JOURNAL OF BACTERIOLOGY, Oct. 2001, p. 5974–5981 Vol. 183, No. 20
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.20.5974–5981.2001

RpoS-Dependent Transcriptional Control of *sprE*: Regulatory Feedback Loop

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Received 1 June 2001/Accepted 16 July 2001

The stationary-phase response exhibited by *Escherichia coli* upon nutrient starvation is mainly induced by a decrease of the ClpXP-dependent degradation of the alternate primary σ factor RpoS. Although it is known that the specific regulation of this proteolysis is exercised by the orphan response regulator SprE, it remains unclear how SprE's activity is regulated in vivo. Previous studies have demonstrated that the cellular content of SprE itself is paradoxically increased in stationary-phase cells in an RpoS-dependent fashion. We show here that this RpoS-dependent upregulation of SprE levels is due to increased transcription. Furthermore, we demonstrate that *sprE* is part of the two-gene *rssA-sprE* operon, but it can also be transcribed from an additional RpoS-dependent promoter located in the *rssA-sprE* intergenic region. In addition, by using an in-frame deletion in *rssA* we found that RssA does not regulate either SprE or RpoS under the conditions tested.

Bacteria are constantly sampling their surroundings and regulating gene expression accordingly. Since many of the environments they encounter often have hazardous conditions (for example, limiting nutrients, high osmolarity, extreme pH, or extreme temperature), bacteria have evolved to survive in such hostile habitats. In particular, the gram-negative bacterium *Escherichia coli* enters a state in its life cycle known as the stationary phase, which renders it highly resistant to unfavorable environmental conditions.

When cells enter stationary phase, they undergo dramatic changes in their morphology and physiology that increase their chance for survival in a wide variety of stresses. This cross-protection results from the global control system regulated by RpoS. RpoS, encoded by the *rpoS* gene, is the second primary σ factor of *E. coli,* and it is required for the transcription of stationary-phase-specific genes. Due to the drastic consequences (i.e., slowed metabolism) of entering stationary phase, RpoS is tightly regulated. In fact, RpoS is regulated at all levels: transcription, translation, protein stability, and activity (for a recent review, see reference 13).

Among the possible stresses that can induce RpoS (the stationary-phase response), starvation of an essential nutrient is perhaps the most widely studied. When nutrients are readily available, the levels of RpoS are very low, mainly due to its efficient degradation by the ATP-dependent ClpXP serine protease. Conversely, when nutrients become limiting for growth, this ClpXP-dependent proteolysis stops and, consequently, RpoS levels increase significantly (26). This mode of RpoS regulation has been shown to occur in response to carbon starvation as well as during growth in Luria-Bertani (LB) medium, although the specific signals sensed in the latter medium remain to be determined (25, 32).

The regulation of RpoS proteolysis is not mediated by controlling either the levels of the ClpXP protease itself or its activity (33). Instead, it is orchestrated by the response regulator SprE (named RssB in *E. coli*, MviA in *Salmonella enterica* serovar Typhimurium, and ExpM in *Erwinia carotovora*) (1, 2, 22, 25). In a recent report, Zhou et al. demonstrated in vitro that SprE plays a catalytic role in the delivery of RpoS to ClpX, the regulatory component of ClpXP that is believed to unfold RpoS and eventually feed it to ClpP, the proteolytic component (34). ClpP then degrades RpoS and SprE is released from the proteolytic complex. Furthermore, Zhou et al. also showed that this in vitro degradation is greatly enhanced upon SprE phosphorylation (34).

To date, it remains unknown how SprE is phosphorylated in vivo; therefore, SprE is an orphan response regulator. Moreover, unphosphorylated SprE can still promote, although less efficiently, RpoS degradation (reference 6 and unpublished results cited in reference 34). This raises the possibility that SprE might be regulated by a mechanism(s) other than phosphorylation.

A possible mechanism for regulating SprE-mediated degradation of RpoS is to control the levels of SprE itself. Paradoxically, the levels of SprE (and MviA) have been shown to increase when cells enter stationary phase (11, 21). Specifically, it has been shown that the translation of *sprE* increases in an RpoS-dependent manner (11). In addition, it was reported that *sprE* (and *mviA*) transcription is also upregulated during the stationary phase (11, 21). Although those studies did not prove the exact location of the *sprE* promoter, their reporter fusion data showed that *sprE* transcription can occur independently from that of its upstream gene, *rssA* (11). Prior to these studies it was assumed, based on DNA sequence analysis, that *rssA* and *sprE* constitute an operon (5, 22). In addition, RssA itself was implicated in the SprE pathway regulating RpoS, although its function has never been clearly demonstrated (unpublished results cited in references 13 and 22).

Since it is unclear how *sprE* transcription is regulated and what role RssA plays in RpoS regulation, we have constructed a series of reporter fusions and *rssA* null alleles that allow us to

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TABLE 1. Bacterial strains

Strain	Genotype	Reference[a]
MC4100	F$^-$ *araD139* Δ*(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	7
AF633	MC4100 λφ(*uspB'-lacZ*$^+$)	9
NR247	AF633 *rpoS*::*kan*	
NR252	AF633 *sprE19*::*cam*	
NR253	AF633 *sprE*::*tet*	
NR260	AF633 *rssA1*::*kan*	
NR270	AF633 *rssA1*::*kan sprE19*::*cam*	
NR286	AF633 *rssA1*::*kan sprE*::*tet*	
NR289	MC4100 *nadA*::Tn*10* Δ*(gal-att-bio)*	
NR501	MC4100 λφ(*rssA'-'lacZ*)	
NR502	NR501 *rpoS*::*kan*	
NR507	MC4100 λφ(*rssA-sprE'-lacZ*$^+$)	
NR508	NR507 *rpoS*::*kan*	
NR509	NR289 λφ(*rssA-sprE'-lacZ*$^+$)	
NR510	NR509 *rpoS*::*kan*	
NR511	MC4100 λφ(*'rssA-sprE'-lacZ*$^+$)	
NR512	NR511 *rpoS*::*kan*	

[a] Strains without a given reference were constructed in this study.

address these issues. In this report we present data demonstrating that although *rssA* and *sprE* constitute an operon, *sprE* can also be transcribed from an RpoS-regulated promoter located in the *rssA-sprE* intergenic region. In addition, while RpoS controls RssA levels, we found no role for RssA in the regulation of either SprE or RpoS under the conditions tested.

