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Identification of *icsA*, a plasmid locus of *Shigella flexneri* that governs bacterial intra- and intercellular spread through interaction with F-actin

(pathogenicity/intracellular movement)

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ABSTRACT **The capacity of *Shigella* to spread within the cytosol of infected epithelial cells and to infect adjacent cells is critical for the development of infection foci, which lead to mucosal abscesses. *Shigella* is a nonmotile microorganism that appears to utilize host cell microfilaments to generate intra- as well as intercellular movements, since this movement was inhibited by cytochalasin D and involvement of F-actin was demonstrated by direct labeling of infected cells with the specific dye *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phallacidin. Such movements led to the formation of extracellular protrusions, which may explain cell to cell spread. *icsA*, a locus necessary for intra- and intercellular spread, was identified on the *Shigella flexneri* virulence plasmid pWR100. This locus was cloned and shown to express a 120-kDa outer membrane protein, which plays an important role in the interactions established between host cell microfilaments and the bacterial surface, thus leading to intracellular movement.**

The major step in the pathogenesis of shigellosis is invasion of the human colonic mucosa (1). Invasion by *Shigella flexneri* can be approximated by infecting monolayers of cultured mammalian cells such as HeLa or Henle cells (1, 2). Entry into cells that requires bacteria and host cells to be metabolically active (2, 3) has recently been shown to involve actin polymerization and myosin accumulation (4). These data provide direct evidence that entry occurs through a mechanism of phagocytosis performed by nonprofessional phagocytes (5). Genetic loci encoding entry are located on a 20-kilobase (kb) portion (6) of pWR100, the virulence plasmid of *S. flexneri* (7). Genetic "dissection" of this region is currently under way (8, 9). Rapid intracellular multiplication is another characteristic of *S. flexneri* that has been correlated with the bacterial capacity to lyse the membrane-bound phagocytic vacuole within 1 hr after entry (10). This property should also allow the pathogen to interact with major structural components of the cell machinery and to disseminate within the cytosol. Whether the bacterium moves passively within the cytosol, uses the regular streams that exist within a cell (11, 12), or generates a movement of its own has yet to be elucidated. After intracellular spread, invasion of adjacent cells allows extension of initial foci of infection within the epithelium. Makino *et al.* (13) have described a *virG* locus on the virulence plasmid of *S. flexneri* that is required for cell to cell spread. This phenotype can be studied in the plaque assay in which virulent shigellae cause a cytopathic effect on a confluent monolayer (14).

This work demonstrates that *S. flexneri* spreads intracellularly by a mechanism involving polymerized actin (F-actin). Intracellular motility was reversibly inhibited in infected HeLa cells by cytochalasin D, which prevents polymerization of G-actin into filaments (15). Staining of F-actin in infected cells (16) showed labeling of the surface of intracellular bacteria as well as trails of F-actin formed behind moving bacteria. A transposon mutant of *S. flexneri* was isolated that did not disseminate within infected cells and did not infect adjacent cells. No evidence of accumulated F-actin was detected in the vicinity of this mutant.

MATERIALS AND METHODS

Bacterial Strains and Media. All *S. flexneri* strains were derived from the wild-type serotype 5 strain M90T (7). This strain carries the virulence plasmid pWR100. BS176 is an avirulent strain that is spontaneously cured of pWR100. Plasmids used are as follows: pRT733, a derivative of pJM7031, is a suicide vector constructed by Miller and Mekalanos (17). It was used to deliver the kanamycin-resistance transposon Tn*phoA* (18). Plasmid pRT733 was maintained in *Escherichia coli* SM10 (*thi, thr, leu, tonA, lacY, supE, recA*::RP4-2-Tc::Mu::). Plasmid pIL22 is a pBR322 recombinant molecule carrying a 6.7-kb insert of the uropathogenic *E. coli* K552 chromosomal DNA encoding the afimbrial adhesin AFA-I (19). Plasmid pIL22 was used to transform *S. flexneri* mutant SC557, thus producing strain SC558. Bacteria were routinely grown in tryptic soy broth (Diagnostics Pasteur, Marnes la Coquette, France) or LB broth (20). When necessary, antibiotics were added at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; nalidixic acid, 40 μg/ml.

