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Molecular cloning of verrucosidin-producing *Penicillium polonicum* genes by differential screening to obtain a DNA probe

E. Aranda [1], M. Rodríguez, M.J. Benito [1], M.A. Asensio, J.J. Córdoba*

Higiene de los Alimentos, Facultad de Veterinaria, Universidad de Extremadura, Avda. de la Universidad s/n, 10071 Cáceres, Spain

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Abstract

A differential molecular screening procedure was developed to obtain DNA clones enriched for verrucosidin-related genes that could be used as DNA probes to detect verrucosidin-producing *Penicillium polonicum.* Permissive and nonpermissive conditions for verrucosidin production were selected to obtain differentiated poly $(A)^+$ RNA for the cloning strategy. *P. polonicum* yielded the highest amount of verrucosidin when cultured in malt extract broth at 25 °C without shaking. These conditions were selected as verrucosidin permissive conditions. When shaking was applied to the verrucosidin permissive conditions, verrucosidin was not detected. Approximately 5000 transformants were obtained for the library of DNA fragments from verrucosidin-producing *P. polonicum* and hybridized with cDNA probes obtained from poly $(A)^+$ RNA of permissive and nonpermissive conditions. A total of 120 clones hybridized only with the permissive cDNA probes. From these, eight representative DNA inserts selected on the basis of size and labelled with fluorescein-dUTP were assayed as DNA probes in the second differential screening by Northern hybridization. Probe SVr1 gave a strong hybridization signal selectively with poly $(A)^+$ RNAs from high verrucosidin production. When this probe was assayed by dot–blot hybridization with DNA of different moulds species, hybridization was detected only with DNA from the verrucosidin-producing strain. The strategy used in this work has proved to be useful to detect unknown genes related to mycotoxins. In addition, the DNA probe obtained should be considered for the detection of verrucosidin-producing moulds.

Keywords: *Penicillium polonicum*; Verrucosidin; DNA probe; Dry-cured ham

1. Introduction

Penicillium polonicum is the most common species of the genus *Penicillium* series *Viridicata* in meat products (Lund and Frisvad, 1994). A high number of isolates of *P. polonicum* produce verrucosidin (Lund and Frisvad, 1994; Nielsen et al., 1999), a potent neurotoxin responsible for a neurological disease in cattle (Wilson et al., 1981) and experimentally in mice (Fink-Gremmels et al., 1991). This species has been isolated from the surface of dry-cured ham during most of the ripening process, and all the isolates showed toxigenic potential (Núñez et al., 1996).

The detection of mycotoxigenic moulds in culture is time-consuming and expensive, since mycotoxin production is needed to obtain presumptive results. More rapid, simple and sensitive methods able to detect

* Corresponding author. Tel.: +34-927-257124; fax: +34-927-257110.

E-mail address: jcordoba@unex.es (J.J. Córdoba).

URL: http://higiene.unex.es/.

[1] Present address: Nutrición y Bromatología, Escuela de Ingenierías Agrarias, Universidad de Extremadura, Badajoz, Spain.

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verrucosidin-producing *P. polonicum* are needed for microbial quality assurance in meat products. The polymerase chain reaction (PCR) and DNA probes that have been used for detecting aflatoxigenic moulds (McAlpin and Mannarelli, 1995; Sweeney et al., 2000) are promising new diagnostic tools to monitor other toxigenic moulds. Knowledge of the genetic basis of toxin production is required to develop PCR or DNA probe techniques. However, no information is available about *P. polonicum* genes related to verrucosidin production.

The advent of molecular cloning procedures has provided a means to study the mechanism of gene expression. To date, no verrucosidin-related genes have been cloned, and molecular tools have been mainly applied to aflatoxigenic moulds (Skory et al., 1990; Yu et al., 2000). A differential molecular screening procedure that has generated DNA clones enriched for aflatoxin-related genes (Feng et al., 1992) could be used to obtain verrucosidin-producing genes. To follow this approach, it is necessary to grow the mould under permissive and nonpermissive conditions for verrucosidin biosynthesis. Verrucosidin production has been reported in malt extract agar and in meat extract peptone agar in the range of 12–30 °C (Núñez et al., 2000).

The aim of this work has been to find a DNA probe to detect verrucosidin-producing *P. polonicum*. For this, it will be necessary to find permissive and nonpermissive conditions for verrucosidin production, as well as to obtain DNA clones enriched for verrucosidin-related activities.

