Repressor activity of the RpoS/σS-dependent RNA polymerase requires DNA binding

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ABSTRACT

The RpoS/σS sigma subunit of RNA polymerase (RNAP) activates transcription of stationary phase genes in many Gram-negative bacteria and controls adaptive functions, including stress resistance, biofilm formation and virulence. In this study, we address an important but poorly understood aspect of σS-dependent control, that of a repressor. Negative regulation by σS has been proposed to result largely from competition between σS and other σ factors for binding to a limited amount of core RNAP (E). To assess whether σS binding to E alone results in significant downregulation of gene expression by other σ factors, we characterized an rpoS mutant of Salmonella enterica serovar Typhimurium producing a σS protein proficient for EσS complex formation but deficient in promoter DNA binding. Genome expression profiling and physiological assays revealed that this mutant was defective for negative regulation, indicating that gene repression by σS requires its binding to DNA. Although the mechanisms of repression by σS are likely specific to individual genes and environmental conditions, the study of transcription downregulation of the succinate dehydrogenase operon suggests that σ competition at the promoter DNA level plays an important role in gene repression by EσS.

INTRODUCTION

In eubacteria, the dissociable σ subunit of RNA polymerase (RNAP) enables specific binding of RNAP to gene promoters and is required for transcription initiation. Besides a primary housekeeping σ, which promotes transcription of genes required for essential functions, one or more alternative σ factors direct transcription of specific subsets of genes (1–3). The alternative sigma σS/38 (RpoS) is a central regulator allowing many Gram-negative bacteria to adapt to stress conditions and specialized environments (4–7). In the wide host-range pathogen Salmonella enterica serovar Typhimurium (S. Typhimurium), σS is not only required for general stress resistance but also for virulence, biofilm formation and development of the red dry and rough (rdar) morphotype, a colony morphology caused by production of amyloid fibers (curli) and cellulose (5,8,9). In contrast to the housekeeping sigma, σ70 (RpoD), σS is almost undetectable in early exponential phase and is induced in stationary phase or in response to various stresses (4–7). S. Typhimurium contains four other alternative σ, σE/24 (rpoE), σH/32 (rpoH), σN/54 (rpoN) and σ24 (fliA) which associate with the core RNAP (E) to form the holoenzyme Eσ (1–3). The cellular concentration of σ70 molecules exceeds that of E, suggesting that σ factors compete for binding to a limiting number of E (2–5). Levels and affinity for E are thus major determinants of σ competitiveness and its ability to form Eσ. However, in vivo, the efficiency of formation of the housekeeping and alternative Eσ is also modulated by regulatory factors that bind E and/or σ (2–5).

In this study, we have addressed an important aspect of σS-dependent control, that of a repressor. σS has a negative effect on the expression of a large number of σ-dependent genes, besides its binding to DNA.
Indeed, $\sigma^S$ controls the expression of sRNAs (12,22–24) and several regulatory proteins and metabolic/signaling enzymes (5–7,10,12,25,26) that might endow $\sigma^S$ with repressor function. RNA polymerase itself is also a DNA binding protein and $\sigma^S$ might theoretically function as a transcriptional repressor through promoter occlusion or transcriptional interference (Figure 1C) (27–35). Stable but transcriptionally inactive RNA polymerase bound to DNA might inhibit transcription by another holoenzyme (27,28,32). Tandem or overlapping promoters and pausing of RNA polymerase outside of the promoter context might also sterically hinder DNA binding by an alternative RNA polymerase and/or transcription factors, producing transcriptional interference and repression (30,31,33–35) (Figure 1C). Few examples of direct negative effect of $\sigma$ have been reported so far (27–29,35) but the extensive overlap between $\sigma$ factor binding sites recently reported in E. coli (36,37) is compatible with antagonisms between $\sigma$ factors at the promoter DNA level. In particular, during the completion of this study, Cho et al. (37) reported that $\sigma^S$ and $\sigma^{70}$ binding regions in the E. coli genome extensively overlap, and many $\sigma^S$ binding promoters showed increased transcription and increased $\sigma^{70}$ binding in a $\Delta rpoS$ strain. Interestingly, these data suggested that direct interference between $\sigma^S$ and $\sigma^{70}$ might occur at the DNA level. However, it was not shown whether the decreased $\sigma^{70}$ binding and transcription, observed in the wild-type strain compared to the $\Delta rpoS$ mutant, was a direct consequence of $\sigma^S$ binding in the same promoter region (according to model C; Figure 1) or an indirect effect, due to $\sigma$ competition for E (according to model A; Figure 1) and/or the synthesis of a negative effector under the control of $\sigma^S$ (according to model B; Figure 1).

To shed light on the molecular mechanisms of negative regulation by $\sigma^S$, we first asked whether $\sigma^S$ binding to E alone results in significant downregulation of gene expression by other $\sigma$. Since the competition model (Figure 1A) solely requires $\sigma^S$ binding to E whereas alternative mechanisms (Figure 1B and C) also require the ability of $\sigma^S$ to bind DNA, we characterized a $\sigma^S$ variant proficient for $\sigma^S$ formation but impaired in binding to promoter DNA. Our data suggest that, under physiological conditions in stationary phase, $\sigma$ competition for E binding alone does not result in detectable gene repression by $\sigma^S$, which instead relies on the ability of $\sigma^S$ to bind DNA. Based on these findings and further analysis of the downregulation by $\sigma^S$ of the $sdh$ operon encoding succinate dehydrogenase, we propose that gene repression by $\sigma^S$ relies on negative effects of $\sigma^S$ at the promoter DNA level and presumably on the action of $\sigma^{70}$-dependent negative effectors.

