Molecular Cloning and Expression of a *Streptomyces* Sarcosine Oxidase Gene in *Streptomyces lividans*

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A genomic library of *Streptomyces* sp. KB210-8SY, prepared in the plasmid vector pACYC184, was screened to obtain the gene encoding sarcosine oxidase with probes based on the amino acid sequence of the protein. A plasmid pSOXS13, which was isolated from a clone identified by hybridization with the probes, contained a 8.4-kb insert of *Streptomyces* DNA. When the 2.0-kb *MluI/EcoRV* DNA fragment of pSOXS13 was inserted into the *Streptomyces* vector pJ680 and introduced into *S. lividans*, the transformants produced 100-fold more sarcosine oxidase intracellularly than KB210-8SY. The nucleotides of the 1.7-kb fragment containing the sarcosine oxidase gene were sequenced. An open reading frame encoded a mature sarcosine oxidase consisting of 388 amino acids, with a calculated molecular mass of 42,107 daltons.

Sarcosine oxidase (EC 1.5.3.1) catalyzes the oxidation of sarcosine to form formaldehyde, glycine, and hydrogen peroxide. The enzyme is produced by microorganisms such as the genera *Corynebacterium*,1) *Cylindrocarpon*,2) *Streptomyces*,3) and *Bacillus*,4–6) and useful for a diagnostic measurement of creatinine in combination with creatinase (creatine amidohydrolase; EC 3.5.2.10) and creatinase (creatine amidohydrolase; EC 3.5.3.3). Sarcosine oxidase from *Streptomyces*3) and *Bacillus*4) species are monomeric enzymes with molecular weights of 44,000 and 42,000, respectively. In the producing microorganisms, the enzymes were isolated from the cells grown in medium containing choline, sarcosine, or creatinine as the carbon source.1,2)

In this study, we cloned and sequenced the sarcosine oxidase gene from *Streptomyces* sp. KB210-8SY,3) and demonstrated high productivity of the sarcosine oxidase without the putative inducers described above by using the *S. lividans* host-vector system.

Materials and Methods

**Bacterial strains and plasmids.** *Streptomyces* sp. KB210-8SY was used as a sarcosine oxidase-producing strain.3) *S. lividans* TK24 and pJ6807) (5.3 kb) were used as a host for cloning and a plasmid vector, respectively. *Escherichia coli* DH110) and the plasmid pACYC18419) (4.2 kb) were used for the construction of a genomic library of *Streptomyces* DNA. *E. coli* MV1184 and the vectors pUC118 and pUC11910) were used for nucleotide sequencing.

Construction of genomic library. Chromosomal DNA of *Streptomyces* sp. KB210-8SY was prepared by the method of Hopwood et al.7) The DNA was digested with *BstE* II ligated into the dephosphorylated BamHI site of pACYC184, and introduced into *E. coli* DH1. The colonies which grew on Brain Heart Infusion (Difco)-1.5% agar medium containing 30 μg/ml of chloramphenicol were maintained for establishment of the genomic library.

Culture media. To produce the sarcosine oxidase, *Streptomyces* species were grown at 28°C for the given times in Tryptic Soy Broth (Difco) medium in the presence or absence of 0.5% choline chloride. Brain Heart Infusion (BHI) medium, instead of Tryptic Soy Broth (TSB), was used for *E. coli*.

**Purification of the sarcosine oxidase.** *Streptomyces* sp. KB210-8SY were grown at 28°C for 3 days in TSB medium supplemented with 0.5% choline chloride. Mycelia were collected by centrifugation and disrupted by sonication to prepare the cell-free extract. Solid ammonium sulfate was added to the extract to give 70% saturation, by the method of Inouye et al.10) The precipitate collected by centrifugation was dissolved in and dialyzed against 10 mm Tris/Cl (pH 8.0). The dialysate was put on a column of Q-Sepharose (4.4×85 cm) equilibrated with the same buffer containing 0.15 M NaCl. Elution was done with a linear gradient of NaCl (0.15–0.6 μl) in 10 mm Tris/Cl (pH 8.0). The active fraction was put on the second Q-Sepharose (4.4×70 cm) column equilibrated with 10 mm Tris/Cl (pH 8.0) containing 0.2 M NaCl. The sarcosine oxidase was eluted with a linear gradient of 0.2–0.5 M NaCl in the same buffer. The active fraction was put on a Sephacryl S-200 column (4.4×86 cm) equilibrated with 10 mm Tris/Cl (pH 8.0) containing 0.15 M NaCl. The partially purified sample was put on a column of Mono-Q HR 10/10 equilibrated with 20 mm Tris/Cl (pH 8.0) containing 0.15 M NaCl and eluted with a linear gradient of NaCl (0.15 to 0.25 μl) in the same buffer. The sarcosine oxidase was finally purified to homogeneity.

