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Characterization of the RpoS Status of Clinical Isolates of *Salmonella enterica*

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The stationary-phase-inducible sigma factor, σ^S (RpoS), is the master regulator of the general stress response in *Salmonella* and is required for virulence in mice. *rpoS* mutants can frequently be isolated from highly passaged laboratory strains of *Salmonella*. We examined the *rpoS* status of 116 human clinical isolates of *Salmonella*, including 41 *Salmonella enterica* serotype Typhi strains isolated from blood, 38 *S. enterica* serotype Typhimurium strains isolated from blood, and 37 *Salmonella* serotype Typhimurium strains isolated from feces. We examined the abilities of these strains to produce the σ^S protein, to express RpoS-dependent catalase activity, and to resist to oxidative stress in the stationary phase of growth. We also carried out complementation experiments with a cloned wild-type *rpoS* gene. Our results showed that 15 of the 41 *Salmonella* serotype Typhi isolates were defective in RpoS. We sequenced the *rpoS* allele of 12 strains. This led to identification of small insertions, deletions, and point mutations resulting in premature stop codons or affecting regions 1 and 2 of σ^S, showing that the *rpoS* mutations are not clonal. Thus, mutant *rpoS* alleles can be found in freshly isolated clinical strains of *Salmonella* serotype Typhi, and they may affect virulence properties. Interestingly however, no *rpoS* mutants were found among the 75 *Salmonella* serotype Typhimurium isolates. Strains that differed in catalase activity and resistance to hydrogen peroxide were found, but the differences were not linked to the *rpoS* status. This suggests that *Salmonella* serotype Typhimurium *rpoS* mutants are counterselected because *rpoS* plays a role in the pathogenesis of *Salmonella* serotype Typhimurium in humans or in the transmission cycle of the disease.

The alternative sigma factor, σ^S (RpoS), plays a key role in the survival of bacteria during starvation or exposure to stress conditions and is required for the expression of many genes in the stationary phase of growth (for reviews see references 13 and 16). σ^S levels are maximal at the onset of the stationary phase and are controlled at several levels, including transcription, translation, and protein turnover (for reviews see references 14 and 16). RpoS plays a key role in the virulence of *Salmonella* in mice (5, 6, 8, 24, 30, 31). Salmonellae are enteric pathogens that cause a wide range of host- and serotype-specific illnesses, including gastroenteritis and enteric fever. In mice, *Salmonella enterica* serotype Typhimurium infection results in a systemic illness similar to human enteric (typhoid) fever caused by *S. enterica* serotype Typhi.

In *Salmonella* serotype Typhimurium, σ^S controls expression of the *Salmonella* virulence plasmid genes, *spvRABCD* (8, 31), which control the growth rate of *Salmonella* in deep organs (for a review see reference 12). The *spvB* gene product is an ADP-ribosyltransferase that may promote the growth of *Salmonella* within macrophages (26, 33, 43). The *spv* genes are not required for the pathogenesis of *Salmonella* serotype Typhimurium gastroenteritis in humans but are thought to be important for the pathogenesis of *Salmonella* serotype Typhimurium bacteremia in humans (11, 28). σ^S also regulates chromosomal genes required for the colonization of Peyer's patches (6, 30) and for persistence in infected mice (24). *Salmonella* serotype Typhi does not contain a virulence plasmid, and the role of *rpoS* in the virulence of this serotype is not known yet. However, *rpoS* might also contribute to the virulence of this serotype because *Salmonella* serotype Typhi *rpoS* mutants appear to be less cytotoxic for macrophages than wild-type *Salmonella* serotype Typhi is (22).

Several groups of workers have described a natural variation of the *rpoS* gene in laboratory strains of *Escherichia coli* (1, 10, 17, 18, 44). In addition, mutant *rpoS* alleles have been detected in clinical isolates of Shiga-like toxin-producing *E. coli* (45) and in natural isolates of enterohemorrhagic *E. coli* O157:H7 (38). *rpoS* allelic variation has also been observed in highly passaged laboratory strains of *Salmonella* (36, 41, 42, 46). However, little information is available concerning the presence of mutant *rpoS* alleles in clinical isolates of *Salmonella* (20, 21). Our aim was to determine the prevalence of *rpoS* mutant alleles in recent human clinical isolates of *Salmonella* serotype Typhi and *Salmonella* serotype Typhimurium. Our results indicated that *rpoS* mutants can frequently be found among isolates of *Salmonella* serotype Typhi but not among isolates of *Salmonella* serotype Typhimurium, suggesting that *Salmonella* serotype Typhimurium *rpoS* mutants are counterselected. This result is consistent with the hypothesis that *rpoS* contributes to the pathogenesis of *Salmonella* serotype Typhimurium in humans or to the transmission cycle of the disease.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. A strain of *Salmonella* serotype Typhimurium that is virulent in mice (SL1344) and an isogenic

