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NAGW-26: Microbial Ecology of Extreme Environments: Antarctic Dry Valley Yeasts and Growth in Substrate-limited Habitats.

H.S. Vishniac, Principal Investigator

Department of Botany and Microbiology

Oklahoma State University

Stillwater, OK 74078

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INTRODUCTION

Antarctic Dry Valley yeasts are still the only biota known to be indigenous to the most highly stressed sites supporting life in earth's most extreme cold desert. Critical examination of the parameters of isolation methods may reveal other indigenous biota. The isolation of indigenes from this area is in any case an exercise in life detection in habitats for which conventional methods are inadequate. The success of the Dry Valley yeasts presumeably results from adaptations to multiple stresses, to low temperatures and substrate-limitation as well as prolonged resting periods enforced by low water availability. Previous investigations have suggested that the crucial stress is substrate limitation. Specific adaptations may be pinpointed by comparing the physiology of the Cryptococcus vishniacii complex, the yeasts of the Tyrol Valley, with their congeners from other habitats. Progress has been made in methods of isolation and definition of ecological niches, in the design of experiments in competition for limited substrate, and in establishing the relationships of the Cryptococcus vishniacii complex with other yeasts. In the course of investigating relationships, a new method for 25SrRNA homology was developed. The results are of wide interest, because for the first time it appears that 25SrRNA homology may reflect parallel or convergent evolution.

Papers published:

Baharaeen, S., and H.S. Vishniac. 1982. A fixation method for visualization of yeast ultrastructure in the electron microscope. Mycopathologia 77: 19-22.

Baharaeen, S., J.A. Bantle, and H.S. Vishniac. 1982. The evolution of Antarctic yeasts: DNA base composition and DNA-DNA homology. Can. J. Microbiol. 28: 406-413.

Baharaeen, S., and H.S. Vishniac. 1982. Cryptococcus lupi sp. nov., an Antarctic Basidioblastomycete. Int. J. Syst. Bacteriol. 32: 229-232.

In Press:

Vishniac, H.S. and S. Baharaeen. . Five new basidioblastomycetous yeast species segregated from Cryptococcus vishniacii emend auct., an Antarctic Yeast species comprising four new varieties. Int. J. Syst. Bacteriol.

Submitted for publication:

Vishniac, H.S. An enation system for the isolation of Antarctic yeasts inhibited by conventional media. (Can. J. Microbiol.)

Baharaeen, S., U. Melcher, and H.S. Vishniac. Complementary DNA - 25S ribosomal RNA hybridization: An improved method for phylogenetic studies. (Can. J. Microbiol.)

THE ISOLATION OF ANTARCTIC MICROBIOTA

A new system (enation) and method of isolation which has been successfully used to isolate additional yeasts unique to the Antarctic as well as to reisolate C. vishniacii var. asocialis is described in Appendix A (ms submitted).This method does not discriminate against yeasts common in other soils. It is now possible to state that reports implying that common soil yeasts dominate in the Dr y Valleys reflect the inadequacy of the methods used. This method also proved suitable for the isolation of heterotrophic bacteria, cyanobacteria, algae and other fungi. Since the investigation of soil samples

collected by Dr. E.I. Friedmann's Antarctic expeditions is a cooperative affair, algal and filamentous fungal cultures of interest were turned over to Dr. Friedmann, bacterial isolates (lyophilized) to Dr. Peter Hirsch.

The biomass/population sizes of these yeasts in the Antarctic remain unknown, as does their contribution to biologically mediated activities in the Dry Valleys. The media (E-1 and 2 MC) described proved unsuitable for enumerating yeasts because they selected against yeasts of biotypes 18 and 19 when small populations were used as inocula in growth and simulated recovery studies. No quantitative studies of their suitability for enumerating other microbiota were made. An enation medium (E-3) which does not discriminate between biotypes 17 (3aY1) and 18 (30bY33) was developed. This medium, however, limits the growth of biotype 19 in both rate and yield. Since non-selective media are required both for enumeration and to provide a base for the definition of ecological niches, I have emphasized studies of the requirements of biotype 19 (3aY86) during this report period. The growth of 3aY86 is approximately equivalent to that of other biotypes if 0.01-0.025% yeast extract is added to medium E-3. Yeast extract is inhibitory in slightly higher concentrations, for reasons which are unknown. When soil samples which may contain the same inhibitory factors are used, there is a risk of inadvertently exceeding the threshold of toxicity. I cannot presently omit yeast extract because vitamins do not substitute for it, nor do the concentrations required suggest requirements for other organic growth factors. No dose/response curve was obtained when

extracts of ashed yeast extract were substituted. While this does not rule out the possibility of a trace inorganic requirement, there was no response to various doses of Ni^{++}, nor to limited trial. of V (as $VOSO_4$, Na_3VO_4). Selenium was not tried; other elements which might be required in trace amounts are already present in the medium. Since this approach has failed to be rapidly rewarding, I shall abandon it. I plan to examine the suitability of E-3 + 0.01% yeast extract (E-3S) by a) determining the recoverability of current isolates (now over 1 year old and possiby adapted to laboratory media) from mixed small inocula, plating on the same medium with increased substrate concentrations (to allow the formation of visible colonies) and b) by using this medium to make fresh isolations from the soil samples of Dr. Friedmann's '81-'82 expedition. The latter should be rewarding even if E-3S is inadequate in simulated recoveries. The isolations made with E-1 were not entirely congruent with the results of recovery experiments.

NICHE PARAMETERS IN THE DRY VALLEYS

The C. vishniacii complex is not more psychrophilic, nor more osmotolerant, than other yeasts which have not successfully colonized the dry valleys. There are many other yeasts capable of growth in the refrigerator: the pink yeasts of cold storage, the Leucosporidium spp. of richer Antarctic soils (and New Zealand slaughter house sites), and most soil yeasts, described and undescribed. Comparison of the reported habitats of these yeasts suggested that substrate concentration was the major niche parameter. If this is so, the Tyrol Valley yeasts, evolved in a habitat essentially lacking measurable organic carbon (Horowitz et al.,1972)may present the first convincing example of oligotrophy in soil microbes. True

oligotrophy has been demonstrated only in aquatic microbes. The dominant organisms of soil adapt to famine by evading the issue. Growth only on very dilute media in the laboratory may result from the use of volatile compounds in the ambient air and the amino acid imbalance of complex media (see, p.e., Hattori et al., 1979)

Within the Dry Valleys, endolithic lichens constitute an oasis, retaining moisture and producing organic carbon (see Friedmann, 1982). The yeasts isolated from lichenized rocks or associated soils (exemplified by isolate 3aY1) cease growing after ca. 60 hours when competing with the Tyrol valley yeast, C. vishniacii var. asocialis, for glucose in batch culture. The social yeasts are not only more substrate limited. The form in which nitrogen is available also constitutes an important niche parameter. Tyrol valley (or asocial) yeasts are inhibited by ammonia and utilize nitrate-N. The predominance of nitrate-N in desert soils is well established. The social yeasts resemble all other yeasts(Phaff, 1978) in utilizing considerably higher ammonia concentrations quite happily, and many other yeasts in failing to assimilate nitrate-N. The presence of an asocial yeast does not allow a social yeast to continue growth after ca. 40 hours in media in which only nitrate-N is supplied.

I have not yet shown that competing populations can be manipulated at will by supplying the limiting C or N source after growth of the unsuccessful competitor has become stationary. Such experiments are planned for the near future, but the available isolates have now been in laboratory culture for over a year. It

may be necessary to use freshly isolated strains (from the experiments planned in "Isolation...."). To ensure continuity of experimental material, I plan to complete the formal description of the '81-'82 yeast isolates. These descriptions now lack primarily G+C mol% and quinone data. Budding morphology has been determined by scanning electron micrography. They appear to be congeners of C. vishniacii by the criteria of the next section.

Adaptations to substrate-limitation and differential N-source utilization should be confirmed by examination of biochemical mechanisms. But the demonstration that these niche parameters are of wider importance (than in the model isolates)and that they have relevance to the in situ fluxes of C and N in the habitats of social and asocial yeasts are of more immediate interest. The flux of organic carbon and ammonia-N in endolithic communities in the Dry Valleys is unknown. The requirements of social yeasts (ie the relationship of yield to dilution rate in chemostat culture) could suggest a minimum figure. A required carbon flux for the growth of asocial yeasts has already been suggested (Vishniac and Hempfling, 1979b). Such experiments, and a screen for substrate-limitation in competitive batch culture with other psychrotolerant yeasts, should follow the demonstration that 3aY1 and C. vishniacii var. asocialis can be controlled by substrate-concentration and N-source. It is anticipated that a doctoral candidate, June Klingler, will undertake such studies beginning Spring'83 in my laboratory.

