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Identification of RpoS (σ^S)-Regulated Genes in *Salmonella enterica* Serovar Typhimurium

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The *rpoS* gene encodes the alternative sigma factor σ^S (RpoS) and is required for survival of bacteria under starvation and stress conditions. It is also essential for *Salmonella* virulence in mice. Most work on the RpoS regulon has been in the closely related enterobacterial species *Escherichia coli*. To characterize the RpoS regulon in *Salmonella*, we isolated 38 unique RpoS-activated *lacZ* gene fusions from a bank of *Salmonella enterica* serovar Typhimurium mutants harboring random Tn*5*B21 mutations. Dependence on RpoS varied from 3-fold to over 95-fold, and all gene fusions isolated were regulated by growth phase. The identities of 21 RpoS-dependent fusions were determined by DNA sequence analysis. Seven of the fusions mapped to DNA regions in *Salmonella* serovar Typhimurium that do not match any known *E. coli* sequence, suggesting that the composition of the RpoS regulon differs markedly in the two species. The other 14 fusions mapped to 13 DNA regions very similar to *E. coli* sequences. None of the insertion mutations in DNA regions common to both species appeared to affect *Salmonella* virulence in BALB/c mice. Of these, only three (*otsA*, *katE*, and *poxB*) are located in known members of the RpoS regulon. Ten insertions mapped in nine open reading frames of unknown function (*yciF*, *yehY*, *yhjY*, *yncC*, *yjgB*, *yahO*, *ygaU*, *ycgB*, and *yeaG*) appear to be novel members of the RpoS regulon. One insertion, that in mutant C52::H87, was in the noncoding region upstream from *ogt*, encoding a O^6-methylguanine DNA methyltransferase involved in repairing alkylation damage in DNA. The *ogt* coding sequence is very similar to the *E. coli* homolog, but the *ogt* 5′ flanking regions were found to be markedly different in the two species, suggesting genetic rearrangements. Using primer extension assays, a specific *ogt* mRNA start site was detected in RNAs of the *Salmonella* serovar Typhimurium wild-type strains C52 and SL1344 but not in RNAs of the mutant strains C52K (*rpoS*), SL1344K (*rpoS*), and C52::H87. In mutant C52::H87, Tn*5*B21 is inserted at the *ogt* mRNA start site, with *lacZ* presumably transcribed from the identified RpoS-regulated promoter. These results indicate that *ogt* gene expression in *Salmonella* is regulated by RpoS in stationary phase of growth in rich medium, a finding that suggests a novel role for RpoS in DNA repair functions.

The alternative sigma factor σ^S (also known as RpoS, KatF, or σ^{38}) plays a key role in the survival of bacteria under starvation or stress conditions (for reviews, see references 14, 16, and 22). Homologs of RpoS have been found in a number of bacteria, but most work on the RpoS regulon has been in *Escherichia coli.* The number of genes shown to be subjected to RpoS regulation has already reached the 50 predicted by two-dimensional gel analysis of cell extracts, yet most RpoS-regulated genes and functions remain unknown.

During rapid growth in the laboratory, *E. coli* contains extremely little RpoS. The RpoS protein is most abundant at the onset of the stationary phase of growth, the maximum level being 30% of that of σ^{70} (for reviews, see references 14, 16, and 22). Indeed, onset of the stationary phase induces the RpoS regulon. Expression of RpoS is also induced when cells are exposed to certain stress conditions even during exponential phase, resulting in the activation of a number of RpoS-dependent promoters (for reviews, see references 14, 16, and 22). The cellular level of RpoS is regulated by mechanisms involving transcription, translation, and posttranslational stability. Different stress conditions differentially affect these various levels of control to create a complex regulatory profile.

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Salmonellae are enteric pathogens that cause a wide range of host- and serotype-specific illnesses, including gastroenteritis and enteric fever. *Salmonella enterica* serovar Typhimurium (serovar Typhimurium) infection of mice results in a systemic illness similar to human enteric (typhoid) fever, with bacteria disseminating to organs rich in phagocytic cells. In serovar Typhimurium, σ^S controls expression of the *Salmonella* virulence plasmid *spv* genes (10, 26). The *spvRABCD* gene cluster controls the growth rate of *Salmonella* in deep organs and is required for systemic infection and bacteremia in animals and humans (for a review, see reference 11). As expected, *Salmonella rpoS* mutants have a severely impaired capacity to colonize spleens of infected mice (4, 7, 20). In addition, *rpoS* mutations reduce the ability of serovar Typhimurium to colonize Peyer's patches of infected mice (7, 25) and decrease the persistence of virulence plasmid-cured strains in the spleen (20). These effects presumably result from the inappropriate expression of one or more unidentified *rpoS*-regulated chromosomal genes. The human-restricted *Salmonella* serovars such as Typhi, which causes typhoid fever, have no virulence plasmid, and the role of *rpoS* in the virulence of these serovars is unknown. However, an *rpoS* mutant of serovar Typhi is less cytotoxic for macrophages than the parental strain, and therefore *rpoS* may be involved in the virulence of serovar Typhi in humans (18). Interestingly, *Salmonella rpoS* mutants are efficient live vaccines (6, 7, 29), and an *rpoS* mu-

tation increases the attenuation of *aroA* serovar Typhimurium live vaccines in BALB/c mice and athymic BALB/c mice (6, 7).

Identification of σ^S-regulated genes in *Salmonella* may lead to characterization of novel factors contributing to the persistence of the pathogen in the environment and hosts. We therefore studied the RpoS regulon of *Salmonella.* This report describes a method for identifying σ^S-regulated genes in serovar Typhimurium by using *lacZ* transcriptional fusions carried on Tn*5*B21 transposon insertions. In the first screening, 38 unique σ^S-regulated *lacZ* gene fusions were isolated. We report a preliminary characterization of 21 of these mutants in *Salmonella* and a comparative analysis with the closely related species *E. coli.* Fourteen of the fusions mapped to genes present in both species, and ten of them are new members of the RpoS regulon. One of these new σ^S-regulated genes has been identified as *ogt*, encoding a O^6-methylguanine (O6MeG) DNA methyltransferase (MTase) involved in the repair of alkylation damage in DNA.

