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Cloning and characterization of *avfA* and *omtB* genes involved in aflatoxin biosynthesis in three *Aspergillus* species

Jiujiang Yu ^{a,*}, Charles P. Woloshuk ^b, Deepak Bhatnagar ^a, Thomas E. Cleveland ^a^a Southern Regional Research Center, USDA, Agricultural Research Service, New Orleans, LA 70179, USA^b Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907, USA

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Abstract

The biosynthesis of aflatoxins (B_1 , G_1 , B_2 , and G_2) is a multi-enzyme process controlled genetically by over 20 genes. In this study, we report the identification and characterization of the *avfA* gene, which was found to be involved in the conversion of averufin (AVF) to versiconal hemiacetal acetate (VHA), in *Aspergillus parasiticus* and *A. flavus*; a copy of *avfA* gene was also cloned from a non-aflatoxin producing strain *A. sojae*. Complementation of an averufin-accumulating, non-aflatoxigenic mutant strain of *A. parasiticus*, SRRC 165, with the *avfA* gene cloned from *A. flavus*, restored the ability of the mutant to convert AVF to VHA and to produce aflatoxins B_1 , G_1 , B_2 , and G_2 . Sequence analysis revealed that a single amino acid replacement from aspartic acid to asparagine disabled the function of the enzyme in the mutant strain SRRC 165. The *A. parasiticus avfA* was identified to be a homolog of previously sequenced, but functionally unassigned transcript, *stcO*, in *A. nidulans* based on sequence homology at both nucleotide (57%) and amino acid (55%) levels. In addition to *avfA*, another aflatoxin pathway gene, *omtB*, encoding for an *O*-methyltransferase involved in the conversion of demethylsterigmatocystin (DMST) to sterigmatocystin (ST) and dihydrodemethylsterigmatocystin (DHDMS) to dihydrosterigmatocystin (DHST), was cloned from *A. parasiticus*, *A. flavus*, and *A. sojae*. The *omtB* gene was found to be highly homologous to *stcP* from *A. nidulans*, which has been reported earlier to be involved in a similar enzymatic step for the sterigmatocystin formation in that species. RT-PCR data demonstrated that both the *avfA* and *avfA1* as well as *omtB* genes in *A. parasiticus* were expressed only in the aflatoxin-conducive medium. An analysis of the degrees of homology for the two reported genes between the *Aspergillus* species *A. parasiticus*, *A. flavus*, *A. nidulans* and *A. sojae* was conducted. Published by Elsevier Science B.V. All rights reserved.

Keywords: Averufin; Methyltransferase; Mycotoxins; *O*-methylsterigmatocystin; SAM-binding motif; Secondary metabolites

1. Introduction

Aflatoxins are a family of polyketide derived secondary metabolites of several *Aspergilli*, but commonly

produced by the fungi *Aspergillus flavus* and *A. parasiticus*. Amongst these, aflatoxin B_1 is one of the most potent natural carcinogens known. Aflatoxin contamination of the agricultural commodities is, therefore, not only a serious health hazard but also an economic issue (Jelinek et al., 1989; Cleveland and Bhatnagar, 1992; Bhatnagar et al., 1997). The formation of aflatoxins involves over 20 enzymatic reactions, and most of the corresponding genes have been isolated and characterized (for reviews, see Bhatnagar et al., 1997; Cleveland et al., 1997; Minto and Townsend, 1997; Payne and Brown, 1998; Woloshuk and Prieto, 1998). These aflatoxin pathway genes are clustered in a 70 kb DNA region in *A. flavus* and *A. parasiticus* (Yu et al., 1995; Cleveland et al., 1997, Fig. 1) under the control of the positive regulatory gene, *aflR* (Chang et al., 1993; Payne et al., 1993), and another gene *aflJ* (Meyers et al., 1998).

Abbreviations: AVF, averufin; AVN, averantin; B_1 , aflatoxin B_1 ; B_2 , aflatoxin B_2 ; cDNA, DNA complementary to RNA; DHDMS, dihydrodemethylsterigmatocystin; DHOMST, dihydro-*O*-methylsterigmatocystin; DHST, dihydrosterigmatocystin; DMST, demethylsterigmatocystin; EMW, ether:methanol:water solvent system; G_1 , aflatoxin G_1 ; G_2 , aflatoxin G_2 ; GMS, glucose-mineral salts; HAVN, 5'-hydroxy-averantin; OMST, *O*-methylsterigmatocystin; PDA, potato dextrose agar; PMS, peptone-mineral salts; SAM, *S*-adenosyl methionine; ST, sterigmatocystin; TLC, thin-layer chromatography; VER A, versicolorin A; VER B, versicolorin B; VHA, versiconal hemiacetal acetate.

* Corresponding author. Tel.: +1-504-286-4405;
fax: +1-504-286-4419.

E-mail address: jiuju@commsvr.srrc.usda.gov (J. Yu)

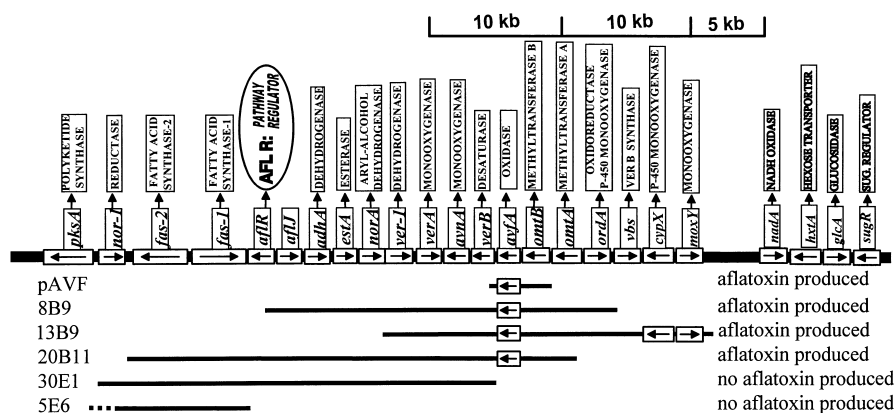


Fig. 1. Cluster of aflatoxin biosynthetic pathway genes and the complementing clones in *A. parasiticus* and *A. flavus*. The 70 kb DNA region harboring over 20 identified aflatoxin pathway genes (open boxes) is shown; arrows inside the boxes indicate the direction of gene transcription; the names of genes are labelled on top of the open boxes; the vertical arrows indicate the relationships from genes to the corresponding enzymes they encode. The aflatoxin pathway regulator, AFLR, was encoded by the *aflR* gene for transcription initiation. The cosmid and plasmid clones used for complementation are presented under the gene cluster.

