The *Leptospira* HemKR two-component system regulates heme/iron homeostasis by sensing 5-aminolevulinic acid

Juan Andrés Imelio[1], Felipe Trajtenberg[1], Leticia Zarantonelli[2], Iakov Vitrenko[3], Laure Lemée[3], Thomas Cokelaer[3,4], Mathieu Picardeau[5], Alejandro Buschiazzo[1,6]

[1]Institut Pasteur de Montevideo, Laboratory of Molecular & Structural Microbiology, Montevideo, Uruguay
[2]Unidad Mixta Pasteur INIA, Institut Pasteur de Montevideo, Montevideo, Uruguay
[3]Institut Pasteur, Université Paris Cité, Plateforme Technologique Biomics, Paris, France
[4]Institut Pasteur, Bioinformatics and Biostatistics Hub, Paris, France
[5] Institut Pasteur, Université Paris Cité, Biology of Spirochetes Unit, Paris, France
[6] Institut Pasteur, Université Paris Cité, Dept of Microbiology, Paris, France

SUMMARY

Two-component systems (TCSs) sense specific signals, regulating countless physiological pathways. Heme concentrations are kept at defined levels in cells by means of exquisite regulatory networks, including TCS-mediated ones. Heme biosynthesis involves energetically costly pathways, which are finely controlled with degradation, avoiding toxicity of heme overabundance. The regulation of heme import/export is also integrated, securing proper homeostasis. In *Leptospira* –Spirochete bacteria–, the TCS HemKR regulates heme metabolism, yet the cues detected by the sensory kinase HemK are still unknown. Here we show that 5-aminolevulinic acid (ALA), a committed porphyrin biosynthesis precursor, is sensed by HemK shutting down the kinase's autophosphorylation activity. In this way, ALA induces HemK to act as a phosphatase towards HemR, its cognate response regulator. HemR dephosphorylation leads to a substantial modulation of the bacterial gene expression profile, including the repression of genes encoding heme-biosynthesis enzymes and TonB-dependent iron-chelate/heme importer systems, combined with a simultaneous induction of heme oxygenase, critically involved in heme degradation. The transcription of several other genes linked to heme and iron homeostasis, is also modulated by HemKR, consistent with phenotypic differences between *hemKR*$^-$ double-knockout *vs wild-type L. biflexa* strains. Considering that heme oxygenase is a virulence factor in pathogenic *Leptospira*, HemKR is now identified as a relevant factor of spirochetal virulence regulation.

INTRODUCTION

Bacteria detect intracellular and environmental stimuli through an array of sensory proteins that transmit the information, triggering physiologic adaptive responses. Such sensory/regulatory proteins constitute networks of signal transduction systems regulating numerous biologic processes (Imelio et al., 2021). Two-component systems (TCSs) are signal transduction systems (Jacob-Dubuisson et al., 2018; Francis & Porter, 2019), most often composed of a transmembrane sensory histidine-kinase (HK) and a cytosolic response regulator (RR). The presence/absence of a specific stimulus regulates the activation of the HK through ATP-mediated histidine autophosphorylation (Buschiazzo & Trajtenberg, 2019). The phosphorylated HK transfers its phosphoryl group to a conserved aspartate residue on its cognate RR (Gao et al., 2019), which can thereafter bind to DNA promoter sites, regulating the transcription of one or more genes. Most HKs are also capable to act as RR-specific phospho-aspartate (P~Asp) phosphatases, critically contributing to keeping the pathways off when appropriate (Huynh et al., 2010; Gao & Stock, 2017; Buschiazzo &

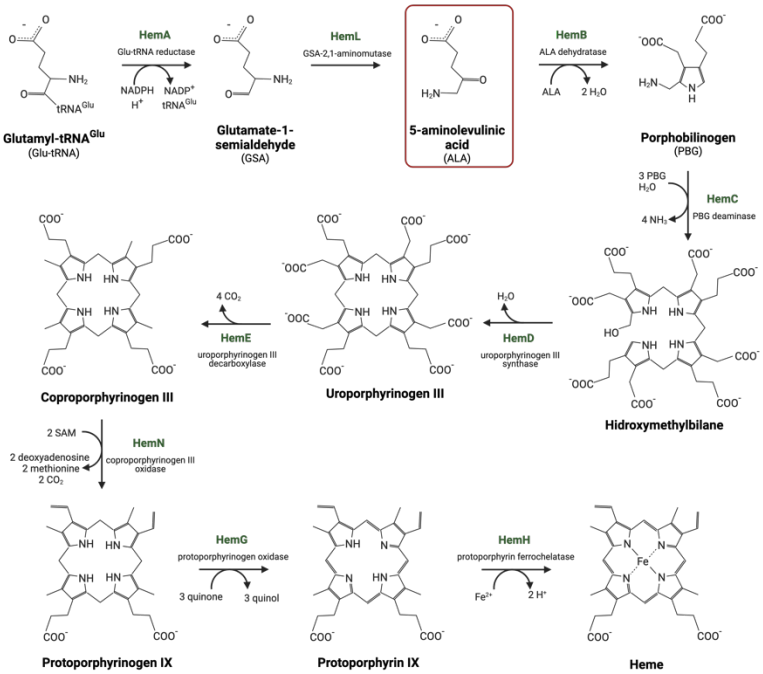
Trajtenberg, 2019). For such down-control function, some TCSs may also rely on dedicated auxiliary phosphatases, distinct from the sensory HK (Silversmith, 2010; Parashar et al., 2011). Except for a few pathogenic and endosymbiotic species such as *Mycoplasma* spp. and *Amoebophylus* spp. (Wuichet et al., 2010), TCSs are present in almost all bacteria, regulating important functions that include motility, osmoregulation, antibiotic resistance, virulence, pathogenicity, and micronutrient metabolism, among many others (Stock et al., 1990; Harshey et al., 2003; Galperin et al., 2010; Bi & Sourjik, 2018). TCSs are also found in plants and fungi, but not in Protists and Metazoans, turning them into attractive antibacterial drug targets (Bem et al., 2015).

The metabolic pathways of iron and heme are linked: heme is a tetrapyrrole that binds iron tightly within its porphyrin ring, allowing for heme's essential functions as an electron transporter in respiratory chains, as well as an iron storage molecule (Dailey et al., 2017). To make the transition metal available, bacteria rely on heme oxygenases that degrade heme in an oxygen-dependent manner, rendering biliverdin, carbon monoxide and free iron (Frankenberg-Dinkel, 2004). Intracellular iron excess is highly toxic, unleashing the production of harmful oxygen radicals through Fenton reactions. Excess of heme has also been reported to be toxic (Anzaldi & Skaar, 2010), even though the mechanisms by which heme kills bacteria remain unclear. In any case, exquisite regulatory mechanisms are essential to integrate iron and heme metabolism, maintaining physiologic concentrations of both, and ultimately ensuring homeostasis. Several TCSs have been implicated in iron and heme metabolism control, often linked to bacterial pathogenicity and virulence. In *Porphyromonas gingivalis*, the TCSs HaeRS controls a regulon that includes genes involved in hemin acquisition. The HK HaeS is activated by hemin, transducing the signal by phosphorylation of its cognate RR HaeR (Scott et al., 2013). FirRS from *Haemophylus influenzae* (Steele et al., 2012) and *Salmonella* PmrBA (Wosten et al., 2000), respond to extracellular iron. In *Staphylococcus aureus* and *Bacillus anthracis*, the HssRS system senses/responds to exogenous heme, regulating the expression of the heme-detoxifying transporter HrtAB to keep intracellular heme concentrations in line (Stauff & Skaar, 2009). Furthermore, in *Corynebacterium glutamicum*, the TCSs HrrSA and ChrSA perceive fluctuations of environmental heme availability, and trigger the expression of genes involved in cellular detoxification and homeostasis coordination of intracellular heme such as the heme efflux system *hrtAB*, which are also regulated by the global iron-dependent regulator DtxR (Bibb et al., 2007; Keppel et al., 2020).

The Spirochetes phylum includes unique bacteria that possess spiral-shaped cells and endo-flagella confined within the periplasm (Wolgemuth et al., 2006). *Borrelia burgdorferi* (the agent of Lyme disease) and *Treponema pallidum* (syphilis), lack the enzymatic machinery to synthesize heme (Panek & O'Brian, 2002). *B. burgdorferi* evolved to use manganese instead of iron altogether (Posey & Gherardini, 2000). The *Leptospira* genus on the other hand, comprises highly motile, aerobic Spirochete species, with saprophytic or pathogenic lifestyles (Picardeau, 2017). The latter are etiologic agents of leptospirosis, the most widespread bacterial zoonosis, infecting over 1 million people every year in all continents and causing >60,000 annual deaths (Costa et al., 2015). The saprophytic species *L. biflexa* has been used extensively as a model to study leptospirosis, more tractable for genetic manipulations and exhibiting faster growth compared to pathogenic species, yet sharing similar genomes and overall cell behaviour in culture (Picardeau, 2017). *Leptospira* cells need iron to survive, with previous reports starting to uncover how this metal is incorporated, and how pathogenic *Leptospira* cope with iron limitations within their

hosts (Louvel et al., 2006; Lo et al., 2010). *Leptospira* is able to synthesize heme *de novo* (Figure 1) and can also acquire it from exogenous sources (Guegan et al., 2003; Louvel et al., 2006).

The TCS HemKR is involved in regulating heme metabolism in *Leptospira* (Louvel et al., 2008; Morero et al., 2014). The knockout strain *hemK-/hemR-* (hereafter *ΔHemKR*) was reported to be auxotrophic, requiring an exogenous source of heme, or of the heme precursor 5-aminolevulinic acid (ALA) (Figure 1), to grow in EMJH medium (Louvel et al., 2008). HemR is phosphorylated by HemK, and P~HemR binds to a *hem*-box (TGACA(N_6)TGACA motif) present upstream of several key heme-metabolism genes (Morero et al., 2014). Acting as a dual regulator, P~HemR activates the expression of genes encoding for the heme biosynthetic pathway, while simultaneously represses the expression of *hmuO*, which encodes the first heme-degradation enzyme heme oxygenase (Morero et al., 2014), a known virulence factor in pathogenic *Leptospira* species (Murray et al., 2009). The *hem*-box is also found just 5' of the genes *exbB1/exbD1* (Morero et al., 2014) that code for an important TonB-dependent energy transducer complex controlling active transporters, including for importing iron/siderophores/heme across the outer membrane (Krewulak & Vogel, 2011; Wandersman & Stojiljkovic, 2000; Ratliff et al., 2022). However, the cognate environmental signal(s) regulating the activity of the HK HemK, and thus controlling HemR-dependent regulation, are still unknown.



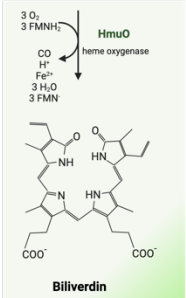


Figure 1. Heme biosynthesis in *Leptospira*. As in many other bacteria, *Leptospira* spp. possess all the genes coding for anabolic enzymes of the so called C5 pathway for *de novo* heme biosynthesis. Starting from glutamyl-tRNA^{Glu}, as initial precursor, the committed substrate 5-aminolevulinic acid (ALA) is generated (boxed in red). Four ALA moieties are needed to build the tetrapyrrole backbone of porphyrins including heme. Note that HemC and HemD are found within a single bifunctional enzyme in *Leptospira* spp (Guegan et al., 2003). The first step of heme degradation, catalysed by heme oxygenase, is highlighted at the end of the pathway.

