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Bacillus subtilis Fur represses one of two paralogous haem-degrading monooxygenases

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Identification of genes regulated by the ferric uptake regulator (Fur) protein has provided insights into the diverse mechanisms of adaptation to iron limitation. In the soil bacterium *Bacillus subtilis*, Fur senses iron sufficiency and represses genes that enable iron uptake, including biosynthetic and transport genes for the siderophore bacillibactin and uptake systems for siderophores produced by other organisms. We here demonstrate that Fur regulates *hmoA* (formerly *yetG*), which encodes a haem monooxygenase. HmoA is the first characterized member of a divergent group of putative monooxygenases that cluster separately from the well-characterized IsdG family. *B. subtilis* also encodes an IsdG family protein designated HmoB (formerly YhgC). Unlike *hmoA*, *hmoB* is constitutively expressed and not under Fur control. HmoA and HmoB both bind haemin *in vitro* with approximately 1 : 1 stoichiometry and degrade haemin in the presence of an electron donor. Mutational and spectroscopic analyses indicate that HmoA and HmoB have distinct active site architectures and interact differently with haem. We further show that *B. subtilis* can use haem as an iron source, but that this ability is independent of HmoA and HmoB.

INTRODUCTION

Almost all bacteria require iron for growth. In most aerobic environments, however, iron availability is limited by the formation of highly insoluble hydroxides. To overcome this scarcity, bacteria have developed several mechanisms for iron acquisition (Andrews *et al.*, 2003). Many bacteria secrete low-molecular-mass iron chelators called siderophores that have a high affinity for ferric iron. Iron–siderophore complexes are transported through high-affinity uptake systems to the cytoplasm, where iron is removed from the complex either by reduction or by cleavage of the ferri–siderophore complex using an esterase.

The ability of bacteria to use haem as an iron source has been best studied in human pathogens (Skaar & Schneewind, 2004). These organisms face a significant challenge, since almost all iron in human tissues is tightly sequestered by haemoproteins and by high-affinity iron binding proteins such as transferrin and lactoferrin. Indeed, the withholding of iron from invading organisms is an important component of the innate immune system (Cornelissen, 2003). Bacterial pathogens have developed elaborate systems to acquire iron from the host, including expression of high-affinity siderophores, transferrin receptors, and proteins implicated in haem and haemoprotein utilization. Although best documented for pathogens, several free-living *Rhizobium* species can also acquire iron from haem (Noya *et al.*, 1997). In *Bradyrhizobium japonicum*, haem uptake genes have been characterized (Nienaber *et al.*, 2001) and two haem-degrading monooxygenases have been identified (Puri & O’Brian, 2006).

The soil bacterium *Bacillus subtilis* serves as a model organism for the investigation of iron homeostasis (Baichoo *et al.*, 2002; Moore & Helmann, 2005). *B. subtilis* has several routes to acquire iron including synthesis and import of the high-affinity siderophore bacillibactin (BB) (May *et al.*, 2001), although laboratory strains (*B. subtilis* 168 and its derivatives) fail to synthesize the intact siderophore due to a mutation in the gene encoding the phosphopantetheinyltransferase Sfp. Upon binding iron, the Fe–BB complex binds with high affinity to the substrate binding protein FeuA (Miethke *et al.*, 2006), and is transported through the ATP binding cassette (ABC) transport system FeuBC in conjunction with the YusV ATPase (Ollinger *et al.*, 2006). In addition to its own siderophore, *B. subtilis* pirates other siderophores through expression of specific uptake systems, efficiently utilizes ferric citrate, and has an oxidase-dependent elemental iron transport (OfeT-type) system (Grosse *et al.*, 2006; Ollinger *et al.*, 2006). It has been shown that haemin can rescue the growth of a *B. subtilis hemA* mutant, which is unable to synthesize haem (Miczák, 1977). Moreover, exogenously added haemin can restore cytochrome *c* function to *ccdA* mutant cells (Schiött *et al.*, 1997). To our knowledge, however, it has never been shown that *B. subtilis* can use haem as an iron source.

Abbreviations: EDDHA, ethylenediamine-*N*,*N′*-bis(2-hydroxyphenylacetic acid); EMSA, electrophoretic mobility shift assay.

A supplementary figure, showing percentage similarities and identities among putative haem monooxygenases, and a supplementary table, listing oligonucleotides used in this study, are available with the online version of this paper.

Here we demonstrate that *B. subtilis* can utilize haem as an iron source and that its genome encodes two haem monooxygenase homologues (HmoA and HmoB). HmoB belongs to the well-characterized IsdG family and is not regulated by iron, while the Fur-regulated HmoA protein represents a previously uncharacterized subgroup of monooxygenases that is also found in several pathogenic bacteria. Although both HmoA and HmoB are haem binding proteins, they are not required for iron utilization from added haem and their *in vivo* role remains to be resolved.

METHODS

Strain construction and growth conditions. All *B. subtilis* strains used in this study are derivatives of CU1065 (*trpC2 attSPβ sfp*^{0}) and are listed in Table 1. For selection, antibiotics were added at the following concentrations: erythromycin (1 μg ml^{-1}) and lincomycin (25 μg ml^{-1}) [for selecting for macrolide–lincosamide–streptogramin B (MLS) resistance], spectinomycin (100 μg ml^{-1}), chloramphenicol (10 μg ml^{-1}), kanamycin (15 μg ml^{-1}), tetracycline (5 μg ml^{-1}) and neomycin (10 μg ml^{-1}). Growth curves were determined using a Bioscreen C MBR system with OD_{600} measurements every 10 min for 24 h.

Routine molecular biology procedures were carried out using *Escherichia coli* DH5α for DNA cloning as described by Sambrook & Russell (2001). Isolation of *B. subtilis* chromosomal DNA, transformation and specialized SPβ transduction were performed as described by Cutting & VanderHorn (1990). Restriction enzymes, DNA ligase and DNA polymerases were used according to the manufacturer's instructions (New England Biolabs). Primers used are listed in Supplementary Table S1.

