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sanN Encoding a Dehydrogenase is Essential for Nikkomycin Biosynthesis in *Streptomyces ansochromogenes*

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Nikkomycins are a group of peptidyl nucleoside antibiotics with potent fungicidal, insecticidal, and acaricidal activities. *sanN* was cloned from the partial genomic library of *Streptomyces ansochromogenes* 7100. Gene disruption and complementation analysis demonstrated that *sanN* is essential for nikkomycin biosynthesis in *S. ansochromogenes*. Primer extension assay indicated that *sanN* is transcribed from two promoters (*sanN*-P1 and *sanN*-P2), and *sanN*-P2 plays a more important role in nikkomycin biosynthesis. Purified recombinant SanN acts as a dehydrogenase to convert benzoate-CoA to benzaldehyde in a random-order mechanism *in vitro*, with respective K_{cat}/K_m values of 3.8 $mM^{-1}s^{-1}$ and 12.0 $mM^{-1}s^{-1}$ toward benzoate-CoA and NADH, suggesting that SanN catalyzes the formation of picolinaldehyde during biosynthesis of nikkomycin X and Z components in the wild-type stain. These data would facilitate us to understand the biosynthetic pathway of nikkomycins and to consider the combinatorial synthesis of novel antibiotic derivatives.

Keywords: *sanN*, dehydrogenase, nikkomycin biosynthesis, *Streptomyces ansochromogenes*

Nikkomycins are a group of peptidyl nucleoside antibiotics produced by *Streptomyces tendae* Tü901 [8] and *Streptomyces ansochromogenes* 7100 [11]. Because of their structural similarity to UDP-*N*-acetylglucosamine, nikkomycins act as potent competitive inhibitors of chitin synthetase and display high fungicidal, insecticidal, and acaricidal activities [13]. Furthermore, nikkomycins can be easily degraded in the natural environment and have no toxicity to mammals, plants, and fishes. Their application in agriculture and development as antifungal drugs in human therapy have been studied extensively [14]. Nikkomycins contain a peptidyl moiety and a nucleoside moiety. Nikkomycin X and Z are the main components produced by the wild-type producer, the peptidyl moiety of which is hydroxypyridyl-homothreonine (HPHT), whereas the nucleoside moiety consists of an aminohexuronic acid with *N*-glycosidically bonded 4-formyl-4-imidazoline-2-one or uracil. The two moieties of nikkomycins are assumed to be formed in separate ways and then combined with peptide bonds [7, 12]. A partial biosynthetic pathway for nikkomycin X and Z is shown in Fig. 1A, in which the conversion of L-lysine to picolinate-CoA was clarified in earlier studies [9, 21, 26]. When picolinate biosynthesis was genetically blocked, benzoate as a substituted precursor could be efficiently incorporated into the biosynthetic pathway, and nikkomycin B_x and B_z were produced instead [6]. Moreover, SanJ, an ATP-dependent picolinate-CoA ligase involved in the conversion of picolinate to picolinate-CoA during nikkomycin biosynthesis, also catalyzes the conversion of benzoate to benzoate-CoA *in vitro* [21] (Fig. 1B). However, still uncharacterized is which gene or enzyme would be responsible for the following step; that is, the formation of picolinaldehyde or benzaldehyde. In a previous study, even though *nikA* in *S. tendae* was deduced to participate in the formation of picolinaldehyde [10], further experimental datum has not been provided so far.

Recently, we cloned the *sanN* gene from a partial genomic library of *S. ansochromogenes*. Sequence analysis indicated that *sanN* is the counterpart of *nikA*. Our preliminary study demonstrated that the SanN protein could activate the aldolase activity of SanM for the formation of 4-pyridyl-2-oxo-4-hydroxyisovalerate during nikkomycin biosynthesis in *S. ansochromogenes* [17]. In this paper, we further report the thorough characterization of *sanN* in the biosynthetic pathway of nikkomycins.