We now show that HemKR plays a central role in the complex signalling network involved in heme/iron homeostasis in *Leptospira*. The *ΔHemKR* knockout strain was found to secrete significantly larger amounts of heme to the extracellular milieu compared to the *wild-type* strain, while also exhibiting marked resistance to iron deprivation. We uncover that ALA, but not iron, acts as a cognate signal for HemKR. ALA, a committed substrate for heme biosynthesis in all domains of life (Dailey et al., 2017; Layer, 2021), shuts down the HemKR TCS by inducing HemK-catalysed P~HemR dephosphorylation. Accordingly, RNASeq analyses showed that ALA downregulates the expression of heme-biosynthesis (*hemACBLENG*) and metal/porphyrin importer genes (*exbB1-exbD1* / *hemTUVS* / putative heme-receptor), while upregulating heme oxygenase (*hmuO*).

RESULTS

Leptospira biflexa ΔHemKR knockout strain secretes excess heme to the extracellular milieu.

In the way of further characterizing the phenotype of the *ΔHemKR* double-knockout *L. biflexa* strain (Louvel et al., 2008; Morero et al., 2014), growth curves showed by serendipity that this strain can grow in the presence or absence of heme and ALA. That *ΔHemKR* is a prototroph strain able to synthesize heme *de novo* was further confirmed by performing eight consecutive passages in EMJH medium with no supplementation of ALA nor hemin, and thorough washing of cells in each passage. The KO genotype confirmed the *hemKR* locus disruption, also consistent with further RNASeq analyses (*vide infra*). *ΔHemKR* cultures displayed a conspicuously darker colour than the *wt* strain (see Figure 3a further below). Considering that *ΔHemKR* has a deregulated expression of heme-metabolizing enzymes (Morero et al., 2014), the coloured cultures could be due to the enrichment of heme or heme-derived compounds. Indeed, UV-visible spectroscopy revealed a 405 nm absorption peak in culture supernatants, significantly enhanced in *ΔHemKR* compared to *wt* (Figure 2a,b). The energy scan profile is consistent with the absorption spectrum of heme, with a Soret band around 405nm (typical of Fe^{+3}-heme) and a Q-band in the 500-600 nm region (Abdelhameed et al., 2021; Moreira et al., 2008). Moreover, these peaks correlated with absorption at ~280nm, suggesting that exported heme was protein-bound (Traore et al., 2021). *ΔHemKR* cell viability was not compromised (dark-field microscopy), ruling out lysis as the source of porphyrin. Overall, *wt* vs *ΔHemKR* Abs_{405} ratios, in the presence or absence of ALA, indicated that (i) *ΔHemKR* secreted 2-3 times more heme to the supernatant than *wt*; and, (ii) extracellular ALA increased heme secretion, further highlighting the HemKR-dependent effect. Additionally, we searched for peaks corresponding to biliverdin, the product of heme oxygenase HmuO activity. Considering that *ΔHemKR* overexpresses *hmuO* (Morero et al., 2014), cultures under conditions that promote oxygen consumption revealed an additional peak at 670 nm – consistent with biliverdin (Soldano et al., 2017)– only in *ΔHemKR* supernatants and not *wt* (Figure 2c,d).

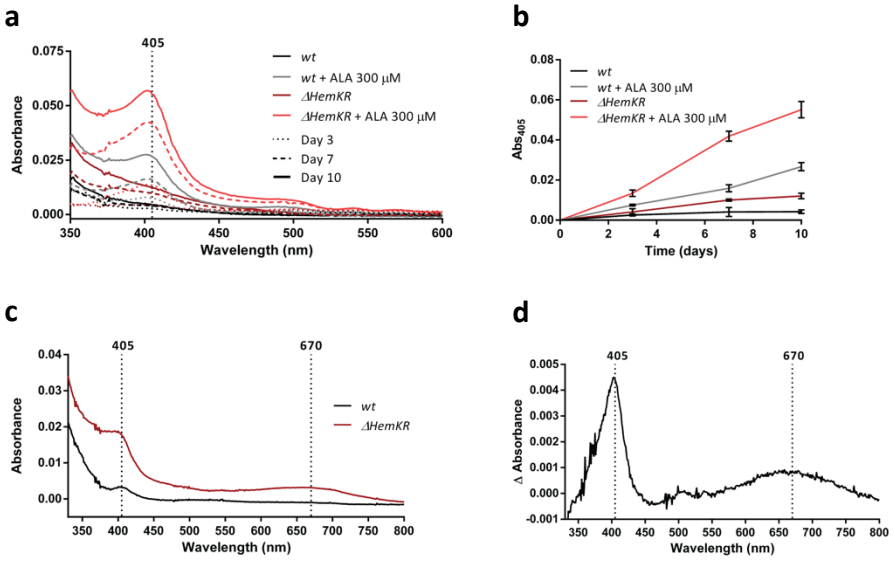


Figure 2. The *ΔHemKR* strain secretes heme and biliverdin in excess to the extracellular milieu. (a) Absorbance scan of supernatants from cultures of *L. biflexa wt* and *ΔHemKR* strains, supplemented or not with 300 µM 5-aminolevulinic acid (ALA) at days 3, 7 and 10 of growth (30ºC, no agitation). The inset highlights the Soret (~405 nm) and Q bands (~500-600 nm), characteristic for heme. **(b)** Summary plot of panel (A)'s data. **(c)** Absorbance energy scan of supernatants from *L. biflexa wt* and *ΔHemKR* cultures (3 days of growth at 30ºC, 100 rpm agitation). Note the 670 nm peak, characteristic of biliverdin. **(d)** Plot derived from panel (C), showing the difference in absorbance (*ΔHemKR* minus *wt*) highlighting peaks corresponding to heme and biliverdin.

The ΔHemKR strain is more resistant to iron deficiency.

As mentioned previously, HemR is a transcriptional repressor of *hmuO* (Louvel et al., 2008; Morero et al., 2014). Exacerbated heme oxygenase activity can lead to increased concentrations of free intracellular iron. We then asked whether HemKR plays a role in iron homeostasis, by limiting extracellular iron availability to different extents. A mild metal deficiency was achieved using non-standard EMJH medium, which lacked supplemented iron; while severe deficiency was obtained by additionally including 35µM 2,2'-dipyridyl (DIP) to that medium, a widely used iron-chelator. While the *wt* showed a very strong growth delay comparing mild and severe iron deprivation, the *ΔHemKR* mutant was significantly more tolerant, exhibiting only a very slight lag when 35µM DIP was present (Figure 3a,b). The mutant's resistance to iron

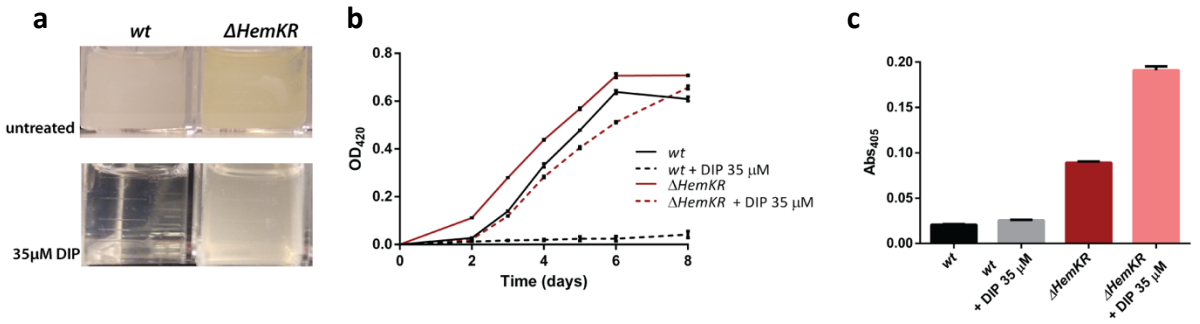


Figure 3. The *ΔHemKR* strain is more tolerant to iron depletion. (a) Representative *L. biflexa wt* and *ΔHemKR* cultures grown in EMJH medium with no supplemented iron, untreated and treated with 35µM 2,2'-dipyridyl (DIP). **(b)** Growth curves of *L. biflexa wt* and *ΔHemKR* cultures grown in EMJH medium without iron either untreated (solid lines) or treated with 35 µM DIP (dotted lines). Measurements of optical density at 420 nm (OD420) were obtained from two biological replicates. Of note, *wt* cultures treated with 35µM DIP grew to 0.384 +/- 0.003 AU after 15 days. **(c)** Absorbance at 405 nm (Abs_{405}) of supernatants from *L. biflexa wt* and *ΔHemKR* cultures, was recorded at day 8 of growth (except for *wt* + 35µM DIP, where this was extended to day 15, maximizing chances to detect potential heme buildup).

deprivation can already be observed under mild iron deficiency (comparing solid lines in Figure 3b): *ΔHemKR* cells exhibited faster growth than the *wt*, a difference that cannot be detected under standard, iron-abundant EMJH conditions. Of note, and in line with results described in the previous section, *ΔHemKR* secreted more heme to the extracellular milieu compared to *wt*, both under normal and iron-deficient conditions (Figure 3c), confirming that this is a HemKR-dependent phenotype.

Complete depletion of iron can be achieved by further increasing the concentration of DIP to 70 µM, at which point both *wt* and *ΔHemKR* cultures arrested growth, only to be reverted –in both strains– by adding the xenosiderophore deferoxamine (data not shown). All evidence considered, the double KO strain *ΔHemKR* lacking the HemKR TCS, is more resistant than the *wt* to iron deficiency.

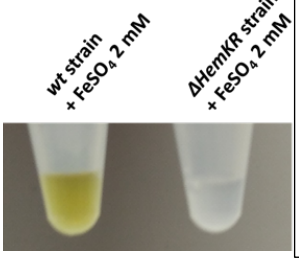
Iron deficiency triggers a strong global regulatory effect, which is only partially HemKR-dependent

Given that HemKR is a heme synthesis-activating/degradation-repressing signalling system (Morero et al., 2014), whose absence exerted heme/iron homeostasis deregulation *in vivo* (this study), we decided to explore for potential HemKR cues among heme-synthesis building blocks: 5-aminolevulinic acid (ALA) and/or iron (Fe^{3+}). Differential gene expression (DGE) analyses were performed comparing *wt* and *ΔHemKR* strains, both grown in the presence and absence of ALA or DIP, extending previous single-gene

studies (Morero et al., 2014) to a whole transcriptome view of HemKR-dependent transcription regulation (Table S1).

In this study, RNA-seq libraries were prepared with 3 replicates for each condition. The libraries were sequenced on an Illumina NextSeq2000 platform, generating single-end reads with an average read length of 107bp. Quality control and data pre-processing were conducted to ensure robust downstream analyses. The number of reads was between 12-20M reads per sample, with an average phred score of 33. Fold-change of individual mRNA species was an obvious figure to analyse DGE. However, sorting the genes also by adjusted p-significance values (Benjamini & Hochberg, 1995), and doing so with all the genes (and not just the ones with $\log_2$ fold-change ≥|1|), was extremely useful. It is well-known that subtle, yet significant changes can be biologically relevant (St Laurent et al., 2013). Indeed, by so including false discovery rate estimates, several genes known to be regulated by HemR were readily identified even with lower fold-change numbers.

To test iron as a direct HemKR signal, we used metal-starving conditions including a strong iron-chelator such as DIP. Iron excess was also attempted by using 2mM $FeSO_4$-supplemented EMJH (instead of 330µM of the iron salt as used in standard EMJH). However, iron excess conditions hindered RNA-purification only in the *wt* strain and not in the *ΔHemKR* mutant (Figure S1). RNASeq analyses comparing cells grown under normal *vs* iron-suppressed conditions, were thus performed to assess the role of iron as a specific cue of the HemKR system.



