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## Iron-Responsive Bacterial Small RNAs: Variations on a Theme

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### Abstract

For most living organisms, iron is both essential and potentially toxic, making the precise maintenance of iron homeostasis necessary for survival. To manage this paradox, bacteria regulate the acquisition, utilization, and storage of iron in response to its availability. The iron-dependent ferric uptake repressor (Fur) often mediates this iron-responsive regulation, by both direct and indirect mechanisms. In 2002, Masse and Gottesman identified a novel target of Fur-mediated regulation in *Escherichia coli*: a gene encoding a small regulatory RNA (sRNA) termed RyhB. Under conditions of iron-limitation, RyhB is produced and functions to regulate the expression of several target genes encoding iron-utilizing enzymes, iron acquisition systems, and iron storage factors. This pivotal finding provided the missing link between environmental iron-limitation and previously observed decreases in certain iron-dependent metabolic pathways, a phenomenon now referred to as an “iron-sparing” response. The discovery of RyhB opened the door to the rapidly expanding field of bacterial iron-regulated sRNAs, which continue to be identified and described in numerous bacterial species. Most striking are findings that the impact of iron-responsive sRNA regulation often extends beyond iron homeostasis, particularly with regard to production of virulence-associated factors by pathogenic bacteria. This review discusses trends in the collective body of work on iron-regulated sRNAs, highlighting both the regulatory mechanisms they utilize to control target gene expression and the impact of this regulation on basic processes controlling bacterial physiology and virulence.

### 1. Introduction

Due to its abundance and broad potential for mediating redox chemistry, iron participates in an array of biological functions and is essential for the survival of most organisms. In anaerobic environments, reduced and soluble ferrous iron is easily attainable by bacteria. In contrast, the insolubility of ferric iron found in aerobic environments limits its bioavailability, presenting a substantial barrier to aerobically growing bacteria that require this element for survival. Pathogenic bacteria face an additional challenge in acquiring iron during the course of an infection. The level of available iron within an animal host is maintained at exceedingly low concentrations by sequestration of the element within iron-binding, host-associated proteins, such as hemoglobin, myoglobin, transferrins and ferritin.<sup>1</sup> To overcome this significant nutritional barrier, microbes have evolved to produce a variety of high-affinity iron uptake systems that are essential for survival and pathogenesis of a broad range of bacterial species.<sup>2</sup>

Despite its essentiality, iron can promote the formation of reactive oxygen species during aerobic metabolism, threatening the survival of the bacterium. To manage their dichotomous relationship with iron, bacterial cells tightly regulate the production and activity of iron uptake and storage systems in response to intra-cellular iron concentrations.<sup>2b</sup> Iron-responsive regulation of bacterial gene regulation is often mediated by Fur (ferric uptake repressor), an iron-dependent transcriptional repressor. Specifically, under iron-replete conditions, ferrated Fur binds in a sequence-specific manner within the promoter region of target genes, efficiently preventing their expression.<sup>3</sup> Under iron-poor conditions, Fur-mediated repression is relieved, thus allowing for the expression of Fur-repressed genes.<sup>4</sup> Fur directly influences the expression of a variety of genes, including those for iron-acquisition and virulence.<sup>5</sup>

Several groups have also observed that Fur mediates an iron-dependent increase in the expression of certain *E. coli* genes, yet the regulatory mechanism underlying this apparent activation remained elusive for several years.<sup>6</sup> In 2002, Masse and Gottesman identified a Fur-regulated sRNA, termed RyhB, and demonstrated that this sRNA was responsible for many of these effects. This seminal finding paved the way for the identification of additionally iron-regulated sRNAs, and also expanded our appreciation for the significant contributions of sRNAs to bacterial physiology and virulence. While *E. coli* RyhB remains the most thoroughly studied example of this growing family of sRNAs, researchers have made large strides in understanding how similar sRNAs in other bacteria contribute to gene regulation and iron homeostasis. These studies have revealed considerable differences in sequence, structure, regulatory mechanisms and targets of these sRNAs, yet some common themes have emerged. A recent review by Salvail and Masse focused on the integral role of RyhB and its homologs in iron homeostasis.<sup>7</sup> Here, we sought to highlight how the variations in function of these RNAs are intimately linked to the lifestyle of the bacteria that encode them. In spite of their diverse roles, each of these sRNAs function to redirect the cell's resources during iron-depleted growth and affect a remarkably broad range of cellular processes.

## 2. The RyhB Paradigm

Appreciation of the significant contribution that iron-responsive sRNAs make to multiple complex regulatory networks began with the characterization of *E. coli* RyhB.<sup>8</sup> Initial characterization of this conserved 90-nucleotide (nt) long sRNA revealed that RyhB functions to coordinate the expression of several genes in response to iron availability. Specifically, under iron-rich conditions, ferric-Fur efficiently represses RyhB production, allowing for increased expression of RyhB-repressed genes (Fig. 1). Under iron-poor conditions, Fur-mediated repression of RyhB is relieved and the sRNA functions to repress the expression of several gene targets. *E. coli* genes subjected to RyhB-dependent repression include those encoding iron storage proteins (*fimA* and *bfr*), enzymes of the tricarboxylic acid cycle (*acnA* and *fumA*), succinate dehydrogenase (*sdh* operon) and superoxide dismutase (*sodB*).<sup>8</sup> Such regulation ensures the production of iron-storage proteins and iron-containing enzymes under iron-replete conditions, when their function is most advantageous to the survival of the bacterium. Conversely, RyhB prevents the production of such factors under iron-poor conditions, when their function is not essential (Fig. 1). By inhibiting the production of iron-binding proteins during growth under iron-limited conditions, RyhB regulation ensures iron is “spared” for use in essential pathways.

