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# The Alternative Sigma Factor, $\sigma^E$, Is Critically Important for the Virulence of *Salmonella typhimurium*

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**In *Escherichia coli*, extracytoplasmic stress is partially controlled by the alternative sigma factor, RpoE ($\sigma^E$). In response to environmental stress or alteration in the protein content of the cell envelope, $\sigma^E$ upregulates the expression of a number of genes, including *htrA*. It has been shown that *htrA* is required for intramacrophage survival and virulence in *Salmonella typhimurium*. To investigate whether $\sigma^E$-regulated genes other than *htrA* are involved in salmonella virulence, we inactivated the *rpoE* gene of *S. typhimurium* SL1344 by allelic exchange and compared the phenotype of the mutant (GVB311) in vitro and in vivo with its parent and an isogenic *htrA* mutant (BRD915). Unlike *E. coli*, $\sigma^E$ is not required for the growth and survival of *S. typhimurium* at high temperatures. However, GVB311 did display a defect in its ability to utilize carbon sources other than glucose. GVB311 was more sensitive to hydrogen peroxide, superoxide, and antimicrobial peptides than SL1344 and BRD915. Although able to invade both macrophage and epithelial cell lines normally, the *rpoE* mutant was defective in its ability to survive and proliferate in both cell lines. The effect of the *rpoE* mutation on the intracellular behavior of *S. typhimurium* was greater than that of the *htrA* mutation. Both GVB311 and BRD915 were highly attenuated in mice. Neither strain was able to kill mice via the oral route, and the 50% lethal dose ($LD_{50}$) for both strains via the intravenous (i.v.) route was very high. The i.v. $LD_{50}$s for SL1344, BRD915, and GVB311 were <10, $5.5 \times 10^5$, and $1.24 \times 10^7$ CFU, respectively. Growth in murine tissues after oral and i.v. inoculation was impaired for both the *htrA* and *rpoE* mutant, with the latter mutant being more severely affected. Neither mutant was able to translocate successfully from the Peyer's patches to other organs after oral infection or to proliferate in the liver and spleen after i.v. inoculation. However, the *htrA* mutant efficiently colonized the livers and spleens of mice infected i.v., but the *rpoE* mutant did not. Previous studies have shown that salmonella *htrA* mutants are excellent live vaccines. In contrast, oral immunization of mice with GVB311 was unable to protect any of the mice from oral challenge with SL1344. Furthermore, i.v. immunization with a large dose (~$10^6$ CFU) of GVB311 protected less than half of the orally challenged mice. Thus, our results indicate that genes in the $\sigma^E$ regulon other than *htrA* play a critical role in the virulence and immunogenicity of *S. typhimurium*.**

*Salmonella* species can infect both warm- and cold-blooded hosts and cause a spectrum of diseases ranging from mild enteritis to severe systemic infections. This range reflects the ability of salmonellae to adapt to a range of different environments, including the interior of macrophages, in the vertebrate host. Although a number of virulence genes have been characterized and there is a basic understanding of how salmonellae cause infection, a number of questions remain unanswered about how the bacterium adapts and survives in different environments in vivo.

A clue to this adaptation is given by the reduced virulence of salmonella strains harboring mutations in the *htrA* gene (6, 29). HtrA (also called DegP) is a stress-induced serine protease. In *E. coli*, where it was first identified, HtrA is required for survival at high temperatures (>42°C). *E. coli htrA* accumulate abnormal periplasmic proteins, indicating that HtrA was active in the periplasm, where it is thought to assist in the degradation of denatured or damaged proteins which may result from enteric bacteria encountering a toxic environment (50). Unlike their *E. coli* counterparts, *S. typhimurium htrA* strains are not temperature sensitive; they are, however, more sensitive to the oxidizing agents $H_2O_2$ and menadione (a superoxide radical generator) than are wild-type strains (29). *S. typhimurium htrA* strains are also less able to survive within macrophages and exhibit a profound reduction in virulence in mice (2, 6). Thus, HtrA is required as part of the salmonella adaptive response to the host environment, in particular the oxidative stress present in the interior of macrophages (2, 29).

Homologues of *htrA* have been identified in a number of different pathogenic and nonpathogenic bacteria, including *Brucella abortus* and *Yersinia enterocolitica* (31, 44, 47, 54). A strain of *Y. enterocolitica* harboring a mutation in the *htrA* gene showed a reduced ability to colonize the livers and spleens of BALB/c mice and an increased sensitivity to oxidative killing (31). Several studies indicate that HtrA expression is upregulated in the interior of eucaryotic cells. LacZ expression was induced in a salmonella strain harboring a *lacZ* reporter gene under the control of the *htrA* promoter when it entered macrophages or epithelial cells (15). A similar finding was obtained by using the promoter of *gsrA*, the *htrA* homologue from *Y. enterocolitica* (55).

*E. coli htrA* mutants are temperature sensitive, indicating that HtrA is part of the heat shock response. However, expres-

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TABLE 1. Bacterial strains and plasmids used or constructed in this study

| Strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| Strains | | |
| SL1344 | *S. typhimurium his* mutant, mouse virulent strain | 28 |
| BRD509 | SL1344 *aroA aroD* mutant | 51 |
| BRD915 | SL1344 Δ*htrA* | 15 |
| GVB311 | SL1344 *rpoE*::Km$^r$ | This study |
| SM10λ*pir* | *E. coli thi-1 thr1 leuB6 tonA21 lacY1 supE44 recA*::RP4-2-Tc::MuKmRλ*pir* | 30 |
| Plasmids | | |
| pCR II | PCR cloning vector | Invitrogen |
| pRDH10 | λ*pir*-based suicide vector: Tet$^r$ Cm$^r$ SacB | R. Haigh (Leicester University, Leicester, United Kingdom) |
| pUC4K | Km$^r$ cassette | 43 |
| pSH101 | *rpoE* in pCR II | This study |
| pSH102 | pCR II *rpoE*::Km$^r$ | This study |
| pWSK29 | Ap$^r$, low copy number vector | 52a |
| pSH103 | pRDH10 *rpoE*::Km$^r$ | This study |
| pSH117 | *rpoE* in pWSK29 | This study |

sion of *htrA* is independent of sigma 32 ($\sigma^{32}$), the classical heat shock sigma factor, but is controlled instead by a novel sigma factor ($\sigma^E$) encoded by the *rpoE* gene (13, 33). Under extreme stress (50°C or 10% ethanol), $\sigma^E$ is also required in *E. coli* for expression of $\sigma^{32}$ (26). Sequence analysis of RpoE reveals a similarity with a family of sigma factors classified as extracytoplasmic sigma factors which control the expression of gene products required in the extracellular compartments (34). The accumulation of misfolded proteins in the periplasm or cytoplasmic membrane is thought to induce $\sigma^E$ to activate expression of *htrA*.

