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Heme acquisition by hemophores

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Abstract Bacterial hemophores are secreted to the extracellular medium, where they scavenge heme from various hemoproteins due to their higher affinity for this compound, and return it to their specific outer membrane receptor. HasR, the outer membrane receptor of the HasA hemophore, assumes multiple functions which require various energy levels. Binding of heme and, of heme-free or heme-loaded hemophores is energy-independent. Heme transfer from the holo-hemophore to the outer membrane receptor is also energy-independent. In contrast, heme transport and hemophore release require basal or high levels of TonB and proton motive force, respectively. In addition, HasR is a component of a signaling cascade, regulating expression of the *has* operon via specific sigma and anti-sigma factors encoded by genes clustered at the *has* operon. The signal is the heme landing on HasR in the presence of the hemophore in its apo form. The *has* system is the only system thus far characterized in which the anti-sigma factor is submitted to the same signaling cascade as the target operon. Specific autoregulation of the *has* system, combined with negative regulation by the Fur protein, permits bacterial adaptation to the available iron source. In the presence of a heme-loaded hemophore, inactive anti-sigma factor is accumulated and can be activated as soon as the heme source dries up. Hence, the *has* system, instead of being submitted to amplification like other systems regulated by sigma anti-sigma factors, functions by pulses triggered by heme availability.

Keywords Heme uptake · Hemophore · Signaling cascade

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Introduction

Heme is a major iron source for bacteria, which are able to internalize it as a whole and to degrade it in the cytosol in order to retrieve iron (Wandersman and Stojiljkovic 2000). In most species, heme uptake occurs by direct recognition of exogenous heme or host hemoproteins by cell surface proteins. However, some Gram-negative bacteria secrete proteins able to acquire heme from diverse sources and to transport it to a specific outer membrane receptor (Wandersman and Delepelaire 2004). HxuA from *Haemophilus influenzae* is one type of hemophore that binds hemopexin (Hanson et al. 1992). A second hemophore system

has been identified in several bacteria, including *Serratia marcescens, Pseudomonas aeruginosa, Pseudomonas fluorescens*, *Yersinia pestis* and *Yersinia enterocolitica* (Wandersman and Delepelaire 2004). These hemophores, called HasA (for heme acquisition system), form a family of highly conserved proteins without homology to any other known proteins. Hemophores, capture free heme or extract heme from hemoglobin in the external medium and present it to specific outer membrane receptors (Ghigo et al. 1997). The *S. marcescens* HasA protein was studied in detail, and it presents certain activities which are described in the present review.

Functions of HasA

HasA is secreted by the Type I secretion pathway

To capture exogenous heme, the bacterial hemophore must reach the extracellular medium. This occurs via the type I secretion pathway (TISS) (Letoffe et al. 1994). The TISS is one of the five major secretion systems identified in Gram-negative bacteria (Delepelaire 2004). It transports widely diverse proteins across the cell envelope without periplasmic intermediates. The TISS is characterized by the presence of the ABC protein belonging to the ATP binding cassette class of proteins implicated in the vectorial movement of solutes across biological membranes, and several of which have essential functions. The TISS transporter comprises two other membrane proteins; one in the inner membrane belonging to the membrane fusion protein family (MFP) (Dinh et al. 1994) and the other in the outer membrane. The outer membrane component belongs to the TolC family (Wandersman and Delepelaire 1990). TolC homologues are characterized by a small β-barrel inserted in the outer membrane and a large protruding domain forming a so-called periplasmic channel; they are multifunctional proteins involved in drug efflux and colicin import (Wandersman and Delepelaire 1990). Genetic and biochemical studies primarily performed on the hemophore have demonstrated that TISS proteins are not permanently associated. The hemophore interacts with the ABC protein and triggers a secretion protein interaction (Letoffe et al. 1996; Thanabalu et al. 1998). This association is ordered: the ABC protein interacts with the MFP protein which, in turn, promotes TolC binding to the complex (Letoffe et al. 1996).