Genetic Procedures. Transformation of *S. flexneri* and *E. coli* was performed as described (21). Conjugations were carried out on membrane filters. Random insertions of Tn*phoA* were generated into pWR100 by use of pRT733, which was conjugally transferred into a nalidixic acid-resistant mutant of *S. flexneri* strain M90T. Selection was obtained by plating mating bacteria on LB agar supplemented with glucose, nalidixic acid, kanamycin, and 5-bromo-4-chloro-3-indolyl phosphate.

Random insertion of Tn*5* into the recombinant plasmid pHS3192 was carried out with λ::Tn*5* (*cI857 rex*::Tn*5* Oam29 Pam f221), at a multiplicity of infection of 5, on *S. flexneri* BS169 (22) carrying pHS3192.

In vitro mutagenesis of pHS3192 was performed by inserting the interposon ω into the *Hin*dIII restriction site (see Fig. 3). This gene cassette encodes spectinomycin resistance and is flanked on each side by T4 translation transcription stop signals.

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Cloning Procedures. Plasmid DNA from SC557 was extracted and digested with *Eco*RV. Since Tn*phoA* has no *Eco*RV cleavage site, we were able to identify a 17-kb plasmid fragment containing Tn*phoA*, which was subsequently cloned into pBR322.

Hybridization Analysis. DNA fragments used as probes were labeled by nick-translation (24) with ^{32}P-labeled 5′-dCTP (Amersham, Little Chalfont, Buckinghamshire, U.K.). A DNA probe consisting of *Xho* I internal fragments of Tn*5* was used to identify the presence of Tn*phoA*. DNA fragments were transferred from agarose gels to nitrocellulose filters (Schleicher & Schüll, Dassel, F.R.G.) and hybridization was carried out by the method of Southern (25).

BAL-31 Exonuclease Digestion. pHS3192 DNA was cleaved with *Sma* I and treated with BAL-31 at 37°C for 15, 30, 45, 60, and 75 min. Religation was done by adding T4 DNA ligase.

Analysis of Bacterial Proteins. Immunoblotting procedures were carried out as described by Burnette (23) with serum from a monkey convalescing from shigellosis. Expression of proteins of minicells was carried out as described for *S. flexneri* (6). Outer membrane proteins were prepared as described by Osborn (26).

Virulence Assays. HeLa cell invasion was performed as described (27). Other tests used to measure virulence were the plaque assay described by Oaks *et al.* (14) and the Sereny test (28).

Treatment of Infected HeLa Cells with Cytochalasin D. Infection of HeLa cells was carried out as described (27) except that after 30 min of infection, following washing, cytochalasin D (Sigma) was added at a final concentration of 0.5 μg/ml to MEM (GIBCO) with gentamicin (20 μg/ml). Incubation was then carried out at 37°C for 2 hr. Preparations were then washed three times in phosphate-buffered saline (PBS), fresh MEM was added with gentamicin, and incubation was again allowed to proceed until infection was stopped by fixation either with formaldehyde or methanol. Infection with adherent strain SC558 was carried out as described (4).

Double Fluorescence Labeling of F-Actin and Bacteria, F-Actin and Myosin. These techniques have been extensively described in a recent paper (4). F-actin was labeled with a solution (10 units/ml) of *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phallacidin (NBD-phallacidin; Molecular Probes) in PBS. Intracellular bacteria were labeled by indirect immunofluorescence using a rabbit polyclonal antiserum directed against *S. flexneri* lipopolysaccharide and a goat anti-rabbit rhodamine-conjugated immunoglobulin G (Miles).

Myosin was labeled by indirect immunofluorescence using the CC-212 monoclonal antibody, which recognizes both muscular and nonmuscular myosin (29) and a rhodamine-conjugated goat anti-mouse immunoglobulin G (Sigma).

Confocal Microscopy. Some preparations described above were observed under ×40 magnification with a Phoibos three-dimensional confocal laser scanning microscope (Saraströ, Bjornnäsvägen, Stockholm). Observations were performed from the adherence zone of the cell to the top. The distance between each scanning plane was 0.3 μm. Polaroid pictures were recorded on the monitor's screen.

