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Central Regulatory Role for the RpoS Sigma Factor in Expression of *Salmonella dublin* Plasmid Virulence Genes

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The plasmid virulence genes *spvABCD* of *Salmonella* spp. are regulated by SpvR and the stationary-phase sigma factor RpoS. The transcription of *spv* genes is induced during the post-exponential phase of bacterial growth in vitro. We sought to investigate the relationship between growth phase and RpoS in *spv* regulation. *rpoS* insertion mutations were constructed in *S. dublin* Lane and plasmid-cured LD842 strains, and the mutants were found to be attenuated for virulence and deficient in *spv* gene expression. We utilized the plasmid pBAD::*rpoS* to express *rpoS* independent of the growth phase under the control of the arabinose-inducible *araBAD* promoter. *SpvA* expression was induced within 2 h after the addition of 0.1% arabinose, even when bacteria were actively growing. This suggested that the level of RpoS, instead of the growth phase itself, controls induction of the *spv* genes. However, RpoS did not activate transcription of *spvA* in the absence of SpvR protein. Using a constitutive *tet* promoter to express *spvR*, we found that the *spvA* gene can be partially expressed in the *rpoS* mutant, suggesting that RpoS is required for SpvR synthesis. We confirmed that *spvR* is poorly expressed in the absence of RpoS. With an intact *rpoS* gene, *spvR* expression is not dependent on an intact *spvR* gene but is enhanced by *spvR* supplied in *trans*. We propose a model for *Salmonella spv* gene regulation in which both RpoS and SpvR are required for maximal expression at the *spvR* and *spvA* promoters.

The virulence of the host-adapted nontyphoid *Salmonella* strains, including *S. dublin*, *S. choleraesuis*, *S. gallinarum-pullorum*, and *S. abortusovis*, is associated with the presence of large plasmids of approximately 50 to 100 kb (12, 15). Many isolates of the broad-host range serovars *S. typhimurium* and *S. enteritidis* also contain virulence plasmids. Elimination of these plasmids results in the loss of virulence in mouse models of systemic *Salmonella* infection. It has been shown that the *S. dublin* plasmid pSDL2 plays an important role in multiplication within the reticuloendothelial system but not in colonization and invasion of the intestine and Peyer's patches in mice (16).

While virulence plasmids of different serovars may vary considerably in size and in overall nucleotide sequences, the core virulence genes, designated *spv* (salmonella plasmid virulence), are highly conserved among all serovars (31). The *spv* coding region consisting of the regulatory gene *spvR* and the structural genes *spvABCD* spans approximately 6 kb (13, 23). It has been shown that *spvABCD* genes form a single operon and are transcribed from the same promoter located upstream of *spvA*. mRNA transcripts terminate at different sites, resulting in messages of various length containing *spvA*, *spvAB*, *spvABC*, and *spvABCD* (22). *spvR* is located directly upstream of the *spvABCD* genes and is transcribed as a distinct message in the same orientation. Mutational analysis of the *spv* region from *S. dublin* indicates that *spvR* and *spvB* are essential for mouse virulence (41). Studies of *S. typhimurium*, however, suggest that mutations in *spvC* and *spvD* have significant effects on virulence in this serovar (5, 13).

The SpvR protein is required for the transcription of *spv* *ABCD* (3, 7). Amino acid sequence analysis reveals that SpvR belongs to the MetR/LysR family of bacterial transcriptional regulators (39, 43). Members of the LysR family possess helix-turn-helix motifs in the N-terminal domain and bind to the promoter regions of the regulated genes. SpvR binds to the *spvA* promoter upstream from the transcriptional start sites (21, 27), and mutations in the helix-turn-helix of SpvR abolish both promoter binding in vitro and *spvA* expression in vivo (21).

The expression of *spv* genes is regulated by the growth phase of the bacterial culture in vitro with the genes being induced during post-exponential growth (5, 7, 22). Growth-phase regulation of *spvABCD* is mediated through transcription from the *spvA* promoter, with *spv* mRNA increasing dramatically in stationary phase (7, 22). It has also been shown that the *spv* genes are rapidly induced following entry of the bacterium into host cells (9, 40). These results suggest that nutrient deprivation associated with the intracellular environment may induce *spv* expression. Other stress conditions, such as iron limitation and low pH, have also been shown to augment *spv* gene expression (44, 46).

It was shown that *spvABCD* gene expression is dependent on the alternative sigma factor, RpoS (also known as KatF, sigma 38) (8, 17, 20, 35). RpoS has been shown to be involved in regulation of many stress- or starvation-inducible genes in *Escherichia coli* as well as in *S. typhimurium* (25, 26, 37). *spvB* expression is significantly reduced in an *S. typhimurium* mutant containing an insertion in the *rpoS* gene (8). The derived RpoS protein sequence from *S. typhimurium* is 99% identical to that from *E. coli* and thus is believed to function similarly in gene regulation (32, 38). *rpoS* itself was shown to be regulated by growth phase, ppGpp, cyclic AMP and starvation conditions (11, 24, 26, 28, 33, 45).

