Identification of a novel two-component system SenS/SenR modulating the production of the catalase-peroxidase CpeB and the haem-binding protein HbpS in *Streptomyces reticuli*

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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. The expression of the *furS-cpeB* operon depends on the redox regulator FurS and the presence of the haem-binding protein HbpS. Upstream of *hbpS*, the neighbouring *senS* and *senR* genes were identified. SenS is a sensor histidine kinase with five predicted N-terminally located transmembrane domains. SenR is the corresponding response regulator with a C-terminal DNA-binding motif. Comparative transcriptional and biochemical studies with a designed *S. reticuli* *senS/senR* chromosomal disruption mutant and a set of constructed *Streptomyces lividans* transformants showed that the presence of the novel two-component system SenS/SenR negatively modulates the expression of the *furS-cpeB* operon and the *hbpS* gene. The presence of SenS/SenR enhances considerably the resistance of *S. reticuli* to haemin and the redox-cycling compound plumbagin, suggesting that this system could participate directly or indirectly in the sensing of redox changes.

Epitope-tagged HbpS (obtained from an *Escherichia coli* transformant) as well as the native *S. reticuli* HbpS interact *in vitro* specifically with the purified SenS fusion protein. On the basis of these findings, together with data deduced from the *S. reticuli* *hbpS* mutant strain, HbpS is suggested to act as an accessory protein that communicates with the sensor protein to modulate the corresponding regulatory cascade. Interestingly, close and distant homologues, respectively, of the SenS/SenR system are encoded within the *Streptomyces coelicolor* A3(2) and *Streptomyces avermitilis* genomes, but not within other known bacterial genomes. Hence the SenS/SenR system appears to be confined to streptomycetes.

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INTRODUCTION

Streptomyces species are found worldwide in soil, exhibit a complex developmental life cycle and are important in soil ecology. These bacteria are metabolically diverse and utilize many carbon sources, including sugars, amino acids, organic acids, aromatic compounds and diverse polymers. The cellulose degrader *Streptomyces reticuli* has been 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, and its transcription is regulated by the FurS protein 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). Downstream of the *cpeB* gene, the *hbpS* gene is located in an opposite transcriptional orientation (Fig. 1; Ortiz de Orué Lucana *et al.*, 2004). Comparative physiological, biochemical and immunological studies have revealed that HbpS is extracellularly located and presents a new haem-binding protein type from *Streptomyces*.

Since *Streptomyces* species are soil-dwelling organisms, they need to respond to highly variable conditions. Bacterial adaptation to a wide variety of environmental stimuli is accomplished through the coordinated regulation of many different signal-processing pathways. Many different

Abbreviations: aa, amino acid; MW, molecular weight; RR, response regulator; SK, sensor kinase; WT, wild-type.

Fig. 1. Restriction and genetic map of a *KpnI*–*Bam*HI fragment on the chromosome of *S. reticuli* strains. The relative positions of the *furS*–*cpeB* operon and *hbpS* gene are presented. The WT genome contains intact *senS* and *senR*. The *senS*/*senR* mutant has a deleted *senS* (*senS'*) followed by a hygromycin-resistance cassette (*hyg*) and a deleted *senR* (*'senR*). The *SrfI*–*Bam*HI fragment is present in plasmid pUBB1 (Zou *et al.*, 1999).

bacterial systems for signal transduction comprise a two-component structure: a sensor kinase (SK), which is an autophosphorylating histidine protein kinase, and a response regulator (RR), which becomes activated by phosphorylation (Parkinson & Kofoid, 1992; Pao & Saier, 1995). The N-terminal part of the SK contains an input domain, and the C-terminal portion comprises a transmitter module with several blocks of amino acid residues which are conserved among SKs. The RR has a receiver domain (generally at its N-terminus) and contains conserved amino acids that define its membership within the response-regulator family. The receiver domain catalyses the transfer of a phosphoryl group from the histidine of the corresponding SK to its conserved aspartic acid residue. The phosphorylation state of the receiver modulates the activity of a unique C-terminally arranged output domain, commonly a transcriptional regulator, eliciting an adaptive response (for a review, see Parkinson, 1993).

The exploration of the *Streptomyces coelicolor* A3(2) genomic sequences has led to the identification of 84 genes which could encode SKs and 80 genes for RRs (Hutchings *et al.*, 2004). In comparison, an analysis of the complete sequence of the *Bacillus subtilis* chromosome revealed the presence of 36 genes for putative SKs and 34 genes encoding putative RRs (Fabret *et al.*, 1999). The genome of the Gram-negative bacterium *Escherichia coli* encodes 32 SKs and 23 RRs, and five hybrid SKs (Mizuno, 1997). The large number of different two-component systems within *Streptomyces* species appears to reflect their responsiveness to a wide range of environmental stimuli in their rapidly changing habitat. To date, however, only a few two-component systems have been investigated experimentally: the ChiS/ChiR (*Streptomyces thermoviolaceus* OPC-520) system is required for the formation of a chitinase (Tsujiibo *et al.*, 1999); the AbsA1/AbsA2 system of *S. coelicolor* A3(2) negatively regulates the production of several antibiotics (Brian *et al.*, 1996; Sheeler *et al.*, 2005); the PhoR/PhoP system from *Streptomyces lividans* is implicated in the regulation of a high-affinity phosphate transporter and in the production of actinorhodin and undecylprodigiosin (Sola-Landa *et al.*, 2003); the VanR/VanS system from *S. coelicolor* A3(2) activates the expression of vancomycin resistance (Hong *et al.*, 2004); and the CutR/S system

(*S. lividans*) negatively affects the synthesis of actinorhodin (Chang *et al.*, 1996).