**Transformation for *Streptomyces*.** Introduction of the plasmid DNA into *Streptomyces* cells was principally done as described by Thompson et al.11)

**Synthesis of oligonucleotides.** The oligonucleotides used in this study were synthesized by the phosphoramidite method using the Cyclone Plus DNA Synthesizer (Milligen Bioresource, U.S.A.).

**Colony hybridization.** This was principally done as described by Davies et al.12) A Magnagraph Nylon Membrane (Micron Separations, Inc., U.S.A.) layered on bacterial colonies was treated with 0.5 N NaOH containing 1.5 M NaCl. After the membrane was neutralized with 0.5 M Tris/Cl (pH 7.0) containing 0.3 M NaCl, DNA was fixed on a membrane filter at 80°C for 2 hr. DNA probes were labeled with 70 Kβq of ([γ-32P]ATP (Amersham). DNA hybridization was done at 42°C overnight.

**DNA sequencing.** DNA fragments were subcloned in pUC118 and pUC119 and sequenced by the dideoxy chain termination method13) using the 7-DEAZA Sequencing kit (Takara Shuzo Co., Ltd.). The sequence data was analyzed with the GENIAS and PRINAS software programs.
Cloning of Sarcosine Oxidase Gene from Streptomyces

Sarcosine oxidase assay. Sarcosine oxidase activity was measured by the method described by Inouye et al.31 The reaction mixture (510 μl), which consisted of 10 μl of the enzyme preparation, 50 μl of 0.2 M Tris-HCl (pH 8.0), 50 μl of 15 mM 4-aminopyrine, 50 μl of 0.2 (w/v)% phenol, 50 μl of horseradish peroxidase (50 units/ml), 100 μl of 1 M sarcosine, and 200 μl of distilled water, was incubated at 37°C for 5 min. The reaction was stopped by adding ethanol (2.5 ml) and the absorbance at 480 nm was measured. One unit of the activity was defined as the amount of enzyme which catalyzed the oxidation of 1 μmol of substrate per min under the conditions described above.

Results

The purified sarcosine oxidase was digested by lysyl endopeptidase. The resulting fragments and the amino (N)-terminus of the protein were analyzed for amino acid sequence by the Edman degradation method. The N-terminus of the undigested protein and a peptide designated as K-13 are shown in Fig. 1. Since the GC-rich

(a) Ser-Pro-Thr-Tyr-Asp-Val-Ile-Val-Ile-Glu-Leu-Gly-Met-Gly-Ser-Ala-Ala-Ala-His-His-Leu-Ser-Ala-Arg-Gly-Ala-Arg-Val-Leu-

(b) Gly-Gly-Thr-Gly-Pro-Phe-Pro-Arg-His-Is-Pro-Val-Tyr-Ile- 
Trp-Glu-Asp-Ala-Asp-Gly-Gly-Leu-Val-Glu-Leu-Glu-Pro-Leu-

(c) Tyr-Ile-Trp-Glu-Asp-Ala-Asp-Gly 

TAC ATG TGG GAG GAC GCA GAC G

Fig. 1. N-Terminus of Sarcosine Oxidase and Amino Acid Sequences of the K-13 Peptide Isolated after Lysyl Endopeptidase Digestion, and Design of the Mixed Probes Corresponding to This Amino Acid Sequence.

(a) N-terminus of sarcosine oxidase; (b) amino acid sequence of K-13 peptide; (c), nucleotide sequence of the mixed probes used in the present study.

Fig. 2. Restriction Endonuclease Map of pSOXS13 Plasmid Carrying Sarcosine Oxidase Gene.

A closed bar indicates the region containing the sarcosine oxidase gene.

Fig. 3. Nucleotide and Amino Acid Sequence of Sarcosine Oxidase Gene.

