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Contact with Cultured Epithelial Cells Stimulates Secretion of *Salmonella typhimurium* Invasion Protein InvJ

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Contact of *Salmonella typhimurium* with cultured epithelial cells results in the assembly of surface appendages termed invasomes which are presumably required for the internalization of these organisms into host cells. The assembly of these structures requires the function of a dedicated protein secretion system encoded in the *inv* locus. We show in this report that contact of wild-type *S. typhimurium* with cultured Henle-407 cells stimulated the secretion of InvJ, a recently identified target of the *inv*-encoded type III protein secretion system. Stimulation of InvJ secretion also occurred upon bacterial contact with bovine calf serum-coated culture dishes but did not occur upon *S. typhimurium* contact with glutaraldehyde-fixed Henle-407 cells. The stimulation of InvJ secretion did not require de novo protein synthesis. Invasion-defective *invC* and *invG* mutants of *S. typhimurium* failed to secrete InvJ upon contact with live Henle-407 cells. In contrast, contact-dependent secretion of InvJ in *S. typhimurium invE* mutants occurred at levels equivalent to those of the wild type. These results indicate that the presence of Henle-407 cells and/or serum is capable of activating the type III secretion system encoded in the *inv* locus, further supporting the notion that *Salmonella* entry into cultured cells is the result of a biochemical cross-talk between the bacteria and the host cells.

Salmonella spp. are the principal etiologic agents of gastroenteritis and enteric fever in a variety of animals, including humans (12). An essential step in the development of these diseases is the entry of the bacteria into nonphagocytic cells, including those that line the intestinal epithelium (23, 24). *Salmonella* entry into host cells is the result of a multistep process that culminates in host cell membrane ruffling and subsequent bacterial uptake. The events that trigger internalization require a battery of bacterial proteins, including the components of a dedicated protein export system, termed type III (reviewed in reference 6), that is distinct from both the type I (*sec*-independent) protein secretion system exemplified by the export of the *Escherichia coli* hemolysin and the type II (*sec*-dependent) general secretory pathway of gram-negative bacteria exemplified by the secretion of pullulanase of *Klebsiella oxytoca*. Similar type III secretion systems are required for the virulence of other pathogenic bacteria, including *Yersinia* spp., *Shigella* spp., and enteropathogenic *E. coli*, as well as a number of plant pathogens from the *Xanthomonas*, *Pseudomonas*, *Aeromonas*, and *Erwinia* genera (reviewed in references 20 and 25). In addition, *trans* complementation and domain swapping experiments have established functional similarity for at least two of the protein homologs (7, 10).

The type III secretion systems are usually encoded by genes that are clustered together, either on the chromosome, in the case of *Salmonella* spp. (reviewed in reference 6), or on large plasmids, in the cases of *Shigella* (reviewed in reference 21) and *Yersinia* (reviewed in references 5 and 22) spp. One protein common to all of the type III systems is an ATPase which presumably energizes the transport system. In *Salmonella* spp., this protein is InvC (4), which is homologous to the products of *spa47* from *Shigella* spp. (26) and *yscN* of *Yersinia* spp. (2, 27). Another common component of the type III systems is an outer membrane-associated translocase that is homologous to PulD from *K. oxytoca* (18). This protein is InvG in *Salmonella* spp. (13), MxiD in *Shigella* spp. (1), and YscC in *Yersinia* spp. (17). The type III systems have typically been identified through the analysis of mutants of pathogenic bacteria that interact with their host cells in a rather intimate manner. For example, contact between bacteria and phagocytes is necessary for *Yersinia* spp. to inactivate the phagocytic process (5). In this case, the bacterium-host cell interactions activate the *Yersinia* type III transport system, stimulating it to transfer YopE into the host cell (19). Entry of *Shigella* spp. into their host cells requires the coordinated transport and release of several Ipa proteins from the bacteria. This process is directed by a type III system whose function appears to be modulated by contact between the bacterium and the host cell (16).