In this report we describe the identification of genes for a novel two-component system named SenS/SenR from *S. reticuli*. Comparative genetic and biochemical experiments revealed that SenS/SenR is important to modulate the level of FurS, CpeB and HbpS.

METHODS

Bacterial strains, plasmids, media and culture conditions. *S. reticuli* T45 (H. Zhner, Tbingen, 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 J. Messing (State University of New Jersey, Piscataway, USA). The *E. coli* strains DH5 (Villarejo *et al.*, 1972), M15 (pREP4) (Qiagen) and XL-1 Blue (Loenen & Blattner, 1983), and the plasmids pWHM3 (Vara *et al.*, 1989), pQE30 (Qiagen) and pASK-IBA7+ (IBA, Gttingen, Germany) were employed for routine cloning purposes (Table 1). The constructs pUKS10, pUBB1 (pUC18 derivatives; Table 1) and pWKS10 (a pWHM3 derivative; Table 1), all of which contain the *furS*–*cpeB* operon, have been described previously (Zou *et al.*, 1999; Ortiz de Oru Lucana & Schrepf, 2000). In addition, plasmids were constructed (Table 1). For cultivation of *S. reticuli* and *S. lividans*, complete or minimal media (Schlochtermeyer *et al.*, 1992) supplemented with the indicated carbon source were used. Suspensions of spores were made as described elsewhere (Hopwood *et al.*, 1985). 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 on solid media, R2 medium (without sucrose) was used (Hopwood *et al.*, 1985). *E. coli* strains were grown in LB medium at 37 or 16 C (Sambrook *et al.*, 1989).

Chemicals and enzymes. Chemicals for SDS gel electrophoresis were obtained from Serva. Molecular weight (MW) markers, diamide, haemin and plumbagin were supplied by Sigma. Hydrogen peroxide (30%, w/v) was bought from Merck. Restriction enzymes, T4 ligase and DNA polymerase for PCR were obtained from Roche, New England Biolabs or Promega.

Isolation of DNA, transformations and hybridization experiments. Chromosomal DNA of *S. reticuli* strains was isolated after growth in a sucrose-containing complete medium for 2 days (Schlochtermeyer *et al.*, 1992). Plasmids were isolated from *E. coli* with the aid of a mini plasmid kit (Qiagen). *E. coli* DH5, XL-1 Blue and M15 (pREP4) were transformed with plasmid DNA by electroporation (Dower *et al.*, 1988). *S. lividans* protoplasts were transformed and regenerated as described by Hopwood *et al.* (1985). Transformants were selected using an overlay of 2 ml 0.4% agarose

Table 1. Plasmids and relevant properties

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

Plasmid	Relevant properties	Source or reference
<i>E. coli</i> plasmids		
pUBB1	pUC18 derivative carrying <i>furS</i> , <i>cpeB</i> , <i>hbpS</i> , <i>senS</i> , <i>senR</i> and <i>amp^r</i>	Zou <i>et al.</i> (1999)
pUKS10	pUBB1 derivative containing <i>furS</i> , <i>cpeB</i> , <i>hbpS</i> and <i>amp^r</i>	Zou <i>et al.</i> (1999)
pUS1	pUKS10 derivative carrying truncated <i>hbpS</i> , truncated <i>senS</i> and <i>amp^r</i>	This work
pUSH1	pUS1 derivative containing <i>amp^r</i> and <i>hyg^r</i>	This work
pUSHS1	pUSH1 derivative used for the generation of a <i>senS/senR</i> disruption mutant and containing truncated <i>hbpS</i> , truncated <i>senS</i> , truncated <i>senR</i> , <i>hyg^r</i> and <i>amp^r</i>	This work
pQE30	Expression vector with a multiple cloning site, six histidine codons for the His-tag and <i>amp^r</i>	Qiagen
pQS1	pQE30 derivative containing <i>senS</i> downstream of the codons for the His-tag	This work
pQH2	pQE30 derivative containing <i>hbpS</i> (without codons encoding the signal sequence) downstream of the codons for the His-tag	This work
pASK-IBA7+	Expression vector with a multiple cloning site and codons for the Strep-tag and <i>amp^r</i>	IBA
pASKS1	pASK-IBA7+ derivative containing <i>senS</i> downstream of the Strep-tag	This work
<i>E. coli</i>/Streptomyces shuttle vectors		
pWHM3	Multiple cloning site, <i>amp^r</i> and <i>tsr^r</i>	Vara <i>et al.</i> (1989)
pWKS10	pWHM3 derivative carrying the 4.6 kb <i>KpnI</i> – <i>SphI</i> fragment from pUKS10 containing <i>furS</i> , <i>cpeB</i> , <i>hbpS</i> , <i>amp^r</i> and <i>tsr^r</i>	Zou <i>et al.</i> (1999)
pWKB1	pWKS10 derivative containing <i>furS</i> , <i>cpeB</i> , <i>hbpS</i> , <i>senS</i> , <i>senR</i> , <i>amp^r</i> and <i>tsr^r</i>	This work

supplemented with 400 µg thiostrepton leading to a final concentration of 30 µg antibiotic ml⁻¹ on the plate (Hopwood *et al.*, 1985). DNA fragments of the restricted *S. reticuli* genome were transferred onto nylon membranes, 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).

PCR, DNA sequencing and computer analysis. PCR was performed with Pfu DNA polymerase. Sequencing was done as described by Zou *et al.* (1999), using the GATC kit (Beck & Pohl, 1984). 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 (<http://www.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 (Nielsen *et al.*, 1997) were deduced as described. The prediction of membrane-spanning regions was supported by the TMpred program (ExPASy server; Gasteiger *et al.*, 2003).

