Anti-sigma factors

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Anti-σ factors modulate the expression of numerous regulons controlled by alternative σ factors. Anti-σ factors are themselves regulated by either secretion from the cell (i.e. FlgM export through the hook–basal body), sequestration by an anti-anti-σ (i.e. phosphorylation regulated partner-switching modules), or interaction with extracytoplasmic proteins or small molecule effectors (i.e. transmembrane regulators of extracytoplasmic function σ factors). Recent highlights include the genetic description of the opposed σ/anti-σ binding surfaces; the unexpected role of FlgM in holoenzyme destabilization and the finding that folding of FlgM is coupled to σ^{28} binding; the first structure determination for an anti-σ antagonist; and the detailed dissection of two complex partner-switching modules in *Bacillus subtilis*.

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Abbreviations

AA	SpoIIAA
AB	SpoIIAB
ECF	extracytoplasmic function

Introduction

Promoter recognition in bacteria requires that RNA polymerase core enzyme ($\beta\beta'\alpha_2$) associates with a sigma (σ) subunit to form a holoenzyme [1,2]. Most bacteria contain multiple σ factors including both a primary σ factor, controlling essential housekeeping functions, and alternative σ factors, activated by specific signals or stress conditions. Most σ factors (the σ^{70} family) are related in sequence and presumably in structure. The exceptions are members of the σ^{54} family, often controlling aspects of nitrogen metabolism, which are structurally and functionally distinct. Genome sequencing has revealed that the numbers of σ subunits vary greatly between different bacteria: *Mycoplasma genitalium* contains a single σ factor, whereas most other bacterial genomes encode at least three. The proliferation of σ factor paralogs is particularly noteworthy in the Gram-positive lineage including 17 in *Bacillus subtilis* and 13 in *Mycobacterium tuberculosis*. Several of these are associated with known or suspected anti-σ factors.

Alternative σ factors are regulated at the transcriptional, translational, and post-translational levels. One common mechanism is the reversible interaction of σ and a protein inhibitor, designated an anti-σ (reviewed in [3•]). Anti-σ factors figure prominently in regulons controlled by σ^{28} (i.e. flagellar biosynthesis), extracytoplasmic function (ECF) σ factors, and *B. subtilis* σ^{F} (i.e. early sporulation) and σ^{B} (i.e. general stress response). In this review, I summarize progress in the characterization of these and related systems, with an emphasis on results reported in 1997 and 1998.

Regulators interacting with *Escherichia coli* σ^{70}

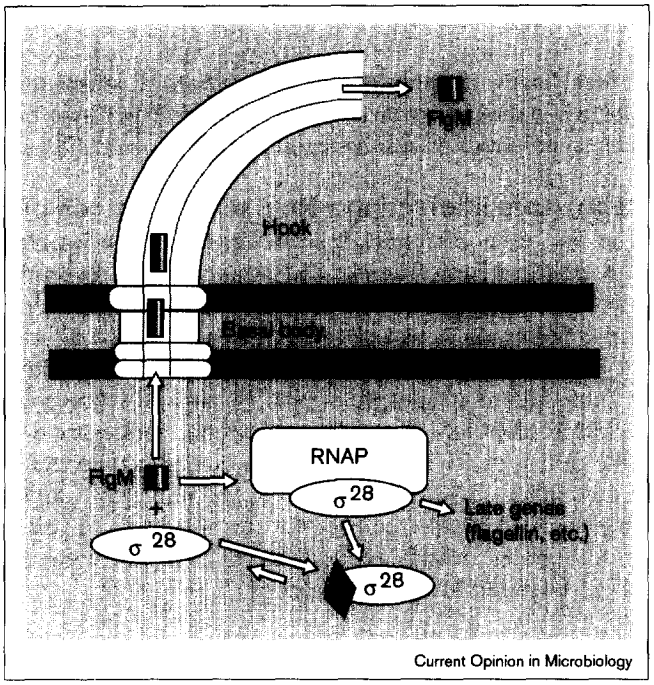
The first demonstration of an inhibitor protein binding to σ emerged from studies of RNA polymerase modification during phage T4 infection (reviewed in [4]). This anti-σ factor, AsiA, associates tightly with *E. coli* σ^{70} and inhibits transcription from both host promoters and early, σ^{70}-dependent phage promoters. Because T4 late transcription depends on a phage-encoded alternative σ (σ^{gp55}), it initially appeared that AsiA might function in σ switching. The story, however, is not this simple, because AsiA is also required for the activation of phage middle genes, which depend on σ^{70} together with the phage-encoded MotA protein [5,6].

