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Macrophage killing is an essential virulence mechanism of *Salmonella typhimurium*

(cytotoxicity/*ompR/envZ*)

SUSANNE W. LINDGREN*, IGOR STOJILJKOVIC, AND FRED HEFFRON

Department of Microbiology and Immunology, L220, Oregon Health Sciences University, Portland, OR 97201

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ABSTRACT **Phagocytic cells are a critical line of defense against infection. The ability of a pathogen to survive and even replicate within phagocytic cells is a potent method of evading the defense mechanisms of the host. A number of pathogens survive within macrophages after phagocytosis and this contributes to their virulence. *Salmonella* is one of these pathogens. Here we report that 6–14 hr after *Salmonella* enters the macrophage and replicates, it resides in large vacuoles and causes the destruction of these cells. Furthermore, we identified four independently isolated MudJ-*lacZ* insertion mutants that no longer cause the formation of these vacuoles or kill the macrophages. All four insertions were located in the *ompR/envZ* regulon. These findings suggest that killing and escape from macrophages may be as important steps in *Salmonella* pathogenesis as are survival and replication in these host cells.**

The diarrheagenic disease Salmonellosis is prevalent throughout the world. Research has shown that the *Salmonella* bacteria adhere to specialized small intestinal epithelial cells called M cells (1) via long polar fimbriae (2). After attachment, these bacteria mediate cytoskeletal and cell surface rearrangements causing internalization within a membrane-bound vesicle (3–5). Once internalized, *Salmonella* destroy these cells (1, 6) and enter macrophages in the mesenteric lymph follicles. Macrophage survival is critical for *Salmonella* because it enables the evasion of the immune system and perhaps dissemination to deeper tissues. Researchers in our laboratory and others have observed that *Salmonella* infection *in vitro* occasionally resulted in macrophage death (D. Monack, S. Falkow, A. D. O'Brien, P. Fields, and F.H., personal communication). In this study, we demonstrate that *Salmonella* is cytotoxic to infected macrophages and report a selection strategy for obtaining noncytotoxic mutants. Using this system, we identified four nontoxic MudJ-*lacZ* mutants. All four mutants had insertions in *ompR/envZ* that resulted not only in abrogation of the cytotoxic phenotype but also in a dramatic attenuation *in vivo*. To our knowledge, this is the first analysis of a direct cytotoxic effect of *Salmonella typhimurium* on infected macrophages.

MATERIALS AND METHODS

Macrophage Cytotoxicity Assays. Bacterial cultures were grown overnight at 37°C in 3 ml Luria–Bertani broth in 13 × 100 borosilicate glass tubes (reduced aeration). The next day, bacteria were washed in phosphate-buffered saline (PBS), and placed on low-passage-number cultured J774 cells at a ratio of 100 bacteria to 1 macrophage [multiplicity of infection (moi) of 100]. The bacteria were not opsonized with normal mouse serum before infection. After addition of the bacteria, the cells were briefly centrifuged to synchronize invasion and incubated for 20 min at 37°C with 5% CO_2. The J774 cells were then washed with PBS three times and fresh media containing 26 μg/ml gentamicin was added to kill any remaining extracellular bacteria. Dulbecco's modified essential medium (DMEM), which contained 0.1 mM MEM nonessential amino acids (GIBCO/BRL), 0.2 mM MEM sodium pyruvate (GIBCO/BRL), and 10% fetal calf serum, was used throughout this study.

To evaluate intracellular growth of the noncytotoxic mutants, the number of bacteria per macrophage at 1–6 hr postinfection was determined. At various times, infected J774 cells were washed once with PBS, and one of the four wells of macrophages infected with each strain was resuspended in PBS, trypan blue was added (0.2% final concentration), and viable cells (those excluding the dye) were counted. The other three wells of infected J774 cells were lysed with 1% deoxycholate and separately plated for bacterial viability.

