Hansenula populi, a New Homothallic Species of Yeast from Exudates of Cottonwood Trees

H. J. PHAFF,* YUZO YAMADA,† JOANNE TREDICK, AND MARY MIRANDA

Department of Food Science and Technology, University of California, Davis, California 95616

The evolutionary affinities among several morphologically and physiologically similar species of the ascogenous yeast genus *Hansenula* were studied by a technique which involved genome comparisons in combination with nuclear deoxyribonucleic acid base composition determinations. The results revealed a new member of the genus *Hansenula* that was recovered five times in 1968 from slime exudates of *Populus trichocarpa* (cottonwood trees) in British Columbia and Alaska. This new species is named *Hansenula populi* because of its specific habitat in exudates of cottonwood trees. *H. populi* is homothallic and occurs naturally in the haploid state. Conjugation between vegetative cells precedes sporulation, and up to four hat-shaped spores are produced in zygotes. The spores are liberated from the asci soon after formation. *H. populi* resembles *Hansenula nonfermentans* and *Hansenula dryadoides* but differs from these species in habitat, guanine-plus-cytosine content of the nuclear deoxyribonucleic acid, maximum growth temperature, and ability to assimilate several carbon compounds. The type strain of *H. populi* is UCD-FS&T 68-628C (= CBS 8094 = ATCC 48773).

In 1968 Phaff et al. (8) isolated five strains of yeast from exudates of cottonwood trees (*Populus trichocarpa* Torrey and Gray) growing in five widely separated locations in British Columbia and Alaska. These strains were identified as *Hansenula nonfermentans* Wickerham by using the key provided by Wickerham (16). All five strains had similar properties. However, these properties differed from those of the type strain of *H. nonfermentans* in the following respects: assimilation of trehalose negative or very weak, assimilation of L-rhamnose positive, assimilation of D-ribose and ribitol negative, assimilation of lactic acid and glucono- δ -lactone positive, and growth at 37°C negative. *H. nonfermentans* was represented by only a single strain, which Wickerham isolated from the water of a mountain stream in Wyoming (16). The guanine-plus-cytosine (G+C) contents of the nuclear deoxyribonucleic acids (DNAs) of two of our strains were close to the value reported by Nakase and Komagata (7) for the type strain of *H. nonfermentans*, approximately 45 mol% G+C. *Hansenula dryadoides*, a more recently described species (11), was also included in our study, because it has a number of phenotypic properties in common with the yeasts isolated from cottonwood exudates. In this study we conducted DNA-DNA complementarity experiments to

determine whether the five strains obtained from *Populus* exudates represent a new species of *Hansenula*. Because there was little complementarity between the DNA of *H. nonfermentans* and the DNAs of the strains isolated from cottonwood trees, we regard these strains as belonging to a new species of *Hansenula*, for which we propose the name *Hansenula populi* (pop'u.li. L. n. *populus* generic name of cottonwood; L. gen. n. *populi* of cottonwood) because its habitat is the exudate of cottonwood trees. A description of this new species is given below.

MATERIALS AND METHODS

Samples of slime fluxes were collected during June and July of 1968 in various locations of Alaska, the Yukon Territory, British Columbia, Washington, Oregon, and California (8). The precise locations of the samples from *Populus* which contained the new species of *Hansenula* are given in Table 1.

The samples were collected in new plastic vials or bags. Usually within 6 to 18 h after collection, a loopful of the slimy exudate was streaked directly onto 5% malt agar acidified to pH 3.7 with a precalculated amount of 1 N HCl. The plates were stored at ambient temperatures (ca. 15 to 25°C) until colonies appeared. Despite the low pH, varying numbers of bacterial colonies usually appeared together with colonies of yeasts. After 3 to 6 days, the plates were inspected with a dissecting microscope, and for identification purposes one yeast colony of each morphological type was brought into pure culture by two successive platings on malt agar. For further details concerning the collecting areas, see Table 1 and the study of Phaff et al. (8).

† Present address: Laboratory of Applied Microbiology, Department of Agricultural Chemistry, Shizuoka University, Shizuoka 422, Japan.

