

## Limited Role for the DsrA and RprA Regulatory RNAs in *rpoS* Regulation in *Salmonella enterica*

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**RpoS, the sigma factor of enteric bacteria that responds to stress and stationary phase, is subject to complex regulation acting at multiple levels, including transcription, translation, and proteolysis. Increased translation of *rpoS* mRNA during growth at low temperature, after osmotic challenge, or with a constitutively activated Rcs phosphorelay depends on two *trans*-acting small regulatory RNAs (sRNAs) in *Escherichia coli*. The DsrA and RprA sRNAs are both highly conserved in *Salmonella enterica*, as is their target, an inhibitory antisense element within the *rpoS* untranslated leader. Analysis of *dsrA* and *rprA* deletion mutants indicates that while the increased translation of RpoS in response to osmotic challenge is conserved in *S. enterica*, dependence on these two sRNA regulators is much reduced. Furthermore, low-temperature growth or constitutive RcsC activation had only modest effects on RpoS expression, and these increases were, respectively, independent of *dsrA* or *rprA* function. This lack of conservation of sRNA function suggests surprising flexibility in RpoS regulation.**

RpoS, the general stress and stationary-phase (SP) sigma factor, is highly conserved among *Escherichia coli*, *Salmonella enterica*, and other related enteric bacteria. The diverse and often harsh conditions encountered by these bacteria, whether residing as pathogens in the gut or as saprophytes in the environment, require the ability to integrate multiple stress signals and initiate the appropriate cellular responses in order to survive. RpoS serves in this capacity as the master regulator of the general stress response. Its levels increase in response to a number of stress signals, including osmotic shock, nutrient depletion, low temperature, and growth into stationary phase (reviewed in reference 19). As RpoS becomes more abundant, it effectively competes with the vegetative sigma factor in binding to core RNA polymerase, leading to increased transcription of genes necessary for mediating the stress response (49).

Regulation of RpoS is complex, with a large posttranscriptional component, and involves *trans*-acting factors (19). These factors include several small regulatory RNAs (28, 39) which target a *cis*-acting antisense element within the *rpoS* mRNA untranslated leader (7). In *E. coli*, two such small RNAs (sRNAs), DsrA and RprA, activate *rpoS* translation by binding to and inhibiting the antisense element (reviewed in reference 30). DsrA is necessary for activation of *rpoS* translation in response to low temperature and osmotic shock (27), while RprA increases RpoS both in response to osmotic shock (29) and in response to a constitutively active *rscC* allele, indicating a role in cell envelope stress (15, 29).

These sRNAs were initially discovered and characterized in *E. coli*, and their gene sequences are  $\approx 90\%$  identical in *S. enterica*. The high degree of sequence conservation shared by *E. coli* and *S. enterica*, in both *rpoS* and the sRNAs, suggested that their regulatory functions are likely to be conserved as

well. Here we describe the results of experiments undertaken to characterize the roles of DsrA and RprA in *S. enterica*, specifically their effects on *rpoS* regulation. Our findings strongly suggest that, under the conditions tested, neither of the two sRNAs is required for optimal RpoS synthesis in *S. enterica*. Mutational analysis of the *rpoS* antisense element in *S. enterica* was also performed to further characterize the role of this element in RpoS synthesis. The results of this analysis are broadly comparable to those in *E. coli*.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The bacterial strains used in this study are listed in Table 1. The *katE-lac* operon [op] fusion used in this work has been described previously and is a reporter of RpoS activity (6, 9, 21). Isolation of Mud insertions and construction of site-specific mutations is described below. The high-frequency generalized transducing bacteriophage P22 mutant HT105/1 *int-201* was used for transduction in *S. enterica* by standard methods (12). Phage P1 *vir* was used for transduction in *E. coli*, also by standard methods (41). Bacteria were grown in media and at temperatures which are described for each individual experiment. LB was used as rich medium (41), and the minimal medium was morpholinepropanesulfonic acid (34) modified as described elsewhere (4). Plates were prepared by using nutrient agar (Difco) with 5 g of NaCl per liter. Antibiotics were added to rich medium to final concentrations as follows: 20  $\mu\text{g}$  of tetracycline hydrochloride per ml, 20  $\mu\text{g}$  of chloramphenicol per ml, 50  $\mu\text{g}$  of kanamycin sulfate per ml, 200  $\mu\text{g}$  of streptomycin sulfate per ml, and either 100  $\mu\text{g}$  of sodium ampicillin per ml for high-copy-number plasmids or 30  $\mu\text{g}$  per ml for low-copy-number plasmids. In minimal medium, kanamycin sulfate was added to a final concentration of 100  $\mu\text{g}$  per ml.

**Isolation and analysis of *rpoS-lac* fusions formed by insertion of Mud transposons.** Insertions of MudJ (MudJ 1734) to form operon fusions and MudK to form protein fusions (1, 8) were obtained by screening large pools of insertions ( $>10^5$  clones) for linkage to the *rpoS* region. Since *rpoS* is very close to *cysC* (6 kb separate the two genes), a phage P22-transducing lysate grown on each insertion pool was used to transduce TE8607 ( $\Delta$  *cysC::tet*) to Kan<sup>r</sup> Cys<sup>+</sup> on plates containing 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal). Blue colonies were picked, purified once on selective medium, patched onto NB agar containing kanamycin, and tested for an *rpoS* mutant phenotype by scoring formation of bubbles when spotted with 5  $\mu\text{l}$  of 3% hydrogen peroxide. Insertions were mapped by PCR, and the precise joint with the Mud element was located by DNA sequencing. The starting insertion pools were isolated by mutagenesis of *S. enterica* LT2 using the method of Hughes and Roth (22). The original host for MudK insertions carried a plasmid unrelated to the current

