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Secretion of *Shigella flexneri* Ipa Invasins on Contact with Epithelial Cells and Subsequent Entry of the Bacterium into Cells Are Growth Stage Dependent

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Upon contact with the surface of epithelial cells, *Shigella flexneri* secretes Ipa proteins through the Mxi-Spa type III secretion apparatus. Among the Ipa proteins, IpaB and IpaC form a soluble complex in the bacterial supernatant which appears to be sufficient to initiate the cellular rearrangements necessary to achieve bacterial entry. Here, we provide the first evidence that efficiency of bacterial entry into cells depends on the stage of bacterial growth. Bacteria in the early phase of exponential growth are six times more invasive than those in the stationary phase. The entry efficiency of the bacteria present on the cell surface appears to correlate with the percentage of those that are able to secrete their invasins. This suggests that the capacity to activate the Mxi-Spa apparatus is a major factor in the regulation of bacterial entry efficiency. Consistent with these observations, we have further shown that bacteria which have reached the stage of division secrete Ipa proteins more often than those that have not. Also, initial secretion occurs essentially in the area of the septation furrow. The Ipa proteins, secreted in the vicinity of the septation furrow, seem to initiate the early stages of reorganization of the host cell cytoskeleton.

Shigella species cause bacillary dysentery, particularly in young children of the developing world, by disrupting the colonic epithelial barrier and invading epithelial cells (26). Invasion of epithelial cells has been studied extensively in vitro by infecting cell lines such as HeLa cells with *Shigella flexneri*. This has permitted the definition of basic phenotypes such as entry, which occurs via a pathogen-directed macropinocytic event (1, 12), intracellular motility (10, 29), and cell-to-cell spread (46). Genes required for all three steps are carried on a 200-kb virulence plasmid (45). The data currently available on these genes have recently been reviewed (37). A large 30-kb fragment of this plasmid is necessary to govern entry (30, 48). This region encompasses two divergently transcribed loci representing a total of 31 genes. Phenotypic characterization of the mutants that have been obtained in the majority of these genes indicates that one locus encodes essentially a set of secreted Ipa proteins which are the effectors of the entry process, and the other locus encodes their specialized Mxi-Spa secretion apparatus.

The four Ipa proteins IpaB (62 kDa), IpaC (42 kDa), IpaD (38 kDa), and IpaA (70 kDa) are encoded by contiguous genes belonging to an operon (7, 47, 50, 51). Characterization of the Ipa mutants indicates that IpaB, IpaC, and IpaD are the actual effectors of the entry (23, 34, 47). More recent experiments have demonstrated that IpaB and IpaC form a complex in the bacterial supernatant, and the complex is sufficient to induce internalization. This was achieved by coating latex beads with immunopurified IpaB-IpaC complexes and observing that these beads were efficiently internalized by HeLa cells (32, 35). In addition, IpaB and IpaD have been shown to form a transient complex associated with the bacterial membranes. This complex may play a primary role in regulating the secretory flux of Ipa proteins (33). Accordingly, deletions of the *ipaB* and *ipaD* genes cause a leaky phenotype in which the mutants constitutively secrete the Ipa proteins as well as several other uncharacterized proteins (38). Secretion of the Ipa proteins is induced upon contact of invasive bacteria with the epithelial cell surface and by low concentrations of serum (33). However, as assessed by quantitating the IpaB and IpaC proteins released in the supernatant of infected HeLa cells, the amount of secreted Ipa proteins appeared limited compared with the total amount of Ipa proteins associated with the bacteria. This may indicate that most of the secreted proteins had been deposited onto the eukaryotic cells and remained associated with them, or at a given time, among the fraction of bacteria interacting with the cell surface in a potential productive manner, only a limited number were actually competent to secrete their Ipa proteins.

Secretion of the Ipa proteins, which lack a signal peptide, requires expression of the specialized Mxi-Spa secretion apparatus (2, 3, 5, 49, 52). The reason for the existence of this secretory system may be to allow one-step secretion of the Ipa proteins from the bacterial cytoplasm to the extracellular medium, upon contact of the pathogenic microorganism with the target cells. These proteins would otherwise remain trapped within the bacterial cytoplasm, or within the bacterial membranes, due to intrinsic biophysical properties that are likely to allow their subsequent interaction with eukaryotic cell membranes and cytoskeletal structures. In addition, IpgC, a 17-kDa molecule, acts as a chaperone of IpaB and IpaC within the bacterial cytoplasm (35), thus preventing them from forming a complex sensitive to proteolytic degradation. This is a major factor that permits bacteria to maintain a cytoplasmic pool of invasins ready for use. This behavior may be a common denominator among the enteric pathogens that share homologous type III secretory pathways. This is particularly obvious in *Salmonella* spp., in which the homologs of the last 13 genes of the *Shigella mxi-spa* locus are present and involved in virulence (15–17, 20). More recent evidence indicates that Ipa homologs

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are secreted via this system (22, 24, 39). Similarly, 11 genes required for the secretion of Yop proteins by *Yersinia* spp. have their homologs in *Shigella* spp. (4, 8, 36, 54). This similarity among the various systems has been emphasized by experiments showing that the *Shigella* IpaB protein was secreted via the *Yersinia* (42, 43) and *Salmonella* (22) secretory machineries, and *Yersinia* YopE was secreted and translocated into epithelial cells via the *Salmonella* secretory machinery.

