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Genetic Evidence for a Novel Interaction Between Transcriptional Activator SoxS and Region 4 of the σ^{70} Subunit of RNA Polymerase at Class II SoxS-dependent Promoters in *Escherichia coli*

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Abstract

Escherichia coli SoxS activates transcription of the genes of the *soxRS* regulon, which provide the cell's defense against oxidative stress. In response to this stress, SoxS is synthesized *de novo*. Because the DNA binding site of SoxS is highly degenerate, SoxS efficiently activates transcription by the mechanism of prerecruitment. In prerecruitment, newly synthesized SoxS first forms binary complexes with RNA polymerase. These complexes then scan the chromosome for class I and class II SoxS-dependent promoters, using the specific DNA-recognition properties of SoxS and σ^{70} to distinguish SoxS-dependent promoters from the vast excess of sequence-equivalent soxboxes that do not reside in promoters. Previously, we determined that SoxS interacts with RNA polymerase in two ways, by making protein-protein interactions with the DNA-binding determinant of the α subunit and by interacting with σ^{70} region 4 (σ^{70} R4) both “on-DNA” and “off-DNA”. Here, we address the question of how SoxS and σ^{70} R4 co-exist at class II promoters, where the binding site for SoxS either partially or completely overlaps the -35 region of the promoter, which is usually bound by σ^{70} R4. To do so, we created a tri-alanine scanning library that covers all of σ^{70} R4. We determined that interactions between σ^{70} R4 and the DNA in the promoter's −35 region are required for activation of class I promoters, where the binding site lies upstream of the −35 hexamer, but they are not required at class II promoters. In contrast, specific three-amino acid stretches are required for activation of class I (*lac*) and class II (*galP1*) cyclic AMP receptor protein-dependent promoters. We conclude from these data that SoxS and σ^{70} R4 interact with each other in a novel way at class II SoxS-dependent promoters such that the two proteins do not accommodate one another in the −35 region but instead SoxS binding there occludes the binding of σ^{70} R4.

Keywords

SoxRS regulon; genetic epistasis; protein-DNA interactions; pre-recruitment

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Introduction

In *Escherichia coli*, transcription is carried out by a single, DNA-dependent RNA polymerase holoenzyme (RNAP) which is comprised of a σ factor that specifies transcription initiation and a core set of five proteins (α_2, β, β′, ω) that carries out transcription elongation. Although *E. coli* encodes seven σ factors, most transcription during exponential growth is dependent on the σ^{70} factor, whose binding to the core is a multistep and cooperative process[1 2]. σ^{70} cannot bind DNA by itself[3].

Members of the σ^{70} family share four regions of amino acid sequence homology (regions 1–4), which are also conserved in structure and function[4–10], Among the four σ^{70} domains, region 4 (σ^{70} R4), and in particular its 4.2 subdomain, play a predominant role in binding of the σ^{70} subunit to the −35 promoter element. Interactions between a hydrophobic pocket in σ^{70} R4 and a hydrophobic patch on the flap-tip helix of the β subunit are required to position region 4.2 properly[11]. These interactions also appear to stabilize the contacts between σ^{70} R4 and the Zn^{2+}-binding domain (ZBD) of the β' subunit, which may further promote binding of subdomain 4.2 to the −35 element[10–12]. Many transcriptional activators are known to bind to, or close to, the −35 element and then recruit RNAP through interactions with σ^{70} R4[13]. Conversely, some bacterial and bacteriophage-encoded proteins, like Rsd and AsiA, are known to repress RNAP activity by binding directly to σ^{70} R4 and other regions of σ^{70} involved in contacting the −35 element[14–19]. To date, however, no transcription factor has been reported that occludes the binding of RNAP to the −35 element during transcription activation.

SoxS, a member of the AraC/XylS family of bacterial regulatory proteins[20], is the direct transcription activator of the genes of the SoxRS regulon,[21–25], whose products provide the cell's defense against the oxidative stress imposed by redox cycling compounds[26] as well as other environmental threats such as antibiotics, heavy metals, organic solvents, bile salts and long chain fatty acids[21; 24; 27–35]. The promoters of the genes whose transcription is activated by SoxS fall into two classes[22; 36; 37] (Figure 1): the SoxS binding site of class I promoters (e.g., *fpr* and *mar*) lies 15–16 bp or 26–27 bp upstream of the −35 promoter hexamer and in the “backward” orientation whereas in class II promoters (e.g., *fumC* and *inaA*) the binding site overlaps the −35 region of the promoter and lies in the “forward” direction[36; 37].

SoxS is endowed with several features that distinguish it from most other bacterial transcription activators. Thus, SoxS functions as a monomer[38] and binds to a highly degenerate 20 bp DNA sequence[36; 37; 39–42]. Moreover, SoxS belongs to a non-canonical two-component system that functions in two stages[43; 44]. In this system, dimeric SoxR plays the role of a sensor-transmitter in that its 2Fe-2S centers are reduced during normal growth and become oxidized during oxidative stress[45–48]. This oxidation activates constitutively expressed SoxR[49], which then activates the transcription of *soxS*. The ensuing transcription of *soxS* leads to the *de novo* synthesis of SoxS, which, as the response-regulator, then activates the transcription of the genes of the SoxRS regulon.

The mechanism of SoxS-dependent transcription activation of the regulon's genes is also unusual: it occurs by pre-recruitment[50; 51], which is also called DNA-scanning[52]. In prerecruitment, newly synthesized SoxS molecules first form binary complexes with RNAP in solution and off DNA and then these complexes scan the chromosome for SoxS-dependent promoters that contain promoter elements recognized by σ^{70} and a properly positioned binding site for SoxS. The physiological advantage of this mechanism is that it enables newly synthesized molecules of SoxS to distinguish the degenerate soxboxes that

reside in SoxS-dependent promoters from the excess of sequence-equivalent, non-functional soxboxes that do not lie in promoters[50; 51].

Although the crystal structure of SoxS has not been determined, the protein has been characterized extensively at the molecular level. Thus, by subjecting SoxS to a comprehensive alanine scanning mutagenesis, the plate phenotypes of the mutants allowed the identification of two classes of mutants: some were defective in specific DNA binding while others were positive control (PC) mutants, which bound soxbox DNA normally but were defective in transcription activation of SoxS-dependent promoters[53]. Importantly, the two types of mutants could be located on a three-dimensional model of SoxS that was based on the co-crystal structure of MarA, a paralog of SoxS, bound to *mar* DNA[54]. The substitutions that confer defects in DNA binding were located within the recognition helices of the two helix-turn-helix (HTH) DNA binding motifs of SoxS[53]. Interestingly, the PC mutants located near but above the HTH motif in the N-terminal region of the protein are defective in SoxS-dependent activation of both class I and class II promoters whereas the PC mutants located near but above the HTH motif in the C-terminal region are only defective in SoxS-dependent activation of class II promoters53.

Moreover, a systematic mutagenesis of the soxbox of two class I promoters allowed the determination of the optimal binding site of SoxS, which contains: an A residue at position 1; recognition element 1 (RE1) with the sequence GCAC at positions 4–7; an A/T rich spacer at positions 8–14; recognition element 2 (RE2) with the sequence CAAA at positions 15–18; and two remaining bp with no information content but necessary for DNA binding *in vitro*[36; 41].

As an “ambidextrous” transcription activator that activates transcription of both class I and class II promoters[22], SoxS was expected to make protein-protein interactions with at least two surfaces of RNAP. Indeed, *in vivo* and *in vitro* evidence has been presented that SoxS contacts both the C-terminal domain (CTD) of the α subunit of RNAP and σ^{70} R4. Using a library of single alanine substitutions in the α-CTD from positions 255 to 329, we determined that alanine substitution of 10 amino acids reduce or enhance transcription activation of class I and/or class II promoters[55] (K.L. Griffith, T.I. Wood and R.E.W., Jr., unpublished results). Moreover, the interactions found between SoxS and the α-CTD were shown to occur in solution and in the absence of specific DNA binding, interactions required for pre-recruitment[50; 52]. These interactions were the first examples of an activator binding to this portion of the α-CTD. .

In addition, we recently identified seven single alanine substitutions of the C-terminal tail of σ^{70} R4 that reduce SoxS-dependent transcription activation of either the class II promoters *fumC* and *micF* or the non-canonical *zwf* promoter. As expected, none of the σ^{70} R4 substitutions reduced activation of the canonical class I promoter, *fpr*, because its soxbox is too far (15 bp) from σ^{70} R4 to interact with it[56]. Furthermore, we determined that SoxS and σ^{70} R4 also interact in solution in the absence of specific DNA binding and that amino acids of the class I/II surface of SoxS are required for these “off-DNA” interactions[56]. In conclusion, these experiments provide evidence that the class I/II surface of SoxS makes protein-protein contacts with domains of two different subunits of RNAP, the α-CTD and σ^{70} R4, with some interactions likely to occur off-DNA and others on-DNA and/or to be dependent on the specific promoter being activated[55; 56].

Since RE2 of the soxboxes of class II promoters (*fumC*, *inaA*, *micF*) either partially or completely overlaps the −35 hexamers, we wished to determine whether SoxS and σ^{70} can co-occupy this region of a promoter. To answer this question, we constructed a library of tri-alanine substitutions of σ^{70} R4 covering positions 531–590 and determined the effects of the

substitutions on transcription at these class II SoxS-dependent promoters. The data obtained suggest that the binding of SoxS to a soxbox that overlaps the −35 hexamer occludes the binding of σ^{70} R4 to the −35 element.

We also conducted experiments to determine whether the position and orientation of SoxS on promoter DNA are similar to those of MarA and Rob. We found that the interactions between SoxS and promoter DNA is similar to the interactions observed in the crystal structure of MarA bound to *mar* DNA[54]. Thus, by determining the specific position of SoxS on soxbox DNA, these data provide further support for the ability of SoxS to interfere with the binding of σ^{70} R4 to the −35 hexamer, as inferred from the experiments with the tri-alanine substitutions of σ^{70} R4.