RESULTS

Effects of Cytochalasin D on Intracellular Spread of Bacteria. After 60 min of infection of HeLa cell monolayers by *S. flexneri*, cytochalasin D was added to the medium. Such treatment of infected cells blocked spread of intracellular bacteria but did not significantly affect bacterial growth over the 4-hr period of observation as shown in Fig. 1 *A* and *B*. Bacteria remained sequestered in close proximity to the nucleus. Observation by transmission electron microscopy (data not shown) confirmed that bacteria were free within the cytosol, thus eliminating interference of the drug with the capacity of *S. flexneri* to lyse the membrane-bound phagocytic vacuole (10). Thirty minutes after removal of cytochalasin D, intracellular bacteria regained full capacity to spread within the cell and infect adjacent cells. This experiment indicated that F-actin was required for *S. flexneri* to spread intracellularly and infect adjacent cells.

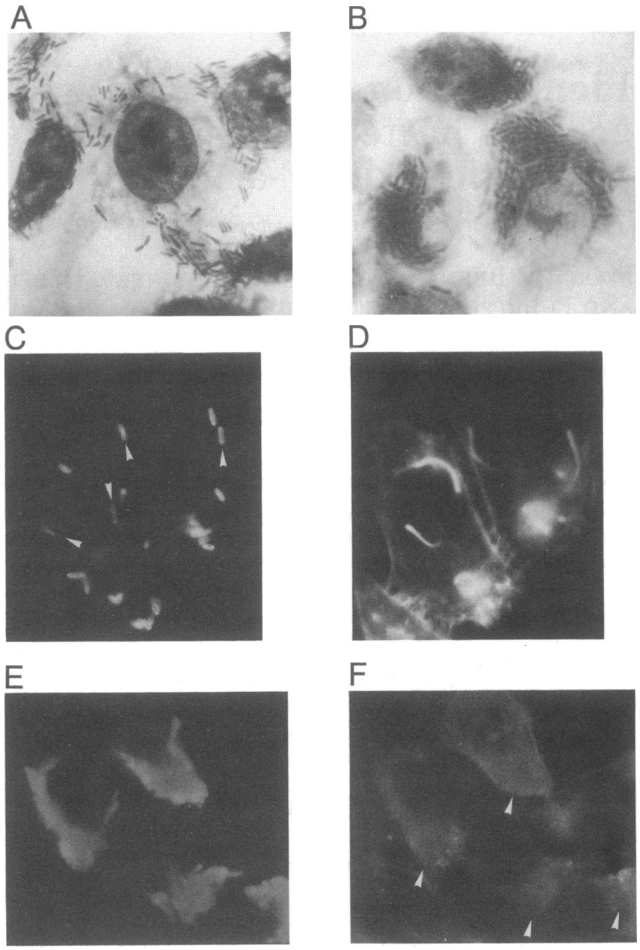

FIG. 1. Bacterial invasion of HeLa cells. (*A*) M90T, 4 hr of incubation without cytochalasin D. (*B*) M90T, 4 hr of incubation with cytochalasin D added 45 min after entry. (*C* and *D*) Spread of M90T within HeLa cells as observed 1 hr after removal of cytochalasin D. Double labeling of infected cells with anti-lipopolysaccharide rhodamine (*C*) and NBD-phallacidin (*D*). Arrowheads in *C* point to bacteria that are followed by a trail composed of F-actin in *D*. (*E* and *F*) HeLa cells invaded by mutant SC557 after 4 hr of incubation. Double labeling with anti-lipopolysaccharide rhodamine (*E*) and NBD-phallacidin (*F*). (×30.)

Analysis of Intracellular Movements of *S. flexneri*. Preparations of HeLa cells infected by M90T were studied by double-fluorescent labeling in the presence or absence of cytochalasin D. F-actin was labeled with NBD-phallacidin and intracellular bacteria were detected by indirect immunofluorescence. Observations within 30 min after removal of cytochalasin D demonstrated no significant intracellular movement of bacteria. After this lag period, they could be seen in the process of moving from their area of sequestration toward another edge of the cell. Labeling with NBD-phallacidin showed that intracellular bacteria were brightly fluorescent with a maximum at their two poles. In addition, some of the bacteria were followed by a bright trail of fluorescent material. The length of these trails varied greatly, whereas their width appeared constant and equivalent to the width of the bacterium. These results are summarized in Fig. 1 *C* and *D* and Fig. 2*A*. In addition, staining of myosin by

