The mycelium-associated *Streptomyces reticuli* catalase-peroxidase, its gene and regulation by FurS

Pei-jian Zou, Ilya Borovok, Darío Ortiz de Orúe Lucana, Dagmar Müller and Hildgund Schrempf

Author for correspondence: Hildgund Schrempf. Tel: +49 541 969 2895. Fax: +49 541 969 2804. e-mail: schrempf@sfbbio1.biologie.uni-osnabrueck.de

Universität Osnabrück, FB Biologie/Chemie, Barbarstraße 11, 49069 Osnabrück, Germany

During early stages of growth, *Streptomyces reticuli* synthesizes a hyphae-associated, haem-containing enzyme which exhibits catalase and peroxidase activities with broad substrate specificity (CpeB). The purified dimeric enzyme (160 kDa) consists of two identical subunits. Using anti-CpeB antibodies and an expression- as well as a mini-library, the corresponding *cpeB* gene was identified and sequenced. It encodes a protein of 740 aa with a molecular mass of 81.3 kDa. The deduced protein shares the highest level of amino acid identity with KatG from *Caulobacter crescentus* and *Mycobacterium tuberculosis*, and PerA from *Bacillus stearothermophilus*. *Streptomyces lividans* transformants carrying *cpeB* and the upstream-located *furs* gene with its regulatory region on the bifunctional vector pWHM3 produced low or enhanced levels of CpeB in the presence or absence of Fe ions, respectively. An in-frame deletion of the major part of *furs* induces increased CpeB synthesis. The data imply that FurS regulates the transcription of *cpeB*. The deduced FurS protein is rich in histidine residues, contains a putative N-terminally situated helix-turn-helix motif and has a molecular mass of 15.1 kDa. It shares only 29% amino acid identity with the *Escherichia coli* ferric uptake regulator (Fur) protein, but about 64% with FurA deduced from the genomic sequences of several mycobacteria. The predicted secondary structures of FurS and FurA are highly similar and considerably divergent from those of the *E. coli* Fur. In contrast to some Gram-negative bacteria, within several mycobacteria an intact *furA* gene or a *furA* pseudogene is upstream of a catalase-peroxidase (*katG*) gene predicted to encode a functional or a non-functional (*Mycobacterium leprae*) enzyme. Thus the data obtained for *Streptomyces reticuli* are expected to serve as an additional model to elucidate the regulation of mycobacterial catalase-peroxidase genes.

Keywords: Fur proteins, catalase-peroxidase genes, streptomycetes, mycobacteria

INTRODUCTION

The dismutation of H₂O₂ to O₂ and H₂O is catalysed by catalases, whereas peroxidases use H₂O₂ to oxidize a variety of compounds (Elstner, 1982). Catalases are protohaem IX proteins of 225–270 kDa, which consist of four identical subunits. The enzymes are active within a broad pH range, resistant to treatment with ethanol and specifically inhibited by 3-amino-1,2,4-triazole (Nadler et al., 1986; Margoliash et al., 1960). Bifunctional catalase-peroxidases constitute a distinct group of proteins exhibiting catalase and peroxidase activities. The ratio of these activities varies among the members of this class of enzymes.

Most microbial catalase-peroxidases contain two or four identical subunits and they carry ferric proto-
protoporphyrin IX (0.5-1 molecule per subunit) as the prosthetic group. Monomeric catalase-peroxidases have been purified from two halophilic archaea (Cendrin et al., 1994; Fukumori et al., 1985).

Proteins deduced from the few known characterized catalase-peroxidase gene sequences share varying but high levels of amino acid identity, but show little sequence similarity with proteins derived from typical catalase genes. It is interesting that bacterial catalase-peroxidases are homologous to eukaryotic, monomeric, haem-containing peroxidases comprising 290-350 aa. Analyses of deduced proteins indicate that the double terminal part, but not the C-terminal half of a subunit from a bacterial catalase-peroxidase binds haem (Welinder, 1991). A study of the crystal structure of cytochrome c peroxidase (Finzel et al., 1984) and modelling data led to the suggestion that the N-terminal part, but not the C-terminal half of a subunit from a bacterial catalase-peroxidase binds haem (Welinder, 1991).

