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Repressor activity of the RpoS/σ^S-dependent RNA polymerase requires DNA binding

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ABSTRACT

The RpoS/σ^S 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 σ^S-dependent control, that of a repressor. Negative regulation by σ^S has been proposed to result largely from competition between σ^S and other σ factors for binding to a limited amount of core RNAP (E). To assess whether σ^S 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 σ^S protein proficient for Eσ^S 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 σ^S requires its binding to DNA. Although the mechanisms of repression by σ^S 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 Eσ^S.

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 $\sigma^{S/38}$ (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), σ^S 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, σ^{70} (RpoD), σ^S 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, $\sigma^{E/24}$ (*rpoE*), $\sigma^{H/32}$ (*rpoH*), $\sigma^{N/54}$ (*rpoN*) and σ^{28} (*fliA*) which associate with the core RNAP (E) to form the holoenzyme Eσ (1–3). The cellular concentration of σ^{70} 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 σ^S-dependent control, that of a repressor. σ^S has a negative effect on the expression of a large number of

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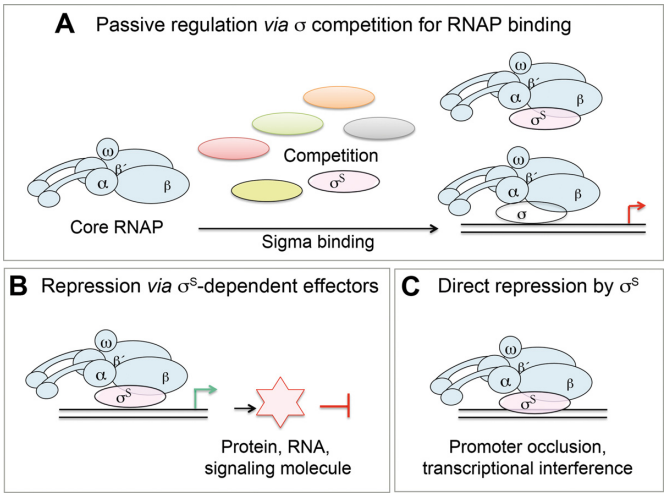

Figure 1. Schematic illustration of the main possible regulatory mechanisms of gene repression by σ^S. Graphical representation of the core RNA polymerase complex (in blue), σ^S (in light pink) and the other sigma subunits (different colors) is shown. Each box represents a possible σ^S repression mechanism. (**A**) Repression model based on competition of σ factors for RNAP core binding. (**B**) Indirect repression model *via* diverse effector molecules. (**C**) Direct repression model where $E\sigma^S$ binding prevents the transcription by other Eσ holoenzymes. Models B and C require the DNA binding activity of σ^S. Note that combinations of different mechanisms are possible.

genes, including genes involved in metabolism and membrane permeability (5,6,10–13). Consequently, the acquisition of stress resistance mediated by σ^S comes at the expense of growth capabilities, causing a trade-off between self-preservation and nutritional competence, the so-called SPANC balance (11,14). This trade-off favors the appearance of non-functional *rpoS* alleles in environments with no stress, where reduced σ^S activity confers a growth advantage (5,11,15–17). The negative control of bacterial growth by σ^S and its contribution to population polymorphisms is a subject of growing interest with relevance in molecular ecology and evolution. However, gene repression by σ^S is not mechanistically well documented and is thought to result primarily from σ^S competing with other σ for E binding (18,19).

Previous studies, using artificial manipulation of σ factor levels and the use of σ mutants with reduced affinity for E, have shown that σ competition for binding to E modulates the activity of promoters controlled by σ^S, σ^{32} and σ^{54} (20,21) and may explain how a σ negatively regulates transcription by other σ (2,18–21). For example, mutations in *rpoD* reducing the σ^{70} affinity for E cause an upregulation of genes controlled by alternative σ, presumably increasing the pool of available E (2,20,21). Conversely, overexpression of σ^S lowers the expression of genes controlled by σ^{70}, presumably reducing the $E\sigma^{70}$ amounts, and affecting promoters that are sensitive to the cellular concentration of $E\sigma^{70}$ (18,19). Although these findings are compatible with a model in which σ^S competition with other σ for E binding alone accounts for negative regulation of gene transcription by σ^S (Figure 1A), they could be explained by alternative regulatory mechanisms (Figure 1B and C). Negative regulation by σ^S might be mediated by negative effectors whose expression is activated by σ^S (Figure 1B) (5,6,12,13). Indeed, σ^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 σ^S with repressor function. RNAP itself is also a DNA binding protein and $E\sigma^S$ might theoretically function as a transcriptional repressor through promoter occlusion or transcriptional interference (Figure 1C) (27–35). Stable but transcriptionally inactive RNAP bound to DNA might inhibit transcription by another holoenzyme (27,28,32). Tandem or overlapping promoters and pausing of RNAP outside of the promoter context might also sterically hinder DNA binding by an alternative RNAP and/or transcription factors, producing transcriptional interference and repression (30,31,33–35) (Figure 1C). Few examples of direct negative effect of σ have been reported so far (27–29,35) but the extensive overlap between σ factor binding sites recently reported in *E. coli* (36,37) is compatible with antagonisms between σ factors at the promoter DNA level. In particular, during the completion of this study, Cho *et al.* (37) reported that $E\sigma^S$ and $E\sigma^{70}$ binding regions in the *E. coli* genome extensively overlap, and many σ^S binding promoters showed increased transcription and increased σ^{70} binding in a Δ*rpoS* strain. Interestingly, these data suggested that direct interference between $E\sigma^S$ and $E\sigma^{70}$ might occur at the DNA level. However, it was not shown whether the decreased σ^{70} binding and transcription, observed in the wild-type strain compared to the Δ*rpoS* mutant, was a direct consequence of σ^S binding in the same promoter region (according to model C; Figure 1) or an indirect effect, due to σ competition for E (according to model A; Figure 1) and/or the synthesis of a negative effector under the control of σ^S (according to model B; Figure 1).

