

PENICILLIN V AMIDOHYDROLASE GENE FROM *FUSARIUM OXYSPORUM*

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The enzyme penicillin V amidohydrolase is used for enzymatic hydrolysis of penicillin V (phenoxy-methylpenicillin) to 6-aminopenicillanic acid (6-APA). 6-APA is the active beta-lactam nucleus used in the manufacture of semi-synthetic penicillins.

10 Various penicillin V amidohydrolase (PVA) enzymes have been found in fungal, streptomyces and bacterial sources (Lowe et al., 1986, Biotechnol. Lett. 8:151-156). PVA enzyme activity is described by Lowe et al. from a strain of *Fusarium oxysporum*; however, the enzyme was not isolated or purified.

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The present invention concerns an isolated nucleic acid molecule coding for all or part of the PVA from *Fusarium oxysporum* strain 435. The PVA gene has been cloned,
20 sequenced and expressed.

In another aspect, the present invention is directed to a novel promoter from *F. oxysporum* strain 435, expression vectors containing all or part of the PVA gene and/or the novel promoter; and host cells containing the expression vectors. In yet another
25 aspect the present invention is directed to the production of PVA, particularly in the presence of phenoxyacetate as an inducer.

Figure 1 - The amino acid sequences of six peptide fragments of the intact PVA enzyme. The amino acid sequence of the N-terminus is SEQ. ID. NO.:1, the amino acid sequence of peptide A is SEQ. ID. NO.:2; the amino acid sequence of peptide B1 is SEQ. ID. NO.:3; the amino acid sequence of peptide B2 is SEQ. ID. NO. 4; the amino acid sequence of peptide C is SEQ. ID. NO.:5; the amino acid sequence of peptide D is SEQ. ID. NO.:6; the amino acid sequence of peptide E is SEQ. ID. NO.:7.

Figure 2 - Oligonucleotide probe. A set of four oligonucleotide probes was derived from the reverse transcription of seven amino acids from peptide C (SEQ. ID. NO.:5). The seven amino acid sequence is SEQ. ID. NO.:8; the DNA sequence reversely transcribed from SEQ. ID. NO.:8 is SEQ. ID. NO.:9; the DNA sequence complementary to SEQ. ID. NO.:9 is SEQ. ID. NO.:10; the oligonucleotide probe is SEQ. ID. NO.:11.

Figure 3 - Various DNA and peptide fragments. The PVA N-terminal amino acid sequence is SEQ. ID. NO.:12; the 2585 translate amino acid sequence is SEQ. ID. NO.:13; the 2585 DNA sequence is SEQ. ID. NO.:14; the 2585-M DNA sequence is SEQ. ID. NO.:15; the 2585-FL translate amino acid sequence is identical to SEQ. ID. NO.:12; the 2585-FL DNA sequence of the coding strand is SEQ. ID. NO.:16; the 2585-FL DNA sequence of the template strand (i.e., complementary to the coding strand) is SEQ. ID. NO.:17. All nucleotide sequences (except SEQ. ID. NO.:17) are shown left to right in the 5' to 3' direction.

Figure 4 - Schematic representation of 2585-FL.

Figure 5 - Schematic representation of cloning of the genomic PVA gene.

Figure 6 - PVA cDNA (SEQ. ID. NO.:18) and corresponding amino acid sequence (SEQ. ID. NO.:19). The underlined DNA sequence (base pairs 1348 to 1368 SEQ. ID. NO.:20) is complementary to a 20mer probe (SEQ. ID. NO.:11). The asterick indicates a termination codon.

Figure 7 - PVA genomic DNA (SEQ. ID. NO.:21) and corresponding amino acid sequence (SEQ. ID. NO.:22). The promoter is base pair nos. 1 to 240 (SEQ. ID. NO.:23). The first asterisk indicates transcription start and the second asterisk indicates a termination codon.

Figure 8 - Schematic representation of construction of pWB19N.

Figure 9 - Schematic representation of construction of pSJC62.

Figure 10 - Schematic representation of construction of pBMFXPVA6.

Figure 11 - Schematic representation of construction of pBMFXPVA7.

Figure 12 - Schematic representation of construction of pF020.

Figure 13 - Schematic representation of construction of pF020-P.

Figure 14 - Schematic representation of construction of pBMPVA-P.

Figure 15 - Schematic representation of construction of pF020-M.

Figure 16 - Schematic representation of construction of pBMPVA-M.

Figure 17 - Schematic representation of construction of pBMPVA-P/DAA02.

Figure 18 - Schematic representation of construction of pBMPVA-M/DAA04.

The present invention concerns an isolated nucleic acid molecule comprising a nucleic acid sequence coding for all or part of PVA from *Fusarium oxysporum*. Preferably, the nucleic acid molecule is a DNA molecule and the nucleic acid sequence is a DNA sequence. All DNA sequences are represented herein by

formulas whose left to right orientation is in the conventional direction of 5' to 3' (with the sole exception of SEQ. ID. NO.:17 as shown in Figure 3). Further preferred is a DNA sequence having all or part of the nucleotide sequence substantially as shown in
5 Figures 6 and 7 (particularly SEQ. ID. NO.:18 and SEQ. ID. NO.:21); or a DNA sequence complementary to one of these DNA sequences; or a DNA sequence which hybridizes to a DNA sequence complementary to one of these DNA sequences. Preferably, the DNA sequence hybridizes under stringent
10 conditions. "Stringent conditions" means conditions no less stringent than described in the "Detailed Examples of Preferred Embodiments" section hereof. In the case of a nucleotide sequence (e.g., a DNA sequence) coding for part of PVA, it is preferred that the nucleotide sequence be at least about 20
15 nucleotides in length.

Preferred DNA fragments are the probe of SEQ. ID. NO.:11 and the promoter of SEQ. ID. NO.:23.

The PVA molecules of the present invention do not necessarily need to be catalytically active. For example,
20 catalytically inactive PVA or fragments thereof may be useful in raising antibodies to the protein.

It is also contemplated that the present invention encompasses modified sequences. As used in the present application, the term "modified", when referring to a nucleotide or
25 polypeptide sequence, means a nucleotide or polypeptide sequence which differs from the wild-type sequence found in nature.

The DNA sequences of the present invention can be obtained using various methods well-known to those of ordinary
30 skill in the art. At least three alternative principal methods may be employed:

- (i) the isolation of a double-stranded DNA sequence from genomic DNA or complementary DNA (cDNA) which contains the sequence;

- (2) the chemical synthesis of the DNA sequence; and
- (3) the synthesis of the DNA sequence by polymerase chain reaction (PCR).

In the first approach, a genomic or cDNA library can be
5 screened in order to identify a DNA sequence coding for all or part
of PVA. For example, a *F. oxysporum* genomic DNA library can be
screened in order to identify the DNA sequence coding for all or
part of PVA. Various techniques can be used to screen the
genomic DNA or cDNA libraries.

10 For example, labeled single stranded DNA probe
sequences duplicating a sequence present in the target genomic
DNA or cDNA coding for all or part of PVA can be employed in
DNA/DNA hybridization procedures carried out on cloned copies
of the genomic DNA or cDNA which have been denatured to
15 single stranded form.

A genomic DNA or cDNA library can also be screened for
a genomic DNA or cDNA coding for all or part of PVA using
immunoblotting techniques.

In one typical screening method suitable for either
20 immunoblotting or hybridization techniques, the genomic DNA
library, which is usually contained in a vector, or cDNA library is
first spread out on agar plates, and then the clones are transferred
to filter membranes, for example, nitrocellulose membranes. A
DNA probe can then be hybridized or an antibody can then be
25 bound to the clones to identify those clones containing the
genomic DNA or cDNA coding for all or part of PVA.

In the second approach, the DNA sequences of the
present invention coding for all or part of PVA can be chemically
synthesized. For example, the DNA sequence coding for PVA can
30 be synthesized as a series of 100 base oligonucleotides that can
be sequentially ligated (via appropriate terminal restriction sites or
complementary terminal sequences) so as to form the correct
linear sequence of nucleotides.

In the third approach, the DNA sequences of the present invention coding for all or part of PVA can be synthesized using PCR. Briefly, pairs of synthetic DNA oligonucleotides at least 15 bases in length (PCR primers) that hybridize to opposite strands of the target DNA sequence are used to enzymatically amplify the intervening region of DNA on the target sequence. Repeated cycles of heat denaturation of the template, annealing of the primers and extension of the 3'-termini of the annealed primers with a DNA polymerase results in amplification of the segment defined by the 5' ends of the PCR primers. See, White et al., Trends Genet. 5, 185-189 (1989).