2. Materials and methods

2.1. Mould strains

P. polonicum strain Pa 21, which produces verrucosidin and was isolated from dry-cured ham (Núñez et al., 2000), was used to obtain clones enriched for verrucosidin-related activities. Another strain of *P. polonicum*, which also produced verrucosidin, and several strains of mould species which do not produce verrucosidin and are usually detected in dry-cured ham (Núñez et al., 1996; Díaz, 1999), were used as reference strains (Table 1) to test the specificity of selected DNA probe.

Table 1
Species used as reference strains to test the specificity of probe SVr1

Strains	Signal with probe SVr1
Verrucosidin producer	
Penicillium polonicum CBS 222.90	yes
Non-verrucosidin producer	
Penicillium echinulatum Pe 352[a]	no
Penicillium commune Pc 351[a]	no
Penicillium solitum Ps 25[a]	no
Penicillium aurantiogriseum CBS 491.74	no
Aspergillus versicolor Av 111[a]	no
Aspergillus niger An 261[a]	no
Aspergillus pullulans Ap 261[a]	no
Eurotium herbariorum Eh 331[a]	no
Eurotium rubrum Eu 261[a]	no
Eurotium repens Er 361[a]	no

CBS, Centraalbureau voor Schimmelcultures, Holland.
[a] Strains isolated from dry-cured ham (Núñez et al., 1996; Díaz, 1999).

2.2. Culture conditions

Mould strains were cultured in malt extract broth (2% malt extract, 2% glucose, 0.1% peptone). To select verrucosidin permissive, low permissive and nonpermissive conditions, several culture conditions were assayed. Malt extract broth was used at 25 °C for 4–30 days: (a) stationary, (b) with shaking (200 rpm), and (c) with shaking (200 rpm) and 0.1% (w/v) $CaCO_3$. Calcium has been reported to stimulate the biosynthesis of other mycotoxins like penitrem A by *P. nigricans* (Mantle et al., 1984).

For DNA or RNA extraction, mycelium was harvested, filtered through Miracloth (Calbiochem, Darmstadt, Germany) washed with TE buffer (10 mM Tris–Cl, 1 mM EDTA), frozen in liquid nitrogen and stored at −70 °C until use.

2.3. Verrucosidin detection

Production of verrucosidin in culture media was investigated by HPLC (Hewlett Packard, Palo Alto, CA, USA) on a Supercosil LC-18 column (Supelco, Bellafonte, Palo Alto, CA, USA) using water and 0.05% trifluoroacetic acid in acetonitrile, as described by Núñez et al. (2000). Detection of verrucosidin by atmospheric pressure ionization-mass spectrometry and confirmation of identity of verrucosidin by ion

spray mass spectrometry analysis were done on a Finnigan LCQ (Finnigan, San José, CA, USA) (Núñez et al., 2000).

2.4. DNA isolation

In liquid nitrogen, 2 g of frozen mycelium was ground to a fine powder, homogenized in 4 ml TES buffer (0.05 M Tris–Cl, pH 8; 0.005 M EDTA; 0.05 M NaCl) and sodium dodecyl sulfate (SDS) to a final concentration of 1% (w/v). Then 200 μl proteinase K (10 μg/μl) was added, incubated at 60 °C for 35 min, cooled on ice, and extracted with phenol–chloroform–isoamyl alcohol (25:24:1). This suspension was centrifuged at 3000×*g* for 2 min. The upper phase containing DNA was transferred to another centrifuge tube and precipitated by adding 3 M sodium acetate to a final concentration of 10% (w/v) and two volumes of cold ethanol. After centrifugation, the pellet was resuspended with sterile water and treated with 50 μl RNAse (10 μg/μl). Finally, DNA was precipitated again as indicated above.

2.5. Construction of a genomic library of *P. polonicum*

Genomic DNA of *P. polonicum* Pa 21 was partially digested with *Sau*3AI (Boehringer, Mannheim, Germany) and electrophoresed on a 1% agarose gel. The fragments in the range of 0.5–7 kbp were excised from the gel and purified with Qiaex gel extraction kit (Qiagen, Hilden, Germany).

