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An Altered *rpoS* Allele Contributes to the Avirulence of *Salmonella typhimurium* LT2

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Virulent *Salmonella typhimurium* strains differ from the attenuated laboratory strain LT2 at the *rpoS* locus. It was previously shown that the *rpoS* gene in strain LT2 contains a rare UUG start codon (I. S. Lee, J. Lin, H. K. Hall, B. Bearson, and J. W. Foster, Mol. Microbiol. 17:155–167, 1995). This difference is responsible for the inability of LT2 to display a sustained log-phase acid tolerance response. We show that the altered *rpoS* allele (*rpoS*^{LT2}) also affects the stationary-phase acid tolerance response in *Salmonella*. By transducing the *rpoS*^{LT2} allele into virulent strain backgrounds and crossing wild-type *rpoS* allele into strain LT2, we demonstrate that the *rpoS*^{LT2} allele contributes to the attenuation of strain LT2. We examined the effect of the *rpoS* allele on invasion and found that the *rpoS* status of the cell had no effect on the ability of the strains to invade intestinal epithelial cells in tissue culture. Enumeration of bacteria from tissues of infected mice indicated that the presence of the *rpoS*^{LT2} allele affected the ability of *S. typhimurium* to reach the liver and spleen and to persist in several tissues at 6 days postinfection. This is likely due, at least in part, to a decrease in *spv* gene expression in these mutants. We demonstrate that strains containing the *rpoS*^{LT2} allele are not only sensitive to pH 3.0 (acid stress) but are also sensitive to the DNA-damaging agent methyl methanesulfonate. However, these strains appear to survive stationary-phase and oxidative stresses as well as strains containing a wild-type *rpoS* allele. Despite an increased sensitivity to acid stress and DNA damage, strains containing either an *rpoS*-null mutation or the *rpoS*^{LT2} allele survived in J774 cells and bone marrow-derived macrophages as well as did otherwise isogenic strains with a wild-type *rpoS* allele.

Members of the genus *Salmonella* are of clinical importance because they are responsible for causing a diversity of illnesses in a wide range of hosts including humans. *Salmonella enterica* serotypes such as *Salmonella typhimurium* generally cause a food-borne infection that results in a limited gastroenteritis (23). However, in some individuals the infection can become systemic and is often life-threatening (23).

The significance of bacterial growth rate and growth phase both prior to and during the course of infection has been the subject of several studies (2, 5, 10, 19, 25, 26). In vitro, entry of the bacterium into stationary phase is accompanied by physiological changes which enable the cell to survive a wide variety of environmental stresses (29). *rpoS* mutants of *S. typhimurium* and *Escherichia coli* display a rapid loss of viability during prolonged starvation (11, 29). They are also highly sensitive to a number of stresses including near-UV radiation, elevated temperature, high osmolarity, low pH, and hydrogen peroxide (29). The *rpoS* gene encodes a stationary-phase sigma factor (σ^s) (33) which is responsible for control of a regulon of over 30 genes (29). As a consequence of the pleiotropic nature of *rpoS* mutants, this gene has been characterized independently by a number of laboratories and is known by several other gene designations including *katF* (for regulation of the catalase hydroperoxidase II), *nur* (for near-UV sensitivity), *appR* (for regulation of *appA*), *csi-2* (for carbon starvation inducible), and *abrD* (for *aidB* regulatory gene) (see reference 29 and references therein). The *rpoS* gene product, σ^s, has been shown to bind to core RNA polymerase and to enhance transcription (30). Due to its striking similarity to the vegetative sigma factor, σ^{70}, and to the fact that many promoters appear to be regulated by both σ^s and σ^{70}, it is believed that similar binding sequences are recognized by the two sigma factors (30, 41). It has been shown that considerable heterogeneity exists in the *rpoS* gene in *E. coli* (24, 41). Furthermore, Robbe-Saule et al. showed that a live oral *Salmonella typhi* vaccine strain, Ty21a, is an *rpoS* mutant (38).

Several groups have shown that transcription of the *Salmonella spv* genes is enhanced upon entry into stationary phase or when cells are nutrient deprived (1, 12, 42). This induction requires the *rpoS* gene product (8, 21). The *spv* locus is located on the *Salmonella* virulence plasmid and consists of a four-gene operon, *spvABCD*, which encodes proteins of unknown function (20). This operon is regulated positively by the product of the *spvR* gene which is located immediately upstream (7, 12). The 8-kb *spv* locus present on the *S. typhimurium* virulence plasmid is essential for these strains to cause a systemic infection (17, 45). Recently, the presence of the virulence plasmid and the *spv* locus has been shown to increase the growth rate of *S. typhimurium* within host cells (19).

Fang et al. constructed an *S. typhimurium rpoS* mutant by insertional inactivation of the gene using a suicide vector (11). They reported that a mutant containing an inactive *rpoS* allele is sensitive to a variety of environmental stresses and is attenuated in mice. In addition, expression of an *spvB-lacZ* fusion is reduced in their mutant. They postulated that the *rpoS* mutant may be attenuated due to increased sensitivity to environmental conditions which are present in the phagosomal environment of a host macrophage (such as low pH or oxidative stress) as well as to decreased levels of *spv* expression.

