Title:

The Diversity of Heme Sensor Systems – Heme-responsive Transcriptional Regulation Mediated by Transient Heme Protein Interactions

Aileen Krüger[1)], Marc Keppel[1)], Vikas Sharma[1)], Julia Frunzke[1)*]

1) Forschungszentrum Jülich GmbH, Institute for Bio- and Geosciences 1, IBG1, 52425 Jülich, Germany

*corresponding author: Prof. Dr. Julia Frunzke (j.frunzke@fz-juelich.de)

One-sentence Summary:

This review covers diverse prokaryotic and eukaryotic heme sensor systems and discusses the mechanism of signal perception, heme binding pockets and network architecture focusing on systems with a role in the control of heme homeostasis.

1. Abstract

Heme is a versatile molecule that is vital for nearly all cellular life by serving as prosthetic group for various enzymes or as nutritional iron source for diverse microbial species. However, elevated levels of heme molecule are toxic to cells. The complexity of this stimulus has shaped the evolution of diverse heme sensor systems, which are involved in heme-dependent transcriptional regulation in eukaryotes and prokaryotes. The functions of these systems are manifold – ranging from the specific control of heme detoxification or uptake systems to the global integration of heme and iron homeostasis. This review focuses on heme sensor systems, regulating heme homeostasis by transient heme protein interaction. We provide an overview of known heme-binding motifs in prokaryotic and eukaryotic transcription factors. Besides the central ligands, the surrounding amino acid environment was shown to play a pivotal role in heme binding. The diversity of heme-regulatory systems therefore illustrates that prediction based on pure sequence information is hardly possible and requires careful experimental validation. Comprehensive understanding of heme-regulated processes is not only important for our understanding of cellular physiology, but also provides a basis for the development of novel antibacterial drugs and metabolic engineering strategies.

Keywords: Heme, heme sensor systems, heme-protein interaction, heme regulatory motifs, heme homeostasis, HrtBA

2. Introduction

Heme - iron bound protoporphyrin IX - is an essential molecule for nearly all cells and is synthesized and used by prokaryotes and eukaryotes alike (Ponka, 1999). It has an important role in critical cellular processes like electron transfer, respiration and oxygen metabolism (Ajioka et al., 2006) where it serves as prosthetic group of cytochromes, hydroxylases, catalases, peroxidases, and hemoglobins (Layer et al., 2010). Since hemoglobin is the most abundant reservoir of iron in the human body, host synthesized heme is often the only reliable source of iron for many pathogenic bacteria (Contreras et al., 2014). However, also many non-pathogenic bacteria rely on the salvage of heme-bound iron in iron-depleted environments (Wilks, 2002).

Similar to iron, heme causes severe toxicity at elevated levels. In human cells, the toxicity is speculated to partly originate from the redox-active iron, triggering the formation of reactive oxygen species via the Fenton and the Haber-Weiss reaction, thereby damaging lipids, proteins, and genomic DNA (Kumar and Bandyopadhyay, 2005). Additionally, heme is capable of promoting oxidation after accumulation in biological membranes inducing cell lysis (Aft and Mueller, 1984). The mechanism of how this molecule affects bacterial cells is not conclusively unraveled (Anzaldi and Skaar, 2010), but previous studies indicated that also non-iron metalloporphyrins can show a significant antibacterial activity (Stojiljkovic et al., 1999). This hints at a general, iron-independent toxicity of certain porphyrin structures. These probably stimulate the generation of reactive oxygen species, especially upon illumination (Nakahigashi et al., 1991).

Heme is chemically versatile and allows a variety of potential interactions with proteins (Brewitz et al., 2017). A schematic overview of some common heme interacting proteins is given in Figure 1. One can in general distinguish between two types of heme sensing systems (Figure 1A): either heme can be sensed directly, or the porphyrin can act as a sensor of gases such as O_2, CO or NO as well as of the redox state of the cell (Girvan and Munro, 2013).

In hemoproteins, heme is bound with high-affinity coordination motifs and serves as prosthetic group (Figure 1B) for enzymes of essential reactions, including electron transfer, cell respiration or oxygen metabolism (Li et al., 2011; Smith et al., 2010). In contrast, transient binding allows for situation-dependent responses and rapid reactions to environmental fluctuations (Granick et al., 1975). Transient heme binding is found for several heme-responsive sensor systems involved in transcriptional regulation. Targets of these systems are highly diverse ranging from genes involved in the circadian rhythm (e.g. NPAS2 (Dioum et al., 2002), Rev-erbα and Rev-erbβ (Raghuram et al., 2007)), demethylases (e.g. Gis1 (Lal et al., 2018)) or glucose metabolism (Rev-erbα (Yin et al., 2007)). Intuitively, another important role of heme sensor systems is the control of heme homeostasis, which is the focus of this review. Heme-protein interactions can also trigger protein degradation (Dent et al., 2019; Ogawa et al., 2001; Qi et al., 1999)), the regulation of K^+-channels (e.g. mammalian Kv1.4 channels (Sahoo et al., 2013; Tang et al., 2003)) or the heme-based regulation of tRNA synthesis, mRNA splicing, or protein-protein interactions, which are further summarized e.g. in the review article by Shimizu et al. (2019).

In this review, we will focus on heme sensor systems directly involved in heme sensing, but will not discuss the role of heme-binding proteins in gas sensing (here, the interested reader is referred to the following articles: Green et al. (2009); Girvan and Munro (2013); Martínková et al. (2013); Shimizu et al. (2015)). Here, we will compare and discuss transient heme sensor systems across the kingdoms, which contribute to the transcriptional regulation of heme/iron homeostasis, including heme utilization, heme biosynthesis, detoxification, or related responses. In general, we will discuss similarities and differences of prokaryotic and eukaryotic heme sensor systems with respect to signal perception, transduction and regulon structure. The examples also highlight the variability in heme binding motifs in the different systems across domains of life. An overview of the heme sensor systems discussed in this review is presented in Figure 2 and Table 1.

3. Heme Sensor Systems in Eukaryotes

In eukaryotes, mainly two different heme sensor systems acting as transient heme sensors have been described: The transcription factor BACH1 balancing the cellular heme content of mammalian cells, and Hap1 of the yeast *Saccharomyces cerevisiae*, which regulates the expression of heme-requiring enzymes like cytochromes in a heme-dependent manner in response to hypoxia.

3.1 BTB and CNC homology 1 (BACH1) in mammalian cells

In mammalian cells, the activity of a conserved transcription factor named BTB and CNC homology 1 (BACH1, Figure 2A) is influenced by heme-binding (Ogawa et al., 2001). This protein acts as a key player in balancing the cellular heme content by regulating *HMOX1*, encoding a heme oxygenase (Sun et al., 2002), as well as the iron storage protein ferritin, a thioredoxin reductase (Hintze et al., 2007), the NAD(P)H:menadione oxidoreductase 1 (Dhakshinamoorthy et al., 2005) and more than 50 other target genes (Warnatz et al., 2011).

In the absence of heme, BACH1 was shown to heterodimerize with small Maf proteins (e.g. MafF, MafG, MafK), which represent conserved transcription factors among vertebrates (Simile et al., 2018). BACH1-Maf heterodimers bind to the promoter regions of Maf recognition elements (MARE) in order to inhibit the transcription of numerous target genes, like e.g. *HMOX1* encoding a heme oxygenase (Igarashi et al., 1998; Igarashi et al., 1994; Motohashi et al., 1997; Oyake et al., 1996). In order to achieve dimerization with Maf proteins, BACH1 contains at least two functional domains: (i) an N-terminal BTB/POZ domain containing the protein interaction motif to form a multivalent DNA-binding complex (Bardwell and Treisman, 1994; Igarashi et al., 1998; Zollman et al., 1994), and (ii) a C-terminal bZip domain which binds DNA and consequently mediates the heterodimerization (Oyake et al., 1996).

The presence of intracellular heme negatively regulates the repressor activities of BACH1 (Oyake et al., 1996). This is achieved by a direct interaction of heme with BACH1, binding with high affinity to the cysteine-proline (CP) motifs of the heme-responsive motifs (HRMs). CP-motifs are well known heme binding motifs (Lathrop and Timko, 1993), which will be discussed in the context of HRMs more detailed throughout chapter 5. BACH1 overall contains six CP-motifs: two downstream the BTB/POZ domain (CP1-2) and four surrounding the bZip domain (CP3-6). However, it was shown that only the latter ones are pivotal for function (Ogawa et al., 2001). Interestingly, no single CP-motif is essential, but simultaneous mutation of all four motifs (CP3, CP4, CP5 and CP6) abolished interaction with heme, suggesting cooperativity between CPs (Ogawa et al., 2001). While the exact molecular mechanism of heme-responsive activation remains to be elucidated, a recent study in mice suggests a binding of a CP-motif to heme via a 5-coordination (Segawa et al., 2019). Upon heme binding, structural changes around the bZip domain are likely, which finally lead to the dissociation of BACH1 from the enhancers and a derepression of target genes (Ogawa et al., 2001). For example, derepressed MafK can bind to the transcription factor Nrf2 that activate several protective antioxidant genes (Dhakshinamoorthy et al., 2005). In addition, a nuclear export signal for BACH1 is activated, as well as HOIL-1-mediated ubiquitination and consequent degradation (Zenke-Kawasaki et al., 2007).

3.2 Heme activator protein 1 (Hap1) in the yeast *Saccharomyces cerevisiae*

In *S. cerevisiae*, the heme activator protein Hap1 (Figure 2B) is important for heme-responsive gene expression (Hon et al., 2005; Zhang and Hach, 1999) and the indirect sensing of hypoxia via the availability of heme (Zitomer et al., 1997). The regulon of Hap1 includes genes encoding various cytochromes like *CYC1* (Guarente and Mason, 1983), *CYC7* (Lowry and Zitomer, 1988) or *CYT1* (Schneider and Guarente, 1991), the *CTT1* gene coding for a catalase coping with oxidative stress (Winkler et al., 1988) and the *YHB1* gene coding for flavohemoglobin

(Buisson and Labbe-Bois, 1998). These targets have in common that all encode proteins requiring heme as prosthetic group.

Since heme biosynthesis is oxygen-dependent in yeast (Hon et al., 2003), Hap1 is connecting heme levels to the oxygen availability. This is also reflected by its regulon, as Hap1 acts as an activator of several genes involved in aerobic metabolism, while genes for the hypoxic metabolism like e.g. *ANB1* (Lowry and Zitomer, 1988) are indirectly repressed by the activation of the transcriptional repressor *ROX1* (Ter Linde and Steensma, 2002).

The Hap1 activity increases with increasing heme concentrations (Zhang and Guarente, 1994a). In the absence of heme, Hap1 was described to be present in a putative high-molecular weight complex (HMC) (Zhang and Guarente, 1994b). Besides Hap1, this HMC encompasses four further cellular proteins, including the heat shock proteins Hsp82 and Ydj1 (Zhang et al., 1998), and probably also Hsp70 and Hsp90 (Zhang and Hach, 1999). To allow Hap1 binding to the DNA, the HMC needs to be disrupted by direct binding of heme to the protein (Zhang and Guarente, 1994b; Zhang et al., 1998).

Considering the molecular mechanism of heme binding and activation, Hap1 can roughly be divided into three domains: (i) a heme-binding domain, (ii) a DNA binding domain, and (iii) an activation domain (Creusot et al., 1988; Pfeifer et al., 1989; Zhang et al., 1998). The heme domain is responsible for the transient binding of heme and comprises seven repeats called heme-responsive motifs (HRMs) 1-7, with HRM1-6 being located near the DNA-binding domain and HRM7 near the activation domain (Zhang and Guarente, 1995). HRM7 was reported to be pivotal for heme binding via a cysteine-proline (CP) motif (Lathrop and Timko, 1993) at heme-sufficient conditions, with cysteine absolutely essential for heme binding and proline increasing the affinity of interaction (Zhang and Guarente, 1995). This leads to conformational changes of Hap1 permitting the disassembly of the HMC and consequent activation (Zhang and Hach, 1999). HRM1-6 are suggested to rather scavenge heme at low levels and are significantly less important in activation of Hap1 (Hon et al., 2000; Lee et al.,

2003). Additionally, Hsp90 was described to be important for Hap1 activation, as it induces conformational changes necessary for its activation (Lee et al., 2003).

The DNA binding domain of Hap1 consists of a C_6 zinc cluster and dimerization domain near its N-terminus (Creusot et al., 1988). The dimerization domain contains the coiled-coil dimerization element and is necessary for Hap1 to bind to the target DNA with high affinity (Zhang et al., 1993). Hap1 binds as a dimer to DNA containing two CGG triplets (Zhang and Guarente, 1994c). Finally, the acidic activation domain located at the C-terminus was reported to be involved in Hap1 activation (Pfeifer et al., 1989). In the absence of heme, Hap1 is inactivated by so-called repression modules (RPM1-3). This process involves the molecular chaperones Hsp70 and further co-chaperones contributing to the formation of the HMC (Hach et al., 1999; Hon et al., 2001).

4. Heme Sensor Systems in Prokaryotes

4.1 Heme-responsive transcriptional regulators in Gram-negative bacteria

Heme-responsive systems in prokaryotes are highly diverse. In this section, we will focus on two well-described types found in Gram-negative bacteria, including the extracytoplasmic function (ECF) σ-factor based system from Pseudomonales and Burkholderiales as well as the Irr protein playing a major role in the control of iron homeostasis in Rhizobiales. Finally, we will briefly touch on the recently reported regulator Har from *Porphyromonas gingivalis*.

4.1.1 The heme assimilation system (Has) in *Pseudomonas* and *Serratia*

The **h**eme **as**similiation (Has) system represents a well-characterized heme-responsive extracytoplasmic function (ECF) σ-factor signal transduction cascade in *Pseudomonas aeruginosa* (Dent et al., 2019; Ochsner et al., 2000) (Figure 2C) and *Serratia marcescens* (Biville et al., 2004; Rossi et al., 2003). These systems play an important role in heme

acquisition and utilization in these pathogenic species. The Has system compromises four important components: (i) the hemophore HasA, (ii) the outer membrane receptor HasR, (iii) the anti-σ-factor HasS and (iv) the σ-factor HasI.

The secreted, extracellular hemophore HasA passively scavenges heme from the environment. HasA was shown to form a heme pocket with two loops, one with a histidine (H^{32}) residue and the other with a tyrosine (Y^{75}) residue in addition to a stabilizing histidine (His^{83}), remarkably in both *P. aeruginosa* and *S. marcescens* (Jepkorir et al., 2010; Létoffé et al., 2001; Yukl et al., 2010). Binding of heme to the hemophore was described to be caused by π-π stacking and van der Waals interactions (Kumar et al., 2014). Heme is then released from this rather high-affinity binding by HasA to the outer membrane receptor HasR with a lower affinity side (Dent et al., 2019). HasA and HasR are encoded in a single operon (Ochsner et al., 2000). This transfer of heme to HasR includes an initial break of the first axial heme coordination in the hemophore at H^{32}, followed by steric displacement of heme by a receptor residue, which ruptures the second axial coordination to T^{75} (Krieg et al., 2009). The tyrosine ligand is thereby weakened on transient protonation by H^{83} (Dent et al., 2021; Dent et al., 2019). The HasR receptor receiving heme encompasses a variant of the known, conserved bacterial heme receptor motif FRAP (W^{609}-R-P-P in *P. aeruginosa*) and NPNL (N^{634}-P-F-L in *P. aeruginosa*) with a typical histidine residue (H^{624} in *P. aeruginosa*, H^{603} in *S. marcescens*) in between these motifs (Dent and Wilks, 2020). FRAP/NPNL motifs are conserved bacterial heme receptor motifs, which are known to be involved in heme binding and transport in several proteins, but are however hardly to predict due to their high variability (Bracken et al., 1999; Nienaber et al., 2001; Stojijkovic et al., 1995). After sensing, conformational changes in HasR lead to a capturing of heme by its N-terminal plug domain, including a crucial H^{221} in *P. aeruginosa*, and H^{189} in *S. marcescens* (Dent and Wilks, 2020). Upon heme binding, this plug interacts with the anti-σ-factor HasS for its inactivation. Such an inactivation leads to a release of the ECF σ-factor HasI, which recruits the core RNA polymerase regulating the transcriptional activation of the whole *hasRA* operon. HasS therefore represents a typical anti-sigma factor, which is involved in the activation of HasI (Dent et al., 2019).

In addition to this heme response for heme acquisition, heme is also transported through the HasR receptor in a TonB-dependent manner. TonB is an energy-transducing protein, and the acquisition of heme as an alternative iron source has already been shown to be TonB-dependent in several Gram-negative bacteria (e.g. (Biswas et al., 1997; Elkins et al., 1998; Occhino et al., 1998)). In *P. aeruginosa*, HasR-imported heme is then further translocated to the cytoplasm by the periplasmic heme import system PhuT-PhuUV. In this case, it was described that the Has system is mainly required for heme sensing, while the *Pseudomonas* heme uptake (Phu) system is additionally required as major heme transporter for efficient import into the cytosol (Dent et al., 2019; Dent and Wilks, 2020; Smith and Wilks, 2015). Comparing it to *S. marcescens*, there is also a non-hemophore heme uptake system (Hem). However, this system appears to have no significant role in heme uptake – in contrast to the *Pseudomonas* system (Benevides-Matos and Biville, 2010; Dent et al., 2019), underlining possible diversity in regulations among this systems.

