

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

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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 σ subunit that associates with the core enzyme. This key role of σ in promoter recognition suggests a mechanism for the coordinate control of gene expression using alternative forms of σ and different subsets of promoters, an idea that was first proposed as soon as the role of σ was established (Burgess *et al.*, 1969). It is now clear that most, if not all, bacteria use alternative σ subunits to control gene expression, and that these σ factors fall into two distinct families: the σ^N (or σ^{54}) family, which is discussed in the preceding chapter, and the σ^{70} family. The σ^{70} family includes those σ factors that, broadly speaking, are related in sequence and domain organization to the primary *Escherichia coli* σ factor, σ^{70} . Although the overall architecture of members of the σ^{70} family appears to be conserved, the σ^{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 σ factors

In the late 1980s, biochemical analysis of RNA polymerase from *Streptomyces coelicolor* and *E. coli* led to the identification of two σ 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^{32} , 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 (*dagAp2*) 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 Fecl 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* Fecl 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 σ factors (σ^E , σ^R and σ^{BldN}) that have been studied in detail in *S. coelicolor*. For each of these three σ factors, the mechanism controlling σ factor activity is different, variously involving *de novo* synthesis, pro- σ processing, and anti- σ factor-directed control. These examples serve to illustrate the fascinating variety of regulatory systems that exist in bacteria to ensure that σ factors are recruited to core RNA polymerase only when appropriate.

THE σ^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 σ^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: σ^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 σ^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 σ^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 than 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).

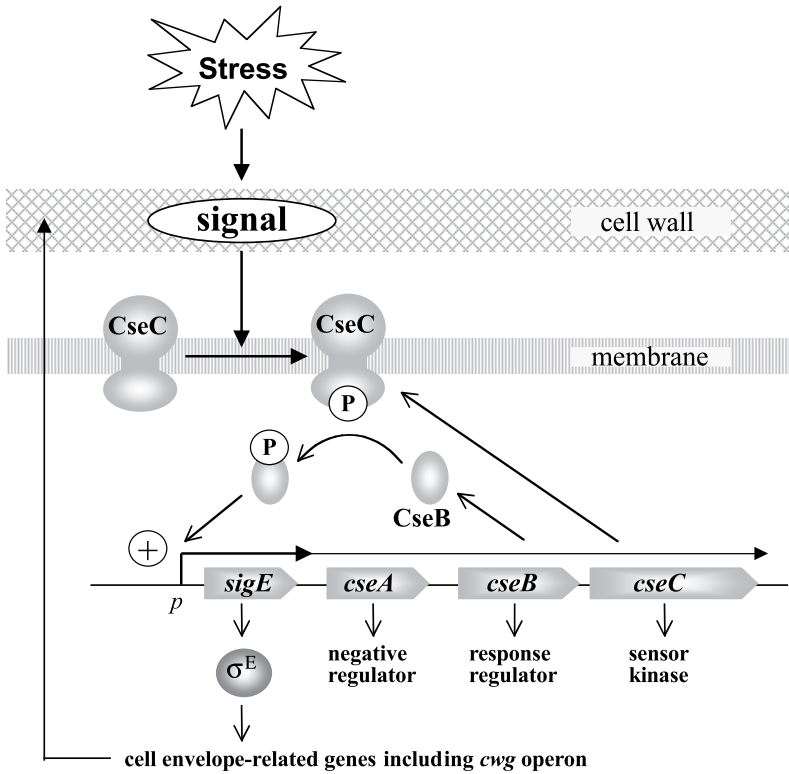


Fig. 1. Model for the regulation of σ^E activity in response to signals from the cell envelope. Expression of σ^E activity is governed at the level of *sigE* transcription by the CseB/CseC two-component signal transduction system. In response to signals that originate in the cell envelope when it is 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 σ^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.