MATERIALS AND METHODS

Bacterial strains and bacteriophages. *E. coli* DH5α (Invitrogen Life Technologies) was used as the host strain for all plasmid constructions. All other bacterial strains used (Table 1) are derivatives of MC4100 (7). Standard microbial techniques were used for strain construction (27). All fusions were recombined with λRZ5 (23).

Media and growth conditions. LB medium was prepared as described previously (27). Unless indicated, all bacterial strains were grown under aeration at 37°C and their growth was monitored by measuring the optical density at 600 nm (OD_{600}).

DNA manipulations. Plasmid DNA was purified by standard techniques and was introduced into the appropriate strains by the method of Kushner (17). All restriction endonucleases (New England Biolabs), *Taq* and *Pfu* DNA polymerases (Roche and Stratagene, respectively), T4 polynucleotide kinase (T4 PNK) (New England Biolabs), and T4 DNA ligase (New England Biolabs) were used according to the recommendations of their respective manufacturers. Primers were synthesized by the Princeton University Department of Molecular Biology Synthesis and Sequencing Facility.

Plasmid construction. The plasmids used in complementation studies were constructed as follows. A 3.06-kbp fragment containing the *ychJ-rssA-sprE* region was amplified from MC4100 chromosomal DNA using the primers RSSAUP (5′-CGGGATCCCGCCAGAGAACGTAAAGTATCG) and 3SPREMBP2 (5′-CCACCAGCCAAGCTTAGCAGG), which introduce *Bam*HI and *Hin*dIII restrictions sites (underlined) at the 5′ and 3′ ends of the PCR products, respectively. After digestion with these restriction enzymes, the PCR fragment was inserted between the *Bam*HI and *Hin*dIII sites of pBluescript KS(+) (Stratagene) and pBBR1MCS (15) to yield pRSSA5 and pRSSA6, respectively.

We also constructed a plasmid (pRSSA9) that contains the *ychJ-rssA* region but not *sprE*. The 2,332-bp *Bam*HI-*Bgl*II fragment from pRSSA5 containing the *ychJ-rssA* region (and the first 332 bp of *sprE*) was introduced into *Bam*HI-digested pBBR1MCS, resulting in pRSSA9.

Insertional inactivation of *rssA*. A null *rssA* allele was constructed by insertion of a kanamycin cassette into the *Pst*I site in the RssA-coding region (580 bp after the first guanine of the GTG start codon). The RssA open reading frame was amplified from MC4100 chromosomal DNA by PCR using primers 5RSSAKPN1 (5′-GCGATATGGGTACCATTGCATTCC) and 3RSSAHIND3 (5′-TTCTGGTCAAGCTTCGGTGCGTACC), which introduce *Kpn*I and *Hin*dIII restriction sites (underlined), respectively. The resulting 0.94-kbp fragment was digested with *Kpn*I and *Hin*dIII and ligated with *Kpn*I-*Hin*dIII-digested pRSETB (Invitrogen Life Sciences), and the new plasmid was named pRSSA2. Then, the 794-bp *Eco*RV-*Hin*dIII fragment containing the 3′ end of *rssA* was introduced into *Sma*I-*Hin*dIII-digested pBluescript KS(+) to generate pRSSA3. The *Pst*I fragment containing the kanamycin cassette from pUC4K (30) was then inserted into the *Pst*I site of pRSSA3 to create pRSSA3::*kan*. The kanamycin cassette was inserted in an orientation opposite to that of *rssA* transcription (confirmed by DNA sequencing). The *Xba*I-*Kpn*I fragment from pRSSA3::*kan*, containing *rssA*::*kan*, was ligated with *Xba*I-*Kpn*I-digested pAMPTS (24), and the resulting plasmid, pRSSA::*kan*Ts, was used for the allelic exchange in MC4100 as previously described (12).

Introduction of an in-frame deletion into *rssA*. A defined in-frame deletion in *rssA* was generated in pRSSA5 (which carries the *ychJ-rssA-sprE* region; see above) by using the method of inside-out PCR previously described (14). Briefly, pRSSA5 served as the template for an inside-out PCR with primers RTRSSA1 (5′-CCTCTCGCCGCGCCAGATCCC) and RTRSSA2 (5′-GCACGATGCTCATTTGATGC), which were designed to create an in-frame deletion of amino acids 30 to 194 of RssA. The 5.47-kbp PCR product was phosphorylated using T4 PNK and self-ligated to yield pRSSA5Δ, which contains the 492-bp in-frame deletion allele of *rssA* named *rssAΔ1*. The pRSSA5Δ plasmid was then digested with the *Bam*HI and *Hin*dIII restriction endonucleases and the fragment containing the *ychJ-rssAΔ1-sprE* region was inserted between the *Bam*HI and *Hin*dIII sites of pBBR1MCS to generate pRSSA6Δ.

Construction of *rssA-lacZ* and *sprE-lacZ* fusions. An *rssA'-'lacZ* translational fusion containing the *ychJ-rssA'* region was constructed as described below. Two *sprE'-lacZ*$^+$ transcriptional fusions were also made: one containing the *ychJ-rssA-sprE'* region (*rssA-sprE'-lacZ*$^+$) and the other containing only an *'rssA-sprE'* fragment (*'rssA-sprE'-lacZ*$^+$). The appropriate regions (see below) were inserted into either pRS414 (for the *rssA'-'lacZ* fusion) or pRS415 (for both *sprE'-lacZ*$^+$ fusions) (28). All fusion-containing plasmids were recombined into the phage λRZ5 (23) as described by Simons et al. (28). For integration of the fusions at the λ attachment (*att*) site, MC4100 was infected with the appropriate recombinant λ phage. For integration of the fusions at the chromosomal *sprE* locus, *att* deletion MC4100 derivative strains (Table 1) were infected with the appropriate recombinant λ phage.

The pRS414 derivative plasmid containing the *rssA'-'lacZ* protein fusion was constructed as follows. A PCR product was amplified from MC4100 chromosomal DNA using primers 5RSSAECOR1 (5′-GGAATTCGCCGCGATTTCGACATCC), which introduces an *Eco*RI site (underlined), and 3RSSAHIND3 (see above). The resulting PCR product was digested with *Eco*RI and *Eco*RV to generate a 1.07-kbp fragment containing the *ychJ-rssA'* region (from 395 bp downstream of *ychJ* to the first 141 bp of *rssA*), which was then inserted between the *Eco*RI and *Sma*I sites of pRS414, resulting in pRSSA11.

