INFECTION AND IMMUNITY, Sept. 1993, p. 3625–3635
0019-9567/93/093625-11$02.00/0

Vol. 61, No. 9

OmpC Is Involved in Invasion of Epithelial Cells by *Shigella flexneri*

MARIA L. BERNARDINI,† MARIA G. SANNA, ANNICK FONTAINE, AND PHILIPPE J. SANSONETTI*

Unité de Pathogénie Microbienne Moléculaire, Institut National de la Santé et de la Recherche Médicale, U 199, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris Cédex 15, France

Received 22 February 1993/Returned for modification 1 April 1993/Accepted 18 June 1993

Osmoregulation of the *Shigella flexneri ompC* gene and the role of OmpC in *Shigella* virulence have been investigated. OmpC was highly expressed when bacteria were grown in medium of either low or high osmolarity. This constitutive expression is in contrast with the regulation observed in *Escherichia coli*, in which the expression of OmpC is repressed at low osmolarity and induced at high osmolarity. In addition, the *Shigella ompC* gene was barely expressed by a Δ*ompB* (Δ*ompR* and Δ*envZ*) mutant. We described in a previous report that such a mutant was severely impaired in virulence both in vitro and in vivo. Starting from this observation, and in order to assess which gene(s) regulated by *ompR* and *envZ* are involved in virulence, we constructed an *S. flexneri* Δ*ompC* mutant. Three *S. flexneri* mutants, *ompF'-lacZ*, Δ*ompC*, and Δ*ompB*, were compared for virulence. The *ompF'-lacZ* mutant behaved like the *S. flexneri* serotype 5 wild-type strain M90T in all in vitro and in vivo virulence tests. On the contrary, the Δ*ompB* and Δ*ompC* strains were considerably impaired in their virulence phenotypes. The ability of these two mutants to spread from cell to cell and to kill epithelial cells was severely affected. Consequently Δ*ompC*, as previously described for Δ*ompB*, was unable to elicit a positive Sereny test. The Δ*ompB* mutant was restored to virulence by introducing a recombinant multicopy plasmid carrying the cloned *E. coli ompC* gene, indicating that a functional OmpC protein was necessary and sufficient to restore virulence to this mutant of *S. flexneri*.

Shigellae are gram-negative enteric bacilli which cause bacillary dysentery by a multistep invasive process. First, bacteria penetrate and replicate within colonic epithelial cells (22). Entry into epithelial cells occurs by induced phagocytosis (9); intracellular bacteria then lyse the phagocytic vacuole and multiply inside host cells (47). This activity can be studied in in vitro invasion and intracellular multiplication assays that use cultivated mammalian cell lines such as HeLa and Caco-2. Another key step of the invasive process is the intra- and intercellular spread of bacteria within epithelial tissues (5, 27). Shigellae move to adjacent cells via protrusions without leaving the intracellular compartment. Similar intra- and intercellular movements have been described for *Listeria monocytogenes* (33, 50). The driving force which promotes the movement of both *Shigella flexneri* and *L. monocytogenes* arises from the production of multiple foci of actin nucleation near the bacterial surface that subsequently localize and elongate at one pole of the bacterium (5, 14, 21). The final consequence of these processes is killing of the host cells. Intra- and intercellular spread and cellular disruption can be approximated in vitro in the plaque assay in which the ability of invasive bacteria to cause a cytopathic effect on a confluent monolayer of HeLa cells is tested.

The *S. flexneri* genetic loci encoding entry into epithelial cells as well as intra- and intercellular spread are located on a 220-kb virulence plasmid (28, 46). Nevertheless the expression of chromosomal genes is also necessary for full expression of virulence (40, 51).

We have previously demonstrated that the two-component regulatory system EnvZ-OmpR is involved in the regulation of genes associated with virulence of shigellae (4). Deletion mutants in the *ompB* locus, which includes the *envZ* and *ompR* genes, were severely affected in in vitro and in vivo virulence assays (4, 45). In *Escherichia coli*, EnvZ is an inner-membrane-bound protein (12) that has both kinase (13) and phosphatase (18) activities, and OmpR is a DNA-binding protein which can be phosphorylated by EnvZ (2, 13) and activates transcription of *ompF* and *ompC* (38). *E. coli* responds to changes in the osmotic strength of the environment by changing the ratio of porins OmpF and OmpC, so that the total level of porin proteins remains approximately constant (26, 53). OmpF, which produces slightly wider pores than does OmpC (1.2 nm), predominates at low osmotic strength, whereas OmpC (1.1 nm) predominates at high osmotic strength (for a review, see reference 37). It has been proposed that the smaller pores formed by OmpC could reduce the diffusion of larger hydrophobic and negatively charged molecules when bacteria encounter high-osmolarity conditions as in host compartments (i.e., 300 mosM). On the contrary, in an external environment which presents lower osmotic strength and lower concentrations of toxic substances and nutrients, the larger channel of OmpF may be better adapted. In addition, higher growth temperature, a condition again found in the bodies of animals and humans, favors the production of OmpC over that of OmpF (37).

In contrast to the situation in *E. coli*, the human pathogen *Salmonella typhi* shows OmpC expression in both high- and low-osmolarity conditions (42). Nevertheless, as described for *E. coli*, expression of *S. typhi* OmpC is dependent on the OmpR transcriptional activator. Furthermore, *Salmonella typhimurium ompR* mutants express an attenuated virulence phenotype (11).

In a previous study, we showed that mutants lacking the

* Corresponding author.

† Present address: Dipartimento di Biologia Cellulare e dello Sviluppo, Sezione di Scienze Microbiologiche, Università di Roma "La Sapienza," Rome, Italy.

TABLE 1. Bacteria and plasmids

Strains and plasmids	Genotype and relevant characteristics	Reference or source
Strains		
E. coli K-12		
MC4100	F⁻ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 fbB5301 ptsF25 deoC thiA1*	7
JMS150	MC4100 (*ompF'-lacZ*) 16-13	4
RAM191	MCR106 Δ*ompC178 zei-198*::Tn*10*	31
S. flexneri		
M90T	Serotype 5; wild type harboring 220-kb invasion plasmid	46
SC433	M90T Δ*ompB*--Ω; Sp^r	4
SC442	M90T *ompF'-lacZ* transductant of JMS150 donor	4
SC445	M90T Δ*ompC zei*::Tn*10* transductant of RAM191 donor; Tc^r	This study
Plasmids		
pJP33	Vector pACYC184, cloned *E. coli ompF* gene	52
pMY150	Vector pBR322, cloned *E. coli ompC* gene	32

expression of EnvZ and OmpR were unable to colonize epithelial tissue cultures. Nevertheless, the step of the invasive process that was affected in such mutants had not been elucidated. On this basis, we analyzed in this study the phases of the invasive process and the virulence-associated activities of the Δ*ompB* strain, particularly intracellular multiplication and intra- and intercellular spreading. In addition, we investigated genes submitted to EnvZ-OmpR regulation which could possibly be involved in *Shigella* virulence. In particular, since Δ*ompB* mutants were not thought to produce porins OmpC and OmpF, we were interested in investigating whether loss of the porins could compromise the ability of the bacteria to survive and grow in the host compartments.

MATERIALS AND METHODS

Strains and media. All *S. flexneri* strains studied here were derived from the wild-type serotype 5 strain M90T (46). The bacterial strains and plasmids used are listed in Table 1. Bacteria were routinely cultured in tryptic casein soy (Diagnostics Pasteur, Marne La Coquette, France) and Luria (30) broth or agar. Nutrient broth (NB; Difco) and NB supplemented with 0.3 M NaCl were used as low- and high-osmolarity media, respectively. Alternatively, K medium was also used as a low-osmolarity medium (20). M9 salts (30) were used for preparing minimal medium. Carbon sources were added to a final concentration of 0.2%, and the medium was supplemented with 10 μg of nicotinic acid per ml to allow shigellae to grow. When necessary, streptomycin, ampicillin, tetracycline, and chloramphenicol were added at 100, 100, 12.5, and 30 μg/ml, respectively.

Genetic procedures. Transformation was performed as described by Dagert and Erlich (10). Generalized transduction with bacteriophage P1 was carried out as described by Miller (30).