The acid tolerance response (ATR) is a complex phenomenon in which exposure to slight or moderate acid stress results in the synthesis of proteins which protect the bacterium against a severe acid challenge (13). Several studies have reported

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Background and/or genotype	Reference or source
Strains		
χ3000	LT2	18
χ3339[a]	SL1344/*rpsL hisG xyl*	18
χ3689	SL1344/*rpsL hisG xyl phoP12*	15
χ3761	UK-1	9
χ4996	LT2/*rpoS*::Ap	This study
χ4997	UK-1/*rpoS*::Ap	This study
χ4998	UK-1/*rpoS*^{LT2} *zgd-5178*::Tn*10*(dTc)	This study
χ4999	LT2/*rpoS*$^{UK-1}$ *zgd-5178*::Tn*10*(dTc)	This study
χ8000	LT2/*rpoS*^{SL1344} *zgd-5181*::Tn*10*(dTc)	This study
χ8012	SL1344/*rpsL hisG xyl rpoS*^{LT2} *zgd-5178*::Tn*10*(dTc)	This study
JF2690	UK-1/*rpoS*::Ap	27
JF2736	LT2/*rpoS*$^{UK-1}$ *zgd-5178*::Tn*10*(dTc)	27
JF2782	LT2/*rpoS*^{SL1344} *zgd-5181*::Tn*10*(dTc)	27
Plasmids		
pGTR075	*tet-lacZ*	7
pGTR311	*spvR spvA-lacZ*	39

[a] Strain χ3339 is a mouse-passaged derivative of SL1344, the latter being obtained from B. A. D. Stocker.

differences in comparing the ATR of virulent strains of *S. typhimurium* with that of the laboratory strain LT2 (16, 27, 46). When Lee et al. examined this phenomenon more closely, they found that LT2 displayed a transient log-phase ATR while the virulent strains displayed a sustained ATR (27). More specifically, LT2 cells acid shocked at pH 4.3 for 20 to 40 min developed tolerance to a challenge with pH 3.3, while a longer period of adaptation (i.e., 60 to 120 min) provided no protection. That is, these cells were rapidly killed when challenged with pH 3.3. On the other hand, virulent strains such as UK-1, SL1344, and 14028S become acid tolerant regardless of the length of adaptation to a moderate acid stress (i.e., pH 4.3 for 30 to 120 min). In fact, increasing the length of time of adaptation to pH 4.3 resulted in enhanced acid resistance for the virulent strains. Lee et al. demonstrated that this sustained ATR requires a wild-type *rpoS* allele and that strain LT2 contains an altered *rpoS* allele (*rpoS*^{LT2}) which is responsible for its inability to exhibit a sustained ATR (27). DNA sequence analysis showed that the *rpoS*^{LT2} allele included a rare UUG start codon.

In this study, we demonstrate that the *rpoS* allele from strain LT2 contributes to the attenuation of this strain. Enumeration of bacteria from infected mice showed that, unlike wild type, a strain containing the *rpoS*^{LT2} allele failed to reach the liver and spleen. This is likely due, at least in part, to a decrease in *spv* gene expression. We demonstrate that strains containing the *rpoS*^{LT2} allele are more sensitive to certain environmental stresses than is wild type in vitro. Surprisingly, despite their increased sensitivity to acid stress and DNA damage, strains containing either an *rpoS*-null mutation or the *rpoS*^{LT2} allele survived in macrophages as well as did otherwise isogenic strains with a wild-type *rpoS* allele.

MATERIALS AND METHODS

Bacterial strains, plasmids, phenotypic screens, and media. *S. typhimurium* strains listed in Table 1 are derivatives of the virulent strain UK-1 or SL1344 or derivatives of the attenuated laboratory strain LT2. Plasmids used in this study are listed in Table 1. Strains generated in this study were constructed by standard P22 HT*int* transduction (40). The *rpoS* insertion mutation (*rpoS*::Ap [11]) present in JF2690 (27) was transduced into other strains selecting for the ampicillin resistance marker. The *rpoS*$^{UK-1}$ and *rpoS*^{LT2} alleles were transduced by using a linked Tn*10* insertion originally from JF2736 (*zgd-5178*::Tn*10*), and the *rpoS*^{SL1344} allele was transduced by using a linked Tn*10* insertion originally from JF2782 (*zgd-5181*::Tn*10*). Tetracycline-resistant (Tc^r) transductants were screened for their RpoS phenotype by assaying catalase activity. This was done by adding 0.1 ml of 30% hydrogen peroxide (H_2O_2) to 1.0 ml of a stationary-phase culture in a 13- by 100-mm culture tube. The tube was vortexed briefly, after which the height of the bubbling was measured after 1 min. Strains containing the *rpoS*$^{UK-1}$ or *rpoS*^{SL1344} allele showed a large amount of bubbling, strains containing the *rpoS*::Ap mutation showed no bubbling, and strains containing the *rpoS*^{LT2} allele demonstrated an intermediate level.

Standard media including Lennox broth (28), Luria-Bertani broth (31), glucose E medium (44), and MacConkey agar (Difco, Detroit, Mich.) were used for cultivation of bacteria. Difco agar was added to Lennox broth at 1.5% for base agar (L agar), and MacConkey agar was supplemented with lactose at 1.0%. Antibiotics (from Sigma, St. Louis, Mo.) were added to media at the indicated concentrations: tetracycline, 15 μg/ml; ampicillin, 25 μg/ml; and chloramphenicol, 12.5 μg/ml.

Measuring β-galactosidase activity. The protocol for measuring β-galactosidase activity was described previously (32). Activity is expressed in Miller units.

Stress survival assays. Cultures of *S. typhimurium* were evaluated for their ability to survive acid stress, prolonged stationary phase, oxidative stress, and DNA damage. To assay sensitivity to acid, 1.0 ml of cells from an overnight culture grown in glucose E medium (pH 7.7) was pelleted and resuspended in 1.0 ml of glucose E medium (pH 3.0). Cells were diluted to approximately 2×10^8 in glucose E medium (pH 3.0), and then samples were removed immediately (t_0) and at timed intervals. At each time point, cells were diluted in buffered saline with gelatin (BSG) to determine CFU per milliliter. To assay stationary-phase survival, overnight cultures were diluted 1:100 in Lennox broth, followed by growth with aeration at 37°C for approximately 6 h. At this time, samples were removed to determine CFU per milliliter (t_0). Viable cell counts were determined at 24-h intervals for 5 additional days. To measure susceptibility to oxidative stress, H_2O_2 (at a final concentration of 25 mM) was added to cultures grown overnight in Lennox broth. Samples were removed at t_0 and at 20-min intervals for 1 h following challenge. Sensitivity to DNA damage was evaluated by adding methyl methanesulfonate (MMS; final concentration, 15 mM for UK-1 derivatives or 30 mM for SL1344 or LT2 derivatives) to cells grown overnight in Lennox broth. Quantification of viable cells was determined immediately prior to and for 90 min after addition of MMS. For each assay, percent survival is the number of CFU per milliliter present at each time point divided by the number of CFU per milliliter present at t_0.

