Deoxyribonucleic Acid Base Composition and Deoxyribonucleic Acid/Deoxyribonucleic Acid Hybrid Formation in Psychrophobic and Related Yeasts

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The deoxyribonucleic acid (DNA) base composition of several strains of the psychrophobic species *Saccharomyces telluris*, *Torulopsis pintolopesii*, *T. bovis*, and *Candida slooffii* was found to be similar, DNA/DNA hybridization experiments by means of the nitrocellulose filter technique revealed high degrees of homology between the nuclear DNAs of all four species. On the basis of these findings, *T. bovis* is considered to be a synonym of *T. pintolopesii*; *C. slooffii*, which has certain distinct and reproducible properties, was reclassified as *T. pintolopesii* var. *slooffii*. *Saccharomycopsis guttulata* has been retained as a separate species because its guanine plus cytosine value is higher than that of the four species mentioned above and it has other distinct properties. The nonpsychrophobic species *Torulopsis glabrata* was included for comparison. The guanosine plus cytosine content of its DNA was approximately 7% higher than that of *T. pintolopesii*.

Psychrophobic enteric yeasts occur in the alimentary canal of certain domestic and wild animals (22). Such yeasts have minimum temperatures for growth ranging from approximately 20 to 30 C, whereas their maximum limits are somewhere between 40 and 46 C, depending on species and strain differences. Since a significant number of yeast species are known with similar maxima but with much lower minimal growth temperatures, the principal characteristic of psychrophobic yeasts is their rather high minimal temperature of growth. The species normally included in this group are *Saccharomyces telluris* (17) and its imperfect form *Torulopsis bovis* (21), *T. pintolopesii* (18, 21), *Candida slooffii* (19), and *Saccharomycopsis guttulata* (13). Species differentiation is based mainly on minimal temperature of growth and ability to form ascospores or pseudomycelia. In addition, some differences have been reported in nutritional requirements (22, 23) and in cell size. Very few carbon sources are utilized for growth, and the fermentative and assimilatory reactions are nearly identical. The salient properties of these species are shown in Table 1. Also included is *Torulopsis glabrata*, not a psychrophobic but a thermophilic species, which is sometimes associated with urinary tract infections in humans. This species superficially resembles the other organisms. It was included in this study for that reason and it also served as a control.

Because of the many apparent similarities among these species, it was decided to study their deoxyribonucleic acid (DNA) base compositions on a comparative basis and to perform DNA/DNA reassociation experiments so that genetic relatedness could be evaluated between strains with similar base compositions. Several strains of each species were included in the survey.

MATERIALS AND METHODS

Organisms and growth conditions. The yeast strains designated IGC or CBS were obtained from the Instituto de Microbiologia of the Universidade Federal do Rio de Janeiro, Brazil. Their original sources are listed in Table 2. Yeasts were grown at 37 C in a medium containing 1% proteose/peptone (Difco), 0.5% yeast extract (Difco), and 2% glucose. The pH was adjusted to 6.0. After 48 h of growth (beginning of stationary phase), cultures were inspected microscopically for bacterial contamination, after which the cells were harvested by centrifugation. *Saccharomycopsis guttulata* was cultivated for 24 h in a fermentor sparged with a mixture of air and 20% CO₂ (4). In this medium, the yeast extract was replaced by 1% yeast autolysate (Albimi-Pfizer).

DNA extraction and purification. The DNA was extracted and purified by a combination of the procedures of Marmur (10) and Bernardi et al. (2). The yeast paste was suspended in sucrose buffer [0.02 M tris(hydroxymethyl)aminomethane-hydrochloride pH 7.8, 0.01 M ethylenediaminetetraacetate 15% sucrose] to form a very thick suspension, after which the cells were broken in a Braun cell homogenizer (Bronwill Scientific, Inc.) operated at maximum speed for 1 min and cooled with liquid CO₂. The

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TABLE 1. *Differentiating characters among psychrophobic and thermophilic yeasts*

