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## A new player in bacterial sulfide-inducible transcriptional regulation

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### Summary

Although hydrogen sulfide (H<sub>2</sub>S) is perhaps best known as a toxic gas, the electron-rich H<sub>2</sub>S functions as an energy source and electron donor for chemolithotrophic and photosynthetic bacteria, via sulfide oxidation, and is a universal substrate for cysteine biosynthesis. These distinct harmful and beneficial roles of H<sub>2</sub>S suggest the need to “sense” prevailing concentrations of sulfide and downstream reactive sulfur species (RSS) and regulate the expression of genes mediating sulfide homeostasis. The paper by Li *et al.* in this issue of *Molecular Microbiology* adds *Cupriavidus* FisR to an expanding repertoire of regulatory mechanisms that bacteria have evolved to sense cellular RSS and mitigate their deleterious effects.

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It is well established that many organisms are capable of using hydrogen sulfide (H<sub>2</sub>S, HS<sup>-</sup>, S<sup>2-</sup>) as a source of reducing equivalents via sulfide oxidation, which provides sufficient energy to power respiration, energy (ATP) production, and for photosynthetic organisms, photosynthetic electron flow. For example, tetrathionate, a sulfide oxidation product formed by the condensation of two thiosulfate molecules (see Fig. 1A) by thiosulfate dehydrogenases (Brito *et al.*, 2015) can function as a terminal electron acceptor; indeed, in the lumen of the inflamed gut, anaerobic respiratory tetrathionate reduction provides a growth advantage for *Salmonella* ssp. (Winter *et al.*, 2010). Recent work in eukaryotes describes H<sub>2</sub>S as a vasorelaxant and signaling molecule (Mustafa *et al.*, 2009, Gadalla & Snyder, 2010) whose biological impact may be intertwined with adaptation to reactive nitrogen species (RNS), including nitric oxide (Kolluru *et al.*, 2015, Cortese-Krott *et al.*, 2015). Although a defined role for microbial H<sub>2</sub>S signaling has yet to be described, H<sub>2</sub>S/RNS crosstalk may provide protection against the effects of antibiotic stress (Shatalin *et al.*, 2011).

H<sub>2</sub>S is a potent inhibitor of cytochrome oxidase (Cooper & Brown, 2008) and forms stable coordination complexes with transition metals (Luther *et al.*, 1996) thus potentially limiting their bioavailability. These beneficial yet toxic attributes of H<sub>2</sub>S therefore suggest the need to regulate intracellular concentrations of this strongly nucleophilic and reducing molecule. This is particularly so since H<sub>2</sub>S is produced endogenously in many organisms (from bacteria to vertebrates) by two enzymes of the transsulfuration pathway, cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) (Yadav *et al.*, 2016). Two additional enzymes, 3-mercaptopyruvate sulfurtransferase, which functions in cysteine catabolism, and

sulfite reductase, which functions in dissimilatory sulfate reduction, also generate H<sub>2</sub>S (Yadav *et al.*, 2013, Santos *et al.*, 2015). Sulfide is a substrate for cysteine synthase, which synthesizes cysteine from an acetyl-CoA activated serine, *O*-acetylserine (OAS); cysteine is the precursor for all S-containing metabolites in the cell, including the major reducing thiol(s), *e.g.*, glutathione or bacillithiol, coenzyme A, thiamine pyrophosphate (TPP), biotin and lipoic acid.

