A Novel RNA Polymerase-binding Protein that interacts with a Sigma-Factor Docking Site

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ABSTRACT

Sporulation in *Bacillus subtilis* is governed by a cascade of alternative RNA polymerase sigma factors. We previously identified a small protein Fin that is produced under the control of the sporulation sigma factor $\sigma^F$ to create a negative feedback loop that inhibits $\sigma^F$-directed gene transcription. Cells deleted for *fin* are defective for spore formation and exhibit increased levels of $\sigma^F$-directed gene transcription. Based on pull-down experiments, chemical crosslinking, bacterial two-hybrid experiments, and nuclear magnetic resonance chemical shift analysis, we now report that Fin binds to RNA polymerase and specifically to the coiled-coil region of the $\beta'$ subunit. The coiled-coil is a docking site for sigma factors on RNA polymerase, and evidence is presented that the binding of Fin and $\sigma^F$ to RNA polymerase is mutually exclusive. We propose that Fin functions by a mechanism distinct from that of classic sigma factor antagonists (anti-$\sigma$ factors), which bind directly to a target sigma factor to prevent its association with RNA polymerase, and instead functions to inhibit $\sigma^F$ by competing for binding to the $\beta'$ coiled-coil.

INTRODUCTION

RNA polymerase (RNAP) holoenzyme in bacteria principally consists of the subunits $\beta$, $\beta'$ and $\alpha$, which constitute the core enzyme, and one of several alternative sigma factors, which mediates promoter recognition. Transcription initiation depends on the interaction between the sigma subunit and the core enzyme in part via the highly conserved $\alpha$ helical region of sigma factors known as 2.2 and a coiled-coil motif in the $\beta'$
subunit (also called the β’ clamp helices). This interaction is essential for holoenzyme formation and is also necessary for sigma region 2.4 to be in the proper orientation to bind to the -10 promoter element and for promoter melting to occur during transcription initiation (Arthur et al., 2000; Arthur and Burgess, 1998; Young et al., 2001; Young et al., 2004). Additionally, region 4 of the sigma subunit must interact with the β-flap domain of the β subunit in order to be positioned correctly to contact the promoter -35 element (Kuznedelov, 2002). Transcription initiation in bacteria can be regulated by DNA-binding proteins that augment or impede the ability of RNAP holoenzyme to bind to, and initiate transcription from, promoters. In addition, transcription initiation can be modulated by a variety of proteins that either influence holoenzyme formation or function in the context of the preassembled holoenzyme, typically by targeting sites of interaction between the core enzyme and the sigma subunit (reviewed in Browning and Busby, 2016).

Here we report on a novel RNAP-binding protein that inhibits the function of a sigma factor by targeting the coiled-coil region of the β’ subunit.

A well-studied example of an RNAP-binding protein that interferes with the function of a sigma factor is the phage T4 protein AsiA. Unlike classic anti-σ factors that function by sequestering a target sigma factor, AsiA binds to the E. coli RNAP holoenzyme containing the house-keeping sigma factor σ70 and alters the enzyme’s promoter recognition properties. In particular, AsiA makes direct contact with both σ70 region 4 and the β-flap (Yuan et al., 2009), thereby preventing σ70 region 4 from engaging the –35 element and so inhibiting transcription from the canonical –10/–35 class of promoters (Baxter et al., 2006; Gregory et al., 2004; Lambert et al., 2004; Simeonov et al., 2003; Yuan et al., 2009). Another phage protein that targets σ70-
containing RNAP is the P7 protein of phage Xp10. P7 inhibits transcription by interacting with both the β-flap and the first 10 residues of the β’ subunit to displace σ70 from core enzyme upon promoter engagement (Liu et al., 2014).

Here we describe how a protein that is produced during the developmental process of sporulation in *Bacillus subtilis* interacts with RNAP to regulate sigma factor utilization. Sporulation involves the formation of an asymmetrically positioned septum that partitions the developing cell into small (forespore) and large (mother cell) compartments. Sporulation is governed by a hierarchical cascade of alternative sigma factors with two of the factors (σF and σG) appearing successively in the forespore compartment and two (σE and σK) appearing successively in the mother cell (reviewed in Piggot and Hilbert, 2004; Piggot and Losick, 2002). We previously reported that σF turns on a gene named fin that feedback-inhibits σF activity. Cells deleted for fin are defective for spore formation and exhibit increased σF-directed gene transcription, suggesting that Fin is an inhibitor of σF (Camp et al., 2011). We now report that Fin interacts with the coiled-coil region of the β’ subunit of RNAP and that binding of Fin and of σF to RNAP appear to be mutually exclusive. We propose that Fin is not a canonical anti-sigma factor but instead inhibits σF by competing for binding to the sigma factor region 2 docking site on RNAP. Fin does not appear to bear significant sequence similarity to other proteins, and its solution structure shows that Fin is structurally distinct from other RNAP-binding proteins.
RESULTS AND DISCUSSION

