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# CbiX-homologous protein (CbiXhp), a metal-binding protein, from *Streptomyces seoulensis* is involved in expression of nickel-containing superoxide dismutase

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**Abstract**

To find the accessory proteins participating in expression and maturation of nickel-containing superoxide dismutase (NiSOD), a metal-binding protein (CbiXhp) homologous to cobaltochelatase (CbiX) of *Bacillus megaterium* was isolated by nickel-nitrilotriacetic acid resin from *Streptomyces seoulensis*. The deduced amino acid sequence of *cbiXhp* showed 87% and 39% identity to CbiX of *Streptomyces coelicolor* and that of *B. megaterium*, respectively. Overexpression of CbiXhp increased the activity and the expression of NiSOD in the presence and absence of nickel, but to a lesser extent in its absence. This result indicates that CbiXhp is involved in the expression of NiSOD.


*Keywords:* *Streptomyces seoulensis*; Nickel-containing superoxide dismutase; CbiX-homologous protein

## 1. Introduction

The divalent metal ions, such as $Ni^{2+}$, $Zn^{2+}$ and $Co^{2+}$, are essential nutrients for bacteria and play various roles in proteins. Nickel, for example, is found in several enzymes including hydrogenase, carbon monoxide dehydrogenase, acetyl-*S*-coenzyme A synthase, methyl-*S*-coenzyme M reductase, urease and nickel-containing superoxide dismutase (NiSOD) [1–4]. It is known that nickel is incorporated into metallocenters through the coordination with surrounding ligands such as histidine and cysteine and for the complete metallocenter assembly, several accessory proteins which participate in the incorporation of nickel ions to the target metalloproteins are required [5,6].

Previously we have shown that a novel NiSOD exists in *Streptomyces seoulensis* (*Streptomyces* sp. IMSNU-1) [7]. In reconstitution experiments, low recovery of NiSOD activity in vitro suggested that accessory proteins might be involved in the activation of NiSOD [7]. In addition, proteolytic cleavage of the NiSOD precursor polypeptide was a prerequisite for the production of active NiSOD [8]. Although the presence of accessory proteins participating in N-terminal cleavage and metallocenter assembly of NiSOD has been suggested, no accessory protein has yet been identified and reported.

In this study, to search for the accessory protein of NiSOD, nickel-chelating affinity column chromatography was used [9], since a protein containing a histidine-rich region could be a candidate for the accessory protein involved in the expression or the maturation of NiSOD. Here we describe the purification of the protein (CbiXhp) homologous to cobaltochelatase (CbiX) of *Bacillus megaterium* from *S. seoulensis* and the cloning and sequencing of the *cbiXhp* gene encoding CbiXhp, and finally the effect of *cbiXhp* on NiSOD expression in *Streptomyces lividans*.

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## 2. Materials and methods

### 2.1. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this work are given in Table 1. *S. seoulensis* were grown in modified Bennett medium (1% glucose, 0.5% yeast extract, 0.3% malt extract, 0.5% malt extract, 0.5% peptone, 0.5% casamino acid). *S. lividans* TK24 cells were grown as described previously [10]. Cells containing pIJ702 or its derivatives were selected and maintained in 50 μg $ml^{-1}$ of thiostrepton (Sigma). *Escherichia coli* DH5α carrying pGEM®-5Zf(+) derivatives and the pET3a-CbiXhp plasmid were grown in LB containing 50 μg $ml^{-1}$ of ampicillin; BL21(DE3)pLysS carrying the pET3a-CbiXhp plasmid in LB containing 50 μg $ml^{-1}$ of ampicillin and 34 μg $ml^{-1}$ of chloramphenicol.

