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Metal-responsive RNA polymerase extracytoplasmic function (ECF) sigma factors

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Summary

Bacteria must adapt to fluctuations in their environment to survive. One of the most interesting challenges they must cope with is changes in metal concentrations. Many metals are essential for viability, since they act as cofactors of indispensable enzymes. But on the other hand, they are potentially toxic because they generate reactive oxygen species or displace other metals from proteins, turning them inactive. This dual effect of metals forces cells to maintain homeostasis by using a variety of systems to import and export them. These systems are usually inducible, and their expression is regulated by metal sensors and signal-transduction mechanisms, one of which is mediated by extracytoplasmic function (ECF) sigma factors. In this review we have focused on the metal-responsive ECF sigma factors, several of which are activated by iron depletion (FecI, FpvI, and PvdS), while others are activated by excess of metals such as nickel and cobalt (CnrH), copper (CarQ and CorE), or cadmium and zinc (CorE2). We focus particularly on their physiological roles, mechanisms of action and signal-transduction pathways.

Due to their exposure to a variable environment, bacteria have developed several adaptive mechanisms to rapidly respond to changes in their habitat and thereby increase their chances of survival. For instance, bacteria must adapt to the presence of metals, as they are required as cofactors of many enzymes. Iron is thought to be one of the first metals used as a cofactor for enzymatic reactions, and nowadays is an essential metal for almost all living organisms, with a few exceptions such as Lactobacilli and *Borrelia* (Posey and Gherardini, 2000). Iron-containing proteins are not only excellent electron carriers (81% of the oxidoreductases use this metal to transfer electrons) (Waldron *et al*., 2009), but they also participate in enzyme catalysis and regulate gene expression as they function as sensors for environmental or intracellular signals (Lill, 2009). Additionally, iron plays a central role in host-pathogen interplay, and the fight between host cells and intercellular or intracellular pathogens for this essential metal will influence the outcome of infectious diseases in favour of either the host or the pathogenic invaders (Nairz *et al*., 2010). When the atmosphere became oxygenated, iron became insoluble and the preference of many enzymes shifted to more bioavailable metals such as copper and zinc, which also became essential cofactors for many organisms (Dupont *et al*., 2010; Festa and Thiele, 2011). Other metals such as nickel are essential for a limited number of proteins, including glyoxalases, ureases, hydrogenases and some superoxide dismutases (Hausinger and Zamble, 2007; Boer *et al*., 2014), while cobalt has no clear physiological role except as a component of several cobalamins and as a cofactor of a few noncorrin-cobalt-containing enzymes (Kobayashi and Shimizu, 1999; Barras and Fontecave, 2011). In contrast, cadmium is not required for any biological function so its presence in the cell, even at low concentrations, is considered toxic (Moulis, 2010).

Nevertheless, all of the abovementioned metals are potentially toxic, and high concentrations can lead to the generation of hydroxyl radicals through Fenton or Fenton-

like reactions and singlet oxygen, damage to DNA and cell membranes by generation or enhancement of oxidative stress, inhibition of enzymes with histidine or cysteine residues in their active sites, and replacement of other metal cofactors on several metalloproteins (Waldron *et al*., 2009; Moraleda-Muñoz *et al*., 2010a; Moraleda-Muñoz *et al*., 2010b; Macomber and Hausinger, 2011; Rensing and McDevitt, 2013; Chandrangsu *et al*., 2017; Cheng *et al*., 2018; Pérez *et al*., 2018; Grosse *et al*., 2019). This capacity to replace other metals is especially important in the case of copper, since it occupies the highest position in the Irving-Williams series ($Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$) and can potentially substitute any other biological metal used as a cofactor inside the cell (Irving and Williams, 1953). It is therefore critical for cells to maintain metal homeostasis both to ensure an adequate supply for their metal requirements and to avoid and alleviate the toxicity of metals. Bacteria have thereby developed a very diverse set of mechanisms for acquiring metals, which are often scarce in their natural habitats, and for exporting and detoxifying them when they reach high concentrations.

Although iron is abundant in natural terrestrial and aquatic niches, under physiological conditions free iron levels are frequently very low. The ferrous form is only soluble in anoxic environments and the presence of oxygen prompts its rapid oxidation to Fe^{3+}, which is poorly soluble. Because of this, bacteria have developed complex strategies to acquire this important metal, including the reduction of ferric to ferrous ions, uptake of iron in the form of exogenous iron chelators such as Fe^{3+}-citrate (Silva *et al*., 2009) or siderophores (compounds that complex the ferric ions) such as pyoverdine (Koster *et al*., 1994), and direct acquisition of iron from other organisms' iron-binding proteins, such as iron- and heme-carrier proteins and hemophores (Wandersman and Delepelaire, 2004).

Because of their high solubility, most metal homeostasis mechanisms for cobalt, nickel, copper, zinc and cadmium are focused on their detoxification rather than on their

acquisition. These mechanisms include exporting metals by P_{1B}-type ATPases, transenvelope CBA transporters and cation diffusion facilitators (CDF), metal oxidation to a less toxic form (as with multicopper oxidases, which turn Cu^{+} into Cu^{2+}), or binding metals to metallochaperones (Sánchez-Sutil *et al*., 2007; Moraleda-Muñoz *et al*., 2010a; Moraleda-Muñoz *et al*., 2010b; Rensing and McDevitt, 2013; Pérez *et al*., 2018; Grosse *et al*., 2019).

These adaptive mechanisms are regulated by several transcription factors and signaling systems that have been traditionally classified in the four pillars of bacterial signal-transduction mechanisms: one-component systems, two-component systems, extracytoplasmic function (ECF) sigma factors, and serine-threonine protein kinases (Staroń *et al*., 2009; Muñoz-Dorado *et al*., 2012).

ECF sigma factors represent group IV of the σ^{70} family of sigma factors, which are directly involved in the transcription process by recognizing the -10 and -35 promoter sequences and, together with the core RNA polymerase (RNAP) enzyme, are responsible for initiating the transcription of the genes they regulate (Helmann, 2002; Gruber and Gross, 2003; Mooney *et al*., 2005; Lee *et al*., 2013). This promoter recognition will only happen after their specific stimulus has been detected (Nies, 2004; Staroń *et al*., 2009; Mascher, 2013; Pinto and Mascher, 2016). They are smaller than other sigma factors, and they only contain the σ2 and σ4 domains (Lonetto *et al*., 1994; Helmann, 2002). Canonical ECF sigma factors are regulated by anti-sigma factors. Anti-sigma factors are usually membrane proteins with a high affinity for their cognate sigma factor, so in the absence of the specific stimulus, the sigma factor is sequestered by the anti-sigma factor. Anti-sigma factors usually act as the sensor part of this signal-transduction system, and upon detection of the stimulus, they release the sigma factors. ECF sigma factors and anti-sigma factors are usually co-transcribed to ensure that no sigma factor is released in the

absence of an appropriate stimulus (Missiakas and Raina, 1998; Ho and Ellermeier, 2012; Muñoz-Dorado *et al*., 2012). However, not all ECF sigma factors function in the same manner. A phylogenetic classification of the ECF sigma factors into 94 different groups has shed some light on the diversity of mechanisms regulating their activity and the stimuli detected (Staroń *et al*., 2009; Mascher, 2013; Pinto and Mascher, 2016).

