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***Pseudomonas aeruginosa* possesses three distinct systems for sensing and using the host molecule heme**

Joaquín R. Otero-Asman, Ana I. García-García, Cristina Civantos, José M. Quesada, and María A. Llamas*

Department of Environmental Protection, Estación Experimental del Zaidín-Consejo Superior de Investigaciones Científicas, 18008 Granada, Spain

***Correspondence:** Dr. María A. Llamas, Department of Environmental Protection, Estación Experimental del Zaidín – CSIC, C/ Profesor Albareda 1, 18008 Granada, Spain. Email: marian.llamas@eez.csic.es

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Originality-Significance Statement

Iron acquisition during infection is essential for pathogens to spread and colonize their host. Because most iron in vertebrates is complexed within heme, this host molecule represents an important source of iron for pathogens. In this work we have identified a previously uncharacterized system in the human pathogen *Pseudomonas aeruginosa* that is used to sense and respond to heme. This finding makes *P. aeruginosa* the only pathogen in which three heme systems have been reported to date, which highlights the importance of this host molecule during *P. aeruginosa* infections. We show that *P. aeruginosa* uses heme not only as a source of iron but also as a signal molecule and report the molecular mechanism that triggers the heme signaling cascade. These findings provide new insights that may be exploited to block heme sensing and utilization, and thus virulence, in this emerging human pathogen.

Summary

Pathogens have developed several strategies to obtain iron during infection, including the use of iron-containing molecules from the host. Heme accounts for the vast majority of the iron pool in vertebrates and thus represents an important source of iron for pathogens. Using a proteomic approach, we have identified in this work a previously uncharacterized system, which we name Hxu, that together with the known Has and Phu systems, is used by the human pathogen *Pseudomonas aeruginosa* to respond to heme. We show that the Has and Hxu systems are functional signal transduction pathways of the cell-surface signaling class and report the mechanism triggering the activation of these signaling systems. Both signaling cascades involve an outer membrane receptor (HasR and HxuA, respectively) that upon sensing heme in the extracellular medium produces the activation of an σ^{ECF} factor in the cytosol. HxuA has a major role in signaling and a minor role in heme acquisition in

37 conditions in which the HasR and PhuR receptors or other sources of iron are present.
38 Remarkably, *P. aeruginosa* compensates the lack of the HasR receptor by increasing the
39 production of HxuA, which underscores the importance of heme signaling for this pathogen.

40 **Keywords:** *Pseudomonas aeruginosa*, signaling, sigma factor, iron, heme, TonB-dependent
41 receptor

Introduction

Iron is essential for the survival of virtually all organisms. This metal is involved in a wide range of cellular processes ranging from DNA replication to important metabolic reactions. For most pathogens, iron acquisition is a key factor in colonization and infection (Becker and Skaar, 2014). Vertebrates have evolved several mechanisms to reduce the availability of iron as a strategy to combat pathogens, a process referred to as ‘nutritional immunity’. The vast majority of iron in vertebrates is complexed within the porphyrin ring of heme in hemoproteins, such as hemoglobin, or intracellularly sequestered by the iron storage protein ferritin. Extracellular iron is, at physiological pH, present in the oxidized form Fe^{3+} , which is insoluble and therefore not available for pathogens. Moreover, most extracellular iron of vertebrates is bound to the serum protein transferrin for transport through the body, or to lactoferrin, a glycoprotein of the transferrin family found in milk, tears, and the mucosa of nasal passages (Cassat and Skaar, 2013; Parrow et al., 2013). Increased synthesis of these iron-scavenging proteins, or of haptoglobin and hemopexin that conjugate free hemoglobin and heme, are some of the strategies used by the host to reduce the amount of iron available for invading microorganisms (Cassat and Skaar, 2013; Parrow et al., 2013). In response, microorganisms synthesize and secrete siderophores, high affinity iron chelating compounds able to scavenge minute quantities of free iron from the environment and the host. Several pathogens also produce hemophores that bind host heme with high affinity, and most successful pathogens can also directly use the host-iron complexes transferrin, lactoferrin and hemoglobin as sources of iron (Wandersman and Delepelaire, 2004). The battle for iron is an important process in the host-pathogen interaction, with evolution shaping host iron-scavenging proteins into variants that are less well recognized by the pathogen, and the bacterium facing this process by counterselecting receptors able to recognize the altered host proteins (Armitage and Drakesmith, 2014; Barber and Elde, 2014).

Pseudomonas aeruginosa is a Gram-negative free-living bacterium commonly found in soil and water. This bacterium is increasingly recognized as an emerging human pathogen of high clinical relevance because it causes a wide array of life-threatening acute and chronic infections, particularly in immunocompromised and cystic fibrosis (CF) patients (Sadikot et al., 2005). This bacterium is moreover one of the leading causes of nosocomial infections affecting hospitalized patients (Rosenthal et al., 2016). *P. aeruginosa* infections are often difficult to treat due to its natural resistance to antibiotics and to its capacity to acquire such resistance (Lister et al., 2009; Breidenstein et al., 2011). *P. aeruginosa* contains a large repertoire of virulence determinants and a complex regulatory network that allows the bacterium to colonize many different host environments (Lyczak et al., 2000; Balasubramanian et al., 2013; Moradali et al., 2017). This is also driven by the ability of this pathogen to scavenge iron during infection. The different strategies used by *P. aeruginosa* to acquire iron include: 1) the production of two siderophores, pyoverdine and pyochelin; 2) the uptake of siderophores produced by other microorganisms (referred as xenosiderophores) with which *P. aeruginosa* shares the niche; 3) the uptake of Fe²⁺ through the Feo system; and 4) the utilization of heme from host hemoproteins (Cornelis and Dingemans, 2013).

Two systems involved in heme acquisition have been identified in *P. aeruginosa*, the Phu (from *Pseudomonas* heme uptake) and the Has (from heme assimilation system) (Ochsner et al., 2000) (Fig. 1A and 1B). In both systems the transport of heme through the highly impermeable outer membrane of *P. aeruginosa* occurs through specialized receptors of the TonB-dependent receptor (TBDR) family in an energy-dependent process (Noinaj et al., 2010) (Fig. 1C). The Phu system mediates the direct acquisition of free heme through the PhuR receptor, whereas the Has system involves the secretion of a heme-binding protein, the hemophore HasAp, and the HasR receptor that extracts the heme from the hemophore and transports it into the periplasm (Fig. 1C). Heme is internalized into the cytoplasm through the

PhuSTUV system and degraded by the heme oxygenase HemO, which results in the release of iron (O'Neill et al., 2012) (Fig. 1C). Expression of both systems occurs in iron restricted and not in iron sufficient conditions, a control exerted by the Fur repressor protein that in high iron conditions binds to the *hasR* and *phu* promoter regions inhibiting gene transcription (Ochsner et al., 2000). Although it was initially proposed that the Has system was the high-affinity transport system for heme (Létoffé et al., 1998; Ochsner et al., 2000), a recent study proposes that the PhuR receptor plays a major role in heme acquisition whereas the primary role of HasR is to sense the presence of heme (Smith and Wilks, 2015). HasR belongs to a special subfamily of TBDR that are not only involved in uptake but also in sensing and signaling. These receptors contain an N-terminal extension of about 90-100 amino acids referred to as the signaling domain that is responsible of the signal transduction function (Koebnik, 2005; Noinaj et al., 2010) (Fig. 1C, red ball of the HasR receptor), and are functionally, and normally also genomically, associated with extracytoplasmic function sigma factor (σ^{ECF}) and anti- σ factor pairs (Llamas et al., 2014; Otero-Asman et al., 2019) (Fig. 1B). These three proteins form a signal transduction pathway known as cell-surface signaling (CSS) (Llamas et al., 2014). In response to the presence of the cognate CSS inducing signal in the extracellular medium, the CSS receptor initiates a cascade that produces the targeted proteolysis of the CSS anti- σ factor and the release and activation of the CSS σ^{ECF} in the cytosol (Draper et al., 2011; Bastiaansen et al., 2014; Llamas et al., 2014; Bastiaansen et al., 2015a). Upon activation, the σ^{ECF} binds to the core enzyme of the RNA polymerase (RNAPc) activating the transcription of the genes required to respond to the signal. One of the genes usually stimulated by CSS σ^{ECF} is the one encoding the CSS receptor (Llamas et al., 2008; Llamas et al., 2014). This positive feedback mechanism increases the amount of the receptor in the outer membrane and, therefore, the capacity of the bacterium to sense and respond to the CSS signal.

By analogy with the well-studied hemophore-mediated CSS pathway of *Serratia marcescens* (Rossi et al., 2003; Biville et al., 2004) we and others had proposed that the *P. aeruginosa* HasR receptor together with the σ^{HasI} /HasS proteins forms a CSS involved in the detection and uptake of heme (Llamas et al., 2014; Smith and Wilks, 2015). The involvement of the hemophore HasAp and the σ^{HasI} factor in this process has been recently shown (Dent et al., 2019), but many features of the *P. aeruginosa* heme signaling process remain unknown. The purpose of this work was to analyze the response of *P. aeruginosa* to the presence of heme, which is important to get new insights to block *P. aeruginosa* iron acquisition during infection and, therefore, virulence.

Results

P. aeruginosa responds to the presence of heme in the extracellular medium.

We focused on the outer membrane composition of *P. aeruginosa* to analyze the response of the bacterium to heme. Cell envelope preparations enriched by outer membrane proteins of *P. aeruginosa* cells grown in low iron without or with heme were analyzed by SDS-PAGE. Three main protein bands were observed in the low iron control sample (Fig. 2A), which, as identified by proteomic in previous work (Llamas et al., 2006), likely consist of the pyoverdine receptor FpvA, the heme receptor PhuR and the pyochelin receptor FptA, respectively (Fig. 2A). A differentially expressed protein band appeared in cells grown in heme (Fig. 2A). Proteomic analyses showed that this band contained the HasR receptor and also the uncharacterized TBDR PA1302 (Fig. 2A). In fact, PA1302 was the major protein within this sample as judged by the higher number of tryptic peptides that matched this protein as compared with those matching HasR (Fig. 2A). PA1302 presents the N-terminal extension typical of TBDR involved in CSS and it is genomically associated with an σ^{ECF} /anti- σ factor pair (PA1300 and PA1301, respectively). Protein sequence analysis and

classification using InterPro revealed that this protein belongs to the TonB-dependent heme/hemoglobin receptor family (IPR011276), of which the *Haemophilus influenzae* HxC heme receptor is the best characterized (Zambolin et al., 2016). Therefore, we re-named PA1302 as HxuA, and the associated σ^{ECF} /anti- σ factor proteins as σ^{HxuI} and HxuR, respectively, following the nomenclature adopted for the components of CSS systems in *Pseudomonas* (Llamas et al., 2014). DIAMOND BLASTp analyses including a total of 4847 *Pseudomonas* genome sequences (2759 of which belonging to *P. aeruginosa* strains) showed that HxuA is present in 98.6% of the *P. aeruginosa* strains analyzed and scarce in other *Pseudomonas* species (Dataset S1).