Fin does not bind to $\sigma^F$

Cells lacking fin exhibit increased $\sigma^F$-dependent transcription and are defective in sporulation (Camp et al., 2011). Use of an insertion of an antibiotic-resistance gene in fin ($\Delta fin::phleo$) had indicated that the sporulation defect was $\sim$50-fold (Camp et al., 2011), but the use of an in-frame (markerless) deletion ($\Delta fin$) now indicates that the defect is $\sim$10-fold (Figure S1). A simple interpretation of the inhibitory effect of Fin is that it acts by binding to $\sigma^F$; indeed, Fin somewhat resembles the anti-sigma factor CsfB (Gin), which interacts with and inhibits both $\sigma^F$ (a sporulation sigma factor active in the mother cell) and $\sigma^G$ (a later-acting sporulation sigma factor in the forespore that is related to $\sigma^F$) (Camp et al., 2011; Karmazyn-Campelli et al., 2008; Serrano et al., 2015). To investigate this possibility, we used a bacterial two-hybrid system (Agilent) in an effort to detect an interaction between Fin and $\sigma^F$. In this system, protein-protein interactions in a reporter E. coli strain are indicated by the expression of a HIS3 reporter cassette, which allows cell growth on selective medium lacking histidine. However, our efforts to detect an interaction between Fin and $\sigma^F$ were unsuccessful (Figure S2A). A yeast two-hybrid assay similarly yielded negative results (Figure S2B). For comparison, the known anti-$\sigma^F$ factor SpoIIAB interacted with $\sigma^F$ and CsfB with $\sigma^G$ in these assays (Figure S2).

Fin binds to RNA polymerase

We therefore undertook an unbiased approach to investigate how Fin acts by attempting to identify proteins that interact with Fin using a pull-down assay with purified His6-tagged Fin (His-Fin) and clarified lysates from mutant cells lacking Fin
(Δfin cells) grown under sporulation-inducing conditions. The His-Fin construct was functional as judged by its ability to complement the Δfin mutation, restoring sporulation to wild-type levels (Figure S1).

Strikingly, His-Fin pulled down RNAP from the lysate and did so with high selectivity as judged by Coomassie staining and as confirmed by immunoblot analysis with anti-RNAP antibodies (Figure 1) and mass spectrometry (data not shown). As a control, a His₆-tagged mutant of Fin bearing a C-terminal truncation (His-FinΔ₆⁴⁷₆) was markedly impaired in its ability to pull down RNAP (Figure 1). The observed interaction of Fin with RNAP did not depend on a sporulation-specific component or modification as similar results were obtained with pull-down experiments done with lysates of vegetatively growing cells (Figure S3). Furthermore, consistent with the two-hybrid results, His-Fin did not pull down σF or σG (or σA), as their presence above background was not detected in the pull-down experiments by mass spectrometry or by immunoblot analysis with antibodies to those sigma factors (data not shown).

**Fin crosslinks to the β' subunit**

We used crosslinking to determine which RNAP subunit(s) is involved in binding Fin. Heterobifunctional crosslinkers were chosen in order to minimize crosslinking Fin to itself and to avoid crosslinking RNAP subunits to each other. We used NHS-diazirine crosslinkers of two different lengths: SDA (succinimidyl 4,4'-azipentanoate, 3.9 Å) and LC-SDA (succinimidyl 6-(4,4'-azipentanamido) hexanoate, 12.5 Å).

First, purified His-Fin was incubated with a crosslinker to crosslink primary amines to the NHS end of the crosslinker. Next, a cleared lysate from sporulating cells
that lacked Fin and harbored a FLAG-tag on the β’ subunit (RpoC-FLAG) was prepared and incubated with the crosslinker-treated His-Fin. The diazirine end of the crosslinker was then activated via UV exposure. His-tag affinity magnetic beads were then used to pull on His-Fin, and elutions were analyzed by SDS-PAGE and immunoblot analysis with antibodies raised against RNAP core enzyme, the β subunit, and the FLAG tag.

Use of either the LC-SDA or SDA crosslinker revealed a shift in the mobility of a portion of the β’ molecules and in a manner that depended on addition of the crosslinker (Figure 2A, B). Similar results were obtained with the His₆ tag at the C-terminus of Fin (Fin-His) and with the His₆ tag separated from the C-terminus with a tri-glycine linker (Fin-GGG-His) (Figure 2A, B). We interpret these results to indicate that RNAP-bound His-Fin was in close enough proximity to β’ for crosslinking to occur and in a manner that was independent of the placement of the His₆ tag. In the case of the β subunit there appeared to be a faint doublet in the SDA-treated samples that was at least partially dependent on the addition of the crosslinker (Figure 2B). Attempts to improve the separation of the apparently shifted band from β were unsuccessful. We do not rule out the possibility that His-tagged Fin can crosslink with β, but the results were not as clear as in the case of β’.

Finally, we were unable to detect any indication of crosslinking with α, as there was no shifted band that appeared above α with either the LC-SDA (Figure 2C) or SDA crosslinker. Also, because of the relatively small size of α as compared to β and β’, a crosslinked product should have readily been detected had it occurred.

**Fin interacts with the coiled-coil region of β’**
To localize the region of interaction of Fin with RNAP, we used a bacterial two-hybrid system (Dove and Hochschild, 2004) with a library of *E. coli* RNAP fragments (“coreome”) fused to αNTD as the “prey” and Fin fused to λCI as the “bait”. A protein-protein interaction results in β-galactosidase production. Although Fin interacted weakly with *E. coli* RNAP (as His-Fin pulled down less RNAP out of an *E. coli* lysate compared to a *B. subtilis fin* lysate), the two-hybrid assay revealed that out of all the fragments tested, the larger of two overlapping *E. coli* (Ec) β’ fragments (Ec β’ residues 249-328) showed a modest level of interaction with Fin (Figure 3). This fragment contained a region of β’ called the coiled-coil (or β’ clamp helices), which is required for sigma binding to RNAP and for sigma to make functional contact with the -10 promoter region (Arthur and Burgess, 1998; Young et al., 2001; Young et al., 2004).

