INFECTION AND IMMUNITY, Apr. 1996, p. 1085–1092
0019-9567/96/$04.00+0
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Vol. 64, No. 4

Role of the Acid Tolerance Response in Virulence of *Salmonella typhimurium*

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Received 25 August 1995/Returned for modification 30 November 1995/Accepted 8 January 1996

During its life cycle, *Salmonella typhimurium* is exposed to a variety of acidic conditions. Survival in the acidic environments within the host may require the adaptive acid tolerance response (ATR), which is characterized by the induction of several *Salmonella* proteins upon exposure to mildly acidic conditions. These induced proteins protect the bacterium from death under severe acid challenge. The goal of this study was to examine the role of the ATR in *Salmonella* pathogenesis. Initially, we observed that differences exist between the virulent *S. typhimurium* strains and the laboratory *S. typhimurium* strain LT2 with respect to their ATR. Mutations affecting the ATR of *S. typhimurium* LT2, including *atrB*, *atrC* (*polA*), *atrD*, *atbR*, and *fur*, were crossed into virulent *Salmonella* strains, and the resultant transductants were screened for virulence in mice and acid sensitivity. Surprisingly, with the exception of the *fur* mutation, none of the mutations had a major effect on acid resistance or virulence in the pathogenic strains. The *fur* mutants showed a 1- to 3-log increase in the 50% lethal dose; however, the magnitude of its effect was dependent on the strain background. Strains containing two or three different *atr* mutations were constructed, and these were also examined for acid sensitivity and virulence. The double and triple mutants that contained an *atrC* mutation no longer displayed an ATR. Those mutants which were more acid sensitive were also highly attenuated, suggesting a strong correlation between the ability to mount an ATR and virulence in *S. typhimurium*. Comparison of the ability of the various *atr* single, double, and triple mutants to survive within macrophages showed that strains containing an *atrC* mutation survived much less well than the wild type in bone marrow-derived macrophages. No differences in survival within J774 macrophage-like cells were detected.

Salmonellosis represents one of the most widespread communicable bacterial diseases in the United States (36). Members of the genus *Salmonella* are responsible for a diversity of illnesses, including typhoid fever, gastroenteritis, and septicemia (21). Although more than 1,500 *Salmonella* serovars exist, the most frequently isolated serotype is *Salmonella typhimurium*, which accounts for about 35% of reported human isolates (6). In the majority of cases, humans acquire *Salmonella* organisms through the ingestion of contaminated food or water or by fecal-oral spread (21).

S. typhimurium is a neutralophilic organism; hence, it grows at a wide range of pH values. However, during the course of infection, *S. typhimurium* is exposed to potentially lethal acid conditions such as those found in the stomach or within the macrophage phagolysosome. To provide an explanation for how *Salmonella* bacteria can survive such harsh, acidic environments within the host, Foster and Hall (13) examined the adaptive ability of *S. typhimurium* to become acid tolerant. They characterized a phenomenon termed the adaptive acid tolerance response (ATR), in which exposure to slight or moderate acid stress results in the synthesis of proteins which then protect the bacterium from more-severe acid challenge. More recent studies have provided evidence that there are at least two distinct pH-dependent ATR systems in *S. typhimurium* LT2 (25). The first, log-phase ATR, is seen in logarithmically growing cells and is characterized as a two-stage system with some proteins induced after a shift to pH 5.8 (pre-acid shock) and others induced following a shift to pH ≤4.5 (acid shock). The proteins induced at the mild pH shift (pH 5.8) function to induce a pH homeostasis which maintains an internal pH when housekeeping homeostasis systems fail. Those induced at or below pH 4.5 include at least 43 proteins, many of which presumably function in the survival of the bacterium by preventing or repairing acid damage to macromolecules (9). A second ATR system is evident when stationary-phase cells are shifted to pH ≤4.5. The stationary-phase ATR is characterized by the synthesis of 15 proteins which are distinct from the log-phase acid shock proteins (25). In contrast to the log-phase ATR, which is only transiently induced, stationary-phase ATR was shown to exhibit sustained induction over the course of several hours at pH 4.3. In addition to these pH-dependent systems, stationary phase appears to confer a pH-independent protection against acid stress (25).

Several *S. typhimurium* LT2 mutants have been identified which are altered in their ability to mount an ATR. These mutants fall into one of two general classes on the basis of their sensitivity to acid pH. The first class consists of mutants which are acid sensitive, including *atp* (9), *fur* (9), *phoP* (13), *atrB*, *atrC* (*polA*), *atrD*, *atrF*, and *atrG* (12). The second class contains mutants which are constitutively acid resistant, including *ace*, *icd*, *atr-1* (14), and *atbR* (12). Some of the mutations conferring an acid-sensitive phenotype to LT2 (*phoP*, *atp*, and *fur*) have also been shown to attenuate virulent *Salmonella* strains to different degrees in the mouse typhoid model (8, 17). However, because of the pleiotropic nature of these mutations, attenuation has not yet been correlated with acid sensitivity.

Since *S. typhimurium* encounters acid conditions within the host, it is likely that the ATR is important in the pathogenesis of this organism. To test this, we examined the effect of mu-