Recent biochemical analyses have clarified this mechanism. The small 10.6 kDa AsiA protein binds in a 1:1 complex with the carboxy-terminal 63 amino acids (conserved region 4) of σ^{70} and thereby blocks recognition of the –35 promoter region [7•,8••]. AsiA modified holoenzyme recognizes middle phage promoters containing bound MotA and lacking a –35 recognition region [5,6,9,10•]. It is not yet known whether AsiA also binds to MotA, and whether this interaction is important for middle gene transcription.

Together with previous studies, these results indicate that phage T4 has evolved the ability to recruit and modify the host RNA polymerase for the transcription of both early and middle phage genes, prior to the eventual σ^{gp55}-dependent activation of late genes. In the first stage of infection, T4 ADP-ribosylates the RNA polymerase α subunits which greatly reduces transcription from strong host promoters that require an upstream (UP) element for optimal activity (cited in [7•]). This increases the pool of RNA polymerase available to transcribe phage early genes. In the second step, AsiA selectively inhibits transcription from other σ^{70}-dependent promoters by blocking recognition of the –35 element.

Remarkably, a variant of this mechanism may also function in uninfected *E. coli*. Biochemical fractionation of σ^{70}-associated proteins from stationary phase cells identified a single, specifically associated polypeptide designated Rsd [11•]. Rsd binds to region 4 of σ^{70} and appears to block association with RNA polymerase core enzyme. The effects of Rsd on *in vitro* transcription are modest and appear to be promoter specific, with a maximum observed inhibition of four fold. Since the levels of Rsd *in vivo* are only sufficient to complex 20% of σ^{70}, the role of this protein is not immediately obvious. Ongoing genetic analyses of *rsd* mutants will be needed to establish the role of Rsd *in vivo*.

Figure 1



Regulation of late flagellar genes by export of the FlgM anti-σ. FlgM (square) inhibits the activity of σ^{28} either by binding to free σ^{28} or by inducing dissociation of the σ^{28} holoenzyme [21••]. When bound to σ^{28}, FlgM adopts a partially folded structure (diamond). Once the hook and basal body structure is complete, FlgM can be exported from the cell by the flagellar export machinery [14–16]. FlgM passes through the hollow inner core of the basal–body, hook, and flagellar filament structures [18]. Once σ^{28} transcription commences, flagellin becomes a competing substrate for this export channel and FlgM levels in the cell again begin to rise.

FlgM and the regulation of flagellar gene expression

One of the most dramatic examples of anti-σ factor regulation emerged from analyses of flagellar biogenesis in *Salmonella typhimurium*. The synthesis of late flagellar genes (encoding flagellin and chemotaxis functions) depends on an alternative σ factor of the σ^{28} subfamily (reviewed in [12]). During the early stages of flagellar biogenesis, σ^{28} is held inactive by tight association with FlgM [13]. Once the hook and basal body is assembled, FlgM is secreted by the flagellar export system thereby freeing active σ^{28} [14–16] (Figure 1).

The mechanisms acting to couple secretion of FlgM to completion of the hook–basal body structure are complex. Genetic studies implicate two proteins, FhlB and RflH, as molecular 'gatekeepers' that act to sense, by an as yet unknown mechanism, the completion of the hook [17•]. Even after the hook is complete and FlgM export commences, the FliD, FliS, and FliT proteins still partially inhibit export, and thereby prevent the complete derepression of σ^{28}-dependent flagellar late genes. Finally, *flgE* (encoding hook protein) missense mutations have been identified that leads to conditional (ionic strength sensitive) FlgM secretion [18], which further supports the notion that secretion of flagellar proteins occurs through the hollow channel inside the hook and flagellar filament.

