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Acidification of Phagosomes Containing *Salmonella typhimurium* in Murine Macrophages

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***Salmonella* species are facultative intracellular pathogens. Following entry into mammalian host cells, they reside in membrane-bound vacuoles, resist killing, and replicate. In this work, we investigated the importance of phagosomal pH in the ability of *Salmonella typhimurium* to survive and replicate within macrophages. Intraphagosomal pH was measured in situ by recording the fluorescence intensity of a pH-sensitive probe, DM-NERF dextran. The majority of vacuoles containing *S. typhimurium* (live, heat killed, or formalin fixed) acidified from pH ≥6.0 to between pH 4.0 and 5.0 within 60 min after formation. In contrast, *Mycobacterium avium*-containing vacuoles failed to acidify even at later time points. Acidification of *S. typhimurium*-containing vacuoles was completely blocked by treatment of host cells with bafilomycin A_1, a specific inhibitor of vacuolar proton-ATPases. Bafilomycin inhibition of vacuolar acidification from the onset of infection significantly decreased the survival of *S. typhimurium* in macrophages. Furthermore, bafilomycin treatment at 2, 4, 8, or even 12 h postinfection decreased the percentage of recoverable bacteria by up to 20-fold. Loss of bacterial viability was seen with several other reagents which, like bafilomycin, raise the pH of phagosomal compartments but are not directly lethal to the bacteria or host cells. Thus, we conclude that *Salmonella*-containing phagosomes acidify soon after formation and hypothesize that an acidic environment is necessary for survival and replication of the bacteria within the macrophage.**

Salmonella species infect both animal and human hosts and are the causative agents of diseases including enteric (typhoid) fever, gastroenteritis, bacteremia, and a variety of localized systemic infections (23). Numerous virulence determinants that play key roles in the ability of *Salmonella* species to infect, colonize, and replicate within a host have been identified (14, 15, 18, 24). Much of this research has been conducted on *Salmonella typhimurium*, a serotype of particular interest since it is one of the leading causes of food poisoning in human beings (7) and also induces a typhoid-like disease in susceptible mice (40). Murine typhoid pathogenesis has served as a model of human typhoid infection and has enabled a detailed analysis of the organism's spread beyond the bowel to deeper tissues and the systemic circulation. One trait of *S. typhimurium* thought to be essential for this disease course and, ultimately, typhoid pathogenesis is its ability to survive in macrophages (13, 29).

To survive within macrophages, pathogens have developed defense mechanisms to counter antibacterial assaults such as toxic oxygen derivatives, reactive nitrogen intermediates, and defensins. Organisms that remain within phagosome membranes must also combat nutrient limitation, fusion with lysosomes, and phagosome acidification. Phagosome acidification is probably not directly toxic to most bacteria (12) but has been suggested to facilitate the microbial killing process by inducing spontaneous generation of hydrogen peroxide from superoxide (17), influencing fusion of lysosomes with phagosomes (21), and providing an optimal environment for the activity of hydrolytic enzymes (8).

Organisms that successfully survive in macrophages vary in the manner with which they deal with the intracellular environment. Progressive phagosome acidification is combatted by bacterial mechanisms of avoidance, prevention, and resistance. *Listeria monocytogenes*, shigellae, and rickettsiae avoid the repercussions of acidification by lysing the phagosome membrane and subsequently replicating in the cytoplasm (35, 37, 39). In contrast, *Legionella pneumophila* and several mycobacteria reside in remodeled vacuoles that do not acidify (10, 21, 36). *Coxiella burnetii*, *Leishmania* spp., and *Francisella tularensis* also remain within phagosomal compartments but survive acidic conditions and apparently resist any detrimental effects inherent to this proton-rich environment (3, 16, 27).

Previous experiments that quantified the pH of *S. typhimurium*-containing vacuoles (STVs) indicated that live bacteria, in contrast to heat-killed organisms, delay and attenuate phagosomal acidification (2). In this study, we characterized phagosomal pH by utilizing a new experimental protocol and a novel pH probe. In addition, the influence of vacuolar pH on survival and replication of *S. typhimurium* within cultured macrophages was analyzed. Our results not only demonstrate that *Salmonella*-containing phagosomes acidify soon after formation but also suggest that an acidic environment is necessary for the survival of *S. typhimurium* in macrophages.

MATERIALS AND METHODS

Materials. Bafilomycin A_1, *N*-ethylmaleimide (NEM), nigericin, monensin, methylamine, and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit immunoglobulin G were purchased from Sigma. DM-NERF dextran (molecular weight, 10,000) is a product of Molecular Probes.

Bacterial strains and growth conditions. The virulent strains *S. typhimurium* SL1344 and *Mycobacterium avium* 104 (serovar 1) were used in this study. To generate SL1344 carrying the *phoP* mutation, a transposon insertion of *phoP* was transduced from *S. typhimurium* LT2 (Salmonella Genetic Stock Centre strain TT13220) into SL1344 by P22 transduction (11). Colonies containing the Tn*10* insertion were selected by screening for tetracycline resistance. The location of the insertion was verified by Southern hybridization of total chromosomal DNA *Hin*dIII fragments with [^{32}P]*phoP* as a probe (26). SL1344 was grown in Luria-

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Bertani broth (26) to stationary phase at 37°C and then subcultured overnight as a standing culture. Upon reaching the mid- to late log phase of growth, the bacteria were pelleted by centrifugation, resuspended in phosphate-buffered saline (PBS), and used immediately. Molecular Probes' LIVE/DEAD *Bac*Light bacteria viability kit was used to analyze bacterial viability. The initial inoculum was determined to be 97% ± 1% viable. Heat-killed organisms were prepared by incubating bacteria at 65°C for 15 to 20 min. *M. avium* was grown in MADCTW liquid as previously described (9). Immediately before use, the bacteria were pelleted by centrifugation and resuspended in PBS.

Cell cultures. Bone marrow-derived macrophages were prepared by culturing bone marrow cells isolated from femurs of BALB/c mice in Dulbecco minimal essential medium containing 20% fetal calf serum, 10% L-cell-conditioned medium, 1 mM glutamine, 50 U of penicillin per ml, and 50 μg of streptomycin per ml for approximately 1 week. RAW 264.7 cells, a murine macrophage-like cell line, were grown in Dulbecco minimal essential medium containing 10% fetal calf serum and 1 mM glutamine. For experiments quantitating vacuolar pH, macrophages were incubated in pH experimental medium (pEM), pH 7.0, containing 5.37 mM KCl, 0.81 mM $MgSO_4 \cdot 7H_2O$, 102.67 mM NaCl, 5.55 mM D-glucose, 0.6 mM L-arginine HCl, 0.1 mM L-cysteine HCl, 2.05 mM L-glutamine, 0.19 mM L-histidine HCl · H_2O, 0.40 mM L-isoleucine, 0.1 mM L-methionine, 0.40 mM L-leucine, 0.40 mM L-lysine HCl, 0.20 mM L-phenylalanine, 0.40 mM L-threonine, 0.05 mM L-tryptophan, 0.29 mM L-tyrosine, 0.40 mM L-valine, 1× MEM vitamin solution (GIBCO), 10.0 mM Na HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 1.80 mM $CaCl_2 \cdot 2H_2O$, and 1.0 mM NaH_2PO_4. Nigericin calibration medium (NCM) differed from pEM only in that the KCl concentration was increased to 132.80 mM and the NaCl concentration was decreased to 4.1 mM; nigericin (10 μM) was added to the medium immediately before use. The media were titrated to pH 4.0, 5.0, or 6.0 by the addition of HCl.