Metals such as iron, nickel, cobalt, copper, zinc, and cadmium activate a number of ECF sigma factors to trigger the specific response in the bacteria to keep metal homeostasis. In this review we will discuss the signaling mechanisms of the best characterized ECF sigma factors involved in the regulation of metal homeostasis genes, covering the iron uptake regulator systems FecI, FpvI and PvdS, the cobalt and nickel resistance regulator system CnrH, the copper-responsive regulator CarQ involved in the biosynthesis of carotenoids, and the CorE-like sigma factors involved in copper, zinc and cadmium resistance.

Iron starvation-responsive ECF sigma factors

In most cases, iron acquisition genes (normally forming operons) are only expressed under conditions of metal deficiency. In the presence of iron, these operons are repressed by the regulator Fur, which forms complexes with ferrous iron and binds to conserved sites of DNA termed Fur boxes, preventing the transcription of iron-uptake genes (Figure 1A) (Baichoo and Helmann, 2002; Cornelis *et al*., 2009; Fillat, 2014). Under iron-depletion, the regulator does not bind the metal, thus preventing Fur from binding to DNA, and transcription of the genes involved in iron acquisition often occurs under the control of transcriptional activators. Several iron supply systems are positively regulated by ECF sigma factors induced by the availability of specific iron sources (Sexton *et al*.,

1996; Leoni *et al*., 2000; Biville *et al*., 2004; Lindeberg *et al*., 2008; Thakur *et al*., 2013; Chevalier *et al*., 2018; Lang *et al*., 2018).

Most iron starvation-responsive ECF sigma factors are regulated by a cell surface signaling (CSS) that transmits the input signal from a substrate-bound outer membrane protein (OMP) across the periplasmic space to a cytoplasmic membrane-spanning anti-sigma factor, releasing the cytoplasmic ECF sigma factor that transcriptionally regulates the expression of the stimulus-responsive genes (Braun *et al*., 2006; Brooks and Buchanan, 2008; Llamas *et al*., 2014). CSS was first described in the transport of ferripyoverdine in *Pseudomonas putida* WCS358 (Koster *et al*., 1994) and in the Fe^{3+}-citrate transport system in *E. coli* (Härle *et al*., 1995). These iron starvation-responsive ECF sigma factors belong to groups ECF05-ECF09 (Staroń *et al*., 2009; Mascher, 2013; Pinto and Mascher, 2016), and the best characterized FecI-like sigma factors, FecI from *Escherichia coli*, and PvdS and FpvI from *Pseudomonas aeruginosa*, are described below.

1. The *Escherichia coli* Fec signaling pathway

The Fe^{3+}-citrate system is regulated by the anti-sigma/ECF sigma factor pair FecR/FecI via CSS (Angerer *et al*., 1995; Härle *et al.* 1995; Mahren and Braun, 2003), which in the presence of iron is repressed by Fur-Fe^{2+} (Figure 1A). Under conditions of iron depletion and presence of Fe^{3+}-citrate, Fur does not bind the DNA, and the OMP receptor protein FecA has a dual role: it transports the Fe^{3+}-citrate complexes across the outer membrane and is involved in signaling the extracellular presence of these complexes to the genetic machinery in the cytoplasm (Brooks and Buchanan, 2008). The C-terminal portion of FecA forms a beta barrel (with twenty-two anti-parallel beta-strands) that spans the outer membrane, modeling a pore that is occluded by a plug domain that prevents the unspecific diffusion of large molecules. The plug must undergo conformational changes to allow the

opening of the pore to facilitate transport. Fe^{3+}-citrate is first adsorbed from the medium by aromatic residues located in the external pocket of FecA and from there it is transferred to its high-affinity binding site, which is formed mainly of several arginine residues that bind the negatively charged ferric citrate (Ferguson *et al*., 2002). Binding of Fe^{3+}-citrate to FecA provokes structural changes in two extracellular loops involved in the iron complex transport (Ferguson *et al*., 2002; Yue *et al*., 2003) and in a small loop in the plug domain that seems to play a role in the transmission of the signal to TonB (Buchanan *et al*., 1999; Brooks and Buchanan, 2008). FecA interacts with TonB through the TonB box domain (which also changes its conformation by Fe^{3+}-citrate binding) and it is able to transport iron through the periplasmic FecB protein and the ABC transporter FecCDE to the cytoplasm (Staudenmaier *et al*., 1989). In the cytoplasm, a ferric reductase catalyzes the release of Fe^{2+} from the citrate complexes (Miethke et al., 2011), although other enzymes may also be implicated in this process (Miethke and Marahiel, 2007).

FecA belongs to the group of TonB-dependent transporters (TBDT) (Noinaj *et al*., 2010), and receives energy from the complex TonB-ExbB-ExbD, which is anchored to the cytoplasmic membrane and extends into the periplasm. Additionally, the N-terminal domain of FecA interacts with the C-terminal domain of the inner membrane anti-sigma factor FecR, which then releases FecI to recruit the core RNAP and binds to the promoters to initiate transcription of the clusters *fecIR* and *fecABCDE* to accelerate the uptake of Fe^{3+}-citrate (Figure 1B) (Van Hove *et al*., 1990; Wriedt *et al*., 1995; Mahren and Braun, 2003).

The pair FecR/FecI does not function as a canonical anti-sigma/sigma pair because FecR is also required for full FecI activity, probably by inducing the binding of FecI to the core RNAP (Mahren and Braun, 2003). The positive role of FecR on FecI could also be due to the fact that the ECF sigma factor might be unstable in the absence of FecR, and under

these conditions, it would be more susceptible to be degraded by proteolysis, probably by the protease RseP (Braun *et al*., 2006).