Contact of *Salmonella* spp. with host cells results in the assembly of peritrichous appendages called invasomes (8). Invasome assembly requires a functioning type III secretion system, since mutations in *invC* or *invG* prevent assembly of the invasome and drastically reduce *Salmonella* entry into the host cells, indicating that there is a close correlation between invasome assembly and the internalization process. We have recently discovered a *Salmonella* protein, InvJ, that is required for bacterial entry and is secreted in the culture medium via the type III secretory pathway encoded in the *inv* locus of these organisms (3). However, in Luria-Bertani broth, under conditions that optimize *Salmonella* spp. for invasion competence, only a minor fraction of the total InvJ is secreted into the culture medium. These observations prompted us to examine the possibility that contact with host cells might stimulate the secretion of InvJ.

MATERIALS AND METHODS

Bacterial strains. The wild-type strain of *S. typhimurium* used throughout our studies was SL1344 (11). Constructions of the isogenic strains SB566 (4), SB132 (9), and SB161 (13), which carry nonpolar insertion mutations in *invC*, *invE*, and *invG*, respectively, have been previously described. Strains were grown in L broth (14) under conditions that stimulate bacterial entry as described elsewhere (8), and when appropriate, either 50 μg of kanamycin ml^{-1} or 100 μg of streptomycin ml^{-1} was added to the growth medium.

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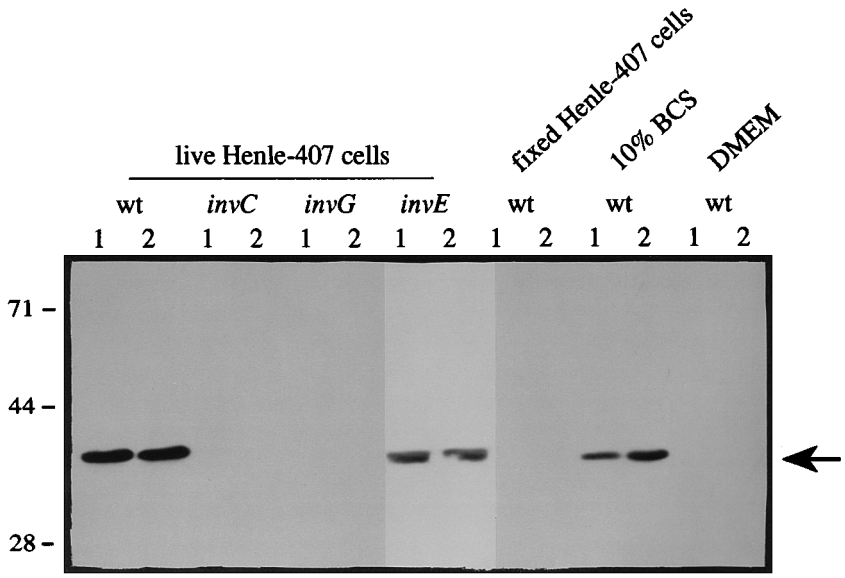

FIG. 1. Contact with Henle-407 cells stimulates InvJ secretion. Wild-type *S. typhimurium* SL1344 and the isogenic strains SB566 (*invC*), SB161 (*invG*), and SB132 (*invE*) were used to infect live or glutaraldehyde-fixed Henle-407 cells or exposed to tissue culture dishes treated with either DMEM containing 10% BCS or DMEM alone. Treatment of culture dishes and detection of InvJ were carried out as described in Materials and Methods. wt, wild-type *S. typhimurium* SL1344. Lanes 1 and 2, two independent repetitions of each treatment. This experiment was repeated at least four times with equivalent results. Arrow, InvJ. The positions of the molecular weight standards are indicated on the left (in thousands).

