Post-transcriptional regulation of the Pseudomonas aeruginosa heme assimilation system (Has) fine-tunes extracellular heme sensing

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Pseudomonas aeruginosa is an opportunist pathogen that utilizes heme as a primary iron source within the host. Extracellular heme is sensed via a heme assimilation system (has) that encodes an extracytoplasmic function (ECF) σ factor system. Herein, using has deletion mutants, quantitative PCR analyses, and immunoblotting, we show that the activation of the σ factor HasI requires heme release from the hemophore HasAp to the outer-membrane receptor HasR. Using RT-PCR and 5′-RACE, we observed that following transcriptional activation of the co-transcribed hasRAp, it is further processed into specific mRNAs varying in stability. We noted that the processing and variation in stability of the hasAp and hasR mRNAs in response to heme provide a mechanism for differential expression from co-transcribed genes. The multiple layers of post-transcriptional regulation of the ECF signaling cascade, including the previously reported post-transcriptional regulation of HasAp by the heme metabolites biliverdin IXβ and IXδ, allow fine-tuning of the cell-surface signaling system in response to extracellular heme levels. We hypothesize that the complex post-transcriptional regulation of the Has system provides P. aeruginosa an advantage in colonizing a variety of physiological niches in the host.

Iron is essential for the survival and virulence of nearly all bacterial pathogens. However, in the human body iron is tightly bound by high-affinity binding proteins such as transferrin, lactoferrin, and ferritin (1). Within the host, iron is further limited by the innate immune response and the up-regulation of lipocalin-2 and ferritin (2). To circumvent the iron-deficient environment of the host, bacterial pathogens have evolved systems that can scavenge iron and heme (3–6). The opportunistic pathogen Pseudomonas aeruginosa is a major cause of infection in immunocompromised patients (7, 8). P. aeruginosa encodes several iron-uptake pathways, including the siderophore-based pyoverdine and pyochelin systems (9, 10), ferrous uptake system (Feo) (11), and the heme assimilation (Has) and Pseudomonas heme uptake (Phu) systems (12, 13). To adapt within the host, the invading pathogen must sense and alter gene expression in response to the extracellular iron source. A mechanism through which bacteria respond to external stimuli are the cell-surface signaling systems that encode alternative σ factors. ECF σ factors are proteins that complex with the core RNA polymerase, direct binding to the promoter region of target genes, and activate transcription (14–17). P. aeruginosa encodes several ECF σ factors associated with iron-uptake systems, including the iron-starvation σ factors, PvdS and FpvI. PvdS and FpvI respond to the presence of extracellular iron—pyoverdine and up-regulate expression of pyoverdine biosynthesis genes and the pyoverdine outer membrane (OM) receptor FpvA, respectively (18–23). Several heme-dependent σ factors associated with the heme uptake systems have also been identified in Gram-negative pathogens, including Bordetella pertussis (24), Serratia marcescens (25, 26), and P. aeruginosa (12). To date, the only study of a heme-dependent ECF σ factor system is the Has system of S. marcescens, which was functionally reconstituted in Escherichia coli (26). In this system, the heme-loaded extracellular hemophore HasA (holo-HasA) on interaction with the OM heme receptor triggers a signal inactivating the anti-σ factor HasS and releasing the σ factor HasI. HasI through recruitment of RNA polymerase up-regulates transcription of the hasR, hasA, and hasS genes. Therefore, positive autoregulation of HasS by HasI allows the anti-σ factor HasS to accumulate in an inactive state in the presence of heme. As extracellular heme levels decrease, the accumulated HasS is activated and rapidly sequesters HasI down-regulating the system. Therefore, the ECF anti-σ/σ factor system serves as both a transcriptional activator, when heme is present in the extracellular media, and a repressor when levels decline. To date, the S. marcescens Has system is the only iron-starvation σ factor either heme- or siderophore-dependent that has been shown to directly autoregulate its anti-σ factor. Although S. marcescens also encodes a

The abbreviations used are: Has, heme assimilation system; Phu, Pseudomonas heme uptake system; BVIX, biliverdin IX; sRNA, small RNA; 5′-RACE, 5′-rapid amplification of cDNA ends; qPCR, quantitative polymerase chain reaction; ITC, isothermal titration calorimetry; Ap, ampicillin; OM, outer membrane; Gm, gentamycin; Cb, carbenicillin; Tc, tetracycline; AAP, abridged anchor primer; UAP, universal amplification primer; ECF, extracytoplasmic function; F, forward; R, reverse.

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This article contains Figs. S1–S4, Tables S1–S2, and supporting Refs. 1–6.

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second nonhemophore-dependent heme uptake system (Hem), it was proposed based on the high affinity of HasA for heme that the Has system was the high capacity transport system compared with the nonhemophore-dependent Hem system (27).

However, recent studies in P. aeruginosa, which also encodes a nonhemophore-dependent Pseudomonas (P.) heme uptake (Phu) system in addition to the Has system, are not consistent with this hypothesis. Specifically, $^{13}$C-heme uptake studies with P. aeruginosa PAO1 and the $\Delta$phuR or $\Delta$hasR strains showed deletion of the gene encoding the outer-membrane receptor PhuR significantly decreased the efficiency of heme uptake, whereas loss of HasR had no effect on the rate of heme uptake (13). Similarly, in vitro $^{13}$C-heme uptake and utilization studies performed with longitudinal chronic lung infection isolates revealed an adaptation over time to heme utilization at the expense of pyoverdine biosynthesis (28). Furthermore, transcriptional analysis of longitudinal P. aeruginosa chronic infection isolates shows over time they adapt to utilize heme through mutations within the promoter that give rise to increased expression of PhuR (29). Additionally, in vitro spectroscopic and kinetic studies indicate that heme binding to P. aeruginosa HasAp is passive and occurs at a similar rate to heme dissociation from hemoglobin (30). The emerging evidence from both in vitro and in vivo studies suggests the Has and Phu systems have nonredundant roles in heme sensing and transport, respectively. Once transported into the cell, heme is degraded by an iron-regulated heme oxygenase HemO to release iron, generating carbon monoxide, BVIX$\beta$, and BVIX$\delta$ as by-products (31).

Given the significance of heme as an iron source during infection, we sought to further investigate the role of the P. aeruginosa Has system in extracellular heme sensing. In this study, we show hasR and hasAp are transcribed in an operon that is subject to heme-dependent activation by the HasI $\sigma$ factor. The hasRAp transcript then undergoes post-transcriptional processing and differential stabilization of the hasR and hasAp mRNAs. We also show that activation of the ECF anti- $\sigma$ factor (HasS/I) system depends on the release of heme from HasAp to the HasR receptor. Furthermore, we recently determined the heme metabolites BVIX$\beta$ and BVIX$\delta$ post-transcriptionally regulate the protein levels of the extracellular hemoaphore HasAp (32). In contrast, the hasS operon encoding the $\sigma$ factor and anti-$\sigma$ factor is solely regulated by iron in a Fur-dependent manner. Taken together, the post-transcriptional regulation of the cell signaling cascade at the level of heme and its metabolites (BVIX$\beta$ and BVIX$\delta$) allows P. aeruginosa to rapidly and efficiently respond to fluctuations in extracellular heme. We propose the complex integration of extracellular heme metabolism into the post-transcriptional regulatory network of the Has system provides a significant advantage for P. aeruginosa adaptation and colonization within the host.