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TABLE 1. Bacterial strains

Strain	Genotype and/or phenotype	Reference or source
S. typhimurium LT2		
SF1	Wild type	13
JF2043	*fur-1 zbf-5123*::Tn*10 iroA1*::MudJ; Tet^r Kan^r Lac$^+$	17
JF2430	*atrB13*::MudJ; Kan^r Lac$^+$	12
JF2433	*atrD16*::MudJ; Kan^r Lac$^-$	12
JF2464	*atrC20* (*polA*)::MudJ; Kan^r Lac$^-$	12
JF2589	*atbR*::Tn*10*; Tet^r	12
TT10251	*pmi-51*::Mu*d*1-8; Amp^r	J. Roth
S. typhimurium SL1344		
χ3339	*rpsL hisG xyl*	20
χ3689	Same as that for χ3339 but *phoP12*	16
χ4659	Same as that for χ3339 but *fur-1 zbf*::Tn*10*; Tet^r	This study
χ4715	Same as that for χ3339 but *atrC20*::MudJ; Kan^r Lac$^-$	This study
χ4716	Same as that for χ3339 but *atrB13*::MudJ; Kan^r Lac$^+$	This study
χ4717	Same as that for χ3339 but *atrD16*::MudJ; Kan^r Lac$^-$	This study
χ4751	Same as that for χ3339 but *atrD16*::MudJ *zxx*::Tn*10*; Kan^r Lac$^-$ Tet^r	This study
χ4752	Same as that for χ3339 but *atrB13*::MudJ *zxx*::Tn*10 atrD16*::MudJ; Kan^r Lac$^+$ Tet^r	This study
χ4753	Same as that for χ3339 but *atrC20*::Mu*d*1-8; Amp^r	This study
χ4754	Same as that for χ3339 but *atrC20*::Mu*d*1-8 *atrB13*::MudJ; Amp^r Kan^r Lac$^+$	This study
χ4755	Same as that for χ3339 but *atrD16*::MudJ *atrC20*::Mu*d*1-8; Kan^r Lac$^-$ Amp^r	This study
χ4756	Same as that for χ3339 but *atrB13*::MudJ *zxx*::Tn*10 atrD16*::MudJ *atrC20*::Mu*d*1-8; Kan^r Lac$^+$ Tet^r Amp^r	This study
χ4855	Same as that for χ3339 but *atbR*::Tn*10*; Tet^r	This study
S. typhimurium UK-1		
χ3761	Wild type	7
χ4224	Same as that for χ3761 but pSTV3::Tn*mini-tet*; Tet^r	30
χ4652	Same as that for χ3761 but *atbR*::Tn*10*; Tet^r	This study
χ4711	Same as that for χ3761 but *atrC20*::MudJ; Kan^r Lac$^-$	This study
χ4712	Same as that for χ3761 but *atrB13*::MudJ; Kan^r Lac$^+$	This study
χ4713	Same as that for χ3761 but *atrD16*::MudJ; Kan^r Lac$^-$	This study
χ4731	Same as that for χ3761 but *atrB13*::MudJ *zxx*::Tn*10 atrD16*::MudJ; Kan^r Lac$^+$ Tet^r	This study
χ4732	Same as that for χ3761 but *atrC20*::Mu*d*1-8 *atrB13*::MudJ; Amp^r Kan^r Lac$^+$	This study
χ4733	Same as that for χ3761 but *atrD16*::MudJ *atrC20*::Mu*d*1-8; Kan^r Lac$^-$ Amp^r	This study
χ4734	Same as that for χ3761 but *atrB13*::MudJ *zxx*::Tn*10 atrD16*::MudJ *atrC20*::Mu*d*1-8; Kan^r Lac$^+$ Tet^r Amp^r	This study
χ4971	Same as that for χ3761 but *fur-1 zbf*::Tn*10*	This study
S. typhimurium ATCC 14028s		
MS3792	*zxx*::Tn*10*; macrophage-sensitive mutant	5

tations which confer an acid-sensitive or constitutive acid-resistant phenotype on virulence in *S. typhimurium*. This was done by crossing the various *atr* mutations into virulent *S. typhimurium* backgrounds, both singly and in combination. The resultant transductants were examined with respect to (i) their ATR, (ii) their virulence in mice (both orally and intraperitoneally), (iii) their ability to invade intestine-407 (Int-407) epithelial cells, and (iv) their ability to survive within different populations of macrophages.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are listed in Table 1. Those generated in this study were constructed by standard P22HT*int* transduction (33). Transductants were purified on EBU plates (4) and subsequently cross-streaked with P22H5 phage (a clear plaque mutant) for identification of and subsequent elimination of pseudolysogens and lysogens. To construct double and triple *atr* mutants, the drug resistance marker for the *atrC* insertion was changed to ampicillin resistance (Amp^r), and a Tn*10* insertion linked to the *atrB*::MudJ (*zxx*::Tn*10*) mutation was identified from a Tn*10* library available in the Curtiss laboratory. The *atrC*::Mu*d*1-8 mutation was generated by infecting JF2464 with P22HT*int* grown on TT10251 (*pmi*::Mu*d*1-8). Amp^r transductants were purified and scored for kanamycin sensitivity (Kan^s), UV sensitivity (UV^s), and the ability to ferment mannose (Man$^+$). One Amp^r transductant was shown to be Kan^s UV^s Man$^+$ and presumably contained a Mu*d*1-8 insertion in the *atrC* (*polA*) gene which resulted in homologous recombination between the two Mu*d* elements.

To construct strains containing two MudJ (Kan^r) insertions, the *atrD*::MudJ was first transduced into a recipient selecting Kan^r. Subsequently, to construct χ4752 and χ4756, the *atrB*::MudJ insertion was crossed into the *atrD*::MudJ or *atrD*::MudJ *atrC*::Mu*d*1-8 recipient, respectively, by selecting for the linked *zxx*::Tn*10* and scoring for the presence of the Lac$^+$ *atrB*::MudJ insertion. The genotypes of χ4752 and χ4756 were verified by use of these strains as donors in subsequent P22HT*int* transductions.

Media and phenotypic screens. Standard media, including Lennox broth (26), Luria-Bertani (LB) broth (27), MacConkey agar (Difco, Detroit, Mich.), glucose E medium (34), Simmon's citrate agar (Difco), and EBU agar, were used for cultivation of bacteria. Difco agar was added to Lennox broth at 1.5% for base agar and 0.4% for motility agar. The dye triphenyltetrazolium chloride was also added to motility agar at a final concentration of 0.25%. MacConkey agar was supplemented with the appropriate carbohydrate at 1.0%. Antibiotics were added at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 15 μg/ml; kanamycin, 50 μg/ml. The chromogenic dye 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used as an indicator for β-galactosidase synthesis and spread onto plates at a final concentration of 40 μg/ml. *fur* mutants are defective in the ability to use citrate as the sole carbon source and were characterized by stabbing strains into Simmon's citrate agar slants. *atrC* (*polA*) mutants tested for UV sensitivity were streaked across the surface of an LB agar plate. The plates were exposed to a 15-W germicidal lamp at a distance of 10 cm for 0, 10, and 20 s and then incubated in the dark. Motility was assayed by stab inoculation of petri dishes containing semisolid agar. The plates were incubated for 24 h.

Measuring the ATR. The protocol for measuring the ATR is described in detail elsewhere (13). Briefly, the ATR was tested by growing parallel cultures in minimal glucose E medium (pH 7.7) until an optical density at 600 nm of 0.2 was reached. One culture was then adjusted to pH 5.8 with HCl until an optical density at 600 nm of 0.4 was reached. The other culture was unadapted and allowed to continue to grow until the optical density had doubled. Both cultures were then adjusted to pH 3.3. Viable counts were determined for both unadapted and adapted cells immediately prior to acid challenge and at 1, 2, and 4 h postchallenge.