In the cytoplasm of *P. aeruginosa*, heme is bound by the heme binding protein PhuS, which further transfers the porphyrin to the heme oxygenase HemO, which cleaves the tetrapyrol ring yielding iron, CO and biliverdin IXβ and IXδ. The latter two heme metabolites then act as positive feedback regulators of the hemophore HasA (Mouriño et al., 2016). Apart from biliverdin IXβ- and IXδ-dependent regulation of protein level, it was also shown in *P. aeruginosa* that post-transcriptional processing of *hasRA* mRNA contributes to the Has systems regulation. This processing is suggested to be performed by small regulatory RNAs (sRNAs) and results in different stabilities for a differentially regulated expression of the co-transcribed genes *hasR* and *hasA* (Dent et al., 2019). Even further, the *hasRA* operon is also regulated by Fur (Ochsner et al., 2000), integrating the utilization of heme in the global iron network of the cell. However, in *S. marcescens*, the Has system is rather regulated at the level of transcription with HasI regulating HasS (Biville et al., 2004). In general, when heme levels decline, HasS is activated and sequesters HasI again, thereby resulting in a down-regulation of the Has systems dependent on availability of heme (Biville et al., 2004; Dent et al., 2019).

4.1.2 RhuR and the sigma factor RhuI in the Burkholderiales

The Gram-negative bacterium *Bordetella avium* responds to heme by controlling expression of the *bhuRSTUV* heme acquisition system also via an ECF σ-factor system. This three-component system includes (i) BhuR, the outer membrane receptor for heme (Murphy et al., 2002), (ii) RhuR, a membrane bound activator protein (Kirby et al., 2004) and (iii) the ECF σ-factor RhuI (Kirby et al., 2001). The components of these systems are homolog to the Has systems of Pseudomonales described in the section before. For HasR-BhuR, HasS-RhuR and HasI-RhuI, there are identities above 26% and significant similarities above 40% (NCBIResourceCoordinators, 2016). However, no homolog to the hemophore HasA of *P. aeruginosa* or *S. marcescens* is present in *B. avium*, implying a significant difference between these systems not only concerning heme acquisition but also regulation. However, a HasA homolog with 35% identity to that of *P. aeruginosa* can be found for *B. petrii*.

Two promoters, P_{rhuIR} and $P_{bhuRSTUV}$ control the expression of the *rhuIR-bhuRSTUV* cluster (King et al., 2005). Overall, this regulatory cascade also responds to both Fe stress and the presence of heme and hemoglobin (Kirby et al., 2001; Murphy et al., 2002). In the presence of iron, transcription of the *bhuRSTUV* operon as well as that of *rhuIR* is repressed by Fur, the global iron-dependent transcriptional repressor (Kirby et al., 2001). Upon iron starvation, P_{rhuIR} is derepressed, and in addition to RhuI and RhuR, low levels of *bhuR* are expressed supporting recognition of heme at the outer membrane. It is assumed that this is the result of an incomplete termination of *rhuIR* transcription. This rare 'read-through transcription' allows a fast response if heme becomes available as an alternative iron source (King et al., 2005; Kirby et al., 2004). In the presence of extracellular heme, the cascade starts by binding of heme to the outer membrane receptor BhuR. This receptor also includes a consensus FRAP- and NPNL motif, with Y^{604}-R-A-P and N^{627}-P-N-L in *B. avium*, respectively (Ahn et al., 2005; Bracken et al., 1999; Fusco et al., 2013). However, unlike in other bacterial outer membrane heme receptors, BhuR does not contain the conserved histidine between the FRAP and the NPNL domain, but a

similar tyrosine at position 616 (Murphy et al., 2002). Both histidine and tyrosine were described to be involved in transient heme binding (Li et al., 2011) (compare section 5). Further, two TonB boxes are present in BhuR, required for the interaction with TonB for functioning of the heme utilization system (Murphy et al., 2002; Richard et al., 2019).

BhuR subsequently transduces a signal across the periplasm to RhuR, which was proposed to contain a C-terminal periplasmically exposed controller domain for signal interception. The exact mechanism of how the signal is actually transduced is not yet known, but the signal is further propagated through the cytoplasmic membrane to RhuI. Activation of RhuI is achieved by its interaction with the N-terminal 97-amino-acid region of RhuR and some conformational changes. Therefore, RhuR represents an untypical anti-sigma factor, which is involved in the activation of RhuI instead of simple sequestration and inactivation. Consequently, activated RhuI escorts the RNA polymerase to P_{bhuR} directly or indirectly, leading to high transcription levels of *bhuR* as well as *bhuSTUV* encoding for the heme uptake system (Kirby et al., 2004; Kirby et al., 2001; Vanderpool and Armstrong, 2003). RhuI is described as an absolute necessary activator of BhuR for a respective response to heme utilization (King et al., 2005). When iron requirements are met again, Fur-dependent regulation allows for a rapid downregulation of heme acquisition.

This three-component signal transduction cascade seems to be highly conserved among *Bordetella* species, as similar loci have been described in the mammalian pathogens *B. pertussis* and *B. bronchiseptica* (*hurIR-bhuRSTUV)* (King-Lyons et al., 2007; Vanderpool and Armstrong, 2001). These systems resemble the control of the ferric dicitrate uptake system *fecIR-fecABCDE* of *E. coli* (Pressler et al., 1988).

4.1.3. Indirect sensing via Irr in Rhizobiales

In contrast to other model organisms, rhizobia feature a unique way of controlling iron homeostasis. Their setup is particularly interesting because these bacteria integrate heme and

298 iron homeostasis into one regulon and thereby sense ‘functional’ iron pools in the form of heme
299 instead of free iron (Johnston et al., 2007; O'Brian, 2015).

300 The **i**ron **r**esponse **r**egulator protein (Irr) (Figure 2D) was originally identified in the nitrogen-
301 fixing, symbiotic bacterium *Bradyrhizobium diazoefficiens* (former *B. japonicum*) (Hamza et al.,
302 1998) and was identified as key transcriptional regulator of iron homeostasis controlling heme
303 biosynthesis, import and utilization (Qi et al., 1999). Further Irr orthologues were analyzed in
304 *Rhizobium leguminosarum* (Singleton et al., 2010), *Bartonella quintana* (Parrow et al., 2009),
305 and *Brucella abortus* (Martinez et al., 2005). Irr proteins were found to have both activator and
306 repressor functions. Upon iron limitation, Irr accumulates and acts as a repressor for, e.g.,
307 *hemB*, which encodes the delta-aminolevulinic acid dehydratase of the heme biosynthesis
308 pathway. Thereby, Irr repression avoids the accumulation of toxic porphyrin precursors due to
309 lack of iron (Hamza et al., 1998). Furthermore, Irr serves as an activator of the *hmuVUT*
310 operon, encoding a heme-import system, and further genes responsible for the uptake of ferric
311 iron, to allow fast uptake when these resources become available (Hamza et al., 1998; Rudolph
312 et al., 2006). Binding to the respective target DNA depends on a *cis*-acting DNA element,
313 called iron control element (ICE) upstream of the promoter (Rudolph et al., 2006).
314 Although Irr is a Fur-like protein, it does not bind to iron directly, but senses the iron pool
315 indirectly via heme. Under iron-replete conditions, heme directly interacts with Irr at a heme
316 regulatory motif (HRM) near its N-terminus in *B. diazoefficiens* (Qi et al., 1999; Qi and O'Brian,
317 2002). This HRM contains the putative motif G^{28}-C-P-W-H-D, with $C^{29}P^{30}$ belonging to the
318 known CP-motif, was shown to be essential for binding (Qi et al., 1999). Apart from this high-
319 affinity binding site at the HRM for ferric (oxidized) heme, further studies postulated that there
320 is an additional low-affinity ferrous (reduced) heme-binding site with a histidine-rich motif ($H^{117-119}$)
321 (Qi et al., 1999; Qi and O'Brian, 2002; Yang et al., 2005). Based on mutational studies,
322 both binding sites were suggested to be required for rapid turnover (Yang et al., 2005). In order
323 to properly respond to the iron availability in the cell, it is important that Irr does not sense the
324 extracellular free heme, but only newly synthesized heme. How can this be accomplished? Qi
325 and O’Brian showed that there is a direct interaction of Irr and ferrochelatase, the enzyme

catalyzing the terminal step of heme biosynthesis by inserting an iron into protoporphyrin IX (Qi and O'Brian, 2002). This Irr-ferrochelatase complex is only formed under high iron conditions i.e. when heme is produced. Consequently, complex formation leads to an inhibition of Irr by the ferrochelatase and allows interaction of Irr with the ferrochelatase-generated heme (Qi and O'Brian, 2002). Heme binding finally leads to a degradation of Irr and the consequent derepression of its target genes. This degradation might be accomplished either by changes in the conformation of Irr upon heme binding in such a way that it becomes accessible to proteolysis or the binding leads to protein damage due to the formation of local reactive oxygen species and subsequent proteolytic degradation (Kitatsuji et al., 2016; Kobayashi et al., 2016; Qi et al., 1999; Yang et al., 2006).

In *Rhodobacter sphaeroides*, Irr was found to be involved in the oxidative stress response, e.g. repressing the expression of the catalase gene *katE* under non-stress conditions (Peuser et al., 2012). It is worth noting that some alphaproteobacteria rely entirely on Irr for their iron-responsive gene regulation, while Fur possesses a rather minor role compared to *E. coli*, where it represents the global regulator of iron homeostasis (Johnston et al., 2007). These findings highlight the special role of this Fur-like protein as heme/iron sensor system in Rhizobia.

4.1.3 Fur orthologue Har in Bacteroidales

A recent study has emphasized the **h**eme-**a**ssociated **r**egulator (Har) protein as a heme-dependent transcriptional regulator in *Porphyromonas gingivalis* – a Gram-negative oral pathogen associated with the biofilm-mediated periodontal disease. Har is a Fur orthologue and plays a role in hemin-responsive biofilm development. In the presence of heme, Har binds it via a CP-motif (C^{97}) triggering structural changes within Har. This consequently affects the expression of several genes, including *hmuY*, which is a hemophore scavenging heme from various hemoproteins, and the replication initiator *dnaA*. Overall, Har was described as a positive regulator of biofilm formation (Butler et al., 2014). However, recent studies claimed that the heme binding cannot be shown for all Har variants of different *P. gingivalis* strains

(Śmiga et al., 2019). Thus, further experimental clarification is needed to understand the role of this system in heme-responsive gene expression depending on the strain background.

4.2 Heme-responsive two-component systems of Gram-positive bacteria

In Gram-positive bacteria, two-component systems (TCS) appear to be the predominant form of heme sensing. Prototypical TCSs consist of a membrane bound histidine kinase and a cytoplasmic response regulator receiving the signal from the sensor kinase (Hoch, 2000; Mascher et al., 2006). Upon stimulus perception, e.g. heme-binding, the histidine kinase undergoes autophosphorylation at a conserved histidine residue. The phosphoryl group is subsequently transferred to a conserved aspartate residue of the response regulator. Typically, the phosphorylated form of the response regulator represents its 'active' state, in which it initiates an appropriate cellular output, commonly by binding to the promoter regions of target genes and thereby acting as a transcriptional regulator (Capra and Laub, 2012; Mascher et al., 2006; Stock et al., 2000). Two different examples of heme sensor systems based on TCSs are known in Gram-positive bacteria: the HssRS system and the paralogous systems HrrSA and ChrSA. Similarities and differences in heme-responsive TCS-signaling are discussed in the following.

4.2.1 The heme exporter HrtBA is a prevalent target of heme-responsive TCSs in Gram-positive bacteria

Strikingly, there is one conserved target controlled by all known heme-responsive TCSs in Gram-positive species: The **h**eme-**r**egulated ABC **t**ransporter HrtBA. This exporter is required to counteract toxic heme levels and it consists of two components: the ATPase component HrtA and the permease HrtB. Until now, it could not be directly shown that heme is the substrate of export for HrtBA, or if it is rather a toxic intermediate (Stauff et al., 2008). However, increased levels of intracellular heme in mutants lacking HrtBA (e.g. *Staphylococcus aureus*

as well as *Lactococcus lactis*) (Joubert et al., 2014; Wakeman et al., 2014), are in favor of the hypothesis that heme is, indeed, the respective substrate of this exporter. Studies confirmed that the activity of HrtAB is essential for e.g. *Staphylococcus aureus* and *Corynebacterium glutamicum* to allow growth at toxic heme levels (Bibb and Schmitt, 2010; Heyer et al., 2012; Stauff et al., 2008; Torres et al., 2007). There are many orthologues of the heme exporter HrtBA in Gram-positives (Bibb and Schmitt, 2010; Heyer et al., 2012; Stauff and Skaar, 2009a; Torres et al., 2006), as well as further efflux systems, e.g. PefAB or PefRCD in *Streptococcus agalactiae* (Fernandez et al., 2010). Therefore, the strategy of efflux to cope with the toxicity of heme seems to be conserved, especially among pathogenic bacteria.

In silico analysis of overall 39,096 bacterial genomes revealed the presence of *hrtBA* orthologues in 3,232 of them, from which almost 99% belong to the phyla of Firmicutes or Actinobacteria (Figure 3A). Among those, overall 4,750 genomic hits were found for the *hrtBA* locus. In approximately half of the analyzed Actinobacteria (443 of 905) even two or more copies of *hrtBA* orthologues are present and the same applies for Firmicutes (1,000 of 2,286). When inspecting 5 kb up- and downstream of the identified *hrtBA* operon using the COG database (clusters of orthologous groups of proteins (Tatusov et al., 2000)), we found a HssRS-like TCS in more than 40% of the loci in Firmicutes and a ChrSA-like TCS in >50% of the actinobacterial genomes containing a *hrtBA* locus (Figure 3B). Among the 20 most abundant domains found at the *hrtBA* locus, we identified three further transcriptional regulator domains – namely AcrR, MarR and LytT (Figure 3B). Although being in close proximity, no conserved pattern in terms of synteny could be identified, as it is evident for the ChrSA and HssRS TCSs.

A schematic genomic organization of the HrtBA locus and a respective TCS encoded in close proximity for different species is shown in Figure 3C. These two-component systems will be described in the following sections.

4.2.2 Two-component system HssRS in Bacillales

The pathogenic species *Bacillus anthracis* and *Staphylococcus aureus* have been the model systems to investigate the function of the TCS called the heme sensor system HssRS, which plays a crucial role in the heme-dependent activation of the expression of the genes encoding the heme exporter HrtBA in these species (Figure 2E) (Stauff and Skaar, 2009a; Stauff and Skaar, 2009b). The TCS is composed of the cytoplasmic response regulator HssR and the histidine kinase HssS that is anchored to the membrane via two transmembrane helices (Torres et al., 2007). The operons of *hssRS* and *hrtAB* are divergently oriented (Torres et al., 2007) (Figure 3C).

The periplasmic sensing domain of HssS consisting of 132 amino acids is flanked by two transmembrane helices (Stauff and Skaar, 2009a). Nevertheless, the actual heme sensing mechanism is not yet fully understood – a blind spot for all heme-responsive TCS so far. No potential heme-binding domain in HssS could be revealed and its sensing domain does not contain any conserved heme-binding site found in other heme proteins (Torres et al., 2007). Interestingly, it was suggested that the *S. aureus* kinase HssS might not sense heme molecules directly. The application of several non-iron metalloporphyrins showed that, instead, secondary effects of heme toxicity (e.g., heme-induced cell-wall damage or membrane disruption) might influence the activity of this system (Stauff and Skaar, 2009b; Wakeman et al., 2014). The presence of heme or its toxic effects triggers autophosphorylation of a histidine residue (H^{249} in *S. aureus*) in HssS, with consequent phosphotransfer to the aspartic acid residue of the response regulator HssR (D^{52} in *S. aureus*) (Stauff et al., 2007). HssR is thereby activated - probably via conformational changes (Bronner et al., 2004) - and binds to the promoter region of *hrtAB* (Torres et al., 2007). *S. aureus* codes for a set of proteins, which are designated as **i**ron-regulated **s**urface **d**eterminants (Isd) as well as the **h**eme **t**ransport **s**ystem (Hts). These proteins are important for the utilization of heme as alternative iron source. However, Stauff et al. claimed that HssRS probably does not regulate the expression of these genes, but is rather specific for *hrtAB* (Stauff et al., 2007).

A recent study demonstrated significant cross-talk between HssRS and a second system called HssRS interfacing TCS (HitRS) in *B. anthracis* (Mike et al., 2014; Pi et al., 2020). HitRS was shown to respond to compounds, which induce cell envelope stress, and consequently influence the expression of the operon *hitPQRS*, which includes a yet unstudied ABC transporter HitPQ (Mike et al., 2014). HitRS cross-regulates P_{hrt} at the transcriptional level, while HssRS cross-regulates P_{hit} at both transcriptional as well as on the level of phosphorylation. This interaction of HssRS and HitRS nicely demonstrates an additional integration of cell envelope stress into the heme-responsive HssRS regulon.

In general, the activation of the transporter by the TCS HssRS was shown to be highly important for coping with heme stress as deletion of either component resulted in drastically elevated heme sensitivity and an attenuated virulence of *S. aureus* (Stauff et al., 2007; Torres et al., 2007). Homologous TCSs can be found in *Lactococcus lactis, Staphylococcus epidermidis* or *Listeria innocua* (Stauff and Skaar, 2009a). Notably, *hssRS* homologs seem to be absent in non-pathogenic Bacillales like *B. subtilis* or *B. lichenformis* and this was attributed to the higher demand of heme tolerance for bacterial species infecting mammalian hosts (Stauff and Skaar, 2009b). However, the relevance of the HrtBA transporter and its control by a heme-responsive TCS was also described for the non-pathogenic actinobacterium *Corynebacterium glutamicum*, which uses heme as an important alternative iron source. Here, control is mediated by a different TCS setup described in the next section.

