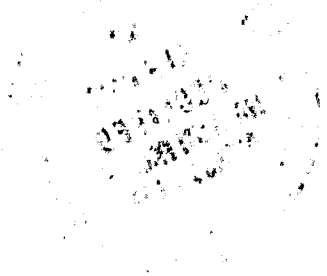
Progress Report (1 Dec 80-30 Nov 81) NAGW-26

MICROBIAL ECOLOGY OF EXTREME ENVIRONMENTS: ANTARCTIC DRY VALLEY YEASTS AND GROWTH IN SUBSTRATE-LIMITED HABITATS

Helen S. Vishniac Ph.D. (Principal Investigator)
Department of Microbiology
Oklahoma State University
Stillwater, OK 74078

Table of contents:



INTRODUCTION

The Dry Valleys of Antarctica are the coldest desert areas on this planet. The multiple stresses--temperature, moisture, and, for chemoheterotrophs, sources of carbon and energy--of the Dry Valley soils allow at best depauperate communities, low in species diversity and population density. The nature of community structure, the operation of biogeochemical cycles, the evolution and mechanisms of adaptation to this habitat are of interest in informing speculations upon life on other planets as well as in modeling the limits of gean life. Yeasts of the Cryptococcus vishniacii complex (Basidioblastomycetes) occupy a unique position in investigations of these topics, as the only known indigenes of the most hostile, lichen-free, parts of the Dry Valleys. We have, in the past grant year, developed methods for isolating these yeasts (methods which do not exclude the recovery of other microbiota), refined the definition of the complex, and established the importance of nitrogen sources as well as substrate-competition in fitness to the Dry Valley habitats.

ISOLATION OF ANTARCTIC MICROBIOTA

The basic dependence of ecological studies upon the isolation and characterization of indigenes cannot be exaggerated. Sophisticated and sensitive indirect techniques for investigating the biota and their activity in ecosystems cannot, when used alone, distinguish between the indigene and the stranded tourist, the living and the dead, biological and non-biological processes with certainty. Their use depends upon a learning spiral which incorporates increasing knowledge of both the physico-chemical and biological parameters in an ecosystem. In the Dry Valleys both climatic-edaphic factors and a sparse and unusual microbiota contribute to the difficulty of their interpretation. ATP, the most often used nucleotide indicator compound (reviewed by Karl, 1980), in soils near lichenized rocks, fails to show reasonable correlation with biomass (Friedmann et al., 1980). No known biochemical taxonomic indicator compound can discriminate at the level required to separate related microbiota at sites where preserved traces of intruded microbes exceed the contribution of living indigenes.

The original isolation of yeasts of the Cr. vishniacii complex, tho a direct result of the brilliantly creative approach of Dr. W. P. Hempfling to the problem of isolating from a completely unknown community, was fortuitous in the sense that the methods used gave few clues to the reasons for successful isolation (Vishniac and Hempfling, 1979b). These yeasts have now undergone selection by up to 8 years of laboratory culture on stock media requiring the use of relatively large inocula.

These yeasts, as degraders, must in some way be part of a larger microbiota. Indeed, micrographs of colonies in Antarctic soils (Uyuess and Vishniac, 1976) provide evidence that other microbes await isolation. For these reasons we have made the development of isolation procedures for yeasts(attempting to avoid the exclusion of other microbes) a primary aim .during the past grant year.

Fresh, continuously frozen, soil samples collected during the 1980-81 Antarctic season were contributed by Dr. E. I. Friedmann (Table 1). These samples were used to develop a method which did allow the isolation of microbes of every major taxonomic group except the protozoa. The technique of enation, the media used, our results and their implications are discussed below. Requirements for aeration and (optimally) a temperature of 10°C, dictated the use of a refrigerated shaker water bath for liquid cultures, relatively unencumbered surface exposure in a refrigerated incubator (unlit) for cultures on solid media and manipulation of samples on ice beds (in the absence of suitable cold room space near enough for routine use) or on dry ice (soil samples).

The isolated microbes have all been screened for psychrotolerant growth. The isolated yeasts have been used to validate the method. Our results indicate that interactions (biological and/or physico-chemical) between components of the soil sample and the medium were essential to the success of the method. The development of more flexible methods and of methods which are more adapted to use in the field, will require additional soil samples. We have no more of the most productive sample. The productivity of the remaining samples is suspect in any case, since our experience with frozen stock cultures and the experience of Dr. Hempfling's laboratory with frozen soils lead me to believe that a year in cold storage markedly reduces the chances of recovering yeasts.

Enation: Sprinkle plates with or without overlays, biphasic cultures, plates spread with material eluted from soil or from liquid enrichments all have their peculiar advantages. Sprinkle plates prepared in vials would be the ideal field system, calling for minimal additional equipment and prepared and shipped with the greatest ease. The only technique initially successful, and therefore pursued, was enation in liquid media followed by spreading on solid media.

The principle of enation differs from that of enrichment. An enrichment culture allows a minor component of a diverse population to become dominant by enhancing its relative growth rate, a component usually assumed to be substantially elutable from solid samples. An enation culture assumes that the desired organisms are bound to particulate matter in the sample, an assumption reasonable for many microbes (Bitton and Marshall, 1980) and certainly for encapsulated yeasts. What is desired is not the dominance of a particular population, but the enation or outgrowth of as great a variety as possible from the soil sample. The strategy most likely to maximize successful enation

Table 1. Soils sampled

A 801-3 Dec. 19, 1980. Linnaeus Terrace, on N slope of terrace facing Wright Valley, alt. ca. 1550 m, under a rock ledge, from which Dr. M. Hale collected lichens. (Hale 80/33-35) 77° 36' X, 161° 08' E.

a = surface-1 cm depth
b = 3 cm depth

A801-8 Dec 21, 1980. W part of Linnaeus Terrace, desert pavement, alt. ca. 1600 m. No lichens in immediate vicinity. 77° 36' S, 161° 02' E.

a = surface
b = 1 cm depth
c = 3 cm depth

A 801-25 Dec. 23, 1980. N slope of Oliver Peak above Linnaeus Terrace, ca. 1800 m alt. Collected by M. Hale (This sample was collected in a sterile bag with a piece of "native" rock, in the absence of sterile spoon. Depth was probably 0-3 cm.). 77° 36' S, 161° 03' E.

A 801-28 Dec 30, 1980. Saddle between Siegfried Peak and Siegmund Peak, 1520 m alt. In the vicinity of sandstone rocks colonized by lichens. 77° 35' S, 161° 46' E.

a = 1 cm depth
b = 3-4 cm depth

A 801-29 Dec. 30, 1980. Depression in valley W of Oliver Peak, 1430 m alt. Few lichens in vicinity. 77° 37' S, 160° 54' E.

a = 1 cm depth
b = 3 cm depth

A 801-30 Dec 30, 1980. Tyrol Valley, center, 1350 m alt. No lichens in vicinity. 77° 35' S 160° 37' E. (This is the area in which Dr. W. V. Vishniac collected.)

a = 1 cm
b = 2 cm

uses the smallest inoculum, minizing the interactions of microbiota with each other and of soil constituents with the enation medium, and the least selective medium and requires sampling before growth is visible (as well as for selective isolations). We have used most successfully about 0.5 g of soil in 25 ml of medium (in a 125 ml Erlenmeyer flask), sampled 1 week after inoculation.

Media: The failure of other investigators (most recently Atlas et al., 1978) to find unique yeasts in the Dry Valleys soils by conventional methods, the demonstrated inadequacy of these methods in other habitats (Fell, 1974), and the paucity of recognizable substrates for yeasts in the experiments of Dr. Hempfling's laboratory suggested diluting media on which Cr. vishniacii was known to grow well. Growth of these yeasts was most rapid and abundant on GPYPi of complex media; more rapid and with higher yields on GPYPi than on synthetic media such as amended Wickerham's or Y-1 (Vishniac and Hempfling, 1979a). Assuming therefore that complex media had the energy-sparing effects commonly seen in microbial growth, as well as the advantage of encouraging possible auxotrophs and damaged cells, we used dilutions of GPYPi in all preliminary experiments (i.e., including with techniques other than enation). The successful enation medium was E-1, in which the concentration ingredients of GPYPi were reduced to glucose 0.05%, peptone 0.05%, yeast extract 0.025%, KPi (pH 6.86) 1 mM (a version consisting of the organic ingredients at 2X with 10mM KPi failed) and supplemented with NaCl 50 mM, $MgSO_4 \cdot 7H_2O$ 0.2 mM, trace metal solution (diluted 1:10 from that used in Y-1) and with or without NH_4Cl 2 mM, with or without streptomycin 15 $\mu g\ ml^{-1}$ and penicillin G 100 $\mu g\ ml^{-1}$ (a version lacking the inorganic additions failed).

E-1 does not allow adequate growth of colonies for counting and isolation. After trials which included a number of conventional media (malt extract agar-MEA, Sabouraud's Dextrose agar -SDA, Cornmeal Agar-CMA) as well as GPYPi we used 2MC, a Medium for Counting developed in our laboratory. 2MC contains glucose 0.2%, peptone 0.1%, yeast extract 0.05%, KPi (pH 6.86) 1.0 mM, NH_4Cl 2.0 mM, $MgSO_4 \cdot 7H_2O$ 0.2 mM, Trace metals 1:10 and agar 1.8%.

Microbiota found: The combination of E-1 and 2MC proved felicitous beyond our expectations. The results are given in Table 2, including the few (non-yeast) isolates recovered by other techniques than enation (in some cases on slightly different media). It is noteworthy that no site, and only one soil sample (A801-28b), was sterile. Enation (though not necessarily for the reasons posited) was required. Yeasts could be eluted (ie, were present in washings or initial samples) only when inocula approaching 1 gram were used. Bacterial activity is better

Table 2. Productivity of Antarctic Soil Samples Shaken at 10° in Enation Medium 1*

Soil Sample	Mass**	Bacteria (initial)	spent pH (init.) (5.6 uninoc.)	Yeasts	Other
A 801-3a	0.260^{7} g	(Antibiotics)	ND	Biotype 17	Algae: 3aA1 (sprinkle plate)
	0.938^{5} g	"	6.53	Biotypes 19, 20	Fungi: 3aFF2-7 (other media)
	0.757^{7} g	ND	7.04	Biotype 19	
-3b	1.112^{3} g	(Antibiotics)	6.60	0	
	1.139^{2} g	ND	6.67	0	3bFF9
A 801-8a	ND	ca. 126 cfu g^{-1}	7.62	0	
	0.43 g	ND	7.27	0	8aFF15
	0.22 g	(Antibiotics)	6.54	0	
-8b	0.41 g	249 cfu g^{-1}	7.63	0	
	0.32 g	ND	7.37	0	8bFF16
	0.43 g	(Antibiotics)	6.56	0	(dematiaceous col., not isolated)
-8c	1.07 g	3.7 cfu g^{-1}	7.42	0	
	0.31 g	ND	6.59	0	
	0.25 g	(Antibiotics)	6.50	0	
A 801-25	0.72 g	0.0 cfu g^{-1}	7.22	0	
	0.27 g	0.0 cfu g^{-1}	6.57	0	25FF18, 19
	0.29 g	(Antibiotics)	6.57	0	25FF16.5, 17.5
A 801-28a	0.59 g	0.0 cfu g^{-1}	7.85	0	
	0.21 g	0.0 cfu g^{-1}	6.55 (6.47)	0	28aFF20
	0.20 g	(Antibiotics)	6.51 (6.40)	0	

(con't)

(Table 2. continuation)

-28b	0.96 g	0.0 cfu g^{-1}	6.65	0	
	0.19 g	0.0 cfu g^{-1}	6.18 (6.39)	0	
	0.20 g	(Antibiotics)	5.90 (6.40)	0	
A 801-29a	0.68 g	93 cfu g^{-1}	7.91	0	29aA15-19
	0.20 g	62 cfu g^{-1}	6.72 (6.34)	Biotype 21	29aA10
	0.33 g	(Antibiotics)	5.83 (6.31)	0	29aA11
-29b	0.41 g	171 cfu g^{-1}	7.60	0	
	0.31 g	128 cfu g^{-1}	6.50 (6.42)	0	29bA12
	0.36 g	(Antibiotics)	6.22 (6.41)	0	
A 801-30a	0.236 g	(Antibiotics)	ND	0	[washings:30aFF10]
	0.8185 g	"	6.41	0	30aFF11,14,13
	0.8608 g	(Fungal overgrowth)	7.53	(Fungal overgrowth)	30aFF1
-30b	0.7589 g	(Antibiotics)	6.68	Biotype 18	
	0.6281 g	0.0 cfu g^{-1}	6.48	0	

*Bracketed isolates made with other techniques or media. ** Dry mass of soil recovered at conclusion of experiment; inoculum estimated to be around 1.5-2X depending upon clay content. pH of spent media taken after 14 days of incubation.

indicated by elevated final pH (in Table 2) than by initial counts. Bacteria were enate from all soil samples (except A 801-28b).

