The ECF sigma factors of *Streptomyces coelicolor* A3(2)

Mark S. B. Paget,¹ Hee-Jeon Hong,² Maureen J. Bibb² and Mark J. Buttner²

¹School of Biological Sciences, University of Sussex, Brighton BN1 9QG, UK
²Department of Molecular Microbiology, John Innes Centre, Colney, Norwich NR4 7UH, UK

INTRODUCTION

In bacteria, gene expression is controlled primarily at the level of transcription initiation. Control can be achieved through the use of DNA-binding proteins (repressors and activators) that affect the efficiency of initiation, but also through the use of alternative forms of RNA polymerase with different promoter recognition characteristics. The promoter specificity of the RNA polymerase holoenzyme depends on the nature of the $\sigma$ subunit that associates with the core enzyme. This key role of $\sigma$ in promoter recognition suggests a mechanism for the coordinate control of gene expression using alternative forms of $\sigma$ and different subsets of promoters, an idea that was first proposed as soon as the role of $\sigma$ was established (Burgess et al., 1969). It is now clear that most, if not all, bacteria use alternative $\sigma$ subunits to control gene expression, and that these $\sigma$ factors fall into two distinct families: the $\sigma^N$ (or $\sigma^N_5$) family, which is discussed in the preceding chapter, and the $\sigma^70$ family. The $\sigma^70$ family includes those $\sigma$ factors that, broadly speaking, are related in sequence and domain organization to the primary *Escherichia coli* $\sigma$ factor, $\sigma^70$. Although the overall architecture of members of the $\sigma^70$ family appears to be conserved, the $\sigma^70$ family can be divided into several phylogenetically distinct subfamilies (Lonetto et al., 1992). Members of each subfamily are often involved in the control of related functions, such as the heat-shock response, flagella biosynthesis, or sporulation.

The ECF subfamily of $\sigma$ factors

In the late 1980s, biochemical analysis of RNA polymerase from *Streptomyces coelicolor* and *E. coli* led to the identification of two $\sigma$ factors that were particularly small in...
size. In *E. coli*, σ^E^ (21.7 kDa) was shown to account for transcription of the gene encoding the heat-shock σ factor, σ^E^, at high temperatures (Erickson & Gross, 1989). In *S. coelicolor*, another small σ factor, also named σ^E^ (20.4 kDa), was shown to direct *in vitro* transcription from one of four promoters (dagA^p2^) of the agarase-encoding gene dagA (Buttner et al., 1988). The cloning of the gene encoding *S. coelicolor* σ^E^ several years later using a reverse genetics approach revealed that it belonged, together with *E. coli* σ^E^, to a new subfamily of the σ^70^ family (Lonetto et al., 1994). Members of this new subfamily were sufficiently different from the previously known σ factors that in many cases they were not identified as σ factors by standard similarity searching methods. As a consequence, several members of the subfamily were present in the protein databases, but their biochemical role was unrecognized. Each had been identified by genetic means, each had a known positive regulatory role, but with no biochemical understanding of mechanism. These included AlgU from *Pseudomonas aeruginosa*, CarQ from *Myxococcus xanthus* and FecI from *E. coli*. The available information about the roles of these σ factors at the time suggested that they functioned as effector molecules responding to extracytoplasmic stimuli, and that they often controlled extracytoplasmic functions, and for this reason, the new subfamily was named the ECF subfamily (Lonetto et al., 1994). For example, *E. coli* σ^E^ is involved in sensing and responding to protein misfolding in the extracytoplasmic space (Ades et al., 1999), *M. xanthus* σ^CarQ^ activates the synthesis of membrane-localized carotenoids in response to light (Gorham et al., 1996), and *E. coli* FecI activates the citrate-dependent iron(III) transport system in response to citrate and iron in the periplasmic space (Härle et al., 1995). The characteristically small size of ECF σ factors (~20–30 kDa) is accounted for by the absence of most or all of both regions 1 and 3 (Lonetto et al., 1994). For a detailed review of σ domain structure and function see Lonetto et al. (1992, 1994).

Since the initial discovery of the ECF subfamily, hundreds of new members have been discovered in a wide variety of Gram-negative and Gram-positive bacteria, mostly through genome sequencing projects. Indeed, for several bacteria, including *Bacillus subtilis*, *Mycobacterium tuberculosis* and *S. coelicolor*, ECF σ factors represent the major class of σ factors. It is striking that relatively few ECF σ factors were discovered by traditional genetic approaches. For example, in *B. subtilis* there are seven ECF σ factor genes, none of which was discovered genetically. This seems to imply that either they are functionally redundant or they control the expression of genes not pertinent to normal laboratory culture conditions (or both).

The genome sequence of *S. coelicolor* has revealed an astonishing 51 ECF σ factors from a total of 65 σ factors, implying that these proteins play a major role in transcriptional regulation in *Streptomyces*. In order to understand the physiological roles of these ECF σ factors, it will be necessary to elucidate the signals to which they respond,
to characterize the regulatory mechanisms involved in their activation, and to identify their regulons (the genes under their control). The aim of this review is to summarize current understanding of the biological roles and regulation of the three ECF $\sigma$ factors ($\sigma^E$, $\sigma^R$ and $\sigma^{BldN}$) that have been studied in detail in *S. coelicolor*. For each of these three $\sigma$ factors, the mechanism controlling $\sigma$ factor activity is different, variously involving de novo synthesis, pro-$\sigma$ processing, and anti-$\sigma$ factor-directed control. These examples serve to illustrate the fascinating variety of regulatory systems that exist in bacteria to ensure that $\sigma$ factors are recruited to core RNA polymerase only when appropriate.

