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Absence of Spermine in Filamentous Fungi[1]

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Polyamines were examined in several yeasts and filamentous fungi. Whereas putrescine, spermidine, and spermine were present in the yeasts, spermine was not detected in any of the filamentous fungi.

Many differences have been observed between prokaryotic and eukaryotic organisms. Among these differences is the distribution of polyamine-synthesizing capacity. Eukaryotic organisms synthesize putrescine, spermidine, and spermine, whereas prokaryotes are unable to synthesize spermine (5). Although the fungi are clearly eukaryotic organisms, they are close enough to the evolutionary interface between prokaryotes and eukaryotes that they do not possess all of the biochemical characteristics of higher eukaryotes. Fungal individuality regarding enzyme regulation (11), cell wall structure (2), and lysine biosynthesis (24) is well known. Indeed, the differences are of such magnitude that Whitaker (25) has suggested that the fungi be placed in their own kingdom.

The assumption that all fungi contain spermine results primarily from the classical proof that the yeast *Saccharomyces cerevisiae* contains (15) and synthesizes (20) spermine. However, the majority of the filamentous fungi have not been examined for the presence of spermine. Significantly, two recent investigations have failed to detect spermine in uredospores of *Puccinia graminis* f. sp. *tritici* (10) and in zoospores and sporangia of *Blastocladiella emersonii* (13). Consequently, we examined representatives of the major classes of filamentous fungi for the presence of spermine.

MATERIALS AND METHODS

Except for *Blastocladiella emersonii* (kindly provided by J. S. Lovett), all the fungi were from the culture collection, Plant Pathology Department, University of Nebraska. Media used to grow the fungi are noted in Table 1. Cells (usually equivalent to 200 mg, dry weight) were suspended in 6% perchloric acid, mixed for 2 min in a Waring blender, homogenized in a Potter-Elvehjem hand homogenizer, and stirred overnight. After centrifugation to remove cellular debris, perchloric acid extracts were routinely analyzed for polyamines by the modification of Dion and Herbst (7) of the procedure of Seiler and Wiechmann (17). The perchloric acid extracts (0.5 ml) were transferred to a conical centrifuge tube and reacted with 0.4 ml of dansyl chloride (30 mg/ml of acetone) and 120 mg of Na_2CO_3. Dansylation proceeded overnight at room temperature in the dark. Excess dansyl chloride was then converted to dansyl proline by reaction with 0.1 ml of proline (100 mg/ml of water) for 30 min in the dark. The dansylated polyamines were extracted with 0.5 ml of spectroquality benzene. Precoated Silica Gel G thin-layer chromatographic (TLC) plates (250 μm) (Analtech, Newark, Del.) were spotted with 5-μl portions of the benzene extract, and the dansylated polyamines were separated by development in ethyl acetate-cyclohexane (2:3, vol/vol). The TLC plates were sprayed with triethanolamine-isopropanol (1:4 vol/vol) and dried in vacuo overnight, and the polyamines were quantitated by fluorometric scanning. Fluorescence intensities were measured as recommended by Dion and Herbst (7) with a Turner model 111 fluorometer equipped with a TLC scanner and recorder. Six samples and three standards were run on each plate.

RESULTS AND DISCUSSION

Table 1 summarizes our findings on the polyamine content of the fungi surveyed. We have confirmed the presence of three polyamines, putrescine, spermidine, and spermine, in *S. cerevisiae* as well as in other yeasts. The spermidine levels that we have detected (Table 1) are in the range of those previously reported (4, 22). However, we were consistently unable to detect spermine in any of the filamentous fungi that we examined. These results were obtained with the fluorescent dansyl derivative procedure of Dion and Herbst (7) as recommended by Cohen (5). In this procedure, the dansylated polyamines are developed in ethyl acetate-cyclohexane (2:3, vol/vol). We have also been unable to detect spermine in perchloric acid extracts from the filamentous fungi, utilizing three alternate procedures: (i) that of Fleisher and Russell (8), wherein the dansylated polyamines are developed in chloroform-triethylamine (100:20, vol/vol); (ii) the two-dimensional procedure of Creveling and Daly (6); and (iii) the fluorometric procedure of Abe and Same-