In *Escherichia coli*, protection against H$_2$O$_2$ involves the enzymes hydroperoxidase HIPI (a typical catalase) and hydroperoxidase HPI (an atypical catalase corresponding to a catalase-peroxidase type) that are encoded by katE and katG, respectively. Transcription of katE requires the sigma factor $\sigma^3$ and occurs when cultures enter stationary phase (Loewen & Hengge-Aronis, 1994). In *E. coli* and *Salmonella typhimurium*, in contrast, katG is transcribed independently of $\sigma^3$, in response to H$_2$O$_2$ and under the control of the positive activator OxyR. OxyR senses oxidative stress (Storz et al., 1990) and is regulated by reversible intramolecular disulphide bond formation (Zheng et al., 1998). The regulation of catalase-peroxidase genes from various other micro-organisms has up to now not been elucidated in detail.

In this paper we report on the characteristics of a mycelium-associated *Streptomyces reticuli* catalase-peroxidase with broad substrate specificity (CpeB) and on the corresponding gene regulated by the newly described gene *furS*.

**METHODS**

**Bacterial strains and plasmids.** *Streptomyces reticuli* Tu45 (H. Zähner, Tübingen, Germany) and *Streptomyces lividans* 66 (D. A. Hopwood, John Innes Institute, Norwich, UK) were used. The plasmids pWHM3 (Vara et al., 1989) and pUC18/pUC19 (Sambrook et al., 1989) were gifts from C. R. Hutchinson (University of Wisconsin, Madison, USA) and J. Messing (State University of New Jersey, Piscataway, USA), respectively. A derivative of pUC18 (named pUDS18) carrying a deletion of the SstI site was constructed after SstI cleavage, Klenow treatment and religation.

**Media and culture conditions.** For cultivation of *Streptomyces* strains, complete and minimal media were used (Schlochtermeier et al., 1992a). Minimal medium was supplemented with glucose or Avicel (1%). Depending on the purpose of the experiments, cultures were grown in baffled Erlenmeyer flasks containing 5-200 ml on a rotary shaker for 2-8 d. *E. coli* strains (JM83 or XL-1 Blue) were cultivated in LB medium at 37 °C (Sambrook et al., 1989).

**Chemicals and enzymes.** Chemicals for SDS gel electrophoresis were obtained from Serva. Molecular mass markers, nitrophenyl, O-dianisidine, 4-chloro-1-naphthol (4ClN) and 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were supplied by Sigma. Columns for FPLC were purchased from Pharmacia. H$_2$O$_2$ (30 % w/v) was from Merck.

**Large-scale protein purification.** Spores of *Streptomyces reticuli* were added to basal minimal medium supplemented with 1 % crystalline cellulose (Avicel TG 104, Sigma) in baffled 1 l Erlenmeyer flasks for 2 d. *Streptomyces reticuli* was grown in minimal medium supplemented with 1 % Avicel in 10 aliquots of 700 ml, as described by Schlochtermeier et al. (1992b). Mycelia were harvested by centrifugation (20 min at 100000 g) and washed three times with 20 mM potassium phosphate buffer, (pH 7)/0.1 % Triton X-100. The combined wash fractions (2.2 l) were loaded onto a DEAE column (Pharmacia 16/10) previously equilibrated in 20 mM sodium phosphate buffer (pH 7.0), supplemented with 0.05 % Triton X-100 and eluted with a 90 ml gradient (0-0.5 M NaCl). The catalase-peroxidase-containing fractions eluted at 0.25-0.3 M NaCl. The samples from the DEAE column were diluted with phosphate buffer and loaded onto a MonoQ (5/5) column. Proteins were eluted with a 50 ml gradient (0-0.5 M NaCl) at a flow rate of 1 ml min$^{-1}$ with the buffer described above. The proteins eluted in the presence of 0.3-0.35 M NaCl displayed catalase and peroxidase activity. These fractions were pooled, dialysed against phosphate buffer with 150 mM NaCl and loaded onto a Superose 6 (HR10/30) column (Pharmacia), equilibrated with 20 mM sodium phosphate buffer containing 150 mM NaCl. The protein concentration of the samples was determined employing the method of Bradford (1976) and the Lowry method.

**Production of antibodies.** Antibodies were raised against the purified catalase-peroxidase. Antisera from rabbits were obtained by immunization with the lyophilized enzymes mixed with Freund’s complete and incomplete adjuvants. The rabbits were bled 10 d after the third booster injection. Cellular particles were removed by centrifugation and the antisera were stored in aliquots at -20 °C.

**Determination of N-terminal amino acids.** The purified mature enzyme and peptides (generated by the LysC protease) were commercially determined by Edman degradation (Wita).

**Native and SDS-PAGE.** For electrophoresis of the native enzyme, a 10 % (w/w) polyacrylamide gel, pH 7.5, was used (Laemmli, 1970). SDS-PAGE was performed in the presence of 0.1 % SDS (Laemmli, 1970).