Animal studies. Fifty percent lethal doses (LD_{50}s) for the *S. typhimurium* strains were determined following peroral or intraperitoneal inoculation of BALB/c mice (Harlan Sprague-Dawley, Indianapolis, Ind.) as described previously (37, 46).

To compare the abilities of *S. typhimurium* strains χ3761 and χ4998 to colonize mice, cells were grown in Luria broth to late log phase (optical density at 600 nm of 0.8 to 1.0). Cells were then pelleted and resuspended in BSG. Cell suspensions were mixed, and mice were infected perorally with approximately 3×10^8 CFU (total) at a ratio of χ3761/χ4998 of approximately 1.0. At 1 h and at 6 days following infection, four mice were euthanized and the small intestinal wall, Peyer's patches, small intestinal contents, liver, and spleen were removed and placed in 2.0 ml of BSG. Samples were homogenized, and bacterial counts were determined on MacConkey agar and MacConkey agar containing tetracycline.

Tissue culture assays. Intestine 407 (Int-407) (22) cells were used for tissue culture invasion assays using a modification of the procedure of Galan and Curtiss (14) as described previously (46).

The in vitro assay used to determine the ability of *Salmonella* to survive within bone marrow-derived macrophages (from either BALB/c mice or Sprague-Dawley rats) or J774 macrophage-like cells (36) was modified slightly from that developed by Buchmeier and Heffron (6) as described previously (46).

RESULTS

Effect of an altered *rpoS* allele on acid resistance. Virulent *S. typhimurium* strains display a sustained ATR in which prolonged exposures of log-phase cells to moderate acid conditions (up to 2 h at pH 4.3) result in a gradual increase in the ability of cultures to survive a challenge at pH 3.3. In contrast, *S. typhimurium* LT2 exhibits an ATR which is transient. Short exposures to a moderate acid shock (pH 4.3 for 20 to 40 min) protect cells from acid challenge, whereas longer exposures to acid shock provide no protection. Lee and coworkers reported that this difference is attributable to an altered *rpoS* allele which is present in strain LT2 (27).

We constructed a series of strains similar to those made by Lee et al., in which the *rpoS* allele from LT2 (*rpoS*^{LT2}) was

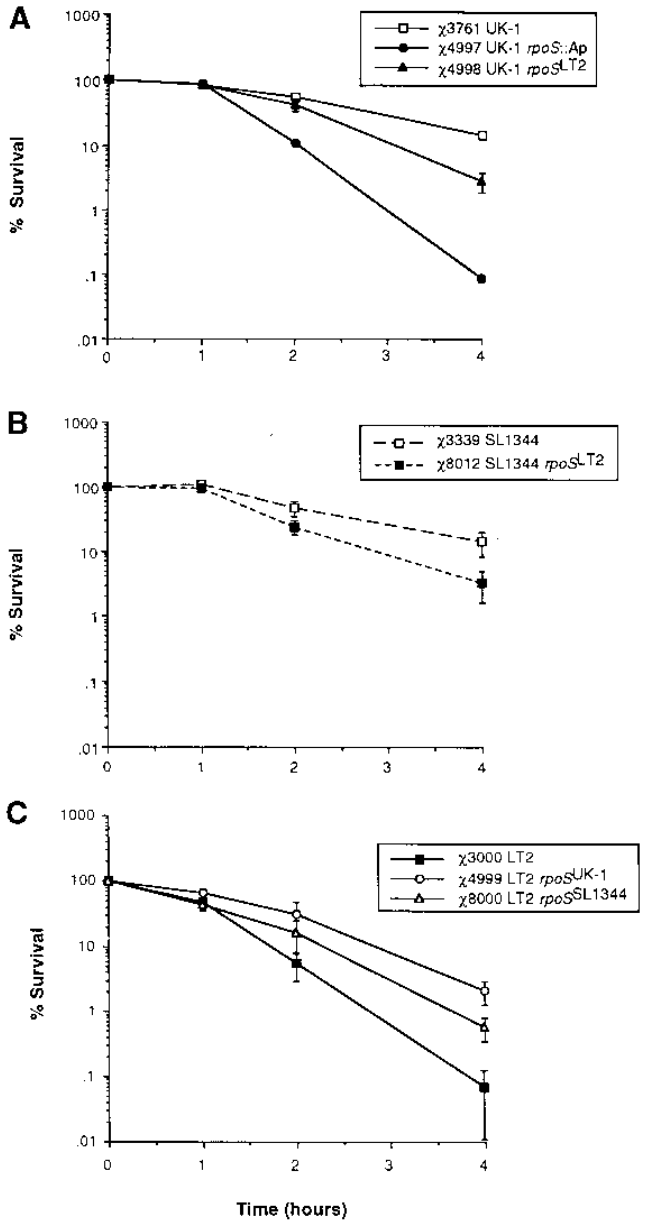

FIG. 1. Survival of stationary-phase *S. typhimurium* UK-1 (A), SL1344 (B), and LT2 (C) derivatives at pH 3.0. The experiment was performed as described in Materials and Methods. Values represent averages of three to five trials. Standard errors are given. Strains containing an *rpoS*::Ap or an $rpoS^{LT2}$ allele survived acid stress significantly less well at 4 h postchallenge than an otherwise isogenic strain containing a wild-type *rpoS* allele ($P \leq 0.05$).

