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The novel extracellular *Streptomyces reticuli* haem-binding protein HbpS influences the production of the catalase-peroxidase CpeB

Darío Ortiz de Orué Lucana, Tanja Schaa and Hildgund Schrempf

Universität Osnabrück, FB Biologie/Chemie, Barbarastraße 11, D-49069 Osnabrück, Germany

Correspondence
Darío Ortiz de Orué Lucana
ortiz@biologie.uni-osnabrueck.de

Hildgund Schrempf
schrempf@biologie.uni-osnabrueck.de

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The Gram-positive soil bacterium and cellulose degrader *Streptomyces reticuli* synthesizes the mycelium-associated enzyme CpeB, which displays haem-dependent catalase and peroxidase activity, as well as haem-independent manganese-peroxidase activity. Downstream of the *cpeB* gene, a so far unknown gene was identified. The new gene and its mutated derivatives were cloned in *Escherichia coli* as well as in *Streptomyces lividans* and a gene-disruption mutant within the chromosome of the original *S. reticuli* host was constructed, comparative physiological, biochemical and immunological studies then allowed the deduction of the following characteristics of the novel gene product. (i) The protein was found extracellularly; the substitution of twin arginines within the signal peptide abolished its secretion. (ii) The highly purified protein interacted specifically with haem and hence was designated HbpS (haem-binding protein of *Streptomyces*). (iii) HbpS contained three histidine residues surrounded by hydrophobic amino acids; one of them was located within the motif $LX_3THLX_{10}AA$, which is related to the motif within the yeast cytochrome *c* peroxidase $LX_2THLX_{10}AA$ whose histidine residue interacts with haem. (iv) The addition of haemin (Fe^{3+} oxidized form of haem) to the *Streptomyces* cultures led to enhanced levels of HbpS which correlated with increased haemin-resistance. (v) The presence of HbpS increased synthesis of the highly active catalase-peroxidase CpeB containing haem. In this process HbpS could act as a chaperone that binds haem and then delivers it to the mycelium-associated CpeB; HbpS could also interact with membrane-associated proteins involved in a signal transduction cascade regulating the expression of *cpeB*. (vi) HbpS shared varying degrees of amino acid identities with bacterial proteins of so far unknown function. This report contributes to the elucidation of the biological function of these proteins.

INTRODUCTION

Haem is synthesized in a multistep biosynthetic pathway with well-defined intermediates. The haems are tetrapyrroles. The tetrapyrrole precursor, δ-aminolaevulinic acid (ALA), is formed either from glutamate by the C_5 pathway or from glycine and succinyl coenzyme A via ALA synthase. The subsequent conversion of ALA to the cyclic intermediate uroporphyrinogen III (UroIII) requires three successive enzymic steps which are catalysed by ALA dehydratase, porphobilinogen deaminase and uroporphyrinogen III synthase. UroIII is the final common precursor for all tetrapyrroles and thus presents the major branch point in haem biosynthesis. The later steps of haem biosynthesis require uroporphyrinogen III decarboxylase, coproporphyrinogen III oxidase, followed by ferrochelatase, which catalyses the protoporphyrin chelation of ferrous iron to form protohaem. This is the prosthetic group of numerous haem proteins, and can be modified further to other types of haem (Panek & O'Brian, 2002; Thony-Meyer, 1997). In some Gram-positive bacteria investigated (*Bacillus subtilis*, *Clostridium josui*, *Mycobacterium tuberculosis* and *Staphylococcus aureus*), the genes for enzymes catalysing the biosynthesis of UroIII are organized in operons (*hemAXCDBL*) (Johansson & Hederstedt, 1999). The compact organization of *hem* genes in Gram-positive bacteria is probably important for concerted regulation and may also be of evolutionary significance. Several Gram-negative bacteria have specific receptors recognizing free haem or haem-containing proteins. Free haem or haem from carrier proteins is actively transported through the outer membrane to the periplasm via an energy-dependent mechanism (Braun & Braun, 2002).

Haem is the prosthetic group of numerous proteins involved in a wide variety of biological processes, including oxygen carriers, redox enzymes and regulatory proteins. Haem-containing enzymes are abundant in many microorganisms and include cytochrome *c* oxidase, catalases,

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different types of peroxidases and catalase-peroxidases (Woloszczuk *et al.*, 1980; Zou *et al.*, 1999). During early stages of growth, the Gram-positive bacterium *Streptomyces reticuli* was found to produce a mycelia-associated, haem-containing enzyme (CpeB), which exhibits a catalase-peroxidase activity with broad substrate specificity and manganese-peroxidase activity (Zou & Schrempf, 2000). The *cpeB* gene and the regulator gene *furS* form an operon which is transcribed under the control of a promoter(s) located upstream of the *furS* gene. Thus FurS also acts as autoregulator in a redox-dependent fashion (Ortiz de Orué Lucana & Schrempf, 2000). The thiol form of FurS contains one zinc ion per monomer and binds in this state to its cognate operator upstream of the *furS* gene. Oxidation of -SH groups within FurS induces Zn^{2+} release (Ortiz de Orué Lucana *et al.*, 2003).

CpeB can use H_2O_2 to oxidize a number of substrates in dependence on an attached haem group (ferric-protophorpyrin) or in a haem-independent reaction which is coupled to Mn(II)/(III) peroxidation (Zou & Schrempf, 2000). The additional haem-dependent catalase activity of the enzyme leads to a disproportionation of H_2O_2 to O_2. Thus the mycelia-associated enzyme also plays an important part in detoxifying H_2O_2 and in minimizing reactions caused by highly reactive oxygen species arising from the interaction of H_2O_2 with certain divalent metal ions (Fe^{2+} and others). Therefore *S. reticuli* is well equipped to minimize the Fenton reaction, the reaction products of which are hazardous to every organism (Fridovich, 1986).

In this report we describe the identification of a gene whose product has been characterized as a novel haem-binding protein from *Streptomyces* (HbpS). Using biochemical studies, we showed that the secretion of HbpS depends on the presence of twin arginine residues within its signal peptide. Chromosomal mutants carrying a disruption of the *hbpS* gene were designed and analysed to gain insights as to the physiological role of HbpS.

METHODS

Bacterial strains and plasmids. *Streptomyces reticuli* Tü45 (H. Zähner, Tübingen, Germany) and *Streptomyces lividans* 66 (D. A. Hopwood, John Innes Institute, Norwich, UK) were used. The plasmid pUC18 (Sambrook *et al.*, 1989) was a gift from J. Messing (State University of New Jersey, Piscataway, USA). *Escherichia coli* strains DH5α and M15 (Villarejo *et al.*, 1972) and plasmids pWHM3 (Vara *et al.*, 1989; Table 1) and pQE32 (Qiagen; Table 1) were used for routine cloning purposes. The constructs pUKS10 (a pUC18 derivative; Table 1) and pWKS10 (a pWHM3 derivative; Table 1), both of which contain the *furS–cpeB* operon, have been described earlier (Zou *et al.*, 1999; Ortiz de Orué Lucana & Schrempf, 2000).