To quantify levels of cytotoxicity, bacteria were titered onto macrophages, and the moi required to cause 50% killing of the macrophages was obtained. To this end, J774 cells (1×10^5) were infected with 2-fold serial dilutions of PBS-washed bacteria from overnight cultures (verified by plate counts of overnight cultures to be moi 2–1000) as described above. At 18–20 hr postinfection, the remaining viable cells were fixed in 10% formalin solution and stained in a 0.13% crystal violet solution. The absorption at A_{620} was measured on a microplate reader (Dynatech); the well containing the dilution of bacteria/macrophage that gave 50% of the absorption recorded for uninfected wells was considered the moi CD_{50} (50% of the cytotoxic dose).

To assess bacterial uptake by macrophages, J774 cells were infected with bacteria as detailed above. However, 20 min after infection, the cells were washed four times with PBS and further incubated for 90 min with medium containing 100 μg/ml gentamicin. Cells were then washed with PBS, lysed with 1% deoxycholate, and plated for viable counts. Bacterial uptake (number of intracellular bacteria/number of bacteria used to infect) was normalized such that the uptake of the parent strain SR-11 was 100%.

Macrophages infected with *Salmonella* were analyzed for the presence of DNA fragmentation using terminal deoxytransferase-mediated dUTP nick end-labeling (TUNEL) reaction. The Fluorescein In Situ Cell Death Detection Kit from Boehringer Mannheim was used to fluorescein label free 3′-OH termini of DNA fragments. J774 cells were seeded onto coverslips and infected at an moi of 50 as detailed above. At 6 hr postinfection, the cells were washed four times with PBS and fixed with buffered picric acid-formaldehyde solution (7). Cells were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate and then treated for TUNEL reaction. The cells were then incubated with polyclonal rabbit anti-*S. typhimurium* antiserum (A0643, 1:500) overnight. After a brief washing,

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Abbreviations: moi, multiplicity of infection; TUNEL reaction, terminal deoxytransferase-mediated dUTP nick end-labeling reaction; CD_{50}, 50% cytotoxic dose; TNFα, tumor necrosis factor α.

*To whom reprint requests should be addressed.

cells were incubated with Texas Red-labeled goat anti-rabbit secondary antibody (1:200; Molecular Probes). Coverslips were treated with slow-fade (Molecular Probes) and inverted onto microscope slides for fluorescence microscopy analysis. A negative control slide of fixed and permeabilized cells treated with labeling solution without the terminal transferase enzyme was done and did not fluoresce green.

Mutagenesis. SR-11(Nal^r, Rif^r) was mutagenized with MudJ-*lacZ* by P22-HT*int* transduction as described (8). Transductants were plated on M9 media with Noble agar to avoid isolation of auxotrophic mutants. Colonies from each transduction (plate of 1,000 colonies) were combined into pools of 6,000–16,000 colonies and stored at −70°C.

Localization of MudJ-*lacZ* Insertions. Pulsed-field gel electrophoresis was done as described (9). Inverse PCR was done as described (10), except that the products were blunt-end ligated into the *Eco*RV site of pBluescript SK$^-$ (Stratagene) and sequenced using reverse and universal primers. Sequence data was compared with GenPept data base by using the program BLASTN (11). For Southern Blot analysis, mutant chromosomal DNA was digested with restriction enzymes, separated by gel electrophoresis, and transferred onto nylon membrane. Blots were hybridized using Renaissance (DuPont) with labeled *ompR/envZ* operon (isolated as described below).

Cloning *ompR/envZ* and Complementation. The entire *ompR/envZ* operon was obtained from strain SR-11 by PCR amplification with Vent polymerase (New England Biolabs). The oligonucleotide primers used were generated to add unique *Kpn* I and *Xba* I sites on the 5′ and 3′ end of the operon for directional cloning into pWSK29 (12). Once made, this construct (pSWLOMP) was transfected by electroporation [BTX (San Diego) electrocell manipulator 600] into each of the four SR-11 noncytotoxic mutants. The vector alone was also transfected into SWL350 as a control.

Electron Microscopy. Transmission electron microscopy was done on J774 cells at both 6 hr and 14 hr postinfection with SR-11, or noncytotoxic mutant SWL350, and on uninfected cells. Cells were washed four times in PBS, fixed in 1.5% gluteraldehyde/paraformaldehyde solution buffered with 0.1 M cacodylate, dehydrated through graded acetone, and imbedded in LR White. Five fields of macrophages were evaluated to quantify vacuole formation.