Construction of an *S. flexneri ompC* deletion mutant. A P1 lysate of RAM191 (*E. coli* Δ*ompC178 zei*::Tn*10*) (31, 48) was used to transfer the *ompC* region into wild-type *S. flexneri* M90T by using the linked selectable marker *zei-198*::Tn*10* (the Tc^r marker is approximatively 60% linked to the *ompC* gene). Tc^r transductants, selected on Luria-tetracycline plates, were screened for OmpC production by means of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the outer membrane proteins (see below). A transductant, named SC445 (M90T Δ*ompC178 zei-198*::Tn*10*), was selected for further studies.

Strain RAM191, constructed by Misra and Benson (31), carries the Δ*ompC178* deletion previously isolated by Schnaitman and McDonald (48). This deletion includes the entire chromosomal *Hin*dIII (2.6-kb) fragment carrying *ompC* and containing all information necessary for normal regulation of OmpC protein. In addition, this deletion extends into flanking DNA sequences on the *gyrA* side of *ompC*.

Plasmid PMY150 (32) carries a 1.1-kb *E. coli* chromosomal DNA fragment cloned in pBR322. This fragment contains the entire *ompC* coding sequence, including the *ompC* promoter and the DNA encoding the 5'-end untranslated region of *ompC* RNA involved in *ompF* regulation (32). In *S. flexneri* SC445 (M90T Δ*ompC178 zei-198*::Tn*10*), OmpC production was restored by complementation with plasmid PMY150.

Protein analysis. Bacterial envelopes were isolated by the method of Achtman et al. (1). Bacteria were cultured to late exponential growth phase in NB or NB–0.3 M NaCl, harvested, and resuspended in 10 mM Tris-HCl (pH 8.0)–5 mM Mg^{2+}. The bacterial suspension was sonicated three times at 4°C for 30 s each time. Unbroken intact cells were removed by low-speed centrifugation for 20 min, and whole membranes were pelleted at 32,000 × *g* for 60 min at 4°C. Outer membranes were extracted with Triton X-100 (final concentration, 2%) in 10 mM Tris-HCl (pH 7.4)–5 mM Mg^{2+} for 30 min at room temperature. Protein samples prepared by the method of Lugtenberg et al. (25) were subjected to urea-SDS-polyacrylamide gel electrophoresis (8 M urea, 7% acrylamide, 0.21% bisacrylamide), and gels were loaded so that each well contained 20 μg of protein.

Immunoblotting was carried out as described by Burnette (6) by using the serum of a monkey experimentally infected with the wild-type *S. flexneri* M90T or an anti-OmpC polyclonal serum kindly provided by J. Tommassen.

Analysis of LPS side chains. Lipopolysaccharide (LPS) samples were prepared essentially as described by Westphal and Jann (54). The component parts were separated as previously described (23) and transferred to a nitrocellulose membrane. Filters were successively incubated with *S. flexneri* anti-LPS serum and ^{125}I-labeled protein A. LPS O side chains were then visualized by autoradiography.

Virulence assays. (i) Infection of HeLa and Caco-2 cells. The HeLa cell invasion assay was performed as previously described (16). The Caco-2 cell invasion procedure was also performed as previously described (34).

(ii) Intracellular multiplication of bacteria within HeLa cells. Multiplication of bacteria within HeLa cells was assayed as previously described (47), with minor modifica-

tions. Nonconfluent monolayers of HeLa cells were inoculated with bacteria suspended in 2 ml of minimal essential medium (GIBCO) at a multiplicity of infection of 100, centrifuged, and incubated for 30 min at 37°C to allow entry. Plates were washed three times with Earle's balanced salt solution (EBSS; GIBCO) and covered with 2 ml of minimal essential medium with gentamicin (50 μg/ml). This point was considered as time zero (T_0). Incubation was usually carried out for 5 h. Two plates were removed every hour (T_1, T_2, T_3, T_4, and T_5). One plate was washed three times with EBSS and Giemsa stained to calculate the percentage of infected HeLa cells. The other plate was washed five times with EBSS to eliminate viable extracellular bacteria. Cells were trypsinized, counted, and then lysed with 0.5% sodium deoxycholate in distilled water. Dilutions of this suspension were plated onto tryptic soy agar. The average number of bacteria per infected cell was calculated according to the following equation: (number of CFU/plate)/(number of HeLa cells × percentage of infected HeLa cells). Experiments were repeated at least five times for each strain tested. Results represent the means of the five experiments.

(iii) Plaque assay. The plaque assay was carried out as originally described by Oaks et al. (39).

(iv) Infection of J774 macrophages. The mouse macrophage cell line J774 was propagated and infected as described previously (8).

(v) Sereny test. The keratoconjunctivitis assay was performed with guinea pigs as originally described (49).

Fluorescence staining of F-actin and bacteria. Cell permeabilization and fixation, labeling of intracellular bacteria by goat anti-rabbit rhodamine-conjugated immunoglobulin G (Sigma Chemical Co., St. Louis, Mo.), and F-actin staining by NBD-phallacidin (Molecular Probes, Inc., Junction City, Ore.) were carried out as previously described (8).

Electron microscopy. HeLa cells were infected according to the procedure described above. After 3 h of incubation at 37°C, cells were fixed for 48 h at 20°C in 0.1 M cacodylate buffer (pH 7.2) containing 5% sucrose and 1.6% glutaraldehyde. Successively, cells were washed in the same buffer containing 7.5% sucrose and postfixed for 2 h with Ferro-Osmium (1:1%). After this procedure, samples were dehydrated in acetone and embedded in Epon. Thin Epon sections were collected on nickel grids, stained with uranyl acetate and lead citrate (43), and observed on a Philips CM12 electron microscope. About 200 infected cells were routinely observed.

RESULTS

Expression of the porins in the *S. flexneri* wild-type strain and in *ompC*, *ompF*, and *ompB* mutant strains. It was previously demonstrated that an *S. flexneri* Δ*envZ*-Δ*ompR* (Δ*ompB*) strain was severely impaired in virulence (4). This strain, named SC433, achieved a low level of entry into eucaryotic cells, did not cause a cytopathic effect on a confluent monolayer, and was unable to provoke keratoconjunctivitis in guinea pigs (Sereny test). To identify *S. flexneri* genes that are involved in virulence under the control of *ompB*, the effects of defined mutations affecting expression of *ompC* and *ompF* in wild-type virulent *S. flexneri* M90T were assessed.

A previously constructed (4) *S. flexneri* M90T *ompF'-lacZ* transductant of JMS150 (*E. coli ompF'-lacZ*), named SC442, was used as an *ompF* mutant in this study. As an *ompC* mutant, we used an M90T Δ*ompC178 zei-198*::Tn*10* mutant, named SC445 (see Materials and Methods). *S. flexneri ompC ompF* double mutants were also constructed. Unfortunately, such mutants always expressed a rough LPS (see below).

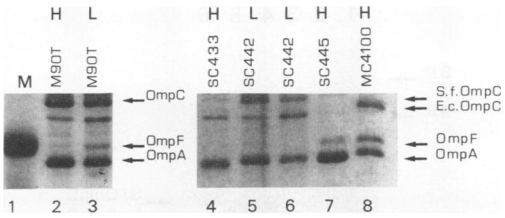

FIG. 1. Electrophoretic pattern of OMP preparations. *S. flexneri* M90T (lanes 2 and 3), SC433 (lane 4), SC442 (lanes 5 and 6), and SC445 (lane 7) and *E. coli* MC4100 (lane 8) were grown in low-osmolarity (L) media (NB [lanes 3 and 5] and K medium [lane 6]) and high-osmolarity (H) media (NB–0.3 M NaCl [lanes 2, 4, and 7] and LB [lane 8]). The positions of the *S. flexneri* (S.f.) and *E. coli* (E.c.) OMPs are indicated by arrows. Lane M, molecular size marker (ovalbumin; 46 kDa).

To assess production of outer membrane proteins OmpC and OmpF by mutant strains SC433 (M90T Δ*ompB*), SC445 (M90T Δ*ompC*), and SC442 (M90T *ompF'-lacZ*), SDS-polyacrylamide gel electrophoresis was used to characterize outer membrane proteins prepared from these strains (Fig. 1). The *ompC* and *ompF* mutant strains were tested in high- and low-osmolarity media, respectively.