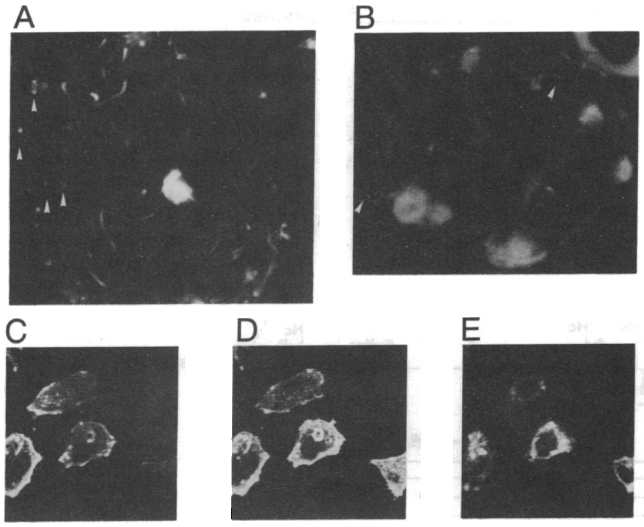

FIG. 2. Spread of M90T within HeLa cells 1 hr after removal of cytochalasin D. Single labeling of infected cells with NBD-phallacidin. (*A*) Polykaryon with numerous intracellular bacteria followed by F-actin trails. Arrows point to some bacteria that are brightly labeled by NBD-phallacidin, thus indicating accumulation of F-actin on their surface. (*B*) Formation of protrusions from the HeLa cell surface by moving M90T. Some of the protrusions are indicated by arrowheads. (×30.) (*C–E*) Confocal microscopy showing the location of an area of bacterial movement underneath the nucleus, in close proximity to the adherence zone of the cell. (*C*) Cell is 0.3 μm from the coverslip. (*D*) Cell is 0.6 μm from the coverslip (arrowhead points to the trail of F-actin). (*E*) Cell is 2.1 μm from the coverslip.

double fluorescence using an anti-myosin monoclonal or a polyclonal antiserum showed no trace of this protein in the trails (data not shown). Such antisera recognized HeLa cell myosin at the site of entry of shigellae in previous experiments (4).

Observations in which a confocal microscope was used allowed accurate location of the zone of movement. This is shown in Fig. 2 *C–E*, in which each view represents a single scanning plane. Appearance and disappearance of an F-actin trail is shown (arrowhead in Fig. 2*D*). Bacteria covered with F-actin as well as trails were always located underneath the nucleus, close to the lower surface of the cell (Fig. 2 *C* and *D*). NBD-phallacidin-labeled trails could usually be observed in four successive planes, thus indicating a diameter of ≈1 μm.

These experiments also provided indications on the way bacteria may spread from one cell to another. As shown in Fig. 2*B*, bacteria could be observed at the end of a fibrillar structure protruding from the surface of host cells. Most of these protrusions originated from areas of the cell corresponding to adhesion plaques. They appeared to contain F-actin.

Selection of a Tn*phoA* Mutant of *S. flexneri* That Has Lost Its Capacity to Spread Intracellularly and Infect Adjacent Cells. SC557, a Tn*phoA* mutant, was obtained that had lost its capacity to spread intracellularly and infect adjacent cells. Such a mutant did not form plaques on a confluent monolayer of HeLa cells (14). It was also negative in the Sereny test (28), although the mutation did not affect the rate of intracellular growth. SC557 displayed early juxtanuclear localization (data not shown) followed by growth on the same site. Neither intracellular spread nor infection of adjacent cells was observed. The intracellular pattern of this mutant appeared strikingly similar to that obtained while treating cells infected with the wild-type strain with cytochalasin D (Fig. 1*B*), thus suggesting that SC557 was impaired in its capacity to interact with cell microfilaments.

Phenotypic Analysis of SC557. Evidence that SC557 was impaired in its capacity to interact with host cell microfilaments was confirmed by double-labeling experiments in which growth of intracellular microcolonies did not elicit significant accumulation of F-actin either on the surface or in the vicinity of bacteria. No protrusions of bacteria could be observed either (Fig. 1 *E* and *F*).