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

MATERIALS AND METHODS

Bacterial strains, bacteriophage, plasmids and growth conditions

Strains and plasmids are listed in Supplementary Table S1. Bacteriophage P22HT105/1*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

(LB) (40) at 37°C under aeration. To assess *Salmonella* growth at the expense of succinate and glucose (20 mM) as carbon sources, M9 minimal medium (40) was used. Stationary phase cultures were washed, resuspended in phosphate-buffered saline (40) to OD600 of 1.0 and 0.05, and 5 μl of each dilution was spotted onto plates that were incubated at 37°C for 48 h. Rdar morphotypes were analyzed on CR plates (LB agar without NaCl supplemented with Congo red 40 μg/ml and Coomassie brilliant blue R250 20 μg/ml), at 28°C as described (8). Antibiotics were used at the following concentrations (in μg per ml): carbenicillin (Cb), 100; chloramphenicol (Cm), 15 for the chromosomal resistance gene and 30 for the plasmid resistance gene; kanamycin, (Km) 50; and tetracycline (Tet) 20.

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 pUCC52-2922K using the QuikChange II Site-directed mutagenesis kit (Stratagene) as recommended by the manufacturer. pVF9793 was obtained by site-directed mutagenesis of pVF9551. Plasmids pQE30*rpoS*$_{R141S}$, pQE30*rpoS*$_{A157T}$ and pQE30*rpoS*$_{db}$ (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 pQE30*rpoS* (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 Δ*rssB* 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 *tet*AR module produced by PCR (12) and (ii) replacement of the *tet*RA module by a DNA fragment obtained by PCR and carrying the desired mutations (Supplementary Table S2) through positive selection of tetracycline-sensitive recombinants (43). The presence of desired mutations in all strains was confirmed by DNA sequencing. Tn*5*B21 insertions creating *lacZ* fusions in σ^S-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 Y* 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 integrants (using standard test primers, such as those described in (45)). We also used flanking locus-specific primers to amplify junction fragments that were subsequently analyzed by DNA sequencing. When required, the Km^R cartridge in chromosomal *lacZ Y* gene fusions was replaced with a Cm^R cartridge as described previously (16).

Construction of isogenic *S.* Typhimurium strains carrying the Δ*rpoS* and *rpoS*$_{db}$ alleles

The *rpoS* allele in *S.* Typhimurium ATCC14028 was replaced by the *rpoS*$_{R141S}$, *rpoS*$_{A157T}$ and *rpoS*$_{db}$ 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^S recombinants containing the *STM2922*::Km mutation. This mutation was then transduced into ATCC14028 and its Δ*rpoS*::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^S substitutions R141S, A157T and R141A-A57T, respectively). These plasmids were electroporated in the ATCC14028 strain containing the *rpoS*$_{420}$::Cm mutation (VF9579) to facilitate screening of strains into which simultaneous recombination of the *STM2922*::Km and *rpoS* point mutations occurred. Cb^S recombinants that were Km^R but Cm^S were selected, and recombination of the *STM2922*::Km mutation and simultaneous replacement of the *rpoS*$_{420}$::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 Δ*rpoS*::Cm background (VF7928) and the resulting Km^R Cm^S strains (VF9849, VF9676 and VF9682) 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 *rpoS*$_{db}$ 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

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 σ^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 σ^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 (NA934, GE Healthcare) and the Pierce ECL2 western blotting substrate (Thermoscientific).

Fractionation of free and RNAP-bound σ^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 Disrupter (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 µl of the supernatant was applied to a gel filtration column (Superdex 200 PC3.2/30, GE Healthcare) using the EttanLC System (GE Healthcare). Elution was performed at a flow rate of 0.04 ml/min at room temperature, gathering fractions of 50 µl. The proteins in the elution fractions were analyzed by SDS-PAGE and electroblotted onto nitrocellulose membranes (Hybond ECL GE Healthcare). Immunoblot analysis of the eluted fractions was carried out using the σ^S rabbit antibody (47) as described above and monoclonal antibodies against the β′ and α subunits of RNAP (WP001 and WP003, Neoclone) 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 Disrupter (Constant System, Daventry, UK). Outer membrane protein preparations were obtained as described by Lobos and Mora (48).

Overproduction and purification of His_6-σ^S variants

His6-σ^S wild-type and variants were purified from JM109 carrying plasmids pQE*rpoS*, pQE$rpoS_{R141S}$, pQE$rpoS_{A157T}$ and pQE$rpoS_{db}$ 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 Eσ^S (σ^S: E = 10) for 1 h at 30°C before $KMnO_4$ addition.

Band shift analysis

Escherichia coli core and *S.* Typhimurium His_6-σ^S (wild-type and/or mutants) in 6-µl buffer (40-mM Hepes pH 8.0, 10-mM $MgCl_2$, 100-mM K-glutamate, 2-mM DTT containing 500-µg/ml bovine serum albumin) were incubated for 10 min at 37°C. Three microliter of [^{32}P]-labeled *katN* fragment prepared as in (49) was then added and incubated for 20 min at 37°C. After addition of 3 µl loading buffer (buffer A containing 50% sucrose, 0.025% xylene cyanol blue and 150 µg/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

β-galactosidase activity was measured as described by Miller (50) and is expressed in Miller units.

RESULTS AND DISCUSSION

Construction of *rpoS* mutants affected in σ^S promoter DNA binding

To assess the molecular basis of σ^S mediated gene repression, we constructed an *rpoS* mutant specifically deficient in promoter DNA binding. Sigma factors from the σ^{70} family, including σ^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 σ_2 and σ_4, respectively (1–3,51,52). σ_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 σ^S-dependent promoters display a poorly conserved -35 element and it is not clear how Eσ^S uses the -35 element (7,53).