**THE $\sigma^E$ PATHWAY FOR SENSING AND RESPONDING TO CELL ENVELOPE STRESS**

Since the initial cloning of the *sigE* gene (Lonetto et al., 1994), extensive analysis suggests that $\sigma^E$ is part of a signal transduction pathway that allows *S. coelicolor* to sense and respond to changes in the integrity of its cell envelope (Paget et al., 1999a, b). A model for the pathway is shown in Fig. 1. The signal transduction system is composed of four proteins, encoded in an operon: $\sigma^E$ itself; CseA, a negative regulator of undefined biochemical function; CseB, a response regulator; and CseC, a sensor histidine protein kinase with two predicted transmembrane helices; (Cse = control of sigma E).

Expression of $\sigma^E$ activity is governed at the level of *sigE* transcription by the CseB/CseC two-component signal transduction system. In response to signals that originate in the cell envelope when it is under stress, the sensor kinase, CseC, becomes autophosphorylated at His-271, and, in accordance with the known mechanism for other two-component regulatory systems, this phosphate is then transferred to Asp-55 in the response regulator, CseB. Phospho-CseB activates the promoter of the *sigE* operon, and $\sigma^E$ is recruited by core RNA polymerase to transcribe genes with cell-envelope-related functions, including a putative operon of 12 genes likely to specify cell wall glycan synthesis.

**Evidence for the model**

*sigE* null mutants were extremely sensitive to cell wall hydrolytic enzymes, and had an altered cell wall muropeptide profile, suggesting that *sigE* is required for normal cell wall integrity. Importantly, the *sigE* mutant was sensitive to both muramidases (for example, lysozyme) and amidases, which cut the peptidoglycan backbone and the peptide side chain, respectively, suggesting that the defect in the *sigE* mutant envelope allowed hydrolytic enzymes increased access, rather then specifically altering their target sites (Paget et al., 1999a). Mg$^{2+}$ ions are known to have stabilizing effects on cell envelopes, and *sigE* null mutants required millimolar levels of Mg$^{2+}$ for normal growth and sporulation, forming crenellated colonies, sporulating poorly, and overproducing the blue antibiotic actinorhodin in its absence (Paget et al., 1999a).
Most transcripts from the $\text{sigE}$ promoter terminates immediately downstream of $\text{sigE}$, but about 10% read through into the downstream genes (Paget et al., 1999b). Analysis of the activity of the $\text{sigE}$ promoter in different mutant backgrounds was highly informative. The $\text{sigE}$ promoter was found to be inactive in a constructed $\text{cseB}$ null mutant, such that $\text{cseB}$ mutants lack $\sigma^E$. This observation explained why $\text{cseB}$ and $\text{sigE}$ mutants had the same phenotype (Paget et al., 1999b). In contrast, the $\text{sigE}$ promoter was substantially up-regulated in a $\text{sigE}$ null mutant, suggesting that the cell envelope defects in $\text{sigE}$ mutants are sensed by CseC, which responds by increasing the level of phospho-CseB in the cytoplasm in a futile attempt to increase expression of $\text{sigE}$ and hence expression of the cell-envelope-related genes under $\sigma^E$ control (Paget et al., 1999b).
What signal is sensed by CseC?

The exact nature of the signal recognized by the sensor kinase is known for relatively few two-component systems. In order to better understand the nature of the signal sensed by CseC, a screening system was developed to test for compounds that induced the sigE promoter (H.-J. Hong, M. S. B. Paget, E. Leibovitz & M. J. Buttner, unpublished). The sigE promoter was placed upstream of a plasmid-borne kanamycin-resistance gene to yield a construct that conferred a basal level of kanamycin resistance on the host. A wide selection of antibiotics was then tested to see which increased kanamycin resistance above the basal level in a plate assay. In agreement with the proposed role for $\sigma^E$ in controlling cell envelope integrity, antibiotics that target the cell envelope induce sigE expression. These included certain $\beta$-lactam antibiotics and, most effectively, glycopeptide antibiotics such as vancomycin and teicoplanin. ‘Negative control’ antibiotics that target the ribosome (e.g. thiostrepton, streptomycin) or DNA gyrase (novobiocin) did not induce sigE expression. In addition to antibiotics, lysozyme was also found to induce sigE expression, making it highly unlikely that CseC senses these inducers directly.

It is important to note that the sigE gene is transcribed under all growth conditions tested, implying that the CseB/CseC signal transduction system may be responding to changes in cell envelope metabolism that occur during ‘normal’ growth, which are amplified by the effects of antibiotics and enzymes that target the cell envelope. Accordingly, CseC could be activated by the accumulation of an intermediate in peptidoglycan degradation or biosynthesis, analogous to the control of $\beta$-lactam-inducible $\beta$-lactamase gene expression in many Gram-negative bacteria (Jacobs et al., 1997). Alternatively, it is conceivable that CseC might be responding to some physical characteristic of the cell envelope (e.g. turgor). The KdpD/KdpE sensor kinase/response regulator pair of E. coli (Walderhaug et al., 1992; Sugiura et al., 1994) has been proposed to sense and respond to physical changes in the cell envelope.