4.2.3 Two-component systems HrrSA and ChrSA in Corynebacteriaceae

A special setup of heme sensor systems can be found in most members of the *Corynebacteriaceae* family. Here, two paralogous TCSs, named HrrSA and ChrSA, represent key players in the control of heme homeostasis. The interaction and regulons of these systems have in particular been studied in the human pathogen *C. diphtheriae* as well as the biotechnologically relevant soil bacterium *C. glutamicum* (Bibb and Schmitt, 2010; Burgos and Schmitt, 2016; Frunzke et al., 2011; Heyer et al., 2012; Schmitt, 1999) (Figure 2F).

Remarkably, this is one example of two paralogous systems reacting to the same stimulus, which is in this case heme; other examples are the NarX-NarL and NarQ-NarP systems of *Escherichia coli*, which both respond to nitrate (Noriega et al., 2010). Nevertheless, Bott and Brocker also reported several exceptions of *Corynebacteriaceae* possessing only one of those TCS e.g. *C. efficiens* (only HrrSA) or *C. jeikeium* (only ChrSA) (Bott and Brocker, 2012).

Since the two TCSs HrrSA and ChrSA share significant sequence identity (*C. glutamicum* HrrS versus ChrS: 39%; HrrA versus ChrA: 57% (Heyer et al., 2012)), cross-talk at the level of phosphorylation and regulation of target genes was no surprise for both *C. glutamicum* (Hentschel et al., 2014) (Figure 4) as well as *C. diphtheriae* (Bibb et al., 2007).

Both systems, ChrSA and HrrSA, show marked differences with respect to their regulons in *C. glutamicum*. While HrrSA acts as a global regulator of heme homeostasis, the function of ChrSA appears to be focused on the detoxification of heme (Frunzke et al., 2011; Hentschel et al., 2014; Heyer et al., 2012) (Figure 4). Recently, genome-wide mapping of HrrA-binding led to the identification of more than 200 different genomic targets of HrrA (Keppel et al., 2020). Among these targets are inter alia *hmuO* encoding the heme oxygenase, as well as genes involved in heme biosynthesis, the respiratory chain, oxidative stress response, and cell envelope remodeling (Keppel et al., 2020). In contrast, the only target promoter of ChrA is the operon encoding the HrtBA heme exporter, which is divergently located to *ChrSA* (Figure 3C) (Heyer et al., 2012; Ito et al., 2009).

However, in contrast to *C. glutamicum*, several studies in *C. diphtheriae* suggest considerable overlap of the HrrSA and ChrSA regulons in this species (Bibb et al., 2005; Bibb and Schmitt, 2010). Here, ChrSA was proposed to be the major TCS required for *hmuO* activation (80 %), with a rather minor contribution of HrrSA (20 %) (Bibb et al., 2007). These examples illustrate the plasticity of heme-responsive TCS signaling which is continuously adapting to the genetic environment and the ecological niche of the particular organism.

Both histidine kinases HrrS and ChrS are embedded into the cytoplasmic membrane via six α-helices and have been proposed to sense the hydrophobic heme molecule via an

intramembrane sensing mechanism (Bibb and Schmitt, 2010; Ito et al., 2009; Keppel et al., 2018). The heme-binding pocket appears to be different for ChrS and HrrS. For HrrS, the three amino acids Y^{112}, F^{115} and F^{118} were identified to be crucial for heme binding. However, mutation of the respective Y^{87}-F^{90}-F^{94} motif in ChrS did not abolish heme sensing but caused only a red shift of the Soret band (Keppel et al., 2018). This underlines differences of heme signal perception in both histidine kinases (compare section 5). For transient heme binding in *C. diphtheriae*, a conserved tyrosine residue Y^{61} located in the second transmembrane helix of ChrS was described to be crucial (Bibb and Schmitt, 2010). However, mutation of the analogous region Y^{74} in HrrS of *C. glutamicum* did not lead to significant effects on heme binding (Keppel et al., 2018).

The high similarity between the paralogous TCS provides the basis for cross-talk. In the case of *C. glutamicum* ChrSA and HrrSA, cross-phosphorylation was shown to facilitate a faster onset of the heme detoxification response upon heme stimulus. Here, the HrrS kinase appears to contribute as a 'kickstarter' by activating the non-cognate response regulator ChrA, allowing a faster induction of the *hrtBA* operon (Keppel et al., 2019).

The fast shut-off of P_{hrtBA} is mediated by the phosphatase activity of the ChrS kinase upon stimulus decline, which is very specific to its cognate response regulator ChrA (Hentschel et al., 2014; Keppel et al., 2019). In contrast, both the gene encoding the response regulator HrrA as well as its target gene *hmuO* are under direct control by the global iron regulator DtxR resulting in a delayed activation of *hmuO* upon heme stimulus due to the repression by DtxR (Keppel et al., 2019; Wennerhold and Bott, 2006). This hierarchy thereby integrates information on iron availability in the regulation of heme utilization and homeostasis.

For *C. diphtheriae* HrrS a study proposed only minimal kinase activity, but it primarily functions as a phosphatase (Burgos and Schmitt, 2016). In summary, several studies have provided insights in the dynamic interplay between ChrSA and HrrSA at the level of phosphorylation, dephosphorylation, and cross-regulation. The interaction with the DtxR regulon provide

insights in the homeostatic network coordinated by these systems integrating responses to heme, iron and related stress responses.

4.2.4 Transcriptional regulator HatRT in anaerobic Clostridia

A new concept of sensing heme in the context of detoxification was recently reported for the obligate anaerobic pathogen *Clostridioides difficile* (Knippel et al., 2020; Knippel et al., 2018). *C. difficile* infection of the host's colon is accompanied by a significant release of heme molecules into the lumen due to erythrocyte lysis and necrotic cell death (Chumbler et al., 2012). In fact, this bacterium is exposed to increased heme levels during infection and is in demand for efficient detoxification strategies. Knippel et al. (2018) identified the **h**eme **a**ctivated **t**ransporter system (HatRT), which is involved in detoxification from heme. This includes the TetR family transcriptional regulator HatR, which binds heme probably transiently (K_D = 9.2 ± 1.8 µM) with H^{99} as critical residue. Formation of the HatR-heme complex derepresses the *hatRT* operon, allowing the expression of the transporter HatT, which functions as a heme efflux pump. Apart from detoxification, this system was also shown to be important for full pathogenicity in a murine model (Knippel et al., 2018).

The same group also identified another **h**eme-**s**ensing **m**embrane protein system HsmRA in *C. difficile* also required for heme tolerance. Heme was shown to bind to the transcriptional regulator HsmR (K_D = 6.6 ± 1.1 µM) at a conserved H^{50} residue, probably placing heme in a cleft between the dimer interfaces and the DNA-binding domain. HsmR then acts as an activator of the *hsmRA* operon. Absorption spectroscopy revealed that the membrane-bound HsmA is able to sequester heme with high-affinity. Heme-bound HsmA then also offers increased resistance to oxidative stress generating compounds, like the clinically relevant antibiotics vancomycin or metronidazole (Knippel et al., 2020) providing another example of the diverse strategies of microbes to deal with elevated heme levels and the resulting oxidative stress.

5. Diversity of transient heme binding in regulatory proteins

Different heme-responsive sensor systems regulating heme homeostasis across kingdoms were presented in the previous sections (summarized in Figure 2 and Table 1). Here, we will take a closer look at the necessity of transient binding of regulatory heme, its general features and challenges and compare the binding mechanisms associated with CP- or non-CP-motifs of the different heme sensor systems.

5.1 The challenge to sense “free” heme

Heme is a versatile signaling molecule. Due to its hydrophobic and poorly soluble nature, it can non-specifically bind to lipids, proteins and further macromolecules causing cytotoxicity. Therefore, it is unlikely that there are large amounts of “free” heme present in the cell. Recent studies claimed that intracellular “free” heme, which has been newly synthesized or released from hemoproteins due to oxidative stress, can only exist transiently in low amounts and is weakly coordinated with water molecules (Chiabrando et al., 2014; Gallio et al., 2021; Walter et al., 2021). In addition to “free” heme and heme bound irreversibly to hemoproteins, further studies adopted the term “exchangeable heme” for another portion of total heme present in the cell. This term describes the part of the heme pool which is transiently bound to proteins or small molecules, so that it can serve as kind of reservoir for heme reflecting its bioavailability (Atamna et al., 2015; de Villiers and Egan, 2021; Gallio et al., 2021). These rather low amounts of the free heme and exchangeable heme can collectively be referred to as regulatory heme, which can engage in transient heme-protein interactions and is therefore accessible to the heme sensor systems discussed in this review (Atamna et al., 2015).

5.2 Heme responsive motifs (HRMs)

While heme binding in hemoproteins or oxygen-sensor systems is well established in terms of structural aspects or sequence features (Gong et al., 2000; Li et al., 2011; Schneider et al., 2007; Smith et al., 2010), the transient binding of heme by regulatory proteins is less understood, as it becomes evident from the examples covered by this review (summarized in Table 1).

Unlike in hemoproteins, the described heme sensor systems do not covalently bind heme moieties or feature high-affinity heme coordination motifs. They rather display a weak but specific heme binding on the protein surface for temporary events and situation-dependent responses with micromolar affinities (e.g. $K_D \approx 13.7$ µM for BACH1 (Segawa et al., 2019)), and the ability to rapidly react to environmental fluctuations (Granick et al., 1975; Qi et al., 1999; Shimizu, 2012; Zhang and Guarente, 1995).

Heme-regulatory motifs (HRMs) are structural characteristics found in several systems. In general, it was described that the central iron ion of heme is coordinated by a heteroatom-containing amino acid side chain at the HRM of the protein. Most often, this amino acid is cysteine (like in Hap1, BACH1 or Irr), but can also be histidine or tyrosine (like in HrrS); nonetheless in few cases it can also be methionine or lysine (Li et al., 2011). However, surrounding amino acids are further contributing to hydrophobic interactions via non-polar amino acids, π-π stacking by the porphyrin ring, electrostatic interactions or hydrogen bonds formed with the propionate side chains (Li et al., 2011; Schneider et al., 2007; Wißbrock et al., 2019). For example, the protein-heme interface was shown to be most often dominated by hydrophobic amino acids, including the aliphatic hydrophobic amino acids leucine, isoleucine, methionine, valine and alanine. The polar amino acids threonine, serine and aspartate are typically not involved as ligands in observed structures in heme proteins (Reedy and Gibney, 2004).

5.3 CP-motif heme binding

589 The most prominent and best-explored HRM encompasses cysteine-proline (CP) motifs (Kühl
590 et al., 2011; Lathrop and Timko, 1993). In CP motifs, the cysteine residue acts as direct ligand
591 of the heme-iron ion (Kühl et al., 2011), while the proline supports the coordination of the
592 cysteine in the thiolate form of the Fe(III) heme complex by incorporating a bend for mainly
593 alpha helices and avoiding the formation of hydrogen bonds (Li et al., 2011; Shimizu, 2012).
594 For the systems discussed in this review, CP-motifs are present in the eukaryotic Hap1 and
595 BACH1 as well as in the prokaryotic Irr protein.

596 CP-motifs are typically located in hydrophobic protein environments, but the heme binding
597 affinities of the particular heme sensing system is of course strongly influenced by the particular
598 amino acids configurations (Figure 5A). The binding affinity of the particular system is the result
599 of adaptation to the prevailing environmental conditions and the physiological function of the
600 heme sensor system. This is, for example, mirrored by the heme binding affinity of human
601 BACH1 ($K_D \approx 13.7$ µM), which is in the range of the heme concentration in human blood (~21
602 µM) (Aich et al., 2015). The binding affinities of the Hap1 protein is in a similar, low-micromolar
603 range ($K_D < 20$ µM) matching the reported intracellular heme concentration of yeast (Hanna et
604 al., 2018).

605 The prokaryotic example of a CP-motif discussed in this review is the rhizobial Irr protein, which
606 is - like BACH1 - degraded upon heme binding. The bulky amino acids tryptophan and histidine
607 right adjacent to the CP-motif of Irr (Figure 5A) were described to assist steric hindrance for
608 preventing hydrogen bond formation (Ishimori and Watanabe, 2014). In contrast to BACH1
609 and Hap1, Irr features significantly higher heme binding affinity than the eukaryotic systems
610 (oxidized heme in *R. leguminosarum*: $K_D \approx 0.01$ µM) (White et al., 2011). Considering the low
611 iron availability of iron in the soil (Colombo et al., 2014), this high affinity is likely required for
612 efficient iron utilization and the establishment of the symbiotic relationships with legumes
613 (O'Hara, 2001). In fact, symbiotic legumes were shown to have a higher iron demand for
614 proper nodule initiation and development (Brear et al., 2013).

Remarkably, all of the three described systems show a heterogeneous heme binding. Analysis of Soret bands displays broad peaks suggested a rather unusual heme binding environment and speaks for multiple configurations of heme binding in BACH1 (Hira et al., 2007), Hap1 (Zhang and Guarente, 1995) and Irr (Ishikawa et al., 2011). As already described in the sections before, Hap1 contains seven HRMs (Zhang and Guarente, 1995), while mammalian BACH1 has four HRMs (Ogawa et al., 2001). This leads to the remaining question if all HRMs of a protein are of equal relevance for the respective function, which is rather assessed as unlikely (Lee et al., 2003). For the regulator BACH1, it was shown that heme binding to the different HRM varies dependent on the heme concentration. Consequently, this leads to two separate activities: DNA-binding at low heme concentrations or nuclear export of BACH1 at high heme concentrations (Hira et al., 2007). Interestingly for Irr there are multiple configurations of low-spin and high-spin heme binding to the cysteine C^{29} residue of Irr, which could influence the respective affinities (Ishikawa et al., 2011). This emphasizes that this labile binding is highly sensitive and shaped by the surrounding amino acid environment as well as the local heme concentration in the particular microenvironment. In addition to the 5-coordinated CP-motif, an additional histidine residue ($H^{117-119}$) acts as secondary transient heme binding site of Irr, suggested with a 6-coordinated heme binding fortifying a rapid turnover (Ishikawa et al., 2011; Yang et al., 2005).

5.4 Non-CP-motif heme binding

Generally, it was reported, that CP motifs are a favored amino acid arrangement for heme binding, but also other, non-CP motifs were described, further highlighting the diversity of transient heme binding modes (Igarashi et al., 2008). The already above-mentioned second heme binding site of Irr in Rhizobiales, which is described to be histidine-rich, represents such an example (Yang et al., 2005). This histidine-rich region is conserved in Irr homologs, while the CP-motif is not present in all of its representatives (Yang et al., 2005). Histidine residues were already early described to be frequently involved in heme binding (Dawson et al., 1982).

642 The shortly presented *C. difficile* HatR and HsmRA systems also contain histidine residues
643 important for heme binding (Knippel et al., 2020; Knippel et al., 2018). Transient heme binding
644 affinities for these systems are however lower than for Irr (HatR: $K_D \approx 9.2$ µM, HsmR: $K_D \approx 6.6$
645 µM), which can be explained by the fact that *C. difficile* is usually found in the host's colon,
646 where heme concentrations are higher than in the soil.

647 The high diversity in heme binding modes is further underlined by the heme-responsive TCS
648 of Gram-positive bacteria. Although the two paralogous histidine kinases HrrS and ChrS of
649 *C. glutamicum* share 39% identity, the N-terminal heme sensor domains share only 8.5%.
650 Regarding the histidine kinase HrrS, three amino acids encompassing tyrosine and
651 phenylalanines (Y^{112}, F^{115} and F^{118}) are crucial for heme binding. Here, Y^{112} was discussed to
652 be important for the interaction with the iron atom of heme, which was also shown for several
653 NEAT proteins (**nea**rly to iron **t**ransport) (Andrade et al., 2002; Grigg et al., 2007; Keppel et
654 al., 2018; Sharp et al., 2007; Villareal et al., 2008). NEAT domains are also suggested to play
655 important roles in heme binding and transport probably generating a heme binding-cleft.
656 However, their high diversity in sequence makes them hard to predict (Grigg et al., 2007). The
657 phenylalanine residues F^{115} and F^{118} of HrrS have been proposed to be involved in aromatic
658 stacking interactions with the porphyrin ring (Li et al., 2011; Schneider et al., 2007; Smith et
659 al., 2010). By contrast, mutation of the corresponding amino acids in Y^{87}, F^{90} and F^{93} in ChrS
660 did not abolish heme binding (Keppel et al., 2018), although the respective amino acid
661 environment shows a high degree of similarity (Figure 5B). While the transcriptional response
662 mediated by ChrSA shows a higher sensitivity towards heme, structural data and the heme-
663 binding affinity are missing.