Results

Effect of tri-alanine substitutions of σ^{70} R4 on transcription from SoxS-dependent promoters

Previous work has shown that SoxS and σ^{70} interact with one another and that amino acids in the distal portion of region 4.2 (residues 590–600) and the C-terminal tail (residues 591–612) are important for SoxS-dependent transcription activation of class II promoters *fumC* and *micF* and the non-canonical class I *zwf* promoter but not the class I *fpr* promoter[56]. Here, we investigated whether amino acids of σ^{70} that reside in the distal portion of region 3.2 (residues 531–540), in region 4.1 (residues 541–570) and in the N-terminal portion of region 4.2 (residues 571–590)[4; 5; 10] play a role in transcription from SoxS-dependent promoters. To accomplish this goal, we used site-directed mutagenesis of plasmid pVR- σ57 to create a tri-alanine- scanning library of σ^{70} from positions 531–590 such that each mutant σ^{70} protein would have three successive amino acid residues substituted with alanine (e.g., P531A, L532A, and D533A). The effect of the tri-alanine substitutions on transcription from the various promoters was determined by assay of β-galactosidase activity. Transcription reduced to 80% or less of wild type transcription was considered to be a significant defect, as defined previously[58; 59].

We first tested our library at three class II SoxS dependent promoters (*fumC, inaA* and *micF*), where the soxbox partially or completely overlaps the −35 hexamer (Fig. 1). Surprisingly, we found that none of the tri-alanine substitutions reduce SoxS-dependent transcription activation at any of these promoters (Table 1). Thus, we infer that the binding of SoxS to the soxbox of class II promoters prevents the binding of σ^{70} R4 to the −35 region of the promoter. The results of these experiments were startling since previous *in vitro* work with *gal*P19*T*, a class II, cyclic AMP receptor protein (CRP)-dependent promoter, indicates that the binding of CRP has no effect on the position of σ^{70} R4 upon its subsequent binding to the −35 element of the promoter[60].

Next, we determined the effect of the library on SoxS-dependent transcription activation of class I promoters *fpr* and *mar*, and on *zwf*, the non-canonical class I promoter. We also determined the effect of the library on basal transcription from these promoters. Table 1 shows that eight tri-alanine substitutions reduce SoxS-dependent transcription of these class I promoters. Of these eight substitutions, two are in region 4.1 and six are in region 4.2. None of the mutants confer a strong phenotype in that transcription was reduced by less than twofold. These substitutions also reduce SoxS-independent transcription (data not shown). We rationalize these data by arguing that since the −35 elements of class I promoters typically reside at least 15–16 bp downstream of the SoxS binding site, they are almost certain to be fully available for contact with σ^{70} R4 under both SoxS-dependent and SoxS-independent conditions. Accordingly, tri-alanine substitutions that confer a defect in the

binding of σ^{70} R4 to the respective −35 elements would be expected to reduce transcription from these promoters under the two conditions.

A few substitutions reduce transcription more under non-inducing than inducing conditions (data not shown). We do not know the basis for this effect. However, one explanation is that the 35° bend in the DNA induced by the binding of SoxS to the soxbox[38] partially compensates for the defects in transcription initiation conferred by the tri-alanine substitutions of σ^{70} R4.

Given these data showing that members of the library can reduce transcription of class I promoters wherein the respective soxboxes do not overlap the −35 element, we expected that some library members would reduce basal, SoxS-independent transcription from the class II promoters. Table 2 shows that six substitutions significantly reduce basal transcription from the *fumC* promoter and three substitutions significantly reduce basal transcription from the *inaA* promoter. Curiously, no substitutions reduce transcription from the *micF* promoter (Table 2). We do not know why none of the substitutions reduces SoxS-independent transcription at the *micF* promoter. One possible explanation is that under non-inducing conditions, the contribution to the overall binding of σ^{70} to the *micF* promoter made by the interaction of σ^{70} R4 with the −35 element is small, because the affinity of σ^{70} R4 for this particular promoter element is low, If so, then loss of contact by a mutant of σ^{70} R4 would be inconsequential.

Regardless of the problem with the *micF* promoter, these data show that members of the library can indeed interfere with functions essential to the basal, SoxS-independent transcription from the class II promoters *fumC* and *inaA*.

Protein-DNA Interactions between SoxS and the soxbox

To explore further the inference that SoxS competes with the binding of σ^{70} R4 to the −35 promoter element at class II SoxS-dependent promoters, we performed *in vivo* genetic epistasis tests[61] between SoxS mutants defective in DNA binding[53] and single base pair substitutions in the soxbox[53]. The goal of this experiment was not only to determine the orientation of SoxS when it is bound to the soxbox, but also to identify some specific contacts between amino acids of SoxS and base pairs within the soxbox so that we could accurately position SoxS on the DNA. The co-crystal structure of MarA bound to the marbox of the *mar* promoter[54] served as a critical reference for SoxS, because MarA is a paralog of SoxS. Moreover, SoxS and MarA bind DNA as monomers[38; 54], they bind to the same degenerate sites[22; 36; 37; 39; 62] and they activate transcription of the same set of genes, although to different degrees28; 63–65. Thus, the amino acid residues of SoxS that make base-specific contacts with soxbox/marbox DNA are likely to be similar, if not identical, to the amino acids of MarA that make base-specific contacts with soxbox/marbox DNA[54]. Accordingly, for our genetic epistasis tests, we selected five alanine substitutions of SoxS (W36A, Q39A, R40A, T87A and R90A) which are homologous to five of the eight amino acids of MarA (W42, Q45, R46, T93 and R96) that are predicted by the MarA/*mar* co-crystal structure to contact specific bases of marbox DNA[54].

For two reasons our epistasis tests were conducted with *fpr*, a canonical class I SoxS-dependent promoter, and the non-canonical *zwf* promoter. First, we did not use canonical class II promoters because RE2 of their soxboxes overlaps the −35 element[22]; as such, transcriptional defects arising by substitutions in that region would be difficult to interpret because they could be due to alterations in binding by SoxS or σ^{70} R4. However, the soxbox of the *zwf* promoter can serve as a surrogate of soxboxes within class II promoters because, like them, it is oriented in the forward direction. Second, we knew from our alanine

scanning mutagenesis of SoxS that the above substitutions are defective in DNA binding and transcription activation *in vivo* at these two promoters[53].

Presuming that SoxS would bind soxbox DNA like the binding of MarA to *mar* DNA in the co-crystal, we set up our genetic epistasis tests by combining each single alanine substitution of SoxS residing in the N-terminal HTH motif (W36A, Q39A and R40A) with a base pair substitution at each of the four positions in RE1 of the *fpr* and *zwf* promoters; similarly we combined each single alanine substitution of SoxS residing in the C-terminal HTH motif (T87A and R90A) with a base pair substitution at each of the four positions in RE2 of the two promoters. At each position in the REs, we chose a base pair substitution that reduces transcription activation of a given promoter by wild type SoxS to an intermediate level[41], since small or large effects are problematic in genetic epistasis tests. We then carried out the epistasis tests by determining the effects of the various combinations of mutations on transcription in each strain.

The criterion for epistasis[61] was that if the defect in transcription conferred by a double mutant is no greater than that conferred by either of the single mutations alone, the two mutations are considered to be epistatic. If two mutations are epistatic to one another, then their wild type counterparts contact each other. On the other hand, if the defect conferred by a double mutant is greater than the defect conferred by the two single mutations, the two mutations are considered to be non-epistatic[61]. If two mutations are non-epistatic, then their wild type counterparts do not contact one another.

Figures 2 and 3 show the results of the epistasis tests with the *fpr* and *zwf* promoters, respectively. With the *fpr* promoter, W36A and R40A in the putative N-terminal HTH motif of SoxS are each epistatic to a base pair in RE1 that is predicted by the co-crystal structure to be contacted by W42 and R46, the homologous amino acids of MarA[54]. Similarly, T87A and R90A in the putative C-terminal HTH motif of SoxS are each epistatic to a base pair in RE2 that is predicted by the co-crystal structure to be contacted by T93 and R96, the homologous amino acids of MarA. In addition, R40A, T87A and R90A are each epistatic to a second base pair which is not predicted from the co-crystal structure to be contacted by the homologous amino acids of MarA. These additional contacts are not surprising because the sequences of the two binding sites are different and the epistasis tests were carried out *in vivo* whereas the co-crystal structure was determined with a purified complex. Regardless, these results show that the position and orientation of SoxS bound to soxbox DNA *in vivo* are the same as those observed in the structure of the MarA/*mar* co-crystal.

Similar results were obtained with the *zwf* promoter (Fig. 3). Thus, R40A and R90A are epistatic to a base pair in RE1 and RE2, respectively; the same base pairs are predicted by the co-crystal structure to be contacted by R46 and R96, the homologous amino acids of MarA[54]. In addition, T87A is epistatic to a base pair in RE2 that is not predicted by the co-crystal structure to be contacted by T93 of MarA. Thus, the epistasis experiments conducted with the *zwf* promoter also place the N-terminal HTH motif on RE1 of a soxbox and the C-terminal HTH motif on RE2.

Effect of tri-alanine substitutions of σ^{70} R4 on CRP-dependent transcription activation of its class I and class II promoters

The surprising results of Table 1 led us to ask: “Is the absence of co-occupancy between SoxS and σ^{70} R4 at the −35 region of class II SoxS-dependent promoters a common property of bacterial transcription activators that activate class II promoters? As the system for answering this important question, we chose the best-studied transcription activator, CRP. Thus, we used the tri-alanine scanning library of σ^{70} R4 to determine whether the binding of CRP to the class II *gal* P1 promoter interferes with the binding of σ^{70} R4 to the

−35 region. We also determined the effect of the tri-alanine substitutions of σ^{70} R4 on CRP-dependent transcription from the *lac* promoter and an on CRP-independent transcription from a *lac* promoter whose CRP binding site has been replaced by an UP element, which enhances transcription by interacting with the DNA-binding determinant of the α-CTD of RNAP[66; 67].

