Chlamydospore Production and Germ-Tube Formation by Auxotrophs of Candida albicans

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Received for publication 16 January 1973

A prototrophic strain and 21 auxotrophic strains of Candida albicans were assessed for their capacity to produce chlamydospores and germ tubes. All of the mutants were able to produce germ-tubes in human serum but only two mutants produced them in defined medium with L-alpha-amino-n-butyric acid as the sole source of nitrogen. Most auxotrophs were not able to produce chlamydospores on corn meal agar with 1% Tween 80, but they could be induced to do so if the medium was supplemented with their growth requirement(s). Although L-cysteine was able to support the growth of two methionine mutants, it did not support chlamydospore formation when added to corn meal agar with 1% Tween 80. Mutants of C. albicans that do not form chlamydospores could be incorrectly identified in laboratories that rely on chlamydospore formation for identification.

In addition to fermentation and assimilation tests (1), two other procedures commonly used in the identification of Candida albicans, in the clinical laboratory, are chlamydospore production and germ-tube formation in human or animal serum (15). Many of the specimens sent to the clinical laboratory for the isolation and identification of C. albicans come from cancer or transplant patients who are undergoing therapy with X rays or radiomimetic or cytotoxic drugs (2, 5, 9, 16). Such therapy could produce auxotrophs of C. albicans since this yeast is a frequent inhabitant of the gastrointestinal tract of man. Mutants of C. albicans, because of their induced nutritional requirements, might be hindered in the production of germ tubes and chlamydospores on media commonly used in the clinical laboratories for this purpose.

In this study auxotrophs of C. albicans obtained by treatment with ultraviolet light (UV), N-nitroso-N-methyl-N-nitrosoguanidine (NG), and N-nitroso-N-methyleurethane (NMU) were assessed for their capacity to produce chlamydospores and germ tubes.

MATERIALS AND METHODS

The prototrophic C. albicans used in these investigations was a clinical isolate from the Medical Division, Oak Ridge Associated Universities, Oak Ridge, Tenn., and is designated here as C. albicans W-3. Strains WD-1 and WD-2, both auxotrophs, were obtained from A. Saracheck, Wichita State University, Wichita, Kan.

The complex media used in these studies were Sabouraud dextrose agar (SDA) or broth (BBL). The defined minimal medium, designated herein as GSB, had the following composition: KH₂PO₄, 3.0 g; K₂HPO₄, 0.5 g; MgSO₄, 0.5 g; MgCl₂, 2.0 g; CaCl₂, 2.0 g; CaCl₂·2H₂O, 0.1 g; Biotin, 30 µg; dextrose, 25 g; NH₄Cl, 1.1 g; and 1,000 ml of distilled water. When L-alpha-amino-n-butyric acid was used in germ-tube studies, it was present at a concentration of 1 g/liter of GSB medium without NH₄Cl.

Stock cultures were maintained on SDA at 25 C and transferred to fresh SDA medium weekly. Cells for chlamydospore and germ-tube experiments were first grown on SDA at 37 C for 16 h, harvested and washed (three times) in saline by centrifugation (5,000 x g), and suspended in saline.

Germ-tube formation. Germ-tube formation was verified by inoculating yeast cells (5 x 10⁹), harvested from 16-h SDA slants, into human serum or into defined GSB medium with L-alpha-amino-n-butyric acid as the nitrogen source (8). The percentage of germ tubes formed was estimated at 0, 1, and 3 h (at 37 C in a water bath) by observations on wet mounts with the high dry objective of a microscope. The number of cells with germ tubes per 100 cells counted was used to indicate the percentage of germ tubes formed; an average of six determinations was used for each time interval.
**Chlamydomspore production.** Chlamydomspore formation was assessed by examining, with the 10x objective of a microscope, corn meal agar plates (with 1% Tween 80) that had been inoculated by deliberately cutting into the agar with a loopful of cells from a 16-h SDA slant. A cover slip was placed over the line of inoculation. The inoculated plates were incubated at 37 C for 24 h and at 25 C for 96 h. Microscope examinations were carried out every 24 h for estimation of chlamydomspores present, and the form of growth was assumed by the dimorphic yeast.