crossed into the virulent strains UK-1 and SL1344. Conversely, we transduced the *rpoS* alleles from UK-1 and SL1344 ($rpoS^{UK-1}$ and $rpoS^{SL1344}$, respectively) into the LT2 background. *rpoS*-null mutants were constructed in the LT2 and UK-1 backgrounds as well. The effect of the *rpoS* allele on stationary-phase acid resistance was evaluated with these strains. Consistent with previous data (11), a strain containing an *rpoS*::Ap insertion is much more acid sensitive than its wild-type parent. As shown in Fig. 1A, there was nearly a 3-log difference in the survival of strain UK-1 (χ3761) compared to its *rpoS*::Ap derivative (χ4997) at 4 h postchallenge. The $rpoS^{LT}$ allele also appeared to affect the ability of stationary-phase cells to survive acid challenge; however, the differences were not as dramatic. That is, χ4998 survived acid challenge fivefold less well than χ3761. Similarly, the $rpoS^{LT}$ mutant in the SL1344 strain background was approximately fivefold more sensitive to pH 3.0 than wild-type SL1344 at the 4-h time point (Fig. 1B). We examined the effect of these alleles in the LT2 background and found that the presence of an *rpoS* allele from a wild-type virulent strain increased the ability of LT2 to survive acid stress (Fig. 1C; compare χ3000 with χ4999 or χ8000). Interestingly, χ4999 ($rpoS^{UK-1}$) appears consistently more acid resistant than χ8000 ($rpoS^{SL1344}$) at 4 h postchallenge ($P < 0.05$). This is surprising because DNA sequence analysis indicated that the *rpoS* alleles from UK-1 and SL1344 are identical (27). We considered that the difference in acid sensitivity between χ4998 and χ8000 may be attributable to the fact that they contain different Tn*10* insertions. However, this is unlikely, as neither Tn*10* insertion affected acid sensitivity in the virulent strain backgrounds (data not shown). Although strains χ4999 and χ8000 show differences in their susceptibility to acid at the later time point, they behave similarly in all other assays in this study, including assays for virulence.

The effect of the $rpoS^{LT2}$ allele on pathogenicity. To assess the role of the $rpoS^{LT2}$ allele on virulence, we infected mice perorally with the strains described above. As shown in Table 2, the presence of the $rpoS^{LT2}$ allele results in a severe attenuation of strains UK-1 and SL1344. Strains containing this *rpoS* mutation in either background demonstrated an oral LD_{50} 3 logs higher than that of their respective parents. In fact, χ4998 (UK-1 $rpoS^{LT2}$) displayed an LD_{50} comparable to that of χ4997 (UK-1 *rpoS*::Ap). Consistent with these data, we found that the *rpoS* alleles from UK-1 and SL1344 increased the virulence of strain LT2. The LD_{50}s for strains χ4999 and χ8000 were at least 1 to 2 logs lower than χ3000. The actual differences may in fact be much greater, because we were unable to determine an exact LD_{50} for χ3000 due to its inability to kill mice. As expected, crossing the $rpoS^{UK-1}$ or $rpoS^{SL1344}$ allele into strain LT2 did not restore this strain to full virulence (compare LD_{50}s for χ4999 and χ8000 with those for χ3761 and χ3339, respectively). This is likely due to the presence of an additional attenuating mutation(s) in the LT2 strain background including, but perhaps not limited to, *mviA* (4).

We considered that strains containing the $rpoS^{LT2}$ allele might be attenuated due to increased sensitivity to gastric acidity. To test this possibility, the LD_{50}s were determined for the *rpoS* mutants following intraperitoneal inoculation. As shown in Table 2, derivatives of UK-1 and SL1344 containing the $rpoS^{LT2}$ allele are attenuated even when the gastric acidity barrier is circumvented. The $rpoS^{LT2}$ mutants have intraperitoneal LD_{50}s about 2 logs higher than those of the wild-type parents. These data suggest that the $rpoS^{LT2}$ mutants are preferentially killed at a site within the animal other than the stomach. LT2 strains containing a wild-type *rpoS* allele were more virulent than LT2 alone; however, the differences were only slight in this background. A larger decrease in the LD_{50} may be difficult to determine in the LT2 background due to the presence of other attenuating mutations.

We examined whether *rpoS* mutants are attenuated due to their inability to invade through the small intestine. This could occur if σ^s was involved in regulating *Salmonella* invasion genes. Strain LT2 has been shown to be much less invasive in tissue culture than virulent strains such as SL1344 and UK-1 (14) (Table 2). However, as shown in Table 2, the *rpoS* status of the cell had no significant effect on the ability of the strains to invade Intestine-407 cells.

The effect of the $rpoS^{LT2}$ allele on *spv* expression and the ability of *Salmonella* to reach deep tissues. The *spvABCD*

TABLE 2. Effects of the different *rpoS* alleles in several strain backgrounds on virulence in mice and on invasion of intestinal epithelial cells

Strain	Background	*rpoS* allele	Peroral LD_{50}[a]	Intraperitoneal LD_{50}	% Invasion[b]
χ3761	UK-1	$rpoS^{UK-1}$	1.3×10^5	<113	10.8 ± 1.6
χ4996	UK-1	*rpoS*::Ap	2.7×10^8	NT[c]	7.0 ± 2.0
χ4998	UK-1	$rpoS^{LT2}$	3.4×10^8	3.0×10^4	6.8 ± 0.3
χ3339	SL1344	$rpoS^{SL1344}$	7.6×10^5	<65	16.9 ± 6.2
χ8012	SL1344	$rpoS^{LT2}$	$>7.0 \times 10^8$	2.6×10^3	11.9 ± 2.5
χ3000	LT2	$rpoS^{LT2}$	$>6.8 \times 10^8$	3.6×10^4	0.6 ± 0.2
χ4999	LT2	$rpoS^{UK-1}$	7.9×10^6	1.7×10^4	0.3 ± 0.04
χ8000	LT2	$rpoS^{SL1344}$	1.7×10^7	4.1×10^3	0.6 ± 0.1

[a] LD_{50}s were determined with BALB/c mice as described in Materials and Methods.
[b] The data represent an average of four trials. Standard errors are given.
[c] NT, not tested.