Significantly, high resolution NMR experiments have established that FlgM is largely unfolded in solution [19••] with, at best, transient helical elements [20•]. Upon interaction with σ^{28}, the carboxy-terminal region of FlgM becomes ordered [19••]. It is postulated that this unfolded state may facilitate the passage of FlgM through the narrow channel of the hook–basal body structure.

The mechanism by which FlgM inhibits the activity of the σ^{28} RNA polymerase involves both the sequestration of σ^{28} and the destabilization of existing σ^{28} holoenzyme [21••]. Using surface plasmon resonance, the equilibrium dissociation constant of σ^{28} and FlgM has been estimated at $\sim 2 \times 10^{-10}$ M [21••]. For unknown reasons, this value is much lower than that inferred from NMR analyses [19••]. Genetic experiments suggest that multiple sites of σ^{28}, involving portions of both regions 2 and 4, bind to FlgM [21••].

σ^{28} homologs are widely, but not universally, present in flagellated bacteria [12], and many of these systems contain FlgM. Although the details differ substantially, *flgM* is under complex control often including a σ^{28}-dependent promoter that provides an auto-repressing circuitry. In *S. typhimurium*, *flgM* is also regulated at the translational level by Flk, which acts as a checkpoint for flagellar ring assembly [22,23]. Thus, the mechanisms of morphogenetic coupling that coordinate flagellar assembly include the control of FlgM synthesis (by Flk) in addition to the previously mentioned hook assembly checkpoint that controls FlgM export. In *B. subtilis*, *flgM* is downstream of a competence operon [24]. As a result, *flgM* is expressed under partial control of the ComK transcription factor and this, in part, accounts for the mutually antagonistic expression of the motility and competence regulons.

Regulation of anti-σ factors by partner-switching modules

The *B. subtilis* σ^F and σ^B regulons are both modulated by partner-switching modules involving the mutually exclusive binding of an anti-σ to either of two partners: the corresponding σ or an anti-anti-σ [25]. Although σ^F functions early during sporulation [26] and σ^B regulates a complex stress response [27], both these σ factors and their regulators are paralogs.

Regulation of σ^F activity during sporulation

Prior to sporulation-specific septation, σ^F is held in an inactive complex with its corresponding anti-σ (SpoIIAB; AB for short) via contacts to three conserved σ factor regions: 2.1, 3.1 and 4.1 [28]. Active σ^F is released when AB instead complexes with the antagonist (anti-anti-σ) protein SpoIIAA (AA). In work reviewed elsewhere [26], it was established that the binding of AA to AB is stabilized

by ADP, whereas the AB•σ^F complex is stabilized by ATP. In addition, AB acts as a protein kinase to phosphorylate AA. Phosphorylation inactivates AA thereby allowing AB to inhibit σ^F. Once the sporulation septum forms, AA is dephosphorylated by the membrane-bound SpoIIE phosphatase. This allows AA to bind AB and is a key event in the release of active σ^F in the forespore.

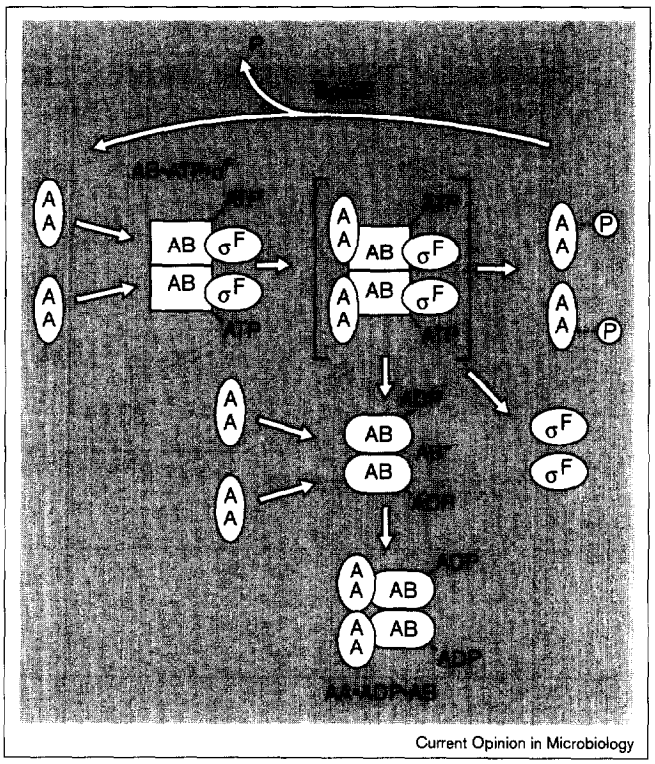
Recent studies provide a more detailed picture of this partner-switching module and support an induced release mechanism for σ^F activation (Figure 2). First, kinetic studies have shown that phosphorylation of AA by AB is very slow (0.005 sec^{-1} [29••,30•]). Once phosphotransfer occurs, AA–P dissociates at a relatively rapid rate (0.017 sec^{-1}) but the remaining AB•ADP complex, designated AB*, is only slowly recycled (0.0002 sec^{-1}; a one hour half-life!) [30•]. This kinetic bottleneck may correspond to the accumulation of stable AA•AB•ADP complexes [31••].