B. subtilis was grown in Luria–Bertani (LB) medium or in a MOPS-based iron starvation minimal medium (Chen *et al.*, 1993). Metals were added from filter-sterilized stocks before inoculation. Haemin was dissolved in 0.1 M NaOH and neutralized by adding an equal volume of 1.0 M Tris buffer (pH 7.5) (Tullius *et al.*, 2011). Haemin solutions were treated overnight with 5 % (w/v) Chelex-100 to remove contaminating iron and filter-sterilized.

Mutants were constructed using long-flanking-homology PCR (LFH-PCR) as described previously (Butcher & Helmann, 2006). To construct *cat–lacZ* transcriptional fusions, regulatory regions were amplified from genomic DNA by PCR and cloned as *Hin*dIII–*Bam*HI fragments into pJPM122 (Slack *et al.*, 1993). The resulting constructs were linearized with *Sca*I and transformed into ZB307A (Zuber & Losick, 1987) selecting for neomycin resistance. A SPβ-transducing lysate was prepared by heat induction and used to transduce different strains as indicated (Slack *et al.*, 1993). β-Galactosidase activity was assayed using a modification of the procedure of Miller (1972), as described by Bsat *et al.* (1998).

DNA binding assays. Fur was purified as described previously (Bsat & Helmann, 1999). The *hmoA* promoter region was amplified from *B. subtilis* chromosomal DNA by PCR using a [γ-^{32}P]ATP-labelled primer, and electrophoretic mobility shift assays (EMSAs) were performed using DNA (<100 pM) essentially as described previously (Baichoo & Helmann, 2002).

Expression and purification of HmoA and HmoB. The *hmoA* and *hmoB* coding sequences were amplified from the *B. subtilis* 168 genome using PCR, cloned into pET16B (Novagen) and used to transform *E. coli* BL21(DE3) pLysS (Novagen). For overexpression, cells were grown in LB with appropriate antibiotics at 37 °C to OD_{600} 0.4. After addition of 1 mM IPTG, cells were further incubated for 2 h and harvested by centrifugation. Purification of His-tagged proteins was done using PrepEase His-tagged protein purification resin (USB) according to the manufacturer's instructions. Purified proteins were dialysed against 50 mM Tris/HCl (pH 8), 150 mM NaCl, 5 % (v/v) glycerol and stored at −20 °C.

Haemin binding to HmoA and HmoB. The binding of haemin to HmoA and HmoB was monitored spectrophotometrically in 50 mM Tris/HCl (pH 8) and 150 mM NaCl, as described by Skaar *et al.*

Table 1. Strains used in the study

Strain	Genotype	Source or reference
CU1065	*trpC2 attSPβ sfp*^{0}	Laboratory stock
HB2501	CU1065 *fur*::*kan*	Fuangthong & Helmann (2003)
HB8402	CU1065 *hmoA*::*spc*	This study
HB8404	CU1065 SPβc2Δ2::Tn*917*::ϕ (*hmoA-cat-lacZ*)	This study
HB8405	HB2501 SPβc2Δ2::Tn*917*::ϕ (*hmoA-cat-lacZ*)	This study
HB8412	CU1065 *hmoB*::*tet*	This study
HB8413	CU1065 *hmoA*::*spc hmoB*::*tet*	This study
HB8419	CU1065 SPβc2Δ2::Tn*917*::ϕ (*hmoB-cat-lacZ*)	This study
HB8420	HB2501 SPβc2Δ2::Tn*917*::ϕ (*hmoB-cat-lacZ*)	This study
HB11078	CU1065 *ywbLMN*::*kan*	This study
HB11080	CU1065 *hmoA*::*spc ywbLMN*::*kan*	This study
HB11081	CU1065 *hmoB*::*tet ywbLMN*::*kan*	This study
HB11082	CU1065 *hmoA*::*spc hmoB*::*tet ywbLMN*::*kan*	This study
HE8253	*E. coli* BL21(DE3) pLysS with *hmoA* in pET16b	This study
HE8255	*E. coli* BL21(DE3) pLysS with *hmoB* in pET16b	This study
HE8261	*E. coli* BL21(DE3) pLysS with *hmoA* R6A in pET16b	This study
HE8262	*E. coli* BL21(DE3) pLysS with *hmoA* R6N in pET16b	This study

(2006). Haemin was added to wild-type and mutant proteins in a 4 : 1 molar ratio. The resulting proteins were repurified using PrepEase His-tagged protein purification resin and dialysed against 50 mM Tris/HCl (pH 8), 150 mM NaCl, 5 % (v/v) glycerol. Some precipitate was observed after dialysis, which was removed by centrifugation.

Haemin degradation assay. Haemin degradation was assayed using ascorbate as reductant in 50 mM Tris/HCl (pH 8) and 150 mM NaCl buffer. Ascorbic acid-dependent degradation of haem bound to HmoA or HmoB was monitored in the presence and absence of catalase, as described by Skaar *et al.* (2006). Site-directed mutagenesis of *hmoA* and *hmoB* was done using PCR and overlap extension (Ho *et al.*, 1989). Different concentrations were used for each protein, since some precipitation occurred during dialysis of the haemin-bound proteins and the resulting concentrations were remeasured after removing the precipitates.

RESULTS AND DISCUSSION

Haem serves as an iron source for *B. subtilis*

To test whether *B. subtilis* can use haemin as an iron source, the wild-type strain CU1065 (which is *sfp*^{0} and cannot produce bacillibactin) was grown in iron-limiting minimal medium containing the strong iron chelator ethylenediamine-*N*,*N*′-bis(2-hydroxyphenylacetic acid) (EDDHA) (Fig. 1). Whereas 1 μM EDDHA caused an almost 50 % reduction in cell density, 2 and 4 μM EDDHA completely abolished growth. As shown previously (Ollinger *et al.*, 2006), growth inhibition by EDDHA can be overcome by addition of siderophores with a higher affinity for iron than EDDHA (e.g. bacillibactin; Ollinger *et al.*, 2006). The addition of 1 μM haemin restored growth to normal levels even in the presence of 4 μM EDDHA (Fig. 1). Thus, haemin provides a form of iron that can be utilized by *B. subtilis* in the presence of the strong Fe(III) chelator EDDHA, which binds ferric iron with an affinity similar to many hydroxamate siderophores (Ollinger *et al.*, 2006; Wong *et al.*, 1983).