### 2.2. Purification of S. seoulensis CbiXhp

*S. seoulensis* were cultured at 28°C with vigorous shaking in modified Bennett medium containing 50 μM $NiCl_2$. After sonication and consecutive centrifugation, supernatants were loaded on a nickel-nitrilotriacetic acid (Ni-NTA) column (Qiagen) and the protein was eluted with a 0–250 mM imidazole gradient in 20 mM Tris–HCl (pH 7.9). The CbiXhp was further purified by Superdex 200 HR 16/60 column (Amersham Bioscience) with 20 mM Tris–HCl (pH 8.0).

### 2.3. Determination of N-terminal and internal amino acid sequences of CbiXhp

The purified CbiXhp was subjected to 12% (w/v) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto polyvinylidene fluoride (PVDF) membrane. The N-terminal amino acid sequence was determined using the Precise Protein sequencing system (Applied Biosystems). The internal amino acid sequences of CbiXhp were determined as follows. The purified enzyme (10 μg) was boiled in 0.01% SDS for 10 min and digested with endoproteinase Lys-C (Boehringer Mannheim) in 25 mM Tris–HCl (pH 8.5) and 1 mM EDTA for 20 min. The resulting peptide fragments were separated by SDS–PAGE and blotted onto PVDF membrane.

### 2.4. Cloning of the cbiXhp gene from S. seoulensis

To generate a genomic library, DNA was prepared from *S. seoulensis* cells, partially digested with *Sau*3AI and cloned into *Bam*HI-digested λEMBL3 (Stratagene). The gene encoding part of the nickel-binding protein was amplified by the polymerase chain reaction (PCR) with primers (A, B) and the genomic DNA of *S. seoulensis* as a template. Primer A, 5′-CCSGCSCTSCTSGTSGCSGGCCAC-3′, was designed from the N-terminal amino acid sequence Pro-Ala-Leu-Leu-Val-Ala-Gly-His. Primer B, 5′-SCGSCCGTASGTGTASGTSACSCC-3′, was designed from the internal sequence Gly-Val-Thr-Tyr-Thr-Tyr-Gly-Arg. The PCR product was used as a hybridization probe to screen the *S. seoulensis* genomic library. A 3.0-kb *Sal*I fragment from one positive phage clone was subcloned into pGEM®-5Zf(+) to generate pKGS500. The nucleotide sequence of *cbiXhp* from *S. seoulensis* has been deposited in the GenBank database under accession number AY338237.

### 2.5. Overproduction and purification of recombinant CbiXhp in E. coli

To overexpress the *cbiXhp* gene in *E. coli*, the 1.1-kb *cbiXhp* gene was amplified from pKGS500 using the two primers P1 (5′-CCGAGGAGAACATATGACCCCCCATTC-3′ [the *Nde*I site is underlined]) and P2 (5′-CGCGCGGCCGGATCCTCCGGGTAGGCC-3′ [the *Bam*HI site is underlined]), generating pGEMTCbiXhp. Then, the *Nde*I/*Bam*HI fragment of pGEMTCbiXhp was ligated into the expression plasmid pET3a (Novagen), yielding pET3a-CbiXhp. This plasmid was transformed into *E. coli* strain BL21(DE3)pLysS. Cells were grown up to an $OD_{600}$ of approximately 0.5 in medium containing 100 μM $NiCl_2$ and induced by 1 mM isopropyl-β-D-thiogalactoside at 37°C. After 3 h of induction, cells were harvested and recombinant protein was purified by the same procedures as the purification step of *S. seoulensis* CbiXhp.

### 2.6. Preparation of antibodies and Western immunoblot analysis

Antibody against CbiXhp was raised in mice using recombinant CbiXhp. For Western blot analysis, proteins were transferred onto nitrocellulose membrane and immunostained with an anti-CbiXhp antibody and an anti-Ni-SOD antibody. The bands were detected by anti-rabbit alkaline phosphatase-linked antibody and visualized by the addition of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

### 2.7. Activity staining of SOD in polyacrylamide gel

Crude extracts of *S. lividans* were separated on a 12% non-denaturing polyacrylamide gel. After soaking in 20 mM sodium phosphate buffer (pH 7.4) containing 28 μM riboflavin and 28 mM *N*,*N*,*N*′,*N*′-tetramethylethylenediamine for 10 min, the gel was incubated in 20 mM sodium phosphate buffer (pH 7.4) containing 2.5 mM nitroblue tetrazolium for 10 min and illuminated with fluorescent light for 3–10 min.