**Results**

**Heme-dependent activation of the has system by the HasI $\sigma$ factor**

qPCR analysis of the hasR mRNA levels in the presence of 2.5 $\mu$M FeCl$_3$ was significantly down-regulated in PAO1 compared with those from cultures grown in low iron, consistent with the previously reported Fur-mediated regulation of the hasR promoter (Fig. 1A) (12). In the aforementioned study, it was also reported that hasR and hasAp were co-transcribed as an operon (12). qPCR analysis of the hasAp mRNA expression profile over time in low iron or when supplemented with 2.5 $\mu$M FeCl$_3$ is similar to that of hasR, suggesting co-transcription from the Fur-regulated hasR promoter (Fig. 1B). Similarly, mRNA levels of hasR and hasAp in a $\Delta$hasI strain lacking the $\sigma$ factor are Fur-repressed in the presence of iron (Fig. 1, A and B). In the presence of heme as an iron source, hasR mRNA levels from PAO1 cultures show a time-dependent increase that peaks at ~5 h (Fig. 2A), whereas in the $\Delta$hasI strain we observe no heme-dependent activation of hasR as a result of the loss of the heme-dependent ECF $\sigma$ factor HasI (Fig. 2A). Similarly, in the presence of heme a similar time-dependent increase in hasAp mRNA levels is observed, whereas in the $\Delta$hasI strain the heme-dependent transcriptional activation is repressed (Fig. 2B). The mRNA expression profile is consistent with co-transcription of hasRAp from the hasR promoter. Western blotting of HasR protein levels in both PAO1 and $\Delta$hasI shows a similar profile over time to the mRNA levels, with a slight difference in the peak levels due to a lag between transcription and translation (Fig. 2C). Similarly, we do not detect HasAp protein until the later time points in keeping with the fact that HasAp following translation has to be secreted to the extracellular media.

**hasR and hasAp are co-transcribed in an operon and subjected to post-transcriptional processing**

Although the overall mRNA expression profiles of hasR and hasAp were similar, we noted that the relative mRNA levels for hasAp are greater than those of hasR in both low iron and heme-supplemented cultures (Figs. 1 and 2). This observation seems inconsistent with a single bicistronic hasRAp transcript. Previous studies by Ochsner and Vasil (12) suggested that in addition to the heme- and iron-dependent regulation of the hasR promoter there was a weak but constitutive promoter within the intergenic region between hasR and hasAp. Employing RT-PCR using nested primers specific for hasAp and hasR, we first confirmed hasR and hasAp are co-transcribed as an operon (Fig. 3A). 5’-RACE mapped the transcriptional start site of hasR 45 bp upstream of the start codon within the intergenic region between hasS and hasR (Fig. 3B and Fig. S1). We also performed 5’-RACE with primers specific for hasAp and identified a 0.7-kb hasAp transcript with a putative weak transcriptional start site 80 bp upstream of the ATG codon (Fig. 3B and Fig. S1).

To investigate the transcriptional regulation of hasR and hasAp, we constructed chromosomal transcriptional reporters for the hasR and putative hasAp promoters. The hasR and hasAp transcriptional construct comprised the 156-bp hasS/hasR intergenic region and 213 bp upstream of the transcriptional start site of hasAp fused to a promoter-less lacZ gene to give PhasR-lacZ and PhasAp-lacZ, respectively. Transcriptional reporter assays were performed on cultures washed and depleted of endogenous HasAp and supplemented with either 2.5 $\mu$M FeCl$_3$, 1 $\mu$M heme, or 2.5 $\mu$M apo-HasAp partially heme loaded (0.25 $\mu$M heme) or fully heme loaded (2.5 $\mu$M heme). PAO1 PhasR-lacZ cultures grown in the presence of 2.5 $\mu$M
Figure 1. Iron regulation of hasAp and hasR in PAO1 WT and PAO1 ΔhasI. A, relative expression of hasR mRNA from cultures in low iron or supplemented with 2.5 μM FeCl₃. mRNA was isolated at 0, 2, 5, and 7 h as described under "Experimental procedures." B, relative expression of hasAp mRNA as described for hasR. mRNA values represent the standard deviation from three independent experiments performed in triplicate and normalized to 0 h for the respective strains. The indicated p values as determined by a Student’s two-tailed t test were normalized to mRNA levels of the respective strains at the same time point, where *, p < 0.05, and **, p < 0.005.

Figure 2. Heme regulation of hasAp and hasR in PAO1 WT and PAO1 ΔhasI. A, relative expression of hasR mRNA from cultures supplemented with 1 μM heme. mRNA was isolated at 0, 2, 5, and 7 h as described under "Experimental procedures." mRNA values represent the standard deviation from three independent experiments performed in triplicate and normalized to 0 h for the respective strains. The indicated p values as determined by a Student’s two-tailed t test were normalized to mRNA levels of the respective strains at the same time point, where *, p < 0.05, and **, p < 0.005. B, relative expression of hasAp performed as described above for hasR. C, Western blot analysis of HasAp and HasR. Western blotting of extracellular HasAp levels in PAO1 WT and ΔhasI were performed on supernatant (5–10 μl) adjusted for differences in A₆₀₀. For HasR, 25 μg of total protein as determined by the Bradford assay was loaded in each well. RNA (polymerase subunit was used as an internal loading control. D, normalized intensity (n = 3) of HasR to the loading control was performed on Western blottings for three separate biological replicates. The indicated p values as determined by a Student’s two-tailed t test for PAO1 ΔhasI were normalized to PAO1 WT at the same time point where *, p < 0.05, and **, p < 0.005.
FeCl₃ show decreased β-gal activity compared with cultures in low iron as a result of Fur repression (Fig. 4A). Cultures supplemented with 1 μM heme in the absence of extracellular HasAp show a decrease in β-gal activity consistent with hemophore-independent heme uptake and iron release. However, cultures supplemented with partially heme-loaded HasAp show a 6-fold increase in β-gal activity as a result of heme-dependent transcriptional activation by the HasI factor (Fig. 4A). Interestingly, we observe a less robust but still significant increase in β-gal activity in cultures supplemented with fully-heme loaded holo-HasAp (Fig. 4A). We attribute this decrease in heme-dependent transcriptional activation to increased intracellular iron levels as a result of active heme uptake and degradation. The data are consistent with previous reports that the hasR promoter is subject to heme-dependent transcriptional activation by the ECF σ factor HasI and iron-dependent repression by Fur (33).