Table 3 shows that five tri-alanine substitutions of σ^{70} R4, D570A-T572A, L573A-E575A, V576A-L578A, V582A-R584A and R588A-I590A, reduce CRP-dependent transcription from the *lac* promoter under inducing conditions. Previous work has shown that one or more amino acids within stretches V582-R584 and R588-I590 interact directly with the −35 promoter element[68] and substitutions therein reduce *lac* transcription[69] while single amino acid substitutions within the other three stretches either reduce or enhance transcription in a sequence-independent manner[69]. In addition, at the *lac* promoter and the related CC-61.5 promoter, amino acids within positions 573–604 of σ^{70} make protein-protein interactions with the 261 determinant of the α-CTD[70]. These contacts, together with the interactions between Activation Region 1 of CRP and the 287 determinant of the α-CTD, allow the α-CTD to serve as a bridge that connects RNAP bound at the *lac* promoter to CRP bound to a site centered at −61.5[70]. Regardless of whether the five tri-alanine substitutions reduce CRP-dependent transcription from the *lac* promoter by interfering with the binding of σ^{70} R4 to promoter DNA or whether they function by disrupting the bridging interactions between the α-CTD and σ^{70} R4, the results obtained with them provide evidence that our data are consistent with previously published results[69; 70]. Importantly, four of the tri-alanine substitutions (L573A-E575A, V576A-L578A, V582A-R584A, R588A-I590A) that reduce CRP activation of the *lac* promoter also reduce SoxS-dependent transcription from at least one of the class I promoters examined (compare Table 1 and Table 3). As described below (Table 4), some of these alanine substitutions confer a growth-defective phenotype.

As a control, we wanted to determine whether the tri-alanine substitutions of σ^{70} R4 that reduce CRP-dependent transcription from the *lac* promoter also reduce CRP-independent transcription from this promoter. However, the level of CRP-independent *lac* transcription in a strain carrying a *crp* deletion and growing in the presence of IPTG[71], is only about one percent of the amount of induced *lac* transcription in a wild type strain; as such, it would be difficult to determine accurately the effect of mutants of σ^{70} R4 on *lac* transcription in such a strain. Accordingly, we used strain RLG4282, wherein DNA upstream of position −37 of the *lac* promoter, which includes the CRP binding site, has been deleted and replaced by the UP element of the *rrnB*P1 promoter[72]; introduction of the UP element enhances CRP-independent transcription by ~30-fold[72]. Following the introduction of the library into strain RLG4282, we found that eight of the tri-alanine substitutions of σ^{70} R4 reduce transcription from the UP^{+}/CRP^{-} *lac* promoter to 80% or less of the amount obtained with the strain carrying the plasmid encoding wild type σ^{70} R4 (Table 3). Of these, five also reduce SoxS-dependent transcription activation of at least one class I promoter (see Table 1).

Interestingly, three of these substitutions, E555A-K557A, M561A-F563A and G564A-F566A, have no effect on CRP-dependent transcription from the wild type *lac* promoter (compare columns 1 and 2 of Table 3). Moreover, all three substitutions reside in region 4.1 of σ^{70}, the region that interacts with the hydrophobic patch of the flap-tip helix of the β subunit and thereby positions region 4.2 so that it can bind a −35 promoter element[11]. Thus, in some way, the ability of CRP to bind to its site in the *lac* promoter masks the effect of these substitutions on *lac* transcription. One possibility is that at the wild type *lac* promoter where Activation Region 1 in the promoter-proximal subunit of DNA-bound CRP interacts with the 287 determinant of the α-CTD[73], the concurrent interaction between the 261 determinant of the α-CTD and amino acids of σ^{70} R4[70] stabilizes the binding of region 4.2 to the −35 promoter element.

None of the 20 tri-alanine substitutions of σ^{70} R4 reduces SoxS-dependent transcription activation of the class II promoters *fumC*, *inaA* and *micF* (Table 1), as if the binding of the activators to its respective binding sites occludes the binding of σ^{70} R4. Thus, we very much wanted to determine whether CRP would have a similar effect on the class II CRP-dependent *gal*P1 promoter, where the CRP binding site partially overlaps the −35 promoter hexamer (Fig. 1). To do this, we introduced the library into strain DM00021, which carries a *gal*P1-*lacZ* fusion, and determined the effects of the substitutions on expression from the CRP-dependent promoter. Significantly, we found that the data obtained with CRP are completely different from those obtained with SoxS in that five tri-alanine substitutions of σ^{70} R4 (A549A-L551A, T552A-R554A, M567A-T569A, D570A-T572A, V582A-R584A) reduce transcription of the class II *gal*P1 promoter under CRP-dependent conditions (Table 3) whereas none of the library's tri-alanine substitutions reduces SoxS-dependent transcription at the class II promoters (Table 1). Thus, the data in Table 3 indicate that CRP and σ^{70} R4 can co-bind the −35 region of the class II *gal*P1 promoter. Indeed, *in vitro* Fe-BABE footprinting studies by Bown *et al*. [60] showed that CRP and σ^{70} R4 can co-exist at the −35 region of the *gal* P1 promoter.

Promoters that contain an extended −10 element, TGn, can initiate transcription without the specific binding of σ^{70} R4 to the −35 region[74–78]. Since the *gal*P1 promoter contains an extended −10 element and since none of the bases in the −35 region match those of the consensus −35 hexamer (see Fig. 1), we do not know how the five tri-alanine substitutions of σ^{70} R4 reduce CRP-dependent transcription from the *gal*P1 promoter. This leads us to point out that just because promoters with an extended −10 element do not require interactions between the −35 region and σ^{70} R4, does not mean that interactions between base pairs within the −35 region and σ^{70} R4 cannot occur and thereby enhance overall transcription[78; 79]. We also note that amino acids R554 and R584 are known to interact directly with DNA within the −35 region[68]. Thus, the absence of an interaction between the −35 region and one or more amino acids within these two tri-alanine stretches could be responsible for the defect.

Effect of the tri-alanine substitutions of σ^{70} R4 on growth

We determined the effect of the tri-alanine substitutions of σ^{70} R4 on growth by introducing the library into strain PB1*rpoD800*, which carries *rpoD800*, a temperature-sensitive allele of the gene encoding σ^{70} [80]. For this complementation analysis, we carried out two assays at the non-permissive temperature of 42°C with the set of partial diploids: growth in liquid medium; and a qualitative determination of the colony-forming units per ml when the cells were grown at 37°C, a permissive temperature[80], and plated on LB agar at 42°C and 37°C.

As expected, parental strain PB1*rpoD800* grew normally in liquid LB medium at 37°C and growth ceased within 30 min after shifting the culture to 42°C (Fig. 4); the growth lethal phenotype conferred by the *rpoD800* mutation was also observed when the parental strain was plated on LB agar at 42°C (Table 4). Also as expected, plasmid pVR-σ^{57} carrying the wild type *rpoD* gene complemented the *rpoD800* mutation during growth of the partial diploid at 42°C in liquid medium (Fig. 4) and on LB agar (Table 4).

With the set of PB1*rpoD800* strains carrying the library of tri-alanine substitutions, we carried out the test of growth in liquid medium at the non-permissive temperature and the test of the ability to form colonies on plates a 42°C. The two tests produced the same results with each partially diploid strain. Thus, plasmids carrying mutants T552A-R554A, D570A-T572A and Q579A-D581A failed to complement the *rpoD800* allele in that the growth of the respective liquid cultures ceased within 30 min when the cultures were shifted from 37°C to 42°C (Fig. 4) and the lethal phenotype of *rpoD800* was also observed when the respective diploid strains were plated at 42°C (Table 4). In addition, mutants V576A-K578A

and R588A-I590A partially complemented the *rpoD800* mutation in the two tests. Thus, each of these five tri-alanine substitutions alter or completely disrupt an essential function of σ^{70} and therefore cell growth. Indeed, amino acids R554 and R588 are known to directly contact DNA in the −35 promoter hexamer[68] and hence transcription of many housekeeping genes would be significantly reduced. Also, mutation G577S is defective in transcription from the *lac* and phage P22 *ant* promoters[69]; accordingly, transcription from many essential genes is likely to be affected by the G577A substitution. The nature of the defects in the function of σ^{70} conferred by the other two tri-alanine substitutions, D570A-T572A and Q579A-D581A, is unknown, but they may alter the proper conformation of the σ^{70} factor.

We note that four of the tri-alanine substitutions, T552A-R554A, V576A-K578A, Q579A-D581A and R588A-I590A, also reduce transcription at the class I SoxS-dependent promoters in the presence of the wild type *rpoD* gene (see Table 1). Importantly, although substitutions T552A-R554A and D570A-T572A reduce transcription from the class II, CRP-dependent *gal*P1 promoter (Table 3), none of the five tri-alanine substitutions reduce transcription of the class II SoxS-dependent promoters, *fumC*, *inaA* and *micF* (Table 1). Thus, these observations provide additional evidence that the binding of σ^{70} R4 to the −35 region of these three class II promoters is inessential for SoxS-dependent activation of their transcription, while binding to the −35 element of class I promoters is required for activation.

Location of the amino acid substitutions of σ^{70} R4 that reduce transcription on structural models of holo-RNAP and of σ^{A} bound to promoter DNA

We sought a means of understanding better the basis of the effects of the tri-alanine substitutions of σ^{70} R4 on growth and on SoxS-dependent transcription of class I promoters. Accordingly, we located the amino acids that confer these defects in growth and transcription on the crystal structures of holo-RNAP of *Thermus thermophilus*[10] and on the binary complex of *Thermus aquaticus* σ^{A} bound to DNA containing a consensus −35 element[68] (Fig. 5).