Δ*rpoS*::*kan* derivative of this strain (SL1344K) were used in this study (6). SL1344::2.4 is a derivative of SL1344 and contains transposon Tn*5*B21 inserted into the HPII catalase-encoding gene *katE* (15). *Salmonella* serotype Typhi and *Salmonella* serotype Typhimurium clinical isolates were provided by F. Grimont and P. Bouvet from the National Reference Center for *Salmonella* (Unité de Biodiversité des Bactéries Pathogènes Emergentes, Institut Pasteur). *E. coli* S17-1 (*pro thi recA hsdR*, chromosomal RP4-2, Tn*1*::ISR*1* Tc::Mu Km::Tn*7*) carries the transfer genes of plasmid RP4 on its chromosome and allows mobilization of plasmids in which the Mob region of plasmid RP4 has been cloned (40). pSTF4 contains a transcriptional *spvRAB'-lacZ* fusion (7). pVK100 is a mobilizable, low-copy-number cloning vector (23). pVKKatF contains the 2-kb *Sal*I fragment carrying the *Salmonella* serotype Typhimurium *rpoS* gene from pSTK5 (24) cloned into the *Sal*I restriction site of pVK100. The 1.1-kb *Hin*dIII-*Sal*I fragment carrying the *cat* cartridge from pAMPCm (37) was cloned into the *Hin*dIII-*Xho*I restriction sites of pVKKatF to obtain pVKKatFCm. pUCK3Km contains the *Salmonella* serotype Typhimurium *rpoS* region in which the 0.8-kb *Hpa*I-*Pst*I fragment in *rpoS* has been replaced by the 1.3-kb *Hin*cII fragment encoding the kanamycin resistance gene (24). Strains were grown at 37°C in Luria-Bertani medium (LB) (39). Minimal medium was M9 (39) containing 0.4% glucose or 0.2% propionate. *Salmonella* serotype Typhi was grown in minimal medium supplemented with tryptophan, cysteine, valine, and isoleucine (20 μg ml^{-1} each). When appropriate, the following antibiotics were added: carbenicillin, 100 μg ml^{-1}; chloramphenicol, 30 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; and tetracycline, 20 μg ml^{-1}.

DNA manipulations and enzyme assays. Standard molecular biological techniques and genetic exchange methods were used (39). PCR-amplified DNA products were sequenced by Genome Express (Paris, France). Plasmid DNA was introduced into *Salmonella* strains by electroporation. Mobilizable plasmids were transferred into *Salmonella* strains by conjugation by using *E. coli* S17-1 as the donor strain. Conjugation between *E. coli* and *Salmonella* was carried out by plate mating overnight at 37°C. Transconjugants were selected on minimal medium containing appropriate antibiotics. To measure catalase activity, cells were grown to the stationary phase, washed, resuspended in phosphate buffer (50 mM potassium phosphate, 0.1 mM EDTA; pH 7.8) to 1/10 the original culture volume, and lysed by sonication. Samples were kept on ice. Cell debris was removed by centrifugation at 4°C for 30 min at 18,000 × *g*. The amount of protein in each whole-cell lysate was determined by the Coomassie brilliant blue assay (Pierce Chemicals). Catalase activity was visualized by the method of Woodbury et al. (47) on 10% nondenaturing polyacrylamide gels. This method is based on reduction of potassium ferricyanide(III) to potassium ferrocyanide(II) by hydrogen peroxide (H_2O_2), which reacts with ferric chloride to form stable, insoluble Prussian blue pigment. Therefore, if H_2O_2 is broken down by catalase, a clear zone is observed. β-Galactosidase activity was measured as described by Miller (27) and was expressed in Miller units (27).

Construction of *Salmonella* serotype Typhi *rpoS* strains. pUCK3Km was used to construct a defined *rpoS* mutant of *Salmonella* serotype Typhi by using strain 5959, a *Salmonella* serotype Typhi strain isolated from human blood, as the parental strain. After electroporation in *Salmonella* serotype Typhi strain 5959, pUCK3Km appeared to be unstable. Recombination of the *kan* cartridge into the host genome, with simultaneous loss of pUCK3Km, resulted in isolation of clones that were resistant to kanamycin and sensitive to carbenicillin. The presence of the *rpoS* mutation at the appropriate site in the genome of one clone, designated 5959K, was confirmed by hybridization with an *rpoS*-specific probe as described previously (24).