RESULTS

Identification and Characterization of the Cytotoxic Phenotype. We found that *S. typhimurium* is cytotoxic to macrophages by infecting J774 cells with strain SR-11 at an moi of 100 (Fig. 1*A*). Cytotoxicity was morphologically apparent by 12–14 hr postinfection. Several other well-characterized *S. typhimurium* strains were tested for cytotoxicity on J774 cells and all of them found to be cytotoxic, whereas *Salmonella typhi* and *Escherichia coli* were not cytotoxic at doses as high as 1000 moi (Fig. 2*A*). Proteose peptone-elicited BALB/c peritoneal macrophages were also found to be susceptible to the cytotoxic effect of SR-11 (data not shown). To evaluate whether the cytotoxicity was due to a soluble protein, J774 cells were treated with SR-11 culture supernatant and extracts from sonicated bacteria. Neither fraction was cytotoxic when added undiluted to J774 cells (data not shown). To determine if the cytotoxicity observed on macrophages could be caused by dead extracellular bacteria, the supernatant from J774 cells at 24 hr postinfection with SR-11 (moi 100) was transferred onto fresh J774 cells, and heat-killed SR-11 were also cultured with J774 cells. Neither the spent tissue culture media nor the dead cells had an effect on the J774 cells (data not shown). Therefore, live bacterial cell association is required for cell death because heat-killed bacteria, culture supernatants, bacterial sonic extracts, or spent tissue culture media did not reproduce the effect on J774 cells. Finally, the possibility that the cytotoxicity was due to tumor necrosis factor α (TNFα) production by the J774 cells in response to bacterial infection was evaluated. To test this, SR-11-infected cells were incubated with an excess of anti-murine TNFα polyclonal neutralizing antisera for 24 hr (100 μl/ml neat; Genzyme), and serial dilutions of mouse rTNFα were also added directly to uninfected J774 cells. Not only did the antisera not neutralize the cytotoxicity, the rTNFα was not cytotoxic to these cultured cells at levels as high as 1×10^5 units/ml. These results indicate that TNFα is not responsible for the cytotoxicity described here.