The DNA sequences of the present invention can be used in a variety of ways in accordance with the present invention. The most apparent use of the DNA sequence is to prepare PVA to be useful for conversion of penicillin V to 6-APA. However, they also can be used as DNA probes to screen other cDNA and genomic DNA libraries as to select by hybridization other DNA sequences that code for proteins related to PVA. In addition, the DNA sequences of the present invention coding for all or part of PVA can be used as DNA probes to screen other cDNA and genomic DNA libraries to select by hybridization DNA sequences that code for PVA molecules from organisms other than *Fusarium oxysporum*.

The DNA sequences of the present invention coding for all or part of PVA can also be modified (i.e., mutated) to prepare various mutations. Such mutations may be either degenerate, i.e., the mutation changes the amino acid sequence encoded by the mutated codon, or non-degenerate, i.e., the mutation does not change the amino acid sequence encoded by the mutated codon. These modified DNA sequences may be prepared, for example, by mutating the PVA DNA sequence so that the mutation results in the deletion, substitution, insertion, inversion or addition of one or more amino acids in the encoded polypeptide using various methods known in the art. For example, the methods of site-

directed mutagenesis described in Morinaga et al., Bio/Technol. 2, 636-639 (1984), Taylor et al., Nucl. Acids Res. 13, 8749-8764 (1985) and Kunkel, Proc. Natl. Acad. Sci. USA 82, 482-492 (1985) may be employed. In addition, kits for site-directed mutagenesis
5 may be purchased from commercial vendors. For example, a kit for performing site-directed mutagenesis may be purchased from Amersham Corp. (Arlington Heights, IL). In addition, disruption, deletion and truncation methods as described in Sayers et al., Nucl. Acids Res. 16, 791-802 (1988) may also be employed. Both
10 degenerate and non-degenerate mutations may be advantageous in producing or using the polypeptides of the present invention. For example, these mutations may permit higher levels of production, easier purification, or provide additional restriction endonuclease recognition sites. All such modified DNA and
15 polypeptide molecules are included within the scope of the present invention.

The present invention is also particularly directed to the novel promoter for PVA which is shown in Figure 7 (SEQ. ID. NO.:23). The novel promoter of the invention can be used with
20 other known DNA sequence coding for other useful proteins or polypeptides.

The present invention further concerns expression vectors comprising a DNA sequence coding for all or part of PVA and/or the promoter for PVA. The expression vectors preferably
25 contain all or part of one of the DNA sequences having the nucleotide sequences substantially as shown in Figures 6 or 7. Further preferred are expression vectors comprising one or more regulatory DNA sequences operatively linked to the DNA sequence coding for all or part of PVA. As used in this context, the
30 term "operatively linked" means that the regulatory DNA sequences are capable of directing the replication and/or the expression of the DNA sequence coding for all or part of PVA.

Expression vectors of utility in the present invention are often in the form of "plasmids", which refer to circular double

stranded DNA loops which, in their vector form, are not bound to the chromosome. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

5 Expression vectors useful in the present invention typically contain an origin of replication, a promoter located in front (i.e., upstream of) the DNA sequence (preferably the promoter of SEQ. ID. NO.:23) and followed by the DNA sequence coding for all
10 or part of a structural protein such as PVA, D-amino acid oxidase, monoclonal antibodies, insulin, interferon, epidermal growth factor, growth hormone, and the like. The DNA sequence coding for all or part of the structural protein is followed by transcription termination sequences and the remaining vector. The expression
15 vectors may also include other DNA sequences known the art, for example, stability leader sequences which provide for stability of the expression product, secretory leader sequences which provide for secretion of the expression product, sequences which allow expression of the structural gene to modulated (e.g., by the
20 presence or absence of nutrients or other inducers in the growth medium), marking sequences which are capable of providing phenotypic selection in transformed host cells, stability elements such as centromeres which provide mitotic stability to the plasmid, and sequences which provide sites for cleavage by restriction
25 endonucleases. The characteristics of the actual expression vector used must be compatible with the host cell which is to be employed. For example, when cloning in a fungal cell system, the expression vector should contains promoters isolated from the genome of fungal cells (e.g., the *trpC* promoter from *Aspergillus*
30 *nidulans* and the PVA promoter from *Fusarium oxysporum*). Certain expression vectors may contain a fungal autonomously replicating sequence (ARS; e.g., ARS from *Fusarium oxysporum* and *Saccharomyces cerevisiae*) which promotes *in vivo* production of self-replicating plasmids in fungal hosts. It is

preferred that the fungal expression vectors of the invention do not have a fungal ARS sequence and thus will integrate into host chromosomes upon plasmid entry of host cells. Such integration is preferred because of enhanced genetic stability. An expression vector as contemplated by the present invention is at least capable of directing the replication in *Escherichia coli* and integration in fungal cells, and preferably the expression, of the PVA DNA sequences of the present invention. Suitable origins of replication in *E. coli* various hosts include, for example, a ColEI plasmid replication origin. Suitable promoters include, for example, the *trpC* promoter from *Aspergillus nidulans*, the PVA promoter from *F. oxysporum*, and the *neo-r* gene promoter from *E. coli*. Suitable termination sequences include, for example, the *trpC* terminator from *A. nidulans*, the PVA terminator for *F. oxysporum*, and the *neo-r* gene terminator from *E. coli*. It is also preferred that the expression vector include a sequence coding for a selectable marker. The selectable marker is preferably antibiotic resistance. As selectable markers, phleomycin resistance (for fungal cells), ampicillin resistance, and neomycin resistance (for bacterial cells) can be conveniently employed. All of these materials are known in the art and are commercially available.

Particularly preferred is the expression vector designated pBMFXPVA6, described herein below and in Figure 10, which contains the DNA sequence coding for PVA, or expression vectors with the identifying characteristics of pBMFXPVA6. Also preferred are the expression vectors designated pWB19N, pSJC62, pBMFXPVA7, pBMPVA-P and pBMPVA-M, described herein below and in Figures 8, 9, 11, 14 and 16, respectively, or expression vectors with the identifying characteristic of pWB19N,, pSJC62, pBMFXPVA7, pBMPVA-P, and pBMPVA-M.

Host cell *Escherichia coli* DH5 α strain containing pBMFXPVA7, pBMPVA-P and pBMPVA-M were deposited with the American Type Culture Collection, Rockville, Maryland on

December 14, 1994 under the Budapest Treaty and assigned ATCC accession nos. 69721, 69722 and 69720, respectively.

Suitable expression vectors containing the desired coding and control sequences may be constructed using standard recombinant DNA techniques known in the art, many of which are described in Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).

The present invention additionally concerns host cells containing an expression vector which comprises a DNA sequence coding for all or part of PVA, and/or the promoter of SEQ. ID. NO.:23. The host cells preferably contain an expression vector which comprises all or part of one of the DNA sequence having the nucleotide sequences substantially as shown in Figures 6 or 7. Further preferred are host cells containing an expression vector comprising one or more regulatory DNA sequences capable of directing the replication and/or the expression of and operatively linked to a DNA sequence coding for all or part of PVA. Additionally included are host cells containing an expression vector which comprises a DNA sequence which has been modified (e.g., disrupted, deleted or truncated) so as to code for a PVA molecule which is not catalytically active. Suitable host cells include both eukaryotic and prokaryotic host cells, for example, *Escherichia coli* cells. Suitable eukaryotic host cells include, for example, *Cephalosporium acremonium*, *Fusarium oxysporum* and *Penicillium chrysogenum* cells.

Particularly preferred as host cells are *Fusarium oxysporum* strains.

Expression vectors may be introduced into host cells by various methods known in the art. For example, transfection of host cells with expression vectors can be carried out by the polyethylene glycol mediated protoplast transformation method. However, other methods for introducing expression vectors into

host cells, for example, electroporation, biolistic injection, or protoplast fusion, can also be employed.

Once an expression vector has been introduced into an appropriate host cell, the host cell may be cultured under
5 conditions permitting expression of large amounts of the desired polypeptide, in the preferred case a polypeptide molecule comprising all or part of PVA.

Host cells containing an expression vector which contains a DNA sequence coding for all or part of PVA may be
10 identified by one or more of the following six general approaches: (a) DNA-DNA hybridization; (b) the presence or absence of marker gene functions; (c) assessing the level of transcription as measured by the production of PVA mRNA transcripts in the host cell; (d) detection of the gene product immunologically; (e)
15 colorimetric detection; and (f) enzyme assay, enzyme assay being the preferred method of identification.

In the first approach, the presence of a DNA sequence coding for all or part of PVA can be detected by DNA-DNA or RNA-DNA hybridization using probes complementary to the DNA
20 sequence.

In the second approach, the recombinant expression vector host system can be identified and selected based upon the presence or absence of certain marker gene functions (e.g., acetamide utilization, resistance to antibiotics, resistance to
25 fungicide, uracil prototrophy, etc.). A marker gene can be placed in the same plasmid as the DNA sequence coding for all or part of PVA under the regulation of the same or a different promoter used to regulate the PVA coding sequence. Expression of the marker gene in response to induction or selection indicates the presence
30 of the entire recombinant expression vector which carries the DNA sequence coding for all or part of PVA.