Western blot analysis. Proteins were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis. Equal amounts of proteins were loaded in the lanes. Proteins were transferred onto reinforced cellulose nitrate membranes (Schleicher & Schuell) and incubated with an anti-σ^S polyclonal rabbit serum as previously described (6). The bound antibodies were detected by using a secondary anti-rabbit antibody linked to peroxidase and an ECL Western blotting reagent kit (Amersham Life Sciences).

Survival assays. For the oxidative shock survival assay, cells were grown overnight in LB, washed, and resuspended in 0.9% NaCl to an optical density at 600 nm of 1.0. H_2O_2 was added to a final concentration of 15 mM. For the heat shock survival assay, cells were grown to the stationary phase in LB, washed, diluted in 0.9% NaCl to a concentration of about 3,000 cells ml^{-1}, and placed in glass tubes that had been prewarmed at 55°C. In both experiments, aliquots of bacteria were removed at timed intervals, and numbers of viable cells were determined on LB plates.

RESULTS AND DISCUSSION

σ^S production by clinical isolates of *Salmonella*. To determine the prevalence of *rpoS* mutant alleles in a natural population consisting of pathogenic *Salmonella* serotype Typhi and *Salmonella* serotype Typhimurium, we first tested 41 *Salmonella* serotype Typhi and 75 *Salmonella* serotype Typhimurium clinical isolates for the production of σ^S protein. All isolates were collected from infected patients; all of the *Salmonella* serotype Typhi strains and 38 of the *Salmonella* serotype Typhimurium strains were isolated from blood, and 37 of the *Salmonella* serotype Typhimurium strains were isolated from feces. Although the exact passage histories of these isolates were not known, we used strains that had been sent to the French National Reference Center for *Salmonella* from clinical institutions following primary isolation and had not been extensively subcultured. In addition, isolates were collected from different geographic areas to avoid analysis of epidemiologically related isolates. Accordingly, it was established that the *Salmonella* serotype Typhi isolates belong to 14 different phage types (F. Grimont, personal communication). All of the *Salmonella* serotype Typhimurium isolates and 32 of the *Salmonella* serotype Typhi isolates tested produced a σ^S protein that migrated like the σ^S protein of the wild-type strains *Salmonella* serotype Typhimurium strain SL1344 and *Salmonella* serotype Typhi strain 5959 (Table 1), as exemplified by strains C1, H1, and Ty04 (Fig. 1). In contrast, 8 of the 41 *Salmonella* serotype Typhi isolates were impaired in the ability to produce a σ^S protein (Table 1), as exemplified by Ty02 (Fig. 1), and one strain, Ty39, produced a truncated σ^S protein (38 instead of 42 kDa) (Fig. 1 and Table 1).

***rpoS* mutants among clinical isolates of *Salmonella*.** σ^S controls the general stress response of stationary-phase bacteria (for reviews see references 13 and 16). In particular, *rpoS* mutants are impaired in the ability to resist hydrogen peroxide (H_2O_2) during the stationary phase. Indeed, two of the three catalases produced by *Salmonella* are expressed in the stationary phase under the control of *rpoS*; these are KatE and KatN, a major catalase and a minor catalase, respectively (4, 35). Therefore, to obtain further information on the presence of *rpoS* mutant alleles, we carried out a semiquantitative visual examination of σ^S expression in these strains by adding H_2O_2 to the bacterial colony mass derived from a nutrient agar plate. The extent of bubbling for most strains was similar to that for the positive controls (*Salmonella* serotype Typhi strain 5959 and *Salmonella* serotype Typhimurium strain SL1344), suggesting that sufficient active σ^S was present for catalase production in these strains. However, in the nine *Salmonella* serotype Typhi strains that were impaired in the production of wild-type σ^S protein and in an additional six *Salmonella* serotype Typhi and five *Salmonella* serotype Typhimurium strains, the extent of bubbling was less than that observed with the positive controls (data not shown). These strains thus appeared to be deficient in expression or activity of the catalase. Consistent with this, these strains had an impaired capacity to resist H_2O_2 stress in the stationary phase of growth (Table 1 and Fig. 2). Twelve of these strains (including those that did not produce σ^S) were highly sensitive to H_2O_2, and eight of them (three *Salmonella* serotype Typhi strains and five *Salmonella*