**Detection of catalase activity.** The native polyacrylamide gel was washed three times for 15 min with distilled H$_2$O$_2$ suspended in a solution of 0.01 ml 30 % H$_2$O$_2$ in 100 ml H$_2$O and gently rocked for 10 min. H$_2$O$_2$ was aspirated and the gel quickly rinsed in H$_2$O. A freshly prepared mixture of 30 ml each of 2% ferric chloride and 2% potassium ferricyanide, both in H$_2$O, was poured into a fresh staining pan and the rinsed gel transferred to the ferricyanide mixture (Wayne & Diaz, 1986). The gel tray was gently but steadily rocked by hand over a light box. As soon as a green colour began to appear in the gel itself, the ferricyanide mixture was rapidly aspirated and replaced with water. The gel was washed twice with water.

**Test for peroxidase activity.** Samples were loaded onto a
native 10% polyacrylamide gel. After the run, the gel was washed twice with acetate buffer (20 mM, pH 5.5) and activity staining was carried out with 4C1N and 5 mM H2O2 (Conyers & Kidwell, 1991). The peroxidase activity was monitored spectrophotometrically at room temperature in a reaction mixture containing 50 mM sodium acetate buffer (pH 5.5), 2 mM H2O2 and 1 mM O-dianisidine by following the rate of oxidation at 460 nM (ε460 = 11.3 × 105 M cm⁻¹). One unit of peroxidase activity is defined as the amount of enzyme causing the disappearance of 1 µmol substrate min⁻¹.

**Isolation of total DNA and plasmids.** Chromosomal DNA of *Streptomyces reticuli* was isolated after growth in a sucrose-containing complete medium for 2 d (Dittrich et al., 1991). The *E. coli* plasmid pUC18 was isolated using the alkaline lysis method (Sambrook et al., 1989) and the multicopy *Streptomyces/E. coli* vector pWHM3 was obtained by a modified alkaline lysis method (Dittrich et al., 1991; Hopwood et al., 1985).

**Cleavage of DNA, ligation and agarose gel electrophoresis.** The DNAs were cleaved with various restriction enzymes according to the suppliers’ instructions. Ligation was performed with T4 ligase (Sambrook et al., 1989). Gel electrophoresis was carried out in 0.8–1% agarose gels using TBE buffer. Fragments were visualized under UV after Staining with ethidium bromide (Sambrook et al., 1989).

**Transformations.** *E. coli* was transformed with plasmid DNA using the CaCl2 method (Sambrook et al., 1989) or by electroporation (Dower et al., 1988). *Streptomyces lividans* 66 protoplasts were transformed and regenerates as described by Hopwood et al. (1985). Transformants were selected using an overlay of 0.4% agarose containing 500 µg thioestrepton ml⁻¹ (Hopwood et al., 1985).

**Screening of a λgt11 gene library.** In our laboratory, a library of genomic DNA fragments (in the range 0.4–4.0 kb) was constructed in the bacteriophage expression vector λgt11 (Schlochtermeier et al., 1992b). Phages were propagated in *E. coli* Y1090 and screened with antibodies. Filters were incubated in PBS containing a 1:105 dilution of the anti-CpeB antibodies and washed three times in PBS. Then they were incubated with goat anti-rabbit IgG F(ab),, diluted 1:3000 in PBS, followed by color development (West et al., 1990). Immunopositive plaques were collected and rescreened to purity.

**Hybridization experiments.** The transfer of DNA fragments from a subgenomic *Streptomyces reticuli* library from BamHI fragments (6–8 kb) and a *Streptomyces reticuli* genomic library in Charon 35 (Schlochtermeier et al., 1992b) onto nylon membranes was performed as described by Sambrook et al. (1989). DNA from phage plaques was transferred to nitrocellulose filters (Allday & Jones, 1987). The inserts isolated from hybrid λgt11 phages were labelled using Klenow enzyme and digoxigenin-11-dUTP. Hybridization and immunological detection were carried out according to the specifications of the DNA Labelling and Detection Kit supplied by Boehringer Mannheim.

**DNA sequencing and computer analysis.** For sequencing, specific DNA fragments were subcloned into pUC18/19. Overlapping deletions were generated by exonuclease III digestion (Henikoff, 1987). Non-radioactive sequencing was performed with the GATC kit (Beck & Pohl, 1984), using standard primers of the lacZ system as well as synthesized primers. Sequence entry, primary analysis and ORF searches were performed using CloneManager 5.0. Database searches using the PAM120 scoring matrix were carried out with BLAST algorithms (BLASTX, BLASTP and TBLASTN) on the NCBI file server (blast@ncbi.nlm.nih.gov) (Altschul et al., 1997). Multiple sequence alignments were generated by means of the CLUSTAL W (1.74) program (Higgins et al., 1992).