Quantitative real-time PCR (qRT-PCR) analyses to measure expression of the identified receptor genes showed that *hasR* and *hxuA* mRNA levels were higher in low than in high iron (12- and 4.5-fold, respectively) conditions and increased when heme was added to the low iron medium (20- and 13-fold, respectively) (Fig. 2B). Expression of the third heme receptor gene *phuR* was also higher in low than in high iron conditions but decreased upon addition of heme (Fig. 2B). This effect is likely due to the supply of iron that *P. aeruginosa* receives through heme, and shows that, in contrast to *hasR* and *hxuA* that both require iron limitation and heme to be fully expressed, iron limitation alone is enough for full expression of *phuR*. This is in agreement with PhuR being detected in the outer membrane of *P. aeruginosa* cells grown solely in low iron (Llamas et al., 2006), but not HasR and HxuA (Fig. 2A).

To know if the heme receptors were differentially produced depending on the amount of heme available, we measured their expression in different concentrations of heme using transcriptional fusions in which the known (Ochsner et al., 2000) or *in silico* predicted (Winsor et al., 2016) promoter-containing region upstream the receptor gene was fused to *lacZ* (Tables S1 and S2). *phuR* promoter activity was maximal in low iron and addition of heme resulted in a concentration-dependent decrease of its activity (Fig. 2C), in accordance

with the previous observation indicating that the supply of iron diminishes the mRNA levels of this receptor (Fig. 2B). In contrast, *hasR* promoter activity was induced by all heme concentrations tested, although maximal induction was observed at 5 μ M (Fig. 2C). At this heme concentration the activity of the *hxA* promoter was only moderately induced and higher amounts of heme (20 μ M) were required to fully induce the expression from this promoter (Fig. 2C). These results suggest that *P. aeruginosa* adapts the production of its three heme receptors to the amount of heme it encounters.

The *P. aeruginosa* PhuR and HasR receptors play a role in heme utilization.

The contribution of the identified heme receptors to the utilization of heme by *P. aeruginosa* was assayed by constructing *phuR*, *hasR*, and *hxA* single, double, and triple mutants and measuring their growth in low iron medium either or not supplemented with heme. All strains grew poorly in low iron conditions but grew at a similar rate as the PAO1 wild-type strain when iron (FeCl_3) was added to the medium (Fig. 3A). Addition of 20 μ M of heme to the low iron medium allowed the growth of the PAO1 wild-type strain, whereas addition of lower amounts of heme (0.5 and 5 μ M) did not (Fig. 3B). The growth of the single *phuR*-Tn and Δ *hasR* mutants upon supplementation of heme was delayed, especially that of the Δ *hasR* mutant (Fig. 3C). However, the Δ *hxA* mutant grew in heme at similar rate as the wild-type strain (Fig. 3C). This indicates that PhuR and especially HasR play a larger role in heme utilization than HxA. Surprisingly, the growth of double and triple receptor mutants in heme, although impaired, was less affected than that of the *hasR* single mutant (Fig. 3C). This could be due to a switch in the predominant use of heme by *P. aeruginosa* to another source of iron when two or more heme receptors are lacking. In fact, production of the *P. aeruginosa* siderophore pyoverdine was higher in the heme receptor mutants than in the wild-type strain, especially in those mutants that were more impaired for heme utilization (i.e. *hasR* and *phuR*

mutants) (Fig. S1). This indicates that *P. aeruginosa* adapts to a situation in which utilization of heme is less efficient by using other sources of iron.

***P. aeruginosa* compensates for a lack of HasR by increasing HxuA production.**

Next, we wondered whether *P. aeruginosa* compensates a lack of one of the three heme receptors by increasing the amounts of the others. Thus, we assayed *hasR*, *hxuA* and *phuR* promoter activity in the single, double and triple receptor mutants (Fig. 4). Because the mutants contain a transposon (Tn) that confers tetracycline (Tc) resistance, a gentamycin (Gm) cassette was cloned in the pMP220-derived plasmids used to measure promoter activity (Table S1). For an unknown reason, presence of the Gm cassette reduced the *lacZ* activity of the original plasmids (without Gm, Fig. 2C) but had no effect on the overall response of the promoters to low iron or heme (Fig. 4, PAO1 strain). The *hasR* promoter activity was unaffected in the *phuR*-Tn mutant, only slightly reduced in the Δ *hxuA* mutant and totally abolished in the Δ *hasR* mutant (Fig. 4A). Complementation of the Δ *hasR* mutation with a copy of the *hasR*-*hasAp* operon (Fig. 1B) restored the promoter activity of *hasR* but not with a copy of the *hasR* gene alone (Fig. S2A). This suggests that the *hasR* deletion affects the expression of its downstream *hasAp* gene and thus the production of the HasAp hemophore, which seems to be necessary for *hasR* expression. Providing the *hasAp* gene alone to Δ *hasR* did not restore the *hasR* promoter activity (Fig. S2A), which indicates that the HasR receptor is also required for its own expression. Besides, our results also indicate that the lack of *PhuR* or *HxuA* is not compensated by increased synthesis of HasR (Fig. 4A). In agreement with this last observation, the activity of the *hasR* promoter was also reduced and not increased in the double mutants, especially the heme-induced activity of this promoter (Fig. 4A). This was more pronounced in the *phuR*-Tn Δ *hasR* double and in the triple mutant where *hasR* promoter activity was almost abolished (Fig. 4A).

Regarding *hxuA*, the promoter activity of this receptor in heme-containing medium was completely null in the $\Delta hxuA$ mutant, unaffected in the *phuR*-Tn mutant and considerably higher (4-fold) in the $\Delta hasR$ mutant (Fig. 4B). Complementation of the $\Delta hxuA$ and $\Delta hasR$ mutations by providing a wild-type copy of the *hxuA* gene or the *hasR*-*hasAp* operon, respectively, restored the promoter activity of *hxuA* in these mutants to wild-type levels (Fig. S2B). Increased activity of the *hxuA* promoter in the $\Delta hasR$ mutant correlates with more HxuA receptor protein detected in the outer membrane of this mutant than in the wild-type strain (Fig. S3). This indicates that *P. aeruginosa* compensates the lack of HasR by increasing the synthesis of HxuA. These results also show that HxuA is essential for its own heme-induced expression. In accordance, *hxuA* expression did not occur in any of the mutants containing the $\Delta hxuA$ deletion (Fig. 4B). However, the promoter activity of *hxuA* was also totally abolished in a *phuR*-Tn $\Delta hasR$ double mutant, despite the presence of an intact HxuA receptor. This suggests that both presence of HxuA and of either the PhuR or the HasR receptor are necessary for *hxuA* expression.

Finally, the promoter activity of the *phuR* receptor gene was only affected in the mutants containing the *phuR*-Tn mutation (Fig. 4C), suggesting that the PhuR receptor is required for its own expression. In contrast, expression of *phuR* does not depend on HasR and/or HxuA and thus loss of these receptors is not compensated by increasing the synthesis of PhuR.

The hemophore HasAp is essential for expression of the *P. aeruginosa* *hasR* receptor gene.

The complementation assay of the $\Delta hasR$ mutant suggested that not only the HasR receptor but also the hemophore HasAp is needed for *hasR* expression (Fig. S2A). In accordance, the promoter activity of *hasR* was completely null in a *hasAp*-Tn mutant (Fig.

5A) but it was restored by providing a copy of the wild-type *hasAp* gene to the mutant (Fig. S2C). This phenotype was observed both in absence and presence of heme, which shows that both the low iron- and the heme-induced expression of *hasR* depend on the production of the hemophore HasAp. In contrast, *hxuA* and *phuR* promoter activities were only slightly lower in the mutant (Fig. 5A), which indicates that the hemophore plays a minor role in the production of these receptors. These results suggest a role for HasAp as a signal molecule able to trigger the synthesis of HasR. This was confirmed using the supernatant of *P. aeruginosa* iron-restricted cultures, which contain the secreted hemophore (Létoffé et al., 1998; Dent et al., 2019). Addition of the supernatant of PAO1 cultures (hemophore-containing supernatant) to the *hasAp*-deficient strain triggered *hasR* promoter activity but not the addition of the *hasAp*-Tn mutant supernatant (hemophore-free supernatant) (Fig. 5B). Interestingly, the supernatant of both PAO1 iron-restricted cultures without heme (-heme sup) and with heme (+heme sup) were able to induce the activity of the *hasR* promoter (Fig. 5B). This indicates that the HasAp hemophore is able to trigger *hasR* expression even in the absence of external heme. Similar to the supernatant of the *hasAp*-Tn mutant, the supernatant of cultures of the Δ *hasR* receptor mutant did not induce *hasR* promoter activity (Fig. 5B). This confirms that the downstream *hasAp* gene is not expressed in the Δ *hasR* mutant, as suggested by the complementation assay (Fig. S2A). In contrast, HasAp production was unaffected in the Δ *hxuA* and *phuR*-Tn receptor mutants (Fig. 5B), which is in line with the wild-type *hasR*-*hasAp* promoter activity measured in these mutants (Fig. 4A).

The HasR and HxuA receptors are part of CSS regulatory pathways.

Both HasR and HxuA, but not PhuR, contain an N-terminal extension typical of TBDR involved in CSS pathways (Fig. 6A) and are genomically associated with σ^{ECF} /anti- σ factor pairs (Llamas et al., 2014) (Fig. 1B). Therefore, we hypothesized that these receptors promote

their own expression in response to the inducing signal (i.e. heme) through the activation of
 their cognate σ^{ECF} . To test this, we measured *hxuA* and *hasR* promoter activity in mutants in
 each one of the three components of the potential CSS systems. Both promoter activities were
 null in the CSS receptor mutant (*hxuA*-Tn and *hasR*-Tn, respectively) and in the σ^{ECF} mutant
 (*hxuI*-Tn and *hasI*-Tn, respectively) (Fig. 6B), which indicates that these proteins are essential
 in the signaling pathway that triggers *hxuA* and *hasR* expression. Complementation of the
 mutants with a wild-type copy of the mutated gene restored the promoter activities (Figs.
 S2A, S2B and S2D). The *hasR*-Tn mutation could be complemented with the *hasR*-*hasAp*
 operon but not with the *hasR* gene alone (Fig. S2A), likely because the Tn insertion affects
 the expression of the downstream *hasAp* gene (as also observed in the Δ *hasR* mutant).
 Constitutive production of σ^{HxuI} and σ^{HasI} resulted in constitutive expression of *hxuA* and
hasR, respectively (Fig. S2D). Thus, overproduction of σ^{HxuI} and σ^{HasI} can bypass the need of
 low iron and heme for signaling, as occurs with other *Pseudomonas* σ^{ECF} factors (Llamas et
 al., 2008; Bastiaansen et al., 2014; Quesada et al., 2016). All these results are in line with
 HxuA and HasR being the sensing protein, and σ^{HxuI} and σ^{HasI} the response protein of a CSS
 pathway (Llamas et al., 2014).

Regarding the CSS anti- σ factor component, mutation of *hxuR* increased *hxuA* promoter
 activity both in absence and presence of heme (Fig. 6B, *hxuR*-Tn mutant). Providing a copy
 of the *hxuR* gene to this mutant restored the wild-type activity (Fig. S2E). Lack of HxuR is
 thus enough to have expression of *hxuA*, likely because σ^{HxuI} is free in this situation and can
 promote *hxuA* transcription even when the signaling system has not been activated by heme.
 In contrast, overproduction of HxuR completely abolished the activity of the *hxuA* promoter
 (Fig. S2E, PAO1 strain). These phenotypes are in line with the anti- σ factor role of this
 protein. However, unlike *hxuR*, mutation of the *hasS* anti- σ factor did not enhance but impair
 the low iron-induced promoter activity of *hasR*, which was lower than in the PAO1 wild-type

strain (although not null), and did not affect the heme-induced activity of this promoter (Fig. 6B). This effect was observed in both a *hasS* mutant that contains the Tn insertion in the *hasI*-*hasS* intergenic region (hasS-Tn1) and in a mutant where the Tn is inserted in the periplasmic domain of the protein (hasS-Tn2) (Table S1 and Fig. 7A). A possible effect of the Tn insertion on the expression of the downstream *hasR*-*hasAp* genes was discarded because the supernatant of both mutants induced *hasR* expression in a *hasAp*-deficient strain (Fig. 5B, hasS-Tn1 and -Tn2), which indicates that production of the HasAp hemophore is not affected.