**MATERIALS AND METHODS**

**Bacterial strains, bacteriophage, plasmids and growth conditions**

Strains and plasmids are listed in Supplementary Table S1. Bacteriophage P22HT105/int was used to transfer mutations and $lacZ$ fusions between Salmonella strains by transduction (38). Green plates, for screening for P22-infected cells or lysogens, were prepared as described previously (39). Bacteria were routinely grown in Luria-Bertani medium...
was confirmed by DNA sequencing. 

**DNA manipulations, inactivation of chromosomal genes and construction of chromosomal lacZ fusions**

Standard molecular biology techniques were used (8,40). Oligonucleotides were obtained from Sigma-Aldrich and are listed in Supplementary Table S2. DNA sequencing was performed by Beckman Coulter Genomics. Plasmids pVF9551, pVF9647 were obtained by site-directed mutagenesis of plasmid pUC62-2922K using the QuickChange II Site-directed mutagenesis kit (Stratagene) as recommended by the manufacturer. pVF9793 was obtained by site-directed mutagenesis of pVF9551. Plasmids pQE30posS141S, pQE30posS157T and pQE30posSdb (Supplementary Table S1) were obtained by cloning polymerase chain reaction (PCR) amplified DNA fragments from strains VF9682 VF9676 and VF9849, respectively, between the BamHI and HindIII sites of pQE30 using primers HK1 and HK2 as described previously for the wild-type rpoS gene in pQE30posS (17). All plasmids were verified by DNA sequencing. Chromosomal deletions in Salmonella ATCC14028 were generated using PCR-generated linear DNA fragments (Supplementary Table S2) and the λ-Red recombination method as described by Datsenko and Wanner (41). Because the ΔrssB mutants were sick and might accumulate compensatory mutations, they were constructed freshly for each experiment by transduction using a P22 lysate prepared on strain VF8293. When required, the resistance cassette was eliminated using a temperature-sensitive helper plasmid pCP20, which encodes the FLP recombinase (41). Point mutations in the sdh promoter region were introduced on the chromosome by a two-step Red-recombinase-based recombineering procedure (42). The procedure involves (i) replacement of the wild-type sequence by a tetAR module produced by PCR (12) and (ii) replacement of the tetRA module by a DNA fragment obtained by PCR and carrying the desired mutations (Supplementary Table S2) through positive selection of tetracycline-resistant recombinants (43). The presence of desired mutations in all strains was confirmed by DNA sequencing. Tn5B21 insertions creating lacZ fusions in σ^7-dependent genes have been previously isolated (16,44). Single-copy transcriptional lacZ fusions were constructed in ompD and ynfM using conditional plasmids containing promoterless lacZ genes and the FLP recognition target site as described (45). PCR assays were used to ensure integration of the plasmids in the correct location and to exclude the presence of multiple plasmid-integrase (Tet) 20.

**Construction of isogenic *S. Typhimurium* strains carrying the ΔrpoS and rpoSdb alleles**

The rpoS allele in *S. Typhimurium* ATCC14028 was replaced by the rpoS_R141S, rpoS_A157T and rpoSdb alleles using the strategy previously described for rpoS allelic exchange (16,17). Briefly, pUCC52-2922K contains the rpoS and downstream sequences including a gene encoding a putative decarboxylase (named STM2922 in *S. Typhimurium* strain LT2 and STM14_3524 in ATCC14028) into which a Km cartridge has been inserted. When introduced into ATCC14028 by electroporation this plasmid was unstable. Recombination of the Km cartridge into the host genome with simultaneous loss of pUCC52-2922K resulted in the isolation of Km^R^ Cb^b^ recombinants containing the STM2922::Km mutation. This mutation was then transduced into ATCC14028 and its ΔrpoS::Cm derivative VF7928, and the resulting strains (VF7969 and VF7975, respectively) were checked by PCR and DNA sequencing. The same strategy was applied with the pUCC52-2922K derivatives containing the rpoS mutations C421A, G469A and C421A-G469A (yielding to σ^2^ substitutions R141S, A157T and R141A-A57T, respectively). These plasmids were electroporated in the ATCC14028 strain containing the rpoSdb::Cm mutation (VF9759) to facilitate screening of strains into which simultaneous recombination of the STM2922::Km and rpoS point mutations occurred. Cb^b^ recombinants that were Km^R^ but Cm^R^ were selected, and recombination of the STM2922::Km mutation and simultaneous replacement of the rpoSdb::Cm mutation by the mutated rpoS alleles were confirmed by PCR and DNA sequencing. The mutated rpoS alleles and STM2922::Km were then co-transduced into a fresh ΔrpoS::Cm background (VF9798) and the resulting Km^R^ Cm^S^ strains (VF9849, VF9676 and VF9862) were checked by DNA sequencing for the presence of the mutated rpoS alleles. The resistance cassette in VF7975 was eliminated to yield strain VF9356 using a temperature-sensitive helper plasmid pCP20, which encodes the FLP recombinase (41). The rpoSdb mutation was also introduced in ATCC14028 using the two-step Red-recombinase-based recombineering procedure (42) mentioned above (Supplementary Table S1).