Although both RpoS and SpvR are required for expression of the *spvABCD* operon, the mechanism of regulation remains

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Reference or source
S. typhimurium		
14028s	Wild-type virulent strain	American Type Culture Collection
CC1000	14028s *rpoS*::*tet*, *rpoS* null mutant	This study
S. dublin		
Lane	Wild-type virulent strain containing pSDL2	4
LD842	Plasmid-cured Lane strain	4
Lane *rpoS*	Lane *rpoS*::*tet*, *rpoS* null mutant	This study
LD842 *rpoS*	LD842 *rpoS*::*tet*, *rpoS* null mutant	This study
E. coli		
DH5α	*endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*) *U169 deoR* ϕ80d*lac* Δ(*lacZ*)*M15*	Lab collection
SY327λ*pir*	Δ(*lac-pro*) *argE*(Am) *recA56 nalA* Rf^r(λ*pir*)	30
MV12	C600 Δ*trpE5 recA*	18
TE1335	*trp* Δ(*lac*)*X74* Str^r [F′ 128(P22 *HT105/1 int-201 sieA44*)]	6
Plasmids		
pBAD::*rpoS*	P*araBAD*::*rpoS*, *ori*pBR322, Pen^r	47
pBC-SK(+)	Cloning vector, Cm^r	Stratagene
pBluescript II-KS/SK(+)	Cloning vector, Pen^r	Stratagene
pBR322	Cloning vector, Pen^r Tc^r	New England Biolabs
pBR::*rpoS*	*S. typhimurium rpoS* cloned into *Eco*RI-*Sca*I-digested pBR322, Tc^r	This study
pCC507	*Eco*RV-*Hin*dIII fragment of pBR::*rpoS* cloned into *Eco*RV-*Hin*dIII digested pBC-SK(+), Cm^r	This study
pCC508	*rpoS*::*tet* on pBC-SK(+), Cm^r Tc^r	This study
pCC509	*rpoS*::*tet* on pKNG101, Sm^r Tc^r	This study
pFF14	*spvRAB′*::*lacZ* translational fusion on pACYC184, Cm^r	7
pKNG101	*oriR6K mob*$_{RK2}$ *sacRB* Sm^r	19
pRK2073	Derivative of pRK2013, *oriT* Tp^r Sm^r	48
pSK::*rpoS*	*S. typhimurium rpoS* cloned into *Eco*RV-digested pBluescript II-SK(+), Pen^r	This study
pSpvA-Z	*spvRA′*::*lacZ* translational fusion on pACYC184, Cm^r	22
pSpvA-Z$_{mutR}$	Similar to pSpvA-Z but with a linker insertion in *spvR*	22
pSpvR-Z	*spvR′*::*lacZ* translational fusion on pACYC184, Cm^r	This study
p*tet*::*spvR*	*Nsi*I-*Pst*I fragment of pFF14 cloned in *Eco*RV-digested pBR322, Pen^r	This study

unclear. *spvR* transcription increases in stationary phase and is at least partially dependent on RpoS. Although variable results have been reported, most studies have been done with *E. coli* rather than the native *Salmonella* host (1, 17). In *S. typhimurium*, transcription of *spvR* requires a functional SpvR protein, implicating a positive control mechanism (20, 36). Overproduction of SpvR restores expression of *spvB* in an *rpoS* mutant (20). However, these studies do not explain the finding that synthesis of SpvR from a constitutive promoter does not abolish the growth-phase regulation of *spvABCD* expression (7). In this study, we define the interaction between RpoS and SpvR in the control of *spv* expression and show that RpoS levels in the cell regulate transcription from both the *spvR* and the *spvA* promoters.

MATERIALS AND METHODS

Strains, plasmids, and media. The strains and plasmids used in this study are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) broth (42) with appropriate antibiotics. M9 medium without Casamino Acids was used when minimal medium was required (29). Bacto-agar (1.5%) was added for solid media. Penicillin (200 μg/ml), chloramphenicol (20 μg/ml), streptomycin (50 μg/ml), and tetracycline (10 μg/ml) were added to the media as indicated.

DNA manipulations. Recombinant DNA techniques were performed as described previously (42). Unless otherwise indicated, plasmids were maintained in *E. coli* DH5α (Table 1). Restriction endonucleases were purchased from New England Biolabs (Beverly, Mass.) and used according to the manufacturer's recommendations.

Construction of plasmid p*tet*::*spvR* and pSpvR-Z. Plasmid pFF14 (Table 1) was digested with *Nsi*I and *Pst*I, and the 3′-overhanging ends were removed with the DNA polymerase I Klenow fragment. The resulting 1.5-kb blunt-ended fragment containing the *spvR* gene was gel purified and then ligated to *Eco*RV-digested pBR322. A clone, designated p*tet*::*spvR*, in which *spvR* is inserted in the same orientation as the *tet* gene was selected. Plasmid pSpvR-Z was constructed by digesting pSpvA-Z with *Sca*I and *Bam*HI and replacing this fragment with the *Eco*RV-*Bam*HI adapter sequence from pBluescript II-KS(+) (Stratagene, La Jolla, Calif.), resulting in the first 80 bp of *spvR* fused in frame to *lacZ*.