TABLE 1. Characteristics of strains used

Strain	σ^s production[a]	H_2O_2 resistance (%)[b]	Growth on propionate[c]	*spvRAB-lacZ* expression[d]
Salmonella serotype Typhi controls				
5959	+	100	−	742 ± 122
5959K (Δ*rpoS::kan*)	−	0[e]	+	9 ± 2
Salmonella serotype Typhi blood isolates				
Ty01, Ty10, Ty13, Ty14, Ty15, Ty16, Ty17, Ty18, Ty20, Ty21, Ty22, Ty24, Ty25, Ty27, Ty29, Ty32, Ty35, Ty36, Ty37, Ty38, Ty40, Ty41, Ty42, Ty43, Ty44, Ty45	+	100	—[f]	473 ± 95
Ty02	−	0	+	8 ± 3
Ty03	+	0	+	11 ± 4
Ty04	+	8	−	81 ± 12
Ty06	+	0	+	ND
Ty08	−	0	+	7 ± 2
Ty09	−	0	+	ND
Ty11	−	0	+	7 ± 1
Ty12	+	76	−	20 ± 6
Ty23	+	0.001	+	159 ± 52
Ty26	−	0	+	5 ± 1
Ty28	+	0	+	21 ± 2
Ty30	−	0	+	9 ± 2
Ty31	−	0	+	ND
Ty33	−	0	+	ND
Ty39	Truncated	0	+	8 ± 2
Salmonella serotype Typhimurium controls				
SL1344	+	100	−	ND
SL1344K (Δ*rpoS::kan*)	−	0	+	ND
Salmonella serotype Typhimurium blood isolates				
H1, H2, H4, H5, H6, H7, H8, H9, H10, H11, H15, H16, H17, H18, H19, H20, H21, H22, H23, H25, H28, H30, H31, H32, H33, H34, H35, H36, H37, H38, H39, H40, H41, H42	+	100	—[g]	ND
H3	+	0.0003	−	ND
H12	+	0.007	−	ND
H24	+	0.0004	−	ND
H26	+	0.001	−	ND
Salmonella serotype Typhimurium fecal isolates				
C1, C2, C3, C4, C5, C8, C9, C10, C11, C12, C13, C14, C15, C16, C17, C18, C19, C20, C21, C22, C23, C24, C25, C26, C27, C28, C30, C31, C32, C33, C34, C35, C36, C37, C38, C39	+	100	—[h]	ND
C6	+	0.0003	−	ND

[a] σ^s production was examined by analyzing total proteins by Western blotting with an anti-σ^s polyclonal rabbit serum.
[b] Level of survival after 60 min of exposure to 15 mM H_2O_2 compared to the survival at time zero. The data are from one representative experiment.
[c] An overnight LB culture was diluted 10^6-fold in 5 ml of minimal medium containing 0.2% propionate and incubated on a shaker at 37°C. Growth was recorded after 3 days for *Salmonella* serotype Typhimurium and after 5 days for *Salmonella* serotype Typhi. Strains were able to grow in minimal medium containing glucose. Similar results were obtained in two independent experiments.
[d] Strains containing the *spvRAB-lacZ* fusion on pSTF4 were grown overnight in LB and assayed for β-galactosidase activity. The results are expressed in Miller units (27). The values are means ± standard deviations from three independent experiments. ND, not determined.
[e] No CFU were detectable when 10^9 bacteria were exposed to 15 mM H_2O_2 for 60 min.
[f] Determined for only Ty10.
[g] Determined for only H1.
[h] Determined for only C1.

serotype Typhimurium isolates) showed intermediate levels of resistance to H_2O_2.

To determine whether the H_2O_2-sensitive *Salmonella* serotype Typhi and *Salmonella* serotype Typhimurium strains had a defect in *rpoS*, complementation studies were performed. A low-copy-number plasmid containing a functional *rpoS* gene (pVKKatF or pVKKatFCm) was introduced into the H_2O_2-sensitive *Salmonella* serotype Typhi and *Salmonella* serotype Typhimurium isolates by conjugation. Transconjugants were tested for H_2O_2 resistance in the stationary phase. All of the *Salmonella* serotype Typhi transconjugants were able to resist H_2O_2 at levels similar to the level resisted by *Salmonella* serotype Typhi wild-type strain 5959, suggesting that these strains contained an altered *rpoS* allele (data not shown). In contrast, the *Salmonella* serotype Typhimurium strains were not complemented for H_2O_2 resistance when the cloned *rpoS* gene was supplied in *trans* (data not shown). This strongly suggested that the H_2O_2 sensitivity of the *Salmonella* serotype Typhimurium

