NEW HOMOTHALLIC TAXA OF HANSENULA

by

LYNFERD J. WICKERHAM

Northern Regional Research Laboratory [1]), *Peoria, Illinois* 61604

(with 1 fig.)

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Starting in 1951, the author has classified the species of *Hansenula* according to a phylogenetic interpretation of this expanding genus (WICKERHAM, 1951; WICKERHAM & BURTON, 1962). Phylogeny is based on change in ploidy of vegetative cells, the most primitive species being haploid and the most recently evolved species being diploid. Intermediate species are isolated from nature as both haploid and diploid cells. Although there are probably some haploid species poised just below the diploid level that have not been isolated from nature in the diploid state, still these species convert readily to the diploid state on laboratory media. One example is *Hansenula polymorpha* MORAIS & DÁLIA MAIA (1959) (see TEUNISSON, HALL & WICKERHAM, 1960).

Phylogeny in *Hansenula* is based secondarily on habitat and on increase or decrease in physiological capacities as correlated with habitat. Most homothallic lines within the genus have comparable heterothallic lines. Lines 1a, 1b, 3 and 5 of Fig. 1 are homothallic and, perhaps largely for this reason, include more species than the heterothallic lines 2 and 4. It is less easy to isolate both sexes of the heterothallic haploid species than the single strain necessary to demonstrate sexuality in homothallic species. This situation is especially true of species that are rare or of common species like *Hansenula holstii* WICKERHAM (1960), very few isolates of which are known to be sexually active. Further, none of the species of line 3 produce a gaseous fermentation, but *H. bimundalis* WICKERHAM & SANTA MARIA and its variety *americana* WICKERHAM (1965b) of line 4 do produce a latent but strong fermentation of glucose. Possibly they may some day be transferred to a heterothallic line comparable

[1]) This is a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

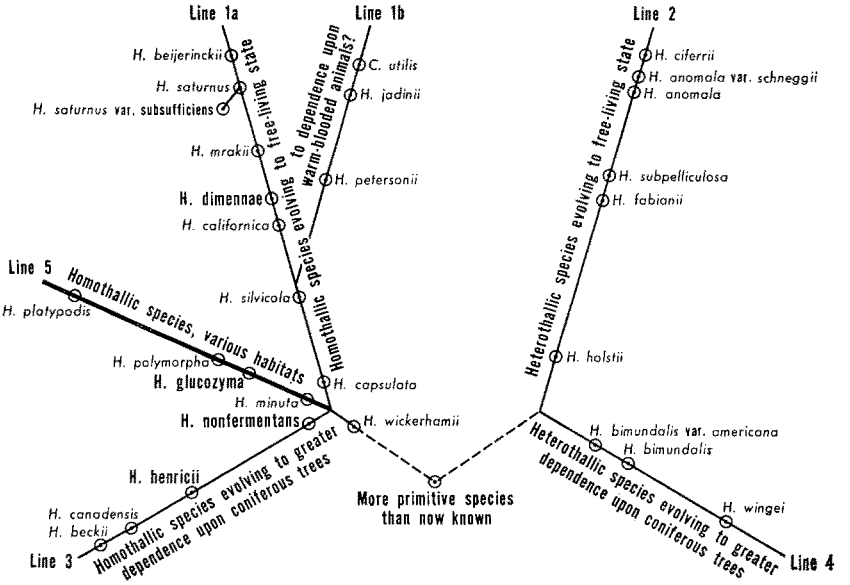

Fig. 1. Diagram of presumed phylogenetic relationships among species of the genus *Hansenula*.

to line 5 (all species of which ferment glucose) when more heterothallic haploid species are known for the genus. Sexual studies led to the discovery of heterothallism in diploid species of *Hansenula* (WICKERHAM & BURTON, 1954; WICKERHAM, 1956, 1958) and to enlargement of the genus by addition of the heterothallic haploid species *H. holstii, H. fabianii* (WICKERHAM, 1965a), and *H. bimundalis*.

The relationship of *Hansenula* to *Pichia* and *Pachysolen* has been strengthened by common ability of primitive species of these three genera to synthesize extracellular phosphomannans (WICKERHAM & BURTON, 1961; SLODKI et al., 1961). Structural and physical differences exist between phosphomannans produced by various species (JEANES et al., 1961; SLODKI, 1962, 1963). Among yeasts, only the three genera mentioned are known to produce extracellular phosphommannans. EDDY (1958) found bound phosphorus-containing mannanprotein complexes in strains of *Saccharomyces cerevisiae* and closely related species. Nearly all the phosphorus was apparently an integral part of the mannans and represented about 60 % of that found in the cell wall. MILL (1966) reported phosphomannans to occur in the cell walls of *S. cerevisiae*, and YELINOV & VITOVSKAYA (1965) also reported a similar occurrence in *Candida pseudotropicalis* (the imperfect form of *Kluyveromyces fragilis*). Presumably, bound phosphomannans may indicate a wider range of related genera than the extracellular phosphomannans.

Media and Procedures

Most of these were published by Wickerham in 1951. For convenience of the reader, a dalmau plate is inoculated at two points and along a line. Sterile cover glasses are placed over one point and across the center of the line. The anaerobic condition caused by the cover glass promotes development of hyphae which may be observed directly under a microscope. The yeast morphology medium (MM) for the dalmau plates is chemically defined as are the following media used for physiological tests: Yeast nitrogen base is for carbon assimilation tests and yeast carbon base for nitrogen assimilation tests. Yeast vitamin-free base is for determining either the ability of a yeast to grow in the absence of vitamins or the specific vitamins required for growth. All these media, in addition to yeast extract-malt extract (YM) agar and broth for general cultivation and maintenance of yeasts, are prepared according to our formulae and sold by Difco Laboratories [1]) of Detroit, Michigan. Malt extract (ME) medium is for the production of ascospores.

