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Review

Redox control in actinobacteria

Chris D. den Hengst *, Mark J. Buttner

Department of Molecular Microbiology, John Innes Centre, Norwich Research Park, Colney, Norwich, NR4 7UH, UK

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Abstract

As most actinobacteria are obligate aerobes, they have to cope with endogenously generated reactive oxygen species, and actinobacterial pathogens have to resist oxidative attack by phagocytes. Actinobacteria also have to survive long periods under low oxygen tension; for example, *Mycobacterium tuberculosis* can persist in the host for years under apparently hypoxic conditions in a latent, non-replicative state. Here we focus on the regulatory switches that control actinobacterial responses to peroxide stress, disulfide stress and low oxygen tension. Other unique aspects of their redox biology will be highlighted, including the use of the pseudodisaccharide mycothiol as their major low-molecular-weight thiol buffer, and the [4Fe–4S]-containing WhiB-like proteins, which play diverse, important roles in actinobacterial biology, but whose biochemical role is still controversial.
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1. Introduction

Most actinobacteria are obligate aerobes, and an inescapable consequence of aerobic metabolism is the production of reactive oxygen species (ROS) that can damage numerous components of the cell, including lipids, nucleic acids, proteins and metal cofactors, leading to a deterioration in cellular function usually termed oxidative stress. Oxidative stress can also be imposed on actinobacteria externally, for example ROS (and reactive nitrogen species) are generated by human phagocytes to kill pathogenic actinobacteria such as *Mycobacterium tuberculosis* (*Mtb*) and *Corynebacterium diphtheriae*.

A 'subset' of oxidative stress relevant to part of this review is the formation of unwanted disulfide bonds, a condition termed 'disulfide stress' by Åslund and Beckwith [1]. The cytoplasm of all organisms is a highly reducing environment in which protein cysteines are maintained in their reduced thiol (−SH) state. This environment is maintained in part by millimolar concentrations of low-molecular-weight thiol buffers such as glutathione, or in the case of actinobacteria, the structurally unrelated mycothiol. In addition to these thiol buffers, living cells also use a variety of reductive enzymatic pathways to remove disulfide bonds from the cytoplasm, including the thioredoxin pathway, which is present in actinobacteria. Despite these protective mechanisms, unwanted thiol oxidation occurs in actinobacterial cells, and these bacteria have evolved a unique mechanism to sense and respond to this disulfide stress.

To counteract oxidative stress, actinobacteria activate functions that detoxify ROS and repair cellular damage. To achieve this, transducing proteins (mostly transcription factors) act as molecular switches that are either activated or inactivated by exposure to ROS. In many cases, these switches exploit the chemistry of the residues and cofactors that have to be protected elsewhere in the cell. Thus, the very vulnerability of, for example, Fe–S clusters and cysteine thiol groups, to ROS is exploited to create regulators that can switch from one state to another. In this way, a chemical signal is transduced into a biological readout through an induced conformational change in a regulatory protein.

Although most actinobacteria are obligate aerobes, they may have to survive long periods under low oxygen tension. For example, *Mtb* can survive for years in the human body in a non-dividing, latent state, and this condition is very likely to be linked to hypoxia. The rapid withdrawal of oxygen from *Mtb* is lethal, but gradual depletion allows the bacteria to adapt and survive [2]. This adaptive response to hypoxia in *Mtb* is mediated, at

* Corresponding author. Tel.: +44 1603 450796; fax: +44 1603 450778.
E-mail address: Chris.den-Hengst@bbsrc.ac.uk (C.D. den Hengst).

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least in large part, by transcriptional regulatory systems that exploit the ability of heme cofactors to monitor oxygen and redox poise.

Here we review current knowledge of redox control in actinobacteria, particularly *Streptomyces coelicolor* and *Mtb*, focusing on the best-characterised regulatory systems and the most conspicuously novel aspects of their redox biology, including mycothiol and the WhiB-like proteins.

2. Thiol-disulfide oxidoreductase systems

2.1. Thioredoxin-dependent systems in actinobacteria

The thioredoxin system (Fig. 1) is a major antioxidant system for protection against oxidative stress and the maintenance of intracellular thiol homeostasis [3] and is composed of thioredoxin (TrxA) and thioredoxin reductase (TrxB). Thioredoxins are small (8–14 kDa), heat-resistant proteins that use their electrons to reduce unwanted disulfide bonds formed in cellular proteins during oxidative stress and that are also required for the function of some enzymes, such as ribonucleotide reductase, which form disulfides at their active site as part of their catalytic cycle [3]. Thioredoxins are characterised by the presence of a highly conserved CXXC motif in their active site. These two neighbouring cysteines are contained in a characteristic protein fold, the thioredoxin fold, and form a redox-active disulfide bond. Thioredoxins are kept in the reduced thiol state by thioredoxin reductase, which is a member of the large family of flavoprotein oxidoreductases. These dimeric enzymes catalyse the transfer of electrons from NADPH, via two FAD prosthetic groups, to two cysteine residues in the TrxB active site that, in turn, can reduce oxidised TrxA.

The ubiquitous thioredoxin system is found in nearly all known living organisms. Of the actinobacteria, the Trx system was first characterised in *Streptomyces clavuligerus*, the producer of clavulanic acid, where it was identified as a NADPH-dependent factor with a broad-range disulfide reductase activity. In addition to reducing protein disulfide bonds *in vitro*, the thioredoxin system of *Streptomyces* can reduce the oxidised disulfide form of the cysteine-containing tripeptide β-lactam precursor ACV. Bis-ACV is not a substrate for isopenicillin-*N*-synthase, which led to suggestions that the thioredoxin system may play a crucial role in the biosynthesis of penicillin and cephalosporin antibiotics in β-lactam-producing streptomycetes [4,5]. To date, the thioredoxin systems of several streptomycetes and mycobacterial species have been cloned and characterised [6–10]. However, in the model organism, *S. coelicolor*, only TrxBA (encoded by SCO3890 and SCO3889) has been biochemically characterised and was shown to constitute an NADPH-dependent redox couple able to efficiently reduce insulin, a widely-used thioredoxin substrate [9,11], as well as the oxidised form of RsrA, a redox-sensing anti-sigma factor [12]. The genome of *S. coelicolor* encodes three other putative thioredoxins (SCO5438, SCO5419 and *trxC*/SCO0885) of which only *trxC* is adjacent to a gene encoding a putative FAD-containing oxidoreductase, suggesting that they could form a second TrxBA-like pair [13,14]. Expression of *trxAB* and *trxC* is controlled by σ^R, an RNA polymerase sigma factor that regulates the thiol-disulfide status in *S. coelicolor* (see below; [11,12,14,15]).

For resistance to hydroperoxides, *Mtb* depends on the activities of two other thioredoxin-dependent enzyme systems. Firstly, they contain an alkyl hydroperoxide reductase, AhpC, which belongs to the peroxiredoxin superfamily and converts organic hydroperoxides to their corresponding alcohols with concomitant formation of an intersubunit disulfide bond [16]. The way in which mycobacterial AhpC receives its reducing equivalents is quite different to AhpCs from other bacteria. Commonly, AhpC is reduced by AhpF, but such an activity could not be detected in *Mtb*. Instead, AhpC is recycled by AhpD. AhpD is a thioredoxin-like molecule and has a CXXC motif in its active site that reduces the disulfide in AhpC using electrons from NADH, transferred through dihydrolipoamide dehydrogenase (Lpd) and dihydrolipoamide succinyltransferase (SucB) [16]:

$$\text{NADH}\rightarrow\text{Lpd}\rightarrow\text{SucB}\rightarrow\text{AhpD}\rightarrow\text{AhpC}\rightarrow\text{ROOH}$$

In contrast to most bacteria, mycobacterial AhpC can also be reduced by thioredoxin C, but not by TrxB [17]. A second thioredoxin-dependent peroxiredoxin system in *Mtb* is encoded by *tpx*. Tpx can react with a wide range of hydroperoxides and is superior to AhpC with respect to the rate constants of most of these reactions. In contrast to AhpC, Tpx can be reduced by both TrxB and TrxC [17]. Transcripts of *ahpC* and *tpx* were detected in *Mtb* in a screening for genes that are differentially expressed under disulfide stress, which was artificially induced with the thiol-specific oxidising agent diamide [18]. As AhpC and Tpx protect the pathogen against ROS that are created in the host phagosome, they are important virulence factors. Expression of *ahpC* has also been linked to resistance to the primary antitubercular drug isoniazid, INH [19]. Some resistant strains contain a mutation in the catalase gene (*katG*), which is essential

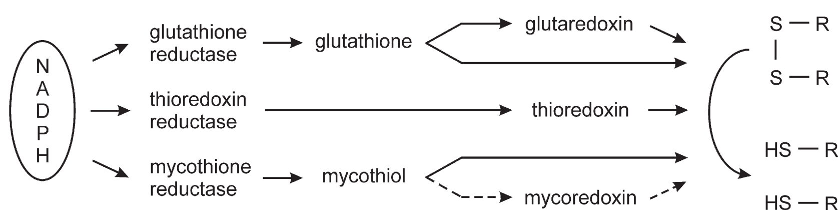

Fig. 1. Thiol-disulfide oxidoreductase pathways. Top, middle and bottom reactions show flow of electrons from NADPH to disulfide substrates via the glutaredoxin, thioredoxin and hypothetical (dashes) mycoredoxin pathways, respectively.

for INH activation. Hyperexpression of *ahpC* is observed in these *katG* deficient strains, suggesting the existence of a compensation mechanism [19].