Genetic, biochemical and structural studies have identified many amino acids that play a variety of functional roles in transcription initiation, either directly or indirectly. With respect to the work presented here, we note that amino acids R541, T544 and L607 of σ^{70} R4 interact with the β-flap of RNAP[81; 82], a critical interaction that helps position σ^{70} R4 on the core RNAP so that it can make specific contacts with the −35 promoter element[11; 83]. Moreover, an interaction between the ZBD of the β' subunit of RNAP and σ^{70} R4 enhances its binding to promoter DNA, in part by stabilizing the β-flap interaction with σ^{70} R4[11]. Additional work conducted by Geszvain *et al.*[11] provided evidence that amino acid R554 of σ^{70} R4 interacts with the ZBD of the β subunit. Lastly, the co-crystal structure of σ^{70} R4 bound to a consensus −35 element indicates that amino acid residues R554, R562, L573, E574, T583, R584, E585, R586, R588 and K593 of σ^{70} R4 make direct or water-mediated contacts with the bases, the phosphate backbone and deoxyribose[68].

Most of the amino acids residues mentioned above that contact the β flap-tip helix, the ZBD of the β' subunit or the −35 hexamer have been substituted with alanine in our library of trialanine substitutions and many members have been shown in this work to alter transcription initiation, or growth, or both. For example, in region 4.1 of σ^{70}, substitution T552A-R554A reduces transcription at the *mar* promoter (Table 1) and also confers a temperature-lethal phenotype (Table 4). In addition, amino acid R554 appears to contact the ZBD of the β′ subunit[11] as well as the phosphate backbone just upstream of the −35 element[68]; the absence of any of these functions could be the basis for the defect in *mar* transcription. Similarly, substitution E555A-K557A reduces SoxS-dependent activation from the *zwf* promoter. This defect could be because these residues can no longer make

productive interactions with the core RNAP[2] or with the ZBD on the β' subunit[11]. Substitution, G564A-D566A, has a mild growth defect in the plate assay (Table 4), but no effects on SoxS-dependent transcription were observed. Although amino acid I565 is known to contact the RNAP core[2], the effect of substituting alanine for isoleucine at this position is rather small, as was the case for several other substitutions examined by Gezvain *et al*.[11]. However, substitution G564A may be responsible for the growth defect because of the change in local protein structure effected by replacing glycine, which disrupts α-helices, with alanine. Interestingly, previous work did not reveal a role in either DNA binding or in interaction with core-RNAP for the amino acids M567-T569 of σ^{70} R4. However, we found that alanine substitutions of these amino acids reduce transcription activation of *mar* (Table 1), basal transcription of *fumC* and *inaA* (Table 2) and CRP-dependent transcription from *gal*P1. In agreement, the crystal structure of holo-RNAP shows that these amino acids are close to the ZBD of the β' subunit[10], which likely accounts for their effects on transcription.

Tri-alanine substitutions in region 4.2 that caused a defect in transcription activation, growth, or both included D570A-T572A, L573A-E575, V576-L578A, Q579A-D581A V582-R584A and R588A-I590A. Previous studies implicate substitution D570A-T572A in reducing transcription at the *lac* promoter[69]. These amino acids are close to the core of RNAP and to promoter DNA and thus are capable of making direct protein-DNA or protein-protein interactions. Alternatively, they may stabilize the functional conformation of the surrounding amino acids so that they can make those contacts. Furthermore, Q579A-D581A conferred a temperature-lethal phenotype, presumably due to the loss of a stabilizing effect of D581 on the interaction of σ^{70} R4 with the β-flap and the −35 hexamer[81; 82]. Two other substitutions, V576AK578A and R588A-I590A, confer a mild growth defect and reduce transcription activation by SoxS. The V576-K578 patch is buried between amino acids that either contact DNA or are involved in contacting the β-flap. Thus, as with G564A in the G564A-D566A patch, the G577A substitution within the V576A-K578A patch may also disrupt the positioning of the surrounding amino acids and thereby inhibit their ability to make protein-DNA or protein-protein interactions. Also, these amino acids may directly contact DNA at SoxS-dependent promoters. Finally, studies have suggested that amino acids L573, E574 and E575 contact DNA[68; 69; 84]. In our experiments, alanine substitutions of these amino acid residues reduced transcription at class I SoxS-dependent promoters (Table 1) but not basal transcription of class II promoters (Table 2). According to the crystal structure determined by Campbell *et al*.[68] and the genetic studies of Siegele *et al*.[69], amino acid R584A should directly contact the C/G bp in the −35 hexamer of the *lac* promoter. At the *zwf* promoter, a C/G bp is located at the same position and, consistent with the crystal structure, substitutions, V582A-R584A reduce transcription from it, suggesting that the patch interacts with the −35 element.

Discussion

The construction described herein of a library of tri-alanine substitutions of σ^{70} from positions 531–590 enabled us to identify yet another novel property of SoxS: when SoxS binds to the soxbox of at least three class II promoters, which partially or completely overlaps the -35 region of the promoter (and resides in the forward orientation), σ^{70} R4 does not bind to this region of the promoter. Thus, neither substitutions of amino acids of σ^{70} R4 that are known to directly contact base pairs in the −35 region[68], nor substitutions of amino acids that are known to be involved in the proper positioning of σ^{70} R4 on core RNAP[10; 11] have any effect on SoxS-dependent transcription activation of the three studied class II promoters (Table 1). However, growth experiments showed that at least five amino acids within five different stretches subjected to tri-alanine scanning mutagenesis are essential for growth (Table 4).

We also determined the effect of the substitutions on basal, SoxS-independent transcription at the three class II promoters. Importantly, several substitutions reduce basal transcription at the *fumC* and *inaA* promoters under non-inducing conditions (Table 2). Two conclusions can be drawn from these data. First, the substitutions of σ^{70} R4 function as expected in that substitutions of amino acids of σ^{70} R4 known to be important to binding to the −35 hexamer[68] reduce transcription when the −35 element is not bound by SoxS, i.e., under SoxS-independent conditions. Second, since the substitutions of σ^{70} R4 function as expected, then the failure of any of the substitutions to reduce SoxS-dependent transcriptions strongly indicates that the binding of SoxS to the class II soxboxes occludes the binding of σ^{70} R4 to the −35 hexamer.

The genetic epistasis tests conducted *in vivo* allowed us to determine whether the binding of SoxS to the soxbox of a class II promoter can actually occlude the binding of σ^{70} R4 to base pairs of the −35 region that would otherwise be bound by σ70 R4 under non-inducing conditions. With these experiments, we determined that amino acids W36 and R40 of SoxS within its N-terminal HTH motif contact base pairs in RE1 of the *fpr* soxbox and that amino acids T87 and R90 of the C-terminal HTH motif contact base pairs in RE2. Our epistasis tests with the *zwf* soxbox produced the same results. Thus, since the DNA sequences of canonical class II promoters show that RE 2 partially or completely overlaps the −35 hexamer and that RE 1 lies upstream, these data fix the orientation of SoxS on soxbox DNA of class II promoters. Moreover, the orientation deduced from these experiments agrees with the orientation of MarA bound to the marbox of the class I *mar* promoter in the crystal structure of the complex[54], i.e., RE 1 is bound by the N-terminal HTH motif and RE 2 is bound by the C-terminal motif. Thus, these experiments support the hypothesis that the binding of SoxS to the soxbox of class II promoters prevents the co-binding of σ^{70} R4 to the −35 region.

To determine if SoxS also prevents the binding of σ^{70} R4 to class I promoters, we tested the canonical class I promoters, *fpr* and *mar*, and the non-canonical *zwf* promoter. We show that members of the library affecting either the binding of σ^{70} R4 to the −5 region and/or the positioning of σ^{70} R4 on core RNAP significantly reduce transcription from these three promoters. Thus, the failure of any of the 20 tri-alanine substitutions of σ^{70} R4 to reduce transcription from the canonical class II promoters strongly indicates that the binding of SoxS to the respective −35 regions blocks the binding of σ^{70} R4 to the region. Moreover, the data also suggest that the normal function of σ^{70} R4, i.e., binding to the −35 region of promoters and facilitating and stabilizing the binding of σ^{70} R4 to core RNAP, is not required for SoxS-dependent transcription activation of these class II promoters.

Is the interference with the binding of σ^{70} R4 to class II promoters by the binding of SoxS to the soxboxes of such promoters a general property of activators of class II promoters? To address this question, we tested the effect of the substitutions on CRP-dependent activation of the class II *gal*P1 promoter fused to *lacZ*. Importantly, and in contrast with the effect of SoxS binding to class II SoxS-dependent promoters on the binding of σ^{70} R4, six members of the library of tri-alanine substitutions significantly reduce transcription from the class II *gal*P1 promoter (Table 3). This genetic result indicating that both CRP and σ^{70} R4 can co-bind to the −35 region of the *gal*P1 promoter is consistent with the FeBABE *in vitro* cleavage experiments of Bown *et al.* [60] who showed that by an unknown mechanism CRP and σ^{70} R4 can accommodate one another in simultaneously binding to the −35 region of the promoter. Thus, the contrasting results between the effects of the binding of SoxS and CRP to their respective sites within the −35 regions of class II promoters on the binding there of σ^{70} R4 demonstrates another unusual characteristic of SoxS in transcription activation.

Is there precedence for an activator binding preferentially at or near the −35 promoter element and excluding σ^{70} R4 from the promoter? Even though there are some examples of proteins that inhibit the binding of σ^{70} R4 to its −35 element, SoxS is to our knowledge the first example of an activator that blocks the binding of σ^{70} R4 to the −35 hexamer by binding to its target binding site in class II promoters. An example of a system with some resemblance to the effect of SoxS on the function of σ^{70} R4 at class II promoters is transcription from the middle promoters of bacteriophage T4. In the T4 system, the binding of phage-encoded AsiA to σ^{70} R4 diverts the binding of host RNAP from host promoters to MotA-dependent “middle genes” of the phage[18; 19; 85; 86]. Thus, like the binding of SoxS to soxbox DNA prevents σ^{70} R4 from exerting its normal DNA binding function, the direct binding of AsiA to σ^{70} R4 has the same effect. The difference between the two systems is that SoxS appears to exert its inhibitory function by physically blocking the binding of σ^{70} R4 to a specific set of promoters whereas AsiA functions by binding to and remodeling σ^{70} R4 such that it cannot bind efficiently to promoters lacking DNA-bound MotA.

