*Biosci. Biotechnol. Biochem.*, **70** (9), 2316–2319, 2006

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# Molecular Cloning of *Streptomyces* Genes Encoding Vanillate Demethylase

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Received April 4, 2006; Accepted May 22, 2006; Online Publication, September 7, 2006
[doi:10.1271/bbb.60180]

**The vanillate demethylase genes from *Streptomyces* sp. NL15-2K were cloned and sequenced. The *van*A and *van*B gene homologs, which encode the terminal oxygenase subunit (VanA) and the ferredoxin-type reductase subunit (VanB) of the enzyme respectively, were found in the sequenced 7.5-kb DNA region. Expression of the *van*AB genes in *Streptomyces lividans* 1326 resulted in *in vivo* demethylation of veratric acid to vanillic acid.**

**Key words:** vanillate demethylase; *van*A; *van*B; *Streptomyces*

Lignin is a cross-linked phenylpropanoid polymer, and is the next major component to cellulose in plants. Hence, it has attracted considerable attention as a natural resource for aromatic compounds such as vanillin and catechol. Depolymerization of lignin by fungi[1)] has been studied. The eventual breakdown products are simple aromatic compounds, which can be metabolized by bacteria through aromatic hydrocarbon degradation pathways. Recently, we isolated *Streptomyces* sp. NL15-2K from forest soil, which degrades veratric acid *via* vanillic acid. Liquid chromatography–mass spectrometry (LC–MS) analysis of the catabolic intermediates of lignin-related aromatic compounds indicated that this strain has a broad catabolic network for these compounds.[2)] In this study, cloning of the gene for vanillate demethylase (VDMase) from *Streptomyces* sp. NL15-2K was conducted to obtain information on the demethylation of veratric acid to vanillic acid.

Vanillate demethylase (EC 1.14.13.82) is a two-component monooxygenase (class I) responsible for demethylation of vanillic acid and veratric acid (Fig. 1). The *van*A and *van*B genes encoding the subunits of this enzyme have been cloned and sequenced from *Pseudomonas*[3–6)] and *Acinetobacter*[7)] and, most recently, *Corynebacterium*.[8)] The *van*A and *van*B genes have been identified as the cistrons for the terminal oxygenase subunit (VanA) containing a conserved Rieske-type [2Fe-2S] domain and the ferredoxin-type reductase subunit (VanB), respectively. Alignment of the deduced amino acid sequences of VanB proteins showed two major conserved regions, an NAD-ribose-binding domain and an iron-sulfur binding domain (Fig. 2A). These domains were also found in an iron-sulfur oxidoreductase $\beta$-subunit from *Streptomyces coelicolor* A3(2). Hence, from their amino acid sequences, AGGIGITP and GTCGTCET, the PCR primers, 5′-GC(C/G)GG(C/G)GG(C/G)ATCGG(C/G)ATCAC(C/G)CC-3′ and 5′-GTCTCGCA(C/G)GT(C/G)CCGCA(C/G)GT(C/G)CC-3′, were synthesized for probe preparation. An approximately 490-bp fragment amplified with the primers using genomic DNA from NL15-2K as a template was labeled with fluorescein-11-dUTP using a Gene Images Random-Prime Labelling Module (Amersham Biosciences, Piscataway, NJ). The resulting probe hybridized to a Southern blot of the *Sac*I-digested (11.5 kb), *Kpn*I-digested (7.6 kb), and *Bgl*II-digested (4.3 kb) NL15-2K DNA. The genomic DNA fragments of NL15-2K digested with *Sac*I were ligated into $\lambda$DASH II phage DNA (Stratagene, La Jolla, CA), digested with *Sac*I and dephosphorylated with thermosensitive alkaline phosphatase. The construct was