operon which is required for *Salmonella* to cause a systemic infection in mice is positively regulated by SpvR in response to growth phase (8). Fang et al. reported that stationary-phase expression of an *spvB-lacZ* fusion was reduced 86% in a strain with an *rpoS*::Ap insertion mutation compared with the wild-type parent (11). To study the effect of the different *rpoS* alleles on expression of the *spv* operon, we used the plasmid pGTR311, which contains an *spvA-lacZ* fusion as well as a wild-type copy of the *spvR* gene (39). This plasmid was introduced into strains containing different *rpoS* alleles in both the UK-1 and LT2 strain backgrounds. In the UK-1 background, stationary-phase *spvA* expression was reduced by 68% in the *rpoS*::Ap mutant compared to wild type (Table 3). The *spvA-lacZ* fusion was expressed at an intermediate level in the $rpoS^{LT2}$ mutant (a 34% reduction compared to wild type). No significant effect of the *rpoS* allele in UK-1 was seen on expression of the *spvA-lacZ* fusion in log-phase cells or on the expression of a *tet-lacZ* fusion in stationary-phase or log-phase cells. A similar pattern of expression of the *spvA-lacZ* fusion was observed in stationary-phase cells of the LT2 background. Strains χ4996 (*rpoS*::Ap) and χ3000 ($rpoS^{LT2}$) displayed an 83 and 58% reduction in expression of *spvA-lacZ*, respectively, compared with χ4999 ($rpoS^{UK-1}$). Interestingly, we found that *spvA* gene expression in the LT2 background was regulated to some extent by σ^s in log-phase cells as well. The significance of this is unknown.

Data presented in Table 2 indicate that *S. typhimurium* $rpoS^{LT2}$ mutants are severely attenuated in mice. Furthermore, *spv* gene expression is reduced in $rpoS^{LT2}$ mutants. In order to determine if the $rpoS^{LT2}$ mutants are capable of colonization and of conferring a systemic infection, mice were coinfected with χ3761 (UK-1) and χ4998 (UK-1 $rpoS^{LT2}$) at a ratio of χ3761/χ4998 of approximately 1.0. Enumeration of live bacteria from the intestinal contents and intestinal wall (with Peyer's patches included) just 1 h postinfection showed that χ3761 and χ4998 survived passage through the stomach equally well. That is, the CFU of χ3761 and χ4998 obtained from these tissues were similar in mice from three different experiments (Table 4). This result supports the data indicating that preferential killing of strains containing the $rpoS^{LT2}$ allele occurs at a site within the animal other than the stomach.

We also determined the CFU for the two strains in several different tissues 6 days following inoculation, a time at which wild-type *Salmonella* is capable of conferring a systemic infection in most mice. As shown in Table 4, large differences were observed in the recovery of χ3761 and χ4998. In particular, χ3761 colonized livers and spleens much better than χ4998, with a greater than 3-log difference in the recovery of these strains. These results are consistent with the decrease in *spvA* expression reported above. χ3761 was also recovered from the Peyer's patches and intestinal walls of mice at significantly higher numbers than χ4998. The reasons for this are unknown, although these differences may reflect a secondary infection of the intestine following systemic spread. Alternatively, it is possible that the $rpoS^{LT2}$ mutant may invade the Peyer's patches and intestinal wall less well than its wild-type parent. However, the in vitro invasion studies showed no difference with respect to the abilities of these strains to invade intestinal epithelial cells.

Effect of the $rpoS^{LT2}$ allele on the ability of strains to survive environmental stresses. It has previously been shown that an *S. typhimurium rpoS* insertion mutant survives conditions mim-

TABLE 3. Effects of different *rpoS* alleles on expression of an *spv-lacZ* fusion

Strain	Background and *rpoS* allele	β-Galactosidase activity[a]			
		Stationary phase		Log phase	
		spvA-lacZ	*tet-lacZ*	*spvA-lacZ*	*tet-lacZ*
χ3761	UK-1/$rpoS^{UK-1}$	1,057 ± 96	147 ± 28	58 ± 19	83 ± 3
χ4997	UK-1/*rpoS*::Ap	335 ± 38	145 ± 28	38 ± 4	106 ± 22
χ4998	UK-1/$rpoS^{LT2}$	696 ± 141	173 ± 33	50 ± 13	87 ± 14
χ3000	LT2/$rpoS^{LT2}$	1,174 ± 122	167 ± 9	101 ± 21	138 ± 35
χ4996	LT2/*rpoS*::Ap	488 ± 72	167 ± 31	74 ± 12	141 ± 33
χ4999	LT2/$rpoS^{UK-1}$	2,792 ± 475	218 ± 38	322 ± 58	172 ± 14

[a] β-Galactosidase activity was measured from strains containing pGTR075 (*tet-lacZ*) or pGTR311 (*spvR spvA-lacZ*). Activity is expressed as Miller units, and values represent averages of three trials. Standard errors are given.

TABLE 4. Recovery of *S. typhimurium* from mice after oral inoculation with a mixture of χ3761 (UK-1) and χ4998 (UK-1 $rpoS^{LT2}$)[a]

Time postinoculation	Tissue	Mean paired difference[b]	*P* value[c]
1 h	Intestinal contents	−0.07 ± 0.44	NS
	Intestinal wall[d]	−0.05 ± 0.18	NS
6 days	Intestinal contents	0.39 ± 1.2	NS
	Intestinal wall	1.22 ± 1.15	<0.005
	Peyer's patches	1.82 ± 1.55	<0.005
	Liver	3.56 ± 1.30	<0.005
	Spleen	3.16 ± 1.61	<0.005

[a] In three separate experiments, mice were inoculated with a mixed suspension of cells at a ratio (χ3761/χ4998) of approximately 1.0. At the indicated times, four mice were euthanized and bacterial counts were determined for the various tissues and organs as described in Materials and Methods.