In contrast, the PhasAp-lacZ transcriptional fusions showed little to no activity above that of the background β-gal activity in

![Diagram](https://example.com/diagram.png)
low iron or on the addition of holo-HasAp or when supplemented with 1 \( \mu M \) heme (Fig. 4B). Furthermore, when supplemented with 2.5 \( \mu M \) FeCl\(_3\), we observed no iron suppression, consistent with the lack of a Fur box within the hasR/hasAp intergenic region. It was previously reported that hasAp is constitutively expressed from a weak promoter upstream of hasAp (12). However, this is difficult to reconcile given the lack of promoter activity upstream of hasAp and the significantly higher hasAp mRNA levels in low iron or heme. We next sought to determine whether the relative difference in hasAp and hasR mRNA levels (Fig. 2) is the result of post-transcriptional processing and regulation of the bicistronic hasAp mRNA. We performed Northern blot analysis on mRNA isolated from PAO1 WT cultures at 2, 5, and 7 h grown in either low iron or supplemented with 1 \( \mu M \) heme. Following hybridization of mRNA with a hasR-specific probe (shown in Fig. 5A), we observe two bands corresponding to hasR (\( \sim 2.7 \) kb) and a faint band corresponding to hasRAp (\( \sim 3.6 \) kb) in low iron (Fig. 5B). The hasR transcript is more clearly visualized on increased exposure time (Fig. S2). For the hasAp-specific probe (shown in Fig. 5A), we observe a band corresponding to hasAp (\( \sim 0.7 \) kb) (Fig. 5B). However, the longer hasR transcript is only detected with the hasAp probe on increased exposure times (Fig. S2). Taken together with the RT-PCR and qPCR data, Northern blot analysis is consistent with co-transcription of hasR and hasAp in an operon, followed by rapid post-transcriptional processing to yield the individual hasR and hasAp mRNAs. To determine whether the differences in hasR and hasAp mRNA levels (Figs. 1 and 2) following the initial processing of the hasRAp transcript are a consequence of mRNA stability, we monitored mRNA decay rates following termination of transcription. As shown in Fig. 5C, the rate of decay of hasAp in low-iron conditions was significantly slower than that of hasR (Fig. 5C), consistent with qPCR analysis (Fig. 1).

As expected, cultures grown in the presence of heme show an increase in the intensity of the hasRAp and hasAp bands on Northern blot analysis compared with low-iron conditions (Fig. 5B). The activation of transcription in the presence of heme is consistent with activation of the ECF \( \sigma \) factor HasI. Interestingly, hasR mRNA time-dependent decay is slowed in the presence of heme compared with low iron (Fig. 5C). In contrast, hasAp mRNA stability is unaffected in the presence of heme (Fig. 5C). However, despite the fact we see no heme effect on the stability of hasAp mRNA (Fig. 5C), consistent with qPCR analysis (Fig. 2B), we observe an increase in band intensity at later time points (Fig. 5B). The increase in intensity of the hasAp band is also consistent with previous studies that correlate the exponential increase in hasAp mRNA with the production of the heme metabolites BVIX\( \beta \) and BVIX\( \delta \) (32). Therefore, the combined effect of increased hasAp mRNA stability and post-transcriptional regulation by the heme metabolites amplifies and maintains extracellular HasAp levels as a function of active heme uptake. We propose post-transcriptional regulation of hasR and hasAp at the level of heme metabolism allows \textit{P. aeruginosa} to rapidly respond to both fluctuations in extracellular heme as well as its iron needs.

**Heme release from HasAp to HasR is required to activate the \( \sigma \) factor HasI**

Previous transcriptional reporter assays of the \textit{S. marcescens} hasR promoter in \textit{E. coli} transformed with a plasmid constitutively expressing hasISR concluded heme release from HasAp to HasR was required for activation of HasI (34). However, several mutants that showed reduced \( \beta \)-gal activity, including the
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Figure 6. Spectroscopic characterization of the holo-HasAp mutant proteins. A, overlay of the apo- and holo-HasAp crystal structures with the His-32 and Tyr-75 loop in apo-HasAp shown in magenta. In holo-HasAp, the His-32 loop and Tyr-75 loop and His-83 are shown in cyan and green, respectively, with the coordinating ligands, and His-83 is shown in stick format (Protein Data Bank codes 3ELL (holo) and 3MOK (apo)). B, absorbance spectra of the holo-HasAp WT, H32A, and H83A mutants. C, absorbance spectra of the holo-HasAp WT, Y75A, and Y75H mutants. Spectra were recorded in 20 mM Tris (pH 8.0) containing 50 mM NaCl at 25 °C with a final protein concentration of 20 μM.

heme ligand mutants H32A and Y75A, were reported to be competent to transport heme (35). Therefore, given the conflicting nature of these results, we sought to further dissect the HasAp–HasR heme signaling complex in the native P. aeruginosa background.

It has previously been reported that HasAp undergoes C-terminal proteolytic cleavage on secretion to the extracellular media (36). The cleaved protein retained biological activity and was proposed to be the major form in the extracellular environment. Consequently, we and others have studied the biochemical and biophysical properties of purified HasAp lacking the C-terminal 21 amino acid residues (37). We constructed a series of mutants targeting the proximal Tyr-75 (Y75A and Y75H) and distal His-32 (H32A) and His-83 (H83A), which contribute a hydrogen bond between the Nε of the histidine and the Oα of Tyr-75 that is proposed to increase its tyrosinate character (Fig. 6A). Rivera and co-workers (30, 38, 39) have previously characterized the structural and spectroscopic contributions of the Tyr-75, His-32, and His-83 residues to heme binding. Kinetic analysis of heme loading to the holo-HasAp WT and the corresponding heme coordination mutant H32A concluded that heme binds rapidly to the Tyr-75 loop, followed by a slower conformational closing of the His-32 loop (38).

Consistent with previous reports, CD spectrophotometry confirmed there were no significant differences in the overall structural fold of the HasAp heme coordination mutants compared with the WT protein (Fig. S3). The HasAp WT, H32A, Y75A, and H83A variants when reconstituted with heme all show absorption spectra identical to those previously reported (Fig. 6, B and C) (30, 37, 39). Compared with holo-HasAp WT, which has a Soret maxima at 406 nm and a typical high-spin marker at 616 nm, the H32A and H83A mutants have slightly blue-shifted Soret maxima at 401 and 404 nm and high-spin markers at 619 and 621 nm, respectively (Fig. 6B). In contrast, the holo-HasAp Y75A mutant has a Soret maxima identical to that of the WT complex, with the major difference being the appearance of Q bands at 535 and 550 nm, consistent with an increase in low-spin character (Fig. 6C). The spectrum of the holo-HasAp Y75H mutant showed a slight red shift in the Soret maxima to 408 nm accompanied by increased intensity in the Q bands at 537 and 567 nm, indicative of a low spin bis-His coordinated heme.

Despite expression of the HasAp proteins in M9 minimal media, the proteins purify with residual heme bound (5–20%). To perform heme-binding assays by ITC, we further separated apo- and holo-HasAp fractions by hydrophobic chromatography (38). Consistent with previously calculated binding affinities (K_D), the HasAp WT has a K_D value in the micromolar range when measured by ITC (Table 1 and Fig. S4) (30). As reported previously for S. marcescens HasA, the free energy (∆G) of heme binding to HasAp is favorable and enthalpy-driven as judged by the large negative ∆H and unfavorable entropy (T∆S) (40). The unfavorable entropy arises from hydrophobic interactions of heme with the Tyr-75 loop and subsequent displacement of water molecules on rearrangement of the His-32 coordinating loop. Perhaps not surprisingly, the HasAp Y75H mutant shows a similar enthalpy-driven process, consistent with heme binding to His-75, driving rearrangement of the His-32 loop. The bis-His heme coordination in holo-HasAp Y75H is also supported by the red-shifted Soret band and increased definition in the Q-band region (Fig. 6B).