Algae: The recovery of algae was surprising, since the refrigerated incubator used was not provided with lights and the shaker water bath was hooded during most experiments. The algal cultures which appeared free of bacteria (3aA1, 29aA10, 11 29aA15) were turned over to Dr. E. I Friedmann (pursuant to an agreement as to the "ownership" of isolates from the soil samples), 3aA1, 29aA10, 11 were identified by Dr. Friedmann as possibly Chlorosarcina, certainly Chlorosarcinaceae-- common soil algae, previously unreported from the Antarctic. The rubbery colonies and failure to grow at room temperature, the low light requirements of these algal x isolates argue for the possibility of activity in the Antarctic soils, though their dependence upon water for sporulation argues against indigenicity in the Dry Valleys; the rubbery colonies taken with their common occurrence elsewhere suggests frequent transport into the area. The algae which failed to come through isolation procedures appeared similar to these. Isolate 29aA15 was identified by Dr. Friedmann as a Trebouxia or Pseudotrebouxia, a lichen phycobiont. The unprecedented isolation of this alga from soil (with "few lichens in vicinity") provides evidence for the (reasonable) postulation that exfoliated lichen material both disseminates and adds to organic matter available.

Bacteria: More than 98 bacterial strains survived the isolation procedure (10°, 2MC). Although these were picked as representing colony types, the dominant colony type (hyaline, blocky) from some enations was not isolated-- these strains cannot be said to represent the procaryote biota of the soil samples. From the 32 strains which grew well enough at low temperatures (4/25° C screen), 19 have been lyophilized (chosen after Gram stain examination of cell morphology) for transmission to Dr. Peter Hirsch (pursuant to agreement with Dr. E. I. Friedmann) for identification. Of these, 14 were red or pink, a proportion roughly that of the total bacterial isolates and to be expected from the studies of others. The temperature preferences shown by these isolates make it possible that they are active in the Antarctic. It will be difficult to demonstrate that any are indigenes. Their coloring suggests carotenoids, well known to protect during aerial transport, but not typical of soil bacteria living below the surface. Bacterial counts required incubation for at least 4 weeks at 10°, notably longer than the yeasts.

Filamentous fungi: Moniliaceous fungi isolated (30aFF13, 28aFF20) were discarded after temperature screening showed clear adaptation to Oklahoma room temperatures. 30aFF1 is Mucor racemosus fide Dr. R. K. Benjamin (deposited in the Rancho Santa Ana Botanic Garden collection as RSA 2578), a common soil fungus (Zygomycete) notable

for the production of chlamydospores, and a wide growth temperature range. Although this fungus produced large colonies in the enation flask and overgrew plates of samples from this flask, the inability of hyphal fungi to survive freezing and the reputation of mucoralean fungi as "sugar fungi" make it highly unlikely to function in the Dry Valleys-- its presence can be accounted for by aerial transport of chlamydospores from common population centers elsewhere. This is the only occasion on which filamentous fungi posed technical problems in our experiments, a testimony to absence of usual soil microflora from Antarctic soils. The remaining fungal isolates were dematiaceous hyphomycetes or "black yeasts"(ie non-budding, but barely hyphal). Even after 4 weeks of incubation, the colonies were barely pinpoint. We did not therefore attempt isolation of more than a few, though we have formed the impression that they are ubiquitous in Antarctic soils. Isolates growing optimally at 25°C (30aFF10, 8aFF15, 8aFF16) were given to Dr. Friedmann (for examination as possible mycobionts by Dr. Hale) and sent to Dr. Bryce Kendrick for possible identification. Authentication would be of value as confirming their origin in temperate soils, suspected from my (inexpert) identification of them as Torula humicola, Rhizoctonia solani, and "X, not Rhizoctonia" respectively, as well as their temperature preferences. Isolates with 10° temperature optima were "black yeasts" falling into two groups: 3aFF3, 4, 6 failing to grow at 25°C, prefering GPYPi to 2MC, PDA (Potato Dextrose Agar) and SDA and 3aFF2, 5, 7 and 30aFF11, 14 growing better at 25° than at 4°, prefering PDA. (30aFF12, 25FF17.5 and 25 FF 19 are in the process of examination). These putative Phaeococcus spp. , though possibly active in Dry Valley soils, could be considered constant imports from milder climes (Phaeococcus spp. being common in soils world wide) were it not that assimilation tests performed with x 3aFF6 and 3aFF7 indicated non-identity with the "black yeasts" studied by de Hoog (1977).

Yeasts: To ensure adequate sampling, 100 yeast isolates were screened. They proved to belong to the following biotypes:

Biotype 17 : 3aY1-14D, type 3aY1 (3aY14B retained). nitrate negative, L-arabinose, cellobiose, glucuronate, 2-ketogluconate(weak), maltose, melezitose, L-rhamnose (slow, weak), sucrose, trehalose, succinate (slow, weak) assimilated. Undescribed.

Biotype 18: 30bY15-42, type 30bY33 (30bY15 retained). nitrate positive, gamma aminobutyric acid (?confirm), glucuronate(slow, weak), 2-ketogluconate, maltose, melezitose (?confirm), L-rhamnose (slow), salicin (?? confirm, late agar contamination), succinate, sucrose, trehalose, xylose assimilated. ?biotype 5 or undescr.

Biotype 19: 3a43-62, 90-91, 94-95 from subsample 2, antibiotics used;3aY63-89 from subsample 3, no antibiotics;type 3aY86 (cultural variants retained). nitrate positive, cellobiose, maltose, alpha methyl-glucoside, melezitose, sucrose, trehalose, xylose assimilated. Undescribed.

Biotype 20: 3aY92, 93. nitrate negative, gamma aminobutyric acid (? confirm), L-arabinose, 2-ketogluconate, glucuronate, L-malate (?confirm), sucrose, trehalose, xylose assimilated. Undescribed.

Biotype 21: 29aY98,99. Not an indigene, barely grows at 10°C, 37° positive. Trichosporon sp. (?cutaneum) (T. cutaneum has been reported from Antarctica previously).

Leaving biotype 21 out of our discussion, these isolates grow well on 2MC but require adaptation to other agars, if they will grow at all. Some fresh isolates were inhibited by the yeast extract of GPYPl, some by the peptone of GPPl (yeast extract omitted), and additionally by the low pH of SDA, MEA. It is obvious then why previous investigators failed to find these yeasts by standard methods. What is more interesting is the relative absence of yeasts isolated by previous investigators from our collection. Since neither the media we have used nor the conditions of culture should have excluded them(vd. Cr. laurentii in Vishniac and Hempfling, 1979b, Trichosporon above and unreported experience in my laboratory), it appears that tourists do not survive well enough through the process of collection and frozen transport to outnumber indigenous yeasts. The yeast biota of the Dry Valleys is NOT a selected, less populous version of that found in other soils! Indeed, its discovery could serve as a paradigm for the investigation of unknown ecosystems.

The greater abundance and diversity of yeasts in A 801-3was expected-- the presence of lichens in the vicinity indicates more favourable climatic conditions and contributes available carbon to adjacent soils. We cannot presently account for the failure to recover yeasts from A801-28, 29 (lichens also present in the vicinity), or from A 801-8, 25. A prelimary attempt to do so by means of recovery experiments was inconclusive. It is possible that tightly packed clay soils (A 801-25) are physically unsuitable for obligately respiratory microbes. The presence of lichens was not correlated with bacterial count (as a crude indicator of fertility)in A801-28 (lichens, no countable bacteria-- while A 801-8 with no lichens, contained up to 249 cfu g^{-1}).

Method validation: Medium E-1 does not, however, entirely meet our specific ations--it. selects among the biotypes of Dry Valley yeasts . The presence of antibiotics had no obvious untoward effects, merely suppressing the growth of bacteria when desired. The presence of 2 mM NH_4Cl suppresses the growth of 30bY33 (Biotype 18) in a density dependent fashion; omitting this nitrogen source makes

enation dependent upon available nitrogen in the soil sample. Dry Valley soils are rich in nitrate-N (see Friedmann and Kibler, 1980). (Samples for soil analysis were not collected from these sites.) Nitrate-negative yeasts such as 3aY1 ((Biotype 17) and 3aY92 (Biotype 20) are selected against if NH_4Cl is omitted. Biotype 17 was enate in E-1 with 2 mM NH_4Cl. That Biotype 20 was isolated at all is presumeably the result of our systematic picking of many colonies from each experiment and the slightly more watery colonies it forms on MEA (trials of isolation media were still going on at that time; MEA is as poor a medium for Biotype 20 as for the other Dry Valley yeasts). Other soil interactions with this medium are presumed to have assisted in the enation of Biotype 18 (3aY86)-- this biotype has grown relatively poorly in all the media we have tried to date, frequently showing stress. The viability of exponentially growing cells of 3aY86 dropped below the limit of detection (ca. 90%) during dilution for recovery experiments. Figure 1 gives the growth curve of a mixed population of Dry Valley yeasts (enation flasks with sterile spent soil samples 8b, 25, 29b and no soil, inoculated with an estimated 7×10^3 cfu ml^{-1} of each biotype) in Medium E-1 with 2 mM NH_4Cl. Biotypes 17 (3aY1) and 20 (3aY92) increased from ca 57% of the initial population (28 colonies picked from initial and final plates of 25 and no soil) to 93-96% of the final population, at the expense of Biotype 18 (30b33). Figure 2 contrasts the density dependent inhibition of 30bY33 by E-1 with NH_4Cl with the growth (separately) of 3aY1 in this medium. Surprisingly, the growth rates of Figure 1 ($k = 0.045, 0.060, 0.038, 0.041$) were in the range seen in enation experiments (Biotype 17 $k=0.042$; Biotype 18 $k=0.053$), suggesting that cells held in frozen soil for up to three months were as ready to go as were exponentially growing cells after dilution in the absence of temperature shock!

DEFINITION OF THE CR. VISHNIACII COMPLEX

A ecologically useful definition of the Cr. vishniacii complex should be founded in a nomenclature which has predictive value-- that is, indicates evolutionary origin and allows experimental contrast with congeners from other habitats-- and should include means of identification. During the past year we have applied the techniques of molecular taxonomy to Biotypes 1-16 of Cr. vishniacii, clarifying relationships at species and varietal levels (Baharaeen and Vishniac, in press; Baharaeen and Vishniac, Appendix A). These biotypes constitute a species complex comprising 7 species (DNA:DNA homology less than 52%), probably having a common ancestor with Cr. bhutanensis (a yeast of Bhutan) and therefore originating outside of the Antarctic. The species Cr. vishniacii (G+C mol % 54.52-55.48, DNA:DNA homology greater than 59%) appears to have undergone

