Galactomannans from the cell walls of species of *Paecilomyces* sect. *Paecilomyces* and their teleomorphs as immunotaxonomic markers

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An alkali-extractable and water-soluble fraction (F15) was obtained from cell walls of *Paecilomyces variotii* and species of the related genera *Talaromyces*, *Byssochlamys* and *Thermoascus*. The structure of the main polysaccharide of these fractions was studied and found to consist of a core of (1 → 6)- α -mannopyranose partially substituted at O-2 by chains of galactofuranose and shorter chains of mannopyranose. The differences in the regularity of the branching points and the length of the galactofuranose side chains are useful to distinguish between species. These differences were detected by immunological methods, since highly specific polyclonal antibodies were raised against these polysaccharides. Mycelium of *P. variotii* CBS 990.73A was stained by indirect immunofluorescence. The polysaccharides studied in this work differ from the one described for species from section *Isarioidea*, and this is another indication of the heterogeneity of the genus *Paecilomyces*.

Keywords: *Paecilomyces*, cell wall, galactomannan, chemotaxonomy, fungal antibodies

INTRODUCTION

Paecilomyces is a genus of filamentous fungi closely related to *Penicillium* (Brown & Smith, 1957). Taxonomically, the genus is subdivided into two sections. Section *Paecilomyces* contains mesophilic, thermotolerant and thermophilic species and colonies with yellow-brown to brownish colours, with perfect states in *Talaromyces*, *Byssochlamys* and *Thermoascus*. Section *Isarioidea* contains mesophilic species with purple-, pink-, green- or yellow-coloured colonies. The type species of section *Paecilomyces* is *P. variotii*. The history of *Paecilomyces*, its delimitation and its relationships to another genera have been reviewed by Samson (1974).

These genera are economically important since some species are responsible for food contamination and spoilage (King *et al.*, 1969; Engel & Teuber, 1991), can infect immunodeficient humans (Byrd *et al.*, 1992) or are pathogenic for invertebrate animals such as the silk-worm (*Bombyx mori*) (Aoki & Yanase, 1970).

Taxonomy based only on the morphology of these genera is sometimes unclear. The use of biochemical and molecular techniques is needed in order to give some insight into the classification of related groups of fungi (Taylor *et al.*, 1990; Berbee *et al.*, 1995). The fungal cell

wall is mainly composed of polysaccharides, which have proved to be useful chemotaxonomic and antigenic markers for identification purposes (Bartnicki-García, 1970; Weijman *et al.*, 1982; Weijman & Golubev, 1987; Weijman & Van der Walt, 1989; Kamphuis *et al.*, 1992; Leal, 1994; Leal & Bernabé, 1998).

The finding that fungal polysaccharides have immunological properties, and that some of them are almost genus-specific (Notermans & Soentoro, 1986), enhanced interest in the elucidation of the structure of these molecules since they might be used as chemotaxonomic characters. Most studies on serological properties of fungal polysaccharides deal with exopolysaccharides (Notermans & Soentoro, 1986; Notermans *et al.*, 1987; De Ruiter *et al.*, 1992) and cell wall glycoproteins (Gailliez & Poulain, 1988; Sridhara *et al.*, 1990; Hearn, 1991). An antigenic water-soluble galactomannan isolated from cell walls of *Paecilomyces fumosoroseus* and *Paecilomyces farinosus* (section *Isarioidea*) has been studied in our laboratory (Domenech *et al.*, 1996). In the present research we report on the structural and immunological characterization of polysaccharides purified from cell walls of species of section *Paecilomyces* and discuss their use as chemotaxonomic characters for the delimitation of the section.

METHODS

Micro-organisms and culture media. Fungal isolates used were: *P. variotii* CBS 990.73A, CBS 323.34, CBS 339.51 and CBS 371.70; *Talaromyces leycettanus* (teleomorph of *Paecilomyces leycettanus*) CBS 275.70; *Talaromyces byssochlamydoides* (teleomorph of *Paecilomyces byssochlamydoides*) CBS 533.71; *Byssochlamys fulva* (teleomorph of *Paecilomyces fulvus*) CBS 604.71; *Byssochlamys nivea* (teleomorph of *Paecilomyces niveus*) CBS 100.11; and *Thermoascus crustaceus* (teleomorph of *Paecilomyces crustaceus*) CBS 114.66. The isolates were maintained on slants of Bacto potato-dextrose agar supplemented with 1 g yeast extract (Difco) l⁻¹. The culture medium and growth conditions were as previously described (Gómez-Miranda *et al.*, 1990).