Some procedures and media, not previously published, have been applied at the Northern Laboratory for many years to isolate yeasts from substrates in which yeasts may be outnumbered by bacteria and molds: Isolation medium (IM) is designed to aid the development of yeast in general but provides additional enhancement of species, such as *Hansenula,* that assimilate a moderate number of carbon compounds. The 10× strength medium consists of 6.7 g of dehydrated yeast nitrogen base; 5.0 g of glucose; 1.0 g each of cellobiose, D-xylose, L-rhamnose, *i*-erythritol, D-mannitol, and calcium 2-ketogluconate; 0.1 g each of yeast extract and malt extract; and 100 ml of distilled water. The pH is not adjusted, and sterilization is by filtration.

Another isolation medium that often gives good results is 20D. It consists of 20 % glucose plus 0.1 % each of yeast extract and malt extract in distilled water, the medium being autoclaved in flasks. Sterile 10× nitrogen base is added. Cultivation is the same as for the IM medium.

One milliliter of 10× isolation medium (IM) is pipetted into 9 ml of sterile water in a 50-ml Erlenmeyer flask. A small amount of frass, soil, or other solid substrate is added and the culture is shaken for 4 to 6 days, usually at 25° C. The flasks are then placed on a flat surface for about 15 to 30 minutes until the yeast cells have settled to the bottom. All flasks are inclined in turn so that the medium and mold growth flow to the lower side, the bacteria having been largely eliminated by the acidic reaction of the medium. Cells of yeasts adhering to the upper part of the bottom of the flask are removed to a second flask of sterile IM, and the second serial culture is shaken until abundant growth occurs. Then cells are streaked on

[1]) Mention of firm names or trade products is for identification only and does not imply endorsement by the Department of Agriculture.

YM plates. Often the plates produce only yeast colonies, but occasionally molds develop abundantly in the first flask and evidently kill any yeasts originally present in the sample.

Hansenula nonfermentans WICKERHAM, sp. nov.

A natura isolata in forma cellularum haploidearum homothallicarum. Conjugatio plerumque inter cellulas maturas gemmasque. Ascosporae plerumque exiguae, pilliformes, 1–4 numero, plerumque 2–3 in asco. Cellulae ellipsoideae aut sphaeroideae, 1.4×2.6 ad 2.1×3.1 μ. Pseudohyphae et hyphae desunt. Pelliculae desunt. Non fermentatur glucosum, galactosum, maltosum, sucrosum, lactosum, nec raffinosum. Assimilatur nitras, glucosum, cellobiosum, trehalosum, D-xylosum, D-ribosum, ethanolum, glycerolum, adonitolum, mannitolum, sorbitolum, salicinum, succinatum, citratumque. Non assimilatur galactosum, L-sorbosum, maltosum, sucrosum, lactosum, melibiosum, raffinosum, melezitosum, inulinum, amylum solubile, L-arabinosum, D-arabinosum, L-rhamnosum, D-glucosamini hydrochloridum, *i*-erythritolum, dulcitolum, α-methyl-D-glucosidum, kalii glyconas, calcii 2-ketogluconas, kalii 5-gluconas, kalii-natrii saccharas, pyruvas, lactas, acetoacetas ethylicus, nec *i*-inositolum. Esteres non fiunt. Vitaminis externis opus est.

Typus: NRRL YB-2203, isolata ab aqua in montibus Medicine Bow in Wyoming collecta mense Augusto, 1950. Deposita in Officina Investigationum Tractus Borealis, Peoria, Illinois.

Source of culture

The only strain known of this species, NRRL YB-2203, was isolated from fast running, clear water taken from Libby Creek, a mountain stream in the Medicine Bow Range of southeastern Wyoming. The water was collected in August 1950, and the yeast was isolated following enrichment in 20D medium.

Sexuality

H. nonfermentans sporulates well on ME, YM, and MM. Usually sporulation of this homothallic yeast is preceded by conjugation between a mature cell and its bud and is less commonly preceded by conjugation of independent cells. When cultures on ME slants are 48 hours old at 25° C, the growth is slightly mucoid and some of the cells near the base flow downward to form pools at the bottom of the slants. Even in cultures so young, nearly all asci liberate their tiny hat-shaped ascospores. Brims, where apparent, are quite reduced. Liberated spores enlarge slightly, increase in refractility only slightly, and agglutinate in large clusters. They measure from 1.2 to 2.4 μ in diameter, and if present at all, oil globules remain tiny. Two spores are the most common in intact asci, but asci with three and four spores are also observed. Ascospores are present on 5-day-old YM slants. The mucoid pools of growth at the bottom of 30-day-old ME slants are pale pink, and the ascospores in them

remain tiny. They are among the smallest in the genus and are about the same size as spores of *H. minuta* WICKERHAM (1951) and *H. wickerhamii* CAPRIOTTI (1961). Spores of all three species are considerably smaller than those of *H. glucozyma* sp. nov. and *H. henricii* sp. nov. (which are described later in this paper). Their phylogenetic positions are indicated in lines 3 and 5 of Fig. 1.

