

IDENTIFICATION, ISOLATION, CLONING AND SEQUENCING A PARTIAL ANNEXIN GENE FROM *AUREOBASIDIUM PULLULANS*

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ABSTRACT

Background and Objectives: Annexin is the common name for genes and proteins that were identified as calcium-dependent phospholipid-binding proteins. Recently a more complex set of functions has been recognized for this superfamily of proteins including in vesicle trafficking, cell division, apoptosis, calcium signalling, mineralization, crystal nucleation inside the extracellular organelles-matrix vesicles (MVs) and growth regulation.

Methods: In the present work *Aureobasidium pullulans* strain PRAFS8 genomic DNA was extracted. Using designed primers from a highly conserved region of annexin genes of *Aspergillus fumigatus* and *Aspergillus niger* a 800 bp PCR product was obtained from degenerated PCR. The 800 bp PCR product was gel purified and cloned into *E. coli* using the suitable plasmid and standard cloning procedures. From grown transformed *E. coli*, plasmid was extracted and the presence of expected insert in the plasmid, was confirmed by digestion of plasmid by *Eco* R1 restriction enzyme.

Results: Gel purified 800 bp band was sequenced and submitted at NCBI gene bank with accession No.: AY848856. A phylogenetic tree for obtained partial gene of annexin was drawn using bioinformatic software in order to understand the evolutionary relationship of annexin genes between some microorganisms. Also southern analysis of 800 bp PCR product using digoxigenin (DIG) labeled probe demonstrated the probability of two copies of annexin genes existence in the *A. pullulans* genome.

Conclusion: This study for the first time presented the presence of annexin gene in yeast-like fungi and this result is important due to the existence of this superfamily of genes in moulds but not in yeasts.

We emphasize for future additional work to clone and sequence the full length of annexin gene(s) from *A. pullulans* and also additional studies for this gene expression and annexin mRNA transcription to understand the effective factors for expression of annexin.

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INTRODUCTION

Basically annexins have been identified and introduced as calcium-dependent phospholipid-binding proteins.^{1,3} Recent investigations confirmed a more complex set of functions for this superfamily of proteins in vesicle trafficking, cell division, apoptosis, calcium signalling, mineralization, crystal nucleation inside the extracellular organelles-matrix vesicles (MVs) and growth regulation.^{4,12} For example annexin 1 can be induced by glucocorticoids (GCs) in inflammatory cells and shares with these drugs many anti-inflammatory effects. This class of annexin proteins can play an anti-inflammatory role by direct PLA2 inhibitor, suppression of cytokine-induced activation of PLA2, functional link between annexin 1 and receptors of the formyl-peptide family and also both leucocyte recruitment and neutrophil apoptosis.^{13,17} Apoptosis of neutrophil granulocytes is a significant determinant of the declaration of inflammation. Apoptotic neutrophils undergo exact modifications in their receptor profiles. Such modifications are expected to supply to the attributed functional silencing of the failing cells.¹⁸ Although the mechanism of annexin 1-dependent glucocorticoids (GCs) signaling in the neuroendocrine system has been considered as a paracrine/juxtacrine mediator,¹⁹ based on the study of annexin A2 expression levels during cellular differentiation in myeloid cell lines, this protein has been identified as an important player in cellular differentiation and its disorders.²⁰ Guzman-Aranguéz has postulated an association between human colon adenocarcinoma cell differentiation with an up-regulation of annexin A1 (AnxA1), annexin A2 (AnxA2), and annexin A5 (AnxA5). Also he detected a correlation between a subcellular relocation of these proteins by changes in AnxA1, AnxA2, and AnxA5 levels and localization in human colon adenocarcinoma cells differentiated by butyrate treatment or by culture in glucose-free inosine-containing medium.²¹ Radiolabeled annexin V with technetium-99m has been employed for the *in vivo* study of apoptosis and is now undergoing clinical trials in both Europe and the United States.^{22,23} Using this technique, the exact monitoring of chemosensitivity as well as the early guess of chemoresistance in response to various pro-apoptotic interventions are critical supplies for the best management of oncology cases. The use of technetium-99m labeled annexin V provides enough evidences of belief for the viability of a non-invasive, *in vivo* discovery of an apoptotic indication and then for the early and inconvenient assessment of tumor response in the course of chemotherapy.²⁴ Moreover there is a hypothesis about the fitting of annexins into the complex molecular machinery that regulates the actin cytoskeleton as a mal-

leable framework of polymerised actin monomers.²⁵ From this point of view there are some suggestions and evidence about the contribution of these proteins in pathological consequences and sequelae of human disease such as cardiovascular disease and cancer.²⁶ Also many patients who present clinically with autoimmune-like pregnancy complications have antibodies against annexin V, phosphatidylserine, or other appropriate antigens.²⁷ Annexin's ubiquitous superfamily consists of more than a thousand proteins in different species of eukaryotic phyla except yeast, but no annexin has been isolated from prokaryotic phyla. At the same time, it is notable that numerous isolations of annexin proteins and genes have been identified from different species of molds.^{28,39}

