CHAPTER 19.3

The iron–sulfur cluster biosynthesis regulator IscR contributes to iron homeostasis and resistance to oxidants in Pseudomonas aeruginosa

Adisak Romsang2,3, James M. Dubbs1, and Skorn Mongkolsuk1–4

1Laboratory of Biotechnology, Chulabhorn Research Institute, Lak Si, Bangkok, Thailand
2Center of Emerging Bacterial Infections, Faculty of Science, Mahidol University, Bangkok, Thailand
3Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok, Thailand
4Center of Excellence for Environmental Health, Toxicology and Management of Chemicals, Bangkok, Thailand

19.3.1 Introduction

Iron–sulfur (Fe-S) cluster-containing proteins are required for many essential biochemical functions across all domains of life. This is due to their structural versatility and chemical reactivity (Py and Barras, 2010). Fe-S cluster proteins play essential roles in a variety of cellular activities, including respiration, central metabolism, biosynthetic pathways, nitrogen fixation, DNA repair, RNA modification, and gene regulation (Kiley and Beinert, 2003; Lill, 2009; Py et al., 2011). Their ability to undergo redox reactions with gaseous molecules allows them to be used by regulatory proteins to sense the intracellular levels of molecular oxygen (O₂) and nitric oxide (NO), which have important biological roles (Crack et al., 2014; Py and Barras, 2010). O₂ serves as a terminal electron acceptor for aerobic respiration and as a precursor of reactive oxygen species (ROS; see Section 10), whereas NO functions as a signaling molecule and as an antimicrobial (see Section 17). Control of gene expression results from conformational changes that affect DNA binding (Crack et al., 2014).

Fe-S centers come in a variety of forms. The most common is an approximately 90% cubic, 4Fe-4S type. Other examples are rhombic 2Fe-2S, 3Fe-4S, and 8Fe-7S, as well as other types (Fontecave, 2006; Py and Barras, 2010). In most Fe-S cluster proteins, cysteine (Cys) residues coordinate the iron ion, but histidine (His) residues can also be involved (Fleischhacker et al., 2012; Nesbit et al., 2009), as is the case in the Fe-S cluster-containing transcriptional regulator, IscR, found in Escherichia coli and Pseudomonas aeruginosa as well as other bacteria (Py and Barras, 2010). In the case of both E. coli and P. aeruginosa, one histidine and three cysteine residues bind the 2Fe-2S cluster (Fleischhacker et al., 2012; Romsang et al., 2014).

Fe-S clusters are thought to be one of the most ancient types of protein prosthetic groups, and the basic process by which they are synthesized is conserved among all organisms (Py and Barras, 2010). The Fe-S cluster is first assembled within a protein scaffold before it is inserted into the target protein (Py and Barras, 2010). In bacteria, three independent Fe-S cluster biosynthetic systems have been identified, namely the NIF (nitrogen fixation), ISC (iron–sulfur cluster), and SUF (mobilization of sulfur) systems (Ayala-Castro et al., 2008; Johnson et al., 2005; Zheng et al., 1998). The ISC and Suf systems are general Fe-S cluster biosynthetic systems. The NIF system is specific for the assembly and insertion of Fe-S clusters in nitrogenase, the enzyme responsible for nitrogen fixation (Py and Barras, 2010). Some bacteria contain only one system, such as the ISC system in the human pathogen P. aeruginosa (Romsang et al., 2014), and the Suf system in Gram-positive bacilli (Santos et al., 2014) and the Gram-negative plant pathogen Xanthomonas campestris pv. campestris (Xcc) (Fuangthong et al., 2015). However, others can contain more than one system, like E. coli and Thermocapulaputens, which possess both the ISC and Suf systems (see Figure 19.3.1) (Fontecave et al., 2005; Outten et al., 2004; Takahashi and Tokumoto, 2002).

Regardless of the Fe-S biosynthetic system(s) that an organism contains, the fundamental mechanisms and key proteins necessary for Fe-S cluster biogenesis are similar between them. Sulfur is provided by Cys via a cysteine desulfurase (IscS, NifS, and SufS), which is a phosphate-dependent enzyme that catalyzes the degradation of the L-cysteine to an enzyme-linked S-sulfanyl cysteine (E-S-SH) and L-alanine (Py and Barras, 2010). Apart from their function in Fe-S cluster biogenesis, some cysteine desulfurases are involved in the biosynthesis of numerous sulfur-containing substances such as thiamine, molybdopterin, and the...
nucleosides in transfer RNAs (tRNAs) (Kessler, 2006; Shigi et al., 2006).

Our understanding of the processes controlling the provision of iron for Fe-S cluster biosynthesis is incomplete. There, are however, a number of proteins that have been proposed as iron donors, including CyaY (a bacterial homolog of eukaryotic frataxin) and iron storage proteins such as ferritins and bacterioferritin (Yao et al., 2011).

Scaffold proteins, such as IscU, IscA, SufU, SufA, and NifU, form an intermediate assembly site for Fe-S cluster precursor assembly. Other scaffold and electron transfer proteins also play a role in the formation of 4Fe-4S clusters from 2Fe-2S precursors (Giel et al., 2013; Santos et al., 2015). The finished clusters are then transferred to target apo-proteins (Boyd et al., 2014; Roche et al., 2013).