Following up on this clue, we tested the corresponding *B. subtilis* (Bsu) β’ coiled-coil fragments: a minimal coiled-coil-containing fragment (residues 251-298) and a larger fragment (residues 238-317; corresponding to Ec β’ residues 249-328). The two-hybrid assay using *B. subtilis* fragments revealed that Fin bound to both *B. subtilis* β’ coiled-coil fragments and in both prey and bait orientations with β’ \(^{238-317}\) (Figure 4). A particularly robust interaction was observed between λCI-Fin and αNTD-β’ \(^{238-317}\) (Figure 4A). We favor the view that the β’ coiled-coil is the main site of interaction because Fin was able to interact with the fragment β’ \(^{251-298}\), which contains only the β’ coiled-coil (Figure 4A). However, we do not rule out contributions from the β’ lid (residues 240-253) or β’ rudder (residues 297-314), as the interaction was more robust with the larger fragment β’ \(^{238-317}\) (Figure 4).
We also confirmed that the *B. subtilis* β’ coiled-coil interacted with region 2 of σ^A, σ^F, and σ^G (Figure S4). Consistent with previous data, no interaction between Fin and region 2 of those sigma factors was detected (Figure S5).

**NMR structure of Fin**

To investigate the interaction between Fin and the β’ coiled-coil further, the solution structure of Fin was solved by NMR. We used a mutant Fin with a three-residue C-terminal truncation (Fin^{Δ74-76}) because it was better behaved in solution than the full-length protein and because NMR analysis confirmed that the last three residues were unstructured (see Supporting Information and Figures S6 and S7). The Fin^{Δ74-76} protein was proficient for interaction with RNAP in pull down assays. The structure (Figure 5A; PDB Accession Number: 5MSL; structural statistics in Table S2) showed a main folded region and a long intrinsically disordered loop that sits apart from the rest of the structure. The folded region resembles a psi-loop motif (Hutchinson and Thornton, 1990) and includes residues 3-15 and 49-64. These residues form a C-terminal alpha helix (α_2: residues 55-64) and a short β-sheet that consists of two well-defined parallel β-strands (β_1: residues 4-8; β_3: residues 49-53) and a distorted antiparallel β-strand (β_2: residues 12-14) (Figure 5A). The distortion is probably due to the presence of two flanking glycine residues Gly11 and Gly15 (Figure 5A). A long loop between the β_2 and β_3 strands that sits apart from the main fold appears disordered except for an alpha helical region in the middle of the loop (α_1: residues 35-40) (Figure 5A).

An interesting feature of the main folded region is the coordination of a zinc molecule by two pairs of cysteine residues (Cys7 and Cys10 in the loop between β_1 and
(β₂ and Cys55 and Cys58 in the first turn of the α₂ helix) (Figure 5B). The presence of a zinc cation at equimolar concentration was confirmed by inductively coupled plasma mass spectrometry (ICP-MS). Additional zinc finger coordination sphere parameters are summarized in Table S3. This confirms our previous prediction that Fin binds zinc due to its perfectly conserved CXXC motifs, an attribute commonly present in zinc-binding proteins like CsfB (Camp et al., 2011).

The structure of Fin appears to be unique as no structural homology matches were detected using the eFOLD program from PDBe.

**NMR chemical shift analysis reveals Fin residues involved in binding to the β’ coiled-coil**

Using the structure, we were able to characterize the interaction between unlabeled β^{238-317} and 15N-labeled Fin^{Δ74-76} by using chemical shift analysis. At low concentrations of β^{238-317}, chemical shift perturbations of Fin^{Δ74-76} residues could be detected, especially for Gly15, Glu45, and the residues near those positions (Figure S8). Residues that exhibited large shifts were located in the N-terminal portion of the protein (highlighted in the cartoon representation of the structure in Figure 5C). In particular, both Gly15 and Glu45 are located in the long unstructured loop between the β₂ and β₃ strands: Gly15 is in the loop directly following the β₂ strand, and Glu45 is located in the loop between the α₁ helix and β₃ strand (Figure 5C). These results reinforce the evidence presented above that Fin directly contacts the β’ coiled-coil region and also indicate the identity of residues that are at or near the contact site.
Purified RNAP bound to Fin does not contain $\sigma^F$

Because Fin binds to the $\beta^*$ coiled-coil, a region critical for holoenzyme formation, we next asked whether Fin binding to RNAP core prevented $\sigma^F$ from binding to RNAP. We compared how much $\sigma^F$ was associated with RNAP versus Fin-bound RNAP by doing a pull-down assay with purified FLAG-tagged or His$_6$-tagged RNAP (RNAP-FLAG and RNAP-His, respectively, in which the tag was fused to the C-terminal end of RpoC), purified His-Fin, and purified $\sigma^F$. In one sample, $\sigma^F$ and RNAP-His were incubated together, and His-affinity magnetic beads were applied to pull down RNAP-His. In another sample, $\sigma^F$, RNAP-FLAG, and His-Fin were incubated together, and His-affinity magnetic beads were applied to pull down His-Fin. Elution fractions were analyzed by immunoblot using a mixture of anti-RNAP and anti-$\sigma^F$ antibodies (Figure 6).