[b] Mean of the paired differences ($\log_{10}$ CFU of strain χ3761 − $\log_{10}$ CFU of χ4998) ± standard deviation ($d \pm s_d$).

[c] *P* values are given if the mean of the paired differences is >0. The test statistic (t) was calculated as follows: $t = \frac{d-0}{s_d\sqrt{n}}$. NS, not significant ($P > 0.1$).

[d] At 1 h postinoculation, intestinal wall contained the Peyer's patches intact.

icking those found within the macrophage phagolysosome (such as starvation, acid stress, oxidative stress, and DNA damage) considerably less well than a strain containing a wild-type *rpoS* allele (11). When we examined stationary-phase acid resistance, we found that strains containing an $rpoS^{LT2}$ allele survived acid stress less well than strains with a wild-type *rpoS* allele. However, loss in viability in the $rpoS^{LT2}$ mutants was not as great as was observed with an *rpoS*::Ap mutant, indicating an intermediate level of acid sensitivity (Fig. 1).

We examined the ability of strains containing the $rpoS^{LT2}$ allele to survive additional environmental stresses including prolonged stationary phase (starvation), challenge with H_2O_2 (oxidative stress), and challenge with MMS (DNA damage). Consistent with the results of Fang et al. (11), our *rpoS*::Ap mutant survived stationary phase less well than wild type, with an eightfold reduction in the percentage of organisms recovered after 5 days of incubation in Lennox broth at 37°C (data not shown). In contrast, each strain containing the $rpoS^{LT2}$ allele survived as well as an otherwise isogenic strain with an *rpoS* allele from UK-1 or SL1344 throughout the entire experiment (data not shown). Similar results were obtained when we examined the sensitivity of our strains to oxidative damage. The strain containing the *rpoS*::Ap allele was significantly more sensitive to hydrogen peroxide, whereas strains containing the $rpoS^{LT2}$ allele were as sensitive as otherwise isogenic strains containing the wild-type *rpoS* allele (data not shown). In contrast, the $rpoS^{LT2}$ allele did appear to have an effect on the ability of strains to survive the DNA-damaging agent, MMS, in all backgrounds tested (Fig. 2). However, as was seen following acid challenge, the $rpoS^{LT2}$ allele conferred an intermediate level of sensitivity to MMS (Fig. 2A).

Effect of the $rpoS^{LT2}$ allele on the ability of *Salmonella* to survive within macrophages. *S. typhimurium rpoS*::Ap mutants survive a variety of in vitro environmental stresses less well than wild type (reference 11 and this study). As described above, strains containing the $rpoS^{LT2}$ allele survive some stresses (i.e., acid and DNA damage) less well than strains with an *rpoS* allele from UK-1 or SL1344. Because mutants containing the $rpoS^{LT2}$ allele appear more sensitive to certain stresses (in particular, those relevant to the intraphagosomal environment of the macrophage), we assessed the ability of both the *rpoS*::Ap and the $rpoS^{LT2}$ mutants to survive within

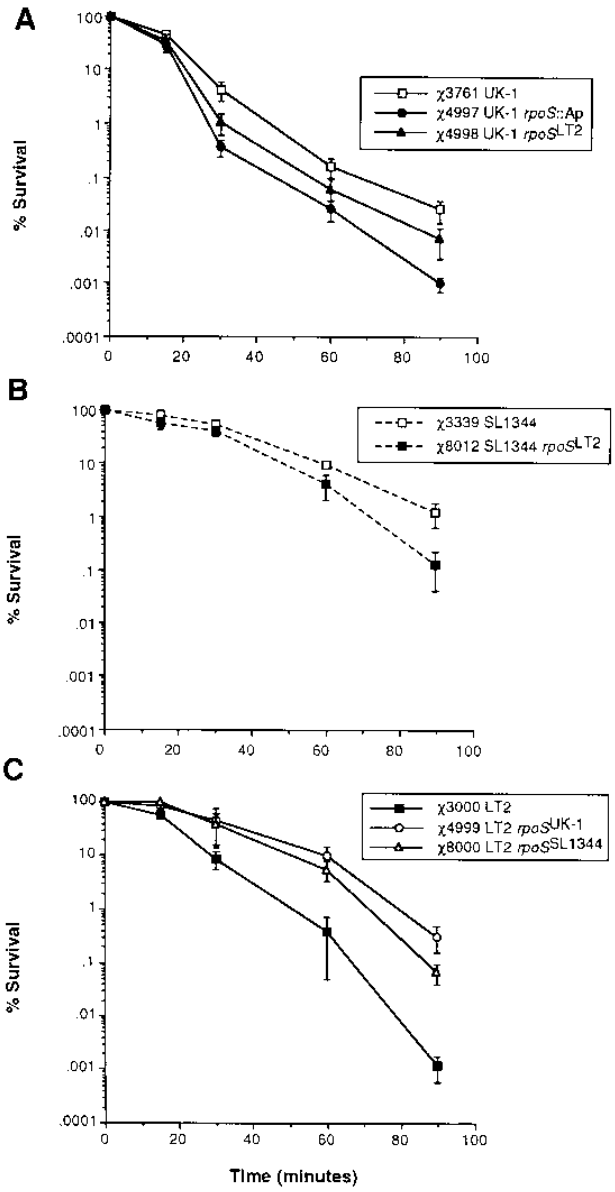

FIG. 2. Survival of *S. typhimurium* UK-1 (A), SL1344 (B), and LT2 (C) derivatives in MMS. The UK-1 derivatives were exposed to 15 mM MMS, and the SL1344 and LT2 derivatives were exposed to 30 mM MMS. The experimental protocol is described in Materials and Methods. Values represent averages of three to five trials, and standard error bars are given. Strains containing an *rpoS*::Ap or an $rpoS^{LT2}$ allele survived MMS significantly less well than an otherwise isogenic strain containing a wild-type *rpoS* allele ($P \leq 0.05$).

different populations of macrophages. As shown in Fig. 3, *S. typhimurium* UK-1, SL1344, and LT2 survive well within J774 macrophage-like cells, increasing in cell number after an initial period of killing. In contrast, a *phoP* mutant appears sensitive, with no significant increase in percent survival for 24 h following phagocytosis (Fig. 3B). Neither the *rpoS*::Ap insertion nor the $rpoS^{LT2}$ allele affected the ability of strains to survive within these macrophage-like cells. Strains containing either of these alleles survived as well as strains containing a wild-type *rpoS* allele in each background (Fig. 3).