Supporting Figure S1. Samples, as labelled, were submitted to standard RNA extraction procedures in preparation for whole mRNA transcriptomic sequencing. For unknown reasons, the *wt* strain –and not the *ΔhemKR* KO– exhibited an abnormal behaviour if previously treated with excess iron: an insoluble yellow precipitate was formed during RNA extraction, which impaired RNA recovery prior to sequencing.

Iron-deficiency indeed elicited a strong regulatory response, largely consistent with previous reports in *L. interrogans* (Lo et al., 2010). In the case of the *wt* strain this stressing treatment resulted in the downregulation of 151 genes and the upregulation of 105 (considering DGEs of $\log_2$>|1|). The overall pattern of this response is similar compared to the *ΔHemKR* KO, even though in the mutant there were fewer genes affected (43 and 72, respectively down- and upregulated). Fold-change values were also often different between the two strains, yet the same regulation trend was observed for the shared set of genes (Figure 4a). Most deregulated genes encode hypothetical proteins, leaving their roles currently uncertain. However, restricting a first analysis to annotated genes linked to heme and/or iron, a list of major players can be summarized (Figure 4b):

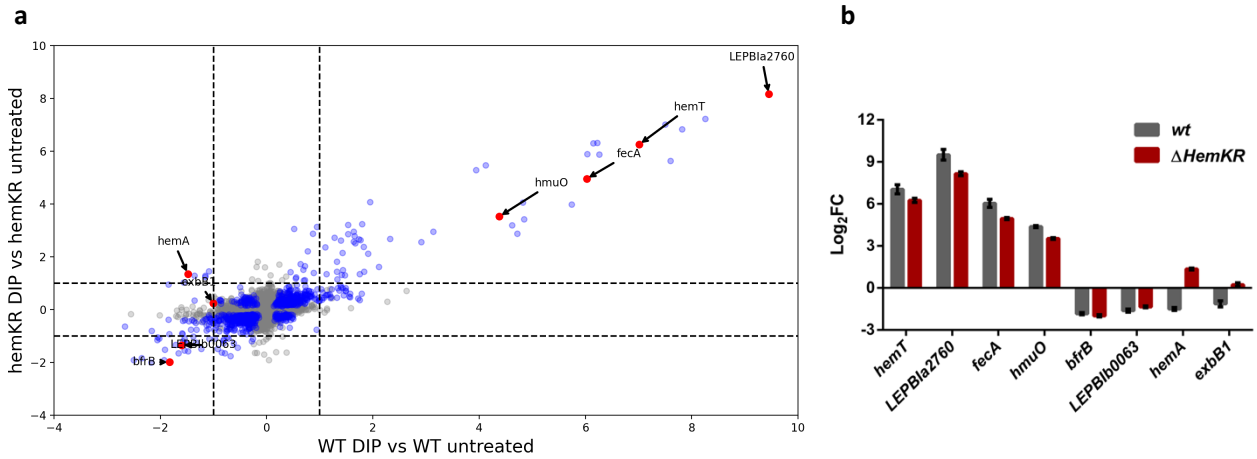


Figure 4. Iron deficit induces transcriptional regulation with mixed HemKR-dependent/independent responses. Transcriptomic differential gene expression analysis comparing *L. biflexa* cells treated *vs* untreated with 35µM DIP for 3 h at 30ºC with no agitation. **(a)** Dual $\log_2$ fold change (Log2FC) plot, comparing differential gene expression values of *ΔHemKR vs wt* strains. Genes with False Discovery Rate-adjusted P-values (Padj) ≤ 0.05 are coloured in blue (dark = in both strains; light = in one strain), and greyed when Padj > 0.05. Selected genes (shown in panel (b)) are indicated with labelled arrows. **(b)** The Log2FC is plotted for selected genes implicated in heme/iron metabolism, for *wt* and *ΔHemKR* strains as indicated. All of them have significant differences (Padj≤0.05) with respect to untreated cells (see Table S1, sheet 3).

a- genes induced by iron deficit:

I- *hemTUVS* (genes p*LEPBI0015/14/13/12*, on the antisense strand, with only *hemT* being plotted in Figure 4, as they all show similar induction behaviour), and *pLEPBI0016/17/18* (on the sense strand adjacent to *hemTUVS*, similarly overexpressed). These plasmid-borne genes encode, on the one hand, a periplasmic heme-carrier protein (HemT) and an inner-membrane heme-permease complex ABC-transporter (HemUV) (Letoffe et al., 2008). On the other hand, *pLEPBI0018* codes for a TonB-dependent outer-membrane receptor/transporter, homologous to the free heme uptake receptor PhuR (Smith & Wilks, 2015), strongly suggesting it could act in concert with HemTUV. *pLEPB0018* seems part of an operon, as *pLEPB0016/17/18* ORFs partly overlap among each other, and the three exhibit consistent transcription levels under all conditions. *pLEPB0016/17* code for proteins annotated as hypothetical, yet interestingly, *pLEPB0017* encodes a protein orthologous to HmuY (Wójtowicz et al., 2009), an outer-membrane heme-recruiter that then delivers heme to outer-membrane heme receptors (such as pLEPB0018). Also worth noting, homologous *hemTUV* operons in other bacteria are repressed by metal-charged Fur (Letoffe et al., 2006; Runyen-Janecky et al., 2010), whereas HemS has been proposed to act as a cytoplasmic heme-sequestering protein, dealing with excess heme toxicity in Gram negatives (Choby & Skaar, 2016).;

II- another putative TonB-dependent outer-membrane receptor/transporter (*LEPBIa2760*), homologous to PhuR, and comprised within an upregulated operon that includes a second iron-dependent membrane protein (*LEPBIa2762*);

III- the outer-membrane TonB-dependent ferric citrate receptor/transporter *fecA* (*LEPBIa1883*); and,

IV- the heme oxygenase *hmuO* (*LEPBIa0669*).

b- *genes repressed by iron deficit:*

I- an operon including the gene that codes for bacterioferritin *bfrB* (*LEPBIa1791*) –a protein that captures/stores intracellular iron;

II- a nitric oxidoreductase (*LEPBIb0063*) –comprising a heme-binding cytochrome C-like domain;

III- the *hemACBLENG* operon (*LEPBIa1171/70/69/68/67/66/65*, only *hemA* is shown, the other genes of the operon similarly exhibit repressed profiles); and,

IV- the *exbB1/exbD1* operon (*LEPBIa0149/48*) coding for the inner-membrane importer subcomplex, part of the TonB-dependent system that supplies energy to drive iron/siderophore/porphyrin outer-membrane transporters.

Most of the activated and repressed genes share the same differential expression trends in both the *wt* and the *ΔHemKR* strains. This is true, except for the *hemACBLENG* and the *exbB1/exbD1* operons, for which the lack of HemKR abolished their signal-triggered behaviour. Intriguingly, in the case of the heme-anabolic genes, iron starvation reversed the normal repression, and instead triggered a ~2-2.5-fold induction only in the *ΔHemKR* KO strain. As for the metal/siderophore/porphyrin importer ExbB1/ExbD1 subcomplex, the KO ceased to exert the significant transcriptional repression of its coding genes. The latter effect could be relevant to explain the larger amounts of heme that remain in the extracellular milieu when comparing KO vs *wt* strains (see Discussion below), especially under iron deprivation conditions (Figure 3). Taken together, this evidence indicates that HemKR plays a role in responding to iron deficiency, but it does so on top of a rich regulatory network likely involving additional transcription factors.

5-aminolevulinic acid triggers a HemKR-dependent regulatory response effecting heme/iron homeostasis

When *Leptospira* bacteria were treated with ALA, a global transcriptional regulatory response was triggered that followed a strong HemKR-dependency. In the *wt* strain, the exposure to 300µM ALA resulted in the downregulation of 128 genes and the upregulation of 51 ($\log_2$>|1|), which contrasted with only 6 and 24, respectively down- and upshifted in the *ΔHemKR* mutant (Figure 5a).
Even though once again most of the regulated genes encode hypothetical proteins, all the differentially expressed genes annotated as heme and/or iron metabolism related, were observed to be regulated only in the *wt* and not in the *ΔHemKR* KO strain (Figure 5b):

a- genes induced by ALA:

I- *hmuO* coding for heme oxygenase; and,

II- a Fur-like/PerR-like regulator (*LEPBIa2461*).

b- genes repressed by ALA:

I- the *hemACBLENG* heme-biosynthetic operon;

II- the *exbB1/exbD1* TonB-dependent transporter operon;

III- an operon including a TonB-dependent outer-membrane receptor/transporter (*LEPBIa3432*), homologous to PhuR; and,

IV- the *hemTUVS* heme-permease, and the neighbouring TonB-dependent PhuR-like receptor *pLEPBI0018*.

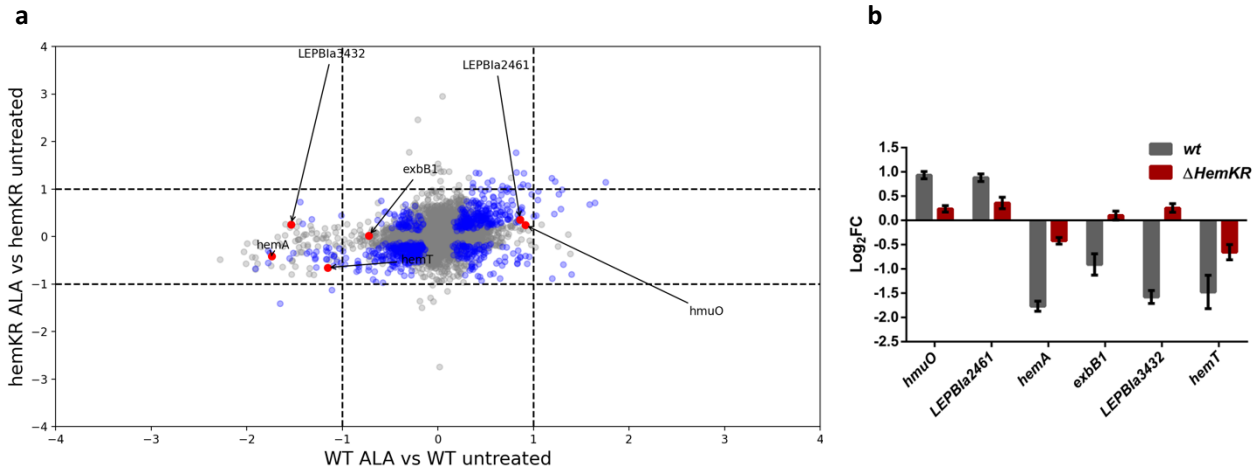


Figure 5. 5-aminolevulinic acid (ALA) induces a largely HemKR-dependent transcriptional regulation response. Transcriptomic differential gene expression analysis comparing *L. biflexa* cells treated *vs* untreated with 300µM ALA for 3 h at 30ºC with no agitation. **(a)** Dual Log2FC plot, comparing differential gene expression values of *ΔHemKR vs wt* strains. Genes with Padj ≤ 0.05 are coloured in blue (dark = in both strains; light = in one strain), and greyed when Padj > 0.05. Selected genes (shown in panel (b)) are indicated with labelled arrows. **(b)** The Log2FC is plotted for selected genes implicated in heme/iron metabolism, for *wt* and *ΔHemKR* strains as indicated. All of them have significant differences (Padj ≤ 0.05) with respect to untreated cells (see Table S1, sheet 4).