**RESULTS**

**Purification of a mycelium-associated *Streptomyces reticuli* catalase-peroxidase.**

When studying the cellulolytic system of *Streptomyces reticuli*, we noticed that the strain produces a mycelium-associated catalase-peroxidase in addition to the previously described mycelium-associated cellulase 1 (Avicelase) (Schlochtermeier et al., 1992b). The isolation procedure was optimized and larger amounts of the protein were purified. To achieve this, proteins were released from the mycelia by three consecutive washings in buffer supplemented with 0.1% Triton X100 and were separated by anion exchange chromatography (DEAE and MonoQ), followed by Superose 6. The size of the enzymes was determined to be 160 and 82 kDa, respectively, by gel filtration and SDS-PAGE (Fig. 1). Thus the native CpeB consists of two identical subunits. Spectroscopical investigations revealed that a solution of the enzyme has a pronounced Soret band at 406 nm and two smaller peaks at 503 and 643 nm. The addition of KCN (10 mM) resulted in a shift of the Soret band to 421 nm, the appearance of a peak at 540 nm and of a shoulder at 637 nm. The ratio A406/A280 for the pure enzyme was 0.525, suggesting that one molecule of protohaem IX was present per CpeB dimer. The enzyme acts as catalase-peroxidase and accepts a broad range of substrates, hence the designation CpeB, including ABTS, benzidine, 3-dimethylaminobenzoic acid, 3-methyl-2-
benzothiazolinone hydrazone (MBTH), 3,3'-diaminobenzidine, 4C1N, NADH, NADPH, ascorbate, guaiacol and veratrylalcohol.

Screening of an expression library and cloning of the gene

Five hundred micrograms of the highly purified 82 kDa enzyme was used to raise antibodies. In addition, the N-terminal amino acids from the LysC protease-generated internal peptides of the purified enzyme were determined by Edman degradation. The antibodies and a previously constructed expression library (in λgt11) of Streptomyces reticuli total DNA (Schlochtermeier et al., 1992b) were used to screen for hybrid phages. Two phages synthesized peptides cross-reacting with anti-CpeB antibodies. The phage DNAs were isolated and their inserts (0.5 and 0.7 kb fragments carrying the EcoRI linkers of λgt11) were sequenced. An analysis of the possible ORFs allowed the deduction of a stretch of amino acids, which shares a high degree of similarity to a block of amino acids, including the proximal histidine ligand involved in haem binding within bacterial catalase-peroxidases.

The 0.5 kb fragment hybridized to a 6 kb BamHI fragment, a 1.8 kb SalI fragment and a 4.6 kb KpnI-SphI fragment from total Streptomyces reticuli DNA (Fig. 2). Subgenomic libraries of BamHI fragments (5-7 kb) and KpnI-SphI fragments (4-5 kb) from Streptomyces reticuli DNA were constructed with pUC18. Ampicillin-resistant E. coli JM83 transformants were screened by colony hybridization. Several clones carrying the correct insert were identified. The 6 kb BamHI insert of one of them, pUBB1, was characterized by restriction enzyme analysis (Fig. 2). The insert of pUKS10 containing a 4.6 kb KpnI–SphI fragment was found to overlap with the main part of the BamHI insert and to stretch beyond one of its ends.

Determination of the sequence and its analysis

The sequence of 3193 bp corresponding to a KpnI–SrfI fragment was determined. Two potential ORFs were found. A small one (furS) starts with a start codon (ATG) at position 385 and ends with a stop codon (TGA) at position 822. The larger ORF (cpeB) comprises...
The deduced proteins

cpeB encodes a protein of 740 aa with a predicted molecular mass of 81,346 Da, corresponding to that of the purified monomeric form (apparent molecular mass 82 kDa) of the Streptomyces reticuli enzyme (Fig. 1).

The calculated isoelectric point is 4.88. The experimentally determined amino acid sequence of the N termini of the internal peptides corresponded to that in the protein deduced from the DNA sequence. No predictable signal peptide could be identified in the deduced CpeB. Within several regions, the deduced protein shares a high number of identical amino acids with several other deduced bacterial catalase-peroxidases (Fig. 3) and includes the regions containing the amino acid residues essential for the catalytic centre within other haem-containing peroxidases (Smulevich, 1995). The number of identical amino acids ranges from 63 to 76% (block I) and from 39 to 72% (block II), depending on the origin of the corresponding enzyme (Fig. 3 and Discussion). Moreover, the relative positions of the amino acid residues Arg105, His109 and His272 correspond to those of the yeast cytochrome c peroxidase (Fig. 3). These residues are essential for the formation of the catalytic centre within other haem-containing peroxidases (Smulevich, 1995; Poulos et al., 1995).