Species	Asco-spores produced	Pseudo-mycelium	Respiratory ability	Cell size ^a (μ m)	Temperature range for growth ^b (C)	Assimilation of trehalose	Requirement for high concentration of gaseous CO ₂
<i>Saccharomyces telluris</i> van der Walt 1957	+ ^c	—	+ (weak)	3.5–6.5 by 4.5 to 8.5	20–22 to 43	—	—
<i>Torulopsis bovina</i> (van Uden and do Carmo-Sousa) van Uden and Vidal-Leiria 1957	— ^c	—	+ (weak)	4–6 by 6–9	20–22 to 43	—	—
<i>T. pintolopesii</i> van Uden 1952	—	—	—	2.5–3 by 3–5	24–42	—	—
<i>Candida slooffii</i> van Uden and do Carmo-Sousa 1957	—	+	—	4–7 by 6–9	28–46	—	—
<i>T. glabrata</i> (Anderson) Lodder and de Vries 1917	—	—	+	2.5–4.5 by 4–6	6–43	+	—
<i>Saccharomycopsis guttulata</i> (Robin) Schiöningg 1853, Synonymous with <i>Cyniclomyces guttulatus</i> (Robin) van der Walt and Scott	+	+	+	4.4–6.1 by 14–21	30–40	— or latent	+

^a Cells (1 to 3 days old) in glucose/yeast extract/peptone water.^b Data from references 13, 17, 19, and 21, except for the minimum temperature of growth for *T. glabrata*, which was determined in this study.^c +, Property present; —, property lacking.TABLE 2. *Yeast strains used in this study*

Species	Strain designations ^a	Original sources
<i>Saccharomyces telluris</i>	IGC 2622 CBS 4055 CBS 2676 CBS 2679 IGC 2663 (= CBS 2685) ^b	Mouse feces (Parle, New Zealand) Cecum of horse Excreta of rats and mice Lung of pullet Soil (van der Walt, South Africa)
<i>Torulopsis bovina</i>	CBS 2760 IGC 2678 IGC 2681	Cecum of a cow Crop of turkey Cecal contents of a horse (van Uden, Portugal)
<i>T. pintolopesii</i>	CBS 3046 IGC 2405 ^b (= CBS 1787) IGC 2616 IGC 2457	Mice injected with soil Liver of a mouse Unknown Peritoneal cavity of a rat (Artagaveytia-Allende, Uruguay)
<i>Candida slooffii</i>	CBS 2419 ^b IGC 2687 IGC 2689 CBS 5040	Cecum of a horse Cecum of a horse (van Uden, Portugal) Cecum of a swine (van Uden, Portugal) Frass of cossidae larvae (van der Walt, South Africa)
<i>T. glabrata</i>	UCD 72-33 CBS 4692 UCD 72-28 UCD 72-27	Gastric juice Case of generalized mycosis Peritoneal fluid (Ahearn, USA) Sputum (Ahearn, USA)
<i>Saccharomycopsis guttulata</i> (synonymous with <i>Cyniclomyces guttulatus</i>)	UCD 73-90	Domestic rabbit (Davis, California)

^a Abbreviations: CBS, Centraal Bureau voor Schimmelcultures, Delft, Netherlands; IGC, Instituto Gulbenkian de Ciencias- LISBON, Portugal; UCD, University of California, Davis.^b Type strain.

