Eeniella, a New Yeast Genus of the Torulopsidales

MAUDY T. SMITH,¹ WILMA H. BATENBURG-VAN DER VEGTE,² AND W. A. SCHEFFERS²

Yeast Division, Centraalbureau voor Schimmelcultures,¹ and Laboratory of Microbiology, Delft University of Technology,² 2628 BC Delft, The Netherlands

We found that three yeast strains previously assigned to the genus *Brettanomyces* differed in reproduction and morphology from the known *Brettanomyces* species, and we transferred them to the new yeast genus *Eeniella*, with the single species *Eeniella nana* sp. nov. The type strain of *E. nana* is CBS 1945.

In the course of a revision of the genus *Brettanomyces* Kufferath et van Laer and its ascigerous state *Dekkera* van der Walt, we reexamined three strains in the Centraalbureau voor Schimmelcultures culture collection which are representatives of the invalidly described species *Brettanomyces nanus* (9). With a view toward validating the name, we performed a more detailed study of the morphology, physiology, and base composition of the deoxyribonucleic acids of these strains.

MATERIALS AND METHODS

Yeast strains. The strains used in this study were all isolated in 1952 by Gillis Een from bottled beer from different breweries in Sweden and included strain CBS 1945 from a brewery in Kalmar, strain CBS 1955 from a brewery in Stockholm, and strain CBS 1956 from a brewery in Göteborg. The reference strains used were the type strain (CBS 72) and strain CBS 3025 of the type species *Brettanomyces bruxellensis* Kufferath et van Laer, the type strain (CBS 6042) of *Brettanomyces naardenensis* Kolschoten et Yarrow, and the type strain (CBS 479) of the type species *Hanseniaspora valbyensis* Klöcker.

Electron microscopy. After the cells were washed with water, they were fixed with 1.5% aqueous KMnO₄ for 30 min at room temperature, washed again with water, and suspended in 2% agar. The cells were dehydrated in an ethanol series, stained with 1.5% uranyl acetate for 1.5 h at the 50% ethanol step, and finally embedded in Epon 812 or Spurr. Thin sections were cut with an LKB 4801A microtome, poststained with Reynolds lead citrate for 5 min, and examined with a Philips model 201 transmission electron microscope at 60 kV.

Physiology. The physiological properties were investigated by the methods described by van der Walt (10). The strains used for the tests on the assimilation of carbon compounds were incubated on a shaker at 30 cycles per min for 28 days at 25°C. We performed five assimilation tests per strain independently in parallel. The diazonium blue B test was performed as described by van der Walt and Hopsu-Havu (12). Acetic acid production was detected by smell and was confirmed by gas chromatography.

Deoxyribonucleic acid base composition. Deoxyribonucleic acid was extracted from the cells by lysis with mercaptoethanol and sodium dodecyl sulfate

and then purified by using the methods of Meyer and Phaff (6). Deoxyribonucleic acid base composition was determined by the thermal denaturation method, using the following formula (5): moles percent guanine plus cytosine = (thermal denaturation temperature - 69.3)/0.41. Deoxyribonucleic acid from the type strain (CBS 604) of *Candida parapsilosis* (Ashf.) Langeron et Talice (thermal denaturation temperature, 85.9°C) was included as the standard in all determinations.

