Amino Acids (2009) 37:681–691
DOI 10.1007/s00726-008-0188-5

ORIGINAL ARTICLE

The heme-binding protein HbpS regulates the activity of the *Streptomyces reticuli* iron-sensing histidine kinase SenS in a redox-dependent manner

Gabriele Bogel · Hildgund Schrempf · Darío Ortiz de Orué Lucana

Received: 26 August 2008 / Accepted: 1 October 2008 / Published online: 19 October 2008

Abstract The SenS/SenR system of *Streptomyces reticuli* regulates the expression of the redox regulator FurS, the catalase-peroxidase CpeB and the heme-binding protein HbpS. SenS/SenR is also proposed to participate in sensing redox changes, mediated by HbpS. Here, we show in vitro that heme-free HbpS represses the autokinase activity of SenS; whereas hemin-treated HbpS considerably enhances SenS autophosphorylation under redox conditions using either H_2O_2 or DTT. The presence of iron ions alone or in combination with H_2O_2 or DTT also leads to significantly increased phosphorylation levels of SenS. Further comparative physiological studies using the *S. reticuli* WT, a *S. reticuli hbpS* mutant and a *S. reticuli senS-senR* mutant corroborates the importance of HbpS and the SenS/SenR system for resistance against high concentrations of iron ions and hemin in vivo. Hence SenS/SenR and HbpS act in concert as a novel three-component system which detects redox stress, mediated by iron ions and heme.

Keywords *Streptomyces* · Two-component system SenS/SenR · Heme-binding protein HbpS · Redox stress

Introduction

Two-component signal transduction systems (TCS) are one of the most important mechanisms by which bacteria sense, respond, and adapt to changes in their environment or in their intracellular state. These signalling systems typically consist of a sensor histidine kinase (SK), which is autophosphorylated upon signal recognition, and one or more cognate response regulators (RR) which alter their DNA binding behaviour upon phosphorylation (Hoch and Varughese 2001). A single bacterium may encode for 10–50 and sometimes even up to 200 TCS (Laub and Goulian 2007). For instance, the complete genome sequence of the soil-dwelling *Streptomyces coelicolor* A3(2) comprises 84 SK- and 80 RR-genes (Hutchings et al. 2004) suggesting that this organism might be well equipped to respond to highly variable environmental conditions.

In the cellulose degrader *Streptomyces reticuli* the TCS SenS/SenR has been recently identified. The SK SenS comprises five predicted transmembrane spanning regions and is phosphorylated at a conserved histidine residue. It is able to transfer its phosphate group to the cognate RR SenR. Upon phosphorylation SenR binds with high affinity to the upstream regions of the *senS-senR* operon and *hbpS*, which encodes for a secreted heme-binding protein (HbpS). The phosphatase activity of SenS leads to the dephosphorylation of SenR, which binds upstream of the *furS-cpeB* operon encoding for the redox regulator (FurS) and the mycelia associated catalase-peroxidase (CpeB), respectively (Bogel et al. 2007; Ortiz de Orué Lucana and Schrempf 2000). The presence of the SenS/SenR system was found to considerably enhance the resistance of *S. reticuli* to hemin, hydrogen peroxide and the redox cycling compound plumbagin, leading to the assumption that this TCS is important for sensing redox changes (Ortiz de Orué Lucana et al. 2005).

Iron is the fourth most abundant element in the earth crust and is essential for nearly all bacteria. The presence of oxygen and highly reactive transition metal ions, such as

G. Bogel · H. Schrempf · D. Ortiz de Orué Lucana (✉)
FB Biologie/Chemie,
Angewandte Genetik der Mikroorganismen,
Universität Osnabrück, Barbarastr. 13,
49069 Osnabrück, Germany
e-mail: ortiz@biologie.uni-osnabrueck.de

iron, frequently leads to formation of redox stress. Thus, while bacteria have to ensure that enough iron ions are present for the diverse biochemical reactions, they also have to avoid their harmful effects. The same is true for the iron-containing porphyrin heme, which is important for a variety of biological processes, including oxygen carriers, redox enzymes and regulatory proteins. However, at higher concentrations hemin is highly toxic due to its ability to catalyze free radical formation (Baker et al. 2003).

Redox-sensing systems are often related to oxygen-sensing mechanisms and are widely distributed along bacteria, yeast and metazoans (Cash et al. 2007). In *E. coli* the TCS ArcA/ArcB was shown to sense anoxic or low redox conditions in dependence on the redox state of its cysteine residues, and the VicR/VicK system of *Streptococcus mutans* was found to be responsible for protection against oxidative stress; but the exact sensing mechanism is still unknown (Malpica et al. 2004; Deng et al. 2007).

In *S. reticuli* the sensing of certain redox changes requires HbpS in addition to the SenS/SenR system. HbpS is an extracellular protein, which is secreted in a Tat-dependent manner (Ortiz de Orué Lucana et al. 2004). It exhibits specific heme-binding ability and is able to interact with the SK SenS in vitro (Ortiz de Orué Lucana et al. 2004, 2005). Therefore, HbpS was proposed to act as an accessory protein which communicates with the sensor protein to modulate the downstream regulatory cascade (Ortiz de Orué Lucana et al. 2005). The influence of accessory molecules or proteins on the activity of SKs has been shown for other TCS. For example, the PhoQ/PhoP system of *Salmonella typhimurium* is activated by binding of antibacterial peptides to the SK, and the activity of the SK CpxA of the *E. coli* CpxA/CpxR system can be regulated by the lipoprotein NlpE (Prost et al. 2008; DiGiuseppe and Silhavy 2003). In many cases also little is known about the specific signals sensed by the diverse TCS, however, some exceptions are the Nar system of *E. coli*, consisting of two SKs and two RRs, which sense independently nitrate and nitrite (Lee et al. 1999).

In this report, we investigate in vitro and in vivo the role of various combinations of redox agents and HbpS on the SenS/SenR system from *Streptomyces reticuli*.

Materials and methods

Bacterial strains, plasmids, media and culture conditions

The wildtype *S. reticuli* Tü54 (*S. reticuli*; H. Zähner, Tübingen, Germany), *S. reticuli senS-senR* mutant (Ortiz de Orué Lucana et al. 2005) and *S. reticuli hbpS* mutant (Ortiz de Orué Lucana et al. 2004) as well as the *E. coli* strains BL21(DE3)pLysS (Novagen) and XL1-blue (Loenen and Blattner 1983) were used. *E. coli* strains were grown in LB medium (Sambrook et al. 1989). Suspensions of *S. reticuli* spores were made as described elsewhere (Hopwood et al. 1985), and inoculated and propagated as described earlier (Ortiz de Orué Lucana et al. 2004).

Chemicals and enzymes

Chemicals for SDS-PAGE were obtained from Serva. Molecular weight markers and hemin were supplied by Sigma. Restriction enzymes, T4 ligase and *Pfu* DNA polymerase were obtained from Roche, New England Biolabs or Promega.

