JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1979, p. 579-582
0095-1137/79/10-0579/04$02.00/0

Vol. 10, No. 4

Rapid Identification of *Prototheca* Species by the API 20C System

ARVIND A. PADHYE,*[1] JOHN G. BAKER,[2] AND RICHARD F. D'AMATO[2]

Mycology Division, Center for Disease Control, Public Health Service, U.S. Department of Health, Education, and Welfare, Atlanta, Georgia 30333,[1] and Analytab Products, Inc., Division of Ayerst Laboratories, Plainview, New York 11803[2]

Received for publication 23 July 1979

The conventional auxanographic method of testing for the assimilation of carbohydrates and alcohols by the various species of *Prototheca* requires at least 2 weeks of incubation at 25 to 30°C before definitive results are obtained. Even though *Prototheca* spp., in culture as well as in fixed tissues, can be identified more rapidly by fluorescent-antibody techniques in which species-specific reagents are used, such diagnostic facilities and reagents are not available in most diagnostic laboratories. The API 20C clinical yeast identification system, a commercially available ready-to-use micromethod, was found to permit the definitive identification of *P. stagnora*, *P. wickerhamii*, and *P. zopfii* within 4 days.

Protothecosis, an infection of humans as well as domestic and wild animals, is caused by two species of the genus *Prototheca*, *P. wickerhamii* and *P. zopfii*. The *Prototheca* species are achlorophyllous algae morphologically similar to the green algae of the genus *Chlorella*. They grow rapidly on routine laboratory media (without the commonly used antimycotic cycloheximide) and produce soft, yeastlike, white to tan colonies. Their identification is based on their morphology and their carbohydrate and alcohol assimilation patterns. Arnold and Ahearn (1) investigated the carbohydrate and alcohol assimilation patterns of the then-described species of *Prototheca* and recognized the validity of five species, *P. filamenta*, *P. moriformis*, *P. stagnora*, *P. wickerhamii*, and *P. zopfii*.

Nadakavukaren and McCracken (4) studied the ultrastructure of *P. filamenta* and pointed out that the genus *Prototheca* was not the proper genus for it. Subsequently, King and Jong (3) proposed a new genus *Sarcinosporon*, and reduced *P. filamenta* to synonymy with *Sarcinosporon inkin*. Their treatment was not accepted by Pore et al. (5), who carried out comprehensive morphological and physiological studies with several isolates of *P. filamenta*. They proposed a new genus, *Fissuricella*, to accommodate *P. filamenta*, with the binomial *Fissuricella filamenta* (5). Morphological, physiological, and immunofluorescent studies by Sudman and Kaplan (6) clearly demonstrated that *P. moriformis* was synonymous with *P. zopfii*. Their studies led them to conclude that there are only three valid species in the genus *Prototheca*, *P. stagnora* Cooke 1968, *P. wickerhamii* Tubaki and Soneda 1959, and *P. zopfii* Kruger 1894.

The auxanographic method of Arnold and Ahearn, although reliable, is time consuming. It may take as long as 2 weeks to make a definitive identification with it. The *Prototheca* spp., in culture as well as in fixed tissues, can be identified more rapidly by fluorescent-antibody techniques, with species-specific reagents (2). Such facilities and reagents are not available in most diagnostic laboratories. The API 20C clinical yeast system is a ready-to-use micromethod that permits the performance of 19 assimilation tests for the identification of most clinically significant yeasts belonging to the genera *Candida*, *Cryptococcus*, *Rhodotorula*, *Saccharomyces*, *Torulopsis*, and *Trichosporon*. Biochemical reactions are complete after 72 h of incubation. The API 20C system provided an opportunity to determine the assimilation patterns of the three *Prototheca* species for possible use in their rapid identification. The results of this investigation are presented here.

MATERIALS AND METHODS

Cultures. Thirty-three isolates of the *Prototheca* spp. and five of *F. filamenta*, from the culture collections of the Mycology Division of the Center for Disease Control and Analytab Products (API), were selected for the study. These 38 cultures had been previously identified by the method of Arnold and Ahearn and also by the fluorescent-antibody technique (6).

The stock cultures were grown on duplicate slants of Sabouraud dextrose agar. The assimilation pattern of each isolate was studied by two laboratories: the Center for Disease Control Mycology Division, which used the API 20C system, and the API Mycology

Laboratory, which used the API 20C and API 50C systems. The API 50C system is also a ready-to-use micromethod which permits the study of the assimilation patterns of 50 carbohydrates. The system is not available commercially for public use. API uses it as a research tool. The 19 and 50 carbohydrates included in the API 20C and API 50C systems are shown in Table 1.

