Molecular Cloning, Sequencing, and Heterologous Expression of the vaoA Gene from Penicillium simplicissimum CBS 170.90 Encoding Vanillyl-Alcohol Oxidase

Benen, Jacques A.E.; Sánchez-Torres, Paloma; Wagemaker, Matthé J.M.; Fraaije, Marco; Berkel, Willem J.H. van; Visser, Jaap

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The cDNA encoding vanillyl-alcohol oxidase (EC 1.1.3.7) was selected from a cDNA library constructed from mRNA isolated from Penicillium simplicissimum CBS 170.90 grown on veratryl alcohol by immunochromatography screening. The vao cDNA nucleotide sequence revealed an open reading frame of 1680 base pairs encoding a 560-amino acid protein with a deduced mass of 62,915 Da excluding the covalently bound FAD. The deduced primary structure shares 31% sequence identity with the 8a-(O-tyrosyl)-FAD containing subunit of the bacterial flavocytochrome p-cresol methyl hydroxylase.

The vao gene was isolated from a P. simplicissimum genomic library constructed in λEMBL3 using the vao cDNA as a probe. Comparison of the nucleotide sequence of the vao gene with the cDNA nucleotide sequence demonstrated that the gene is interrupted by five short introns.

Aspergillus niger NW156 prf2 pyrA leuA cspA transformed with the pyrA containing plasmid and a plasmid harboring the complete vao gene including the promoter and terminator was able to produce vao mRNA and active vanillyl-alcohol oxidase when grown on veratryl alcohol and anisyl alcohol. A similar induction of the vao gene was found for P. simplicissimum, indicating that similar regulatory systems are involved in the induction of the vao gene in these fungi.

Introduction of a consensus ribosome binding site, AGAAGGAG, in the vao cDNA resulted in elevated expression levels of active vanillyl-alcohol oxidase from the lac promoter in Escherichia coli TG2. The catalytic and spectral properties of the purified recombinant enzyme were indistinguishable from the native enzyme.

Vanillyl-alcohol oxidase (EC 1.1.3.7) is a novel type of flavoprotein oxidase that was first isolated from Penicillium simplicissimum CBS 170.90 grown on veratryl alcohol (1). The enzyme is a homo-octamer with each 65-kDa subunit harboring an 8a-(N3-histidyl)-FAD (2). Vanillyl-alcohol oxidase has a broad substrate specificity. In addition to the conversion of vanillyl alcohol to vanillin (Equation 1), the enzyme catalyzes the conversion of a wide range of phenolic compounds bearing side chains of variable size at the para-position of the aromatic ring (3, 4). Due to its broad substrate spectrum, vanillyl-alcohol oxidase may be applied in the fine chemical industry (5). Based on induction experiments, 4-(methoxymethyl)phenol has been proposed to represent the physiological substrate (6). Recently, from rapid reaction kinetics conclusive spectral evidence was obtained that the vanillyl-alcohol oxidase-mediated oxidative demethylation of 4-(methoxymethyl)phenol proceeds through the formation of a quinone-methide product intermediate (4). In the absence of oxygen, this intermediate is stabilized in the active site of the reduced enzyme. Upon flavin reoxidation, the quinone methide of 4-(methoxymethyl)phenol readily reacts with water, yielding 4-hydroxybenzaldehyde, methanol, and hydrogen peroxide as final products.

Recently, the three-dimensional structure of vanillyl-alcohol oxidase was elucidated (7). The crystallographic analysis corroborated earlier observations (2, 8) that the vanillyl-alcohol oxidase octamer can be described as a tetramer of tightly interacting dimers. Each vanillyl-alcohol oxidase monomer consists of two domains. The larger domain creates a binding site for the ADP moiety of the FAD, whereas the smaller cap domain covers the active site that is located between the two domains. Furthermore, from the structures of several vanillyl-alcohol oxidase inhibitor complexes, it could be deduced that the shape of the active site cavity controls substrate specificity (7).