RNA isolation and analysis of transcripts. Spores (1.2×10^7) of *S. reticuli* [wild-type (WT) and *senS/senR* mutant] and *S. lividans* (containing the plasmids pWKB1 or pWKS10) were inoculated into 50 ml complete medium (Schlochtermeier *et al.*, 1992) and grown as a standing culture at 30 °C for 8 h and afterwards on a rotary shaker for 16 h. The cultures were washed four times in minimal medium (MM) without supplement. The mycelia from *S. reticuli* strains were suspended in 50 ml MM supplemented with Avicel (1 % final concentration) and cultivated for 6 h. Mycelia from *S. lividans* and its transformants were suspended in the same medium in which Avicel had been replaced by 0.5 % yeast extract (since they cannot use crystalline cellulose as sole carbon source) and cultivation was continued for 14 h. Samples were taken to analyse RNA and proteins (FurS, CpeB and HbpS). Genomic RNA was isolated as previously described (Ortiz de Orué Lucana & Schrempf, 2000), electrophoretically separated on 2 % agarose gels containing 2 % formamide and 1 × MOPS buffer, and subsequently transferred to a positively charged nylon membrane. For DNA–RNA hybridizations, *furS* and *hbpS* probes were radioactively labelled with the ‘Rediprime DNA labelling system’ (Amersham) using Klenow polymerase and [³²P]dCTP. Prior to this procedure, the unlabelled probe had been obtained by amplifying DNA regions corresponding to *furS* and *hbpS*, respectively, with the primer combinations Fur1

(5'-GGCGCATGCCCGCTCCCGACACCG-3') and Fur2 (5'-GGAAGCTTCCGAACTGTGCGGAACCTCG-3'), and HBP6 (5'-CAACGGATCCGACACCACGGAGGCC-3') and HBP7 (5'-GGGCAAGC TTGTGGCCGAGCACGG-3'). Following incubation at 64 °C, the membranes were washed as described previously (Ortiz de Oru Lucana & Schrempf, 2000) and then subjected to autoradiography. The corresponding transcript signals were quantified by comparing their intensities using 'Gel-Pro Analyser' software (INTAS, Germany).

Cloning of genes in *E. coli*. The longer *SrfI*-*EcoRI* fragment (comprising *hbpS*, *senS* and *senR*) of plasmid pUBB1 (Zou *et al.*, 1999) (Table 1) was ligated with the shorter *SrfI*-*EcoRI* DNA fragment (containing *furS* and *cpeB*) of plasmid pWKS10 (Zou *et al.*, 1999). The obtained plasmid was named pWKB1 (Table 1). The *senS*-coding region of pWKB1 was amplified by PCR using the primers: SenS1, 5'-CTTTGGATCCGTGCCGACGGTGGCCGTAC-3', consisting of an *Bam*HI restriction site (underlined), followed by the sequence encoding the N-terminal amino acids of SenS; and SenS2, 5'-GTATAAGCTTTCATCTCGGCTCCAACG-3', determining the C-terminal amino acids of SenS, followed by the *Hind*III restriction site (underlined). The PCR product was digested with *Bam*HI and *Hind*III and ligated with *Bam*HI/*Hind*III-digested pQE30 (Table 1) or *Bam*HI/*Hind*III-digested pASK-IBA7+ (Table 1). The resulting plasmids pQS1 or pASKS1 (Table 1), respectively, were transformed into *E. coli* XL-1 Blue. Having sequenced the plasmids, the correctness of the designed *senS* gene and its in-frame fusion with the His-tag or Strep-tag codons were confirmed.

The *hbpS*-coding region (without the codons encoding the signal peptide) of the above-described construct pWKB1 was amplified by PCR with primers: HBP6, 5'-CAACGGATCCGACACCACGGAGGCC-3', consisting of a *Bam*HI restriction site (underlined), followed by the sequence encoding the N-terminal amino acids of the mature HbpS (without the signal peptide); and HBP7, 5'-GGGCAAGCTTGTGGCCG AGCACGG-3', determining the C-terminal amino acids of HbpS, followed by the *Hind*III restriction site (underlined). The PCR product was digested with *Bam*HI and *Hind*III, ligated with *Bam*HI/*Hind*III-cleaved pQE30 (Table 1) and subsequently transformed into *E. coli* XL-1 Blue. Having sequenced the resulting plasmid pQH2, the correctness of the *hbpS* gene and its in-frame fusion with the His-tag codons were confirmed.

Purification of the fusion proteins and generation of antibodies. Each of the above-described plasmids pQS1 and pQH2 was transformed into *E. coli* M15 (pREP4). A selected transformant containing the pQS1 plasmid was inoculated in LB medium with ampicillin (100 µg ml⁻¹) and kanamycin (50 µg ml⁻¹). Cultivation was carried out at 16 °C to avoid the formation of inclusion bodies, which had been found to occur at higher temperatures (data not shown). The synthesis of the His-tag-SenS fusion protein was induced by the addition of 0.5 mM IPTG after the culture had reached an OD₆₀₀ of 0.6. The cells were grown for 8 h, harvested, washed with chilled solution A (10 mM HEPES, 60 mM KCl, pH 8.0) and disrupted by ultrasonication (Branson sonifier, 5 × 10 s, with 10 s intervals). Purification of the fusion protein was performed under native conditions using Ni²⁺ affinity chromatography according to the instructions of the manufacturer (Qiagen). From 1 l of culture, 0.05 mg His-tag-SenS was obtained. For further purification and concentration, the His-tag-SenS protein was recovered from a preparative SDS-polyacrylamide gel by using an electro-elution cell according to the instructions of the manufacturer (Bio-Rad). The purified protein (150 µg) was used to generate antibodies in guinea pigs (EUROGENTEC, Belgium). The antisera were then directly used for protein-protein interaction studies (see below).