BASIDIOBLASTOMYCETE PHYLOGENETICS

It has generally been intuitively believed that classification based on phylogeny has predictive value and is therefore preferable to phenetic taxonomy. Congeneric material, with a common phylogenetic background, is more reliable when specific adaptations to different habitats are to be inferred from laboratory studies of physiology. The Antarctic yeasts are anamorphic, that is, they lack sexual reproduction. Since sexual characters have been the primary basis for "natural"classification in the fungi, anamorphic taxa are very poorly based. The achievements of nucleic acid homologies in providing a phylogenetic basis for bacterial taxonomy make such procedures a natural choice for examining anamorphic yeasts. Since homologies between DNAs are useful only in defining fungal species, and those between the more conservative small rRNAs seemed likely to be useful only in defining higher taxa, we have studied the range of taxa definable by 25S rRNA homology in trying to determine the congeners of the Antarctic yeasts. For this purpose, the methods of modern molecular genetics were used to develop a new, biochemically preferable, method of homology determination by hybridization of 25S rRNA with cDNA prepared from appropriately sized fragments (Appendix B, ms submitted).

The results of hybridizations performed with three ^{3}H-cDNA probes are given in Tables 1,2,3, and 4. Figure 1 maps the positions of yeasts showing 50 rb% or more homology with all three probes. This material is being prepared for publication. These data indicate that teleomorphic (sexy) genera of basidiomycetous yeasts (Filobasidiella, Filobasidium, Leucosporidium, and

Table 1. cDNA:25S rRNA hybridization; *Cryptococcus vishniacii* probe cDNA

Organisms	% actual binding ± SD	% relative binding
Cryptococcus vishniacii	78.01 ± 0.98	(100.00) ± 1.26
Cryptococcus lupi	76.92 ± 1.18	98.60 ± 1.53
Cryptococcus bhutanensis	76.64 ± 0.54	98.24 ± 0.70
Cryptococcus himalayensis	74.99 ± 0.53	96.13 ± 0.71
Filobasidiella neoformans	71.71 ± 2.31	91.92 ± 3.22
Phaffia rhodozyma	63.05 ± 1.16	80.82 ± 1.84
Leucosporidium scottii	60.00 ± 0.57	76.91 ± 0.95
Rhodosporidium toruloides	59.88 ± 0.53	76.76 ± 0.89
Candida fujisanensis	55.69 ± 0.69	71.39 ± 1.24
Candida humicola	54.92 ± 0.69	70.40 ± 1.26
Candida acutus	54.74 ± 1.27	70.17 ± 2.32
Aessosporon salmonicolor	20.83 ± 0.44	26.70 ± 2.11
Candida curvata	19.89 ± 0.36	25.50 ± 1.81
Pityrosporum ovale	19.69 ± 0.81	25.24 ± 4.11
Candida aquatica	19.37 ± 0.76	24.83 ± 3.92
Candida podzolica	14.38 ± 0.24	18.43 ± 1.67
Candida zeylanoides	9.49 ± 1.00	12.17 ±10.54
Hansenula saturnus	8.25 ± 0.52	10.58 ± 6.30

The actual binding values were not corrected for 1.01 ± 0.23 % actual binding (self reannealing) of the probe cDNA.

Table 2. Complementary DNA-25S ribosomal RNA homology: *Rhodosporidium toruloides* probe complementary DNA.

Source of 25S rRNA	% actual binding ± SD	% relative binding
Rhodosporidium toruloides	82.99 ± 2.23	100 ± 2.69
Rh. infirmo-miniatum	82.80 ± 0.97	99.77 ± 1.17
Rh. dacryoidum	82.51 ± 0.84	99.42 ± 1.02
Rh. malvinellum	80.47 ± 1.52	96.97 ± 1.89
Leucosporidium antarcticum	72.84 ± 0.40	87.77 ± 0.55
Candida fujisanensis	72.56 ± 1.04	87.43 ± 1.43
L. nivalis	71.57 ± 1.28	86.24 ± 1.79
L. gelidum	71.32 ± 0.63	85.94 ± 0.88
L. scottii	71.23 ± 1.04	85.83 ± 1.46
Can. humicola	70.26 ± 1.22	84.66 ± 1.74
L. stokesii	70.11 ± 1.05	84.48 ± 1.50
L. frigidum	70.06 ± 1.08	84.42 ± 1.54
Can. acutus	68.50 ± 2.88	82.54 ± 4.20
Cryptococcus himalayensis	67.96 ± 0.92	81.89 ± 1.35
Cr. laurentii	67.31 ± 0.57	81.10 ± 0.85
Filobasidiella neoformans	66.84 ± 0.78	80.54 ± 1.17
Cr. albidus	65.96 ± 0.41	79.48 ± 0.62
Phaffia rhodozyma	64.41 ± 1.17	77.61 ± 1.82
Cr. bhutanensis	63.81 ± 0.65	76.89 ± 1.02
Cr. lupi	62.82 ± 2.63	75.69 ± 4.19
Filobasidium uniguttulatum	60.99 ± 0.54	73.48 ± 0.89
Cr. vishniacii	59.36 ± 1.33	71.52 ± 2.24
Filob. capsuligenum	58.31 ± 1.12	70.26 ± 1.92
Aessosporon salmonicolor	37.10 ± 0.47	44.70 ± 1.27
Can. curvata	36.80 ± 0.52	44.34 ± 1.41
Can. aquatica	36.38 ± 0.51	43.83 ± 1.40
Can podzolica	21.64 ± 0.17	26.07 ± 0.79
Can. zeylanoides	18.80 ± 0.21	22.65 ± 1.12
Tremella mesenterica	13.04 ± 0.14	15.71 ± 1.07
Pityrosporum ovale	12.16 ± 0.23	14.65 ± 1.89
Agaricostilbum palmicolum	9.59 ± 0.29	11.56 ± 2.78
Hansenula saturnus	8.32 ± 0.10	10.03 ± 1.20
Ustilago maydis	7.73 ± 0.47	9.31 ± 6.08

Table 3. Complementary DNA - 25S ribosomal RNA homology: *Filobasidiella neoformans* probe complementary DNA.

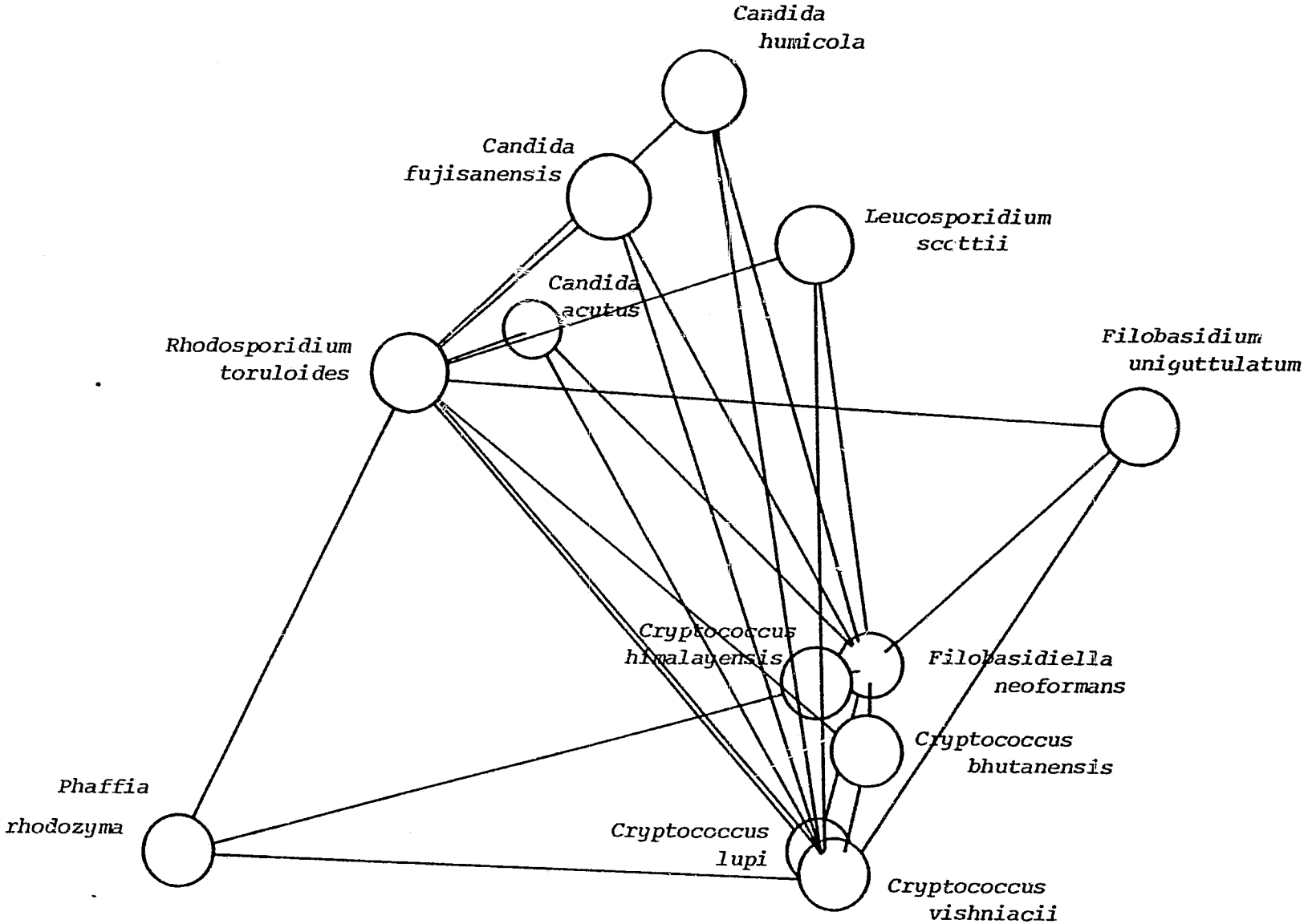
Source of 25S rRNA	% actual binding ± SD	% relative binding
Filobasidiella neoformans	83.81 ± 1.09	100 ± 1.30
Cryptococcus bhutanensis	80.87 ± 1.32	96.50 ± 1.63
Cryptococcus himalayensis	79.80 ± 1.47	95.21 ± 1.84
Cryptococcus lupi	78.48 ± 2.14	93.64 ± 2.73
Cryptococcus vishniacii	78.45 ± 2.01	93.61 ± 2.56
Leucosporidium antarcticum	71.35 ± 0.79	85.14 ± 1.11
Leucosporidium scottii	71.10 ± 0.91	84.84 ± 1.28
Leucosporidium frigidum	70.38 ± 0.78	83.98 ± 1.11
Filobasidium uniguttulatum	70.32 ± 1.00	83.90 ± 1.42
Filobasidium capsuligenum	70.14 ± 0.59	83.68 ± 0.84
Candida fujisanensis	66.61 ± 2.08	79.48 ± 3.12
Candida humicola	65.22 ± 1.70	77.82 ± 2.91
Rhodosporidium toruloides	65.13 ± 1.65	77.71 ± 2.53
Candida acutus	59.93 ± 0.73	71.50 ± 1.78
Phaffia rhodozyma	59.43 ± 1.05	70.91 ± 2.19