RESULTS

Morphological characteristics. Light microscope examination revealed that after 3 days at 25°C in glucose yeast extract peptone water the cells were usually ogival to apiculate (1.8 to 4.5 by 2.2 to 7.5 µm), seldom spherical or cylindrical, and occurred singly or in pairs. Pear-shaped (pyriform) cells also occurred (Fig. 1). After 1 month, a sediment was formed. Pseudohyphae were rarely observed in slide cultures on potato agar, rice agar, or cornmeal agar, and the cell form on agar was the same as the form in liquid medium. The shape of the cells indicated that conidiogenesis was bipolar, a characteristic observed in the Saccharomycodaceae, as well as in the genus *Brettanomyces*, which belongs to the order Torulopsidales. However, in the Saccharomycodaceae conidiogenesis is of the percurrent type (e.g., in *H. valbyensis* CBS 479 [19]) (Fig. 2), and in the Torulopsidales it is random in the apical zones, resulting in ogival to apiculate cells in young cultures of *Brettanomyces* strains and in rectangular (pillow-shaped [19]) cells in old *Brettanomyces* cultures (Fig. 3). These light microscope observations were complemented by electron microscope studies. In these studies, the reference strains *B. bruxellensis* CBS 72 and *B. naardenensis* CBS 6042 were included, as well as *H. valbyensis* CBS 479 (a representative of the Saccharomycodaceae). In strains CBS 1945, CBS 1955, and CBS 1956, multiple scar ridges were clearly confined to two opposite areas (Fig. 4 and 6). The bipolar conidiogenesis was of the percurrent type with progressive loci, as in *H. valbyensis* (3) (Fig. 5). We often observed a preference for budding on one

FIG. 1. *E. nana* CBS 1956 in 2% glucose-0.5% yeast extract-1% peptone water after 3 days at 25°C. Bipolar, percurrent budding. Pyriform cells present (arrow).

FIG. 2. *H. valbyensis* CBS 479 in 2% glucose-0.5% yeast extract-1% peptone water after 3 days at 25°C. Bipolar, percurrent budding.

FIG. 3. *B. bruxellensis* CBS 3025 in 2% glucose-0.5% yeast extract-1% peptone water after 3 days at 25°C. One ogival and one rectangular cell.

side, which caused extreme elongation of one end of the cell (Fig. 7). In *B. bruxellensis* (Fig. 8) and *B. naardenensis* (Fig. 9), buds and bud scars were observed side by side in the apical zones, indicating multilateral conidiogenesis.

Formation of ascospores. No ascospores were observed in individual cultures or in mixtures of cultures on normal sporulation media or media supplemented with vitamins (13).

Physiological characteristics. Fermentation tests were positive with D-glucose, and D-galactose, negative with sucrose, maltose, lactose, raffinose, trehalose, melibiose, melezitose, inulin, soluble starch, and α -methyl-D-glucoside, and variable between strains with cellobiose.

Assimilation tests were positive with D-glucose, D-galactose, cellobiose, L-rhamnose, ethanol, ribitol, D-mannitol, D-glucitol, salicin, and arbutin, negative with L-sorbose, sucrose, maltose, melibiose, raffinose, melezitose, inulin, soluble starch, L-arabinose, D-arabinose, D-ribose, glycerol, *i*-erythritol, galactitol, α -methyl-D-glucoside, DL-lactic acid, succinic acid, citric acid, *i*-inositol, glucono- δ -lactone, 2-ketogluconate, and D-glucosamine hydrochloride, and variable within strains with trehalose, lactose, and D-xylose.

Assimilation of KNO₃: negative.

Assimilation of ethylamine hydrochloride: positive.

Growth in vitamin-free medium: negative.

Growth on medium with 50% (wt/wt) glucose: negative.

Growth at 37°C: negative. Growth at 30°C: weakly positive. Growth at 3°C: positive.

Reaction with diazonium blue B: negative.

Additional properties included slow growth in malt extract and on malt agar, short-lived cultures, production of a characteristic aroma, production of acetic acid from glucose and ethanol under aerobic conditions, fermentation stimulated by molecular oxygen, and resistance to 1,000 μ g of cycloheximide (actidione) per ml.

Deoxyribonucleic acid base composition. Table 1 shows the results of the thermal denaturation determinations and the calculated guanine-plus-cytosine values of the deoxyribonucleic acids.

