Cryptococcus lupi sp. nov., an Antarctic Basidioblastomycete

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Cryptococcus lupi sp. nov. was isolated from soil samples from the Dry Valleys of South Victoria Land, Antarctica. The guanine-plus-cytosine content (53.3 mol%) of *C. lupi* deoxyribonucleic acid differs by more than 1.5 mol% from the guanine-plus-cytosine contents of biotypes originally described under the name *Cryptococcus vishniacii*. The type strain of *C. lupi* (MYSW 202Y252 = ATCC 44529) differs phenotypically from the type strain of *C. vishniacii* in that it assimilates cellobiose, methyl- α -D-glucoside, and salicin and fails to assimilate γ -amino butyric acid, gluconate, 2-ketogluconate, 5-ketogluconate, L-rhamnose, and succinate; it also differs by having a higher maximum growth temperature and in its cell size and ratio of cell width to length.

The only heterotrophs demonstrably indigenous to the barren (lichen-free) sites of the Dry Valleys of Antarctica are yeasts belonging to the *Cryptococcus vishniacii* complex. Determinations of the guanine-plus-cytosine (G+C) contents of the deoxyribonucleic acids (DNAs) of the organisms in this complex allowed the segregation of biotypes 12 and 13 as a new species, for which we propose the name *Cryptococcus lupi* (lu'pi. L. noun *lupus* wolf; L. gen. noun *lupi* of a wolf; in honor of Wolf Vladimir Vishniac, 1922-1973, who collected the soil samples from which these yeasts were isolated).

MATERIALS AND METHODS

The methods of collection, isolation, and characterization of the yeasts have been described elsewhere (14, 15). For additional physiological studies we used the following methods.

Cycloheximide resistance was determined by visual estimation of growth on GPYPi (0.5% glucose, 0.5% peptone, 0.3% yeast extract, 10 mM potassium phosphate buffer, pH 6.86) agar to which an appropriate amount of cycloheximide stock solution in 95% ethanol was added when the agar was still hot from autoclaving. No growth during the 19-day observation period was referred to as suppressed, and growth delay was referred to as inhibition.

The method of determining the temperature limits for growth has been described previously (14, 15). The temperature for optimum growth was determined by transferring to fresh GPYPi medium a volume of exponentially growing (at 10°C) cells sufficient to yield an optical density at 650 nm of 0.2. These secondary cultures were grown at the desired temperatures on a refrigerated Gyrotory water bath shaker (New Brunswick Scientific Co.) at 190 rpm. At appropriate intervals, samples (1 ml) were removed without stopping the shaker for determinations of the change in optical density (Bausch & Lomb Spectronic 70 spectrophotometer) with time.

The other methods used in the morphological studies have been described previously (1a).

Coenzyme Q (CoQ) was isolated by the method of Yamada and Kondo (16). The CoQ type was identified by cochromatography with standard CoQs (Sigma Chemical Co.) in a reverse-phase system, using petrolatum-coated (2.5% in toluene) Whatman no. 1 paper and *N,N*-dimethyl formamide (97:3) in water as the solvent system.

Reactions to diazonium blue B were determined by applying freshly prepared, chilled diazonium reagent to 3-week-old colonies on GPYPi agar, as described by van der Walt and Hopsu-Havu (13). A positive reaction was recorded when the colonies developed a dark red color within 1 to 2 min at room temperature.

DNA extraction and purification were accomplished by a combination of the procedures of Marmur (8) and Bernardi et al. (3), as described by Price et al. (10). DNA purification was repeated if the preparation deviated more than 0.05 u from a ratio of absorbance at 260 nm to absorbance at 280 nm of 1.86 and a ratio of absorbance at 230 nm to absorbance at 260 nm of 0.50 (9). The G+C content of the nuclear DNA was calculated from three separate determinations of buoyant density in cesium chloride (optical grade; Sigma Chemical Co.) (11, 12) in a Beckman model L5-50 preparative ultracentrifuge equipped with a Prep UV scanner (Beckman) and an An-F analytical rotor modified for use in the L5-50 ultracentrifuge (Beckman). *Micrococcus luteus* (kindly provided by E. A. Grula, Department of Microbiology, Oklahoma State University) DNA was used as a reference; this DNA had a buoyant density of 1.731 g/ml compared with DNA from *Escherichia coli* K-12 strain 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 from the formula given in the *Prep UV Scanner Instruction Manual* (2).