A sterigmatocystin pathway gene cluster (sterigmatocystin is a precursor of aflatoxins) in *A. nidulans* has also been reported (Brown et al., 1996) containing most of the genes required for aflatoxin synthesis. However, the organization of the genes in the *A. nidulans* cluster is quite different from that in *A. parasiticus* and *A. flavus*. The homology of the clustered genes between *A. parasiticus* and *A. flavus* is about 90–99%, but between the *A. parasiticus* and *A. nidulans* is 55–75%. The identified genes in the aflatoxin pathway gene cluster cannot account for all of the enzymatic steps in the aflatoxin biosynthesis, and the function of some of the identified open reading frames remains undefined.

In the early step of aflatoxin biosynthesis, the conversion from averufin (AVF) to versiconal hemiacetal acetate (VHA) may involve more than one enzyme (Bhatnagar et al., 1992). So far, no enzyme or its corresponding gene has yet been identified. In the later stages of aflatoxin biosynthesis, two critical steps involve *O*-methyltransferase activities for the conversion of demethylsterigmatocystin (DMST) and dihydrodemethylsterigmatocystin (DHDMST) to sterigmatocystin (ST) and dihydrosterigmatocystin (DHST), followed by ST and DHST to *O*-methylsterigmatocystin (OMST) and dihydro-*O*-methylsterigmatocystin (DHOMST). Two distinct *O*-methyltransferases were detected in aflatoxin biosynthesis (Bhatnagar et al., 1988; Yabe, et al., 1989). The *O*-methyltransferase for the conversion of ST to OMST and DHST to DHOMST was purified, and the corresponding gene (*omtA*) was cloned and characterized (Yu et al., 1993). The other methyltransferase has also been purified (Yabe, et al., 1998), and the homologous gene coding for this enzyme in *A. nidulans*, *stcP*, was characterized (Kelkar et al., 1996). While this manuscript was in review, the cloning of *stcP* homolog, *dmtA*, in *A. parasiticus* strain NIAH26, was reported (Motomura et al., 1999).

We report here the identification and characterization of the *avfA* gene from *A. parasiticus*, *A. flavus*, *A. sojae*, and the mutant allele, *avfA1*, from AVF-accumulating, non-aflatoxigenic, mutant *A. parasiticus* strain SRRC 165. We have also compared the sequence of *avfA* to a homologous transcript, *stcO*, in *A. nidulans* and determined the function of the *avfA* gene product in aflatoxin biosynthesis. We also report the cloning and sequence comparison of the *omtB* gene from these *Aspergillus* species required for the conversion of demethylsterigmatocystin (DMST) and dihydrodemethylsterigmatocystin (DHDMST) to sterigmatocystin (ST) and dihydrosterigmatocystin (DHST).

2. Materials and methods

2.1. Fungal strains and culture conditions

The fungal strains used were as follows:

1. *A. parasiticus* wild-type strain SRRC 143 (ATCC 56775), which produces aflatoxins B₁, B₂, G₁, and G₂;
2. *A. parasiticus* mutant strain SRRC 165 (ATCC 24551), which accumulates averufin and does not produce any of the four aflatoxins; For transformation purpose, a *pyrG* mutation was introduced into this strain using previously described methods (Woloshuk et al., 1989) and was used as the recipient strain in the complementation experiment by fungal transformation;
3. aflatoxigenic strain *A. flavus* CRA01-2B (Payne et al., 1993), a benomyl-resistant mutant of NRRL 3357, which produces aflatoxins B₁ and B₂; and
4. non-aflatoxigenic *A. sojae* strain SRRC 1126 (ATCC 42251), which does not produce aflatoxins or any of the aflatoxin pathway intermediates.

Fungal strains were maintained on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI.); PDA was also used for detection of aflatoxin production. Glucose-mineral salts (GMS; Adye and Mateles, 1964) (50 g of sucrose, 3 g of ammonium sulfate, 10 g of potassium phosphate, 2 g of magnesium sulfate, and 1 ml of micro-nutrient mixture per liter of medium, pH 4.5) was used as aflatoxin-conducive medium for growth of fungal mycelia as submerged cultures. Peptone-mineral salts (PMS; Adye and Mateles, 1964), in which glucose in GMS medium was replaced with an equal amount (weight) of peptone, was used as the non-aflatoxin-conducive medium.

2.2. Vector construction

The cosmid library constructed from *A. flavus* strain CRA01-2B was as described previously (Payne et al., 1993; Yu et al., 1995), and genetically mapped cosmid clones 8B9, 13B9, 20B11, 30E1, and 5E6 were used in the complementation experiments. In addition, a 2.5 kb DNA fragment harboring a functional copy of *avfA* gene from *A. flavus* was subcloned into pBluescript vector (pAVF) for complementation of *A. parasiticus* mutant strain SRRC 165.

2.3. Complementation of *avfA* gene

A. parasiticus strain SRRC 165 was transformed by *A. flavus* cosmid clones 8B9, 13B9, 20B11, 30E1, 5E6 and subclone pAVF. The fungal transformation was performed by the polyethylene glycol–calcium method, as described previously (Woloshuk et al., 1989). Czapek solution agar (Difco Laboratories, Detroit, MI) supplemented with 0.6 M KCl and Cove's micro-nutrients (Cove, 1966) was used as the protoplast regeneration medium. Transformants were selected, based on their ability to grow on medium lacking uracil.

2.4. Solvent extraction and thin-layer-chromatography (TLC) assays

Aflatoxin-producing transformants (*avfA* complemented mutants) were analyzed by TLC. Three-day-old mycelia were harvested by filtration and extracted by acetone and chloroform, as described previously (Dutton, 1988). The extracted metabolites were assayed on a thin-layer-chromatography (TLC) plate (catalog no. 7001-04; 20 cm × 20 cm silica gel; J.T. Baker, Inc.) in a EMW (ether: methanol: water, 96:3:1, v/v/v) solvent system.