In vitro calibration of DM-NERF dextran. The DM-NERF dextran was resuspended in pEM or NCM at a final concentration of 2.5 μg/ml and a pH of 4.0, 5.0, 6.0, or 7.0 at 37°C. The excitation spectra were monitored at 490 and 440 nm with a fluorescence spectrophotometer (Perkin-Elmer LS5B). Emission was set at 535 nm with narrow ±10-band-pass slits. The ratios of the values collected at 490 and 440 nm were then calculated and plotted as a function of pH.

Confocal analysis of phagosomes containing DM-NERF dextran. To confirm the colocalization of the pH probe and phagosomes containing live *S. typhimurium*, confocal images of infected cells were collected. RAW 264.7 macrophages were infected in the presence of DM-NERF dextran for 10 min and washed extensively with PBS. Next the tissue culture dishes containing the cells were mounted on a Molecular Dynamics MultiProbe 2010 confocal fluorescence microscope. The dishes were secured to the microscope stage and left in place for the duration of the experiment. Analysis was begun by collecting a scanning confocal image of DM-NERF fluorescence (excitation at 488 nm; emissions collected with a 510-nm long-pass filter). The cells were then fixed for 10 min with 1% formaldehyde in PBS and permeabilized for 10 min with 0.1% saponin in PBS containing 2% bovine serum albumin (BSA). Next, the cells were incubated for 20 min with rabbit anti-*S. typhimurium* immunoglobulin G polyclonal antiserum diluted in the saponin-BSA-PBS solution, washed, and incubated for a final 20 min with tetramethyl rhodamine isothiocyanate-conjugated goat anti-rabbit immunoglobulin G antiserum diluted in the saponin-BSA-PBS solution. A scanning confocal image of rhodamine fluorescence was collected from precisely the same cells that had been used to measure DM-NERF fluorescence (excitation at 568 nm; emissions collected with a 590-nm long-pass filter). Finally, colocalization of the pH probe with intracellular bacteria was analyzed by merging the DM-NERF and rhodamine fluorescent images. The percentage of fluorescent vacuoles containing *S. typhimurium* was calculated by tabulating the results from three different experiments in which 10 to 15 large vacuoles were analyzed per experiment.

Measurements of phagosomal pH. Macrophages were cultured in pEM, seeded in Nunc chambered coverglass tissue culture dishes, and infected at a ratio of approximately 20 bacteria or latex beads to 1 macrophage in the presence of 2 mg of DM-NERF dextran per ml. Following a 10-min infection period, the macrophages were washed extensively with PBS and incubated in pEM. Finally, the tissue culture dishes were mounted on a Nikon Diphot 300 inverted microscope equipped for digitized ratiometric epifluorescence analysis. The temperature of the tissue culture dishes was maintained at approximately 37°C, and fresh pEM was perfused into the cultures at regular intervals. Vacuoles containing DM-NERF were identified by their epifluorescence. Bacteria within these vacuoles could be visualized by switching from epifluorescence to bright-field Nomarski optics. The bacteria appeared as motile nonfluorescent rods surrounded by fluorescent halos. Digitized images of DM-NERF excitation at 490 and 440 nm were collected every 10 to 30 s with an intensified CCD camera (Dage MTI, Michigan City, Ind.). A C-Imaging 1280 system equipped with Simca ratiometric software (Compix Inc., Mars, Pa.) was used to visualize and process the signals of individual vacuoles. At the end of each experiment, the pH values were determined by an in situ calibration technique (25). Solutions of NCM at pH 7.0, 6.0, 5.0, and 4.0 were successively perfused into the dish at approximately 10- to 15-min intervals. Fluorescent intensities were measured every 30 s. Images of background fluorescence were subsequently collected from regions of the dish where cells were removed with sterile cotton swabs. Finally, 490 nm/440 nm excitation spectrum ratios were calibrated and plotted as a function of time.

Inhibition of host cell vacuolar proton-ATPases was accomplished by incubating the macrophages with 100 nM bafilomycin A_1 for 15 min prior to infection. For long-term experiments, macrophages were infected for 10 min in the presence of DM-NERF dextran, washed, and incubated in medium containing gentamicin (50 μg/ml) at 37°C for up to 5 h before analysis.

Bacterial survival and replication in bafilomycin-treated macrophages. Macrophages were seeded at approximately 3.0×10^5 cells per well in 24-well tissue culture dishes and incubated overnight. The cells were pretreated with 100 nM bafilomycin for 15 min and then infected for 20 min with wild-type or *phoP*::Tn*10* *S. typhimurium* at a multiplicity of infection of 10 bacteria to 1 macrophage. The cells were washed three times with PBS and incubated for 0 to 4 h in medium containing gentamicin (100 μg/ml for the first 2 h and 10 μg/ml for the remainder of the experiment). At designated time intervals, the macrophages were washed with PBS and lysed with 1% Triton X-100. The number of bacteria recovered was divided by the number of input bacteria, and this percentage was plotted as a function of time. Control cells which had not been treated with bafilomycin were infected and processed in parallel. Each experiment was performed in triplicate. Additional experiments in which bafilomycin was added 2, 4, 8, or 12 h postinfection were performed. The inhibitor was left in the medium for the duration of the experiment, and the number of viable bacteria was quantitated every 2 h.

Addition of vacuolar-pH-neutralizing reagents postinfection. At 2 h postinfection, one of the following was added to infected cells: 100 nM bafilomycin, 25 μM NEM, 50 μM monensin, 10 mM methylamine, 100 mM methylamine, 10 mM NH_4Cl, or 100 mM NH_4Cl. The effect of each of these reagents on *S. typhimurium* viability was analyzed by quantitating the number of surviving bacteria 2 h after addition of the substance. Control cells were infected and processed in parallel. Each experiment was performed in triplicate. In conjunction, the direct bacterial toxicity of each reagent was tested by in vitro growth assays and macrophage viability was verified by Trypan blue dye exclusion assays.