Construction of *rpoS* mutations in *S. typhimurium* and *S. dublin*. An *Eco*RV-*Hin*dIII fragment from pBR::*rpoS*, which contains the *S. typhimurium rpoS* coding region and 360 bp of the upstream sequence, was subcloned into *Eco*RV and *Hin*dIII-digested pBC-SK(+) (Stratagene), resulting in pCC507. pBR::*rpoS* was constructed by digesting pSK::*rpoS* (Table 1) with *Hin*dIII and filling-in the 3′-recessed end with the Klenow fragment, followed by *Eco*RI digestion and cloning of the 1.5-kb fragment into *Eco*RI- and *Sca*I-digested pBR322.

The *tet* gene was removed from pBR322 as an *Aat*II-*Msc*I fragment and cloned into *Aat*II-*Hpa*I-digested pCC507 in an orientation opposite the direction of *rpoS* transcription. The resulting plasmid is pCC508. The *rpoS*::*tet*::*rpoS* cassette was excised as an *Xho*I-*Xba*I fragment and cloned into *Sal*I-*Xba*I-digested pKNG101 (Table 1). The resulting suicide vector, pCC509, was transformed into SY327λ*pir* in order to support plasmid replication. pCC509 was mobilized into *S. typhimurium* 14028s by triparental mating with MV12(pRK2073) and plated on minimal M9 agar containing tetracycline (10 μg/ml) to select for integration of the entire plasmid into the *Salmonella* chromosome.

The *sacB* gene of pCC509 encodes levansucrase from *Bacillus subtilis*. Levans, synthesized from sucrose by levansucrase, are lethal to gram-negative bacteria (10). Loss of the *sacB* gene and the vector through a second recombination event was detected by selecting sucrose-resistant colonies. Overnight cultures of clones containing the entire plasmid integrated into the chromosome were diluted, and 10^5 to 10^6 bacteria were plated on LB agar without NaCl, containing 6% sucrose and tetracycline (10 μg/ml), and incubated at 30°C (2). The number of clones that lost the *sacB* gene was approximately 10^{-4} to 10^{-5}, close to the number reported previously (2, 19). The resulting sucrose-resistant colonies were screened for sensitivity to streptomycin (50 μg/ml) and reduced catalase activity. The desired *rpoS* mutant had streptomycin sensitivity, tetracycline resistance,

and much weaker catalase activity than that of the wild type. The new 14028s *rpoS* mutant was named CC1000. To move the mutation into *S. dublin* strains, phage P22 was grown in CC1000 by zygotic induction with TH1335 (6), and the mutant *rpoS* allele was transduced into Lane and LD842, resulting in Lane *rpoS* and LD842 *rpoS*, respectively. Southern hybridization by using the Amersham (Arlington Heights, Ill.) ECL direct nucleic acid labeling and detection system was performed with *Eco*RI- or *Bam*HI-digested chromosomal DNA from the newly constructed *rpoS* mutants to confirm the correct gene structure of all clones (data not shown).

Virulence testing in mice. BALB/c mice (6 to 8 weeks old) in groups of four were injected intraperitoneally with approximately 10^3 bacteria in 0.2 ml of sterile phosphate-buffered saline (PBS) (4). Survivors were sacrificed on day 7, spleens were taken aseptically and homogenized in 2 ml of sterile PBS, and appropriate dilutions were plated on LB agar alone and LB agar containing 10 μg of tetracycline per ml. Colonies were counted the next day, and the number of viable bacteria per spleen was calculated. Representative colonies were assayed for catalase activity to eliminate the possibility of contamination.

Measurement of *spv* expression. Overnight cultures of strains containing the appropriate plasmid(s) were diluted 1:5,000 into fresh LB broth with the addition of antibiotics. Cultures were allowed to grow, and aliquots were taken at different time points and assayed for β-galactosidase activity (29). For arabinose induction of strains containing pBAD::*rpoS*, overnight cultures were diluted 1:5,000 into LB broth plus 0.2% glucose with appropriate antibiotics and allowed to grow to early log phase (an optical density at 600 nm [OD_{600}] of approximately 0.2). Bacteria were collected by centrifugation and diluted to an OD_{600} of approximately 0.03 to 0.05 in prewarmed LB broth plus 0.2% glucose (noninducing control) or LB broth plus 0.1% arabinose (inducing culture). Samples were taken every hour and assayed for β-galactosidase activity. All experiments were repeated at least three times, and the data from a representative experiment was plotted.