### 2.8. Overexpression of CbiXhp in S. lividans

A 510-bp portion of the *sodN* promoter encoding the

Table 1
Strains and plasmids used in this study

| Strain or plasmid | Relevant genotype/phenotype or description | Source or reference |
|---|---|---|
| *E. coli* DH5α | *F⁻ u80dlac DM15 D(lacZYA-argF) U169 supE44 k⁻ thi-1 gyrA recA1 endA1 hsdR17* | [11] |
| *E. coli* ET12567 | *dam⁻ dcm⁻ hsdS⁻* | [12] |
| *E. coli* BL21(DE3) pLysS | *F⁻ ompTr$_{B-}$ m$_{B-}$ (DE)/pLysS* | Novagen |
| *E. coli* XL1-Blue MRA | *Δ(mcrA)183, (mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 gyrA96 relA1 lac⁻* | Stratagene |
| *S. seoulensis* | isolate | [13] |
| *S. lividans* TK24 | wild-type strain | [10] |
| pGEMTCbiXhp | pGEM®-T Easy with 1.1-kb PCR fragment of CbiXhp | This work |
| pGEMTpsodN | pGEM®-T Easy with 0.5-kb PCR fragment of *sodN* promoter | This work |
| pNC02 | pGEMTCbiXhp with 0.5-kb fragment from pGEMTpsodN | This work |
| pKGS500 | pGEM®-5Zf(+) with about 3.0-kb *S. seoulensis* DNA insert isolated from genomic library containing CbiXhp | This work |
| pET3a-CbiXhp | pET3a with 1.1-kb fragment from pGEMTCbiXhp | This work |
| pIJ702 | *Streptomyces* vector derived from pIJ101 | [14] |
| pIJ-psodN | pIJ702 with 0.5-kb fragment from pGEMTpsodN | This work |
| pIJ-CbiXhp | PIJ702 with 1.6-kb fragment from pNC02 | This work |

promoter of NiSOD was amplified from the *S. seoulensis* λEMBL3 genomic library using the two primers, sodN-pro-SacI (5′-ACCCAGCGCACGAGCTCCAGCGGCG-3′ [the *Sac*I site is underlined]) and sodNpro-NdeI (5′-GGCGGGAAAGCATATGGCGTTCCTT-3′ [the *Nde*I site is underlined]), and cloned into pGEMT, generating pGEMTpsodN. Then the 1.1-kb *Nde*I/*Sac*I fragment of pGEMTpsodN was cloned into pGEMTCbiXhp, generating pNC02. To express the *cbiXhp* gene in *S. lividans*, the 0.5-kb *Sac*I/*Sph*I fragment from pGEMTpsodN and the 1.6-kb *Sac*I/*Sph*I fragment from pNC02 were cloned into the *Sac*I/*Sph*I sites of pIJ702, generating pIJ-psodN containing the *sodN* promoter only and pIJ-CbiXhp containing the *cbiXhp* gene under control of the *sodN* promoter in pIJ702. pIJ702, pIJ-psodN and pIJ-CbiXhp were transformed into *S. lividans*.