Morphology

Colonies on YM plates are mucoid, becoming butyrous with age as commonly happens with colonies that are mucoid because they contain a phosphomannan. The more isolated colonies become butyrous when 4 days old, sooner than less isolated colonies. Colonies are small when compared with colonies of diploid species of the genus. They are highly glistening, convex to conical, and hyphae are absent. Esters with a strong odor of ethyl acetate are not produced, but a slight sweet odor is sometimes noticeable when the colonies are about 4 days old.

The dalmau colony is smooth, gray-white, and highly glistening; it lacks hyphae, is stiff mucoid when young, and becomes butyrous with age. Cells at the edge of the colony occur singly, in pairs, and in small clusters. Most of the cellls are ellipsoidal, varying from 1.4×2.0 to 2.1×3.1 μ, but a few of the larger cells are spheroidal. Ascospores are present.

Cells at the edge of the growth under the cover glass of the dalmau streak are ellipsoidal, cylindroidal, and very small; but after some 10 cells nearer the center of the line of inoculation, the cells are much larger and spheroidal. Neither pseudohyphae nor true hyphae are produced.

Pellicles are not produced on media used for the assimilation tests.

Physiology

H. nonfermentans does not ferment glucose, galactose, maltose, sucrose, lactose, melibiose, or raffinose.

Compounds assimilated are as follows:

Glucose *)	+	D-Xylose	+	α-Methyl-D-glucoside	—
Galactose	—	L-Arabinose	—	Salicin	+
L-Sorbose	—	D-Arabinose	—	K-D-Gluconate	—
Maltose	—	D-Ribose	+	Ca-2-Ketogluconate	—
Sucrose	—	L-Rhamnose	—	K-5-Ketogluconate	—
Cellobiose	+	D-Glucosamine . HCl	—	NaK-Saccharate	—
Trehalose	+	Ethanol	W	Pyruvate	—
Lactose	—	Glycerol	+	DL-Lactate	—
Melibiose	—	i-Erythritol	—	Succinate	V
Raffinose	—	Adonitol	+	Citrate	V
Melezitose	—	Dulcitol	—	Ethylacetoacetate	—
Inulin	—	D-Mannitol	+	i-Inositol	—
Starch	—	D-Sorbitol	+	Nitrate	+

*) The + sign indicates abundant growth; V, variable growth; W, weak growth; and —, no growth.

An external source of vitamins is required, growth does not occur in yeast nitrogen base containing 10 % sodium chloride and 5 % glucose, typical esters are not produced, growth occurs at 37° C, and starch is not synthesized.

Phylogenetic position

Phylogenetic lines 3 and 4 (Fig. 1) are composed of species that produce either a latent or no gaseous fermentation, that depend upon their environment for vitamins, and that fail to develop the strongly oxidative mat colony so characteristic of *Hansenula mrakii* (WICKERHAM, 1951), *Hansenula petersonii* (WICKERHAM, 1964), and *Hansenula anomala* (HANSON) H. & P. SYDOW.

Of the currently known species of *Hansenula*, the one that is most closely related to *H. nonfermentans* is *H. minuta* of line 5. *H. minuta* possesses three abilities that *H. nonfermentans* lacks. These are the production of a gaseous fermentation of glucose, the ability to assimilate potassium gluconate in shaken cultures, and the ability to grow at 37° C. The two species differ also in the ratio of phosphate to mannose in their respective phosphomannans. *H. minuta* produces a polymer with only one phosphate group to 27 mannose units, but *H. nonfermentans* constructs a polymer with one phosphate to approximately 10 mannose units.

Hansenula glucozyma WICKERHAM, sp. nov.

Cellulae haploideae homothallicae a natura isolatae. Conjugatio plerumque inter cellulas maturas gemmasque. Asci plerumque continent ascosporas 2 pilliformes, aliquando 3 vel 4. Cellulae ellipsoideae aut sphaeroideae 2.4×3.1 ad 6.6×7.3 μ. Nec hyphae nec pseudohyphae fiunt. Desunt pelliculae. Glucosum fermentatur. Non fermentatur galactosum, maltosum, sucrosum, lactosum nec raffinosum. Assimilatur nitras, glucosum, cellobiosum, trehalosum, D-xylosum, D-ribosum, L-rhamnosum, ethanolum, glycerolum, *i*-erythritolum, adonitolum, D-mannitolum, D-sorbitolum, salicinum, citratumque. Non assimilatur galactosum, L-sorbosum, maltosum, sucrosum, lactosum, melibiosum, raffinosum, melezitosum, inulinum, amylum solubile, L-arabinosum, D-arabinosum, D-glucosamini hydrochloridum, dulcitolum, α-methyl-D-glucosidum, kalii gluconas, calcii 2-ketogluconas, kalii 5-ketogluconas, kalii-natrii saccharas, pyruvas, lactas, succinas, acetoacetas ethylicus, nec *i*-inositolum. Esteres non fiunt. Vitaminis externis opus est.

Typus: NRRL YB-2185, isolata a dejectis coleopterorum in cortice *piceae engelmannii* PARRY, collectis in montibus Medicine Bow in Wyoming. Deposita in Officina Investigationum Tractus Borealis, Peoria, Illinois.

Source

Strain NRRL YB-2185, the only strain known of this species, was isolated from frass of a live tree of the species *Picea engelmannii*

PARRY serving as host to bark beetles of the genus *Ips*. Live beetles were present when the frass was collected in August 1950 in the Medicine Bow Mountains of Wyoming. Small amounts of frass were enriched in various shaken media. The medium that yielded the selected colony isolate was 20D.

