Nectrisine Biosynthesis Genes in *Thelonectria discophora* SANK 18292: Identification and Functional Analysis

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ABSTRACT The fungus *Thelonectria discophora* SANK 18292 produces the iminosugar nectrisine, which has a nitrogen-containing heterocyclic 5-membered ring and acts as a glycosidase inhibitor. In our previous study, an oxidase (designated NecC) that converts 4-amino-4-deoxyarabinitol to nectrisine was purified from *T. discophora* cultures. However, the genes required for nectrisine biosynthesis remained unclear. In this study, the nectrisine biosynthetic gene cluster in *T. discophora* was identified from the contiguous genome sequence around the *necC* gene. Gene disruption and complementation studies and heterologous expression of the gene showed that *necA*, *necB*, and *necC* could be involved in nectrisine biosynthesis, during which amination, dephosphorylation, and oxidation occur. It was also demonstrated that nectrisine could be produced by recombinant *Escherichia coli* coexpressing the *necA*, *necB*, and *necC* genes. These findings provide the foundation to develop a bacterial production system for nectrisine or its intermediates through genetic engineering.

IMPORTANCE Iminosugars might have great therapeutic potential for treatment of many diseases. However, information on the genes for their biosynthesis is limited. In this study, we report the identification of genes required for biosynthesis of the iminosugar nectrisine in *Thelonectria discophora* SANK 18292, which was verified by disruption, complementation, and heterologous expression of the genes involved. We also demonstrate heterologous production of nectrisine by recombinant *E. coli*, toward developing an efficient production system for nectrisine or its intermediates through genetic engineering.

Iminosugars, which are sugar analogues with the ring oxygen replaced by nitrogen, have been isolated from natural sources, including microorganisms and plants. Many of them are known to show inhibitory activity against glycosidases by mimicking the corresponding substrates or their hydrolysis transition states (1–5). Because of that biological activity, iminosugars are considered to have powerful therapeutic potential for the treatment of many diseases, such as diabetes mellitus, viral infections, lysosomal storage disorders, and cancers (1, 2, 4, 6–9).

Nectrisine (compound 1) (Fig. 1), also known as FR-900483 (10), is an iminosugar that has a five-membered nitrogen-containing ring and shows inhibitory activity against α- and β-glucosidases, α- and β-mannosidases, β-*N*-acetylglucosaminidase, and other glycosidases (11). Nectrisine can be biologically produced by *Nectria lucida* F-4490 (12), which is synonymous with *Thelonectria lucida*, as described by Chaverri et al. (13), and *Thelonectria discophora* SANK 18292 (JCM 30947), as determined in our screening program for nectrisine-producing organisms (14). Nectrisine, having three stereogenic centers, can also be chemically synthesized from chiral starting materials, such as D-arabinose or D-serine (15–19). However, the costs of synthesizing nectrisine as a pharmaceutical compound might be high, because of low yield and the high costs of the starting materials. For cost reduction, microbial *de novo* production through fermentation or *in vitro* enzymatic synthesis is attractive. In order to begin to identify enzymes or genes required for nectrisine biosynthesis, the biosynthetic pathway in *T. discophora* was studied using stable isotope labeling techniques, with 4-amino-4-deoxyarabinitol (compound 2) (Fig. 1) being identified as a potential late intermediate (14). Moreover, a potential enzyme, designated NecC, that catalyzes the conversion of

4-amino-4-deoxyarabinitol to nectrisine was purified from the fungus and showed characteristics of glucose-methanol-choline oxidoreductase (20); however, it was not verified that the *necC* gene was involved in nectrisine biosynthesis. Furthermore, the other genes required for nectrisine biosynthesis remain unclear.

In the present study, a cosmid library of *T. discophora* genomic DNA was screened using *necC* genomic DNA. Then, the functions of isolated genes were confirmed through deletion and subsequent complementation and heterologous expression.

MATERIALS AND METHODS

Fungal strain and culture conditions. *T. discophora* SANK 18292, deposited in the Japan Collection of Microorganisms (Tsukuba, Japan) under accession number JCM 30947, was used in this study. The spore suspension was inoculated into A1 medium (14) and incubated for 5 days at 23°C on a rotary shaker. Then glucose-potato-yeast extract-calcium carbonate no. 3 (GPYC-3) medium, containing 8% glycerol, 1% potato granules,
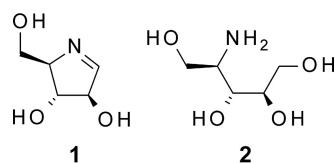
FIG 1 Structures of nectrisine (compound 1) and 4-amino-4-deoxyarabinitol (compound 2).

2.6% yeast extract, and 0.2% $CaCO_3$, was inoculated with 1% (vol/vol) culture and cultivated at 23°C on a rotary shaker. *T. discophora* colonies were maintained on potato dextrose agar (PDA) (Nissui Pharmaceutical, Tokyo, Japan) or yeast mold agar (YMA) (Becton, Dickinson, Franklin Lakes, NJ, USA).

Bacterial strains, nucleic acid manipulations, and PCR primers. *Escherichia coli* JM109 from Takara Bio (Shiga, Japan) and *E. coli* XL1-Blue MR from Agilent Technologies (Santa Clara, CA) were used for the construction, propagation, and amplification of plasmids. *E. coli* BL21(DE3) (Merck Millipore, Billerica, MA) was used for protein production. Media, growth conditions, and general recombinant DNA techniques for *E. coli* were used according to standard methods (21). PCR primers used for this work are listed in Table 1.

Construction and screening of the *T. discophora* genomic library. A cosmid library of *T. discophora* genomic DNA was constructed and *necC*-positive cells were screened as described previously (20). Briefly, the cosmid library was constructed using SuperCos1 cosmid vector (Agilent Technologies), with *E. coli* XL1-Blue MR as the host strain. It was screened by hybridization with digoxigenin (DIG)-labeled partial *necC* DNA fragments. The positive cosmids were isolated, digested with EcoRI and BamHI separately, and subcloned into pUC118 EcoRI/BAP vector or pUC118 BamHI/BAP vector (Takara Bio).