2.2. Mycothiol, the major thiol in actinobacteria

In addition to the thioredoxin system, most organisms contain millimolar concentrations of low-molecular-weight (LMW) thiol compounds that serve as a buffer to avert disulfide stress. The most well-known LMW thiol buffer is the cysteine-containing tripeptide glutathione (GSH), that can reduce disulfide bonds by (*i*) directly forming a mixed disulfide with a substrate or (*ii*) indirectly, via glutaredoxin enzyme (Fig. 1). GSH is present in animals, plants, Gram-negative bacteria and some Gram-positive bacteria, but is absent from the actinobacteria [20,21]. Instead, actinobacteria contain millimolar concentrations of mycothiol (MSH; 1-D-*myo*-inositYl-2-(*N*-acetyl-L-cysteinyl)-amido-2-deoxy-α-D-glucopyranoside; Fig. 2) [20]. MSH is a pseudodisaccharide containing a cysteine moiety as a reactive thiol [20,22–24]. Importantly, MSH undergoes metal ion-catalysed autoxidation to its disulfide, mycothione (MSSM), at a much slower rate than GSH (GSSG) and cysteine do, while the redox potential of MSH/MSSM is believed to be similar to that of GSH/GSSG, thereby making it a very suitable antioxidant [25].

Oxidised MSH is recycled by mycothione reductase (Mtr). Mtr was first characterised in *Mtb* and catalyses the reduction of MSSM with a concomitant oxidation of NADPH [26,27]:

$$\mathrm{NADPH} + \mathrm{H}^+ + \mathrm{MSSM} \rightarrow \mathrm{NADP}^+ + 2\mathrm{MSH}$$

Active Mtr is a homodimer composed of two 50-kDa subunits and, like glutathione reductase (GR), uses an FAD group to shuttle electrons from NADPH to two cysteines present in the active site [26]. Although Mtr was identified as a homologue of human GR, it cannot catalyse the reduction of GSSG. It is currently not known whether Mtr and MSH form part of a system analogous to the glutaredoxin pathway, which could be designated the “mycoredoxin pathway” (Fig. 1). Strikingly, despite the absence of GSH, many species of actinobacteria (e.g. *Mtb* and *S. coelicolor*) possess one or more homologues of *E. coli* glutaredoxin-1. The genome of *S. coelicolor* encodes three Grx-like proteins that all contain the CXXC motif conserved in the active site of Grx1 [13,14]. It is not known what role these proteins serve but, by analogy with the glutaredoxin pathway, one formal possibility is that they could act as ‘mycoredoxins’, transferring electrons from mycothiol to disulfide substrates.

Mycothiol biosynthesis is a multi-step process (Fig. 2) requiring the products of the *mshA*, a postulated *mshA2*, *mshB*, *mshC* and *mshD* genes [28–31]. With the biochemical characterisation of the initial steps in *Mycobacterium smegmatis*, the MSH biosynthetic pathway is now fully elucidated [29]. MshA is a glycosyltransferase that makes the disaccharide from 1L-Ins-1-P and UDP-GlcNAc [32], after which MshA2 (encoded by a yet unidentified gene) and MshB catalyse the removal of a phosphate and acetyl group, respectively [29,33]. Subsequently, the ligase MshC attaches a cysteine moiety at the expense of ATP [34]. In the final step the acetyltransferase MshD transfers an acetyl group from acetyl CoA to yield MSH [35]. Recently, the homologous MSH biosynthetic pathway in *S. coelicolor* was identified [36]. *S. coelicolor* mutants of *mshA*, *C* and *D* produce no detectable levels of MSH, showing that they encode unique activities in this bacterium. In contrast, deletion of *mshB* only resulted in a 90% reduction in MSH levels, indicating that at least one other protein must have acetylase activity [36]. Similarly, it was shown that *mshC* is absolutely required for MSH biosynthesis in both *M. smegmatis* and *Mtb*, whereas all the other mutants produced lowered levels of MSH [37–39]. Since *S. coelicolor* and *M. smegmatis msh* mutants lacking MSH can be constructed, these bacteria clearly do not require MSH for viability, in contrast to *Mtb* for which a certain level of MSH is essential [39]. Interestingly, when *mshD* is mutated in *M. smegmatis*, two novel thiols, *N*-formyl-Cys-GlcN-Ins and *N*-succinyl-Cys-GlcN-Ins are produced that may function as a substitute for MSH [40].

In addition to MSH (derivates), actinobacteria produce another LMW thiol buffer called ergothioneine (ESH), but ESH does not appear to play a role similar to that of MSH [20,41]. Whereas MSH is kept at millimolar levels, the amount of ESH is ~10-fold lower in these bacteria [25,42].

2.3. Cellular role of mycothiol

Since its discovery in 1993, MSH has been shown to be involved in a wide variety of processes. There are indications that MSH is important for virulence of *Mtb*, as the *mshD* gene appears to be essential for growth in macrophages [43]. MSH mutants are hypersensitive to oxidative stress, caused by reactive oxygen compounds such as cumene hydroperoxide, hydrogen peroxide as well as nitric oxide, free radicals and diamide [20,37–39,42,44,45]. Thus, MSH is required to sustain the redox balance of the cell and its level increases during late exponential/stationary phase in *Mtb* [46]. Interestingly, in *S. coelicolor*, MSH levels are reduced four-fold in a null mutant

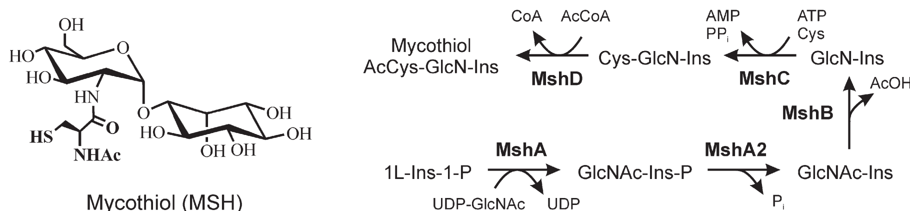

Fig. 2. Structure (left) and biosynthetic pathway (right) of mycothiol.

of *sigR*, suggesting that one or more of the MSH biosynthetic genes is a σ^R target and that MSH biosynthesis becomes induced under disulfide stress [14].

The first MSH-dependent enzyme that was characterised is the NAD/MSH-dependent formaldehyde dehydrogenase, MscR, which is involved in detoxification of formaldehyde. MSH, like GSH, spontaneously reacts with formaldehyde, after which the resulting molecule is converted to MSH and formate by the combined action of MscR and a thiol esterase [47]. More recently, it was found that MscR operates primarily as an MS-nitroso reductase, suggesting a role for the enzyme in the protection against oxidative stress, which leads to the formation of reactive nitrogen intermediates (RNI) [48].

MSH also serves a key function in a novel system for the detoxification of alkylating agents [49], which was identified using the fluorescent alkylating agent monobromobimane (mBBr) that can enter cells and react with thiols. Mycobacteria incubated with mBBr form *S*-conjugates of MSH and mBBr. The enzyme mycothiol-*S*-conjugate amidase (Mca) rapidly converts the conjugate into GlcN-ins plus AcCySmB. Finally, this mercapturic acid derivate is expelled into the medium [49]. Organisms depending on glutathione utilise the well-characterised glutathione *S*-transferase to form the analogous *S*-conjugate. For actinobacteria it is not known whether such an enzyme is required or whether MSH spontaneously reacts with the alkylating agent (although the reaction with mBBr is spontaneous, this may not be true for less reactive electrophiles). Mycobacterial mutants for MSH are more sensitive to a wide variety of antibiotics, such as erythromycin, azithromycin, penicillin G and vancomycin [39,46]. It has been proposed that the MSH *S*-conjugate amidase pathway is responsible for detoxification of these antibiotics itself, which, if true, would make the amidase an attractive candidate for drug development against pathogenic mycobacteria [38].

Recently, an additional role of mycothiol has been reported: MSH can actively be imported by *M. smegmatis* and can function as a resource for metabolic precursors and for energy production [50]. For example, MSH can be used as a storage form of cysteine, the intracellular accumulation of which is undesirable because it rapidly oxidises, which can lead to the formation of peroxides as toxic by-products [50].

