

# Expression and purification of SenX3 from the *Mycobacterium tuberculosis* strain H37Rv in *Escherichia coli*

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**Abstract.** Infection with the bacterium *Mycobacterium tuberculosis* (MTB) causes tuberculosis, a pulmonary infection that may be fatal if left untreated. Misuse or mismanagement of tuberculosis drugs may lead to drug-resistant pathogen forms that are difficult to treat and contribute to a global health problem. The MTB SenX3/RegX3 signal transduction system allows bacteria to externally sense the environment and mediate an appropriate internal response; SenX3 is also associated with MTB virulence, suggesting that this protein may provide a potential therapeutic target. To investigate the role of SenX3 and MTB drug resistance, SenX3 was cloned, expressed and purified in *Escherichia coli*. SenX3 was cloned from the genome of the MTB strain H37Rv by polymerase chain reaction and an internal *NcoI* restriction site was destroyed by site-directed mutagenesis to allow cloning into the pET-28b prokaryotic expression vector. SenX3 expression from the resulting pET-28b-mSenX3 plasmid was induced with isopropyl  $\beta$ -D-thiogalactoside and the protein was purified using Ni-NTA agarose affinity chromatography. A pure protein of the expected size was identified. The examination of purified SenX3 protein is considered to enable the in-depth investigation of SenX3-mediated drug resistance.

## Introduction

Tuberculosis is a chronic infectious disease caused by *Mycobacterium tuberculosis* (MTB) infection (1,2). Although drugs are capable of effectively treating tuberculosis, misuse or mismanagement of drug courses may lead to drug-resistant tuberculosis (DR-TB) or multidrug-resistant tuberculosis (MDR-TB). These drug-resistant disease forms are difficult to

treat and underlie a new global prevalence of tuberculosis (3,4). A better understanding of MTB pathogenesis is required to ameliorate this threat.

Two-component signal transduction systems (TCSSs) are pathogen junction systems that monitor external environmental stimuli and coordinate cellular responses, particularly the modulation of gene expression. TCSSs are important bacterial mechanisms for pathogenesis (5) and typically consist of two proteins; a transmembrane histidine kinase and a corresponding cytoplasmic response regulator. The MTB genome encodes 30 TCSS proteins (6), several of which are strongly associated with MTB drug resistance (4,5). Thus, inhibiting TCSSs of resistant bacteria may be an effective strategy to combat DR-TB and MDR-TB. SenX3/RegX3 was the first MTB TCSS to be reported, where SenX3 is a transmembrane histidine kinase and RegX3 is the corresponding DNA-binding response regulator (6). SenX3 is associated with MTB virulence and, thus, its investigation may further elucidate the molecular mechanisms of MTB drug resistance (7-9).

In the present study, we purified the MTB strain H37Rv recombinant SenX3 following expression in *Escherichia coli* (*E. coli*) in order to provide reagents for the future investigation of SenX3 and its role in MTB pathogenesis.

## Materials and methods

*Cloning of MTB strain H37Rv SenX3 into pMD18-T.* According to the published sequence (GenBank accession no. Y13628.1), the primers were designed to incorporate an *NcoI* restriction site upstream and an *XhoI* restriction site downstream of *SenX3* in the genomic DNA of the MTB strain H37Rv, which was provided by Dr Wen-Tao Shen (Chinese Academy of Tropical Agricultural Sciences, Hainan, China). The primers were synthesized by Yingjun Life Technologies (Shanghai, China) and the following sequences were used, with the restriction sites underlined: P1, 5'-CA CCATGGCAACTGTGTTCTCGGCGCTGTTGC-3' and P2, 5'-TCCTCGAGTCGGCTCAGCTCTTCTCTCGTTG-3'. *SenX3* was amplified from the genomic DNA of the MTB strain H37Rv by polymerase chain reaction (PCR) using Pyrobest DNA polymerase (Takara Biotechnology, Dalian, China) as well as forward and reverse primers. PCR cycling conditions were as follows: 94°C for 2 min; 30 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 90 sec and 72°C for 10 min.

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The amplicon was separated by gel electrophoresis. Correctly sized products were excised and purified with a gel extraction kit (CoWin Biotech Co., Ltd., Beijing, China). The purified and confirmed PCR product was ligated into the pMD18-T vector using a DNA ligation kit (D6023; Takara Biotechnology). The resulting pMD18-T-SenX3 construct was transformed into *E. coli* DH5 $\alpha$ -competent cells. The cells were then cultured at 37°C overnight on LB-ampicillin (100 mg/l) plates. Individual colonies were selected and screened by colony PCR using a vector sequencing primer.