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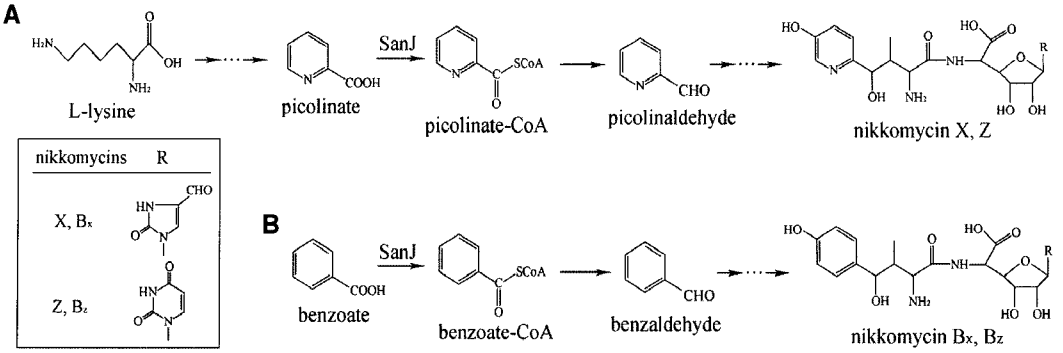

Fig. 1. Partial biosynthetic pathways for nikkomycin X/Z (**A**) and nikkomycin B_x/B_z (**B**).
The inset shows the structures of the R-group for the X and Z series of nikkomycins.

Materials and Methods

Strains, Plasmids, and Media

Streptomyces ansochromogenes 7100 (wild-type strain), a nikkomycin producer, was isolated from the soil of Northeastern China [11]. *Escherichia coli* DH5α was used for propagating plasmids. Methylation-deficient *E. coli* strain ET12567/pUZ8002 was used for the conjugal transfer of DNA from *E. coli* to *Streptomyces* [19, 22]. *Candida albicans* was used as the indicator strain in the nikkomycin bioassay. *S. lividans* TK24 was used as the host strain for gene expression [15]. Plasmids pBluescript II KS-(Invitrogen) and pIJ8600 [15] served as the gene cloning and expressing vectors, respectively. pKC1139 and pSET152 were used for gene disruption and complementation in *Streptomyces* strain [5]. pNL5400, derived from pBluescript II KS- by insertion of a 9.8 kb PstI DNA fragment containing the *sanN-I* transcriptional unit, was isolated from the partial genetic library of *S. ansochromogenes*.

All media for *Streptomyces*, *E. coli*, and *C. albicans* growth were prepared as previously described [10, 15]. When necessary, antibiotics were used at the following concentrations: ampicillin (Amp), 100 μg/ml in LB; apramycin (Apr), 10 μg/ml in YEME or MM, 30 μg/ml in MS, and 50 μg/ml in LB; kanamycin (Kan), 10 μg/ml in YEME or MM, 50 μg/ml in YPD or YPDA; tetracycline and chloramphenicol, 10 μg/ml and 25 μg/ml in LB, respectively.

DNA Manipulations and Sequence Analysis

Pfu high fidelity DNA polymerase (Shanghai Sangon) was used to amplify targeted DNA fragments. DNA isolation and transformation of *E. coli* and *Streptomyces* were performed by established techniques [15, 23]. The digoxigenin-11-dUTP labeling kit (Roche) was used for DNA labeling and detection in Southern hybridization following the instructions provided. DNA sequencing was performed by Takara Biotechnology Corporation (Dalian, China). Database searching and sequence analysis were made using the BLAST program [2].

Gene Disruption and Complementation

For disruption of *sanN*, a 3.0 kb PstI-KpnI fragment carrying *sanN* from pNL5400 was inserted into the PstI/KpnI sites of pBluescript II KS- to generate pM13-N. Then, a 400 bp BstEII fragment of *sanN* in pM13-N was replaced by a 1.0 kb kanamycin resistance gene (*kan*^{R}) to generate pM13-N-*kan*^{R}. A PstI-KpnI fragment carrying *sanN-kan*^{R} was further isolated from pM13-N-*kan*^{R}, end-blunted, and inserted into the EcoRV site of pKC1139 to yield pKC1139-N-*kan*^{R}. Then, the resulting plasmid was introduced into *S. ansochromogenes* 7100 by conjugal transfer, and the *sanN* disruption mutant with Apr^{S}Kan^R was selected *via* homologous recombination (double crossover) as described elsewhere [16]. The *sanN* disruption mutant was further identified by Southern hybridization.

For the complementation of *sanN* disruption, a 1.4 kb DNA fragment carrying *sanN* and its native promoter region from pNL5400 was amplified using following primers: P1 (5'-CTGGATCCTGCCGG-TTCTCGCTGTTC-3') and P2 (5'-AGGGATCCCGGGTCGTGGAT-CTGGA-3') (both containing the BamHI site underlined). The PCR product was digested with BamHI and then inserted into the same site of pSET152 to generate pSET152-N. The resulting plasmid was introduced into the *sanN* disruption mutant, and complementary strains were obtained with Apr as resistance selection.