## 3. Results

### 3.1. Purification of CbiXhp from S. seoulensis and determination of N-terminal and internal amino acid sequences

A nickel-binding protein (CbiXhp) was purified by a Ni-NTA column from cell lysates of *S. seoulensis* which had been cultured in the presence of 50 μM $NiCl_2$. The purified nickel-binding protein (CbiXhp) at the position of about 35 kDa in SDS–PAGE eluted at 100 mM imidazole (Fig. 1). CbiXhp was further purified by Superdex 200 HR 16/60 column chromatography and the N-terminal amino acid sequence was determined using Edman degradation. The nickel-binding protein was digested by endoproteinase Lys-C and subjected to SDS–PAGE. Six peptide frag-

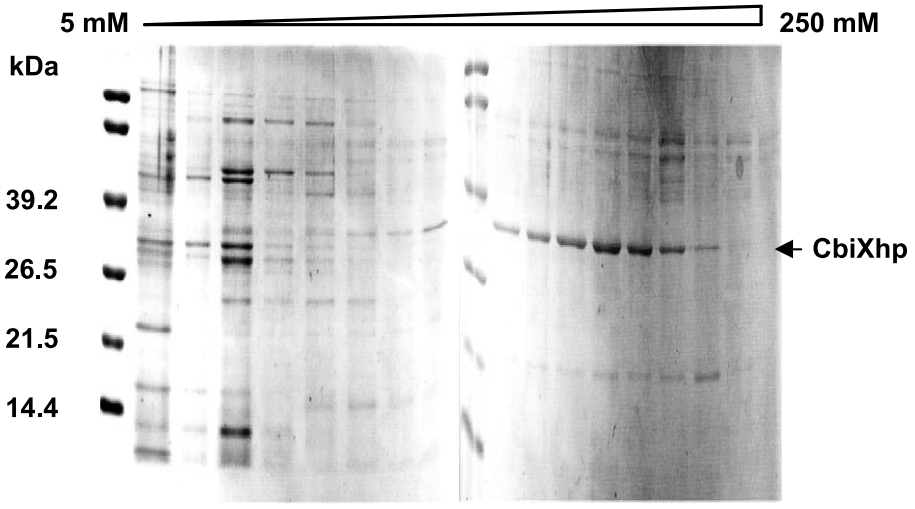


Fig. 1. Purification of *S. seoulensis* CbiXhp by the Ni-NTA column. Crude extracts of *S. seoulensis* were loaded on a Ni-NTA column and elution was performed with a gradient from 5 mM to 250 mM imidazole in Tris–HCl (pH 8.0) and 0.5 M NaCl. Fractions were analyzed by 12% SDS–PAGE and visualized with Coomassie blue.

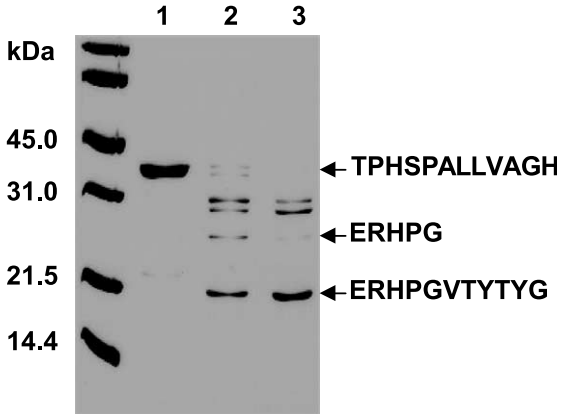


Fig. 2. SDS–PAGE of purified CbiXhp and its fragments digested by endoproteinase Lys-C. The uppermost arrow indicates the predetermined N-terminal sequence of CbiXhp. Lane 1: purified CbiXhp; lane 2: 10 min after Lys-C digestion; lane 3: 20 min after Lys-C digestion.

ments were generated from the proteolytic digestion of the protein (Fig. 2). Two bands corresponding to molecular masses of 28 and 22 kDa were subjected to amino acid sequence analysis. The N-terminal amino acid sequences of the two fragments were identical but different from that of the purified CbiXhp. Therefore, this sequence was taken as an internal amino acid sequence.