In the third approach, the production of PVA mRNA transcripts can be assessed by hybridization assays. For example, polyadenylated RNA can be isolated and analyzed by

Northern blotting or nuclease protection assay using a probe complementary to the RNA sequence. Alternatively, the total nucleic acids of the host cell may be extracted and assayed for hybridization to such probes.

5 In the fourth approach, the expression of all or part of PVA can be assessed immunologically, for example, by Western blotting.

10 In the fifth approach, the expression of PVA protein can be assessed by complementation analysis. For example, in cells known to be deficient in this enzyme, expression of PVA activity can be detected on the enzymatic hydrolysis of a colorless substrate, phenoxyacetic-*p*-nitroanilide, to a yellow colored *p*-nitroaniline on the media plate.

15 In the sixth approach, expression of PVA can be measured by assaying for PVA enzyme activity using known methods. For example, the assay described in the "Detailed Examples of the Preferred Embodiments" section hereof may be employed.

20 The DNA sequences of expression vectors, plasmids or DNA molecules of the present invention may be determined by various methods known in the art. For example, the dideoxy chain termination method as described in Sanger et al., Proc. Natl. Acad. Sci. USA 74, 5463-5467 (1977), or the Maxam-Gilbert method as described in Proc. Natl. Acad. Sci. USA 74, 560-564 (1977) may
25 be employed.

30 It should, of course, be understood that not all expression vectors and DNA regulatory sequences will function equally well to express the DNA sequences of the present invention. Neither will all host cells function equally well with the same expression system. However, one of ordinary skill in the art may make a selection among expression vectors, DNA regulatory sequences, and host cells using the guidance provided herein without undue experimentation and without departing from the scope of the present invention.

The present invention further concerns a method for producing PVA comprising culturing a host cell containing an expression vector capable of expressing PVA. Preferably the expression vector is pBMFXPVA6 or pBMFXPVA7. It has been
5 surprisingly found that the production of PVA is substantially enhanced in the presence of phenoxyacetate which acts as an inducer.

The present invention further concerns polypeptide molecules comprising all or part of PVA, said polypeptide
10 molecules preferably having all or part of one of the amino acid sequences substantially as shown in Figures 6 or 7. In the case of polypeptide molecules comprising part of PVA, it is preferred that polypeptide molecules be at least about 10 amino acids in length.

All amino acid residues identified herein are in the
15 natural L-configuration. In keeping with standard polypeptide nomenclature, J. Biol. Chem. 243, 3557-3559 (1969), abbreviations for amino acid residues are as shown in the following Table of Correspondence:

TABLE OF CORRESPONDENCE

<u>SYMBOL</u>		<u>AMINO ACID</u>
<u>1-Letter</u>	<u>3-Letter</u>	
Y	Tyr	L-tyrosine
G	Gly	L-glycine
F	Phe	L-phenylalanine
M	Met	L-methionine
A	Ala	L-alanine
S	Ser	L-serine
I	Ile	L-isoleucine
L	Leu	L-leucine
T	Thr	L-threonine
V	Val	L-valine
P	Pro	L-proline
K	Lys	L-lysine
H	His	L-histidine
Q	Gln	L-glutamine
E	Glu	L-glutamic acid
W	Trp	L-tryptophan
R	Arg	L-arginine
D	Asp	L-aspartic acid
N	Asn	L-asparagine
C	Cys	L-cysteine

All amino acid sequences are represented herein by formulas whose left to right orientation is in the conventional direction of amino-terminus to carboxy-terminus.

The polypeptides of the present invention may be obtained by synthetic means, i.e., chemical synthesis of the polypeptide from its component amino acids, by methods known to those of ordinary skill in the art. For example, the solid phase
5 procedure described in Houghton et al., Proc. Natl. Acad. Sci. 82, 5131-5135 (1985) may be employed. It is preferred that the polypeptides be obtained by production in prokaryotic or eukaryotic host cells expressing a DNA sequence coding for all or part of PVA, or by *in vitro* translation of the mRNA encoded by a
10 DNA sequence coding for all or part of PVA. For example, the DNA sequence of Figure 6 or 7 may be synthesized using PCR as described above and inserted into a suitable expression vector, which in turn may be used to transform a suitable host cell. The recombinant host cell may then be cultured to produce PVA.
15 Techniques for the production of polypeptides by these means are known in the art, and are described herein.

The polypeptides produced in this manner may then be isolated and purified to some degree using various protein purification techniques. For example, chromatographic
20 procedures such as ion exchange chromatography, gel filtration chromatography and immunoaffinity chromatography may be employed.

In addition to preparing 6-APA, the polypeptides of the present invention may be used in a wide variety of other ways. For
25 example, the polypeptides may be used to prepare in a known manner polyclonal or monoclonal antibodies capable of binding the polypeptides. These antibodies may in turn be used for the detection of the polypeptides of the present invention in a sample, for example, a cell sample, using immunoassay techniques, for
30 example, radioimmunoassay or enzyme immunoassay. The antibodies may also be used in affinity chromatography for purifying the polypeptides of the present invention and isolating them from various sources.

The polypeptides of the present invention have been defined by means of determined DNA and deduced amino acid sequencing. Due to the degeneracy nature of the genetic code, which results from there being more than one codon for most of the amino acid residues and stop signals, other DNA sequences which encode the same amino acid sequence as depicted in Figures 6 and 7 may be used for the production of the polypeptides of the present invention. In addition, it will be understood that allelic variations of these DNA and amino acid sequences naturally exist, or may be intentionally introduced using methods known in the art. These variations may be demonstrated by one or more amino acid differences in the overall sequence, or by deletions, substitutions, insertions, inversions or additions of one or more amino acids in said sequence. Such amino acid substitutions may be made, for example, on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphiphatic nature of the residues involved. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; amino acids with uncharged polar head groups or nonpolar head groups having similar hydrophilicity values include the following: leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, tyrosine. Other contemplated variations include salts and esters of the aforementioned polypeptides, as well as precursors of the aforementioned polypeptides, for example, precursors having N-terminal substituents such as methionine, N-formylmethionine used and leader sequences. All such variations are included within the scope of the present invention.

The following examples are further illustrative of the present invention. These examples are not intended to limit the scope of the present invention, and provide further understanding of the invention.

Detailed Examples of Preferred Embodiments

In the examples, microbial strains, plasmids, buffer, growth media and common methods are described as follows.

5 Microbial Strains and Plasmids

The plasmids, bacterial and fungal strains used are listed in Table 1.

TABLE 1

10

Strains and Plamids	Relevant Characteristics	Reference
<i>Escherichia coli</i> DH5 α	F- ϕ 80d <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> ($r_K^- m_K^-$) <i>supE44 λ-thi-1 gyrA96 relA1</i>	Life Technologies, Inc.
<i>Fusarium oxysporum</i> strain 435	Penicillin V amidase producer	novel isolate
<i>Fusarium oxysporum</i> f.sp. <i>lycopersici</i>	ATCC strain	ATCC 16322
pBM11/M5	Neo ^R , 5.6 kb	ATCC 67436
pES200	Amp ^R , HygB ^R , 6.2 kb	(1)
pUC19	Amp ^R , 2686 bp	(2)
pUT715	Amp ^R , 3337 bp	(3)
pUT715/ <i>trpC</i>	Amp ^R , <i>Phl</i> ^R , 4.6 kb	*, Fig. 9
pWB19N	Neo ^R , 2.9 kb	*, Fig. 8
pSJC62	Neo ^R , <i>Phl</i> ^R , 5.3 kb	*, Fig. 9
pF020	Amp ^R , PVA ⁺ , 6.3 kb	*, Fig. 12

pF020-P	Amp ^R , PVA ⁺ , 6.3 kb	*, Fig. 13
pF020-M	Amp ^R , PVA ⁺ , 6.3 kb	*, Fig. 15
pF021	Neo ^R , PVA ⁺ , 15 kb	*, Fig. 5
pF023	Neo ^R , PVA ⁺ , 9.2 kb	*, Fig. 5
pBMFXPVA6	Neo ^R , Phi ^R , PVA ⁺ , 10.3 kb	*, Fig. 10
pBMFXPVA7	Neo ^R , Phi ^R , PVA ⁺ , 14.6 kb	*, Fig. 11
pBMPVA-P	Amp ^R , PVA ⁺ , 6.3 kb	*, Fig. 14
pBMPVA-M	Amp ^R , PVA ⁺ , 6.3 kb	*, Fig. 16
pBMPVA-P/DAAO1	Amp ^R , DAAO ⁺ , 6.8 kb	*, Fig. 17
pBMPVA-P/DAAO2	Neo ^R , Phi ^R , DAAO ⁺ , 8.7 kb	*, Fig. 17
pBMPVA-M/DAAO3	Amp ^R , DAAO ⁺ , 6.9 kb	*, Fig. 18
pBMPVA-M/DAAO4	Neo ^R , Phi ^R , DAAO ⁺ , 8.8 kb	*, Fig. 18

(1) Staben et al., 1989, Fungal Genet. Lett. 36:79-81.