The pRS415 derivative plasmid containing the *rssA-sprE'-lacZ*$^+$ fusion (pCNP1) was created by introducing into the *Sma*I site of pRS415 a 2.11-kbp fragment that had been generated by PCR amplification from MC4100 DNA by using primers 5RSSAECOR1 (see above) and 3SPREBAMH1 (5′-CGGGATCCGCCAGTACCGTTGTCGCTC). The amplified region extends from 395 bp downstream of *ychJ* to 112 bp into the *sprE* coding region. To construct the pRS415 derivative plasmid containing the *'rssA-sprE'-lacZ*$^+$ fusion (pRSSA10), the 10,660-bp *Eco*RV fragment from pRSSA9 (see above) containing the region from the last 798 bp of *rssA* to the first 174 bp of *sprE* was introduced into the *Sma*I site of pRS415.

β-Galactosidase assays. After growing overnight in LB broth, cells were diluted 1:100 into fresh LB broth and grown to an OD_{600} of ~0.3 to 0.4 for logarithmic-phase samples or to an OD_{600} of ~3.0 for stationary-phase samples. β-Galactosidase assays were performed using a microtiter plate assay as described previously (29). The β-galactosidase activities were expressed as $\Delta OD_{420}/(OD_{600} \times \text{volume})$, where volume refers to the amount (in milliliters) of cell lysate used. For each experiment, every sample was assayed three times and the average activity and standard deviation (SD) were obtained. The data shown resulted from a single experiment representative of at least three other independent experiments.

Western blot analysis. Cells were grown as indicated above for the β-galactosidase assays to obtain both logarithmic- and stationary-phase samples. Once cells reached the indicated OD_{600}, 1-ml samples were pelleted. To standardize samples, the pellets were resuspended in a volume of sodium dodecyl sulfate (SDS) sample buffer (18) equal to the $OD_{600}/6$. Samples were boiled for 5 min and equal volumes were subjected to SDS–12% polyacrylamide gel electrophoresis as described by Laemmli (18). The proteins were transferred to nitrocellulose membranes (Schleicher & Schuell), and Western blot analyses were performed as previously described (10). When appropriate, polyclonal sera against RpoS or SprE were used as primary antibodies at a dilution of 1:6,000 and 1:4,000,

respectively. Donkey anti-rabbit immunoglobulin G-horseradish peroxidase conjugate (Amersham Pharmacia Biotech) was used as secondary antibody at a 1:6,000 dilution. For visualization of bands, the ECL antibody detection kit (Amersham Pharmacia Biotech) and X-Omat film (Kodak) were used.

Primer extension analysis. AF633 cells were grown to stationary phase as described above for the β-galactosidase assays. Total RNA was extracted using Trizol (Invitrogen Life Sciences). Primer RTSPRE1 (5′-AGCCGCCAGTACC GTTGTCGC) was labeled with [γ-^{33}P]ATP (ICN) using T4 PNK prior to primer extension. For the primer extension reaction, 5 μg of total RNA, labeled primer, and 100 U of Moloney murine leukemia virus reverse transcriptase (U.S. Biochemicals) were used as directed by the manufacturer. For the sequencing reaction, 3 μg of pRSSA5 plasmid was digested with *Hin*dIII for 15 min. After the digestion, pRSSA5 was mixed with 0.5 pmol of unlabeled RTSPRE1 primer and denatured by boiling for 3 min. After cooling on ice for 20 min, the sequences were determined by using a Sequenase DNA sequencing kit (version 2; U.S. Biochemicals) with [α-^{33}P]dATP (ICN) according to the directions of the manufacturer. All reactions were subjected to electrophoresis in an 8.3 M urea–6% polyacrylamide gel. The reaction products were visualized on X-Omat film (Kodak).

RESULTS

Disruption of *rssA* increases RpoS-mediated transcription. Sequence homology suggests that RssA belongs to a family of serine esterases/proteases found from bacteria to humans (20). Unfortunately, the only members of this family with a characterized function are those found in *Drosophila* and humans; their function pertains to neuronal development and they contain additional domains not found in their bacterial counterparts (16, 20).

To clarify RssA's role in RpoS regulation, we constructed a null allele (*rssA1*::*kan*) by insertionally inactivating *rssA* with a kanamycin resistance (*kan*) cassette after nucleotide 576 (where 1 corresponds to the first guanine of the GTG start codon). This *rssA1*::*kan* allele was introduced into strain AF633, which carries the RpoS-dependent *uspB'-lacZ*$^+$ fusion (Table 1) (9), and β-galactosidase activity was measured to monitor RpoS-mediated transcriptional activity. After growing in LB medium, stationary-phase cultures carrying the *rssA1*::*kan* allele had a 2.4-fold increase in the levels of β-galactosidase activity above those of the wild-type parent strain. Similar results were found using other known RpoS-dependent LacZ transcriptional fusions (data not shown). Although the increase in RpoS activity caused by the *rssA1*::*kan* allele was significant, it must be pointed out that it was not as high as that caused by a *sprE* null allele tested under the same conditions (a threefold increase with respect to the wild type). This difference in RpoS-dependent activities between the *sprE* and *rssA* null strains was also detectable on lactose MacConkey indicator media, and it was further confirmed by the fact that while the *sprE* null strain cannot grow in minimal succinate medium (due to its high levels of RpoS [see reference 24]), the *rssA* null strain can. Moreover, since a strain carrying both *rssA* and *sprE* null alleles has levels of RpoS activity equivalent to those of the *sprE* null strain, the *rssA* and *sprE* null alleles do not function in additive fashion. Thus, these results show that disruption of *rssA* increases RpoS-dependent transcription and that a mutation in *sprE* is epistatic to *rssA*.

Disruption of *rssA* increases RpoS levels by altering SprE levels. Since SprE regulates RpoS at the level of protein stability, it is likely that the increase in RpoS-mediated transcription caused by the *rssA1*::*kan* allele reflects increased RpoS levels rather than increased specific activity of RpoS. To test this, we determined the relative levels of RpoS by Western blot analysis in both logarithmic- and stationary-phase cultures of various strains grown in LB medium.

