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Production of Extracellular Ribonuclease by Yeasts and Yeastlike Fungi, and Its Repression by Orthophosphate in Species of *Cryptococcus* and *Tremella*

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A strain of *Cryptococcus laurentii* and a haploid isolate of *Tremella foliacea* were shown to produce orthophosphate-repressible ribonuclease in liquid culture. Addition of as little as 1 mM K_2HPO_4, pH 7.0, completely repressed enzyme production by both fungi. The orthophosphate-repressible enzyme was not produced by other species of the two genera tested. These results, together with other findings, suggest a close phylogenetic relationship between *Cryptococcus laurentii* and *Tremella foliacea*. The ability of other yeasts and yeastlike fungi to hydrolyze ribonucleic acid in a solid test medium was assessed. Based on the limited number or organisms available for study, extracellular ribonuclease activity was found in species having close affinity to the *Basidiomycetes* and in yeasts classified in the ascomycetous genera, *Endomycopsis, Hansenula,* and *Kluyveromyces*. Other ascomycetous yeasts did not exhibit extracellular ribonuclease.

Slodki et al. (28) suggested that species of the genus *Cryptococcus* might represent haploid states of the life cycle of certain species of the basidiomycetous genus *Tremella*. Their conclusions were based on similarities of extracellular heteropolysaccharides produced by species of the two genera and on other morphological and physiological criteria. In a previous report (4), production of extracellular deoxyribonuclease (DNase) was found to be a characteristic common to yeasts that were considered to have close affinity to *Basidiomycetes*. DNase production was demonstrated in all species tested of the imperfect genera *Cryptococcus* and *Rhodotorula,* and the basidiomycetous genus *Tremella*. With the single exception of *Endomycopsis fibuligera,* all ascosporogenous yeasts failed to hydrolyze the deoxyribonucleic acid (DNA) in the solid test medium.

While examining the nucleases obtained from representative species of *Cryptococcus* and *Tremella*, it was noted that isolates of *Cryptococcus laurentii* and *Tremella foliacea* secreted abundant amounts of ribonuclease (RNase) when grown in an orthophosphate-deficient yeast extract broth culture medium. The presence of orthophosphate in culture medium was found to repress RNase production significantly.

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Experiments reported here were carried out to determine whether the ability to produce extracellular RNase might be a unique characteristic of basidiomycetous yeasts and yeastlike organisms, and to study further the repression of RNase production by orthophosphate in species of *Cryptococcus* and *Tremella*.

MATERIALS AND METHODS

Yeast strains. Haploid isolates of species of *Tremella* were supplied by L. J. Wickerham, then at Northern Regional Research Laboratories, Peoria, Ill. All *Tremella* species grew as typical yeastlike cells without production of pseudomycelium. *C. laurentii* strain 614 was identified as *C. laurentii* var. *flavescens* by its ability to assimilate melibiose but not erythritol (25). Cultures of all other yeasts and yeastlike organisms used in this study were obtained from the fungus culture collection maintained by the Department of Microbiology, University of Iowa. Yeast cultures were stored at 4 C on Sabouraud dextrose agar slants and subcultured several times on the same medium before use.

Media for RNase production. For production of extracellular RNase in liquid culture, yeasts were grown in an orthophosphate-free medium similar to that formulated by Weimberg and Orton (34). Inorganic phosphate present in yeast extract was precipitated from solution as $MgNH_4PO_4 \cdot 6H_2O$ by the addition of magnesia reagent (55 g of $MgCl_2 \cdot 6H_2O$ and 100 g of NH_4Cl dissolved in 500 ml of distilled water) (31). A 24-ml volume of magnesia reagent was added to each 100-ml volume of 20% yeast extract previously adjusted to pH 8.0 with 1 N KOH. The precipi-

tate, after settling for 4 h at room temperature, was removed by filtration, and the supernatant fluid was adjusted to pH 7.0 with 1 N HCl and diluted to 10% with distilled water. Final composition of the liquid growth medium was 2.5% dextrose, 0.1% urea, and 0.5% orthophosphate-free yeast extract. All ingredients were autoclaved separately and combined after sterilization. In some experiments, concentrations of sterile K_2HPO_4, pH 7.0, were added to the above medium.