MATERIALS AND METHODS

Bacterial strains, plasmids, phages, and growth conditions. The *E. coli* strains used were S17-1 (*pro thi recA hsdR*, chromosomal RP4-2; Tn*1*::ISR*1* Tc::Mu Km::Tn*7*) (34) and MC1061 [*araD139* Δ(*ara-leu*)*7697 rpsL galU galK* Δ(*lacI POZY*)FX74] (3). The serovar Typhimurium mouse virulent strains used were C52 and SL1344 and their isogenic Δ*rpoS*::*kan* derivatives C52K and SL1344K, respectively (7). Bacteriophage P22HT105.1/int was used to transduce mutations between *Salmonella* strains (33). Green plates for screening for P22-infected cells or lysogens were prepared as described previously (37). Strains were routinely cultured at 37°C in Luria-Bertani (LB) medium (32). Antibiotics were used at the following concentrations (micrograms per milliliter): carbenicillin, 100; chloramphenicol, 30; kanamycin, 50; streptomycin 100; and tetracycline, 20.

Plasmids. Plasmid pBDJ103 was used as a source of transposon Tn*5*B21 (17). Plasmid pUC19 (Cb^r) (42) and the mobilizable plasmid pVK100 (Tet^r Km^r) (19) were used as cloning vectors. Plasmid pVKCm (Tet^r Cm^r) contains the 1.1-kb *Hin*dIII-*Sal*I fragment carrying the *cat* gene from pAMPCm (30) inserted into the *Hin*dIII-*Xho*I sites of pVK100. The 2-kb *Sal*I fragment carrying the serovar Typhimurium *rpoS* gene from pSTK5 (20) was ligated into the *Sal*I site of pVKCm to yield pVKRpoS (Cm^r). DNA sequences flanking Tn*5*B21 insertions in serovar Typhimurium mutants were cloned by digesting genomic DNA and vector pUC19 with either *Hin*dIII or *Pst*I (for cloning of DNA 5′ to the insertion) and with *Eco*RI (for cloning DNA 3′ to the insertion), ligating, and selecting for *E. coli* MC1061 transformants carrying the tetracycline resistance gene from Tn*5*B21. To construct pUCogt1 carrying the *ydaL-ogt* intergenic region from serovar Typhimurium, a 413-bp DNA sequence was amplified from C52 total DNA by PCR using primers YDAL1 (5′-AGGCTCGGATCCCAGCGGCTGG ACATCCTCCATGGC-3′) and OGT1 (5′-TTCCGAAAGCTTCTGTTCCCAC TCAATGGCCCGC-3′) such that it acquired *Bam*HI and *Hin*dIII restriction sites at its 5′ and 3′ ends, respectively. The PCR-amplified fragment was then ligated into the *Bam*HI-*Hin*dIII sites of pUC19 to give pUCogt1. The nucleotide sequence of the PCR-amplified fragment in pUCogt1 was checked by DNA sequencing.

Transposon mutagenesis of serovar Typhimurium. Transposon Tn*5*B21 is a Tet^r derivative of Tn*5* which was constructed to make *lacZ* gene fusions (35). pBDJ103 is an Amp^r ColE1 derivative which carries Tn*5*B21 and an Sm^s allele for the ribosomal protein S12 (17). The presence of the S12 gene on this plasmid can be used to select positively (in a strain carrying the *rpsL* allele) for loss of the plasmid. Strain C52K-Sm^r is a spontaneous Sm^r mutant of serovar Typhimurium C52K selected for growth on LB agar containing 100 μg of streptomycin per ml. pDBJ103 was introduced into serovar Typhimurium C52K-Sm^r by electroporation, the resulting transformants were Km^r Tet^r Cb^r Sm^s. The protocol used to generate pools of Tn*5*B21 insertion mutants from C52K-Sm^r has been described previously (17). An exponentially growing culture, derived from a single colony of serovar Typhimurium C52K-Sm^r(pBDJ103), was diluted, and approximately 5,000 CFU were plated onto LB agar containing 20 μg of tetracycline per ml and grown overnight at 30°C. The colonies were then replica plated onto LB agar containing 100 μg of streptomycin per ml and 20 μg of tetracycline per ml and grown overnight at 37°C to select for transposition and loss of plasmid pDBJ103. Each Tet^r Sm^r colony obtained represents at least one unique Tn*5*B21 transposon insertion. All of the Tet^r and Sm^r colonies from a single plate were pooled. Eight independent pools of serovar Typhimurium Tn*5*B21 mutants (from eight plates labeled 1 to 3 and E to I) were generated by this procedure. More than 99% of the CFU in each pool were Amp^s, confirming the loss of plasmid pDBJ103. The serovar Typhimurium pools were then screened for mutants containing *rpoS*-regulated *lacZ* fusions as depicted in Fig. 1.

DNA and RNA manipulations and enzyme assays. Standard molecular biology techniques were used (30, 32). Oligonucleotides were purchased from Genset (Paris, France). Double-stranded DNA was sequenced with a Thermo Sequenase radiolabeled terminator cycle sequencing kit and Redivue 5′ α-^{33}P-labeled dideoxyribonucleotide triphosphates (Amersham Pharmacia). Primers TnlacZ and IS50R (Fig. 1), which anneal to the left and right ends of Tn*5*B21, respectively, and read into the flanking serovar Typhimurium genomic DNA insert, were used for sequencing. Homology searches were performed with both BLAST and FASTA computer programs on the website at the Institut Pasteur (http://www.pasteur.fr/). Feature annotations for *E. coli* sequences were found through the Colibri World Wide Web server provided by the Institut Pasteur (http:/genolist.pasteur.fr/Colibri/).