Initial investigations into the molecular mechanism underlying RyhB-mediated regulation focused primarily on the regulation of *sodB*, a gene encoding the superoxide dismutase enzyme. These studies demonstrated that efficient RyhB-dependent repression of *sodB* expression requires all of the following: nucleic acid complementarity between RyhB and

the *sodB* transcript, the RNA-binding protein Hfq, and the RNA degrading enzymes RNaseE and RNase III.<sup>8-9</sup> Specifically, nucleic acid complementarity and the activity of Hfq mediate the direct binding of RyhB to the *sodB* transcript.<sup>8-9</sup> Following this specific interaction, the *sodB* mRNA molecule and RyhB are rapidly degraded in a process mediated by RNaseE and RNase III (Fig. 2A).<sup>9a, 9c</sup> Together these studies provided the first detailed evaluation of the molecular mechanism underlying RyhB-dependent repression of a specific gene target.

Subsequent studies of *E. coli* RyhB have revealed additional regulatory targets of the sRNA, many of which appear to be controlled by the regulatory mechanism detailed for the control of *sodB* expression.<sup>10</sup> However, other targets of RyhB-dependent regulation are controlled by unique regulatory mechanisms, summarized in Fig. 2. The impact of RyhB on *E. coli* physiology, as well as the variety of molecular mechanisms by which this regulation is achieved, is described below with the discussion of select targets of RyhB-dependent regulation.

### Indirect regulation of *fur* translation

RyhB-dependent regulation of *fur* expression is mediated by a fascinating, novel molecular mechanism. Weak auto-regulation of *fur* expression allows for a basal level of Fur synthesis during growth of *E. coli* under iron-rich conditions.<sup>11</sup> Under low iron conditions, auto-regulation by the Fur protein is relieved, yet *fur* transcript levels remain relatively unchanged.<sup>9b</sup> This paradoxical observation is explained by the fact that *fur* is co-transcribed with a small open reading frame termed *uof*, and that RyhB binds within this region of the transcript and negatively impacts the stability of this polycistronic message (Fig. 2B).<sup>9b</sup> Such regulation ensures that *E. coli* produces a relatively constant level of Fur during growth under both high and low iron conditions. In addition, these studies provide the first example of indirect translational regulation mediated by a trans-acting bacterial sRNA molecule.

### Activation of *shiA* expression

Studies by Prevost, et al, implicate RyhB in directly activating the expression of *shiA*, a gene encoding a permease for the uptake of shikimate.<sup>12</sup> Shikimate is utilized by *E. coli* for the synthesis siderophores, secreted iron scavenging molecules that facilitate bacterial survival in low iron environments.<sup>13</sup> Specifically, RyhB binds to nucleic acid sequences within the 5' untranslated region of *shiA*, and by doing so prevents the formation of an inhibitory structure that otherwise occludes the ribosomal binding site within the *shiA* transcript (Fig. 2C).<sup>12</sup> Such regulation implicates RyhB in controlling the production of siderophores that promote growth under iron-limiting conditions. Additionally, this study was the first to show direct activation of target gene expression by *E. coli* RyhB.

### Dis-coordinate regulation of genes within the polycistronic *iscRSUA* transcript

Two different studies have shown that RyhB mediates differential control over the expression of genes within the polycistronic *iscRSUA* transcript, encoding machinery required for the synthesis of Fe-S clusters.<sup>10, 14</sup> This unique regulatory mechanism is governed by binding of RyhB to complementary sequences over-lapping the ribosomal binding site of *iscS*, an interaction that results in degradation of the portion of the message encoding the Fe-S cluster synthesis machinery (*iscSUA*, Fig. 2D). The *iscR* portion of the message, encoding a Fe-S responsive transcriptional regulator, is protected from RyhB-dependent degradation by the presence of a strong RNA structure within the transcript. Such regulation prevents the production of Fe-S cluster synthesis machinery in the absence of available iron, while maintaining production of the transcriptional regulator that facilitates survival under conditions of iron-limitation. In addition to expanding the reach of RyhB into regulatory pathways directly controlling the biosynthesis of Fe-S clusters, these studies were

the first to document differential degradation of a polycistronic transcript mediated by a bacterial sRNA molecule.<sup>14</sup>

Collectively, these studies highlight RyhB's influence over several key aspects of *E. coli* physiology by a variety of mechanisms (Fig. 2). Through these diverse mechanisms, RyhB exerts control over every facet of iron homeostasis in *E. coli*. Moving forward with an appreciation for the breadth of RyhB regulatory mechanisms (and the potential of others yet to be characterized) may well facilitate the identification of additional regulatory targets of RyhB, as well as similar iron-regulated sRNAs encoded by a broad range of bacterial species.

### 3. RyhB homologs in pathogenic bacteria

The first publication characterizing *E. coli* RyhB noted that the gene was conserved in several bacterial genera, including *Salmonella*, *Klebsiella*, *Yersinia* and **Vibrio**<sup>8</sup>. Subsequent studies have identified RyhB in these and other species of bacteria, shown in Table 1. In this section we present the major findings of the past decade of RyhB research, which highlight the role of this conserved sRNA in multiple pathogenic relatives of *E. coli*. Combined, these studies demonstrate how RyhB affects a wide variety of important, and often virulence-associated, processes within a collection of closely related pathogens, each employing unique life-styles and virulence strategies. For the purpose of this review, an sRNA will be considered to be a RyhB homolog if (1) the molecule contains no less than 55% sequence identity to *E. coli* RyhB and (2) production of the molecule is regulated in response to iron availability. If the RyhB homolog is referred to by more than one name within published literature, both names are noted.