Aureobasidium pullulans is a ubiquitous saprophyte with medical and agricultural importance and also biotechnological applications.^{40,41,42} This yeast like fungus has been classified among dematiaceous (feo) fungi because of its ability to generate a dark, melanin-like pigment that accumulates in the fungal cell wall.⁴³ This fungi can be easily isolated from the philosopher and from plant residues, soil, wood, air, and even the surface of stone.^{44,48} Also its isolation from clinical samples is normally considered as an environmental contaminant. Of the 14 species of *Aureobasidium* known to date, *A. pullulans* is the best known to medical mycologists, since it has been indicated as a rare etiologic agent of pheohyphomycosis, mycetoma, keratomycosis, fungemia, systemic mycosis particularly in immunodeficient patients, peritoneal sepsis, and dermatological infections.^{49,62}

During the last two decades with advances in medicine, mycoses due to opportunistic fungi are increasing dramatically and *A. pullulans* is considered one of them. The increased frequency of organ transplantations, the improvement of effective therapies and new surgical techniques for severe diseases such as cancer, have increased the chances of recovery for many patients otherwise destined not to survive. It is therefore not surprising that the rates of fungal opportunistic infections have increased among these severely ill patients. Indeed, there are no limits to the amount of fungal species in the environment that can potentially invade and disseminate in host organs, increasing the already high number of opportunistic microorganisms.^{63,64} With regard to differences on the presence of annexin between yeasts and moulds and due to the important role of these group of proteins in cell physiology the present assay was carried out for detection, isolation, cloning and sequencing of partial annexin gene(s) in this yeast-like fungus.

MATERIAL AND METHODS

Fungus and bacterial strains, source and maintenance

Aureobasidium pullulans strain PRAFS8 was donated kindly by Dr. Jeremy S. Webb from University of Manchester. *Escherichia coli* TOP 10 F' (Genotype: F {lacI^qTet^R} mcrA (mrr-hsdRMS-mcrBC) 80lacZM15lacX74 deo R rec A1 araD139 (ara-leu) 7697 galU galK rpsL endA1 nupG) competent cells were obtained from Invitrogen and were used for all cloning procedures. Competent cells in aliquots of 100 µL were stored at -80°C until required. Prior to use for transformation, cells were thawed slowly on ice.

For long-term storage of fungus, equal volumes of 1×10^6 mL spore suspensions and 40% (v/v) glycerol were mixed and 1 mL aliquots stored at -80°C.

Media

Potato Dextrose Agar (Oxoid) was used for routinely growing the fungus strain and Modified Vogel's Medium was prepared by filter sterilising (0.2 µm) a 50x stock of Vogel's salt solution which was stored at 25°C until required. For agar plates, 1.5 % (w/v) agarose (Lucas Meyer) was included with sugar to solidify the medium.

Luria-Burtani Medium (LB) was prepared by autoclaving AND for solid medium, 1.5 % (w/v) agarose (Lucas Meyer) was also included. When required, 0.1 % (v/v) of a filter sterilised solution of 50 mg ml⁻¹ ampicillin was added after autoclaving the medium.

Growth of Fungus

Spore production and viability

For the production of *A. pullulans* blastospore, fungi were grown at 25°C for 5 days in Vogel's modified agar media (1% w/v glucose replaced sucrose). To prepare blastospore, 10 mL of sterile citric acid buffer (0.05 M, pH 5), was spread on the surface of the colony and spores were collected by gently scraping the surface of the colonies with a flame-sterilised glass spreader. The spore suspension was filtered through Whatman lens tissue to remove mycelial fragments and sterile water diluted spores were counted using an Improved Neubauer hemocytometer to give a spore concentration of 1×10^8 mL⁻¹ and stored at 4°C. For long-term storage, blastospores were frozen at -80°C in a 15% (vol/vol) sterile glycerol solution.

Growth in liquid and solid media culture

For liquid cultures, 50 mL of modified Vogel's medium was inoculated with 0.1 ml of a 1×10^8 mL⁻¹ spore suspension in a 250 mL Erlenmeyer flask. Flasks were incubated for up to 5 days on a rotary shaker (250 rpm) at 25°C. For obtaining of biomass the culture broth was filtered and the mycelium washed with citrate buffer (0.05 M, pH 5.5) twice before suspending in buffer to bring to a concentration of 100 g wet cell mass L⁻¹.

Aureobasidium pullulans was grown on modified Vogel's medium as described above, except that the medium was solidified with 1.5% agarose (Luca Meyer).

