GERM-TUBE FORMATION BY ORAL STRAINS OF CANDIDA TROPICALIS

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PLATE VIII

Candida species are common commensals in the mouth, alimentary canal, and vagina (Cruickshank et al., 1973), and can also act as opportunistic pathogens to cause local and systemic disorders (Ahearn, 1975). Of the various Candida species that have been implicated in disease, Candida albicans is most often isolated and the best studied. Its rapid recognition and differentiation from other species are usually based on its growth on selective media and its formation of characteristic germ tubes. These are filamentous outgrowths produced by blastospores, usually within 3 h, when C. albicans is incubated at 37°C in mammalian serum (Ahearn, 1970). Germ-tube formation is often used as the sole criterion for identifying C. albicans cultured from clinical specimens (Law et al., 1972; Budtz-Jørgensen, Stenderup and Grabowski, 1975), but its reliability for this purpose has recently been questioned after the isolation of germ-tube-positive strains of Candida tropicalis from four patients with pulmonary disease (Tierno and Milstoc, 1977).

During routine examination of oral strains of Candida species, 26 germ-tube-positive strains of C. tropicalis were identified among 937 Candida colonies examined. This report describes some of their properties.

MATERIALS AND METHODS

Isolation of Candida strains. Strains of C. albicans and C. tropicalis were isolated from the mouths of seven patients suffering from denture stomatitis and from the mouths of 19 healthy children from 7 to 12 years of age. Samples were taken from the surface of the palate on plain cotton swabs; the site sampled lay on the imaginary line between the approximal surfaces of the upper first deciduous molar and canine, midway between the gingival margin and the palatal midline. In patients suffering from denture stomatitis the affected area only was swabbed.

Each swab was inoculated on blood agar (Oxoid Blood Agar Base No. 1 containing 5% v/v of Oxoid Defibrinated Horse Blood) and incubated for 24 h at 37°C. Colonies resembling Candida species were selected and each inoculated on blood agar and Sabouraud’s Agar (Oxoid) and incubated for 24 h at 37°C.

Tests for germ-tube formation. Each of the cultures was then tested for its ability to form germ tubes in horse serum (Horse Serum No. 5, Wellcome Reagents Ltd, London), by inoculating a loopful of organisms into 0.5 ml of serum and incubating for 3 h at 37°C. Germ-tube formation was sought by microscopic examination, once at the start of incubation.
to ensure that only blastospores were present, and again after 3 h. To aid the identification of germ tubes an equal amount of 0.5% (w/v) nigrosin in 0.5% (v/v) formalin in water was added as a counterstain. For the purposes of this investigation a germ tube was defined as a filamentous outgrowth from a blastospore at least twice as long as the parent cell (fig. 1). Great care was taken to exclude pseudohyphae, elongated blastospores, or basally constricted hyphae of the kind described by Mackenzie (1964).

Each germ-tube-positive colony was then examined in tests for sugar assimilation and fermentation and was tested for growth on Tween 80 corn-meal agar by the methods described by Dolan (1971). Strains that fermented and assimilated glucose, sucrose, galactose, and trehalose, but not lactose or raffinose, were classified as *C. tropicalis*. The fermentation of glucose and sucrose was accompanied by gas production and in addition such strains were urease-negative on Christensen's agar and failed to produce chlamydospores on Tween 80 corn-meal agar. Strains that assimilated glucose, galactose, sucrose, and trehalose, and fermented glucose and maltose but not sucrose, were classified as *C. albicans*; these were also urease-negative on Christensen's agar but produced chlamydospores on Tween 80 corn-meal agar; they did not produce gas in the fermentation tests.

Further examination of selected strains. From the first subcultures 26 germ-tube-positive strains of *C. tropicalis* were selected, one strain of each species from each subject to avoid duplication. Each strain was subcultured weekly for 9 weeks, starting one week after first isolation. Strains isolated on Sabouraud's agar were subcultured on Sabouraud's agar and those isolated on blood agar were subcultured on blood agar. After subculture, strains were incubated for 24 h at 37°C and then stored at 4°C until required.

Immediately before each subculture was made each strain was tested for its ability to produce germ tubes and the number formed was measured by a modification of the method of Chattaway et al., (1973), in which approximately $4 \times 10^4$ blastospores were harvested from the blood or Sabouraud's agar and washed once in distilled water; the washed cells were then added to 0.5 ml of serum, and immediately thereafter a small sample was taken aseptically and examined microscopically to ensure that only blastospores were present. The blastospores in serum were incubated for 3 h at 37°C. Portions of the incubation mixture were then removed, germ-tube production measured in a counting chamber (Improved Neubauer, Hawksley Instruments, London), and the percentage of blastospores forming germ tubes was then calculated. Great care was taken in these estimates to avoid counting pseudohyphae or elongated buds as germ tubes.

After nine weekly serial subcultures each strain was subjected again to sugar fermentation and assimilation tests and tested for growth on selective media. The performance of each strain in these tests and in those done at the start of the investigation were compared.

Control strains. *C. albicans* strains NCTC3091 and 3118C, and *C. tropicalis* strain NCTC 3111 (all from the National Mycological Reference Laboratory) were subcultured and tested at the same time and in the same way as the test strains.