**Induction of mutants.** Procedures for the induction of mutants have been described in detail by Savage and Balish in a previous publication (13). Briefly, UV, UV, NG, and NMU were used as mutagens. Survival was determined by plate counts on samples appropriately diluted and plated prior to exposure (to the mutagen) so that a suitable survival was obtained with a given exposure. Nutritionally deficient organisms were detected by replica plating, to minimal medium, colonies developing from those cells surviving the mutagenic treatment. The nutritional requirements of the mutants were determined by streaking the isolates on the minimal medium containing individual supplements.

**RESULTS**

The mutants and their growth requirements are listed in Table 1. Twenty-one mutants were used in this study.

The prototrophic strain (W-3) and the mutants that required adenine (red pigmented, 1-NMU-5), arginine, histidine, arginine-ornithine, or arginine-ornithine-citrulline-asparagine all formed abundant chlamydomspores within 96 h on corn meal agar with 1% Tween 80 (Table 2). Those strains that required adenine (nonpigmented strain 5-NG-45), adenine-methionine, guanine, leucine, isoleucine, tryptophan, or methionine did not form chlamydomspores under exactly the same growth conditions. These strains would probably not be classified as C. albicans on the basis of a chlamydomspore test alone.

Germ-tube production in serum (15) or in L-alpha-amino-n-butyric acid can also be used to rapidly identify C. albicans: 3 h for the germ-tube test versus 48 to 72 h for chlamydomspore production. Table 3 shows that the prototrophic strain (W-3) and all of the auxotrophic strains of C. albicans formed a substantial number of germ tubes in human serum within 3 h at 37 C. However, as would be expected, many of the auxotrophic strains were not able to form germ tubes in the GSB plus L-alpha-amino-n-butyric acid medium of Mardon et al. (8). Good germ-tube production was observed with only two mutants (5-NG-45 and 5-NG-24) in GSB medium with L-alpha-amino-n-butyric acid (Table 3).

It should be pointed out that all of the auxotrophs were able to show some growth on corn meal agar although not all of them were able to produce chlamydomspores. The auxotrophs produced chlamydomspores only when the corn meal agar (with 1% Tween 80) was supplemented with the compound(s) required for growth. This is demonstrated by the data on the methionine auxotrophs shown in Table 4 and in Fig. 1 which reveal the morphology manifested by a methionine auxotroph of C. albicans after 96 h of growth on corn meal agar (with 1% Tween 80) with and without various amino acid supplementations.

Tables 5 and 6 represent attempts to detail the deficiencies of the methionine auxotrophs further. Table 5 shows the capacity of three sulfur amino acid auxotrophs to grow in GSB.
medium supplemented with known intermediates of methionine biosynthesis in yeasts (3, 14). Strain 7-NMU-1 grew when L-cystathionine, L-methionine, or S-adenosyl-L-methionine was added to the GSB medium. In addition to the latter compounds, strains WD-1 and WD-2 also grew in the presence of L-cysteine or L-cysteine and L-homocysteine. However, none of the strains were able to form chlamydospores on corn meal agar with 1% Tween 80 (Table 6). However, when corn meal agar with 1% Tween 80 was supplemented with L-methionine, S-adenosyl-L-methionine, L-homocysteine, L-cystathionine, or a combination of L-cysteine and L-homoserine, all three mutants formed typical chlamydospores. One of the mutants, 7-NMU-1, was also able to form chlamydospores when L-homoserine or L-cysteine plus L-homoserine was added to the corn meal agar with 1% Tween 80. Mutants WD-1 and WD-2, although they grew in the presence of L-cysteine, were not able to form chlamydospores when cysteine was added to corn meal agar with 1% Tween 80 (Table 6). Clearly, some of the supplements were able to augment chlamydospore production on corn meal agar with 1% Tween 80, but they were not able to support the growth of the mutant in GSB medium (as shown in Table 5), and cysteine was able to support yeastlike growth only. The latter differences can probably be explained by the more complex nutritional makeup of corn meal in comparison to GSB medium. It is also known that cysteine enhances yeastlike growth of C. albicans (10).