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TABLE 1. Strains

Strain <sup>a</sup>	Description	Source or reference
<i>S. enterica</i> strains		
TE6134	<i>hfq-1</i> ::Mud-Cam	6
TE6153	<i>putPA1303</i> ::Kan <sup>r</sup> - <i>katE-lac</i> [op]	6
TE6266	<i>hfq-1</i> ::Mud-Cam <i>putPA1303</i> ::Kan <sup>r</sup> - <i>rpoS-lac</i> [pr]	7
TE6369-2	<i>hfq-1</i> ::Mud-Cam <i>putPA1303</i> ::Kan <sup>r</sup> - <i>rpoS-lac</i> [pr] (C470G)	7
TE6369-3	<i>hfq-1</i> ::Mud-Cam <i>putPA1303</i> ::Kan <sup>r</sup> - <i>rpoS-lac</i> [pr] (G550C)	7
TE6382	<i>hfq-1</i> ::Mud-Cam <i>putPA1303</i> ::Kan <sup>r</sup> - <i>rpoS-lac</i> [pr] (C470G) (G550C)	7
TE6557	<i>hfq-1</i> ::Mud-Cam <i>putPA1303</i> ::Kan <sup>r</sup> - <i>rpoS-lac</i> [pr] (C469G)	7
TE6558	<i>hfq-1</i> ::Mud-Cam <i>putPA1303</i> ::Kan <sup>r</sup> - <i>rpoS-lac</i> [pr] (G551C)	7
TE6590	<i>hfq-1</i> ::Mud-Cam <i>putPA1303</i> ::Kan <sup>r</sup> - <i>rpoS-lac</i> [pr] (C469G) (G551C)	7
TE6850	<i>clpX1</i> ::Tn10d-Cam	10
TE6851	<i>mviA22</i> ::Tn10d-Cam	10
TE8007	JF3490 <i>dxsA4</i> ::Tn10d-Tet	J. Foster
TE8012	TE8007 LT2A <i>putPA1303</i> ::Kan <sup>r</sup> - <i>katE-lac</i> [op]	
TE8544	<i>putPA::katE-lac</i> [op] <i>rpoS1074::tetAR</i> (A.S., deletes nt 461–464 of <i>rpoS</i> leader)	
TE8546	<i>putPA::katE-lac</i> [op] <i>rpoS1076::cat</i> (deletes from bp 110 upstream to 469 of <i>rpoS</i> leader)	
TE8566	<i>putPA::katE-lac</i> [op] $\Delta$ <i>dsrA::tetAR</i>	
TE8567	<i>putPA::katE-lac</i> [op] $\Delta$ <i>rprA::tetAR</i>	
TE8587	<i>putPA::katE-lac</i> [op] $\Delta$ <i>dsrA::cat</i>	
TE8588	<i>putPA::katE-lac</i> [op] $\Delta$ <i>cysC::tetAR</i>	
TE8589	<i>putPA::katE-lac</i> [op] <i>rpoS1084::tetAR</i> (AGGA, deletes nt 554–557 of <i>rpoS</i> leader)	
TE8607	$\Delta$ <i>cysC::tetAR</i>	
TE8608	<i>putPA1303</i> ::Kan <sup>r</sup> - <i>katE-lac</i> [op] $\Delta$ <i>dsrA::cam</i>	
TE8610	<i>putPA1303</i> ::Kan <sup>r</sup> - <i>katE-lac</i> [op] $\Delta$ <i>rprA::tetAR</i>	
TE8613	<i>putPA1303</i> ::Kan <sup>r</sup> - <i>katE-lac</i> [op] $\Delta$ <i>dsrA::cam</i> $\Delta$ <i>rprA::tetAR</i>	
TE8622	$\Delta$ <i>cysC::tetAR</i> <i>rpoS1076::cat</i> (deletes from bp 110 upstream to 469 of <i>rpoS</i> leader)	
TE8701	<i>putPA::katE-lac</i> [op] <i>rpoS1080::tetAR</i> ( $\Delta$ A.S.-AGGA, deletes nt 461–557)	
TE8737	<i>rpoS1082</i> ::MudJ (codon 66)	
TE8794	<i>rpoS1083</i> ::MudJ (codon 222)	
TE8804	<i>nlpD</i> ::MudJ ( <i>nlpD</i> codon 213, nt 9 of <i>rpoS</i> leader)	
TE8805	<i>rpoS1079</i> ::MudK (codon 187)	
TE8807	<i>rpoS1077</i> ::MudK (codon 22)	
TE8808	<i>rpoS1078</i> ::MudK (codon 216)	
TE8810	<i>rpoS1081</i> ::MudK (codon 250)	
TE8815	<i>rpoS1078</i> ::MudK (codon 216) <i>hfq-1</i> ::Mud-Cam	
TE8852	TE8815 <i>rpoS</i> (C469G)	
TE8853	TE8815 <i>rpoS</i> (C470G)	
TE8854	TE8815 <i>rpoS</i> (G471C)	
TE8855	TE8815 <i>rpoS</i> (C549G)	
TE8856	TE8815 <i>rpoS</i> (G550C)	
TE8857	TE8815 <i>rpoS</i> (G551C)	
TE8858	TE8815 <i>rpoS</i> (C469G) (G551C)	
TE8859	TE8815 <i>rpoS</i> (C470G) (G550C)	
TE8860	TE8815 <i>rpoS</i> (G471C) (C549G)	
TE8935	<i>nlpD</i> ::MudJ ( <i>nlpD</i> codon 302, nt 276 of <i>rpoS</i> leader)	
TE8936	<i>rpoS</i> ::MudJ (codon 36)	
TE8983	<i>rpoS1077</i> ::MudK (codon 22) $\Delta$ <i>dsrA::cat</i>	
TE9049	<i>nlpD</i> ::[FRT- <i>lacZY</i> pKG137 Kan <sup>r</sup> ] ( <i>nlpD</i> codon 302, nt 276 of <i>rpoS</i> leader)	
TE9050	<i>rpoS</i> ::[FRT- <i>lacZY</i> pKG137 Kan <sup>r</sup> ] ( <i>rpoS</i> codon 36)	
TE9051	<i>rpoS</i> ::[FRT- <i>lacZY</i> pKG137 Kan <sup>r</sup> ] ( <i>rpoS</i> codon 222)	
TE9052	<i>nlpD</i> ::[FRT- <i>lacZY</i> pKG137 Kan <sup>r</sup> ] ( <i>nlpD</i> codon 213, nt 9 of <i>rpoS</i> leader)	
TE9053	<i>rpoS</i> ::[FRT- <i>lacZY</i> pKG137 Kan <sup>r</sup> ] ( <i>rpoS</i> codon 4)	
TE9160	<i>rpoS1081</i> ::MudK (codon 250) (TTG→ATG start codon)	
TE9179	MS1868 <i>StrA1</i> <i>rpoS</i> ::[ <i>tetAR</i> <i>rpsL</i> <sup>+</sup> ] (AGGA)	S. Maloy
TE9213	<i>rpoS1081</i> ::MudK (codon 250) (TTG→ATG start codon) $\Delta$ <i>dsrA::cat</i>	
TE9219	<i>rpoS1081</i> ::MudK (codon 250) (TTG→ATG start codon) $\Delta$ <i>dsrA::cat</i> $\Delta$ <i>rprA::tetAR</i>	
TE9236	TE8815 <i>rpoS</i> (G461C)	
TE9237	TE8815 <i>rpoS</i> (G461A)	
TE9238	TE8815 <i>rpoS</i> (G461T)	
TE9239	TE8815 <i>rpoS</i> (G462C)	
TE9240	TE8815 <i>rpoS</i> (G462A)	
TE9241	TE8815 <i>rpoS</i> (G462T)	
TE9242	TE8815 <i>rpoS</i> (G463C)	
TE9243	TE8815 <i>rpoS</i> (G463A)	
TE9244	TE8815 <i>rpoS</i> (G463T)	
TE9245	TE8815 <i>rpoS</i> (G464C)	
TE9246	TE8815 <i>rpoS</i> (G464A)	
TE9247	TE8815 <i>rpoS</i> (G464T)	

Continued on following page

TABLE 1—Continued

Strain <sup>a</sup>	Description	Source or reference
TE9316	<i>rpoS1077::MudK</i> (codon 22) <i>zef-6829::tetAR rcsC55</i> (T903A)	
TE9317	<i>rpoS1077::MudK</i> (codon 22) <i>zef-6829::tetAR rcsC<sup>+</sup></i>	
TE9318	<i>rpoS1077::MudK</i> (codon 22) <i>zef-6829::tetAR rcsC64</i> (F4731)	
TE9333	<i>gmm-21::MudJ zef-6829::tetAR rcsC55</i> (T903A)	
TE9334	<i>gmm-21::MudJ zef-6829::tetAR rcsC<sup>+</sup></i>	
TE9352	<i>rpoS1077::MudK</i> (codon 22) <i>zef-6829::tetAR rcsC55</i> (T903A) $\Delta$ <i>rprA::cam</i>	
TE9353	<i>rpoS1077::MudK</i> (codon 22) <i>zef-6829::tetAR rcsC<sup>+</sup> ?rprA::cam</i>	
TE9354	<i>rpoS1077::MudK</i> (codon 22) <i>zef-6829::tetAR rcsC64</i> (F4731) $\Delta$ <i>rprA::cam</i>	
TE9368	<i>gmm-21::MudJ zef-6829::tetAR rcsC64</i> (F4731)	
TE9394	<i>gmm-21::MudJ zef-6829::tetAR rcsC<sup>+</sup> ?rprA::cam</i>	
TE9395	<i>gmm-21::MudJ zef-6829::tetAR rcsC55</i> (T903A) $\Delta$ <i>rprA::cam</i>	
TE9396	<i>gmm-21::MudJ zef-6829::tetAR rcsC64</i> (F4731) $\Delta$ <i>rprA::cam</i>	
TE9426	TE8807/pNM12 (vector)	
TE9427	TE8983/pNM12 (vector)	
TE9428	TE8983/pNM3 (P <sub>BAD</sub> - <i>E. coli dsrA<sup>+</sup></i> )	
TE9429	TE8807/pBAD18 (vector)	
TE9430	TE8983/pBAD18 (vector)	
TE9431	TE8983/pTE780 (P <sub>BAD</sub> - <i>S. enterica dsrA<sup>+</sup></i> )	
TE9452	TE8815 <i>rpoS</i> (T468C)	
TE9453	TE8815 <i>rpoS</i> (C476A) (C477A)	
<i>E. coli</i> strains		
SG22182	MC4100 <i>ara mal::lacI<sup>Q</sup></i>	D. Sledjeski
DDS1365	MC4100 <i>ara mal::lacI<sup>Q</sup> dsrA1::cat</i>	D. Sledjeski
TE6897	MC4100 <i>ara mal::lacI<sup>Q</sup> trpDC700:: putPA1303::Kan<sup>r</sup>-katE-lac</i> [op]	
TE6898	MC4100 <i>ara mal::lacI<sup>Q</sup> trpDC700:: putPA1303::Kan<sup>r</sup>-rpoS-lac</i> [pr]	
TE6913	MC4100 <i>ara mal::lacI<sup>Q</sup> trpDC700:: putPA1303::Kan<sup>r</sup>-katE-lac</i> [op] <i>dsrA1::cat</i>	
TE6914	MC4100 <i>ara mal::lacI<sup>Q</sup> trpDC700:: putPA1303::Kan<sup>r</sup>-rpoS-lac</i> [pr] <i>dsrA1::cat</i>	
TE8096	BW26678 <i>E. coli</i> K-12/pKD46 [AmpR, pSC101 <i>rep</i> (Ts) <i>araC<sup>+</sup></i> P <sub>BAD</sub> - $\lambda$ <i>red</i> ]	B. Wanner
TE9062	<i>zgd::tetAR</i> (just upstream of <i>rpsL<sup>+</sup></i> )	
TE9416	TE6898/pBAD18 (vector)	
TE9418	TE6914/pBAD18 (vector)	
TE9419	TE6914/pTE780 (P <sub>BAD</sub> - <i>S. enterica dsrA<sup>+</sup></i> )	
TE9422	TE6898/pNM12 (vector)	D. Sledjeski
TE9424	TE6914/pNM12 (vector)	
TE9425	TE6914/pNM3 (P <sub>BAD</sub> - <i>E. coli dsrA<sup>+</sup></i> )	D. Sledjeski

<sup>a</sup> Numbering corresponds to the position of the last nucleotide retained from the *rpoS* leader or the codon within which the fusion occurs (i.e., the last intact codon is one previous).

project (pRF1) (14); therefore, backcrossed candidate insertions were checked to confirm that this plasmid had been lost as expected.