The principal pathway for hydrogen sulfide detoxification and homeostasis is via step-wise oxidation to less toxic products (Libiad *et al.*, 2014). The first step in this process is the two-electron oxidation of sulfide to sulfane or “sulfur-bonded” sulfur (Fig. 1) conjugated to a suitable acceptor nucleophile. When a low molecular weight (LMW) thiolate is the acceptor, sulfide:quinone oxidoreductase (SQR) generates a LMW thiol persulfide (Fig. 1B), with the reducing equivalents shuttled into the quinone pool. Flavocytochrome c:sulfide dehydrogenases (FCSD) catalyze precisely the same reaction as SQRs, but use cytochrome c instead of an oxidized quinone as the electron acceptor (Marcia *et al.*, 2010). Inorganic polysulfides can also be biosynthesized by SQRs when H<sub>2</sub>S is used as the nucleophilic acceptor (Jackson *et al.*, 2012) (Fig. 1C). LMW thiol persulfides are then subject to further oxidation to sulfite or thiosulfate (see Fig. 1A,B), with the release of the free LMW thiol, by non-heme Fe<sup>II</sup> persulfide dioxygenases (PDO) (Kabil & Banerjee, 2012, Liu *et al.*, 2014, Sattler *et al.*, 2015, Shen *et al.*, 2015, Pettinati *et al.*, 2015), enzymes often mis-annotated as metallo- β-lactamase-like hydrolases (Blh) (Sattler *et al.*, 2015). Further oxidation of sulfite and thiosulfate to sulfate and tetrathionate, respectively, can occur in some organisms (Fig. 1A). Sulfurtransferases or rhodanases often function as persulfide (activated sulfur) carriers in these processes (Higgins *et al.*, 2015, Mueller, 2006), likely facilitating the production of LMW thiol persulfides (Xin *et al.*, 2016) (Fig. 1A). LMW thiol persulfides and inorganic polysulfides, collectively termed reactive sulfur species (RSS) (Fig. 1C), are detectable in bacterial cells (Shen *et al.*, 2016, Peng *et al.*, 2017) and may well engage in persulfide or RSS transfer reactions (Mishanina *et al.*, 2015) with other small molecule or proteome thiolate acceptors (Wedmann *et al.*, 2016, Gao *et al.*, 2015) (Fig. 1D).

In new work published in this issue of *Molecular Microbiology*, Liu, Xun and coworkers describe the characterization of FisR from the heterotroph *Cupriavidus pinatubonensis* JMP134, a Gram-negative β-proteobacterium of industrial and biotechnological importance (Li *et al.*, 2017) (Fig. 2A). FisR is shown to be a transcriptional activator of the divergently transcribed operon, encoding FisR itself and a two-gene cluster of *sqr* and *pdo* in *C. pinatubonensis*. The *sqr-pdo* cluster, encoding a *bona fide* SQR and PDO as characterized previously by the same group (Xin *et al.*, 2016), is transcriptionally inducible in FisR-dependent manner by Na<sub>2</sub>S and inorganic polysulfides (see Fig. 1C; primarily HSSH in this work) and *C. pinatubonensis* lacking *pdo* and *sqr* fails to oxidize Na<sub>2</sub>S and exhibits a growth defect on sulfide-containing media. Finally, FisR-like regulators are somewhat widespread among proteobacteria and could be further classified into two major clusters on the basis of amino acid similarity, corresponding to *Burkholderia* spp. and *Pseudomonas* spp. clades (Li *et al.*, 2017).

The remarkable findings that connect and contrast the work of Li *et al.* to previous work are two-fold. First, the striking contrast: unlike RSS-sensing transcriptional repressors already

characterized (*vide infra*), FisR is a member of the bacterial enhancer binding protein (bEBP) family (Fig. 2B). These are  $\sigma^{54}$ -dependent transcription factors that harbor three consensus domains: an N-terminal regulatory or receiver (R) domain that is capable of “sensing” the inducer, a middle AAA+ domain (AAA+) that hydrolyzes ATP, and a C-terminal DNA binding domain (D) which directs a specific interaction with an operator (enhancer) sequence far upstream of the promoter (Bush & Dixon, 2012) (Fig. 2B, *inset*). This “action at a distance” is mediated by integration host factor (IHF), which introduces a bend in the DNA, positioning bound FisR in close proximity to the promoter-bound RNA polymerase, with ATP hydrolysis stimulating open complex formation and transcription initiation (Hartman *et al.*, 2016). The striking similarity to previous work (Luebke *et al.*, 2014, Shimizu *et al.*, 2017) is that FisR contains three conserved Cys residues (C53, C64 and C71) in the R domain and reduced FisR is activated by exposure to glutathione persulfide and inorganic polysulfide RSS (see Fig. 1C). Major products of the reaction as analyzed by tandem mass spectrometry (Luebke *et al.*, 2014) include a disulfide linkage between C53 and C64, but not C53 and C71, as well as tetrasulfide linkage between the same two cysteine residues (Fig. 2B). These oxidative modifications do not impact the DNA-binding activity or oligomerization state of FisR [FisR, like other bEBPs is an obligatory homohexameric ring structure (Bush *et al.*, 2015), but in this case is in equilibrium with tetramers, dimers and monomers], but do increase the ATPase activity modestly, a stimulation that appears to be abolished in the C53S, C64S and C71S mutants (Li *et al.*, 2017). Left for future work is the mechanism by which RSS-dependent oxidative modification of FisR in the R domain impacts transcriptional activation of the *pdo-sqr* promoter (Fig. 2B).