A strong signal for $\sigma^F$ was detected when RNAP was pulled down directly and in the absence of Fin (lane 4), whereas there were only background levels of $\sigma^F$ associated with RNAP when RNAP was pulled down via His-Fin (lanes 5 and 6). Similar results were observed in a variation of this experiment that used sporulation lysates instead of purified RNAP and purified $\sigma^F$. Specifically, there was more endogenous $\sigma^F$ associated with RNAP when RNAP-His was pulled down directly from a sporulation lysate than when comparable amounts of RNAP were pulled down with His-Fin (Figure S9).

These results are consistent with the idea that Fin binding and $\sigma^F$ binding (and presumably the binding of other sigma factors) are mutually exclusive. Efforts to test this inference functionally via in vitro transcription assays met with limited success in part because Fin is prone to aggregate and undergo degradation, making it difficult to work with biochemically.
Nonetheless, given that Fin binds to a site critical for the binding of sigma factors to RNAP and given that the production of Fin during sporulation inhibits σ^F-directed gene expression, the simplest interpretation of our findings is that Fin restricts σ^F activity during sporulation in whole or in part by interfering with the binding of the sporulation sigma factor to RNAP core enzyme. Thus, we propose that Fin, rather than acting as a canonical anti-sigma factor that binds to its cognate sigma factor, is part of a negative feedback loop that antagonizes σ^F in the forespore by competing with σ^F for binding to a common docking site on RNA polymerase.

That Fin acts by binding to the sigma factor docking site on core RNAP leaves unresolved the remaining issue of how σ^F is replaced by σ^G in the forespore (Camp et al., 2011). One possibility is that σ^G outcompetes Fin more effectively than σ^F does. σ^G is under positive autoregulation (Karmazyn-Campelli et al., 1989; Sun et al., 1991) and may outcompete Fin by enhancing its own synthesis and/or by having a higher binding affinity for core RNAP than σ^F has. Finally, it is possible that other yet-to-be identified sporulation proteins help mediate the switch to the later-acting transcription factor.

**EXPERIMENTAL PROCEDURES**

**General methods**

*Escherichia coli* strain XL1-Blue was used for propagating plasmids, and grown and transformed using standard procedures (Sambrook et al., 1989). *E. coli* BL21 (DE3) was used for the expression and purification of recombinant proteins. *B. subtilis* strains used in this work are listed in Table 1. Transformation of *Bacillus* was done as previously described (Wilson and Bott, 1968).
Bacterial strains were propagated in Luria-Bertani medium. When appropriate, antibiotics were included at the following concentrations: chloramphenicol (5 µg/ml for *B. subtilis* or 25 µg/ml for *E. coli*), erythromycin plus lincomycin (MLS) (1 µg/ml and 25 µg/ml respectively), spectinomycin (100 µg/ml), kanamycin (5 µg/ml for *B. subtilis* or 50 µg/ml for *E. coli*), phleomycin (0.4 µg/ml), and ampicillin (100 µg/ml).

**Sporulation assays**

To measure sporulation efficiency, cells were induced to sporulate by nutrient exhaustion for 25 hours at 37°C in Difco (Schaeffer’s) sporulation medium (DSM) (Nicholson and Setlow, 1990; Schaeffer et al., 1965). The number of colony-forming units (CFUs) that survived heat treatment (80°C for 20 minutes) was determined and normalized to the number of heat-resistant CFUs obtained in parallel from the wild-type strain. For all other experiments, sporulation was induced at 37°C by the Sterlini-Mandelstam resuspension method (Nicholson and Setlow, 1990; Sterlini and Mandelstam, 1969) with the modification of using 25% LB instead of CH medium. β-galactosidase activity was measured as previously described measured in a Synergy 2 plate reader (BioTek) (Camp and Losick, 2009). β-galactosidase activity is reported in arbitrary (AU) units as the rate of 2-nitrophenyl β-D-galactopyranoside (ONPG) hydrolysis (i.e. $V_{\text{max}}$, with units of OD$_{420}$ per minute) divided by the optical density at 600 nm (OD$_{600}$) of the culture at the time of collection.

**Strain and plasmid construction**

*B. subtilis* strains used in this study were derived by transformation of the prototrophic laboratory strain PY79 (Youngman et al., 1984) or derivatives thereof with chromosomal DNA, plasmids, or polymerase chain reaction (PCR) products. The genes
used to confer resistance of *B. subtilis* to antibiotics are as follows: *cat* (chloramphenicol), *erm* (erythromycin plus lincomycin), *spc* (spectinomycin), *kan* (kanamycin), and *phleo* (phleomycin). Competent *B. subtilis* cells were prepared as previously described (Wilson and Bott, 1968). Unless otherwise noted, PY79 chromosomal DNA served as a template for PCR amplification. Plasmids were cloned and propagated in the *E. coli* strain XL1-Blue. Plasmid mutagenesis was performed with the QuikChange II XL site-directed mutagenesis kit (Stratagene).

