A SIMPLE TECHNIQUE FOR OBTAINING MATING TYPES IN HETEROTHALLIC DIPLOID YEASTS, WITH SPECIAL REFERENCE TO THEIR USES IN THE GENUS HANSENULA

LYNFERD J. WICKERHAM AND KERMIT A. BURTON

Fermentation Division, Northern Regional Research Laboratory, Peoria, Illinois

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A procedure has been devised by which large numbers of ascospore isolates may be obtained from yeasts without the use of a micromanipulator and auxiliary costly laboratory equipment or a need for the exacting skills which such tools require. The procedure is being continually applied in this laboratory to the genus Hansenula. Some of the species of this genus produce many ascospores, but so few are viable that isolation of mating types by micromanipulation is often impractical. Hence for genetical studies different experimental procedures are required. In this genus the ascus ruptures when it is mature and the liberated ascospores often agglutinate in large masses. This habit of early escapement from the ascus also tends to thwart the usual tetrad analysis that starts with isolation of the four ascospores by micromanipulation. It is intended that the new procedure for isolating mating types and simple ancillary techniques for genetical analyses will be modified to whatever extent is necessary so that they may be applied to a genus like Saccharomyces where viability of ascospores is usually high and the ascus usually remains intact until the ascospores germinate. Various uses of the new procedure will be presented with examples taken from results obtained for the most part with diploid species of Hansenula.

The principal procedure for the isolation of cultures derived from single ascospores is based on the fact that ascospores can withstand slightly more heat than vegetative cells. For most species of Hansenula, 57 or 58 C is employed.

MATERIALS AND METHODS

Isolation of cultures derived from ascospores.

First, heat resistance of a culture of rapidly growing vegetative cells is determined. A loopful of cells from a culture growing on a yeast extract-malt extract agar slant (vegetation medium, Wickerham, 1951) is suspended in two ml of yeast extract-malt extract broth in a 16 mm test tube. The cotton plug is discarded and the tube placed in a water bath at the desired temperature. At 0 time, 1, 2, 3, 4, 6, 8, 10, 15, and 20 minutes a loopful of suspension is streaked on the agar surface of petri dishes. The plates are examined after incubation to determine how many minutes are required to kill the vegetative cells. Then a suspension of a sporulated culture of the same strain is prepared. The suspension is heated at the same temperature but for a longer time than was required to kill the vegetative cells. If only 12 to 20 ascospore isolates are desired, duplicate plates are streaked at intervals of 1, 2, 3, 4, 5, and 10 minutes in excess of the time required to kill the vegetative cells. The plates are incubated at 28 C until colonies have developed, usually 3 to 8 days. The colonies may vary from pin point to normal in size. The desired number of colonies are picked of those that arose from cells which survived the longest periods of heat. The larger colonies generally are avoided, for they may have arisen from diploid vegetative cells which survived heating; moderate sized colonies are suspended in sterile water and restreaked on plates; tiny colonies are restreaked directly without suspending. A typical colony is transferred to a slant from each plate of this second streaking. These colony isolates are preserved for further study.

Examples. Hansenula anomala, strain NRRL Y-366. Vegetative cells of this strain were heated at 58 C. Heavy growth occurred on plates streaked at 0 time and 1 minute. Approximately 200 colonies grew on the plate streaked after 2 minutes, but none grew on the plates streaked after longer heating intervals. A sporulated culture which was heated at 58 C gave heavy growth on plates streaked at 0 time and 2 minutes. Sixteen colonies developed on the 4 minute plate.

303
and 6 colonies on the 6 minute plate. Colonies from the 6 and 4 minute plates were restreaked, and a typical colony from each plate was selected for further work.

*Hansenua anomala*, strain NRRL Y-2153. Vegetative cells heated at 58 C gave heavy growth when streaked on plates at 0 and 1 minute times. The 2 minute plate had 8 colonies, but none of the plates of greater time intervals had any colonies. A sporulated culture heated at the same temperature had 2 colonies on the 10 minute plate (isolates Y-2153-1 and Y-2153-2), 6 colonies on the 8 minute plate (isolates -3 through -8), and 4 colonies on the 6 minute plate (isolates -9 through -12). The isolates were replated, and typical colonies were picked for preservation.