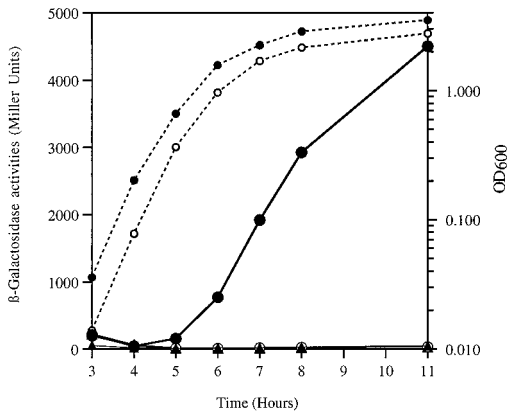

FIG. 1. Expression of *spvA*::*lacZ* fusion in the presence or absence of *spvR* (pSpvA-Z and pSpvA-Z_{mutR}, respectively) in *S. dublin* LD842 *rpoS* and wild-type strains. Symbols: filled circle, LD842(pSpvA-Z); open circle, LD842 *rpoS* (pSpvA-Z); filled triangle, LD842(pSpvA-Z_{mutR}); open triangle, LD842 *rpoS* (pSpvA-Z_{mutR}). Solid lines, β-galactosidase activities; dotted lines, OD_{600}. The growth curves of the four strains were similar; thus, the OD_{600} values for only LD842(pSpvA-Z) and LD842 *rpoS*(pSpvA-Z) were plotted. In cultures incubated overnight, expression of pSpvA-Z in LD842 *rpoS* strain eventually reached 5 to 10% of that of LD842, consistent with the results of earlier studies with *S. typhimurium* (8).

RESULTS

Mouse virulence of *S. dublin rpoS* mutants. We constructed the *rpoS* mutants in *S. dublin* Lane and LD842 by allelic exchange, using a suicide vector containing *sacB*. The *sacB* gene enabled us to positively select for the loss of the vector sequences, leaving a *tet* insertion in *rpoS* without other exogeneous DNA sequences introduced at the site of replacement (see Materials and Methods).

Mouse virulence testing was performed with wild-type *S. dublin* Lane (containing virulence plasmid pSDL2), LD842 (plasmid-cured strain), the newly constructed Lane *rpoS*, and LD842 *rpoS*. Approximately 10^3 bacteria per mouse were administered intraperitoneally (four mice in each group). All mice infected with Lane died by 6 days following infection, while the rest of the mice appeared to be healthy until they were sacrificed on the seventh day, and the numbers of viable bacteria in the spleens were determined.

Mice infected with the *rpoS* mutant of *S. dublin* Lane had (mean ± standard deviation) $1.47 \times 10^4 \pm 0.05 \times 10^4$ bacteria per spleen, indicating markedly attenuated virulence compared with that of the wild-type parent (16). In addition, the *rpoS* mutation further decreased the virulence of the plasmid-cured LD842 strain: the bacterial count for LD842 in spleens was $3.25 \times 10^4 \pm 0.04 \times 10^4$, while two mice infected with the LD842 *rpoS* mutant had sterile spleens and the other two had fewer than 6×10^3 bacteria. The effect of the *rpoS* mutation on mouse virulence in the absence of the virulence plasmid was confirmed in an independent experiment. These results suggest that the *rpoS* gene controls not only the essential *spv* operon on the virulence plasmid but also other unidentified chromosomal virulence genes that contribute to the ability of the organism to survive and grow in vivo.

Role of RpoS and SpvR in *spvABCD* regulation. The expression of the *spvABCD* genes has been shown to be regulated by RpoS in *S. typhimurium* (8, 20). Since the *spv*-mediated virulence phenotype is more pronounced in *S. dublin* than in *S. typhimurium* (4, 14), we investigated the role of RpoS in *spv* regulation using *S. dublin*. We examined *spv* expression using an *spvA*::*lacZ* translational fusion in the presence or absence of *spvR* (pSpvA-Z and pSpvA-Z_{mutR}, respectively) in LD842 and the newly constructed *rpoS* null mutant (Fig. 1). In the LD842 containing pSpvA-Z, *spvA* expression was induced during the transition from logarithmic to stationary phase. The stationary-phase induction was greatly diminished in strains lacking *rpoS*, *spvR*, or both (Fig. 1). This result confirms that both the RpoS and SpvR proteins are required for the expression of *spvABCD* in *S. dublin*. However, these data do not indicate whether RpoS functions through induction of *spvR* transcription or acts directly at the *spvABCD* promoter.