A $\sigma^E$ homologue, AlgU, was isolated from *Pseudomonas aeruginosa* prior to its identification in *E. coli* (35). *P. aeruginosa algU* mutants show increased sensitivity to chemically or enzymatically produced halogenated reactive oxygen intermediates and increased sensitivity to phagocytic killing, indicating a similar function for the AlgU and $\sigma^E$ regulons in protecting bacteria from environmental stress (56). Strangely, an *algU* mutant had a slightly lower 50% lethal dose ($LD_{50}$) in normal inbred mice and killed neutropenic mice faster than wild-type *P. aeruginosa* (56). Both *P. aeruginosa* AlgU and *E. coli* $\sigma^E$ negatively, as well as positively, affect the expression of a number of polypeptides (45, 56).

We were interested in investigating the involvement of $\sigma^E$ and $\sigma^E$-regulated genes in salmonella virulence. In particular, we wished to determine whether the involvement of the *rpoE* regulon was confined to *htrA* or if it involved other genes. To this end, we constructed an *rpoE* mutant of *S. typhimurium* and compared its phenotype in vitro and in vivo with that of its parent or an isogenic *htrA* mutant. Compared to its wild-type parent and an isogenic *htrA* mutant, the *S. typhimurium rpoE* mutant was more sensitive to oxidizing agents and antimicrobial peptides, survived less well in eucaryotic cells, and was highly attenuated in mice, thus implicating other genes in the $\sigma^E$ regulon in *S. typhimurium* virulence.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** Bacterial strains and plasmids used or constructed in this study are listed in Table 1. All strains were maintained on Luria-Bertani (LB) or M9 minimal media that was prepared as described earlier (49). Where required, media were supplemented with 1.5% agar; 100 μg of ampicillin (Ap), 50 μg of kanamycin (Km), 100 μg of streptomycin (Sm), or 50 μg of chloramphenicol (Cm) per ml; 0.4% glucose, or 0.4% succinate.

**PCR and template preparation.** All PCR reactions were carried out with *Taq* polymerase (Gibco BRL) with the manufacturer's buffer and deoxynucleoside triphosphate mix. The *S. typhimurium rpoE* gene was isolated by PCR with the primers 181 (5′-GTCTACAACATGACAAACAAAAACAAATGC) and 182 (5′-CCTTTTCCAGTATCCCGCTATCGTCAACGC) from an *S. typhimurium* BRD509 colony template (25). The amplified DNA fragment was cloned into the vector pCR II (Invitrogen) to create plasmid pSH101.

**Recombinant DNA manipulations.** Standard methods were used for the preparation of plasmid or chromosomal DNA, for restriction analysis, and for ligation (49). DNA hybridization was carried out by using the Gene Images labelling and detection kit (Amersham Life Sciences). DNA for sequencing was isolated by using Qiagen plasmid preparation columns. Sequencing reactions were carried out by using the Thermo Sequenase kit (Amersham) and run on an LI-COR 4000L automated DNA sequencer. Standard methods were used for the transformation and conjugation of plasmid DNA from *E. coli* to *S. typhimurium*.

**Construction of an *rpoE* mutant.** The *rpoE* gene isolated by PCR was mutated by insertion of a kanamycin antibiotic resistance cassette. The kanamycin antibiotic resistance cassette was isolated from pUC4K by digestion with *Hin*cII and inserted into a unique *Stu*I site within the coding sequence of the *rpoE* gene to form pSH102. The mutated copy of the *rpoE* gene (*rpoE*::Km$^r$) was isolated from pSH102 by digestion with *Bam*HI and *Sal*I and ligated into similarly digested pRDH10 to form pSH103. The suicide vector pRDH10 (25a) requires the *pir* product for replication and possesses *sacB*, from *Bacillus subtilus*, which allows positive selection of allelic exchange (11). pSH103 was introduced into *S. typhimurium* SL1344 from *E. coli* SM10λ*pir* by conjugation. Merodiploids were isolated by selection with Km, Cm, and Sm. Chromosomal DNA was isolated from a number of putative merodiploids (Km$^r$, Cm$^r$, and Sm$^r$) for Southern analysis. Southern hybridization with a DNA probe consisting of the last 528 bp of the *rpoE* gene revealed that the *rpoE* locus had been altered, indicating that the plasmid had inserted into the correct region of the chromosome (data not shown). The merodiploids were resolved by growing the bacteria in the presence of 6% sucrose. Km$^r$ Cm$^s$ colonies were isolated, and mutation of the *rpoE* gene was confirmed by Southern blotting and PCR with primers 181 and 182. Both techniques revealed that in the mutant the size of the *rpoE* gene had increased by ~1.2 kb, which could be attributed to the presence of the Km$^r$ gene (data not shown).

**Complementation.** The *rpoE* gene, along with its natural P2 promoter, was excised from pSH101 by digestion with *Xba*I and *Hin*dIII and ligated into the low-copy-number vector pWSK29 (52a), which had been similarly digested. The resulting plasmid, pSH117, was used in complementation studies.

**Analysis of bacterial growth.** Strains were grown overnight in the appropriate medium. To analyze the growth curve of the strains, the overnight cultures were diluted 100-fold into 50-ml portions of fresh media. Bacteria were incubated at 30, 37, or 42°C with aeration, 1-ml samples were removed at intervals, and the absorbance at 600 nm was recorded. The number of CFU per milliliter was determined by plating out serial dilutions of bacterial samples.

**Disk diffusion assay.** Bacteria cultured overnight in M9-glucose (M9-G) media were diluted to an optical density at 600 nm of 0.25 in fresh M9-G and grown for 2 h at 30 or 37°C. Then, 100 μl was used to inoculate 3 ml of top agar to form a lawn on M9-G plates. Next, 6-mm filter paper disks were soaked in 10 μl of the toxic agent (3% $H_2O_2$ or 2% paraquat [methyl viologen]; both from Sigma). These disks or disks containing 300 U of polymyxin B (Oxoid) were added to the agar surface. Plates were incubated aerobically overnight at 37 or 30°C. The diameter of the zones of inhibition were measured.

**Invasion and persistence of *S. typhimurium* strains in phagocytic and nonphagocytic cells.** The ability of the different *S. typhimurium* strains to invade and survive in phagocytic and nonphagocytic cells was assessed by using the macrophage-like cell line RAW264.7 and the epithelial cell line HEp-2, respectively.