Detection of Nikkomycin Production

To detect nikkomycin production, *S. ansochromogenes* 7100 and its derivatives were inoculated in liquid 2×YT medium and incubated at 28°C on a rotary shaker for 36 h. Then, 1 ml of culture was inoculated to 50 ml of fresh SP medium and further incubated at 28°C for 6 days. The fermentation broth was harvested by centrifugation and the supernatant was filtered through a minipore membrane (pore diameter 0.2 μm). HPLC analysis and bioassay against *C. albicans* for detecting the presence of nikkomycins in culture filtrates were performed as described previously [21].

Primer Extension

Total RNA was isolated from *S. ansochromogenes* 7100 grown in SP medium for 24 h and 72 h incubation with Trizol reagent (Invitrogen) as protocol instructed. The transcription start point (TSP) was determined by primer extension as previously described [15], except that the reaction was incubated at 42°C for 1 h with *sanN*-specific primer P3 (5'-GGTCCATACCGATGAGTCCG-3').

Construction of Expression Plasmid for SanN

The open reading frame (ORF) of *sanN* was amplified using primers P4 (5'-GGAATTCCATATGCAAGCAACT- GGACGA-3', with NdeI site underlined) and P5 (5'-TTACTCGAGTGCCGT-CTCCCCTGCC-3', with XhoI site underlined), digested with NdeI and XhoI, and inserted into the same sites of pET23b to generate pET23b-N. Then, *sanN* fusion with his-tag was further amplified from pET23b-N using primers P6 (5'-CGATCCCGCGAAATTA-ATAC-3') and P7 (5'-CCGGATCCAAAAAACCCCTCAAGACC-

3') with BamHI site underlined, respectively. The PCR product was digested with NdeI and BamHI, and ligated with the NdeI-BamHI digested pIJ8600 to generate pIJ8600-N.

Determination of the Relative Molecular Mass (M_r)

The M_r of purified protein was determined by size-exclusion chromatography on a Superdex 75 column (Amersham Phamarcia Biotech). The column was equilibrated and eluted with 50 mM Tris-Cl (pH 8.0) at a flow rate of 0.5 ml/min, and calibrated with bovine serum albumin (M_r=66,000), chicken ovalbumin (M_r=44,000), and cytochrome *c* (M_r=12,400).

Enzyme Assay and Kinetics Analysis

Enzyme assay was performed at 28°C. The standard reaction mixture (1 ml) for detecting the dehydrogenase activity of SanN contained 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 2 mM benzoate-CoA, 600 μM NADH, and an appropriate amount of purified SanN. Purified SanM protein served as the negative control. After incubation for 3 h, 20 μl of reaction products was loaded onto a reverse-phase HPLC column (Zorbax Eclipse XDB-C18, 4.6 mm× 250 mm, 5 μm; Agilent) to analyze the presence of benzaldehyde. Samples were separated at 30°C at a flow rate of 1 ml/min with an isocratic elution of 5% methanol from 0 to 8 min, followed by a gradient elution of 5% methanol at 8 min to 50% methanol at 15 min. The elution was monitored with a UV/Vis diode array at 260 nm. For kinetics analysis, the reaction was determined by monitoring the oxidation of NADH (OD_{340}) in the enzyme coupling assay under similar conditions, except that the substrate concentrations were varied. Initial velocities were determined by three separate measurements at each point. The K_{cat}/K_m values were calculated using a Lineweaver-Burk plot.

Nucleotide Sequence Accession Number

The nucleotide sequence of *sanN* was deposited in the GenBank database under the accession number AY188795.

RESULTS AND DISCUSSION

Cloning and Sequence Analysis of *sanN*

A 9.8 kb PstI DNA fragment was isolated from the partial genomic library of *S. ansochromogenes* 7100 using a 0.8 kb KpnI-PvuII DNA fragment of a previously cloned gene (*sanL*) as a probe, and was sequenced. It was revealed