The sequence complement to the oligonucleotide probe is underlined (---). A peptide, K-13, isolated after lysyl endopeptidase digestion is underlined with a heavy line (-----). The amino acid sequences which were found for other peptides and the N-terminus of the undigested sarcosine oxidase are underlined (-----). The inverted repeat sequences are indicated by arrows. The "rhs" indicates the consensus Shine-Dalgarno sequence. The candidate for a nucleotide-binding region is indicated by a rectangle (----).
genome of streptomycetes rarely contains A or T in the third position of codons within structural genes, we designed and synthesized the mixture of the 23-mer oligonucleotide probes corresponding to this amino acid sequence, as shown in Fig. 1.

A genomic library of *Streptomyces* sp. KB210-8SY, prepared in the *E. coli* vector pACYC184, was screened with this mixed probe. By using the technique of colony hybridization, a candidate was obtained in a population of about 11,000 colonies. A partial restriction endonuclease cleavage map of the 8.4-kb insert contained in the plasmid designated as pSOXS13 is shown in Fig. 2. *E. coli* harboring the plasmid did not express the sarcosine oxidase activity even upon the addition of choline. To locate the sarcosine oxidase structural gene, the probes were hybridized to a Southern blot of *MluI/EcoRV-, SphI-, or MluI/Hpal-* digested pSOXS13 DNA. The fragments which hybridized with the probes overlapped in a region of 2.0 kb between the *MluI* and *EcoRV* sites, as shown in Fig. 2, suggesting that the sarcosine oxidase gene was within this 2.0 kb region.

**Nucleotide sequence of the sarcosine oxidase gene**

To sequence nucleotides for the sarcosine oxidase gene, we analyzed the 2.0 kb *MluI/EcoRV* DNA fragment in detail with restriction endonucleases and found a signal *salI* site within the fragment. The nucleotides of the sarcosine oxidase gene between the *MluI* and *SalI* sites (1.7 kb) were sequenced by the dideoxy-chain termination method. The nucleotide sequence of the structural gene, as well as 5’ and 3’ adjacent regions, is shown in Fig. 3. An open reading frame consisting of 1167 bp starts at GTG codon and ends with the stop codon TGA. The analysis of the *N*-terminal sequence of the sarcosine oxidase verified that the open reading frame was surely the structural gene and encoded a protein of 42,107 daltons. A consensus Shine–Dalgarno sequence was found in the nucleotide sequence 9 bp upstream of the translational initiation codon. However, we were unable to identify the promoter sequences upstream from this ribosome-binding sequence. Analysis of the sequence showed that two inverted repeats are present downstream from the stop codon TGA. One or the other of these putative hairpin-loop structures might represent a transcription-termination signal.

**Subcloning and expression of the sarcosine oxidase gene in *S. lividans***

To express the sarcosine oxidase gene in *S. lividans*, pSOXS13 was digested with *MluI/EcoRV*. The resulting 2.0-kb fragment was filled in to make a blunt end with T4 DNA polymerase and ligated with the pIJ680 digested with *EcoRV*, to create pSOXS102 (Fig. 4). When pSOXS102 was introduced into *S. livivivans* TK24, the sarcosine oxidase activity was surely expressed (data not shown).

**Production of sarcosine oxidase in KB210-8SY strain and *S. lividans* carrying pSOXS102**

*Streptomyces* sp. KB210-8SY, *S. lividans* TK24, and *S. lividans* harboring pSOXS102 were grown aerobically at 28°C for 3 days in 10 ml of TSB medium in the presence or absence of 0.5% choline chloride (Table 1). After a 1-ml portion of the culture broth was centrifuged at 15,000 × *g* for 3 min, the cell pellet was suspended in 0.9 ml of 20 mM Tris/HCl (pH 8.0) and sonicated. The resulting supernatant obtained by centrifugation of the cell extract at 15,000 × *g* for 1 min was used as a enzyme solution. When choline was

<table>
<thead>
<tr>
<th>Strain</th>
<th>Enzyme activity (unit/ml)</th>
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<tbody>
<tr>
<td>KB210-8SY</td>
<td>0.00</td>
</tr>
<tr>
<td><em>E. coli</em> DH1</td>
<td>0.00</td>
</tr>
<tr>
<td><em>E. coli</em> DH1 (pSOXS13)</td>
<td>0.00</td>
</tr>
<tr>
<td><em>S. lividans</em> TK24</td>
<td>0.00</td>
</tr>
<tr>
<td><em>S. lividans</em> TK24 (pSOXS102)</td>
<td>1.30</td>
</tr>
</tbody>
</table>