RESULTS

Germ-tube formation by strains of *C. albicans*

The results of testing the strains of *C. albicans* for germ-tube formation are shown in figs. 2a and 2b. Strains of *C. albicans* subcultured on either blood agar or Sabouraud's agar retained their ability to form germ tubes for 9 weeks. Strains maintained on blood agar produced a higher mean number of germ tubes throughout the 9 weeks, although the range was similar to that of strains maintained on Sabouraud's agar. At least 52% of blastospores of all 26 strains maintained on either blood or Sabouraud's agar produced germ tubes. All 26 strains at the end of the 9 weeks of subculture were still identifiable as *C. albicans*. 
Fig. 1.—Approximately $4 \times 10^4$ blastospores were inoculated into 0.5 ml. of serum. The germinating blastospores were harvested after 2, 3, and 4 h, PAS stained and photographed $\times 1000$. (a) Early germ-tube formation by \textit{C. tropicalis} after 2 h incubation. (b) Germ-tube formation by \textit{C. tropicalis} after 3 h incubation. (c) Two germ tubes originating from one blastospore of \textit{C. tropicalis} after incubation for 4 h.
Fig. 2.—Germ-tube formation during 9 weeks' incubation of: (a) C. albicans on blood agar; (b) C. albicans on Sabouraud's agar. Each point represents the mean percentage of blastospores forming germ tubes among the 26 strains tested; vertical bars indicate the percentage ranges observed.
Germ-tube production by strains of *C. tropicalis*

In contrast, the 26 strains of *C. tropicalis* lost their ability to produce germ tubes (figs. 3a and 3b). Strains subcultured on blood agar at first produced a higher mean number of germ tubes than did strains subcultured on Sabouraud's agar, but during the first 5 weeks all 26 strains gradually lost their ability to produce germ tubes. After 6 weeks none of these strains produced germ tubes.
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During the first 4 weeks many pseudohyphae were formed in the germ-tube tests, but were rarely seen after the strains had lost their ability to produce germ tubes.

**Germ-tube production by control strains**

*C. tropicalis* strain 3111 never produced germ tubes although it formed a few elongated blastospores. In contrast, at least 56% of blastospores of *C. albicans* strains 3091 and 3118C formed germ tubes before and after serial subculturing, whether on blood or Sabouraud’s agar.

**DISCUSSION**

Germ-tube formation by *C. albicans* has received a great deal of attention and variables affecting it have been reviewed by Evans et al., (1974). In contrast, germ-tube formation by *C. tropicalis* has not been closely studied. Tierno and Milstoc (1977) reported the formation of true germ tubes by strains of *C. tropicalis*; this observation was challenged by Sandstrom and Stockman (1978) but has been confirmed by Tierno and Milstoc (1978). Mackenzie (1964) was able to demonstrate budding and the formation of “pseudo-germ tubes” by one strain of *C. tropicalis in vivo*, but drew attention to basal constrictions in these filaments. The observations reported here, that selected oral strains of *C. tropicalis* did indeed form germ tubes (see fig. 1), confirm the observations of Tierno and Milstoc (1977, 1978).

In this study when the number of germ tubes formed by selected oral strains of *C. albicans* and *C. tropicalis* was measured it was found that 26 strains of *C. tropicalis* rapidly lost the ability to form germ tubes during repeated subculture on two widely used media. There are several possible explanations for this finding. A substance or substances present *in vivo* may be necessary for the production of germ tubes by *C. tropicalis*. The germ-tube-forming strains of *C. tropicalis* described by Tierno and Milstoc (1977) and those described in this report were derived from areas rich in mucous secretions. It may be that substances present in such secretions stimulate *C. tropicalis* to produce germ tubes and it is possible that they were removed by the washing that preceded counting or that they were diluted during serial subculture. Another possible explanation is that each isolate of *C. tropicalis* includes distinct variants, one able to form germ tubes and the other not. The latter variant may be selected during subculture, thus explaining the loss of germ-tube formation which was observed.

In addition, although strains of *C. tropicalis* can be preserved by conventional freeze-drying techniques, to date all germ-tube-positive strains that have been freeze-dried have failed to produce germ tubes when rehydrated and cultured (M. V. Martin unpublished results). However, all strains used in the present investigation and designated *C. tropicalis* had reproducible identifying characteristics at the start and end of the investigation, as well as before and after freeze-drying.

To assess fully the relevance of germ-tube formation by strains of *C.
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tropicalis in the differentiation of Candida species it would be necessary to

determine the incidence of germ-tube-positive strains of C. tropicalis in a

much larger sample of isolates. However, it should be noted that because
germ-tube-positive strains of C. tropicalis can be found among isolates of
Candida species from the mouth and lung, germ-tube formation should not be

used as the sole or main criterion for the identification of C. albicans from these

sites. A combination of chlamydospore formation on corn-meal agar, sugar

assimilation, and fermentation tests should be used to identify strains of this

species.

SUMMARY

Candida species isolated from the mouths of healthy children and of patients

with denture stomatitis included strains of Candida tropicalis that formed germ
tubes when incubated in serum.

Twenty-six germ-tube-forming strains of C. albicans and of C. tropicalis

were subcultured weekly for 9 wk on blood agar and on Sabouraud's agar and
the ability of each subculture to form germ tubes was measured. All the strains
of C. albicans formed almost as many germ tubes after nine weekly subcultures
as they did when first isolated. By contrast, although all 26 strains of C. tropicalis
formed germ tubes when first isolated, all had lost the ability to do
so after six serial weekly subcultures.

Germ-tube formation should not be the sole criterion for the identification
of oral C. albicans strains.

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