(2) Yanisch-Perron et al., 1985, Gene 33:103-119.

(3) Jain et al., 1992, Mol. Gen. Genet. 234:489-493.

*Described hereinafter.

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Buffers and Media

Luria broth: 1% Difco Bacto tryptone, 0.5% Difco Bacto yeast extract, 0.5% sodium chloride.

10 Luria agar: Luria broth supplemented with 1.5% Difco Bacto agar.

SOC media: 2% Difco Bacto tryptone, 0.5% Difco Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl. After autoclaving, add

one hundredth volume of 1 M MgCl_2 , 1 M MgSO_4 , and 20% glucose to the medium.

Fusarium vegetative growth medium: 6% starch, 4% Pharmamedia (Trader Protein, Memphis, TN), 0.3% $(\text{NH}_4)_2\text{SO}_4$,
5 0.75% KH_2PO_4 , 0.75% K_2HPO_4 , adjusted to pH 6.8 with 10 N NaOH.

PVA production medium: *Fusarium* vegetative growth medium supplemented with 0.4% phenoxyacetate.

Tris-EDTA buffer (TE): 10 mM Tris-HCl (pH 7.4), 1 mM
10 EDTA.

Tris-acetate electrophoresis buffer (TAE): 40 mM Tris-acetate (pH 8.0), 1 mM EDTA.

20X SSC: 3 M NaCl, 0.3 M sodium citrate, adjusted to pH 7.0 with 10 N NaOH.

15 20X SSPE: 3 M NaCl, 0.2 M NaH_2PO_4 , 20 mM EDTA, adjusted to pH 7.4 with 10 N NaOH.

50X Denhardt solution: 1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin (BSA).

30X NET: 4.5 M NaCl, 0.45 M Tris-HCl (pH 7.5), 30 mM
20 EDTA.

Methods

General cloning techniques, DNA sequencing and plasmid DNA extraction from *E. coli* were used as described in
25 Sambrook et al. 1989, "Molecular Cloning: A Laboratory Manual", 2nd edition, Cold Spring Harbor Laboratory, CSH, New York. All restriction enzymes and DNA modifying enzymes were obtained from commercial supplies. They were used according to the manufacture's instructions. Other frequently used methods were
30 carried out as follows.

**1. Electro-Transformation of *E. coli*
(Electroporation)**

Four ml of a fresh overnight culture of *E. coli* DH5 α cells was inoculated into 400 ml of Luria broth and grown by shaking at 37°C to an OD₆₀₀ of 0.6. Cells were harvested by chilling on ice for 15 minutes and centrifuging in a cold rotor at 7,000 x g for 10 minutes. The cell pellet was washed twice in 200 ml of 0°C water and once in 10 ml of 0°C 10% glycerol. The cell pellet was resuspended to a final volume of 1 ml (1 to 3 x 10¹⁰ cells/ml) in 0°C 10% glycerol. The cell suspension was frozen in 40 μ l aliquotes per 1.5 ml polypropylene tube on dry ice and stored at -70°C.

The frozen cells were thawed at room temperature and then immediately placed on ice. About 2-3 μ l of DNA in TE or in ligation mixture was added to the cells, gently mixed and set on ice for 1 minute. The mixture was then transferred to a prechilled 0.2 cm electroporation cuvette (Bio-Rad catalog #165-2086, Bio-Rad Laboratories, Inc.). The Bio-Rad Gene Pulser apparatus (Bio-Rad catalog #165-2075) was set at 25 μ F for capacitance and 2.50 KV for voltage. The Pulse Controller (Bio-Rad catalog #165-2098) was set to 200 ohms for resistance. The cuvette was pulsed once at these settings. After electroporation, 1 ml of SOC medium was added to the cuvette. The cell suspension was transferred with a Pasteur pipet to a 17 x 100 mm polypropylene tube and incubated at 37°C for 1 hour. Cells were plated on a selective medium (Luria agar with neomycin or ampicillin at a concentration of 40 μ g/ml or 100 μ g/ml, respectively).

2. *Fusarium* Protoplast Transformation

Fusarium oxysporum strains were cultured in 20 ml potato dextrose broth (PDB; Difco Laboratories) at 24°C. Microconidia were filtered through a layer of 30 μ m mesh nylon filter (Spectra/Mesh Nylon N, Spectrum Medical Industries, Inc.). The microconidia were pelleted by centrifugation at 1,500 x g for 8

minutes at room temperature. The microconidia were washed twice in sterile distilled water. Approximately 1×10^{10} microconidia were germinated in 100 ml of PDB by shaking for 15 hours at 24°C. The germinated microconidia were collected by centrifugation at 1,500 x g for 8 minutes at room temperature in sterile 50 ml conical tubes. The pellet was washed twice with O buffer (1.4 M MgSO_4 , 50 mM sodium citrate, pH 5.8). The germinated microconidia were treated with 20 ml of O buffer containing 2% of Novozyme 234 (BiosPacific, Inc.) at 24°C for 1 to 2 hours. When greater than 90% of the protoplasts were formed, the mixture were centrifuged in two 15 ml conical tubes at 900 x g at 4°C for 30 minutes. The protoplasts were gently pipeted from the top of the suspension and washed twice in T buffer (1.2 M sorbitol, 50 mM CaCl_2 , 10 mM Tris-HCl, pH 7.4) at 4°C. The protoplasts were suspended at 1×10^9 per ml in T buffer containing 6% polyethylene glycol-4000 and 1% dimethylsulfoxide. The protoplasts were frozen in small aliquotes per 1.5 ml polypropylene tube on dry ice and stored at -70°C.

The protoplasts were transformed by thawing a frozen suspension at room temperature and adding a nuclease inhibitor, aurintricarboxylate, to a final concentration of 2 mM. Ten to twenty μg of plasmid DNA in 10 μl TE was added to 200 μl of the protoplast suspension and incubated for 30 minutes on ice. A solution of 60% polyethylene glycol-4000/50 mM CaCl_2 was added in two 200 μl and one 800 μl volumes with gentle mixing between each addition and was incubated for 30 minutes on ice. Then 10 ml of 1.2 M sorbitol/0.5X PDB was added and gently mixed. The protoplasts were pelleted by centrifugation at 900 x g for 8 minutes at 4°C. The pellet was suspended in 0.5 ml of 1.2 M sorbitol/0.5X PDB. One tenth ml of the protoplast suspension was spread on a 1.2 M sorbitol/0.1X PDB/1.5% agar plate which had been freshly poured in two layers. The bottom layer contained 12.5 ml medium with phleomycin at a concentration of 75 μg per ml, which slowly diffused into the 12.5 ml medium in the top layer.

The plates were incubated at 24°C for 7 days. The transformants were verified by the growth on a 1X PDA medium containing phleomycin at a concentration of 20 µg/ml and by DNA dot-blot hybridization with the plasmid DNA probe.

5

3. Extraction of Chromosomal DNA from *Fusarium* Strains

Fusarium protoplasts were prepared as described in the *Fusarium* protoplast transformation section. After protoplasts were collected from the top of the suspension and washed twice in T buffer at 4°C, the protoplasts were resuspended in 4 ml of lysis buffer [0.7 M NaCl, 10 mM Tris-HCl, pH 8.0, 10 mM EDTA and 1% sodium dodecyl sulfate (SDS)], gently mixed and incubated at 37°C for 5 minutes. One tenth volume of 10% hexadecyltrimethylammonium bromide (CTAB) in 0.7 M NaCl was added to the protoplast lysate, gently mixed and incubated at 65°C for 15 minutes. Chromosomal DNA was extracted by the addition of an equal volume of chloroform:isoamyl alcohol mixture (24:1). The aqueous solution was collected and six tenths volume of isopropanol was added to precipitate the chromosomal DNA. The DNA was collected by centrifugation at 1,500 x g for 5 minutes at room temperature. The DNA was washed once with 70% ethanol; dried under vacuum. The DNA was resuspended in 1 ml of TE with 200 µg of ribonuclease A (EC 3.1.27.5, from Sigma Chemical Co.) per ml and incubated at 37°C for 20 minutes. A preparation of proteinase K solution (EC 3.4.21.14, from Boehringer Mannheim, GmbH) was added to a final concentration of 400 µg/ml and the DNA solution was incubated at 37°C for 20 minutes. One tenth volume of 3 M NaCl was added and DNA solution was extracted two times with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) mixture. The aqueous solution was collected and two volumes of ethanol was added to precipitate the chromosomal DNA. The DNA was then collected by centrifugation at 1,500 xg for 5 minutes at room

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temperature. The DNA was washed once with 70% ethanol, dried under vacuum and resuspended in 1 ml TE.