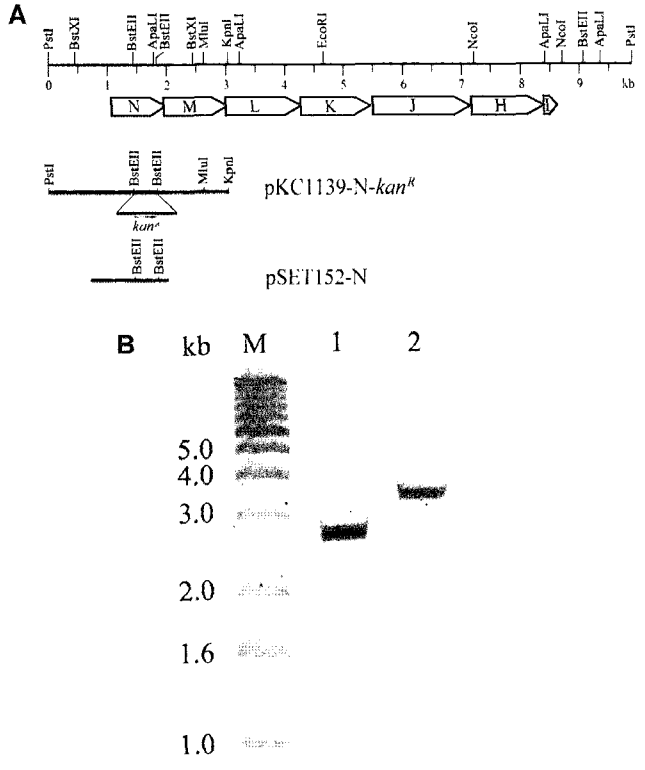

Fig. 2. Organization of the *sanN-I* transcriptional unit and identification of the *sanN* disruption mutant by Southern hybridization.

A. Organization of the *sanN-I* transcriptional unit. Boxes indicate the size and orientation of genes. Lower bars indicate plasmids pKC1139-N-*kan*^{R} and pSET152-N for *sanN* disruption and complementation, respectively. **B.** Identification of the *sanN* disruption mutant by Southern hybridization. DNAs of both wild-type strain (lane 1) and *sanN* disruption mutant (lane 2) were digested with PstI and MluI, and the 0.9 kb *sanN* fragment served as a probe. M, DNA marker.

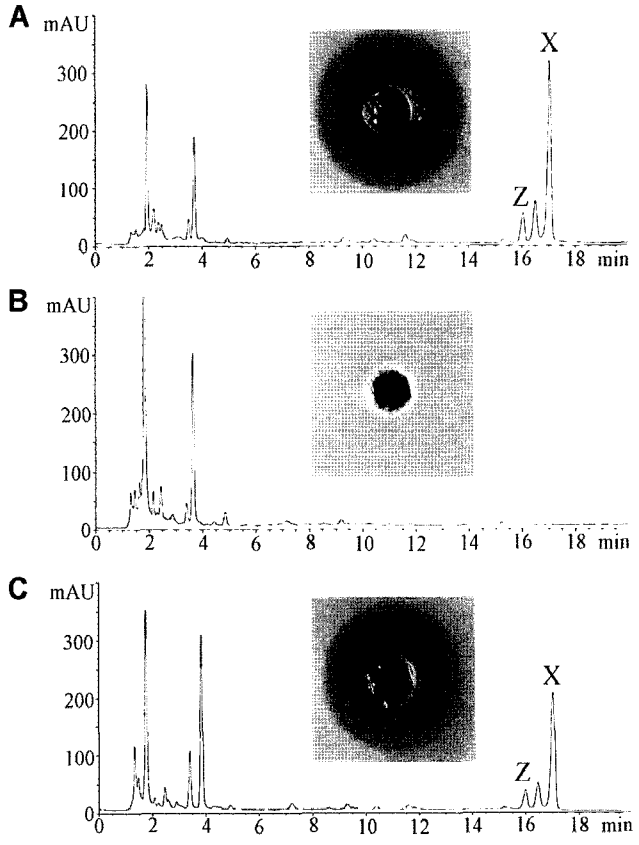

Fig. 3. Detection of nikkomycin production.