Most transcripts from the *sigE* promoter terminate immediately downstream of *sigE*, but about 10% read through into the downstream genes (Paget *et al.*, 1999b). Analysis of the activity of the *sigE* promoter in different mutant backgrounds was highly informative. The *sigE* promoter was found to be inactive in a constructed *cseB* null mutant, such that *cseB* mutants lack σ^E . This observation explained why *cseB* and *sigE* mutants had the same phenotype (Paget *et al.*, 1999b). In contrast, the *sigE* promoter was substantially up-regulated in a *sigE* null mutant, suggesting that the cell envelope defects in *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 *sigE* and hence expression of the cell-envelope-related genes under σ^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 σ^E in controlling cell envelope integrity, antibiotics that target the cell envelope induce *sigE* expression. These included certain β -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 β -lactam-inducible β -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 σ^E -specific anti- σ factor (σ^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 containing purified σ^R initiated transcription from *trxBp1*, one of the two promoters that transcribe *trxB*, 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 uninduced in the *sigR* null mutant (Paget *et al.*, 1998).

Regulation of σ^R activity

The second σ^R target promoter to be identified, *sigRp2*, lay upstream of its own structural 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 σ^R synthesis, there must be a negative regulator in place to ensure that σ^R is only switched on when necessary and to ensure that its activity is effectively switched off when the disulphide stress has been dealt with. This key negative regulator was identified as RsrA (regulator of sigR), a σ^R -specific anti- σ factor that is encoded by the gene lying immediately 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 promoter binding (Hughes & Mathee, 1998; Helmann, 1999). Purified RsrA can bind tightly to σ^R and inhibit σ^R -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 σ^R nor inhibit σ^R -directed transcription (Kang *et al.*, 1999). Moreover, if *rsrA* is deleted from the *S. coelicolor* chromosome, σ^R target promoters are constitutively expressed at the fully induced level (Paget *et al.*, 2001a). In other words, the regulation of σ^R 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 σ^R , 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 σ^R activity is presented in Fig. 2. σ^R protein is present in the hyphae all the time, but σ^R activity is not, because, in the absence of oxidative stress, RsrA sequesters σ^R in an