4. DNA Labeling

- 5 DNA probes were made from plasmid DNA or agarose purified DNA fragments and labeled with α -³²P-dCTP (Amersham Corp.) using the Nick translation System (Life Technologies, Inc.). Oligonucleotide probes were phosphorylated with γ -³²P-ATP (Amersham Corp.) using T4 polynucleotide kinase enzyme.
- 10 Before each use of the labeled probes, DNA probes were heat denatured at 100°C for 5 minutes and quickly cooled on ice for 2 minutes.

5. DNA Dot-Blot Hybridization

- 15 DNA samples were heat denatured in a total volume of 0.4 ml with 0.4 M NaOH, 10 mM EDTA at 100°C for 10 minutes and loaded on a Bio-Rad Zeta-probe GT membrane (Bio-Rad Laboratories, Inc.) or a nitrocellulose membrane (Minifold, Schleicher & Schnell, Inc.) under vacuum in a dot-blot apparatus
- 20 (Minifold, Schleicher & Schnell, Inc.). Hybridization was carried out in a hybridization oven (Laboratory Products Sales, Inc.). The Zeta-probe GT membrane was placed in a bottle and prehybridized with 5 ml of hybridization buffer (0.5 M Na₂HPO₄, pH 7.2, 7% SDS) at 65°C for 2 hours with rotation. Then the
- 25 solution was replaced with 5 ml of freshly prepared hybridization buffer. The labeled DNA probe was added and hybridization was carried out for 16 to 24 hours at 65°C with rotation. The hybridized membrane was washed twice at 65°C for 30 minutes each in buffer consisting of 40 mM Na₂HPO₄ (pH 7.2) and 5% SDS. The
- 30 membrane was subsequently washed twice at 65°C in buffer consisting of 40 mM Na₂HPO₄ (pH 7.2) and 1% SDS. When nitrocellulose membrane was used, the hybridization was carried out in a similar condition, except that the composition of the hybridization buffer was 6X SSC, 5X Denhardt solution, 10 mM

potassium phosphate (pH 7.2), 0.1% SDS and 250 µg of denatured salmon sperm DNA per ml. The hybridized membrane was washed twice at 65°C for 30 minutes each with 2X SSC-0.1% SDS, followed by two 30 minutes washes at 65°C with 0.1X SSC-0.1% SDS. If oligonucleotide probe was used, the temperature of hybridization and wash were maintained at 50°C. After washing, the membrane was exposed to Kodak XAR-5 X-ray film (Eastman Kodak Co.) for autoradiography.

10 **6. Southern DNA Hybridization**

Fusarium chromosomal DNA was digested to completion with each of several different restriction enzymes and electrophoresed through a 0.8% agarose gel in TAE buffer with 0.25 µg ethidium bromide per ml. The gel was treated by acid
15 depurination, alkaline denaturation and neutralization as described in Sambrook et al. and transferred to a Bio-Rad Zeta-probe GT membrane or a nitrocellulose membrane using Bio-Rad Model 785 Vaccum Blotter (Bio-Rad catalog #165-5000). DNA hybridization and autoradiography were carried out as described
20 above.

7. Penicillin V Aldohydrolase Enzyme Assay

 Conidia from *Fusarium oxysporum* strains were isolated for 1-week-old potato dextrose agar (PDA, Difco Laboratories)
25 plates by washing the surface with 5 ml of sterile distilled water. One half ml of conidia solution was inoculated into 25 ml *Fusarium* vegetative growth medium in a 125 ml flask. The vegetative culture was grown for 72 hours on a rotary shaker (250 rpm) at 24°C and two tenth ml aliquote of the negetative culture was
30 inoculated into 25 ml PVA production medium in a 125 ml flask. The culture was carried out on a rotary shaker at 24°C fore 144 hours.

 Production cultures were first diluted in a 5 mM potassium phosphate buffer (pH 7.5). One ml of an appropriately

diluted culture was added to 1 ml of 3% penicillin V solution made in 25 mM potassium phosphate buffer (pH 7.5). The reaction mixture was shaken at 35°C for 15 minutes and stopped by adding 1 ml of 6% (w/v) trichloroacetic (TCA) solution. The mixture was

5 centrifuged at 1,500 x g for 8 minutes. One ml of the cleared supernatant was added to 3 ml *p*-dimethylaminobenzaldehyde (*p*-DAB) working solution which was made by mixing 1 part of 1% *p*-DAB in 100% methanol with 6 parts of glacial acetic acid: H₂O: 1 N NaOH mixture (40:19:1 ratio). The mixture was incubated at room

10 temperature for 5 minutes and taken optical density measurement at a wavelength of 415 nm. A control tube containing 1 ml of 6-APA at 100 µg/ml and 3 ml of *p*-DAB working solution yielded a 0.27 optical density reading at 415 nm. The enzyme activity (IU/ml) is calculated by using the optical density reading at 415 nm

15 multiplied by the dilution factor and multiplied by 0.34. Penicillin V amidohydrolase activity is expressed as international units (IU), one unit equivalent to the conversion of 1 µmole of penicillin V per minute under the above assay condition.

Example 1

Penicillin V Amidase Amino Acid Sequence

The secreted form of the PVA enzyme was isolated, purified and characterized. It is a glycoprotein with a molecular weight of 65 kilodaltons. Purified enzyme was digested with cyanogen bromide. Gel filtration chromatography of cyanogen bromide-digested PVA on sephadex G-100 allowed the purification of six major fragments, A, B1, B2, C, D, and E. The N-terminal amino acid sequence of six peptide fragments as well as the intact PVA molecule was determined by automated Edman degradation method (Hunkapiller and Hood, 1983, Science 219:650-659). The peptide sequence information obtained is as shown in Figure 1.

Example 2

Penicillin V Amidase cDNA Cloning

1. Extraction of mRNA from *Fusarium oxysporum* strain 435

Fusarium oxysporum strain 435 cells were grown in PVA production medium, washed with resuspended guanidine isothiocyanate buffer, and sonicated to break open the cells. Total RNA was isolated by centrifugation through a CsCl cushion (Glisin et al, 1974, Biochem. 13:2633-2637). mRNA was prepared by oligo(dT) chromatography (Sambrook et al., 1989, Molecular Cloning - A Laboratory Manual, 2nd edition) followed by sucrose gradient fractionation. mRNA in each fraction was determined by *in vitro* translation and immunoprecipitation.

2. Synthesis of cDNA and Preparation of cDNA Library

Complementary DNA (cDNA) was prepared from mRNA following the Gubler-Hoffman procedure (Gubler and Hoffman, 1983, Gene 25:263-269). The first strand was synthesized in a 50 µl reaction mixture containing 3 µg mRNA, 50 mM Tris-HCl (pH

7.5), 65 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.5 µg oligo(dT), 2.5 µg Actinomycin D, 1 mM each of 4 dNTP's and 2 units of Murine Leukemia Virus (MLV) reverse transcriptase. The reaction was incubated at 0°C for 3 minutes, then 20°C for 5 minutes and 37°C for 1 hour. Two µl 0.5 M EDTA and 5 µl 3 M NaCl was added at the end of reaction. The mixture was extracted with phenol/chloroform and precipitated with 2 volumes of ethnaol. The second strand was prepared by a repair synthesis reaction using RNase H, DNA polymerase, and DNA ligase. The DNA obtained from first strand synthesis was dissolved in 100 µl repair buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 10 mM ammonium sulfate, 100 mM KCl, 150 µM β-NAD, 40 µM each of 4 dNTP's, 5 µg BSA, 1 unit *E. coli* RNase H, 20 units DNA polymerase I, and 1 unit *E. coli* DNA ligase. The mixture was incubated at 12°C for 1 hour and then 1 hour at 20°C. At the end of the reaction, 10 µl of 3 M NaCl was added and the mixture was extracted with phenol/chloroform. The DNA was precipitated by adding 2 volumes of ethanol. The cDNA was C-tailed using terminal deoxynucleotidyl transferase (TdT) and dCTP. One ng of C-tailed cDNA was annealed and ligated to 25 ng of G-tailed plasmid pUC19. The ligated DNA was used to transform and prepare a cDNA library in *E.coli*.

3. Preparation of Oligonucleotide Probes

A set of four oligonucleotide probes (Figure 2, SEQ. ID. NO.:11) was derived from the reverse translation of seven amino acids (SEQ. ID. NO.:8) from the peptide C (Figure 1, SEQ. ID NO.:5), which is near the carboxyl end of the PVA enzyme.

4. Identification of the PVA cDNA Clone and Generation of A Full Length cDNA

The colonies of cDNA library were screened with a degenerate oligonucleotide probe (Figure 2, SEQ. ID. NO.:11). Colony blots were screened by hybridization to ³²P end-labeled

probes in hybridization buffer (6X NET/10X Denhardts/1% SDS) at 50°C.