DISCUSSION

Considering properties such as slow growth, brief life, production of a characteristic aroma, and, in particular, production of acetic acid from glucose under aerobic conditions, the strains examined fit the diagnosis of the genus *Brettanomyces*. Stimulation of fermentation of glucose

by molecular oxygen was demonstrated by Scheffers (9) in these strains. This phenomenon is considered a characteristic feature of *Brettanomyces* species (14; M. T. J. Custers, Ph.D. thesis, Delft University of Technology, Delft, The Netherlands, 1940), although it also has been observed in some other yeast genera (W. A. Scheffers and T. O. Wikén, *Antonie van Leeuwenhoek J. Microbiol. Serol.* **35**[Suppl.]:A31-A32, 1969). These strains are physiologically very similar to *B. naardenensis* Kolfshoten et Yarrow (1), from which they differ only in their inability to assimilate maltose, soluble starch, and succinic acid. However, in the manner of vegetative reproduction and, consequently, in the shape of the cells, there is an important difference from the species of *Brettanomyces*. From light microscopy as well as from electron microscopy, it is evident that the strains examined reproduce vegetatively by bipolar budding of the percurrent type, whereas *Brettanomyces* species reproduce vegetatively by multilateral budding localized in the apical zones. This difference in the manner of reproduction can be overlooked easily in young cultures, since in both types of reproduction, the young cells are ogival to apiculate after a few buddings. However, with the percurrent type of reproduction, the cells in old cultures keep their ogival to apiculate form, whereas the cells of *Brettanomyces* strains turn into rectangles (pillow-shaped [19]) by a somewhat restricted sympodial conidiogenesis at opposite sides. Since in generic differentiation the mode of vegetative reproduction and, consequently, cell morphology are more important than physiological properties (2, 7), the three strains examined cannot be considered members of the genus *Brettanomyces*; they must be recognized as representatives of a yeast genus with bipolar budding. The diazonium blue B test and the morphology of the cell wall indicate the ascomycetous nature of these strains. Since they are asporogenous and fermentative, these strains might be assigned to the imperfect ascomycetous yeast genus *Kloeckera* Janke, the anamorph of the genus *Hanseniaspora*. However, in view of some properties, such as slow growth, brief life, production of a characteristic aroma, and, in particular, production of acetic acid from glucose under aerobic conditions (a feature to which importance is attached as a generic characteristic [11]), it seems appropriate to set the strains CBS 1945, CBS 1955, and CBS 1956 apart from the genus *Kloeckera* and to create a new genus to accommodate them.

This opinion is supported by the findings of Yamada et al. (18) concerning the coenzyme Q

FIG. 4. *E. nana* CBS 1955. Two scar ridges are visible on both sides (arrows).

FIG. 5. *H. valbyensis* CBS 479. Scar ridges present on both sides (arrows).

FIG. 6. *E. nana* CBS 1956. Several scar ridges on one side and one scar and one bud on the opposite side are visible.

FIG. 7. *E. nana* CBS 1945 cell with one elongated end having multiple scar ridges.

system in the genus *Brettanomyces*. All nine recognized species of *Brettanomyces*, as well as the invalid species *B. nanus* have the Q-9 system, whereas the species of the genus *Kloeckera*

and its ascigerous state, *Hanseniaspora*, are characterized by the Q-6 ubiquinone system (15). Since the coenzyme Q system is considered to be a valuable generic parameter (4, 8, 16, 17)

FIG. 8. *B. bruxellensis* CBS 72 cell showing three buds at one apical zone.

FIG. 9. *B. naardenensis* CBS 6042 cell showing three single bud scars at one apical zone (arrows).

and in view of the above-mentioned characteristics, we feel justified in separating the three strains described above from the imperfect yeast genus *Kloeckera* and in creating a new genus.

For this genus we propose the name *Eeniella* gen. nov. in honor of Gillis Een, who isolated the strains, with the single species *Eeniella nana* sp. nov.

The epithet *nana* (Gr. for dwarf) was chosen because of the small size of the cells.

Since the genus *Eeniella* resembles the genus *Kloeckera* in its morphology and the genus *Brettanomyces* in its physiology, elucidation of the possible phylogenetic relationships among these genera will require further study. The salient generic characteristics of *Brettanomyces*, *Eeniella*, and *Kloeckera* are summarized in Table 2.

Latin diagnosis and description of the taxa. (i) *Eeniella* gen. nov.

Gemmatio bipolaris percurrents. Cellulae obtuso-acuminatae vel apiculatae, raro rotundatae ad cylindricae.