Few biochemical studies have been performed on Eσ^S (7,53–56) and the three-dimensional structure of σ^S is unknown. Although the overall sequence of the σ^S and σ^{70}

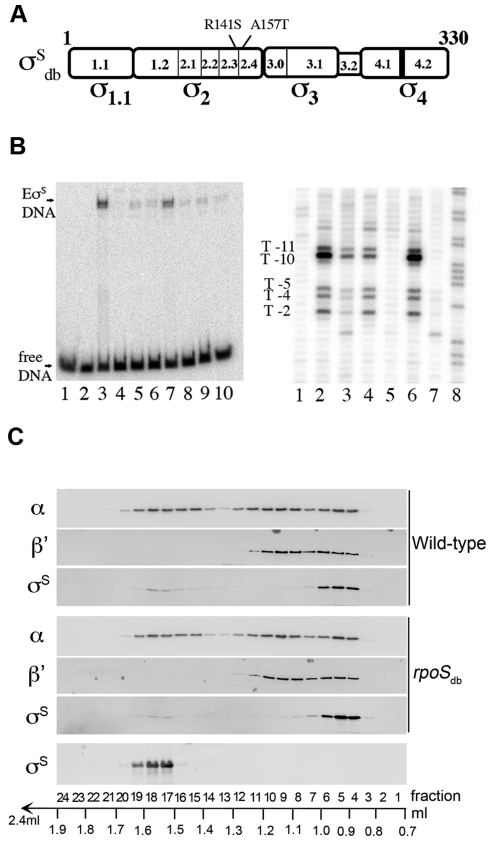

Figure 2. σ^S_{db} is impaired in DNA promoter binding but not in RNAP core binding. (**A**) Schematic representation of the four regions of the σ^S protein (17,51) and location of the amino-acid substitutions in σ^S_{db} in regions involved in binding and melting the -10 promoter element (54,55). (**B**) The σ^S variants containing one or both substitutions are deficient in DNA promoter binding. When bound to core RNA polymerase (E), σ^S makes sequence-specific promoter contacts and plays a crucial role in DNA melting (54–56). Left panel: electrophoretic mobility shift assay indicating binding of $E\sigma^S$, but not $E\sigma^S_{db}$, to the *katN* promoter region. The [^{32}P]-labeled *katN* promoter fragment was incubated for 20 min at 37°C with buffer (lane 1), E (lane 2) or holoenzymes reconstituted with His$_6$-σ^S wild-type (lanes 3 and 7), His$_6$-σ^S_{R141S} (lanes 4 and 8), His$_6$-σ^S_{A157T} (lanes 5 and 9) and His$_6$-σ^S_{db} (lanes 6 and 10) before heparin challenge. The E:σ^S ratios were 1:33 (lanes 3–6) and 1:4 (lanes 7–10). Right panel: $KMnO_4$ probing of the $E\sigma^S$ holoenzymes (E:σ^S of 1:10) on the 5′-[^{32}P]-labeled template strand *katN* fragment. $KMnO_4$ preferentially oxidizes exposed unstacked thymines of RNAP–promoter complexes and gives rise to marked $KMnO_4$ reactivity at the *katN* promoter as previously reported (49). The positions of the reactive Ts with respect to the transcription start are indicated (T-11, T-10, T-5, T-4 and T-2). Lane 1: control with no protein; lanes 2 and 6: E His$_6$-σ^S wild-type; lane 3: E His$_6$-σ^S_{R141S}; lane 4: E His$_6$-σ^S_{A157T}; lane 5: E His$_6$-σ^S_{db}; lane 7: E only. Lane 8 is a G+A reaction performed on the same DNA fragment according to Maxam and Gilbert (58). (**C**) Distribution of σ^S between free and holoenzyme forms in the wild-type and $rpoS_{db}$ strains. Whole cell lysates from wild-type strain VF7969 and $rpoS_{db}$ mutant VF9849 were fractionated by size exclusion chromatography and the relative concentration of RNAP subunits was subsequently analyzed in the fractions by immunoblot using monoclonal antibodies against the β' and α subunits of RNA polymerase and a polyclonal anti-σ^S antibody. Purified σ^S was used to pinpoint fractions containing free σ^S. The percentage of total σ^S in fractions corresponding to free σ^S was very low (and appeared slightly lower in $rpoS_{db}$ than in wild-type) suggesting that most σ^S molecules are associated with RNAP in stationary phase. Two independent experiments were performed with similar results (the elution profiles were similar for the wild-type strain and the $rpoS_{db}$ mutant, and the percentages of total σ^S in fractions 4–8 (bound σ^S) were 69 and 83% for the wild-type strain and 83 and 93% for the $rpoS_{db}$ mutant, calculated using the IMAGEJ software).

DNA-binding regions is well conserved (51,54), the corresponding holoenzymes are distinct in some mechanistic features and residues important for DNA recognition by $E\sigma^S$ appear to be significantly different from those of $E\sigma^{70}$ (5,7,53–56). Amino-acid substitutions R141S and A157T (Figure 2A) have been shown to impair σ^S promoter binding but not E binding (54). These residues are not conserved in σ^{70} (Supplementary Figure S1A). However, the corresponding residues in σ^{70} are located in a DNA binding α helix, and residue K426, corresponding to R141 in σ^S (Supplementary Figure S1A), interacts with nucleotides in the -10 element of the promoter (57). The *rpoS* alleles encoding σ^S_{R141S}, σ^S_{A157T} (54) and $\sigma^S_{R141SA157T}$ (named σ^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 σ^S_{R141S} and σ^S_{db}, respectively) and to a lesser extent $rpoS_{A157T}$ (encoding σ^S_{A157T}), were unable to display the σ^S-dependent rdar 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 σ^S-dependent gene (16,49,58). Since σ^S_{R141S}, σ^S_{db} and to a lesser extent σ^S_{A157T} were produced in increased amounts compared to wild-type σ^S (Supplementary Figure S2C), these variant σ^S proteins were likely impaired in their activity. Consistent with this hypothesis, RNAP holoenzymes containing the σ^S variants were affected for binding at the *katN* promoter, as assessed by band shift assays and potassium permanganate reactivity footprinting (Figure 2B). The σ^S_{db} variant was more strongly affected than the σ^S_{R141S} and σ^S_{A157T} variants, especially *in vivo* (Supplementary Figure S2B), and the corresponding mutant was thus selected for further studies.