The pBSK (+) plasmid (Stratagene, Heidelberg, Germany) was made linear with *Bam*HI (Boehringer) at 37 °C for 4–5 h. Linear plasmid was dephosphorylated with calf intestinal alkaline phosphatase (Boehringer) at 37 °C for 1 h. The reaction was stopped with 0.5 M EDTA. The mixture was incubated at 65 °C for 10 min to denaturate alkaline phosphatase.

The ligation was performed with 200 ng each of size-selected *Sau*3AI digested genomic DNA and the linear dephosphorylated pBSK (+), and 1 U of T4 DNA ligase (Boehringer) at 16 °C overnight. The hybrid plasmids obtained were used to transform *Escherichia coli* DH5 (Gibco, Grand Island, NY, USA). The transformants were spread on Luria Bertani plates containing 50 μg/ml ampicillin (Sambrook et al., 1989) and incubated at 37 °C for 24–36 h. Approximately 1% of colonies were randomly tested to confirm DNA insertion using restriction enzyme *Pvu*II (Boehringer) which cuts upstream and downstream of the *Bam*HI site. The transformants obtained were isolated in Luria Bertani broth containing 50 μg/ml ampicillin and transferred directly to nylon membranes Hybond H^+ (Amersham-Pharmacia-Biotech, Uppsala, Sweden) by slot blot milliblot-S apparatus (Millipore, Bedford, MA, USA).

2.6. Poly $(A)^+$ RNA isolation and fluorescein-dUTP-labelled cDNA preparation

Total RNA was extracted from mycelium according to the method of Timberlake (1980). Poly $(A)^+$ RNA was isolated with an oligo(dT)-cellulose column kit (Amersham-Pharmacia-Biotech) and precipitated with ethanol and sodium acetate. The concentration of poly $(A)^+$ RNA was determined by separating on an agarose gel, stained with ethidium bromide. For cDNA synthesis, the Moleney murine leukemia virus reverse transcriptase (Boehringer) was used. The resulting first cDNA strands were randomly labelled with fluorescein-dUTP using a fluorescein-dUTP kit (Amersham-Pharmacia-Biotech) according to the manufacturer's instructions.

2.7. First differential screening by colony hybridization

The colonies on nylon membranes were lysed with denaturation buffer (0.5 M NaOH, 1.5 M NaCl) and then treated with neutralization buffer (1.5 M NaCl, 0.5 M Tris–HCl pH 7.4). After washing with 2×SSC (1×SSC is 0.15 M NaCl and 0.015 M sodium citrate), DNA was fixed by alkali treatment (0.4 M NaOH). Each membrane was hybridized with the above-labelled fluorescein-dUTP cDNA in a solution containing 5×SSC, 0.1% SDS, 5% dextrane sulfate and 1/20 blocking liquid (Amersham-Pharmacia-Biotech). Hybridization was carried out overnight at 60 °C in a shaker bath. After hybridization, membranes were washed twice in 1×SSC–0.1% SDS at 60 °C for 15 min and then in 0.5×SSC–0.1% SDS at 60 °C for 15 min. The hybridization was detected using an enhanced chemiluminescence kit (Amersham-Pharmacia-Biotech). The membranes were exposed to X-ray film at 20 °C for 20 min.

Clones hybridizing only with the cDNA probe obtained from verrucosidin permissive conditions were selected for the second differential hybridization screening.

2.8. Second differential screening: Northern hybridization

Selected clones were digested with restriction endonucleases *Xba*I and *Sma*I to obtain the DNA inserts. Restriction fragments were separated on agarose gel, excised from the gel and purified with Qiaex gel extraction kit (Qiagen). A fluorescein-dUTP probe was elaborated as above with each insert from selected clones. Different size inserts were chosen to obtain probes. These probes were assayed with poly $(A)^+$ RNA obtained from high, low and nonpermissive conditions for verrucosidin production. For this, denatured poly $(A)^+$ RNA was separated in a 1% agarose gel. Gels were washed with water treated with diethylpyrocarbonate, immersed in 10×SSC for 30 min and transferred using a capillary onto nylon membranes with 10×SSC solution and held overnight. Hybridization with each probe was performed overnight at 60 °C in a shaker bath. Then, membranes were washed and exposed to X-ray film as indicated above. The probe showing strong hybridization signal only with poly $(A)^+$ RNA obtained from high permissive conditions for verrucosidin production was finally selected for the assay of specificity.