3. Iron–sulfur cluster assembly in actinobacteria

Because of their electrochemical properties, iron–sulfur [Fe–S] clusters are present in many proteins involved in redox homeostasis. [Fe–S] clusters, which exist in [2Fe–2S], [4Fe–4S], [3Fe–4S] or more complex forms, can serve as redox-active cofactors in processes such as electron transfer and enzyme activation (for excellent reviews see [51,52]). Because the actual configuration of the cluster depends on the redox status of the cell, they are well-suited to serve as a switch that controls the activity of transcriptional regulators involved in oxidative stress. The classical example of such a regulator is FNR, a homodimeric protein containing one $[4Fe–4S]^{2+}$ cluster per monomer, coordinated by four cysteine residues. FNR regulates >100 genes in response to oxygen limitation: it represses genes needed for respiration and activates genes involved in the use of alternative terminal electron acceptors. When exposed to oxygen, the cluster converts to a $[2Fe–2S]^{2+}$ form and dimerisation and high-affinity DNA binding are abolished, resulting in derepression of FNR target genes [53].

In vitro, [Fe–S] clusters can be reconstituted spontaneously, from free Fe^{2+} and S^{2-} in the presence of a reducing agent, but it is generally believed that, *in vivo*, [Fe–S] proteins depend on a dedicated machinery to assemble the [Fe–S] cluster and transfer it to the apoprotein. In bacteria, at least three different such systems are active. The first one to be discovered was the NIF system, consisting of NifSU, identified as a factor needed for maturation of nitrogenase in *Azotobacter vinelandii* [54]. NifS is a pyridoxal phosphate-containing desulfurase that liberates inorganic sulfur from L-cysteine. NifU serves as a scaffolding protein on which the [Fe–S] cluster is assembled prior to its transfer to nitrogenase. A second assembly system is encoded in the *isc* (*i*ron–*s*ulfur *c*luster) locus [55]. Like NIF, ISC utilizes the activities of a desulfurase (IscS) and a scaffolding protein (IscU). In addition, the *isc* gene cluster encodes two heat shock chaperones and contains a gene, *cysE*, needed for cysteine biosynthesis [55]. Whereas NifS is dedicated to nitrogenase maturation, the ISC machinery is thought to serve a much wider, housekeeping role in [Fe–S] assembly [51]. SUF (*s*ulfur *u*tilisation *f*actor) forms a third conserved system involved in [Fe–S] cluster formation and was first identified in *E. coli* [56]. This system, composed of six proteins encoded by *sufABCDSE*, was found to play a crucial role during oxidative stress and iron limitation and is regulated by both the ferric-uptake regulator Fur and OxyR, the latter of which is responsive to H_2O_2 (see below).

These three [Fe–S] cluster assembly machineries have been studied extensively and appear to have partially overlapping functions, as removing parts of these systems does not necessarily lead to severe growth defects [57]. Relatively little is known about the systems involved in [Fe–S] cluster formation in actinobacteria. Recently, a SUF system was identified in *Mtb*, of which the highly conserved *sufB* gene is interrupted by an intein coding sequence [58]. It was shown that formation of a protein complex involving SufBCD, which are proposed to function as an ATPase complex providing energy for the cluster assembly steps [59], is dependent on splicing of SufB [60]. Attempts to delete the *suf* region of *M. smegmatis* failed, and it was suggested that the SUF system is the unique and exclusive way to assemble and repair the [Fe–S] clusters in these bacteria [58]. Although a genome survey revealed that no *isc* and *nif* gene clusters are present in available mycobacterial sequences [58], they do contain homologues of *nifS*/*iscS*. At least one of these proteins (Rv3025c) is a functional L-cysteine desulfurase *in vitro* [61]. However, it is not known whether Rv3025c functions in [Fe–S] cluster formation in *Mtb in vivo*. In *S. coelicolor* the *suf* operon is similarly conserved and, like *Mtb*, includes a copy of a *nifU*-like gene as the penultimate gene in the operon. In addition, the *S. coelicolor* genome encodes five other NifS-like proteins [13].

In *Streptomyces lividans*, a NifS/IscS-like protein, DndA, was shown to be involved in a highly novel DNA modification system

that can sensitise DNA to degradation [62,63]. The corresponding gene is located in the *dndA-E* gene cluster that is contained in a genomic island conferring the Dnd (DNA degradation) phenotype. The Dnd system was initially discovered as a type of modification that makes DNA susceptible to site-specific double-strand cleavage during electrophoresis in Tris buffers contaminated with ferrous iron [64–66]. Recently, it was shown that this post-replicative modification results in the replacement of one of the phosphate oxygen atoms in the DNA backbone with sulfur (i.e. phosphorothioation) at a cGGCCgccg consensus site [62,67,68]. Part of the biochemical pathway leading to *S*-modification has some similarity with the 4-thiouridine tRNA modification pathway in *E. coli*, which depends on only two proteins, IscS and ThiI [62,63]. The IscS-like DndA is a cysteine desulfurase required for the formation of an [Fe–S] cluster in apo-DndC, the *S. lividans* homologue of ThiI. The other proteins involved in the Dnd system (DndB, DndD and DndE) are believed to catalyse later steps in the *S*-modification reaction, but their exact functions are currently not understood [62,63]. Eleven counterparts of the *dnd* cluster were identified in distantly related bacteria [69]. In addition, about 10% of 74 investigated actinobacterial strains were found to possess a *dnd* cluster, which are all located on mobile elements [69]. Although no biological function has been recognised for the Dnd system, it has been proposed that *S*-modification could work as a host defence system similar to that of DNA methylation for protection against nucleases [67].

4. Regulatory systems

4.1. OhrR — a sensor of lipid hydroperoxides

Lipid hydroperoxides, generated during oxidative stress, can oxidise other molecules, promote the formation of highly reactive lipid radicals and adversely affect membrane function [70,71]. To counteract these effects, bacterial cells have evolved multiple mechanisms to detoxify hydroperoxides. The best characterised of these is the enzyme alkyl hydroperoxide reductase (AhpC), which is activated by thioredoxin-like proteins (see above). A second, more recently identified system, confined to bacteria, is the enzyme Ohr (*o*rganic *h*ydroperoxide *r*esistance), which reduces organic hydroperoxides to their corresponding alcohols in a thiol-dependent manner [72]. Ohr proteins are widely distributed in both Gram-negative and Gram-positive bacteria and, where characterised, their expression is controlled by a redox-regulated transcription factor, OhrR, which is frequently genetically linked. OhrR regulators are members of the MarR family of transcription factors, and have been studied in species of *Bacillus*, *Xanthomonas* and *Streptomyces* [73–76,79]. OhrR functions as a repressor of *ohr* and its activity is controlled by organic hydroperoxide-induced thiol oxidation. OhrR proteins can be divided into two classes: the multiple cysteine subfamily and the single cysteine subfamily. All OhrR proteins are homodimeric and sense lipid hydroperoxides via initial oxidation of a family-wide conserved cysteine near the N-terminus of the protein that creates a transient cysteine sulfenic acid derivative. However, although all OhrR proteins are homologous, subsequent reactions following the initial oxidation event appear not to be conserved. In the multiple cysteine subfamily, which is the major class and exemplified by the *Xanthomonas* protein, the N-terminal cysteine sulfenic acid goes on to form an intersubunit disulfide with a different cysteine in the apposing subunit, leading to decreased binding affinity and induction of *ohr* expression [76,77]. However, in the single cysteine subfamily, exemplified by the *B. subtilis* protein, no intersubunit disulfide forms. The N-terminal cysteine sulfenic acid derivative is relatively stable *in vitro* [75], but recent evidence suggests that sulfenic acid formation is not sufficient for induction and that formation of a cyclic sulfenamide or formation of a mixed disulfide with a low-molecular-weight intracellular thiol (such as cysteine or CoASH) is required for efficient induction in the single cysteine subfamily [78]. The cyclic sulfenamide can be reduced back to cysteine by DTT *in vitro*, implying that the OhrR redox switch is likely to be reversible *in vivo*, regardless of whether DNA binding is abolished by the formation of a disulfide bond or a cyclic sulfenamide.

Among the actinobacteria, OhrR has only been studied in streptomyces [79]. In *S. coelicolor* there are three paralogous *ohr* genes, *ohrA*, *ohrB*, and *ohrC*. Of these three, only expression of *ohrA* is induced by organic hydroperoxides such as *tert*-butyl hydroperoxide (t-BHP) the induction of which is mediated by OhrR, encoded by an adjacent, divergent gene. *ohrB* lies near a gene encoding another MarR homologue that lacks the critical cysteine conserved in OhrR orthologues, and *ohrC* is flanked by a putative ferredoxin gene (*fdxA*) and an ECF (extracytoplasmic function) sigma factor gene [79]. *S. coelicolor* OhrR is a member of the single cysteine subfamily, and a Cys to Ser mutant binds to its operator site without any change in its binding affinity in response to organic peroxides. Intriguingly, *S. coelicolor* OhrR acts as a repressor of *ohrA* but as an activator of *ohrR*. Reduced OhrR binds to the *ohrA–ohrR* intergenic region which contains a central (primary) and two adjacent (secondary) inverted repeat motifs that overlap with promoter elements. Oxidation of OhrR by organic peroxides leads to derepression of the *ohrA* promoter but when oxidised, OhrR can remain weakly bound to stimulate transcription from the *ohrR* promoter [79].