The deduced protein (Furs) encoded by the small ORF

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<tr>
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<tr>
<td>Archful</td>
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<tr>
<td>Synecys</td>
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<tr>
<td>Rhocaps</td>
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<td>Synecys</td>
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<tr>
<td>Rhocaps</td>
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**Fig. 3.** Comparisons of block motifs. Blocks I and II from the deduced Streptomyces reticuli catalase-peroxidase (Stretic) containing both the distal and the proximal histidine ligand (black) were aligned with the corresponding regions of deduced catalase-peroxidases from: Caulobacter crescentus, Caulcre (AF027168); Archaeoglobus fulgidus, Archful (AE000591); Mycobacterium tuberculosis, Myctube (X68081); Mycobacterium smegmatis, Mycintr (X88718); Mycobacterium intracellulare, Mycintr (M86741); Bacillus stearothermophilus, Bacstea (X62422); E. coli (chromosomal) Ecoli C (M21516) and E. coli (plasmid) Ecoli P (X89017); Salmonella typhimurium, Saltyph (X53001); Synechococcus sp. PCC 7942, Syneec (D61378); Synechocystis sp. PCC 6803, Synecys (D90910); Rhodobacter capsulatus, Rhocaps (X71420); and the cytochrome c peroxidase from Saccharomyces cerevisiae, Cytoper (X62422; aa numbering from aa 68 of the precursor). All sequences were deduced from sequences deposited in GenBank. Identical (*) and similar (.) amino acid residues are marked. The additional lane of identical residues is shown at the bottom shows identical and similar amino acids between the alignment of bacterial KatG sequences and the cytochrome c peroxidase sequence.

a start codon (ATG) at position 856 and a stop codon (TGA) at position 3078. The G+C content of cpeB is 69.6 mol% and that of furS is 73.5 mol%.
comprises 145 aa; it has a molecular mass of 15,123 Da and a calculated pI of 5.35. FurS shares 29% identical and 44% similar amino acids with the *E. coli* Fur (ferric uptake regulator) protein. The relative location of the His91/His93 and Cys96/Cys99 residues of FurS corresponded to that of the predicted metal-binding sites (His88/His90 and Cys93/Cys96) of the deduced *E. coli* Fur protein (Bagg & Neilands, 1987).

The multiple sequence alignment analysis of more than 45 known Fur and Fur-like proteins (used to generate the phylogenetic tree in Fig. 7) showed that TyrS9 (aa numbering for FurS) is completely conserved (alignment not shown).

**Expression of the enzyme in *Streptomyces lividans***

The 4.6 kb *KpnI–SphI* fragment from pUKS10 was cloned into pWHM3, inducing the formation of pWKS10, which in turn was cloned into *Streptomyces lividans* as host. Like *Streptomyces reticuli*, the *Streptomyces lividans* transformant carrying pWKS10 produced a mycelium-associated catalase-peroxidase that could be reproducibly released by washing the mycelia with buffer supplemented with Triton X100. Moreover, antibodies raised against the enzyme purified from *Streptomyces reticuli* cross-reacted with one of the proteins released from the mycelia of *Streptomyces lividans* (pWKS10). However, no cross-reacting protein was detected among the proteins released from the control strain *Streptomyces lividans* (pWHM3) (Fig. 4). In conclusion, the data show that the cloned gene encoded the enzyme characterized from *Streptomyces reticuli* (Fig. 1).