Constitutive expression of *spvR* partially bypasses the requirement for RpoS in *spvABCD* regulation. We sought to examine the relationship between RpoS and SpvR in the regulation of *spv* expression. We first determined whether constitutive expression of *spvR* can restore *spvA* expression in an *rpoS* mutant. We used the *tet* promoter of pBR322 to drive the expression of *spvR* on plasmid p*tet*::*spvR* (Table 1). This plasmid was able to fully complement *spvA* expression in LD842 containing pSpvA-Z_{mutR} (Fig. 2). The level of expression was higher than that of LD842(pSpvA-Z), consistent with overproduction of SpvR. When the same plasmids were tested in the *rpoS* mutant, strains containing p*tet*::*spvR* and one of the *spvA*::*lacZ* reporter plasmids expressed higher levels of β-galactosidase activity than those of LD842(pSpvA-Z_{mutR}), LD842 *rpoS*(pSpvA-Z), and LD842 *rpoS*(pSpvA-Z_{mutR}) (Fig. 1 and 2). The *spvA*::*lacZ* expression in *rpoS* mutants LD842 *rpoS*(pSpvA-Z)(p*tet*::*spvR*) and LD842 *rpoS*(pSpvA-Z_{mutR})(p*tet*::*spvR*) increased at a low linear rate throughout the growth curve and did not exhibit a marked growth-phase induction (Fig. 2). The maximal activities of the above strains were approximately 50% of that of LD842(pSpvA-Z) and less than 30% of that of LD842(pSpvA-Z)(p*tet*::*spvR*). These experiments demonstrate that overproduction of *spvR* from an RpoS-independent promoter results in significant but submaximal expression of *spvA* in the absence of RpoS. This finding suggests that a major role of RpoS in *spv* gene expression is to regulate the production of SpvR, since the defect in *spvA* expression in the *rpoS* mutant is partially due to the lack of sufficient SpvR.

***spvR* expression is controlled by RpoS.** Regulation of the *spvR* promoter was investigated by using the pSpvR-Z plasmid

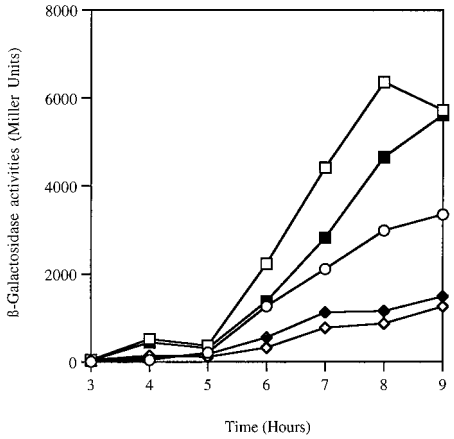

FIG. 2. Expression of *spvA*::*lacZ* fusion in *S. dublin rpoS* mutant and wild-type strains carrying plasmid p*tet*::*spvR*, which overexpresses *spvR* from a *tet* promoter. Symbols: open circle, LD842(pSpvA-Z); open square, LD842 (pSpvA-Z)(p*tet*::*spvR*); filled square, LD842(pSpvA-Z_{mutR})(p*tet*::*spvR*); open diamond, LD842 *rpoS*(pSpvA-Z)(p*tet*::*spvR*); filled diamond, LD842 *rpoS* (pSpvA-Z_{mutR})(p*tet*::*spvR*).

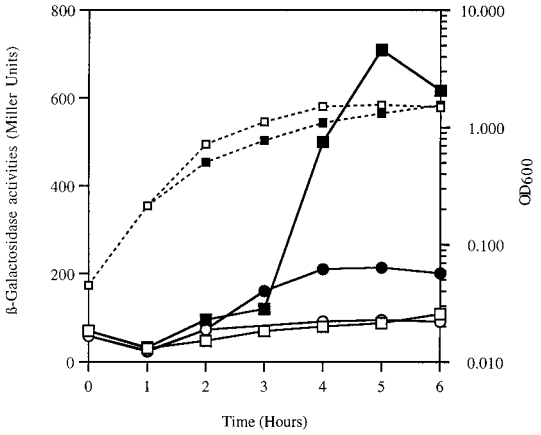

FIG. 4. Arabinose induction of LD842 *rpoS* strains containing pBAD::*rpoS* and pSpvR-Z. Log-phase cultures were induced at time zero in LB plus 0.1% arabinose or in LB plus 0.2% glucose as a control. Symbols: squares, Lane *rpoS* (pSpvR-Z)(pBAD::*rpoS*); circles, LD842 *rpoS*(pSpvR-Z)(pBAD::*rpoS*); open symbols, LB plus 0.2% glucose; filled symbols, LB plus 0.1% arabinose. Solid lines, β-galactosidase activities; dotted lines, OD_{600}. The growth curves of the four strains were similar; thus, the OD_{600} values for only Lane *rpoS*(pSpvR-Z) (pBAD::*rpoS*) in LB plus 0.2% glucose and in LB plus 0.1% arabinose were plotted.

containing an *spvR*::*lacZ* translational fusion. *spvR* expression increased in the post-exponential-growth phase with both wild-type *S. dublin* Lane and the plasmid-cured LD842 as the host strains, with a difference of less than 25% in the levels of β-galactosidase activity (Fig. 3). This result suggests that intact SpvR protein is not essential for its own expression. On the other hand, the expression of *spvR*::*lacZ* in the *rpoS* mutant was about fourfold lower than that of the wild type in the presence (in *S. dublin* Lane) or absence (in LD842) of a functional copy of *spvR* (Fig. 3). This suggests that *rpoS* plays a crucial role in *spvR* regulation. It is interesting to note that there is a significant level of basal expression of *spvR* in the absence of *rpoS* and *spvR* (Fig. 3).