Consistent with previous data (6, 46), *S. typhimurium* UK-1 is efficiently killed following phagocytosis by rat bone marrow-derived macrophages with less than 2% of the bacteria recov-

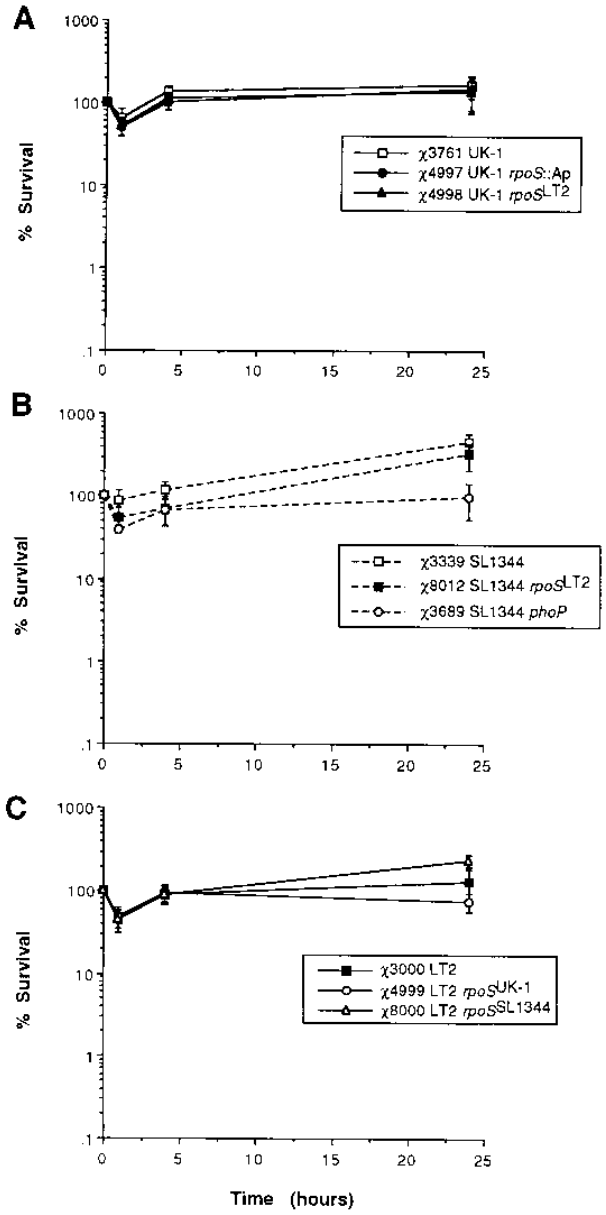

FIG. 3. Survival of *Salmonella* containing different *rpoS* alleles within J774 macrophage-like cells. The experimental protocol is described in Materials and Methods. Values represent averages of two to four trials done in duplicate. Standard errors are indicated. The difference between survival of χ3689 and χ3339 was significant at the 24-h time point ($P \leq 0.05$).

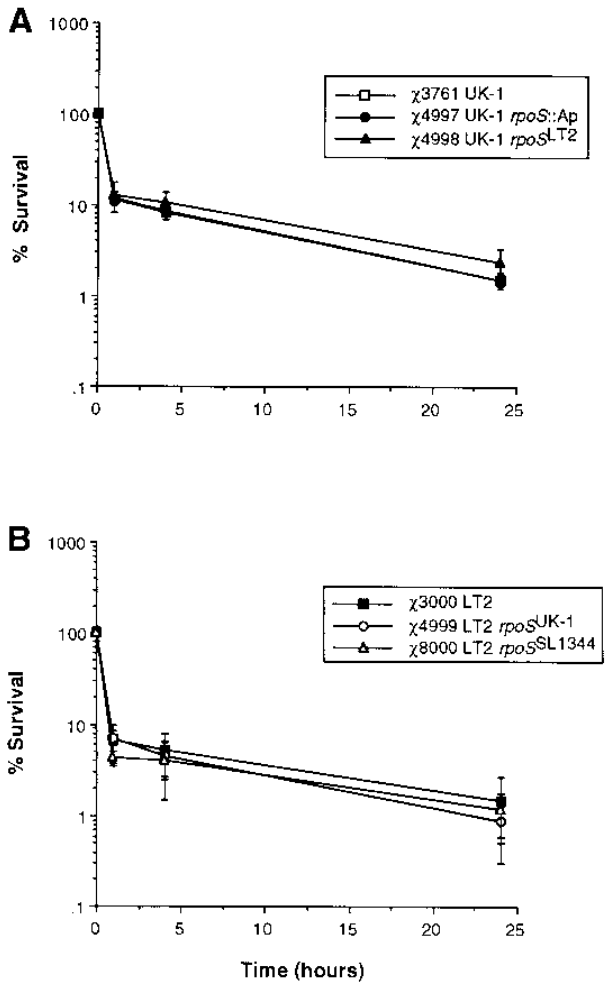

FIG. 4. Survival of *Salmonella* containing different *rpoS* alleles within rat bone marrow-derived macrophages. The experimental protocol is described in Materials and Methods. Values represent averages of three trials done in duplicate. Standard error bars are indicated.

ered at the 24-h time point (Fig. 4A). Surprisingly, despite their sensitivity to a variety of environmental stresses, UK-1 derivatives containing the *rpoS*::Ap or the *rpoS*^{LT2} allele were no more sensitive to these macrophages than their wild-type parent (Fig. 4A). There also was no effect of the *rpoS* allele on macrophage survival in the LT2 background (Fig. 4B). *S. typhi*, which is known to be more sensitive to murine macrophages than *S. typhimurium* (43), survived approximately 30-fold less well than UK-1 (data not shown). Qualitatively similar results were obtained with mouse bone marrow-derived macrophages (data not shown).