Although the overall free energy (∆G) of heme binding to the HasAp Y75A, H32A, and H83A mutants is favorable, all three mutants show significant increases in entropy at the expense of enthalpic contributions. The gain in entropy and disorder is consistent with a greater contribution of hydrophobic interactions of the heme scaffold with HasAp and increased conformational flexibility of the Tyr-75 and His-32 loops in the absence of the heme-coordinating ligands. Interestingly, the thermodynamic parameters of the H83A HasAp mutant are almost identical to Y75A consistent with its role in stabilizing heme coordination to Tyr-75. For all three mutants, the increase in hydrophobic contributions is reflected in the increased heme-binding affinities (K_D). Taken together, the data are consistent with previous reports that hydrophobic interactions of the heme scaffold with the HasAp Tyr-75 loop contribute to heme capture, whereas heme ligation through Tyr-75 and His-32 controls heme release to HasR (39).

To assess the contributions of heme coordination to heme release and activation of the cell-surface signaling cascade, we
employed a ΔhasAp strain exogenously supplemented with the purified holo-HasAp WT or mutant proteins. Transcriptional activation from the hasR promoter was determined by qPCR analysis of hasR mRNA levels following addition of holo-HasAp WT or mutant proteins to the cultures. As expected, addition of heme alone in the absence of extracellular HasAp shows no time-dependent increase in hasR mRNA levels (Fig. 7). In contrast, addition of 1 μM holo-HasAp WT to the cultures results in induction of hasR mRNA levels, consistent with transcriptional activation of the ECF σ factor HasI (Fig. 7). Interestingly, addition of the holo-HasAp Y75A, H83A, or H32A proteins shows an even greater increase (~30-fold) in hasR mRNA levels between 2 and 5 h compared with holo-HasAp WT. As the heme content in all of the proteins is identical, we interpret this increase in hasR mRNA to result from the formation of a non-physiological heme HasAp–HasR-coordinated intermediate that is less efficiently transported and is thus “kinetically” trapped in the signaling mode. In contrast, on supplementation of ΔhasAp cultures with holo-HasAp Y75H, we observed no transcriptional activation of hasR. These data suggest that by introducing the stronger bis-His ligation, the conformational rearrangement and free energy gained on holo-HasAp–HasR complex formation are not sufficient to drive release of the heme-coordinating ligands.

**hasI and hasS are co-transcribed as an operon and subject to Fur-dependent iron regulation**

In *S. marcescens*, the ECF σ factor HasI and anti-σ factor HasS are co-transcribed as operons. Furthermore, hasS was shown to be autoregulated via HasI (26). Therefore, in *S. marcescens* hasS and hasI have different expression profiles depending on the presence or absence of heme. Sequence analysis of the *P. aeruginosa* hasI–hasS intergenic region revealed no obvious terminator suggesting the genes are co-transcribed as an operon. RT-PCR analysis using primers designed within the intergenic region that also includes the previously identified Fur-box (41). The Fur-dependent regulation of the hasS construct was confirmed in PAO1 cultures supplemented with 2.5 μM FeCl₃, where mRNA levels of hasS and hasI are repressed as a function of time (Fig. 8, B and C). The similar hasI and hasS mRNA profiles are consistent with their co-transcription from the hasI promoter. PAO1 cultures grown in the presence of 1 μM heme show similar decreases in the relative expression of both hasS and hasI when compared with low-iron conditions (Fig. 8, B and C). The decrease in the relative expression of hasS in PAO1 WT cultures supplemented with either iron or heme suggests the hasS gene is not subject to autoregulation by HasI (Fig. 8B). The lack of autoregulation over the hasS gene was further confirmed in the ΔhasI strain, where hasS mRNA levels in PAO1 WT and the ΔhasI strain are similarly repressed in the presence of heme (Fig. 8B). Therefore, in contrast to *S. marcescens* the ECF σ factor/anti-σ system in *P. aeruginosa* is subject to iron regulation by the Fur-repressor and is not autoregulated by HasI.

**Discussion**

As is the case for the majority of bacteria, including *P. aeruginosa*, the maintenance of iron homeostasis is globally regulated by the iron-dependent repressor Fur. However, many of the individual iron uptake systems, both siderophore and heme-dependent, are regulated by their respective substrates. This regulation is mediated by specific σ factors belonging to the ECF family of regulators (14–16). In *P. aeruginosa*, the siderophore-dependent signaling systems have been well characterized, including the iron–pyoverdine system, which regulates divergent pathways through the ECF σ factors PvdS (pyoverdine biosynthesis, exotoxin A, and PrpL endoprotease) and FpvI (outer-membrane receptor FpvA) (21). However, to date the *P. aeruginosa* heme-dependent holo-HasAp signaling cascade has not been well characterized.

In this study, we have elucidated the molecular mechanisms underlying the regulation of the Has system. 5′-RACE confirmed hasR and hasAp are co-transcribed in an operon under

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**Table 1**

Thermodynamic parameters for heme binding to HasAp WT and mutant proteins

Experiments were performed as described under "Experimental procedures." The data were fit to a one-site binding model with the MicroCal PEAQ-ITC analysis software supplied by the manufacturer. The data were averaged from three independent experiments.

| Protein       | Kₐ (μM) | N(sites) | ΔH kcal mol⁻¹ | ΔG kcal mol⁻¹ | ΔAS kcal mol⁻¹ |
|---------------|---------|----------|---------------|---------------|---------------|
| HasAp         | 1.2 ± 0.8 | 1.3 ± 0.6 | -99.7 ± 0.5 | -8.5 ± 1.0 | -91.2 ± 0.4 |
| HasAp Y75H    | 1.1 ± 0.7 | 1.4 ± 0.7 | -100 | -8.1 ± 4.0 | -91.8 ± 0.2 |
| HasAp Y75A    | 0.012 ± 0.006 | 0.95 ± 0.03 | -7.1 ± 0.59 | -10.9 ± 0.4 | 3.8 ± 0.9 |
| HasAp H32A    | 0.079 ± 0.003 | 0.55 ± 0.05 | -22.3 ± 1.4 | -9.7 ± 0.2 | -126 ± 1.3 |
| HasAp H83A    | 0.0012 ± 0.003 | 0.92 ± 0.03 | -7.1 ± 0.34 | -10.8 ± 0.15 | 3.7 ± 0.24 |

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**Figure 7.** Effect of purified holo-HasAp WT, His-32, Tyr-75, and His-83 proteins on transcriptional activation of hasR in a PAO1 ΔhasAp strain. Relative expression of hasR mRNA from PAO1 hasAp cultures supplemented with holo-HasAp WT or mutant proteins. mRNA was isolated at 4 h as described under "Experimental procedures." mRNA values represent the standard deviation from three independent experiments performed in triplicate and normalized to 0 h for the respective strains. The indicated p values as determined by a Student’s two-tailed t test were normalized to mRNA levels of the respective variants to HasAp WT at the same time point, where *, p < 0.05, **, p < 0.005.
**Figure 8. Heme and iron regulation of hasI and hasS in PAO1 WT and PAO1 ΔhasI.**