Isolation of DNA and transformations

Plasmids were isolated from *E. coli* with the aid of a Mini or Midi plasmid kit (Qiagen). *E.coli* XL1-blue was transformed with plasmid DNA by electroporation (Dower et al. 1988), whereas BL21(DE3)pLysS was transformed with the $CaCl_2$ method as described (Sambrook et al. 1989).

PCR, DNA sequencing and computer analysis

PCR was performed with *Pfu* DNA polymerase. Sequencing was done using the Ready Reaction mix and ABI PRISM equipment by the departmental sequence service (U. Coja, FB Biologie/Chemie, Botany, University of Osnabrück). Sequence entry, primary analysis and ORF searches were performed using Clone manager 5.0. Multiple sequence alignments were generated by means of the CLUSTAL W (1.74) program (Thompson et al. 1994).

Cloning of genes in *E. coli*

The *senS*-coding region of the previously described construct pQS1 (Ortiz de Orué Lucana et al. 2005) was amplified by PCR using the primers SenSfor (5′-CGACCATGG TGCCGACCGTGGCCGTAC-3′) with an internal *Nco*I restriction site (underlined), followed by the sequence encoding the N-terminal part of SenS and SenSrev (5′-GG GAAGCTTTCATCTCGGCTCCAACGG-3′) encoding the C-terminal amino acids of SenS followed by an *Hin*dIII restriction site (underlined). The PCR-product was digested with *Nco*I and *Hin*dIII, ligated with *Nco*I/*Hin*dIII-cleaved pETM11, and subsequently transformed into *E. coli* XL1-blue. The correctness of the *senS* gene and its in frame fusion with the His-tag was confirmed by sequencing. The resulting plasmid was named pETS1. To overproduce His_6-SenS (SenS), pETS1 was transformed in *E. coli* BL21(DE3)pLysS.

Purification of the fusion proteins

An *E. coli* BL21(DE3)pLysS transformant containing the plasmid pETS1 was inoculated in LB medium with kanamycin (25 μg/ml) and chloramphenicol (34 μg/ml) and cultivated at 37°C up to an OD_{600} of 0.4. The cultures were cooled and further cultivation took place at 20°C. The synthesis was induced by the addition of 0.2 mM isopropyl thio-β-D-galactosid (IPTG) after the culture had reached an OD_{600} of 0.6. The cells were grown for 6 h, harvested, washed with chilled solution A (10 mM HEPES, 60 mM KCl, pH 8.0) and disrupted by ultrasonification (Branson sonifier: 8 × 10 s, with 10 s intervals). Purification of the fusion proteins was performed in buffer A with 10 mM dodecylmaltoside using Ni^{2+}-NTA affinity chromatography according to the instructions of the manufacturer (Qiagen).

The heterologously overexpressed His_6-HbpS and, respectively, the C-terminal abbreviated Strep-tagged SenS protein ($SenS_C$) was purified to homogeneity as described previously (Zou et al. 2008; Bogel et al. 2007). For phosphorylation analyses, the His_6-tag of HbpS was removed using TEV protease as described (Zou et al. 2008).

The protein concentrations of the samples were determined by previously established methods (Layne 1957; Bradford 1976). Analysis of proteins was performed by SDS-PAGE (Laemmli 1970).

In vitro phosphorylation assays

To investigate the autokinase reaction, SenS (5 μM) was incubated in phosphorylation buffer containing 25 mM Tris-HCl (pH 7.5), 5% glycerol, 10 mM $MgCl_2$ and 500 mM NaCl. $FeCl_2$, $FeCl_3$, DTT, H_2O_2 and/or hemin, respectively, were added in the indicated amounts. To prepare hemin solutions, hemin (10 mM) was dissolved in 0.2 M KOH. The pH was adjusted to 7.5 using 50 mM Tris-HCl. The reaction mixture was incubated at 30°C for 10 min with 0.05 μCi [γ-^{32}P] ATP (Hartmann-Analytik), and directly spotted onto a polyvinylidenfluorid (PVDF) membrane (Immobilon P, Millipore). The membrane was subsequently washed with phosphate buffer saline (PBS; 40 mM disodium hydrogen phosphate, 8 mM sodium dihydrogen phosphate, 150 mM sodium chloride, pH 7.4) containing 0.1% (v/v) Tween 20 for three times of 10 min each. As negative control, phosphorylation buffer with 0.05 μCi [γ-^{32}P] ATP lacking SenS was used. After drying, the membranes were imaged on a PhosphorImager system and quantified using the software ImageQuant 5.2. The negative control was subtracted from each dot blot. The amount of the radioactivity was averaged from three to five dot blots per assay, with at least three independent repetitions. The diagrams shown display the integrated radioactivity of the probe with the indicated added reagents divided by the value for the integrated radioactivity of SenS and ATP alone in the same phosphorylation buffer.

In further studies HbpS protein was first incubated with different quantities of the indicated reagents in 20 mM Tris-HCl pH 7.0 for different time intervals. Then, SenS (in phosphorylation buffer) was added and the sample incubated for 10 min at room temperature, and 0.05 μCi [γ-^{32}P] ATP was added. After incubation at 30°C for 10 min the reaction mixture was directly spotted on a PVDF membrane and further analyzing was performed as described above.

HbpS/SenS interaction

The co-purification studies of the truncated version of $SenS_C$ (lacking the transmembrane as well as intra and extracellular parts) and HbpS were done with slight variations as described previously (Ortiz de Orué Lucana et al. 2005). Purified His_6-HbpS protein was loaded onto a streptactin column with immobilized Strep-tagged-$SenS_C$ and washed several times. The bound proteins were eluted using desthiobiotin according to the instructions of the manufacturer (IBA). As control the same procedure was done with the immobilized Strep-tagged full-length SenS protein (containing transmembrane as well as intra and extracellular parts).

Inhibition tests

The sensitivity of the *S. reticuli* WT, the *senS-senR* disruption mutant and the *hbpS* disruption mutant against the indicated substances was determined using a disc inhibition assay. Spores (5×10^8) were distributed in 3 ml soft agar onto R2 plates and allowed to solidify (Sambrook et al. 1989). On the top of these plates sterile 6 mm diameter paper discs (Schleicher and Schuell) were placed, which were soaked with 20 μl of the indicated solutions in 20 mM Tris-HCl pH 7.0. The zones of inhibition were measured after incubation of the plates for 48 h at 30°C. The tests were repeated four times. The showed data represent their arithmetic average.

Mass spectrometry

The N-terminal amino acids and internal peptides of selected proteins were determined by ESI LC/MS (Bruker Daltonics, Karlsruhe, Germany) analysis. For this purpose the proteins were first separated by SDS-PAGE. After staining (with Coomassie Brilliant Blue) the protein containing band was excised. The gel piece was subsequently cleaned, dehydrated, dried and digested with trypsin

(modified, sequencing grade from Roche) following standard procedures (Wilm et al. 1996).