Strains with markerless deletions were constructed using derivatives of pMiniMad2 according to methods adapted from that which were previously described (Arnaud et al., 2004; Patrick and Kearns, 2008). Briefly, the recipient *B. subtilis* strain was transformed with a derivative of pMiniMad2 harboring sequences homologous to regions in the chromosome flanking the intended site of deletion. ~10 colonies selected on LB/MLS were picked and grown together in LB at 25 °C for 1-3 days to allow the plasmid to loop out. Cells were then grown for ~1 days at 37 °C in LB to cure the cells of the plasmid completely and then plated on LB agar. Single colonies were picked and the deletion was verified by sequencing. MLS sensitivity was also checked by patching onto LB/MLS plates.

Plasmid construction and cloning was done using either traditional restriction enzyme methods or with isothermal assembly (Gibson et al., 2009). Plasmid digests were done with restriction enzymes (NEB or Thermo) for ~2-3 h at the appropriate temperature and also treated with calf intestinal phosphatase (NEB) for 30 min at 37 °C.

The genotypes, features, and sources of strains and plasmids used in this study are listed in Table 1. Primers used in strain and plasmid construction were synthesized by
IDT or Thermo and sequences are provided in Table S1. Strain and plasmid construction details are described in Supporting Information.

**Preparation of clarified lysates**

500 ml of *B. subtilis* culture was pelleted at 5000 g for 10 min and was stored at -80 °C. To lyse, pellets were incubated in 1/10th culture volume of lysis buffer (200 mM NaCl, 50 mM Tris pH 8, 2 mM β-mercaptoethanol (β-ME), 5 mM imidazole, 1 mg/mL lysozyme, 1 cOmplete ULTRA mini EDTA-free protease inhibitor tablet (Roche) per 10 ml buffer) for 1-2 h at room temperature with rocking. The lysate was then sonicated on ice for up to 15 min total of sonication time, alternating in 1 min intervals between sonication and rest. Cell debris was cleared by centrifugation at 14,000 rpm for 30 min at 4 °C in a SS-34 or F21S-8x50y rotor and stored overnight at 4 °C. The supernatant was passed through a 0.2 µm filter before use. Clarified lysate protein concentration was measured by Bradford assay (Bio-Rad, (Bradford, 1976)). Lysates compared to each other in pull down assays had protein concentrations adjusted to match with filtered lysis buffer if necessary.

**Protein expression and purification**

*E. coli* BL21 (DE3) derivative strains were used for the overexpression and purification of recombinant Fin and Fin derivatives. The His6-tagged Fin could functionally replace native Fin in vivo. For details on the expression and purification of σF and proteins used for NMR studies, please see Supporting Information. Cells were grown at 37 °C until OD600 ~ 0.8-1 and protein expression was induced with 1 mM IPTG. Cells were then grown at 25 °C and harvested after 4 h by centrifugation at 5000 g for 10 min.
Cells were lysed using BugBuster Master Mix® (Novagen) and incubated at room temperature for 2-3 h with rocking. Lysates were cleared by centrifugation at 16,000 g for 25 min at 4 °C. The cleared lysate was also filtered through a 0.2 µm filter. His-tagged proteins were purified by incubating with Ni-NTA resin (Qiagen or Thermo) for 1 h at 4 °C rotating and then transferred to Polyprep columns (Bio-Rad). Resin was washed with at least 10 column volumes (CV) of buffer A (200 mM NaCl, 50 mM Tris pH 8, 2 mM β-ME) + 5 mM imidazole, 10 CV of buffer A + 20 mM imidazole, and 1 CV of buffer A + 50 mM imidazole. Protein was eluted with buffer A + 200 mM imidazole in 0.5 CV fractions. Fractions were analyzed by SDS-PAGE, pooled, buffer exchanged to buffer A using Zeba 7K spin desalting columns (Thermo), and stored at 4 °C. Proteins were typically prepared fresh the day before use.

**His-tagged RNAP**

*B. subtilis* strain AWB220, which encodes a His6-tagged β' subunit as the only copy in the cell in a ∆fin background, was constructed, and RNAP-His was purified from crude lysates (from vegetative or cells harvested at 3 hours into sporulation) similar to previously described (Anthony et al., 2000). Protein purification was done using Ni-NTA (Qiagen or Thermo) affinity chromatography purification protocols followed by ion exchange chromatography using a MonoQ 5/50 GL column (GE Healthcare Life Sciences) equilibrated with 240 mM NaCl, 50 mM Tris pH 8, 0.1 mM DTT, 5% glycerol and eluted with a linear gradient from 240 mM NaCl to 1 M NaCl over 45 ml at 0.5 ml/min. Fractions were analyzed by SDS-PAGE, pooled, concentrated using a 10K Amicon Ultra centrifugal filter (EMD Millipore), and then dialyzed overnight into 200 mM NaCl, 50 mM Tris pH 8, 2 mM β-ME, 50% glycerol and stored at -20°C.
FLAG-tagged RNAP

*B. subtilis* strain AWB218, which encodes a FLAG-tagged β’ subunit as the only copy in the cell in a Δfin background, was constructed, grown under sporulating conditions, and harvested at 3 hours into sporulation. RNAP-FLAG was purified from crude lysates made from a 2 L culture using 4.4 ml FLAG magnetic beads (Sigma) equilibrated in wash buffer (300 mM NaCl, 50 mM Tris pH8, 0.01% Tween-20). Beads were washed 4 times with a total of 20 column volumes of wash buffer. Beads were eluted with 3x FLAG peptide (Apexbio) by incubating for 30 min at 4 °C, 3 times with a total of 5 column volumes of elution buffer (0.3 mg/ml 3x FLAG peptide in 200 mM NaCl, 50 mM Tris pH8). Elutions were pooled, concentrated, and further purified using ion exchange chromatography as described above. Dialysis and storage of purified protein was performed as described above.