To test whether RpoS activity can regulate *spvR* expression independent of the growth phase, the arabinose-inducible *araBAD* promoter was used to express *rpoS* on plasmid pBAD::*rpoS*. When induced with arabinose, the pBAD::*rpoS* plasmid was able to complement *spvR* expression in Lane *rpoS* and LD842 *rpoS* strains containing pSpvR-Z (Fig. 4). However, the induction of *spvR*::*lacZ* was much more rapid in the presence of a wild-type copy of *spvR* on the virulence plasmid (strain Lane *rpoS*). These results suggest that the rapid induction of *spvR* associated with rising levels of RpoS requires a functional SpvR protein in the cell.

We then determined the effect of overproducing *spvR* on *spvR*::*lacZ* expression (Fig. 5). The level of expression of *spvR*::*lacZ* in LD842(pSpvR-Z)(p*tet*::*spvR*) was approximately 15- to 20-fold higher than that in Lane(pSpvR-Z) or LD842 (pSpvR-Z), an effect much more prominent than that observed by using the pSpvA-Z reporter plasmid. When tested in the *rpoS* mutant LD842 *rpoS*(pSpvR-Z)(p*tet*::*spvR*), the level of *spvR* expression was about three- to fourfold higher than that of LD842 *rpoS*(pSpvR-Z) (Fig. 5) and was similar to that of Lane(pSpvR-Z) (data not shown). These findings show that

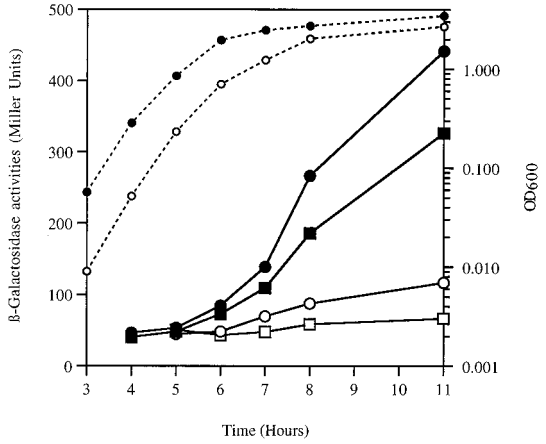

FIG. 3. Expression of *spvR*::*lacZ* in *rpoS* mutant and wild-type *S. dublin* Lane (containing virulence plasmid pSDL2) or LD842 (cured of virulence plasmid). Symbols: filled circle, Lane(pSpvR-Z); open circle, Lane *rpoS*(pSpvR-Z); filled square, LD842(pSpvR-Z); open square, LD842 *rpoS*(pSpvR-Z). Solid lines, β-galactosidase activities; dotted lines, OD_{600}. The growth curves of the four strains were similar; thus, the OD_{600} values for only Lane(pSpvR-Z) and Lane *rpoS*(pSpvR-Z) were plotted.

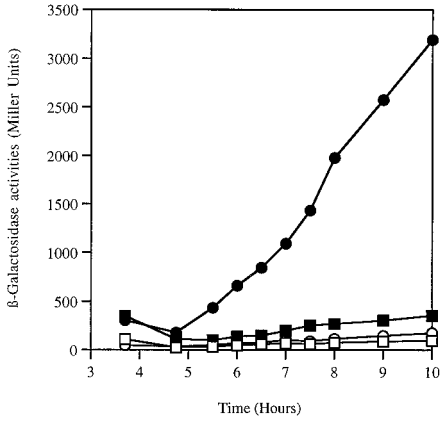

FIG. 5. Expression of *spvR*::*lacZ* fusion in *S. dublin* strains carrying p*tet*::*spvR*, which overexpresses *spvR* from a *tet* promoter. Symbols: open circle, LD842 (pSpvR-Z); filled circle, LD842(pSpvR-Z)(p*tet*::*spvR*); open square, LD842 *rpoS* (pSpvR-Z); filled square, LD842 *rpoS*(pSpvR-Z)(p*tet*::*spvR*).

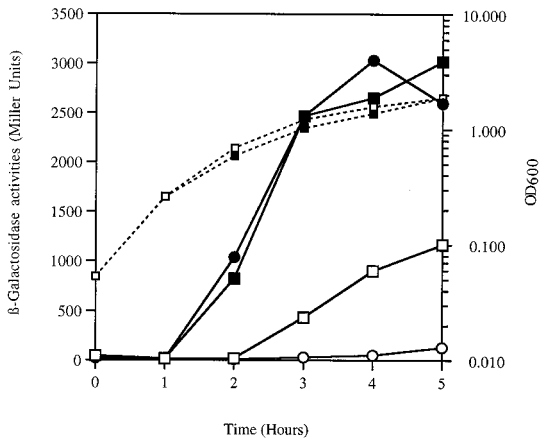

FIG. 6. Arabinose induction of *S. dublin* LD842 or LD842 *rpoS* strains containing pBAD::*rpoS* and pSpvA-Z. Log-phase cultures were induced at time zero in LB plus 0.1% arabinose or in LB plus 0.2% glucose as control. Symbols: squares, LD842(pSpvA-Z)(pBAD::*rpoS*); circles, LD842 *rpoS*(pSpvA-Z)(pBAD::*rpoS*); open symbols, LB plus 0.2% glucose; filled symbols, LB plus 0.1% arabinose; solid lines, β-galactosidase activities; dotted lines, OD_{600}. The growth curves of the four strains were similar; thus, the OD_{600} values for only LD842 (pSpvA-Z)(pBAD::*rpoS*) in LB plus 0.2% glucose and in LB plus 0.1% arabinose were plotted.