In E. coli, as well as many other pathogenic bacteria, Fe-S clusters are normally synthesized using the ISC machinery encoded by the isc operon (iscRSUA-hscBA-fdx-iscX), which is expressed constitutively, but upregulated in response to oxidative stress (see Section 10) and iron-limiting conditions (Fleischhacker et al., 2012). As previously mentioned, iscS encodes cysteine desulfurase, while iscU and iscA encode scaffold proteins for 2Fe-2S cluster assembly (Ayala-Castro et al., 2008). Chaperone proteins encoded by hscA and hscB are involved in the formation of 4Fe-4S clusters. They function to destabilize the IscU-Fe-S complex and facilitate cluster delivery to acceptor proteins by regulating cluster release and transfer (Füzéry et al., 2011; Vickery and Cupp-Vickery, 2007). Fdx contains a 2Fe-2S cluster and supplies electrons to reduce sulfane sulfur ($S^0$) produced by IscS to sulfide ($S_{2-}$), as required for the assembly of Fe-S clusters on the scaffold protein (IscU), and it has been shown that Fdx directly interacts with IscS and competes with IscU and CyaY for binding to IscS (Kim et al., 2013). iscR encodes a dimeric transcriptional regulator of the MarA–SoxS–Rob family (Fleischhacker et al., 2012) and contains one 2Fe-2S cluster per subunit (see Figure 19.3.2) (Fleischhacker et al., 2012; Rajagopalan et al., 2013). In E. coli, IscR is capable of binding DNA at two distinct consensus binding sites, denoted type 1 (ATASYGACTRWYWYAGTCRSTAT) and type 2 (AWARCCCTNSGTTTGMNGKKTWA) (Giel et al., 2006). The presence or absence of Fe-S clusters in the protein affects the binding site preference of the regulator (Rajagopalan et al., 2013). IscR containing Fe-S clusters binds DNA at both type 1 and type 2 IscR-binding motifs (Crack et al.,

Figure 19.3.1 Organization of the genes encoding Fe-S biogenesis proteins in several bacterial genomes. The double lines separating the two gene clusters in T. potens indicate that these clusters are encoded at different positions in the genome.
2014; Schwartz et al., 2001). If no Fe-S clusters are present in the dimer, apo-IscR shifts binding site specificity to type 2 IscR-binding motifs exclusively (Nesbit et al., 2009; Rajagopalan et al., 2013).

The two forms of IscR coordinate the regulation of Fe-S cluster biosynthesis systems. During normal growth, when the Fe-S cluster supply is sufficient, IscR dimers containing Fe-S clusters repress the transcription of the isc operon by binding to two type 1 binding sites present in the promoter–operator region (Schwartz et al., 2001). Under conditions where Fe-S cluster production is insufficient, a buildup of apo-IscR leads to the derepression of isc operon expression and the activation of the suf-operon (sufABCDSE) via specific binding to type 2 IscR-binding motifs within the suf-operator region (Nesbit et al., 2009).

Cellular Fe-S cluster content is the key signal for IscR-mediated transcriptional control through these two different types of IscR-binding motifs. It follows that environmental stresses that reduce Fe-S content, such as Fe limitation and Fe-S cluster damage due to oxidative stress (Crack et al., 2012a,b; Imlay, 2006), would affect the expression of the ISC and SUF systems (Fuangthong et al., 2015; Outten et al., 2004; Romsang et al., 2014; Yeo et al., 2006). This occurs through IscR (Fuangthong et al., 2015; Outten et al., 2004; Romsang et al., 2014; Yeo et al., 2006) as well as via direct links with other stress regulators. For example, in the Enterobacteriaceae, the control of isc expression is directly linked to Fe starvation through the regulatory RNA, RyhB (Desnoyers et al., 2009; Massé and Gottesman, 2002; see Section 5), and to oxidative stress via the oxidant-sensing transcriptional regulator, OxyR (Outten et al., 2004; Yeo et al., 2006). Both iron limitation and oxidative stress are encountered during infection (Atichartpongkul et al., 2010; Cornelis and Dingemans, 2013; Tan et al., 1999), which makes the regulation of Fe-S cluster synthesis a potentially important aspect of the physiological adjustment necessary for the shift to growth within a host organism (Kim et al., 2009; Miller et al., 2014; Runyen-Janecyk et al., 2008).

### 19.3.2 Iron–sulfur cluster biogenesis in Pseudomonas aeruginosa

*P. aeruginosa* is an aerobe with the capacity for facultative anaerobic growth utilizing nitrate as a respiratory electron acceptor (Hunt and Phibbs, 1983). It is of interest mainly because it is a common opportunistic human pathogen infecting burn patients, immunocompromised patients, and people with pneumonias or cystic fibrosis (Lavoie et al., 2011; Lyczak et al., 2000).

The *P. aeruginosa* PAO1 genome contains an isc gene cluster consisting of iscRSUA–hscBA–fdx2–iscX (PA3815–PA3808) (Romsang et al., 2014). As shown in Figure 19.3.1, this basic gene organization is conserved among isc operons of the Enterobacteriaceae (*E. coli*, *Shigella flexneri*, *Salmonella enterica* serovar Typhimurium, and *Klebsiella pneumonia*) as well as other Gram-negatives (*Azotobacter vinelandii*, *Erwinia chrysanthemi*, *Yersinia pseudotuberculosis*, and *Vibrio vulnificus*). By contrast, *T. potens* contains iscR linked to iscS and iscU without other ISC-component genes, while *X. campestris* carries iscR linked to genes of the suf system (see Figure 19.3.1).

Comparison of the deduced amino acid sequences indicates that *P. aeruginosa* IspR shares 77% identity with the IscR from *A. vinelandii* and approximately 60% identity with the IscR from *V. vulnificus*, *Y. pseudotuberculosis*, *E. chrysanthemi*, and *Enterobacteriaceae* (Figures 19.3.3 and 19.3.4). *P. aeruginosa* IscR shares only 39% identity with the IscR from the Gram-positive bacterium *T. potens* and about 20% identity with the IscR from *X. campestris*. This is reflected in the rooted phylogenetic tree shown in Figure 19.3.3, in which *T. potens* and *X. campestris* IscRs do not branch with the others.