Tissue culture cells and treatment of dishes. Henle-407 cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat inactivated bovine calf serum (BCS) or, when appropriate, in the serum-free medium HyQ-CCM 5 (HyClone Laboratories, Logan, Utah). Growth was at 37°C in a humidified 5% CO_2–95% air incubator. Prior to infection, Henle-407 cells were transferred to 100-mm petri dishes and grown for 24 to 48 h, to 75% confluency. Cells were washed three times with Hanks balanced salt solution (HBSS) before infection. Some cells were fixed with 2.5% glutaraldehyde in phosphate-buffered (PBS) for 1 h at room temperature and then extensively washed with HBSS before infection. Other experimental petri dishes were prepared as follows. Laminin (final concentration, 0.01%), fibronectin (0.01%), ovalbumin (0.1 and 0.01%), BCS (10, 1, 0.1, and 0.01%), bovine serum albumin (0.1 and 0.01%), and poly-L-lysine (0.01%) were added to 4 ml of DMEM in 100-mm petri dishes. The dishes were incubated for 2 h at 37°C in a humidified 5% CO_2–95% air incubator, washed three times with HBSS, and infected with bacteria. Control culture dishes were covered with DMEM for 24 to 48 h at 37°C in a humidified 5% CO_2–95% air incubator and washed three times with HBSS before addition of bacteria.

Infection protocol and preparation of secreted proteins. Bacteria were grown under conditions that optimize competency for entry into eukaryotic cells (3). When the bacteria reached an optical density at 600 nm of 0.2 to 0.3, they were pelleted by centrifugation at 3,200 × *g* for 15 min, resuspended in 10 to 30 ml of HBSS, pelleted again, resuspended in HBSS to a final density of 10^9 bacteria per 2.5 ml, and used immediately. Tissue culture dishes containing live or glutaraldehyde-fixed Henle-407 cells or dishes previously treated with either DMEM only, DMEM containing BCS, or DMEM containing purified proteins were infected by adding 2.5 ml of bacteria per dish. Infections were carried out at 37°C in a humidified 5% CO_2–air incubator. After 1 h (unless otherwise indicated), the infection medium was recovered, dishes were washed twice with HBSS, and washes and medium were pooled (total volume, 7.5 ml) and passed through a 0.45-μm-pore-size syringe filter (Gelman Science) to remove the bacteria. Protein in the cell-free medium was precipitated by the addition of cold trichloroacetic acid to 10% (vol/vol) and deoxycholic acid to 0.03% (vol/vol) and incubation for 1 h on ice. The protein was collected by centrifugation at 4°C, 10,000 × *g* for 20 min. Pellets were washed in 0.8 ml of cold acetone, dried, and resuspended in PBS buffered with 77 mM Tris-HCl, pH 8.0.

Western blotting (immunoblotting). The samples were resolved by discontinuous sodium dodecyl sulfate-polyacrylamide amide gel electrophoresis and transferred to nitrocellulose. InvJ and 6-phosphogluconate dehydrogenase were detected by immunoblot analysis using monoclonal antibody J33.13 (3) and a polyclonal antibody kindly provided by Donald Oliver (Wesleyan University), respectively. The immunocomplexes were visualized by using horseradish peroxidase coupled to either sheep anti-mouse immunoglobulin, which recognizes J33.13, or donkey anti-rabbit immunoglobulin, which recognizes anti-6-phosphogluconate dehydrogenase, followed by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, Ill.).