Unexpectedly, studies of AB mutants altered near the ATP-binding domain indicate that kinase activity is needed during the process of AA-mediated release of σ^F (Figure 2). In this induced release model [31••,32], AA is phosphorylated directly by the AB•ATP•σ^F ternary complex, leading to an unstable AB•ADP•σ^F complex that dissociates to liberate σ^F and AB•ADP. The AB•ADP is ultimately trapped in a long-lived complex with AA. Interestingly, genetic experiments have revealed that certain missense mutations in the amino-terminal region of AB specifically impair binding to AA, whereas alanine substitutions at the same positions affect binding to both AA and σ^F [33•]. This suggests that the binding surface used by AB to bind AA overlaps with that used to bind σ^F.

Further work will be needed to sort out the remaining details in this mechanism. Specifically, a direct comparison of the slow rate of AA phosphorylation by AB•ATP with that of the AB•ATP•σ^F ternary complex is needed. In addition, AB is a dimer [34], but AA and σ^F are not. Thus, one can imagine that two AA molecules are needed to fully discharge σ^F from an AB_2•2ATP•2σ^F complex (Figure 2). The resulting AB_2•2ADP complex can either undergo ADP/ATP exchange reactions and rebind σ^F, or bind another two AA monomers to form the 'dead-end' AB_2•2AA•2ADP complex. Inspection of this pathway suggests that inactivation of the dimeric anti-σ, AB_2, requires net consumption of four unphosphorylated AA monomers. Therefore, activation of σ^F may be highly cooperative with respect to the concentration of unphosphorylated AA generated by SpoIIE.

Recent inroads have also been made into the structural analysis of these regulatory components. NMR spectroscopy has allowed the three-dimensional structure of AA to be visualized [35••]. This small protein contains a four-stranded β sheet flanked by four α helices. This structural information, together with genetic data on AA and AB mutant proteins, provides a preliminary glimpse of the likely interaction surfaces (e.g. [33•]). Further structural analyses of AA, and other components of this and related systems, will allow the biochemical basis of partner switching to be elucidated.