Morphology. The colonial micromorphology of each isolate was studied on Sabouraud dextrose agar and corn meal agar with 10% Tween 80 at 30°C.

Urease activity and nitrate assimilation. Urease activity was determined on Christensen urea agar at 30°C and read after 7 days. Authenticated isolates of *Cryptococcus albidus* and *Candida albicans* were run simultaneously as positive and negative controls, respectively.

Nitrate assimilation was determined by suspending a small amount of growth of each isolate on Sabouraud dextrose agar in tubes of warm liquefied 2% Noble agar with yeast carbon base to match a density of slightly less than that of a Wickerham no. 1 standard. The suspension was poured into a petri dish and allowed to solidify. Paper disks with 3% KNO_3 and 3% peptone were set on the agar surface at opposite sides, and the plates were incubated at 30°C for 7 days. Positive growth was interpreted as increased turbidity around the disks; the peptone served as a positive control. An isolate of *Cryptococcus albidus* served as a positive control for nitrate assimilation.

Picked colonies of each isolate (from 3-day-old growth on Sabouraud dextrose agar at 30°C) were suspended in the basal medium (0.7% agar with yeast nitrogen base) to match a density slightly less than that of a Wickerham no. 1 standard. The suspension was then pipetted into wells containing 19 and 50 carbohydrates (API 20C and API 50C, respectively). These strips were incubated in moist chambers at 30°C and were read daily for a total of 4 days.

RESULTS

Morphology. Colonies of the three *Prototheca* spp., on Sabouraud dextrose agar, were smooth, heaped, mucoid, yeastlike, and white to tan. Grossly, they looked so much like yeasts that they could have been considered to be such without microscopic examination. An unknown isolate should be examined first microscopically to identify it as belonging to the genus *Prototheca* before its assimilation pattern is determined by the API 20C or API 50C system. The generic identification can be made without any difficulty because these organisms have such a distinctive morphology.

Microscopically, the cells were hyaline and globose to ovoid, and they ranged from 1.3 to 13.4 by 1.3 to 16.1 nm. Asexual reproduction in mature cells began with the nuclear division of the mother cell, generally more than once, followed by cleavage of the cytoplasm surrounding each daughter nucleus. The endospores, so produced, formed their own walls while still enclosed within the mother cell, which eventually ruptured to release them (Fig. 1). The freed endospores enlarged and went through the same process of reproduction. The production of endospores inside the mother cell is characteristic of the genus *Prototheca*, and differentiates it from other genera (Fig. 1). Even though there were some differences in the size of sporangia and endospores among the three species, these differences were not consistent enough to definitely identify the species on the basis of morphology alone.

Colonies of *F. filamenta* were creamy to tan, dry to slightly moist, raised and pitted to rugose. Microscopically, all isolates showed sarcina-like clusters of cells formed by repeated fission of the parent cell in a number of planes. Hyphal development was evident in all five isolates. The hyphal filaments broke easily into arthroconidia that again divided into sarcina-like clusters.

Growth at 37°C. The isolates of *P. wickerhamii*, *P. zopfii*, and *F. filamenta* grew well at 30 and 37°C. Three isolates of *P. stagnora* showed good growth at 30°C but did not grow at 37°C.

Urease activity and nitrate assimilation. All isolates of the three *Prototheca* spp. failed to hydrolyze urea or assimilate KNO_3; on the other hand, the *F. filamenta* isolates hydrolyzed urea within 7 days, but did not utilize KNO_3 as a nitrogen source.

Carbohydrate assimilation. The assimilation patterns of the API 20C and the API 50C

TABLE 1. *Carbohydrates of the API 20C and the API 50C systems*

Temoin	Amygdaline
Glycerol[a]	Arbutin
Erythritol	Esculin
D-Arabinose	Salicin
L-Arabinose[a]	D-Cellobiose[a]
Ribose	Maltose[a]
D-Xylose[a]	Lactose[a]
L-Xylose	D-Melibiose
Adonitol[a]	Saccharose[a]
Methyl xyloside	Trehalose[a]
Galactose[a]	Inulin
D-Glucose[a]	D-Melezitose[a]
D-Fructose	D-Raffinose[a]
D-Mannose	Amidon
L-Sorbose	Glycogene
Rhamnose	Xylitol[a]
Dulcitol	β-Gentobiose
Inositol[a]	D-Turanose
Mannitol	D-Lyxose
Sorbitol[a]	D-Tagatose
Methyl-D-mannoside	D-Fucose
Methyl-D-glucoside[a]	L-Fucose
N-Acetyl-D-glucosamine[a]	D-Arabitol
L-Arabitol	2-Keto-gluconate[a]
Gluconate	5-Keto-gluconate

[a] Carbohydrates included in the API 20C system.