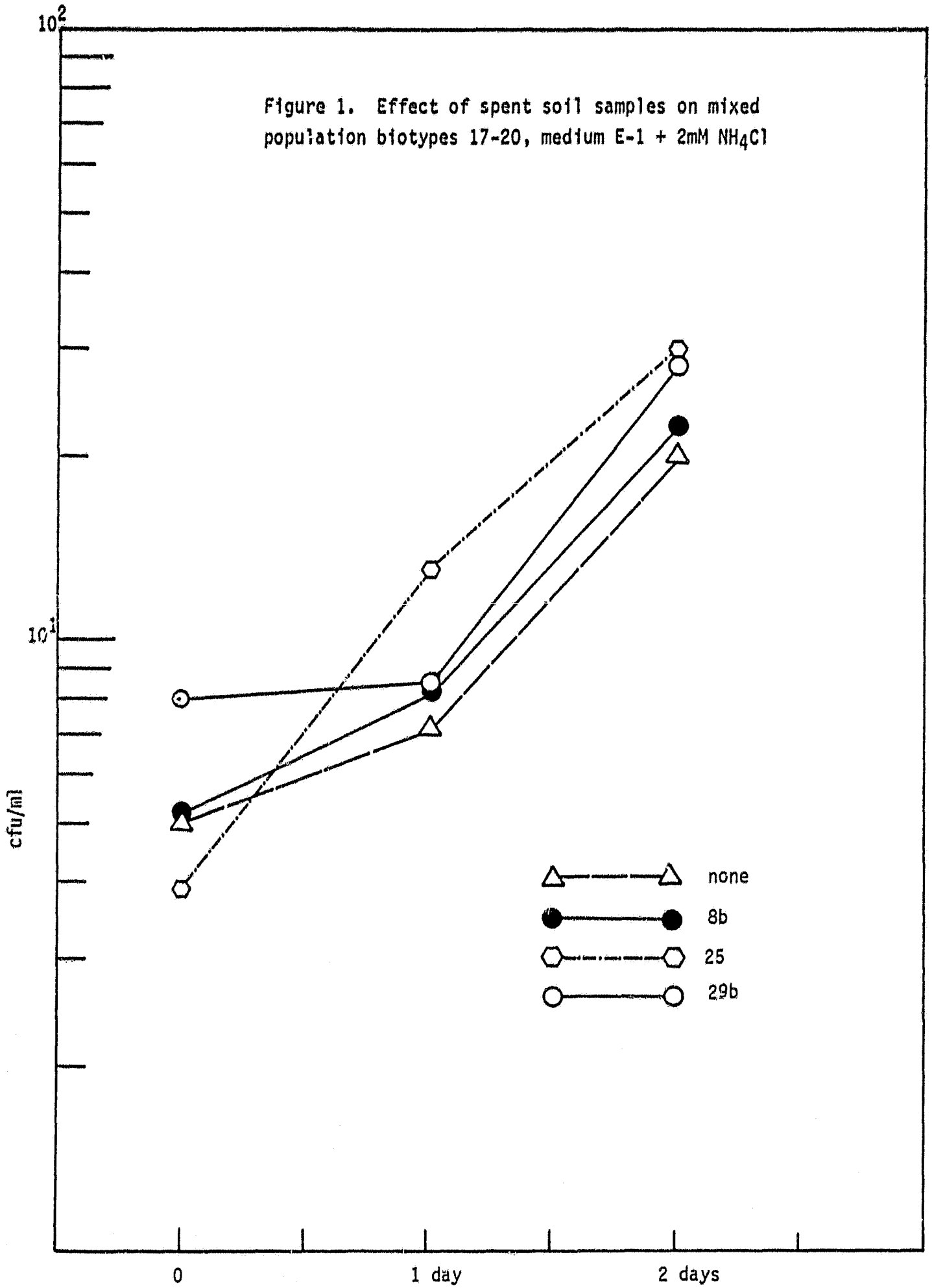
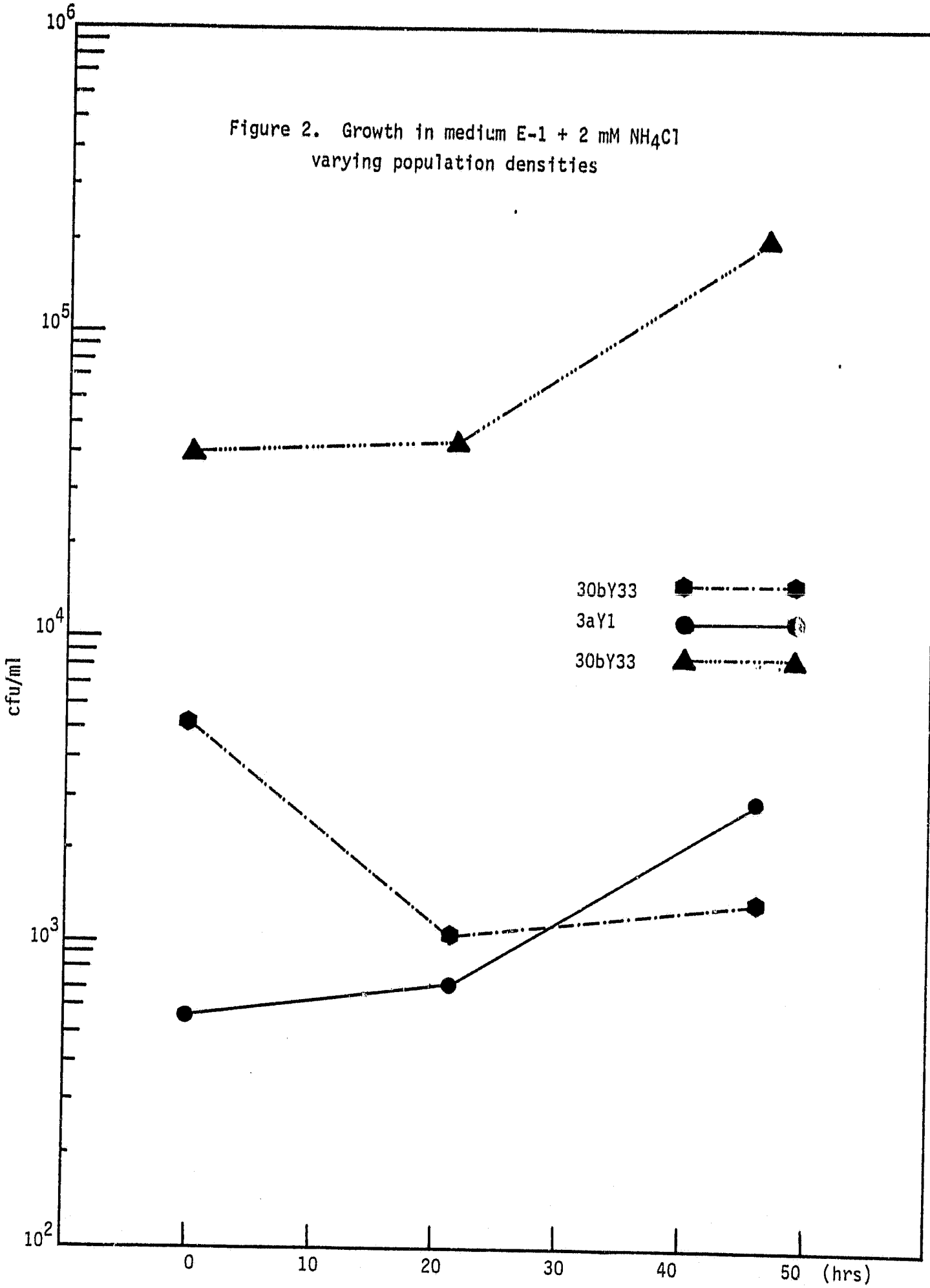


Figure 1. Effect of spent soil samples on mixed population biotypes 17-20, medium E-1 + 2mM NH_4Cl

Figure 2. Growth in medium E-1 + 2 mM NH_4Cl varying population densities



further evolution in the Dry Valleys, differentiating 7 varieties.

The use of these genetic definitions in site analysis (Table 3) confirms the conclusions reached through phenetic grouping of biotypes (Vishniac and Hempfling, 1979b), the greatest diversity being seen in soil from the foot of a glacier (therefore with most reliable moisture access) and highest carbon content rather than the soil allowing greatest tourist survival.

The species and varieties defined genetically are not easily identified by other means. Species are identifiable by assimilation reactions only if keyed at the varietal level; the standard reactions do not separate Cr. vishniacii varieties biotypes 4 and 5 or biotypes 10 and 11. The failure of a scheme used with fair success in identifying Ascomycetous yeasts to distinguish Basidiomycetous ones has been noted before (Rhodotorula graminis is the anamorph of two species of Rhodosporidium). The desireability of finding new reactions for the identification of Basidioblastomycetes is obvious. The use of molecular genetic techniques is cumbersome and expensive. The description of our new isolates will require the use of these techniques.

The definition of genera and higher taxa in the Basidioblastomycetes is not based on molecular genetics and is, in its present state, nearly useless to microbial ecology. Few studies of DNA:rRNA homology have been performed with yeasts but it seems likely (see Bicknell and Douglass, 1970) that such studies can extend molecular taxonomy above the species level in the Basidioblastomycetes, as in other biota. S. Baharaeen is completing his Ph. D. thesis research by examining DNA:rRNA homology in and between Basidiomycetes which bud from one pole (Cryptococcus spp., Malassezia spp., Phaffia), from two poles (Rhodotorula, Leucosporidium ("Vanrija") scottii) and are multipolar (Vanrija spp). Melissa Morgan (M. S. candidate) has begun a study of budding morphology in Basidioblastomycetes kindly sent by Dr. C. P. Kurtzman and collected from other sources. The result should allow identification of the congeners of the Cr. vishniacii complex.

NICHE PARAMETERS IN THE DRY VALLEYS

The Cr. vishniacii complex is not more psychrophilic, nor more osmotolerant, nor does it have any other character which would explain its occurrence in Dry Valley soils to the exclusion of other ~~xxxx~~ indigenous yeasts. We have suggested that superior ability to compete in substrate-limited habitats could account for their dominance, i.e., that the yeasts of this complex have a lower J value (Hanson and Hubbell, 1980) than say Leucosporidium scottii, the yeast most common in better provisioned Antarctic sites (di Menna, 1966).

Table 3. Distribution of the Cr. vishniacii complex in Dry Valley soils

Sample no.	Cr. vishniacii var.: wolfii	vladimirii	4	5	vishniacii	10	11	Cr. spp.: lupi	6	9	14	15	16
202			+				+	+					
302			+	+		+		+			+		+
303*	+	+	+		+			+	+	+		+	
304					+			+					
306**								+	+				
309			+										

* foot of glacier; 17.8 mmol C g^{-1}

** site allowing greatest survival of tourists

The determination of $J_{glucose}$ requires that the limiting concentrations of glucose not change significantly , a condition which can only be met in chemostat culture. Penelope Boston (a U. Colorado Ph. D. candidate) studied the use of a commercial chemostat (New Brunswick Bioflo C30) to determine appropriate parameters for L. scottii and for competition experiments between the type of L. scottii alpha (LSA) and the type of Cr. vishniacii (CV7) in my laboratory during the summer of 1981. A suitable reservoir medium and differential plating system was developed. LSA was able to maintain steady state at a dilution rate (= μ) of 0.019 with a $Y_{glucose}$ of around 125 mg dry weight and 94 mg protein (Bio-Rad) per mM. This dilution rate is presumeably near the minimum specific growth rate of LSA (15% of μ_{max} 0.13, GPYPi, 10°) but not of CV7 (equivalent μ_{max} 0.066; μ_{min} 0.006) . We therefore introduced batch grown cells of CV7 (in excess because of the possibility of decimation by culture shock and the lower growth rate of CV7; a second chemostat was not available) to determine whether this dilution rate (the lowest reliably maintained by Bioflo C30 in our experience) was below the crossover point characterizing dilution-rate-dependent displacement in aquatic bacteria (Jannasch, 1977). At the (premature, technical problems) termination of the experiment both competitors were present. CV7 did experience the anticipated lag, the large, relatively thick-walled cells of batch culture apparently dying as well as undergoing (multiple!) budding to produce much smaller, more elongated cells. LSA underwent a marked change in cell size on being down-shifted from exponential growth or in the chemostat. Our results are therefore inconclusive. The commercial chemostat proved to have several drawbacks which require remediation before it will be worthwhile to repeat the experiment. Chemostat experiments will be necessary in the future in order to obtain cells adapted to constant, substrate-limited, conditions. In the meantime, I believe we have found a way to demonstrate the existence of substrate-competition, and its role in the exclusion of L. scottii , which is both less time consuming than chemostat experiments at low D and temperature and technically simpler.

Resources other than substrate may be limiting in Dry Valley soils. In most soils ammonium-N, in some soils phosphate may be below the limit of detection. Ammonium-N is present in soils largely as a result of the action of "ammonifying"bacteria upon organic debris, debris which is notably lacking in the Dry Valleys except downwind of stromatolite producing lakes (reviewed by Parker et al., 1981) and lichenized rocks (review by Friedmann, In Press). These factors probably do not exclude L. scottii (a nitrate utilizer), but may operate in niche definition among indigenes, allowing the success of Brand X in one location but Brand Y in another. The type of nitrogen resource available appears to do so. In the course of conducting batch competition experiments with various nitrogen sources, we have found circumstantial evidence of substrate-limited competition between yeasts from soils

near to and far from lichenized rocks.