**Isolation of total RNA from *S. Typhimurium*, cDNA library preparation, sequencing and analysis of sequences**

Total RNA from three biological replicates of strains VF7969, VF9356 and VF9849 was isolated from late stationary phase cultures (18 h growth in LB at 37°C) and the rRNA depleted fraction was used for construction of strand specific single end cDNA libraries as described recently (12). Libraries were sequenced using an Illumina HiSeq2000 sequencer (multiplexing 3 samples per lane). One replicate of each strain was sequenced in each lane. Analysis

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**Grants**

(data from https://academic.oup.com/nar/article-abstract/43/3/1456/2411267 by guest on 06 April 2019)
of sequences and statistical analyses were performed as described (12).

Quantitative real-time PCR
Total RNA was extracted from cells grown to stationary phase in LB and reverse-transcribed as described (12). Quantitative real-time PCR was performed using Applied Biosystems 7300 Real-Time PCR system and methods recently detailed (12). Three biological replicates were analyzed in duplicate each. The rpoZ gene was used as a reference (12). Gene expression levels were calculated using the comparative Ct method \(2^{-\Delta\Delta CT}\) as previously described (12,46).

Electrophoresis and immunoblot analysis of proteins
Whole-cell extracts were prepared and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described (16,17). For detection of \(\sigma^S\) proteins during growth, exponential-phase cultures of Salmonella in LB at 37°C were diluted into LB prewarmed at 37°C to prolong the exponential phase and aliquots were removed during the exponential phase and stationary phase. The amount of protein in whole-cell lysates was determined using the DC Protein Assay kit (Bio-Rad). Equal amounts of protein were loaded in each slot. The molecular sizes of the proteins were estimated using Precision Plus Protein Standard (Bio-Rad). Proteins were transferred to nitrocellulose blotting membranes (Hybond ECL membranes, GE Healthcare) and incubated with rabbit antibody against the \(\sigma^S\) protein of S. enterica serovar Typhimurium as previously described (16,17,47). Bound antibodies were detected using a secondary anti-rabbit antibody linked to peroxidase (A4416, Sigma-Aldrich).

Fractionation of free and RNAP-bound \(\sigma^S\) from cellular extracts and immunoblot analysis
Gel filtration of cellular extracts from stationary phase cultures of strains VF7969 and VF9849 was performed as previously described (20). Crude cell extracts were obtained using a Cell Disruptor (Constant Systems, Daventry, UK) in 10-mM Tris-HCl pH 7.9, 0.1-mM DTT, 0.1-mM ethylenediaminetetraacetic acid, glycerol 5%, 300-mM NaCl supplemented with antiprotease (Roche). A total of 20 \(\mu\)l of the supernatant was applied to a gel filtration column (Superdex 200 PC3.2/30, GE Healthcare) using the EtanLC System (GE Healthcare). Elution was performed at a flow rate of 0.04 ml/min at room temperature, gathering fractions of 50 \(\mu\)l. The proteins in the elution fractions were analyzed by SDS-PAGE and electrophlocted onto nitrocellulose membranes (Hybond ECL GE Healthcare). Immunoblot analysis of the eluted fractions was carried out using the \(\sigma^S\) rabbit antibody (47) as described above and monoclonal antibodies against the \(\beta^\prime\) and \(\alpha\) subunits of RNAP (WP001 and WP003, Neolclone) and a secondary anti-mouse antibody linked to peroxidase (A4416, Sigma-Aldrich).

Preparation of outer membrane proteins
Salmonella strains were grown for 18H in LB at 37°C. Cells were centrifuged and washed with 10 mM, Tris-HCl pH 8.0 and crude cell extracts were obtained using a Cell Disruptor (Constant System, Daventry, UK). Outer membrane protein preparations were obtained as described by Lobos and Mora (48).

Overproduction and purification of His6-\(\sigma^S\) variants
His6-\(\sigma^S\) wild-type and variants were purified from JM109 carrying plasmids pQEExpoS, pQERpoS\(_{R141S}\), pQERpoS\(_{A157T}\) and pQERpoS\(_{DB}\) as described (17).

KMnO\(_4\) reactivity
The assays were performed as described in (49) with the following modifications: the katN promoter fragment was incubated with 60 nM reconstituted Er\(S^5\) (\(E = 10\) for 1 h at 30°C before KMnO\(_4\) addition.

Band shift analysis
Escherichia coli core and S. Typhimurium His6-\(\sigma^S\) (wild-type and/or mutants) in 6-\(\mu\)l buffer (40-mM Heps pH 8.0, 10-mM MgCl\(_2\), 100-mM K-glutamate, 2-mM DTT containing 500-\mu\)g/ml bovine serum albumin) were incubated for 10 min at 37°C. Three microliter of \([^{32P}]\)-labeled katN fragment prepared as in (49) was then added and incubated for 20 min at 37°C. After addition of 3 \(\mu\)l loading buffer (buffer A containing 50% sucrose, 0.025% xylene cyanol blue and 150 \(\mu\)g/ml of heparin) the mixture was loaded onto a 5.5% native polyacrylamide gel run in TG buffer (25-mM Tris, 192-mM Glycine pH 8.5) at 10 V/cm.