CseA has a negative role in sigE expression

The gene immediately downstream from sigE, cseA, appears to play a negative role in sigE expression. The basal level of transcription from the sigE promoter was substantially higher in a constructed, in-frame cseA deletion mutant, and the maximal level of transcription from sigEp following induction with vancomycin was also several fold higher than in the wild-type (H.-J. Hong, M. S. B. Paget, E. Leibovitz & M. J. Buttner, unpublished). Although CseA has no similarity with any other proteins in the databases, its first 21 N-terminal amino acids (MAVFVALGVSLAGCGTGGTGA) are predicted to form a single transmembrane domain. Since CseA cannot function as a $\sigma^E$-specific anti-$\sigma$ factor ($\sigma^E$ does not direct transcription from the sigE promoter), perhaps it modulates the CseB/CseC signal transduction pathway, for example as an inhibitor of the kinase activity of CseC, or as a CseB-specific phosphatase.
**σ^E** directs transcription of a putative operon of 12 genes likely to specify cell wall glycan synthesis

Although **σ^E** was discovered by virtue of its ability to direct transcription of **dagAp2** in *vitro*, when genetic analysis of **sigE** began, the activity of this promoter was found to be unaffected in a constructed **sigE** null mutant (Paget *et al.*, 1999a). Presumably this reflects relaxed promoter specificity *in vitro*, and the existence of a closely related ECF **σ** that recognizes **dagAp2** *in vivo*. The first bona fide **σ^E**-dependent promoter identified was **hrdDp1** (Paget *et al.*, 1999a; Kang *et al.*, 1997), one of two promoters of the **hrdD** gene, which itself encodes a **σ** factor. However, this discovery was relatively uninformative because the physiological function of **σ^HrdD** is unknown (**hrdD** null mutants have no apparent phenotype; Buttner *et al.*, 1990). To identify further **σ^E**-dependent promoters, computer-searching methods were used to identify sequences in the emerging *S. coelicolor* genome sequence that closely resemble the **hrdDp1** promoter (GCAAC – 17 bp – CGTCT). An initial search identified a perfect match lying upstream of 12 genes that are likely to form an operon (H.-J. Hong, M. S. B. Paget & M. J. Buttner, unpublished). The predicted functions of the enzymes encoded by this operon strongly suggest that the operon specifies the synthesis of a species of cell wall glycan (hence the operon has been named **cwg**). High-resolution S1 nuclease mapping showed that the putative **−10 and −35** sequences identified by computer searching do indeed correspond to a bona fide promoter, and that the **cwg** promoter is induced by vancomycin in a **sigE**-dependent manner (H.-J. Hong, M. S. B. Paget & M. J. Buttner, unpublished). Thus a set of genes under **σ^E** control has been identified that has a clear cell-envelope-related function, and transcription of these genes has been shown to be induced by vancomycin and, presumably therefore, other cell-wall-targeted antibiotics and enzymes. A constructed mutant in which the **cwg** operon was deleted did not show any of the phenotypes associated with **sigE** mutants, showing that other, as yet unknown, **σ^E** target genes play critical roles in maintaining cell envelope integrity.

**THE **σ^R** PATHWAY FOR SENSING AND RESPONDING TO OXIDATIVE STRESS**

**σ^R** was the second ECF **σ** factor to be discovered in *S. coelicolor*. Like **σ^E**, it was first identified in purified RNA polymerase holoenzyme preparations isolated from liquid-grown cultures (Kang *et al.*, 1997; Paget *et al.*, 1998). The role of **σ^R** as a key regulator of the oxidative stress response was discovered after phenotypic analysis of a constructed **sigR** null mutant. This mutant was sensitive to oxidizing agents such as the superoxide-generating, redox cycling compounds menadione and plumagin, and was particularly sensitive to a thiol-specific oxidant called diamide. The cytoplasm of all organisms is a reducing environment where thiol groups are maintained in their reduced state. The diamide-sensitive phenotype suggested that **sigR** mutants may be unable to respond to adverse changes in the thiol–disulphide redox balance, a condition
termed disulphide stress (Åslund & Beckwith, 1999). This hypothesis was borne out by
the demonstration of lowered levels of cytoplasmic disulphide reductase activity in sigR
mutants (Paget et al., 1998). The major system for controlling the thiol–disulphide
balance in Streptomyces spp. is the thioredoxin system, which consists of the disulphide
reductase thioredoxin and its reactivating enzyme thioredoxin reductase (Aharonowitz
et al., 1993; Cohen et al., 1993). These enzymes use the reducing power of NADPH to
remove unwanted disulphide bonds in oxidized cellular proteins, and to reduce
enzymes, such as ribonucleotide reductase, that form disulphide bonds at their active
site as part of their catalytic cycle. Reconstituted RNA polymerase holoenzyme con-
taining purified σR initiated transcription from trxBp1, one of the two promoters that
transcribe trxBA, the operon that encodes thioredoxin reductase and thioredoxin.
Most importantly, trxBp1 activity was rapidly and massively induced by the addition of
the thiol-specific oxidizing agent diamide to wild-type mycelium, but remained unin-
duced in the sigR null mutant (Paget et al., 1998).