broken-cell suspension was diluted with a detergent solution containing 0.02 M tris(hydroxymethyl)amino-methane-hydrochloride buffer, pH 7.8, 0.01 M ethylenediaminetetraacetic acid, 1% sodium lauryl sarcosate, and made to 1 M sodium perchlorate. An equal volume of a mixture of chloroform/isoamyl alcohol (24:1, vol/vol) was added, and the mixture was shaken at room temperature for 3 h. The emulsion was then separated by centrifugation at $20,000 \times g$ for 20 min. The aqueous upper layer was removed and chilled, and 1.3 volumes of cold ethanol were added. The precipitate was collected by centrifugation for 5 min at $5,000 \times g$. The pellet was dissolved in $0.1 \times$ SSC (SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), and the solution was made to $1 \times$ SSC. Pancreatic ribonuclease (Calbiochem) solution (boiled for 10 min) and α -amylase (Sigma Chemical Co.; *Bacillus subtilis*) solution were then added to final concentrations of 100 $\mu\text{g/ml}$ each and placed at room temperature for 4 h. This was followed by the addition of Pronase (nuclease free; Calbiochem) to a final concentration of 50 $\mu\text{g/ml}$, and the solution was stored for 4 h at room temperature. The preparation was again extracted with chloroform/isoamyl alcohol for 30 min and separated as previously described. Ethanol was added to the aqueous phase, and the DNA was spooled out, dissolved in 1 mM phosphate buffer at pH 6.8, and dialyzed against the same buffer. The DNA was then purified by hydroxyapatite (Bio-Rad) chromatography. Proteins, carbohydrates, and ribonucleic acids (RNAs) were eluted with 0.15 M phosphate buffer at pH 6.8, and the DNA was eluted with 0.5 M phosphate buffer at pH 6.8. The absorbance at 260 nm (A_{260}) of the column eluate was monitored automatically with a model 2400 Gilford spectrophotometer. The ratios A_{260}/A_{280} and A_{230}/A_{260} of the samples containing peak absorbances were measured. Hydroxyapatite chromatography was repeated if the preparation showed a deviation of more than 0.05 from the ideal ratios: $A_{260}/A_{280} = 1.86$ and $A_{230}/A_{260} \leq 0.5$.

Labeling of DNA. DNA prepared as described above was labeled with ^{125}I based on the procedure described by Commerford (6). Carrier-free ^{125}I was obtained as NaI in 0.1 N NaOH from New England Nuclear Corp., and thallium trichloride was obtained from K & K Laboratories. Unlabeled DNA was sheared at 40,000 lb/in² in a small French pressure cell made of titanium. Sheared, double-stranded DNA (50 to 150 μg) and 5 to 10 μCi of ^{125}I were used for labeling. The specific activities obtained varied between 10^4 and 6×10^4 dpm/ μg of DNA. Although there was a slight broadening of the DNA band in the ultracentrifuge, the quality of the DNA did not seem to be affected significantly.

Determination of guanine plus cytosine (G+C) content. The methods of Schildkraut et al. (14) and Szybalski (15) were used for the determination of the buoyant densities of the DNA samples. *Micrococcus lysodeikticus* DNA was used as reference DNA, whose buoyant density was taken to be 1.7310 g/cm³. Cesium chloride, code 62, was purchased from the Rare Earth Division of American Potash and Chemical Corp. *M. lysodeikticus* DNA was obtained from Miles Labora-

tories. Each DNA preparation was centrifuged at least four times in a Spinco analytical ultracentrifuge equipped with ultraviolet optics.

Preparation of DNA filters. DNA filters were prepared by the method of Gillespie and Spiegelman (8). Eight milliliters of an unlabeled, high-molecular-weight DNA solution in $0.1 \times$ SSC (50 $\mu\text{g/ml}$) was denatured by heating to 100 C for 10 min and quenched by pouring them into 72 ml of ice-cold $6 \times$ SSC. The solution was filtered without vacuum through a 50-mm BAC-T-Flex B₈ Schleicher and Schüll nitrocellulose filter presoaked with $6 \times$ SSC. The DNA was washed with 10 ml of $6 \times$ SSC, and the filter was dried at room temperature overnight and then placed for 3 h in a vacuum oven at 80 C. About 90% of the DNA in solution was retained by the filter. Filters 10 mm in diameter were punched out of the larger filter, each one containing approximately 16 μg of DNA, as determined by the Burton procedure (5). The small filters were then incubated for 3 h at 60 C in the preincubation medium specified by Denhardt (7) to reduce background binding.

DNA/DNA duplex formation. Labeled, sheared DNA in $0.1 \times$ SSC used for duplex reactions was denatured by heating to 100 C for 10 min, after which it was poured into ice-cold $4 \times$ SSC to give a final concentration of $2 \times$ SSC. The radioactive DNA solution was then added to vials containing the filters and incubated at 60 C for 14 h. The ratio of DNA on the filter to that in solution was approximately 30:1.