Animal studies. Six- to 8-week-old female BALB/c mice were used for all studies. The mice were obtained from Harlan Sprague-Dawley (Indianapolis, Ind.) and kept for 1 week prior to inoculation. The virulence of SL1344 and its derivatives was determined by peroral and intraperitoneal inoculation. Bacteria for inoculation were grown overnight in Luria broth from a frozen stock and diluted 1:200 in fresh Luria broth the following morning. Cultures were aerated at 37°C for approximately 3 h until an optical density at 600 nm of 0.8 to 1.0 was reached. The cells were then pelleted and resuspended in buffered saline with gelatin (BSG; 0.85% NaCl, 0.01% gelatin, 2.2 mM KH_2PO_4, and 4.2 mM Na_2HPO_4). Dilutions were made in BSG to obtain the appropriate doses. To determine bacterial titers, dilutions in BSG were made as appropriate, and cells were plated on LB agar.

For peroral inoculations, mice were deprived of food and water for 4 to 6 h prior to infection. No sodium bicarbonate was given to neutralize gastric acidity. Peroral inoculations were done with a micropipette tip placed immediately behind the incisors and by injecting 20 μl of *Salmonella* suspension. Food and water were returned to the mice 30 min postinoculation. Intraperitoneal inoculations were performed by injecting each mouse with 100 μl of *Salmonella* suspension with a 26-gauge needle. For each 50% lethal dose (LD_{50}) experiment, mice were observed for a 4-week period. LD_{50} determinations were done by the method of Reed and Muench (32) with three or four mice per inoculum dose.

In vitro invasion assays. Int-407 (19) cells were used for tissue culture invasion assays which employed a modification of the procedure of Galan and Curtiss (15). Briefly, Int-407 cells were seeded into 24-well tissue culture plates and grown in minimal essential medium (MEM; Gibco BRL, Grand Island, N.Y.) supplemented with 10% fetal calf serum (FCS; HyClone Laboratories, Inc., Logan, Utah), 2 mM glutamine, 100 U of penicillin (Gibco BRL) per ml, and 100 μg of streptomycin (Gibco BRL) per ml. The medium was removed and replaced with the same medium but lacking antibiotics 1 day prior to the assay and, again approximately 1 h prior to the assay. Log-phase *S. typhimurium* (optical density at 600 nm of 0.2 to 0.4) grown in Luria broth was added to the monolayers (70 to 90% confluent) in triplicate at a multiplicity of infection of 1 to 10. The plates were centrifuged at 800 rpm in a Beckman GS6R centrifuge with a GH-3.7 rotor to facilitate contact between the bacteria and the monolayer. The bacteria were allowed to adhere to and invade the Int-407 cells for 2 h, after which the monolayers were washed twice with Hanks' balanced salt solution (Gibco BRL). MEM supplemented with 10% FCS, 2 mM glutamine, and 10 μg of gentamicin per ml was added for 1 h more to kill extracellular bacteria. The monolayers were washed twice with BSG, and then BSG containing 0.1% sodium deoxycholate was added to lyse the Int-407 cells. The bacteria were diluted in BSG and plated on LB agar to assess the total number of bacteria associated with the monolayer.

Macrophages. Bone marrow-derived macrophages were from BALB/c mice (Harlan Sprague-Dawley) or Sprague-Dawley rats (Harlan Sprague-Dawley). Bone marrow-derived macrophages were prepared by washing bone marrow cells from the femur and tibia with Dulbecco's MEM (DMEM; Gibco BRL) supplemented with 10% FCS, 5% horse serum (Sigma Chemical Co., St. Louis, Mo.), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% L-cell conditioned medium (DMEM containing 10% FCS, 100 U of penicillin per ml, and 100 μg of streptomycin per ml, which was used for the growth of L929 cells and which was removed after 8 to 10 days of growth and filter sterilized). The bone marrow exudate was incubated for 24 h in a tissue culture flask to allow the macrophages to adhere; after this time, the nonadhered cells were removed and the spent medium was replaced. The cells were incubated for 5 additional days, at which time the macrophages were scraped from the surface of the flask and suspended in 3 to 5 ml of fresh medium. With a hemacytometer, the concentration of macrophages was determined, after which the macrophage suspension was adjusted to approximately 5×10^5 cells per ml with DMEM containing 10% FCS, 5% horse serum, and 10% L-cell conditioned medium (no antibiotics). One milliliter of macrophage suspension was then added to each well of a 24-well tissue culture plate, and the cells were incubated overnight prior to the assay.

J774 cells, a macrophage-like cell line (31), were maintained in MEM containing 10% FCS, 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. The cells were scraped from a confluent lawn in a 75-cm^2 flask and then seeded into 24-well tissue culture plates. The cells were grown for several days with medium changes every 2 to 3 days. When the cells reached approximately 70 to 80% confluency, the medium was replaced with MEM containing 10% FCS and 2 mM glutamine (no antibiotics) and the survival assay was performed the following day.

In vitro macrophage survival assay. The in vitro assay used to determine the ability of *S. typhimurium* to survive within bone marrow-derived macrophages and J774 macrophage-like cells was described previously (5). This protocol was altered in two minor ways. First, to remove nonphagocytosed bacteria, the macrophages were washed with Hanks' balanced salt solution instead of phosphate-buffered saline. Second, to kill extracellular bacteria, macrophage monolayers were incubated with RPMI 1640 containing 5% FCS and 10 μg of gentamicin per ml instead of 6 μg of gentamicin per ml.