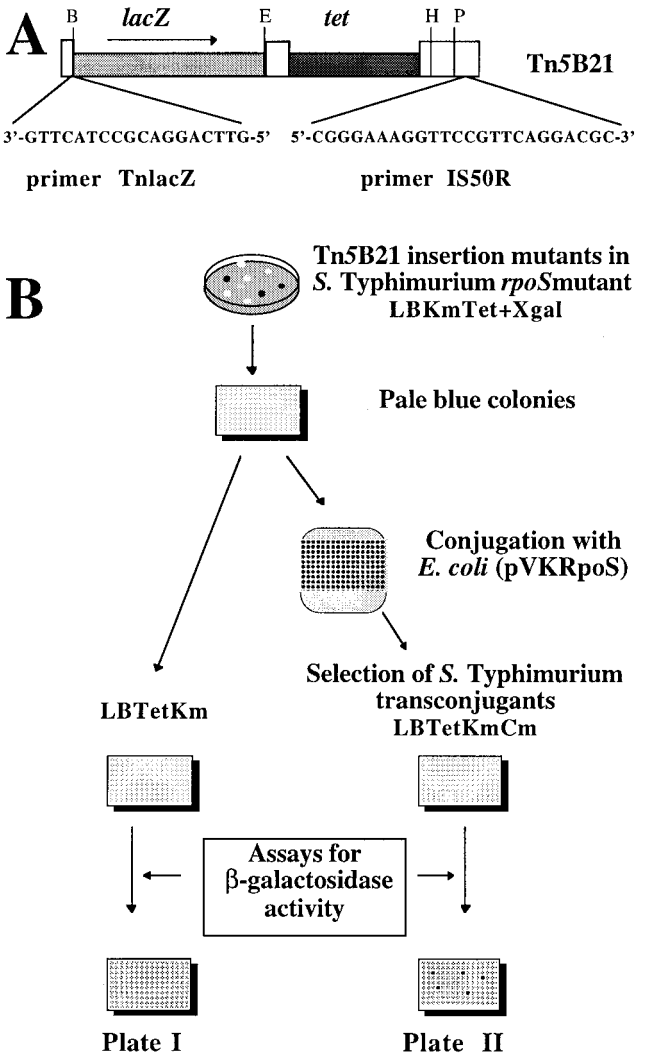

FIG. 1. Strategy used to select Tn*5*B21 insertion mutants of serovar Typhimurium carrying σ^S-dependent promoter-*lac*Z fusions. (A) Transposon Tn*5*B21 (35) and primers used for DNA sequencing. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I. (B) Transposon Tn*5*B21 insertion mutagenesis of the Sm^r Km^r serovar Typhimurium *rpoS* mutant C52K-Sm^r is described in Materials and Methods. Mutants containing poorly expressed *lacZ* gene fusions were identified as having a pale blue colony phenotype after growth in LB agar containing X-Gal. Such strains were used to inoculate 96-well enzyme-linked immunosorbent assay (ELISA) plates (0.2 ml of LB per well) and grown overnight at 37°C. Conjugation between these mutant strains and *E. coli* strain S17-1 carrying the mobilizable plasmid pVKRpoS (Cm^r) was carried out on LB agar plates for 4 to 5 h at 37°C (donor and recipient cells were mixed in a 1:1 ratio). Serovar Typhimurium transconjugants carrying pVKRpoS were selected in LB broth supplemented with 20 μg of tetracycline per ml 25 μg of kanamycin per ml, and 30 μg of chloramphenicol per ml (LBTetKmCm). β-Galactosidase activity of serovar Typhimurium Tn*5*B21 mutants with and without pVKRpoS (plates II and plate I, respectively) was assayed using 96-well ELISA plates and the method of Miller (24). An automated ELISA plate reader (Labsystems Multiskan RC) was used to measure A_{420} after 1 h of incubation at room temperature. Mutant candidates showing increased gene fusion activity on acquisition of plasmid pVKRpoS were selected for further studies.

Total RNA was extracted and primer extension experiments were performed as previously described (20). An oligonucleotide complementary to the 5′ end of the coding region of the *ogt* gene (OGT2; 5′-CCACCCATAACGGTCCTAAT GGCGTGGC-3′) was used in primer extension experiments. β-Galactosidase activity was measured as described by Miller (24) and is expressed in Miller units (ΔOD_{420} [optical density at 420 nm] per minute per OD_{600}).

Mouse infection. Female BALB/c mice, which are innately susceptible to serovar Typhimurium, were obtained from the Centre d'Elevage IFFA CREDO (Domaine des Oncins, L'Arbresle, France) and were used when approximately 7 to 8 weeks old. For inoculation of mice, bacteria were freshly streaked onto LB agar plates and the antigenic formulae of serovar Typhimurium strains were confirmed by slide agglutination using rabbit antisera specific for O- and H-antigen factors (Bio-Rad). Single colonies were used to inoculate LB broth, and the cultures were incubated overnight at 37°C with gentle shaking. Each culture was then diluted in fresh medium and incubated at 37°C until reaching an OD_{600} of approximately 0.5. The culture was centrifuged, and cells were resuspended in phosphate-buffered saline (pH 7.2). Dilutions of this suspension in phosphate-buffered saline were used to inoculate mice. The number of CFU per milliliter in suitable dilutions was determined by plate counts. For oral inoculation, 0.2-ml aliquots were administered to mice, lightly anesthetized with ether, with 1-ml disposable syringes to which polyethylene catheters (Biotrol) were attached. Animal care and handling were in accordance with institutional guidelines.