This study by Li, Xun and colleagues provides new insights into the evolution of sulfide sensing, homeostasis and detoxification in microbes (Li *et al.*, 2017) (Fig. 2A). Earlier work on the regulation of the sulfur oxidation (*sox*) genes in the  $\alpha$ -proteobacterium *Pseudaminobacter salicylatoxidans* and related bacteria (Mandal *et al.*, 2007) led to the identification of SoxR, a member of the ubiquitous arsenic repressor (ArsR) family of the homodimeric winged helical repressors that specifically harbors two conserved cysteine residues (Ma *et al.*, 2009). SoxR was next essentially rediscovered and structurally characterized as BigR from the non-sulfur oxidizing plant pathogens *Xylella fastidiosa* and *Agrobacterium tumefaciens* where it regulates the expression of what is predicted to be a type II PDO (Blh) (Barbosa & Benedetti, 2007, Sattler *et al.*, 2015) (Fig. 2A). BigR and regulated gene products are reported to impact hypoxic growth in biofilms via unknown chemical and physiological mechanisms (Guimaraes *et al.*, 2011). This was followed by our characterization of *Staphylococcus aureus* CstR (Luebke *et al.*, 2014, Grosseohme *et al.*, 2011), a dithiol-containing transcriptional repressor like BigR, but derived from an entirely different family of homotetrameric disc-shaped bacterial repressors evolutionarily related to the copper-sensitive operon repressor (CsoR) (Liu *et al.*, 2007) and the *E. coli* Ni<sup>II</sup> efflux regulator RcnR (Higgins & Giedroc, 2014, Iwig *et al.*, 2006) (Fig. 2A). The CstR-regulated *cst* operon encodes a sulfurtransferase CstA, a PDO-rhodanese fusion protein CstB, and an SQR (Fig. 2A) that is strongly induced by sulfide or inorganic polysulfides added to cells (Luebke *et al.*, 2014). Reduced CstR reacts directly and rapidly with LMW thiol persulfides and sodium tetrasulfide (S<sub>4</sub>) to form a mixture of di-, tri- and tetrasulfide crosslinked species

involving two conserved cysteines, thus establishing the scope of chemistry that was possible with these RSS (Luebke *et al.*, 2014). Strikingly, a recently described master regulator of sulfide-dependent photosynthesis in *Rhodobacter capsulatus*, sulfide:quinone oxidoreductase repressor (SqrR), a BigR- and SoxR-like repressor, carries out precisely analogous chemistry when incubated with glutathione persulfide by forming di-, tri- and tetrasulfide crosslinked species between the two conserved Cys residues (Shimizu *et al.*, 2017).

This new work establishes that FisR engages in CstR- and SqrR-like dithiol oxidative chemistry, on yet a third distinct molecular scaffold and therefore raises a number of important questions (Li *et al.*, 2017). For example, to what degree and what conserved aspects of protein microenvironment render the cysteine pair in these three structural scaffolds selective for RSS? Clearly, a minimum of two cysteine residues must be involved, including an attacking thiol, which is selective for RSS over other potential oxidants, and a resolving thiol which would create a disulfide linkage with the release of H<sub>2</sub>S; further rounds of per- and polysulfide attack of this regulator disulfide or direct initial attack of a polysulfide species would create a range of di-, tri- and tetrasulfide crosslinked products (Mishanina *et al.*, 2015) as experimentally observed (Luebke *et al.*, 2014, Shimizu *et al.*, 2017, Li *et al.*, 2017). The fact that three Cys are functionally required and conserved in most FisRs suggests the possibility of thiol-disulfide exchange after initial formation of a non-native disulfide, as pointed out by the authors (Li *et al.*, 2017). In fact, *R. capsulatus* SqrR harbors a third cysteine, which although not required in cells for RSS sensing, changes the scope of oxidative reaction products obtained upon incubation with glutathione persulfide (Shimizu *et al.*, 2017). Do organic or inorganic per- and polysulfide species (see Fig. 1) play the major cellular role in RSS sensing? Does this vary in different bacteria? Li *et al.* argue that polysulfides are likely physiological *pdo-sqr* inducers in *C. pinatubonensis* given the low *K<sub>m</sub>* for sulfide by microbial SQRs (Xin *et al.*, 2016, Shen *et al.*, 2016) and the potent electrophilicity of a tautomer of HSSH, thiosulfoxide HS(=S)H, that is capable of reacting directly with thiolates (Ono *et al.*, 2014).