Regulation of σR activity
The second σR target promoter to be identified, sigRp2, lay upstream of its own structu-
ral gene, sigR, thereby establishing a positive feedback loop for its own synthesis (Paget
et al., 1998). It thus became clear that, in order to prevent an upward spiral of σR syn-
thesis, there must be a negative regulator in place to ensure that σR is only switched on
when necessary and to ensure that its activity is effectively switched off when the disul-
phide stress has been dealt with. This key negative regulator was identified as RsrA
(regulator of sigR), a σR-specific anti-σ factor that is encoded by the gene lying immedi-
ately downstream of sigR. Anti-σ factors are proteins that inhibit σ factor activity
either by binding to it and preventing its interaction with core RNA polymerase, or by
binding to the σ factor when it is part of the holoenzyme form, thereby preventing pro-
moter binding (Hughes & Mathee, 1998; Helmann, 1999). Purified RsrA can bind
tightly to σR and inhibit σR-directed transcription in vitro. However, RsrA can only
perform this function when the in vitro conditions are sufficiently reducing. In the
absence of strong thiol-reducing agents such as dithiothreitol (DTT), RsrA can neither
bind to σR nor inhibit σR-directed transcription (Kang et al., 1999). Moreover, if rsrA is
deleted from the S. coelicolor chromosome, σR target promoters are constitutively
expressed at the fully induced level (Paget et al., 2001a). In other words, the regulation
of σR activity by disulphide stress appears to be mediated solely by RsrA, with RsrA
itself acting as the direct sensor of the thiol–disulphide redox status of the cell. Indeed,
unlike σR, which contains no cysteines, RsrA, a protein of only 105 residues, contains
seven cysteines and rapidly forms intramolecular disulphide bonds in the absence of
thiol-reducing compounds (Kang et al., 1999). A model for how RsrA regulates σR
activity is presented in Fig. 2. σR protein is present in the hyphae all the time, but σR
activity is not, because, in the absence of oxidative stress, RsrA sequesters σR in an
RsrA : $\sigma^R$ complex. $\sigma^R$ is released during oxidative stress as a direct consequence of the inactivation of RsrA through intramolecular disulphide bond formation. $\sigma^R$ is then free to associate with core RNA polymerase and activate transcription of its target genes, including $trxBA$ and other thiol–disulphide oxidoreductase genes (see ‘The $\sigma^R$ regulon’ below). At least in vitro, oxidized RsrA is a direct biochemical substrate for purified thioredoxin, the product of the $trxA$ gene (Kang et al., 1999). If the thioredoxin system

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**Fig. 2.** Model for the regulation of $\sigma^R$ activity in response to disulphide stress. The thiol–disulphide status of *S. coelicolor* is controlled by a novel regulatory system consisting of a $\sigma$ factor, $\sigma^R$, and RsrA, a redox-sensitive, $\sigma^R$-specific, anti-$\sigma$ factor. Under reducing conditions, RsrA binds to $\sigma^R$ and prevents it from activating transcription. Exposure to disulphide stress induces the formation of one or more intramolecular disulphide bonds in RsrA, which causes it to lose its affinity for $\sigma^R$, releasing $\sigma^R$ to activate transcription of $>30$ genes and operons, including $trxBA$. Increased $trxBA$ expression in turn leads to the thioredoxin-dependent reduction of oxidized RsrA back to its $\sigma^R$-binding conformation, thereby shutting off $\sigma^R$-dependent transcription. In addition, $\sigma^R$ positively autoregulates expression of the $sigR$–$rsrA$ operon. As a consequence, disulphide stress not only activates $\sigma^R$ post-translationally, but also induces its *de novo* synthesis.
also reduces (reactivates) RsrA in vivo, this would allow it to rebind $\sigma^R$ and shut down the response, thereby creating a simple homeostatic feedback loop in which the $\sigma^R$ regulon is regulated in response to changes in the thiol-disulphide redox status of the hyphae.

This model raises several important questions, including the exact nature of the redox event that inactivates RsrA. Attempts to identify which of the seven cysteines in RsrA form the disulphide bond switch have not been straightforward. In principle, the loss of a cysteine residue that is involved in inactivating RsrA might be expected to lock RsrA in a constitutively active conformation, causing it to bind $\sigma^R$ irrespective of the redox conditions. However, the individual substitution of each of seven RsrA cysteines did not reveal such mutants. Four of the cysteines in RsrA could be substituted, individually or collectively, still leaving a protein that could both inhibit $\sigma^R$ activity and release it during disulphide stress. The remaining three individual cysteine mutants (C11, C41 and C44) had no $\sigma^R$-binding activity, preventing analysis of their ability to sense redox (Paget et al., 2001a). There is now good evidence to suggest that, in their reduced state, these three cysteines play an important role in the $\sigma^R$-binding activity of RsrA by coordinating a zinc cofactor (see below).

**The ZAS family of anti-$\sigma$ factors**

Since the discovery of rsrA, many related genes have been uncovered by genome sequencing in both Gram-positive and Gram-negative bacteria. Although the sequence similarity between the products of these genes is often very low, certain residues are highly conserved, especially an invariant HisXXXCysXXCys motif (see, for example, Fig. 3). Furthermore, each rsrA-related gene is located near (typically downstream and
immediately adjacent to) an ECF σ-factor gene, strongly suggesting that the corresponding pair of proteins interact. Metal content analysis of RsrA (Paget et al., 2001a) and ChrH (an RsrA-related anti-σ factor from Rhodobacter sphaeroides; see below) (Newman et al., 2001) revealed that they are zinc metalloproteins. This, together with the absolute conservation of HisXXXCysXXCys, a potential zinc-binding motif, strongly suggests that all RsrA-related proteins are likely to bind zinc. This new family of proteins was therefore named the ZAS (zinc-binding anti-σ factor) family of anti-σ factors (Paget et al., 2001a).