RESULTS

DM-NERF dextran is an accurate reporter of pH values present within a phagosome. Previous studies of phagosomal pH have relied on the pH-sensitive probe fluorescein isothiocyanate-conjugated dextran. In contrast, we quantitated vacuolar pH by using DM-NERF dextran. DM-NERF dextran is a 2′,7′-dimethyl derivative of rhodol characterized by an excitation spectrum which is sensitive to pH fluctuations at 490 nm but insensitive to changes at 440 nm (20). This type of pH excitation spectrum allows for a ratiometric analysis of fluorescence intensities. Ratiometric analysis eliminates ion-independent factors that can affect signal intensities. These factors include the amount of dye internalized into the phagosome, the thickness of the host cell, and leakage of probe from the vacuole. DM-NERF is an ideal reporter of the pH of acidic environments because of its low pK_a (4.5 to 6.0) and pH-dependent spectrum that is optimal at values between 4.0 and 6.0. In addition, the inherent photostability of DM-NERF dextran allows for continual microscopic analysis of phagosomal changes, a benefit that is not afforded by fluorescein isothiocyanate-conjugated dextran.

Initial experiments were conducted to verify that (i) DM-NERF dextran accurately reports the range of pH values likely to be present within a phagosome and (ii) ratios derived from the fluorescence spectra of DM-NERF dextran are consistent whether the probe is prepared in pEM or NCM. The fluorescence spectrum of DM-NERF dextran was found to efficiently record pH values that range from 4.0 to 7.0 (Fig. 1). In addition, the 490 nm/440 nm excitation ratios remained constant whether the DM-NERF dextran was resuspended in pEM or NCM.

The majority of DM-NERF dextran taken up by macrophages infected with live *S. typhimurium* colocalizes in the phagosome. Previous studies have shown that dextran-conjugated fluorescent probes are incorporated into spacious phagosomes containing *S. typhimurium* if such probes are present during the infection period (2). However, time-lapse video microscopy studies of phagocytosis have shown that, in contrast to heat-killed organisms, live *S. typhimurium* cells induce macropinocytosis in infected host cells (1). Hence, it was necessary to establish that the majority of the DM-NERF dextran

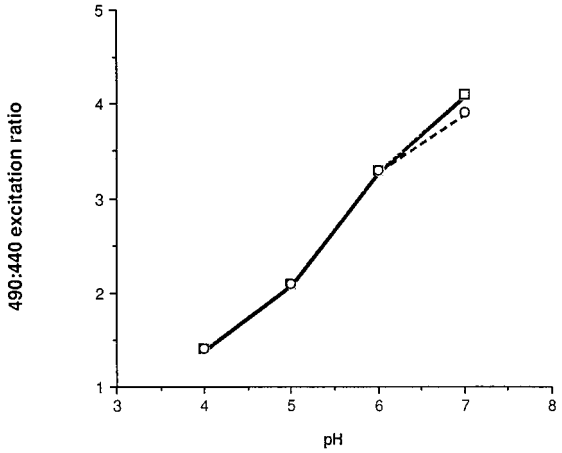


FIG. 1. DM-NERF dextran in vitro calibration. DM-NERF dextran was resuspended in solutions (pH 4.0, 5.0, 6.0, and 7.0 at 37°C) of either pEM (□) or NCM, (○), and the excitation spectra at 490 and 440 nm were monitored with a fluorescence spectrophotometer. The 490 nm/440 nm ratio was calculated and plotted as a function of pH. Error bars are smaller than the depicted symbols.

internalized by macrophages infected with live *S. typhimurium* localizes to vacuoles containing bacteria rather than to vacuoles formed via macropinocytosis. As stated in Materials and Methods, phagosomes containing both DM-NERF dextran and motile bacteria can be visualized on the ratiometric imaging system by switching from epifluorescence to bright-field Nomarski optics. To further confirm that in vitro DM-NERF-containing vacuoles colocalize with intracellular *S. typhimurium*, confocal images of infected cells were collected. Fluorescent images of DM-NERF-containing vacuoles were merged with images of rhodamine-labeled bacteria. Although very small vacuoles containing minor amounts of DM-NERF but no bacteria could be detected in infected macrophages by confocal microscopy, similar small vacuoles were not seen with the ratiometric imaging system, most likely because of differences in microscope detection sensitivities. Of the larger DM-NERF-containing vacuoles detected by confocal microscopy, 82.9% ± 4.8% colocalized with bacteria (Fig. 2). Hence, the majority of DM-NERF dextran taken up by macrophages infected with live *S. typhimurium* is taken up in large phagosomes that also contain bacteria. As depicted in Fig. 2, some bacteria that do not colocalize with DM-NERF dextran can be detected by confocal analysis. A portion of these bacteria were clearly not cell associated and appeared to be sticking to the tissue culture dish (data not shown). Other detectable bacteria did appear to be cell associated but may have been extracellular since the protocol used does not differentiate between intra- and extracellular bacteria. Nevertheless, there does remain the possibility that some internalized bacteria reside in vacuoles that have not incorporated enough DM-NERF dextran to be detected and analyzed by fluorescent ratiometric imaging.

FIG. 2. Merged confocal images of DM-NERF dextran (green) and *S. typhimurium* (red) in infected RAW 264.7 macrophages. The bacteria that colocalize with the DM-NERF dextran appear yellow.

Phagosomes containing *S. typhimurium* (live, heat killed, or formalin fixed) acidify within 20 to 30 min after formation. Previous experiments suggested that live *S. typhimurium* delays and attenuates phagosomal acidification (2). Our preliminary immunoelectron microscopy studies, however, indicate that the vacuolar proton-ATPases responsible for vacuolar acidification are present in phagosomes containing live, virulent *S. typhimurium* at the earliest times analyzed (30 min postinfection) (34). These results suggest that the machinery for vacuolar acidification is present soon after phagosome formation (25). For this reason, we sought to reanalyze the acidification of STVs in murine macrophages.

Before analyzing the pH of phagosomes containing live *S. typhimurium*, we determined the acidification kinetics of phagosomes containing nonviable particles. The pH of vacuoles containing latex beads or nonviable bacteria (heat-killed or formalin-fixed *S. typhimurium*) decreased from ≥6.0 to values between 4.0 and 5.0 (Fig. 3A). The majority of these phagosomes acidified 20 to 30 min after formation (Table 1). The pH of vacuoles containing live *S. typhimurium* also decreased from ≥6.0 to values between 4.0 and 5.0 soon after formation; this pattern was observed in both bone marrow-derived murine macrophages and the murine macrophage-like cell line RAW 264.7 (Fig. 3B and C). The acidification kinetics of STVs varied only slightly from those of phagosomes containing beads or nonviable organisms in that the process was slightly slower, but only on the order of minutes rather than hours (Table 1). The pH of STVs remained below 5.0 for at least 5 h postinfection (Fig. 3D). Analysis of STVs at time points beyond 5 h was impeded by inadequate signal from the probe, possibly due to degradation or gradual exclusion of the compound from the phagosome. Prior experiments have shown that vacuoles containing *M. avium* fail to acidify below pH 6.3 to 6.5 (36). These organisms were used as a control for our experimental system. Our observations are consistent with those already reported: *M. avium*-containing phagosomes failed to acidify even 5 h after formation (Fig. 4A and B).