Nucleotide sequence accession numbers. The sequence data reported in this communication will appear in the EMBL/GenBank/DDBJ nucleotide sequence databases under accession numbers AJ291321 to AJ291334.

RESULTS AND DISCUSSION

Isolation of strains with transposon insertions in σ^S-activated genes. It may be possible to isolate RpoS-regulated *lacZ* gene fusions in serovar Typhimurium by using an inducible promoter to control σ^S expression. However, the functional similarities of σ^{70} and σ^S (for a review, see reference 22) might result in competition effects between the two sigma factors, leading to artifacts during the selection procedure of fusions when σ^S is overexpressed (for example, selection of σ^{70}-dependent fusion artifactually regulated by high levels of σ^S). Thus, to ensure levels of σ^S in the physiological range, we devised a strategy based on functional complementation with an *rpoS* allele on a low-copy-number vector (Fig. 1). A set of strains with *lacZ* gene fusions was generated in the Sm^r derivative of the serovar Typhimurium *rpoS* mutant C52K from eight independent Tn*5*B21 mutagenesis experiments (labeled 1 to 3 and E to I; see Materials and Methods and Fig. 1A). To isolate genes whose expression was highly dependent on RpoS, we selected strains whose basal level of *lacZ* expression was low in the absence of RpoS (Fig. 1B). Strains with poorly expressed *lacZ* gene fusions were identified as those with a pale blue colony phenotype after growth on LB agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). These strains were then screened for increased fusion gene expression following acquisition of plasmid pVKRpoS, a low-copy-number vector which contains the *rpoS* gene from strain C52 (Fig. 1B). A total of 39 Tn*5*B21 insertion strains repeatedly exhibited increased β-galactosidase activity upon introduction of plasmid-borne *rpoS* (Fig. 1B and data not shown).

To determine whether the 39 Tn*5*B21 insertions mutants were independent mutants, preliminary physical maps of the genomic regions 5′ and 3′ to the Tn*5*B21 insertions were determined by Southern hybridization of total DNAs from the mutants, using as probes the *lacZ* gene and the Tn*5*B21-bearing plasmid pBDJ103 (data not shown); 38 of the 39 strains appeared to be independent mutants. The two mutants that show similar physical maps were isolated from the same mutagenesis experiment and were subsequently found to contain Tn*5*B21 inserted in the same position in their genomes (data not shown).

Each of these putative RpoS-dependent transcriptional fusions was transduced into the *rpoS* mutant C52K and into the wild-type strain C52 by using phage P22 and retested for σ^S dependency (Table 1). In addition, Southern hybridization experiments were conducted to check that Tn*5*B21 insertions mapped at the correct position in the genome of transductants (data not shown). β-Galactosidase activity of the transductant strains was assayed in stationary-phase cultures in LB medium (Table 1). Expression of the fusions was in all cases lower in the *rpoS* mutant C52K than in the wild-type strain C52. However, expression of the fusions in strain C52K could be restored upon introduction of plasmid pVKRpoS (Table 1). In contrast, there was no variation in the levels of β-galactosidase activity expressed by the fusions in C52K upon introduction of the control vector pVKCm (data not shown). The difference in expression of σ^S-regulated gene fusions varied from 3- to 95-fold when isogenic strains with wild-type and null *rpoS* loci were compared. Thus, the RpoS protein can regulate transcription over a wide range. The strategy used to screen the series of fusions would be expected to prevent the selection of putative RpoS-regulated genes, for which the basal level of expression is high in the absence of RpoS and further increased upon introduction of an *rpoS* allele. Consistent with this, the basal levels of β-galactosidase activity in the absence of a wild-type *rpoS* allele were very low in the growth conditions used (Table 1).

Cellular levels of RpoS increase during entry into stationary phase in rich medium, under starvation conditions, or when strains are exposed to various stress conditions (for reviews, see references 14 and 22). However, the pattern of induction of genes regulated by RpoS may be narrower due to dependence on other global additional regulators. We monitored gene fusion activity in strains grown to stationary phase in LB rich medium (Fig. 1B), a condition allowing RpoS-dependent expression of a variety of RpoS-regulated genes in *E. coli*. As expected, expression of all the RpoS-regulated fusions in the $rpoS^+$ strain C52 was induced in stationary phase of growth in rich medium (data not shown). In some cases, however, the level of expression of the fusion in C52 was low (<50 Miller units) even in the stationary phase of growth (Table 1). Growth conditions other than those used may be required for maximal expression of the RpoS-regulated gene which has been mutated in these strains.

Molecular characterization of σ^S-regulated genes. We determined the σ^S-regulated loci defined by 21 of the Tn*5*B21 insertion mutants. We cloned the DNA fragments harboring the junction between the left end of Tn*5*B21 and the *Salmonella* chromosome and determined the nucleotide sequence of the region adjacent to Tn*5*B21 (upstream of the promoterless *lacZ* gene). The sequences were then used to search sequence databases for related genes or proteins.