σ^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 σ^S to bind E (54). Since we used a combination of these two substitutions in σ^S_{db}, and given that *in vivo* modulators of RNAP formation (2–5,52) might differentially affect the interaction between E and the σ^S and σ^S_{db} variants, it was important to compare the ability of σ^S and σ^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 σ has been used previously to assess the effect on the assembly of $E\sigma^{70}$ and $E\sigma^S$ holoenzymes of ppGpp (20), Crl (59) and the ω subunit of RNAP (60). Here we used the same methodology to separate and compare levels of free σ^S from σ^S bound to RNAP in the wild-type strain and its $rpoS_{db}$ derivative in stationary phase (Figure 2C). Two populations of σ^S were found in both strains, the major one (fractions 4–8) co-eluted with the β and α subunits of core RNAP and was interpreted to represent holoenzyme-associated σ^S, while the other, in fractions 17–19, represented free (unbound) σ^S. Indeed, free σ^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 σ^S_{db} was not impaired in RNAP

binding, compared to wild-type σ^S. Thus, the defect of $\sigma^S{}_{db}$ in gene activation was likely due to its inability to bind the promoter DNA.

σ^S, but not $\sigma^S{}_{db}$, downregulates expression of *sdh* and *ompD* genes

To further characterize the activity of $\sigma^S{}_{db}$ on different gene targets besides *katN*, a collection of 17 σ^S-activated *lacZ* gene fusions was used. Expression of all the fusions was downregulated in the *rpoS*$_{db}$ mutant, compared to the wild-type strain (Figure 3A), even though it is worth noting that $\sigma^S{}_{db}$ 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 *rpoS*$_{db}$ mutant (Figure 3B and C, respectively).

We previously showed that, in stationary phase of growth in rich medium, σ^S downregulates most *S.* Typhimurium genes involved in the TCA cycle (12). For example, the *sdhCDAB* operon encoding succinate dehydrogenase, a membrane bound complex that directly connects the TCA cycle with the respiratory electron transport chain, is downregulated by σ^S in *Salmonella* (12) and *E. coli* K-12 (10). Interestingly, *sdhB* transcript levels were strongly increased in both the *rpoS*$_{db}$ and Δ*rpoS* mutants, compared to that in the wild-type strain (Figure 4A). Consistently, the Δ*rpoS* and *rpoS*$_{db}$ 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 *rpoS*$_{R141S}$ and to a lesser extent *rpoS*$_{A157T}$ (Supplementary Figure S2D). The *ompD* (*nmpC*) gene is another target of negative regulation by σ^S (12), encoding a major porin of *Salmonella* and a target of a protective antibody response (61). In the *rpoS*$_{db}$ and Δ*rpoS* 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 σ^S binding to DNA is required for downregulation of *sdh* and *ompD*.

The *rpoS*$_{db}$ and Δ*rpoS* mutations alleviate growth restriction of a Δ*rssB* mutant

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

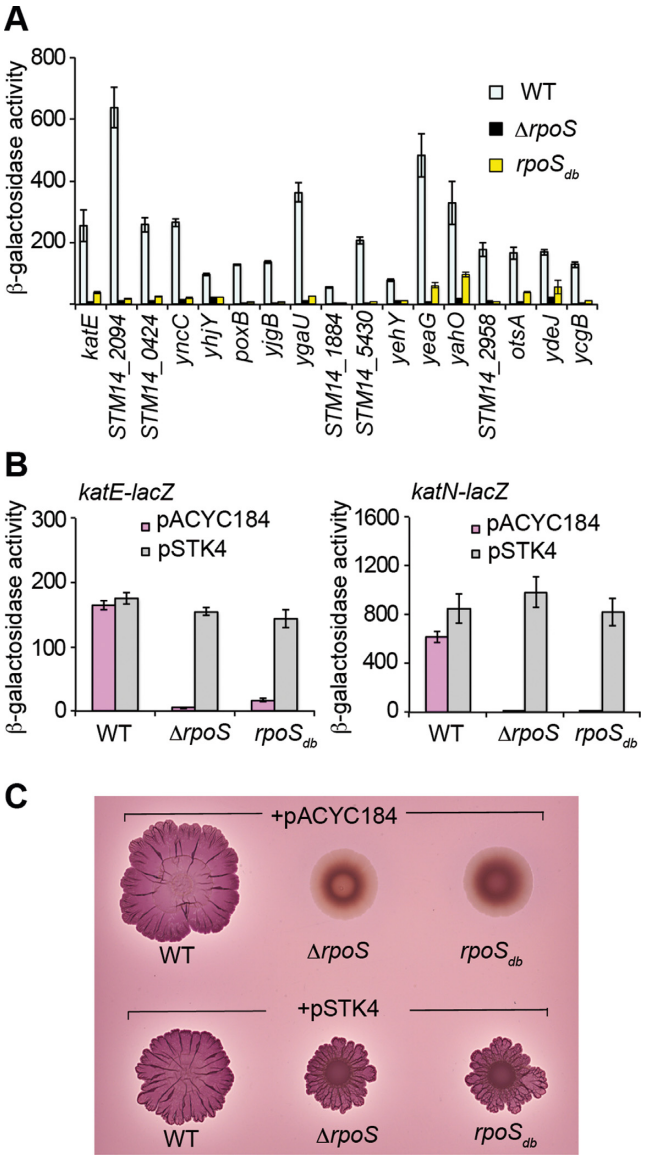

Figure 3. Effects of the Δ*rpoS* and *rpoS*$_{db}$ mutations on σ^S-activated genes and the rdar morphotype. (**A, B**) Expression of transcriptional *lacZ* fusions in σ^S-dependent genes in the wild-type (WT) strain VF7969 and its *rpoS* derivatives VF9356 and VF9849, grown for 18 h in LB at 37°C. Bar graphs represent the mean β-galactosidase activity and error bars represent standard deviation of at least three independent experiments. (**C**) Development of the red dry and rough (rdar) morphotype of the wild-type strain and *rpoS* mutants carrying the plasmid indicated was visualized on CR plates at 28°C. Empty vector pACYC184 and plasmid pSTK4 carrying the *rpoS* gene were used in complementation experiments (B, C).