### DISCUSSION

The formation of large thick-walled structures known as chlamydospores takes place when C. albicans is inoculated on nutritionally deficient growth media such as corn meal agar (7), Zein agar (4), potato carrot agar (12), or the purified polysaccharide medium commonly known as chlamydospore agar (11). Kelly and Funigielo (7) reported that the addition of Tween 80 to corn meal and Zein agar stimulated chlamydospore production by C. albicans. A description of various media, inoculation procedures, incubation temperatures, and other mechanisms to enhance chlamydospore production by C. albicans has been the topic of many reports.

Many of the patients that C. albicans invades are undergoing therapy with radiation or radi-

### TABLE 3. Germ-tube formation by prototrophic and auxotrophic strains of C. albicans in human serum and in defined medium with L-alpha-amino-n-butyric acid as sole nitrogen source

| Organism          | Germ tubes at 3 h (%) | GSB medium + l-alpha-amino-n-butyric acid | Human serum |
|--------------------|-----------------------|------------------------------------------|-------------|
| Prototroph; W-3    |                       |                                          |             |
| Auxotrophs         |                       |                                          |             |
| Adenine; 5-NG-45   | 90-95                 |                                         | 95-100       |
| Guanine; 5-NG-17   | 50-70                 |                                         | 80-90       |
| Histidine; 5-NG-12, 4-NG-10, 5-NG-6, 5-NG-7, 7-NG-23, 5-NMU-21 | 10-20                 |                                         | 70-80       |
| Tryptophan; 5-NG-20, 7-NG-22 | 5-10                 |                                         | 80-90       |
| Leucine; 7-NG-26   | 5-10                  |                                         | 80-90       |
| Isoleucine; 2-NMU-7 | 5-10                  |                                         | 80-90       |
| Arginine; 2-NMU-55, 5-NMU-24 | 5-10                  |                                         | 80-90       |
| Arginine-ornithine; 5-NG-24, 2-NMU-24 | 40-50                 |                                         | 80-90       |
| Arginine-ornithine-citrulline-asparagine; 5-NG-35 | 5-10                  |                                         |             |
| Methionine; 7-NMU-1, WD-2 |            |                                         |             |
| Methionine-adenine; WD-1 |            |                                         |             |
| Adenine; 1-NMU-5   | 5-10                  |                                         |             |

### TABLE 4. Chlamydospore production by sulfur amino acid auxotrophs on corn meal agar (with 1% Tween 80) with and without L-methionine supplementation

| Auxotroph          | Morphology at 72 h |
|---------------------|--------------------|
|                     | Corn meal agar with 1% Tween 80 | Corn meal agar with 1% Tween 80 + L-methionine (30 mg/100 ml) |
| WD-1 (methionine-adenine) | Many yeast cells, rare hyphae, and blastospores | Many hyphae, blastospores, and chlamydospores |
| WD-2 (methionine)    | Hyphae and blastospores | Many hyphae, blastospores, and chlamydospores |
| 7-NMU-1 (methionine) | Mostly yeast cells, rare hyphae | Many hyphae, blastospores, and chlamydospores |
FIG. 1. Morphology of an auxotrophic strain (methionine) of C. albicans after growth on corn meal agar (with 1% Tween 80) with and without amino acid supplementation. A, Corn meal agar with 1% Tween 80: hyphae and blastospores; ×400. B, Corn meal agar with 1% Tween 80 and 30 mg of L-methionine/100 ml of medium: hyphae, fewer blastospores and many chlamydomspores; ×400. C, Corn meal agar with 1% Tween 80 and 30 mg of L-leucine/100 ml: hyphae, many blastospores, very rare chlamydospores; ×400. D, Corn meal agar with 1% Tween 80 plus 15 mg of L-methionine and 15 mg L-leucine/ml: hyphae, blastospores, many chlamydospores; ×400.