The hemophore, like most substrates secreted by the TISS, lacks an N-terminal signal peptide, but has an α-helical C-terminal secretion signal ending by a motive conserved in many substrates secreted by this pathway, consisting of a negatively charged residue followed by several hydrophobic residues (Delepelaire 2004). The signal interacts with the ABC protein and modulates its ATPase activity. Since the secretion signal is synthesized last, it must remain accessible on the full protein. In the case of hemophore secretion, the general chaperone SecB binds to HasA and maintains it in a secretion-competent state prior to transport (Delepelaire and Wandersman 1998). SecB slows down folding and facilitates secretion of hemophores during the course of translation (Wolff et al. 2003). When synthesis and secretion are uncoupled, the hemophore folds in the cytoplasm and becomes incompetent for secretion, a status which is not reversed by SecB overexpression (Debarbieux and Wandersman 2001).

The folded HasA inhibits secretion of newly synthesized HasA molecules (Debarbieux and Wandersman 2001). This strongly suggests that folded molecules still interact with the transporter in vivo, as already demonstrated in vitro by hemin affinity copurification of HasA with the ABC protein HasD which per se does not bind to hemin (Letoffe et al., 1996).

HasA is a heme binding protein

HasA is a monomer of 19 kD, and the crystal structure of the holo-HasA resolved at 1.9Å revealed that it is a globular protein with a fold showing two faces: 4 α-helices on one face and 7 β-sheets on the other (Arnoux et al. 1999). Iron in the heme-hemophore complex is in the oxidized form with a very low redox potential (–550 mV) (Izadi et al. 1997).

The heme pocket was identified from the crystal structure, and is highly exposed to the solvent. Amino acids histidine 32 and tyrosine 75 are the iron axial ligands in this pocket, and histidine 83 is very close to the heme pocket (Deniau et al. 2003). Site-directed mutagenesis was used to construct proteins in which these three residues, H32, Y75 and H83, were replaced by alanine. The heme binding constants were determined by absorption spectrometry and isothermal micro-calorimetry. The wild-type protein has a Kd of 10^{-11} M for heme, and each of the single alanine mutants has a lower Kd. Thus, a single axial iron ligand is sufficient to ligate heme, albeit less efficiently. The phenolate of Y75 is tightly hydrogen-bonded to the Nδ atom of H83. This hydrogen bond strengthens the bond between Y75 and iron. Disruption of this bond might play a role in the heme release mechanism. In addition, H83 may serve as an alternative iron ligand in the absence of Y75, or of both H32 and Y75 (Deniau et al. 2003). NMR analysis of the apo HasA revealed that the apo protein has the same overall structure, with wide displacement of the upper loop carrying the H32 iron ligand (Fig. 1).

Heme sources for HasA

HasA is able to capture heme from hemoglobins of diverse species (human, bovine, leg-hemoglobin, etc.) (Wandersman and Delepelaire 2004). It captures heme regardless of the heme redox status (Fe^{+++} or Fe^{++}). HasA is also able to capture heme from hemopexin and myoglobin (Wandersman and Delepelaire 2004). This wide range of substrates suggests a passive transfer of heme from the heme carrier protein to the hemophore due to its higher affinity, rather than a protein–protein interaction between the hemophore and the heme carrier protein (Letoffe et al. 2001). This hypothesis is strengthened by analytical ultracentrifugation experiments which were unable to identify a stable complex between hemoglobin and HasA (Letoffe et al. 1999).

HasA binds to its specific receptor HasR with high affinity in vivo

HasR-expressing cells have been shown to specifically bind HasA with high affinity by dot-blot. Further quantitative measurements using radiolabeled HasA and live cells have clearly established the following points:

Binding is independent of the heme-loaded status of HasA: dissociation constants of apo- and holo-HasA are around 5 nM; indeed, by using a heme pocket HasA mutant with very low heme affinity so as to avoid heme transfer between hemophores, we were able to show that both apo- and holo-HasA bound HasR at the same or at an overlapping site (Letoffe et al. 2001). Binding is also both TonB complex-, energy- and temperature-independent (Letoffe et al. 1999).