Metal analysis

The detection of metal ions was done using described chemiluminiscence and colorimetric assays (Högbom et al. 2005) with slight variations. Purified SenS (10–15 µg) as well as 100 nmol of $FeCl_2$, $MnCl_2$, $CuCl_2$ or $ZnCl_2$, respectively, were first incubated with freshly prepared luminol solution. As negative controls 20 mM Tris-HCl pH 8.0 or H_2O were used. The luminescence data were acquired using a Jasco FP-6500 Fluorescens Spectrophotometer (Analytik Jena, Germany). After additional 2 h incubation with 4-(2-pyridylazo)rescorcinol (PAR) at room temperature, the absorbance was measured using a Specord 205 UV-Vis spectrophotometer (Analytik Jena, Germany).

Results

The autokinase activity of SenS is influenced in vitro by iron ions and redox stress

In order to investigate which signals are sensed by the *S. reticuli* SK SenS, autokinase activity tests were done under different redox-stressing conditions. Dithiothreitol (DTT) and hydrogen peroxide (H_2O_2) were used in combination with iron and hemin as redox-stressing agents. They represent natural stressors that can greatly harm the organism at higher concentrations (Gonzales-Flecha and Demple 1995). In the absence of any of the stressors SenS showed a low level of autokinase activity, which was subsequently defined as a basal activity and served as reference value. Up to 5 mM concentrations of neither DTT nor H_2O_2 showed any effect on the autokinase activity of SenS (data not shown). In contrast, $FeCl_2$ or $FeCl_3$ was able to enhance the phosphorylation level of SenS by a factor of ten relative to the basal level (Fig. 1a). The combination of DTT and $FeCl_2$ or DTT and $FeCl_3$ had no effect. However, a combination of $FeCl_2$ and H_2O_2 increased autophosphorylation activity by a factor of twenty (Fig. 1a). In contrast, a combination of $FeCl_3$ and H_2O_2 had no effect, indicating that the peroxide prevents the activation by $FeCl_3$. To corroborate this assumption a set of different concentrations of H_2O_2 was used in the phosphorylation assays. Fig. 1b shows clearly that increasing concentrations of H_2O_2 block the activation of SenS phosphorylation by $FeCl_3$.

Hemin, the oxidized form of heme, did not influence the phosphorylation state of SenS and neither the addition of DTT nor H_2O_2 caused hemin to influence the autokinase activity of SenS (Fig. 1a).

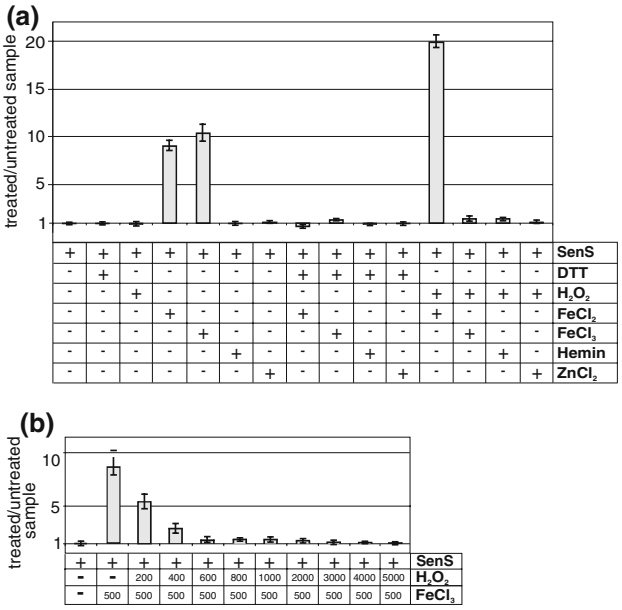

Fig. 1 Autokinase activity of the sensor kinase SenS under different conditions. **a** SenS (5 µM) was incubated with the indicated additives for 10 min before the addition of [γ-^{32}P] ATP. The amount of autophosphorylation of SenS was determined using a PhosphorImager System and ImageQuant software 5.2. (for details see "Materials and methods"). DTT or H_2O_2 were added to 5 mM, and $FeCl_2$, $FeCl_3$, $ZnCl_2$ or hemin to 500 µM. **b** SenS (5 µM) was incubated with the indicated micromolar concentrations of $FeCl_3$ and H_2O_2 before the addition of [γ-^{32}P] ATP. The diagrams show the quantified results representing the amount of radioactivity of the treated (stressed) sample divided by the basal activity of the untreated (unstressed) sample. The amount of radioactivity is averaged from three to five dot blots per assay, with at least three independent repetitions

Zinc is, in contrast to iron, not redox active in chemical terms and functions as an antioxidant in several systems (Bray and Bettger 1990). As expected, phosphorylation tests demonstrated that $ZnCl_2$ is not able to change the autokinase activity of SenS (Fig. 1a). Further chemiluminiscence and colorimetric assays revealed that the used SenS protein does not contain neither iron nor manganese nor cobalt nor copper (data not shown).

Thus the presented data show clearly that the *S. reticuli* SK SenS responds to redox-stressing conditions induced or mediated by iron ions.

HbpS influences the autokinase activity of SenS in vitro

HbpS has been proposed to participate in the signal cascade that modulates the phosphorylation of SenS/SenR (Ortiz de Orué Lucana et al. 2005). Indeed, experiments combining HbpS and SenS in different molar ratios revealed that the autophosphorylation activity of the SK was reduced dependent on their molar ratio (Fig. 2a). The autokinase activity of SenS (5 µM) was reduced to 75% upon addition of 2 µM HbpS, whereas stoichiometrical quantities (5 µM)