2.5. cDNA cloning by reverse-transcriptase polymerase chain reaction (RT-PCR)

Fungal wild-type strains SRRC 143, mutant strain SRRC 165, and SRRC 165 complemented by 8B9 and

13B9 were grown in PMS medium for 48 h and shifted to GMS medium. Total RNA was isolated using RNeasy Total RNA Kit (Qiagen, Valencia, CA) from the mycelia after 48 h growth in PMS medium and 24 and 48 h growth after shifting to GMS medium. Polyadenylated RNAs were purified subsequently using PolyAtract mRNA isolation System (Promega, Madison, WI). First-strand cDNA was synthesized by Advantage RT-for-PCR Kit (Clontech, Palo Alto, CA) and used as the template in PCR. The PCR amplification was carried out as described earlier (Yu et al., 1998). The resulting cDNA fragments were sequenced and analyzed.

3. Results

3.1. *avfA* gene involved in the conversion of AVF to VHA

In the search for the gene involved in the conversion of AVF to VHA, we complemented the AVF-accumulating, non-aflatoxigenic mutant strain, *A. parasiticus* SRRC 165, with several previously mapped *A. flavus* cosmid clones (Fig. 1). When the AVF-accumulating mutant (Fig. 2, lane 2) was transformed with the cosmid clones 8B9 (Fig. 2, lane 3), 13B9, and 20B11, the aflatoxin (B₁, B₂, G₁, and G₂)-producing ability of *A. parasiticus* mutant strain was restored. When the AVF-accumulating mutant SRRC 165 was transformed with the cosmid clones 30E1 or 5E6, no change in pigment-accumulating profile was observed, and no aflatoxin was produced. These complementation experiments revealed that the gene responsible for the conversion from AVF to VHA, and ultimately to aflatoxins, may reside on cosmid clones 8B9, 13B9 and 20B11 within the DNA region between *omtA* and *avnA* (Fig. 1).

The DNA sequence of the region between *omtA* (Yu et al., 1993) and *avnA* (Yu et al., 1997) in *A. parasiticus*

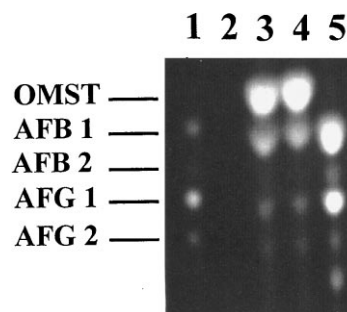


Fig. 2. Thin-layer chromatographic assays (TLC) of toxin production by the *avfA* complemented SRRC 165 transformants. Aflatoxins were extracted by acetone/chloroform from the *avfA* complemented mutant strain SRRC 165. One-fourth of the total extracted metabolites from each sample was loaded onto each lane. lane 1, aflatoxin standard (B₁, B₂, G₁, and G₂); lane 2, SRRC 165 mutant without *avfA* complementation; lane 3, SRRC 165 mutant complemented with *A. flavus* cosmid 8B9; lane 4, SRRC 165 mutant complemented with *A. parasiticus* pAVF; lane 5, SRRC 143 wild type as positive control.

-296 CCCCAACCCAGGCTAGGTFAGTCACTACTTGGTATTCTCGCAAGTCGCGGGGATCATCACCGTGAATCCGCCACACTTGGCTCAGCGACACAT-----CTCCCGAGAGCATCTAGCTCTGAATGGTTTCCCATGAGTTCCGGTATACAG
A.f.T.....C.....A..GT.....T...TCG..G...CGA.....ACTT.....C.....G.....
A.s.C.....C.....TCG.....CGA.....

-150 GCGGGAGTAGCAAGAAGCGTTAATAAAGGTTAATAACAAACAATAACCCCTGGCTCAGGGACCAGTCTTCAAGCCCTGACCCCTCGTGCATCTTAATTGACTGTACAAATTTGACCGCCTGTGAAAGTGGTAGGTTACAAAGG
A.f.C.....C.....C.....G.....C.....A.....G.....T.....T.....A.....
A.s.C.....C.....C.....G.....C.....A.....G.....T.....T.....A.....

0001 ATGACGGGCCCTTGACATGGAACCATCTTCGCGAAAATTAAGAAGATATGCCAGAACAGCATGTGGCAACCGSAAATTAAGGCCCATATCCCGAGCTTACGGGTGGGATTTACTCGGATGGGATGTGGTATGCGATTGACC
A.f. ATG.....C.....T.....G.....T.....A.....
A.s. ATG.....C.....T.....G.....G.....G.....
A.p. M T G L D M E T I F A K I K E E Y A R T D V G K R Q I Q G H I R E L Q V G F Y S D W D V V M R L S

0151 AGTGGAgtagtctctatgtttgaagccctagctatggttgagaatgaaatgtaataatcctctagCCCCACAAAGTCCCTCTCACGAAGTGGCATCGATCTGGGCATCTCCGACCCCTCAAGAGAGGACGACACGCCCAATTAACC
A.f.t.....C.....G.....G.....C.....T.....G.....T.....G.....T.....G.....
A.s.t.....C.....G.....G.....C.....T.....G.....T.....G.....T.....G.....
A.p. S G P L Q V A L T K V G I D L G I F R S L K E S D T P I T

0301 CTAGCGGAAATGTCAAGAAGACGGCGCTTCCAGACTCCTTGGTggttacctatctacctggttaaccaggaatgatttaaccaggaatagTCGCATTTACGGACCCAGCGGCTTTTGGCCTGATCAAGGAGACAGGTCGCC
A.f.T.....G.....A.....a.....T.....t.....t.....t.....t.....t.....t.....T.....
A.s.T.....G.....A.....a.....T.....t.....t.....t.....t.....t.....t.....T.....
A.p. L A E I V K K T G A S P R L L G R I L R T Q A A F G L I K E T G P

0451 AAGAGTATACCTCAGTGCAATTTACCGATGCTTTGGCAACTCGCCAGTGGGTGCAACTgtatggtttatccacagtcggcgttgagtagtataactaataactctgtggcctgttttagATTTCGACATCTCGGACC
A.f.G.....A.....A.....a.....T.....t.....t.....t.....t.....t.....t.....T.....
A.s.G.....A.....A.....a.....T.....t.....t.....t.....t.....t.....t.....T.....
A.p. Q E Y T S S A F T D V F A N S D A A G A V V Q L F D I S G P