**Determination of whether a diploid yeast is homothallic or heterothallic.** Twelve to twenty colony isolates are transferred individually to slants of malt extract sporulation medium (Wickerham, 1951). The slants are incubated for 7 to 14 days at 25 C. If all, or nearly all, of the slants show good sporulation, the culture is homothallic; but if the reverse is true, the culture is heterothallic. Where only a few sporulated isolates are found, they represent either diploid vegetative cells which survived the heating or ascospore isolates which undergo self diploidization. Diploids which survived heat treatment are recognized as such because they show no conjugation either when grown by themselves or in mixtures with either mating type. Ascospore isolates which self diploidize are rather infrequent. The number of spores they produce when grown alone on sporulation medium is usually small, and conjugations are rare. Their chief distinguishing features are the better sporulation and particularly the more numerous conjugations they produce when mixed with one of the mating types as compared to the mixture with the other mating type.

By this procedure it was learned that all species of *Hansenua* producing Saturn shaped ascospores are homothallic. Diploid species of *Hansenua* which produce hat shaped ascospores are heterothallic, namely *H. subpelliculosa*, *H. anomala*, and *H. ciferrii*.

**Examples.** *H. anomala*, strain Y-366. After 6 days' incubation on sporulation slants, only 1 of 12 colony isolates produced ascospores and it therefore was diploid. From strain Y-2153, 1 of the 12 isolates was likewise diploid, the other 11 being haploid. These two strains of *H. anomala* are heterothallic.

*H. saturnus*, strains Y-2165 and Y-2166. From each strain 15 isolates were grown separately on sporulation slants and produced between 50 and 90 per cent ascospores. These strains are homothallic.

**Isolation of mating types from diploid, heterothallic species.** As stated previously, if all or nearly all of the colony isolates fail to produce spores when grown separately on sporulation medium the yeast under study is heterothallic. The non-ascosporogenous colony isolates then are mixed by fours, taking approximately equal amounts of vegetative cells from rapidly growing cultures of each of four isolates and mixing the cells on the center of a slant of sporulation medium. The mixed inoculum is spread then over the entire surface of the slant. Those mixtures of four which contain both sexes will show conjugating cells within a day or two, and ascospores usually will be present in three or four days.

The colony isolates which showed the most ascospores when mixed by fours are selected for analysis. The four isolates of which it is composed are mixed in the 6 different combinations of two isolates. All pairs producing conjugations or ascospores consist of opposite mating types. The pair giving the most spores is selected, and its two members are mated against each of the 20 colony isolates to determine the sex of each as well as to record the approximate number of spores produced in each mating. If desired all the better sporulating isolates of opposite sexes may be paired and the best sporulating pair of mating types then may be selected to represent the strain.

Once mating types have been obtained for one strain of a diploid species, ascospore isolates of other strains of the same species generally can be paired with them to determine sex and fruitfulness, thus eliminating the mixtures by fours.

**Examples.** *H. anomala*, strain Y-2153. Mixtures were made of various combinations of 3 or 4 haploid isolates. All mixtures produced ascospores. The mixture containing the highest percentage of ascospores was analyzed by mixing all of the isolates it contained in all possible combinations of two to each mixture. The best sporulating pair was selected. Each member was mated against all the other haploid isolates from this strain to determine their sex. The most fruitful pair of isolates was Y-2153-4 and Y-2153-6.
Five days after mixing on sporulation medium they had produced about 20 per cent ascospores. Aci consisting of single cells outnumbered those which consisted of conjugated cells. Aci which consist of conjugated cells are produced immediately after the fusion of two haploid cells, and aci which consist of single cells (simple aci) are formed from diploid cells which arose originally as buds from conjugated cells.

*H. anomala*, strain Y-1656. Instead of mixing 20 isolates of this strain by fours, each was mixed directly with known opposite mating types Y-2153-4 and Y-366-8 to determine sex and relative yields of ascospores. Nine of the isolates of Y-1656 mated with Y-366-8 to produce 2 to 50 per cent spores depending upon the individual isolate. Eleven of the isolates mated with Y-2153-4 to produce from 30 to 90 per cent spores. The diploid parent strain Y-1656 normally produces about 20 per cent spores at 5 days.