In contrast to the effect triggered by iron starvation, which resulted in regulatory responses of similar trend in both *wt* and *ΔHemKR* strains, ALA provoked more significant effects in the *wt*, pinpointing this porphyrin-precursor as a specific signal for the HemKR TCS pathway. This global effect is highlighted in dual log2_fold-change plots, in which a diagonal of most upregulated and downregulated genes under iron deprivation conditions, reflects a similar tendency in both mutant and *wt* (Figure 4a). Whereas in the case of the ALA treatment (Figure 5a), a flattened distribution pattern reveals the stronger regulation effect in the *wt*. Consistent with a direct implication of HemR as an ALA-responsive regulator, the promoter regions of several modulated genes (Figure 5) with functional annotation (or reliable homology to annotated genes), contain *hem*-box motifs (Morero et al., 2014). Either identical to the consensus TGACA$(N)_6$TGACA sequence (*e.g.* in *hmuO*, the *hemA* operon, and *exbB1/exbD1*), or including observed minor variations (Morero et al., 2014) from the consensus (in *LEPBIa3432*, and in *hemTUVS/pLEPB0018*; the latter two extend in opposite ways from their promoter, respectively on the anti-sense and sense strands). Contrarywise, several of the genes regulated by iron starvation (Figure 4), do not comprise *hem*-boxes in their promoters (p*LEPBI0015, LEPBIa2760, LEPBIa1883, LEPBIa1791* or *LEPBIb0063*).

Finally, and in line with a heme-homeostatic role of this TCS, the *ΔHemKR* KO mutant exhibited significantly higher transcription levels of the *hemTUVS* and *hmuO* operons, while lower for those encoding *exbB1/exbD1* TonB-dependent transporter, and the *acrA/acrB* that codes for a promiscuous efflux pump (Figure 6).

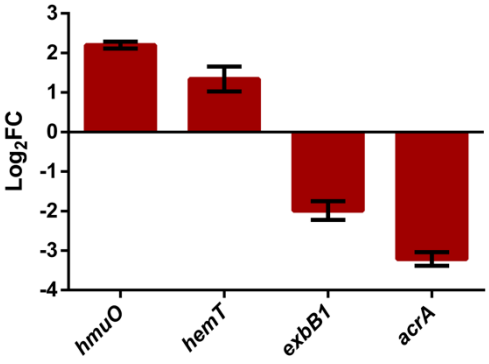


Figure 6. The presence of HemKR is involved in normal regulation of genes linked to heme/iron homeostasis. Transcriptomic differential gene expression analysis comparing *L. biflexa ΔHemKR* relative to *wt* cells, grown under standard conditions (EMJH for 3 h at 30ºC with no agitation). The log_2 fold change (Log2FC) is plotted using three biological replicates.

In summary, the HemKR system senses 5-aminolevulinic acid, ultimately inhibiting heme synthesis and import, while promoting heme degradation. The overall physiologic outcome of such regulation is a feedforward homeostatic effect on intracellular heme.

5-aminolevulinic acid is sensed by HemK triggering its P~HemR-specific phosphatase activity

Considering that variable iron or ALA concentrations produced HemKR-dependent transcriptional responses, we then asked whether the HemKR phosphorylation status is directly modified by those signals. *L. biflexa* cells were grown untreated in EMJH, or subjected to different treatments: 300µM ALA, 35µM DIP or 2mM $FeSO_4$ (Figure 7). Whole protein extracts were then separated with PhosTag SDS-PAGE, and HemR was revealed by Western blot with anti-HemR polyclonal antibodies.

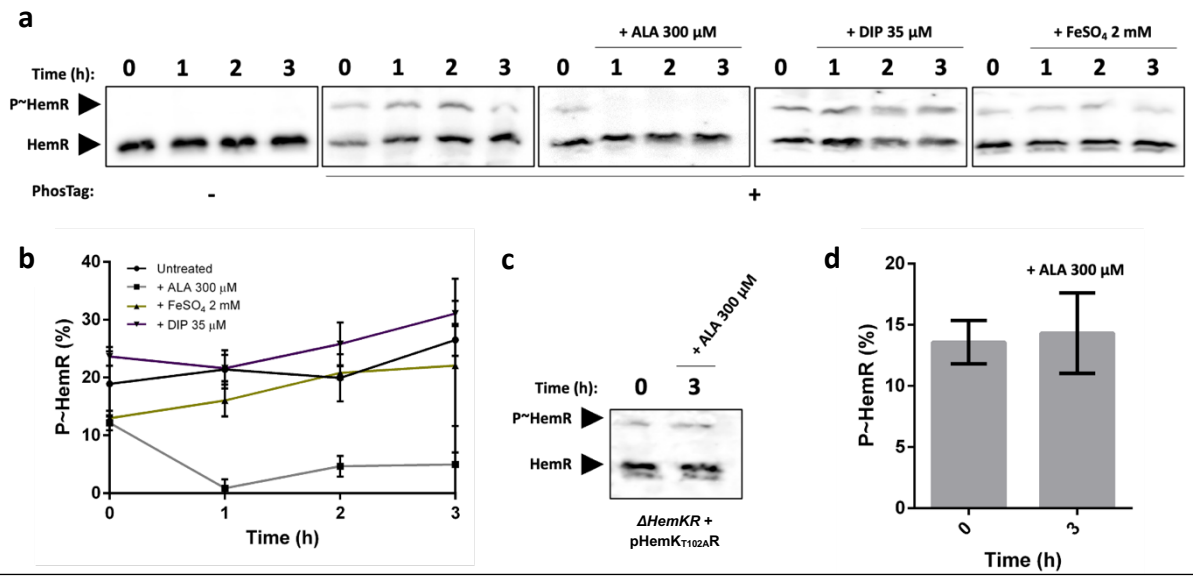
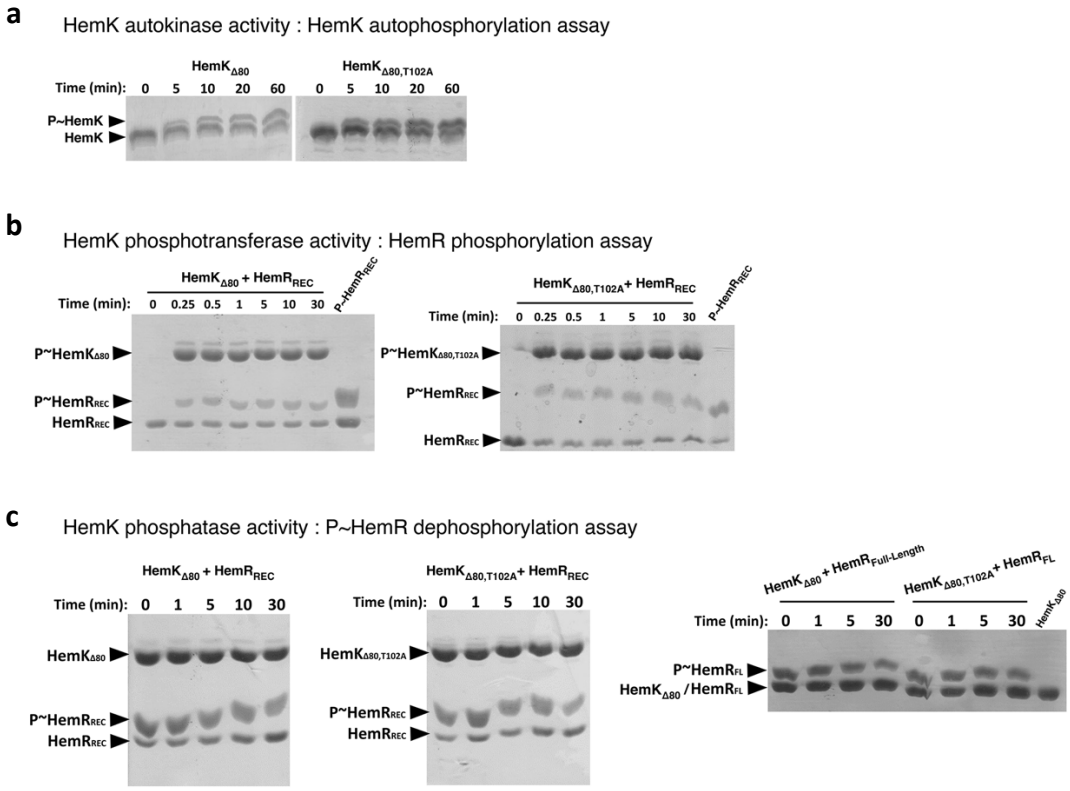


Figure 7. 5-aminolevulinic acid ALA, and not Fe^{3+}, shuts-down HemK-mediated phosphorylation of HemR *in vivo*. (a) PhosTag-SDS-PAGE of whole protein extracts from *L. biflexa wt* cultures, untreated or treated with 5-aminolevulinic acid (ALA), 2,2-dipyridyl (DIP), or an excess of iron ($FeSO_4$), as indicated. Gels were Western blotted and revealed with anti-HemR antibody. Black arrows indicate the electrophoretic mobility position of phosphorylated (P~HemR) and unphosphorylated HemR species. **(b)** Percentage of P~HemR as quantified from panel (a). Measurements were obtained from three biological replicas. **(c)** PhosTag-SDS-PAGE + Western blot as in panel (A) from *ΔHemKR L. biflexa* strain back-complemented by conjugation with plasmid P*hemK*$_{Thr102Ala}$/*hemR*. **(d)** Percentage of P~HemR as quantified from panel (c). Measurements were obtained from three biological replicas.

In untreated *Leptospira* cultures P~HemR represents ~12-25% of the total pool of HemR in the cells at mid exponential phase (Figure 7b). This ratio did not change over time in the conditions of the experiments. While excess or deficit of iron did not produce detectable changes of HemR phosphorylation, exposure to ALA provoked a sustained depletion of P~HemR (Figure 7a,b). Diving deeper into the molecular mechanism, the *ΔHemKR* mutant strain was complemented with a pMAORI (shuttle replicative expression) plasmid containing the *hemK/hemR* operon under its own promoter, encoding for *wt* HemR and the point mutant Thr102Ala of HemK. The latter lacks the sidechain of a key residue for His-kinases to act as a phosphatase toward their cognate response regulator (Buschiazzo & Trajtenberg, 2019; Willett & Kirby, 2012). Confirming the HemKR-mediated mechanism triggered by ALA, the phosphatase-null HemK$_{T102A}$/HemR variant exhibited *wt* levels of HemR and P~HemR but was no longer able to sense ALA and trigger P~HemR dephosphorylation (Figure 7c,d).

A soluble construct of HemK (periplasmic and trans-membrane regions truncated) exhibited autokinase activity (Figure S2a), as well as phosphotransferase catalysis from HemK to HemR (Figure S2b). Consistent with our previous findings in other HKs (Lima et al., 2023), reverse phosphotransfer to HemK was not observed starting the reactions with phosphorylated HemR (Figure S2c). Unexpectedly, HemK-dependent P~HemR dephosphorylation was not detected *in vitro* under different conditions, including presence/absence of ADP (Figure S2c), strongly suggesting that the truncated form of HemK is trapped in

the kinase-active state, and that the periplasmic+transmembrane region is required to shift this HK to a phosphatase state (*e.g.* triggered by ALA sensing).