RESULTS AND DISCUSSION

InvJ secretion is stimulated by bacterium-host cell contact. To test if InvJ secretion from *Salmonella* spp. was altered by contact between bacteria and host cells, Henle-407 intestinal epithelial cells were infected with wild-type and mutant strains of *S. typhimurium*, and the relative levels of InvJ in the medium were determined. Wild-type *S. typhimurium* SL1344 (11) and the isogenic strains SB566 (4), SB132 (9) and SB161 (13), which carry nonpolar insertion mutations in *invC*, *invE*, and *invG*, respectively, were grown under conditions that optimize competency for entry into eukaryotic cells (3), washed in HBSS, and exposed to tissue culture dishes containing Henle-407 cells or previously treated with either the culture medium DMEM alone or DMEM containing 10% BCS. InvJ secretion from wild-type *S. typhimurium* was detected in medium from dishes containing Henle-407 cells or coated with DMEM containing 10% BCS but not from dishes coated with DMEM alone (Fig. 1). Mutant strains SB566 (*invC*) and SB161 (*invG*) were not stimulated by Henle-407 cells to secrete InvJ. In contrast, the mutant strain SB132 (*invE*) secreted InvJ at wild-type levels in the presence of Henle-407 cells. Interestingly, this mutant had shown contact-dependent assembly of the invasome, albeit the architecture of the surface structures was aberrant (8). These results indicate that the presence of Henle-407 cells and/or serum is capable of activating the type III secretion system encoded in the *inv* locus. The appearance of InvJ in the culture supernatant of infected cells could not have been due to nonspecific bacterial lysis since (i) the very abundant cytoplasmic protein 6-phosphogluconate dehydrogenase could not be detected by Western blot analysis of these preparations and (ii) mutations in at least two *inv* genes prevented the contact-stimulated secretion of InvJ. Infection of Henle-407 cells that had been fixed with 2.5% glutaraldehyde did not result in the stimulation of InvJ secretion (Fig. 1), indicating that the stimulatory effect requires live cells or that the activity of the stimulant is sensitive to the fixative.

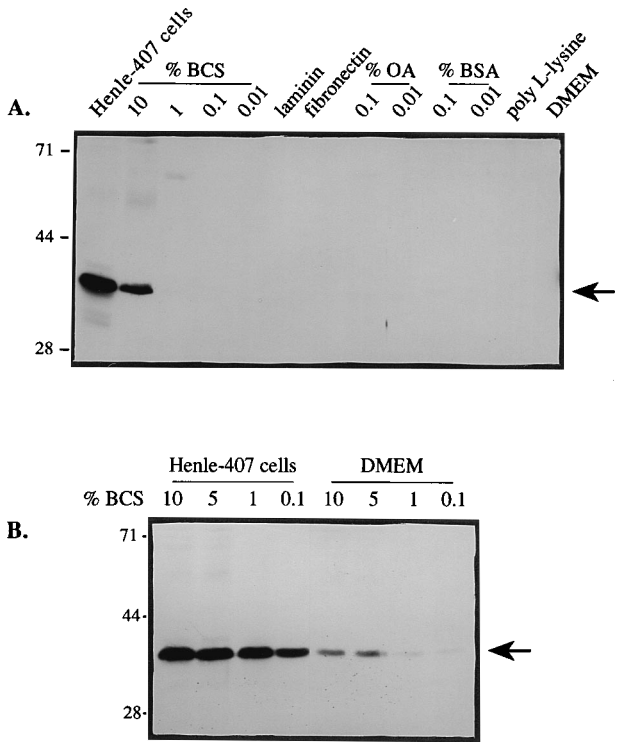

FIG. 2. Effects of serum and other proteins on InvJ secretion. (A) Wild-type *S. typhimurium* SL1344 grown to invasion competency was exposed for 1 h to tissue culture dishes containing live Henle-407 cells or treated with the indicated concentrations of BCS, laminin (0.01%), fibronectin (0.01%), ovalbumin (OA), bovine serum albumin (BSA), poly-L-lysine, or DMEM alone. (B) Wild-type *S. typhimurium* SL1344 grown to invasion competency was exposed for 1 h to tissue culture dishes containing live Henle-407 cells that had been grown in DMEM containing the indicated concentrations of BCS or culture dishes treated with DMEM and the indicated concentrations of BCS. Treatment of culture dishes and detection of InvJ were carried out as described in Materials and Methods. This experiment was repeated at least four times with equivalent results. Arrows, InvJ. The positions of the molecular weight standards are indicated on the left (in thousands).