These experiments were conducted with 3aY1 (the yeast first isolated from A 801-3, under lichenized rock ledge, Linnaeus Terrace) and 30bY33 (from Tyrol Valley, no lichens, the home of the Cr. vishniacii complex). Exponentially growing cells of both isolates were diluted to give ca. 3.5×10^2 cfu ml^{-1} of each (7×10^2 cfu ml^{-1} total) in flasks containing enation base 3 (E-3) with various nitrogen sources (2 mM NH_4Cl, KNO_3, NH_4NO_3 or NH_4Cl + glutamate), or 2MC(glucose 0.2% rather than 0.05%, N as 0.1% peptone, 0.05% yeast extract) + NH_4Cl. E-3 contains glucose and inorganic constituents only of E-1+ HBO_3 5 µg /100 ml, KI 1 µg/100 ml and 1:10 Wickerham's vitamins (without riboflavin and folic acid). Samples were plated on media containing glucose (total count), cellobiose (3aY1) and xylose (30bY33) as sole substrates. The results are shown in figures 3-7. A comparison of the growth of 3aY1 in figures 3 and 5 (limiting factor 0.05% glucose) with that in figure 6 (glucose + 0.011% glutamate as substrates) and figure 7 (glucose 0.2% + est. 0.015% glutamate in peptone) suggests strongly that 3aY1 is unable to compete with 30bY33 (from the more impoverished habitat) for glucose. We plan competition experiments of similar design between L. scottii and the Cr. vishniacii complex yeasts (of which 30bY33 appears to be one).

The exhaustion of 3aY1 (nitrate negative) at 43 hours in media without added ammonium-N (figure 4) was expected. The increased lag phase (to ca. 40 hours) of 30bY33 in the presence of 2 mM NH_4Cl is evident when Figures 3 and 5 are compared to Figure 4 (nitrate-N). The growth rate of 30bY33 becomes negative in 2MC (Figure 7) but the addition of organic -N in the form of glutamate alone (Figure 6) ameliorates the effect of 2mM NH_4Cl. Ammonium-lag is completely abolished by glutamate at this concentration if NH_4Cl is lowered to 0.2 mM (Figure 8. 30bY33 alone, NH_4Cl 0.2 mM + glutamate 2 mM, HBO_3 and KI omitted from E-3). One may speculate that the use of ammonium-N (the universally preferred inorganic source for yeasts (Phaff, 1978)as for other fungi) places a greater demand on anapleurotic reactions which are limited at low population densities by CO_2 availability. Since glutamate is unlikely to occur in situ unaccompanied by other amino acids and forms of organic nitrogen, 30bY33 seems specialized for an asocial existence, life in a desert environment rich in nitrate and lacking other inhabitants which could produce organic-N and from it ammonia. 3aY1 is by contrast a social yeast. The subsequent appearance of (nitrate-positive) Biotype 19, with a minor component of (nitrate-negative) Biotype 20, in soil sample A801-3 suggests succession during frozen storage.

We wish to thank all those who made this work possible: NASA, Dr. E.I. Friedmann (soil samples), graduate students Siavash Baharaeen, Penelope Boston and Melissa Morgan, colleagues who contributed advice Dr. J.A. Bantle and Dr. U. Melcher, technicians Mary Gray and Teri McElroy, and dishwashing work/study students J.D. Walker and Amelia Gormley. We are also grateful for the contribution of expertise from Dr. R.K. Benjamin and cultures from Dr. C.P. Kurtzman and Dr. Kwon-Chung.

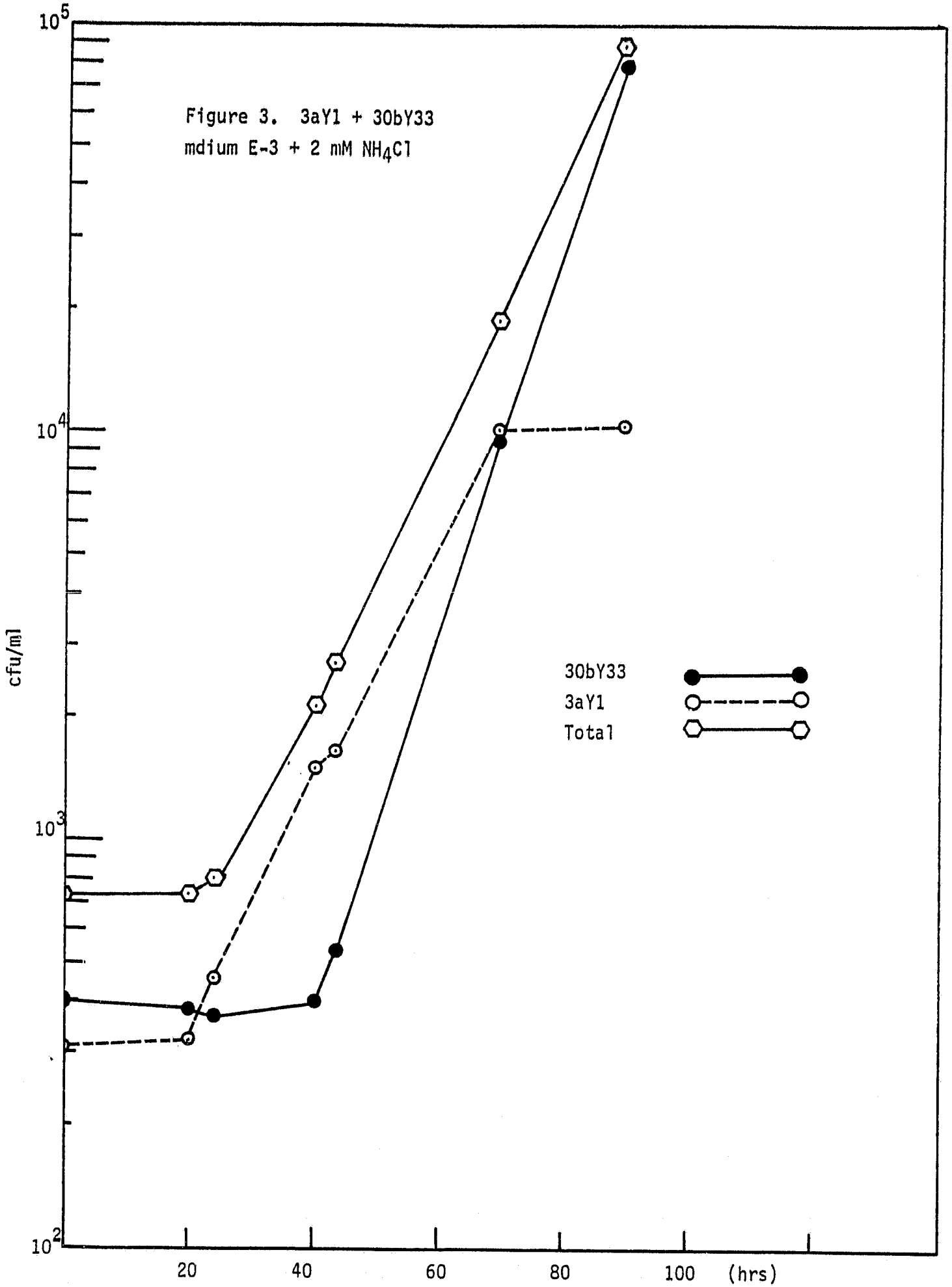


Figure 3. 3aY1 + 30bY33
mdium E-3 + 2 mM NH_4Cl
10^5
10^4
10^3
10^2
cfu/ml
20
40
60
80
100
(hrs)
30bY33
3aY1
Total

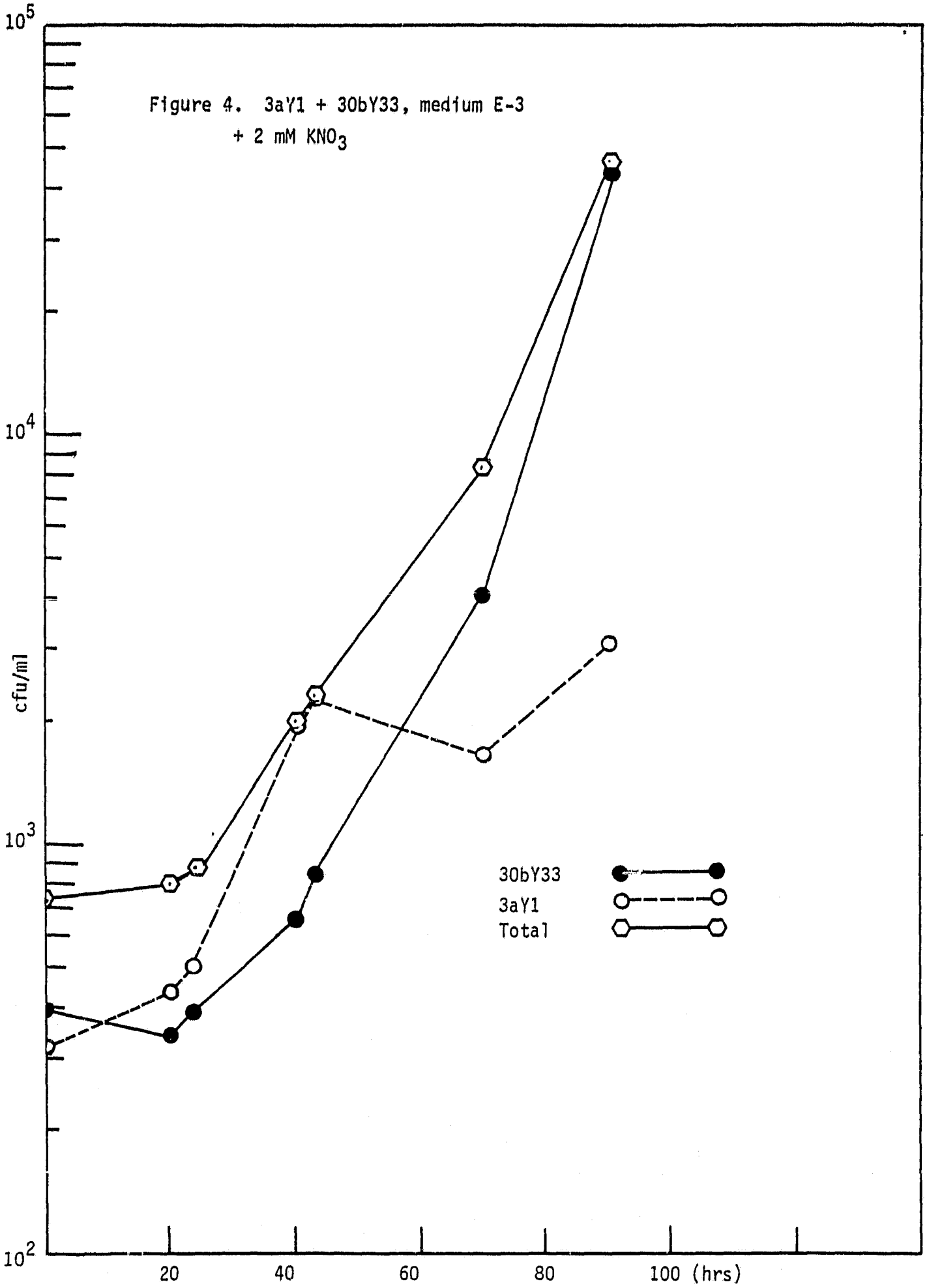


Figure 4. 3aY1 + 30bY33, medium E-3
+ 2 mM KNO3
10^5
10^4
10^3
10^2
cfu/ml
20
40
60
80
100 (hrs)
30bY33
3aY1
Total