In this work, we have further characterized the mode of secretion of the two key invasins of *S. flexneri*, IpaB and IpaC. We have shown that, at a given time, only a small proportion of the bacteria in contact with the host cell surface secrete their invasins and that induction of this secretory process is growth phase dependent. We have also demonstrated that IpaB and IpaC are predominantly secreted by dividing bacteria in an area adjacent to the septation furrow and that early recruitment of cytoskeleton-associated proteins occurs in close association with the secreted Ipa proteins. These data emphasize the major role played by these two invasins in triggering *Shigella* entry into epithelial cells.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strain SC301 is an invasive isolate of *S. flexneri* belonging to serotype 5. It contains the 200-kb virulence plasmid pWR100 which encodes proteins required for the cell invasion. It also carries plasmid pIL22, which encodes the human-specific AFA1 adhesin of uropathogenic *Escherichia coli* (25). Expression of this adhesin increases the efficiency of *S. flexneri* adherence on HeLa cells, thus facilitating synchronization of entry experiments and increasing chances for the bacterium to generate productive entry foci (12). SC300 is a noninvasive equivalent of SC301 that does not harbor pWR100.

Bacteria were routinely grown at 37°C in Trypticase soy broth (Diagnostics Pasteur, Marnes la Coquette, France) in a shaking incubator. They were used in the stationary phase, after overnight culture, at an optical density at 600 nm of 2 and in the mid-exponential phase at an optical density at 600 nm of 0.3. To equalize the multiplicity of infection, bacterial cultures were diluted to a final optical density of 0.03 before infection experiments were carried out.

HeLa cell culture and infection. HeLa cells were routinely grown at 37°C, in 6% CO_2, in minimal essential medium containing L-glutamine (GIBCO BRL, Gaithersburg, Md.) and supplemented with 10% fetal bovine serum (GIBCO BRL). Semiconfluent cell monolayers grown on glass coverslips in 35-mm-diameter tissue culture dishes were infected with SC301 and SC300 at a standardized concentration of 10^7 bacteria/ml. Bacterial suspensions were incubated over HeLa cells for 15 min at room temperature to bring bacteria in contact with cells under noninvasive conditions. Dishes were then placed on the surface of a 37°C water bath for 15 min to allow entry (1). Invasion was stopped by three washes in phosphate-buffered saline (PBS) at room temperature.

Immunofluorescence labelling of infected cells. Cells were fixed in 3.7% (wt/vol) paraformaldehyde for 20 min, quenched in PBS–0.1 M glycine for 5 min at pH 7.4, and permeabilized in PBS–0.5% (vol/vol) Triton X-100 for 2 min. To label the secreted IpaB or IpaC proteins, preparations were then incubated for 20 min with a mouse monoclonal antibody (MAb) specific to IpaB (clone H16, diluted 1/50), a rabbit polyclonal antibody (PcAb) directed against IpaB (diluted 1/200), or with a mouse MAb specific to IpaC (clone J22, diluted 1/50). These various antibodies have been described previously (6, 40). To label the host cell cytoskeleton to define bacterial entry sites, preparations were similarly incubated with a mouse anticortactin MAb (clone 4F11, diluted 1/400; Upstate Biotechnology Incorporated, Lake Placid, N.Y.) or with a rabbit PcAb directed against actin (diluted 1/200; Sigma Chemical Co., St. Louis, Mo.). Bacteria themselves, when necessary, were labelled under similar conditions with either a rabbit PcAb directed against the *S. flexneri* serotype 5 somatic specificity (diluted 1/100) or with a mouse monoclonal immunoglobulin G (IgG) directed against *S. flexneri* serotype 5 lipopolysaccharide (LPS; clone C20, diluted 1/200). The secondary antibodies used were tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG (diluted 1/100; Sigma), TRITC-conjugated anti-rabbit IgG (diluted 1/100; Sigma), fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (diluted 1/200; Sigma), and a FITC-conjugated anti-rabbit IgG serum (diluted 1/200; Amersham). Observations were essentially performed with a confocal laser scanning microscope (Leica Inc., Deerfield, Ill.). To disconnect the capacity to secrete Ipa proteins on the host cell surface and the entry process itself, invasion experiments were carried out on cells treated with 0.5 μg of cytochalasin D (Sigma) per ml 30 min prior to infection and also during the 15-min incubation period in the presence of bacteria.

Quantification of intracellular and extracellular bacteria. After infection, cells were fixed in paraformaldehyde and quenched in glycine. For staining extracellular bacteria, nonpermeabilized cells were incubated with the anti-LPS C20 mouse MAb described above and TRITC-labelled secondary antibody. The cells were then permeabilized with 0.5% (vol/vol) Triton X-100 for 2 min, and all bacteria, now including intracellular ones, were labelled with a rabbit PcAb directed against the *S. flexneri* serotype 5 somatic specificity and FITC-labelled secondary antibody. Preparations were mounted in triethylenediamine (DABCO) and observed with a conventional fluorescence microscope (BH2-RFCA; Olympus Optical Co. Ltd.). By use of a dual-band filter which allows both FITC and TRITC emission (515 to 550 nm and 580 nm, respectively) and excitation (460 to 490 nm and 520 to 550 nm, respectively), it was possible to distinguish the intracellular bacteria that stained green (i.e., FITC) from the extracellular bacteria that stained yellow (i.e., TRITC plus FITC). A few bacteria appeared bicolor due to their intermediate position (i.e., ongoing entry process).