HPLC analysis was used to identify the nikkomycins of culture filtrates from the wild-type strain (**A**), *sanN* disruption mutant (**B**), and the complementary strain (**C**). The elution was monitored at 290 nm. Peaks corresponding to nikkomycins X and *Z* are indicated. X, nikkomycin X; Z, nikkomycin Z; mAU, milli absorbance units. The insets show the bioassay of culture filtrates against *C. albicans*.

that the DNA fragment contains seven complete ORFs in linked arrangement, designated as *sanN-I* (*Streptomyces ansochromogenes* nikkomycin gene N-I) transcriptional unit (Fig. 2). Among them, *sanN* is the first gene, which is composed of 891 bp with a typical (G+C) content of *Streptomyces* (69.14%) and a potential ribosome binding site (GGAAAGG) separated from the ATG start codon by 9 bp. In searches of databases, the deduced product of *sanN* (SanN) contains a semialdehyde dehydrogenase domain and an AcetDehyd-dimer at its N-terminus and C-terminus, respectively, showing 46% end-to-end identities to acetaldehyde dehydrogenases (acylating) from *Streptomyces coelicolor* A3 (2) (GenBank Accession No. NP_639629) and semialdehyde dehydrogenase from *Serratia proteamaculans* 568 (GenBank Accession No. ZP_01534271).

sanN is Essential for Nikkomycin Biosynthesis in *S. ansochromogenes*

Plasmid pKC1139-N-*kan*^{R} was constructed by replacing the 400 bp BstEII fragment with 1.0 kb *kan*^{R} in the ORF of *sanN*, as shown in Fig. 2A, and introduced into the wild-type strain by conjugal transfer to generate the *sanN* disruption mutant *via* homologous recombination. The *sanN* disruption mutant was further identified by Southern hybridization. It was revealed that a positive 3.2 kb signal band appeared in the genomic DNA of the *sanN* disruption mutant, whereas a 2.6 kb signal appeared in that of the wild-type strain (Fig. 2B), indicating that *sanN* had been expectedly disrupted. The confirmed *sanN* disruption mutant and wild-type strain were inoculated in SP medium for 5 days of fermentation and culture filtrates were subjected to HPLC analysis and bioassay. In contrast to the wild-type strain, the *sanN* disruption mutant abolished nikkomycin production (Figs. 3A and 3B). To further confirm that the abolishment of nikkomycin production is only due to the disruption of *sanN*, plasmid pSET152-N was introduced into the *sanN* disruption mutant by conjugal transfer to generate the complementary strain. HPLC

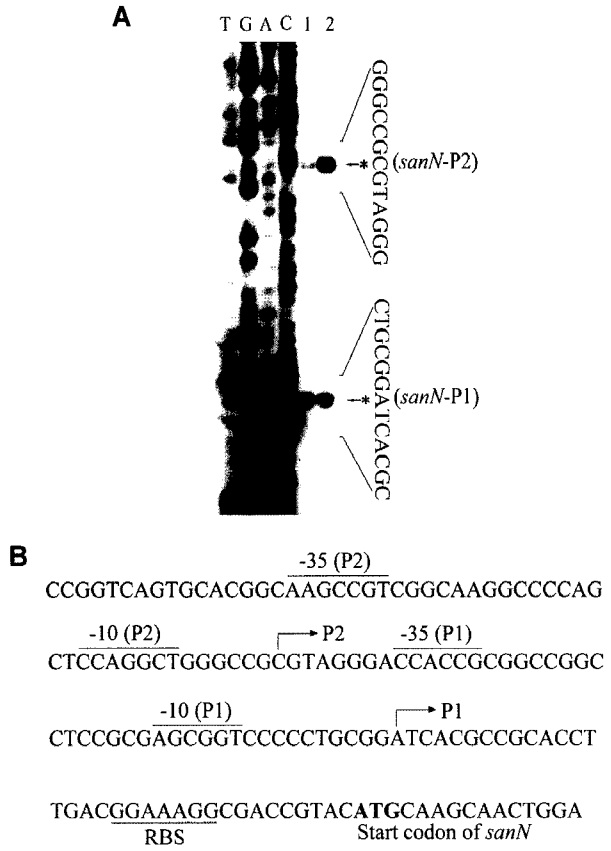

Fig. 4. Determination of TSP of *sanN* by primer extension.
A. Primer extension. Lanes C, A, G, and T, DNA sequence ladder; lanes 1 and 2, reverse transcriptional products (arrowhead) of RNAs isolated from the strain after incubation for 24 h and 72 h; asterisks indicate the TSPs. **B**. Nucleotide sequences covering the promoter region of the *sanN* gene. TSPs are indicated by arrowheads; the translational start codon (ATG) is shown in bold type and the potential ribosomal binding site (RBS) is underlined; the deduced -10 and -35 regions of *sanN*-P1 and *sanN*-P2 are overlined.