Wall material preparation and fractionation. Wall material was obtained as reported by Gómez-Miranda *et al.* (1990). Cell wall material (8 g) was repeatedly extracted with 1 M NaOH (300 ml) at 20 °C. After centrifugation, the supernatants were combined and an equal volume of 96% ethanol was added. The precipitate was collected by centrifugation, dialysed against running tap water and then freeze-dried. This polysaccharidic material (500 mg) was suspended in distilled water (100 ml), stirred at room temperature for 2 h and centrifuged. The precipitate was extracted again as described above and the supernatant solutions were combined and freeze-dried (F1S).

Isolation of polysaccharides from F1S. Fraction F1S (190 mg) was dissolved in 3 ml distilled water and centrifuged at 13000 g for 15 min to eliminate insoluble material. The supernatant was added to a column (90 × 3 cm) of Sepharose CL-6B and eluted with distilled water (flow rate 21.4 ml h⁻¹). Fractions of 2.5 ml were collected and monitored for carbohydrate by the phenol-sulphuric acid method (Dubois *et al.*, 1956). Appropriate fractions were combined, concentrated to a small volume (15 ml) and freeze-dried.

Isolation of the mannan core. A sample of each polysaccharide (100 mg) was treated with 20 ml 0.05 M H₂SO₄ at 100 °C for 6 h. The degraded polysaccharide was dialysed against distilled water (exclusion limit 3000 Da), concentrated to a small volume (10 ml) and freeze-dried.

Chemical analyses. Monosaccharides were released by Saeman hydrolysis (Adams, 1965) and analysed by GLC as their corresponding alditol acetates as previously described (Gómez-Miranda *et al.*, 1981). Methylation analysis and GLC-MS of the derivatives were performed as described by Domenech *et al.* (1996). Permethylated samples were subjected to reductive cleavage as described by Gray (1987) in order to identify the ring size (pyranose or furanose) of the sugar residues. The partially methylated anhydroalditol acetates obtained were analysed by GLC-MS using a fused silica SPB-1 column and a temperature range of 150 to 200 °C (3 min initial hold and ramp rate 3 °C min⁻¹). Absolute configuration was determined according to Gerwig *et al.* (1978) by GLC-MS of the tetra-O-TMSi-(+)-2-butyglycosides obtained. Protein was determined by the Lowry method.

NMR spectroscopy. The polysaccharides (approx. 20 mg) obtained after column chromatography were dissolved in D₂O (1 ml) followed by centrifugation (10000 g for 20 min) and lyophilization of the supernatant. The process was repeated twice and the final sample was dissolved in D₂O (0.7 ml, 99.98% D). ¹H- and ¹³C-NMR spectra were recorded at 40 °C on a Varian INOVA 300 spectrometer. Proton chemical shifts refer to residual HDO at δ 4.61 p.p.m. Carbon chemical shifts refer to internal acetone at δ 31.07 p.p.m.

Antibody production and serology. Antibodies were obtained by immunization of rabbits with F1S-B polysaccharide (the major fraction isolated by gel permeation chromatography of F1S on Sepharose CL-6B) of *P. variotii* CBS 990.73A, *P. fumosoroseus* CBS 375.70 and *Eupenicillium crustaceum* CBS 635.70. The protocols for immunization and titration by ELISA were as previously described (Domenech *et al.*, 1996) except that the ELISA plates were coated with different concentrations of polysaccharide (40–400 g ml⁻¹) depending on the antigens. The specificity of the antisera obtained was tested by inhibition studies (Domenech *et al.*, 1996). The polysaccharides isolated from all species tested in this work and the water-soluble polysaccharide from *E. crustaceum* (Leal *et al.*, 1993) were used as inhibitors. Staining of *P. variotii* CBS 990.73A was carried out by immunofluorescence under the same conditions as reported by Domenech *et al.* (1996), but in this case it was not necessary to adsorb the secondary antibody with mycelium since nonspecific staining was not observed.