Preliminary characterization showed both expected and unexpected properties for strains carrying these fusions. Fusions were recovered within *rpoS* but also in the upstream region encoding the C-terminal part of *nlpD*. Polarity of the insertions lying within this part of *nlpD* on expression of *rpoS* is consistent with the location of the major *rpoS* promoter roughly in the middle of the *nlpD* gene (21, 23, 46). Also as expected, all *lac* protein fusions to *rpoS* tested were found to be substantially ( $\approx$ 4- to 5-fold) dependent on function of the *hfq* and *dkxA* genes, known from previous studies to affect *rpoS* translation but not transcription (6, 33, 48). Sensitivity to *clpX* and *mviA* mutations was tested in the LT2A background (10). The effect of mutations blocking the protein turnover pathway was variable, depending on the location of the insertion site within *rpoS*. The MudK insertion at *rpoS* codon 22 was unaffected by loss of *clpX* or *mviA*, whereas expression of the insertion at codon 250 was increased five- to sixfold in both *clpX* and *mviA* mutant backgrounds during exponential phase in LB medium. This behavior is consistent with identification of K173 as a critical residue in the RpoS "turnover element" in *E. coli* (2). In stationary phase, expression of sensitive *rpoS-lac* protein fusions became independent of *clpX* and *mviA*. Other results in *S. enterica* (21) show that this behavior is not characteristic of the RpoS protein itself or of RpoS-dependent reporters. We can explain this result by postulating that the *rpoS* dependence of *mviA* (*rssB/sprE*) expression during stationary phase found in *E. coli* (40) is conserved in *S. enterica*.

One unexpected result was that several MudK insertions in *rpoS* (screened as dark blue Lac<sup>+</sup> colonies on X-Gal plates) were found to be out of frame but expressed *lac* as strongly as in-frame fusions. Remarkably, an insertion in the +1 frame at codon 36 expressed *lac* at a threefold-higher level during exponential phase than any in-frame fusion recovered. Several high-expressing out-of-frame

fusions were confirmed to have the predicted sequence across Mu and into the first 300 bp of *lacZ*. The explanation for this unusual situation is likely that: (i) exponential-phase expression of in-frame *rpoS-lac* protein fusions is quite low ( $\approx$ 10-fold lower than any *rpoS-lac* operon fusion), due at least in part to the action of the antisense element; and (ii) the sequence at the joint with MudK consists of the sequence XTG, where X is contributed by *rpoS* and TG is from Mu. This XTG codon is in frame with *lacZ*. The sequences of all high-expressing out-of-frame insertions contained a plausible ribosome-binding site (RBS) upstream from an initiation codon. This suggests that the high relative expression of out-of-frame fusions is an artifact due to the novel sequence at the insertion joint.

A second puzzling and cautionary result comes from comparison of MudJ (operon) fusions upstream and downstream of the *rpoS* initiation codon. We found that two early fusions, one within *nlpD* at +9 with respect to the *rpoS* transcriptional start and the other at +276 showed no SP induction in LB medium, whereas later fusions at codon 36 and codon 222 showed normal induction. This behavior indicated that normal transcriptional regulation depends on sequences substantially downstream of the promoter. However, leader-dependent regulation is not consistent with our previous study of SP induction (21), which showed that a short segment surrounding the promoter region displayed the full range of SP regulation of transcription.

To resolve this issue and map the apparent discontinuity, a number of precisely targeted *lac* operon fusions were constructed using the method of Ellermeier et al. (13). In this method, the  $\lambda$  Red recombination system is used to direct insertion of a drug resistance marker flanked by directly repeated FLP recombination target (FRT) sites into the bacterial chromosome. The insertions are then resolved to leave a single unmarked FRT site, which is targeted for FLP/FRT-mediated integration of a replication-defective Kan<sup>r</sup> plasmid to provide the

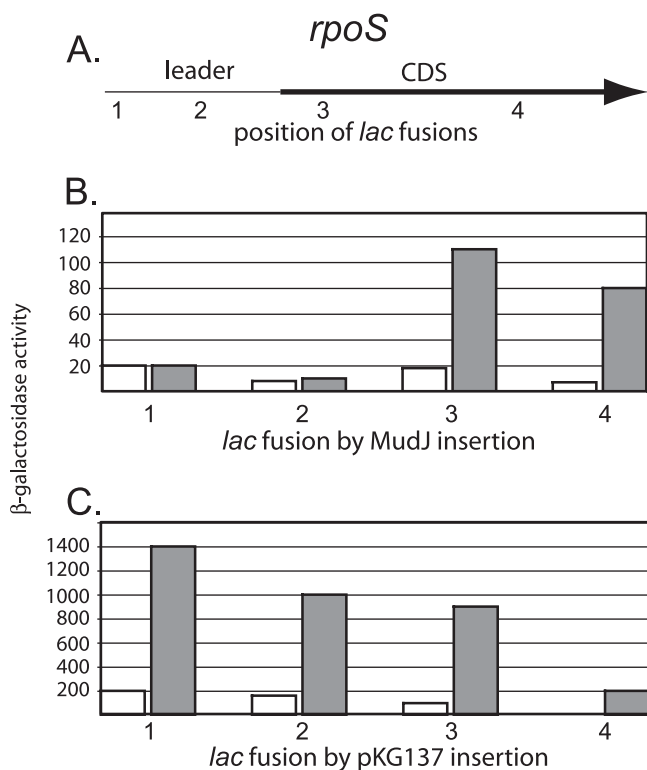


FIG. 1. Comparison of the regulation of *lac* operon fusions formed by MudJ insertion or plasmid integration. A. The *rpoS* coding sequence (bold line) and upstream leader are indicated, with labels indicating four sites at which fusions were isolated or constructed as described in the text. These positions are: 1 (+9 of the leader), 2 (+276 of the leader), 3 (codon 36 of *rpoS*), and 4 (codon 222 of *rpoS*). B. Analysis of *lac* expression from MudJ insertions at sites 1 to 4. Open bars indicate exponential-phase cultures, and filled bars indicate SP cultures, as defined in the text. SP induction of *rpoS* transcription is measured as the ratio of the value from the filled bars to open bars. C. The same experiment as in panel B, except that each fusion was made by integration of plasmid pKG137, as described in Materials and Methods. Strains for panel B were TE8804, TE8935, TE8936, and TE8794; strains for panel C were TE9052, TE9049, TE9050, and TE9051. Each bar represents the average of at least three experiments. Standard deviations were within 15% of the mean.

*lac* genes and form either an operon or a protein fusion. The *lac* fusions used here were constructed with pKG137 (M. Garsha and J. Schlauch, personal communication), a plasmid that forms *lac* operon fusions including a strong RBS for *lacZ*. As shown in Fig. 1, all the fusions made by this method were regulated, including those at +9 and +276 with respect to the transcriptional start, in contrast to the behavior of MudJ fusions inserted at exactly the same sites. The different behavior of MudJ insertions is currently unexplained but presumably depends on the nature of the extra sequences present at the *attR* end of MudJ, including about 400 bp from Mu as well as a substantial segment of the *E. coli* *trp* operon.

Plasmid pTE780 ( $P_{BAD}$ -*S. enterica dsrA*) was constructed by PCR using the following primers: 5'-GCGGGATCCTACCTGACGCTTTTATCGCAACTCTCTACTGTTTCTCCATCACATCAGATTTCTGGTGT-3' and 5'-GCGTCTAGAACCCTAAAAGGCCGAAA-3' on LT2 DNA as template. Sequences from *dsrA* are shown in italics. The PCR product was purified, digested with BamHI and XbaI, and cloned into pBAD18 (18).

**Construction of insertion and point mutations using  $\lambda$  Red recombination.** Other point mutations and insertion/deletions were made by direct transformation of *S. enterica*, either with oligonucleotides or with DNA segments amplified by PCR, utilizing the  $\lambda$  Red recombination system as provided on plasmid pKD46 (11). Exponential-phase recipient cells carrying pKD46, grown at 30°C with selection for ampicillin resistance (Amp<sup>r</sup>), were induced by treatment with

0.2% arabinose for 1 hour before electroporation, after which transformants were grown out in liquid medium before plating and selection at 37°C. A few experiments utilized pSIM5, a plasmid for mutant construction based on the method of Yu et al. (51) and obtained from D. Court.

Most of the unmarked point mutations were obtained as follows. First, an insertion of *tetAR* near or at the target site was isolated. Subsequently, a recipient bearing that insertion and induced for  $\lambda$  Red was transformed with a mutated oligo and selection applied for loss of Tet<sup>r</sup> (Bochner selection [5, 31]). For unknown reasons, this selection has a high background for insertions at certain sites. For insertion within the *rpoS* RBS, it was important that the recipient strain also contained *katE-lac* [op], an RpoS-dependent reporter fusion. Replacement of the *tetAR* cassette restored a Lac<sup>+</sup> phenotype, visualized by subsequent single colony isolation on MacConkey lactose agar. Some double mutants with lesions affecting both the antisense element and the RBS region were constructed by an iterative procedure in which a *tet* insertion derivative of an existing point mutant was constructed as an intermediate. A second, more rapid method used a singly mutant DNA template (lesion in the RBS) for PCR to introduce the second mutation, which was recovered by transformation of a recipient deleted for the region between the antisense element and the RBS. This deletion, marked with *tetAR*, was from strain TE8701 ( $\Delta rpoS1080::tetAR$ ).

A mutation changing the *rpoS* TTG start codon to ATG was obtained by oligo transformation as described above, but a second mutation changing the TTG to TCG could not be screened in the same way since it does not confer a Lac<sup>+</sup> phenotype. To make this change, we designed and constructed a different counterselectable insertion in the RBS region of *rpoS*, based on the known dominance of wild-type *rpsL* (Str<sup>r</sup>) in merodiploids containing one Str<sup>r</sup> and one Str<sup>s</sup> allele, as exploited by others (42). First, *tetAR* was inserted just upstream of the *rpsL*<sup>+</sup> gene of an Str<sup>r</sup> *E. coli* strain. Chromosomal DNA from this strain (TE9062) was used as template to amplify a *tetAR-rpsL*<sup>+</sup> cassette, and this DNA segment was then inserted at the RBS of *S. enterica rpoS*. TE9179, carrying both the *rpoS*::[*tetAR-rpsL*<sup>+</sup>] insertion and the *strA1* (Str<sup>r</sup>) allele, was found to be Str<sup>r</sup>, as expected. For unknown reasons the strain forms small colonies on NB agar lacking streptomycin, but this slow growth phenotype appears to be stable. Transformants were easily obtained by selecting Str<sup>r</sup> in this background, again using  $\lambda$  Red recombination. The vast majority of these were Tet<sup>r</sup> when mutated oligonucleotide DNA was added to the transformation mixture; three candidate transformants were sequenced, and all contained the TTG-to-TCG change.