4.2. OxyR — a sensor of peroxides

In response to hydrogen peroxide, *E. coli* OxyR activates a regulon of >20 antioxidant genes, including loci encoding detoxifying enzymes such as hydroperoxidase I (*katG*), alkyl hydroperoxide reductase (*ahpCF*), components of disulfide reductase pathways such as glutathione reductase (*gorA*) and glutaredoxin 1 (*grxA*), and the regulatory locus *oxyS* (encoding a regulatory RNA). *E. coli* OxyR is frequently cited as an archetypal example of a redox regulator, but the exact mechanism by which it senses peroxides is still controversial [80,81]. The conventional model is that hydrogen peroxide oxidises C_{199} to a sulfenic acid and that this intermediate reacts with C_{208} to form a disulfide bond, resulting in a change in the DNA-binding specificity of OxyR, recruitment of RNA polymerase to OxyR-dependent promoters and transcriptional activation of the OxyR

regulon [82–85]. Subsequently, the OxyR-mediated response is attenuated through the reduction of oxidised OxyR by glutaredoxin 1, which together form a homeostasis feedback loop because the glutaredoxin 1 structural gene, *grxA*, is a direct target for OxyR activation. This model is disputed by the Stamler laboratory, who failed to detect disulfide bond formation in OxyR. Instead, they presented evidence that OxyR is activated by peroxide stress through the formation of a stable sulfenic acid at C_{199}, and by disulfide stress through the formation of a mixed disulfide between C_{199} and glutathione [86]. This controversy, and the recent exciting revisions to the model for the control of OhrR activity in *Bacillus* [78], highlight the difficulties inherent in analysing complex redox-active regulatory proteins, both *in vivo* and *in vitro*.

Among the actinobacteria, OxyR has only been studied in *Streptomyces* [87]. In *S. coelicolor* OxyR regulates expression of its own structural gene and of the divergently oriented *ahpCD* operon. This gene organisation is conserved in several mycobacterial species including *M. leprae* and *Mtb*, but in *Mtb oxyR* is a pseudogene inactivated by multiple mutations [88]. *S. coelicolor* OxyR binds the *ahpC–oxyR* intergenic region *in vitro*, and the *oxyR* and *ahpCD* promoters are both induced by H_2O_2 in an *oxyR*-dependent manner. This shows that OxyR acts as a positive regulator of *ahpCD*, as expected, but also of its own structural gene. This is in contrast to *E. coli* OxyR, which acts as an activator of *ahpC* but represses its own expression. The mechanism by which H_2O_2 controls *S. coelicolor* OxyR activity has not been investigated, but the two cysteines involved in disulfide bond formation in the *E. coli* protein (C199 and C208 in *E. coli* OxyR) are conserved in the *Streptomyces* protein.

4.3. Rex — a sensor of NADH/NAD$^+$ redox poise

During aerobic respiration, NADH generated by the oxidation of substrates is reoxidised by oxygen via an electron transport chain. Because the turnover of NADH/NAD$^+$ is very high, oxygen depletion can lead to rapid changes in the NADH/NAD$^+$ redox poise. The first transcriptional regulator to be described that responds directly to the poise of the NADH/NAD$^+$ redox couple is Rex, initially characterised in the actinomycete *S. coelicolor* [89]. Rex is widely distributed through Gram-positive bacteria, including *Streptomyces*, *Bacillus*, *Clostridium*, *Staphylococcus*, *Streptococcus* and *Listeria*, but is absent from the mycobacteria and from Gram-negative bacteria. Rex is a repressor that controls expression of the cytochrome *bd* terminal oxidase operon (*cydABCD*), which is predicted to have a high affinity for oxygen. Transcription of the *cydABCD* operon is rapidly induced when *S. coelicolor* cultures are limited for oxygen or when respiration is poisoned with cyanide [89]. In addition to the *cydABCD* operon, Rex also controls its own transcription as part of the *rex–hemACD* operon (also encoding three heme biosynthetic enzymes).

Rex binds to an inverted repeat sequence known as a ROP (Rex operator) site, which, in the case of the *cydABCD* operon, is located downstream of the transcription initiation site [89]. Rex binds both NAD$^+$ and NADH, but only NADH causes Rex to lose its affinity for DNA [89]. As a consequence, competition between NAD$^+$ and NADH for the pyridine nucleotide binding site allows Rex to monitor the NADH/NAD$^+$ ratio, rather than the absolute concentration of NADH (the larger the NAD(H) pool, the higher the levels of NADH required to displace Rex from the DNA). Thus, Rex repressor activity reflects the poise of the NADH/NAD$^+$ couple which in turn reflects the flow of electrons through the respiratory chain and the availability of oxygen. The structure of *Thermus aquaticus* Rex (T-Rex) bound to NADH has been solved [90]. T-Rex is a homodimeric protein with each monomer having two domains: an N-terminal winged helix DNA-binding domain and a C-terminal redox-sensing domain. The C-terminal domain, which binds NAD(H), has a Rossmann fold that is characteristic of many NAD(H)-dependent dehydrogenase enzymes such as lactate dehydrogenase. Mutation of a highly conserved residue in the Rossmann fold in *S. coelicolor* Rex, predicted to abolish NADH binding, creates a variant that constitutively binds ROP DNA, even in the presence of high concentrations of NADH [89].

4.4. σ^R–RsrA — a sensor of disulfide stress

Unlike many of the redox regulators described here, σ^R–RsrA is a redox switch that was first discovered in the actinobacteria, and so far, appears to be confined to these bacteria. The σ^R–RsrA system has been studied most extensively in *S. coelicolor* but has also been analysed in *Mtb* [91,92], where it is called σ^H–RshA, and is also present in *Corynebacterium glutamicum* [93].

The σ^R–RsrA redox switch appears to have evolved specifically to sense and to respond to disulfide stress. σ^R is an RNA polymerase sigma subunit and RsrA is a small, cysteine-rich Zn^{2+} metalloprotein that functions as a σ^R-specific anti-sigma factor and as the redox sensor. Under reducing conditions, σ^R is sequestered by RsrA in a 1:1 complex that prevents σ^R interacting with core RNA polymerase (Fig. 3). In response to disulfide stress, an intramolecular disulfide bond forms between C11 and C44 of the anti-sigma factor, resulting in the expulsion of Zn^{2+} and loss of σ^R binding (Fig. 3). As a consequence, σ^R is then free to bind core RNA polymerase and activate expression of its target genes. *S. coelicolor* σ^R controls a regulon of approximately 60 genes that help re-establish normal cytoplasmic thiol-disulfide redox poise and to repair damage caused by disulfide stress ([14]; M. Paget, pers. comm.).

Among the genes under σ^R control are those encoding thioredoxin and thioredoxin reductase and, at least *in vitro*, the thioredoxin system will reduce oxidised RsrA, creating a homeostasis feedback loop (Fig. 3) in which the σ^R regulon is regulated in response to changes in the thiol-disulfide redox status of the hyphae [11,12]. This is analogous to the homeostasis feedback loop created by the actions of OxyR and glutaredoxin 1 in *E. coli* (see above). Another key target of σ^R is the *sigR* gene itself [11]. *sigR* and *rsrA* form an operon that is transcribed from a low level constitutive promoter (*sigRp1*) and from a second, disulfide stress-inducible σ^R target promoter (*sigRp2*) that creates a positive autoregulatory feedback loop for its own synthesis (Fig. 3). As a consequence, disulfide stress not only regulates σ^R activity post-translationally through RsrA, it also leads to *de novo*

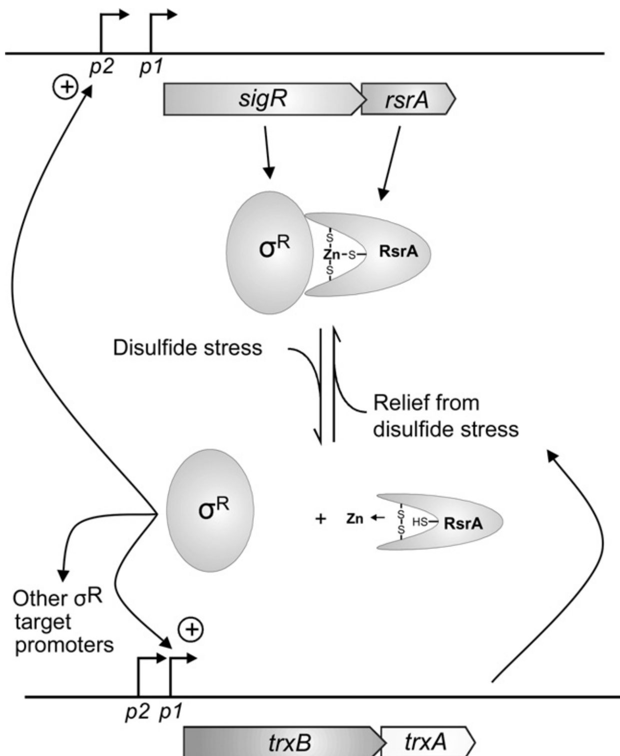

Fig. 3. Regulation of σ^R activity in response to disulfide stress (adapted from [81]). The thiol-disulfide status of *S. coelicolor* is controlled by a regulatory system consisting of σ^R and the σ^R-specific, zinc-containing, redox-sensitive anti-σ factor, RsrA. In the reduced state, σ^R is held inactive in a complex with RsrA. Disulfide stress induces the formation of an intramolecular disulfide bond in RsrA, resulting in liberation of σ^R. Subsequently, σ^R induces the expression of >60 genes, including *trxBA*. Increased *trxBA* expression, in turn, leads to Trx-dependent reduction of oxidised RsrA, which causes RsrA to re-associate with σ^R, thereby completing a homeostasis feedback loop. In addition, σ^R positively autoregulates expression of the *sigR–rsrA* operon. As a consequence, disulfide stress not only regulates σ^R activity post-translationally, it also induces its *de novo* synthesis.

synthesis of both σ^R and RsrA, and this circuitry means that the transcriptional response to disulfide stress is dramatic.

