CHAPTER 19.3

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

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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 (O_2) and nitric oxide (NO), which have important biological roles (Crack *et al*., 2014; Py and Barras, 2010). O_2 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 *Thermincola potens*, 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

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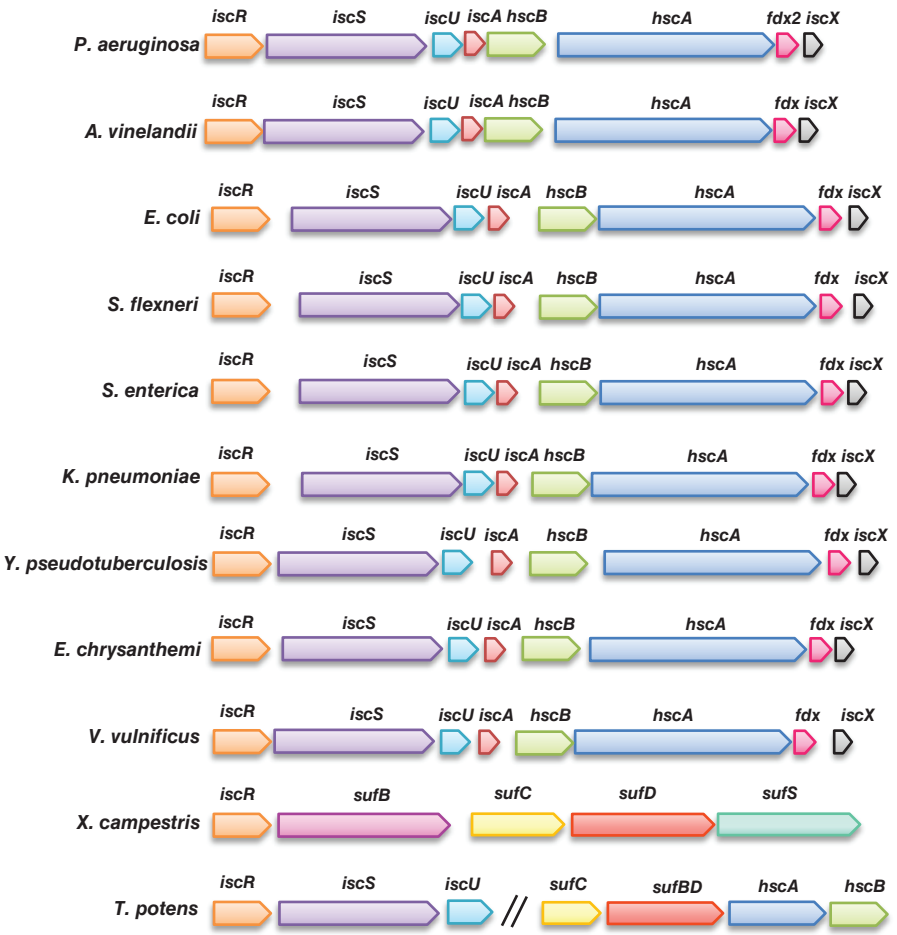


Figure 19.3.1 Organization of the genes encoding Fe-S biogenesis proteins in several bacterial genomes. The double lines separating the two gene clusters in *T. potens* indicate that these clusters are encoded at different positions in the genome.

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 (ATASYYGACTRWWWYAGTCRRSTAT) and type 2 (AWARCCCYTSNGTTTGMNGKKKTKWA) (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.*,