Spore viability

To determine spore viability, spores of *A. pullulans* were diluted in sterile distilled water to 10^{-4} , 10^{-5} and 10^{-6} spores mL⁻¹. 50 µL of each dilution was spread onto three petri dishes on modified Vogel's medium and incubated overnight at 37°C. The total numbers of colonies were counted on plates which gave between 30 to 300 colonies and spore viability expressed and colony forming units (cfu) mL⁻¹.

Growth and maintenance of bacteria

For plasmid preparations, 2 mL or 4 mL of LB-broth (containing 1 or 2 µl respectively of a filter sterilised 50 mg mL⁻¹ ampicillin stock solution), was inoculated with *E. coli* TOP 10 F' either from a colony using a sterile toothpick or from a glycerol stock culture and incubated overnight at 37° (250 rpm) in a sterile universal tube.

For long term storage of bacterial strains, glycerol stocks were prepared by mixing an equal volume of overnight culture and 40% (v/v) glycerol and stored at -80°C.

Fungal genomic DNA extraction

Approximately 1.4 g of mycelium (wet weight) from 18 h (mid-log phase) liquid cultures was harvested by filtration, washed with ddH₂O twice and squeezed gently to remove excess liquid. Harvested mycelium were immediately frozen in liquid nitrogen, and if not required immediately, stored at -80°C.

Genomic DNA was extracted using a modified version of the method described by Griffin et al.⁶⁵ and quantified either by comparison with a standard of known DNA concentration on agarose gels (1 kb ladder, GIBCO BRL) or from the OD₂₆₀ : OD₂₈₀ ratio.

Degenerate primer design

Forward and reverse degenerate primers (AATATCCYCCATITCTTGAG and GAIAGAAITCACAGCGTG) for annexin gene were designed from highly conserved regions of amino acids from an alignment of the translated sequences of annexin gene from *Aspergillus fumigatus* (NCBI Accession numbers AY598938.1, AY598939.1, AY598940.1), *Aspergillus niger* (NCBI Accession number AY033935.1), *Aspergillus fumigatus* annexin ANXC4 (ANXC4) gene, complete cds gi|47059734|gb|AY598940.1|[47059734], *Aspergillus fumigatus* annexin ANXC3.2 (ANXC3.2) gene, complete cds gi|47059732|gb|AY598939.1|[47059732], *Aspergillus fumigatus* annexin ANXC3.1 (ANXC3.1) mRNA, complete

cds gi|47059730|gb|AY598938.1|[47059730], *Aspergillus niger* annexin XIV-like protein gene, complete cds gi|15809583|gb|AY033935.1|[15809583].

Degenerate PCR

PCR was performed in ready-made reaction tubes containing 1.5 units either Taq or Expand DNA polymerase, 10 mM Tris-HCl (pH 9), 50 mM KCl, 1.5 mM MgCl₂, 200 μM of each dNTP and stabilisers, and BSA in a 25 μL final volume (Amersham Pharmacia Biotech) including target DNA and designed primers. Optimum annealing temperature and magnesium concentrations were determined by individual reactions. A typical reaction mixture contained 100 ng genomic DNA, 10 pmol of each primer and 5 mM final concentration of reactions were carried out in 0.2 mL thin walled tubes (Alpha) and appropriate controls were included in conjunction with these reactions. For annexin gene, reactions were carried out with *A. pullulans* genomic DNA and degenerate primers. PCR reactions with each primer set were optimised by carrying out the reactions with MgCl₂ concentrations in the range 4-8 mM and varying the annealing temperatures. The optimal magnesium concentration was found to be 4 mM in the PCR conditions as shown below.

3 min 94°C x 1, 1 min 47°C x 1, 2 min 72°C x 1, 1 min 94°C x 29, 1 min 47°C x 29, 2 min 72°C x 29, 7 min 72°C x 1.

Restriction digestion of DNA and agarose gel electrophoresis

Digestion of DNA was carried out using restriction endonucleases (Boehringer Mannheim) and the appropriate 1x buffer, usually in a volume of 10 μL. For genomic DNA, the digestion was performed overnight at 37°C. For plasmid DNA, digests were usually performed at 37°C for 2 h. Generally agarose gel electrophoresis was carried out in BioRad horizontal gel kits for submerged electrophoresis using 1% (w/v) agarose (ICN Biochemicals) gels for the visualisation of PCR products and 0.8% (w/v) agarose gels for plasmid and genomic DNA following restriction digestion. After electrophoresis, gels were stained in an ethidium bromide bath before visualisation under a short wave UV light. Purification of DNA fragments from agarose gels was carried out using a QIAquick gel extraction kit (QIAGEN) according to the manufacturer's instructions.