In this paper we describe the cloning, sequencing, and expression of the gene encoding vanillyl-alcohol oxidase from P. simplicissimum CBS 170.90, providing the necessary amino acid sequence information which together with the three-dimensional structure establishes the basis for future protein engineering studies.

**EXPERIMENTAL PROCEDURES**

**Strains and Media—**Growth conditions for P. simplicissimum (CBS 170.90; ATCC 90172) have been described before (6). Aspergillus niger NW156 (pyF28, leuA6, pyrA1, cspA1) a derivative of A. niger N400 (CBS 120.49) from the laboratory collection was used for transformation and was grown in minimal medium (9), pH 6.0, containing 1% (mass/volume) fructose, 0.1% (mass/volume) yeast extract, 0.02% (mass/volume) leucine, 0.02% (mass/volume) uridine, and a trace element solution according to Ref. 10. Escherichia coli DH5α F’ [pλendA1 hsdR17 (rK mK) supE44 thi-1 recA1 gyrA (Nal+) relA1 ΔlacIZY-argF] U169 deoR (Δ80lacΔIacZΔM15) (11) was used for cloning throughout. λ phages were propagated in E. coli LE392 [F’ e44 (McrA-) hasR514 (rK mK) supE44 supF58 ΔlacIZY6 galK2 galT22 metB1 trpR55] (12), E. coli BB4 [F’ lacFΔZM15 proAB Tn10 (tetR)] and
poly(A) tail mRNA, isolated from *P. simplicissimum* using an *E. coli* Immunoscreen Kit instruction manual (Stratagene) using oxidase, detection limit less than 10 pg (15), as described in the manual supplied with the cDNA Kit. The cDNA library was screened with purified antibodies raised against vanillyl alcohol oxidase. *E. coli* strains were grown in Luria-Bertani medium supplemented with the appropriate antibiotic with the exception of *E. coli* LE392 which was grown in NZCYM medium. Media were solidified using 1.5% (mass/volume) bacto-agar. Top agar was prepared using NZCYM medium solidified with 0.7% agarose (electrophoresis grade).

Preparation of Cell Extracts—Cell extracts were prepared by sonication as described (6).

Enzyme Activity—Vanillyl-alcohol oxidase activity was determined at 25 °C in 50 mM potassium phosphate, 1 mM vanillyl alcohol, pH 7.5, by measuring the production of vanillin at 340 nm (16).

Protein Sequence Analysis—Protein sequence analysis of vanillyl-alcohol oxidase was performed by Edman degradation at Eurosequence, Groningen, The Netherlands.

Manipulation of DNA—Isolation of plasmid and cDNA DNA and other molecular manipulations were carried out essentially as described (12). *A. niger* and *P. simplicissimum* chromosomal DNA was isolated according to de Graaff et al. (14). Restriction enzymes were used as recommended by the supplier (Life Technologies, Inc., Gaithersburg, MD).

The nucleotide sequence was determined using either the Cy5™ AutoCycle™ Sequencing Kit (Pharmacia Biotech, Uppsala, Sweden) or the Cy5™-dATP Labeling Mix (Pharmacia Biotech). The reactions were analyzed with an ALFRed™ DNA Sequencer. Computer analysis was done using the program DNAStar (Madison, WI).

Isolation of RNA—Total RNA was isolated by grinding frozen mycelium in liquid nitrogen-cooled shake flasks and grinding balls using a mini dismembrator (Braun Melsungen AG, Melsungen, Germany). Ground mycelium was extracted with TRIzol™ Reagent according to the suppliers recommendations (Stratagene Cloning Systems, La Jolla, CA). *E. coli* TG2 (Δlacr-pro) thi supE recA (Res’ Mod’-kl) F’ (trd368 proA B’ lacZAM15) (13) was used for expression of vanillyl-alcohol oxidase.

Ground mycelium was extracted with TRIzol™ Reagent according to the instructions of the manufacturer and rehydrated with a 900-μl EcoRI fragment encoding part of the 3’ end of the 28 S rDNA of *Agaricus bisporus* (18) to provide a loading control.