At least two distinct pathways for the utilization of haem-bound iron have been described (Anzaldi & Skaar, 2010; Létoffé *et al.*, 2009). In many organisms, haem is transported into the cell, where cleavage of the porphyrin ring by haem monooxygenases facilitates iron release. An alternative pathway in *E. coli* has been described in which iron is removed from surface-bound haemin and imported by the EfeUOB system (Létoffé *et al.*, 2009). The *E. coli* EfeUOB system was originally described as an acid-induced, low-pH Fe(II) uptake pathway (Cao *et al.*, 2007; Grosse *et al.*, 2006) homologous to the *B. subtilis* YwbLMN elemental iron uptake system (Ollinger *et al.*, 2006). These elemental iron uptake systems share similarities with the *Saccharomyces cerevisiase* Ftr1p/Fet3p copper oxidase-dependent iron uptake pathway. We hypothesized that the YwbLMN (EfeUOB-type) pathway might be responsible for haem utilization in *B. subtilis.* However, a *ywbL* : : *kan* mutant grew as well as the wild-type strain using haemin as an iron source (in the presence of EDDHA), suggesting that *B. subtilis* may instead (or additionally) use a haem monooxygenase-dependent pathway.

Identification of two candidate haem monooxygenases in *B. subtilis*

The *B. subtilis* genome encodes two candidate haem monooxygenases (YetG and YhgC) with similarity to *Staphylococcus aureus* IsdG (Skaar *et al.*, 2004) (see Supplementary Fig. S1). Based on the results presented herein, we rename YetG as HmoA and YhgC as HmoB. Neither the *hmoA* nor the *hmoB* gene had been previously linked to iron homeostasis. HmoA and HmoB are annotated in the *B. subtilis* genome as a putative monooxygenase and conserved hypothetical protein, respectively.

Comparison of HmoA with IsdG and other homologous proteins suggests that HmoA starts with a methionine

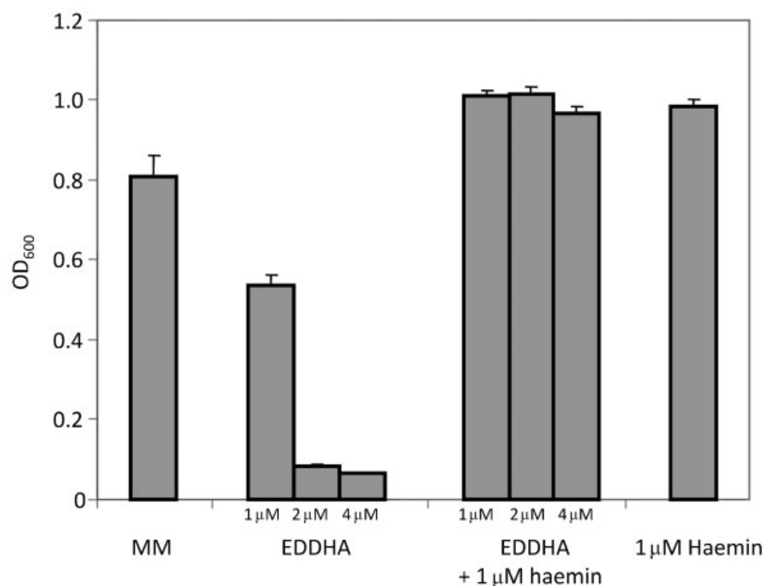

Fig. 1. *B. subtilis* uses haemin as an iron source. Cells were grown in minimal medium supplemented with 1, 2 or 4 μM EDDHA in the absence or presence of 1 μM haemin overnight at 37 °C. The results are the mean and SD of at least three independent replicates.

corresponding to position 18 within the originally annotated protein sequence. This reassignment reveals a candidate σ^A-type promoter and overlapping Fur box (Fig. 2a). Analysis of transcripts from this region using 5′-random amplification of cDNA ends (RACE) confirms that this promoter drives expression of *hmoA*: the dominant cDNA product detected corresponded to initiation at an adenine residue 26 nt upstream of the reassigned ATG start codon. HmoA is 33 % identical (61 % similar) to *S. aureus* IsdG (Supplementary Fig. S1).

Unlike *hmoA*, the *hmoB* gene is not associated with an apparent Fur box. Sequence analyses and tiling array transcriptome datasets (Rasmussen *et al.*, 2009) suggest that *hmoB* is constitutively expressed as a monocistronic transcript initiated from a σ^A-type promoter element (Fig. 2c). HmoB is 25 % identical (42 % similar) to HmoA and 33 % identical (61 % similar) to *S. aureus* IsdG (Supplementary Fig. S1).

Expression of HmoA is regulated by Fur and iron

Genes involved in iron uptake are frequently regulated by the ferric uptake repressor Fur (Bsat *et al.*, 1998; Lee & Helmann, 2007). Under iron sufficiency, Fur binds to inverted repeat sequences in the operator regions of iron-regulated genes (Bsat *et al.*, 1998; Bsat & Helmann, 1999). The Fur regulon has been extensively characterized using transcriptome analysis (Baichoo *et al.*, 2002), and the Fur binding site defined by mutational and DNA binding studies (Baichoo & Helmann, 2002).

The presence of a candidate Fur box upstream of the reannotated *hmoA* (*yetG*) coding sequence (Fig. 2a) suggests that HmoA is a member of the Fur regulon. Indeed, analysis of previous microarray data showed that *hmoA* is derepressed sixfold in a *fur* mutant and induced 8.5-fold under conditions of iron deficiency in the presence of dipyridyl (Baichoo *et al.*, 2002). However, in this previous study the *hmoA* gene was not assigned as a direct target of Fur regulation due, in retrospect, to the misannotation of the gene and the resulting lack of an associated upstream Fur box.