Media and culture conditions. For cultivation of *S. reticuli* and *S. lividans* complete or minimal media (Schlochtermeier *et al.*, 1992a) supplemented with the indicated carbon source were used. Depending on the purpose of the experiments, cultures were grown in baffled Erlenmeyer flasks containing 5–200 ml on a rotary shaker for 1–4 days. For cultivation of *S. reticuli* strains in solid media, R2 medium (without sucrose) (Hopwood *et al.*, 1985) containing 0·25 g K_2SO_4 l^{-1}, 10·12 g $MgCl_2.6H_2O$ l^{-1}, 10 g glucose l^{-1}, 0·10 g Casamino acids l^{-1}, 5 g yeast extract l^{-1}, 3·03 g Tris-base l^{-1} (adjusted with HCl to pH 7·5) and 14 g agar l^{-1} was used. *E. coli* strains (DH5α or M15) were grown in LB medium at 37 °C (Sambrook *et al.*, 1989).

Chemicals and enzymes. Chemicals for SDS-gel electrophoresis were obtained from Serva. Molecular mass markers, nitrophenyl, *o*-dianisidine, 4-chloro-1-naphthol, haemin and 3,3′,5,5′-tetramethylbenzidine were supplied by Sigma. Hydrogen peroxide (30 %, w/v) was bought from Merck.

Test for peroxidase activity. CpeB was released from the mycelium using acetate buffer (pH 5·5) containing 0·1 % Triton X-100 (Zou *et al.*, 1999), and aliquots (30 µl) were tested for activity. Samples were loaded onto a native 10 % polyacrylamide gel. After the run, the gel was washed twice with acetate buffer (20 mM, pH 5·5) and activity staining was carried out with 4-chloro-1-naphthol and 5 mM H_2O_2 (Conyers & Kidwell, 1991).

Cleavage of DNA, ligation and agarose gel electrophoresis. DNA was cleaved with various restriction enzymes according to the suppliers' instructions. Ligation (Sambrook *et al.*, 1989) was performed with T4 ligase (New England Biolabs). Gel electrophoresis was carried out in 0·8–2 % agarose gels using TBE buffer.

Transformation and isolation of plasmids. *E. coli* was transformed with plasmid DNA by electroporation (Dower *et al.*, 1988). Plasmids were isolated from *E. coli* with the aid of a mini plasmid kit (Qiagen).

Hybridization experiments. The transfer of DNA fragments of the restricted genomic *S. reticuli* DNA onto nylon membranes was performed as described by Sambrook *et al.* (1989). The hybridization probes were labelled using Klenow enzyme and digoxigenin-11-dUTP (Roche). Hybridization and immunological detection were carried out according to standard procedures (Sambrook *et al.*, 1989).

RNA isolation. To obtain well-grown mycelia, *S. reticuli* (wild-type and *hbpS* mutant) spores [20 µl in 80 %, (v/v) glycerol] were inoculated in 10 ml complete medium (Schlochtermeier *et al.*, 1992b) and grown as a standing culture at 30 °C for 16 h. The culture was diluted (1 : 10) in the same medium, and cultivation was continued on a rotary shaker at 30 °C for 12 h. For further scaling up, the culture was diluted to 1 l, and cultivation was continued (16 h). The cultures were washed twice in minimal medium (MM) without supplement, and kept shaking after resuspension in the same medium. The mycelia were suspended in 1 l MM, divided into 100 ml portions and supplemented with Avicel (1 % final concentration). Samples (100 ml) were taken for enzyme activity tests, immunoblotting and RNA isolation. Genomic RNA was isolated as previously described (Ortiz de Orué Lucana & Schrempf, 2000).

Analysis of transcripts. Total RNA was electrophoretically separated on a 2 % agarose gel containing 2 % formamide and 1 × MOPS buffer and was transferred over 3 h to a positively charged nylon membrane under vacuum using 20 × SSC. RNA size marker I (0·3–6·9 kb; Roche) was used for size determination. The membrane was dried at 80 °C for 30 min and subsequently exposed to UV radiation for 3 min to fix the RNA. Hybridization was performed in a solution containing 5 × SSC, 0·1 % SDS, 100 µg salmon sperm DNA ml^{-1} and 5 × Denhardt's reagent (Sambrook *et al.*, 1989) at 64 °C for 2 h. The ^{32}P-labelled probe was added and hybridization was continued for another 20 h.

The *cpeB* probe was radioactively labelled with the Rediprime DNA labelling system (Amersham Biosciences) using Klenow polymerase

Table 1. Plasmids

Plasmid	Relevant properties*	Source or reference
***E. coli* plasmids**		
pUKS10	pUC18 derivative containing *furS*, *cpeB*, *hbpS* and *amp*^{r}	Zou *et al.* (1999)
pUKS15	pUKS10 derivative carrying *hbpS* with six histidine codons at the 3′ end and *amp*^{r}	This work
pUKS16	pUKS10 derivative with *hbpS* containing two lysine substitutions instead of two arginine (R9 and R10) codons and *amp*^{r}	This work
pUCH1	pUKS10 derivative carrying truncated *cpeB*, *hbpS* and *amp*^{r}	This work
pUCH2	pUCH1 derivative used for the generation of a *hbpS* disruption mutant and containing *amp*^{r} and *hyg*^{r}	This work
pQE32	Expression vector with a multiple cloning site and six histidine codons for the His-tag and *amp*^{r}	Qiagen
pQH1	pQE32 derivative containing *hbpS* downstream of the His-tag	This work
***E. coli*/*Streptomyces* shuttle vectors**		
pWHM3	Multiple cloning site, *amp*^{r} and *tsr*^{r}	Vara *et al.* (1989)
pWKS10	pWHM3 derivative carrying the 4·6 kb *Kpn*I–*Sph*I fragment from pUKS10 containing *furs*, *cpeB*, *hbpS*, *amp*^{r} and *tsr*^{r}	Zou *et al.* (1999)
pWKS15	pWKS10 derivative carrying *hbpS* with six histidine codons at the 3′ end, *amp*^{r} and *tsr*^{r}	This work
pWKS16	pWKS10 derivative with *hbpS* containing two lysine substitutions instead of two arginine (R9 and R10) codons, *amp*^{r} and *tsr*^{r}	This work
pWKS17	pWKS10 derivative lacking *hbpS* and containing *amp*^{r} and *tsr*^{r}	This work

*amp^r, ampicillin resistance gene; *tsr^r*, thiostrepton resistance gene; *hyg*, hygromycin resistance gene.

and [^{32}P]dCTP. Firstly, the DNA region of *cpeB* was amplified by PCR. The following primers were used: CD, 5′-GAGTTCCGCACAGTT-CGGAAGG-3′, and CE, 5′-CGTTGGCACCGCCGCGCTTGTCGC-3′.

The membrane was washed twice with 2 × SSC containing 0·1 % SDS at room temperature for 20 min and subsequently with 0·1 × SSC supplemented with 0·1 % SDS at 64 °C for 45 min, and subjected to autoradiography at −70 °C.