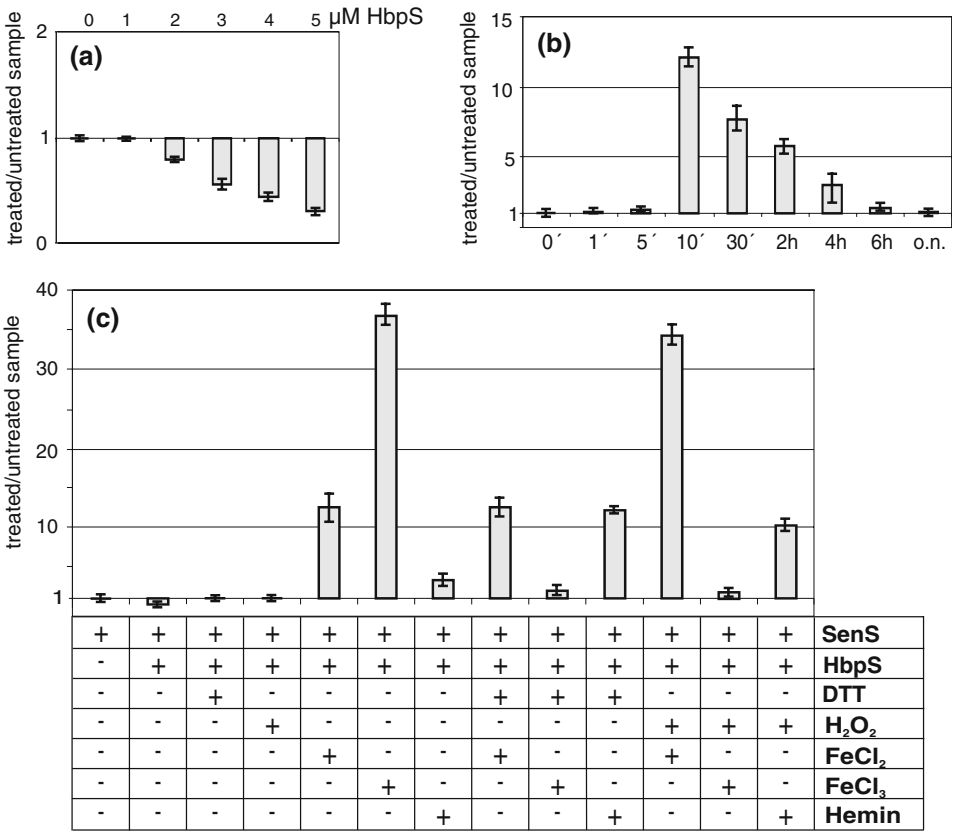

Fig. 2 Influence of HbpS on the autokinase activity of SenS. **a** Increasing quantities of HbpS (as indicated) were incubated with SenS (5 µM) for 10 min prior starting of the autokinase reaction. **b** The autokinase activity of SenS was measured over the indicated length of incubation with HbpS (5 µM), DTT (5 mM) and hemin (500 µM). Similar results were obtained by incubation of HbpS with H_2O_2 and hemin (data not shown). **c** HbpS was incubated with the indicated reagents for 10 min. DTT or H_2O_2 were added to 5 mM, and $FeCl_2$, $FeCl_3$ or hemin to 500 µM concentrations prior to the addition of SenS and [γ-^{32}P] ATP. All diagrams (**a**, **b**, **c**) show the measured activity of the treated sample divided by the basal activity of SenS (untreated sample). The amount of radioactivity was averaged over three to five dot blots per assay, with at least three independent repetitions

of both proteins led to nearly complete inhibition of the SenS autokinase activity.

The simultaneous incubation of HbpS and hemin immediately prior to the addition of SenS and ATP induced a slight (~twofold) elevation of autokinase activity (Fig. 2c). However, pre-incubation of HbpS with hemin and H_2O_2 or DTT induced a significantly stronger enhancement (Fig. 2c). Maximum induction of autokinase activity was achieved with a 10 min pre-incubation of HbpS with hemin and H_2O_2 or DTT, prior to the addition of SenS and [γ-^{32}P] ATP (Fig. 2b). This length of pre-incubation time was therefore used in the subsequent experiments. Pre-incubation of HbpS with H_2O_2 or DTT and no hemin showed no induction of autokinase activity (Fig. 2c).

As shown above (Fig. 1), $FeCl_3$ stimulates autokinase activity up to tenfold. Addition of HbpS resulted in a 30-fold increase (Fig. 2c), but this enhancement was abolished in the presence of DTT or H_2O_2. Incubation of HbpS with $FeCl_2$ induced the kinase activity about 12-fold and addition of DTT had no further effect (Fig. 2c). The presence of H_2O_2, $FeCl_2$ and HbpS increased the autophosphorylation level of SenS again over 30-fold (Fig. 2c). In contrast to $FeCl_2$ and $FeCl_3$, $ZnCl_2$ had no effect on the SenS autokinase activity mediated by HbpS (data not shown).

Taken together, these data demonstrate that the presence of HbpS strongly influences the autophosphorylation activity of SenS and enables the SK to react to redox-stressing conditions.

The N-terminal domain of SenS responds to iron ions and HbpS in vitro

To investigate the sensing properties within SenS in more detail comparative phosphorylation analyses were done with both full-length SenS and its truncated form ($SenS_C$: which lacks 168 N-terminal amino acids; corresponding to the transmembrane domains interspaced by extra- and intracytoplasmic loops (Bogel et al. 2007)). The results clearly show that in the presence of $FeCl_3$ as well as $FeCl_2$ and H_2O_2 the autokinase activity of $SenS_C$ increased only twofold (Fig. 3a). This value is considerably lower than the one mediated by the full-length protein (9–20-fold; see Fig. 1a). Interestingly, the autokinase activity of $SenS_C$ is even repressed if combinations of hemin or $FeCl_3$ with DTT or H_2O_2 were applied to the reaction mixtures (Fig. 3a). This result could be explained as a consequence of possible chemical/oxidative damage to the catalytic residues of $SenS_C$ under the experimental conditions.

As shown above (Fig. 2b, c), HbpS is able to considerably enhance the autokinase activity of the full-length

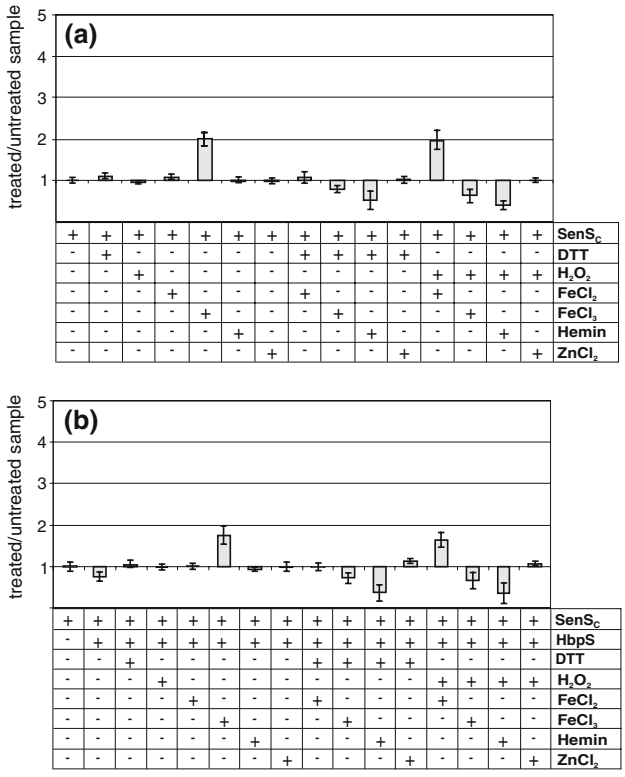

Fig. 3 Autokinase activity of the truncated SenS protein ($SenS_C$) under different conditions. Autophosphorylation of $SenS_C$ (5 μM) alone (**a**) or in the presence of HbpS (5 μM) (**b**) was measured with the indicated reagents as described under "Materials and methods". Different molar ratios of HbpS and $SenS_C$ did not change the obtained results (data not shown). Both diagrams show the quantified results representing the amount of phosphorylation under the indicated conditions divided by the amount of radioactivity of the untreated sample. The radioactivity values are averaged from three to five dot blots per assay and at least three independent repetitions

SenS protein in combination with hemin and DTT or H_2O_2. Under the experimental conditions used for SenS, HbpS only marginally influenced the autokinase activity of $SenS_C$ (Fig. 3b).