STV acidification is mediated by vacuolar proton-ATPases. A variety of mechanisms have been suggested to play a role in vacuolar acidification, including increased lactic acid production during phagocytosis, proton generation during superoxide formation, carbonic anhydrase-coupled proton transport, Na^+-H^+ exchange, vacuolar proton-ATPase activity, and phagosome-lysosome fusion (25). It has been shown that phagosomes containing *M. avium* fail to acidify because of the exclusion or removal of vacuolar proton-ATPases (36). To examine the role of vacuolar proton-ATPases in the acidification of STVs, we analyzed the effects of bafilomycin A_1, a specific vacuolar proton pump inhibitor. Pretreatment of host cells with this reagent completely blocked STV acidification (Fig. 3E and Table 1). Thus, proton pumping via vacuolar proton-ATPases appears to be the predominant mechanism of STV acidification.

Bafilomycin inhibits survival and replication of *S. typhimurium* in murine macrophages. As described above, STVs acidify soon after formation and remain acidified for at least 5 h postinfection. We investigated the importance of this phenomenon to the survival and replication of *S. typhimurium* in macrophages. After pretreating RAW 264.7 macrophages with

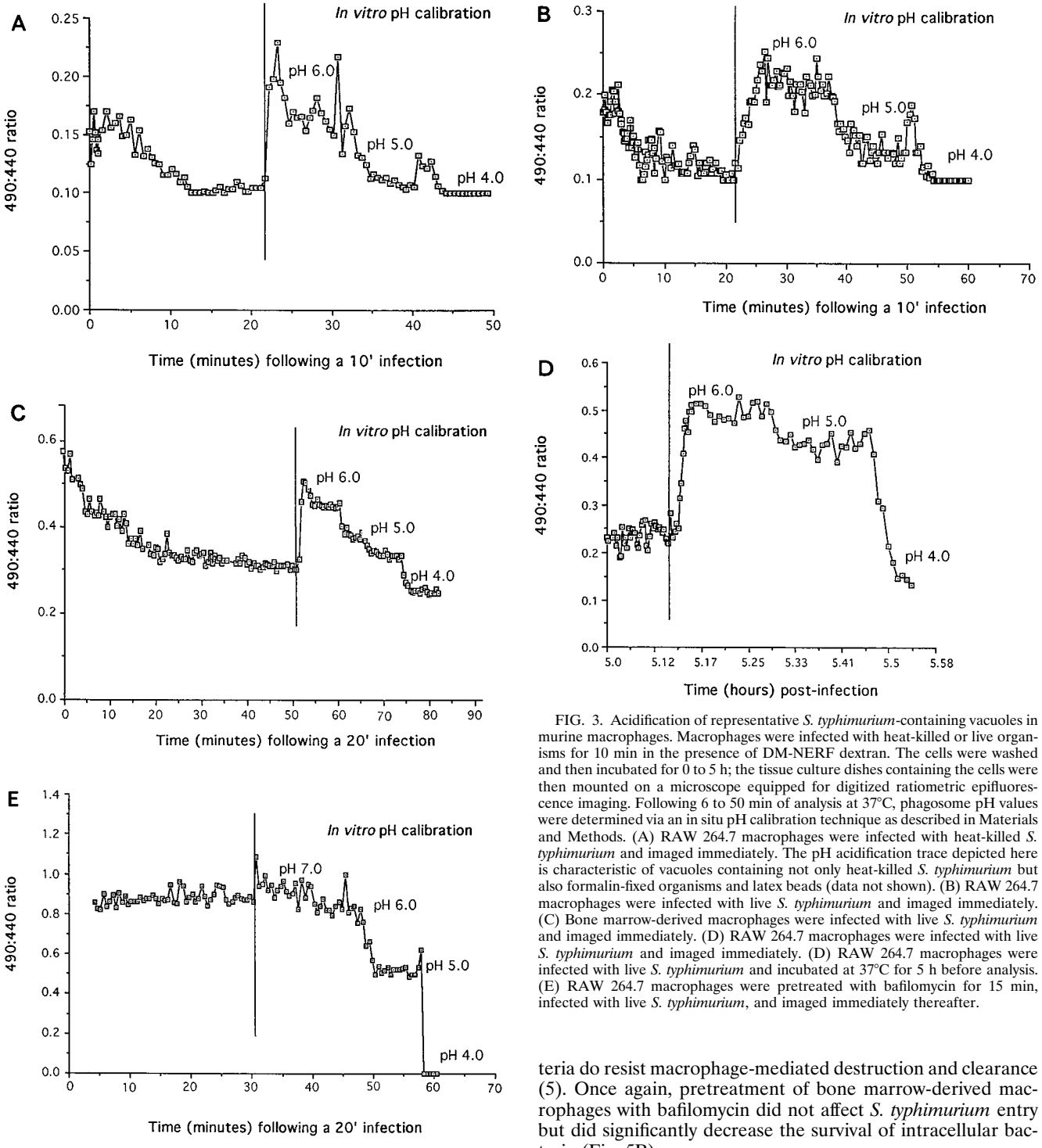


FIG. 3. Acidification of representative *S. typhimurium*-containing vacuoles in murine macrophages. Macrophages were infected with heat-killed or live organisms for 10 min in the presence of DM-NERF dextran. The cells were washed and then incubated for 0 to 5 h; the tissue culture dishes containing the cells were then mounted on a microscope equipped for digitized ratiometric epifluorescence imaging. Following 6 to 50 min of analysis at 37°C, phagosome pH values were determined via an in situ pH calibration technique as described in Materials and Methods. (A) RAW 264.7 macrophages were infected with heat-killed *S. typhimurium* and imaged immediately. The pH acidification trace depicted here is characteristic of vacuoles containing not only heat-killed *S. typhimurium* but also formalin-fixed organisms and latex beads (data not shown). (B) RAW 264.7 macrophages were infected with live *S. typhimurium* and imaged immediately. (C) Bone marrow-derived macrophages were infected with live *S. typhimurium* and imaged immediately. (D) RAW 264.7 macrophages were infected with live *S. typhimurium* and imaged immediately. (D) RAW 264.7 macrophages were infected with live *S. typhimurium* and incubated at 37°C for 5 h before analysis. (E) RAW 264.7 macrophages were pretreated with bafilomycin for 15 min, infected with live *S. typhimurium*, and imaged immediately thereafter.

bafilomycin to block STV acidification, we assayed the number of recoverable bacteria at various times postinfection. Bafilomycin pretreatment had no deleterious effects on the entry of *S. typhimurium*. However, by 4 h postinfection, the number of bacteria recovered from bafilomycin-treated macrophages was two- to threefold lower than the number recovered from untreated cells (Fig. 5A). A similar result was observed in bone marrow-derived macrophages. Although salmonellae do not replicate as readily in these cytokine-activated cells, the bacteria do resist macrophage-mediated destruction and clearance (5). Once again, pretreatment of bone marrow-derived macrophages with bafilomycin did not affect *S. typhimurium* entry but did significantly decrease the survival of intracellular bacteria (Fig. 5B).