**Site-directed mutagenesis of an internal *NcoI* site in *SenX3*.** Site-directed mutagenesis was used as previously described (10) to mutate an internal *NcoI* site within *SenX3* (Fig. 1A). The primers were synthesized by Yingjun Life Technologies and the sequences used were as follows: P3, 5'-GAGTAGAGCCATCGCACCGACGGG-3' and P4, 5'-CCCGTTCGGTGCATGGCTCTACTC-3'. The PCR cycling conditions for P1-P3 primer amplification from pMD18-T-SenX3 were as follows: 94°C for 2 min; 30 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec and 72°C for 10 min. The cycling conditions for P2-P4 primer amplification from pMD18-T-SenX3 were as follows: 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 35 sec and 72°C for 10 min. The P1-P2 primers were then used to amplify a 1:1 mixture of the P1-P3/P2-P4 PCR products in the following conditions: 94°C for 2 min; 30 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 90 sec and 72°C for 10 min. The final PCR products were separated by gel electrophoresis and purified with a gel extraction kit (CoWin Biotech Co., Ltd.). The purified product was ligated back into pMD18-T to yield pMD18-T-mSenX3, which was then transformed into *E. coli* DH5 $\alpha$ -competent cells (11). Individual colonies were screened by colony PCR.

**Cloning *SenX3* into the prokaryotic expression vector pET-28b.** pMD18-T-mSenX3 and the prokaryotic expression vector pET-28b(+) were digested with *NcoI* and *XhoI* restriction endonucleases (Takara Biotechnology) to produce compatible ends. The products were separated by gel electrophoresis, purified with a gel extraction kit (CoWin Biotech Co., Ltd.) and ligated together with T<sub>4</sub> DNA ligase (Takara Biotechnology) at 16°C overnight. The ligated vector was transformed into *E. coli* DH5 $\alpha$ -competent cells and grown overnight on LB-kanamycin (50 mg/l) plates at 37°C. Individual colonies were selected and the recombinant pET-28b-mSenX3 plasmid was purified and confirmed by restriction digestion. Following identification, pET-28b-mSenX3 was transformed into *E. coli* BL21 (DE3) and grown overnight on LB-kanamycin (50 mg/l) plates at 37°C. Individual colonies were selected and confirmed by PCR with P1 and P2 primers (11).

**Expression of recombinant *SenX3*.** Recombinant expression strains were inoculated in 20 ml of LB-kanamycin (50 mg/l) broth and grown at 37°C overnight. Overnight cultures were diluted 1:100 in 50 ml of fresh LB-kanamycin (50 mg/l) broth and cultured with agitation at 37°C until the OD<sub>600</sub> was 0.6-0.8. The cultures were then divided: Protein expression was induced in one half with 0.1 mM isopropyl  $\beta$ -D-thiogalactoside (IPTG) the other half was an uninduced control. The cultures were induced at either 28 or 37°C to

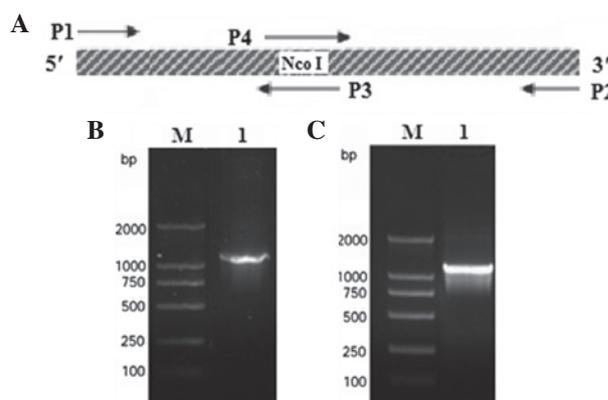


Figure 1. PCR amplification of *SenX3*. (A) Schematic overview of site-directed mutagenesis of *SenX3* by PCR overlap extension. (B) PCR amplification of *SenX3*. (C) PCR amplification of *SenX3* by overlap extension. M, DL2000 marker; 1, PCR product. PCR, polymerase chain reaction.

optimize protein expression. For the analysis of protein induction, 1-ml samples were collected after 2 h and centrifuged at 5,000 x g at room temperature for 5 min. Pellets were resuspended in buffer [50 mM Tris-HCl, 5 mM EDTA, 100 mM NaCl (pH 8.0)] and the cells were lysed by ultrasonic homogenization. The lysates were centrifuged at 12,000 x g at 4°C for 10 min. The supernatant and precipitate were collected, denatured at 100°C for 5 min and analyzed by SDS-PAGE and Coomassie Blue.