0601 TTGCACCTCAGATATTGCCCTGACTTCCTGGCGAGAGGAATTAACAAGATATCACCTTAACAAGACTGCGCTTCCAAAAGGTTTCGGCAGTGACCTAACCATGTTGAGTGGATGCCAGCATCCCAAGCATGGAAATCACTCGG
A.f.C.....A.....T.....
A.s.C.....A.....T.....
A.p. C T Q I L P D F L A E R N Y Q D I T S N K D C V F Q K A F G S D L T M F E W M P Q H P K H M E S L G

0751 TCAFTTAATGGCTTTGGAGGCTCCCGTTTCCTGGTGACCACTACCTGTTTCGGGCTCCTGACAGGTATTAATGGTTGATATCGGTGGAGGCTTTGGGAGGAGTCCGAAGCCCTCGCGCGGAAGTT
A.f.C.....T.....T.....T.....A.....A.....A.....A.....A.....A.....A.....A.....
A.s.C.....T.....T.....T.....A.....A.....A.....A.....A.....A.....A.....A.....
A.p. H L M A L E R P V S W V D H Y P V L E E L G G F P A P D K V L M V D I G G F G Q Q S K A L R A K F

0901 TCCCGACCTTCCTGGCTTATAGTTCAAGATATCCCTCAACTCTGGCCCAAGCCGACACCAGCGCGGATCGAGTTCATGGACACAACCTTCTCGAGCCACAGCCCTATTCCAGAAATGCCAAAGTTCTACTATTTCCGTCATGCTCT
A.f.A.....C.....
A.s.A.....C.....
A.p. P D L P G R L I V Q D I P Q T L A N A Q P A A G I E F M E H N F E P Q I O N A K F Y Y L R H V F

1051 CCATGACTGGCCAGATGAACAATCGCTTCATCTTGAAGCAGATATCCAGGATGGACCGGATGCCAGATTTGATTGACGAGATGGTGAATCCAGTCCGGGGTCCCGGTGGCAAGCGGGTTTACAGATCTCTCATGATGAA
A.f.A.....
A.s.A.....
A.p. H D W P D E Q C V L I L K Q I I P A M G P E S Q I L I D E M V I P S T G V P W Q A A F T D L L M M N

1201 CTCTCTCGGGGAGTAGAGGCCACACCGCGGAATTTGAGGACGAGTGGCAATTTCCAGTAAAGGTTTACACAGTAAAGAGGAGCCATCTGGTCTCTACTAAAGGACCTAAGGGTTTCCCGAGAC
A.f.G.....C.....
A.s.G.....C.....G.C.G.A...TAAAC.....T.....
A.p. S I G G V E R T R A E W D D L M E Q V G L E I I Q S K V Y D S K E Q A I L V A V P K R T *
TAA

A	<i>A.p. avfA</i>	MVTYALLGATGATGSSILRHLLQKSPDS-LHIQVLRSKVKLLQAFPDL	49
	<i>A.f. avfA</i>	MHE.....-R..I.....N.Q	49
	<i>A.s. avfA</i>	M-	49
	165 avfA1	M-	49
	<i>A.n. stcO</i>	MPSV.....YSGSS.D.TVN.....S...A...S.D	50
	<i>A.p. avfA</i>	-----TTRRPQVHVIQGMSTSDALSECLRNASIVFMCVAQNGSPIGTT	93
	<i>A.f. avfA</i>	-----...N.....T...P.....	93
	<i>A.s. avfA</i>	-----...Q.....R.....A.....	93
	165 avfA1	-----	93
	<i>A.n. stcO</i>	KPRPSV.SSI.TIRIFE.D..NP.V.CAV.QD..L.....M...	100
	<i>A.p. avfA</i>	LCQDSAR P IISVLQQQQSEGASYQPCTIVQLRSASLNPALAAQVPFVH	143
	<i>A.f. avfA</i> AQ.....	143
	<i>A.s. avfA</i> A	143
	165 avfA1 A	143
	<i>A.n. stcO</i>	.V.NT. AAL .EARRR.A.PR.E---L.VI.....V..V..R...	146
	<i>A.p. avfA</i>	RIVSFCLFANYADIKQACQYSEAKQGTLEYIILV D PPTLHDANGTHPTG	193
	<i>A.f. avfA</i>K.....Q.....I..... DQ...	193
	<i>A.s. avfA</i> D	193
	165 avfA1 N	193
	<i>A.n. stcO</i>	.V.C...A.G...LRR..VL.EA.ATE.L.Q.V.. DR..QT..	196
<i>A.p. avfA</i>	YRLIST-----EPQATALSADLGAAMCEIAHRESEFHGRAVGVAT	235	
<i>A.f. avfA</i>-----S.....Q.....	235	
<i>A.s. avfA</i>-----S.....H.....	235	
165 avfA1-----	235	
<i>A.n. stcO</i>D.TDMKDKENQR..IC.....V.....S.AD.L..QG.....	246	
<i>A.p. avfA</i>	GRVRQ T WGVLLRHLLGGSSRLRETI A KEAVVVRVLCIFLVILACLMSSL*	285	
<i>A.f. avfA</i>A.....A...K..Q.T..DG.VFA...V..Y..*	282	
<i>A.s. avfA</i>G.....*	285	
165 avfA1*	285	
<i>A.n. stcO</i>	.P....A..AGF....LGH.DYRYGR.N...LGV..L.LLGLL.Y.IKR*	297	
B	<i>A.p. omtB</i>	MTGLDMETIFAKIKEEYARTDDVGKRQIQGHIRELQVGFYSDWDVVMRLS	50
	<i>A.f. omtB</i>	M ...M.....D.....	50
	<i>A.s. omtB</i>	M ...M.....A.....	50
	<i>A.n. stcP</i>	---- MDA ..KQ..D...A.EH...Q...Y.....	45
	<i>A.p. omtB</i>	SGPLQVALTKVGIDLGIFRSLKESDTPITLAEIVKKTGASPRLLGRILRT	100
	<i>A.f. omtB</i>A..A.....F.....	100
	<i>A.s. omtB</i>	100
	<i>A.n. stcP</i>A.IA...DL..T...EA.LS..QLAE.....K.....	95
	<i>A.p. omtB</i>	QAAFGLIKETGPQEYTS A FTDVFANS D AAGAVVQLFDISGPCTQILPDF	150
	<i>A.f. omtB</i>	150
	<i>A.s. omtB</i>	150
	<i>A.n. stcP</i>S.P.....IA.....L...Y	145
	<i>A.p. omtB</i>	LAERNYQDITSNKDCV F QKAFGSDLT M FEWMP Q HPKH M ESLGH L MA L ER P	200
	<i>A.f. omtB</i>	200
	<i>A.s. omtB</i>	200
	<i>A.n. stcP</i>	..TG..E.V...E.P.....HTSQ.L... MK.....Q..	195
	<i>A.p. omtB</i>	VSWVDHYPVLEELGGFPAPDKV MVDIGGGFG QSKALRAKFPDLPGR L I	250
	<i>A.f. omtB</i>F.I.....K..... VDIGGGFGN.....	250
	<i>A.s. omtB</i> VDIGGGFG	250
	<i>A.n. stcP</i>	TV...F...Q..E..M...T.. VDIGGGFGSRC.NVE.KI.	245
	<i>A.p. omtB</i>	VQDIPQTLANA Q PAAGIEFMEHNFFEPQPIQNAKFYILRHVFHDWPDE Q C	300
	<i>A.f. omtB</i>	300
	<i>A.s. omtB</i>	300
	<i>A.n. stcP</i>	..M.....S.E..E.V..S..D..Q..VKG.....L.....	295
	<i>A.p. omtB</i>	VLILKQIIPAMGPESQILIDEMVIPSTGVPW Q AAFTDLLMNSLGGV E RT	350
	<i>A.f. omtB</i>	350
	<i>A.s. omtB</i>	350
	<i>A.n. stcP</i>	.Q..Q.V...A...R...V..V.....M.....E.FASI...	345
	<i>A.p. omtB</i>	RAEWDDLMEQV G LEIIQSKVYDSKEQAILVAVPK R T*	386
	<i>A.f. omtB</i>A.....A...*	386
	<i>A.s. omtB</i>A.....*	386
	<i>A.n. stcP</i>EA..DKA..K..EYY..G.....VI..*	379