Cloning PCR products and screening of transformant cells

A range of cloning vectors was used for cloning of DNA fragments. The use of a particular cloning vector was dependent on what type of cloning procedure was being carried out. Usually, PCR products were gel purified and cloned into *E. coli* Top-10F' (Stratagene) using

standard cloning procedures using the pGEM[®]-T easy vector system (Promega). This vector contains a multiple cloning site (MCS) containing a range of restriction enzyme recognition sites, the *lacZ* gene for blue-white selection and an ampicillin resistance gene for antibiotic selection.

Ligation reactions were typically carried out by incubating 1 μL of 10x reaction buffer, 1 μL vector (50 ng μL⁻¹), 100 ng μL⁻¹ purified PCR product and sterile ddH₂O (to make a final volume of 10 μL) and incubated at 4°C overnight. The insert to vector ratio was 2:1. After ligation, 3 μL of the ligation reaction was added to 100 μL of competent cells (*E. coli* Top 10 F', Stratagene), and incubated on ice for 20 min. Competent cells were provided in 100 μL aliquots and were stored at -80°C until required when they were left to thaw on ice before use. Following incubation with the ligation products, cells were incubated at 42°C for 2 min and returned to ice for a further 5 min. After the addition of 900 μL of LB, cells were incubated at 37°C for 1 h on a rotary shaker (250 rpm). 50, 100 and 150 μL of this culture was then spread directly onto X-gal / IPTG agar plates supplemented with 50 μg mL⁻¹ of ampicillin and incubated at 37°C overnight.

For the next step the blue/white colony selection was carried out on ampicillin agar plates containing 0.0265 mg mL⁻¹ X-gal (prepared as a stock solution at 400 mg mL⁻¹ in ddH₂O) and 0.3 mg mL⁻¹ Isopropyl β-D-thiogalactopyranoside (IPTG, prepared as a 50 mg mL⁻¹ stock solution in formamide) according standard method.

Screening of transformant cells was carried out using standard alkaline lysis protocol and for the rapid screening of recombinant transformants, plasmid DNA was prepared from cultures using the boiling method of Holmes and Quigley.⁶⁶

When a particularly clean plasmid preparation was required (e.g. for sequencing or subcloning), plasmid DNA was prepared using a QIAprep Spin Miniprep kit (Qiagen).

For southern analysis the 800 bp insert was cut from the plasmid with Eco RI, labeled with digoxigenin using the DIG-High Prime DNA Labeling kit (Roche) and was used to probe *A. pullulans* DNA that was restricted with *Hind* III, *Sma* I, *Xba* I and *Xho* I. Southern blots were washed at stringency condition (5×SSC washes) and blotting showed that the probe hybridised to a single band with each digest except *Xba* I (Line 3, Fig. 7). The *Xba* I digest produced a band of 1.1 Kb and a second band of 9.4 Kb, the *Hind* III digest a 5.1 Kb band, the *Sma* I digest a 3.9 Kb band and the *Xho* I digest a 1.6 Kb band (Fig.3).

DNA sequencing

All DNA products that required sequencing were

cloned using the pGEM[®]-T easy vector and sent either to MWG or Lark Technology for automated sequencing. The pGEM[®]-T easy vector contains T7 and SP6 sequencing sites 5' and 3' to the insertion site allowing direct sequencing of cloned PCR products from the vector (Fig.2).

Bioinformatics tools

A range of web-based tools were used for analysis of DNA and protein sequences. Novel DNA sequences were submitted for analysis through the Basic Local Alignment Search Tool (Basic BLAST version 2.0). BLAST was accessed through the National Centre for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/>) or through the Bioinformatics Centre, University of Kyoto (<http://blast.genome.ad.jp/>) to search for sequence similarity with sequences held on the BLAST database. Novel sequences were submitted in FASTA format through blastn (database nr) and BLASTX (database nr). In the case of BLASTN, the nucleotide sequence submitted was compared for similarity with all the nucleotide sequences held in the database. In the case of BLASTX, the six frame translation of the nucleotide sequence was compared with the translated nucleotide sequences held in the database. Standard/default parameter values were used in all cases.

DNA-to-protein translation of DNA sequences was performed using the EXPASY translate tool (<http://au.expasy.org/tools/dna.html>) using the standard genetic code.

For comparing reverse and forward strand sequences, one strand was reverse complimented prior to alignment using the reverse and compliment program at Lipper Centre for Computational Genetics, Harvard (<http://>

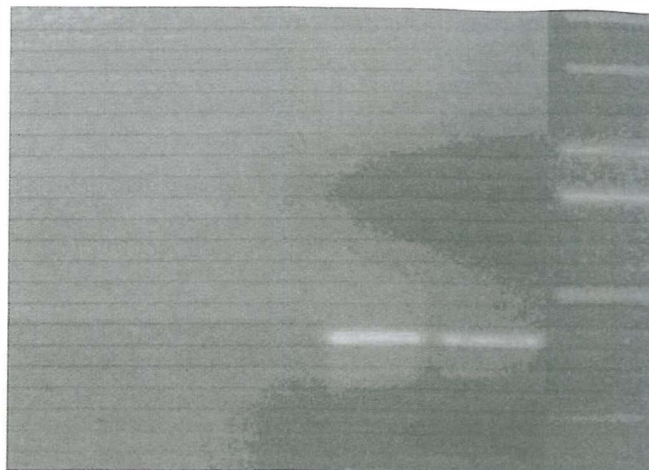


Fig. 1. Agarose gel electrophoresis of PCR products.