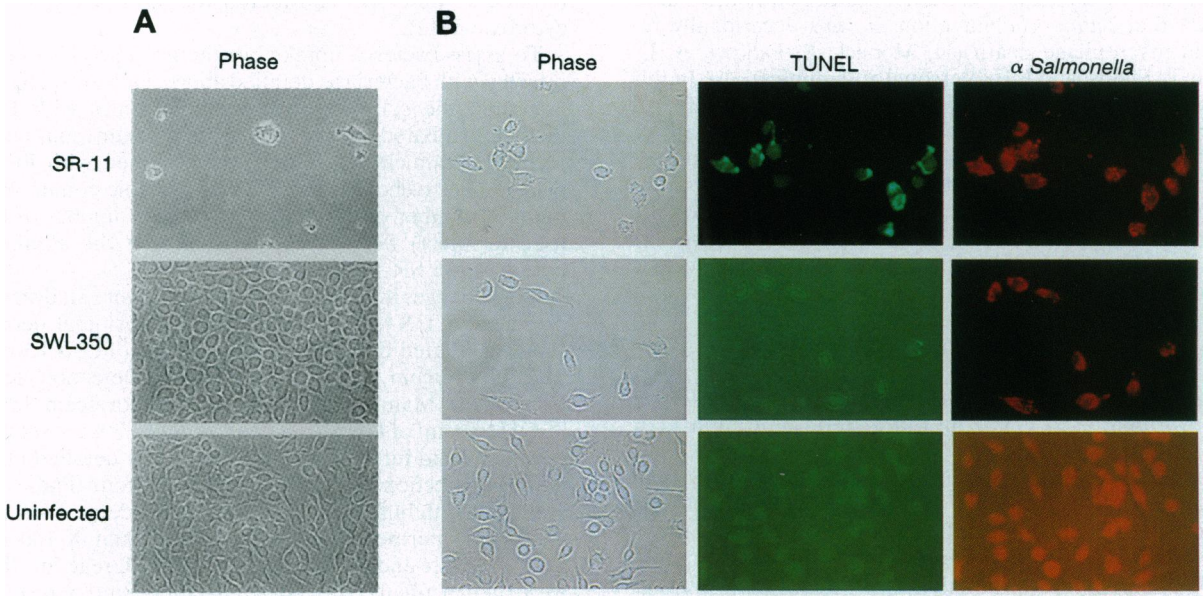

FIG. 1. (*A*) Light micrographs of J774 cells demonstrating the cytotoxic effect of *S. typhimurium*. Macrophages were infected with an moi of 50. At 14 hr postinfection, cells were washed in PBS and fixed with buffered picric acid–formaldehyde solution (7). Mutant SWL350 is shown as a representative of the four mutants. (*B*) Colocalized detection of fragmented DNA and *Salmonella* in SR-11-infected J774 cells. Cells were lightly seeded onto coverslips and infected at an moi of 50 with SR-11 or *ompR* mutant SWL350, or were left uninfected. Cells were fixed as above at 6 hr postinfection and treated for TUNEL reaction as described in *Materials and Methods*. Fluorescence controls and SWL350 fluorescein panel were overexposed for cell delineation. In this reproduction, individual bacteria are not visible. Red staining indicates presence of bacteria. (×40.)

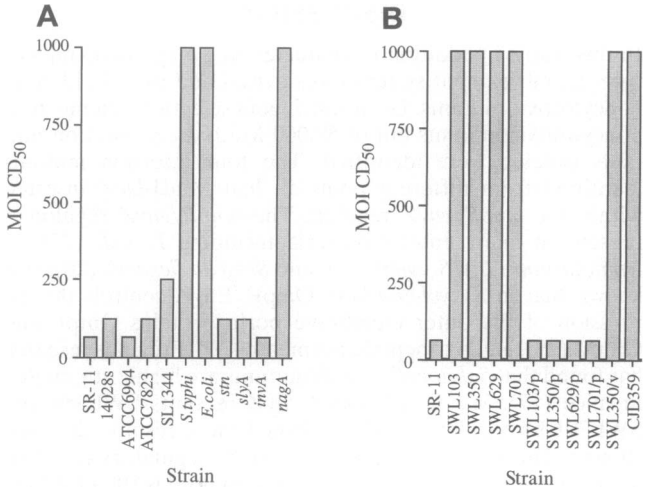

FIG. 2. Quantified cytotoxicity of *Salmonella* strains on J774 cells. The moi given is that observed to kill 50% of the cells by 18 hr postinfection (moi CD_{50}). Short bars indicate higher level of cytotoxicity. Data from each graph are representative of at least three separate experiments with no more than a twofold variation in values between each of the trials. (*A*) *S. typhimurium* strains: SR-11, 14028s, ATCC6994, ATCC7823, SL1344. Clinical isolates of *S. typhi*, and *E. coli* strain C600 were also tested. Mutants: 1–5 (*stn*::MudJ-*lacZ*, 14028s) (13); SL2161 (*slyA*::pRR10Δ*trfA*, 14028s) (14); χ3642 (*invA*::Tn*phoA*-61, SR-11) (15); MS3792 (*nagA*::Tn*10*, 14028s) (9, 16). (*B*) Complementation of cytotoxin mutants. The "/p" in SWL103/p, SWL350/p, SWL629/p, and SWL701/p indicates presence of pSWLOMP. SWL350/v indicates presence of vector alone. CJD359 (ompR1009::Tn10, SL1344) (17) was also tested.

Several previously described *Salmonella* mutants were tested to identify genes involved in cell killing. A mutant in the enterotoxin gene *stn* (18) and a mutant in the salmolysin gene *slyA* (14) were both found to be cytotoxic to J774 cells (Fig. 2*A*). Therefore, the cytotoxicity is not caused by these toxins. An *invA* mutant that is known to be deficient in epithelial cell invasion (15) was cytotoxic to J774 cells (Fig. 2*A*), although this mutant may enter the macrophage by a mechanism that does not require the *inv* locus. However, a mutant in the *N*-acetylglucosamine-6-phosphate deacetylase gene (*nagA*), which is taken up poorly by macrophages (19), was defective in cytotoxicity (Fig. 2*A*). These data suggest that uptake of *Salmonella* by macrophages may be required for cytotoxicity, and that the route of uptake may be a critical factor. It is also interesting to note that a mutant in *sipB* [homolog of *ipaB* in *Shigella* (32) which is required for apoptosis of macrophages (33)] had no effect on *S. typhimurium* cytotoxicity on macrophages nor did loss of the *S. typhimurium* virulence plasmid (S.W.L. and F.H., unpublished observations).

