# A two-subunit bacterial $\sigma$-factor activates transcription in *Bacillus subtilis*

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The $\sigma$-like factor YvrI and coregulator YvrHa activate transcription from a small set of conserved promoters in *Bacillus subtilis*. We report here that these two proteins independently contribute $\sigma$-region 2 and $\sigma$-region 4 functions to a holoenzyme-promoter DNA complex. YvrI binds RNA polymerase (RNAP) through a region 4 interaction with the $\beta$-subunit flap domain and mediates specific promoter recognition but cannot initiate DNA melting at the -10 promoter element. Conversely, YvrHa possesses sequence similarity to a conserved core-binding motif in $\sigma$-region 2 and binds to the N-terminal coiled-coil element in the RNAP $\beta'$-subunit previously implicated in interaction with region 2 of $\sigma$-factors. YvrHa plays an essential role in stabilizing the open complex and interacts specifically with the N-terminus of YvrI. Based on these results, we propose that YvrHa is situated in the transcription complex proximal to the -10 element of the promoter, whereas YvrI is responsible for -35 region recognition. This system presents an unusual example of a two-subunit bacterial $\sigma$-factor.

RNA polymerase | initiation | promoter | open complex

Transcription is catalyzed by an evolutionarily conserved enzyme complex comprising a core catalytic complex together with accessory specificity factors. In bacteria, the RNA polymerase (RNAP) core enzyme contains, minimally, the $\beta\beta'\alpha_2$-assembly, but promoter recognition and transcription initiation additionally require a $\sigma$-factor. The $\sigma$-factor binds RNAP and targets the resulting holoenzyme to cognate promoter sites (1).

All bacteria encode a primary $\sigma$-factor (called RpoD or $\sigma^{70}$ in *Escherichia coli* and $\sigma^A$ in *Bacillus subtilis*) that is responsible for the transcription of most genes expressed during vegetative growth. Primary (or group 1) $\sigma$-factors consist of four functional regions called regions 1, 2, 3, and 4 (2–5). Group 2 $\sigma$-factors are structurally similar to group 1 $\sigma$-factors but are not essential to the cell. Group 3 $\sigma$-factors retain only regions 2–4, whereas group 4 [or extracytoplasmic function (ECF)] $\sigma$-factors carry only the two most functionally important and strongly conserved regions, 2 and 4. Regions 2 and 4 are responsible for recognition of the -10 and -35 sequence elements found in most bacterial promoters (6).

Region 2 is the most highly conserved region in bacterial $\sigma$-factors (4, 7) and can be further subdivided into regions 2.1, 2.2, 2.3, and 2.4. Amino acids in regions 2.1 and 2.2 form a pair of $\alpha$-helices that mediate core binding by virtue of interaction with a coiled-coil element in the amino terminus of the $\beta'$-subunit of core RNAP (8). This interaction is critical for holoenzyme formation, because a number of mutations in these interacting elements negatively influence $\sigma$-binding to core (9–15). This interaction is also important for $\sigma$-region 2.3–2.4 recognition of the single- and double-stranded -10 element within promoter DNA (16, 17). Finally, amino acids in region 2.3 play a critical role in recognizing and binding single-stranded nontemplate DNA encompassing the -10 region (18–20). In doing so, region 2.3 stabilizes the transcription bubble during open-complex formation (19).

Region 4 is also a highly conserved and functionally important region in $\sigma$-factors. Region 4.2 includes a helix-turn-helix (HTH) domain that recognizes and binds the -35 element. Region 4 also interacts with hydrophobic amino acids in the $\beta$-flap domain of the core RNAP $\beta$-subunit (21), and this interaction properly orients the HTH domain for interaction with -35 region DNA (22). Additionally, region 4 is a frequent target for regulators that modulate RNAP activity through interaction with $\sigma$ (23).

Recently, an alternative $\sigma$-like regulator named YvrI was identified in *B. subtilis* (24). YvrI carries a carboxy-terminal $\sigma^{70}$-family region 4 but is quite divergent in its amino terminus. Indeed, domain prediction programs fail to identify a $\sigma^{70}$-family region 2 in YvrI despite this region being the most highly conserved segment in $\sigma$-factors (4, 7). In contrast, the other 17 $\sigma^{70}$-family $\sigma$-factors in *B. subtilis* all carry a predicted region 2. YvrI nevertheless copurifies with RNAP and activates transcription from at least three conserved promoters in *B. subtilis* (24). YvrI activation in the cell is linked both to challenge with certain cell wall-acting antibiotics (25) and to growth under acidic conditions (24, 26). Activation of YvrI during growth under low pH conditions results in the accumulation of a secreted oxalate decarboxylase (OxdC) as the most abundant protein in the cell wall (27). YvrI-activated transcription additionally requires the 9.5-kDa YvrHa protein (24), although the nature of this requirement has not been previously defined.

Here, we demonstrate that YvrHa carries a $\sigma^{70}$-family region 2, the same region poorly represented in YvrI, and that this region interacts with the $\beta'$-coiled-coil element. YvrI carries region 4, which interacts with the $\beta$-flap-tip helix. In single-subunit $\sigma$-factors, the homologous regions are known to contact these same regions of $\beta$ and $\beta'$. Thus, YvrI and YvrHa reconstitute $\sigma$-factor form and function, enabling efficient promoter melting and transcription initiation.