PCR was performed using the degenerate primers and products were visualised by agarose gel electrophoresis. Lanes 1, 2 and 3 show the results of the PCR in the absence of genomic DNA or forward primer (lane 4) or reverse (lane 5). Lanes 4 and 5 show the PCR products obtained with both primers with a concentration of MgCl₂ of either 4 or 6 mM respectively. Lane 6 shows the molecular weight marker, with band sizes (Kb) indicated at the right. The + at the bottom of the gel indicates the direction of the positive electrode during electrophoresis.

arep.med.harvard.edu/labgc/adnan/projects/Utilities/revcomp.html.

Multiple alignments was carried out using the <http://prodes.toulouse.inra.fr/multalin/multalin.html> and imported into boxshade. A phylogenetic tree was constructed using the <http://prodes.toulouse.inra.fr/multalin/>

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1  cccccatttc ttgagtaaga atttccacat ggccttccac ccaggcacgc attatcggcg
61  ggtgaaccgc cacctaataa gcataaaaga gaacaccgac cgcaacagcg gtatagtgct
121 gctatgcect tgtctcatgt ttaatgttct ccctattgcc gacatctccg acatggtcag
181 aatcgaccgc atggtcccta ggccctcatt aagccgtttg attatectct ccatcaggct
241 cttcgtctca ttaatcctgt gccattgttt gtctatgacc acggagaatt tgctcgccaa
301 gtcacatctg gttaaggett ctgctccga aaagcaacc tccgcttcga aaggtcacga
361 accagcgtgc ttaatacgcc taccaccaca cgcacgatgt acttggtctaa gagatgaaac
421 agcttattaa agactaccgc acgcccactcc gaaagcgacc cacagtcaaa gccctcgtct
481 tgccctgaaa acggccccgc tccatgaacc agagccccgc gcaagccaaa cagcaactaca
541 taacatatc ctcatgccag cettttctact tcctgtatat catcacacaat tatacgagtc
601 tgtatggcga atggcatcga cggccaata gggaggact acgaacaaaa gtaccaacca
661 tccttgatgg attatatgga agcaaaaagg aagaaccada tgcagcaaat gctgtctacg
721 actcttcaag cagccgctta tgagagcgg acgtatgact attagccaca ggcttaaacac
781 gctgtgattt ctttc

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Fig. 2. Sequence of partial annexin gene from *A. pullulans* (apanx1). Results of nucleotide sequence analysis have been highlighted as described in discussion.

multalin.html computer software using standard/default parameter values.

RESULTS

Amplification and cloning of a predicted 800 bp band from *A. pullulans* genomic DNA was carried out successfully and during the PCR procedure control reactions with individual primers, or with no template DNA, did not produce any amplification products (Fig. 1).

After extraction, the PCR products were analysed on a 1% (w/v) agarose gel electrophoresis, and bands with expected size (about 800 bp) were subcloned into pGEM®-T easy vector using a 3:1 ratio. Clones containing an insert were identified by blue/white selection and 12 clones that appeared to contain an insert were isolated at random. Following plasmid isolation, plasmids were digested with *Eco* R I and subjected to agarose gel elec-

trophoresis. Of the 12 clones isolated, 10 were found to contain an insert of ca. 800 bp (Data not shown).

Southern analysis

Southern blotting was carried out as described in Material and Methods. The result of southern analysis demonstrated the probability of two copies of annexin genes existence in the *A. pullulans* genome (Fig. 4).

A. pullulans genomic DNA was extracted, restriction digested and subjected to agarose gel electrophoresis. Following blotting, the membrane was hybridised with probe. Lane 1 shows the molecular weight marker, with band sizes (Kb) indicated to the left. Lanes 1-4 show *A. pullulans* genomic DNA restricted with *Hind* III, *Sma* I, *Xba* I and *Xho* I respectively. The position and size of the molecular weight markers are indicated on the left of the blot. The + shows the direction of the positive electrode during electrophoresis.