Other FecI homologues, such as FiuR/FiuI, FoxR/FoxI, HasS/HasI and FemR/FemI, are present in many bacterial species belonging to the Proteobacteria phylum. Most of them are often clustered with genes coding for a FecA-like OMP and a putative anti-sigma factor containing a FecR-like domain (Sexton *et al*., 1996; Biville *et al*., 2004; Braun *et al*., 2006; Brooks and Buchanan, 2008; Thakur, *et al*., 2013; Llamas *et al*., 2014; Chevalier *et al*., 2018; Lang *et al*., 2018). The great variety of associated domains, organized in almost 200 different architectures according to the Pfam database (El-Gebali *et al*., 2019), seems to indicate that this FecIR system might be a generalized signal-transduction mechanism used to regulate the entry of different types of products (Staroń *et al*., 2009; Karlsson *et al*., 2011; Mascher, 2013; Pinto and Mascher, 2016).

2. Mechanism of action of *Pseudomonas aeruginosa* PvdS and FpvI

The *P. aeruginosa* PAO1 genome encodes 19 ECF sigma factors, 14 of which are regulated by iron starvation and are involved in the expression of TBDTs for siderophores, heme or iron citrate uptake (Llamas *et al*., 2008; Chevalier *et al*., 2018). The best-studied system in this bacterium is the pyoverdine CSS system (Ravel and Cornelis, 2003; Visca *et al*., 2007). Although the pyoverdine signaling pathway is similar to the Fe^{3+}-citrate system described above, it differs in several aspects. First, it responds to the endogenously produced siderophore pyoverdine complexed with Fe^{3+}, as opposed to an exogenous source as in the case of Fe^{3+}-citrate; second, one anti-sigma factor controls two sigma factors (Beare *et al*., 2003); and third, genes involved in this system are not adjacent in the genome, and even the sigma and anti-sigma factors are not co-transcribed (Beare *et al*., 2003; Llamas *et al*., 2014).

This CSS consists of the ferripyoverdine receptor FpvA (energized by the complex TonB-ExbB-ExbD), the anti-sigma factor FpvR, and the two ECF sigma factors FpvI and PvdS, which remain sequestered in the membrane by FpvR in the absence of ferripyoverdine (Figure 2A). In this cascade, the interaction of ferric siderophore with the FpvA binding pocket, formed by at least 14 amino acid (most of them aromatic residues), transmits a signal to TonB that facilitates the import of ferripyoverdine (Schalk *et al*., 1999; Schalk *et al*., 2002; Schalk *et al*., 2004; Cobessi *et al.*, 2005; Wirth *et al*., 2007). The conformational changes in FpvA also result in the proteolytic cleavage of FpvR by the RseP/MucP protease (Visca *et al*., 2007). The subsequent liberation of FpvI and PvdS allows the expression of the target regulons (Figure 2B). The FpvI regulon includes the *fpvA* gene (Beare *et al.,* 2003), some genes involved in pyoverdine biosynthesis (*pvdA*, *pvdIJD*, and *fpvGHIJ*), and other genes such as the heme-uptake transporter *hasR* or the small RNA gene *prrF1* (Ravel and Cornelis, 2003; Schulz *et al*., 2015). PvdS controls the transcription of a regulon of about 80 genes (Ochsner *et al*., 2002; Schulz *et al*., 2015), including genes involved in pyoverdine biosynthesis, secretion and utilization, other virulence genes such as those encoding exotoxin A, the extracellular protease PrpL, and several type III secretion systems that function as toxins, such as ExoT and ExoS (Wilderman *et al*., 2001; Gaines *et al*., 2007). Moreover, PvdS regulates the expression of the sigma factor PA14_21540 (PA3285), the ferrochalatase *hemH*, and other genes involved in cellular functions unrelated to iron uptake (for a review see Chevalier *et al*., 2018).

As happens with most iron-starvation ECF sigma factors, *pvdS* (and *fpvI*) is primarily controlled by Fur (not shown in Figure 2), but its expression is very complex and it is modulated by several environmental signals and different factors, such as regulators related to the response to oxidative stress or sulfur homeostasis. Moreover, *pvdS* is also

under the control of another FecI-like ECF sigma factor, FecI2 (Llamas *et al*., 2014; Chevalier *et al*., 2018). The complexity of the activation mechanism of these ECF sigma factors, and the cross-talk between them and other regulatory elements, such as two-component systems, have been recently reviewed by Chevalier *et al*. (2018).

Nickel- and cobalt-responsive ECF sigma factors

A co-founder of the ECF sigma factor family (Lonetto *et al*., 1994), CnrH (group ECF20), controls nickel and cobalt resistance in *Cupriavidus metallidurans* CH34, an aerobic β-proteobacterium that prevails in heavy metal-rich environments.

The activity of CnrH is regulated by a complex of two transmembrane (TM) proteins: the metal-sensor CnrX and the anti-sigma factor CnrY. At the resting state, CnrH is sequestered at the membrane by CnrY (Figure 3A), whereas binding of nickel or cobalt to CnrX frees CnrH from CnrY, allowing the expression of genes coding for proteins that extrude metals to the periplasm and the exterior (Figure 3B) (Grass *et al*., 2005; Trepreau *et al*., 2011; Maillard *et al*., 2014; Trepreau *et al*., 2014).

The sensor CnrX is a membrane-anchored dimeric protein with a C-terminal periplasmic metal-sensor domain. This portion binds Ni^{2+} or Co^{2+} and discriminates against the other transition metal cations, displaying subtle allosteric modifications depending on the nature of the metal ion (Trepreau *et al*., 2011; Trepreau *et al*., 2014). The affinity of CnrX for Ni^{2+} is 10- to 100-fold higher than its affinity for Co^{2+}, which is consistent with the expression observed in the genes under control of CnrH (Grass *et al*., 2000; Grass *et al*., 2005; Monchy *et al*., 2007; Trepreau *et al*., 2011; Maillard *et al*., 2015). These affinities are also in good agreement with the trend in the Irving-Williams series. Accordingly, the complex CnrYXH plays a minor role in the resistance of *C. metallidurans* to Co^{2+} (Nies *et al*., 2006). At the resting state of this complex, zinc ions bind CnrX in a 3N2O

coordination sphere (formed by His42, His46, Glu63, and His119). However, since nickel and cobalt exhibit a stronger affinity for CnrX, these ions displace the zinc ions at the metal binding site. The antagonistic effect of zinc on nickel and cobalt may be explained by the ability of nickel and cobalt to recruit the only methionine (Met123) of the sensor domain of CnrX as an additional ligand. This residue lies at the bottom of the cavity that harbors the metal ion and is central to the CnrX protomer architecture. Recruitment of Met123 to the coordination sphere of the metal results in a dramatic change in the geometry of the metal-binding site that remodels the four-helix bundle where this residue is located and elicits the biological response (Trepreau *et al*., 2011; Trepreau *et al*., 2014; Maillard *et al*, 2015).