## Results

Previously, we demonstrated that both YvrI and YvrHa are required for activation of transcription from a group of conserved promoters in *B. subtilis* and other closely related species (24, 26). Sequence analysis suggests a model in which YvrHa contains $\sigma^{70}$-like regions 2.1, 2.2, and 2.3, whereas YvrI carries a typical region 4 and a highly divergent region 2 (possibly including a functional region 2.4). Here, we present an analysis of protein–protein and protein–DNA interactions that, together with in vitro and in vivo transcription results, support this model.

**YvrI Region 4 Interacts with $\beta$-Flap.** YvrI binds RNAP (24) and is predicted to carry a $\sigma$-region 4. Previous results have demonstrated that region 4 of $\sigma^{70}$ specifically contacts the $\beta$-flap domain in the core enzyme. To test whether similar contacts are made by YvrI, we used a bacterial two-hybrid (BACTH) system.
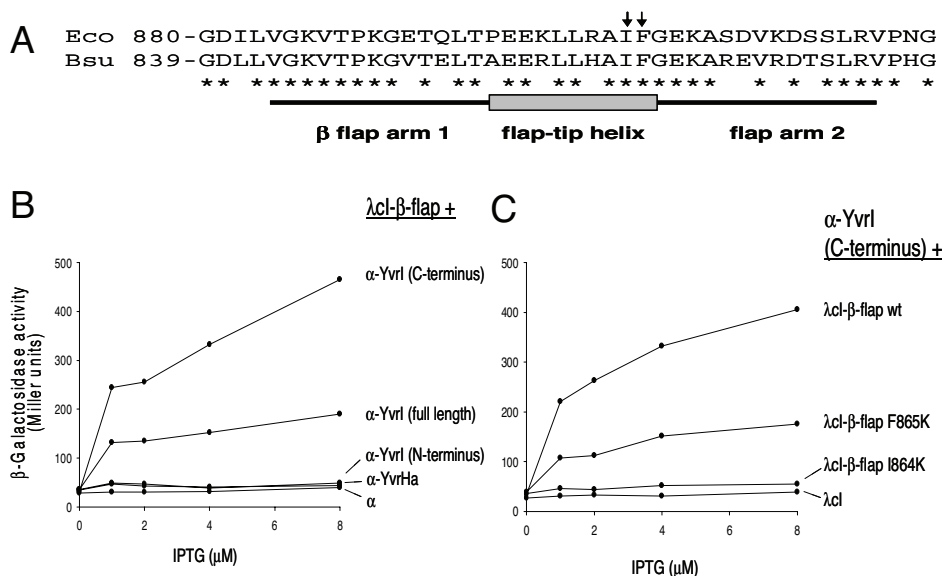
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**Fig. 1.** YvrI region 4 interacts with the β-flap-tip helix. (*A*) Relevant amino acid sequence of *E. coli* and *B. subtilis* β-flap-tip regions (21). Two tip helix mutations (I905K and F906K) that impair in *E. coli* binding by σ⁷⁰-region 4 (21) are indicated (arrows). For interaction assays (BACTH), the region from amino acid 817 to 896 was fused to λcI. (*B*) BACTH analysis to test YvrI and YvrHa binding to *B. subtilis* β-flap. (*C*) BACTH analysis of the effect of I864K and F865K flap-tip mutations on the *B. subtilis* β-flap–YvrI region 4 interaction.

In this system, test polypeptides are expressed as fusions with either a DNA-binding domain (λcI) or the amino-terminal domain (NTD) of the α-subunit, and positive interactions are manifest as an increase in β-galactosidase activity from an *E. coli* host strain (28).

To monitor interactions with the *B. subtilis* β-flap domain, amino acids 817–896 (homologous to *E. coli* β-flap amino acids 858–937) were fused to λcI and tested for interacting partners fused to the α-subunit NTD (Fig. 1*A*). YvrI, but not YvrHa, interacts with β-flap (Fig. 1*B*). Genetic bisection of YvrI into amino- and carboxy-terminal segments [supporting information (SI) Fig. S1] revealed that this interaction is specifically mediated through the C-terminal portion of YvrI corresponding to region 4 (Fig. 1*B*). Because the β-subunit sequence is largely conserved between *E. coli* and *B. subtilis* (Fig. 1*A*), we introduced mutations in the *B. subtilis* β-flap that are known to affect interaction negatively between the *E. coli* β-flap (I905K and F906K; Fig. 1*A*) and σ⁷⁰-region 4 (21). As predicted, the corresponding *B. subtilis* flap mutations (I864K and F865K) dramatically reduced the observed interaction between region 4 of YvrI and the β-flap (Fig. 1*C*). These results support a model in which the YvrI-core interaction is analogous to that observed between conventional σ-factors and the β-subunit of core RNAP.

YvrI region 4.2 carries a predicted HTH motif that aligns with those in σ-factors (e.g., *B. subtilis* σ^X and σ^W; Fig. S2*A*). We mutated 2 residues (Q166 and S169) in the YvrI HTH recognition helix to alanine (based on their strong conservation among YvrI homologs; Fig. S3), and, despite similar expression levels (Fig. S2*C*), both mutations strongly impaired in vivo transcription (Fig. S2*B*). These data, coupled with the observation that YvrI-dependent promoters possess a conserved -35 promoter motif (TTGACW) (24, 26), suggest that YvrI region 4.2 mediates recognition of -35 promoter DNA and that this is critical for transcription.