2.9. Assay of specificity of the selected probe

The selected probe was hybridized with DNA of both verrucosidin- and non-verrucosidin-producing moulds. For this, DNA of reference strains was extracted following the procedure indicated above. A weight of 30 μg of DNA from each mould species and 30 and 15 μg of DNA from *P. polonicum* CBS 222.90 were denatured by boiling for 5 min in 1×SSC, transferred to a Nylon membrane Hybond H^+ (Amersham-Pharmacia-Biotech) by slot blot milliblot-S apparatus (Millipore), and fixed in 0.4 M NaOH. The blot was hybridized with the selected probe overnight at 60 °C in a shaker bath. Then, it was washed and exposed to X-ray film as indicated for colony hybridization.

3. Results

3.1. Expression of verrucosidin at different culture conditions

The calibration curve for verrucosidin quantification by high-pressure liquid chromatography-mass spectrometry revealed a linear relationship (r^2= 0.9929) between detector response and amount of verrucosidin from 0.1 to 100 ng. Verrucosidin was only detected when incubated at 25 °C for at least 14 days (Table 2). Verrucosidin production was high under static incubation in the latter conditions. When *P. polonicum* was cultured on malt extract broth under continuous shaking, verrucosidin was not detected, except for a very low production after 30 days at 25 °C with 0.1% (w/v) $CaCO_3$ (Table 2).

3.2. Cloning and screening of the genomic DNA library

Approximately 5000 transformants were obtained for the library of genomic DNA fragments from verrucosidin-producing *P. polonicum*.

Two sets of poly $(A)^+$ RNAs were isolated from *P. polonicum* grown on malt extract broth: the first one, after incubating at 25 °C for 15 days without shaking, as permissive incubation for verrucosidin production; the second one, also at 25 °C for 15 days with no $CaCO_3$ added but under continuous shaking, as nonpermissive conditions. From these two sets of RNAs, the respective cDNAs were obtained and these were labelled with fluorescein-dUTP, to be used as probes for the first screening by colony hybridization.

Table 2
Verrucosidin production by *P. polonicum* Pa 21 growing on malt extract broth at 25 °C for 4–30 days

Incubation time (days)	Shaking	$CaCO_3$ added (%)	Verrucosidin production (ng/ml)
4–12	no	no	Nd
14	no	no	1850
15	no	no	1950
7–30	200 rpm	no	Nd
14, 21	200 rpm	0.1	Nd
30	200 rpm	0.1	0.0052

Nd: Not detected.

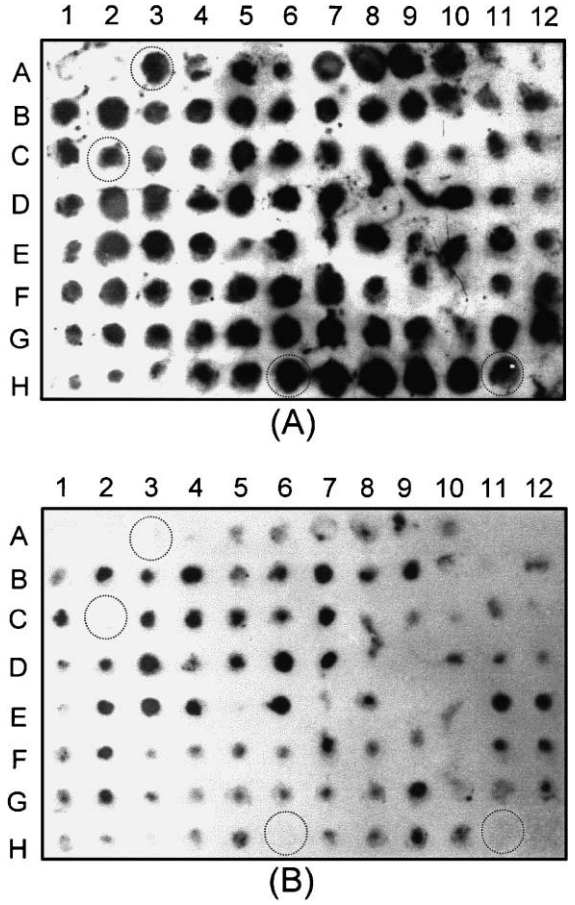

Fig. 1. First differential hybridization screening of a part of the 5000 clones with cDNA probes obtained from poly $(A)^+$ DNA at: (A) verrucosidin permissive and (B) non-verrucosidin permissive conditions. The circled clones located in positions A3, C2, H6 and H11 represent presumptive verrucosidin-related genes, which hybridized with verrucosidin permissive cDNA probe but not with non-verrucosidin permissive cDNA probe at all.