In all cases, Cys92, Cys98, and Cys104 (*E. coli* numbering), which are known to participate in the coordination of the 2Fe-2S cluster in *E. coli* IscR, are conserved (Figure 19.3.4). His107 is also involved in coordinating the 2Fe-2S cluster in *E. coli* IscR (Giel et al., 2013; Rajagopalan et al., 2013), but is absent in IscR from *T. potens* and *X. campestris*. It is thought that...
motif containing three conserved Cys residues (C-X$_{24}$ to $26$-C-).

A U-type scaffold protein that contains a characteristic sequence

The second gene in the operon, iscS, encoding cysteine desulphurase, contains the sequence motif, SSGSGC(T/S)S, spanning the conserved C-terminal catalytic Cys residue characteristic of type 1 enzymes such as NifS and NFS (Liu et al., 2009; Urbina et al., 2001). IscS mobilizes sulfur from L-cysteine for the synthesis of several biomolecules, including Fe-S clusters, molybdopterin, thiamin, lipoic acid, biotin, and the thiolation of tRNAs (Dahl et al., 2013). The sulfur transfer from IscS to various biomolecules is mediated by different interaction partners such as IscU, Fdx, IscX, and CyaY for Fe-S cluster biogenesis; TusA for thiomodification of tRNAs; and Thl for thiamine biosynthesis and tRNA thiolation (Dahl et al., 2013).

The third gene in the operon, iscU, encodes a Fe-S scaffold protein (Fontecave and Ollagnier-de-Choudens, 2008; Py and Barras, 2010; Urbina et al., 2001). There are three known types of scaffold proteins: U-type, NFU-type, and A-type (Fontecave and Ollagnier-de-Choudens, 2008; Rouault and Tong, 2008). IscU is a U-type scaffold protein that contains a characteristic sequence motif containing three conserved Cys residues (C-X$_{24}$ to $26$-C-X$_{42}$ to $43$-C) that are required for assembly of the Fe-S cluster (Urbina et al., 2001). There are no other homologs of IscU in the PAO1 genome.
IscA is an iron-binding A-type carrier protein, as are IscA, NfuA, SufA, and ErpA (Py and Barras, 2010; Rouault and Tong, 2008). IscA is thought to be responsible for the transfer of completed Fe-S clusters to target apo-proteins. This is because it has been demonstrated to accept Fe-S clusters from IscU, but transfer in the opposite direction is not possible (Chandramouli et al., 2007).

Both hscB and hscA encode chaperones that mediate the transfer of finished Fe-S clusters from IscU to target apo-proteins. The product of hscB is a J-type co-chaperone protein of approximately 20 kDa that directs IscU to the HscA substrate-binding domain and stimulates the adenosine triphosphate (ATP) hydrolysis activity of HscA (Füzéry et al., 2011; Py and Barras, 2010; Vickery and Cupp-Vickery, 2007). HscA, a homolog of the chaperone DnaK, is required for 4Fe-4S formation. It interacts with the IscU–HscB complex, where it facilitates Fe-S cluster transfer at the expense of ATP hydrolysis (Füzéry et al., 2011).

fdx2 encodes a ferredoxin that contains a 2Fe-2S cluster and is involved in the formation of 4Fe-4S clusters (Chandramouli et al., 2007; Py and Barras, 2010; Roche et al., 2013). The product of fdx2 is a ferredoxin that contains a 2Fe-2S cluster that is necessary for the reductive coupling of two 2Fe-2S clusters to form a single 4Fe-4S2 cluster in homodimeric IscU (Yan et al., 2013).

The last gene in the isc operon, iscX, is a Fe-S cluster assembly protein composed of only 66 amino acids. IscX has been shown to interact with IscU and IscS both individually and in a ternary complex containing all three (Dahl et al., 2013). IscX inhibits IscS desulfurase activity and functions as an iron donor for Fe-S assembly (Kim et al., 2014).

The isc operon appears to encode the primary Fe-S cluster biosynthesis system in P. aeruginosa since no homologs of the suf genes are present in the genome (Romsang et al., 2014). However, a number of genes that have Fe-S cluster biogenesis-related function are present. These include nfuA, erpA, cyaY, and yggX (Daung-nkern et al., 2010; A Romsang and S Mongkolsuk, unpublished observations).

NfuA is a fused protein between an Nfu domain and a degenerate A-type carrier domain, which is thought to be a Fe-S carrier involved in the maturation of certain Fe-S proteins during oxidative stress (Angelini et al., 2008; Bandyopadhyay et al., 2008; Zimblter et al., 2012). In E. coli, NfuA is required for survival under oxidative stress and during iron limitation (Angelini et al., 2008; Zimblter et al., 2012), and it is required for full activity of aconitases in A. vinelandii (Bandyopadhyay et al., 2008). In P. aeruginosa, inactivation of nfuA enhances susceptibility to fluoroquinolone antibiotics, which are known to induce oxidative stress (Daung-nkern et al., 2010).

The open reading frame (ORF) PA0665 is predicted to encode the Fe-S insertion protein, ErpA. In E. coli, ErpA is an A-type scaffold protein that is essential for both aerobic and anaerobic respiratory growth (Bolstad et al., 2010; Py and Barras, 2010). This may also be the case for P. aeruginosa PAO1 erpA since efforts to inactivate the gene under aerobic conditions have been unsuccessful (A Romsang and J Duang-nkern, unpublished observations).