At low TonB complex concentrations, HasA impedes further heme entry via HasR, by binding

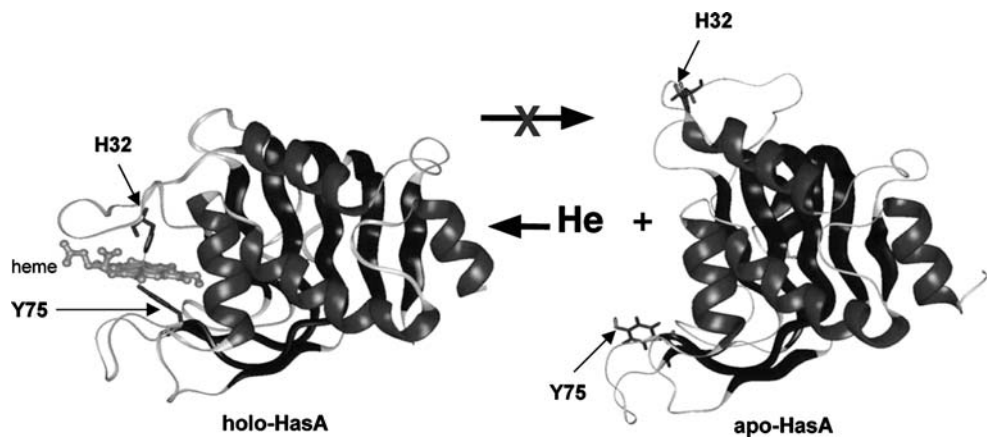

Fig. 1 Structure of holo-HasA and apo-HasA

to HasR. This observation was the basis for a selection of HasA mutants allowing heme entry via HasR at low TonB complex concentrations. Two point mutants were isolated in such a way, HasAS51P and HasAG95V (Letoffe et al. 2003). Both of them were unaffected in their heme binding properties and were still able to bind HasR, although with a somewhat greater dissociation constant (about 15 nM). At a low TonB complex concentration, they were, however, unable to inhibit heme uptake, and could still serve as a heme source for HasR. Double mutant HasAS51P, G95V was no longer able to bind HasR to a detectable extent under our conditions. To identify residues or regions of HasA potentially involved in HasA–HasR interactions, systematic pentapeptide insertion mutagenesis was undertaken. Two beta strands of HasA, 51-60 and 95-105 were identified in which pentapeptide insertion led to the same phenotype of a single HasAS51P or G95V mutant. No other position was identified in which pentapeptide insertion led to this phenotype. Finally, a peptide corresponding to the 51-60 region was able to interact with HasR, as detected by its ability to make wild-type HasA behave like a single mutant and to abrogate the interaction of HasAG95V, but not HasAS51P, with HasR. We therefore concluded that the HasA–HasR interaction is mediated by two independent beta strands, most likely making contact with two distinct regions on the extracellular part of the receptor (Letoffe et al. 2003).

Heme transfer from HasA to HasR is driven by protein–protein interactions

The presence of the hemophore greatly enhances the efficiency of heme uptake by HasR, but requires higher amounts of the TonB complex and of pmf (see below). Given the very high affinity of HasA for heme and the much lower affinity of HasR for heme, we sought to determine whether heme transfer from HasA to HasR is TonB-complex-dependent or not. The same question would apply to other receptors using host proteins as iron source, as is the case for transferrin/lactoferrin receptors, hemopexin and hemoglobin/haptoglobin receptors. To study this question, we used an in vitro approach. The HasR receptor was purified and shown to be functional, both in terms of heme binding ($Ka = 5 \times 10^6\ M^{-1}$, with a 1:1 stoichiometry) and HasA binding (over $10^9\ M^{-1}$ for apoHasA, $2.5 \times 10^8\ M^{-1}$ for holo-HasA, with a 1:1 stoichiometry in both cases). The heme environment was assessed by both UV-visible absorption spectroscopy and resonance Raman spectroscopy, which is a very sensitive probe of the local environment. We were able to show that heme is in the same environment in holo-HasR and in the complex formed by the holo-HasA–apo-HasR interaction; this environment is clearly distinct from that found in holo-HasA (Izadi-Pruneyre et al. 2006). We concluded that, in vitro, upon complex formation, the protein–protein interaction drives heme transfer from HasA to HasR. Binding of the hemophore might distort it in such a way that the hydrogen bound between Y75 and H83 is disrupted, thus weakening Y75 binding to iron of heme (Caillet-Saguy et al. 2006). Several other lines of evidence also strongly support this conclusion. HasR, like many other heme receptors, has two conserved histidine residues, one in the plug, the other in the barrel, most likely close to each other and exposed to the medium. The first characterized mutations of those residues in HemR from *Y. enterocolitica* abrogated heme transport (Bracken et al. 1999). A mutation of both conserved histidine residues in HasR also completely abrogated heme transport and heme binding, but only marginally affected hemophore binding. The complex formed upon the holo-HasA interaction with this mutant did not display any spectral change compared to holo-HasA. Likewise, in systematic pentapeptide HasA mutagenesis, a mutant was isolated which has an insertion close to the heme binding pocket (HasA76t), active both in heme and receptor binding. This mutant is unable to serve as a heme source for HasR, and the stoichiometric complex formed following the holo-HasA76t–HasR interaction displays the same spectral characteristics as holo-HasA76t. We thus concluded that heme transfer from its binding site in HasA to its binding site in HasR occurs in vitro; it is driven by the protein–protein interaction and is critically dependent