ELISA for titration of the secreted IpaC. An enzyme-linked immunosorbent assay (ELISA) was performed by use of a two-antibody sandwich assay. Briefly, polystyrene plates were coated with J22, a MAb specific to IpaC (40). Bacteria in the exponential and stationary stages of growth were incubated with 10% fetal calf serum at 37°C for 30 min, and the supernatant of these bacteria was added to the wells. After 1 h of incubation at 37°C, a PcAb specific to IpaC was added, and IpaC was quantitated by measuring the amount of bound PcAbs with a horseradish peroxidase anti-rabbit IgG (28).

RESULTS

The experiments presented in this manuscript were carried out with *S. flexneri* SC301 and SC300, which are derivatives of the serotype 5 invasive isolate M90T and its noninvasive plasmidless mutant, BS176, respectively. Both are transformants expressing the afimbrial adhesin of uropathogenic *E. coli* (AFA1), a human cell-specific adhesin (25). Expression of this adhesin facilitates efficient adhesion of *Shigella* to epithelial cells of human origin, thus allowing synchronization of the entry process and dramatically increasing the chances to observe early stages of interaction between the bacteria and their cell targets.

The first series of experiments were aimed at studying how bacterial growth stage could affect entry into cells and whether secretion of the Ipa proteins could be a growth-dependent modulator of entry efficiency.

Growth phase dependency of *S. flexneri* entry into HeLa cells. A double fluorescence labelling method was developed to detect intra- and extracellular bacteria to quantify the invasive process. Briefly, an initial anti-LPS labelling (i.e., with TRITC) was performed with nonpermeabilized infected cells, thus labelling only extracellular bacteria. A second anti-LPS labelling (i.e., with FITC) was performed with the same preparation after permeabilizaton of the cells, thus labelling both extracellular and intracellular bacteria. As a consequence, under microscopic analysis through a dual-band filter allowing observation of both TRITC- and FITC-stained structures, extracellular bacteria appeared yellow whereas intracellular bacteria appeared green. As illustrated in Fig. 1, regardless of the growth phase, the noninvasive strain SC300 appeared uniformly yellow (Fig. 1C and D). On the other hand, infection with the invasive strain SC301 resulted in the presence of both green (intracellular) and yellow (extracellular) bacteria (Fig. 1A and B). Clearly, fewer intracellular bacteria are observed in cells infected with a bacterial inoculum that has reached the stationary phase compared with an inoculum taken at an early stage of exponential growth, indicating that entry of *Shigella* is a growth phase-dependent event.

To quantify the difference in entry efficiency, the intra- and extracellular bacteria were counted in four independent experiments. For each preparation, a total of 1,000 bacteria associated with 100 to 200 cells were counted. Figure 2 shows the results of these counts. A small percentage of bacteria appeared half green and half yellow (intermediate), indicating that they were deeply inserted into the host cell membrane. Strain SC300 remained consistently extracellular after 15 min of incubation with HeLa cells, regardless of the stage of the growth. In addition, the stage of growth did not quantitatively