**YvrI Binds Core and Acts as a Promoter-Binding Specificity Factor.** We reasoned that if YvrI interacted with core RNAP (E) at the same site as conventional σ-factors, this binding should competitively inhibit recognition of σ^A-dependent promoters. Indeed, YvrI inhibited promoter interaction, as judged by monitoring DNA melting using KMnO₄ footprinting, by either Eσ⁷⁰- or Eσ^A-holoenzymes at three different promoters (Fig. 2*A*). In contrast, YvrHa did not competitively appear to inhibit promoter recognition and melting by holoenzyme. Thus, although YvrI-dependent activation of transcription relies on YvrHa, YvrI binding to RNAP does not.

To test whether YvrI acts as a promoter-binding specificity factor, we mixed increasing concentrations of RNAP containing either YvrI or YvrHa (or both) with end-labeled P_oxdC DNA (or control DNA) and monitored binding using an EMSA assay (Fig. 2*B*). As expected, RNAP core binds both target DNAs nonspecifically (Fig. 2*B*, *a*). Interestingly, YvrHa largely abolished this nonspecific binding (Fig. 2*B*, *b*), an activity reminiscent of σ⁷⁰ (29, 30). In contrast, YvrI alone resulted in a bias toward shifting P_oxdC DNA vs. the control DNA (Fig. 2*B*, *c*), suggesting that the E-YvrI complex has significant promoter recognition capability. Finally, inclusion of both YvrI and YvrHa in the binding reactions further increased the specificity of binding (Fig. 2*B*, *d*), probably because YvrHa is reducing residual nonspecific binding of the control DNA by RNAP itself. These data suggest that YvrI (in the absence of YvrHa) can act as a specificity factor and direct binding to P_oxdC DNA, even though, as shown previously (24), it is insufficient to activate transcription.

**YvrHa Is Also a σ-Factor-Related Protein.** YvrHa is a 9.5-kDa protein encoded by the gene downstream of, and cotranscribed with, *yvrI*. A multiple sequence alignment of YvrHa and its orthologs with various σ-factors (Fig. 3) demonstrates a remarkable similarity in the region corresponding to the most highly conserved portions of region 2 (including the originally defined "RpoD box") (31). The amino acid sequence EDLxQE within region 2.2 is the principal point of conservation and is thought to be part of a conserved σ-factor core interface (4, 7). This EDLxQE motif in YvrHa is also the most strongly conserved region among its homologs (Fig. 3, *Bottom*), implying functional importance. Previously, mutations in σ-factor that negatively influence holoenzyme formation (presumably by disrupting interaction with the β′-subunit coiled coil) were mapped to this subsequence (13–15). To determine if this region is also critical for YvrHa function, we engineered mutations in this motif. As predicted, mutations in this conserved region of YvrHa
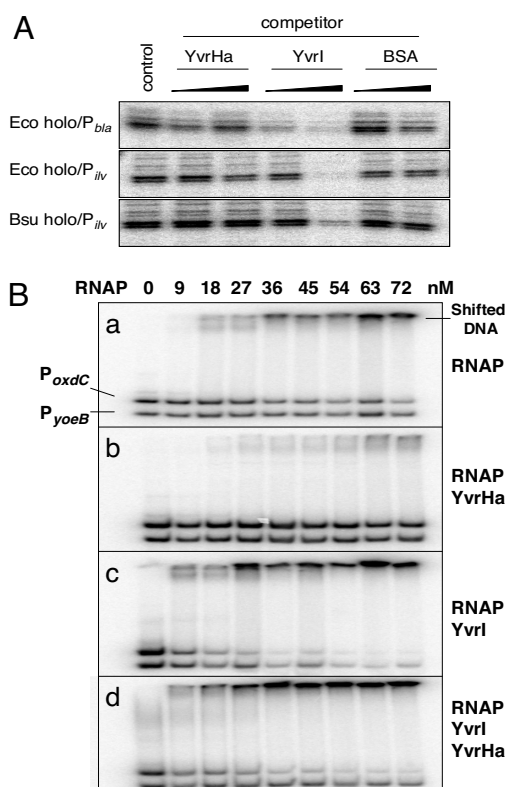
**Fig. 2.** σ-Factor activities of YvrI. (*A*) YvrI competitively inhibits DNA melting by *E. coli* σ70- and *B. subtilis* SigA. Reactions include *E. coli* σ70-holoenzyme melting of the *E. coli bla* and *B. subtilis ilv* promoters and *B. subtilis* SigA holoenzyme melting of the *ilv* promoter. Competitor proteins [YvrHa, YvrI, and a control protein (BSA)] were either not added (control lane) or added at a concentration 5-fold and 10-fold over the concentration of holoenzyme (62 nM) before oxidation reaction using KMnO$_4$. (*B*) YvrI is a promoter specificity factor determined using EMSA and end-labeled YvrI-dependent *oxdC* promoter DNA (P$_{oxdC}$) and a control promoter DNA (P$_{yoeB}$). Each binding reaction contained the indicated concentration of RNAP (*a*) and 500 nM YvrHa (*b*), 500 nM YvrI (*c*), and 500 nM YvrHa and 500 nM YvrI (*d*). Note that the initial binding reaction in *b*, *c*, and *d* containing 0 nM RNAP includes the indicated amount of YvrI and/or YvrHa to control for nonspecific binding by these proteins.

strongly impaired transcriptional activation from a target promoter in vivo (Fig. S4).