RESULTS

Purification and chemical composition of the polysaccharides

In all species, two polysaccharides were isolated from the alkali- and water-soluble material (F1S, 3–6% of dry cell walls) by gel permeation through Sepharose CL6B. A small unretained fraction (F1S-A, 3.3–10%), mainly composed of glucose, was not further analysed. The major polysaccharide (F1S-B, 70–80%) was rechromatographed and eluted as a single peak. Monosaccharide analysis of the purified polysaccharides gave D-mannose (23–45%), D-galactose (16–43%), and D-glucose (3–9%). No protein was detected.

Table 1. Methylation analysis of F1S-B polysaccharides from *Paecilomyces* species

THC, *Th. crustaceus* CBS 144.66; PVA, *P. variotii* CBS 990.73A; BFU, *B. fulva* CBS 604.71; BNI, *B. nivea* CBS 100.11; TBY, *Ta. byssochlamydoides* CBS 533.71; TLE, *Ta. leycettanus* CBS 275.70; –, none detected. All values are means of at least three determinations.

Linkage type	Relative percentage					
	THC	PVA	BFU	BNI	TBY	TLE
Glcp-(1 →	–	2.2	1.6	–	–	1.9
Galp-(1 →	11.2	6.3	9.2	5.9	6.6	6.3
→ 5)-Galp-(1 →*	30.5	34.0	27.5	15.8	44.2	16.5
→ 6)-Galp-(1 →	3.4	4.0	3.5	2.5	–	–
Manp-(1 →	4.3	2.2	4.0	3.7	9.0	16.0
→ 2)-Manp-(1 →	7.4	7.0	10.9	11.4	8.7	12.5
→ 6)-Manp-(1 →	30.1	32.5	33.6	44.0	20.2	21.2
→ 2,6)-Manp-(1 →	10.5	11.6	9.5	14.1	11.8	26.0

* The identity of → 5)-Galp-(1 → was determined by reductive cleavage.

Fig. 1. ^1H - (a) and ^{13}C - (b) NMR spectra of the anomeric region of fractions F1S-B from the fungal species studied. The spectrum of fraction F1S-B from *B. fulva* (not shown) was similar to that depicted for *P. variotii*.

Structural studies of the polysaccharides

The water-soluble polysaccharides F1S-B were studied using a combination of chemical and spectroanalytical techniques. The methylation results (Table 1) demonstrated that all the polysaccharides contained almost identical substituted galactofuranose and mannopyranose residues, although in different proportions. Three of them had also very small amounts (2%) of terminal glucopyranose. All the ^1H -NMR spectra (Fig. 1a) showed broad uneven signals coexisting with small signals, indicative of irregular polysaccharides. In addition, the ^{13}C -NMR spectra (Fig. 1b) contained sharp

and broad signals, suggesting the presence of a low mobility backbone with side chains of higher flexibility (Rath *et al.*, 1995; Prieto *et al.*, 1997). The chemical shifts of the sharp signals had values over 105 p.p.m., indicative of β -galactofuranose units, while the broad signals were assigned to the mannopyranose residues. These values are in agreement with the existence of β -galactofuranose chains linked to a mannan core (Prieto *et al.*, 1997). Some of the polysaccharides were analysed by comparison with known polysaccharides. The signals for the galactofuranose residues of *P. variotii* and *B. fulva* were found to be closely related to those of *Penicillium expansum* (Parra *et al.*, 1994) since (1 $\rightarrow$ 5)-

Table 2. Methylation analysis of the mannan core obtained from F1S-B of *Paecilomyces* species

THC, *Th. crustaceus* CBS 144.66; PVA, *P. variotii* CBS 990.73A; BNI, *B. nivea* CBS 100.11; TBY, *Ta. byssoschlamydoides* CBS 533.71; TLE, *Ta. leycettanus* CBS 275.70. All values are means of at least three determinations.