Additional experiments were conducted to analyze the viability of *S. typhimurium* in macrophages treated with bafilomycin postinfection. Infected cells were treated with the inhibitor 2, 4, 8, or 12 h postinfection, and the number of viable intracellular organisms was quantitated at 2-h intervals. Bafilomycin treatment at any time postinfection decreased the number of viable intracellular *S. typhimurium* (Fig. 6). At 12 h postinfection, the number of bacteria recovered from cells treated with the inhibitor was 20-fold lower than the number of bacteria recovered from untreated cells.

TABLE 1. Percentage of phagosomes that have acidified to pH 4.0 to 5.0

Time of analysis (min postinfection)[a]	% of phagosomes that have acidified in cells infected with[b]:			
	Live *M. avium*	*Salmonella typhimurium*		
		Live	Heat killed or formalin fixed	In bafilomycin-treated cells
20	4 (1/23)	57 (21/37)	81 (22/27)	0 (0/15)
30	4 (1/23)	82 (30/37)	89 (24/27)	0 (0/15)
60	4 (1/23)	95 (35/37)	100 (27/27)	0 (0/15)

[a] After a 10-min infection period.
[b] Each value represents data accumulated from three to eight experiments. The numbers in parentheses indicate the ratios of the number of phagosomes at pH 4.0 to 5.0 to the total number of phagosomes analyzed.

We wanted to verify that the observed inhibition of bacterial survival and replication in bafilomycin-treated macrophages was due to blockage of vacuolar acidification rather than to a direct antibacterial effect of the drug. Gram-negative organisms have previously been shown to be insensitive to bafilomycin (4). To confirm this finding, we analyzed the viability of *S. typhimurium* in media containing bafilomycin at 25 times the concentration used in the experiments described above. *S. typhimurium* not only survived but also replicated under these conditions (Fig. 5C), indicating that the observed decrease in the number of recoverable bacteria is not due to any direct antibacterial effect of bafilomycin. Furthermore, bacteria released from infected macrophages and immediately incubated for 1 or 2 h in medium containing 100 nM bafilomycin showed no decrease in viability (data not shown), ruling out the possibility that adaptation of *S. typhimurium* to life within the macrophage increases its sensitivity to bafilomycin.

We next tested the possibility that the loss of bacterial viability was an artifact of our assay conditions. Gentamicin, a membrane-impermeant antibiotic, was used in the experiments described above to prevent extracellular bacterial replication postinfection. In order to determine whether bafilomycin treatment compromises the integrity of host cell membranes and, in turn, increases the accessibility of gentamicin to intracellular bacteria, we repeated the experiments but did not include gentamicin in the medium during bafilomycin treatment. Bafilomycin treatment was effective at decreasing the number of recoverable bacteria whether or not gentamicin was included in the medium (data not shown). An additional requirement of our experimental system is the release of intracellular bacteria via lysis of host cells with Triton X-100 postinfection. To eliminate the possibility that increased bacterial susceptibility to detergent is responsible for the observed decrease in numbers of recoverable bacteria in bafilomycin-treated cells, we repeated the experiment, but instead of lysing the cells with Triton X-100, we incubated the infected cells with either water or PBS containing 0.5 mM EDTA for 15 min and then lysed the cells by passing them through a 27-gauge needle. Bafilomycin reduced the number of recoverable bacteria when either one of these protocols was used (data not shown), demonstrating that bacterial recovery is independent of the method of cell lysis.

A variety of vacuolar-pH-neutralizing reagents decrease the survival of *S. typhimurium* in macrophages. In addition to bafilomycin, there are available a variety of reagents that raise the pH of acidic vacuolar compartments but do so through different mechanisms. NEM inhibits vacuolar proton-ATPases, monensin acts as a cationic ionophore, and methylamine and NH_4Cl are lysosomotrophic reagents that are membrane permeative in their nonprotonated forms but impermeative when protonated in acidic vacuoles. *S. typhimurium*-infected macrophages were incubated with each of these reagents at 2 h postinfection for an additional 2 h, and the numbers of recoverable bacteria were quantitated. None of the reagents tested was directly lethal to either the bacteria or the host cells at the concentrations used (data not shown), but all of them significantly decreased the survival of intracellular *S. typhimurium* (Fig. 7). Higher concentrations of NEM and monensin resulted in lower numbers of recoverable bacteria, but these concentrations also decreased the viability of the host cells and, therefore, are not reported here. These results confirm that the loss of *S. typhimurium* viability is not specific to bafilomycin treatment but can be accomplished with any one of several reagents that neutralize the vacuolar compartment.