TABLE 1. Strains used

Strain ^a	Source	G+C content of nuclear DNA (mol%)
<i>H. populi</i> UCD-FS&T 68-628C ^T	Exudate of <i>P. trichocarpa</i> , Alcan Highway 55 miles (ca. 90 km) northwest of Dawson Creek, British Columbia, Canada	44.2 ± 0.35 ^b
<i>H. populi</i> UCD-FS&T 68-809C	Exudate of <i>P. trichocarpa</i> , Alcan Highway 2 miles (ca. 3.2 km) south of Fort Nelson, Canada	44.0 ± 0.27
<i>H. populi</i> UCD-FS&T 68-676B	Exudate of <i>P. trichocarpa</i> , Delta Junction, Alaska	43.9 ± 0.12
<i>H. populi</i> UCD-FS&T 68-644A	Exudate of <i>P. trichocarpa</i> , Kenai Peninsula, Alaska	43.9 ± 0.06
<i>H. populi</i> UCD-FS&T 68-603	Exudate of <i>P. trichocarpa</i> , Prince Rupert, British Columbia, Canada	43.8 ± 0.40
<i>H. nonfermentans</i> UCD-FS&T 72-15 ^T	Stream water, Wyoming	45.3 ± 0.16
<i>H. dryadoides</i> UCD-FS&T 80-296 ^T	Insect tunnels in <i>Ficus</i> spp., South Africa	31.0 ± 0.09
<i>Saccharomyces cerevisiae</i> UCD-FS&T C-146	Industrial champagne yeast	39.5 ± 0.19

^a UCD-FS&T, Collection of the Department of Food Science and Technology, University of California, Davis.

^b Calculated from the average of at least three buoyant density determinations. Mean ± standard deviation.

Identification of the isolates involved characters determined by standard methods currently used in yeast taxonomy (14). DNA extraction and purification were done by a combination of the procedures of Marmur (6) and Bernardi et al. (1), as described by Price et al. (9). The G+C contents of the DNAs were calculated from buoyant density values in cesium chloride (10, 13) and were based on three separate determinations. The DNA of strain 2039 of *Micrococcus luteus* (synonym, "*Micrococcus lysodeikticus*" [not on the Approved Lists of Bacterial Names {12}]), which was obtained from the International Collection of Phytopathogenic Bacteria, Department of Bacteriology, University of California, Davis, was used as a reference (buoyant density, 1.7311 g/ml). The buoyant density of the "*M. lysodeikticus*" DNA was determined from a comparison with plasmid-free *Escherichia coli* K-12 DNA, whose buoyant density was taken to be 1.7100 g/ml.

DNA reannealing reactions. For the reassociation experiments, purified DNA was concentrated by electrophoresis in an ISCO concentrator and subsequently adjusted to a concentration of 700 µg/ml before shearing. Reference DNA that was to be labeled with ¹²⁵I was further purified by ultracentrifugation with a Beckman model L ultracentrifuge to eliminate residual protein and ribonucleic acid before iodination. Peak fractions, as determined by absorbance at 260 nm, were pooled and dialyzed against 0.113 M ammonium acetate-0.053 M acetic acid buffer (pH 5) for 48 h. Labeling with ¹²⁵I was done after the DNA solution was denatured in a glycol bath at 110°C by the method of Commerford (5), using approximately 200 µCi of ¹²⁵I per 200 µg of DNA. After subsequent shearing and removal of rapidly renaturing sequences (9), the activity of the labeled DNA was approximately 5 × 10⁵ cpm/µg of DNA.

Unlabeled DNA was sheared to an average molecular weight of approximately 300,000 by two passages through a miniature French press at 13,500 lb/in².

Reference DNA (concentration, approximately 150 µg/ml) was sheared after iodination by the same method. After shearing, all DNAs were filtered through membrane filters (pore size, 0.45 or 1.2 µm; Metricel) to remove debris. Rapidly renaturing sequences were removed from the labeled DNA by the method of Price et al. (9).

The final concentrations of unlabeled and labeled DNAs were determined by the diphenylamine test (4). Subsequently, unlabeled DNA was diluted to a concentration of 500 µg/ml, and labeled DNA was diluted to a concentration of 2 µg/ml for renaturation experiments.