#### ***Shigella* species RyhB regulates virulence-associated traits**

Not long after the discovery of RyhB, homologs were identified in several pathogens, including the closely related species of *Shigella flexneri*<sup>15</sup> and *Shigella dysenteriae*,<sup>16</sup> both of which cause bacillary dysentery. Given that RyhB of *Shigella* and *E. coli* are identical in nucleic acid sequence, it is not surprising that the regulatory targets of each overlap extensively.<sup>15-16</sup> There are however, two notable exceptions to this rule, and each highlights the impact that RyhB has on virulence of *Shigella* species. Studies in *S. flexneri* have demonstrated that RyhB represses the expression of *ydeP*, a gene encoding a conserved, putative oxidoreductase required for the virulence-associated phenotype of extreme acid resistance. This regulation appears to occur via decreased expression of *evgA*, the upstream transcriptional regulator of *ydeP* and other genes required for extreme acid resistance.<sup>15</sup> As a result of this regulation, the molecular machinery required to survive extreme acid stress is optimally produced by *S. flexneri* during growth under iron-rich conditions, when Fur actively inhibits the synthesis of RyhB. Such regulation should be advantageous for *Shigella* within the extremely low pH environment of the stomach, where ferric iron is soluble and therefore more bio-available. These findings indicate that RyhB promotes *S. flexneri* virulence by facilitating survival of the pathogen during transit through the stomach, en route to the colon where infection becomes established.

Studies in *S. dysenteriae* have demonstrated that RyhB represses the expression of *virB*, a gene encoding the transcriptional activator of several virulence-associated genes.<sup>16</sup> RyhB-mediated repression of *virB* expression results in the inhibition of virulence-associated phenotypes due to decreased expression of genes encoding the type three secretion system that is required for eukaryotic cell invasion, and IcsP, a protease required for actin-based motility.<sup>16-17</sup> Although the precise regulatory mechanism remains elusive, it is clear that RyhB represses the transcription of *virB* and that this regulation is independent of the upstream transcriptional activator VirF.<sup>18</sup> Importantly, RyhB-mediated regulation of *virB*

expression provides an example of a conserved, chromosomally-encoded sRNA molecule controlling the production of a genus specific, horizontally acquired virulence-determinant.

Acid resistance, eukaryotic cell invasion and cell-to-cell spread are all important virulence-associated processes that contribute to the success of *Shigella* species as primary pathogens and, remarkably, are all influenced by the activity of RyhB.

### ***Vibrio cholerae* RyhB regulates motility, chemotaxis, and bio-film formation**

RyhB is less well conserved between *E. coli* and *Vibrio cholerae*, an environmental microbe and the causative agent of cholera.<sup>8</sup> Two separate studies have demonstrated that in addition to a relatively conserved core sequence, *V. cholerae* RyhB possesses an additional 69 and 54 nucleotides at the 5' and 3' ends of the RNA, respectively.<sup>19</sup> Despite these significant differences, *V. cholerae* RyhB represses expression of many of the same genes as *E. coli* RyhB, including *sodB* (superoxide dismutase), *sdhCDAB* (succinate dehydrogenase), *fum* (fumarase), and *acnA* (aconitase).<sup>19</sup> In addition to these conserved targets, *V. cholerae* RyhB modulates the expression of several genes that facilitate the unique life-style of this pathogen, including those controlling motility, chemotaxis and biofilm formation.<sup>19</sup> For these reasons, while RyhB is not required by *V. cholerae* for colonization in an infant mouse model of infection,<sup>19</sup> it is reasonable to conclude that RyhB is important for environmental survival of this pathogen.

### ***Salmonella enterica* encodes two differentially-regulated homologs of RyhB**

As predicted by Masse and Gottesman,<sup>8</sup> *Salmonella* encodes two RyhB homologs, originally termed RfrA and RfrB.<sup>20</sup> RfrA shares 82% sequence identity with *E. coli* RyhB and is encoded in the same genetic context as RyhB, while RfrB shares only 53% sequence identity with RfrA and is encoded within a *Salmonella* specific genetic locus.<sup>20-21</sup> While the production of both sRNAs is influenced in response to iron-availability by the activity of Fur, production of RfrB is primarily regulated by the activity of the stationary phase sigma factor RpoS.<sup>20, 22</sup> Recent analysis of the regulatory targets of RfrA and RfrB, in this study termed RyhB-1 and RyhB-2, reveals that they each influence the expression of a set of genes that is similar, but not identical, to that regulated by *E. coli* RyhB.<sup>22</sup> Moreover, RfrA and RfrB play overlapping yet distinct roles in protecting against oxidative stress, bactericidal antibiotics and acid resistance, as inactivation of each has varying effects on these virulence-associated phenotypes.<sup>22</sup> Additionally, both RfrA and RfrB appear to influence the survival of *Salmonella* within the unique environments of a eukaryotic epithelial cells and macrophages.<sup>20, 23</sup> Combined, these studies suggest that *Salmonella* RfrA and RfrB are produced during growth under unique environmental conditions and have overlapping yet distinct roles in regulating *Salmonella* physiology and virulence-associated phenotypes.

### ***Klebsiella pneumoniae* RyhB regulates capsular biosynthesis genes**

Recent studies show RyhB modulates two important virulence-associated phenotypes in *K. pneumoniae*: biosynthesis of capsular polysaccharide and iron acquisition.<sup>24</sup> Increased production of RyhB, as would be seen under iron-limiting conditions (Fig. 1), is associated with increased capsular polysaccharide synthesis and increased expression of multiple genes encoding components of three separate iron acquisition systems.<sup>24</sup> Such regulation coordinates the production of capsular polysaccharide and iron acquisition systems, two factors required for survival within the human host, to growth within the iron-poor environments endured by the pathogen during the course of a natural infection. Of note, a recent study suggests that, like *S. enterica* and *Y. pestis*, *K. pneumoniae* encodes two RyhB homologs.<sup>25</sup> The full extent to which each RyhB homolog influences *K. pneumoniae* physiology and virulence is yet to be determined.