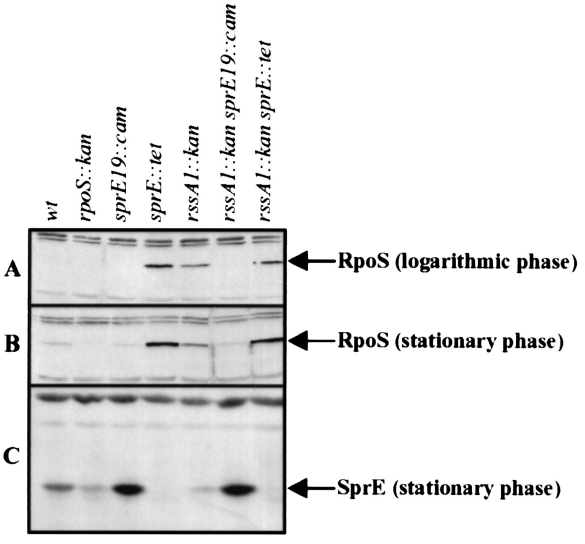

FIG. 1. Disruption of *rssA* increases the levels of RpoS by reducing those of SprE, owing to polarity. (A and B) Western blot analysis was used to monitor the levels of RpoS in total cell lysates recovered during the logarithmic (A) and stationary (B) phases of growth in LB medium. (C) The same stationary-phase samples were used to determine the levels of SprE. The bands corresponding to RpoS and SprE are marked with labeled arrows. Lanes in each panel: wt, strain AF633; *rpoS*::*kan*, strain NR247; *sprE19*::*cam*, strain NR252; *sprE*::*tet*, strain NR253; *rssA1*::*kan*, strain NR260; *rssA1*::*kan sprE19*::*cam*, strain NR270; *rssA1*::*kan sprE*::*tet*, strain NR286. The sample orders are the same in all panels.

In wild-type cells, the levels of RpoS increased as cells entered stationary phase (Fig. 1A and B, compare wt lanes). This increase in RpoS levels is largely dependent on SprE-mediated regulation of its proteolysis by ClpXP. Therefore, altering SprE levels affects the content of RpoS in the cell. As shown previously (25), cells carrying the *sprE19*::*cam* allele contain higher levels of SprE than do wild-type cells (Fig. 1C, compare wt and *sprE19*::*cam* lanes), and this results in lower levels of RpoS (Fig. 1B, compare wt and *sprE19*::*cam* lanes) and RpoS activity (a ca. threefold decrease in *uspB'-lacZ*$^+$ activity). Accordingly, depleting cells of SprE (Fig. 1C, *sprE*::*tet* lane) increased RpoS levels throughout the entire life cycle (Fig. 1A and B, *sprE*::*tet* lanes).

As shown in Fig. 1A and B, both logarithmic- and stationary-phase cells carrying the *rssA1*::*kan* allele contained increased levels of RpoS above that of the wild-type parent strain. This increase in RpoS levels correlated with the increase in RpoS-dependent transcription discussed above. Also in line with the results found for RpoS-mediated transcription, the levels of RpoS in the *rssA1*::*kan* strain were not as high as those found in the *sprE*::*tet* strain (Fig. 1A and B). In addition, RpoS levels did not further increase in a strain carrying both *rssA1*::*kan* and *sprE*::*tet* alleles with respect to those found in one carrying only *sprE*::*tet* (Fig. 1A and B, compare *rssA1*::*kan sprE*::*tet* and *sprE*::*tet* lanes). More importantly, the increased levels of RpoS detected in the *rssA* null strain correlated with reduced levels of SprE (Fig. 1C, compare wt and *rssA1*::*kan* lanes). In sum-

mary, the results presented here show that disruption of *rssA* decreases, but does not eliminate, SprE levels in the cell. This most likely accounts for the increase in the cellular RpoS content seen in the *rssA1*::*kan* strain.

The decreased levels of SprE in the *rssA1*::*kan* null strain are the result of polarity. The decreased levels of SprE reported above that occurred upon disruption of *rssA* can be explained as follows. If *rssA* and *sprE* are cotranscribed, disruption of *rssA* would result in lowered *sprE* expression. Alternatively, RssA could be a positive regulator of SprE levels. In addition, both of these scenarios could be true.

In order to address these issues, we first tested whether the effects of *rssA1*::*kan* on *sprE* expression are the result of polarity. To accomplish this, we uncoupled *sprE* transcription from that of *rssA* by using the *sprE19*::*cam* allele, which carries a mini-Tn*cam* cassette 27 bp upstream from the adenine of the ATG start codon of *sprE* (25). As stated in the previous section, strains carrying the *sprE19*::*cam* allele contain high levels of SprE and, therefore, low levels of RpoS (Fig. 1). Interestingly, when the *sprE19*::*cam* allele was present in *cis* with the *rssA1*::*kan* allele, the levels of SprE, RpoS, and RpoS-dependent transcription (as assessed by measuring the β-galactosidase activity of *uspB'-lacZ*$^+$) did not change from those found in the strain carrying the *sprE19*::*cam* allele alone (Fig. 1A to C, compare *sprE19*::*cam* and *rssA1*::*kan sprE19*::*cam* lanes). Thus, when the transcription of *sprE* is uncoupled from that of *rssA*, disruption of *rssA* has no effect on either SprE or RpoS, suggesting that RssA has no role in the posttranscriptional regulation of these proteins under the conditions tested.

The notion that RssA does not regulate either SprE or RpoS was further supported by the following complementation studies. We introduced into a low-copy-number plasmid (pBBR1MCS) either the entire region encompassing *ychJ-rssA-sprE* (pRSSA6) or just *ychJ-rssA* (pRSSA9). The presence of the plasmid carrying the entire *ychJ-rssA-sprE* region (Fig. 2A, *ychJ rssA sprE* lanes) caused a significant decrease of RpoS levels in both the wild-type and *rssA1*::*kan* strains from those found in the same strains carrying the control pBBR1MCS vector (Fig. 2A, vector lanes). On the contrary, the presence of the plasmid-encoded *ychJ-rssA* region (Fig. 2A, *ychJ rssA* lanes) did not alter RpoS levels in either the wild-type or *rssA1*::*kan* strain with respect to the pBBR1MCS vector (Fig. 2A, vector lanes). Therefore, the presence of *rssA* in multicopy does not affect RpoS levels.