Enzymatic assays
\(\beta\)-galactosidase activity was measured as described by Miller (50) and is expressed in Miller units.

RESULTS AND DISCUSSION
Construction of rpoS mutants affected in \(\sigma^S\) promoter DNA binding
To assess the molecular basis of \(\sigma^S\) mediated gene repression, we constructed an rpoS mutant specifically deficient in promoter DNA binding. Sigma factors from the \(\sigma^70\) family, including \(\sigma^S\), are composed of globular domains divided into functional regions (Figure 2A). They direct the RNAP holoenzyme to promoter elements -10 and -35, recognized by domains \(\sigma_2\) and \(\sigma_4\), respectively (1–3,51,52). \(\sigma_2\) regions 2.3–2.4 are critical for recognition and melting of the -10 element, the most highly conserved and essential promoter motif (1–3,51,52). In contrast, most \(\sigma^S\)-dependent promoters display a poorly conserved -35 element and it is not clear how Er\(S^5\) uses the -35 element (7,53).

Few biochemical studies have been performed on Er\(S^5\) (7,53–56) and the three-dimensional structure of \(\sigma^S\) is unknown. Although the overall sequence of the \(\sigma^S\) and \(\sigma^70\)
DNA-binding regions is well conserved (51,54), the corresponding holoenzymes are distinct in some mechanistic features and residues important for DNA recognition by $\sigma^S$ appear to be significantly different from those of $\sigma^{70}$ (57,53–56). Amino-acid substitutions R141S and A157T (Figure 2A) have been shown to impair $\sigma^S$ promoter binding but not E binding (54). These residues are not conserved in $\sigma^{70}$ (Supplementary Figure S1A). However, the corresponding residues in $\sigma^{70}$ are located in a DNA binding $\alpha$ helix, and residue K426, corresponding to R141 in $\sigma^S$ (Supplementary Figure S1A), interacts with nucleotides in the -10 element of the promoter (57).

The $rpoS$ alleles encoding $\sigma^S_{R141S}, \sigma^S_{A157T}$ (54) and $\sigma^S_{R141SA157T}$ (named $\sigma^S_{db}$) were generated and introduced in the chromosome of S. Typhimurium ATCC14028 as described in the Materials and Methods section. The mutant derivatives, $rpoS_{R141S}$ and $rpoS_{db}$ (encoding $\sigma^S_{R141S}$ and $\sigma^S_{db}$, respectively) and to a lesser extent $rpoS_{A157T}$ (encoding $\sigma^S_{A157T}$), were unable to display the $\sigma^3$-dependent $rdr$ morphotype (8,9) of Salmonella wild-type (Supplementary Figure S2A). They were also impaired in expression of a transcriptional $lacZ$ fusion to $katN$ (Supplementary Figure S2B), a well-characterized $\sigma^3$-dependent gene (16,49,58). Since $\sigma^S_{R141S}, \sigma^S_{db}$ and to a lesser extent $\sigma^S_{A157T}$ were produced in increased amounts compared to wild-type $\sigma^S$ (Supplementary Figure S2C), these variant $\sigma^S$ proteins were likely impaired in their activity. Consistent with this hypothesis, RNAP holoenzymes containing the $\sigma^S$ variants were affected for binding at the $katN$ promoter, as assessed by band shift assays and potassium permanganate reactivity footprinting (Figure 2B). The $\sigma^S_{db}$ variant was more strongly affected than the $\sigma^S_{R141S}$ and $\sigma^S_{A157T}$ variants, especially in vivo (Supplementary Figure S2B), and the corresponding mutant was thus selected for further studies.

**$\sigma^S_{db}$ is not impaired in RNAP core binding**

As mentioned above, Lee and Gralla established that substitutions R141S and A157T do not affect the ability of $\sigma^S$ to bind E (54). Since we used a combination of these two substitutions in $\sigma^S_{db}$, and given that in vivo modulators of RNAP formation (2–5,52) might differentially affect the interaction between E and the $\sigma^S$ and $\sigma^S_{db}$ variants, it was important to compare the ability of $\sigma^S$ and $\sigma^S_{db}$ to form E$\sigma^S$ holoenzyme in vivo.

Separation by size exclusion chromatography and immuno-detection in crude cell extracts of free and E-associated $\sigma$ has been used previously to assess the effect on the assembly of $E^{70}$ and $E_{db}$ holoenzymes of ppGpp (20), Crl (59) and the $\omega$ subunit of RNAP (60). Here we used the same methodology to separate and compare levels of free $\sigma^S$ from $\sigma^S_{db}$ bound to RNAP in the wild-type strain and its $rpoS_{db}$ derivative in stationary phase (Figure 2C).

Two populations of $\sigma^S$ were found in both strains, the major one (fractions 4–8) co-eluted with the $\beta$ and $\alpha$ subunits of core RNAP and was interpreted to represent holoenzyme-associated $\sigma^S$, while the other, in fractions 17–19, represented free (unbound) $\sigma^S$. Indeed, free $\sigma^S$ used as a control eluted in fractions 17–19 only (Figure 2C). The elution profiles were similar for the wild-type and $rpoS_{db}$ strains, showing that $\sigma^S_{db}$ was not impaired in RNAP.
binding, compared to wild-type σS. Thus, the defect of σSdb in gene activation was likely due to its inability to bind the promoter DNA.