Bacterial frataxin (CyaY) is involved in the donation of the iron ions during Fe-S cluster assembly (Py and Barras, 2010; Roche et al., 2013). Finally, YggX, also an iron-binding protein, has been suggested to function in Fe-S cluster repair in E. coli due to the observation that, under exposure to paraquat, a yggX deletion mutant showed a deficiency in the activity of the Fe-S cluster–containing enzyme, aconitase, compared to the wild type (Gralnick and Downs, 2001; Justino et al., 2007). In E. coli, cyaA and yggX might be functionally redundant since individual mutations in cyaY or yggX are not associated with phenotypic changes (Justino et al., 2007; Li et al., 1999). However, in S. enteric serovar Typhimurium, CyaY and YggX play distinct supporting roles (Velayudhan et al., 2014). Cya functions in Fe-S cluster biosynthesis and the repair of labile clusters damaged by oxidants, while YggX functions in Fe-S cluster synthesis, protection, and repair during severe oxidative stress (Velayudhan et al., 2014).

19.3.3 IscR as a transcriptional regulator for Fe-S biogenesis in Pseudomonas aeruginosa

Northern blotting and reverse transcription polymerase chain reaction (RT-PCR) analyses indicate that the P. aeruginosa isc operon (iscRSUA–hscBA–fdx2–iscX) is transcribed as a single polycistronic messenger RNA (mRNA) of approximately 5 kb (Romsang et al., 2014). Unlike E. coli, in which the large polycistronic mRNA is processed to leave a stable mRNA-encoding iscR (Fleischhacker et al., 2012), no stable iscR mRNA segments have been detected in P. aeruginosa (Romsang et al., 2014). Transcription of the P. aeruginosa isc operon is constitutive but shows significant induction in the presence of redox-cycling and superoxide-generating agents (menadione, plumbagin, and paraquat) as well as H2O2 and organic peroxides (tert-butyl hydroperoxide and cumene hydroperoxide) (Romsang et al., 2014).

Initial indications that IscR is a repressor that controls the expression of itself and the isc operon in P. aeruginosa came from the observation that an iscR deletion mutant (ΔiscR) had a 20-fold higher level of isc operon transcription, relative to wild-type PAO1, as measured using real-time RT-PCR that targeted a region 5′ to the deletion site (Romsang et al., 2014). Expression of plasmid-borne iscR returned transcription to wild-type levels, indicating the direct involvement of IscR (Romsang et al., 2014).

The sequence upstream of P. aeruginosa iscR contains two putative type 1 IscR-binding motifs denoted site A (5’AATCTGAGTATTTGCATGCTT3’), between positions −43 and −67, and site B (5’ATAAGTTGACCTAA TACTCGGATA3’), spanning positions −18 to −42 relative to the iscR transcription start (Romsang et al., 2014). These two
motifs share 68% and 76% identity with the consensus sequence for the *E. coli* type 1 IscR-binding motif (see Figure 19.3.5). The results of gel mobility shift assays confirm that purified His-tagged IscR binds to a DNA fragment spanning both sites. Additionally, the presence of multiple shifted bands suggests the binding of multiple IscR to this isc promoter fragment (A Romsang and S Mongkolsuk, unpublished results). These IscR-binding motifs overlap a putative sigma 70 RNA polymerase consensus promoter sequence. Thus, the binding of IscR to these sites would likely prevent RNA polymerase binding to the promoter and repress the transcription of the *isc* operon, which is consistent with the observed increased *isc* transcription in *P. aeruginosa ΔiscR* that is reversed with the expression of functional IscR (Romsang et al., 2014).

A recent report in *E. coli* highlights differences in *iscR* binding site organization in its *isc* operon promoter compared to that in *P. aeruginosa* (Figure 19.3.5). DNase I protection analyses of the *E. coli* *isc* promoter revealed that IscR binds to three individual sites within the *isc* promoter region (Giel et al., 2006). One region of protection, which encompasses nucleotides −67 to −14 relative to the +1 transcription start site, includes two type 1 IscR binding sites: site A (−65 to −41) and site B (−40 to −16) (Giel et al., 2006). A remaining IscR site, denoted site C, is located from +9 to +26. It does not share sequence similarity with other known IscR-binding sites and has no known function (Giel et al., 2006). The two adjacent type 1 IscR-binding sites, A and B, span the consensus RNA polymerase binding site. Each of these two sites binds the IscR holocomplex and is responsible for *isc* repression *in vivo*, where they appear to contribute equally (Giel et al., 2013). Thus, the mechanism of IscR repression in *E. coli* can be simply explained by promoter occlusion.

The *P. aeruginosa* *isc* promoter is similar to that of *E. coli* in that it contains two IscR-binding sites at positions corresponding to sites A and B in the *E. coli* promoter. However, no site corresponding to site C is present (Figure 19.4.5). In *P. aeruginosa*, site B is primarily responsible for repression. This is based on the observations that site B exhibits higher affinity for IscR binding *in vitro* and that site-directed mutations in site B increased *isc* promoter activity (approximately fourfold), while mutations in site A had only a small effect on repression (A Romsang and S Mongkolsuk, unpublished results). Moreover, comparison of IscR-binding sites A and B present in the *P. aeruginosa* *isc* promoter with those in *E. coli* shows that the B site shares 81% DNA sequence identity between the two organisms, while site A shares only 42% identity. The function of site A, if any, in the regulation of the *P. aeruginosa* *isc* operon is unclear. Site A in *E. coli* is clearly a type 1 binding site, while site A in *P. aeruginosa* shows roughly equal sequence identity to the type 1 (68%) and type 2 (65%) IscR consensus binding motifs (see Figure 19.3.5). Thus, the function of site A likely differs significantly from that in *E. coli*.

Recent IscR DNA-binding and target gene expression studies in *E. coli* have shown that three cysteine residues (Cys92, Cys98, and Cys104) and one histidine residue (His107), which are ligands for 2Fe-2S coordination (Fleischhacker et al., 2012; Santos et al., 2015), are required for repression of target gene transcription (Rajagopalan et al., 2013; Santos et al., 2015).