Fourteen of the twenty-one mutations mapped to DNA regions very similar to *E. coli* regions (Table 1). These insertions mapped to open reading frames (ORFs) with the exception of insertion H87, which is located in a noncoding region (promoter region of *ogt* [see below]). Two insertions (F1 and 1.39) are in the same ORF (Table 1). The degree of conservation between *E. coli* and serovar Typhimurium in the sequenced regions of the 12 identified ORFs was from 66 to 100% identity at the protein level (Table 1) and from 71 to 87% identity at the DNA level (data not shown). These sequences are presumably orthologs. These ORFs are scattered throughout the *E. coli* chromosome. This approach identified the serovar Typhimurium homologs of the *E. coli katE*, *otsA*, and *poxB* genes, three well-characterized σ^S-regulated genes (22), thereby validating the method (Table 1). *katE*, *otsA*, and *poxB* encode catalase HPII, trehalose-6-phosphate synthase, and a pyruvate oxidase, respectively. Our partial sequence of *otsA* from serovar Typhimurium strain C52 is 100% identical to the corresponding region of the serovar Typhimurium *otsA* sequence

TABLE 1. Molecular characterization of σ^S-activated gene fusions

Tn*5*B21 insertion	β-Galactosidase activity[a] (mean ± SD)				*E. coli* homolog[c]	
	rpoS$^+$	*rpoS*	*rpoS* pVKRpoS	Fold reduction[b]	Gene (% identity/ % similarity)	Position/length
1.32	196 ± 9	20 ± 14	199 ± 6	10	*otsA* (85/89)	236/474
1.36	309 ± 21	11 ± 0.1	319 ± 8	28	*ygaU* (92/94)	119/149
1.39	977 ± 133	65 ± 3	1,840 ± 121	15	*yciF* (83/96)	30/166
2.4	266 ± 18	8 ± 1	212 ± 8	33	*katE* (82/90)	109/753
2.9	126 ± 6	6 ± 1	137 ± 10	21	*ycgB* (97/98)	109/510
2.10	431 ± 24	12 ± 0.6	450 ± 77	36	*yeaG* (100)	550/644
2.11	34 ± 8	7 ± 0.1	40 ± 2	5	No	
3.3	146 ± 4	27 ± 0.1	125 ± 6	5	NI	
3.6	119 ± 5	8 ± 0.1	142 ± 3	15	NI	
3.7	473 ± 80	5 ± 0.1	527 ± 59	95	NI	
E18	136 ± 9	4 ± 1	107 ± 27	34	NI	
E26	61 ± 14	16 ± 11	45 ± 2	4	No	
E44	85 ± 2	14 ± 5	17 ± 2	6	*yehY* (80/89)	287/385
E45	253 ± 36	17 ± 1	195 ± 39	15	No	
E46	95 ± 12	18 ± 9	92 ± 15	5	*yhjY* (67/84)	106/232
F1	602 ± 223	89 ± 15	786 ± 86	7	*yciF* (96/96)	134/166
F2	82 ± 3	16 ± 4	81 ± 2	5	No	
F3	391 ± 33	69 ± 8	436 ± 80	6	NI	
F4	103 ± 13	23 ± 18	95 ± 8	4	*poxB* (94/95)	200/572
F7	135 ± 3	20 ± 6	124 ± 26	7	NI	
F8	38 ± 3	15 ± 1	44 ± 3	3	No	
F9	449 ± 157	32 ± 15	533 ± 5	14	No	
F10	420 ± 155	29 ± 13	343 ± 120	14	NI	
F11	159 ± 18	23 ± 11	193 ± 17	7	No	
F12	318 ± 7	43 ± 16	441 ± 67	7	*yncC* (82/87)	34/221
F13	65 ± 4	18 ± 3	86 ± 15	4	NI	
F79	154 ± 39	6 ± 2	106 ± 6	26	*yjgB* (93/97)	133/339
G10	354 ± 38	23 ± 7	276 ± 3	15	*yahO* (66/79)	66/91
G20	185 ± 26	36 ± 4	175 ± 29	5	NI	
G57	356 ± 113	21 ± 6	303 ± 67	17	NI	
G87	58 ± 7	10 ± 2	57 ± 10	6	NI	
H5	40 ± 10	7 ± 1	34 ± 1	6	NI	
H21	163 ± 33	5 ± 1	126 ± 14	33	NI	
H30	146 ± 28	24 ± 1	119 ± 22	6	NI	
H87	165 ± 7	29 ± 6	183 ± 46	6	*ogt* promoter region	
I8	101 ± 28	20 ± 5	76 ± 6	5	NI	
I28	371 ± 88	44 ± 9	330 ± 14	8	NI	
I36	326 ± 69	77 ± 35	252 ± 25	4	NI	

[a] Comparison of β-galactosidase activities (in Miller units [24]) in strains with wild-type and null *rpoS* loci. Values (averages of at least three independent experiments) were calculated from stationary-growth-phase cultures in LB. *rpoS*$^+$ denotes the Tn*5*B21 insertions in strain C52 containing a wild-type *rpoS* locus. *rpoS* denotes an isogenic strain carrying the Δ*rpoS*::*kan* locus (C52K). pVKRpoS carries the *rpoS* gene from strain C52 cloned into the low-copy-number vector pVK100 (19).

[b] Decrease in β-galactosidase activity on acquisition of the null *rpoS* allele in strain C52. Values are rounded to the nearest whole number.

[c] Junction fragments containing chromosomal sequences 5′ to the *lacZ* gene of Tn*5*B21 were cloned and sequenced; extents of amino acid identity and similarity were determined. No, no homology with *E. coli*; NI, insertion not identified. The position of each identified insertion and length of the *E. coli* homolog (in amino acids) are indicated. Accession numbers for the *E. coli* proteins were P33361 (YehY), P37663 (YhjY), P07003 (PoxB), P76114 (YncC), P27250 (YjgB), P75694 (YahO), P39169 (YgaU), P31677 (OtsA), P77391 (YeaG), P21179 (KatE), P21362 (YciF), and P29013 (YcgB).

AF213176, and *otsA* has been previously shown to be regulated by σ^S in serovar Typhimurium (8, 13).