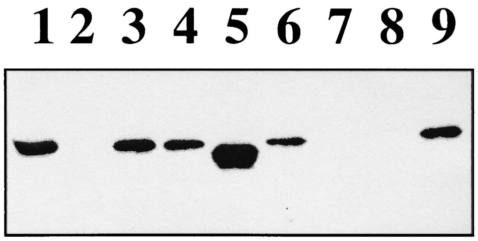

FIG. 1. Detection of σ^S in *Salmonella* strains. Whole-cell extracts, prepared from stationary-phase LB cultures, were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10 μg per lane) and subjected to immunoblot analysis with an anti-σ^S polyclonal antibody. *Salmonella* serotype Typhimurium strain SL1344 and the isogenic Δ*rpoS* mutant SL1344K were used as controls. Lane 1, SL1344; lane 2, SL1344K; lane 3, C1; lane 4, H1; lane 5, Ty39; lane 6, 5959; lane 7, 5959K; lane 8, Ty02; lane 9, Ty04.

isolates was not the result of a mutated *rpoS* allele but more likely was the result of another defect.

We observed that *Salmonella rpoS* mutants grow more rapidly than wild-type strains grow in minimal medium containing propionate as the sole carbon source. After 3 days of incubation in minimal medium containing propionate, no growth of wild-type strain SL1344 and the H_2O_2-sensitive strains of *Salmonella* serotype Typhimurium was apparent (Table 1). In contrast, *rpoS* mutant SL1344K was able to grow in these conditions (Table 1). To confirm that the H_2O_2-sensitive *Salmonella* serotype Typhimurium strains did not contain a mutant *rpoS* allele, the *rpoS* gene from these strains was PCR amplified and sequenced. The nucleotide sequences of *rpoS* in these five strains were identical to that of the *rpoS* gene of *Salmonella* serotype Typhimurium strain C52 (24), indicating that these strains indeed contained a wild-type *rpoS* gene. To investigate the H_2O_2 sensitivity phenotype of these strains further, we measured their catalase activities on native polyacrylamide gels. The five strains expressed the *katG* catalase and the *rpoS*-controlled *katN* catalase but lacked the *rpoS*-controlled *katE* catalase (Fig. 3). PCR amplification of total DNA with primers specific for the *katE* open reading frame indicated that the strains contained a *katE* sequence. Therefore, the H_2O_2-sensitive *Salmonella* serotype Typhimurium strains probably have mutations in *katE* or in a gene required for KatE activity or *katE* expression.

To investigate this issue further, the *katE* genes from three of these strains (H3, H24, and H26) were sequenced. The three *katE* alleles were different from each other and different from the *katE* allele of *Salmonella* serotype Typhimurium strain SL1344 (accession no. AJ289167). Compared to SL1344, *Salmonella* serotype Typhimurium strains H3 and H24 contained point mutations at codons 140 (AAC [Asn] instead of GAC [Asp]), 141 (CTA [Leu] instead of CTG [Leu]), and 189 (TAA [OCH] instead of GAA [Glu]). Strain H24 had an additional mutation at codon 254 (ATA [Ile] instead of ATG [Met]). The stop codon at position 189 in the *katE* sequence of *Salmonella* serotype Typhimurium strains H3 and H24 probably results in a truncated and inactive KatE product. The nucleotide sequence of the *katE* open reading frame in strain H26 was identical to that in *Salmonella* serotype Typhimurium strain SL1344 except for a mutation at codon 117 (GGG [Gly] instead of GAG [Glu]). The activity of the mutant protein may