Sexuality

Growth on ME slants when 48 hours old is mucoid, and cells collect at the base of the slant. The cells, tending to remain in small clusters, are spheroidal; conjugations usually occur between cells and their buds; but quite often independent cells conjugate. Unconjugated asci that arise from diploid vegetative cells are occasionally observed. Such asci often have one or more live buds. Ascospores number from two, predominating in young cultures, to four, in older cultures. The abundantly produced spores are hat-shaped with a reduced brim. They measure from 2.0 to 3.5 μ and are much larger than those of *H. minuta*, its more primitive relative on line 5 (Fig. 1). The free ascospores of *H. glucozyma* generally occur in clusters of three or four spores but after liberation enlarge and become rather strongly refractile. Growths on young ME and YM slant cultures are more mucoid than similar cultures of *H. minuta* and *H. nonfermentans*. The growth of *H. glucozyma* changes from mucoid to butyrous as the slants age, the growth on 9- to 12-day-old slant cultures generally being butyrous. Of these three species, sporulated cultures of *H. glucozyma* are a deeper pink. This color is especially noticeable in the aerobic streak on MM.

Because ascospores and vegetative cells of strain YB-2185 are so much larger than spores and cells of the more primitive species *H. minuta* and *H. nonfermentans* and because some unconjugated asci have been observed, it was assumed that *H. glucozyma* approaches sufficiently the diploid level to permit easy isolation of diploid vegetative cells. To confirm this assumption, cells from a sporulated slant culture were streaked on several plates containing ME agar. When the plates had incubated 13 days at 25° C, the colonies were mucoid and pinkish, but intensity of color varied little. The center of a well-isolated colony that was slightly more pinkish than most of the others consisted almost exclusively of free ascospores. At its edge were many intact asci containing two to four ascospores. Since none of the asci were conjugated the colony had arisen from a diploid cell.

Cells from the extreme edge of the colony were streaked on YM plates. Cells from the edges of four of the resulting young colonies were transferred to YM and ME slants; the YM slants were refrigerated and the ME slants were incubated at 25° C. When 48 hours old, the growth on all four ME slants contained asci that were exclusively unconjugated. The refrigerated YM slants of colony isolates 1 and 4 were incubated 1 day and lyophilized the next day as sources of diploid vegetative cultures of the species.

Morphology

Colonies on streak plates of YM agar are smooth, gray-white, convex to conical, and without hyphae; esters are not produced. The colonies may be mucoid, submucoid, or butyrous and may vary from highly glistening to glazed in appearance; consistency and texture vary with age of the colonies and degree of isolation among the colonies. Haploid colonies that are 15 days old on YM agar remain gray-white, whereas diploid colonies are pale pink.

Young dalmau colonies on MM are twice the diameter of dalmau colonies of *H. minuta* and *H. nonfermentans*. The colony of *H. glucozyma* at 7 days is flat, slightly conical, and slightly raised in the center. Partly because it is the only colony on the dalmau plate, it is only moderately glistening; it is butyrous, entire, and pink; and it is without hyphae. Cells from the edge of the colony occur singly and in clusters of two, three, or more cells. They vary in size from 2.4×3.1 to 6.6×7.3 μ and are much larger than cells of the more primitive species, *H. minuta* and *H. nonfermentans*. Also present are many zygotes formed by conjugation of independent cells. Most intact asci contain three or four ascospores. Two millimeters in from the edge of the dalmau colony are many free ascospores in clusters of three and four spores. The aerobic streak is more deeply pigmented than the colony.

Neither pseudohyphae nor true hyphae are produced by *H. glucozyma*. Pellicles are not produced on the surface of assimilation media.

Physiology

Glucose is fermented so rapidly that the insert is filled with gas in 3 or 4 days. Galactose, maltose, sucrose, lactose, and raffinose are not fermented.

Compounds assimilated are as follows:

Glucose	+	D-Xylose	— (still) + (shaken)	α-Methyl-D-glucoside	—
Galactose	—	L-Arabinose	—	Salicin	+ (shaken)
L-Sorbose	—	D-Arabinose	—	K-D-Gluconate	—
Maltose	—	D-Ribose	+	Ca-2-Ketogluconate	—
Sucrose	—	L-Rhamnose	+	K-5-Ketogluconate	—
Cellobiose	+	D-Glucosamine · HCl	—	NaK-Saccharate	—
Trehalose	+	Ethanol	+	Pyruvate	—
Lactose	—	Glycerol	+	DL-Lactate	—
Melibiose	—	*i*-Erythritol	+	Succinate	—
Raffinose	—	Adonitol	+	Citrate	V
Melezitose	—	Dulcitol	—	Ethylacetoacetate	—
Inulin	—	D-Mannitol	+	*i*-Inositol	—
Starch	—	D-Sorbitol	+	Nitrate	+

The ability of *H. glucozyma* to assimilate nitrate deserves special mention. Growth in still nitrate medium in the first and second serially transferred cultures is weak, but since the chemical test for

nitrite is quite strong, it is evident that the nitrate has been attacked. When the nitrate tests are incubated on a shaker, good grow is obtained.

H. glucozyma requires an external source of vitamins, growth is absent or slight in yeast nitrogen base containing 10 % of sodium chloride and 5 % glucose, esters are not produced, growth is moderate at 37° C, and starch is not synthesized.