Supporting Figure S2. Autokinase, phosphotransferase and phosphatase activities of the soluble portion of HemK. (a) Autophosphorylation of HemK$_{\Delta 80}$ (left panel) and the phosphatase-null point-mutant HemK$_{\Delta 80,T102A}$ (right), incubated for indicated times with 5mM ATP-Mg^{2+}. **(b)** Phosphoryl-transfer kinetics from P~HemK$_{\Delta 80}$ (left) or P~HemK$_{\Delta 80,T102A}$ (right) to HemR$_{REC}$, in the presence of 5mM ATP-Mg^{2+}. **(c)** HemK-catalysed phosphatase activity, assayed on P~HemR$_{REC}$, using HemK$_{\Delta 80}$ (left panel) or HemK$_{\Delta 80,T102A}$ (centre). The dephosphorylation of the regulator was not detectable under any condition. 5mM ADP-Mg^{2+} was included in the reactions, as some HisKA His-kinases are known to require nucleotide to exert their phosphatase activity (indistinguishable results were obtained with no nucleotide added). The right-most panel reproduces the previous panels' assays but using a full-length construct of HemR (HemR$_{FL}$), ruling out the potential need of its DNA-binding domain to detect HemK phosphatase activity. The phosphorylated species of HemK and HemR are indicated (P~HemK and P~HemR) and suffer a mobility shift during electrophoresis due to the co-polymerization of PhosTag into the polyacrylamide gels. Each experiment was done by duplicate, representative Coomassie-stained gels are shown. Gels were scanned and bands were quantified by densitometry using ImageJ (data not shown).

All the evidence considered, HemK is a sensor of 5-aminolevulinic acid, reacting to it by inducing its phosphatase activity toward its cognate substrate HemR, and ultimately shutting down the signalling pathway.

Discussion

Heme, as well as many other tetrapyrroles —including the corrin macrocycle of vitamin B12; bilanes; siroheme; chlorophyll in photosynthetic organisms; etc— play central roles in Biology. Heme is an iron-complexed porphyrin (one of the families of cyclic tetrapyrroles), which acts as an essential cofactor for many proteins and enzymes. The heme macrocycle encompasses a complete system of conjugated double bonds, resulting in its characteristic red pigmentation (Layer, 2021). Heme b is one of the most ubiquitous variants of the porphyrin, enabling heme-proteins to carry out electron transport in respiration, diverse oxidoreductive enzymatic catalyses (catalase, nitric oxide synthase, etc), or yet oxygen storage/transport functions. Heme is also involved in signalling and regulation, such as through gas sensing, microRNA processing and cell differentiation (Faller et al., 2007; Shelver et al., 1997; Chen & London, 1981). Heme and iron homeostasis are intimately linked. Even though iron is an essential micronutrient for most living organisms (Crichton & Pierre, 2001), allowing for respiration, nitrogen fixation, hydrogen evolution, and a myriad of other processes, the very oxidoreductive chemistry that lies at the basis of iron's functions (Pierre & Fontecave, 1999), transforms the free metal into a potentially deadly toxic, capable of damaging nucleic acids and proteins irreversibly. Among the many ways that cells possess to maintain a healthy iron homeostasis (Bradley et al., 2020), binding the metal to heme, both in its ferrous and ferric oxidations states, is a widespread means to sequester the cation out of solution.

The *Leptospira* genus is no exception, requiring iron to live (Faine, 1959) and adapting to its extracellular availability (Lo et al., 2010). *Leptospira* is also able to synthetise heme *de novo* (Guegan et al., 2003), leading to the identification (Louvel et al., 2008) and further characterization (Morero et al., 2014) of the two-component system HemK/HemR (HemKR) as a regulator of heme metabolism. HemKR activates and represses genes coding for heme-metabolism enzymes, a heme regulon whose promoters include a *hem*-box to which phosphorylated HemR binds (Morero et al., 2014). However, until now the signal(s) perceived by HemK was not known.

To further confirm the involvement of the HemKR TCS in controlling heme homeostasis, we now extended previous studies characterizing the live phenotype of a double knock-out *L. biflexa* strain *ΔHemKR*. The lack of this signalling TCS not only results in this mutant secreting significantly more heme to the extracellular milieu compared to a *wt* strain, but it showcases the expected link to iron homeostasis in that it renders the cells more resistant to iron deficit. The latter behaviour correlates to the upregulated expression of metal and/or metal-chelate transporters as revealed by the transcriptomic analyses that we performed. Considering the known effect of P~HemR in activating the transcription of heme-anabolic genes while simultaneously repressing the catabolic heme oxygenase (Morero et al., 2014), potential signals sensed by HemK could lie among heme's building blocks. On the one hand, 5-aminolevulinic acid (ALA), a committed substrate towards porphyrin biosynthesis (heme, vitamin B12 and siroheme); on the other hand, iron itself that, catalysed by ferrochelatase HemH, is added to protoporphyrin IX producing heme, along the classical heme synthesis pathway (Figure 1). The approach we followed was based on RNASeq transcriptomics to study differential gene expression (DGE) in response to treatments that modified ALA or Fe^{3+} availabilities. Comparing DGE profiles of *wt vs ΔHemKR* strains was a means to pinpoint HemKR-dependent signalling/regulation.

Most differentially regulated genes triggered by excess ALA or Fe^{3+} deprivation, encode hypothetical proteins, underscoring the need for further investigations. Nonetheless, the annotated sequences consistently correspond to proteins related to heme metabolism and porphyrin-binding, as well as

iron/iron-chelate transport and storage, revealing valuable information. While the transcriptomic response to iron revealed a rich regulatory network, in which HemKR plays an important role together with other unidentified factors, ALA triggered a more discriminated response, largely dependent on HemKR.

The response to iron deficit is largely consistent with the results of a previous study on the pathogenic *L. interrogans* (Lo et al., 2010), strongly suggesting that these essential metabolic networks are broadly conserved within the genus. Metal deprivation triggers a very strong regulatory response overall leading to maximizing the entry of iron via heme and iron transporters, as well as mobilizing the intracellular iron pool by inducing heme demetallation –via heme oxygenase HmuO– while repressing bacterioferritin-sequestered iron storage and heme biosynthesis (Figure 4). A direct role of the HemKR TCS is evidenced, in that the *ΔHemKR* strain not only changes the fold-change expression of several of these target genes, but even more strikingly, in that it inverts the effect on the key heme-biosynthetic *hemA* operon: in coordination with yet unidentified factors, the lack of HemKR results in an abnormal overexpression of the *hemACBLENG* gene cluster, which might explain why the mutant ends up secreting excess of the tetrapyrrole, to minimize its toxic effects. The response to iron availability was not only more complex in terms of the mixed dependence on HemKR and additional transcriptional modulators, but also proved irrelevant to modify the phosphorylation level of HemR *in vivo* (Figure 7).
The reaction of *L. biflexa* cells to 5-aminolevulinic acid (ALA) was, in contrast, sharper, and directly signalled through HemKR (Figure 5). We discovered that under standard culture conditions HemK is constitutively turned on, auto-phosphorylating and transferring the phosphoryl moiety to HemR, such that a basal ~12-25% of total RR is kept in its phosphorylated, DNA-binding competent state (Figure 7). The presence of an external source of ALA, a committed substrate toward porphyrin synthesis, is sensed by HemK, shutting down its phosphoryl-transfer activity and uncovering its P~HemR-specific phosphatase function (Figure 8).

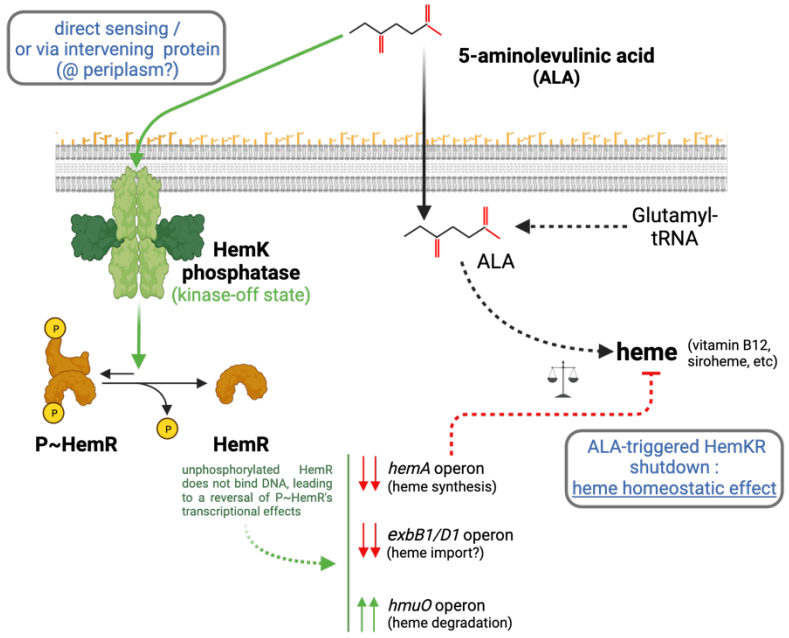


Figure 8. Schematic model of the effect of ALA on shutting down HemKR. Inducing the dephosphorylation of P~HemR, the consequent transcriptional effects on several heme-related genes/operons ultimately contributes to heme homeostasis.

Cues that turn off TCSs are well known to play important and diverse biological roles including in quorum sensing (Neiditch et al., 2006), chemotaxis (Parkinson et al., 2015), virulence regulation (Lesne et al.,

2018), among many others (Gao & Stock, 2017). ALA provoked a prolonged dephosphorylation of P~HemR *in vivo*, which was dependent on HemK's phosphatase activity. Four residues away from the phosphorylatable histidine, HisKA family HKs (such as HemK) harbour an extremely conserved residue —Thr or Asn—, critical to catalysing the hydrolytic dephosphorylation of their cognate P~RR partner (Buschiazzo & Trajtenberg, 2019). In HemK that position is occupied by Thr102, which after substitution by an alanine, confirmed that $HemK_{T102A}$'s phosphorylation toward HemR is similar to the *wt*'s, yet abolishing its phosphatase activity and with it, HemK's capacity to sense and respond to ALA (Figure 7). It is worth noting that ALA induces a similar-trend effect compared to the lack of Fe^{3+}, inducing higher HmuO and lower HemA expression. HemKR might be controlling the porphyrin/Fe ratio —a constant ratio in the cells to avoid toxicity—, rather than just the separate concentration of any of those two molecules on their own. However, these hypotheses need to be tested with designed target gene mutants, and step-by-step functional analyses, considering that other genes are inversely regulated in response to iron and ALA, such as several iron and heme importer systems.

Further investigations shall uncover whether ALA is directly bound to HemK, which possesses an unusually short periplasmic loop instead of a full-blown sensory domain. Alternatively, ALA could bind to a third protein component, mimicking *e.g.* the quorum sensing system in *Vibrio harveyi* (Neiditch et al., 2006), in which the signalling molecule autoinducer-2, binds to periplasmic LuxP, which in turn associates to the transmembrane HK LuxQ triggering its HK shut-down response. Intriguingly, a third gene (*LEPBIa1421*) is present very close to the *hemK/hemR* pair in *L. biflexa*'s genome, perhaps within an operon. This third gene codes for a hypothetical protein particularly rich in tyrosine residues, with predicted periplasmic location and an all-alpha 3D structure. Highly conserved in the *Leptospira* genus, knocking its gene out has so far proved unsuccessful, this third protein could play a role in binding ALA and/or other cognate signals for the HemKR TCS.