To determine hydrolysis of ribonucleic acid (RNA) in a solid test medium, RNA and Noble agar (Difco) were added in concentrations of 0.4% and 1.5%, respectively, to the liquid medium. The medium was adjusted to pH 7.0 with 1 N NaOH and sterilized in an autoclave, and 15-ml portions of the test medium were distributed into sterile glass petri plates and allowed to solidify.

RNase assays. Quantitative enzymatic hydrolysis of RNA was assayed similar to the method devised by Kunitz (13) to measure DNase activity. The procedure measures increase in absorbance of ultraviolet light produced by acid-soluble reaction products enzymatically from the RNA substrate. Each reaction mixture, in a total volume of 1.1 ml, contained 0.05 M sodium acetate, pH 5.0, 0.01 M ethylenediaminetetraacetic acid, 1 mg of yeast RNA (Calbiochem), and enzyme solution. The reaction was allowed to proceed for 1 h in a 37 C water bath. After hydrolysis, 0.5 ml of cold uranyl reagent (0.75% uranyl acetate in 25% perchloric acid) was added to the mixture to precipitate unhydrolyzed RNA. Control mixtures received an appropriate amount of enzyme after the acid reagent precipitation procedure. Precipitated reaction and control mixtures were centrifuged at 1,500 × *g* for 15 min in the cold, and the supernatant fluids were decanted and diluted fivefold with distilled water. Absorbance readings were made at 260 nm with either a Gilford model 240, or a Zeiss model PMQ II spectrophotometer using silica cells with a 1-cm light path. Increase in absorbance at 260 nm was determined by subtracting absorbance of the control from that obtained for the test mixture. A unit of enzyme activity is defined as that amount of enzyme producing an increase in absorbance of 1.0 under the above conditions.

Hydrolysis of RNA by growing cultures was determined on the solid test medium (10). Fresh cultures were inoculated onto the test medium, making both a streak and a stab inoculation, and then incubated at 25 C for 7 days. A single plate was adequate for testing two organisms. At the end of the incubation period, the agar surface of each plate was flooded with 1 N HCl to precipitate unhydrolyzed RNA. Hydrolysis of RNA was detected by a zone of clearing around the areas of yeastlike growth.

RESULTS

Effect of K_2HPO_4 on the production of RNase by C. laurentii and T. foliacea. Preliminary data suggested that both *C. laurentii* and *T. foliacea* were capable of producing a phosphate-repressible RNase. Each organism was inoculated into 100 ml of the orthophosphate-free yeast extract medium contained in a 500-ml flask and incubated at 25 C with constant shaking. Individual flasks containing the basal medium were supplemented with 0.0, 0.1, 1.0, and 10.0 mM K_2HPO_4, pH 7.0. At specified time intervals, culture samples were removed, centrifuged, and assayed for RNase activity as outlined previously. An inverse relationship between the concentration of K_2HPO_4 and RNase production by both organisms is shown in Fig. 1 and 2. As little as 0.1 mM K_2HPO_4 was sufficient to significantly reduce RNase production by both fungi when compared to production of enzyme in media containing no orthophosphate. Complete repression of extracellular RNase was evident at 1.0 and 10 mM K_2HPO_4.