**Regulation**

Secondary structure predictions suggest that the deduced protein FurS contains a putative HTH (helix–turn–helix) motif within the N-terminal region (Fig. 5b). Therefore, we concluded that it may represent a regulatory DNA-binding protein. To test whether the upstream gene affects expression of *cpeB*, the *KpnI–HindIII* insert of pWKS10 was cloned into pUDS18 (a derivative of pUC18 carrying an SstI deletion). The SstI site of the resulting construct was removed (after cleavage, Klenow treatment and religation in plasmid pUKD30). By sequencing it could be shown that plasmid DNA of several transformants containing pUKD30 carry the SstI site deletion. The deduced truncated protein contains only 11 of the N-terminal amino acids of FurS. The *KpnI–HindIII* fragment of pUKD30 containing the in-frame deletion was then recloned in the bifunctional vector pWHM3 (Fig. 2), entailing the formation of pWKD30. The level of peroxidase activity was compared in *Streptomyces lividans* transformants containing either pWKS10 or pWKD30. In the presence of Fe ions, the peroxidase activity of *Streptomyces lividans* (pWKD30) was several times higher than that of *Streptomyces lividans* (pWKS10). Immunological tests and analyses of protein-stained gels showed that *Streptomyces lividans* (pWKD30) produced low levels of the enzyme in the presence of Fe ions and enhanced levels in the presence of chelators for Fe$^{3+}$ and Fe$^{2+}$ ions, respectively (diethylenetriaminepentaacetic acid and EDTA). *Streptomyces lividans* (pWKD30), on the other hand, produced higher quantities of CpeB than *Streptomyces lividans* (pWKS10) and this amount was not affected by the presence of Fe$^{3+}$/$^{2+}$ chelators (Fig. 6).
Regulation of *Streptomyces reticuli* cpeB by FurS

**(a)**

<table>
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<th>Location</th>
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**Fig. 5.** Comparison of FurS and Fur proteins. (a) Deduced amino acid sequence of *Streptomyces reticuli* (Streptic) furS aligned with Fur and Fur-like proteins deduced from sequences deposited in GenBank: *Mycobacterium tuberculosis*, Myctube (X68801); *Staphylococcus epidermidis*, Sepider (X97011); *Bacillus subtilis*, Bsubtil (D84432); Synechocystis sp. PCC 6803, Synecys (D90909); *E. coli*, E. coli (X02589); *Pseudomonas aeruginosa*, Psaerug (L06004). (b) Secondary structure prediction. Comparison of predicted secondary structures of FurS (*Streptomyces reticuli*) and Fur (*E. coli*) proteins; four secondary structural elements, coils (C), α-helices (H), β-sheets (S) and turns (T) are shown as differently shaded boxes.

Data imply that FurS is a repressor and regulates the transcription of cpeB.

**DISCUSSION**

During the early stages of growth, *Streptomyces reticuli* was found to synthesize a mycelium-associated dimeric catalase-peroxidase with broad substrate specificity. After its purification, the corresponding gene was characterized. An analysis of the deduced CpeB protein (81.3 kDa) revealed that it shares a high degree of amino acid identity with enzymes of similar size, including KatG from *Caulobacter crescentus* (Steinman et al., 1997), *Mycobacterium tuberculosis* (Zhang et al., 1992; Nagy et al., 1995) and *Mycobacterium smegmatis* (Marcinkeviciene et al., 1995; Magliozzo & Marcinkeviciene, 1997), KatP from *E. coli* (Brunder et al., 1996), and PerA from *Archaeoglobus fulgidus* (GenBank AE000951) and *Bacillus stearothermophilus* (Loprasert et al., 1988) (Fig. 3). The unrooted phylogenetic tree (data not shown) confirms that CpeB belongs to the plant type of peroxidases. Subfamily 1, to which it belongs, contains yeast cytochrome c peroxidase, ascorbate peroxidase and bacterial catalase-peroxidases (Welinder, 1991). Key residues required for catalytic activities are highly conserved among all members of the catalase-peroxidases. Gapped multiple sequence alignments (data not shown) disclosed variations in the length of amino acid ‘stretches’ separating the distal and the proximal histidine residues. Only the introduction of a gap of 37–39 aa leads to an alignment of these enzymes with haem-dependent peroxidases. This variability may explain the substrate plasticity of catalase-peroxidases, as compared to monofunctional...
peroxidases. In this context, it is interesting that the *Streptomyces reticuli* CpeB enzyme, unlike all other tested catalase-peroxidases, also oxidizes ascorbate, guaiacol and veratrylalcohol. All known catalase-peroxidases studied to date, except KatP, have been found within the cytoplasm. The *Streptomyces reticuli* CpeB enzyme, in contrast, is extracellularly loosely associated with the mycelia. As we could not predict a corresponding signal peptide on the basis of known signal peptide sequences of bacteria, we will investigate whether CpeB may be exported by a sec-independent secretion machinery which has been identified in other bacteria (Wattiau et al., 1996; Pugsley et al., 1991). *E. coli* KatP, on the other hand, has a predicted signal peptide and was detected within the periplasm (Brunder et al., 1996).