Binding assays

50 µl (2mg) His-tag Dynabeads (Life Technologies) were incubated with 700 µl clarified lysate for at least 1 h at 4 °C rotating to allow binding. Manipulation of the beads was done using a magnetic tube rack half-submerged in ice-water to keep the protein samples cold. Wash steps were done according to manufacturer’s protocols with wash buffer (200 mM NaCl, 50 mM Tris pH8, 5 mM imidazole, 0.01% Tween-20). Samples were eluted off the His beads by incubation in 100 µl elution buffer (200 mM NaCl, 50 mM Tris pH8, 400 mM imidazole, 0.01% Tween-20) and gentle agitation by hand for 5 min at room temperature, followed by 10 min incubation on ice. 1 µl of load and flow-through fractions and 9 µl of wash and elution fractions were mixed with protein sample buffer (Amresco) and water (if necessary) to a final volume of 12 µl per
sample, run on SDS-PAGE Next Gels (Amresco), transferred onto PVDF membranes (Millipore) and probed with primary antibodies anti-RNAP (Traag et al., 2013), anti-σF (Decatur and Losick, 1996), anti-FLAG (Sigma), or anti-β [8RB13] (Abcam) and the secondary antibody goat anti-rabbit-HRP (Bio-Rad). Images were developed using film, the ChemiDoc™ XRS+ System (Bio-Rad), or the Azure Imaging System (Azure Biosystems).

**Crosslinking**

NHS-Diazirine crosslinkers (Thermo) of two lengths were used: SDA (succinimidyld 4,4'-azipentanoate, 3.9 Å) and LC-SDA (succinimidyld 6-(4,4'-azipentanamido) hexanoate, 12.5 Å). His-Fin and derivatives were purified as described above but then buffer exchanged into phosphate buffered saline (PBS) (Lonza). SDA was solubilized in DMSO at 5 mg/ml, and LC-SDA was solubilized in DMSO at 7 mg/ml. Purified proteins were treated with 20-fold molar excess of crosslinker by incubation for 2 h at 4 °C rotating and light-protected. The reaction was quenched with adding 10% reaction volume of 1 M Tris pH 8 and 15 min incubation on ice. Excess crosslinker was removed by Zeba 7K spin desalting column and exchanging the crosslinker treated proteins into buffer A. Crosslinker-treated His-Fin and derivatives were then incubated with *B. subtilis* lysate for 1 h at 4 °C rotating light protected. The protein + lysate solution was then distributed into a clear 96-well plate placed on ice and exposed to UV irradiation at 365 nm for 15 min using a 8 W lamp. Pull downs using His-tag Dynabeads (Life Technologies) were then performed as described above.

**Two-hybrid assays**
Two-hybrid assays (Dove and Hochschild, 2004) using the reporter strain FW102 OL2-62 (Nickels, 2009) were done as previously described (Deighan et al., 2008; Montero-Diez et al., 2013). β-galactosidase assays to measure protein-protein interaction were done as previously described (Thibodeau et al., 2004). See Supporting Information for details for the BacterioMatch II (Agilent) and Matchmaker Gold yeast two-hybrid systems (Clontech).