To retrieve genomic DNA from the 3′ end of *necC*, a *T. discophora* genomic library using a plasmid vector was also constructed, as follows. Genomic DNA was digested with HindIII, gel electrophoresed, blotted on positively charged nylon membranes (Roche Diagnostics, Tokyo, Japan), and hybridized with the DIG-labeled 3′-terminal region of cosmid RB185 (199 bp) as a probe. The positive band on the agarose gel was retrieved,

ligated to the pUC118 HindIII/BAP vector, and screened by colony hybridization with the probe.

Sequence analysis. The resulting positive clones were sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific, Yokohama, Japan) and a 3730xl DNA analyzer (Thermo Fisher Scientific). The fragments were assembled using Genetyx software (version 11; Genetyx, Tokyo, Japan). A homology search of the resulting sequences was carried out with the BLAST program (22), using the EMBL/GenBank/DDBJ database. Ratios of sequence identity and similarities to proteins were calculated using Discovery Studio software (version 4.0; Dassault Systemes Biovia, Tokyo, Japan).

Preparation of primary vectors for gene disruption and complementation. Modification of plasmid pSAK2000 (23) to make its multicloning site in reverse order yielded pSAK451, which was kindly provided by Satoshi Baba (Daiichi Sankyo Co., Ltd., Japan) and was used for construction of pNEC001 for targeted gene disruption and complementation. The promoter region of the phosphoglycerol kinase (*pgk*) gene in *T. discophora* was cloned and amplified from *T. discophora* genomic DNA by PCR using an Expand High Fidelity PCR system (Roche Diagnostics), employing the forward and reverse primers EcoRI-Ppgk1-f and HindIII-Ppgk2-r, respectively, with the following program: 94°C for 2 min; 10 cycles of 94°C for 15 s, 50°C for 30 s, and 72°C for 55 s; 15 cycles of 94°C for 15 s, 50°C for 30 s, and 72°C for 55 s plus 5 s per cycle; and 72°C for 7 min. The promoter region of pSAK451 was then replaced with the EcoRI- and HindIII-digested PCR fragment of the promoter region of *T. discophora pgk*. The resulting plasmid, designated pNEC001, contained the neomycin phosphotransferase gene for G418 (Geneticin) resistance. Plasmid pNEC002 was constructed by replacing the neomycin phosphotransferase gene of pNEC001 with an *E. coli* hygromycin B phosphotransferase gene from plasmid pSAK1000 (24) for hygromycin resistance.

Construction of disruption vectors. DNA fragments of *necC* (1.3 kbp), *necA* (1.2 kbp), and *necB* (1.1 kbp) were amplified by PCR with the primer sets KpnI-dis1-f/BglII-dis2-r, KpnI-ATdis1-f/BglII-ATdis2-r, and KpnI-AKdis1-f/BglII-AKdis2-r, respectively. The PCR products were digested with KpnI and BglII and introduced into the KpnI-BglII sites of pNEC001 to yield pNEC001dis*necC*, pNEC001dis*necA*, and pNEC001dis*necB*. The resulting plasmids were digested with XhoI to convert them to linear forms.

TABLE 1 PCR primers

Primer name	Sequence (5′ to 3′)[a]
EcoRI-Ppgk1-f	CCCGAATTCATGCCAAGTGAGATGAAG
HindIII-Ppgk2-r	CCCAAGCTTGATTGTTTGTGGAGGAGA
KpnI-dis1-f	ATATGGTACCACTTCAACCGCGGAGCGAGCAACTC
BglII-dis2-r	ATATAGATCTAGATCGGTATCGACGACTCCGTCTC
KpnI-ATdis1-f	ATATGGTACCCAACGTAGCCTACTGCCACCCAGGC
BglII-ATdis2-r	ATATAGATCTGCGTAGCCGTCGTACGTGCCCCCCG-
KpnI-AKdis1-f	CGATATAATGGTACCACCACGCCGAGATTCGCGTCAAGGC
BglII-AKdis2-r	GCATTATATAGATCTAGTAAAAGCCAGGGAAGCCCCGGAA
MluI-AOcomp1-f	ATATACGCGTTGATGGGGGACACGGGAAAGAATGT
AOcomp2-BglII-r	ATATAGATCTAGGTGGATGAATTTGATGGCGGGGA
NotI-ATcomp1-f	ATATGCGGCCGCTGGCCATTGTGACTCGACTCACTCG
ATcomp2-Kpn1-r	ATATGGTACCAGAGGCCAGACCGTTCCAACCAACC
KpnI-AKdis1-f	CGATATAATGGTACCACCACGCCGAGATTCGCGTCAAGGC
BglII-AKdis2-r	GCATTATATAGATCTAGTAAAAGCCAGGGAAGCCCCGGAA
BamHI-AK-f	GCATATAATGGATCCGATGACCGACATCCCGTTCGTGCAGC
AK-HindIII-r	CGATTATATAAGCTTTCACGGGGCTGCCCATCTTTCCTCT
NdeI-AT-f2	CGATATAATCATATGGGTTCCCAAGGTCCAAAGAACA
AT-HisKpnI-r	CGATTATATGGTACCTCAGTGGTGGTGGTGGTGGTGAACGGCGCACACCTTGGTAGTCACC
BamHI-AO-f	ATATGGATCCAATGGATCATCTTCTCCATATCGACA
AO-NotI-r	ATATGCGGCCGCCTATTGAGCGTCGTTTCCAATCTTC

[a] Underlining denotes restriction site sequences.

Construction of complementation vectors. A 3.6-kbp fragment containing the complete *necC* gene was amplified by PCR using Phusion High-Fidelity DNA polymerase (New England BioLabs, Tokyo, Japan) and the primers MluI-AOcomp1-f and AOcomp2-BglII-r, with the following program: 98°C for 30 s; 30 cycles of 98°C for 10 s, 64°C for 20 s, and 72°C for 2 min; and 72°C for 7 min. The fragment was then digested with MluI and BglII and introduced into the MluI-BglII sites of pNEC002. The resulting plasmid was designated pNEC002comp*necC*. This plasmid was digested with NheI to convert it to a linear form. A 3.1-kbp fragment containing the complete *necA* gene was amplified by PCR with the primers NotI-ATcomp1-f and ATcomp2-KpnI-r, with the following program: 98°C for 30 s; 30 cycles of 98°C for 10 s, 66°C for 20 s, and 72°C for 2 min; and 72°C for 7 min. The fragment was digested with NotI and KpnI and introduced into the NotI-KpnI sites of pNEC002. The resulting plasmid was designated pNEC002comp*necA*. The plasmid was digested with XhoI to convert it to a linear form.