The increased level of $\sigma^S{}_{db}$ compared to that of σ^S (Figure 5A) might contribute to the observed residual activity of $\sigma^S{}_{db}$ at some promoters in stationary phase (Figure 3A and see below Table 1).

Global gene expression in wild-type, Δ*rpoS* and *rpoS*$_{db}$ *Salmonella* strains

The above results suggested that σ^S binding to DNA is required for downregulation of *sdh* and *ompD* genes. To assess regulation of additional gene targets, and attempt to

Table 1. Genes differentially expressed in the $rpoS_{db}$ and Δ*rpoS* mutants ($P < 0.001$)

Name	Normalized read counts[a]			Product
	WT	$rpoS_{db}$	Δ*rpoS*	
STM14_0422	1203	25	3	Cytochrome BD2 subunit I
yahO	961	112	15	Hypothetical protein
ybgS	4003	116	6	Hypothetical protein
yeaG	5730	352	26	Putative serine protein kinase
STM14_1559[b]	589	30	1	Hypothetical protein
osmC	453	26	4	Putative envelope protein
otsA	865	161	11	Trehalose-6-phosphate synthase
otsB	832	99	6	Trehalose-6-phosphate phosphatase
yohC	1820	79	8	Putative transport protein
STM14_2680[c]	415	23	3	Hypothetical protein
ygdI	1871	229	23	Putative lipoprotein
yghA	716	18	1	Oxidoreductase
STM14_5096[d]	1830	117	2	Putative cytoplasmic protein
yjbJ	8161	571	25	Putative stress-response protein
STM14_5129[e]	9767	195	21	Hypothetical protein
ecnB	3219	114	12	Entericidin B membrane lipoprotein
osmY	2396	333	17	Hypothetical protein
STM14_5481	419	46	3	Hypothetical protein
oat	6006	288	35	Putrescine-2-oxoglutarate aminotransferase
ynfM	190	13	220	Putative transport protein

[a]see also Supplementary data set S1.
[b]*STM14_1559* or 5′non-coding region of *yeaG*.
[c]*STM14_2680* or the partially overlapping STnc1330 sRNA (63).
[d]*STM14_5096* or 5′non-coding region of *yjbJ*.
[e]Reads from the SraL sRNA, partially overlapping *STM14_5129* (12).

identify a subset of genes downregulated by σ^S primarily *via* σ competition for E binding (Figure 1A), the activity of $\sigma^S{}_{db}$ was determined at the genome level. Transcript levels of the wild-type strain and the $rpoS_{db}$ and Δ*rpoS* 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 Δ*rpoS* strains (12). Six hundred and seven genes were differentially expressed in the wild-type strain and the Δ*rpoS* mutant with a high probability ($P < 0.001$), of which 145 were repressed by σ^S (12), including many genes also repressed by σ^S 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 Δ*rpoS* control strains, the $rpoS_{db}$ and Δ*rpoS* 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 Δ*rpoS* 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 $E\sigma^S$ (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 $E\sigma^S$ 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 $E\sigma^S{}_{db}$ (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 Δ*rpoS* 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 Δ*rpoS* and wild-type strains (12) (Table 1), whereas expression of a transcriptional *ynfM-lacZ* fusion was downregulated in the Δ*rpo*S and $rpoS_{db}$ strains, compared to the wild-type strain (Supplementary Figure S4A). Complementation of the Δ*rpo*S 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 Eσ efficiently but display a low rate of transcription initiation, so that they are occupied by Eσ most of the time and are

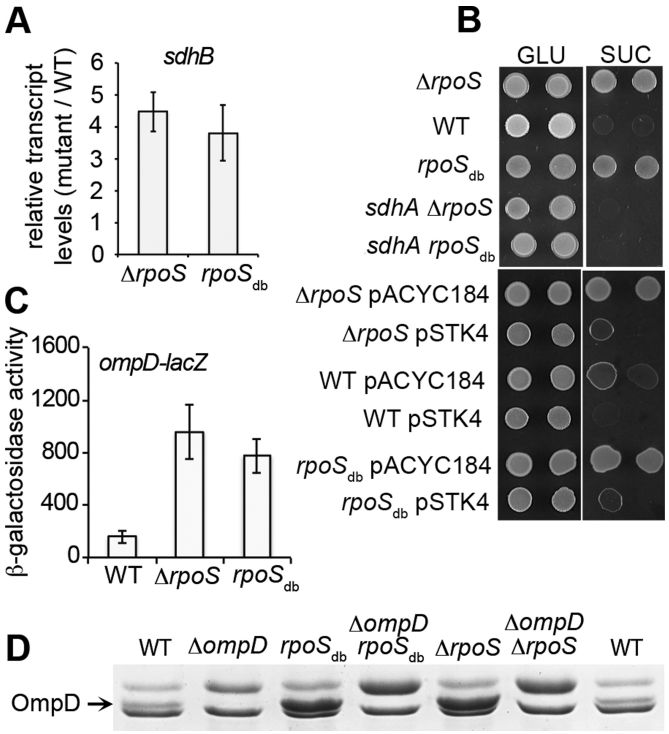

Figure 4. Effects of the Δ*rpoS and rpoS*$_{db}$ mutations on *sdh* and *ompD* expression. (**A**) *sdhB* transcript levels using quantitative real-time PCR in the Δ*rpoS* and *rpoS*$_{db}$ mutants, VF9356 and VF9849, respectively, were compared to that in the wild-type strain VF7969. Strains were grown 18 h in LB at 37°C. Three biological replicates were used. Results shown are the mean and standard deviation. (**B**) The wild-type (WT) and *rpoS* strains carrying the mutations and/or plasmid indicated were spotted on minimal medium with succinate (SUC) and glucose (GLU) as the sole carbon source (5 μl of cultures diluted to OD600 of 1.0 and 0.05 were spotted). The *rpoS* mutations were complemented by the *rpoS* gene on pSTK4 but not by the empty vector pACYC184. (**C**) *ompD-lacZ* expression in the *rpoS* mutants compared to the wild-type strain. Strains were grown 18 h in LB at 37°C. Results shown are the mean and standard deviation of at least three independent experiments. (**D**) Detection of the OmpD porin in membranes from the wild-type and *rpoS* strains carrying the indicated mutations (Supplementary Table S1) and grown for 18 h in LB at 37°C.