To further investigate the regulation of the *hmoA* gene, we cloned the putative promoter and regulatory region to generate an ectopically integrated reporter fusion (carried within the SPβ prophage). This *hmoA–cat–lacZ* transcriptional fusion was highly expressed in iron starvation minimal medium and was fully derepressed in a *fur* null mutant (Fig. 3a). While *hmoA* expression was repressed by addition of 100 μM iron, it was not affected by addition of 1 μM haemin (Fig. 3a). In an EMSA, purified Fur bound with high affinity [dissociation constant (K_d) <20 nM] to the *hmoA* regulatory region (Fig. 3b). Together with the previous transcriptome data (Baichoo *et al.*, 2002), these results demonstrate that *hmoA* is a member of the Fur regulon. Unlike *hmoA*, *hmoB* was constitutively expressed and not regulated by Fur or iron. Addition of haem did not

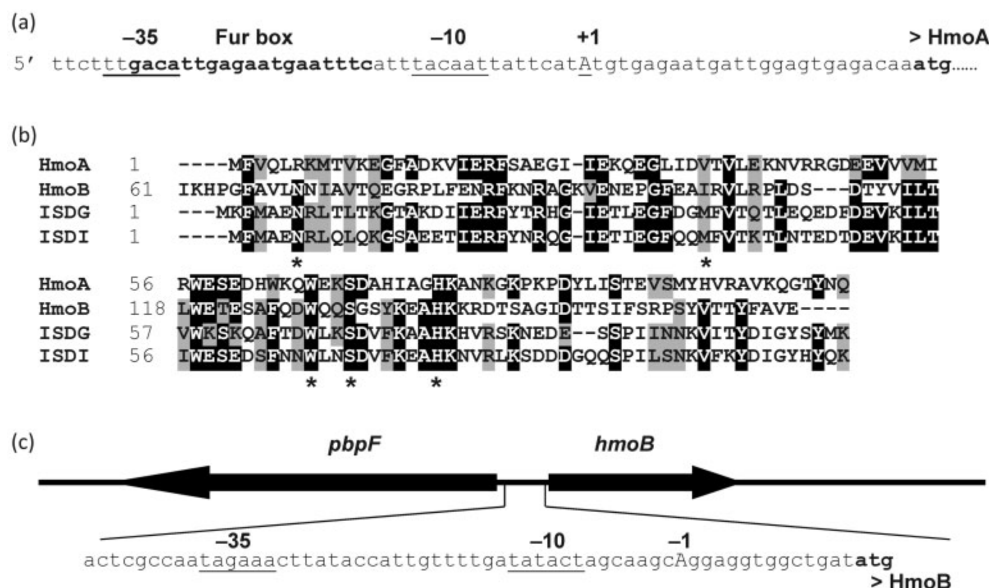

Fig. 2. HmoA and HmoB regulatory region of the haem monooxygenases. (a) Operator region of the *hmoA* gene with −35 and −10 promoter elements, and the experimentally determined +1 start site indicated by underlined upper-case type. The Fur box is indicated by bold type. (b) Amino acid sequence alignment of HmoA and HmoB from *B. subtilis* with IsdG and IsdI from *S. aureus.* Residues suggested to bind haem are indicated by asterisks. (c) Organization of the *pbpF–hmoB* intergenic region, showing the putative divergent promoter for *hmoB*.

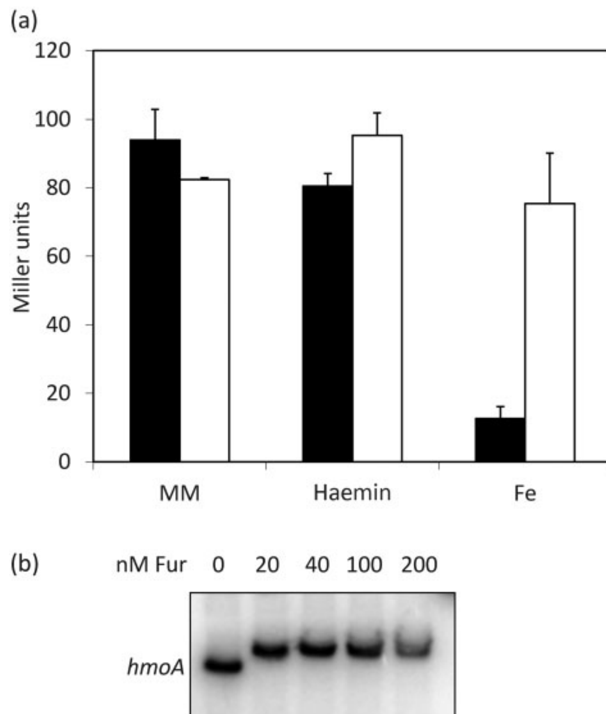

Fig. 3. *hmoA* is under Fur regulation. (a) Regulation of *hmoA* as determined using a *hmoA–cat–lacZ* fusion. Cells of the wild-type strain (black bars) or a *fur* mutant (white bars) were grown in minimal medium (MM) or MM containing either 100 μM Fe or 1 μM haemin. The results are the mean and SD of at least three independent replicates. (b) EMSA showing binding of Fur to the *hmoA* promoter region.

affect expression of either *hmoA* or *hmoB* (data not shown).

HmoA and HmoB proteins bind haem

Haem monooxygenases bind haemin *in vitro* and, in the presence of a suitable reductant, haem is oxidatively degraded (Frankenberg-Dinkel, 2004; Skaar *et al.*, 2004; Wilks & Schmitt, 1998). HmoA and HmoB were over-expressed and purified from *E. coli* as His-tagged fusion proteins. Both proteins as purified showed very low absorbance in the range characteristic of haem, indicating that they are largely free of contaminating haem (Fig. 4). Addition of increasing concentrations of haemin to HmoA or HmoB at pH 8.0 increased the difference absorption spectrum (relative to haemin added to buffer) with a maximum at 414 nm, and a broad peak at either 560 nm (HmoA) or 543 nm (HmoB) (Fig. 4a, b). These titration studies suggest that both proteins bind haemin with approximately 1 : 1 stoichiometry (Fig. 4a, b, insets). These results are consistent with what was found for the haem monooxygenases IsdG from *Bacillus anthracis* and *S. aureus* (Skaar *et al.*, 2004, 2006). To ensure that the haem binding was not affected by the His-tag used for purification, the tag (2.5 kDa) was cleaved from the protein sequence using factor Xa followed by dialysis using 6–8 kDa dialysis tubes. Binding of haem to the resulting native HmoA and HmoB proteins was indistinguishable from binding to His-tagged proteins (data not shown).