664 Besides cysteine residues in CP motifs and histidine residues, tyrosine represents a frequently
665 found ligand of proteins involved in transient heme sensing. This is also exemplified by the
666 RhuR/RhuI heme sensor of Burkholderiales where a tyrosine residue (T^{616}) is flanked by a
667 FRAP motif and a NPNL motif (Figure 5B). In general, histidine residues in between these
668 motifs were described in context of heme binding, like it is also the case for the herein

669 described HasR of *P. aeruginosa* (Figure 5B). These conserved motifs represent a common
670 structure in heme receptors of several proteins found in Gram-negative bacteria, like e.g.
671 HemR of *Y. enterocolitica* (Bracken et al., 1999; Murphy et al., 2002; Richard et al., 2019).
672 However, this type of transient heme binding is less described in the context of heme sensor
673 systems, but rather heme utilization proteins or heme transporters, which do not propagate a
674 signal but the heme molecule itself. The general mechanism of transient heme binding and
675 transport around FRAP/NPNL-motifs is not completely established, as they are also highly
676 variable with respect to their surrounding protein environment (Bracken et al., 1999; Fusco et
677 al., 2013; Murphy et al., 2002).

678 Comparing heme sensor systems, it is striking, that Gram-positive bacteria seem to dedicate
679 at least one TCS to heme-responsive gene regulation, which seems not to be the case for
680 Gram-negative bacteria. Here, ECF σ-factor based systems appear to present a predominant
681 form of heme sensing, as described for the systems found in Burkholderiales (Murphy et al.,
682 2002), *P. aeruginosa* (Dent and Wilks, 2020) or *S. marcescens* (Biville et al., 2004).

683 Comparable to CP-motif based transient heme binding it becomes evident that for all of these
684 systems structural information and detailed biochemical data are still missing. In some cases,
685 the residues involved in heme binding have not yet been identified, illustrating the large number
686 of blind spots in our understanding of transient heme-protein interactions. However, it can be
687 summarized that there are several similarities as well as variations between CP-motif and non-
688 CP-motif HRMs in the context of heme homeostasis shaped by the environmental niche.

689

690 **5.5 Sense and sensitivity – the challenge to predict transient heme-protein interactions**
691 **and their physiological function**

692 Known heme sensor systems already cover astonishing diversity of heme binding modes and
693 physiological functions. While some systems act as global regulators of heme homeostasis,
694 like e.g. corynebacterial HrrS or the mammalian BACH1, other systems display a specific

control of heme uptake or detoxification. Across kingdoms, global systems typically integrate further responses, including responses to oxidative stress, oxygen availability, cell envelope damage or iron availability (Johnston et al., 2007; Keppel et al., 2020; Warnatz et al., 2011). The example of the paralogous TCS HrrSA and ChrSA of *C. glutamicum* nicely illustrate that simple prediction of the physiological function based on pure sequence homology is not possible – but the context provides more insights. While the ChrSA system is genetically linked to its probably only target operon (*hrtBA*), the expression of the response regulator *hrrA* is repressed by the global iron regulator DtxR, thereby creating a link between iron and heme homeostasis (Keppel et al., 2019).

Can we actually predict heme-protein interactions based on the amino acid sequence of a protein? The simple answer is: not with sufficient certainty. For some systems, which do not contain typical heme-binding motifs, the heme-binding ligands have not yet been identified. However, towards this goal, several heme-binding proteins were predicted using combinatorial peptide library screens and database searches or the implemented prediction tool ‘SeqD-HBM‘ for heme-binding motifs described by Wißbrock et al. (2019). This includes e.g. bacterial proteins like FeoB (Schubert et al., 2015), hemolysin C (Peherstorfer et al., 2018) or the human dipeptidylpeptidase 8 (Kühl et al., 2013). Specific sequence features could be revealed which have positive effects on heme-protein-binding, like a positive net charge or hydrophobic residues (Wißbrock et al., 2019).

However, experimental studies will always be needed for verification of transient heme binding and the physiological function of the respective system. Since binding motifs are highly diverse and the above described examples probably just represent the tip of the iceberg, it is almost impossible to predict transient heme-binding based on sequence information only (Kühl et al., 2013; Schubert et al., 2015; Wißbrock et al., 2019). When it comes to heme-binding affinities, it becomes even more difficult.

In addition to that, it is also important to note that currently, we rely on the ‘bulk’ measurements of heme pools, but do account for the potential spatial differences and gradients within cells

and communities. This is particularly relevant when we consider the hydrophobic nature of the molecule heme, which can concentrate in cellular membranes and at hydrophobic protein environments. In this context, advanced imaging technologies and fluorescent sensors might bring light into the spatial biology of heme sensing (Song et al., 2015).

5.6 From static regulons to dynamic networks

Heme-regulatory systems discussed in this review are controlled by a transient interaction with the effector molecule heme. This already emphasizes a dynamic nature in their response to heme availability. For most systems, however, we only know a limited number of genomic targets that are controlled by a specific regulator under defined conditions. In a recent study, Keppel et al. analyzed the dynamic response controlled by the HrrSA TCS in response to a heme stimulus in *C. glutamicum* (Keppel et al., 2020). Chromatin affinity purification of the response regulator HrrA followed by sequencing of its bound genomic targets revealed a dynamic binding to more than 200 different genomic targets involved in heme biosynthesis, respiration, stress responses and cell envelope remodeling. This genome-wide and time-resolved analysis provided unprecedented insights in the timing and hierarchy of this systemic response coordinated by HrrSA in response to heme.

Insights gained from these approaches have also the potential to challenge canonical models of transcriptional regulation revealing substantial binding of regulatory proteins to regions outside of promoter regions and the surprisingly high number of weak and so far unknown binding sites (Galagan et al., 2013). While some may be considered as experimental artefacts, the high reproducibility rather suggests that binding of transcription factors exits along a continuum of weak, medium and high affinity sites, raising the question of physiological significance (Rhee and Pugh, 2011). Can we define a proper threshold for physiological significance, if we appreciate that cells are analog systems? This is especially relevant for binding sites for which no impact on gene expression has been observed – under the defined experimental conditions. However, genome-wide approaches including different transcription

749 factors revealed a high level of context dependency. Coming back to the example of the
750 corynebacterial HrrSA system, it is the interplay with the global iron regulator DtxR which
751 enables a proper integration of iron and heme regulatory networks fine-tuning the cellular
752 behavior to the actual environmental conditions (including iron/heme availability, cell envelope
753 and oxidative stress responses) (Keppel et al., 2020). Here, heme-triggered HrrA binding to
754 the promoter of *hmuO*, encoding the heme oxygenase, will not further enhance gene
755 expression as long as the iron-bound form of DtxR is repressing it.

756

757 **6. Importance of heme sensor systems for medicine and biotechnology**

758 The heme-regulatory systems discussed in this review highlight the diversity of mechanisms
759 organisms have evolved to respond to the multifaceted stimulus heme. The study of these
760 systems not only provides important insights into cellular physiology and bacterial virulence,
761 but also has a high potential for the development of novel antimicrobial drugs. Due to the fact
762 that TCSs are found in nearly all sequenced bacterial genomes, but are absent in animals and
763 humans, several studies have emphasized them as an attractive targets for antimicrobials
764 (Hirakawa et al., 2020; Ma and Phillips-Jones, 2021). As a specific example, decreased clinical
765 efficacy of metronidazole in patients suffering from a *C. difficile* infection was recently
766 described to be heme-associated (Gonzales-Luna et al., 2021; Wu et al., 2021). This further
767 underlines the clinical relevance of the herein described heme sensor systems as a potential
768 antimicrobial drug target and the importance of a mechanistic understanding of their role in
769 microbial pathogenicity.

770 However, heme sensor systems are not only important in fighting bacterial diseases, but also
771 for example in cancer progression. The mammalian heme sensing transcription factor BACH1
772 was shown to play a role in tumorigenesis as it is involved in the expression of genes e.g.
773 associated with breast-, colon- or prostate cancer metastasis (Igarashi et al., 2021; Liang et
774 al., 2012; Shajari et al., 2018; Zhu et al., 2018). Several studies already suggested BACH1 as
775 a potential therapeutic target for adjuvant cancer therapy, e.g. by silencing therapies

(Davoudian et al., 2016; Shajari et al., 2018). Recently, BACH1 was also considered to be of interest as target for developing COVID-19 candidate drugs. SARS-CoV-2 was shown to inhibit NRF2, which could be potentially counteracted by the inhibition of BACH1 leading to a derepression of Maf proteins and consequent activation of NRF2 (Cuadrado et al., 2020; Liu et al., 2019; Olagnier et al., 2020). Given the tight link between iron/heme metabolism and microbial growth and virulence, an integrated view on the function of heme sensor systems is highly relevant for medical applications and drug development.

Besides medical applications, another example where knowledge about heme sensor systems bear potential for the industry is the food sector. For instance, the animal-free production of heme has recently attracted attention as ingredient mimicking the meat flavor in artificial meat (Fraser et al., 2017; Waltz, 2019), which could contribute to a necessary reduction of meat consumption (González et al., 2020). Hence, engineering of microbial cell factories is required to achieve high-yield production of animal-free heme. A recent systems metabolic engineering approach resulted in the establishment of animal-free heme production using the biotechnological production strain *C. glutamicum* (Ko et al., 2021). Further future metabolic engineering approaches will therefore strongly benefit from a comprehensive understanding of heme-regulation to foster efficient development of microbial cell factories.

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9. References

Aft, R.L., and Mueller, G.C. (1984). Hemin-mediated oxidative degradation of proteins. Journal of Biological Chemistry *259*, 301-305.

Ahn, S.H., Han, J.H., Lee, J.H., Park, K.J., and Kong, I.S. (2005). Identification of an iron-regulated hemin-binding outer membrane protein, HupO, in *Vibrio fluvialis*: effects on hemolytic activity and the oxidative stress response. Infection and Immunity *73*, 722-729.

Aich, A., Freundlich, M., and Vekilov, P.G. (2015). The free heme concentration in healthy human erythrocytes. Blood Cells, Molecules, and Diseases *55*, 402-409.

Ajioka, R.S., Phillips, J.D., and Kushner, J.P. (2006). Biosynthesis of heme in mammals. Biochimica et Biophysica Acta *1763*, 723-736.

Andrade, M.A., Ciccarelli, F.D., Perez-Iratxeta, C., and Bork, P. (2002). NEAT: a domain duplicated in genes near the components of a putative Fe^{3+}siderophore transporter from Gram-positive pathogenic bacteria. Genome Biology *3*, Research0047.

Anzaldi, L.L., and Skaar, E.P. (2010). Overcoming the Heme Paradox: Heme Toxicity and Tolerance in Bacterial Pathogens. Infection and Immunity *78*, 4977-4989.

Atamna, H., Brahmbhatt, M., Atamna, W., Shanower, G.A., and Dhahbi, J.M. (2015). ApoHRP-based assay to measure intracellular regulatory heme. Metallomics *7*, 309-321.

Bardwell, V.J., and Treisman, R. (1994). The POZ domain: a conserved protein-protein interaction motif. Genes & Development *8*, 1664-1677.

Benevides-Matos, N., and Biville, F. (2010). The Hem and Has haem uptake systems in *Serratia marcescens*. Microbiology (Reading) *156*, 1749-1757.

Bibb, L.A., King, N.D., Kunkle, C.A., and Schmitt, M.P. (2005). Analysis of a heme-dependent signal transduction system in *Corynebacterium diphtheriae*: deletion of the *chrAS* genes results in heme sensitivity and diminished heme-dependent activation of the *hmuO* promoter. Infection and immunity *73*, 7406-7412.

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*. Infection and Immunity *75*, 2421-2431.

Bibb, L.A., and Schmitt, M.P. (2010). The ABC Transporter HrtAB Confers Resistance to Hemin Toxicity and Is Regulated in a Hemin-Dependent Manner by the ChrAS Two-Component System in *Corynebacterium diphtheriae*. Journal of Bacteriology *192*, 4606-4617.

Biswas, G.D., Anderson, J.E., and Sparling, P.F. (1997). Cloning and functional characterization of *Neisseria gonorrhoeae tonB, exbB* and *exbD* genes. Molecular Microbiology *24*, 169-179.

Biville, F., Cwerman, H., Létoffé, S., Rossi, M.S., Drouet, V., Ghigo, J.M., and Wandersman, C. (2004). Haemophore-mediated signalling in *Serratia marcescens*: a new mode of regulation for an extra cytoplasmic function (ECF) sigma factor involved in haem acquisition. Molecular Microbiology *53*, 1267-1277.

Bott, M., and Brocker, M. (2012). Two-component signal transduction in *Corynebacterium glutamicum* and other corynebacteria: on the way towards stimuli and targets. Applied Microbiology and Biotechnology *94*, 1131-1150.

Bracken, C.S., Baer, M.T., Abdur-Rashid, A., Helms, W., and Stojiljkovic, I. (1999). Use of heme-protein complexes by the *Yersinia enterocolitica* HemR receptor: histidine residues are essential for receptor function. Journal of Bacteriology *181*, 6063-6072.

Brear, E., Day, D., and Smith, P. (2013). Iron: an essential micronutrient for the legume-rhizobium symbiosis. Frontiers in Plant Science *4*.

Brewitz, H.H., Hagelueken, G., and Imhof, D. (2017). Structural and functional diversity of transient heme binding to bacterial proteins. Biochimica et Biophysica Acta *1861*, 683-697.

Bronner, S., Monteil, H., and Prévost, G. (2004). Regulation of virulence determinants in *Staphylococcus aureus*: complexity and applications. FEMS Microbiol Rev *28*, 183-200.

Buisson, N., and Labbe-Bois, R. (1998). Flavohemoglobin expression and function in *Saccharomyces cerevisiae*. No relationship with respiration and complex response to oxidative stress. Journal of Biological Chemistry *273*, 9527-9533.
Burgos, J.M., and Schmitt, M.P. (2016). The ChrSA and HrrSA Two-Component Systems Are Required for Transcriptional Regulation of the *hemA* Promoter in *Corynebacterium diphtheriae*. Journal of Bacteriology *198*, 2419-2430.
Butler, C.A., Dashper, S.G., Zhang, L., Seers, C.A., Mitchell, H.L., Catmull, D.V., Glew, M.D., Heath, J.E., Tan, Y., Khan, H.S.G*., et al.* (2014). The *Porphyromonas gingivalis* ferric uptake regulator orthologue binds hemin and regulates hemin-responsive biofilm development. PloS one *9*.
Capra, E.J., and Laub, M.T. (2012). Evolution of two-component signal transduction systems. Annual Review of Microbiology *66*, 325-347.
Chapman, S.K., Daff, S., and Munro, A.W. (1997). Heme: The most versatile redox centre in biology? In Metal Sites in Proteins and Models: Iron Centres, H.A.O. Hill, P.J. Sadler, and A.J. Thomson, eds. (Berlin, Heidelberg: Springer Berlin Heidelberg), pp. 39-70.
Chiabrando, D., Vinchi, F., Fiorito, V., Mercurio, S., and Tolosano, E. (2014). Heme in pathophysiology: a matter of scavenging, metabolism and trafficking across cell membranes. Frontiers in Pharmacology *5*.
Chumbler, N.M., Farrow, M.A., Lapierre, L.A., Franklin, J.L., Haslam, D., Goldenring, J.R., and Lacy, D.B. (2012). *Clostridium difficile* Toxin B Causes Epithelial Cell Necrosis through an Autoprocessing-Independent Mechanism. PLoS Pathogens *8*.
Colombo, C., Palumbo, G., He, J.-Z., Pinton, R., and Cesco, S. (2014). Review on iron availability in soil: interaction of Fe minerals, plants, and microbes. Journal of Soils and Sediments *14*, 538-548.
Contreras, H., Chim, N., Credali, A., and Goulding, C.W. (2014). Heme uptake in bacterial pathogens. Current Opinion in Chemical Biology *19*, 34-41.
Creusot, F., Verdiere, J., Gaisne, M., and Slonimski, P.P. (1988). CYP1 (HAP1) regulator of oxygen-dependent gene expression in yeast. I. Overall organization of the protein sequence displays several novel structural domains. Journal of molecular biology *204*, 263-276.
Cuadrado, A., Pajares, M., Benito, C., Jiménez-Villegas, J., Escoll, M., Fernández-Ginés, R., Garcia Yagüe, A.J., Lastra, D., Manda, G., Rojo, A.I*., et al.* (2020). Can Activation of NRF2 Be a Strategy against COVID-19? Trends in Pharmacological Sciences *41*, 598-610.
Davoudian, S., Shajari, N., Kazemi, T., Mansoori, B., Salehi, S., Mohammadi, A., Shanehbandi, D., Khaze Shahgoli, V., Asadi, M., and Baradaran, B. (2016). BACH1 silencing by siRNA inhibits migration of HT-29 colon cancer cells through reduction of metastasis-related genes. Biomedicine & Pharmacotherapy *84*, 191-198.
Dawson, J.H., Andersson, L.A., and Sono, M. (1982). Spectroscopic investigations of ferric cytochrome P-450-CAM ligand complexes. Identification of the ligand trans to cysteinate in the native enzyme. Journal of Biological Chemistry *257*, 3606-3617.
de Villiers, K.A., and Egan, T.J. (2021). Heme Detoxification in the Malaria Parasite: A Target for Antimalarial Drug Development. Acc Chem Res *54*, 2649-2659.
Dent, A.T., Brimberry, M., Albert, T., Lanzilotta, W.N., Moënne-Loccoz, P., and Wilks, A. (2021). Axial Heme Coordination by the Tyr-His Motif in the Extracellular Hemophore HasAp Is Critical for the Release of Heme to the HasR Receptor of *Pseudomonas aeruginosa*. Biochemistry *60*, 2549-2559.
Dent, A.T., Mouriño, S., Huang, W., and Wilks, A. (2019). Post-transcriptional regulation of the *Pseudomonas aeruginosa* heme assimilation system (Has) fine-tunes extracellular heme sensing. Journal of Biological Chemistry *294*, 2771-2785.
Dent, A.T., and Wilks, A. (2020). Contributions of the heme coordinating ligands of the *Pseudomonas aeruginosa* outer membrane receptor HasR to extracellular heme sensing and transport. Journal of Biological Chemistry *295*, 10456-10467.
Dhakshinamoorthy, S., Jain, A.K., Bloom, D.A., and Jaiswal, A.K. (2005). BACH1 competes with Nrf2 leading to negative regulation of the antioxidant response element (ARE)-mediated NAD(P)H:quinone oxidoreductase 1 gene expression and induction in response to antioxidants. Journal of Biological Chemistry *280*, 16891-16900.