RESULTS

Latin diagnosis of *Cryptococcus lupi* sp. nov.
Coloniae in agaro colore cremeo, aut politae et mucosae aut asperae et durae; in mediis liquidis difficiliter crescit, pelliculam haud, anulum exi-

FIG. 1. Scanning electron micrograph of *C. lupi* strain 202Y252 multiparous budding cell showing the characteristic frayed collar of basidioblastomycetes. The capsule, collapsed as a result of critical-point drying, appears as nodules and lines on the surface.

gue, et sedimentum lente formans. Cellulae crescentes forma ova vel lagonae, ca. $5 \times 6 \mu\text{m}$, cum capsulis exiguis, e situ cicatricis natalis iterumque iterumque generatae. Parietes typici Basidioblastomycetium. Nec pseudomycelium nec mycelium formatur. Non generant sed per modo blastico asexuali, calore minus 4°C usque ad 25°C .

Fermentatio nulla. Assimilat L-arabinosum, cellobiosum, acidum citricum, fructosum, glucosum, acidum D-glucuronicum, acidum D-glutamicum (variabile), maltosum, mannosum, melizitosum, methyl- α -D-glucosidum, raffinolum, salicinum, sucrosum, trehalosum, D-xylosumque. Amylosum formatum est. Crescit cum nitrato; crescit sine vitaminis. Gelatinum et urea non finduntur. CoQ_9 continens; diazonium blue B respondens. G+C ca. 53.3 mol%.

Standard description of *Cryptococcus lupi* sp. nov. This species is typified by isolate MYSW 202Y252, which was isolated from coarse, weathered dolerite gravel from the north flank of Mount Baldr (South Victoria Land, Antarctica) at 6,220 feet (1,896 m). The species comprises isolates originally described as *C. vishniacii* biotypes 12 (the type isolate and, from other Dry Valley sites, isolates 302Y253, 303Y267, and 306Y250) and 13 (isolates 202Y375 and 303Y368)

(14). The type strain (MYSW 202Y252) has been deposited in the American Type Culture Collection, Rockville, Md., as ATCC 44529.

Colonies on glucose-peptone-yeast extract agar are cream colored, entire, glistening, and somewhat mucoid; on malt extract agar, they are tough and granular. Growth in liquid media is limited unless agitation and aeration are provided; in tubes, a scanty annulus (no pellicle) and slowly produced sediment provide the only evidence of growth. Exponentially growing cultures may produce very small amounts of rudimentary pseudomycelium; pseudomycelium and mycelium are not formed. Sexual reproduction is unknown.

Cells in exponentially growing GPYPi cultures are ovoid to flask shaped, averaging 5.06 (standard deviation, ± 0.71) by 6.11 (standard deviation, ± 0.71) μm , with a ratio of width to length of 0.83 ± 0.07 . Cells are thinly encapsulated (Fig. 1). Budding is monopolar and repetitive through the birthscar site (1); primary buds are holoblastic (Fig. 2), and secondary buds are enteroblastic, with the septum formed at the level of the characteristic frayed collar of basidioblastomycetes (Fig. 3).

Fermentation does not occur.

Carbon compounds assimilated: L-arabinose, cellobiose, citrate, fructose, glucose, D-glucuronate, L-glutamate (variable response), maltose, mannose, melezitose, methyl- α -D-glucoside, raffinose, salicin, sucrose, tartrate (variable response), trehalose, and D-xylose. Amylose is produced during growth on L-arabinose, glucose, or xylose. The following compounds were not assimilated as sole sources of carbon and energy (arbutin and methanol were not tested): acetate, amino acids other than glutamate (L-alanine, γ -amino butyrate, L-arginine, L-aspartate, glycine, L-isoleucine, L-lysine, L-methionine, L-ornithine, L-phenylalanine, L-proline, and L-valine), D-arabinose, D-arabitol, L-arabitol, butyrate, decane, erythritol, ethanol, D-fucose, fumarate, galactitol, D-galactose, D-glucitol, D- δ -gluconolactone, glycerol, *myo*-inositol, inulin, 2-ketogluconate, 5-ketogluconate, DL-lactate, lactose, L-malate, D-mannitol, melibiose, L-rhamnose, ribitol, D-ribose, L-sorbose, starch, succinate, and xylitol.

Nitrogen sources utilized: NH_4Cl , KNO_3 , L-amino acids (L-alanine, L-arginine, L-aspartate, L-glutamate, glycine, L-histidine, L-isoleucine, L-lysine, L-methionine, L-ornithine, L-phenylalanine, L-proline, and L-valine). Not utilized: D-alanine, creatinine, cytosine, ethylamine, nicotinic acid, thiamine, thymine, and uracil.

Gelatin and urea are not hydrolyzed.

Externally supplied vitamins are not required.

Growth occurs from less than 4 to 25°C ; optimal growth is at 17°C .

Growth is inhibited by $0.05 \mu\text{g}$ of cyclohexi-

FIG. 2. Transmission electron micrograph of *C. lupi* strain 202Y252 cell with the primary bud still attached. Note the continuation of the whole mother cell wall with that of the bud (holoblastic). CW, Cell wall; Mt, mitochondrion; Nu, nucleus; v, vacuole.