On the other hand, SC558, the adhesive variant of SC557, has conserved its capacity to penetrate into cells through directed phagocytosis. When transformed with pIL22, a recombinant plasmid encoding the uropathogenic *E. coli* adhesin AFA-1, SC557 acquired the capacity to adhere strongly to HeLa cells thus allowing synchronization of the entry process. As shown in *S. flexneri* (4), entering microorganisms triggered local polymerization of actin.

***icsA*, a Genetic Region of pWR100 Responsible for Intra- and Intercellular Spread.** The site of insertion of Tn*phoA* within SC557 was localized on a 9.5-kb *Eco*RV fragment of pWR100. A 6.5-kb *Eco*RI/*Sal* I fragment was then subcloned into pBR325, thus producing the recombinant plasmid pHS3192 (Fig. 3). This plasmid could restore the wild-type invasive phenotype of SC557 and particularly the capacity to spread intracellularly. Immunoblots performed with the serum of a monkey convalescing from shigellosis showed that among the five protein antigens predominantly recognized in invasive strains, a 120-kDa outer membrane protein was missing (Fig. 3 *A* and *B*). As expected, pHS3192 could restore the capacity of SC557 to produce p120 (Fig. 3*B*). This protein was also expressed by pHS3192 in BS169, a noninvasive plasmidless derivative of M90T (Fig. 3*B*) as well as in *S. flexneri* minicells (Fig. 3*C*). Analysis of polypeptides synthesized in minicells by immunoblotting (Fig. 3*C Right*) demonstrated bands at apparent molecular masses of 100, 96, 88, 82, 76, 70, and 66 kDa. The 70-kDa polypeptide was also encoded by pBR325. Since pHS3192 could not account for the synthesis of these additional polypeptides, we believe that these are either proteolytic products of p120 or results of the translation of additional transcripts starting at different initiation codons within the sequence. To locate the structural gene of p120 on pHS3192, Tn*5* mutagenesis on BS169(pHS3192), insertion of the ω cassette at the single *Hin*dIII restriction site of the sequence and BAL-31 digestion of the right end of the sequence were combined. Insertions of Tn*5* allowed us to locate the left end of the p120 structural gene quite precisely. On the other hand, BAL-31 deletions were necessary to locate the right end. The structural gene of p120 can be located near a *Hin*cII restriction site on the left end and between a *Cla* I and *Hin*cII restriction site on the right end (Fig. 3). Mapping of the Tn*phoA* insert of SC557 is perfectly consistent with these data. It appears that this *icsA* locus (intra- and intercellular spread) is similar to the *VirG* locus of Makino *et al.* (13).

DISCUSSION

Although *Shigella* is a nonmotile microorganism, evidence exists that it has the capacity to move intracellularly. Using phase-contrast time-lapse cinematography, Ogawa *et al.* (30) demonstrated that *S. flexneri* bacilli moved independently of the cellular organelles and could be seen at the tip of microfibrillar structures protruding from the host cell surface. More recently, Makino *et al.* (13) have identified a region (*virG*) of the virulence plasmid that is required for cell to cell spread of the bacterium. This region encodes a 130-kDa (now 120 kDa) protein that had previously been shown to be recognized by sera from convalescent monkeys and humans (14). Expression of this antigen is positively regulated at the transcriptional level by the 30-kDa product of *virF*, another plasmid gene (31). Finally, Pal *et al.* (32) have observed that *E. coli* K-12 transconjugants containing the *S.*