*H. anomala*, strain Y-366. Forty-nine isolates obtained by heating at 58 C were mixed with opposite mating types Y-2153-4 and Y-2153-6. All 49 isolates mated with Y-2153-4, and none mated with Y-2153-6. All the ascosporic isolates of the diploid strain Y-366 were of one sex.

**Obtaining more highly sporogenous pairs of mating types for diploid heterothallic yeasts.**

Opposite mating types from two different diploid strains of a species of *Hansenula* generally are more sexually active than the best pair of mating types stemming from a single strain. The pair from the different strains, when mixed on sporulation medium, usually produces predominantly diploid zygotes before sporulation is complete, whereas mixtures of opposite sexes made from one or the other of these two strains, but not from both, often lack the vigor to produce many diploid vegetative cells. Most of the conjugated cells may be converted directly to asc. Thus, crossing of strains generally lends vigor to the offspring, as shown by the production of more ascospores than shown by matings of isolates from a single strain.

More highly sporulating pairs may be obtained for a diploid species represented by only one strain by isolating a larger number of ascospore cultures from it. It is common for one or two unusually prolific matings to be obtained from a large number of matings. The prolific matings are repeated each time the two isolates are mixed.

**Examples.** *H. anomala*. Isolates Y-1656-11 and 1656-18, on a sporulation slant for 5 days, produced approximately 10 per cent spores with all the intact asc i conjugated. Isolates Y-2153-4 and Y-2153-6, on a sporulation slant for 6 days, produced approximately 20 per cent spores. Although simple aci predominated, many conjugated aci were present. Mixtures of isolates Y-1656-11 and Y-366-8 produced 90 per cent ascospores at 5 days, and nearly all of the aci were simple. Thus, mating types from two different strains sporulated more abundantly than mating types from either of these two strains.

*Hansenula ciferrii*. The only ascosporogenous strain of *H. ciferrii* in our collection is NRRL Y-1031. It normally produces from 8 to 20 per cent ascospores at 5 days. Thirty-five isolates were obtained by heat treatment of a sporulated culture. Fourteen were of one sex, and 17 were of the opposite sex. Four isolates mated with neither sex. Sporulation was very poor with most of the positive pairs even at 9 days having conjugations but no spores. One pair, isolates Y-1031-11 and Y-1031-27, was outstanding. It produced approximately 70 per cent spores. They have been mated several times and consistently give so many healthy ascospores in such large agglutinated masses that it is difficult to estimate percentages. Most of the aci are formed from diploid cells. The next most fruitful pair was 1031-11 and 1031-18 which produced approximately 4 per cent ascospores. When isolate 1031-11 was mated with each of the other isolates of opposite sex, maximum sporulation was 3 per cent. Isolate 1031-27 was mated similarly with isolates of the opposite sex. In no case were ascospores produced although in nearly all pairs conjugations were observed.

**Recognition of sexually active haploid species classified in both ascosporogenous and nonascosporogenous genera.** *Candida pelliculosa* is the name currently used for strains of yeasts which would be classified as *H. anomala* except that *C. pelliculosa* is nonascosporogenous. Most strains of *C. pelliculosa* show weaknesses in assimilation or fermentation of sucrose and raffinose not shown by strains of *H. anomala*. All of our stock culture strains of *C. pelliculosa* mate with one sex of *H. anomala* to form typical hat shaped ascospores.

*Hansenula schneggi* has been ascosporogenous ever since its original description by Weber (1922). It is identical with certain strains of *C. pelliculosa* which show reduced action on raffinose and sucrose. It reacts sexually with *H. anomala* and with strains of *C. pelliculosa*,
for its sex is opposite to that of all our stock strains of *C. pelliculosa*. Thus, the stock culture of *H. schneggi* Y-993 when mixed with *H. anomala* mating type Y-365-8 produced ascospores in both simple and conjugated ascii. All stock cultures of *C. pelliculosa* (NRRL Y-98, Y-120, Y-317, Y-840, Y-945, etc) are of the opposite sex from Y-993 and mate with it and with *H. anomala* mating type Y-2153-4.

Actually, most strains of *C. pelliculosa* and *H. schneggi* vary away from *H. anomala* by small transitional degrees of physiology, and the strains of maximum difference should be considered as a variety, with those of intermediate physiology being regarded as intermediates. The remaining strains of *C. pelliculosa* are typical of *H. anomala* except that they are haploid.