Table 4.

cDNA-25S rRNA hybridization: Cryptococcus vishniacii probe cDNA

	% actual	% relative binding
Cryptococcus vishniacii	78.02 ± 0.71	100.00 ± 0.91
Filobasidium capsuligenum	59.87 ± 0.67	76.73 ± 0.86
Filobasidium uniguttulatum	61.28 ± 0.68	78.54 ± 0.87
Leucosporidium antarcticum	58.41 ± 0.57*	74.87 ± 0.73

* determined from duplicate vials.



Rhodosporidium) possess 25S rRNAs so closely similar within each genus as to be indistinguishable (given the values for standard error of difference).

A teleomorphic genus can therefore be defined as a group of species having 96.5 to 100 rb% homology in 25S rRNA. An anamorphic genus, as expected from the paucity of characterizing features, cannot be so closely circumscribed. The anamorphic genus Vanrija (= basidiomycetous Candida spp.) spans nearly the entire range of homology values. Species of this genus do have phenetic characters which have not been utilized appropriately; it will probably be possible to split Vanrija into more "natural" genera. The anamorphic genus Cryptococcus appears to be appropriately, if empirically, defined, since its spp. (including those originally described as the Cryptococcus anamorphs of Filobasidium) occupy a finite, mappable, range of homology values. The distance from Filobasidium to C. vishniacii is roughly 21.5 rb% units. The historical definition of Cryptococcus as inositol-assimilating, non-fermenting encapsulated yeasts, lacking sexual reproduction, mycelium/pseudomycelium, and red colors, should be replaced with: basidiomycetous anamorphic yeasts, typically lacking mycelium/pseudomycelium and red colors, more or less encapsulated, budding repetitively and only at the birth scar site (monopolar) during uninterrupted growth, cell wall not pitted or grooved. All of the Dry Valley yeasts are Cryptococcus by this definition.

25S rRNA homology can also define family in basidiomycetous yeasts. An examination of Fig. 1 shows that the families

Filobasidiaceae (Filobasidiella, Filobasidium) and Sporidiaceae (Leucosporidium, Rhodosporidium can be defined by the ca. 83-90 rb% homology between their genera. A better definition may be ca. 78 - 90 rb% homology, since Cryptococcus spp are in all likelihood Filobasidiaceae.

However, the definition of family requires at least 3-dimensional mapping rather than simply the determination of an homology value. Filobasidium and Leucosporidium, Filobasidiella and Rhodosporidium, are pairs as homologous as those belonging to the same family. Since these families are well defined, by several conventional criteria, it is evident that the basic concept of molecular phylogeny, that rRNA homology values indicate phylogenetic distance, must be amended. rRNAs are conserved because they are constrained. These constraints may evolve differently in two families, but limitation in the position and variety of nucleotide substitutions has led to the parallel or convergent evolution seen in our map!!!!!

This work has constituted the Ph.D. thesis of Siavash Baharaeen (degree granted Aug '82). It leaves unanswered several questions of great interest to molecular evolution. These questions are not related directly to the problem of adaptation to extreme environments. I am asking therefore for a split in NASA support for my laboratory to include 1) ecology of extreme environments: Dry Valley yeasts and substrate-limitation and 2) Molecular evolution: 25S rRNA homology in basidiomycetous yeasts as a test of molecular phylogeny and the morphology of primaeval eufungi.

As a test of molecular phylogeny, our investigation is incomplete. What is lacking are: 1) more definitive evidence that our method of homology determination includes all of the information of the 25S rRNA molecule; 2) demonstration that 3 dimensions (ie three probes) adequately define the relative positions of taxa not used as probes-- and within what degree of homology this is possible. (DNA-DNA homologies by similar procedures become unreliable--unmappable-- below roughly 50 rb%); 3) extension of the map to include the Sporidiobolaceae. Aessosporon, the only genus of this family included in our study, is off the map. Its phylogenetic distance from the 2 probes used is far greater than would have been expected from Moore's inclusion of the Sporidiobolaceae and the Sporidiaceae in the same order-- Sporidiales-- and a division --Ustomycota-- different from tha t of the Filobasidiaceae. More than a single pair of families is needed to examine the phenomenon of parallel rRNA evolution and the hierarchical depths of 25S rRNA homology as a tool in eucaryote phylogeny.

Extension of our study of 25S rRNA homology to other yeasts would also contribute to the question of the morphology of the urfungus. A variety of evidence suggests that the Ascomycota and Basidiomycota are closely related and probably of different evolutionary origin from the other divisions rather loosely included in the Kingdom Fungi. Many mycologists believe that the first fungus of this group was cellular - a yeast - rather than mycelial. I think this unlikely. The adaptive zone

penetrated by the first fungus (the urfungus) of this group probably required mycelial growth. Mycelial fungi compete successfully in many habitats against procaryotes, yeasts only in a specialized few. Furthermore, yeasts appear in the developmental cycles of fungi which seem to be unrelated. The question is therefore one which could be settled by examining the relationships of fungi which have yeast stages. Our present data show only that ascomycetous (Hansenula) and basidiomycetous yeasts are related at a hierarchical level beyond the scope of our experiments. However, the question requires the evolutionary detail available only with 25S rRNA homology, because it is necessary to know not at what point yeasts share a common ancestor, but whether yeast morphology is a primitive or derived homology. It is derived if it appears on several branches of a mycelial lineage, examined at the hierarchical level of genus.

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In addition to Siavash Baharaeen, the following have contributed to the work of this report period: Dr. E.I. Friedmann (soil samples, algal identifications), my colleagues Drs. Bantle and Melcher (advice on nucleic acid chemistry), students Penelope Boston (Colorado U., soil samples) and Jana Collins (inorganic nutrition of yeasts), technician Teresa McElroy and work/study student Amelia Gormly. Cultures were kindly contributed by Dr. R.J. Bandoni, Dr. C.P. Kurtzman, and Dr. K.J. Kwon-Chung.

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An enation system for the isolation of Antarctic yeasts inhibited by conventional media.

H. S. Vishniac

Department of Microbiology, Oklahoma State University, Stillwater OK 74078, U.S.A.

Abstract

Yeasts peculiar to the Dry Valleys of Antarctica can be recovered by allowing them to become enate (grow out) from soil particles. The enation system was successful in liquid medium E-1 (glucose, 0.05%; peptone, 0.05%; yeast extract, 0.025%; potassium phosphate buffer, pH 6.86, 1 mM; NaCl, 50 mM; $MgSO_4 \cdot 7H_2O$, 0.2 mM; Vishniac and Santer (Bacteriol. Rev. 21:195-213) trace metal mixture, 1.0 mL/L), shaken at 160 rpm in a 10° water bath. Samples were spread on 2 MC (glucose, 0.2%; peptone, 0.1%; yeast extract, 0.05%; NH_4Cl, 2.0 mM; trace metal mixture, 1.0 mL/L; agar, 1.8%). This system also allowed the isolation of typical soil yeasts from Colorado soils, and of cyanobacteria, heterotrophic bacteria, filamentous fungi, and chlorophyta. Marked inhibitory effects of conventional yeast media account for the failure of previous investigators to isolate these Antarctic yeasts. Their abundance remains unknown, since E-1 and 2 MC also have some inhibitory effects.