We also tested expression of porins OmpC and OmpF in M90T grown in low-osmolarity (NB) and high-osmolarity (NB–0.3 M NaCl) conditions (Fig. 1). As recently observed in *S. typhi* (42), *S. flexneri* M90T OmpC was highly expressed under conditions of low and high osmolarity (Fig. 1). In contrast, the expression of *E. coli* OmpC is inhibited at low osmotic strength of the medium (26). We used OmpC and OmpF expression of *E. coli* MC4100 grown in LB medium as a control, since under such conditions this strain produces both OmpC and OmpF. The *S. flexneri* OmpC and *E. coli* OmpC porins did not comigrate (Fig. 1). Electrophoretic profiles indicated that the apparent molecular weight of *S. flexneri* OmpC was greater than that of *E. coli* OmpC. SC445 (Δ*ompC*) and SC442 (*ompF'-lacZ*) did not express OmpC and OmpF, respectively (Fig. 1). We tested SC442 in two low-osmolarity media, K medium (~125 mosM) (20) and NB medium (~176 mosM). This mutant strain showed no expression of OmpF in either of these media, whereas OmpC expression was observed under both conditions.

SC433 (Δ*ompB*) had no detectable expression of OmpF in low-osmolarity (data not shown) or high-osmolarity medium. In contrast, when bacteria were grown in a high-osmolarity medium, a thin band was present in the region of the gel where the *S. flexneri* wild-type OmpC protein migrates. To assess OmpC expression in mutant strains, the OmpC profile was also investigated by means of Western blotting (immunoblotting) with a polyclonal serum directed against *E. coli* OmpC protein. Outer membranes from M90T, SC433 (Δ*ompB*), and SC445 (Δ*ompC*) grown in high-osmolarity conditions were tested in this analysis (Fig. 2). Expression of *E. coli* OmpC was used as a control. SC445 did not show any OmpC expression. SC433 was observed to produce a low amount of the OmpC protein. This residual production of OmpC in the absence of the *ompB* locus could be due to a regulatory effect exerted by DNA supercoiling (15).

Cell invasion rates of mutant strains assessed in in vitro assays. Since *Shigella* rough strains are avirulent when tested in a more definitive assay system such as the Sereny

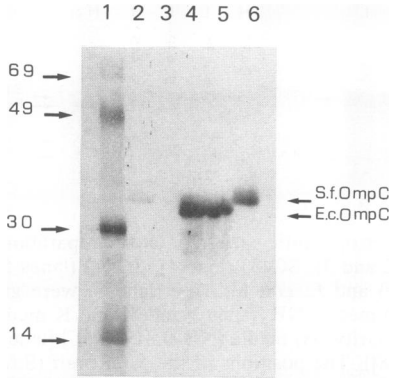

FIG. 2. Immunoblot analysis of OmpC expression. A Western blot of OMP preparations was resolved by polyacrylamide gel electrophoresis and probed with a polyclonal serum against *E. coli* OmpC. Lanes: 1, protein standards; 2, SC433 grown in high-osmolarity medium (NB–0.3 M NaCl); 3, SC445 grown in high-osmolarity medium (NB–0.3 M NaCl); 4, *E. coli* JMS150 grown in LB; 5, MC4100 grown in LB; 6, M90T grown in high-osmolarity medium (NB–0.3 M NaCl). Positions of *S. flexneri* (S.f.) and *E. coli* (E.c.) OmpC proteins are shown at the right; positions of protein standards (sizes in kilodaltons) are indicated at the left.

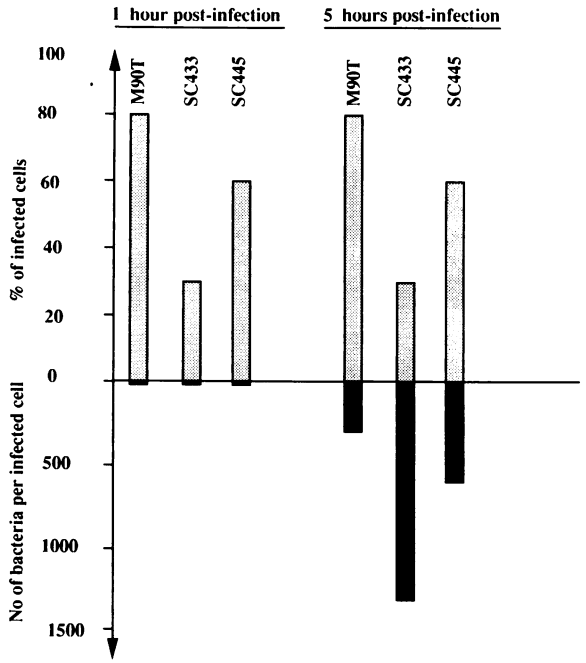

FIG. 3. Bacterial growth and percentage of infected HeLa cells after 1 and 5 h of incubation postinfection. All relevant data are shown and represent the means of five experiments. Standard deviations were less than 10% of the mean.

test, LPS was prepared from each mutant strain. Each strain was shown to possess complete O-polysaccharide side chains when compared with M90T (data not shown). SC433 (Δ*ompB*), SC445 (Δ*ompC*), and SC442 (*ompF'-lacZ*) were tested for expression of virulence in the HeLa cell invasion assay. As previously described, SC433 showed a reduced invasive phenotype. On average, SC433 infected only 30% of the cells, whereas M90T, the wild-type strain, infected 80% of them. SC445 also showed a lowered rate of invasion of HeLa cells (60% infected HeLa cells). In contrast, SC442 was observed to invade HeLa cells at a rate similar to that of wild-type strain M90T.

Intracellular multiplication of mutant strains. In a previous study (45), M90T was found to reach a peak of ca. 300 bacteria per infected HeLa cell after 5 h of infection and then to exhibit a decrease corresponding to the disruption of the monolayer. In this study, following intracellular multiplication, M90T reached a peak of 280 bacteria per infected cell in 5 h. Surprisingly, peaks of 1,290 and 650 bacteria per infected cell were observed with mutants SC433 and SC445, respectively (Fig. 3). In contrast, SC442 showed an intracellular multiplication rate similar to that of the wild-type strain.

After only 3 h of infection, the number of bacteria per infected cell in mutant strains and the wild type appeared similar. Striking differences in intracellular bacterial accumulation to reach the peak values mentioned above became obvious only between 3 and 5 h.

Intracellular mutants were observed by Giemsa staining at different times of infection. One hour after infection, intracellular SC433 and SC445 appeared to be altered in shape (Fig. 4A). In particular, SC433 showed a different length-to-width ratio, resulting in a round morphology (4.3 for the wild type and 1.7 for SC433). Electron microscopy confirmed this observation (Fig. 4B). The altered shape of SC433 and SC445 was not due to the high osmolarity encountered in the cellular compartment. Indeed, observation by electron microscopy of bacteria grown in low- and high-osmolarity media did not reveal any difference in morphology between mutants and the wild-type strain (data not shown). In addition, when all of these strains were grown in conditions of high osmolarity, they displayed a characteristic rod shape. In contrast, the bacterial shape became round in low-osmolarity medium.

After 5 h of infection, Giemsa staining highlighted that intracellular SC433 remained trapped inside HeLa cells, in agreement with the high number of bacteria reported in the experiment described above (Fig. 5). The wild-type strain showed a characteristic random distribution inside HeLa cells, whereas SC445 was less randomly distributed. As expected, the phenotype of SC442 was similar to that of M90T.

These results suggested that mutants SC433 and SC445 could be impaired in intra- or intercellular spreading, thus multiplying and accumulating within the cell that was initially invaded. To assess this hypothesis, bacterial activities associated with these phenotypes (intra- and intercellular spreading) were investigated.