Two types of CSS anti- σ factor proteins have been identified, those that function as mere anti- σ factors and those that, next to the anti- σ function, also contain pro- σ activity (Mettrick and Lamont, 2009; Bastiaansen et al., 2015a). The pro- σ activity lies within the cytosolic N-terminal domain (N-tail) of the anti- σ factor protein (Fig. 7A), which has been proposed to be bound to the σ^{ECF} in the σ^{ECF} -RNAP complex (Mahren and Braun, 2003; Llamas et al., 2014). Complete deletion of the first class of anti- σ factors results in constitutive σ^{ECF} activity whereas that of the second class results in a lack of σ^{ECF} activity. The position of the Tn insertion in the *hXuR*-Tn and hasS-Tn1 mutants (Table S1) produces the complete absence of the corresponding anti- σ factor protein, including the N-tail domain. According to the phenotype of these mutants (Fig. 6B), HxuR seems to function as a mere anti- σ factor but HasS could also have pro- σ activity. To investigate this, the N-tail of these proteins was overexpressed, because overproduction of this domain has been shown to promote σ^{ECF} inhibition in the case of mere anti- σ factors and activation in the case of anti- σ factors with pro- σ activity (Mettrick and Lamont, 2009). *hXuA* promoter activity was significantly lower in PAO1 wild-type cells overexpressing the N-tail of HxuR, an effect that was more pronounced in cells lacking the HxuR anti- σ factor (Fig. 6C). This confirms that the N-tail of HxuR only contains anti- σ activity. Similarly, overexpression of the N-tail of HasS

protein reduced the activity of the *hasR* promoter (Fig. 6C), indicating that HasS also solely contains anti- σ activity.

***hasS* is expressed from its own promoter in a σ^{HasI} -dependent manner.**

The phenotype of the *hasS* mutants in which decreased instead of increased σ^{HasI} activity was observed (Fig. 6B) prompted us to investigate whether *hasS* is expressed differently than other anti- σ factors. Most *P. aeruginosa* CSS σ^{ECF} /anti- σ factor genes overlap and are thus co-transcribed from a single transcriptional unit (Llamas et al., 2014), as observed for the *hxuI* and *hxuR* genes that overlap 4 bp (Fig. S4). However, *hasI* and *hasS* are separated by 64 bp and RT-PCR analyses showed that these genes are not co-transcribed (Fig. S4). Thus, we constructed a *lacZ* transcriptional fusion of this intergenic region to analyze the presence of an active promoter. Activity of this fusion was null in iron sufficient conditions and was ~10-fold induced in iron deficient conditions (Fig. 6D). This indicates that the *hasI*-*hasS* intergenic region contains an active promoter for transcription of *hasS* that is regulated by iron. Addition of heme to the low iron medium resulted in increased promoter activity (~2-fold) (Fig. 6D). This pattern of activity resembles that of the *hasR* promoter (Figs. 2C and 6B), suggesting a role of the Has CSS system and σ^{HasI} also in the expression of *hasS*. In agreement, *hasS* promoter activity was extremely high in cells in which σ^{HasI} was constitutively produced, even in iron sufficient conditions, and was totally abolished in a *hasR* mutant (Fig. 6D). Because constitutive production of CSS σ^{ECF} is enough to produce expression of target genes (Llamas et al., 2008; Bastiaansen et al., 2014; Quesada et al., 2016), these results indicate that transcription of *hasS* is initiated by σ^{HasI} upon activation of this σ factor by HasR and the CSS pathway.

The HxuR and HasS anti- σ factors are subject to proteolysis.

Activation of CSS pathways requires the targeted proteolysis of the CSS anti- σ factor (Draper et al., 2011; Bastiaansen et al., 2014; Bastiaansen et al., 2015a; Bastiaansen et al., 2017). *Pseudomonas* CSS anti- σ factors are usually cytoplasmic membrane proteins that contain a short cytosolic N-domain that binds the σ^{ECF} , a large periplasmic C-domain that receives the CSS signal from the receptor, and a transmembrane domain that connects both regions (Llamas et al., 2014; Otero-Asman et al., 2019) (Fig. 7A). To determine if proteolysis of the anti- σ factor is also responsible for Has and Hxu CSS activation, processing of N-terminally HA-tagged versions of HxuR and HasS was analyzed by Western-blot. A predominant protein band of ~21-22 kDa was obtained for both proteins in heme-free and heme-containing medium (Fig. 7B, PAO1 strain). This fragment, which is considerably smaller than the size of the full protein (~35-36 kDa), likely corresponds to the N-domain that is produced after the spontaneous cleavage of CSS anti- σ factors. We have recently determined that most *Pseudomonas* CSS anti- σ factors undergo self-cleavage immediately upon production (Bastiaansen et al., 2015a; Bastiaansen et al., 2015b). This spontaneous cleavage occurs in a conserved Gly-Thr (GT) cleavage site located in the periplasmic domain of CSS anti- σ factors by a protease-independent chemical reaction known as N-O/S acyl rearrangement (Bastiaansen et al., 2015b). This cleavage produces two functional N- and C-anti- σ factor domains (Fig. 7A) that interact with each other in the periplasm and are required for proper CSS function (Bastiaansen et al., 2015a). Both HasS and HxuR proteins contain GT residues in the cleavage site (Fig. 7C), and it is therefore not surprising that these proteins undergo the spontaneous cleavage.

In response to heme, the N-domains of HxuR and HasS were further processed producing protein subfragments of about 12-13 kDa (Fig. 7B, PAO1), which is the expected size of the cytosolic tail (N-tail) of the proteins (Fig. 7A). Two proteases, RseP and Prc have been identified to be involved in CSS anti- σ factor processing in response to the CSS signal

(Bastiaansen et al., 2014; Bastiaansen et al., 2015a). RseP cuts within the transmembrane (TM) domain of CSS anti- σ factors generating the N-tail subfragment (Bastiaansen et al., 2015a). This cleavage allows the release and activation of the σ^{ECF} factor and it is therefore indispensable for CSS activity (Bastiaansen et al., 2014; Bastiaansen et al., 2015a). In accordance with this model, the N-tails of HxuR and HasS were not detected in a Δ rseP mutant (Fig. 7B). Instead, larger HxuR and HasS protein fragments were accumulated (Fig. 7B). These fragments likely are the RseP substrates and their accumulation in the mutant indicates that the N-domains of HxuR and HasS undergo another cleavage prior the RseP cleavage. In agreement with RseP being essential for CSS σ^{ECF} activation, the activity of the *hxuA* and *hasR* promoters was completely abolished in the Δ rseP mutant in both absence and presence of heme (Fig. 7D). The HxuR and HasS protein fragments that accumulated in the *rseP* mutant were absent in a Δ prc mutant (Fig. 7B). This suggests that Prc is responsible of the proteolytic event that generates these RseP substrates or acts upstream of it, thus being essential for this event to occur. Activity of the Hxu and Has CSS systems in the Δ prc mutant was considerably lower than in the wild-type strain, especially that of the Hxu system, although not null (Fig. 7D). In fact, small amounts of the HxuR and HasS N-tail subfragments, usually indicative of CSS activation (Bastiaansen et al., 2015a; Bastiaansen et al., 2015b), could still be detected in the Δ prc mutant (Fig. 7B). This indicates that the Hxu and Has systems can be partially activated in a Prc-independent manner.

Transcription of *phuR*, *phuS* and *hemO* is not promoted by σ^{HasI} or σ^{HxuI} .

We next wondered whether expression of the heme utilization genes *phuR*, *phuSTUVW* and *hemO* (Fig. 1A and 1C) depends on the Has and/or Hxu CSS pathways. Therefore, we tested the ability of the CSS response proteins σ^{HasI} and σ^{HxuI} to activate expression from the promoter of these genes. However, constitutive production of σ^{HasI} or σ^{HxuI} did not induce the

activity of *phuR*, *phuS* and *hemO* promoters while it did induce the activity of the σ^{HasI} -dependent promoter *hasR* and that of the σ^{HxuI} -dependent promoter *hxA* (Fig. 8). This indicates that the transcription of *phuR*, *phuS* and *hemO* is not mediated by these σ factors and thus that their expression does not depend on the Has and Hxu signaling pathways. In accordance, the *phuR*, *phuS* and *hemO* promoter activities did not increase in response to the Has and Hxu CSS inducing signal heme (Fig. 8). As previously observed for *phuR* (Fig. 2B and 2C), expression of *phuS* and *hemO* seem to be controlled solely by the intracellular concentration of iron and thus occurs in low but not in high iron conditions and diminishes in presence of the iron-supplier heme (Fig. 8).

Besides, analysis of *hasR* expression in cells producing σ^{HxuI} and that of *hxA* in cells producing σ^{HasI} showed that there is no cross-talk between the Has and Hxu signaling systems at the σ factor level because σ^{HasI} did not induce *hxA* promoter activity and σ^{HxuI} did not affect that of *hasR* (Fig. 8). In contrast, a considerably reduction in *hxA* expression was observed in cells overproducing σ^{HasI} (Fig. 8). This suggests that high amounts of σ^{HasI} impair the σ^{HxuI} -mediated transcription of *hxA* likely due to the competition of these σ factors for binding to the RNAPc.

Discussion

Acquisition of iron by pathogens is key for a successful infection (Ratledge and Dover, 2000; Sheldon and Heinrichs, 2015). Because heme accounts for the vast majority of the iron pool in mammalian hosts (Parrow et al., 2013), the capacity of a pathogen to use this host molecule as source of iron represents an important advantage. In the human pathogen *P. aeruginosa*, the Phu and Has systems discovered by Ochsner and coworkers in 2000 (Ochsner et al., 2000) (Fig. 1) have since been considered the only heme utilization systems (Smith and

406 Wilks, 2015; Huang and Wilks, 2017). Although the presence of a third TBDR for heme in
 407 this bacterium was suggested before (Cornelis and Bodilis, 2009), this has not been shown
 408 experimentally. We report here the identification of a third heme system in *P. aeruginosa*, the
 409 Hxu system, which makes this bacterium the only pathogen in which three heme systems
 410 have been identified. The three systems involve a receptor of the TBDR family. These outer
 411 membrane proteins have an N-terminal plug domain that is sequestered inside a C-terminal
 412 22-stranded β -barrel domain in the absence of ligand and is ejected from the β -barrel upon
 413 ligand binding, thereby allowing its transport (Noinaj et al., 2010). Most bacterial heme
 414 receptors bind heme through two His residues located within the plug domain and the
 415 FRAP/PNPPL extracellular loop of the protein, respectively (Bracken et al., 1999; Krieg et
 416 al., 2009). The *P. aeruginosa* HasR receptor presents this bis-His coordination (His²²¹ and
 417 His⁶²⁴) but not the PhuR receptor that binds heme through His¹²⁴ and Tyr⁵¹⁹ (Smith et al.,
 418 2015) (Fig. S5). The HxuA receptor contains a conserved His residue within the plug domain
 419 (His²²³) but not within the extracellular loop where a Tyr residue is present instead (Tyr⁵⁹⁶)
 420 (Fig. S5). It has been proposed that the emergence of the His-Tyr coordination in heme
 421 receptors provides an advantage in scavenging and acquiring heme over the bis-His
 422 coordination (Smith et al., 2015). These authors suggest that this is in line with PhuR being
 423 the main heme utilization receptor of *P. aeruginosa*, a suggestion based on the impaired heme
 424 utilization phenotype and the decreased rate of heme uptake they observed in a *phuR* mutant
 425 but not in a *hasR* mutant (Smith and Wilks, 2015). However, HxuA, which seems to
 426 coordinate heme through His-Tyr residues (Fig. S5) does not appear to play a predominant
 427 role in heme acquisition (Fig. 3C), although further analyses are needed to confirm this
 428 observation. Our results suggest that *P. aeruginosa* adapts the production of its three heme
 429 receptors to the amount of heme encountered. In low iron conditions PhuR is fully and HasR
 430 partially produced, making the bacterium ready to use heme if this source of iron becomes

available. Production of PhuR goes down when iron is being supplied as heme and that of HasR when high amounts of heme are encountered. This correlates with increased synthesis of HxuA, which would allow *P. aeruginosa* to continue using heme to obtain iron and/or to sense heme and, therefore, the host.