Production of RNase by species of Cryptococcus and Tremella. The ability of various species of *Cryptococcus* and *Tremella* to produce extracellular RNase is shown in Table 1. Organisms were inoculated into the orthophosphate-free medium (50 ml) contained in 200-ml flasks and incubated at 25 C with constant shaking. Similarly, a second set of flasks containing medium, supplemented with 1.0 mM K_2HPO_4, pH 7.0, were inoculated with the same organisms. After 4 days of growth, RNase activity in the culture supernatant fluids was measured. To insure safety, fluids from cultures of *Cryptococcus neoformans* were filter-sterilized before use in the enzyme assay. Only *C. laurentii* and *T. foliacea* were capable of producing phosphate-repressible RNase in relatively high amounts. Enzyme production by *Cryptococcus albidus*, *Cryptococcus diffluens*, and *Cryptococcus luteolus* was considerably less under the conditions of the experiment, and did not appear to be affected greatly by K_2HPO_4. There was very little or no significant evidence of RNase activity in supernatant fluids from cultures of *C. neoformans* or in culture fluids from the species of *Tremella* other than that of *T. foliacea*.

RNase production by yeasts on solid test medium. All 19 species of the genus *Candida* tested produced wide zones of RNA hydrolysis after 7 days of growth on the solid test medium (Table 2). Similarly, all species of the imperfect yeast and yeastlike genera tested, *Crytococcus*, *Geotrichum*, *Rhodotorula*, *Sporobolomyces*, and *Trichosporon*, were RNase positive. RNA was hydrolyzed by *Torulopsis colliculosa* but not by *Torulopsis glabrata*. All *Tremella* species appeared to grow rather poorly on the solid test medium, and only two of the four species (*Tremella encephala* and *T. foliacea*) produced extracellular RNase, as evidenced by relatively small zones of hydrolysis. Hydrolysis