### 3.2. *Identification and characterization of the* cbiXhp *gene encoding CbiXhp of* S. seoulensis

A portion of *cbiXhp* was amplified by PCR using two degenerate oligonucleotide primers designed by the N-terminal and internal sequence of CbiXhp and a 309-bp PCR product was obtained. A 3.0-kb DNA fragment containing the *cbiXhp* gene was cloned from a λEMBL3 genomic library of *S. seoulensis* using the 309-bp PCR product as a probe. The partially sequenced 3.0-kb DNA fragment contained three open reading frames. One of them was identified to encode CbiXhp based on the fact that its deduced amino acid sequence matched 12 N-terminal and 12 internal amino acid residues of the purified CbiXhp. The *cbiXhp* gene resided between genes encoding homologues of the *Mycobacterium tuberculosis* putative aminotransferase CobC [15] and the *B. megaterium* precorrine-8× methylmutase CbiC [16], suggesting that CbiXhp may be involved in cobalamin biosynthesis. The *cbiXhp* gene consisted of 306 amino acid residues with a calculated molecular mass of 33 241 Da, which was smaller than the molecular mass determined by SDS–PAGE. Multiple alignments showed that *S. seoulensis* CbiXhp exhibited 87% and 39% identity to CbiX of *Streptomyces coelicolor* [17] and of *B. megaterium*, respectively (Fig. 3). *S. seou-*

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SSCbiXhp  MTP-------------------HSPALLVAGHGTRDEAGAEAFRDFVKELGR-RHPEIPV : 40
SC-CbiX   MT--------------------TPPALLIAGHGTRDEAGAEAFRDFVRELGR-RHPELPV : 39
SY-CbiX   MTLTSVPAPVSLFPELELPPLPYHRPLLMIGHGTRDEDGRQTFLDFVAQYQA-LDHSRPV : 59
BM-CbiX   MGG-----------------HYMKSVLFVGHGSRDPEGND--REFISTMKHDWDASILV : 40

SSCbiXhp  AGGFIELSPPPLGDAVTELVERGVRRFAAVPLMLVSAGHAKGDIPAALAREKERHPGVTY :100
SC-CbiX   AGGFIELSPPPLGDAVAELVERGVRRFAAVPLMLVSAGHAKGDIPAALAREKERHPGITY : 99
SY-CbiX   IPCFLELTEPNIQAGVQQCVDQGFEEISALPILLFAARHNKFDVTNELDRSRQAHPQINF :119
BM-CbiX   ETCFLEFERPNVSQGIDTCVAKGAQDVVVIPIMLLPAGHSKIHIPAAIDEAKEKYPHVNF :100

SSCbiXhp  TYGRPLGQHPALLRVLERRLDEALAGTPR------EDVTVLLVGRGSTDPDANAEVCKAA :154
SC-CbiX   TYGRPLGPHPGLLRVLERRLAEAVDPAWDP-----AEVTVLLVGRGSTDPDANAEVFKAA :154
SY-CbiX   FYGRHFGITPAILDLWKARLNQLDSPEANPQGIDRQDTVLLFVGRGSSDPDANGDVYKMA :179
BM-CbiX   VYGRPIGVHEEALEILKTRLQESGENLETP----AEDTAVIVLGRGGSDPDANSDLYKIT :156

SSCbiXhp  RLLWEGRGYAGVETAFVSLAAPDVPSGLDRVARLGARRVVVLPYFLFTGILPERVRRQTE :214
SC-CbiX   RLLWEGRGYAGVETAFVSLAEPDVPGGLERCVRLGARRVVVLPYFLFTGILPDRVRHQTE :214
SY-CbiX   RMLWEGSGYQTVETCFIGISHPRLEEGFRRARLYQPKRIIVLPYFLFMGALVKKIFTITE :239
BM-CbiX   RLLWEKTNYKIVETSFMGVTAPLIDEGVERCLKLGAKKVVILPYFLFTGVLIKRLEEMVK :216