YvrHa also displays similarity to conserved region 2.3, which characteristically carries positionally conserved aromatic amino acids known, in the vegetative factors of *B. subtilis* and *E. coli*, to play a role in stabilizing open-complex formation (18, 19). This region in YvrHa might also play a role in DNA melting. Note that the sequence of YvrHa ends after region 2.3, and therefore does not allow for a region 2.4-like segment.

**YvrHa Copurifies with RNAP.** Previously, it was shown that overexpressed YvrI copurifies with RNAP (24). To examine whether YvrHa stably associates with RNAP in vivo, we partially purified hexahistidine-tagged RNAP (12) from *B. subtilis* after coinduction of epitope-tagged YvrI and YvrHa. Indeed, both YvrI and YvrHa were copurified with RNAP using nickel affinity chromatography (Fig. S5B). YvrI and YvrHa was not detected in control purifications from cells not expressing YvrI and YvrHa (Fig. S5A) or expressing WT RNAP (Fig. S5D). We also tested a variant of YvrHa carrying a mutation (D43A) in its conserved region 2.2 EDLxQE motif that resulted in decreased transcription activation in vivo (Fig. S4). Although the mutant protein was readily detected in crude lysate (Fig. S5C), it copurified with

RNAP to a much lesser extent than WT YvrHa. This supports the hypothesis that this region 2.2 motif in YvrHa is involved in core binding, consistent with results for conventional σ-factors (13–15).

**YvrHa Interacts with the β′-Subunit Coiled-Coil Element.** Because YvrHa carries what appears to be a σ-region 2.2 (including the EDLxQE motif), we tested whether it could interact with the initial 303 aa of the β′-subunit from *B. subtilis* that, by analogy to *E. coli* β′ (amino acids 1–314) (12), should contain the coiled-coil element critical for region 2 core interaction. Indeed, YvrHa interacted with this region (Fig. 4*A*). Several point mutations in this conserved region of YvrHa, many of which strongly decrease transcription in vivo (Fig. S4), also decreased the magnitude of this apparent interaction (Fig. 4*B*). In repeated experiments, the most dramatic impairment resulted from mutation of amino acid L44 in the YvrHa EDLxQE motif. Mutations E42A and E47A had no significant impact on this interaction, whereas E45A, D43R, and a double-mutant D43A/Q46A slightly increased the apparent interaction. In contrast, we were unable to detect an interaction between β′ (amino acids 1–303) and YvrI, despite the possible presence of a divergent region 2 in the amino terminus of YvrI (Fig. 4*A*).

Several mutations in the *E. coli* β′-subunit coiled-coil element impair its interaction with σ-region 2 (12), including (*E. coli* numbering) R275Q, E295K, and A302D (Fig. 4*C*). The introduction of two analogous mutations in the *B. subtilis* β′- (amino acids 1–303) fusion protein (R264Q and A291D) reproducibly and significantly impaired the interaction with YvrHa in the BACTH system (Fig. 4*D*). In contrast, an E284K mutation had no significant effect. These results support a model in which the region 2.2 motif of YvrHa makes a specific interaction with the coiled-coil element of the *B. subtilis* β′-subunit.

**YvrHa Is Required to Stabilize Open-Complex Formation.** Collectively, our data indicate that the E-YvrI complex can mediate promoter binding (Fig. 2*B*), but previous data indicate that it cannot activate transcription (24). Therefore, we monitored the effects of YvrI and YvrHa alone and together on DNA melting of P$_{oxdC}$ using KMnO$_4$ footprinting and negatively supercoiled promoter DNA (Fig. 5). Despite acting as a promoter specificity factor, YvrI itself is unable to stabilize open-complex formation (Fig. 5, lane 4). In contrast, the E-YvrI-YvrHa holoenzyme melts (minimally) a ≈12-nt region of the *oxdC* promoter (Fig. 5, lane 6, and Fig. S6). Thus, YvrHa is essential for DNA melting, and this can account for its essential function in transcription initiation.

**YvrHa Interacts with the Amino Terminus of YvrI.** Because both YvrHa and YvrI are required for transcription (24) and both make contacts with core subunits characteristic of σ-factors, we hypothesized that they might also interact with one another. In fact, a BACTH analysis demonstrated that they do and that YvrHa interacts with the amino but not carboxy (region 4) segment of YvrI (Fig. 6*A*). Further, using purified YvrHa (and a control protein, lysozyme) conjugated to NHS-activated sepharose beads (Fig. 6*B*), we found that both full-length YvrI and the amino segment of YvrI, but not the carboxy segment, were pulled down by immobilized YvrHa, thus corroborating the BACTH data.