The redox regulation of RsrA is not a paradigm for all ZAS anti-σ factors
Importantly, although all RsrA-related anti-σ factors probably bind zinc, it is already clear that their activities are likely to be regulated in diverse ways, so the regulation of RsrA activity by a reversible thiol–disulphide redox switch is not a paradigm for the whole family. Thus a gene encoding a ZAS anti-σ factor lies immediately downstream of the sigW gene in B. subtilis, but σW-dependent gene expression is not induced by diamide and the known σW target genes have no obvious connection to thiol–disulphide metabolism (Huang et al., 1999; Cao et al., 2001; Wiegert et al., 2001; J. Helmann, pers. comm.). Similarly, the ZAS anti-σ factor ChrR controls the activity of σE in Rhodobacter sphaeroides, but σE directs expression of the cytochrome c2 structural gene (Newman et al., 1999, 2001). Further, deletion of chrR or the σE-encoding rpoE does not affect the resistance of R. sphaeroides to diamide, and diamide does not induce σE-dependent gene expression (Newman et al., 2001; T. Donohue & J. Newman, pers. comm.). Eleven of the 51 ECF σ factors in S. coelicolor are encoded by genes located near (typically upstream and immediately adjacent to) zas genes, and are therefore likely to be regulated by a ZAS anti-σ factor (Fig. 3). Several of these proteins differ from RsrA in having predicted transmembrane helices C-terminal to the HisXXXCysXXCys motif, suggesting that these ZAS proteins may regulate their cognate σ factor in response to extracytoplasmic signals.

The σR regulon
Searches for further σR target genes were made possible by the generation of a consensus target promoter sequence (GGAAT – 18 bp – GTT) using for comparison trxBp1 and sigRp2, together with the sequence of hrdDp2, another promoter recognized by σR in vitro. Computer searches showed that this sequence occurred more than 60 times in the S. coelicolor genome, although only 34 of these were appropriately positioned just upstream from a gene. Each of these 34 sequences was examined experimentally for promoter activity; including sigRp2, trxBp1 and hrdDp2, 30 were bona fide promoters that were induced by diamide in a σR-dependent manner (Paget et al., 2001b).
than half of the $\sigma^R$ target genes associated with these promoters have no known biological function.

Unsurprisingly, several $\sigma^R$ target genes are likely to play important roles in thiol metabolism, including a second thioredoxin, $trxC$, and a glutaredoxin-like gene. Together with the $trxBA$ operon, the induction of these genes by $\sigma^R$ presumably helps to restore the thiol–disulphide balance following disulphide stress. Apart from cysteine thiols in proteins, low-molecular-mass thiols are also likely to become oxidized during disulphide stress, and the induction of the $\sigma^R$ targets $cysM$ and $moeB$ is likely to act to restore levels of reduced cysteine and the dithiol-containing cofactor molybdopterin, respectively (Paget et al., 2001b). Unlike Gram-negative bacteria and eukaryotes that use the cysteine-containing tripeptide glutathione as their major thiol buffer, Streptomyces and mycobacteria use a structurally unrelated, sugar-containing monothiol compound called mycothiol (Newton et al., 1996). Although no target genes were found that were predicted to play a role in mycothiol biosynthesis, the $\sigma^R$ mutant was found to have significantly lowered levels of mycothiol (Paget et al., 2001b). The root cause of diamide sensitivity in $\sigma^R$ mutants could therefore be due to any one of these $\sigma^R$-dependent mechanisms for coping with disulphide stress, or a combination of all of them.

At least three $\sigma^R$ targets encode ribosome-associated products, including $relA$, $ssrA$ and the ribosomal protein gene $rpmE$, suggesting that ribosome composition and function are modified in response to disulphide stress (Paget et al., 2001b). RelA catalyses the production of ppGpp when ribosomes stall due to an uncharged tRNA entering the ribosome A-site. This intracellular signalling molecule then elicits the stringent response by selectively inhibiting transcription of rRNA genes, thereby acting to slow growth (Cashel et al., 1996; Chatterji & Ojha, 2001). In Streptomyces spp., ppGpp also elicits antibiotic production in response to nutritional stress, and plays a role in differentiation (Chakraburttty & Bibb, 1997). $ssrA$ encodes an unusual small stable tRNA–mRNA hybrid called tmRNA, which also acts when ribosomes stall, either at a rare codon or when ribosomes reach the end of a 3' truncated mRNA that lacks a stop codon. tmRNA rescues the ribosome by acting as a surrogate mRNA to tag the nascent peptide with a hydrophobic tag that targets the protein for degradation (Keiler et al., 1996; Roche & Sauer, 1999; Karzai et al., 2000). It is tempting to speculate that disulphide stress inhibits some aspect of the translation process causing ribosomes to stall. A possible ribosomal target for disulphide stress is the product of the $\sigma^R$ target gene $rpmE$, ribosomal protein L31, which contains a CysXXCys motif. The induction of $relA$ and $ssrA$ may then provide pathways to rescue stalled ribosomes and to slow ribosome production and growth, respectively, thereby focusing available resources on stress survival.
Another interesting $\sigma^R$ target, *rbpA*, encodes a newly discovered RNA polymerase-binding protein, which may well be a novel low-molecular-mass RNA polymerase subunit (Paget et al., 2001b). RbpA appears to exist only in the actinomycetes, including the mycobacteria. Although the role of RbpA is not known, the induction of *rbpA* transcription by $\sigma^R$ suggests that the composition and function of RNA polymerase may also be modified in response to disulphide stress. Like the ribosome subunit L31, RbpA contains a CysXXCys motif, suggesting that it too may undergo thiol–disulphide redox reactions and may be a target of disulphide stress.