Protoplast preparation and PEG-mediated DNA transformation. Fungal protoplast generation and transformation were performed as follows. For preparation of *T. discophora* protoplasts, a suspension containing 6×10^7 spores of *T. discophora* was grown in GY medium, containing 5% glycerol and 1.6% yeast extract, at 23°C for 16 h at 210 rpm. The young mycelium was recovered by filtration and washed with KC buffer, containing 1 M KCl and 50 mM $CaCl_2$ (osmotic stabilizer). The recovered hyphae of 0.5 g to 1.0 g were incubated with 30 ml of 2% Yatalase enzyme (Takara Bio) in KC buffer at 30°C for 1 h at 50 rpm, filtered through a sterile 3GP160 glass filter (Sibata Scientific Technology, Saitama, Japan), and washed with KC buffer. The resulting protoplasts were gently mixed with 15 μg of vector and maintained on ice for 10 min. A polyethylene glycol (PEG) solution (100 μl) containing 5% PEG 3350, 50 mM $CaCl_2$, and 10 mM Tris-HCl (pH 8.0) was added, and the mixture was placed on ice for 20 min. Subsequently, 500 μl of PEG solution was added and the mixture was incubated at room temperature for 10 min (25). After being washed with 5 ml of SCT buffer, containing 1 M sucrose, 50 mM $CaCl_2$, and 10 mM Tris-HCl (pH 8.0), the protoplasts were resuspended in 1 ml of SCT buffer. The resulting solution was added to 5 ml of YMA, containing yeast mold (YM) broth, 0.6% agar, and 1 M sucrose, at 42°C and then was overlaid on YMA plates, containing YM broth, 2% agar, and 1 M sucrose, and the plates were incubated at 23°C for 16 h to regenerate the mycelium. The plates were then overlaid with 5 ml of YMA containing 80 mg/liter of G418 or hygromycin B and were incubated at 23°C for 3 days.

Southern blotting. Genomic DNA of *T. discophora* transformants was digested with BglII, SphI, BamHI, ScaI, or HindIII. The digested DNA was electrophoresed through an agarose gel in 1× TAE buffer (Wako Pure Chemical Industries) and transferred to a positively charged nylon membrane (Roche Diagnostics). Hybridization was performed using a DIG system (Roche Diagnostics), according to the supplier's instructions, with DIG-labeled *necC*, *necA*, or *necB* as the probe.

Extraction of metabolites. *T. discophora* metabolites were extracted from the mycelium with freezing and thawing and then heating at 60°C for 10 min. This procedure was repeated twice. The extracts were centrifuged, and the supernatants were filtered. They were then diluted to one-tenth their concentration with deionized water and incubated for 3 h at room temperature to convert 4-amino-4-deoxyarabinitol to nectrisine. Nectrisine and 4-amino-4-deoxyarabinitol were purified from the extracts of *T. discophora* or recombinant *E. coli* with cation-exchange solid-phase extraction columns (Oasis MCX Plus Short cartridge, 225 mg; Waters). Samples were acidified with 0.02 ml of formic acid and loaded onto the extraction column, which had been preconditioned according to the manufacturer's instructions. After washing with 0.2% formic acid and 4 ml of methanol, metabolites were eluted with 3 ml of 2% ammonium hydroxide in methanol, evaporated to dryness, and then reconstituted with 0.2 ml of deionized water.

LC-MS analysis of metabolites. To detect and to efficiently separate nectrisine and 4-amino-4-deoxyarabinitol by high-performance liquid chromatography (HPLC), samples were reduced with NaBH₄ and de-

rivatized with 4-fluoro-7-nitrobenzofurazan (NBD-F) (Dojindo Laboratories, Kumamoto, Japan) (26, 27); liquid chromatography (LC)-photodiode array (PDA)-positive electrospray ionization (ESI) mass spectrometry was performed as described previously (20). The peak areas of the PDA signal at 500 nm for the samples and the nectrisine standard were compared to quantify the concentrations.

Bacterial coexpression of *necA*, *necB*, and *necC*. Total RNA was extracted from ground cells of *T. discophora* with RNeasy Plant kits (Qiagen) and was reverse transcribed with ReverTraAce-α- reverse transcriptase and oligo(dT)₂₀ primer (Toyobo, Osaka, Japan), according to the supplier's instructions. Then, *necB* cDNA was amplified by PCR with the forward and reverse primers BamHI-AK-f and AK-HindIII-r, respectively. The PCR product was gel purified and digested with BamHI and HindIII. The resulting product was introduced into the same restriction sites of the pACYCDuet-1 vector (Merck), carrying the P15A replicon and the chloramphenicol resistance gene, and the vector was designated pACYCDuet-*necB*. Next, a vector for coexpression of *necA* and *necC* was constructed by using the pETDuet-1 vector containing two multiple cloning sites (MCSs) under the T7 promoter and the *lac* operator and carrying the ColE1 replicon and the ampicillin resistance gene. The *necA* and *necC* genes were amplified by PCR using the primer sets BamHI-AO-f/AO-NotI-r and NdeI-AT-f2/AT-HisKpnI-r and were gel purified. The *necC* product was digested with BamHI and NotI and then introduced into the BamHI-NotI site of the pETDuet-1 vector located in MCS1, generating pETDuet*necC*. Subsequently, the *necA* product was digested with NdeI and KpnI and then introduced into the NdeI-KpnI site of the pETDuet*necC* vector located in MCS2, generating pETDuet*necCnecA*. For coexpression of *necA*, *necB*, and *necC*, *E. coli* BL21(DE3) was transformed with the pACYCDuet-*necB* and pETDuet*necCnecA* vectors. The cells were grown at 37°C in shake flasks containing LB medium (Wako Pure Chemical Industries), supplemented with 1 g/liter D-ribose, 100 mg/liter carbenicillin, and 30 mg/liter chloramphenicol, until the optical density at 600 nm (OD_{600}) reached 0.6 to 1.0. After immediate cooling, production of the His-tagged proteins was induced by adding isopropyl-β-D-thiogalactoside (IPTG) at a final concentration of 0.3 mM, and the cultivation was continued at 18°C for another 36 h at 210 rpm. Next, the cells were pelleted by centrifugation and resuspended in 100 mM NaH_2PO_4, 300 mM NaCl (pH 7.8). After disruption of the cells with an ultrasonic homogenizer, soluble fractions were collected by centrifugation and analyzed by SDS-PAGE under reducing conditions. The gels were stained with Coomassie brilliant blue R250 or transferred to a membrane for Western blotting. His-tagged proteins were detected with Penta-His-horseradish peroxidase (HRP) conjugate (Qiagen) and visualized with ECL Prime Western blotting detection reagent (GE Healthcare). A supernatant of the culture was reduced and labeled with NBD-F, and then nectrisine in the solution was quantified by HPLC as described previously (14).