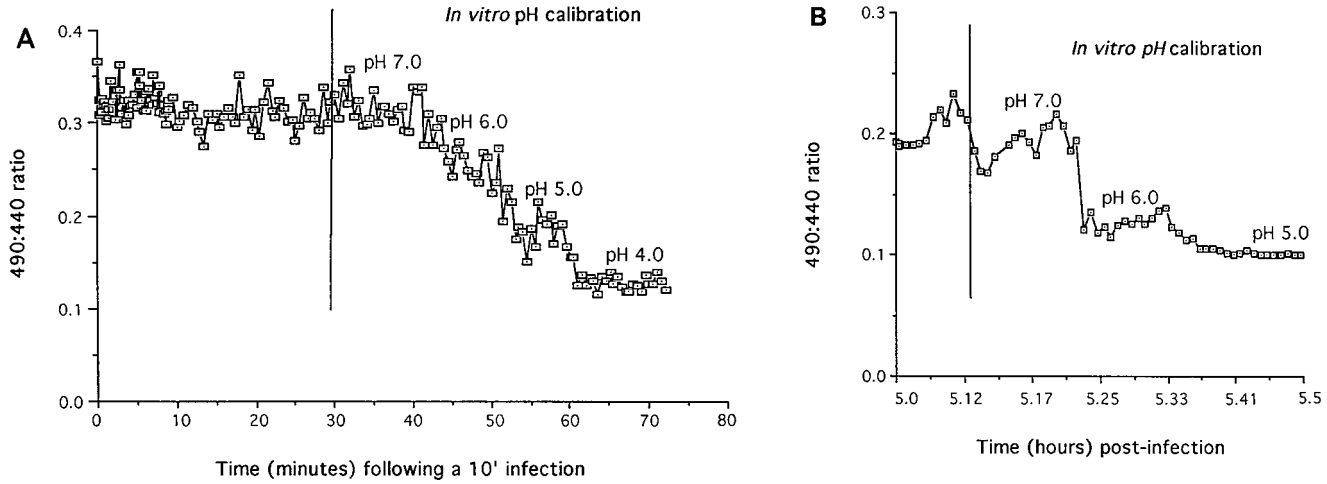
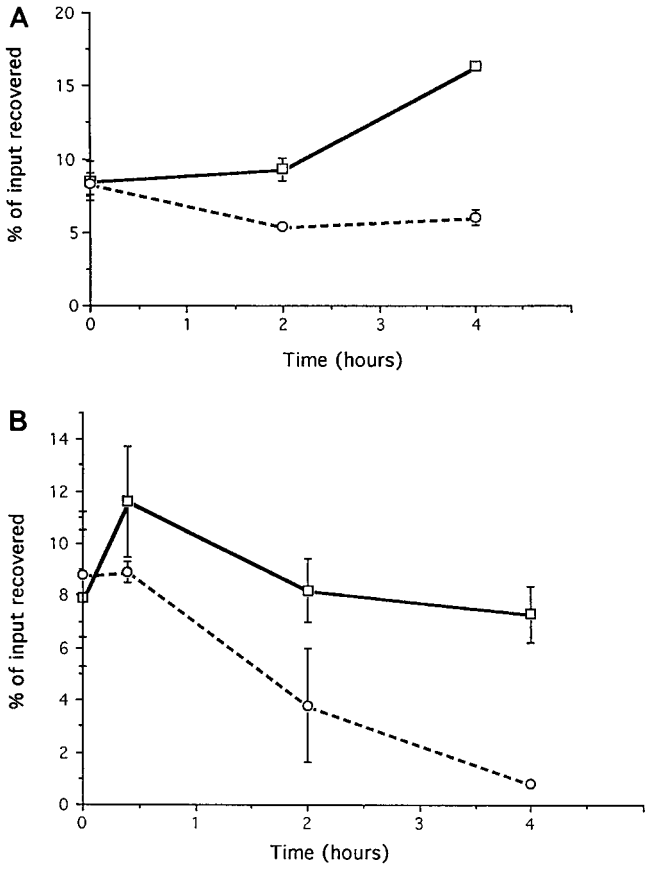


FIG. 4. Acidification of representative vacuoles containing *M. avium* in RAW 264.7 macrophages. Macrophages were infected with live organisms for 10 min in the presence of DM-NERF dextran. The cells were washed and then incubated for 0 to 5 h; the tissue culture dishes containing the cells were then mounted on a microscope equipped for digitized ratiometric epifluorescence imaging. Following 5 to 30 min of analysis at 37°C, phagosome pH values were determined via the in situ pH calibration technique described in Materials and Methods. (A) Macrophages were infected with live *M. avium* and imaged immediately. (B) Macrophages were infected with live *M. avium* and incubated at 37°C for 5 h before analysis.



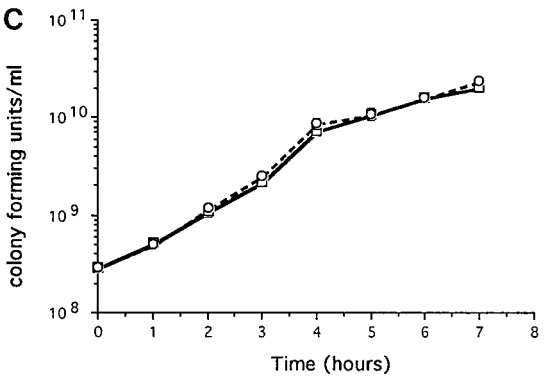


FIG. 5. Inhibition of bacterial survival and growth in murine macrophages by bafilomycin. (A and C) Macrophages, either untreated (□) or pretreated with 100 nM bafilomycin for 15 min (○), were infected with *S. typhimurium* for 10 to 20 min. The cells were washed, and the number of intracellular organisms was quantitated at 0, 2, and 4 h postinfection. (A) Percentage of bacteria recovered from RAW 264.7 macrophages. (B) Percentage of bacteria recovered from bone marrow-derived macrophages. Error bars indicate the standard deviations of the means. (C) *S. typhimurium* growth in media containing 2.5 μM bafilomycin. Media with (○) and without (□) 2.5 μM bafilomycin were inoculated with approximately 3×10^8 organisms per ml and incubated at 37°C for 0 to 7 h. The number of CFU per milliliter was quantitated every hour.

A subpopulation of *S. typhimurium* survives neutralization of the phagosomal environment and replicates when the phagosome is allowed to reacidify. Results show that bafilomycin treatment of host cells significantly decreases the number of recoverable intracellular bacteria; however, a subpopulation of bacteria does survive this treatment (Fig. 6 and 7). We sought to determine whether this subpopulation replicates in phagosomes that are allowed to reacidify. Bafilomycin binds to and inhibits vacuolar proton-ATPases irreversibly. Methylamine, on the other hand, is as effective as bafilomycin at decreasing intracellular bacterial viability (Fig. 7), but the effects are reversible (22). Methylamine, therefore, was used to test the replicative ability of bacteria that survive vacuolar neutralization. Methylamine was added to infected host cells at 2 h postinfection, and the cells were incubated for 2 h. At 4 h postinfection, the cells were washed and then incubated for 6 h in the absence of methylamine. At this time point, methylamine was again added to the medium and the cells were incubated for an additional 2 h (Fig. 8). The first 2-h methylamine incubation resulted in a three- to fourfold decrease in the number of recoverable bacteria compared with that of untreated cells. Nevertheless, a small percentage of the original bacterial input survived this treatment and, upon removal of methylamine and vacuole reacidification, was capable of replicating. When methylamine was once again added to the host cells, the number of recoverable bacteria decreased significantly. Although it remains unclear how and why a subpopulation of bacteria survives the initial methylamine treatment, these data indicate that this subpopulation does not represent bacteria selected for resistance to vacuolar neutralization.