HmoA and HmoB behave as haem monooxygenases

Degradation of haem in the presence of an electron donor can be monitored by the decrease in the characteristic haem absorption peak (Wilks, 2002). Using absorption spectroscopy in the presence of ascorbate, both the haemin–HmoA (Fig. 5a) and haemin–HmoB (Fig. 6a) complexes displayed a time-dependent loss of haem absorbance. Their overall rate of degradation was similar to that reported for IsdG from *B. anthracis* and *S. aureus* (Skaar *et al.*, 2004, 2006), but with HmoA and HmoB the reaction did not result in a complete loss of haemin absorption. This may mean that the reaction did not go to completion or that the product retains absorption in this region. To ensure that this reaction was not the result of non-enzymic degradation of haem (which can occur through a coupled oxidation reaction triggered by hydrogen peroxide; Sigman *et al.*, 2001), the same reaction was carried out in the presence of catalase and similar results were obtained (in the presence of catalase in a 0.5 : 1 ratio with HmoA–haemin or HmoB–haemin). These results indicate that both HmoA and HmoB bind haemin in an approximately 1 : 1 stoichiometry, and that in the presence of reductant, the haemin is chemically altered, as reflected by the decrease of absorbance.

HmoA is a founding member of an uncharacterized subfamily of Pfam03992 proteins

S. aureus possesses two paralogous haem monooxygenases, IsdG and IsdI, which have been characterized both functionally (Skaar *et al.*, 2004) and structurally (Lee *et al.*, 2008). Significant sequence similarity is apparent between HmoA, HmoB and the IsdG and IsdI enzymes (Fig. 2b). These proteins all belong to the antibiotic biosynthesis monooxygenase or ABM domain family (pfam03992), and the true enzymic activity has only been established for a few selected examples.

The IsdG and IsdI proteins are dimeric enzymes (Lee *et al.*, 2008). The haem binding site in IsdG was identified on the basis of mutational analysis as involving residues N7, W67 and H77 (Wu *et al.*, 2005). This critical NWH triad is conserved in HmoB but, surprisingly, only partially conserved in HmoA: the residue corresponding to asparagine 7 (N7) is substituted with arginine (Fig. 2b). HmoB differs from other homologous proteins in that it contains an additional 60 aa N-terminal domain with no similarity to known motifs.

To further investigate the possible implications of the N to R substitution within the putative active site, we modelled

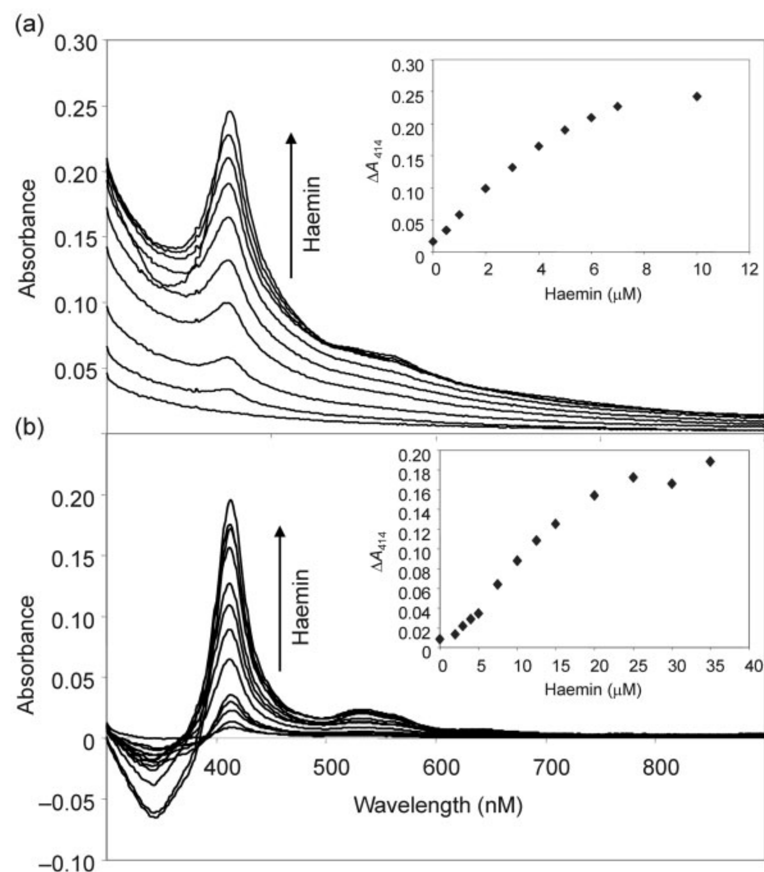

Fig. 4. HmoA and HmoB bind and degrade haem. Difference absorption spectroscopy of haemin binding to 8 μM HmoA (a) and to 20 μM HmoB (b). Haemin was added to both the sample and reference cuvettes and the differences in absorbance were recorded. Differential absorption showed that both proteins bind haemin with approximately 1 : 1 stoichiometry (insets).

the HmoA sequence on the IsdG crystal structure (Fig. 7). The overall structure of the haem binding pocket, as visualized in IsdG, is conserved in HmoA. Not surprisingly, the HmoA H76 (equivalent to IsdG H77) is also predicted to be spatially conserved, consistent with its reported role in the binding of the ferric ion of the haem group. However, the essential IsdG N7 residue (Wu *et al.*, 2005), is positionally substituted with R6, as predicted from the sequence alignment (Fig. 2b). Since N7 is predicted to interact directly with the haem iron in the active site and is essential for enzymic function (Wu *et al.*, 2005), this substitution is surprising. The replacement of N7(IsdG) with R6(HmoA) suggests that the details of substrate binding or catalysis may differ between the IsdG/HmoB type enzymes and HmoA.