After metal sensing in the periplasm, signal propagation proceeds through a modulation of the CnrX–CnrY interaction. On the periplasmic side, the C-terminal portion of the anti-sigma factor CnrY is docked in the hydrophobic cavity of the CnrX dimer facing the membrane. Within the membrane, interactions between the TM helices would be sensitive to any movement depending on the metal status of CnrX, affecting the interaction between the CnrX and CnrY TM domains (Figure 3) (Trepeau *et al*., 2011). CnrY is a single-pass TM protein with a 45 amino acid cytoplasmic domain that generates two helices that embrace the σ2 and σ4 domains of CnrH in a closed conformation so that the σ4 domain is buried against the -10 interaction surface of σ2, blocking the CnrH RNAP-binding determinants (Grass *et al*., 2005; Maillard *et al*., 2014; Paget, 2015). CnrY belongs to class II of anti-sigma domains (Grass *et al*., 2005; Maillard *et al*., 2014). Members of this class of anti-sigma factors carry out their function via a short N-terminal cytoplasmic domain that displays helical propensity but no canonical structure on its own (Staroń *et al*., 2009; Campagne *et al*., 2012; Campagne *et al*., 2015; Huang *et al*., 2015).

Genes regulated by the CnrYXH complex are part of the cobalt-nickel resistance (*cnr*) determinant *cnrYXHCBAT* borne in the megaplasmid pMOL28 of *C. metallidurans* CH34, consisting of two operons: *cnrCBAT* and *cnrYXH*. *cnrCBAT* is under control of the *cnrCp* promoter and encodes the transenvelope heavy-metal efflux pump complex CnrCBA and the inner membrane exporter CnrT. At high concentrations of nickel, this metal is removed by CnrT from the cytoplasm to the periplasm, and from there to the exterior by CnrCBA (Figure 3). On the other hand, *cnrYXH* is under the control of the *cnrYp* promoter and encodes the signal-transduction system that regulates the expression of *cnrCBAT* (Liesegang *et al*., 1993; Grass *et al*., 2000; Grass *et al*., 2005; Monchy *et al*., 2007).

In addition to CnrH, the adaptive mechanism to nickel and cobalt in *C. metallidurans* is also regulated by two other ECF sigma factors, RpoE and RpoP. However, they do not respond to metals and their role covers the maintenance of cell wall integrity and the repair of nickel- and cobalt-mediated damage (Grass *et al*., 2000; Tibazarwa *et al*., 2000; Grass *et al*., 2005; Grosse *et al*., 2019).

Copper-, cadmium- and zinc-responsive ECF sigma factors

Whereas in most bacteria the expression of copper, cadmium and zinc resistance mechanisms is under control of one- and two-component systems, in the case of the myxobacterium *Myxococcus xanthus* three ECF sigma factors have been found to be involved in the adaptive mechanism to toxic concentrations of these metals. One of them is CarQ, an ECF sigma factor that responds to light and copper and regulates the biosynthesis of carotenoids. The others are members of the group ECF44, which respond to copper, zinc and/or cadmium, and are known as CorE-like sigma factors.

1. **Mechanism of action of CarQ**

M. xanthus is able to synthesize carotenoids in response to light and copper (Pérez *et al*., 2018) to quench singlet oxygen and other reactive oxygen species that are generated by these two environmental stimuli (Ziegelhoffer and Donohue, 2009). Genes involved in the synthesis of carotenoids are located in the operon *carB*, which contains six genes, and the gene *crtIb* (Figure 4). Expression of these seven genes is directly or indirectly regulated by the ECF sigma factor CarQ, which in the dark and in the absence of copper is sequestered in the membrane by the anti-sigma factor CarR (Figure 4A) (Gorham *et al*., 1996). Copper and light act at different levels to inactivate CarR and release CarQ. Light is indirectly sensed through CarF, which mediates signaling by the singlet oxygen generated via photoexcited protoporphyrin IX (Fontes *et al*., 2003; Elías-Arnanz *et al*., 2011; Galbis-Martínez *et al*., 2012). In this condition, CarF acts as an anti-anti-sigma factor to inactivate CarR by an unknown mechanism and release CarQ (Figure 4B). In contrast, copper does not require CarF to release CarQ (Figure 4B) (Moraleda-Muñoz *et al*., 2005), although the mechanism by which CarR is inactivated by this metal remains to be elucidated. Once CarQ is released, the signal-transduction pathway triggered is the same for both stimuli, which eventually up-regulates the expression of *carB* (which was repressed by CarA and CarH, both encoded in the *carA* operon, consisting of five genes) and *crtIb* (directly regulated by CarQ) to synthesize carotenoids (see Figure 4B for details) (Moraleda-Muñoz *et al*., 2005; Elías-Arnanz *et al*., 2011; Pérez *et al*., 2018).

Although the CarR/CarQ pair mainly functions as a canonical anti-sigma/sigma pair, one peculiarity is that CarQ requires additional proteins to bind to the promoters: IhfA and the CarD-CarG pair to bind to P*carQRS* (P_{QRS} in Figure 4), and only CarD-CarG to bind to P*crtIb* (P_I in Figure 4). IhfA is the α subunit of the integration host factor, which seems to be an essential architectural element of the appropriated macromolecular complex at the *carQRS* promoter (Moreno *et al*., 2001). CarD is a DNA-binding architectural factor

with similarities to the eukaryotic high mobility group A proteins (Padmanabhan *et al*., 2001). CarD always functions in conjunction with CarG, a zinc-binding protein that regulates gene expression without binding to DNA. Instead, CarG interacts with the N-terminal domain of CarD to function as a transcriptional regulatory unit (Peñalver-Mellado *et al*., 2006). Interestingly, the CarD-CarG complex is also required for proper activity by other *M. xanthus* ECF sigma factors, none of which are known to respond to metals (Abellón-Ruiz *et al*., 2014).

2. Mechanism of action of the CorE-like sigma factors

CorE-like sigma factors (group ECF44) represent the best understood sigma factors regulated by C-terminal extensions (Mascher, 2013; Pinto and Mascher, 2016). This mechanism provides a mode of action independent of an anti-sigma factor, where the sensor domain responsible for triggering the response is part of the DNA-binding sigma factor. Therefore, only one protein participates in this adaptive mechanism, whose activity is directly modulated by metals.

Even though only two members of the CorE-like sigma factors have been characterized, 67 sigma factors have been predicted to fall within this group, with most of them encoded in a metal-related genetic environment (Marcos-Torres *et al*., 2016). These metal-sensing ECF sigma factors can be identified because they contain two highly conserved regions: a CxC motif, located between the σ2 and σ4 domains, and a C-terminal cysteine rich domain (CRD). Cysteines present in both regions are predicted to coordinate the metal, thus determining its activation state (Gómez-Santos *et al*., 2011a; Marcos-Torres *et al*., 2016; Pérez *et al*., 2018).