Phylogenetic position

H. glucozyma occurs in a phylogenetic line, line 5, that now appears for the first time in phylogenetic relationships diagrammed in Fig. 1. This line of homothallic species follows a trend midway between line 1a and line 3. Line 1a consists of species that gained strength in their fermentative and vitamin-synthesizing capacities as they evolved from a habitat in tunnels of bark beetles in coniferous trees, then through associations with gums of deciduous trees, and finally to a free-living state in soil and water. Species of line 3 became increasingly dependent upon coniferous trees for their habitat; they lack completely the ability to produce a gaseous fermentation; and all require an external source of vitamins. All species of line 5 ferment only glucose, all require vitamins, and the two most highly evolved species occur in the frass of trees: *H. polymorpha* in the frass of deciduous trees and *Hansenula platypodis* FIOL 1967 (*Endomycopsis platypodis* BAKER & KREGER-VAN RIJ, 1964) in the frass of oaks in Europe and of hemlock in North America. Line 5 is the only line that contains species throughout its length that produce a pink or red color when cultures are heavily sporulated. Some species of other lines possess this ability, but they occur only in the primitive ends of the lines. Line 5, more than any of the other four lines, is notable for relationships with other genera. Line 5, through a distant but definite relationship (I believe) with *Cephaloascus* through *Hansenula platypodis*, is the one line that so far suggests continuity with a genus that is not now considered to be within the domain of the yeasts.

The following species are involved in indicating relationships to other genera:

H. glucozyma could be classified as *Pichia pinus* (HOLST) PHAFF (1956) except for the ability of *H. glucozyma* to assimilate nitrate. KREGER-VAN RIJ (1964) reported that some strains of *P. pinus* assimilate nitrite, that others did not, and that none assimilated nitrate. Since the only characteristic separating these two genera is the ability to assimilate nitrate, the genera are evidently very close together at the phylogenetic points occupied by these two species.

According to serological studies of TSUCHIYA et al. (1965), the species of *Pichia* that they studied lack the ability to assimilate nitrate and have the group specific antigen 11; but species of *Hansenula* assimilate nitrate, and most have the group antigen 16.

H. polymorpha, however, has group antigen 11. This inconsistency also indicates a relationship of this taxonomic area of *Hansenula* to the genus *Pichia*. *Hansenula holstii* was reported to be serologically similar to *Candida pulcherrima*. It will be of interest to learn how the species of *Pichia* and *Hansenula* not yet studied by TSUCHIYA and coworkers relate to one another and to species of other genera.

H. platypodis is an unusually interesting yeast that yields yeastlike colonies when freshly isolated from nature. It produces more highly filamentous variants at a slow rate on laboratory media, the variants becoming increasingly more filamentous and less sporogenous. The yeastlike colonies develop on their surfaces hyphae that are in dense palisade formation. Many of the hyphae branch at their distal ends, each branch producing at its apex an ascus or asci, generally three in a chain. Nearly all asci are produced by such ascophores. Anastomoses are occasionally observed between hyphae. Though the erect ascophores would seemingly indicate that this species should be included in the Cephaloascoideae (SCHIPPERS-LAMMERTSE & HEYTING, 1962), it seems improbable that the narrow ascophores could support their large heads were it not for the dense packing of hyphae in palisade arrangement. The narrow ascophores of *H. platypodis* are indeed less capable of supporting asci than are the strong ascophores of *Cephaloascus fragrans* HANAWA, the only species currently placed in the subfamily.

Before leaving the subject of relationship of line 5 to other genera, it may be noted that WICKERHAM & BURTON (1961) have pointed out the biochemical and morphological similarity of *Pachysolen* and *Hansenula*. Both genera assimilate nitrate, and primitive species of each produce extracellular phosphomannans, a characteristic limited to these two genera and *Pichia*. As *Pachysolen tannophilus* (BOIDIN & ADZET, 1957) is homothallic, the present area of association of the two genera may be considered to be near the confluence of lines 1a, 3, and 5, though the isolation of additional species of *Pachysolen* may some day indicate a much earlier separation of the two genera.

Hansenula henricii WICKERHAM, sp. nov.

Haec species homothallica est, plerumque diploidea. Asci plerumque 2, aliquando 3 aut 4 ascosporas pilliformes continent; cellulae ellipsoideae aut sphaeroideae 3.5×5.0 ad 5.2×6.6 μ. Hyphae non fiunt, sed catenae efficiuntur e cellulis sphaeroideis aut ellipsoideis. Pelliculae desunt. Non fermentatur glucosum, galactosum, maltosum, sucrosum, lactosum, nec raffinosum. Assimilatur nitras, glucosum, cellobiosum, D-xylosum, D-ribosum, L-rhamnosum, ethanolum, glycerolum, *i*-erythritolum, adonitolum, D-mannitolum, D-sorbitolum, salicinumque. Trehalosum plerumque, succinas aliquando assimilatur. Non assimilatur galactosum, L-sorbosum, maltosum, sucrosum, lactosum, melibiosum, raffinosum, melezitosum, inulinum, amylum solubile, L-arabinosum, D-arabinosum, D-

glucosamini hydrochloridum, dulcitolum, α-methyl-D-glucosidum, kalii gluconas, calcii 2-gluconas, kalii 5-gluconas, kalii-natrii saccharas, pyruvas, lactas, citras, acetoacetas ethylicus, nec *i*-inositolum. Esteres consueti non fiunt. Vitaminis externis opus est.