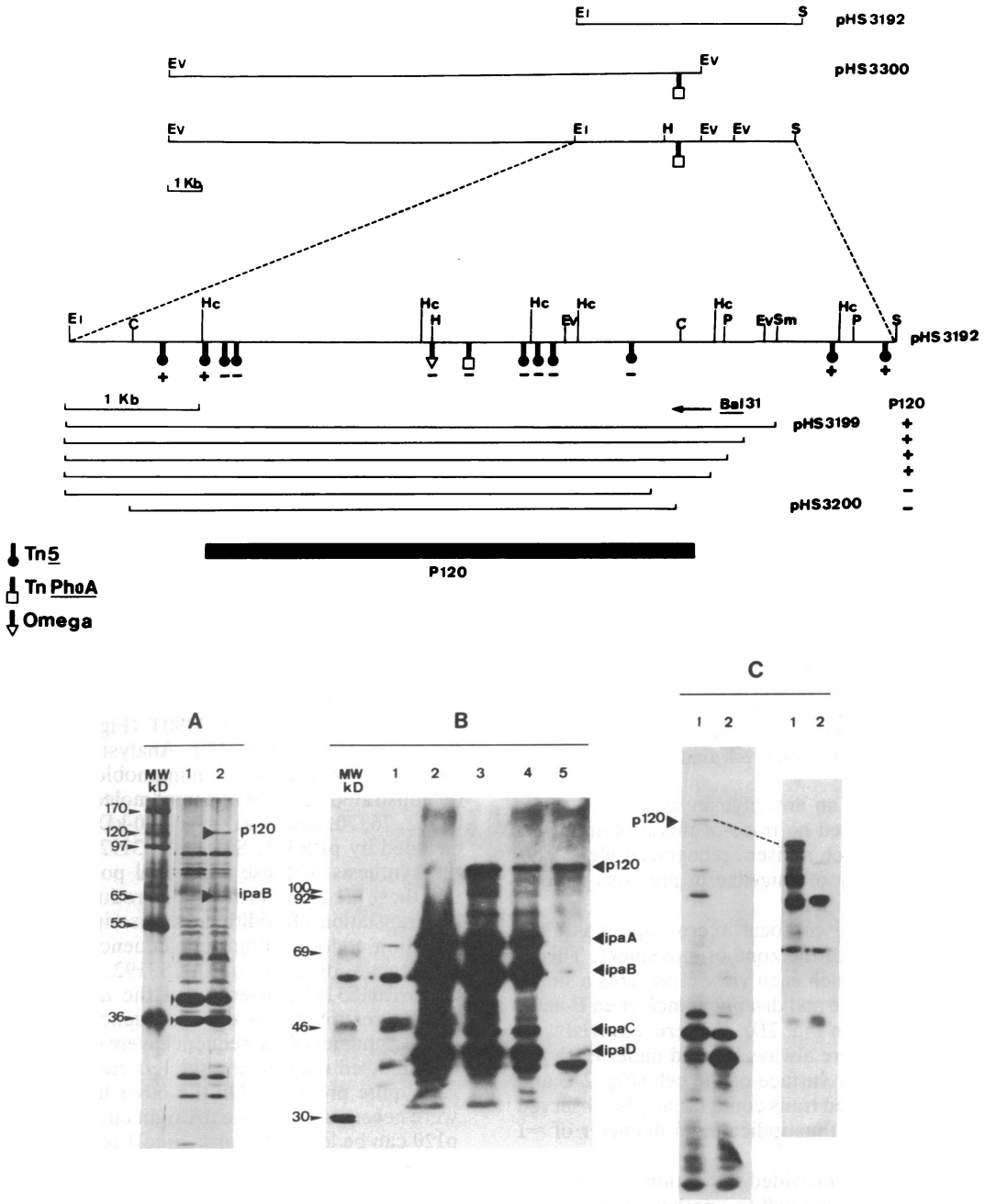

FIG. 3. (*Upper*) Genetic map of pHS3192, carrying a 6.5-kb insert that contains the *icsA* locus. Line 3 shows the portion of pWR100 reconstructed with data from pHS3192 and pHS3300. Line 4 shows the detailed restriction map of the 6.5-kb insert of pHS3192. E1, *Eco*RI; C, *Cla* I; Hc, *Hin*cII; H, *Hin*dIII; Ev, *Eco*RV; P, *Pvu* II; Sm, *Sma* I; S, *Sal* I. In addition to location of Tn*5* insertions and ω *in vitro* integration, location of Tn*phoA* in pHS3300 is shown. Lines 5 and 10 show two subclones of the pHS3192 insert. *Eco*RI/*Sma* I (pHS3199) and *Cla* I/*Cla* I (pHS3200), respectively. In between, BAL-31 deletions of pHS3199 are shown along with expression of p120. (*Lower*) Expression of the *icsA* gene product. (*A*) Outer membrane protein preparations stained with Coomassie brilliant blue. Lanes: 1, BS176; 2, M90T. (*B*) Total protein extract, immunoblot with serum of convalescent monkey. Lanes: 1, BS176; 2, SC557; 3, M90T; 4, SC557(pHS3192); 5, BS176(pHS3192). (*C*) (*Left*) *S. flexneri* minicells labeled with [^{35}S]methionine. Lanes: 1, pHS3192; 2, pBR325. (*Right*) Immunoblot with serum of convalescent monkey. Lanes: 1, pHS3192; 2, pBR325.