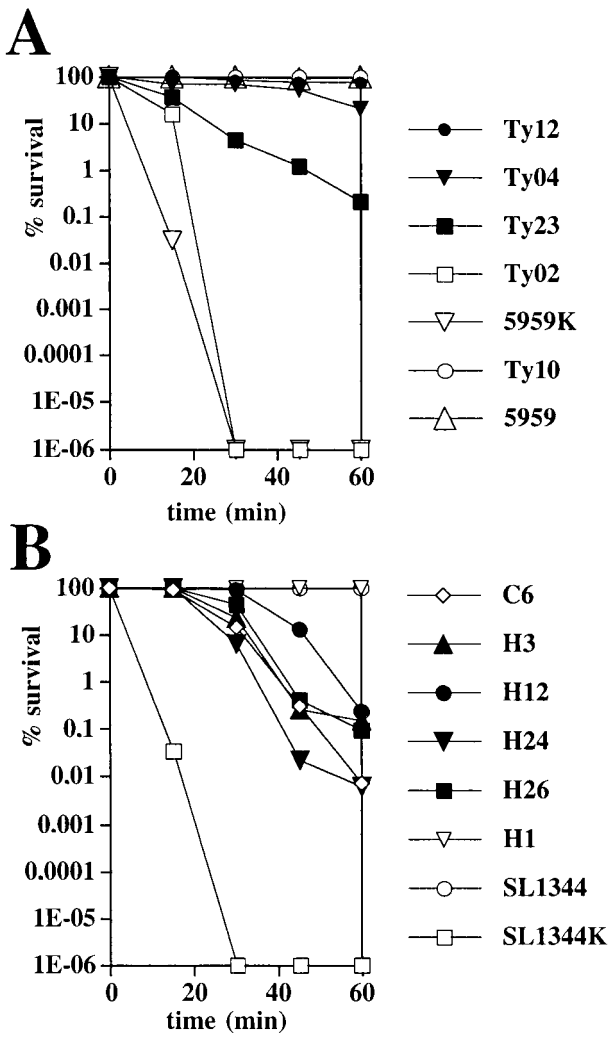

FIG. 2. Resistance to hydrogen peroxide. The viabilities of *Salmonella* serotype Typhi (A) and *Salmonella* serotype Typhimurium (B) stationary-phase LB cultures were determined in the presence of 15 mM hydrogen peroxide (H_2O_2). The results of representative experiments are shown. Wild-type strains of *Salmonella* serotype Typhi (strain 5959) and *Salmonella* serotype Typhimurium (strain SL1344) and the corresponding *rpoS* mutants (strains 5959K and SL1344K, respectively) were used as controls.

have been affected because the mutation was located within the catalase proximal active site signature (seven codons upstream of the putative active site His residue, PS00438).

***rpoS* allelic variation in *Salmonella* serotype Typhi.** To determine the nature of the *rpoS* mutations occurring in *Salmonella* serotype Typhi isolates, the *rpoS* genes from one σ^S-positive, H_2O_2-resistant strain (Ty10), four σ^S-negative, H_2O_2-sensitive strains (Ty02, Ty08, Ty26, and Ty30), the σ^S-positive H_2O_2-sensitive strains (Ty03, Ty04, Ty06, Ty12, Ty23, and Ty28), and strain Ty39 producing the truncated σ^S protein were PCR amplified and sequenced. The nucleotide sequence of the *rpoS* open reading frame of strain Ty10 was identical to that of *Salmonella* serotype Typhimurium strain C52 (24) except for a neutral C-to-T mutation at nucleotide 474. This mutation is present in all of the *Salmonella* serotype Typhi *rpoS*

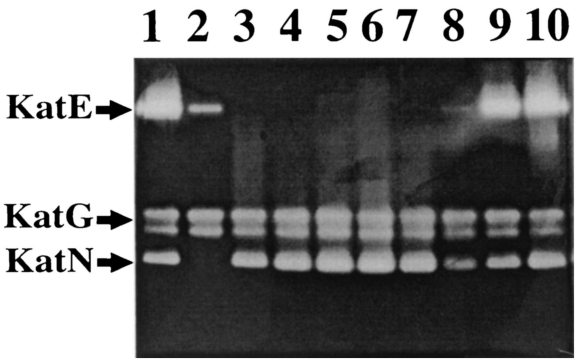

FIG. 3. Visualization of *Salmonella* serotype Typhimurium catalases on polyacrylamide gels. Catalase activity was detected in nondenaturing 10% polyacrylamide gels as described by Woodbury et al. (47). The positions of the double band corresponding to the *katG* (HPI) catalase and the single bands corresponding to the RpoS-regulated catalases *katE* (HPII) and *katN* are indicated by arrows. Wild-type *Salmonella* serotype Typhimurium strain SL1344 and the isogenic *rpoS* and *katE* mutants (strains SL1344K and SL1344::2.4, respectively) were used as controls. Each lane was loaded with 80 μg of total protein. Lane 1, SL1344; lane 2, SL1344K; lane 3, SL1344::2.4; lane 4, C6; lane 5, H3; lane 6, H12; lane 7, H24; lane 8, H26; lane 9, H1; lane 10, C1.