**Purification of recombinant *SenX3*.** For protein purification, pellets were resuspended in buffer [20 mM Tris-HCl, 150 mM NaCl (pH 7.4)] and the cells were lysed by ultrasonic homogenization. Inclusion bodies were collected by high-speed centrifugation (12,000 rpm) and dissolved in 8 M carbamide buffer, followed by centrifugation at 12,000 rpm for 1 h. The supernatant was removed and purified using Ni-NTA affinity chromatography (Invitrogen Life Technologies, Carlsbad, CA, USA). The proteins were eluted with 50, 150 or 250 mM imidazole and fractions were collected and analyzed by SDS-PAGE to determine the optimal elution conditions. Eluted proteins were diluted in renaturation buffer [50 mM Tris-HCl, 200 mM carbamide, 1% glycerol, 0.5 mM EDTA (pH 7.9)], incubated at 4°C overnight and centrifuged at 12,000 rpm for 10 min. Precipitates were removed and the solution was concentrated by ultrafiltration.

**Western blot analysis.** Purified recombinant *SenX3* was separated by SDS-PAGE and transferred onto nitrocellulose membranes for western blot analysis with an anti-6X His Tag primary antibody (CoWin Biotech Co., Ltd.). Signals were detected by conjugation to a horseradish peroxidase (HRP)-labeled goat anti-mouse IgG secondary antibody (CoWin Biotech Co., Ltd.) and visualized with an HRP-3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate chromogenic kit (CoWin Biotech Co., Ltd.).

## Results

**Site-directed mutagenesis to eliminate an internal *NcoI* site within *SenX3*.** In accordance with GenBank (accession

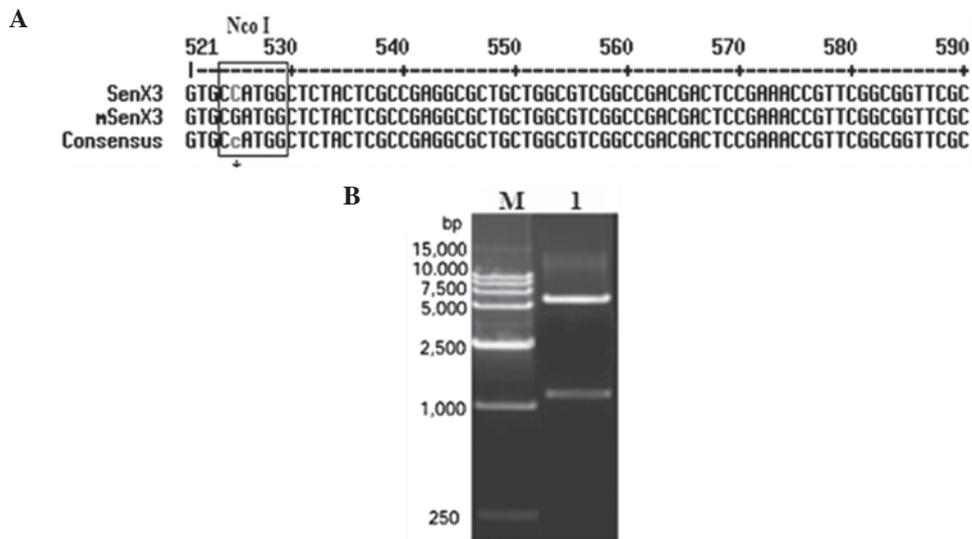


Figure 2. Restriction digestion analysis of pET-28b-mSenX3 with *NcoI* and *XhoI*. (A) Sequence alignment of *SenX3* and *mSenX3* with the *NcoI* restriction site boxed. (B) Restriction digestion analysis of pET-28b-mSenX3 with *NcoI* and *XhoI*. M, DL15000 marker; 1, restriction-digested pET-28b-mSenX3.