Both characterized CorE-like sigma factors belong to the model myxobacterium *M. xanthus*: the copper-regulated ECF sigma factor CorE (Gómez-Santos *et al*., 2011a) and

its homologue CorE2, which is regulated by cadmium and zinc (Marcos-Torres *et al*., 2016; Pérez *et al*., 2018).

Although CorE and CorE2 are highly similar in sequence, they exhibit significant differences. CorE is involved in the immediate response to copper in *M. xanthus*, regulating the expression of the *corE* gene itself, genes for the P_{1B}-type ATPases CopA and CopB, and the multicopper oxidase CuoB (Sánchez-Sutil *et al*., 2007; Moraleda-Muñoz *et al*., 2010b; Gómez-Santos *et al*., 2011a). Genes regulated by CorE exhibit a characteristic expression profile, in which expression levels rapidly increase after copper addition, reaching a peak at 2 h. Thereafter, expression rapidly decreases to basal levels in spite of the fact that copper is still present in the medium (Gómez-Santos *et al*., 2011a). The use of chelators of Cu^{+}, reducing agents, and metals that mimic Cu^{+} and Cu^{2+} has revealed that this quick on/off molecular switch is caused by the ability of CorE to distinguish between the two oxidation states of copper, with Cu^{2+} acting as an activator and Cu^{+} as an inactivator (Figure 5) (Gómez-Santos *et al*., 2011a). Therefore, the resting state of CorE would be in the absence of copper (Figure 5A) and in the presence of Cu^{+} (Figure 5C), while it will be activated only in the presence of Cu^{2+} (Figure 5B), which explains the typical expression profile of the genes regulated by CorE.

In contrast, CorE2 is activated only in the presence of cadmium and zinc, and genes regulated by this sigma factor, among them a CDF and a hypothetical protein with a glyoxal oxidase domain (Marcos-Torres *et al*., 2016; Pérez *et al*., 2018), exhibit a different expression profile, in which expression rapidly increases after the metal addition, reaching a maximum. Expression levels remain nearly constant thereafter (Marcos-Torres *et al*., 2016). The difference between the expression profile exhibited by genes regulated by CorE2 and that of genes regulated by CorE seems to be related to the fact that cadmium and zinc are divalent metals with only one oxidation state. Therefore,

the resting state of CorE2 will be in the absence of cadmium and zinc, while it will be active when bound to either of these metals (similar to the situation depicted in Figure 5A and B, respectively, for CorE).

CorE and CorE2 share features that may be common to all members of the group ECF44 that differ from the canonical ECF sigma factors. One of those common traits is that they are not completely auto-regulated. Even though there is some auto-regulation in the case of *corE* expression, genes for both regulators have metal-independent expression patterns (Gómez-Santos *et al*., 2011a; Marcos-Torres *et al*., 2016). Another common feature of CorE-like sigma factors is the absence of an anti-sigma factor that modulates their activity. Instead, the activation and inactivation of these sigma factors is controlled by the CxC motif and the CRD located at the carboxyl terminus. These two motifs are reminiscent of those present in the ZAS domains of the zinc-binding anti-sigma factors responsible for redox sensing (Jung *et al*., 2011; Devkota *et al*., 2017). However, neither the CRD nor CxC regions act as anti-sigma factors, since deletion of the CRD or substitution of any cysteine in the CxC motif results in inactivation (instead of activation) of the sigma factor, despite keeping the σ2 and σ4 domains present in all ECF sigma factors (Gómez-Santos *et al*., 2011a; Marcos-Torres *et al*., 2016). Therefore, they are essential for activity.

Although the CRD domains of CorE and CorE2 are very similar, investigations into the role of each cysteine in both sigma factors have revealed that only one (Cys189 in CorE and 178 in CorE2) plays the same role in both regulators (Gómez-Santos *et al*., 2011a; Marcos-Torres *et al*., 2016). Interestingly, only one residue of each CRD seems to be determinant for the metal specificity. Thus, the metal affinity of CorE and CorE2 (copper for CorE, and cadmium and zinc for CorE2) could be exchanged just by mutating a single

amino acid of their CRD to the one found in the paralogous regulator (CorE Ala185 into a Cys and CorE2 Cys174 into an Ala) (Marcos-Torres *et al*., 2016).

Whereas the CxC motif is strictly conserved in all ECF44 sigma factors, the length and cysteine distribution of the CRD vary between the different members. Its composition ranges from 21 to 50 residues and it may contain other metal-binding residues such as methionines, aspartates and histidines. In view of the shift in metal recognition discussed previously, the differences in the CRDs of uncharacterized sigma factors in this group suggest that they may sense other metals (Marcos-Torres et al., 2016).

Concluding remarks and future perspectives

Here we have reviewed the mechanisms developed by bacteria to adapt to fluctuations in metal concentrations that are mediated by ECF sigma factors. Interestingly, metal responsive ECF sigma factors share few common traits apart from participating in signal-transduction mechanisms that are activated by the availability of metals and from regulating the expression of genes that modulate metal homeostasis.

Although metal-responsive ECF sigma factors are small proteins that contain two conserved domains, they also exhibit sufficient differences in their sequences to be phylogenetically classified in different groups (Staroń *et al*., 2009; Mascher, 2013; Pinto and Mascher, 2016). Moreover, while the activity of most of these sigma factors is regulated by an anti-sigma factor, CorE-like sigma factors function in a different manner, as they are not sequestered by an anti-sigma factor and require the presence of a metal (even in a specific redox state) to become activated. However, the most striking differences in the mechanism of action of these regulators are found in the proteins that function as metal sensors in each signal-transduction pathway. Although all these sensor proteins can bind metals, their sequences do not exhibit significant similarities. In the

pathways of the iron starvation-responsive ECF sigma factors, the sensor is a TBDT located in the outer membrane. Both TBDTs, FecA and FpvA, bind iron, which is complexed with citrate and pyoverdine, respectively. The residues involved in binding these complexes are different in the two proteins due to the different chemical natures of these chelators. However, both TBDTs share a common domain structure consisting of a 22-stranded β-barrel with an inserted plug domain (Ferguson et al., 2002; Yue et al., 2003; Cobessi et al. 2005). Although the rest of sensors seem to directly bind specific metals, they do not exhibit sequence similarities either. Moreover, in the CnrH pathway, the sensor is an inner membrane protein (CnrX) that interacts with the anti-sigma factor CnrY. In the carotenogenesis pathway, copper seems to directly inactivate the inner membrane anti-sigma factor CarR. And in the CorE-like sigma factors, these loner cytoplasmic proteins directly bind the metal, bypassing the usual multiprotein complexes displayed by the other signaling mechanisms. These differences between sensor domains may be related to the type of metal that each protein recognizes, although the location of the protein sensor may also play a crucial role. For instance, it is known that proteins mainly use cysteines to bind copper in reducing intracellular compartments (cytoplasm), whereas they use methionines and histidines in the oxidizing compartments (periplasm) and the extracellular milieu (Frausto da Silva and Williams, 1991; Davis and O'Halloran, 2008; Sánchez-Sutil et al., 2016).