Accession number(s). The nucleotide sequences of *necA*, *necB*, and *necC*, with assigned open reading frames (ORFs), were deposited in the EMBL/GenBank/DDBJ database under accession numbers LC150594 for *necA*, LC150595 for *necB*, and LC056029 for *necC*. The *gabT1*, *yktC1*, and *gutB1* genes are included in the *Bacillus amyloliquefaciens* genome, for which the accession number in the database is CP000560.

RESULTS

Sequence analysis of genomic DNA around *necC*. We hypothesized that *necC* is involved in nectrisine biosynthesis, since it catalyzes the conversion of 4-amino-4-deoxyarabinitol, which can be produced by *T. discophora*, to nectrisine. We also assumed that nectrisine biosynthesis genes might be clustered, since many of the biosynthesis genes for fungal secondary metabolites, including the iminosugar deoxynojirimycin (28, 29), are known to be clustered (30–33). Thus, in order to find nectrisine biosynthesis genes, cosmid clones of *T. discophora* genomic DNA containing *necC* were retrieved and sequenced (Fig. 2). Inserts of cosmids RB185 and RB246 and plasmid RB237 represent a contiguous portion of
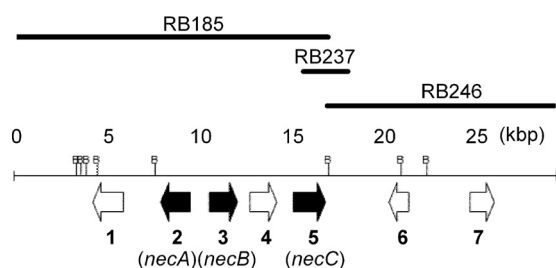
FIG 2 Nectrisine biosynthetic gene cluster. The restriction endonuclease map, inserted positions of cosmid RB185, cosmid RB246, and plasmid RB237, and locations of genes are shown. The arrows indicate the putative direction of transcription, based on sequence analysis and homology searches. Black arrows indicate the biosynthesis genes. B, BamHI.

genomic DNA that includes the *necC* gene. Database searches revealed at least five ORFs near *necC*, predicting the presence of a fungus-specific transcription factor domain (ORF1), an aminotransferase (ORF2), a phosphotransferase (ORF3), and a transporter (ORF4) (Table 2). ORF5 corresponded to the *necC* gene, which encodes an oxidase that converts 4-amino-4-deoxyarabinitol to nectrisine. The functions of ORF6 and ORF7, located downstream of ORF5, could not be assigned because of their low levels of homology with known amino acid sequences. Aminotransferases could be classified into five subgroups, based on sequence alignments in the Pfam database (34). ORF2 showed homology to class III aminotransferases, including 4-aminobutyrate aminotransferases, acetylornithine aminotransferases, and ω-amino acid aminotransferases (35). ORF3 showed homology to choline/ethanol amine kinases. Our previous study suggested that, besides oxidation by NecC, amination and dephosphorylation should be required for nectrisine biosynthesis (14). Thus, in addition to ORF5 (*necC*), ORF2 and ORF3, designated *necA* and *necB*, respectively, were expected to be genes potentially required for nectrisine biosynthesis.

The NecA protein, consisting of 487 amino acid residues with a molecular mass of 52,583 Da, and NecB protein, consisting of 381 amino acid residues with a molecular mass of 42,600 Da, were predicted on the basis of cDNA sequences. Comparison of the DNA fragment with the corresponding cDNA revealed that the coding regions were interrupted by two introns, with lengths of 66 bp and 63 bp, for *necA* and five introns, with lengths of 64, 52, 63, 66, and 139 bp, for *necB*.

Disruption and complementation of *necC*. Targeted gene disruption and subsequent complementation experiments were conducted to confirm that the genes were involved in a nectrisine

biosynthetic pathway in *T. discophora*. First, *necC* was disrupted by homologous recombination with pNEC001dis*necC*. Southern analysis confirmed that the digestion patterns for the genomic DNA with BglII, SphI, and BamHI were consistent with a single insertion of pNEC001dis*necC*, as shown in Fig. 3. Subsequently, the *necC* disruptant was complemented by homologous recombination with pNEC002comp*necC*, containing a full *necC* sequence. Southern analysis confirmed that the digestion patterns for the genomic DNA with BglII, SphI, and BamHI were consistent with a single insertion of pNEC002comp*necC*, as shown in Fig. 3.

The inactivation of *necC* abolished nectrisine production and instead led to production of substantial amounts of 4-amino-4-deoxyarabinitol, which was confirmed by extracted ion chromatograms for reduced and 7-nitrobenzofurazan (NBD)-labeled nectrisine (m/z 297.08, $[M+H]^+$) and NBD-labeled 4-amino-4-deoxyarabinitol (m/z 315.09, $[M+H]^+$) (see Fig. S1 in the supplemental material). Nectrisine production by the Δ*necC* mutant was recovered with complementation. Production of 4-amino-4-deoxyarabinitol by the Δ*necC* mutant and nectrisine production by the *necC*-complemented mutant were verified by the retention times in HPLC analysis and the positive ESI mass spectra, which were consistent with those of authentic 4-amino-4-deoxyarabinitol and nectrisine (Fig. 3C). These results demonstrated that NecC, converting 4-amino-4-deoxyarabinitol to nectrisine, is involved in nectrisine biosynthesis.

Disruption and complementation of *necA*. Southern analysis of one of the pNEC001dis*necA* transformants confirmed that the digestion patterns for the genomic DNA with ScaI, HindIII, and BamHI were consistent with a single insertion of the plasmid copy, as shown in Fig. 4, indicating that the plasmid had been introduced homologously into the *necA* locus. Subsequently, the *necA* gene of the disruptant was complemented by homologous recombination with pNEC002comp*necA*, containing a full *necA* sequence. Southern analysis of the complementation mutant confirmed that the digestion patterns for the genomic DNA with ScaI and BamHI were consistent with a single insertion of the plasmid copy, as depicted in Fig. 4.