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Cells were routinely cultured in Dulbecco modified Eagle medium (DMEM; Gibco BRL) supplemented with 4 mM L-glutamine, 10% (vol/vol) fetal bovine serum and 1× Antibiotic-Antimycotic mix (Gibco-BRL). For invasion assays, cells were seeded into 24-well tissue culture plates (Costar) at $2 \times 10^5$ cells per well and incubated overnight at 37°C with 5% $CO_2$. Prior to infection, the monolayers were washed twice in antibiotic-free DMEM. Bacteria from overnight culture in LB-glucose (LB-G) broth were diluted in DMEM to give $2 \times 10^5$ CFU/ml. Then, 1 ml of bacterial suspension was added to each well to give an approximate 1:1 multiplicity of infection. Bacterial invasion was synchronized by centrifugation at 1,000 rpm for 10 min followed by incubation for 1 to 2 h as described above. The monolayers were then washed twice with sterile phosphate-buffered saline (PBS) and overlaid with 1 ml of DMEM containing 100 μg of polymyxin B per ml and incubated for 1 h as described above. The monolayers were washed twice with PBS, and either the cells were lysed with sterile water or they were overlaid with 1 ml of DMEM containing 10 μg of polymyxin B per ml and incubated for a further 21 h before being washed and lysed as described above. After lysis of the cells, the number of viable bacteria released from the cells was determined by plating serial dilutions on LB-agar (LA) plates.

**Analysis of salmonella virulence, in vivo growth, and immunity in mice.** Virulence and immunity studies were carried out as previously described (6, 50). *S. typhimurium* strains grown statically overnight in LB-G were recovered by centrifugation and resuspended in sterile PBS (pH 7.2) to approximately $1 \times 10^{10}$ to $10^{10}$ CFU/ml. The actual number of bacteria present was determined by a viable counting. Female BALB/c mice (6 to 8 weeks old; Charles River, Margate, United Kingdom) were challenged either orally with a gavage tube or intravenously (i.v.) by injection into the tail vein as described previously (6, 50). For the virulence studies, mice were challenged with different numbers of organisms and were then closely observed, and deaths were recorded for 28 days. The $LD_{50}$s of the strains were determined by the method of Reed and Muench (46). To study the in vivo growth and survival of *S. typhimurium* strains, groups of mice were inoculated by the oral or i.v. route as described above. On various days after infection, groups of four mice were sacrificed and the spleen, liver, mesenteric lymph nodes, and Peyer's patches were removed from each animal. Then, 10 ml of sterile water was added to each organ in a separate sterile bag, and the organs were homogenized with a Stomacher 80 (Steward Lab System). To determine the number of viable organisms present, the homogenates were serially diluted and plated onto LA-Sm plates. To examine the immunogenicity of the mutant strain, mice were immunized either orally or i.v. with different doses of this strain. The mice were challenged orally 28 days later with $2 \times 10^8$ CFU of SL1344 and deaths were recorded for 6 weeks.

## RESULTS

**Construction of a *S. typhimurium rpoE* mutant.** The complete *S. typhimurium rpoE* gene was isolated by PCR. The sequence was found to be 100% identical to the published *S. typhimurium rpoE* gene (36). The *rpoE* gene was insertionally inactivated in vitro by using a Km resistance cassette, and this construct was used to produce an *S. typhimurium* SL1344 *rpoE* mutant (GVB311) by allelic exchange as described above. The disruption of the *rpoE* gene in GVB311 was confirmed by PCR and Southern blotting as described above.

**The *S. typhimurium rpoE* mutant (GVB311) exhibits aberrant growth under certain conditions.** Inactivation of the *E. coli rpoE* gene confers a temperature-sensitive phenotype such that the bacteria begin to lyse at growth temperatures above 43°C (26). Therefore, we analyzed GVB311 for any temperature-dependent effects on growth. The mutant formed normal-sized colonies on LA after overnight growth at 30, 37, or 42°C (data not shown). When the bacteria were grown in LB at 30, 37 (data not shown), or 42°C (Fig. 1B), GVB311 exhibited a longer lag phase than SL1344 or an SL1344 *htrA* mutant (BRD915). The growth rate during the log phase for GVB311 was the same as for SL1344 and BRD915; however, GVB311 did not reach the same final optical density after overnight growth as the other two strains. This reflected fewer CFU of GVB311 per milliliter in the overnight culture than with SL1344 and BRD915 (data not shown). Introduction of the wild-type *rpoE* gene into GVB311 on a low-copy-number plasmid complemented the growth defect in LB (Fig. 1B).

In contrast to the growth in LB, GVB311 grew normally in M9-G medium (data not shown). However, when glucose was replaced with succinate as the carbon source, the lag period of GVB311 was greatly protracted (4 to 6 h [data not shown]). If LB medium was supplemented with glucose, then the growth curve of GVB311 was normal (Fig. 1A [note that our LB medium does not normally contain glucose]). These results suggest that GVB311 is defective in its ability to utilize carbon sources other than glucose.

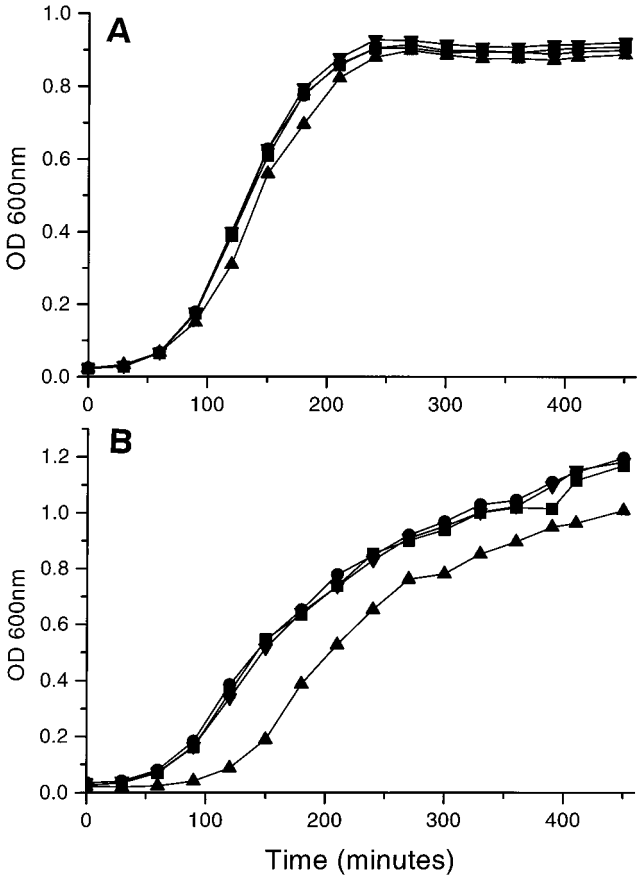


FIG. 1. Effect of media and temperature on the growth of *S. typhimurium rpoE* mutant. Overnight cultures of the *S. typhimurium* strains SL1344 (■), BRD915 (●), GVB311 (▲), and GVB311(pSH117) (▼) were used to inoculate fresh media, which was then incubated aerobically. Growth was followed spectrophotometrically. (A) Growth in LB-G at 42°C. (B) Growth in LB at 42°C.