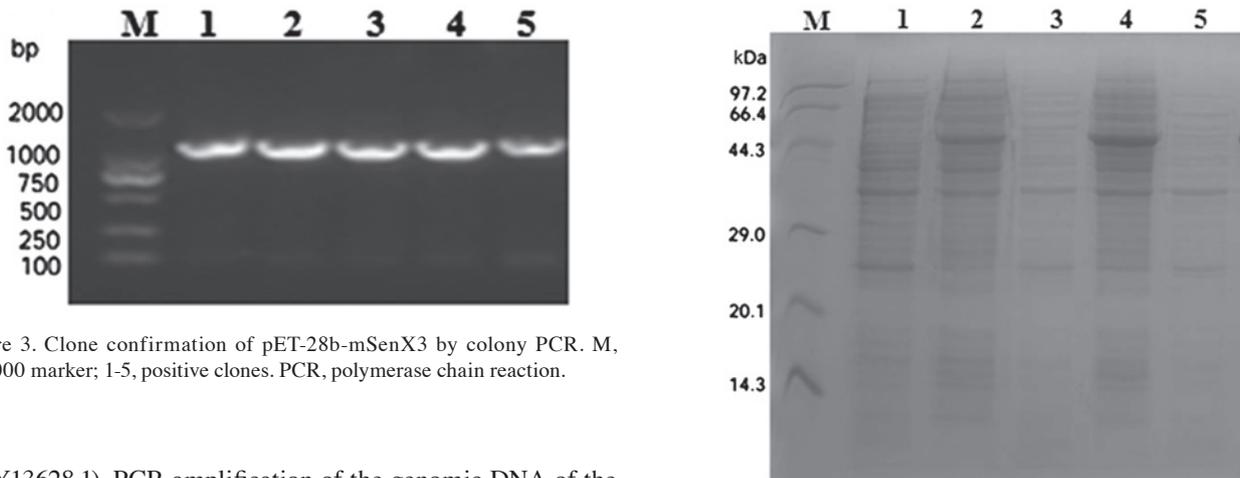


Figure 3. Clone confirmation of pET-28b-mSenX3 by colony PCR. M, DL2000 marker; 1-5, positive clones. PCR, polymerase chain reaction.

no. Y13628.1), PCR amplification of the genomic DNA of the MTB strain H37Rv generated a 1,233-bp *SenX3* DNA fragment (Fig. 1B). This product was cloned into the pMD18-T vector to generate pMD18-T-*SenX3*, which was confirmed by colony PCR identification and sequencing. Analysis of the *SenX3* fragment with Vector NIT 9 software revealed an *NcoI* restriction site at 524-529 bp; to clone the *SenX3* fragment within the pET-28b(+) *NcoI* and *XhoI* sites, the internal *NcoI* site within *SenX3* had to be destroyed by site-directed mutagenesis. Overlapping PCRs were designed to induce a synonymous mutation into the internal *NcoI* site (Fig. 1C). Diagnostic cloning and sequencing results demonstrated that the restriction site was mutated (Fig. 2A); the mutated vector was named pMD18-T-mSenX3.

**Identification of the prokaryotic expression vector pET-28b-mSenX3.** After mutating the internal *NcoI* site, *mSenX3* was digested from the pMD18-T-mSenX3 vector by double digestion with *NcoI* and *XhoI* restriction endonucleases. The isolated fragment was then ligated into the prokaryotic expression vector pET-28b(+) following similar digestion with *NcoI* and *XhoI* to create compatible ends, generating pET-28b-mSenX3. Diagnostic digestion with *NcoI* and *XhoI* confirmed this construct (Fig. 2B). Sequencing results also confirmed that

Figure 4. SDS-PAGE analysis of *SenX3* induction from *E. coli* using Coomassie Blue. M, low molecular weight protein marker; 1, pre-induction sample; 2, ultrasonic precipitate post-induction at 28°C; 3, ultrasonic supernatant post-induction at 28°C; 4, ultrasonic precipitate post-induction at 37°C; 5, ultrasonic supernatant post-induction at 37°C. *E. coli*, *Escherichia coli*.

the open reading frame of *mSenX3* was correct. Finally, the confirmed pET-28b-mSenX3 vector was transformed into *E. coli* BL21 (DE3) and confirmed by colony PCR identification (Fig. 3).

**Expression of recombinant *SenX3*.** Expression of *SenX3* was induced from *E. coli* by treatment with IPTG, and soluble (supernatant) and insoluble (precipitate) proteins were separated by ultrasonication. Analysis by SDS-PAGE and Coomassie staining indicated the appearance of a band ~46 kDa (expected size) following IPTG induction in the precipitate lanes only (Fig. 4).

**Purification of recombinant *SenX3*.** Following induction, the ultrasonicated precipitate was collected, solubilized and purified by Ni-sepharose affinity chromatography, from which

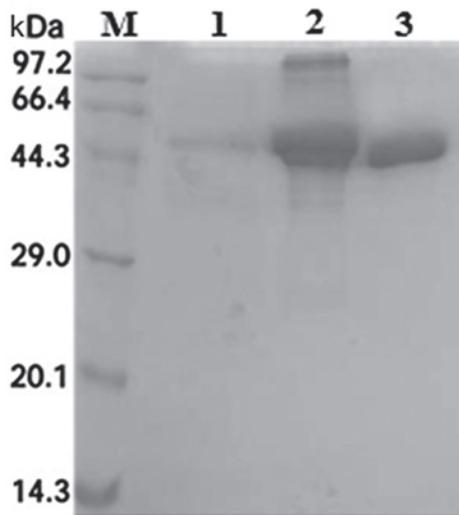


Figure 5. SDS-PAGE analysis following purification of recombinant SenX3 by Ni-sepharose affinity chromatography using Coomassie Blue. M, low molecular weight protein marker; 1-3, fractions eluted with 50, 150 or 250 mM imidazole, respectively.