RsrA is the founding member of a family of anti-sigma factors termed the *z*inc-binding *a*nti-*s*igma (ZAS) proteins. ZAS proteins are typically encoded adjacent to Group IV (ECF) sigma factor genes, and are characterised by an invarient HxxxCxxC 'ZAS motif' (H37, C41 and C44 in RsrA). Along with a variable N-terminal residue (C11 in RsrA), the three residues of the ZAS motif coordinate the Zn^{2+} ion (Fig. 4; but note that an alternative model for zinc coordination in RsrA has been put forward in [94]). In RsrA, all four residues are essential for anti-sigma function *in vivo* [15,95–97]. Because C11 and C44 are Zn^{2+} ligands in the reduced state but form the trigger disulfide, oxidation causes expulsion of the metal from RsrA (Fig. 4). Zn^{2+} serves to modulate (dampen) the reactivity of the C11–C44 switch; apo-RsrA oxidises far more readily than does the Zn^{2+}-containing protein [95].

ZAS proteins respond to a variety of different stresses and it is clear that the RsrA regulatory mechanism is not a paradigm for all ZAS anti-σ factors. For example, in *Rhodobacter sphaeroides* the σ-ZAS couple σ^E–ChrR does not respond to disulfide stress but, instead, controls the response to the toxic ROS, singlet oxygen, which is generated when *Rhodobacter* undergoes photosynthesis aerobically [98]. Our understanding of ZAS proteins in general is greatly advanced by the recent determination of the crystal structure of the σ^E–ChrR complex [97]. Initially, ZAS proteins were predicted to represent a novel family of anti-sigma factors because they did not show significant amino acid sequence similarity to any previously characterised anti-sigma factor [15]. However, the crystal structure of ChrR reveals that the three-helix bundle containing the ZAS motif is structurally related to the anti-sigma domain of RseA, which controls σ^E in *E. coli*. ChrR makes only seven direct contacts between the helices, but an additional 17 via the Zn^{2+} ion ligands. In contrast, *E. coli* RseA, which lacks both Zn^{2+} and the HxxxCxxC ZAS motif, makes 40 non-polar contacts between the helices [97]. Thus, the structure of the three-helix bundle in RseA is maintained by a well-packed hydrophobic core, whereas ChrR uses Zn^{2+} coordination to achieve the same end [97].

RseA/ZAS-related anti-sigma factors are very widespread. Group IV sigma factors are by far the most abundant class of sigma factors and approximately one-third of all Group IV sigmas in the databases are encoded next to RseA/ZAS-related proteins [97]. In *S. coelicolor*, 11 of the 51 Group IV sigma genes lie next to ZAS genes.

4.5. σ^B — a general stress sensor

S. coelicolor contains some 65 sigma factors, including nine closely related to the σ^B "general stress response" σ factor of *B. subtilis* [99–101]. In *B. subtilis*, σ^B activates >200 genes (~5% of the genome) that confer general stress resistance on the cell. In *S. coelicolor*, analysis of the function of these σ^B-like σ factors is extremely complex for several reasons [99–101]. First, the nine σ^B-like σ factors (σ^B, σ^F, σ^G, σ^H, σ^I, σ^K, σ^L, σ^M, σ^N) are likely to have very similar promoter specificity, such that single promoters are recognised by multiple paralogues. Second, in the actinobacteria, single genes frequently have multiple promoters recognised by different sigma factors [14]. Third, the regulatory networks frequently involve cascades in which one σ factor directly activates expression of other σ factors. Fourth, disruption of one σ factor gene can result in a stress condition that can indirectly activate or repress expression of other σ factor genes [14]. For all these reasons, separating direct from indirect effects is not trivial. Studies of at least four σ^B-like σ factors have been concerned with osmotic shock, but it is clear that some of these σ factors influence oxidative stress responses in *Streptomyces*. Thus, *sigB* disruption in *S. coelicolor* results in increased carbonylation of proteins (an irreversible metal-catalysed oxidation of the side chains of proline, lysine, arginine and threonine) [101]. Further, disruption of *sigB* influences expression of genes involved in both cysteine and mycothiol biosynthesis in *S. coelicolor*, and this is reflected in decreased levels of cysteine and mycothiol in a *sigB* mutant [101]. The promoter of the *catB* catalase gene of *S. coelicolor* was originally reported to be a direct σ^B target, but later evidence suggests that *catB* expression is indirectly influenced by σ^B [101,102]. Gene disruption experiments have shown that a σ^B-like σ factor contributes to virulence in *Mtb* [103].

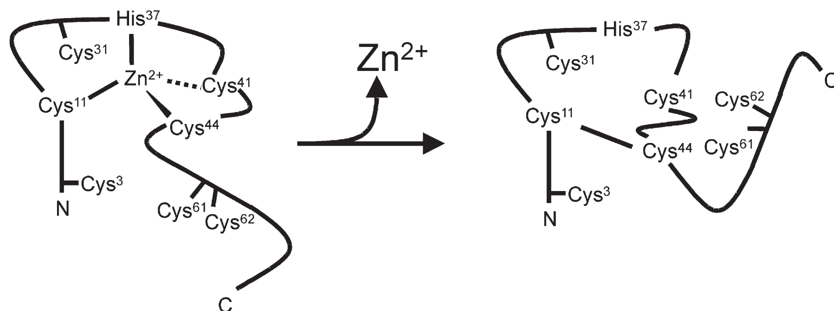

Fig. 4. Redox inactivation of RsrA (adapted from [95]). When reduced, the ZAS protein RsrA coordinates zinc through residues C11, H37, C41, and C44. Exposure to oxidants results in the formation of an intramolecular disulfide between C11 and C41 and expulsion of Zn from the protein, causing a large conformational change in the protein and concomitant dissociation of the RsrA–σ^R complex. Note that an alternative model for zinc coordination in RsrA has been put forward in [94].

4.6. DosS and DosT — sensors of NO, CO and O_2

Mtb is an obligate aerobe, yet it can survive for years in the host, presumably under hypoxic conditions, in a non-replicative, metabolically quiescent state, termed persistence. Almost all the *Mtb* genes induced in response to hypoxia or nitric oxide (NO) are controlled by the DosRST two-component system (originally called the DevRST system), which is thought to assist *Mtb* to enter and maintain a persistent state. Several independent studies [104–106] have shown a dramatic overlap in gene expression profiles of *Mtb* treated with NO, or *Mtb* cells cultured under hypoxic conditions. Thus, NO and hypoxia can be regarded as two distinct signals that can trigger the dormancy programme in *Mtb*. The *Mtb* Dos system consists of the DosR response regulator that is activated by phosphorylation by two sensor kinases (DosS and DosT), both of which bind Fe-containing heme cofactors via an N-terminal GAF domain. Recently, the ligands that bind the heme groups of DosS and DosT have been characterised and their effects on kinase activity determined [107,108]. In both cases, the state of the heme iron determines the activity of the kinase. For DosT, the deoxy form is a highly active kinase, and binding of O_2 to the heme iron (the oxy form) turns off the kinase activity. Sousa et al. [107] conclude that DosS works in the same way as DosT, with O_2 binding acting to inhibit kinase activity. In contrast, Kumar et al. [108] conclude that O_2 inactivates DosS by rapidly oxidising the Fe^{2+} form of the heme to the Fe^{3+} state. In other words, Kumar et al. [108] conclude that the kinase activity of DosS is modulated via oxidation of its heme iron by O_2, as opposed to DosT that is inactivated by O_2 binding. If this is true then DosT functions as a sensor of hypoxia, whereas DosS functions as a redox sensor. It has been shown that NO binds ~5000 times more avidly to DosT than oxygen and that NO binding does not turn off DosT kinase activity. As a consequence, NO induces the DosR regulon under aerobic conditions [107,108]. Intriguingly, CO was shown to significantly upregulate key members of the *Mtb* Dos regulon, suggesting that CO can also function as a dormancy signal.