Point mutations were backcrossed by transduction of recipient strain TE8607 ( $\Delta cysC::tetAR \Delta rpoS1076::cat$ ), selecting Cys<sup>+</sup> and screening for loss of the Cam<sup>r</sup> marker in the *rpoS* leader. Double mutants carrying both a *lac* fusion and linked point mutation were constructed by transduction using a donor strain carrying the *lac* fusion (marked with Kan<sup>r</sup>) and the  $\Delta rpoS1084::tetAR$  insertion into a recipient bearing the desired point mutation. The desired class of transductants was Kan<sup>r</sup> Lac<sup>+</sup> Tet<sup>s</sup>. The *rpoS* leader genotype of each recombinant strain was confirmed by DNA sequencing.

Targeted insertion/deletions of several genes were made by the  $\lambda$  Red method. The extent of deletion for each construct was as follows:  $\Delta cysC$  deletes 11 bp of the leader including the RBS precisely to the stop codon;  $\Delta dsrA$  deletes from the -35 hexamer of the promoter through the terminal poly(U) sequence (2068766 to 2068651 of GenBank NC\_003197);  $\Delta prpA$  also deletes from the -35 hexamer of the promoter through the terminal poly(U) sequence (1444972 to 1444822 of GenBank NC\_003197). For reference, the *rpoS* leader extends from 3067051 to 3066487 of GenBank NC\_003197.

**$\beta$ -Galactosidase assays.** Cells were centrifuged, resuspended in Z buffer (100 mM KPO<sub>4</sub> [pH 7.0], 10 mM KCl, 1 mM MgSO<sub>4</sub>), and then permeabilized by treatment with sodium dodecyl sulfate (SDS) and chloroform (32). Assays were performed in Z buffer containing 50 mM  $\beta$ -mercaptoethanol. Usually, reactions were carried out in microtiter (96-well) plates and read in a Molecular Devices plate reader. Activities (change in optical density at 420 nm [OD<sub>420</sub>] per minute) were normalized to cell density (OD<sub>650</sub>) and were always compared with activities of appropriate controls assayed at the same time. For experiments involving cultures grown to different densities, the number of cells harvested was adjusted to provide approximately equal cell densities in the assay. One unit of activity, as determined by this method, is equivalent to  $\approx$ 23 units as measured by the Miller assay (32). The latter assay is much more sensitive and was employed in selected experiments for this reason.

**Immunological detection of proteins.** Cultures were grown as described in the figure legends. Exponential-phase samples were taken at an OD<sub>600</sub> of  $\approx$ 0.13, and SP samples were taken after 24 h or 48 h for cultures growing at 37°C or 18°C, respectively. Protein samples were prepared from 1-ml culture volumes by centrifugation and resuspension in 100  $\mu$ l Tris-SDS buffer (5 mM Tris-HCl, 2% SDS). Samples were vortexed, then boiled for 10 min, and centrifuged for 30 min, and the supernatants were collected and stored at -20°C. The total protein

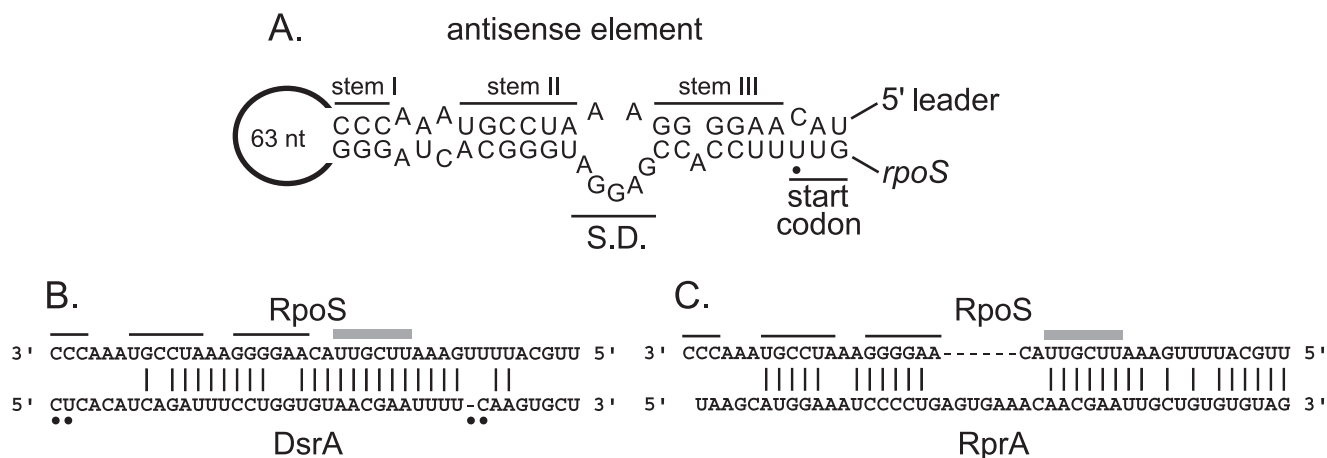


FIG. 2. A. RNA sequence of a segment of the 565-nt *S. enterica rpoS* leader RNA, starting at nt 456 (110 nt upstream from the start codon), and folded to show pairing between the antisense element and the *rpoS* RBS region (stems I, II, and III). Paired regions flank the Shine-Dalgarno (S.D.) complementarity to 16S rRNA and extend to the start codon (UUG in *S. enterica*, AUG in *E. coli*). Nucleotides that differ between *S. enterica* and *E. coli* are marked by filled circles. The antisense element and the RBS region are connected by 63 nt, which are not shown but are indicated by the oval. B. Pairing is shown between the antisense element (extended by an additional 18 nt on the upstream side) and two different sRNA regulators of RpoS: DsrA and RprA. Paired nucleotides are indicated by vertical lines, and spaces have been introduced where needed to facilitate the alignment. The stems of the antisense element are overlined for reference. The gray boxes indicate a hexameric sequence whose complementarity with the target RpoS RNA is essential for sRNA function, as shown for *E. coli* (28, 29). The DsrA sequence shown starts at +1, and the RprA sequence starts at +28.

concentration of each sample was determined using a Lowry-based protein assay (Bio-Rad) according to the manufacturer's protocol. For each gel, protein samples were diluted in sample buffer to an equal concentration and then boiled for 5 min prior to loading onto 10 or 12% polyacrylamide mini-gels. Gels were loaded with either 10 µg of total protein per well (SP protein samples) or 50 µg per well (exponential-phase samples). Electrophoresis was carried out at 75 V for 2.5 h, and resolved proteins were transferred to a Sequi-blot polyvinylidene difluoride membrane (Bio-Rad) at 100 V for 50 min using Bio-Rad's Mini Trans-Blot transfer cell. Subsequent steps and RpoS detection, using the R12 monoclonal antibody (6), were carried out as previously described (21) with the following exception. Membranes prepared from stationary-phase protein samples, containing relatively higher levels of RpoS, were incubated for 2 to 3 h with a secondary antibody directly conjugated to horseradish peroxidase.

**Northern blot assays.** To detect DsrA, a probe (designated SL1) specific for the first stem-loop of DsrA, was designed based on a probe used to study DsrA in *E. coli* (28). The SL1 probe is a single-stranded biotinylated DNA oligo and has the following sequence: 5'-biotin-AATCGTTACACCAGGAAATCTGATGTG.

Cultures were grown overnight, diluted 1:1,000 into LB containing ampicillin and 0.02% L-arabinose, and incubated at either 18 or 32°C. Total RNA was isolated from 1 ml of culture grown to mid-log phase using an RNeasy mini kit (QIAGEN) according to the manufacturer's protocol. RNA samples were diluted to 1 µg in a final volume of 15 µl in glyoxal load dye (Ambion) and incubated at 50 to 55°C for at least 1 hour prior to loading onto 6% urea-polyacrylamide mini-gels. Electrophoresis was carried out at 100 V for 80 min in 1× Tris-borate-EDTA (TBE) buffer.

Gels were assembled into a Bio-Rad Mini Trans-Blot unit, and RNA was transferred to a BrightStar membrane (Ambion) at 100 V for 50 min in 1× TBE. The RNA was cross-linked with a UV cross-linker (Stratagene) using the autocrosslink function. Cross-linked membranes were incubated at 37°C in 10 ml of UltraHyb-Oligo buffer (Ambion) for 2 to 4 h and then with the same buffer containing the SL1 probe at a concentration of 185 ng/ml. Hybridization was carried out overnight at 37°C. The blots were washed and developed using the BrightStar Biodetect nonisotopic kit (Ambion) according to the manufacturer's protocol.