omimetic drugs (2, 5, 9, 16). Such toxic compounds or X-ray therapy could produce mutants of C. albicans in these patients. The behavior of C. albicans auxotrophs on laboratory media that are routinely used to identify C. albicans has, to my knowledge, only been mentioned briefly in one report (13). Auxotrophs of C. albicans could produce very few, or atypical chlamydospores or germ tubes, or both, and this could be the source of incorrect laboratory identifications of the pathogenic yeasts. Especially since the major test, i.e., chlamydomspore production, is routinely carried out on nutritionally deficient medium such as corn meal agar or Zein agar.

This study showed that the production of germ tubes in human serum was a consistently reproducible laboratory phenomenon with a
TABLE 5. Growth of sulfur amino acid auxotrophs in supplemented minimal medium*

| Supplement          | 7-NMU-1 | W-D-1 | W-D-2 |
|---------------------|---------|-------|-------|
| None                | -       | -     | -     |
| L-cysteine          | -       | +     | +     |
| L-homoserine        | -       | -     | -     |
| L-cysteine + L-homo-serine* | -     | +     | +     |
| L-serine            | -       | -     | -     |
| L-homocysteine      | -       | -     | -     |
| L-serine + L-homo-cysteine* | -     | -     | -     |
| L-cystathionine     | +       | +     | +     |
| L-methionine        | +       | +     | +     |
| S-adenosyl-L-methionine | +     | +     | +     |
| S-adenosylhomocysteine  | -     | -     | -     |

* Each supplement was at a concentration of 20 μg/ml in GSB medium.

A 5-μg amount of each amino acid per ml.

TABLE 6. Chlamydosporereation by sulfur amino acid auxotrophs*

| Supplement          | Chlamydosporereation |
|---------------------|----------------------|
|                     | 7-NMU-1 | W-D-1 | W-D-2 |
| None                | -       | -     | -     |
| L-Cysteine          | -       | -     | -     |
| L-Homoserine        | +       | -     | -     |
| L-Cysteine + L-homo-serine* | +     | +     | +     |
| L-Serine            | -       | -     | -     |
| L-Homocysteine      | +       | +     | +     |
| L-Serine + L-homo-cysteine* | +     | +     | +     |
| L-Cystationine      | +       | +     | +     |
| L-Methionine        | +       | +     | +     |
| S-Adenosyl-L-methionine | +     | +     | +     |
| S-Adenosylhomocysteine* | -     | -     | -     |

* 15 mg/ml of supplement was added to corn meal plus 1% Tween 80 agar.

A 100-μg amount of each per ml.

C A 4-μg amount per ml.

prototrophic and 21 auxotrophic strains of C. albicans. This was not unexpected since serum does have a vast variety of amino acids and other compounds that could satisfy the growth requirements of the mutants. However, the production of germ tubes in L-alpha-amino-n-butyric acid, as described by Mardon et al. (8) was not, as could be expected from the lack of compounds required by the auxotroph in GSB medium, as good as in serum.

These studies further reveal that many auxotrophic strains, even though they are capable of some growth on corn meal agar with 1% Tween 80, do require the presence of their growth factor for abundant, typical, chlamydosporereation. Thus, if chlamydosporereation is relied on for the identification of C. albicans in the clinical laboratory, it should be kept in mind that some auxotrophs will not form chlamydosporeres on corn meal agar with 1% Tween 80.

That such auxotrophs do occur in patients undergoing therapy with radiation or radiomimetic drugs is a good possibility. However, they are probably missed since C. albicans is usually isolated on complex media (usually SDA) and our auxotrophs grew very well on the latter nutritionally complex medium. A hint of the presence of such auxotrophs would come from those strains of C. albicans with typical antigenic makeup and typical sugar fermentation patterns, and, yet, they do not form chlamydosporeres on the nutritionally deficient media that are used for their production.