Typus: NRRL YB-2194, isolata a fecibus avium (?) in montibus Medicine Bow in Wyoming collectis, 1950. Deposita in Officina Investigationum Tractus Borealis, Peoria, Illinois.

This species is named in honor of Dr. ARTHUR T. HENRICI, who had a broad interest in yeasts. In 1940, he sent me a culture isolated from fermenting mushrooms bearing his number 18 and the designation *Saccharomyces* 39*ff*. This culture is now strain NRRL Y-411, the type and only known culture of the species *H. minuta*.

Three strains of *Hansenula henricii* have been isolated from materials collected in mountainous areas of the western United States. Strain NRRL YB-2194 was isolated from bird (?) feces collected in the Medicine Bow Mountains of Wyoming in 1950 and strain YB-2259 was isolated in 1950 from frass of lodgepole pine (*Pinus contorta* DOUGL.) collected in Hope Valley near Markleeville, Alpine County, California, at an elevation of approximately 7,000 feet. Both of these strains were isolated in the diploid state. A third strain, NRRL YB-2224, was isolated as a haploid colony in 1950 from soil in an area where lodgepole pines were growing along Libby Creek in the Medicine Bow Mountains. It readily produces the diploid state of the species. All three strains were isolated following enrichment in IM.

Sexuality

The three strains of *H. henricii* sporulate abundantly on ME agar slants producing from 40 to 60 % of cells as asci in 2 to 4 days. Growth becomes increasingly pink as sporulation becomes increasingly abundant. The diploid strains produce exclusively, or nearly exclusively, unconjugated asci. The ellipsoidal and spheroidal asci formed on ME slants contain from two to four ascospores with two usually predominating, three quite common, and four rather rare except on MM agar where four spores per ascus are common. Asci commonly rupture when mature, liberating the ascospores. The spores are hat-shaped with narrow brims and increase in size and become refractile after liberation from the asci. The spores measure 2.1 to 2.4 μ high by 3.1 to 3.8 μ diam, but occasionally reach 5 μ in diam.

Diploid strains YB-2194 and YB-2259 are readily converted to the haploid form by sporulating them on ME agar, and haploid cultures are readily isolated by streaking cells of heavily sporulated cultures on YM or ME agar. Haploid colonies are produced in abundance and can readily be recognized by the reduced intensity of color when the colonies are several days old or by the type of ascus in the colonies. The haploid cultures so obtained produce few unconjugated asci among the numerous conjugated asci.

As previously mentioned, a 2-day-old ME slant culture of haploid strain YB-2224 produced few asci from diploid cells. Cells from the slant were transferred to a second ME slant and to a flask of ME liquid medium which was then shaken. When the second serial slant was 2 days old, its growth contained about 5% of conjugated asci. The shaken flask culture, when 2 days old, had many cells with tubes and many asci and clusters of ascospores up to 50–100. Many intact asci were diploid, and an estimated 30 to 50 % of them contained three or four spores.

Cells from the flask culture were streaked on several plates containing ME agar, and 7 days later among some 60 isolated colonies appeared two redder colonies. Both contained a large number of exclusively unconjugated asci with three or four spores. Cells from the very edges of the two diploid colonies were streaked on YM plates. As soon as colonies developed but before they contained ascospores, cells from selected colonies were transferred to YM and ME slants. The YM slants were immediately refrigerated and the ME slants were incubated at 25° C. Cultures of the diploids on ME slants produced exclusively unconjugated asci at the bottom of the slant, but a few conjugated asci at the top arose from germinated ascospores. Growth on the slants were a pinkish red when 4 days old. A refrigerated counterpart was incubated 1 day at 25° C and lyophilized as a diploid culture in the vegetative state.

Morphology

Colonies on YM agar are smooth, glistening, and butyrous and they lack hyphae. Colonies on ME agar may be submucoid when about 4 days old and may become butyrous and pink with age. Red pigment develops more intensely on MM than on ME agar, and the aerobic streaks of dalmau preparations may become a deep reddish brown when 7 to 10 days old.

Cells at the edge of haploid colonies on MM may be spheroidal to ellipsoidal measuring 3.5×5.0 to 5.2×6.6 μ. Cells of diploid colonies have similar shape and likewise occur singly, in pairs, and in small clusters; the diploid cells average somewhat larger in size than the haploid cells. Asci formed from diploid cells are spheroidal and measure 7.0 to 7.8 μ wide.

No hyphae are produced aerobically in the dalmau preparations, but infrequent chains of spherical or ellipsoidal cells containing 10 to 15 cells may occur under the cover glass. True hyphae are not produced.

Neither rings nor pellicles are produced on the surface of assimilation media.

Physiology

H. henricii ferments no sugars.

Compounds assimilated are as follows:

Glucose	+	D-Xylose	+	α-Methyl-D-glucoside	—
Galactose	—	L-Arabinose	—	Salicin	+
L-Sorbose	—	D-Arabinose	—	K-D-Gluconate	—
Maltose	—	D-Ribose	+	Ca-2-Ketogluconate	—
Sucrose	—	L-Rhamnose	+	K-5-Ketogluconate	—
Cellobiose	+	D-Glycosamine · HCl	—	NaK-Saccharate	—
Trehalose	V	Ethanol	+	Pyruvate	—
Lactose	—	Glycerol	+	DL-Lactate	—
Melibiose	—	*i*-Erythritol	+	Succinate	V
Raffinose	—	Adonitol	+	Citrate	—
Melezitose	—	Dulcitol	—	Ethylacetoacetate	—
Inulin	—	D-Mannitol	+	*i*-Inositol	—
Starch	—	D-Sorbitol	+	Nitrate	+

Haploid strain YB-2224 consistently failed to assimilate trehalose whether the cultures were still or shaken. The diploid strains readily assimilated this sugar. The haploid strain is the only one that assimilated succinic acid and even in shaken cultures assimilation was slow.