Using Strep-tag-SenS affinity chromatography, it could be shown that the entire SenS interacts specifically with HbpS (Ortiz de Orué Lucana et al. 2005). Similar co-purification experiments were performed using Strep-tagged $SenS_C$ immobilized on Streptactin resin and purified His_6-HbpS suspension. His_6-HbpS remained in the flow-through and wash fractions, demonstrating no direct interaction with SenS. After the addition of desthiobiotin $SenS_C$ proteins were released from the resin (Fig. 4). As a control, the same procedure was performed in the presence of the entire Strep-tagged-SenS protein, in which case HbpS co-eluted with SenS (data not shown).

In summary the data show that the N-terminal domain of SenS is crucial for sensing redox stress inducing agents and is required for the interaction with HbpS.

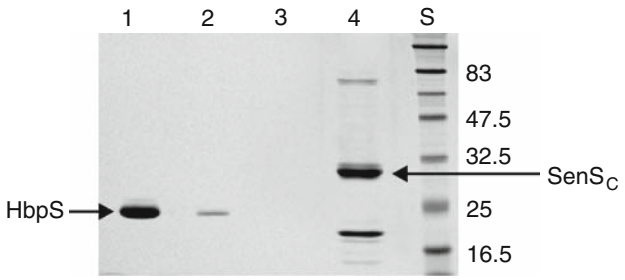

Fig. 4 Interaction studies of $SenS_C$ and HbpS. Purified His_6-HbpS protein was loaded onto a Streptactin column with immobilized Strep-tag-$SenS_C$. The flow-through (*1*) and washing steps (*2*, *3*) were collected. Bound proteins were eluted in the presence of desthiobiotin (*4*) and fractions were analyzed by SDS-PAGE and subsequent Coomassie brilliant blue staining. Molecular weights of markers are indicated in kilodaltons (*S*). The protein bands migrating as ~80 and ~20 kDa, respectively, represent non-specific interacting proteins, as indicated by the lack of immunoreactivity with anti-SenS- and anti-HbpS antibodies (data not shown)

The presence of SenS/SenR leads to the synthesis of extracellular redox active proteins

During cultivation in minimal media with cellulose and $FeCl_3$ or hemin, but not $FeCl_2$, the pattern of extracellular proteins of the *S. reticuli* WT shows two extra proteins, which are missing in the *senS-senR* disruption mutant (Fig. 5a). Tryptic digestion, HPLC followed by LC/MS and sequence information revealed that one of them displays characteristics of the xylanase B from *Streptomyces lividans* (Biely et al. 1993). Additionally, a homologue of a tyrosinase from *Streptomyces galbus* (Zhu et al. 2005) could be identified. Sequence alignments allowed us to identify motifs with high similarity (58 and 85%, respectively) to the previously determined *S. reticuli* SenR DNA-binding sites (site II and III; Bogel et al. 2007) in a corresponding position upstream of the xylanase B gene. Further sequence alignments of the upstream region of the *S. galbus* tyrosinase *melC* gene revealed a DNA region with 67% identity to the SenR DNA-binding site I, which is located upstream of the *furS-cpeB* operon (Fig. 5b).

It can therefore be concluded that the SenS/SenR system is involved in controlling the transcription of genes encoding a xylanase and a tyrosinase in the melanin producing strain *S. reticuli*.

The *S. reticuli* WT and the *senS-senR* mutant as well as the *hbpS* mutant differ in sensitivity to different compounds

The in vivo effect of different stressors (dropped on paper discs) was tested on spore lawns on agar plates. Compared to the WT strain, the relative sensitivity of the mutant strains to hemin alone increased 66% for the

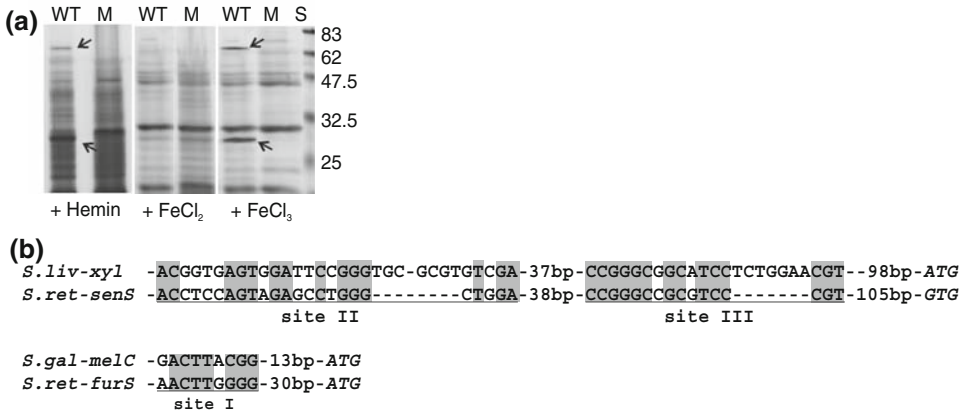

Fig. 5 Proteins under the control of SenS/SenR and alignments. The *S. reticuli* wild type (*WT*) and a *senS-senR* disruption mutant (*M*) were cultivated in minimal media containing 1% Avicel and $FeCl_2$ (250 mM), $FeCl_3$ (250 mM) or hemin (500 µM), respectively. **a** Extracellular proteins from the culture filtrate were precipitated and analyzed by SDS-PAGE. The protein bands (marked with an *arrow*) were subsequently analyzed using tryptic digestion and LC–MS (as described under "Materials and methods"). The molecular weight of the standard proteins (*S*) is indicated in kilodaltons. **b** The regulatory DNA regions of the xylanase B (*S.liv-xyl*) and the tyrosinase *melC* gene (*S.gal-melC*) were aligned with the SenR DNA-binding sites (I, II, III) upstream of the *senS-senR* operon (*S.ret-senS*) or the *furS-cpeB* operon (*S.ret-furS*), respectively. The SenR DNA-binding sites are underlined. Identical bases are marked by *gray boxes* and the start codons (ATG or GTG) are written in *italics*

senS-senR mutant and 42% for the *hbpS* mutant which is comparable to previous results. Further addition of DTT or H_2O_2 to hemin did not change this effect. Exposure of both mutant strains of *S. reticuli* to $FeCl_2$ demonstrated an increased sensitivity (14% for the *senS-senR* mutant and 23% for the *hbpS* mutant) that was elevated only slightly by further addition of H_2O_2 or DTT (Table 1). The mutants and the wild type had the same sensitivity to $FeCl_3$, which was only slightly elevated upon addition of DTT or H_2O_2 (Table 1).

Taken together, it can be concluded that in vivo the presence of SenS/SenR as well as HbpS is essential for resistance against high concentrations of hemin and Fe(II).