We have shown that the *P. aeruginosa* HasR and HxuA receptors are involved in sensing and signaling and that, together with an σ^{ECF} /anti- σ pair, form part of functional signal transduction pathways of the CSS class. Our results are in accordance with a model in which sensing of free heme by HxuA or the hemophore HasAp by HasR, induces a signaling cascade that promotes the activation of σ^{HxuI} and σ^{HasI} in the cytosol, which then initiates transcription of *hxuA* and *hasR*, respectively (Fig. 9). Most *has* and *hxu* gene promoter regions (except that of *hasS*) contain a Fur binding-site (Fig. S6), which suggests that the regulation exerted by iron occurs through the Fur repressor protein that inhibits gene transcription when high intracellular amounts of iron are encountered and allows it when the iron levels are low (Escolar et al., 1999). The anti- σ factor *hxuR* is co-transcribed with *hxuI* (Fig. S4) and therefore also expressed under iron starvation, which produces the inhibition of σ^{HxuI} (Fig. 9A). The basal amount of HxuA produced under iron starvation allows heme sensing when heme becomes available, which then results in σ^{HxuI} activation and maximal HxuA production (Fig. 9B). Activation of the Has system is slightly different because this signaling pathway is already active under iron starvation. This activation depends completely on the hemophore HasAp (Fig. 5A), which is co-transcribed with *hasR* under iron starvation (Ochsner et al., 2000; Dent et al., 2019) (Fig. 1B). This suggests that HasAp is able to activate the Has CSS pathway even in the absence of external heme. In fact, the supernatant of heme-free low iron cultures of *P. aeruginosa* induces the activity of the *hasR* promoter (Fig. 5B, - heme sup), which further indicates that the hemophore produced and secreted by *P. aeruginosa* in absence of external heme can signal through the Has system. In *S. marcescens*

456 a HasA hemophore protein mutant impaired in heme binding is unable to signal (Rossi et al.,
457 2003). However, some degree of signaling is observed with purified HasA, probably due to
458 the traces of heme that the purified protein contains (Rossi et al., 2003). The HasAp
459 hemophore of *P. aeruginosa* purifies with 5-20% of residual heme bound (Dent et al., 2019),
460 which supports that activation of the Has system in absence of external heme could occur
461 through HasAp molecules loaded with endogenous heme. Activation of the Has signaling
462 pathway requires the release of heme from HasAp to the HasR receptor (Dent et al., 2019). In
463 fact, HasAp protein variants that are more efficient in releasing heme than the wild-type
464 protein increase CSS activation while variants unable to release heme do not activate the
465 signaling system (Dent et al., 2019). Probably HxuA also needs to be loaded with heme to
466 trigger activation of the signaling cascade.

467 Furthermore, activation of σ^{HasI} and σ^{HxuI} factors requires the targeted proteolysis of the
468 cognate anti- σ factor HasS and HxuR, respectively. The first proteolytic event takes place in
469 the absence of heme (Fig. 9A). Presence of GT residues in the cleavage site of HasS and
470 HxuR suggests that this step occurs by a protease-independent mechanism known as N-O acyl
471 rearrangement, as we demonstrated for the *P. aeruginosa* FoxR anti- σ factor (Bastiaansen et
472 al., 2015a; Bastiaansen et al., 2015b). This spontaneous cleavage produces a HasS and HxuR
473 N-domain that by analogy with FoxR (Bastiaansen et al., 2015a) likely interacts with its
474 corresponding C-domain in the periplasm (Fig. 9A). However, this proteolytic step is not
475 responsible for the liberation and activation of either σ^{HasI} , as proposed by other authors
476 (Huang and Wilks, 2017), or σ^{HxuI} , because it takes place in situations in which σ^{HasI} and σ^{HxuI}
477 are inactive (e.g. absence of heme or in the ΔrseP mutant) (Fig. 7). Activation of these σ
478 factors involves the targeted proteolysis of, at least, the N-domain of its cognate anti- σ factor
479 (Fig. 9B). Proteolysis of CSS anti- σ factors in response to the inducing signal occurs through
480 regulated intramembrane proteolysis (RIP), a conserved mechanism in which a

transmembrane protein is subjected to several processing steps in order to liberate and activate a cytosolic effector (Brown et al., 2000). The RIP of CSS anti- σ factors always involves the site-2 metalloprotease RseP, which cuts within the transmembrane domain of the protein generating the cytosolic N-tail (Draper et al., 2011; Bastiaansen et al., 2014; Llamas et al., 2014; Bastiaansen et al., 2015a; Bastiaansen et al., 2017). Production of the N-tail coincides with CSS σ^{ECF} activation, likely due to the release of the σ factor in the cytosol (Fig. 9B). In accordance with being activated by RIP of their cognate anti- σ factor, σ^{HasI} and σ^{HxuI} activities are null in the Δ rseP mutant (Fig. 7D). RseP substrate recognition and cleavage occurs through the size-filtering function of the two PDZ domains of RseP rather than by the recognition of a specific sequence/motif (Hizukuri et al., 2014), and the RseP substrate is normally generated by a site-1 protease (Brown et al., 2000). The first evidence that a site-1 cleavage of CSS anti- σ factors takes place is the accumulation of a slightly larger fragment than the RseP product in an *rseP* mutant (Bastiaansen et al., 2014; Bastiaansen et al., 2015a), which we have also observed for HasS and HxuR (Fig. 7B). The periplasmic protease Prc is required for the generation of the RseP substrate of the HasS and HxuR proteins (Fig. 7B). However, whether or not Prc cuts directly on these anti- σ factors needs to be investigated.

A difference of the Has CSS pathway with respect to the Hxu system is the functioning and expression of the anti- σ factor component. Whereas HxuR is totally dispensable for σ^{HxuI} activity, σ^{HasI} activity seems to require HasS (Fig. 6B), although no pro- σ activity has been observed for any of these anti- σ factors (Fig. 6C). Besides, *hasS* is not co-transcribed with *hasI* although transcription of *hasS* is promoted by σ^{HasI} . These observations are not in line with the conclusions drawn by Dent and coworkers in a very recent work (Dent et al., 2019). Based on the similarity of the *hasI* and *hasS* mRNA profiles and the lack of an obvious transcriptional terminator between these two genes, these authors proposed that the *hasI*-*hasS*

genes are co-transcribed (Dent et al., 2019). However, we have not detected such a mRNA co-transcript by RT-PCR (Fig. S4). Furthermore, our results clearly show that the *hasI*-*hasS* intergenic region contains an active promoter that is stimulated by σ^{HasI} and needs activation of the Has CSS cascade (i.e. low iron or heme and an intact HasR receptor) to be active (Fig. 6D). Thus, the similarity of the *hasI* and *hasS* mRNA profiles observed by Dent and coworkers (Dent et al., 2019) seems to be due to the σ^{HasI} -dependent transcription of *hasS*, rather than to co-transcription of these genes. Moreover, these authors observed a decrease instead an increase of *hasS* mRNA levels upon addition of heme (Dent et al., 2019). This discrepancy with our results can be due to the different growth time at what *hasS* expression has been measured (2-7 hours in their study and 18-22 hours in ours). Indeed, we observed that activation of the *hasS* promoter either in low iron or with heme occurred after 8-9 hours of *P. aeruginosa* growth. This is likely due to the time the bacterium needs to produce and secrete the HasAp hemophore, which is needed for σ^{HasI} activation and thus *hasS* transcription. In fact, Dent and coworkers have shown that maximum levels of HasAp in *P. aeruginosa* supernatants are reached after ~7 hours of bacterial growth and that activation of σ^{HasI} is not time-dependent when purified hemophore is provided to the *P. aeruginosa* culture (Dent et al., 2019). Nevertheless, although this dependency on σ^{HasI} for *hasS* transcription is not a common feature of CSS anti- σ factors (Llamas et al., 2014), it has also been shown in *S. marcescens* (Biville et al., 2004). In this way σ^{HasI} , which in *S. marcescens* does not promote its own expression, autoregulates its activity, and this mechanism fine-tunes the response to the heme-hemophore signal (Biville et al., 2004).

Liberation and activation of σ^{HasI} and σ^{HxuI} result in the transcription of target genes by the σ^{HasI} - or σ^{HxuI} -loaded RNAP holoenzyme (Fig. 9B). The regulons of these σ factors have not been identified yet, but we have shown here that the σ^{HasI} regulon includes the *hasR* and *hasS* genes, and the σ^{HxuI} regulon the *hxA* gene, and that none of them include the *phuR*,

phuSTUVW and *hemO* genes (Figs. 1 and 8), as proposed for σ^{HasI} previously (Smith and Wilks, 2015). Actually, these authors observed a considerably higher expression of *phuS* in a *hasR* receptor mutant (Smith and Wilks, 2015). Because activation of CSS σ^{ECF} does not occur in CSS receptor mutants (Llamas et al., 2014), increased expression of *phuS* in a *hasR* mutant is the opposite of what would be expected if σ^{HasI} would indeed be involved in the transcription of this gene. In fact, the σ^{HasI} -transcribed genes *hasR* and *hasS* are not expressed in the *hasR* mutant (Figs. 6B and 6D). σ^{HasI} and σ^{HxuI} do also not promote expression of the *phuR* receptor gene and thus the reduced *phuR* expression observed in a *phuR* mutant (Fig. 4C) seems not to be related with the Has/Hxu signaling pathways. The pyochelin receptor FptA of *P. aeruginosa*, which is not a CSS receptor, is also partially required for its own expression in a process that involves Fur and PchR, a transcriptional regulator of the AraC/XylS family (Michel et al., 2005; Michel et al., 2007). Besides Fur (Ochsner et al., 2000), other regulators involved in *phuR* expression have not been identified yet and thus the molecular mechanism by which PhuR may be involved in its own expression is at present unknown. This mechanism may be required to tightly regulate production of PhuR only when required and/or to avoid an excess of heme into the cell.

