Amino acid residues involved in reversible thiol formation and zinc ion binding in the *Streptomyces reticuli* redox regulator FurS

**Abstract** *Streptomyces reticuli* produces a mycelium-associated enzyme, CpeB, whose N-terminal and C-terminal portions mediate heme-dependent catalase-peroxidase and heme-independent manganese-peroxidase activities, respectively. The regulator FurS governs transcription of the furS-cpeB operon. The thiol form of FurS contains one zinc ion per monomer and binds in this state to its cognate operator. Oxidation of SH groups within FurS induces the release of the zinc ion. Substitution of the codons for the amino acids cysteine 96, histidine 92 and 93, and tyrosine 59 in furS disrupts the in vivo repressor activity of FurS and results in enhanced synthesis of CpeB in corresponding *S. lividans* transformants. Biochemical and footprinting studies with FurS and its mutant derivatives revealed that the cysteine residues 96 and 99 in furS are involved in reversible S-S bond formation, while cysteine 96 and the histidine residues 92 and 93 are required for zinc coordination, and tyrosine 59 is necessary for the binding of FurS to DNA. On the basis of these data, functional predictions can be made for the mycobacterial regulator FurA, a close homologue of FurS.

**Keywords** Streptomycetes · Thiol-reduced FurS · Redox regulator protein · Metalloregulator · CpeB

**Introduction**

During early stages of growth, *Streptomyces reticuli* synthesizes a mycelium-associated enzyme called CpeB. The N-terminal portion of the protein is required for heme-dependent catalase and peroxidase activities, while the C-terminal part mediates heme-independent manganese-peroxidase activity (Zou and Schrempf 2000). An analysis of the protein sequence (81.3 kDa) deduced from the sequence of the *cpeB* gene revealed that it shares a high degree of similarity (65% to 70%) with several other bacterial catalase-peroxidases, including KatG proteins from *Caulobacter crescentus* (Steinman et al. 1997), *Mycobacterium tuberculosis* (Zhang et al. 1992; Nagy et al. 1995) and *M. smegmatis* (Marcinkeviciene et al. 1995; Magliozzo and Marcinkeviciene 1997).

A previous study from this laboratory (Zou et al. 1999) and further transcriptional analyses have demonstrated that the *cpeB* gene forms an operon with the *furS* gene. Upstream of the transcriptional start site of the *furS* gene is a putative promoter which is similar to the one previously encountered upstream of the cellulase (Avicelase) gene of *S. reticuli* (Schlochtermeier et al. 1992b; Walter and Schrempf 1996). In vivo studies have revealed that FurS acts as a transcriptional repressor. In vitro only the thiol-reduced form of the protein was found to bind specifically to the operator of the *furS* gene (Ortiz de Orué Lucana and Schrempf 2000). The deduced amino acid sequence and the predicted secondary structure of the *S. reticuli* FurS are closely related to those of FurA proteins – the predicted products of genes which have been found in the genomic sequences of several mycobacteria – and they diverge considerably (Zou et al. 1999) from those of the global Fur regulator of *Escherichia coli* (Hantke 2001). In the mycobacterial genomes, as in *S. reticuli*, a putative gene for a catalase-peroxidase (KatG) is located adjacent to the *furA* gene. An operator site resembling the one identified for the *S. reticuli* FurS is also located upstream of the *katG* gene in mycobacteria (Ortiz de Orué Lucana and Schrempf 2000).

In this report we present data on several mutant *furS* gene constructs and their corresponding protein products. These studies have allowed us to localize residues that participate in reversible thiol formation and zinc ion coordination in FurS.
Materials and methods

Bacterial strains and plasmids

Streptomyces lividans 66 (provided by D. A. Hopwood, John Innes Institute, Norwich, UK) was used as a host for transformations. In the literature this type strain (with the original designation S. lividans 1326, which is the number of the strain in the John Innes Strain Collection. For convenience we omit the number in the following. The E. coli strains DH5α and M15 (Zamenhof and Villarcejo 1972) and plasmids pWHM3 (Vara et al. 1989) and pQE32 (Qiagen, Hilden, Germany) were used for routine cloning purposes. The constructs pUKS10 (a pUC18 derivative) and pWKS10 (a pWHM3 derivative), both of which contain the furS-cpeB operon (Fig. 1A), have been described earlier (Zou et al. 1999; Ortiz de Oriu Lucana and Schrempf 2000).

Media and culture conditions

S. lividans was cultivated in complete medium and the washed mycelia were shifted to minimal medium supplemented with 0.5% yeast extract as carbon source (Schlochtermeier et al. 1992a; Ortiz de Oriu Lucana and Schrempf 2000). Depending on the purpose of the experiment, cultures were grown on a rotary shaker for 2-4 days, in baffled Erlenmeyer flasks containing 5-200 ml of medium. E. coli strains (DH5α or M15, Zamenhof and Villarcejo 1972) were grown in LB medium at 37°C (Sambrook et al. 1989).

Chemicals and enzymes

Chemicals for SDS-polyacrylamide gel electrophoresis were obtained from Serva. Molecular weight markers, nitrophenyl, O-dianisidine, 4-chloro-1-naphthol (4CN), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and diamide (a thiol-specific oxidant) were supplied by Sigma. Hydrogen peroxide (30% w/v) was purchased from Merck. For footprinting studies, a 6% PA gel (containing 7 M urea and 1% TBE) was used (Biometra). Trypsin (sequencing grade) and the endoproteinase Lys-C were supplied by Roche Diagnostics. Restriction enzymes were obtained from New England Biolabs.

Test for peroxidase activity

CpeB was released from the mycelium using phosphate buffer (pH 7) containing 0.1% Triton X-100 (Zou et al. 1999), and 30-μl aliquots were tested for activity. The peroxidase activity (Zou et al. 1999) 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 (E460 = 11.3×103 M cm⁻¹). One unit of peroxidase activity is defined as the amount of enzyme required to convert 1 μmol of substrate.

Cleavage of DNA, ligation, and agarose gel electrophoresis

DNAs were cleaved with restriction enzymes according to the suppliers' instructions. Ligation (Sambrook et al. 1989) was performed with T4 ligase (New England Biolabs). Gel electrophoresis was carried out in 0.8-2% agarose gels containing TBE buffer.

Mutagenesis of the furS gene, transformation and isolation of mutant plasmids

Each of the mutant constructs was assembled from two fragments (A + B, C + D and E + F), obtained by PCR using the furS gene in pWKS10 (Zou et al. 1999; see Fig. 1A) as the template, with appropriate oligonucleotide primers.

To replace tyrosine 59 in the FurS protein by phenylalanine, fragments A and B were linked together. To amplify fragment A, the primer N1eco (5'-CAAGGCGATTAAGTTGGGTACCGC-3') was combined with C1aSuII (5'-GTGAAGGGCTTCCGAGCAGCGGCTGAGGAGATG-3'). For fragment B, primers N2aSuII (5'-CAAGGCGATTCGAGCTGAGGAGATG-3') and C2nor (5'-CCCGGCCTGGCAGGAGATGACCC-3') were used. The primer N1aSuII (5'-CGACGCGGCTGAGCAGCGGCTGAGGAGATG-3') anneals upstream of the furS promoter and ligates to the 3' end of fragment A. The primer C2aSuII (5'-CGACGCGGCTGAGCAGCGGCTGAGGAGATG-3') anneals in the cpeB gene downstream of furS in pWKS10 and ligates to the 5' end of fragment B. After cleavage with AsuII, the two fragments were ligated. The resulting DNA fragment was then cleaved with EcoRI and StuI, and ligated to the longer EcoRI-StuI fragment.