Discussion

In this study we show for the first time the regulation of the SenS autokinase activity. Using a comparative phosphorylation analysis of the full-length and truncated form of SenS it could be demonstrated that its N-terminal domain is necessary for the signal detection in vitro. An increased phosphorylation of full-length SenS was detected in the presence of iron ions ($FeCl_2$ or $FeCl_3$), which we propose as signals for the TCS. Iron is essential for nearly all living organisms and an important co-factor in many enzymes. However, iron is also potentially toxic in the presence of oxygen (Galaris and Pantopoulos 2008). The PmrA/PmrB system of several bacteria, including species of *Salmonella,*

Table 1 Sensitivity of the *S. reticuli* WT and mutant strains to SenS autokinase activating substances

Substance (concentration)	Diameter of zone of inhibition (mm)			Relative increase of sensitivity (%)	
	WT	*senS-senR* mutant	*hbpS* mutant	*senS-senR* mutant	*hbpS* mutant
Hemin (300 µM)	12.0	19.9	17.1	66	42
Hemin (300 µM) + DTT (5 mM)	11.8	19.2	17.6	63	49
Hemin (300 µM) + H_2O_2 (5 mM)	11.6	18.6	16.9	60	46
$FeCl_2$ (250 mM)	12.0	13.7	14.8	14	23
$FeCl_2$ (250 mM) + DTT (5 mM)	11.5	13.3	14.4	16	25
$FeCl_2$ (250 mM) + H_2O_2 (5 mM)	11.8	13.6	14.8	15	25
$FeCl_3$ (250 mM)	14.0	14.0	14.1	0	0
$FeCl_3$ (250 mM) + DTT (5 mM)	14.2	14.6	14.6	3	3
$FeCl_3$ (250 mM) + H_2O_2 (5 mM)	13.8	14.1	14.0	2	1

S. reticuli strains were exposed to the indicated high concentrations of hemin, $FeCl_2$ and $FeCl_3$ alone or in combination with DTT or H_2O_2 (for details see "Materials and methods"). The diameter of inhibition zones was then measured in at least four independent assays

Klebsiella and *E. coli*, is responsible for protection against iron-mediated toxicity. With this system, signal recognition is provided by binding of Fe^{3+} directly to the periplasmic sensing domain of the SK PmrB (Wösten et al. 2000). The PhoQ/PhoR system from different Gram-negative bacteria including *E. coli*, *P. aeruginosa* and *S. typhimurium* is activated by limiting concentrations of extracellular divalent cations (Mg^{2+} or Ca^{2+}), which are suggested to bind directly to the sensor domain of PhoQ leading to a conformational change that influences the enzymatic activities of the cytoplasmic domain (Prost et al. 2008). We expect that iron might play a similar role in the activation of SenS. Further biophysical and biochemical approaches should demonstrate this assumption.

To further characterize the recognition of iron ions by SenS, in vitro phosphorylation analyses in combination of $FeCl_2$/$FeCl_3$ with DTT were done and showed no effect on the autokinase activity of SenS. Interestingly, higher levels (about 20-fold) of SenS autophosphorylation are recorded under oxidizing conditions (with H_2O_2 and $FeCl_2$). As Fe^{2+} and H_2O_2 are precursors for the Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow \cdot OH + -OH + Fe^{3+}$; Fenton 1986), it could be proposed that not only iron ions alone but also hydroxyl radicals formed via this reaction may be involved in the induction of the autophosphorylation activity of SenS. Surprisingly, H_2O_2 prevents the activation of the autokinase activity of SenS by $FeCl_3$. It can be assumed that the reaction products can modify the conformation of SenS leading to its inactivation. It was shown that peroxide can coordinate to the Fe(III) center to produce active catalytic species, which immediately oxidize the corresponding substrate (Theodoridis et al. 2008). To clarify this process in SenS further analyses are required. Recognition of hydroxyl radicals has been otherwise shown for the Sln1/Ssk1p TCS in *Saccharomyces cerevisae*. This is specifically induced by a currently unknown mechanism in the presence of H_2O_2 and thus differs from the *S. reticuli* SenS/SenR (Singh 2000). The SK ArcB from *E. coli* has two cytosolic cysteine residues which form intermolecular disulphide bridges between the ArcB dimers under aerobic conditions. The formation of disulphide bonds provokes the silencing of the autokinase activity (Malpica et al. 2004). Analyses of the amino acid sequence of SenS revealed that it contains two cysteine residues, one of which is predicted to be cytosolic. However, currently no disulphide bond formation has been observed between two SenS monomers.

We demonstrated previously that SenS can specifically interact with the heme-binding protein HbpS in vitro, leading to the assumption that HbpS plays a role in the corresponding signal cascade in vivo (Ortiz de Orué Lucana et al. 2005). Phosphorylation analyses of SenS in the presence of HbpS revealed that high quantities of heme-free HbpS inhibit in vitro the autophosphorylation of the SK under non-stressing conditions (Fig. 6). Thus, interaction with heme-free HbpS is predicted to lead to an inactive conformation of SenS. In the additional presence of $FeCl_2$ alone or combined with DTT or H_2O_2, however, HbpS was able to enhance the autokinase activity of SenS. As mentioned above, $FeCl_2$ and H_2O_2 are precursors of the Fenton reaction leading to the formation of hydroxyl radicals and Fe^{3+}, which could induce the SenS autokinase activity. Surprisingly, the greatest enhancement (over 35-fold) took place in the presence of HbpS and $FeCl_3$. Comparative physiological studies in the presence of $FeCl_3$ or hemin revealed two proteins which are only produced if the intact *senS-senR* operon is present. One of them shows sequence identities to a xylanase B from *S. lividans*, and the other to a tyrosinase from *S. galbus*. Xylanases have been found to be important soil redox effectors as xylans can modify the redox potential of the soil (Polizeli et al. 2005). Tyrosinases belong to the family of oxidoreductases and can incorporate and reduce molecular oxygen (Claus and Decker 2006). Sequence alignments revealed that the regulatory DNA regions upstream of each of the corresponding genes contain motifs displaying high sequence similarity (58–85%) to the previously identified SenR DNA-binding sites suggesting that SenS/SenR regulates directly the production of these redox active proteins.

Growth inhibition assays revealed that the SenS/SenR system as well as HbpS is required for *S. reticuli* to have increased resistance against high concentrations of $FeCl_2$, but not of $FeCl_3$. Iron ion-mediated stress differs in dependence on the oxidative state of the ion. The oxidized form of iron (Fe^{3+}) is known to lead to killing independent of oxygen whereas Fe^{2+}-ions have always oxygen-dependent microbicidal effects (Chamnongpol et al. 2002). Thus, the SenS/SenR system in concert with HbpS seems to play an important role during the oxygen induced iron stress in vivo. This assumption is in accordance with our previous

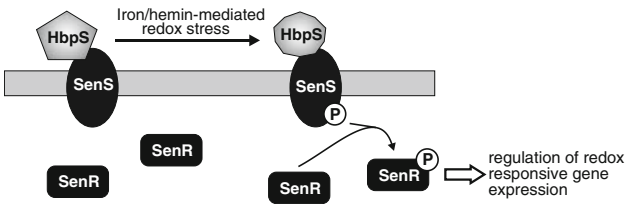

Fig. 6 Model presenting the regulation of the autokinase activity of SenS by HbpS in a redox-dependent manner. Under non-stressing conditions HbpS inhibits SenS autophosphorylation activity. In combination with HbpS, however, SenS responds to hemin- and iron-mediated redox stress with enhanced autokinase activity and therewith SenR phosphorylation enabling the regulation of genes encoding redox proteins

findings showing that SenS/SenR and HbpS modulate the transcription of the *furS-cpeB* operon encoding the redox regulator FurS and the mycelia-associated catalase-peroxidase CpeB, respectively (Ortiz de Orué Lucana et al. 2004, 2005). CpeB oxidizes in the presence of H_2O_2 a number of substrates either in dependence on an attached heme group or a heme-independent reaction which is coupled to Mn(II)/(III) peroxidation. The additional heme-dependent catalase activity of the enzyme leads to disportionation of H_2O_2 to O_2 (Zou and Schrempf 2000). Thus, FurS and CpeB together with SenS/SenR and HbpS provide *S. reticuli* with an efficient protection system minimizing reactions caused by highly reactive oxygen species.