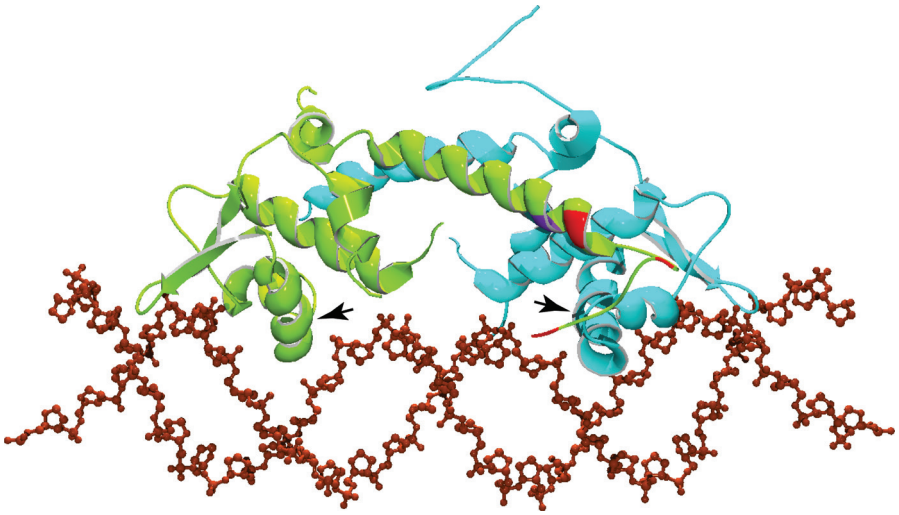


Figure 19.3.2 The crystal structure of *E. coli* Apo–IscR, carrying Ala substitutions at Cys92, Cys98, Cys104, and His107, bound to DNA. Monomers of the IscR dimer are green and blue. Arrows indicate the DNA-binding helices. In the green monomer, positions corresponding to the Fe-S cluster coordinating residues Cys92, Cys98, and Cys104 are red, with the position of His107 indicated in purple. The sugar–phosphate backbone of the DNA is brown. The PDB ID for *E. coli* IscR is 4hf1.

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*-operon promoter (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 *Enterobacteriacea*, 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-Janecky *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* IscR 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

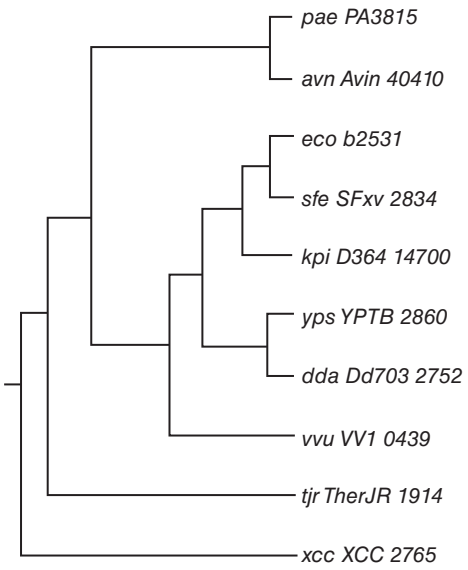


Figure 19.3.3 Neighbor-joining phylogenetic analysis of the deduced amino acids sequences of IscR from the bacteria shown in Figure 19.3.1. The cladogram was prepared using the online Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis tool, Cladogram. The default parameters of the program were used.

S104 functions as the fourth iron ligand in these organisms (Santos *et al*., 2015). Figure 19.3.4 shows that residues proposed to constitute the DNA–IscR interface, such as Ser40, Glu43, Gln44, Arg59, and Gly60, are also conserved (Santos *et al*., 2015), with the exceptions of Glu43, which prevents apo-IscR from binding to the type 1 binding sites (Giel *et al*., 2013; Rajagopalan *et al*., 2013), as well as Ser40 and Gln44, which are absent in *X. campestris* IscR.

The second gene in the operon, *iscS*, encoding cysteine desulfurase, contains the sequence motif, SSGSAC(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 ThiI 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.