Infection of Caco-2 cell islets. When grown in culture dishes under appropriate conditions, confluent Caco-2 cells become well differentiated, showing the presence of apical microvilli. Under these conditions, Caco-2 cells form roughly circular islets consisting of 20 to 100 cells. It has been previously reported that *S. flexneri* strains enter the Caco-2 islets only through the basolateral pole of these cells (34). Only bacteria able to move intracellularly and from cell to cell can reach the center of the infected islet without passage in the extracellular medium. Giemsa-stained preparations of Caco-2 cells infected with the wild-type strain and with SC433 (Δ*ompB*), SC445 (Δ*ompC*), and SC442 (*ompF'-lacZ*$^+$) showed that mutant strains SC433 and SC445 were not able to reach the center of the islet. After 4 h of infection, M90T had colonized the entire islet. In contrast, SC433 had entered and disseminated only within the peripheral cells of the islet,

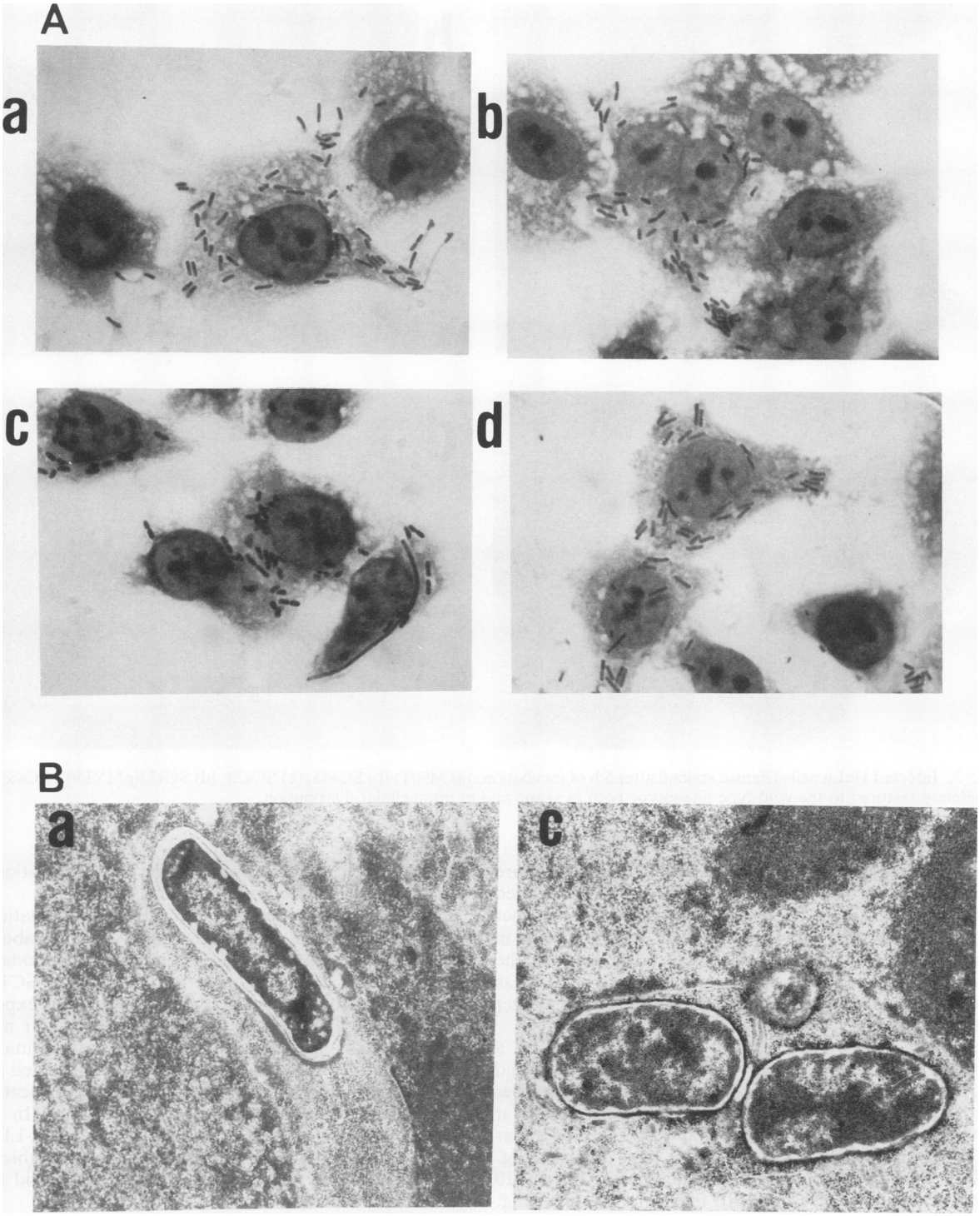

FIG. 4. (A) Infected HeLa cells incubated for 1 h postinfection and Giemsa stained. (a) M90T; (b) SC445; (c) SC433; (d) SC442. One hour after infection, SC445 (b) and SC433 (c) showed a shape different from that of M90T (a). In particular, SC433 appeared round and formed long chains. (B) Electron micrographs of HeLa cells infected with M90T (a) and SC433 (c). Samples were processed after 2 h of infection. Bar, 0.5 μm.

so that the centrally located cells remained uninfected. SC445 had a phenotype similar to that of SC433: although bacteria did not reach the center of the islet, they seemed to be less impaired in inter- and intracellular movement than was SC443 (Fig. 6). SC442 colonized the Caco-2 cells as well as the wild-type strain M90T did.

Analysis of intracellular spread in HeLa cells. The plaque assay reflects the ability of bacteria to spread within a cell

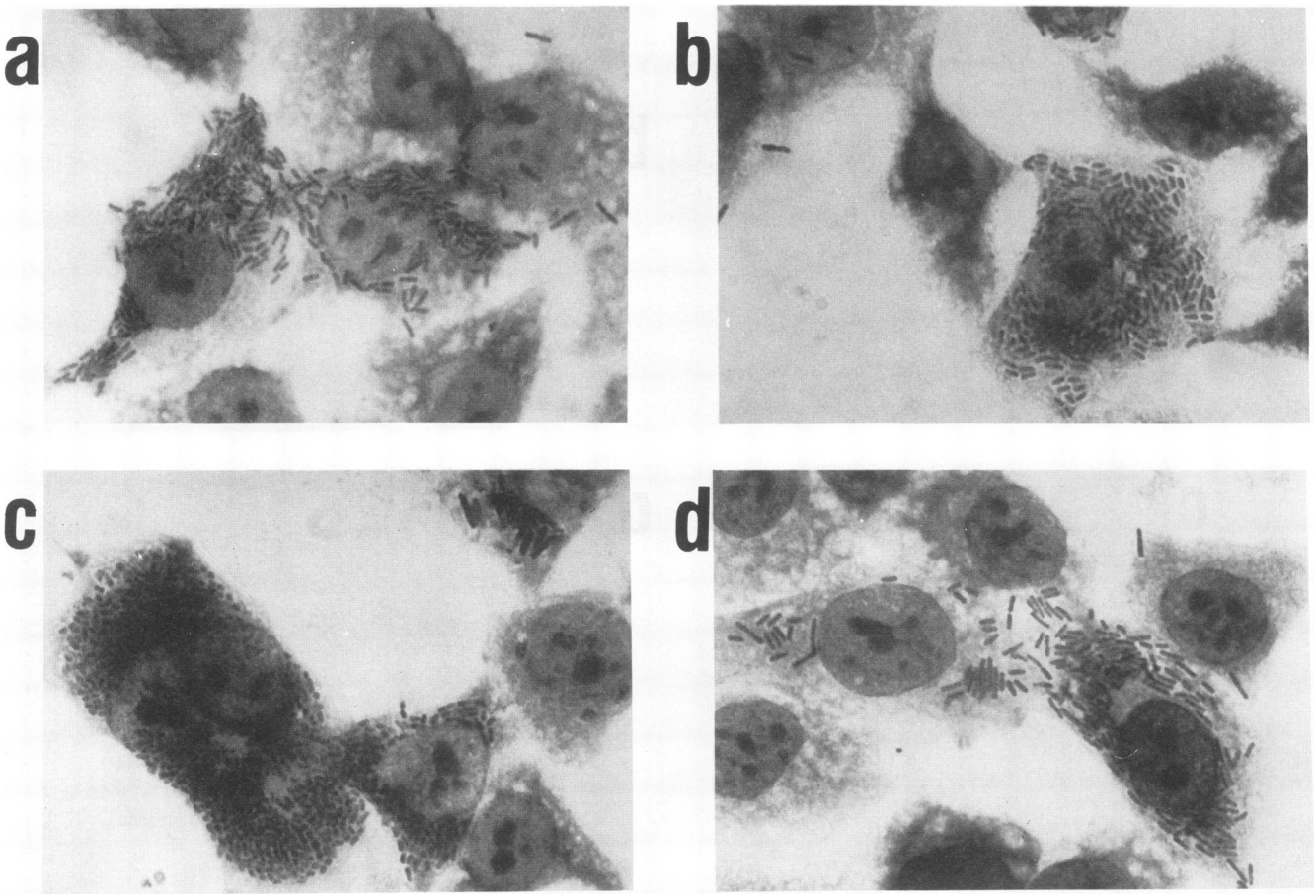

FIG. 5. Infected HeLa cells Giemsa stained after 5 h of incubation. (a) M90T; (b) SC445; (c) SC433; (d) SC433(pMY150). SC433(pMY150) is completely restored to the wild-type phenotype both in shape and in intracellular distribution.

monolayer and kill host cells. The results of this process are observable as zones of dead cells that have been destroyed by bacterial infection, or plaques (39). To determine whether mutant strains SC433, SC445, and SC442 were impaired in the ability to move intercellularly, we tested them in the plaque assay (Fig. 7). As previously published, SC433 was not able to form plaques on a confluent HeLa cell monolayer (4).