**σS, but not σSdb, downregulates expression of sdh and ompD genes**

To further characterize the activity of σSdb on different gene targets besides katN, a collection of 17 σS-activated lacZ gene fusions was used. Expression of all the fusions was downregulated in the rpoSdb mutant, compared to the wild-type strain (Figure 3A), even though it is worth noting that σSdb retained basal activity at some promoters (e.g. yeaG and yahO). The cloned rpoS gene restored expression of the lacZ fusions as shown for katE and katN and of the rdar morphotype in the rpoSdb mutant (Figure 3B and C, respectively).

We previously showed that, in stationary phase of growth in rich medium, σS downregulates most S. Typhimurium genes involved in the TCA cycle (12). For example, the sdhCDA operon encoding succinate dehydrogenase, a membrane bound complex that directly connects the TCA cycle with the respiratory electron transport chain, is downregulated by σS in Salmonella (12) and E. coli K-12 (10). Interestingly, sdhB transcript levels were strongly increased in both the rpoSdb and ΔrpoS mutants, compared to that in the wild-type strain (Figure 4A). Consistently, the ΔrpoS and rpoSdb strains grew better than wild-type in minimal medium with succinate and this phenotype was complemented by wild-type rpoS expressed from plasmid pSTK4 (Figure 4B). The ability of Salmonella to grow on succinate was also improved by the single rpoS mutation rpoSΔr141s and to a lesser extent rpoSΔs157t (Supplementary Figure S2D). The ompD (ompC) gene is another target of negative regulation by σS (12), encoding a major porin of Salmonella and a target of a protective antibody response (61). In the rpoSdb and ΔrpoS strains, the levels of ompD-lacZ fusion expression and OmpD production were higher than in the wild-type strain (Figure 4C and D). Overall, these results suggested that σS binding to DNA is required for downregulation of sdh and ompD.

**The rpoSdb and ΔrpoS mutations alleviate growth restriction of a ΔrssB mutant**

The levels of σS are low in exponential phase due to proteolysis by the ClpXP protease and to RssB, a protein required for σS recognition by ClpXP (4, 5) (Figure 5A). Consistently, the levels of σSdb and σS were higher in the ΔrssB mutant than the wild-type strain (Figure 5A). In ΔrssB mutants, the accumulation of σS in exponential phase resulted in growth defects that were alleviated by the rpoSdb and ΔrpoS mutations (Figure 5B). Thus, in contrast to wild-type σS, σSdb did not impair Salmonella growth when produced to high levels in exponential phase. The increased levels in stationary phase of σS variants with reduced activity, compared to that of wild-type σS (Figure 5A and Supplementary Figure S2C), might result from the inactivation of auto-regulatory circuits. Indeed, σS controls transcript levels of numerous genes that modulate its expression (5, 7, 12),

The increased level of σSdb compared to that of σS (Figure 5A) might contribute to the observed residual activity of σSdb at some promoters in stationary phase (Figure 3A and see below Table 1).

**Global gene expression in wild-type, ΔrpoS and rpoSdb Salmonella strains**

The above results suggested that σS binding to DNA is required for downregulation of sdh and ompD genes. To assess regulation of additional gene targets, and attempt to
identify a subset of genes downregulated by σ^S primarily via σ competition for E binding (Figure 1A), the activity of σ^S-db was determined at the genome level. Transcript levels of the wild-type strain and the rpoS_db and ΔrpoS mutants were measured by directional RNA-seq using three biological replicates of strains grown to stationary phase in LB medium. We recently reported the comparative analysis of expression profiles between the wild-type and ΔrpoS strains (12). Six hundred and seven genes were differentially expressed in the wild-type strain and the ΔrpoS mutant with a high probability (P < 0.001), of which 145 were repressed by σ^S (12), including many genes also repressed by σ^E in *E. coli* (5, 10). It is worth noting that whereas some σ^S-activated genes are directly regulated through binding of σ^S to σ^S-dependent promoters, others are likely regulated indirectly by σ^S. When the expression profile of the rpoS_db mutant was compared to that of the wild-type and ΔrpoS control strains, the rpoS_db and ΔrpoS mutants showed similar expression profiles (Figure 6 and Supplementary data set S1). Besides rpoS, only 19 genes showed expression levels higher in the rpoS_db mutant than in the ΔrpoS strain (P < 0.001; Figure 6 and Table 1). These σ^S-activated genes exhibited high expression levels in the wild-type strain and strong σ^S-dependency (12) (Table 1). Consistent with this finding, promoter sequences of those genes (62, 63) show typical features of σ^S-promoters (7, 53, 56) (Figure 7A and B and Supplementary Figure S3). In particular, the conserved -12T, -11A and -7T are of paramount importance for promoter recognition and use by Er^S (7, 53, 56). In contrast, the data did not show any conserved nucleotides corresponding to a -35 region (Figure 7A), in agreement with previous findings that Er^S promoters display low level of sequence conservation near -35 (7, 53, 56). It is likely that these σ^S-dependent promoters are functional to some extent with Er^S-db (Table 1). Basal expression levels in the rpoS_db mutant of yahO, yeaG and otsA were also detected using lacZ fusions (Figure 3A).