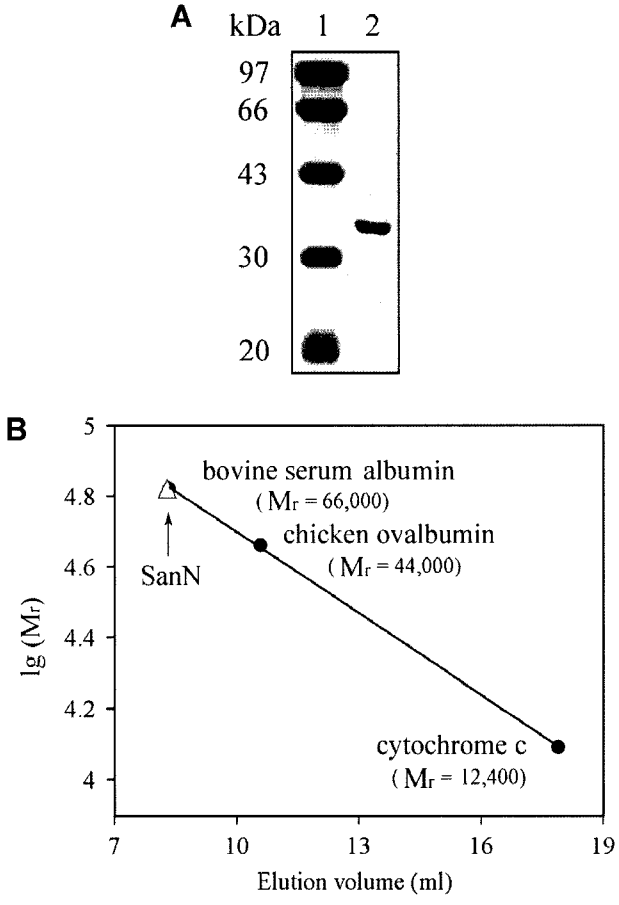

Fig. 5. SDS-PAGE and determination of the M_r of purified SanN by size-exclusion chromatography.
A. SDS-PAGE. Lane 1, standard M_r protein markers. Lane 2, purified SanN. **B**. Determination of the M_r of purified SanN. The column was equilibrated and eluted with 50 mM Tris-Cl (pH 8.0) at a flow rate of 0.5 ml/min. The M_r values of protein standards were linearly regressed against elution volumes to generate a calibration curve for the column, and the M_r of purifed SanN was calculated using this calibration curve. The arrow indicates the position of the purified SanN.

analysis and bioassay of the culture filtration demonstrated that the complementary strain restored the nikkomycin production (Fig. 3C). These results suggested that *sanN* is essential for the nikkomycin biosynthesis in *S. ansochromogenes*.

Determination of TSP of *sanN*

The TSP of *sanN* was determined by primer extension with a *sanN*-specific primer (P3). Results revealed that the *sanN* has two TSPs located at A (*sanN*-P1) and C (*sanN*-P2) at the positions of 34 nt and 79 nt upstream of the translation start codon (ATG) of *sanN*, respectively (Fig. 4A). The nucleotide sequences covering the promoter region of *sanN* are shown in Fig. 4B. The deduced promoters, *sanN*-P1 and *sanN*-P2, share homology in their −10 regions with *tsr*-P1 and *afsR*-P2, and in their −35 regions with *rep*-P2 and *orfI*-P1, respectively [25]. It was also demonstrated that the transcriptional activity of *sanN*-P1 was nearly identical during the *S. ansochromogenes* growth in SP medium for 24 h and 72 h. In contrast, the transcriptional activity of *sanN*-P2 increased rapidly when the strain was incubated from 24 h to 72 h. The variable trend of transcriptional level is consistent with the velocity of nikkomycin production in *S. ansochromogenes*, indicating that *sanN*-P2 might play a more important role in nikkomycin production than *sanN*-P1. Previously, our study had revealed that transcription of *sanN* is controlled by the pathway-specific regulator SanG [18]. Further studies on the regulatory mechanism of *sanN* transcription are under way in our laboratory.