σ^{HasI} and σ^{HxuI} are activated under the same condition, i.e. presence of external heme, and they thus likely compete for binding to the RNAPc in this situation. We have observed increased production of the HxuA receptor upon heme induction when the Has CSS pathway, and therefore σ^{HasI} , is inactive (i.e. in the ΔhasR mutant) (Figs. 4B and S3). This can be due to the formation of more σ^{HxuI} -RNAP complexes in conditions in which σ^{HasI} is not active, which results in increased *hxuA* transcription. The opposite effect is not observed, which suggests that σ^{HasI} binds more strongly to the RNAPc than σ^{HxuI} . Indeed, overproduction of σ^{HasI} considerably reduces the σ^{HxuI} -dependent activity of the *hxuA* promoter whereas overproduction of σ^{HxuI} does not affect the promoter activity of *hasR* (Fig. 8). Because HasR

and HxuA are both involved in heme sensing, this mechanism of increasing HxuA production when HasR is lacking would ensure the ability of *P. aeruginosa* to continue recognizing this host molecule. Identification of the regulons of σ^{HasI} and σ^{HxuI} , an important topic that we will undertake in the near future, will help to understand how *P. aeruginosa* uses heme not only as a source of iron but also to respond to the host.

In summary, we report here that the human pathogen *P. aeruginosa* contains three heme systems. Orthologues to the PhuR receptor are present in most *Pseudomonas* species while HasR and HxuA seems to be more restricted to *P. aeruginosa* (Dataset S1). A previous study already suggested that *phuR* is a ‘universal’ TBDR gene in *Pseudomonas* but not *hasR* and *hxuA* that seem to be restricted to pathogenic species (Cornelis and Bodilis, 2009). Moreover, σ^{HxuI} and σ^{HasI} are present in more than 98% of the *P. aeruginosa* strains sequenced to date being scarce in other *Pseudomonas* (Otero-Asman et al., 2019). Remarkably, the proteins and genomic organization of the Has and Hxu CSS systems are highly conserved among *P. aeruginosa* clinical isolates (Dataset S2), which highlights the importance that sensing heme during infection may have for this pathogen. In accordance, expression of most *P. aeruginosa* heme genes is considerably induced in murine models of acute and chronic infections (Turner et al., 2014; Damron et al., 2016). Because a mutant lacking the HasR heme receptor has attenuated virulence (Damron et al., 2016), blocking heme sensing and acquisition may be a promising strategy to combat *P. aeruginosa* infections, and our work has revealed important new information that could be used to achieve this aim.

Experimental Procedures

Bacterial strains and culture media.

Strains used in this study are listed in Table S1. *P. aeruginosa* PAO1 wild-type strain and all the transposon insertion mutants used were from the comprehensive *P. aeruginosa* transposon

mutant library at the University of Washington Genome Center (Jacobs et al., 2003). The locations of the mutations were confirmed by PCR with primers flanking the insertion sites. Construction of null mutants was performed by allelic exchange using the suicide vector pKNG101 as described before (Bastiaansen et al., 2014). Southern blot analyses to confirm the transposon insertion or the chromosomal gene deletion were performed as described (Llamas et al., 2000). Bacteria were routinely grown in liquid LB (Sambrook et al., 1989) on a rotatory shaker at 37°C and 200 rpm. For low iron conditions, cells were cultured in CAS medium (Llamas et al., 2006) containing 400 µM of 2,2'-bipyridyl. The low iron medium used in growth experiments and to measure pyoverdine production contained 800 µM of 2,2'-bipyridyl. The low iron medium was supplemented with 20 µM of heme (Sigma-Aldrich) (other amounts used are indicated) or 100 µM of FeCl₃ (high iron medium). The concentration of heme in the stock solutions was determined by a pyridine hemochromagen assay (Barr and Guo, 2015). When required, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the medium to induce full expression from the pMMB67EH *Ptac* promoter. Antibiotics were used at the following final concentrations (µg/ml): ampicillin (Ap), 100; gentamycin (Gm), 10; piperacillin (Pip), 25; streptomycin (Sm), 100; tetracycline (Tc), 20.

Plasmid construction and molecular biology.

Plasmids used are described in Table S1 and primers listed in Table S2. PCR amplifications were performed using Phusion[®] Hot Start High-Fidelity DNA Polymerase (Finnzymes) or Expand High Fidelity DNA polymerase (Roche). All constructs were confirmed by DNA sequencing and transferred to *P. aeruginosa* by electroporation (Choi et al., 2006).

Outer membrane proteins isolation and proteomics.

603 *P. aeruginosa* PAO1 was cultured overnight in 1 liter of low iron medium without or with 20
604 μ M heme at 37 °C. Cells were harvested and centrifuged at $10.000 \times g$ for 30 min. Pellets
605 were frozen at -80 °C during 15 min and then resuspended in 30 ml of buffer A (20 mM Tris,
606 0.1 mM EDTA, 300 mM NaCl, 10 mM imidazole, 5% (v/v) glycerol, pH 7.35) supplemented
607 with 1x Complete protease inhibitor cocktail (Roche) and benzonase (Merck). Cells were
608 lysed by repeated French Press passages at 1.000 psi. Cell lysates were centrifuged at
609 $10.000 \times g$ for 20 min to remove unbroken cells. Supernatants were then centrifuged at
610 $68.000 \times g$ in an ultracentrifuge (Model ULTRA-100, Thermo Scientific) during 1 h 30 min.
611 Outer membrane proteins from pellets were extracted with 15 ml of buffer A containing 1%
612 (v/v) Triton X-100 at room temperature for 4 h. Proteins were then concentrated in an
613 ultrafiltration device (Amicon Ultra, Millipore) by centrifugation at $8.000 \times g$ and separated
614 by SDS-PAGE containing 7.5% (w/v) acrylamide. Proteins were visualized by Coomassie blue
615 staining and bands of interest excised for proteomic analyses at the Proteomics facility of the
616 University Complutense of Madrid (Spain). Proteins were digested with trypsin and the
617 resulting peptides analyzed by matrix-assisted laser desorption/ionization-time of flight
618 (MALDI-TOF) to obtain the peptide fingerprint. Proteins ambiguously identified by matching
619 experimental masses with those of theoretical digests of bacterial proteins were subjected to
620 MS/MS sequencing analyses. The identification of proteins by peptide mass footprints were
621 carried out with MASCOT version 2.3 (Matrix Science) in NCBI database without and with
622 taxonomic restriction to bacteria. A Mascot's MOWSE (for molecular weight search) score
623 >89 and a sequence coverage $\geq 16\%$ was used as cut off for protein identification. The
624 identified proteins exceeded the score that the mascot's MOWSE algorithm estimated to be
625 statistically significant with a P-value < 0.05 .

626 **Enzyme assay.**

627 β -galactosidase activities in soluble cell extracts were determined using *o*-nitrophenyl-b-D-

galactopyranoside (ONPG) (Sigma-Aldrich) as described before (Llamas et al., 2006). Each condition was tested in duplicate in at least three biologically independent experiments and the data given are the average of the three biologically independent experiments with error bars representing standard deviation (SD). Activity is expressed in Miller units.

Real-time PCR analyses.

Total RNA from late exponentially grown *P. aeruginosa* PAO1 cells in low iron medium or low iron medium supplemented with 100 μ M FeCl₃ (high iron) or 20 μ M heme was extracted by the hot phenol method using the TRI® Reagent protocol (Ambion) as described elsewhere (Llamas et al., 2008). The isolated RNA was subjected to two DNase I treatments with the Turbo DNA-free kit (Ambion) and RNaseOUT (Invitrogen) to eliminate contaminated DNA. RNA quality, including purity, integrity and yield, was assessed by electrophoresis of 1 μ g of total RNA and by UV absorption at 260 nm in a ND-1000 spectrophotometer (NanoDrop Technologies, USA). RT-PCR to assess the co-transcription of the *hasI-hasS* and *hxuI-huxR* genes was performed on total RNA using the Titan One-Tube RT-PCR system (Roche) in accordance with the manufacturer's recommendations and primers that amplify the *hasIS* or *hxuIR* intergenic regions (Table S2). For each reaction, 50 ng of total RNA from three biologically independent PAO1 cultures was used. Thermal cycling conditions were the following: one cycle at 42°C for 30 min, one cycle at 95°C for 2 min, and then 35 cycles at 95°C for 30 sec, 58°C for 30 s and 68°C for 30 s, with a final cycle at 68°C during 10 min. DNA contamination of the RNA samples was ruled out by performing a PCR with Taq DNA polymerase (Roche). A positive control using *P. aeruginosa* genomic DNA was included. For quantitative Real-time PCR (qRT-PCR), cDNA was produced in triplicate by reverse transcription reactions of 0.5 μ g of total RNA using SuperScript II reverse transcriptase (Invitrogen) and random hexamers as primers according to the protocol supplied. Amplifications were carried out on a MyiQ2 system (Bio-Rad) associated with iQ5 optical

system software (version 2.1.97.1001) in 11,5 µl reaction mixture containing 6,25 µl of iQ SYBR green Supermix (Bio-Rad), 0.4 µM of each primer (Table S2) and 1 µl of the template cDNA (diluted 1000-fold when measuring the 16S rRNA reference gene). Thermal cycling conditions were the following: one cycle at 95°C for 5 min and then 40 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 20 s, with a single fluorescence measurement per cycle according to the manufacturers' recommendations. Melting curve analyses were performed by gradually heating the PCR mixture from 55 to 95°C at a rate of 0.5°C per 10 s for 80 cycles. The relative expression of the genes was normalized to that of 16S rRNA and the results were analyzed by means of the comparative cycle threshold ($\Delta\Delta\text{ct}$) method (Pfaffl, 2001). Three biologically independent experiments were performed each one including three technical replicates and the data given are the average.

Growth curves.

P. aeruginosa strains were cultured overnight in LB medium. Cultures were then diluted and inoculated in a Honeycomb[®] plate (Oy Growth Curves AB Ltd) containing 200 µl of CAS medium with 800 µM of 2,2'-bipyridyl (low iron medium) supplemented or not with 100 µM FeCl₃ or the indicated concentration of heme. Plates were placed in a Bioscreen C Microbiology Reader[™] and incubated at 37 °C with shaking during 24 h. Turbidity measurement were performed every 30 min using a wideband filter (420-580 nm). Three technical repeats were performed in three biologically independent experiments and the data given are the average.

Preparation of hemophore containing supernatants.

Hemophore containing supernatants were obtained from iron-restricted cultures of *P. aeruginosa* PAO1 and hemophore free supernatants from *P. aeruginosa* hasAp-Tn cultures in the absence (-heme sup) or presence (+heme sup) of 20 µM heme. Supernatants were filtered

through a 0.2 μ m filter (GE Healthcare) and added in 1:1 proportion to iron restricted cultures. Presence of the hemophore in the supernatants was analyzed by measuring the activity of the *hasR* promoter (pMPhasRGm plasmid) in the *P. aeruginosa* hasAp-Tn hemophore mutant. At least three biologically independent experiments were performed by using supernatants from three different cultures on a given strain.

Pyoverdine production.

Overnight cultures of *P. aeruginosa* strains in LB medium were diluted and inoculated in CAS medium containing 800 μ M of 2,2'-bipyridyl supplemented with 20 μ M heme. The production of pyoverdine was measured spectrophotometrically as described before (Wilderman et al., 2001). Briefly, 1 mL of the culture at the indicated growth time was centrifuged and the supernatant filtered through a 0.22 μ m pore size filter. The absorbance at 380 nm was measured and normalized for differences in cell density with the OD at 660 nm of the cultures. Values were normalized to that obtained with the PAO1 wild-type strain. Data given are the average of three biologically independent experiments with error bars representing standard deviation (SD).