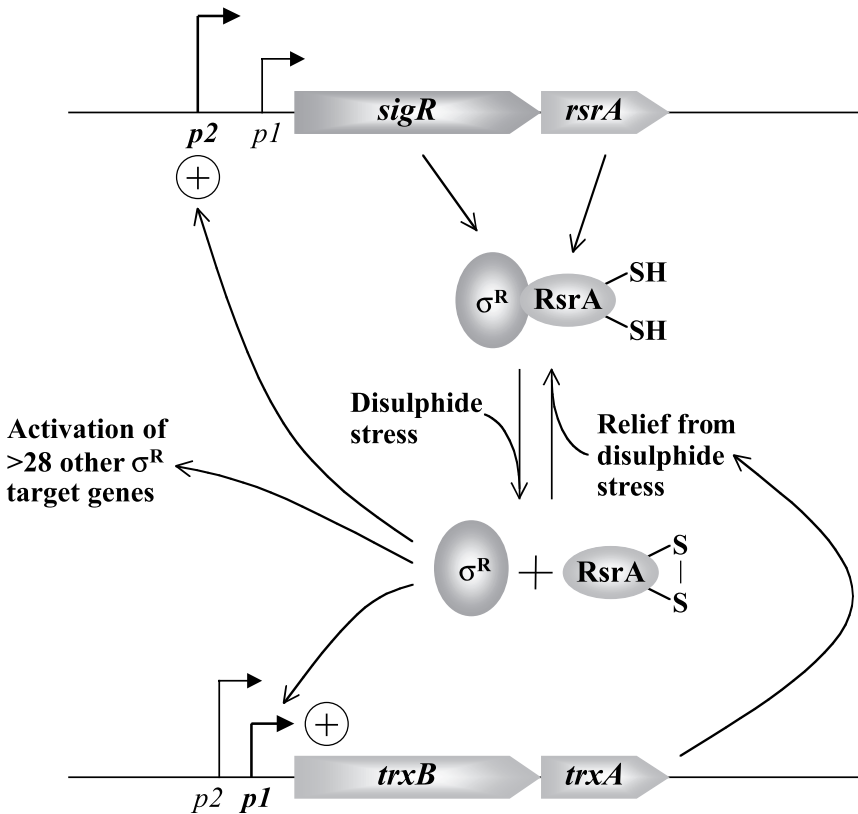


Fig. 2. Model for the regulation of σ^R activity in response to disulphide stress. The thiol–disulphide status of *S. coelicolor* is controlled by a novel regulatory system consisting of a σ factor, σ^R , and RsrA, a redox-sensitive, σ^R -specific, anti- σ factor. Under reducing conditions, RsrA binds to σ^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 σ^R , releasing σ^R to activate transcription of >30 genes and operons, including *trxB*. Increased *trxB* expression in turn leads to the thioredoxin-dependent reduction of oxidized RsrA back to its σ^R -binding conformation, thereby shutting off σ^R -dependent transcription. In addition, σ^R positively autoregulates expression of the *sigR-rsrA* operon. As a consequence, disulphide stress not only activates σ^R post-translationally, but also induces its *de novo* synthesis.

RsrA : σ^R complex. σ^R is released during oxidative stress as a direct consequence of the inactivation of RsrA through intramolecular disulphide bond formation. σ^R is then free to associate with core RNA polymerase and activate transcription of its target genes, including *trxB* and other thiol–disulphide oxidoreductase genes (see ‘The σ^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

RsrA (SC7E4.14)	18	IYEFLLDKEMPDSDCVKFEHHFEECSPCLEKY	48
RstA (SCH24.13c)	10	IADLAEGLLPTRTRTTEVRQHLESCELCADVY	40
RsuA (SCE59.12c)	13	VGAYALGILDDAEATAFEAHLATCEWCAQQL	43
SCM10.32	10	VGAYALGVLDDEAEAFRFEDHLMCCPRCAAQV	40
SCF56.17	10	TGAYALHALPDDEREAFERHLAGCATCEQEA	40
SCI11.11c	9	AAPYALDALEGAERVRFERHLEGCAAAEV	39
SC6F11.06c	71	LGAWALAACSAPEAAAVEEHLGECDSCADEA	101
SCD84.14	19	LRAYARGELAAPALWSTDAHLTACATCRGVL	49
SCE46.08	29	VEAYADGQLTGAHRMQVAAHIAACWACSGSL	59
SCJ12.08	9	LVELALGHASGEADVGLRHAASCPCREEL	39
SCP8.11	21	LSALVDGELGHDARERVLAVATCPKCKAEV	51
SCE46.06c	24	LQSYLDGETDEVTTARRVAAHLEDCCRRGLEA	54

Fig. 3. Alignment of the HisXXXCysXXCys motif in RsrA and 11 other putative ZAS anti- σ factors from *S. coelicolor*. All of the genes encoding these proteins are located near (typically downstream and immediately adjacent to) genes encoding ECF σ factors.

also reduces (reactivates) RsrA *in vivo*, this would allow it to rebind σ^R and shut down the response, thereby creating a simple homeostatic feedback loop in which the σ^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 σ^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 σ^R activity and release it during disulphide stress. The remaining three individual cysteine mutants (C11, C41 and C44) had no σ^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 σ^R -binding activity of RsrA by coordinating a zinc cofactor (see below).

The ZAS family of anti- σ 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 σ^W -dependent gene expression is not induced by diamide and the known σ^W 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 σ^E in *Rhodobacter sphaeroides*, but σ^E directs expression of the cytochrome *c*₂ structural gene (Newman *et al.*, 1999, 2001). Further, deletion of *chrR* or the σ^E -encoding *rpoE* does not affect the resistance of *R. sphaeroides* to diamide, and diamide does not induce σ^E -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 σ^R regulon

Searches for further σ^R 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 σ^R *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 σ^R -dependent manner (Paget *et al.*, 2001b). More

than half of the σ^R target genes associated with these promoters have no known biological function.