**GVB311 shows increased sensitivity to $H_2O_2$ and paraquat compared to SL1344.** It was previously reported that an *S. typhimurium htrA* mutant was more sensitive to $H_2O_2$ and menadione (a superoxide generator) than its wild-type parent, indicating a role for HtrA in the defense against oxidative stress (29). If, as well as *htrA*, *S. typhimurium rpoE* regulates other genes required to repair the damage caused by oxidative stress, then the *rpoE* mutant should be at least as sensitive as, if not more sensitive than, an *htrA* mutant to oxidative agents. The sensitivity of GVB311 and other *Salmonella* strains to $H_2O_2$ and paraquat (a superoxide anion generator) was assessed by a disk diffusion assay. The assays were performed at 30 and 37°C to analyze whether temperature affected the ability of the bacteria to adapt to oxidative stress. The rationale for this is that at high temperatures both the $\sigma^{32}$ and $\sigma^E$ regulons will be upregulated (13, 14); therefore, the bacteria may be better adapted to survive oxidative stress. The results are shown in Fig. 2. BRD915 was not more sensitive to $H_2O_2$ than SL1344 at either temperature. However, BRD915 and SL1344 were both significantly ($P < 0.05$) more sensitive to $H_2O_2$ at 30°C than at 37°C. GVB311 was significantly more sensitive to $H_2O_2$ than SL1344 and BRD915 at both temperatures and, unlike the other two

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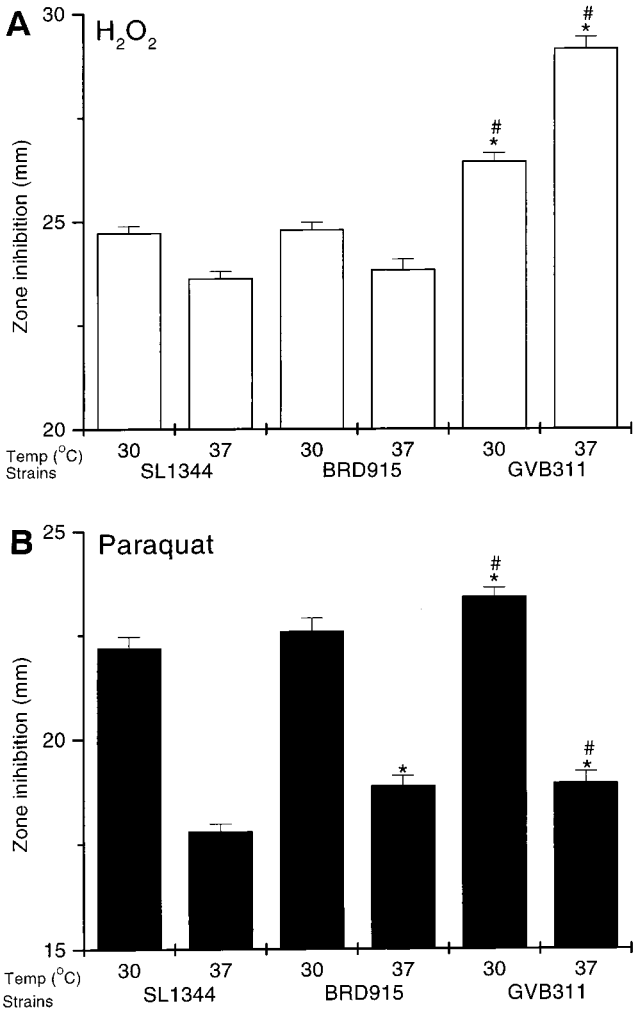


FIG. 2. Effect of the *rpoE* and *htrA* mutations on the sensitivity of *S. typhimurium* to oxidizing agents. The salmonella strains were tested for sensitivity to 3% $H_2O_2$ (A) or 2% paraquat (B) by disk diffusion assay. The plates were incubated overnight at 30 or 37°C as indicated in the figure below each lane. Each bar represents the mean diameter of the zone of inhibition, and the error bar shows the standard deviation (SD) of 30 replica assays. The asterisk indicates that the mean diameter of the zones is statistically different ($P < 0.05$) from that of SL1344 incubated at the same temperature; the number sign (#) indicates that the mean is statistically different from BRD915 grown at the same temperature (one-way analysis of variance [ANOVA]).

strains, GVB311 is more sensitive to the effect of $H_2O_2$ at 37 than at 30°C.

All three strains were more sensitive to paraquat at 30°C than at 37°C. At 37°C, there was no significant difference in the paraquat sensitivities of BRD915 and GVB311, but both were significantly more sensitive to paraquat than SL1344. At 30°C, GVB311 (but not BRD915) was again more sensitive to paraquat than SL1344. The effect of the *rpoE* and *htrA* mutations on the sensitivity of *S. typhimurium* to reactive nitrogen intermediates was assayed by disk diffusion assay by using SIN-1. SIN-1 spontaneously generates peroxynitrate radicals under aerobic conditions. There was no significant difference in the sensitivities of the three strains to SIN-1 (500 mM [Sigma]; data not shown).

**GVB311 is more sensitive to the antimicrobial peptide polymyxin B than SL1344 and BRD915.** The sensitivity of GVB311 to polymyxin B was analyzed by disk diffusion assay. GVB311 was significantly more sensitive to killing by polymyxin B than were SL1344 and BRD915, as shown in Fig. 3. This peptide binds to the lipid A core of lipopolysaccharide (LPS). Analysis of the LPS of GVB311 and SL1344 on silver-stained gels revealed no obvious differences between the LPS profiles (data not shown).

The sensitivity of the *rpoE* and *htrA* mutants to other membrane-damaging agents was also assessed. The ability of the strains to grow in the presence of detergents was assessed by growing the organisms on MacConkey and desoxycholate-citrate agar containing 0.5% bile salts and 0.5% sodium deoxycholate, respectively. There was no difference in the plating efficiencies or in the sizes of the colonies of the three strains on these two media. The sensitivity of the strains to killing by complement was assessed by exposing the strains to 10% normal human serum. There was no difference in the sensitivities of the three strains to complement killing (data not shown). These studies indicate that the *rpoE* and *htrA* mutations do not cause a generalized defect in outer-membrane integrity.