Dioum, E.M., Rutter, J., Tuckerman, J.R., Gonzalez, G., Gilles-Gonzalez, M.A., and McKnight, S.L. (2002). NPAS2: a gas-responsive transcription factor. Science *298*, 2385-2387.
Elkins, C., Totten, P.A., Olsen, B., and Thomas, C.E. (1998). Role of the *Haemophilus ducreyi* Ton system in internalization of heme from hemoglobin. Infection and Immunity *66*, 151-160.
Fernandez, A., Lechardeur, D., Derré-Bobillot, A., Couvé, E., Gaudu, P., and Gruss, A. (2010). Two coregulated efflux transporters modulate intracellular heme and protoporphyrin IX availability in *Streptococcus agalactiae*. PLoS Pathog *6*.
Fraser, R., Davis, S.C., and O ' Reilly Brown, P. (2017). Secretion of heme-containing polypeptides, R.C. Impossible Foods Inc . , CA (US) ed. (United States).
Frunzke, J., Gätgens, C., Brocker, M., and Bott, M. (2011). Control of heme homeostasis in *Corynebacterium glutamicum* by the two-component system HrrSA. Journal of Bacteriology *193*, 1212-1221.
Fusco, W.G., Choudhary, N.R., Council, S.E., Collins, E.J., and Leduc, I. (2013). Mutational analysis of hemoglobin binding and heme utilization by a bacterial hemoglobin receptor. Journal of Bacteriology *195*, 3115-3123.
Galagan, J., Lyubetskaya, A., and Gomes, A. (2013). ChIP-Seq and the complexity of bacterial transcriptional regulation. Current Topics in Microbiology and Immunology *363*, 43-68.
Gallio, A.E., Fung, S.S.P., Cammack-Najera, A., Hudson, A.J., and Raven, E.L. (2021). Understanding the Logistics for the Distribution of Heme in Cells. JACS Au *1*, 1541-1555.
Girvan, H.M., and Munro, A.W. (2013). Heme sensor proteins. The Journal of biological chemistry *288*, 13194-13203.
Gong, W., Hao, B., and Chan, M.K. (2000). New Mechanistic Insights from Structural Studies of the Oxygen-Sensing Domain of *Bradyrhizobium japonicum* FixL. Biochemistry *39*, 3955-3962.
Gonzales-Luna, A.J., Olaitan, A.O., Shen, W.-J., Deshpande, A., Carlson, T.J., Dotson, K.M., Lancaster, C., Begum, K., Alam, M.J., Hurdle, J.G*., et al.* (2021). Reduced Susceptibility to Metronidazole Is Associated With Initial Clinical Failure in *Clostridioides difficile* Infection. Open Forum Infect Dis *8*, ofab365-ofab365.
González, N., Marquès, M., Nadal, M., and Domingo, J.L. (2020). Meat consumption: Which are the current global risks? A review of recent (2010-2020) evidences. Food Res Int *137*, 109341-109341.
Granick, S., Sinclair, P., Sassa, S., and Grieninger, G. (1975). Effects by heme, insulin, and serum albumin on heme and protein synthesis in chick embryo liver cells cultured in a chemically defined medium, and a spectrofluorometric assay for porphyrin composition. Journal of Biological Chemistry *250*, 9215-9225.
Green, J., Crack, J.C., Thomson, A.J., and LeBrun, N.E. (2009). Bacterial sensors of oxygen. Current Opinion in Microbiology *12*, 145-151.
Grigg, J.C., Vermeiren, C.L., Heinrichs, D.E., and Murphy, M.E.P. (2007). Haem recognition by a *Staphylococcus aureus* NEAT domain. Molecular Microbiology *63*, 139-149.
Guarente, L., and Mason, T. (1983). Heme regulates transcription of the CYC1 gene of *S. cerevisiae* via an upstream activation site. Cell *32*, 1279-1286.
Hach, A., Hon, T., and Zhang, L. (1999). A new class of repression modules is critical for heme regulation of the yeast transcriptional activator Hap1. Molecular and Cellular Biology *19*, 4324-4333.
Hamza, I., Chauhan, S., Hassett, R., and O'Brian, M.R. (1998). The bacterial Irr protein is required for coordination of heme biosynthesis with iron availability. Journal of Biological Chemistry *273*, 21669-21674.
Hanna, D.A., Hu, R., Kim, H., Martinez-Guzman, O., Torres, M.P., and Reddi, A.R. (2018). Heme bioavailability and signaling in response to stress in yeast cells. The Journal of biological chemistry *293*, 12378-12393.
Hentschel, E., Mack, C., Gatgens, C., Bott, M., Brocker, M., and Frunzke, J. (2014). Phosphatase activity of the histidine kinases ensures pathway specificity of the ChrSA and HrrSA two-component systems in *Corynebacterium glutamicum*. Molecular Microbiology *92*, 1326-1342.

Heyer, A., Gatgens, C., Hentschel, E., Kalinowski, J., Bott, M., and Frunzke, J. (2012). The two-component system ChrSA is crucial for haem tolerance and interferes with HrrSA in haem-dependent gene regulation in *Corynebacterium glutamicum*. Microbiology *158*, 3020-3031.
Hintze, K.J., Katoh, Y., Igarashi, K., and Theil, E.C. (2007). Bach1 repression of ferritin and thioredoxin reductase1 is heme-sensitive in cells and in vitro and coordinates expression with heme oxygenase1, beta-globin, and NADP(H) quinone (oxido) reductase1. Journal of Biological Chemistry *282*, 34365-34371.
Hira, S., Tomita, T., Matsui, T., Igarashi, K., and Ikeda-Saito, M. (2007). Bach1, a heme-dependent transcription factor, reveals presence of multiple heme binding sites with distinct coordination structure. IUBMB Life *59*, 542-551.
Hirakawa, H., Kurushima, J., Hashimoto, Y., and Tomita, H. (2020). Progress Overview of Bacterial Two-Component Regulatory Systems as Potential Targets for Antimicrobial Chemotherapy. Antibiotics *9*, 635.
Hoch, J.A. (2000). Two-component and phosphorelay signal transduction. Current Opinion in Microbiology *3*, 165-170.
Hon, T., Dodd, A., Dirmeier, R., Gorman, N., Sinclair, P.R., Zhang, L., and Poyton, R.O. (2003). A mechanism of oxygen sensing in yeast. Multiple oxygen-responsive steps in the heme biosynthetic pathway affect Hap1 activity. Journal of Biological Chemistry *278*, 50771-50780.
Hon, T., Hach, A., Lee, H.C., Cheng, T., and Zhang, L. (2000). Functional analysis of heme regulatory elements of the transcriptional activator Hap1. Biochem Biophys Res Commun *273*, 584-591.
Hon, T., Lee, H.C., Hach, A., Johnson, J.L., Craig, E.A., Erdjument-Bromage, H., Tempst, P., and Zhang, L. (2001). The Hsp70-Ydj1 molecular chaperone represses the activity of the heme activator protein Hap1 in the absence of heme. Molecular and Cellular Biology *21*, 7923-7932.
Hon, T., Lee, H.C., Hu, Z., Iyer, V.R., and Zhang, L. (2005). The heme activator protein Hap1 represses transcription by a heme-independent mechanism in *Saccharomyces cerevisiae*. Genetics *169*, 1343-1352.
Igarashi, J., Murase, M., Iizuka, A., Pichierri, F., Martinkova, M., and Shimizu, T. (2008). Elucidation of the Heme Binding Site of Heme-regulated Eukaryotic Initiation Factor α; Kinase and the Role of the Regulatory Motif in Heme Sensing by Spectroscopic and Catalytic Studies of Mutant Proteins. Journal of Biological Chemistry *283*, 18782-18791.
Igarashi, K., Hoshino, H., Muto, A., Suwabe, N., Nishikawa, S., Nakauchi, H., and Yamamoto, M. (1998). Multivalent DNA Binding Complex Generated by Small Maf and Bach1 as a Possible Biochemical Basis for Globin Locus Control Region Complex. The Journal of biological chemistry *273*, 11783 - 11790.
Igarashi, K., Kataokat, K., Itoh, K., Hayashi, N., Nishizawa, M., and Yamamoto, M. (1994). Regulation of transcription by dimerization of erythroid factor NF-E2 p45 with small Maf proteins. Nature *367*, 568-572.
Igarashi, K., Nishizawa, H., Saiki, Y., and Matsumoto, M. (2021). The transcription factor BACH1 at the crossroads of cancer biology: From epithelial-mesenchymal transition to ferroptosis. Journal of Biological Chemistry *297*, 101032.
Ishikawa, H., Nakagaki, M., Bamba, A., Uchida, T., Hori, H., O'Brian, M.R., Iwai, K., and Ishimori, K. (2011). Unusual Heme Binding in the Bacterial Iron Response Regulator Protein: Spectral Characterization of Heme Binding to the Heme Regulatory Motif. Biochemistry *50*, 1016-1022.
Ishimori, K., and Watanabe, Y. (2014). Unique Heme Environmental Structures in Heme-regulated Proteins Using Heme as the Signaling Molecule. Chemistry Letters *43*, 1680-1689.
Ito, Y., Nakagawa, S., Komagata, A., Ikeda-Saito, M., Shiro, Y., and Nakamura, H. (2009). Heme-dependent autophosphorylation of a heme sensor kinase, ChrS, from *Corynebacterium diphtheriae* reconstituted in proteoliposomes. FEBS Letters *583*, 2244-2248.
Jepkorir, G., Rodríguez, J.C., Rui, H., Im, W., Lovell, S., Battaile, K.P., Alontaga, A.Y., Yukl, E.T., Moënne-Loccoz, P., and Rivera, M. (2010). Structural, NMR Spectroscopic, and Computational Investigation of Hemin Loading in the Hemophore HasAp from Pseudomonas aeruginosa. Journal of the American Chemical Society *132*, 9857-9872.

Johnston, A.W.B., Todd, J.D., Curson, A.R., Lei, S., Nikolaidou-Katsaridou, N., Gelfand, M.S., and Rodionov, D.A. (2007). Living without Fur: the subtlety and complexity of iron-responsive gene regulation in the symbiotic bacterium Rhizobium and other α-proteobacteria. BioMetals *20*, 501-511.
Joubert, L., Derré-Bobillot, A., Gaudu, P., Gruss, A., and Lechardeur, D. (2014). HrtBA and menaquinones control haem homeostasis in *Lactococcus lactis*. Molecular Microbiology *93*, 823-833.
Keppel, M., Davoudi, E., Gätgens, C., and Frunzke, J. (2018). Membrane Topology and Heme Binding of the Histidine Kinases HrrS and ChrS in *Corynebacterium glutamicum*. Frontiers in Microbiology *9*, 183.
Keppel, M., Hünnefeld, M., Filipchyk, A., Viets, U., Davoudi, C.F., Krüger, A., Mack, C., Pfeifer, E., Polen, T., Baumgart, M*., et al.* (2020). HrrSA orchestrates a systemic response to heme and determines prioritization of terminal cytochrome oxidase expression. Nucleic Acids Research *48*, 6547–6562.
Keppel, M., Piepenbreier, H., Gätgens, C., Fritz, G., and Frunzke, J. (2019). Toxic but tasty – temporal dynamics and network architecture of heme-responsive two-component signaling in *Corynebacterium glutamicum*. Molecular Microbiology *111*, 1367-1381.
King-Lyons, N.D., Smith, K.F., and Connell, T.D. (2007). Expression of *hurP*, a gene encoding a prospective site 2 protease, is essential for heme-dependent induction of bhuR in *Bordetella bronchiseptica*. Journal of Bacteriology *189*, 6266-6275.
King, N.D., Kirby, A.E., and Connell, T.D. (2005). Transcriptional control of the *rhuIR-bhuRSTUV* heme acquisition locus in *Bordetella avium*. Infection and immunity *73*, 1613-1624.
Kirby, A.E., King, N.D., and Connell, T.D. (2004). RhuR, an extracytoplasmic function sigma factor activator, is essential for heme-dependent expression of the outer membrane heme and hemoprotein receptor of *Bordetella avium*. Infection and Immunity *72*, 896-907.
Kirby, A.E., Metzger, D.J., Murphy, E.R., and Connell, T.D. (2001). Heme utilization in *Bordetella avium* is regulated by RhuI, a heme-responsive extracytoplasmic function sigma factor. Infection and immunity *69*, 6951-6961.
Kitatsuji, C., Izumi, K., Nambu, S., Kurogochi, M., Uchida, T., Nishimura, S.-I., Iwai, K., O'Brian, M.R., Ikeda-Saito, M., and Ishimori, K. (2016). Protein oxidation mediated by heme-induced active site conversion specific for heme-regulated transcription factor, iron response regulator. Scientific Reports *6*, 18703.
Knippel, R.J., Wexler, A.G., Miller, J.M., Beavers, W.N., Weiss, A., de Crécy-Lagard, V., Edmonds, K.A., Giedroc, D.P., and Skaar, E.P. (2020). *Clostridioides difficile* Senses and Hijacks Host Heme for Incorporation into an Oxidative Stress Defense System. Cell Host Microbe *28*, 411-421.e416.
Knippel, R.J., Zackular, J.P., Moore, J.L., Celis, A.I., Weiss, A., Washington, M.K., DuBois, J.L., Caprioli, R.M., and Skaar, E.P. (2018). Heme sensing and detoxification by HatRT contributes to pathogenesis during *Clostridium difficile* infection. PLoS pathogens *14*, e1007486-e1007486.
Ko, Y.J., Kim, M., You, S.K., Shin, S.K., Chang, J., Choi, H.J., Jeong, W.Y., Lee, M.E., Hwang, D.H., and Han, S.O. (2021). Animal-free heme production for artificial meat in *Corynebacterium glutamicum* via systems metabolic and membrane engineering. Metabolic Engineering *66*, 217-228.
Kobayashi, K., Nakagaki, M., Ishikawa, H., Iwai, K., O'Brian, M.R., and Ishimori, K. (2016). Redox-Dependent Dynamics in Heme-Bound Bacterial Iron Response Regulator (Irr) Protein. Biochemistry *55*, 4047-4054.
Krieg, S., Huché, F., Diederichs, K., Izadi-Pruneyre, N., Lecroisey, A., Wandersman, C., Delepelaire, P., and Welte, W. (2009). Heme uptake across the outer membrane as revealed by crystal structures of the receptor–hemophore complex. Proceedings of the National Academy of Sciences *106*, 1045-1050.
Kühl, T., Sahoo, N., Nikolajski, M., Schlott, B., Heinemann, S.H., and Imhof, D. (2011). Determination of Hemin-Binding Characteristics of Proteins by a Combinatorial Peptide Library Approach. ChemBioChem *12*, 2846-2855.
Kühl, T., Wissbrock, A., Goradia, N., Sahoo, N., Galler, K., Neugebauer, U., Popp, J., Heinemann, S., Ohlenschläger, O., and Imhof, D. (2013). Analysis of Fe(III) Heme Binding to Cysteine-Containing Heme-Regulatory Motifs in Proteins. ACS chemical biology *8*, 1785-1793.

Kumar, R., Matsumura, H., Lovell, S., Yao, H., Rodríguez, J.C., Battaile, K.P., Moënne-Loccoz, P., and Rivera, M. (2014). Replacing the Axial Ligand Tyrosine 75 or Its Hydrogen Bond Partner Histidine 83 Minimally Affects Hemin Acquisition by the Hemophore HasAp from *Pseudomonas aeruginosa*. Biochemistry *53*, 2112-2125.

Kumar, S., and Bandyopadhyay, U. (2005). Free heme toxicity and its detoxification systems in human. Toxicology Letters *157*, 175-188.

Lal, S., Comer, J.M., Konduri, P.C., Shah, A., Wang, T., Lewis, A., Shoffner, G., Guo, F., and Zhang, L. (2018). Heme promotes transcriptional and demethylase activities of Gis1, a member of the histone demethylase JMJD2/KDM4 family. Nucleic Acids Research *46*, 215-228.

Lathrop, J., and Timko, M. (1993). Regulation by heme of mitochondrial protein transport through a conserved amino acid motif. Science *259*, 522-525.

Layer, G., Reichelt, J., Jahn, D., and Heinz, D.W. (2010). Structure and function of enzymes in heme biosynthesis. Protein Science *19*, 1137-1161.

Lee, H.C., Hon, T., Lan, C., and Zhang, L. (2003). Structural environment dictates the biological significance of heme-responsive motifs and the role of Hsp90 in the activation of the heme activator protein Hap1. Molecular and Cellular Biology *23*, 5857-5866.