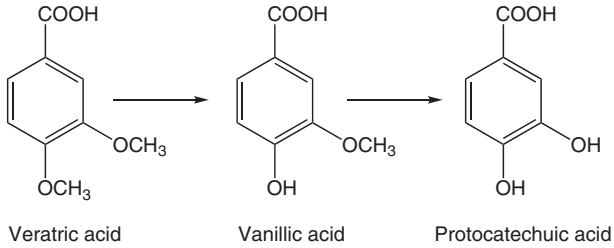


**Fig. 1.** Demethylation of Veratric Acid and Vanillic Acid by Vanillate Demethylase.

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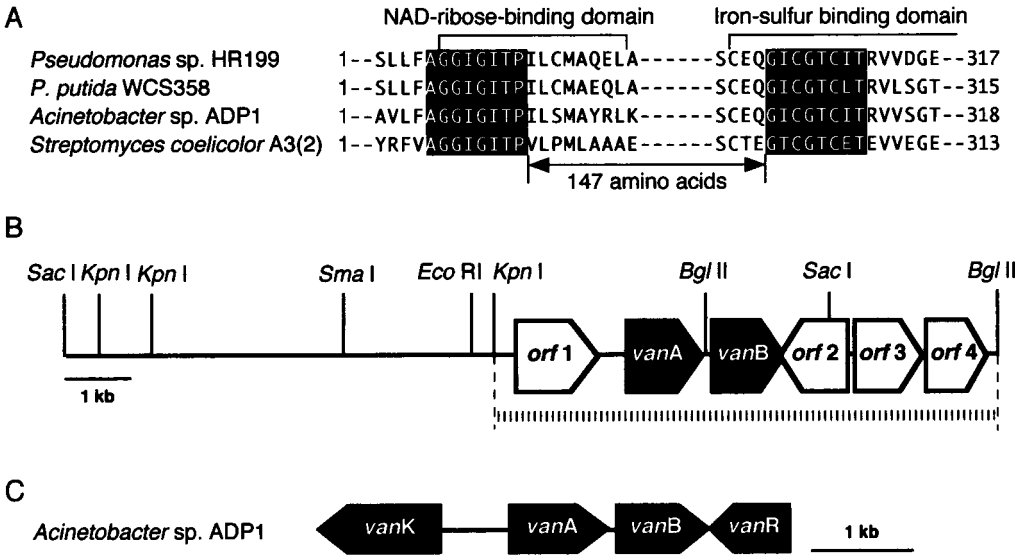


**Fig. 2.** Conserved Sequence Motifs of VanB Proteins (A), Restriction Map of the Cloned 14.3-kb Fragment from *Streptomyces* sp. NL15-2K (B), and Organization of the Genes Involved in Vanillic Acid Demethylation in *Acinetobacter* sp. ADP1 (C).

A, PCR primers were designed from two conserved sequences (shaded), and used for preparation of a probe for detection of the streptomycete vanillate demethylase gene. B and C, The hatched line indicates the 7,532-bp region sequenced in this study. The pentagon indicates the size and orientation of the open reading frame.

packaged *in vitro* with Gigapack III Gold Packaging Extract (Stratagene). *Escherichia coli* XL-1 Blue MRA(P2) (Stratagene) was infected with the packaging products to generate a genomic library of NL15-2K. In addition, another genomic library of *Bgl*II-digests, prepared in the pUC118 plasmid vector (Takara Bio, Otsu, Japan), was constructed to obtain clones containing the flanking regions of the *van*AB genes from NL15-2K. An 11.5-kb *Sac*I insert and a 4.3-kb *Bgl*II insert were obtained from the positive clones by plaque hybridization and colony hybridization with the PCR fragment probe, respectively. Restriction enzyme mapping of these inserts indicated that the putative *van*B gene was located on a 1.8-kb *Bgl*II-*Sac*I overlap between the inserts (Fig. 2B). The 7.5-kb *Kpn*I-*Bgl*II region in the 14.3-kb *Sac*I-*Bgl*II DNA stretch of the combined inserts was sequenced by the dideoxy chain termination method[9] using a Dye Primer Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an ABI377 DNA sequencer (Applied Biosystems). The sequence data and the deduced amino acid sequences have been deposited in the DDBJ database (accession no. AB252870).