Cys92, Cys98, Cys104, and His107 are also conserved in *P. aeruginosa* IscR, where they perform a similar function. This is based on the results of complementation experiments performed in *P. aeruginosa ΔiscR* mutant strains expressing IscR mutants carrying single alanine substitutions at Cys92, Cys98, Cys104, or His107 (Romsang et al., 2014). The C92A and H107A *isc* mutants failed to repress transcription of the *isc* operon in the *ΔiscR* background, while the IscR-C98A and IscR-C104A partially restored repression relative to wild-type IscR. Moreover, oxidant treatment of *isc* mutants expressing IscR-C98A and IscR-C104A de-repressed *isc* operon transcription to wild-type levels (Romsang et al., 2014). The inability of IscR-C92A and IscR-H107A to repress *isc* operon transcription is thought to be due to an inability of these proteins to ligate 2Fe-2S clusters, thus preventing binding of the C92A and H107A mutants to the type 1 binding motifs in the *isc*-operon promoter and operator (Romsang et al., 2014). By contrast, it is thought that IscR-C98A and IscR-C104A are capable of ligating 2Fe-2S clusters but that the IscR-C98A and C104A holocomplexes are present at
much lower levels than the wild-type protein. This is supported by spectroscopic analyses of purified His-tagged IscR wild-type and C92A, C98A, C104A, and H107A mutants (Romsang et al., 2014). All of the IscR variant proteins (C98A, C104A, H107A, and C92A) contained a reduced amount of 2Fe-2S clusters relative to wild-type IscR (Romsang et al., 2014). Consistent with the complementation results, the IscR-C92A and H107A contained the lowest levels of 2Fe-2S clusters, while the levels of 2Fe-2S clusters bound by the C98A and C104A mutants were intermediate between those of the C92A and H107A mutants and wild-type IscR (Romsang et al., 2014). The lower levels of the IscR-C92A and IscR-H107A mutant holo-forms could be due to weakened 2Fe-2S cluster attachment, rendering it susceptible to oxidation under aerobic conditions or 2Fe-2S cluster insertion, which may be less efficient. Complementation experiments performed under anaerobic conditions gave results that were similar to those obtained under aerobic conditions (A Romsang and S Mongolsuk, unpublished observations), perhaps arguing against increased susceptibility of the C98A and C104A mutants to oxidation; however, this particular point has yet to be resolved.

IscR regulatory targets are not limited to Fe-S cluster biosynthesis genes. Recently, E. coli IscR has been shown to be a global regulator that controls the transcription of at least 50 genes, apart from the well-known isc and suf operons (Giel et al., 2006; Lim et al., 2014; Schwartz et al., 2001; Wu and Outten, 2009). Most of these genes encode Fe-S cluster proteins and include transcriptional regulators such as IscR, SoxR, and Fnr; the Fe-S cluster carriers NfuA and ErpA; TCA cycle enzymes (aconitase and succinate dehydrogenase); and proteins involved in anaerobic respiration (periplasmic nitrate reductase and hydrogenases-1 and −2), among others (Schwartz et al., 2001). IscR also controls some non-Fe-S cluster protein–encoding genes, such as the fim and fla operons (Wu and Outten, 2009), both of which encode cell surface adhesins involved in surface attachment and aggregation that can affect virulence (Schwan, 2011; Wu and Outten, 2009; see Section 22). In P. aeruginosa, IscR is a repressor of tpx encoding a thiol-peroxidase that plays a role in hydrogen peroxide resistance (Somprasong et al., 2012). Moreover, P. aeruginosa IscR indirectly modulates catalase activity at the posttranslational level through its effect on intracellular iron–heme levels (Kim et al., 2009; Romsang et al., 2014).

19.3.4 Involvement of IscR in the oxidative stress response and virulence in Pseudomonas aeruginosa

Fe-S clusters are susceptible to damage due to reactive oxygen intermediates (ROS), including hydroxyl radicals, superoxide anion, and hydrogen peroxide, resulting in loss of the functional clusters (Crack et al., 2012a,b; D’Autréaux and Toledano, 2007; Imlay, 2006; Py and Barras, 2010; see Section 10). These ROS directly oxidize the Fe-S clusters in proteins, converting the 4Fe-4S$^{2+}$ form to an unstable 4Fe-4S$^{3+}$ state, which releases Fe$^{2+}$. The resultant 3Fe-4S$^{3+}$ cluster (Djaman et al., 2004; Imlay, 2006) is metastable and rapidly degrades, as shown in Reactions 1 and 2 (Djaman et al., 2004):

\[
\begin{align*}
\text{Reaction 1:} & \quad \text{Ox} + 4\text{Fe-4S}^{2+} \rightarrow \text{Red} + 4\text{Fe-4S}^{3+} \\
\text{Reaction 2:} & \quad 4\text{Fe-4S}^{3+} \rightarrow 3\text{Fe-4S}^{3+} + \text{Fe}^{2+}
\end{align*}
\]

Damage to clusters can also occur upon reactions with strongly coordinating species such as sodium hypochlorite (NaOCl) and nitrosylating agents such as nitric oxide (NO) (Crack et al., 2012a,b; Zumft, 2002; see Section 17). NO reacts readily with Fe-S proteins, leading to S-nitrosylation of the iron atom in the Fe-S cluster–containing proteins (Imlay, 2006). This sensitivity to certain types of damage allows Fe-S clusters to be used by regulatory proteins as environmental sensors. Examples of this in E. coli can be seen in the aerobic-anaerobic sensing regulator, Fnr, and the oxidative stress sensor, SoxR. Fnr binds a 4Fe-4S cluster under anaerobic conditions that stimulates the formation of an active DNA-binding dimeric form. Exposure to O$_2$ oxidizes the cluster to a 2Fe-2S form, leading to protein monomerization and hence loss of DNA-binding ability (Kiley and Beinert, 1998, 2003; Saini et al., 2012). Another example is the transcriptional regulator, SoxR, a 2Fe-2S cluster–containing protein that functions as a sensor of oxidative stress and NO in bacteria (Demple et al., 1999; Kiley and Beinert, 2003; Kobayashi et al., 2014). A reversible one-electron oxidation of the 2Fe-2S cluster converts SoxR to a transcriptional activator of SoxS, which in turn activates stress response genes in the soxRS regulon. NO also activates SoxR by direct nitrosylation of the iron atoms in the 2Fe-2S cluster (Kobayashi et al., 2014; Saini et al., 2012).