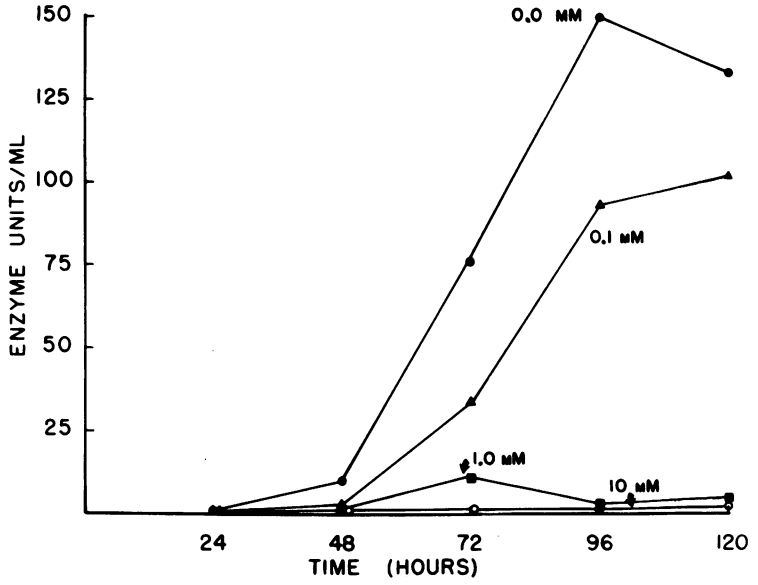

FIG. 1. *Effect of K_2HPO_4 concentration on RNase production by T. foliacea.*

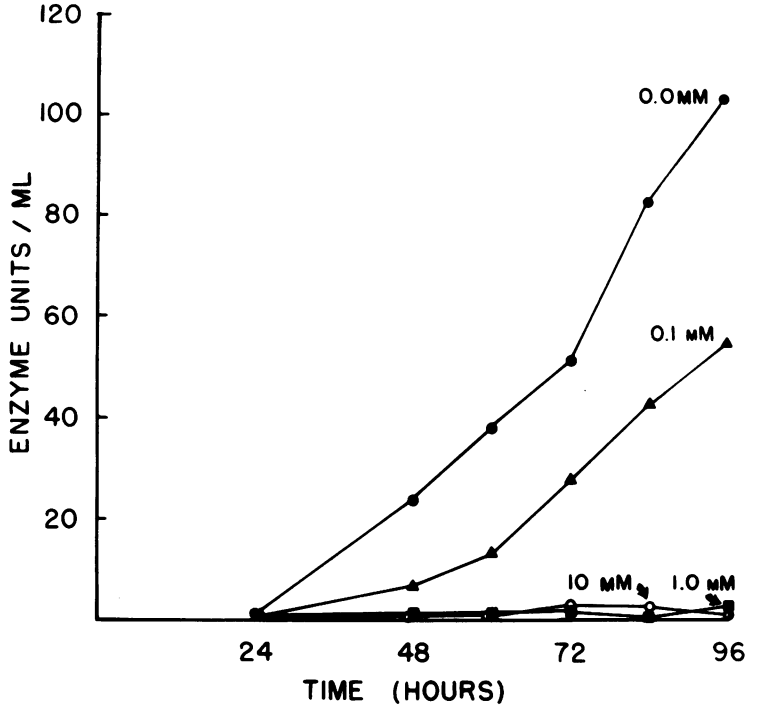

FIG. 2. *Effect of K_2HPO_4 concentration on RNase production by C. laurentii.*

zones produced by *Cryptococcus* species also were of relatively limited size even though growth of the cryptococci did not appear to be inhibited on the solid test medium.

A correlation between extracellular RNase production in the liquid orthophosphate-free medium and the solid test medium was not apparent, since some species of *Crytococcus* and *Tremella* produced the enzyme on the solid test medium but not in the liquid culture medium. Differences between the cultural conditions or the sensitivities of the two assays may have

TABLE 1. *Extracellular RNase production by Tremella and Cryptococcus species grown in liquid media with and without orthophosphate*

Organism	Strain	Enzyme units/ml	
		K_2HPO_4[a]	No K_2HPO_4
Cryptococcus albidus	615	13	0
C. diffluens	627	0	6
C. diffluens	632	19	24
C. laurentii	614	0	292
C. luteolus	626	28	72
C. neoformans	602	0	8
C. neoformans	606	0	0
C. neoformans	609	0	0
Tremella aurantia	NRRL Y-6321	0	0
T. encephala	NRRL Y-6318	0	2
T. foliacea	NRRL Y-6315	0	216
T. mesenterica	NRRL Y-6151	0	2

[a] K_2HPO_4 (1.0 mM), pH 7.0.

accounted for this apparent lack of correlation.

Of seven ascomycetous genera tested, only three species (*Endomycopsis fibuligera, Hansenula saturnus,* and *Kluyveromyces fragilis*) appeared to hydrolyze RNA, producing wide zones of hydrolysis on the solid test medium.

DISCUSSION

The ability of both *C. laurentii* and *T. foliacea* to produce an orthophosphate-repressible RNase has been shown. At present, it is not clear whether phosphate in the growth medium caused repression of enzyme synthesis by the cell or whether the enzyme was prevented from being released from the cell into the culture fluid. Other investigators have shown that addition of phosphate to a growth medium will repress production of nuclease by *Neurospora crassa* (8), and will similarly repress production of phosphomonoesterase by both this organism (9) and *Saccharomyces mellis* (34). Production of RNase might also be influenced by the presence of inducer molecules, such as oligoribonucleotides in the culture medium. Increased yield of an extracellular RNase U_1, from the heterobasidiomycetous fungus *Ustilago sphaerogena,* was found when cells were grown in a medium in which RNA served as the carbon source (7). Growth of the same organism in a medium containing RNA as the only source of phosphate induced synthesis of a different RNase, designated U_4 (3). In the present study, *Cryptococcus* and *Tremella* species that were negative for RNase production in the liquid culture medium, but which were positive when grown on the solid test medium, might have been induced to produce the enzyme by the RNA contained in the agar medium.