Figure 2

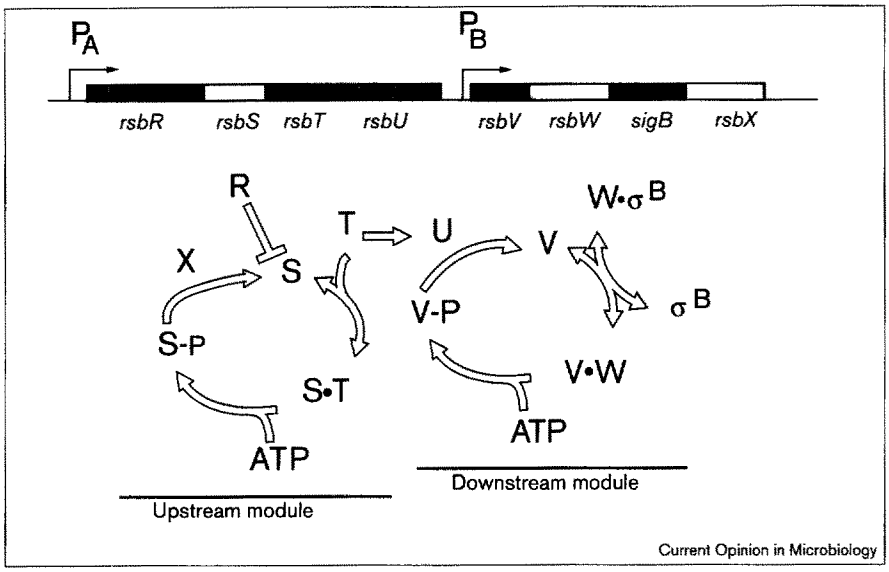


Regulation of early forespore gene expression by the SpoIIAB anti-σ. In the predivisional cell, σ^F is held in an inactive complex with the dimeric SpoIIAB (AB; rectangle) anti-σ and ATP [26]. The anti-anti-σ, SpoIIAA (AA), is presumably phosphorylated and therefore inactive. As sporulation proceeds, the SpoIIE phosphatase triggers a rise in unphosphorylated AA levels and the resulting AA binds to the AB•ATP•σ^F complex to induce the release of active σ^F [32]. This induced release reaction requires the kinase activity of AB, suggesting that AA phosphorylation and σ^F release are coupled events [31••]. AB is subsequently trapped as a long-lived species containing ADP [29••,32]. This is presumably an altered conformation of AB (AB*) [30•] and may also include AA (the AA•ADP•AB complex) [31••].

Regulation of the σ^B-controlled stress responses

Remarkably, the regulation of σ^B, controlling the expression of more than 60 proteins in response to several stress conditions (reviewed in [27]), is even more complex than that described for σ^F. Activation of the σ^B regulon is governed by two, sequentially linked partner-switching modules [36,37]. The regulatory components are encoded in a large, complex operon containing *sigB* and seven regulator of sigma-B (*rsb*) genes (Figure 3). The output of the upstream module, which integrates environmental stress signals [38], is RsbT, which stimulates σ^B activity indirectly by activating the RsbU phosphatase of the downstream module. Interestingly, with the significant exception of RsbX (see below), there is correspondence between the genetic and functional order of these genes.

Figure 3



Regulation of the σ^B stress response by two coupled partner-switching modules. The *Bacillus subtilis sigB* gene is co-transcribed with seven regulatory genes from a σ^A-dependent promoter (P_A). Genes that positively affect σ^B activity (and *sigB* itself) are shaded grey, whereas negative regulators are white. The downstream portion of the operon, which can also be expressed from a σ^B-dependent autoregulatory promoter (P_B), encodes the downstream partner-switching module [36,37]. The RsbW protein (W) functions as an anti-σ and binds to either σ^B or to RsbV (V). W also functions as a protein kinase and phosphorylates V. This reaction is postulated to sense the energy status of the cell: when ATP levels drop, W will become sequestered by V and the σ^B regulon will be induced [38,39•]. The upstream module (proteins R, S, and T) controls the activity of the U phosphatase, which can regenerate active V protein [36,37]. T is an allosteric activator of U and also has protein kinase activity targeted against its antagonist S [41•]. Active S, in turn, can be regenerated by X, which serves to downregulate signals from the upstream module [40].

Recent genetic and biochemical analyses have confirmed and extended the essential features of the dual partner-switching model [36,37]. Genetic analyses of *rsbX* mutants, and their suppressors, confirm that this gene encodes a negative regulator of *sigB* that acts upstream of RsbU and RsbT but that is not required for the response to at least some stresses [39•,40]. As originally proposed [37], RsbX appears to act as a homeostatic feedback signal that functions to downregulate σ^B. Thus, signals that liberate active σ^B from its anti-σ (RsbW) also lead to increased transcription of the genes for the downstream module from an internal, autoregulatory promoter. This up-regulation of σ^B levels may initially magnify the response, but the concomitant up-regulation of RsbX will act to reduce the signal generated by the upstream module. Specifically, RsbX dephosphorylates RsbS that thereby sequesters RsbT preventing activation of the RsbU phosphatase. Intriguingly, mutational studies of RsbT reveal that kinase activity is not essential for stimulation of RsbU, but is required for the transmission of stress signals to the downstream module [41•].

Recent analyses have also shed light on the heretofore mysterious RsbR protein [42•]. RsbR appears to act as a modulator of RsbS activity in the upstream module and represents a new branch in this signal transduction cascade.