Numerous methods are available to evaluate cell death. DNA fragmentation is indicative of apoptosis and end-stage necrosis (20). We used the TUNEL reaction (21) to determine whether DNA fragmentation occurs in *Salmonella* infected cells. As early as 6 hr postinfection, cells infected with wild-type *Salmonella* had fragmented DNA, whereas uninfected cells did not (Fig. 1*B*). Therefore, the cellular machinery of the infected cells is adversely affected as early as 6 hr postinfection, although dramatic morphological changes are not observed until 12–14 hr postinfection.

Selection of Noncytotoxic Mutants. For identification of the genes involved in cell death, seven pools of SR-11 MudJ-*lacZ* insertion mutants (a total of 58,000 independent mutants) were made. Enrichment for noncytotoxic mutants was done by serial passage through macrophages at an moi of 0.1–1 to ensure only one mutant entering each macrophage. Fresh gentamicin (26 μg/ml) was added throughout the 48-hr assay to kill bacteria that were cytotoxic to the macrophages and had escaped into the extracellular melieu. At 48 h postinfection, the remaining macrophages were lysed with 1% deoxycholate, intracellular bacteria were isolated, and the procedure repeated four times for enrichment of noncytotoxic bacteria. Each initial pool of mutants was isolated on separate occasions and, after passage through macrophages, one mutant was chosen per pool to obviate the risk of isolating siblings. Four noncytotoxic mutants were identified through this method: SWL103, SWL350, SWL629, and SWL701. To be certain that we had not isolated gentamicin-resistant strains, the minimum inhibitory concentration of gentamicin for the four mutants was obtained and found to be the same as the parent strain (10 μg/ml). Each of the mutants was retransduced with KB1-*int* (22) into wild-type SR-11(χ3041) for further analysis.

Characterization of Mutants. As demonstrated with mutant SWL350, the four mutants had no morphological effect on J774 cells at an moi of 100 (Fig. 1*A*). Furthermore, although the macrophages were clearly infected with SWL350, DNA fragmentation indicative of cell death was not observed (Fig. 1*B*). Additionally, the difference in cytotoxicity between the mutant and the parent strains did not appear to be due to a difference in growth rate or capacity to enter macrophages because the *in vitro* growth rate of the mutants in Luria–Bertani broth (data not shown), the intracellular growth of the four mutants (Fig. 3) and the uptake of the mutants into J774 cells was comparable with the parent strain SR-11. Mutants SWL103 and SWL350 were found to enter the macrophages at levels comparable with parent strain SR-11 at 100% and 96%, respectively.

The mutants were mapped by pulsed-field gel electrophoresis. All four mutants were located between 75 and 80 centisomes on the *S. typhimurium* chromosome where *ompR/envZ* is located (23). Regions of DNA adjacent to the MudJ-*lacZ* insertions in SWL350, SWL629, and SWL701 were isolated by inverse PCR, cloned, and sequenced. Mutant SWL350 contains a MudJ-*lacZ* insertion 276 bp into the coding region of *ompR*, and SWL629 and SWL701 each contain a MudJ-*lacZ* insertion in the promoter region of this same regulatory gene (Fig. 4). The insertion in SWL103 is also located in the promoter region of *ompR/envZ* as determined by Southern blot hybridization (data not shown).