### ***Yersinia pestis* encodes Hfq-dependent and Hfq-independent RyhB homologs**

As originally predicted by Masse and Gottesman,<sup>8</sup> *Y. pestis* encodes two RyhB homologs, designated RyhB1 and RyhB2.<sup>26</sup> Studies have demonstrated that while Fur regulates the production of both RyhB1 and RyhB2 in response to iron-availability, Hfq differentially influences the stability of each.<sup>26</sup> As observed in *E. coli*,<sup>9a</sup> Hfq stabilizes RyhB1, but surprisingly has no effect on the stability of RyhB2.<sup>9a, 26</sup> The insensitivity of RyhB2 to Hfq is a feature that makes this molecule unique among RyhB homologs characterized to date. While much is left to learn about the role of RyhB1 and RyhB2 in controlling *Y. pestis* physiology, the inaugural investigation demonstrates that production of each sRNA is higher following growth of the pathogen in a mouse lung as compared to that measured following *in vitro* growth, an observation that suggests that the sRNAs may facilitate the progression of pneumonic plague in mice.<sup>26</sup>

## **4. Functional analogs of RyhB**

Several bacteria encode for iron-responsive sRNAs that mediate a similar iron-sparing response to RyhB, yet share no significant homology with this paradigmatic sRNA. While these divergent sRNAs lack homology to RyhB, they are considered functional analogs, as (1) the production of each is repressed in response to iron via Fur, and (2) they function to regulate a similar set of genes as RyhB. As described below, these sRNAs play a significant role in modulating the iron-sparing response of the bacteria that encode them, while also controlling several other key biological processes.

### **The duplicated PrrF RNAs of *Pseudomonas aeruginosa***

Shortly after the identification of RyhB, the Fur-regulated PrrF (*Pseudomonas* RNA responsive to Fe) sRNAs, PrrF1 and PrrF2, were identified in the opportunistic Gram-negative pathogen *Pseudomonas aeruginosa*.<sup>27</sup> While sharing no significant sequence homology with RyhB, the PrrF RNAs contribute to an analogous iron-sparing response via the repression of a similar set of gene targets.<sup>27–28</sup> In addition to controlling the expression of regulatory targets shared with *E. coli* RyhB, PrrF affects the production of several factors involved in a variety of metabolic pathways, including one for the degradation of anthranilate.<sup>28</sup> Anthranilate is a precursor for the *Pseudomonas* quinolone signal (PQS) quorum sensing molecule,<sup>29</sup> and deletion of the genes encoding PrrF1 and PrrF2 results in loss of PQS production due to the constitutive degradation of anthranilate (Fig. 3).<sup>28</sup> Thus, the PrrF RNAs spare anthranilate for PQS production during iron-restricted growth. PQS can also induce synthesis of pyoverdine, the primary siderophore of *P. aeruginosa*, via PQS's ability to chelate and sequester extracellular ferric iron (Fig. 3).<sup>30</sup> These findings have led to the proposal that PQS functions in extracellular iron entrapment to facilitate pyoverdine-mediated iron acquisition by *P. aeruginosa*. As such, these findings suggest a role for PrrF1 and PrrF2 in controlling iron acquisition by *P. aeruginosa*. Furthermore, as a quorum sensing molecule PQS is proposed to play an important role in virulence,<sup>31</sup> suggesting these duplicated sRNAs may directly impact *P. aeruginosa* virulence.

Similar to what was described for certain RyhB-encoding pathogens above, *P. aeruginosa* species encode for two PrrF RNAs. *Pseudomonas* presents an interesting twist on the duplicated RNA theme, however, in that the PrrF RNAs in *P. aeruginosa* are encoded in tandem by two virtually identical genes (93% identity): *prrF1* and *prrF2*. Each PrrF RNA appears to be capable of nearly complete repression of most of its target mRNAs, as genetic deletion of both the *prrF1* and *prrF2* genes is required for significant derepression of target gene expression in iron-depleted growth conditions.<sup>27–28</sup> The *prrF* region encodes for an additional, heme-responsive 325-nt sRNA, designated PrrH, providing a likely rationale for the duplicated *prrF* genes.<sup>32</sup> Transcription of PrrH initiates at the 5' end of *prrF1*, proceeds

through the *prfF1-prfF2* intergenic sequence (95 nt), and terminates at the 3' end of *prfF2*. Thus, expression of *prfH* is dependent on read-through transcription at the *prfF1* Rho-independent, or intrinsic, terminator. PrfH regulates genes involved in heme biosynthesis,<sup>32a</sup> suggesting this tandem *prfF* organization imparts unique heme-responsive regulatory activities to *P. aeruginosa*. The advantage that this tandem arrangement imparts to *P. aeruginosa*, as compared to other Pseudomonads where *prfF1* and *prfF2* are encoded distally, is unknown. However, it may play a role in *P. aeruginosa*'s unique ability to cause disease in humans, as heme is an abundant source of iron encountered during infection.