5. The Fur superfamily of regulators — sensors of metals and peroxides

Members of the Ferric Uptake Repressor (Fur) superfamily of metallo-regulatory proteins consist of an N-terminal DNA-binding domain that contains a helix-turn-helix motif, and a C-terminal domain that usually contains two metal ions coordinated by cysteines. One of the metal-binding sites coordinates a structural zinc ion [109,110], while the other site tends to be more promiscuous, probably reflecting its regulatory role. Members of this superfamily can act as sensors of iron (Fur), zinc (Zur), nickel (Nur), manganese (Mur) and peroxide (Per, CatR). In the case of PerR, there is good evidence that the regulatory site can be occupied either by Fe^{2+} or Mn^{2+} and that, crucially, the metal content dictates the ability of PerR to sense peroxides. The Mn^{2+} form of PerR is relatively insensitive to H_2O_2, but when PerR is exposed to H_2O_2 and Fe^{2+} is present in the regulatory site, the Fe^{2+} ion catalyses *in situ* formation of a hydroxyl radical through Fenton chemistry and the hydroxyl radical oxidises either His37 or His91, two of the ligands that coordinate the bound Fe^{2+} ion [111]. This oxidation event generates 2-oxo-histidine, and available evidence suggests that formation of 2-oxo-histidine at either His37 or His91 is sufficient to cause irreversible loss of DNA binding [111]. Thus, PerR senses H_2O_2 through metal-catalysed oxidation of the protein, and although this has been shown previously to contribute to enzyme inactivation, this is the first time metal-catalysed oxidation of a protein has been identified as a regulatory device. There is an intimate connection between metals and oxidative stress, arising from the ability of metals to generate ROS when exposed to oxygen or peroxides. As a consequence, it is perhaps not surprising that members of the Fur superfamily have evolved to regulate both metal homeostasis and oxidative stress responses [112].

There are four members of the Fur superfamily encoded in the *S. coelicolor* chromosome (FurA, CatR Nur and Zur). CatR and FurA have been characterised as regulators of catalase genes and Nur has been shown to regulate expression of three superoxide dismutase genes.

5.1. FurA

In *S. coelicolor*, a gene designated *furA* lies upstream of, and is cotranscribed with, the *catC* catalase–peroxidase gene and CatC expression is upregulated in a *furA* null mutant. Expression of the *furA–catC* operon is not induced by H_2O_2 and FurA appears to act as a metal-sensing repressor of the operon [113]. *Mtb* contains a similar *furA–katG* operon and *katG* expression is derepressed in a *furA* null mutant. However, in contrast to the

S. coelicolor system, expression is induced by H_2O_2, consistent with FurA acting as a peroxide-sensing repressor [114–116].

5.2. CatR

S. coelicolor produces three catalases and CatA, the major catalase, is required for efficient aerobic growth and resistance to H_2O_2 [117]. CatA expression is induced by H_2O_2 but the regulator that mediates this regulation (CatR) is not genetically linked to the *catA* locus. The *catR* locus was defined by a mutant that overexpressed CatA and, when isolated, was found to encode a PerR-like member of the Fur superfamily of repressors [118]. *catR* mutants show high levels of transcription of both *catA* and *catR* itself, implying that CatR acts as a repressor of both. DTT-treated CatR binds specifically to the *catA* and *catR* promoters, and this binding is abolished when exposed to H_2O_2 [118]. Preliminary experiments suggest that the mechanism of inactivation of CatR by H_2O_2 might involve the formation of intramolecular disulfide bond(s), but this needs to be investigated further, especially in the light of the subsequent realisation that H_2O_2-inactivation of *B. subtilis* PerR involves the oxidation of His37 or His91, which are both conserved in CatR [118,119].

5.3. Nur

Nickel-dependent enzymes are comparatively rare in biology, but include some superoxide dismutases (SODs). *S. coelicolor* has three SOD enzymes: the Ni-containing SodN and the Fe-containing SodF and SodF2. Their expression responds to nickel in opposite ways: nickel represses expression of the two Fe-containing SODs and induces expression of the Ni-containing enzyme [120–122]. This regulation is mediated by a novel Ni-sensing member of the Fur superfamily named Nur; deletion of the *nur* gene causes constitutive expression of *sodF* and *sodF2* encoding the Fe-containing enzymes and a failure to induce *sodN* in the presence of nickel [112]. However, whereas Nur prevents expression of the Fe-SODs in the presence of nickel by directly repressing the *sodF* and *sodF2* promoters, Nur does not interact directly with the *sodN* promoter. Given that Nur does not induce expression of the Ni-SOD by acting as an activator of the *sodN* promoter, it seems likely that Nur acts indirectly as a repressor of a (as-yet-unidentified) repressor of *sodN* [112]. By analogy, in *E. coli*, Fe-responsive Fur activates expression of the *sodB*-encoded Fe-SOD indirectly by repressing expression of a small regulatory RNA called RyhB, which acts to destabilise the *sodB* mRNA [123]. However, this exact mechanism is unlikely to account for Nur's indirect influence on SodN expression since nickel does not appear to change the half-life of the *sodN* mRNA in *S. coelicolor* [122]. In addition to its role in regulating the three superoxide dismutase genes, Nur directly represses expression of the *nikABCDE* nickel uptake system in response to nickel, and as a result, the *nur* mutant shows elevated levels of intracellular nickel [112].

5.4. Zur

S. coelicolor contains paralogous versions of seven ribosomal genes (S14, S18, L28, L31, L32, L33 and L36). One set of paralogues, designated C^+, carry predicted Zn ribbon motifs consisting of two pairs of conserved cysteine residues that, in several cases, have been demonstrated to bind Zn. The second set, designated C^-, conspicuously lack these cysteine Zn ligands. Because ribosomes are major components of rapidly growing cells (e.g. a single *E. coli* can contain up to 70,000 ribosomes), it is likely that a large proportion of total cellular zinc would be present in ribosomes containing the seven C^+ paralogues, potentially placing a great demand on the zinc resources of the cell. In *S. coelicolor*, expression of four transcription units encoding C^- ribosomal proteins is elevated under conditions of zinc deprivation [124,125]. Expression of three of these transcription units is directly controlled by a Zn-responsive member of the Fur superfamily of repressors called Zur. In addition, Zur represses expression of a high-affinity zinc uptake system encoded by the *znuABC* operon [124,125]. In contrast, the response of the fourth transcription unit encoding C^- ribosomal proteins (*rpmG3–rpmJ2*) to zinc is not controlled by Zur. Instead, the zinc-responsive regulation of *rpmG3–rpmJ2* is mediated by the σ^R–RsrA redox switch, which controls the disulfide stress response in *S. coelicolor* (see above). The promoter of the *rpmG3–rpmJ2* operon is a direct target for RNA polymerase containing σ^R. Because the RsrA anti-sigma factor is a zinc metalloprotein and zinc is required for anti-sigma function, depleting *S. coelicolor* cultures of zinc leads to the release of σ^R from the σ^R–RsrA complex and induction of the σ^R regulon [124,125]. These results establish a clear and important link between zinc homeostasis and thiol-disulfide metabolism in *Streptomyces*. *Mtb* carries paralogous versions of 4 ribosomal genes, and Zur regulates expression of the C^- versions of these genes, which are encoded in a single operon [126].

6. The WhiB-like family of proteins

Even though WhiB-like (Wbl) proteins were discovered more than 15 years ago, and despite their evident importance in actinomycete biology, the biochemical role of these proteins is still uncertain and controversial. Most papers, based on indirect, circumstantial evidence, have suggested that Wbl proteins are likely to function as transcription factors. However, in 2007, the group of Agrawal has published two papers in which they present data consistent with Wbl proteins functioning as disulfide reductases, which, if true, would profoundly change the view of Wbl biology [127,128]. A further important connection to redox biology is that, in 2005, Jakimowicz et al. [129] demonstrated that WhiD, a member of the WhiB family, binds a redox-sensitive [4Fe–4S] cluster, and other groups have subsequently found that other members of the WhiB family from the mycobacteria also bind redox-sensitive [4Fe–4S] clusters [61,127].

Members of the *wbl* family, of which the *S. coelicolor whiB* gene was the first characterised [130], are found exclusively in actinobacteria (Fig. 5). To date, genome sequencing has revealed over 270 Wbl homologues. Including *whiB* and *whiD*, there are a total of 14 *wbl* genes in *S. coelicolor* itself, 11 on the chromosome and three on the giant linear plasmid, SCP1 [13,131].