## RESULTS

**Conservation of *dsrA* and *rprA* gene sequences between *E. coli* and *S. enterica*.** As noted previously by the authors who first reported the functions of the *dsrA* and *rprA* genes in *E. coli* (28,

29), these genes are highly conserved between *E. coli* K-12 and *S. enterica* LT2 as well as in certain other enteric species. In fact, conservation of sequence is thought to have predictive value in the search for new small regulatory RNA genes (16, 47). Both the *dsrA* and *rprA* genes reside at similar positions in the two bacterial chromosomes. Each gene is flanked by the same neighboring genes. The primary sequence of *S. enterica dsrA* shows eight substitutions and three missing nucleotides compared to its *E. coli* counterpart (≈90% identity), and these changes can be accommodated with limited effect on the folded structure (24, 43). Furthermore, the nucleotides of DsrA RNA that are predicted to pair with *rpoS* mRNA are completely conserved, as shown in Fig. 2. The *S. enterica rprA* gene shows even higher identity to its *E. coli* counterpart (three substitutions in 107 nucleotides [nt]), and its interface with *rpoS* mRNA is completely conserved as well. The promoter sequences of the two genes are also nearly identical.

This conservation of sequence suggests that the functions of these two genes are important and also implies these functions should be conserved between the two species. Therefore, we investigated the role of these two sRNAs in regulation of *S. enterica rpoS* under three conditions shown to activate *rpoS* expression in *E. coli*: low temperature (DsrA), osmotic shock (DsrA and RprA), and activation by RcsC (RprA).

**Effect of low temperature.** Sledjeski et al. reported that growth at low temperature (20°C) has a dramatic effect on *rpoS* in *E. coli*. Expression in exponential phase was reported to increase by ≈100-fold compared to 42°C (44). This increase specifically required DsrA (44) and was a posttranscriptional effect on synthesis, consistent with the known mode of action of overexpressed DsrA on RpoS at 37°C (28). The effect of a *dsrA* mutation was less dramatic in stationary phase but still significant (8- to 10-fold).

To study the role of *dsrA* and *rprA* in *S. enterica*, we char-

acterized the effect of the deletion of each gene separately, or both genes together, on expression of the RpoS-dependent reporter, *katE-lac* [op]. This reporter was used because the level of expression of *rpoS-lac* [pr] fusions is very low in exponential phase, particularly since the *rpoS* gene has a TTG start codon in *S. enterica* (see below). Results obtained by assay of *katE-lac* were confirmed by Western blotting of RpoS itself. As a positive control, we used the *katE-lac* reporter fusion in an *E. coli* MC4100 derivative that was either wild type for *dsrA* or carried the *dsrA1::cat* mutation (44). As expected, little effect of *dsrA* loss was seen for cells in exponential phase at 37°C, either in *E. coli* or in *S. enterica* (Fig. 3A). For *dsrA*<sup>+</sup> *E. coli* cells in exponential phase at 18°C, expression of *katE-lac* [op] was about 10-fold higher than at 37°C (compare Fig. 3A and B; note different scale). The *E. coli dsrA* mutant showed ≈6.5-fold-lower expression compared to wild type at 18°C. A modest increase in reporter expression at low temperature was also seen in *S. enterica* (≈2-fold), but there was no effect of deleting *dsrA* or *rprA*, either singly or together. Western blot analysis using a monoclonal antibody specific for RpoS confirmed this finding (Fig. 3C and D). A *dsrA* mutation has a large effect on both RpoS levels and expression of *katE-lac* [op] at 18°C in *E. coli*, but not in *S. enterica*. Additionally, the effect of *dsrA1::cat* on *rpoS-lacZ* [pr] activity was assessed in *E. coli* as performed previously by Sledjeski et al. (44). In exponential phase at 18°C, the *rpoS-lacZ* activity of the *dsrA*<sup>+</sup> cells was ≈12-fold higher than at 37°C, and the activity of the *dsrA1::cat* cells was ≈14-fold less than wild type at 18°C (data not shown).

The temperature response in *E. coli* results from a combination of ≈6-fold-increased expression from the *dsrA* promoter at 25°C compared to 37°C, as well as stabilization of the RNA at low temperature (37). A *lac* operon fusion to the chromosomal *S. enterica dsrA* gene was constructed to test whether there was a response similar to that of *E. coli*. We observed 2.5- to 3-fold-higher expression at 18°C compared to 37°C (Fig. 3E).

**TTG start codon for *rpoS*.** Expression of *S. enterica rpoS* is particularly low because the gene starts with a TTG initiation codon (26), compared to the ATG start found in *E. coli*. In strain LT2, this is partially compensated at the level of the RpoS protein and reporters such as *katE-lac* [op] by the *mviA* V102G mutation, which eliminates regulated RpoS protein turnover (3, 10). We are not aware of any protein sequence analysis that would confirm the assignment of the initiation codon in either *E. coli* or *S. enterica*.

The origin of this difference between *E. coli* and *S. enterica* lineages is not clear. The DNA sequences of several LT2 isolates (stored frozen for many years) all show the TTG start (unpublished data). On the other hand, *rpoS* from a virulent *Salmonella* strain (ATCC 14028s) has an ATG start (50). Since it has recently been found that N-terminal deletions of *rpoS* may retain substantial function (17, 36), we tested the function of the *S. enterica* TTG initiation codon. Site-directed mutations changing the start codon were substituted at the native *rpoS* locus in the bacterial chromosome as described in Materials and Methods. Substitution of TTG with ATG increased expression of an *rpoS-lac* protein fusion (codon 250) by ≈10-fold, whereas substitution of TTG by TCG decreased expression by more than 50-fold. This genetic test confirms that the predicted TTG start codon carries out this role for *rpoS* in *S. enterica*.

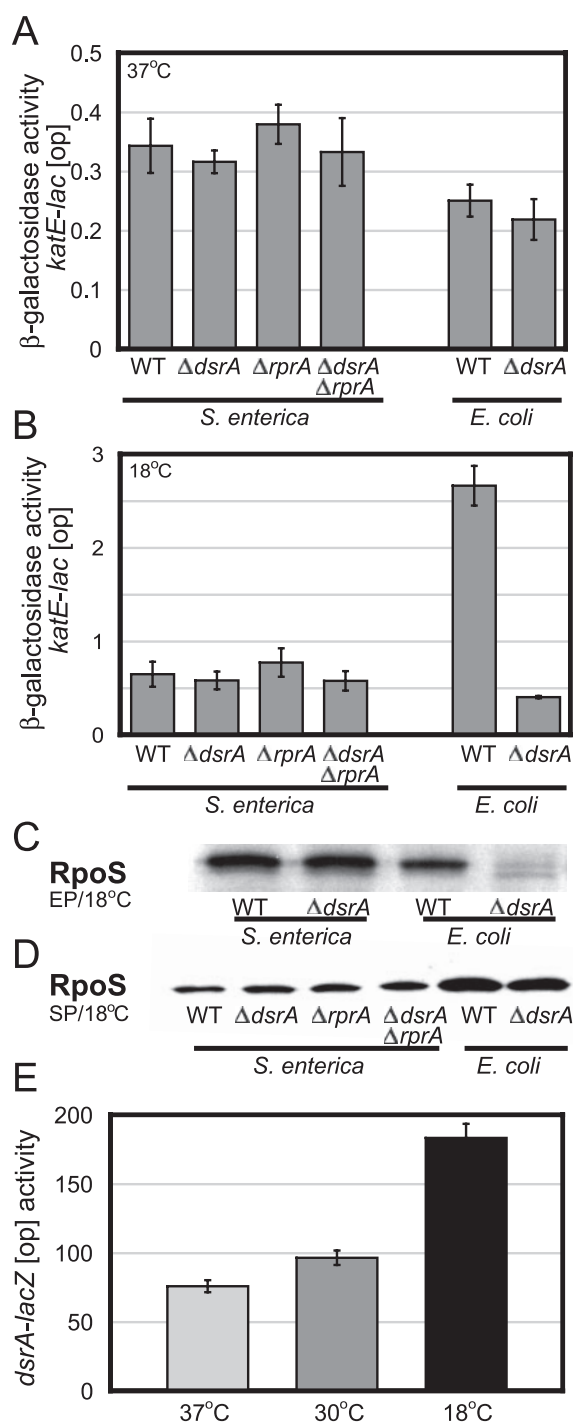


FIG. 3. Effect of low temperature on RpoS. Cells carrying the RpoS-dependent reporter *katE-lac* [op] were grown in LB medium at either 37°C (A) or 18°C (B) to early exponential phase (OD<sub>600</sub> of 0.18) and assayed for β-galactosidase activity. The *S. enterica* strains used were wild type (TE6153),  $\Delta dsrA$  (TE8608),  $\Delta rprA$  (TE8610), and  $\Delta dsrA \Delta rprA$  (TE8613). The *E. coli* strains were wild type (TE6897) and *dsrA1::cat* (TE6913). The indicated strains were also analyzed for RpoS protein by Western blotting as described in Materials and Methods. Cells were grown to exponential phase (C) or stationary phase (D) in LB medium at 18°C. (E) A *lac* operon fusion to +11 of the *S. enterica dsrA* gene was constructed as described in Materials and Methods, and its expression was assayed by measuring β-galactosidase activity after growth to exponential phase in LB medium at the indicated temperatures.