The plaque assay was performed in most cases at a multiplicity of infection of one bacterium per cell. To avoid the possibility that SC433 would not produce plaques because of its low capacity to infect, this test was carried out at multiplicities of infection of 10, 100, and 1,000. Even under these conditions, SC433 was unable to form plaques (Fig. 7c). SC445, at a multiplicity of infection of at least 10 bacteria per cell, was observed to form tiny plaques (Fig. 7b). Even after prolonged incubation, these tiny plaques did not increase in diameter. SC442 produced plaques similar in number and size to those produced by M90T (Fig. 7a).

The intra- and intercellular movement of shigellae is due to a mechanism involving the formation of multiple foci of actin nucleation, followed by the formation of actin filaments which are reorganized at the extremity of the bacterial body and tightly bundled by fibrin (14, 41). Moving bacteria leave behind trails of F-actin and become incorporated into cytoplasmic protrusions which are internalized by adjacent cells. The *S. flexneri icsA* gene encodes a 120-kDa outer membrane protein which is essential for the organization of actin that pushes the bacterium forward (5, 14).

It is possible to demonstrate actin polymerization during bacterial infection of HeLa cells by double labeling with NBD-phallacidin for F-actin and anti-LPS-rhodamine for bacteria (5). To assess whether SC433 and SC445 could cause actin to polymerize, we performed two experiments. Western blot analysis of cytoplasmic and outer membrane fractions was used to determine whether the mutant strains expressed the 120-kDa protein. All mutants tested produced a wild-type level of this protein, which was located in the outer membrane fraction (data not shown). In addition, double labeling by NBD-phallacidin and anti-LPS-rhodamine showed that intracellular bacteria were able to polymerize actin as well as the wild-type strain did (data not shown).

Infection of J774 macrophages and Sereny test. To assess whether killing of macrophages was impaired, J774 macrophages were infected with the mutant strains and the wild type. No significant differences between mutant strains and M90T in destruction of macrophages were observed (data not shown). As previously demonstrated, SC433 exhibits a weak response in the Sereny test. SC445 provoked a mild and delayed reaction, whereas SC442 elicited a positive Sereny test.

Phenotypical complementation of SC433. Complementation with pMY150 carrying the *E. coli ompC* gene restored SC445

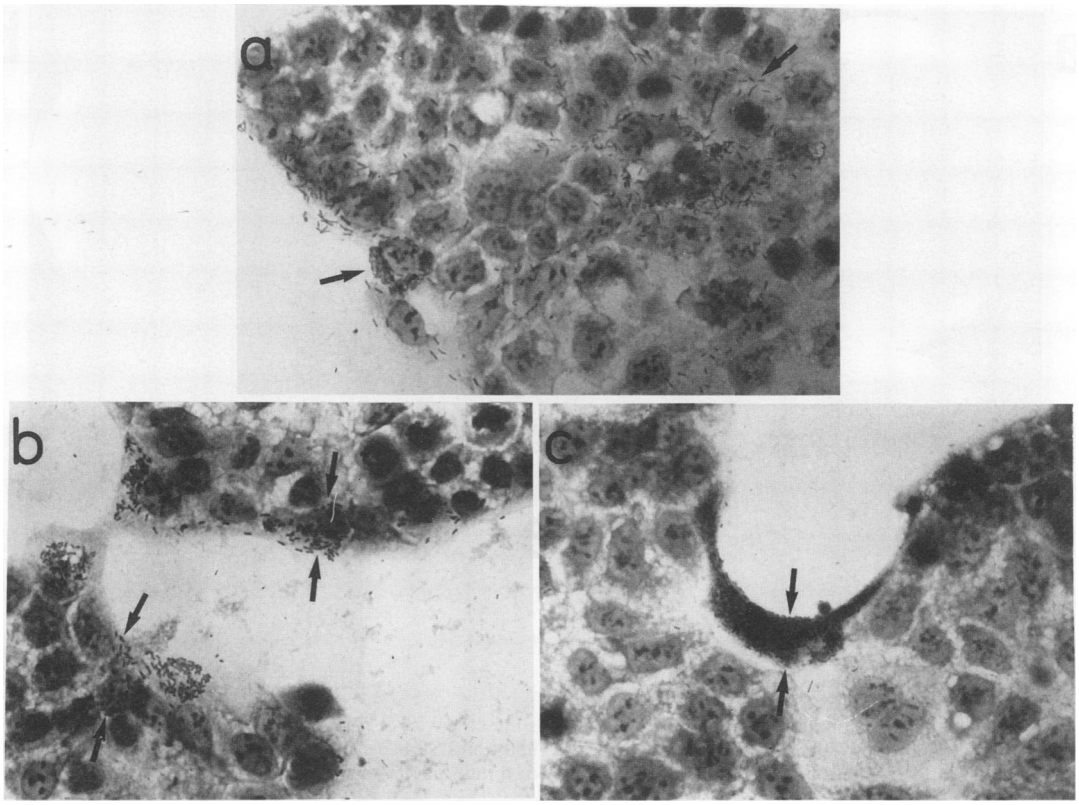

FIG. 6. Giemsa stains of confluent islets of Caco-2 cells infected for 3 h with *S. flexneri* and mutants. M90T (a) colonized the entire islet, whereas SC445 (b) and SC433 (c) remained inside the peripheral cells of the islet. Arrows indicate the limits of bacterial dissemination inside the islets.

to full virulence in the Sereny test. In particular, SC445 (pMY150) elicited a positive plaque assay at a multiplicity of infection of one bacterium per cell. Most of the plaques obtained were similar in size to those produced by the wild-type strain, and some were small (Fig. 7d). We reasoned that the presence of *ompC* cloned on a multicopy plasmid might be toxic for bacteria. Thus, the smaller plaques could be due to the overproduction of OmpC in SC445(pMY150). For these reasons, pMY150 was also introduced into the wild-type strain M90T, and the resulting strain was tested in the plaque assay. Also in this case, both small and normal-size plaques were observed. SC433 was cotransformed with pMY150 (*E. coli ompC*) and pJP33 (*E. coli ompF*). SDS-polyacrylamide gel electrophoresis showed that both *E. coli* OmpC and OmpF overexpressed by multicopy plasmids were produced in an *S. flexneri ompB* background (data not shown).

The resulting strain, when assessed in the virulence assays, appeared to be restored to full virulence. Only the cellular invasion rate was not completely restored to the wild-type level. The shape and the number of intracellular bacteria were the same as those of the wild-type strain (data not shown). SC433(pMY150, pJP33) was positive in the Sereny test and formed plaques on a confluent monolayer of HeLa cells. Unlike SC433, SC433(pMY150, pJP33) produced plaques under the same conditions as the wild-type strain did, although in lower number than found for the wild-type strain. Complementation with pJP33 alone did not change the avirulent phenotype of SC433, whereas introducing pMY150 alone restored the virulence phenotype to SC433 (Fig. 5d). This strain yielded plaques that were similar in number and size to those observed with SC433(pMY150, pJP33) (Fig. 7e).

DISCUSSION

The aim of this study was to examine the expression, osmoregulation, and role in virulence of the *S. flexneri* OmpC and OmpF porins.

We first studied the regulation of OmpC and OmpF expression in response to different conditions of osmotic strength. In *E. coli*, OmpC production is induced by high osmolarity, high temperature, and anaerobiosis (35, 53). These conditions reflect the situation encountered by enterobacteriaceae in the human bowel lumen. Under the same conditions, the production of OmpF is turned off. Conversely, OmpF is abundantly produced when osmolarity is low, and this production is not greatly affected by oxygen tension. In contrast to *E. coli*, expression of OmpC in *S. typhi* is not regulated by osmolarity. This porin is highly expressed when bacteria are grown in medium of either low or high osmolarity (42).