NMR

Please see Supporting Information for all methods for NMR studies.
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<tr>
<td>AWB209</td>
<td>Δfin</td>
<td>This study</td>
</tr>
<tr>
<td>AWB218</td>
<td>rpoC::rpoC-FLAG spec Δfin</td>
<td>This study</td>
</tr>
<tr>
<td>AWB220</td>
<td>rpoC::rpoC-his&lt;sub&gt;6&lt;/sub&gt; spec Δfin</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pET28a</td>
<td>IPTG inducible protein expression vector with N or C terminal His&lt;sub&gt;6&lt;/sub&gt; tag and thrombin cleavage site. This vector has no insert.</td>
<td>Novagen</td>
</tr>
<tr>
<td>pMiniMad2</td>
<td>For markerless mutations in the chromosome</td>
<td>(Patrick and Kearns, 2008), (Arnaud et al., 2004)</td>
</tr>
<tr>
<td>pACαCI-β-flap (831-1057)</td>
<td>P&lt;sub&gt;lacUV5&lt;/sub&gt;-directed synthesis of the λCI protein fused via three alanines to residues 831-1057 of the β subunit of E. coli RNAP</td>
<td>(Deighan et al., 2008)</td>
</tr>
<tr>
<td>pBRα-β flap (831-1057)</td>
<td>P&lt;sub&gt;lacUV5&lt;/sub&gt; and P&lt;sub&gt;lpp&lt;/sub&gt;-directed synthesis of the αNTD (residues 1-248 of the α subunit of E. coli RNAP) fused via three alanines to residues 831-1057 of the β subunit of E. coli RNAP.</td>
<td>(Deighan et al., 2008)</td>
</tr>
<tr>
<td>pAW163</td>
<td>Expression vector for N-terminally His&lt;sub&gt;6&lt;/sub&gt;-tagged Fin</td>
<td>This study</td>
</tr>
<tr>
<td>pAW199</td>
<td>Vector to make markerless Δfin</td>
<td>This study</td>
</tr>
<tr>
<td>pAW203</td>
<td>Expression vector for N-terminally His&lt;sub&gt;6&lt;/sub&gt;-tagged FinΔ64-76</td>
<td>This study</td>
</tr>
<tr>
<td>pAW226</td>
<td>Expression vector for periplasmic expression of N-terminally StrepII-tagged SigF</td>
<td>This study</td>
</tr>
<tr>
<td>pAW301</td>
<td>Expression vector for C-terminally His&lt;sub&gt;6&lt;/sub&gt;-tagged Fin with a triglycine linker before the tag</td>
<td>This study</td>
</tr>
<tr>
<td>pAW302</td>
<td>Expression vector for C-terminally His&lt;sub&gt;6&lt;/sub&gt;-tagged Fin with no linker before the tag</td>
<td>This study</td>
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<td>pAW306</td>
<td>P&lt;sub&gt;lacUV5&lt;/sub&gt;-directed synthesis of the λCI protein fused via three alanines to residues 251-298 of the β’ subunit of B. subtilis RNAP</td>
<td>This study</td>
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<tr>
<td>pAW307</td>
<td>P&lt;sub&gt;lacUV5&lt;/sub&gt; and P&lt;sub&gt;lpp&lt;/sub&gt;-directed synthesis of the αNTD (residues 1-248 of the α subunit of E. coli RNAP) fused via three alanines to residues 251-298 of the β’ subunit of B. subtilis RNAP</td>
<td>This study</td>
</tr>
<tr>
<td>pAW308</td>
<td>P&lt;sub&gt;lacUV5&lt;/sub&gt;-directed synthesis of the λCI protein fused via three alanines to Fin</td>
<td>This study</td>
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<td>pAW309</td>
<td>P&lt;sub&gt;lacUV5&lt;/sub&gt; and P&lt;sub&gt;lpp&lt;/sub&gt;-directed synthesis of the αNTD (residues 1-248 of the α subunit of E. coli RNAP) fused via three</td>
<td>This study</td>
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alanines to Fin

pAW316

\( P_{\text{lac}} \)-directed synthesis of the \( \lambda CI \) protein fused via three alanines to residues 238-317 of \( \beta' \) subunit of \( B. subtilis \) RNAP

This study

pAW317

\( P_{\text{lac}} \) and \( P_{\text{pp}} \)-directed synthesis of the \( \alpha \text{NTD} \) (residues 1-248 of the \( \alpha \) subunit of \( E. coli \) RNAP) fused via three alanines to residues 238-317 of \( \beta' \) subunit of \( B. subtilis \) RNAP

This study

\( ^a \) All \( B. subtilis \) strains are isogenic with PY79
REFERENCES


Youngman, P., Perkins, J.B., and Losick, R. (1984). Construction of a cloning site near one end of Tn917 into which foreign DNA may be inserted without affecting
transposition in *Bacillus subtilis* or expression of the transposon-borne *erm* gene. Plasmid 12, 1-9.

FIGURE LEGENDS

**Figure 1. His-tagged Fin interacts with RNAP.** Clarified sporulation lysate of cells harboring a fin deletion (∆fin, strain AWB209) harvested at 3 hours into sporulation (T₃) was incubated with 80 µg purified His₆-Fin or His₆-Fin with a C-terminal truncation (His-Fin₁⁶⁴₋₇₆). His-affinity magnetic beads were added to pull down His₆-Fin or His₆-Fin₁⁶⁴₋₇₆. Beads were washed and eluted. Samples were run on 12.5% SDS-PAGE Sprint Next Gel stained with Coomassie (top) or transferred to PVDF membranes and immunoblotted by co-incubation with anti-RNAP and anti-σ⁹⁷ antibodies on the same membrane (bottom). Lanes: (1) MW marker in kDa; (2-4) Load (lysate + His-Fin or His-Fin₁⁶⁴₋₇₆); (5-7) Final wash; (8-10) Elution.