Ten fusions mapped to serovar Typhimurium homologs of *E. coli* ORFs not previously known to require RpoS for expression (Table 1). Our partial sequence of *yahO* is 100% identical to nucleotides 13 to 299 of the serovar Typhimurium DNA sequence U51879 carrying the propionate catabolism operon *prpBCDE* and the divergently transcribed regulatory gene *prpR* (15). This suggests that *yahO* is located downstream from *prpR* in serovar Typhimurium as in *E. coli*. The functions of *yehY*, *yhjY*, *yncC*, *yjgB*, *yahO*, *ygaU*, *yeaG*, *yciF*, and *ycgB* are unknown. The YciF and YhjY proteins have been classified by Blattner et al. (2) as a putative structural protein and a putative protein involved in fatty acid and phospholipid metabolism, respectively. Features in the predicted amino acid sequences of *yehY*, *yjgB*, and *yncC* suggest that they encode an ABC transporter permease, a zinc-type alcohol dehydrogenase-like protein, and a putative transcriptional regulator of the GntR family, respectively.

Interestingly, nucleotide sequence data from the junction fragment of the other seven insertions (F2, F8, F9, F11, E26, E45 and 2.11 [Table 1]) do not match any known *E. coli* sequence. Preliminary examination of the remaining 17 RpoS-regulated fusions indicates that more than one-third of the RpoS-regulated fusions mapped to DNA regions not present in *E. coli* (data not shown). This suggests that the composition of the RpoS regulon differs markedly in the two species. This in turn is consistent with preliminary genome sequence analysis of *Salmonella* indicating that the genomes of *E. coli* and *Salmonella* are more different than might be suggested by the considerable concordance of their genetic maps (23, 44).

More than 50 genes have been found to be positively regulated by RpoS in *E. coli* (for a review, see reference 22). Previous works have identified RpoS-regulated genes in sero-

var Typhimurium (8, 9, 13, 31, 36), including the *E. coli* homologs *narZ* (nitrate reductase), *cfa* (cyclopropane fatty acid synthase), *otsA* (trehalose synthetase), *sodC* (superoxide dismutase), *csg* (curli biosynthesis), ORFO186 (U18997; unknown) and *yohF* (oxidoreductase). Of the 20 RpoS-regulated sequences that this work has identified in *Salmonella*, 13 are present in *E. coli*, and only 3 of these are known members of the RpoS regulon. The RpoS regulon may thus be larger than initially predicted. With one exception (the *ogt* gene, [see below]), the new members of the RpoS regulon identified in this study are homologs of ORFs in *E. coli* which have not been studied and not been assigned any function. This suggests that a large proportion of the bacterium's genome may be involved in adaptation to particular growth phase- or stress-related stimuli (or suboptimal conditions) and not involved in growth under optimal conditions such as those generally used in laboratories (e.g., rich or glucose-based minimal medium under aerobic growth conditions). Work is in progress in our lab to analyze phenotypes of these mutants under a variety of nonstandard growth conditions and in various genetic backgrounds.

Genetic rearrangements in the *yda-ogt* region of the enterobacterial chromosome. The nucleotide sequence of the 96-bp DNA region upstream from the *lacZ* gene of Tn*5*B21 in mutant C52::H87 is identical to the 5′ end of the serovar Typhimurium DNA sequence U23465. This 96-bp sequence in U23465 is located 71 nucleotides upstream from the start codon of the serovar Typhimurium *ogt* gene encoding O6MeG DNA MTase (46). A DNA sequence that matches the partial sequence of the *ydaL* gene of *E. coli* (89% nucleotide identity) was found 186 bp upstream from the Tn*5*B21 insertion H87, with the *lacZ* and *ydaL* genes being divergently transcribed. This was surprising because in *E. coli*, *ydaL* and *ogt* are separated by four ORFs, namely, *ydaKJIH* (Fig. 2A). To check whether the *ogt* gene was present downstream from Tn*5*B21 in strain C52::H87, we cloned and sequenced the DNA fragment harboring the junction between the right end of Tn*5*B21 and the *Salmonella* chromosome fragment in mutant strain C52::H87. Insertion H87 is indeed located 71 bp upstream from the translational start codon of the *ogt* gene of *Salmonella* strain C52 (Fig. 2B). The sequence of the 257-bp DNA region between the *ydaL* and *ogt* ORFs in serovar Typhimurium does not match the DNA sequence upstream from *ogt* in *E. coli*. In contrast, the nucleotide sequences of the coding regions of *ogt* were very similar in the two species (77% nucleotide identity and 88% amino acid identity). This shows that the *ydaL-ogt* DNA region of the enterobacterial chromosome has undergone genetic rearrangements during species divergence.

The *ogt* gene belongs to the RpoS regulon in *Salmonella*. A major mutagenic adduct induced in DNA by methylating agents is O6MeG. This altered base mispairs with thymine during DNA replication, resulting in GC-to-AT transition mutations. To counteract such mutagenic effects, *E. coli* and serovar Typhimurium possess two DNA MTases, Ada and Ogt, that repair O6MeG lesions by directly transferring the methyl group from the methylated base to specific cysteine residues in the MTase (references 12, 27, and 46) and references therein). Exposure of *E. coli* cells to sublethal concentrations of DNA-methylating agents triggers the expression of a set of genes which confer increased resistance to the effects of these agents. This process, called the adaptive response, requires the Ada protein, which plays a dual role, being both a DNA repair enzyme and a transcription activator of the adaptive response. The response consists of induction of at least the *ada-alkB* operon, the *alkA* gene, and the *aidB* gene (for a review, see reference 21). The Ogt protein is not inducible by DNA alkylation damage and is the major MTase in unadapted cells (27, 28).

The adaptive response of serovar Typhimurium to alkylating agents seems to be less efficient than that in *E. coli* (12, 41). The Ada protein appears to play a major role in serovar Typhimurium tolerance to organic acid stress (1), but unlike an *E. coli ada* mutant, an *ada* mutant of serovar Typhimurium is not sensitive to the mutagenic action of DNA-methylating agents (45, 46). In contrast, an *ogt* mutant of serovar Typhimurium is much more sensitive than the corresponding wild-type strain to the mutagenic action of alkylating agents and to spontaneous mutagenesis, suggesting that the Ogt protein plays a major role in protecting serovar Typhimurium from the mutagenic action of both endogenous and exogenous alkylating agents (46).