At the end of the incubation period, the filters were washed by two consecutive immersions in beakers containing about 200 ml of $2 \times$ SSC at 60 C. The filters were dried at room temperature and counted in a Packard Gamma model 578 scintillation spectrometer. Filters containing *Escherichia coli* DNA (and in one experiment calf thymus DNA) were used as controls to determine nonspecific binding. These control filter values were subtracted from the counts obtained with the yeast DNA. Relative binding was calculated as follows:

Relative binding

$$= 100 \times \frac{\text{dpm of heterologous filters} - \text{dpm of control filters}}{\text{dpm of homologous filters} - \text{dpm of control filters}}$$

Experiments showing 15 to 40% binding of the homologous, labeled input DNA to the homologous, unlabeled filter DNA were considered acceptable. One problem with ^{125}I -labeled DNA is its erratic and often relatively high nonspecific binding (up to approximately 10%). Only those experiments are reported in which the binding of labeled input DNA to control filters was not higher than 2 to 3%.

All hybridizations were done in triplicate, by using one filter per vial. In general, the variation among the hybridization values was $\pm 5\%$ (disintegrations per minute per filter); the values reported are averages for three filters. After the filters were counted, the residual DNA on the filters was determined by the Burton procedure (5) to establish losses due to leaching during incubation. If the filters lost more than 25%

of the DNA originally placed on them, the values for hybridization were not used, and the experiment was repeated. Usually the loss was around 20 to 25% of the filter DNA.

RESULTS

Base composition of the DNAs. The mol% G+C of the DNA preparations of the organisms studied are listed in Table 3. The results for the three strains of *C. slooffii* showed good agreement. Also, the DNAs of four strains of *T. pintolopesii* were similar in G+C content. With one exception, the strains of *S. telluris* and its imperfect form *T. bovina* were all comparable in their DNA base composition. The exceptional strain, *S. telluris* IGC-2622, had a much higher mol% G+C. The various *T. glabrata* strains showed good agreement among themselves; their G+C levels were approximately 7% higher than those of the other species so far mentioned. Only one strain of *S. guttulata*, recently isolated in our laboratory, was studied. This organism is most difficult to maintain in laboratory cultures due to the very short life of the cells. Its G+C content was somewhat higher than those of the other psychrophobic yeasts studied.

The most striking result was the similarity in G+C values among the strains of *S. telluris*, *T. bovina*, *T. pintolopesii*, and *C. slooffii* (excluding the one with aberrant G+C content). Thus the possibility was considered that these four species might represent simply morphological (ability to form pseudomycelium and cell size) or physiological (minimum temperature of growth) variants of a single taxon. To verify this hypothesis, DNA/DNA hybridizations were performed in various combinations.

Intraspecific hybridization of *C. slooffii* DNAs. Table 4 shows the results obtained with the three *C. slooffii* strains investigated. Similar results were obtained with two species of labeled DNA. It is evident that the strains with an average of 32.37 mol% G+C are representatives of the same species.

Intraspecific hybridization of *T. pintolopesii* DNAs. Results from these experiments are shown in Table 5. The four strains with an average base composition of 32.98 ± 0.5 mol% G+C all demonstrated high degrees of relative binding of their DNAs with that of the reference strain no. 2616 (Table 5).

