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

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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_10AA, which is related to the motif within the yeast cytochrome *c* peroxidase L_{2}THLX_{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.

The *GenBank/EMBL/DDBJ* accession number for the sequence of hbpS from *Streptomyces reticuli* reported in this paper is Y14336.

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 prothetic 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 prothetic 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,
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 Zn²⁺ release (Ortiz de Orué Lucana et al., 2003).

CpeB can use H₂O₂ to oxidize a number of substrates in dependence on an attached haem group (ferric-protoporphyrin) 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 H₂O₂ to O₂. Thus the mycelia-associated enzyme also plays an important part in detoxifying H₂O₂ and in minimizing reactions caused by highly reactive oxygen species arising from the interaction of H₂O₂ with certain divalent metal ions (Fe²⁺ 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. Zahnér, Tübingen, Germany) and *S. lividans* 66 (D. A. Hopwood, John Innes Institute, Norwich, UK) were used. The plasmid pUC18 (Sambrook et al., 1989) was a gift from I. 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 (Schlochermeier 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 K₂SO₄ l⁻¹, 10-12 g MgCl₂·6H₂O 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 H₂O₂ (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 (Schlochermeier 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 32P-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
and $[^{32}P]dCTP$ Conversely, the DNA region of $cpeB$ was amplified by PCR. The following primers were used: CD, 5'-AGTTCGCCGACAGTTCCGGAAGG-3', and CE, 5'-CTTGGCCACCGCCGCGCGTCG-3'. The membrane was washed twice with 2 x SSC containing 0.1% SDS at room temperature for 20 min and subsequently 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 pWK10 (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: HB1P1, 5'-CTTGGCCACCGCCGCGCGTCG-3', annealing upstream of the BamHI restriction site, followed by the sequence encoding N-terminal amino acids of Hbps; and HB2P1, 5'-AGTTCGCCGACAGTTCCGGAAGG-3', determining the C-terminal amino acids of Hbps, followed by a BamHI restriction site. The PCR product was digested with *SfiI* and BamHI. After digestion with BamHI the DNA was treated with the Klenow polymerase to get a blunt-ended fragment which was ligated with *SfiI*-Smal-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 hbps gene was amplified using the oligonucleotides HB3P1, 5'-AGTTCGCCGACAGTTCCGGAAGG-3', annealing upstream of the *BsiWI* restriction site in the middle of hbps, and HB4P1, 5'-CTTGGCCACCGCCGCGCGTCG-3', containing a *SfiI* restriction site, a stop codon and six histidine codons. Following the PCRs, the generated product was cleaved with *BsiWI* and ligated with the large *BsiWI*–*SfiI* fragment of the plasmid pUKS10 (Zou et al., 1999; Table 1). The 4.6 kb *EcoRI*–*HindIII* fragment of the resulting plasmid pUKS15 (Table 1) was ligated with *EcoRI*/*HindIII*-digested pWHM3 vector. The resulting plasmid pUKS15 (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 pUKS15 (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: HB5P1, 5'-CTTGGCCACCGCCGCGCGTCG-3', containing a *BbvI* restriction site and two lysine-encoding codons replacing the two arginine codons of the hbps wild-type gene, and HB4P1 (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 *BbvI* and *BsiWI*, resulting in a 0.32 kb mutagenized hbps fragment which was ligated with the large *BbvI*–*BsiWI* fragment of plasmid pUKS10 (Table 1). The 4.6 kb *EcoRI*–*HindIII* fragment of the resulting plasmid pUKS16 (Table 1) was ligated with *EcoRI*/*HindIII*-digested pWHM3 vector. The resulting plasmid pUKS16 (Table 1) carried an hbps 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 sequencing.