We also examined the effects on SprE levels caused by the presence of these plasmids and found that they are changed only in cells carrying *sprE* in multicopy. Specifically, the presence of the plasmid carrying the entire *ychJ-rssA-sprE* region caused a significant increase in the levels of SprE in both wild-type and *rssA1*::*kan* backgrounds (Fig. 2B, *ychJ rssA sprE* lanes) with respect to the vector control (Fig. 2B, vector lanes), while no changes were detected in those strains carrying the plasmid containing only the *ychJ-rssA* region (Fig. 2B, *ychJ rssA* lanes). Furthermore, overexpression of an MBP-SprE hybrid protein (composed of the maltose binding protein lacking the signal sequence and SprE from a high-copy-number plasmid (pMBPSprE) alone is sufficient to increase RpoS degradation to the same extent in both the wild-type and *rssA1*::*kan* strains compared to that in the respective strains carrying the control vector pMALc-2 (Fig. 2C, compare *sprE* and vector lanes).

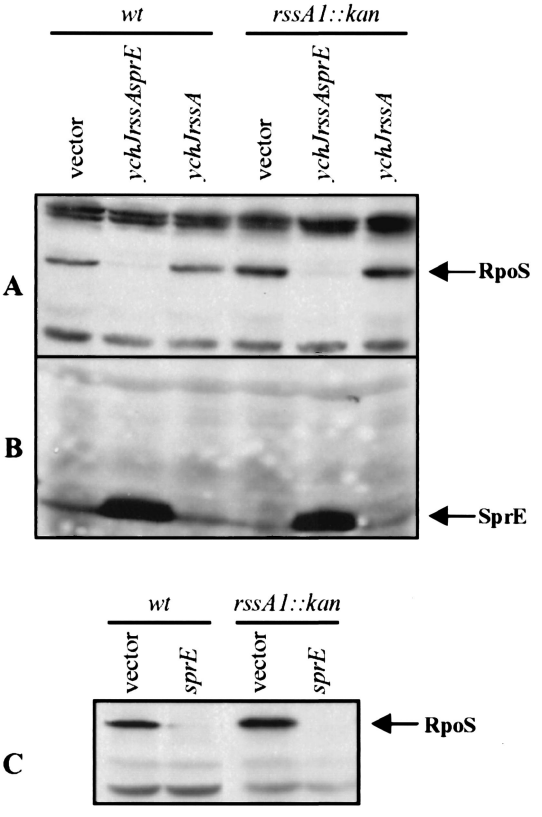

FIG. 2. RssA does not regulate SprE or RpoS. Complementation studies were done in stationary-phase cells grown in LB medium. RpoS and SprE levels (corresponding bands marked with labeled arrows) were monitored by Western blot analysis. (A) RpoS levels present in wild-type (wt) AF633 and *rssA1*::*kan* NR260 strains carrying the vector plasmid (pBBR1MCS), a plasmid encoding the *ychJ-rssA-sprE* region (pRSSA6), or one carrying the *ychJ-rssA* region (pRSSA9) (as marked above the lanes). (B) SprE levels present in wild-type AF633 and *rssA1*::*kan* NR260 strains carrying either pBBR1MCS, pRSSA6, or pRSSA9. The sample order is the same as for panel A. (C) RpoS levels in wild-type (wt) AF633 and *rssA1*::*kan* NR260 strains carrying either the vector plasmid pMAL-c2 (vector) or a plasmid encoding the MBP-SprE hybrid protein pMBPSprE (SprE), respectively.

Thus, under the conditions tested, RssA does not appear to participate in the regulation of either RpoS or SprE.

***rssA* and *sprE* constitute an operon.** To further support that polarity alone is responsible for the decreased levels of SprE in the *rssA1*::*kan* strain, we examined the levels of SprE in a cell depleted of RssA by an in-frame deletion in *rssA*. We introduced an allele (*rssAΔ1*) that carries an internal in-frame deletion (encompassing amino acids 30 to 194) in *rssA* into a low-copy-number plasmid (pRSSA6Δ) and determined its effects on SprE levels by Western blot analysis.

The presence of the plasmid carrying the wild-type *ychJ-rssA-sprE* region (Fig. 3, wt lanes) increased the levels of SprE in an *rssA1*::*kan* null strain in both the logarithmic and stationary phases of growth compared to cells containing the vector control (Fig. 3, vector lanes). In addition, depleting cells of RssA by an in-frame deletion did not have an effect on SprE levels (Fig. 3, compare wt and *rssAΔ1* lanes). The same was observed when these plasmids were introduced into our wild-type strain, AF633: there was no detectable difference in the

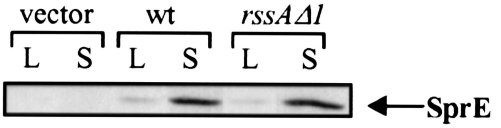

FIG. 3. In-frame deletion in *rssA* has no effect on SprE levels. A plasmid carrying the in-frame deletion allele *rssAΔ1* was introduced into the *rssA1*::*kan* strain NR260 and the levels of SprE in logarithmic (L) and stationary (S) phase cells were monitored by Western blot analysis. The relevant genotype from the plasmid is shown above the lanes. Vector, samples from strain NR260(pBBR1MCS); wt, NR260 (pRSSA6); *rssAΔ1*, samples from NR260(pRSSA6Δ).

levels of SprE, RpoS, or RpoS activity between strains carrying the wild-type gene versus those carrying the in-frame-deletion *rssAΔ1* allele (data not shown). Together with the results presented above, these data further demonstrate that *rssA* and *sprE* constitute an operon and that, under the conditions tested, RssA does not function in the regulation of either SprE or RpoS.

Interestingly, *rssA1*::*kan* does not result in total depletion of SprE (Fig. 1C), suggesting that under certain conditions *sprE* can be transcribed independently of *rssA*. The fact that there is still some SprE made in the strain carrying the *rssA1*::*kan* allele indicates that there is an additional promoter in the region between the *kan* cassette insertion and the translational start of *sprE*, as has been previously suggested (11). Furthermore, the residual levels of SprE cannot be attributed to an artifact created by the insertion of the *kan* cassette at the *Pst*I site of *rssA*, since another independently isolated insertion (*rssA3*::*kan*) located upstream of *rssA1*::*kan* behaves similarly (C. W. Bowers, unpublished results).