BCS stimulates InvJ secretion. To investigate further the stimulation of InvJ secretion by BCS, we coated dishes with different concentrations of BCS prior to *Salmonella* infection. The amount of InvJ released into the medium decreased as the level of BCS dropped from 10 to 1% and was virtually undetectable when BCS concentrations were below 1% (Fig. 2A). We also tested several purified proteins (including two from serum) and poly-L-lysine for their ability to stimulate InvJ secretion. Culture dishes coated with either mouse fibronectin, bovine serum albumin fraction V, the basement membrane protein laminin, crude ovalbumin, or poly-L-lysine failed to stimulate InvJ secretion following infection of these culture dishes with *S. typhimurium* (Fig. 2A). It is possible that the stimulation of InvJ secretion by *Salmonella* spp. during infection of Henle-407 cells might be due to the accumulation of serum factors on the surface of the eukaryotic cells. To test this possibility, Henle-407 cells were grown for 48 h in DMEM containing different amounts of BCS. The concentration of BCS present did not affect the rate of growth of the Henle-407 cells. The monolayers were washed and infected with *S. typhimurium*. As shown in Fig. 2B, the amount of InvJ secreted by *Salmonella* spp. was independent of the amount of serum used in the Henle-407 cell growth medium. However, the level of InvJ secreted in the absence of epithelial cells (Fig. 2B, DMEM) was directly proportional to the BCS concentration in the medium, and little InvJ secretion was observed with BCS concentrations lower than 1%. In any case, InvJ secretion was always greater in the presence of cultured Henle-407 cells, regardless of the serum concentration. If the component responsible for stimulating secretion is solely of serum origin, then these results suggest that the eukaryotic cells efficiently extract the factor from the BCS. On the other hand, the ability of serum to stimulate InvJ secretion may be serendipitous, and the Henle-407 cells may produce a compound(s) that acts independently of serum to stimulate InvJ secretion. The latter possibility is more likely since Henle-407 cells grown in the serum-free medium HyQ-CCM 5 efficiently stimulated InvJ secretion although the medium alone did not (Fig. 3). We are in the process of identifying the factor(s) in serum and in the host cell that is responsible for stimulating the secretion of InvJ. It has been recently reported that fetal bovine serum stimulates the secretion of the *Shigella flexneri* IpaB protein (16). It remains to be determined if *Salmonella* spp. and *Shigella* spp. utilize similar inducing molecules to activate their respective type III secretion systems.

Contact-stimulated InvJ secretion does not require de novo protein synthesis. To determine if the contact-stimulated secretion of InvJ required de novo protein synthesis, we assayed the release of InvJ during infection in the presence of 100 μg of chloramphenicol per ml, an amount that immediately halts protein synthesis in *Salmonella* spp. (15). *S. typhimurium* SL1344 was used to infect Henle 407 cells in the absence or presence of chloramphenicol added at the beginning of infection. Infections were allowed to proceed for 30 or 60 min, and the secreted proteins were prepared as indicated in Materials and Methods. Chloramphenicol had virtually no effect on the amount of secreted InvJ released upon contact with either Henle-407 cells or tissue culture dishes coated with 10% BCS (Fig. 4). Thus, InvJ appears to be stockpiled in *Salmonella* spp. and is subsequently released into the medium during the interaction with the host cells.

InvJ secretion by wild-type *S. typhimurium* correlates with the invasome assembly process. Previous work has shown that the assembly of invasomes on the surface of wild-type *S. typhi-*

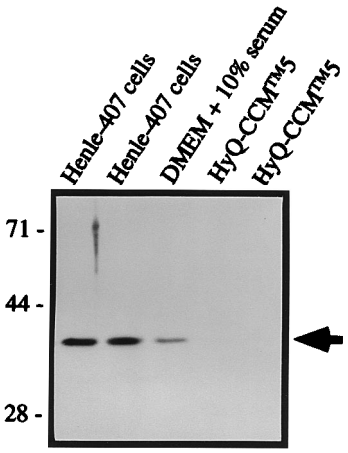

FIG. 3. Contact with Henle-407 cells grown in serum-free medium stimulates InvJ secretion. Wild-type *S. typhimurium* SL1344 was used to infect Henle-407 cells grown in the serum-free medium HyQ-CCM 5 or exposed to tissue culture dishes treated with either DMEM containing 10% BCS or HyQ-CCM 5 medium alone for 1 h at 37°C. Treatment of culture dishes and detection of InvJ were carried out as described in Materials and Methods. Repeated lanes represent two independent experiments. This experiment was repeated at least four times with equivalent results. Arrow, InvJ. The positions of the molecular weight standards are indicated on the left (in thousands).