**RpoE is involved in intracellular survival within macrophages and nonphagocytic cells.** Previous studies with *S. typhimurium* showed that an *htrA* mutant strain survived less well in macrophages than did a wild-type bacteria. It is therefore likely that a strain that lacks *rpoE* would also survive less well in macrophages (2). Furthermore, if $\sigma^E$-regulated genes, in addition to *htrA*, are involved in intramacrophage survival, then an *rpoE* mutant may be more attenuated in macrophages than an *htrA* strain. In order to address this SL1344, BRD915, GVB311, and GVB311(pSH117) were assayed to assess their abilities to invade and survive in the murine macrophage cell line RAW264.7. We also examined the capacity of the strains to invade and survive in nonphagocytic HEp-2 cells. GVB311 invaded macrophages as well as had SL1344 and BRD915 (Fig. 4). However, after 24 h the number of GVB311 inside

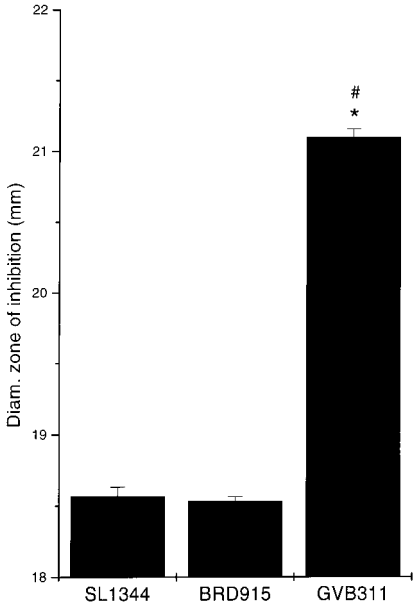


FIG. 3. Effect of the *rpoE* and *htrA* mutations on the sensitivity of *S. typhimurium* to polymyxin B. SL1344, BRD915, and GVB311 were tested for sensitivity to polymyxin B by disk diffusion assay. Disks containing 300 U of polymyxin B (Oxoid) were placed on the plates and incubated overnight at 37°C. The diameter of the zone of inhibition was then measured in millimeters. Each bar represents the mean diameter of the zone of inhibition, and the error bar shows the SD of 30 replica assays. The asterisk indicates that the mean diameter of the zones is statistically different ($P < 0.05$) from that of SL1344, and the number sign (#) indicates that the mean is statistically different from BRD915 (one-way ANOVA).

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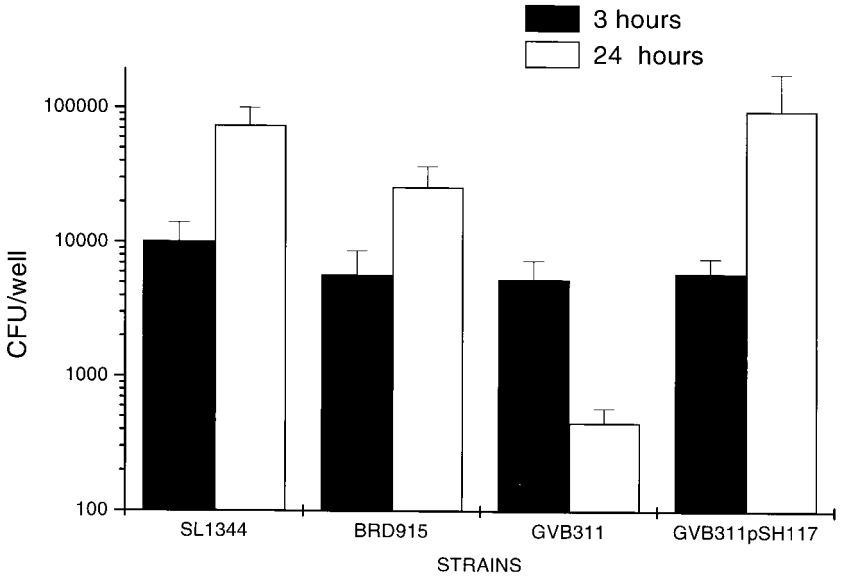


FIG. 4. Effect of the *rpoE* and *htrA* mutations on the ability of *S. typhimurium* to invade and survive in macrophages. The ability of the different *S. typhimurium* strains to invade and survive in macrophages was examined by using the murine macrophage-like cell line RAW264.7. RAW264.7 cells in the wells of tissue culture plates were infected with bacteria at a multiplicity of infection of ~1:1. The assay was performed as described in the text. The graph shows the number of viable bacteria inside macrophages at 3 and 24 h after infection. Each bar represents the mean of CFU from triplicate experiments, and the error bars indicate the SD.

RAW264.7 cells had decreased ~6-fold, whereas the numbers of BRD915, SL1344, and GVB311(pSH117) had increased ca. 6- to 40-fold (Fig. 4). This indicates that *rpoE*-regulated genes other than *htrA* do participate in intramacrophage survival. GVB311 was also able to invade HEp-2 normally. However, by between 3 and 24 h the number of GVB311 organisms present intracellularly had decreased 5-fold, whereas the numbers of SL1344 and BRD915 had increased 10- and 5-fold, respectively (data not shown).

Recently, it has been reported that *Salmonella* spp. can induce apoptosis in infected macrophages (7, 32, 42). We compared the cytotoxicity of the three strains for RAW264.7 cells by using a commercial assay (Cytotox; Promega). No difference was found between the cytotoxicity induced in RAW264.7 cells by these three strains (data not shown). This suggests that the *rpoE* and *htrA* mutations are not affecting the different effector mechanisms that are reported to be responsible for the induction of apoptosis.

**RpoE is critical for the virulence and immunogenicity of *S. typhimurium*.** The virulence of GVB311 in mice was compared with SL1344 and BRD915 after oral or parenteral (i.v.) challenge. As shown in Table 2, GVB311 and BRD915 were unable to kill mice after oral challenge, even at a dose of ca. $10^{10}$ CFU. Whereas SL1344 had an $LD_{50}$ at least 4 logs lower, the $LD_{50}$ of GVB311 after i.v. inoculation was approximately 20-fold higher than that of the *htrA* mutant and $10^6$ times greater than the $LD_{50}$ of the wild-type strain.

Although highly attenuated, *S. typhimurium htrA* mutants are excellent live vaccines that can induce solid immunity to lethal challenge with wild-type *S. typhimurium* (5). To determine whether inactivation of *rpoE* affected the ability of *S. typhimurium* to induce a protective immune response, we immunized BALB/c mice with different doses of GVB311 by the oral or i.v. routes and 28 days later challenged the mice with 100× $LD_{50}$s (~$2 \times 10^8$ CFU) of SL1344; the results are shown in Table 3. Even at the highest dose of ~$10^{10}$ CFU, none of the mice immunized orally were protected. The i.v. immunization usually induces a stronger immunity than an oral immunization. Nevertheless, an i.v. immunization with a dose of $10^6$ CFU of GVB311 protected fewer than half of the mice. This inactivation of *rpoE* severely compromised the immunogenicity of *S. typhimurium*.