upon the two conserved histidine residues. Those two conserved histidine residues are also implicated in heme/iron liganding in HasR (Izadi-Pruneyre et al. 2006).

Hemophore recycling is energy-dependent

HasR-mediated heme entry into the cell is TonB-complex-dependent, whether taken up as free heme or as HasA-bound heme. In the absence of an energy source, and at 4 or 30°C, HasA which is bound to the receptor either in its apo or holo form cannot be displaced by an excess of hemophore (Letoffe et al. 2001). This led us to propose that heme might be exchanged from free hemophores to receptor-bound hemophores in order to ensure heme entry into the cell.

It was also conceivable that hemophore was exchanged at the receptor to ensure sustained heme entry. To study this, a radiolabeled hemophore was used and its release from HasR-expressing cells measured under different conditions. We were able to show that hemophore release from the receptor only occurs in the presence of energy, high TonB-complex concentrations and heme. Comparatively more energy and TonB complexes are required for HasA-mediated HasR functioning (Letoffe et al. 2004). Thus, the TonB complex acts at both the heme entry step and apo-HasA recycling in the extracellular medium. It is thus likely that HasA-mediated heme entry per se requires more energy than free heme entry, and that both steps (heme entry and HasA ejection) are tightly linked (Fig. 2).

The HasR barrel constitutes a heme-specific pore and contains the HasA binding site

The HasR barrel can be stably expressed in cells where it is incorporated into the outer membrane. The barrel maintains several functionalities of full-length HasR: it is still able to bind HasA both in whole cells, although with reduced affinity, and in vitro; it is still able to associate with the plug expressed separately (without reconstituting a functional receptor); it constitutes a specific low-affinity heme ''porin'', the function of which is abrogated by mutation of the conserved barrel histidine (Letoffe et al. 2005).

Hemophore-dependent signal transduction

The has *signaling cascade*

Bacterial iron homeostasis is mainly achieved by tightly regulated iron uptake systems that are negatively controlled by the iron-loaded Fur repressor (McHugh et al. 2003). However, some iron acquisition systems including ferric dicitrate, siderophores, heme and hemophore are also positively regulated by their specific substrates (Braun et al. 2003). Binding of the iron source to its cognate receptor triggers a signaling cascade which leads to the activation of a specific sigma factor belonging to the ECF family, involved in the perception of extracytoplasmic stimuli.

The genes of the Has system are organized into an operon (Ghigo et al. 1997) encoding HasR (the hemophore-specific outer membrane receptor), HasA (the hemophore), HasD and HasE