**A.** Map of the transcriptional start site and promoter of hasI. The hasI transcriptional start site and start codon are in **boldface type**. Positions of primers for 5’-RACE and primer-specific extension of hasAp and hasR are shown by sequence position. **B.** hasS mRNA levels as a function of time in PAO1 and PAO1 ΔhasI in the presence of heme or iron. mRNA was isolated at 0, 2, 5, and 7 h following supplementation with 1 μM heme (left panel) or 2.5 μM FeCl₃ (right panel). **C.** hasI mRNA levels as a function of time in PAO1. mRNA isolated as in **B**. mRNA values represent the mean from three biological experiments each performed in triplicate and normalized to their respective strains in low-iron conditions at 0 h. Error bars represent the standard deviation from three independent experiments performed in triplicate. The indicated *p* values as determined by a Student’s two-tailed *t* test were normalized to mRNA levels of PAO1 at the same time point, where *, *p* < 0.05; ***, *p* < 0.005.
the control of the global iron regulator Fur (12). However, the observed difference in hasR and hasAp mRNA levels in low-iron conditions is not consistent with a single co-transcript. A previous report on the basis of translational reporter assays suggested a potential weak but constitutive promoter upstream of hasAp (12). Although we confirmed by 5′-RACE a second transcript specific for hasAp, we determined this is the result of post-transcriptional processing of hasRAp and differential stabilities of the resulting hasAp and hasR mRNAs (Fig. 5). However, during active heme uptake hasR mRNA is stabilized when compared with decay rates in low iron, whereas hasAp mRNA decay rates are similar in both conditions. Interestingly, RNA endonuclease cleavage within multigene operons leading to differential stability of the resulting mRNAs has been well documented in bacterial systems, including the glycolysis operon cggR-papA of Bacillus subtilis (42, 43) and the arginine deimination pathway in P. aeruginosa (44, 45). Thus, processing of polycistronic mRNAs by RNA endonucleases provides a mechanism to differentially regulate expression of co-transcribed genes. In the context of infection, the secreted extracellular hemophore HasAp would be expected to be expressed at high levels to combat diffusion from the site of infection, as well as opsonization and degradation by proteases. Similarly, on active heme uptake, the heme-dependent stabilization of hasR mRNA allows for increased levels of HasR to further amplify the signaling cascade as a function of the intracellular iron needs of the bacteria.

At the present time, the specific RNA endonuclease involved in the processing of hasRAp, as well as the mechanism by which the resulting hasR and hasAp mRNAs are differentially stabilized, is not known. An intriguing possibility is the potential involvement of small regulatory RNAs (sRNAs). The largest class of sRNAs in P. aeruginosa function through direct binding to the mRNA target affecting stability or translational efficiency. Indeed, the iron- and heme-dependent sRNAs PrrF1/F2 and PrrH present an interesting target for further investigation given their significant role in iron homeostasis and secondary metabolism (46–49). The processing of the hasRAp transcript and differential stabilization of the upstream and downstream products is the focus of ongoing studies.

We have previously also shown the increase in HasAp protein levels over those of HasR at later time points is a function of the accumulation of the heme metabolites BVIXβ and/or BVIXδ (32). The integration of heme-dependent post-transcriptional and stabilization of hasR and hasAp mRNA with the BVIXβ- and IXδ-dependent regulation of HasAp protein levels allows P. aeruginosa to rapidly fine-tune and regulate heme signaling independent of iron. Furthermore, post-transcriptional regulation of extracellular heme signaling via modulation of HasAp and HasR levels is distinct from that previously reported for the S. marcescens Has system. In S. marcescens, the Has system is regulated at the level of transcription via autoregulation of the anti-σ factor HasS by its σ factor HasI (26). The HasI-dependent regulation of HasS allows inactive anti-σ factor to accumulate when the system is active, and as extracellular heme levels decrease, HasS is activated sequestering HasI and down-regulating the signaling cascade. We hypothesize the post-transcriptional integration of heme metabolism into the ECF σ factor system contributes to the adaptability of P. aeruginosa in colonizing a range of physiological niches within the host.

In addition to elucidating the transcriptional and post-transcriptional regulation of the Has system, we further investigated the mechanism by which the holo-HasAp–HasR complex triggers activation of the Has σ factor itself. Previous spectroscopic and structural analysis of axial ligand mutants H32A and Y75A confirmed they retained the overall structural fold as the HasAp WT protein and the ability to bind heme with similar affinity (30, 39). Similarly, loss of His-83, which contributes a hydrogen bond between the Nω with the Oα of Tyr-75 increasing the tyrosinate character of the heme ligand, does not decrease the heme-binding affinities or overall structural fold of the protein (39). Based on these findings, the authors concluded the heme axial ligands are primarily required for slowing the release of heme from the hemophore, whereas the association of heme with the protein is primarily governed by noncovalent hydrophobic interactions. However, given previous reports, these same mutations in S. marcescens HasA are compromised in heme signaling but are competent to transport heme, and we sought to clarify the role of the heme-coordinating ligands in P. aeruginosa Has signaling.

Based on previous spectroscopic and crystallographic studies of the S. marcescens HasA–HasR complex, a sequential mechanism of heme release was proposed. In the first step, the interaction of holo-HasA with HasR displaces the His-32 ligand of HasA, while heme remains coordinated to HasA through Tyr-75 (50, 51). In a second step, the displacement of His-83 weakens the tyrosinate character of Tyr-75 promoting heme release to HasR. If the holo-HasA His-32 “off”-state is an intermediate in the sequential transfer of heme to HasR, then the H32A variant would be expected to show a heme-dependent transcriptional activation profile similar to the holo-HasA WT protein. However, transcriptional reporter assays following reconstitution of the signaling system in E. coli showed a decrease in promoter activity for the HasA H32A mutant (34). In contrast, our analysis of hasR transcriptional activation on supplementation of a P. aeruginosa ΔhasAp strain with holo-HasAp H32A showed a significant increase (~30-fold) in hasR mRNA at 5 h compared with holo-HasAp WT. We interpret these findings to be the result of a nonphysiological kinetically trapped “signaling” holo-HasApH32A/HasR intermediate following protein–protein interaction (Fig. 9B). A similar profile was observed for the H83A and Y75A mutants suggesting mutation of the Tyr-75 axial ligand, or weakening of the tyrosinate character of Tyr-75 on loss of His-83, leads to a similar kinetically trapped heme intermediate. In contrast, the holo-HasAp Y75H mutant shows no increase in hasR mRNA levels above those in the absence of holo-HasAp. This is consistent with the stronger bis-His coordination inhibiting heme release to HasR (Fig. 9C). Tyrosine is a relatively weak heme ligand that in heme transport proteins is strengthened through a hydrogen bond with a neighboring His residue increasing the tyrosinate character. The conserved Tyr–His motif has been exploited in bacterial heme transport proteins where protein–protein interaction drives a histidine-driven protonation of the tyrosinate ligand that weakens the coordination promoting release of
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Figure 9. Proposed mechanism for heme transfer from holo-HasAp to HasR. A, holo-HasAp WT interaction with HasR. On complex formation, a conformational change and the free energy gained drives the concerted release of the HasAp Tyr-75 and His-32 ligands transferring heme to HasR. B, holo-HasAp Y75A (or H32A) interaction with HasR. The open heme coordination site in Y75A (or H32A) mutant is occupied by a H2O molecule that on interaction with HasR is displaced forming a kinetically trapped intermediate. The kinetically trapped intermediate leads to increased heme signaling activity and decreased heme transport. C, holo-HasAp Y75H interaction with HasR. The free energy gained on protein–protein interaction of holo-HasAp with HasR is not sufficient to drive release of the low spin six coordinate bis-His ligation in holo-HasAp Y75H blocking heme signaling and transport. D, heme bound to HasR is transported into the periplasm in a TonB-dependent manner.

heme (52–54). We conclude that on interaction of holo-HasAp Y75H with HasR, the stronger bis-His coordination inhibits the required conformational rearrangement and free energy gain required to drive heme release to HasR.