In a previous study by Savage and Balish (13), methionine and adenine auxotrophs were shown to retain their virulence for white mice. It is possible that some auxotrophs could be more virulent than the prototrophic strains that usually colonize man. Auxotrophic strains could be an important cause of serious and persistent infections. We also have no information on the susceptibility or resistance of these auxotrophs to polyene antibiotics.

In a recent paper, Jansons and Nickerson (6) concluded that chlamydosporereation formation was accomplished through the endogenous metabolism of C. albicans. In our studies, auxotrophs, harvested from SDA, were able to manifest some growth on corn meal agar. However, many auxotrophs were not able to form chlamydosporeres unless the growth-promoting compound was added. This would indicate that compounds other than endogenous reserves, unless the accumulation of endogenous reserves was defective in our mutants, are needed for chlamydosporereation production. This was especially true of the methionine auxotrophs studied and is further evidence that sulfur amino acid metabolism plays an important role in the morphogenesis and control of cellular form in C. albicans.

ACKNOWLEDGMENTS

I thank Brenda Joan Marks for her able assistance in this study. This investigation was supported by a grant from the National Science Foundation (GB-30110X).1. Ahearn, D. G. 1970. Systematics of yeasts of medical interest, p. 64-70. In Proceedings International Symposium on Mycoses. Pan American Health Organization, Washington, D.C.

LITERATURE CITED
2. Bodey, G. P. 1966. Fungal infections complicating acute leukemia. J. Chron. Dis. 19:667-672.
3. Botsford, J. L., Jr., and L. W. Parks. 1969. Serine transhydroxymethylase in methionine biosynthesis in Saccharomyces cerevisiae. J. Bacteriol. 97:1176-1183.
4. Duncan, J., and J. Gloeher. 1966. Fungal infections complicating acute leukemia. J. Chron. Dis. 19:667-672.
5. Hart, P. D., E. Russell, Jr., and J. S. Remington. 1969. A comparison of media for the production of chlamydospores by Candida albicans. Amer. J. Med. Technol. 29:199-206.
6. Jansons, V. K., and W. J. Nickerson. 1970. Induction, morphogenesis, and germination of the chlamydospore of Candida albicans. J. Bacteriol. 104:910-921.
7. Kelly, J. P., and F. Funigliello. 1959. Candida albicans: a study of media designed to promote chlamydospore production. J. Lab. Clin. Med. 53:907-909.
8. Mardon, D. N., S. K. Hurst, and E. Balish. 1971. Germ-tube production by Candida albicans in minimal liquid culture media. Can. J. Microbiol. 17:851-856.
9. Mirsky, H. S., and J. Cuttner. 1972. Fungal infections in acute leukemia. Cancer 30:348-352.
10. Nickerson, W. J., and C. W. Chung. 1954. Genetic block in the cellular division mechanism of a morphological mutant of a yeast. Amer. J. Botany 41:114-120.
11. Nickerson, W. J., and Z. Mankowski. 1953. A polysaccharide medium of known composition favoring chlamydospore formation in Candida albicans. J. Infect. Dis. 92:20-25.
12. Paulatou, M., and U. Marselou. 1961. Rapid identification of Candida albicans with PCB medium. J. Trop. Med. Hyg. 64:268-272.
13. Savage, N., and E. Balish. 1971. Morphology, physiology and virulence of some mutants of Candida albicans. Infect. Immunity 3:141-148.
14. Shapiro, S. K., D. A. Yphantis, and A. Almenas. 1964. Biosynthesis of methionine in Saccharomyces cerevisiae: partial purification and properties of S-adenosylmethionine; homocysteine methyltransferase. J. Biol. Chem. 239:1551-1556.
15. Taschdjian, C. L., J. J. Burchall, and P. J. Kozinin. 1960. Rapid identification of Candida albicans by filamentation of serum and serum substitutes. J. Dis. Child. 99:212-215.
16. Viola, M. V. 1967. Acute leukemia and infection. J. Amer. Med. Ass. 201:103-107.