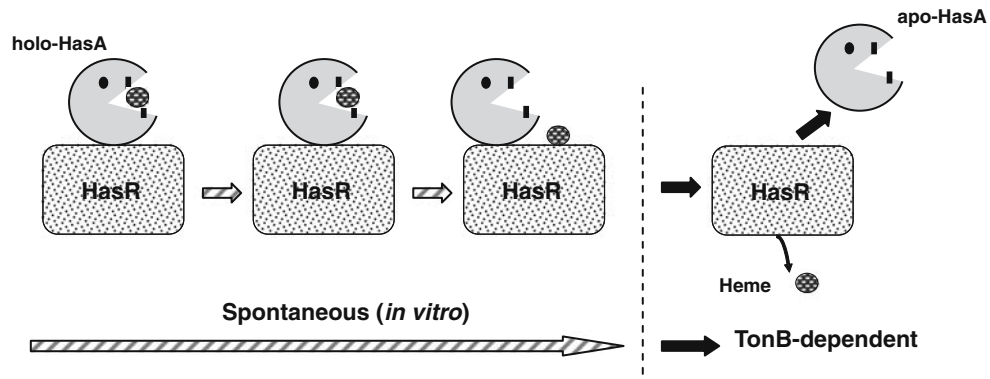

Fig. 2 TonB requirement of the hemophore-dependent heme uptake process steps

(the specific inner membrane hemophore secretion proteins). HasD and HasE, together with the outer membrane component (TolC or HasF) encoded by an unlinked gene, constitute the HasA secretion apparatus for extracellular release of the hemophore. The last gene of the *has* operon, *hasB*, encodes a TonB homologue specializing in heme uptake via the Has system (Paquelin et al. 2001). Consistent with most of the other bacterial iron/heme acquisition systems, a Fur box is located upstream from *hasR*, thus suggesting regulation of *hasRADEB* operon expression by the iron-loaded Fur repressor. Two genes located upstream from *hasR* are homologous to specific sigma and sigma-modulator factors. These genes, called *hasI* and *hasS*, (Rossi et al. 2003), share significant homologies with the *Escherichia coli fecI* and *fecR* genes and the *Pseudomonas putida pupI* and *pupR* genes which encode ECF sigma factors and their modulators, respectively (Koster et al. 1994; Harle et al. 1995). The deduced HasI protein is a 166 amino acids molecule with a calculated molecular weight of 18.957 kD. The HasI sequence exhibits 30.3% and 25.7% identity with the FecI and PupI proteins, respectively. The deduced HasS protein is a 317 amino acids molecule with a calculated molecular weight of 34.719 kD. It exhibits 23.9% and 27.4% identity with the FecR and PupR proteins, respectively. The hydropathic profile of HasS suggests the existence of one transmembrane segment, as is the case for FecR and PupR (Rossi et al. 2003). A typical Fur-Fe (II) DNA binding site (Fur box) is located 45 bp upstream from the *hasI* start codon, suggesting that it is a Fur-regulated gene. Thus, according to its sequence, its neighborhood genes and regulatory sequences, expression of the *has* operon can be repressed by the iron-loaded Fur repressor and can also be induced by a substrate binding the outer membrane receptor HasR. Regulation of *has* operon expression was investigated both in *Serratia marcescens* and in *Escherichia coli*. In *S. marcescens* we first introduced, in the chromosome of a control strain and its *hasA*, *hasI*, *hasR*, *hasS* derivatives, a *hasR–lacZ* transcriptional fusion and then assayed its expression in iron-deplete or iron-replete medium, and in the absence or in the presence of heme. To study *hasR* regulation in the system reconstituted in *E. coli*, *hasR–lacZ* transcriptional fusion was inserted into the chromosome of strain POP3 (POP3 *att λ::hasR–lacZ*) and its isogenic *fur* mutant (POP3 *fur::Cm attλ::hasR–lacZ*). These two strains expressed *β*-galactosidase activity at a basal level. They were transformed with a plasmid containing either *hasI* (pAM*rbs*HasI), *hasI* and *hasS* (pAM *rbs*HasIS) or *hasI*, *hasS*, and *hasR* (pAM*rbs*HasISR). In *S. marcescens*, as in *E. coli*, the *hasR* promoter was (i) sensitive to Fur-mediated iron repression, (ii) activated by HasI, and (iii) repressed by HasS (Rossi et al. 2001; Biville et al. 2004).

Hemophore-dependent initiation of the has *signaling cascade*

At least three different molecules can bind to HasR: apo-hemophore, holo-hemophore, and heme. To determine the nature of the inducer, we compared the inducing abilities of these compounds on the *has* signaling cascade both in *S. marcescens* and in *E. coli*. In *S. marcescens*, disruption of *hasA* abolished induction of the *has* signaling cascade evidenced when bacteria were grown in the presence of hemoglobin in an iron-depleted medium. In the *S. marcescens hasA* mutant, as in *E. coli* expressing *hasR*, *hasI*, and *hasS*, addition of free or hemoglobin-bound heme had no effect on *hasR–lacZ* expression (Rossi et al. 2001; Biville et al. 2004). This result indicates that, although transported and provided at a growth-promoting concentration, heme is not an inducer of the *has* operon. Also, addition of a heme-free HasA did not induce the system. In contrast, addition of heme-loaded HasA induced the *has* signaling cascade and increased 10-fold the expression of *hasR–lacZ* fusion (Rossi et al. 2001; Biville et al. 2004). Thus, the *has* signaling cascade can exist in two states: the sleeping state and the working state in the presence of the heme-loaded hemophore (Fig. 3).