One gene only, ynfM, was upregulated in the ΔrpoS strain compared to the rpoS_db mutant (Table 1), even when a P-value cut-off of 0.05 was used (Supplementary data set S1). The ynfM gene was poorly transcribed under the growth condition used (Table 1 and Supplementary Figure S4A). Surprisingly, its transcript levels were not significantly different in the ΔrpoS and wild-type strains (12) (Table 1), whereas expression of a transcriptional ynfM-lacZ fusion was downregulated in the ΔrpoS and rpoS_db strains, compared to the wild-type strain (Supplementary Figure S4A). Complementation of the ΔrpoS and rpoS_db mutations was observed when σ^S was produced in *trans* from plasmid pSTK4 (Supplementary Figure S4B), confirming that ynfM-lacZ transcription was activated by σ^S. Activation of the ynfM promoter by σ^S might be masked, at the ynfM transcript level, by compensatory negative effects of σ^S on ynfM mRNA elongation and/or stability, resulting in a complex regulatory pattern.

Altogether, these results strongly suggested that, under the conditions used, the negative effects of σ^S on gene expression require its binding to DNA and are unlikely to result solely from competition between σ^S and other σ factors for E binding. Our finding, that σ^S binding to E alone did not result in detectable gene repression by σ^S, might be explained if promoters are saturated, i.e. they bind Er efficiently but display a low rate of transcription initiation, so that they are occupied by Er most of the time and are

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Table 1. Genes differentially expressed in the rpoS_db and ΔrpoS mutants (P < 0.001)

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<th>Normalized read counts</th>
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*see also Supplementary data set S1.
*SSTM14.1359 or S-non-coding region of yeaG.
*STM14.2680 or the partially overlapping STnc1330 sRNA (63).
*STM14.5096 or S-non-coding region of yjbJ.
*Reads from the SraL sRNA, partially overlapping STM14.5129 (12).
weakly affected by \( \sigma^70 \) competition. It is also possible that, in stationary phase under physiological conditions (as opposed to conditions where a factor is over-expressed), \( \sigma \) competition for E is weakened and/or its effects on gene transcription are not detected by the methods used here.

In stationary phase, the stop of transcription of ribosomal RNA increases the availability of E (the activities of these promoters sequester 60–70% of the transcriptional machinery during rapid growth in rich media; (2)). In addition, molecules produced in stationary phase (such as anti-\( \sigma \), 6SRNA, ppGpp and metabolites) also alter \( \sigma \) competition by sequestering \( \sigma \) and/or modulating E formation (2,52). Variations in the efficiency of promoter escape and transcript elongation in stationary phase may also alter the distribution of E, \( \sigma \) and Eor and thus \( \sigma \) competition. To determine whether elimination of \( \sigma^8 \) favors the formation of Eor\(^{70} \) in the conditions of our study, levels of \( \sigma^70 \) bound to RNAP were compared in the wild-type strain and the \( \Delta \text{rho}S \) and \( \text{rho}S_{\text{db}} \) mutants, by size exclusion chromatography of crude extracts and immuno-detection of \( \sigma^70 \) and the \( \beta \)’ subunit of RNAP. The percentage of total \( \sigma^70 \) co-eluting with the \( \beta \)’ subunit of core RNAP was 94–98% and similar for the three strains, suggesting that most \( \sigma^70 \) molecules were associated with RNAP in stationary phase (Supplementary Figure S5). A more detailed study and the use of complementary techniques, to assess the concentrations of the different holoenzymes at different time points upon entry to stationary phase, in different media and bacterial genetic backgrounds, are required to carefully address the relevance of the \( \sigma \) competition for E model during stationary phase. Nevertheless, these data reinforced our conclusion that the extensive gene repression by \( \sigma^8 \) in stationary phase (Supplementary data set S1 and Figure 6) (12) does not rely on the regulatory model depicted in Figure 1A.

\( \sigma^8 \) and \( \sigma^70 \) factor antagonism at the \( \text{sdh} \) promoter

The \( \text{sdh} \)CDAB succinate dehydrogenase operon is negatively controlled at the post-transcriptional level by the RyhB sRNAs (22,64). Expression of the Salmonella homologous RyhB1 and RyhB2 sRNAs is positively controlled by \( \sigma^8 \) in the growth conditions used in the present study (i.e. late stationary phase in rich medium) (12,22), thus making these sRNAs possible intermediates in the downregulation of \( \text{sdh} \) expression by \( \sigma^8 \) (according to the regulatory model in Figure 1B). However, \( \text{sdh} \) transcription levels were similar in the wild-type strain and the \( \text{ryh}B1/2 \) strain containing mutations in both \( \text{ryh}B \) genes (Supplementary Table S1) and were increased to similar levels in the \( \Delta \text{rho}S \) and \( \Delta \text{rho}S_{\text{db}} \) strains, compared to that in wild-type (Figure 8A).
Figure 6. Transcriptome profile of wild-type and *rpoS* Salmonella strains. Scatterplot was used to compare gene expression (log2-transformed normalized read counts) in the *rpoS*mutant and the wild-type (WT) and Δ*rpoS* strains grown to stationary phase in LB. Transcriptome profiles of the wild-type and Δ*rpoS* strains, used as controls in the experiment, have been recently reported (12). Red and green dots represent genes differentially expressed (P < 0.001) and black dots represent genes not differentially expressed.