RESULTS

ATR of virulent and nonvirulent *S. typhimurium* strains. Studies characterizing the ATR of *S. typhimurium* involved the use of an avirulent laboratory strain of *S. typhimurium*, LT2 (9, 10, 12–14). We examined the ability of LT2 to mount an effective ATR and compared it with that of two mouse-virulent strains, SL1344 and UK-1. Figure 1 illustrates a typical ATR for LT2. Adapted cultures, that is, those shifted to pH 5.8, were much more acid resistant than the unadapted cells to a challenge with pH 3.3. As shown in Fig. 1, the adapted cells were about 10-fold more acid resistant just 1 h postchallenge than the unadapted cells, and this difference became even greater with longer exposure to acid conditions (nearly 3 logs by 4 h postchallenge). When we examined the ability of the virulent strains to survive at pH 3.3, we found that the adapted cultures survived the acid challenge much better than the unadapted cultures, verifying that these strains also display an ATR. Interestingly, the adapted cultures for both virulent strains were strikingly more acid resistant than the corresponding LT2 cultures. This was also true for unadapted cultures of SL1344 and UK-1 (but only for earlier time points for the latter strain) when compared with unadapted cultures of LT2. These virulent strains appear to be naturally more acid resistant than LT2, which may account, at least in part, for their virulence. These results are consistent with a similar observation made by Garcia-del Portillo et al. (17), who also reported that strain SL1344 appeared more acid resistant than LT2.

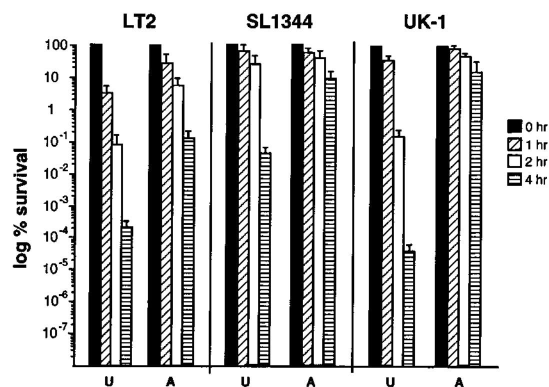

FIG. 1. ATR of avirulent (LT2) and virulent (SL1344 and UK-1) strains. The ATR was measured as described in Materials and Methods. The data represent an average of four independent trials. A, adapted cultures; U, unadapted cultures.

Effect of *atr* mutations on pathogenicity and acid tolerance in virulent *S. typhimurium* backgrounds. To assess the role of the ATR in the pathogenesis of *S. typhimurium*, we transduced mutations affecting acid resistance into the virulent strains SL1344 and UK-1. Initially, these mutations included those which confer an acid-sensitive phenotype on LT2, such as *atrB*, *atrC* (*polA*), *atrD*, and *fur*. We tested the effect of these mutations on virulence by orally infecting mice. As shown in Table 2, the *atrB* and *atrD* mutations had virtually no effect on virulence. The *atrC* and *fur* mutations resulted in an approximately 10-fold increase in the LD_{50} value. (The result obtained with the *fur* mutant is consistent with that reported previously [17], where a different *fur* mutant was shown to confer a 2-log increase in the LD_{50}.) Similar results were seen when each of these mutations was crossed into the virulent strain UK-1, except for the *fur* mutant which showed a 3-log increase in the LD_{50} (data not shown). The differences with respect to the effect of the *fur* mutation on virulence are currently under investigation.

Since these mutations had little or no effect on virulence (with the exception of the *fur* mutation in the UK-1 back-

TABLE 2. Effect of several mutations in the SL1344 strain background on peroral and intraperitoneal LD_{50}s in BALB/c mice and invasiveness in Int-407 cells

Strain	Relevant genotype	Peroral LD_{50}[a]		Intraperitoneal LD_{50}[a]	Relative level of invasion[b]
		Expt 1	Expt 2		
χ3339	Wild type	2.1×10^5	8.7×10^5	<41	1.0 ± 0
χ4715	*atrC*::Mu*d*J	1.8×10^6	—	—	1.1 ± 0.1
χ4753	*atrC*::Mu*d*1-8	9.0×10^5	—	220	0.8 ± 0.2
χ4716	*atrB*::Mu*d*J	3.9×10^5	—	120	1.1 ± 0.1
χ4717	*atrD*::Mu*d*J	1.7×10^5	—	<43	1.1 ± 0.2
χ4751	*atrD*::Mu*d*J *zxx*::Tn*10*	$<6.9 \times 10^4$	—	—	1.2 ± 0.3
χ4752	*atrB*::Mu*d*J *zxx*::Tn*10* *atrD*::Mu*d*J	4.1×10^5	—	<38	1.0 ± 0.2
χ4754	*atrC*::Mu*d*1-8 *atrD*::Mu*d*J	$>2.5 \times 10^8$	—	4.6×10^4	1.1 ± 0.2
χ4755	*atrB*::Mu*d*J *atrC*::Mu*d*1-8	$>2.2 \times 10^8$	—	3.1×10^4	1.0 ± 0.1
χ4756	*atrB*::Mu*d*J *zxx*::Tn*10* *atrD*::Mu*d*J *atrC*::Mu*d*1-8	9.9×10^7	—	—	0.9 ± 0.1
χ4659	*fur-1 zbf*::Tn*10*	1.8×10^6	—	—	0.5 ± 0.1
χ4855	*atbR*::Tn*10*	—	1.9×10^5	—	1.9 ± 0.5

[a] LD_{50} values (numbers of bacteria) were determined as described in Materials and Methods. —, experiment not performed.
[b] To compare data generated on different days, the values for tissue culture invasion are normalized and expressed as a fraction of the wild-type control value ± the standard error of the mean. The data represent an average of four trials.

ground), we examined the sensitivity of both adapted and unadapted cultures to pH 3.3. As shown in Fig. 2, when adapted, the SL1344 *atr* mutants displayed ATR profiles very similar to that of the wild-type control. The unadapted cultures appeared more sensitive to pH 3.3 than the wild-type control at some time points; however, the effect was not as dramatic as is evident with *atrB*, *atrC*, and *atrD* mutants in the LT2 background. As shown previously, the LT2 mutants are extremely acid sensitive, with at least a 5-log drop in percent survival just 2 h postchallenge at pH 3.3 for both unadapted and adapted cells (12). These data suggest that the *atr* mutations do not confer an acid-sensitive phenotype in the virulent backgrounds, i.e., SL1344 (Fig. 2) and UK-1 (data not shown).