Loss of *Salmonella* viability in neutralized vacuolar compartments is *phoP* independent. The *Salmonella* PhoP and PhoQ proteins constitute a two-component regulatory system that controls the expression of genes involved in virulence and survival of the bacterium in macrophages (28). It was originally reported that PhoP-activated gene transcription is induced in macrophages and that this increase in transcription is induced in macrophages and that this increase in transcription can be abolished by adding compounds that raise the pH of acidic compartments (2). These observations led to the hypothesis that phagosome acidification is an intracellular inducer of the expression of PhoP-regulated genes that are essential for bacterial survival in macrophages. Thus, a possible explanation for the loss of *S. typhimurium* viability in bafilomycin-treated macrophages is that PhoP-regulated gene transcription was abrogated in neutralized phagosomes and that factors required for

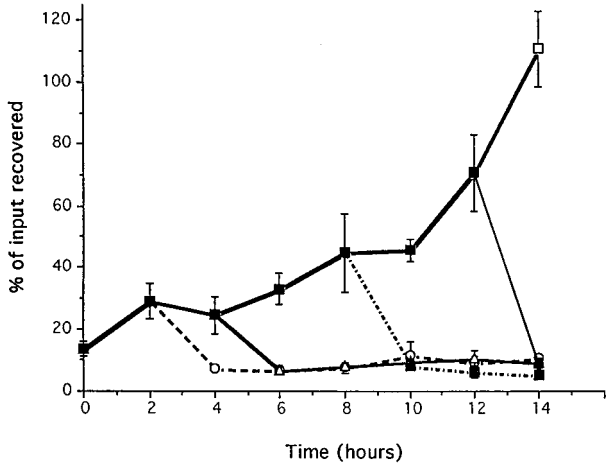


FIG. 6. Time course study of the effect of bafilomycin treatment on survival of *S. typhimurium* in macrophages. RAW 264.7 macrophages were infected with *S. typhimurium* for 20 min, washed, and incubated at 37°C. Bafilomycin either was added at 2 h (○), 4 h (△), 8 h (■), or 12 h (●) postinfection or was not added (□), and the number of recoverable bacteria was quantitated every 2 h. Error bars indicate the standard deviations of the means.

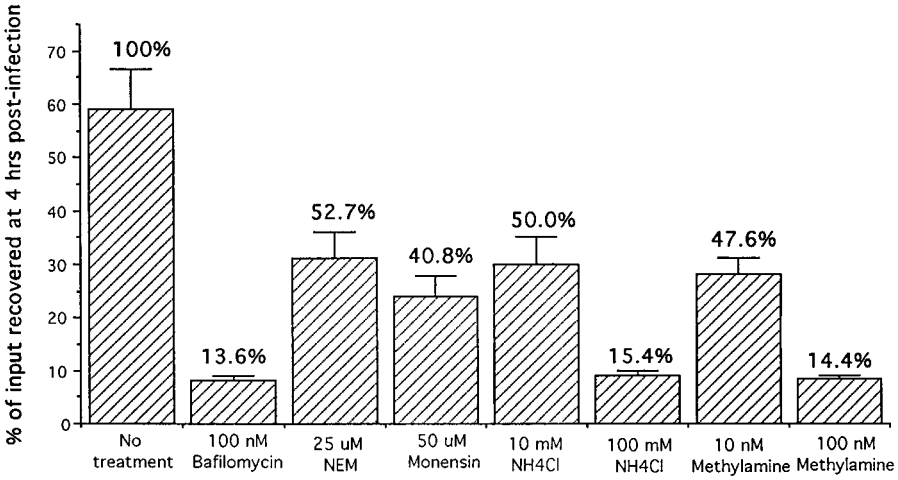


FIG. 7. Effects of vacuolar-pH-neutralizing reagents on survival of *S. typhimurium* in macrophages. RAW 264.7 macrophages were infected for 2 h before the addition of bafilomycin, NEM, monensin, NH_4Cl, or methylamine. The cells were incubated at 37°C, and the number of surviving bacteria was quantitated 2 h later. Percentages are with respect to control (no treatment). Error bars indicate the standard deviations of the means.

intravacuolar survival were no longer expressed. A test of this hypothesis involves determining whether wild-type *S. typhimurium* in bafilomycin-treated macrophages resemble *Salmonella phoP* mutants in untreated cells. Thus, we compared the survival of wild-type and *phoP*::Tn*10 S. typhimurium* in bafilomycin-treated and untreated macrophages. No significant differences were observed in the growth of these two strains in treated and untreated cells during the first 4 h of infection (Fig. 9). Other investigators have also demonstrated that *phoP* mutants survive as well as wild-type organisms during the early stages of macrophage infection but are attenuated at later time points (13, 38). We found that the *phoP* mutant survived better in untreated cells than wild-type bacteria did in bafilomycin-treated cells. In addition, wild-type and *phoP*::Tn*10 S. typhimurium* were equally susceptible to bafilomycin treatment. When combined, these observations indicate that lack of transcription from the *phoP* locus is not responsible for the loss of *S. typhimurium* viability in bafilomycin-treated cells.

DISCUSSION

The present study provides evidence that in murine macrophages the majority of vacuoles containing live *S. typhimurium* acidify within 20 to 30 min after formation. These vacuoles acidify to a pH between 4.0 and 5.0, and this phenomenon can be blocked by inhibiting host cell vacuolar proton-transporting ATPases. Phagosomes containing nonviable particles acidify to comparable pH values of 4.0 to 5.0 and do so at a rate that is only a few minutes faster than STV acidification. This slight difference in acidification kinetics may simply reflect the size of the phagosomes. Time-lapse video microscopy studies have shown that *S. typhimurium* enters murine macrophages in spa-

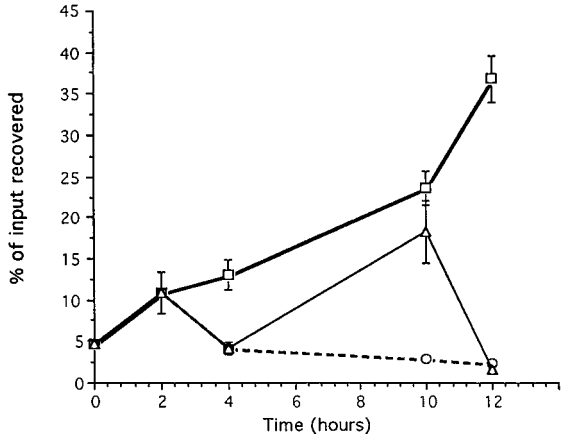


FIG. 8. Recovery of *S. typhimurium* from methylamine inhibition. Methylamine was added to infected RAW 264.7 macrophages at 2 h postinfection, and the cells were incubated for 2 h. At 4 h postinfection, the cells were washed and then incubated for 6 h in medium devoid of methylamine; at this time point, methylamine was again added to the medium, and the cells were then incubated for a final 2 h. □, no treatment; ○, methylamine added at 2 h, followed by a 2-h incubation; △, methylamine added at 2 h, followed by a 6-h incubation without methylamine and a subsequent 2-h incubation with methylamine. The error bars indicate the standard deviations of the means.