The inactivation of *necA* abolished the production of both nectrisine and 4-amino-4-deoxyarabinitol, as verified by NBD-derived signals (Fig. 4C) and extracted ion chromatograms (see Fig. S1 in the supplemental material). The nectrisine production of the Δ*necA* mutant was recovered with the subsequent complementation, as verified by the retention times in HPLC analysis and the mass spectra (Fig. 4C). These results indicated that *necA* is related to 4-amino-4-deoxyarabinitol biosynthesis.

Disruption of *necB*. Transformation with a disruption vector,

TABLE 2 Deduced functions of enzymes encoded in the nectrisine biosynthetic cluster

Gene	Homologous protein (GenBank or NCBI reference sequence accession no.)	Putative function	No. of amino acids	Identity[a] to homologous protein (%)	Similarity[b] to homologous protein (%)
ORF1	FVEG_12058 from *Fusarium verticillioides* (EWG53678)	Transcription factor			
ORF2 (*necA*)	FPSE_08018 from *Fusarium pseudograminearum* (XP_009259411)	Aminotransferase	487	72	86
ORF3 (*necB*)	AOL_s00078g295 from *Arthrobotrys oligospora* (XP_011121860)	Phosphotransferase	382	48	69
ORF4	Predicted protein from *Nectria haematococca* (XP_003042865)	MFS transporter[c]			
ORF5 (*necC*)	FVEG_12711 from *Fusarium verticillioides* (EWG54502)	Oxidase	559	65	80

[a] Percentage of alignment positions that have identical residues.
[b] Percentage of alignment positions that have similar residues.
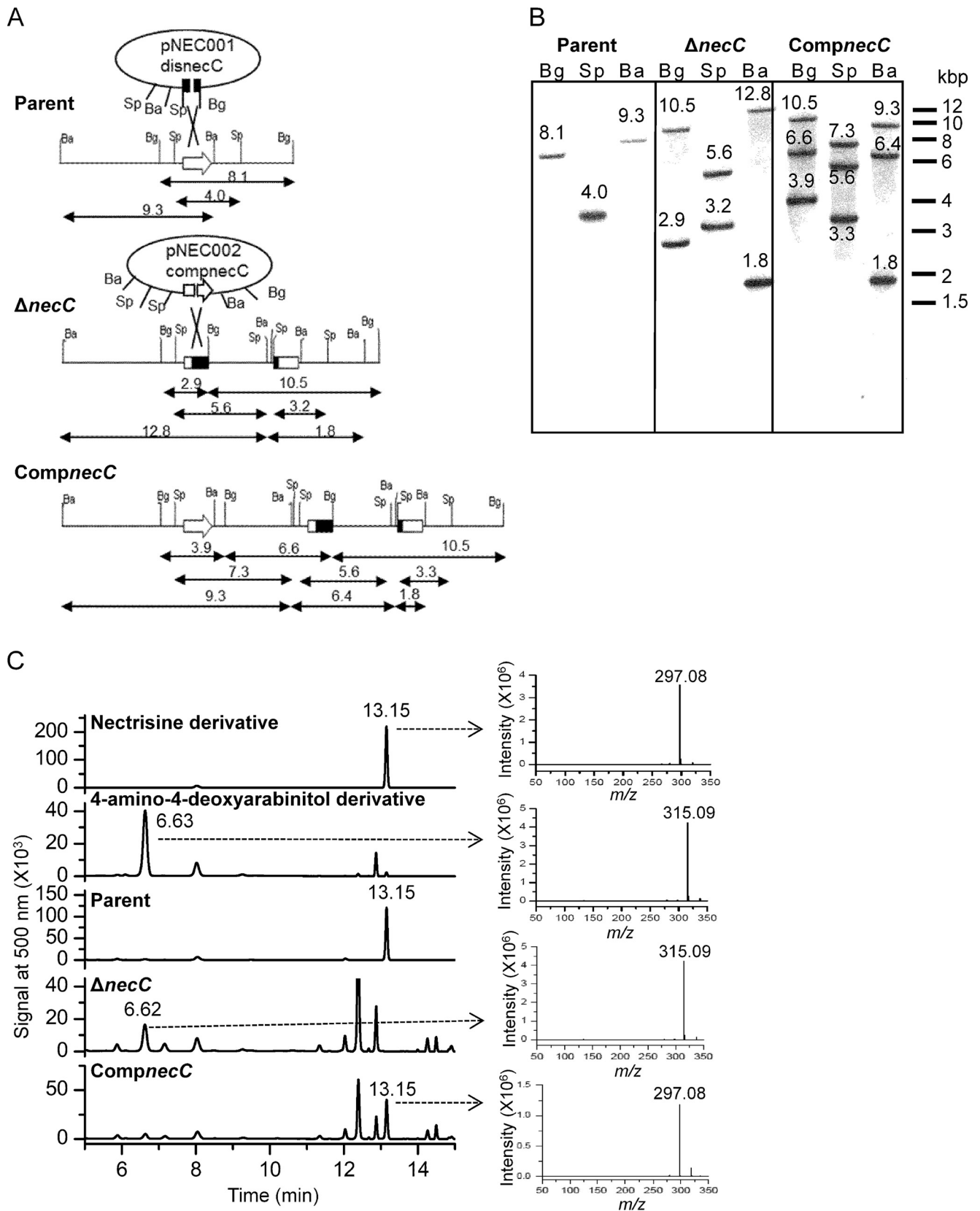[c] MFS, major facilitator superfamily.