Our data revealed that the synthesis of the *Streptomyces reticuli* catalase-peroxidase is under the control of a regulatory gene, whose gene product Fur5 shares 29% identical and 44% similar amino acids with the *E. coli* Fur protein. Homologues of *E. coli* Fur have been characterized predominantly in various Gram-negative bacteria (Bourke et al., 1996; Hantke, 1984). Recently, fur-like genes have also been reported in three Gram-positive bacteria: *Staphylococcus epidermidis* (Heidrich et al., 1996), *Bacillus subtilis* (GenBank Z82044, D84432) and *Streptococcus pyogenes* (GenBank U76538). Fur proteins are rich in histidine and cysteine residues and contain an N-terminally located DNA-binding region, as well as a C-terminal domain binding metal ions (Fe²⁺ or other divalent first row transition elements) (Bagg & Neilands, 1987). Upon binding of the metal ions to the C-terminal domain, a conformational change is induced within the N-terminal part of the protein, leading subsequently to the interaction of the Fur homodimer with the corresponding operator sequence. When complexed with Fe³⁺, *E. coli* Fur and its close bacterial homologues act as repressors of a number of genes (Ochsner & Vasil, 1996; Hantke, 1984, 1981). Recent studies with *Salmonella typhimurium* have indicated that the global regulatory protein Fur can regulate gene expression both in a positive and in a negative way and that regulation also occurs in the absence of the iron-sensing function of Fur (Hall & Foster, 1996).

When grown in the presence of varying concentrations of Fe ions, *Streptomyces lividans* (pWKSlO), containing the intact furS gene, synthesized low levels of CpeB. However, if the strain is cultivated with compounds chelating Fe²⁺ or Fe³⁺ ions, higher amounts of CpeB protein are synthesized, as shown by immunological studies. In contrast, *Streptomyces lividans* (pWKD30), carrying an in-frame deletion in furS, produced enhanced levels of CpeB protein. These data suggest that in the presence of Fe ions, FurS acts as repressor of *cpeB*. OxyR regulates the transcription of *E. coli* katG (encoding hydroperoxidase I) (Storz et al., 1990; Zheng et al., 1998). Within *Mycobacterium tuberculosis*, however, oxyR is inactivated by multiple lesions. These findings refuted the initial assumption that the transcription of the mycobacterial katG gene is under the control of an OxyR-type regulator (Deretic et al., 1995).

In the course of sequencing projects, several furA genes could be identified within different mycobacteria, the deduced proteins of which are closely related to *Streptomyces reticuli* Fur5. FurS and FurA (*Mycobacterium tuberculosis*) show 64% identity and 77% similarity; the internal FurS region (Gln56 to Ser140) even shares 80% identical and 94% similar amino acids with the corresponding part of the *Mycobacterium tuberculosis* homologue (Fig. 5A). It is intriguing that all katG genes found within the genomes of different mycobacteria contain an upstream-located furA gene (Fig. 7). Moreover, a recently sequenced genomic region within *Mycobacterium leprae*, which is linked to a non-

**Fig. 6.** Regulation of enzyme synthesis. *Streptomyces lividans* (pWKSlO) (column/lanes 1) and *Streptomyces lividans* (pWKD30) (column/lanes 2) were cultivated for 40 h and shifted to medium containing 5 μM Fe ions for 30 h. Aliquots of released mycelium-associated proteins were tested for peroxidase activity (a), analysed on SDS-PAGE gels stained with Coomassie blue (b) or were tested with anti-CpeB antibodies after transfer of the SDS-gel to a membrane (c). In addition, each strain was shifted to a medium containing the chelator for Fe³⁺ and Fe²⁺ ions (diethylenetriaminepentaacetic acid and EDTA). The proteins released from the mycelia were analysed on SDS-PAGE gels stained with Coomassie blue (d) or with anti-CpeB antibodies after transfer (e).