Previous and the presented comparative physiological studies showed that SenS/SenR and HbpS are also important for the resistance of *S. reticuli* against high concentrations of hemin (Ortiz de Orué Lucana et al. 2004, 2005). Hemin, the oxidized form of heme, possesses significant antibacterial activity that is augmented by the presence of physiological concentrations of H_2O_2 or reducing agents (Stojilikovic et al. 2001). Phosphorylation analyses revealed that the autokinase activity of SenS is not affected under redox-stressing conditions mediated by hemin alone or in combination with H_2O_2 or DTT. Other TCS like ChrS/ChrA from *Corynebacterium diphtheriae* and the HssS/HssR system of *Staphylococcus aureus* are involved in sensing of heme-mediated signals, but the exact mechanisms by which the SKs sense heme are still unknown (Schmitt 1999; Stauff et al. 2007). Recognition of hypoxia and nitric oxide by the *Mycobacterium tuberculosis* SK DevS of the DevS/DevR system also requires heme which is bound to one of its aminoterminal GAF domains (Ioanoviciu et al. 2007). A corresponding domain is absent in SenS. Analysis of the amino acid sequence of the *S. reticuli* SenS revealed that it has none of the typical heme-binding motifs and is unable to interact with heme (data not shown). The heme-binding protein HbpS, however, is an essential component for recognizing hemin-mediated redox stress by SenS (Fig. 6); phosphorylation analyses of SenS in the presence of HbpS and hemin alone or hemin in combination with DTT or H_2O_2 revealed that HbpS enhanced significantly the autokinase activity. Thus, HbpS, SenS and SenR can be concluded to form a novel sensing system consisting of a typical TCS and a third component; together they are all required for optimal recognition of redox stress processes mediated by iron ions and heme. A role of accessory proteins for TCS has been postulated, although direct interaction and the kind of influence have only been described in a few cases. In *Rhodobacter capsulatus* the cytoplasmic metalloprotein HupUV was identified to interact specifically with the SK HupT playing an important regulatory role during recognition of H_2. Interaction of these proteins is provided by the HupT PAS (Period circadian protein, Ah receptor nuclear translocator protein, Single minded protein) domain, which is not present in the SK of the SenS/SenR system (Elsen et al. 2003). Further more precisely analyses should clarify the mechanisms of the signal transduction between HbpS and SenS.

The best-studied system to date with a third component regulating the SK is the FixL/FixT/FixJ system of *Sinorhizobium meliloti*. The SK FixL is regulated by FixT through direct binding of FixT to the catalytic cytoplasmic domain of FixL resulting in a reduced level of autophosphorylation of the SK. This regulatory system is conserved among several rhizobial species (Crosson et al. 2005). Homologues of the *S. reticuli senS-senR* system in combination with *hbpS* are present within sequenced genomes of several *Streptomyces* species, *Arthrobacter aurescens* TC1, *Leifsonia xyli subsp. xyli* and *Rhodococcus species* RHA1. Thus, the presented data serve as model for the HbpS/SenS/SenR-like systems in various actinobacteria.

Acknowledgments We are very grateful to Dr. Matthew R. Groves (EMBL, Hamburg, Germany) for his comments on the manuscript and improvements in the language. We also thank Dr. Stefan Walter (in our group) for LC-MS analysis. This work was supported by grant OR 224/1-1 of the Deutsche Forschungsgemeinschaft (DFG).

References

Baker HM, Anderson BF, Baker EN (2003) Dealing with iron: common structural principles in proteins that transport iron and heme. Proc Natl Acad Sci USA 100:3579–3583. doi:10.1073/pnas.0637295100

Biely P, Kluepfel D, Morosoli R, Shareck F (1993) Mode of action of three endo-beta-1, 4-xylanases of *Streptomyces lividans*. Biochim Biophys Acta 1162:246–254

Bogel G, Schrempf H, Ortiz de Orué Lucana D (2007) DNA-binding characteristics of the regulator SenR in response to phosphorylation by the sensor histidine autokinase SenS from *Streptomyces reticuli*. FEBS J 274:3900–3913. doi:10.1111/j.1742-4658.2007.05923.x

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of the protein-dye binding. Anal Biochem 72:248–254. doi:10.1016/0003-2697(76)90527-3

Bray TM, Bettger WJ (1990) The physiological role of zinc as an antioxidant. Free Radic Biol Med 8:281–291. doi:10.1016/0891-5849(90)90076-U

Cash TP, Pan Y, Simon MC (2007) Reactive oxygen species and cellular oxygen sensing. Free Radic Biol Med 43:1219–1225. doi:10.1016/j.freeradbiomed.2007.07.001

Chamnongpol S, Dodson W, Cromie MJ, Harris ZL, Groisman EA (2002) Fe(III)-mediated cellular toxicity. Mol Microbiol 45:711–719. doi:10.1046/j.1365-2958.2002.03041.x

Claus H, Decker H (2006) Bacterial tyrosinases. Syst Appl Microbiol 29:3–14. doi:10.1016/j.syapm.2005.07.012

Crosson S, McGrath PT, Stephens C, McAdams HH, Shapiro L (2005) Conserved modular design of an oxygen sensory/signalling network with species-specific output. Proc Natl Acad Sci USA 102:8018–8023. doi:10.1073/pnas.0503022102

Deng DM, Liu MJ, ten Cate JM, Crielaard W (2007) The VicRK system of *Streptococcus mutans* responds to oxidative stress. J Dent Res 86:606–610

DiGiuseppe PA, Silhavy TJ (2003) Signal detection and target gene induction by the CpxRA two-component system. J Bacteriol 185:2432–2440. doi:10.1128/JB.185.8.2432-2440.2003

Dower WJ, Miller JF, Ragsdale CW (1988) High efficiency transformation of *E.* coli by high voltage electroporation. Nucleic Acids Res 16:6127–6145. doi:10.1093/nar/16.13.6127

Elsen S, Dúche O, Colbeau A (2003) Interaction between the sensor HupUV and the histidine kinase HupT controls HupSL hydrogenase synthesis in *Rhodobacter capsulatus*. J Bacteriol 185:7111–7119. doi:10.1128/JB.185.24.7111-7119.2003