We conducted a multiple sequence alignment and phylogenetic analyses to determine whether this asparagine residue is present in any other pfam03992 proteins (Fig. 8). All known IsdG-like haem monooxygenases (including HmoB) form one cluster, whereas HmoA and related proteins form a distinct cluster. Interestingly, there is a perfect correspondence between these two protein families (as defined by alignments based on the full-length protein sequences) and the presence of either a conserved asparagine (IsdG/HmoB subfamily) or an arginine or lysine residue (HmoA subfamily) (Fig. 8). This suggests that a substitution of this active site residue occurred early during the divergence of these two protein groups. To our knowledge, HmoA is the first member of this second group to be characterized. Thus, the results presented here lead to the hypothesis that other members of this HmoA subfamily (including, for example, *B. anthracis* BA2987) may also function as haem monooxygenases, albeit with possible differences in the mechanisms of haem recognition and degradation relative to the better-studied IsdG family proteins.

Mutational analysis of the variable Asn residue of the conserved NWH catalytic triad

The roles of the residues corresponding to IsdG N7 in HmoA (R6) and HmoB (N70) were tested by using site-directed mutagenesis either to truncate the corresponding side chain (by mutation to A) or to substitute with the amino acid found in the other subfamily. The resulting HmoA R6A and R6N and HmoB N70A and N70R mutant proteins were purified and tested for haem binding as described above for wild-type proteins. All four mutant proteins bound haem with approximately 1 : 1 stoichiometry, as noted for wild-type HmoA and HmoB. Before, an N7A mutant of *S. aureus* IsdG has been shown to still bind haem but to have lost all capacity for haem degradation (Wu *et al.*, 2005). In contrast, both the

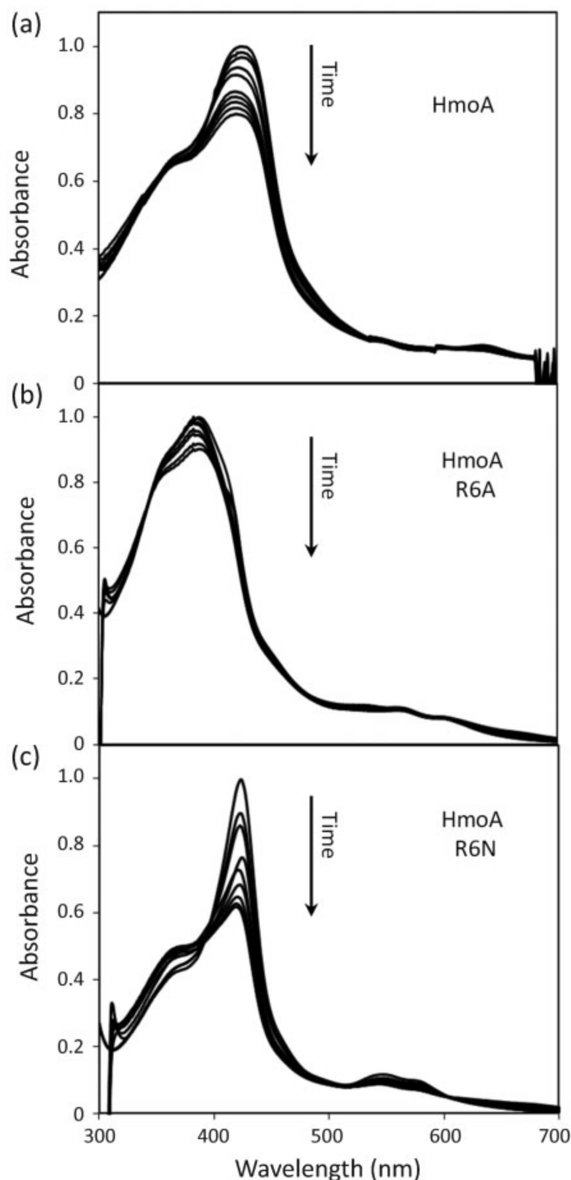

Fig. 5. Absorption spectra of haemin complexes of 9 μM HmoA wild-type (a), 13 μM HmoA R6A (b) and 13 μM HmoA R6N (c), after addition of ascorbic acid. Spectra were taken immediately before addition of ascorbic acid and then every 10 min. All maximum absorption values (0.56, 0.93 and 0.804, respectively) were normalized to 1.0 for clearer comparison.

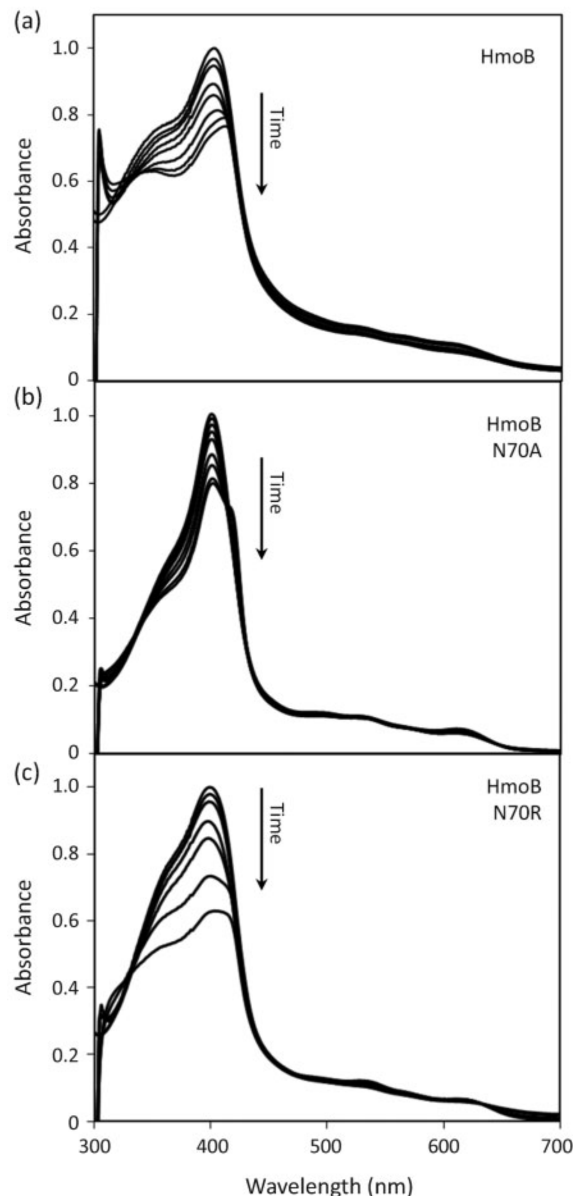

Fig. 6. Absorption spectra of haemin complexes of 6 μM HmoB wild-type (a), 15 μM HmoB N70A (b) and 6 μM HmoB N70R (c), after addition of ascorbic acid. Spectra were taken immediately before addition of ascorbic acid and then every 10 min. All maximum absorption values (0.494, 1.0 and 0.548, respectively) were normalized to 1.0 for comparison.