E. coli M15 (pREP4, pQH2) was cultivated in LB medium with ampicillin (100 µg ml⁻¹) and kanamycin (50 µg ml⁻¹) at 37 °C. After

the culture had reached an OD₆₀₀ of 0.6, the synthesis of His-tag-HbpS was induced by adding 0.5 mM IPTG and the cultivation was continued for 3 h. His-tag-HbpS was purified from the cytoplasm by affinity chromatography, as described previously (Ortiz de Oru Lucana & Schrempf, 2000). From 1 l of culture, 2 mg His-tag-HbpS was obtained.

SenS was purified as a Strep-tag fusion protein from an *E. coli* XL-1 Blue transformant (carrying the plasmid pASKS1 containing the *senS* gene), which had been cultivated in LB medium containing ampicillin (100 µg ml⁻¹) at 16 °C. The synthesis of the protein was induced (at an OD₆₀₀ of 0.6) by adding anhydrotetracycline (200 ng ml⁻¹). The produced protein was purified from cleared cell lysates using streptactin affinity chromatography, according to the instructions of the manufacturer (IBA). Per 1 l of culture, 0.1 mg Strep-tag-SenS was obtained.

Protein-protein interactions. The Strep-tag-SenS fusion protein was used to test the interaction with HbpS by affinity chromatography. Firstly, Strep-tag-SenS was purified as described above and dialysed against buffer W (100 mM Tris/HCl, pH 8.0, 150 mM NaCl) and loaded onto a streptactin column. Non-specifically bound proteins were washed off and the Strep-tag-SenS protein was kept immobilized. In parallel, the synthesis of His-tag-HbpS was induced in the recombinant *E. coli* M15 (pREP4, pQH2). Its cytoplasmic extract (comprising His-tag-HbpS) and concentrated proteins of the culture filtrate from *S. reticuli* were then separately loaded onto the streptactin column containing Strep-tag-SenS. As a control, the protein extracts were loaded onto the streptactin column without immobilized Strep-tag-SenS. Each column was then washed five times with buffer W, and bound proteins were eluted using desthiobiotin according to the instructions of the manufacturer (IBA).

For affinity blotting (named 'Far Western' or 'Blot overlay analysis'; Hall, 2004) 1 µg of the purified proteins (His-tag-SenS and His-tag-HbpS) was fractionated separately via SDS-PAGE, transferred to a PVDF membrane and preincubated for 2 h in PBS buffer (40 mM disodium hydrogen phosphate, 8 mM sodium dihydrogen phosphate, 150 mM sodium chloride, pH 7.4) containing 5% skimmed milk powder. Purified His-tag-SenS was added in PBS buffer to a final concentration of 1 µg ml⁻¹ and incubated for 16 h at room temperature. As a control, one membrane containing lanes of the transferred proteins was incubated with PBS buffer without the addition of His-tag-SenS. Membranes were then washed three times with PBS buffer and incubated with anti-SenS antibodies in PBS buffer. After treatment with secondary anti-guinea-pig antibodies conjugated with alkaline phosphatase, the membrane was stained with 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium (Blake *et al.*, 1984).

Generation of the *S. reticuli* *senS*/*senR* disruption mutant.

The plasmid pUKS10 (Zou *et al.*, 1999) was digested with *Bsi*WI and *Sph*I. The 1142 bp DNA fragment (containing a portion of *hbpS* and part of *senS*; Fig. 3) was treated with Klenow enzyme and then ligated with the vector pUC18, which had been digested with *Hinc*II and *Sph*I. The resulting construct was named pUS1 (Table 1). A *Hind*III fragment (2.3 kb) of a pBR322 derivative with a hygromycin-resistance cassette (*hyg*) containing terminator sequences (Blondelet-Rouault *et al.*, 1997) was blunt-ended using the Klenow enzyme and ligated with the *Sph*I-digested and blunt-ended pUS1. The resulting plasmid, pUSH1 (Table 1), containing *hyg* was then digested with *Hind*III, blunt-ended and ligated with the blunt-ended *Pst*I-*Pst*I fragment (1105 bp) from the pWKB1 plasmid containing part of *senR* and its downstream region (Fig. 3). The ligation mixture was added to electrocompetent *E. coli* DH5α cells. Hygromycin-resistant *E. coli* transformants were selected, and the correctness of their plasmid constructs was analysed with restriction enzymes. One of the correct constructs, named pUSHS1, was isolated. A 10 µg

portion thereof was denatured (0.2 M NaOH, 10 min, 37 °C), chilled on ice and neutralized by rapid addition of HCl. The DNA was then used to transform 50 µl of protoplasts ($\sim 10^9$ ml⁻¹) generated from *S. reticuli*, which were spread onto osmotically stabilized medium (R2 medium containing 15% sucrose) 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), leading to a final concentration of 75 µg antibiotic ml⁻¹ on the plate. Hygromycin-resistant colonies were restreaked several times, and then their genomic DNA was analysed with respect to the size of fragments carrying the hygromycin-resistance gene.