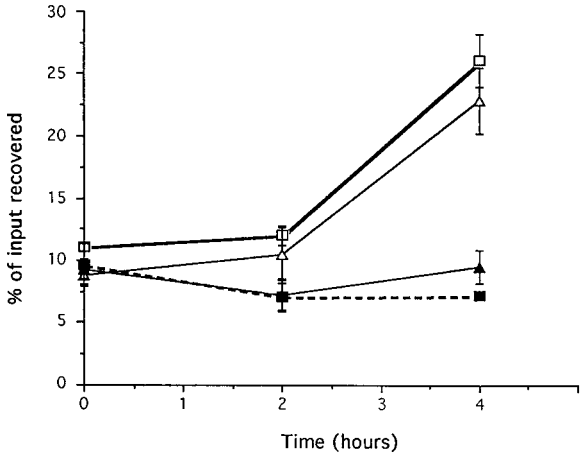


FIG. 9. Survival of wild-type and *phoP S. typhimurium* in bafilomycin-treated macrophages. RAW 264.7 macrophages were pretreated with 100 nM bafilomycin for 15 min and then infected with wild-type or *phoP*::Tn*10 S. typhimurium* for 20 min. The cells were washed, and the number of intracellular organisms was quantitated at 0, 2, and 4 hours postinfection. □, wild-type SL1344 in untreated macrophages; ■, wild-type SL1344 in bafilomycin-treated macrophages; △, *phoP* SL1344 in untreated macrophages; ▲, *phoP* SL1344 in bafilomycin-treated macrophages. The error bars indicate the standard deviations of the means.

cious phagosomes whereas other bacteria, such as *Yersinia enterocolitica*, and, presumably, nonviable particles are taken up in comparably smaller phagosomes (1). The data presented here and the observations of others indicate that proton-ATP ases accumulate either during or shortly after phagosome formation (25, 32, 36). Rates of phagosome acidification probably depend, in part, on how quickly proton-ATPases accumulate. Rates may, in addition, reflect the volume into which the protons are being pumped, with larger vacuoles requiring more protons and, hence, more time to fully acidify. If this hypothesis is true, then the larger size of phagosomes containing the live *S. typhimurium* may explain their slightly lower acidification rate.

The findings reported here contrast with those of Alpuche-Aranda et al. (2), who reported that the pH of vacuoles containing live *S. typhimurium* does not fall below 5.0 and that the rate of acidification is low compared with that of vacuoles containing heat-killed organisms. Although different strains of *S. typhimurium* and two distinct but related pH probes were used in the two experimental systems, it is highly unlikely that the disagreement is a result of these parameters. One explanation for experimental discrepancies was differential cytokine activation of the macrophages. Our initial experiments and those of Alpuche-Aranda et al. were conducted in bone marrow-derived macrophages which are activated through cultivation in media containing cytokines produced by L cells. However, we subsequently showed that STV acidification is as rapid and complete in nonactivated RAW 264.7 cells as it is in bone marrow-derived macrophages. Hence, the state of macrophage activation does not seem to play a role in the rate of STV acidification. A possible explanation for the attenuated and delayed acidification observed by our colleagues involves the bacterial load. The Alpuche-Aranda protocol, in which macrophages are infected for 45 to 60 min with opsonized bacteria, certainly results in significantly higher numbers of intracellular *S. typhimurium* than does our own protocol, in which cells are infected with nonopsonized organisms for only 10 min. High bacterial loads are toxic to macrophages (30). It is possible that normal cellular functions, including the activities of vacuolar proton-ATPases, are compromised in intoxicated macrophages.

The results reported here indicate that *S. typhimurium* resides in vacuoles that rapidly acidify and that intracellular bacterial viability is compromised by addition of reagents that neutralize acidic compartments. One explanation for this phenomenon is that the inhibitors used have side effects, independent of the neutralization of acidified endocytic compartments, that compromise cell functions required to maintain an environment compatible with *S. typhimurium* survival. Examples of such effects include changes in cytosolic pH, neutralization of acidified compartments of the exocytic pathway, inhibition of the interactions between endocytic compartments, and dissipation of membrane potentials that are necessary for uptake of amino acids.

An alternate hypothesis that may explain the loss of *S. typhimurium* viability in cells treated with drugs such as bafilomycin is that the bacterium actually requires an acidic environment for survival and growth within the macrophage. Two organisms that have already been shown to fall within this category are *C. burnetii* and *Leishmania donovani* amastigotes (19, 31). These pathogens require a proton-rich environment for optimal transport of catabolic substrates and for metabolic processes. It seems unlikely that this biochemical stratagem can also be applied to intracellular *S. typhimurium* since this organism, unlike *C. burnetii* and *L. donovani* amastigotes, replicates outside of cells not only in low-pH L broth but also in L broth at pH 7.0. *F. tularensis* is another intracellular pathogen that requires an acidic compartment for intracellular replication (16). Recent studies have shown that localization in an acidic environment facilitates the dissociation of iron from transferrin and, in turn, the availability of iron essential for growth of *F. tularensis*. Thus, growth-inhibitory effects of reagents that neutralize the otherwise acidic *Francisella* phagosomes can be completely reversed by addition of ferric PP_i, a transferrin-independent iron source. The addition of ferric PP_i to *S. typhimurium*-infected macrophages, on the other hand, is ineffective at reversing the effects of bafilomycin, monensin, or lysosomotrophic agents. Furthermore, the addition of deferoxamine, an iron chelator that inhibits the growth of *F. tularensis* in macrophages, has no effect on the replication of *S. typhimurium* in RAW 264.7 macrophages (33). Thus, the survival and replication of *S. typhimurium* in acidic phagosomes do not appear to be linked to a requirement for iron.

If an acidic environment is indeed required for *S. typhimurium* survival and replication in macrophages, then the question as to why that may be the case arises. We suggest that low pH serves as an intracellular signal that plays an integral part in transcriptional regulation of genes whose products are essential for intramacrophage survival. PhoP, as already noted, has been shown to be a transcriptional activator of gene transcription in macrophages. Our results, however, indicate that lack of transcription of PhoP-regulated genes does not account for the reported effects of bafilomycin treatment on bacterial survival. Nevertheless, numerous investigations have shown that over 30 *Salmonella* proteins are selectively synthesized in macrophages and that many of these proteins are not under the control of the PhoP-PhoQ regulatory system (6, 41). Perhaps one or more of these pH-induced proteins are essential for bacterial survival and growth within macrophages. We are currently testing this hypothesis by investigating pH regulation of bacterial gene transcription in macrophages.

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