SSCbiXhp  EWAAAHPDVEVRSADVIGPEPELLDLVMERYAEAVAGDLRMNCDSCVYRIALPGFEDKVG :274
SC-CbiX   EWAAAHPETDVRSADVIGPEPELLDLVWERYEEAVGGDLRMNCDSCVYRIALPGFEDKVG :274
SY-CbiX   EQRATFPEIEIQSLSEMGIQPELLALVRERREIETQLGQVAMNCEACKFRLAFKNQGHGHD :299
BM-CbiX   QYKMQHENIEFKLAGYFGFHPKLQTILKERAEEGLEGEVKMNCDTCQYRLGI-MEHIDH :274

SSCbiXhp  QPQQPHFHPDDDGHDHGHGHHHHGGHSHSHAH----- :306
SC-CbiX   LPQQPHFHPDDDG-DHPHGHHHGHGHAHSHAH----- :305
SY-CbiX   HGHGHHHHGHDHGHSHGEWVDTYIEPTAYHEKIWQAP :336
BM-CbiX   HHHHDHDHDHDHGHHHDHHHDHHEDKVGELK----- :306
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Fig. 3. Alignment of amino acid sequences of *S. seoulensis* CbiXhp (SSCbiXhp) with those of *S. coelicolor* CbiX (SC-CbiX), *Synechocystis* sp. CbiX (SY-CbiX) and *B. megaterium* CbiX (BM-CbiX). Residues identical in all four proteins are shaded in dark gray and similar residues in light gray. The conserved histidine-rich region and the possible CXXC metal-binding motif are boxed. GenBank accession numbers are as follows: *S. seoulensis* (AY338237), *S. coelicolor* CbiX (CAC38793), *Synechocystis* sp. CbiX (BAA10794) and *B. megaterium* CbiX (CAA04308).

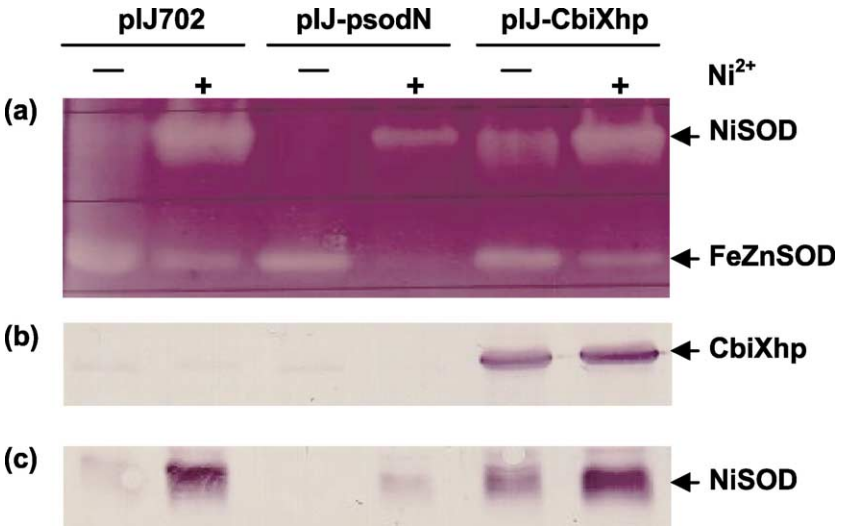


Fig. 4. Expression of multiple copies of the *sodN* promoter and *cbiXhp* gene in *S. lividans* TK24 in the absence and presence of 100 μM $NiCl_2$. a: SOD activity staining in a non-denaturing gel. b: Western blot with anti-CbiXhp antibody. c: Western blot with anti-NiSOD antibody. pIJ702, pIJ-psodN and pIJ-CbiXhp refer to *S. lividans* cells transformed with each vector construct as described in Section 2.

*lensis* CbiXhp had a histidine-rich metal-binding motif in its C-terminal region (13 histidines out of 27 amino acid residues) and a potential CXXC motif which was also likely to participate in the binding of metals. This C-terminal histidine-rich region and the CXXC motif are conserved in several, but not all, bacterial CbiXs (Fig. 3). Domain search analysis using Protein families database (Pfam) [18] revealed that *S. seoulensis* CbiXhp has a CbiX domain and ferrochelatase domain at its N-terminus, suggesting that it might be involved in metal chelation.