### Azotobacter iron-sparing response is mediated by ArrF

*Azotobacter vinelandii* is a non-pathogenic, nitrogen-fixing bacteria and close relative of *P. aeruginosa*. Similar to *P. aeruginosa*, *A. vinelandii* requires an abundance of iron for respiratory pathways, and displays a similar iron-sparing response mediated by an iron-regulated sRNA. *A. vinelandii* encodes a single homolog of PrfF, termed ArrF, which shares considerable sequence homology with both PrfF1 and PrfF2 RNAs (81% and 80% respectively) of *P. aeruginosa*.<sup>33</sup> Like previously described iron-regulated sRNAs, ArrF represses the expression of *sodB*, likely via binding of the sRNA to the target RNA molecule within a region of nucleic acid complementary.<sup>33</sup> Unlike PrfF and RyhB however, ArrF does not appear to influence the expression of the genes encoding TCA cycle enzymes. The lack of repression of TCA cycle enzymes is likely a consequence of that fact that *A. vinelandii* is an obligate aerobe. As such, enzymes in the TCA cycle are essential during growth of the organism under any condition, regardless of iron availability. To maintain iron stores for aerobic respiration, ArrF represses expression of the *feSII* gene,<sup>33</sup> encoding a complex that protects the iron-loaded nitrogenase enzyme required for nitrogen fixation, a non-essential function. ArrF additionally represses expression of the *phbBAC* operon,<sup>34</sup> which converts acetyl-CoA to the carbon storage molecule poly- $\beta$ -hydroxybutyrate. As such, ArrF spares the use of acetyl-CoA for metabolism via the TCA cycle during growth in iron-poor conditions. These variations in iron-responsive sRNA targets provide a clear example of how two closely related bacteria have evolved to utilize highly similar regulatory molecules, such as PrfF/ArrF, to uniquely promote the fitness of each organism.

### Iron-sparing response of *Neisseria* is only partially due to NrrF

*Neisseria meningitidis*, a Gram-negative diplococci that causes meningitis epidemics in infants and young adults,<sup>35</sup> also displays an iron sparing response, with Fur exerting a positive effect on the expression of several genes.<sup>36</sup> In some cases this regulation occurs directly via binding of Fur to nucleic acid sequences within the operator regions of the activated genes.<sup>37</sup> However, *N. meningitidis* also encodes a 169-nt Fur-repressed sRNA, NrrF, that is responsible for the Fur-dependent activation of the genes encoding succinate dehydrogenase.<sup>38</sup> Despite sharing very little homology with either PrfF or RyhB, NrrF regulates succinate dehydrogenase in an Hfq-dependent manner similar, yet not identical, to that described for RyhB.<sup>38b</sup> Interestingly, Hfq is also required for the Fur-dependent activation of *sodB* and *fumB*, yet these genes are unaffected by deletion of *nrrF*,<sup>38b</sup> suggesting that a separate, as of yet unidentified sRNA is responsible for the repression of these regulatory targets. Sequence for a potential NrrF homolog sharing 93% identity with *N. meningitidis* NrrF and 195 nt in size has been identified in the related pathogen *Neisseria gonorrhoeae*,<sup>39</sup> although no targets have yet been characterized for this sRNA. Also yet to be determined is the role that the NrrF sRNA plays in controlling the expression of virulence-related gene expression and/or the production of virulence factors in each of these bacterial pathogens.

## Iron-responsive RNAs in Gram-positive bacteria (*Bacillus subtilis* FsrA and Fbp)

Fur homologs and iron sparing responses have been identified and characterized in several Gram-positive organisms, including *Bacillus subtilis*.<sup>40</sup> In particular, the *B. subtilis* iron-sparing response is dependent on Fur-regulated expression of the 84-nt sRNA FsrA (Iron [Fe]-responsive sRNA).<sup>41</sup> Similar to RyhB, FsrA negatively affects the expression of genes encoding iron-containing TCA cycle enzymes (succinate dehydrogenase - *sdhABC*; aconitase - *citB*), as well as related metabolic enzymes involved in amino acid biosynthesis (glutamate synthase - *gltAB*; isopropyl malate dehydrogenase - *leuCD*), a dicarboxylate permease important for succinate and fumarate utilization (*dctP*), and three iron-sulfur containing lactate utilization genes (*lutABC*). An intriguing component of the FsrA regulatory paradigm is the involvement of three small basic proteins, encoded by two distinct transcriptional units (*fbpAB* and *fbpC*), which are hypothesized to chaperone the FsrA RNA.<sup>41b</sup> The requirement of the Fbp proteins for FsrA regulation is not clearly understood, and appears to vary with FsrA regulatory target (Fig. 4). For example, FsrA repression of the *lutABC* genes for lactate utilization specifically requires the FbpB protein,<sup>42</sup> while repression of *leuCD* is dependent on FbpC.<sup>41b</sup> As such, the Fbp proteins are proposed to function as chaperones that direct FsrA activity to mediate iron homeostasis in *B. subtilis*.

Several exceptions to this chaperone model exist, however, including the repression of *sdhCDAB*, *citB*, and *gltAB* by FsrA, which is not dependent on either of the Fbp proteins.<sup>41b</sup> Neither does Hfq appear to have a role in FsrA repression of *sdhCDAB*,<sup>41b</sup> presenting the possibility that FsrA regulation of some targets is independent of chaperone activity. The authors postulate that extensive sequence complementarity between *sdhC* and *fsrA* allows this regulation to occur in the absence of chaperone. Alternatively, a constitutively expressed chaperone, similar to the Fbp's, may function as a FsrA chaperone for this message. Further, the Fbp proteins appear to regulate gene expression independent of FsrA, a trait reminiscent of the Hfq protein in Gram-negative bacteria. Studies into the structural basis for FsrA, FbpAB and FbpC regulation will undoubtedly be required to understand the contribution of these different RNA chaperones in the iron sparing response. The genes encoding the FsrA RNA and Fbp proteins have been identified in two other *Bacillus* species – *Bacillus amyloquefaciens* and *Bacillus licheniformis*, however, neither their expression nor their activity have been validated. It will also be of interest to determine if these or analogous RNAs are present in other Gram-positive bacteria that exhibit the typical Fur-dependent iron-sparing response, including *Staphylococcus aureus*<sup>43</sup> and *Listeria monocytogenes*.<sup>44</sup> In particular, it will be interesting to determine if the iron-sparing responses regulate genes that contribute to virulence, as has been demonstrated for many Gram-negatives that encode for iron-regulated sRNAs.