Role of TonB, ExbB and HasB in heme-loaded hemophore-mediated hasR induction

In surface signaling systems, the dual receptor function of sensor and transporter requires the

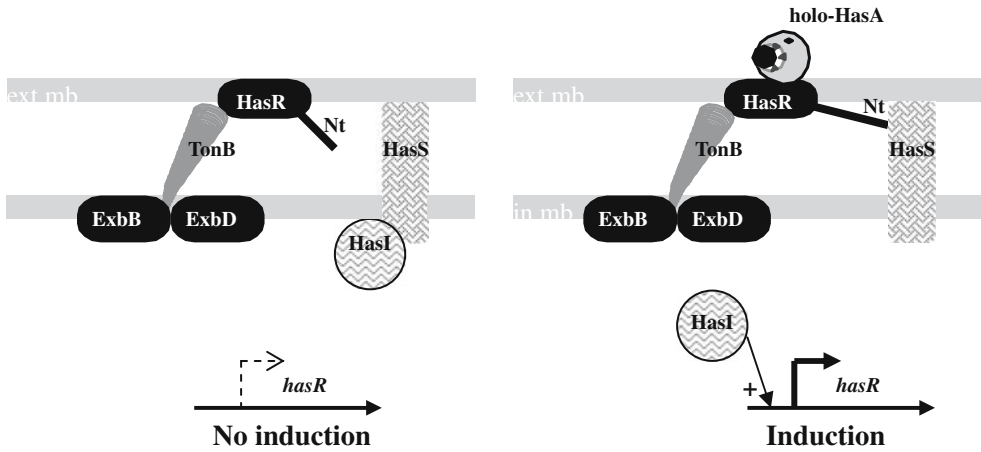

Fig. 3 The *has* signaling cascade

inner membrane multiprotein complex TonB–ExbB–ExbD (Braun et al. 2003). Heme transport via the Has system requires either TonB or HasB, the TonB homolog specialized in heme uptake and encoded by the *has* operon. In *S. marcescens*, HasB is not strictly required for induction of the *has* signaling cascade, probably because HasB and TonB can replace each other for this function (Paquelin et al. 2001). In *E. coli* expressing *hasR*, *hasI*, and *hasS*, disruption of *tonB* or *exbB* abolished heme-loaded hemophore-mediated *hasR* induction (Biville et al. 2004). Thus, a functional TonB–ExbB–ExbD complex was required for hemophore-mediated signal transduction. The defect of a *tonB* mutant in inducing the *has* signaling cascade in *E. coli* can be complemented by a plasmid containing either TonB or HasB6, a *hasB* mutant affected in its transmembrane domain and functional in *E. coli* for heme uptake (Paquelin et al. 2001)

The HasR N-terminal extension is required for hasR induction

HasR, like the other characterized autoregulated receptors, has an N-terminal extension located upstream from the putative TonB box. This extension was shown in FecA to be necessary for signal transduction, but not for ferric dicitrate transport through FecA (Kim et al. 1997). To test the involvement of this extension in *hasR* induction, a Δ 45–125 deletion of the unprocessed HasR was constructed, leaving the entire signal peptide. The absence of the N-terminal extension abolished heme-loaded hemophore-mediated *hasR* induction but not heme transport (Biville et al. 2004)

hasI is iron-regulated and not autoregulated

Iron-starvation ECF sigma factors, described previously, are repressed by the iron-loaded Fur protein, but are not autoregulated. The presence of a putative Fur binding site at positions –20 to –2 with respect to the *hasI* transcriptional start point and the assay of expression of a *hasI-lacZ* fusion in fur^+ and fur^- isogenic backgrounds have evidenced Fur-dependent iron repression for *hasI* (Biville et al. 2004). Expression of a *hasI-lacZ* fusion does not differ significantly according to the presence of *hasI*, *hasIS*, or *hasISR*. Therefore, HasI does not regulate its own expression (Biville et al. 2004).