In order to understand further the reduced virulence of GVB311, the ability of the organism to colonize, survive, and replicate in different murine tissues was compared with BRD915 after oral or i.v. inoculation (Fig. 5). After oral inoculation, BRD915 was able to colonize Peyer's patches (PPs) efficiently in high numbers but was slowly eliminated from this organ after day 3; however, hundreds of bacteria were still detected in the PPs at day 21. GVB311 could not be detected in the PPs on day 3, although levels comparable to BRD915 were present on day 7. Thereafter, GVB311 was cleared rapidly from the PPs and was detectable in only one of four mice on day 21. Both BRD915 and GVB311 translocated very poorly from the PPs to deeper tissues.

After i.v. infection, strain BRD915 colonized both livers and spleens well, but it did not demonstrate an increase in numbers over the course of the study; instead it slowly cleared from both organs as previously described (6). GVB311 was present in the liver and spleen on day 2 but in much lower numbers than BRD915. For example, there were ca. 100 CFU in the livers and spleens of GVB311-infected mice but ca. 15,000 CFU in the corresponding organs of BRD915-infected mice. On day 17 only one of the four mice sampled had bacteria in the liver, as

TABLE 2. Effect of the *rpoE* mutation on *S. typhimurium* virulence[a]

| Strain | Genotype | $LD_{50}$ | |
|---|---|---|---|
| | | Oral | i.v. |
| SL1344 | Wild type | $1.20 \times 10^6$ | <10 |
| BRD915 | *htrA* | >$10^{10}$ | $5.5 \times 10^5$ |
| GVB311 | *rpoE* | >$10^{10}$ | $1.24 \times 10^7$ |

[a] Groups of five mice were challenged with different doses of each strain of *S. typhimurium* by the route indicated, and the deaths were recorded for 6 weeks.

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TABLE 3. Effect of the *rpoE* mutation on *S. typhimurium* immunogenicity[a]

| Immunization route | Dose (CFU) | Protection (no. of survivors/ no. challenged) |
|---|---|---|
| Oral | $8.75 \times 10^9$ | 0/5 |
| | $9.63 \times 10^7$ | 0/5 |
| | $1.0 \times 10^6$ | 0/5 |
| i.v. | $1.11 \times 10^6$ | 2/5 |
| | $1.0 \times 10^4$ | 2/5 |

[a] Mice were immunized as indicated; 28 days later they were challenged orally with $2 \times 10^8$ CFU of SL1344. Mice were observed for 6 weeks, and the number of deaths were recorded.

was also the case on day 21, by which point the organism was undetectable in the spleen.

## DISCUSSION

*E. coli* $\sigma^E$ is one of the factors that control the response of the bacterium to extracytoplasmic stress (26, 45, 48). Our results indicate that $\sigma^E$ plays a similar role in *S. typhimurium* and also that it is intimately involved in salmonella virulence. However, *S. typhimurium* $\sigma^E$ is not required for survival at high temperatures as it is in *E. coli* (26). GVB311 appeared to grow normally at different temperatures on solid medium. However, aberrant growth was noted in liquid media. In general, the growth rate of GVB311 was very similar to SL1344 and BRD915, but in media lacking glucose the lag period was extended and the final cell yields were lower. Growth was normal in M9 containing glucose as the sole carbon source, but if glucose was replaced with the nonfermentable carbon source succinate it took up to 7 h for GVB311 to begin growing (data not shown). Supplementing LB with glucose abolished the extended lag period of GVB311. These results suggest that *rpoE* mutants may be defective in their ability to utilize nonfermentable carbon sources or carbon sources other than glucose.

A clue to understanding this phenomena may come from studies with *E. coli* mutants that are hypersensitive to redox-cycling drugs, such as paraquat, because they lack cytosolic superoxide dismutase (20). Such mutants can only utilize fermentable carbon sources because $O_2^-$ generated normally during aerobic respiration inactivates enzymes involved in the tricarboxylic acid cycle (5). The *rpoE* strain is more sensitive to $O_2^-$ than wild-type *S. typhimurium*, and so it may grow poorly

