolar localization of IcsA. First, LPS may play a direct role in guiding the IcsA molecules to the bacterial pole. This model would have to take into account the fact that different *Shigella* species display a wide variety of O-antigen types containing different sugar groups. Thus, it is unlikely that specificity for recognition of IcsA and directed transport out to the pole lies solely in the O-antigen component of LPS. In addition, since LPS is distributed circumferentially, the nature of how it might function to restrict the distribution of IcsA to a single pole must be addressed. Our alternative model is that LPS plays an indirect role by maintaining the proper unipolar distribution of IcsA. In this model, we propose that IcsA is initially correctly directed to the pole in the *galU* and *rfe* mutants. Subsequently, in the absence of a complete LPS molecule, increased membrane fluidity might permit the unipolarly localized IcsA molecules to freely diffuse along the outer membrane and become distributed around the bacteria. Thus, a separate, as yet undefined mechanism which directs the unipolar localization of IcsA would exist.

Finally, we are investigating the possibility of using a rough variant such as BS109 as a live vaccine against shigellosis. Vaccine candidates currently under study are mutated in expression of one or more of their virulence determinants in order to attenuate the strain. BS109 would have the advantage of expressing all of the known virulence determinants of *Shigella* spp. but be attenuated as a result of a mutation which affects proper functioning of a key virulence gene, *icsA*. Such a vaccine strain would therefore be expected to express all of the *Shigella* virulence-associated antigens in vivo while being unable to spread and cause disease.

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