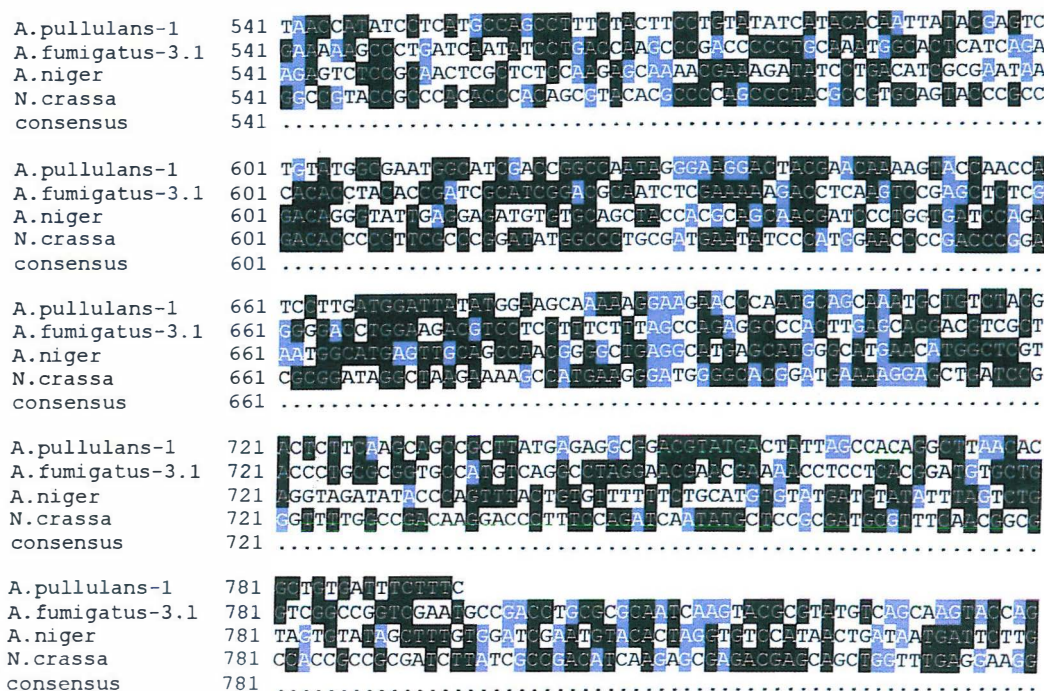


Fig. 3. Nucleotide sequence alignment of some fungal putative annexin gene fragments using multiple alignment software.

