Corynebacterium sp. U-96 Contains a Cluster of Genes of Enzymes for the Catabolism of Sarcosine to Pyruvate

Haruo SUZUKI,1,2,1 Ryou TAMAMURA,1 Satoshi YAJIMA,2 Miho KANNO,1 and Masaya SUGURO1

1Division of Biosciences, Graduate School of Fundamental Life Science, Kitasato University, Kitasato 1-15-1, Sagamihara, Kanagawa 228-8555, Japan
2Department of Biosciences, School of Science, Kitasato University, Kitasato 1-15-1, Sagamihara, Kanagawa 228-8555, Japan

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The sarcosine oxidase gene and nearby genes from Corynebacterium sp. U-96 were determined. The genes for serine hydroxymethyltransferase, serine dehydratase, and 10-formyltetrahydrofolate hydrolase are arranged in this order. This suggests that the bacteria contain a cluster of genes for the catabolism of sarcosine to pyruvate. The possibility that the gene cluster is a merit for the cellular energy demands of the bacteria is discussed. Functional expression of sarcosine oxidase in Escherichia coli was accomplished, but the β subunit and the βδ complex were expressed at a low level as a soluble protein.

Key words: sarcosine oxidase; nucleotide sequence; gene organization; sarcosine metabolism; flavoprotein

The concentration of serum creatinine is widely used as an indicator of renal function.1) Sarcosine oxidase (SO) [sarcosine: oxygen oxidoreductase (demethylating), EC 1.5.3.1] has been used in the enzymatic determination of creatinine in the clinical laboratory. SO has been isolated from various bacterial strains.1) It was first purified from Corynebacterium sp. U-96 and shown to be a heterotetrameric enzyme.2) Since then, heterotetrameric SO has been purified from various sources: C. sp. P-1,3) Anthrobacter ureafaciens,4) and A. sp. 1-IN.5) We have been studying SO from C. sp. U-96, and have characterized it.2,6) A chemical modification study of SO-U966,7) indicated that iodoacetamide (IAM) reacts with two lysine residues, one at the sarcosine-binding site and the other at the covalent FMN-binding site. The amino acid sequence around the IAM-reactive lysine residues has been identified,6,7) but the positions of the residues in the whole sequence of SO-U96 are not known, since the amino acid sequence of the enzyme is not known. The sequence of the tetrameric SO gene and nearby genes was first reported for C. sp. P-18) and then for A. sp. 1-IN.9) The gene arrangements in the SO operon and nearby genes from these bacteria are quite similar. The genes arranged are glyA (serine hydroxymethyltransferase, SHMT), soxBDA (SO), and purU (10-formyltetrahydrofolate hydrolase, FTH). The two genes contain no ORF between soxG and purU, but rather a putative stem-loop structure.8,9)

The present study reports the nucleotide sequence of SO-U96 gene and nearby genes, the expression of SO-U96 in E. coli, and its characterization. The gene arrangement was found to be similar to that of Corynebacterium sp. P-1, but different in that the serine dehydratase (SDH) gene was observed between soxG and purU. The presence of a cluster of genes for the catabolism of sarcosine to pyruvate is discussed.

Materials and Methods

Materials. Pfx DNA polymerase and Taq DNA polymerase were obtained from Gibco BRL and Takara respectively. All restriction endonucleases were from Roche. Bacto-tryptone, Bacto-peptone, and Bacto-yeast extract were from Difco. His-Bind Resin was from Novagen, and DEAE-Sepharose was from Pharmacia/ LKB. A Thermo Sequenase Fluorescent Labeled Primer cycle sequence kit was purchased from Amersham Pharmacia Biotech. Isopropyl-β-D-thiogalacto-pyranoside (IPTG) and 5-bromo-4-chloro-3-indoyl β-D-galactoside (X-Gal) were from Takara Biochemicals. All other chemicals were obtained from Wako.

Buffers. Phosphate-buffered saline (PBS): 8 g NaCl, 0.2 g KCl, 0.2 g KH2PO4, 1.15 g Na2HPO4 in 1-liter deionized water. Buffer A: 20 mM Tris–HCl, pH 7.9, 500 mM NaCl.