Expression and Purification of SanN

We had constructed the pET23b and pGEX-4T-3 derived plasmids, pET23b-N and pGEX-4T-3-N, to express His_6 and GST tagged SanN in *E. coli*, respectively. However, the SanN protein is insoluble under various expression conditions, including different concentrations of IPTG and inductive temperatures. SanN was insoluble even though it was coexpressed with chaperonin GroESL in *E. coli*. Hence, we tried to express the protein in *S. lividans* instead. The plasmid pIJ8600-N was introduced into *S. lividans* TK24 by conjugal transfer to generate strain *S. lividans* TK24/pIJ8600-N. The recombinant strain was grown in YEME at 28°C for 36 h, and further induced by 5 μg/ml thiostrepton for another 12 h. Then, cultures were collected, washed, and sonicated until completely lyzed. SanN protein was purified from the supernatants by Ni^{2+} affinity chromatography and showed the expected size of 32.5 kDa on SDS-PAGE (Fig. 5A). The M_r of native recombinant SanN was estimated to be about 66 kDa by size-exclusion chromatography, suggesting that the recombinant SanN is a dimeric protein (Fig. 5B).

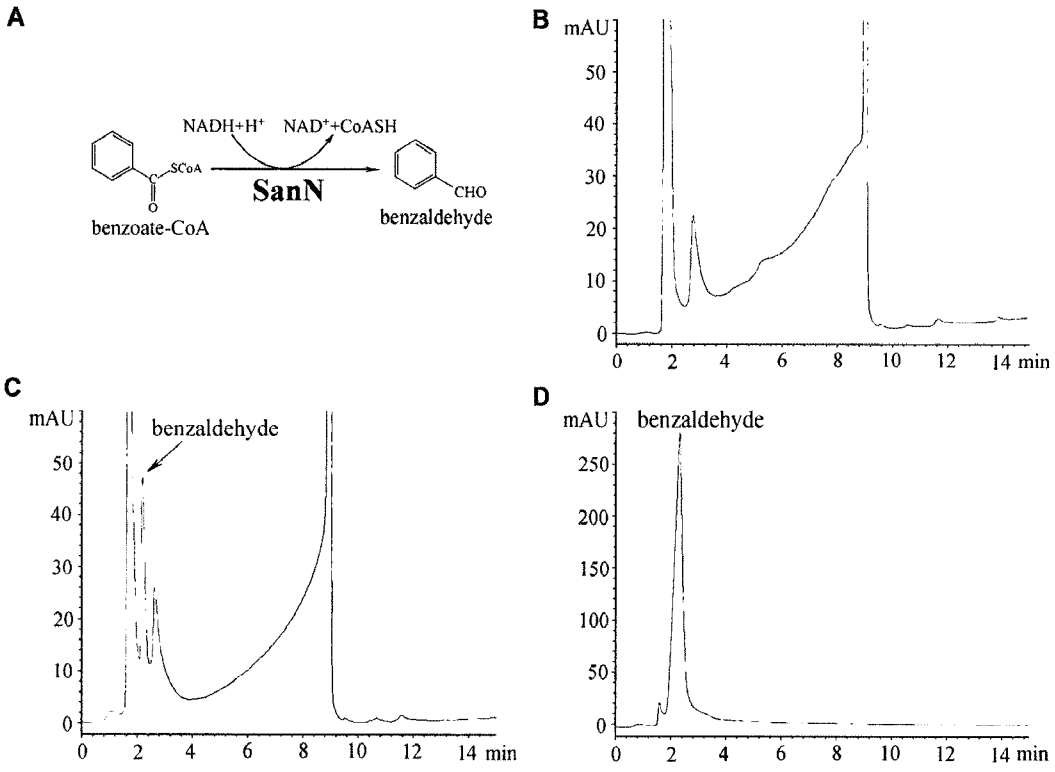

Fig. 6. Conversion of benzoate-CoA to benzaldehyde catalyzed by SanN.

The dehydrogenation reaction catalyzed by SanN in this experiment is schemed in panel (**A**). Reaction products catalyzed by SanM (control protein) (**B**) and SanN (**C**) were loaded onto an HPLC column and eluted, as described in Materials and Methods. Authentic benzaldehyde (2 mM) was subjected to HPLC analysis under the same condition (**D**).

Characterization of SanN as a Dehydrogenase

Sequence analysis showed that SanN displays significant similarities to acetaldehyde dehydrogenases (acylating) and semialdehyde dehydrogenase. Therefore, we proposed that SanN might function as a dehydrogenase to catalyze the conversion of picolinate-CoA/benzoate-CoA to picolinaldehyde/benzaldehyde during nikkomycin biosynthesis. Since picolinate-CoA is commercially or preparatively unavailable, here we used benzoate-CoA as the substrate to investigate the dehydrogenase activity of SanN (Fig. 6A). The reaction mixtures were subjected to HPLC analysis. Compared with the reaction product catalyzed by control protein (SanM), a novel peak appeared in that by SanN at 2.4 min corresponding to authentic benzaldehyde (Figs. 6B–6D). These results demonstrated that SanN could convert benzoate-CoA to benzaldehyde, hence suggesting that SanN catalyzes the formation of picolinate during nikkomycin biosynthesis in the wild-type strain.