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TABLE 1. *Polyamines detected in perchloric acid extracts of fungi*

Species	Class	Medium (reference)	Polyamines[a]		
			Putrescine	Spermidine	Spermine
Filamentous fungi					
Blastocladiella emersonii[b]	Chytridiomycetes	Difco Cantino PYG (12)	+	+	−
Pythium ultimum[b]	Oomycetes	Modified Fries (14)[c]	±	±	−
Pythium debaryanum[b]	Oomycetes	Modified Fries (14)[c]	±	±	−
Rhizopus stolonifer[b, d]	Zygomycetes	Glucose-asparagine (21)	+	+	−
Mucor hiemalis[b]	Zygomycetes	Glucose-asparagine (21)	+	−	−
Neocosmospora vasinfecta[e]	Ascomycetes	Vogel minimal medium N (23)	+	+	−
Neurospora crassa (FGSC988A)[f]	Ascomycetes	Vogel Minimal medium N (23)	+	+	−
Sclerotinia sclerotiorum[f, g]	Ascomycetes	Glucose-asparagine plus (9)	+	+	−
Botryodiplodia theobromae[b, d]	Deuteromycetes	Modified Westergaard-Mitchell, (R. H. Knight and J. L.Van Etten, J. Gen. Microbiol., in press)	+	+	−
Fusarium moniliforme[e]	Deuteromycetes	Vogel minimal medium N (23)	+	+	−
Helminthosporium maydis[f]	Deuteromycetes	Modified Fries (14)	+	+	−
Periconia circinata[f]	Deuteromycetes	Modified Fries (14)	+	+	−
Macrophomina phaseoli[e, g]	Deuteromycetes	Glucose-asparagine plus (9)	+	+	−
Rhizoctonia solani[e]	Deuteromycetes	Glucose-asparagine plus (9)	+	+	−
Ustilago sphaerogena[b]	Basidiomycetes	Glucose-yeast extract (9)	+	+	−
Yeasts					
Debaryomyces globosus	Ascomycetes	Burkholder medium (3)	+	+	+
Hansenula subpelliculosa	Ascomycetes	Burkholder medium (3)	+	+	+
Saccharomyces cerevisiae	Ascomycetes	Burkholder medium (3)	+	+	+

[a] Symbols: +, 0.1 to 10 μg/mg, dry weight; ±, 0.001 to 0.1 μg/mg, dry weight; −, less than 0.001 μg/mg, dry weight.
[b] Analyzed mycelia from shake cultures.
[c] Medium included 100 μg of thiamine per liter and 5 μg of biotin per liter.
[d] Analyzed both spores and mycelia.
[e] Analyzed mycelia from both shake and stationary cultures.
[f] Analyzed mycelia from stationary cultures.
[g] Analyzed both sclerotia and mycelia.

jima (1), wherein the nonderivatized polyamines are chromatographed and subsequently sprayed with fluorescamine.

We explored several possibilities as to why procedures that were adequate for the detection of spermine in yeast might fail to detect spermine in filamentous fungi. One possibility is that 6% perchloric acid might extract putrescine and spermidine, but not the more highly charged spermine. However, several lines of evidence refute this possibility. Spermine was not detected in fungal spores disrupted in a Braun MSK cell homogenizer prior to extraction. Likewise, extraction of mycelia in either 12% perchloric acid, hot 5% trichloroacetic acid (95°C for 20 min), 5% trichloroacetic acid containing 0.5 M HCl, or 6% perchloric acid supplemented with 0.1 M $MgCl_2$ failed to detect cryptic spermine. The presence of 0.01 M $MgCl_2$ in the extraction medium is considered sufficient to displace ribosomal polyamines (5), and the hot 5% trichloroacetic acid treatment would degrade any nucleic acids to which spermine might be bound.