Létoffé, S., Deniau, C., Wolff, N., Dassa, E., Delepelaire, P., Lecroisey, A., and Wandersman, C. (2001). Haemophore-mediated bacterial haem transport: evidence for a common or overlapping site for haem-free and haem-loaded haemophore on its specific outer membrane receptor. Molecular Microbiology *41*, 439-450.

Li, T., Bonkovsky, H.L., and Guo, J.-t. (2011). Structural analysis of heme proteins: implications for design and prediction. BMC Structural Biology *11*, 13.

Liang, Y., Wu, H., Lei, R., Chong, R.A., Wei, Y., Lu, X., Tagkopoulos, I., Kung, S.-Y., Yang, Q., Hu, G.*, et al.* (2012). Transcriptional Network Analysis Identifies BACH1 as a Master Regulator of Breast Cancer Bone Metastasis. Journal of Biological Chemistry *287*, 33533-33544.

Liu, Q., Gao, Y., and Ci, X. (2019). Role of Nrf2 and its activators in respiratory diseases. Oxid Med Cell Longev *2019*.

Lowry, C.V., and Zitomer, R.S. (1988). *ROX1* encodes a heme-induced repression factor regulating *ANB1* and *CYC7* of *Saccharomyces cerevisiae*. Molecular and Cellular Biology *8*, 4651-4658.

Ma, P., and Phillips-Jones, M.K. (2021). Membrane Sensor Histidine Kinases: Insights from Structural, Ligand and Inhibitor Studies of Full-Length Proteins and Signalling Domains for Antibiotic Discovery. Molecules *26*, 5110.

Martinez, M., Ugalde, R., and Almiron, M. (2005). Dimeric *Brucella abortus* Irr protein controls its own expression and binds haem. Microbiology (Reading) *151*, 3427-3433.

Martínková, M., Kitanishi, K., and Shimizu, T. (2013). Heme-based Globin-coupled Oxygen Sensors: Linking Oxygen Binding to Functional Regulation of Diguanylate Cyclase, Histidine Kinase, and Methyl-accepting Chemotaxis. Journal of Biological Chemistry *288*, 27702-27711.

Mascher, T., Helmann, J.D., and Unden, G. (2006). Stimulus Perception in Bacterial Signal-Transducing Histidine Kinases. Microbiology and Molecular Biology Reviews *70*, 910-938.

Mike, L.A., Choby, J.E., Brinkman, P.R., Olive, L.Q., Dutter, B.F., Ivan, S.J., Gibbs, C.M., Sulikowski, G.A., Stauff, D.L., and Skaar, E.P. (2014). Two-component system cross-regulation integrates *Bacillus anthracis* response to heme and cell envelope stress. PLoS Pathog *10*, e1004044.

Motohashi, H., Shavit, J.A., Igarashi, K., Yamamoto, M., and Engel, J.D. (1997). The world according to Maf. Nucleic Acids Research *25*, 2953-2959.

Mouriño, S., Giardina, B.J., Reyes-Caballero, H., and Wilks, A. (2016). Metabolite-driven Regulation of Heme Uptake by the Biliverdin IXβ/δ-Selective Heme Oxygenase (HemO) of *Pseudomonas aeruginosa*. Journal of Biological Chemistry *291*, 20503-20515.

Murphy, E.R., Sacco, R.E., Dickenson, A., Metzger, D.J., Hu, Y., Orndorff, P.E., and Connell, T.D. (2002). BhuR, a virulence-associated outer membrane protein of *Bordetella avium*, is required for the acquisition of iron from heme and hemoproteins. Infection and immunity *70*, 5390-5403.

Nakahigashi, K., Nishimura, K., Miyamoto, K., and Inokuchi, H. (1991). Photosensitivity of a protoporphyrin-accumulating, light-sensitive mutant (*visA*) of *Escherichia coli* K-12. Proc Natl Acad Sci U S A *88*, 10520-10524.
NCBIResourceCoordinators (2016). Database resources of the National Center for Biotechnology Information. Nucleic acids research *44*, D7-D19.
Nienaber, A., Hennecke, H., and Fischer, H.-M. (2001). Discovery of a haem uptake system in the soil bacterium *Bradyrhizobium japonicum*. Molecular Microbiology *41*, 787-800.
Noriega, C.E., Lin, H.Y., Chen, L.L., Williams, S.B., and Stewart, V. (2010). Asymmetric cross-regulation between the nitrate-responsive NarX-NarL and NarQ-NarP two-component regulatory systems from *Escherichia coli* K-12. Molecular Microbiology *75*, 394-412.
O'Brian, M.R. (2015). Perception and Homeostatic Control of Iron in the Rhizobia and Related Bacteria. Annual Review of Microbiology *69*, 229-245.
O'Hara, G.W. (2001). Nutritional constraints on root nodule bacteria affecting symbiotic nitrogen fixation: a review. Australian Journal of Experimental Agriculture *41*, 417-433.
Occhino, D.A., Wyckoff, E.E., Henderson, D.P., Wrona, T.J., and Payne, S.M. (1998). *Vibrio cholerae* iron transport: haem transport genes are linked to one of two sets of *tonB*, *exbB*, *exbD* genes. Molecular Microbiology *29*, 1493-1507.
Ochsner, U.A., Johnson, Z., and Vasil, M.L. (2000). Genetics and regulation of two distinct haem-uptake systems, phu and has, in *Pseudomonas aeruginosa*. Microbiology (Reading) *146 (Pt 1)*, 185-198.
Ogawa, K., Sun, J., Taketani, S., Nakajima, O., Nishitani, C., Sassa, S., Hayashi, N., Yamamoto, M., Shibahara, S., Fujita, H.*, et al.* (2001). Heme mediates derepression of Maf recognition element through direct binding to transcription repressor Bach1. EMBO J *20*, 2835-2843.
Olagnier, D., Farahani, E., Thyrsted, J., Blay-Cadanet, J., Herengt, A., Idorn, M., Hait, A., Hernaez, B., Knudsen, A., Iversen, M.B.*, et al.* (2020). SARS-CoV2-mediated suppression of NRF2-signaling reveals potent antiviral and anti-inflammatory activity of 4-octyl-itaconate and dimethyl fumarate. Nat Commun *11*, 4938.
Oyake, T., Itoh, K., Motohashi, H., Hayashi, N., Hoshino, H., Nishizawa, M., Yamamoto, M., and Igarashi, K. (1996). Bach proteins belong to a novel family of BTB-basic leucine zipper transcription factors that interact with MafK and regulate transcription through the NF-E2 site. Molecular and cellular biology *16*, 6083-6095.
Parrow, N.L., Abbott, J., Lockwood, A.R., Battisti, J.M., and Minnick, M.F. (2009). Function, Regulation, and Transcriptional Organization of the Hemin Utilization Locus of *Bartonella quintana*. Infection and Immunity *77*, 307-316.
Peherstorfer, S., Brewitz, H.H., Paul George, A.A., Wißbrock, A., Adam, J.M., Schmitt, L., and Imhof, D. (2018). Insights into mechanism and functional consequences of heme binding to hemolysin-activating lysine acyltransferase HlyC from *Escherichia coli*. Biochimica et Biophysica Acta *1862*, 1964-1972.
Peuser, V., Remes, B., and Klug, G. (2012). Role of the Irr Protein in the Regulation of Iron Metabolism in *Rhodobacter sphaeroides*. PloS one *7*, e42231.
Pfeifer, K., Kim, K.S., Kogan, S., and Guarente, L. (1989). Functional dissection and sequence of yeast HAP1 activator. Cell *56*, 291-301.
Pi, H., Chu, M.L., Ivan, S.J., Latario, C.J., Toth, A.M., Carlin, S.M., Hillebrand, G.H., Lin, H.K., Reppart, J.D., Stauff, D.L.*, et al.* (2020). Directed evolution reveals the mechanism of HitRS signaling transduction in *Bacillus anthracis*. PLoS Pathogens *16*, e1009148.
Ponka, P. (1999). Cell biology of heme. American Journal of Medical Sciences *318*, 241-256.
Pressler, U., Staudenmaier, H., Zimmermann, L., and Braun, V. (1988). Genetics of the iron dicitrate transport system of *Escherichia coli*. Journal of Bacteriology *170*, 2716-2724.
Qi, Z., Hamza, I., and O'Brian, M.R. (1999). Heme is an effector molecule for iron-dependent degradation of the bacterial iron response regulator (Irr) protein. Proceedings of the National Academy of Sciences *96*, 13056-13061.

Qi, Z., and O'Brian, M.R. (2002). Interaction between the bacterial iron response regulator and ferrochelatase mediates genetic control of heme biosynthesis. Molecular Cell *9*, 155-162.
Raghuram, S., Stayrook, K.R., Huang, P., Rogers, P.M., Nosie, A.K., McClure, D.B., Burris, L.L., Khorasanizadeh, S., Burris, T.P., and Rastinejad, F. (2007). Identification of heme as the ligand for the orphan nuclear receptors REV-ERBα and REV-ERBβ. Nat Struct Mol Biol *14*, 1207-1213.
Reedy, C.J., and Gibney, B.R. (2004). Heme Protein Assemblies. Chemical Reviews *104*, 617-650.
Rhee, H.S., and Pugh, B.F. (2011). Comprehensive genome-wide protein-DNA interactions detected at single-nucleotide resolution. Cell *147*, 1408-1419.
Richard, K.L., Kelley, B.R., and Johnson, J.G. (2019). Heme Uptake and Utilization by Gram-Negative Bacterial Pathogens. Frontiers in Cellular and Infection Microbiology *9*, 81.
Rossi, M.S., Paquelin, A., Ghigo, J.M., and Wandersman, C. (2003). Haemophore-mediated signal transduction across the bacterial cell envelope in *Serratia marcescens*: the inducer and the transported substrate are different molecules. Molecular Microbiology *48*, 1467-1480.
Rudolph, G., Semini, G., Hauser, F., Lindemann, A., Friberg, M., Hennecke, H., and Fischer, H.-M. (2006). The Iron Control Element, Acting in Positive and Negative Control of Iron-Regulated *Bradyrhizobium japonicum* Genes, Is a Target for the Irr Protein. Journal of Bacteriology *188*, 733-744.
Sahoo, N., Goradia, N., Ohlenschläger, O., Schönherr, R., Friedrich, M., Plass, W., Kappl, R., Hoshi, T., and Heinemann, S.H. (2013). Heme impairs the ball-and-chain inactivation of potassium channels. Proc Natl Acad Sci U S A *110*, E4036-4044.
Schmitt, M.P. (1999). Identification of a two-component signal transduction system from *Corynebacterium diphtheriae* that activates gene expression in response to the presence of heme and hemoglobin. Journal of Bacteriology *181*, 5330-5340.
Schneider, J.C., and Guarente, L. (1991). Regulation of the yeast *CYT1* gene encoding cytochrome c1 by HAP1 and HAP2/3/4. Molecular and Cellular Biology *11*, 4934-4942.
Schneider, S., Marles-Wright, J., Sharp, K.H., and Paoli, M. (2007). Diversity and conservation of interactions for binding heme in b-type heme proteins. Natural Products Reports *24*, 621-630.
Schubert, E., Florin, N., Duthie, F., Henning Brewitz, H., Kühl, T., Imhof, D., Hagelueken, G., and Schiemann, O. (2015). Spectroscopic studies on peptides and proteins with cysteine-containing heme regulatory motifs (HRM). Journal of Inorganic Biochemistry *148*, 49-56.
Segawa, K., Watanabe-Matsui, M., Matsui, T., Igarashi, K., and Murayama, K. (2019). Functional Heme Binding to the Intrinsically Disordered C-Terminal Region of Bach1, a Transcriptional Repressor. Tohoku Journal of Experimental Medicine *247*, 153-159.
Shajari, N., Davudian, S., Kazemi, T., Mansoori, B., Salehi, S., Khaze Shahgoli, V., Shanehbandi, D., Mohammadi, A., Duijf, P.H.G., and Baradaran, B. (2018). Silencing of BACH1 inhibits invasion and migration of prostate cancer cells by altering metastasis-related gene expression. Artificial Cells, Nanomedicine, and Biotechnology *46*, 1495-1504.
Sharp, K.H., Schneider, S., Cockayne, A., and Paoli, M. (2007). Crystal Structure of the Heme-IsdC Complex, the Central Conduit of the Isd Iron/Heme Uptake System in *Staphylococcus aureus*. Journal of Biological Chemistry *282*, 10625-10631.
Shimizu, T. (2012). Binding of cysteine thiolate to the Fe(III) heme complex is critical for the function of heme sensor proteins. Journal of Inorganic Biochemistry *108*, 171-177.
Shimizu, T., Huang, D., Yan, F., Stranava, M., Bartosova, M., Fojtíková, V., and Martínková, M. (2015). Gaseous O_2, NO, and CO in Signal Transduction: Structure and Function Relationships of Heme-Based Gas Sensors and Heme-Redox Sensors. Chemical Reviews *115*, 6491-6533.
Shimizu, T., Lengalova, A., Martínek, V., and Martínková, M. (2019). Heme: emergent roles of heme in signal transduction, functional regulation and as catalytic centres. Chemical Society Reviews *48*, 5624-5657.
Simile, M.M., Latte, G., and Pascale, R.M. (2018). MAF proteins: a family of regulating and regulated molecules. Digestive Medicine Research *1*.

Singleton, C., White, G., Todd, J., Marritt, S., Cheesman, M., Johnston, A., and Le Brun, N. (2010). Heme-responsive DNA Binding by the Global Iron Regulator Irr from *Rhizobium leguminosarum*. The Journal of biological chemistry *285*, 16023-16031.
Śmiga, M., Bielecki, M., Olczak, M., and Olczak, T. (2019). *Porphyromonas gingivalis* PgFur Is a Member of a Novel Fur Subfamily With Non-canonical Function. Frontiers in Cellular and Infection Microbiology *9*.
Smith, A.D., and Wilks, A. (2015). Differential contributions of the outer membrane receptors PhuR and HasR to heme acquisition in *Pseudomonas aeruginosa*. Journal of Biological Chemistry *290*, 7756-7766.
Smith, L.J., Kahraman, A., and Thornton, J.M. (2010). Heme proteins—Diversity in structural characteristics, function, and folding. Proteins: Structure, Function, and Bioinformatics *78*, 2349-2368.
Song, Y., Yang, M., Wegner, S.V., Zhao, J., Zhu, R., Wu, Y., He, C., and Chen, P.R. (2015). A Genetically Encoded FRET Sensor for Intracellular Heme. ACS Chemical Biology *10*, 1610-1615.
Stauff, D.L., Bagaley, D., Torres, V.J., Joyce, R., Anderson, K.L., Kuechenmeister, L., Dunman, P.M., and Skaar, E.P. (2008). *Staphylococcus aureus* HrtA Is an ATPase Required for Protection against Heme Toxicity and Prevention of a Transcriptional Heme Stress Response. Journal of Bacteriology *190*, 3588-3596.
Stauff, D.L., and Skaar, E.P. (2009a). *Bacillus anthracis* HssRS signalling to HrtAB regulates haem resistance during infection. Molecular Microbiology *72*, 763-778.
Stauff, D.L., and Skaar, E.P. (2009b). The heme sensor system of *Staphylococcus aureus*. Contributions to Microbiology *16*, 120-135.
Stauff, D.L., Torres, V.J., and Skaar, E.P. (2007). Signaling and DNA-binding activities of the *Staphylococcus aureus* HssR-HssS two-component system required for heme sensing. Journal of Biological Chemistry *282*, 26111-26121.
Stock, A.M., Robinson, V.L., and Goudreau, P.N. (2000). Two-Component Signal Transduction. Annual Review of Biochemistry *69*, 183-215.
Stojijkovic, I., Hwa, V., de Saint Martin, L., O'Gaora, P., Nassif, X., Heffron, F., and So, M. (1995). The *Neisseria meningitidis* haemoglobin receptor: its role in iron utilization and virulence. Molecular Microbiology *15*, 531-541.
Stojiljkovic, I., Kumar, V., and Srinivasan, N. (1999). Non-iron metalloporphyrins: potent antibacterial compounds that exploit haem/Hb uptake systems of pathogenic bacteria. Molecular Microbiology *31*, 429-442.
Sun, J., Hoshino, H., Takaku, K., Nakajima, O., Muto, A., Suzuki, H., Tashiro, S., Takahashi, S., Shibahara, S., Alam, J.*, et al.* (2002). Hemoprotein Bach1 regulates enhancer availability of heme oxygenase-1 gene. EMBO J *21*, 5216-5224.
Tang, X.D., Xu, R., Reynolds, M.F., Garcia, M.L., Heinemann, S.H., and Hoshi, T. (2003). Haem can bind to and inhibit mammalian calcium-dependent Slo1 BK channels. Nature *425*, 531-535.
Tatusov, R.L., Galperin, M.Y., Natale, D.A., and Koonin, E.V. (2000). The COG database: a tool for genome-scale analysis of protein functions and evolution. Nucleic Acids Research *28*, 33-36.
Ter Linde, J.J., and Steensma, H.Y. (2002). A microarray-assisted screen for potential Hap1 and Rox1 target genes in *Saccharomyces cerevisiae*. Yeast *19*, 825-840.
Torres, V.J., Pishchany, G., Humayun, M., Schneewind, O., and Skaar, E.P. (2006). *Staphylococcus aureus* IsdB is a hemoglobin receptor required for heme iron utilization. Journal of Bacteriology *188*, 8421-8429.
Torres, V.J., Stauff, D.L., Pishchany, G., Bezbradica, J.S., Gordy, L.E., Iturregui, J., Anderson, K.L., Dunman, P.M., Joyce, S., and Skaar, E.P. (2007). A *Staphylococcus aureus* regulatory system that responds to host heme and modulates virulence. Cell Host Microbe *1*, 109-119.
Vanderpool, C.K., and Armstrong, S.K. (2001). The Bordetella *bhu* locus is required for heme iron utilization. Journal of Bacteriology *183*, 4278-4287.
Vanderpool, C.K., and Armstrong, S.K. (2003). Heme-responsive transcriptional activation of Bordetella *bhu* genes. Journal of bacteriology *185*, 909-917.