Abstract

Yeasts peculiar to the Dry Valleys of Antarctica can be recovered by allowing them to become enate (grow out) from soil particles. The enation system was successful in liquid medium E-1, shaken at 160 rpm in a 10^{o} water bath. Samples were spread on a richer medium, 2 MC. This system also allowed the isolation of typical soil yeasts from Colorado soils, and of cyanobacteria, heterotrophic bacteria, filamentous fungi, and chlorophyta. Marked inhibitory effects of conventional yeast media account for the failure of previous investigators to isolate these Antarctic yeasts. Their abundance remains unknown, since E-1 and 2 MC also have some inhibitory effects.

The use of standard methods for enumerating the microbiota of an ecosystem for which these methods have not been validated may give very erroneous impressions. The Antarctic cold desert is the most extreme environment of its type on earth. The Dry Valleys of South Victoria Land approach in some respects the conditions of Mars (Vishniac and Mainzer, 1973; Cameron et al., 1976; Friedmann, 1982). Endolithic communities are visible in climatically favored sites, but it is generally believed that indigenous populations in soils of the Dry Valleys are very sparse (Friedmann, 1982). This belief is based partly upon the experience of investigators using standard methods of enumeration but is also a result of the difficulty of establishing indigenicity. Microbes isolated in small numbers from soil may actually be tourists, incapable of establishing local colonies. The only microbes demonstrably indigenous to the soils of highly stressed sites are the yeasts of the Cryptococcus vishniacii complex (Vishniac and Hempfling, 1979a,b). These yeasts were isolated serendipitously, in the course of a number of non-specific procedures. Other investigators (see Atlas et al., 1978) have reported only yeasts common in soils elsewhere. The C. vishniacii complex appears to have undergone subspecific evolution in the Dry Valleys (Baharaeen et al., 1982). It is likely to display significant adaptations to the environmental stresses of this habitat. Its importance in the Dry Valley ecosystem cannot be assessed in the absence of quantative methods of isolation. Attempts

to develop such methods are reported here.

Materials and Methods

Soil samples:

The soil samples used are listed in Table 1. Antarctic samples were aseptically collected by Dr. E.I. Friedmann's expedition during the austral summer of '80-'81. Colorado soil samples were collected during the winter of '81-'82 by Penelope Boston. Soil samples were transported in a continuously frozen state, were stored at temperatures not exceeding -30°C, and were subsampled while resting on a bed of dry ice in a laminar flow hood. The logistic problems of obtaining frozen samples from remote and inaccessible areas have prevented complete characterization of these soils. The mass of Antarctic inocula was "guesstimated"; the masses given are those of inocula collected by centrifugation and filtration at the end of enation experiments. Varying amounts of clay particles were lost during this procedure. Inocula of Colorado soils were weighed in tared culture flasks at room temperature and quickly transferred to 10°C.

Conditions of culture and identification:

All experiments were conducted at 10°C, using either a refrigerated water bath shaker (New Brunswick Scientific Co., Model G-76)at 160-190 rpm, or a refrigerated incubator (Precision Scientific Co., Model 815). Small subsamples of Antarctic soils were sprinkled onto agar (with and without a soft agar

overlay, with and without antibiotics) and were used to inoculate biphasic cultures and liquid media. The liquid media used were GPYPi (glucose, 0.5%; peptone, 0.5%; yeast extract, 0.3%; potassium phosphate buffer, pH 6.86, 10 mM), GPYPi diluted 1:10, and diluted GYPPi amended with a mineral supplement (E-1, Table 2). Solid media used included those customary for yeast isolation (GPYPi agar, malt extract agar, Sabouraud's dextrose agar, cornmeal agar), as well as E-1 agar and 2 MC (Table 3), a medium developed for more efficient counting of yeasts of the C. vishniacii complex (unpublished data). Liquid cultures were sampled at weekly intervals for a month. Since yeasts were recovered by the end of the first week from Antarctic soils, Colorado soil cultures were sampled every other day for 8 days. Liquid cultures were sampled by spreading 0.2 mL on each of 3-5 plates of each agar used. Final counts of yeasts could be made after 14 days of incubation; other fungi, algae and bacteria required ca. 28 days incubation.

Yeasts were at first maintained on the medium of isolation, subsequently on YC medium (glucose, 0.4%; sodium glutamate, pH 6.0, 2.0 mM; NH_4Cl, 2.0 mM; potassium phosphate buffer, pH 6.0, 5 mM; Vishniac and Santer (1957) trace metal mixture, 1.0 mL/L (= 1/10 of recommended concentration); H_3BO_4, 50 µg/L; KI, 10 µg/L; Wickerham's vitamins (van der Walt, 1970); yeast extract, 0.05%; agar, 1.8%). Stock cultures were maintained at 4°C.

Antarctic yeasts (104 isolates) were characterized by

temperature preference (YC medium), by the diazonium blue B procedure of Hagler and Ahearn (1981), and by nitrate utilization and carbon assimilation tests using previously published methods (Vishniac and Hempfling, 1979a) but medium Y-2 (mineral base containing potassium phosphate buffer, pH 6.0, 1 mM; NaCl, 50 mM; $MgSO_4.7H_2O$, 0.2 mM; Vishniac and Santer (1957) trace metal mixture, 1.0 mL/L; to which was added vitamins as required, 2 mM NH_4Cl or other nitrogen source, and 0.2% glucose or other carbon source). Colorado yeasts (72 isolates) were identified only as far as necessary (Barnett et al., 1979) to ascertain the absence of the C. vishniacii complex and the presence of common soil yeasts (colony morphology, temperature preference , diazonium blue B reaction, nitrate utilization,and carbon assimilation on commercial Wickerham's media (Difco). The carbon sources tested were: cellobiose, citrate, erythritol, dulcitol (galactitol), galactose, glucuronate, inositol, lactose, maltose, mannitol, melezitose, melibiose, α-methyl-D-glucoside, raffinose, succinate, sucrose, trehalose, and xylose. Isolates appearing similar in all respects were assigned to the same biotype number, pending identification or formal description.

Growth rate and competition experiments:

Growth rates on various media were determined either by following turbidity spectrophotometrically at 650nm (Bausch and Lomb Spectronic 70) or by spreading dried plates of 2 MC with appropriately diluted samples. The inocula for growth rate (k_{max}) studies were cells growing exponentially under the cultural conditions being tested. Cells growing exponentially in 2 MC broth were diluted appropriately for studies of the initial phases

of growth in E-1.

When biotypes 17 and 18 competed in mixed culture, their proportion in the culture was calculated from the number of colonies appearing on a synthetic medium containing glucose (total count) vs. the number appearing on media containing cellobiose (biotype 17) and xylose (biotype 18) as substrates. Total counts of mixed cultures containing biotypes 17-20 were made on 2 MC; isolates were subsequently identified by picking at random colonies for assimilation tests (L-arabinose, cellobiose, but not xylose = 3aY1, biotype 17; xylose but not L-arabinose,cellobiose = 30bY33, biotype 18; cellobiose, xylose, but not L-arabinose = 3aY86, biotype 19; L-arabinose, xylose, but not cellobiose = 3aY92, biotype 20.

Results and Discussion

The enation system:

The term "enation" is used here for the technique in which microbes adhering to soil surfaces are allowed to grow out into non-selective media. The success of this technique depends upon the presence of an initially sparse microbiota and upon sampling liquid enation cultures before growth has resulted in significant alteration in the conditions of culture. This technique avoids the technical difficulties of percolation (Uydess and Vishniac, 1976) and sonications (Martini et al., 1980) necessary for the maximal recovery of yeasts from soil.

Enation in shaken liquid medium E-1 was the only technique which allowed the recovery of yeasts from Antarctic soils. Medium 2 MC, but neither E-1 with agar nor conventional yeast media, allowed relatively rapid and unrestricted growth of all the yeast isolates recovered. The richer medium 2 MC is not suitable for enation. Biotypes 17-20 do not grow at the same k_{max} in 2 MC broth from large inocula. When small inocula of two biotypes with nearly comparable k_{max} competed in 2 MC broth, their growth rates were no longer comparable. One biotype grew more rapidly; the other disappeared (Fig. 1).

It is not surprising that Antarctic yeasts prefer a dilute enation medium. Dry Valley soils are low in carbon (Cameron et al., 1976; Vishniac and Hempfling, 1979b). The non-specific procedures which yielded the first isolates of the C. vishniacii complex cannot have provided much higher substrate concentrations. It was surprising that GPYPi was suitable neither for enation nor isolation, because GPYPi agar had been our standard maintenance medium for the C. vishniacii complex. This contradiction was resolved by the discovery that it was possible to adapt some isolates to growth on GPYPi broth and agar, or to Sabouraud's dextrose agar, by using larger inocula than appear to occur in Antarctic soils. The k_{max} of isolate 3aY86 (biotype 19) in GPYPi broth at ca. 4.7 generations was 0.064 g/h. A subculture grew at k_{max} = 0.102 after ca. 8.7 (total) generations in GPYPi broth.

Soil productivity data obtained with this system are given in Table 4. Media E-1 and 2 MC appear to be non-selective. It is clear that the microbiota of the Antarctic soil samples were initially sparse. The use of antibiotics may be required to prevent significant changes in pH, since yeasts are inhibited at the pH values which typically resulted from unchecked bacterial growth. Filamentous fungi very rarely caused problems.