When the two sets of labelled cDNA probes were hybridized consecutively to the same membranes carrying the DNA library, each one was found to hybridize to approximately 80% of the clones. However, only 120 transformants showed a strong signal with the permissive probe and weak or no signal with the non-permissive probe (Fig. 1).

After digestion with *Xba*I and *Sma*I, most of the plasmid DNAs from these 120 clones showed inserts of 0.5–2 kbp (data not shown). Eight of these DNA inserts were selected on the basis of size ranging from 0.5 to 2 kbp, for the second screening. After labelling with fluorescein-dUTP, the eight DNA probes were assayed by Northern hybridization with three sets of poly $(A)^+$ RNA obtained at permissive, nonpermissive and low permissive verrucosidin production. Only one probe, of about 0.6 kbp named SVr1, gave a strong signal with poly $(A)^+$ RNAs from permissive incubation, and no signal with poly $(A)^+$ RNAs from low and nonpermissive incubation (Fig. 2). The remaining probes hybridized nonspecifically with poly $(A)^+$ RNAs obtained at high, low and nonpermissive incubation.

3.3. Specificity of SVr1 probe

When the SVr1 probe was assayed by dot–blot hybridization with DNA of different mould species tested, hybridization was detected only with DNA from the verrucosidin-producing strain (Fig. 3; Table 1).

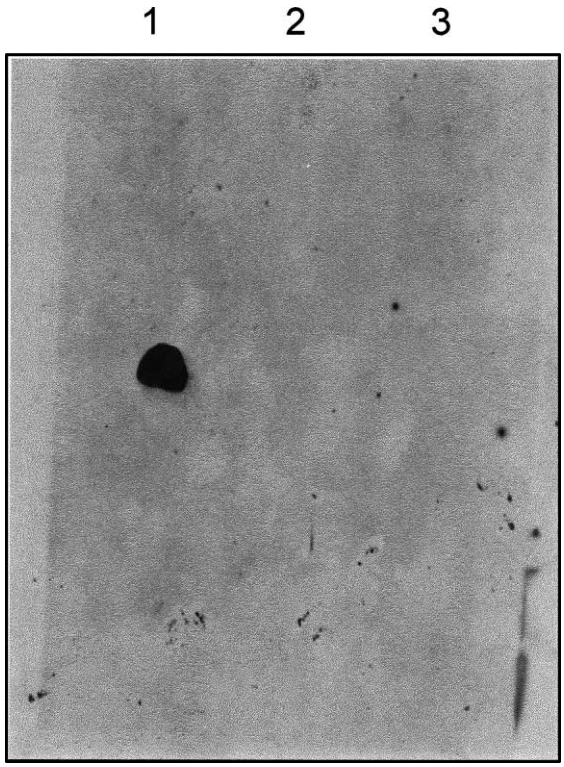

Fig. 2. Northern hybridization of SVr1 probe with poly $(A)^+$ RNA from *P. polonicum* grown under permissive (lane 1), low permissive (lane 2) and nonpermissive (lane 3) conditions for verrucosidin production.

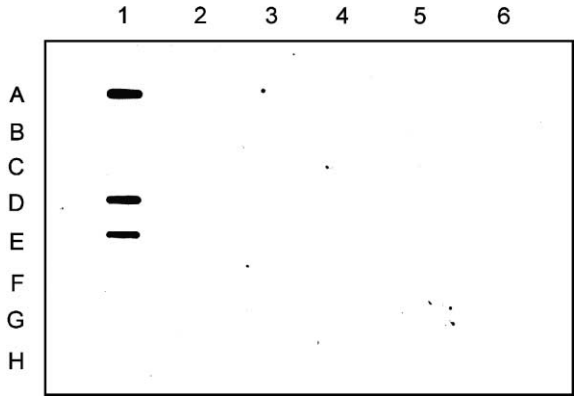

Fig. 3. Dot–blot hybridization of DNA from different moulds with probe SVr1. A1: fluorescein-dUTP positive control; B1: *P. aurantiogriseum* CBS 491.74 (30 μg); B2: *P. echinulatum* Pe 352 (30 μg); B3: *P. commune* Pc 351 (30 μg); B4: *P. solitum* Ps 25 (30 μg); B5: *A. versicolor* Av 111 (30 μg); C1: *A. niger* An 261 (30 μg); C2: *A. pullulans* Ap 261 (30 μg); C3: *E. herbariorum* Eh 331 (30 μg); C4: *E. rubrum* Eu 261 (30 μg); C5: *E. repens* Er 361 (30 μg); D1: *P. polonicum* CBS 222.90 (30 μg); E1: *P. polonicum* CBS 222.90 (15 μg).