FIG 3 Disruption and complementation of *necC*. (A) Predicted disruption and complementation events caused by homologous recombination with the XhoI-digested plasmids pNEC001dis*necC* and pNEC002comp*necC*. The deduced restriction patterns for genomic DNA from the parent strain (top), a *necC* disruptant (Δ*necC*) (middle), and a *necC*-complemented mutant (Comp*necC*) (bottom) are shown. Bars and white arrows indicate *necC* genes. *necC* fragments from a plasmid (pNEC001dis*necC*) are shown as black bars. Bg, BglII; Sp, SphI; Ba, BamHI. (B) Genomic Southern hybridization patterns with DIG-labeled *necC* for genomic DNA from the parent strain, the *necC* disruptant, and the *necC*-complemented mutant, digested with the indicated enzymes. (C) HPLC chromatograms (left) and ESI mass spectra (right) of reduced and NBD-labeled authentic nectrisine, authentic 4-amino-4-deoxyarabinitol, and metabolites produced by the parent strain, the *necC* disruptant, and the *necC*-complemented mutant.
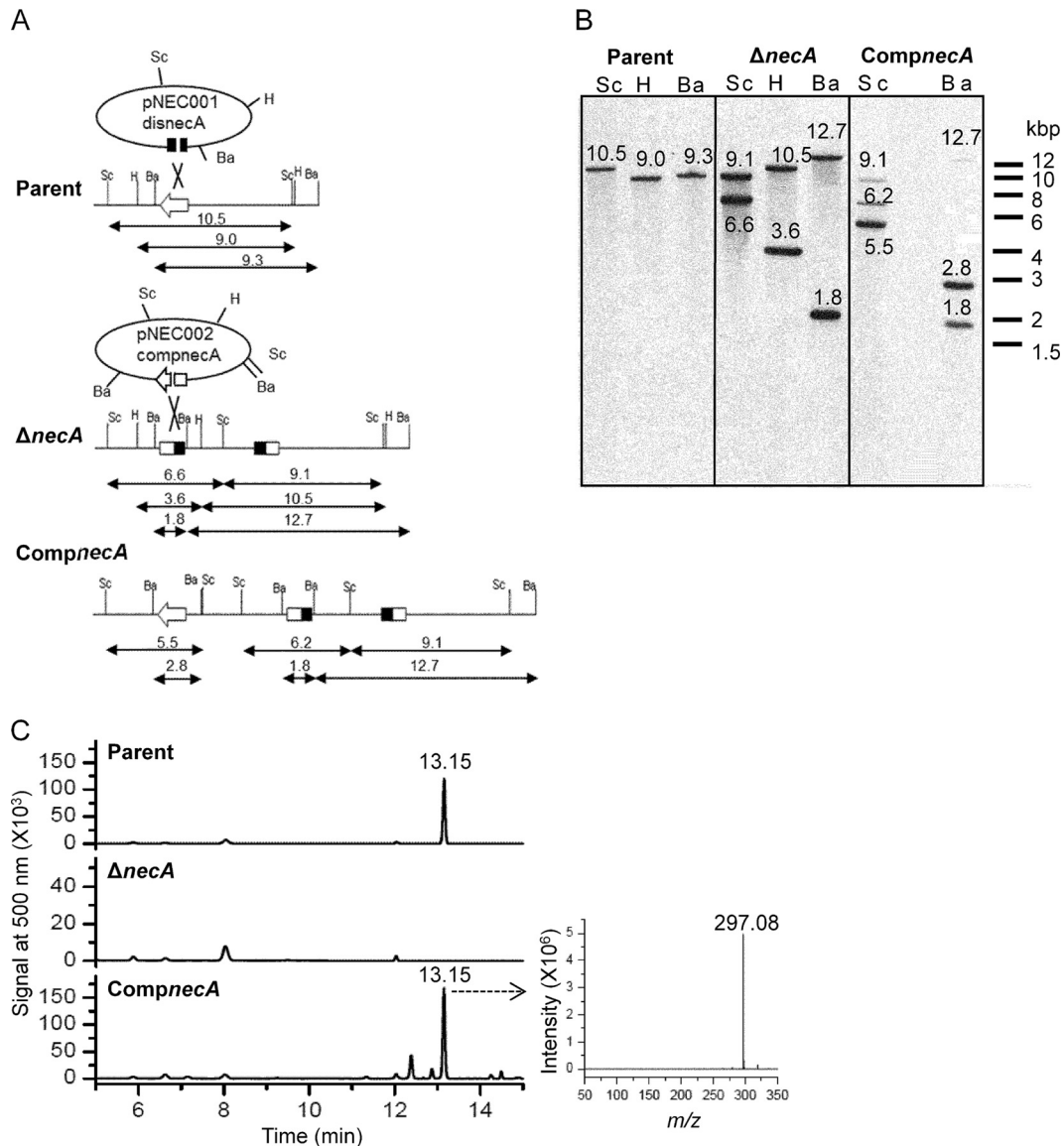
FIG 4 Disruption and complementation of *necA*. (A) Predicted disruption and complementation events caused by homologous recombination with the XhoI-digested plasmids pNEC001dis*necA* and pNEC002comp*necA*. The deduced restriction patterns for genomic DNA from the parent strain (top), a *necA* disruptant (Δ*necA*) (middle), and a *necA*-complemented mutant (Comp*necA*) (bottom) are shown. Bars and white arrows indicate *necA* genes. *necA* fragments from a plasmid (pNEC001dis*necA*) are shown as black bars. Sc, ScaI; H, HindIII; Ba, BamHI. (B) Genomic Southern hybridization patterns with DIG-labeled *necA* for genomic DNA from the parent strain, the *necA* disruptant, and the *necA*-complemented mutant, digested with the indicated enzymes. (C) HPLC chromatograms (left) and ESI mass spectrum (right) of reduced and NBD-labeled metabolites produced by the parent strain, the *necA* disruptant, and the *necA*-complemented mutant.

pNEC001dis*necB*, yielded at least 8 transformants. Southern analysis of HindIII digests of the genomic DNAs confirmed that the plasmid was inserted singly for 6 transformants and doubly for 2 transformants (data not shown). The gene disruption of one of the clones was confirmed using 3 restriction enzymes, as shown in Fig. 5. The nectrisine level of the deletion mutant was significantly decreased, to 24% of the level of the parent clone, in a single experiment.

Coexpression of *necA*, *necB*, and *necC* by recombinant *E. coli* and effects of D-ribose supplementation. In order to confirm that *necB* is involved in nectrisine biosynthesis, *necA* and *necC* or *necA*, *necB*, and *necC* were coexpressed as His-tagged proteins by recombinant *E. coli*. Western blot analysis of soluble

fractions from the *E. coli* cultures confirmed the production of the proteins (Fig. 6A). Comparison with individually expressed *necA*, *necB*, or *necC* indicated that the upper, middle, and lower bands for the *necA*-, *necB*-, and *necC*-expressing strain corresponded to NecC, NecA, and NecB, respectively, with the molecular weights measured by gel electrophoresis being consistent with the theoretical molecular weights. Nectrisine from the supernatants of the cultures was verified by its retention times in HPLC analysis and mass spectra, not only for the mutant expressing *necA*, *necB*, and *necC* but also for the mutant expressing *necA* and *necC* but not *necB* (Fig. 6B), indicating that *necB* is not likely to be necessary for nectrisine biosynthesis.