To check whether binding to σ-factors might be a general property of YvrHa, we repeated the in vitro interaction experiment (Fig. 6*C*) with purified *B. subtilis* σ^A and an ECF σ-factor, σ^M. Unlike with YvrI, there was no indication that YvrHa specifically interacted with either protein. Therefore, YvrHa interacts specifically with the amino region of YvrI but not with other σ-factors tested.
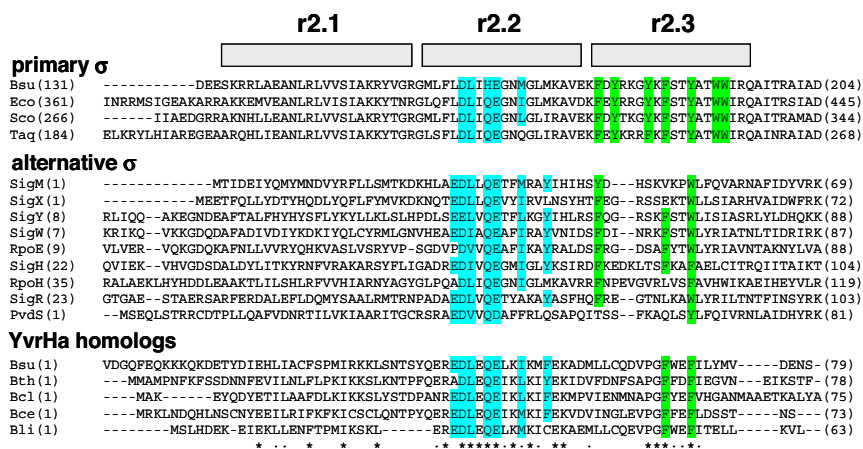
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**r2.1          r2.2          r2.3**

**primary σ**
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Bsu(131) -----------DEESKRRLAEANLRLVVSIAKRYVGRGMLFLDLIHEGNMGLMKAVEKFDYKGVKFSTYATWWIRQAITRAIAD(204)
Eco(361) INRRMSIGEAKARRAKKEMVEANLRLVISIAKKYTNRGLQFLDLIQEGNIGLMKAVDKFEYRRGYKFSTYATWWIRQAITRSIAD(445)
Sco(266) ------IIAEDGRRAKNHLLEANLRLVVSLAKRYTGRGMLFLDLIQEGNLGLIRAVEKFDYTKGYKFSTYATWWIRQAITRAMAD(344)
Taq(184) ELKRYLHIAREGEAARQHLIEANLRLVVSIAKKYTGRGLSFLDLIQEGNQGLIRAVEKFDYKRRFKFSTYATWWIRQAINRAIAD(268)
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**alternative σ**
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SigM(1)  -----------MTIDEIYQMYMNDVYRFLLSMTKDKHLAEDLLQETFMRAYIHIHSVD---HSKVKPWLFQVARNAFIDYVRK(69)
SigX(1)  -----------MEETFQLLYDTYHQDLYQFLFYMVKDKNQTEDLLQEVYIRVLNSYHTEG--RSSEKTWLLSIARHVAIDWFRK(72)
SigY(8)  RLIQQ--AKEGNDEAPTALFHYHYSFLYKYLLKLSLHPDLSEELVQETFLKGYIHLRSQG--RSKFSTWLISIASRLYLDHQKK(88)
SigW(7)  KRIKQ--VKKGDQDAFADIVDIYKDKIYQLCYRMLGNVHEAEDIAQEAFIRAYVNIDSDI--NRKFSTWLYRIATNLTIDRIRK(87)
RpoE(9)  VLVER--VQKGDQKAFNLLVVRYQHKVASLVSRYVP-SGDVDVVQEAFIKAYRALDSFRG--DSAFYTWLYRIAVNTAKNYLVA(88)
SigH(22) QVIEK--VHVGDSDALDYLITKYRNFVRAKARSYFLIGADREDIVQEGMIGLYKSIRDFKEDKLTSFKAFAELCITRQIITAIKT(104)
RpoH(35) RALAEKLHYHDDLEAAKTLILSHLRFVVHIARNYAGYGLPQADLIQEGNIGLMKAVRRFNPEVGVRLVSFAVHWIKAEIHEYVLR(119)
SigR(23) GTGAE--STAERSARFERDALEFLDQMYSAALRMTRNPADAEDLVQETYAKAYASFHQFRE--GTNLKAWLYRILTNTFINSYRK(103)
PvdS(1)  --MSEQLSTRRCDTPLLQAFVDNRTILVKIAARITGCRSRAEDVVQDAFFRLQSAPQITSS--FKAQLSYLFQIVRNLAIDHYRK(81)
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**YvrHa homologs**
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Bsu(1)   VDGQFEQKKKQKDETYDIEHLIACFSPMIRKKLSNTSYQEREDLEQELKIKMFEKADMLLCQDVPGFWEFILYMV-----DENS-(79)
Bth(1)   ---MMAMPNFKFSSDNNFEVILNLFLPKIKKSLKNTPFQERADLEQEIKLKIYEKIDVFDNFSAPGFFDFIEGVN---EIKSTF-(78)
Bcl(1)   ----MAK------EYQDYETILAAFDLKIKKSLYSTDPANREDLEQEIKLKIFEKMPVIENMNAPGFYRFVHGANMAAETKALYA(75)
Bce(1)   ----MRKLNDQHLNSCNYEEILRIFKFKICSCLQNTPYQEREDLEQEIKMKIFEKVDVINGLEVPGFFEFLDSST-----NS---(73)
Bli(1)   --------MSLHDEK-EIEKLLENFTPMIKSKL------EREDLEQELKMKICEKAEMLLCQEVPGFWEFITELL-----KVL--(63)
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         * :: *    *. *   .  :*.*****:*:*:.** :.      ***:::*:
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**Fig. 3.** σ-Factor region 2-like sequence in YvrHa includes functionally important residues. Composite alignments of the relevant sequences [regions 2.1, 2.2, and 2.3 as previously defined (4)] from a selection of primary and alternative σ-factors and the complete sequences of YvrHa and four YvrHa homologs. Highly conserved amino acids in regions 2.2 are highlighted in blue, whereas positionally conserved aromatic amino acids among the subgroups are highlighted in green. ClustalW was used to generate each individual alignment, and conservation symbols (asterisks) pertain to YvrHa homolog alignment only. For the alternative σ-factors: sequences SigM, SigX, SigW, SigY, and SigH are from *B. subtilis*; RpoE and RpoH are from *E. coli*; SigR is from *Streptomyces coelicolor*; and PvdS is from *Pseudomonas aeruginosa*. For the vegetative and YvrHa proteins, abbreviations are as follows: Bsu, *B. subtilis*; Eco, *E. coli*; Sco, *S. coelicolor*; Taq, *Thermus aquaticus*; Bth, *Bacillus thuringiensis*; Bcl, *Bacillus clausii*; Bce, *Bacillus cereus*; Bli, *Bacillus licheniformis*.