It seemed likely that the *lacZ* fusion in strain C52::H87 was under the control of the *ogt* promoter. To verify this and identify the *ogt* promoter in *Salmonella*, we conducted primer extension experiments with RNAs isolated from stationary-phase cultures of wild-type strains (C52 and SL1344), *rpoS* mutants (C52K and SL1344K), and mutant C52::H87. A major extended product was detected with RNAs from the wild-type strains C52 and SL1344 but not with RNAs from the *rpoS* mutants (Fig. 2C). Therefore, the identified promoter (*ogtp1*) was under the control of RpoS. No extended product was detected with RNA from mutant C52::H87 (Fig. 2C). This result is consistent with the location of Tn*5*B21 in mutant C52::H87, just downstream from the putative RpoS-dependent promoter *ogtp1* (Fig. 2B), and suggests that *lacZ* expression in mutant C52::H87 is under the control of *ogtp1*. Additional faint bands were detected in the *rpoS* strain C52K (Fig. 2C).

The *ogtp1* −10 region (CTATCTT [Fig. 2B]) closely resembles the σ^S consensus sequence. Indeed, 33 σ^S-dependent promoters have a possible consensus sequence in the −10 region of CTATACT, which is very similar to the corresponding σ^{70} sequence of TATAAT (for a review see reference 22). No common −35 sequence element can be discerned in the σ^S-dependent promoter group, and the −35 sequence of *ogtp1* does not closely resemble the corresponding σ^{70} consensus sequence TTGACA (Fig. 2B).

These results demonstrate that RpoS regulates expression of the serovar Typhimurium *ogt* gene during the stationary phase in rich medium. This is consistent with previous findings suggesting that alkylating agents can accumulate in stationary phase or starved cells (references 27 and 39 and references therein). Moreover, expression of the Ada protein in *E. coli* has been shown to be dependent on RpoS in stationary phase (39). In *Salmonella*, it is not known whether RpoS controls Ada expression, and the Ada MTase does not seem to contribute to protection against mutagenesis by alkylating agents (46). Thus, RpoS may play a role in the ability of *Salmonella* to repair DNA damage caused by alkylating agents during stationary phase via the control of the Ogt MTase.

In preliminary experiments, transposon insertion H87 increased only two- to fivefold the number of rifampin- and nalidixic acid-resistant mutants recovered after *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) mutagenesis of serovar Typhimurium C52 (data not shown). The *ogt* gene is not disrupted in mutant C52::H87, and it cannot be excluded that in this mutant, basal levels of *ogt* mRNAs are produced from a promoter-like sequence located within the transposon. Previous work, showing that *ogt* plays a major role in protecting serovar Typhimurium from the mutagenic action of MNNG, used the serovar Typhimurium strain TA1535 (46). This strain is a derivative of the nonpathogenic strain LT2 with increased sensitivity to mutagens, and many LT2 isolates show altered