Taken together, the data suggest that in contrast to the sequential release of His-32 to yield the holo-HasAp His-32 “off”-intermediate, heme release occurs via a concerted mechanism where both His-32 and Tyr-75 are simultaneously released (Fig. 9). This model is consistent with the data obtained for the H32A and Y75A HasAp mutants where loss of either heme ligand yields a kinetically trapped off-pathway intermediate that while competent in heme signaling is less efficiently transported (Fig. 9B). Further in vitro spectroscopic studies in combination with 13C-heme uptake assays utilizing the holo-HasAp WT and mutant proteins will allow us to dissect the mechanistic steps in heme signaling and transport by the Has system. Although we cannot fully explain the differences between our data and that previously reported for the S. marcescens Has system, it is worth noting the transcriptional reporter assays were performed in a heterologous E. coli strain expressing hasRSI on a plasmid (34). Furthermore, heme transport by HasR requires the TonB paralog HasB that is not present in E. coli. It is unclear at the present time whether HasB plays a role in allowing or facilitating conformational changes in HasR required for heme signaling as well as transport (55).

Based on this study, we propose the following model for the transcriptional and post-transcriptional activation of the Has signaling cascade by heme and its metabolites. 1) In low iron, the hasRAp operon is transcribed and processed to yield the individual hasR and hasAp mRNAs. 2) The increased mRNA stability of hasAp versus hasR allows for accumulation of high levels of extracellular HasAp. 3) In the presence of heme, holo-HasAp interacts with HasR releasing heme and triggering activation of HasI up-regulating hasRAp. 4) Active heme uptake leads to post-transcriptional stabilization of hasR by heme and the up-regulation of hasAp by the heme metabolites BVIXβ and BVIXδ. Therefore, post-transcriptional regulation of the signaling cascade at the level of active heme uptake and metabolism allows the bacteria to rapidly respond to extracellular heme levels as well as its iron needs. Furthermore, the integration of heme metabolism into the post-transcriptional regulation of the Has system represents a new paradigm in iron-starvation ECF σ factor–dependent cell signaling.

Experimental procedures

Bacterial strains, plasmids, and protein

Bacterial strains and plasmids used in this study are listed in Table S1 and oligonucleotide primers and probes in Table S2. E. coli strains were routinely grown in Luria Bertani (LB) broth (American Bioanalytical) or on LB agar plates, and P. aeruginosa strains were freshly streaked and maintained on P. isolation agar (PIA) (BD Biosciences). Brain heart infusion agar and TYS10 (10 g/liter tryptone, 5 g/liter yeast extract, 10% (w/v) filtered sucrose) were used in generating the ΔhasI and ΔhasAp deletion strains as described previously (56, 57). All strains were stored frozen at −80 °C in LB broth with 20% glycerol. For qPCR, Western blotting, or β-gal assays, singly isolated colonies from each Pseudomonas strain were picked, inoculated into 10 ml of LB broth, and grown overnight at 37 °C. The bacteria were then harvested and washed in 10 ml of M9 minimal medium (Nalgene). The iron levels in M9 medium were determined by inductively coupled plasma-mass spectrometry to be
less than 1 nm. Following centrifugation, the bacterial pellet was resuspended in 10 ml of M9 medium and used to inoculate 50 ml of fresh M9 low-iron medium to a starting A600 of 0.04. Cultures were grown at 37 °C with shaking for 3 h before the addition of supplements (0 h) and incubated for a further 7 h. When required, antibiotics were used at the following final concentrations (μg ml⁻¹): ampicillin (Ap), 100; tetracycline (Tc), 10 (for E. coli) and 150 (for P. aeruginosa); gentamicin (Gm), 250; and carbenicillin (Cb), 500.

Construction of the P. aeruginosa Δhasl and ΔhasAp PAO1 strains

The P. aeruginosa Δhasl unmarked deletion was generated by a method developed in our laboratory (56). Briefly, partial overlapping flanking primer pairs were designed to amplify the 500-bp upstream (hasl, upstream F/hasl, upstream R) and downstream (hasl, downstream F/hasl, downstream R) flanking sequences of hasl (Table S2). The resulting upstream and downstream fragments were cloned by Gibson assembly into the pEX18Tc suicide plasmid (57), and the resulting plasmid (pEX18Tc-Δhasl) was transformed into P. aeruginosa PAO1 strain as described previously (56). Clones in which a double event of homologous recombination resulted in the allelic exchange of the Δhasl gene were checked by PCR with primers flanking the genomic sequence of the deleted locus (Δhasl-F/Δhasl-R) and further verified by DNA sequencing (Eurofins MWG Operon).

The in-frame P. aeruginosa hasAp mutant was constructed using two primer pairs, ΔhasAp-A/ΔhasAp-B and ΔhasAp-C/ΔhasAp-D (Table S2), designed to amplify the upstream and downstream regions of the gene from the genomic DNA. The sequence of PCR products was confirmed by DNA sequencing (Eurofins MWG Operon). Following BamHI–HindIII digestion, the fragments were cloned into the counter-selective suicide plasmid pEX18Tc (57). The resulting pEX18Tc-ΔhasAp was transformed into E. coli S17-1-Apir, and a 0.8-kb KpnI fragment containing the FRT–Gm–FRT resistance cassette from pPS858 was inserted into the KpnI site of pEX18Tc-ΔhasAp. The resulting pEX18Tc-ΔhasAp-GmFRT plasmid was transferred from E. coli S17-1-Apir into P. aeruginosa PAO1 by conjugation. Clones in which a double event of homologous recombination resulted in the chromosomal integration of the ΔhasAp::GmFRT allele were selected on PIA plates containing Gm and 5% sucrose. To obtain the unmarked ΔhasAp deleted strain, the pFLP2 plasmid was mobilized into PAO1 ΔhasAp::GmFRT strain. This plasmid encoding the flippase recombinase (Flp) promotes recombination between FRT sequences flanking the Gm cassette. Isolated colonies were selected on PIA plates containing Cb, and loss of the hasAp gene was confirmed by screening for Gm5. Plasmid pFLP2 was then cured by streaking Cb5 Gm5 colonies on PIA plates supplemented with 5% sucrose. Loss of pFLP2 was tested by selecting for sucrose resistance and Cb5. Southern blotting hybridization, PCR, and sequencing analysis were used to verify allelic exchange of the parental gene and to ensure that the construct was nonpolar (data not shown).