Finally, both FisR and CstR orchestrate what is essentially an acute phase response to H<sub>2</sub>S toxicity, with rapid induction following sulfide exposure, with a return to basal transcription 30 minutes post-induction (Luebke *et al.*, 2014, Li *et al.*, 2017). The origin of these induction kinetics is unknown, and may not track with RSS clearance in cells (Peng *et al.*, 2017). Perhaps cells have an overcapacity to “buffer” sulfide as RSS so as to protect critical physiology under conditions of sulfide toxicity while deploying persulfide or RSS transfer chemistries (see Fig. 1D) to rapidly remodel sulfur speciation as needed. This would allow an organism to harness the chemical potential of H<sub>2</sub>S while mitigating collateral damage associated with sulfide toxicity. Precisely what that damage is, however, is currently unknown in any organism. In any case, the characterization of this new proteobacterial sulfide-responsive regulator suggests that sulfide homeostasis is evolutionarily conserved and provides an organism with a means to utilize both exogenous sulfide sources and endogenous RSS that may be important at the host-bacterial pathogen interface (Luhachack & Nudler, 2014).

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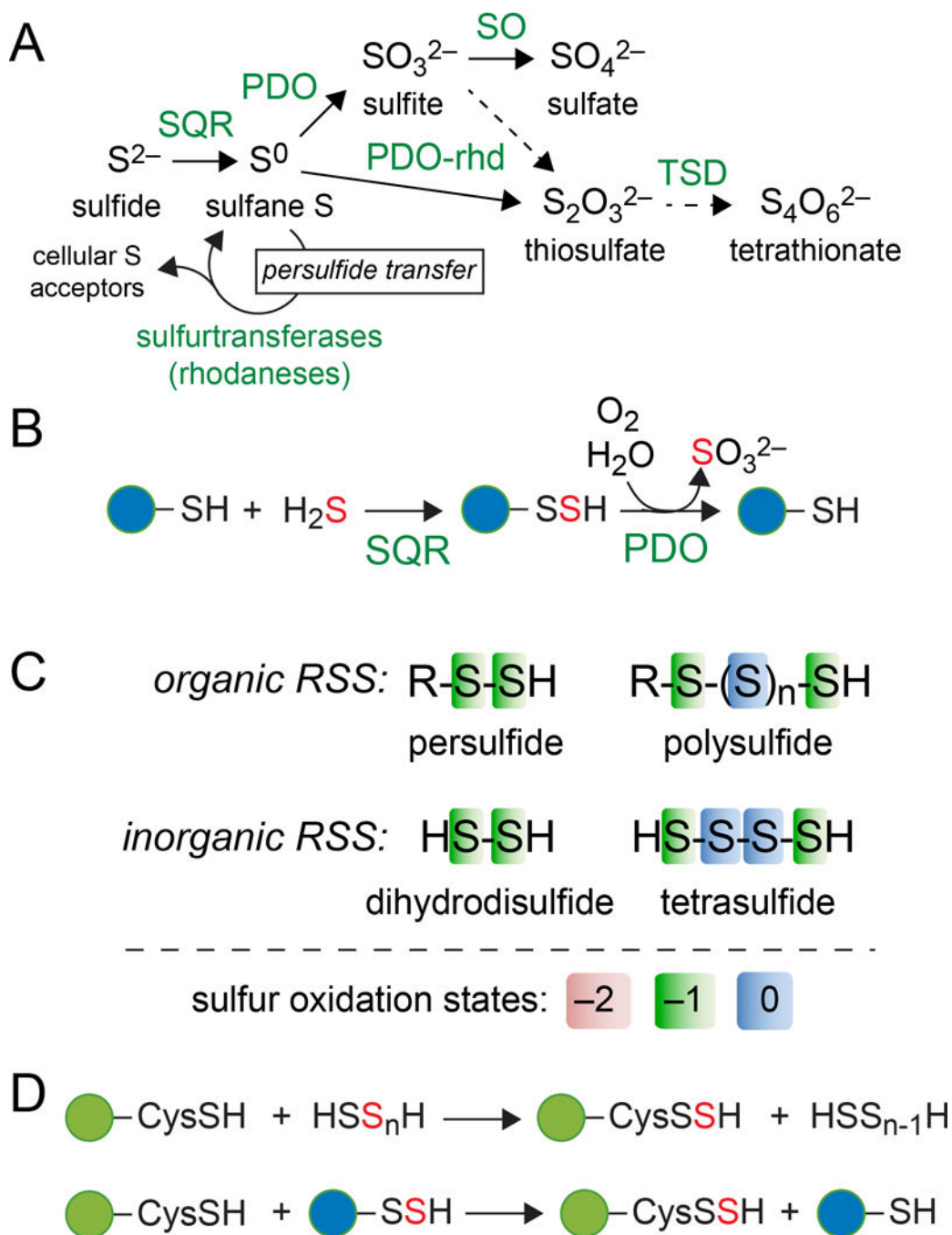
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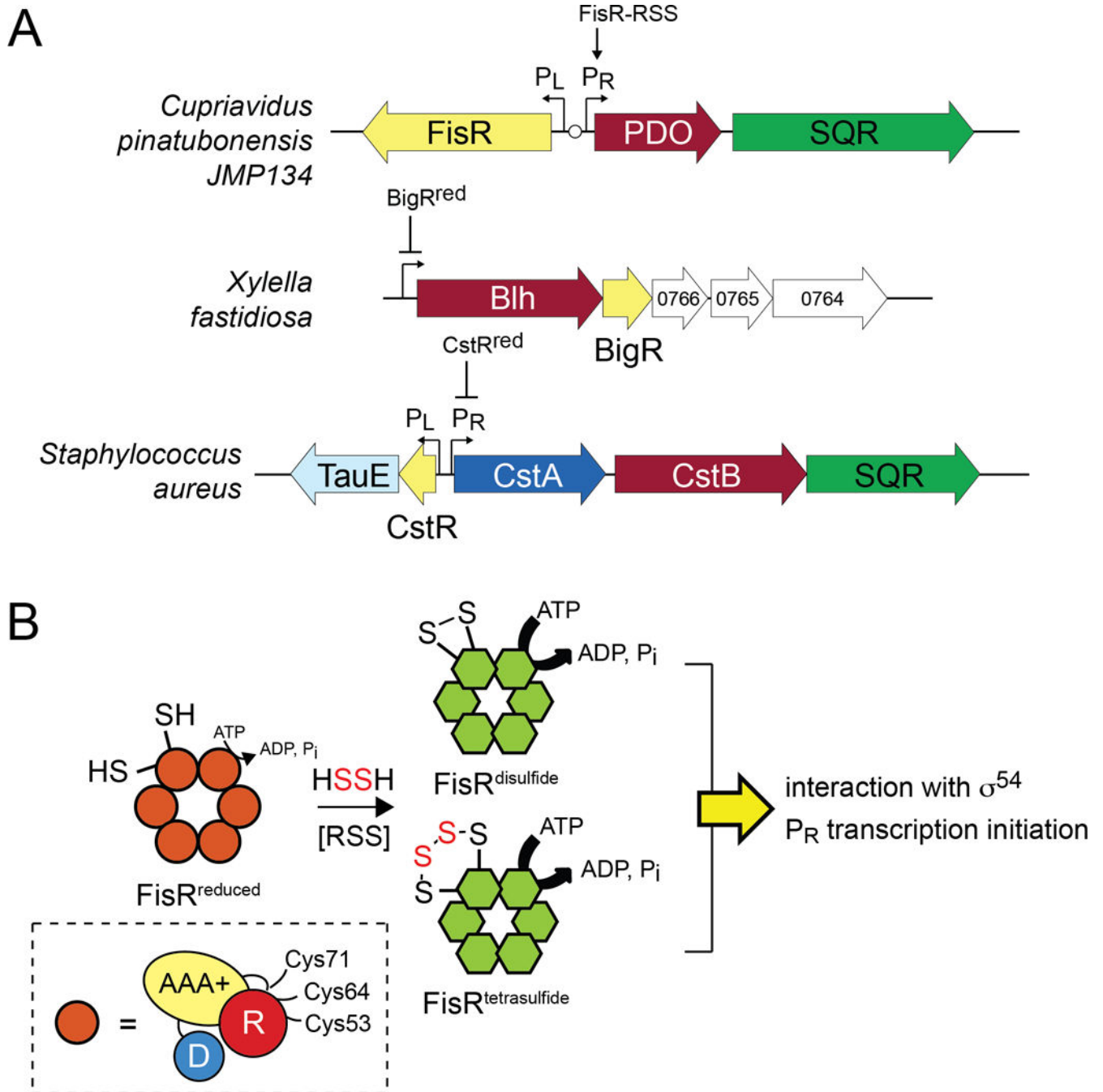
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**Figure 1.**