Table 1
Percentage identities among several *Aspergillus* species

	nt (%) ^a	aa (%) ^b
<i>avfA</i> gene		
<i>A. parasiticus</i> and <i>A. flavus</i>	92.4	89
<i>A. parasiticus</i> and <i>A. sojae</i>	99	98
<i>A. flavus</i> and <i>A. sojae</i>	92.5	88.4
<i>A. parasiticus</i> wild type and mutant	99.8%	99.3
<i>A. parasiticus avfA</i> and <i>A. nidulans stcO</i>	57	55
<i>omtB</i> gene		
<i>A. parasiticus</i> and <i>A. flavus</i>	96	97
<i>A. parasiticus</i> and <i>A. sojae</i>	99	99
<i>A. flavus</i> and <i>A. sojae</i>	96.1	97
<i>A. parasiticus</i> wild type and mutant	100	100
<i>A. parasiticus omtB</i> and <i>A. nidulans stcP</i>	68	75

^a nt, at the nucleotide level in the gene.

^b aa, at the amino acid level in the protein.

was determined in this study. A search for a possible sequence homology in the GenBank database demonstrated that there were three open reading frames within this region: (1) *verB* (Accession No. AF106958), with homology to a desaturase involved in the conversion of versicolorin B (VER B) to versicolorin A (VER A) (Kelkar et al., 1997); (2) *omtB*, showing homology to *stcP* in *A. nidulans* for an *O*-methyltransferase activity (Kelkar et al., 1996); and (3) an as-yet uncharacterized transcript encoding for an oxidase or dehydrogenase named *avfA* in this study (note that part of the *avfA* cDNA sequence was formerly named *ord2*, Yu et al., 1995). As discussed later, these transcripts were expressed only in aflatoxin-conducive medium (GMS) and not in the non-aflatoxin-conducive medium (PMS). A careful comparison of the cosmid clones used in the transformation experiments revealed that the cosmid clones 8B9, 13B9, and 20B11 contained the DNA region harboring *omtA*, *omtB* and *avfA* genes while 30E1 and 5E6 did not (Fig. 1). Also, the functions of the *omtA* and *omtB* genes are known, suggesting that the complementation of the AVF-accumulating mutant was the result of the *avfA* gene for the conversion of AVF to VHA. In further complementation studies, using subcloned DNA region (Fig. 1, pAVF) containing only the functional copy of *avfA* gene sequence, the transformants were also able to convert AVF to VHA and produce aflatoxins (Fig. 2, lane 4). Thus, the *avfA* gene

was confirmed to be involved in the conversion of AVF to VHA in aflatoxin biosynthesis.

3.2. *A. parasiticus avfA* homologs in *A. flavus*, *A. sojae*, and *A. nidulans*

The sequences of genomic DNA and cDNA between *omtA* and *avnA* genes from *A. flavus* and *A. sojae* were also determined in this study. Fig. 3 shows a comparison of the genomic DNA and cDNA sequences of *avfA* from the three *Aspergillus* species, together with the translated amino acid sequences. The *avfA* coding sequence of the three *Aspergillus* species from translation start to translation stop consists of 858 nt with no intron identified and is capable of encoding 285 aa with a calculated molecular mass of 30.9 kDa (Fig. 3). The *avfA* gene is located very close to the *omtB* gene with only a 173 bp intergenic region between the *omtB* translation stop and the *avfA* start codons. No regulatory protein-binding motif was found in the *avfA* promoter region; however, two putative TATA sequences were identified at –55 and –150 positions to the *avfA* translation start (Fig. 3). The *A. parasiticus avfA* genomic DNA sequence showed 92.4 and 99% identity to that of *A. flavus* and *A. sojae* (Fig. 3, Table 1). At the aa level, the *A. parasiticus avfA* gene product showed 89% identity to that of *A. flavus* and 98% identity to that of *A. sojae* (Fig. 4A; Table 1).