The levels of SprE are growth phase regulated at the level of transcription. Previous reports have shown that in both *E. coli* and *S. enterica* serovar Typhimurium, SprE (MviA) is growth phase regulated (11, 21). Consistent with this, we find that there is a significant increase in SprE levels between samples prepared from logarithmic- versus stationary-phase cells (Fig. 3). Paradoxically, this increase in SprE is RpoS dependent (11). This explains why an *rpoS*::*kan* null strain contains less SprE than its parent wild-type strain (Fig. 1C, compare wt and *rpoS*::*kan* lanes).

To better understand the growth phase regulation of *sprE*, we constructed *lacZ* fusions to both *rssA* and *sprE*. First, we constructed a *rssA'-'lacZ* fusion that included 929 bp of sequence upstream of the translational start of *rssA* to ensure that all regulatory sites were present (see below). Interestingly, when this *rssA'-'lacZ* fusion is recombined onto a λ phage and integrated at the λ attachment site (*att* site), it produces such low levels of β-galactosidase activity that this lysogen is unable to grow in lactose minimal medium. Possibly, the low activity of this fusion is the result of *rssA* having a GTG start codon instead of the more efficiently translated ATG codon. However, the activity of the fusion is still much higher than that derived from a promoterless fusion located at the λ *att* site (which yields values comparable to background levels [data not shown]). As shown in Fig. 4, the activity of the *rssA'-'lacZ* fusion increases about twofold when cells enter the stationary phase. Furthermore, the levels of β-galactosidase in samples obtained from stationary-phase cells decreased about fivefold

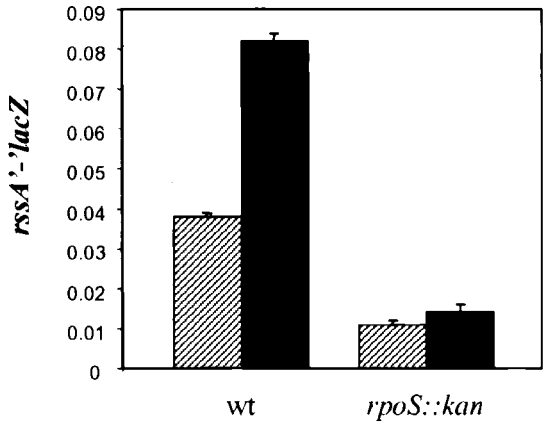

FIG. 4. RssA levels are growth phase regulated by RpoS. The β-galactosidase levels of an *rssA'-'lacZ* fusion were measured in a wild-type (NR501) and an *rpoS*::*kan* (NR502) strain during logarithmic (hatched bars) and stationary (black bars) phases of growth in LB medium. The relative levels of β-galactosidase activity are shown as the average and SD of three measurements per sample and they are representative of at least three independent experiments.

in an *rpoS*::*kan* null strain (Fig. 4), indicating that *rssA* is growth phase regulated in an RpoS-dependent fashion.

We also constructed a *lacZ* transcriptional fusion to *sprE* that included all of *rssA* (and 929 bp upstream) fused to the first 112 bp of *sprE*. This fusion was either recombined into the native *rssA-sprE* chromosomal locus or integrated at the λ *att* site, and the β-galactosidase levels from both strains were compared. Both strains contained equivalent levels of β-galactosidase under all conditions tested (Fig. 5), suggesting that all

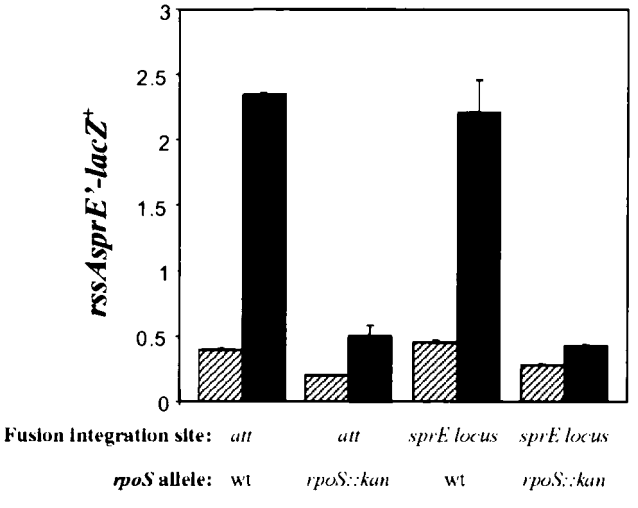

FIG. 5. RpoS regulates SprE at the transcriptional level. The β-galactosidase levels of an *rssA-sprE'-lacZ*$^+$ fusion were measured in wild-type strains (NR507 carries the fusion at the *att* site and NR509 carries the fusion at the *sprE* chromosomal locus) and *rpoS*::*kan* strains (NR508 carries the fusion at the *att* site and NR510 carries the fusion at the *sprE* chromosomal locus) during logarithmic (hatched bars) and stationary (black bars) phases of growth in LB medium. The location where the fusion was integrated (*att* site or *sprE* locus) and the *rpoS* genotype are indicated. The relative levels of β-galactosidase activity are shown as the average and SD of three measurements per sample, and they are representative of at least three independent experiments.

of the regulatory sites necessary for the transcriptional regulation of *sprE* are present in the region used to construct the fusion. In addition, a promoterless fusion did not generate any significant amount of β-galactosidase activity (data not shown). As also shown in Fig. 5, transcription of *sprE* (when the fusion was either at the *sprE* locus or at the *att* site) increased about sixfold in stationary-phase cells compared to that in logarithmically growing cells in an RpoS-dependent fashion (Fig. 5, compare LacZ activity in wild-type and *rpoS*::*kan* strains). Thus, RpoS regulates *sprE* transcription in a positive manner.

As predicted, recombination of the *sprE'-lacZ*$^+$ fusion into the *rssA-sprE* locus in *cis* with the *rssA1*::*kan* allele yielded two LacZ phenotypes (as determined using lactose MacConkey agar) (data not shown), since the fusion could have recombined before or after the *kan* cassette (confirmed by PCR analysis). β-Galactosidase assays showed that the presence of the *kan* cassette upstream of *lacZ* did not abolish activity of the fusion but significantly reduced it (ca. 50%) with respect to those strains in which the *kan* cassette was either absent or located downstream of *lacZ*. This correlates with the results reported above showing that disruption of *rssA* with the *kan* cassette decreased but did not eliminate SprE levels.