The hybridized membrane was washed twice at room temperature for 30 minutes each with 2X NET-0.1% SDS, followed by two 30 minutes washes at 50°C with 0.5X NET-0.1% SDS. A strongly hybridizing colony (#362) was selected for further study. It contained a 500 bp insert which was used as a probe to rescreen the library. A second clone (#2585) contained a 1.7 kb insert and hybridized strongly to the probe. Comparison of the N-terminal amino acid sequence of the PVA to the translation of the 5' end of the insert indicated that the entire coding region of the mature enzyme except for five amino acids had been cloned (Figure 3). A PstI restriction site was created at the 5' end by site specific mutagenesis to generate #2585-M. A synthetic linker was added to convert #2585-M clone to a full-length cDNA clone, 2585-FL (Figures 3 and 4).

5. DNA Sequence Analysis of 2585-FL cDNA Clone

Both strands of the cDNA insert 2585-FL were sequenced by the Maxam-Gilbert method (Maxam and Gilbert, 1977, Proc. Natl. Acad. Sci. USA 74:560-564). The insert contains a 1521 base pair (bp) open reading frame which can code for a 55,000 dalton protein (Figure 6). All of the amino acid sequence data from the N-terminus of the intact PVA molecule and cyanogen bromide digested peptide fragments are found in this sequence. The region (base pairs 1348 to 1368, SEQ. ID. NO.:20) corresponding to the 20mer oligonucleotide probe (SEQ. ID. NO.:11) is underlined in Figure 6.

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Example 3
Penicillin V Amidase Genomic DNA Cloning

**1. Identification of the Genomic PVA Gene
Fragment**

High molecular weight chromosomal DNA from *F. oxysporum* strain 435 was digested with several restriction enzymes, electrophoresed through a 0.6% agarose gel in TAE buffer, and transferred to nitrocellulose. A probe prepared from 2585 insert DNA was hybridized to the Southern blot to determine the size of the fragments containing the PVA gene. The gene was found on a ~12 kb EcoRI fragment.

2. Construction of Plasmid pWB19N

pBM11/M5 (ATCC 67436) is derived from pBM11 (ATCC 67366) in which a NcoI site present in the neomycin-resistance gene has been removed by site-specific mutagenesis. Plasmid pBM11/M5 DNA was digested with HindIII and SmaI. The 5' protruding ends of the HindIII cut were converted to blunt ends using the Klenow fragment of *E. coli* DNA polymerase I. The DNA mixture was subjected to electrophoresis on a 0.8% preparative agarose gel. The 1.3 kb fragment containing the neomycin-resistance gene was excised from the gel and subjected to electroelution at 100 V for 1 hour at room temperature. The resulting eluate was collected and extracted 3 times with an equal volume of TE-saturated phenol. DNA was recovered from the aqueous phase by ethanol precipitation in the presence of 0.3 M NaCl. The recovery of the DNA fragment was analyzed by agarose gel electrophoresis.

The pUC19 plasmid DNA (Yanisch-Perron et al., 1985, Gene, 33:103-119) was cleaved with BspHI and the 5' protruding ends were converted to blunt ends with Klenow fragment. The 1.6 kb fragment (base pair No. 2639 to No. 2686 and No. 1 to No. 1526) containing the polylinker sites and the origin of replication

was isolated and ligated to the 1.3 kb neomycin-resistance gene fragment in the presence of ligation buffer (50 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 1 mM ATP, 1 mM DTT, 5% polyethylene glycol-8000) and T4 DNA ligase.

5 The resultant plasmid, designated pWB19N, was electroporated into *E. coli* DH5 α cells. Neomycin resistant colonies were screened for the presence of pWB19N plasmid which has one restored BspHI site at the junction point of the Klenow filled-in HindIII and BspHI sites. The colonies were also
10 screened for ampicillin sensitivity and α -complementation of β -galactosidase activity on X-gal plates. The construction of pWB19N is depicted in Figure 8.

3. Cloning of the Genomic PVA Gene Fragment

15 One hundred μ g of high molecular weight chromosomal DNA from *F. oxysporum* strain 435 was cleaved with EcoRI. The DNA was electrophoresed through a 0.6% agarose gel in TAE buffer. DNA in the size range of 11.5 kb to 12 kb was excised from the gel and subjected to electroelution at 100V for 1 hour at room
20 temperature. The resulting eluate was collected and extracted 3 times with an equal volume of TE-saturated phenol. DNA was recovered from the aqueous phase by ethanol precipitation in the presence of 0.3 M NaCl. The ~12 kb DNA fragment was then cloned into the pWB19N vector to form pF021 (Figure 5).

25 Restriction analysis of pF021 DNA revealed that the cloned fragment contains the entire PVA gene, with an additional 3.7 kb upstream and 6.8 kb downstream of the PVA coding sequences.

4. DNA Sequence Analysis

30 The coding region of the genomic clone was also sequenced by the Maxam-Gilbert method (Figure 7). The cDNA and genomic sequences were identical except for the codon usage at the 5' end where a synthetic linker had been inserted in the cDNA clone. In the genomic clone, an additional 315 bp of

sequence information was obtained at the 5' end. This data suggests that the translation of PVA mRNA begins at base 241 of Figure 7 and that a "pre-PVA" containing a 25 amino acid signal sequence is synthesized and then cleaved to form the secreted form of PVA enzyme.

The transcription start site was determined by S1 mapping (Berk and Sharp, 1977, Cell 12:721-732,; Sambrook et al., 1989, Molecular Cloning - A Laboratory Manual, 2nd edition). It is located at or near base 162. No other potential ATG translation start signals are found between this site and the ATG at base 241.

Example 4

PVA Gene Expression in Heterologous Hosts

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1. Construction of plasmid pSJC62

Plasmid pES200 (Staben et al., 1989, Fungal Genet. Lett. 36:79-81) has the *trpC* promoter sequence from *Aspergillus nidulans* to direct the transcription of the bacterial hygromycin B-resistance gene. The pES200 DNA was cleaved with EcoRI and ClaI enzymes; the 5' protruding ends were converted to blunt ends with Klenow fragment. The 1250 bp *trpC* promoter fragment was isolated by agarose gel electrophoresis and recovered by electroelution. Plasmid pUT715 (Jain et al., 1992, Mol. Ge. Genet. 234:489-493) is a promoter probe vector carrying a promoter-less phleomycin-resistance gene from *Streptoalloteichus hindustanus*. The pUT715 DNA was digested with EcoRV and ligated to the 1250 bp *trpC* promoter fragment. The ligation mixture was transformed into *E. coli* DH5 α and the resultant plasmid was designated as pUT715/*trpC*.

The pUT715/*trpC* DNA was digested with BamHI and BglII enzymes. The 2.4 kb fragment consisting of the *trpC* promoter and the phleomycin-resistance gene was isolated after agarose gel electrophoresis. The pWB19N DNA was cleaved with

BamHI enzyme and ligated to the 2.4 kb fragment. The resultant plasmid was designated pSJC62 (Figure 9).

2. Construction of pFO23

5 The plasmid pFO21 has an insert of a ~12 kb EcoRI fragment from the *F. oxysporum* strain 435 genomic DNA. The pFO21 DNA was cleaved by Sall enzyme and recirculized with T4 DNA ligase. The resultant plasmid pFO23 has a 5.8 kb deletion of the non-coding *Fusarium* DNA from pFO21 (Figure 5).

3. Construction of pBMFXPVA6

10 The pSJC62 DNA was cleaved with EcoRI and XbaI enzymes. The resulting 2.4 kb fragment consisting of the *Aspergillus trpC* promoter plus the phleomycin-resistance gene was isolated. The pFO23 DNA was digested with EcoRI and XbaI enzymes. The DNA mixture was separated on agarose gel. A 7.9 kb fragment containing the PVA gene was isolated and ligated to the 2.4 kb fragment consisting the *Aspergillus trpC* promoter and the phleomycin-resistance gene. The resultant plasmid was designated pBMFXPVA6 (Figure 10).

4. Construction of pBMFXPVA7

25 The pBMFXPVA6 DNA was cleaved with NcoI and XbaI enzymes. The cohesive ends were converted to blunt ends with Klenow fragment. The DNA mixture was separated on an agarose gel and 4.3 kb fragment containing the PVA gene was isolated. The pBMFXPVA6 DNA was cleaved with XbaI and treated with Klenow fragment. The linearized pBMFXPVA6 DNA was ligated to the 4.3 kb PVA gene fragment to form pBMFXPVA7 (Figure 11).