SDS-PAGE and Western-blot.

P. aeruginosa was grown in iron-limited medium containing 1 mM IPTG and without or with 20 μ M heme until late log phase. Cells were pelleted by centrifugation and heated for 10 min at 95 °C following solubilization in SDS-PAGE sample buffer. Sample normalization was done according to the OD₆₆₀ of the bacterial culture. Proteins were separated by SDS-PAGE containing 12 or 15% (w/v) acrylamide and electrotransferred to nitrocellulose membranes (Millipore). Ponceau S (Serva) staining was performed as a loading control. HA-tagged proteins were detected using a monoclonal antibody directed against the influenza hemagglutinin epitope (HA.11, Covance) as previously described (Bastiaansen et al., 2014).

Computer-assisted analysis.

Protein sequence analysis and classification were performed using InterPro (Finn et al., 2017) and alignments with ClustalW (Thompson et al., 1994). BLASTn and DIAMOND BLASTp analyses were performed at the Pseudomonas database (<http://www.pseudomonas.com/>) (Winsor et al., 2016) (70% of query coverage was used in all analyses). The 4847 complete and incomplete *Pseudomonas* genomes contained in this database were used to analyze the presence of the three heme receptors (Dataset S1, 60% of identity cutoff). A total of 93 *P. aeruginosa* complete genome sequences were included in the analyses of heme promoter regions and proteins (Dataset S2, 70% of identity cutoff). Statistical analyses are based on *t*-test in which two conditions are compared independently. P-values from raw data (i.e. miller units from β -galactosidase assays) were calculated by independent two-tailed *t*-test and from ratio data to the control (i.e. qRT-PCR and pyoverdine production) by one-sample *t*-test using GRAPHPAD PRISM version 5.01 for Windows and are represented in the graphs by *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

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906

Figures legends

Fig. 1. Genetic organization and schematic representation of the *P. aeruginosa* Phu and Has heme utilization systems. (A, B) Block arrows represent the different genes and their transcriptional orientation, and small arrows identified (solid line) or proposed (dashed line) promoters. (A, C) The Phu system is encoded by two divergently transcribed operons (Ochsner et al., 2000). PhuR transports free heme to the periplasm in a process that requires the energy provided by the ExbBD-TonB complex (Noinaj et al., 2010). In the periplasm the heme binding protein PhuT delivers the heme to the PhuUV ABC transporter that translocates it into the cytoplasm where heme is sequestered by the heme-trafficking protein PhuS. PhuS forms a complex with the heme oxygenase HemO that degrades heme to biliverdin IX and CO releasing the ferric ion (O'Neill et al., 2012). (B, C) The Has system is arranged in three potential operons, with the first encoding a σ^{ECF} /anti- σ factor pair (*hasI*-*hasS*). The second operon encodes the HasR receptor together with the hemophore HasAp (Ochsner et al., 2000; Dent et al., 2019). The last operon encodes a potential type I secretion system that, by homology with the *S. marcescens* HasD-HasE-HasF exporter system, is likely involved in the secretion of HasAp (Ochsner et al., 2000). HasAp binds and delivers heme to HasR, which, in an energy-requiring process extracts and translocates the heme into the periplasm (Smith et al., 2015), which is then internalized in the cytoplasm by the components of the Phu system (Smith and Wilks, 2015). The N-terminal extension of HasR potentially involved in signaling is shown in red.

Fig. 2. Response of *P. aeruginosa* to heme. (A) SDS-PAGE and proteomic analyses of *P. aeruginosa* PAO1 outer membrane proteins after growth in iron-restricted conditions without (-) or with (+) 20 μ M heme. The arrow indicates the position of the protein band that was excised and subjected to proteomic analysis by MALDI-TOF. The results of the proteomics analyses are shown and include the number of peptides matching each protein, coverage (%) of the protein, and the score expressed as the $-10 \log(P)$ where P is the probability that the observed match is a random event. Protein scores shown are significant ($P < 0.05$). The positions of the receptors FpvA, PhuR and FptA, identified by proteomics in a previous work (Llamas et al., 2006), are indicated on the right and those of the molecular size markers on the left. SDS-PAGE is representative of two biological replicates ($N=2$). (B) Analysis of the expression of the heme receptors by qRT-PCR. *P. aeruginosa* PAO1 wild-type strain was grown under iron-restricted conditions (low Fe) or iron-restricted medium supplemented with FeCl_3 (high Fe) or 20 μ M heme (+heme). qRT-PCR on total RNA was performed with primers hybridizing the indicated gene (Table S2). Data are means $\pm$ SD from three biological replicates ($N=3$) each one including three technical replicates. P-values were calculated by

944 one-sample *t*-test to a hypothetical value of 1 as described in Materials and Methods. (C) β -
945 galactosidase activity of the indicated *lacZ* promoter fusion gene in the *P. aeruginosa* PAO1
946 wild-type strain after growth under iron-restricted conditions (low Fe) or iron-restricted
947 medium supplemented with FeCl₃ (high Fe) or the indicated amount of heme. Data are means
948 $\pm$ SD from three biological replicates (N=3). P-values were calculated as described in
949 Materials and Methods and brackets indicate the comparison to which the P-value applies.

Fig. 3. Growth of *P. aeruginosa* heme receptor mutants. (A) Growth of *P. aeruginosa* PAO1 wild-type strain and the indicated mutants in low iron (dashed line) or low iron supplemented with FeCl₃ (solid line). (B) Growth of *P. aeruginosa* PAO1 wild-type strain under iron-restricted conditions (low Fe) or iron-restricted medium with the indicated amount of heme. (C) Growth of *P. aeruginosa* PAO1 wild-type strain and the indicated single, double and triple heme receptor mutant under iron-restricted conditions (dashed line) or iron-restricted medium supplemented with 20 μM heme (solid line). In all panels data are means from three biological replicates (N=3) with SD below 0.18 in all cases (error bars are not included to allow a better visualization of the data).

Fig. 4. Expression of the *hasR*, *hxA* and *phuR* receptors in the absence of one, two or the three heme receptors. β -galactosidase activity of the *hasR* (A), *hxA* (B) or *phuR* (C) *lacZ* promoter fusion gene in the *P. aeruginosa* PAO1 wild-type and the indicated mutants. Strains were grown under iron-restricted conditions without (-) or with (+) 20 μ M heme. Data are means $\pm$ SD from three biological replicates (N=3). P-values were calculated as described in Materials and Methods by comparing the value obtained for the mutant with that of the PAO1 wild-type strain in the same growth condition.

Fig. 5. Expression of the *P. aeruginosa* heme receptors in the absence of hemophore. (A) β -galactosidase activity of the indicated *lacZ* promoter fusion gene in the *P. aeruginosa* PAO1 wild-type strain or the hasAp-Tn hemophore mutant after growth under iron-restricted conditions without (-) or with (+) heme. (B) β -galactosidase activity of *hasR::lacZ* promoter fusion in the *P. aeruginosa* hasAp-Tn hemophore mutant after growth under iron-restricted conditions supplemented with the supernatant of the indicated strain cultured in the absence (-heme sup) or the presence (+heme sup) of 20 μ M of heme. In both panels data are means $\pm$ SD from three biological replicates (N=3). P-values were calculated as described in Materials and Methods by comparing the value obtained for the mutant with that of the PAO1 wild-type strain in the same growth condition or by comparing different growth conditions of a same strain (indicated by brackets in this case).

Fig. 6. Analysis of the Has and Hxu CSS pathways. (A) N-terminal extension of mature *P. aeruginosa* HasR and HxuA proteins compared to mature PhuR protein. The numbering of amino acid residues is indicated. Colors indicate identical (red), strongly similar (green) and weakly similar (blue) residues. **(B)** β -galactosidase activity of the indicated *lacZ* promoter fusion gene in the *P. aeruginosa* PAO1 wild-type and the indicated transposon mutant. Strains were grown under iron-restricted conditions without (-) or with (+) 20 μ M heme. **(C)** β -galactosidase activity of the indicated *lacZ* promoter fusion gene in the PAO1 wild-type and the indicated transposon mutant bearing the empty pMMB67EH plasmid (- N-tail) or the pMMB67EH-derived plasmid expressing the N-tail (+ N-tail) of the indicated anti- σ factor (Table S1). Strains were grown under iron-restricted conditions with IPTG and 20 μ M heme. **(D)** β -galactosidase activity of the *hasS::lacZ* promoter fusion in the *P. aeruginosa* wild-type strain bearing the pMMB67EH empty plasmid (PAO1) or the pMMB-hasI plasmid (PAO1- σ^{HasI}), and in the Δ hasR receptor mutant. Strains were grown under iron-restricted conditions with IPTG and in the absence (low Fe) or presence of FeCl_3 (high Fe) or 20 μ M heme (+heme). Data in all panels are means $\pm$ SD from three biological replicates (N=3). P-values were calculated as described in Materials and Methods by comparing the value obtained for the mutant or in cells overproducing σ^{HasI} with that of the PAO1 wild-type strain in the same growth condition, or by comparing different growth conditions of a same strain (indicated by brackets in this case).

Fig. 7. Processing of the HasS and HxuR anti- σ factors and role of the Prc and RseP proteases. (A) Schematic representation of the HxuR and HasS proteins. The proteins have been drawn to scale, and the cytosolic, transmembrane (TM), and periplasmic domains are shown. Numbers indicate amino acid positions. The spontaneous cleavage site between Gly¹⁸⁸ and Thr¹⁸⁹ is indicated as well as the respective N- and C-domains generated from this cleavage and their molecular masses. The estimated size of the N-tail is also indicated. (B) *P. aeruginosa* PAO1 wild-type strain and their isogenic Δprc and ΔrseP mutants expressing an N-terminally HA-tagged HasS or HxuR protein were grown to late log-phase under iron-restricted conditions with IPTG and in the absence (-) or presence (+) of 20 μ M heme. Proteins were immunoblotted for HA. Position of the protein fragments and the molecular size marker (in kDa) is indicated. Presence of the HA-tag adds ~1 kDa to the molar mass of the protein fragments. Blots are representative of four biological replicates (N=4). (C) Alignment of the region of the indicated *P. aeruginosa* anti- σ factor proteins that contains the conserved Gly and Thr residues (GT) (in bold) where the spontaneous cleavage of these proteins occurs. (D) Activity of the Has and Hxu CSS systems in the Δprc and ΔrseP protease mutants. β -galactosidase activities were determined after bacterial growth in iron-restricted conditions without (-) or with (+) 20 μ M heme. Data are means $\pm$ SD from three biological replicates (N=3). P-values were calculated as described in Materials and Methods by comparing the value obtained for the mutant with that of the PAO1 wild-type strain in the same growth condition.

Fig. 8. Expression of heme genes in cells overexpressing σ^{HasI} or σ^{Hxul} . β -galactosidase activity of the indicated *lacZ* promoter fusion gene in the *P. aeruginosa* PAO1 wild-type strain bearing the pMMB67EH empty plasmid (WT), the pMMB-hasI plasmid (σ^{HasI}), or the pMMB-hxulI plasmid (σ^{Hxul}). Strains were grown in IPTG-containing iron-restricted medium (low Fe or -heme) supplemented with FeCl_3 (high Fe) or 20 μM heme (+heme). Data are means $\pm$ SD from three biological replicates (N=3). P-values were calculated as described in Materials and Methods by comparing the value obtained for the cells overexpressing the σ factor with that of the PAO1 wild-type strain in the same growth condition, or by comparing different growth conditions of a same strain (indicated by brackets in this case).