Cloning of the *hbpS* gene in *E. coli*. The *hbpS*-encoding region of the previously described construct pWKS10 (Zou *et al.*, 1999; Table 1), harbouring *S. reticuli furS*, *cpeB* and the newly identified *hbpS* genes, was amplified by PCR using the following primers: HBP1, 5′-CCTGAGCATGCCCAGCCGCAAGAAGCCGTCCC-3′ consisting of an *Sph*I restriction site, followed by the sequence encoding N-terminal amino acids of HbpS; and HBP2, 5′-GCCGGATCCTGGCCGAGCACGGCCGCGCC-3′, determining the C-terminal amino acids of HbpS, followed by a *Bam*HI restriction site. The PCR product was digested with *Sph*I and *Bam*HI. After digestion with *Bam*HI the DNA was treated with the Klenow polymerase to get a blunt-ended fragment which was ligated with *Sph*I/*Sma*I-digested pQE32 (Table 1). The resulting plasmid pQH1 (Table 1) was transformed into *E. coli* M15(pREP4). The plasmid was sequenced and the correctness of the designed *hbpS* gene and its in-frame fusion with the His-tag codons were confirmed.

Cloning of a *hbpS* fusion gene in *S. lividans*. The downstream region of the *hbp*S gene was amplified using the oligonucleotides HBP3, 5′-GTCGGTGGCCGTCGTCGACCGCAACGGCAACACCC-TGGTCACCCTG-3′, annealing upstream of the *Bsi*WI restriction site in the middle of *hbpS*, and HBP4, 5′-CTCGGCCCGGGCGG-GTCAGTGGTGGTGGTGGTGGCCGAGCACGG-3′, containing an *Srf*I restriction site, a stop codon and six histidine codons. Following the PCRs, the generated product was cleaved with *Bsi*WI and *Srf*I and ligated with the large *Bsi*WI–*Srf*I fragment of the plasmid pUKS10 (Zou *et al.*, 1999; Table 1). The 4·6 kb *Eco*RI–*Hin*dIII fragment of the resulting plasmid pUKS15 (Table 1) was ligated with *Eco*RI/*Hin*dIII-digested pWHM3 vector. The resulting plasmid pWKS15 (Table 1) carried a *hbpS* gene with six histidine codons in front of its stop codon. The correctness of the in-frame insertion was confirmed by restriction and sequencing. The plasmid pWKS15 (Table 1) was transformed into *S. lividans* protoplasts, which were generated as described by Hopwood *et al.* (1985). Transformants were selected using an overlay of 0·4 % agarose containing 200 μg thiostrepton ml^{-1} (Hopwood *et al.*, 1985).

Site-directed mutagenesis of *hbpS*. The codons for arginines (R9 and R10) from the identified twin arginines within the signal sequence of HbpS (Fig. 1b) were replaced by two lysine-encoding codons. Lysines and arginines are highly basic with a long side-chain and possess similar hydrophobicity coefficients; thus, it is less likely that the substitutions induce unfavourable steric interactions in the resulting protein. For this purpose the upstream region of *hbpS* was amplified using the following oligonucleotides: HBP5, 5′-CTTCCT-CAGCATGTCCAGCCLGCAAGAAGCCGTCCAAGAAGACCCGCG-TCCTCGT-3′, containing a *Bbv*CI restriction site and two lysine codons replacing the two arginine codons of the *hbpS* wild-type gene, and HBP4 (see above). For the PCR and further ligation the plasmid pUKS10 (Zou *et al.*, 1999; Table 1) was used. The PCR product was digested with *Bbv*CI and *Bsi*WI, resulting in a 0·328 kb mutagenized *hbpS* fragment which was ligated with the large *Bbv*CI–*Bsi*WI fragment of plasmid pUKS10 (Table 1). The 4·6 kb *Eco*RI–*Hin*dIII fragment of the resulting plasmid pUKS16 (Table 1) was ligated with the *Eco*RI/*Hin*dIII-digested pWHM3. The resulting plasmid pWKS16 (Table 1) carried an *hbp*S gene containing mutations in the codons encoding arginines 9 and 10 within HbpS. The correctness of the in-frame replacement was checked by restriction and

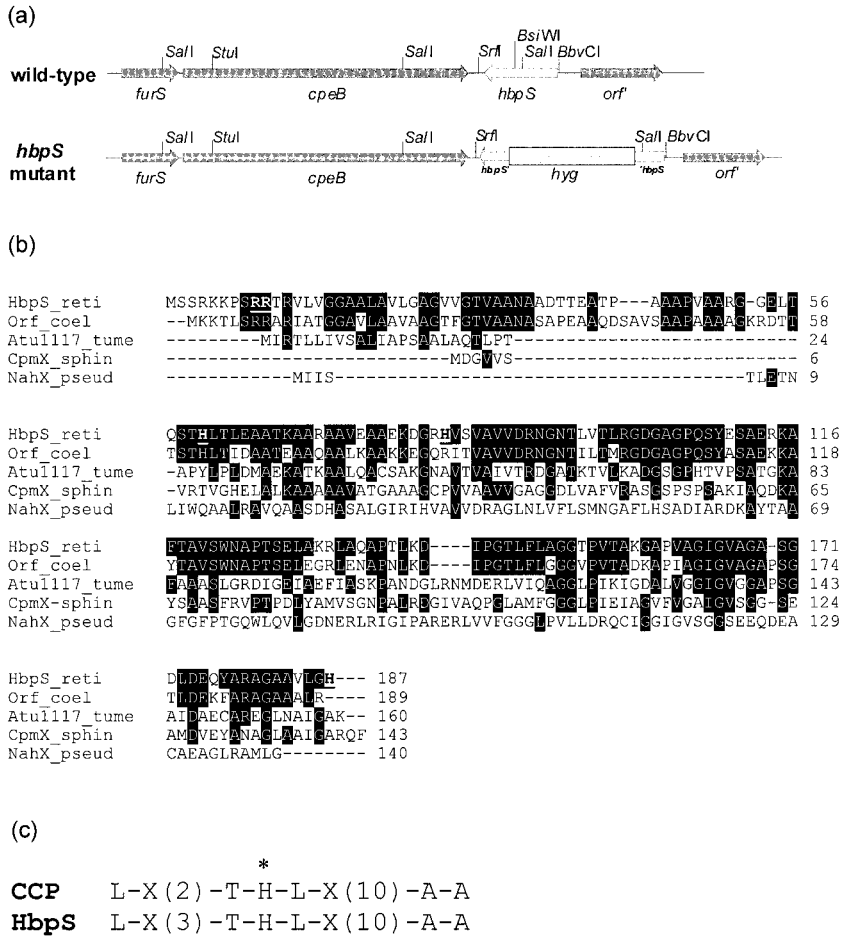


Fig. 1. Relative location of the *hbpS* gene and alignments. (a) Gene locations. In the wild-type, the *hbpS* gene, and one other ORF located within the *Srf*I–*Sph*HI fragment of pUKS10 (Zou *et al.*, 1999) are presented downstream of the *furS–cpeB* operon (GenBank/EMBL accession no. Y14336). In the *hbpS* mutant, the *hbpS* gene within the chromosomal DNA has been disrupted by the insertion of the hygromycin resistance cassette (*hyg*). (b) The HbpS protein from *S. reticuli* (HbpS_reti) was aligned to an ORF from *S. coelicolor* A3(2) (Orf_coel), Atu1117 from *A. tumefaciens* C58 (Atu1117_tume), CpmX from *Sphingomonas aromaticivorans* (CpmX_sphin) and NahX from *P. putida* G7 (NahX_pseud). The arginine residues at positions 9 and 10 as well as the three histidine residues in HbpS are in bold, white letters and are underlined. (c) The region surrounding the histidine residue at position 60 (indicated by *) of HbpS was aligned to the region containing the histidine residue involved in haem binding by the yeast cytochrome *c* peroxidase (CCP).

sequencing. The plasmid pWKS16 was transformed into *S. lividans* protoplasts as described above.