Fenton HJH (1986) Oxidation of tartaric acid in the presence of iron. J Chem Soc 65:899–910

Galaris D, Pantopoulos K (2008) Oxidative stress and iron homoeostasis: mechanistic and health aspects. Crit Rev Clin Lab Sci 45:1–23. doi:10.1080/10408360701713104

Gonzales-Flecha B, Demple B (1995) Metabolic sources of hydrogen peroxide in aerobically growing *Escherichia coli*. J Biol Chem 270:13681–13687. doi:10.1074/jbc.270.23.13681

Hoch JA, Varughese KI (2001) Keeping signals straight in phosphorelay signal transduction. J Bacteriol 183:4941–4949. doi:10.1128/JB.183.17.4941-4949.2001

Högbom M, Ericsson UB, Lam R, Bakali HMA, Kuznetsova E, Nordlund P, Zamble DB (2005) A high throughput method for the detection of metalloproteins on a microgram scale. Mol Cell Proteomics 4:827–834. doi:10.1074/mcp.T400023-MCP200

Hopwood DA, Bibb MJ, Chater KF, Kieser T, Bruton CJ, Kieser HM, Lydiate DJ, Smith CP, Ward JM, Schrempf H (1985) Genetic manipulation of *Streptomyces*: a laboratory manual. John Innes Foundation, Norwich

Hutchings MI, Hoskisson PA, Chandra G, Buttner MJ (2004) Sensing and responding to diverse extracellular signals? Analysis of the sensor kinases and response regulators of *Streptomyces coelicolor* A3(2). Microbiology 150:2795–2806. doi:10.1099/mic.0.27181-0

Ioanoviciu A, Yukl ET, Moënne-Loccoz P, de Montellano PR (2007) DevS, a heme-containing two-component sensor of *Mycobacterium tuberculosis*. Biochemistry 46:4250–4260. doi:10.1021/bi602422p

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227:680–685. doi:10.1038/227680a0

Laub MT, Goulian M (2007) Specificity in two component signal transduction pathways. Annu Rev Genet 41:121–145. doi:10.1146/annurev.genet.41.042007.170548

Layne E (1957) Spectrophotometric and turbidimetric methods for measuring proteins. Methods Enzymol 3:447–455. doi:10.1016/S0076-6879(57)03413-8

Lee AI, Delgado A, Gunsalus RP (1999) Signal-dependent phosphorylation of the membrane-bound NarX two-component sensor-transmitter protein of *Escherichia coli*: nitrate elicits a superior anion ligand response compared to nitrite. J Bacteriol 181:5309–5316

Loenen WA, Blattner FR (1983) Lambda charon vectors (Ch32, 33, 34 and 35) adapted for DNA cloning in recombinant-deficient hosts. Gene 26:171–179. doi:10.1016/0378-1119(83)90187-7

Malpica R, Franco B, Rodriguez C, Kwon O, Georgellis D (2004) Identification of a quinone-sensitive redox switch in the ArcB sensor kinase. Proc Natl Acad Sci USA 101:13318–13323. doi:10.1073/pnas.0403064101

Ortiz de Orué Lucana D, Schaa T, Schrempf H (2004) The novel extracellular *Streptomyces reticuli* haem-binding protein HbpS influences the production of the catalase-peroxidase CpeB. Microbiology 150:2575–2585. doi:10.1099/mic.0.27091-0

Ortiz de Orué Lucana D, Schrempf H (2000) The DNA-binding characteristics of the *Streptomyces reticuli* regulator FurS depend on the redox state of its cysteine residues. Mol Gen Genet 264:341–353. doi:10.1007/s004380000328

Ortiz de Orué Lucana D, Zou P, Nierhaus M, Schrempf H (2005) 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*. Microbiology 151:3603–3614. doi:10.1099/mic.0.28298-0

Polizeli ML, Rizzatti AC, Monti R, Terenzi HF, Jorge JA, Amorim DS (2005) Xylanases from fungi: properties and industrial applications. Appl Microbiol Biotechnol 67:577–591. doi:10.1007/s00253-005-1904-7

Prost LR, Daley ME, Bader MW, Klevit RE, Miller SI (2008) The PhoQ histidine kinases of *Salmonella* and *Pseudomonas* spp. are structurally and functionally different: evidence that pH and antimicrobial peptide sensing contribute to mammalian pathogenesis. Mol Microbiol 69:503–519. doi:10.1111/j.1365-2958.2008.06303.x

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbour Laboratory, Cold Spring Harbor

Schmitt MP (1999) Identification of a two-component signal transduction system from *Corynebacterium diphteriae* that activates gene expression in response to the presence of heme and hemoglobin. J Bacteriol 181:5330–5340

Singh KK (2000) The *Saccharomyces cerevisae* Sln1-Ssk1p two-component system mediates response to oxidative stress and in an oxygen dependent fashion. Free Radic Biol Med 29:1043–1050. doi:10.1016/S0891-5849(00)00432-9

Stauff DL, Torres VJ, Skaar EP (2007) Signaling and DNA-binding activities of the *Staphylococcus aureus* HssR-HssS two-component system required for heme sensing. J Biol Chem 282(36):26111–26121. doi:10.1074/jbc.M703797200

Stojilikovic I, Evavold BD, Kumar V (2001) Antimicrobial properties of porphyrins. Expert Opin Investig Drugs 10:309–320. doi:10.1517/13543784.10.2.309

Theodoridis A, Maigut J, Puchta R, Kudrik EV, van Eldik R (2008) Novel iron(III) porphyrazine complex. Complex speciation and reactions with NO and H_2O_2. Inorg Chem 47:2994–3013. doi:10.1021/ic702041g

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680. doi:10.1093/nar/22.22.4673

Wilm M, Shevchenko A, Houthaeve T, Breit S, Schweigerer L, Fotsis T, Mann M (1996) Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. Nature 379:466–469. doi:10.1038/379466a0

Wösten MMSM, Kox LFF, Chamnongpol S, Soncini FC, Groisman EA (2000) A signal transduction system that responds to extracellular iron. Cell 103:113–125. doi:10.1016/S0092-8674(00)00092-1

Zhu D, He X, Zhou X, Deng Z (2005) Expression of the *melC* operon in several *Streptomyces* strains is positively regulated by AdpA, an AraC family transcriptional regulator involved in morphological development in *Streptomyces coelicolor*. J Bacteriol 187:3180–3187. doi:10.1128/JB.187.9.3180-3187.2005

Zou P, Groves MR, Viale-Bouroncle SD, Ortiz de Orué Lucana D (2008) Cristallization and preliminary characterization of a novel haem-binding protein of *Streptomyces reticuli*. Acta Crystallogr Sect F Struct Biol Cryst Commun 64:386–390. doi:10.1107/S1744309108008348

Zou P, Schrempf H (2000) The heme-independent manganese-peroxidase activity depends on the presence of the C-terminal domain within the *Streptomyces reticuli* catalase-peroxidase CpeB. Eur J Biochem 267:2840–2849. doi:10.1046/j.1432-1327.2000.01259.x