Fig. 9. Schematic models of the *P. aeruginosa* Has and Hxu CSS systems. (A) The three proteins of the Hxu and Has CSS systems (receptor, anti- σ factor and σ^{ECF}) are shown. Immediately upon production and in the absence of heme, the HxuR and HasS anti- σ factors undergo a spontaneous cleavage that results in the generation of two protein domains. By analogy with other anti- σ factor (Bastiaansen et al., 2015a), it is likely that these domains interact with each other in the periplasm. The N-domain of the anti- σ factor binds to and keeps sequester the σ^{ECF} . **(B)** Detection of free heme by HxuA or the heme-hemophore (HasAp) complex by HasR generates a signal that is transmitted by the signaling domain of these receptors (in red). This triggers the regulated intramembrane proteolysis (RIP) of the N-domain of HxuR and HasS by the action of, at least, the Prc and RseP proteases. Liberation of σ^{HxuI} results in *hxA* gene transcription and that of σ^{HasI} in *hasS* and *hasR-hasAp* transcription. Uptake of heme through HasR occurs in an energy-dependent process involving the ExbBD-TonB complex (Smith et al., 2015). OM, outer membrane; CM, cytoplasmic membrane. Figure adapted from (Llamas et al., 2014) with the results from this work.

1046

Supporting Information

1047

***Pseudomonas aeruginosa* possesses three distinct systems for sensing and using**

1048

the host molecule heme

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Joaquín R. Otero-Asman, Ana I. García-García, Cristina Civantos, José M. Quesada, and María A.

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Llamas

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Fig. S1. Production of pyoverdine by *P. aeruginosa* heme receptor mutants.

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Fig. S2. Complementation of key heme mutant phenotypes.

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Fig. S3. Outer membrane composition of *P. aeruginosa* *hxuA* and *hasR* receptor mutants.

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Fig. S4. Analysis of the co-transcription of the *has* and *hxu* σ^{ECF} /anti- σ genes by RT-PCR.

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Fig. S5. Sequence alignment of the heme-coordination extracellular loop of *P. aeruginosa*

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heme receptors.

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Fig. S6. Predicted Fur boxes in the promoter region of the *P. aeruginosa* heme utilization

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genes.

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Dataset S1. Occurrence of the PhuR, HasR and HxuA receptors in *Pseudomonas*.

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Dataset S2. Heme promoter regions and proteins in *P. aeruginosa* clinical isolates.

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Table S1. Bacterial strains and plasmids used in this study.

Table S1. Bacterial strains and plasmids used in this study^a

Strain	Characteristics	Reference
<i>E. coli</i>		
CC118 λ pir	$\Delta(ara-leu) araD \Delta lacX74 galE galK phoA20 thi-1 rpsE rpoB argE recA1$, lysogenized with λ pir; Rif ^R	(Herrero et al., 1990)
DH5 α	<i>supE44</i> $\Delta(lacZYA-argF)U169$ $\phi 80 lacZ\Delta M15 hsdR17$ (r _K ⁻ m _K ⁺) <i>recA1 endA1 gyrA96 thi1 relA1</i> ; Nal ^R	(Hanahan, 1983)
<i>P. aeruginosa</i>		
PAO1	Wild-type strain	(Jacobs et al., 2003)
hasAp-Tn	PAO1 carrying a ISphoA/hah transposon in the <i>hasAp</i> (PA3407) gene (insertion in codon 79, ID: PW6748); Tc ^R	(Jacobs et al., 2003)
hasR-Tn	PAO1 carrying a ISlacZ/hah transposon in the <i>hasR</i> (PA3408) gene (insertion in codon 354, ID: PW6750); Tc ^R	(Jacobs et al., 2003)
hasS-Tn1	PAO1 carrying a ISlacZ/hah transposon at the -10 position upstream the start codon of the <i>hasS</i> (PA3409) gene (ID: PW6754); Tc ^R	(Jacobs et al., 2003)
hasS-Tn2	PAO1 carrying a ISphoA/hah transposon in the <i>hasS</i> (PA3409) gene (insertion in codon 155, ID: PW6751); Tc ^R	(Jacobs et al., 2003)
hasI-Tn	PAO1 carrying a ISphoA/hah transposon in the <i>hasI</i> (PA3410) gene (insertion in codon 137, ID: PW6755); Tc ^R	(Jacobs et al., 2003)
hxA-Tn	PAO1 carrying a ISlacZ/hah transposon in the <i>hxA</i> (PA1302) gene (insertion in codon 200, ID: PW3356); Tc ^R	(Jacobs et al., 2003)
hXR-Tn	PAO1 carrying a ISphoA/hah transposon in the <i>hXR</i> (PA1301) gene (insertion in codon 8, ID: PW3355); Tc ^R	(Jacobs et al., 2003)
hXI-Tn	PAO1 carrying a ISlacZ/hah transposon in the <i>hXI</i> (PA1300) gene (insertion in codon 147, ID: PW3353); Tc ^R	(Jacobs et al., 2003)
phuR-Tn	PAO1 carrying a ISphoA/hah transposon in the <i>phuR</i> (PA4710) gene (insertion in codon 417, ID: PW8935); Tc ^R	(Jacobs et al., 2003)
Δ hasR	Markerless PAO1 null mutant in the <i>hasR</i> (PA3408) gene	This study
Δ hxA	Markerless PAO1 null mutant in the <i>hxA</i> (PA1302) gene	This study
phuR-Tn Δ hasR	phuR-Tn with the <i>hasR</i> markerless mutation (double mutant); Tc ^R	This study
phuR-Tn Δ hxA	phuR-Tn with the <i>hxA</i> markerless mutation (double mutant); Tc ^R	This study
Δ hxA Δ hasR	Δ hxA with the <i>hasR</i> markerless mutation (double mutant)	This study
phuR-Tn Δ hxA Δ hasR	phuR-Tn Δ hxA with the <i>hasR</i> markerless mutation (triple mutant); Tc ^R	This study
Δ pvdD	Allelic unmarked mutant deficient in pyoverdine (<i>pvdD</i>) production	(Ghysels et al., 2004)
Δ pvdD pchEF	Allelic unmarked mutant deficient in pyoverdine (<i>pvdD</i>) and pyochelin (<i>pchEF</i>) production	(Ghysels et al., 2004)
Plasmid		
pBSL141	Source of the Gm cassette; Ap ^R , Gm ^R	(Alexeyev et al., 1995)
pKNG101	Gene replacement suicide vector, <i>oriR6K</i> , <i>oriTRK2</i> , <i>sacB</i> ; Sm ^R	(Kaniga et al., 1991)
pK Δ hasR	pKNG101 carrying in XbaI-BamHI a 2.4-Kb PCR fragment containing the regions up- and downstream the <i>P</i> .	This study

	<i>aeruginosa hasR</i> (PA3408) gene; Sm ^R	
pKΔhxA	pKNG101 carrying in XbaI-BamHI a 2.5-Kb PCR fragment containing the regions up- and downstream the <i>P. aeruginosa hxA</i> (PA1302) gene; Sm ^R	This study
pMMB67EH	IncQ broad-host range plasmid, <i>lacI^q</i> ; Ap ^R	(Furste et al., 1986)
pMMB/hasI	pMMB67EH carrying a 0.62-Kb EcoRI-XbaI PCR fragment expressing the <i>P. aeruginosa hasI</i> (PA3408) gene; Ap ^R	This study
pMMB/hxA	pMMB67EH carrying a 0.76-Kb EcoRI-XbaI PCR fragment expressing the <i>P. aeruginosa hxA</i> (PA1300) gene; Ap ^R	This study
pMMB/HA-hxA	pMMB67EH carrying a 1.1-Kb XbaI-HindIII PCR fragment containing an N-terminally HA-tagged <i>P. aeruginosa hxA</i> (PA1301) gene; Ap ^R	This study
pMMB/HA-hasS	pMMB67EH carrying a 1.3-Kb EcoRI-XbaI PCR fragment containing an N-terminally HA-tagged <i>P. aeruginosa hasS</i> (PA3409) gene; Ap ^R	This study
pMMB/N-tail HasS	pMMB67EH carrying a 0.48-Kb EcoRI-XbaI PCR fragment expressing the N-tail of the <i>P. aeruginosa HasS</i> (PA3409) protein; Ap ^R	This study
pMMB/N-tail HxA	pMMB67EH carrying a 0.42-Kb XbaI-HindIII PCR fragment expressing the N-tail of the <i>P. aeruginosa HxA</i> (PA1301) protein; Ap ^R	This study
pMMB/hasAp	pMMB67EH carrying a 0.84-Kb EcoRI-XbaI PCR fragment containing the <i>P. aeruginosa hasAp</i> (PA3407) gene; Ap ^R	This study
pMMB/hasR	pMMB67EH carrying a 3-Kb EcoRI-XbaI PCR fragment containing the <i>P. aeruginosa hasR</i> (PA3408) gene; Ap ^R	This study
pMMB/hasR-hasAp	pMMB67EH carrying a 3.7-Kb EcoRI-XbaI PCR fragment containing the <i>P. aeruginosa hasR-hasAp</i> (PA3408-PA3407) genes; Ap ^R	This study
pMMB/hxA	pMMB67EH carrying a 2.8-Kb EcoRI-XbaI PCR fragment containing the <i>P. aeruginosa hxA</i> (PA1302) gene; Ap ^R	This study
pMP220	IncP broad-host-range <i>lacZ</i> fusion vector; Tc ^R	(Spaink et al., 1987)
pMPhasR	pMP220 carrying a 387-bp EcoRI-XbaI PCR fragment containing the <i>P. aeruginosa hasR</i> (PA3408) promoter region upstream the <i>lacZ</i> gene; Tc ^R	This study
pMPhasRGm	pMPhasR carrying in BglII-EcoRI, and in the opposite direction to the <i>lacZ</i> gene, a 0.97-kb BamHI-EcoRI fragment of pBSL141 containing a Gm cassette; Gm ^R , Tc ^R	This study
pMPHxA	pMP220 carrying a 331-bp EcoRI-XbaI PCR fragment containing the <i>P. aeruginosa hxA</i> (PA1302) promoter region upstream the <i>lacZ</i> gene; Tc ^R	This study
pMPHxAGm	pMPHxA carrying in BglII-EcoRI, and in the opposite direction to the <i>lacZ</i> gene, a 0.97-kb BamHI-EcoRI fragment of pBSL141 containing a Gm cassette; Gm ^R , Tc ^R	This study
pMPHemO	pMP220 carrying a 401-bp BglII-KpnI PCR fragment containing the <i>P. aeruginosa hemO</i> (PA0672) promoter region upstream the <i>lacZ</i> gene; Tc ^R	This study
pMPphuR	pMP220 carrying a 430-bp EcoRI-XbaI PCR fragment containing the <i>P. aeruginosa phuR</i> (PA4710) promoter region upstream the <i>lacZ</i> gene; Tc ^R	This study

pMPphuRGm	pMPphuR carrying in BglII-EcoRI, and in the opposite direction to the <i>lacZ</i> gene, a 0.97-kb BamHI-EcoRI fragment of pBSL141 containing a Gm cassette; Gm ^R , Tc ^R	This study
pMPphuS	pMP220 carrying a 243-bp EcoRI-XbaI PCR fragment containing the <i>P. aeruginosa</i> <i>phuR</i> (PA4709) promoter region upstream the <i>lacZ</i> gene; Tc ^R	This study

^a Ap^R, Gm^R, Nal^R, Sm^R and Tc^R, resistance to ampicillin, gentamycin, nalidixic acid, streptomycin and tetracycline, respectively.