Sulfide oxidation relevant to cellular sulfide homeostasis and sensing of the reactive sulfur species (RSS) in cells. (A) Step-wise oxidation of the most reduced form of S, sulfide ( $\text{H}_2\text{S}$ ,  $\text{HS}^-$  and  $\text{S}^{2-}$ ) to increasingly more oxidized forms of S, sulfite and tetrathionate, with the enzymes that catalyze these oxidations indicated: SQR, sulfide:quinone oxidoreductase; PDO-persulfide dioxygenase; PDO-rhd, PDO-rhodanese fusion; SO, sulfite oxidase; TSD, thiosulfate dehydrogenase. Sulfurtransferases catalyze persulfide transfer reactions (see panel D) to assimilate bioactive sulfur. (B) Coordinate activity of the SQR and PDO catalyze



the oxidation of H<sub>2</sub>S to sulfite or thiosulfate, with a LMW thiol (*blue* circle) as an intermediate. (C) Reactive sulfur species organized as organic and inorganic species. The simplest organic RSS is a LMW thiol persulfide, while the simplest inorganic polysulfide is dihydrodisulfide, HSSH (Li *et al.*, 2017). Organic and inorganic polysulfide anions feature S chains ( $n - 1$ ) with the internal S atoms formally S<sup>0</sup> (see panel A). Not shown are organic polydisulfides of the general formula, RS-S<sub>n</sub>-SR ( $n - 1$ ), sulfite and thiosulfate. (D) Examples of potential sulfur transfer reactions between proteome (*green* circles) or LMW thiols and inorganic (*top*) and organic (*bottom*) RSS.



**Figure 2.** (A) Schematic representations of the operons regulated by FisR (*top*) (Li *et al.*, 2017), BigR (*middle*) (Barbosa & Benedetti, 2007) and CstR (*bottom*) (Grossoehme *et al.*, 2011) (shaded yellow). FisR is an RSS-activated transcriptional activator, while BigR and CstR are repressors in their reduced states; RSS mediates transcriptional depression. All three operons encode a known or presumed persulfide dioxygenase (PDO, shaded maroon), while two encode SQRs (shaded green). Blh, metallo- $\beta$ -lactamase-like hydrolase. (B) Cartoon representation of hexameric FisR, which in its reduced state has lower ATPase activity, and

upon incubation with HSSH (and likely other RSS), forms a mixture of disulfide and tetrasulfide crosslinked species with higher ATPase activity; this in turn activates transcription from the  $P_R$  promoter (see panel A) via a presumed interaction with RNA polymerase bound  $\sigma^{54}$ . All subunits within the hexamer are anticipated to form crosslinks, but just one is shown for clarity. *Inset*, domain organization of FisR, with the N-terminal regulatory (R), middle ATPase domain (AAA+) and C-terminal DNA binding domain (D) indicated (based on Bush *et al.*, 2015). The three conserved cysteine residues are indicated located in the R domain or a linker that connects the R and AAA+ domains, with a major site of intrasubunit crosslinking shown to be between Cys53 and Cys64 (Li *et al.*, 2017).