An LT2 *fur* mutant is also extremely acid sensitive, with a 4- to 5-log drop in percent survival at 90 min postchallenge (9). We examined our SL1344 *fur* mutant, and the results indicate that it may be only slightly more acid sensitive than the wild-type parent. That is, the SL1344 *fur* mutant displayed an ATR profile nearly identical to that of the parent except that the unadapted cells appeared more acid sensitive after several hours of exposure to pH 3.3 (there was a 3-log difference at the 4-h time point). Again, this is consistent with previous data in which an SL1344 *fur* mutant was shown to be more acid sensitive than the parent but only after several hours of exposure

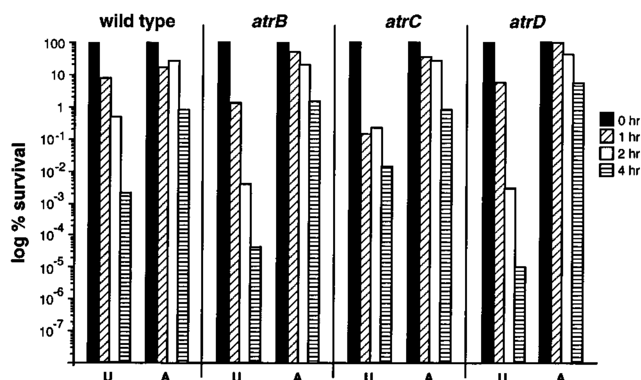

FIG. 2. ATR of SL1344 and the *atr* single mutants. The ATR was tested as described in Materials and Methods. The experiment was performed two or three times; representative data are presented. A, adapted cultures; U, unadapted cultures.

to pH 3.3 (17). The results obtained with the UK-1 *fur* mutant are consistent with those described above (data not shown).

We also assessed the effect of the *atbR* mutation on virulence in mice. In LT2, *atbR* mutants are constitutively acid resistant. Furthermore, the *atbR* gene product is involved in regulating several genes, including *atrB* (12). This mutation appeared to have only a slight effect, if any, on virulence in the SL1344 background, with an LD_{50} approximately fourfold lower than that of the wild-type control (Table 2). In the UK-1 background, there was essentially no difference in the LD_{50} between the *atbR* mutant and the wild-type control (3.4×10^4 bacteria for UK-1 and 2.3×10^4 bacteria for UK-1 *atbR*). Interestingly, these mutants do appear to be more acid resistant than their wild-type parents but only at later time points (data not shown).

Virulence and ATR of double and triple *atr* mutants. The *atr* mutations had little or no effect on virulence or acid resistance when crossed into the SL1344 or UK-1 strain background. This led us to consider that the virulent *S. typhimurium* backgrounds may have more than one pathway functioning to protect the bacterium from acid damage. Therefore, we constructed strains containing two or three different *atr* mutations (the construction of these strains is outlined in Materials and Methods). The double and triple mutants that contained an *atrC* mutation were extremely acid sensitive, particularly when the cultures were adapted (Fig. 3). Those mutants that were acid sensitive were also highly attenuated (Table 2). In particular, strains χ4754 (*atrC atrD*), χ4755 (*atrB atrC*), and χ4756 (*atrB atrC atrD*) displayed essentially no ATR, with a greater than 6-log drop in percent viability just 2 h postchallenge, for both unadapted and adapted cultures. In addition, the oral LD_{50} values for strains χ4754, χ4755, and χ4756 were 3 or more logs greater than the values of the wild-type parent. In contrast, χ4752 (*atrB atrD*), which had an LD_{50} value comparable to that of the wild type, appeared to display an ATR (Fig. 3). This strain was nearly as resistant as the wild type when adapted but still showed considerable acid sensitivity when unadapted. These data indicate that the *atrC* gene may play an important role in the ATR and in virulence. In addition, our data show that the ability to mount an effective acid tolerance response is strongly correlated with virulence. In particular, *atr* mutants that survive acid challenge in a manner comparable to that of the wild type (when acid adapted) also display an LD_{50} similar to that of the wild type.

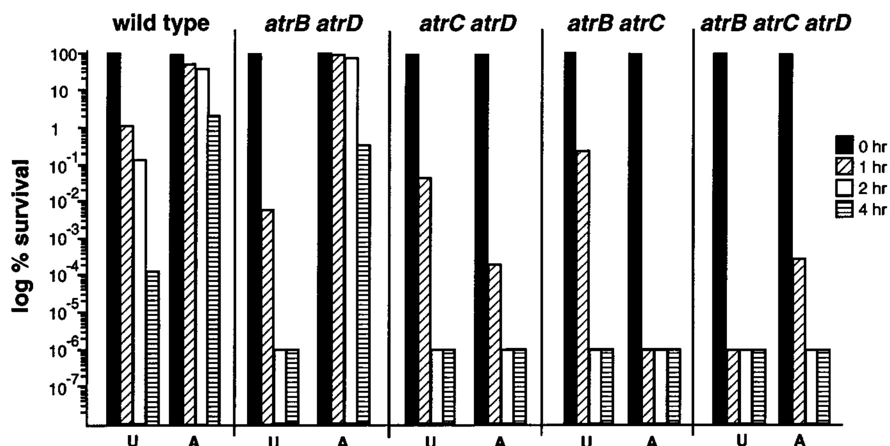

FIG. 3. ATR of the *atr* double and triple mutants. The ATR was tested as described in Materials and Methods. The experiment was performed twice; representative data are presented. A, adapted cultures; U, unadapted cultures.

Invasion capacity of the *atr* mutants. We considered that the double and triple *atr* mutants might be attenuated as a result of a decreased ability to invade intestinal cells. Therefore, the capacity of the *atr* single, double, and triple mutants to invade the intestinal epithelial cell line Int-407 was examined. In this experiment, centrifugation of the bacteria onto the monolayer was necessary because several of the *atr* mutants are less motile than the wild-type parent, and it was previously shown that a nonmotile *fli* mutant appeared to be less invasive than the wild type unless centrifuged onto the monolayer (22). As shown in Table 2, all of the *atr* mutants invaded Int-407 cells as well as the wild-type parent did. The *fur* mutant demonstrated a two-fold reduction in invasion, consistent with what has been reported previously (17).