While continued research will undoubtedly add to the growing number of iron-regulated sRNAs, it is important to point out that some bacteria appear to carry out iron-sparing responses independent of sRNAs. In *Helicobacter pylori*, for example, Fur directly activates *sodB* expression.<sup>45</sup> Although not required for iron-dependent regulation of *sodB* expression, numerous sRNAs have been identified in *H. pylori*,<sup>46</sup> and the role these regulators play in iron-responsive gene regulation remains to be seen. Additionally, it is not yet known if bacteria utilize sRNAs to respond to other biologically important metals, such as manganese. This is a particularly interesting question with regard to the  $\alpha$ -proteobacteria, which respond to manganese instead of iron via a Fur homolog (Mur).<sup>47</sup>

## 5. *cis*-antisense RNAs regulating iron homeostasis

Even before the identification of RyhB, the iron-responsive *cis*-encoded antisense RNA $\alpha$  was identified in the fish pathogen *Vibrio anguillarum*.<sup>48</sup> RNA $\alpha$  is transcribed from the

noncoding strand of the *fatABCD* operon, which encodes a transport system for the siderophore anguibactin.<sup>49</sup> As such, the 650-nucleotide RNA $\alpha$  is complementary to almost two-thirds of the *fatB* gene.<sup>48a</sup> While binding of RNA $\alpha$  to the *fatABCD* operon has not yet been experimentally demonstrated, expression of RNA $\alpha$  correlates closely with loss of FatA translation.<sup>48a, 50</sup> In contrast to RyhB and its homologs, however, the Fur protein promotes transcription initiation of RNA $\alpha$  in an iron-independent manner, and RNA $\alpha$  is posttranscriptionally stabilized under iron-replete conditions.<sup>50</sup> The mechanism of this regulation is unknown, and is likely distinct from earlier mechanisms described in this review; yet the outcome is reminiscent of the effect of *E. coli* RyhB on siderophore biosynthesis,<sup>12</sup> and provides yet another example of how an iron-responsive RNA can mediate gene expression and iron homeostasis.

The Fur proteins of cyanobacteria control the expression of genes for iron uptake systems and oxidative stress, as well as photosynthesis.<sup>51</sup> Photosynthesis, in particular, places a particularly high iron demand on the bacterium. To compensate for loss of light-harvesting systems under low iron conditions, cyanobacteria express *isiA* (iron stress-induced protein A), encoding a Fur-repressed photosystem (PS) I accessory protein.<sup>52</sup> In *Synechocystis*, the IsiA protein forms an additional ring around the photosystem I complex, which enhances light absorption during periods of iron depletion to compensate for the reduction in the number of PSI complexes.<sup>53</sup> The 177-nt IsiR RNA is transcribed constitutively from the non-coding strand of *isiA* and appears to bind to complementary portions of the mRNA, causing coupled degradation of the RNA hybrid.<sup>54</sup> Thus, transcription of the *isiA* mRNA must outcompete that of IsiR in order to function as template for IsiA protein synthesis. This competitively-based RNA regulatory network is thought to ensure quick turnover of residual *isiA* mRNA when intracellular iron levels are restored.

Another intriguing *cis*-antisense regulatory scheme exists in the cyanobacterium *Anabaena*, which encodes three putative Fur homologs.<sup>55</sup> Expression of the *Anabaena* FurA protein is repressed by the antisense  $\alpha$ -FurA RNA under high iron conditions.<sup>56</sup> Mutants defective for  $\alpha$ -FurA RNA display increased levels of the FurA protein, and are disrupted for iron homeostasis, growth, and photosynthetic efficiency.<sup>57</sup> Thus, the paradigmatic roles of Fur and regulatory RNA appear to be reversed in *Anabaena*, yet work to similarly affect the overall physiology of this cyanobacterium.

## 6. Closing remarks

The studies presented in this review have made significant contributions to the ever-expanding appreciation for the role that iron-regulated sRNAs play in influencing overall cellular physiology and virulence in a broad range of bacterial species. Although it is obvious that iron-regulated sRNAs are important regulators, continued investigation into the regulatory targets and molecular mechanisms of iron-regulated sRNAs will be needed to reveal the full impact of these regulators on bacterial physiology and virulence. These studies also demonstrate the diversity of regulatory mechanisms and mRNA targets by which iron-responsive sRNAs function, variations that are often linked to the unique lifestyles of the organisms that encode them. Thus, it is important that research in this area move forward with awareness for the possible exceptions to the RyhB paradigm. Future studies in this field will undoubtedly yield a more comprehensive understanding of the complex role of iron in bacterial physiology.