Intraspecific hybridization of *T. bovina* DNAs. The three strains with similar base compositions of 33.3 to 33.9 mol% G+C (average 33.5) revealed high degrees of relative binding with the reference strains 2681 and 2678 (Table 6). In this case, an unrelated yeast

TABLE 3. DNA base compositions (mol% G+C) of the strains studied based on buoyant density equilibrium centrifugations

Species	Strains	Buoyant densities (g/cm ³) ^a	Mol% G+C
<i>Saccharomyces telluris</i>	IGC-2622	$1.6990 \pm 6 \times 10^{-4}$	39.8 ± 0.6
	CBS-4055	$1.6931 \pm 2 \times 10^{-4}$	33.8 ± 0.2
	CBS-2676	$1.6921 \pm 1 \times 10^{-4}$	32.8 ± 0.1
	CBS-2679	$1.6925 \pm 2 \times 10^{-4}$	33.2 ± 0.2
	IGC-2663	$1.6933 \pm 2 \times 10^{-4}$	34.0 ± 0.2
<i>Torulopsis bovina</i>	CBS-2760	$1.6926 \pm 1 \times 10^{-4}$	33.3 ± 0.1
	IGC-2678	$1.6926 \pm 1 \times 10^{-4}$	33.3 ± 0.1
	IGC-2681	$1.6932 \pm 3 \times 10^{-4}$	33.9 ± 0.3
<i>T. pintolopesii</i>	CBS-3046	$1.6928 \pm 1 \times 10^{-4}$	33.5 ± 0.1
	IGC-2405	$1.6921 \pm 1 \times 10^{-4}$	32.8 ± 0.1
	IGC-2616	$1.6924 \pm 2 \times 10^{-4}$	33.1 ± 0.2
	IGC-2457	$1.6919 \pm 4 \times 10^{-4}$	32.5 ± 0.4
<i>Candida slooffii</i>	CBS-2419	$1.6918 \pm 2 \times 10^{-5}$	32.4 ± 0.02
	IGC-2687	$1.6917 \pm 3 \times 10^{-4}$	32.3 ± 0.3
	IGC-2689	$1.6918 \pm 2 \times 10^{-4}$	32.4 ± 0.2
<i>T. glabrata</i>	CBS-5040	$1.6994 \pm 4 \times 10^{-4}$	40.2 ± 0.4
	UCD 72-33	$1.6990 \pm 5 \times 10^{-4}$	39.8 ± 0.5
	CBS-4692	$1.6994 \pm 4 \times 10^{-4}$	40.2 ± 0.4
	UCD 72-28	$1.6990 \pm 5 \times 10^{-4}$	39.8 ± 0.5
	UCD 72-27	$1.6988 \pm 4 \times 10^{-4}$	39.6 ± 0.4
<i>Saccharomycopsis guttulata</i>	UCD 73-90	$1.6938 \pm 3 \times 10^{-4}$	34.5 ± 0.3

^a Average of four determinations.

control was included. This species, whose DNA contained 40.2 mol% G+C, revealed a very low degree of relative binding.

Intraspecific hybridization of *S. telluris* DNAs. Three of the five strains (base compositions 32.8 to 34.0 mol% G+C) were used for this experiment. Significant duplex formation was observed between the reference strain and the two test strains (Table 7). Although the degree of relative binding of *S. telluris* strain 2676 DNA was somewhat lower than that observed in other

TABLE 4. Degree of relative binding between various strains of *Candida slooffii*^a

Unlabeled filter DNA from <i>C. slooffii</i> strain:	Labeled DNA from:			
	dpm/filter minus control ^b	<i>C. slooffii</i> 2687 ^c	dpm/filter minus control ^b	<i>C. slooffii</i> 2689 ^c
2419	1564	100.3 ^e	440	98 ^e
2687	1558	100	420	94
2689	1646	105	450	100

^a Incubation was at 60 C for 14 h.^b Control filter with *E. coli* DNA gave 200 dpm.^c *C. slooffii* 2687 and *C. slooffii* 2689 DNAs had specific activities of 2.5×10^4 and 1×10^4 dpm/ μ g, respectively.^d Control filter with *E. coli* DNA gave 38 dpm.^e The results are expressed in percentages of the homologous reaction.TABLE 5. Degree of relative binding between various strains of *Torulopsis pintolespii*^a

Unlabeled filter DNA from <i>T. pintolespii</i> strain:	dpm/filter minus control ^b	Labeled DNA from <i>T. pintolespii</i> 2616 ^c
3046	850	89 ^d
2405	940	99
2616	950	100
2457	900	95

^a Incubation was at 60 C for 14 h.^b Control filter with *E. coli* DNA gave 100 dpm.^c *T. pintolespii* 2616 had a specific activity of 10^4 dpm/ μ g.^d The results are expressed in percentages of the homologous reaction.

combinations, we interpret the results of intra-specific binding as significant since unrelated yeasts normally give values close to 20% or lower (compare Table 6).