The production of an orthophosphate-repressible RNase by *C. laurentii* and *T. foliacea* may have taxonomic and phylogenetic implications. This finding might lend additional weight to the suggestion that species of *Cryptococcus* are,

TABLE 2. *Production of extracellular RNase by yeasts and yeastlike organisms grown on a solid test medium*

Organism	No. of isolates tested	RNase production
Deuteromycetes		
Candida albicans	2	+
C. brumptii	1	+
C. catenulata	1	+
C. curvata	1	+
C. guilliermondii	1	+
C. japonica	1	+
C. krusei	2	+
C. lipolytica	1	+
C. membraneafaciens	1	+
C. mycoderma	1	+
C. natalensis	1	+
C. obtusa	1	+
C. parapsilosis	2	+
C. pseudotropicalis	1	+
C. rugosa	1	+
C. stellatoidea	1	+
C. tropicalis	1	+
C. utilis	1	+
C. zeylanoides	1	+
Cryptococcus albidus	1	+
C. diffluens	2	+
C. laurentii	2	+
C. luteolus	1	+
C. neoformans	21	+
Geotrichum species	2	+
Rhodotorula aurantiaca	1	+
R. glutinis	1	+
R. lactosa	2	+
R. pallida	1	+
R. rubra	4	+
Sporobolomyces species	1	+
Torulopsis colliculosa	1	+
T. glabrata	1	−
Trichosporon cutaneum	1	+
Basidiomycetes		
Tremella aurantia	1	−
T. encephala	1	+
T. foliacea	1	+
T. mesenterica	1	−
Ascomycetes		
Dipodascus uninucleatus	1	−
Endomycopsis fibuligera	1	+
Hansenula saturnus	1	+
Kluyveromyces fragilis	1	+
Lipomyces starkeyi	1	−
Pachysolon tannophilus	1	−
Saccharomyces cerevisiae	1	−
S. delbrueckii	1	−
S. globosus	1	−

in fact, haploid forms of various *Tremella* species. Based on identification of identical components making up the extracellular heteropolysaccharides of *C. laurentii* and of several species of *Tremella*, Slodki et al. (28) arrived at this conclusion. Other characteristics common to both genera are their similarities in morphology, production of starch-like compounds at low pH (28), urease and DNase production (4), and content of guanine plus cytosine (G+C) in their DNA (15, 16, 30).

To date, a complete sexual life cycle for species of *Cryptococcus* has not been described. An isolate identified as *C. neoformans* has been found to produce hyphae with clamp connections (27). Recently, Kurtzman (14) was able to show conjugation between different strains of *C. laurentii* var. *laurentii*, isolated from corn and wheat. Conjugation gave rise to hyphae not unlike those produced in mating experiments with species of *Tremella* (1). The complete sexual cycle of *C. laurentii* was not observed however. Attempts to cross several of the corn and wheat isolates with other strains of the same species from other sources and with strains of *Tremella aurantia* and *T. encephala* failed to induce conjugation tube formation.

A previous study (4) suggested that production of extracellular DNase on a solid test medium was characteristic of basidiomycetous yeasts. All ascomycetous yeasts tested, with the single exception of *E. fibuligera* were unable to hydrolyze DNA under the test conditions. Although a limited number of yeasts were available for the present study, it seems likely that extracellular RNase production might be a property not only of yeasts having close basidiomycetous affinity, but also of yeasts classified in several ascomycetous genera.

Information from various sources indicates a basidiomycetous relationship for many anascosporogenous yeasts.

Sexual life cycles, similar to those exhibited by fungi classified in the order *Ustilaginales*, have been demonstrated for several yeasts and yeastlike fungi. These organisms, representing species of the imperfect genera *Rhodotorula, Candida, Sporobolomyces*, and *Bullera*, most of which were found to be RNase positive in the present study, have been placed in the perfect basidiomycetous yeast genera *Rhodosporidium* (2, 5, 20, 21), *Leucosporidium* (6), *Filobasidium* (23, 26), *Sporidiobolus* (22, 24), and *Aessosporon* (32, 33).