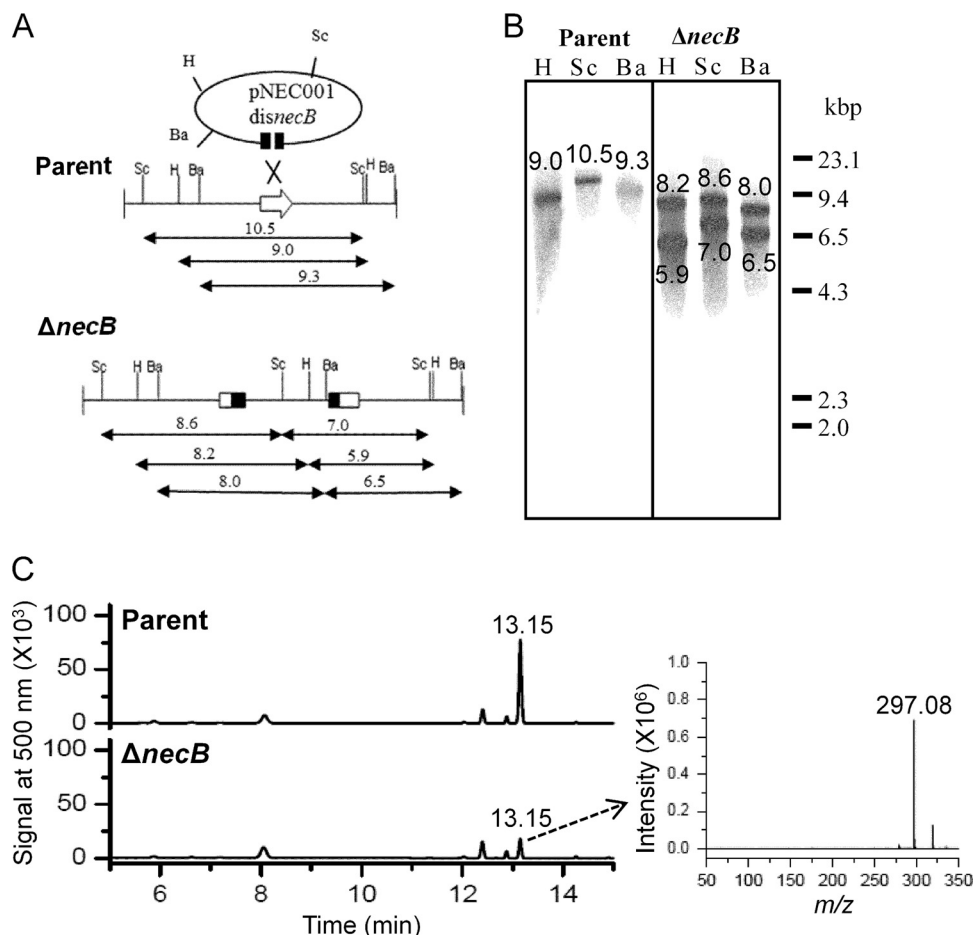Experiments feeding D-ribose to the *T. discophora* culture in

FIG 5 Disruption of *necB*. (A) Predicted disruption events caused by homologous recombination with the XhoI-digested plasmid pNEC001dis*necB*. The deduced restriction patterns for genomic DNA from the parent strain (top) and a *necB* disruptant (Δ*necB*) (bottom) are shown. Bars and white arrows indicate *necB* genes. *necB* fragments from a plasmid (pNEC001dis*necB*) are shown as black bars. H, HindIII; Sc, ScaI; Ba, BamHI. (B) Genomic Southern hybridization patterns with DIG-labeled *necB* for genomic DNA from the parent strain and the *necB* disruptant, digested with the indicated enzymes. (C) HPLC chromatograms (left) and ESI mass spectrum (right) of reduced and NBD-labeled metabolites produced by the parent strain and the *necB* disruptant.

our previous study suggested that D-ribose could be an intermediate for nectrisine biosynthesis and enhanced nectrisine production (14). As expected, addition of D-ribose to the recombinant *E. coli* culture expressing *necA*, *necB*, and *necC* enhanced nectrisine production, from a concentration of 1 mg/liter to 3 mg/liter, in a single experiment.

DISCUSSION

In the present study, we identified genes required for nectrisine biosynthesis in *T. discophora*. Based on our study, a biosynthetic pathway of nectrisine in *T. discophora* could be proposed (Fig. 7). The *necC* disruption and complementation experiments demonstrated that NecC, converting 4-amino-4-deoxyarabinitol (compound 2) to nectrisine (compound 1), is involved in nectrisine biosynthesis. Since NecC is an alcohol oxidase, 4-amino-4-deoxyarabinitol is considered to be first converted to an aldehyde (compound 3) with intramolecular addition of the amino group to the carbonyl group, resulting in a cyclic compound (compound 4) that could be spontaneously dehydrated (without an enzymatic reaction) to afford nectrisine (20). Our results also indicated that *necA*, encoding a putative aminotrans-