Another system that somewhat resembles that of SoxS involves the effect of transcription activator Spo0A on the precise positioning of region 4 of the sigma A (σ^{A} R4) subunit of *Bacillus subtilis* RNAP on the −35 region of the *spoIIG* promoter. With RNAP containing FeBABE covalently bound to the amino acid of σ^{A} that is homologous to R588 of σ^{70} R4, Kumar *et al.* [87] found that in the absence of Spo0A, σ^{A}-containing holo-RNAP forms a closed complex that leads to the cleavage of DNA at a consensus −35 sequence of the *spoIIG* promoter whose 3' end is 22 bp upstream of the −10 hexamer. However, when cleavage was carried out after the introduction of Spo0A to the *in vitro* reaction mixture, a primary cleavage site was 4 bp downstream of the site obtained in the absence of the activator. With σ^{A} R4 now residing only 18 bp upstream of the −10 element, σ^{A} is better able to interact with the −10 hexamer and thereby form a stable closed complex that can subsequently lead to open complex formation[87]. Thus, in this system, the binding of the activator repositions σ^{A} so that transcription initiation can proceed. In contrast, the binding of SoxS to a soxbox at class II promoters prevents the binding of σ^{70} R4 to the DNA, while protein-protein interactions between amino acids of the two positive control surfaces of SoxS and the distal end of region 4.2 of σ^{70} anchor the C-terminal region of σ^{70} in a position that allows region 2.4 to bind the −10 hexamer and form the open complex that leads to transcription initiation (Fig. 6).

In conclusion, we can now ask whether the above described interaction between SoxS and the C-terminal region of σ^{70} form a binary complex that lands on class II promoters during prerecruitment. Alternatively, a different binary complex might find a class II promoter and then the complex would rearrange to the above-described complex between a positive control surface(s) of SoxS and the distal end of region 4.2 of σ^{70}. FeBABE cleavage experiments would help determine whether the binding of SoxS to the soxbox of a class II promoter prevents the binding of σ^{70} R4 to the −35 region, while *in vitro* transcription experiments[22] using SoxS positive control mutants[53] and cross-linking experiments may help determine whether either of the above two models is correct.

Methods and Materials

Bacterial strains, plasmids, and tri-alanine scanning mutagenesis

Table 5 lists the *Escherichia coli* strains used in this study and Table S1 lists the plasmids carrying members of the library of tri-alanine substitutions of σ^{70} R4 as well as other plasmids used in this study. The tri-alanine substitutions of σ^{70} R4 from positions 531–590 were introduced into the *rpoD* gene of plasmid pVR-σ^{57} by QuikChange site-directed mutagenesis (Stratagene) using primer pairs whose sequences are given in Table S2. The DNA sequences of the mutants were confirmed by the UMBC core sequencing facility using

sense and antisense sequencing primers 5`-GTTGGCAAGCTTTTA-3' and 5`-GCTTTTAATCGTCCA-3', respectively. Each member of the library of tri-alanine substitutions of σ^{70} R4 was transformed by electroporation into derivatives of strain N7840 [pBAD33-his$_6$-SoxS] carrying transcriptional fusions of *lacZ* to the *fpr*, *mar*, *zwf*, *fumC*, *micF*, and *inaA* promoters. The chromosome of N7840 carries the wild type allele of *rpoD* and thus constitutively expresses wild type σ^{70}. Members of the library of tri-alanine substitutions are expressed constitutively from a truncated *gal*P1 promoter carried on pVR-σ. The library was also introduced into strains W3110, DM0021 and RLG4282.

Assay of β-galactosidase activity in strains carrying a promoter-*lacZ* transcriptional fusion, a member of the pVR-σ^{70} library of tri-alanine substitutions and in some cases a plasmid expressing his$_6$-SoxS

Cultures of the set of derivatives of strain N7840 [pBAD33-his$_6$-SoxS] each carrying a member of the library of tri-alanine substitutions and one of the six SoxS-dependent promoters fused to *lacZ* were grown overnight in LB medium containing chloramphenicol (25 μ-g/ml) and ampicillin (100 mg/ml) at 37°C, diluted 1:100 in the same medium and grown at 37°C to a density at A_{600} of 0.1–0.2, at which point 0.2% arabinose was added to induce SoxS expression from the P_{BAD} promoter of the plasmid. After growth under inducing conditions for 1 hr, expression of the transcriptional fusions in each strain was determined by duplicate assays of the β-galactosidase activity within duplicate samples taken from duplicate cultures using the high-throughput method of Griffith and Wolf[88]. Three independent experiments were carried out. The averages were determined and the standard errors of the mean were calculated. As described in the text, average values of β-galactosidase activity produced from a strain expressing a mutant of σ^{70} R4 that were ≤ 80% of the wild type activity were taken as being significantly different from the strain expressing wild type σ^{70}; these values are shown in bold and are highlighted in the respective tables. In addition, the experiments determining the effect of the library on expression from the *lac* promoter (strain W3110), the *gal*P1 promoter (strain DM0021) and the UP^+ CRP-*lac* promoter (RLG 4282) were carried out as above, except that the cultures of W3110 and DM0021were induced with 1 mM IPTG or 0.2% D-galactose, respectively, when the A_{600} of the cultures reached ~0.2; cultures of strain RLG4282 were untreated.

Genetic Epistasis Tests

The epistasis experiments to define specific *in vivo* interactions between amino acids of SoxS and base pairs within the *fpr* and *zwf* soxboxes were conducted in strain GC4468 (Δ *lac*) carrying two compatible plasmids. One was pBAD33-his$_6$-SoxS encoding wild type his$_6$-SoxS or alanine substitutions of it, with expression of his$_6$-SoxS being under the control of the arabinose-inducible P_{BAD} promoter[55; 88]. The second plasmid was either pfpr-6 carrying the *fpr* promoter fused to *lacZ* with wild type[22] or mutant[41] soxboxes or pZ5 carrying the *zwf* promoter fused to *lacZ* with wild type[40] or mutant[41] soxboxes. The pBAD33-his$_6$-SoxS plasmids carrying the single alanine substitutions of SoxS were prepared by digesting pBAD18-his$_6$-SoxS carrying the respective substitutions[53] with HindIII and XbaI, purifying the resulting SoxS-containing fragments and cloning them into pBAD33 digested with the same two enzymes. The plasmids are listed in Table S1.

The pairs of compatible plasmids were introduced into strain N7840 by electroporation and grown at 37°C in LB broth containing ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml) to A_{600} of ~0.2, at which point 0.2% arabinose was added to induce SoxS synthesis. After incubation for 1 hr, samples were taken and assayed for β-galactosidase activity as described above.

The following plasmid combinations were tested for epistasis: (i) plasmid pZ5 carrying a *zwf-lac* fusion with the wild type, G4A and C7T alleles of the *zwf* soxbox together with plasmid pBAD33-his$_6$-SoxS carrying the wild type, W36A, Q39A and R40A alleles of SoxS; (ii) plasmid pZ5 carrying a *zwf-lac* fusion with the wild type, C15T, A16C, A17C, A18T and A18C alleles of the *zwf* soxbox together with plasmid pBAD33-his$_6$-SoxS carrying the wild type, T87A and R90A alleles of SoxS; plasmid pfpr6 carrying an *fpr-lac* fusion carrying the wild type, G4A, T6C and C7T alleles of the *fpr* soxbox together with plasmid pBAD33-his$_6$-SoxS carrying the wild type, W36A, Q39A and R40A alleles of SoxS; and (iv) plasmid pfpr-6 carrying the wild type, C15T, A17C, A18C and A18T and A18G alleles of SoxS together with plasmid pBAD33-his$_6$-SoxS carrying the wild type, T87A and R90A alleles. The cells were grown, SoxS synthesis was induced and samples were taken and assayed for β-galactosidase activity as described above.

Assays of overexpression toxicity on plates and measurement of growth in liquid medium

To determine whether any of the tri-alanine substitutions in the library of substitutions of σ^{70} R4 confer a general defect on growth, we transformed the plasmids into strain PB1*rpoD800*^{80} (Table 5) whose *rpoD800* allele produces an in-frame deletion within σ^{70} that renders the cell temperature-sensitive for growth at 42°C; we then carried out two growth tests on each partially diploid strain. For the assay of toxicity upon overexpression of the members of the library of trialanine substitutions of σ^{70} R4, overnight cultures of each partially diploid strain grown in LB medium containing ampicillin (100 μg/ml) at the permissive temperature of 37°C were subjected to six serial tenfold dilutions. Then, using a multi-channel pipettor, 10μl of each dilution of a given culture was spotted onto two LB agar plates containing ampicillin (100μg/ml). One plate was incubated overnight at 37°C and the other at 42°C. This procedure was carried out for the strains carrying each member of the library. The results were recorded with a digital camera. For determining the effect of the substitutions on growth in liquid medium, the overnight cultures were diluted 1:100 into the same medium and incubated at 37°C. Initially, the A_{600} value for each culture was determined every 30 min but when the A_{600} reached a value of 0.1–0.2, the cultures were divided into two portions. One culture was incubated at 37°C and the other at 42°C, with measurements of the A_{600} values being taken every 20 min. The data were collected and the growth curves for each substitution at the permissive and non-permissive temperatures were plotted semi-logarithmically on the same graph.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

RNAP	RNA polymerase
σ^{70} R4	region 4 of σ^{70}
CTD	C-terminal domain

NTD	N-terminal domain
HTH	helix-turn-helix
RE	Recognition Element
CRP	cyclic AMP receptor protein
ZBD	zinc binding domain

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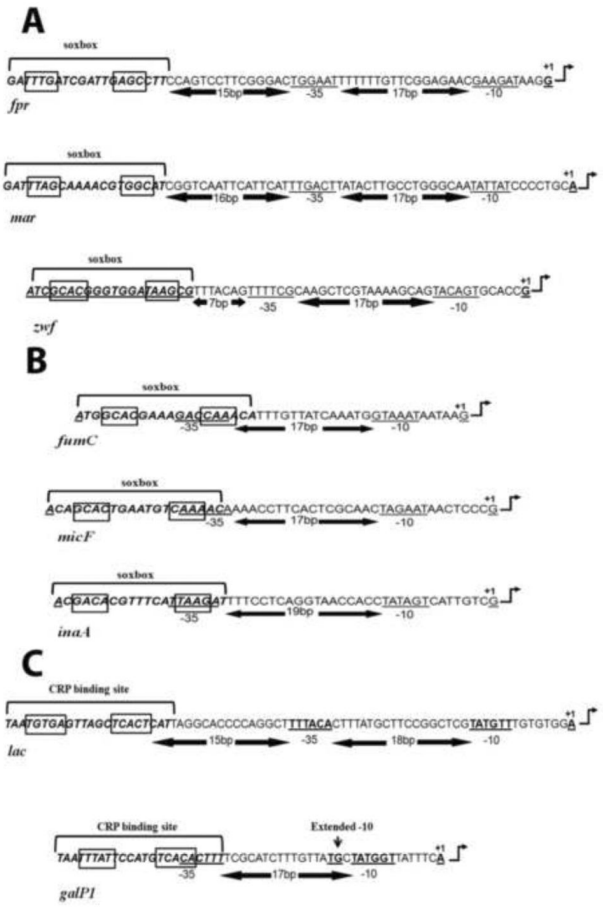


Figure 1.