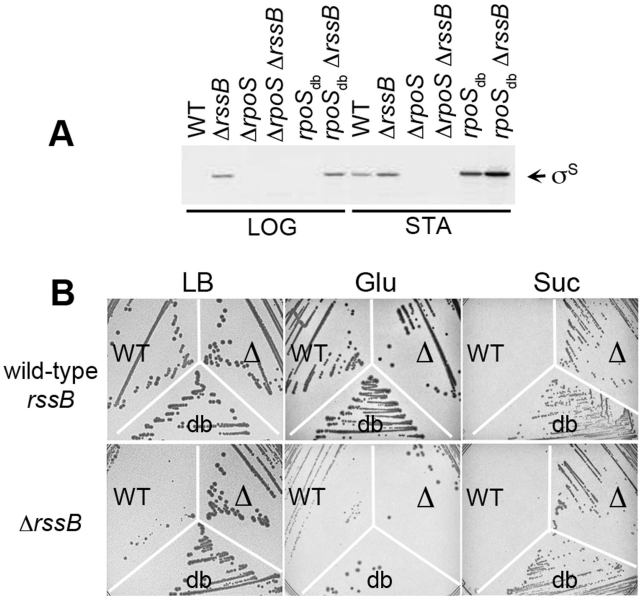

Figure 5. Effect of a Δ*rssB* mutation on σ^S expression and *Salmonella* growth. (**A**) Immunodetection of σ^S and σ^S_{db} in exponential (LOG, OD600 of 0.3) and stationary phase (STA, OD600 of 4.5) LB cultures of the wild-type (WT) strain VF7969 and its *rpoS* mutants VF9356 and VF9849, carrying wild-type and Δ*rssB* alleles. (**B**) Growth, on LB and minimal medium with succinate (Suc) and glucose (Glu) as a carbon source at 37°C, of the wild-type and Δ*rssB* strains carrying a wild-type (WT) or a mutated *rpoS* allele (Δ, deletion of *rpoS*; db, *rpoS*$_{db}$).

weakly affected by σ competition. It is also possible that, in stationary phase under physiological conditions (as opposed to conditions where a σ factor is over-expressed), σ 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-σ, 6SRNA, ppGpp and metabolites) also alter σ competition by sequestering σ and/or modulating Eσ formation (2,52). Variations in the efficiency of promoter escape and transcript elongation in stationary phase may also alter the distribution of E, σ and Eσ and thus σ competition. To determine whether elimination of σ^S favors the formation of $E\sigma^{70}$ in the conditions of our study, levels of σ^{70} bound to RNAP were compared in the wild-type strain and the Δ*rpoS* and *rpoS*$_{db}$ mutants, by size exclusion chromatography of crude extracts and immuno-detection of σ^{70} and the β′ subunit of RNAP. The percentage of total σ^{70} co-eluting with the β′ subunit of core RNAP was 94–98% and similar for the three strains, suggesting that most σ^{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 σ competition for E model during stationary phase. Nevertheless, these data reinforced our conclusion that the extensive gene repression by σ^S in stationary phase (Supplementary data set S1 and Figure 6) (12) does not rely on the regulatory model depicted in Figure 1A.

σ^S and σ^{70} factor antagonism at the *sdh* promoter

The *sdhCDAB* 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 σ^S 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 *sdh* expression by σ^S (according to the regulatory model in Figure 1B). However, *sdhB* transcript levels were similar in the wild-type strain and the *ryhB*1/2 strain containing mutations in both *ryhB* genes (Supplementary Table S1) and were increased to similar levels in the Δ*rpoS* and Δ*rpoS ryhB*1/2 strains, compared to that in wild-type (Figure 8A).

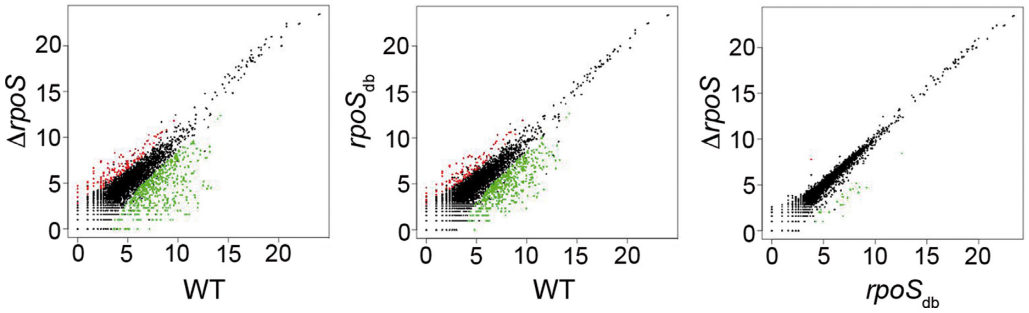

Figure 6. Transcriptome profile of wild-type and *rpoS Salmonella* strains. Scatterplot was used to compare gene expression (log_2-transformed normalized read counts) in the $rpoS_{db}$ mutant and the wild-type (WT) and Δ*rpoS* strains grown to stationary phase in LB. Transcriptome profiles of the wild-type 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.