5. PVA Gene Expression in Heterologous Host

30 Recombinant plasmid pBMFXPVA6 was derived from pFO23 (Figure 5) with the addition of a phleomycin-resistance gene which is regulated by the *trpC* promoter of *Aspergillus*

nidulans (Figure 10). Recombinant plasmid pBMFXPVA7 was derived from pBMFXPVA6 with an addition of a second copy of the PVA gene (Figure 11). These recombinant plasmids were introduced into ATCC 16322 strain (*F. oxysporum* f. sp. *lycopersici*), which is a heterologous host (i.e., a different subspecies of *F. oxysporum*) of the PVA gene, via polyethylene glycol mediated protoplast transformation (Powell and Kistler, 1990, J. Bacteriol. 172:3163-3171). DNA analysis of the phleomycin-resistant transformants confirmed that the transforming DNAs were integrated into the host chromosomes. At this time, *Fusarium oxysporum* strain 435 could not be transformed using this polyethylene glycol-mediated protoplast transformation protocol.

The transformants were then fermented in shake flasks and assayed for PVA activity. The *F. oxysporum* strain 435 produced 20 IU/ml. The untransformed ATCC 16322 strain produced 1.0 IU/ml, the transformants with the pBMFXPVA6 plasmid (one PVA gene) produced 10 IU/ml and the transformants with the pBMFXPVA7 plasmid (two PVA genes) produced 130 IU/ml (Table 2). Overall, the PVA activity expressed in a heterologous host was increased 5 fold over the original *F. oxysporum* strain 435.

TABLE 2

Penicillin V Amidohydrolase Enzyme Activity

STRAINS	SHAKE FLASK TITER (IU/ml)
<i>Fusarium oxysporum</i> strain 435	20
ATCC16322	1
ATCC16322(pBMFXPVA6)+ (one PVA gene)	10
ATCC16322(pBMFXPVA7)+ (two PVA genes)	130

Furthermore, expression of the recombinant PVA can be induced to the same extent as *F. oxysporum* strain 435 in the presence of phenoxyacetate (Table 3).

5 **TABLE 3**
Phenoxyacetate Induction of the Penicillin V
Amidohydrolase Gene Expression

STRAIN	-phenoxy- acetate TITER (IU/ml)	+phenoxy- acetate TITER (IU/ml)	INDUCTION LEVEL
<i>F. oxysporum</i> strain 435	0.49	21.13	43X
ATCC16322 (pBMFXPVA7) ⁺	2.73	135.32	50X

10 **Example 5**
Construction of Fungal Expression Vectors

Expression of the PVA gene is inducible by the addition of phenoxyacetate. It appears that expression vectors can be developed from the PVA transcriptional and translational regulatory region, referred as a promoter, to use for foreign gene expression in *Fusarium oxysporum*. The following constructions were used to generate plasmid pBMPVA-P for expression of intracellular proteins and plasmid pBMPVA-M for expression of secretable proteins.

20 **1. Construction of pF020**

The pF023 DNA was cleaved with BamHI and XhoI enzymes. The DNA mixture was separated on agarose gel. A 2.1 kb fragment containing the transcriptional and translational regulatory sequences upstream of the 5' end of the PVA gene was isolated. The pF023 was also cleaved with XhoI and HindIII

enzymes. An 1.5 kb DNA fragment containing the latter part of the the PVA gene was gel-purified. Both fragments were ligated to pUC19 at HindIII and BamHI sites. The resulted plasmid was designated pF020. The construction of pF020 is diagrammed in Figure 12.

2. Construction of pF020-P

Site-specific mutagenesis protocols derived from Morinaga et al. (1984, Bio/Technol. 2:636-639) was used to generate a MluI site at the translation start site of the PVA gene (base pair no. 241 of SEQ. ID. NO.:21).

ATGCGCGTC (SEQ. ID. NO.:24)

site-specific mutagenesis

ATACGCGTC (SEQ. ID. NO.:25)

MluI site

The generation of the MluI site allows the removal of the coding region of the PVA gene by MluI and BamHI cleavage and the replacement with a coding sequence of a gene to be expressed under the PVA promoter control. A 15mer mutagenic oligonucleotide (GGCACTATACGCGTC SEQ. ID. NO.:26) was synthesized.

The construction of pF020-P was diagrammed in Figure 13. Two µg of pF020 was digested with XmaI, followed by treatment with bacterial alkaline phosphatase (BAP) to prevent self-ligation of the resulting fragment I. Two µg of pF020 was cleaved with EcoRI and SphI to remove the mutation target region (e.g., the upstream region of the PVA gene). The resulting 5 kb fragment (fragment II) was gel-purified. Equal molar amounts of fragments I and II were mixed with a 200 fold molar excess of the 15mer mutagenic oligonucleotide. The mixture was incubated at 100°C for 3 minutes in order to completely denature the DNA

fragments in the mixture. After the denaturation, mixture was gradually cooled in a step-wise manner to allow the denatured DNA fragment to reanneal. During this reannealing procedure, two new species of DNAs, DNA-a and DNA-b were formed in addition to the original fragments I and II. As shown in Figure 13, the 15mer mutagenic oligonucleotide hybridized with only one of the two species of circular DNAs to form the heteroduplex, since the oligonucleotide was complementary to only one of the two strands of the target region. The DNAs were incubated with the Klenow fragment, T4 DNA ligase and four dNTP's. This treatment converted the open circular DNAs to closed circular DNAs. After the reaction, the mixture was used for transformation of *E.coli* DH5 α . Plasmids DNAs of transformants were screened by MluI clavage analysis. Plasmid pF020-P was identified for the creation of a MluI site.

3. Construction of pBMPVA-P

Since the pF020-P has two BamHI sites which prevent the use of this expression vector, the BamHI site from the region of the pUC19 vector needs to be removed. pF020-P was treated with a limited amount of BamHI enzyme to generate a partial cleavage condition and then cleaved with EcoRI. The 5' protruding ends were filled in with the Klenow fragment. A 6.3 kb fragment was gel-purified, ligated with T4 DNA ligase and transformed into DH5 α . The resulting plasmid pBMPVA-P (Figure 14) can be used as an expression vector to insert a coding sequence of the desired gene at the MluI/BamHI sites under the PVA promoter control.

4. Construction of pF020-M

A SmaI site was generated at the junction of the PVA secretion signal sequence and mature PVA protein (amino acid No. 24-25, SEQ. ID. NO.:22).

AACAAA/GGCAAC (SEQ. ID. NO.:27)

N K G N (SEQ. ID. NO.:28)

site-specific mutagenesis

AACCCC/GGGAAC (SEQ. ID. NO.:29)

SmaI site

The creation of the SmaI sites allows the insertion of a coding sequence after the PVA secretion signal sequence and thus will allow secretion of the expressed protein. A 28mer mutagenic oligonucleotide

(GCAGCTCCCAACCCCGGGAACGATGATT, SEQ. ID. NO.:30) was synthesized.

The construction of pF020-M was shown in Figure 15. The process was similar to the construction of pF020-P. Plasmid pF020 was cleaved with XmaI, followed by BAP treatment to generate fragment I. pF020 was then digested with SphI and HindIII to remove the target region and generate a 6 kb fragment II. The conditions of heteroduplex formation and DNA extension was similar to the pF020-P construction. Plasmids DNAs of transformants were screened for the creation of an additional SmaI site.

5. Construction of pBMPVA-M

Plasmid pF020-M has two BamHI and two SmaI sites which prevent the use of this vector. The extra BamHI and SmaI sites from the region of pUC19 need to be removed. pF020-M was partially cleaved with BamHI and then cleaved with EcoRI. The cohesive ends were filled with the Klenow fragment. A 6.3 kb fragment was gel-purified, ligated and transformed into *E. coli* DH5 α . The resulting plasmid pBMPVA-M (Figure 16) can be used as a secretable expression vector to insert a coding sequence of

the desired gene at the SmaI/BamHI sites under the PVA promoter control.

Example 6

5 Expression of the D-Amino Acid Oxidase Gene by the PVA Expression Vectors

A D-amino acid oxidase gene which was cloned from *Trigonopsis variabilis* was used as an example of a foreign gene to be expressed by the *Fusarium* PVA expression vectors.

10

1. Expression of the D-Amino Acid Oxidase Gene By pBMPVA-P

The D-Amino acid oxidase (DAAO) gene of *Trigonopsis variabilis* was cloned by Bristol-Myers Squibb scientists. The DNA
15 sequence analysis indicated that a 38 bp intron exists near the 5' end of the coding sequence. Oligonucleotide directed site-specific mutagenesis method was used to remove the intron and create a NcoI site at the translation start site of the DAAO gene. Therefore, the DAAO coding sequence plus a 700 bp 3' non-transcriptional
20 region can be purified as a 2.1 kb NcoI/BamHI fragment.