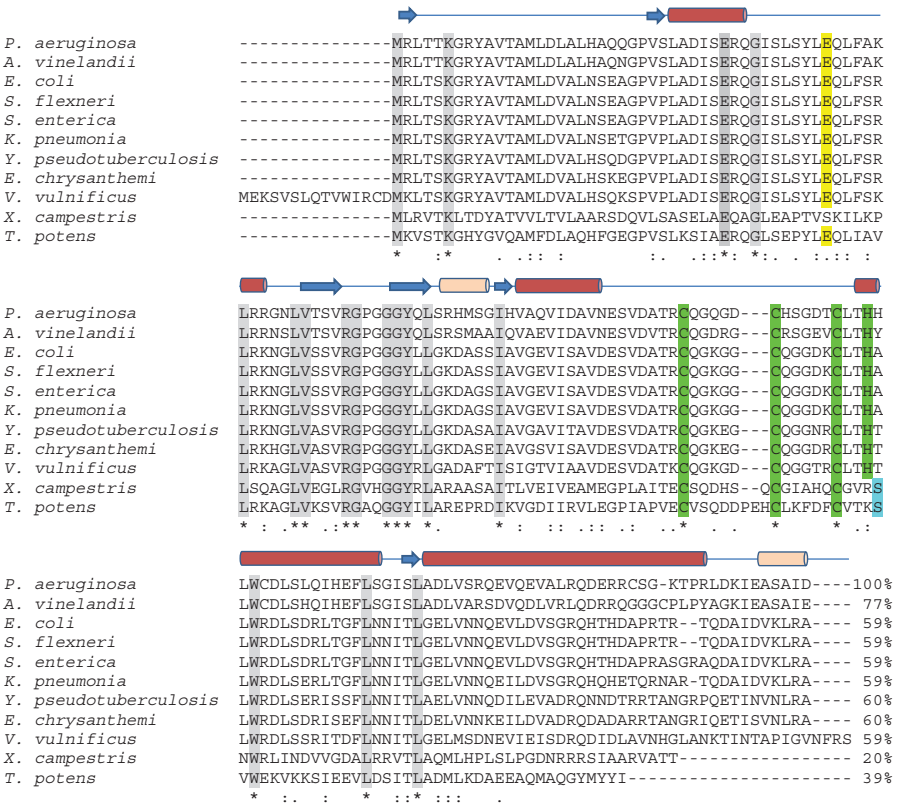


Figure 19.3.4 The ClustalW multiple alignment of the deduced amino acid sequences of the IscR proteins in Figure 19.3.1. Multiple alignment of the amino acid sequences was performed using the ClustalW program linked from the online Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis tools. Conserved cysteine and histidine residues for Fe-S ligation (green), the conserved glutamic acid in DNA binding (yellow), nonconserved serine residues (blue), and strictly conserved amino acids (gray) are highlighted.

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-$2S^{2+}$ clusters to form a single 4Fe-$4S^{2+}$ 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; Zimbler *et al*., 2012). In *E. coli*, NfuA is required for survival under oxidative stress and during iron limitation (Angelini *et al*., 2008; Zimbler *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).

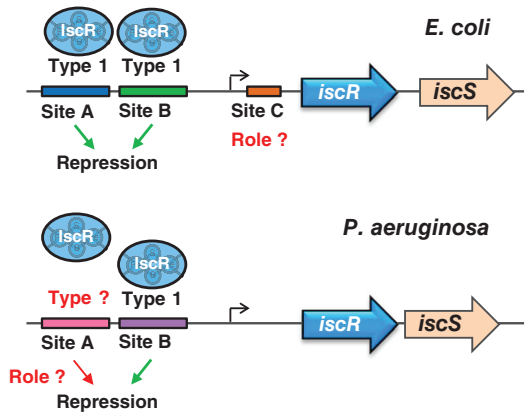
Bacterial frataxin (CyaY) is involved in the donation of the iron ions during Fe-S cluster assembly (Py and Barras, 2010; Roche *et al*., 2013). Finally, YggX, also an iron-binding protein, has been suggested to function in Fe-S cluster repair in *E. coli* due to the observation that, under exposure 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). CyaA 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 *isc* 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 H_2O_2 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 (Δ*iscR*) 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 cite (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′AATCCTGAGTAATTTGATCGGTCTT3′), between positions −43 and −67, and site B (5′ATAGTTGACCTAAT TACTCGGATAA3′), spanning positions −18 to −42 relative to the *iscR* transcription start (Romsang *et al*., 2014). These two



E. coli	**Type 1**	**ATASYYGACTRWWWYAGTCRRSTAT**
E. coli	**Type 2**	**AWARCCCYTSNGTTTGMNGKKKTKWA**
E. coli	P_{iscR} (A)	ATACCCGACTAAATCAGTCAAGTAAA
E. coli	P_{iscR} (B)	ATAGTTGACCAATTTACTCGGGAATG
P. aeruginosa	P_{iscR} (A)	AATCCTGAGTAATTTGATCGGTCTTA
P. aeruginosa	P_{iscR} (B)	ATAGTTGACCTAATTACTCGGATAAG

Figure 19.3.5 Comparison of the IscR binding site organization and sequences in the *E. coli* and *P. aeruginosa iscR* promoters. Type 1 and type 2 IscR binding sites are indicated by boxes. The relative positions of the transcription starts are indicated by bent arrows. A comparison of the sequences of each of the IscR binding sites is shown along with the consensus sequences for the *E. coli* IscR type 1 and type 2 binding sites. Consensus sequences are expressed using base notations that are consistent with the rules of the International Union of Pure and Applied Chemistry (IUPAC): A (adenine), C (cytosine), G (guanine), T (thymine), R (A or G), Y (T or C), K (T or G), M (A or C), S (C or G), W (A or T), B (all but A), D (all but C), H (all but G), V (all but T), and N (any).