In IscR, the presence or absence of a 2Fe-2S cluster determines target gene promoter specificity as well as whether the protein functions as an activator or repressor (Giel et al., 2006; Nesbit et al., 2009; Otsuka et al., 2010; Yeo et al., 2006). During oxidative stress, damage to the 2Fe-2S cluster leads to a decrease in the level of the IscR holo-form and an increase in IscR apo-form levels. Thus, redox conditions modulate the ligation status of the labile IscR cluster that, in turn, determines a switch in DNA sequence specificity of the regulator. This results in the selective regulation of subsets of genes within the IscR regulon in both E. coli and P. aeruginosa (Giel et al., 2013; Outten et al., 2004; Romsang et al., 2014; Somprasong et al., 2012; Wu and Outten, 2009; Yeo et al., 2006).

Several studies have shown that inactivation of iscR in P. aeruginosa results in increased sensitivity to H$_2$O$_2$ and organic hydroperoxides, as well as to the superoxide generators: plumbagin, menadione, and paraquat (Choi et al., 2007; Kim et al., 2009; Romsang et al., 2014). Similar defects in oxidative stress resistance have also been observed in iscR inactivation mutants of other bacteria (Fuangthong et al., 2015; Jones-Carson et al., 2008; Lim et al., 2014). All of these phenotypes can complement functional iscR, indicating that IscR plays an important role in the oxidative stress response (see Section 10) and suggesting that misregulation of genes in the IscR regulon...
is responsible for the increased oxidant sensitivity of the ΔiscR mutant (Fuangthong et al., 2015; Lim et al., 2014; Romsang et al., 2014).

The observed H$_2$O$_2$ sensitivity of the ΔiscR mutant is due, at least in part, to reductions in the levels and activities of H$_2$O$_2$ detoxification enzymes. In P. aeruginosa PAO1, IscR directly regulates tpx, which plays a role in hydrogen peroxide resistance (Somprasong et al., 2012), whereas iscR inactivation has also been shown to reduce catalase activity in P. aeruginosa (Kim et al., 2009) as well as the plant pathogen Xanthomonas campestris (Fuangthong et al., 2015), Burkholderia mallei (Jones-Carson et al., 2008), and Vibrio vulnificus (Lim et al., 2014). The basis for the iscR mutant's sensitivity to organic peroxides remains to be determined.

The basis for the increased sensitivity to the redox-cycling drugs plumbagin, menadione, and parquat is unknown. There are several possible pathways for toxicity. These drugs generate superoxide anion (O$_2^-$) through a redox-cycling reaction in which single electrons are removed from cellular redox cofactors and transferred to oxygen. Toxicity can result from either the oxidation of cellular components, including Fe-S clusters, or the disruption of normal electron flow via redox-cycling reactions that oxidize the cellular electron carrier pool (Gu and Imlay, 2011; Romsang et al., 2013). These drugs can also irreversibly oxidize NAD cofactors in proteins (Gu and Imlay, 2011; Romsang et al., 2013).

The sensitivity of the P. aeruginosa ΔiscR mutant to redox-cycling drugs does not appear to be due to O$_2^-$ since anaerobic respiratory growth with NO$_3^-$ as an electron acceptor did not affect sensitivity relative to that observed during aerobic growth (Romsang et al., 2013, 2014). The question remains as to how the presence of these redox-cycling drugs is sensed by IscR if superoxide-mediated damage to its Fe-S cluster is not involved. Is the Fe-S cluster in IscR damaged in some other way, or does the disruption of cellular redox status affect Fe-S cluster biogenesis pathways?

IscR clearly plays a role in virulence. The inactivation of P. aeruginosa iscR attenuates virulence in Drosophila melanogaster and mouse peritonitis models (Kim et al., 2009) and has been shown to reduce virulence in other pathogens such as E. chrysanthemi (Rincon-Enriquez et al., 2008), S. flexneri (Runyen-Janecky et al., 2008), Burkholderia mallei (Jones-Carson et al., 2008), and V. vulnificus (Lim et al., 2014). IscR is also known to be involved in virulence related processes in E. coli where it modulates the formation of biofilms via the regulation of type 1 fimbria expression (Wu and Outten, 2009). In the foodborne pathogen Y. pseudotuberculosis, IscR is critical for virulence and expression of the type III secretion system through direct regulation of the T3SS master regulator, LcrF (Miller et al., 2014). Recently, it has been shown that IscR senses iron starvation via a shift in the protein to the IscR apo-form, which activates prx3 expression (Lim et al., 2014). Prx3 is a 1-Cys peroxiredoxin that reduces H$_2$O$_2$ to H$_2$O using reducing equivalents supplied via glutaredoxin 3 (Grx3) and glutathione (GSH). This contributes to the survival and virulence of V. vulnificus during pathogenesis (Lim et al., 2014). IscR not only is a key factor for pathogenicity in mammalian pathogens, but also is important for virulence in plant pathogens such as X. campestris (Fuangthong et al., 2015).