DISCUSSION

S. typhimurium LT2 contains an altered *rpoS* allele which was previously shown to be responsible for the transient nature of its log-phase ATR. In this report, we show that this mutation contributes to the attenuation of strain LT2. We evaluated the effect of the *rpoS*^{LT2} allele on virulence in BALB/c mice. Our results show that UK-1 and SL1344 derivatives containing the *rpoS*^{LT2} allele showed a 3-log increase in the oral LD_{50} compared to the wild-type parents, while LT2 derivatives containing a wild-type *rpoS* allele are considerably more virulent than the LT2 parent. The increase in LD_{50} seen with the *rpoS*^{LT2} allele is similar in magnitude to that which is seen with an *rpoS*-null mutation (reference 11 and this study). Strains containing an *rpoS*^{LT2} allele are also attenuated following intraperitoneal infection of mice, suggesting that these mutants are preferentially killed at a site within the animal other than the stomach. The recovery of similar numbers of χ3761 (UK-1) and χ4998 (UK-1 *rpoS*^{LT2}) organisms from the intestinal wall and intestinal contents at 1 h following a coinfection supports this hypothesis.

Tissue culture invasion assays show no effect of the *rpoS* allele on the ability of *S. typhimurium* to invade intestinal

epithelial cells. Interestingly, LT2 invades less well than a virulent strain of *S. typhimurium* (reference 14 and this study). This suggests that LT2 contains an additional mutation reducing its ability to invade intestinal epithelial cells. Such a defect would likely result in reduced virulence and may be responsible for the fact that an LT2 strain containing a wild-type *rpoS* allele is not fully restored for virulence. It is unlikely that any additional attenuating mutations are present on the virulence plasmid of LT2. This is based on the results of Gulig and Curtiss (18), who showed that replacing the 100-kb plasmid of a virulent strain of *S. typhimurium* with the virulence plasmid from LT2 had no effect on the ability of the resultant strain to kill mice when administered orally.

Several laboratories have shown that RpoS is involved in regulating the *spvABCD* operon (11, 18, 35), and Chen and coworkers showed that RpoS also regulates expression of the *spvR* gene (8). When we examined the effect of the $rpoS^{LT2}$ allele on expression of an *spvA-lacZ* fusion, our results indicated that the $rpoS^{LT2}$ allele resulted in reduced expression of the *spv* operon. The reduction of *spv* expression in the $rpoS^{LT2}$ mutant is likely due to lower amounts of σ^s protein in this mutant during stationary phase (3), as Chen et al. have shown that the level of RpoS protein in the cell controls the induction of the *spv* genes (8). However, it is unlikely that the small decrease in *spv* expression seen in strains containing the $rpoS^{LT2}$ allele is entirely responsible for the attenuation of these strains. In support of this, Fang et al. showed that their *rpoS*-null mutant was more attenuated than a strain lacking the virulence plasmid (11). The authors proposed that RpoS controls not only the plasmid-encoded *spv* genes but also unidentified chromosomal genes that are necessary for virulence.

Experiments in which mice are coinfected with UK-1 and a derivative containing the $rpoS^{LT2}$ allele clearly show that large differences exist in the abilities of these strains to reach deep tissues. These data are consistent with the decrease in *spv* expression seen in the $rpoS^{LT2}$ mutants. However, as suggested above, it is possible that the reduced virulence of the $rpoS^{LT2}$ mutant may be due to decreased expression of other RpoS-regulated virulence genes as well. Although not as great, we also saw significant differences in the numbers of χ3761 and χ4998 organisms recovered from intestinal walls and Peyer's patches of infected mice. This is consistent with the data of Nickerson and Curtiss, who showed that an *rpoS*-null mutant is recovered at lower levels from Peyer's patches compared with a wild-type strain (34). These data suggest that the RpoS protein may play a role in several stages of the infection process.

Our *rpoS*::Ap mutant was shown to be highly sensitive to acid stress, nutrient deprivation, oxidative stress, and DNA damage. In contrast, $rpoS^{LT2}$ mutants survive as well as strains containing a wild-type *rpoS* allele when subjected to nutrient deprivation or oxidative stress and display an intermediate level of sensitivity to acidic pH and MMS. However, despite their sensitivity to various environmental stresses, these mutants were no more sensitive than strains with a wild-type *rpoS* allele to J774 macrophage-like cells or bone marrow-derived macrophages from rats or mice.

The results presented in this report show a lack of complete correlation between the in vivo and in vitro virulence assays. That is, our in vivo animal model clearly shows that the *rpoS*-null and $rpoS^{LT2}$ mutants are attenuated. Furthermore, an $rpoS^{LT2}$ mutant is significantly reduced in its ability to reach or persist in several tissues at later stages of infection. However, despite these striking differences, we have been unable to elucidate the reason(s) for the attenuation of $rpoS^{LT2}$ or *rpoS*-null mutants. We did see an effect of these mutations on expression of the *spv* operon; however, the decrease in *spv* expression was small, particularly in the $rpoS^{LT2}$ mutants. In vitro assays show no effect of either *rpoS* allele on invasion of epithelial cells or on survival within macrophages. The latter result is particularly surprising in light of the fact that strains containing an *rpoS* mutation, in particular, the *rpoS*-null mutation, are very sensitive to a variety of environmental stresses. These inconsistencies point out either deficiencies in the assays used, in particular, the macrophage survival assay, or perhaps an inadequate understanding of the disease process in mice. If the latter is true, then there may be another site within the animal host which provides an environment which is inhospitable to the *rpoS*::Ap and $rpoS^{LT2}$ mutants. This site could be a different type of macrophage or a different cell type.

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