Interspecific hybridizations among *S. telluris*, *T. bovina*, *T. pintolespii*, and *C. slooffii* DNAs. The percentages of hybrid DNA formation are related to *T. bovina* 2678 or to *S. telluris* 2679 (Table 8). It is evident that the DNAs from all four species show high percentages of duplex formation as compared to the homologous reaction. It may, therefore, be concluded that the four species mentioned above are all members of a single taxon.

Intraspecific hybridization of *T. glabrata* DNAs. Although the DNA base compositions of the five strains tested (Table 9) were very close (range 39.6 to 40.2 mol% G+C), one of the strains (CBS 4692) gave a low degree of relative binding in comparison to the other four strains. Although the usual taxonomic criteria (21) were

typical of this strain of *T. glabrata*, the colony morphology was atypical in that the surface was dull rather than soft and shiny. The low degrees of homology found suggest that this strain belongs to a species different from *T. glabrata*.

Since strain IGC-2622 of *S. telluris* had a DNA base composition very close to that of *T. glabrata*, this strain was included in the hybridization experiment. Strain IGC-2622 DNA showed no evidence of hybridization with *T. glabrata* 72-28 DNA although their base compositions were identical (Table 3). This aberrant strain was reidentified by the criteria discussed by van der Walt (16) and was shown to be a typical strain of *Candida parapsilosis*. The G+C value found for this strain is in good agreement with values reported for *C. parapsilosis* in the literature (11). It is possible

TABLE 6. Degree of relative binding between various strains of *Torulopsis bovina*^a

Unlabeled filter DNA from:	Labeled DNA from			
	dpm/filter minus control ^b	<i>T. bovina</i> 2681 ^c	dpm/filter minus control ^b	<i>T. bovina</i> 2678 ^c
<i>T. bovina</i> 2760	6271	87 ^e	432	87
2678	7222	100.2	496	100
2681	7208	100		
<i>Saccharomyces cerevisiae</i>	1441	20	74	15

^a Incubation was at 60 C for 14 h.^b Control filter with calf thymus DNA gave 500 dpm.^c *T. bovina* 2681 and *T. bovina* 2678 DNAs had specific activities of 5.5×10^4 and 1.6×10^4 dpm/ μ g, respectively.^d Control filter with *E. coli* DNA gave 50 dpm.^e The results are expressed in percentages of the homologous reaction.TABLE 7. Degree of relative binding between various strains of *Saccharomyces telluris*^a

Unlabeled filter DNA from <i>S. telluris</i> strain:	dpm/filter minus control ^b	Labeled DNA from <i>S. telluris</i> 2679 ^c
2676	777	72 ^d
2679	1080	100
2663	1004	93

^a Incubation was at 60 C for 14 h.^b Control filter with *E. coli* DNA gave 96 dpm.^c *S. telluris* 2679 DNA had a specific activity of 1.6×10^4 dpm/ μ g.^d The results are expressed in percentages of the homologous reaction.

TABLE 8. Degree of relative binding between strains of four psychrophobic species of yeast^a

Unlabeled filter DNA from:	Labeled DNA from:			
	dpm/ filter minus control ^b	<i>T.</i> <i>bovina</i> 2678 ^c	dpm/ filter minus control ^b	<i>S.</i> <i>telluris</i> 2679 ^c
<i>Candida slooffii</i> 2689	1169	95 ^e		
<i>Torulopsis pintolopesii</i> 3046 2457	1193	97	4001	80
<i>T. bovina</i> 2760 2678	1045 1230	85 100	4000 4252	80 85
<i>Saccharomyces telluris</i> 2676 2679 2663	1242 1143 1142	101 93 93	5000	100

^a Incubation done at 65 C for 14 h.^b Control filter with *E. coli* DNA gave 130 dpm.^c *T. bovina* 2678 and *S. telluris* 2679 DNAs had specific activities of 1.6×10^4 and 5×10^4 dpm/ μ g, respectively.^d Control filter with *E. coli* DNA gave 430 dpm.^e The results are expressed in percentages of the homologous reaction.

that this strain was mislabeled while kept in the Brazilian culture collection.