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Fig. 7. Unrooted phylogenetic tree of the deduced amino acid sequences of bacterial fur and fur-like genes. All sequences used were obtained from the GenBank database [Mycobacterium tuberculosis FurA, AF002194; Mycobacterium leprae pseudo-FurA, AF13983; Mycobacterium smegmatis FurA, AF012631; Mycobacterium fortuitum FurA1, Y07865*; Mycobacterium fortuitum, FurA2, Y07866*; Mycobacterium tuberculosis FurB, 295208; Mycobacterium leprae FurB, L78812*; Streptomyces venezuelae, X74791; Desulfovibrio vulgaris, U82323; E. coli K-12, X02589; Klebsiella pneumoniae, L23871; Yersinia pestis, S47625; Vibrio cholerae, M86623; Haemophilus influenzae, L42023; Neisseria gonorrhoeae, L11361; Bordetella pertussis, L31851; Pseudomonas aeruginosa, L00604; Pseudomonas fluorescens, AF505677; Legionella pneumophila, U06072; Rahtiaa eutropha, AJ001224; Campylobacter jejuni, Z35165; Campylobacter upsaliensis, L77075; Synechococcus sp. PCC 7942, L41065; Synechocystis sp. PCC 6803 Fur1, D90909; Synechocystis sp. PCC 6803 Fur2, D90903; Synechocystis sp. PCC 6803 Fur3, D90917; Staphylococcus epidermidis, X97011; Bacillus subtilis Fur (YqkL), D84432; Bacillus subtilis Fur2 (YqkV), Z99116; Bacillus subtilis Per (YqkG), Z99108; Desulfomonile tiedjei, AF015192*; Aquifex aeolicus FurR1, AE000728; Aquifex aeolicus FurR2, AE000739; Brucella abortus, AF23177; Rhizobium leguminosarum, Y13657; Helicobacter pylori, AE006611; Streptococcus pyogenes Per (Fur) U76538), the TIGR database [contig gmt7891 encoding the FurC protein (database for the Mycobacterium tuberculosis clinical isolate #CSU94 genome), contigs gdr 186 and gdr 157 encoding Fur1 and Fur2, respectively, in the Deinococcus radiodurans genome; contigs BTMCUS4R, BTMDR61R and BTMDH20F encoding Fur1, Fur2 and Fur3, respectively, in the Thermotoga maritima genome], the Oklahoma University (OU_ACGT) database [contig 874 (Actinobacillus actinomycetemcomitans genome) and the Genome Therapeutics Corp. database [contig 112 (Clostridium acetobutylicum ATCC 824 genome)]. An asterisk next to the above accession numbers indicates the presence of a fur-like gene that has not been annotated by the authors. The CLUSTAL W (1.74) software on the EMBL file server (http://www2.ebi.ac.uk) was used for multiple sequence alignments and computation of the phylogenetic tree. The scale represents 0.1 aa changes per position. The Fur and Fur-like proteins (and the corresponding bacteria) whose properties have been elucidated are printed in larger type.

The functional katG gene, represents a furA-like pseudogene (Eiglmeier et al., 1997; Nakata et al., 1997). Using primers deduced from the genomic Mycobacterium tuberculosis sequence, a homologous furA-katG locus was cloned from the non-tuberculous pathogen Mycobacterium marinum (Pagan-Ramos et al., 1998). In contrast, the upstream regions of catalase-peroxidase genes sequenced to date from various other bacteria do not contain fur or fur-like genes (Fig. 7). It therefore seems likely that the katG genes from several mycobacteria are, contrary to those from other bacteria, regulated by FurA in a fashion similar to that ascertained for FurS from Streptomyces reticuli. Thus it would be interesting to test whether clinical mycobacterial strains carry varying mutations in the corresponding furA genes, leading to differing levels of the catalase-peroxidase KatG and subsequently to different degrees of isoniazid resistance.

Sequencing data indicate that the genome of Mycobacterium tuberculosis harbours, apart from the Streptomyces reticuli furS homologue, a furB and a furC gene whose deduced gene products are quite closely related to each other, but significantly differ from FurA and Streptomyces reticuli FurS (Fig. 7). The Bacillus
**subtilis** genome contains three fur-like genes which encode the repressors Fur (formerly YqfK), PerR (formerly YgaG) and YqfV whose functions have not yet been explored (Bsat et al., 1998). It will also be of interest to investigate whether the genomes of streptomycetes contain two or three fur-like genes, like those of *Mycobacterium tuberculosis*, *Bacillus subtilis*, *Synechocystis* sp. PCC 6803, *Deinococcus radiodurans*, *Thermotoga maritima* and *Aquifex aeolicus*. The analyses (unrooted phylogenetic tree) of all known deduced Fur-like proteins from different bacteria allow us to classify four additional types (Fig. 7) in addition to the 'classic' *E. coli* Fur type.

Previously a homologue (desR) of *dtXR* was found in *Streptomyces pilosus* (Günter-Seeboth & Schupp, 1995). DtxR had been identified as an iron repressor in *Corynebacterium diphteriae* (Tao et al., 1994) and it had been assumed that fur genes are missing in streptomycetes. Our data show for the first time that a *Streptomyces* strain harbours a fur-like gene encoding a functional, regulatory protein. It will be of great interest to define the regulatory protein. It will be of great interest to define the principle of protein-dye binding.


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