Linkage type	Relative percentage				
	THC	PVA	BNI	TBY	TLE
Manp-(1 →	21.4	20.6	19.1	28.1	27.7
→ 2)-Manp-(1 →	16.3	17.1	16.2	17.0	15.8
→ 6)-Manp-(1 →	50.1	48.2	50.2	24.6	26.7
→ 2,6)-Manp-(1 →	12.2	13.7	14.3	23.0	29.7

β - and (1 → 6)- β - linkages appeared in a 3:1 proportion. F1S-B of *Ta. leycettanus* and *Ta. byssoschlamydoides* had outer chains of β -Galf O-5-substituted, giving signals comparable to those found for the (1 → 5)- β -galactan isolated from *E. crustaceum* (Leal *et al.*, 1993).

Methylation and reductive cleavage analyses (Table 1) of the polysaccharides gave terminal Galf, O-5-substituted and O-6-substituted Galf, terminal Manp, O-2-substituted, O-6-substituted and di-O-2,6-substituted Manp in different proportions.

The peaks observed in the ^1H - and ^{13}C -NMR spectra (Fig. 1) are consistent with these residues (Leal *et al.*, 1993; Parra *et al.*, 1994; Prieto *et al.*, 1997). Signals corresponding to the mannan residues appeared poorly resolved in most of the cases. Hence, taking advantage of the lability of the glycosidic linkages of the furanoid rings as compared with those of the pyranoid rings, we further investigated them by selectively hydrolysing the galactofuranan moieties of all the polysaccharides to leave only the mannan skeleton.

The methylation results demonstrated that all the mannan cores also had identical residues (Table 2), although in different proportions. The ^1H -NMR spectra of the mannans obtained are depicted in Fig. 2, the mannan from *B. fulva* and *Th. crustaceus* (not shown) being very similar to that of *P. variotii*.

By comparison with known mannan derivatives (Gorin & Spencer, 1970; Cohen & Ballou, 1980; Vliegenthart *et al.*, 1983; Prieto *et al.*, 1997) it can be deduced that all cores are constituted of (1 → 6)- α -mannan chains, irregularly substituted at position 2 of some of the residues either by terminal α -mannopyranose units or else by small chains containing Manp-(1 → 2)- α - and Manp-(1 → 6)- α - linkages. To such cores, different chains of β -galactofuranose are attached in an irregular way.

Immunological studies

The titre of the sera after the fifth injection reached 1/8000 for *P. variotii* and *P. fumosoroseus*, and 1/6000 for *E. crustaceum*. The results of inhibition studies are

shown in Fig. 3. F1S-B polysaccharides of all isolates of *P. variotii* inhibited the serum anti-F1SB of *P. variotii* CBS 990.73A (Fig. 3b) at low concentrations (3.2–69.3 g ml⁻¹), but the polysaccharide F1S-B from the remaining species of section *Paecilomyces* studied did not block the immunological reaction (results not shown).

Fifty percent inhibition of the binding of serum anti-F1SB of *E. crustaceum* to its specific antigen (Fig. 3a) was achieved with 4.8, 39.0 and 78.0 g ml⁻¹ of the F1S-B polysaccharides of *E. crustaceum*, *Ta. byssoschlamydoides* and *Th. crustaceus*, respectively. Polysaccharides F1S-B from other species of section *Paecilomyces* also inhibited the reaction, but at higher concentrations. Finally, the serum anti-F1SB of *P. fumosoroseus* did not react with any of the polysaccharides used in this study.

The hyphae of *P. variotii* CBS 990.73A reacted with polyclonal antibodies raised against fraction F1S-B of this micro-organism as demonstrated by immunofluorescence (Fig. 4).