Sequence analysis revealed that the 7.5-kb *Kpn*I-*Bgl*II region contained six putative open reading frames (ORFs), of which two genes were identified, as shown in Fig. 2B. The *van*A was an ORF encoding a protein of 356 amino acids. The deduced amino acid sequence showed 87.9% identity to an oxidoreductase $\alpha$-subunit from *S. coelicolor* A3(2) (accession no. O88036), 34% identity to VanA from *Corynebacterium*,[8] 31 to 33% identity to four VanA proteins from pseudomonads,[3–6] and 30% identity to VanA from *Acinetobacter*.[7] The consensus regions for the Rieske-type [2Fe-2S] domain[10] and the mononuclear iron (II)-binding domain[10] of the class IA terminal oxygenase components were present in the amino acid sequence of *Streptomyces* VanA at positions $^{53}CXHX_{18}CX_2H^{77}$ and $^{162}DX_2HX_4H^{170}$, respectively. The *van*B was an ORF encoding a protein of 311 amino acids. The deduced amino acid sequence showed 74.9% identity to an iron-sulfur oxidoreductase $\beta$-subunit from *S. coelicolor* A3(2) (accession no. O88034), whose amino acid sequence was used for the design of the primers described above, 31 to 37% identity to four VanB proteins from pseudomonads,[3–6] 28% identity to VanB from *Acinetobacter*,[7] and 26% identity to VanB from *Corynebacterium*.[8] The consensus regions for the NAD-ribose-binding domain[10] and the ferredoxin [2Fe-2S]-binding domain[10] were found in the amino acid sequence of *Streptomyces* VanB at positions $^{115}GGIGITPX_3MX_3(A)^{129}$ and $^{260}CX_2GXCGXCXTX_4$-$GX_3HRDX_2LX_{13}C^{298}$, respectively. The consensus sequence for the FMN isoalloxazine-binding domain,[10] $^{52}RXYSL^{56}$, was also present in the N-terminal half of VanB. These three consensus sequences and their arrangement were conserved in the reductase components of the class IA oxygenases. Immediately downstream of the *van*B gene there was *orf*2, which encoded a putative protein of 283 amino acids. The deduced amino acid sequence showed 54.7 and 36% identity to PcaR, a regulatory protein of the $\beta$-ketoadipate pathway, from *Rhodococcus opacus* 1CP[11] and *P. putida* WCS358,[12] respectively. As shown in Fig. 2B, *orf*2 and *van*B genes were oriented convergently, and the 3′ ends of their coding regions overlapped by 86 bp. This

overlapping arrangement of genes was also observed between *van*B and *van*R, which encodes a repressor of *van*AB expression, from *Acinetobacter* sp. ADP1 (Fig. 2C).[13] Although the function of this organization is unknown, we speculate that it might play an additional role in the regulation of the expression of *van*AB, since elevated transcription of one of the two might impair the expression of the other. No significant homology was detected between the amino acid sequences of *orf*2 and *van*R (12% identity). The organization of *van*AB and *van*R in *Acinetobacter* was also found in *Pseudomonas* species.[7] Hairpin structures containing stems composed of 14 nucleotides were found 82 bp and 85 bp downstream of the stop codons (TGA) of *van*B and *orf*2. Their structures had free energies[14] of −13.8 kcal/mol and −15.9 kcal/mol, respectively. No significant hairpin structure was found between *van*A and *van*B. In addition, a putative promoter sequence, containing a −35 region (TTGCGC; nucleotide positions 2007 to 2012) and a −10 region (CAGGA; nucleotide positions 2028 to 2032) similar to the streptomycete consensus promoter sequence,[15] was found about 61 bp upstream of the possible start codon (ATG at nucleotide position 2094) in *van*A. These results suggest that *van*A and *van*B might be organized as an operon in NL15-2K, as with other *van*AB operons.[3–7] In the flanking regions of the streptomycete *van*AB and *orf*2, there were three additional ORFs that had the same transcriptional orientation as *van*AB. The first ORF (*orf*1) was 1,284 bp in length and was located 599-bp upstream of *van*A, and the predicted protein from this ORF exhibited 75% identity to a putative integral membrane protein from *S. coelicolor* A3(2) (accession no. Q9F2S7), belonging to the major facilitator superfamily (MFS) of transporters. Similarly, *van*K, which is located divergently upstream of the *van*AB operon of *Acinetobacter* sp. ADP1 (Fig. 2C), is also a member of the MFS transporter family.[7] *Orf*3 was 969 bp in size and was located immediately upstream of *orf*2, and the predicted protein showed 43% identity to a putative extracellular solute-binding protein from *Arthrobacter* sp. FB24 (accession no. Q4NH63). *Orf*4 (771 bp) was predicted to code for a protein that shared 58.4% identity to a binding-protein-dependent transport system inner membrane component from *Arthrobacter* sp. FB24 (accession no. Q4NH64).