The renaturation kinetics of the reference DNA was determined by a C₀t analysis (3). Reannealing reactions were done by using the protocol of Price et al. (9). Separation of single- and double-stranded DNAs was performed by the hydroxylapatite batch method (2), as modified by Price et al. (9). The resulting samples were transferred to counting tubes and counted with a Nuclear Chicago model 1185 gamma ray counter (90% efficiency). Calculations of sequence complementarity were performed by the method of Price et al. (9).

RESULTS

The nuclear DNA base compositions of the organisms studied are shown in Table 1. We included *H. dryadoides* (11) in this study because this species has a significant number of phenotypic properties in common with the strains isolated from cottonwood trees. It is clear that of the two *Hansenula* species that have a significant number of phenotypic traits in common with *H. populi*, *H. dryadoides* obviously shows little relationship to *H. populi*. Thus far, in our laboratory, yeasts with base compositions differing by more than about 1.5 mol%

G+C (as determined by the buoyant density equilibrium centrifugation technique) have not been found to be closely related. On the other hand, *H. nonfermentans* could be conspecific with *H. populi*.

Strain UCD-FS&T 68-628C^T (type strain) of *H. populi* was chosen as the reference organism for DNA base sequence comparisons. The renaturation kinetics of homologous reference DNA from strain UCD-FS&T 68-628C^T were followed to a C₀t value of 100 mol · s/liter (C₀t is the initial nucleotide concentration [in moles per liter] multiplied by the time [in seconds] [3, 9]). At this stage renaturation was 67.5% complete. A modified Wetmur-Davidson plot (15) of the early data points of the C₀t curve showed that only 2.3% of the rapidly renaturing sequences remained in the radiolabeled preparation.

Table 2 shows the results of heterologous reannealing reactions when DNA from the type strain of *H. populi* was used as the reference DNA. Three strains of *H. populi* shared more than 90% sequence complementarity with the *H. populi* reference strain. The fourth strain, strain UCD-FS&T 68-603, shared only 77% of its base sequences with the reference strain. Our results also clearly show that neither *H. nonfermentans* nor *H. dryadoides* had significant DNA sequence similarity to *H. populi*, thus precluding conspecificity.

H. populi strain UCD-FS&T 68-603 seemed to have undergone significant divergence from the other four strains studied. To determine whether this divergence was real or due to experimental imperfection, a reverse experiment was done, in which strain UCD-FS&T 68-603 was labeled and used as a reference for the other strains. *H. dryadoides* was used as a negative control. Table 3 demonstrates that the other four strains of

H. populi all showed approximately corresponding lower degrees of homology with the reference strain. Although the results of reciprocal labeling experiments suggested that there may be a slight difference in genome size between strain UCD-FS&T 68-603 and the other four strains of *H. populi*, the main difference appears to be a change in base sequences and a resulting genetic divergence.

The DNA reannealing experiments described above were the basis for regarding *H. populi* as a new species, and a description of this species is given below.

Latin diagnosis of *Hansenula populi* sp. nov. In extracto malti post dies 3 cellulae subovoideae aut rotundae, 2.0–5.0 × 2.5–5.6 μm, singulae, binae, aut in catenis brevis; sedimentum et annulus tenuis formantur. Post dies 21 sedimentum formatur et annulus tenuis. Pellicula non formatur.

Cultura in agaro malti post 1 mensem (20°C) crenea, mollis, nitida, glabra, demissa convexa aut plana; margo lobata aut glabra.

In agaro farinae Zea mays post dies 14 pseudomycelium vel mycelium verum nullum.

Species homothallica, haploideae. Orientur asci ex conjugatione inter cellulas haploideae. Pileiformae ascosporae formantur ad quattuor in quoque asco; asci rumpuntur post 4–6 dies.

Fermentatio glucosi nullum.