In this paper we report that in contrast to *E. coli*, *S. flexneri*, like *S. typhi*, expresses OmpC in both high- and low-osmolarity media, whereas OmpF is barely expressed only in media of low osmotic strength. Both *S. flexneri* and *S. typhi* are human pathogens that colonize the gut. They may therefore have adapted to life in conditions of high osmolarity, high temperature, and anaerobiosis. Additionally, even though pathogens such as shigellae are transmitted

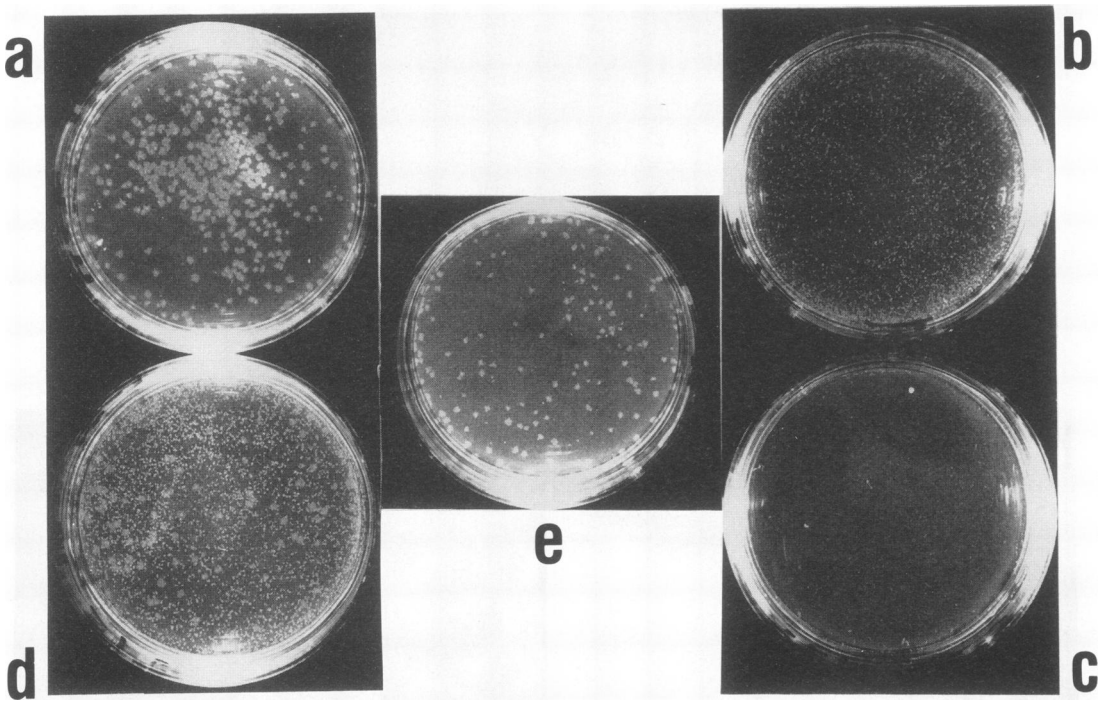

FIG. 7. Formation of plaques on confluent monolayers of HeLa cells infected with *S. flexneri* and mutants. (a) M90T; (b) SC445; (c) SC433; (d) SC445(pMY150); (e) SC443(pMY150). M90T, SC433, SC443(pMY150), and SC445(pMY150) were tested at a multiplicity of infection of one bacterium per cell. To reveal the tiny plaques produced by SC445, a multiplicity of infection of 10 bacteria per cell was used.

by the fecal-oral route, they do not survive well in the environment. Thus, it can be speculated that in *E. coli*, regulation of porin expression has evolved according to the different environmental niches that this species must adapt to. This same regulation would not provide a distinct advantage to *S. flexneri*, which has a more restricted niche and a very short phase of transition from an external reservoir to the host.

Second, we demonstrated that in *S. flexneri*, as also observed in *E. coli* (17) and *Salmonella* spp. (24, 42), the two-component regulatory system EnvZ-OmpR regulates the expression of porins OmpC and OmpF. An *S. flexneri* Δ*ompB* (Δ*envZ-ompR*) mutant became unable to produce a significant amount of OmpC in response to high-osmolarity conditions. Residual expression of OmpC in the absence of *envZ-ompR* can still be induced by changes in DNA supercoiling in response to environmental stress (15). This has not been investigated for *S. flexneri*.

We have also investigated the role of OmpC and OmpF in *S. flexneri* virulence. We have previously reported that an *S. flexneri envZ-ompR* deletion mutant is severely impaired in virulence (4). This mutant, as shown in this study, is unable to produce OmpC and OmpF. Starting from these observations, we have analyzed the effects of specific mutations introduced into *ompC* and *ompF* on *S. flexneri* virulence. This was achieved by studying the virulence phenotypes of Δ*ompC* and *ompF'-lacZ* mutants. The virulence phenotypes of Δ*ompC* and *ompF'-lacZ* mutants were compared with the virulence phenotype of the Δ*ompB* mutant. Results indicated that the absence of OmpC caused a reduction of virulence. Conversely, OmpF did not appear to be involved in virulence. These data are in contrast to those reported for *S. typhimurium* (11), in which mutations in *ompC* do not affect virulence. When the Δ*ompB* mutant was restored to producing OmpC by introduction of a multicopy recombinant plasmid carrying the *E. coli ompC* gene, virulence was fully recovered, thus indicating that expression of OmpC alone accounts for virulence changes.

However, virulence of the Δ*ompC* mutant was impaired less dramatically than was that of the Δ*ompB* strain. In particular, the rate of cell invasion of the Δ*ompC* mutant appeared to be less severely affected. The difference observed raises two points. First, as previously observed for the *E. coli* Δ*ompC* original parental strain (48), the *S. flexneri* Δ*ompC* mutant produces the OmpF porin in conditions of high osmolarity, whereas the Δ*ompB* mutant does not. Thus, in the Δ*ompC* strain, OmpF can partially replace the function of OmpC pores since *S. flexneri* does not produce the PhoE porin (51a). Second, as previously reported, the *ompB* locus osmoregulates other genes involved in the entry process.

Both the Δ*ompB* and Δ*ompC* mutants appeared to be affected in two key steps of *Shigella* pathogenesis: spreading from one epithelial cell to another and host cell killing. We investigated these activities by means of the plaque assay. The Δ*ompB* mutant had previously been reported (4) to be unable to form plaques, while in this study, the Δ*ompC* mutant produced tiny plaques.

Neither the Δ*ompB* nor the Δ*ompC* mutant was able to spread from one cell to the adjacent ones, as demonstrated by the lack of capacity to colonize islets of Caco-2 cells. Nevertheless, both of these mutants moved intracellularly via actin polymerization, as monitored by means of fluorescence staining of intracellular bacteria and F-actin. The absence of intercellular spreading did not result from the inability to lyse the phagocytic vacuole, since transmission electron microscopic examination of HeLa cells infected with Δ*ompB* and Δ*ompC* mutants revealed that membrane-

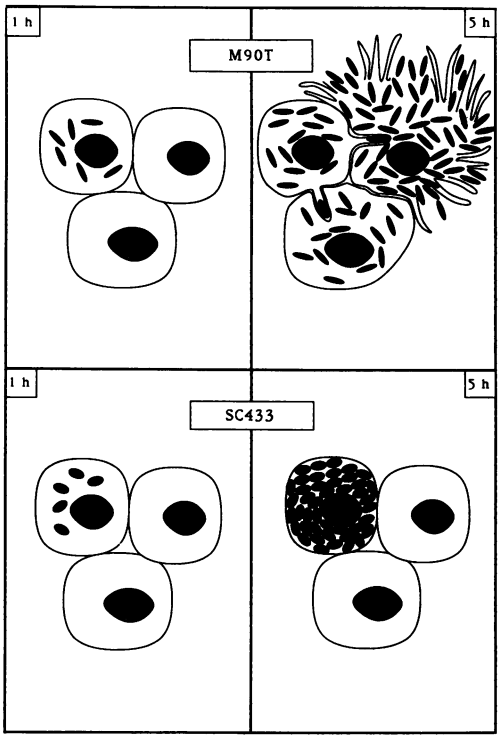

FIG. 8. Schematic drawing of HeLa cells infected by M90T and SC433. After 1 h of incubation postinfection, the numbers of bacteria per infected cell are comparable for the two strains. After 5 h of incubation, because the wild-type strain is able to pass into adjacent cells by way of extracellular protrusions and to kill the host cell, there is little accumulation of multiplying bacteria inside the cell that was initially invaded. In contrast, at this same time, the Δ*ompB* mutant, which is impaired in intercellular spread, multiplies within the initially invaded cell, so that the number of bacteria per infected cell appears fourfold higher than that of the wild-type strain.

bound vacuoles could be lysed as efficiently and as quickly as those of cells infected by the wild-type strain (data not shown).