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Gene expression and mRNA stability analysis using quantitative real-time PCR (qRT-PCR)

To analyze gene expression, total RNA was purified from 1-ml aliquots collected at several time points from cultures grown under various conditions. RNA was stabilized by the addition of 250 μl of RNALater Solution (Ambion), and the samples were stored at −80 °C until further use. Total RNA was isolated from each cell pellet using the RNeasy mini spin columns according to the manufacturer’s directions (Qiagen). 6 μg of total RNA was treated with RNase-free DNase I (New England Biolabs) for 2 h at 37 °C to remove contaminating chromosomal DNA and precipitated with 0.1× volume of 3 M sodium acetate (pH 5.2) and 2× volume of 100% (v/v) ethanol. RNA quantity and quality were assessed by UV absorption at 260 nm in a NanoDrop 2000c spectrophotometer (ThermoFisher Scientific). cDNA was generated using the GoScript™ reverse transcriptase kit (Promega) from RNA (250 ng) and random primers (0.5 μg). cDNA (10 ng) was analyzed with gene-specific primers (Table S2) using the StepOnePlus real-time PCR system (Applied Biosystems) and FastStart Universal Probe Master (Roxygen Applied Science). The relative gene expression was calculated using the ΔΔCt method, and the cycle threshold (Ct) values at each time point were normalized to the constitutively expressed oprF gene. mRNA values represent the standard deviation of three independent experiments performed in triplicate.

For RNA stability assays, PAO1 cultures were grown for 4 h as described previously. Aliquots (1 ml) were collected following transcriptional inhibition with rifampicin (200 μg/ml) at 0, 5, 15, 25, 35, 45, and 55 min and mixed with 250 μl of RNALater solution. Total RNA was isolated as above and analyzed by qRT-PCR. rRNA 16S was used as an endogenous reference and analyzed with TB Green Premix Ex TaqII (Tli RNaseH Plus) (Takara). Estimated mRNA levels were calculated using the ΔΔCt method, and the cycle threshold (Ct) values at each time point were normalized to the rRNA 16S gene. mRNA values represent the standard deviation of three independent experiments performed in triplicate, and data are presented as the percentage of hasF and hasAp mRNA levels relative to the amount of these transcripts at time 0. All primers and probes used for qPCR studies are listed in Table S2.

5’-RACE and RT-PCR

5’-RACE analysis was performed according to the manufacturer’s instructions (Invitrogen) with ~3 μg of total RNA extracted from P. aeruginosa PAO1 cells grown in M9 media. A gene-specific primer for the hasl (hasl-GSP1) transcript was used to initiate the first-strand cDNA synthesis from hasl mRNA (Table S2). A 5-μl aliquot of cDNA was amplified using the abridged anchor primer (AAP) and the nested gene-specific primer hasl-GSP2. The amplified product was purified by agarose gel electrophoresis and used as a template for a second round of amplification with the universal amplification primer (UAP) and hasl-GSP3–specific primer (Table S2). The cDNA product resulting from this last amplification step was sequenced to determine the transcriptional start site following the AAP. Reverse transcriptase (RT)-PCR analysis was per-
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formed to confirm the hasI co-transcript. Gene-specific hasS primer RT-hasS R was used to initiate the first-strand cDNA synthesis. The resulting cDNA was used as a template to amplify the co-transcribed genes hasI (primers hasI F/hasI R) and hasS (primers hasS F/hasS R) and the intergenic region between hasS-hasS genes (primers intergenic F/intergenic R).

Specific 5′-RACE analysis of hasRAp was performed as described previously using a gene-specific primer for hasAp mRNA (cDNAhasAp) to initiate the first-strand cDNA synthesis. Subsequent PCRs were performed with the AAP primer and the nested gene-specific primers hasR-RACE and hasAp-RACE (Table S2). Finally, the amplified product was purified and re-amplified with the UAP primer and same nested gene-specific primers used previously. To confirm co-transcription of hasR-hasAp by RT-PCR analysis, the same 5′-RACE cDNA sample was used as a template to amplify specific regions of hasR and hasAp ORFs with primers hasR F/hasR R and hasAp F/cDNAhasAp, respectively. Amplification of the intergenic region between hasR-hasAp was carried out with primers PhasAp/hasAp-R (Table S2).

Northern blot analysis

RNA probes complementary to hasR or hasA mRNA were obtained by in vitro transcription. The specific hasR and hasAp DNA fragments were amplified by PCR with primers hasR F/hasR R and hasAp F/cDNAhasAp, respectively (Table S2), and cloned into the pCR2.1-TOPO vector (Invitrogen). Resulting constructs in which the amplification products were cloned in opposite orientation to the T7 promoter were selected by PCR and sequenced. Following digestion with BamHI, 1 µg of the linearized plasmid was used as a template to obtain the specific biotin-labeled RNA probe with the HiScribe T7 high yield RNA synthesis kit (New England Biolabs) and biotin-14-dCTP (Invitrogen). RNA probes were DNase-treated (RNase-free DNase I, New England Biolabs), cleaned, and concentrated with RNA Clean & Concentrators-5 (ZymoResearch), respectively.

Total RNAs (3 µg) isolated from PAO1 cultures grown under different conditions were heat-denatured at 65 °C for 15 min and then separated by electrophoresis in 1.2% agarose formaldehyde gels. The RNA marker lane was separately stained with ethidium bromide and aligned with the membrane to mark off a molecular weight reference following transfer. RNA was then transferred to a positively charged nylon membrane (BrightStar-Plus Positively Charged Nylon Membranes, Ambion) by capillary transfer (Northern Max Transfer Buffer, ThermoFisher Scientific). After UV cross-linking, membranes were hybridized overnight at 68 °C in ULTRAhyb ultrasonic hybridization buffer (ThermoFisher Scientific) with RNA-biotinylated probe 0.1 µm. The membranes were washed twice in low- and high-stringency buffers (NorthernMax low/high-stringency wash buffers, ThermoFisher Scientific), and the specific biotin-labeled RNA–RNA complex was visualized by chemiluminescence detection with the Chemiluminescent Nucleic Acid Detection Module (ThermoFisher Scientific) and Amersham Biosciences hyperfilm ECL (Amersham Biosciences).

Construction of the hasAp-lacZ and hasR-lacZ transcriptional fusions

To generate the chromosomal fusions, the hasR promoter region, including 156 nucleotides upstream of the transcriptional start site, and the hasAp promoter, including 213 nucleotides upstream of the transcriptional start site, were amplified with primer pairs PhasR/PhasR R and PhasAp/PhasAp R, respectively (Table S2). Each fragment was digested with Smal–PstI and BamHI–PstI, respectively, and fused to the promoterless lacZ gene cloned into the miniCTX-1-LacZ vector (58). Recombinant plasmids, PhasR-lacZ and PhasAp-lacZ, were then transferred from E. coli S17-1-λpir into P. aeruginosa PAO1 by conjugation. Screening for TcR yielded derivatives containing the plasmid integrated into the CTX phase attB site of the chromosome. Excision of the unwanted plasmid DNA sequences was achieved by expressing the Flp recombinase as described previously and confirmed by PCR and DNA sequencing utilizing the P_Ser-up and P_Ser-down primers, as described previously (58). For all β-gal assays, the P. aeruginosa strains were grown in triplicate in 25 ml of M9 minimal medium. Growth cultures were left untreated or supplemented with 1 µm heme, 2.5 µm FeCl₃, or 2.5 µm holo-HasAp (partially 0.25 µm or fully 2.5 µm heme-loaded). Aliquots (1 ml) at various time points were harvested and assayed for β-gal activity as described previously (59).

Cloning expression and purification of WT and mutant HasAp proteins

The His-32, Tyr-75, and His-83 mutations were introduced into the previously constructed pET11a harboring the truncated hasAp gene using the QuikChange II site-directed mutagenesis kit (Agilent) (37). All mutations were verified by DNA sequencing (Eurofins MWG Operon). All primers for site-directed mutagenesis are listed in Table S2.