***sprE* is transcribed from an RpoS-dependent promoter located in the *rssA-sprE* intergenic region.** The last result described above confirms that although *rssA* and *sprE* constitute an operon, there is an additional promoter(s) from which *sprE* can be transcribed. This promoter must lie between the location of the *kan* cassette insertion and the translational start of *sprE*, since SprE is still made in the presence of the *rssA1*::*kan* allele. We have also observed that the already-decreased levels of SprE present in an *rssA1*::*kan* strain are growth phase regulated, since they are undetectable in the logarithmic growth phase by Western blot analysis (data not shown), suggesting that this second *sprE* promoter is also growth phase regulated. To better understand the nature of this promoter, we constructed an additional *sprE* transcriptional fusion and conducted primer extension analysis.

The new *sprE'-lacZ*$^+$ fusion differs from the one described in the previous section in two ways. First, the fusion junction (i.e., the 3′ end of the cloned fragment) is 62 bp downstream from that of the previously described fusion. Second, the region before the translational start of *sprE* that is contained in this new fusion is only 893 bp long (i.e., it contains the last 798 bp of *rssA*). After analyzing the levels of β-galactosidase produced by this fusion throughout the growth curve, we conclude that it is growth phase regulated, because stationary-phase cells carrying this fusion contained about eightfold more β-galactosidase than their logarithmic counterparts (Fig. 6). Furthermore, transcription from this fusion is considered RpoS dependent, because introducing the *rpoS*::*kan* allele reduced the levels of β-galactosidase activity present in stationary-phase cells to that found in wild-type logarithmic-phase cells (Fig. 6). In addition, introduction of other mutations known to alter the levels of RpoS (either increasing or decreasing the levels) caused directly proportional changes in expression from this *sprE-lacZ*$^+$ fusion.

To determine the location of this additional RpoS-dependent *sprE* promoter, primer extension analysis was performed using RNA isolated from stationary-phase wild-type cells. As shown in Fig. 7A, we determined that there is a transcriptional

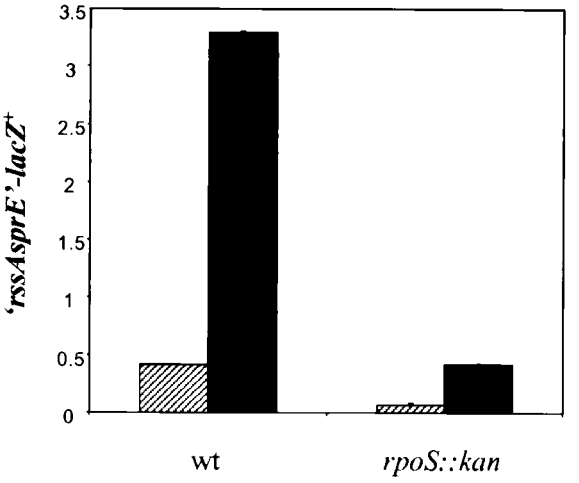

FIG. 6. The intergenic *rssA-sprE* region contains an RpoS-dependent promoter for *sprE*. The β-galactosidase levels of an '*rssA-sprE'-lacZ*$^+$ fusion were measured in a wild-type strain (NR511) and an *rpoS*::*kan* strain (NR512) during logarithmic (hatched bars) and stationary (black bars) phases of growth in LB medium. The relative levels of β-galactosidase activity are shown as the average and SD of three measurements per sample, and they are representative of at least three independent experiments.

start site for *sprE* expression 21 bp upstream of the adenine of its ATG start codon. Sequence analysis revealed that the region immediately upstream of this transcriptional start site has features found in the consensus for promoters recognized by RpoS recently proposed by Becker and Hengge-Aronis (3). Therefore, we propose that this additional promoter, located as shown in Fig. 7B, be named P_2.

DISCUSSION

The developmental commitment that *E. coli* makes when RpoS levels increase is immense; therefore, the cellular content of RpoS is tightly regulated. RpoS is controlled at multiple levels and regulation of its degradation by the ClpXP protease is considered a major level of control (13, 32). Although it has been known for several years that the response regulator SprE orchestrates this degradation of RpoS (22, 25), a critical question remains to be answered: how is SprE's activity regulated? We believe that the knowledge gained from understanding how SprE expression is regulated will help us to answer this question.

The studies presented here were aimed at examining *sprE* expression. By using *rssA* null alleles as well as reporter fusions and primer extension analysis, we have demonstrated that *rssA* and *sprE* are cotranscribed from a promoter, P_1, that is regulated by RpoS. We have also identified an additional promoter, P_2, located in the *rssA-sprE* intergenic region from which *sprE* is transcribed in an RpoS-dependent fashion. Furthermore, we have shown that under the conditions tested in this report, RssA is not involved in the regulation of either SprE or RpoS.

Interestingly, the P_2 promoter has features found in the consensus for promoters recognized by RpoS recently proposed by Becker and Hengge-Aronis (3). Specifically, it lacks a recognizable −35 region but it contains a run of A/T between the −30 and −14 positions. It also contains the GC motif at the