DISCUSSION

The polysaccharides F1S-B of the species studied from section *Paecilomyces* have similar structural features. All of them have a core of (1 → 6)- α -mannopyranose irregularly substituted at O-2 by a single α -mannopyranose residue or small chains of mannopyranose. The degree of branching of this core depends on the species, being higher in the two *Talaromyces* spp. studied here. The galactofuranose residues of these polysaccharides are located in side chains of variable length (less than eight residues), O-2-linked to the mannan core. The polysaccharides studied differed in the relative proportion of the mannose and galactofuranose residues and in the presence of small amounts (2.5–4.0%) of O-6-substituted galactofuranose (Table 1). The signals in the region of 5.0–5.25 p.p.m. of the ^1H -NMR spectra of F1S-B from *P. variotii* and *B. fulva* are similar to that of F1S-B isolated from *Penicillium expansum*, which is a linear polysaccharide with a highly regular structure whose repetitive unit consists of three residues of (1 → 5)- β - and one of (1 → 6)- β -galactofuranose (Parra *et al.*, 1994). These residues from *Paecilomyces* and *Byssoschlamys* are components of the side chains which differ from *Penicillium expansum*, as shown by the shape of signals in the spectrum, because they form shorter chains that appear irregularly linked to the mannan core. In *Penicillium expansum* the mannan core is so small that it was not detected by NMR, although small amounts of mannose were detected in the methylation analysis. The side chains of the polysaccharides from *Ta. leycettanus* and *Ta. byssoschlamydoides*, which lack the O-6-galactofuranose residues, are similar to the galactofuranan described in cell walls of *E. crustaceum* (Leal *et al.*, 1993) and some species of *Penicillium* (Leal *et al.*, 1997). Nevertheless, when the complete polysaccharides are compared, the mannose content is much higher in F1S-B from these teleomorphic species of *Paecilomyces* than in other species of *Talaromyces* which are teleomorphs of

Fig. 2. ^1H -NMR spectra of the mannan cores obtained from fraction F1S-B after partial hydrolysis. The spectrum of the mannan core from F1S-B of *B. fulva* (not shown) was similar to that depicted for *P. variotii*.

Penicillium. Mannans with different structures are the main components of fraction F1S of several species of *Aphanoascus* and of various dermatophytes (Jiménez-Barbero *et al.*, 1993) included in the order Onygenales within the ascomycetous fungi. This order was rearranged by Fennell (1973), who included two families of this order in the Eurotiales, together with the family Trichocomaceae (where the genera *Byssochlamys*, *Talaromyces* and *Thermoascus* are included). The fruiting bodies of Eurotiales are similar to those of genera included in Onygenales, differing in the mode of conidiation (Currah, 1985). It seems that the galactomannans of the fungi of section *Paecilomyces* are an intermediate step in the evolution of the mannans of Onygenales towards the mannogalactans of Eurotiales, and therefore the polysaccharides may show the evolution of these micro-organisms. As Bartnicki-García (1987) stated, 'the nature of the polysaccharides in any particular fungus is not capricious, but is related to its taxonomic position and thus reflects its evolutionary history'.

Polyclonal antibodies raised against F1S-B polysaccharide from *P. variotii* CBS 990.73A were highly

specific, since the binding to their specific antigen was only inhibited by different isolates of the same species (Fig. 3b). Polysaccharides with a structure similar to that of the antigen used, such as those from other species of section *Paecilomyces*, did not inhibit the immunochemical reaction. This could be related to the differences in the length of the side chains and the irregularity of the polysaccharides F1S-B of this group. Inhibition experiments were also performed using serum anti-F1S-B from *E. crustaceum*, which is a linear (1 → 5)- β -galactofuranan (Leal *et al.*, 1993), in order to check if these antibodies recognized the galactofuranan side chains of the polysaccharides studied here. In this case, the polysaccharides F1S-B from *Ta. byssochlamydoides*, *P. variotii*, *Th. crustaceus* and *B. fulva* inhibited the binding of the antiserum to its specific antigen at concentrations that varied from 9 to 300 $\mu\text{g ml}^{-1}$. F1S-B fractions from these species have a higher content of galactofuranose than those from *Ta. leycettanus* and *B. nivea*, which reacted only at concentrations higher than 300 $\mu\text{g ml}^{-1}$. This may indicate that the (1 → 5)- β -galactofuranose residues are immunodominant as in the extracellular polysaccharides of *Penicillium* and *Aspergillus* (Notermans *et al.*, 1988). On the other hand,