Inhibition tests. The sensitivity of *S. reticuli* WT and *senS/senR* mutant to haemin, H₂O₂, the thiol-specific oxidant diamide and the redox-cycling substance plumbagin was determined using a disc inhibition assay. Spores (5×10^8) in a 100 µl suspension were added to 3 ml soft agar (Sambrook *et al.*, 1989), poured onto the respective R2 plates and allowed to solidify. On the top of this plate, sterile 6 mm diameter paper discs (Schleicher & Schuell) were placed which contained 20 µl of each of the following solutions: haemin (300 µM), H₂O₂ (250 mM), diamide (250 mM) and plumbagin (100 mM). Plates were incubated overnight at 30 °C and 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 10% SDS-PAGE and transferred to a PVDF membrane (Sambrook *et al.*, 1989), which was blocked for 1 h at room temperature with PBS containing 5% skimmed milk powder and subsequently incubated overnight at 4 °C with the anti-CpeB antibodies previously described (Zou *et al.*, 1999). After treatment with secondary anti-rabbit antibodies conjugated with alkaline phosphatase, the membrane was stained with 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium (Blake *et al.*, 1984). The corresponding protein signals were quantified by comparing their intensities using 'Gel-Pro Analyser' software (INTAS).

RESULTS

senS and *senR* genes encode a novel two-component system

Sequence analysis of a previously cloned stretch of genomic *S. reticuli* DNA (within the plasmid pUBB1; Zou *et al.*, 1999) revealed upstream of the *hbpS* gene the presence of two neighbouring *Orfs* of 1196 bp and 653 bp (see Fig. 1 and EMBL: Y14336). These were named *senS* and *senR*, respectively. The *senS* gene is separated by 191 bp from the *hbpS* gene, which is located in an opposite orientation. The deduced SenS protein has 398 amino acids with a predicted MW of 42.2 kDa, a theoretical isoelectric point (pI) of 7.96 and five possible membrane-spanning domains (Fig. 2a). Multiple sequence alignments (Fig. 2a) suggested that the deduced protein may encode a histidine kinase (SenS) which carries, in addition to individually conserved amino acids, two shorter regions with several identical amino acids, including two histidine residues (positions 199 and 315) and an asparagine residue (position 311). SenS shares 68% and 34% amino acid (aa) identity, respectively, with a putative histidine kinase (OrfS) from *S. coelicolor* A3(2) and with the described histidine kinase ChrS from *Corynebacterium diphtheriae* (Schmitt, 1999). Using the sequence alignments (Parkinson, 1993), the histidine

residue at position 199 within SenS was predicted to be the phosphorylation site.

The stop codon (TGA) of *senS* overlaps the start codon of *senR* (ATG). The deduced SenR protein has 217 aa, with a predicted MW of 23.2 kDa and a theoretical pI of 6.60. The deduced N-terminal region of SenR shares aspartic acid residues (in positions 19, 20, 60 and 65), as well as their relative positioning, with characterized RRs, including ChrA (Schmitt, 1999). A phosphoryl group is predicted to be transferable to aspartic acid residue 65 in SenR. The C-terminal region of SenR contains a predicted helix–turn–helix (HTH) (Fig. 2b) which has similarities to members of the HTH_LUXR_FAMILY; thus SenR is probably the RR for the predicted SK SenS. SenR shares the highest (80%) aa identity to a putative RR (OrfR) from *S. coelicolor* A3(2) and 50% aa identity to the described RR ChrA from *C. diphtheriae* (Schmitt, 1999). The genes *chrA* and *orfR* are located downstream of the genes *chrS* and *orfS*, respectively (data not shown), and are predicted to encode the corresponding RRs for ChrS and OrfS, respectively.

Role of SenS/SenR in *S. lividans* transformants

The *senS* and *senR* genes are located upstream of the *hbpS* gene encoding the haem-binding protein HbpS and downstream of the *furS–cpeB* operon encoding the redox-dependent FurS regulator protein and the haem-containing catalase-peroxidase CpeB. Thus, it has been speculated that SenS and SenR as a two-component system might influence the production of these proteins. Due to the relatively low abundance of FurS, CpeB and HbpS in *S. reticuli* (Ortiz de Oru Lucana & Schrempf, 2000; Ortiz de Oru Lucana *et al.*, 2004), the multicopy plasmids pWKS10 containing a truncated *senS* without *senR* (Fig. 3, Table 1) and pWKB1 carrying *senS* and *senR* (Fig. 3, Table 1) were transformed in *S. lividans* protoplasts.

Each of the selected transformants containing the correctly designed plasmids was cultivated in minimal medium supplemented with yeast extract, as described in Methods. Interestingly, comparative RNA analysis showed that the amount of *furS* mRNA (Fig. 4a) and *hbpS* mRNA (Fig. 4b) was considerably higher in *S. lividans* containing pWKS10 than in *S. lividans* with pWKB1. Further immunological studies revealed that *S. lividans* containing pWKS10 produced clearly higher amounts of the proteins FurS (Fig. 4c), CpeB (Fig. 4d) and HbpS (Fig. 4e). The data imply that the *senS* and *senR* genes negatively influence the expression of the *furS–cpeB* operon and the *hbpS* gene.