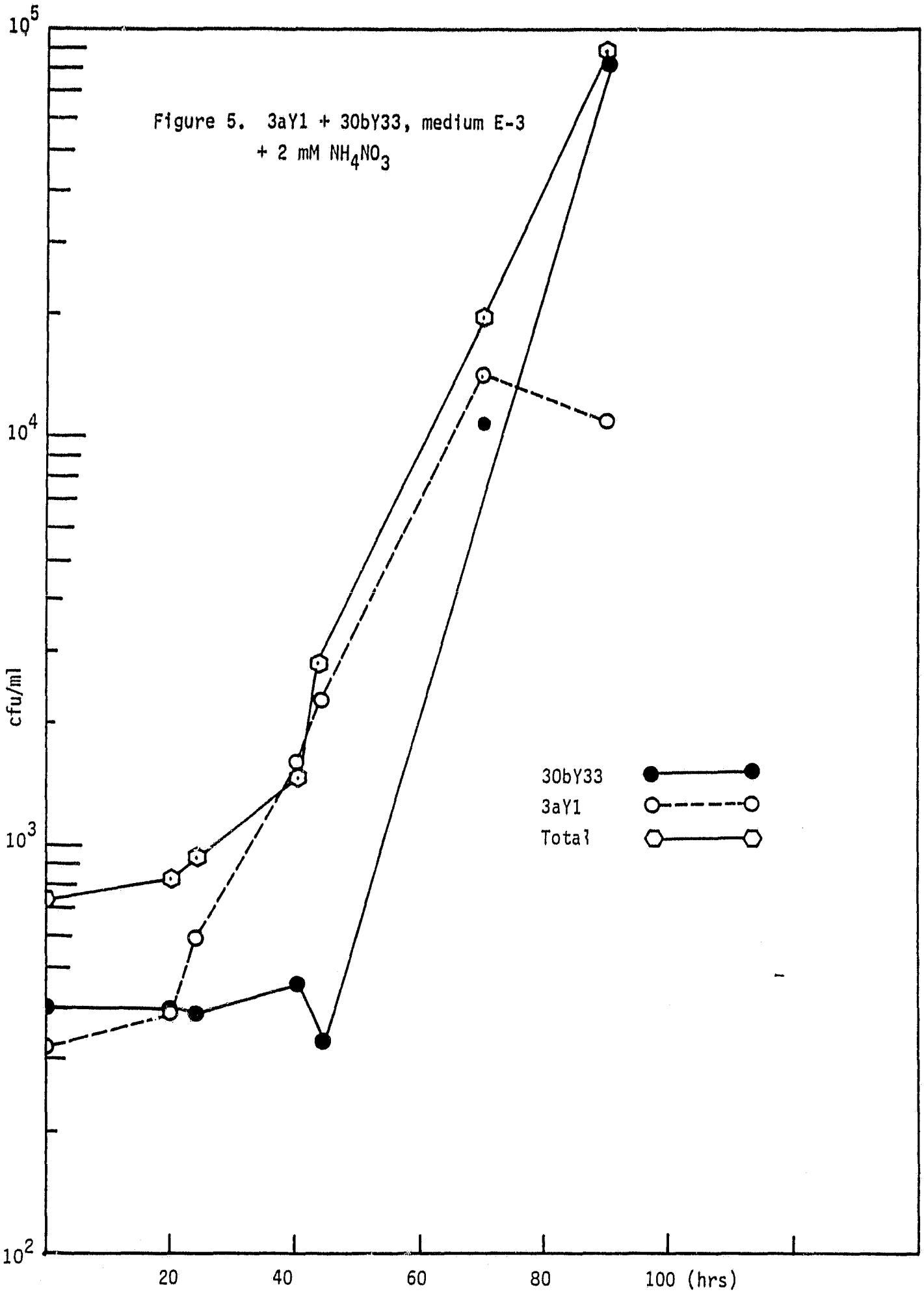


Figure 5. 3aY1 + 30bY33, medium E-3
+ 2 mM NH_4NO_3
10^5
10^4
10^3
10^2
cfu/ml
20
40
60
80
100 (hrs)
30bY33
3aY1
Total

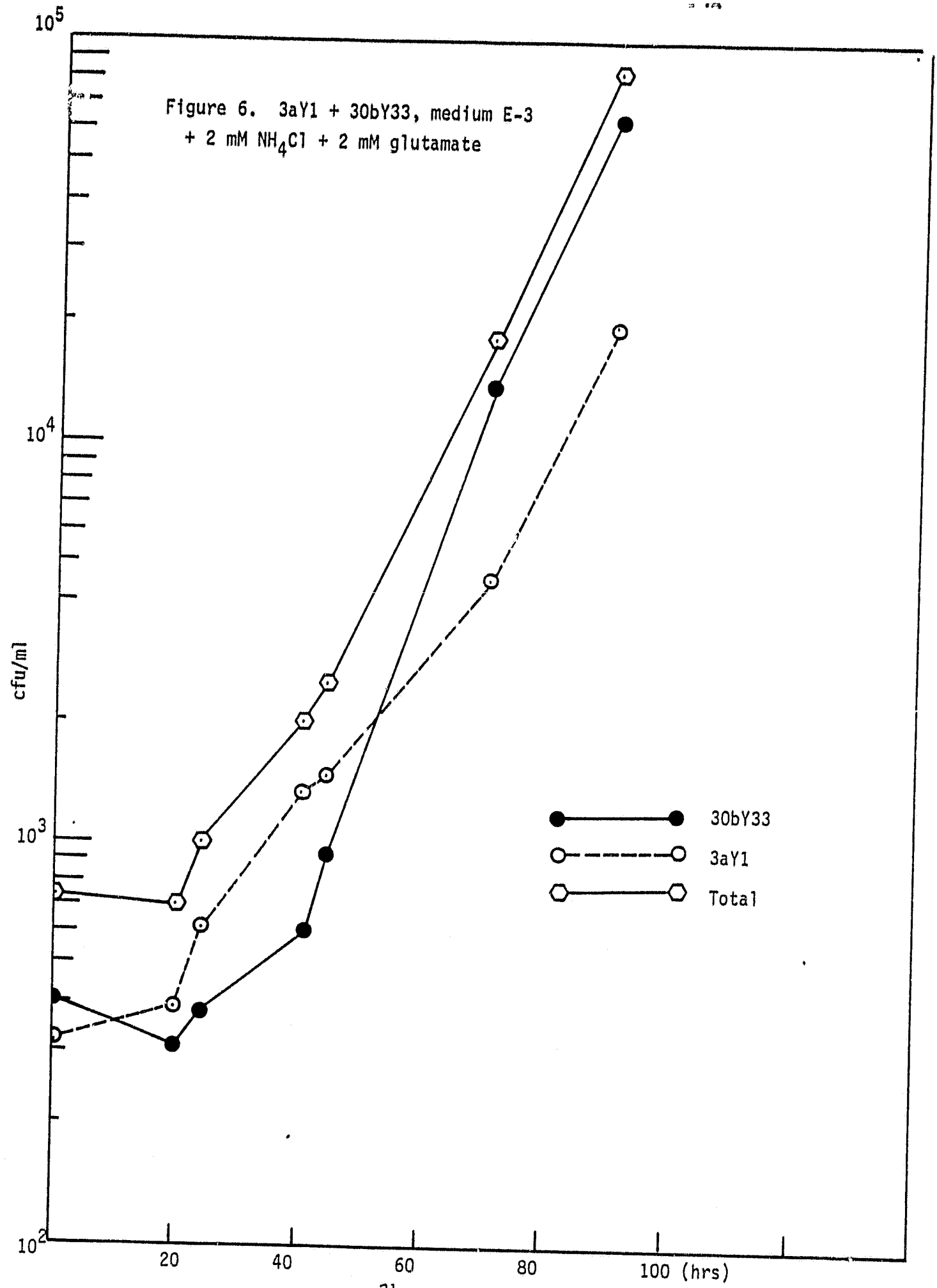


Figure 6. 3aY1 + 30bY33, medium E-3
+ 2 mM NH_4Cl + 2 mM glutamate
10^5
10^4
10^3
10^2
cfu/ml
20
40
60
80
100 (hrs)
30bY33
3aY1
Total

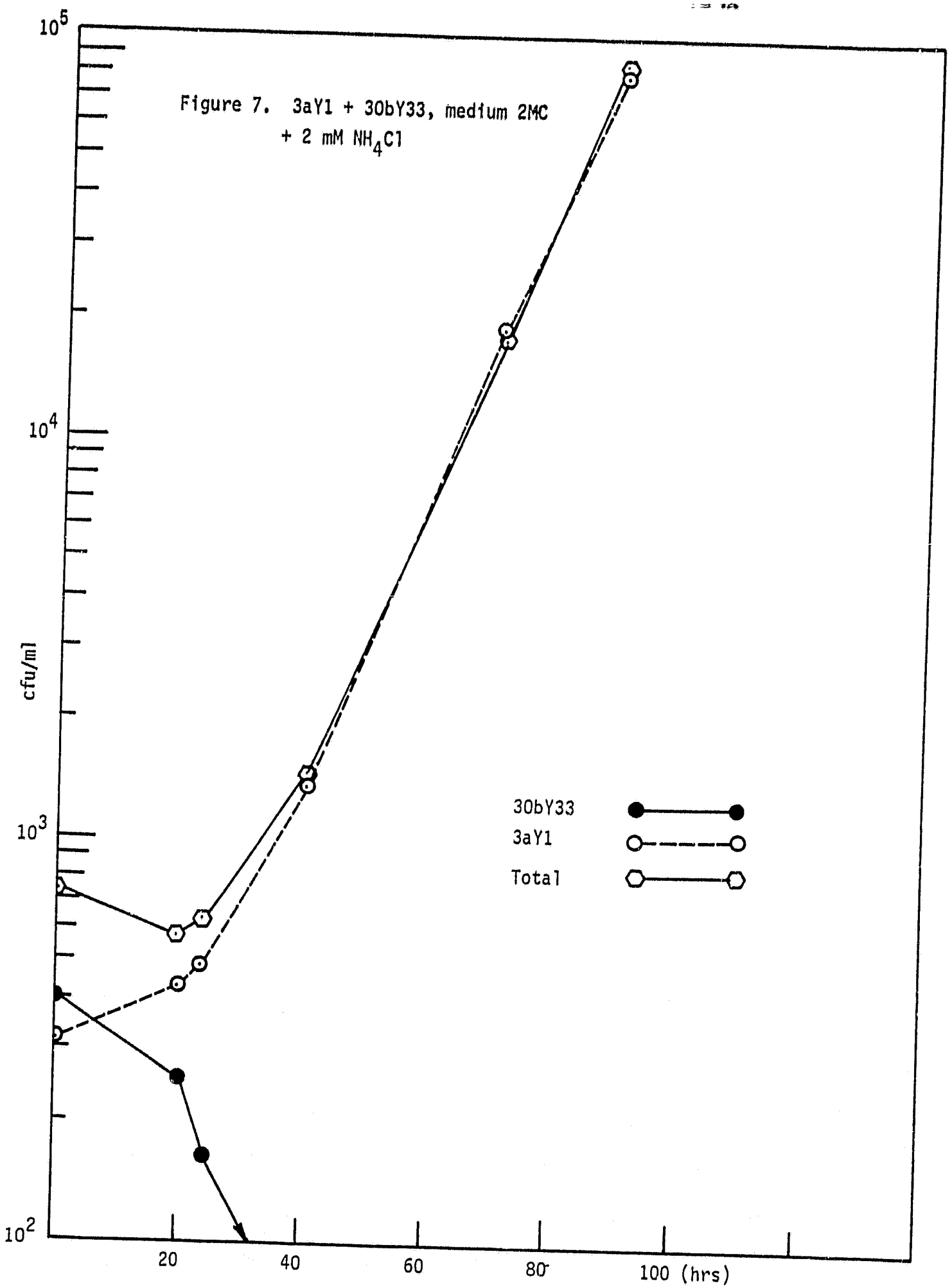


Figure 7. 3aY1 + 30bY33, medium 2MC + 2 mM NH_4Cl
10^5
10^4
10^3
10^2
cfu/ml
20
40
60
80
100 (hrs)
30bY33
3aY1
Total

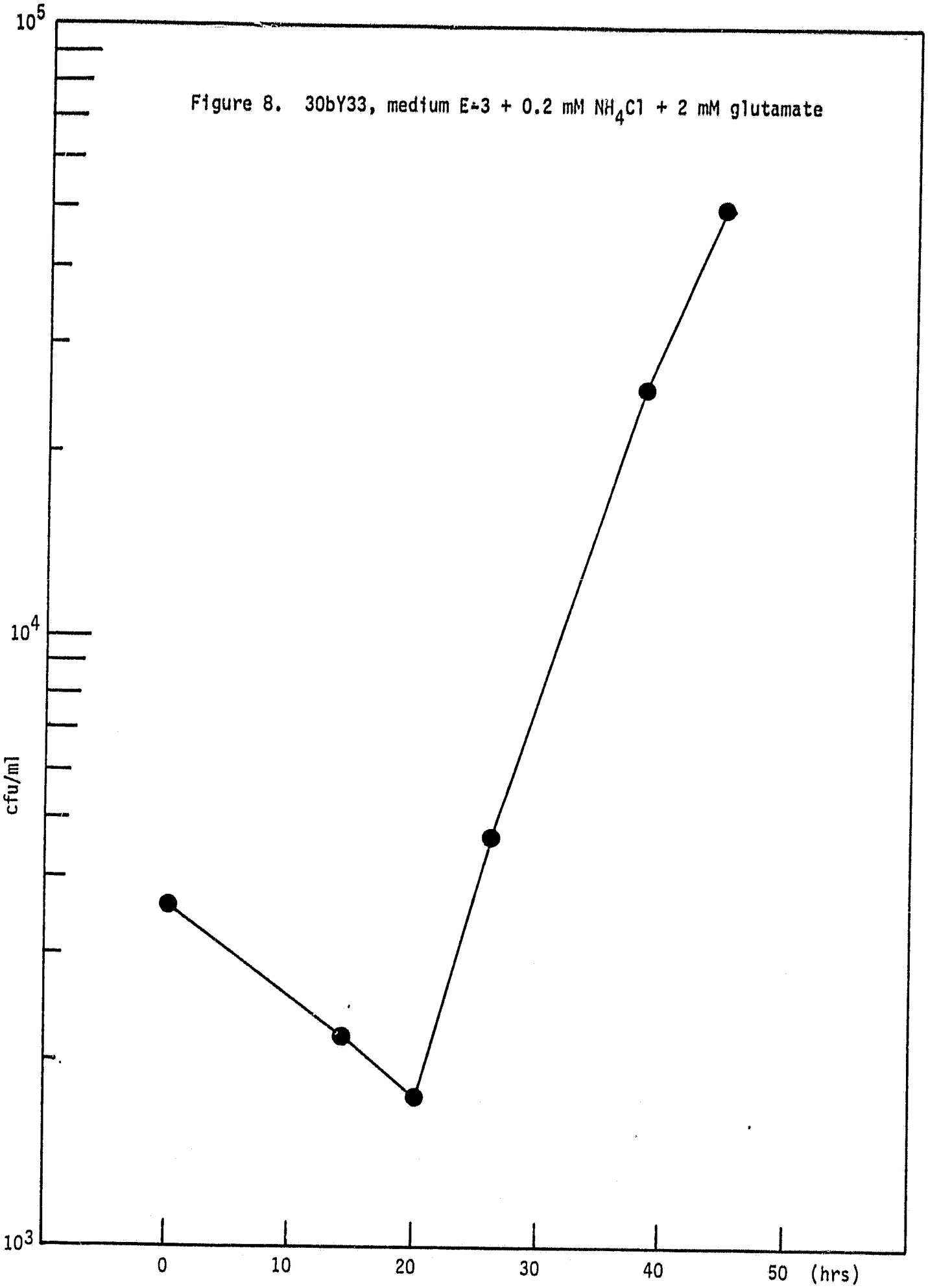


Figure 8. 30bY33, medium E-3 + 0.2 mM NH_4Cl + 2 mM glutamate

Papers produced:

Baharaeen, S., and H.S. Vishniac. 1981. Budding morphology of a psychrophilic Cryptococcus and related species compared with Leucosporidium scottii. Mycologia 73(4): 618-633.

Baharaeen S., and H.S. Vishniac. IN PRESS. A fixation method for visualization of yeast ultrastructure in the electron microscope. Mycopathologia.

Baharaeen, S., and H.S. Vishniac. IN PRESS. Cryptococcus lupi sp. nov., an Antarctic Basidioblastomycete. International Journal of Systematic Bacteriology.

Bantle, J.A.,

Baharaeen, S., and H.S. Vishniac. (received by Canadian Journal of Microbiology 6 Oct 81-- not returned by reviewer 14 Dec 81!!!!!) The evolution of Antarctic yeasts: DNA base composition and DNA:DNA homology. Appendix A

Reports on method for isolation of Antarctic microbiota in preparation.

Literature cited:

Atlas, R.M., M. Di Menna and R.E. Cameron. 1978. Ecological Investigations of Yeasts in Antarctic Soils. Antarctic Research Series 30: Terrestrial Biology III: 27-34.

Bicknell, J.N., and H.C. Douglas. 1970. Nucleic acid homologies among species of Saccharomyces. J. Bacteriol. 101:505-512.

Bitton, G., and K.C. Marshall. 1980. Adsorption of microorganisms to surfaces. 439 pp. John Wiley & Sons, New York.

deHoog, G.S. 1977. Rhinocladiella and allied genera. Studies in Mycology no. 15 (The black yeasts and allied hyphomycetes): 1-140.

diMenna, M.E. 1966. Yeasts in Antarctic Soils. Antonie van Leeuwenhoek 32:29-38.

Fell, J.W. 1974. Distribution of yeasts in the water masses of the southern oceans. IN Effect of the Ocean Environment on Microbial Activities, pp. 510-523. R.R. Colwell and R.Y. Morita, eds. University Park Press, Baltimore.

Friedmann, E.I. IN PRESS. Endolithic Microorganisms in the Antarctic Cold Desert. Science.

Friedmann, E.I., and A.P. Kibler. 1980. Nitrogen Economy of Endolithic Microbial Communities in Hot and Cold Deserts. Microb. Ecol. 6: 95-108.

Friedmann, E.I., P.A. LaRock, and J.O. Brunson. 1980. Adenosine triphosphate (ATP), chlorophyll, and organic nitrogen in endolithic microbial communities and adjacent soils in the dry valleys of southern Victoria Land. Antarct. J. U.S. 15(5):164-167.

Hansen, S. R., and S. P. Hubbell. 1980. Single-nutrient microbial competition: qualitative agreement between experimental and theoretically forecast outcomes. Science 207: 1491-1493.

Jannasch, H. W. 1977. Growth Kinetics of Aquatic Bacteria. IN Aquatic Microbiology, pp. 55-68. F. A. Skinner and J. M. Shewan, eds. Academic Press, N. Y.

Karl, D. M. 1980. Cellular Nucleotide Measurements and Applications in Microbial Ecology. Microbiol. Revs. 44(4):739-796.

Parker, B. C., G. M. Simmons, F. G. Love, R. A. Wharton, and K. G. Seaburg. 1981. Modern Stromatolites in Antarctic Dry Valley Lakes. Bioscience 31(9): 656-661.

Phaff, H. J., M. W. Miller and E. M. Mrak. 1978. The life of yeasts. 3nd ed. Harvard University Press, Cambridge.

Uydess, I. L., and W. V. Vishniac. 1976. Electron Microscopy of Antarctic Soil Bacteria. IN Extreme Environments, pp. 29-56. M. R. Heinrich, ed. Academic Press, N. Y.

Vishniac, H. S. and W. P. Hempfling. 1979a. Cryptococcus vishniacii sp. nov., an Antarctic Yeast. Intern. J. Syst. Bacteriol. 29:153-158.

Vishniac, H. S. and W. P. Hempfling. 1979b. Evidence of an Indigenous Microbiota (Yeast) in the Dry Valleys of Antarctica. J. Gen. Micro. 112:301-314.

Appendix A

The evolution of Antarctic yeasts: DNA base composition and DNA:DNA homology

Siavash Baharaeen[1], John A. Bantle[2], and Helen S. Vishniac[1,3]

[1]Department of Microbiology and [2]Department of Zoology,
Oklahoma State University, Stillwater, OK, U.S.A. 74078

3 Author to whom reprint request should be addressed.

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

The 16 biotypes of the Cryptococcus vishniacii complex of anamorphic yeasts (Basidioblastomycetes), unique to the Dry Valleys of Antarctica, include 7 species separated by DNA:DNA homologies of less than 52%. Since species belonging to the complex can be as distantly related as C. bhutanensis (a Himalayan yeast, G+C 54.18 mol %) is to these species, a common ancestor probably originated and speciated outside of the Dry Valleys. The species C. vishniacii (G+C 54.52-55.48 mol %) comprises 7 varieties with greater than 59% DNA:DNA homology and must therefore have been established in the Dry Valleys long enough to have evolved these divergent genomes. In the C. vishniacii complex, G+C mol % values differing by more than 1 mol % are accompanied by DNA:DNA homologies of less than 23%.

Introduction

The Cryptococcus vishniacii complex (yeasts of basidiomycetous affinity), isolated from the soil samples of Dr. W. V. Vishniac's 1973 expedition, is, so far as known, peculiar to the Dry Valleys of Antarctica, constituting the only heterotrophic biota demonstrably indigenous to the most severe cold desert on earth (Vishniac and Hempfling, 1979a, 1979b). The examination of genetic relationships can illuminate the nature of microbial adaptation to such extreme environments, as well as the mode of evolution of adapted populations. The characteristics of the Dry Valley yeasts are presumptively adaptive if they are absent in congeners from other habitats or present in yeasts of other evolutionary origins from cold or desert habitats.

Are the 16 biotypes of the C. vishniacii complex the result of evolutionary divergence from a single ancestral type which became dominant as the Dry Valleys developed their present climate? While a polyphyletic origin is not entirely incompatible with in situ evolution, it would suggest that these yeasts are more likely to be descendants of successive establishments of airspora in niches vacated by catastrophic temperature changes or failure of water and substrate supplies in a multiply stressed, variable, environment.

We have determined the guanine + cytosine (G + C) mol % of nuclear DNA and the degree of DNA:DNA homology in the biotypes of the C. vishniacii complex and C. bhutanensis, the yeast most similar to the complex (Vishniac and Hempfling, 1979a; Baharaeen and Vishniac, 1981), in order to establish the extent of relationship and evolutionary divergence which may have occured.

Materials and Methods

Cryptococcus bhutanensis Goto et Sugiyama (ATCC 22461), C. himalayensis Goto et Sugiyama (IAM 4963), and isolates representing each biotype of the C. vishniacii complex were grown in 6 liters of GPYPi medium (Vishniac and Hempfling, 1979a) supplemented with 10^{-4} % (v/v) of antifoam A concentrate (Sigma Chemical Co.) in a cold room (8-11°C), with vigorous stirring and aeration, for about 5-6 generations. Carboys were inoculated to an optical density (650 nm) of about 0.2, from exponentially growing cultures prepared at 10°C in a New Brunswick Scientific Company gyrotory water bath shaker operated at 190 rpm. Cells were harvested by centrifugation at 3 000 x g for 5 minutes in a refrigerated centrifuge at 4°C, washed once in glass-distilled water and resuspended in sucrose buffer (Price et al., 1978) for isolation of DNA.

DNA was isolated and purified by a combination of the procedures of Marmur (1961) and Bernardi et al. (1970) as described by Price et al. (1978). DNA purification was repeated if the preparation deviated more than 0.05 from the absorbance ratios A_{260}/A_{280}=1.86 and A_{230}/A_{260}= 0.50 (Mendonça-Hagler and Phaff, 1975). The 80-100 g wet weight of cells obtained yielded about 5-8 mg of highly purified DNA. Analytical ultracentrifugation of the DNA in cesium chloride showed no significant contamination with mitochondrial DNA.

The G+C mol % of nuclear DNA was calculated from three separate determinations of buoyant density in cesium chloride (optical grade, Sigma Chemical Co.) (Schildkraut et al., 1962; Szybalski, 1969) in

a Beckman Model L5-50 preparative ultracentrifuge equipped with Beckman Prep UV Scanner and an An-F analytical rotor modified for the use in the L5-50. Micrococcus luteus (obtained from Dr. E.A. Grula, Department of Microbiology, Oklahoma State University) DNA was used as reference. This DNA had a buoyant density of 1.731 g/mL when compared with the DNA from Escherichia coli K-12, NX-185, plasmid-free (obtained from A. Rashtchian, Department of Medical Microbiology, University of Nebraska Medical Center), the density of which was calculated to be 1.710 g/mL, using the formula given in the Beckman Prep UV Scanner instruction manual LUV-IM-2, 1975.

Purified native DNA was sheared before radioactive labeling by one passage through a French pressure cell (Aminco Model J4-3339) at 36 000 psi, after adjusting to a concentration of 250 µg/mL in Tris-EDTA buffer (tris(hydroxymethyl)-aminomethane hydrochloride, 10 mM; ethylenediamine tetraacetic acid, disodium salt, 1 mM; pH 8.2). The sheared DNA was passed through a 0.45 µm pore-size Metricel filter. The number average nucleotide size of sheared DNA was determined by sucrose density gradient centrifugation (Bantle and Hahn, 1976), using ϕX-174 DNA and λ phage DNA (Miles Laboratories) as references, to be about 500 base pairs (1.5×10^5 daltons). Gradients were centrifuged at 38 000 rpm, using a Beckman SW-41 rotor, for 20 hours at room temperature.

The sheared DNA was labeled with methyl,1',2'-^{3}H-thymidine in vitro by nick translation techniques described by Rigby et al. (1977) and Balmain and Birnie (1979), using the New England Nuclear ^{3}H-Nick Translation Kit (#NEK-005). After incubation for 2.5 hours

at 13°C (determined to allow maximum tracer incorporation), 80 µL of carrier DNA (400 µg/mL of sheared calf thymus DNA in 300 mM sodium acetate) was added to the 20 µL reaction mixture and the reaction was immediately stopped by the addition of an equal volume of a chloroform:phenol preparation (1:1; the phenol Bethesda Research Laboratories, ultrapure, saturated in Tris-EDTA buffer and treated with 0.01 % 8-hydroxyquinoline). The mixture was incubated on ice for 15 minutes with occasional vortexing, then separated into two phases by centrifugation at 12 800 x g (Eppendorf microcentrifuge) for 10 minutes. The upper (aqueous) layer was added to 1 mL of cold 95% ethanol and incubated overnight at -20°C. The DNA precipitate was collected by centrifugation, dried in a vacuum desiccator, and redissolved in 50 µL of sodium phosphate buffer (140 mM, pH 6.86). The resulting preparations of DNA had a number average nucleotide size of 380 base pairs (1.14×10^5 daltons) and specific activities of 2.2 to 2.7 $\times 10^6$ cpm/µg.