hasS is subject to the signaling cascade mediated by holo-HasA

Using primer extension analysis, we identified two different transcriptional starts for the *hasI* and *hasS* genes. The *hasS* transcriptional start point is not preceded by any Fur box consensus sequence but contains a sequence, also found upstream from the *has* operon and characterized as a target of HasI. A

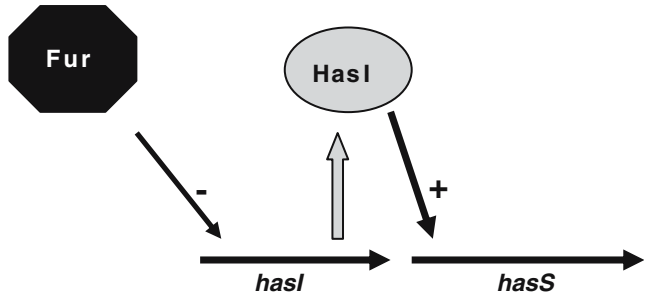

Fig. 4 Regulation of *hasI* and *hasS* expression

mutation in this ''HasI box'' significantly decreases or abolishes induction of *hasS-lacZ* fusion expression by HasI (Biville et al. 2004) (Fig. 4). Also, *hasS* expression is subject to the TonB-dependent signaling cascade induced by binding of holo-HasA to HasR (Biville et al. 2004).

Hemophore regions required for hasR *induction*

The interaction between HasA (heme-free or heme-loaded) and HasR involves two regions of the hemophore: those located between positions 51 and 60 and those situated between positions 95 and 105 (Letoffe et al. 2003). With respect to induction of the *has* signaling cascade, the status of the two binding regions was quite different. Holo-HasA G95V strongly induced the *has* signaling cascade, which was only slightly induced by holo-HasA S51P (Cwerman et al. 2006). Pentapeptide insertions at other locations in HasA had only a marginal effect on induction of the *has* signaling cascade (Cwerman et al. 2006). The failure of the HasA S51P mutant to induce *hasR–lacZ* suggested that the region between residues 51 and 60 in HasA is important for initiation of *has* signaling. We studied a collection of mutants obtained by insertion of a pentapeptide into this

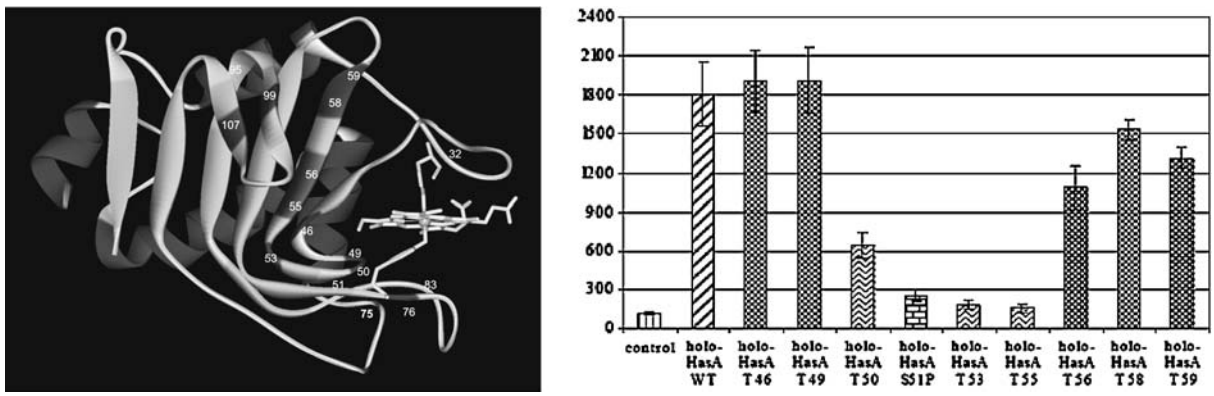

Fig. 5 Residues of the 50-60 HasA binding region required for initiation of the *has* signaling cascade

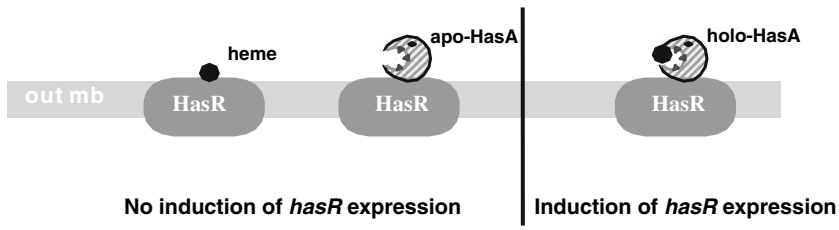

Fig. 6 Induction activity of the three HasR ligands

region for precise identification of the residues important for holo-HasA-dependent *hasR–lacZ* induction. Pentapeptide scanning analysis implicated residues 50–55 of the 51-60 β-strand in the initiation of *has* signaling (Fig. 5).