Metal-responsive ECF sigma factors may also have biotechnological applications. For instance, due to the specificity of several ECF sigma factors, bacteria could be designed to function as metal biosensors. Moreover, metal-responsive promoters recognized by ECF sigma factors could be used to engineer plasmids that allow cheap heterologous gene expression, in a similar manner to how they are already available using promoters that

451 are activated by two-component systems that respond to copper (Gómez-Santos *et al*.,
452 2011b) or to non-metal-responsive ECF sigma factors (Pinto *et al*., 2019).
453
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457
458 **Conflict of interest**
459 The authors declare no conflict of interest.
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Figure legends

Figure 1. The *E. coli* CSS Fe^{3+}-citrate transport is regulated by the FecR/FecI system. A. In the absence of Fe^{3+}-citrate and in the presence of Fe^{2+}-Fur, the repressor binds to the region upstream of the operons *fecIR* and *fecABCDE*. Moreover, the FecR anti-sigma factor sequesters the FecI ECF sigma factor by interaction of the N-terminal region of the anti-sigma factor and the σ4 domain of the ECF sigma factor, preventing transcription of these two operons. **B.** Signaling pathway in the presence of Fe^{3+}-citrate and under low iron availability. The Fur repressor is not bound to Fe^{2+} and cannot bind DNA. The outer membrane protein receptor FecA suffers conformational changes, interacts with TonB through the TonB box domain, and allows the transport of the substrate to the periplasm, and through FecB and the FecCDE transporter to the cytoplasm. FecA changes also allow interaction between the FecA N-terminal region and the C-terminal region of the anti-sigma factor FecR, releasing the ECF sigma factor FecI. This sigma factor can now up-regulate the transcription of *fecIR* and *fecABCDE* after recruiting the core RNAP.

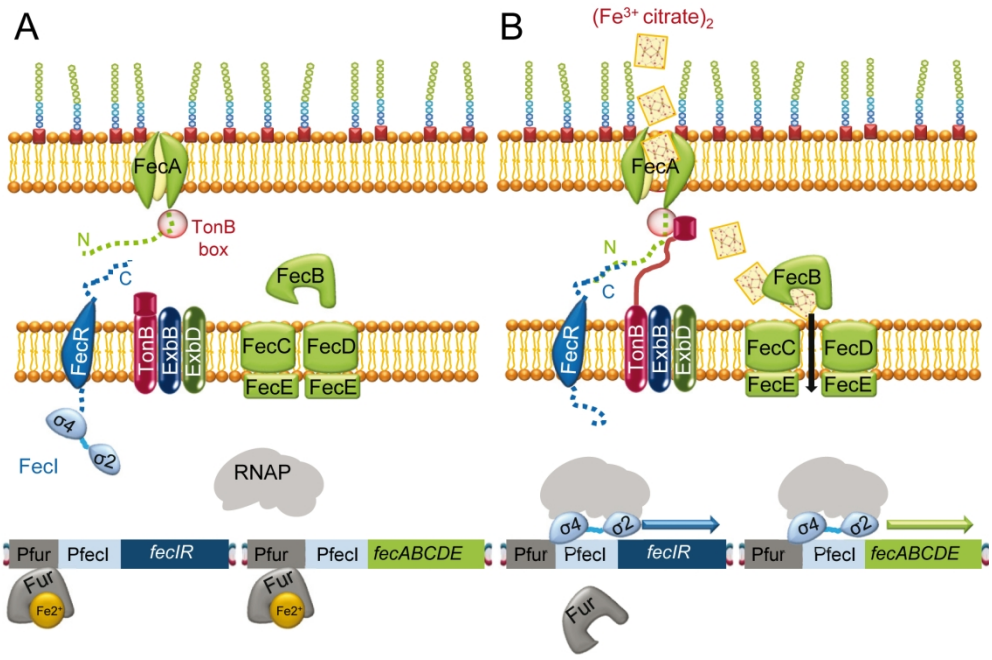
Figure 2. The *P. aeruginosa* ferripyoverdine CSS is regulated by FpvR/FpvI/PvdS. A. In the absence of ferripyoverdine, the anti-sigma factor FpvR sequesters the sigma factors FpvI and PvdS, preventing them from binding to the core RNAP and DNA. The expression of both ECF sigma factors is also repressed by Fur (in a similar way to that shown for FecI in Figure 1 and not shown here). In these conditions, the target regulons for each sigma factor are not transcribed. **B.** When pyoverdine binds to Fe^{3+} to form ferrypioverdine, a signal is transmitted to FpvA and TonB, enabling the import of ferripyoverdine and the proteolysis of FpvR by the RseP/MucP protease, liberating FpvI and PvdS, and allowing expression of the two regulons.

818 **Figure 3. Mechanism of action of the *C. metallidurans* CnrH sigma factor. A.** In the
819 absence of nickel, CnrH is sequestered at the inner membrane by the protein complex
820 CnrYX, where CnrX is an inner membrane protein that possesses a periplasmic metal
821 sensor domain and CnrY is a transmembrane anti-sigma factor. In this condition, CnrY
822 wraps around CnrH and blocks the sites where the beta subunit of the RNAP binds. **B.**
823 Nickel-binding to CnrX results in a modification of the interaction between CnrX and
824 CnrY that provokes a conformational change in CnrY, releasing CnrH to initiate
825 transcription from the promoters *cnrYp* and *cnrCp*. Transcription from these promoters
826 leads to synthesis of CnrH, CnrYX, the transenvelope complex CnrCBA, and the exporter
827 of cytoplasmic nickel ions CnrT.
828 **Figure 4. Mechanism of action of the *M. xanthus* CarQ sigma factor. A.** In the absence
829 of light and copper, the ECF sigma factor CarQ is sequestered at the membrane by the
830 anti-sigma factor CarR. Genes involved in carotenogenesis (located in the gene *crtIb* and
831 the operon *carB*) are not expressed because *crtIb* is regulated by CarQ and the operon
832 *carB* is repressed by CarA and CarH, encoded in the *carA* operon. **B.** Light is sensed via
833 CarF, which is activated by singlet oxygen generated by photoexcited protoporphyrin IX
834 (PPIX). In this condition, CarF functions as an anti-anti-sigma factor, inactivating CarR
835 and releasing CarQ. Copper does not require CarF to inactivate CarR. Free CarQ by any
836 of the two stimuli can bind to two promoters, P_I, with the participation of CarD-CarG, to
837 express the gene *crtIb*, and P_{QRS}, in conjunction with CarD-CarG and IhfA, to express the
838 operon *carQRS*. The operon *carB* can now be expressed after eliminating the repression
839 by CarA and CarH. The repressor CarA is inactivated by CarS, which is encoded in the
840 operon *carQRS*. CarH is a photoreceptor that is directly inactivated by light. Although it
841 is not known how copper inactivates CarH, most likely this metal displaces cobalt in the
842 vitamin B12 used as a cofactor by this repressor, thus allowing transcription of *carB*.