Comparative studies of *S. reticuli* WT and *senS/senR* disruption mutant

To analyse the role of SenS/SenR within the *S. reticuli* WT strain, the corresponding chromosomal genes were disrupted by insertional inactivation. To achieve this, the plasmid pUSHS1 containing the hygromycin-resistance cassette (*hyg*) flanked by residual portions of *senS* and

Fig. 2. Alignments of comparisons of SenS and SenR with members of other two-component systems. (a) The SenS protein from *S. reticuli* (SenS_retic; EMBL Y14336) was aligned to the putative sensor kinase OrfS from *S. coelicolor* (OrfS_coeli; UniProt/TrEMBL Q9KXW6) and the sensor kinase ChrS from *C. diphtheriae* (ChrS_dipht; EMBL AF161327). The transmembrane domains (TM1–TM5) are underlined. The extracytoplasmic loops (ECL1 and ECL2) are shown in white type on a black background. The cytoplasmic domains are marked with colons. The histidine (H) residues at positions 199 and 315, as well as the asparagine (N) residue at position 311 in the cytoplasmic domain of SenS and the corresponding positions in OrfS and ChrS are written in bold white type on a black background. (b) The SenR protein from *S. reticuli* (SenR_retic; EMBL Y14336) has been aligned with the deduced response regulators OrfR from *S. coelicolor* (OrfR_coeli; UniProt/TrEMBL Q9KXW5) and ChrA from *C. diphtheriae* (ChrA_dipht; EMBL AF161327). The aspartic acid residues at positions 19, 20, 60 and 65 in SenR and the corresponding ones in OrfR and ChrA are written in bold white type on a black background. The DNA-binding domain is indicated and underlined.

senR was constructed (see Methods and Table 1). The *S. reticuli* WT strain was transformed with the denatured plasmid pUSHS1; subsequently, several hygromycin-

resistant colonies were obtained. As revealed by Southern hybridization (data not shown), *hyg* was found to disrupt the reading frame of *senS* and *senR* (Fig. 1), and hence the

Fig. 3. Characteristics of the plasmids. The plasmids are derived from the bifunctional pWHM3 vector. pWKB1 contains the genes *furS*, *cpeB*, *hbpS*, *senS* and *senR*, whereas pWKS10 differs in having a truncated *senS* (shown by the dashed line) and lacking *senR*.

Fig. 4. Analysis of transcripts and gene products in *S. lividans* transformants and *S. reticuli* strains. *S. lividans* transformants (left) carrying the plasmids pWKS10 and pWKB1 and the *S. reticuli* *senS/senR* mutant (right) and *S. reticuli* WT (right) were cultivated as described in Methods and used for the detection of specific mRNA (a, b, f and g) and proteins (c, d, e, h, i and j). Aliquots (15 µg) of total RNA were separated by gel electrophoresis (a, b, f and g, left) transferred to a nylon membrane and hybridized with the *furS* gene (a and f, right) or the *hbpS* gene (b and g, right). Cytoplasmic proteins were precipitated with (NH₄)₂SO₄ (fraction 30–60%, w/v) and separated by SDS-PAGE followed by Coomassie staining (c and h, left), and then analysed by Western blot using anti-FurS antibodies (c and h, right). Aliquots of released mycelia-associated proteins (d and i, left) were tested for the presence of CpeB by Western blot analysis using anti-CpeB antibodies (d and i, right). Precipitated proteins from the culture filtrates were separated by SDS-PAGE followed by Coomassie staining (e and j, left) and analysed for the presence of HbpS (Western blot) using anti-HbpS antibodies (e and j, right).

independent colonies must have arisen by double crossover. The characteristics of substrate and aerial mycelia, as well as spore formation, were identical in the mutant and WT strains.

Further comparative studies were performed with a representative *S. reticuli* *senS/senR* mutant and the WT strain. Hybridization studies (Northern blot) showed that among equivalent quantities of total RNA, the level (about 12-fold; for estimation see Methods) of specific *furS*–*cpeB* transcripts was enhanced (Fig. 4f) and the level (about sevenfold; for estimation see Methods) of specific *hbpS* transcripts was enhanced (Fig. 4g) in the *S. reticuli* *senS/senR* disruption mutant. Immunological studies using anti-FurS antibodies revealed that the proteins of the cytoplasmic fraction [enriched by fractionated (NH₄)₂SO₄ precipitation] from the *S. reticuli* *senS/senR* disruption mutant contained higher amounts (about eightfold; for estimation see Methods) of FurS than the WT strain (Fig. 4h). As detected with anti-CpeB antibodies, the *S. reticuli* *senS/senR* mutant produced considerably higher quantities (about eightfold; for estimation see Methods) of the mycelium-associated CpeB than the WT strain (Fig. 4i). Further immunological studies using anti-HbpS antibodies revealed that the proteins of the culture filtrate [enriched by (NH₄)₂SO₄ precipitation] of the *S. reticuli* *senS/senR* disruption mutant contained considerably higher amounts (about eightfold; for estimation see Methods) of HbpS than those of the WT (Fig. 4j). These data clearly revealed that the presence of the two-component system SenS/SenR dictates relatively low levels of FurS, CpeB and HbpS within *S. reticuli*.

S. reticuli WT and the *senS/senR* mutant differ in sensitivity to different compounds

As shown recently, the production of HbpS in *S. reticuli* WT is induced by haemin (the Fe³⁺ oxidation product of haem) (Ortiz de Oru Lucana *et al.*, 2004). Haemin possesses significant antibacterial activity that is augmented by the presence of physiological concentrations of hydrogen peroxide or a reducing agent (Stojiljkovic *et al.*, 2001). Compared to the WT strain, the relative sensitivities of the mutant strain to haemin (300 µM) and the redox-cycling substance plumbagin (100 mM) showed increases of 66 and 41 %, respectively (Table 2). The presence of H₂O₂ (250 mM) or the thiol-specific oxidant diamide (250 mM) led to a relatively small enhancement of sensitivity (26 and 12 %, respectively; Table 2).