It should be noted that the method used to identify $\sigma^R$ target promoters means that there may be many other, unidentified targets having promoter sequences that differ slightly from the consensus sequence used in the computer searches. The total $\sigma^R$ regulon may therefore be considerably larger than the current total. Nonetheless, the identification of 30 genes and operons under $\sigma^R$ control is a very significant step towards understanding the cellular response to disulphide stress in *S. coelicolor*.

**Is $\sigma^R$ a checkpoint in development?**

A completely unexpected consequence of *rsrA* inactivation was a block in sporulation, and there is some circumstantial evidence to suggest that *S. coelicolor* may use $\sigma^R$ as a checkpoint to inhibit development under conditions of oxidative stress, which may make sporulation undesirable. *S. coelicolor* differentiates on solid agar plates by forming aerial hyphae that grow out of the aqueous environment of the substrate mycelium into the air. These multigenomic aerial hyphae eventually undergo synchronous septation to produce chains of unigenomic exospores. Developmental mutants that are unable to raise an aerial mycelium have a shiny appearance on agar plates and are termed ‘bald’ (*bld*) mutants. Mutants that raise an aerial mycelium in the normal way but are unable to complete the developmental process by sporulating are termed white (*whi*) mutants, because the colonies fail to develop the characteristic grey pigment associated with mature spores.

A constructed *rsrA* mutant had a classical ‘early’ white phenotype, forming aerial mycelium, but failing to initiate sporulation septation. In contrast, a constructed *sigR rsrA* double mutant sporulated normally, showing that the inability of the *rsrA* single mutant to sporulate was a consequence of uncontrolled $\sigma^R$ activity. One possible explanation for these observations is that the high level of free $\sigma^R$ out-competes a sporulation-specific $\sigma$ factor, such as $\sigma^{WhiG}$ (Chater et al., 1989), for core RNA polymerase (Paget et al., 2001a). However, recent analogous experiments with $\sigma^U$ and RsuA, another ECF $\sigma$ factor: ZAS anti-$\sigma$ factor pair in *S. coelicolor*, provided circumstantial evidence against this model (Gehring et al., 2001). Disruption of *rsuA* caused a bald phenotype, but a *sigU rsuA* double mutant developed normally, again showing that the
block in differentiation was a consequence of uncontrolled $\sigma$ activity. As pointed out by Gehring et al. (2001), it seems unlikely that $\sigma^R$ and $\sigma^I$ could differentially compete with different $\sigma$ factors, one required for aerial mycelium formation and one required for spore formation.

An alternative hypothesis is that the developmental phenotype of the rsrA null mutant is physiologically significant, that $\sigma^R$ directs transcription of a ‘sporulation inhibitor gene(s)’, and that S. coelicolor uses this mechanism as a checkpoint to arrest development under conditions of disulphide stress, which make sporulation undesirable (Gehring et al., 2001; Paget et al., 2001a). If this latter hypothesis is valid, it should be possible to identify mutations in the proposed ‘sporulation inhibitor gene’ that suppress the white phenotype of rsrA mutants, provided that there is only one $\sigma^R$ target gene that mediates the arrest of development, and that this gene is non-essential. However, the four rsrA suppressor mutations characterized to date all map to sigR (Paget et al., 2001a).

The $\sigma^R$–RsrA system also exists in pathogenic actinomycetes

The $\sigma^R$–RsrA system appears to exist in other actinomycetes. It is certainly present in mycobacteria, where it is named $\sigma^H$–RshA (Fernandes et al., 1999; Paget et al., 1998; I. Smith, pers. comm.), and analysis of the near-complete genome sequence of Corynebacterium diphtheriae (http://www.sanger.ac.uk/Projects/C_diphtheriae/) suggests that it also exists in this important actinomycete pathogen (M. S. B. Paget, unpublished). Of the 30 S. coelicolor $\sigma^R$ target genes and operons so far identified, 13 of the homologues in M. tuberculosis have sequences upstream that resemble the consensus for $\sigma^R$-dependent promoters and may therefore be regulated by $\sigma^H$ in M. tuberculosis (Paget et al., 2001b). These include homologues of the S. coelicolor genes sigR, trxBA, ssrA, rpmE and rbpA. These observations make it likely that the $\sigma^H$–RshA system contributes to the well known resistance of M. tuberculosis to oxidative killing by white blood cells during human infection.