Unsurprisingly, several σ^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 σ^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 σ^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 *sigR* mutant was found to have significantly lowered levels of mycothiol (Paget *et al.*, 2001b). The root cause of diamide sensitivity in *sigR* mutants could therefore be due to any one of these σ^R -dependent mechanisms for coping with disulphide stress, or a combination of all of them.

At least three σ^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 (Chakraborty & 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 σ^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 σ^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 σ^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 σ^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 σ^R regulon may therefore be considerably larger than the current total. Nonetheless, the identification of 30 genes and operons under σ^R control is a very significant step towards understanding the cellular response to disulphide stress in *S. coelicolor*.

Is σ^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 σ^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 σ^R activity. One possible explanation for these observations is that the high level of free σ^R out-competes a sporulation-specific σ factor, such as σ^{WhiG} (Chater *et al.*, 1989), for core RNA polymerase (Paget *et al.*, 2001a). However, recent analogous experiments with σ^U and RsuA, another ECF σ factor: ZAS anti- σ 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 σ activity. As pointed out by Gehring *et al.* (2001), it seems unlikely that σ^R and σ^U could differentially compete with different σ 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 σ^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 σ^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 σ^R -RsrA system also exists in pathogenic actinomycetes

The σ^R -RsrA system appears to exist in other actinomycetes. It is certainly present in mycobacteria, where it is named σ^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* σ^R target genes and operons so far identified, 13 of the homologues in *M. tuberculosis* have sequences upstream that resemble the consensus for σ^R -dependent promoters and may therefore be regulated by σ^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 σ^H -RshA system contributes to the well known resistance of *M. tuberculosis* to oxidative killing by white blood cells during human infection.

THE σ^{BldN} PATHWAY TO AERIAL MYCELIUM FORMATION

Unlike σ^E and σ^R , which were discovered biochemically, σ^{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 σ^{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 σ factor (Bibb *et al.*, 2000). That both these mutants retained partial σ^{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 σ^{BldN} , while in the 'weak' mutant, R650, the σ^{BldN} produced carries a glycine to aspartate substitution in region 2.1 (Bibb *et al.*, 2000). In other σ factors, region 2.1 has been implicated in the interaction of σ with core RNA polymerase (Burgess & Anthony, 2001), and it is therefore likely that the mutant σ^{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- σ factor from *B. subtilis*, implying that the role of *bldG* is indirect. Anti-anti- σ factors are proteins that inhibit the activity of anti- σ factors, thereby stimulating the activity of its cognate σ factor. One possibility, therefore, is that *bldG* mutants have reduced activity of the σ factor that is required for transcription of the *bldN* promoter, caused by the uncontrolled activity of the respective anti- σ 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, *whiG*, 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 σ^{BldN} in *Streptomyces griseus*, have raised some intriguing possibilities for another mechanism