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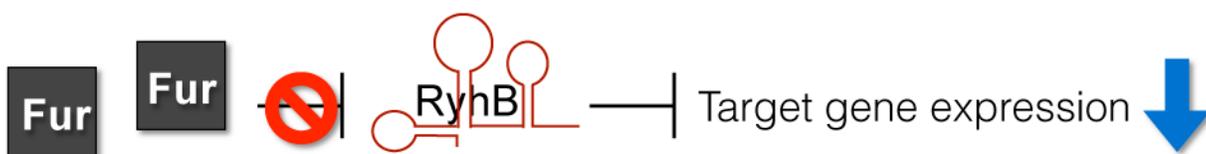
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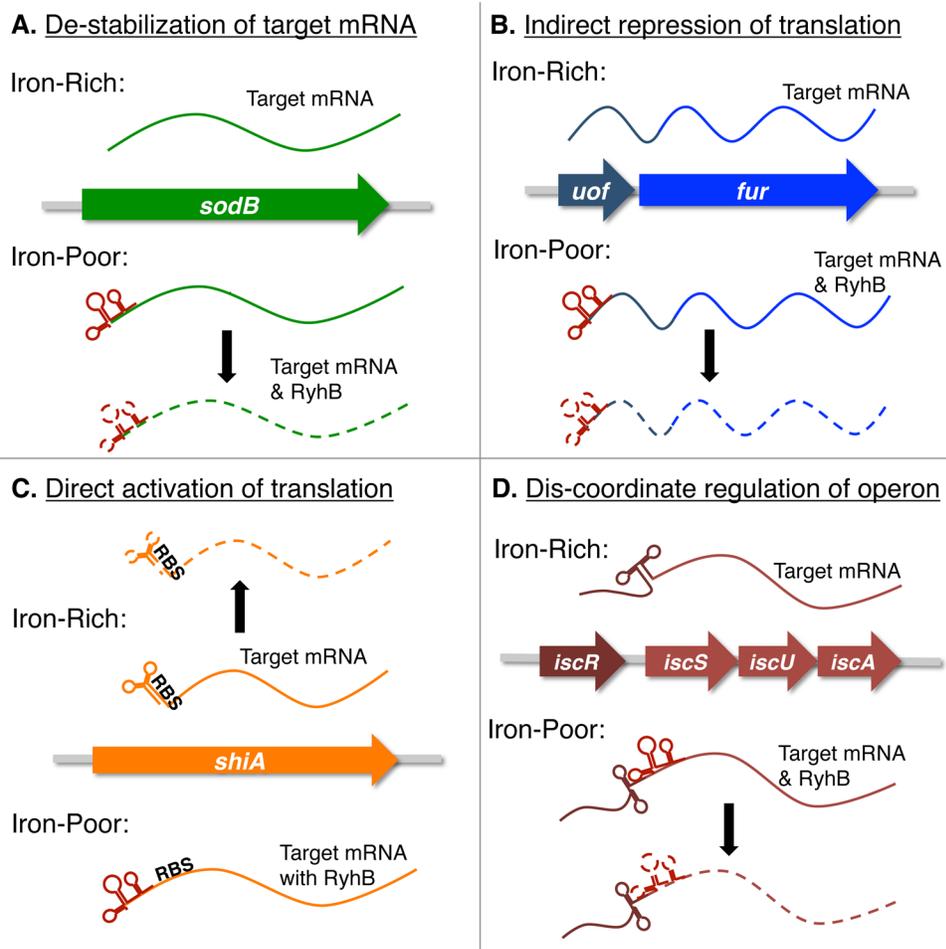
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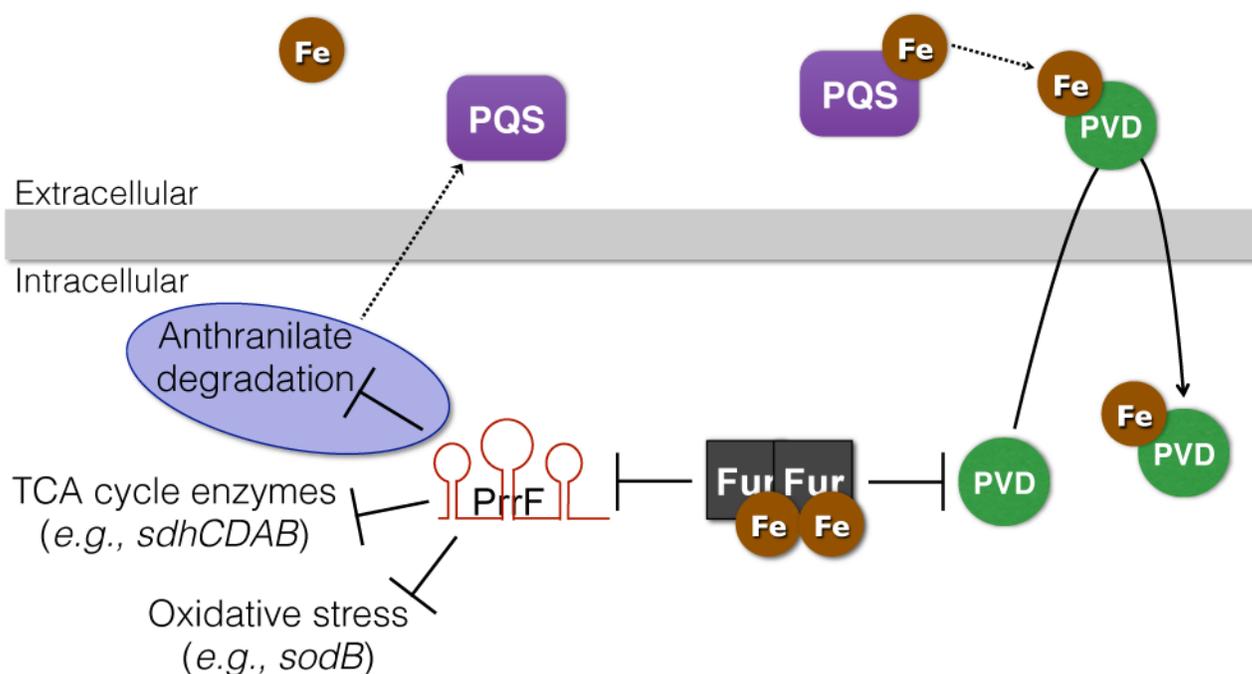
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**A. Iron-rich:****B. Iron-poor:**