A second possibility is that the spermine present in filamentous fungi was converted metabolically to a spermine derivative such as acetylspermine or glutathionylspermine. Prolonged exposure to low temperatures during harvesting by centrifugation at 4°C or anaerobic culture conditions causes *Escherichia coli* to convert a substantial portion of its spermidine to acetylspermidine (18) or glutathionylspermidine (19), respectively. However, we were unable to detect spermine in the filamentous fungi, regardless of the growth conditions (stationary and shake culture) or the temperature at which the cultures were harvested or extracted. Additionally, HCl hydrolysis of the perchloric acid extracts (6 N HCl at 110°C for 24 h) did not release spermine from spermine conjugates. Such reaction conditions did not destroy the putrescine and spermidine present in the extracts.

A third possibility is that the synthesis of spermine is repressed under the culture conditions chosen. We feel this situation is unlikely because of the wide variety of organisms and culture media employed. In addition, we examined fungal spores and sclerotia as well as mycelia, and cultures were examined periodically for 5 days after spore germination. We also excluded the presence of a spermine-degrading enzyme system in the filamentous fungi; exogenous spermine added prior to extraction was detected in undiminished concentration. No extraneous spots were observed as a result of the added spermine. The inability to detect spermine was not due to insufficient dansylating reagent or excess ammonium ions, since spermine dansylation was unimpaired when 0.1 M NH_4Cl was added to the reaction mixture.

The conclusion that fungi, like all other eukaryotes, possess the polyamine spermine rests not only on its presence in yeast (15, 20) but also on its reported presence in *Neurospora* (22) and *Aspergillus* (4). We were unable to detect spermine in *Neurospora* when using identical procedures (7, 22) (Table 1). Instead, in agreement with Mennucci et al. (13) we detected in all of the filamentous fungi examined an unidentified compound which migrates with an R_f value very close to that of spermine and which could easily have been mistaken for spermine. This possibility was rendered more likely since the amount of the unidentified compound present in all of our fungal extracts (about 0.25 μg/mg, based on the fluorescence constant of authentic spermine) was almost identical to levels of spermine reported in filamentous fungi (4, 22). However, this compound is not spermine. (i) Its R_f value is consistently slightly less than that of spermine, i.e., 0.20 versus 0.22. When authentic spermine is added, two distinct spots are evident. (ii) Its dansylated derivative fluoresces slightly more orange than authentic dansylated spermine. (iii) The compound is not visible when the fluorescamine spray procedure (1) is used to detect polyamines. (iv) An ethyl acetate-cyclohexane ratio in the developing solvent of 2:3 was originally chosen (7) because it readily separates putrescine from ammonia and cadaverine. Fortuitously, this solvent system causes the unknown compound to migrate similarly to authentic spermine. If the ethyl acetate-cyclohexane ratio is altered to 3:2, a distinct separation of spermine and the unknown compound is achieved. (v) Most convincingly, the unknown compound is not derivatized via a sulfonamide linkage; it is not a dansylated amine. Seiler and Deckardt (16) have reported that dansylated amines can be distinguished from oxygen-linked derivatives such as dansylated sucrose by hydrolysis in 5 M methanolic KOH for 30 min at 50°C. When this procedure was applied to the extracts from the filamentous fungi, the dansylated putrescine and spermidine spots remained, whereas the unknown compound disappeared.

In conclusion, we have been unable to detect spermine in filamentous fungi by procedures adequate for its detection in yeast. The concentrations of spermine and spermidine in yeast are roughly equivalent. If the filamentous fungi do contain spermine, it must be present at less than 0.001 μg/mg, dry weight, or less than 0.1% of the spermidine present since our techniques can detect 10^{-12} mol of spermine.

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