1 To whom correspondence should be addressed. Tel/Fax: +81-42-778-9401; E-mail:suzuki@sci.kitasato-u.ac.jp

Abbreviations: FTH, 10-formyltetrahydrofolate hydrolase; IAM, iodoacetamide; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; rec, recombinant; SDH, serine dehydratase; SHMT, serine hydroxymethyltransferase; SO, sarcosine oxidase; SO-U96, SO from Corynebacterium sp. U-96; X-Gal, 5-bromo-4-chloro-3-indoyl β-D-galactoside
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Bacterial cells, plasmids and culture conditions. C. sp. U-96 was kindly supplied by Dr. M. Suzuki, Kikkoman Ltd. pBlue-script II (KS+) was from Stratagene, and pET-31b was from Novagen. E. coli strains JM109 and BL21(DE3) were from Novagen. E. coli cells were grown in LB medium or on an LB agar plate. LB medium contained 5 g of Bacto-yeast extract, 10 g of Bacto-trypton, and 0.1 ml of 10 M NaOH in 1-liter H2O.

Preparation of Corynebacterium sp. U-96 genomic DNA. All common DNA manipulations were performed by standard procedures.10) The bacterial strain C. sp. U-96 was cultivated as described previously,2) and genomic DNA was prepared from the cells by the standard procedures.

Strategy of DNA sequencing of the SO-U96 gene. DNA fragments (approximately 400 bp) were obtained by PCR using primers designed from the partial amino acid sequences of SO-U966,7) and from the DNA sequence of the SO-P1 gene.8) PCR was performed using B5Nde as the sense primer and pET vector 31b. At least 3 clones were selected for each PCR product. DNA sequencing of the insert in the plasmid was done using a Thermo Sequenase fluorescent-labeled primer sequencing kit (Amersham Pharmacia Biotech) and Shimadzu DNA Sequencer DSQ-1000L. The downstream region of the SO-U96 gene was sequenced by the Genomic Research Department of Shimadzu-Biotech. Sequences were assembled, edited, and analyzed using GENETYX-MAC (Software Development). The complete sequence of the SO-U96 gene and nearby genes was deposited with DDBJ (accession no. AB186138).

Construction of the expression vectors for SO-U96 and subunits. Since the SO-U96 gene was found to contain only one Pvu I restriction enzyme site, the gene was divided into two DNA fragments by PCR using the genomic DNA as a template. PCR primers were designed on the basis of the sequence of the SO-U96 gene. The 5′-terminal half fragment (approximately 2,300 bp) was amplified using B5Nde, 5′-CATATGCGTGATCTGCTCCCGGA-3′ (the Ndel site is underlined) as a sense primer, and sar29, 5′-GGGCGGTTGTTGTTCTCAGA-3′ as an antisense primer. The 3′-terminal half fragment (approximately 2,800 bp) was amplified using sar27, 5′-TGGCCACATCCCGCGCCAAAGCA-3′ as a sense primer, and C3Xho, 5′-CTCGAGGGGGCCTCCCGGGAAG-3′ (the XhoI site is underlined) as an antisense primer. PCR fragments were inserted into EcoRV site of pBluescript vector. The plasmids were designated p2300 and p2800 respectively. Plasmid p2300 was digested with Ndel and PvuI, and p2800 with PvuI and XhoI. These restriction fragments were inserted into the Ndel–XhoI site of pET vector 31b, and the vector was transformed into E. coli BL21(DE3) competent cells. The expression vector obtained was designated pSO.

The expression vectors for the βδ complex and the β subunit were constructed by a method similar to that described for the construction of the expression vector, pSO. The primers for the βδ complex were B5Nde as a sense primer and D3Xho, 5′-CTCGAGGTGGGACCCACGGGCGGCGGCGGCCC-3′ (the XhoI site is underlined) as an anti-sense primer. The primers for the β subunit were B5Nde as the sense primer and B3Xho, 5′-CTCGAGGTGGGACCCACGGGCGGCGGCGGCCC-3′ (the XhoI site is underlined) as an antisense primer. The PCR fragment was inserted into the EcoRV site of pBluescript, and the endonuclease fragment of the pBluescript was inserted into the Ndel–XhoI site of the pET vector, 31b. The expression vectors constructed were designated pSOβδ and pSOβ for the βδ complex and the β subunit, respectively.