Purification of the fusion protein and generation of antibodies. The complete *hbpS*-encoding region from the construct pUKS10 described previously (Zou *et al.*, 1999) was amplified using PCR, and subsequently cloned into pQE32 (Table 1). The resulting construct pQH1 (Table 1) was transformed into *E. coli* M15(pREP4). The corresponding transformant containing the pQH1 plasmid was inoculated in LB medium with ampicillin (100 μg ml^{-1}) and kanamycin (50 μg ml^{-1}) and during its exponential growth phase (OD_{600} 0·6–0·8) was induced with 1 mM IPTG for 3 h. The cells were washed with chilled solution A (10 mM HEPES, 60 mM KCl, pH 8·0) and disrupted in the same solution by ultrasonication (Branson sonifier, 5 × 10 s, with 10 s intervals). After centrifugation (16 000 ***g***), the debris and the inclusion bodies (constituting about 50 % of the insoluble fusion protein) were separated from the cytoplasm. The inclusion bodies were denatured with solution A containing 6 M urea and supplemented with 30 mM imidazole. After centrifugation (16 000 ***g***) the supernatant containing the fusion protein was mixed with Ni^{2+}-NTA (Qiagen) on a wheel at 4 °C for 2 h. The agarose column containing the bound proteins was washed with solution A supplemented with 30 mM imidazole (using about 10-fold of the column volume), and eluted with the same solution containing 200–500 mM imidazole. The whole procedure was repeated. To remove imidazole, the sample was dialysed against solution A and the protein was concomitantly concentrated (to about 0·45 mg ml^{-1}) by using an Amicon device. The protein concentrations of the samples were determined by the methods of Lowry, and Bradford (1976). The isolated protein (150 μg) was used to generate antibodies in a guinea pig (Eurogentec). The antisera were stored in aliquots at −20 °C.

Localization of HbpS in *Streptomyces*. *S. reticuli* (wild-type), *S. reticuli hbpS* disruption mutant, *S. lividans* pWKS10 (Zou *et al.*, 1999), *S. lividans* pWKS17 (derivative of pWKS10 lacking *hbpS*, see below and Table 1) and *S. lividans* pWHM3 (control strain with vector) were grown in complete medium. The pWKS17 (Table 1) plasmid was generated by self-ligation of the longer *Srf*I–*Bbv*CI (Fig. 1a) restriction fragment of pWKS10. Thus, pWKS17 lacks the *hbpS* gene. The cultures were transferred to a minimal medium containing 1 % Avicel (for *S. reticuli*) or 0·5 % yeast extract (for *S. lividans*) and cultivated on a rotary shaker at 30 °C. *S. reticuli* was incubated for 6 h, *S. lividans* for 14 h. Mycelia were centrifuged (4000 ***g***). The extracellular proteins from the culture filtrate were precipitated by adding $(NH_4)_2SO_4$ (90 % saturation). The precipitated proteins were tested for immuno-reactivity with anti-HbpS antibodies.

Purification of HbpS–His-tag from *Streptomyces*. *S. lividans* transformants carrying the plasmid pWKS15 (Table 1) or the construct pWKS17 (derivative of pWKS10 lacking *hbpS*) were grown as described above. After precipitation [using $(NH_4)_2SO_4$, 90 % saturation] of proteins from the culture filtrate, the protein solutions were dialysed against solution A (see above). Afterwards, the HbpS–His-tag protein was purified using Ni–NTA-affinity chromatography as described previously (Ortiz de Orué Lucana & Schrempf, 2000).

Detection of haem binding by HbpS. Haem binding in HbpS was identified in SDS-polyacrylamide gels as described by Moore *et al.* (1978) and Smalley *et al.* (2001) using tetramethylbenzidine/H_2O_2, which detects the presence of haem in proteins. For the preparation of gels, loading buffer and electrophoresis buffer with 0·1 % SDS was used. The HbpS–His-tag-containing sample was free from DTT and was not boiled prior to electrophoresis, which was carried out at 4 °C in the dark. Then, the gel was incubated for 30 min in 20 mM Tris/HCl (pH 7·3) buffer containing 50 % methanol to fix the protein and to lower the concentration of SDS within the gel. This was consecutively stained for 45 min using the following solutions: 0·25 mM sodium acetate, pH 5·3; 0·25 % (w/v) 3,3′,5,5′-tetramethylbenzidine, 25 % (v/v) methanol, 0·75 % H_2O_2 [2·5 % (v/v) of a commercial 30 % solution]. H_2O_2 was added immediately prior to use. After staining, the gel was incubated for 10 min in 25 % (v/v) methanol to remove excess of tetramethylbenzidine. The gel was then stored in 0·1 M Tris/HCl, pH 7·3.

Generation of disruption mutant. The plasmid pUKS10 (Zou *et al.*, 1999) was digested with *Eco*RI. The linearized DNA was then partially digested with *Sal*I to obtain a 4·6 kb DNA fragment containing part of *cpeB*, the *hbpS* gene and its downstream region. After treatment with the Klenow enzyme, the DNA was circularized by self-ligation. The resulting construct was named pUCH1 (Table 1). Using a pBR322 derivative with a hygromycin-resistance gene containing terminator sequences (*hyg*) (Blondelet-Rouault *et al.*, 1997), a *Hin*dIII fragment (2·3 kb) containing this *hyg* was generated. This fragment was blunt-ended using the Klenow enzyme and ligated with the *Bsi*WI-digested pUCH1 which was also blunt-ended. The resulting plasmid pUCH2 (Table 1) contains *hyg*, which is flanked on the left by 0·87 kb and on the right by 1·15 kb of the above-described plasmid pUCH1. The ligation mixture was added to electrocompetent *E. coli* DH5α. Hygromycin-resistant *E. coli* transformants were selected, and the correctness of their plasmid constructs was analysed by restriction. One of the correct constructs was isolated. Ten micrograms thereof was denatured (0·2 M NaOH, 10 min, 37 °C), chilled on ice and neutralized by rapid addition of HCl. Then the DNA was used to transform 50 µl protoplasts ($\sim 10^9$ ml^{-1}) generated from *S. reticuli*, which were spread onto osmotically stabilized medium, as described by Hopwood *et al.* (1985) and incubated at 30 °C for 19 h. The plates were overlaid with 2 ml molten agarose (40 °C) containing hygromycin (1 mg ml^{-1}). Hygromycin-resistant colonies were restreaked several times, and their genomic DNA was analysed as to the size of fragments carrying the hygromycin gene.