Glucosum, cellobiosum, D-xylosum, L-rhamnosum, ethanolum, glycerolum, D-mannitolum, D-glucitolum, salicinum, glucono-delta-lactonum, acidum gluconicum, acidum lacticum, et acidum succinicum assimulantur, at non D-galactosum, L-sorbosum, maltosum, saccharosum, trehalosum, lactosum, melibiosum, raffinolum, melezitolum, inulinum, amyllum solubile, L-arabiosum, D-arabiosum, D-ribosum, i-erythritum

TABLE 2. Reannealing between labeled DNA from *H. populi* UCD-FS&T 68-628C^T and DNAs from seven strains of yeast and calf thymus^a

Organism or tissue	Actual binding (%) ^b	Corrected actual binding (%) ^c	Relative binding (%) ^d
<i>H. populi</i> UCD-FS&T 68-628C ^T	67.5 ± 0.03 ^d	66.4	100.0
<i>H. populi</i> UCD-FS&T 68-809C	63.6 ± 0.6	62.4	94.0
<i>H. populi</i> UCD-FS&T 68-676B	62.3 ± 0.7	61.1	91.9
<i>H. populi</i> UCD-FS&T 68-644A	63.6 ± 0.8	62.4	94.0
<i>H. populi</i> UCD-FS&T 68-603	52.6 ± 0.5	51.0	76.8
<i>H. nonfermentans</i> UCD-FS&T 72-15 ^T	5.6 ± 1.6	2.5	3.8
<i>H. dryadoides</i> UCD-FS&T 80-296 ^T	4.7 ± 0.4	1.6	2.4
<i>Saccharomyces cerevisiae</i> UCD-FS&T C-146	5.6 ± 0.4	2.5	3.7
Calf thymus	4.1 ± 0.4	1.0	1.4

^a ¹²⁵I-labeled DNA (0.2 μg) and unlabeled DNA (200 μg) were incubated in 0.5 ml of 280 mM phosphate buffer at 65.1°C.

^b Average of three samples, corrected for zero-time binding.

^c Corrected for self-renaturation (3.3 ± 0.7%) (9).

^d Mean ± standard deviation.

TABLE 3. Reannealing between labeled DNA from *H. populi* UCD-FS&T 68-603 and DNAs from five strains of yeast and calf thymus^a

Organism or tissue	Actual binding (%) ^b	Corrected actual binding (%) ^c	Relative binding (%) ^c
<i>H. populi</i> UCD-FS&T 68-603	58.9 ± 0.5 ^d	56.5	100
<i>H. populi</i> UCD-FS&T 68-628C ^T	39.2 ± 2.0	35.7	63.1
<i>H. populi</i> UCD-FS&T 68-809C	42.4 ± 0.7	39.0	69.0
<i>H. populi</i> UCD-FS&T 68-676B	41.7 ± 0.3	38.2	67.7
<i>H. populi</i> UCD-FS&T 68-644A	42.1 ± 0.9	38.7	68.4
<i>H. dryadoides</i> UCD-FS&T 80-296 ^T	9.8 ± 1.2	4.5	8.0
Calf thymus	10.2 ± 0.9	4.9	8.7

^a ¹²⁵I-labeled DNA (0.2 µg) and unlabeled DNA (200 µg) were incubated in 0.5 ml of 280 mM phosphate buffer at 64.3°C.

^b Average of three samples, corrected for zero-time binding.

^c Corrected for self-renaturation (5.9 ± 1.4%) (9).

^d Mean ± standard deviation.

lum, ribitolum, galactitolum, α-methyl-D-glucosidum, 2- et 5-ketogluconatum, acidum citricum, i-inositolum, methanolum, glucosaminum, nec hexadecanum.

Kalium nitricum, natrium nitrosum, ethyl aminum, et lysinum assimilantur.

Ad crescentium vitaminarum externarum necessariae sunt.

Crescere potest in 30°C; nullum incrementum calore 33°C.

Crescere potest in 1 µg cyclohexamidum/ml; nullum incrementum in 10 µg cyclohexamidum/ml.

G+C acidi deoxyribonucleati, 43.8–44.2 mol% (quinque stirpes) (Table 1).

Habitatio in exudati Populi trichocarpi.

Typus: stirps UCD-FS&T 68-628C (= CBS 8094) ex exudato P. trichocarpi Canadensis isolata est.

In collectione zymotica Centraalbureau voor Schimmelcultures, Delphi Batavorum, sub no. 8094 deposita est.

Description of *H. populi*: growth in malt extract. After 3 days at 24°C, the cells are short ovoid, sometimes spheroid, and measure 2.0 to 5.0 by 2.5 to 5.6 µm. They occur usually singly or in pairs, but sometimes in small clusters or short chains. A thin ring and a small amount of sediment are present, but no pellicle is formed. After 3 weeks a moderate amount of sediment is present, the ring is very thin or absent, and a film is lacking.