A His-tag was attached to the C-terminal end of the γ, δ, and β subunits in the expression of SO-U96, the βδ complex, and the β subunit, respectively.

Expression and purification of recSO-U96. E. coli BL21(DE3)/pSO cells were grown in LB medium containing ampicillin (50 μg/ml) and 0.5 mM IPTG for 4 h at 30 °C. The E. coli cells were harvested by centrifugation and used for the preparation of recSO-U96. The cells (8 g wet weight from 2-liter culture) were washed with PBS, suspended in 50 ml of buffer A, and lysed for 40–60 min by sonication using a Branson sonicator (Model 250, 15 output, constant duty cycle) below 7 °C with constant mixing. To the supernatant of the lysate, 10 ml His·Bind resin pre-equilibrated with buffer A was added, and the whole was mixed overnight at 4 °C. Then the resin packed in a glass column was washed with buffer A and further washed with buffer A + 5 mM imidazole. recSO-U96 was eluted with buffer A + 50 mM imidazole. The eluates were combined and recSO-U96 was further purified by DEAE-Sephacel column chromatography. The purified enzyme was kept in 10 mM potassium phosphate buffer (pH 8.0) containing 0.3 mM EDTA.

Cofactor analysis. Cofactors, FAD and NAD+ were analyzed as described previously.11) Free NAD+ content was also determined using yeast alcohol dehydrogenase, as described by Eschenbrenner et al.12) Absorption spectra were measured using a double-beam spectrophotometer, type Ubest-50, from Japan Spectroscopic.

Assay of enzyme activity. The activity of the enzyme was determined by measuring oxygen uptake, using an oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH) in the presence of 5 mM sarcosine in 20 mM potassium phosphate buffer (pH 8.0), 0.1 mM EDTA at 25 °C.
Results and Discussion

Organization of the SO-U96 gene and nearby genes
Tetrameric SO was first purified from C. sp. U-96, but the complete primary structure of the enzyme is not known. Hence we determined the nucleotide sequence of the gene for the enzyme, as described in “Materials and Methods”, and deduced the primary structure. A schematic diagram of the genetic organization of the SO gene and nearby genes is shown in Fig. 1. The genes encoding SO-U96 are arranged in the order soxB, soxD, soxA, and soxG, coding for the β, δ, α, and γ subunits, respectively. These genes are highly homologous to those of C. sp. P-1. The nucleotide sequence of the upstream and downstream regions of the SO-U96 gene were also determined. The sequence of the upstream region agreed with the glyA gene coding the C-terminal sequence of SHMT, as in the gene of C. sp. P-1.

To determine the downstream region of the SO-U96 gene, PCR was performed using the genomic DNA as a template. The primers were sar35, 5′-ATCGCGCAA-GTCTCAAGAC-3′ as a sense primer and sar46, 5′-CGGTGCGTAAAGGGACACC-3′ as an anti-sense primer. The positions of the primers are shown roughly in Fig. 1. The antisense primer was designed on the basis of the sequence of the C. sp. P-1 gene. Sequencing analysis of the PCR product indicated that the fragment contains one ORF. This result is in contrast to the genes of C. sp. P-1 and of A. sp. 1-IN, since these sequences contain no ORF, but rather a putative stem-loop structure. A BLAST search of the ORF hits SDHs. For example, the amino acid sequence of the ORF is 61.2% identity in a 464 amino acid overlap with that of SDH from Pseudomonas aeruginosa strain PAO1. They are homologous to each other in their overall structure. A BLAST search of the ORF hits SDHs. They are homologous to each other in their overall structure.

Fig. 1. Gene Arrangement in the SO Gene and Nearby Genes.

Numbering begins at the G in the sequence, which has been deposited with DDBJ, accession no. AB186138. Thick arrows indicate gene position and junction. The positions of PCR primers are shown. The positions of gene products are also shown. SO, sarcosine oxidase (soxBDAG); SHMT, serine hydroxymethyltransferase (glyA); SDH, serine dehydratase (sdh); FTH, 10-formyltetrahydrofolate hydrolase (purU).

SDH was concluded that the ORF codes FTH.