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* Δ*iscR* 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 *iscR* 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 holo-form and is responsible for *iscR* 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 *in vivo* 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 iscR* 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* Δ*iscR* mutant strains expressing IscR mutants carrying single alanine substitutions at Cys92, Cys98, Cys104, or His107 (Romsang *et al.*, 2014). The C92A and H107A *iscR* mutants failed to repress transcription of the *isc* operon in the Δ*iscR* background, while the IscR-C98A and IscR-C104A partially restored repression relative to wild-type IscR. Moreover, oxidant treatment of Δ*iscR* strains 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 holo-forms 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 Mongkolsuk, 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 *flu* 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-$4S^{1+}$ cluster (Djaman *et al*., 2004; Imlay, 2006) is metastable and rapidly degrades, as shown in Reactions 1 and 2 (Djaman *et al*., 2004):

Reaction 1: $Ox + 4Fe\text{-}4S^{2+} \rightarrow Red + 4Fe\text{-}4S^{3+}$
Reaction 2: $4Fe\text{-}4S^{3+} \rightarrow 3Fe\text{-}4S^{1+} + Fe^{2+}$

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_2O_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 Δ*iscR* mutant (Fuangthong *et al*., 2015; Lim *et al*., 2014; Romsang *et al*., 2014).

The observed H_2O_2 sensitivity of the Δ*iscR* mutant is due, at least in part, to reductions in the levels and activities of H_2O_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 paraquat is unknown. There are several possible pathways for toxicity. These drugs generate superoxide anion ($O_2^{\bullet-}$) 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* Δ*iscR* mutant to redox-cycling drugs does not appear to be due to $O_2^{\bullet-}$ 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 I 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_2O_2 to H_2O using reducing equivalents supplied via glutaredoxin 3 (Grx3) and

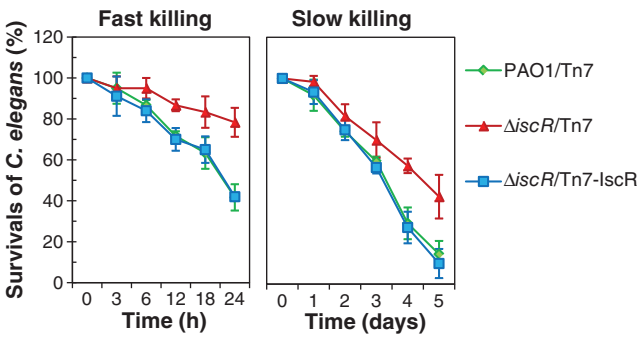


Figure 19.3.6 Effect of *iscR* deletion on *P. aeruginosa* virulence using *a Caenorhabditis elegans* host model system. The results of fast- and slow-killing assays are shown between *P. aeruginosa* wild-type PAO1 and the Δ*iscR* mutant carrying Tn7 genomic insertions as well as the Δ*iscR* mutant carrying a Tn7 genomic insertion containing an *iscR* expression cassette. The worms were scored as live or dead after 3, 5, 7, and 9 h for fast killing and 1, 2, 3, and 4 days for slow killing. The data presented are means and standard deviations of three biologically independent experiments.

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 as a lawn on rich media (Cezairliyan *et al*., 2013). When grown on minimal media, killing occurs more slowly due to the buildup of cyanide that is generated by the bacteria during growth in the worm gut (Cezairliyan *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* Δ*iscR* 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 Δ*iscR* 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 Δ*iscR* 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 (Cezairliyan *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 Δ*iscR* 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 (Fe^{2+}), which has the potential to react with H_2O_2 and catalyze the formation of cell-damaging ROS, particularly the highly reactive hydroxyl radical ($OH^{\bullet}$) via the Fenton reaction (see Section 10):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-} \text{ (Fenton reaction)}$$

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'Autréaux and Toledano, 2007). This is reflected by the fact that the expression of genes encoding iron-binding proteins such as *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 ferritins 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 *sufABCDSE* (*suf*) operon in response to peroxide stress. Activation also required integration host factor (IHF) and apo-IscR (Outten *et al*., 2004). Induction of the *suf* 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 *iscR* shifts cells into an apparent iron-starved state. For example, a *P. aeruginosa* Δ*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, *iscR* overexpression reduced siderophore levels. Complementation experiments using wild-type *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 Δ*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* Δ*iscR* mutant exhibits a reduced intracellular iron pool and is more sensitive to H_2O_2 due to a posttranslational reduction in KatA catalase activity (Kim *et al*., 2009). Catalase is a heme-containing enzyme that detoxifies H_2O_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 Δ*iscR* 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 H_2O_2 sensitivity and increased catalase activity in the Δ*iscR* 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 Δ*iscR* 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 (Angelini *et al*., 2008; Outten *et al*., 2004), which interferes 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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