Single stranded labeled DNA was prepared and renaturation kinetics and sequence complementarity determined by the following modifications of the method of Price et al. (1978). Sealed siliconized reaction vials containing 0.02 µg of labeled ssDNA unique sequence probe (separated on hydroxyapatite after incubation to E_{Cot} of 0.7 moles.sec/L) and 20 µg of sheared homologous or heterologous DNA (in 280 mM sodium phosphate buffer pH 6.86, to a final volume of 50 µL) were denatured at 105°C for 10 minutes. The temperature was then reduced to 67°C (25°C below the thermal denaturation point, calculated form G+C mol % values) and incubation continued to an E_{Cot} of 280.

E_{Cot} values were calculated by the method of Britten et al. (1974).

The remaining ssDNA was removed with S_1 nuclease by the method of Maxwell et al. (1978). The vial contents were diluted with 450 µL of S_1 nuclease buffer, 200 µL removed for determination of total radioactivity, and 5 µL of S_1 nuclease (Bethesda Research Laboratories, 99.995% single stranded specific) added to a total concentration of 8 000 U/mL. After 60 minutes incubation at 37°C, a second 200 µL sample was removed. Samples were applied onto DE-81 DEAE filter discs (Whatman, Inc.) and left undisturbed for 5 minutes at room temperature before washing, drying, and counting.

Filters with "total radioactivity" samples were washed with 5 mL of 140 mM sodium phosphate buffer for 5 minutes, eluting about 2.9% of their radioactivity. Filters with S_1 nuclease treated samples were washed for 15 minutes with 3 five mL changes of 480 mM sodium phosphate buffer and with glass-distilled water. All filters were then washed with 95% ethanol. Washing is known to remove very small pieces of DNA (below 15 base pairs) which react non-specifically with heterologous DNA (McConaughy and McCarthy, 1967). The size of the fragments removed in our procedure was determined by horizontal agarose gel electrophoresis modified from the method of McDonnel et al. (1977). Samples (40-50 µL) and tracking dye mixture (10 µL; polyethylene glycol, 10%; orange G, 1%; xylene-cyano-fluorophosphate, 0.05%; bromophenol blue, 0.02%) were run into gel (1.2% agarose in Tris-borate buffer: Tris-HCl, 8.9 mM; boric acid, 8.9 mM; EDTA, 2.5 mM, pH 8.0) at 50 mAmp for 1 hour and separated at 30 mAmp for an additional 5-6 hours. The gel was then sliced (0.5 x 1 cm pieces) and counted.

Water washing removed fragments up to and including 7.4×10^4 daltons (245 base pairs) from "total radioactivity" filters; 140 mM sodium phosphate buffer did not remove additional fragments. Washing with 480 mM sodium phosphate buffer removed fragments mostly sized between 1.0 and 2.7×10^4 daltons (50-100 base pairs) from S_1 nuclease treated homologous hybridized samples. Since the size of the 245 base pair peak from washings of nuclease treated and untreated samples was the same, we concluded that the small loss was immaterial, probably representing unattached DNA fragments.

Washed filters were dried under an infrared lamp, placed in 10 mL of a toluene based scintillation fluid (Beckman, Ready-Solv, HP), vortexed vigorously and counted at the 2% error level in a Beckman LS-7500 liquid scintillation counter equipped with Texas Instruments Silent 700 electronic terminal. Counting efficiency was about 30-35%.

Hybridization experiments were conducted in triplicate, unless otherwise indicated. The results were not corrected for duplex formation in probe DNA at zero time or at E_{Cot} 280. Duplex formation at zero time in biotype 7 probe DNA was 1.77 ± 0.05 % actual binding, in biotype 10 probe DNA 1.69 ± 0.00 % actual binding. "Self-reannealing" in biotype 7 probe DNA was 3.99 ± 0.01 % actual binding.

Results

Base ratios of nuclear DNA of the 16 biotypes of the C. vishniacii complex and of C. bhutanensis and C. himalayensis are shown in Table 1.

The renaturation kinetics of homologous DNA (C. vishniacii,

biotype 7, type of the species) are shown in Figure 1. The Britten-Kohne (1968) plot (Figure 1a) shows that the reaction was essentially complete at E_{Cot} 210 with 88.14% renaturation. Genome size, estimated from the $E_{Cot\ ½}$ value of 41.8 mol.sec/L by the method of Britten and Kohne (1968), was 13.0 x 10^9 daltons. The modified Wetmur-Davidson (1968) plot (Figure 1b) of early data points in renaturation indicated that about 1.98% rapidly reannealing sequences remained in the radiolabeled preparation, a value similar to duplex formation at zero time as determined above.

Tables 2-9 present the results of hybridization experiments with appropriate radiolabeled DNAs as % actual and relative (to the homologous DNA) binding.

Discussion

These data show that more than one species is represented in the C. vishniacii complex. Since speciation is an ongoing process in nature, there can be no absolute level of DNA:DNA homology which separates the specific and varietal taxonomic levels. Price et al. (1978) have suggested that 80% (or more) homology should be considered evidence of conspecificity and 20% (or less) homology a conclusive bar to conspecificity, in part because intermediate values were, at the time, rare in yeasts. The correlation of DNA homology and the production of fertile offspring (as an indicator of conspecificity) is further complicated by the probability that very minor changes in total genome may interdict mating while far more extensive changes can leave the mating process intact. Kurtzman et al. (1980) found

that base sequence complementarity averaging only 24% between Issatchenkia scutullata varieties scutullata and exigua permitted the production of 3-6% viable, fertile, ascospores which did not appear to be amphidiploids. While the cytology of meiosis at this level of complementarity should be very interesting, the low fertility of this mating suggests that its products are not likely to be found in nature.

The usefulness of G+C mol % is limited to the exclusion of conspecificity. Among the ascomycetous yeasts, a difference of 1 mol % implies a level of DNA homology which will not permit normal meiosis (Price et al., 1978). The basidiomycetous yeasts are fewer and less extensively investigated. The G+C mol % values for Rhodosporidium spp. reported by Nakase and Komagata (1972) are consonant with the 1% exclusion rule, but other reports are not. The interfertility of strains of Filobasidiella neoformans differing by more than 1 mol % G+C (Aulakh et al., 1981) could conceivably be an artifact of the method of G+C determination; the apparent interfertility of strains CBS 490, G+C 63.5 mol % (Storck et al., 1969), and CBS 2630, G+C 65.0 mol % (Storck et al., 1969), of Aessosporon salmonicolor (Fell and Tallman, 1980) was not followed through meiosis (teliospore germination).

The low DNA:DNA homology between biotypes 12 and 13 of the C. vishniacii complex and the remaining biotypes (Tables 2-4, 6-8) indicates that the 1% exclusion rule holds in this case. These biotypes, differing by about 1.2 mol % from the rest of the complex (Table 1), have been described as C. lupi (Baharaeen and Vishniac,

In Press). Cryptococcus bhutanensis is confirmed as a distinct species by the low DNA:DNA homology between the type strain and C. vishniacii complex biotypes differing from it by less than 1 G+C mol % (Table 9).

The DNA:DNA homology between biotypes of the C. vishniacii complex frequently had values between 21 and 79%. The fertility of sexual hybrids cannot be determined, since sexual reproduction is unknown in these (anamorphic) yeasts. We consider, somewhat arbitrarily, that homology values of 60% and above indicate conspecificity with the type strain of C. vishniacii. Above 59% homology, the results of the three probes (biotypes 3, 7, and 10) triangulate neatly into a pattern (Figure 2). Comparison of reciprocal hybridizations indicates the precision with which these genetic distances have been determined. The mean of differences in the 11 reciprocal hybridizations was 2.74 ± 1.79 %, indicating a variation considerably greater than that of the individual homology experiments. While the smallest differences (0.59, 0.68 %) were found in reciprocal hybridizations between the biotypes with homology above 74%, differences were not correlated with genetic distances. In the group thus circumscribed as the species C. vishniacii no single biotype had less than 74% homology with some other biotypes in the group.

Homology between C. vishniacii and the remaining biotypes of the complex, and among the remaining biotypes, was lower than 52%. Closely related, but sexually isolated, species of Filobasidiella have been reported to show 55-63% relatedness, including 9% relatedness

due to base pair mismatch (Aulakh et al., 1981). Base pair mismatch and random fragment match (e.g., the 6.34 % homology between calf thymus and biotype 7 probe DNA in Table 2) may be assumed to account for larger proportions of the perceived relatedness at greater genetic distances. The unit of distance must be sized differently as distance values become larger; biotypes 9 and 15, when positioned from the two most homologous probes, are much farther from the other two probes used than the measured genetic distance. For these reasons, we consider biotypes 6, 9, 14, 15, and 16, as well as C. bhutanensis, C. lupi, and C. vishniacii, distinct species.

The tools which we have used are inadequate for evaluating relationships at taxonomic levels above species. The informed intuition which has defined the form-genus Cryptococcus has not excluded species subsequently found to differ at the class level (reviewed in Baharaeen and Vishniac, 1981). Our data do not reliably demonstrate any greater evolutionary distance between C. bhutanensis and the Antarctic yeasts than between C. lupi and other species of the C. vishniacii complex. Cryptococcus bhutanensis is a Himalayan (Laya, Bhutan) yeast (Goto and Sugiyama, 1970). This argues strongly that the Antarctic yeasts are at least diphyletic and that a generic prototype existed and speciated outside of the Dry Valleys.

The biotypes within the species C. vishniacii are clearly of monophyletic origin. The biotypes which fail to differ by 6% (the largest reciprocity difference) in responding to one of the alternative probes should not be considered genetically distinct. Biotypes 7 and 8 differ at most by 2.5% (in response to biotype 7 probe DNA);

the only phenetic difference was seen in a growth/temperature screen. Biotypes 1 and 2, differing by 5.3% in response to biotype 10 probe DNA, differ phenetically only in ammonium sensitivity (not quantitated) (Vishniac and Hempfling, 1979a). Biotypes 3, 4, 5, 10, and 11 appear genetically distinct from each other and from 1,2 and 7,8, and should be accorded varietal status. Since the adaptive fit between observed varietal differences and microhabitat is unknown, we cannot differentiate between selection and drift in intraspecific evolution. Since we have not distinguished between functional and non-functional base sequence changes, the time scale of intraspecific evolution can only be guessed at. It is, however, evident that C. vishniacii is a species which may well have originated in the Dry Valleys of Antarctica and that it has been established there long enough to have evolved to at least 7 more or less divergent genomes.

Acknowledgments

This research was supported by a National Aeronautics and Space Administration (NASA) grant, NAGW-26. We are grateful to Dr. Ulrich Melcher of the Department of Biochemistry of this university for advice.

Aulakh, H. S., S. E. Straus, and K. J. Kwon-Chung. 1981. Genetic relatedness of Filobasidiella neoformans (Cryptococcus neoformans) and Filobasidiella bacillispora (Cryptococcus bacillisporus) as determined by deoxyribonucleic acid base composition and sequence homology studies. Int. J. Syst. Bacteriol. 31: 97-103.

Baharaeen, S., and H. S. Vishniac. 1981. Budding morphology of a psychrophilic Cryptococcus and related species compared with Leucosporidium scottii. Mycologia 73: 618-633.

Balmain, A., and G. D. Birnie. 1979. Nick translation of mammalian DNA. Biochem. Biophys. Acta 561: 155-166.

Bantle, J. A., and W. E. Hahn. 1976. Complexity and characterization of polyadenylated RNA in the mouse brain. Cell 8: 139-150.

Bernardi, G., M. Faures, G. Pipero, and P. P. Slominski. 1970. Mitochondrial DNAs from respiratory-sufficient and cytoplasmic respiratory-deficient mutants of yeasts. J. Mol. Biol. 48: 23-43.

Britten, R. J., and D. E. Kohne. 1968. Repeated sequences in DNA. Science 161: 529-540.

Britten, R. J., D. E. Graham, and B. R. Neufeld. 1974. Analysis of repeating DNA sequences by reassociation. Meth. Enzymol. 29: 363-418.

Fell, J. W., and A. S. Tallman. 1980. Mating between strains of the yeast Aessosporon salmonicolor and Sporobolomyces spp. Int. J. Syst. Bacteriol. 30: 206-207.

Goto, S., and J. Sugiyama. 1970. Studies on Himalayan yeasts and molds. IV. Several asporogenous yeasts including two new taxa of Cryptococcus. Can. J. Bot. 48: 2097-2101.