Growth assays. The sensitivity of *S. reticuli* wild-type and *S. reticuli hbpS* mutant to haemin was determined using a disc inhibition assay. A sample of 100 µl spores (5×10^8) was added to 3 ml soft agar (Sambrook *et al.*, 1989) poured onto the respective R2 plates and allowed to solidify. Sterile 6 mm-diameter blank papers discs (Schleicher & Schuell) were added to the bacteria-overlaid plates and saturated with 20 µl of different concentrations (100, 200, 300 and 400 µM) of haemin. Plates were incubated overnight at 30 °C before zones of inhibition were measured.

SDS-PAGE and Western blotting. SDS-PAGE was performed in the presence of 0·1 % SDS (Laemmli, 1970). Proteins were separated by 12·5 % SDS-PAGE and transferred to a fluorotrans membrane (Sambrook *et al.*, 1989). The membrane was blocked for 1 h at room temperature with PBS (40 mM Na_2HPO_4, 8 mM NaH_2PO_4, 150 mM NaCl, pH 7·4) containing 5 % skimmed milk powder, and subsequently incubated overnight at 4 °C with the generated anti-CpeB or anti-HbpS antibodies. After treatment with secondary anti-rabbit or anti-guinea pig antibodies, respectively, conjugated with alkaline phosphatase, the membrane was stained with 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium (Blake *et al.*, 1984).

PCR, DNA sequencing and computer analysis. PCR was performed using *Pfu* DNA polymerase (Invitrogen). To test the correctness of cloned genes, sequencing was done using the Ready Reaction mix and ABI PRISM equipment (PE Biosystems) by the departmental sequence service (U. Coja, FB Biologie, University of Osnabrück). Sequence entry, primary analysis and ORF searches were performed using Clone Manager 5.0. Database searches using the PAM120 scoring matrix were carried out with BLAST algorithms (BLASTX, BLASTP and TBLASTN) on the NCBI file server (BLAST@ncbi.nlm.nih.gov) (Altschul *et al.*, 1997). Multiple sequence alignments were generated by means of the CLUSTAL W (1.74) program (Higgins *et al.*, 1992). Putative Shine–Dalgarno (ribosome-binding) sites (Gold *et al.*, 1981; Strohl, 1992) and signal peptide cleavage sites were predicted as described by Nielsen *et al.* (1997).

RESULTS

Analysis of the *hbpS* gene and the deduced protein

S. reticuli produces the mycelia-associated, haem-containing catalase-peroxidase CpeB; the corresponding *cpeB* gene is regulated by the redox regulator FurS encoded by *furS*, which is located upstream of *cpeB* (Zou *et al.*, 1999). The genomic DNA from *S. reticuli* downstream of *cpeB* had been cloned in plasmid pUBBI previously (Zou *et al.*, 1999), and was subsequently sequenced (see Fig. 1a and GenBank/EMBL accession no. Y14336). The *cpeB* gene was found to be followed by an intergenic region and by an ORF comprising 566 bp. The deduced protein has 188 aa with a predicted molecular mass of 18·5 kDa and theoretical pI of 9·6. Fig. 1(a) (wild-type) displays the physical map of the *furS–cpeB* operon and the newly identified ORF, which in the course of additional studies was subsequently named *hbpS* (for nomenclature, see results of functional tests and Discussion). The *hbpS* gene is separated by 117 bp from the *cpeB* gene, which is located in the opposite orientation. The deduced HbpS protein contains an N-terminally located signal peptide which includes twin arginines (at positions 9 and 10; Fig. 1b).

Comparative analysis revealed that the amino acid sequence of HbpS shows the highest identity (69·9 %) to an ORF from *Streptomyces coelicolor* A3(2). HbpS also shares a limited number of identical amino acids with a few other deduced proteins with so far uncharacterized features, including Atu1117 from *Agrobacterium tumefaciens* C58 (38·3 %) (Wood *et al.*, 2001), CpmX from *Sphingomonas aromaticivorans* (36·6 %) (Yrjala *et al.*, 1997) and NahX from *Pseudomonas putida* G7 (33 %) (Grimm & Harwood, 1999) (Fig. 1b). The genes *cpmX* and *nahX* are each located in an operon, which is responsible for degradation of chlorinated or methylated aromatic compounds via the meta-cleavage pathway. The N-terminal region of Atu1117 shows a predictable signal peptide cleavage site, however without twin arginines.

The following investigations were focused on analysing the function of the protein encoded by the *hbpS* gene.

Secretion of HbpS requires a twin arginine in its signal peptide

A *S. lividans* transformant was generated previously which has the plasmid pWKS10 (Table 1); this containing the *furS–cpeB* operon and *hbpS* (Zou *et al.*, 1999). As outlined in Methods, the *hbpS* fusion gene with six histidine codons was cloned and overexpressed in *E. coli*. The subsequently purified His-tag–HbpS protein molecules consist predominantly of the monomeric form, including its signal peptide (25 kDa), and smaller quantities of the dimeric form (50 kDa) (Fig. 2). After generation of antibodies, immunological studies revealed that the culture filtrate of *S. lividans* pWKS10 contained the mature monomeric (apparent molecular mass 16·5 kDa) and dimeric (apparent molecular mass 33 kDa) forms of HbpS (Fig. 3b, lanes 3 and 6). The control strains *S. lividans* pWKS17 (without *hbpS*) and *S. lividans* pWHM3 (vector alone) lacked any extracellular protein immuno-reacting with anti-HbpS antibodies (Fig. 3b, lanes 4 and 5). N-terminal sequencing of HbpS (secreted by *S. lividans* pWKS15) showed that the cleavage site in the signal peptide could be located between the residues Asp37 and Thr38 and not between the alanine residues (positions 35 and 36) which would have been predicted for a signal peptidase cleavage site (Nielsen *et al.*, 1997). Thus, it has to be assumed that the N-terminus of the mature HbpS was further modified via action of an extracellular peptidase. The predicted mature secreted protein has a molecular mass of 15·1 kDa and a pI of 5·9.

To determine the role of the twin arginine signature in HbpS, the codons for the twin arginines (R9 and R10) in the *hbpS* gene (located within the construct pWKS10) were replaced by two for lysine (see Methods). The resulting plasmid pWKS16 (Table 1) was transformed into *S. lividans*. The transformant *S. lividans* pWKS16 lacked any extracellular HbpS protein (Fig. 3b, lane 7), but it contained several immuno-reacting (using anti-HbpS antibodies) proteins intracellularly (Fig. 3b, lane 9), one dominant at 33 kDa and others at 41 kDa and 60 kDa. This result indicates that the secretion of HbpS depends on the presence of the RR-motif within the signal peptide.