DISCUSSION

The work reported here has shown that four recognized species of psychrophobic yeasts, which are all inhabitants of the alimentary canal of various warm-blooded animals (mammals and birds), are closely related by virtue of the similar base compositions and base sequences of their nuclear DNAs. This conclusion is based on the high percentages of heterologous DNA/DNA duplex formation as compared to homologous controls. Unrelated yeasts generally give DNA/DNA hybrid percentages around 20% or lower while closely related strains give values in the 80 to 100% range (3, 12). The data obtained here are in good agreement with these ranges.

It might be mentioned here that relatively few publications have appeared dealing with yeast systematics based on nucleic acid hybridization, and these are limited to the nitrocellulose filter disk technique. Therefore, brief mention of these methods with some comments on their advantages and disadvantages may be useful. Bak and Stenderup (1) used reciprocal binding to nitrocellulose filters, between in vitro-synthesized radiolabeled RNA (messenger RNA-like) and filter-bound nuclear (not whole-

cell) DNA. The advantages of this method are: (i) no mitochondrial sequences are present; (ii) the in vitro labeling of messenger is simpler than in vivo labeling of nuclear DNA (but more laborious than in vitro labeling of DNA with ¹²⁵I); and (iii) reciprocal binding experiments give excellent control over the data. On the other hand, a disadvantage of the method is the uncertainty that the entire genome is represented in RNA transcribed in vitro; some portions of the DNA may be selectively transcribed.

Bicknell and Douglas (3) performed DNA/DNA and ribosomal RNA/DNA reannealing experiments on nitrocellulose filter disks by the competition technique; they used in vivo labeling of the required nucleic acids. The advantages of the method are: (i) uniform labeling of the nucleic acids; (ii) a greater portion of the genome is compared with competition than by direct binding; (iii) ribosomal RNA/DNA hybridization allows comparison of organisms which are not closely related because there is a significant degree of conservation of the genes coding for ribosomal RNA in different species of yeast. A disadvantage of this approach is the poor in vivo labeling of the DNA of a number of species with tritium or ¹⁴C-labeled bases, unless radiophosphorus with its rather short half-life is used. ¹²⁵I is not only very inexpensive, but the in vitro labeling of DNAs from different yeasts was

TABLE 9. Degree of relative binding between strains of *Torulopsis glabrata* and other suspected strains of this species^a

Unlabeled filter DNA from:	Labeled DNA from:			
	dpm/ filter minus control ^b	<i>T.</i> <i>glabrata</i> 72-27 ^c	dpm/ filter minus control ^b	<i>T.</i> <i>glabrata</i> 72-28 ^c
<i>T. glabrata</i> 5040	5350	99 ^e		
72-33	4486	83		
4692	1405	26	764	21
72-28	4540	84	3637	100
72-27	5404	100	3201	88
<i>Saccharomyces tel-</i> <i>luris</i> 2622			38	1

^a Incubation was at 65 C for 14 h.^b Control filter with *E. coli* DNA gave 350 dpm.^c *T. glabrata* 72-27 and *T. glabrata* 72-28 had specific activities of 2.5×10^4 and 1×10^4 dpm/ μ g of DNA, respectively.^d Control filter with *E. coli* DNA gave 280 dpm.^e The results are expressed in percentages of the homologous reaction.

quite reproducible and gave high counts. A disadvantage of this method is the need for a gamma ray counter.