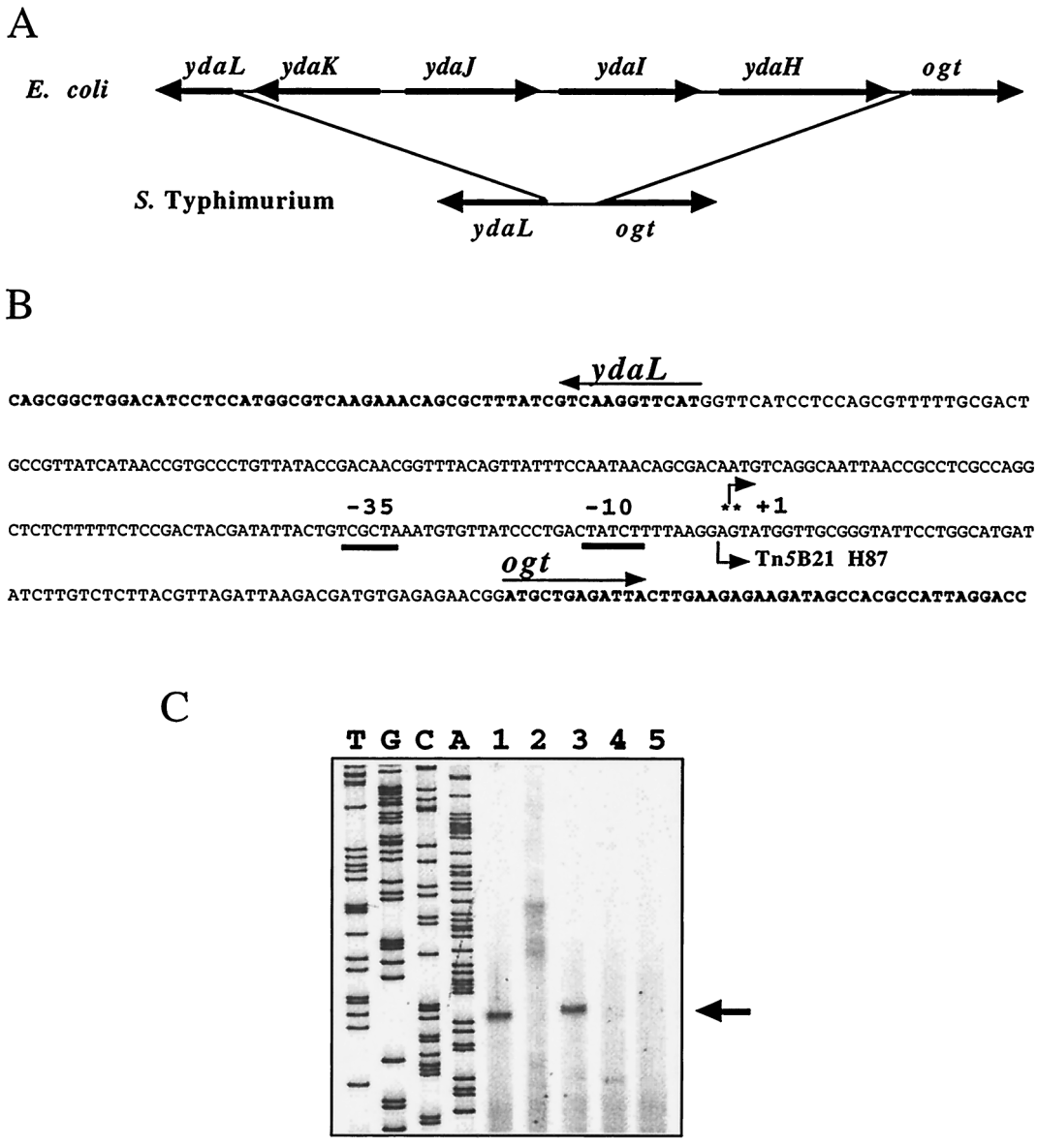

FIG. 2. Genomic organization of the *ogt* region and mapping of the σ^S-regulated promoter of *ogt*. (A) Comparison of the gene order near *ogt* in serovar Typhimurium and *E. coli*. (B) DNA sequence in the *ydaL-ogt* region in serovar Typhimurium. The 5′ ends of the *ydaL* and *ogt* genes are shown in bold. The nucleotide sequence was that from serovar Typhimurium C52 (this study). The position of the putative RpoS-dependent transcriptional start site of *ogt* (+1) is indicated by asterisks. The −10 and −35 regions of the *rpoS*-regulated promoter of *ogt* (*ogtp1*) are underlined. The position of insertion of Tn*5*B21 in mutant C52::H87 is indicated by a broken arrow. (C) Mapping of the 5′ end of *ogt* mRNA in serovar Typhimurium. A 5′ ^{32}P-labeled primer complementary to the *ogt* coding strand was annealed to total RNAs isolated from LB-grown stationary-phase cultures of serovar Typhimurium wild-type (C52 and SL1344) and *rpoS* mutant (C52K and SL1344K) strains and of the Tn*5*B21 insertion mutant C52::H87. The primer was extended with reverse transcriptase, and the products were resolved by electrophoresis on a sequencing gel. The DNA sequencing ladder (lanes A, C, G, and T) was prepared using the primer as used to sequence pUCogt1. The major extended product is indicated by an arrow. Lane 1, C52; lane 2, C52K; lane 3, SL1344; lane 4, SL1344K; lane 5; C52::H87.

rpoS expression (38, 43). It would be of interest to evaluate the impact of an *ogt* deletion on the resistance of a wild-type virulent strain of serovar Typhimurium to the effects of alkylating agents.

In conclusion, the transcription of *ogt* appears to be regulated by *rpoS* in serovar Typhimurium during the stationary phase of growth in rich medium, but further investigation is required to determine the physiological meaning of this finding.

Virulence assay. None of the 14 strains studied, harboring Tn*5*B21 insertions in the conserved σ^S-regulated genes (Table 1), were attenuated for virulence in Ity^s BALB/c mice. All mice (four per group) given either a mutant strain or wild-type strain C52 (10^8 bacteria orally) were dead within 12 days. Consistent with previous reports (7, 20), no animal died after receiving equivalent inocula of *rpoS* mutant strain C52K. *rpoS* mutants of *Salmonella* are thus highly attenuated in mice, with a 50% lethal dose at least 4 logs higher that of wild-type strains (7, 10). This presumably results largely from the reduced expression in the *rpoS* strain of the virulence plasmid genes *spv*, required for efficient growth of *Salmonella* in spleens and livers of infected mice (10, 20, 26). However, analysis of intestinal and splenic colonization in mice by wild-type and *rpoS Salmonella* strains cured for the virulence plasmid (7, 20, 25) suggest-

ed that *rpoS* regulates some unidentified chromosomal gene(s) involved in colonization and persistence of *Salmonella* in spleens and Peyer's patches.

In addition to the *spv* genes, at least three σ^S-regulated loci may contribute to *Salmonella* virulence: the *agf* genes (analogous to the *E. coli csg* genes) for curli biosynthesis, *narZ* (analogous to *E. coli narZ*, the first gene of an operon encoding a nitrate reductase), and *sodCII* (analogous to *E. coli sodC*), encoding a periplasmic Cu,Zn-superoxide dismutase (5, 9, 31, 36, 40). However, mutations in these genes result in only a weak attenuating effect on *Salmonella* lethality for mice. Mutations in the *agfB* and *narZ* genes cause 3- and 10-fold, respectively, increases in the oral 50% lethal dose of *S. typhimurium* for *Salmonella*-susceptible Ity^s mice (36, 40). A *sodCII* mutation appears to decrease *S. typhimurium* lethality in *Salmonella*-resistant (Ity^r) but not *Salmonella*-sensitive (Ity^s) mice (9). Interestingly, a second gene (*sodCI*) encoding a periplasmic Cu,Zn-superoxide dismutase is present in *Salmonella*, and mutants lacking both *sodC* genes are less lethal for mice than mutants possessing either *sodC* locus alone (9). This is an example of the potential difficulties in the analysis of gene products with redundant functions.

Therefore, although none of the conserved σ^S-regulated genes identified in this study are essential for *Salmonella* lethality in mice, some may contribute to the mouse infection process. In addition, characterization of the *Salmonella* mutants carrying Tn*5*B21 insertions in DNA regions not present in *E. coli* will help elucidate the physiological function of the RpoS regulon in this pathogen.

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