To facilitate purification of larger amounts of HbpS for further analysis the *hbpS* gene was extended by six histidine

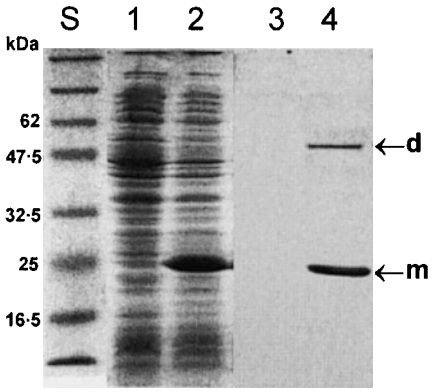


Fig. 2. Purification of the HbpS fusion protein from *E. coli*. *E. coli* M15(pREP4) containing plasmid pQH1 (lane 2) or the control plasmid pQE32 (lane 1) were grown and induced as described in Methods. Total proteins (lanes 1 and 2) were separated by SDS-PAGE. The desired HbpS fusion protein was purified using affinity chromatography (lane 4). No protein could be eluted from the control strain (lane 3). Protein markers are in lane S.

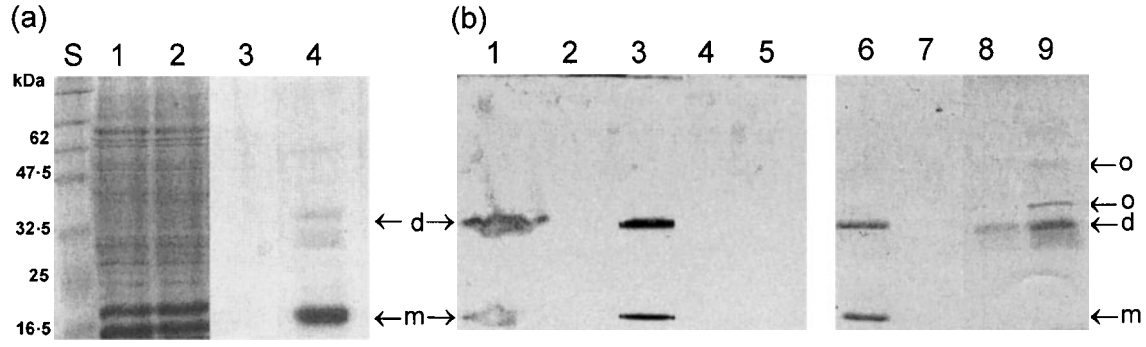
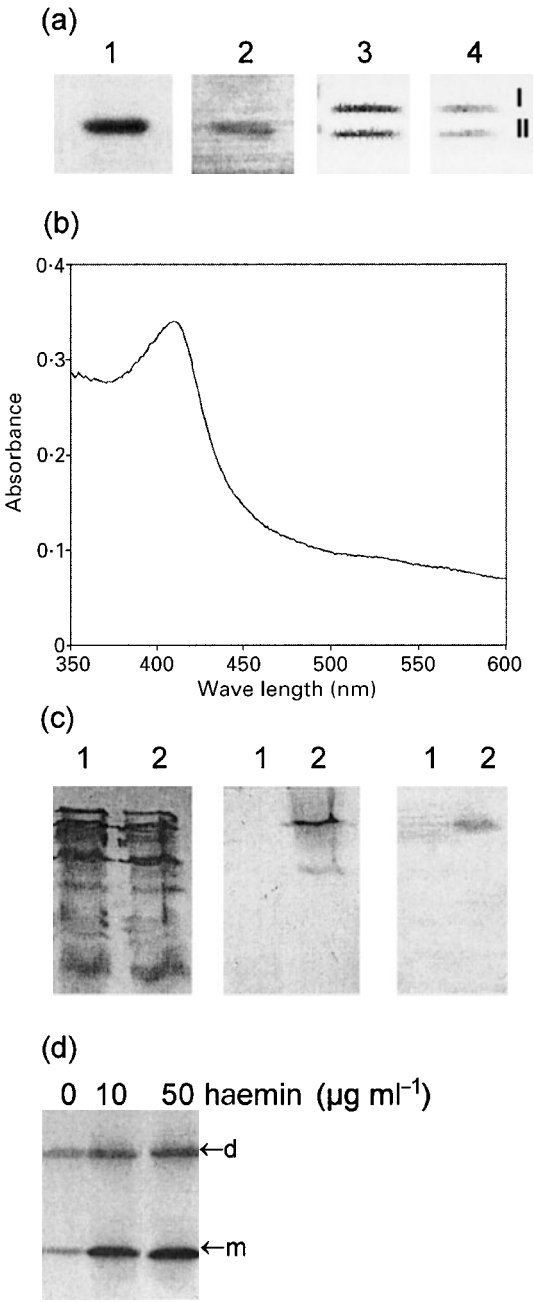


Fig. 3. Detection of HbpS in *Streptomyces* strains. (a) *S. lividans* containing pWKS15 (lane 2) or the control plasmid pWKS17 (lane 1) was grown as described in Methods. The HbpS fusion protein was purified from the precipitated proteins using affinity chromatography (lane 4). No protein could be eluted from the control extracts (lane 3). Protein markers are in lane S. (b) Proteins were precipitated from the culture filtrate of *S. reticuli* wild-type (lane 1), *S. reticuli hbpS* disruption mutant (lane 2), *S. lividans* pWKS10 (lanes 3 and 6), *S. lividans* pWKS17 lacking *hbpS* (lane 4), *S. lividans* pWHM3 (lane 5) and *S. lividans* pWKS16 containing mutated *hbpS* (lane 7) or from a cytoplasmic extract of *S. lividans* pWKS10 (lane 8) and *S. lividans* pWKS16 containing mutated *hbpS* (lane 9). After their separation by SDS-PAGE proteins were analysed for the presence of HbpS (Western blot) using anti-HbpS antibodies. Monomeric (m), dimeric (d) and higher oligomeric (o) forms are indicated.

codons (corresponding to the codon usage of *Streptomyces*). This was cloned in such a way that it replaced the original *hbpS* gene in pWKS10 and resulted in the plasmid pWKS15 (Table 1). The HbpS fusion protein with the His-tag predicted at the C-terminus could be concentrated by affinity chromatography from the culture filtrate of *S. lividans* pWKS15 (Fig. 3a, lane 4), but not from the control strain *S. lividans* pWKS17 (lacking the *hbpS* gene) (Fig. 3a, lane 3). The isolated protein has an apparent molecular mass of 17·2 kDa corresponding to the secreted form of the His-tag protein. Small quantities of its dimeric form (34 kDa) were also observed (Fig. 3a). Proteins corresponding to the non-mature form (20 kDa) and to the mature form (17·2 kDa) were purified from the cytoplasm of *S. lividans* pWKS15 (Fig. 4a, lane 3).