We also found that the optimal pH of SanN dehydrogenase is 7.5, and metal ions Mn^{2+}, Mg^{2+}, Ca^{2+}, or Zn^{2+} (1 mM) had no effect on its activity. Moreover, initial velocities versus varied benzoate-CoA concentration at three fixed NADH concentrations, and versus varied NADH concentration at three fixed benzaldehyde concentrations, were further determined. Lineweaver-Burk plots describing the dehydrogenation reaction are shown in Fig. 7. In both panels, straight lines intersect at one point ($1/K_m$), indicating that a random-order mechanism might be suitable for the dehydrogenation reaction catalyzed by SanN. The calculated K_{cat}/K_m values toward benzoate-CoA and NADH are 3.8 $mM^{-1}s^{-1}$ and 12.0 $mM^{-1}s^{-1}$, respectively.

Many homologous proteins of SanN were found in the degradation pathway of aromatic compounds, which convert acetyl-CoA to aldehyde [1, 3, 4, 20, 24]. Although they all belong to the dehydrogenase family, SanN and its homologous proteins catalyze dehydrogenation reactions in exactly reverse directions in their respective physiological pathways. More detailed physiological, biochemical, and structural studies are needed to understand the catalytic mechanism of SanN.

In conclusion, we first cloned the *sanN* gene from *S. ansochromogenes*, and characterized that *sanN* encodes a dehydrogenase that is responsible for the conversion of picolinate-CoA/benzoate-CoA to picolinaldehyde/benzaldehyde during nikkomycin biosynthesis. This study would provide insights into elucidating the biosynthetic pathway of nikkomycins and give clues to the combinatorial synthesis of novel antibiotic derivatives.

Acknowledgments

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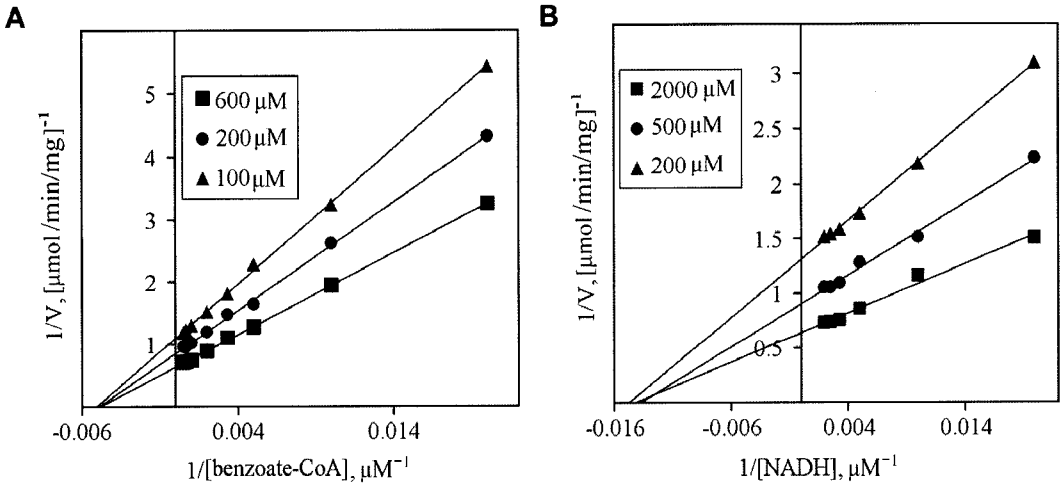

Fig. 7. Kinetics analysis of SanN as a dehydrogenase.
A. Lineweaver-Burk plot of the reciprocal of initial velocities versus the reciprocal of benzoate-CoA concentration. Concentrations of NADH were fixed at 600 μM (■), 200 μM (●), and 100 μM (▲), respectively. **B.** Lineweaver-Burk plot of the reciprocal of the initial velocities versus the reciprocal of NADH concentration. Concentrations of benzoate-CoA were fixed at 2,000 μM (■), 500 μM (●), and 200 μM (▲), respectively.

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