Base composition analysis of fungal DNA has shown to be of considerable taxonomic significance (29). A G+C content in excess of 50% was found to be characteristic of fungi classified in the *Basidiomycetes* whereas, ascomycetous fungi possess DNA with a G+C content usually below 50%. Laminar cell wall structure (12) and urease production (15) also have been shown to be properties characteristic of basidiomycetous yeasts. Based on G+C content and urease production, the genera *Candida* (18), *Torulopsis* (17), and *Trichosporon* (19) most likely include both ascomycetous and basidiomycetous forms. Some *Candida* species are known to be imperfect forms of *Leucosporidium* species, while others are perfect forms of species in the ascomycetous genera *Hansenula, Kluyveromyces, Pichia* (11), and *Endomycopsis* (35). It is of interest that all *Candida* species tested were RNase positive as were the ascomycetous yeasts *Hansenula saturnus, Kluyveromyces fragilis*, and *E. fibuligera*.

LITERATURE CITED

1. **Bandoni, R. J.** 1963. Conjugation in *Tremella mesenterica*. Can. J. Bot. 41:467–474.
2. **Banno, I.** 1967. Studies on the sexuality of *Rhodotorula*. J. Gen. Appl. Microbiol. 13:167–196.
3. **Blank, A., and C. A. Dekker.** 1972. Ribonuclease U_4 from *Ustilago sphaerogena*. Purification and physical properties. Biochemistry 11:3956–3962.
4. **Cazin, J., Jr., T. R. Kozel, D. M. Lupan, and W. R. Burt.** 1969. Extracellular deoxyribonuclease production by yeasts. J. Bacteriol. 100:760–762.
5. **Fell, J. W.** 1970. Yeasts with heterobasidiomycetous life cycles, p. 49–66. *In* D. G. Ahearn (ed.), Recent trends in yeast research. Georgia State University Press, Atlanta.
6. **Fell, J. W., A. C. Statzell, I. L. Hunter, and H. J. Phaff.** 1969. *Leucosporidium* gen. n., the heterobasidiomycetous state of several yeasts of the genus *Candida*. Antonie van Leeuwenhoek J. Microbiol. Serol. 35:433–462.
7. **Glitz, D. G., and C. A. Dekker.** 1964. Studies on a ribonuclease from *Ustilago sphaerogena*. I. Purification and properties of the enzyme. Biochemistry 3:1391–1399.
8. **Hunsunuma, K.** 1973. Repressible extracellular nucleases in *Neurospora crassa*. Biochim. Biophys. Acta 319:288–293.
9. **Jacobs, M. M., J. F. Nyc, and D. M. Brown.** 1971. Isolation and chemical properties of a repressible acid phosphatase in *Neurospora crassa*. J. Biol. Chem. 246:1419–1425.
10. **Jeffries, C. D., D. F. Holtman, and D. G. Guse.** 1957. Rapid method for determining the activity of microorganisms on nucleic acids. J. Bacteriol. 73:590–591.
11. **Kreger-van Rij, N. J. W.** 1969. Taxonomy and systematics of yeasts, p. 5–78. *In* A. H. Rose, and J. S. Harrison (ed.), The yeasts, vol. 1. Academic Press Inc., New York.
12. **Kreger-van Rij, N. J. W., and M. Veenhuis.** 1971. A comparative study of the cell wall structure of basidiomycetous and related yeasts. J. Gen. Microbiol. 68:87–95.
13. **Kunitz, M.** 1950. Crystalline desoxyribonuclease. I. Isolation and general properties. J. Gen. Physiol. 33:349–362.
14. **Kurtzman, C. P.** 1973. Formation of hyphae and chlamydospores by *Cryptococcus laurentii*. Mycologia 65:388–395.
15. **Nakase, T., and K. Komagata.** 1968. Taxonomic signifi-