The killing of Caenorhabditis elegans by P. aeruginosa is a good example of how iscR can affect virulence in a number of ways. P. aeruginosa can kill C. elegans quickly because of intoxication due to diffusible toxins, such as phenazines, released by the bacteria when grown on a lawn on rich media (Cezarlityan et al., 2013). When grown on minimal media, killing is more slowly due to the buildup of cyanide that is generated by the bacteria during growth in the worm gut (Cezarlityan et al., 2013; Glasser et al., 2014; Tan et al., 1999). The results of C. elegans killing assays of worms grown on bacterial lawns of P. aeruginosa strains on rich (panel a) and minimal media (panel b) are shown in Figure 19.3.6. In both cases, worms grown on the P. aeruginosa ΔiscR strain exhibited an increase in mortality in both fast- and slow-killing assays compared to the wild-type PAO1. Wild-type mortality levels were restored by iscR expression from a Tn7 expression construct inserted into the genome in single copy, confirming that the absence of functional iscR was responsible for the phenotype.

The observed virulence attenuation in the ΔiscR mutant in the slow-killing assays is likely due, at least in part, to reduced oxidative stress defense since inactivation of oxidative stress defense genes has previously been shown to reduce virulence in similar assays (Atichartpongkul et al., 2010; Romsang et al., 2013). Attenuation of virulence in the fast-killing assay may be related to the ΔiscR mutant's increased sensitivity to redox-cycling agents. Phenazines are a class of toxins that are excreted by P. aeruginosa when grown on rich media (Cezarlityan et al., 2013), and these compounds are thought to act as redox-cycling agents (Cornelis and Dingemans, 2013; Glasser et al., 2014). Moreover, when grown on rich media, the ΔiscR mutant shows
an approximately twofold reduction in phenazine production (A Romsang and S Mongkolsuk, unpublished results). While the participation of iscR in oxidant stress resistance and virulence is clear, going forward it will be important to identify exactly which genes in the IscR regulon contribute to virulence and the sensitivities to oxidative stress and redox-cycling agents.

### 19.3.5 IscR modulates intracellular iron homeostasis in *Pseudomonas aeruginosa*

Biologically, iron is an essential, albeit potentially toxic, element that is important for bacterial growth and survival owing to its capacity as a cofactor that mediates electron transfer and acid–base reactions (Andrews et al., 2003). However, free iron is dangerous since in the intracellular reducing environment it exists in the ferrous form ($\text{Fe}^{2+}$), which has the potential to react with $\text{H}_2\text{O}_2$ and catalyze the formation of cell-damaging ROS, particularly the highly reactive hydroxyl radical ($\text{OH}^-$) via the Fenton reaction (see Section 10):

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^- \quad (\text{Fenton reaction})$$

Since iron-containing proteins are very sensitive to oxidative damage, one of the effects of oxidative stress is an increase in intracellular free iron (D’Autr´eaux and Toledano, 2007). This is reflected by the fact that the expression of genes encoding iron-binding proteins such as $\text{dps}$ is commonly induced as part of the bacterial oxidative stress response (Haikarainen and Papageorgiou, 2010).

Clearly, too much free iron can amplify the effect of oxidative stress, thereby increasing a cell’s sensitivity to oxidants (Troxell and Hassan, 2013), but too little iron can also increase sensitivity since peroxide detoxification enzymes, such as heme-containing catalases and peroxidases, require iron for their activity (Chiancone et al., 2004; Cornelis et al., 2011). It is therefore crucial that cells tightly control the levels of intracellular iron under all growth conditions (Andrews et al., 2003; Chiancone et al., 2004; Cornelis et al., 2011; Troxell and Hassan, 2013).

Bacteria employ a variety of mechanisms for regulating and maintaining intracellular iron homeostasis. In general, this involves balancing iron import via the excretion and uptake of iron-chelating siderophores with iron storage by proteins like ferritin and general ferric iron-buffering pathways such as heme and Fe-S cluster biosynthesis (Cornelis et al., 2011; Touati, 2000). In order to orchestrate this complex task, a number of regulators have been identified. The most widespread of these is Fur (ferric uptake regulator), a well-characterized iron-dependent transcriptional regulatory protein that is a global regulator of the expression of more than 100 genes involved in functions including iron uptake, metabolism, cofactor assembly, and efflux, as well as many genes encoding iron-containing proteins (Cornelis et al., 2009; Troxell and Hassan, 2013; Vasil, 2007; see Chapter 19.1). Depending on the target, Fur can function as either an activator or repressor by binding to a specific DNA sequence called a Fur-box (Ahmad et al., 2009; Katigbak and Zhang, 2012; Mills and Marletta, 2005). Fur also regulates the expression of certain target genes indirectly via the iron-dependent repression of small noncoding regulatory RNAs (Oglesby-Sherrouse and Vasil, 2010; Vasil, 2007; Wilderman et al., 2004; see Section 5).

In *E. coli*, the regulator IscR lies at the core of Fe-S cluster homeostasis regulation, but additional regulators, such as Fur and the peroxide sensing regulator, OxyR, are also involved (Py and Barras, 2010; Roche et al., 2013; Xu and Moller, 2011). *E. coli* OxyR, in its oxidized form, is known to activate the $\text{su}f\text{ABCDSE (suf)}$ operon in response to peroxide stress. Activation also required integration host factor (IHF) and apo-IscR (Outten et al., 2004). Induction of the $\text{su}f$ operon by iron limitation has been proposed to be mediated through the inactivation of Fur (Yeo et al., 2006).