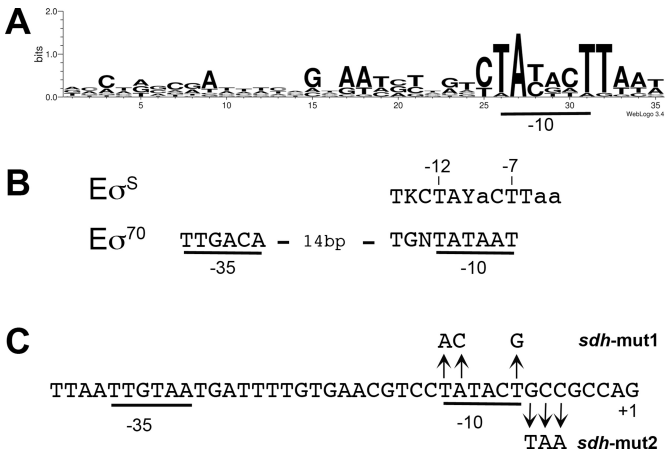

Figure 7. Promoter sequences and features. (**A**) Sequence logo generated with promoter sequences of genes in Table 1 (Supplementary Figure S3) and the WebLogo application (http://weblogo.threeplusone.com/create.cgi). (**B**) Possible consensus sequences for the -10 element of promoters preferentially recognized and transcribed by $E\sigma^{70}$ and $E\sigma^{S}$ (7,53). The -35 sequence of $E\sigma^{70}$-dependent promoters is also indicated. The -35 element is less conserved in $E\sigma^{S}$-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 (P_{sdh}) region and base substitutions generated in the chromosome of *Salmonella* to yield *sdh*-mut1 and *sdh*-mut2.

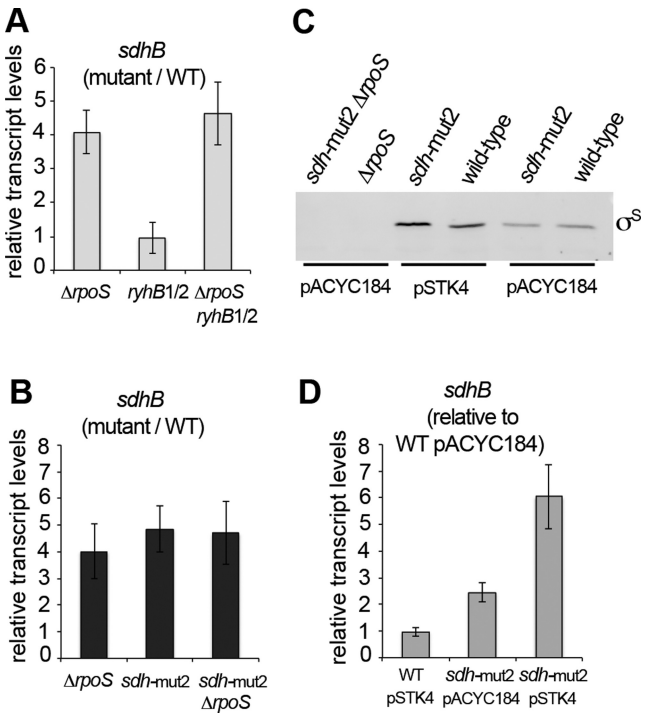

Figure 8. Role of the RyhBs sRNAs and discriminator region of P_{sdh} in *sdhB* gene repression by $E\sigma^{S}$. (**A**) *sdhB* transcript levels in the Δ*rpoS*, *ryhB*1/2 and Δ*rpoS ryhB*1/2 strains (VF8158, VFB867 and VFB868, respectively; Supplementary Table S1), relative to *sdhB* transcript levels in the wild-type strain ATCC14028. (**B**) *sdhB* transcript levels in the Δ*rpoS, sdh*-mut2 and Δ*rpoS sdh*-mut2 strains (VF8158, VFD984 and VFD987, respectively; Supplementary Table S1), relative to *sdhB* transcript levels in the wild-type strain ATCC14028. (**C**) Immunodetection of σ^{S} in stationary phase LB cultures of wild-type and mutant strains carrying the vector pACYC184 and *rpoS* on pSTK4. (**D**) *sdhB* transcript levels in strains containing the plasmid vector pACYC184 or the *rpoS* gene on pSTK4, relative to *sdhB* transcript levels in the wild-type strain containing pACYC184. Three biological replicates were used for qRT-PCR experiments in panels (A), (B) and (D), and results shown are the mean and standard deviation.

These data suggested the existence of a RyhB-independent mechanism of negative regulation of *sdh* by σ^{S}.

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 (P_{sdh}) at positions -12T, -11A and -7T that are of paramount importance for promoter recognition by both $E\sigma^{70}$ and $E\sigma^{S}$ (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 P_{sdh}. *In vitro*, $E\sigma^{70}$ and $E\sigma^{S}$ were both able to bind the *sdh* promoter region (data not shown). This finding was not unexpected since (i) the DNA sequence specificity of σ^{S} is very similar to that of σ^{70} and many promoters are bound *in vitro* by both holoenzymes (5,7,53), and (ii) the −10 sequence of P_{sdh} is identical to that of promoters preferentially recognized by $E\sigma^{S}$ and $E\sigma^{70}$ (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 $E\sigma^{S}$ or $E\sigma^{70}$ (5,7,53). In-