Western Analysis—Western blot analysis was done as described previously (6) using the same antibodies as used for the cDNA library screening. Bound antibodies were detected by an alkaline phosphatase based immunassay.

UV Visualization of Vanillyl-Alcohol Oxidase—Vanillary-alcohol oxidase was visualized after SDS-PAGE as described (6).

PCR Mutagenesis to Introduce a Shine-Dalgarno Sequence—To enhance the expression of the *vao*-cDNA a consensus Shine-Dalgarno sequence was introduced via PCR mutagenesis. This was done using the *E. coli* harboring plasmid pM3970, taking advantage of the XbaI site at position 451 (numbering according to Fig. 1) and the KpnI site from the polymerase of the vector pBlueScript SK downstream of the *vao*-cDNA. Two oligonucleotides were used, primer 1, a 51-mer with the following sequence: 5’ GGCGGACGTCGTGTTAGAAAGGATATACAATGTCCAGACACGACAGAGA3’ and primer 2, a 17-mer with the sequence 5’ CGAGAATTCTGTCGCTCC3’. Primer 1 is a mutagenic oligonucleotide. The sequence shown in boldface is identical to the N-terminal coding sequence, and the italicized sequence represents the Shine-Dalgarno sequence. Primer 2 is complementary to the sequence from positions 587 to 603 in Fig. 1. The fragment was amplified in *E. coli* TG1 cells using the primers and conditions described under “Results.” At regular intervals mycelia were harvested and processed for RNA extraction or preparation of cell extract as described above.

**Northern and Southern Analysis**—Northern and Southern blots were carried out as described previously (12). [32P]dATP-labeled *vao*-cDNA was used as a probe (see above). The membranes were stripped according to the instructions of the manufacturer and rehybridized with a 900-μl EcoRI fragment encoding part of the 3’ end of the 28 S rDNA of *Agaricus bisporus* (18) to provide a loading control.

**Ura**—Mutants of *P. simplicissimum* were selected on minimal medium plates supplemented with uracil at 0.5 mg/l.

**Xeast**—Transformation of *P. simplicissimum* was performed by the electroporation method as described previously (16). Mycelia were harvested from plates and suspended in 100 μl of transformation buffer (100 mM CaCl2, 10 mM Tris, pH 7.5) by pipetting and vortexing. The suspension was placed in an electroporation cuvette (1.5 ml capacity) and 20 μl of the mycelial suspension was added. The cuvette was placed in a medium containing 1 μg of the plasmid pIM3970, followed by electroporation (500 V, 20 μF, 1.5 kOhm) and the mycelia were plated on minimal medium plates supplemented with uracil at 0.5 mg/l.

**Xcall**—A single colony was inoculated into 50 ml of minimal medium containing 0.5 mg/l uracil and cultures were grown at 30 °C on a rotary shaker (225 rpm) to an *A*. *optical density* of 0.5. Twenty microliters of the culture were streaked onto minimal plates containing 0.5 mg/l uracil and grown at 30 °C for 100 h. The plates were then treated with the restriction enzyme indicated (see Table 1) and transformed with 200 ng of plasmid DNA prepared as described (16). The plates were incubated at 30 °C for 3 days, after which the *P. simplicissimum* colonies were isolated and grown on minimal medium plates.

**Xact**—A single colony was used to inoculate 50 ml of minimal medium containing 0.5 mg/l uracil and 100 μg/ml acriflavine. Cultures were grown at 30 °C on a rotary shaker (225 rpm) to an *A*. *optical density* of 0.5. Twenty microliters of the culture were streaked onto minimal plates containing 0.5 mg/l uracil and grown at 30 °C for 2 days. The plates were then treated with the restriction enzyme indicated (see Table 1) and transformed with 200 ng of plasmid DNA prepared as described (16). The plates were incubated at 30 °C for 3 days, after which the *P. simplicissimum* colonies were isolated and grown on minimal medium plates.