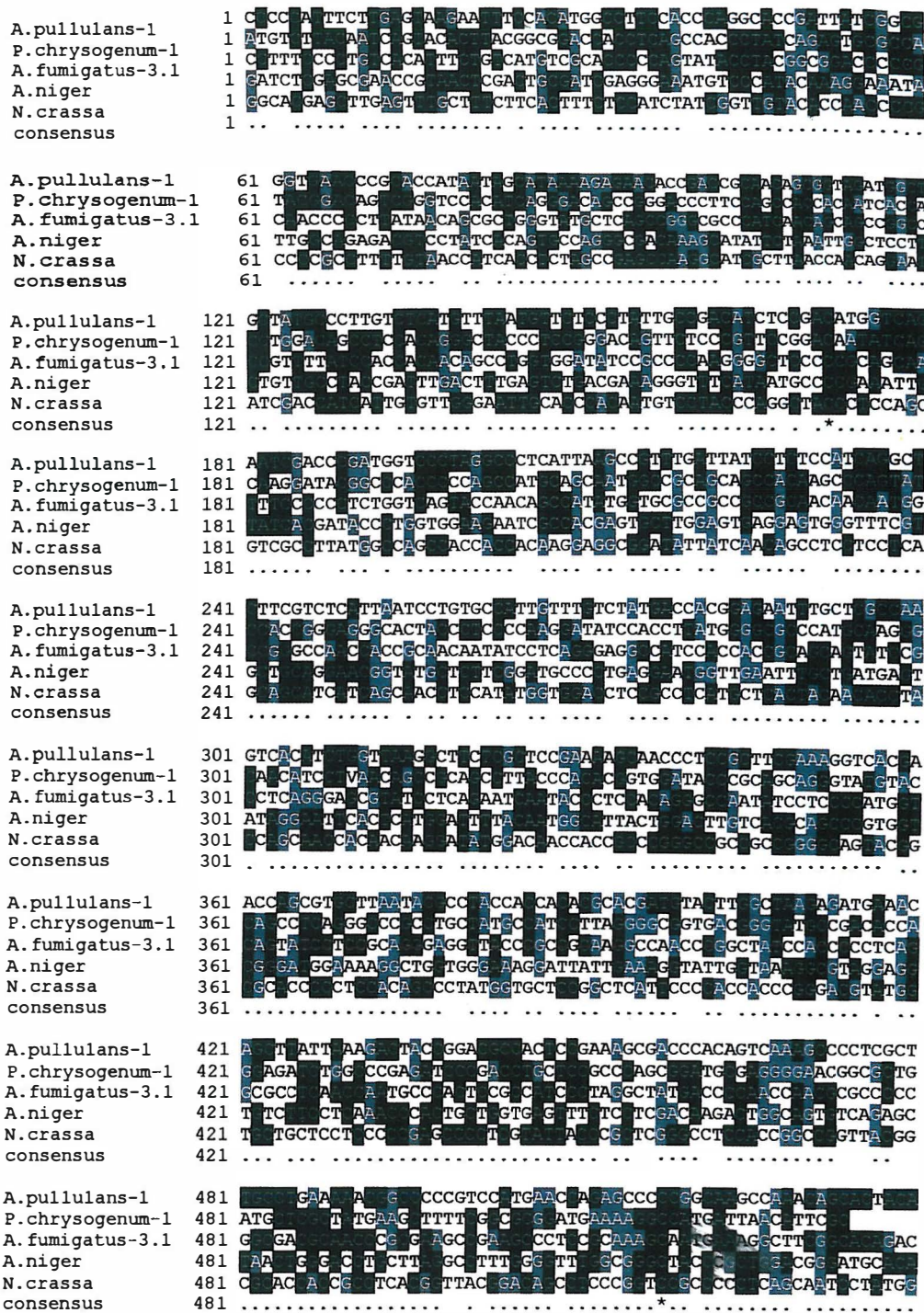


Fig. 3. Cont.

DISCUSSION

Aureobasidium pullulans mainly is an industrial fun-

gus with biotechnological applications and also medical and agriculture importance. This yeast like fungus has been classified among dematiaceous (feo) fungi and can

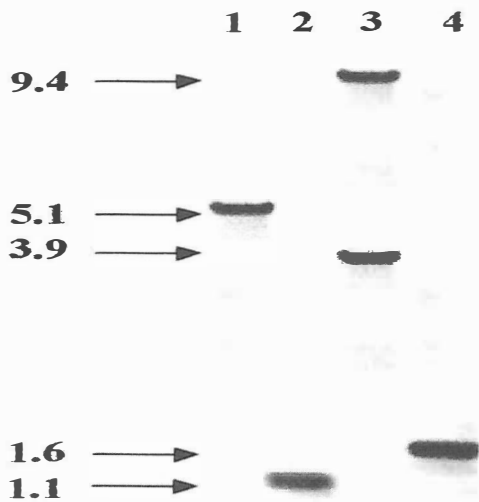


Fig. 4. Southern analysis of *A. pullulans* genomic DNA.

be easily isolated from the environment. Also its isolation from clinical samples is normally considered as an environmental contaminant. This is notable that the term “yeast like” is only a phrase for identification of this group of fungi and there is no specific relationship between this group and yeast.

Nucleotide sequence analysis

The nucleotide sequence of the cloned partial gene revealed a 908 bp without any intron that encodes a 264-amino-acid protein. From this 302 amino acid sequence only a 110 amino acid motif look-like a valid motif. The amino acid sequence exhibited high levels of homology with the amino acid sequences of two annexin genes at NCBI (USA) gene bank.

In the partial sequence we were able to recognize three TATAA-like boxes⁶⁸ at position 576 (TATAT), 590 (TTATA) and 672 (TTATAT) bp (red highlighted sequences) and three CAATs (CAAT box) at positions 626, 698 and 706 (blue italic highlighted CAAT, CAAT and CAAAT respectively). However we could not find 5' and 3' intron(s) flanking sequence, GTATGT and CAG respectively, and consequently the presence of any intron(s) and its consensus TACTCAC sequence that are commonly found in fungal intron(s). Here, we postulated that annexin genes are not intronless genes and usually in the full length of annexin genes there are intron(s) present: therefore, probable intron(s) may be located at an undefined part of this gene. Poly (A) sequences and also either predicted start or stop codon and CT rich sequence before start codon therefore; we were not able to hypothesize the C- and N terminal of

peptide. Three possible CREA binding sites (5'-[G/C][C/T]GG[G/A]-3')⁶⁹ were present at positions 58, 437 and 747 bp (under lined green sequences) in the nucleotide sequence of motif suggesting significant carbon catabolite repression of the annexin gene and deacylase activity can affect different carbon sources (Fig. 2).

Also no binding site was found for the transcriptional regulator PacC⁷⁰ that regulates gene expression in response to external pH (GCCARG) suggesting that the annexin gene is not pH regulated.

Analysis of the obtained sequences for the presence of GATA sequences which are recognized by the AREA nitrogen repression transcription factor⁷¹ did not show this element. Therefore, accordingly the obtained sequences would not appear to be under nitrogen repression but full length sequencing of the gene and also more studies on the expression level under strong (ammonium) and weak (nitrate) nitrogen repression would need to be conducted to confirm this.