Figure 7. Promoter sequences and features. (A) Sequence logo generated with promoter sequences of genes in Table 1 (Supplementary Figure S3) and the WebLogo application (http://weblogo.threeplusone.com/create.cgi). (B) Possible consensus sequences for the -10 element of promoters preferentially recognized and transcribed by Eo/s/H9268 and Eo/s/H9268 S (7,53). The -35 sequence of Eo/s-dependent promoters is also indicated. The -35 element is less conserved in Eo/s-dependent promoters and is variable in its sequence and location (7,53). The most conserved nucleotides are indicated in capital letters. Y denotes a pyrimidine (T/C). K stands for T/G. (C) DNA sequence of the *sdh* promoter (Psdh) region and base substitutions generated in the chromosome of *Salmonella* to yield *sdh*-mut1 and *sdh*-mut2.

These data suggested the existence of a RyhB-independent mechanism of negative regulation of *sdh* by σS.

A single promoter has been identified upstream of *sdhC* in wild-type *Salmonella* (62,63) (Figure 7C). Chromosomal mutations were introduced in the -10 element of this promoter (Psdh) at positions -12T, -11A and -7T that are of paramount importance for promoter recognition by both Eo/s and Eo/s S (7,52,53,55) (*sdh*-mut1, Figure 7C). These mutations strongly reduced *sdhC*, *sdhD* *sdhA* and *sdhB* mRNAs levels in the wild-type strain and in the Δ*rpoS* mutant (Figure 9A) and impaired the ability of these strains to grow on succinate (Figure 9B), suggesting that *sdh* expression in both wild-type and Δ*rpoS* strains is driven mainly from Psdh. In vitro, Eo/s and Eo/s S were both able to bind the *sdh* promoter region (data not shown). This finding was not unexpected since (i) the DNA sequence specificity of σS is very similar to that of σS S and many promoters are bound

in vitro by both holoenzymes (5,7,53), and (ii) the -10 sequence of Psdh is identical to that of promoters preferentially recognized by Eo/s S and Eo/s S (7,53,55) (Figure 7C). In vivo however, variable combinations of intrinsic promoter features and regulatory proteins determine whether a promoter is recognized and transcribed by Eo/s S or Eo/s S (5,7,53).
σ factors of the σ^70 family are involved in promoter recognition to position RNAP, but also in the formation of the open promoter complex, in which melted DNA includes the −10 region and extends downstream to the transcription start site (3,52). In housekeeping RNAP region 1.2 of domain 2 of σ interacts with three non-template strand nucleotides immediately downstream the −10 element (GG and G of the discriminator sequence GGGA (3,52,57)). The strength of interaction between the discriminator and σ^70 region 1.2 influences open complex formation/stability and its consequence on transcriptional output and regulation depends on the intrinsic kinetics of the promoter (3,52,65). Although the discriminator sequence likely influences σ^5′-dependent transcription in concert with the −10 hexamer (7.53,66), its exact role in σ^5′-transcription is unknown. In addition, residues of σ^70 interacting with the discriminator (57) are not all conserved in σ^5 (Supplementary Figure S1B) raising the possibility that σ^5 and σ^70 use different discriminator sequences. The nature of the P_dh discriminator may influence different steps in σ^5-dependent transcription, such as positioning of σ^5 on the promoter DNA, formation/stability of the open complex and/or promoter clearance. As σ^5 binds duplex (unmelted) DNA promoters more weakly than σ^70, AT-rich discriminator sequences may well optimize promoter melting near the transcription site (7.53,55). When the level of σ^5 was increased in Salmonella by providing additional copies of rpoS in trans on plasmid pSTK4 (Figure 8C), sdhB transcript levels increased in the sdh-mut2 strain but not in wild-type (Figure 8D). Altogether, these data suggest that σ^5 (i) represses transcription from the wild-type P_dh promoter, but not from the sdh-mut2 promoter (Figure 8B), and (ii) activates transcription from the wild-type P_dh promoter, at least when it is over-produced (Figure 8 CD). It is thus possible that the sdh promoter is poised by σ^5 engaged and having difficulties to escape and that sdh-mut2 allows to some extent poised σ^5 to escape into elongation mode.

The discriminator sequence has been implicated in the regulation of σ^70-dependent promoters by ppGpp and its cofactor DksA (2.52,65). Since a GC-rich discriminator favors promoter repression by ppGpp (2.52,65), it would be interesting to determine whether ppGpp and DksA have a direct role in the regulation by σ^5 of the sdh promoter. In a recent transcriptome analysis, the sdh promoter was shown to be upregulated in a relAspoT mutant of Salmonella deficient for the production of ppGpp (62). However, this effect may be indirect, via σ^5, since ppGpp has positive effects on rpoS transcription, σ^5 stability and the formation/performance of σ^5 (2.4,20). Changing the GC-rich discriminator to a TA-rich one in sdh-mut2 had no significant effect on σ^70 binding to P_dh, suggesting that this modification had no major impact on σ^70 transcription in these conditions. It remains to be determined how it affects σ^5 transcription and whether ppGpp is involved.