Media E-1 and 2 MC appeared to be non-selective to an unusual degree. Bacteria, yeasts, filamentous fungi and algae were recovered. Although the algae have been tentatively identified as the common soil alga Chlorosarcina and the lichen phycobiont Trebouxia and therefore might have been expected in these soil samples, they were not expected to grow in these media or in unlighted incubators. However, the only yeast isolates identifiable were biotype 18, C. vishniacii var. asocialis (in press), and biotype 21, Trichosporon cutaneum. The yeasts found most prevalent in Antarctic soils by other investigators were notably absent. These are: Aureobasidium pullulans and Cryptococcus albidus (Atlas et al., 1978), C. laurentii, Leucosporidium nivale, and L. scottii (di Menna, 1960,1966). Although T. cutaneum has been reported from the Antarctic (Goto et al., 1969), it is common in temperate rather than Antarctic soils.

The possibility that these media do discriminate against common soil yeasts was tested by examining frozen Colorado soils, from sites and depths expected to be depauperate. The bacterial counts attest to a much richer microbiota than that of Antarctic

soils but do not indicate a rich soil. Total counts of soil bacteria are higher on dilute than on the usual commercial media (see, p. e., Dabek-Szreniawska and Hattori, 1981). All of the Colorado soils yielded isolates identifiable as Cryptococcus albidus. Other common soil yeasts were identifiable from some samples: LPN contained C. laurentii, UMRS and HSR T. cutaneum, and HSR Aureobasidium pullulans. Isolates identifiable as yeasts of the C. vishnacii complex, or as the unidentifiable biotypes of the Antarctic soil samples were not found. The enation system does not discriminate against common soil yeasts.

The enation technique is, further, at least as efficient as the combined agitation, percolation, and sonication recommended by Martini et al. (1980) in recovering diverse yeasts from soil. Their recommended procedure recovered 12 species from several grams of vineyard soil. Although the tests used for identification were very limited, 7-16 biotypes were distinguishable in much smaller samples of each of the Colorado soils.

Quantitative recovery:

The enation technique could readily be adapted to a most probable number procedure, utilizing the program of Koch (1982) for analysis. Varying masses of soil, rather than the usual dilutions, would be required as inocula. The results of varying soil mass from roughly a quarter of a gram to a gram, and the isolation of 2 biotypes from the largest inoculum (Table 4), argue that medium E-1 was both buffered against soil constituents and capable of non-selectively

supporting the growth of at least 2 yeast strains to levels allowing isolation.

Determination of initial growth rates of small yeast inocula showed that medium E-1 does not support equally the growth of all Antarctic yeasts. The early stages of growth of 3aY1 (biotype 17) and 30bY33 (biotype 18) are shown in Fig. 2. In another experiment, an inoculum of 3.6×10^4 cfu/mL of 30bY33 had not achieved a positive growth rate by 44 h. Unless an initial population of nearly 10^5 cfu/mL is present, 30bY33 should become a decreasing fraction of a mixed culture during enation. The isolation of this biotype seems to have depended upon the absence of competition rather than lack of selection during enation culture.

Recovery from soil samples containing mixed populations was simulated by placing 0.2 mL of a suspension containing equal numbers of exponentially growing cells representing biotypes 17-20 on sterile dry fiberglass filters (with and without sterile soil collected from completed enation experiments) and allowing the cells to rest for 4.5 h before the addition of equilibrated enation medium. One such experiment is summarized in Fig 3. The results were the same in the presence and absence of soil. Isolate 3aY86 (biotype 19) was not recovered after the addition of medium; 30bY33 (biotype 18) was detectable 2 days later in proportions implying negative growth; 3aY1 (biotype 17) had grown, but was outstripped by 3aY92 (biotype 20). The actual recovery of biotypes 19 and 20 from a single enation flask is inexplicable.

The population of biotype 19 in this soil sample is unlikely to have been large. The only yeast recovered on initial sampling of an enation flask was biotype 20 (59 cfu/g).

The application of the enation technique to the enumeration of Antarctic yeasts is clearly premature. One may conclude that yeasts peculiar to the Antarctic dominate in Dry Valley soils, and that at least two microcolonies of yeasts may be present in a gram of soil in relatively sheltered, productive sites. But it remains possible, given the improbability of our isolation of biotypes 18 and 19 with the media used, that the total biomass of yeasts in these inhospitable soils rivals that of the endolithic communities. The results of applying more suitable methods for yeast isolation imply that other Antarctic microbiota await recovery by as yet undiscovered techniques.

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Table 1. Soils Sampled

South Victoria Land, Antarctica:

A 801-3 19Dec80. Linnaeus Terrace, N slope facing Wright Valley, ca. 1550 m alt. 77° 36' S, 161° 08' E. Under rock ledge with lichens. a = surface - 1 cm; b = 3 cm depth.

A 801-8 21Dec80. Linnaeus Terrace, W part, desert pavement, ca. 1600 m alt. 77° 36' S, 161° 02' E. No lichens in immediate vicinity. a = surface; b = 1 cm; c = 3 cm depth.

A 801-25 23Dec80. N slope Oliver Peak, above Linnaeus Terrace, alt. ca. 1800 m. 77° 36' S, 161° 03' E. 0 - 3 cm depth.

A 801-28 30Dec80. Saddle between Siegfried Peak and Siegmund Peak, alt. 1520 m. 77° 35' S, 161° 46' E. Sandstone rocks colonized by lichens in vicinity. a = 1 cm; b = 3 - 4 cm depth.

A 801-29 30Dec80. Depression in valley W of Oliver Peak, 1430 m alt. 77° 37' S, 160° 54' E. Few lichens in vicinity. a = 1 cm; b = 2 cm depth.

A 801-30 30Dec80. Tyrol Valley (site of Dr. W.V. Vishniac's collections), center, 1350 m alt. 77° 35' S, 160° 37' E. No lichens in vicinity. a = 1 cm; b = 2 cm depth.

Front Range, Colorado, U.S.A.:

UMRS 26Oct81. Mountain Research Station, University of Colorado, alt. ca. 3570-3600 m. No vegetation in vicinity; largely well-sorted silty clay. Soil temp. 0°C. 15.2 mg H_2O/g. 6 - 10 cm depth.

(Table 1. con't.)

LPN 4Nov81. North slope of Long's Peak, ca. 3900 m alt. Lichens present on boulders, no other vegetation; sandy blow-out. Soil temp - 2°C. 3.67 mg H_2O/g. 7-11 cm depth.

HSR 3Jan82. Horse Shoe Ridge, ca. 2820 - 2880 m alt. Grasses and coniferous underbrush present but sampled area clear; sandy with small pebbles. Soil temp. - 7°C. 8.12 mg H_2O/g. 5 - 7 cm depth.

Table 2. Enation medium 1 (E-1)

glucose (aseptically added)	0.05%
peptone	0.05%
yeast extract	0.025%
potassium phosphate buffer, pH 6.86	1.0 mM
NaCl	50.0 mM
$MgSO_4.7H_2O$	0.2 mM
Trace metal mixture*	1.0 mL/L
with or without antibiotics aseptically added:**	
streptomycin sulfate	15 µg/mL
penicillin G	100 µg/mL

*Vishniac and Santer (1957) at 1:10 recommended concentration.

** This concentration of antibiotics was adequate to suppress bacterial populations below 10^4 cfu/g, but not those at 10^6 cfu/g.

Table 3. Second medium for counting (2 MC)

glucose (aseptically added)	0.2%
peptone	0.1%
yeast extract	0.05%
potassium phosphate buffer, pH 6.86	1.0 mM
NH_4Cl	2.0 mM
$MgSO_4.7H_2O$	0.2 mM
Trace metal mixture*	1.0 mL/L
Agar	1.8%

*Vishniac and Santer (1957) at 1:10 recommended concentration.

Table 4. Soil sample productivity.

Sample	Mass	Bacteria cfu/g	pH at 14 days (initial pH)*	Yeasts (other eucaryotes**)
Antarctica:				
A 801-3a	0.27 g***	ND[a]	ND	biotype 17
	0.94 g	ND[a]	6.53	biotype 19,20
	0.76 g	ND	7.04	biotype 19
-3b	1.11 g	ND[a]	6.60	0
	1.14 g	ND	6.67	0 (DH)
A 801-8a	1.17 g	8.1×10^3	7.62	0
	0.43 g	6.1×10^3	7.27	0 (DH)
	0.22 g	ND[a]	6.54	0
-8b	1.35 g	5.7×10^3	7.62	0
	0.32 g	14.6×10^3	7.27	0 (DH)
	0.43 g	ND[a]	6.56	0 (DH)
-8c	1.07 g	2.7×10^3	7.42	0
	0.31 g	10.6×10^3	6.59	0
	0.25 g	ND[a]	6.50	0
A 801-25	0.72 g	0.0	7.22	0
	0.27 g	0.0	6.57	0 (DH)
	0.29 g	ND[a]	6.57	0 (DH)
A 801-28a	0.59 g	0.0	7.85	0
	0.21 g	0.0	6.55 (6.47)	0 (DH)
	0.20 g	ND[a]	6.51 (6.40)	0

Table 4. con't.