## Discussion

Collectively, these genetic, biochemical, and BACTH experiments support a model in which YvrI and YvrHa functionally reconstitute σ-factor activity on interaction with core RNAP. YvrHa binds the coiled-coil element in the β′-subunit and the amino-terminal segment of YvrI. The carboxy-terminal region 4 domain of YvrI interacts with the β-flap domain and contains an HTH motif postulated to mediate -35 region recognition. These results lead to a provisional model for the subunit architecture of the E-YvrI-YvrHa holoenzyme (Fig. 7).

Because YvrI is about the size (23 kDa) of many alternative σ-factors and includes a divergent region 2, this model postulates that YvrI is broadly extended along a single face of core RNAP and that the amino terminus of YvrI is situated toward the leading edge of the holoenzyme. The ability to direct specific promoter DNA binding suggests that YvrI recognizes promoter DNA. This presumably includes the conserved -35 element
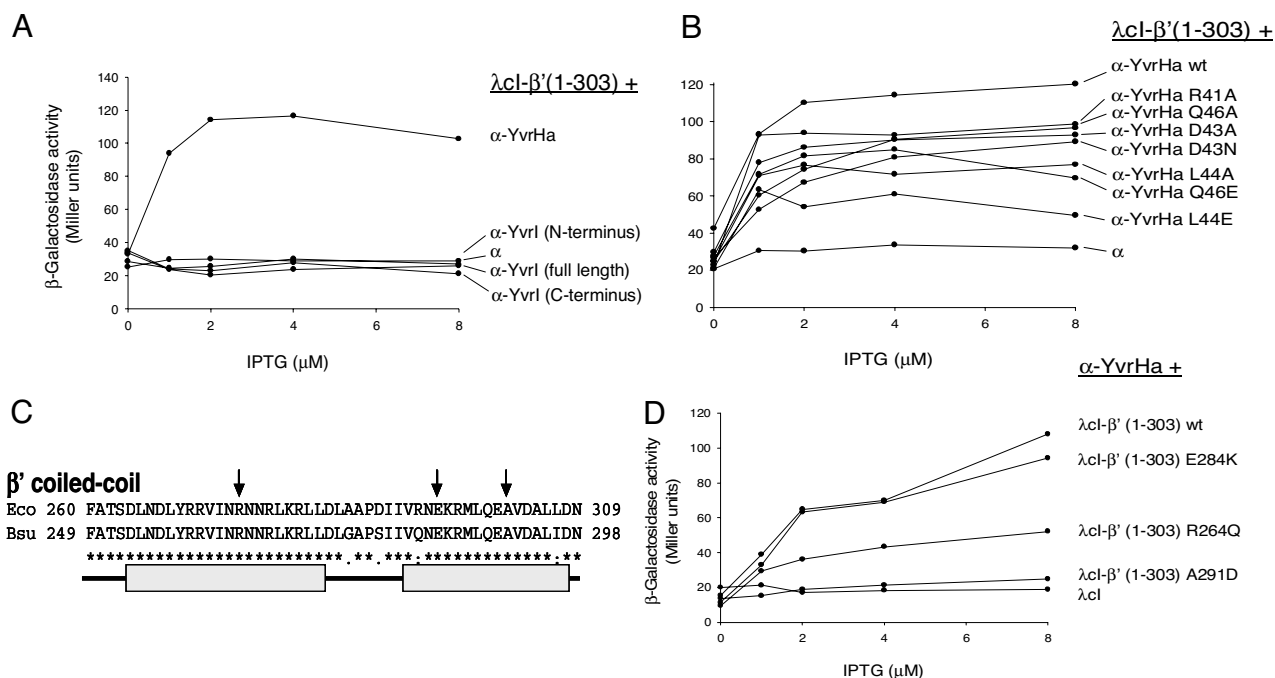