**Synthesis of diploid strains from haploid isolates of intermediate evolutionary development.** In the writers' opinion, based on the phylogenetic diagram of the genus *Hansenula* (Wickerham, 1951, p. 46), any species of this genus which ferments a disaccharide with the production of gas may be expected to exist to some extent in the vegetative state as a diploid yeast. Five isolates of an unnamed, sucrose fermenting species have been studied. All but one strain are sexually active mating types. The most active pair of opposite sexes gave rise to some conjugations which immediately sporulated, and to other conjugations which budded off diploid zygotes, as shown by the observation that some ascii were not conjugated but consisted of single cells. Diploid zygotes were isolated by streaking and selecting large colonies. In this species the diploid state can be maintained pure only so long as sporulation is prevented. Perhaps it should be noted here that diploid strains synthesized from haploid isolates of species that exist in nature predominantly as diploids have a greater tendency to maintain the diploid state than does the species just discussed.

Were a stable diploid desired for industrial purposes, it should be a relatively simple task to obtain, by selection, a nonsporulating diploid colony isolate.

**Example.** Opposite mating types, Y-2167 and Y-2168, were mixed on sporulation medium. When numerous conjugations were present, but before many ascii were formed, the culture was streaked on plates of yeast extract-malt extract agar. Four of the largest colonies were transferred directly to slants of malt extract agar (sporulation medium). Colony isolate-2 on the sporulation medium produced 30 to 40 per cent ascospores. The ascii were formed exclusively from diploid cells, and there were no conjugations. Colony isolate-2 from the sporulation slant was streaked on plates, and four colonies were picked and then sporulated. Colony isolate-2-4 produced 50 to 60 per cent ascospores at 3 days of incubation. It was entirely diploid. This isolate also sporulated well on malt extract-yeast extract agar on which it had been grown with the intention of lyophilizing it. If a sporulated culture were lyophilized and subsequently subcultured, some of the ascospores would germinate to give cultures which were no longer purely diploid. Therefore, colony isolate-2-4 was streaked on plates, each colony selected was put on both sporulation and on vegetative media, and the malt extract-yeast extract slants were put in the refrigerator immediately after inoculation. The malt extract agar slants were incubated at 25 C, and at 3 days colony isolate-2-4-4 showed 50 to 70 per cent sporulation with the ascii formed exclusively from diploid cells. Then the refrigerated slant of colony isolate 2-4-4 was taken from the refrigerator, incubated at 28 C for 24 hours, observed microscopically for the absence of ascospores, and then lyophilized.

**Isolation and analysis of diploid hybrids.** Results indicate that in the genus *Hansenula* hybridization does not occur between different heterothallic species, no matter how closely related the species may be. Hybridization does occur among physiologically different strains of a single species. The maximum amount of physiological variation occurs in the species *H. subpelliculosa* and *H. anomala*. In the case of *H. subpelliculosa*, mating types may be obtained for two diploid strains representing extremes of physiological (or morphological) differences. When opposite sexes from the two strains are mated, matings are very fruitful. When the mixture is only 2 or 3 days old, and many conjugating forms are present, it is streaked on plates. Some of the largest colonies are transferred to both malt extract-yeast extract agar, which may be refrigerated immediately to prevent growth, and to sporulation medium. If all of the resulting ascii on the sporulation medium consist of single cells, with conjugation absent, the culture is composed of a diploid hybrid. The culture on malt extract-yeast extract agar, corresponding to the best sporulating diploid isolate on malt extract agar, is removed then from the refrigerator and grown at
28 C for immediate lyophilization and studied to determine which characteristics it has received from each parent. Colony isolates of diploid hyrbrids having the same haploid parents are quite uniform in their reactions.

Example. Mating types were obtained by heat treatment for _H. subpelliculosa_, strain NRRL Y-1683, which assimilates cellobiose strongly, melezitose latently, and soluble starch strongly. Mating types were similarly obtained for _H. subpelliculosa_, strain NRRL Y-1822, which assimilates neither cellobiose or melezitose and has latent action on soluble starch. Opposite mating types, having the reactions of the parent diploid cultures, were strains Y-1683-11 and Y-1822-12. They were mixed on a slant of sporulation medium, and at 5 days numerous budding zygotes were present. The culture was streaked on plates which were incubated at 28 C to minimize sporulation. Medium sized and large colonies appeared, the smaller colonies presumably being composed of one or the other mating type, the large colonies consisting of diploid cells. Seven large and two moderate sized colonies were transferred to sporulation slants. After 8 days of incubation at 25 C, all cultures stemming from large colonies produced 40 to 80 per cent ascospores in asci arising exclusively from diploid cells, and the cultures stemming from small colonies produced no ascospores. The diploid hyrbrids were tested for their ability to assimilate cellobiose, melezitose, and soluble starch. All seven assimilated cellobiose strongly, melezitose and soluble starch latently.