Based on the above considerations, the organization of the genes can be drawn (Fig. 1). The genes of C. sp. P-1 and of A. sp. 1-IN contain no ORF between the genes soxG and purU, but have an approximately 340 bp untranslated region. In contrast, C. sp. U-96 has one ORF, SDH gene in the region. Moreover, the untranslated region of the upstream of the purU gene is short (40 nucleotides long), and no stem-loop structure was found in the region. Therefore all seven ORFs are clustered in the same orientation, suggesting transcriptional coupling, and the genes for the enzymes SO, SHMT, SDH, and FTH perhaps constitute a sox operon. This led us to propose the pathway of sarcosine catabolism in C. sp. U-96 (Fig. 2).

Many genes of enzymes involved in bacterial catabolic pathways are clustered on the chromosomes and regulated as operons. Several models for the origins of gene clusters have been presented, in which gene clusters allow dissemination of functionally related genes via horizontal transfer. According to this model, many selfish operons can encode degradative pathways, and the encoded enzymes degrade a particular substance to produce a metabolite that easily enters the central metabolism. All the genes involved in the metabolism of creatinine and sarcosine in A. sp. TE1826 are clustered, and the clustering has been explained by horizontal gene transfer. There are no data on the origin of the gene cluster in C. sp. U-96. But cellular energy demands might be met by a sequential conversion of sarcosine to pyruvate using the enzymes regulated by a single operon. If this is correct, the growth rate of C. sp. U-96 must be higher than that of sp. P-1 grown in a medium using sarcosine as a sole carbon source. Using the data obtained under similar conditions, the bacteria harvested from 1-liter medium were calculated to be 40 g (wet weight) for C. sp. U-96, and 6 g for sp. P-1, and the amount of SO in 1 g (wet weight) of bacteria was estimated to be 11.2 mg for C. sp. U-96 and 8.4 mg for sp. P-1. Perhaps these values support the above idea.
Purification and characterization of recSO-U96 Recombinant SO was purified from E. coli/pSO cells, and the kinetic parameters and an optimum pH were determined, as described in “Materials and Methods”. recSO-U96 showed an optimum pH of 8 to 9, and $k_{\text{cat}}$ 18.0 $s^{-1}$ and $K_m$ 2.0 mM at 25 $^\circ$C and pH 8.0. Metal ions, sodium acetate and IAM inhibited recSO-U96 to an extent similar to the native enzyme. Moreover, recSO-U96 was found to contain 1 mol of noncovalent FAD and 1 mol of covalent flavin, and 1.5 mol NAD$^+$ /mol of enzyme. These properties are similar to those of the native enzyme. The subunit of SO-U96 contains covalently bound FMN and possibly noncovalent FAD, and has high sequence identity (approximately 97%) with that of other tetrameric SO, SO from Corynebacterium sp. P-1 and Antrobacter SO. Moreover, the $\delta$ subunit is believed to be contact the $\beta$ subunit. Hence, we tried to express the $\beta$ subunit and the $\beta\delta$ complex in E. coli. The E. coli cells harboring pSO$\beta$ or pSO$\delta$ were cultured in LB medium at 25 $^\circ$C in the presence of 1 mM IPTG for 3 h. The cells were harvested from the culture medium, and the $\beta$ subunit and the $\beta\delta$ complex were purified using His-bind resin. The typical yield was 0.4 mg $\beta$ subunit and 0.07 mg of $\beta\delta$ complex (mostly the $\delta$ subunit, as described below) from 500 ml culture medium. SDS–PAGE analysis of the eluates showed a single staining pattern for the expression of the $\beta$ subunit, but stronger staining for the $\delta$ subunit for the expression of the $\beta\delta$ complex. By Scion Image analysis (http://www.scioncorp.com/) of the protein-staining of SDS-PAGE of the expression products of pSO$\beta\delta$, a 12 to 14 mol excess of the $\delta$ subunit over the $\beta$ subunit was estimated, indicating low affinity of the $\delta$ to the $\beta$ subunit. Attempts to purify these further by the method used for the purification of recSO-U96 were not successful, since the proteins became insoluble by these procedures. Moreover, no flavin was found to be bound to these protein samples. This indicates that the coordinate synthesis of four subunits is important in the incorporation of both covalent FMN and noncovalent FAD to the $\beta$ subunit.

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