ALA is very similar to glutamic acid, from which it is metabolically derived along the C5 biosynthetic pathway (Figure 1). An important metabolite in life, ALA plays key roles from within the extracellular milieu, notably in plants where it is used as a stress-protective factor and growth-promotor (Akram & Ashraf, 2013), or yet in medical applications associated to its photosensitive properties (Fukuda et al., 2005). Porin-dependent and -independent translocation, anticipate straightforward penetration of ALA into the periplasm as with other amino acids (Ude et al., 2021). How could *Leptospira* cells be confronted to increased extracellular ALA concentrations, especially in the case of *L. biflexa* that is a free-living bacterium? *Leptospira* is known to form biofilms, and in these community-driven organizations, different molecular cues are relevant to modulate biofilm formation/disassembly, or yet to trigger disparate metabolic profiles of different groups of cells in the colony. Indeed, secreted amino acids modulate biofilm biology in both Gram-positive (Kolodkin-Gal et al., 2010) and Gram-negative bacteria (Bernier et al., 2011). On the other hand, pathogenic *Leptospira* species encode a functional HemKR TCS, whose genes are intriguingly translocated to a different locus, in the middle of the *hemA* biosynthetic operon (Louvel et al., 2008). In pathogens ALA could play obvious roles in host tropism/infection such as other amino acids do (Robinson et al., 2021). Further studies of HemK-dependent ALA-sensing in pathogenic *Leptospira* spp. shall also be valuable, considering that the heme oxygenase HmuO belongs to the HemR regulon and is strongly regulated by the HemKR TCS (Morero et al., 2014); and this study). HmuO is a known virulence factor for leptospirosis agents (Murray et al., 2008; Murray et al., 2009), eventually linking heme homeostasis to pathogenesis by intriguing and still unknown mechanisms.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

Leptospira biflexa serovar Patoc strain Patoc 1, and a derived double-knockout mutant strain *hemK*$^-$/*hemR*$^-$ (or *ΔHemKR*) (National Reference Center for Leptospirosis, Institut Pasteur, Paris, France) were grown at 30 ºC in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid media (Goarant et al., 2020). For growth measurements of *wt* and *ΔHemKR* strains, cells were grown with no agitation, optical density at 420 nm was measured every 24 h, and cell viability confirmed under dark field microscopy (AxioImager A2.LED, Zeiss). In indicated cases, 300 µM 5-aminolevulinic acid (ALA), 2 mM $Fe_2(SO_4)_3$, or 35µM / 70µM 2,2'-dipyridyl (DIP), were added to the culture. Agitation was used only to grow cultures in the search of biliverdin, under 2 mM $Fe_2(SO_4)_3$ as indicated in the text. For all cultures, genotypes were confirmed by PCR with oligonucleotides HemK_Fw and HemR_Rev (Table S2).

Supporting Table S2

List of Oligonucleotides	
Genotyping	
HemK_Fw	GAGATTTCGTCGAAGTTCAAAGTTG
HemR_Rev	CAAGCACCGAGAGAAACCGAAAAC
HemK2_Rev	CTGAAATGGTTTTGGCTTGGCG
Mutagenesis	
HemR_pMAORI_Fw	CCTCACTAAAGGGAACAAAAGCTGATGAAACCAAGAATTTTACTCGTAGAAG
HemR_pMAORI_Rev	GTGGGAATATGCTATACGGATTTGCCTCATACCAAGGGTTGAAGAGG
p_{hemKR}_pMAORI_Fw	CCTCACTAAAGGGAACAAAAGCTGGAGTGTTCGATTCCTTGGTCTACTG
p_{hemKR}_pMAORI_Rev	CTTCTACGAGTAAAATTCTTGGTTTCATTTTTGCCTCTCAAAACAAGATCAGG
HemK_pMAORI_Fw	CCTGATCTTGTTTTGAGAGGCAAAATTGAGAGGATCTGCACAATTTC
HemK_pMAORI_Rev	CTTCTACGAGTAAAATTCTTGGTTTCATGTGACCTCGTTCAAACTCAGTTTGAC
$HemK_{T102A}$_Fw	CTCACGAGATGAAAGCGCCTATCGCCAGCTTAC
HemK1_Rev	CATGTGACCTCGTTCAAACTCAG

The shuttle expression plasmid pMAORI (Spc^R) (Pappas et al., 2015) was used to express mutated HemK-encoding gene constructs in *Leptospira*. Cloning and pMAORI manipulation/preparation was done in *E. coli* strain π1 (*ΔthyA*) cells (Demarre et al., 2005). For *Leptospira* conjugation, pMAORI was transformed into *E. coli* strain β2163 (*ΔdapA*) (Demarre et al., 2005) and then introduced by conjugation into *L. biflexa* (Picardeau, 2008). *E. coli* strains π1 and β2163 were grown in LB containing 50 µg/mL spectinomycin, the former strain supplemented with 0.3 mM thymidine, whereas the latter with 0.3 mM diaminopimelic acid (Demarre et al., 2005).

Spectroscopic analyses

L. biflexa wt and *ΔhemKR* strains were grown at 30ºC with no agitation (or when indicated, agitated at 100 rpm), either untreated or treated with 300 µM 5-aminolevulinic acid. At the third (mid-exponential phase), seventh (late-exponential phase) and tenth (stationary phase) day of growth, cells were harvested by centrifugation at 3220 g for 20 min., and supernatants thereof further centrifugated at 18000 g for 30 min. Cells grew faster if agitated, in which case they reached stationary phase at day 3. To monitor growth 200 µL of culture supernatants were transferred to plates and absorbance scans measured with a spectrophotometer (230 to 800 nm wavelength).

Cloning of complementation plasmids

To complement the *L. biflexa ΔhemKR* mutant, the wildtype *hemKR* allele harbouring its own promoter region (349 bp upstream *hemK*'s start codon), was amplified from *wt L. biflexa* using primers HemR_pMAORI_Fw, HemR_pMAORI_Rev, HemK_pMAORI_Fw, HemK_pMAORI_Rev p_{hemKR}_pMAORI_Fw and p_{hemKR}_pMAORI_Rev (Table S2). Site-directed mutagenesis was performed to generate a $hemK_{Thr102Ala}$ mutant with primers $HemK_{T102A}$_Fw and HemK1_Rev (Table S2) and inserted into pMAORI (Pappas et al., 2015) by restriction-free cloning (van den Ent & Lowe, 2006). Correctly recombined pMAORI constructs were confirmed by BamHI/EcoRI digestion profile. Cloned pMAORI-*hemKR* and pMAORI-$hemK_{T102A}R$ plasmids were then transformed into *E. coli* strains π1 and β2163 for downstream conjugation experiments.

Conjugation

E. coli strain β2163 cells harbouring the pMAORI-*hemKR* and pMAORI-$hemK_{T102A}R$ plasmids were coincubated with *L. biflexa* cells in EMJH liquid media supplemented with 0.3 mM DAP (when required), until OD_{420} 0.3, according to standard procedures (Picardeau, 2008). Conjugants were incubated in solid EMJH agar plates supplemented with 50 µg/mL spectinomycin, at 30 ºC for 7-10 days. Colonies obtained were grown in liquid EMJH medium supplemented with 50 µg/mL spectinomycin, and the presence of the plasmid was confirmed by PCR with primers HemK_Fw and HemK2_Rev (Table S2).

RNA extraction

Cultures of wt and *L. biflexa* serovar Patoc strain Patoc and *L. biflexa* serovar Patoc $hemKR^-$ double knockout mutant were grown in liquid EMJH media with no shaking at 30 ºC until mid-exponential phase was reached (OD_{420} ~0.2). Untreated cultures and cultures treated with iron sulfate 2 mM, δ-aminolevulinic acid 300 µM and 2,2'-dipyridyl 35 µM were further incubated 3 h at 30 ºC with no shaking. RNA extraction was then performed as previously described (Zavala-Alvarado & Benaroudj, 2020).

RNA Seq

The bioinformatic analyses of whole transcriptomes and differential gene expression were performed with Sequana (Cokelaer et al., 2017). Specifically, the RNA-seq pipeline (v0.15.1) available at https://github.com/sequana/sequana_rnaseq was employed, built upon the Snakemake framework

(Koster & Rahmann, 2012). To prepare the data, reads were trimmed for adapter sequences and low-quality bases using fastp software v0.20.1 (Chen et al., 2018) and were subsequently mapped to the Leptospira genome using bowtie2 (Langmead & Salzberg, 2012). The nucleotide sequence and annotation were downloaded from the MicroScope platform (Vallenet et al., 2020) using the *Leptospira biflexa serovar Patoc* Patoc 1 entry. The count matrix was generated using FeatureCounts 2.0.0 (Liao et al., 2014), which assigned reads to relevant features based on the previously mentioned annotation. To identify differentially regulated genes, statistical analysis was performed with the DESeq2 library 1.30.0 (Love et al., 2014), and HTML reports were generated using the Sequana RNA-seq pipeline. Key parameters for the statistical analysis encompassed significance, measured by Benjamini-Hochberg adjusted p-values with a false discovery rate (FDR) threshold of less than 0.05, as well as the effect size, quantified through fold-change calculations for each comparison.

PhosTag-SDS-PAGE and Western blot of whole protein extracts

L. biflexa cultures were grown in 30 mL EMJH until OD_{420} ~0.2. Prior to 5-aminolevulinic acid or 2,2'-dipyridyl incubation, an aliquot of 6.5 mL of untreated culture was harvested at 10000 g for 10 mins at 4 ºC. When indicated, 300 µM 5-aminolevulinic acid or 35 µM 2,2'-dipyridyl were added to the cultures, and 6.5 mL aliquots were harvested at 1, 2 and 3 hs incubation. Cell pellets were lysed resuspending with BugBuster and Lysonase according to the manufacturer's protocol (Merck). Lysed cells were fractionated by centrifugation at 16000 g for 20 min at 4 ºC, and an aliquot of the soluble fraction was resuspended in SDS buffer with 25 µM dithiotreitol and incubated 10 min at RT, followed by 50 µM iodoacetamide for extra 10 min at RT. Samples were run in SDS-PAGE 12% bis-acrylamide, co-polymerized with 100µM PhosTag (Wako) and 200µM $ZnCl_2$. PhosTag in the presence of transition metals, shifts the electrophoretic mobility of phosphorylated proteins (Gao & Stock, 2018; Kinoshita et al., 2006), allowing to quantify P~HemR/HemR ratios. Electrophoreses were run at <10 ºC. Before Western blotting (WB), PhosTag was eliminated by washing gels with SDS-PAGE running buffer and 2mM EDTA for 10 min, and then with no EDTA for extra 10 min. Gels were blotted to nitrocellulose HyBond (Cytiva) membrane overnight with a TE 22 Mighty Small Transphor Tank Transfer Unit (Amersham) at 20 V.

Polyclonal monospecific anti-HemR antibodies (αHemR) were produced in rabbits. Immunizations were done at Instituto Polo Tecnológico de Pando (Uruguay) respecting ethical guidelines for the use of animals in research. Briefly, 100µg pure recombinant HemR (produced in *E. coli*, see (Morero et al., 2014) for construct and purification procedures) were injected subcutaneously with complete Freund's adjuvant, followed by two boosters of 50µg HemR in incomplete Freund's at 14 and 28 days post-first immunization. Animals were bled for intermediate titration assays, and at ~60 days post-immunization for sera separation, stored at -20ºC until use. WB membranes were pre-blocked with 3% bovine serum albumin in 0.1% PBS-Tween for 3.5 h at RT and gentle agitation. αHemR anti-serum was diluted 1/100 in blocking solution, and added to the WB membrane, with gentle agitation for 2 h RT. Membranes were washed four times with 0.1% PBS-Tween 15 min/each, with strong agitation. Secondary anti-rabbit IgG (Sigma) antibody diluted 1/20000 in blocking solution was added to the WB membrane, with gentle agitation for 1 h RT. Four washing steps were repeated as before, and WB membranes finally incubated with bioluminescent reagents (BM Chemiluminescence Western Blotting Kit, Roche) for 5 min. Protein bands were revealed and quantified using an ImageQuant™ 800 (Cytiva) WB imaging system.