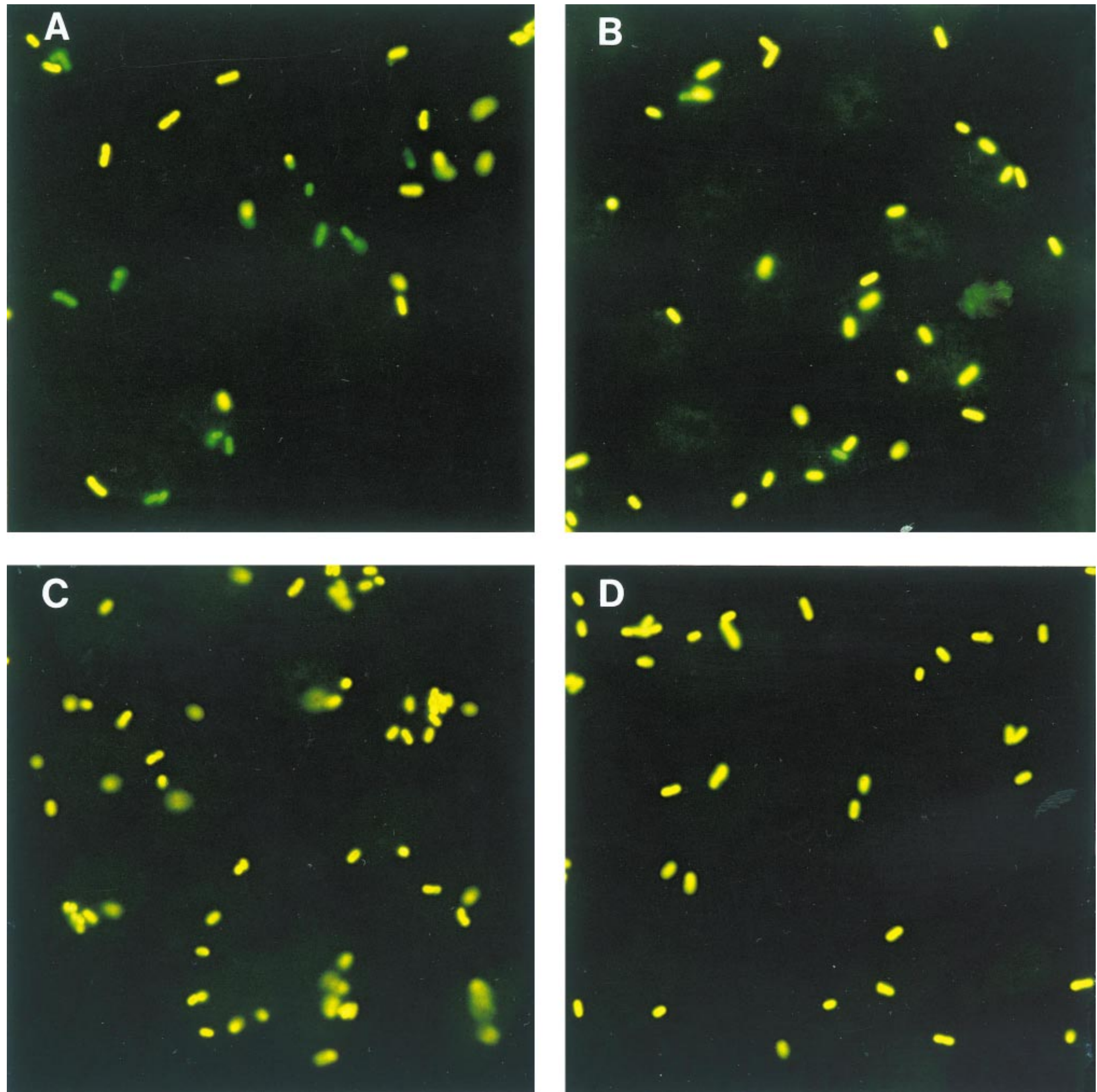

FIG. 1. Infection of HeLa cells with *S. flexneri* invasive strain SC301 and noninvasive strain SC300. After 15 min of incubation, double labelling of bacterial LPS was performed by TRITC labelling of a nonpermeabilized preparation, followed by FITC labelling after subsequent permeabilization. Microscopical observation of the preparation with a dual-band filter shows extracellular bacteria fluorescing in yellow and intracellular bacteria fluorescing in green. Equal numbers of SC301 (A and B) and SC300 (C and D) bacteria were used for infection at an early stage of the exponential phase of growth (A and C) and during the stationary phase (B and D).

affect the number of adhering bacteria (data not shown). This observation indicated that expression of AFA1 does not affect growth phase-dependent entry of *Shigella*. Strain SC301 entered cells after an incubation period similar to that of M90T, and entry was dependent on the stage of growth. While 41% ± 8% (mean ± standard deviation) of the bacteria at an early stage of the exponential phase of growth were intracellular, only 7% ± 2% of bacteria at the stationary phase entered the cells. Therefore, the ratio of entry efficiency of the exponential-phase bacteria to the stationary-phase bacteria was 6. These data were further confirmed in a gentamicin assay which allows quantitation of intracellular CFU. In this case, the ratio of exponential-phase intracellular bacteria to stationary-phase bacteria varied between 7.2 and 11.6 in different experiments (data not shown). When bacteria were used after a 24-h incubation period, infections carried out at similar multiplicity of infection showed no invasion with SC301, thus confirming the importance of the growth phase in modulating entry efficiency (data not shown).

Labelling of IpaB and IpaC invasins associated with adhering bacteria and frequency of secretion associated with the stage of growth. Double fluorescence labelling of SC301 bacteria for LPS and IpaC (Fig. 3A and B) indicated that secretion of IpaC, at a given time, was a relatively rare event since only

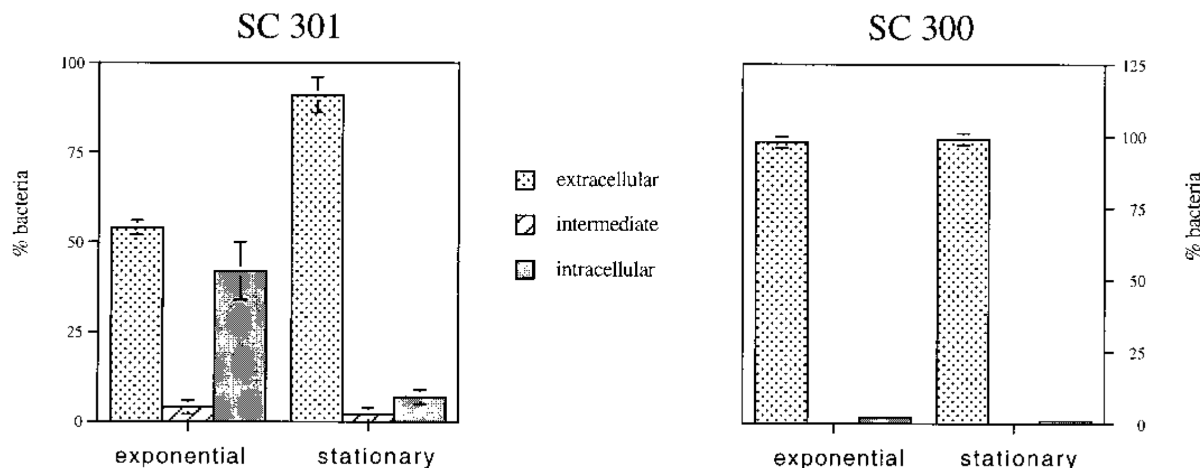

FIG. 2. Quantitative analysis of entry of invasive SC301 bacteria and noninvasive SC300 bacteria into cells at different stages of bacterial growth.

4% ± 1% of the cell-associated bacteria appeared doubly labelled when inoculation was performed with the bacteria in the exponential phase of growth (Fig. 4). This frequency decreased to 0.8% ± 0.5% when cells were inoculated with the bacteria in the stationary phase. The ratio of secretion efficiency of the exponential-phase bacteria to the stationary-phase bacteria was 5. This corresponds to the ratio of the entry efficiency, suggesting that growth phase-controlled entry is actually dictated by a growth phase-dependent induction of secretion of the invasins. This phenomenon was further confirmed in another experiment using serum as the inducer of the secretion of the Ipa proteins. Bacteria grown to the exponential and the stationary phases were put in contact with 10% fetal calf serum, and the level of IpaC in the supernatant was measured by ELISA. As shown in Fig. 5, upon contact with serum, there was an approximately 18-fold increase in the level of IpaC secretion by bacteria in the exponential phase of growth. However, no increase in secretion by overnight culture was detected.