Anti-σ factors that regulate ECF σ factors

ECF σ factors [43] are a large and growing subfamily of σ factors that typically regulate functions related, in the broadest sense, to the cell envelope (see [44] for a recent review). Typically, the genes encoding ECF σ factors are positively autoregulated and are transcriptionally coupled to the expression of a cognate anti-σ factor. In *E. coli*, ECF σ factors include σ^{FecI}, an activator of ferric citrate transport (reviewed in [45•]), and σ^E, a mediator of a periplasmic stress response. ECF σ factors are also well represented in other bacteria with seven in *B. subtilis* and 10 in *M. tuberculosis*.

Most ECF σ factors are regulated by anti-σ factors. In the absence of an external signal, the σ is held in an inactive, stoichiometric complex with an anti-σ, often located in the cytoplasmic membrane. By virtue of its transmembrane disposition, the anti-σ is poised to activate a transcriptional response signaled by the presence of molecules external to the cytoplasmic membrane. Systems regulated in this manner include various stress responses, uptake systems for ferri-siderophore complexes, carotenoid biosynthesis in response to light, and synthesis of the exopolysaccharide alginate [44].

Several recent studies have investigated the topological arrangement of anti-σ factors in the cytoplasmic membrane as well as their interactions with regulatory ligands. In *E. coli*, ferric citrate transport requires σ^{FecI}, which is regulated by FecR. Although initially thought to function as an anti-σ, some results suggest that FecR may be required to activate σ^{FecI} [45•]. FecR appears to have a single membrane spanning segment with a cytoplasmic amino-terminal domain and a periplasmic carboxy-terminal domain [46]. Activation of FecR requires that the periplasmic domain interacts with the amino-terminal domain of the outer-membrane FecA transporter [47•]. In *B. subtilis*, σ^X is also regulated by an anti-σ factor [48,49]. Despite the report that this σ activates a σ^{FecI}-dependent

gene in *E. coli* [48], in *B. subtilis* σ^X modulates cell wall structure and does not control iron transport [50]. The *B. subtilis* σ^W regulon is also regulated by an anti-σ that senses perturbations of cell wall structure (JD Helmann, unpublished data).

Anti-ECF σ factors respond to a wide variety of signals transmitted either by other proteins or by small molecules. In the case of *E. coli* σ^E, release from the transmembrane anti-σ (RseA) requires signals perceived in the periplasm that may be modulated by RseB and RseC [51•,52•]. In a closely related system regulating alginate secretion in *Pseudomonas aeruginosa*, a σ^E homolog (σ^{22}) is regulated by an anti-σ MucA. MucA contains a single transmembrane domain and interacts with the periplasmic MucB protein [53]. Not all ECF σ factors are controlled by signals external to the cell: the *S. coelicolor* σ^R regulon is induced by oxidants perceived by the redox activity of a cytoplasmic anti-σ factor (cited in [54•]).

Conclusions

Genomic analyses highlight the widespread occurrence of both *alternative σ factors and anti-σ factors as regulators* of gene expression. The systems reviewed range from the relatively simple, the secretion of the anti-σ^{28} FlgM as a mechanism to sense the completion of the flagellar hook–basal body structure, to the incredibly elaborate signal transduction cascade ultimately regulating the RsbW *anti-σ factor. The mechanisms by which anti-σ* factors exert their effects are still an active area of study, but seem to involve both sequestration and holoenzyme dissociation. To date, the pathways serving to control *anti-σ activity can be broadly grouped into three categories: export from the cell (i.e. FlgM),* partner-switching modules as found in the σ^F and σ^B regulons, and interaction with small molecule or protein ligands as found in systems regulated by ECF σ factors.

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A genetic screen was conducted to identify AB mutants that were able to inhibit σ^F activity, but unable to release σ^F properly during sporulation. An AB mutant (E104K) is described that is defective in the AA phosphorylation. Unlike the corresponding AA mutant that cannot be phosphorylated (and therefore leads to high σ^F activity), this mutant leads to low σ^F activity. The results suggest that phosphorylation of AA by the AB•ATP•σ^F complex is necessary for σ^F release (induced release, see [32]).

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