Plasmid pBMPVA-P DNA was treated with MluI cleavage and the cohesive ends were filled in with the Klenow fragment. The DNA was then cleaved with BamHI. A 4.7 kb fragment containing the PVA promoter region was gel-purified. The DAAO
25 gene fragment was ligated with the 2.1 kb NcoI-Klenow/BamHI fragment of DAAO gene to generate plasmid pBMPVA-P/DAA01. A 3.4 kb PVA promoter/DAAO gene fusion fragment was prepared by NdeI cleavage and Klenow filled-in of plasmid pBMPVA-P/DAA01 and gel-purified. The fragment was inserted into pSJC62 DNA
30 and the Klenow filled-in XbaI site. The resulting plasmid was designated as pBMPVA-P/DAA02. The entire construction scheme is depicted in Figure 17.

The pBMPVA-P/DAA02 DNA was introduced into ATCC 16322 strain by polyethylene glycol mediated protoplast

transformation. Transformants were selected from its resistance to phleomycin. Several transformants were grown in PVA production medium and assayed for D-amino acid oxidase activity. The results indicated that the D-amino acid oxidase gene was expressed in ATCC 16322 transformants at a level of 0.2-0.8 IU/ml:

2. Expression of the D-Amino Acid Oxidase Gene by pBMPVA-M

Plasmid pBMPVA-M DNA was cleaved with SmaI and BamHI enzymes. A 4.8 kb fragment containing the PVA promoter region as well as the PVA secretable signal sequence was gel-purified. The DAAO gene fragment was prepared by NcoI cleavage plus mung bean nuclease treatment to remove the 5' cohesive ends and maintain a proper reading frame of the DAAO gene, and then BamHI digestion to generate a 2.1 kb fragment. The 4.8 kb PVA promoter/signal sequence fragment was ligated to the 2.1 kb DAAO gene fragment to generate plasmid pBMPVA-M/DAA03. The pBMPVA-M/DAA03 DNA was cleaved with NdeI and the 5' cohesive ends were filled in with the Klenow fragment. The 3.5 kb PVA promoter-signal sequence/DAAO gene fusion fragment was purified and inserted into pSJC62 DNA at Klenow filled-in XbaI site. The resulting plasmid was designated as pBMPVA-M/DAA04 (Figure 18).

The pBMPVA-M/DAA04 DNA was transformed into ATCC 16322 strain. Several phleomycin-resistant transformants were assayed for D-amino acid oxidase activity. Unfortunately, none of the transformants showed D-amino acid oxidase activity. The failure of the expression of the D-amino acid oxidase gene by pBMPVA-M vector may be due to the intracellular nature of the D-amino acid oxidase enzyme. It is widely recognized that genes encoding intracellular protein can not be expressed and translocated extracellularly by a secretable expression system (Model and Russel, 1990, Cell 61:739-741). Even if the gene is

expressed intracellularly, the attachment of the PVA signal sequence may inactivate the D-amino acid oxidase activity. Therefore, the pBMPVA-M vector should still be useful for expression of other secretable protein genes.

What We Claim Is:

1. An isolated nucleic acid molecule having a sequence coding for the amino acid sequence of SEQ. ID. NOS.: 19 or 22.
2. An isolated nucleic acid molecule having a sequence complementary to a nucleic acid sequence coding for the amino acid sequence of SEQ. ID. NOS.:19 or 22.
3. An isolated nucleic acid molecule having a sequence capable of hybridizing to a nucleic acid having a sequence complementary to a nucleic acid sequence coding for the amino acid sequence of SEQ. ID. NOS.:19 or 22.
4. The nucleic acid molecule of Claim 1 which is a DNA molecule.
5. The nucleic acid molecule of Claim 2 which is a DNA molecule.
6. The nucleic acid molecule of Claim 3 which is a DNA molecule
7. An isolated DNA molecule having a sequence coding for the amino acid sequence of SEQ. ID. NO.:19.
8. An isolated DNA molecule having a sequence capable of hybridizing to a nucleic acid having a sequence complementary to a nucleic acid sequence coding for the amino acid sequence of SEQ. ID. NO.:19.
9. An isolated DNA molecule having a sequence coding for the amino acid sequence of SEQ. ID. NO.:22.



10. An isolated DNA molecule having a sequence capable of hybridizing to a nucleic acid having a sequence complementary to a nucleic acid sequence coding for the amino acid sequence of SEQ. ID. NO.:22.

11. An isolated DNA molecule having the nucleotide sequence of SEQ. ID. NO.:18.

12. An isolated DNA molecule having the nucleotide sequence of SEQ. ID. NO.:21.

13. An isolated DNA molecule having the nucleotide sequence of SEQ. ID. NO.: 11.

14. An isolated DNA molecule having the nucleotide sequence of SEQ. ID. NO.: 23.

15. An isolated DNA molecule having the nucleotide sequence of SEQ. ID. NOS.: 14, 15, 16, or 17.

16. An isolated DNA molecule having the nucleotide sequence of SEQ. ID. NOS.:9 or 10.

17. An isolated DNA molecule having the nucleotide sequence of SEQ. ID. NOS.:26 or 30.

18. An isolated polypeptixde having the amino acid sequence of SEQ. ID. NOS.:1, 2, 3, 4, 5, 6, 8, or 12.

19. The polypeptide of Claim 18 having the amino acid sequence of SEQ. ID. NOS.:1 or 12.



20. An expression vector comprising a nucleic acid sequence coding for part of the amino acid sequence of SEQ. ID. NOS.:19 or 22.

21. The expression vector of Claim 20 comprising a DNA sequence coding for the amino acid sequence of SEQ. ID. NO.:19.

22. The expression vector of Claim 20 comprising a DNA sequence coding for the amino acid sequence of SEQ. ID. NO.:22.

23. The expression vector of Claim 20 further comprising an origin of replication, a promoter, and a transcription termination sequence.

24. The expression vector of Claim 23 further comprising a selectable marker sequence.

25. The expression vector of Claim 24 wherein the selectable marker is phleomycin resistance.

26. The expression vector of Claim 23 which is capable of integrating into fungal chromosomes.

27. The expression vector of Claim 20 having the DNA sequence of SEQ. ID. NOS.:18 or 21.

28. The expression vector of Claim 20 which is a plasmid.

29. An expression vector comprising a promoter having the DNA sequence of SEQ. ID. NO.:23.



30. The expression vector of Claim 29 further comprising an origin of replication, DNA coding for a structural protein, and a transcription termination sequence.

31. The expression vector of Claim 30 wherein the DNA coding for a structural protein codes for PVA.

32. The expression vector of Claim 30 further comprising a selectable marker sequence.

33. The expression vector of Claim 32 wherein the selectable marker is phleomycin resistance

34. The expression vector of Claim 30 which is capable of integrating into fungal chromosomes.

35. An expression vector having the designation pBMFXPVA6.

36. An expression vector having the designation pBMFXPVA7.

37. An expression vector having the designation pBMPVA-P.

38. An expression vector having the designation pBMPVA-M.

39. A host cell containing the expression vector of Claim 20.

40. A host cell containing the expression vector of Claim 23.



41. A host cell containing the expression vector of Claim 25.

42. A host cell containing the expression vector of Claim 26.

43. A host cell containing the expression vector of Claim 27.

44. A host cell containing the expression vector of Claim 28.

45. A host cell containing the expression vector of Claim 29.

46. A host cell containing the expression vector of Claim 30.

47. A host cell containing the expression vector of Claim 31.

48. A host cell containing the expression vector of Claim 32.

49. A host cell containing the expression vector of Claim 33.

50. A host cell containing the expression vector of Claim 34.

51. A host cell containing the expression vector of Claim 35.



* C L M S *

52. A host cell containing the expression vector of Claim 36.

53. A host cell containing the expression vector of Claim 37.

54. A host cell containing the expression vector of Claim 38.

55. The host cell of Claim 39 which is eukaryotic.

56. The host cell of Claim 45 which is eukaryotic.

57. The host cell of Claim 39 which is a *Fusarium* species.

58. The host cell of Claim 45 which is a *Fusarium* species.

59. The host cell of Claim 39 which is *Fusarium oysporum*.

60. The host cell of Claim 45 which is *Fusarium oysporum*.

61. The host cell of Claim 51 which is *Fusarium oysporum*.

62. The host cell of Claim 52 which is *Fusarium oysporum*.

63. The host cell of Claim 53 which is *Fusarium oysporum*.



64. The host cell of Claim 54 which is *Fusarium oysporum*.

65. A biologically pure culture of *Escherichia coli* ATCC 69720.

66. A biologically pure culture of *Escherichia coli* ATCC 69721.

67. A biologically pure culture of *Escherichia coli* ATCC 69772.

68. A method for producing a polypeptide having the amino acid sequence of SEQ. ID. NOS.:19 or 22 comprising culturing the host cell of Claim 39 under conditions resulting in expression of the polypeptide.

69. The method of Claim 68 wherein expression is induced by culturing in the presence of phenoxyacetate.

70. A method for producing a polypeptide comprising culturing the host cell of Claim 45 under conditions resulting in expression of the polypeptide.

71. The method of Claim 70 wherein expression is induced by culturing in the presence of phenoxyacetate.