In addition, when bacteria were counted with regard to the presence of a septation furrow, it appeared that regardless of the stage in growth phase, the majority of the bacteria that secreted IpaC (i.e., ca. 75%) displayed a clear septation furrow, indicating that they were reaching the stage of division. This percentage remained the same in the exponential and stationary phases, although the percentages of bacteria with a visible septation furrow in the exponential and stationary phases were 25.5% ± 3.7% and 14% ± 4%, respectively. A similar observation was made in equivalent experiments in which bacteria were doubly labelled for LPS and IpaB (Fig. 3C and D). The percentages of cell-associated bacteria that were doubly labelled were absolutely identical (data not shown).

Double labelling of bacteria for IpaB and IpaC, as shown in Fig. 3E to G, indicated that these two proteins always colocalized. Figure 3E and F show IpaB and IpaC labelling of the same secreting bacterium, respectively. The use of a dual-band filter, which allows observation of both FITC and TRITC labelling, demonstrated the presence of the two invasins associated with a single bacterium (Fig. 3G). This control was essential to confirm that fluorescence labelling was showing the actual secretory process. In addition, similar immunostaining experiments carried out on bacteria immobilized on glass coverslips without HeLa cells showed no significant labelling, regardless of the growth phase. For this control, several thousands bacteria were screened in each experiment (data not shown). This indicated that the fixation-permeabilization technique did not lead to leakage of the Ipa proteins from the bacteria or labelling of the bacterial cytoplasmic compartment. Infections were also carried out with strain SC301 on HeLa cells treated with cytochalasin D to block the actin-mediated entry process, thus disconnecting the internalization event and the capacity of bacteria to initiate Ipa secretion on the cell surface. The percentage of bacteria binding on epithelial cell surfaces and secreting IpaC in these experimental conditions was 9%, thus indicating that Ipa secretion was not a characteristic of intracellular bacteria. Also, in the absence of entry, the number of bacteria seen secreting their Ipa proteins at a given moment was doubled.

Characteristics of Ipa secretion on the cell surface. The next series of experiments was aimed at better characterizing Ipa secretion on the cell surface and at demonstrating its correlation with early reorganization of the cell cytoskeleton. Considering the consistent colocalization of the secreted IpaB and IpaC proteins, most of the following experiments were based on IpaC labelling as an indicator of the secretory process. Confocal microscopy analysis permitted the study of the early phase of Ipa secretion on the epithelial cell surface. These results are summarized in Fig. 6. The left side of the figure shows FITC labelling of bacteria located on the HeLa cell surface by an anti-LPS PcAb. The right side of the figure shows rhodamine labelling of IpaC secreted by the same bacterium. In the middle, the association of both stainings is visible. Figure 6A, from top to bottom, shows various stages of secretion. In the top two lanes, which correspond to an early phase of secretion, IpaC is clearly released into an area corresponding to the septation furrow of a bacterium reaching its division phase. As shown in the lower four lanes of Fig. 6A, IpaC is further released from the bacterium and appears closely localized to the bacterial body. A reinforcement remains, however, in the area of the bacterial septation furrow. Figure 6B shows the confocal analysis of a *Shigella* secreting its Ipa invasins in x and z coordinates. To further assess the relevance of the observed secretory process, and to confirm that the few bacteria secreting their Ipa proteins were interacting with the host cell surface, double fluorescence labelling was performed on IpaC and two key components of the host cell cytoskeleton that are intimately associated with the entry focus, actin (1) and cortactin (14, 55). Clear colocalization of secreted IpaC, actin, and