Heme transfer and has *signaling cascade induction*

The *has* signaling cascade is initiated by the binding of a heme-loaded hemophore to HasR, its cognate receptor, leading to specific sigma factor activation. HasR has a number of ligands, heme, apo-hemophore and holo-hemophore, but only holo-hemophore initiates the signaling cascade (Fig. 6). This raises questions concerning the nature of the inducing stimulus. This stimulus may be a particular holo-HasA conformation. Indeed, the three-dimensional (3D) structures of holo-HasA and apo-HasA indicate that heme binding to HasA substantially modifies the conformation of the heme pocket, without changing the α-β fold of the protein (Arnoux et al. 1999) (PDB accession number PDB ID 1YBJ). Alternatively, induction may result from the extraction of heme from HasA, heme landing on HasR or empty hemophore recycling.

Heme transfer from HasA to HasR is required for induction of the has *signaling cascade*

(*a*) *Study of a HasA mutant affected in heme release.* One hemophore mutant, HasA76T, which had conformations similar to those of the wild-type hemophore, was affected in heme release, since it was unable to supply heme in vivo (cells expressing HasR) or in vitro (heme transfer from holo-HasA to apo-HasR). The heme-loaded form of HasA 76T was shown to be unable to induce the *has* signaling cascade.
(*b*) *HasR mutant study* A HasR mutant carrying mutations H189A and H603A (H1H2), that had no affinity for heme, was unable to use free or hemophore-bound heme in vivo or in vitro (Izadi-Pruneyre et al. 2006). Addition of the wild type holo-hemophore to a strain expressing the H1H2 HasR mutant did not induce the *has* signaling cascade.

With both types of mutants, holo-HasA-mediated *has* signaling cascade was abolished. Thus, no particular holo-HasA conformation acted as the stimulus for induction. An interaction between heme and the receptor is necessary for induction. However, no induction was observed with heme alone, suggesting that induction requires either heme transfer from the hemophore to HasR or the concomitant presence of heme and apo-HasA on the receptor.

Induction of the *has* signaling cascade by non-interacting heme and HasA

The addition of heme, together with a HasA mutant unable to bind heme (HasA H32AY75A-H83A), led to full induction of the *has* signaling cascade (Cwerman et al. 2006). This demonstrates that a direct interaction between heme and the hemophore is not required for triggering induction. HasA H32AY75AH83A binding to HasR does not prevent heme from reaching HasR. Purified heme-free HasA H32AY75AH83A–HasR complexes bind heme in vitro, confirming that heme can reach a hemophore-loaded receptor (N. Izadi, *personal communication*). In contrast, the concomitant addition of heme and the heme-loaded form of the HasA HasA76t does not induce the *has* signaling cascade even if heme is added prior to the holo-hemophore. The latter result suggests that only the apo form of the hemophore can induce the *has* signaling cascade in the presence of heme. The return to the apo form structure of HasA after the release of heme from the hemophore is required to initiate the *has* signaling cascade when heme lands on HasR.

HasA H32AY75AH83A binding to the receptor blocks free heme uptake (Letoffe et al. 2004). HasA H32AY75AH83A may remain on the receptor, blocking heme transport but not heme binding. The mechanism by which heme transport is inhibited remains unclear. It is possible that the wild-type hemophore dissociates from the receptor after its heme is transferred, whereas no such dissociation is observed with a mixture of heme and the H32AY75AH83A mutant hemophore. Thus, hemophore recycling may require heme

transfer from the hemophore to the receptor. Hemophore recycling was shown to be the only step in hemophore-dependent heme uptake that required high TonB levels (Letoffe et al. 2004). Since induction of the *has* signaling cascade seems to be independent of hemophore recycling, it is suggested that a basal TonB level, also required for heme transport, may also be sufficient for induction of the *has* signaling cascade.

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