ferase, is involved in 4-amino-4-deoxyarabinitol biosynthesis. Unexpectedly, *necB* disruption could not completely inactivate nectrisine production, suggesting that *necB* is unlikely to be necessary for 4-amino-4-deoxyarabinitol biosynthesis. It was confirmed in heterologous expression experiments that *E. coli* coexpressing *necA* and *necC* produced nectrisine without *necB* expression. However, nectrisine production of *necB* disruptants was remarkably suppressed. These results indicated that *necB* is not necessary but is related to 4-amino-4-deoxyarabinitol biosynthesis. Since 4-amino-4-deoxyarabinitol should be generated by NecA and NecB, NecB is considered to catalyze the dephosphorylation of D-xylulose 5-phosphate (compound 5) (route 1) or 4-amino-4-deoxyarabinitol phosphate (compound 7) (route 2). D-Xylulose 5-phosphate (compound 5), which is supplied via the pentose phosphate pathway, could be a potential intermediate (14). Route 2 is expected to be reasonable because *necB*, a member of the choline/ethanolamine kinase family, is likely to prefer an alcoholamine (compound 7) to D-xylulose 5-phosphate (compound 5) as a substrate. Also, route 2 is similar to the proposed biosynthetic pathway for the iminosugar deoxynojirimycin, with a 6-membered ring, in *Bacillus amyloliquefaciens*, in which amina-
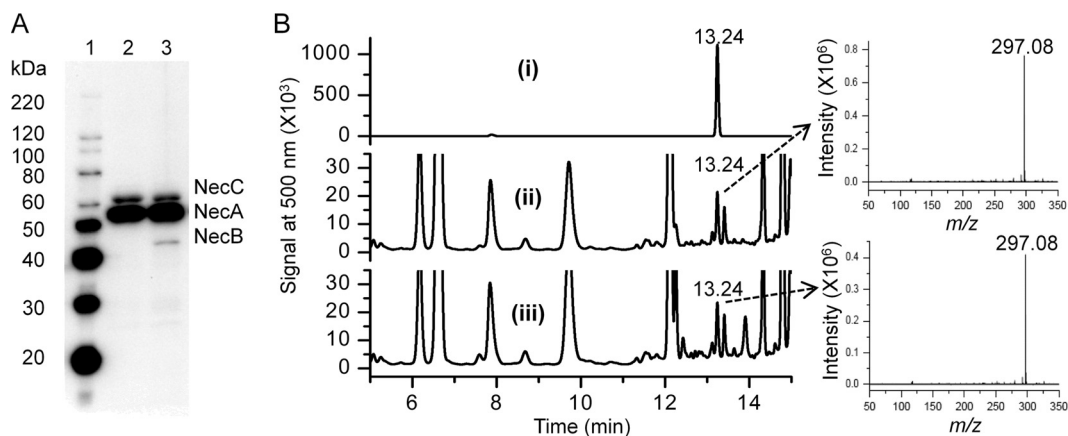
FIG 6 Coexpression of *necA*, *necB*, and *necC* by recombinant *E. coli*. (A) Western blot of soluble fractions probed with anti-His antibodies. Lane 1, molecular weight markers; lane 2, *E. coli* BL21(DE3) harboring the plasmid pETDuet*necCnecA*; lane 3, *E. coli* BL21(DE3) harboring the plasmids pETDuet*necCnecA* and pACYCDuet*necB*. (B) HPLC chromatograms (left) and ESI mass spectra (right) of reduced and NBD-labeled supernatants of the *E. coli* cultures. (i) Reduced and NBD-labeled authentic nectrisine, for which the calculated molecular mass ([M+H]) is 297.08. (ii) *E. coli* BL21(DE3) harboring the plasmid pETDuet*necCnecA*. (iii) *E. coli* BL21(DE3) harboring the plasmids pETDuet*necCnecA* and pACYCDuet*necB*.

tion, dephosphorylation, and oxidation occur in the same order as in route 2 (29).

Clustered genes for iminosugar biosynthesis are not yet known except for those in deoxynojirimycin biosynthesis, for which the gene cluster could contain *gabT1*, *yktC1*, and *gutB1*, coding for a putative aminotransferase, phosphatase, and dehydrogenase, respectively (28, 29), analogous to *necA*, *necB*, and *necC* for nectrisine biosynthesis. However, *necA*, *necB*, and *necC* showed relatively low levels of identity (25%, 14%, and 17%, respectively) and similarity (48%, 31%, and 33%, respectively) with respect to *gabT1*, *yktC1*, and *gutB1* from *Bacillus amyloliquefaciens*.

Nectrisine production by fungal *necB* disruptants and by recombinant *E. coli* without *necB* expression might be assisted by other enzymes catalyzing the NecB reaction with a variety of substrate specificities, such as choline kinases, most of which were shown to utilize both choline and ethanolamine as substrates (36–38). The existence of semiessential genes like *necB* in biosynthetic gene clusters has been reported previously; the aminotransferase gene *tdiD* was semiessential for bis-indolylquinone biosyn-

thesis in *Aspergillus nidulans* (39). That report also implied alternative enzymes for TdiD, which was supported by the existence of genes similar to *tdiD* in the *A. nidulans* genome. Also, analysis of a genome-scale model of *E. coli* metabolism showing that about 37% of enzymes act on a variety of substrates (40) could support the existence of alternative enzymes.

ORF1 is proposed to be a member of the GAL4-type zinc cluster proteins, a typical class of positive transcriptional regulators found in a variety of fungal organisms (41, 42). Thus, it is possible that ORF1 is a transcriptional regulator for nectrisine biosynthesis genes. This should be investigated in future work.

Bacterial production is attractive as a commercial manufacturing process, compared with fungal production, since production by filamentous fungi sometimes encounters technical problems, such as low productivity due to insufficient control of growth and morphology and difficult handling for filtration and extraction due to high viscosity (43–47). The gene cluster for nectrisine biosynthesis could provide a foundation to develop a microbial production system for nectrisine and its intermediates, such as 4-amino-4-deoxyarabinitol, through genetic engineering.

FIG 7 Proposed pathway for nectrisine biosynthesis in *T. discophora*.

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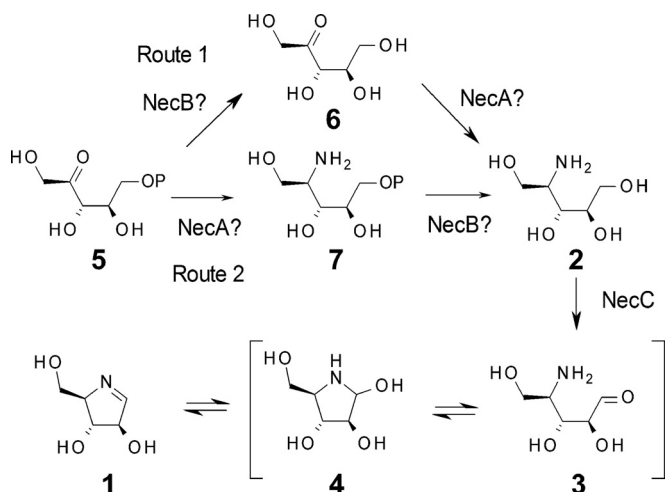5. **Winchester BG.** 2009. Iminosugars: from botanical curiosities to licensed drugs. Tetrahedron Asymmetry **20**:645–651. http://dx.doi.org/10.1016/j.tetasy.2009.02.048.

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