To demonstrate that the MudJ-*lacZ* insertions in *ompR/envZ* were solely responsible for lack of cytotoxicity, the *ompR/envZ* operon was cloned from SR-11 by PCR. The resultant plasmid (pSWLOMP) was transfected by electroporation into each of the four mutants and found to complement

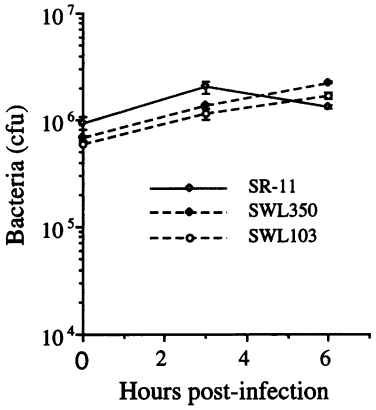

FIG. 3. Growth of cytotoxin mutants within J774 cells. Growth of the bacteria was assessed as described in *Materials and Methods* at time points where there would be no concomitant cell death. The colony-forming unit (cfu) values indicate the number of intracellular bacteria that were isolated from 1.4×10^5 viable macrophages at various times postinfection. Error bars represent SEM ($n = 3$). Data are representative of two experiments.

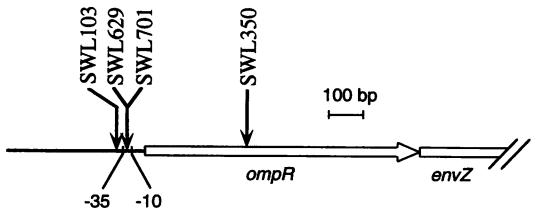

FIG. 4. Location of MudJ-*lacZ* insertions in the four noncytotoxic mutants of SR-11. Mutant SWL103 was not precisely mapped, but thought to be in the operon region based upon restriction fragment length of band observed by Southern blot analysis.

the cytotoxicity to wild-type levels in each of the mutants (Fig. 2*B*). Additionally, previously characterized *S. typhimurium ompR::Tn10* mutant CJD359 [from C. J. Dorman (17)] was tested and found to have comparable moi CD_{50} values to the *ompR/envZ* mutant strains isolated in this study (Fig. 2*B*).

Morphological data of J774 cells infected at an moi of 50 with SR-11 or SWL350 were obtained by transmission electron microscopy at 6 hr and 14 hr postinfection. At 14 hr postinfection, greater than 20% of the J774 cells infected with SR-11 demonstrated large vacuole formation (Fig. 5*A*) that appeared to be formed by fusion of numerous smaller vacuoles that contained bacteria (Fig. 5*B*). It is possible that these vacuoles play a role in macrophage cytotoxicity because no large vacuoles were observed in J774 cells infected with the noncytotoxic mutant SWL350 (Fig. 5*C* and *D*). No large vacuoles were observed at the earlier 6 hr time point; however, some cells containing SR-11 demonstrated initial stages of vacuolar fusion (data not shown). It does not appear that these large vacuoles are spacious phagosomes which have been described to form in *Salmonella*-infected macrophages by 30 min postinfection and shrink within 2 hr postinfection (24).

DISCUSSION

In this study, in addition to characterizing a cytotoxic phenotype, an enrichment system was devised and used to identify noncytotoxic mutants. Using a stringent selection scheme, four noncytotoxic mutants out of 58,000 MudJ-*lacZ* insertion mutants isolated were identified. The four insertion mutants identified from different pools all had MudJ-*lacZ* inserted within the *ompR/envZ* regulon. The *ompR/envZ* regulon is present in many enteric bacteria including *E. coli* (25), *S. typhimurium* (26), *S. typhi* (27), and *Shigella flexnerii* (28). It is known that in *S. typhimurium*, OmpR/EnvZ controls the expression of the outer membrane porin proteins OmpF and OmpC (29), and a tripeptide permease (30). Previous research has found that *Salmonella ompR* mutants are highly attenuated in mice (17). We tested *ompR/envZ* mutants SWL350 and SWL701 for virulence and confirmed these results (data not shown). This attenuation is not due to the regulatory effect on *ompF*, *ompC*, and *tppB* because a triple mutant is 1000-fold less attenuated than a mutation in *ompR* itself (31). These data indicate not only that OmpR is essential for virulence but also that it may be required for the regulation of some other gene(s) for the systemic spread of *Salmonella* such as one involved in the cytotoxic phenotype observed on macrophages.