Survival of *atr* mutants in acidic environments within the host. We wished to assess the means by which the animal host preferentially kills the acid-sensitive mutants. Acidic environments within the host in which *S. typhimurium* might find itself include the stomach and the macrophage phagolysosome. We used two approaches to test whether the ATR is important for survival within the stomach. First, the LD_{50} was determined for strain UK-1 following growth under conditions in which the ATR was induced or not induced. In particular, strain UK-1 was grown in (i) glucose E broth, (ii) glucose E broth buffered with MOPS (morpholinepropanesulfonic acid), or (iii) glucose E broth with a pH 5.8 adaptation to induce the ATR. No major differences were observed in the LD_{50} values for UK-1 grown under these different conditions. (The LD_{50} for UK-1 grown in glucose E broth was 1.9×10^5 bacteria, that in glucose E broth buffered with MOPS was 3.0×10^5 bacteria, and that in glucose E broth with pH adaptation was 1.3×10^5 bacteria.) A parallel experiment was performed with Luria broth instead of glucose E broth, and again, no significant difference was seen in the LD_{50} values for UK-1 grown under these different conditions. (The LD_{50} for UK-1 grown in Luria broth was 3.4×10^4 bacteria, that in Luria broth buffered with MOPS was 2.4×10^4 bacteria, and that in Luria broth with pH adaptations was 3.1×10^4 bacteria.)

A second approach that was used to determine whether the ATR is important for surviving gastric acidity involved coinfecting mice with strains χ3761 (UK-1) and χ4224 (UK-1 containing a mini-Tn*10* which is located on the 100-kb plasmid but which has no effect on virulence). In these experiments, one strain was grown in glucose E broth with no acid adaptation while the other was grown in glucose E broth and adapted to pH 5.8 to induce the ATR. One hour postinoculation, their ability to survive gastric acidity was ascertained by determining the number of viable *Salmonella* bacteria present in the small intestine for each strain. The data in Fig. 4 indicate that the ratio of the two strains obtained from the small intestinal contents was not significantly different from the ratio in the inoculum in each of four separate experiments.

We also assessed the ability of wild-type salmonellae and *Salmonella atr* mutants to survive within different populations of macrophages. Survivability in J774 macrophage-like cells (see Fig. 6) and bone marrow-derived macrophages from mice (see Fig. 7) and rats (see Fig. 8) was examined for 24 h following phagocytosis. Consistent with previous results (5), *Salmonella* bacteria survive well within J774 macrophages. In fact, wild-type SL1344 apparently replicates within J774 cells, since we found an approximately fourfold increase in the number of intracellular bacteria 24 h after phagocytosis (Fig. 5). The number of intracellular bacteria of an attenuated *S. typhimurium phoP* mutant, χ3689, was reduced fivefold, relative to

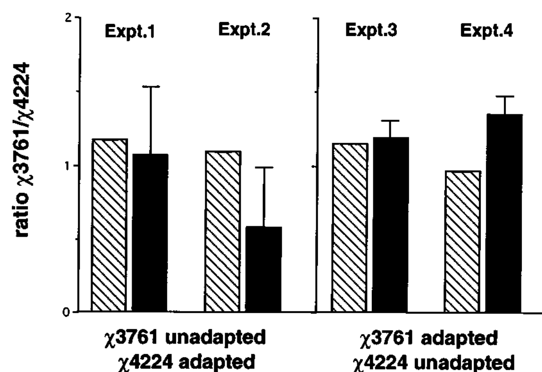

FIG. 4. Effect of acid adaptation on the ability of χ3761 and χ4224 to survive gastric acidity. Mice were coinfected with strains χ3761 and χ4224. In experiments 1 and 2, χ4224 was grown in glucose E medium and then adapted to pH 5.8 (to induce the ATR). In experiments 3 and 4, χ3761 was grown under conditions in which the ATR was induced. In each experiment, the ratio of the strains was determined for the inoculum, and for the intestinal contents 1 h after infection, by plating cells on LB agar and LB agar with tetracycline. The means given for experiments 1 and 3 are from three mice each, and the means in experiments 2 and 4 were determined with four mice each. Standard errors are shown for each experiment. Symbols: ▧, ratio of bacteria present in inoculum; ■, ratio of bacteria present in intestinal contents (mean + standard deviation).

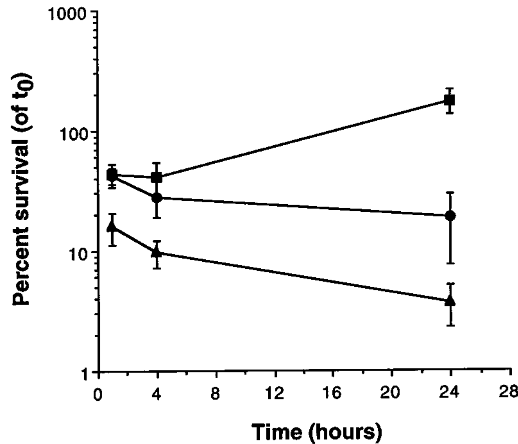

FIG. 5. Comparison of the abilities of wild-type SL1344 to survive within J774 macrophage-like cells and rat bone marrow-derived macrophages. Monolayers of J774 cells (■) (approximately 80% confluent, representing about 10^6 cells) or bone marrow-derived macrophages (about 5×10^5 cells) from mice (▲) or rats (●) were infected with opsonized bacteria at a multiplicity of infection of approximately 5 to 10. The plates were incubated for 20 min to allow phagocytosis, and then the monolayers were washed and the medium was replaced with RPMI 1640 supplemented with 5% FCS and 10 μg of gentamicin per ml. The number of viable intracellular bacteria was determined immediately (t_0) and then 1, 4, and 24 h later. For each strain at each time point, the number of bacteria recovered was expressed as a percentage of the number which was present at t_0. Values represent a mean of three or five trials in duplicate. Standard errors of the means are indicated.

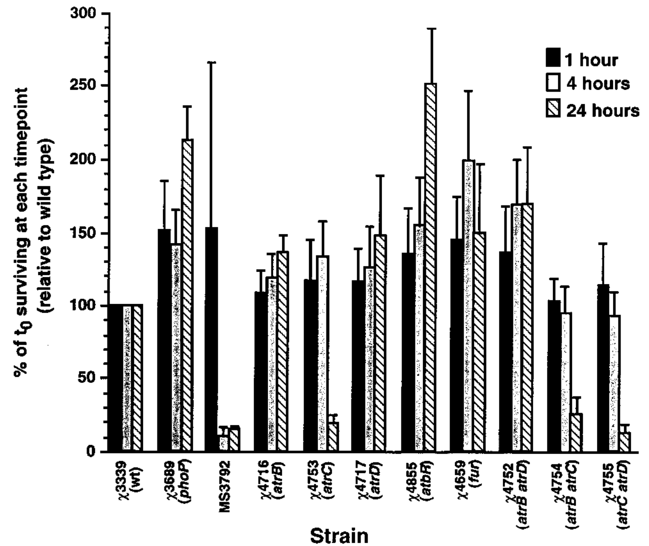

FIG. 7. Comparison of the abilities of wild-type SL1344 and several *atr* mutants to survive within mouse bone marrow-derived macrophages. The experiment was performed as described in the legend to Fig. 5. For each strain at each time point, the number of bacteria recovered was expressed as a percentage of the number which was present at t_0. To compare data from different experiments, the values for percent recovery were then normalized relative to that of the wild-type control, which was designated 100% at each time point. The results represent an average of three or four trials done in duplicate. Standard error bars are indicated.

that of the wild type, 24 h after phagocytosis. Surprisingly, the *atr* single, double, and triple mutants all appeared to survive and grow as well or better than the wild type in the J774 cells (Fig. 6).