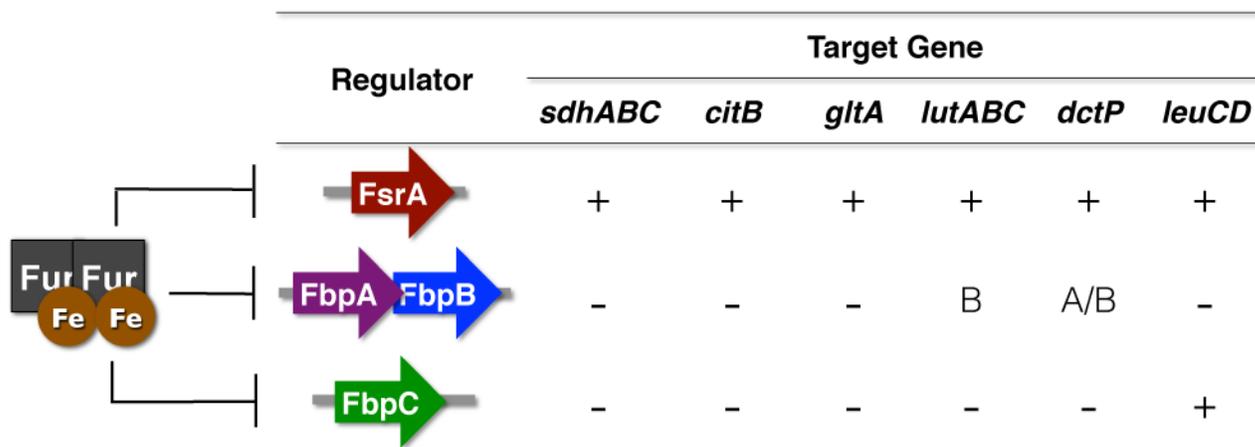
**Fig. 1.** Schematic of RyhB-mediated repression of gene expression. A. In iron-rich environments, RyhB expression is repressed by the iron-bound Fur protein, resulting in derepression of target genes. B. In iron-poor conditions, Fur repression is relieved and RyhB is expressed, leading to decreased expression of specific target genes.



**Fig. 2.** *E. coli* RyhB controls the expression of target genes via multiple regulatory mechanisms. Presented are simplified schematics of the molecular mechanisms governing RyhB-dependent regulation of target gene expression in *E. coli*. Only the relevant RNA molecules are depicted in each panel, and each are presented as a dashed line under conditions in which they are destabilized. RyhB is red in each panel.



**Fig. 3.** Putative role of PrrF sRNAs in pyoverdine-mediated iron uptake by *P. aeruginosa*. When bound to iron, the Fur protein represses expression of genes for synthesis of the pyoverdine (PVD) siderophore, which has a very high affinity for ferric iron. Fur also represses expression of the PrrF1 and PrrF2 sRNAs. The PrrF sRNAs themselves regulate a number of cell processes, most notably TCA cycle enzymes and factors that protect against oxidative stress. The PrrF sRNAs also contribute to production of the Pseudomonas quinolone signal (PQS) in low iron conditions by blocking the degradation of a vital precursor (anthranilate - regulation highlighted in purple). Additionally, PQS may enhance PVD production by chelating extracellular iron, which is subsequently scavenged by the PVD siderophore.



**Fig. 4.**

FsrA sRNA and Fbp proteins mediate iron sparing response in *B. subtilis*. The FsrA RNA is expressed under iron-poor conditions, when iron-dependent Fur repression is relieved. FsrA in turn represses several target genes, including the six examples shown in this figure. Fur also represses expression of three small basic proteins, FbpAB and FbpC; the *fsrA*, *fbpAB*, and *fbpC* transcripts are unlinked on the chromosome. The Fbp proteins make variable contributions to FsrA regulation, as indicated by the – and + signs. For one *fbpAB*-dependent target, *lutABC*, FbpB specifically has been shown to be required for FsrA-mediated regulation.

Table 1

## Iron-responsive sRNA regulation in bacteria

Bacterial Species	RNA Reference	Affected Functions
<i>Escherichia coli</i>	RyhB <sup>8</sup>	Iron storage; oxidative stress protection; metabolism; siderophore biosynthesis; Fur
<i>Vibrio cholerae</i>	RyhB <sup>19</sup>	Iron storage; biofilm formation; chemotaxis; OmpT
<i>Shigella flexneri</i>	RyhB <sup>15</sup>	Iron storage; oxidative stress protection; acid resistance
<i>Shigella dysenteriae</i>	RyhB <sup>16</sup>	Iron storage; oxidative stress protection; virulence
<i>Salmonella enterica</i> serovar Typhimurium	RfrA/RyhB1, RfrB/RyhB2 <sup>20-21</sup>	Oxidative stress protection; virulence
<i>Klebsiella pneumoniae</i>	RyhB1, RyhB2 <sup>24-25</sup>	None identified
<i>Yersinia pestis</i> biovar microtus	RyhB1, RyhB2 <sup>26</sup>	None identified
<i>Pseudomonas aeruginosa</i>	PrrF1, PrrF2 <sup>27-28</sup>	Iron storage; oxidative stress protection; metabolism; quorum sensing (PQS)
	PrrH <sup>32a</sup>	Heme biosynthesis
<i>Neisseria meningitidis</i>	NrrF <sup>38</sup>	Succinate dehydrogenase
<i>Neisseria gonorrhoea</i>	NrrF <sup>39</sup>	None identified
<i>Azotobacter vinelandii</i>	ArrF <sup>33-34</sup>	Oxidative stress protection; carbon storage; nitrogen fixation
<i>Bacillus subtilis</i>	FsrA <sup>41-42</sup>	Metabolism; amino acid biosynthesis
<i>Vibrio anguillarum</i>	RNA <sub>α</sub> <sup>50</sup>	Iron acquisition
<i>Synechocystis</i>	IsrR <sup>54</sup>	Photosynthesis
<i>Anabaena</i>	α-furA <sup>58</sup>	Photosynthesis; iron uptake