JOURNAL OF CLINICAL MICROBIOLOGY, July 1998, p. 2153–2156 Vol. 36, No. 7
0095-1137/98/$04.00+0

Differentiation of *Rhizomucor* Species by Carbon Source Utilization and Isoenzyme Analysis

MÁRIA VASTAG, TAMÁS PAPP, ZSOLT KASZA, AND CSABA VÁGVÖLGYI*

Department of Microbiology, Attila József University, H-6701 Szeged, Hungary

Received 18 December 1997/Returned for modification 11 February 1998/Accepted 2 April 1998

Nineteen *Rhizomucor miehei* and *Rhizomucor pusillus* isolates were assayed for their ability to utilize 87 various substrates as a single carbon source. Besides a difference in sucrose utilization, distinctive differences were found in the utilization of glycine, phenylalanine, and β-alanine. Five isoenzyme systems also proved useful for the determination of markers of distinctive value at a species level. Data were used to obtain information about the genetic polymorphism of these species: a high degree of variability was found among the *R. pusillus* isolates, whereas the group of *R. miehei* isolates was more homogeneous genetically.

The zygomycoses comprise a diverse group of rare mycotic diseases; the term mucormycoses is preferred for zygomycoses caused by some members of the order *Mucorales* (e.g., *Absidia*, *Rhizomucor*, *Rhizopus*, and *Mortierella* [7, 10]). These infections are most frequently associated with diabetic ketoacidosis, immunosuppressive conditions, extreme malnutrition, or neutropenia (7). Though these mycoses are relatively rare, they attract special attention, as they are rapidly progressive and frequently fatal (12, 14).

The genus *Rhizomucor* includes three species: *Rhizomucor pusillus*, *Rhizomucor miehei*, and *Rhizomucor tauricus*; these are clearly distinct from *Mucor* by virtue of their thermophilic nature and some morphological features (8). While the legitimacy of regarding *R. tauricus* as an autonomous species (represented by a single isolate only) is sometimes questioned, the other two *Rhizomucor* species are well represented in nature (8).

Fungi belonging in this genus are found among the etiological agents of human and animal mucormycosis. The role of *R. tauricus* in such infections is unknown, but there are both clinical and experimental data concerning infections caused by *R. pusillus* or *R. miehei* (7, 11). However, there are two reasons why the exact number of infections caused by one or another of the *Rhizomucor* species can only be guessed at. One is that in several cases the fungal pathogen has not been identified properly at low taxonomic levels (genus and species), and the other is that there is some uncertainty in the differentiation of *R. miehei* and *R. pusillus* isolates. Though *R. miehei* has been found to be homothallic, while *R. pusillus* is mainly heterothallic, the discovery of the rarely occurring homothallic *R. pusillus* isolates did not help to simplify the scheme (8). Certain approaches, such as determination of the number of nuclei in the sporangiospores (15) or mating studies coupled with determination of the morphological traits of the zygospores (8, 18), could supply characteristics for the delimitation of these species. However, the differences are not clear-cut in every case (15), or the procedure requires a prolonged time (8).

Both isoenzyme analysis and the determination of carbon source utilization patterns have proved to be valuable tools in the handling of taxonomic questions in the genus *Mucor* (16, 17). Analysis of proteases by immunoelectrophoretic techniques underlined the connection of the homo- and heterothallic strains of *R. pusillus* and their difference from *R. miehei* (2). Assimilation abilities on a limited number of compounds have also been checked: the growth-stimulative effect of thiamine and the inability to assimilate sucrose were found to be characteristic of *R. miehei* (10, 11), although these differences seem to be insufficient to allow species delineation in every case. These characteristics, together with the colony color (brownish for *R. pusillus* and grayish for *R. miehei*) and the sizes of the zygospores (the diameter is below 50 μm for *R. miehei* and over 50 μm for *R. pusillus*), are now used to identify *Rhizomucor* isolates (8, 11).