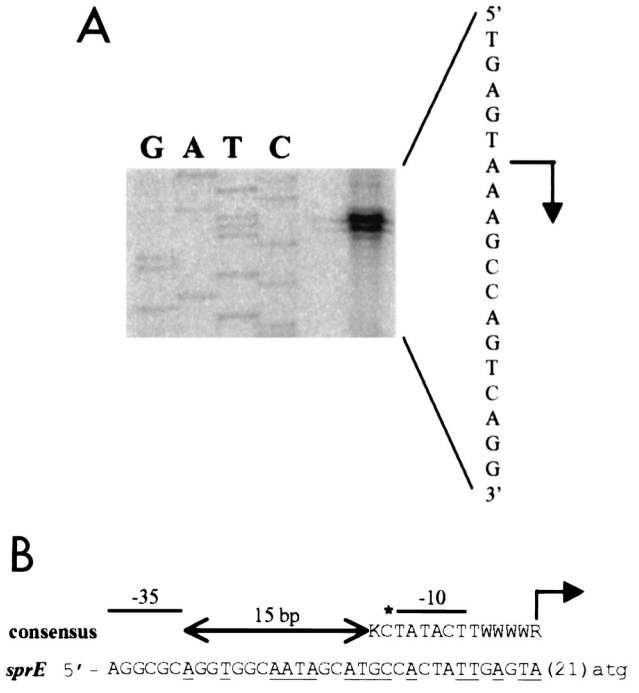

FIG. 7. Location of the *sprE* promoter in the *rssA-sprE* intergenic region. (A) Primer extension analysis on RNA prepared from stationary-phase wild-type cells (AF633) showed that there is a transcriptional start site located 21 bp upstream of the adenine of the ATG start codon of *sprE*. The primer extension reaction (shown on the right) was performed using the RTSPRE1 primer as described in Materials and Methods. A sequencing reaction was also performed using pRSSA5 as the template and RTSPRE1 as the primer. The section of the gel corresponding to the sequence of the noncoding strand for the relevant region is shown. The order of loading in the sequencing gel is marked above the lanes and the corresponding sequence of the coding strand appears on the right, with the transcriptional start site marked with an arrow pointing to the direction of transcription. (B) Sequence of the putative promoter (coding strand alone) and comparison with the consensus sequence (similarities appear underlined) of RpoS-dependent promoters (top line) as described by Becker and Hengge-Aronis (3). The location of the transcriptional start is marked with an arrow and it corresponds to +1. The *sprE* start codon is shown in lower case. The locations of the putative −10 and −35 regions are marked, but because RpoS-dependent promoters do not have a recognizable −35 consensus region, none is shown. The conserved −13 position is also marked with an asterisk. In RpoS-dependent promoters, the 15-bp-long sequence between the −10 and −35 regions is AT rich. K, G or T; W, A or T; R, A or G.

−14 and −13 positions and the highly conserved T at position −6, besides a −10 region partially homologous to the proposed TATACT consensus. Although the existence of an RpoS-specific consensus is somewhat controversial (see below), it is important to emphasize that Becker and Hengge-Aronis have shown both allelic suppression between a C at position −13 and residue 173 of RpoS (i.e., proving direct interaction) and the necessity of having either a G or a T at position −14 for maximal expression of RpoS-dependent promoters (3). The *sprE* P_2 promoter fulfills both criteria, having the highly conserved C at the −13 position and a G at the −14 position. More experiments are required, though, to demonstrate the specific role of these positions in *sprE* expression.

Although many RpoS-dependent promoters have been identified through the years, deriving a consensus from them has not been an easy task. This difficulty arises because RpoS and σ^{70} are so similar (19). In a recent report, in vitro selection studies searching for the promoter sequences best recognized by RpoS showed that both RpoS and σ^{70} prefer the same consensus sequences. These studies propose that the specificity of these sigma factors is dictated by how they differ in tolerating binding to nonpreferred sequences (i.e., tolerance to binding to sites with deviations from the well-characterized σ^{70} consensus sequences) (T. Gaal, W. Ross, S. T. Estrem, L. H. Nguyen, R. R. Burgess, and R. L. Gourse, submitted for publication). Regardless of whether there is a clear RpoS promoter consensus sequence or not, depletion of RpoS significantly decreases transcription of *sprE* from both P_1 and P_2 promoters, proving RpoS-dependent transcription of *sprE*.

Previously, it was reported that although transcription from a fusion containing only the P_2 promoter (i.e., it did not include all of *rssA*) was upregulated in stationary phase, this growth phase regulation was RpoS independent (11). Furthermore, an analogous translational fusion was shown to be RpoS regulated, suggesting translational but not transcriptional control of *sprE* by RpoS. Additional evidence supporting this RpoS-dependent translational regulation of *sprE* showed that the levels of SprE present in a strain carrying the *sprE19*::*cam* allele were also growth phase regulated (i.e., they increased in stationary phase) (11). Note that the *sprE19*::*cam* allele carries a mini-Tn*cam* cassette inserted 27 bp upstream of the translational start of *sprE* and it is believed to be constitutively transcribed (25).

In contrast, we found that the *sprE* P_2 promoter is RpoS regulated. We have recently isolated a strain that contains a mini-Tn*cam* cassette inserted 95 bp from the translational start of *sprE* and, in this strain, *sprE* is constitutively expressed regardless of the growth phase or the presence or absence of RpoS (data not shown), which argues against an RpoS-dependent translational control. To resolve this controversy, we sequenced the region upstream of *sprE* and found that several strains previously used (11) contain an IS*1E* element in the *rssA-sprE* intergenic region which interferes with native *sprE* expression and regulation.

The paradox of RpoS being necessary for the expression of its negative regulator SprE remains (11). We speculate that by having this feedback loop, the cell ensures a proper amount of RpoS at all times. It is known that under certain conditions, too much RpoS is not beneficial to the cell and can even be fatal. For example, Zambrano et al. showed that during prolonged starvation, cells with an altered form of RpoS, which is less active as sigma factor, results in growth advantage (31). In addition, cells that have exceptionally high levels of RpoS (i.e., *sprE* null strains) cannot grow in media containing either succinate or acetate as the sole carbon source (24). Thus, coupling the levels of SprE to those of RpoS might serve as a safety mechanism to ensure that the levels of RpoS are appropriate in the cell at all times. Interestingly, it has been reported that in addition to its role in orchestrating RpoS degradation, SprE has anti-sigma factor activity (4, 33). Thus, it is possible that in stationary-phase cells, when SprE-mediated degradation of RpoS does not occur, SprE itself might be acting as an anti-RpoS factor. This could explain, at least in part, why SprE levels need to increase when ClpXP is not degrading RpoS.

In addition, as previously proposed, having SprE already present in stationary phase might be beneficial to cells once they encounter more favorable conditions, thus providing a growth advantage (11). High levels of SprE could ensure rapid degradation of RpoS when nutrients become available. RpoS-dependent transcription would cease, and the cell would then focus its transcriptional and translational machinery on producing proteins that are necessary for rapid growth (σ^{70}-dependent promoters). Reports showing that cells deficient in ClpP suffer a growth disadvantage during competition experiments (i.e., repeated rounds of glucose starvation and recovery) support this idea (8). Alternatively, growth phase regulation of SprE might be necessary if SprE plays a role, as yet to be identified, during stationary phase.

ACKNOWLEDGMENTS

We are grateful to the members of the Silhavy lab for their critical reading of the manuscript. Special thanks to Susan DiRenzo for her assistance in the preparation of this paper. We also thank Weihong Hsing for her gift of pMBPSprE and for her work, in addition to that of Katherine Gibson, in generating the RpoS and SprE antisera. We thank T. Nystrom for his gift of strain AF633. We are also grateful to R. L. Gourse and C. W. Bowers for sharing their unpublished results.

T.J.S. was supported by a grant from the National Institute of General Medical Sciences (GM35791).

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