-28b	0.96 g	0.0	6.65	0
	0.19 g	0.0	6.18 (6.39)	0
	0.20 g	ND[a]	5.90 (6.40)	0
A 801-29a	0.67 g	7.0×10^3	7.91	0 (A)
	0.20 g	7.7×10^3	6.72 (6.34)	biotype 21 (A)
	0.33 g	ND[a]	5.83 (6.31)	0 (A)
-29b	0.41 g	10.0×10^3	7.60	0
	0.31 g	12.1×10^3	6.50 (6.42)	0 (A)
	0.36 g	ND[a]	6.22 (6.41)	0
A 801-30a	0.24 g***	ND[a]	ND	0
	0.82 g	ND[a]	6.41	0 (DH, MH)
	0.86 g	ND	7.53	? (Z)
-30b	0.76 g	ND[a]	6.68	biotype 18
	0.63 g	0.0	6.48	0
Colorado:				
UMRS	0.27 g	5.4×10^6	ND	12 biotypes (C,FF)
LPN	0.25 g	2.7×10^6	ND	7 biotypes (FF)
HSR	0.30 g	30×10^6	ND	16 biotypes (FF)

* pH after addition of soil sample; pH of equilibrated medium before inoculation was 5.60. ** other eucaryotes included A, algae; C, cyanobacteria; DH, dematiaceous hyphomycetes; FF, filamentous fungi; MH, moniliaceous hyphomycetes; Z, zygomycete (Mucor racemosus, deposited in the Rancho Santa

Table 4. con't.

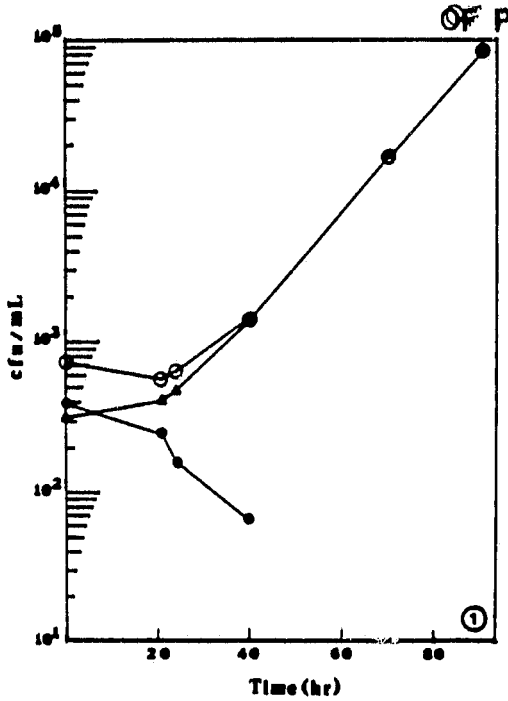
Ana Botanic Garden collection as RSA 2578. Mucor racemosus overgrew both enation flask and sample plates, providing the only occasion on which filamentous fungi interfered in the enation procedure. *** E-1 + 4 mM NH_4Cl was used in these enation flasks. ND, not determined; ND[a], antibiotics present in medium. Antibiotics were added to enation flasks containing Colorado soil samples after initial sampling for bacterial counts.

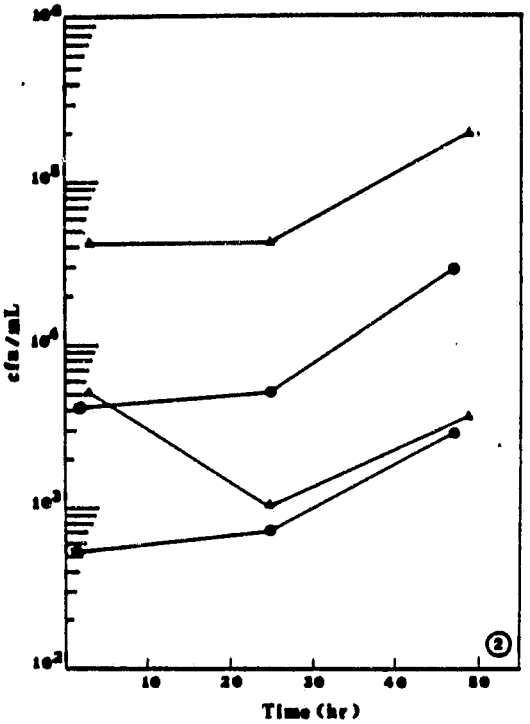
Legends to figures:

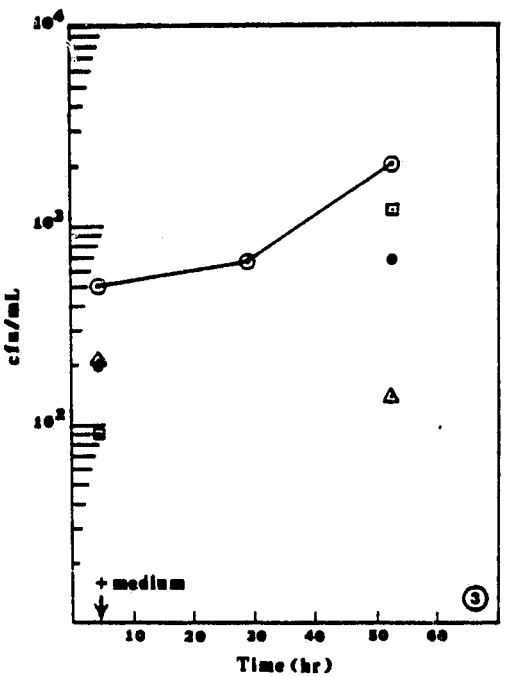
Fig. 1. Growth on 2 MC: ○, total count; ▲, 3aY1 (biotype 17); ●, 30bY33 (biotype 18). Isolate 30bY33 was undetectable at 70.6 and 90.6 h. Isolate 3aY1 grew at 0.12 generations / h during the latter part of this experiment.

Fig. 2. Initial phases of growth in E-1: ●, 3aY1 (biotype 17); ▲, 30bY33 (biotype 18). The growth of 30bY33 is density dependent.

Fig. 3. Simulated recovery by enation in E-1 + 2.0 mM NH_4Cl of biotypes 17-20. The inoculum (0.2 mL) was estimated (from $OD_{650\ nm}$) to yield, after addition of medium, a total count (○) of 4.4 cfu/mL as 3aY1 (●), 0.95 x 10^3 cfu/mL; 30bY33 (△), 1.2 x 10^3 cfu/mL; 3aY86 (not recovered), 1.3 x 10^3 cfu/mL; and 3aY92 (□), 1.0 x 10^3 cfu/mL.







Complementary DNA - 25S ribosomal RNA hybridization: An improved method for phylogenetic studies

Siavash Baharaeen[†]
Department of Botany and Microbiology, Oklahoma State University,
Stillwater, Oklahoma, U.S.A. 74078

Ülrich Melcher
Department of Biochemistry, Oklahoma State University,
Stillwater, Oklahoma, U.S.A. 74078

Helen S. Vishniac*
Department of Botany and Microbiology, Oklahoma State University,
Stillwater, Oklahoma, U.S.A. 74078

[†]Present address: Department of Biochemical and Biophysical Sciences, University of Houston Central Campus, Houston, Texas, U.S.A. 77004.

*Author to whom reprint request should be addressed.

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In a new combination of techniques for ribosomal RNA hybridization, complementary DNA is synthesized on 25S ribosomal RNA fragments generated by mild alkali treatment, by the enzymatic addition of polyadenylic acid tails, hybridization of these tails with oligo d(T), and reverse transcription in the presence of tritiated TTP. Hybridization reactions are performed in solution. Heteroduplexes are collected on diethylaminoethyl cellulose filter discs after treatment with S_1 nuclease. The problems presented by secondary rRNA structure are avoided by denaturation before reverse transcription and before hybridization. The high percentage of duplex formation (to 84%), the low standard deviation of relative binding (averaging 1.93% relative binding), and small differences in reciprocal hybridizations (1.69-5.24% relative binding), as well as the elimination of complications resulting from differences in the proportion of rRNA cistrons in nuclear DNA, make this method preferable to the membrane filter technique commonly used in phylogenetic classifications based on large rRNA homology.

Introduction

Ribosomal RNA (rRNA) cistrons are among the most conserved regions in the genomes of bacteria (Doi and Igarashi, 1965; Moore and McCarthy, 1967) and of higher organisms (Gibson, 1967; Matsuda and Siegel, 1967; Bicknell and Douglas, 1970) and can be used as an indicator of evolutionary relationships among organisms (Fox *et al.*, 1980). The simplest and least time consuming means by which these sequences can be utilized for comparisons of the evolutionary relationships of large numbers of organisms is through DNA-rRNA hybridization.