The purpose of the present study was to broaden the basis of knowledge, affording a methodically simple, quick, and more unambiguous identification of the two *Rhizomucor* species. As part of this, carbon source assimilation patterns were determined and isoenzyme analysis was carried out with 19 *Rhizomucor* strains obtained from various sources and three other zygomycetous strains (Table 1).

The abilities of the *Rhizomucor* and selected *Absidia*, *Mucor*, and *Rhizopus* strains to utilize 87 individual compounds as their sole carbon source were tested basically as described earlier for *Mucor* isolates (16), except that the incubation was at 37°C (20°C for *Mucor*).

Of the 87 carbon substrates tested, 13 yielded uniformly positive results; these included D-lyxose, L-xylose, maltose, lactose, melibiose, starch, sorbitol, L-alanine, L-proline, L-tyrosine, L-asparagine, raffinose, and glycerol-α-monoacetate. Thirteen other compounds gave only negative results; these were xylan, γ-butyrolactone, vanillin, α-methyl-D-xyloside, ascorbic acid, L-glutamine, L-malic acid, methanol, orotic acid, L-lysine, protocatechuic acid, inulin, and gallic acid. Certain carbon substrates led to ambiguous results with low reproducibility. These were L-rhamnose, L-isoleucine, L-serine, cytosine, thymine, dihydroxyacetone, α-methyl-D-galactoside, D-glucosamine, fumaric acid, succinic acid, gluconic acid, L-ornithine, and uracil.

The remaining 48 carbon substrates were found to be utilized to various extents by the investigated strains (Table 2). Most of these variations were intergeneric and related to the differences observed between *Rhizomucor*, *Absidia*, *Mucor*, and *Rhizopus* strains. Among them, however, four compounds whose utilization showed a clear difference between the *R. miehei* and *R. pusillus* isolates were identified. These were sucrose, glycine, phenylalanine, and β-alanine; this is the first example of the distinctive value as biochemical markers of the

* Corresponding author. Mailing address: Department of Microbiology, Attila József University, P.O. Box 533, H-6701 Szeged, Hungary. Phone: (36) (62) 455 992. Fax: (36) (62) 432 488. E-mail: csaba@bio.u-szeged.hu.

TABLE 1. Fungal strains investigated in the present study

Species name	Code[a]	Original code[b]	Source and other code[c]
R. pusillus[d]	R2A	NRRL 2543	Animal mycosis, United Kingdom; ATCC 22064 as *R. miehei*
R. pusillus[d]	R2B	NRRL 2543	Animal mycosis, United Kingdom; ATCC 22064 as *R. miehei*
R. pusillus	R4	NRRL A-23448	US
R. pusillus	R5	NRRL A-23504	US
R. miehei	R6	NRRL 3169	US, United States; ATCC 46343
R. miehei	R8	NRRL 5282	Composted peppermint hay, India; ATCC 46344
R. miehei	R9	CBS 370.71	Human sputum, The Netherlands
R. miehei	R10	NRRL 5284	Rotting apple, United States; ATCC 46346
R. miehei	R11	NRRL 5901	Cow placenta, United States
R. miehei	R12	NRRL 6303	Corn crop, United States; A-16207
R. pusillus[d]	R13	NRRL A-13100	US
R. miehei	R14	NRRL A-18359	US
R. miehei	R15	CBS 360.92	Human mycosis, Australia
R. pusillus[d]	R16	ETH M4920	Human tracheal discharge, Switzerland
R. miehei	R17	ETH M4918	Composted litter, Switzerland
R. pusillus	R18	WRL CN(M)231	Suspected pathogen from lamb
R. pusillus	R19	FRR 2490	US, Australia
R. pusillus	R20	IBP M.p./1	US, Poland
R. pusillus[d]	R21	FRR 1652	US
Absidia glauca	Ab1	CCM F450	Soil; NCAIM F00658
Mucor piriformis	N6	NRRL 26212	Rotting pear, United States
Rhizopus stolonifer	Rp11	SzMC 1123	Soil, United States