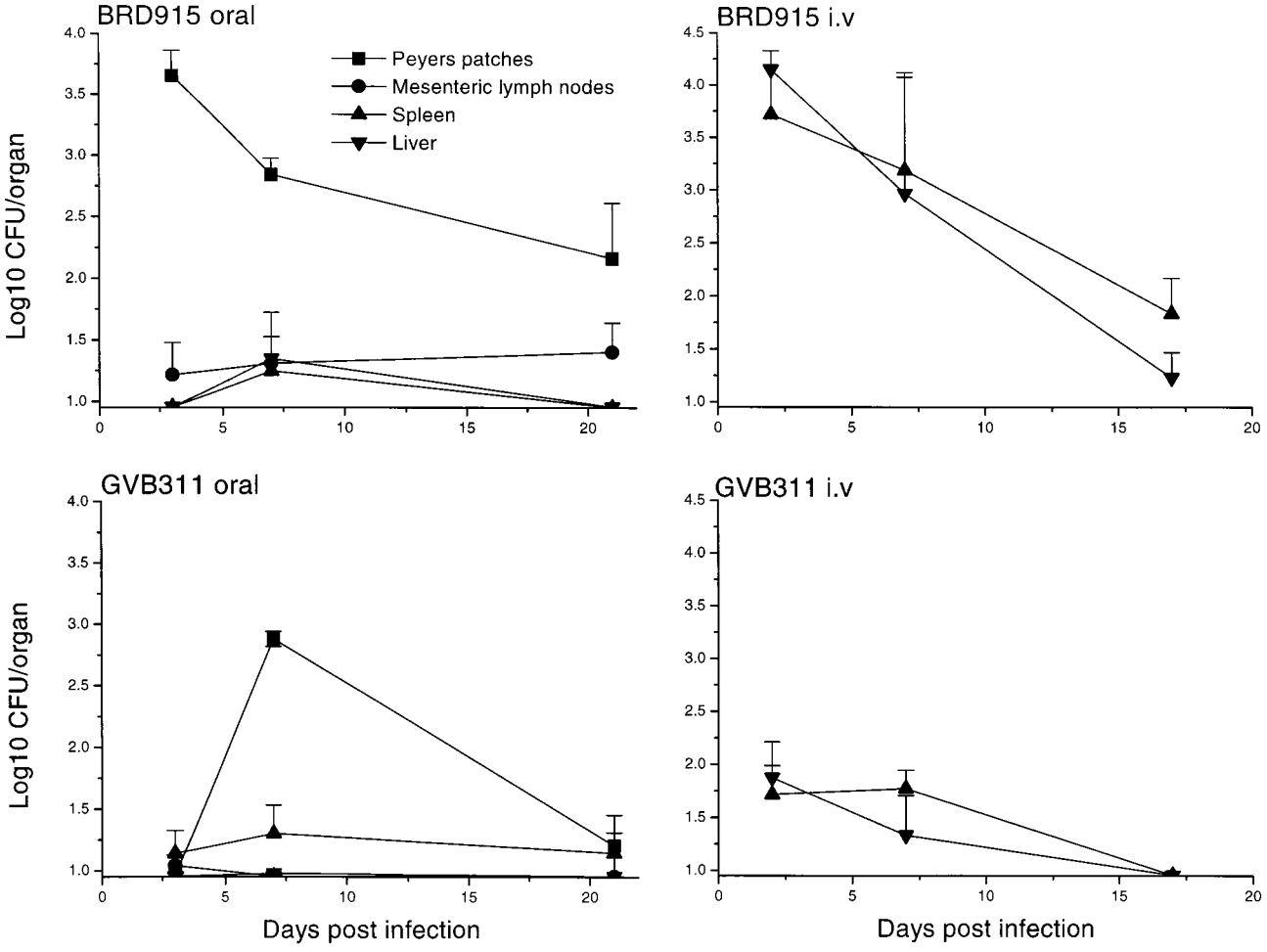


FIG. 5. Colonization, growth, and survival of BRD915 and GVB311 in murine tissues after oral or i.v. inoculation. Groups of mice were infected with BRD915 or GVB311 orally ($10^{10}$ CFU) or i.v. ($10^6$ CFU). At the indicated periods after infection, organs were removed from groups of four mice and homogenized and the number of viable organisms present was determined. Symbols: ■, PPs; ●, mesenteric lymph nodes; ▲, spleen; and ▼, liver. Each point represents the mean CFU/organ for four mice, and the error bars indicate one standard error of the mean.

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in the presence of nonfermentable carbon sources because it cannot detoxify the endogenously produced $O_2^-$. Alternatively, the products of one or more $\sigma^E$-regulated genes other than *htrA* may be involved in the correct folding or integrity of one or more polypeptides involved in the transport of certain carbon sources across the outer and/or inner membranes into the cell.

GVB311 is more sensitive than SL1344 and BRD915 to oxidative stress imposed by both $H_2O_2$ and paraquat. Although the differences were not large, they were statistically significant. This indicates that genes in the $\sigma^E$ regulon other than *htrA* are required for defense against $H_2O_2$ and paraquat. Both SL1344 and BRD915 are more resistant to $H_2O_2$ at 37 than at 30°C but, interestingly, the reverse was true for GVB311. We hypothesized that strains may be more resistant to oxidants at the higher temperatures because of the increased activity of $\sigma^E$ and RpoH ($\sigma^{32}$). Lower sensitivity of the strains to the oxidant at the higher temperature would indicate that there is involvement of the intracellular stress regulon (controlled by $\sigma^{32}$) and/or the extracellular stress regulon (controlled by $\sigma^E$). The *rpoE* mutation also had a bigger effect on the sensitivity of GVB311 to $H_2O_2$ than on its sensitivity to paraquat. This suggests that $\sigma^E$ is more important for the defense against $H_2O_2$ than for the defense against paraquat. This is as expected because paraquat causes the generation of superoxide in the cytoplasm where it exerts its effect, whereas $H_2O_2$ readily crosses biological membranes and is likely to cause damage in both the cytoplasmic and extracytoplasmic compartments (37). It may be that $\sigma^E$-regulated genes are required to combat damage caused by $H_2O_2$ in the cell envelope. Also, $\sigma^E$ may be involved in controlling $\sigma^{32}$-mediated responses to $H_2O_2$ damage in the cytoplasm. $H_2O_2$ is known to induce a subset of the classical ($\sigma^{32}$-dependent) heat shock genes. $\sigma^E$ can activate the transcription of *rpoH* from the *rpoH3* promoter under particular conditions, such as higher temperature (>42°C) and 20% ethanol (14). It may be that exposure of salmonellae to $H_2O_2$ also stimulates $\sigma^E$-dependent *rpoH* transcription and, because this response would be absent in GVB311, this may account for the results we obtained. Unlike an earlier study, we did not find here that *htrA* was required for resistance to $H_2O_2$ (29). The previous work used an *S. typhimurium* C5 *htrA* mutant produced by *TnphoA* mutagenesis, and it may be that variation in the strain background (SL1344 versus C5), the nature of the mutations, or the assay methodology may account for the different results. Salmonella has a number of genes that are involved in the defense against oxidizing agents, including the *kat* genes, the *sod* genes, *oxyR*, *rpoS*, *soxS*, *slyA*, *htrA*, and now *rpoE* (3, 4, 9, 16–18, 29, 37).

Unlike the other genes listed above, some of which are also transcriptional regulators, *rpoE* also contributes towards the ability of salmonella to resist the activity of antimicrobial peptides. GVB311 was more sensitive to the antibacterial activity of the peptide antibiotic polymyxin B, and preliminary experiments also indicate that GVB311 is more sensitive to cecropin P1 and a peptide (P2) derived from the bactericidal-permeability increasing protein (reference 53 and data not shown). These peptides bind to the negatively charged phosphoryl groups on the lipid A core of LPS. After this binding, the inner and outer membranes are permeabilized, resulting in cell death (21, 22). A number of genes have been reported to influence the sensitivity of salmonella to antibacterial peptides, including polymyxin B, which is probably the most well studied and is regulated by the two-component regulators PhoPQ and PmrAB (23, 24). PhoPQ activates or represses a total of 40 genes, and the regulon is essential for virulence (38, 39). As regards its involvement in peptide resistance, PhoPQ activates the *pmrAB* genes and, in turn, PmrAB positively regulates the expression of two genes, *pmrE* and *pmrF*, that mediate antimicrobial peptide resistance. The function of the product of these genes is to add an aminoarabinose residue onto the lipid A core of LPS, reducing the anionic charge and thereby lowering its ability to be bound by cationic peptides (23).

GVB311 does not have a generalized defect in membrane integrity because it is no more sensitive to detergents or serum than SL1344 or BRD915 and there were no apparent defects in its LPS profile. How $\sigma^E$ affects peptide resistance is unknown, but our results indicate that it is not via activation of *htrA*. Interestingly, it has been suggested that proteases in the bacterial envelope may also mediate peptide resistance (21). It may be that $\sigma^E$ controls the expression of an extracytoplasmic protease other than HtrA that mediates peptide resistance.