**Xpro**—A single colony was used to inoculate 50 ml of minimal medium containing 0.5 mg/l uracil, 50 μg/ml acriflavine and 50 μg/ml cephalosporin C. Cultures were grown at 30 °C on a rotary shaker (225 rpm) to an *A*. *optical density* of 0.5. Twenty microliters of the culture were streaked onto minimal plates containing 0.5 mg/l uracil and grown at 30 °C for 2 days. The plates were then treated with the restriction enzyme indicated (see Table 1) and transformed with 200 ng of plasmid DNA prepared as described (16). The plates were incubated at 30 °C for 3 days, after which the *P. simplicissimum* colonies were isolated and grown on minimal medium plates.
LB medium supplemented with 80 μg/ml ampicillin and 0.1 mM isopro- 
pyl-l-thio-β-μ-galactopyranoside in 2-liter flasks in a rotary shaker 
set at 250 rpm at 37 °C. From 5-liter batch cultures cells were harvested by 
centrifugation and resuspended in 55 ml of 50 mM potassium phosphate 
buffer, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 0.5 
mM EDTA, 0.5 mM MgSO4, 10 mg of DNase I, pH 7.0. Cells were 
disrupted by passing three times through a precooled French pressure 
cell press operated at 10,000 p.s.i. Following centrifugation for 15 min 
at 15,000 × g to remove cellular debris, the supernatant was made 0.5% 
in protamine sulfate from a 2% mass/volume stock solution. Subse-
quenty, the protamine sulfate aggregates were precipitated by centri-
figuration for 15 min at 15,000 × g followed by adjustment of the super-
natant to 25% ammonium sulfate saturation. After centrifugation for 15 
min at 15,000 × g, the supernatant was adjusted to 65% ammonium 
sulfate saturation. The precipitate was collected by centrifugation at 
identical settings as before. The pellet was dissolved in 50 mM potas-
sium phosphate buffer, 0.5 mM EDTA, 0.5 mM ammonium sulfate, pH 7.0, 
and loaded onto a phenyl-Sepharose column (30 × 2.6 cm) pre-equi-
librated in the same buffer. The enzyme was eluted with a linear de-
scending gradient from 0.5 to 0 mM ammonium sulfate in the same buffer. 
Fractions were assayed for vanillyl-alcohol oxidase activity, pooled, and 
dialyzed against 25 mM potassium phosphate, pH 7.0. Next the dialy-
sate was loaded onto a hydroxyapatite column (30 × 2.6 cm) pre-
equilibrated with 25 mM potassium phosphate, pH 7.0. After washing 
the column with 3 volumes of starting buffer, the enzyme was eluted 
with a linear gradient of 25–300 mM potassium phosphate, pH 7.0. Fractions 
containing vanillyl-alcohol oxidase were pooled and concen-
trated in an Amicon ultrafiltration unit equipped with a YM-30 
membrane.

RESULTS

Construction of the cDNA Library, Selection, and Sequence of the vaoA-cDNA—To ensure high abundance of the vanillyl-

alcohol oxidase mRNA, total RNA was isolated from P. simplicissimum after 54 h of growth on minimal medium containing 

veratryl alcohol when vanillyl-alcohol oxidase activity was at 

75% of its maximum (not shown). The primary library of 7·10⁶ 

plaque-forming units was amplified to a titer of 1.4·10⁹ plaque-

forming units/ml. The first screening of the amplified library 

revealed that approximately 4.5% of the phages reacted with 

the vanillyl-alcohol oxidase-specific antibodies. From the sec-

ond screening five positive phages were selected, and the pha-
gemid was excised. Restriction enzyme analysis showed that 

the five phagemids contained identical inserts. From two such 

phagemids the nucleotide sequence of the insert was deter-

mined over both strands by sequencing subclones and by the 

use of specific oligonucleotides. The vaoA-cDNA sequence and the 
derived amino acid sequence are presented in Fig. 1 to-

gether with the complete gene (see below). The vaoA-cDNA 
nucleotide sequence revealed an open reading frame of 1680 bp 
encoding a protein of 560 amino acids with a deduced mass of 
62,915 Da excluding FAD. Amino acid sequences obtained by 
automated Edman degradation of the N terminus of purified 
vanillyl-alcohol oxidase and of a purified internal peptide ob-
tained by tryptic digestion of the enzyme were both identified in the 
deduced primary amino acid sequence (bold type in Fig. 1). 
The nucleotide sequence revealed that the first five amino 
acids were missing from the N-terminal peptide sequence sug-

suggesting some limited proteolytic processing of the enzyme in P. simplicissimum.