Since a packed cell volume (PCV) in a 1-ml portion of culture broth was almost the same among *Streptomyces* sp. KB210-8SY, *S. lividans* TK24, and *S. lividans* TK24 harboring pSOXS102 in the presence or absence choline chloride, the sarcosine oxidase activity was expressed as units per 1 ml of enzyme solution obtained by the method described in the text. The PCV of *E. coli* was approximately one-fifth of that of *Streptomyces*. The PCV for 3 min, the cell pellet was suspended in 0.9 ml of 20 mM Tris/HCl (pH 8.0) and sonicated. The resulting supernatant obtained by centrifugation of the cell extract at 15,000 × *g* for 1 min was used as a enzyme solution. When choline was
added to the cultivation medium, KB210-8SY strain produced intracellularly 0.03 unit/ml of sarcosine oxidase, while enzyme activity was scarcely detected in the absence of choline (Table I). In S. lividans TK24 as a host cell, though no enzyme activity was detected, the cells harboring pSOXS12 produced large amount of sarcosine oxidase, regardless of the presence or absence of choline. The production yield of the clone was 100-fold more activity than that of KB210-8SY (Table I).

**Discussion**

In this study, we cloned and sequenced the sarcosine oxidase gene from *Streptomyces* sp. KB210-8SY. Previously, the enzyme was found in culture broth of the strain and purified to homogeneity.\(^3\) The molecular mass of the enzyme was estimated to be about 44,000 daltons by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration on Sephadex G-150.\(^3\) This agrees with the molecular weight of the putative protein encoded by the sarcosine oxidase gene sequenced in this study. We found in this work that the sarcosine oxidase is essentially an intracellular enzyme and is secreted into the culture broth after the cell lysis. In fact, N-terminal sequences of the enzymes isolated intracellularly and extracellularly were completely the same (data not shown). In adjacent regions of the structural gene coding for the enzyme isolated from the inside of the cells, we could not find a signal sequence for secretion of enzymes. These results indicate that sarcosine oxidase of *Streptomyces* sp. KB210-8SY is an intracellular enzyme.

The codon usage for translation of the sarcosine oxidase gene product are given in Table II. As might be expected for a *Streptomyces* gene, the high G–C content is reflected noticeably in the high proportion (96%) of codons that have a G or C in the third position.

Recently, the gene coding for the sarcosine oxidase from *Bacillus* sp. NS-129 was cloned and sequenced.\(^5\) We compared the translated protein sequence of the sarcosine oxidase gene from *Streptomyces* sp. KB210-8SY with that from *Bacillus* sp. NS-129. As shown in Fig. 5, highly conserved regions were recognized at both the amino- and carboxyl-terminus.

Nucleotide-binding proteins such as flavoproteins have a common structural domain.\(^15\) Thus, we searched for homology in the peptide sequences of flavoproteins\(^16,17\) and the sarcosine oxidase from *Streptomyces* sp. KB210-8SY and *Bacillus* sp. NS-129.\(^9\) As shown in Table III, we found the sequence-Gly-X-Gly-X-Gly- conserved highly in the dinucleotide-binding region of flavoproteins. A detailed structural comparison awaits the elucidation of the three-dimensional structure of sarcosine oxidase, which is under investigation.

*E. coli* harboring pSOXS13 did not express the sarcosine oxidase activity. We can not identify a consensus sequence corresponding to the –35 or –10 regions as a promoter signal of *E. coli*. These results suggest that a promoter of the *Streptomyces* sarcosine oxidase gene is not functional in *E. coli*.

To obtain the sarcosine oxidase, the microorganisms have been cultured in the medium containing choline, sarcosine, or creatinine.\(^1,2\) In this study, we found that the sarcosine oxidase of *Streptomyces* sp. KB210-8SY was induced by choline. Since these inducers are expensive, it is not suitable to use them on an industrial scale. Our study demonstrated high productivity of the sarcosine oxidase without the inducers with the *S. lividans* host-vector system.

<table>
<thead>
<tr>
<th>Enzyme (origin)</th>
<th>Sequence</th>
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| Sarcosine oxidase (KB210-8SY)* | Gly-Leu-Gly-Gly-Met-Gly-Gly
| Sarcosine oxidase (NS-129)* | Gly-Ala-Gly-Ser-Met-Gly-Gly
| p-Hydroxybenzoate hydroxylase (Pseudomonas fluorescens)* | Gly-Ala-Gly-Pro-Ser-Gly-Gly

* This study.
Thus it is now possible to make large amounts of the sarcosine oxidase by recombinant DNA technology.

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References