The specific mechanisms by which IscR affects iron homeostasis in *P. aeruginosa* are unclear. What is clear is that inactivation of $\text{iscR}$ shifts cells into an apparent iron-starved state. For example, a *P. aeruginosa* $\text{iscR}$ mutant shows reduced intracellular iron content as measured by inductively coupled plasma mass spectrometry (ICP-MS) (Romsang et al., 2014). Consistent with this, sensitivity to the intracellular iron chelator, 2,2′-dipyridyl, was increased (Romsang et al., 2014). Another indication of iron starvation is the fact that siderophore levels also slightly increased relative to wild-type strain PAO1 (Romsang et al., 2014). Siderophores are low-molecular-weight, high-affinity, ferric-iron-specific chelators involved in the binding and uptake of extracellular iron that are produced when iron is limiting for growth (Vasil, 2007). Conversely, $\text{iscR}$ overexpression reduced siderophore levels. Complementation experiments using wild-type $\text{iscR}$ and the site-directed mutants (C92A, C98A, C104A, or H107A) indicated that Fe-S cluster ligation to IscR was not required for complementation of the increased siderophore phenotype (Romsang et al., 2014), indicating that Apo-IscR is involved.

The physiological basis for the iron starvation phenotype remains to be elucidated, but one line of evidence suggests that it may be due to a defect in iron uptake. This is because the ∆$\text{iscR}$ mutant shows increased resistance to high extracellular iron in the form of either FeCl$_3$ or FeSO$_4$, relative to wild-type PAO1 (Romsang et al., 2014). All of the available evidence seems to indicate that at least one of the roles of IscR may be to upregulate iron acquisition pathways when iron is limiting. It remains to be determined what gene targets are involved, as well as whether the regulation is direct or indirect.

Alterations in the intracellular iron level have an indirect effect on oxidative stress defenses (see Section 10). A *P. aeruginosa* $\text{iscR}$ mutant exhibits a reduced intracellular iron pool and is more sensitive to $\text{H}_2\text{O}_2$ due to a posttranslational reduction in KatA catalase activity (Kim et al., 2009). Catalase is a heme-containing enzyme that detoxifies $\text{H}_2\text{O}_2$ and is crucial for peroxide resistance (McLeod, 1925). In addition, the activity levels of the heme-containing respiratory electron transport chain...
enzyme, cytochrome c oxidase, were dramatically reduced in the ΔiscR mutant (Romsang et al., 2014). Evidence clearly indicates that the reduced catalase level is due to heme depletion. The addition of heme to the culture medium restored the wild-type levels of H2O2 sensitivity and increased catalase activity in the ΔiscR mutant (Romsang et al., 2014). Addition of 2,2′-dipyridyl to the heme-supplemented cultures had no effect on the level of the superoxide dismutase, SodB, a nonheme Fe-S cluster–containing protein, indicating that heme was not acting as a source of free iron and was directly incorporated into catalases and likely other heme-containing enzymes (Romsang et al., 2014). Taken together, the results indicate defects in heme synthesis and the activity of heme-containing enzymes in a ΔiscR mutant.

In *P. aeruginosa*, the iscR target genes involved in iron homeostasis are unknown, and it is unclear whether the regulation that occurs is direct. An example of IscR-mediated regulation in response to iron availability is seen in *Klebsiella pneumoniae*, where IscR directly regulates the expression of genes encoding proteins involved in capsular polysaccharide biosynthesis and iron acquisition in response to environmental iron availability (Wu et al., 2014). Indirect regulation through links with other iron-responsive regulators is also a possibility. In this regard, it is interesting to note that the promoter–operator region of *P. aeruginosa* IscR contains a sequence similar to a consensus Fur-box. Conversely, *P. aeruginosa* fur contains a putative IscR consensus binding sequence in its promoter–operator region. Furthermore, RT-PCR analyses indicate that inactivation of IscR results in the loss of oxidant-inducible transcription of fur (A Romsang and S Mongkolsuk, unpublished results). In *E. coli*, one of the regulatory links between fur and the isc operon is through the Fur-regulated regulatory RNA, RhyB (Massé and Gottesman, 2002), which directs the processing of the full-length iscRSUA–hscBA–fdx2–iscX mRNA down to a stable segment encoding iscR (Massé and Gottesman, 2002). *P. aeruginosa* does not possess rhyB, but it does express an iron-inducible regulatory RNA encoded by prrF (Oglesby-Sherrouse and Vasil, 2010; Wilderman et al., 2004). However, at this time, there is no evidence for processing of the iscRSUA–hscBA–fdx2–iscX mRNA.

### 19.3.6 Conclusion

*P. aeruginosa* IscR directly represses its own transcription along with that of the isc operon in response to the intracellular level of Fe-S clusters. Conditions that result in Fe-S cluster damage, such as exposure to oxidants and redox-cycling drugs (Gu and Imlay, 2011; Imlay, 2006; Romsang et al., 2014), or Fe starvation (Angeliní et al., 2008; Outten et al., 2004), which interfere with Fe-S cluster synthesis, cause induction of isc operon transcription. Like the case in *E. coli*, *P. aeruginosa* iscR likely also functions as a global regulator. Indications of this are seen in the fact that iscR inactivation affects virulence, expression of extracellular toxins, oxidant sensitivity, and possibly iron uptake (Choi et al., 2007; Kim et al., 2009; Romsang et al., 2014; Somprasong et al., 2012). The effect on virulence is explainable, in part, by a reduction in the expression level of thiol peroxidase and reduced levels of catalase activity since these oxidative stress protective enzymes are known virulence factors (Romsang et al., 2014; Somprasong et al., 2012). The effect on catalase activity is the result of heme depletion probably due to iron starvation. The effect on heme availability extends to other heme-containing enzymes such as cytochrome oxidase (Romsang et al., 2014; Wharton et al., 1976), which would affect the redox state of the cellular electron donor pools. The task now is to determine if the observed phenotypic effects are due to IscR directly or through the action of other regulators. Already, there is evidence that IscR and Fur may regulate one another, potentially shedding light on the effect of IscR on iron homeostasis. Other IscR regulatory targets need to be identified and characterized in order to gain an understanding of the linkages between IscR and other cellular pathways. This will provide insights that may aid drug development and the design of strategies for the control of both human and plant pathogens.

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