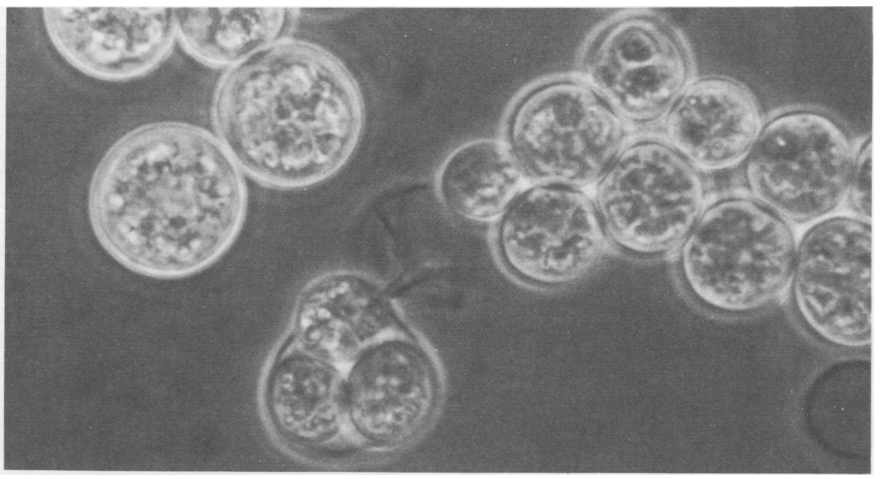

FIG. 1. *Photomicrograph of Prototheca zopfii showing characteristic sporangia containing endospores; magnification ×1,300.*

carbohydrates by the *Prototheca* spp. are summarized in Tables 2 and 3. The identifications of the 38 isolates obtained by the two systems were identical with those originally obtained by the method of Arnold and Ahearn and by the fluorescent-antibody technique.

With the API 20C system, *P. stagnora* showed positive assimilation of only 3 of the 19 carbohydrates. *P. wickerhamii* isolates used 4 of the 19 carbohydrates, whereas *P. zopfii* utilized only two carbohydrates (Table 2). In some isolates of *P. wickerhamii*, the assimilation of galactose was weak and was not conclusive by the end of 3 days. In 3 of the 19 isolates of *P. wickerhamii*, galactose assimilation was negative at the end of a 4-day incubation period. The variable assimilation of galactose, however, did not interfere with the identification of the *P. wickerhamii* isolates, because its differentiation from the other two species was based on the assimilation of trehalose, which was conclusive in all of the isolates at the end of 3 days.

The assimilation patterns obtained with the API 50C system were almost identical to those obtained by the API 20C system, except that the 38 isolates showed positive assimilation of an additional carbohydrate, fructose. Sixteen of the 19 isolates of *P. wickerhamii* assimilated mannose when tested with the API 50C, but the remaining three isolates of *P. wickerhamii* showed weak assimilation of mannose by the end of a 4-day incubation period. None of the remaining 44 carbohydrates was assimilated by any of the three *Prototheca* spp. (Table 3).

The *F. filamenta* isolates assimilated a wide range of carbohydrate substrates. The tests with the API 20C system showed that the five isolates assimilated glucose, 2-keto-gluconate, arabinose, xylose, galactose, methyl-D-glucoside, *N*-acetyl-glucosamine, cellobiose, lactose, maltose, sucrose, and trehalose. Assimilation of glycerol was evident in four isolates, that of sorbitol was evident in three isolates, and that of melezitose was evident in four of the five isolates. When tested by the API 50C system, 100% of the isolates assimilated erythritol, L-arabinose, ribose, D-xylose, galactose, D-glucose, D-fructose, D-mannose, methyl-D-glucoside, *N*-acetyl-glucosamine, D-cellobiose, maltose, lactose, trehalose, β-gentiobiose, D-turanose, L-fucose, gluconate, and 2-keto-gluconate. Assimilation of glycerol was observed in four of the five isolates, that of arbutin was observed in three isolates, that of D-arbitol was observed in two isolates, and that of 5-keto-gluconate was found in four of the five isolates.