[a] A code which is used throughout this paper for clarity.
[b] CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; ETH, Swiss Federal Institute of Technology Culture Collection, Zurich, Switzerland; FRR, CSIRO Food Research Culture Collection, North Ryde, New South Wales, Australia; IBP, Institute of Fermentation Industry Culture Collection, Warsaw, Poland; SzMC, Szeged Microbial Collection, University of Szeged, Szeged, Hungary; WRL, Wellcome Bacterial Collection, Beckenham, United Kingdom.
[c] US, unknown source.
[d] Isolate whose original species name was found to be incorrect.

last three of these. The difference in sucrose utilization was described earlier (10); this and the stimulative effect of thiamine on the growth of *R. miehei* were the only two biochemical markers of some practical value for the differentiation of these species (11). The use of these four carbon sources allows a clear delineation of these two species.

To provide further characteristics of distinctive value, isoenzyme analysis of the isolates was also carried out. Sporangiospores collected from malt extract agar slants incubated at 37°C for 5 days were inoculated (10^7 spores/150 ml) in yeast extract-glucose liquid medium. Cultivation was carried out in 500-ml Erlenmeyer flasks at 37°C on a rotary shaker at 200 rpm. Protein extraction and electrophoresis were performed as described earlier (17). The resolvable enzyme systems used were acid phosphatase (EC 3.1.3.1) (4), β-esterase (EST) (EC 3.1.1.2) (4), glutamate dehydrogenase (EC 1.4.1.4) (1), glucose-6-phosphate dehydrogenase (G6D) (EC 1.1.1.49) (5), and malate dehydrogenase (EC 1.1.1.38) (3). All incubations took place at 21°C, in the dark. The gels were then rinsed, first in distilled water and next in 7% acetic acid, and read.

The highest variability was found for G6D; eight different electrophoretic patterns (electromorphs) were detected, while for EST three electromorphs were found. The least variable were the acid phosphatase, glutamate dehydrogenase, and malate dehydrogenase staining patterns; these each showed two different electromorphs. These two electrophoretic patterns correlated perfectly with the two investigated *Rhizomucor* species. A similar correlation was found in the case of the EST pattern; however, this is less easily scored because of the more complex pattern of this activity stain (Fig. 1).

This study was started with 18 different *Rhizomucor* strains. However, after preliminary experiments, it turned out that the strain NRRL 2543 (isolated from an animal mycosis) is not homogeneous; it contains two morphologically slightly different fungi. Otherwise, this strain clearly illustrates the problems with the species identification in the genus; it is maintained under different names in two international collections (as *R. pusillus* in the Northern Regional Research Laboratory Agricultural Research Service Culture Collection, Peoria, Ill. [NRRL], and as *R. miehei* in the American Type Culture Collection, Rockville, Md. [ATCC]). On the basis of our results, it was identified as *R. pusillus*. While the carbon source assimilation patterns of these separated strains were the same, their G6D patterns were different.

Besides determining easily countable characteristics for the identification of *Rhizomucor* isolates, the experimental data provide preliminary information concerning the genetic variability of these species. After enzyme staining, each independent band with a defined relative mobility was coded with a binary state character; this was 1 if the band was present and 0 if the band was absent for an isolate (variations in staining intensity were not taken into account). The results of the carbon source utilization experiments were coded as follows: 0, negative; 0.5, weak, ambiguous reaction; 1, positive reaction. Matrices created from these data were used for the calculation of Jaccard coefficients. Dendrograms were produced with an unweighted pair-group method by using arithmetic averages (UPGMA) (13) linkage. All computations were performed with the SYNTAX 5.0 software package (6).