Although additional experiments are required to highlight the mechanistic implication of the σ^5iscriminator interaction in σ^5-dependent transcription and how sdh-mut2 might influence transcriptional outputs at P_dh, our

Interestingly, genome-wide mapping of σ^70 and σ^5 binding sites in E. coli (37) shows that both σ^70 and σ^5 bind the sdh promoter region, in vivo as well. These findings raised the possibility that σ^5 binding to the sdh promoter in vivo competes with σ^70-dependent transcription initiation (as proposed in the regulatory model in Figure 1C).

A striking feature of P_dh is the presence of a GC-rich region just downstream the −10 box (Figure 7C), in the discriminator region of the promoter (3.52.57). In contrast, the discriminator of σ^5-dependent promoters is frequently TA rich (7.53.55) (Figure 7B). Interestingly, a TA motif is conserved in the discriminator region of promoters retaining basal activity in the rpoS Δs mutant (Figure 7A), and may favor residual activity of σ^5 rh. When the GCC sequence in P_dh was substituted by TAA (sdh-mut2; Figure 7C), sdhB transcript levels were upregulated in the wild-type strain (Figure 8B). In stark contrast, sdh-mut2 had no significant effect on sdhB transcript levels in the ΔrpoS strain (Figure 8B), indicating a σ^5-specific effect of the TAA motif. These data suggested that sdh-mut2 mutations eliminate the negative effects of σ^5 on sdhB transcription (Figure 8B) and are consistent with a model in which in which σ^5 binding to P_dh poises the promoter due to an unfavorable discriminator region and disrupts normal transcription by competing with σ^70 binding.

Altogether, the results suggest that σ competition at the promoter DNA plays an important role in gene repression by σ^5 and open new field of investigation regarding the role of the promoter discriminator region in σ^5 activity.
data suggest that negative regulation of sdh transcription by $\sigma^S$ is mediated by competition between $\sigma^S$ and $\sigma^{70}$ at the DNA rather than E level, a finding consistent with data from experiments using $\sigma^S_{dh}$. It must be emphasized that the presence of a perfect $-10\sigma^S$-consensus followed by GC-rich region is not a general characteristic of promoters of genes negatively controlled by $\sigma^S$ (data not shown) and the repression mechanism used by $\sigma^S$ likely adapts to promoter characteristics. Also, we cannot exclude a role for additional factors helping $\sigma^S$ to form unproductive complex at $P_{sdh}$ in vivo. Indeed, there are a few examples of poised RNAP generated by transcriptional regulators and/or inappropriate environmental condition (27,29,52,67).

**CONCLUSION**

Our study provides novel insights into mechanisms of downregulation of gene expression by $\sigma^S$ by showing that, under physiological conditions in stationary phase, gene repression requires $\sigma^S$ binding to DNA. Furthermore, we suggest that $\sigma^S$ can function as a transcriptional repressor. Our data are likely the tip of the iceberg and will inspire future studies to decipher the underlying molecular mechanisms. These mechanisms are likely to be specific to individual genes and environmental conditions and to rely on two main categories of regulatory processes: direct effects of $\sigma^S$ at the promoter DNA level, as shown in the present study (Figure 1C), and the action of $\sigma^S$-dependent negative effectors (Figure 1B).

Direct negative regulation of transcription by $\sigma$ factors (Figure 1C) is not well documented and the possibility that $\sigma^S$ blocks active gene transcription as a repressor calls for further investigation. Our data suggest that $\sigma^S$ occludes the sdh promoter and this lowers transcription by competition with $\sigma^{70}$ binding. Other genes downregulated by $\sigma^S$ are transcribed by more than one promoter and $\sigma^S$ binding at one promoter or pausing might sterically hinder binding of $\sigma^{70}$ or an alternative RNAP and/or transcription factors at a second promoter, resulting in promoter interference and gene repression. Due to the extensive overlap between promoter regions bound by housekeeping and alternative RNAP in vivo, especially $\sigma^{70}$ and $\sigma^S$ (36,37), $\sigma$ factor antagonisms at the promoter DNA level might be more frequent in vivo than initially thought.

A second main category of regulatory processes requiring the DNA binding activity of $\sigma^S$ might endow $\sigma^S$ with repressor functions: the involvement of $\sigma^S$-dependent effectors (Figure 1B). Future experiments will assess whether some of the $\sigma^S$-controlled secondary regulators (12) are intermediates in regulatory cascades (Figure 1B), or co-repressors in feed-forward regulatory loops (Figure 1C), to control gene transcription negatively. Another exciting issue will be to determine to which extent $\sigma^S$-dependent transcriptional and post-transcriptional control mechanisms are combined to allow for dynamic and flexible regulatory patterns and additional signal inputs. The RhbS sRNAs are another possible tool for $\sigma^S$ to downregulate sdh expression. It is possible that the expression levels of these sRNAs and/or the growth conditions used in the present study are not appropriate to observe a significant impact of the rhbB mutations on sdhB transcript levels (Figure 8A). Experiments are underway to determine how the different regulatory components of the $\sigma^S$ control cooperate to adjust the levels and dynamics of sdh expression in response to different environmental conditions, and whether these controls contribute to the cell fitness.

**ACCESSION NUMBER**

The RNA-seq data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession GSE46380 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=rhedtgougouiuri&acc=GSE46380).

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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**REFERENCES**


**AUTHOR CONTRIBUTIONS**