There are several explanations why only mutants in *ompR/envZ* were isolated. It is possible that this operon is a master regulator of a redundant system. If so, then individual mutants of the system would remain partially cytotoxic due to other cytotoxic genes still present. Partial cytotoxic mutants would not be isolated with the selection system used in this study. It is also clear that mutants reduced in macrophage uptake (such as the *nagA* mutant MS3792 described above) or macrophage survival would not have been isolated because the initial selection was done at a very low moi. It is most likely that OmpR/EnvZ does not have a direct cytotoxic effect but that

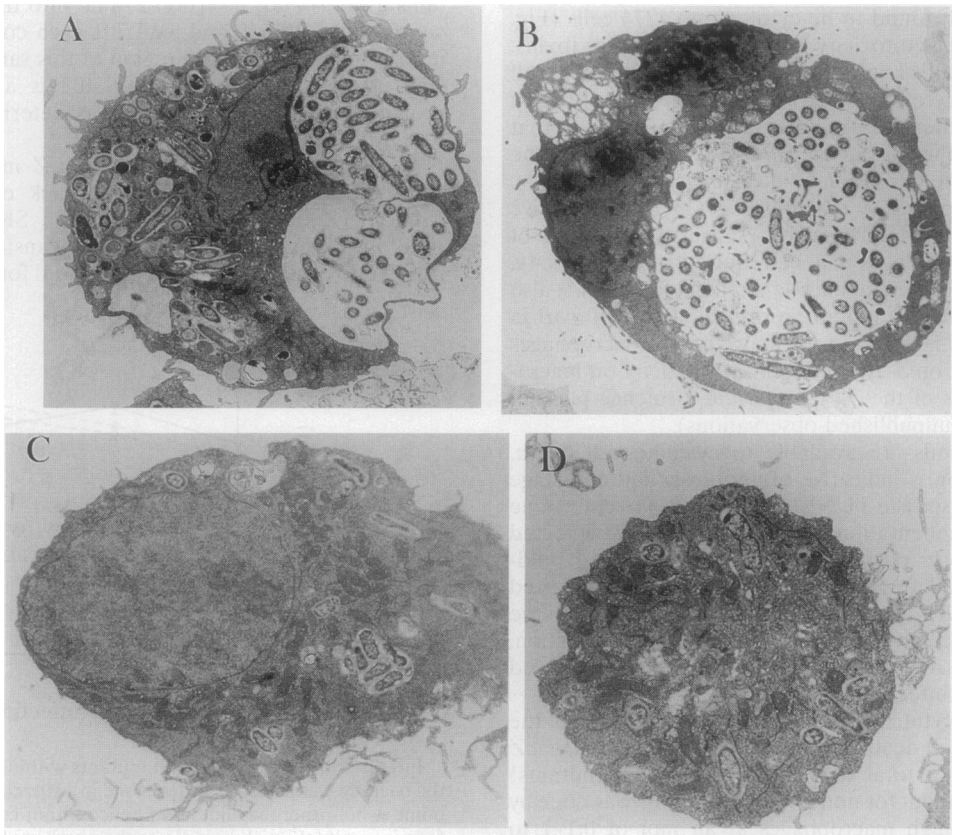

FIG. 5. Transmission electron microscopy of J774 cells 14 hr postinfection with (*A* and *B*) SR-11 or (*C* and *D*) SWL350. Five fields of macrophages were evaluated for quantification of vacuole formation. In the SR-11-infected cells, 95.2 ± 1.6 (SEM) cells out of 400 cells per field demonstrated vacuole formation.

it regulates some other gene(s) that is involved in this phenotype.

Our study clearly shows that *S. typhimurium* once replicated, form large vacuoles, and kill host cell macrophages. Our hypothesis to explain these results is that during uptake, a portion of *Salmonella* enter the macrophage into an alternative phagosome (for which the *nagA* gene product may be required and *invA* not required), replicate, and fuse with other bacteria-filled phagosomes to form large vacuoles. During this time (6–14 hr), the host cell machinery is disrupted and the macrophage dies. The bacteria remain within the cell, perhaps feasting on cellular nutrients, and then escape to infect adjacent cells and spread infection. Other bacteria enter into a classical phagosome and are destined for death by the macrophage. It is not clear what triggers vacuole formation or cell death. Results from this study indicate that the *ompR/envZ* regulon is required.

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