H. henricii requires an external source of vitamins; growth is slight or absent in yeast nitrogen base containing 10 % of sodium chloride and 5 % glucose; typical esters are not produced, but a sweet odor is occasionally present in aged ME plates; growth is moderate at 37° C; and starch is not synthesized.

Phylogenetic position

As *H. henricii* is homothallic, is probably associated with coniferous trees, and does not produce a gaseous fermentation, it evidently belongs in line 3 of Fig. 1. Two of the three strains isolated are diploid so *H. henricii* would occupy a position between *H. nonfermentans*, which is haploid, and *H. canadensis*, which is exclusively diploid. Conjugation predominantly by independent cells rather than by cells with their buds is another indication of the higher phylogenetic development reached by *H. henricii* than attained by the more primitive haploids, *H. nonfermentans* and *H. minuta*. Cell size and ascospore size of *H. henricii* compare more favorably which *H. canadensis* than with *H. nonfermentans*, and its position on line 3 is therefore selected to show this relationship. Like *H. nonfermentans*, *H. henricii* produces a phosphomannan that has a moderately low ratio of phosphorus and has a high dextro rotation of light (SLODKI et al., 1961). The production of phosphomannans is most abundant among haploid species of *Hansenula*, and as one might expect, the degree to which growth of *H. henricii* on ME agar is mucoid is slight even at its maximum. It is not at all mucoid on YM agar.

The species most likely to be confused with *H. henricii* is *H. glucozyma*. They are separated by ability to produce a gaseous fermentation of glucose; the former lacks the capacity, the latter has it.

Hansenula dimennae WICKERHAM, sp. nov.

Cellulae haploideae homothallicae isolatae a natura. Conjugatione facta asci gignunt 1–4 ascosporas similes stellae Saturni, id est, ellipsoideas sed annulo exiguo cinctas. Cellulae vegetativae ellipsoideae, 2.0×3.4 ad 3.4×6.5 μ. Nec pseudohyphae nec hyphae verae fiunt. Pelliculae plerumque desunt. Glucosum fermentatur; non fermentatur galactosum, maltosum, sucrosum, lactosum, nec raffinosum. Assimilatur nitras, glucosum, L-sorbosum, cellobiosum, D-xylosum, ethanolum, glycerolum, D-mannitolum, salicin, kalii gluconas, pyruvas, lactas, et succinas. Aliquando assimilantur L-rhamnosum, D-sorbitolum, α-methyl-D-glucosidum, kalii 2-ketogluconas, acidumque citricum. Non assimilantur galactosum, maltosum, sucrosum, trehalosum, lactosum, melibiosum, raffinosum, melezitosum, inulinum, D-glucosamini hydrocholoridum, *i*-erythritolum, adonitolum, dulcitolum, kalii 5-ketogluconas, kalii-natrii saccharas, acetoacetas ethylicus, nec *i*-inositolum. Esteres fiunt; vitaminis externis opus est.

Typus: NRRL YB-3239 isolata est anno 1952 a solo in superficie terrae in vicinia Delhi Novae, India. Deposita in Officina Investigationum Tractus Borealis, Peoria, Illinois.

Source

Strain NRRL YB-3239 was isolated by IM enrichment of soil collected in March 1952 in Uttar Pradesh, India, by R. N. MATHUR and sent to the Northern Regional Research Laboratory by C. R. RANGANATHAN, President of the Forest Research Institute at Dehra Dun, U.P. Strains NRRL Y-5863 and Y-5864 were received from Dr. MARGARET DI MENNA, her respective numbers 2F14 and 3F6. They were received in November 1961 from the Soil Bureau, Taita Experimental Station, Lower Hutt, New Zealand. Dr. DI MENNA correctly believed her isolates to be related to *H. mrakii*. The two species have similar assimilation patterns.

Sexuality

H. dimennae closely resembles *H. californica* in sexual reactions and appearances of the asci and ascospores. Since conjugations occur between cells and their buds, and between independent cells, the species is homothallic. Conjugation tubes may be exceptionally long. Unconjugated asci that arise from diploid cells are rarely observed. The asci contain one to four Saturn-shaped ascospores. The spores are elongated as are ascospores of the haploid species *H. californica* and are less spherical than spores of its diploid neighbor, *H. mrakii* (Fig. 1, line 1a). The ascospores of *H. dimennae* have thin rings and one or more globules of oil that are smaller than the single large globules usually found in the diploid species of line 1a. The asci of *H. dimennae* usually liberate mature, somewhat refractile, ascospores. At 8 days of incubation on ME slants, 5–10 % of

all cells present are ascospores, and when the culture is 20 days old, 70 % of the cells are ascospores. A longer time is required by the haploid than by the diploid Saturn-spored species to reach maximum sporulation. Highly sporulated cultures are not pigmented.