Pseudohyphae parcae. Ascosporae absentes. In extracto malti et agar malti culturae lente crescunt et ad mortem praematuram propensae. Odor insignitus aromaticus. In conditione aerobia acidum aceticum e glucoso plerumque vehementur. Fermentatio glucosi plerumque oxygeno moleculari augetur.

Systema ubiquinoni typi Q-9.

Species typica: *Eeniella nana* sp. nov.

Vegetative reproduction is by bipolar budding of the percurrent type. Cells are ogival to apiculate, seldom spherical to cylindrical. Pseudo-

hyphae are seldom formed. Ascospores are not produced. Slow growth occurs in malt extract and on malt agar. Cultures are generally short-lived. A characteristic aroma is produced. Usually there is vigorous production of acetic acid from glucose under aerobic conditions. Fermentation is present and usually is stimulated by molecular oxygen. The Q-9 ubiquinone is present in the electron transport system. The type species of the genus is *Eeniella nana* sp. nov.

(ii) *Eeniella nana* sp. nov. Synonym: *Brettanomyces nanus* nom. inval. Art. 36 (Scheffers 1966).

Cellulae ovoidae vel apiculatae ($1.8-4.5 \times 2.2-7.5 \mu\text{m}$), rarius rotundatae vel cylindricae, singulae vel binae. Cultura butyrosa, glabra, nitida, eburnea.

Pseudohyphae parcae. Ascosporae nullae.

Glucosum et galactosum fermentantur. Glucosum, galactosum, cellobiosum, trehalosum (variabiliter), lactosum (variabiliter), D-xylosum (variabiliter), L-rhamnosum, ethanolum, ribitolum, D-mannitolum, D-glucitolum, salicinum, et arbutinum assimilantur, neque L-sorbosum, saccharum, maltosum, melibiosum, raffinolum, melezitosum, inulinum, amyllum solubile, L-arabinosum, D-arabinosum, D-ribosum, glycerolum, i-erythritolum, dulcitolum, α -methyl-D-glucosidum, DL-acidum lacticum, acidum succinicum, acidum citricum, i-inositolum, glucono- δ -lactonum, calcii-2-keto-gluconatum, et D-glucosaminum hydrochloricum.

Kalii nitratum non assimilatur.

Ethylaminum hydrochloricum assimilatur.

Crescere non potest 37°C.

Ad crescentiam vitamina externa necessaria sunt.

In conditione aerobia acidum aceticum ex-glucoso et ethanollo formatur.

Proportio molaris guanini + cytosini in acido deoxyribonucleinico: 40.2–41.4 mol%.

Typus isolatus ex cerevisiae in Suecia depositus in collectione Centraalbureau voor Schimmelcultures, Delphi (CBS 1945).

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TABLE 1. DNA base compositions of *E. nana* strains

Strain	Thermal denaturation temp (°C)	Guanine-plus-cytosine content (mol%)
CBS 1945	86.10 $\pm$ 0.19 ^a	41.0 $\pm$ 0.5 ^a
CBS 1955	86.26 $\pm$ 0.19	41.4 $\pm$ 0.5
CBS 1956	85.80 $\pm$ 0.15	40.2 $\pm$ 0.4

^a Mean $\pm$ standard deviation (average of five determinations).

TABLE 2. Salient generic characteristics of *Brettanomyces*, *Eeniella*, and *Kloeckera*

Genus	Vegetative reproduction	Production of acetic acid	Fermentation stimulated by molecular O ₂	Coenzyme Q system
<i>Brettanomyces</i>	Multilateral budding in apical zones	+	+	9
<i>Eeniella</i>	Percurrent budding, bipolar	+	+	9
<i>Kloeckera</i>	Percurrent budding, bipolar	—	—	6

REPRINT REQUESTS

Address reprint requests to: M. Th. Smith, Yeast Division, Centraalbureau voor Schimmelcultures, Laboratory of Microbiology, Delft University of Technology, Julianalaan 67a, 2628 BC Delft, The Netherlands.

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