DNA sequences of the SoxS-dependent and CRP-dependent promoters used in this study and location of their activator binding sites and promoter elements. The sequence of the non-template strand of each promoter is shown. The −10 hexamer of each promoter is underlined and in bold. The −35 hexamers of the canonical SoxS-dependent class I promoters *fpr* and *mar* are underlined, as is the −35 hexamer of the CRP-dependent class I *lac* promoter. The −35 regions of the *zwf* promoter, the class II SoxS-dependent promoters *fumC*, *micF* and *inaA* and the class II CRP-dependent *gal*P1 promoter are also underlined and in bold. The number of bp between the two promoter elements is given between opposing arrows, as is the number of bp between the – 35 element and the activator binding site of the *fpr*, *mar*, *zwf* and *lac* promoters. The binding sites for SoxS and CRP are enclosed within brackets. (A) The sequences of the canonical SoxS-dependent class I promoters *fpr* and *mar* and the non-canonical class I *zwf* promoter. The binding sites of SoxS of both class I and class II promoters contain two conserved recognition elements, RE1 and RE2, which are enclosed within boxes and an "invariant A" at position 1, which is not enclosed[41]. Note, then, that position 1 of the soxbox of canonical class I promoters is at the promoter-proximal end of the binding site, with RE1 lying four bp upstream. (B) The sequences of the canonical class II SoxS-dependent promoters *fumC*, *micF* and *inaA*. Here, the invariant A at position 1 of these class II promoters is at the promoter-distal end of the soxbox, with RE1 lying four bp downstream. (C) The sequences of a class I (*lac*) and a class II (*gal*P1) CRP-dependent promoter. The two most conserved elements of the CRP binding sites are enclosed within boxes.

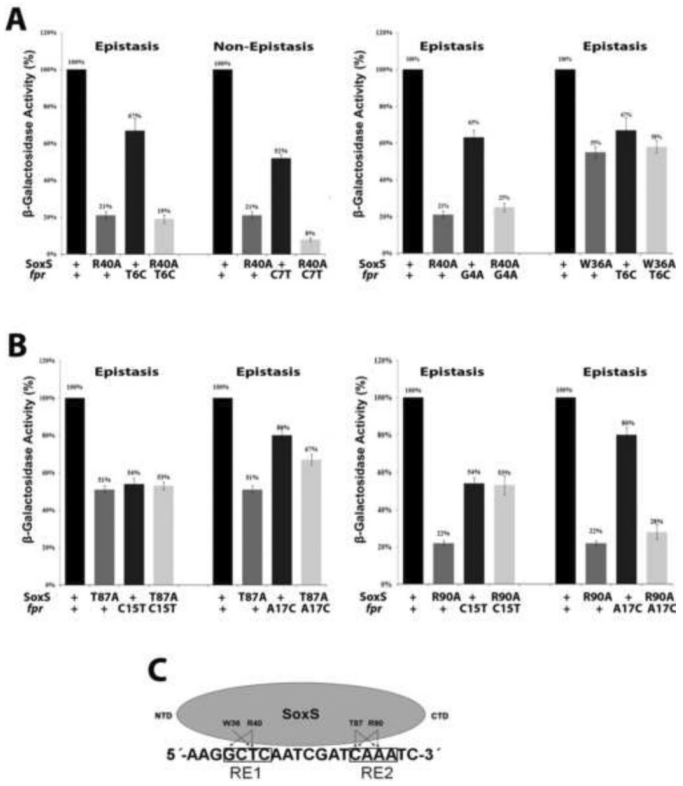


Figure 2.

Genetic epistasis experiments between single base pair substitutions of the soxbox of the *fpr* promoter and DNA binding mutants of SoxS. The sequence of the template strand of the *fpr* soxbox is shown in panel C; the invariant A of the soxbox is at the 5' end of the sequence. (A) (Left) Epistatic and non-epistatic interactions between the R40A substitution in the N-terminal HTH motif of SoxS and base pair substitutions T6C and C7T of RE 1, respectively. (Right) Epistatic interactions between the W36A and R40A substitutions in the N-terminal HTH motif of SoxS and substitutions T6C and G4A of RE 1, respectively. (B) (Left) Epistatic interactions between the T87A substitution in the C-terminal HTH motif of SoxS and base pair substitutions C15T and A17C of RE 2. (Right) Epistatic interactions between the R90A substitution in the C-terminal HTH motif of SoxS and substitutions C15T and A17C of RE 2. (C). A schematic representation of the interactions between amino acid residues of SoxS and nucleotide bases within the *fpr* soxbox, as determined by the genetic epistasis tests shown in (A) and (B). The epistasis data show that amino acids of the N- and C-terminal HTH motifs of SoxS contact one or two base pairs within RE 1 and RE 2, respectively, but whether the contacted base is on the template or non-template strand cannot be determined by these tests.

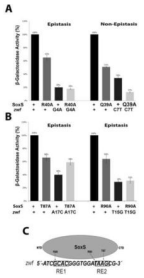


Figure 3.

Genetic epistasis experiments between single base pair substitutions of the soxbox of the *zwf* promoter and DNA binding mutants of SoxS. The sequence of the non-template strand of the *zwf* soxbox is shown in panel C; the invariant A of the soxbox is at the 5' end of the sequence. (A) (Left) Epistatic interaction between the R40A substitution in the N-terminal HTH motif of SoxS and base pair substitution G4A of RE 1. (Right) Non-epistatic interaction between the Q39A substitution in the N-terminal HTH motif of SoxS and substitution C7T of RE 1. (B) (Left) Epistatic interaction between the T87A substitution in the C-terminal HTH motif of SoxS and base pair substitution A17C of RE 1. (Right) Epistatic interaction between the R90A substitution of the C-terminal HTH motif of SoxS and substitution T15G of RE 2. (C) A schematic representation of the interactions between amino acid residues of SoxS and nucleotide bases within the *zwf* soxbox, as determined by the genetic epistasis tests shown in (A) and (B). The epistasis data show that amino acids of the N- and C-terminal HTH motifs of SoxS each contact one base pair within RE 1 and RE 2, respectively, but whether the contacted base is on the template or non-template strand cannot be determined by these tests.

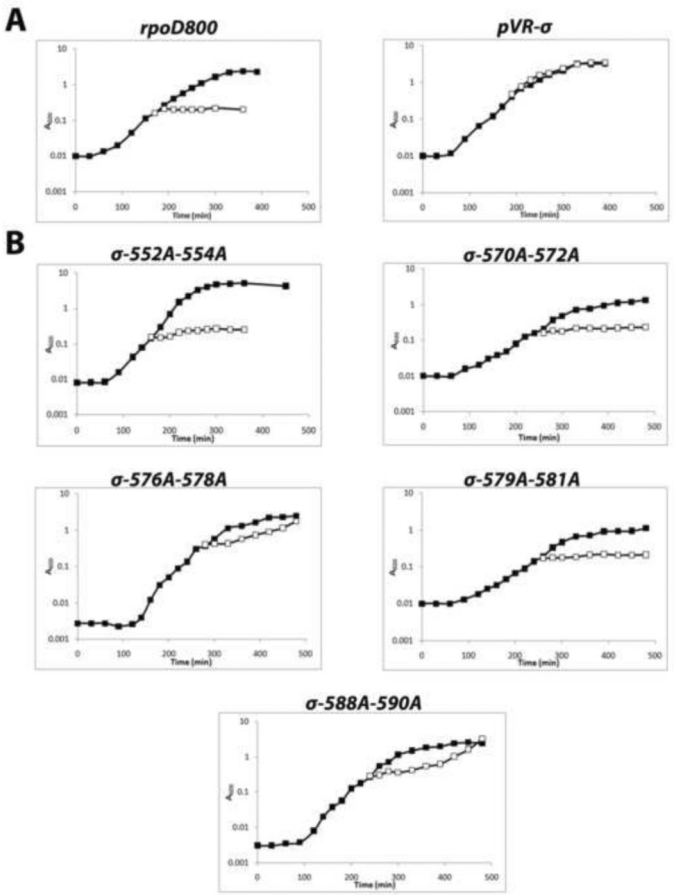


Figure 4.