**Figure 2. Fin crosslinks to the β’ subunit of RNAP.** Purified N-terminally His-tagged Fin (His-Fin), C-terminally His-tagged Fin with or without a tri-glycine linker before the tag (Fin-GGG-His, Fin-His, respectively) were attached to the NHS end of the LC-SDA (panel A, C) or SDA (panel B) crosslinker. Clarified lysate from cells at hour 3 of sporulation that harbored a FLAG-tag at the C-terminus of the β’ subunit (rpoC::rpoC-FLAG spc ∆fin, strain AWB218) was incubated with 7.16 nmol crosslinker-treated His-Fin, Fin-GGG-His, or Fin-His. The diazirine end of the crosslinker was then activated by UV exposure. His-affinity magnetic beads were added to pull down His-Fin, Fin-GGG-His, or Fin-His. Beads were washed and eluted. Samples were run on 7.5% SDS-PAGE Next Gel, transferred onto PDVF membrane, and immunoblotted with anti-FLAG, anti-β, or anti-RNAP antibodies. Lanes: AWB218 lysate, Load (1); Elutions after pulling on His-Fin (2-3), Fin-GGG-His (4-5), or Fin-His (6-7). (A) Fin crosslinks to β’ subunit with
the LC-SDA crosslinker. Anti-FLAG (top) or anti-β (middle; a shorter exposure (s.e.) shown at bottom) immunoblots of elutions after pulling down His-Fin, Fin-GGG-His, or Fin-His not treated (-) or treated (+) with LC-SDA. (B) Fin crosslinks to β’ subunit with the SDA crosslinker. Anti-FLAG (top) or anti-β (bottom) immunoblots of elutions after pulling down His-Fin, Fin-GGG-His, or Fin-His not treated (-) or treated (+) with SDA. (C) Fin does not crosslink to α subunit. Anti-RNAP immunoblot of His-Fin elutions not treated (-) or treated (+) with LC-SDA.

Figure 3. Fin interacts with the E. coli β’ coiled-coil. Top is a schematic of the bacterial two-hybrid assay. Reporter cells were co-transformed with a plasmid producing Fin fused to λCI and plasmids producing E. coli RNAP fragments fused to the α-NTD (numbered 1-33 in the table), or the pBRα control plasmid. Cells were cultured in the presence of 20 µM IPTG. A protein-protein interaction results in lacZ reporter gene expression, which was quantified by assaying β-galactosidase activity and is graphed. An interaction was detected between Fin and RNAP fragment #5 (E. coli β’ residues 249-328), a fragment that contains the β’ coiled-coil.

Figure 4. Fin interacts with the B. subtilis (Bsu) β’ coiled-coil. (A) Plasmids producing λCI only or λCI fused to Fin were co-transformed with plasmids producing α-NTD fused to two different β’ coiled-coil fragments (residues 251-298 or residues 238-317) into reporter cells and induced with 50 µM IPTG. Protein-protein interaction results in lacZ reporter gene expression, which was quantified by assaying β-galactosidase activity. (B) Plasmids producing λCI only, or λCI fused to β’ coiled-coil fragments
(residues 251-298 or residues 238-317) were co-transformed with plasmids producing α-NTD fused to Fin or the pBRα control, into reporter cells and induced with 50 µM IPTG. Protein-protein interaction results in lacZ reporter gene expression, which was quantified by assaying β-galactosidase activity.

**Figure 5. Use of NMR to determine the structure of FinΔ74-76 and identify residues that undergo a chemical shift perturbation in response to β’238-317** (A) Orthogonal views of ensemble backbone and cartoon representations showing the 20 lowest energy ARIA-calculated structures as deposited in the PDB (Accession number: 5MSL). (B) Detailed view of the zinc finger coordination shell showing the residues coordinating the zinc cation; hydrogen bonds in the second coordination shell are shown as cyan dashed lines. (C) Representation of Fin residues that undergo a chemical shift perturbation in the presence of β’238-317 using a gradient color scheme from unperturbed to most perturbed (yellow to red).

**Figure 6. Purified RNAP bound to His6-Fin does not contain σF.** FLAG-tagged RNAP (RNAP-FLAG) and His6-tagged RNAP (RNAP-His) were purified from T3 sporulating cells that harbor a FLAG-tag or a His6-tag at the C-terminus of the β’ subunit (rpoC::rpoC-FLAG spc Δfin, strain AWB218; rpoC::rpoC-His6 spc Δfin, strain AWB220) using affinity resin and ion exchange chromatography to remove endogenous σF. 0.12 nmol RNAP-His was incubated with 1.2 nmol purified σF in a total volume of 700 µl. 0.12 nmol RNAP-FLAG was incubated with 1.2 nmol purified σF and 7.16 nmol His-Fin in a total volume of 700 µl. 2 mg His-affinity magnetic beads were added to pull
down either RNAP-His or His-Fin. Beads were washed and bound proteins eluted.

Samples were run on 12.5% SDS-PAGE Next Gel, transferred to PVDF, and

simultaneously immunoblotted with anti-RNAP and anti-$$\sigma^F$$ antibodies on the same

membrane. Lanes: (1) 1 µg purified RNAP-FLAG; (2) 1 µg purified RNAP-His; (3) 0.05

µg purified $$\sigma^F$$; (4) RNAP-FLAG elution; (5) His-Fin elution; (6) non-specific binding of

$$\sigma^F$$ to His magnetic beads.
Figure 1

279x361mm (300 x 300 DPI)
Figure 2

279x361mm (300 x 300 DPI)
Figure 3

279x361mm (300 x 300 DPI)
Figure 5

279x361mm (300 x 300 DPI)
Figure 6

279x361mm (300 x 300 DPI)
Abbreviated summary

During the developmental process of sporulation in *Bacillus subtilis* a cascade of alternative sigma factors associate with RNA polymerase to direct gene expression. We report that Fin, a novel RNAP-binding protein produced during sporulation, inhibits the function of the sporulation sigma factor $\sigma^F$ by competing with the binding of region 2 (Rg2) of $\sigma^F$ to the coiled-coiled region of the $\beta'$ subunit of RNA polymerase ($\beta'$ CC).