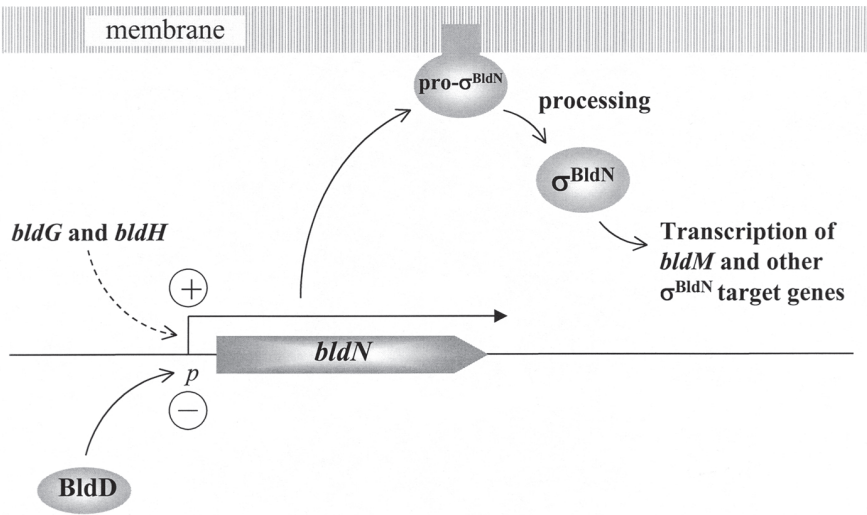


Fig. 4. Model for the regulation of σ^{BldN} activity during development. σ^{BldN} activity is regulated at the level of transcription of the *bldN* gene and by post-translational processing of the primary translation product, pro- σ^{BldN} . The *bldN* promoter shows little or no activity during vegetative growth, but is dramatically up-regulated during differentiation. This developmental control is mediated in part by BldD, which binds the *bldN* promoter and represses *bldN* transcription during vegetative growth. In contrast, the products of the *bldG* and *bldH* genes are required, directly or indirectly, for the activation of *bldN* transcription during development. The primary translation product of the *bldN* gene is a pro- σ factor, which is processed to a smaller, mature form through the proteolytic removal of an unusual N-terminal extension. The amino acid sequence of this N-terminal extension suggests that it might cause pro- σ^{BldN} to associate with the membrane. Release of mature σ^{BldN} allows the activation of its target genes, which include *bldM*.

by which *bldN* transcription might be regulated. In *S. griseus*, the γ -butyrolactone signalling molecule A-factor (2-isocaprolyl-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 *strR*, the gene encoding the pathway-specific activator of the streptomycin cluster (Ohnishi *et al.*, 1999). Until recently, no targets for AdpA have been identified to explain the morphological defects of an *adpA* mutant. However, Yamazaki *et al.* (2000) isolated new AdpA-binding sites from *S. griseus* chromosomal DNA, one of which was the promoter of an ECF σ factor gene they named *adsA* (AdpA-dependent σ factor), the *S. griseus* orthologue of *bldN*. As is true for *S. coelicolor bldN*, transcription of *S. griseus adsA* begins approximately at the time of aerial mycelium formation, and disruption of *adsA* also results in loss of aerial mycelium formation. Neither *S. coelicolor bldN* nor *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 σ^{BldN}

Most ECF σ factors either completely lack conserved region 1 or have only a few residues upstream of region 2.1 (Lonetto *et al.*, 1994). σ^{BldN} is unusual in having an N-terminal extension of approximately 86 amino acids that is not present in other σ 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- σ 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 σ factors σ^{E} and σ^{K} are synthesized as inactive pro- σ 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 sporangium (Errington, 1996; Stragier & Losick, 1996). The pro sequences of both pro- σ^{E} and pro- σ^{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 σ^{BldN} contains a stretch of 20 hydrophobic amino acids (YAVPALAAA AV-PAGPCYALA). It will be interesting in the future to determine if pro- σ^{BldN} is membrane-associated, to identify the pro- σ^{BldN} protease, and to define the signals responsible for triggering the processing event.

The σ^{BldN} regulon

To date, only one σ^{BldN} target gene has been identified (Bibb *et al.*, 2000). Given the involvement of σ^{BldN} in the control of aerial mycelium formation, it seemed likely that other *bld* genes might be regulated by σ^{BldN} and would therefore have promoter sequences related to the consensus sequences of other ECF σ 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 σ^{BldN} -containing holoenzyme *in vitro*, showing that *bldM* is a direct biochemical target for σ^{BldN} 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* σ^{E} target *rpoHp3* and the *S. coelicolor* σ^{E} 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 σ^{R} target promoters known, at least 13 retained some activity in a *sigR* null mutant. Furthermore, this σ^{R} -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 σ^{R} target promoters to be recognized by additional holoenzyme forms while other promoters are recognized uniquely by σ^{R} ? Analysis of the 30 known σ^{R} target promoters indicates that most promoters that are recognized by additional σ factors contain the -10 sequence CGTT, whereas those recognized only by σ^{R} have the -10 sequence TGTT or GGTT. Although the importance of the -10 region of σ^{R} 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 σ^{X} or σ^{W} target promoters switched their recognition characteristics such that promoters that were usually recognized by σ^{W} were recognized by σ^{X} , 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 – σ^E , σ^R and σ^{BldN} – 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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