Most DNA-rRNA hybridization studies (Pace and Campbell,1971a,1971b; Palleroni *et al.*, 1973; Johnson and Francis, 1975; De Smedt and De Ley, 1977; Mordarski *et al.*, 1980; Moss and Bryant, 1982) have utilized the method of Gillespie and Spiegelman (1965), later modified by De Ley and De Smedt (1975), in which denatured DNA (50 µg) is immobilized on nitrocellulose membrane filters and reacted with *in vivo* labeled rRNA (10 µg) in solution. Alternative methods in which both nucleic acids are in solution and the heteroduplexes formed are separated on hydroxylapatite (Green *et al.*, 1971) or on diethylaminoethyl (DEAE) cellulose filter discs (Salzberg *et al.*, 1977; Maxwell *et al.*, 1978) have not been used in phylogenetic studies.

The major complication inherent in hybridization with rRNA stems from the extensive base pairing and base stacking of these molecules (Woese *et al.*, 1980); Noller and Woese, 1981; Noller *et al.*, 1981; Mankin *et al.*, 1981; Veldman *et al.*, 1981). Regardless of the methodology used, these "helices" interfere with proper binding of

DNA with rRNA. As a result, the annealing of DNA and rRNA reaches an apparent steady state with a low percentage of duplex formation. This effect is prominent in the membrane filter technique; maximum duplex formation in homologous reactions reaches only about 45% (*cf.* Spiegelman *et al.*, 1973). In phylogenetic studies, this limited percentage of reannealing in homologous reactions leaves a narrow range available for the detection of differences in heterologous reactions. This lack of sensitivity is compensated for by the increased discrimination obtained when percent hybridization is plotted against the denaturation temperature mid point ($T_{m(e)}$) of the heteroduplexes (De Smedt and De Ley, 1977).

We have employed a new combination of techniques to perform cDNA-25S rRNA hybridization for phylogenetic studies among basidiomycetous yeasts. The radiolabeled cDNA used as the probe is synthesized by reverse transcription (Verma, 1981) on enzymatically polyadenylated, denatured 25S rRNA fragments generated by partial alkaline hydrolysis (Engel and Davidson, 1978). The cDNA and 25S rRNA fragments are denatured prior to hybridization. The hybridization reactions are performed in solution and the heteroduplexes formed are treated with S_1 nuclease prior to collection on DEAE cellulose filter discs (Salzberg *et al.*, 1977; Maxwell *et al.*, 1978) for determination of the extent of duplex formation. The method overcomes the problem of secondary structure formation in rRNA molecules and yields a high percentage of duplex formation in homologous reactions. The obvious disadvantage of hybridization reactions performed in solution (i.e. pairing of DNA molecules) is minimized by the use of unlabeled rRNA in

excess of the radiolabeled cDNA.

Materials and methods

Yeast strains and culture conditions:

The yeast strains used and their sources are listed in Table 1. *Cryptococcus vishniacii* was grown at 10°C in GPYPi medium (glucose, 0.5%, peptone, 0.3%, yeast extract, 0.3%, potassium phosphate buffer pH 6.86, 10 mM). *Filobasidiella neoformans* and *Rhodosporidium toruloides* were grown at room temperature (24-26°C) in YM medium (glucose, 1%, peptone, 0.5%, malt extract, 0.3%, yeast extract, 0.3%). Primary cultures grown in a New Brunswick Scientific Company Gyrotory water bath shaker, were transferred to carboys containing 6 liters of the appropriate medium to an optical density (650 nm) of 0.20, and incubated with vigorous stirring and aeration at the appropriate temperature.

25S ribosomal RNA isolation and purification

Exponentially growing yeast cells were harvested, resuspended in TSM buffer (Tris-HCl, 10 m*M*; NaCl, 100 m*M*, $MgCl_2$, 30 m*M*, pH 7.4) containing 0.5% bentonite (Sigma Chemical Co.), and mechanically disrupted in a Braun cell homogenizer (Braunwill Scientific Co.) as described previously (Baharaeen *et al.* 1982). The broken cell suspension was centrifuged at 25 000 x g for 20 min to remove cell debris. Ribosomes were then pelleted from the supernatant at 90 000 x g for 12 h on 5 mL of a 15% sucrose solution in TSM buffer containing

5% ammonium sulfate. The ribosome pellet was resuspended in TSM buffer containing 0.2% SDS and phenol extracted (phenol was freshly prepared by saturation with Tris-HCl (10 m*M*)-EDTA (1 m*M*) buffer pH 7.0). Phenol was removed from the aqueous phase by ether extraction and the nucleic acids were alcohol precipitated from the aqueous phase of centrifugation at 20 000 x g for 20 min. The nucleic acids were dissolved in TSM buffer and treated with deoxyribonuclease I (Sigma Chemical Co., 100 $\mu g.mL^{-1}$) for 1 h and then with pronase (Calbiochem, 50 $\mu g.mL^{-1}$) for 2 h at 25°C. After phenol extraction, purified rRNA was alcohol precipitated and redissolved in 140 mM sodium phosphate buffer (pH 6.86). The classes of rRNA were separated on 5-20% sucrose gradients by centrifugation at 90 000 x g for 18 h. The 25S rRNA peak was pooled and sucrose was removed by gel filtration chromatography using Sephadex G-50.

Partial hydrolysis of 25S rRNA and recovery of fragments

The purified 25S rRNA was partially hydrolyzed by mild alkali treatment (sodium borate buffer, 500 m*M*, pH 9.2, 70°C, 12 min)(Engel and Davidson, 1978). The 400-600 nucleotide long fragments were separated on a 1.75% agarose Sub-Gel system (BioRad Laboratories) and recovered from the gel by the freeze-squeeze method of Thuring *et al.* (1975).

Synthesis of polyadenylic acid tails on 25S rRNA fragments

Ribosomal RNA fragments were treated with bacterial alkaline

phosphatase (Collaborative Research, 0.5 U.μg^{-1} RNA, 65°C, 1 h). The reaction was stopped by phenol extraction (ultrapure phenol, Bethesda Research Laboratories, was saturated with Tris-EDTA buffer (see above) and mixed with chloroform (1:1) just before use). The phenol was removed by ether extraction of the aqueous layer and the rRNA fragments were recovered by ethanol precipitation and centrifugation. The pellet was dried in a vacuum desiccator and redissolved in polynucleotide phosphorylase buffer (Tris-HCl, 50 m*M*, sodium citrate, 10 m*M*, $MgCl_2$, 5 m*M*, pH 8.0). Poly(A) was then synthesized by polynucleotide phosphorylase (0.4 U.μg^{-1} RNA) using rADP as substrate under the conditions which yielded short (about 10) nucleotide long tails (Engel and Davidson, 1978). The poly(A) tailed fragments were treated with phenol-chloroform and recovered as described above.

Synthesis of complementary DNA by reverse transcription

The poly(A) tailed 25S rRNA fragments were denatured at 100°C for 10 min and cooled rapidly on ice before cDNA synthesis. The reverse transcription mixture included 25 µCi of (methyl-^{3}H)-deoxythymidine-5'-triphosphate, tetrasodium salt (New England Nuclear; dried in a vacuum desiccator to remove ethanol), 10 µg of poly(A) tailed 25S rRNA fragments dissolved in 40 µL of deionized, glass-distilled water, 10 µL each of 10 m*M* dATP, dCTP, and dGTP and 2.5 µL of 10 m*M* TTP (Sigma Chemical Co.), 20 µL of reverse transcriptase buffer (Tris-HCl, 50 m*M*, KCl, 100 m*M*, $MgCl_2$, 10 m*M*, pH 8.3), 5 µL of 1 *M* dithiothreitol, 10 µL of 1 mg.mL^{-1} oligo $d(T)_{10}$ (Miles Laboratories), and 5 µL of 12 U.μg^{-1}

of AMV reverse transcriptase (Bethesda Research Laboratories). The mixture was incubated at 35°C for 10 min to assure A-T base pairing and then at 43°C for 70 min. The cDNA synthesis was terminated by the addition of 20 µL of 1 *M* EDTA solution. After alkaline hydrolysis of the rRNA strand (20 µL of 3 *N* NaOH, 55°C, 40 min), the cDNA was recovered on a Sephadex G-50 column equilibrated with 140 m*M* sodium phosphate buffer (pH 6.86) and prewashed with sheared (to 500 base pair long fragments), denatured calf thymus DNA to avoid binding of the trace amount of cDNA to the glass walls of the column. The specific activity of the cDNA was about 5.7×10^6 dpm.µg^{-1}. The yield of cDNA was between 60-70% of rRNA input.

cDNA - 25S rRNA hybridization

The cDNA-25S rRNA hybridization experiments were carried out in 500 µL polypropylene microfuge vials each containing 20 µg of 400-600 nucleotide long 25S rRNA fragments and 0.02 µg of homologous or heterologous labeled cDNA in 50 µL of 280 m*M* sodium phosphate buffer (pH 6.86). The contents were denatured at 100°C for 10 min and incubated at 65°C to E_{Rot} (the product of time in seconds and RNA concentration in moles of nucleotides per liter, corrected for phosphate buffer concentration by the method of Britten *et al.*, 1974) of 220 mol.sec.L^{-1}. For kinetic studies, vials were removed at various time intervals and the extent of duplex formation was determined. The contents of each vial were diluted to a total of 250 µL with S_1 nuclease buffer (Maxwell *et al.*, 1978), a 100 µL

aliquot was removed and dried on a DE-81 (Whatman, Inc.) DEAE cellulose filter disc. The remaining 150 µL were treated with S_1 nuclease (Bethesda Research Laboratories, 100 U per vial) at 37°C for 60 min. A 100 µL aliquot from the S_1 nuclease digestion was applied onto DE-81 filter discs and washed with 480 m*M* sodium phosphate buffer, glass-distilled water, and ethanol as described by Maxwell *et al.*.(1978). Dried filters were placed in 10 mL of a toluene based scintillation fluid and counted at the 2% error level in a Beckman LS 7500 liquid scintillation counter. Comparisons of counts on S_1 nuclease untreated (total) and S_1 nuclease treated (duplexes) samples determined the extent of duplex formation. The data for heterologous hybridizations were expressed as % actual and relative (to homologous reactions) binding with the homologous system normalized to 100%. The data were not corrected for 0.02% (actual binding) self-pairing of the cDNA.