The ability of salmonella strains to invade and survive in macrophages in vitro usually correlates with the virulence of the strain in vivo (2, 19). Many genes are involved in salmonella intramacrophage survival and growth (1). Our results indicate that $\sigma^E$-regulated genes other than *htrA* are also involved in this process because GVB311 is less able to survive in macrophages than is BRD915. One candidate $\sigma^E$-regulated gene is *fkpA*. FkpA is a peptidyl-proly-*cis-trans* isomerase involved in protein folding in the periplasm of *E. coli* (40). Expression of *fkpA* in *E. coli* is positively regulated by $\sigma^E$, making it the second such gene, along with *htrA*, that is involved in extracytoplasmic protein folding or stability (8). FkpA shows homology to Mip proteins, which were identified as important for intracellular survival in *Legionella pneumophilia* and *Chlamydia trachomatis* (27). Recently, an *S. typhimurium fkpA* mutant was shown to survive less well in macrophages and epithelial cells (27). GVB311 showed reduced survival in HEp-2 cells compared to wild type, indicating a similar phenotype as the *fkpA* mutant. The affect of the *fkpA* mutation on salmonella virulence in vivo was not investigated. Whether HtrA and FkpA can account for all of the intracellular survival (and virulence) functions regulated by $\sigma^E$ will require the construction and analysis of *S. typhimurium htrA fkpA* mutants.

The *rpoE* mutant strain was able to invade eucaryotic cells as well as did the wild-type strain. This indicates that $\sigma^E$ is not involved in either regulating the genes required for invasion or the correct assembly or translocation of the invasion factors. The same applies to the ability of salmonellae to kill macrophages, since we found no difference in ability of the three strains to mediate macrophage lysis.

The *rpoE* mutation greatly reduced the ability of *S. typhimurium* to cause disease in BALB/c mice by either the natural or parenteral routes of infection. As expected, the *htrA* mutant BRD915 was also highly attenuated compared with SL1344. It was not possible to differentiate between the virulence levels of GVB311 and BRD915 when administered via the oral route because all mice that received the largest dose survived. However, the $LD_{50}$ of GVB311 was ca. 20-fold higher than that of BRD915 delivered via the i.v. route. The number of CFU of GVB311 that need to be given to kill mice is very high, and it is likely that some of the mice are dying directly from endotoxinemia rather than from salmonella infection per se.

Analysis of the growth and survival of BRD915 and GVB311 in murine tissues after oral or parenteral challenge confirmed that both strains are defective in survival and/or replication in vivo and that the *rpoE* mutation has a greater effect than the *htrA* mutation. Both strains were able to enter PPs after oral inoculation. BRD915 was able to persist at high levels in this tissue throughout the course of the experiment but appears unable to translocate efficiently to deeper tissues as has been previously reported (12). GVB311 was only found in high num-

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bers in the PPs at day 7 postinfection. This result is not due to large numbers of organisms present in a single mouse because the PPs of the four mice sampled contained a similar number of organisms. The reason that only low numbers of GVB311 were found at day 3 is not known. It may be that higher numbers were present but that the organisms were fragile and were damaged during homogenization or that they required prolonged incubation in vitro for the colonies to appear.

The *htrA* mutant was able to colonize the liver and spleen efficiently after inoculation directly into the bloodstream, but thereafter the numbers of organisms slowly decreased, indicating that the organism cannot replicate in these tissues or that bacterial killing exceeds replication as previously reported (6). In contrast only a tiny percentage of the GVB311 inoculated i.v. remained 2 days after challenge. This may be because the organism is unable to enter the liver and spleen or, more likely, because the organisms are rapidly killed, as suggested from the studies in macrophages. Our results suggest that genes regulated by $\sigma^E$ other than *htrA* are required for full virulence of salmonella (at least in mice) after oral and systemic infection. The low levels of GVB311 in murine tissues following oral or i.v. immunization probably accounts for the poor immunogenicity of this strain.

We cannot conclude definitively why the *rpoE* strain is so attenuated at present. This is probably due to a combination of the phenotypes that it exhibits in vitro. As mentioned previously, the ability of strains to survive and grow within macrophages correlates with virulence. GVB311 is defective in this regard, but whether this is because of (i) its increased sensitivity to oxidizing agents, (ii) antimicrobial peptides, (iii) the reduced ability of GVB311 to grow when carbon sources other than glucose are used, (iv) a combination of these effects, or (v) some other defect(s) is not known. The sensitivity of salmonella strains to oxidizing agents in vitro does not necessarily correlate with the loss of virulence. For example, mutations in *soxS* and *kat* render *S. typhimurium* more sensitive to paraquat and $H_2O_2$, respectively, but does not affect the ability of the organism to survive in macrophages or cause infection in mice (4, 16).

The structural genes so far known to be positively regulated by $\sigma^E$, *htrA* and *fkpA*, both affect intramacrophage survival and are involved with protein folding and degradation outside of the cytoplasm. Presumably, the inability to degrade, fold, or refold extracytoplasmic proteins that have been damaged in vivo is a major reason why *rpoE* strains are attenuated.

It will be interesting to learn which signals lead to activation of $\sigma^E$ in vivo. The $\sigma^E$-dependent *htrA* promoter is activated upon the entry of salmonellae into epithelial and macrophage cell lines (5). However, the cue(s) from the eucaryotic cell that trigger this response are unknown. A strong local and serum antibody response to the tetanus toxin fragment C antigen is seen in mice orally immunized with attenuated salmonellae expressing fragment C from the *htrA* promoter. These data suggest that $\sigma^E$ and $\sigma^E$-regulated genes are highly upregulated within salmonellae in vivo.

In *E. coli*, $\sigma^E$ activity is regulated positively at the transcriptional level and negatively at the posttranslational levels. Negative regulation of $\sigma^E$ is mediated by the anti-sigma factors RseA and RseB (and possibly RseC) (41). RseA is situated in the cytoplasmic membrane and may have periplasmic and cytoplasmic domains. It is thought that the cytoplasmic domain binds $\sigma^E$ and prevents it from interacting with RNA polymerase, whereas the periplasmic domain interacts with RseB. RseB is thought to sense changes in protein content in the cell envelope that are brought about by environmental stress. This is transmitted to RseA, causing a conformational change that releases $\sigma^E$, which can then bind to specific recognition sequences, allowing transcription of *htrA* and other genes in its regulon (10, 41). We are currently analyzing how Rse regulation of $\sigma^E$ affects the ability of salmonellae to interact with their host.

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