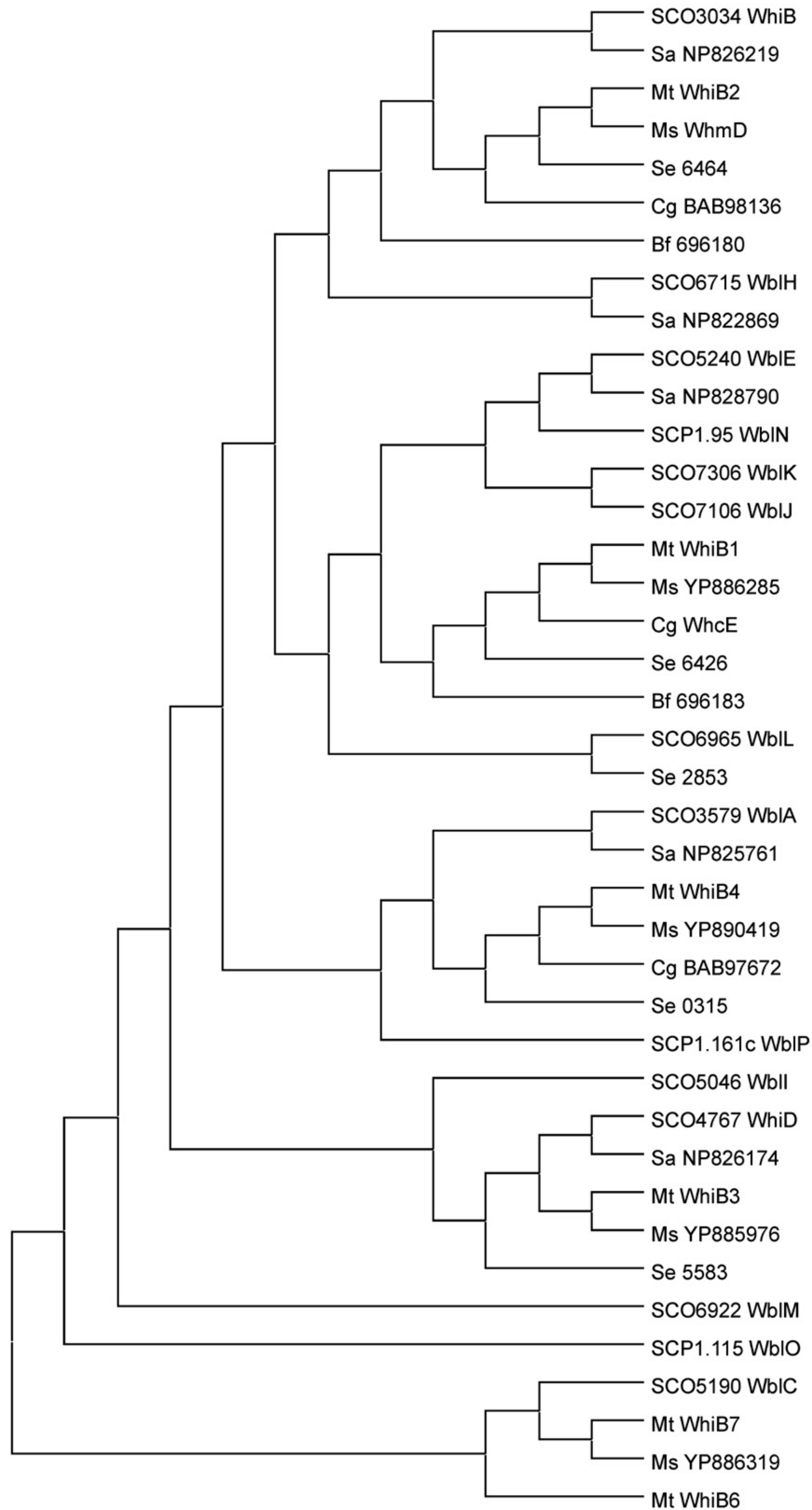

Fig. 5. Phylogenetic tree of selected WhiB-like proteins. Protein sequences were retrieved after BLAST analysis of the genomes of *S. coelicolor* (SCO), *Mtb* H37Rv (Mt), *Mycobacterium smegmatis* MC2 155 (Ms), *Corynebacterium glutamicum* ATCC13032 (Cg), *Streptomyces avermitilis* MA-4680 (Sa), *Saccharopolyspora erythraea* NRRL233 (Se) and *Bifidobacterium longum* NCC2705 (Bf). Phylogenetic analysis was conducted following the Neighbour-Joining method using MEGA. The names of *S. coelicolor* WhiB-like proteins are those allocated by Soliveri et al. [155] or K. Chater (pers. comm.).

With the exception of WblP (see below), all the Wbl proteins are relatively small and vary in length from 81 to 122 residues. A key feature of Wbl proteins is the presence of four highly conserved cysteines, of which the central two form a CXXC motif (Fig. 6). Below we review the available evidence concerning the biochemical function of Wbl proteins, the discovery

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ScWhiB       GWQERALCAQTDPESFFPEKG--GS-----TREAKKVCLACEVRSECLEYALANDERFGIWGGLSERERRRLKKAAV-
ScWhiD       -WQLLAACRGVDSSLFFHPEGERGAARSARENSAKEVCMRCPVRAECAAHALAVREPYGVWGGLTEDEREELMGRARN
MtWhiB1      -WRHKAVCRDEDPELFFPVGN--SGPALAQIADAKLVCNRCPVTTECLSWALNTGQDSGVWGGMSEDERRALKRRN--
MtWhiB7      ------PCHVGDPDLWFADTP-------AGLEVAKTLCVSCPIRRQCLAAALQRAEPWGVWGGEIFDQGSIVSHKRPR
MsWhmD       QWQERALCAQTDPEAFFPEKG--GS-----TREAKRICQGCEVRDACLEYALAHDERFGIWGGLSERERRRLKRGII-
MsYP886285   -WRHKAVCRDEDPELFFPVGN--SGPALAQIADAKLVCNRCPVTTECLSWALESGQDAGVWGGMSEDERRALKRRN--
Cgl0743      -WQEQALCAQTDPEAFFPEKG--GS-----TREAKRICQGCPVRDECLEFALEHDERFGIWGGLSERERRRLKREIS-
CgWhcE       -WRHEAICREEDPELFFPVGN--SGPALAQIASAKMVCNRCPVTSQCLAWALETGQDAGVWGGMSEDERRALKRRKNR
SaNP826174   -WQLLAACRGVDSSLFFHPEGERGAARSARENSAKEVCMRCPVRAQCAAHALAVREPYGVWGGLTEDEREELMGRARN
Se5583       -WQLKAACRDLASSRFFHPDHERGNSRDERERNAKRVCHTCPVIRECRAHALATREPYGVWGGLSEHERRTLVAS---
Bf696183     DWRAKAACRDKDPELFFPVGN--TGAAYQQIEEAKAVCRTCKVIDACLKCALDTNQDYGVWGGLSEDERRALKRRA--
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Fig. 6. Protein sequence alignments of WhiB-like proteins. Shown are partial amino acid sequences of Wbl proteins of *S. coelicolor* (Sc), *Mtb* (Mt), *M. smegmatis* (Ms), *C. glutamicum* (Cg), *S. avermitilis* MA-4680 (Sa), *S. erythraea* (Se) and *B. longum* NCC2705 (Bf). Dots indicate the positions of four cysteines present in nearly all Wbl proteins. The alignment was created using ClustalW.

that they bind a [4Fe–4S] cofactor, and the biological roles of these proteins in actinomycete biology.

6.1. Circumstantial evidence that Wbl proteins function as transcription factors

Bioinformatic analysis of Wbl protein sequences shows that they lack a recognised DNA-binding motif and, at the time of writing, there are no published data demonstrating DNA binding for any Wbl protein. Nevertheless, various data have been taken as circumstantial evidence that Wbl proteins are transcription factors. *Mtb* WhiB3 was identified in a yeast two-hybrid screen in which the C-terminal domain (region 4.2) of the principal RNA polymerase sigma factor of *Mtb* was used as bait, and this interaction was confirmed biochemically using SELDI-TOF mass spectrometry [132]. This observation led Nakunst et al. [133] to propose that that the product of *whcE*, a *wbl* gene required for expression of *trxA* in *C. glutamicum*, recruits SigM, which is a sigma factor involved in thiol-oxidative stress, to its target promoters [134].

Further, the *wblP* gene of *S. coelicolor* (carried on the 356 kb plasmid, SCP1) encodes a protein having a Wbl at its N-terminus and an RNA polymerase sigma factor at its C-terminus [131]. In addition, the very fact that mutations in different *wbl* genes have been found to cause highly pleiotropic phenotypes in both *Streptomyces* and *Mtb* has been seen as consistent with a proposed role as transcription factors, as has the fact that *wbl* mutations can lead to the downregulation of specific gene sets, as revealed in microarray experiments (e.g. [135,136]). The discovery that these proteins bind an oxygen-sensitive [4Fe–4S] cluster is recent [61,127,129]. If it subsequently emerges that Wbl proteins are transcription factors and the [4Fe–4S] cofactor is required for function, the current absence of biochemical data may seem more explicable, given the need to run the experiments anaerobically.