Expression and purification of recombinant proteins

pQE80L (Qiagen) plasmids harbouring $HemK_{\Delta 80}$ (encoding a truncated version of the kinase, lacking the first 80 amino acids), $HemK_{\Delta 80,T102A}$ ($HemK_{\Delta 80}$ with Thr102 substituted by Ala), $HemR_{REC}$ (recever domain only) and $HemR_{FL}$ (full-length) ORFs, each with an encoded Tobacco Etch Virus (TEV) protease cleavage site for N-terminal His-tag removal, were transformed into *E. coli* RosettaGammi(DE3) —for HemK— or TOP10F' —for HemR— cells. Transformed RosettaGammi(DE3) cells were grown with agitation (250 rpm) in 2xYT supplemented with 100 µg/mL ampicillin at 37ºC until OD_{600} ~ 1. Induction was carried with 1 mM IPTG at 30ºC for 6 h. Transformed TOP10F' cells grown similarly, until OD_{600} ~ 0.6, when induction using 1 mM IPTG was performed at 37ºC for 3 h. Cells were harvested by centrifugation at 4000g, and pellets resuspended in 50 mM Tris pH 8, 500 mM NaCl, EDTA-free protease inhibitors (Roche) and 0.1 mg/mL DNase I (Sigma). Cells were disrupted with 1 mg/mL lysozyme followed by 1 cycle of freezing/thawing and high-pressure homogenisation (Emulsiflex-C5, Avestin).

Soluble fractions were obtained by centrifugation at 30000g for 30 min, and supernatants subjected to Ni^{2+} affinity chromatography (HisTrap, Cytiva) in buffer A (50 mM buffer Tris pH 8, 500 mM NaCl and 120 mM imidazole for recombinant $HemK_{\Delta 80}$ and $HemK_{\Delta 80,T102A}$ purification, or 20 mM imidazole for $HemR_{REC}$ and $HemR_{FL}$). Elution was performed with a gradient of buffer B (buffer A with added 750 mM imidazole for $HemK_{\Delta 80}$ and $HemK_{\Delta 80,T102A}$ purification, and 500 mM imidazole for $HemR_{REC}$ and $HemR_{FL}$). Eluted fractions were pooled and incubated overnight with TEV protease in dialysis buffer (50 mM Tris pH 8, 500 mM NaCl). Digested $HemR_{REC}$ and $HemR_{FL}$ pools were subjected to a second Ni^{2+} column purification step to separate cleaved His-tag and TEV protease from the sample. All samples were finally subjected to size-exclusion chromatography (Superdex 16/60 75 prep column, Cytiva), previously equilibrated with 25 mM Tris pH 8 and 500 mM NaCl. The peak fractions were pooled, concentrated, and stored at -80ºC until use.

Autophosphorylation, phosphoryl-transfer and dephosphorylation catalysis assays

For autophosphorylation assays, 20 µM $HemK_{\Delta 80}$ or $HemK_{\Delta 80,T102A}$ were incubated with 5 mM ATP, 10 mM $MgCl_2$. For phosphotransfer assays, 20 µM $HemK_{\Delta 80}$ or $HemK_{\Delta 80,T102A}$ previously incubated with 5 mM ATP, 10 mM $MgCl_2$ for 60 min at RT, were then incubated with equimolar concentrations of $HemR_{REC}$ or $HemR_{FL}$. For phosphatase assays, 600 µM $HemR_{REC}$ or $HemR_{FL}$ were phosphorylated by incubating with 5 mM acetyl phosphate, 10 mM $MgCl_2$; then dimeric, phosphorylated $HemR_{REC}$ or $HemR_{FL}$, were purified from monomeric, non-phosphorylated forms, by size-exclusion chromatography (Superdex 10/300 75 prep, Cytiva). 20 µM of phosphorylated $HemR_{REC}$ or $HemR_{FL}$ were then incubated with equimolar concentrations of $HemK_{\Delta 80}$ or $HemK_{\Delta 80,T102A}$. At defined time intervals, reactions were stopped with SDS buffer and 25 mM DTT for 10 min at RT, followed by further incubation with 50 mM IAA for additional 10 min. All samples were then separated by PhosTag-SDS-PAGE, as described above. Gels were stained with Coomassie blue, scanned with a CanoScan Lide 110 (Canon) scanner, and bands quantified by densitometry using ImageJ (Schindelin et al., 2012).

ACKNOWLEDGMENTS

JAI had PhD fellowships from ANII (POS_NAC_2016_1_ 129903) and CAP/Udelar. AB was supported by grants FCE_1_2017_1_136291 (ANII-Uruguay); and, together with MP, by the IMiZA Pasteur International

Joint Research Unit (Inst Pasteur/Inst Pasteur Montevideo). Members of the Biomics Platform, C2RT, Institut Pasteur, Paris, France, were supported by France Génomique (ANR-10-INBS-09) and IBISA.

REFERENCES

Abdelhameed, S. A. M., Ly, H. G. T., Moons, J., de Azambuja, F., Proost, P., and Parac-Vogt, T. N. (2021) Expanding the reactivity of inorganic clusters towards proteins: the interplay between the redox and hydrolytic activity of Ce(iv)-substituted polyoxometalates as artificial proteases. *Chem Sci* **12**: 10655-10663.

Akram, N. A., and Ashraf, M. (2013) Regulation in Plant Stress Tolerance by a Potential Plant Growth Regulator, 5-Aminolevulinic Acid. *Journal of Plant Growth Regulation* **32**: 663-679.

Anzaldi, L. L., and Skaar, E. P. (2010) Overcoming the heme paradox: heme toxicity and tolerance in bacterial pathogens. *Infect Immun* **78**: 4977-4989.

Bem, A. E., Velikova, N., Pellicer, M. T., Baarlen, P., Marina, A., and Wells, J. M. (2015) Bacterial histidine kinases as novel antibacterial drug targets. *ACS Chem Biol* **10**: 213-224.

Benjamini, Y., and Hochberg, Y. (1995) Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B-Statistical Methodology* **57**: 289-300.

Bernier, S. P., Ha, D. G., Khan, W., Merritt, J. H., and O'Toole, G. A. (2011) Modulation of Pseudomonas aeruginosa surface-associated group behaviors by individual amino acids through c-di-GMP signaling. *Res Microbiol* **162**: 680-688.

Bi, S., and Sourjik, V. (2018) Stimulus sensing and signal processing in bacterial chemotaxis. *Curr Opin Microbiol* **45**: 22-29.

Bibb, L. A., Kunkle, C. A., and Schmitt, M. P. (2007) The ChrA-ChrS and HrrA-HrrS signal transduction systems are required for activation of the hmuO promoter and repression of the hemA promoter in Corynebacterium diphtheriae. *Infect Immun* **75**: 2421-2431.

Bradley, J. M., Svistunenko, D. A., Wilson, M. T., Hemmings, A. M., Moore, G. R., and Le Brun, N. E. (2020) Bacterial iron detoxification at the molecular level. *J Biol Chem* **295**: 17602-17623.

Buschiazzo, A., and Trajtenberg, F. (2019) Two-Component Sensing and Regulation: How Do Histidine Kinases Talk with Response Regulators at the Molecular Level? *Annual review of microbiology* **73**: 507-528.

Chen, J. J., and London, I. M. (1981) Hemin enhances the differentiation of mouse 3T3 cells to adipocytes. *Cell* **26**: 117-122.

Chen, S., Zhou, Y., Chen, Y., and Gu, J. (2018) fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* **34**: i884-i890.

Choby, J. E., and Skaar, E. P. (2016) Heme Synthesis and Acquisition in Bacterial Pathogens. *Journal of molecular biology* **428**: 3408-3428.

Cokelaer, T., Desvillechabrol, D., Legendre, R., and Cardon, M. (2017) 'Sequana': a Set of Snakemake NGS pipeline. *J Open Source Softw* **2**: 352.

Costa, F., Hagan, J. E., Calcagno, J., Kane, M., Torgerson, P., Martinez-Silveira, M. S., . . . Ko, A. I. (2015) Global Morbidity and Mortality of Leptospirosis: A Systematic Review. *PLoS Negl Trop Dis* **9**: e0003898.

Crichton, R. R., and Pierre, J. L. (2001) Old iron, young copper: from Mars to Venus. *Biometals* **14**: 99-112.

Dailey, H. A., Dailey, T. A., Gerdes, S., Jahn, D., Jahn, M., O'Brian, M. R., and Warren, M. J. (2017) Prokaryotic Heme Biosynthesis: Multiple Pathways to a Common Essential Product. *Microbiol Mol Biol Rev* **81**.

Demarre, G., Guerout, A. M., Matsumoto-Mashimo, C., Rowe-Magnus, D. A., Marliere, P., and Mazel, D. (2005) A new family of mobilizable suicide plasmids based on broad host range R388 plasmid (IncW) and RP4 plasmid (IncPalpha) conjugative machineries and their cognate Escherichia coli host strains. *Res Microbiol* **156**: 245-255.

Faine, S. (1959) Iron as a growth requirement for pathogenic Leptospira. *J Gen Microbiol* **20**: 246-251.

Faller, M., Matsunaga, M., Yin, S., Loo, J. A., and Guo, F. (2007) Heme is involved in microRNA processing. *Nat Struct Mol Biol* **14**: 23-29.

Francis, V. I., and Porter, S. L. (2019) Multikinase Networks: Two-Component Signaling Networks Integrating Multiple Stimuli. *Annual review of microbiology* **73**: 199-223.

Frankenberg-Dinkel, N. (2004) Bacterial heme oxygenases. *Antioxid Redox Signal* **6**: 825-834.

Fukuda, H., Casas, A., and Batlle, A. (2005) Aminolevulinic acid: from its unique biological function to its star role in photodynamic therapy. *The International Journal of Biochemistry & Cell Biology* **37**: 272-276.

Galperin, M. Y., Higdon, R., and Kolker, E. (2010) Interplay of heritage and habitat in the distribution of bacterial signal transduction systems. *Mol Biosyst* **6**: 721-728.

Gao, R., Bouillet, S., and Stock, A. M. (2019) Structural Basis of Response Regulator Function. *Annual review of microbiology* **73**: 175-197.

Gao, R., and Stock, A. M. (2017) Quantitative Kinetic Analyses of Shutting Off a Two-Component System. *mBio* **8**: e00412-00417.

Gao, R., and Stock, A. M. (2018) Overcoming the Cost of Positive Autoregulation by Accelerating the Response with a Coupled Negative Feedback. *Cell Rep* **24**: 3061-3071 e3066.

Goarant, C., Girault, D., Thibeaux, R., and Soupé-Gilbert, M.-E., (2020) Isolation and Culture of Leptospira from Clinical and Environmental Samples. In: Leptospira spp. Methods and Protocols N. Koizumi & M. Picardeau (eds). New York: Humana New York, pp. 1-9.

Guegan, R., Camadro, J. M., Saint Girons, I., and Picardeau, M. (2003) Leptospira spp. possess a complete haem biosynthetic pathway and are able to use exogenous haem sources. *Mol Microbiol* **49**: 745-754.

Harshey, R. M., Kawagishi, I., Maddock, J., and Kenney, L. J. (2003) Function, diversity, and evolution of signal transduction in prokaryotes. *Dev Cell* **4**: 459-465.

Huynh, T. N., Noriega, C. E., and Stewart, V. (2010) Conserved mechanism for sensor phosphatase control of two-component signaling revealed in the nitrate sensor NarX. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 21140-21145.

Imelio, J. A., Trajtenberg, F., and Buschiazzo, A. (2021) Allostery and protein plasticity: the keystones for bacterial signaling and regulation. *Biophys Rev* **13**: 943-953.

Jacob-Dubuisson, F., Mechaly, A., Betton, J. M., and Antoine, R. (2018) Structural insights into the signalling mechanisms of two-component systems. *Nat Rev Microbiol* **16**: 585-593.