Identification of HbpS as a haem-binding protein

Domain analysis using the Pfam protein families database (Bateman *et al.*, 2002) showed homologies with small stretches within the N-terminal regions from the NapC/NirT cytochrome *c* family, which bind four or five haem groups (Cartron *et al.*, 2002). The haem-binding motif (CXXCH) of these regions was, however, not present in HbpS. Nevertheless, the ability of HbpS to bind haem was tested. The mature HbpS–His-tag protein which was purified from the culture filtrate of *S. lividans* pWKS15 (Fig. 3a) was shown to contain haem by using tetramethylbenzidine/H_2O_2 staining (see Methods) (Fig. 4a, lane 2). The mature HbpS–His-tag protein also had a characteristic ferric high-spin spectrum with a very strong Soret peak at 409 nm (Fig. 4b), which is typical for haem-containing proteins including the mycelium-associated *S. reticuli* CpeB (Zou & Schrempf, 2000), albumin and the mouse haem-binding protein p22 HBP (Taketani *et al.*, 1998). Among the extracellular proteins of *S. lividans* pWKS10 carrying wild-type *hbpS* (without additional histidine codons) one protein was detected which immuno-reacted with anti-HbpS antibodies and was stained with tetramethylbenzidine/H_2O_2 (Fig. 4c, lane 2) indicating the presence of haem-containing HbpS. As expected, HbpS was absent in the culture filtrate of *S. lividans* pWKS17 (without *hbpS*) (Fig. 4c, lane 1).

To investigate haem-binding properties of the non-mature HbpS–His-tag protein, it was purified from the cytoplasmic fraction of *S. lividans* pWKS15 (Fig. 4a, lane 3).

Fig. 4. Identification of HbpS as a haem-binding protein and influence of haemin on HbpS production. (a) The HbpS fusion protein purified from the extracellular (lane 1 and 2) or the cytoplasmic fraction (lane 3 and 4) of *S. lividans* pWKS15 was loaded (without reducing agents and without boiling) on to SDS-polyacrylamide gels and analysed. The lanes were stained with Coomassie brilliant blue (lane 1), silver (lane 3) or treated with tetramethylbenzidine/H_2O_2 (lane 2 and 4). The non-mature form of the cytoplasmic protein is indicated by **I** and the corresponding mature form by **II**. (b) The absorption spectrum of a solution containing purified HbpS fusion protein (100 μg in 10 mM HEPES buffer, pH 8·0). (c) Proteins from the culture filtrate from *S. lividans* pWKS10 containing *hbpS* (lane 2) and *S. lividans* pWKS17 lacking *hbpS* (lane 1) were analysed as described under (a) and treated with Coomassie brilliant blue (left), anti-HbpS antibodies (middle) or tetramethylbenzidine/H_2O_2 (right). (d) *S. lividans* pWKS10 was grown as described in Methods. Different concentrations of haemin (0, 10 or 50 μg ml^{-1}) were added to the culture medium. After separation of proteins by SDS-PAGE, the proteins were transferred to a nylon membrane for Western analysis using anti-HbpS antibodies. The monomeric (m) and dimeric (d) forms of HbpS are indicated.

Tetramethylbenzidine/H_2O_2 staining showed that the non-mature (20 kDa) form of HbpS also contained haem (Fig. 4a, lane 4).

Analysis of HbpS production in *S. reticuli* wild-type and *hbpS* disruption mutant

Using antibodies, *S. reticuli* was found to secrete small amounts of HbpS (Fig. 3b, lane 1). As expected, the levels were considerably lower than those from *S. lividans* pWKS10 carrying the *hbpS* gene on the multicopy plasmid. As a basis for further investigations, the *hbpS* gene within the *S. reticuli* genome was disrupted according to the strategy outlined in Methods. Southern hybridizations using *hbpS*- and *hyg*-probes (data not shown) revealed that a double crossover between the genomic *S. reticuli hbpS* gene and the residual *hbpS* portions flanking the *hyg* in pUCH2 (Table 1) had occurred as desired (Fig. 1a, *hbpS* mutant). Immunological studies (Western blot analysis) revealed that the culture filtrate of the chromosomal *S. reticuli hbpS* disruption mutant lacked HbpS (Fig. 3b, lane 2).

Resistance to haemin correlates with HbpS production

It is well known that haemin, as a natural porphyrin, possesses significant antibacterial activity that is augmented by the presence of physiological concentrations of hydrogen peroxide or a reducing agent (Stojiljkovic *et al.*, 2001). Increasing concentrations (100–400 μM) of haemin (the Fe^{3+} oxidation product of haem) in the culture medium led to a higher growth-inhibition of the *S. reticuli hbpS* mutant strain (Table 2) than of the *S. reticuli* wild-type strain. *S. lividans* pWKS10 also produced considerably greater amounts of HbpS in the presence of haemin (0–50 μg ml^{-1}) than in the absence of haemin (Fig. 4d). The data imply that HbpS plays an important role in defence against the high toxicity of haemin, which catalyses free radical formation.

Table 2. Haemin sensitivity of *S. reticuli* strains

Haemin (μM)	Zone of inhibition (mm)	
	Wild-type	*hbpS* mutant
100	8·0	9·0
200	9·5	13·0
300	12·0	17·0
400	15·0	22·0

Effect of HbpS on *cpeB* expression

The *hbpS* gene is located downstream of the *cpeB* gene encoding the haem-containing catalase-peroxidase CpeB. Thus, it was speculated that HbpS might be required to form active, haem-containing CpeB. To test this assumption, *S. reticuli* wild-type and *S. reticuli hbpS* mutant were cultivated in minimal medium containing crystalline cellulose (1 % Avicel), in which high levels of the mycelium-associated enzyme are produced (Zou *et al.*, 1999). Enzymic analysis (test for peroxidase activity; Fig. 5c) as well as immunological studies (using anti-CpeB antibodies; Fig. 5b) revealed that the amount of CpeB enzyme released from the mycelia (see Methods) of the *S. reticuli* wild-type was considerably greater than from the *S. reticuli hbpS* disruption mutant strain. Further hybridization studies (Northern blot) showed that among equivalent quantities of total RNA, the level of specific *cpeB* transcripts was considerably reduced in the *S. reticuli hbpS* disruption mutant (Fig. 5d). The wild-type strain also secreted a 45 kDa (Fig. 5a) protein, which was either absent or produced at undetectable levels in the *hbpS* mutant strain. This mutant strain, however, secreted a 28 kDa (Fig. 5a) protein which was not found in the wild-type strain. The nature of these two proteins still has to be characterized.