Sequence homology analysis identified that the functionally undefined *stcO* transcript in *A. nidulans* may be a homolog of *A. parasiticus avfA* gene. The 55% identity at the aa level (Fig. 4A; Table 1) between the *avfA* gene product in *A. parasiticus* and that of *stcO* in *A. nidulans* is significant and is consistent with the degree of homology observed for other aflatoxin/sterigmatocystin (ST) pathway genes between the two organisms. The gene products of *avfA* and *stcO* were similar in polypeptide size and in molecular mass. Since the three species *A. parasiticus*, *A. flavus* and *A. nidulans* carry out the synthesis of aflatoxins/ST, it is safe to assume that the *avfA/stcO* genes are functional in the three organisms. However, the functionality of the *avfA* gene in *A. sojae* was not determined, but it can be assumed to be functional since the *avfA* gene product of *A. sojae* showed a 98% aa sequence homology to that of *A.*

Fig. 4. Comparisons of aa sequence homologies of *avfA* and *omtB* gene products from *A. parasiticus*, *A. flavus*, *A. sojae*, *A. parasiticus* mutant strain SRRC 165, and *A. nidulans*. The deduced aa sequences of *avfA* (A) and *omtB* (B) from *A. parasiticus* SRRC 143 (labelled as A.p), *A. flavus* (A.f.), *A. sojae* (A.s.), *A. parasiticus* mutant strain SRRC 165 (Ap165) and *A. nidulans* (A.n.) were aligned [the *omtB* gene product from SRRC 165 is 100% identical to the wild type and is therefore omitted in (B)]. The identical aa residues to the *A. parasiticus* wild-type strain SRRC 143 (A.p.) are represented by a dot (.), and gaps are represented by a dash (-). Numbers to the right refer to the aa positions to the right. The first amino acid methionine (M), the reported first amino acid methionine of *stcP* gene in *A. nidulans*, and the translation termination (*) are shown in bold. The aa replacement at positions #101 and #179 (A) and the highly conserved amino acid residues of the *omtB* SAM binding motif (B) are shown in bold as well.

parasiticus with no apparent genetic defects in the *A. sojae avfA* promoter and coding region.

3.3. Point mutation disables the functionality of the *avfA1*

To examine further why *A. parasiticus* mutant strain SRRC 165 was unable to carry out the conversion from AVF to VHA, we cloned and sequenced the entire region of the mutant allele, *avfA1*, gene from mutant strain SRRC 165 (Fig. 3, where only the region containing the two point mutations is presented and the identical sequences omitted). Sequence comparison of the non-functional *avfA1* allele from the mutant strain to the functional *avfA* allele in wild-type strain revealed that, in the entire *avfA1* DNA sequence, only two nt mutations resulted in two amino acid substitutions (Figs. 3 and 4A). A C→G transversion at nt 1810 resulted in alanine (A) instead of proline (P) at aa position 101, and a G→A transition at nt position 2045 resulted in asparagine (N) instead of an aspartic acid (D) at aa position 179. Sequence comparison showed that the *avfA* gene from *A. flavus*, *A. sojae*, and *A. nidulans* contained the mutated alanine (A) at aa position 101 (Figs. 3 and 4A). The *avfA* gene in *A. flavus* and *A. nidulans* is functional, so the amino acid change at position 101 from proline (P) to alanine (A) apparently does not affect the enzymatic activity of the *avfA* gene product. The *avfA* gene and its gene product in *A. flavus*, *A. sojae*, and *A. nidulans* at nt position 2045 were found to be identical to the *avfA* gene from the aflatoxigenic *A. parasiticus* wild-type strain (Figs. 3 and 4A). The change of amino acid residue at position 179 in the *avfA1* gene product of the *A. parasiticus* mutant SRRC 165 (Fig. 4A) suggested that the point mutation at this position may be responsible for the loss of the enzymatic activity in the mutant strain.

3.4. *omtB* gene from *A. parasiticus*, *A. flavus* and *A. sojae* is a homolog of *stcP* in *A. nidulans*

In addition to *avfA*, the *omtB* gene was sequenced from *A. parasiticus*, *A. flavus* and *A. sojae* (Figs. 1 and 3). The *omtB* gene is located 1.3 kb away downstream from the *omtA* gene (Yu et al., 1995) on the aflatoxin biosynthetic pathway gene cluster. The *omtB* coding region of the three species consists of 1650 bp genomic DNA sequence (Fig. 3, base # –296 to #1350). There are three introns of 63, 50, and 62 bp each within the coding region (Fig. 3). The 1161 bp *omtB* cDNA sequence is capable of encoding a polypeptide of 386 aa with a calculated molecular mass of 43.1 kDa. In the promoter region, a typical aflatoxin regulatory protein (AFLR) binding motif (TCGN₅CGA, Fig. 3, Ehrlich et al., 1999) was located at –216 to –206. Two putative TATA sequences were also identified (Fig. 3, underlined).

The *A. parasiticus omtB* genomic DNA coding region showed 96% identity to that in *A. flavus*; 99% identity

to that in *A. sojae*. At the aa level, the *A. parasiticus omtB* gene product, *O*-methyltransferase B, showed 97% identity to that of *A. flavus* and 99% identity to that of *A. sojae* (Fig. 4B). The comparisons of homology among several *Aspergillus* species are summarized in Table 1.

Sequence comparison demonstrated that the *omtB* gene showed significant homology at the genomic DNA level (68% identity) to that of the *stcP* gene in *A. nidulans*, with a 75% identity between their gene products (Table 1). The *stcP* gene product has been characterized to encode an *O*-methyltransferase for the conversion of DMST to ST (Kelkar et al., 1996). The *omtB* gene showed much lower sequence homology (less than 25%) to the gene, *omtA*, encoding for another methyltransferase in aflatoxin pathway gene cluster both at nt and aa levels in *A. parasiticus* and *A. flavus*. However, a typical SAM binding motif found in over 90 methyltransferases, including the *omtA* gene product, with the conserved aa residues VDXGGGXG was identified at aa #225 to #232 in the *omtB* gene product (Fig. 4B). Thus, it is reasonable to assume that the *omtB* in *A. parasiticus* is a homolog of *stcP* in *A. nidulans*. A recent study (Motomura et al., 1999), published while this manuscript was in review, shows that *omtB* (named *dmtA* in that study) is required for the conversion of DMST to ST and DHDMST to DHST since the sequence of *dmtA* is 100% identical to *omtB*.

It should be pointed out that the total length of the *O*-methyltransferase gene, *stcP*, in *A. nidulans* reported earlier (Kelkar et al., 1996) was incomplete. Comparisons with the *omtB* sequence reported here from *A. parasiticus*, *A. flavus* and *A. sojae* as well as an independent determination by Motomura et al., 1999 (*dmtA*) show that the coding sequence of *stcP* should be 1140 nt instead of 612 nt reported earlier and should code for a protein consisting of 397 aa instead of 204. Based on our observation with the coding region of *omtB*, the discrepancy may have arisen from defining the real translation start of the *stcP* gene, which should have been much earlier than that reported (Fig. 3; nt # 716 in bold), and consequently, they reported a protein 175 aa shorter than that reported in this study for the *omtB* gene product (Fig. 4B).