### 3.3. *Effect of CbiXhp overexpression on NiSOD activity in* S. lividans

To investigate the effect of CbiXhp overexpression on NiSOD, *S. lividans* TK24 cells were transformed with pIJ-psodN containing the *sodN* promoter only and pIJ-CbiXhp containing the *cbiXhp* gene under control of the *sodN* promoter in pIJ702. SOD activities of the transformants containing parental pIJ702, pIJ-psodN and pIJ-CbiXhp were analyzed by activity staining in the gel (Fig. 4a). It was reported that *S. lividans* TK24 cells produced two types of SOD whose electrophoretic mobility and antigenic properties were very similar to NiSOD and iron- and zinc-containing superoxide dismutase (FeZnSOD) of *S. coelicolor*, and when sufficient $Ni^{2+}$ was supplemented, NiSOD was induced and FeZnSOD was repressed [8]. Even though the $Ni^{2+}$-dependent regulation of the two SODs was conserved in all *S. lividans* transformants, a significant decrease of NiSOD activity was seen in *S. lividans* harboring pIJ-psodN compared to cells transformed with parental pIJ702. However, when CbiXhp was overexpressed under the promoter of *sodN*, the activity and amount of NiSOD were greatly increased compared to the cells harboring pIJ-psodN in the presence and absence of nickel (Fig. 4a,c). The observation that the introduction of multiple copies of the *sodN* promoter into *S. lividans* resulted in a decrease of the amount and activity of NiSOD in the absence of supplemented nickel could be explained by the existence of a nickel-dependent transcriptional activator. Since many copies of the *sodN* promoter were introduced into the cell, a limited amount of *sodN* activator could not act on the genomic *sodN* gene.

## 4. Discussion

In the present study, we purified CbiXhp and characterized the *cbiXhp* gene in *S. seoulensis*. CbiXhp had a histidine-rich motif and potential CXXC motif at its C-terminus. The observation that the *cbiXhp* gene resided between two genes involved in cobalamin biosynthesis and CbiXhp contained CbiX and a ferrochelatase domain at its N-terminus suggested that CbiXhp may function as a cobaltochelatase.

Based on the result that the overexpression of CbiXhp increased the activity and amount of NiSOD compared to cells harboring pIJ-psodN (Fig. 4a,c) and the fact that the regulation by $Ni^{2+}$ occurs at the level of transcription, it is suggested that CbiXhp could be involved in the expression of NiSOD. For example, *Bradyrhizobium japonicum* HypB [19], which contains a histidine-rich region at its N-terminus, is required for the nickel-dependent transcriptional regulation of hydrogenase [20]. There is another plausible function that CbiXhp might act as a nickel chelatase in NiSOD maturation. *B. megaterium* CbiX can chelate ferrous iron [21]. Raux et al. [16] suggested that MJ0908 in *Methanococcus jannaschii*, which has similarity to the cobalt chelatase CobN of *Pseudomonas denitrificans* [22], could also function as a nickel chelatase in the synthesis of coenzyme $F_{430}$ containing nickel. Furthermore, it was

reported in *Helicobacter pylori*, which contains two different nickel-containing enzymes, hydrogenase and urease, that in a *hypB* deletion mutant lacking a metallochaperone for hydrogenase the urease activity is 200-fold lower than that of the wild-type. It suggested that the metallochaperone reveals a pleiotropic effect on the other metalloenzymes [23]. Its histidine-rich motif could also render CbiXhp a candidate for sequestering nickel. To investigate the in vivo role of CbiXhp in NiSOD maturation in *Streptomyces* spp., the *cbiXhp* deletion mutant is being constructed.

In conclusion, we have demonstrated that CbiXhp from *S. seoulensis* affects the expression and activity of NiSOD via several possible ways. Therefore, our results would provide a clue to understand the mechanism of NiSOD synthesis and maturation.

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