There was no basic difference between the dendrograms created from the unified data matrix or from the matrix of carbon source utilization data (results not shown) and those created from the isoenzyme data alone (Fig. 2). All these dendrograms revealed the presence of three clusters. One of these (cluster C) corresponds to the isolates used as outgroups in this study (Ab1, N6, and Rp11), while clusters A and B contain the investigated *R. pusillus* and *R. miehei* isolates, respectively (Fig. 2). While the reading of the extent of colony growth inevitably involves some inconsistencies (resulting in a higher variation of characteristics), the evaluation of isoen-

TABLE 2. Characteristics of growth on carbon sources variably used by zygomycetous strains[a]

Compound	*R. miehei*	*R. pusillus*	*Rhizopus* (Rp11)	*Absidia* (Ab1)	*Mucor* (−4)
D-Ribose	++	++	(−)	+	−
D-Arabinose	+	+	−	(−)	−
D-Xylose	++	++	+	+++	+ (i)
D-Glucose	+++	+++	+	+++	+++
D-Mannose	+++	++	+	+++	+++
D-Galactose	++	++	+	+++	+++
D-Fructose	++	++	+	+++	+++
L-Arabinose	++	++	+	++	+
L-Sorbose	(−)	(+)	−	(+)	+
Sucrose	−	+	−	+++	−
Cellobiose	++	++	+	+++	+++
Melezitose	(−)	(−)	−	+	+
Dextran	+	+	−	−	(−)
Glycerol	(−)	+	+	(+)	−
i-Erythritol	(−)	+	−	−	(−)
D-Mannitol	++	+	+	++	+
Galactitol	(−)	−	−	(+)	(−)
Ribitol	+	+	(−)	+++	(+)
myo-Inositol	(+)	(−)	−	−	(−)
Xylitol	+	+	+	++	+
Glycine	−	+	+	+	(−)
L-Valine	(+)	+	− (i)	(+)	(−)
L-Leucine	−	−	−	+	(−)
L-Methionine	(−)	−	− (i)	−	(−)
L-Phenylalanine	−	+	(−)	(+)	(−)
L-Tryptophan	−	−	−	−	(−)
L-Threonine	(−)	(−)	−	(−)	−
L-Cysteine	(+)	(−)	−	(−)	(−)
L-Aspartic acid	++	+	+	+++	++
L-Glutamic acid	++	++	+	+++	+
L-Arginine	−, +	+	(−)	+	(−)
L-Histidine	(−) (i)	−	− (i)	+	−
β-Alanine	−	+	−	(+)	(−)
L-Citrulline	−	(−), +	(+)	(+)	(−)
Uridine	−	(+), −	−	−	−
Cytidine	−	(−)	(−)	(−)	(−)
Adenosine	(−)	(+)	(−)	+	+
L-Lactic acid	−	−, (+)	(−)	−	+
L-Hydroxybutyric acid	−	(−), +	−	−	(+)
β-Methyl-D-galactoside	+, (+)	++	−	−	(+)
α-Methyl-D-mannoside	− (i), +	− (i), +	−	−	(+)
5-Keto-D-gluconic acid	−, (+)	+	−	−	(+)
2-Keto-D-gluconic acid	(−), +	+	−	+	(−)
Gentisic acid	−, +	+	−	−	−
D-Glucuronic acid	−, +	−, +	−	−	−
D-L-Isocitric acid	−	−, +	+	+	−
Pyruvic acid	(+), +	(−), +	(+)	+	(−)
β-Methyl-D-glucoside	−, (−)	−, (−)	−	(−)	−

[a] Growth for a particular strain is designated on the basis of the strength of colony development as follows: +++, vigorous growth; ++, average growth; +, slight growth [very weak mycelial growth was designated (+) or (−) if it suggested an ambiguous slight growth or an ambiguous lack of growth, respectively]; −, no growth; (i), inhibition of spore germination or reduced (compact) colony morphology.

zyme patterns is less problematic. Therefore, we suggest that this discloses the intraspecific variability of *R. miehei* and *R. pusillus* more precisely than the other two dendrograms. Three important features could be observed in this dendrogram. First, all *R. pusillus* isolates segregate from the *R. miehei* isolates at a very high level of dissimilarity ($D = 0.90$); this is practically the same as the level of dissimilarity found for the representatives of the other three genera involved in the outgroup (*Absidia*, *Mucor*, and *Rhizopus*; $D = 0.87$, 0.89, and 0.92, respectively). The second feature is that the extent of genetic