**Fig. 4.** YvrHa interacts with the coiled-coil domain in β′-subunit. (*A*) BACTH analysis of YvrHa and YvrI interactions with β′ (amino acids 1–303). (*B*) Influence of mutations in the region 2.2-like motif (Fig. 3) of YvrHa on binding with β′ (1–303). (*C*) Sequence of the *E. coli* and *B. subtilis* coiled-coil element in β′ as previously defined (8). Mutations in *E. coli* β′ (R275Q, E294K, and A302D) that impair β′-binding to σ⁷⁰-region 2 are indicated with arrows. Approximate dimensions of helices that contribute to the coiled-coil domain are indicated by gray bars. (*D*) Influence of mutations in *B. subtilis* β′ (analogous to mutations shown in *C*) on β′-binding to YvrHa.
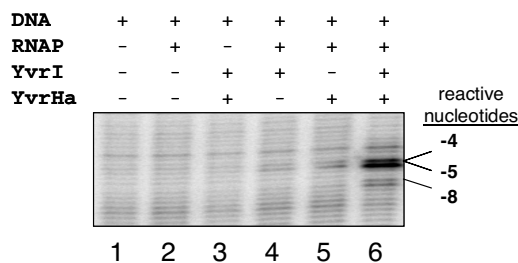
**Fig. 5.** YvrI and YvrHa are required for promoter melting. Permanganate sensitivity of nucleotides in the template strand of P$_{oxdC}$ as influenced by the addition of indicated proteins is shown. Nucleotide coordinates are shown in Fig. S6. Reactions contained *B. subtilis* RNAP (60 nM) with other proteins added to a final concentration of 300 nM.
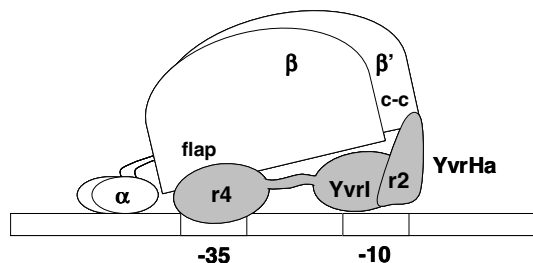


**Fig. 7.** Provisional model for a YvrI-YvrHa holoenzyme–promoter DNA closed transcription complex. Nonessential *B. subtilis* polymerase subunits (δ- and ω-subunits) are not included. The model depicts known interactions of YvrHa region 2-like segment (r2) with β′-coiled-coil domain (c-c), YvrHa with the amino terminus of YvrI, and YvrI region 4 (r4) with the β-subunit flap domain (flap). Drawing not to scale.

(recognized by region 4) but may additionally include contacts to the -10 element mediated, in conventional σ-factors, by region 2.4.

Our model further proposes that YvrHa (which has sequence determinants highly similar to those of σ-factor region 2) is



**Fig. 6.** YvrHa interacts with the amino terminus of YvrI. (*A*) BACTH analysis of interactions between YvrHa and either full-length YvrI or amino- and carboxyl-terminal segments of YvrI (Fig. S1). (*B*) In vitro pull-down assay using NHS-conjugated YvrHa or a control protein (lysozyme). Binding reactions included either full-length YvrI or the amino- or carboxy-terminal segment of YvrI. Arrows indicate bound amino-terminal fragment of YvrI. (*C*) In vitro pull-down assay testing interaction between immobilized YvrHa and either YvrI, SigA, or SigM. All test proteins were added to binding reactions to a final concentration of ≈1 μM.

situated proximal to the -10 element of promoter DNA by virtue of its contacts with the N-terminal regions of YvrI and the β′-subunit (Fig. 7). In this position, it plays an essential role in open-complex formation, perhaps analogous to that played by σ-region 2 in conventional holoenzyme, which is situated proximal to, and acts on, DNA sequence in the -10 element during the transcription initiation process. Future analyses are to be directed at mapping the precise contacts between YvrHa and YvrI and promoter region DNA.

YvrI and YvrHa coregulate transcription from at least three conserved promoters in *B. subtilis* driving the expression of five genes, including the *yvrI-yvrHa* operon. Taken together, the sequence similarity of YvrHa to σ, its protein interaction patterns, and its required involvement in activating open-complex formation indicate that this "accessory" protein differs from previously characterized conventional transcription activators and also from more recently characterized regulators that function by interacting with RNAP but not DNA (32). The YvrI-YvrHa system is reminiscent of certain phage σ-factor systems that involve two proteins. For example, *B. subtilis* phage SPO1 proteins gp33 and gp34 are both required for activation of late genes, and both act as promoter-binding specificity factors (33), although the precise role of these proteins in transcription activation was never elucidated (34). The more thoroughly studied T4 phage regulators gp55 and gp33 participate in reconstituting σ-factor activity at late-phage promoters lacking -35 subsequences (35). In this system, gp33 (which carries no obvious sequence similarity to σ-factor) stimulates transcription by the divergent truncated σ-factor gp55 through a critical interaction with the flap domain of the β-subunit (36). Thus, gp33 partially substitutes for σ-region 4 function.