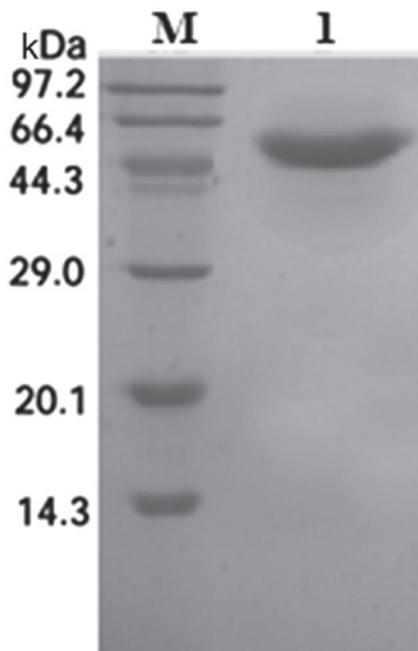


Figure 6. SDS-PAGE analysis of concentrated and refolded SenX3 using Coomassie Blue. M, low molecular weight protein marker; 1, concentrated and refolded SenX3.

protein fractions were collected and analyzed by SDS-PAGE. SenX3 was eluted at an optimal concentration of 250 mM imidazole (Fig. 5). SDS-PAGE analysis and Coomassie staining indicated one band of the expected size of 46 kDa (Fig. 6). Western blot analysis with an anti-6X His Tag antibody also detected a band of ~46 kDa (Fig. 7).

## Discussion

SenX3/RegX3 is a TCSS transmembrane sensor that coordinates pathogen environmental responses. Parish *et al.* (7)

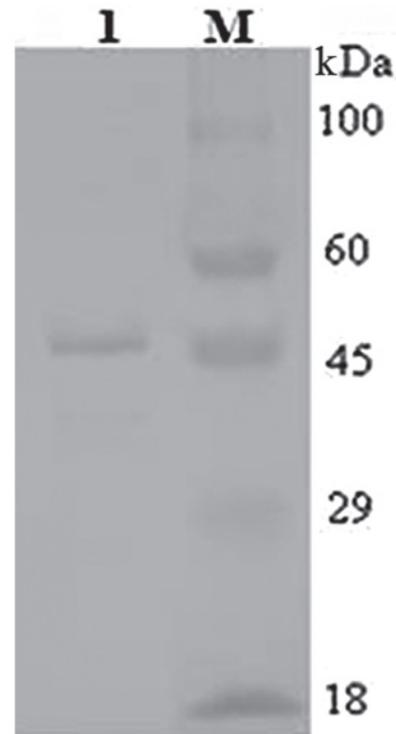


Figure 7. Western blot analysis of purified SenX3 using an anti-6X His Tag antibody. 1, Purified SenX3; M, prestained protein marker.

constructed MTB strains mutated for SenX3/RegX3 that were shown to infect human THP-1 macrophage-like cells and to gradually grow in  $\gamma$ -interferon-activated, bone marrow-derived murine macrophages. These strains were also shown to infect the lungs of mice and significantly decrease splenic function.

MTB strains mutated for either SenX3 or RegX3 have been shown to exhibit decreased virulence similar to MTB strains mutated for SenX3 and RegX3 (8), suggesting that regulatory mechanisms of the SenX3/RegX3 signaling pathway are important for MTB virulence. Furthermore, understanding the functions of SenX3/RegX3 and additional MTB TCSS proteins is of great significance for enabling the identification of the underlying molecular mechanisms of MTB resistance, the clinical treatment of tuberculosis, the identification of novel drugs and controlling the prevalence of drug-resistant strains (12,13). According to Himpens *et al.* (13), cytoplasmic SenX3 from *Mycobacterium smegmatis* was expressed using the pQE30 prokaryotic expression system. To the best of our knowledge, this was the first study to report the exogenous expression of MTB SenX3.

In the present study, PCR techniques were used to clone the genomic sequence of MTB *SenX3*, and SenX3 was purified from *E. coli* using affinity chromatography. The results of the present study provide important data for the future investigation of SenX3 and the molecular mechanisms of MTB resistance.

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