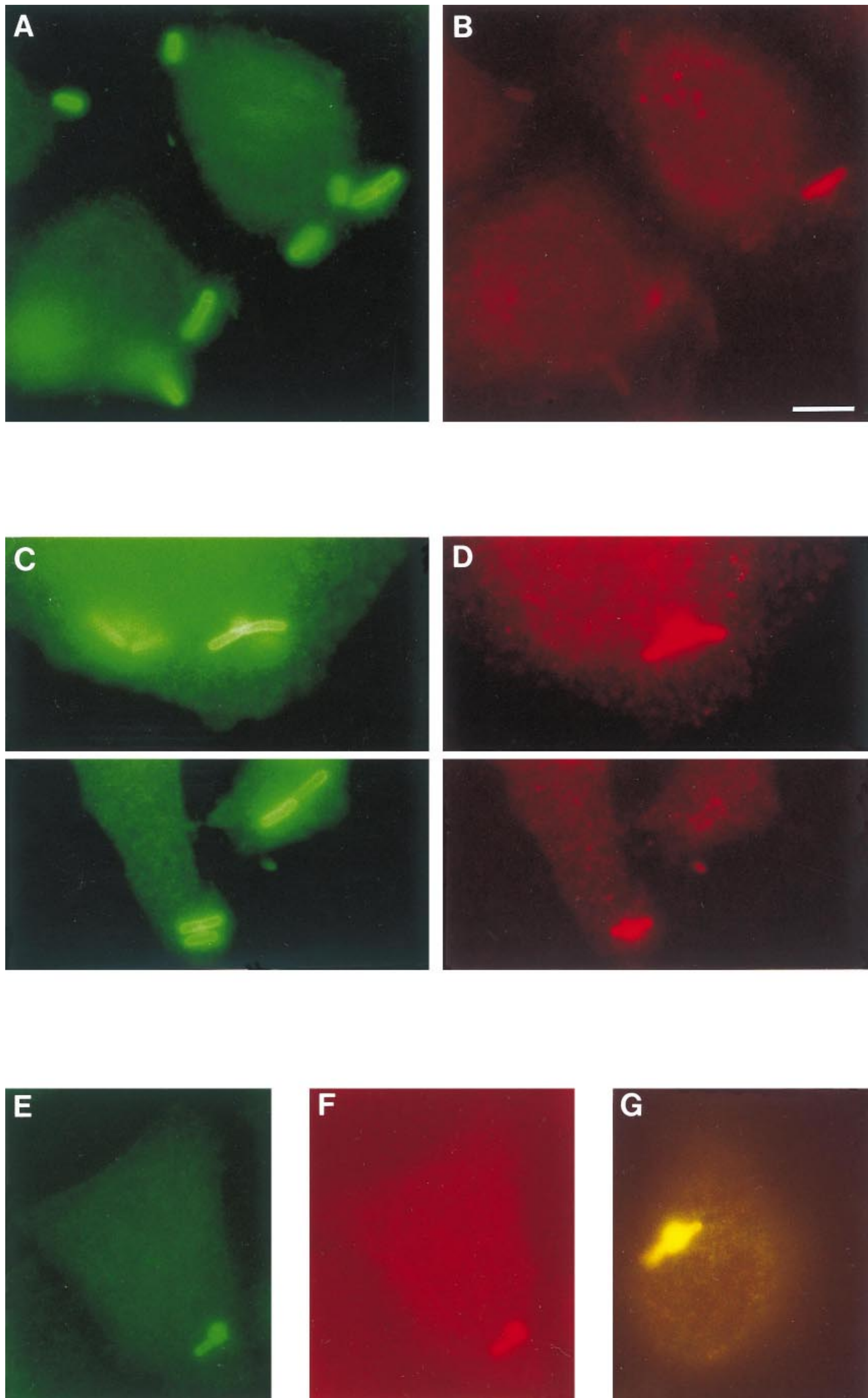

FIG. 3. Double fluorescence FITC and TRITC labelling of SC301 bacteria adhering to HeLa cells. (A and B) LPS and IpaC, respectively; (C and D) LPS and IpaB, respectively; (E and F) IpaB and IpaC, respectively. In panel G, labelling of both IpaB and IpaC is visible on the same bacterium by use of a dual-band filter. Bars, 5 μm.

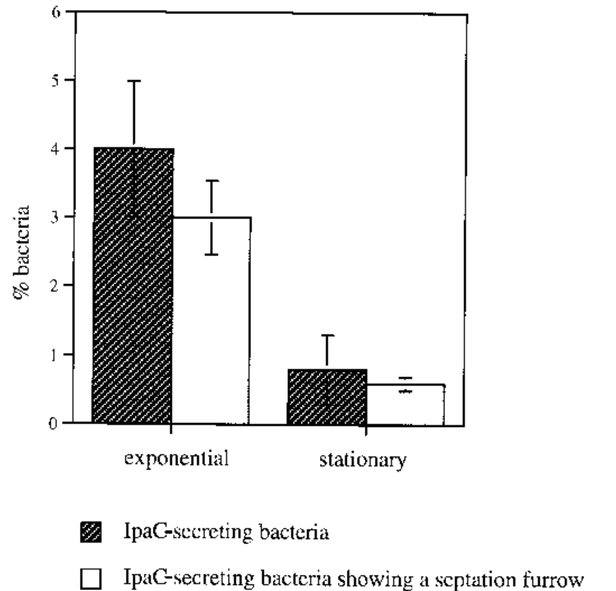

FIG. 4. Frequency of IpaC-secreting SC301 bacteria on the surface of HeLa cells in different stages of growth. The subgroup of these bacteria that displays a septation furrow is also shown.

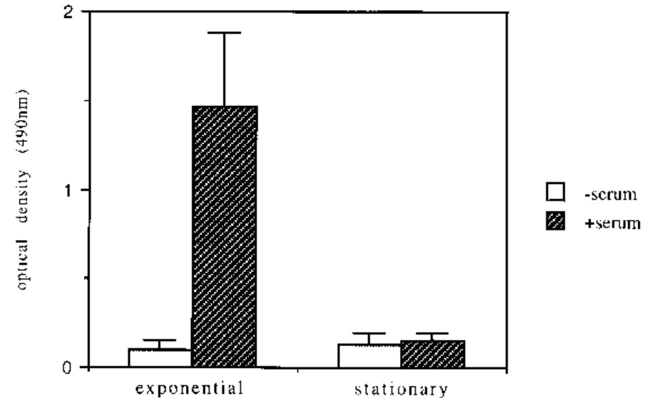

FIG. 5. Secretion of IpaC following contact of the bacteria in the exponential and stationary phases of growth in the presence of fetal calf serum. IpaC levels in the bacterial supernatant were measured by ELISA in three different experiments.

cortactin was regularly observed in confocal microscopy (data not shown), thus confirming the functional significance of Ipa protein release with regard to formation of the entry focus.

To eliminate the possibility that assembly of AFA1 on the bacterial surface may direct Ipa secretion towards the septation zone, a similar series of experiments was carried out with the wild-type isolate M90T. Although the percentage of bacteria secreting Ipa proteins on the cell surface was extremely low, the few positive bacteria observed also showed predominant septal secretion (data not shown).