Comparison of nucleic acid sequences

Three clones containing product inserts of ca. 800 bp were sent externally for sequencing following plasmid DNA isolation. After removal of 5' and 3' vector sequences and primers, sequences were subjected to multiple alignments. All three clones were identical except that one clone was on the opposite strand to the others. This was confirmed by multiple alignments of the nucleotide sequences after reverse complementing the third clone. Also multiple alignments of *A. pullulans* partial annexin gene with some fungal annexin genes using bioinformatics software showed similarity between aligned genes (Fig.3). For multiple alignment, sequences were aligned using the <http://prodes.toulouse.inra.fr/multalin/multalin.html> and imported into boxshade. When the sequence of the 795 bp PCR product was analysed by BLASTX, the translated sequence showed a significant homology to fungal and other eukaryotic annexin genes (Fig. 4). The partial annexin sequence was named *apanx1* and shared a 31% identity with annexin I gene from *Gallus gallus* (NCBI accession No. NM_206906) and 24% with annexin ANXC3.1. gene (NCBI accession No. AY598938) from *Aspergillus fumigatus* at the amino acid level.

Phylogenetic analyses

Phylogenetic analysis of sequences was performed using the <http://prodes.toulouse.inra.fr/multalin/multalin.html> computer software. The tree (Fig. 5) shows that annexin and potential annexin analogues are contained in a large cluster of the annexin super family in eukaryotes.

The first reported fungal annexin gene was *anx14*

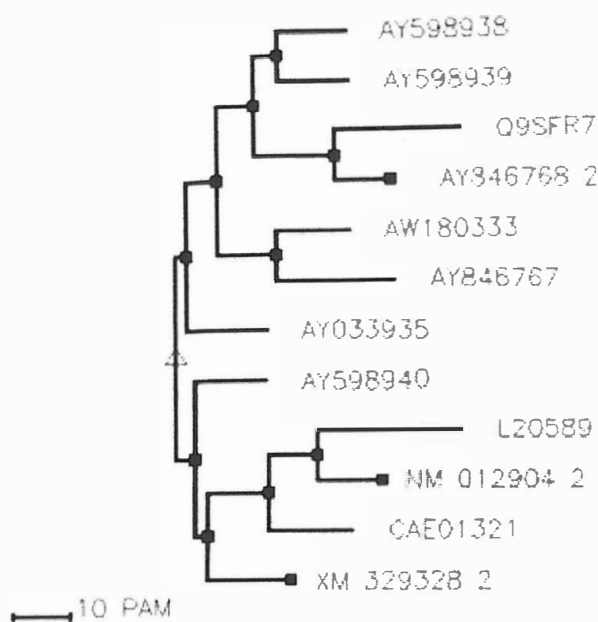


Fig. 5. Phylogenetic tree was drawn after multiple alignments of selected annexin sequences from NCBI gene bank with accession No(s). (*Aspergillus fumigatus*), AY598939 (*A. fumigatus*), Q9SFR7 (Soybean), AY598938 (*A. niger*), AW180333 (*Ciona intestinalis*), AY846767 (*Aureobasidium pullulans*), AY033935 (*Mycosphaerella graminicola*), AY846769 (*Penicillium chrysogenum*), AY846768 (*P. chrysogenum*), AY848856 (*A. fumigatus*), AY598940 (*A. fumigatus*), L20589 (Soybean), 012904 (Human), CAE01321 (*Neurospora crassa*), BQ627874 (Soybean), AABX01000038 (*N. crassa*), XM_329328 (*Rattus norvegicus*).

gene of the filamentous ascomycete *Neurospora crassa* by Braun et al. 1998.³⁹ Survey of Annexins phylogenetic tree in eukaryotic kingdom shows a complex evolutionary background and displays a large number of gene duplications and gene losses in different taxa, including the absolute defeat of annexin sequences from *Saccharomyces cerevisiae* as ascomyceteous yeast. Unexpectedly, the *N. crassa* annexin homologue is most strongly correlated to the annexin homologue of the slime mould *Dictyostelium discoideum*, postulating a phylogenetic relation between cellular slime moulds and true fungi. Both of these annexin homologues are strongly correlated to the family of annexin homologues existing in animals, an observation consistent with the existence of the animal-fungal clade.^{36,72} These data further suggest that the gene duplications that generated the family of annexin sequences existing in animals, fungi, and slime moulds began earlier to the divergence of these taxa.

Nucleotide sequence accession number

The nucleotide sequence reported in this paper has been submitted to NCNBI GenBank under accession number: AY848856.

We emphasize that additional experiments are needed to clone and sequence the full length of the annexin gene from *A. pullulans* and then more fully understand the factors that regulate the activity and control the destination of annexin. Also additional studies of annexin mRNA transcription in solid and broth cultures are needed to understand the effective factors for expression of annexin.

In conclusion this work for the first time revealed the presence of the annexin gene in yeast like fungi and this result is important due to the existence of this superfamily of genes in moulds but not in yeasts.

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