The analysis of intracellular multiplication within HeLa cells revealed that certain aspects of this activity were modified in the Δ*ompB* and Δ*ompC* mutants. Three hours after infection, intracellular multiplication of the Δ*ompB* and Δ*ompC* mutants occurred at the same rate as did that of the wild type. After 5 h of infection, the number of intracellular bacteria was threefold (with the Δ*ompC* strain) and fourfold (with the Δ*ompB* strain) higher than that observed with the wild-type strain. As the wild-type strain starts spreading intra- and extracellularly after about 2 h of infection, the enormous accumulation of bacteria within the initially invaded cell observed after 5 h of infection may have resulted from the absence of spreading (Fig. 8). On the other hand, the defective spread of mutants does not by itself account for the high number of intracellular bacteria, because *icsA* mutants, which are not able to polymerize actin (5) and which are completely impaired in intra- and intercellular spread, multiplied intracellularly at a rate similar to that of the wild-type strain (data not shown). Consequently, it can be deduced that Δ*ompB* and Δ*ompC* mutants may be affected not only in the ability to spread intercellularly but also in the ability to kill host cells. Intracellular mutants unable to escape from the host cells by a mixed mechanism of spreading and killing remain trapped inside living cells (Fig. 8). Macrophage killing (8, 55) was not affected, thus indicating that the two phenotypes, epithelial cell and macrophage killing, are caused by different mechanisms.

The results obtained in this study on mutant spreading and host cell killing suggest that a common molecular basis may support both activities. Changes in the microenvironment around intracellular bacteria could account for the alterations in the killing and the spreading observed for Δ*ompB* and Δ*ompC* mutants. Electron microscopy revealed that intracellular mutants were completely surrounded by a spongy material (data not shown). The composition of this material has not yet been elucidated. Perhaps intracellular bacteria coated with this material cannot directly interact with host cell membranes. As a consequence, mutant strains would become impaired in all mechanisms necessitating direct interaction between bacteria and the host cell membrane. Physical contact between bacterial surfaces and the inner side of host cell membranes therefore appears necessary for both intracellular spreading and the killing of the host cell.

Mutant strains appeared to be affected primarily in the capacity to form extracellular protrusions (44). Generation of the protrusions could be the result of different steps involving a number of virulence factors not yet identified. Recent studies have reported that several cytoskeletal proteins such as vinculin (19) and fimbrin/plastin (41) are involved in *S. flexneri* intra- and intercellular spreading. Consequently, bacterial proteins could interact with these molecules. In this respect, OmpC could be involved in the cytoskeletal reorganization that occurs during the formation of protrusions. Recently it has been reported (3) that IcsB, a plasmid-encoded protein, is involved in the lysis of the two membranes surrounding the bacteria within the protrusion, lysis which allows the bacteria to escape from the phagosome and to invade an adjacent cell.

Whether OmpC plays a role in *Shigella* virulence in its function as a porin or whether it has an additional function, directly interacting with cellular structures, is presently under investigation. It has been reported that alteration in the spreading phenotype can be due to modifications of the bacterial surface, including LPS (40). However, we did not find any alteration of the LPS side chains in the Δ*ompB* and Δ*ompC* mutants. Nevertheless, as there are approximately 10^5 porin molecules per cell, we cannot rule out the possibility that modifications of the outer membrane resulting from the absence of these molecules contribute to making the Δ*ompB* and Δ*ompC* mutants impaired in virulence.

Although proteins encoded by plasmid genes affect the early steps of the invasive process (28), recently a number of studies (29, 40, 51) have highlighted the role of factors encoded by chromosomal genes, most of which act as transcriptional or translational regulators of genes located on plasmids. In conclusion, the protein OmpC in an innocuous commensal like *E. coli* participates in the adaptive response to a number of environmental parameters (osmolarity, carbon source, etc.); in contrast, in a pathogen like *S. flexneri*, it acts as a virulence factor at a key step of the invasive process.

ACKNOWLEDGMENTS

We thank M. Inouye, S. Benz, and J. Tommassen for gifts of strains and plasmids; we are also pleased to acknowledge J. Tommassen for the kind gift of the polyclonal anti-OmpC serum, A.

Pugsley for helpful discussion, and K. Pepper for reading of the manuscript.

M.L.B. is a fellow of the Institut Pasteur-Cenci Bolognetti Foundation.