Morphology

Colonies on YM and MM agar are rather small, smooth, glistening, butyrous; are rather flatly conical; and lack hyphae. Growth is not mucoid on ME agar. Cells at the edge of colonies on dalmau plates occur singly and in pairs, are ellipsoidal, and measure 2.0×3.4 to 3.4×6.5 μ. No hyphae are produced either anaerobically nor aerobically.

Very thin pellicles are sometimes produced on some of the assimilated carbon sources.

Physiology

Glucose is fermented, sometimes latently but strongly. Galactose, maltose, sucrose, lactose, and raffinose are not fermented.

The following compounds are assimilated:

Glucose	+	D-Xylose	+	α-Methyl-D-glucoside	V
Galactose	—	L-Arabinose	—	Salicin	+
L-Sorbose	+	D-Arabinose	—	K-D-Gluconate	+
Maltose	—	D-Ribose	—	Ca-2-Ketogluconate	— or W
Sucrose	—	L-Rhamnose	V	K-5-Ketogluconate	—
Cellobiose	+	D-Glucosamine · HCl	—	NaK-Saccharate	—
Trehalose	—	Ethanol	+	Puryvate	+
Lactose	—	Glycerol	+	DL-Lactate	+
Melibiose	—	*i*-Erythritol	—	Succinate	+
Raffinose	—	Adonitol	—	Citrate	— or W
Melezitose	—	Dulcitol	—	Ethylacetoacetate	—
Inulin	—	D-Mannitol	+	*i*-Inositol	—
Starch	—	D-Sorbitol	V	Nitrate	+

H. dimennae produces typical esters, requires biotin, and does not grow in yeast nitrogen base to which 5 % glucose and 10 % sodium chloride have been added; there is no growth at 37° C; and starch is not synthesized.

Phylogenetic position

H. dimennae is closely related morphologically and sexually to the haploid species *H. californica* and is biochemically similar to the diploid species *H. mrakii*. Differentiation between species in line la is largely based on differences in ability to assimilate or ferment sucrose, which is a strong characteristic, and on ability to assimilate or ferment alpha-glucosides, which occasionally are a less dependable means of identification than sucrose. To confirm or disprove the validity of *H. californica* and *H. dimennae* as autonomous haploid

species and of *H. mrakii*, *H. saturnus* (KLÖCKER) H. & P. SYDOW, and *H. beijerinckii* VAN DER WALT (1957) as autonomous diploid species, a study of the reliability of alpha-glucoside and sucrose assimilations was made and ability of hybrids to produce mature ascospores was determined. A short account of the hybridization study was presented at the Second International Symposium on Yeasts held in Bratislava, 1966, and will be reported in the proceedings when published.

Hansenula saturnus (KLÖCKER) SYDOW var. *subsufficiens* WICKERHAM, var. nov.

Maxima parte similis speciei, sed opus est vitaminis externis.

Typus: NRRL YB-1657, isolata a solo mense Martio 1950 in Liberia collecta. Deposita in collectione culturarum Officinae Investigationum Tractus Borealis, Peoria, Illinois.

In accordance with articles 25 and 26 of the International Code of Botanical Nomenclature (LANJOUW, 1961), the former species must be designated as *H. saturnus* var. *saturnus* and both varieties now comprise the species *H. saturnus*.

In March 1950, J. T. BALDWIN sent from Monrovia 48 soil samples bearing no designation. They were assigned numbers at the Northern Regional Research Laboratory. Several strains of *Hansenula saturnus* were isolated from them, and two of these after IM enrichment were typical of the species in all respects except for inability to grow in vitamin-free medium. Growth was abundant, however, when biotin was added. The two strains have retained this requirement and have not yielded biotin-independent mutants when incubated for long periods of time in vitamin-free medium.

The two dependent strains, NRRL YB-1657 and YB-1718, were isolated, respectively, from samples L-5 and L-24. Sample L-5 was recorded as wet, heavy, semisandy soil containing leaves, twigs, and roots; sample L-24, as heavily packed, wet, dark soil, leaves, etc. Strains of *H. saturnus* having no requirements for any vitamins were YB-1740 from sample L-44 and YB-1748 from sample L-43. One of these two samples was described as dry and there was no mention of moisture content of the other one.

Ability to grow in the absence of vitamins is an important taxonomic characteristic that is most useful in classifying the genus *Hansenula*, as well as other yeasts. Therefore the two biotin-requiring strains are set apart from other strains as the variety *subsufficiens*. It is of interest to consider the vitamin requirements of phylogenetic line 1a to which this new taxon belongs. One of the prime indicators of phylogenetic relationship within the genus is change or lack of change in the vitamins required by succeeding species in a phylogenetic line. According to FURUTANI et al. (1953) the most primitive species of the line, *Hansenula capsulata* WICKERHAM (1951), requires biotin, thiamine, and pyridoxine as does also *H. silvicola*. *H. californica* and *H. dimennae* require only biotin,

whereas all species above *H. dimennae* require no vitamins. Thus, *H. saturnus* var. *subsufficiens* appears to be reversing the normal evolutionary trend.

Because the variety differs from other strains of the species only in complete inability to grow in vitamin-free medium, there is no need to describe it at length.

Acknowledgements

Strain YB-2259 of *H. henricii* was isolated from frass of a lodgepole pine collected by WYNN MAULE and GILBERT DOLL. The efforts of these men and of C. E. FAVRE, forest supervisor, are appreciated. It is noteworthy that Mr. MAULE and Mr. DOLL also collected the frass from which *H. bimundalis* var. *americana* was first isolated.

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