DISCUSSION

It is now established that several gram-negative bacteria which are pathogenic for humans, animals, and plants have evolved similar strategies to deliver toxic factors to their cellular targets and possibly to translocate them into the intracellular compartments where they achieve their toxic effect by interacting with as-yet-undefined components. Typical examples are the YopE cytotoxin (41) and the YopH tyrosine phosphatase (11, 21) of *Yersinia* spp. The Ipa proteins that trigger entry of *S. flexneri* into cells (32) belong to this category, although they may not all be translocated into the cell cytosol. Bacteria belonging to this class have a similar specialized secretory apparatus called type III (44), whose genes are conserved among these different bacterial species. It seems that the logic behind the expression of such secretory systems is in the necessity for bacteria, upon contact with their cellular targets, to deliver a pool of proteins which lack a signal peptide from the cytoplasm to the extracellular milieu (33, 35, 43). Whether these secreted proteins form an even-transient supramolecular structure on the bacterial surface, following their secretion, is still a matter of debate (18). In microorganisms such as yersiniae, salmonellae, and shigellae, the secretory systems appear to be functionally exchangeable (20, 22, 43).

Inducer molecules that trigger activation of the type III secretory system are currently being investigated. In *Yersinia* spp., it has long been known that at 37°C, in the absence of Ca^{2+}, bacteria stop growing and start producing and secreting Yop proteins (13). However, Ca^{2+} depletion may not be the actual signal that triggers Yop secretion in vivo. In *Shigella*, contact with the surface of HeLa cells as well as low concentrations of serum have been shown to trigger secretion of the Ipa proteins (33). As already mentioned, the IpaB and IpaC molecules form an extracellular complex (35) which alone is sufficient to induce internalization of latex particles (32). Unlike directional injection of YopE into cytoplasm of the cells (41), secretion of the Ipa proteins does not seem to be strictly polarized because these proteins can be recovered from the supernatant of infected cells (33). Matrix proteins such as fibronectin and laminin may be among the molecules which activate the Mxi-Spa secretory apparatus of *Shigella* (53). Bacterial targets of these inducers have not yet been identified.

In this paper, we have tried to answer some of the questions mentioned above by characterizing the secretory process of the IpaB and IpaC proteins by *S. flexneri* on the surface of HeLa cells. We have established a system allowing accurate numeration of intracellular and extracellular bacteria and shown that the potential to invade HeLa cells is dependent on the growth phase of the microorganism. This is reminiscent of the growth phase-dependent entry of salmonellae in the cells, since salmonellae also lose their invasiveness in the stationary phase of growth (27). Bacteria in an early phase of their exponential growth appeared five to six times more invasive than the bacteria during the stationary phase. To date, growth temperature via the *virR* gene (31) and osmolarity via the *envZ-ompR* locus (9) have been shown to regulate the efficiency of *Shigella* entry. This is due in both cases to transcriptional control of expression of the proteins involved in the entry process. In the course of the present work, by performing Western blot experiments, we have observed the same amounts of the IpaB and IpaC proteins (representative of invasins) and of MxiG (representative of the Mxi-Spa secretory complex), regardless of the stage of growth (data not shown). This is, in the case of the Ipa proteins, in agreement with results presented in a recent article (38). As a consequence, growth phase-dependent efficiency of entry into cells seems to correlate with the capacity of the preestablished type III Mxi-Spa system to be activated and to secrete a pool of preexisting Ipa proteins and not with a growth-dependent expression of the two categories of components of the entry system. Activation of Ipa secretion, therefore, appears to be more efficient in actively dividing bacteria.