Cloning and Sequencing of the vaoA Gene.—By using meth-

ods and conditions described under “Experimental Procedures” a 
genomic library of P. simplicissimum was constructed and 
subsequently screened with the [32P]dATP-labeled vaoA-cDNA as a probe. Two unsubcloned single colonies were 
selected from the positive colonies that were characterized by restriction enzyme analysis and 
Southern blot analysis (results not shown). It was con-

cluded that the entire gene should be located on an 8-kilobase 
pair Sall fragment which was subsequently subcloned into 
Sall-digested pUC18 yielding pLM3971. pLM3971 was used for 
sequencing either using specific oligonucleotides based on the vaoA-cDNA sequence and on sequences from the gene or by 
subclones generated. Fig. 1 shows the complete vaoA gene, sequenced over both strands, including 582-bp promoter se-

and 293 bp of downstream sequence.

Comparison of the cDNA sequence with the genomic se-

quence revealed that the coding region is interrupted by five 
introns. The intron sequences follow the rules for filamentous 
fungi as proposed earlier (19): they are short, 61, 60, 70, 52, and 
75 bp for introns i, ii, iii, iv and v, respectively, and the introns 
have consensus 5’ and 3’ splice sites, GTPuNGPy and PyAG, 
respectively, and lariat sequences, NNCTPuAPy (where Pu 
indicates purine and Py indicates pyrimidine), with only slight 
deviations.

The promoter region was analyzed for the presence of se-

quences involved in transcription regulation. The sequences 5’ 
GATA 3’ and 5’ GCCARG 3’ involved in nitrogen (20) and pH 
regulation (21), respectively, were not present. The context 

independent CreA binding site of A. nidulans 5’ GYGGGG 3’ 
(22) which is probably also recognized in Penicillium (23) was 
found once (position −557 to −552 in Fig. 1). The 5’ CAAT 3’ 
sequence, shown to be involved in transcription activation in 
Saccharomyces and other fungi (24), was found at positions 
−38, −61, −112, and −373 (lowercase italic letters). No TATA 
box was found in the sequence immediately upstream of the 
start codon. However, CT-rich sequences thought to direct 
transcription initiation (19) were present.

Vanillyl-Alcohol Oxidase Induction in P. simplicissimum and A. niger—Earlier studies have shown that vaoA expression is 
gratuitously induced in P. simplicissimum when grown on 

veratryl alcohol (1) or anisyl alcohol (6). We have readdressed 
this issue in a transfer experiment in which veratryl alcohol 
was used as the sole source of carbon. Analysis was carried out 
by Northern and Western blotting and by vanillyl-alcohol oxy-
dase activity determinations. In Fig. 2 the time course of in-
duction is presented. Panel A shows the Northern blot probed 
with [32P]-labeled vaoA-cDNA. In panels B and C the Western 
blot probed with the vanillyl-alcohol oxidase antibodies and the 
relative vanillyl-alcohol oxidase activity are presented, respec-

A. niger NW156-T10, a pLM3971 multicopy transformant 
harboring 25–30 vaoA copies, was used to study vanillyl-alco-
hol oxidase expression. Southern analysis using the 

vaoA-cDNA as a probe demonstrated that A. niger NW156 does 
not contain a vaoA gene with sufficient homology with the P. simplicissimum vaoA gene to be detected under the conditions 

applied. In transfer experiments with A. niger NW156-T10 the 
inducing properties of veratryl alcohol, anisyl alcohol, vanillyl 
alcohol, veratric acid, vanillic acid, and 4-hydroxybenzoic acid were assessed 3 h after transfer (see Table I and Fig. 3). The 