### Table 1. Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant properties*</th>
<th>Source or reference</th>
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<tr>
<td><strong>E. coli plasmids</strong></td>
<td></td>
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<tr>
<td>pUKS10</td>
<td>pUC18 derivative containing furS, cpeB, hbps and <em>amp</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Zou et al. (1999)</td>
</tr>
<tr>
<td>pUKS15</td>
<td>pUKS10 derivative carrying hbps with six histidine codons at the 3' end and <em>amp</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pUKS16</td>
<td>pUKS10 derivative with hbps containing two lysine substitutions instead of two arginine (R9 and R10) codons and <em>amp</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pUCH1</td>
<td>pUKS10 derivative carrying truncated cpeB, hbps and <em>amp</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pUCH2</td>
<td>pUCH1 derivative used for the generation of a hbps disruption mutant and containing <em>amp</em>&lt;sup&gt;+&lt;/sup&gt; and <em>hyg</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
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<tr>
<td>pQE32</td>
<td>Expression vector with a multiple cloning site and six histidine codons for the His-tag and <em>amp</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Qiagen</td>
</tr>
<tr>
<td>pQH1</td>
<td>pQE32 derivative containing hbps downstream of the His-tag</td>
<td>This work</td>
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<tr>
<td><strong>E. coli Streptomyces shuttle vectors</strong></td>
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<tr>
<td>pWHM3</td>
<td>Multiple cloning site, <em>amp</em>&lt;sup&gt;+&lt;/sup&gt; and <em>tsr</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Vara et al. (1989)</td>
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<tr>
<td>pWKS10</td>
<td>pWHM3 derivative carrying the 4.6 kb <em>KpnI</em>–<em>SphiI</em> fragment from pUKS10 containing <em>furs</em>, cpeB, hbps, <em>amp</em>&lt;sup&gt;+&lt;/sup&gt; and <em>tsr</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Zou et al. (1999)</td>
</tr>
<tr>
<td>pWKS15</td>
<td>pWKS10 derivative carrying hbps with six histidine codons at the 3' end, <em>amp</em>&lt;sup&gt;+&lt;/sup&gt; and <em>tsr</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
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<tr>
<td>pWKS16</td>
<td>pWKS10 derivative with hbps containing two lysine substitutions instead of two arginine (R9 and R10) codons, <em>amp</em>&lt;sup&gt;+&lt;/sup&gt; and <em>tsr</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
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<tr>
<td>pWKS17</td>
<td>pWKS10 derivative lacking hbps and containing <em>amp</em>&lt;sup&gt;+&lt;/sup&gt; and <em>tsr</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
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* *amp*<sup>+</sup>, ampicillin resistance gene; *tsr*<sup>+</sup>, thiostrepton resistance gene; *hyg*<sup>+</sup>, hygromycin resistance gene.
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 (OD₆₀₀ 0.6–0.8) it 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 (containing 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 Ni²⁺-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* pWK17 (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 *hbpS* gene within *S. lividans* pWKS10 (Zou *et al.*, 1999). The chromosomal DNA was disrupted by the insertion of the hygromycin resistance cassette (hyg). (b) The HbpS protein from *S. reticuli* (HbpS_ret) was aligned to an ORF from *S. coelicolor* A3(2) (Orf_coel), Atu1117 from *A. tumefaciens* C58 (Atu1117_tune), 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).
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/H₂O₂, 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 7.5; 0.25% (w/v) 3,3',5,5'-tetramethylbenzidine, 25% (v/v) methanol, 0.75% H₂O₂ [2.5% (v/v) of a commercial 30% solution]. H₂O₂ 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 EcoRI. The linearized DNA was then partially digested with Sall to obtain a 4.6-kb DNA fragment containing part of cepB, 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 HindIII fragment (2.3 kb) containing this hyg was generated. This fragment was blunt-ended using the Klenow enzyme and ligated with the BsiWI-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 (~10⁶ 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 × 10⁸) 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 paper 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 Na₂HPO₄, 8 mM NaH₂PO₄, 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 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 cepB gene is regulated by the redox regulator FurS encoded by furS, which is located upstream of cepB (Zou et al., 1999). The genomic DNA from S. reticuli downstream of cepB 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 cepB 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 pl of 9.6. Fig. 1(a) (wild-type) displays the physical map of the furS–cepB 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 cepB 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 (38-9%) (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 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.

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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/H2O2 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/H2O2 (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 haem 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/H2O2 (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/H2O2 (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$_2$O$_2$ 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**

<table>
<thead>
<tr>
<th>Haemin (μM)</th>
<th>Wild-type Zone of inhibition (mm)</th>
<th><em>hbpS</em> mutant Zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>8·0</td>
<td>9·0</td>
</tr>
<tr>
<td>200</td>
<td>9·5</td>
<td>13·0</td>
</tr>
<tr>
<td>300</td>
<td>12·0</td>
<td>17·0</td>
</tr>
<tr>
<td>400</td>
<td>15·0</td>
<td>22·0</td>
</tr>
</tbody>
</table>

**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)
was as well as immunological studies (using anti-CpeB anti-
bodies; Fig. 5b) revealed that the amount of CpeB enzyme
released from the mycelium (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
strain (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.
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 SRRTRV, which diverges from the consensus sequence of the Tat motif S/TRRXFL (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 MeIC1 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 CXCH 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 LX3THLX10AA, which exhibits a similarity to the region LX3THLX10AA 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 (Fe3+-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 (Wolosczuk 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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