Only one of the species, *S. telluris*, is ascogenous. *T. bovina* (synonymous with *C. bovina*) has been regarded as the imperfect stage of *S. telluris* (9), and the close relationship between these species has been confirmed in this study. *T. pintolopesii* and *C. slooffii* are differentiated from *S. telluris* (*T. bovina*) by minor differences in cell size, by minimal growth temperatures, and by pseudomycelium formation (Table 1). When van Uden and do Carmo-Sousa (20) described *C. bovina*, they considered this species to be closely related to *T. pintolopesii*, described by van Uden five years earlier (18). They felt that using the ability of pseudomycelium formation to distinguish between organisms with very similar properties might lead to their artificial separation in different genera. Similarly, Kreger-van Rij (9), in her restudy of the various psychrophobic species, questioned the separation of *C. bovina* and *T. pintolopesii*, although she retained them provisionally as separate species.

Our work has now given clear evidence that all four species, i.e., *S. telluris*, *T. bovina*, *T. pintolopesii*, and *C. slooffii*, are representatives of the same taxon. The criterion of pseudomycelium formation is therefore of little or no value for species differentiation. The irrelevancy of pseudomycelium-forming ability in the diagnosis of species is supported by the earlier finding that *Candida guilliermondii* (with a well-developed pseudomycelium) revealed a high degree of DNA/DNA homology with *C. guilliermondii* var. *carpophila*, whose pseudomycelium-forming ability is either nonexistent or rudimentary (Sally A. Meyer, Ph.D. thesis, University of California, Davis, 1970). Relatively small variations in minimal growth temperature, which is the main criterion in differentiating these three imperfect yeasts (Table 1), also seem to be of doubtful taxonomic value for species differentiation. For example, Kreger-van Rij (9) reported one strain of *S. telluris* which grew slowly at 25 C, but not at 20 C as did other strains of this species. In general, variations in both minimal and maximal growth temperatures for strains of the same species have been observed by many investigators.

On the basis of this study, we are proposing to retain *S. telluris* and *T. pintolopesii* as the perfect and imperfect stages, respectively, of these psychrophobic yeasts. The name *T. pintolopesii* has priority over *T. bovina* (*C. bovina*) and *C. slooffii*.

Although the evidence presented here clearly shows that *C. slooffii* and *T. pintolopesii* are

closely related and belong to the same species, we propose to retain *C. slooffii* as a variety of *T. pintolopesii* on the following grounds. Strains of *C. slooffii* are very homogeneous, unlike those of the other three "species." The minimal temperature of *C. slooffii* is 28 C with a very short range of variation, while the minimal temperatures of strains of *T. bovina*, *S. telluris*, and *T. pintolopesii* may vary by as much as 5 C (L. R. Travassos, personal communication). In contrast to the other species, *C. slooffii* has an absolute requirement for inositol, and requirements for other vitamins show almost no variation among strains. Furthermore, none of the other species showed a response to certain nutritional imbalances similar to that of *C. slooffii* (23). These characteristics, together with a more strongly developed pseudomycelium and a somewhat more restricted host range (swine and horses), induced us to retain *C. slooffii* as a variety of *T. pintolopesii*. Thus, *T. pintolopesii* var. *slooffii* differs from *T. pintolopesii* var. *pintolopesii* by having a minimal growth temperature of 28 C, by exhibiting an absolute requirement for inositol in Wickerham's medium, and by formation of pseudomycelium.

T. glabrata is clearly a distinct species. The G+C content of its nuclear DNA is much higher than that of *T. pintolopesii* (Table 3), it is not a psychrophobic yeast (Table 1), and its cells are not short-lived in culture.

S. guttulata, on the other hand, has several features in common with the other psychrophobic organisms. Its principal points of difference are: (i) the small but significantly higher G+C content of its nuclear DNA; (ii) its absolute requirement for a high concentration of gaseous CO₂ in the environment; (iii) the very much larger vegetative cells; and (iv) the highly specific habitat in the intestinal tract of rabbits (13). It is possible that the psychrophobic species, now reduced to two ascosporeogenous species and one asporogenous species, are derived from a common ancestor and have subsequently diverged by adapting to specialized habitats.

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