TABLE 2. *Assimilation patterns of the Prototheca spp. by the API 20C system after 3 days at 30°C*[a]

Species	No. of isolates	Glucose	Glycerol	Galactose	Trehalose	Growth at 37°C
P. stagnora	3	+	+	+	−	−
P. wickerhamii	16	+	+	+/w	+	+
	3	+	+	−	+	+
P. zopfii	10	+	+	−	−	+
P. zopfii (= P. moriformis)	1	+	+	−	−	+

[a] +, Positive assimilation; +/w, weakly positive assimilation; −, negative assimilation.

DISCUSSION

Colonies of an unknown clinical isolate should be examined microscopically for the presence of sporangia and endospores, to identify it as a *Prototheca* sp., before its assimilation pattern is determined. The identification should not rest

TABLE 3. *Assimilation patterns of the Prototheca spp. by the API 50C system after 4 days at 30°C*[a]

Species	No. of isolates	Glucose	Glycerol	Galactose	Fructose	Mannose	Trehalose	Growth at 37°C
P. stagnora	3	+	+	+	+	−	−	−
P. wickerhamii	16	+	+	+	+	+	+	+
	3	+	+	−	+	+/w	+	+
P. zopfii	10	+	+	−	+	−	−	+
P. zopfii (= P. moriformis)	1	+	+	−	+	−	−	+

[a] +, Positive assimilation; +/w, weakly positive assimilation; −, negative assimilation.

upon the assimilation pattern alone because many of the assimilation profiles of *Prototheca* spp. are identical with those of other species of yeast. For example, the assimilation profile of *P. zopfii* is identical with profiles of *Trichosporon capitatum*, *Candida krusei*, and *Candida lipolytica*. Some isolates of *P. wickerhamii* showed assimilation patterns identical to those of *Torulopsis glabrata*. A generic identification of an isolate as being a *Prototheca* sp. should first be made on the basis of characteristic micromorphology; its specific identification can then be rapidly obtained by determining its assimilation pattern with the API 20C or API 50C system.

In the method of Arnold and Ahearn, the technique of Wickerham is used to determine the carbohydrate assimilation. Detection of assimilation of sucrose by *P. stagnora*, or of trehalose by *P. wickerhamii*, may require incubation of up to 2 weeks. The present study clearly demonstrates that the *Prototheca* spp. can be identified in 3 days with the API 20C or API 50C system. Identification does not depend upon sucrose or propanol assimilations but rather effectively makes use of the differential assimilation of trehalose and growth at 37°C to separate the three species.

Assimilation patterns of *P. zopfii* and *P. moriformis* are identical (Table 2). In a previous study, Sudman and Kaplan (6) tested five isolates that were received as *P. moriformis* by the fluorescent-antibody technique. They reidentified four of them as *P. wickerhamii*. The remaining isolate, according to them, was synonymous with *P. zopfii*. Our present study confirmed that four of those five isolates were *P. wickerhamii*, and the remaining isolate had an assimilation pattern identical to those of *P. zopfii* isolates. The distinctive assimilation patterns of the five isolates of *F. filamenta* further supported the view that they were not related to the genus *Prototheca*.

LITERATURE CITED

1. **Arnold, P., and D. G. Ahearn.** 1972. The systematics of the genus *Prototheca* with a description of a new species *P. filamenta*. Mycologia **64**:265-275.
2. **Kaplan, W.** 1977. Protothecosis and infections caused by morphologically similar green algae. Proceedings of the IV International Conference on the Mycoses, 1977, Brasilia, Brazil. Pan American Health Organization Scientific Publ. No. 356, p. 218-232, Washington, D.C.
3. **King, D. S., and S. C. Jong.** 1975. *Sarcinosporon*: a new genus to accomodate *Trichosporon inkin* and *Prototheca filamenta*. Mycotaxon **3**:84-94.
4. **Nadakavukaren, M. J., and D. A. McCracken.** 1973. *Prototheca*: an alga or fungus? J. Phycol. **9**:113-116.
5. **Pore, R. S., R. F. D'Amato, and L. Ajello.** 1977. *Fissuricella* gen. nov.: a new taxon for *Prototheca filamenta*. Sabouraudia **15**:69-78.
6. **Sudman, M. S., and W. Kaplan.** 1973. Identification of the *Prototheca* species by immunofluorescence. Appl. Microbiol. **25**:981-990.