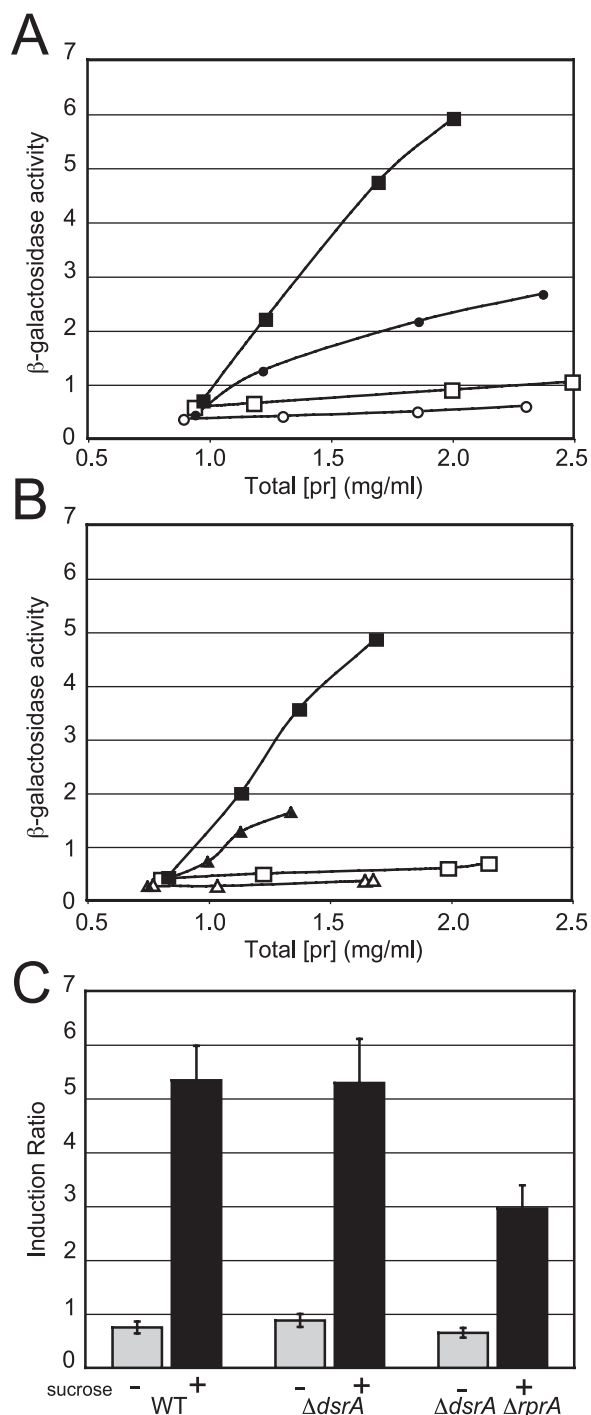


FIG. 4. Effect of osmotic challenge on *rpoS-lac*. The *lac* protein fusion employed was formed by insertion of the transposon MudK at codon 250 of the *S. enterica rpoS* gene (see Materials and Methods for details). Cultures were grown in LB medium at 30°C to early exponential phase, split into duplicate cultures, and grown to an OD<sub>600</sub> of 0.12. Cultures were challenged with prewarmed aliquots of either sucrose dissolved in LB medium (addition of  $\approx 1/5$  volume of 2 M sucrose to give 0.464 M final concentration;  $\approx 16\%$ ) or LB alone. Samples were removed at time zero and at 15, 30, and 45 min after challenge. Cells were concentrated and assayed for total protein and for  $\beta$ -galactosidase activity. (A) Comparison of wild-type cells (squares) and a *dsrA* mutant (circles); sucrose-challenged cells are represented by filled symbols. (B) Comparison of the wild type to the  $\Delta dsrA \Delta rprA$  double

**Osmotic shock.** Sucrose challenge experiments were carried out following the protocol described by Majdalani et al. (27). Cells with a single-copy *rpoS-lac* [pr] fusion were grown in LB medium at 30°C and challenged with sucrose during early exponential phase. The *lac* fusion used for this experiment was formed by insertion of MudK at codon 250 of the *rpoS* gene. In other backgrounds, this fusion can be subject to the protein turnover control exerted by *mviA/rssB/sprE* and the ClpXP protease (data not shown), but the “wild-type” LT2 strain employed for this experiment is defective for this pathway (3, 10). The signal from this *lac* fusion was increased by substituting an ATG start codon, as found in *E. coli*. This change should not affect pairing with the antisense element, and it also did not affect induction of *rpoS* by osmotic shock (data not shown).

Addition of sucrose causes cells to plasmolyse, reducing their cross-section for light scattering and thereby resulting in decreased turbidity. Even without a change in *lac* expression, this decrease in turbidity would result in an artifactual increase in  $\beta$ -galactosidase activity if it were normalized to OD<sub>650</sub> as is typically done. For this reason, following Majdalani et al., we plotted the total  $\beta$ -galactosidase activity from a fixed volume of cells versus the amount of protein in the same sample, as determined at various times after challenge. In Fig. 4A, the results from a representative experiment are given for osmotic challenge of a *dsrA* single mutant, compared to wild type. In panel B, the same experiment was carried out with a *dsrA rprA* double mutant, also compared to wild type. It can be seen that the response to sucrose was reduced in the *dsrA* single mutant but did not appear to be further reduced in the *dsrA rprA* double mutant. Growth of the double mutant was apparently sensitive to sucrose, based on decreased protein accumulation in the challenged culture, and this observation was reproducible.

The incomplete effect in the *S. enterica* double mutant contrasts with the results obtained for *E. coli*. There, a *dsrA* knock-out reduced basal *rpoS-lac* expression by about sevenfold (at 32°C), but the relative induction of *rpoS* by osmotic shock was still nearly as high as in the wild type (28). In contrast, induction was almost eliminated for the *dsrA rprA* double mutant (calculated from Fig. 6 of reference 27). Combined data from a set of trials in *S. enterica* are shown in Fig. 4C. Each bar represents the ratio of enzyme activity normalized to protein, comparing values from 45 min postchallenge to time zero. It can be seen that the double mutant retained substantial induction by osmotic shock, although it was noticeably decreased from the wild-type level. The normalization to protein levels employed here is technically demanding because of limited sensitivity of the small-scale protein assay as well as high background (data not shown) and because the comparison made is between ratios of ratios (activity divided by protein, at two different times). Therefore, we also analyzed the data in a

mutant (triangles) under the same conditions. (C) Data from a set of such experiments. Each bar represents the ratio of enzyme activities from the 45-min and time-zero samples, where both values have been normalized to the corresponding protein concentration. Dark bars represent sucrose-challenged cultures, and light bars represent untreated controls. The strains used were wild type (TE9160),  $\Delta dsrA$  (TE9213), and  $\Delta dsrA \Delta rprA$  (TE9219).

simpler fashion, by calculating the ratio of total  $\beta$ -galactosidase activity in sucrose-challenged and control cultures at 45 min postchallenge. This alternative approach confirms that the double mutant retained about two-thirds the induction seen in the wild type (data not shown). Since growth of this mutant appears somewhat more sensitive to osmotic shock than the wild type, the value of two-thirds is actually a lower limit for the relative inducibility of the mutant. In summary, our results indicate that in *S. enterica* osmotic induction of *rpoS* was decreased by about one-third in a *dsrA rprA* double mutant, while the defect in the *E. coli* double mutant was reported to be nearly complete (27).

**Effect of constitutive RcsC.** The third characterized small RNA-mediated regulation of *rpoS* in *E. coli* involves induction of RprA via the Rcs phosphorelay system, which also regulates capsule synthesis. As described by Majdalani et al. (29), in *E. coli* RprA RNA levels are increased about 50-fold by the constitutive *rscC137* allele of the gene encoding the transmembrane sensor kinase RcsC. The recessive nature of this allele (A904V) suggests that it affects a negative regulatory activity contributed by the response regulator domain of this hybrid sensor kinase. RpoS-LacZ levels are increased more than 20-fold by the same mutation, and most of this increase is *rprA* dependent. We tested two similar constitutively active alleles of *rscC* in *S. enterica*, *rscC55* (T903A) and *rscC64* (F473I), described by Garcia-Calderon et al. (15). Otherwise-isogenic strains were constructed carrying an *rpoS*::MudK insertion (*lac* protein fusion at codon 22 of *rpoS*) and were either wild type for *rscC* or carried one of the two constitutive *rscC* alleles. Subsequently, a deletion of *rprA* was introduced into each strain. Expression of *rpoS-lac* was elevated 2.5- to 3-fold by both *rscC* alleles (Fig. 5); however, deletion of *rprA* had almost no effect on the activation. As a positive control for the effect of constitutive *rscC*<sup>+</sup> mutations, similar strains were constructed by substituting the *lac* fusion with an insertion of MudJ (forms operon fusions) in the *gmm/wcaH* gene to monitor transcription of the capsule biosynthesis cluster (15). Activation of *wcaH* by the mutant alleles of *rscC* was at least 50-fold (expression in the wild-type *rscC*<sup>+</sup> strain was below the limit of detection for this assay). Although RprA was not previously observed to regulate capsule synthesis, introduction of the *rprA* deletion reduced expression of *wcaH-lac* to about 60% of the level seen with wild-type *rprA*. In summary, we found  $\approx$ 3-fold activation of *rpoS* expression by both of these constitutive *rscC* alleles, but there is no evidence for involvement of the RprA RNA in this response.