Keppel, M., Hunnefeld, M., Filipchyk, A., Viets, U., Davoudi, C. F., Kruger, A., . . . Frunzke, J. (2020) HrrSA orchestrates a systemic response to heme and determines prioritization of terminal cytochrome oxidase expression. *Nucleic Acids Res* **48**: 6547-6562.

Kinoshita, E., Kinoshita-Kikuta, E., Takiyama, K., and Koike, T. (2006) Phosphate-binding tag, a new tool to visualize phosphorylated proteins. *Mol Cell Proteomics* **5**: 749-757.

Kolodkin-Gal, I., Romero, D., Cao, S., Clardy, J., Kolter, R., and Losick, R. (2010) D-amino acids trigger biofilm disassembly. *Science* **328**: 627-629.

Koster, J., and Rahmann, S. (2012) Snakemake--a scalable bioinformatics workflow engine. *Bioinformatics* **28**: 2520-2522.

Krewulak, K. D., and Vogel, H. J. (2011) TonB or not TonB: is that the question? *Biochem Cell Biol* **89**: 87-97.

Langmead, B., and Salzberg, S. L. (2012) Fast gapped-read alignment with Bowtie 2. *Nature methods* **9**: 357-359.

Layer, G. (2021) Heme biosynthesis in prokaryotes. *Biochim Biophys Acta Mol Cell Res* **1868**: 118861.

Lesne, E., Dupre, E., Lensink, M. F., Locht, C., Antoine, R., and Jacob-Dubuisson, F. (2018) Coiled-Coil Antagonism Regulates Activity of Venus Flytrap-Domain-Containing Sensor Kinases of the BvgS Family. *mBio* **9**: e02052-02017.

Letoffe, S., Delepelaire, P., and Wandersman, C. (2006) The housekeeping dipeptide permease is the Escherichia coli heme transporter and functions with two optional peptide binding proteins. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 12891-12896.

Letoffe, S., Delepelaire, P., and Wandersman, C. (2008) Functional differences between heme permeases: Serratia marcescens HemTUV permease exhibits a narrower substrate specificity (restricted to heme) than the Escherichia coli DppABCDF peptide-heme permease. *Journal of bacteriology* **190**: 1866-1870.

Liao, Y., Smyth, G. K., and Shi, W. (2014) featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**: 923-930.

Lima, S., Blanco, J., Olivieri, F., Imelio, J. A., Nieves, M., Carrion, F., . . . Trajtenberg, F. (2023) An allosteric switch ensures efficient unidirectional information transmission by the histidine kinase DesK from Bacillus subtilis. *Sci Signal* **16**: eabo7588.

Lo, M., Murray, G. L., Khoo, C. A., Haake, D. A., Zuerner, R. L., and Adler, B. (2010) Transcriptional response of Leptospira interrogans to iron limitation and characterization of a PerR homolog. *Infect Immun* **78**: 4850-4859.

Louvel, H., Betton, J. M., and Picardeau, M. (2008) Heme rescues a two-component system Leptospira biflexa mutant. *BMC Microbiol* **8**: 25.

Louvel, H., Bommezzadri, S., Zidane, N., Boursaux-Eude, C., Creno, S., Magnier, A., . . . Picardeau, M. (2006) Comparative and functional genomic analyses of iron transport and regulation in Leptospira spp. *Journal of bacteriology* **188**: 7893-7904.

Love, M. I., Huber, W., and Anders, S. (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* **15**: 550.

Moreira, L. M., Poli, A. L., Costa-Filho, A. J., and Imasato, H. (2008) Ferric species equilibrium of the giant extracellular hemoglobin of Glossoscolex paulistus in alkaline medium: HALS hemichrome as a precursor of pentacoordinate species. *Int J Biol Macromol* **42**: 103-110.

Morero, N. R., Botti, H., Nitta, K. R., Carrion, F., Obal, G., Picardeau, M., and Buschiazzo, A. (2014) HemR is an OmpR/PhoB-like response regulator from Leptospira, which simultaneously effects transcriptional activation and repression of key haem metabolism genes. *Mol Microbiol* **94**: 340-352.

Murray, G. L., Ellis, K. M., Lo, M., and Adler, B. (2008) Leptospira interrogans requires a functional heme oxygenase to scavenge iron from hemoglobin. *Microbes Infect* **10**: 791-797.

Murray, G. L., Srikram, A., Henry, R., Puapairoj, A., Sermswan, R. W., and Adler, B. (2009) Leptospira interrogans requires heme oxygenase for disease pathogenesis. *Microbes Infect* **11**: 311-314.

Neiditch, M. B., Federle, M. J., Pompeani, A. J., Kelly, R. C., Swem, D. L., Jeffrey, P. D., . . . Hughson, F. M. (2006) Ligand-induced asymmetry in histidine sensor kinase complex regulates quorum sensing. *Cell* **126**: 1095-1108.

Panek, H., and O'Brian, M. R. (2002) A whole genome view of prokaryotic haem biosynthesis. *Microbiology (Reading)* **148**: 2273-2282.

Pappas, C. J., Benaroudj, N., and Picardeau, M. (2015) A replicative plasmid vector allows efficient complementation of pathogenic Leptospira strains. *Appl Environ Microbiol* **81**: 3176-3181.

Parashar, V., Mirouze, N., Dubnau, D. A., and Neiditch, M. B. (2011) Structural basis of response regulator dephosphorylation by Rap phosphatases. *PLoS biology* **9**: e1000589.

Parkinson, J. S., Hazelbauer, G. L., and Falke, J. J. (2015) Signaling and sensory adaptation in Escherichia coli chemoreceptors: 2015 update. *Trends Microbiol* **23**: 257-266.

Picardeau, M. (2008) Conjugative transfer between Escherichia coli and Leptospira spp. as a new genetic tool. *Appl Environ Microbiol* **74**: 319-322.

Picardeau, M. (2017) Virulence of the zoonotic agent of leptospirosis: still terra incognita? *Nat Rev Microbiol* **15**: 297-307.

Pierre, J. L., and Fontecave, M. (1999) Iron and activated oxygen species in biology: the basic chemistry. *Biometals* **12**: 195-199.

Posey, J. E., and Gherardini, F. C. (2000) Lack of a role for iron in the Lyme disease pathogen. *Science* **288**: 1651-1653.

Ratliff, A. C., Buchanan, S. K., and Celia, H. (2022) The Ton Motor. *Front Microbiol* **13**: 852955.

Robinson, C. D., Sweeney, E. G., Ngo, J., Ma, E., Perkins, A., Smith, T. J., . . . Guillemin, K. (2021) Host-emitted amino acid cues regulate bacterial chemokinesis to enhance colonization. *Cell Host Microbe* **29**: 1221-1234 e1228.

Runyen-Janecky, L. J., Brown, A. N., Ott, B., Tujuba, H. G., and Rio, R. V. (2010) Regulation of high-affinity iron acquisition homologues in the tsetse fly symbiont Sodalis glossinidius. *Journal of bacteriology* **192**: 3780-3787.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., . . . Cardona, A. (2012) Fiji: an open-source platform for biological-image analysis. *Nature methods* **9**: 676-682.

Scott, J. C., Klein, B. A., Duran-Pinedo, A., Hu, L., and Duncan, M. J. (2013) A two-component system regulates hemin acquisition in Porphyromonas gingivalis. *PLoS One* **8**: e73351.

Shelver, D., Kerby, R. L., He, Y., and Roberts, G. P. (1997) CooA, a CO-sensing transcription factor from Rhodospirillum rubrum, is a CO-binding heme protein. *Proceedings of the National Academy of Sciences of the United States of America* **94**: 11216-11220.

Silversmith, R. E. (2010) Auxiliary phosphatases in two-component signal transduction. *Curr Opin Microbiol* **13**: 177-183.

Smith, A. D., and Wilks, A. (2015) Differential contributions of the outer membrane receptors PhuR and HasR to heme acquisition in Pseudomonas aeruginosa. *J Biol Chem* **290**: 7756-7766.

Soldano, A., Klinke, S., Otero, L. H., Rivera, M., Catalano-Dupuy, D. L., and Ceccarelli, E. A. (2017) Structural and mutational analyses of the Leptospira interrogans virulence-related heme oxygenase provide insights into its catalytic mechanism. *PLoS One* **12**: e0182535.

St Laurent, G., Shtokalo, D., Tackett, M. R., Yang, Z., Vyatkin, Y., Milos, P. M., . . . Kapranov, P. (2013) On the importance of small changes in RNA expression. *Methods* **63**: 18-24.

Stauff, D. L., and Skaar, E. P. (2009) Bacillus anthracis HssRS signalling to HrtAB regulates haem resistance during infection. *Mol Microbiol* **72**: 763-778.

Steele, K. H., O'Connor, L. H., Burpo, N., Kohler, K., and Johnston, J. W. (2012) Characterization of a ferrous iron-responsive two-component system in nontypeable Haemophilus influenzae. *Journal of bacteriology* **194**: 6162-6173.

Stock, J. B., Stock, A. M., and Mottonen, J. M. (1990) Signal transduction in bacteria. *Nature* **344**: 395-400.

Traore, E. S., Li, J., Chiura, T., Geng, J., Sachla, A. J., Yoshimoto, F., . . . Liu, A. (2021) Heme Binding to HupZ with a C-Terminal Tag from Group A Streptococcus. *Molecules* **26**.

Ude, J., Tripathi, V., Buyck, J. M., Soderholm, S., Cunrath, O., Fanous, J., . . . Bumann, D. (2021) Outer membrane permeability: Antimicrobials and diverse nutrients bypass porins in Pseudomonas aeruginosa. *Proceedings of the National Academy of Sciences of the United States of America* **118**.

Vallenet, D., Calteau, A., Dubois, M., Amours, P., Bazin, A., Beuvin, M., . . . Medigue, C. (2020) MicroScope: an integrated platform for the annotation and exploration of microbial gene functions through genomic, pangenomic and metabolic comparative analysis. *Nucleic Acids Res* **48**: D579-D589.

van den Ent, F., and Lowe, J. (2006) RF cloning: a restriction-free method for inserting target genes into plasmids. *J Biochem Biophys Methods* **67**: 67-74.

Wandersman, C., and Stojiljkovic, I. (2000) Bacterial heme sources: the role of heme, hemoprotein receptors and hemophores. *Curr Opin Microbiol* **3**: 215-220.

Willett, J. W., and Kirby, J. R. (2012) Genetic and biochemical dissection of a HisKA domain identifies residues required exclusively for kinase and phosphatase activities. *PLoS Genet* **8**: e1003084.

Wójtowicz, H., Guevara, T., Tallant, C., Olczak, M., Sroka, A., Potempa, J., . . . Gomis-Rüth, F. X. (2009) Unique structure and stability of HmuY, a novel heme-binding protein of Porphyromonas gingivalis. *PLoS Pathog* **5**: e1000419.

Wolgemuth, C. W., Charon, N. W., Goldstein, S. F., and Goldstein, R. E. (2006) The flagellar cytoskeleton of the spirochetes. *J Mol Microbiol Biotechnol* **11**: 221-227.

Wosten, M. M., Kox, L. F., Chamnongpol, S., Soncini, F. C., and Groisman, E. A. (2000) A signal transduction system that responds to extracellular iron. *Cell* **103**: 113-125.

Wuichet, K., Cantwell, B. J., and Zhulin, I. B. (2010) Evolution and phyletic distribution of two-component signal transduction systems. *Curr Opin Microbiol* **13**: 219-225.

Zavala-Alvarado, C., and Benaroudj, N., (2020) The Single-Step Method of RNA Purification Applied to Leptospira. In: Leptospira spp. Methods and Protocols N. Koizumi & M. Picardeau (eds). New York: Humana New York, pp. 41-51.