Protein expression was performed by slight modification of the previously published method following transformation of the resulting plasmid constructs into E. coli BL21(DE) competent cells (37). HasAp WT and mutant proteins were expressed by culturing a single colony from freshly transformed cells for 16 h in LB medium (100 ml) containing 100 µg/ml ampicillin at 37 °C with shaking. The cells were harvested by centrifugation at 6000 rpm at 4 °C for 5 min and resuspended in M9 media. The cells were washed with M9 twice, and the final cell pellet was resuspended in M9 and used to inoculate 4-liter (1 cultures) in M9 containing 100 µg/ml ampicillin to a final A600 of 0.05. The cells were grown to an A600 of ~1.0 and induced with 1 mM final concentration of isopropyl β-D-thiogalactopyranoside and grown for a further 16 h at 30 °C. Cells were harvested by centrifugation at 7000 rpm for 15 min and stored at −80 °C until further use. Pellets were thawed and resuspended in 40 ml of lysis buffer (20 mM Tris-HCl (pH 7.5), 20 mM NaCl, 1 mM EDTA) containing a protease inhibitor tablet (Roche Applied Science), 1 mg/ml DNase, and 25 mg/ml lysozyme and stirred at 4 °C for 1 h and passed through an LM-20 microfluidizer at 20,000 p.s.i. The lysis suspension was centrifuged at 25,000 rpm for 1 h to separate the cell debris. The supernatant was applied to a Q-Sepharose column (3 × 10 cm) pre-equilibrated with
equilibration buffer (20 mM Tris-HCl (pH 7.5) and 20 mM NaCl). The column was washed (3–5 column volumes) with equilibration buffer and the protein eluted over a gradient from 20 to 600 mM NaCl in 20 mM Tris-HCl (pH 7.5). The purity of the eluted fractions was determined by SDS-PAGE, and those containing HasAp were pooled and dialyzed in 4 liters of 50 mM Tris-HCl (pH 7.5) containing 50 mM NaCl. The protein was then concentrated (10 ml) and applied to a 26/60 Superdex 200 pg size-exclusion column. Fractions containing purified protein were pooled and concentrated to 10 mg/ml. The holo-HasAp samples were prepared with hemin prepared immediately prior to use by dissolving in 0.1 mM NaOH and buffered with 10 mM Tris-HCl (pH 8), and the final concentration was determined by the pyridine hemochrome method (60). HasAp WT and mutant protein were reconstituted 1:1 with hemin and incubated on ice for 30 min and concentrated via Amicon ultra-centrifuge filters (30 MWCO).

The integrity of protein secondary structure was determined by CD spectroscopy recorded on a Jasco J-810 spectropolarimeter. All samples were recorded in 10 mM potassium phosphate (pH 7.4) at 25 °C from 190 to 260 nm at a scan rate of 20 nm/min, with each spectrum representing 10 accumulations. Data were acquired at 0.2-mm resolution and 1.0-cm bandwidth. The mean residue ellipticity (degrees cm² dmol⁻¹) was calculated using CDPRO software supplied by the manufacturer.

**Transcriptional activation in the PAO1 ΔhasAp strain in the presence of exogenous holo-HasAp mutant proteins**

Overnight cultures (50 ml) were harvested by centrifugation at 5000 rpm at 4 °C for 5 min and resuspended in M9. The resulting cell pellet was washed twice, resuspended in M9, and used to inoculate fresh 50 ml of M9 cultures to a final A600 of 0.05. The cultures were grown for 3 h at 37 °C, with shaking to deplete the bacterial iron stores. After 3 h, samples (1 ml) were collected, and the cultures were supplemented with 1 μM HasAp WT or mutant proteins containing 0.1 μM heme to induce HasI-dependent transcriptional activation of hasR promoter by HasI, while ensuring low levels of iron as a result of heme transport and degradation in preventing Fur repression. Aliquots (1 ml) were collected at 2, 5, and 7 h. RNA isolation and qRT-PCR analysis of hasR mRNA levels was performed as described above with primers listed in Table S2.

**ITC experiments**

ITC was performed on a MicroCal PEAQ-ITC (Malvern Panalytical) instrument at 25 °C. All protein solutions were in 10 mM sodium phosphate (pH 7.5). Samples were degassed before use, and injections (0.4 μl) were carried out at 2.5-min intervals. For all experiments, protein concentrations ranged from 20 to 50 μM, and the titrated heme was set to 10–20 times this value. The heat of dilution of the protein was obtained by injecting into the buffer alone. The resulting integrated heats were baseline-corrected and then fitted to a one-site binding model using the MicroCal PEAQ-ITC software. A nonlinear least-squares algorithm (minimization of χ²) was used to fit the heat flow per injection to an equation corresponding to an equilibrium binding model, which provides best fit values for the stoichiometry (nITC), change in enthalpy (HITC), and binding constant (KITC). Kd values and Gibbs free energy were calculated according to $K_d = 1/K_{ITC}$ and $ΔG = RT \ln K_d$. The thermodynamic relationship $ΔG = ΔH - TΔS$ was used to find the entropic contribution to binding. The data were averaged from three independent ITC experiments.

**SDS-PAGE and Western blot analysis**

Aliquots (1 ml) from PAO1 and mutant cultures were harvested at the 0-, 2-, 5-, and 7-h time points. Cell pellets were resuspended in 200 μl per 1.0 A600 of BugBuster (Novagen). Cells were incubated at room temperature for 30 min with occasional agitation to ensure complete cell lysis, and total protein concentrations were determined using the Bio-Rad RCDC assay. Samples of the cell lysate (25 μg of total protein) in SDS-PAGE loading buffer were run on a 7.5% SDS-PAGE for HasR detection. Samples of the supernatant at 2-, 5-, and 7-h time points were collected for analysis of the extracellular HasAp levels and run on 12.5% SDS-PAGE. Following normalization between strains for differences in A600 at each time point, the supernatants (final volume 10 μl) were loaded in SDS-PAGE loading buffer. Proteins were transferred by electrophoresis to polyvinylidene difluoride membranes (Bio-Rad) for Western blot analysis. Membranes were blocked with blocking buffer (5% w/v skim milk in Tris-buffered saline (TBS) with 0.2% v/v Tween 20), washed, and probed with a 1:750 dilution of anti-HasR or anti-HasAp primary antibodies in hybridization buffer (1% w/v skim milk in TBS with 0.2% v/v Tween 20). Antibodies were obtained from Covance custom antibodies and generated from purified proteins supplied by our laboratory. Antibody sensitivity was checked against the respective purified proteins prior to use, and all experimental Western blottings were run with molecular weight markers as standards. Membranes were rinsed three times in TBS with 0.2% (v/v) Tween 20, washed and probed with goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (KPL) at a dilution of 1:10,000 in hybridization buffer. Proteins were visualized and enhanced by chemiluminescence detection using the SuperSignal chemiluminescence kit (Pierce) and hyperfilm ECL (Amersham Biosciences). The normalized density represents the relative abundance of each protein compared with the RNA polymerase α subunit as the loading control for n = 3 independent biological replicates. The RNA polymerase α subunit was detected with the anti-E. coli RNA polymerase α antibody (BioLegend). Densitometry analysis was performed on an Alphalmager HP system using the manufacturer’s supplied AlphaView software.

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