Furthermore, hybridization signal was directly related to the DNA concentration.

4. Discussion

The highest yield of verrucosidin on malt extract broth was obtained at 25 °C without shaking. These levels are similar to those reported for this strain on malt extract agar at 25 °C by Núñez et al. (2000). Thus, these conditions were selected as verrucosidin permissive conditions. When *P. polonicum* was incubated under continuous shaking, no verrucosidin was detected. Therefore, shaking seems to interfere with verrucosidin biosynthesis, which could be related to submerged growth of the mycelium in malt extract broth. For this reason, continuous shaking was applied to obtain nonpermissive conditions for verrucosidin production. To obtain incubation conditions where verrucosidin synthesis was limited to a low value, $CaCO_3$ was added to the nonpermissive conditions. After 30 days of incubation in this culture condition, only a small amount of verrucosidin was detected. Calcium may activate enzymes of verrucosidin biosynthesis which are inhibited when malt extract broth is incubated under continuous shaking.

The permissive and nonpermissive conditions incubation were used to obtain differentiated poly $(A)^+$ RNA for the cloning strategy. Genes expressing as a consequence of different growth conditions can be cloned either by differential screening (Feng et al., 1992) or by subtraction hybridization (Grunstein and Hogness, 1975), resulting from variable levels of extractable poly $(A)^+$ RNA. The former approach was developed by Feng et al. (1992) to find aflatoxin-related genes. These authors used different carbon sources to find permissive and nonpermissive conditions for aflatoxin production. However, poly $(A)^+$ RNA isolated from these two sets of conditions might come from genes specifically related to medium composition, rather than to aflatoxin biosynthesis. In the present work, shaking was the only difference between permissive and nonpermissive verrucosidin conditions, which could allow the finding of poly $(A)^+$ RNA related to verrucosidin production.

When the 5000 clones of the DNA library from *P. polonicum* were screened with the cDNA probes obtained from permissive and nonpermissive conditions, 120 clones hybridized only with the permissive cDNA probes. These clones were further investigated as they could have DNA inserts from genes involved in verrucosidin biosynthesis.

In the second differential screening, the eight DNA inserts selected were hybridized with poly $(A)^+$ RNA from permissive and low permissive conditions. Furthermore, poly $(A)^+$ RNA from nonpermissive incubation was tested as a control. Only the probe from insert SVr1 hybridized with poly $(A)^+$ RNA obtained from verrucosidin permissive incubation but not with those obtained from low or nonpermissive conditions. Since the amount of poly $(A)^+$ RNA related to verrucosidin biosynthesis should be higher in permissive than in low permissive and nonpermissive conditions, this DNA insert was selected as a possible DNA fragment of a gene related to verrucosidin biosynthesis.

The probe obtained from the selected DNA insert was able to detect an amount of DNA from *P. polonicum* as low as 15 μg. In addition, no hybridization was detected with 30 μg DNA from the non-verrucosidin-producing moulds tested. These results confirmed that the selected DNA insert could be used to detect verrucosidin-producing moulds.

As has been indicated by Feng et al. (1992), this cloning strategy is a good alternative approach to other

strategies used to obtain genes related to mycotoxin biosynthesis, such as purifying pathway enzymes (Chuturgoon et al., 1990) and complementation of pathway mutants (Woloshuk et al., 1994). In the cloning strategy followed, it is not necessary to know the enzymology and mechanistic details of many of the pathway steps that would be necessary if a strategy based on purifying pathway enzymes is used. In addition, there are no difficulties due to the lack of an efficient transformation system, as would be the case if the mutant complementation strategy was followed.

In conclusion, the strategy used in this work has proved to be useful in detecting verrucosidin-related genes. In addition, a DNA probe of about 0.6 kbp has been obtained. This DNA probe showed high specificity and should be considered for the detection of verrucosidin-producing moulds.

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