overproduction of SpvR leads to a large increase in *spvR* expression in the presence of RpoS. Without RpoS, SpvR can also increase its own expression but at much lower levels. This result combined with the earlier findings suggest that the presence of *rpoS* is important mainly for the initial induction of *spvR*. Once some SpvR protein is present, it can either efficiently interact with σ^{38} (RpoS)-RNA polymerase for higher levels of *spvR* expression or less efficiently with σ^{70}-RNA polymerase when RpoS is not available.

***rpoS* induced during growth can activate transcription of *spvA*.** In order to determine whether RpoS levels in the cell have a direct regulatory role in *spvA* expression, we used the *rpoS* gene under the control of the arabinose-inducible *araBAD* promoter on pBAD::*rpoS*. When actively growing cells were induced with 0.1% arabinose, both wild-type and *rpoS* strains containing pSpvA-Z and pBAD::*rpoS* plasmids expressed *spvA*::*lacZ* within 2 h after arabinose induction, while *spvA* expression in the control LD842(pSpvA-Z)(pBAD::*rpoS*) culture lagged behind (Fig. 6). This result suggests that stationary-phase or starvation conditions are not required for *spvABCD* expression and that the limiting factor in these actively growing cells is the low level of RpoS activity.

We also tested the effect of pBAD::*rpoS* in strain LD842 *rpoS*(pSpvA-Z_{mutR})(pBAD::*rpoS*), which lacks a functional copy of *spvR*. The expression of *spvA*::*lacZ* was essentially the same as that in LD842*rpoS*(pSpvA-Z_{mutR}), suggesting that RpoS alone was not able to activate the transcription of the *spvABCD* genes in the absence of SpvR (data not shown).

DISCUSSION

In this report, we elucidate the regulatory mechanism of the *Salmonella spv* genes, using constitutive and arabinose-inducible promoters to express *spvR* and *rpoS*, respectively. In this way we were able to examine the effects of SpvR and RpoS independent of the normal growth-phase regulation of *rpoS*. Our results indicate that RpoS is the central regulator of *spv* gene expression. When *rpoS* is expressed from an arabinose-inducible promoter (pBAD::*rpoS*), the *spvA* gene can be induced during active growth (Fig. 6), while *spvR* induction was observed earlier than that in strains containing the native copy of *rpoS* (induction was seen at OD_{600} of 0.8 to 1.0 instead of at the normally higher than 1.2 to 1.5) (Fig. 3 and 4). These findings, combined with the earlier observation that stationary-phase induction of *spv* genes is severely diminished in the *rpoS* mutant (Fig. 1), indicate that RpoS levels are an important determinant of the previously observed growth-phase regulation of *spv* expression. This agrees with the finding that *E. coli* and *S. typhimurium rpoS* genes are regulated by growth-phase and other starvation conditions at the transcriptional and post-transcriptional levels (24, 26, 28, 33). These posttranscriptional controls appear to limit the ability to express RpoS very early in exponential growth. Therefore, the results of arabinose induction of pBAD::*rpoS* clearly show that RpoS activity in the cell regulates *spvR* and *spvA*, but we cannot rule out the possibility of other putative stationary-phase inducers that might contribute to *spv* induction.

Role of RpoS in *spv* gene regulation. A primary site of action of RpoS is at the *spvR* promoter. In Fig. 3, we showed that RpoS is required for the stationary-phase induction of *spvR*, while the SpvR protein is helpful but not essential. The *rpoS* mutation affects the *spvR*::*lacZ* expression more severely than the absence of a functional copy of *spvR* [(Fig. 3, compare Lane(pSpvR-Z), Lane *rpoS*(pSpvR-Z), and LD842(pSpvR-Z)]. The extremely high levels of β-galactosidase activity in LD842 (pSpvR-Z)(p*tet*::*spvR*) compared with the level in LD842*rpoS* (pSpvR-Z)(p*tet*::*spvR*) again supports the finding that RpoS, in conjunction with SpvR, is more important in *spvR* expression than the SpvR protein by itself (Fig. 5).