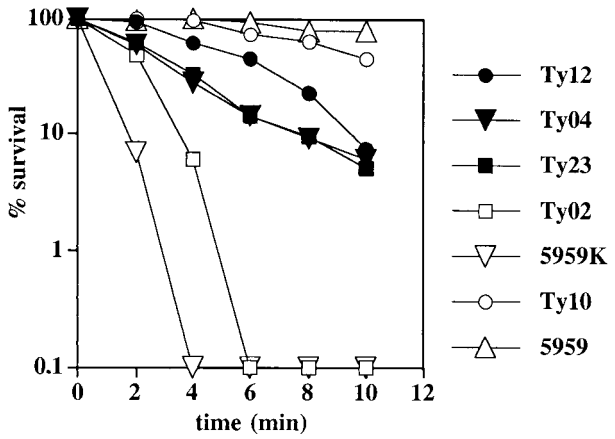

FIG. 4. Heat resistance of *Salmonella* serotype Typhi strains. The viabilities of *Salmonella* serotype Typhi stationary-phase LB cultures were determined at 55°C. The results of representative experiments are shown. Wild-type *Salmonella* serotype Typhi strain 5959 and the corresponding *rpoS* mutant, strain 5959K, were used as controls.

alleles that we have sequenced so far. Compared to Ty10, Ty08 contained an 18-bp insertion downstream of nucleotide 774, Ty26 contained a 68-bp deletion downstream of nucleotide 367, Ty30 contained a 10-bp deletion downstream of nucleotide 109, and Ty02 contained a point mutation at codon 148 (TAG [AMB] instead of TGG [Trp]). In each case, the mutation resulted in the appearance of a stop codon before the end of the *rpoS* gene. These stop codons probably result in a truncated σ^S, which could be less stable and active than full-length σ^S. Strains Ty03, Ty06, and Ty28 contained point mutations at codons 123 (GAC [Asp] instead of GGC [Gly]), 175 (CCG [Pro] instead of CTG [Leu]), and 118 (AAC [Asn] instead of GAC [Asp]), respectively. These mutations did not modify the production of σ^S. However, the activities of the mutant proteins may have been affected because the mutations were clustered in the following two important regions of the sigma factor: region 2.2, which is essential for binding to the RNA polymerase core (48), and region 2.5, which is involved in the DNA-binding activity of the sigma factor (3, 29). Furthermore, strain Ty39, which produced a truncated σ^S protein, contained a 78-bp in-frame deletion from nucleotide 303 to nucleotide 380 that removed most of regions 2.1 and 2.2.

Three *Salmonella* serotype Typhi strains (Ty04, Ty12, and Ty23) showed intermediate levels of H_2O_2 resistance (Fig. 2 and Table 1) and catalase activity (data not shown), in contrast to the *Salmonella* serotype Typhi *rpoS* mutants identified above. Therefore, these strains may contain attenuated *rpoS* alleles. DNA sequence analysis revealed that these strains contained mutations in region 1.2 of σ^S. Strains Ty04 and Ty23 contained point mutations at codon 61 (AAC [Asn] and TCC [Ser] instead of TAC [Tyr], respectively), whereas strain Ty12 contained an additional GAA (Glu) codon at position 77. In σ^{70}, region 1.2 is important for the DNA-binding and transcriptional activities of the sigma factor (2). The function of this region in σ^S is not known, and a recent study indicated that a σ^S protein lacking the first 50 residues is active in vivo (34). To determine the extent of inactivation of σ^S in these strains, we tested additional phenotypic traits whose expression is dependent on *rpoS*. These traits included growth on propionate as the sole carbon source, heat resistance, and expression of the *Salmonella* serotype Typhimurium *rpoS*-regulated *spvRAB-lacZ* gene fusion. The three strains showed similar heat resistance levels and were significantly more resistant to heat than *rpoS* null mutants were (Fig. 4). The expression level of the *spvRAB-lacZ* fusion in strain Ty12 was similar to that in the *rpoS* null mutant (Table 1). However, the fusion was expressed at a higher level in strains Ty04 and Ty23 (11 and 21% compared to wild-type strain 5959 [Table 1]). Thus, *spv* gene expression was affected by *rpoS* mutations less in Ty04 and Ty23 than in Ty12. Finally, strains Ty04 and Ty12 were not able to grow on propionate, unlike strain Ty23 and the *Salmonella* serotype Typhi *rpoS* null mutants (Table 1). This result was surprising because Ty04 and Ty23 harbor mutations at the same position. The nature of the amino acid substitution at codon 61 (Asn in Ty04 and Ser in Ty23 instead of Tyr in wild-type strains) may play an important role in the ability of a strain to grow on propionate. Alternatively, the observed phenotype may be associated with another gene, and not *rpoS*, in at least one of the strains. In conclusion, σ^S was not fully inactivated in these three strains, and it is likely that the three mutations are not equivalent and confer different phenotypes to *Salmonella* serotype Typhi. However, the corresponding *rpoS* mutant alleles need to be studied in otherwise isogenic strains to investigate this issue further.