Once *carB* and *crtIb* are expressed, carotenoids are synthesized. Arrows indicate positive regulation and blunt-ended lines indicate negative regulation.

Figure 5. Mechanism of action of the *M. xanthus* CorE sigma factor. A. In the absence of copper, CorE remains inactive. **B.** When copper enters the cell, CorE is activated by binding this metal in its divalent oxidation state, initiating the transcription of genes involved in the immediate response to this metal. The immediate response includes the P_{1B}-type ATPases CopA and CopB, which will extrude copper to the periplasm, and the multicopper oxidase CuoB, which will oxidize Cu^{+} to Cu^{2+} in the periplasm. **C.** Due to the strongly reducing environment of the cytoplasm, Cu^{2+} will be quickly reduced to Cu^{+}, inactivating the ECF sigma factor (presumably via a conformational change), and stopping the immediate response even when copper is still present in the medium.

Abbreviated summary: Bacteria use ECF sigma factors to maintain metal homeostasis. They regulate the expression of genes either to import metals under conditions of metal depletion or to reduce toxicity when metal concentrations are excessive. These adaptive mechanisms can be complex, consisting of several proteins with different cell locations, or very simple, consisting of only the sigma factor capable of sensing the metal and triggering the response.



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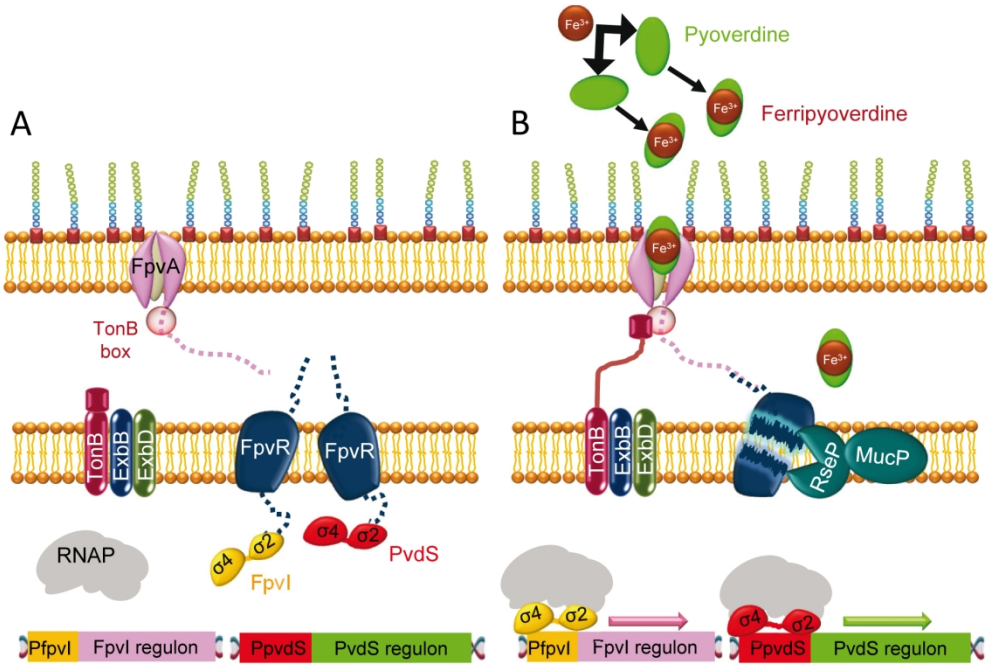

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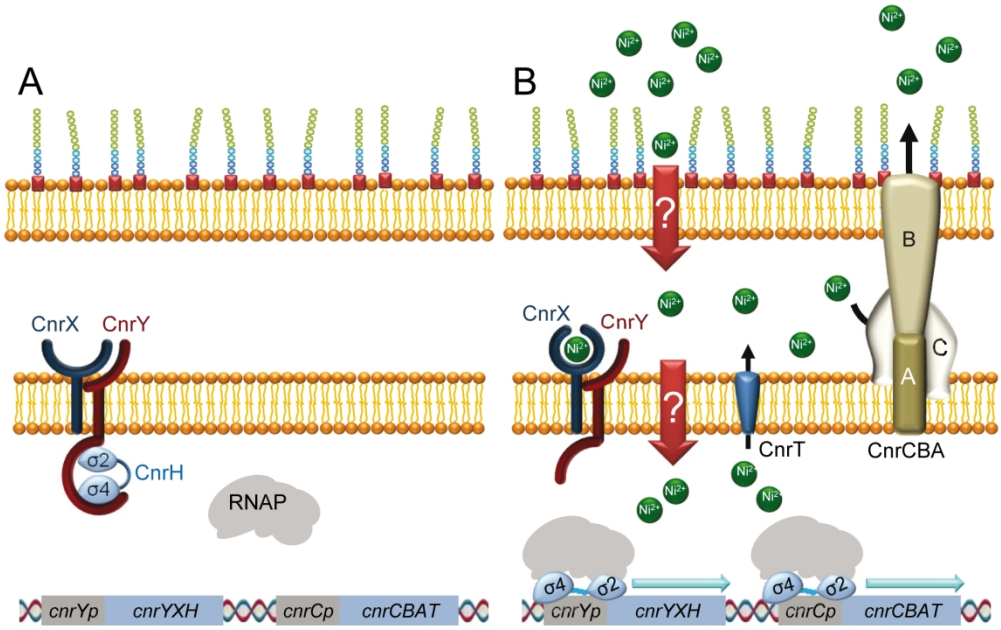