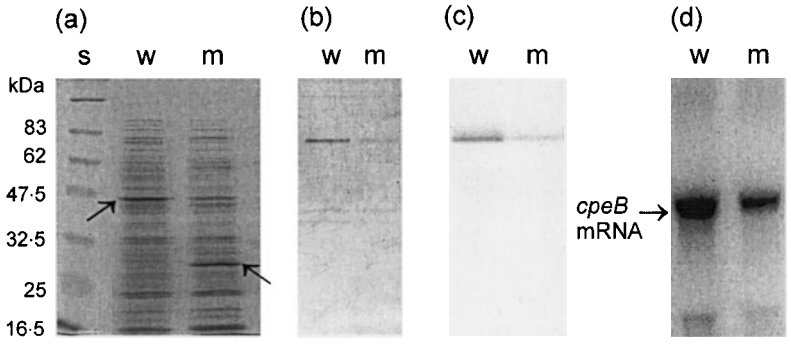


Fig. 5. Expression of *cpeB* in the *S. reticuli hpbS* mutant. *S. reticuli* wild-type (w) and *S. reticuli hbpS* mutant (m) were cultivated as described in Methods. Proteins were released from the mycelium, subjected to SDS-PAGE and either stained with Coomassie brilliant blue (a) or transferred to a nylon membrane for Western analysis using anti-CpeB antibodies (b). Protein-containing bands of 45 and 28 kDa are indicated in (a) by arrows. (c) Mycelium-associated proteins were loaded on to native polyacrylamide gel and tested for peroxidase activity as described in Methods. Protein markers are in lane S. (d) RNA was isolated from the same cultures and each aliquot (10 μg) was fractionated by gel electrophoresis, transferred to a nylon membrane and hybridized using a fragment of the *cpeB* gene as probe.

DISCUSSION

We have described herein the molecular cloning and functional characterization of a novel *S. reticuli* extracellular protein, which was subsequently shown to bind haem and therefore was named HbpS (haem-binding protein from *Streptomyces*). HbpS contains an N-terminal signal peptide with a twin arginine signature within the sequence S**RR**TRV, which diverges from the consensus sequence of the Tat motif S/T**RR**XFL (Brink *et al.*, 1998; Berks *et al.*, 2000), with an additional arginine in position +3 and a valine in position +4. Results of previous experiments had revealed that not only the twin arginines (position 0 and +1) but also the hydrophobic residues at position +3 and +4 (Sargent *et al.*, 1998) are important for translocation of substrates via the Tat pathway. The designed *S. lividans* transformant carrying an *hbpS* gene with substitutions of the two adjacent arginine codons no longer secreted HbpS but accumulated it in the cytoplasm, suggesting that HbpS may be secreted in a Tat-dependent manner. The most remarkable characteristic of the Tat pathway is that it apparently functions to transport folded proteins of variable dimensions across the cytoplasmic membrane. In most cases, the Tat substrates bind their corresponding cofactors in the cytoplasm and are thus already folded prior to export. Such proteins function predominantly in respiratory and photosynthetic electron transport chains and are vital for many types of bacterial energy metabolism (Berks *et al.*, 2000). The Tat translocation pathway in *S. lividans* was found to be encoded by *tatA*, *tatB* and *tatC* genes (Schaerlaekens *et al.*, 2001) encoding proteins which are close homologues to those characterized within *E. coli* (Bogsch *et al.*, 1998). Until now, only two *Streptomyces* proteins have been experimentally proven to be secreted via the Tat pathway. The transactivator protein MelC1 of a tyrosinase (*Streptomyces antibioticus*) and xylanase C (*S. lividans*) were not secreted in the *S. lividans tatC* mutant carrying the corresponding genes on the cloned plasmids (Schaerlaekens *et al.*, 2004). Further studies in a recently described *S. lividans* mutant lacking a functional Tat pathway (Schaerlaekens *et al.*, 2001) could help to elucidate whether HbpS is indeed a Tat substrate. Computer analysis of the *S. coelicolor* A3(2) genome reveals the existence of 230 putative Tat substrates, which are members of a variety of protein classes including a high number functioning in degradation of macromolecules, in binding and transport, and in secondary metabolism (Schaerlaekens *et al.*, 2004).

Whereas some haem-binding proteins (e.g. those of the NapC/NirT cytochrome *c* family) have the CXXCH motif, which is necessary for attaching of haem, others lack this motif. Albumin can bind several haem groups per molecule due to the presence of a hydrophobic region (Shin *et al.*, 1994). Similarly, the mouse p22 haem-binding protein contains a hydrophobic region, which is speculated to bind haem (Taketani *et al.*, 1998). The chaperone CcmE interacts with haem transiently in the periplasm of *E. coli* and delivers it to newly synthesized and exported *c*-type cytochromes. Alanine scanning mutagenesis of conserved amino acids revealed that only H130 is strictly required for haem-binding and delivery. Mutation of the hydrophobic amino acids (F37, F103, L127 and Y134) to alanine affected the interaction with haem of CcmE more than the mutation of other charged and polar amino acids. The data suggest that haem is bound to a hydrophobic platform at the surface of the protein and then attached to H130 by a covalent bond (Enggist *et al.*, 2003). In this context, it is important to mention that HbpS is rich in hydrophobic residues and contains three histidine residues (H60, H83 and H187) surrounded by leucines and valines (Fig. 1b). The crystal structure of yeast cytochrome *c* peroxidase (CCP) shows that the histidine (H) residue at position 181 interacts with haem (Finzel *et al.*, 1984). This residue is located within the motif $LX_2THLX_{10}AA$, which exhibits a similarity to the region $LX_3THLX_{10}AA$ including the histidine residue at position 60 within HbpS (Fig. 1c).

The *S. reticuli hbpS* mutant strain was found to be more sensitive than the wild-type strain to higher concentrations of haemin (Fe^{3+}-oxidized form of haem). It is known that haemin at higher concentrations is highly toxic because of its ability to catalyse free radical formation (Baker *et al.*, 2003). The enhanced sensitivity to haemin in the mutant strain correlated with the lack of HbpS. This result suggested that within the wild-type strain haemin is titrated by HbpS, leading to a reduction of free haemin.

Haem has been found to be necessary for the accumulation and assembly of cytochrome *c* oxidase in *Saccharomyces cerevisiae*, which is able to synthesize haem (Woloszczuk *et al.*, 1980). In addition, it was shown that haem is important not only for the formation of active catalases but also for the synthesis, or at least accumulation, of the apoproteins of catalases (A and T) in yeast. During these processes, haem could either act as a positive regulator of the synthesis of apocatalases or it could prevent rapid degradation of the enzyme precursors (Howe & Merchant, 1994).

Physiological studies showed that the amount of the catalase-peroxidase CpeB in the *S. reticuli hbpS* disruption mutant in comparison to *S. reticuli* wild-type was considerably lower. Within the mutant strain the amount of *cpeB* mRNA is reduced, suggesting that HbpS influences the expression of *cpeB* in a positive manner. These data lead to the following explanations: HbpS could act as chaperone that binds haem and delivers it to the mycelium-associated CpeB, or HbpS could be involved in a signal transduction cascade regulating the expression of *cpeB*. As HbpS is an extracellular protein, it could interact with extracellular or membrane-associated proteins involved in such signal transduction. Interestingly, downstream of *hbpS* two ORFs are located, which are divergently transcribed from *hbpS* and encode a predicted sensor kinase and a putative response regulator (data not shown). These proteins could be the components for the corresponding signal transduction system.

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