**Testing the effect of overexpression of DsrA on *rpoS-lac*.** The experiments presented in this paper do not support a significant role for the DsrA and RprA RNAs in the regulation of *rpoS* in *S. enterica*. One possible explanation is that the few sequence differences between the *dsrA* and *rprA* genes of *S. enterica* and *E. coli* are responsible for this unexpected result. Therefore, we tested the function of *S. enterica dsrA* more extensively by using the overexpression phenotype described by Majdalani et al. (28). The *dsrA* gene from *S. enterica* was expressed from the pBAD promoter in plasmid pTE780, and a similar plasmid, pNM3, carrying the *E. coli dsrA* gene was tested in parallel (28). These plasmids together with vector controls were introduced into *E. coli* and *S. enterica* strains bearing appropriate *rpoS-lac* [pr] fusions. Cultures were grown

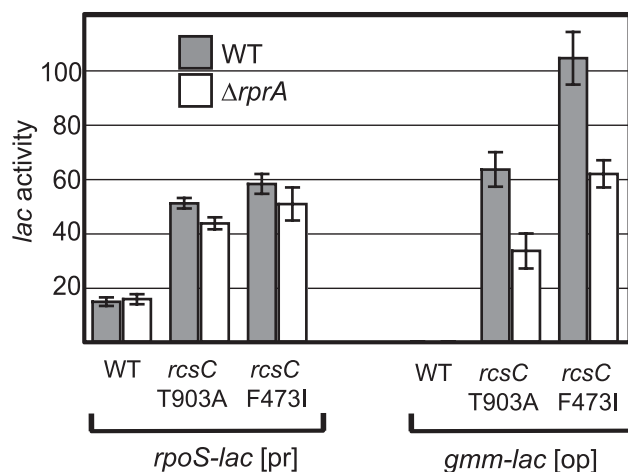


FIG. 5. Effect of activated *rscC* on *rpoS-lac*. In the left half of the figure, the *lac* protein fusion was formed by insertion of the transposon MudK at codon 22 of the *S. enterica rpoS* gene. In the right half, the *lac* fusion was the *gmm-21*::MudJ insertion (also called *wcaH*) (15). Two activated alleles of *rscC* were compared to wild type, and each set consisted of strains either wild type or mutant for *rprA*. Cells were grown overnight to stationary phase in LB medium at room temperature (23 to 25°C) and assayed for  $\beta$ -galactosidase activity, normalized to the OD<sub>600</sub> as described in Materials and Methods. Results shown are the averages and standard deviations for at least seven independent experiments. Strains for *rpoS-lac* were *rscC*<sup>+</sup> *rprA*<sup>+</sup> (TE9317), *rscC*<sup>+</sup>  $\Delta rprA$  (TE9353), *rscC55* *rprA*<sup>+</sup> (TE9316), *rscC55*  $\Delta rprA$  (TE9352), *rscC64* *rprA*<sup>+</sup> (TE9318), and *rscC64*  $\Delta rprA$  (TE9354). The corresponding strains with the *gmm-21*::MudJ insertion were TE9334, TE9394, TE9333, TE9395, TE9368, and TE9396.

overnight at 32°C in LB medium with ampicillin, then diluted into the same medium containing arabinose as the inducer, and grown overnight to stationary phase. Preliminary experiments testing various levels of inducer showed that at high levels (0.2% arabinose) the empty vector controls displayed a significant negative effect on *rpoS* expression. Because of this, the experiments described here were performed with intermediate levels of arabinose (0.02%).

The results are shown in Fig. 6A. Expression of either *E. coli* or *S. enterica dsrA* in *E. coli* resulted in significant induction of *rpoS*. There was an  $\approx$ 8-fold increase in activity of *rpoS-lac* [pr] with overexpression of the *E. coli dsrA* gene; for *S. enterica dsrA* the increase was slightly less,  $\approx$ 6-fold. In contrast, neither gene was able to activate *rpoS* expression in the *S. enterica* strain background. This negative result in *S. enterica*, obtained using a MudK insertion in the native *rpoS* locus as the reporter, was confirmed using a fusion construct identical to the reporter tested in *E. coli*: *E. coli rpoS* fused to *lac* (6) (data not shown). These results show that *S. enterica dsrA* is capable of activating *rpoS* expression in *E. coli*.

Northern blot analysis of RNA purified from induced exponential cultures showed that DsrA RNA expressed from P<sub>BAD</sub> was clearly visible in *E. coli*, but no signal could be detected in *S. enterica* (Fig. 6B and C). DsrA expressed from the chromosome was also detected in the *E. coli* vector control strains at 18°C, but not at 32°C (Fig. 6B and C). The pattern of rRNA in all samples was normal (data not shown) (35), and it was determined that the plasmids were maintained in all strains under these conditions (data not shown). Furthermore, arabi-



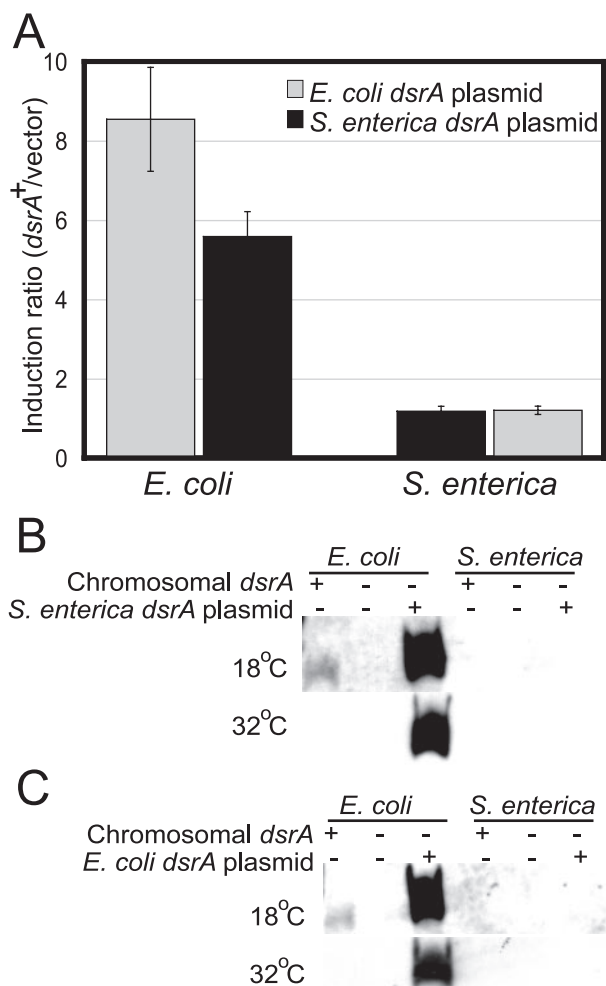


FIG. 6. Expression of *dsrA* from  $P_{BAD}$ . A. Strains of *E. coli* or *S. enterica* with an *rpoS-lac* [*pr*] fusion in the bacterial chromosome, deleted for *dsrA*, and also carrying a plasmid  $P_{BAD}$ -*dsrA* construct from the species indicated, were induced with 0.02% arabinose and grown overnight to stationary phase in LB-Amp medium at 32°C. Activity of  $\beta$ -galactosidase is plotted for each strain, normalized to the expression seen with a vector control. B and C. Northern analysis of DsrA accumulation in *E. coli* and *S. enterica* strains carrying plasmids expressing either *S. enterica dsrA* (B) or *E. coli dsrA* (C) under the control of the  $P_{BAD}$  promoter. Strains carrying the appropriate empty vector were used as controls. Culture conditions, RNA purification, and Northern blotting were carried out as described in Materials and Methods. Strains were TE9416, TE9418, TE9419, TE9422, TE9424, TE9425, TE9426, TE9427, TE9428, TE9429, TE9430, and TE9431.

nose induction of a  $P_{BAD}$ -*lacZ* construct was observed in *S. enterica*, to approximately the level of a fully induced single-copy *lac* operon (data not shown). Failure to overexpress DsrA RNA of either type in *S. enterica* suggests that the defect lies with DsrA RNA turnover, but it allows the formal possibility that *S. enterica rpoS* also fails to respond.

**Mutations affecting the antisense element and its RBS target in *S. enterica rpoS*.** All previous studies on function of the antisense element employed a *lac* fusion to the *E. coli rpoS* gene and its upstream sequences (6). The effects of point mutations on *rpoS* expression were studied primarily in the *S. enterica* background (7), while the effects of deletion and over-

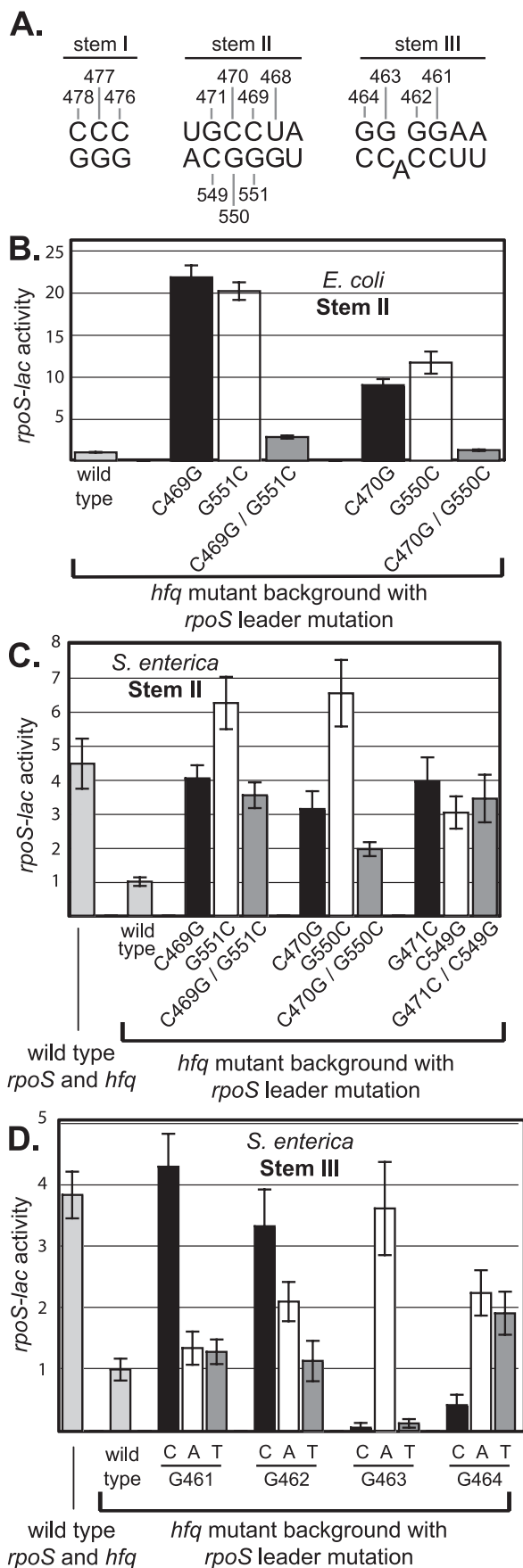
expression of small RNA genes have been studied in *E. coli* (28, 29, 44, 52). Therefore, we tested the effects of mutations changing either the antisense element or the *rpoS* RBS in *S. enterica*. As described above, the sequence of the *rpoS* leader is highly conserved in *S. enterica* compared to *E. coli*, particularly in the region between the antisense element and the start codon (Fig. 2). Even in the 63-nt connector region, which is predicted to form two stem-loops (20), the eight substitutions found in *S. enterica* would affect loop nucleotides rather than paired stem nucleotides (data not shown).