Identification of members of the library of tri-alanine substitutions of σ^{70} R4 carried on plasmid pVR-σ that fail to complement the temperature-sensitive *rpoD800* mutation in strain PB1*rpoD800* during growth at 42°C, the non-permissive temperature. (A) (Left) Demonstration of the growth-lethal effect of the *rpoD800* mutation of strain PB1*rpoD800* upon a shift from growth at the permissive temperature of 37°C (clear square) to 42°C (black square). (Right) Demonstration of the ability of plasmid pVR-σ carrying the wild type *rpoD* allele to complement the growth-lethal effect of the chromosomal *rpoD800* mutation during growth at 42°C. (B)Growth curves demonstrating the failure of five members of the library of tri-alanine substitutions of σ^{70} R4 to complement the growth-lethal effect of the *rpoD800* mutation during growth at 42°C. Note that the growth curves of the partial diploids carrying the other 15 members of the library looked like that of PB1*rpoD800*[pVR-σ], i.e., full complementation (data not shown).

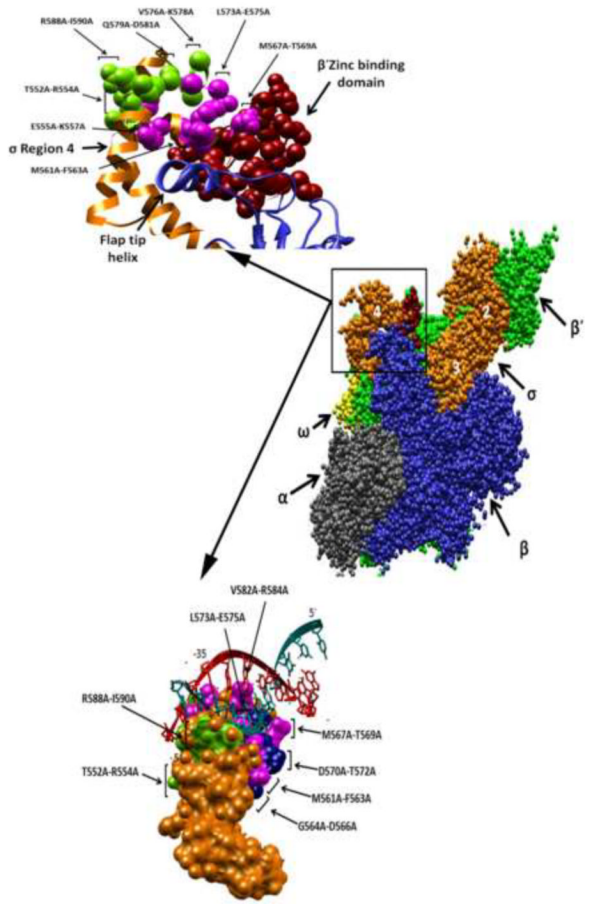


Figure 5.

Location of the equivalent positions of amino acids substituted with alanine in the trialanine scanning library of σ^{70} R4 on the crystal structures of holo-RNAP from *Thermus thermophilus*[10] and on the crystal structure of *Thermus aquaticus* σ^{A} bound to DNA containing a consensus −35 element[68]. Only substitutions that reduce SoxS-dependent transcription activation of class I promoters or confer a defect in growth in liquid medium or on plates are identified. A space-filling model of the *T. thermophilus* RNAP holoenzyme is shown in the center, with the different subunits labeled and color-coded: σ^{A} subunit, orange; β subunit, blue; β' subunit, green; α subunit, grey; and ω subunit, yellow. The region displaying the interaction between σ^{A} R4 and the β flap-tip helix is enlarged in the upper left corner. The colors of the subunits are same as those in the model of holo-RNAP. Shown in magenta are the amino acid residues of σ^{A} that are equivalent to those within four (E555-K557, M561-F563, M567-T569 and L573-E575) of the five tri-alanine stretches of σ^{70} R4 that reduce SoxS-dependent transcription activation of class I promoters but have no effect on growth; the amino acids equivalent to those in the other stretch (V582-R584) do not appear in the crystal structure. The amino acids of σ^{A} that are equivalent to those within the four stretches of tri-alanine substitutions σ^{70} R4 that reduce SoxS-dependent transcription activation and also confer a defect in growth (T552-R554, V576-K578, Q579-D581 and R588-I590) are shown in green. Not appearing in this crystal structure are the amino acids of σ^{A} that are equivalent to those within the two tri-alanine stretches that confer a defect in growth (G564-D566, D570-T572) but not in SoxS-dependent transcription of class I promoters. The lower left corner displays the model of the crystal structure of σ^{A} R4 bound to DNA containing a −35 promoter hexamer. The amino acids residues of σ^{A} that are

equivalent to those within four (M561-F563, M567-T569, L573-E575, V582-R584) of the five stretches of trialanine substitutions of σ^{70} R4 that reduce SoxS-dependent transcription activation of class I promoters but have no effect on growth are shown in magenta; the amino acids equivalent to those within the other stretch (E555-K557) do not appear in the crystal structure. The amino acids σ^{A} that are equivalent to two (T552-R554 and R588-I590) of the four stretches of trialanine substitutions that reduce transcription activation and also confer a growth defect are shown in green; the amino acids of σ^{A} that are equivalent to those within the other two tri-alanine stretches do not appear in this crystal structure. The amino acids of σ^{A} that are equivalent to those within the two tri-alanine stretches that confer a defect in growth (G564-D566, D570-T572) but not in SoxS-dependent transcription of class I promoters are shown in blue. The modeling was carried out with the Chimera molecular modeling software[89], as described in Materials and Methods.

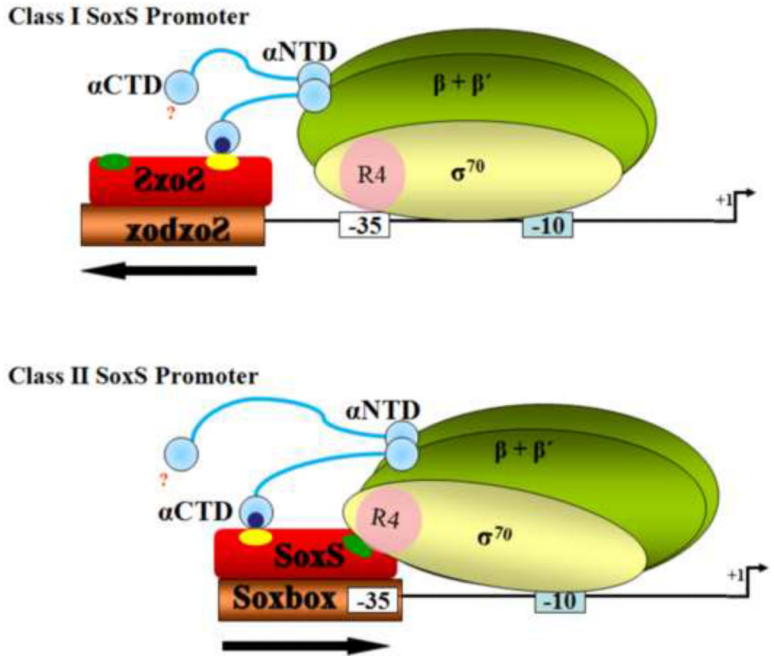


Figure 6.

Cartoon representation of protein-protein interactions at class I and class II SoxS-dependent promoters. The N-terminal and C-terminal domains of the two α subunits, which are connected by a flexible linker, and the β, β' and σ^{70} subunits of holo-RNAP are labeled and colored as are the −10 and −35 promoter hexamers; the relative position of the start site of transcription is numbered and identified by an arrow. Region 4 and the C-terminal tail of the σ^{70} subunit are denoted by a pink circle lying within the σ^{70} subunit and labeled "R4". SoxS is colored red and the yellow and green circles lying on it represent the class I/II and class II positive control surfaces, respectively[53]. The soxbox is colored bronze. The backward orientation of SoxS bound to the soxbox and the soxbox itself at canonical class I promoters is denoted by writing "SoxS" from right to left. The protein-protein interactions between the class I/II surface of SoxS and the DNA-binding (265) determinant of the α-CTD[55] are denoted by the intersection of the yellow and dark blue circles, respectively. The location of the second α-CTD is unknown and thus is denoted by a question mark. The ability of σ^{70} R4 to bind to the −35 promoter element of class I promoters and the absence of effects of tri-alanine substitutions on SoxS-dependent transcription activation at these promoters (Table 1) is denoted by the intersection of σ^{70} R4 (a pink circle) and the promoter hexamer (a colorless rectangle). At canonical class II promoters, the binding of SoxS to the soxbox prevents amino acid residues within σ^{70} R4 from binding to the −35 region (Table 1). However, amino acid residues within the distal end of R4 and the C-terminal end of σ^{70} make specific contacts with amino acids within the class I/II surface of SoxS[56]. For clarity, the interaction between the class II surface of SoxS (colored green) and the amino acids σ^{70} R4 with which it interacts are not positioned accurately.

Table 1

The effects of tri-alanine substitutions of σ^{70} R4 on SoxS-dependent transcription activation of class I and class II promoters.