Fig. 3. Inhibition experiments. (a) Inhibition of the binding of serum anti-F1SB of *E. crustaceum* to its specific polysaccharide by several F1S-B polysaccharides. ---○---, *E. crustaceum*; ---○---, *B. fulva*; ---●---, *B. nivea*; ---□---, *Ta. byssochlamydoides*; ---△---, *Th. crustaceus*; ---▽---, *P. variotii*; ---◇---, *Ta. leycettanus*. (b) Inhibition of the binding of serum anti-F1SB of *P. variotii* CBS 990.73A to its specific polysaccharide by F1S-B polysaccharides from other isolates of the same species. ---●---, *P. variotii* CBS 323.34; ---○---, *P. variotii* CBS 371.70; ---◇---, *P. variotii* CBS 339.51; ---▽---, *P. variotii* CBS 990.73A. All values are means of triplicate determinations.

the polysaccharides of fungi from section *Paecilomyces* did not inhibit the binding of the serum anti-F1SB of *P. fumosoroseus* to its specific antigen, a (1 → 6)- α -mannan substituted at O-4 by one residue of β -galactopyranose or α -glucopyranose.

Fig. 4. Immunofluorescence studies. Hyphae of *P. variotii* CBS 990.73A were grown on coverslips, heat-fixed and stained by reaction with serum anti-F1S-B followed by goat anti-rabbit IgG-rhodamine conjugate. No immunofluorescence was observed when mycelium was treated with preimmune serum (not shown). Bar, 10 μ m.

Mycelium of *P. variotii* reacted specifically with serum anti-F1S-B of the same species (Fig. 4), indicating the accessibility of the epitope in the hyphae. This would allow the detection of fungal contamination in food and biological samples.

The structure of the polysaccharides F1S-B from fungi of section *Paecilomyces* presented in this work differs from the one described for *P. fumosoroseus* and *P. farinosus* from section *Isarioidea* (Domenech *et al.*, 1996) indicating the heterophyletic character of the genus *Paecilomyces*. The available data support the division of the genus into two sections. The characteristic polysaccharides from both sections are different from those described for *Penicillium* species. The existing classification of the genus was proposed by Samson (1974) on the basis of the morphology and the structure of the phialides and conidiophores. More recent studies using molecular techniques, such as the cladistic analysis of PCR-based DNA markers, clearly showed that morphological characteristics of asexual structures are insufficient to yield a cohesive taxonomy of *Paecilomyces*, and that classifications based on morphological criteria alone had problems in distinguishing among species of closely related genera (Tigano-Milani *et al.*, 1995). It is interesting to note that F1S-B polysaccharides from the two species of *Talaromyces* included in this work (*Ta. leycettanus* and *Ta. byssochlamydoides*) are clearly distinct from the complex glucogalactans with small amounts of mannose found in species of the same genus which are closely related to *Penicillium* (Prieto *et al.*, 1995). Our data reinforce the results of rDNA analysis (Taylor *et al.*, 1990) showing that *Ta. leycettanus* and *Ta. byssochlamydoides* are closely related to *Byssochlamys* and *Thermoascus* and phylogenetically separated from other *Talaromyces*, and agree with the proposal of Pitt (1993) that *Talaromyces* species

with *Paecilomyces* anamorphs should be transferred to *Byssoschlamys*.

As has been shown in other fungal taxa, polysaccharides seem to be useful characters for delimiting genera and establishing relationships among anamorphs and teleomorphs (Leal *et al.*, 1992, 1997; Domenech *et al.*, 1994, 1996; Leal, 1994; Jiménez-Barbero *et al.*, 1995; Prieto *et al.*, 1995, 1997; Leal & Bernabé, 1998).

ACKNOWLEDGEMENTS

We thank Mr. J. López for technical assistance. This work was supported by Grants PB 95/0078 and 93/1027 from Dirección General de Investigación Científica y Técnica.

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Received 8 February 1999; revised 10 June 1999; accepted 18 June 1999.