cance of base composition of yeast DNA. J. Gen. Appl. Microbiol. 14:345–357.
16. **Nakase, T., and K. Komagata.** 1971. Significance of DNA base composition in the classification of yeast genera *Cryptococcus* and *Rhodotorula*. J. Gen. Appl. Microbiol. 17:121–130.
17. **Nakase, T., and K. Komagata.** 1971. Significance of DNA base composition in the classification of yeast genus *Torulopsis*. J. Gen. Appl. Microbiol. 17:161–166.
18. **Nakase, T., and K. Komagata.** 1971. Significance of DNA base composition in the classification of yeast genus *Candida*. J. Gen. Appl. Microbiol. 17:259–279.
19. **Nakase, T., and K. Komagata** 1971. DNA base composition of some species of yeasts and yeastlike fungi. J. Gen. Appl. Microbiol. 17:363–369.
20. **Newell, S. Y., and J. W. Fell.** 1970. The perfect form of a marine-occurring yeast of the genus *Rhodotorula*. Mycologia 62:272–281.
21. **Newell, S. Y., and I. L. Hunter.** 1970. *Rhodosporidium diobovatum* sp. n., the perfect form of an asporogenous yeast (*Rhodotorula* sp.) J. Bacteriol. 104:503–508.
22. **Nyland, G.** 1949. Studies on some unusual Heterobasidiomycetes from Washington State. Mycologia 41:686–701.
23. **Olive, L. S.** 1968. An unusual new heterobasidiomycete with *Tilletia*-like basidia. J. Elisha Mitchell Sci. Soc. 84:261–266.
24. **Phaff, H. J.** 1970. *Sporidiobolus*, Nyland, p. 822–830. *In* J. Lodder (ed.), The yeasts, a taxonomic study. North-Holland Publishing Co., Amsterdam.
25. **Phaff, H. J., and J. W. Fell.** 1970. *Cryptococcus* Kutzing emend. Phaff et Spencer, p. 1088–1145. *In* J. Lodder (ed.), The yeasts, a taxonomic study. North-Holland Publishing Co., Amsterdam.
26. **Rodrigues de Miranda, L.** 1972. *Filobasidium capsuligenum* nov. comb. Antonie van Leeuwenhoek J. Microbiol. Serol. 38:91–99.
27. **Shadomy, H. J.** 1970. Clamp connections in two strains of *Cryptococcus neoformans*, p. 67–79. *In* D. G. Ahearn (ed.), Recent trends in yeast research. Georgia State University Press, Atlanta.
28. **Slodki, M. E., L. J. Wickerham, and R. J. Bandoni.** 1966. Extracellular heteropolysaccharides from *Cryptococcus* and *Tremella*: a possible taxonomic relationship. Can. J. Microbiol. 12:489–494.
29. **Storck, R., and C. J. Alexopoulos.** 1970. Deoxyribonucleic acid of fungi. Bacteriol. Rev. 34:126–154.
30. **Storck, R. C., C. J. Alexopoulos, and H. J. Phaff.** 1969. Nucleotide composition of deoxyribonucleic acid of some species of *Cryptococcus*, *Rhodotorula*, and *Sporobolomyces*. J. Bacteriol. 98:1069–1072.
31. **Umbreit, W. W., R. H. Burris, and J. F. Stauffer (ed.).** 1957. Manometric techniques, 2nd ed., Burgess Publishing Co., Minneapolis.
32. **van der Walt, J. P.** 1970. The perfect and imperfect states of *Sporobolomyces salmonicolor*. Antonie van Leeuwenhoek J. Microbiol. Serol. 36:49–55.
33. **van der Walt, J. P.** 1973. *Aessosporon dendrophilum* sp. nov., the perfect state of *Bullera dendrophila*. Antonie van Leeuwenhoek J. Microbiol. Serol. 39:455–460.
34. **Weimberg, R., and W. L. Orton.** 1963. Repressible acid phosphomonoesterase and constitutive pyrophosphatase of *Saccharomyces mellis*. J. Bacteriol. 86:805–813.
35. **Wickerham, L. J., C. P. Kurtzman, and A. I. Herman.** 1970. Sexuality in *Candida lipolytica*, p. 81–94. *In* D. G. Ahearn (ed.), Recent trends in yeast research. Georgia State University Press, Atlanta.