Kurtzman, C. P., M. J. Smiley, and C. J. Johnson. 1980. Emendation of the genus Issatchenkia Kudriavzev and comparison of species by deoxyribonucleic acid reassociation, mating reaction, and ascospore ultrastructure. Int. J. Syst. Bacteriol. 30: 503-513.

Marmur, J. 1961. A procedure for the isolation of DNA from microorganisms. J. Mol. Biol. 3: 208-218.

Maxwell, J. H., J. van Ness, and W. E. Hahn. 1978. Assay of DNA-RNA hybrids by S_1 nuclease digestion and adsorption to DEAE cellulose filters. Nucleic Acid Res. 5: 2033-2038.

McConaughy, B. L., and B. J. McCarthy. 1967. The interaction of oligodeoxynucleotides with denatured DNA. Biochem. Biophys. Acta 149: 180-189.

McDonell, M. W., M. N. Simon, and F. W. Studier. 1977. Analysis of restriction fragments of T-7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. J. Mol. Biol. 110: 119-146.

Mendonça-Hagler, L. C., and H. J. Phaff. 1975. Deoxyribonucleic acid base composition and deoxyribonucleic acid/deoxyribonucleic acid hybrid formation in psychrophobic and related yeasts. Int. J. Syst. Bacteriol. 25: 222-229.

Nakase, T., and K. Komagata. 1972. Significance of DNA base composition in the classification of the genera Rhodotorula and Rhodosporidium. In A. Kocková-Kratochvílová and E. Minárik

(Eds.). Yeasts; models in science and technics. Proceedings of the first specialized international symposium on yeasts. Smolenic, June 1-4, 1971. Bratislava Pub. House of the Slovack Academy of Science. pp. 399-411.

Price, C. W., G. B. Fuson, and H. J. Phaff. 1978. Genome comparison in yeast systematics: delimitation of species within the genera Schwanniomyces, Saccharomyces, Debaryomyces, and Pichia. Microbiol. Revs. 42: 161-193.

Rigby, P. W., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific acitivity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. 113: 237-251.

Schildkraut, C. L., J. Marmur, and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. J. Mol. Biol. 4: 430-443.

Storck, R., 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.

Szybalski, W. 1968. Use of cesium sulfate for equilibrium density gradient centrifugation. Meth. Enzymol. 12B : 330-360.

Vishniac, H. S., and W. P. Hempfling. 1979a. Cryptococcus vishniacii sp. nov., an Antarctic yeast. Int. J. Syst. Bacteriol. 29: 153-158.

_____ 1979b. Evidence of an indigenous microbiota (yeast) in the Dry Valleys of Antarctica. J. Gen. Microbiol. 112: 301-314.

Wetmur, J. G., and N. Davidson. 1968. Kinetics of renaturation of DNA. J. Mol. Biol. 31: 349-370.

Table 1. Base composition of nuclear DNA from *Cryptococcus vishniacii* complex and related species.

Organism	Strain designation[a]	Biotype	Mol % G+C	± SD[b]
Cryptococcus vishniacii				
complex	MYSW 302Y216	1	54.52	0.32
	" 303Y365	2	54.86	0.31
	" 303Y200	3	55.03	0.06
	" 309Y215	4	55.48	0.21
	" 202Y212	5	55.44	0.16
	" 306Y212	6	55.34	0.06
	" 304Y268 (type)	7	54.97	0.16
	" 303Y338	8	54.63	0.06
	" 303Y206	9	55.68	0.06
	" 302Y265	10	54.80	0.18
	" 202Y345	11	55.27	0.31
	" 202Y252	12	53.27	0.21
	" 202Y375	13	53.30	0.16
	" 302Y259	14	54.76	0.21
	" 303Y336	15	55.82	0.27
	" 302Y310	16	55.07	0.12
C. bhutanensis	ATCC-22461 (type)	-	54.18	0.21
C. himalayensis	IAM-4963 (type)	-	57.97	0.20

a MYSW: Dr. Helen S. Vishniac, Department of Microbiology, Oklahoma State University, Stillwater, OK; ATCC: American Type Culture Collection, Rockville, MD.; IAM: Institute of Applied Microbiology University of Tokyo, Tokyo, Japan.

b Standard deviation.

Table 2. DNA:DNA hybridization: Cryptococcus vishniacii complex biotype 7 probe DNA.[a]

Source of unlabeled DNA		% actual binding ± SD	% relative binding
Strain	Biotype		
304Y268	7	87.74 ± 0.13	(100)
303Y338	8	85.55 ± 1.17	97.50
202Y312	5	80.51 ± 0.44	91.76
303Y200	3	77.16 ± 0.26	87.94
303Y365	2	74.57 ± 0.28	84.99
302Y216	1	74.30 ± 0.25	84.38
309Y215	4	70.82 ± 0.35	80.72
202Y349	11	66.10 ± 0.35	75.34
302Y265	10	65.06 ± 0.39	74.15
302Y259	14	29.13 ± 0.03	33.20
306Y212	6	24.56 ± 0.21	27.99
303Y206	9	19.80 ± 0.13	22.57
302Y310	16	18.75 ± 0.11	21.37
303Y336	15	17.51 ± 0.10	19.96
202Y252	12	13.63 ± 0.13	15.53
202Y375	13	13.63 ± 0.11	15.53
Calf thymus	-	5.56 ± 0.05	6.34

a "total radioactivity" filters were not washed in this experiment.

Table 3. DNA:DNA hybridization: Cryptococcus vishniacii complex biotype 7 probe DNA.

Source of unlabeled DNA		% actual binding ± SD	% relative binding
Strain	Biotype		
304Y268	7	91.09 ± 0.34	(100)
302Y309	4	75.16 ± 0.69	82.51
303Y202	6	24.63 ± 0.07	27.04
306Y205	6	23.94 ± 0.31	26.28
202Y256	12	13.88 ± 0.12	15.24
C. bhutanensis			
ATCC-22461	-	11.00 ± 0.04	12.08

Table 4. DNA:DNA hybridization: Cryptococcus vishniacii complex biotype 10 probe DNA.

Source of unlabeled DNA		% actual binding ± SD	% relative binding
Strain	Biotype		
302Y265	10	93.69 ± 0.12	(100)
202Y345	11	77.72 ± 0.48	82.95
303Y338	8	70.28 ± 0.11	75.01
304Y268	7	70.02 ± 0.41	74.74
302Y216	1	62.25 ± 0.43	66.44
303Y200	3	61.13 ± 0.06	65.25
303Y365	2	57.28 ± 0.35	61.14
202Y312	5	56.55 ± 0.72	60.36
309Y215	4	56.27 ± 0.11	60.06
302Y259	14	44.93 ± 0.09	47.96
306Y212	6	42.97 ± 0.52	45.86
303Y336	15	40.38 ± 0.46	43.10
303Y206	9	32.14 ± 0.13	34.30
302Y310	16	26.92 ± 0.06	28.73
202Y252	12	19.36 ± 0.07	20.66
202Y375	13	19.34 ± 0.08	20.64
C. bhutanensis			
ATCC-22461	-	12.75 ± 0.09	13.61

Table 5. DNA:DNA hybridization: Cryptococcus vishniacii complex biotype 3 probe DNA.

Source of unlabeled DNA		% actual binding ± SD	% relative binding
Strain	Biotype		
303Y200	3	94.30 ± 0.98	(100)
309Y215	4	91.38 ± 0.28	96.90
303Y216	1	90.70 ± 1.24	96.18
303Y365	2	90.53 ± 0.48	96.00
202Y312	5	85.65 ± 0.43	90.83
304Y268	7	83.57 ± 1.44	88.62
202Y349	11	64.53 ± 0.34[a]	68.43
302Y265	10	58.93 ± 0.54[a]	62.49

a determined from duplicate vials.

Table 6. DNA:DNA hybridization: Cryptococcus vishniacii complex biotype 14 probe DNA.

Source of unlabeled DNA		% actual binding ± SD	% relative binding
Strain	Biotype		
302Y259	14	94.36 ± 0.36	(100)
303Y336	15	48.64 ± 0.47	51.55
302Y265	10	48.05 ± 0.23	50.92
302Y310	16	35.55 ± 0.13	37.67
303Y206	9	32.06 ± 0.31	33.98
306Y212	6	26.69 ± 0.22	28.29
202Y252	12	21.65 ± 0.17[a]	22.94

a determined from duplicate vials.

Table 7. DNA:DNA hybridization: Cryptococcus vishniacii complex biotype 6 probe DNA.

Source of unlabeled DNA		% actual binding ± SD	% relative binding
Strain	Biotype		
306Y212	6	90.59 ± 1.52	(100)
302Y265	10	38.22 ± 0.92	42.19
302Y259	14	30.26 ± 0.65	33.40
303Y206	9	28.56 ± 0.40	31.53
303Y336	15	25.83 ± 0.14	28.51
303Y310	16	25.76 ± 0.07	27.09
202Y252	12	18.92 ± 0.52[a]	20.89

a determined from duplicate vials.

Table 8. DNA:DNA hybridization: Cryptococcus vishniacii complex biotype 16 probe DNA.

Source of unlabeled DNA		% actual binding ± SD	% relative binding
Strain	Biotype		
303Y310	16	93.37 ± 1.08	(100)
302Y259	14	29.53 ± 1.72	31.63
306Y212	6	26.56 ± 0.42	28.45
303Y206	9	26.08 ± 0.62	27.93
302Y265	10	26.00 ± 0.52	27.85
303Y336	15	24.60 ± 1.50	26.35
202Y252	12	19.86 ± 0.23[a]	21.27

a determined from duplicate vials.

Table 9. DNA:DNA hybridization: Cryptococcus bhutanensis probe DNA[a].

Source of unlabeled DNA		% actual binding ± SD	% relative binding
Strain	Biotype		
C. bhutanensis			
ATCC-22461	-	92.76 ± 1.67	(100)
C. vishniacii complex			
202Y252	12	15.73 ± 0.31	16.97
202Y375	13	15.61 ± 0.21	16.83
302Y265	10	15.21 ± 0.11	16.40
302Y259	14	14.77 ± 0.33	15.92
302Y310	16	14.68 ± 0.47	15.83
304Y268	7	14.30 ± 0.08	15.42

a rapidly reannealing sequences were not removed from the radiolabeled DNA in this experiment and the homology values were determined from duplicate vials.

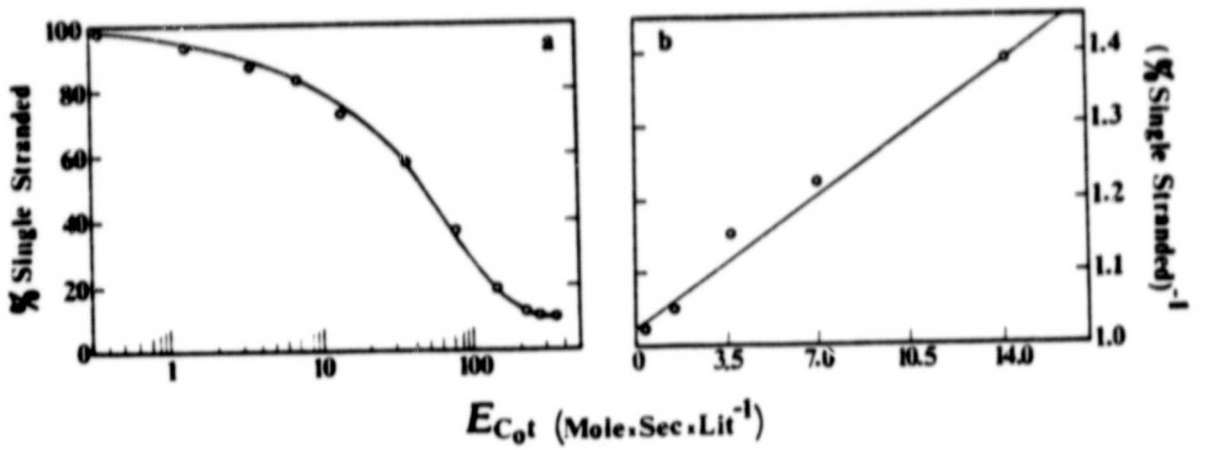


Figure 1. Renaturation kinetics of homologous DNA, Cryptococcus vishniacii biotype 7. (a) Britten_Kohne plot. (b) Wetmur-Davidson plot.

Figure 2. Genetic relationships between biotypes of the species Cryptococcus vishniacii. The length of the connecting sticks is proportional to the genetic distance (100 minus relative binding percentage of heterologous hybridization) between biotypes.