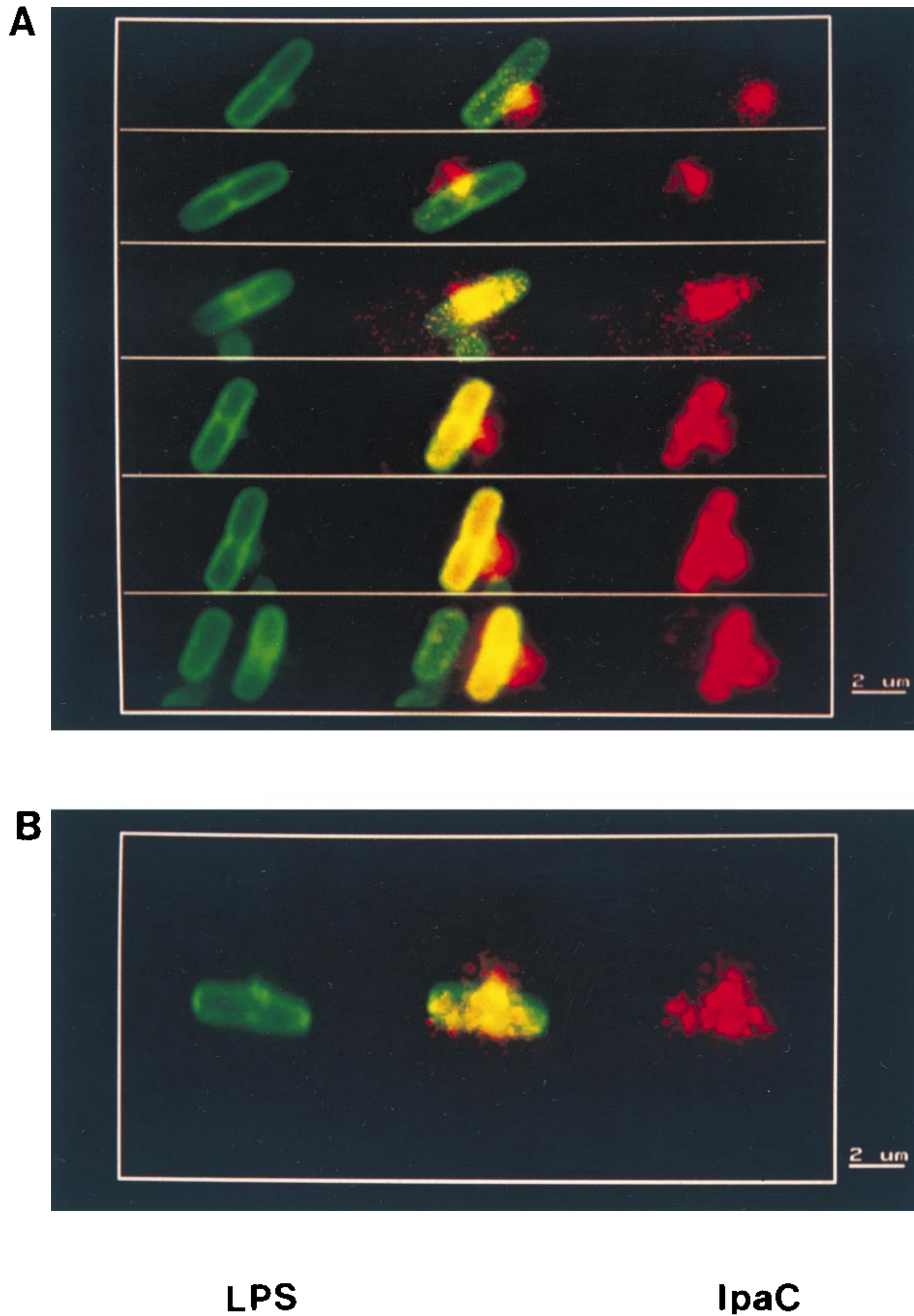

FIG. 6. Double fluorescence labelling of infected HeLa cells for bacterial LPS (FITC) and IpaC (TRITC). Observations were made by confocal microscopy. (A) Top to bottom, various stages of IpaC secretion; (B) confocal analysis in terms of x and z coordinates. Bars, 2 μm.

This is actually true both for bacteria that have established contact with the epithelial cell surface and for bacterial cultures exposed to serum which seems to mimic the signal the bacterium is sensing on the cell surface.

The molecular basis for enhanced secretion during the exponential phase in the presence of eukaryotic cells is as yet unknown. However, the observation that secretion is initiated in the area of the septation furrow suggests that bacterial division may provide physical facilitation for the secretory process. One can hypothesize that the Mxi-Spa system is somewhat linked to the constitution of the septation furrow, with its components becoming predominantly localized in this area, a location opposite to that of IcsA (19). Alternatively, weakness of the peptidoglycan layer in this area in which the bacterial wall is immature may facilitate the construction of the Mxi-Spa apparatus and/or the passage of Ipa proteins. These hypotheses should be tested by combining the use of bacterial division mutants and immunoelectron microscopy.

In spite of the higher efficiency of entry observed for bacteria collected during their exponential growth phase (i.e., about six times more efficient) and of the correlated higher frequency of bacteria secreting their Ipa proteins on the cell surface (i.e., five times greater), the actual frequency of bacteria that secrete their Ipa proteins after 15 min of incubation on the cell surface remained very low (i.e., 4% for exponentially growing bacteria). This percentage may actually be higher considering that at this time, 45% of the bacteria are already intracellular. If such a correction is introduced, this percentage increases to 6%. This does not introduce a dramatic change and even increases the ratio of the secreting bacteria in the exponential and stationary phases of growth. In agreement with the immunofluorescent data, Western blot analysis demonstrated that only less than 10% of the total cytoplasmic pool of the Ipa proteins could be recovered from the supernatant of infected cells after bacterial contact (data not shown).

We were expecting a higher level of detection of the Ipa proteins based on the observation that as early as 15 min after establishing contact with the cells, about 45% of the bacteria already appeared to be intracellular. The most likely explanation is that bacteria establishing contact with the host cell surface are flushing out their pool of Ipa proteins in a very short period of time. That is why at the time that fixation is carried out, only a small fraction of adhering bacteria are seen releasing Ipa proteins, or secretion of the Ipa proteins by a small population of bacteria might be sufficient to mediate the entry of the rest of the bacteria. This represents an early stage of the interaction process in which Ipa proteins cause rapid rearrangement of the host cell cytoskeleton, which is originally at its maximum in the area of contact with the septation furrow where secretion is initiated.

Further observation indicates that IpaB remains associated with the surface of the membrane ruffle, which characterizes the entry process until this structure disappears. IpaB and IpaC may therefore be released very rapidly from the bacteria and immediately bind to, or insert themselves into, the HeLa cell membrane. As soon as the entry process reaches its completion, these proteins may be released from the HeLa cell surface or destroyed on it. Alternatively, they may be internalized; this is currently being investigated. Regardless of these later stages, the current observation is consistent with our recent demonstration that IpaB and IpaC are sufficient to induce entry of latex particles (32).

The next steps will encompass identification of the factors which determine the apparently tightly regulated process of secretion of the Ipa proteins upon contact between the bacterium and the epithelial cell surface. In parallel, the mode of interaction of IpaB and IpaC with the cell surface that leads to generation of the entry process is currently being studied in our laboratory.

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