Villareal, V.A., Pilpa, R.M., Robson, S.A., Fadeev, E.A., and Clubb, R.T. (2008). The IsdC Protein from *Staphylococcus aureus* Uses a Flexible Binding Pocket to Capture Heme. Journal of Biological Chemistry *283*, 31591-31600.
Wakeman, C.A., Stauff, D.L., Zhang, Y., and Skaar, E.P. (2014). Differential Activation of *Staphylococcus aureus* Heme Detoxification Machinery by Heme Analogues. Journal of Bacteriology *196*, 1335-1342.
Walter, E.R.H., Ge, Y., Mason, J.C., Boyle, J.J., and Long, N.J. (2021). A Coumarin–Porphyrin FRET Break-Apart Probe for Heme Oxygenase-1. Journal of the American Chemical Society *143*, 6460-6469.
Waltz, E. (2019). Appetite grows for biotech foods with health benefits. Nature Biotechnology *37*, 573-580.
Warnatz, H.J., Schmidt, D., Manke, T., Piccini, I., Sultan, M., Borodina, T., Balzereit, D., Wruck, W., Soldatov, A., Vingron, M*., et al.* (2011). The BTB and CNC homology 1 (BACH1) target genes are involved in the oxidative stress response and in control of the cell cycle. Journal of Biological Chemistry *286*, 23521-23532.
Wennerhold, J., and Bott, M. (2006). The DtxR regulon of *Corynebacterium glutamicum*. Journal of Bacteriology *188*, 2907-2918.
White, G.F., Singleton, C., Todd, J.D., Cheesman, M.R., Johnston, A.W., and Le Brun, N.E. (2011). Heme binding to the second, lower-affinity site of the global iron regulator Irr from *Rhizobium leguminosarum* promotes oligomerization. FEBS Journal *278*, 2011-2021.
Wilks, A. (2002). Heme Oxygenase: Evolution, Structure, and Mechanism. Antioxidants & Redox Signaling *4*, 603-614.
Winkler, H., Adam, G., Mattes, E., Schanz, M., Hartig, A., and Ruis, H. (1988). Co-ordinate control of synthesis of mitochondrial and non-mitochondrial hemoproteins: a binding site for the HAP1 (CYP1) protein in the UAS region of the yeast catalase T gene (CTT1). EMBO J *7*, 1799-1804.
Wißbrock, A., Paul George, A.A., Brewitz, H.H., Kühl, T., and Imhof, D. (2019). The molecular basis of transient heme-protein interactions: analysis, concept and implementation. Biosci Rep *39*, BSR20181940.
Wu, X., Shen, W.-J., Deshpande, A., Olaitan, A.O., Palmer, K.L., Garey, K.W., Hurdle, J.G., and Simner, P.J. (2021). The Integrity of Heme Is Essential for Reproducible Detection of Metronidazole-Resistant *Clostridioides difficile* by Agar Dilution Susceptibility Tests. Journal of Clinical Microbiology *59*, e00585-00521.
Yang, J., Ishimori, K., and O'Brian, M.R. (2005). Two heme binding sites are involved in the regulated degradation of the bacterial iron response regulator (Irr) protein. Journal of Biological Chemistry *280*, 7671-7676.
Yang, J., Panek, H.R., and O'Brian, M.R. (2006). Oxidative stress promotes degradation of the Irr protein to regulate haem biosynthesis in *Bradyrhizobium japonicum*. Molecular Microbiology *60*, 209-218.
Yin, L., Wu, N., Curtin, J.C., Qatanani, M., Szwergold, N.R., Reid, R.A., Waitt, G.M., Parks, D.J., Pearce, K.H., Wisely, G.B*., et al.* (2007). Rev-erbα, a heme sensor that coordinates metabolic and circadian pathways. Science *318*, 1786-1789.
Yukl, E.T., Jepkorir, G., Alontaga, A.Y., Pautsch, L., Rodriguez, J.C., Rivera, M., and Moënne-Loccoz, P. (2010). Kinetic and spectroscopic studies of hemin acquisition in the hemophore HasAp from *Pseudomonas aeruginosa*. Biochemistry *49*, 6646-6654.
Zenke-Kawasaki, Y., Dohi, Y., Katoh, Y., Ikura, T., Ikura, M., Asahara, T., Tokunaga, F., Iwai, K., and Igarashi, K. (2007). Heme induces ubiquitination and degradation of the transcription factor Bach1. Molecular and Cellular Biology *27*, 6962-6971.
Zhang, L., Bermingham-McDonogh, O., Turcotte, B., and Guarente, L. (1993). Antibody-promoted dimerization bypasses the regulation of DNA binding by the heme domain of the yeast transcriptional activator HAP1. Proceedings of the National Academy of Sciences *90*, 2851-2855.
Zhang, L., and Guarente, L. (1994a). Evidence that TUP1/SSN6 has a positive effect on the activity of the yeast activator HAP1. Genetics *136*, 813-817.

Zhang, L., and Guarente, L. (1994b). HAP1 is nuclear but is bound to a cellular factor in the absence of heme. Journal of Biological Chemistry *269*, 14643-14647.
Zhang, L., and Guarente, L. (1994c). The yeast activator HAP1-a GAL4 family member-binds DNA in a directly repeated orientation. Genes & Development *8*, 2110-2119.
Zhang, L., and Guarente, L. (1995). Heme binds to a short sequence that serves a regulatory function in diverse proteins. EMBO J *14*, 313-320.
Zhang, L., and Hach, A. (1999). Molecular mechanism of heme signaling in yeast: the transcriptional activator Hap1 serves as the key mediator. Cellular and Molecular Life Sciences *56*, 415-426.
Zhang, L., Hach, A., and Wang, C. (1998). Molecular mechanism governing heme signaling in yeast: a higher-order complex mediates heme regulation of the transcriptional activator HAP1. Molecular and cellular biology *18*, 3819-3828.
Zhu, G.D., Liu, F., OuYang, S., Zhou, R., Jiang, F.N., Zhang, B., and Liao, W.J. (2018). BACH1 promotes the progression of human colorectal cancer through BACH1/CXCR4 pathway. Biochem Biophys Res Commun *499*, 120-127.
Zitomer, R.S., Carrico, P., and Deckert, J. (1997). Regulation of hypoxic gene expression in yeast. Kidney International *51*, 507-513.
Zollman, S., Godt, D., Privé, G.G., Couderc, J.L., and Laski, F.A. (1994). The BTB domain, found primarily in zinc finger proteins, defines an evolutionarily conserved family that includes several developmentally regulated genes in Drosophila. Proc Natl Acad Sci U S A *91*, 10717-10721.

Figures

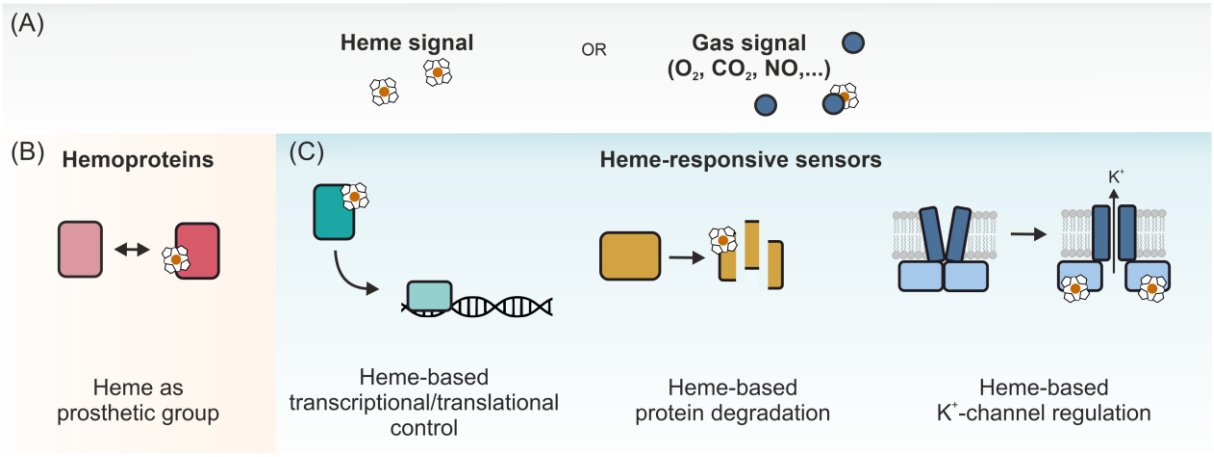

Figure 1: Diversity of heme-binding proteins. Schematic overview of the most common types of heme proteins. (A) In general, we can distinguish between two types of heme sensors: proteins, which directly sense heme and proteins, where heme serves as sensor for gases or redox stress. (B) Hemoproteins are enzymes that use heme as prosthetic group and have diverse functions, including electron transfer, oxygen transport, oxygen storage, reduction of peroxides, etc. (Chapman et al., 1997). (C) Transient Heme-protein interaction may have diverse physiological consequences, including regulation of transcription, proteasome-dependent or -independent protein degradation, regulation of K^+-channels. Further examples are the regulation of tRNA synthetases, mRNA splicing, or other protein-protein interactions, which are further summarized e.g. in Shimizu et al. (2019).

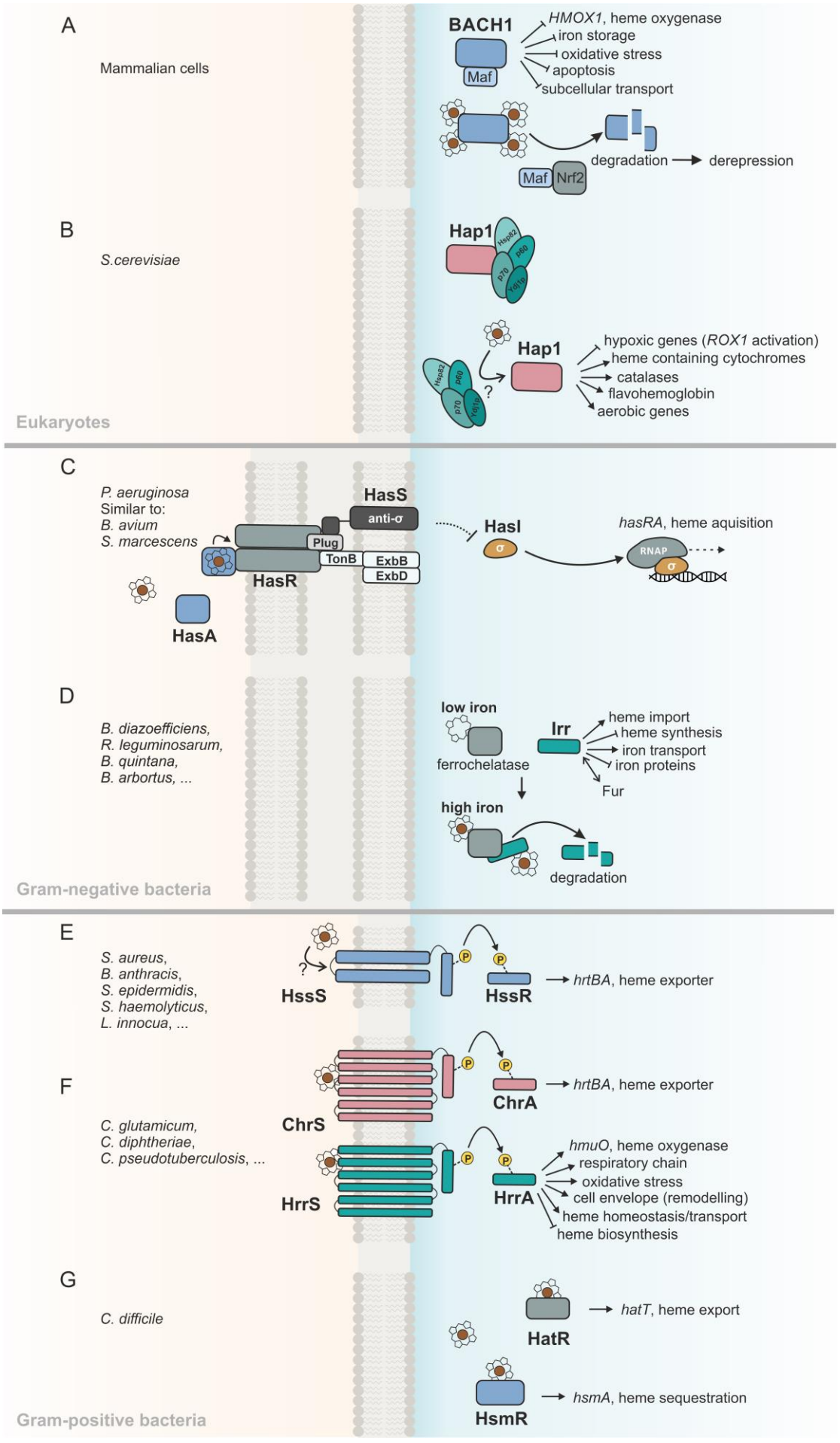
A
Mammalian cells
BACH1
Maf
HMOX1, heme oxygenase
iron storage
oxidative stress
apoptosis
subcellular transport
degradation
derepression
Maf
Nrf2
B
S.cerevisiae
Hap1
Hap1
hypoxic genes (ROX1 activation)
heme containing cytochromes
catalases
flavohemoglobin
aerobic genes
Eukaryotes
C
P. aeruginosa
Similar to:
B. avium
S. marcescens
HasS
anti-σ
Plug
TonB
ExbB
ExbD
HasR
HasA
HasI
σ
hasRA, heme aquisition
RNAP
σ
D
B. diazoefficiens,
R. leguminosarum,
B. quintana,
B. arbortus, ...
low iron
ferrochelatase
Irr
heme import
heme synthesis
iron transport
iron proteins
Fur
high iron
degradation
Gram-negative bacteria
E
S. aureus,
B. anthracis,
S. epidermidis,
S. haemolyticus,
L. innocua, ...
HssS
HssR
hrtBA, heme exporter
F
C. glutamicum,
C. diphtheriae,
C. pseudotuberculosis, ...
ChrS
ChrA
hrtBA, heme exporter
HrrS
HrrA
hmuO, heme oxygenase
respiratory chain
oxidative stress
cell envelope (remodelling)
heme homeostasis/transport
heme biosynthesis
G
C. difficile
HatR
hatT, heme export
HsmR
hsmA, heme sequestration
Gram-positive bacteria

Figure 2: Heme sensor systems controlled by transient heme binding. Schematic overview on eukaryotic and prokaryotic heme sensor systems controlled by transient heme-protein interaction: (A) In *Saccharomyces cerevisiae*, Hap1 (pink) regulates heme containing enzymes like e.g. cytochromes in response to hypoxia sensed indirectly via the availability of heme. Upon heme binding to the protein, Hap1 is released from the high-molecular weight complex (shades of turquoise) allowing it to bind to target promoter regions and control gene expression. (B) In mammalian cells, BACH1 (dark blue) regulates more than 50 genes involved in heme homeostasis. BACH1 forms heterodimers with Maf proteins (light blue) to inhibit transcription of its targets. Upon heme binding, BACH1 is dissociated and Maf proteins can bind to the transcription factor Nrf2 (grey) which activates several genes encoding protective antioxidants. (C) In *Pseudomonas aeruginosa*, the Has system responds to the presence of heme for the regulation of its acquisition. The system consists of the outer membrane receptor HasR (grey) with a N-terminal plug domain (light grey), the membrane bound anti-sigma factor HasS (dark grey) shown with TonB-ExbBD (white) and the extracytoplasmic function (ECF) σ factor HasI (yellow). HasR senses heme with the help of the hemophore HasA, transduces a signal to HasS, which releases HasI initiating transcription. Imported heme further activates PhuTUV for uptake into the cytoplasm, where heme is transported by the heme binding protein PhuS to the heme oxygenase HemO. (D) The Irr protein (turquoise) represents the global regulator of iron/heme homeostasis in rhizobia, by sensing iron pools indirectly via heme. When iron is present, the ferrochelatase (grey) inserts iron into protoporphyrin IX yielding heme. The heme-loaded ferrochelatase binds to Irr, thereby inhibiting it. Heme binding to Irr leads to proteins degradation and derepression of its target genes. (E) In Gram-positive bacteria, two-component systems represent a common mode of heme sensing. In Bacillales the two-component system HssSR (blue) activates the expression its target in a heme-dependent manner. Upon sensing of heme, autophosphorylation (yellow circles) of the histidine kinase HssS allows phosphotransfer to the response regulator HssR for its activation. HssR then functions as a transcriptional regulator for the activation of the expression of *hrtBA* encoding a heme exporter. (F) In Corynebacteria, the two paralogous two-component systems

ChrSA (pink) and HrrSA (turquoise) show a high level of cross-talk and control heme detoxification and heme homeostasis, respectively. (G) In anaerobic Clostridia, the two transcriptional regulators HatR (grey) and HsmR (blue) are necessary for heme detoxification. Upon heme binding, HatR activates the heme efflux pump hat, while HsmR activates HsmA important for heme sequestration. For more detailed information on these systems refer to Figure 4.