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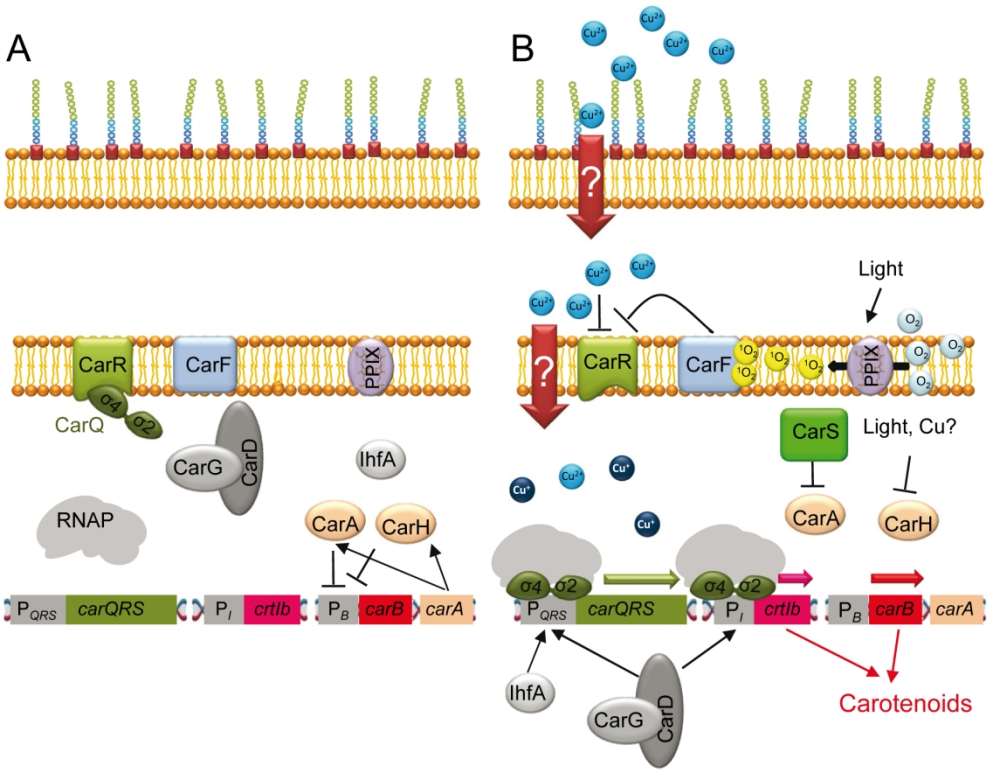

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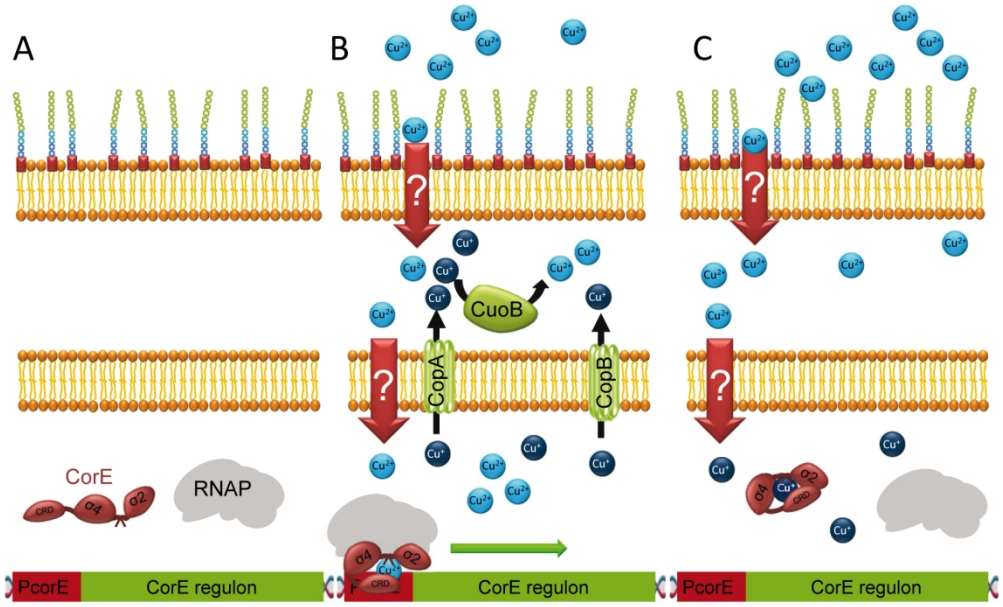

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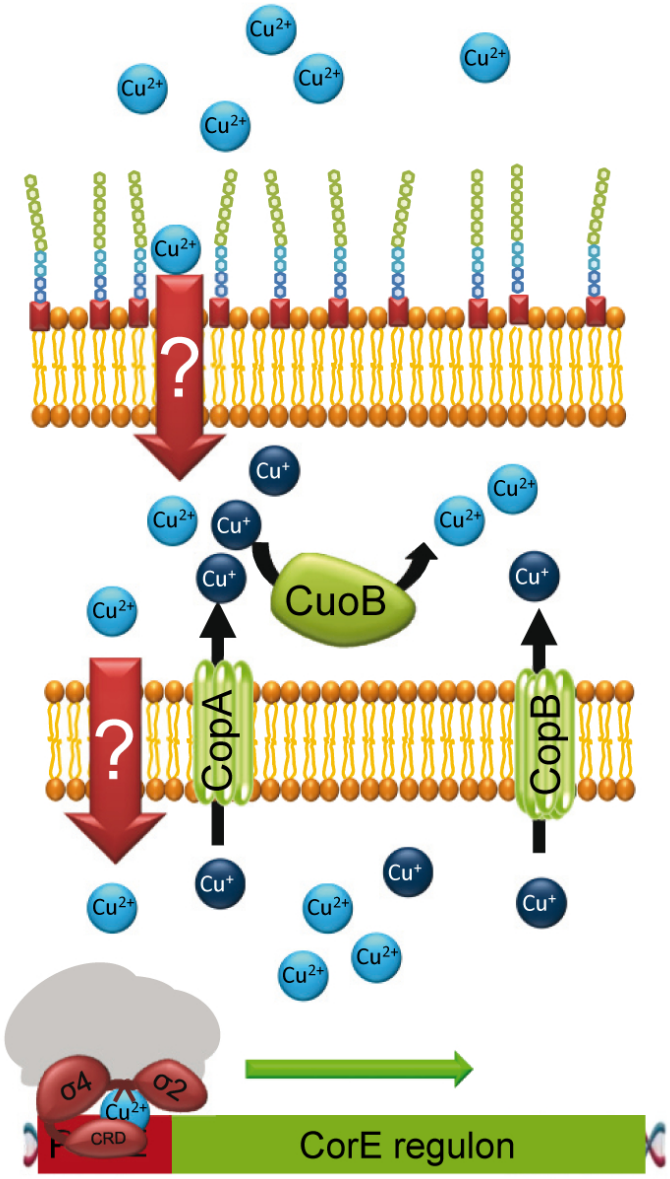

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