THE $\sigma^{BldN}$ PATHWAY TO AERIAL MYCELIUM FORMATION

Unlike $\sigma^E$ and $\sigma^R$, which were discovered biochemically, $\sigma^{BldN}$ was identified genetically in a screen for new genes involved in morphological differentiation (Ryding et al., 1999; Bibb et al., 2000). Two NTG-induced point mutants were isolated in the gene encoding $\sigma^{BldN}$, the two mutants having strikingly different phenotypes. One, R650, had a white colony phenotype, and microscopic examination showed that the colony produced aberrant spores that were longer than those of the wild-type. The second, R112, had a more severe phenotype, producing substantially less aerial mycelium than the parental strain and only very rare spore chains, sometimes showing highly irregular sporulation septum placement (Ryding et al., 1999). Shotgun complementation of...
R650 and R112, followed by subcloning and sequencing, showed that this new developmental gene encoded an ECF $\sigma$ factor (Bibb et al., 2000). That both these mutants retained partial $\sigma^{BldN}$ activity became clear when a constructed null mutant was found to have a bald phenotype, devoid of aerial hyphae. Therefore, the gene was named bldN. Sequence analysis of the two NTG-induced bldN mutant alleles revealed that the more ‘severe’ mutant, R112, carries a mutation in the ribosome-binding site and presumably produces reduced amounts of wild-type $\sigma^{BldN}$, while in the ‘weak’ mutant, R650, the $\sigma^{BldN}$ produced carries a glycine to aspartate substitution in region 2.1 (Bibb et al., 2000). In other $\sigma$ factors, region 2.1 has been implicated in the interaction of $\sigma$ with core RNA polymerase (Burgess & Anthony, 2001), and it is therefore likely that the mutant $\sigma^{BldN}$ produced by R650 interacts less efficiently with core RNA polymerase than the wild-type protein.

**Control of bldN transcription**

The bldN promoter is temporally regulated, showing little or no activity during vegetative growth, but increasing dramatically during aerial mycelium formation and remaining highly active during sporulation (Bibb et al., 2000). Clues as to the mechanism that controls this temporal regulation in S. coelicolor have come from the analysis of bldN transcription in other bld mutants. No bldN transcripts were detectable in bldG and bldH mutant backgrounds, indicating that bldN expression depends on these two genes, either directly or indirectly (Fig. 4; Bibb et al., 2000). bldH has not been characterized, but bldG encodes a homologue of the SpoIIAA anti-anti-$\sigma$ factor from B. subtilis, implying that the role of bldG is indirect. Anti-anti-$\sigma$ factors are proteins that inhibit the activity of anti-$\sigma$ factors, thereby stimulating the activity of its cognate $\sigma$ factor. One possibility, therefore, is that bldG mutants have reduced activity of the $\sigma$ factor that is required for transcription of the bldN promoter, caused by the uncontrolled activity of the respective anti-$\sigma$ factor.

In contrast to the wild-type, bldN transcripts were readily detectable during vegetative growth in a bldD mutant, indicating that bldD acts to repress bldN transcription during vegetative growth (Fig. 4; Elliot et al., 2001). *In vitro* biochemical experiments showed that this effect is direct; BldD is a repressor of the bldN promoter, binding to two operator sites, one either side of the transcription start site (Elliot et al., 2001). Interestingly, BldD also represses transcription of another key developmental gene, wbiG, during vegetative growth (Elliot et al., 2001), and of the development-specific promoter (p2) of the sigH gene in vegetative hyphae (Kelemen et al., 2001), suggesting that one of BldD’s roles is to prevent premature expression of developmental genes.

Investigations by Yamazaki et al. (2000), working on the orthologue of $\sigma^{BldN}$ in *Streptomyces griseus*, have raised some intriguing possibilities for another mechanism
by which \textit{bldN} transcription might be regulated. In \textit{S. griseus}, the γ-butyrolactone signalling molecule A-factor (2-isocaproyl-3R-hydroxymethyl-γ-butyrolactone) triggers a regulatory cascade required for both aerial mycelium formation and production of the antibiotic streptomycin (Horinouchi & Beppu, 1994). A-factor causes expression of a transcriptional activator called AdpA, which induces streptomycin biosynthesis by activating transcription of \textit{strR}, the gene encoding the pathway-specific activator of the streptomycin cluster (Ohnishi \textit{et al.}, 1999). Until recently, no targets for AdpA have been identified to explain the morphological defects of an \textit{adpA} mutant. However, Yamazaki \textit{et al.} (2000) isolated new AdpA-binding sites from \textit{S. griseus} chromosomal DNA, one of which was the promoter of an ECF \(\sigma\) factor gene they named \textit{adsA} (AdpA-dependent \(\sigma\) factor), the \textit{S. griseus} orthologue of \textit{bldN}. As is true for \textit{S. coelicolor bldN}, transcription of \textit{S. griseus adsA} begins approximately at the time of aerial mycelium formation, and disruption of \textit{adsA} also results in loss of aerial mycelium formation. Neither \textit{S. coelicolor bldN} nor \textit{S. griseus adsA} is required for antibiotic production.
S. coelicolor does not produce A-factor, but it does produce several closely related γ-butyrolactone molecules (Efremenkova et al., 1985; Kawabuchi et al., 1997; Takano et al., 2000). These molecules are involved in a signalling pathway for antibiotic production, and there is evidence to suggest that some of them may also be involved in morphological development in S. coelicolor. The predicted AdpA-binding site is not clearly conserved in the promoter region of S. coelicolor bldN, but there is a very close relative of adpA in the S. coelicolor genome sequence, and it will be interesting to see whether it has a role in the control of bldN transcription.