In contrast to these phage models, the YvrI-YvrHa system consists of two σ-factor-related proteins of bacterial origin that activate transcription from promoters with well-defined and conserved -10 and -35 elements (24, 26). The smaller accessory protein in this system (YvrHa) interacts with an N-terminal coiled-coil element in the β′-subunit, contacts the N-terminus of YvrI, and plays an essential and potentially direct role in DNA melting. Significantly, the *yvrI-yvrHa* gene order is not consistent with a simple frameshift-mediated splitting of an ancestral σ into two separately encoded polypeptides: in this system, the region 4-containing polypeptide (YvrI) is encoded upstream of the region 2-containing protein (YvrHa). We believe that YvrI-YvrHa represents the first known bipartite bacterial σ-factor. An investigation of the mechanism by which these proteins activate transcription should extend our understanding of noncanonical σ-factor systems and unusual transcription initiation pathways in bacteria.
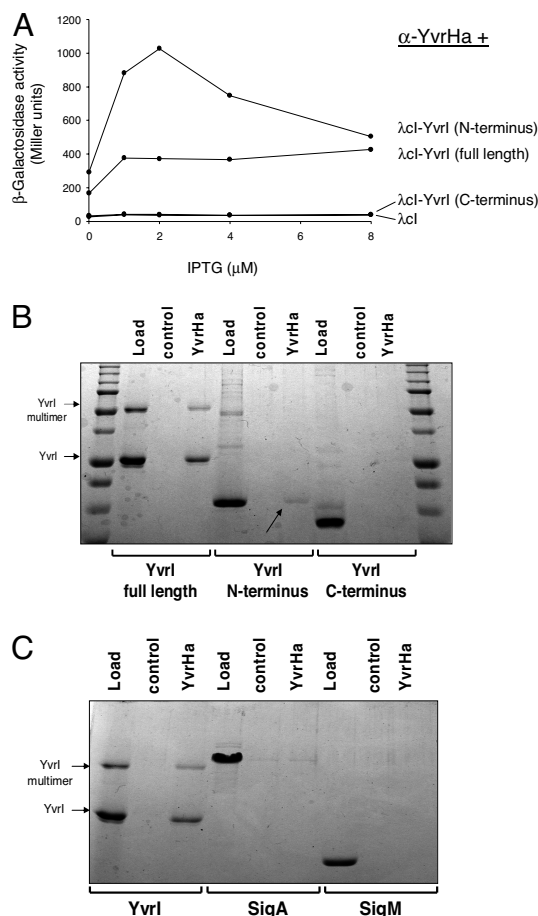
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## Materials and Methods

**Bacterial Strains and Plasmids.** The bacterial strains and plasmids used in this study are listed in Table S1.

**In Vivo Expression and Protein Pull-Down Assays.** Relevant strains were grown overnight, and this growth was used to inoculate fresh 100-mL volumes of LB broth to $OD_{600}$ of 0.05. After incubation at 37 °C with shaking to $OD_{600}$ of 0.4, YvrI and YvrHa expression was induced with 2% (vol/vol) xylose for 1 h. Crude lysate was supplemented with 0.5 mL of Ni NTA beads (Qiagen) and incubated at 4 °C with slow rotation for 1 h. Bound His-tagged RNAP and cobinding proteins were eluted with a step gradient of increasing imidazole. Crude lysate and eluate samples were separated on 12% (vol/vol) SDS/PAGE gels, and proteins were visualized by Coomassie staining or were transferred to PVDF membrane and visualized by immunoblotting.

**Electrophoretic Mobility Assays.** Each 10-$\mu$L binding reaction included <100 pmol end-labeled target DNA carrying either $P_{oxdC}$ (150 nt) or a control promoter ($\sigma^A$-dependent $P_{yoeB}$) on a 110-nt fragment, constant amounts of YvrI and YvrHa, and increasing concentrations of RNAP (see legend for Fig. 2). Samples were loaded onto a 6% (vol/vol) nondenaturing polyacrylamide gel running at 100 V. After electrophoresis for 45 min, the gel was dried and exposed to a phosphor screen.

**In Vitro Pull-Down Assays.** For in vitro interaction assays, 50 $\mu$g of YvrHa or a control protein was conjugated to NHS-activated beads (GE Lifesciences). After extensive washing in 100 mM Tris-HCl (pH 8.0), conjugated beads were stored in this same buffer as a 50% (vol/vol) bead slurry at 4 °C. A typical experiment involved resuspending 50 $\mu$L of the bead slurry in binding buffer and supplementing the suspension with 1 $\mu$M test protein. After extensive washing, the beads were resuspended in 40 $\mu$L of SDS/PAGE sample buffer,

beads were sedimented, and 30 $\mu$L of sample buffer containing dissolved proteins was analyzed by SDS/PAGE using a 14% (vol/vol) polyacrylamide gel. For each experiment, conjugated lysozyme beads were used to control for incidental protein carryover and nonspecific binding of test proteins to conjugated protein, the sepharose bead matrix, or the reaction tube wall.

**In Vivo Two-Hybrid Assay (BACTH).** The _E. coli_ reporter strain FW Kan $O_L 2$–62 lac (37) was cotransformed with relevant recombinant bait and prey plasmids. Single colonies from each strain were used to inoculate a series of 2-mL volumes of LB broth containing ampicillin (100 $\mu$g/mL); kanamycin (50 $\mu$g/mL); chloramphenicol (25 $\mu$g/mL); and either 0, 1, 2, 4, or 8 $\mu$M isopropyl-$\beta$-D-thiogalactopyranoside (IPTG). After overnight growth at 37 °C, these cultures were used to inoculate fresh 5-mL volumes of the same broth series to $OD_{600}$ of 0.05. When cultures reached $OD_{600}$ of 0.3 to 0.5, 1-mL volumes were extracted, cells were collected by centrifugation, and pellets were frozen overnight. $\beta$-galactosidase assays were conducted as previously described (38). Each strain was tested in duplicate, and each experiment was conducted at least two times.

**DNA Footprinting.** Potassium permanganate footprinting (39) was conducted using supercoiled plasmid (pSM106) DNA in a 20-$\mu$L reaction. Each reaction contained 285 nM _B. subtilis_ RNAP and equimolar concentrations of YvrI and YvrHa. Reactions were started by the addition of 1 $\mu$L of 40 mM $KMnO_4$ and were stopped after 3 min by the addition of 1 $\mu$L of $\beta$-mercaptoethanol. Oxidized nucleotides were detected using primer extension as previously described (24).

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