Ultraviolet absorption - melting experiments

The extent of base pairing and base stacking in the intact 25S rRNA and 400-600 nucleotide long fragments was determined from their melting profile. Samples containing about 20 $\mu g.mL^{-1}$ of intact or partially hydrolyzed rRNAs in TSM buffer were denatured and the melting profiles were recorded in a Gilford Model 2000 spectrophotometer according to Mandel and Marmur (1968).

Results

Hybridization kinetics

The hybridization kinetics of homologous cDNA and 25S rRNA (from *C. vishniacii*) are shown in Fig. 1. The reactions were essentially complete at E_{Rot} of approximately 100 mol.sec.L^{-1} with 72% duplex formation; continuation to E_{Rot} of 220 mol.sec.L^{-1} increased the percent hybridization to 84%.

Heterologous hybridizations

The results of reciprocal hybridization of the three probes used in this study are presented in Table 1. The level of saturation of homologous reactions ranged from 78% to 84% heteroduplex formation. The largest differences observed in the members of the three pairs of heterologous hybridizations was 5.24% relative binding (between *C. vishniacii* and *R. toruloides*, Table 1).

Melting profile of the 25S rRNA

The results of thermal denaturation of the rRNA molecules (intact 25S rRNA and 400-600 nucleotide long fragments used for cDNA synthesis and hybridization studies) of *R. toruloides* are shown in Fig. 2. While the intact 25S rRNA shows a melting behavior characteristic of extensive base pairing and base stacking, melting was virtually absent in the fragments. This indicates that denaturation and partial alkaline hydrolysis used to prepare the fragments (see Materials and Methods) have eliminated these structures.

The total hypochromism (i.e. change in absorbance at 260 nm on unfolding of secondary and tertiary structures) for the intact 25S

rRNA is 0.14 A_{260} units corresponding to 25.58% hypochromism. Comparison of this value with that obtained for 5S rRNA of *Saccharomyces cerevisiae* (25.97%, Luoma *et al.*, 1980) indicates that the 25S rRNA is as extensively base paired and base stacked as 5S rRNA.

The T_m (the temperature at which the hypochromism has reached half of its maximum value) is about 61°C. This value is 5°C lower than that of the 5S rRNA of *S. cerevisiae* (Luoma *et al.*, 1980); this lower thermodynamic stability probably reflects a lower G + C content.

The melting range (i.e. the temperature range over which the hypochromism changes from 25% to 75% of its total value) reflects the amount of single stranded stacking that contributes to the total hypochromism (Boedtker and Kelling, 1967). Comparison of the melting range of the 25S rRNA obtained in our experiments (i.e. 15°C, Fig. 2) and that of the 5S rRNA (21°C, Luoma *et al.*, 1980) reveals that the 25S rRNA contains relatively less single stranded stacking.

Discussion

Phylogenetic studies involving DNA-rRNA hybridizations are complicated by the interference imposed by the rRNA secondary structures on proper pairing of the two nucleic acid strands. In our experimental approach, secondary structures are also of concern because they may interfere with cDNA synthesis. If the 25S rRNA fragments generated by the mild alkali treatment possessed double stranded regions, the synthesis of cDNA would continue until the growing chain confronted an already double stranded region.

Although a "nick translation" function has been proposed for reverse transcriptase (Verma, 1981), under the experimental conditions employed here, the cDNA synthesis is more likely to be terminated. If this were occurring then the cDNA synthesized would represent only the single stranded regions of the 25S rRNA.

From the melting profile of the 25S rRNA (25.58% hypochromism) and the observation that a duplex DNA molecule shows about 30% hypochromism upon complete thermal denaturation (Boedtker and Kelling, 1969), it can be concluded that about 85% of the 25S rRNA nucleotides are involved in secondary and/or tertiary structure formation. This value does not agree with the secondary structure model of the 25S rRNA of *S. cerevisiae* proposed by Veldman *et al.* (1981) in which about 2000 nucleotides of a total of 3393, corresponding to 59.6%, are paired. Part of this disagreement can be attributed to the presence of mismatches within the helices which are counted as single stranded even though they contribute to hypochromism. The value obtained from the model also does not consider the hypochromism caused by single stranded base stacking, known to be about 10% of total hypochromism (Boedtker and Kelling,1967; Cantor, 1968).

The absence of hypochromism in the 25S rRNA fragments used for cDNA synthesis (Fig. 2) clearly indicates that denaturation and alkaline hydrolysis have successfully rendered these sequences devoid of double stranded regions. Thus, secondary structure in the 25S rRNA fragments cannot interfere with reverse transcription. Since the cDNA and 25S rRNA fragments are again denatured in the

reaction vessel before hybridization, secondary structure should also not interfere with pairing in hybridization.

The random cleavage when fragments are generated by mild alkali treatment makes it unlikely that preferential copying for other reasons or incomplete transcription results in cDNA which is not representative of the whole 25S rRNA molecule. The homology values obtained can therefore be considered useful for the purpose of constructing phylogenetic trees.

This hybridization technique yields nearly double the duplex formation possible in homologous hybridizations by the membrane filter technique. The usefulness of this improved range depends upon the precision of determinations. Variations in reciprocal hybridization and standard deviations in replicate determinations indicate the precision with which homology values have been determined. The reciprocal hybridizations among the three probes (Table 1) have considerably lower variation (1.69 - 5.24% relative binding) than is seen in some studies employing the membrane filter technique (up to 21% in the study of Johnson and Francis, 1975). The average standard deviation of relative binding in 70 sets of hybridization reactions performed in triplicate in our laboratory (unpublished data) is $1.93 \pm 1.56\%$. The determination of heteroduplex stability ($T_{m(e)}$) required for added discrimination between homology values in the membrane filter technique does not seem to be necessary.

It is probable that some of the imprecision of the membrane filter technique results from the use of total DNA. Results are

reported as μg ^{14}C-labeled rRNA per 100 μg of DNA (de Ley and de Smedt, 1975). The genomes of *Escherichia coli* and *Bacillus subtilis* are 2.6×10^9 daltons (Cairns, 1963) and 2.0×10^9 daltons (Dennis and Wake, 1966) respectively. *B. subtilis* has at least 10 copies of the rRNA cistrons compared to 7 in *E. coli* (Kobayashi and Osawa, 1982); the binding per 100 μg by *B. subtilis* total DNA could therefore be nearly twice (5.00/2.69) that by *E. coli* total DNA. The use of cDNA avoids this complication, as well as that which may be posed by pseudogenes known to occur in fungal nuclear rRNA cistrons (Selker *et al.*, 1981).

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Table 1. cDNA-25S rRNA hybridization: Reciprocal reactions among three probes: *Cryptococcus vishniacii*, *Filobasidiella neoformans*, and *Rhodosporidium toruloides*.

Probe cDNA source	Source of unlabeled 25S rRNA					
	Cryptococcus vishniacii		*Filobasidiella neoformans*		*Rhodosporidium toruloides*	
	A[a]	R[b]	A	R	A	R
Cryptococcus vishniacii ATCC 36649	78.01 ±0.98	100.00 ±1.26	71.71 ±2.31	91.92 ±3.22	59.88 ±0.53	76.76 ±0.89
Filobasidiella neoformans NIH 12	78.45 ±2.01	93.61 ±2.56	83.81 ±1.09	100.00 ±1.30	65.13 ±1.65	77.71 ±2.53
Rhodosporidium toruloides NRRL-Y-1091	59.36 ±1.33	71.52 ±2.24	66.84 ±0.78	80.54 ±1.17	82.99 ±2.23	100.00 ±2.69

[a] % actual binding ± standard deviation (triplicate vials)

[b] % relative (to homologous reactions) binding ± standard deviation.

Fig. 1. Kinetics of hybridization of *Cryptococcus vishniacii* 25S ribosomal RNA with homologous complementary DNA.

Fig. 2. Ultraviolt absorbance - melting profiles (normalized to unit absorbance at 260 nm at 25°C) for *Rhodosporidium toruloides* intact 25S ribosomal RNA (●) and fragments generated by partial alkali hydrolysis (○).