flexneri virulence plasmid usually remained localized within discrete areas of the HeLa cell cytoplasm. On the other hand, hybrids that had also received the *kcpA* chromosomal locus (33) spread within cells, thus indicating that, in addition to the *virG* locus, a chromosomal locus may be necessary for intracellular movement. This work demonstrates that intracellular movement of *S. flexneri* occurs through interaction of bacteria with the host cell cytoskeleton. Using Tn*phoA* to select in one step a mutant altered in a surface component that no longer interferes with the cell cytoskeleton, SC557 was obtained by screening for isolates that did not form plaques on confluent monolayers of HeLa cells. This mutant had lost its capacity to spread intracellularly and infect adjacent cells. SC557 localized within a cytoplasmic area close to the nucleus and grew locally, thus forming a microcolony in spite of the absence of a surrounding phagocytic membrane (electron microscopy data not shown) (10). However, the rate of intracellular growth was not affected by the mutation. The Sereny test performed with SC557 was negative, indicating that the capacity to spread intracellularly and infect adjacent cells is as important as bacterial growth for proper development of *S. flexneri* infection within epithe-

lium. After infection with M90T, cytochalasin D led to the reversible formation of intracellular microcolonies similar to that observed with SC557. On the other hand, no effect was observed on intracellular spread when infected cells were treated with nocodazole, which blocks polymerization of microtubules. Both bacterial and host cell factors, including F-actin, are therefore necessary to generate intracellular movement. Direct evidence was provided by labeling with the fluorescent dye NBD-phallacidin. Starting 30 min after removal of cytochalasin D, bacteria moving from their area of sequestration appeared to be followed by a bright fluorescent trail indicating the presence of F-actin. Moreover, bacteria appeared enveloped with F-actin, with a maximum on their two poles, particularly when located in the proximity of adherence plaques of the cell. As already suggested by microcinematography (30), bacterial movements occurred randomly and did not follow the highly organized network of stress fibers (34). Confocal microscopy allowed us to locate movements essentially within a volume limited by the zone of attachment to the coverslip and the nucleus. On the other hand, mutant strain SC557 did not show concentration of F-actin regardless of the time after infection of cells. Observation of membrane protrusions brightly labeled internally by NBD-phallacidin and containing a bacterium at their tip, suggested that they were a consequence of intracellular movements of bacteria. Such protrusions were particularly frequent in the vicinity of adherence plaques. It is currently assumed that such protrusions may account for cell to cell spread, even within intestinal epithelia, thus allowing extension of infection foci. These observations raise several questions. Is concentration of F-actin due to recruitment of the pool of F-actin available in the vicinity of the bacterium or to *de novo* polymerization of actin subunits? The presence of a receptor for F-actin on the bacterial surface or interaction with an actin binding protein that would induce formation of a gel (i.e., filamin, α-actinin) or of bundles (i.e., fimbrin) may be considered (35). Alternatively, a bacterial product interacting directly with actin monomers to induce polymerization or elicit rapid changes in the cytosolic conditions within the immediate vicinity of the bacterium may be envisioned. A rapid decrease of cytosolic calcium may induce local inhibition of gelsolin severing activity (36) and/or activate binding of bundling proteins such as α-actinin to actin filaments (37).

The 120-kDa outer membrane (19) immunogenic protein encoded by the *icsA* locus certainly plays a major role in one of these mechanisms. Present evidence indicates that *icsA* and *virG* (13) are similar loci. Further work is required to define the molecular interactions between the *icsA* (*virG*) gene product and actin. Finally, the way bacterial movement is generated by F-actin must be discussed. It is generally considered, based on initial work in *Nitella* (38) and *in vitro* assay systems (12), that myosin is the driving force of actin-based intracellular movements. The absence of myosin within the network of F-actin indicates that either rapid polymerization of actin or brisk formation of a gel or bundle is sufficient to generate intracellular movement of bacteria.

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