highest level of vanillyl-alcohol oxidase activity was obtained 
with veratryl and anisyl alcohol, whereas no activity was seen 
when A. niger NW156-T10 was grown on fructose and when an 
untransformed A. niger strain was transferred to veratryl or 
anisyl alcohol (not shown). Both 4-methoxybenzyl alcohol 
was used alone and in combination with fructose to study the
time course of vanillyl-alcohol oxidase induction (results not shown). With anisyl alcohol already after 3 h strong induction was observed both by Northern and Western analysis, whereas after 6 h the induction decreased. When fructose was included the induction was weaker both at 3 and 6 h. With veratryl alcohol induction was retarded when compared with anisyl alcohol. Fructose alone or a mixture of veratryl alcohol and fructose resulted in no detectable vanillyl-alcohol oxidase A-mRNA, and no vanillyl-alcohol oxidase was found by Western analysis.

Expression of vanA-cDNA in E. coli TG2 and Purification of Recombinant Vanillyl-Alcohol Oxidase—Although vanillyl-alcohol oxidase could easily be detected with the vanillyl-alcohol oxidase-specific antibodies during screening of the cDNA library, the expression of the gene was quite low in E. coli (less than 0.5% of total protein, based on the specific activity of vanillyl-alcohol oxidase). In addition to the codon usage (see “Discussion”), the apparent lack of a good ribosome binding site may be the cause of this. To enhance the expression level of vanillyl-alcohol oxidase, a consensus Shine-Dalgarno sequence was introduced via PCR mutagenesis as described under “Experimental Procedures.” Cell extracts of E. coli TG2 harboring pIM3972 showed a 7-fold increase of expression of vanillyl-alcohol oxidase when compared with E. coli TG2 transformed with pIM3970.

Recombinant vanillyl-alcohol oxidase was purified from E. coli TG2 harboring pIM3972 in a two-column procedure (Table II). The recombinant enzyme migrated as a single band in SDS-PAGE (Fig. 4) and was identical with vanillyl-alcohol oxidase.
oxidase from *P. simplicissimum* (1) in all aspects tested: spectral properties (250–520 nm), steady state kinetic parameters for vanillyl alcohol, and the association into octamers.

**DISCUSSION**

In this paper we have described the cloning and sequencing of the gene encoding vanillyl-alcohol oxidase from *P. simplicissimum*, the first 8a-(N²-histidyl)-FAD containing enzyme of known three-dimensional structure (7). The gene is strongly induced in *P. simplicissimum* when the fungus is grown on 4-methoxybenzyl alcohols (6). This high amount of enzyme is conserved. In vanillyl-alcohol oxidase, the 8 valently bound FAD, but the mode of covalent linkage is not oxidase and alcohol oxidase, although at a lower rate (8). Vanillyl-alcohol oxidase from *P. simplicissimum*, the first 8a-carbon of the flavin is bound to the phenolic oxygen of Tyr-384 (25). Furthermore, as can be seen from Fig. 5, Tyr-384 of *p*-cresol methylhydroxylase is shifted 8 residues toward the N terminus compared with His-422 of vanillyl-alcohol oxidase.

The crystal structure of vanillyl-alcohol oxidase shows that each monomer is composed of two domains (7). The larger domain (residues 6–270 and 500–560) binds the ADP part of each monomer is composed of two domains (7). The larger domain (residues 6–270 and 500–560) binds the ADP part of the enzyme subunit of the flavoprotein oxidoreductase subunit closely resembles that of the flavoprotein subunit of *p*-cresol methylhydroxylase (27, 28). From this and the data presented in Fig. 5, it is clear that the most conserved parts of the sequence (101–147, 178–219, and 245–271) concern residues that are located in the FAD binding domain. Several active site residues are also conserved. These include Tyr-108, Tyr-503, and Arg-504 that are involved in binding the phenolic moiety of the substrate. In vanillyl-alcohol oxidase, these residues facilitate substrate deprotonation upon binding (3). Asp-170, which is thought to play a crucial role in the catalytic mechanism of vanillyl-alcohol oxidase (7), is not conserved (Fig. 5). This might explain the different reactivities of both enzymes toward *p*-cresol (8). However, a detailed comparison of the active sites of vanillyl-alcohol oxidase and *p*-cresol methylhydroxylase requires the completion of the crystallographic refinement of the *p*-cresol methylhydroxylase structure. Besides the 31% sequence identity with *p*-cresol methylhydroxylase, no strong sequence identity was found between vanillyl-alcohol oxidase and other enzymes. However, from the crystal structure determination (7), it has become apparent that the folding topology of the FAD-binding domain of vanillyl-alcohol oxidase resembles that of MurB, a flavoenzyme involved in the biosynthesis of the bacterial cell wall (29). Moreover, it has been suggested that this unusual FAD-binding fold is shared by other flavoprotein oxidoreductases (30, 31).