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Table S2. Sequence of the primers used in this study

<i>P. aeruginosa</i> gene (or promoter region)	Plasmid	Primer name	Primer sequence (5'→ 3') ^a
<i>hemO</i> (PA0672)	pMPhemO	PrhemOF-Bg	<u>AATAGATCTCCGCCGGGTAGCAGTGCGTC</u>
		PrhemOR-Kp	<u>ATTGGTACCTTTGCCGAGTGGATTTCAGGG</u>
<i>hxl</i> (PA1300)	pMMB/hxl	hxl2F-E	<u>AAAGAATTCAGGAACGCATTTCCCAGTC</u>
		hxl2R-X	<u>AAATCTAGATCATCCTTCGCCTCCCTGGC</u>
	<i>hxl-hxuR</i> RT-PCR	RThxlF	<u>AGCATGGTGGAAAAGCACAT</u>
		RThxlR	<u>GCTCGGGAATATCGTCCA</u>
<i>hxr</i> (PA1301)	pMMB/HA-hxr	HA-hxrF-X	<u>AAATCTAGAATGTACCCGTACGACGTGCCGG</u> <u>ACTACGCGAAGCCGCTGGAACGACTGG</u>
		Δ hxrAR-H	<u>CACAAGCTTAGGTCATGCTGGAACCTCTCC</u>
	pMMB/N-tail Hxr	N-tail hxrF-X	<u>AAATCTAGACGTTTCGTCCTGCACAAGTT</u>
		N-tail hxrR-H	<u>AAAAAGCTTTTCAGAAGAACGCCGCCGCCAG</u>
<i>hxA</i> (PA1302)	pK Δ hxA	hxrF-X	<u>AAATCTAGAGGAAAAGCACATCGCCAGCG</u>
		Δ hxrAR-H	<u>CACAAGCTTAGGTCATGCTGGAACCTCTCC</u>
		Δ hxrAF-H	<u>TTCAAGCTTACAGCCAGGGACGCAACGC</u>
		PA1303R-B	<u>ATTGGATCCGCTTCGTCTTTGCCATCGCC</u>
	pMPhxA	PrPA1302F-E	<u>AAAGAATTCCTCGACGATTTTCTCCTCGC</u>
		PrPA1302R-X	<u>AAATCTAGAGGCAGGTCGAAGCGTCGTTT</u>
	pMMB/hxA	PrPA1302F-E	<u>AAAGAATTCCTCGACGATTTTCTCCTCGC</u>
		hxrAR-X	<u>AAATCTAGATCAGAAGAACTGGGTGACGC</u>
	qRT-PCR	qhxrAF	<u>GTGTTTCATCGAACC GGAAAT</u>
		qhxrAR	<u>TCGTTGGAGTGATAGCCGTA</u>
<i>hasAp</i> (PA3407)	pMMB/hasAp	hasApF-E	<u>AAAGAATTCGTGAGCGATGAACTGACC</u>
		hasApR-X	<u>AAATCTAGATCAGGCCGCCAGGGCCAGGT</u>
<i>hasR</i> (PA3408)	pK Δ hasR	hasSF-X	<u>AACTCTAGACAGCAGATCCGCCAGCAGGC</u>
		Δ hasRR-E	<u>AAAGAATTCATCCACGGCTCGAAAATGCG</u>
		hasApF-E	<u>AAAGAATTCGTGAGCGATGAACTGACC</u>
		hasApR-B	<u>AAAGGATCCGCCGAGCAGCAGGTAGAGG</u>
	pMPhasR	PrhasRF-E	<u>ATAGAATTCGGCATTGCTGGTGGCTCCC</u>
		PrhasRR-X	<u>AAATCTAGACGCACGGCACTCCATCCACG</u>
	pMMB/hasR	PrhasRF-E	<u>ATAGAATTCGGCATTGCTGGTGGCTCCC</u>
		hasRR-X	<u>AAATCTAGATCAGAAGTGGTATTCGAGGG</u>
	pMMB/hasR-hasAp	PrhasRF-E	<u>ATAGAATTCGGCATTGCTGGTGGCTCCC</u>
		hasApR-X	<u>AAATCTAGATCAGGCCGCCAGGGCCAGGT</u>
	qRT-PCR	qhasRF	<u>CGGTGGAATACCGCTTCTC</u>
		qhasRR	<u>CGTCTGGTAGACCCAGTCGT</u>
<i>hasS</i> (PA3409)	pMMB/HA-hasS	HA-hasSF-E	<u>AAAGAATTCATGTACCCGTACGACGTGCCGG</u> <u>ACTACGCGAACAGCCCCCAGGAACAACAGC</u>
		PrhasRR-X	<u>AAATCTAGACGCACGGCACTCCATCCACG</u>
	pMMB/N-tail HasS	N-tail hasSF-E	<u>AAAGAATCCCGTGAGATCTTCCGTCTCAA</u>
		N-tail hasSR-X	<u>AAATCTAGATCACGCGGCCAGCCGCG</u>
<i>hasI</i> (PA3410)	pMMB/hasI	hasIF-E	<u>AAAGAATTCCTGAGCCCGGCATCTCACAA</u>
		hasIR-X	<u>AAATCTAGAGAACCGGCAAGACTCGGTAA</u>
	<i>hasI-hasS</i> RT-PCR	RThasIF	<u>CCTGGAAATCTCCGATAGCTC</u>
		RTHasSR	<u>ATCCCAGCTGTTTCCAGGTC</u>
<i>phuS</i> (PA4709)	pMPphuS	PrphuS2F-E	<u>AAAGAATTCCTGCCAGAAAAACGCTAATG</u>
		PrphuS2R-X	<u>AAATCTAGACTTCGCTGACCTGCAACAG</u>
<i>phuR</i> (PA4710)	pMPphuR	PrPAphuRF-E	<u>TATGAATTCGCTCGCCTTCGCTGACCTGC</u>
		PrPAphuRR-X	<u>TATTCTAGACGGGTGGCGGTGATGGTGG</u>

	qRT-PCR	qphuRF	<u>GATCACCGGCTACAACATCC</u>
		qphuRR	<u>TTTACGATGTCCGGATCGAC</u>

^aThe sequences of the restriction sites are indicated in bold and the annealing region is underlined

Fig. S1. Production of pyoverdine by *P. aeruginosa* heme receptor mutants. *P. aeruginosa* strains were grown in low iron medium with 20 μ M heme. The production of pyoverdine (PVD) relative to the production in the PAO1 wild-type strain after 3, 7 and 24 hours of growth of each mutant is shown. Mutants deficient in pyoverdine (*pvdD*) production were used as control. Data are means $\pm$ SD from three biological replicates (N=3). P-values were calculated by one-sample *t*-test to a hypothetical value of 1 as described in Materials and Methods.

Fig. S2. Complementation of key heme mutant phenotypes. Complementation of (A, B) the HasR and HxA receptor mutants, (C) the hemophore hasAp-Tn mutant, (D) the hxA-Tn and hasI-Tn σ factor mutants, and (E) the hxA-Tn anti- σ factor mutant. β -galactosidase activity of the indicated *lacZ* promoter fusion gene in the *P. aeruginosa* PAO1 wild-type strain and the indicated mutant bearing the empty (-) or the pMMB67EH-derived plasmid

1078 (Table S1) expressing the indicated gene. Bacteria were grown in iron-restricted conditions
1079 without (-) or with (+) 20 μ M heme. The expression of the *hasAp* and HA-*hxrR* genes from
1080 the pMMB67EH Ptac promoter was induced by adding IPTG to the cultures. Data are means
1081 $\pm$ SD from three biological replicates (N=3). P-values were calculated as described in
1082 Materials and Methods by comparing the value obtained for the strain bearing the empty
1083 plasmid (-) with that of the same strain expressing the indicated gene in the same growth
1084 condition.

Fig. S3. Outer membrane composition of *P. aeruginosa* *hxA* and *hasR* receptor mutants. SDS-PAGE of *P. aeruginosa* PAO1 outer membrane proteins after growth in iron-restricted conditions without (-) or with (+) 20 μM heme. The position of the band corresponding to a mixture of the HasR and HxA receptor proteins is indicated on the right and that of the molecular size marker on the left. SDS-PAGE is representative of two biological replicates (N=2).

Fig. S4. Analysis of the co-transcription of the *has* and *hxA* σ^{ECF} /anti- σ genes by RT-PCR. Total RNA was isolated from the *P. aeruginosa* PAO1 wild-type strain grown under iron-restricted conditions supplemented with 20 μM heme. Reactions were performed with primers amplifying the *hasI-hasS* or *hxAI-hxUR* intergenic regions (Table S2, expected sizes 327 and 312 bp, respectively). Positive controls using *P. aeruginosa* genomic DNA and negative controls containing either H₂O instead of RNA or Taq polymerase (PCR) instead of the reverse transcriptase (RT-PCR) were included. Next to the expected cDNA product, an unspecific product was also obtained as result of the *hxAI-hxUR* RT-PCR. Data show is representative of two biological replicates (N=2).

Receptor	
HasR	602 FATYGKG WRPP AVTESLITGR P HGGGAENMY PNPFL SPERSKA 644
PhuR	503 YGQYAQ GFRTPT AKAL Y GRFENLQAGYHIE PNPNL KPEKSQSF 545
HxuA	580 FADYTET WRAP VIDEQ Y EVQNSSTIGG SSRD LD AERIHAIRGG 622

Fig. S5. Sequence alignment of the heme-coordination extracellular loop of *P. aeruginosa* heme receptors. The conserved FRAP/PNPFL loop required for heme uptake (Krieg et al., 2009) is shown in bold and the His or Tyr coordination residues within this loop are grey boxed.

Promoter	Fur box (consensus): GATAATGATa/tATCATTATC
<i>hasI</i>	CATCTCACAAAAAT ACGAT TGA AATCATTATC CATAAAAAATATGCTGCTGCCCTTCTGAAAGGACGTGGGTG
<i>hasR-hasAp</i>	ATCGTTCTCTTAAC GAGACTGATTCT ATTACA CAAAACGATGGAGTGTAGGCGCTGTCATG
<i>hxuI-hxuR</i>	TTACCTTGTGAAAT GATATGTATTATCATT CGC ATATTTGCAAGGACTTCCGTATCCGACG-65-ATG
<i>hxuA</i>	GGCGCGTCGTCAGC ATTAAATGA AATATTTTC ATTTCGCATATGTGGGTTTTCTCCCTGCTGCGTCT-60-ATG
<i>hemO</i>	GCATCCTTTCTGT GACAT TGAGATTCA ATA CGAATATAATTGAGAATCGTTATTGATCCTG-50-ATG
<i>phuR/phuSTUVW</i>	<i>phuS</i> CAT-50-GTGAACCTTGCCAC AAAACGCATATCTGAATC CATTT GATAAT TATTGCATTAGC GTTTTTCTGGCAGT-60-ATG <i>phuR</i>

Fig. S6. Predicted Fur boxes in the promoter region of the *P. aeruginosa* heme utilization genes. Predicted Fur binding sites are grey boxed with residues matching the consensus shown in bold. The translational-start sites are indicated with arrows. The intergenic region between the divergent *phuR* and *phuS* genes contains a strong and a weak Fur box (Ochsner et al., 2000).