In contrast, when we examined the ability of wild-type SL1344 to survive within bone marrow-derived macrophages, we found that the number of intracellular bacteria decreased nearly twofold between the 4- and 24-h time points within mouse macrophages and nearly threefold in rat macrophages (Fig. 5). These results are also consistent with those reported by Buchmeier and Heffron (5). As expected, the macrophage-sensitive mutant MS3792 (5) was efficiently killed by the bone marrow-derived macrophages (Fig. 7). Strains which survived less well in the bone marrow-derived macrophages, relative to the wild type, include χ4753 (*atrC*), χ4754 (*atrB atrC*), and χ4755 (*atrC atrD*). This result supports the conclusion that the *atrC* (*polA*) gene product is important for virulence; in particular, it appears to be necessary for survival within macrophages.

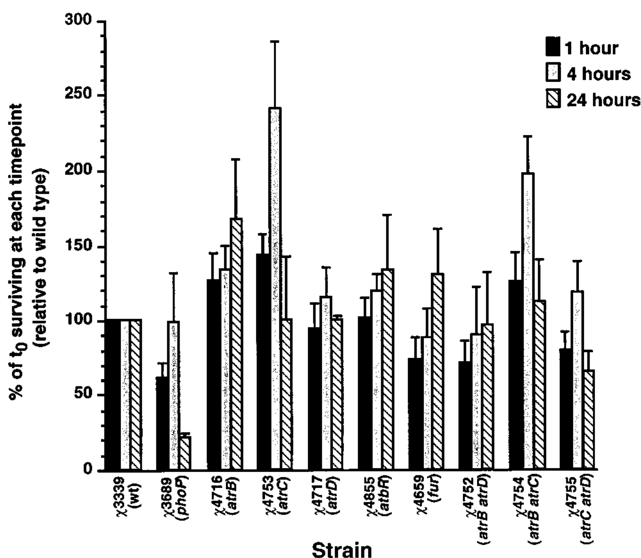

FIG. 6. Comparison of the abilities of wild-type SL1344 and several *atr* mutants to survive within J774 macrophage-like cells. The experiment was performed as described in the legend to Fig. 5. For each strain at each time point, the number of bacteria recovered was expressed as a percentage of the number which was present at t_0. To compare data from different experiments, the values for percent recovery were then normalized relative to that of the wild-type control, which was designated 100% at each time point. The results represent an average of three trials done in duplicate. Standard error bars are indicated.

Contradictory to previous reports (29), our *phoP* mutant, χ3689, survived as well as or better than the wild type within both mouse (Fig. 7) and rat (Fig. 8) macrophages. This was surprising because this strain is attenuated in mice (16). It is unknown why our *phoP* mutant appears to be resistant to these primary macrophages; however, it is possible that differences may exist with respect to the strain backgrounds that have been studied. Furthermore, the ATR profile of the SL1344 *phoP* mutant indicates that this strain may be only slightly more acid sensitive than the wild type (11). This is in contrast to what is seen with LT2 *phoP* mutants, which are extremely acid sensitive (13).

DISCUSSION

The host-pathogen interaction is a dynamic process in which an invading microbe encounters diverse and often harsh environments within the host. As a result, survival and growth in the various locations within the host require that the bacterium constantly adapt to its changing environment. In fact, there are numerous examples in the literature of virulence determinants

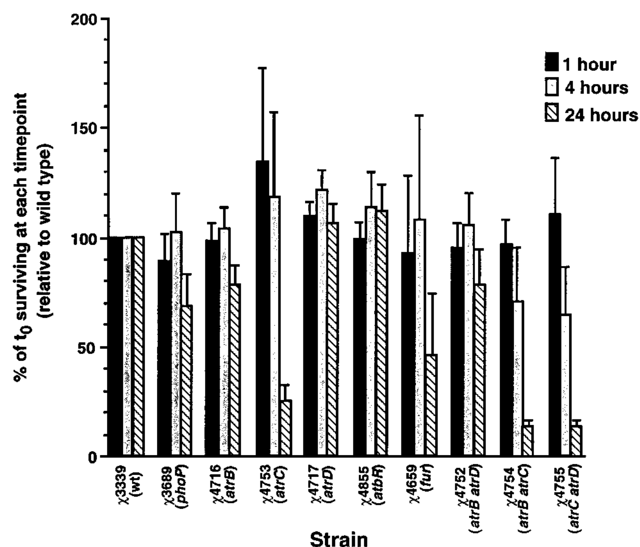

FIG. 8. Comparison of the abilities of wild-type SL1344 and several *atr* mutants to survive within rat bone marrow-derived macrophages. The experiment was performed as described in the legend to Fig. 5. For each strain at each time point, the number of bacteria recovered was expressed as a percentage of the number which was present at t_0. To compare data from different experiments, the values for percent recovery were then normalized relative to that of the wild-type control, which was designated 100% at each time point. The results represent an average of three trials done in duplicate. Standard error bars are indicated.

that are regulated in response to different environmental factors (18, 28).

We show that mouse-virulent *S. typhimurium* strains are much more acid resistant than the avirulent strain LT2. This observation is most striking when one compares the survival of cultures that have been adapted to an intermediate pH prior to acid challenge. It is tempting to speculate that the difference in sensitivity to acid pH may be responsible, at least in part, for the avirulence of LT2. In fact, we recently found a mutational difference between the LT2 and the SL1344 and UK-1 backgrounds which appears to be responsible for the increased acid sensitivity of LT2 (24). Preliminary data also indicate that this mutation, which is not *mviA* or *mviB* (2, 3), is responsible, at least in part, for the avirulence of LT2 (35).