HmoA (R6A) and HmoB (N70A) mutants retained the ability to degrade haem (Figs 5b and 6b), albeit at a reduced rate relative to the wild-type. Moreover, the HmoA R6N and HmoB N70R mutants retained activity which was, in each case, at least as great as that for the corresponding wild-type proteins. In addition, there was a change in the spectrum of the degradation reaction that was more pronounced for HmoA mutants (Fig. 5) than for HmoB mutants (Fig. 6). This might reflect a change in the nature of the haem–protein interaction or in the resulting degradation products. We conclude that either residue (R or N) at this position can support near wild-type rates of haem degradation (Figs 5c and 6c).

Consistent with earlier studies of haem monooxygenases (Kunkle & Schmitt, 2007; Puri & O'Brian, 2006; Skaar *et al.*, 2004, 2006; Skaar & Schneewind, 2004), we observed apparent degradation of the bound haem substrate as

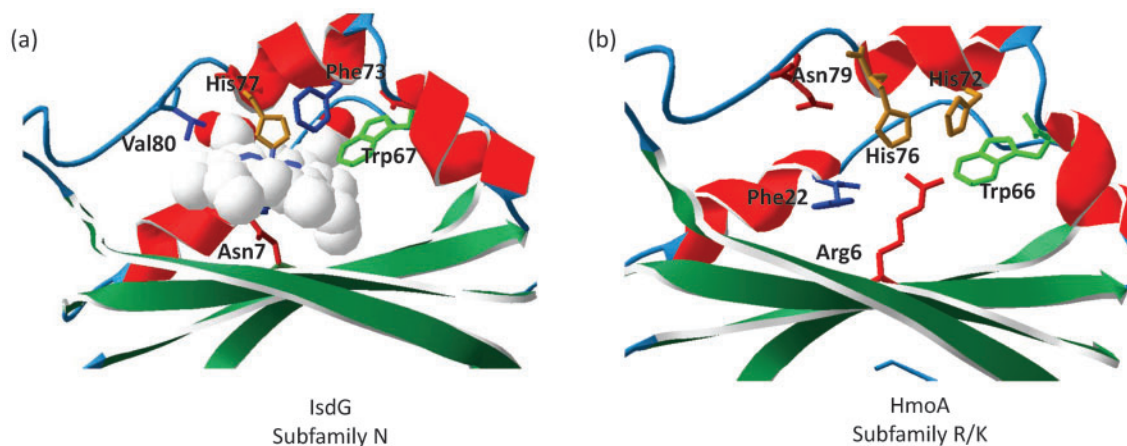

Fig. 7. (a) Structure of *S. aureus* haem-bound IsdG (Lee *et al.*, 2008), showing residues involved in haem binding and enzymic activity. (b) Structure of HmoA modelled on IsdG using SWISS-MODEL (Arnold *et al.* 2006), showing conservation of the haem binding pocket except for IsdG N7. The sequence identity between the two proteins was 57.55 %, with a QMEAN6 score of 0.66 and a QMEAN Z score of −1.34.

monitored spectroscopically, but we have been unable to demonstrate turnover (catalytic activity). Moreover, and despite several attempts, we have been unable to recover sufficient quantities of the degradation product for chemical analysis, apparently due to very tight binding to the protein. Similar results have been reported for other haem monooxygenases (Puri & O'Brian, 2006; Skaar *et al.*, 2004, 2006). This suggests that an additional factor may be needed *in vivo* to dissociate the degradation product from the protein. Recently, sufficient amounts of product were recovered from IsdG- and IsdI-dependent haemin degradation for structural characterization. The resulting products, designated staphylobilins, were identified as 5-oxo-δ-bilirubin and 15-oxo-β-bilirubin (Reniere *et al.*, 2010).

Growth on haem is not dependent on HmoA and HmoB

To determine whether *hmoA* and *hmoB* play a role in haem utilization by *B. subtilis*, single and double mutants were constructed by replacing the relevant coding sequences with an antibiotic gene cassette (Table 1). Since the phenotypes of haem utilization mutants are often very subtle (typically, quite small differences in growth rate and/or colony size have been reported; Reniere & Skaar, 2008), we tested the mutants in both liquid and solid media supplemented with EDDHA (to induce iron stress) and/or with haemin. In these assays, neither the *hmoA* and *hmoB* single mutants nor the *hmoA hmoB* double mutant displayed robust and reproducible phenotypes relative to the wild-type. A number of possible explanations could account for these negative results. It is frequently observed that iron uptake pathways are redundant, in which case a lack of HmoA and HmoB may not preclude haem utilization. For example, it is possible that haem is not internalized under these growth conditions (the iron may be removed outside the cells and then transported) (Létoffé *et al.*, 2009). However, even a triple mutant lacking *hmoA*, *hmoB* and the *ywbLMN* operon (homologous to the *efeUOB* operon implicated in uptake of haem-associated iron in *E. coli*; Létoffé *et al.*, 2009) was still able to grow using haem as an iron source. We also considered the possibility that *hmoA* and *hmoB* might be involved in haem detoxification, but we did not observe any differences in sensitivity to high levels of haem. To date, haem detoxification systems for intracellular haem seem to be closely correlated with a lifestyle that involves exposure to or growth within haem-rich environments such as mammalian blood (Skaar, 2010). It is also possible that HmoA and HmoB function primarily as haem chaperones or delivery proteins, and that their abilities to apparently degrade haem *in vitro* are not related to their *in vivo* function. Finally, we considered the possibility that these proteins might be involved in utilization of other types of metalloporphyrins, although no evidence to support this idea has emerged. Thus, the physiological roles of HmoA and HmoB remain unclear, although the direct regulation of HmoA by Fur certainly supports the notion that utilization of haem as an iron source is one function.