Tri-Alanine Stretches of σ^{70} R4	β-galactosidase activity (% of wild type)					
	Class I Promoters			Class II Promoters		
	fpr	*mar*	*zwf*	*fumC*	*inaA*	*micF*
P531A-D533A	127 ±5	127 ±9	93 ±4	123 ±13	102 ±5	102 ±4
S534A-T536A	126 ±8	97 ±9	109 ±6	102 ±8	110 ±3	98 ±11
T537A-S539A	88 ±6	88 ±5	98 ±8	156 ±4	86 ±12	93 ±6
L540A-A542A	116 ±9	112 ±8	97 ±5	104 ±8	116 ± 8	104 ±4
A543A-H545A	112 ±8	97 ±9	93 ±6	105 ±10	105 ±6	106 ±3
D546A-L548A	120 ±4	97 ±5	89 ±7	85 ±6	113 ±4	116 ±9
A549A-L551A	127 ±5	86 ±6	99 ±4	98 ±12	114 ±2	113 ±8
T552A-R554A	123 ±5	85 ±5	**69 ±4**	149 ±18	100 ±8	86 ±4
E555A-K557A	116 ± 3	**77 ±5**	83 ±5	93 ±12	83 ±2	89 ±8
V558A-R560A	109 ±3	83 ±8	84 ±10	101 ±11	92 ±4	111 ±10
M561A-F563A	**78 ±7**	84 ±7	115 ±5	154 ±8	105 ±5	116 ±8
G564A-D566A	87 ±8	91 ±9	86 ±2	158 ±7	120 ±12	108 ±6
M567A-T569A	101 ±8	83 ±6	**70 ±1**	134 ±15	113 ±7	112 ±7
D570A-T572A	89 ±9	95 ±4	83 ±2	121 ±16	130 ±13	88 ±7
L573A-E575A	**70 ±4**	**79 ±7**	101 ±2	159 ±14	90 ±3	91 ±3
V576A-K578A	**78 ±6**	83 ±7	**69 ±1**	95 ±8	151 ±14	131 ±4
Q579A-D581A	119 ±14	**69 ±8**	**76 ±2**	95 ±8	113 ±2	126 ±5
V582A-R584A	109 ±5	**74 ±8**	85 ±2	89 ±8	96 ±5	98 ±4
E585A-I587A	112 ± 2	85 ±5	85 ±1	88 ±3	101 ±3	121 ±5
R588A-I590A	**70 ±5**	88 ±9	91 ±3	104 ±7	138 ±8	127 ±12

The library of tri-alanine substitutions of σ^{70} R4 carried on plasmid pVR-σ^{70} was introduced into derivatives of *E. coli* strain N7840 (Δ*mor*) [pBAD33-his6-SoxS] carrying transcriptional fusions of the above-named promoters to *lacZ*. SoxS expression was induced with 0.2% arabinose when the A600 reached 0.1–0.2; wild type and mutant σ^{70} proteins are constitutively expressed from a truncated *gal*P1 promoter carried on plasmid pVR-σ^{70}. The host chromosome contains *rpoD* and expresses wild type σ^{70} from it. Expression of the fusions was determined by assay of β-galactosidase activity as described in Materials and Methods. The data are expressed as the % of the wild type value and the standard deviations are given as the % of the mean. Wild type Miller units at SoxS dependent promoters were *fpr* (2110), *mar* (2980), *zwf* (2450), *fumC* (1196), *inaA* (1601) and *micF* (443). As described in the text, values for mutants that are ≤ 80% of the wild type activity are taken as a meaningful difference; these values are in bold and are highlighted in grey.

Table 2

The effect of tri-alanine substitutions of σ^{70} R4 on basal transcription from the class II fumC, inaA and micF promoters.

Tri-Alanine Stretches ofσ^{70}R4	β-galactosidase activity (% of wild type)		
	fumC	***micF***	***inaA***
P531A-D533A	134 ±6	132 ±*18*	124 ±*4*
S534A-T536A	98 ±15	88 ±*10*	118 ±*5*
T537A-S539A	81 ±13	84 ±*11*	137 ±*8*
L540A-A542A	88 ±22	125 ±*9*	116 ±*4*
A543A-H545A	116 ±18	105 ±*14*	98 ±*4*
D46A-L548A	**56 ±5**	100 ±*7*	117 ±*6*
A549A-L551A	174 ±18	121 ±*10*	92 ±*4*
T552A-R554A	177 ±*13*	107 ±*4*	97 ±*6*
E555A-K557A	10 ±12	122 ±*10*	110 ±*3*
V558A-R560A	151 ±8	108 ±*8*	89 ±*9*
M561A-F563A	**59 ±5**	130 ±*6*	120 ±*6*
G564A-D566A	82 ±6	130 ±*4*	124 ±*3*
M567A-T569A	**72 ±10**	137 ±*5*	**73 ±*8***
D570A-T572A	215 ±14	183 ±*13*	126 ±*5*
L573A-E575A	90 ±17	163 ±*8*	104 ±*5*
V576A-K578A	**62 ±3**	209 ±*24*	86 ± *7*
Q579A-D581A	290 ±14	208 ±*8*	100 ±*3*
V582A-R584A	**64 ±7**	135 ±*4*	108 ±*6*
E585A-I587A	**54 ±5**	156 ±*7*	**75 ±*4***
R588A-I590A	219 ±18	169 ±*7*	**78 ±*3***

The experiments were carried out with the same class II fusion strains and under the same conditions as those described in Table 1, except that no arabinose was added to the cultures such that SoxS expression was not induced. Expression of the fusions was determined by assay of β-galactosidase activity as described in Materials and Methods. The data are expressed as the % of the wild type value and the standard deviations are given as the % of the mean. Wild type Miller units at SoxS dependent promoters were *fumC* (287), *inaA* (77) and *micF* (123). As described in the text, values for mutants that are ≤ 80% of the wild type activity are taken as a meaningful difference; these values are in bold and are highlighted in grey.

Table 3

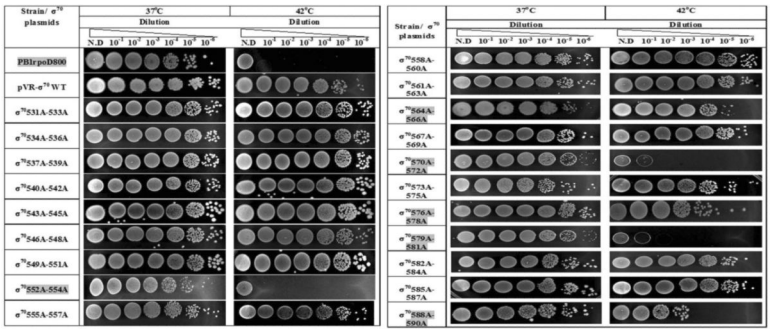
The effects of tri-alanine substitutions of σ^{70} R4 in strains containing transcriptional fusions of *lacZ* to a CRP-dependent class I promoter (strain W3110), a CRP-dependent class II promoter (strain DM00021) and a CRP^{-} class I promoter where the CRP binding site has been replaced by an UP element (strain RLG4282).

Tri-Alanine Stretches of σ^{70} R4	β-galactosidase activity (% of wild tvpe)		
	***lac* (Class I)**	**UP^{+} CRP^{-} *lac***	***gal* (Class II)**
P531A-D533A	116 ±*5*	81 ±*5*	85 ±*3*
S534A-T536A	116 ±*4*	84 ±*4*	90 ±*1*
T537A-S539A	122 ±*5*	82 ±*6*	80 ±*1*
L540A-A542A	111 ±*13*	94 ±*4*	82 ±*2*
A543A-H545A	124 ±*4*	94 ±*2*	84 ±*3*
D546A-L548A	95 ± *9*	86 ±*1*	80 ±*3*
A549A-L551A	92 ± *10*	94 ±*3*	**77 ±*3***
T552A-R554A	98 ± *8*	85 ±*1*	**77 ±*1***
E555A-K557A	95 ±*11*	**67 ±*3***	97 ±*3*
V558A-R560A	107 ±*2*	80 ±*2*	87 ±*7*
M561A-F563A	103 ±*7*	**68 ±*3***	85 ±*3*
G564A-D566A	103 ±*10*	**78 ±*3***	86 ±*5*
M567A-T569A	117 ±*5*	82 ±*5*	**71 ±*4***
D570A-T572A	**67 ±*8***	90 ±*5*	**78 ±*3***
L573A-E575A	**72 ±*7***	88 ±*10*	80 ±*4*
V576A-K578A	**55 ±*6***	**57 ±*9***	85 ±*5*
Q579A-D581A	83 ±*11*	**67 ±*3***	106 ±*5*
V582A-R584A	**63 ±*3***	**77 ±*7***	**78 ±*6***
E585A-I587A	94 ±*14*	83 ±*3*	93 ±*5*
R588A-I590A	**68 ±*4***	**58 ±*4***	104 ±*2*

The experiments were carried out as described in the legend to Table 1, except that the cultures of strains W3110 and DM00021 were treated with 1 mM IPTG to induce transcription from the *lac* promoters and cultures of strain RLG4282 were treated with 0.2% D-galactose to induce transcription from the *gal*P1 promoter. Wild type Miller units were *lac* (5740), *UP^{+} lac* (928) and *gal* (460).

Table 4

Effect of overexpression of the tri-alanine substitutions of σ^{70} R4 on growth at 42°C in strain PB1*rpoD800*.



Several mutants conferred temperature-lethal phenotypes (T552A-R554A, D570-T572-Q579-D581) while others conferred partial defects (G564A-T566A, V576A-K578A, D588A-I590A). The mutants conferring growth defects are highlighted in grey. N.D., no dilution.

Table 5

The strains used in this study.

Strains	Relevant Genotype	Source or Reference
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA relAl Δ(argF-lac)U169 deoR(Φ80 lacZΔM15)*	Lab stock
N7840 – *fumC**	λ *fumC* :: *lacZ Δmar Δ(argF-lac)U169*	R. G. Martin [36]
N7840 – *micF**	λ *micF* :: *lacZ Δmar Δ(argF-lac)U169*	R.G. Martin[36]
N7840 – *inaA**	λ *inaA* :: *lacZ Δmar Δ(argF-lac)U169*	R.G. Martin[36]
N7840 – *fpr**	λ *fpr* :: *lacZ Δmar Δ(argF-lac)U169*	R.G. Martin[36]
N7840 – *zwf**	λ *zwf* :: *lacZ Δmar Δ(argF-lac)U169*	R.G. Martin[36]
N7840 – *marA**	λ *mar* :: *lacZ Δmar Δ(argF-lac)U169*	R.G. Martin[36]
RLG4282 (*lac* UP$^+$)	*NK5031/λ rrnB P1(−88 to −38,Δ72)-lac(−37 to +52)-lacZ*	R.L. Gourse[72]
W3110	λ$^-$ *IN (rrnD-rrnE)1 rph-1*	Lab stock
DM0021	*gal*P1 *galE::lacZΔ(galT)galKgalM*	Lewis *et al.*[90]
PB1*rpoD800*	*galK*$^-$ *rpoD800 zgh/Tn10*	Liebke *et al.*[80]
GC4468	Δ *lacU169 rpsL*	B. Demple[91]

The asterisks denote the name of the promoter to which *lacZ* is transcriptionally fused.