3.5. Expression of *avfA*, *avfA1* and *omtB*

Aflatoxin pathway genes are demonstrated to be expressed only in some media such as the aflatoxin-conductive GMS medium but not in the others such as the non-conductive PMS medium. The aflatoxin pathway gene expression is known to be regulated by *aflR* when AFLR binds onto the specific sequence, *aflR* binding motif, in the promoter region of the aflatoxin pathway genes (Ehrlich et al., 1999). To investigate the expression pattern of the *avfA* and *omtB* genes, RT-PCR experiments were carried out (Fig. 5). The *avfA* gene from *A. parasiticus* wild-type strain SRRC 143 (Fig. 5A, lanes

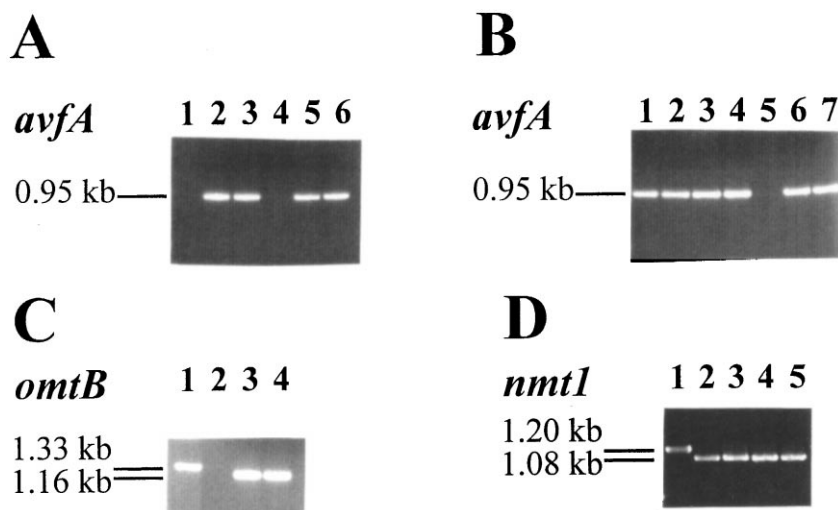


Fig. 5. Detection of gene expression by RT-PCR. RT-PCR experiments were performed to detect *avfA* (A and B) and *omtB* (C) mRNAs from mycelia grown in GMS and PMS media. The constitutively expressed gene, *nmt1* (D) in thiamine biosynthetic pathway, was included as a positive control. Template RNAs were purified from mycelia grown in PMS medium for 48 h and from mycelia grown in GMS medium for 24 and 48 h after shifting from PMS medium. PCR products were separated in 2% agarose gel under 80 V for 90 min. (A) Primers specific to the *avfA* coding region. *avf-723F*: 5' **ATGGTCACATACGCCCTCCTCGGG** 3' *avf-1675R*: 5' **GCCTCGCATTCTCTCGGCGACCGAA** 3' Lanes 1–3, *A. parasiticus* wild-type strain SRRC 143 cDNA as template; lanes 4–6, *A. parasiticus* mutant strain SRRC 165 cDNA as template; lanes 1 and 4, amplified from mycelia grown in PMS medium for 48 h; lanes 2 and 5, amplified from mycelia grown in GMS medium for 24 h; lanes 3 and 6, amplified from mycelia grown in GMS medium for 48 h. (B) Primers specific to the *avfA* coding region same as in (A). Lanes 1–4, genomic DNA as templates. Lane 1, *A. parasiticus* wild-type strain SRRC 143; lane 2, *A. parasiticus* mutant strain SRRC 165; lane 3, SRRC 165 complemented with cosmid 8B9; lane 4, SRRC 165 complemented with subclone pAVF; lanes 5–7, cDNA of *A. parasiticus* mutant strain SRRC 165 complemented by cosmid 8B9 as template; lane 5, PMS medium; lane 6, GMS medium grown for 24 h; lane 7, GMS medium grown for 48 h. (C) Primers specific to the *omtB* coding region in *A. parasiticus* wild-type strain SRRC 143. *OMTB-F*: 5' **GTTACAAGCTTGACGGGCCTTG** 3' *OMTB-R*: 5' **AGGTACAGCAACCAAGATGGC** 3' Lane 1, genomic DNA as template; lanes 2–4, cDNA as template. Lane 2, PMS medium grown for 48 h; lane 3, GMS medium grown for 24 h; lane 4, GMS medium grown for 48 h. (D) primers specific to the *nmt1* gene coding region in *A. parasiticus* wild-type strain SRRC 143. *nmt-35F*: 5' **CTGTGCCAAGATGTCTACTGACAAG** 3' *nmt-1096R*: 5' **CCTCAGGACGTGGTTCGTAGCTACTT** 3'. Lane 1, genomic DNA as template; lane 2, cDNA from PMS medium grown for 48 h; lane 3, 4, and 5, cDNA from GMS medium after 12, 24, and 48 h growth, respectively.

1–3) and the *avfA1* mutant allele from *A. parasiticus* mutant strain SRRC 165 (Fig. 5A, lanes 4–6), as well as the *A. parasiticus* mutant strain SRRC 165 complemented with *A. flavus* cosmid clones 8B9 (*avfA1* plus *avfA*, Fig. 5B, lane 5–7), were expressed only in GMS medium but not PMS medium (Fig. 5A, lanes 1 and 4; Fig. 5B, lane 5). Complementations of SRRC 165 with cosmid clones 13B9 and 20B11 were also performed and gave the same results as that of 8B9 (data not shown). A 0.95 kb *avfA* cDNA fragment was amplified from RNA isolated from the mycelia grown for 24 and 48 h in GMS medium (Fig. 5A, lanes 2 and 3 from SRRC 143, lanes 5 and 6 from SRRC 165; Fig. 5B, lane 6 and 7 from SRRC 165 complemented with cosmid 8B9) but not from the PMS medium (Fig. 5A, lanes 1 and 4; Fig. 5B, lane 5). The 0.95 kb *avfA* genomic DNA fragments were amplified from *A. parasiticus* wild-type strain SRRC 143 (Fig. 5B, lane 1), mutant strain SRRC 165 (Fig. 5B, lane 2), SRRC 165 complemented with *A. flavus* cosmid 8B9 (Fig. 5B, lane 3) and SRRC 165 complemented with *A. flavus* subclone pAVF (Fig. 5B, lane 4). They were found to be the same length as the corresponding cDNA fragments since there was no intron sequence in *avfA* gene. The PCR bands in Fig. 5B,

lanes 3, 4, 6, and 7 are more intense than those in lanes 1 and 2 due to an additional copy of the *avfA* gene in the genome contributed from the *A. flavus* cosmid clones.