To confirm that the *van*AB genes from *Streptomyces* sp. NL15-2K encode VDMase, expression of the *van*AB was performed using *S. lividans* 1326, which is incapable of demethylating veratric acid to vanillic acid, as a host strain. For this purpose, the *Kpn*I-*Sac*I fragment containing the *orf*1 and *van*AB genes was inserted into the high-copy-number vector pIJ702[16] at the *Kpn*I/*Sst*I site to generate pVANAB. The transformant strains carrying pVANAB or pIJ702 were grown at 30 °C in YEME medium[16] (100 ml) for approximately 36 h, at which time the cells were centrifuged, washed twice

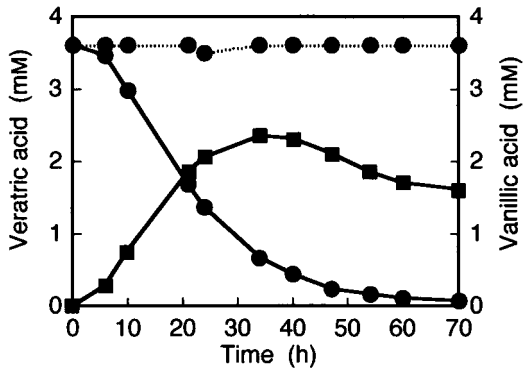


**Fig. 3.** Demethylation of Veratric Acid (solid circle) to Vanillic Acid (solid square) by Recombinant *S. lividans* 1326 Strain Carrying pVANAB (solid line) or pIJ702 Plasmid (dotted line).

The culture was performed at 30 °C in MSMYE medium containing 3.6 mM veratric acid, and the inoculum size was 1–1.5% packed cell volume. Production of vanillic acid was confirmed with an LC–MS system (LCMS-2010; Shimadzu, Kyoto, Japan) on a Shim-pack VP-ODS column (2.0 × 150 mm; Shimadzu) with 30% methanol containing 0.1% acetic acid at a flow rate of 0.2 ml/min at 40 °C. The operation parameters of MS spectrometry were as follows: probe voltage, −3.5 kV; nebulizer gas flow, 4.5 l/min; curved desolvation line (CDL) temperature, 250 °C. The concentrations of the residual veratric acid and its degraded products were measured with an HPLC system (Shimadzu) on a TSK-GEL ODS-80Ts column (4.6 × 250 mm; Tosoh, Tokyo).

with MSMYE medium (0.1 g of $(NH_4)_2SO_4$, 0.1 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2$, 1.0 g of $K_2HPO_4$, 0.5 g of $KH_2PO_4$, and 0.5 g of yeast extract per liter, pH 7.2), and then resuspended in 5 ml of MSMYE. The seed suspension (4 ml) was transferred into 46 ml of MSMYE containing 3.9 mM of veratric acid in a 300-ml baffled flask, and grown at 30 °C on a rotary shaker (180 rpm). Cultivation of the transformed cells was carried out in the presence of thiostrepton (25 μg/ml). The culture fluids were sampled at appropriate times and subjected to HPLC or LC–MS analysis. Figure 3 shows the culture profiles of *S. lividans* 1326 strains carrying pVANAB or pIJ702. In the culture of the cells harboring pVANAB, veratric acid was gradually degraded, releasing vanillic acid as its demethylated product, and almost completely disappeared in 70 h. No accumulation of protocatechuic acid, the demethylation product of vanillic acid, was observed in the culture medium, although vanillic acid was degraded gradually; the same was found during the cultivation of *Streptomyces* sp. NL15-2K.[2] On the other hand, the cells harboring the parental vector (pIJ702) did not demethylate veratric acid. Since the recombinant plasmid pVANAB was constructed by replacing the *Kpn*I-*Sst*I region containing the tyrosinase (*mel*) promoter in pIJ702 with the 5-kb *Kpn*I-*Sac*I fragment containing *orf*1 and *van*AB, the transcription of *van*AB in *S. lividans* 1326 carrying pVANAB was probably driven by the putative promoter of *van*AB, described above. It is unlikely that the putative protein from *orf*1 converted veratric acid to vanillic acid, because its amino acid sequence shares

75% identity to a membrane protein from *S. coelicolor* A3(2) and lacks any conserved domain responsible for the demethylation of aromatic methyl ethers.

In this paper, we describe the first molecular genetic study of vanillate demethylase from a streptomycete. To our knowledge, this enzyme, encoded by *van*AB genes, has not previously been purified and characterized. This might be due to the fact that vanillate demethylase is oxygen-sensitive and unstable, so that direct measurement of the enzyme activity in cell-free extracts is difficult to achieve. Our next goal is to elucidate the characteristics of vanillate demethylase using the enzyme produced by overexpression of the *van*AB genes from *Streptomyces* sp. NL15-2K.

## Acknowledgments

This work was funded by the Natural Sciences and Engineering Research Council of Canada (NSERC). Part of this work was also supported by a Grant-in-Aid for Exploratory Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (no. 12875159). We thank Rumi Asano for technical support throughout this study.

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