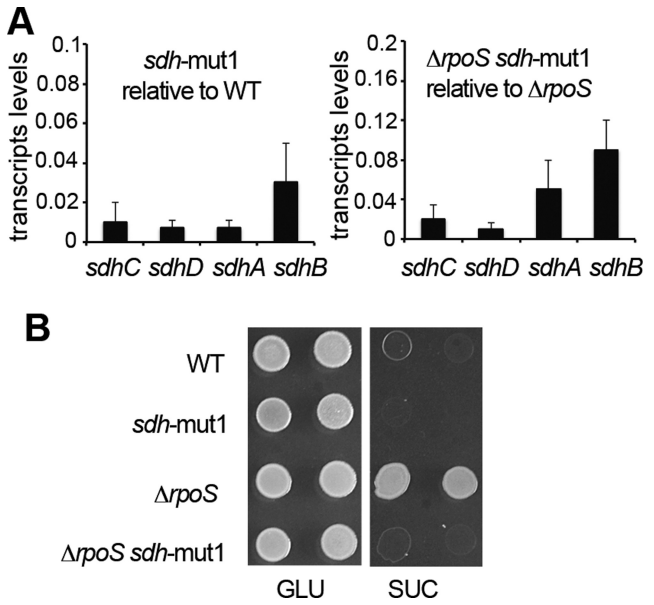

Figure 9. Mutational analysis of the -10 P_{sdh} promoter element. (**A**) Relative transcript levels of *sdhC, sdhD, sdhA* and *sdhB* in wild-type (left) and Δ*rpoS* (right) strains carrying the *sdh*-mut1 mutations (Figure 7C and Supplementary Table S1). Three biological replicates were used and results shown are the mean and standard deviation. *sdh* transcripts levels were reduced at least 10-fold by the *sdh*-mut1 mutations and were close to the background level of detection, leading to variations in the values. (**B**) Effect of *sdh*-mut1 on the ability of the wild-type strain and Δ*rpoS* mutant to grow at the expense of glucose (GLU) and succinate (SUC) as a sole carbon source (5 μl of cultures diluted to OD600 of 1.0 and 0.05 were spotted).

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

A striking feature of P_{sdh} 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 $E\sigma^{S}$-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 $rpoS_{db}$ mutant (Figure 7A), and may favor residual activity of σ^{S}_{db}. When the GCC sequence in P_{sdh} 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 Δ*rpoS* strain (Figure 8B), indicating a σ^{S}-specific effect of the TAA motif. These data suggested that *sdh*-mut2 mutations eliminate the negative effects of σ^{S} on *sdhB* transcription (Figure 8B) and are consistent with a model in which $E\sigma^{S}$ binding to P_{sdh} poises the promoter due to an unfavorable discriminator region and disrupts normal transcription by competing with $E\sigma^{70}$ binding.

Altogether, the results suggest that σ competition at the promoter DNA plays an important role in gene repression by σ^{S} and open new field of investigation regarding the role of the promoter discriminator region in $E\sigma^{S}$ activity. 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 sigma 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^{S}-dependent transcription in concert with the -10 hexamer (7,53,66), its exact role in $E\sigma^{S}$-transcription is unknown. In addition, residues of σ^{70} interacting with the discriminator (57) are not all conserved in σ^{S} (Supplementary Figure S1B) raising the possibility that $E\sigma^{S}$ and $E\sigma^{70}$ use different discriminator sequences. The nature of the P_{sdh} discriminator may influence different steps in $E\sigma^{S}$-dependent transcription, such as positioning of $E\sigma^{S}$ on the promoter DNA, formation/stability of the open complex and/or promoter clearance. As $E\sigma^{S}$ binds duplex (unmelted) DNA promoters more weakly than $E\sigma^{70}$, AT-rich discriminator sequences may well optimize promoter melting near the transcription site (7,53,55). When the level of σ^{S} 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^{S} (i) represses transcription from the wild-type P_{sdh} promoter, but not from the *sdh*-mut2 promoter (Figure 8B), and (ii) activates transcription from the *sdh*-mut2 promoter, but not from the wild-type P_{sdh} promoter, at least when it is overproduced (Figure 8 CD). It is thus possible that the *sdh* promoter is poised by $E\sigma^{S}$ engaged and having difficulties to escape and that *sdh*-mut2 allows to some extent poised $E\sigma^{S}$ to escape into elongation mode.

The discriminator sequence has been implicated in the regulation of $E\sigma^{70}$-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^{S} 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^{S}, since ppGpp has positive effects on *rpoS* transcription, σ^{S} stability and the formation/performance of $E\sigma^{S}$ (2,4,20). Changing the GC-rich discriminator to a TA-rich one in *sdh*-mut2 had no significant effect on *sdhB* transcript levels in the absence of σ^{S} (Figure 8B), suggesting that this modification had no major impact on $E\sigma^{70}$ transcription in these conditions. It remains to be determined how it affects $E\sigma^{S}$ transcription and whether ppGpp is involved.

Although additional experiments are required to highlight the mechanistic implication of the $E\sigma^{S}$-discriminator interaction in $E\sigma^{S}$-dependent transcription and how *sdh*-mut2 might influence transcriptional outputs at P_{sdh}, our

data suggest that negative regulation of *sdh* transcription by σ^S is mediated by competition between σ^S and σ^{70} at the DNA rather than E level, a finding consistent with data from experiments using $\sigma^S{}_{db}$. It must be emphasized that the presence of a perfect −10 σ^S-consensus followed by GC-rich region is not a general characteristic of promoters of genes negatively controlled by σ^S (data not shown) and the repression mechanism used by Eσ^S likely adapts to promoter characteristics. Also, we cannot exclude a role for additional factors helping Eσ^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 σ^S by showing that, under physiological conditions in stationary phase, gene repression requires σ^S binding to DNA. Furthermore, we suggest that Eσ^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 process: direct effects of Eσ^S at the promoter DNA level, as shown in the present study (Figure 1C), and the action of Eσ^S-dependent negative effectors (Figure 1B).

Direct negative regulation of transcription by σ factors (Figure 1C) is not well documented and the possibility that Eσ^S blocks active gene transcription as a repressor calls for further investigation. Our data suggest that Eσ^S occludes the *sdh* promoter and this lowers transcription by competition with Eσ^{70} binding. Other genes downregulated by σ^S are transcribed by more than one promoter and Eσ^S binding at one promoter or pausing might sterically hinder binding of Eσ^{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 Eσ^{70} and Eσ^S (36,37), σ 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 σ^S might endow σ^S with repressor functions: the involvement of σ^S-dependent effectors (Figure 1B). Future experiments will assess whether some of the σ^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 σ^S-dependent transcriptional and post-transcriptional control mechanisms are combined to allow for dynamic and flexible regulatory patterns and additional signal inputs. The RyhB sRNAs are another possible tool for σ^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 *ryhB* mutations on *sdhB* transcript levels (Figure 8A). Experiments are underway to determine how the different regulatory components of the σ^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 number 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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