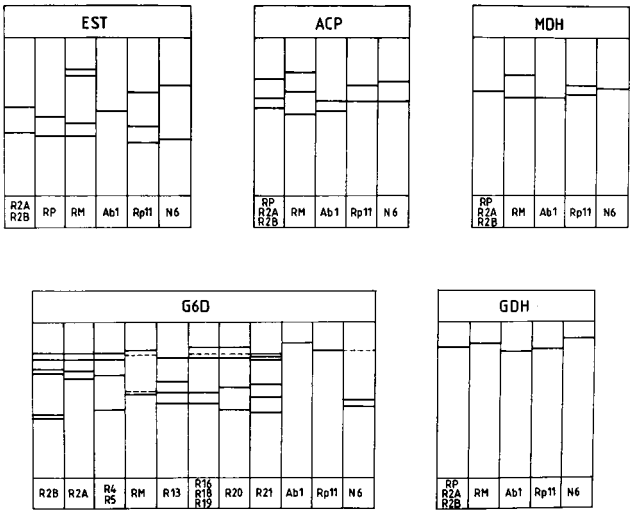

FIG. 1. Isoenzyme patterns observed for various *Rhizomucor*, *Absidia*, *Rhizopus*, and *Mucor* isolates. The direction of migration is towards the anode. RP, heterothallic *R. pusillus* strains; RM, *R. miehei* strains. For other strain codes, see Table 1.

polymorphism is different in the two *Rhizomucor* species: while substantial polymorphism was found among the *R. pusillus* strains, the investigated *R. miehei* strains proved to be homogeneous; no difference was revealed with the five enzyme systems investigated. One explanation could be that this phenomenon is connected with the different, homothallic and (mainly) heterothallic natures of *R. miehei* and *R. pusillus*, respectively. In this respect, it would be interesting to examine if there is any difference in genetic variability in the homo- and heterothallic *R. pusillus* strains. Unfortunately, homothallic *R. pusillus* strains are rare; e.g., of the strains investigated, only R2A and R2B (of common origin) were found to be homothallic. This number of strains is too limited to provide a basis for any conclusion about their different genetic variability. However, as a third observation, it could be seen that the homo- and heterothallic *R. pusillus* strains segregate into two subclusters at a rather high level ($D = 0.56$). This clustering is caused by their different EST and G6D patterns. Further investigations, with the involvement of more *R. pusillus* isolates, should be

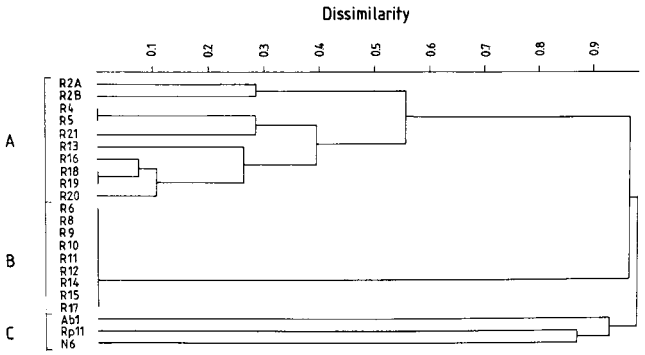

FIG. 2. UPGMA dendrogram of the *Rhizomucor* and outgroup strains produced by UPGMA linkage of the Jaccard coefficients. It was based on the 57 characteristics obtained from their isoenzyme analysis. The scale represents dissimilarity (squared distance). The cophenetic correlation was 0.9975. The labels A, B, and C mark clusters containing the *R. pusillus*, the *R. miehei*, and the outgroup strains, respectively.

performed to clarify the background of this high degree of intraspecific polymorphism and its connection (if any) with the sexual characteristic.

We thank Kerry O'Donnell (U.S. Department of Agriculture, Peoria, Ill.) for providing some of the *Rhizomucor* strains.

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