6.2. Are Wbl proteins disulfide reductases?

Recently, data have been presented by the Agrawal group that *Mtb* WhiB1 and WhiB4 can reduce the disulfide bonds bridging the α- and β-chains of insulin *in vitro*, indicating that they can function as protein disulfide reductases [127,128]. The authors demonstrate that protein containing the [4Fe–4S] cluster is enzymatically inactive. Upon release of the cluster, under aerobic conditions, two intra-molecular disulfide bonds are formed that, in the presence of a reductant (e.g. DTT), are reduced to the thiol form, creating an active enzyme. These data are strongly reminiscent of recent observations that many glutaredoxins can bind an [Fe–S] cluster and that oxidative destruction of the cluster is required to liberate glutaredoxin activity [137–140]. If Wbls function as disulfide reductases, then an important further question is what provides reducing equivalents to the Wbl proteins *in vivo*? Given that thioredoxin reductase is incapable of reducing WhiB1 [128] and a GSH-dependent system is absent from actinobacteria, one possibility is a dedicated Wbl reductase enzyme. Alternatively, it might be that Wbls function as 'mycoredoxins', accepting electrons directly from MSH, in a manner analogous to glutaredoxins accepting electrons from glutathione. This could be seen as consistent with the fact that Wbl proteins and mycothiol are always found together (i.e. they are both confined to, and present in, all actinobacteria). However, if Wbl proteins functioned solely as mycoredoxins, they would presumably be inactive in the absence of MSH, which is not consistent with the known phenotypes of MSH-less mutants [36]. Finally, individual actinomycete species typically contain multiple *wbl* genes, and yet individual *wbl* mutations confer strong phenotypes (see below), implying a lack of functional redundancy. In part, this might be accounted for by temporal and/or spatial separation of the different Wbl proteins in a given actinobacterial species. However, if Wbl proteins function as disulfide reductases *in vivo*, this must surely also imply that they have strong substrate specificity. To date, no cellular substrates have been identified.

6.3. Wbl proteins carry an iron–sulfur cluster

A striking feature of the Wbl-protein family is the presence of four near-invariant cysteines in a $C(X_{29})C(X_2)C(X_5)C$ motif. In the case of *S. coelicolor* WhiD, each of these four residues is essential for function *in vivo*, which suggested that they might act as ligands for a metal cofactor [129]. To investigate this possibility, Jakimowicz et al. [129] purified WhiD and found that it had a reddish-brown colour. The protein contained sub-stoichiometric amounts of iron with an absorption spectrum characteristic of a [2Fe–2S] cluster [129]. After Fe–S cluster reconstitution under anaerobic conditions, WhiD contained a

[4Fe–4S] cluster. Reconstituted WhiD gave no EPR signal as prepared, but, after reduction with dithionite, gave an EPR signal ($g \sim 2.06$, 1.94), consistent with a one electron reduction of a $[4Fe–4S]^{2+}$ cluster to a $[4Fe–4S]^{1+}$ state with electron spin of $S = 1/2$ [129]. The anaerobically reconstituted [4Fe–4S] cluster was oxygen sensitive; exposure to air induced a change from a [4Fe–4S] to a [2Fe–2S] cluster, followed by complete loss of cluster from the protein. More recently, similar properties have been attributed to *Mtb* WhiB1, WhiB3, and WhiB4 [61,127,128], suggesting that all Wbl protein family members bind a [4Fe–4S] cluster. It is not known how the [4Fe–4S] cluster is incorporated into the Wbl proteins *in vivo*. It has been shown, however, that the cluster can be reconstituted *in vitro*, in the presence of Na_2S and $FeCl_3$ or by generating sulfide ion enzymatically using L-cysteine and L-cysteine desulfurase, NifS [61,129].

In the disulfide reductase model of Wbl function, the role of the [4Fe–4S] cluster seems clear; it holds the enzyme inactive until oxidative stress destroys the cluster and releases the enzymatic activity. In contrast, the role of the cluster is not well-defined in the transcription factor model, but the most obvious suggestion would be that the configuration of the [Fe–S] cluster dictates the DNA-binding properties of the protein, as is the case for FNR (see above). While the biochemical function of Wbl proteins is highly controversial, what cannot be disputed are the pivotal roles these proteins play in diverse aspects of actinobacterial biology, as revealed by the phenotypes of *wbl* mutants.

6.4. Diverse phenotypes of wbl mutants

S. coelicolor whiB was identified in a screen for mutations that abolish sporulation and, hence, synthesis of the grey polyketide spore pigment, resulting in a 'white' (*whi*) phenotype [130]. *S. coelicolor whiB* mutants produce abnormally long, tightly-coiled aerial hyphae that are completely blocked in their ability to form sporulation septa [130,141,142]. The closest homologues of *S. coelicolor whiB* in *M. smegmatis* (*whmD*) and in *Mtb* (*whiB2*) are both essential, but controlled depletion experiments have shown that they are required for proper septation and cell division [143–145].

Among the *S. coelicolor* white mutants another *wbl* gene (*whiD*) was identified. A mutant of *whiD* can make sporulation septa, but the strain forms spores that differ from those of the wild type in being heat-sensitive, prone to lysis, irregular in size, and extremely variable in the deposition of spore cell wall material [146,147]. Moreover, *whiD* spores are frequently partitioned into irregular smaller units through the deposition of additional septa that are often laid down in several different planes, very close to the spore poles [146,147]. These 'minicompartments' appear to be devoid of chromosomal DNA.

WblA of *Streptomyces peucetius* was recently identified as a down-regulator of doxorubicin biosynthesis, a clinically very important anti-cancer drug [136]. Moreover, *wblA* overexpression in *S. coelicolor* inhibited biosynthesis of the antibiotics actinorhodin, calcium-dependent antibiotic and undecylprodigiosin. In this strain, expression of the activators genes of the antibiotics biosynthetic gene clusters was reduced, suggesting that *wblA* acts as a pleiotropic regulator of antibiotic biosynthesis in these organisms.

The *wblC* locus of *S. lividans* was identified genetically in a screen for mutants pleiotropically sensitive to a wide range of clinically important, but chemically and functionally unrelated, antibiotics. More recently, WblC was shown to control innate multi-drug resistance, not only in *Streptomyces*, but also in the major human pathogen *Mtb*, in which overexpression of the orthologous gene, *whiB7*, led to enhanced multi-drug resistance [135]. The remarkable antibiotic tolerance of *Mtb* is the main cause of treatment failure in patients with tuberculosis, which makes WhiB7 an attractive target for therapeutic intervention [135]. The expression of *whiB7* is regulated; incubation of *Mtb* with sub-inhibitory concentrations of antibiotics (i.e. erythromycin, tetracycline, kanamycin and streptomycin) leads to a strong induction of expression [135,148]. Similarly, *Mtb whiB2* is induced by capreomycin [149]. Although these antibiotics have different modes of action (the first four target the ribosome whereas capreomycin targets the cell wall), they elicit a similar transcriptional response, suggesting they are sensed indirectly. In addition to antibiotics, *whiB7* is also induced after exposure to fatty acids that *Mtb* accumulates within eukaryotic hosts during infection.

A systematic analysis of the transcriptional pattern of all seven *wbl* genes of *Mtb* revealed that they are differentially regulated in response to various stresses (such as heat shock and oxidative stress) and antibiotics. Expression of *Mtb whiB3* (the closest homologue of *S. coelicolor whiD*) was found to be dependent on the pH of the growth medium, as was *whiB2* of *Mycobacterium avium* [150]. Phagolysosomes of macrophages acidify after activation and it has been shown that *whiB3* is induced in the mouse lung and in macrophages [151,152]. A *Mtb whiB3* mutant is attenuated, but the gene is dispensable for *in vivo* growth [132,153], supporting the view that Wbl proteins may play a role in survival within the host.

7. Conclusions and future perspectives

Actinobacterial antioxidant genes appear to be controlled by a strikingly wide variety of regulatory mechanisms, more than is the case for other bacteria that have been examined. For example, the work of the Roe lab has shown that peroxide-removing enzymes in *S. coelicolor* are under the control of at least five separate regulators; AhpC is under the control of OxyR, OhrA is under the control of OhrR, CatA is under the control of CatR, CatC is under the control of FurA, and CatB is somehow indirectly regulated by σ^B. Another transcription factor, Nur, directly regulates expression of two Fe-containing SODs and indirectly regulates a Ni-containing SOD. Further, actinobacteria have a regulatory system (σ^R–RsrA/σ^H–RshA) that appears to be uniquely dedicated to disulfide stress, whereas *E. coli* OxyR mediates responses to both disulfide and peroxide stress [82].

With some exceptions (e.g. bifidobacteria and corynebacteria), actinobacteria have always been classified as obligate aerobes. Consequently, an unexpected discovery in the *S. coelicolor*

genome sequence was the number of genes that are typically associated with microaerobic or anaerobic metabolism in facultative anaerobes. These include genes encoding respiratory nitrate and nitrite reductases, formate dehydrogenase, NarK, and cytochrome *d* oxidase. Prior to the sequencing of its genome, *B. subtilis* was also thought to be a strict aerobe, but was subsequently found to grow anaerobically using nitrate or nitrite as a terminal electron acceptor, or by fermentation [154]. An important future challenge will be to understand the significance of the 'anaerobic genes' in *S. coelicolor*. Other important challenges for the field include developing a full understanding of the role of mycothiol, assigning an unambiguous biochemical function to the WhiB-like proteins, and identifying more of the redox regulatory systems that undoubtedly still await discovery in actinobacteria.

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