Concluding remarks

Bacterial haem monooxygenases degrade haem and thereby facilitate iron acquisition from this ubiquitous cofactor. The product of haem degradation has, in most cases, not been well defined but is generally presumed to be biliverdin. Haem monooxygenases fall into two major families. The first is characterized by a GXXXG motif, a conserved histidine residue required for activity (Wilks, 2002) and a HemeO superfamily motif. Members of this

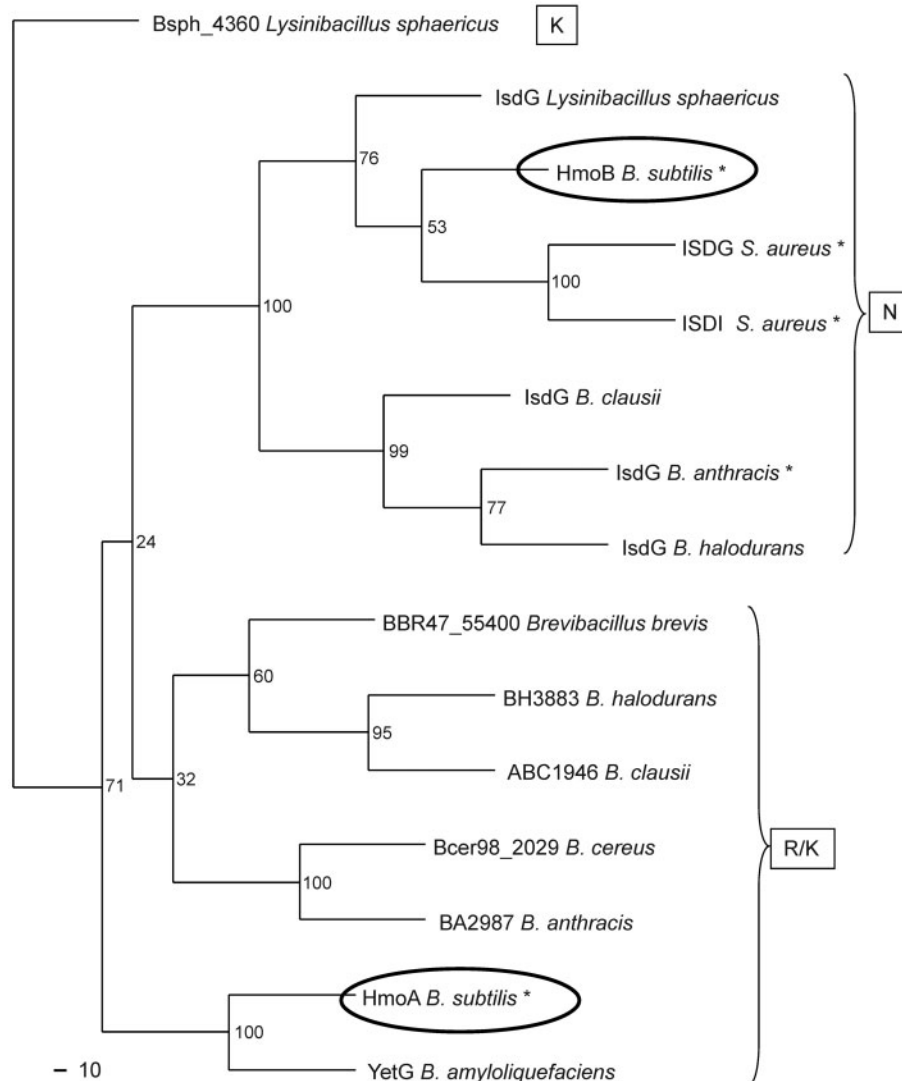

Fig. 8. Phylogenetic analysis of haem monooxygenases using CLUSTAL W and PHYLIP programs at the PhylOgenetic WEb Repeater (POWER) server using standard options (Lin *et al.*, 2005). Asterisks indicate proteins with demonstrated activity. Letters within boxes identify subgroups based on amino acid identity at the first position of the catalytic triad (see text).

family have been characterized in several bacteria, including *Neisseria* spp. (Zhu *et al.*, 2000), *Corynebacterium diphtheriae* (Wilks & Schmitt, 1998) and *Pseudomonas aeruginosa* (Ratliff *et al.*, 2001). The second family of bacterial haem monooxygenases are part of the large and diverse family of Pfam03992 proteins that share an ABM motif (Puri & O'Brian, 2006; Skaar *et al.*, 2004, 2006; Wu *et al.*, 2005). As shown here, HmoA and HmoB also contain ABM motifs (Marchler-Bauer *et al.*, 2007), but define two distinct subfamilies. HmoA and other members of this subfamily (Fig. 1b) contain a basic amino acid (R or K) in place of the N7 in IsdG (Wu *et al.*, 2005). Importantly, members of the HmoA subfamily are found in the human pathogens *B. anthracis* and *Bacillus cereus*, where they may play a role in iron acquisition during infection. The overall sequence conservation with *B. anthracis* and *S. aureus* IsdG (Skaar *et al.*, 2006), and the retention of most of the known active site residues, suggests a mode of activity similar to IsdG (Reniere *et al.*, 2010). Indeed, both HmoA and HmoB bind haemin with approximately 1 : 1 stoichiometry, and the characteristic haemin absorbance decreases upon addition of reductant. In addition, transcription of *hmoA* is regulated by Fur, consistent with a role in iron acquisition. Together, these results support a model in which HmoA, and perhaps also HmoB, functions to oxidatively release iron from haem. Ultimately, further biochemical and physiological studies will be required to define the nature of the haem degradation product(s) and the role of HmoA and HmoB in cell physiology.

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