A number of point mutations were constructed directly on the bacterial chromosome using the  $\lambda$  Red recombination system and selection for Tet<sup>s</sup> transformants as described in Materials and Methods and combined with a MudK insertion in the *rpoS* gene to measure their effects on expression. Mutations in the *rpoS* leader were studied in a strain carrying an insertion in the *hfq* gene for several reasons. First, an *hfq* mutation is known to sensitize *E. coli* to the effects of similar mutations (7). This may be due to the existence of other, as-yet-uncharacterized Hfq-dependent activating sRNAs, or it may be due to far-upstream elements of the *rpoS* leader which also have Hfq-dependent effects (9). A second reason to use an *hfq* mutant is that interactions with other molecules might confound the interpretation of phenotypes for strains with compensatory mutations.

The results of these experiments are shown in Fig. 7. The effects observed for mutations in *S. enterica* are broadly, but not completely, compatible with what was reported previously in *E. coli*. For three positions of stem II lying within the antisense element and the corresponding three positions in the RBS region, each mutation tested showed a mutant phenotype with elevated *rpoS* expression (Fig. 7C). Two differences from *E. coli* were noted. First, the magnitude of the effect on expression in the mutants was somewhat lower in *S. enterica* than in *E. coli*. Second, the effect of compensatory mutations was not as complete or dramatic as observed in *E. coli* and, for one position, compensation was not observed at all (compare panels C and B).

A comprehensive set of mutations in the top strand of stem III was also constructed (Fig. 7D). The pattern for this set is complex: of 12 mutations, 3 have a strong mutant phenotype with elevated expression, 3 are weakly mutant with elevated expression, and 3 are mutant but show decreased expression (as much as 10-fold decreased), while 3 are wild type (Fig. 7D). We do not currently have an explanation for this complex pattern. However, we emphasize that precisely the same pattern was observed in a similar panel of mutations affecting *E. coli rpoS* at positions 461 to 464 (C. Cuning and T. Elliott, unpublished data). The sole exception was G461A, which is mutant in *E. coli* and normal in *S. enterica*.

The conservation of all nucleotides involved in the folded structure as well as the phenotypes of single mutations affecting paired nucleotides are both consistent with a similar function for the antisense element in *S. enterica* as in *E. coli*. As a final support for this interpretation, we sought and obtained additional mutations, including some that have a down phenotype with decreased expression of *rpoS*. These include a double mutation in stem I (C476G C477G, sevenfold elevated) and T468C (fourfold decreased). Phenotypes for both of these mutants are consistent with the model.



**DISCUSSION**

High overall sequence conservation of the DsrA and RprA small regulatory RNAs in *S. enterica* and *E. coli*, as well as complete conservation of their interface with the target in the *rpoS* leader, suggests conservation of their function. Working with *S. enterica*, we tested the role played by these two sRNAs in *rpoS* expression under three specific conditions: growth at low temperature, after osmotic shock, and with constitutive activation of the Rcs phosphorelay by an RcsC\* mutation. Only osmotic shock showed a specific requirement for the two RNAs, and even this dependence was partial, whereas in *E. coli* it is complete (27).

The intrinsic competence of *S. enterica* DsrA for *rpoS* regulation was tested in an *E. coli* host, where it was found to be nearly as effective as *E. coli* DsrA. Conservation of the ability to stimulate *rpoS* expression is not surprising, given the sequence identity. The reciprocal test, of *E. coli* DsrA in an *S. enterica* host, showed no stimulation of *rpoS* but was inconclusive, since Northern blot assays did not show any accumulation of the sRNA.

Mutations affecting the antisense element of the *S. enterica* *rpoS* leader region have phenotypes generally consistent with those seen in *E. coli*, except that predicted compensatory double mutants in stem II are not clearly wild type. The reason for this difference from *E. coli* is not known. Although suppression in double stem II mutants of *E. coli* was a striking finding in support of the antisense model, weak or absent suppression in *S. enterica* is not necessarily a strong argument against the model. To explain this, we can invoke additional (as-yet-unknown) factors which interact (or fail to interact) to give elevated expression even with the paired structure present. There is abundant additional evidence that function of the antisense element is important in *S. enterica*. First, the single top-strand (antisense element) mutants do have a mutant phenotype. Second, for the 469/551 and 470/550 pairs, the double mutant is reduced over expression in the bottom strand mutant alone. So, there is some suppression, but it is weak. Third, we carried out an extensive genetic analysis of the top strand of stem III, and although an explanation for the detailed expression pattern is not available, it is clear that in this respect *S. enterica* does look almost exactly like *E. coli*.

In summary, DsrA and RprA functions in regulation of *rpoS* are not conserved, despite sequence conservation of the sRNA regulators and no change in the sRNA-target interface. The

FIG. 7. Effects of point mutations in the antisense element and RBS region on expression of *rpoS* in *E. coli* and *S. enterica*. *S. enterica* strains with mutations in the *rpoS* leader on the bacterial chromosome were constructed as described in Materials and Methods. The *rpoS*::MudK (codon 216) fusion and an *hfq*::Mud-Cam insertion were introduced by P22 transduction. *E. coli* fusion strains have been described elsewhere or were made in the same way (7). Cultures were grown overnight in LB medium at 23 to 25°C to SP and assayed for  $\beta$ -galactosidase activity. A. Locations of point mutations within the antisense element. B. Results with strains TE6266, TE6557, TE6558, TE6590, TE6369-2, TE6369-3, and TE6382. C. Results with strains TE8808, TE8815, and TE8852 to TE8860. D. Results for strains TE8808, TE8815, and TE9236 to TE9247.

most likely explanation for this is RNA instability in *S. enterica*, though this was not demonstrated directly.

It does not seem too surprising that the sRNAs are not important for *S. enterica rpoS*, since *rpoS* induction by low-temperature growth and activated RcsC\* are both much reduced compared to *E. coli*. The *dsrA* promoter remains modestly sensitive to temperature in *S. enterica*, albeit less so than in *E. coli* (38). The *E. coli* response is reported to be largely dependent on an unusual  $-10$  sequence (TAAGGT) and an AT-rich 17-bp spacer between the  $-35$  and  $-10$  hexamers (38). The  $-10$  sequence as well as the length and AT-rich motif of the spacer are completely conserved in *S. enterica*. A primary difference between the two promoters is found between  $-10$  and  $+1$ . In *E. coli*, replacement of the *dsrA* promoter's native start site with that of *lac UV5p* also decreased the temperature response. Although mutant *dsrA* promoter constructs were not assessed in the context of *rpoS* translation activation, this difference may explain the less dramatic effect of temperature on the *dsrA* promoter in *S. enterica*. Regulation of the response to osmotic shock is also clearly different in the two species. The magnitude of the response is similar, but dependence on both sRNAs is much reduced in *S. enterica*.

Despite partial conservation of thermoregulation for the *dsrA* promoter and strong expression of the *dsrA-lac* [op] fusion, DsrA does not accumulate in *S. enterica* to a level detectable by Northern blotting even when overproduced from the  $P_{BAD}$  promoter. Why would *S. enterica* transcribe DsrA only to degrade it? It might be that this sRNA is more susceptible to RNase activity in *S. enterica* or has lower affinity for Hfq, which stabilizes DsrA in *E. coli* (45). A more interesting possibility would be that, since the action of sRNAs is often coupled with degradation (16), there might exist additional or more competitive targets for DsrA action in *S. enterica*.

How can the nonconservation of DsrA and RprA functions with respect to *rpoS* regulation be reconciled with high sequence conservation of these genes in *E. coli*, *S. enterica*, and other enteric bacteria? For the sRNAs, the simplest explanation is that they have additional targets where their function is adaptive. Some evidence has been presented for additional DsrA functions in *E. coli* (25, 43), although these studies have relied on overexpression of DsrA and thus may not reflect normal physiology. There is no obvious sign that these sequences have been recently exchanged between the two species. Conservation of the antisense element might be explained by the presence of additional sRNAs, like those described in *E. coli* (47), or upstream leader sequences with Hfq-dependent action on this target (9). To our knowledge, a search for unique sRNAs in *S. enterica* has not been made.

Alternatively, activities of other regulatory elements may be tuned to the presence of the antisense element in the same way as they rely on a strong promoter and RBS region. However, this argument is somewhat less persuasive, given the counterexample of the change to a TTG start codon in *S. enterica* and loss of the protein turnover pathway in many strains of the LT2 lineage.

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