The *omtB* gene was also shown to be expressed in aflatoxin-conducive medium. An 1.16 kb cDNA fragment was amplified from RNA isolated from mycelia grown for 24 and 48 h in GMS medium (Fig. 5C, lanes 3 and 4) but not from the PMS medium (Fig. 5C, lane 2). The cDNA fragment was smaller than the genomic DNA (Fig. 5C, lane 1) due to the three introns detected in the *omtB* gene. For positive control, the constitutively expressed (independent of sugar source) *nmt1* gene (Cary and Bhatnagar, 1995) in thiamine biosynthesis pathway was included. A 1.08 kb cDNA fragment was amplified from both PMS and GMS media (Fig. 5D, lanes 2, 3, 4, and 5 for PMS 48 h, GMS 12, 24, and 48 h respectively).

4. Discussion

4.1. Gene copy number

In this study, we have presented the cloning and characterization of *avfA* from *A. parasiticus*, *A. flavus*,

and *A. sojae* as well as *avfAI* from *A. parasiticus* mutant strain SRRC 165. Complementation of the mutant strain SRRC 165 by a single copy of *avfA* gene, the aflatoxin-producing ability was restored. This implies that there is only one copy of the *avfA* gene in the *A. parasiticus* genome. We have also cloned the *omtB* gene in these *Aspergillus* species and found it to be a homolog of the *stcP* gene characterized in *A. nidulans*. PCR experiments, using multi-pair primers inside and outside the coding regions of the *avfA* and *omtB* genes, also demonstrated that there is only one copy of both *avfA* and *omtB* genes in the *A. parasiticus* genome (data not shown).

4.2. Expression of *avfA*, *avfAI* and *omtB*

Aflatoxin biosynthesis is a complex process involving over 20 structural genes and probably under complex regulation. So far, it is understood that the coordinated expression of these structural genes is regulated by the positive regulator, AFLR protein, which is encoded by the *aflR* gene located in the middle of the aflatoxin pathway gene cluster. Upon binding of the AFLR protein to the specific sequence, TCGN₅CGA, in the promoter region (Ehrlich et al., 1999), the transcription is initiated. In the promoter regions of the aflatoxin biosynthetic pathway genes, there is normally a typical AFLR binding motif in addition to TATA boxes. Two AFLR binding motifs are shown in Fig. 3: one in the *omtB* promoter region for *omtB* gene activation, and the other located downstream of the *avfA* gene in the *verB* promoter region. However, no AFLR binding site was found in the *avfA* promoter region. One possible scenario is that the *avfA* gene is transcribed together with the *omtB* gene and processed afterwards into two mRNA species. It may also be the case that the AFLR binding is important but not absolutely required. As in most of the primary pathway gene transcription, TATA sequence alone may serve a purpose for transcription initiation of some aflatoxin pathway genes under certain conditions. However, this latter scenario seems unlikely because most of the genes in the aflatoxin biosynthetic pathway have an *aflR* binding motif (Ehrlich et al., 1999). It may also be possible that the *aflR* binds to some promoter component other than the identified motif. The expression pattern of both *avfA* and *omtB* (Fig. 5B and C) was consistent with the typical aflatoxin pathway gene, *omtA*, indicating that both genes were under the positive regulation by *aflR*.

RT-PCR data demonstrated that the *avfAI* mutant allele was expressed in aflatoxin-conducive medium (GMS) similar to the wild-type allele *avfA* (Fig. 5). Sequence comparison indicated that there was only one critical aa substitution at aa position 179 between the non-functional *avfAI* gene product in the mutant strain SRRC 165 and the functional *avfA* gene product in *A. parasiticus*, *A. flavus*, and *A. nidulans*. We, therefore,

conclude that the aa replacement at position 179 from the acidic aspartic acid (D) to the neutral asparagine (N) is critical to the function of the enzyme, the substitution rendering the *avfAI* gene product in the mutant strain *A. parasiticus* SRRC 165 (Fig. 4A) non-functional, rather than a defect at transcriptional level.

4.3. Role of the *avfA* gene in the conversion of AVF to VHA

The conversion from AVF to VHA may involve more than one enzymatic activity (Bhatnagar et al., 1992). The proposed enzymatic reactions may involve AVF oxidase or dehydrogenase and monooxygenase activities. Yeast expression of the *avfA* gene was unable to convert exogenously supplied AVF to VHA (data not shown). This suggests that the conversion of AVF to VHA may require more than one enzymatic reaction. The following experimental evidence obtained from complementation of *A. flavus* strain 649, which lacks the entire aflatoxin gene cluster, suggests that other enzyme activities may be involved in the conversion of AVF to VHA. When cosmids 5E6 and 8B9 (Fig. 1) were introduced together into strain 649, the transformants accumulated AVF (Prieto et al., 1996). The addition of cosmid 13B9 to the above 5E6 and 8B9 complemented transformant restored aflatoxin biosynthesis, suggesting that additional gene(s) involved in the conversion of AVF to VHA were located in 13B9 (Fig. 1). A fragment from 13B9 was subsequently found to restore aflatoxin production in transformants containing 5E6 and 8B9. This complementing DNA fragment contained two additional genes *cypX* and *moxY* (Fig. 1; Yu et al., 2000), suggesting that one or both of these genes are involved in the conversion of AVF to VHA (Prieto et al., 1996). The *cypX* gene encodes for a cytochrome P450 monooxygenase and is a homolog of *stcB* in *A. nidulans* with a 67.4% aa homology. The *moxY* encodes for a monooxygenase and is a homolog of *stcW* in *A. nidulans* with a 69% aa homology. Without providing details, a recent report has postulated that the *stcB* and *stcW* may be involved in the conversion from AVF to VHA (Keller et al., 2000). It may be possible that all three of these genes, *avfA*, *cypX* and *moxY*, are responsible for the conversion from AVF to VHA. The exact role of the complementary effects of *avfA*, *cypX* and *moxY* genes in the bioconversion AVF to VHA is yet to be determined.

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