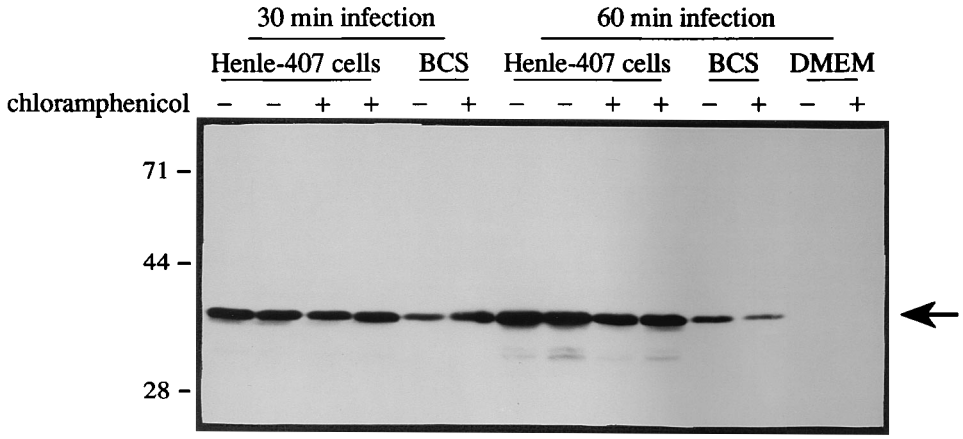

FIG. 4. Effect of protein synthesis inhibition on the contact stimulation of InvJ secretion. Wild-type *S. typhimurium* SL1344 grown to invasion competency was exposed for 30 or 60 min to tissue culture dishes containing live Henle-407 cells or tissue culture dishes treated with DMEM containing 10% BCS or DMEM alone. When indicated, chloramphenicol (100 μg/ml) was added immediately before infection. Treatment of culture dishes and detection of InvJ were carried out as described in Materials and Methods. This experiment was repeated at least four times with equivalent results. Arrow, InvJ. The positions of the molecular weight standards are indicated on the left (in thousands).

murium requires contact with cultured epithelial cells and that the surface appendages are shed upon bacterial signaling to the host cell (8). Furthermore, InvJ secretion in the invasion-defective mutants tested also closely correlated with the formation of the invasome surface appendages. *invC* and *invG* mutants, which failed to secrete InvJ, also failed to assemble the invasome (8). *invE* mutants, which secreted InvJ upon contact with cultured cells, were capable of assembling morphologically aberrant surface appendages but did not stimulate host cell membrane ruffling (8). These results also suggest that InvJ secretion per se, although required, is not sufficient to stimulate bacterial entry into host cells.

Contact between *Salmonella* spp. and epithelial cells appears to be critical for inducing biochemical and subcellular processes in the host cell that result in bacterial internalization. Evidence is growing that two of these events, the secretion of the virulence protein InvJ and the assembly of the invasomes on the surface of *Salmonella* spp., may be linked. Both are induced upon contact with epithelial cells, both require an operating type III export system, and neither event requires de novo protein synthesis following infection. InvJ is therefore a candidate to be a structural component of the invasome appendages. Alternatively, InvJ secretion might precede invasome formation, serving as a signal to assemble the appendages. With the monoclonal antibody J33.13, it will be possible to determine if InvJ forms a part of the invasome and whether InvJ secretion and invasome assembly are sequential events, and if so, what is their order of progression following bacterium-host cell contact. In addition, the results presented in this article further support the notion that *Salmonella* entry into cultured cells is the result of a biochemical cross-talk between the bacteria and the host cells.

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