Growth on malt agar. After 1 month streak cultures are cream colored, smooth, shiny, soft, and low convex to flat in cross-section; the border is entire to finely lobed.

Dalmau plate culture on corn meal agar. After 12 days a pseudomycelium is absent or very rudimentary under aerobic conditions. Under a cover glass spotty clusters of undifferentiated cells growing into the agar are occasionally observed.

Formation of ascospores. Vegetative cells are mostly haploid and homothallic. Some diploid cells may occur. Conjugation takes place between two haploid cells of approximately equal size, and normally four derby hat-shaped spores develop in one of the cells, forming a dumbbell-shaped ascus. Occasionally spores are formed in diploid cells. Asci lyse early, releasing the spores in groups of two, three, or four (Fig. 1). Good sporulation was obtained on malt agar after 2 to 4 days.

Other characteristics.

Glucose is not fermented.

The following carbon compounds are assimilated: D-glucose, cellobiose, D-xylose, L-rhamnose, ethanol, glycerol, D-mannitol, D-glucitol, salicin, glucono-δ-lactone, gluconic acid, lactic acid, and succinic acid. D-Galactose, L-sorbose, maltose, sucrose, trehalose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, L-arabinose, D-arabinose, D-ribose, i-erythritol, ribitol, galactitol, methyl-α-D-glucoside, 2-ketogluconate, 5-ketogluconate, citrate, i-inositol, methanol, glucosamine, and hexadecane are not assimilated.

The following nitrogen compounds are assimilated: potassium nitrate, sodium nitrite, ethyl amine, and lysine.

Vitamin requirements: biotin and pyridoxine are required for growth; two strains (including the type strain) are stimulated by thiamine, whereas the other strains require thiamine.

Growth on 50% (wt/wt) glucose–yeast extract agar: absent.

Growth on 10 and 7.5% (wt/vol) sodium chloride–glucose–yeast extract agars: absent. Growth on 5% NaCl agar: positive (latent).

Maximum temperature for growth: 30°C.

Acid formation on chalk agar: positive (weak).

Synthesis of starchlike compounds: negative.

Gelatin liquefaction: negative.

Casein hydrolysis: negative.

FIG. 1. *H. populi* sp. nov. strain UCD-FS&T 68-628C^T. After 1 to 2 days at room temperature on malt extract agar, vegetative cells of *H. populi* UCD-FS&T 68-628C^T undergo conjugation. The zygote has been observed to bud; diploid asci rapidly differentiate ascospores (a). However, the zygote usually differentiates ascospores immediately after conjugation (b and c). Asci dehisce soon after formation, and clusters of hat-shaped ascospores may be observed within 48 h (d). Bar = 5 μ m. Other strains of *H. populi* behave very similarly.

Hydrolysis of urea: urease test negative.

Lipolytic activity: negative.

Growth in the presence of 100 or 10 μ g of cycloheximide per ml: negative. Growth in the presence of 1 μ g of cycloheximide per ml: positive (weak).

G+C content of the nuclear DNA: 43.8 to 44.2 mol% (range for five strains [Table 1]).

Type strain. The type strain, UCD-FS&T 68-628C, was isolated from a slime flux of a cottonwood tree (*P. trichocarpa* Torrey and Gray) along the Alcan Highway, 55 miles (ca. 90 km) northwest of Dawson Creek, British Columbia, Canada. Strain UCD-FS&T 68-628C^T has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures in Delft, the Netherlands, as strain CBS 8094 and in the American Type Culture Collection, Rockville, Md., as strain ATCC 48773.

Sources. Table 1 lists the substrates, localities, and G+C contents of the nuclear DNAs of the five strains isolated. It appears that *P. tricho-*

carpa growing in the northwestern part of North America is the host for *H. populi*.

DISCUSSION

We have no explanation for the genetic divergence of strain UCD-FS&T 68-603 compared with the type strain and the other three strains (Tables 2 and 3). Phenotypically, the only differences observed for strain UCD-FS&T 68-603 compared with the other strains were lack of growth at 30°C and latent rather than strong growth on media containing D-xylose and D-mannitol. Allopatry does not appear to play a significant role because the strains were isolated from trees that were growing very large distances from each other in British Columbia and Alaska.

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