SenS interacts with HbpS

Our previous data have shown that the haem-binding protein HbpS is secreted (Ortiz de Oru Lucana *et al.*, 2004). Due to the relative positioning of *hbpS* and *senS/senR* (see Fig. 1) and the above outlined results we considered the possibility that HbpS could interact with SenS and therefore could participate in the signal transduction cascade. To obtain SenS and HbpS, the corresponding genes were fused with codons encoding a His-tag or a Strep-tag (only in the

Table 2. Sensitivity of *S. reticuli* strains to haemin, plumbagin, H₂O₂ and diamide

Substance (concentration)	Diameter of zone of inhibition (mm)*		Relative increase of sensitivity for the <i>senS/senR</i> mutant
	WT	<i>senS/senR</i> mutant	
Haemin (300 µM)	12	20	66 %
Plumbagin (100 mM)	24	34	41 %
H ₂ O ₂ (250 mM)	30	38	26 %
Diamide (250 mM)	32	36	12 %

*The assays were repeated four times.

case of *senS*) (see Methods) located on a pQE30 or pASK-IBA7+ vector, respectively. Having optimized expression conditions (see Methods), each of the fusion proteins was isolated in soluble form from the cytoplasm of the corresponding *E. coli* transformant. The Strep-tag-SenS fusion protein (data not shown) was immobilized on the streptactin resin. Proteins from the cytoplasm of the induced *E. coli* M15 (pREP4, pQH2) producing the His-tag-HbpS were added onto this resin. Detachment (with desthiobiotin) of Strep-tag-SenS led to an additional release of HbpS but no other proteins (Fig. 5a). As a control, the same procedure was done in the absence of Strep-tag-SenS; in this case, no His-tag-HbpS was released (data not shown). Using the same strategy, the Strep-tag-SenS fusion protein was shown to interact with an extracellular *S. reticuli* protein (16 kDa; Fig. 5b) that reacts with the anti-HbpS antibody (Fig. 5c).

For affinity blotting, each of the purified proteins (Fig. 5d) was separated by SDS-PAGE and transferred to PVDF membranes. One strip was then incubated with the purified His-tag-SenS protein (Fig. 5e) and the second one (Fig. 5f) without additional His-tag-SenS. Having washed the strips (to remove unbound His-tag-SenS), the results of immunological studies (with anti-SenS antibodies) revealed that the immobilized His-tag-HbpS interacts with His-tag-SenS protein (Fig. 5e). As expected, no cross-reactivity was obtained with HbpS alone (Fig. 5f). Taken together, the presented results clearly show that the HbpS protein interacts *in vitro* specifically with the sensor kinase SenS.

DISCUSSION

The studies described here provide evidence that the novel two-component system SenS/SenR influences the production of the proteins FurS, CpeB and HbpS by *S. reticuli*. The deduced SenS shares some similar features with known SKs. Among the characterized SKs, ChrS (from *C. diphtheriae*; Schmitt, 1999) is most related (34 % aa identity). SenS shows high aa identity (68 %) to a so-far-uncharacterized protein (OrfS) encoded within the *S. coelicolor* A3(2) genome (Bentley *et al.*, 2002) and 40 % aa identity with one

Fig. 5. Interaction between SenS and HbpS. Soluble protein extracts containing His-tag-HbpS obtained from *E. coli* M15 (pREP4, pQH2) (a) and precipitated extracellular proteins from *S. reticuli* WT (b and c) were loaded onto a streptactin column containing the isolated Strep-tag-SenS. Fractions containing unbound proteins (a, b and c, lane 1) and the four wash fractions (a, b and c, lanes 2–5) were collected. Bound proteins were eluted with buffer W containing 2.5 mM desthiobiotin (a, b and c, lane 6). Samples were subjected to SDS-PAGE and stained with Coomassie brilliant blue (a) or silver (b). The separated proteins from (b) were also transferred onto a PVDF membrane and incubated with anti-HbpS antibodies (c). (d–f) Affinity blotting studies. (d) His-tag-SenS (lane 1) and His-tag-HbpS (lane 2) were isolated by Ni^{2+} -NTA affinity chromatography as described in Methods, subjected to SDS-PAGE and stained with Coomassie brilliant blue. (e) His-tag-SenS (lane 1) and His-tag-HbpS (lane 2) were separated by SDS-PAGE, transferred to a PVDF membrane and incubated with His-tag-SenS. Bound His-tag-SenS was detected with anti-SenS antibody. (f) As a control, the same procedure [see (e)] was repeated, but the incubation with His-tag-SenS was left out. The double SenS band (e and f, lane 1) represents the complete protein and a slightly N-terminally truncated form.

encoded in the *S. avermitilis* genome (Ikeda *et al.*, 2003). SenS, ChrS and OrfS contain five predicted transmembrane domains. The deduced extracytoplasmic loop regions of SenS and ChrS do not share relevant aa identities, and their predicted membrane-spanning regions have in common some short, related motifs (Fig. 2a). Extracytoplasmic loop regions between transmembrane helices have been proposed to be involved in the detection of environmental stimuli by other SKs (Parkinson & Kofoed, 1992), and it is also possible that regions between the putative membrane-spanning regions play a role. The cytoplasmic domains of SenS and ChrS show a few related motifs (Fig. 2a). It has been assumed that the SK ChrS is involved in the detection of haem and the transduction of this signal, via a phospho-transfer mechanism, to the RR ChrA (Schmitt, 1999).