Conclusion. σ^S is a master regulator of stationary-phase survival and stress resistance. However, there may be a selective advantage in losing σ^S function during growth in nonstressful conditions. The loss of σ^S in *E. coli* growing in glucose-limited chemostat conditions results in increased expression of σ^{70}-dependent genes encoding glucose uptake that contribute to fitness in these conditions (32). As expected, there is a trade-off between increased fitness and reduced stress resistance in *rpoS* mutants (32). *rpoS* attenuated muta-

tions can also confer a selective advantage to *E. coli* growing on amino acids as carbon sources (49). In addition, *rpoS* mutants of *Salmonella* grow more rapidly than wild-type strains grow in minimal medium containing propionate as the sole carbon source (Table 1). The increased fitness of *rpoS* mutants in these conditions may result from a sigma factor competition phenomenon. Indeed, it has been suggested that RNA polymerase is limiting and that σ^S and σ^{70} compete for RNA polymerase binding (9, 16, 19). According to this model, more σ^{70} proteins are able to bind the RNA polymerase core in the absence of any competing σ^S and more resources are directed towards growth-related functions depending on the σ^{70} activity. This might explain why mutations in *rpoS* are so common in laboratory strains. In addition, mutant *rpoS* alleles may confer an advantage in some natural environments. *E. coli rpoS* mutants may have a competitive advantage for growth in the large intestine of mice (25). In addition, *rpoS* mutants have been found among clinical isolates of Shiga-like toxin-producing *E. coli* (about 20%) (45) and in natural isolates of enterohemorrhagic *E. coli* O157:H7 (38).

Although *rpoS* allelic variation has been demonstrated in archival cultures of *Salmonella* (36, 41, 42, 46), there is little information available concerning the presence of mutant *rpoS* alleles in clinical isolates of *Salmonella*. Jordan et al. (20) examined *rpoS* variability in 18 environmental isolates of different serotypes of salmonellae by single-strand conformation polymorphism analysis. Sequence variations were detected, but phenotypic differences were not sought. In another study (21), 1 of 38 strains of *Salmonella* serotype Typhimurium (DT104) and 2 of 40 *S. enterica* serotype Enteritidis phage type 4 strains were found to be *rpoS* mutants and were more sensitive to stresses than other strains. We found that *rpoS* mutants having a null or attenuated phenotype can frequently be found among *Salmonella* serotype Typhi clinical isolates (36%). Different mutations showing that *rpoS* mutant alleles are not clonal in nature were identified. These strains may display altered virulence properties because the ability of a strain to cause infection also depends on factors such as host susceptibility and infectious dose. However, more data are necessary before we can fully address this aspect of *Salmonella* serotype Typhi virulence. In contrast to the situation in *Salmonella* serotype Typhi, no *rpoS* mutants were found among the 75 *Salmonella* serotype Typhimurium strains isolated from patients with systemic or intestinal infections. This strongly suggests that *rpoS* mutants of *Salmonella* serotype Typhimurium are counterselected. This is consistent with the hypothesis that *rpoS* contributes to the pathogenesis of *Salmonella* serotype Typhimurium in humans. RpoS controls expression of the virulence plasmid genes, *spvRABCD* (8, 31). These genes do not contribute to the pathogenesis of *Salmonella* gastroenteritis but appear to be associated with systemic infections in animals and humans (11, 28). In addition, RpoS regulates chromosomal genes involved in the colonization of Peyer's patches and persistence in infected mice (6, 24, 30), which might play a role during intestinal or systemic infections in humans. Alternatively, as contamination with *Salmonella* serotype Typhimurium usually results from ingestion of food or water contaminated by infected feces from humans or animals, *Salmonella* serotype Typhimurium *rpoS* mutants may be counterselected during the transmission cycle of the disease.

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