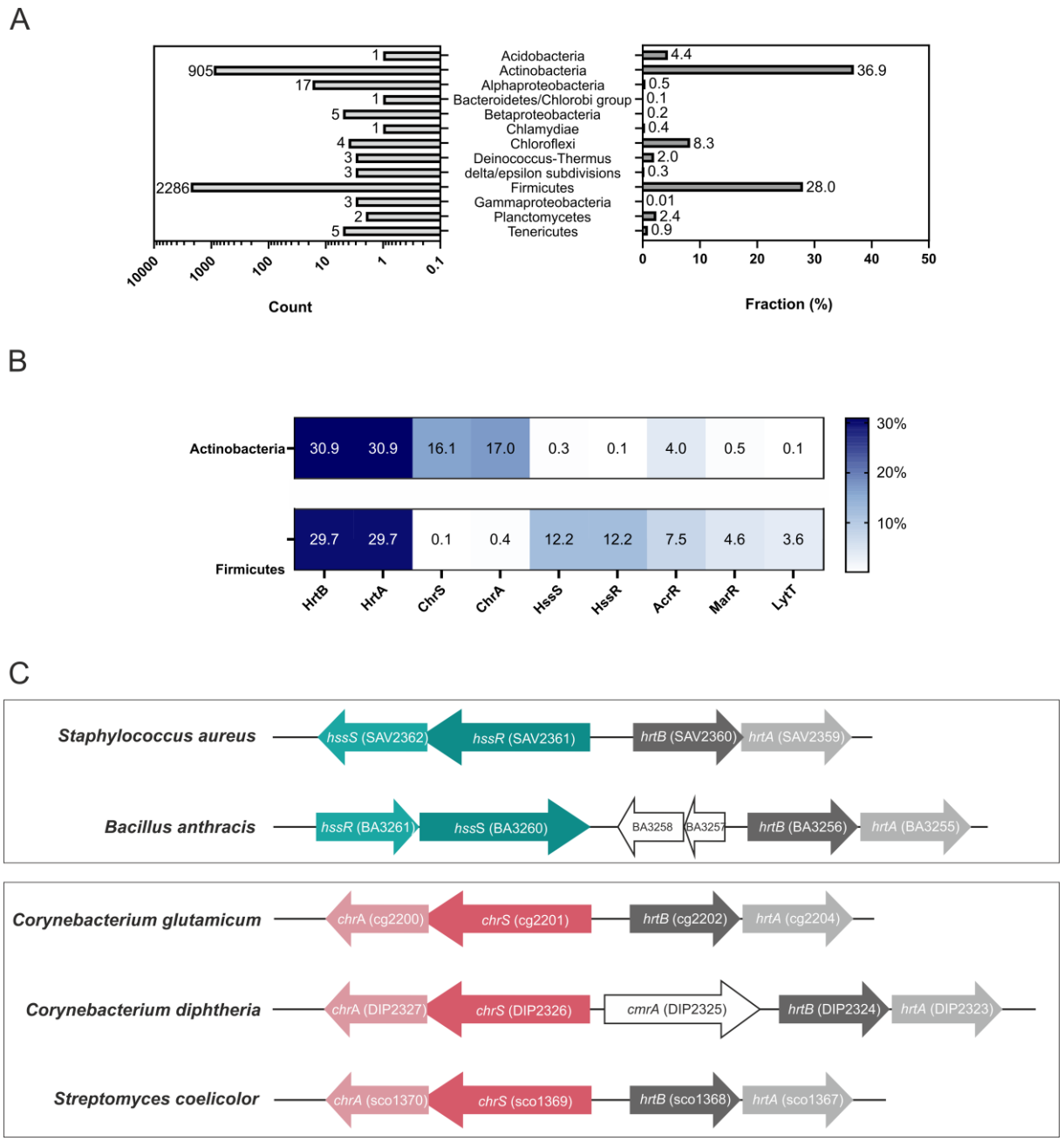

Figure 3: Analysis of *hrtB* synteny. The heme exporter HrtBA is a conserved target of two-component systems (TCS) in Gram-positive bacteria. (A) In silico analysis revealed 3232 bacterial genomes possessing *hrtBA* orthologues. On the y-axis the phyla are listed. Bars to the left indicate the count of bacterial genomes having a hit in the respective phylum; bars to the right represent the fraction of genomes of the particular phylum harboring *hrtBA* orthologues (B) Analysis of co-occurences of *hrtBA* and genes encoding transcriptional regulators, including TCSs. Overall 4704 *hrtBA* loci were found among 2286 Firmicutes and 905 Actinobacteria genomes. Numbers represent the percentage of the respective

transcriptional regulator domain found 5 kb up- and downstream of *hrtBA* in Actinobacteria and Firmicutes. The analysis confirms a conserved synteny of *hssRS* with *hrtBA* in Firmicutes, while *chrSA* is predominantly found in vicinity to *hrtBA* in actinobacterial genomes (C) Genomic organization of the *hrtBA* loci in different Gram-positive species. The *hrtBA* operon is shown in shades of grey, the TCS operons in shades of green and pink.

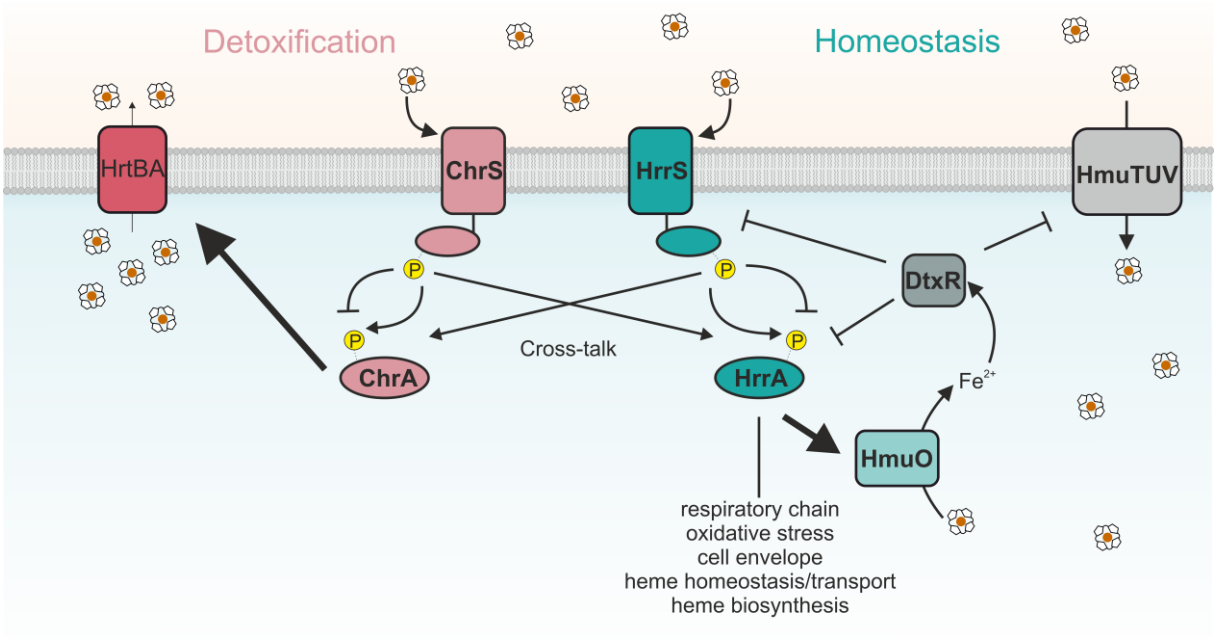

Figure 4: Heme homeostasis and detoxification is mediated by the two paralogous two-component systems HrrSA and ChrSA in *C. glutamicum*. The simplified schematic representation shows the interaction of the two TCS HrrSA (blue) and ChrSA (pink) and their targets *hmuO* and *hrtBA* respectively. HrrSA was recently shown to act as a global regulator of heme homeostasis controlling genes involved in the respiratory chain, heme biosynthesis, oxidative stress or cell envelope remodeling (Keppel et al., 2020). The histidine kinases ChrS as well as HrrS undergo autophosphorylation in response to heme. Both systems display cross-phosphorylation of the respective non-cognate response regulator, but no other TCS of *C. glutamicum* has been shown to activate HrrA or ChrA. In contrast, phosphatase activity of the kinases is specific towards the respective cognate response regulator. The HrrSA-regulated heme oxygenase (HmuO) can degrade heme to release iron for important cellular processes. The released iron can be sensed by DtxR, which consequently represses the expression of *hmuO*, *hrrA* and also *hmuTUV*.

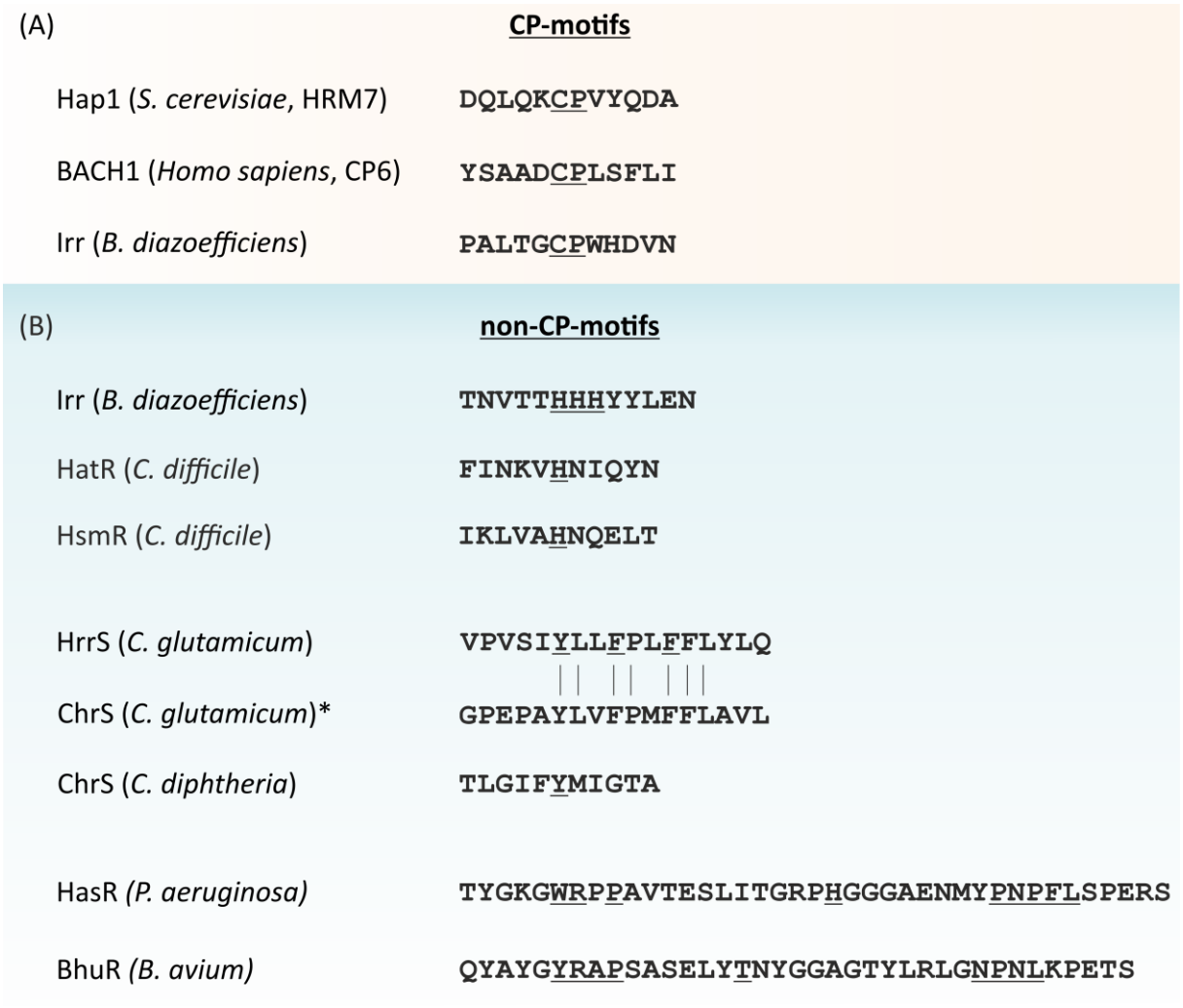

Figure 5: Overview of CP- and non-CP heme-binding motifs. Shown are the heme-responsive motifs of the systems summarized in this review, including five amino acids up- and downstream of the respective residues. (A) Most important CP-motif of eukaryotic Hap1 and BACH1 and prokaryotic Irr proteins. (B) Non-CP-motif like histidine-rich residues in Irr of Rhizobia or HatR and HsmR in Clostridia, tyrosine (and phenylalanine) motifs of Corynebacteria TCS and FRAP/NPNL like motifs of HasR of Pseudomonales or BhuR of Burkholderiales are shown. Vertical lines between the two paralogous systems HrrS and ChrS emphasize same amino acids. * = in contrast to HrrS, mutations of these residues in ChrS does not fully abolish heme binding.

1421 ## Tables

1422 **Table 1:** Overview of heme sensor systems.

Heme sensor	Organisms	Heme effect[1)]	Components[2)]	Heme binding	Mode of action/Function	Target sequence	Selected references
Hap1	*S. cerevisiae*	+	Transcription factor **Hap1**, Hsp82, p70, p60, Ydj1p	➢ Mainly C-terminal HRM7 with CP motif (C^{1193}-P) ➢ additionally HRM1-6	Transcriptional regulator in cell respiration (cytochromes), also redox stress and oxygen sensing; K_D < 20 µM (with peptide)	CGGnnnTAnCGG	Hon et al. (2000), Lee et al. (2003), Zhang and Guarente (1995)
BACH1	Mammalian cells	-	**BACH1**, Maf	➢ Four pivotal, cooperative CP motifs (CP3-6) out of six ➢ CP6: C^{649}-P ➢ binding via 5-coordinated bond	Repressor for heme oxygenase, iron storage, oxidative stress, etc.; K_D ≈ 13.7 µM	TGCTGAC(G)TCAGCA (MARE)	(Ogawa et al., 2001; Segawa et al., 2019; Zenke-Kawasaki et al., 2007)
HasR/ HasS/ HasI/ HasA	*P. aeruginosa* *S. marcescens*	+	Hemophore **HasA**, sensor domain **HasR**, anti-σ-factor HasS, σ-factor HasI	In *P. aeruginosa*: ➢ HasA: H^{32}, Y^{75}, H^{83} ➢ HasR: consensus FRAP domain (W^{609}-R-P-P); NPNL domain (N^{634}-P-F-L); histidine residue in between (H^{624})	Transcriptional activator for heme acquisition and utilization	n.d.	Dent and Wilks (2020)
BhuR/ RhuR/ RhuI	*B. avium* *B. pertussis*	+	sensor domain **BhuR**, extracytoplasmic sigma factor RhuI, regulator RhuR	In *B. avium*: ➢ consensus FRAP domain (Y^{604}-R-A-P); NPNL domain (N^{627}-P-N-L); tyrosine residue in between (H^{616})	Transcriptional activator for heme acquisition (and utilization)	n.d.	Murphy et al. (2002)
Irr	*B. diazoefficiens,* *R. leguminosarum,* *B. quintana,* *B. arbortus*	-	**Irr**	In *B. diazoefficiens:* ➢ High-affinity side for oxidized heme: CP motif (G^{28}-C-P-W-H-D) ➢ Low-affinity side for reduced heme: His-rich motif ($H^{117-119}$)	Iron sensing via heme, transcriptional regulator for heme synthesis, import and utilization, oxidative stress	TTTAGAAnnnTTCTAAA	(Qi et al., 1999; Rudolph et al., 2006; Singleton et al., 2010;

					K_D ≈ 0.01 µM for oxidized heme		Yang et al., 2005)
HssSR	*S. aureus,* *B. anthracis,* *S. epidermis,* *S. haemolyticus,* *L. innocua*	+	Histidine kinase **HssS** and response regulator HssR ABC-transporter HrtBA	➢ ? ➢ suggested to be indirect	Detoxification from heme Signalling/DNA binding	GTTCATAT TnnGTTCA TATT	(Stauff and Skaar, 2009b; Stauff et al., 2007)
ChrSA/ HrrSA	*C. glutamicum,* *C. diphtheria,* *C. pseudotuberculosis*	+	Histidine kinases **ChrS/HrrS** and response regulators ChrA/HrrA	➢ ChrS potentially extracellular sensing ➢ HrrS rather inside the membrane ➢ HrrS in *C. glutamicum*: Y^{112}, F^{115}, F^{118}	Detoxification from/Utilization of heme	n.d.	Bibb et al. (2007), Keppel et al. (2018)
HatR/ HsmR	*C. difficile*	+	Transcriptional regulators **HatR** and **HsmR**	➢ Involved histidine residues: H^{99} in HatR, H^{50} in HsmR	Detoxification from heme	n.d.	Knippel et al. (2020)

[1)] + = heme leads to higher levels of this protein; - = heme leads to lower levels/degradation of this protein. [2)] Proteins, which sense the heme, are bold.