A second RpoS site of action is at the *spvA* promoter. However, at this promoter, SpvR is essential and residual transcription can occur in the absence of RpoS. In Fig. 2, RpoS-independent *spvA* expression was detected when *spvR* was expressed from the constitutive *tet* promoter. Induction of RpoS from the arabinose promoter (pBAD::*rpoS*) does not lead to significant *spvA* expression in the absence of SpvR (data not shown). These observations suggest that RpoS alone is unable to induce the *spvA* promoter, in contrast to the *spvR* promoter. The RpoS-independent expression of *spvA* also suggests that SpvR, when overexpressed, is able to interact with the σ^{70} RNA polymerase. Kowarz and colleagues (20) showed that the *spv* transcripts can be detected in the *rpoS* strain containing overexpressed *spvR* but not in the *rpoS* mutant without the *spvR*-overproducing plasmid. The in vitro transcription experiments using purified *E. coli* RNA polymerase core enzyme and sigma factors (σ^{70} and σ^{38}) also showed a cross-reaction between σ^{70}- and σ^{38}-regulated promoters (34, 45).

These results demonstrating the dual action of *rpoS* clarify several issues raised by earlier studies on *spv* regulation. Previous results with *rpoS* mutants in *S. typhimurium* demonstrated significant residual *spv* expression in stationary phase (8, 20). From our data, we conclude that the residual expression of *spv*::*lacZ* fusions in *rpoS* mutants measured after prolonged incubation in stationary phase is due to accumulation of the fusion protein resulting from a low level of *rpoS*-independent transcription from the *spvA* promoter. Another related issue concerns the report that SpvR supplied in *trans* from a constitutive promoter can restore *spvA* expression in the *rpoS* mutant (20). Our data indicate that *spvR* expressed from a constitutive promoter can only partially suppress the *rpoS* mutation for *spvA* expression, since optimal induction of the *spvA* promoter requires RpoS (Fig. 2). A third issue concerns reports that SpvR is required for its own expression, raising the question of how *spvR* can be induced in cells containing very low levels of SpvR protein. Figures 3 and 4 show that *spvR* can

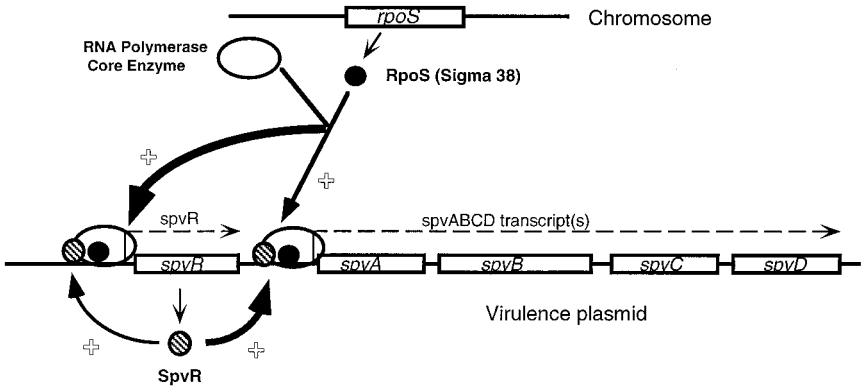

FIG. 7. Model of *Salmonella spv* gene regulation. The thickness of the arrows indicates the importance of each step. The diagram is not drawn to scale.

be induced by increasing levels of RpoS, even in the absence of functional SpvR proteins. Along with data from Fig. 5, we conclude that the stimulatory effect of the SpvR protein for *spvR* expression is RpoS dependent.

Model of *spv* gene regulation. We propose the model of *spv* gene regulation illustrated in Fig. 7. In early-log-phase cells, the level of RpoS activity is low, resulting in little *spvR* expression and essentially no synthesis of the *spvABCD* gene products. Increases in the RpoS concentration (controlled by growth-phase or other inducing conditions) lead to increased *spvR* expression. Positive autoregulation of *spvR* further augments the SpvR concentration. SpvR and RpoS act in concert at the *spvA* promoter to rapidly induce expression of the *spvABCD* genes. In this model, SpvR could act to amplify the RpoS regulation. The significant basal expression of *spvR* in the absence of RpoS and SpvR proteins may be a safety feature for bacteria. Expression of the *spv* gene is detected within 1 h of *Salmonella* entry into host cells (9). According to the present model, the initial induction of the *spv* structural genes can be explained by the interaction of RpoS with already-existing SpvR. Existing SpvR protein can also enhance SpvR synthesis in conjunction with RpoS, resulting in even stronger *spv* expression.

This model is supported by recent studies showing that SpvR binds to sites in both the *spvR* and *spvA* promoter regions (11a, 21, 27). Mutations that disrupt the SpvR helix-turn-helix motif abolish both DNA binding and *spvA* expression, indicating that the direct interaction of SpvR with the *spvA* promoter is required for transcription. On the basis of the genetic evidence presented here, we propose that RpoS is the preferred sigma factor for RNA polymerase interacting with both the *spvR* and *spvA* promoters together with the SpvR protein. However, we cannot rule out an indirect mechanism of RpoS action through expression of other genes. Since RpoS promoters are defined largely on the basis of genetic data, the biochemical approach by using purified RpoS in vitro has not been generally successful for identifying RpoS-specific transcription initiation sites.

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