In *P. simplicissimum* the vao gene is induced by a limited amount of aromatic compounds (6). Apart from 4-methoxymethylphenol, which may represent the natural substrate, the non-vanillyl-alcohol oxidase substrates anisyl alcohol and veratryl alcohol are potent inducers. Also in *A. niger* NW156-T10,
transformed with 25–30 copies of the vao gene, strong expression of the gene was observed with these methoxybenzyl alcohols. With 4-hydroxybenzoic acid, ferulic acid, vanillic acid, and vanillyl alcohol vanillyl-alcohol oxidase was detected as well. This indicates that the vaoA gene in A. niger NW156-T10 is rendered under the control of at least one regulator involved in regulation of genes involved in the metabolism of aromatic compounds. Furthermore, the vaoA gene is both in A. niger NW156-T10 and P. simplicissimum under the control of carbon catabolite repression. Since the vaoA gene is expressed from its own promoter, this means that both in A. niger NW156-T10 and P. simplicissimum similar regulation mechanisms must be operative. In both organisms a surprisingly high expression level of vanillyl-alcohol oxidase is observed in the presence of veratryl and anisyl alcohol. This may be explained by assuming that these compounds, or one of their metabolites, have a high affinity for the common aromatic pathway regulator(s), most likely repressor(s). The affinity must be much higher than the

FIG. 2. Time course of induction of P. simplicissimum vaoA transcription and translation. P. simplicissimum was pregrown on fructose and transferred to medium containing veratryl alcohol (see “Experimental Procedures”). Samples were taken at times indicated. Panel A, Northern blot probed with 32P-labeled vaoA-cDNA (I) and reprobed with 32P-labeled A. bisporus 28 S rDNA as a loading control (II). Panel B, Western blot screened with antibodies raised against vanillyl-alcohol oxidase. Panel C, vanillyl-alcohol oxidase relative activity profile.

Specific activity of vanillyl-alcohol oxidase from the multicopy vaoA transformant Aspergillus niger NW156-T10

A. niger NW156-T10 was pregrown on fructose and transferred to different substrates. Cell extract was prepared by sonication as described previously (6). Untransformed A. niger NW156 showed no vanillyl-alcohol oxidase activity when transferred to veratryl alcohol and anisyl alcohol.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose</td>
<td>0.000</td>
</tr>
<tr>
<td>Anisyl alcohol</td>
<td>0.484</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>0.079</td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid</td>
<td>0.124</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>0.175</td>
</tr>
<tr>
<td>Vanillyl alcohol</td>
<td>0.136</td>
</tr>
<tr>
<td>Veratric acid</td>
<td>0.209</td>
</tr>
<tr>
<td>Veratryl alcohol</td>
<td>0.346</td>
</tr>
</tbody>
</table>

TABLE II

Purification of recombinant vanillyl-alcohol oxidase from E. coli TG2 transformed with pLM3972 harboring the P. simplicissimum vaoA-cDNA