In this report, we examined the role of the ATR in the virulence of *S. typhimurium*. Initially, mutations conferring an acid-sensitive phenotype to LT2 (including *atrB*, *atrC*, *atrD*, and *fur*) were crossed into virulent strains and their effect on acid sensitivity and virulence was assessed. Surprisingly, the ATR profiles of the transductants indicated that these strains were as resistant to acid as the wild type when the cultures were adapted. When unadapted, the *atr* mutants were somewhat more acid sensitive than the wild type; however, the effect of these mutations was much less dramatic than what is seen in the LT2 strain background (12). As indicated above (24, 35), this may be due largely to a mutational difference in the strain backgrounds. The *atrB* and *atrD* mutations had little or no individual effect on virulence as measured by oral and intraperitoneal LD_{50}s in the mouse typhoid model. The mutations which had modest effects on virulence in SL1344 (increasing the LD_{50} value 10-fold) were the *atrC* and *fur* mutations. The *atrC* gene was found to be the same as the *polA* gene which encodes DNA polymerase I (23). This polymerase functions in DNA repair, and it is known that there is a higher spontaneous mutation rate in *polA* mutants. Also, the *fur* gene product regulates a number of genes in response to iron (1). The effects which the *atrC* (*polA*) and *fur* mutations have on virulence may be related not to their role in the ATR but rather to the pleiotropic nature of these mutations. Interestingly, the degree to which the *fur* mutation affects virulence seems to be strain dependent (a 1-log increase in the LD_{50} with the SL1344 mutant and a 3-log increase with the UK-1 mutant). We have also found that independent UK-1 *fur* isolates behave differently with respect to overproduction of enterochelin and acid sensitivity. The reason for these differences is unknown, although it is possible that a mutation linked to the *fur* gene and involved in virulence may have been transduced into some of the *fur* mutants but not others. Alternatively, *fur* mutants may also accumulate additional mutations. These possibilities are currently under investigation.

The *atrB* and *atrD* mutations are in genes of unknown function; however, the map location of the *atrD* gene suggests that the Mu*d* insertion may be in the *exb* operon whose products are involved in iron uptake (12). An additional mutation, *atrF*, also appears to have no effect on acid resistance or virulence in the SL1344 background on the basis of preliminary data. Similarly, this mutation appears to map within an operon involved in iron uptake, *fep* (12). Consistent with the mapping data, the *atrD* and *atrF* mutants produce brownish colonies, indicating that these cells overproduce the siderophore enterochelin. A possible link between acid resistance and iron acquisition and iron-regulated gene expression is also currently under investigation.

Our results obtained with the *atbR* mutants were somewhat surprising. Because these mutants are more acid resistant that the wild-type parents at later time points, we anticipated that they would also be more virulent. In fact, only a small increase in the LD_{50} was seen, and this was only observed for the SL1344 background. Apparently, the nominal increase in acid resistance has little effect on the ability of the mutant to colonize and cause systemic disease, at least by the means which we have tested. Because strains UK-1 and SL1344 are more acid resistant than LT2 and phenotypically similar to an LT2 *atbR* mutant, it is possible that the virulent strains differ genetically from LT2 at this locus.

Strains containing two or more *atr* mutations were constructed, and it was found that the double and triple mutants containing an *atrC* mutation were extremely acid sensitive and unable to mount an ATR. These strains were also completely attenuated in mice, indicating a correlation between the ability to mount an ATR and virulence. We used several approaches to determine why the acid-sensitive mutants are attenuated. We examined the ability of the various *atr* single, double, and triple mutants to invade cells in culture and showed that all of the mutants invade as well as the SL1344 parent does. The ability of the various mutants to survive within different populations of macrophages was also assessed. We found that there were no differences in the survivability of the various strains within J774 macrophages. Only the *phoP* control appeared more sensitive. Because the *atrB atrC* and *atrC atrD* mutants are extremely acid sensitive in vitro, this suggests that J774 macrophages are incapable of effectively killing *Salmonella* bacteria with low pH. In contrast, when survival in bone marrow-derived macrophages was examined, it was found that the *atrC*, *atrB atrC*, and *atrC atrD* mutants survived less well than the wild-type parent did. It is unlikely that the inability of these mutants to survive within macrophages is attributable entirely to acid sensitivity because the *atrC* mutant appears to be nearly as acid resistant as the wild-type parent. Furthermore, macrophages use other means to kill bacteria such as defensins and reactive oxygen radicals. The *atrC* (*polA*) mutant

and presumably the *atrB atrC* and *atrC atrD* mutants would likely be more sensitive to these DNA-damaging molecules.

The LD_{50} of an *atrC* mutant is about 1 log higher than that of the wild type. This modest attenuation is probably due to the decreased ability of the *atrC* mutant to survive within macrophages. The inability to survive within macrophages also is responsible, at least in part, for the avirulence of the *atrB atrC* and *atrC atrD* double mutants. However, because the latter strains are completely attenuated (displaying an LD_{50} at least 2 logs higher than that of the *atrC* mutant alone), we believe that these mutants are blocked at another stage of infection. Because the *atrB atrC* and *atrC atrD* double mutants are unable to mount an ATR, it is possible that these mutants are killed by low pH at another site within the animal host. It is unlikely that these strains are simply more sensitive to gastric acidity, however, because experiments determining the LD_{50} values following intraperitoneal inoculation show that the acid-sensitive double mutants are highly attenuated even when the gastric acidity barrier is circumvented. Furthermore, we were unable to demonstrate a role for the ATR in the survival of wild-type *S. typhimurium* in the stomach.

In this study, we examined the survival of *S. typhimurium* in both mouse and rat bone marrow-derived macrophages. Figure 5 shows that macrophages from mice are two- to fourfold more effective at killing *S. typhimurium* than those isolated from rats. However, macrophages from both sources appear to kill with similar kinetics. Other investigators may be interested in using rats as the source of bone marrow-derived macrophages because these primary cells kill *S. typhimurium* effectively. They are also more easily obtained from and more abundant in rats because rats have larger bones than mice. As a consequence, fewer animals need to be used in each study, saving time, money, and animals' lives.

ACKNOWLEDGMENTS

We thank Daniel Piatchek and Jack Diani for assistance with the animal experiments and Teresa Doggett for technical assistance and helpful discussions.

This work was supported by National Institutes of Health grant AI24533.

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Editor: A. O'Brien