FIG. 3. Transmission electron micrograph of *C. lupi* strain 202Y252 showing secondary budding. The bud cell wall is continuous only with the inner mother cell wall layer(s) (enterblastic). The arrow-heads indicate the frayed outer layers making up the bud scar collar. A septum is formed at the level of the frayed collar. CP, Remains of the capsule; CW, cell wall; er, endoplasmic reticulum; Mt, mitochondrion; SP, septum.

midic acid per ml and is suppressed by 0.5 μg of cycloheximide per ml.

Ubiquinone CoQ_9 is produced.

Diazonium blue B: positive.

G+C content of nuclear DNA: 53.27 ± 0.21 mol%.

These characters code in the widely used system of Barnett et al. (1) as follows: 1, negative; 2 through 4, ?; 5, negative; 6 through 11, ?; 12 through 14, negative; 15 and 16, positive; 17 and 18, negative; 19 through 24, positive; 25, ?; 26 and 27, negative; 28 and 29, positive; 30 through 43, negative; 44, positive; 45, ?; 46 through 48, negative; 49, positive; 50, ?; 51 through 59, positive; 60 and 61, ?; 62, negative; 63, ?; 64, negative; 65, positive; 66, negative; 67, negative; 69 and 70, positive; 71 through 78, negative.

DISCUSSION

Observation of colony color (not red or pink) and determination of assimilation of L-arabinose (positive), galactose (negative), 2-ketogluconate (negative), lactose (negative), and D-mannitol (negative) under conditions (temperature and media) appropriate for *C. lupi* allow presumptive

identification of isolates with this species rather than any other yeast species described previously. *C. lupi* is phenotypically most similar to *C. vishniacii* and to biotypes 10, 11, and 16 of that species. *C. lupi* differs from the type strain of *C. vishniacii* by the assimilation of cellobiose, methyl- α -D-glucoside, and salicin, by the failure to assimilate γ -amino butyric acid (type slow), gluconate (type slow) 2-ketogluconate, 5-ketogluconate (type slow), L-rhamnose (type weak), and succinate, by higher optimal and maximal growth temperatures, and in cell size and the ratio of cell width to length (1). *C. lupi* differs from *C. vishniacii* biotypes 10, 11, and 16 in the following characters: failure to assimilate γ -amino butyric acid (biotypes 10 and 11, slow), L-aspartate (biotypes 10, 11, and 16, slow), 2-ketogluconate (biotypes 10, 11, and 16, weak), L-rhamnose (biotype 16, positive), or succinate (biotypes 10 and 11, positive) and lower susceptibility to cycloheximide (biotype 16, suppressed by 0.05 $\mu\text{g}/\text{ml}$).

The use of the G+C content of the nuclear DNA to exclude conspecificity has become a standard procedure in the taxonomy of yeasts known only as anamorphs. A difference of more than 1 mol% is considered to preclude signifi-

cant DNA-DNA homology in yeasts (10); that is, such a difference is associated with a failure to mate with the production of fertile recombinant offspring in teleomorphic strains. This information is based on studies with ascomycetous yeasts and ascoblastomycetes and has yet to be confirmed for the less-well-known basidioblastomycetes. The biotypes assigned here to *C. lupi* show no significant differences in phenotype (14, 15) or in G+C content (isolate 202Y252, 53.27 ± 0.21 mol%; isolate 202Y375, 53.30 ± 0.16 mol%) but differ by at least 1.5 mol% from the values determined for other biotypes of *C. vishniacii*. The DNA of the type strain of *C. vishniacii* contains 54.97 ± 0.16 mol% G+C; biotype 10 (the closest biotype) contains 54.80 ± 0.19 mol% G+C. DNA-DNA homology is less than 20% (unpublished data).

Our interest in the taxonomy of these yeasts derives from their importance in analyses of the Antarctic ecosystem. We need genetic measures of relatedness because lack of equivalence of taxonomic levels has been a major problem in the application of theoretical ecology to ecosystems containing both macrobes and microbes. We need (and have attempted to find) easily determined descriptors other than those generally used in yeast identification both because these fail to distinguish recognized species (7) and because we need to identify diversity at subspecific levels. Finally, although there are practical limits to the number of assimilation tests that can be performed, as well as the theoretical objection that habitats may not provide unmixed substrates, extended test series provide a major source of data on resource utilization. Apart from airspora, the closest resources for heterotrophs in the more barren areas of the Dry Valleys of Antarctica are endolithic lichens (*Buellia*) (5) and cyanobacteria (6). The failure of yeasts belonging to the *C. vishniacii* complex to utilize any commercially available polyols makes it unlikely that these organisms can derive immediate benefit from association with the more common chlorophycean lichens (4).

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REPRINT REQUESTS

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