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein Activity</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td>2640</td>
<td>46</td>
<td>1.7 x 10^-2</td>
</tr>
<tr>
<td>Protamine sulfate</td>
<td>1648</td>
<td>42</td>
<td>2.5 x 10^-2</td>
</tr>
<tr>
<td>Ammonium sulfate treatment</td>
<td>801</td>
<td>37</td>
<td>4.6 x 10^-2</td>
</tr>
<tr>
<td>Phenyl-Sepharose chromatography</td>
<td>88</td>
<td>38</td>
<td>0.4</td>
</tr>
<tr>
<td>Hydroxyapatite chromatography</td>
<td>24</td>
<td>35</td>
<td>1.5</td>
</tr>
</tbody>
</table>

FIG. 3. Effect of various carbon sources on P. simplicissimum vaoA expression in A. niger NW156-T10. A. niger NW156-T10 transformed with 25–30 copies of the P. simplicissimum vaoA gene pregrown on fructose was transferred to the following substrates: fructose (lane 2), anisyl alcohol (lane 3), ferulic acid (lane 4), 4-hydroxybenzoic acid (lane 5), vanillic acid (lane 6), vanillyl alcohol (lane 7), veratric acid (lane 8), and veratryl alcohol (lane 9) and analyzed 3 hours after transfer. Extracts were prepared by sonication. P. simplicissimum grown on veratryl alcohol (lane 10) served as a control. Lane 1, marker. Panel A, 10% SDS-PAGE gel stained with Coomassie Brilliant Blue. Panel B, UV-visualized vanillyl-alcohol oxidase. Panel C, Western blot screened with vanillyl-alcohol oxidase-specific antibodies.

FIG. 4. SDS-PAGE of purification steps of recombinant vanillyl-alcohol oxidase from E. coli TG2.

The Coomassie Brilliant Blue-stained 10% SDS-PAGE gel contains the following: lanes 1 and 7, marker S4; lane 2, cell extract; lane 3, 65% ammonium sulfate precipitate; lane 4, phenyl-Sepharose pool; lane 5, hydroxyapatite pool; lane 6, vanillyl-alcohol oxidase purified from P. simplicissimum.
affinity of 4-hydroxybenzoic acid, ferulic acid, and vanillyl alcohol or their metabolites. These latter compounds induce vanillyl-alcohol oxidase expression in *A. niger* NW156-T10 but not in *P. simplicissimum*. The discrepancy in the level of vanillyl-alcohol oxidase expression between *A. niger* NW156-T10 and *P. simplicissimum* during growth on 4-hydroxybenzoic acid, ferulic acid, and vanillyl alcohol may be accounted for by the following: (i) in *A. niger* NW156-T10 25–30 copies of the vaoA gene are present versus 1 copy in *P. simplicissimum*, and (ii) in the present study *A. niger* NW156-T10 mycelia were harvested 3 or 6 h following transfer, whereas in the studies with *P. simplicissimum* mycelia were allowed to grow 2 days after transfer which may have caused degradation of vanillyl-alcohol oxidase due to toxic effects and/or the poor carbon sources these aromatic compounds represent (6).

Although expression of vaoA-cDNA in *E. coli* TG2 cells was evident, since specific antibodies could be used to select the cDNA, the expression level was low. It was previously observed that *E. coli* TG2 cells are capable of producing relatively high amounts (>50 mg/liter of culture) of recombinant enzymes from multicopy plasmids like pBlueScript and pUC under the direction of the plasmid-encoded lac promoter (32, 33). Inspection of the vaoA-cDNA sequence revealed two possible explanations for the low expression. The first reason may be the codon usage. Codons that are considered modulator codons in *E. coli*, suppressing high expression (34), occur with a relatively high frequency in the cDNA. The second reason may be the apparent absence of a good ribosome binding site.
introduction of a consensus E. coli ribosome binding site at the correct distance from the start codon increased the expression level only 7-fold indicating that the low expression is related to the codon usage.

Finally, this study has clearly established that expression of the vanA gene in a prokaryotic or eukaryotic host results in active, fully covalently flavinylated enzyme. This suggests that the flavinylation is an autocatalytic process as shown for 6-hydroxy-D-nicotine oxidase (35, 36). However, for p-cresol methylyhydroxylase it was shown that autocatalytic flavinylation only occurred after binding of the cytochrome subunit (28).

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