REFERENCES

1. **Achtman, M., S. Schwuchow, R. Helmuth, G. Morelli, and P. Manning.** 1978. Cell-cell interactions in conjugating *Escherichia coli*: con$^-$ mutants and stabilization of mating aggregates. Mol. Gen. Genet. **164:**171–183.
2. **Aiba, H., T. Mizuno, and S. Mizushima.** 1989. Evidence for physiological importance of the phosphotransfer between the two regulatory components, EnvZ and OmpR, in *Escherichia coli*. J. Biol. Chem. **264:**14090–14094.
3. **Alloui, A., J. Mounier, M. C. Prevost, P. J. Sansonetti, and C. Parsot.** 1992. *icsB*: a *Shigella flexneri* virulence gene necessary for the lysis of protrusions during intercellular spread. Mol. Microbiol. **6:**1605–1616.
4. **Bernardini, M. L., A. Fontaine, and P. J. Sansonetti.** 1990. The two-component regulatory system OmpR-EnvZ controls the virulence of *Shigella flexneri*. J. Bacteriol. **172:**6274–6281.
5. **Bernardini, M. L., J. Mounier, H. d'Hauteville, M. Coquis-Rondon, and P. J. Sansonetti.** 1989. Identification of *icsA*, a plasmid locus of *Shigella flexneri* that governs intra- and intercellular spread through interaction with F-actin. Proc. Natl. Acad. Sci. USA **86:**3867–3871.
6. **Burnette, W. N.** 1981. Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112:**195–203.
7. **Casadaban, M. J.** 1976. Transposition and fusion of *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. J. Mol. Biol. **104:**541–555.
8. **Clerc, P., A. Ryter, J. Mounier, and P. J. Sansonetti.** 1987. Plasmid-mediated early killing of eucaryotic cells by *Shigella flexneri* as studied by infection of J774 macrophages. Infect. Immun. **55:**521–527.
9. **Clerc, P., and P. J. Sansonetti.** 1987. Entry of *Shigella flexneri* into HeLa cells: evidence for directed phagocytosis involving actin polymerization and myosin accumulation. Infect. Immun. **55:**2681–2688.
10. **Dagert, M., and S. D. Erlich.** 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli*. Gene **6:**23–28.
11. **Dorman, C. J., S. Chatfield, C. F. Higgins, C. Hayward, and G. Dougan.** 1989. Characterization of porin and *ompR* mutants of a virulent strain of *Salmonella typhimurium*: ompR mutants are attenuated in vivo. Infect. Immun. **57:**2136–2140.
12. **Forst, S., D. Comeau, S. Norioka, and M. Inouye.** 1987. Localization and membrane topology of EnvZ, a protein involved in osmoregulation of OmpF and OmpC in *Escherichia coli*. J. Biol. Chem. **262:**16433–16438.
13. **Forst, S., J. Delgado, and M. Inouye.** 1989. Phosphorylation of OmpR by the osmosensor EnvZ modulates expression of the *ompF* and *ompC* genes in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **86:**6052–6056.
14. **Goldberg, M. B., O. Bârzu, C. Parsot, and P. J. Sansonetti.** 1993. Unipolar localization and ATPase activity of IcsA, a *Shigella flexneri* protein involved in intracellular movement. J. Bacteriol. **175:**2189–2196.
15. **Graeme-Cook, K. A., G. May, E. Bremer, and C. F. Higgins.** 1989. Osmotic regulation of porin expression: a role for DNA supercoiling. Mol. Microbiol. **3:**1287–1294.
16. **Hale, T. L., and S. B. Formal.** 1981. Protein synthesis in HeLa or Henle 407 cells infected with *Shigella dysenteriae* 1, *Shigella flexneri* 2a, or *Salmonella typhimurium* W118. Infect. Immun. **32:**137–144.
17. **Hall, M. N., and T. J. Silhavy.** 1981. The *ompB* locus and the regulation of the major outer membrane porin proteins of *Escherichia coli* K-12. J. Mol. Biol. **146:**23–43.
18. **Igo, M. M., A. J. Ninfa, J. B. Stock, and T. J. Silhavy.** 1989. Phosphorylation and dephosphorylation of a bacterial activator by a transmembrane receptor. Gene Dev. **3:**1725–1738.
19. **Kadurugamuwa, J. L., M. Rohde, J. Wehland, and K. N. Timmis.** 1991. Intercellular spread of *Shigella flexneri* through a monolayer mediated by membranous protrusions and associated with reorganization of the cytoskeletal protein vinculin. Infect. Immun. **59:**3463–3471.
20. **Kennedy, E. P.** 1982. Osmotic regulation and the biosynthesis of membrane-derived oligosaccharides in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **79:**1092–1095.
21. **Kocks, C., E. Gouin, M. Tabouret, P. Berche, H. Ohayon, and P. Cossart.** 1992. *Listeria monocytogenes*-induced actin assembly requires the *actA* gene product, a surface protein. Cell **68:**521–531.
22. **Labrec, E. H., H. Schneider, T. J. Magnani, and S. B. Formal.** 1964. Epithelial cell penetration as an essential step in pathogenesis of bacillary dysentery. J. Bacteriol. **88:**1503–1518.
23. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
24. **Liljestrom, P., I. Laamanen, and E. T. Palva.** 1988. Structure and expression of the *ompB* operon, the regulatory locus for the outer membrane porin regulon in *Salmonella typhimurium* LT-2. J. Mol. Biol. **201:**663–673.
25. **Lugtenberg, B., J. Meijers, R. Peters, P. Hoek, and L. Alphen.** 1975. Electrophoretic resolution of the major outer membrane protein of *Escherichia coli* K-12 into four bands. FEBS Lett. **58:**254–258.
26. **Lugtenberg, B., R. Peters, M. Bernheimer, and W. Berendson.** 1976. Influence of cultural conditions and mutations on the composition of the outer membrane proteins of *Escherichia coli*. Mol. Gen. Genet. **147:**251–262.
27. **Makino, S., C. Sasakawa, K. Kamata, T. Kurata, and M. Yoshikawa.** 1986. A genetic determinant required for continuous reinfection of adjacent cells on large plasmid in *Shigella flexneri* 2a. Cell **46:**551–555.
28. **Maurelli, A. T., B. Baudry, H. d'Hauteville, T. L. Hale, and P. J. Sansonetti.** 1985. Cloning of plasmid DNA sequences involved in invasion of HeLa cells by *Shigella flexneri*. Infect. Immun. **49:**164–171.
29. **Maurelli, A. T., and P. J. Sansonetti.** 1988. Identification of a chromosomal gene controlling temperature-regulated expression of *Shigella* virulence. Proc. Natl. Acad. Sci. USA **85:**2820–2824.
30. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Misra, R., and S. A. Benson.** 1988. Isolation and characterization of OmpC porin mutants with altered pore properties. J. Bacteriol. **170:**528–533.
32. **Mizuno, T., M. Y. Chou, and M. Inouye.** 1984. A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA). Proc. Natl. Acad. Sci. USA **81:**1966–1970.
33. **Mounier, J., A. Ryter, M. Coquis-Rondon, and P. J. Sansonetti.** 1990. Intracellular and cell-to-cell spread of *Listeria monocytogenes* involves interaction with F-actin in the enterocyte-like cell line Caco-2. Infect. Immun. **58:**1048–1058.
34. **Mounier, J., T. Vasselon, R. Hellio, M. Lesourd, and P. J. Sansonetti.** 1992. *Shigella flexneri* enters human colonic Caco-2 epithelial cells trough the basolateral pole. Infect. Immun. **60:**237–248.
35. **Ni Bhriain, N., C. J. Dorman, and C. F. Higgins.** 1989. An overlap between osmotic and anaerobic stress responses: a potential role for DNA supercoiling in the coordinate regulation of gene expression. Mol. Microbiol. **3:**933–942.
36. **Nikaido, H.** 1992. Porins and specific channels of bacterial outer membranes. Mol. Microbiol. **6:**435–442.
37. **Nikaido, H., and M. Vaara.** 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. **3:**933–942.
38. **Norioka, S., G. Ramakrishnan, K. Ikenaka, and M. Inouye.** 1986. Interaction of a transcriptional activator, OmpR, with reciprocally osmoregulated genes *ompF* and *ompC* of *Escherichia coli*. J. Biol. Chem. **261:**17113–17119.
39. **Oaks, E. V., M. E. Wingfield, and S. B. Formal.** 1986. Plaque

formation by virulent *Shigella flexneri*. Infect. Immun. **48**:124–129.

40. **Okada, N., C. Sasakawa, T. Tobe, M. Yamada, S. Nakai, K. A. Talukder, K. Komatsu, S. Kanegasaki, and M. Yoshikawa.** 1991. Virulence-associated chromosomal loci of *Shigella flexneri* identified by random Tn*5* insertion mutagenesis. Mol. Microbiol. **5**:187–195.
41. **Prevost, M. C., M. Lesourd, M. Arpin, F. Vernel, J. Mounier, R. Hellio, and P. J. Sansonetti.** 1992. Unipolar reorganization of F-actin layer at bacterial division and bundling of actin filaments by plastin correlate with movement of *Shigella flexneri* within HeLa cells. Infect. Immun. **60**:4088–4099.
42. **Puente, J. L., A. Verdugo-Rodriguez, and E. Calva.** 1991. Expression of *Salmonella typhi* and *Escherichia coli* OmpC is influenced differently by medium osmolarity; dependence on *Escherichia coli* OmpR. Mol. Microbiol. **5**:1205–1210.
43. **Reynolds, E. S.** 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. **17**:208–217.
44. **Sansonetti, P. J.** Unpublished observation.
45. **Sansonetti, P. J., J. Arondel, A. Fontaine, H. d'Hauteville, and M. L. Bernardini.** 1991. *ompB* (osmo-regulation) and *icsA* (cell-to-cell spread) mutants of *Shigella flexneri*: vaccine candidates and probes to study the pathogenesis of shigellosis. Vaccine **9**:416–422.
46. **Sansonetti, P. J., D. J. Kopecko, and S. B. Formal.** 1982. Involvement of a plasmid in the invasive ability of *Shigella flexneri*. Infect. Immun. **35**:852–860.
47. **Sansonetti, P. J., A. Ryter, P. Clerc, A. T. Maurelli, and J. Mounier.** 1986. Multiplication of *Shigella flexneri* within HeLa cells: lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. Infect. Immun. **51**:461–469.
48. **Schnaitman, C. A., and G. A. McDonald.** 1984. Regulation of outer membrane protein synthesis in *Escherichia coli* K-12: deletion of *ompC* affects expression of the OmpF protein. J. Bacteriol. **159**:555–563.
49. **Sereny, B.** 1955. Experimental *Shigella* conjunctivitis. Acta Microbiol. Acad. Sci. Hung. **2**:293–296.
50. **Tilney, L. G., and D. A. Portnoy.** 1989. Actin filaments and the growth, movement and spread of the intracellular bacterial parasite *Listeria monocytogenes*. J. Cell Biol. **109**:1597–1608.
51. **Tobe, T., C. Sasakawa, N. Okada, Y. Honda, and M. Yoshikawa.** 1992. *vacB*, a novel chromosomal gene required for expression of virulence genes on the large plasmid of *Shigella flexneri*. J. Bacteriol. **174**:6359–6367.

51a. **Tommassen, J.** Personal communication.

52. **Tommassen, J., P. van der Ley, A. van der Ende, H. Bergmans, and B. Lugtenberg.** 1982. Cloning of *ompF*, the structural gene for an outer membrane pore protein of *E. coli* K-12: physical localization and homology with the *phoE* gene. Mol. Gen. Genet. **185**:105–110.
53. **van Alphen, W., and B. Lugtenberg.** 1977. Influence of osmolarity of the growth medium on the outer membrane protein pattern of *Escherichia coli*. J. Bacteriol. **131**:623–630.
54. **Westphal, O., and K. Jann.** 1985. Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure. Methods Carbohydr. Chem. **5**:83–91.
55. **Zychlinsky, A., M. C. Prevost, and P. J. Sansonetti.** 1992. *Shigella flexneri* induces apoptosis in infected macrophages. Nature (London) **358**:167–169.