The central part of SenS contains a histidine residue (H199), which is predicted to be phosphorylated, as its position corresponds to that of other SKs. The SKs have been classified in five groups (I, II, IIIA, IIIB and IV) according to the relative positioning of the 16 aa residues surrounding the conserved histidine residue (Fabret *et al.*, 1999). Taking these criteria into account, SenS belongs to group II (data not shown). Based on the relatedness of output domains (Fabret *et al.*, 1999), SenR is a member of RR group II. Each

known group II SK gene is upstream of the gene encoding the corresponding RR and in the same orientation within the transcription unit. SenR harbours aspartic acid residues (in positions 19, 20, 60 and 65), which are conserved among RRs (Baikalov *et al.*, 1996) and which probably form an acid pocket that plays a role in the signal-transduction cascade; thus, the phosphoryl group of SenS is predicted to be transferable to aspartic acid residue (D) 65. The relative organization of the *senS* gene and the *senR* gene is identical to that of homologous genes within the *S. coelicolor* A3(2) genome (Bentley *et al.*, 2002), which, interestingly, are preceded by an uncharacterized gene that is closely related to *hbpS*. However, the *furS* and *cpeB* homologues (*furA* and *catC*) from *S. coelicolor* A3(2) are located far apart from the *hbpS* homologue (Fig. 6a).

The analysis of *S. reticuli* WT, the corresponding *senS/senR* disruption mutant and the designed *S. lividans* transformants revealed that the presence of SenS/SenR leads to a significant reduction of *furS*, *cpeB* and *hbpS* transcript levels and of the corresponding proteins. Interestingly, the *S. reticuli* mutant strain exhibits an increased sensitivity to haemin as well as the redox-cycling compound plumbagin, which is known to generate reactive oxygen species. Thus, the SenS/SenR system could participate directly or indirectly

Fig. 6. Gene organization and comparison of DNA regions. (a) The gene organization of *furS*, *cpeB*, *hbpS* and *senS/senR* on the *S. reticuli* genome is compared with a region from the *S. coelicolor* A3(2) genome containing the deduced homologues (not studied) *orf*, *orfS* and *orfR*. Homologous genes to *furS* and *cpeB* are located elsewhere in the genome. (b) The upstream DNA region (0.2 kb) of the *S. reticuli furS* gene (up_ *furS*) was compared with the region (0.2 kb) upstream of the *S. reticuli hbpS* gene (up_ *hbpS*). The corresponding start codons (ATG) are shown in bold type; identical bases are marked by an asterisk.

in sensing signals generated by redox-triggered events. This conclusion is in line with the observation that the sensitivity to H_2O_2 and diamide differs only a little between the WT and the mutant strain.

The upstream regions of *furS* and *hbpS* (Fig. 6b) both share a few motifs (i.e. ATTGTGC, CATC, AGGTTC and GCATG) that could participate in the interaction with a common regulator protein; this could be SenR (or its modified form) or a so-far-unknown regulatory protein, whose transcription would be predicted to be under the control of SenR. For instance, the two-component system ResD/ResE from *B. subtilis* influences indirectly the expression of *arfM* (anaerobic respiration and fermentation modulator), being initially activated by a signal cascade in which the corresponding RR (ResE) activates firstly the synthesis of the redox regulator Fnr (Marino *et al.*, 2001), which subsequently activates the transcription of *arfM*. Finally, ArfM modulates the expression of genes encoding proteins which further sustain nitrate respiration, such as genes for haem biosynthesis.

Previously, we reported that the presence of the *hbpS* gene increases the synthesis of the highly active CpeB (Ortiz de Oru Lucana *et al.*, 2004). The catalase and peroxidase activities of CpeB are also dependent on a properly attached prosthetic haem group (Zou & Schrempf, 2000). Therefore, it can be suggested that HbpS could act as a chaperone that binds haem and then delivers it to CpeB, and/or that it might also interact with extracellular, membrane-associated or membrane-integrated protein(s) involved in a signal

transduction cascade. The present analysis revealed that HbpS interacts specifically with SenS. Thus, the specific interaction of HbpS with SenS is expected to be of importance for the corresponding signal-transduction cascade *in vivo*. This conclusion is supported by our previous findings (Ortiz de Oru Lucana *et al.*, 2004) showing that an *S. reticuli* mutant lacking a functional HbpS has a considerably reduced level of CpeB. To date, only a few bacterial two-component systems have been shown to be modulated by accessory proteins. In *Sinorhizobium meliloti*, the protein FixT acts as an inhibitor of the sensor haemoprotein kinase FixL, preventing the production or accumulation of its phosphorylated form (Garnerone *et al.*, 1999). The FixL/J system plays a major role in inducing genes for nitrogen fixation and respiration in response to a low O_2 concentration within the nodule. The gene *fixT* clusters with *fixJ/fixL*. As shown previously (Ortiz de Oru Lucana *et al.*, 2004), HbpS shares aa identities (38.3 %) to a predicted protein Atu1117 from *Agrobacterium tumefaciens*. The corresponding gene is located in the direct vicinity of the genes *nwsB* (former name *atu1116*), encoding a putative RR, and *fixL* (former name *atu1115*), encoding a putative SK, which together are homologous to the two-component system FixL/FixJ (Fischer, 1994). Sensing of the signal and the kinase activity of FixL depends on the spin state of the Fe ion within the haem. A change from a high to low spin state within the Fe ion is induced upon binding of oxygen and this is thought to result in a slight shift in its position within the porphyrin ring (Bauer *et al.*, 1999). Further genes, encoding the two-component systems *compB/compA* (*B. subtilis*) and

comD/comE (*Streptococcus pneumoniae*), are preceded by *comX* (Magnuson *et al.*, 1994) and *comC* (Pestova *et al.*, 1996), respectively, which encode small peptides playing a part in the signal-transduction cascade.

As mentioned above, the sensor kinase FixL utilizes haem as a co-factor to facilitate a conformational change for adapting to redox changes and control the corresponding gene expression. The bound haem group in the HbpS-SenS complex could assist in sensing redox changes. In the future it will be interesting to gain new insights about a special role for SenS/SenR within streptomycetes.

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