Post-translational processing of $\sigma^{BldN}$

Most ECF $\sigma$ factors either completely lack conserved region 1 or have only a few residues upstream of region 2.1 (Lonetto et al., 1994). $\sigma^{BldN}$ is unusual in having an N-terminal extension of approximately 86 amino acids that is not present in other $\sigma$ factors (Bibb et al., 2000). Using a combination of immunoblotting and mutational analysis of the N-terminal extension, we have obtained substantial evidence that the primary translation product of the bldN gene is a pro-$\sigma$ factor, which is processed to a smaller, mature form through the proteolytic removal of most of the N-terminal extension (Fig. 4; M. J. Bibb & M. J. Buttner, unpublished). During B. subtilis development, the mother-cell-specific $\sigma$ factors $\sigma^E$ and $\sigma^K$ are synthesized as inactive pro-$\sigma$ factors that are subsequently activated by proteolysis of the N-terminal 29 and 20 amino acids, respectively, by membrane-localized proteases (Errington, 1996; Stragier & Losick, 1996). In both cases, the activation of this processing event is triggered by signals derived from the forespore, and this ‘crosstalk’ serves to coordinate the divergent programs of gene expression between the two cellular compartments within the spore (Errington, 1996; Stragier & Losick, 1996). The pro sequences of both pro-$\sigma^E$ and pro-$\sigma^K$ promote membrane association, whereas the mature forms of these proteins are found in the cytoplasm associated with core RNA polymerase (Hofmeister, 1998; Ju & Haldenwang, 1999; Ju et al., 1997; Zhang et al., 1998). The putative pro sequence of $\sigma^{BldN}$ contains a stretch of 20 hydrophobic amino acids (YAVPALAAAAVPAGPCYALA). It will be interesting in the future to determine if pro-$\sigma^{BldN}$ is membrane-associated, to identify the pro-$\sigma^{BldN}$ protease, and to define the signals responsible for triggering the processing event.

The $\sigma^{BldN}$ regulon

To date, only one $\sigma^{BldN}$ target gene has been identified (Bibb et al., 2000). Given the involvement of $\sigma^{BldN}$ in the control of aerial mycelium formation, it seemed likely that other bld genes might be regulated by $\sigma^{BldN}$ and would therefore have promoter sequences related to the consensus sequences of other ECF $\sigma$ factors. Analysis of the promoter regions of known bld genes revealed a possible ECF consensus-like promoter upstream of bldM. bldM encodes an apparently typical member of the FixJ subfamily.
of response regulators, although, surprisingly, aspartate-54, the putative site of phosphorylation, is not required for BldM function (Molle & Buttner, 2000). Transcript mapping experiments identified two promoters, one of which, bldMp1, corresponded to the putative ECF σ factor consensus-like sequence. Like the bldN promoter, bldMp1 was developmentally regulated, being inactive during vegetative growth, but strongly up-regulated during aerial mycelium formation and sporulation. Furthermore, bldMp1 was inactive in a bldN null mutant and was recognized by reconstituted σBldN-containing holoenzyme in vitro, showing that bldM is a direct biochemical target for σBldN holoenzyme (Bibb et al., 2000).

### Overlapping promoter specificity between ECF σ factors

Prior to the discovery of the ECF subfamily, sequence similarity had already been noted between the E. coli σE target rpoHp3 and the S. coelicolor σE target dagAp2 (Erickson & Gross, 1989). Following the characterization of many more promoters under the control of different ECF σ factors, it became clear that there was a significant degree of sequence conservation between them. This fact, together with the existence of multiple ECF σ factors in many bacteria, suggested that some promoters might be recognized by more than one ECF σ in vivo, and it is now clear that this is indeed the case. For example, of the 30 σR target promoters known, at least 13 retained some activity in a sigR null mutant. Furthermore, this σR-independent transcription was constitutive for some promoters but stimulated by diamide (but with delayed kinetics) for others, implying that it represented more than one additional ECF σ factor (Paget et al., 2001b). What are the key DNA sequence features that allow some σR target promoters to be recognized by additional holoenzymes forms while other promoters are recognized uniquely by σR? Analysis of the 30 known σR target promoters indicates that most promoters that are recognized by additional σ factors contain the −10 sequence CGTT, whereas those recognized only by σR have the −10 sequence TGTT or GGTT. Although the importance of the −10 region of σR target promoters in σ selectivity has not been proven, Helmann and colleagues have demonstrated that this region plays a critical role in σ selectivity between two ECF σ factors in B. subtilis. Single or double nucleotide changes in the −10 region of σX or σW target promoters switched their recognition characteristics such that promoters that were usually recognized by σW were recognized by σX, and vice versa (Qiu & Helmann, 2001). Recognition of a single promoter by multiple holoenzyme forms provides a very attractive mechanism for integrating different signal transduction pathways at single promoter elements. Overlapping specificity may be particularly useful in stress responses because different physical insults can often lead to the same physiological stress. For example, both oxidative stress and heat shock can induce protein misfolding. Nevertheless, target promoter sequence constraints must presumably ensure that, within the total subfamily of 51 ECF σ factors in S. coelicolor, each individual ECF σ factor has a distinct regulon and a
distinct biological role. The future identification of the complete regulons for each of these ECF σ factors using DNA microarrays will begin to address these intriguing issues.

**CONCLUSIONS**

The ECF subfamily of σ factors has emerged as a major class of regulatory proteins in *Streptomyces* spp. Detailed analysis of just three of these proteins – σE, σR and σBldN – has already revealed their involvement in a fascinating range of biological processes and shown that control of their activity can be exerted at several different levels, variously involving *de novo* synthesis, pro-σ processing, and anti-σ factor-directed regulation. Understanding the role and regulation of each of the remaining 48 ECF σ factors promises to be an absorbing task.

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