Isolation and analysis of haploid hyrbrids. An ascospore isolate from each of two physiologically (or morphologically) different diploid strains is mated. The sporulated culture is heat treated, and ascospore isolates of the hybrid are obtained in the usual way. Each isolate is mixed with each parent (backcrossed) to determine its sex, and it is grown also by itself on sporulation medium to prove by its inability to sporulate that it is a haploid isolate. Each isolate may be studied then for inheritance of characteristics from its parents. Considerable variation in patterns may be found in haploid hyrbrids if the parents differed in three or more characteristics. In addition to expected patterns of variation, some of the hybrids may show unexpected variations.

Example. Sixty-two isolates were obtained by heat treatment of a sporulated culture of _H. subpelliculosa_ strain NRRL Y-1683 by strain Y-1822. The ability of the parent mating types to assimilate cellobiose, melezitose, and soluble starch is given in the preceding discussion. Twenty-six of the isolates were of one sex, 26 were of the other sex, and 46 of these 52 isolates showed no self diploidization. Six showed self diploidization in pure culture on malt extract agar, as indicated by the presence of a few spores and extremely few conjugations. These six also mated with one or the other of the parent mating types with which each was mixed, as revealed by a larger number of spores and particularly by the larger number of conjugations produced. Seven of the remaining ten isolates were diploids, as shown by ascii which were exclusively single cells and by the absence of conjugations in mixtures with mating types. Three isolates showed no sexual reaction.

All isolates were inoculated into assimilation test media containing either cellobiose, melezitose, or soluble starch. The cultures were incubated at 25 C for 47 days, during which time 3 readings were made. There was a great deal of variation in the intensity with which the isolates attacked the carbon sources. The strong growth of almost all isolates on cellobiose was notable. One isolate was negative for all three carbon sources; two isolates of opposite sexes were positive for starch and negative for the other two; seven isolates were positive only for cellobiose; and seven were negative only for melezitose. Twenty-four isolates assimilated all three carbon sources moderately or strongly. The remainder of the isolates could not be placed in definite groups because of weak reactions. Two isolates showed a clear-cut growth factor deficiency by complete failure to grow in carbon assimilation medium containing glucose, though they grew well on malt extract-yeast extract agar.

It may be assumed that diploid cultures having a variety of reactions could be produced simply by mixing opposite sexes of the same or different reactions in these three carbon sources.

DISCUSSION

Procedures are described that have demonstrated for the first time which of the diploid species of _Hansenula_ are heterothallic and which are homothallic. The procedures permit easy isolation of mating types for use in hybridization studies. They are of value to taxonomists for determining relationships through hybridization, and they should be of value to geneticists who desire to ascertain the range of variation in a
large number of haploid hybrids. The procedures also should be of use to industrial zymologists who desire to mate certain yeasts and obtain hybrids having certain characteristics from each parent.

**SUMMARY**

Simple procedures have been devised for studying the genetics of yeasts which produce asci that rupture when mature. They consist of: Isolation of cultures derived from single ascospores; determination of whether a diploid yeast is homothallic or heterothallic; isolation of mating types from diploid, heterothallic species; obtaining more highly sporogenous pairs of mating types for diploid heterothallic yeasts; production of perfect forms of sexually active haploid species classified in both ascosporogenous and nonascosporogenous genera; synthesis of diploid strains from haploid isolates of intermediate evolutionary development; isolation and analysis of diploid hybrids; isolation and analysis of haploid hybrids.

Of the predominantly diploid species of *Hansenula* studied thus far, all having hat shaped ascospores are heterothallic; all of the species having Saturn shaped ascospores are homothallic. *Hansenula schneegii* and *Candida pelliculosa* are sexually active mating types of *Hansenula anomala*.

**REFERENCES**
